

Inhibition of the Binding of Penicillin to the Pneumococcal Penicillin-binding Proteins (PBPs) by Exogenous Cell Wall Peptides

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Incubation of pneumococci with D-alanine-containing peptides naturally occurring in peptidoglycan protected cells against lysis and killing by β -lactam antibiotics near MIC. Such peptides caused decreased binding of the antibiotic to penicillin-binding proteins (PBPs), primarily PBP 2B. This provides direct evidence *in vivo* for the hypothesis that β -lactams act as substrate analogues and identifies PBP 2B as a killing target in pneumococci.

INTRODUCTION

All antibacterial effects of β -lactam antibiotics require the presence of an intact β -lactam ring. The antibacterial activity of β -lactams is thought to result from the structural similarity between this moiety and the carboxy-terminal D-alanyl-D-alanine residue of the cell wall precursor disaccharide pentapeptide, which is the natural substrate of the cell wall synthetic enzymes (penicillin-binding proteins – PBPs) (Tipper & Strominger, 1965, 1968). However, effective competition between the β -lactam antibiotic and cell wall precursors for binding the PBPs has not been demonstrated *in vivo*. In this report we demonstrate in growing cells that such a competitive mechanism exists and that its consequences include interference with antibiotic-induced bacterial lysis and death.

METHODS

D-Alanine or D-alanine-containing peptides (0.1 to 5 mg ml⁻¹; Serva) listed in Table 1 were added to cultures of *Streptococcus pneumoniae* strain R6 (Tomasz & Waks, 1975a) growing exponentially in a chemically defined medium (Tomasz, 1964). Antibiotics listed in Table 2 were added 15 min later at concentrations ranging from $1 \times$ to $10 \times$ MIC and culture turbidity and viability were followed over 8 h.

For PBP assays *in vivo*, culture samples were incubated with D-amino acids for 15 min at 37 °C. Competition between D-amino acid and penicillin was best demonstrated when the standard PBP assay (Zigheboim & Tomasz, 1980) was modified such that samples were incubated with [³H]benzylpenicillin (ethylpiperidinium salt; obtained from Merck Research Division) at low dose (1.3 kBq, 0.03 μ g ml⁻¹) for a short time (3 min) at low temperature (32 °C), and then rapidly chilled on ice. The bacteria were recovered by centrifugation (1100 g for 15 min at 4 °C) and resuspended in 10 mM-sodium phosphate buffer, pH 7.0 (50 μ l) containing 1% Sarkosyl NL-97 (10 min at 37 °C). Lysates were prepared for slab gel electrophoresis as described by Williamson *et al.* (1980). PBP band densities were determined by scanning densitometry (Helena Instruments).

For PBP assays *in vitro*, pneumococcal membranes (Williamson *et al.*, 1980) (100 μ g protein) in 100 μ l 10 mM-sodium phosphate buffer, pH 7.0, were preincubated with the D-amino acids for 10 min at 37 °C followed by [³H]penicillin (13.3 kBq, 0.006 μ g) for 3 min at 32 °C. The mixture was chilled and 1% Sarkosyl (final concentration) was added to stop the acylation reaction. The mixture was prepared for polyacrylamide electrophoresis as described by Williamson *et al.* (1980).

RESULTS AND DISCUSSION

Addition of D-alanine to cultures of pneumococci completely prevented the lysis normally induced by $2 \times$ MIC of cephamycin C and nocardicin A, and lysis due to benzylpenicillin was delayed (Fig. 1). Results were similar for D-alanine-containing peptides (listed in Table 1) but

Table 1. *Quantification of competition between murein peptides and penicillin for binding to pneumococcal PBPs*

Competitor	Concn		Percentage of control level of [³ H]penicillin binding to PBPs				
	mg ml ⁻¹	mM	1A	1B	2A	2B	3
<i>Ineffective</i>							
L-Ala	0.5	5.6	85	101	84	96	96
L-Ala-D-Ala	0.5	3.1	85	97	96	95	99
D-Ala-L-Ala	0.5	3.1	74	91	79	95	83
<i>Effective</i>							
D-Ala	0.5	5.6	79	97	97	28	90
D-Ala-D-Ala	0.5	3.1	69	68	59	20	83
Lysyl-D-Ala-D-Ala	0.2	0.5	70	53	52	42	93
Lysyl-D-Ala-D-Ala	0.5	1.3	14	< 10	31	< 10	35

Table 2. *Variation in drug and concentration range permissive for prevention of lysis of pneumococci by D-alanine*

Antibacterial agent	Source	MIC ($\mu\text{g ml}^{-1}$)	Multiple of MIC where 1 mg ml ⁻¹ D-alanine could prevent lysis
Cephameycin C	Merck, Sharp & Dohme	50	2
Cephalosporin C	Hoechst Roussel	50	2
Penicillin G	Eli Lilly	0.006	1
Cefoxitin	Merck, Sharp & Dohme	1.5	1
Cephaloridine	Eli Lilly	0.05	1
Piperacillin	Bristol Myers	0.25	2
Nocardicin A	Fujisawa-Smith Klein	50	2

not L-alanine-containing peptides (listed in Table 1). Bacterial viability paralleled the turbidimetric data, indicating that under these conditions antibiotic-induced killing was also prevented (not shown). Each β -lactam tested appeared to have a specific concentration range near its MIC within which D-alanine could interfere with lysis induction (Table 2). Cephameycin C exhibited the broadest such range, while penicillin G demonstrated a very narrow range around the MIC.

Decreasing concentrations ($< 200 \mu\text{g ml}^{-1}$) of D-alanine produced progressively shorter delays in β -lactam-induced lysis (data not shown). On the other hand, increasing the concentration of D-alanine above 2 mg ml^{-1} stimulated the lytic activity of β -lactam antibiotics, as has been previously reported (Lark & Lark, 1961). D-Alanyl-D-alanine was as effective as D-alanine in interfering with cephameycin-C-induced lysis, while L-alanine was completely inactive.

Two mechanisms for the protective effect of D-alanine and its peptides against antibiotic-induced lysis were tested: (i) interference with the functioning of the autolytic amidase that is essential for lysis; and (ii) interference with the binding of the β -lactam antibiotics to PBPs. The first mechanism could be excluded since D-alanine-containing peptides had no effect on two amidase-dependent processes: (a) the autolysis of pneumococci in stationary phase (Tomasz & Waks, 1975b), and (b) lysis by inhibitors of early steps in wall synthesis (data not shown). The D-alanine effect occurred independent of the synthesis of autolysin. For example, cephameycin C ($2 \times \text{MIC}$) did not lyse an autolysis-defective derivative of strain R6 (strain lyt 4-4, Tomasz & Waks, 1975b) in the presence of exogenous autolysin if D-alanine (1 mg ml^{-1}), was also added: $< 5\%$ change in turbidity and $< 25\%$ release of cell wall radiolabel over 4 h in the presence of D-alanine vs $> 90\%$ and $> 95\%$ respectively for drug alone.

Eighty percent inhibition of [³H]penicillin binding to PBP groups 1, 2 and 3 occurred at a comparable concentration of about 30 mM for all D-alanine-containing peptides tested with the

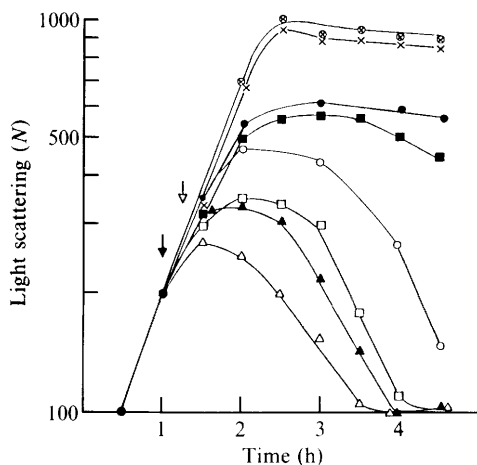


Fig. 1

Fig. 1. D-Alanine protection from lysis by β -lactam antibiotics. Cultures of *S. pneumoniae* (Tomasz & Waks, 1975a) were exposed to 1 mg D-alanine ml⁻¹ (filled symbols, added at filled arrow) followed by antibiotic at $2 \times$ MIC (added at open arrow): cephamycin C (squares), nocardicin A (circles), penicillin G (triangles). No antibiotic was added to control cultures (\otimes , plus D-alanine. \times , minus D-alanine). Representative experiment repeated in triplicate.

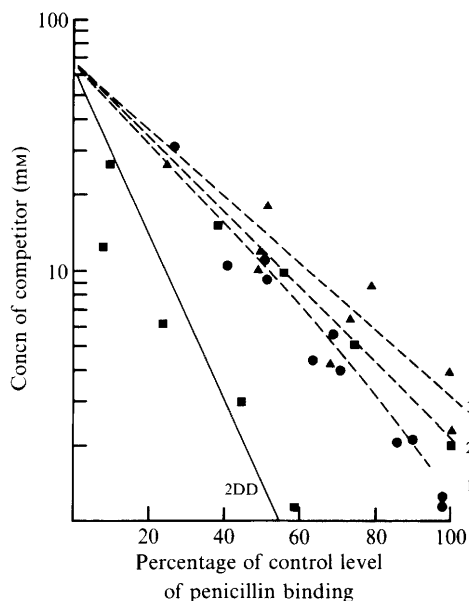


Fig. 2

Fig. 2. Similarity in the molar concentration of peptides required to inhibit penicillin binding to pneumococcal PBPs 1, 2, 3. Each data point is the mean of triplicate readings of PBP band densities. \bullet , PBP 1; \blacksquare , PBP 2; \triangle , PBP 3; in competition assays with D-alanine, D-alanyl-D-alanine and lysyl-D-alanyl-D-alanine. Regression analysis showed no differences between values except for PBP 2 vs D-alanyl-D-alanine (solid line, 2DD). Each dashed line is the regression line (90% confidence limits) calculated for all values for all peptides for an individual PBP (designated by number) except for PBP 2 data plotted as solid line.

exception of the interaction between D-alanyl-D-alanine and PBP 2, where 80% inhibition was achieved at about 10 mM (Fig. 2). Similar data were obtained when membrane preparations were used in the competition assays instead of the *in vivo* labelling of cells.

The protective effect of D-alanine derivatives vis-à-vis individual PBPs was demonstrated to be selective (Fig. 3, Table 1). L-Alanine, L-alanyl-D-alanine and D-alanyl-L-alanine had no effect on the binding of [³H]penicillin to any of the PBPs. On the other hand, D-alanine and the D-alanine-containing di- or tripeptide suppressed penicillin binding primarily to PBP 2B and somewhat less effectively to PBP 1B. The high degree of inhibition of the acylation of PBP 2B (and 1B) strongly suggests that the prevention of penicillin-induced lysis and killing under these conditions is the consequence of this selectivity of the protective effect and indicates that PBP 2B (and, possibly, 1B) is a killing target in pneumococci. This is consistent with the finding that several β -lactam antibiotics that do not bind to PBP 2B of pneumococci (aziridine ring on the C2 position in cefotaxime, ceftazidime and azthreonam) cause inhibition of growth without culture lysis (Hakenbeck *et al.*, 1987; Laible & Hakenbeck, 1987). It is also consistent with the suggestion that PBP 2B may be the rate-limiting enzyme in peptidoglycan synthesis (Williamson & Tomasz, 1985).

Our interpretation of the PBP-binding experiments is as follows: cell wall peptides form reversible complexes with the PBPs by occupying substrate-specific sites or subsites within the PBPs. This results in a decrease in the fraction of the PBP available for irreversible binding and acylation by the β -lactam. Competition between β -lactam antibiotics and peptide substrates

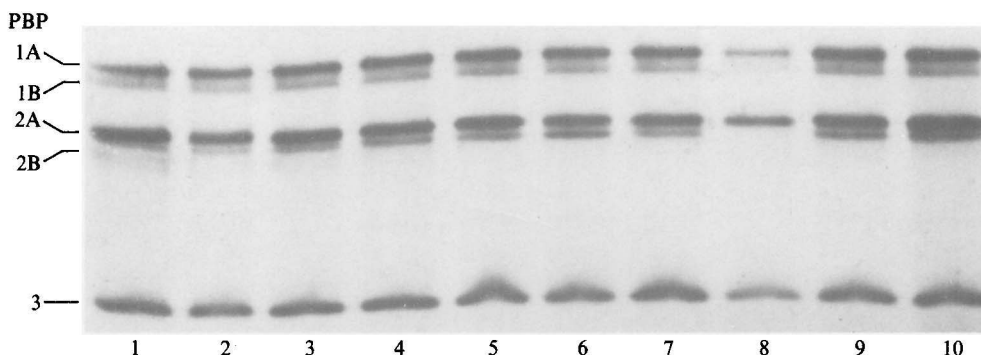


Fig. 3. Titration of *in vivo* [^3H]penicillin binding to pneumococcal PBPs in the presence of various alanine-containing peptides. Peptides were used at concentrations of 200 and 500 $\mu\text{g ml}^{-1}$ in the left and right lanes respectively of each pair. Lane 1, D-alanine; lane 2, D-alanyl-D-alanine; lane 3, no peptide; lane 4, D-alanine (500 $\mu\text{g ml}^{-1}$); lanes 5 and 6, D-alanyl-D-alanine; lanes 7 and 8, lysyl-D-alanyl-D-alanine; lanes 9 and 10, L-alanyl-D-alanine. Representative experiment repeated in triplicate.

used in model transpeptidation and carboxypeptidation reactions and catalysed by soluble enzymes has been demonstrated *in vitro* (Hammes, 1978; Tamura *et al.*, 1976). The lack of competitive inhibition by D-alanyl-L-alanine or L-alanyl-D-alanine in our PBP protection effect shows a striking analogy to the finding that these same peptides had no substrate activity in the model carboxypeptidase assay (Ghuysen *et al.*, 1974). It has also been demonstrated that several radioactively labelled β -lactam antibiotics and the cell wall peptide analogue diacyl-lysyl-D-alanine-D-alanine-formyl ester bind to the same serine residue at the active site of some bacterial PBPs (Ghuysen *et al.*, 1974). Our observations demonstrate that competition between cell wall peptides and β -lactam antibiotics occurs *in vivo* with live, growing bacteria and that this effect can be linked to physiological consequences. The somewhat wider concentration range for cephamycin C and nocardicin A (as compared to benzylpenicillin) within which the protective effect of D-alanine could be demonstrated is presumably due to the fact that these two antibiotics are poor acylating agents and therefore the D-amino acids can compete more effectively (Kunugita *et al.*, 1981; Stapley & Birnbaum, 1981).

Our finding represents an example of penicillin tolerance arising from modulation of antibiotic effects at the level of bacterial PBPs. A selective tuning down of the antibiotic affinities of 'lytic' PBPs may be the mechanism of the drug-specific tolerance described for a group of clinical pneumococcal strains (Liu & Tomasz, 1985; Moreillon & Tomasz, 1988; Tuomanen *et al.*, 1988). It also may explain the protective activity of D-amino acids against β -lactam-induced lysis of *Escherichia coli* (Tuomanen & Tomasz, 1984).

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