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Glycopeptide tolerance in *Staphylococcus aureus*

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Treatment failures with vancomycin prompted us to investigate the phenomenon of tolerance to glycopeptides in recent clinical isolates of *Staphylococcus aureus*. We used both MBC/MIC determinations and time–kill measurements to study tolerance to vancomycin and teicoplanin in 35 blood or heart valve isolates of *S. aureus* from patients with endocarditis or bacteraemia. There was generally good agreement between vancomycin tolerance indicated by an MBC:MIC ratio of ≥32 and by ≤90% kill after 6 h incubation in the presence of 20 mg/L vancomycin. However, two isolates were tolerant according to their MBC:MIC ratios but non-tolerant as judged by time–kill measurements. Seven of 15 methicillin-resistant *S. aureus* (MRSA) isolates but only two of 20 methicillin-susceptible ones were tolerant as judged by time–kill experiments (χ^2 = 4.27 with Yates' correction, *P* = 0.04). Seven of the 16 isolates from patients with endocarditis were tolerant, compared with only two of the 19 isolates from patients with other conditions (χ^2 = 3.43 with Yates' correction, *P* = 0.06). Within the endocarditis and non-endocarditis subgroups, tolerance was associated more frequently with methicillin resistance than with susceptibility, but the numbers were too small for the differences to be statistically significant. Most of the vancomycin-tolerant isolates were also tolerant to teicoplanin. We conclude that glycopeptide tolerance is a real phenomenon in *S. aureus*, particularly amongst MRSA isolates, and can be reliably determined by our method of time–kill analysis. Tolerance may compromise glycopeptide therapy of serious *S. aureus* infection and should be taken into account when deciding treatment.

Introduction

High-level resistance to the glycopeptides has not yet appeared in *Staphylococcus aureus*, and vancomycin and teicoplanin are often the therapeutic drugs of last resort for serious staphylococcal sepsis with methicillin- and multiply-resistant *S. aureus* (MRSA) strains. The glycopeptides are usually regarded as bactericidal for *S. aureus*[1] and are often used for the treatment of endocarditis. However, there are several reports of sporadic glycopeptide-tolerant strains of *S. aureus*[2–9] in which the isolates remain susceptible as judged by MIC determinations but show increased resistance to killing. These tolerant strains may be responsible for treatment failures.

Assessment of the importance of tolerance has been hampered by the unreliability of methods for its detection.[10] For example, problems with MBC determinations include adhesion of cells to test-tube walls above the meniscus of the antibiotic-containing medium, antibiotic carry-over on subculture and the Eagle effect.[10,11] Because of the potential clinical importance of this phenomenon, we have examined tolerance to vancomycin and teicoplanin in recent blood isolates of *S. aureus*, including some from patients with endocarditis, paying particular attention to the methodology of tolerance detection.

Materials and methods

Microorganisms

We examined 35 *S. aureus* isolates from patients presenting at St Thomas' Hospital or Guy's Hospital during 1993–1997. Sixteen isolates were obtained from the blood or valve cultures of patients with clinically defined endocarditis, and seven of these were MRSA. There were 19 blood isolates of *S. aureus* from patients with bacteraemia but no evidence of endocarditis, and of these seven were MRSA.

S. aureus was identified on the basis of its colonial and Gram stain morphology and catalase and coagulase production. Methicillin-susceptibility was determined by broth microdilution[12] and defined with a breakpoint of

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4 mg/L. Phage typing results, where available, were obtained from the Laboratory of Hospital Infection, Central Public Health Laboratory, Colindale, London, UK. *S. aureus* ATCC 29213 was used as the control organism in all susceptibility test procedures and time–kill studies. All organisms were stored at −70°C in glycerol broth. Fresh cultures were used for each experiment.

Antibiotics

Vancomycin was obtained from Sigma Chemical Co. (Poole, UK) and teicoplanin from Hoechst Marion Roussel (Milton Keynes, UK).

Media

IsoSensitest broth (Oxoid, Basingstoke, UK) was used in both the broth microdilution susceptibility tests and the time–kill studies. All colony counts were performed on Columbia base agar (Oxoid) supplemented with 7% defibrinated horse blood (TCS, Botolph Clayton, UK). Medium was pre-warmed for time–kill experiments. The initial inoculum was grown in Brain Heart Infusion (BHI) broth (Oxoid).

Preparation of inocula

Inocula for the MBC/MIC and time–kill determinations were prepared following NCCLS guidelines.[13] For the broth macrodilution method, organisms were inoculated from an overnight agar culture into BHI broth and shaken for 3 h at 37°C. The turbidity of the logarithmic phase culture was adjusted to a 0.5 McFarland standard in IsoSensitest broth. Sufficient was inoculated into the tubes containing 2 mL of the antibiotic dilution to give a final inoculum of $1–5 \times 10^5$ cfu/mL. The inoculum used was controlled by performing a colony count for each test. Ten-fold dilutions were made in physiological saline and a pipette used to deliver 10 μL samples for each dilution to a blood agar plate. The inoculum was spread over the surface of the plates, which were incubated for 48 h at 37°C. The colonies were counted and the inoculum size was calculated.

In preparation for the time–kill method, organisms were grown overnight in BHI broth. After an initial dilution in 10 mL of IsoSensitest broth, a further dilution was made in glass flasks containing 20 mL of broth and shaken for 90 min at 37°C. The dilution was calculated to achieve a final concentration of $1–5 \times 10^5$ cfu/mL.

Broth macrodilution method for MBC/MIC determination

To determine MICs and MBCs we used the NCCLS broth macrodilution method for aerobic bacteria.[13] The experiments were performed with strict adherence to the NCCLS guidelines, standardizing the technique by inoculating all tubes after the initial inoculum had been shaken for 3 h.

Time–kill studies

Time–kill studies were performed according to NCCLS methodology.[13] Each isolate was inoculated into three flasks, one as a growth control and one for each antibiotic. The antibiotic concentrations (chosen to reflect serum levels *in vivo*) were 20 mg/L vancomycin and 10 mg/L teicoplanin.[14] Antibiotics were added to the flasks after the 90 min inoculum preparation. The flasks were shaken at 150 rpm at 37°C (Certomat, B. Braun Biotech, Aylesbury, UK) and subcultured at 0, 2, 4, 6 and 24 h. Colony counts were performed by making appropriate dilutions in physiological saline, plating 100 μL of each dilution on pre-warmed blood agar and incubating for 48 h at 37°C. Viable counts were calculated to give cfu/mL and kill curves were plotted with time against the logarithm of the viable count.

Definitions of tolerance

Standard definitions of tolerance are an MBC:MIC ratio of ⩾32 in susceptibility tests or a kill of ⩽99.9% after 24 h incubation in time–kill experiments. We assessed tolerance by the standard definitions for MBC/MIC ratios but followed the suggestion of Handwerger & Tomasz[10] for time–kill studies and determined a kill at 6 h. If a constant logarithmic rate of killing is assumed, 90% kill at 6 h is equivalent to 99.9% kill at 24 h. We therefore used a kill of ⩽90% at 6 h as the criterion for tolerance. These changes to the methodology eliminated the problem of regrowth between 6 and 24 h that occurred in some isolates.

Kill measurements were made by determining the actual reduction in viable counts at 6 h and by calculating the kill at 6 h from the slope of the decline in the viable count as suggested by the NCCLS.[13] For this latter calculation, regression analysis of time versus logarithm of viable count was performed with a computer program,[15] and a slope of −0.167 (which corresponds to a kill of 90% in 6 h) was used as the breakpoint to define tolerance and non-tolerance.

Results

MBC:MIC ratios

The NCCLS[13] recommends an incubation time of 3–5 h for the inoculum for the determination of MBCs. However, we observed that there were significant differences in the number of viable cells after overnight incubation in the presence of various concentrations of vancomycin when

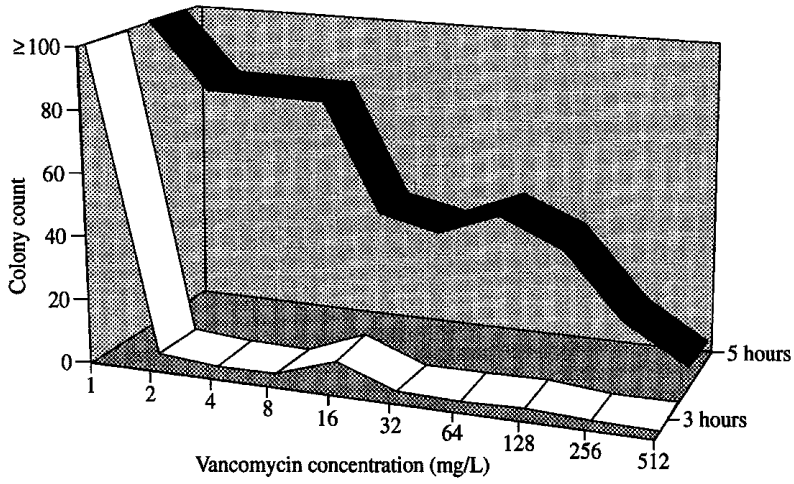

Figure 1. The effect of incubation time before inoculation on colony counts obtained in MBC determinations for *S. aureus* ATCC 29213.

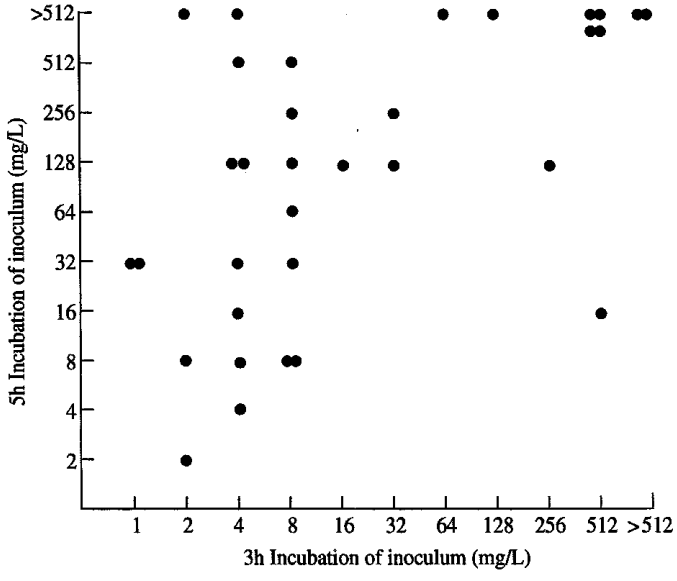

Figure 2. Comparison of vancomycin MBCs (mg/L) determined with 3 h and 5 h incubation of the inoculum for clinical isolates of *S. aureus*, showing a lack of correlation.

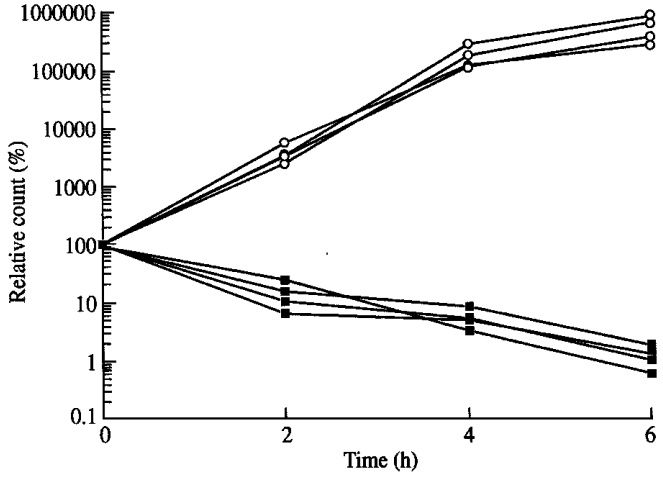

Figure 3. Reproducibility of killing of *S. aureus* ATCC 29213 by vancomycin (20 mg/L) (■) compared with control (○).

inocula prepared after 3 h and 5 h were compared (Figure 1). Furthermore. there was considerable variability in the MBCs determined with an inoculum made from a 5 h incubation. ATCC 29213 would have been designated vancomycin-tolerant in four of the seven experiments with this inoculum. In contrast, a 3 h incubation for the inoculum gave better reproducibility and classified ATCC 29213 as non-tolerant. With clinical isolates of *S. aureus* there was little correlation between vancomycin MBCs determined with 3 h and 5 h inocula (Figure 2); MBCs determined with the 5 h inoculum were frequently higher than those for the 3 h inoculum. For all these reasons we used an incubation time of 3 h for the preparation of inocula for the determination of MICs and MBCs.

Time–kill curves

Four independent killing curve determinations for the control strain, ATCC 29213, incubated in the presence of 20 mg/L vancomycin showed good reproducibility (Figure 3). Results for two representative clinical isolates are shown in Figure 4. Isolate 112 was not tolerant and was readily killed by both vancomycin and teicoplanin (Figure 4a), whereas the tolerant isolate 119 was much less readily killed by these compounds (Figure 4b). Teicoplanin (10 mg/L) had a slightly greater effect than vancomycin (20 mg/L) on the viability of both these isolates, although this was not always the case with other isolates.

Vancomycin and teicoplanin tolerance

The results of tests for vancomycin tolerance on all isolates are summarized in Table I. There were no discrepancies

Table I. Vancomycin tolerance of *S. aureus* isolates

Isolate	Vancomycin		Slope of killing curve	Calculated kill (%) after 6 h	Kill (%) after 6 h	Tolerant on basis of		
	MIC (mg/L)	MBC (mg/L)				MBC[a]	slope[b]	survival[c]
From patients with encocarditis								
methicillin-susceptible								
112	1	4	−0.38	99.4	99.9	−	−	−
105	1	2	−0.35	99.2	99.3	−	−	−
101	1	16	−0.28	98.0	97.8	−	−	−
108	1	2	−0.27	97.5	97.1	−	−	−
102	1	2	−0.23	96.0	96.0	−	−	−
109	1	1	−1.20	94.0	94.7	−	−	−
113	1	1	−0.20	93.4	93.1	−	−	−
107	1	128	−0.15	87.0	88.5	+	+	+
116	1	512	−0.14	86.3	85.0	+	+	+
methicillin-resistant								
117	1	4	−0.40	99.6	99.7	−	−	−
124	2	>512	−0.24	96.3	96.4	+	−	−
103	1	>512	−0.13	83.8	88.0	+	+	+
120	2	>512	−0.12	80.5	77.8	+	+	+
115	1	512	−0.11	77.5	79.5	+	+	+
119	2	>512	−0.09	70.3	68.0	+	+	+
111	2	512	−0.07	59.9	58.0	+	+	+
Not from patients with endocarditis								
methicillin-susceptible								
209	1	4	−0.60	99.9	99.8	−	−	−
210	1	8	−0.39	99.5	99.6	−	−	−
201	1	8	−0.33	99.0	98.7	−	−	−

202	1	8	−0.33	99.0	98.5	−	−	−
206	1	8	−0.27	97.6	97.2	−	−	−
212	1	8	−0.27[d]	91.3[d]	91.3[d]	−	−	−
205	1	16	−0.23	95.8	95.4	−	−	−
207	1	4	−0.23	95.8	95.6	−	−	−
215	1	1	−0.22	95.3	95.1	−	−	−
204	1	8	−0.18	97.7	92.2	−	−	−
213	1	2	−0.18	92.1	99.7	−	−	−
methicillin-resistant								
216	1	4	−0.53	99.9	99.6	−	−	−
220	0.5	128	−0.31	98.7	98.8	−	−	−
217	1	4	−0.27	97.6	97.8	−	−	−
211	2	4	−0.26	97.4	97.3	−	−	−
208	1	4	−0.25	96.8	97.0	−	−	−
214	1	8	−0.18	92.1	91.3	−	−	−
218	1	128	−0.12	82.7	79.5	+	+	+
203	1	>512	−0.09	71.2	60.6	+	+	+
Control strain								
ATCC 29213	1	1	−0.30	98.4	98.0	−	−	−

[a]Tolerant if MBC:MIC ratio is ⩾32.
[b]Tolerant if slope of killing curve is ⩽0.167 (or calculated kill at 6 h ⩽ 90%).
[c]Tolerant if kill at 6 h is ⩽90%.
[d]Because of regrowth between 4 and 6 h, actual kill at 4 h is given, and viable counts at 0–4 h used for estimation of slope and calculated kill (at 4h).

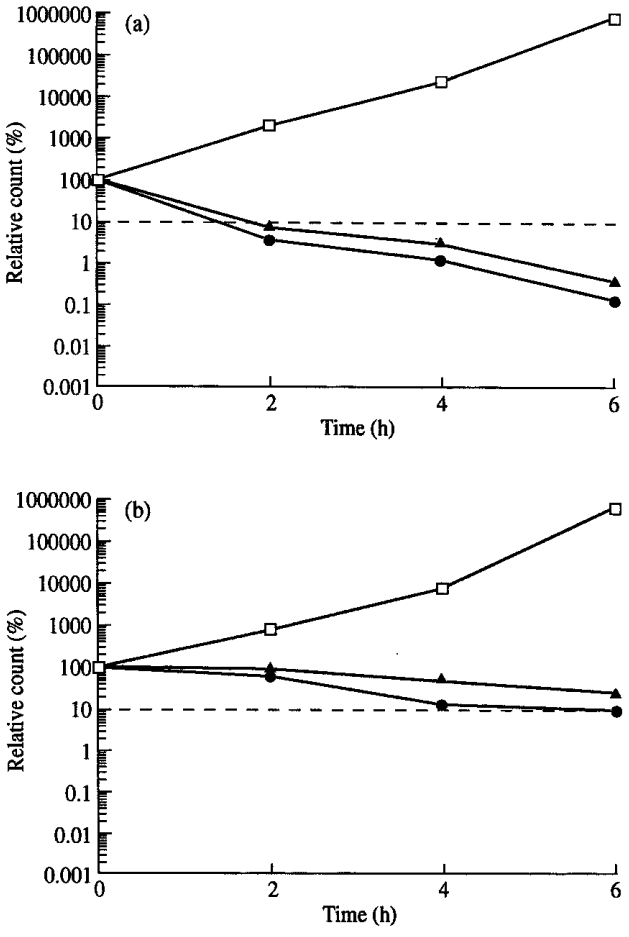

Figure 4. Killing of *S. aureus* isolates 112 (a) and 119 (b) by glycopeptides. Key to symbols: □, growth control; ▲, vancomycin (20 mg/L); ●, teicoplanin (10 mg/L)

between the methods except that isolates 124 and 219 were tolerant on the basis of MBC:MIC ratios but non-tolerant if the rate of killing was used. When tolerance was assessed by measuring the rate of killing by 20 mg/L vancomycin, seven of the 15 MRSA, but only two of the 20 methicillin-susceptible isolates, were tolerant (Table II; $\chi^2 = 4.27$ with Yates' correction, $P = 0.04$). Seven of the 16 isolates from patients with endocarditis were tolerant, compared with only two of the 19 isolates from patients without endocarditis ($\chi^2 = 3.43$ with Yates' correction, $P = 0.06$). Within both endocarditis and non-endocarditis subgroups, tolerance was associated more frequently with methicillin-resistant than with methicillin-susceptible isolates (Table II), but the numbers were too small for the differences to be statistically significant.

Tolerance to teicoplanin was assessed for isolates from patients with endocarditis and the control strain (Table III). One isolate (no. 102) was tolerant on the basis of MBC:MIC ratio but non-tolerant if the rate of killing by 10 mg/L teicoplanin was used. There was also one isolate (no. 119) that was tolerant on the basis of MBC:MIC ratio and the rate of killing but non-tolerant on the basis of survival at 6 h (i.e. if the counts at 2 and 4 h were not taken into account).

When tolerance was assessed by measuring the rate of killing, eight isolates were non-tolerant to both vancomycin and teicoplanin, five were tolerant to both compounds and one (no. 107) was tolerant to vancomycin but not to teicoplanin.

Typing results

Phage typing results from the Central Public Health Laboratory were available for 14 of the 16 isolates from patients with endocarditis. These indicated that these organisms were not multiple isolates of epidemic strains and that there was no association of type with glycopeptide. Of tolerant isolates, one each were of type II+, I/II, IV, and EMRSAvar, two were non-typable and for one there was no result.

Discussion

Tolerance is usually considered in relation to β-lactam antibiotics, and was first described in a mutant strain of *Streptococcus pneumoniae* with an impaired autolytic system.[16] However, Sabath *et al.*,[17] in the paper in which tolerance in *S. aureus* was first reported, stated that most β-lactam-tolerant strains of *S. aureus* showed cross-tolerance to vancomycin. Such cross-tolerance is not universal and strains can have tolerance to β-lactams but not to vancomycin.[10] The mechanism of tolerance in clinical strains remains unclear. It is often associated with

Table II. Association of vancomycin tolerance with methicillin susceptibility test result and underlying disease

	Number of vancomycin-tolerant isolates/total number of isolates		
Underlying disease	methicillin-susceptible	methicillin-resistant	susceptible or resistant
Endocarditis	2/9	5/7	7/16
Not endocarditis	0/11	2/8	2/19
All isolates	2/20	7/15	9/35

Table III. Teicoplanin tolerance of *S. aureus* isolates

Isolate	Teicoplanin		Slope of killing curve	Calculated kill (%) after 6 h	Kill (%) after 6 h	Tolerant on basis of		
	MIC (mg/L)	MBC (mg/L)				MBC[a]	slope[b]	survival[c]
From patients with encocarditis								
methicillin-susceptible								
112	1	8	−0.44	99.8	99.8	−	−	−
105	1	8	−0.36	99.3	99.0	−	−	−
108	1	4	−0.37	99.4	99.4	−	−	−
102	1	64	−0.32	98.9	98.7	+	−	−
109	1	8	−0.17	90.6	91.7	−	−	−
113	1	4	−0.43	99.7	99.7	−	−	−
107	1	4	−0.38	99.5	99.3	−	−	−
methicillin-resistant								
117	0.5	8	−0.28	97.8	97.5	−	−	−
124	4	16	−0.19	92.6	93.1	−	−	−
103	4	>256	−0.12	79.7	82.6	+	+	+
120	4	>256	−0.13	84.1	83.6	+	+	+
115	0.5	128	−0.15	87.7	87.5	+	+	+
119	4	256	−0.17	88.6	91.1	+	+	−
111	1	>256	−0.13	83.3	83.1	+	+	+
Control strain								
ATCC 29213	1	4	−0.44	99.8	99.7	−	−	−

[a] Tolerant if MBC:MIC ratio is ⩾32.
[b] Tolerant if slope of killing curve is ⩽0.167 (or calculated kill at 6 h is ⩽90%).
[c] Tolerant if kill at 6 h is ⩽90%.

autolysin deficiency in both clinical isolates and laboratory mutants,[10,16–18] but this is not always so[19] and other mechanisms may be present in tolerant strains from clinical sources.[10]

There are many conflicting observations in the literature on tolerance to antibiotics that interfere with peptidoglycan synthesis in staphylococci. This is probably a consequence of the various definitions and techniques used to define and determine tolerance, and the effect of the physiological state of the organism at the time it encounters the antibiotic on the rate and extent of lysis.[10]

The macrodilution method for the determination of MICs and MBCs is preferred to the micro method, which is more prone to error.[20] Our results indicate that the range of incubation times prescribed by the NCCLS[13] for preparation of the inocula (3–5 h) is too wide for reproducible MBC determinations. Because of this, we standardized the incubation of the inoculum at 3 h. We found that vancomycin MICs can be determined reliably for *S. aureus*, but confirm that, because of the poor reproducibility of MBC determinations, MBC:MIC ratios should be used with caution for the detection of vancomycin tolerance.

Following the suggestions of Handwerger & Tomasz,[10] we used time–kill determinations as the definitive method for the detection of tolerance. With this method tolerance is often assessed at 24 h, but these authors stated that, "within 2–6 h after the addition of antibiotic, tolerant and non-tolerant strains can be differentiated by their relative rates of killing". Because some of our isolates showed regrowth between 6 and 24 h in some experiments, we used killing during the first 6 h to detect tolerance. We used a reduction in viable count to $<90\%$ of the initial inoculum to indicate tolerance. There was good agreement between kills predicted from the slope of the rate of kill and the actual value measured at 6 h (Tables I and III). The use of the slope for the assessment of tolerance was preferred because it reduced the effects of errors in single viable count determinations. Regrowth occurred between 4 h and 6 h with one isolate, and in this case we used the substantial kill over the first 4 h to indicate lack of tolerance. This is another example of the advantage of assessing kill over several time intervals rather than extrapolating from a single measurement.

Although there was generally good agreement between tolerance as determined by time–kill experiments and that determined by MBC:MIC ratios, there were some discrepancies. However, our findings indicate that a low MBC:MIC ratio can be used to exclude tolerance.

Since we found glycopeptide tolerance mostly in MRSA isolates, it was usually not possible to assess cross-tolerance with β-lactam antibiotics. Although there are β-lactam-tolerant organisms that are not tolerant to vancomycin,[10] we are not aware of reports of vancomycin tolerance without β-lactam tolerance. The high degree of correlation between vancomycin and teicoplanin tolerance is to be expected, since both are glycopeptides.

We found glycopeptide tolerance much more frequently in methicillin-resistant strains of *S. aureus* than in methicillin-susceptible ones, confirming the observations of Mlynarczyk *et al.*[7] in Poland. However, the glycopeptide-tolerant MRSA isolates in the present study did not represent multiple isolations of a small number of epidemic strains since the typing results indicated that the strains were different. The association of glycopeptide tolerance with methicillin resistance could result from the different penicillin-binding proteins of MRSAs caused by the expression of *mecA*, and hence differences in peptidoglycan metabolism. The growth rate of MRSAs in lag phase is slower than that of methicillin-susceptible staphylococci[21] and, since the mode of action of glycopeptides is on the cell wall, this may play a contributing role in tolerance.

Tolerance was also significantly more common in isolates from cases of endocarditis. We do not know the reason for this, but it is possible that tolerant mutants are readily selected during therapy of foreign body infections or that tolerant strains evade prophylaxis. Chuard *et al.*[22] provided some experimental evidence for this when they found that MBCs of vancomycin for two strains of *S. aureus* increased by more than 100 times after culture for 3–6 weeks in subcutaneous tissue cages in rats. Voorn *et al.*[23] supported the concept that tolerance may emerge during clinical therapy by showing that vancomycin tolerance can be lost or gained *in vitro* during repeated subculture in the presence or absence of antibiotic.

In the present study it was not possible to determine the effect of glycopeptide tolerance on the effectiveness of therapy since this was not followed prospectively. However, there are a number of reports in the literature indicating that tolerance does adversely affect the outcome of serious *S. aureus* infections treated with vancomycin or teicoplanin. Some of these are case reports in which additional antibiotics were needed for bactericidal therapy.[2,3] Others are larger studies. Rajashekaraiah *et al.*[4] reported vancomycin tolerance (defined as MIC:MBC ratios $\geqslant 16$) in 32/50 strains of *S. aureus* from patients with endocarditis and 35/54 strains from those with bacteraemia alone. There was no difference in outcome in bacteraemic cases, but in patients with endocarditis tolerance was associated with a poorer response to therapy and an increased mortality (25% compared with 11%). In other studies tolerance was associated with failure of vancomycin therapy of MRSA bacteraemia[5,8] and with failure of teicoplanin therapy of *S. aureus* endocarditis.[9] Finally, tolerance impaired vancomycin and teicoplanin prophylaxis (but not treatment) of experimental *S. aureus* endocarditis in rats.[24]

In conclusion, we have demonstrated that, although glycopeptides are usually bactericidal against *S. aureus*, tolerance is common amongst MRSA isolates. There is evidence in the literature that glycopeptide tolerance

adversely affects the outcome of antimicrobial therapy for staphylococcal endocarditis and septicaemia, and this should be taken into account when deciding therapy.

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References

1. Cooper, G. L. & Given, D. B. (1986). *Vancomycin: Comprehensive Review of 30 Years of Clinical Experience*. Park Row, New York.

2. Faville, R. J., Zaske, D. E., Kaplan, E. L., Crossley, K., Sabath, L. D. & Quie, P. G. (1978). *Staphylococcus aureus* endocarditis. Combined therapy with vancomycin and rifampin. *Journal of the American Medical Association* **240**, 1963–5.

3. Gopal, V., Bisno, A. L. & Siverblatt, F. J. (1976). Failure of vancomycin treatment in *Staphylococcus aureus* endocarditis. In vivo and in vitro observations. *Journal of the American Medical Association* **236**, 1604–6.

4. Rajashekaraiah, K. R., Rice, T., Rao, V. S., Marsh, D., Ramakrishna, B. & Kallick, C. A. (1980). Clinical significance of tolerant strains of *Staphylococcus aureus* in patients with endocarditis. *Annals of Internal Medicine* **93**, 796–801.

5. Sorrell, T. C., Packham, D. R., Shanker, S., Foldes, M. & Munro, R. (1982). Vancomycin therapy for methicillin-resistant *Staphylococcus aureus. Annals of Internal Medicine* **97**, 344–50.

6. Leport, C., Perronne, C., Massip, P., Canton, P., Leclercq, P., Bernard, E. *et al*. (1989). Evaluation of teicoplanin for treatment of endocarditis caused by Gram-positive cocci in 20 patients. *Antimicrobial Agents and Chemotherapy* **33**, 871–6.

7. Mlynarczyk, G., Mlynarczyk, A. & Meisel-Mikolajczyk, F. (1995). Evaluation of susceptibility of methicillin resistant *Staphylococcus aureus* strains to vancomycin. *Medycyna Doswiadczalna I Mikrobiologia* **47**, 133–9.

8. Reis, A. G., Eisencraft, A. P., Ejzenberg, B., Baldacci, E. R., Kodaira, M. S., Domingues, P. P. *et al*. (1995). *Staphylococcus aureus* septicemia in children: bacterial tolerance to vancomycin and serum bactericidal activity. *Revista Da Associacao Medica Brasileira* **41**, 47–52.

9. Wilson, A. P. & Gaya, H. (1996). Treatment of endocarditis with teicoplanin: a retrospective analysis of 104 cases. *Journal of Antimicrobial Chemotherapy* **38**, 507–21.

10. Handwerger, S. & Tomasz, A. (1985). Antibiotic tolerance among clinical isolates of bacteria. *Reviews of Infectious Diseases* **7**, 368–86.

11. Pelletier, L. L. (1984). Lack of reproducibility of macrodilution MBCs for *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy* **26**, 815–8.

12. Phillips, I., King, A., Gransden, W. R. & Eykyn, S. J. (1990). The antibiotic sensitivity of bacteria isolated from the blood of patients in St Thomas' Hospital, 1969–1988. *Journal of Antimicrobial Chemotherapy* **25**, *Suppl. C*, 59–80.

13. National Committee for Clinical Laboratory Standards. (1992). *Methods for Determining Bactericidal Activity of Antimicrobial Agents: Tentative Guideline. M26-T*. NCCLS, Villanova, PA.

14. Lorain, V. (1996). *Antibiotics in Laboratory Medicine*, 4th edn. Williams & Wilkins, Baltimore, MD.

15. Shannon, K. & Phillips, I. (1990). A computer program for the storage and analysis of minimum inhibitory concentrations of antimicrobial agents. *Binary* **2**, 89–95.

16. Tomasz, A., Albino, A. & Zanati, E. (1970). Multiple antibiotic resistance in a bacterium with suppressed autolytic system. *Nature* **227**, 138–40.

17. Sabath, L. D., Lavadiere, M., Wheeler, N., Blazevic, D. & Wilkinson, B. J. (1977). A new type of penicillin resistance in *Staphylococcus aureus. Lancet i*, 443–7.

18. Mani, N., Tobin, P. & Jayaswal, R. K. (1993). Isolation and characterization of autolysis-defective mutants of *Staphylococcus aureus* created by Tn*9l7–lacZ* mutagenesis. *Journal of Bacteriology* **175**, 1493–9.

19. Williamson, R. & Tomasz, A. (1980). Antibiotic-tolerant mutants of *Streptococcus pneumoniae* that are not deficient in autolytic activity. *Journal of Bacteriology* **144**, 105–13.

20. James, P. A. (1990). Comparison of four methods for the determination of MIC and MBC of penicillin for viridans streptoccoci and the implications of penicillin tolerance. *Journal of Antimicrobial Chemotherapy* **25**, 209–16.

21. Mizobuchi, S., Minami, J., Jin, F., Matsushita, O. & Okabe, A. (1994). Comparison of the virulence of methicillin-resistant and methicillin-sensitive *Staphylococcus aureus. Microbiology and Immunology* **38**, 599–605.

22. Chuard, C., Lucet, J. C., Rohner, P., Herrmann, M., Auckenthaler, R., Waldvogel, F. A. *et al*. (1991). Resistance of *Staphylococcus aureus* recovered from infected foreign body *in vivo* to killing by antimicrobials. *Journal of Infectious Diseases* **163**, 1369–73.

23. Voorn, G. P., Thompson, J., Goessens, W. H., Schmal-Bauer, W. C., Broeders, P. H. & Michel, M. F. (1994). *In vitro* development and stability of tolerance to cloxacillin and vancomycin in *Staphylococcus aureus. European Journal of Clinical Microbiology and Infectious Diseases* **13**, 741–6.

24. Voorn, G. P., Kuyvenhoven, J., Goessens, W. H., Schmal-Bauer, W. C., Broeders, P. H., Thompson, J. *et al*. (1994). Role of tolerance in treatment and prophylaxis and experimental *Staphylococcus aureus* endocarditis with vancomycin, teicoplanin, and daptomycin. *Antimicrobial Agents and Chemotherapy* **38**, 487–93.

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