Studies of Cephalothin: Aminoglycoside Synergism Against Enterococci

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Combinations of cephalothin and aminoglycoside antibiotics are not currently used in the therapy of serious enterococcal infections, because clinical trials of these combinations have been unsuccessful. Studies of 28 enterococci isolated from patients with enterococcal bacteremia suggested three possible mechanisms for this in vivo antibiotic failure: (i) a relatively high level of resistance to cephalothin among all enterococci and especially those characterized as *Streptococcus faecium*, (ii) a significant incidence of high-level resistance to the aminoglycosides among certain strains of enterococci, and (iii) a failure of synergism to occur when cephalothin concentrations fall below the minimal inhibitory concentration of the enterococcus, as occurs during the in vivo metabolism and excretion of this antibiotic when given in standard doses for endocarditis.

Serious infections (including endocarditis) caused by the enterococci are difficult to treat because of the unique resistance of enterococci to antibiotics (19). Therapy with single, nontoxic antibiotics, such as penicillin or ampicillin, has resulted in an unacceptably high rate of failure (8). The combination of penicillin with various aminoglycosidic aminocyclitol antibiotics results in a synergistic killing effect against enterococci (13). As a result, current recommendations are that penicillin and an aminoglycoside be used to treat enterococcal endocarditis and other serious infections (8). Penicillin and streptomycin have been most widely used for this purpose. However, recent studies have suggested that other aminoglycosides, including kanamycin and gentamicin, can be substituted for streptomycin and may be effective against more strains (14, 22).

Clinical problems arise in the treatment of enterococcal infections in patients who are allergic to penicillin or who develop severe allergic manifestations during treatment with penicillin and an aminoglycosidic aminocyclitol antibiotic. There is currently no effective substitute for penicillin which does not carry a significant risk of toxicity when used for the prolonged periods of time necessary for the therapy of enterococcal endocarditis. Vancomycin has had limited use in the treatment of enterococcal

endocarditis (5, 23) and appears to be effective, but is potentially oto- and nephrotoxic.

Previous studies have demonstrated that antibiotics which inhibit bacterial cell wall synthesis produce a synergistic effect when combined with aminoglycoside antibiotics against enterococci (13). The cephalosporin antibiotics are included among the agents which have been shown to produce such an effect (4). Because of their low toxicity, the drugs in this group would appear to be ideal agents to substitute for penicillin or ampicillin in patients with enterococcal endocarditis who are allergic to the penicillins. However, the cephalosporins are not currently used to treat enterococcal infections, for trials of cephalothin alone (16) and in combination with streptomycin (20) have not been successful.

The purpose of the present study was to confirm the in vitro synergistic effects of cephalothin and aminoglycosides against enterococci, and to attempt to explain the paradoxical ineffectiveness of such combinations in vivo. Based on these studies, several possible clinical approaches to the solution of this problem are suggested. (This report was presented in part at the Eastern Section Meeting, American Federation for Clinical Research, 11 January 1974, Boston, Mass.)

MATERIALS AND METHODS

Antibiotics used in this study included cephalothin sodium (supplied as Keflin by Eli Lilly and Co.),

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gentamicin sulfate (supplied as garamycin by the Schering Corp.), kanamycin sulfate (supplied as Kantrex by Bristol Laboratories), and streptomycin sulfate (supplied as streptomycin sulfate U.S. Pharmacopeia by E. R. Squibb and Sons). Appropriate antibiotic dilutions as indicated were made in sterile water without preservatives. Desacetylcephalothin (24) was supplied by Eli Lilly and Co.

The 27 strains of enterococci used in this study were isolated from blood cultures submitted to the Bacteriology Laboratory of the Massachusetts General Hospital. One strain was isolated from a blood culture submitted to the Bacteriology Laboratory of the New England Medical Center Hospitals. These isolates represented individual blood stream infections. All organisms were identified as streptococci by standard bacteriological methods. The isolates were then serologically grouped, and detailed physiological identification was made according to the methods of Medrek and Barnes (9).

Cephalosporinase production was assessed by a modification of the method of Mildvan et al. (10). An inoculum of cephalothin-susceptible *Bacillus globigii* (minimal inhibitory concentration [MIC] < 0.1 μg/ml) and cephalothin in a final concentration of 1 μg/ml were incorporated into Trypticase soy agar (BBL). Extracellular cephalosporinase was indicated by the appearance of satellite colonies of the organisms after overnight incubation at 37 C.

Induction of cephalosprinase production by enterococci was attempted by the method of Stroy and Preston (18). An overnight culture of the organism to be induced was diluted 1:100. One milliliter of the diluted culture was then added to 100-cc dextrose-phosphate broth and incubated at 37 C. A subinhibitory concentration of cephalothin (in most cases 10 μg/ml) was added to the culture bottle at 2, 4, 6, 8, 24, 26, 28, 30, and 32 h. At 48 h, the cultures were centrifuged for 20 min at 2,000 rpm. The supernatant fluid was removed and filtered through a membrane filter with a pore size of 0.45 μm (Falcon). The filtrate was tested for cephalosporinase activity by spotting 0.025-ml aliquots on Trypticase soy agar plates containing *B. globigii* and cephalothin.

Studies of possible inactivation of cephalothin by enterococci were carried out by serial determinations of the degradation of microbiologically active cephalothin in the presence and absence of enterococci. Cephalothin concentrations were measured by an agar diffusion method (25) using *Sarcina lutea* ATCC 9341 as the test organism.

Tests of synergism were performed in dextrose-phosphate broth medium (Albimi-Pfizer). A 1-ml aliquot of an overnight culture of the organism to be tested was added to 19 ml of dextrose-phosphate broth, producing a final concentration of approximately 10^7 organisms per ml. Appropriate antibiotics were added in clinically achievable concentrations (cephalothin, 1 to 25 μg/ml; streptomycin, 25 μg/ml; kanamycin, 20 μg/ml; gentamicin, 20 μg/ml) and the cultures were incubated at 37 C. Samples of 0.5 ml were removed at 0.4 and 24 h for determination of viable organisms by a standard serial dilution technique (11) and subsequent subculture on brucella

agar plates with 5% horse blood. Synergism was defined as a 100-fold increase in killing after 24 h of incubation by the combination as compared with the most effective antibiotic (cephalothin or desacetylcephalothin in all instances) used alone.

MIC and minimal bactericidal concentrations (MBC) of cephalothin and desacetylcephalothin against enterococci were determined by the standard broth dilution technique (1). The initial concentration of organisms for these determinations was adjusted to approximately 10^5/ml by appropriate dilution of an overnight broth culture of each strain tested. The MIC was defined as the lowest concentration of antibiotic which prevented growth as evidence by visible turbidity after 24 h of incubation. After the 24-h incubation a loopful of medium from each of the tubes without visible growth was streaked onto a blood agar plate. The lowest concentration of antibiotic which prevented all growth on subculture to solid media was considered to be the MBC.

RESULTS

The MIC and MBC of cephalothin for the 28 enterococci are presented in Fig. 1. Although virtually all strains had MICs which were within the clinically achievable range, four strains were significantly more resistant to cephalothin. These four organisms were the only ones in the group that had been identified as *Streptococcus faecium*; the remaining 24 were *S. faecalis*. The MBCs for all of the organisms were outside the range that can be achieved clinically (21). A subsample of four strains was tested against desacetylcephalothin. The MICs of desacetylcephalothin were all 125 μg/ml, whereas the MICs of cephalothin for these organisms ranged from 8 to 31 μg/ml. The MBCs of desacetylcephalothin were all greater than 500 μg/ml.

All 28 strains were tested for the production of an extracellular cephalosporinase. None of the strains demonstrated the elaboration of this enzyme spontaneously. The induction of cepha-
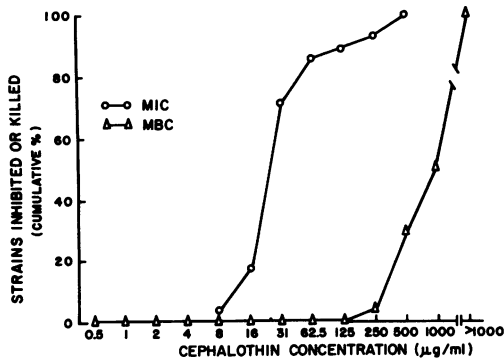

FIG. 1. *MIC and MBC of cephalothin against 28 strains of enterococci.*

losporinase by means of repeated exposure of the organism to subinhibitory concentrations of cephalothin was attempted, and similarly no cephalosprinase production could be detected. However. a strain of *Enterobacter cloacae* (18), known to contain an inducible cephalosporinase, routinely produced the enzyme when exposed to the identical induction method and the same procedure for detecting cephalosporinase activity.

Table 1 shows cephalothin concentrations at various times when incubated in dextrose-phosphate broth at 37 C in the presence and absence of strains of enterococci having a range of MICs for cephalothin from 8 to 32 μg/ml. There was a significant loss of cephalothin activity with time. However, this also occurred in the absence of organisms and there was no evidence that any of the strains tested enhanced the degradation of cephalothin.

Studies of synergism between cephalothin and each of the aminoglycosides (streptomycin, kanamycin, and gentamicin) were carried out. For these studies, cephalothin was used in concentrations equal to the MICs of cephalothin which had previously been determined. The concentrations of the various aminoglycoside antibiotics used were those which had been shown in earlier studies to act synergistically with penicillin against these organisms (12). Synergism between cephalothin and streptomycin was demonstrated with 17 out of 28 strains, between cephalothin and kanamycin with 23 out of 28 strains, and between cephalothin and gentamicin with all 28 strains.

The ability of desacetylcephalothin to act synergistically with aminoglycosides against enterococci was assessed with four strains. One of these strains (L-1) had been isolated from a patient with enterococcal endocarditis who had not responded to cephalothin-aminoglycoside therapy. This strain was synergistically killed in vitro when cephalothin was added to either streptomycin, kanamycin, or gentamicin. The three remaining strains had varying patterns of aminoglycoside susceptibility. As had been noted, the MICs of desacetylcephalothin for all four strains were significantly higher than those of cephalothin. Whenever desacetylcephalothin was substituted in a concentration identical to that of cephalothin which had produced synergism with one or more of the aminoglycosides, synergism failed to occur, regardless of the aminoglycoside susceptibility of the organism. However, when desacetylcephalothin, in a concentration equal to its MIC for the organism, was combined with an appropriate aminoglycoside, synergism could be demonstrated (Fig. 2).

TABLE 1. *Effect of various strains of enterococci on cephalothin concentration*[a]

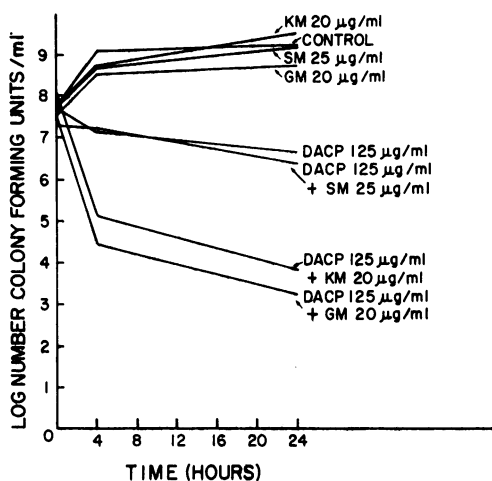| Time (h)	Cephalothin concn (μg/ml)			
	Control (no organisms)	Strain 4927	Strain 7509	Strain 9948
0	10.2	10.4	9.8	9.4
4	9.3	9.5	9.3	8.9
24	6.4	6.5	6.2	6.0

[a] Incubated overnight at 37 C.

FIG. 2. *The effect of desacetylcephalothin and three aminoglycoside antibiotics against a strain of enterococci ribosomally resistant to streptomycin. Abbreviations: DACP, desacetylcephalothin; SM, streptomycin; KM, kanamycin; GM, gentamicin.*

A mixture of one-half cephalothin and one-half desacetylcephalothin whose sum equal the MIC of cephalothin for the organism was studied for its effect, both alone and in combination with streptomycin, kanamycin, and gentamicin, on three of the enterococcal strains. In all situations enhanced killing was noted to occur; however, the amount of bactericidal activity, as depicted for one of the strains in Fig. 3, barely satisified the criteria for synergism.

Strain L-1 was studied in greater detail. The results are presented in Fig. 4. Desacetylcephalothin alone in a concentration of 25 μg/ml was inactive against the organism. The combination of desacetylcephalothin and gentamicin had no significant effect on bacterial growth. Mixtures of cephalothin and desacetylcephalothin totaling 25 μg/ml were combined with gentamicin and examined for evidence of antibiotic synergism. When the mixture contained one-third cephalothin and two-thirds desacetylcephalo-

thin, a synergistic effect was not demonstrated. A mixture of one-half cephalothin and one-half desacetylcephalothin produced an enhancement of bactericidal activity which met the minimal criteria for synergism. When the mixture contained two-thirds cephalothin definite synergism could be demonstrated.

Figure 5 represents the effect of varying concentrations of cephalothin added to gentamicin and studied for antibiotic synergism against strain L-1. Desacetylcephalothin was not included in the mixture. As the quantity of cephalothin was decreased, synergism became progressively less apparent.

DISCUSSION

Previous studies have documented synergism between cephalothin and aminoglycoside antibiotics (4). However, the results of therapy with such combinations have not been satisfac-

tory. It is possible that cephalothin-aminoglycoside combinations are ineffective in vivo because of certain factors which are not yet defined and which are unique to cephalothin (i.e., poor tissue or vegetation penetration, etc.). These could render standard in vitro-in vivo comparisons invalid for this antibiotic. However, such correlations do appear to be useful for penicillin-aminoglycoside combinations (6) against enterococci. Moreover, our studies indicate several potential mechanisms for the failure of cephalothin-aminoglycoside therapy of enterococcal endocarditis.

The MICs of cephalothin for the enterococci that were investigated in this study were all significantly higher than those of a wide-range of cephalothin-susceptible organisms (21). Most, however, with the exception of four strains of S. faecium, were in the range which can be achieved by the high-dose parenteral
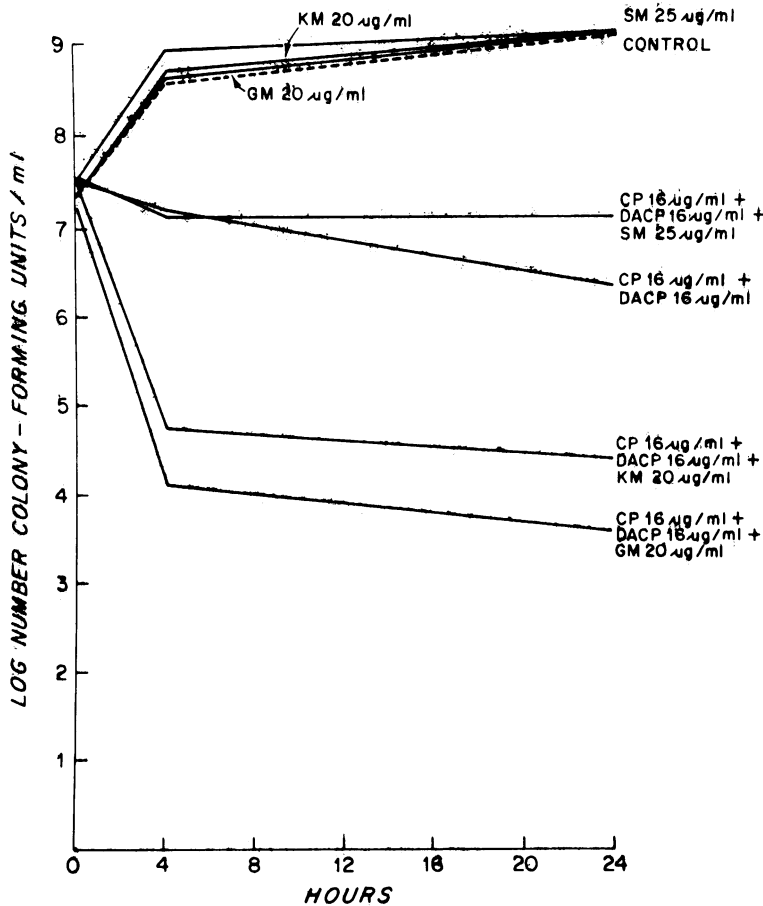

FIG. 3. The effect of a mixture of cephalothin and desacetylcephalothin in combination with three aminoglycosides against an enterococci ribosomally resistant to streptomycin. Abbreviations: CP, cephalothin; DACP, desacetylcephalothin; SM, streptomycin; KM, kanamycin; GM, gentamicin.
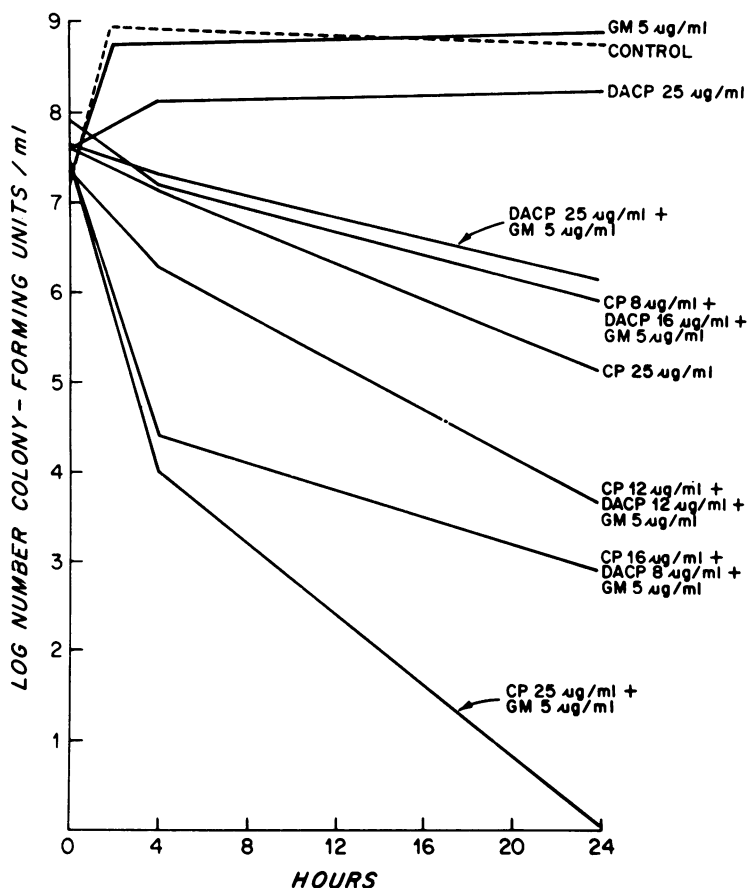
FIG. 4. *The effect of various combinations of cephalothin and desacetylcephalothin in combination with gentamicin against enterococcal strain L-1. Abbreviations: CP, cephalothin; DACP, desacetylcephalothin; GM, gentamicin.*

therapy with cephalothin which would be utilized for the treatment of serious infections such as bacterial endocarditis (21). The extremely high MBCs of cephalothin are consistent with the susceptibilities of enterococci to all other antibiotics which act upon the bacterial cell wall. However, synergism with appropriate aminoglycoside antibiotics occurred whenever cephalothin was used in concentrations equal to or greater than the MIC. For all the strains of *S. faecalis* (which cause the majority of human cases of enterococcal endocarditis [3]) such concentrations fell within clinically achievable ranges. This was not the case with four strains of *S. faecium* tested. Thus, cephalothin resistance per se may explain the in vivo failure of cephalothin-aminoglycoside combinations against *S. faecium*. It does not, however, account for the clinical ineffectiveness of such combinations against the majority of strains of

enterococci. Alternative explanations must be sought for these cases.

It has been suggested that cephalosporinase production by enterococci may be responsible for the clinical ineffectiveness of cephalosporin-aminoglycoside combinations (2). Our in vitro studies consistently failed to demonstrate any significant evidence of diminution of cephalothin activity in the presence of enterococci other than that related to the expected loss of potency of the drug, when it was incubated for prolonged periods at 37 C in culture medium. Similarly, a microbiological assay for cephalosporinase activity gave no indication of any extracellular cephalosporinase spontaneously produced by any of the 28 organisms studied. Attempts to induce the organisms to elaborate cephalosporinase, by using a method which is efficient in stimulating a reference strain of enterobacter to produce the enzyme, were also unsuccessful.

Therefore, clinical ineffectiveness does not appear to be due to enzymatic destruction of cephalothin by enterococci.

Recent studies (12, 17) have demonstrated that the susceptibility of enterococci to a given aminoglycoside is of primary importance in determining whether synergism will occur when that aminoglycoside is combined with an antibiotic which inhibits bacterial cell wall synthesis. Thus, the failure of streptomycin or kanamycin to produce a synergistic effect against enterococci can be correlated with a high level of resistance (>2,000 μg/ml) to the aminoglycoside. All 28 strains studied had previously been investigated for high-level resistance to aminoglycosides. Eleven strains had been shown to be resistant to high levels of streptomycin, five to high levels of kanamycin, and none to high levels of gentamicin. Synergism between penicillin and the aminoglycosides failed to occur in those strains where high-level resistance to the

aminoglycoside was present. In the current study cephalothin was substituted for penicillin. The concentration of cephalothin utilized was equal to the MIC which had previously been determined for the organism. In all instances, the in vitro effect of cephalothin precisely duplicated the earlier experience with penicillin; i.e., the efficacy of synergism was dependent upon the aminoglycoside susceptibility of the organism being studied.

In the present study 11 out of 28 strains of enterococci were resistant to cephalothin-streptomycin synergism. In view of this, it would appear possible that the well-documented failure of cephalothin and streptomycin in the treatment of enterococcal endocarditis may have been due to high-level streptomycin resistance of the organism rather than to any defect in the antibacterial effectiveness of cephalothin. The isolation of an enterococcal strain (L-1) from a patient who had persistent bacteremia
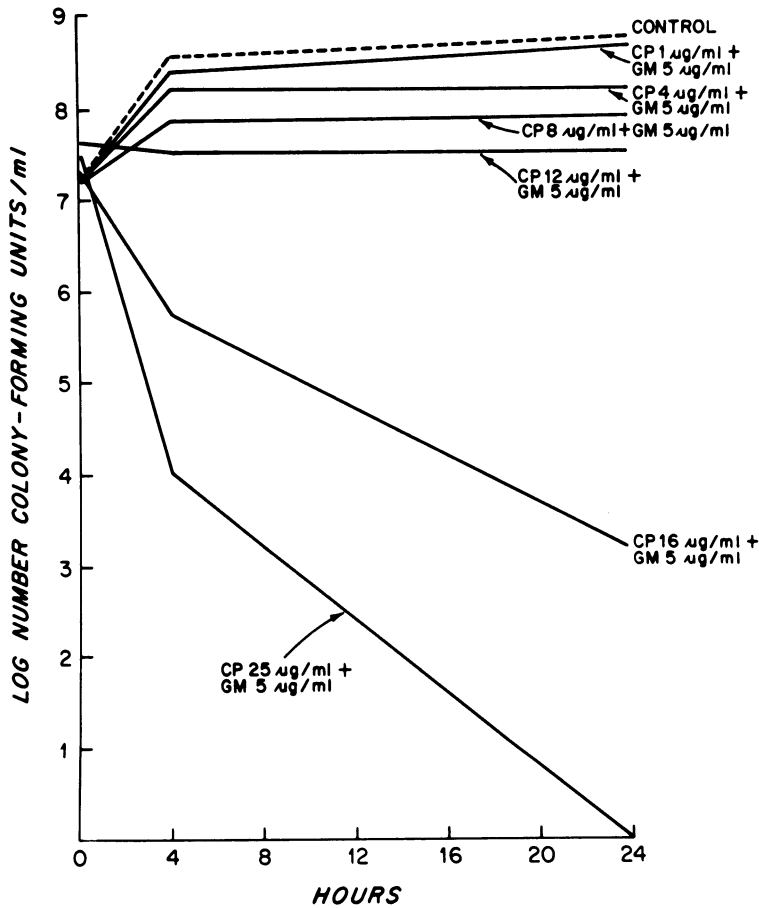

FIG. 5. *The effect of varying amounts of cephalothin in combination with gentamicin against enterococcal strain L-1. Abbreviations: CP, cephalothin; GM, gentamicin.*

despite adequate doses of an apparently effective antibiotic combination suggested, however, that aminoglycoside resistance was not sufficient to explain the treatment failure. Strain L-1 was determined to be susceptible to high levels of streptomycin, kanamycin, and gentamicin, and combinations of cephalothin with each of these three antibiotics produced in vitro synergism against this organism. Despite therapy with large doses of cephalothin and streptomycin, and subsequently cephalothin and gentamicin over many days, the patient's enterococcal bacteremia persisted. That this represented primary failure of cephalothin-streptomycin and cephalothin-gentamicin therapy (and was not due to the presence of an abscess or other sequestered focus) was shown by the subsequent cure of the patient with alternative antibiotic therapy (L. Weinstein and A. J. Weinstein, unpublished data).

In a further effort to understand the paradoxical in vivo ineffectiveness of such combinations of antimicrobial agents, we have attempted to simulate in vitro the normal metabolic fate and excretion of cephalothin to ascertain whether these factors may help to explain its failure to eradicate enterococcal infections.

Cephalothin is partially converted, by cleavage of the acetyl ester at the 3 position of the cephalosporin nucleus (24), to desacetylcephalothin by esterases in the liver and various other organs (7). The antibacterial activity of desacetylcephalothin is only one-half to one-sixteenth that of cephalothin (24). The rate of conversion of cephalothin to desacetylcephalothin varies in different individuals. Between one-third and 90% of each dose of cephalothin has been noted to be converted to the less active form (7).

Studies of strain L-1 and three other enterococci demonstrated that cephalothin was at least four times more active than desacetylcephalothin against the organisms. Neither agent when used alone produced a bactericidal effect. However, when cephalothin and an appropriate aminoglycoside were combined, the organisms were killed. Similar amounts of desacetylcephalothin in combination with the aminoglycoside resulted in less bactericidal effect than cephalothin alone (Fig. 4). As the concentration of cephalothin was decreased and that of desacetylcephalothin increased there was a progressive decrease in synergistic activity. Concentrations of cephalothin lower than 50% of the amount necessary to inhibit the organism failed to produce synergism with the aminoglycoside. When concentrations of desacetylcephalothin equal to its MIC for a given

organism were combined with an effective aminoglycoside, synergism occurred. However, concentrations of desacetylcephalothin this high would not be achieved in vivo.

These data suggest that desacetylcephalothin does not inhibit the effect of cephalothin, but that as cephalothin is normally metabolized to a compound with significantly less antibacterial activity, the serum concentration of the more active parent compound falls rapidly to a level inadequate to produce synergism.

Cephalothin, when administered parenterally, is also rapidly excreted in the urine in patients with normal renal function (21). This rapid excretion of the antibiotic, resulting in a lowering of serum levels below those required for effective synergism, may also contribute to the ineffectiveness of cephalothin against the enterococcus. Thus, the normal metabolic fate and normal renal excretion of cephalothin, both of which contribute to a rapid decline in serum cephalothin levels below those needed for antibiotic synergism against enterococci, may explain its paradoxical ineffectiveness in the therapy of enterococcal endocarditis.

Our current studies, however, suggest several potential solutions to the problem. It is possible that the use of larger or more closely spaced doses of cephalothin (perhaps in combination with probenecid) may result in more sustained or higher levels of the biologically more active form of the drug, thus assuring synergism with an effective aminoglycoside antibiotic. Alternatively, the use of cephalosporins which do not contain the acetyl ester and, thus, are not metabolized to desacetylcephalothin, might also be expected to yield more effective synergism against enterococci, both in vitro and in vivo. Such agents include cephaloridine, cephalexin, and cefazolin (15). Further studies of these possibilities both in vitro and in vivo (especially in animal models) are necessary before they can be recommended for clinical use.

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