

Synergy of Penicillin-Netilmicin Combinations Against Enterococci Including Strains Highly Resistant to Streptomycin or Kanamycin

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The *in vitro* activity of combinations of penicillin and netilmicin was determined against 20 clinical isolates of enterococci and compared with that obtained in simultaneous tests with penicillin/sisomicin, penicillin/streptomycin, and penicillin/kanamycin. Synergy between the two drugs in each combination was determined by the use of quantitative kill curves and was defined as a killing by the combination at least 100-fold greater than that produced by the most effective drug alone. Penicillin/netilmicin and penicillin/sisomicin combinations were found to be synergistic against the majority of isolates tested, including strains resistant to penicillin/streptomycin or penicillin/kanamycin combinations. This synergy with penicillin could be demonstrated at a concentration of ≤ 7 $\mu\text{g/ml}$ for either netilmicin or sisomicin. Studies on the kinetics of killing produced by these combinations showed the rate and extent of killing to be directly dependent upon the organism's relative susceptibility to the aminoglycoside alone and the aminoglycoside concentration in the combination. Results also indicated that the interaction between penicillin and netilmicin was true synergy; i.e., rapid and complete killing was produced by combinations containing each drug at concentrations insufficient to produce any killing alone, and the killing observed could not be produced by either drug alone at a concentration equivalent to the total drug concentration in the combination. The potential clinical application of this synergistic interaction should be investigated further, especially in view of recent reports showing netilmicin to be considerably less toxic than gentamicin in experimental animals.

Since the first report by Hunter in 1947 of the successful use of penicillin plus streptomycin in treatment of enterococcal endocarditis (4), combination therapy with a penicillin and an aminoglycoside has been the recommended treatment for enterococcal infections in man (7, 11, 12, 19). The clinical efficacy of such combinations has been shown to be due to a true synergistic interaction between the two drugs, which can be readily demonstrated *in vitro* (2, 3, 15, 19, 24). After the discovery that combinations of penicillin plus streptomycin or kanamycin would not act synergistically *in vitro* against strains highly resistant to the aminoglycoside (14, 15, 21, 26), numerous synergy studies have been performed with combinations of penicillins and newer aminoglycosides (5, 14, 16, 17, 22, 25). To date, only combinations of penicillin plus gentamicin have been shown to be synergistic *in vitro* and effective therapy against enterococci highly resistant to both streptomycin and kanamycin (14, 16, 22, 23). Previous studies in this laboratory with sisomicin, a new amino-

glycoside most closely related to gentamicin C_{1a} (18), indicated that sisomicin was more active than gentamicin, tobramycin, amikacin, or kanamycin when tested alone against enterococci and may act synergistically with penicillin against these organisms (1, 20). Since recent studies have shown netilmicin, a sisomicin derivative, to be considerably less toxic than gentamicin in animals (10, 13) and more active than sisomicin when tested alone against enterococci (C. Sanders, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 16th, Chicago, Ill., Abstr. no. 408, 1976), the purpose of the current study was to evaluate the activity of this derivative in combination with penicillin against enterococci. For comparative purposes, the activity of combinations containing sisomicin, kanamycin, or streptomycin was evaluated simultaneously.

MATERIALS AND METHODS

Bacterial strains. All organisms used in this study were clinical isolates and were identified as

enterococci based upon the ability to ferment mannitol and to grow in 6.5% NaCl broth and on sodium azide-methylene blue agar. All belonged to Lancefield group D as determined in precipitation tests with specific antisera (9). Strains were designated highly streptomycin resistant if they were capable of growing in broth containing 2,000 μg of the drug per ml (14, 21). All other strains were designated streptomycin susceptible.

Antibiotics. Solutions of the following drugs were prepared (weight corrected for potency) on the day of use: potassium penicillin G (E. R. Squibb and Sons, Inc.), streptomycin sulfate (Eli Lilly and Co.), kanamycin sulfate (Bristol Laboratories), and sisomicin sulfate and netilmicin sulfate (Schering Corp.). Combinations of penicillin and aminoglycoside were prepared immediately before testing.

Tests for synergy. Mueller-Hinton broth (BBL), containing each drug alone or a combination of penicillin and an aminoglycoside, was inoculated with cells (10^8 colony-forming units [CFU]/ml) from an overnight Mueller-Hinton broth culture and incubated at 37°C in air. At 6 and 24 h, a sample was removed and the number of CFU per milliliter was determined by plate counts in brain heart infusion agar (Difco). Penicillinase (BBL) was added to each sample containing penicillin before counting. All results shown are averages of duplicate determinations. The concentration of each aminoglycoside used was below the minimal bactericidal concentration (MBC, determined by the standard dilution assay with an inoculum of 10^5 CFU/ml) for each strain. This was (i) 25 $\mu\text{g}/\text{ml}$ for streptomycin with all but highly resistant strains, for which 2,000 $\mu\text{g}/\text{ml}$ was used, (ii) 25 $\mu\text{g}/\text{ml}$ for kanamycin, and (iii) 3 to 7 $\mu\text{g}/\text{ml}$ for sisomicin and netilmicin. Penicillin G was used in a concentration of 3.1 $\mu\text{g}/\text{ml}$ (1 μg = 1.6 U), as preliminary tests had shown this to be the highest concentration with no bactericidal activity against any of the strains.

Interpretation of synergy. Since much controversy exists over criteria for establishing *in vitro* synergy between antibiotics, synergy was defined at two levels. The first and widely used definition (designated hereafter as relative synergy) was a decrease of 100-fold or more in the number of viable bacteria caused by the combination as compared with the most effective of the drugs when tested alone (14, 21). The second and more stringent definition (designated hereafter as complete synergy) was a complete killing of the inoculum (<10 CFU/ml) by the combination with no killing occurring by either drug tested alone. Data obtained after a 24-h incubation were used to determine synergy.

Kinetics of killing by penicillin/aminoglycoside combinations. The synergistic interaction between netilmicin and penicillin was evaluated further by determining the rate of killing by varying concentrations of netilmicin alone and in combination with penicillin. These tests were performed as described above, except that portions for counting were removed at more frequent time intervals. Sisomicin, streptomycin, and kanamycin were evaluated similarly for comparative purposes. The isolate used in these studies (strain 49) was a streptomycin-resist-

ant strain that was also moderately resistant to kanamycin (MBC = 50 $\mu\text{g}/\text{ml}$).

RESULTS

Streptomycin-susceptible stains. Among the 20 strains evaluated, 14 were inhibited by 2,000 μg of streptomycin per ml and thus were designated streptomycin susceptible. Results of synergy studies with these strains are shown in Table 1. Streptomycin, kanamycin, and sisomicin in combination with penicillin demonstrated relative synergy against each of these strains. The one strain against which relative synergy could not be demonstrated for penicillin/netilmicin was highly susceptible to netilmicin alone. Although relative synergy could not be demonstrated with penicillin plus 3 μg of netilmicin per ml, netilmicin alone at 5 $\mu\text{g}/\text{ml}$ was completely bactericidal for this strain. Complete synergy could be demonstrated in a higher percentage of tests with penicillin/kanamycin than with any other combination (Table 1). The percentage of tests demonstrating complete synergy was 91, 75, 55, and 50 when the aminoglycoside in the combination was kanamycin, sisomicin, netilmicin, and streptomycin, respectively.

Highly streptomycin-resistant strains. Results of synergy studies with six highly streptomycin-resistant strains are shown in Table 2. Combinations of penicillin/netilmicin demonstrated relative synergy against all six strains and complete synergy against four of the five strains for which netilmicin alone was not bactericidal. Combinations of penicillin/sisomicin were only slightly less active. As before, the single strain against which relative synergy could not be demonstrated with penicillin/sisomicin was highly susceptible to sisomicin alone. Although relative synergy could

TABLE 1. Synergy between penicillin (3.1 $\mu\text{g}/\text{ml}$) and four aminoglycosides against 14 streptomycin-susceptible enterococci

Aminoglycoside in combination ($\mu\text{g}/\text{ml}$)	No. of tests showing	
	Relative synergy ^a	Complete synergy ^b
Netilmicin (≤ 7)	13	6 (11) ^c
Sisomicin (≤ 7)	14	9 (12)
Kanamycin (25)	14	10 (11)
Streptomycin (25)	14	6 (12)

^a Increased kill by combination at least 100-fold over most effective drug alone.

^b Complete kill by combination; no kill by either drug alone.

^c Number in parentheses indicates number of strains for which neither drug alone was bactericidal.

TABLE 2. Synergy between penicillin (3.1 $\mu\text{g/ml}$) and four aminoglycosides against six highly streptomycin-resistant enterococci

Aminoglycoside in combination ($\mu\text{g/ml}$)	No. of tests showing	
	Relative synergy ^a	Complete synergy ^b
Netilmicin (≤ 7)	6	4 (5) ^c
Sisomicin (≤ 7)	5	3 (4)
Kanamycin (25)	4	2 (4)
Streptomycin (2,000)	4	0 (6)

^a Increased kill by combination at least 100-fold over most effective drug alone.

^b Complete kill by combination; no kill by either drug alone.

^c Number in parentheses indicates number of strains for which neither drug alone was bactericidal.

not be demonstrated with penicillin plus 3 μg of sisomicin per ml, sisomicin alone at 5 $\mu\text{g/ml}$ was completely bactericidal for this strain. No synergy could be demonstrated between penicillin/kanamycin against two strains. Both strains were resistant to kanamycin (MBCs, 50 and $>200 \mu\text{g/ml}$). Combinations of penicillin plus netilmicin or sisomicin (7 $\mu\text{g/ml}$) demonstrated complete synergy against these strains. As expected, complete synergy could not be demonstrated for penicillin/streptomycin (2,000

$\mu\text{g/ml}$) against any of the six strains, and relative synergy could be demonstrated only when this very high streptomycin concentration was employed.

Kinetics of killing by penicillin/aminoglycoside combinations. A series of tests were performed with strain 49 to determine the rate of killing by (i) increasing concentrations of netilmicin or penicillin alone and (ii) penicillin/netilmicin combinations in which the concentration of one drug remained constant while the other was varied. Sisomicin, streptomycin, and kanamycin were evaluated similarly. The rate of killing by each drug alone is shown in Fig. 1. Penicillin at 6.3 $\mu\text{g/ml}$ produced a slow bactericidal effect that was slightly decreased when higher concentrations were used. Complete killing ($<10 \text{ CFU/ml}$) of the inoculum did not occur in any tests (Fig. 1A). Results for streptomycin are not shown, as growth was equivalent to drug-free controls in all tests containing streptomycin up to and including 10,000 $\mu\text{g/ml}$. With the other three aminoglycosides, the rate of killing gradually increased as the concentration of each drug increased (Fig. 1B-D). Complete killing of the inoculum at 24 h was achieved with 250 μg of kanamycin per ml, and 20 μg of sisomicin or netilmicin per ml.

To examine the influence of the aminoglycoside concentration on the rate of killing by peni-

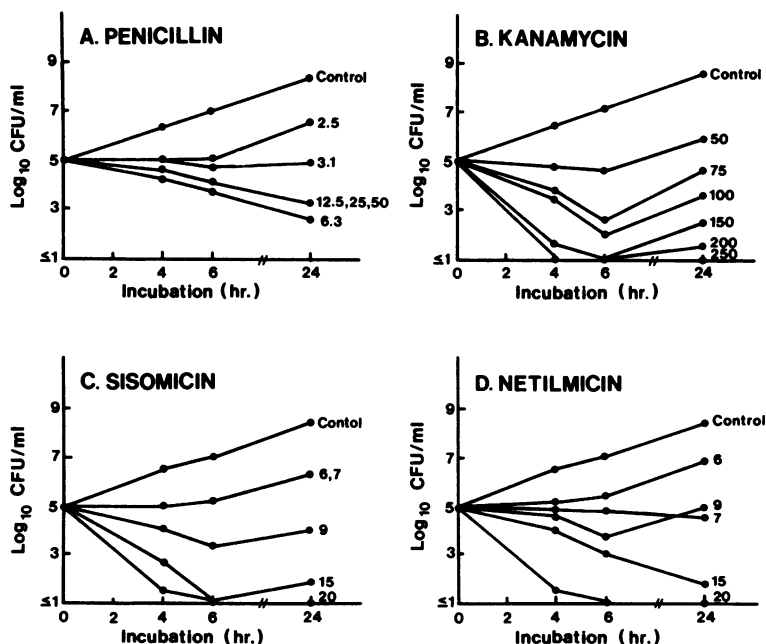


FIG. 1. Rate of killing of strain 49 by increasing concentrations of penicillin, kanamycin, sisomicin, or netilmicin. Numbers at the end of each line indicate the concentration of the drug in micrograms per milliliter.

cillin/netilmicin combinations, tests were performed with combinations containing a single concentration of penicillin ($3.1 \mu\text{g/ml}$) and varying concentrations of netilmicin. To facilitate interpretation of results, the netilmicin concentration in the combinations was below the bactericidal level when tested alone. As shown in Fig. 2A, combinations of penicillin plus $3 \mu\text{g}$ of netilmicin per ml exerted no bactericidal effect, and results were similar to those obtained with the penicillin alone (Fig. 1A). Increasing the netilmicin concentration in the combination to $5 \mu\text{g/ml}$ resulted in a marked increased rate of killing, which was increased even further when $7 \mu\text{g/ml}$ was employed in the combination. Similar results were observed with penicillin/sisomicin combinations (Fig. 2B). Penicillin plus $12 \mu\text{g}$ of kanamycin per ml was less effective than the penicillin alone (Fig. 1A), suggesting a slight antagonism between the two drugs (Fig. 2C). The number of CFU per milliliter did not change over 24 h with $3.1 \mu\text{g}$ of penicillin per ml alone, whereas it increased 1.6 logs over the same time period with $3.1 \mu\text{g}$ of penicillin per ml plus $12 \mu\text{g}$ of kanamycin per ml. Increasing the kanamycin concentration to $25 \mu\text{g/ml}$ resulted in a very slow bactericidal effect. Penicillin plus $50 \mu\text{g}$ of kanamycin per ml was rapidly bactericidal. As shown in Fig. 2D, combinations of penicillin

plus streptomycin showed the same slow rate of killing regardless of the streptomycin concentration employed.

A final series of tests were performed to determine whether the rate of killing observed with the most effective concentration of netilmicin plus $3.1 \mu\text{g}$ of penicillin per ml could be increased by raising the penicillin concentration. Results with either 6.3 or $50 \mu\text{g}$ of penicillin per ml were the same; thus, only results for combinations with $50 \mu\text{g/ml}$ will be presented. As shown in Fig. 2A, the rate of killing by penicillin/netilmicin combinations containing $50 \mu\text{g}$ of penicillin per ml was slightly slower than that observed for combinations containing only $3.1 \mu\text{g}$ of penicillin per ml. This same decreased rate of killing was observed with $50 \mu\text{g}$ of penicillin per ml plus sisomicin (Fig. 2B), kanamycin (Fig. 2C), or streptomycin (Fig. 2D).

DISCUSSION

Results of these studies indicated that combinations of penicillin and netilmicin were synergistic in vitro against enterococci, and their activity was similar to that observed with penicillin/sisomicin combinations. With combinations, synergy could be demonstrated with lower concentrations of sisomicin or netilmicin than of streptomycin or kanamycin in tests

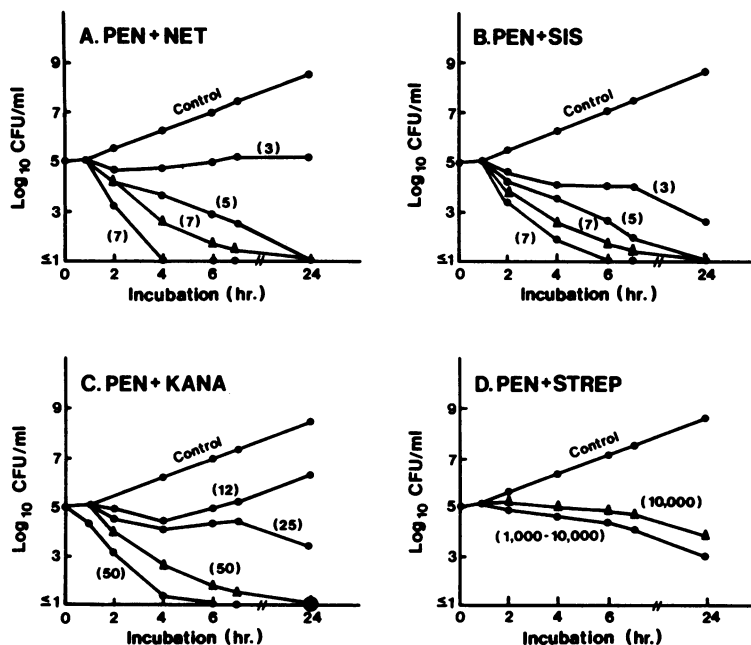


FIG. 2. Rate of killing of strain 49 by penicillin/aminoglycoside combinations. Penicillin concentration in each combination was either $3.1 \mu\text{g/ml}$ (●) or $50 \mu\text{g/ml}$ (▲). Aminoglycoside concentration (micrograms per milliliter) in each combination is indicated by numbers in parentheses.

with streptomycin-susceptible strains. This synergy could also be demonstrated against strains resistant to penicillin/streptomycin or penicillin/kanamycin combinations. Due to the high intrinsic activity of the two newer aminoglycosides alone against some strains, synergy could not be detected in every test with penicillin/sisomicin or penicillin/netilmicin. Although combinations containing low concentrations of the aminoglycoside did not appear synergistic, combinations containing slightly higher concentrations could not be evaluated, as the aminoglycoside alone was completely bactericidal.

Studies on the kinetics of killing produced by penicillin/netilmicin combinations indicated that the interaction between the drugs represented true synergy (6). Rapid and complete killing could be demonstrated by combinations containing each drug at a concentration insufficient to produce any killing alone, and this effect could not be explained by mere summation of the two concentrations employed. These studies also showed the aminoglycoside concentration to be the single most important factor determining the rate of killing, and the early initiation of killing suggested that the aminoglycoside, not penicillin, was responsible for the event lethal to the cell. Results of simultaneous studies with penicillin/streptomycin and penicillin/kanamycin further indicated the importance of the aminoglycoside in the synergistic effect. Although strain 49 was moderately resistant to kanamycin, synergy could be demonstrated with penicillin/kanamycin, with the rate of killing dependent on the aminoglycoside concentration. However, the kanamycin concentration (50 $\mu\text{g/ml}$) required to produce complete killing was above that achievable with safety in man (8). Strain 49 was highly resistant to streptomycin, and the amount of killing produced by penicillin (3.1 $\mu\text{g/ml}$) plus streptomycin (10,000 $\mu\text{g/ml}$) was no greater than that produced by 6.2 μg of penicillin per ml alone. Furthermore, the rate of killing by the combination was very slow and independent of the streptomycin concentration. These findings are consistent with earlier studies on synergy between penicillin-streptomycin or penicillin-kanamycin (2, 3, 15) and suggest a similar interaction between penicillin and netilmicin or sisomicin.

Further studies must be performed to completely assess the potential clinical implications of this synergistic interaction between penicillin and netilmicin. However, it should be noted that netilmicin has been shown to be considerably less toxic than gentamicin in long-term studies in cats (13) and less nephrotoxic than gentamicin in rats (10). Thus, netilmicin, with

its improved therapeutic index over gentamicin and an activity with penicillin against enterococci similar to that reported by others for penicillin/gentamicin (14, 16, 22), may provide a favorable alternative to penicillin/streptomycin or penicillin/kanamycin, which are currently used in combination as therapy for enterococcal infections.

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