Acquired VanD type glycopeptide resistance in Enterococcus faecium is characterized by moderate levels of resistance to vancomycin (MICs from 16 to 256 μg/ml) and to teicoplanin (MICs, 2 to 64 μg/ml). Characterization of the first VanD type E. faecium BM4339 clinical isolate (13) indicated that resistance resulted from acquisition of the constitutively expressed vanD gene for the D-alanine:D-alanine (D-Ala:D-Ala) ligase (12, 13). This combination of genetic events leads to synthesis of precursors terminating in D-Ala:D-lactate (D-Ala:D-Lac) instead of the dipeptide D-Ala: D-Ala in susceptible bacteria (2, 12, 13).

The purpose of this work was to evaluate the impact of VanD type resistance in E. faecium BM4339 on the activity of glycopeptides in vitro and in rabbit aortic endocarditis. The efficiency of vancomycin and teicoplanin was assessed by the reduction of viable bacteria, both in vitro and in the valvular vegetations, and by their ability to select subpopulations with increased glycopeptide resistance.

MATERIALS AND METHODS

Bacterial strains. E. faecium BM4339 is a VanD type glycopeptide-resistant clinical isolate resistant to vancomycin (MIC, 64 μg/ml) and to low levels of teicoplanin (MIC, 4 μg/ml). It is also resistant to the penicillins, tetracyclines, macrolides-lincosamide-streptogramin B and high levels of aminoglycosides and streptomycin. E. faecium BM4459 is a susceptible derivative of BM4339 obtained by insertion in the chromosome of a copy of the ddl gene for the D-Ala:D-Ala ligase from E. faecium BM1447 (3). E. faecium BM4461 and 1096A are two unrelated VanD type clinical strains (4, 12). Enterococcus faecalis JH2-2 is susceptible to glycopeptides and β-lactams, intrinsically resistant to low levels of aminoglycosides, and resistant to rifampin and fusidic acid following mutations (7). Cultures and antibiotic susceptibility testing were performed in brain heart infusion (BHI) broth or agar (Difco Laboratories, Detroit, Mich.) at 37°C.

In vitro susceptibility testing. The MICs of vancomycin or teicoplanin at fourfold the MIC of the strain to screen for selection of mutants (data not shown). The resistance of bacteria growing on antibiotic-containing agar was confirmed by determination of the MICs. Mutation frequencies were determined by dividing the number of CFU obtained on selective media by the number of those obtained on media devoid of antibiotic after 48 h of incubation. Each in vitro experiment was performed at least three times.

Experimental endocarditis. Aortic endocarditis was induced in female New Zealand White rabbits (2.2 to 2.5 kg) by insertion of a polyethylene catheter through the right carotid artery into the left ventricle, as previously described (9). Twenty-four hours after catheter insertion, each rabbit was inoculated by the ear vein with 10^8 CFU of E. faecium BM4339 or BM4459 in 1 ml of 0.9% NaCl. The catheter was left in place throughout the experiment. Forty-eight hours after inoculation, animals received vancomycin intramuscularly at 50 mg/kg thrice a day or teicoplanin 20 mg/kg twice a day for 5 days after a loading dose of 40 mg/kg. Control animals were left untreated.

At sacrifice, the vegetations of each rabbit were excised, pooled, weighed, homogenized in 1 ml of sterile distilled water, and plated after serial dilutions onto agar to determine the surviving bacteria and onto agar containing vancomycin or teicoplanin at fourfold the MIC of the strain to screen for selection of subpopulations with increased resistance to glycopeptides. The MICs of vancomycin and teicoplanin for the bacteria recovered from the selective media were determined. The results were expressed as log_{10} CFU per gram of vegetation.

Statistics. The results were expressed as means ± standard deviations. Comparison of bacterial counts in the vegetations of rabbits treated with various...
Table 1. MICs of glycopeptides for standard (105) and high (106) inocula against enterococcal strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Standard inoculum</th>
<th>High inoculum</th>
<th>Standard inoculum</th>
<th>High inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vancomycin</td>
<td>Teicoplanin</td>
<td>Vancomycin</td>
<td>Teicoplanin</td>
</tr>
<tr>
<td>BM4339 (VanD)</td>
<td>64</td>
<td>4</td>
<td>512</td>
<td>256</td>
</tr>
<tr>
<td>BM4459</td>
<td>1</td>
<td>1</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>BM44416 (VanD)</td>
<td>128</td>
<td>8</td>
<td>&gt;1,024</td>
<td>&gt;1,024</td>
</tr>
<tr>
<td>10/96A (VanD)</td>
<td>128</td>
<td>8</td>
<td>&gt;1,024</td>
<td>&gt;1,024</td>
</tr>
<tr>
<td>JH2-2</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

In vitro experiments. The vancomycin and teicoplanin MICs against VanD type E. faecium BM4339 and its susceptible derivative BM4459 determined on agar with the standard inoculum of 105 CFU per spot are shown in Table 1. Insertion of a ddi gene for the D-Ala-D-Ala ligase in the chromosome of BM4339 restores vancomycin and teicoplanin susceptibility. Subpopulations with increased resistance to glycopeptides were observed at mean frequencies of 7.5 × 10-5 and 6.0 × 10-7 for BM4339 and BM4459, respectively, on agar containing fourfold the vancomycin MIC. These frequencies were 4.2 × 10-6 and 1.1 × 10-7 for BM4339 and BM4459, respectively, when the bacteria were plated on agar containing fourfold the MIC of teicoplanin.

The in vitro selection of teicoplanin-resistant mutants of VanD type enterococci has been reported previously (11). Time-kill curves at 24 h showed that vancomycin or teicoplanin at 40 μg/ml did not diminish the bacterial counts of BM4339 and led to a decrease in bacterial counts of BM4459 (1.7 and −2.3 log10 CFU/ml, respectively) (Fig. 1). Lack of vancomycin activity against VanD type BM3339 was expected at the concentration of 40 μg/ml, which is lower than the MIC (64 μg/ml). By contrast, the lack of in vitro activity of teicoplanin at the same concentration, which corresponds to 10-fold the MIC, was surprising.

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 2. The glycopeptides displayed excellent bactericidal activity against susceptible BM4459, leading to sterilization of eight of nine (89%) and four of eight (50%) of the vegetations from rabbits treated with vancomycin and teicoplanin, respectively. In addition, none of the animals infected with this strain and treated with glycopeptides retained resistant subpopulations. By contrast, vancomycin at 50 mg/kg thrice a day had no activity against BM4339, as shown by bacterial counts in the vegetations similar to those of untreated animals at sacrifice (Table 2). This result could be anticipated from the peak and trough serum concentrations obtained with this regimen (36.3 ± 2.1 μg/ml and 15.0 ± 8.3 μg/ml, respectively) (8) that remained lower than the MIC.

Surprisingly, teicoplanin at a dose producing peak and trough serum levels of 43.0 ± 4.0 μg/ml and 21.0 ± 4.0 μg/ml (8), respectively, five times higher than the MIC during the entire dosing interval, was also ineffective and led to an increase of 1.5 log10 CFU/g of vegetation in comparison to animals sacrificed at the start of therapy (data not shown). Moreover, subpopulations with increased resistance to glycopeptides were recovered from animals either untreated or treated with glycopeptides. The proportion of resistant subpopulations (10-5 to 10-6 of the entire surviving population) was similar in controls and in treated animals, which is consistent with lack of in vivo activity of the two glycopeptides. The MICs () for these subpopulations were from 256 μg/ml to >1,024 μg/ml for vancomycin and from 16 μg/ml to >1,024 μg/ml for teicoplanin. Thus, the in vivo results confirmed the in vitro data indicating lack of glycopeptide activity against VanD type BM4339. A possible explanation could be an inoculum effect of VanD type strains for glycopeptides.

MICs of glycopeptides depending on bacterial inoculum. To screen for a putative inoculum effect as a cause for glycopeptide inactivity, the MICs of vancomycin and teicoplanin were determined on agar and in broth at standard (105) and high (106) inocula, representative of the inocula observed in bacterial endocarditis. As shown in Table 1, there were wide variations in glycopeptide MICs against BM4339 and BM4459 depending on inoculum size that were more pronounced for teicoplanin than for vancomycin, as already reported (5, 6). When the inoculum varied from 105 to 106, the MICs of vancomycin and teicoplanin increased 8- and 64-fold, respectively, by the agar dilution method, and 32- and 512-fold, respectively, by broth dilution. Similar results were obtained with the VanD type E. faecium BM4416 and 10/96A isolates, which indicated that the inoculum effect was not strain-de-
The effect was greater with VanD type isolates than with susceptible *E. faecium* BM4459 and *E. faecalis* JH2-2 (Table 1), suggesting a link with the VanD phenotype.

We have shown that the activity of vancomycin and teicoplanin against VanD type *E. faecium* BM4339 was abolished, both in vitro and in the rabbit model of aortic endocarditis. The lack of in vivo activity of teicoplanin, despite apparent activity in vitro, may be explained by an important inoculum effect which was observed in vitro for the three VanD type strains. The inoculum effect may not be due to the presence of more glycopeptide-resistant subpopulations in the larger inoc-
ulum. Indeed, the proportion of such subpopulations represented only $10^{-5}$ to $10^{-6}$ of the entire surviving subpopulation and was similar in controls and treated animals. It is also not due to heteroresistance, since the phenotype of the resistant population appeared homogenous on agar plates. Rather, the inoculum effect observed for VanD type isolates may be related to that previously observed for teicoplanin against *E. faecalis* (1).

The rabbit model of aortic endocarditis is characterized by a high inoculum, which varies between $10^8$ and $10^9$ CFU per g of vegetation at start of therapy. Although only a handful of VanD type isolates have been described, none of which appear to transfer the resistance genes to other organisms (11, 13), our results emphasize the need to detect VanD type resistance, which abolishes the activity of both vancomycin and teicoplanin and favors the emergence of mutants highly resistant to glycopeptides.

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