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Pharmacodynamics of Vancomycin Alone and in Combination with Gentamicin at Various Dosing Intervals against Methicillin-Resistant *Staphylococcus aureus*-Infected Fibrin-Platelet Clots in an In Vitro Infection Model

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We compared the pharmacodynamic activities of vancomycin with or without gentamicin in an in vitro infection model with methicilin-resistant *Staphylococcus aureus*-infected fibrin-platelet clots. Infected fibrin-platelet clots (FPCs) were prepared with human cryoprecipitate, human platelets, thrombin, and the organism (~10^9 CFU of MRSA-494/g) and were suspended with monofilament line in an infection model capable of simulating human pharmacokinetics. Antibiotics were bolused to simulate vancomycin regimens of 2 g every 24 h (q24h), 1 g q12h, 500 mg q6h, and continuous infusion (steady-state concentration of 20 μg/ml) and gentamicin regimens of 1.5 mg/kg of body weight q12h and 5 mg/kg once daily (q.d.). Model experiments were performed in duplicate over 72 h. FPCs were removed from the models in quadruplicate at 0, 8, 24, 32, 48, 72 h, weighed, homogenized, diluted, and plated to determine colony counts. The inoculum density at 72 h was used to compare bactericidal activities between the regimens. All regimens containing vancomycin significantly decreased the bacterial inoculum compared to the growth control ($P < 0.001$). Vancomycin monotherapy regimens were similar in bacterial kill regardless of dosing frequency. The addition of gentamicin (either q12h or q.d.) significantly improved the bactericidal activity of the vancomycin q6h, q12h, and q24h regimens ($P < 0.001$). The greatest reduction in bacterial density at 72 h ($P < 0.001$) and the most rapid rate of kill (time to 99.9% killing) were achieved with the regimen consisting of 2 g of vancomycin q24h plus gentamicin (q.d. or q12h).

Few options, with the exception of vancomycin, exist for the treatment of beta-lactam-resistant gram-positive infections. Agents such as the investigational antimicrobial daptomycin, teicoplanin, and several fluoroquinolones have been studied for use against methicillin-resistant *Staphylococcus aureus* bacteremia and endocarditis but have not proved useful (8, 12, 14–16, 18, 29).

Vancomycin, although efficacious, has limitations. Its pharmacodynamics are affected by inoculum size and whether organisms are in exponential or stationary growth phase (28). It has also been shown to have slower killing than beta-lactam antibiotics (30). Vancomycin exhibits concentration-independent killing in vitro, and peak concentrations do not correlate with rapid killing or increased microbiologic response (1). The currently accepted dosing regimens of 1 g every 12 h (q12h) and 500 mg q6h achieve a peak of approximately 25 to 30 μg/ml and a trough of 5 to 10 μg/ml. The recommendations for these levels are based predominantly on earlier reports of toxicity and clinical observations (11). It has been suggested that the traditional emphasis on vancomycin peak and trough concentrations should be reexamined (1, 14).

Combination therapy is an additional method employed to improve therapy with vancomycin. Rifampin has been shown to demonstrate both synergism and antagonism when combined with vancomycin in vitro (32, 34). Although there have been occasional case reports indicating a benefit by adding rifampin (6, 20), there is also data indicating no benefit (19). Studies of vancomycin in combination with aminoglycosides appear to demonstrate synergism against some isolates of *S. aureus* (21, 33, 34). Many clinicians may opt to combine vancomycin with another agent (such as gentamicin), expecting an increased bactericidal response when treating complicated methicillin-resistant *S. aureus* infections. However, aminoglycosides are nephrotoxic, and there is an increased incidence of nephrotoxicity when vancomycin and aminoglycosides are combined (27).

Recently, a novel method of administering aminoglycosides, once-daily (q.d.), has been advocated. The rationale behind these large single doses is to attain maximal antibacterial activity while sparing renal function. Studies comparing the nephrotoxicities of traditional and q.d.-dosed aminoglycoside regimens have shown similar or decreased nephrotoxicities with q.d.-dosed aminoglycosides (13).

Although tobramycin appears to quickly penetrate the interior of the vegetation (4), currently there are no recommendations endorsing q.d.-dosed aminoglycosides for bacterial endocarditis due to *S. aureus*. The major reason is the lack of supporting data (2, 26). There have been recent published studies looking at q.d. dosing of aminoglycosides in combination with beta-lactam antibiotics for the treatment of experimental streptococcal and enterococcal endocarditis (7, 9, 10). In two studies comparing q.d.- to traditionally dosed aminoglycosides in combination with a beta-lactam against streptococci

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investigators reported that the synergistic activities were similar regardless of the aminoglycoside dosing frequency (7, 10). One study comparing netilmicin dosing frequencies when the drug was combined with vancomycin against methicillin- and gentamicin-resistant *S. aureus* found that the addition of the aminoglycoside dosed either q.d. or three times a day did not significantly improve the activity of vancomycin in animals with experimental endocarditis (24).

The objective of this study was to examine the effect of dosing frequency on vancomycin's killing activity and to determine the effect of once- versus twice-daily administration of gentamicin when given in combination with vancomycin against methicillin-resistant *S. aureus.*

MATERIALS AND METHODS

Bacterial isolate. A clinical isolate of methicillin-resistant *S. aureus* (MRSA-494) which was isolated from a patient with endocarditis was utilized for all experiments.

Antimicrobial agents. Vancomycin and gentamicin analytical powders were purchased from Sigma Chemicals (St. Louis, Mo.), vancomycin for injection was purchased from Eli Lily & Co. (Indianapolis, Ind.), and gentamicin for injection was purchased from Fujisawa USA, Inc. (Deerfield, Ill.).

MIC determination and synergy testing. MICs and minimum bactericidal concentrations were determined by the microdilution technique with an inoculum of 5×10^5 CFU/ml, following National Committee for Clinical Laboratory Standards guidelines (22). Synergy studies were performed by the checkerboard technique using a fractional inhibitory concentration (FIC) index with an inoculum of 5×10^6 CFU/ml. Synergy, additivity, indifference, and antagonism were defined by FIC indices of ≤0.5, 0.5 to 1.0, 1 to 4, and ≥4, respectively (5). Synergy was also determined by time-kill curve testing using standard (5×10^5 CFU/ml) and high-level (1×10^8 CFU/ml) inocula and was defined as a >2 log decrease in the bacterial inoculum at 24 h more than that produced by the most active agent used alone.

Medium. Mueller-Hinton broth (Difco, Detroit, Mich.) supplemented with 12.5 mg of magnesium and 25 mg of calcium per ml was used for all model experiments and susceptibility testing.

Kill curve tests. Preliminary kill curve tests were conducted in test tubes to assess the bactericidal activities of each antimicrobial agent with an inoculum of approximately 5×10^5 CFU/ml. The concentration of vancomycin was 30 μg/ml and those of gentamicin were 5 and 15 μg/ml in supplemented Mueller-Hinton broth (SMHB). All experiments were performed in duplicate, and samples of 0.1 ml were removed at 0, 2, 4, 8, and 24 h. Samples were serially diluted with cold 0.9% sodium chloride (NS), 20 μl of each sample was plated onto tryptic soy agar (TSA) in triplicate and incubated at 37°C for 24 h, and the colonies were counted. Potential drug carryover samples (0.1 ml) were filtered through a 0.45-μm-pore-size filter (Millipore), aseptically placed onto TSA plates, and incubated for 24 h, and the colonies were counted. Time-kill curves over 24 h were plotted as $\log_{10}$ CFU per milliliter versus time. The time to achieve a 99.9% reduction and the total reduction in the value of $\log_{10}$ CFU per milliliter over 24 h were determined by visual inspection.

Preparation of infected clots. Briefly, bacteria inocula were prepared by inoculating two or three colonies of bacteria from a fresh overnight TSA plate into 10 ml of SMHB and then by incubating the bacterial solution at 37°C for 24 h on a rotator. After centrifugation at 3,500 × *g* for 15 min, the supernatant was removed and the pellet was resuspended with a small quantity of SMHB to achieve an inoculum of approximately 10^{10} CFU/ml. Infected fibrin-platelet clots (FPC) of approximately 1 g were prepared by mixing 0.9 ml of cryoprecipitate antihemolytic factor from volunteer donors (obtained from the American Red Cross), 0.1 ml of organism suspension, 0.05 ml of platelet suspension (platelets mixed with normal saline; 250,000 to 500,000 platelets per clot), and 0.05 ml of aprotinin solution (2,000 KIU/ml) in a sterile, siliconized, 1.5-ml Eppendorf tube. A sterile monofilament line was placed into the cryoprecipitate-bacterium mixture. Bovine thrombin (5,000 U) was reconstituted with 5.0 ml of sterile calcium chloride (50 mmol), and 0.1 ml of the thrombin was added to the cryoprecipitate-bacterium mixture (31). The gelatinous mixture was removed from the Eppendorf tube with a sterile 21-gauge needle. The final inoculum obtained in the clots was approximately 10^9 CFU/g (23).

In vitro model. A one-compartment in vitro infection model capable of simulating human pharmacokinetics in the presence of viable bacteria was utilized (17, 21, 23). The one-compartment glass model (500 ml) is fitted with sample ports from which FPC were suspended and sealed with a rubber stopper. Fresh SMHB was supplied and removed from the model via a peristaltic pump set to achieve a 6-h half-life for vancomycin and a 3-h half-life for gentamicin. The model contained a magnetic stir bar for continual mixing of the antibiotics. Vancomycin was administered as boluses into the model q6h, q12h, q24h, and by continuous infusion (with a simulated loading dose of 500 mg to reach a peak of 20 μg/ml). Gentamicin was administered also as boluses q12h and q24h. The target antibiotic concentration for the vancomycin continuous-infusion regimen was 20 μg/ml; for vancomycin q6h, q12h, and q24h regimens, the targets were peaks of approximately 20 to 25, 30, and 60 μg/ml, respectively, and troughs of approximately 5 to 10 μg/ml. The target antibiotic concentrations for the gentamicin q12h regimen were a peak of approximately 5 μg/ml and a trough of approximately 1.0 μg/ml; for the gentamicin q24h regimen the targets were a peak of approximately 15 μg/ml and a trough of <1.0 μg/ml. For combination regimen experiments, the elimination rate was set for the drug with the shorter half-life and the drug with the longer half-life was supplemented (3). The entire model apparatus was placed in a water bath and maintained at 37°C. Each experiment was conducted over 72 h and performed in duplicate to ensure reproducibility.

Pharmacokinetic analysis. Samples (0.5 ml) from the model were obtained at 0.25, 1, 2, 4, 6, 8, 24, 32, 48, and 72 h for determination of antibiotic concentrations and were stored at −70°C until analysis. The antibiotic peak and trough and half-lives were calculated from the concentration-versus-time plots. The area under the concentration-time curve (AUC) was calculated from the 0-to-24-h portion of each plot by the trapezoid method with the LAGRAN program, version 2.1 (25).

Pharmacodynamic analysis. Two FPCs were removed from each model at 0, 8, 24, 32, 48, and 72 h. The clots were weighed and homogenized by using a minibeadbeater (Biospec Products, Bartlesville, Okla.) with trypsin solution (62.5 mg of trypsin in 5.0 ml of NS). Cold NS was used for dilution of the samples; 20 μl was plated on TSA plates in triplicate, the plates were incubated for 24 h at 37°C, and the colonies were counted. Potential drug carryover samples (0.1 ml) were filtered through a 0.45-μm-pore-size filter (Millipore) and placed aseptically onto TSA plates; the plates were incubated for 24 h, and the colonies were counted. The four samples were averaged for each time point and plotted as $\log_{10}$ CFU per gram versus time. The total reduction in the $\log_{10}$ CFU per gram over 72 h was determined, and the time to achieve a 99.9% reduction was calculated by linear regression. Values for the ratios of AUC from 0 to 24 h (AUC_{0-24}) to the MIC and for the time drug concentration was greater than the MIC were determined for all vancomycin regimens.

Antibiotic assays. Vancomycin and gentamicin model concentrations were determined by fluorescence polarization immunoassay (Abbott Diagnostics TDx), which has a sensitivity of 2.0 μg/ml for vancomycin and 0.27 μg/ml for gentamicin and interday coefficients of variation (%) of ≤12% for vancomycin and ≤11% for gentamicin. Control samples were prepared in SMHB and varied by ≤10% of the assay standards prepared in serum. Vancomycin and gentamicin clot concentrations were determined by well diffusion bioassay methodology with *Bacillus subtilis* spore suspension no. 2 (Difco Laboratories). The assay limits of detection are 2.5 μg/ml for vancomycin and 0.32 μg/ml for gentamicin, with intraday coefficients of variation of ≤13 and ≤9.1%, respectively. The values of r^2 for both assays were ≥0.94 (range, 0.940 to 0.999) over the concentration range of 1.25 to 10 μg/ml for vancomycin and 0.63 to 10 μg/ml for gentamicin. Concentrations outside those of the standard curve were diluted in sterile water and then multiplied by the appropriate dilution factor to determine the final concentrations. All standards and unknowns were run in quadruplicate.

Statistical analysis. The changes in $\log_{10}$ CFU per gram over 72 h were compared by analysis of variance using Tukey's test for analysis. *P* values of ≤0.05 were considered significant.

RESULTS

Susceptibility testing. The MIC and minimum bactericidal concentration of vancomycin for this isolate are 0.5 and 1.0 μg/ml, respectively; those of gentamicin are 0.5 and 2.0 μg/ml, respectively. In synergy studies using the checkerboard technique, the combination of gentamicin with vancomycin was indifferent (FIC index, 0.75). In kill curve testing, all combinations appeared synergistic, resulting in kills greater than 3 log units more than those of the most active agent alone.

Pharmacokinetics and pharmacodynamics. Pharmacokinetic and pharmacodynamic parameters measured in the model are shown in Table 1. Overall, clot concentrations of vancomycin at the time of a trough in relation to the dosing interval (with the exception of the continuous-infusion regimen) were higher for the continuous-infusion and q6h regimens than for the q12h and q24h regimens. Clot concentrations of gentamicin were undetectable at the end of the dosing interval for both the q.d. and q12h regimens, indicating a lack of drug accumulation. The AUC_{0-24} and the AUC_{0-24}/MIC values were similar for the vancomycin regimens, likely because the total daily doses were the same, and did not correlate with reduction in bacterial density. Although the continuous-infusion and q6h regimens achieved trough/MIC ratios two to four times higher than those of the q12h and q24h regimens,

TABLE 1. Pharmacokinetics and pharmacodynamics in the in vitro model

Antibiotic regimen	Concn (μg/ml) of:		$t_{1/2}$[a] (h)	Time>MIC (%)[b]	AUC_{0-24}/MIC	Trough clot concn (μg/ml)
	Peak	Trough				
Vancomycin CI[c]	20.6 ± 0.87[d]		ND	100	902.4	37.7 ± 4.8[d]
Vancomycin 500 mg q6h	24.8 ± 3.6	11.9 ± 0.2	4.9	100	954.4	41.8 ± 9.1
Vancomycin 1 g q12h	33.8 ± 3.6	6.1 ± 1.3	6.1	100	837.2	21.5 ± 4.5
Vancomycin 2 g q24h	64.8 ± 2.1	4.4 ± 0.4	5.8	100	1067.8	13.0 ± 4.1
Gentamicin 1.5 mg/kg[e] q12h	5.98 ± 0.1	0.42 ± 0.1	2.4	ND[f]	ND	UD[g]
Gentamicin 5 mg/kg q24h	15.0 ± 0.7	0.23 ± 0.1	2.7	ND	ND	UD

[a] $t_{1/2}$, half-life.
[b] Time >MIC, percentage of time that the drug concentration was above the MIC.
[c] CI, continuous infusion.
[d] Steady-state concentration.
[e] 1.5 mg/kg, 1.5 mg/kg of body weight.
[f] ND, not done.
[g] UD, undetectable.

this did not result in greater reduction in bacterial density. Vancomycin concentrations were above the MIC for the entire experiment for all regimens, and gentamicin concentrations were less than the MIC prior to the next dosing interval for both the q.d. and q12h regimens. Overall, there was no difference in reductions of bacterial density in FPCs for any vancomycin monotherapy regimens tested (Fig. 1). The addition of gentamicin significantly ($P < 0.001$) improved bactericidal activity whether given as a single daily dose or by traditional dosing, with the exception of the continuous-infusion regimens. However, there was a trend for increased killing with the continuous-infusion vancomycin regimen, although it was not statistically significant (Table 2). For the combination regimens, no apparent correlation to CFU-per-gram reduction at 72 h was found when the gentamicin q.d. and q12h regimens were compared, although one model combination favored q12h gentamicin administration ($P < 0.01$) and one combination favored q.d. gentamicin administration ($P < 0.01$); the other two combinations were not statistically different (Fig. 2). Vancomycin at 2 g q24h combined with gentamicin (q.d. or q12h) achieved the greatest bacterial density reduction (>7 $\log_{10}$ CFU/g) at 72 h ($P < 0.001$). These combinations were the only drug regimen that reduced bacterial density in the vegetation at 72 h to our limit of detection (10^2 CFU/g) (Fig. 3). After two repeat experiments to verify these results we performed an additional experiment by administering a large loading dose (simulating 2 g) of vancomycin at the start of the experiment followed by the continuous-infusion regimen, with gentamicin given as a single daily dose. The results were identical to those obtained with the previous combination regimens in which vancomycin was given q24h with gentamicin (Fig. 3). Combination regimens in which vancomycin was given q24h were more effective than any other monotherapy or combination regimens ($P < 0.001$). The combination of gentamicin and vancomycin in some of the model regimens (seven of nine) was synergistic against the isolate, although this was not evident by checkerboard synergy testing.

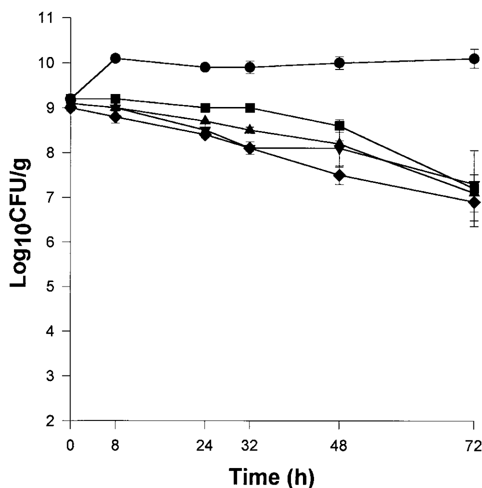

FIG. 1. Vancomycin monotherapy regimens in the in vitro model. —▲—, continuous infusion; —■—, q6h; —▼—, q12h; —◆—, q24h; —●—, growth control.

DISCUSSION

Vancomycin remains the only viable option for the treatment of bacterial endocarditis caused by MRSA. Although vancomycin therapy is similar in overall outcome to that of

TABLE 2. Residual organisms at 72 h and time to 99.9% reduction in bacterial inocula in the in vitro model

Antibiotic regimen	Time to 99.9% reduction in CFU/g (h)	CFU/g at 72 h
Growth control	NA[a]	10.0 ± 0.21
Gentamicin q12h	NA	10.7 ± 0
Gentamicin q.d.	NA	10.6 ± 0.14
Vancomycin CI[b]	NA	7.0 ± 0.42[c]
Vancomycin CI + gentamicin q12h	NA	6.3 ± 0.14[c]
Vancomycin CI + gentamicin q.d.	NA	6.2 ± 0.07[c]
Vancomycin 500 mg q6h	NA	7.2 ± 0.85[c]
Vancomycin 500 mg q6h + gentamicin q12h	45.4 ± 4.6	4.9 ± 0.71[c,d]
Vancomycin 500 mg q6h + gentamicin q.d.	36.8 ± 5.5	4.1 ± 0.78[c,d]
Vancomycin 1 g q12h	NA	7.3 ± 0.21[c]
Vancomycin 1 g q12h + gentamicin q12h	45.3 ± 2.3	4.1 ± 0.35[c,d]
Vancomycin 1 g q12h + gentamicin q.d.	54.8 ± 0.97	5.1 ± 0.07[c,d]
Vancomycin 2 g q24h	NA	6.9 ± 0.42[c]
Vancomycin 2 g q24h + gentamicin q12h	27.0 ± 0.81	2.0 ± 0[c,d,e]
Vancomycin 2 g q24h + gentamicin q.d.	20.9 ± 0.22	2.0 ± 0[c,d,e]
Vancomycin 2 g bolus, then CI + gentamicin q.d.	32.1 ± 0.71	2.0 ± 0[c,d,e]

[a] NA, not achieved.
[b] CI, continuous infusion.
[c] Regimens statistically ($P < 0.001$) different from growth control.
[d] Regimens statistically ($P < 0.001$) different from all vancomycin monotherapy regimens.
[e] Regimens statistically ($P < 0.001$) better than all other monotherapy and combination regimens.

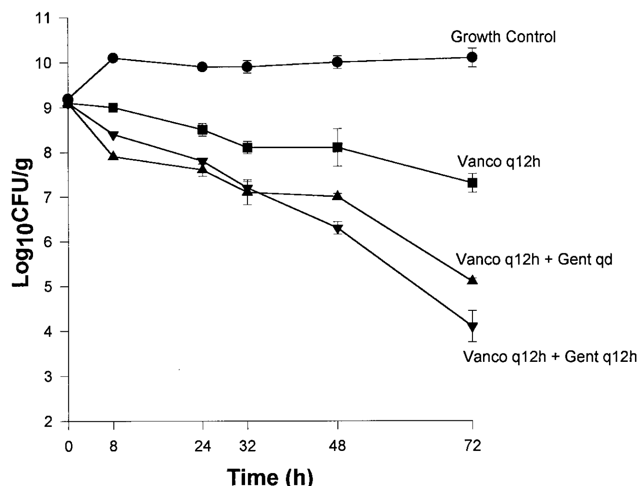

FIG. 2. Vancomycin alone and combined with gentamicin in the in vitro model. Vanco, vancomycin; Gent, gentamicin.

nafcillin, examinations of clinical studies with vancomycin and beta-lactams have shown that the bacteremia period is considerably longer with vancomycin (30). As illustrated by killing curve and in vitro model experiments, vancomycin killing activity is considerably hampered by inoculum size, the stationary growth phase of the organism, and fibrin-tissue barriers. Autoradiographic studies of teicoplanin (a glycopeptide similar to vancomycin) show that penetration is initially concentrated at the periphery, in contrast to what is found for beta-lactams, aminoglycosides, and fluoroquinolones (4). Our data indicates that the treatment of aminoglycoside-sensitive MRSA may benefit from a combination of vancomycin and gentamicin. Although we did not achieve synergy by the standard 2-log-unit-reduction definition for all regimens, the additive effect of the combination was impressive. Consistent with animal studies examining the potential for q.d. dosing of aminoglycosides (7, 9, 10), we did not find a substantial difference when gentamicin was administered either q.d. or q12h in combination with vancomycin. Therefore, the combination of q.d. gentamicin plus vancomycin may have both therapeutic and reduced-toxicity benefits. Curiously, when gentamicin was added to the

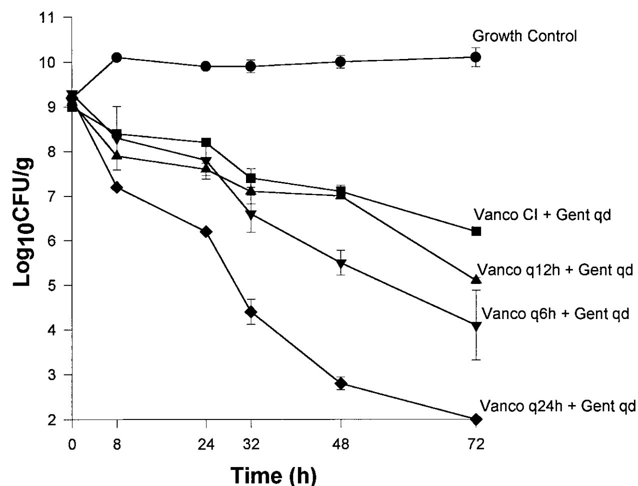

FIG. 3. Regimens of vancomycin combined with gentamicin in the in vitro model. Vanco, vancomycin; Gent, gentamicin.

2-g-q24h vancomycin regimen, the reduction in bacterial inocula was significantly greater than that of any other combination regimen. Since we found no difference in any of the vancomycin monotherapy regimens, this may suggest that the differences noted are due to a step function in either penetration or an interaction, resulting in a synergistic or additive effect. To test this hypothesis, we repeated the continuous-infusion model, increasing the simulated loading dose from 500 mg to 2 g prior to the start of the infusion. Consistent with our findings with the intermittent 2-g vancomycin combination regimens, this resulted in a dramatic decrease in bacterial density over the 72-h period compared with those resulting from all monotherapy regimens (Fig. 3 versus Fig. 1). Our results indicated that vancomycin killing is significantly enhanced by the addition of gentamicin in a gentamicin-sensitive strain. This result occurs regardless of whether gentamicin is administered once or twice daily. The finding that the killing was the greatest when vancomycin was administered by a large 2-g bolus dose is most interesting. Further investigations of this method of administration should be completed with animal models to verify the results.

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