Bacteriolytic effect of teicoplanin

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The glycopeptide antibiotic teicoplanin belongs to the same group as vancomycin and ristocetin and is a valuable tool for studying the autolytic system of sensitive Gram-positive bacteria. Teicoplanin, at a concentration of 1 μg ml⁻¹, caused rapid lysis of exponential phase cells of Streptococcus faecalis. Bacillus spp. were most sensitive to the antibiotic; effective lysis occurred at 0.1 μg teicoplanin ml⁻¹. The bacteriolytic effect depended on the antibiotic concentration, the growth phase and growth rate of the target organism. Antibiotic added to overnight cultures did not cause lysis. Mg²⁺ (50 mM) was unable to prevent lysis. Mutants with decreased autolytic activity were more resistant to teicoplanin and lysed more slowly than the wild-type. Growth of bacteria in slightly acidic medium protected the cells against the lytic effect of teicoplanin typically observed at pH 7 or 8. This pH-dependent antibiotic tolerance was demonstrated with both bacilli and streptococci. Bacterial lysis was prevented by the presence of Ac-L-Lys(Ac)-D-Ala-D-Ala and normal growth was observed when this peptide was added simultaneously with teicoplanin. Bacteria pretreated with teicoplanin, washed and transferred to fresh medium or buffers behaved as if the antibiotic was still present; in neutral or slightly alkaline conditions strong lysis occurred, whereas in acidic buffer only bacteriostasis was observed. In contrast to vancomycin, teicoplanin induced some lysis of bacteria in hypertonic media, presumably by affecting the integrity of the cell membrane.

Introduction

Teicoplanin is an antibiotic chemically related to the group of glycopeptides which also includes vancomycin and ristocetin. It was isolated from the fermentation broth of Actinoplanes teichomyceticus sp. nov. (Parenti et al., 1978; Bardonne et al., 1978) and has outstanding bactericidal activity against Gram-positive pathogenic bacteria (Pallanza et al., 1983; Neu & Labthavikul, 1983; Fietta et al., 1983). The antibiotic is a mixture of five components of very similar polarity and a sixth, more polar component, all with a similar Mₙ of about 1900 (Borghi et al., 1984). The name teicoplanin describes the mixture of six components.

The structural unit common to all teicoplanin components consists of a linear heptapeptide aglycone in which two chlorinated β-hydroxytyrosine units and five substituted phenylglycine residues are interconnected in a tetracyclic structure, carrying D-mannose and N-acetyl-D-glucosamine moieties. An additional acyl glucosamine residue is present in some components and all contain one of five fatty acid residues. The main feature distinguishing teicoplanin from the other glycopeptide antibiotics is the presence of glucosamine and of aliphatic acid residues (Barna et al., 1984).

The mode of action of teicoplanin was investigated by Somma et al. (1984), who presented data showing that: (a) teicoplanin inhibits peptidoglycan synthesis in intact cells and in 'cell-free' systems; (b) inhibition by teicoplanin is reversed by addition of cell wall material; (c) the antibiotic binds to walls and whole cells, rendering them resistant to the action of lysozyme; and (d) a complex is formed between teicoplanin and the peptide Ac-L-Lys(Ac)-D-Ala-D-Ala, a synthetic analogue of the terminal peptid moiety of the pentapeptide peptidoglycan precursor. Greenwood et al. (1987) reported that teicoplanin induced lysis of growing Staphylococcus aureus and Streptococcus faecalis and caused morphological changes in these bacteria.

Teicoplanin should be a valuable biochemical tool in the study of peptidoglycan metabolism and its correlation with the autolytic process. The aim of the present study was to investigate some factors that influence teicoplanin-induced lysis in sensitive bacteria such as Bacillus and Streptococcus.

Methods

Bacterial strains. Bacillus subtilis 168 and the autolysis-defective mutant B. subtilis F36 were from the Bacillus Genetic Stock Center.
optical density of the culture at 660 nm with a Zeiss spectrophotometer. Organisms (Bratislava, Czechoslovakia) CCM 1875 were obtained from the Czechoslovak Collection of Microorganisms. This medium was called MBD LYC. In "poor" medium, for slow growth of a culture (10 ml) of exponential phase cells (approx. 2 x 10^8 ml^-1) growing in MBD LYC medium, teicoplanin (100 μl of an aqueous solution of appropriate concentration) was added at time zero. Untreated cells; cells treated with teicoplanin at the indicated concentration (μg ml^-1).

**Results and Discussion**

*Teicoplanin-induced lysis in exponential phase bacteria*

It has often been observed that the addition of antibiotics inhibiting cell wall biosynthesis to exponential phase bacterial cultures results in a rapid and extensive lysis (Boman & Erikson, 1963; Kitano & Tomasz, 1979; Chmara & Borowski, 1986; van Heijenoort et al., 1987).

The addition of teicoplanin to cultures of *Strep. faecalis* CCM 1875 in the early exponential growth phase caused complete lysis. Lysis was not instantaneous and did not begin until at least 15 min after antibiotic addition (Fig. 1a). Effective lysis was observed at antibiotic concentrations ranging from 1 to 10 μg ml^-1. Teicoplanin at a concentration of 0.1 μg ml^-1 caused lysis for 45 min followed by regrowth. Teicoplanin at 10 μg ml^-1 caused total lysis of *Strep. faecalis* with no regrowth, even after 24 h of incubation.

The clinical strain of *Strep. faecalis*, 8462 Vanco^8, did not lyse in the presence of teicoplanin even at a concentration of 250 μg ml^-1. Only minor growth inhibition was observed (data not shown). *B. pumilus* CCM 1697 was extremely sensitive to teicoplanin: it lysed rapidly at teicoplanin concentrations of 0.1 to 1 μg ml^-1 (Fig. 1b). A concentration of 0.05 μg ml^-1 caused only partial inhibition of growth followed by regrowth after 75 min. After 24 h incubation in the presence of 0.1 μg teicoplanin ml^-1, *B. pumilus*...
showed some regrowth, but not exceeding 25% of the
growth of a control culture without the antibiotic.
Teicoplanin at 2 μg ml⁻¹ caused total lysis of B. pumilus
with no regrowth, even after 48 h of incubation.

Effect of growth phase and growth rate on lysis

Teicoplanin-induced lysis was dependent on the growth
phase of B. pumilus at the time of addition of the
antibiotic. Up to 15 min after the transfer of overnight
culture to fresh medium, addition of 2 μg teicoplanin
ml⁻¹ caused only growth inhibition (data not shown).
This phenomenon, i.e. lack of lysis of non-growing cells,
seems to be a feature common to all bacteria and was
described by Tuomanen et al. (1986) as phenotypic
tolerance.

During the early and late exponential growth phase,
teicoplanin, as expected, induced rapid lysis. However,
addition of the antibiotic towards the end of exponential
growth resulted in an increased delay (45 min) before
lysis started. When the growth rate was clearly declining,
addition of the antibiotic caused only partial inhibition
of growth instead of lysis (data not shown).

In their experiments on the lysis of Escherichia coli
by ampicillin, Boman & Erikson (1963) showed that the
time of lysis was strongly dependent on the growth rate.
As shown in Fig. 2, teicoplanin induced lysis after a
longer lag phase with slowly growing Strept. faecalis
CCM 1875 and lysis was not very extensive.

Our results confirm the finding of Tuomanen et al.
(1986) that the rate of bacterial lysis following exposure
to cell-wall-inhibitory antibiotics appears to be a direct
function of bacterial growth prior to the addition of the
antibiotic.

Effect of teicoplanin on growth in hypertonic medium.

It is of interest that in hypertonic media, teicoplanin at
concentrations of 1 μg ml⁻¹ and 10 μg ml⁻¹, respectively,
induced lysis of B. pumilus CCM 1697 and Strept. faecalis
CCM 1875 (Fig. 3). In contrast only a bacteriostatic
effect was observed with vancomycin and methicillin.
This observation suggests that vancomycin and teico-
planin, although closely related, differ in their mode of
action. The mechanism of teicoplanin-induced lysis in
hypertonic media is unknown. It is possible that
teicoplanin, as well as inhibiting peptidoglycan synthe-
sis, exerts some effect on the cytoplasmic membrane.

Reynolds (1971), studying the effect of vancomycin at
concentrations of 10 and 50 μg ml⁻¹ on growth of
reconditioned B. megaterium KM protoplasts, and
Rosenthal et al. (1975), carrying out similar experiments
with Strept. faecalis ATCC 9790 protoplasts, did not
observe any lytic effects.

Effect of teicoplanin on growth of an autolysis-defective
mutant

Bacteriolysis is not a direct effect of the primary action
of cell-wall antibiotics which inhibit the biosynthesis
of peptidoglycan at various stages. It has been shown that a
secondary effect is a deregulation of bacterial autolysins,
enzymes which hydrolyse the existing peptidoglycan,
thereby leading to bacterial lysis (Rogers & Forsberg,
Subsequently it was shown (Rogers & Forsberg, 1971; Ayusawa et al., 1975; Fein & Rogers, 1976; Rogers et al., 1983) that strains of various micro-organisms deprived of autolysins are lysed less rapidly by antibiotics that inhibit cell wall synthesis than are the parent organisms. The term ‘tolerant’ was used to describe such strains.

In our studies we used *B. subtilis* 168 and the autolysis defective mutant FJ6. Compared to the corresponding wild-type strain, the autolysis defective mutant FJ6 exhibited much slower rates of lysis in the presence of 1 or 10 μg teicoplanin ml⁻¹ (data not shown). The lysis was not fully inhibited because the mutant is not completely deprived of autolysins but retains some activity (Rogers et al., 1983). The main lytic enzyme of *B. subtilis* 168 is an N-acetylmuramoyl-L-alanine amidase (Herbold & Glaser, 1975). Similar results were obtained when the teicoplanin-induced lysis of *Strep. faecium* ATCC 9790 and its autolysis-defective mutant LYT 14 were compared. The mutant showed much slower rates of lysis in the presence of teicoplanin (1 and 10 μg ml⁻¹).

**Effect of chloramphenicol on lysis**

Further evidence supporting the essential role of autolysins in teicoplanin-induced bacteriolysis was obtained by studying the effect of protein synthesis inhibition by chloramphenicol.

As can be seen from Fig. 4, the addition of 1 μg teicoplanin ml⁻¹ caused rapid lysis of *B. pumilus* CCM 1697 cells. Chloramphenicol at a concentration of 100 μg ml⁻¹ inhibited growth, although not completely. When chloramphenicol was added together with teicoplanin the bacteria lysed, but not so extensively as with...
teicoplanin alone. A complete inhibition of lysis was observed when chloramphenicol was added 30 min prior to the addition of teicoplanin. The effect of lysis inhibition lasted for at least 4 h. Quite similar effects were described by Rogers & Forsberg (1971), who recorded complete inhibition of lysis of \textit{B. licheniformis} by vancomycin (10 \(\mu\)g ml\(^{-1}\)) when chloramphenicol (50 \(\mu\)g ml\(^{-1}\)) was added at least 60 min before the addition of vancomycin.

The mechanism of chloramphenicol protection is not yet understood. As reported by Tomasz & Waks (1975) several experiments strongly suggest that this phenomenon is not caused by a deficiency in the number of hydrolase molecules at the cell surface, but rather by a disturbance of the metabolic regulation of the activity of the autolysins.

\textit{Induction of lysis of bacterial cells growing in media at different pH}

The response of \textit{B. pumilus} CCM 1697 and \textit{Strep. faecalis} CCM 1875 to teicoplanin was dependent upon the pH of the growth medium. At pH 7.0 or 8.0 the bacteria lysed rapidly after a short lag phase, but only slow lysis could be observed for \textit{B. pumilus} at pH 6.2 and no lysis was detected at pH 5.3. In the case of \textit{Strep. faecalis}, rapid lysis was observed at pH 7.0 or 8.0 but no lysis was detected at pH 6.2. Additional bacterial strains tested (\textit{Staphylococcus aureus}, \textit{Micrococcus luteus}, \textit{Corynebacterium} sp.) exhibited a similar response to the pH of the medium. These results (data not shown) indicate that appropriate modulation of the pH of the growth medium produces the typical features of antibiotic tolerance. The cellular response changes from lysis to bacteriostasis. It should be noted that the inhibition of lysis was not associated with slower cell growth in media of low pH. All bacterial strains examined grew equally well at both pH 6.2 and 7.0. Slower growth was observed only in the medium of pH 5.3; however, the growth was only about 30\% slower as compared to growth at pH 7.0. The tolerance of cells for teicoplanin in media of low pH was fully reversible. After 90 min exposure to 1-0 \(\mu\)g teicoplanin ml\(^{-1}\) \textit{B. pumilus} cells growing at pH 5.3 started to lyse rapidly after a lag phase of 30 min when washed and resuspended in medium of pH 7-0. This was also found with \textit{Strep. faecalis} pretreated with 10 \(\mu\)g teicoplanin ml\(^{-1}\) at pH 6.2.

This phenomenon of bacterial tolerance for teicoplanin at low pH corroborates the results of Goodell \textit{et al.} (1976) and Lopez \textit{et al.} (1976), who found that cell-wall antibiotics, irrespective of their site(s) of action in the biosynthetic pathway of peptidoglycan, exhibit suppressed lytic activity on bacteria growing in media of low pH.

\textit{Inhibition of teicoplanin-induced lysis by the peptide Ac-L-Lys(Ac)-D-Ala-D-Ala-Ac-L-Lys(Ac)-D-Ala-D-Ala}

Somma \textit{et al.} (1984) reported that teicoplanin, like vancomycin (Best & Durham, 1965; Nieto & Perkins, 1971), binds to cell walls and forms a complex with peptides related to bacterial peptidoglycan, such as Ac-L-Lys(Ac)-D-Ala-D-Ala. This peptide when used at a 250-fold molar excess with respect to teicoplanin (2 \(\mu\)g ml\(^{-1}\)) and when added simultaneously with the antibiotic to cultures of \textit{B. pumilus} CCM 1687 (Fig. 5) not only protected the cells from lysis but allowed bacterial growth indistinguishable from the control. Broadly similar results were obtained with \textit{M. luteus} (data not shown).

The peptides Ac-L-Lys(AC)-D-Ala or Ac-L-Lys(AC)-L-Ala-D-Ala in a 250-fold molar excess relative antibiotic did not exert any inhibitory influence on teicoplanin-induced lysis of \textit{B. pumilus} and \textit{Strep. faecalis} (data not shown). On the other hand the peptides Ac-L-Lys(AC)-D-Ala-Gly and Ac-L-Lys(AC)-D-Ala-D-Leu, tested under the same conditions, protected the cells from lysis but allowed cell growth to reach only 30-40\% of a control culture.
Fig. 6. Effect of a washing step on the lysis of B. pumilus CCM 1697 cells pretreated with teicoplanin. To a culture (40 ml) of exponential phase cells growing in MBD LYC medium, teicoplanin (2 \( \mu g \) ml\(^{-1} \)) was added at the time indicated by the arrow. After 15 min, the cells were harvested, rapidly washed and resuspended in 10 ml of: •, fresh medium; ○, fresh medium (pH 7.0) containing Ac-L-Lys(Ac)-D-Ala-D-Ala in a 250-fold molar excess; △, ▲, 100 mM Bistris/HCl buffer, adjusted to the indicated pH values.

Nieto et al. (1972) have similarly observed that the inhibition of growth of B. megaterium K,M, Staph. aureus and M. lysodeicticus NCTC 2665 by iodovancomycin could be reversed by diacetyl-\( \gamma \)-diaminobutyryl-D-alanyl-D-alanine added in a 38-fold molar excess relative to the antibiotic.

**Effect of teicoplanin pretreatment of bacterial cells on lysis**

A consequence of the binding of teicoplanin to the bacterial cell wall (Somma et al., 1984) is that simple washing of cells pretreated with the antibiotic is not sufficient to prevent lysis. B. pumilus CCM 1697 pretreated with 2 \( \mu g \) teicoplanin ml\(^{-1} \) for 15 min, washed and transferred to fresh medium or buffers behaved as if the antibiotic was still present (Fig. 6). Cells suspended in Bistris/HCl buffer of pH 7.5 lysed rapidly, but when the buffer was slightly acidic, no extensive lysis was observed. For B. pumilus a pH value of 6.0 was enough to prevent lysis. On the other hand, cells resuspended in fresh medium at pH 7.0 lysed to the same degree as if the antibiotic was still present. Cells transferred to fresh medium containing Ac-L-Lys(Ac)-D-Ala-D-Ala in a 250-fold molar excess relative to the antibiotic did not undergo lysis and, moreover, immediately started to grow. Untreated cells of B. pumilus, when suspended in buffer at pH 7.5, exhibited only slight lysis, not exceeding 15\% of the initial optical density. Peptides such as Ac-L-Lys(Ac)-L-Ala-D-Ala and Ac-L-Lys(Ac)-D-Ala-L-Ala, when added to teicoplanin-pretreated and washed cells, did not protect the cells from lysis (data not shown) indicating that the D-D-sequence is indispensable for a reversal of teicoplanin-induced lysis.

The reversal of teicoplanin-induced bacteriolysis by Ac-L-Lys(Ac)-D-Ala-D-Ala occurred only with growing cells. The peptide, at a 250-fold molar excess, did not confer protection from lysis in buffer (pH 7.5) (data not shown).

**Specific effects of teicoplanin**

The results of our studies on the response of bacterial cells to the action of teicoplanin largely coincide with the generally known effects of cell-wall-inhibitory antibiotics including phenomena such as the dependence of the lysis rate on the growth rate and lack of lysis of non-growing cells.

We have made, in addition, the more unusual observation that teicoplanin, unlike vancomycin and methicillin, induces some lysis in hypertonic medium. This suggests that the antibiotic, as well as inhibiting cell wall biosynthesis, also exhibits some membrane-damaging activity. The molecule of the antibiotic contains acyl residues of C\(_9\)-C\(_{10}\) fatty acids (Borghi et al., 1984). This structural feature, making teicoplanin more lipophilic than vancomycin, may favour penetration into the bacterial cytoplasm membrane, thus causing membrane-damaging effects.

Another interesting phenomenon is the rather high tolerance of Strep. faecalis to teicoplanin in slightly acidic media. A similar effect of tolerance to penicillin in acidic medium was earlier described by Goodell et al. (1976). Correspondent with this phenomenon is the tolerance of autolysis-defective strains to teicoplanin. The tolerance of autolysis-defective bacteria to penicillin has been reported many times (Rogers & Forsberg, 1971; Rogers et al., 1983). The clinical importance of these aspects of penicillin action has been underlined by a recent report describing the isolation of penicillin-tolerant autolysis-defective pathogenic bacteria from patients with recurring infections (Horne & Tomasz, 1977). The teicoplanin tolerance of bacteria in a slightly acidic environment may have comparable clinical implications.

The presence of cell-bound teicoplanin is indispensable for its lytic action in growing cells, as shown by the experiments with the specific peptide Ac-L-Lys(Ac)-D-
Ala-d-Ala. In buffer alone (with permissive pH) the peptide did not protect the cells pretreated with teicoplanin from progressive lysis. The elucidation of this phenomenon requires further studies.

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References


