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Consequences of VanE-Type Resistance on Efficacy of Glycopeptides In Vitro and in Experimental Endocarditis Due to *Enterococcus faecalis*

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The consequences on glycopeptide activity of low-level resistance to vancomycin due to VanE-type resistance were evaluated in vitro and in experimental endocarditis caused by *Enterococcus faecalis* BM4405 (MICs of vancomycin and teicoplanin: 16 and 0.5 μg/ml, respectively), its susceptible derivative BM4405-1, and susceptible *E. faecalis* JH2-2. After 24 h of incubation, vancomycin at 8 μg/ml was not active against *E. faecalis* BM4405 whereas it was bacteriostatic against strains BM4405-1 and JH2-2. Against all three strains, vancomycin at 30 μg/ml and teicoplanin at 8 or 30 μg/ml were bacteriostatic but bactericidal when combined with gentamicin. In rabbits with aortic endocarditis due to VanE-type resistant strain BM4405, treatment with a standard dose of vancomycin generated subinhibitory plasma concentrations (i.e., peak of 36.3 ± 2.1 μg/ml and trough of 6.0 ± 2.2 μg/ml) and led to no significant reduction in mean aortic valve vegetation counts compared to no treatment of control animals. In contrast, a higher dosing regimen of vancomycin (i.e., resulting in a peak of 38.3 ± 5.2 μg/ml and a trough of 15.0 ± 8.3 μg/ml), providing plasma concentrations above the MIC for the entire dosing interval, led to significant and similar activities against all three strains, which were enhanced by combination with gentamicin. Treatment with teicoplanin led to results similar to those obtained with vancomycin at a high dose. No subpopulations with increased resistance to glycopeptides were selected in vitro or in vivo. In conclusion, the use of a high dose of vancomycin was necessary for the treatment of experimental enterococcal endocarditis due to VanE-type strains.

Acquired resistance to vancomycin and teicoplanin in enterococci is due to synthesis of new peptidoglycan precursors which bind glycopeptides with reduced affinity. In VanA-, VanB-, and VanD-type strains, the precursors end in depsipeptide D-alanyl-D-lactate in place of dipeptide D-alanyl-D-alanine (1). VanA-type enterococci exhibit inducible resistance to high levels of both vancomycin and teicoplanin (12). VanB-type strains display inducible resistance to various levels of vancomycin but remain susceptible to teicoplanin (19). VanD-type strains are constitutively resistant to intermediate levels of vancomycin and to low levels of teicoplanin (18).

A fourth type of acquired resistance to glycopeptides, VanE, in a clinical strain of *Enterococcus faecalis* has been described recently (9). VanE-type resistance is characterized by peptidoglycan precursors terminating in D-alanyl-D-serine. This dipeptide has a sevenfold-reduced affinity for vancomycin (4), leading to low-level resistance to vancomycin, while susceptibility to teicoplanin is maintained. VanE-type resistance is biochemically and phenotypically similar to VanC-type resistance, although the latter is intrinsic and specific to *Enterococcus gallinarum* (13), *Enterococcus casseliflavus*, and *Enterococcus flavescens* (20).

The purpose of this work was to evaluate the impact of VanE-type resistance on the activity of glycopeptides in vitro and in experimental endocarditis. The efficacy of glycopeptides, alone or in combination with gentamicin, was assessed by the reduction of bacterial loads in vitro and in the valvular vegetations and by selection of subpopulations with increased resistance to glycopeptides.

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MATERIALS AND METHODS

Bacterial strains and media. *E. faecalis* JH2-2 is susceptible to glycopeptides and intrinsically resistant to low levels of β-lactams and aminoglycosides (11). VanE-type *E. faecalis* BM4405 was isolated from an infected peritoneal dialysis fluid in a patient who had received vancomycin (9). *E. faecalis* BM4405-1 is a susceptible derivative of BM4405 obtained after treatment with novobiocin (12.5 μg/ml). Cultures and antibiotic susceptibility testing were performed in brain heart infusion (BHI) broth and agar (Difco Laboratories, Detroit, Mich.) at 37°C.

In vitro susceptibility testing. Antibiotic susceptibility tests were performed by disk-agar diffusion with disks containing 30 μg of vancomycin, 30 μg of teicoplanin, or 500 μg of gentamicin (Bio-Rad, Marnes-la-Coquette, France) (5) MICs of vancomycin (Eli Lilly & Co., Saint-Cloud, France), teicoplanin (Aventis, Levallois Perret, France), gentamicin (Unilabo, Levallois Perret, France), and penicillin G (Roussel Diamant, Paris, France) were determined by the method of Steers et al. (22) with 10^5 CFU per spot on BHI agar after 24 h of incubation (5). For time-kill curves, exponentially growing cultures of *E. faecalis* were diluted in 10 ml of broth to obtain 5×10^7 CFU/ml and incubated with vancomycin (8 and 30 μg/ml), teicoplanin (8 and 30 μg/ml), and gentamicin (4 μg/ml) alone and in combination. After 0, 3, 6, and 24 h of incubation, 0.1-ml aliquots were plated onto agar for the determination of surviving bacteria. As previously shown at these glycopeptide concentrations, antibiotic carryover does not interfere with counts of surviving bacteria (2, 7). Bactericidal activity was defined by a decrease of 3 $\log_{10}$ CFU/ml or more after 24 h of incubation (14). Synergy was defined as a ≥2-$\log_{10}$ decrease in CFU per milliliter between the combination of antibiotics and its most active constituent after 24 h. The number of surviving bacteria in the presence of the combination had to be ≥2 $\log_{10}$ CFU/ml below the starting

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TABLE 1. Efficacy of 5-day antibiotic regimens for treatment of experimental endocarditis due to *E. faecalis*

Treatment	Regimen	Mean ± SD $\log_{10}$ CFU/g of vegetation (no. of rabbits with sterile vegetations/total rabbits) for strain:		
		BM4405	BM4405-1	JH2-2
None (end-of-therapy controls)		8.7 ± 1.3 (0/10)	9.8 ± 0.8 (0/5)	9.8 ± 1.0 (0/12)
Vancomycin standard dose	50 mg/kg q12h	8.1 ± 1.1 (0/7)	7.8 ± 0.7 (0/6)[a]	7.4 ± 1.2 (0/8)[a]
Vancomycin high dose	50 mg/kg q8h	7.2 ± 0.5 (0/10)[a]	8.1 ± 0.4 (0/7)[a]	7.8 ± 0.8 (0/7)[a]
Teicoplanin	20 mg/kg q12h[c]	7.4 ± 0.7 (0/6)[a]	8.1 ± 0.9 (0/5)[a]	6.5 ± 0.7 (0/9)[a,b,d]
Gentamicin	2 mg/kg q8h	9.8 ± 0.7 (0/6)	Not done	8.4 ± 0.3 (0/5)[a]
Vancomycin + gentamicin	50 mg/kg q8h + 2 mg/kg q8h	6.2 ± 1.0 (0/7)[a]	6.9 ± 1.1 (0/7)[a,b]	6.6 ± 1.3 (0/8)[a,b]

[a] $P < 0.05$ versus controls.
[b] $P < 0.05$ versus vancomycin at 50 mg/kg q8h.
[c] After a loading dose of 40 mg/kg.
[d] Results previously published (14) and provided here for comparison.

inoculum, and one of the drugs had to be present in a subinhibitory concentration.

In vitro subpopulation screening. An inoculum of 10^9 CFU of *E. faecalis* BM4405 or BM4405-1 was plated on agar containing serial dilutions of vancomycin (4 to 1,024 μg/ml) or teicoplanin (0.12 to 64 μg/ml), and CFU were counted after 48 h of incubation at 37°C.

Experimental endocarditis. A polyethylene catheter was inserted through the right carotid artery into the left ventricle of New Zealand White rabbits (2.0 to 2.5 kg), as previously described (15). Twenty-four hours after catheter insertion, rabbits were inoculated through the ear vein with approximately 5×10^8 CFU of *E. faecalis* in 1 ml of 0.9% NaCl. Each catheter was left in place throughout the experiment. Treatment was started 48 h after inoculation. Animals were injected intramuscularly for 5 days by using one of the following regimens: vancomycin standard dose, 50 mg/kg of body weight every 12 h (q12h); vancomycin high dose, 50 mg/kg q8h; teicoplanin, 20 mg/kg q12h after a loading dose of 40 mg/kg; gentamicin, 2 mg/kg q8h; combination of gentamicin with a high dose of vancomycin. These regimens were chosen because they reproduce plasma concentrations similar to those obtained in humans (2, 14) and vancomycin standard dose corresponds closely to a standard 1-g q12h vancomycin regimen in humans. Control animals were sacrificed either 48 h after inoculation (start of therapy) for BM4405 and BM4405-1 or at the same time as treated animals (end of therapy). Rabbits were killed by intravenous injection of pentobarbital 8 and 12 h after the last injection in animals treated every 8 or 12 h, respectively. At the time of sacrifice, the heart was removed, and valvular vegetations from individual rabbits were excised, pooled, weighed, and homogenized in 1 ml of 0.9% NaCl. The homogenates were plated onto BHI agar to count surviving bacteria and onto BHI agar containing teicoplanin or vancomycin at two and four times the MIC to count resistant subpopulations after 48 h of incubation. Colony counts were expressed as means ± standard deviations of $\log_{10}$ CFU per gram of vegetation. The vegetations were considered sterile when no growth occurred with 0.1 ml of the undiluted homogenate. For a more accurate analysis the results of a teicoplanin regimen (20 mg/kg q12h for 5 days after a loading dose of 40 mg/kg) against susceptible strain *E. faecalis* JH2-2, which have been previously reported (14), were included in the results.

Antibiotic assays. Antibiotic plasma concentrations were measured in animals with endocarditis by fluorescence polarization immunoassay. The sensitivities of the procedure were 0.3, 1.7, and 2 μg/ml for gentamicin (AxSYM; Abbott Diagnostics, Rungis, France), teicoplanin (TDx; Abbott), and vancomycin (AxSYM; Abbott), respectively. For determination of peak antibiotic plasma levels, 2 ml of blood was sampled by the ear vein on day 5 of antimicrobial therapy, 1 and 2 h after antibiotic injection in 6 animals treated with teicoplanin, in 10 animals treated with vancomycin at 50 mg/kg q8h, and in 6 animals treated with vancomycin at 50 mg/kg q12h and 20 and 30 min after injection in 4 animals treated with gentamicin. Blood was also sampled at the time of sacrifice for determination of trough antibiotic levels.

Statistics. Variance analysis followed by the Fisher test for multiple comparisons was used to compare bacterial counts in vegetations from groups of animals infected with the same strain and treated with different antibiotic regimens (21). A P value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

In vitro susceptibility to antibiotics. *E. faecalis* JH2-2, BM4405-1, and BM4405 displayed intrinsic low-level resistance to gentamicin (MIC: 32 μg/ml for all three strains). The MIC of penicillin G for the three strains was 2 μg/ml. MICs of teicoplanin were 1 μg/ml for JH2-2, 0.5 μg/ml for BM4405-1, and 1 μg/ml for BM4405. VanE-type *E. faecalis* BM4405 was resistant to low levels of vancomycin (MIC, 16 μg/ml), whereas *E. faecalis* BM4405-1 and *E. faecalis* JH2-2 were both susceptible to vancomycin (MICs, 4 and 2 μg/ml, respectively). No subpopulations with increased glycopeptide MICs were recovered from agar containing serial concentrations of either vancomycin or teicoplanin.

In vitro bactericidal activity of glycopeptides alone or combined with gentamicin. Vancomycin alone at a low concentration (8 μg/ml, comparable to trough serum concentrations of the drug during standard therapy in humans) did not produce any effect against VanE-type BM4405, whose growth was similar to that of the control. In contrast, the same concentration resulted in a bacteriostatic effect against *E. faecalis* BM4405-1 (Fig. 1A). However, when vancomycin was used at a higher concentration (30 μg/ml), a bacteriostatic activity against *E. faecalis* BM4405 was obtained, similar to that observed against *E. faecalis* BM4405-1 (Fig. 1A). As observed for *E. faecalis* BM4405-1, vancomycin alone exerted a bacteriostatic effect against *E. faecalis* JH2-2 at all the concentrations tested (data not shown).

Vancomycin at a low concentration (8 μg/ml) combined with gentamicin was not effective against *E. faecalis* BM4405 (Fig. 1B). The same combination resulted in a 2.0-$\log_{10}$ reduction of BM4405-1 CFU per milliliter and was bactericidal against JH2-2. In contrast, vancomycin at the highest concentration (30 μg/ml) in combination with gentamicin resulted in bactericidal synergism against all three strains, although the effect was less pronounced for strains BM4405 and BM4405-1 than for *E. faecalis* JH2-2 (Fig. 1B).

It therefore appears that vancomycin at concentrations above the MIC was bacteriostatic, and vancomycin at concentrations above the MIC and combined with gentamicin was bactericidal irrespective of the presence or absence of the *vanE* gene cluster.

Teicoplanin led to the same effect against the three strains. The antibiotic was bacteriostatic when used alone and bactericidal when combined with gentamicin against *E. faecalis* BM4405, BM4405-1, and JH2-2 at both the 8- and 30-μg/ml concentrations tested (data not shown).

The VanE-type glycopeptide resistance in *E. faecalis* was responsible for in vitro decreased activity of vancomycin when

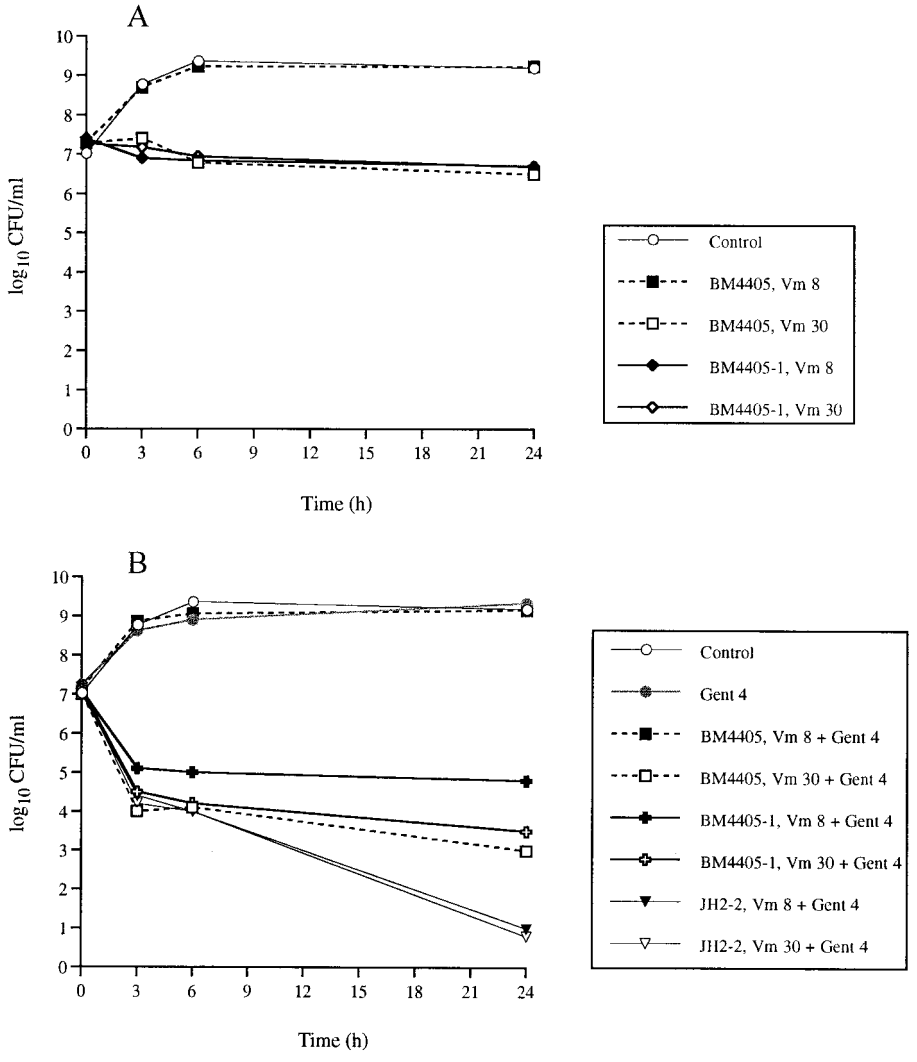

FIG. 1. Time-kill curves. VanE-type *E. faecalis* BM4405 and susceptible BM4405-1 and JH2-2 (data not shown for the last strain) were incubated in BHI broth devoid of antibiotic (control), (A) containing 8 (Vm 8) or 30 (Vm 30) μg of vancomycin per ml, (B) 4 μg of gentamicin per ml (Gent 4), or the combination of 4 μg of gentamicin/ml with 8 (Vm 8 + Gent 4) or 30 (Vm 30 + Gent 4) μg of vancomycin/ml. Surviving bacteria were enumerated on agar plates after 0, 3, 6, and 24 h of incubation at 37°C. Since the three tested strains had similar growth patterns when incubated with gentamicin at 4 μg/ml, a single curve (Gent 4) is shown.

used at a low concentration. However, vancomycin alone at concentrations above the MIC led to a bacteriostatic effect, and it exhibited bactericidal activity when combined with gentamicin against the VanE-type BM4405 strain (Fig. 1).

Antibiotic levels in plasma. Peak plasma concentrations of teicoplanin were 43.0 ± 4.0 μg/ml, and trough concentrations were 21.0 ± 4.0 μg/ml. Peak levels of gentamicin were 7.0 ± 0.7 μg/ml, and trough concentrations were 0.4 ± 0.1 μg/ml at 8 h and <0.3 μg/ml at 12 h. All values were in the range of achievable levels in humans. Peak levels of vancomycin at 50 mg/kg q8h and q12h were 36.3 ± 2.1 and 38.3 ± 5.2 μg/ml, respectively. Eight hours after intramuscular injection of 50 mg of vancomycin/kg, the plasma antibiotic concentration was approximately equal to its MIC for VanE-type *E. faecalis* BM4405 (15.0 ± 8.3 μg/ml), whereas 12 h after injection the plasma drug level was similar to the MIC of vancomycin for the isogenic *E. faecalis* BM4405-1 strain (6.0 ± 2.2 μg/ml). Therefore, in animals infected with BM4405, the plasma vancomycin concentrations remained above the MIC during the entire dosing interval only when the antibiotic was administered every 8 h. However, when vancomycin was given every 12 h, its levels in plasma were below the MIC for *E. faecalis* BM4405 approximately 4 h before each injection. In contrast, in animals challenged with susceptible strains, the plasma vancomycin concentrations remained constantly above the MIC for both strains, whatever the interval (8 or 12 h) between the injections.

Experimental endocarditis. Bacterial counts in the vegetations from animals treated for 5 days and from controls sacrificed at the end of therapy are shown in Table 1. Bacterial titers of controls sacrificed at the start of therapy were similar to those of controls sacrificed at the end of therapy for BM4405 and BM4405-1 (8.7 ± 1.2 and 9.6 ± 0.6 $\log_{10}$ CFU/g of vegetation for BM4405 and BM4405-1, respectively).

Activity of vancomycin depending on dose regimens. Vancomycin at a standard dose (i.e., 50 mg/kg q12h) was not significantly more effective against VanE-type BM4405 than against the controls. In contrast, the same vancomycin regimen produced a significant effect against the two susceptible strains, with an approximately 2-$\log_{10}$ reduction of bacterial counts in the vegetations (Table 1). High-dose vancomycin (i.e., 50 mg/kg q8h) resulted in a significant activity against the VanE-type strain with a 1.5-$\log_{10}$ reduction in the bacterial titers in the vegetations versus the controls (Table 1). The in vivo effect of vancomycin at a high dose against the susceptible strains was similar to that obtained with a standard dose of vancomycin. No subpopulation with increased vancomycin MICs was recovered from any experiment when vancomycin was used alone against the three strains.

The results obtained with the experimental model were consistent with the in vitro data. VanE-type resistance was responsible for reduced in vivo activity when vancomycin was given alone every 12 h (standard dose), but when vancomycin was administered every 8 h, a significant antimicrobial activity was observed (Table 1). These results could be accounted for by the fact that vancomycin given every 8 h achieves concentrations in plasma constantly above the MIC for the VanE-type strain. Thus, VanE-type resistance affected the activity of vancomycin against enterococci only when a standard dose of vancomycin was used, leading to subinhibitory concentrations of vancomycin during approximately 4 out of 12 h. The availability of an isogenic pair of strains allowed us to attribute the differences in the behaviors of *E. faecalis* BM4405 and BM4405-1 in the presence of a low dose of vancomycin to the presence or absence of the *vanE* gene cluster. However, since the vancomycin MIC for susceptible *E. faecalis* BM4405-1 was relatively high, we included fully susceptible *E. faecalis* JH2-2 in our studies. The results for both susceptible strains were similar (Table 1).

Activity of teicoplanin. The activity of teicoplanin (at 20 mg/kg q12h after a loading dose of 40 mg/kg) against *E. faecalis* BM4405 and BM4405-1 was comparable to that of vancomycin at a high dose. No clones resistant to teicoplanin were detected in the vegetations. VanE-type *E. faecalis* BM4405 is susceptible to teicoplanin (5), and this antibiotic had both in vitro (data not shown) and in vivo (Table 1) activity against that strain, similar to that of vancomycin at high dosage (Table 1). The lack of superiority of teicoplanin over vancomycin despite high trough levels in plasma (20 to 40 times the MIC) has been previously reported (2, 16). This can be explained by the low penetration of teicoplanin into the core of the vegetation (6), its high affinity for plasma albumin (90% versus 65% for vancomycin) (3, 17), and the fact that teicoplanin is more sensitive to the inoculum than vancomycin (8, 10).

Activity of the vancomycin-gentamicin combination. A high dose of vancomycin combined with gentamicin was the most effective regimen and produced 2.5- to 3.2-$\log_{10}$ reductions of titers compared with the controls for the three strains (Table 1). This result was in agreement with the time-kill curve study showing that vancomycin when used at a high concentration (30 μg/ml) in combination with gentamicin exerted a synergistic and bactericidal effect in vitro against the three strains, including VanE-type BM4405 (Fig. 1B).

In conclusion, the use of optimal doses of vancomycin was necessary to achieve a significant in vivo activity against VanE-type vancomycin-resistant *E. faecalis*, although no subpopulations with increased resistance to vancomycin were selected either in vitro or in vivo. Since VanE-type strains may be phenotypically indistinguishable from certain VanB-type strains and also from VanC-type isolates, we recommend the use of a high dose of vancomycin in combination with gentamicin for the treatment of enterococcal infections when penicillin G cannot be used.

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