Effects of prolonged vancomycin administration on methicillin-resistant Staphylococcus aureus (MRSA) in a patient with recurrent bacteraemia

George Sakoulas1*, Howard S. Gold2,3, Robert A. Cohen2,3, Lata Venkataraman2, Robert C. Moellering2,3 and George M. Eliopoulos2,3

1Westchester Medical Center and New York Medical College, Valhalla, NY, USA; 2Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA, USA; 3Harvard Medical School, Boston, MA, USA

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Objectives: To evaluate microbiological properties of methicillin-resistant Staphylococcus aureus (MRSA) during prolonged vancomycin therapy.

Methods: We evaluated vancomycin susceptibility and heteroresistance, accessory gene regulator (agr) function, autolysis, biofilm production and in vitro vancomycin killing in serial MRSA bloodstream isolates obtained over a 30 month period from a patient with a chronic endovascular infection.

Results: Despite the fact that the MRSA in this patient had the same genetic background as other clinical glycopeptide intermediate-resistant S. aureus (GISA) isolates, vancomycin administered for 9 months, maintaining serum concentrations >10 mg/L, did not select for GISA. Minimal changes in vancomycin susceptibility were detected using agar dilution and population analysis methods. We noted increases in delta haemolysin production, autolysis and the bactericidal effects of vancomycin in vitro against the MRSA obtained after prolonged vancomycin suppressive therapy was discontinued.

Conclusions: Despite the lack of development of detectable resistance, MRSA exposed to vancomycin for prolonged periods may begin to develop vancomycin tolerance and decreased autolysis. In addition, suppression of agr function appears to end after vancomycin is stopped. Whether these changes are prerequisites for attenuated vancomycin efficacy and the development of glycopeptide resistance warrants further study. The development of vancomycin resistance may be more difficult under conditions where vancomycin serum concentrations are maintained >10 mg/L.

Keywords: glycopeptides, autolysis, biofilms, agr

Introduction

Staphylococcus aureus is an increasingly common pathogen in community-acquired and nosocomial infections. The emergence of glycopeptide intermediate-resistant S. aureus (GISA)1–3 and, most recently, vancomycin-resistant S. aureus4 further limits therapeutic options for clinicians. In addition, some authors have reported suboptimal clinical efficacy of vancomycin in the treatment of fully vancomycin-susceptible methicillin-resistant S. aureus (MRSA) bloodstream infections since their emergence in the clinical mainstream in the mid-1980s.5–9 The development of GISA may to some extent simply reflect the climax of a sequence of adaptive responses of the organism under increased vancomycin selection pressure. Early changes in the physiology of S. aureus in the pathway towards vancomycin resistance may be undetectable by current methods of susceptibility testing.

We were provided with a unique opportunity to study the physiological effects of prolonged vancomycin exposure on MRSA in a patient with a chronic endovascular infection. We noted significant physiological alterations in the organism with minimal changes in vancomycin susceptibility.
The organism, which eventually became linezolid-resistant (A8830, vancomycin (A8826) led to a switch to linezolid for ongoing suppression. In addition, above 10 mg/L. Breakthrough bacteraemia after 9 months of vancomycin therapy. However, the bacteraemia relapsed four times (isolates A8822–A8825) and each was treated with 6 weeks of vancomycin therapy. However, the bacteraemia relapsed four times (isolates A8822–A8825) and each was treated with 6 weeks of vancomycin with varying durations of gentamicin, after which it was decided to maintain the patient on vancomycin therapy long term. Vancomycin troughs were monitored after dialysis and maintained above 10 mg/L. Breakthrough bacteraemia after 9 months of vancomycin (A8826) led to a switch to linezolid for ongoing suppression. The organism, which eventually became linezolid-resistant (A8830, A8831), was characterized by our laboratory. In the month between isolation of A8830 and A8831, the patient received both vancomycin and linezolid. All bloodstream isolates from this patient were analysed and found to be indistinguishable by pulsed-field gel electrophoresis (PFGE) using methods described previously. Susceptibility testing for vancomycin, oxacillin and linezolid was performed using agar dilution techniques as described by the NCCLS. The increase in delta haemolysin expression was seen in bloodstream isolates obtained before and after therapy with vancomycin (Figure 1).

Analysis of isolates
Vancomycin bactericidal assays were performed using a starting inoculum of 10⁶ cfu/mL bacteria in brain heart infusion (BHI) broth (Becton Dickinson) containing 16 mg/L vancomycin (vancomycin hydrochloride, American Pharmaceutical Partners, Los Angeles, CA, USA) as described previously. Aliquots were obtained at 0, 4, 24 and 72 h for assessment of viable bacterial density as described previously. Vancomycin population analyses were performed using a suspension of ~10¹⁰ cfu/mL of bacteria that were serially diluted at 10-fold intervals and plated in duplicate (25 μL) on BHI agar containing varying concentrations of vancomycin as described previously. Adherence to polystyrene (marker for biofilm production) was performed as described previously by diluting overnight cultures 1:200 in Trypticase soy broth (Difco Laboratories, Detroit, MI, USA) supplemented with 1% glucose and dispensing 200 μL aliquots in triplicate into wells of a sterile 96-well flat-bottom plastic tissue culture plate (Costar Corporation, Cambridge, MA, USA). Bacteria were grown aerobically for 20 h at 35°C. Comparable bacterial growth in each well was confirmed by measuring the optical density at 630 nm (OD₆₃₀) with an MRXII Microplate Reader (Dynex Technologies, Chantilly, VA, USA). The supernatant was removed, and each well was washed four times with 300 μL of sterile phosphate-buffered saline. The adherent cells were fixed by heating at 65°C for 1 h. Cells were stained with 200 μL of Gram crystal violet (Becton Dickinson), and residual stain was removed with tap water. Plates were air dried, and the OD₅₄⁰ of the stained adherent bacterial films was measured.

A semi-qualitative assessment of agr function using delta haemolysin expression was performed on sheep blood agar plates as described previously. Autolysis in Triton X-100 was determined using a modification of methods described previously. Briefly, bacteria were grown in BHI broth to an approximate optical density at 630 nm (OD₆₃₀) of 0.8. Cells were spun down in a microcentrifuge tube for 20 s, washed twice with cold water and resuspended to an OD₆₃₀ of 1.0 in 50 mM glycerol, 0.01% Triton X-100, pH = 8.0. The suspension was aliquoted in triplicate into a sterile 96-well flat-bottom plastic tissue culture plate (Costar Corporation) and incubated at 35°C with gentle agitation. OD₆₃₀ was measured at 4 h using an MRXII Microplate Reader (Dynex Technologies) and expressed as a fraction of the OD₆₃₀ at 0 h.

**Methods**

**Bacterial isolates**
Table 1 demonstrates the temporal relationship of MRSA isolates obtained from a haemodialysis-dependent patient with a persistently bacteraemic endovascular infection that was not amenable to surgical therapy, as described previously. The patient developed the first MRSA bacteraemia with isolate A8821 and responded to 6 weeks of vancomycin therapy. However, the bacteraemia relapsed four times (isolates A8822–A8825) and each was treated with 6 weeks of vancomycin with varying durations of gentamicin, after which it was decided to maintain the patient on vancomycin therapy long term. Vancomycin troughs were monitored after dialysis and maintained above 10 mg/L. Breakthrough bacteraemia after 9 months of vancomycin (A8826) led to a switch to linezolid for ongoing suppression. The organism, which eventually became linezolid-resistant (A8830, A8831), was characterized by our laboratory. In the month between isolation of A8830 and A8831, the patient received both vancomycin and linezolid. All bloodstream isolates from this patient were analysed and found to be indistinguishable by pulsed-field gel electrophoresis (PFGE) using methods described previously. Susceptibility testing for vancomycin, oxacillin and linezolid was performed using agar dilution techniques as described by the NCCLS.

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**Results**
All MRSA bloodstream isolates from this patient demonstrated SmaI macrorestriction enzyme digest patterns by PFGE similar to the predominant MRSA subtype at our hospital (as demonstrated previously) as well as to clinical GISA. All isolates from this patient were susceptible to vancomycin and resistant to oxacillin (Table 1). Vancomycin population analysis of a subset of isolates demonstrated no increase in vancomycin heteroresistance in this patient’s MRSA despite repetitive and continuous exposure to vancomycin (Figure 1).

Analysis of agr function of the initial bloodstream isolate A8821 demonstrated low baseline activity of delta haemolysin (Figure 2). With the sequential administration of vancomycin over 9 months, delta haemolysin activity remained low but was not abolished (Figure 2b). None of the isolates obtained from vancomycin treatment relapse demonstrated a complete lack of delta haemolysin expression. However, a noticeable increase in delta haemolysin expression was seen in bloodstream isolates obtained after therapy was switched from vancomycin to linezolid (Figure 2c–e).

Given the findings by us and others showing an inverse relationship between agr function and adherence to polystyrene, a marker of biofilm production, we investigated polystyrene adherence of sequential bloodstream isolates from this patient. We found that the polystyrene adherence ability of MRSA remained stable during vancomycin administration. MRSA obtained after the discontinuation of vancomycin and switching to linezolid showed a sharp decline in polystyrene adherence (Figure 3).

Sequential bloodstream isolates obtained from this patient during prolonged vancomycin administration demonstrated diminished autolysis in Triton X-100 (Figure 4). Isolates obtained

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**Table 1. Antimicrobial susceptibility of MRSA bloodstream isolates**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Months</th>
<th>VAN</th>
<th>OXA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A8821</td>
<td>0</td>
<td>0.5</td>
<td>128</td>
</tr>
<tr>
<td>A8822</td>
<td>+1</td>
<td>1.0</td>
<td>128</td>
</tr>
<tr>
<td>A8823</td>
<td>+2</td>
<td>1.0</td>
<td>128</td>
</tr>
<tr>
<td>A8824</td>
<td>+4</td>
<td>1.0</td>
<td>&gt;128</td>
</tr>
<tr>
<td>A8825</td>
<td>+6</td>
<td>1.0</td>
<td>&gt;128</td>
</tr>
<tr>
<td>A8826</td>
<td>+9</td>
<td>1.0</td>
<td>&gt;128</td>
</tr>
<tr>
<td>A8827</td>
<td>–1</td>
<td>1.0</td>
<td>&gt;128</td>
</tr>
<tr>
<td>A8828</td>
<td>–3</td>
<td>1.0</td>
<td>128</td>
</tr>
<tr>
<td>A8829</td>
<td>–4</td>
<td>1.0</td>
<td>&gt;128</td>
</tr>
<tr>
<td>A8830</td>
<td>–19</td>
<td>1.0</td>
<td>128</td>
</tr>
<tr>
<td>A8831</td>
<td>+1</td>
<td>1.0</td>
<td>128</td>
</tr>
</tbody>
</table>

VAN, vancomycin; OXA, oxacillin.

*All isolates were resistant to erythromycin and quinolones. All isolates were susceptible to clindamycin, gentamicin and tetracycline.

*A positive number represents months on VAN therapy. A negative number represents months off VAN therapy.

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**700**
after the switch in therapy from vancomycin to linezolid showed a significant increase in Triton-induced autolysis (Figure 4).

To determine whether the prolonged exposure to vancomycin would have an effect on the bactericidal activity of vancomycin \textit{in vitro}, we performed bactericidal assays on a subset of serial isolates from this patient and found that prolonged vancomycin resulted in attenuated bactericidal activity of vancomycin in A8824 and further attenuated activity in A8826. (Figure 5) This effect was reversed in A8830 after vancomycin administration was stopped. In A8831, the isolate obtained after vancomycin was re-administered, reduced killing was again seen at 72 h of inoculate in the presence of vancomycin (Figure 5).
readily yielded GISA. The infecting organism in this report was related to clinical GISA and predominating MRSA clones in the United States by PFGE and agr group II genotype (data not shown). Resistance quickly emerged in vitro when the organism was subjected to subinhibitory concentrations of vancomycin. It is important to note that the infection in this patient was felt to be endovascular, so desirable vancomycin concentrations were reliably achievable with appropriate dosing. Exposing organisms in abscess fluid, biliary system, lung tissue and bone to desirable levels of vancomycin would have been more challenging. On the basis of the ability to prevent the emergence of GISA in this patient,
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previously published in vitro data and the adverse clinical outcomes that are observed in patients in whom GISA or hetero-GISA emerge, we feel it is important to monitor vancomycin serum levels when duration of treatment is expected to be prolonged and to maintain trough concentrations >10 mg/L.

Despite not selecting for GISA, prolonged vancomycin therapy was ultimately unsuccessful for this patient, necessitating the switch to linezolid. In fact, the patient experienced breakthrough bacteremia despite an appropriate vancomycin serum concentration. Although this was not completely surprising given the prosthetic material in association with this infection, it highlights the fact that not all MRSA strains that test susceptible to vancomycin in the microbiology laboratory will respond adequately to vancomycin. This may even occur, as in this case, without the development of the hetero-GISA or GISA phenotypes. It is important to point out that the isolates in this study are still considered susceptible to vancomycin according to the new susceptibility breakpoint of ≤2 mg/L. The breakthrough organism demonstrated decreased in vitro killing in response to vancomycin at 72 h, a characteristic that was accompanied by decreased autolysis in Triton X-100. Both phenotypes have been associated directly or indirectly with prolonged bacteremia on vancomycin therapy.20,23 These phenotypes appeared to be unstable in vivo because they were lost in subsequent isolates obtained after the patient stopped receiving vancomycin. The decreased bactericidal activity of vancomycin quickly returned when vancomycin was re-administered. This instability of phenotype suggests an effect not due to genetic mutations but rather due to changes in differential gene expression or the emergence and submergence of variable bacterial subpopulations depending on the antibiotic selection pressure.

We found it noteworthy that the ability to adhere to polystyrene was influenced by antimicrobial therapy. Specifically, MRSA obtained after vancomycin was replaced by linezolid demonstrated reduced binding to polystyrene as delta haemolysin expression increased. Although we cannot definitively rule out effects of linezolid on decreasing polystyrene adherence, we believe that this phenomenon was due to decreased agr expression induced by vancomycin, resulting in increased biofilm formation in isolates obtained while the patient was receiving vancomycin therapy. We and others have previously shown that agr mutants demonstrate a marked increase in biofilm formation. More recent work by Yao et al.24 provides evidence that in Staphylococcus epidermidis this occurs through decreased bacterial production of phenol-soluble modulins (PSMs), small peptides that serve to inhibit the formation of thick biofilms. Production of these peptides is under the control of agr, and agr mutants demonstrate severely compromised PSM production. Given the marked increase in implantable biomedical devices and the consequential increasing relevance of biofilms in bacterial pathogenesis and resistance, the study of antimicrobial effects on bacterial biofilm formation is of interest.

In summary, this study shows that (i) although this strain showed a propensity to evolve into a hetero-GISA phenotype on exposure in vitro to subinhibitory concentrations of vancomycin, this was not observed in vivo after 9 months on vancomycin. We believe this was due to meticulous maintenance of serum vancomycin concentrations to avoid excessively low serum trough concentrations. (ii) Despite minimal changes in susceptibility by agar dilution and the failure to develop a hetero-GISA phenotype by population analysis, MRSA isolates obtained from clinical failure of vancomycin showed physiological changes when compared with the parent strain, including decreased autolysis, reduced killing by vancomycin in vitro, decreased delta haemolysin expression and increased biofilm production. These phenotypic changes were reversible when vancomycin was discontinued. Whether detecting these physiological changes as a means of more universally predicting vancomycin treatment failure in MRSA bacteremia warrants prospective study.

Transparency declarations

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References


