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Mechanism of Vancomycin Resistance in Methicillin Resistant *Staphylococcus aureus*

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Abstract

A collection of laboratory mutants and clinical MRSA strains, additionally exhibiting resistance to glycopeptide antibiotics, was studied in detail. The nature of resistance to glycopeptides was found to be different from that existing in vancomycin resistant (VR) enterococci. The mutants produced abnormal murein in which the level of highly oligomeric muropeptides was drastically reduced. Biochemical and genetic analyses of Penicillin Binding Proteins (PBPs) showed inactivation of PBP4. Changes in other PBPs were not apparent, except for PBP2a that was inactivated in the highly VR mutant VM. Transposon inactivation of the *pbpB* gene and several other genes involved in synthesis of staphylococcal peptidoglycan all caused dramatic reduction of glycopeptide resistance in the staphylococcal mutants. While inactivation of PBP2a slightly increased the levels of glycopeptide resistance, a combination of vancomycin or teicoplanin with β-lactam inhibitors, chosen on the basis of their relatively selective affinities for individual staphylococcal PBPs completely inhibited the expression of glycopeptide resistance in MRSA. Glycopeptide antibiotics caused a virtually complete inhibition of cell wall turnover and autolysis and massive overgrowth of cell wall material in the glycopeptide resistant mutants. Bacteria were able to remove quantitatively glycopeptide molecules from the growth medium, and sequestered antibiotic could be recovered in biologically active form from the purified cell walls. These observations and the results of the vancomycin binding studies suggest alterations in the structural organization of the mutants' cell wall such that access of glycopeptide molecules to the sites of wall biosynthesis is blocked by steric hindrance.

Key words: vancomycin, *Staphylococcus aureus*, MRSA, VRE

The ubiqitous resistance of clinical *Staphylococcus aureus* strains to methicillin, frequently accompanied by simultaneous resistance to aminoglycosides, chinolones, linkosamides, macrolides and tetracyclines creates a situation in which the therapy of contemporary staphylococcal infections is frequently limited to glycopeptides, as the last effective group of antibiotics against these infections. Consequently, the appearance of vancomycin resistant enterococci (VRE), in which genes determining resistance are plasmid-borne (for review on enterococcal type of resistance see: Kłoszewska and Markiewicz, 1999), has raised fears that the possible transfer of the "enterococccal" mechanism of vancomycin resistance to multiply-resistant MRSA strains will make them omniresistant, and thus untreatable human pathogens. These fears were further enhanced by the results of laboratory experiments (Noble *et al.*, 1992) in which the *vanA* operon of *Enterococcus faecalis* was transferred to *S. aureus*, resulting in vancomycin resistance of the latter. However, as shown by Sieradzki *et al.* (1998a), the intergeneric transfer of enterococcal genetic determinants of vancomycin resistance may not occur that easily in the clinical environment. Even after a several months' colonization of the peritoneum of a patient by both a vancomycin resistant (VR) strain of *Enterococcus faecalis* (carrying the *vanA* operon) and *Staphylococcus epidermidis*, the observed reduced susceptibility of consecutive staphylococcal isolates to vancomycin was caused by mutations in undetermined chromosomal genes occurring under conditions of strong selective pressure and not by interspecies genetic exchange. Though the first VRE were isolated close to 20 years ago (Leclercq *et al.*, 1988), this is probably the reason that the first cases of clinical staphylococcus strains demonstrating vancomycin resistance resulting from

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the uptake of enterococcal vancomycin resistance genes were described only recently (Chang *et al.*, 2003). On the other hand, the number of cases from which intermediately glycopeptide resistant (GR) staphylococcal strains, both coagulase-negative and *S. aureus* that are characterized by a mechanism of resistance distinct from the enterococcal one, is constantly on the rise. One report even suggests that heterogeneous teicoplanin resistance-predisposing for resistance to vancomycin – may be an intrinsic feature of coagulase-negative staphylococci (Sieradzki *et al.*, 1998b).

Although many *S. aureus* strains, showing reduced susceptibility to glycopeptides have been identified by now, only a handful of them have been analyzed beyond mere exclusion of the enterococcal type of resistance (Sieradzki and Tomasz, 1996, 1997, 1998, 2003, Sieradzki *et al.*, 1999a). In this article we will focus on some of those few GR strains that have been biochemically characterized in greater detail.

A series of GR *Staphylococcus aureus* strains described herein, both laboratory mutants (VM50, TM, TNM) and clinical strain PC3, also demonstrate a mechanism of resistance to glycopeptide antibiotics other than that present in enterococci. This is indicated by the absence in these strains of genes characteristic for enterococcal resistance to vancomycin and consequently they do not synthesize murein precursors carrying tetrapeptide-D-lactate (Sieradzki and Tomasz, 1997, Sieradzki *et al.*, 1999b). The substitution of C-terminal D-alanine by D-lactate results in a drastic drop in the affinity and therefore binding of vancomycin to thus modified murein precursor (Fig. 1). Consequently, bacterial cell walls formed by incorporating the modified precursor Mur*N*Ac-tetrapeptide-D-lactate, and thus lacking the D-alanylo-D-alanine moiety, should also lose their capacity to specifically bind glycopeptide antibiotics (Markiewicz and Kwiatkowski, 2001). The results obtained for control VRE strains, with which the above-mentioned studied *S. aureus* strains were compared, confirm this dependence – under experimental conditions in which the cell walls of susceptible *E. faecalis* JH2-2 strain bound significant amounts of vancomycin, no binding of the antibiotic was observed by cell walls isolated from VR strains of *Enterococcus faecalis*, or *Enterococcus faecium* (Sieradzki and Tomasz, 1997). Also VRE cultures, growing in the presence of vancomycin, did not cause any detectable changes in the concentration of the antibiotic in the medium. In the case of the analyzed *S. aureus* strains the reverse was observed. Growth of the mutants in the presence of sub-critical concentrations of glycopeptides was paralleled by gradual disappearance of the antibiotics from the medium. Heat-inactivated

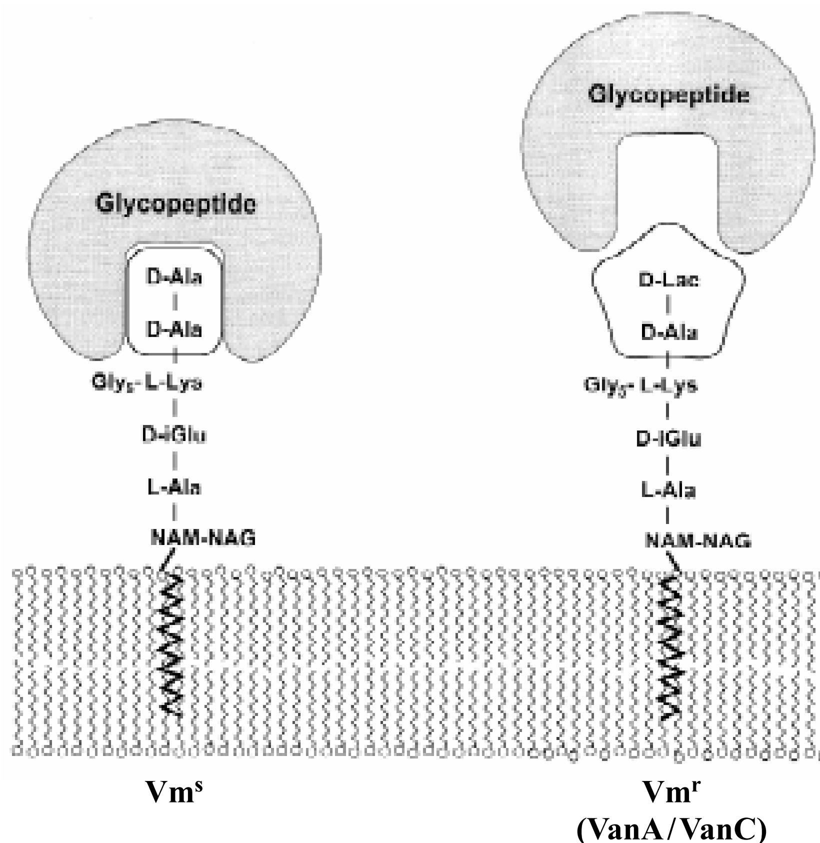

Fig. 1. Schematic depiction of the enterococcal mechanism of resistance to glycopeptide antibiotics conferred by the *vanA*/*vanB* operon

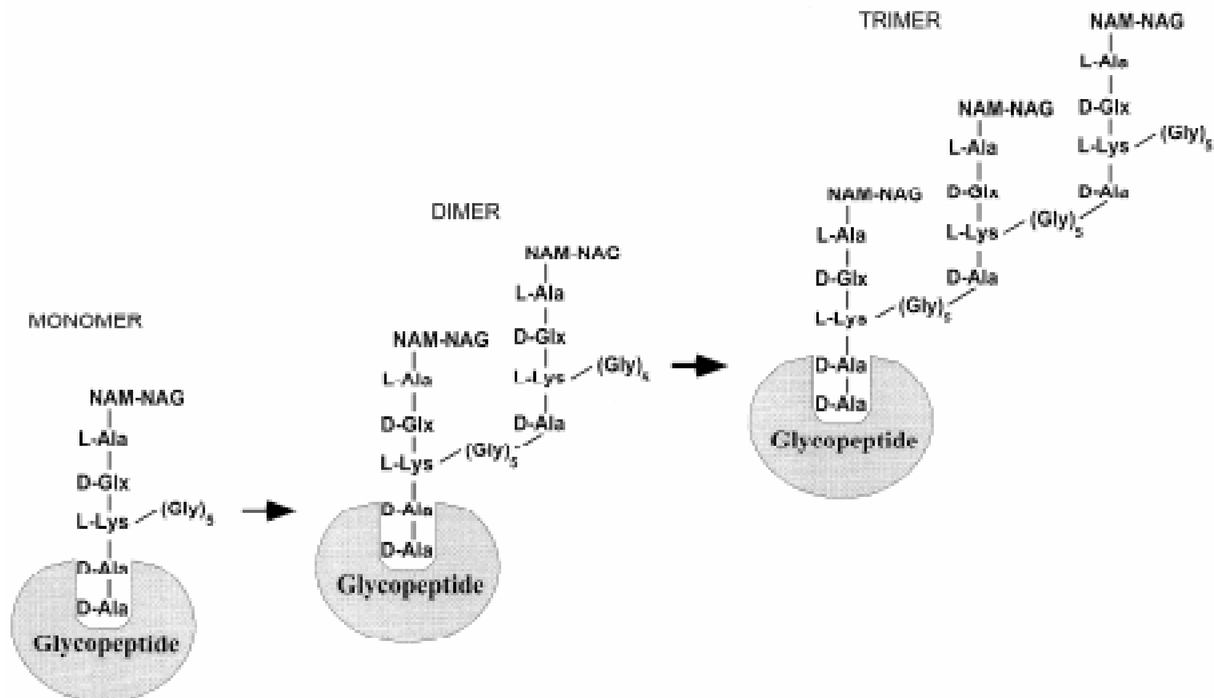

Fig. 2. Relative efficacy for binding glycopeptide molecules by the staphylococcal muropeptides, depending on the molar proportions of unmodified D-alanyl-D-alanine C-termini

cells of the mutants, suspended in buffered saline, were also able to remove significant amounts of vancomycin from the medium, compared to the parental cells. That the decreased concentration of glycopeptides in the cultures of the mutant strains was caused by their binding by cell walls and not by enzymatic inactivation was indicated by the fact that the antibiotic removed from the medium could be isolated in biologically active form from isolated cell walls. The increased binding of glycopeptide antibiotics by cell walls of glycopeptide resistant (GR) mutants was confirmed in experiments *in vitro*: cells walls isolated from highly GR mutants were able to bind several-fold greater amounts of vancomycin than the susceptible strain RN450 and parental strain COL (Sieradzki and Tomasz, 1996, 1997, 1998). This fact once again proves the distinctness of the staphylococcal mechanism of resistance to glycopeptide antibiotics.

As mentioned, unmodified C-terminal D-alanyl-D-alanine residues play an important role in the specific binding of glycopeptides. Consequently, the ability of GR mutants to bind increased amounts of vancomycin compared to susceptible strains, suggests that they may synthesize thickened (or with modified chemical structure) cell walls that are enriched in muropeptides with unaltered D-alanyl-D-alanine moiety. Analysis of cellular structures, involving electron microscopy, did not reveal any significant differences in morphology between the COL strain and GR mutants when they were grown under the same conditions. However, as shown by chemical analysis using HPLC, the murein of the mutants is characterized by drastically reduced degree of cross-linking, causing the content of highly polymerized muropeptides to drop from 60% in the murein of the parental strain to 32, 17 and 15% for mutants VM50, TNM and TM, respectively (Sieradzki *et al*., 1999a). The reduced content of highly polymerized muropeptides in thus modified murein results in relative increase of non-polymerized muropeptides and those with low degree of polymerization, thus retaining relatively high molar proportions of unmodified D-alanyl-D-alanine moiety (Fig. 2). The ability of the cell walls of the mutants to bind increased amounts of vancomycin therefore results from modification of their chemical structure, involving reduced degree of murein crosslinkage.

The structure of murein, which is formed, amongst others, by the so-called penicillin binding proteins (PBP), depends to a large extent on the enzymatic activity of these proteins. The changes in murein structure in all highly GR *S. aureus* mutants results were found to be unidirectionally related to the inactivation of PBP4 (Sieradzki *et al*., 1999a), which is believed to be a secondary transpeptidase (Snowden and Perkins, 1990). In the case of strains VM50 and TM, the inactivation of gene *pbpD* was a consequence of the duplication of a stretch of 51 nucleotides, modifying encoded PBP4 in the area of the active center loop, whereas *pbpD* of strain TNM was inactivated by a point mutation introducing the termination codon TGA into the KTG motif that is characteristic for the DD-peptidase domain (Ghuysen, 1991).

The observed dependence between degree of murein cross-linking and activity (or level of expression) of PBP4 is not accidental since the enzyme is responsible for the high extent of murein crosslink formation (Wyke *et al*., 1981). A *S. aureus* strain with enzymatically inactive PBP4 and drastically reduced extent of murein cross-linking has been described earlier (Curtis *et al*., 1980, Wyke *et al*., 1981). In turn, Domanski *et al.* (1997) and independently Henze *et al.* (1996) have demonstrated that elevated expression of *pbpD* leads to excessive cross-linking of murein.

Taking the above into account, can it be said that the resistance of the discussed GR staphylococcal strains is caused by reduced cross-linking of their murein, thus allowing the removal of greater amounts of antibiotic from the medium? The gradual decrease in cross-linking of murein, which corresponds to gradual decrease in the cellular amounts of PBP4 and increasing degree of resistance to vancomycin in a series of successively selected mutants VM3 – VM50, as well as drastically reduced cross-linking that occurred in the independently selected strain TNM, indicate that a dependence between murein structure and mechanism of resistance to glycopeptide antibiotics cannot be ruled out. Nevertheless, in the case of *e.g.* strain VM50, the close to 100-fold increase in resistance to vancomycin cannot be attributed to reduced cross-linking alone, which would allow the binding of only approximately two-fold greater amount of vancomycin compared to the parental strain. It is therefore not possible to explain the protection of single bacterial cells by more efficient capture of antibiotic in the medium by cell wall.

If this is the case, what is the actual mechanism of resistance to glycopeptide antibiotics? GR mutants continue to synthesize non-modified murein precursors meaning that any contact between them and glycopeptide has to end in inhibition of cell wall synthesis. Furthermore, unmodified precursors are not synthesized with greater efficiency, which would enhance the chance of their incorporation into pre-existing murein. The level of expression of enzymes (PBPs) responsible for the final stages of murein synthesis, especially PBP2, which is considered to be a transglycosylase (TG) and major transpeptidase (TP) (Murakami *et al*. 1994) does not seem to be enhanced. Sequence analysis of the genes coding PBPs also did not reveal any changes resulting in enhanced activity (Sieradzki, 2003).

Taking the above observations into consideration, it can be hypothesized that the resistance of the discussed strains to glycopeptide antibiotics is a consequence of the spatial blocking of glycopeptide molecules, thus not allowing (or strongly impeding) their cell wall penetration towards the sites of synthesis. As a barrier, the cell walls of the mutants with modified secondary structure, additionally enriched in D-alanyl-D-alanine groups being "false" acceptor sites, is suggested. Changes in the structure of the cell walls (regardless of reduced cross-linking) impeding their penetration by glycopeptides, seem to be confirmed by several observations:

1. the addition of vancomycin ($200\ \mu g \times ml^{-1}$) to the growth medium of strain VM50, corresponding to 2-fold the MIC and aimed at the accumulation of cytoplasmic fraction of murein precursors, did not cause such accumulation of UDP-Mur-*N*Ac-pentapeptide precursor as in the case of strain COL.

2. Cell wall preparations from strain VM50, previously grown in the presence of saturating vancomycin concentrations were able to bind additional amounts of the antibiotic upon their mechanical disruption, exposing inner layers to the antibiotic.

3. Differences between amounts of vancomycin bound *in vitro* by cell walls and by muramidase digestion products, were greater for mutant VM50 than for the parental strain.

4. Glycopeptides appear to inhibit the action of murein hydrolases, since the growth of strain VM50 in the presence of concentrations of vancomycin saturating the cell wall completely blocked its enzymatic degradation *in vivo*. Partial hydrolysis of such saturated walls was possible *in vitro* only following their mechanical breakdown. This observation can be interpreted as follows – the mechanical breaking of cell walls allows hydrolases to gain access to inner layers of the wall that are not saturated with antibiotic.

5. Modifications of the murein precursor (in effect of inactivation of such genes as *femB*, *femC* or *murE*), that lead to modification of murein structure and possibly alter the secondary structure of the cell walls of the mutants, resulting in a drastic drop in their resistance to vancomycin and teicoplanin.

6. Finally, the increased mean length of the polysaccharide chains in the murein of GR mutants is yet another example of structural differences in murein that can also modify the organization of the cell wall. On the other hand, an apparent increase in average glycan chain length can also be a method for retaining the integrity of cell walls in which drastic reduction of cross-linking has occurred. The results of hydrolysis *in vitro*, indicating that the cell walls of GR mutants become relatively sensitive to the action of M1 muramidase and resistant to the action of lysostaphin seem to confirm this. The length of the sugar chains of murein has to be considered as a function of the activity of both synthetases (TGs) and hydrolases nicking mature chains. Thus, in a situation in which no significant changes in the activity of autolytic enzymes have

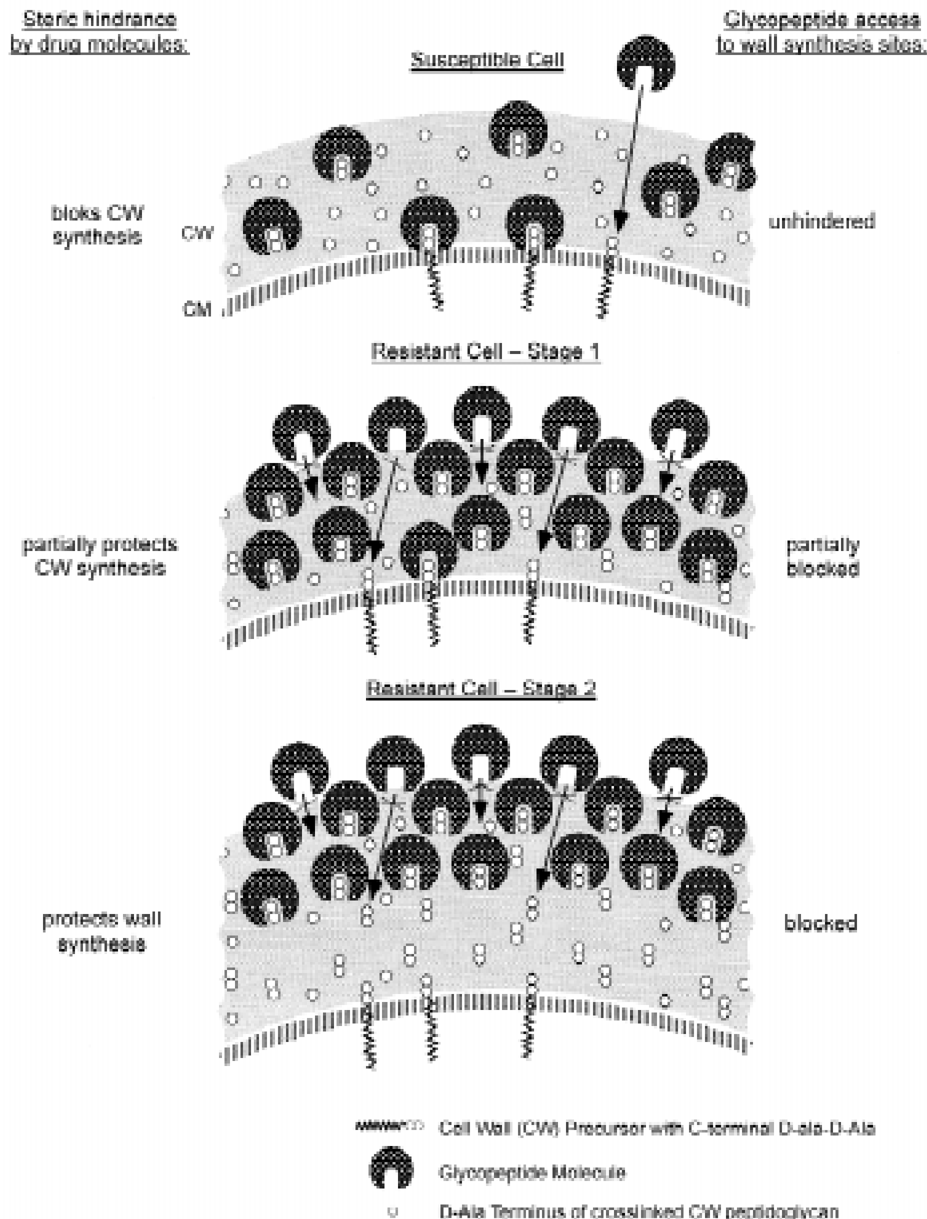

Fig. 3. The mechanism of staphylococcal resistance to glycopeptides

Penetration of drug molecules to the plasma membrane is presumed to occur nearly unhindered through the cell wall of susceptible strains. In contrast, the cell walls of resistant mutants, enriched for the muropeptide monomers, may slow down and prevent access to the cell wall synthetic sites by attachment of the antibiotic molecules to the D-alanyl-D-alanine termini of murein monomers (stage 1). The captured antibiotic molecules may then become part of the mechanism of resistance through the steric hindrance they pose to the penetration of free glycopeptide molecules (stage 2).

been determined, it can be assumed that the increased length of sugar chains is a result of increased TG activity. In turn, the absence of changes affecting PBP2 points to the role of monofunctional TGs.

One of the functions of a bacterial cell wall can be compared to a molecular sieve, whose permeability shows a sharp drop for molecules with mass over 1.2 kDa (Scherrer and Gerhardt, 1971). Thus, in accordance with the suggested resistance mechanism, glycopeptide molecules attacking a susceptible bacterial cell first of all bind to the rare C-terminal D-alanyl-D-alanine moieties of “mature” but for physiological reasons not cross-linked murein. After saturating all “false” acceptor sites, unbound molecules of the antibiotic penetrate to the direct vicinity of the cytoplasmic membrane where they bind to murein precursors, inhibiting the biosynthesis of the macromolecule. In the case of cells of resistant strains, unaltered cell walls enriched in monomeric muropeptides can capture the molecules of the antibiotics in such a way that by blocking the porous channels in the structure of the cell wall, the glycopeptides can sterically hinder the penetration of further molecules through the wall (Fig. 3). This hypothesis seems to be additionally

confirmed by the fact that as the resistant cells grow in the presence of the antibiotic, inhibition of autolytic enzyme activity brings about a gradual increase in the thickness of the cell wall, which correlates well with a gradual increase in growth rate. Since this process begins in a situation when no changes of the concentration of antibiotic in the medium are observed, this may reflect the gradual increase in the effectiveness of the thickening walls as a barrier.

The relatively not very high MIC values of glycopeptide antibiotics for the discussed strains, which is particularly true for vancomycin, seems to be at odds with the proposed resistance mechanism. However, this discrepancy can be explained as follows – the binding of relatively large and strongly positively charged vancomycin molecules by the cell wall, whose nature resembles an amphoteric gel, can lead to changes in the overall charge of wall, alteration in the three-dimensional structure of the wall polymers, changes in their mutual orientation as well as with regard to the surface of the cytoplasmic membrane and alterations in polarity and thus permeability of the membrane itself, in effect drastically impeding the secretion of metabolic products and the uptake of nutrients, ultimately leading to the "choking" of the bacterial cells. Therefore, the growth of the mutant cells in the presence of high concentrations of glycopeptides, which would probably entail complete blocking of porous channels in their cell walls, may be impossible for physiological reasons not directly related to the inhibition of murein synthesis. This somewhat resembles the case of β-lactam antibiotics in which a direct dependence between inhibition of cell wall synthesis and the death of bacterial cells has yet to be elucidated (Moreillon *et al*., 1990).

Certain doubts in connection with the proposed mechanism can also be raised in view of the observed differences in the levels of resistance to the individual glycopeptide antibiotics. Selection of resistance to vancomycin endowed strain VM50 with simultaneous resistance to teicoplanin. On the other hand, in strain TNM, selection for resistance to teicoplanin was accompanied by rather moderate increase in resistance to vancomycin. Nevertheless, this discrepancy can be explained by the different mode of action of both glycopeptides reflecting their different chemical properties. Whereas the activity of vancomycin increases after dimerization, teicoplanin molecules use their lipid moiety to anchor directly in the cytoplasmic membrane (Beauregard *et al*., 1995; Cooper and Williams 1999; Groves *et al*., 1994; Mackay *et al*., 1994). Besides different relative hydrophobicity, vancomycin and teicoplanin also differ in mass (1448 Da *vs.* average of over 1900 Da), and thus also by dimensions as well as by ionic nature. These differences can affect the greater or smaller penetration of the molecule of a given glycopeptide through the barrier of the cell wall of gram-positive bacteria. Thus, in accordance with the suggested mechanism, it seems highly plausible that mutants selected for resistance to teicoplanin strain TNM) may be more susceptible to the smaller and more mobile vancomycin molecules.

The mentioned above effect of the inactivation of several auxiliary genes (*e.g. fem*, indispensable for optimal expression of methicillin resistance) on the drastic drop in resistance to vancomycin or teicoplanin points to the complexity of the glycopeptide resistance phenotype and suggests that a high level of resistance to these antibiotics is determined by the efficient functioning of numerous genes involved in the biosynthesis of the cell wall. This in a way is analogous to the mechanism of methicillin resistance, since the same genes whose full functionality is indispensable for optimal expression of methicillin resistance (de Lencastre *et al*., 1994) are also crucial for high level of resistance to glycopeptide antibiotics. Consequently, strain TNM-632 (*fmtC*::Tn*551*), selected on the basis of drastically reduced resistance to teicoplanin (Sieradzki and Tomasz, 1998), turned out to be yet another mutant, in which not a "central" gene responsible for resistance to teicoplanin, but an auxiliary gene with as yet undetermined function (Komatsuzawa *et al*., 1997) was inactivated (Sieradzki, 2003).

As mentioned above, the growth of GR staphylococcal strains was accompanied by changes (induced by the presence of subcritical concentrations of glycopeptides) in morphology: a gradual increase in large agglomerations of fully formed cells that do not separate from one other and are surrounded by unnaturally thickened cell walls. This phenomenon had been described earlier by Sanyal and Greenwood (1993) who did not follow up their observations. It is common knowledge that growth, division and cell separation depend on the activity of hydrolases that precisely split specific bonds in murein. These enzymes are also responsible for regulating the turnover of murein, which determines the thickness of the cell walls. For this reason, the observed morphological changes in the cells of the mutants caused by the presence of glycopeptide antibiotics indicate that they must somehow impair the functioning of the autolytic system of the cells.

One of the factors that can regulate the activity of hydrolases are teichoic acids (TA). The increased participation of TA in the cell wall or altered degree of their estrification with D-alanine can have an inhibitory effect on the activity of hydrolases (Wecke *et al*., 1997). It can thus be speculated that the presence of glycopeptides in the growth medium induces changes in the structure of TA, or directly affect level of expression

(activity) of the autolysins as such. A series of experiments carried out *in vitro*, allows to unequivocally reject these possibilities (Sieradzki and Tomasz, 1997). The very similar enzymatic activity of hydrolases obtained from cells of the parental strain and the mutants grown with or without glycopeptides against the same substrate, as well as comparable enzymatic profiles allowed to determine that glycopeptide antibiotics do not induce significant changes as regards autolytic enzymes. Moreover, the saturation of cell walls with vancomycin *in vitro*, that is in a situation in which the modification of teichoic acids was ruled out, resulted in complete inhibition of their enzymatic degradation. Taking these facts into account, as well as the observation that glycopeptides form stable complexes with cell walls, it can be explicitly stated that the inhibition of the analyzed autolytic systems was a result of the spatial protection provided by glycopeptide molecules.

An unusual phenomenon that accompanies the acquisition of relatively high level of resistance to vancomycin by strain VM50 is the drastic drop in the resistance to β-lactam antibiotics. A similar phenomenon, though not as pronounced, was observed in the case of the teicoplanin resistant (TR) strain TNM. A possible explanation of this phenomenon could be that altered genetic background involving altered metabolism of the cell wall, may affect gene *mecA* itself or chromosomal genes not related to *mecA*, *b*ut necessary for optimal expression of methicillin resistance. However, analysis of the *mecA* nucleotide sequence of strain VM50 revealed its inactivation, involving the premature introduction of the stop codon TAA and thus resulting in the synthesis of shortened, non-functional peptide (Sieradzki *et al*., 1999). The complementation of VM50 carried out using functional gene *mecA* and restoring a high level of methicillin resistance to the strain, proved the strain's ability to fully express PBP2a. The restoration of methicillin resistance in VM50 cells subjected to complementation with *mecA* or selection with methicillin, resulted in simultaneous reduction in resistance to vancomycin. The reasons for this incompatibility remain obscure but are, however, a fortunate circumstance, allowing for the use of combinations of *â*-lactam antibiotics with glycopeptides, which, indeed, together show strong synergistic action (Sieradzki and Tomasz, 1999, Sieradzki *et al*., 1999b).

Studies by Sieradzki *et al*. (1999b) also embraced an analysis of clinical strains. The identical restriction patterns of genomic DNA of strains: PC1, PC2 and PC3, indicating their close relationship, suggest that PC3 is a selected *in vivo* VR derivative of earlier isolates PC1 and PC2 recovered from the same patient in which vancomycin therapy had failed. Characterization of PC3 showed that is has several traits in common with the laboratory mutants described herein, *i.e.*: drop (compared to the susceptible parental strain PC1) in degree of murein cross-linking, disappearance of PBP4 and lowered resistance to β-lactam antibiotics, which suggests that resistance is mediated by the same mechanism. Similar results were obtained recently for yet another set of isogenic MRSA isolates recovered from a bacteremic patient (Sieradzki *et al*., 2003). However, in this case, some changes in the structure and/or metabolism of their teichoic acids (in addition to reduced amounts of PBP4) appear to participate in the mechanism of their decreased susceptibility to vancomycin (Sieradzki and Tomasz, 2003). A broad epidemiological study revealed that the strain PC3 is closely related to a multi-drug resistant MRSA clone (Sieradzki *et al.*, 1999b), which is commonly isolated in hospital infections throughout New York (de Lencastre *et al*., 1996, Roberts *et al*., 1998).

In conclusion, it can be said that the presented mechanism of staphylococcal resistance to glycopeptide is most probably mediated by the accumulation of numerous mutations with a given genotype – different genes in different regions of the chromosome, which co-operate with one other. This makes the horizontal transfer of this type of resistance unlikely. The appearance of new forms of resistance in the clinical environment is in all likelihood a result of strong selective pressure as a consequence of the excessive and/or frequently improper use of antibiotics.

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