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Synergistic Activity of Ceftriaxone Combined with Netilmicin Administered Once Daily for Treatment of Experimental Streptococcal Endocarditis

PATRICK B. FRANCIOLI* AND MICHEL P. GLAUSER

Division of Infectious Diseases, Department of Internal Medicine, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland

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We have conducted experiments to determine if one daily injection of netilmicin (NET) would be synergistic with the broad-spectrum cephalosporin ceftriaxone (CRO) in the treatment of experimentally induced endocarditis. Rats with catheter-induced aortic vegetations were infected intravenously with 3×10^7 CFU of a beta-lactam-sensitive strain of *Streptococcus sanguis* or a beta-lactam-resistant strain of *Streptococcus mitis*. Treatment with the antibiotics alone (CRO, 10 mg/kg of body weight every 8 h; NET, 18 mg/kg every 24 h) or in combinations which had proved synergistic in in vitro time-kill curves was commenced 48 h postinfection and continued for 72 h. The results show that the combination was markedly effective against *S. sanguis* and moderately effective against *S. mitis*, while, with the protocol used here, the agents alone were not. The results suggest that CRO-NET should be an effective combination for treating streptococcal endocarditis in humans and may permit a shorter duration of treatment and once-a-day dosing to be used.

Penicillin-aminoglycoside combinations often display marked synergy against enterococci and streptococci associated with infective endocarditis (11, 18, 20, 24) and have proved highly successful in the prophylaxis (11, 20) and treatment (4, 8) of experimentally induced and human diseases. Problems associated with high-level resistance to older aminoglycosides (streptomycin, kanamycin) have been overcome by the use of penicillin combined with newer agents such as tobramycin, sisomicin, or netilmicin (NET) (18, 24), while patients with penicillin allergy may be treated with a cephalosporin or vancomycin.

Despite this apparently favorable picture, the treatment of human streptococcal endocarditis usually requires hospitalization: the American Heart Association recommends prolonged (4-week) high-dose therapy with penicillin or a 2-week course in combination with an aminoglycoside (3) necessitating several daily intravenous (i.v.) or intramuscular (i.m.) injections. There is, therefore, considerable interest in the use of new combinations and in the development of protocols which permit once-a-day dosing (13, 23, 26) and/or outpatient treatment.

Ceftriaxone (CRO) is especially appropriate as a candidate for once-a-day treatment because of its very high activity against viridans group and non-enterococcal streptococci (7, 10), its long half-life, and its time-dependent bactericidal effect (17, 21). The efficacy of 2 g of CRO administered i.v. or i.m. once a day for 4 weeks in streptococcal endocarditis in humans has already been demonstrated (12). It may be possible to shorten this time or reduce the dose by combining CRO with an aminoglycoside. In this context, once-daily dosing with the CRO-NET combination in treating a range of serious bacterial infections other than endocarditis (23) and *Escherichia coli*-induced endocarditis in the rabbit (9) has already been assessed with very favorable results.

The purpose of the present study was to determine whether the combination of CRO and NET was more effective than either agent alone in the rat model of infective endocarditis caused by beta-lactam-sensitive and beta-lactam-resistant strains of viridans group streptococci.

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MATERIALS AND METHODS

Bacterial strains. Experimental studies employed a penicillin- and cephalosporin-sensitive strain of *Streptococcus sanguis* originally isolated from a patient with endocarditis (14) and used subsequently in experimental endocarditis in rats (11, 20) and a highly resistant strain of *Streptococcus mitis* provided by R. Moellering.

Antibiotics. CRO was supplied by F. Hoffmann-La Roche Ltd., Basel, Switzerland, and NET was supplied by Essex Chemie, Luzern, Switzerland.

Susceptibility studies and in vitro killing curves. MICs were determined by dilution tests in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.), with inocula of 10^6 CFU/ml, read at 24 h.

Killing curves were determined in Mueller-Hinton broth incubated at 37°C for 24 h with an inoculum of approximately 10^6 to 10^8 CFU/ml from an overnight Mueller-Hinton broth culture. Samples were removed at 2, 4, 6, 12, and 24 h, and the bacterial count was determined by plating 0.1 ml of an appropriately diluted sample onto blood agar plates supplemented with a broad-spectrum β-lactamase (penicillin amido-beta-lactam hydrolase [EC 3.5.2.6]; Genzyme Diagnostics, Kent, England). The plates were incubated at 37°C (in a 10% CO_2 atmosphere for CRO and in anaerobic conditions when NET was tested) and read after 24 h. The concentrations of antibiotic used (in micrograms per milliliter) were selected to approximate those found in the sera of rats 2 h after injection. Synergy was defined as at least a 2 log decrease in count after 24 h of incubation compared with that achieved by the most effective agent alone (19).

Pharmacokinetics. Antibiotic concentrations in sera were

* Corresponding author.

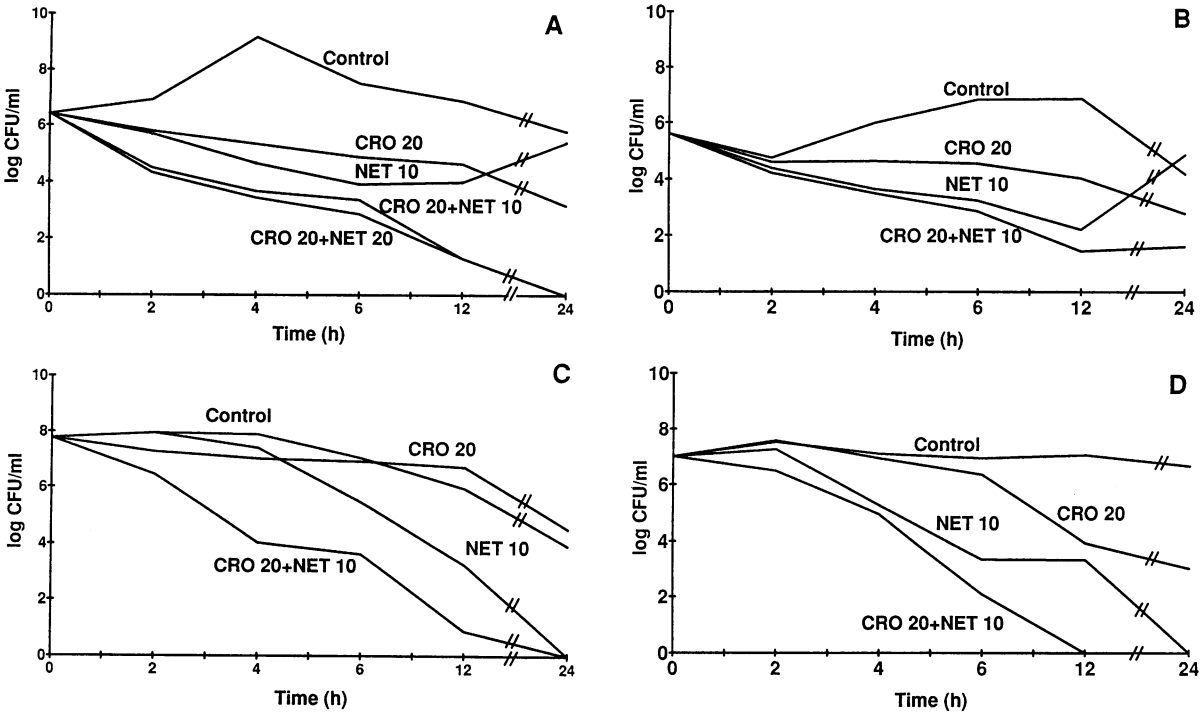

FIG. 1. In vitro killing curves for *S. sanguis* (A and C) and *S. mitis* (B and D) in the presence of CRO, NET, and combinations of the two at concentrations approximately equivalent to those found in rat plasma 2 h after s.c. dosing with CRO (10 mg/kg) and NET (18 mg/kg). The inocula were approximately 10^6 (A and B) and $10^{7.5}$ to 10^8 (C and D) CFU/ml.

determined in groups of four infected rats by using an established agar diffusion technique (1) with *Bacillus subtilis* ATCC 6633 (Difco) as the indicator strain. This method allows the detection of an antibiotic concentration above 0.1 μg/ml. Samples were taken from each animal at 0.5, 2, 4, 6, and 8 h after subcutaneous (s.c.) injection of antibiotic, and mean values and standard deviations were calculated.

SBTs. For the two strains, serum bactericidal titers (SBTs) were determined in three rats of each treatment group, by using standard methods with plates supplemented with a broad-spectrum β-lactamase (penicillin amido-beta-lactam hydrolase; Genzyme Diagnostics) (25). Plates were duplicated and incubated either in a 10% CO_2 atmosphere or in anaerobic conditions. Samples were obtained at 1 and 24 h after initiation of antibiotic therapy (CRO alone [three doses of 10 mg/kg of body weight], NET alone [18 mg/kg in one dose], or a combination of CRO and NET).

Endocarditis model. Sterile vegetations were produced in 162 female Wistar rats (180 to 200 g) by a modification of a previously described method (15). Briefly, a polyethylene catheter (PP10; Portex Ltd., Hythe, Kent, England) was inserted across the aortic valve through the right carotid artery and secured with a silk ligature. Twenty-four hours after catheterization, rats were injected with 0.5 ml of saline containing 1.5×10^7 CFU of the test organism.

Treatment protocol and evaluation of infection. Treatment was commenced 48 h after infection. Antibiotics were administered s.c. for 72 h by using one of the following five protocols: ceftriaxone alone, 10 mg/kg every 8 h; netilmicin alone, 18 mg/kg every 24 h; and ceftriaxone at 10 mg/kg every 8 h plus netilmicin at either 18 or 6 mg/kg once every 24 h or at 6 mg/kg every 8 h.

To evaluate infection, control rats were sacrificed at the commencement of treatment, i.e., at 48 h postinfection. Surviving treated rats were killed 18 h after the last drug dose at a time when no residual antibiotic could be detected in plasma. Rats found dead during the last day of antibiotic treatment were refrigerated, autopsied within 12 h of death, and included in the study. Aortic vegetations were excised, weighed, homogenized in 1 ml of saline, serially diluted, and plated onto blood agar plates supplemented as described above. Colonies were counted after 48 h of incubation at 37°C in a 10% CO_2 atmosphere, and the results were calculated as $\log_{10}$ CFU/g of vegetation. The dilution technique permitted the detection of $\geq 10^2$ CFU/g of vegetation.

Statistical evaluation. The chi-square test with the Yates correction was used for proportional variables. Continuous variables were analyzed by one-way analysis of variance. The Student *t* test was used to compare each group with the others. A *P* value of <0.05 was considered significant.

For calculation of the continuous variables, the value of 10^2 CFU/g was assigned to sterile vegetations (limit of detection).

RESULTS

In vitro studies. The MICs of penicillin G and CRO for *S. sanguis* were 0.032 and 0.064 μg/ml, respectively. *S. mitis* was considerably more resistant, with MICs of penicillin and CRO of 2.0 μg/ml to this organism. Both organisms were equally susceptible to NET, which had an MIC of 8 μg/ml.

The in vitro killing curves for CRO, NET, and their combinations are shown in Fig. 1. The concentrations selected for study are approximately those observed in the sera of treated infected animals 2 h after administration of antibiotic (see Fig. 2). Against *S. sanguis* (Fig. 1A), at an inoculum of 10^6 CFU/ml, CRO alone at 20 μg/ml produced a fall in viable count of just over 2 $\log_{10}$ by 24 h. NET, in

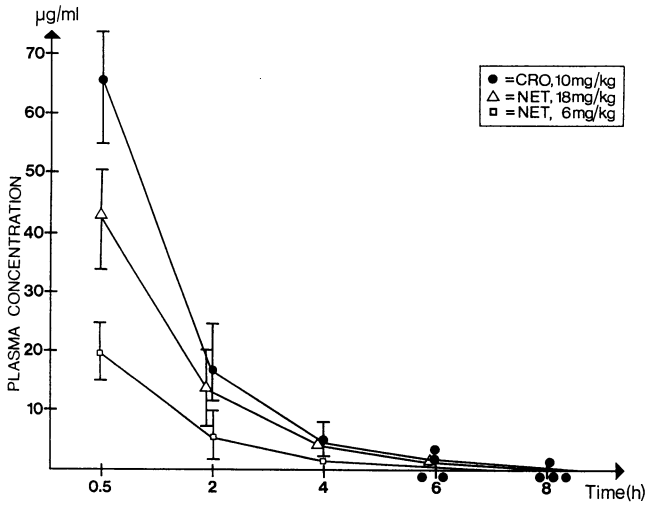

FIG. 2. Plasma concentration-versus-time curves for CRO administered at 10 mg/kg and NET administered at 18 and 6 mg/kg s.c. to streptococcus-infected rats. Points represent means of four animals, and bars represent the standard deviations.

contrast, produced an initial fall, but the viable count had recovered to control values by 24 h. The combination (Fig. 1A, CRO 20+NET 10) resulted in a more profound reduction in the count at all time points, with values more than 3 $\log_{10}$ lower than those of either agent alone by 24 h, indicative of a marked synergistic action. Against *S. mitis*, at an inoculum of 10^6 CFU/ml, similar curves resulted for each agent alone (Fig. 1B), but the combination was less effective, with a reduction of only 1 to 1.5 $\log_{10}$ at 24 h. Hence, although some interaction between the agents was apparent against *S. mitis*, true synergy was not demonstrated. With inocula of 10^7 to 10^8 CFU/ml, the combination also had a more rapid killing effect against both strains than either drug used alone during the first 12 h (Fig. 1C and D).

In vivo drug levels. Monoexponential concentration-time curves for CRO and NET in the plasma of infected rats are shown in Fig. 2. The concentration of NET in plasma after a dose of 18 mg/kg s.c. fell below the MIC for both *S. sanguis* and *S. mitis* within 3 to 4 h, while a dose of 6 mg/kg sustained MIC levels for less than 2 h. In the case of CRO, 10 mg/kg resulted in CRO levels in plasma above the MIC for *S. sanguis* for almost 8 h while levels in excess of the MIC for *S. mitis* were sustained for 5 to 6 h.

SBTs. For *S. sanguis*, the geometric mean SBTs at 1 and 24 h were 1:32 and 1:4 for CRO, 1:4 and 1:4 for NET, and >1:64 and 1:8 for CRO-NET, respectively. For *S. mitis*, the titers were 1:4 and >1:2 for CRO, 1:4 and >1:2 for NET, and 1:8 and >1:2 for CRO-NET. There was no difference between the results observed on the plates incubated in a 10% CO_2 atmosphere and those incubated anaerobically. Although the SBTs of rats receiving CRO-NET differed only by one tube dilution from the SBTs of those receiving CRO alone, this difference was repeatedly observed in all animals. Moreover, if the usual cut-off of 99.9% killing was increased to 99.99%, the SBTs with CRO alone were >1:2 and >1:2 at 1 and 24 h for both strains, as compared to 1:64 and 1:8 for *S. sanguis* and 1:8 and 1:2 for *S. mitis* with CRO-NET.

Treatment of established endocarditis. All untreated control animals had extensive infection of the aortic vegetations by 48 h (Fig. 3), with bacterial counts ranging from 6.5 to 10.5 $\log_{10}$ CFU/g (mean ± standard deviation, 8.87 ± 1.22 and 8.88 ± 0.55 $\log_{10}$ CFU/g for *S. sanguis* and *S. mitis*, respectively). Treatment of both *S. sanguis* and *S. mitis* endocarditis with three doses of CRO per day (at 8-h intervals) for 72 h at 10 mg/kg or with a single daily dose of NET at 18 mg/kg failed to produce a statistically significant reduction in the bacterial count of the infected aortic vegetations (Fig. 3 and Table 1). In the case of *S. sanguis*, the combination of CRO and NET (CRO at 10 mg/kg three times daily plus NET at 18 mg/kg once daily) resulted in the elimination of the infection in 8 of 13 animals (61%) and a statistically significant reduction in the mean bacterial count in vegetations, with values more than 3.5 to 5 to $\log_{10}$ lower than those of the other groups (Table 1). The NET dose divided into three 6-mg/kg doses given at 8-h intervals was equally effective, although a single dose of 6 mg/kg in combination with CRO was slightly less so (Fig. 3A). In the case of a beta-lactam-resistant strain, *S. mitis*, the combination also produced a statistically significant mean reduction in count (Table 1), and in 9 of 26 animals (35%), the infection was below the detection limit by the time of sacrifice (Fig. 3B).

DISCUSSION

The present experiments explored whether CRO plus NET was more effective in the treatment of established streptococcal endocarditis in the rat than the compounds used alone. For a susceptible strain, *S. sanguis*, this proved to be the case, and a statistically significant improvement with the combination was achieved in terms of the number of animals cleared of infection by the time of sacrifice and the reduction in bacterial count of infected vegetations in the animals remaining. Although the results with *S. mitis* were less striking, a beneficial interaction was also observed.

The lack of effect of CRO and NET when used alone in these experiments may be due to a number of factors. First, the initiation of treatment was delayed for 48 h. It has been shown that a delay in treatment initiation from 12 to 48 h has a considerable adverse effect on the outcome of experimental *Staphylococcus aureus* endocarditis treated with beta-lactam antibiotics (21), and the model may therefore be regarded as a rigorous test. Second, the length of treatment (72 h) may have been too short. Studies with cloxacillin and imipenem showed that 5 days of treatment produced a more marked reduction in bacterial count in aortic valve vegetations than 3 days, while 10 days of treatment eliminated infection in most animals (2). Third, considering the relatively short half-life of these drugs in rats, the doses of CRO and NET may have been too low: for penicillin at least, the concentration should be maintained above the MIC throughout the dosing interval to prevent loss of efficacy as a result of bacterial regrowth (8, 16). Indeed, in other experiments with *S. sanguis* in which CRO was administered at 50 or 100 mg/kg/day for 72 h, 11 of 14 rats were clear of infection while the three vegetations still showing infection had a mean count of only 3.3 $\log_{10}$ CFU/g (data not shown).

The ecology of the microenvironment within aortic vegetations may also affect the outcome of therapy in this model. Modified microbial metabolism has been shown to occur within vegetations (6), possibly resulting in changed susceptibility. In any event, the rate of bactericidal activity in broth cultures may be greater than the bacteriological response obtained in infected vegetations (5), and direct extrapolation from in vitro to in vivo settings is not always possible.

Despite the inactivity of CRO and NET when used alone

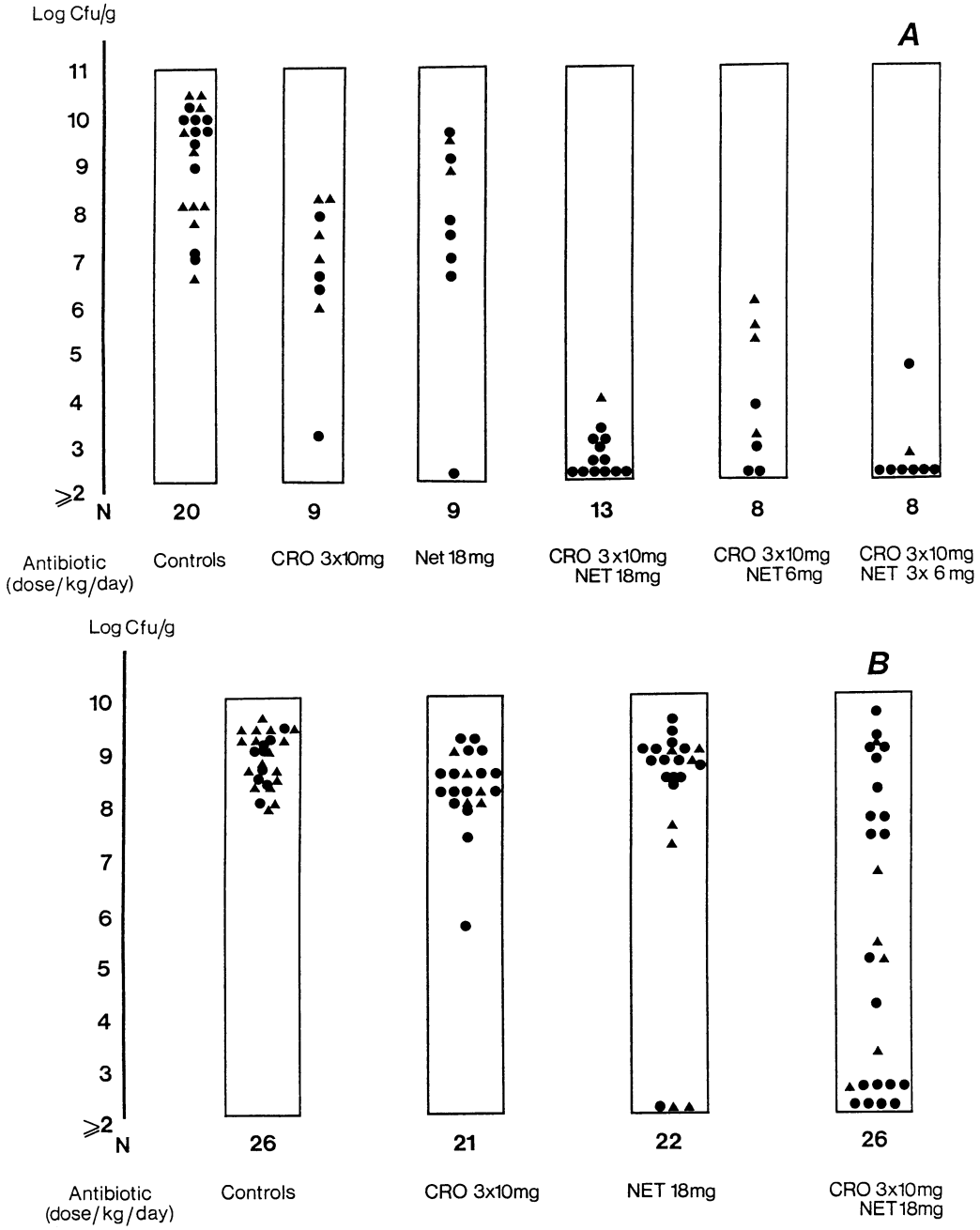

FIG. 3. Counts of *S. sanguis* (A) and *S. mitis* (B) in the aortic vegetations of rats after 72 h of treatment with each antibiotic alone or in combination at the regimens shown. Each point represents a single animal. Circles, animals killed at 72 h; triangles, animals found dead at 72 h.

in the present experiments, the combination was strikingly active against *S. sanguis*. This may be accounted for by genuine bactericidal synergy between the two drugs, as shown in the killing curves presented here and by others (9), or by an important early bactericidal contribution of the aminoglycoside component (24). In addition, drugs may be retained within vegetations at levels higher than those in the plasma (9), resulting in prolonged inhibition or possibly a postantibiotic effect, i.e., a persistent suppression of bacterial growth after exposure to antibiotic. Aminoglycosides, but not penicillin, show a concentration-dependent postantibiotic effect for 2.7 to 6.5 h against gram-negative rods (27), although studies with *Enterococcus faecalis* suggested that an in vitro postantibiotic effect (observed with penicillin G plus gentamicin) was not present in rats with experimental left-sided endocarditis (16). In the results reported here for *S. sanguis*, levels of CRO in plasma would have remained above the MIC for virtually the whole 8-h interval between doses; in the case of *S. mitis*, the MIC would have been exceeded for only 5 to 6 h. With NET (18 mg/kg), the MIC for *S. sanguis* and *S. mitis* (8 μg/ml) would have been exceeded in plasma for only 3 to 4 h.

When administering drugs in animal models, it is never possible to match precisely the half-life and pharmacokinetic

TABLE 1. Effect of treatment of rats with *S. sanguis* and *S. mitis* endocarditis for 72 h with CRO (10 mg/kg every 8 h), NET (18 mg/kg every 24 h), or a combination on the number of animals infected and the bacterial count in aortic vegetations

Treatment	*S. sanguis*		*S. mitis*	
	No. infected/total (%)	Log_{10} CFU/g of infected vegetations	No. infected/total (%)	Log_{10} CFU/g of infected vegetations
None	20/20 (100)	8.87 ± 1.22	26/26 (100)	8.88 ± 0.55
CRO	9/9 (100)	6.82 ± 1.57	21/21 (100)	8.32 ± 0.72
NET	8/9 (89)	8.26 ± 0.81	19/22 (86)	8.62 ± 0.52
CRO-NET[a]	5/13 (38)	3.08 ± 0.56	17/26 (65)	7.01 ± 1.82

[a] $P < 0.001$ compared with other groups by chi-square test with the Yates correction.

profile observed in humans and the choice of dose is inevitably a compromise. CRO in humans has a half-life ranging from 5.8 to 8.7 h (mean, about 6.5 h) (22), and a 2-g dose i.v. or i.m. would sustain MIC levels in plasma above those for the organisms used in the present study for well over 24 h. To provide a similar 24-h cover in rats, CRO was administered in the present study at 10 mg/kg at 8-h intervals, which generated a plasma level of about 20 μg/ml at 2 h. This concentration was used to generate the in vitro killing curves which demonstrated synergy for *S. sanguis* and a weaker interaction for *S. mitis*. This was also observed in the SBTs. On the basis of these data, it is reasonable to infer that the efficacy of the combination in the present rat study is due to a synergistic bactericidal effect. Synergy between beta-lactam–aminoglycoside combinations is, in any case, well documented in a range of animal models and human infections (26).

It was recently demonstrated that a single daily dose of 2 g i.v. or i.m. of CRO for 4 weeks was as safe and effective as conventional penicillin therapy, with or without an aminoglycoside, in treating streptococcal endocarditis in humans (12). The data presented here, in which synergism occurred even at doses of NET well below those required to give 24-h antibacterial levels in serum, suggest that the combination of CRO plus NET given once a day might be more effective than CRO given alone. Such data provide a rationale for human clinical trials of the use of this combination in one daily dose of an aminoglycoside, at least with streptococcal strains highly sensitive to CRO. If successful, they may permit the use of a shorter treatment course, thus reducing the likelihood of aminoglycoside-related adverse events (23), obviate the need for a permanent i.v. line, and allow the ambulatory treatment of selected patients.

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