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Effect of Protein Binding on the Activity of Penicillins in Combination with Gentamicin Against Enterococci

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To assess the effect of protein binding by human serum on the synergistic interaction of penicillins with gentamicin, time-kill curves were determined for four penicillins alone and in combination with gentamicin against 10 blood isolates of enterococci. Killing curves demonstrated synergism with penicillin G plus gentamicin against all 10 strains in either broth or 50% human serum. In broth the combinations of nafcillin plus gentamicin and oxacillin plus gentamicin were synergistic against 10 of 10 strains and 4 of 10 strains, respectively. However, in serum, nafcillin plus gentamicin was synergistically bactericidal against only two strains and oxacillin plus gentamicin against none. Methicillin plus gentamicin was synergistic against none of the enterococci in either medium. Thus, the semisynthetic, penicillinase-resistant penicillins are unlikely to be effective in the therapy of patients with enterococcal endocarditis.

Although enterococci are relatively resistant in vitro to many individual antibiotics (18, 19), combinations of antimicrobial agents (particularly penicillins or cephalosporins plus aminoglycosides) have exhibited in vitro synergistic killing of these organisms (7-10). Furthermore, clinical experience has documented the efficacy of penicillin plus one of the aminoglycosidic aminocyclitols in the therapy of enterococcal endocarditis (11, 15). Previous in vitro studies in our laboratories evaluated the relative synergistic bactericidal potential of three semisynthetic, penicillinase-resistant penicillins in combination with gentamicin against enterococci (8). Using the method of time-kill curves performed in broth, we found that at clinically achievable concentrations the combination of nafcillin plus gentamicin produced enhanced killing against nearly all strains of enterococci tested, whereas the combinations of either oxacillin or methicillin plus gentamicin were ineffective against most strains tested. However, these semisynthetic penicillins vary widely in the extent to which they bind to serum proteins (12). Since it is thought that only free, unbound penicillin exerts antimicrobial activity (13), it is possible that the greater effectiveness of the highly protein-bound nafcillin in broth might not obtain in serum due to the inhibitory effect of serum on highly protein-bound penicillins. Therefore, we elected to examine the effect of human serum and its binding of these penicillins on the synergistic interaction of penicillin-gentamicin combinations against enterococci. Preliminary results of these experiments suggested that the addition of serum made no difference in the synergistic killing effects of the semisynthetic, penicillinase-resistant penicillins in combination with gentamicin against enterococci (R. H. Glew, R. C. Moellering, Jr., and K. Buettner, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 16th, Chicago, Ill., Abstr. no. 73, 1976). While this is true for a few strains of enterococci, it is not the case with the majority, for which the addition of serum to the antibiotic-containing medium has a significant deleterious effect on the in vitro activity of certain of the penicillinase-resistant penicillins plus gentamicin against enterococci, as will be shown in this paper.

MATERIALS AND METHODS

The organisms used in this study had been isolated from blood cultures submitted to the Bacteriology Laboratory of the Massachusetts General Hospital, and each represented a separate infection. These 12 isolates had been identified as enterococci by the usual growth criteria (6, 21), were grouped serologically by using extracts prepared by the method of Rantz and Randall (16), and were identified to species by using a composite of reactions as suggested by Deibel (5). All isolates were group D *Streptococcus faecalis*.

Antibiotics used in this study included sodium nafcillin (Wyeth Laboratories), sodium oxacillin (Bristol Laboratories), sodium methicillin (Bristol Laboratories), potassium penicillin G (Pfizer Laboratories), and

gentamicin sulfate (Schering Corp.). Appropriate dilutions were made in sterile water without preservative. Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) for each antibiotic were determined according to standard tube dilution methods (22) in dextrose-phosphate broth (Pfizer) with and without the addition of pooled human serum at a concentration of 50% by volume. The inoculum for each organism consisted of 1 ml of a 1:1,000 dilution of an overnight broth culture and contained 10^5 to 10^6 organisms per ml. The final volume in each tube was 2 ml.

Tests for antibiotic synergism were performed by the method of time-kill curves, as described previously (8), using a starting concentration of 10^7 organisms per ml diluted from a culture grown overnight in broth. Antibiotics were added as follows: penicillin G, 6.2 μg/ml; nafcillin, oxacillin, and methicillin, 15 μg/ml; gentamicin, 5 μg/ml. The cultures (20-ml final volume) were incubated without agitation in 250-ml Erlenmeyer flasks at 37°C, and samples (0.5 ml) were removed at 0, 4, and 24 h for determination of colony counts. For each strain of enterococcus, killing curves were determined simultaneously in dextrose-phosphate broth and in pooled human serum (50% by volume in broth). Synergistic interaction between gentamicin and one of the penicillins was defined as a decrease of 100-fold or greater in the number of colony-forming units at 24 h caused by the combination as compared with the more effective of the antibiotics alone. In all instances, the more effective antibiotic alone was the penicillin, since gentamicin in the concentrations employed in this study had no effect on the growth of the enterococcus when it was used alone. With certain strains of enterococci, virtually uninhibited growth of organisms occurred in both the flasks containing the penicillin derivative alone and the gentamicin alone; this was most commonly noted with methicillin or oxacillin. In such instances, the extent of killing at 24 h by the combination was compared with the starting colony count in the flask (time 0 h) rather than the number of colony-forming units present at 24 h in the flasks containing individual antibiotics.

Although whole serum rapidly reaches alkaline pH with incubation (2), the dextrose-phosphate broth exerted moderate buffering action in the 50% serum-broth medium. However, to eliminate any effect of pH on antibacterial activity, the serum-broth was buffered to pH 7.4 to 7.5 with HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid) at a final concentration of 50 mM.

The serum protein binding of penicillin, nafcillin, oxacillin, and methicillin was determined in pooled human serum and in pooled human serum in dextrose-phosphate broth (50% by volume) by the ultrafiltration method of Bennett and Kirby (2). The concentrations of antibiotic in the serum preparations and in the filtrate were determined by the use of an agar well diffusion method using as the test organism a strain of *Bacillus globigii* obtained from R. Winters (24).

RESULTS

In these comparative studies, we employed 50% serum (in dextrose-phosphate broth) rather than 100% human serum to provide optimal growth conditions for the enterococcus. Colville and Quinn demonstrated that the inhibitory effect of serum on the activity of the penicillins against *Staphylococcus aureus* was nearly as pronounced in 50% serum as in 100% serum (3). Similarly, our ultrafiltration studies (Table 1) showed that with the highly protein-bound penicillins (nafcillin and oxacillin) protein binding was nearly as extensive in 50% serum as in 100% serum, whereas with the lesser protein-bound agents (penicillin G and methicillin) the percent binding in 50% serum was about one-half that in 100% serum.

In Fig. 1 are shown the frequency distributions of MICs for the four penicillins against 12 strains of enterococci, as determined simultaneously in broth and in 50% human serum. In both media, the order of decreasing inhibitory activity of the penicillins was: penicillin G > nafcillin > oxacillin > methicillin. When MICs in the two media were compared, the MIC for each strain of enterococcus was higher in serum than in broth. Overall, for each of the 12 strains, the mean increase in MIC in serum as compared with broth was approximately twofold (that is, one tube) for methicillin and penicillin and fourfold (two tubes) for nafcillin and oxacillin. In general, the most active drug at most concentrations in either medium was penicillin G, followed in order by nafcillin, oxacillin, and methicillin. For example, in broth at the clinically achievable concentration of 16 μg/ml, 100% of strains were inhibited by penicillin G or by nafcillin, 67% by oxacillin, and 8% by methicillin. In 50% serum at 16 μg/ml, 100% of strains were inhibited by penicillin G, 42% by nafcillin, and 0% by oxacillin or by methicillin.

Similar to the results in our previous study (8), the MBCs in broth for the 12 strains of enterococci were ≥1,000 μg/ml for 8% of the strains with penicillin G, 33% with nafcillin, 67% with oxacillin, and 50% with methicillin. In serum, MBCs generally were higher than in broth for each strain of enterococcus, particularly with

TABLE 1. *Extent of protein binding (by ultrafiltration) of four penicillins*

Antibiotic	Concn (μg/ml)	% Protein bound in[a]:	
		50% serum	100% serum
Penicillin	6.2	29	69
Oxacillin	15	83	93
Methicillin	15	23	50
Nafcillin	15	89	97

[a] In 100% human serum and in 50% serum in dextrose-phosphate broth.

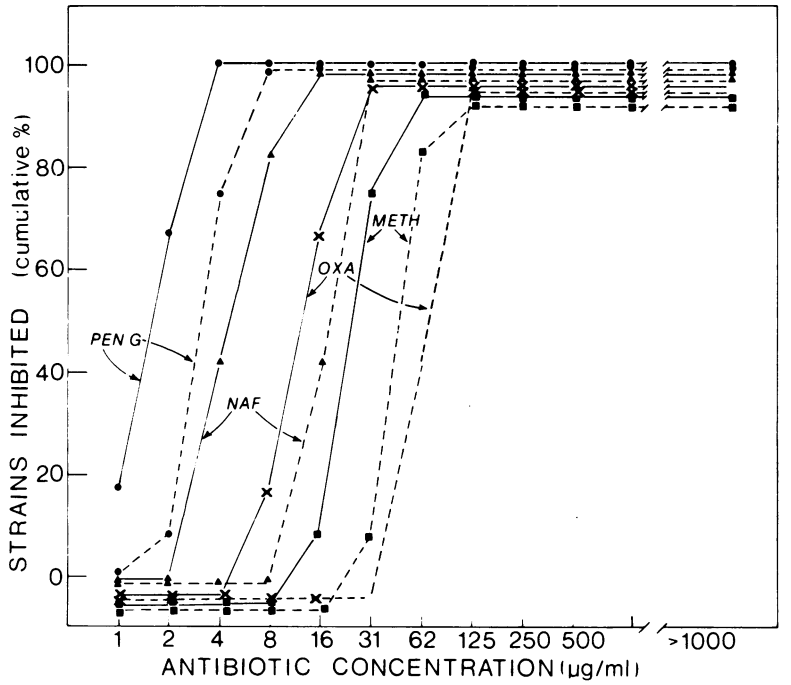

FIG. 1. *Frequency distribution of MICs of various penicillins against 12 strains of enterococci either in dextrose-phosphate broth* (——) *or in 50% serum-broth* (----). *Abbreviations: PEN G, penicillin G; NAF, nafcillin; OXA, oxacillin; METH, methicillin.*

the semisynthetic penicillins; for example, MBCs in serum were ≥1,000 μg/ml for 83% of the strains with oxacillin and 67% with methicillin. In light of the higher MBCs (particularly in serum), end points of bactericidal activity were obtainable with only a few strains using oxacillin or methicillin, so that comparisons between MBCs in broth and serum could not be made with these semisynthetic penicillins. Where bactericidal end points were obtainable (12 strains with penicillin G and 8 strains with nafcillin), the mean increase in MBC in serum as compared with broth was 4-fold (two tubes) with penicillin G and 16-fold (four tubes) with nafcillin.

To evaluate the relative effectiveness in vitro of each of the four penicillins in combination with gentamicin, time-kill curves (Fig. 2) were determined for 10 strains of enterococci (Table 2). Similar to the results in our previous studies (8), killing curves performed in broth demonstrated synergism (greater than 100-fold increased killing by the combination as compared with the more effective of the two antibiotics used alone) against 10 of 10 strains of enterococci tested with penicillin G plus gentamicin and with nafcillin plus gentamicin, but against only 4 of 10 strains with oxacillin plus gentamicin and none of 10 strains with methicillin plus gentamicin.

In 50% serum, results were similar for the penicillins that exhibited relatively little protein binding. That is, in 50% serum, synergism was demonstrated against 10 of 10 strains with penicillin plus gentamicin. Furthermore, there were no statistical differences (using Student's *t* test) between broth and serum determinations in the magnitude of increased killing by penicillin plus gentamicin and by methicillin plus gentamicin against the 10 strains of enterococci. On the other hand, in 50% serum synergism occurred against 2 of 10 strains with nafcillin plus gentamicin and against none of 10 strains with oxacillin plus gentamicin. The differences between synergistic killing in broth and serum were statistically significant for these two combinations ($p < 0.005$ for nafcillin plus gentamicin and $P < 0.05$ for oxacillin plus gentamicin).

DISCUSSION

The various beta-lactam antibiotics are characterized by different degrees of protein binding, and, in many bacterial systems, extensive protein binding of these agents interferes with the biological activity of the drug (4). For many gram-positive cocci, including *S. aureus, Streptococcus pyogenes,* and *Streptococcus pneumoniae,* the antibacterial effect of the penicillins in vitro has been demonstrated to be directly re-

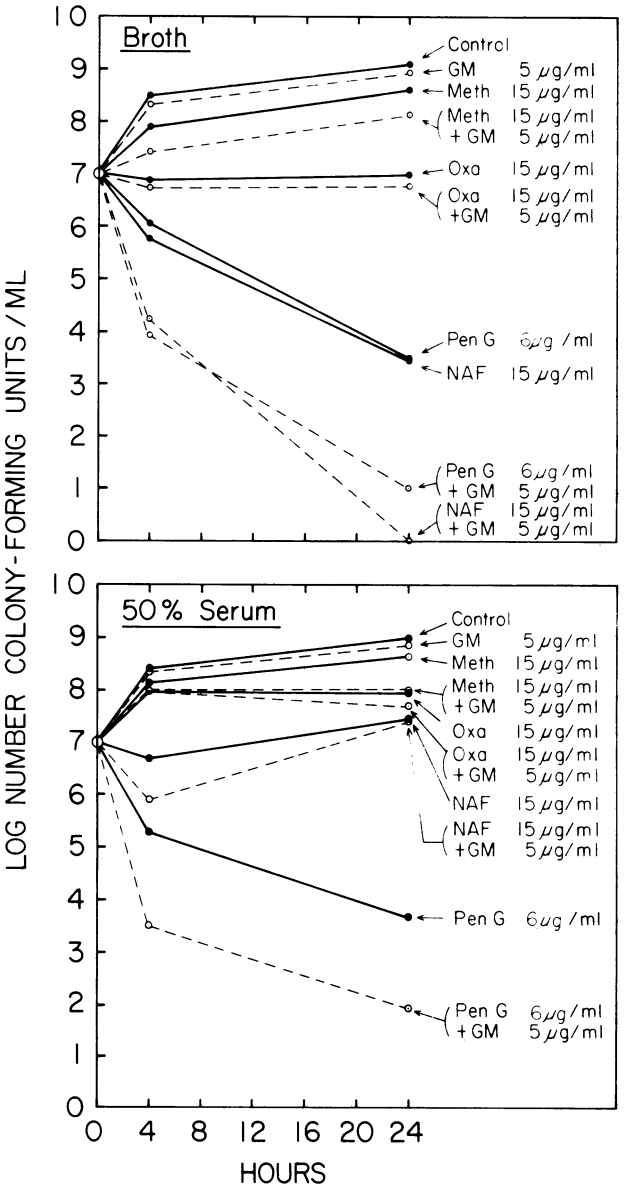

FIG. 2. *Effect of various penicillins alone and in combination with gentamicin against Enterococcus strain 447. Time-kill curves were determined simultaneously in dextrose-phosphate broth (top) and 50% human serum in broth (bottom). Abbreviations: GM, gentamicin; PEN G, penicillin G; NAF, nafcillin; OXA, oxacillin; METH, methicillin.*

lated to the level of free antibiotic in the medium (3, 17, 20). Therefore, the antibacterial effect of the penicillins against these organisms is inhibited in vitro by the presence of serum to an extent that is directly proportional to the degree of protein binding of the respective penicillins (4).

Similarly, an inhibitory effect of serum on the action of four penicillins alone against enterococci was noted. For the highly protein-bound penicillins (nafcillin and oxacillin), MICs were fourfold higher in 50% serum than in broth, whereas MICs for the less highly protein-bound penicillins (penicillin G and methicillin) were only twofold higher in serum than in broth. In addition, the MBCs for penicillin G were 4-fold

TABLE 2. *Effectiveness of four penicillin-gentamicin combinations*[a]

Combination (concn, μg/ml)	No. of strains demonstrating synergism		Mean $\log_{10}$ kill		50% HS vs. DPB	
	DPB	50% HS	DPB	50% HS	*t*	*P*
Penicillin G (6.2) + Gentamicin (5)	10	10	2.6 ± 0.7	2.5 ± 0.8	0.42	>0.60
Nafcillin (15) + Gentamicin (5)	10	2	2.5 ± 1.1	0.9 ± 1.6	3.79	<0.005
Oxacillin (15) + Gentamicin (5)	4	0	1.3 ± 1.4	0.1 ± 0.3	2.57	<0.05
Methicillin (15) + Gentamicin (5)	0	0	0.2 ± 0.4	0.0 ± 0.1	1.38	>0.20

[a] Effectiveness against 10 strains of enterococci in dextrase-phosphate broth (DPB) and 50% human serum in dextrose-phosphate broth (50% HS).

[b] Comparison using Student's *t* test of increased killing in 50% HS and DPB.

higher in serum than in broth, whereas the MBCs for nafcillin were 16-fold higher in serum than in broth.

More importantly, the synergistic bactericidal interaction between the penicillins and gentamicin against enterococci was inhibited in vitro by the presence of serum to a degree proportional to the extent of protein binding of each of the penicillins studied (Fig. 2). Thus, for the two penicillins with relatively little protein binding (in 50% serum, 29% for penicillin and 23% for methicillin) the mean log increased kill was statistically not different in broth and 50% serum. Effectively, this meant that penicillin G plus gentamicin produced synergistic killing against 10 of 10 strains of enterococci in either broth or serum, whereas methicillin plus gentamicin was equally ineffective (none of 10 strains killed) in either medium (see Fig. 2).

With the two highly protein-bound penicillins (in 50% serum, 89% for nafcillin and 83% for oxacillin) there was a significant inhibitory effect of serum on bactericidal synergism between the penicillin derivative and gentamicin. Nafcillin plus gentamicin was synergistically bactericidal against all 10 strains of enterococci in broth and against only 2 strains of serum, and oxacillin plus gentamicin was synergistic against 4 strains in broth and none in serum.

These in vitro studies support results from a recent report by Lincoln and associates on the therapy of experimental enterococcal endocarditis in rabbits (14). Using a relatively susceptible strain of enterococcus (against which in vitro synergistic killing in dextrose-phosphate broth could be demonstrated [using time-kill curves] with gentamicin plus either nafcillin, oxacillin, or methicillin), they noted that 21 days of therapy with high doses of semisynthetic penicillins plus gentamicin resulted in valve sterilization and survival in only 20% of rabbits with nafcillin plus gentamicin, 10% with oxacillin plus gentamicin, and 40% with methicillin plus gentamicin. Although these authors did not measure the binding of the various penicillins to rabbit serum proteins, studies by Barza et al. (1) have shown similar protein binding of methicillin (21%), oxacillin (75%), and nafcillin (74%) as compared with our findings in 50% human serum. Previous studies from that laboratory had demonstrated uniformly excellent therapeutic results in this model system using penicillin G plus gentamicin (23).

In conclusion, our studies as well as the in vivo experiments of Lincoln and associates (14) suggest that the semisynthetic, penicillinase-resistant penicillins are of dubious value in the therapy of enterococcal endocarditis. In part, the inefficacy of the penicillinase-resistant penicillins is evidenced by the higher MICs of these agents as compared with penicillin G, even when determinations are performed in broth (8). Even more important is the inhibitory effect of protein binding in serum, which strikingly raises the MIC of the penicillin and reduces the bactericidal interaction between the highly protein-bound semisynthetic penicillins and gentamicin against enterococci. Furthermore, in our studies, time-kill curves in 50% serum demonstrated synergism against all 10 strains of enterococci with penicillin G plus gentamicin, against 2 of 10 strains with nafcillin plus gentamicin, and against none of 10 with either oxacillin or methicillin plus gentamicin.

Accordingly, semisynthetic, penicillinase-resistant penicillins should not be employed as primary agents in the therapy of patients with known or suspected enterococcal endocarditis. In such patients, penicillin G plus an aminoglycoside should be used as initial treatment.

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