Introduction

Enterococci are frequent causes of nosocomial infections, including urinary tract infections and bacteraemia. The development of vancomycin resistance, often associated with multiple resistance to other antimicrobial agents, has severely compromised the choice of therapies and there are now infections for which no effective therapeutic option is available. While Enterococcus faecalis has been the predominant pathogen in the past, the spread of vancomycin resistance has been associated with a shift in species selection, with Enterococcus faecium being ten-fold more common than E. faecalis among vancomycin-resistant enterococci.

Neutrophils (PMNs) play a critical role in human host defence. We have previously demonstrated that 50% of unrelated clinical isolates of E. faecium are resistant to phagocytosis and PMN-mediated killing, and that a bacterial surface carbohydrate, which may be a component of a capsule, is probably responsible for this effect. A nitrobiotic exposure may alter the surface properties of bacteria, and could potentially affect resistance to phagocytosis. Both quinupristin/dalfopristin, a new semisynthetic injectable streptogramin that is a 30:70 (w/w) combination of quinupristin and dalfopristin, and sparfloxacin, a new fluoroquinolone with two fluorinated substituents, have excellent in-vitro activity against E. faecium. Using two antimicrobial agents with good in-vitro activity against E. faecium, namely quinupristin/dalfopristin and sparfloxacin, we found that exposure to quinupristin/dalfopristin at concentrations both below and above the MIC promoted bacterial adherence to neutrophils (PMNs) for all of three strains of vancomycin-susceptible E. faecium, while sparfloxacin was similarly effective in two of these three strains. In contrast, neither antibiotic was effective in promoting PMN adherence for three vancomycin-resistant strains of E. faecium. The variability amongst strains in response to antibiotic exposure suggests that either the mechanisms of resistance to phagocytosis, or its regulation, may be different amongst different strains of E. faecium.

Materials and methods

Reagents

Quinupristin/dalfopristin and sparfloxacin were obtained from Rhône-Poulenc Rorer (Vitry sur Seine, France and Collegeville, PA, USA). Other reagents were obtained from Sigma (St Louis, MO, USA). Stock antibiotic solutions were freshly prepared in sterile distilled water (for quinupristin/dalfopristin) or 0.05 M NaOH (for sparfloxacin) at 10 g/L.
Bacterial strains and susceptibility testing
Strains of *E. faecium* used in this investigation are listed in Table I. Strains were clonally distinct by pulsed field gel electrophoresis, and were resistant to PMN-mediated phagocytosis in the presence of 10% normal human serum. MICs were determined by microdilution as described by the National Committee for Clinical Laboratory Standards except for the use of brain heart infusion (BHI) broth (Difco, Detroit, MI, USA).

Neutrophil isolation
PMNs were isolated from EDTA-anticoagulated blood of healthy volunteers by dextran sedimentation, Ficoll-Hypaque centrifugation and hypotonic lysis of residual erythrocytes, and resuspended in Hank's balanced salts solution (HBSS). Cells were >95% neutrophils by Diff-Quick staining (Baxter Scientific Products, Miami, FL, USA) and viability was >96% as judged by Trypan Blue exclusion.

Labelling and opsonization of bacteria
Bacteria were grown overnight at 37°C in BHI broth, then diluted 1:100 in fresh BHI broth and grown at 37°C with tumbling for 3 h. Bacteria were incubated at 37°C for 1 h with 0.25, 1 or 4 × the MIC of antibiotic (or HBSS as control). Bacteria were then collected by centrifugation and washed twice in HBSS, and the bacterial density was adjusted spectrophotometrically. Bacteria were labelled by incubation with 0.1% fluorescein isothiocyanate (FITC) in 50 mM sodium carbonate buffer, pH 9.6, for 30 min at 37°C while protected from light. Enterococci were washed twice, suspended in HBSS, then opsonized with 10% autologous serum at 37°C for 15 min. After opsonization, bacteria were washed and resuspended in HBSS plus 2 mM Ca²⁺ and 2 mM Mg²⁺.

Adherence to PMNs
Two hundred microlitres of opsonized FITC-labelled enterococci (2 × 10⁸ cfu/mL) were mixed with 200 µL of neutrophils (2 × 10⁷ cfu/mL) and incubated for 30 min at 37°C. Aliquots of 100 µL were removed and 5 µL of ethidium bromide was added to produce a final concentration of 50 µg/mL. Ten microlitres of the mixture was placed on a slide with a coverslip, and samples were viewed using a Nikon Optiphot fluorescence microscope. Unattached or extracellular attached organisms appear orange, or green with orange centres, while intracellular organisms appear green. Twenty-five consecutive individual PMNs per sample were examined and the number of adherent bacteria was measured as the combined number of ingested and attached organisms per PMN.

Statistical analysis
Differences between groups for each strain were assessed by ANOVA, followed by Student's two-tailed t-test.

Results
A against these six strains of *E. faecium*, the MICs of quinupristin/dalfopristin ranged from 0.25 to 1.0 mg/L and those of sparfloxacin from 0.5 to 8.0 mg/L (Table I). The MICs against the three vancomycin-resistant strains were not appreciably different from the three vancomycin-susceptible strains.

Antibiotic pretreatment enhanced bacterial adherence to PMNs for the three strains of vancomycin-susceptible *E. faecium*, namely MCV161, FA191 and TX0016 (Table II). For MCV161, adherence to PMNs was proportional to the concentration of either quinupristin/dalfopristin or sparfloxacin, with increasing antibiotic concentrations yielding higher PMN adherence. This was also true when MCV161 was pretreated with vancomycin, as exposure to 0.25, 1 or 4 × MIC increased bacterial adherence to 3.74 ± 2.60, 8.96 ± 3.43 and 12.46 ± 2.54 bacteria/PMN, respectively. For the other two vancomycin-susceptible strains of *E. faecium*, antibiotic pretreatment also increased adherence to PMNs, but this was not as prominent as that seen with MCV161. Quinupristin/dalfopristin

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**Table I. Bacterial strains and MICs**

<table>
<thead>
<tr>
<th>E. faecium strain</th>
<th>quinupristin/dalfopristin (mg/L)</th>
<th>sparfloxacin (mg/L)</th>
<th>vancomycin (µg/mL)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCV161</td>
<td>0.25</td>
<td>4</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>FA191</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>TX0016</td>
<td>0.25</td>
<td>1</td>
<td>1</td>
<td>29</td>
</tr>
<tr>
<td>TX0218</td>
<td>0.5</td>
<td>8</td>
<td>&gt;256</td>
<td>J. Patterson</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(personal communication)</td>
</tr>
<tr>
<td>D399</td>
<td>1</td>
<td>0.5</td>
<td>1000</td>
<td>30</td>
</tr>
<tr>
<td>TH1-7</td>
<td>0.25</td>
<td>2</td>
<td>500</td>
<td>7</td>
</tr>
</tbody>
</table>

* MICs were determined by microdilution in BHI broth.
E. faecium–PMN interactions

Table II. Bacterial adherence to PMNs after antibiotic pretreatment

<table>
<thead>
<tr>
<th>E. faecium strain</th>
<th>control</th>
<th>0.25 × MIC</th>
<th>1 × MIC</th>
<th>4 × MIC</th>
<th>0.25 × MIC</th>
<th>1 × MIC</th>
<th>4 × MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCV 161 (VS)</td>
<td>0.9 ± 1.2</td>
<td>6.5 ± 3.8b</td>
<td>10.4 ± 5.0b</td>
<td>13.1 ± 5.2b</td>
<td>2.4 ± 2.5b</td>
<td>4.8 ± 3.9b</td>
<td>7.3 ± 4.4b</td>
</tr>
<tr>
<td>FA 191 (VS)</td>
<td>3.3 ± 2.4</td>
<td>4.5 ± 4.1b</td>
<td>4.9 ± 4.0b</td>
<td>8.4 ± 6.5b</td>
<td>3.0 ± 2.6</td>
<td>3.1 ± 3.2</td>
<td>4.0 ± 3.6</td>
</tr>
<tr>
<td>TX 0016 (VS)</td>
<td>0.8 ± 1.4</td>
<td>2.4 ± 2.9b</td>
<td>2.8 ± 4.5b</td>
<td>1.4 ± 2.6</td>
<td>2.0 ± 3.0b</td>
<td>2.6 ± 3.7b</td>
<td>0.9 ± 1.5</td>
</tr>
<tr>
<td>TX0218 (VR)</td>
<td>0.2 ± 0.7</td>
<td>0.3 ± 0.8</td>
<td>0.7 ± 2.3</td>
<td>0.5 ± 1.7</td>
<td>0.2 ± 0.8</td>
<td>0.4 ± 1.0</td>
<td>3.2 ± 5.0b</td>
</tr>
<tr>
<td>D399 (VR)</td>
<td>0.1 ± 0.5</td>
<td>0.4 ± 1.1</td>
<td>0.3 ± 0.7</td>
<td>0.0 ± 0.1</td>
<td>0.1 ± 0.4</td>
<td>0.4 ± 1.0</td>
<td>0.2 ± 0.9</td>
</tr>
<tr>
<td>TH1-7 (VR)</td>
<td>0.4 ± 1.0</td>
<td>0.8 ± 1.6</td>
<td>1.3 ± 2.0b</td>
<td>0.6 ± 1.3</td>
<td>0.3 ± 1.3</td>
<td>1.2 ± 2.3b</td>
<td>1.0 ± 2.7</td>
</tr>
</tbody>
</table>

a E. faecium was pretreated with 0.25, 1 or 4 × MIC of antibiotic (quinupristin/dalfopristin or sparfloxacin), or with HBSS alone as a control, before exposure to PMNs. Data are expressed as the number of organisms bound per PMN (mean ± standard deviation of two or three experiments).
b P < 0.05 vs control.
VR, Vancomycin-resistant; VS, vancomycin-susceptible; HBSS, Hank’s buffered salts solution.

significantly increased PMN adherence of FA191 at all concentrations tested and promoted adherence of TX0016 at 0.25 and 1 × MIC though not at 4 × MIC. Sparfloxacin similarly increased PMN adherence of TX0016 at the two lower concentrations, but not at 4 × MIC. Surprisingly, sparfloxacin was ineffective in promoting adherence of FA191 at any of the concentrations examined. Overall, a comparison of the data with these three strains reveals that there may have been slightly greater adherence after pretreatment with quinupristin/dalfopristin than with sparfloxacin but the significance of this is unknown.

In contrast to the above data, with the three vancomycin-resistant strains, namely TX0218, D399 and TH1-7, neither quinupristin/dalfopristin nor sparfloxacin was very effective in promoting PMN adherence, with significant differences seen only after exposure of TX0218 to 4 × MIC of sparfloxacin, and of TH1-7 to 1 × MIC of either quinupristin/dalfopristin or sparfloxacin (Table II). Overall, the amount of adherence to PMNs was markedly lower after antibiotic pretreatment for the three vancomycin-resistant strains of E. faecium than for the vancomycin-susceptible strains.

Discussion

The interactions of antibiotics, bacteria and the host defence system are quite complex. A number of studies with a variety of antibiotics, including sparfloxacin and several different species of bacteria, have demonstrated that treatment of bacteria with antibiotics at either sub-inhibitory or suprainhibitory concentrations may make them more susceptible to subsequent PMN-mediated phagocytosis and killing. Our present results indicate that, at least for some strains of E. faecium, both quinupristin/dalfopristin and sparfloxacin can promote binding to PMNs. While we did not specifically quantify phagocytosis of these strains in this study, ingested organisms were clearly visible inside PMNs. The mechanism whereby antibiotics promote phagocytosis is not well understood. A nitrocellular pretreatment may alter bacterial surface properties, including the amount of capsule present, leading to increased deposition of complement. We have demonstrated that complement functions as the primary opsonin for enterococci and, as we suspect that a capsule may be involved in the resistance to phagocytosis of these E. faecium strains, alterations in the amount or composition of their capsules with the subsequent increased complement deposition could explain their increased PMN adherence after exposure to antibiotics. However, we have no data regarding the amount of capsule present in these strains, nor the composition of their bacterial polysaccharides after antibiotic exposure.

It is interesting to note the marked difference in this study between vancomycin-susceptible and vancomycin-resistant E. faecium and, to a lesser extent, between vancomycin-susceptible strains. Vancomycin-resistant strains had very little change in their binding to PMNs after antibiotic pretreatment, much less than that seen with vancomycin-susceptible strains. The reason for this disparity is unknown, but it does not appear to be related to the amount of antibiotic used, as the MICs of both quinupristin/dalfopristin and sparfloxacin for the vancomycin-resistant strains were generally similar to those of the vancomycin-susceptible strains. A vancomycin-resistant enterococci contain altered peptidoglycan, it is possible that changes in the bacterial cell wall associated with vancomycin resistance could affect the activity of other antibiotics, or the interaction of opsonins, with these bacteria. Alternatively, the variability amongst strains in PMN adherence suggests that either the mechanism of resistance to phagocytosis, or its regulation,
may be different amongst different strains of E. faecium.

Resistance to phagocytosis is a likely virulence factor of E. faecium, as it commonly correlates with increased virulence for other microorganisms. The ability to alter this resistance may be an important component of antibiotic activity in vivo. Thus, antibiotic treatment may favourably affect the outcome of infection not only by directly killing, or inhibiting the growth, of bacteria, but also by making bacteria more susceptible to the antimicrobial mechanisms of the human host defence system.

Acknowledgements

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References


