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Penicillin and Netilmicin in Treatment of Experimental Enterococcal Endocarditis

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Successful therapy of enterococcal endocarditis requires the use of a combination of penicillin plus an aminoglycoside. The effectiveness of penicillin (Pen), streptomycin (Str), and netilmicin (Net), a new aminoglycoside, alone and in combination, were studied in vitro and in the treatment of left-sided enterococcal endocarditis in rabbits. In vitro Pen+Str or Net resulted in a more rapid and more complete bactericidal effect than Pen, Str, or Net alone against a Str-susceptible strain of enterococcus (strain 1). Against a highly Str-resistant strain (strain 2), Pen+Net showed an advantage over Pen, Str, or Net alone, or Pen+Str. Endocarditis was produced in rabbits with strain 1 or 2, and treatment was initiated 24 h later. Rabbits were treated for 48 h or 5 days with procaine Pen, Pen+Str, or Pen+Net. With strain 1, numbers of enterococci in the vegetations decreased more rapidly with Pen+Str or Pen+Net treatment than with Pen, Str, or Net alone. With strain 2, Pen+Net showed a clear advantage over Pen, Str, Net, or Pen+Str. Net in combination with Pen showed synergistic in vitro activity and was more effective than Pen alone in the treatment of enterococcal endocarditis in rabbits caused by both Str-susceptible and Str-resistant strains.

Penicillin (Pen) and streptomycin (Str) are synergistic in vitro against most enterococci (14, 15), and it is clear that therapy with this combination has given the best results in the treatment of enterococcal endocarditis (2, 5, 7, 12). However, Pen and Str are not synergistic against all enterococci in vitro (9, 14, 15). In fact, in one study, 40% of enterococci isolated from clinical cultures did not demonstrate synergism (14). Enterococci that are highly resistant to Str (i.e., not inhibited by 2,000 μg of Str per ml) have been shown not to demonstrate synergism (9, 14). The combined use of Pen and gentamicin (Gen) has been shown to result in synergism in vitro against essentially all enterococci (including highly Str-resistant strains) (3, 10).

In previous studies (1, 4), the results of treatment of enterococcal endocarditis were investigated in rabbits with endocarditis caused by Str-susceptible or highly Str-resistant strains of enterococci. There was a close correlation between in vitro and in vivo results, with a large advantage of all Pen-aminoglycoside combinations (Pen plus Str, Gen or sisomicin) over Pen alone against endocarditis produced by Str-susceptible strains. In contrast, in endocarditis produced by Str-resistant strains, Pen plus Gen or sisomicin showed a clear advantage over Pen alone or Pen plus Str.

Netilmicin (Net) is a new aminoglycoside with in vitro activity very similar to that of Gen. It demonstrates synergism with Pen against enterococci that are resistant to Str as well as those that are susceptible (13). In several reported studies in animals, Net is less nephrotoxic and ototoxic than Gen (6, 8). The present study was undertaken to evaluate Pen plus Net in the therapy of enterococcal endocarditis in rabbits produced by Str-susceptible and Str-resistant strains of enterococci. Therapy included Pen alone or in combination with Str or Net.

MATERIALS AND METHODS

Organisms. Two previously described clinical isolates of enterococci (1), both *Streptococcus faecalis*, were incubated in heart infusion broth (Difco) at 37°C for 24 h, and 1-ml samples were stored at −20°C. For each experiment, a sample was subcultured into heart infusion broth with sheep blood, incubated at 37°C for 24 h, and diluted in heart infusion broth.

Enterococcus strain 1 was inhibited by 3.1 μg of Pen G per ml, 125 μg of Str per ml, 6.3 μg of Gen per ml, and 12.5 μg of Net per ml. Enterococcus strain 2 was inhibited by 3.1 μg of Pen per ml, 6.3 μg of Gen per ml, and 6.3 μg of Net per ml and was not inhibited by 7,500 μg of Str per ml. All these minimal inhibitory concentrations were tested by an antibiotic dilution method in heart infusion broth with an inoculum of 10^6 enterococci per ml.

Study of synergism in vitro. Flasks were prepared with heart infusion broth containing Pen, Str, Gen, Net, and combinations of Pen+Str, Pen+Gen, or

Pen+Net. The inoculum in the broth was added to each flask and incubated at 37°C. Samples were removed from the flasks at the start of the experiment and periodically during incubation. A portion of penicillinase (Difco; 0.05 ml containing 1,000 U) was added to each milliliter of the sample. Each sample was serially diluted in 10-fold steps in broth, and 0.1 ml of each dilution and 1 ml of undiluted sample were plated on the surfaces of sheep blood agar plates. After incubation at 37°C for 48 h, the numbers of colonies on the plates were counted, and the colony-forming units (CFU) in the flasks were calculated.

Animal experiments. Female white New Zealand rabbits (West Jersey Biological Supply Farm, Wenonah, N.J.) weighing 2.3 to 3.1 kg were anesthetized with intravenous sodium pentobarbital. The right carotid artery was exposed and cannulated with a polyethylene catheter that was introduced into the heart and secured; 3 days later, the animal was injected intravenously with 10^8 CFU of enterococci as previously described (1).

Antimicrobial therapy was started 24 h after infection. Rabbits received intramuscularly either 1,200,000 U of aqueous procaine Pen, Str (15 mg/kg), or Net (2.5 mg/kg) alone at 9 a.m. and 5 p.m. or the same doses of Pen+Str or Pen+Net administered simultaneously at a different site at 9 a.m. and 5 p.m.

After 2 or 5 days of therapy, after a period of at least 12 h without treatment, the rabbits were killed by intravenous injection of sodium pentobarbital. The hearts were removed, and the aortic valve vegetations were excised and weighed. A 1:10 suspension of each vegetation was made in heart infusion broth containing 1,000 U of penicillinase per ml; the vegetations weighed 0.02 to 0.4 g. The suspensions were homogenized in tissue grinders and diluted in 10-fold steps in broth; then 0.1 and 1 ml (if quantities were sufficient) of the suspension and 0.1 ml of each dilution were plated on the surfaces of blood plates. After incubation at 37°C for 48 h, the number of CFU of enterococci per gram of vegetation was calculated and expressed as $\log_{10}$ CFU.

In performing statistical analyses, sterile vegetations were counted as 1 or 2 $\log_{10}$ CFU, depending on whether or not there was enough of the 1:10 suspension of vegetation to plate 1 ml. With large vegetations (over 0.1 g), there was at least 1 ml of suspension containing 0.1 g of vegetation to plate, and a sterile culture indicated 9 or fewer CFU/g (counted as 1 $\log_{10}$ CFU/g). With small vegetations (under 0.1 g), only 0.1 ml of suspension containing 0.01 g of vegetation could be plated, and a sterile culture indicated 99 or fewer CFU/g (counted as 2 $\log_{10}$ CFU/g). All probability values were calculated by the *t* test.

Assays for antibiotic levels in serum were accomplished after taking blood from the ear veins and removing serum after clot formation. Concentrations of Pen in rabbit serum were assayed by an agar diffusion method with paper disks (11). Concentrations of Str or Net in serum were assayed by the same method after the addition of 1,000 U of penicillinase per ml of serum.

RESULTS

In vitro studies. The rate of decrease in numbers of enterococci was evaluated in broth containing Pen, Gen, Str, and Net alone and combinations of Pen plus Str, Gen, or Net. Figures 1 and 2 show the results of typical experiments with strains 1 and 2 with inocula of 8.3 $\log_{10}$ CFU/ml.

The use of 10 μg of Pen per ml resulted in a decrease in CFU of strain 1 to 5.2 $\log_{10}$ CFU/ml after 72 h of incubation. The combinations of 10 μg of Pen per ml plus 12.5 μg of Str per ml or 5 μg of Gen or Net per ml produced a much more striking decrease in CFU. Cultures were sterile by 24 h with Pen plus Str and by 48 h with Pen plus Gen or Net. Str in a concentration of 12.5

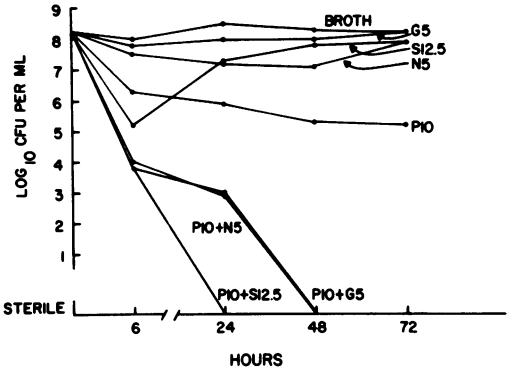

FIG. 1. *Decrease in numbers of Str-susceptible enterococci (strain 1) incubated in broths containing various drugs. Symbols (drug content of broth): P10, Pen G (10 μg/ml); P10+S12.5, Pen (10 μg/ml) plus Str (12.5 μg/ml); P10+G5, Pen (10 μg/ml) plus Gen (5 μg/ml); P10+N5, Pen (10 μg/ml) plus Net (5 μg/ml); S12.5, Str (12.5 μg/ml); G5, Gen (5 μg/ml); N5, Net (5 μg/ml); broth, no drug added.*

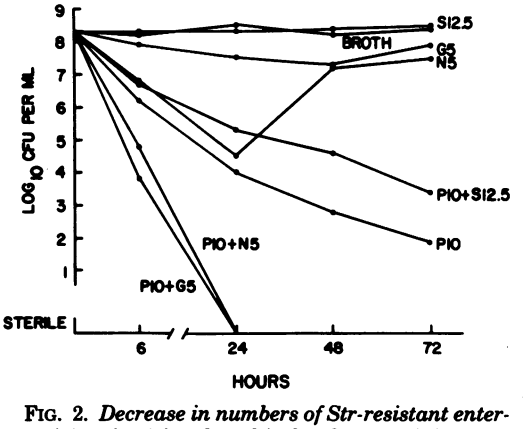

FIG. 2. *Decrease in numbers of Str-resistant enterococci (strain 2) incubated in broths containing various drugs. Symbols (drug content of broth): P10, Pen G (10 μg/ml); P10+S12.5, Pen (10 μg/ml) plus Str (12.5 μg/ml); P10+G5, Pen (10 μg/ml) plus Gen (5 μg/ml); P10+N5, Pen (10 μg/ml) plus Net (5 μg/ml); S12.5, Str (12.5 μg/ml); G5, Gen (5 μg/ml); N5, Net (5 μg/ml); broth, no drug added.*

μg/ml and Gen and Net in concentrations of 5 μg/ml resulted in either no decrease in titers or only a transient decrease. Results were similar with strain 2, except that the combination of Pen with Str was no more effective in reducing the number of enterococci than Pen alone.

Animal experiments. Serum levels of Pen determined 1 h after the first injection were 10.5 to 49.3 μg/ml (mean ± standard deviation, 31.5 ± 11.4). Serum levels of Str 1 h after the first injection were 27 to 31 μg/ml (28.9 ± 1.5). Serum levels of Net at 1 h were 4.5 to 7.4 μg/ml (5.7 ± 1.1).

Table 1 shows the results of experiments in which therapy was started 24 h after infection. With strain 1, the numbers of enterococci decreased more rapidly in rabbits treated with Pen+Str or Pen+Net than in rabbits receiving Pen alone. After 48 h of treatment, the mean $\log_{10}$ CFU/g of vegetation was lower in the Pen+Str- and the Pen+Net-treated groups than in the Pen or untreated control groups ($P < 0.05$ by t test for Pen versus Pen+Str or Pen+Net; $P < 0.01$ for controls versus Pen+Str and Pen+Net; and $P > 0.05$ for Pen+Str versus Pen+Net and controls versus Pen). After 5 days of treatment, titers in animals treated with Pen+Str and Pen+Net were lower than in animals treated with Pen alone ($P < 0.05$ for Pen versus Pen+Str and Pen+Str versus Pen+Net, but $P > 0.05$ for Pen versus Pen+Net). Titers in rabbits treated with Pen, Pen+Str, or Pen+Net were lower than in controls or Str- or Net-treated rabbits ($P < 0.01$ for all comparisons). Net and Str treatment did not affect titers as compared with controls ($P > 0.05$).

TABLE 1. *Number of enterococci per gram of vegetation*[a]

Treatment group	$\log_{10}$ CFU/g (±SD)	
	2[b]	5[b]
Strain 1		
Untreated controls	7.7 ± 0.5	8.7 ± 0.4
Pen	7.0 ± 0.8	3.3 ± 1.8
Pen+Str	5.8 ± 1.3	2.0 ± 0.6
Pen+Net	6.0 ± 1.0	2.9 ± 1.2
Str		8.5 ± 0.7
Net		7.8 ± 1.2
Strain 2		
Untreated controls	8.5 ± 0.5	9.1 ± 0.2
Pen	5.1 ± 1.0	2.1 ± 1.3
Pen+Str	4.6 ± 1.4	2.6 ± 1.5
Pen+Net	3.3 ± 1.0	1.2 ± 0.6
Str		8.5 ± 0.8
Net		5.3 ± 0.5

[a] Only one vegetation from each rabbit was studied. SD, Standard deviation.

[b] Days of therapy.

With strain 1, no vegetations (eight to nine per group) were sterile after 2 days of treatment. After 5 days of treatment, 2 of 14 were sterile with Pen treatment, 3 of 12 were sterile with Pen+Str, and 3 of 15 were sterile with Pen+Net. There were no differences by chi-square analysis ($P > 0.05$) among the groups just mentioned, rabbits treated with Net or Str (no vegetations sterile), and untreated controls (no vegetations sterile).

After 2 days of treatment, in rabbits infected with strain 2, the mean $\log_{10}$ CFU/g of vegetation was lower in the Pen+Str- and the Pen+Net-treated groups than in the Pen or untreated control groups ($P < 0.01$ for the comparison of Pen versus Pen+Net; $P > 0.05$ for Pen+Str versus Pen and for Pen+Str versus Pen+Net; and $P < 0.01$ for controls versus Pen, Pen+Str, or Pen+Net). After 5 days of treatment, rabbits treated with Pen+Net had lower numbers of enterococci in the vegetations than Pen or Pen+Str animals ($P < 0.05$ for Pen+Net versus Pen; $P < 0.01$ for Pen+Net versus Pen+Str; and $P > 0.05$ for Pen+Str versus Pen). Titers in rabbits treated with Pen, Pen+Str, or Pen+Net were lower than in untreated, Str-treated, or Net-treated animals ($P < 0.01$ for all comparisons). Str treatment did not affect titers ($P > 0.05$) as compared with controls, but Net treatment significantly reduced titers ($P < 0.01$).

With strain 2 after 2 days of treatment, one vegetation of eight was sterile in rabbits treated with Pen+Net, but no vegetations were sterile in the other groups (eight to nine per group). After 5 days of treatment, 6 of 11 Pen animals, 4 of 11 Pen+Str animals, and 11 of 12 Pen+Net animals had sterile vegetations. By chi-square analysis, $P < 0.05$ for Pen+Str versus Pen+Net; $P < 0.01$ for Pen+Net versus untreated, Net-treated, and Str-treated rabbits; and $P > 0.05$ for all comparisons between Pen, Pen+Str, controls (no vegetations sterile), and Str- or Net-treated animals (no vegetations sterile).

DISCUSSION

In these studies, peak serum levels of Pen, Str, and Net in rabbits were comparable to levels achieved in patients receiving these antibiotics (16, 17). In vitro studies using these or lower concentrations of antibiotics demonstrated synergism between Pen and Net against both the Str-susceptible and Str-resistant strains of enterococci studied, and the activity was equivalent to that of the Pen-Gen combination. Synergism with Pen and Str was demonstrated only against the Str-susceptible strain. Sanders (13) recently reported that Pen+Net was synergistic against virtually all 20 enterococci studied, including 6 that were highly resistant to Str.

In the present studies, there was also an advantage of Pen-aminoglycoside combinations over Pen alone in the animal model. Against the Str-susceptible strain, both Pen+Str and Pen+Net were more active than Pen alone. In contrast, against the Str-resistant strain, Pen+Net showed a clear advantage over Pen+Str, with no advantage of the Str combination over Pen alone.

Therefore, there was a good correlation between the results of in vitro studies and the results of the treatment of endocarditis in rabbits. Both in vivo and in vitro, Net showed synergistic activity with Pen against Str-susceptible and Str-resistant strains of enterococci.

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LITERATURE CITED

1. **Carrizosa, J., and D. Kaye.** 1976. Antibiotic synergism in enterococcal endocarditis. J. Lab. Clin. Med. **88**:132-141.
2. **Geraci, J. E.** 1958. The antibiotic therapy of bacterial endocarditis. Med. Clin. North Am. **42**:1101-1140.
3. **Harwick, H. J., G. M. Kalmanson, and L. B. Guze.** 1973. In vitro activity of ampicillin or vancomycin combined with gentamicin or streptomycin against enterococci. Antimicrob. Agents Chemother. **4**:383-387.
4. **Hook, E. W., III, R. B. Roberts, and M. A. Sande.** 1975. Antimicrobial therapy of experimental enterococcal endocarditis. Antimicrob. Agents Chemother. **8**:564-570.
5. **Koenig, M. G., and D. Kaye.** 1961. Enterococcal endocarditis. N. Engl. J. Med. **264**:257-264.
6. **Luft, F. C., M. N. Yum, and S. A. Kleit.** 1976. Comparative nephrotoxicities of netilmicin and gentamicin in rats. Antimicrob. Agents Chemother. **10**:845-849.
7. **Mandell, G. L., D. Kaye, M. E. Levison, and E. W. Hook.** 1970. Enterococcal endocarditis. Arch. Intern. Med. **125**:258-264.
8. **Miller, G. H., G. Arcieri, M. J. Weinstein, and J. A. Waitz.** 1976. Biological activity of netilmicin, a broad-spectrum semisynthetic aminoglycoside antibiotic. Antimicrob. Agents Chemother. **10**:827-836.
9. **Moellering, R. C., Jr., C. Wennersten, T. Medrek, and A. N. Weinberg.** 1971. Prevalence of high-level resistance to aminoglycosides in clinical isolates of enterococci, p. 335-340. Antimicrob. Agents Chemother. 1970.
10. **Moellering, R. C., C. Wennersten, and A. N. Weinberg.** 1971. Synergy of penicillin and gentamicin against enterococci. J. Infect. Dis. **124**:S207-S209.
11. **Ries, K., M. E. Levison, and D. Kaye.** 1973. Clinical and in vitro evaluation of cefazolin, a new cephalosporin antibiotic. Antimicrob. Agents Chemother. **3**:168-174.
12. **Robbins, W. C., and R. Tompsett.** 1951. Treatment of enterococcal endocarditis and bacteremia. Am. J. Med. **10**:278-299.
13. **Sanders, C. C.** 1977. Synergy of penicillin-netilmicin combinations against enterococci including strains highly resistant to streptomycin or kanamycin. Antimicrob. Agents Chemother. **12**:195-200.
14. **Standiford, H. D., J. B. deMaine, and W. M. M. Kirby.** 1970. Antibiotic synergism of enterococci. Arch. Intern. Med. **126**:255-259.
15. **Watanakunakorn, C.** 1971. Penicillin combined with gentamicin or streptomycin. Synergism against enterococci. J. Infect. Dis. **124**:581-586.
16. **Weinstein, L.** 1970. Antibiotics. II. Streptomycin. *In* L. S. Goodman and A. Gilman (ed.), The pharmacological basis of therapeutics, 4th ed., p. 1242-1252. The Macmillan Co., New York.
17. **Welling, P. G., A. Baumueller, C. C. Lau, and P. O. Madsen.** 1977. Netilmicin pharmacokinetics after single intravenous doses to elderly male patients. Antimicrob. Agents Chemother. **12**:328-334.