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Ceftriaxone-Netilmicin Combination in Single-Daily-Dose Treatment of Experimental *Escherichia coli* Endocarditis

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We evaluated the activities of ceftriaxone (15 mg/kg), netilmicin (6 mg/kg), and their combination given intramuscularly once daily for 4 days for the treatment of experimental *Escherichia coli* endocarditis in rabbits. In vitro, a greater rate of killing and an increased trough serum bactericidal titer ($P < 0.01$) were achieved with the combination. In vivo, the combination had a greater bactericidal effect ($P < 0.01$) and resulted in a greater number of sterile vegetations ($P < 0.05$) than single-drug therapy. Thus, in vivo, an increased effect can be obtained despite a single daily dose of a long-acting cephalosporin and an aminoglycoside.

Optimization of antibiotic administration is of major clinical importance. Utility of single-daily-dose treatment has been well established for ceftriaxone in animal models and in clinical trials because of its long half-life and time-dependent bactericidal effect (7, 12). Single daily doses with aminoglycosides have been shown to be less toxic than multiple daily doses (1, 17) and are at least as effective in different animal models (1, 8, 17). The latter point can be explained by the dose-dependent in vivo lethal activity and by the postantibiotic effect of the aminoglycosides (3, 16). However, no study has evaluated the combination in vivo of a long-acting cephalosporin with an aminoglycoside administered once daily to animals. If such a combination could provide better in vivo activity, it could reduce both the length of treatment and the total daily dose of each antibiotic. To assess the in vivo effects of the combination, we used an experimental *Escherichia coli* endocarditis model in rabbits, which, although using an infection uncommon in humans, provides a rigorous test of antibiotic efficacy.

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A strain of *E. coli* isolated from the blood of a patient with endocarditis was used for this study. This strain, which was resistant to rabbit serum, has been described previously (4). MICs and MBCs of ceftriaxone and netilmicin were determined by the tube macrodilution method in Mueller-Hinton broth (produced by Diagnostics Pasteur) with log-phase organisms at final concentrations of 10^5 and 10^7 CFU/ml in order to study susceptibility under routine conditions and also to reproduce the high bacterial concentrations present in vegetations (7, 11, 15). Rates of killing were studied in Mueller-Hinton broth in a final volume of 10 ml with log-phase inocula of 10^5 and 10^7 CFU/ml. Antibiotics were used at concentrations equal to their levels in infected vegetations at the time of killing (0.37 μg/ml for ceftriaxone and 0.21 μg/ml for netilmicin). At 1, 3, 6, and 24 h after inoculation of bacteria into the antibiotic-containing broth, serial dilutions of 0.1-ml samples (up to 10^{-6}) were subcultured in duplicate on agar plates containing β-lactamase as previously reported (7, 11), and these subcultures were incubated at 37°C for 24 h prior to CFU counts. In vitro synergism was defined as a 100-fold increase in killing by the combination over that obtained with the single-drug therapy after 24 h of incubation (9). Serum bactericidal titers (SBTs) were determined in a final volume of 1 ml in 50% normal pooled rabbit serum plus 50% Mueller-Hinton broth with an inoculum of 10^5 to 10^6 in log-phase growth (14). Serial twofold dilutions of serum were made (range, 1/2 to 1/256). After a 24-h incubation at 37°C, 0.01-ml portions were removed from all tubes and were subcultured onto Mueller-Hinton agar plates containing β-lactamase. After 24 h of incubation at 37°C, the number of viable CFU was determined. The SBT was defined as the highest dilution that killed at least 99.9% of the original inoculum.

Antibiotic concentrations in serum and in cardiac vegetations were measured by the agar diffusion method. For ceftriaxone, samples were assayed with *E. coli* ATCC 25922 for concentrations above 5 μg/ml and with *E. coli* 1346 for those lower than 5 μg/ml, as previously described (7, 11). *Bacillus subtilis* ATCC 6633 was used for netilmicin assays. For serum samples, assay standards were prepared in normal rabbit serum. Antibiotic levels in vegetations were determined by assay of supernatant of vegetation samples homogenized in 0.3 ml of 0.1 M phosphate buffer, as described previously (4, 7, 11). Assay standards were prepared in 0.10 M phosphate buffer (pH 7.4).

A total of 72 female New Zealand White rabbits (weight range, 1.8 to 3.0 kg) were used for in vivo experiments. Aortic endocarditis was induced as previously described (2, 4). At 24 h after catheter insertion, 1 ml of 10^7 CFU of *E. coli* per ml was injected into an ear vein. At 72 h after inoculation, animals received one of the following antibiotic regimens for 4 days: ceftriaxone, 15 mg/kg of body weight intramuscularly (i.m.), once daily; netilmicin, 6 mg/kg i.m., once daily; or ceftriaxone plus netilmicin. A control group was left untreated. Serum was sampled for bactericidal titer determination 1 h (peak) and 24 h (trough) after the last injection. Animals were killed by chloroform inhalation 24 h after the last antibiotic injection or 6 days after the last injection in the group evaluated for posttherapy results. At the time of killing or of spontaneous death after the 4-day treatment period, the heart was removed. Each vegetation was excised, weighed, and rinsed in sterile saline. Each vegetation was homogenized in 0.5 ml of sterile saline (7,

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TABLE 1. SBTs obtained 1 and 24 h after i.m. antibiotic injection 4 in rabbits with *E. coli* endocarditis

Antibiotic (dose)	Median SBT[a] (range) after:	
	1 h	24 h
Ceftriaxone (15 mg/kg, once daily) (n^b = 8)	≥1/256 (≥1/256–≥1/256)	1/2 (<1/2–1/4)
Netilmicin (6 mg/kg, once daily) (n = 7)	1/8 (1/4–1/8)	<1/2 (<1/2–<1/2)
Ceftriaxone plus netilmicin (n = 8)	≥1/256 (≥1/256–≥1/256)	1/8 (1/2–1/16)[c]

[a] Highest dilution that killed at least 99.9% of the original inoculum.
[b] Number of sera tested.
[c] $P < 0.01$ in comparison with value for ceftriaxone or netilmicin alone.

11), and 0.1-ml portions were quantitatively subcultured onto agar plates containing β-lactamase and incubated at 37°C for 24 h. Colony counts were made, and results were expressed as $\log_{10}$ CFU per gram of vegetation. Vegetations were considered sterile when no growth occurred from a 0.1-ml subculture of the undiluted tissue homogenate. For calculation of the mean number of CFU per gram of vegetation, such vegetations were considered to contain 2 $\log_{10}$ CFU/g. Ten infected rabbits were used exclusively for pharmacokinetic studies. Each received a single i.m. dose of ceftriaxone (15 mg/kg; n = 6) or netilmicin (6 mg/kg; n = 4). Serum samples were collected through a femoral artery catheter at 0.5, 1, 2, 4, 6, 8, 12, and 24 h after injection. Half-lives at β phase were determined by linear regression analysis of the terminal portion of the serum concentration-versus-time curves. The exact Fischer test was used for the comparison of differences between proportions. SBT and $\log_{10}$ CFU per gram of vegetation of various groups of animals were compared by nonparametric tests, including the Kruskall and Wallis test followed by the Wilcoxon test (5, 6). A P value of <0.05 was considered significant.

The MIC and MBC (in micrograms per milliliter) were 0.06 and 0.06 for ceftriaxone and 0.5 and 4 for netilmicin, irrespective of the inoculum (i.e., 10^5 or 10^7). The ceftriaxone-netilmicin combination exhibited a greater rate of killing than did single-drug therapy after 6 h of incubation, but no synergy was observed after 24 h of incubation (Fig. 1). A regrowth was observed with netilmicin alone but did not occur when netilmicin was studied in combination with ceftriaxone. Similar observations were made with the lowest inoculum (data not shown).

Peak and trough SBTs, obtained 1 and 24 h after the last antibiotic injection, are presented in Table 1. High peak SBTs (≥1/256) were obtained for ceftriaxone used alone or in combination with netilmicin. The ceftriaxone-netilmicin combination produced a significantly higher trough SBT (1/8) than did ceftriaxone (1/2) or netilmicin (<1/2) used alone (P < 0.01).

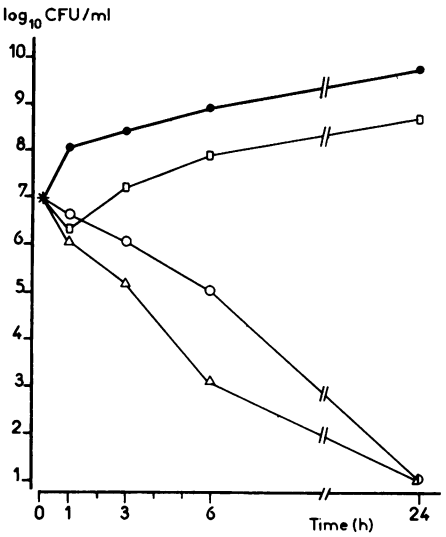

FIG. 1. In vitro killing rates of *E. coli* incubated with antibiotics at concentrations equivalent to those measured in infected vegetations. Symbols: ●, control; ○, ceftriaxone (0.37 μg/ml); □, netilmicin (0.21 μg/ml); △, ceftriaxone (0.37 μg/ml) plus netilmicin (0.21 μg/ml).

Antibiotic levels in serum at different times and in vegetations at the time of killing are shown in Table 2. Serum elimination half-lives (mean ± standard deviation) determined after a single injection were 3.7 ± 0.6 h for ceftriaxone and 1.0 ± 0.2 h for netilmicin. Ceftriaxone and netilmicin concentrations in infected vegetations were also measured (Table 2). Mean ceftriaxone residual concentration/MIC ratios were approximately 2 and 6 in sera and in vegetations, respectively. Therefore, ceftriaxone concentration in serum and in vegetation was always above the MIC. In contrast, for netilmicin the mean residual concentration in vegetation/MIC ratio was around 0.4 and time above MIC was approximately 6 h in serum.

In vivo results are shown in Table 3. When animals were sacrificed 1 day after the last antibiotic injection, the ceftriaxone-netilmicin combination sterilized 4 of 8 animals versus 0 of 15 treated with ceftriaxone or netilmicin alone (P = 0.016). The combination exhibited a reduction 3.1 $\log_{10}$ CFU/g of vegetation greater than the most effective antibiotic used alone (2.6 ± 0.9 versus 5.7 ± 2.0; $P < 0.01$). This enhanced activity persisted for 6 days after the last injection when the combination showed a reduction 4 $\log_{10}$ CFU/g of vegetation greater than the most effective antibiotic used

TABLE 2. Antibiotic concentrations in serum and vegetations

Antibiotic (dose)	Mean antibiotic concn (± SD) in:								
	Serum (μg/ml)[a] at:								Vegetations[b] (μg/g)
	0.5 h	1 h	2 h	4 h	6 h	8 h	12 h	24 h	
Ceftriaxone (15 mg/kg)	52.7 ± 10.3	66.7 ± 22.3	47.3 ± 2.9	38.2 ± 4.6	27.5 ± 3.8	17.6 ± 3.3	7.8 ± 2.8	0.13 ± 0.5	0.37 ± 0.3 (n^c = 8)
Netilmicin (6 mg/kg)	9.1 ± 1.8	10.8 ± 1.4	5.6 ± 1.6	1.9 ± 0.8	0.6 ± 0.4	0.3 ± 0.2	0.08 ± 0.05	0.07 ± 0.05	0.21 ± 0.2 (n = 7)

[a] Concentrations measured in infected rabbits after a single i.m. injection of ceftriaxone (n = 6) or netilmicin (n = 4).
[b] Concentrations measured in infected vegetations 24 h after the last injection of each drug, administered i.m., once daily for 4 days.
[c] Number of samples tested.

TABLE 3. Results of therapy in rabbits with *E. coli* endocarditis

Regimen	Log_{10} CFU/g of vegetation ± SD on[a]:	
	Day 1	Day 6
Control	8.8 ± 0.5 (0/7)	8.1 ± 1.4 (0/8)
Ceftriaxone (15 mg/kg, once daily)	6.7 ± 1.1[b] (0/8)	7.6 ± 1.5 (0/7)
Netilmicin (6 mg/kg, once daily)	5.7 ± 2.0[b] (0/7)	8.3 ± 1.1 (0/9)
Ceftriaxone plus netilmicin	2.6 ± 0.9[b,c] (4/8)	3.6 ± 2.3[b,c] (3/8)

[a] Time of sacrifice after a 4-day therapy. Values in parentheses are sterile vegetations/total vegetations.
[b] Significantly different from control value.
[c] Significantly different from single-drug-therapy value.

alone (3.6 ± 2.3 versus 7.6 ± 1.5; $P < 0.01$). A total of 3 of 8 animals retained sterile vegetations 6 days after the combination therapy versus 0 of 16 for single-drug therapy ($P = 0.028$). Single-drug therapy was minimally effective: ceftriaxone or netilmicin alone reduced bacterial titers in vegetations 1 day after the last injection, but they never sterilized vegetation and were no more effective when sacrifice occurred 5 days later.

We studied the potential advantage of a ceftriaxone-netilmicin combination in an experimental model of *E. coli* endocarditis which may be considered a model of severe infection. Our study demonstrated a clear benefit of the ceftriaxone-netilmicin combination in terms of bacterial concentrations in the vegetations and sterilization of the focus of infection, despite a single daily dose of both drugs. This result was achieved with a 15-mg/kg ceftriaxone regimen previously shown to be ineffective alone, in contrast to the 30-mg/kg dosage shown to be effective in other studies (7, 11). In rabbits, 6 mg of netilmicin per kg gave peak levels in serum comparable to those achieved in humans (13). This dosage of netilmicin given alone was moderately active in vivo despite a peak level of drug in serum/MIC ratio of >20, a value reported to be predictive of a high rate of efficacy in humans (10). This could be explained by the long dosing interval used in our study (24 h), probably allowing in vivo regrowth since levels in serum exceeded the MIC during only 6 h and the duration of aminoglycoside in vivo postantibiotic effect for this strain did not exceed 5 h (B. Fantin and W. A. Craig, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 774, 1988). This regrowth was not demonstrated in vivo, but an in vitro regrowth was shown by the time-kill-curve method when netilmicin concentration equaled the residual concentrations in the vegetations. Importantly, the combination with ceftriaxone prevented the regrowth noted with netilmicin alone, emphasizing the importance of ceftriaxone residual levels above the MIC in serum and in vegetation to achieve a greater activity with the combination.

Mean peak SBTs were high (≥1/256) for ceftriaxone alone or in combination with netilmicin. However, since these titers were not endpoints, we cannot exclude higher titers with the combination than with ceftriaxone alone. The higher SBTs achieved at trough with the combination were associated with improved efficacy in vivo, suggesting that residual bactericidal activity during the latter part of the dosing interval is important in the therapeutic outcome, perhaps because it prevents bacterial regrowth.

In conclusion, we demonstrated a greater rate of killing in vitro and an increased trough SBT with the ceftriaxone-netilmicin combination. In vivo, the greater bactericidal effect and the increased number of sterile vegetations achieved with the combination in comparison with single-drug therapy, despite single daily doses (i.e., one injection every 6 half-lives at β phase for ceftriaxone and every 24 half-lives at β phase for netilmicin), might be of potential clinical relevance.

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