Journal of Antimicrobial Chemotherapy (2001) **47**, 163–170

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In vivo emergence of subpopulations expressing teicoplanin or vancomycin resistance phenotypes in a glycopeptide-susceptible, methicillin-resistant strain of *Staphylococcus aureus*

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Several reports indicate the emergence of subpopulations resistant to glycopeptides in some clinical isolates of *Staphylococcus aureus*. While the development of glycopeptide resistance in *S. aureus* is easily observed *in vitro*, the *in vivo* conditions promoting emergence of glycopeptide-resistant subpopulations are unknown. Using a rat model, subcutaneous implants were chronically infected with a methicillin-resistant strain of *S. aureus*, MRGR3, devoid of a significant (>10^{-7}) glycopeptide-resistant subpopulation at 2 mg/L of either teicoplanin or vancomycin. After 3 weeks of infection in antibiotic-untreated animals, subpopulations emerged, growing on agar containing 10 mg/L of either glycopeptide. These subpopulations were detected in all tissue cage fluids containing >7 log cfu/mL at average frequencies of 4×10^{-5} and 2×10^{-5} on teicoplanin- and vancomycin-containing agar, respectively. While teicoplanin MICs increased two- to 16-fold, vancomycin MICs increased by less than two-fold. Population analysis and survival kinetic studies of three teicoplanin-selected subclones indicated that transfer from solid to liquid medium conditions decreased expression of teicoplanin resistance in the bacterial population. In Mueller–Hinton broth, >90% of cells remained fully resistant to antibiotic, but did not grow in the presence of teicoplanin for an initial period of at least 6 h. All three teicoplanin-resistant subclones expressed stable teicoplanin resistance with slight cross-resistance to vancomycin after a few transfers on teicoplanin-supplemented agar. These data suggest that some *in vivo* conditions may lead to selection of *S. aureus* subpopulations exhibiting decreased glycopeptide susceptibility and growing in the presence of otherwise inhibitory concentrations of these antimicrobial agents.

Introduction

The recent emergence of multi-drug-resistant clones of methicillin-resistant *Staphylococcus aureus* (MRSA) expressing decreased susceptibility to glycopeptides (GISA) brings challenges for hospital infection control,[1–4] antimicrobial therapy[4–11] and antimicrobial susceptibility testing.[11–16] Detection of glycopeptide resistance is particularly difficult in the vast majority of clinical isolates detected so far, which express a highly heterogeneous mode of resistance to glycopeptides (hGISA). While some more elaborate techniques such as population analysis profiles have been proposed for detection of staphylococcal subpopulations resistant to glycopeptides,[6,7,9] this approach cannot be proposed for routine testing and is not suitable for large-scale clinical and epidemiological studies.[17,18]

A number of *in vitro* studies have documented the stepwise development of resistance to either vancomycin or teicoplanin in *S. aureus*,[6,7,9,19–25] but *in vivo* conditions promoting emergence of glycopeptide resistance have not been described experimentally. The molecular mechanisms of glycopeptide resistance in *S. aureus* have not yet been elucidated, yet they are clearly different from those found in enterococcal strains.[5,12] *In vivo* emergence of glycopeptide resistance in *S. aureus* was first reported during teicoplanin therapy of either severely infected patients[22,26,27] or rabbits with experimental endocarditis.[22,28] Such *in vivo* teicoplanin-resistant isolates frequently exhibit a greater

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increase in teicoplanin compared with vancomycin MICs. A similar observation was made after *in vitro* selection of resistant organisms to teicoplanin compared with vancomycin.[22,23,27] Despite these glycopeptide-specific differences, teicoplanin- and vancomycin-resistant mutants of *S. aureus* resulting from *in vivo* or *in vitro* exposure have some biochemical and morphological changes in common, in particular significant cell wall thickening, increased penicillin-binding protein 2 (PBP2) production and an increased binding capacity for glycopeptides by peptidoglycan.[6,7,9,19,23–25,27,29]

We reported previously that a methicillin-resistant strain of *S. aureus* recovered from subcutaneous implant exudates in a rat model of chronic foreign body infection included subpopulations that would grow on agar containing 10 times the teicoplanin MIC.[30] The subpopulations growing on teicoplanin-supplemented agar represented $>10^{-5}$ of the total number of organisms cultivated from tissue cage fluids, after 3 weeks of infection, in contrast to the same strain grown *in vitro*, which yielded a $<10^{-7}$ teicoplanin-resistant colony. Emergence of subpopulations of *S. aureus* growing on teicoplanin-supplemented agar during experimental foreign body infection occurred in the absence of any prior antibiotic exposure and was not promoted further by high-dose teicoplanin therapy. Furthermore, conventional MIC testing of colonies removed from teicoplanin-supplemented agar failed to demonstrate any significant increase in teicoplanin MICs following subcultures in antibiotic-free liquid medium.

This report describes further characteristics of the emerging *in vivo* glycopeptide-resistant subpopulations. Conditions affecting the stability of the resistance phenotype of these subpopulations are described. Finally, a selection of subclones derived from the *in vivo*-selected teicoplanin-resistant subpopulations and expressing stable high levels of glycopeptide resistance is also reported.

Materials and methods

Bacterial strain

The MRSA strain MRGR3,[31,32] used for *in vitro* and animal studies, and expressing heterogeneous resistance to methicillin, was isolated in 1979 from a patient with catheter-related sepsis and selected for its virulence properties in the rat model of chronic *S. aureus* tissue cage infection.[31] Strain MRGR3 is also resistant to penicillin, gentamicin, chloramphenicol, erythromycin, tetracycline and polymyxin, but not to fluoroquinolones. The average MIC and MBC of teicoplanin (Lepetit Research Center, Varese, Italy) for strain MRGR3 grown in cation-adjusted Mueller–Hinton broth (MHB; Difco Laboratories, Detroit, MI, USA) were reported previously as 1 and 2 mg/L, respectively, as determined by a macrodilution method.[30] Identical values were found with vancomycin (Laboratory Lilly, Giessen, Germany).[31] Standard overnight cultures in MHB of strain MRGR3 showed the absence of any glycopeptide-resistant subpopulation growing on Mueller–Hinton agar (MHA) containing 2 mg/L of teicoplanin or vancomycin at a limit of detection of 10^{-7}.

Detection of bacterial subpopulations growing on glycopeptide-supplemented agar

Experiments involving rats were approved by the Ethics Committee of the Faculty of Medicine of the University of Geneva and by the Veterinary Office of the State of Geneva. The *in vivo* emergence of subpopulations of *S. aureus* growing on either teicoplanin- or vancomycin-supplemented agar was studied in a rat model of *S. aureus* chronic tissue cage infections, composed of four tissue cages subcutaneously implanted in Wistar rats as described previously.[31] At 3 weeks post-implantation, tissue cage fluid was aspirated and checked for sterility, then tissue cages were inoculated with 0.1 mL of saline containing $0.2–2 \times 10^6$ cfu of strain MRGR3 as described previously.[31–33] Three weeks later, all tissue cages were punctured and quantitative cultures of 10-fold serially diluted tissue cage fluids performed on either glycopeptide-free MHA or MHA containing 10 mg/L of either teicoplanin or vancomycin. To optimize the yield of viable bacteria, tissue cage fluids were briefly (60 W, 1 min) sonicated (model 2200; Brandson Ultrasonics, Branburry, CT, USA) to disrupt the biofilm and phagocytic cells before the serial dilutions and plating. Plates were incubated for 24–48 h at 37°C. The detection limit was one colony equivalent to 2 $\log_{10}$ cfu/mL of tissue cage fluid.

A very similar procedure was used to record the population analysis profiles of tissue cage fluid bacteria. In this case, quantitative cultures of tissue cage fluid organisms were performed on MHA containing either 0, 2, 4 or 8 mg/L of teicoplanin as described above.

Determination of MICs

MICs of teicoplanin and vancomycin for tissue cage bacterial colonies grown on glycopeptide-supplemented MHA were determined by the broth microdilution method in cation-adjusted MHB according to the standards of the National Committee for Clinical Laboratory Standards (NCCLS).[34] Suspensions of one to several bacterial colonies removed from either teicoplanin- or vancomycin-supplemented agar, or of strain MRGR3 grown on glycopeptide-free agar as a control, were prepared in phosphate-buffered saline (PBS) and adjusted to a turbidity equal to McFarland 0.5 (*c.* 10^8 cfu/mL). Thereafter, 100 μL portions of 100-fold diluted bacterial suspensions, containing an average inoculum of 10^5 cfu as checked by routine plating, were added to 100 μL portions containing increasing concentrations (0.5–16 mg/L) of either teicoplanin or vancomycin in microtitre plates. MICs were read after 48 h of incubation at 37°C.

Population analysis

Suspensions of one to several bacterial colonies removed from teicoplanin-supplemented agar, or of strain MRGR3 grown on glycopeptide-free MHA, were prepared in PBS and adjusted to a turbidity equal to McFarland 0.5. One hundred microlitre portions of either 10- or 10^4-fold diluted bacterial suspensions were spread on MHA plates containing teicoplanin in doubling concentrations ranging from 1 to 16 mg/L or glycopeptide-free MHA, and enumerated after 48 h of incubation at 37°C.

Survival kinetic studies

Suspensions of one to a few bacterial colonies removed from teicoplanin-supplemented agar, or of strain MRGR3 grown on glycopeptide-free MHA, were prepared in PBS and adjusted to a turbidity equal to McFarland 0.5. Thereafter, 50 μL portions of each bacterial suspension were added to glass tubes containing 10 mL of MHB containing 8 mg/L of teicoplanin in a shaking waterbath at 37°C. The total number of viable organisms was determined by subculturing 50 μL of 10-fold serially diluted portions on MHA after 0, 2, 4, 6 and 24 h of incubation. For each time point, 50 μL of 10-fold serially diluted portions were spread in parallel on agar plates containing 2, 4 or 8 mg/L of teicoplanin for population analysis of surviving bacteria. Colonies were enumerated after 48 h of incubation at 35°C. The detection limit was 2 $\log_{10}$ cfu/mL.

Results

Emergence of tissue cage bacterial subpopulations growing on glycopeptide-supplemented agar

Among 24 tissue cages that developed a significant MRSA infection at 3 weeks, none of the 10 cages with the lowest numbers of plated bacteria ($<4 \times 10^4$ cfu), yielded any colony growing on MHA containing 10-fold the MIC of teicoplanin or vancomycin for strain MRGR3. All the other 14 cages in which numbers of plated bacteria exceeded 10^5 cfu yielded colonies on teicoplanin- or vancomycin-supplemented agar with average frequencies of 4×10^{-5} or 2×10^{-5}, respectively. For eight cages with intermediate bacterial titres, the number of bacteria growing on either teicoplanin-supplemented (Figure 1a) or vancomycin-supplemented (Figure 1b) MHA was directly correlated with the number of plated bacteria (range: 4×10^4 to 5×10^5 cfu), with average frequencies of 1.5×10^{-4} and 1.3×10^{-4} on teicoplanin- ($r = 0.84$, $P < 0.01$) and vancomycin-containing ($r = 0.86$, $P < 0.01$) MHA, respectively. Paradoxically, much lower average frequencies of bacteria growing on either teicoplanin-supplemented (8.1×10^{-6}) or vancomycin-supplemented (3.2×10^{-6}) MHA were recorded in six cages with the highest titres of strain MRGR3 (not shown). Thus, the average frequency of tissue cage bacteria growing on glycopeptide-supplemented MHA was not artificially increased by spreading a larger number of organisms on agar plates.

In an independent experiment, population analysis profiles of tissue cage bacteria plated on different concentrations of teicoplanin were also determined from nine infected cages, yielding average frequencies of 2.4×10^{-4}, 1.1×10^{-4} and 2.2×10^{-5} on MHA containing 2, 4 and 8 mg/L of teicoplanin, respectively.

Stability of the glycopeptide resistance phenotypes of tissue cage bacteria

Direct loop transfers of colonies from 12 different cages cultured on teicoplanin-supplemented MHA, then subcultured on to equivalent antibiotic-containing media, led to positive subcultures in all cases. Identical results were

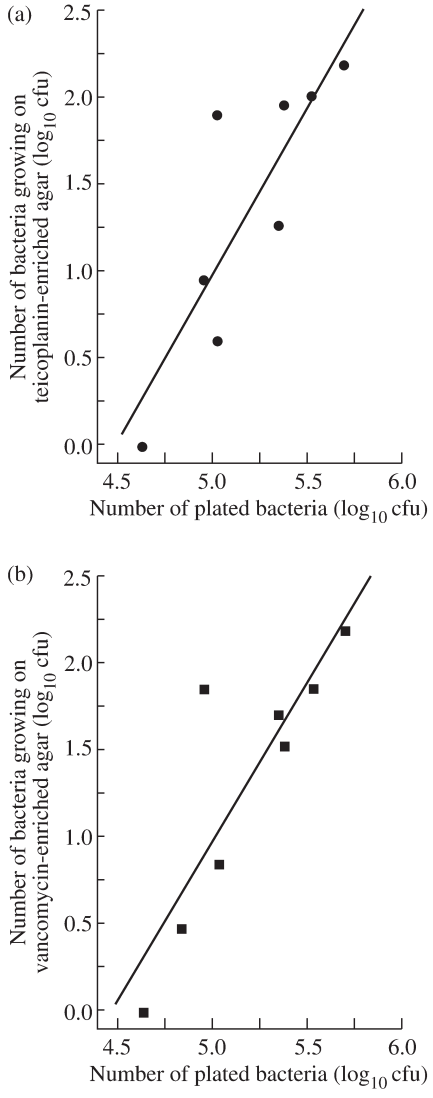

Figure 1. Number of tissue cage bacteria of MRSA strain MRGR3 growing on agar containing 10 mg/L of either teicoplanin (a) or vancomycin (b) as a function of the number of plated bacteria.

obtained with colonies grown on vancomycin-supplemented MHA. In contrast, when the stability of glycopeptide resistance was tested on subcultures of the glycopeptide-selected colonies that were first briefly suspended in saline and then plated at a concentration of *c.* 10^6 cfu, none of these subcultures was positive on either teicoplanin- or vancomycin-containing MHA. This indicated that the glycopeptide resistance phenotypes were unstable and strongly influenced by the methodology used for subcultures.

To analyse in a different way the stability of the resistance phenotypes of the 12 tissue cage bacterial subpopulations grown on glycopeptide-supplemented agar, these colonies were briefly suspended in saline and their teicoplanin or vancomycin MICs evaluated by the broth microdilution method. Compared with the parent strain MRGR3 grown on glycopeptide-free agar, the teicoplanin MIC of which was 0.5 mg/L, all subclones of teicoplanin-selected tissue cage bacteria showed four- to 16-fold increases in teicoplanin MICs, namely 2 mg/L for four subclones, 4 mg/L for five subclones and 8 mg/L for three subclones. In contrast, only three out of 12 vancomycin-selected subclones showed a minor two-fold increase in vancomycin MICs (2 mg/L), whereas nine other subclones showed MICs identical to that of the parent strain MRGR3 (1 mg/L).

These data indicated that expression of the resistance phenotype by tissue cage bacterial subpopulations selected on glycopeptide-containing agar was more frequent and more stable with teicoplanin than vancomycin.

Further characteristics of teicoplanin-selected subpopulations

Four subclones of tissue cage bacterial subpopulations grown on glycopeptide-supplemented MHA that showed the highest increase in teicoplanin MICs (4–8 mg/L) were tested further by population analysis profiles. Figure 2 demonstrates that all four subclones survived much better than the parental strain MRGR3 in teicoplanin concentrations ranging from 2 to 8 mg/L. Despite almost identical population analysis profiles, subclones 14-4 and 15-4 exhibited a markedly different colonial morphology. Subclone 15-4 was the only one to exhibit a uniform small colony variant morphology and was not studied further, in contrast to subclones 14-4, 16-3 and 17-2, which showed heterogeneous colonies ranging from small to essentially normal size.

Survival and population analysis profiles of teicoplanin-selected subclones exposed to the glycopeptide in the liquid phase

Selected colonies from subclones 14-4, 16-3 and 17-2 that were removed from teicoplanin-supplemented agar were briefly suspended in saline and evaluated for survival or killing kinetics in the presence of 8 mg/L teicoplanin in

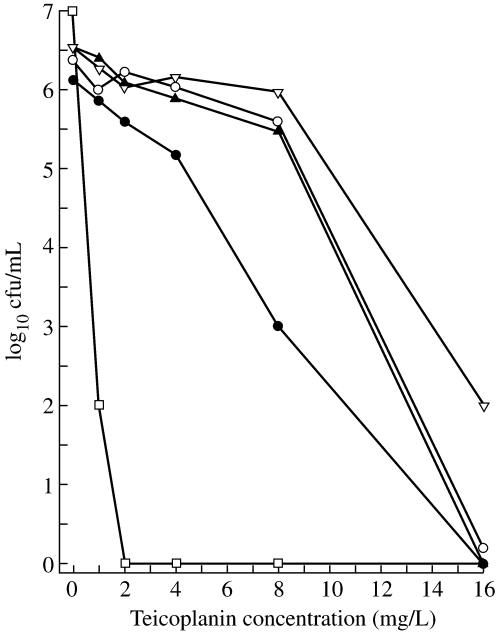

Figure 2. Population analysis of MRSA strain MRGR3 or subclones of tissue cage bacterial subpopulations selected on agar containing 10 mg/L of teicoplanin. *In vitro*-grown strain MRGR3 (□); subclone 14-4 (▲); subclone 15-4 (●); subclone 16-3 (○); subclone17-2 (▽).

MHB. For subclones 14-4 and 17-2, which grew on agar containing 8 mg/L of teicoplanin, a complete growth arrest but no significant killing was observed for at least 6 h in the liquid medium having an equivalent glycopeptide concentration, followed by significant growth from 6 to 24 h (Figure 3a). In contrast, subclone 16-3, which was initially selected on agar containing 4 mg/L of teicoplanin, remained growth-arrested for 24 h.

The population analysis profiles of subclones 14-4, 16-3 and 17-2 were also evaluated during their exposure to teicoplanin in liquid phase. The most interesting profiles were observed with subclone 14-4 (Figure 3b). After 2 h incubation, >90% of cells of this subclone had already lost the ability to develop detectable colonies, even on MHA containing as little as 2 mg/L teicoplanin. Similar results were also recorded with subclones 16-3 and 17-2 (data not shown). These data indicated that transfer of teicoplanin-selected subclones from a solid to liquid teicoplanin-containing growth medium converted a homogeneously resistant (see time zero) into a heterogeneously resistant population (see times 2, 4 and 6 h), composed of >90% of cells still resistant to teicoplanin but unable to grow in the presence of the glycopeptide for a period of at least 6 h.

Selection of stable teicoplanin-resistant subclones

The stability of subclones 14-4 and 17-2 was tested further for a period of 3 months by weekly passages on to three different media, namely MHA supplemented with 8 mg/L

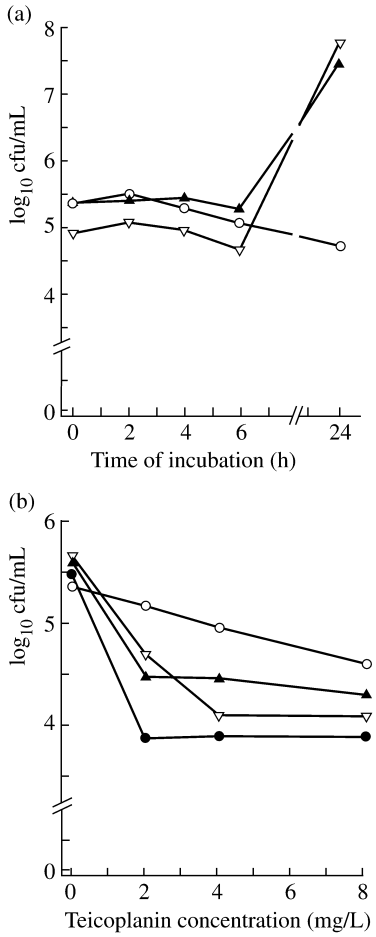

Figure 3. (a) Survival kinetics of teicoplanin-selected subclones in MHB containing 8 mg/L of teicoplanin. Subclone 14-4 (▲); subclone 16-3 (○); subclone 17-2 (▽). (b) Population analysis profiles of subclone 14-4 after increasing periods of incubation in the survival kinetics experiment; time zero (○); 2 h exposure (●); 4 h exposure (▲); 6 h exposure (▽).

teicoplanin or 2 mg/L vancomycin, or glycopeptide-free MHA. This latter medium was used to assay the stability of the resistance phenotype during repeated passages. For each passage, the saline-suspended subclones, plated at an average inoculum of 10^6 cfu on either glycopeptide-supplemented or plain MHA, yielded consistently positive subcultures on either growth medium. After 1, 2 and 3 months of weekly subculture on either glycopeptide-containing or plain MHA, teicoplanin and vancomycin MICs of passaged subclones were assayed by the macrodilution method and found to be consistently equivalent on glycopeptide-containing and glycopeptide-free MHA. The average teicoplanin and vancomycin MICs of the stable subclones 14-4 and 17-2 were 16 and 4 mg/L, respectively.

Discussion

Glycopeptide resistance in *S. aureus* is not acquired exogenously, but seems to result from multiple endogenous changes affecting cell wall synthesis and composition. These metabolic changes appear to trigger overproduction of false target sites that may promote removal of either vancomycin or teicoplanin from the medium, thus decreasing their access to their common lethal target, the D-alanyl-D-alanine of the lipid-II-linked muropeptide precursor.[6,7,9,19,23–25,27] An additional problem complicating molecular studies of glycopeptide resistance is the heterogeneous phenotype of such resistance within cell populations of clinical isolates, referred to as hGISA. The heterogeneous expression of glycopeptide resistance has been studied more extensively with vancomycin than teicoplanin. The detection of hGISA strains is difficult by standard techniques such as disc diffusion, or determination of MICs on either solid or liquid media. While some authors consider population analysis the only reliable technique for detecting glycopeptide resistance in hGISA, this procedure cannot be considered for routine testing.[6,7,9,17,18] Detection of heterogeneous glycopeptide resistance by population analysis profiles is poorly reproducible between laboratories, even with the help of reference GISA strains. This is due not only to the lack of any standardized procedure used for the population analysis profiles, but also to the variability in expression and stability of glycopeptide-resistance phenotypes of clinical and laboratory strains of *S. aureus*.[15,16,18] Finally, expression of glycopeptide resistance is also influenced by the composition of microbiological growth media.[17]

In view of the intensive use of vancomycin and teicoplanin for several years, the rarity with which GISA strains are isolated is surprising. Two opposing explanations might be considered. First, detection of heterogeneous resistance to glycopeptides may be viewed as essentially an *in vitro* phenomenon, *in vivo* expression of which is poorly documented and clinical relevance uncertain (see discussions in references 11 and 18), with the exception of sporadic cases of clinical isolates highly resistant to teicoplanin.[22,26,27] Conversely, it has been suggested that some *in vivo* conditions might promote glycopeptide resistance and compromise the outcome of antimicrobial therapy. *In vivo* expression of glycopeptide resistance might be too unstable to be detected by laboratory antimicrobial assays, because of the reversion of glycopeptide-resistant into glycopeptide-susceptible organisms after repeated *in vitro* passages in glycopeptide-free growth media.[15,16,18] The results recorded in the tissue cage model of *S. aureus* infection seem to support the latter hypothesis. First, we found in infected fluids of the subcutaneous implants the emergence of subpopulations characterized by growth on agar containing 10-fold the MICs of either teicoplanin or vancomycin for the original MRSA strain MRGR3, which under *in vitro* growth conditions was devoid of any glycopeptide-resistant subpopulation. *In vivo* emergence of subpopulations growing on teicoplanin-containing agar was repeatedly observed in several experiments performed between 1993 and 1999, and the frequency of these sub-

populations was consistently $>10^{-5}$ on MHA containing eight- to 10-fold the MIC of teicoplanin for strain MRGR3. Further characterization of the subpopulations growing on glycopeptide-containing agar was hampered by the unstable phenotypes of subclones, which reverted to a teicoplanin-susceptible state, as defined by MIC testing, after passage in antibiotic-free media, as reported previously.[30]

To improve the characterization of the resistance phenotypes of the tissue cage bacterial subpopulations grown on glycopeptide-supplemented MHA, we tried to avoid or minimize passages in antibiotic-free growth media that were suspected of promoting reversion of glycopeptide-resistance expression. MIC testing and population analysis profiles were performed on colonies removed directly from glycopeptide-containing agar, and suspended in saline. This procedure yielded MICs of teicoplanin that increased consistently, whereas those of vancomycin were much less affected. These data fit well with the more frequent emergence of teicoplanin- versus vancomycin-resistant isolates occurring *in vivo* or in *in vitro* conditions used to select glycopeptide-resistant organisms. We can speculate that this *ex vivo* phenomenon of increased teicoplanin resistance may result from some *in vivo* stimulation of cell wall production, with the resulting thickened cell wall affording protection.

The striking difference between plating and survival of the resistant subclones in liquid versus solid phase may reflect either differential expression of cell wall-associated genes in planktonic versus sessile bacteria, or be due to the dilution of an inducer of teicoplanin resistance in liquid medium, which would not occur on agar surfaces in the colonial mode of growth. More than 90% of cells in suspension were unable to grow, but still survived quite well for a prolonged period of time in the presence of teicoplanin, and were thus defined as glycopeptide-tolerant rather than glycopeptide-resistant. These data may indicate an alternative expression of tolerance versus resistance in liquid versus solid growth medium by subpopulations of *S. aureus*. Two recent reports indicate that glycopeptide tolerance is a frequent phenomenon in *S. aureus*, particularly amongst MRSA isolates, which might compromise glycopeptide therapy for serious staphylococcal infection.[35,36] In our tissue cage model of *S. aureus* infection, we also found that *in vivo*-grown organisms exhibited a broad-spectrum tolerance to different antibiotics in several therapeutic studies.[30,31,33,37,38] This *in vivo*-induced tolerance, which is either not expressed or rapidly disappears under *in vitro* conditions, was therefore referred to as phenotypic tolerance.[39] In therapeutic studies, the highest phenotypic tolerance expressed by strain MRGR3 infecting tissue cages was against teicoplanin.[30] Thus, expression of phenotypic tolerance and emergence of *in vivo* glycopeptide-resistant subpopulations may explain to some extent the poor therapeutic activity of teicoplanin recorded previously in tissue cages chronically infected with *S. aureus*.[30]

In conclusion, we have provided evidence that emergence of resistance to glycopeptides can occur *in vivo* in a well-defined experimental model of *S. aureus* infection. These data suggest that some *in vivo* conditions may exert a selective pressure on *S. aureus*, thus leading to emergence of subpopulations exhibiting reduced glycopeptide susceptibility and allowing their growth in the presence of otherwise inhibitory concentrations of glycopeptide. The conditions leading eventually to emergence of stable teicoplanin- or vancomycin-resistant subpopulations are still unknown and deserve further investigation. We hope that this experimental infection model will help to identify some of the up- and down-regulated genes either induced or constitutively expressed *in vivo* by glycopeptide-resistant *S. aureus*, as reported recently under *in vitro* conditions.[29]

Acknowledgements

We thank Manuela Bento for technical assistance and Paul Majcherczyk for helpful comments. This work was supported by research grant 3200-045810.95/2 from the Swiss National Foundation.

References

1. Edmond, M. B., Wenzel, R. P. & Pasculle, A. W. (1996). Vancomycin-resistant *Staphylococcus aureus*: perspectives on measures needed for control. *Annals of Internal Medicine* **124**, 329–34.

2. Tabaqchali, S. (1997). Vancomycin-resistant *Staphylococcus aureus*: apocalypse now? *Lancet* **350**, 1644–5.

3. Wenzel, R. P. & Edmond, M. B. (1998). Vancomycin-resistant *Staphylococcus aureus*: infection control considerations. *Clinical Infectious Diseases* **27**, 245–9.

4. Waldvogel, F. A. (1999). New resistance in *Staphylococcus aureus*. *New England Journal of Medicine* **340**, 556–7.

5. Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T. & Tenover, F. C. (1997). Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *Journal of Antimicrobial Chemotherapy* **40**, 135–6.

6. Hiramatsu, K., Aritaka, N., Hanaki, H., Kawasaki, S., Hosoda, Y., Hori, S. *et al.* (1997). Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet* **350**, 1670–3.

7. Hiramatsu, K. (1998). Vancomycin resistance in staphylococci. *Drug Resistance Updates* **1**, 135–50.

8. Hiramatsu, K. & Hanaki, H. (1998). Glycopeptide resistance in staphylococci. *Current Opinion in Infectious Diseases* **11**, 653–8.

9. Sieradzki, K., Roberts, R. B., Haber, S. W. & Tomasz, A. (1999). The development of vancomycin resistance in a patient with methicillin-resistant *Staphylococcus aureus* infection. *New England Journal of Medicine* **340**, 517–23.

10. Smith, T. L., Pearson, M. L., Wilcox, K. R., Cruz, C., Lancaster, M. V., Robinson-Dunn, B. *et al.* (1999). Emergence of vancomycin resistance in *Staphylococcus aureus*. Glycopeptide-Intermediate Staphylococcus aureus Working Group. *New England Journal of Medicine* **340**, 493–501.

11. Moellering, R. C. (1999). Editorial response: Staphylococci vs. glycopeptides—how much are the battle lines changing? *Clinical Infectious Diseases* **29**, 768–70.

12. Tenover, F. C., Lancaster, M. V., Hill, B. C., Steward, C. D., Stocker, S. A., Hancock, G. A. *et al.* (1998). Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. *Journal of Clinical Microbiology* **36**, 1020–7.

13. Howe, R. A., Wootton, M., Bennett, P. M., MacGowan, A. P. & Walsh, T. R. (1999). Interactions between methicillin and vancomycin in methicillin-resistant *Staphylococcus aureus* strains displaying different phenotypes of vancomycin susceptibility. *Journal of Clinical Microbiology* **37**, 3068–71.

14. Wong, S. S., Ho, P. L., Woo, P. C. & Yuen, K. Y. (1999). Bacteremia caused by staphylococci with inducible vancomycin heteroresistance. *Clinical Infectious Diseases* **29**, 760–7.

15. Boyle-Vavra, S., Berke, S. K., Lee, J. C. & Daum, R. S. (2000). Reversion of the glycopeptide resistance phenotype in *Staphylococcus aureus* clinical isolates. *Antimicrobial Agents and Chemotherapy* **44**, 272–7.

16. Aeschlimann, J. R., Hershberger, E. & Rybak, M. J. (1999). Analysis of vancomycin population susceptibility profiles, killing activity, and postantibiotic effect against vancomycin-intermediate *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **43**, 1914–8.

17. Hubert, S. K., Mohammed, J. M., Fridkin, S. K., Gaynes, R. P., McGowan, J. E. & Tenover, F. C. (1999). Glycopeptide-intermediate *Staphylococcus aureus*: evaluation of a novel screening method and results of a survey of selected U.S. hospitals. *Journal of Clinical Microbiology* **37**, 3590–3.

18. Howe, R. A., Wootton, M., Walsh, T. R., Bennett, P. M. & MacGowan, A. P. (1999). Expression and detection of heterovancomycin resistance in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* **44**, 675–8.

19. Daum, R. S., Gupta, S., Sabbagh, R. & Milewski, W. M. (1992). Characterization of *Staphylococcus aureus* isolates with decreased susceptibility to vancomycin and teicoplanin: isolation and purification of a constitutively produced protein associated with decreased susceptibility. *Journal of Infectious Diseases* **166**, 1066–72.

20. Biavasco, F., Giovanetti, E., Montanari, M. P., Lupidi, R. & Varaldo, P. E. (1991). Development of in-vitro resistance to glycopeptide antibiotics: assessment in staphylococci of different species. *Journal of Antimicrobial Chemotherapy* **27**, 71–9.

21. Watanakunakorn, C. (1990). In-vitro selection of resistance of *Staphylococcus aureus* to teicoplanin and vancomycin. *Journal of Antimicrobial Chemotherapy* **25**, 69–72.

22. Kaatz, G. W., Seo, S. M., Dorman, N. J. & Lerner, S. A. (1990). Emergence of teicoplanin resistance during therapy of *Staphylococcus aureus* endocarditis. *Journal of Infectious Diseases* **162**, 103–8.

23. Shlaes, D. M., Shlaes, J. H., Vincent, S., Etter, L., Fey, P. D. & Goering, R. V. (1993). Teicoplanin-resistant *Staphylococcus aureus* expresses a novel membrane protein and increases expression of penicillin-binding protein 2 complex. *Antimicrobial Agents and Chemotherapy* **37**, 2432–7.

24. Sieradzki, K. & Tomasz, A. (1996). A highly vancomycin-resistant laboratory mutant of *Staphylococcus aureus*. *FEMS Microbiology Letters* **142**, 161–6.

25. Moreira, B., Boyle-Vavra, S., deJonge, B. L. & Daum, R. S. (1997). Increased production of penicillin-binding protein 2, increased detection of other penicillin-binding proteins, and decreased coagulase activity associated with glycopeptide resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **41**, 1788–93.

26. Manquat, G., Croize, J., Stahl, J. P., Meyran, M., Hirtz, P. & Micoud, M. (1992). Failure of teicoplanin treatment associated with an increase in MIC during therapy of *Staphylococcus aureus* septicaemia. *Journal of Antimicrobial Chemotherapy* **29**, 731–2.

27. Mainardi, J. L., Shlaes, D. M., Goering, R. V., Shlaes, J. H., Acar, J. F. & Goldstein, F. W. (1995). Decreased teicoplanin susceptibility of methicillin-resistant strains of *Staphylococcus aureus*. *Journal of Infectious Diseases* **171**, 1646–50.

28. Kaatz, G. W., Seo, S. M., Reddy, V. N., Bailey, E. M. & Rybak, M. J. (1990). Daptomycin compared with teicoplanin and vancomycin for therapy of experimental *Staphylococcus aureus* endocarditis. *Antimicrobial Agents and Chemotherapy* **34**, 2081–5.

29. Kuroda, M., Kuwahara-Arai, K. & Hiramatsu, K. (2000). Identification of the up- and down-regulated genes in vancomycin-resistant *Staphylococcus aureus* strains Mu3 and Mu50 by cDNA differential hybridization method. *Biochemical and Biophysical Research Communications* **269**, 485–90.

30. Schaad, H. J., Chuard, C., Vaudaux, P., Waldvogel, F. A. & Lew, D. P. (1994). Teicoplanin alone or combined with rifampin compared with vancomycin for prophylaxis and treatment of experimental foreign body infection by methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **38**, 1703–10.

31. Lucet, J. C., Herrmann, M., Rohner, P., Auckenthaler, R., Waldvogel, F. A. & Lew, D. P. (1990). Treatment of experimental foreign body infection caused by methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **34**, 2312–7.

32. Chuard, C., Herrmann, M., Vaudaux, P., Waldvogel, F. A. & Lew, D. P. (1991). Successful therapy of experimental chronic foreign-body infection due to methicillin-resistant *Staphylococcus aureus* by antimicrobial combinations. *Antimicrobial Agents and Chemotherapy* **35**, 2611–6.

33. Schaad, H. J., Chuard, C., Vaudaux, P., Rohner, P., Waldvogel, F. A. & Lew, D. P. (1994). Comparative efficacies of imipenem, oxacillin and vancomycin for therapy of chronic foreign body infection due to methicillin-susceptible and -resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* **33**, 1191–200.

34. National Committee for Clinical Laboratory Standards. (2000). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically—Fifth Edition: Approved Standard M7-A5*. NCCLS, Villanova, PA.

35. May, J., Shannon, K., King, A. & French, G. (1998). Glycopeptide tolerance in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* **42**, 189–97.

36. Perry, J. D., Jones, A. L. & Gould, F. K. (1999). Glycopeptide tolerance in bacteria causing endocarditis. *Journal of Antimicrobial Chemotherapy* **44**, 121–4.

37. Chuard, C., Lucet, 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.

38. Cagni, A., Chuard, C., Vaudaux, P. E., Schrenzel, J. & Lew, D. P. (1995). Comparison of sparfloxacin, temafloxacin, and ciprofloxacin for prophylaxis and treatment of experimental foreign-body infection by methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **39**, 1655–60.

39. Vaudaux, P. (1998). Phenotypic antibiotic tolerance of *Staphylococcus aureus* in implant related infections: relationship with in vitro colonization of artificial surfaces. *Drug Resistance Updates* **1**, 352–7.

Received 6 June 2000; returned 24 August 2000; revised 4 October 2000; accepted 6 November 2000