Interaction of Non-lytic β -Lactams with Penicillin-binding Proteins in *Streptococcus pneumoniae*

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The monobactam aztreonam and the cephalosporin ceftazidime, β -lactam antibiotics that possess the same side chain R1, showed unusual effects on exponentially growing pneumococci compared to other β -lactams. Both antibiotics did not induce lysis even at concentrations up to 2 mg ml⁻¹, values well above the respective MICs. However, morphological alterations and growth inhibition of the cells were observed at much lower concentrations. Binding to penicillin-binding proteins (PBPs) *in vitro* could be monitored directly by using anti-aztreonam antiserum and the Western blot technique. Both antibiotics showed high affinity for PBP 3, but had an extremely low affinity for PBP 2b. It is suggested that the failure to bind to PBP 2b is responsible for the failure to induce lysis in pneumococci.

INTRODUCTION

In pneumococci several attempts have been made to establish a correlation between the biologically effective concentration of a β -lactam and its binding to any of the penicillin-binding proteins (PBPs). Williamson *et al.* (1980) compared binding of various β -lactams to PBPs *in vivo* to their MIC values. Acylation of the PBPs was determined indirectly by postlabelling of the treated cells with radioactive penicillin after removal of the unlabelled drug. The problems of this 'indirect competition' assay were extensively discussed by Frère & Joris (1985), and the data should be interpreted with the appropriate care. Nevertheless, it became clear that the antimicrobial target could not be the PBP with the highest affinity, since binding to PBP 3, for example, occurred in many cases far below the MIC level. The study revealed no antibiotic that bound exclusively to only one PBP, at least over a wide concentration range. The only relationship that seemed to exist was between 50% saturation of PBP 2b *in vivo* and the MIC values with 13 out of 18 antibiotics tested.

In another approach, a correlation was found between the residual amount of peptidoglycan synthesized during benzylpenicillin treatment and the amount of PBP 2b that was not acylated (Williamson & Tomasz, 1985). The importance of PBP 2b had also been deduced from analyses of penicillin-resistant clinical isolates of pneumococci. This PBP was the first to have a changed affinity towards benzylpenicillin in bacteria resistant to low levels of β -lactam followed by changes in the other high- M_r PBPs in highly resistant strains (Hakenbeck *et al.*, 1980; Zighelboim & Tomasz, 1980).

In order to get further information about the function of PBP 2b, we performed experiments with two antibiotics that did not show significant interaction with PBP 2b *in vitro*, ceftazidime and aztreonam (Fig. 1). In other words, rather than asking what happens to the cell when PBP 2b is inhibited, we looked at the reverse situation in which PBP 2b is the only PBP still functioning. Since antiaztreonam antibodies were available, which are directed against the side

Abbreviation: PBP, penicillin-binding protein.

Fig. 1. Structures of the antibiotics used in this study. I, ceftazidime; II, aztreonam.

chain of the two drugs (Adkinson *et al.*, 1984), the PBP- β -lactam complexes could be determined directly on Western blots.

METHODS

Bacterial strains and growth conditions. *Streptococcus pneumoniae* R6 is a derivative of the Rockefeller University laboratory strain R36A. Cells were grown in C medium (Lacks & Hotchkiss, 1960), supplemented with 0.1% yeast extract at 37 °C without shaking. For antibiotic treatment, samples of exponentially growing cultures were added to tubes containing the antibiotics to give the final concentrations as indicated in the text. Growth was monitored by measuring light scattering in a Coleman nephelometer. $N = 20$ corresponds to 3×10^7 cells ml⁻¹.

Labelling of PBPs with antibiotics *in vivo*. Samples of antibiotic-treated cultures (1 ml) were centrifuged in a Sorvall SS34 rotor (10 s, 39 000 g). Cells were resuspended in lysis buffer (20 mM-sodium phosphate buffer pH 7.2, 0.2% Triton X 100) and incubated at 30 °C for 5 min. The lysates were immediately prepared for SDS-PAGE by boiling in sample buffer. For post-labelling the PBP with [³H]propionylampicillin (Schwarz *et al.*, 1981; Hakenbeck & Kohiyama, 1982*a*), the radioactive antibiotic was added to the resuspended bacteria and incubated at 30 °C for 20 min. No turnover of *in vivo* acylated PBP was observed under these conditions.

SDS-PAGE, Western blotting and immunostaining. Details of the procedures have been described recently (Hakenbeck *et al.*, 1986). Rabbit anti-aztreonam antiserum was used in a 1 in 10000 dilution in PBS/0.05% Tween on Western blots. Antibodies against aztreonam were raised in rabbits using aztreonam-bovine thyroglobulin (36–92 aztreonam residues per thyroglobulin molecule) as described by Adkinson *et al.* (1984). PBS contains 1.44 g Na₂HPO₄, 0.2 g KH₂PO₄ and 0.8% NaCl l⁻¹.

Antibiotics. Aztreonam was obtained from Squibb-von Heyden (München, FRG), and ceftazidime from Glaxo (Greenford, UK).

RESULTS

Growth response to various concentrations of aztreonam and ceftazidime

Different concentrations of either aztreonam or ceftazidime were added to exponentially growing cultures of *S. pneumoniae*, and growth was followed by nephelometry. An untreated culture served as the negative, non-lysing, control and for a positive, lysing, control, one culture received benzylpenicillin at a concentration corresponding to 10 times the MIC value (Fig. 2*a, b*). Lytic response was not observed with either aztreonam or ceftazidime, even at 2 mg ml⁻¹, although growth inhibition occurred above 0.1 µg ml⁻¹ (ceftazidime) and 100 µg ml⁻¹ (aztreonam). This result has been confirmed in at least five independent experiments.

The MIC was determined by overnight incubation of diluted cultures with twofold dilutions of the antibiotics, and values of 0.25 µg ml⁻¹ and 125 µg ml⁻¹ for ceftazidime and aztreonam, respectively, were obtained. Thus, even far above 10 times the MIC, a concentration where β -lactams usually induce rapidly proceeding lysis in pneumococci, aztreonam- or ceftazidime-treated cultures merely stopped growth. Three hours after the addition of the drugs the plating efficiency of the penicillin-treated cells had dropped by a factor of 10⁵, whereas aztreonam (2 mg ml⁻¹) and ceftazidime (20 µg ml⁻¹) led to a decrease of only a factor of 20–50 compared with the untreated control culture.

*Binding of aztreonam and ceftazidime to PBP *in vivo**

After exposure of intact cells to the drugs, samples of the treated cultures representing similar amounts of cells were harvested by centrifugation, the cells lysed within a few minutes upon

Fig. 2. Growth response of *S. pneumoniae* to ceftazidime and aztreonam. At zero time, samples of an exponentially growing culture of *S. pneumoniae* were added to tubes containing (a) ceftazidime or (b) aztreonam or penicillin at the following concentrations ($\mu\text{g ml}^{-1}$): (a) $\circ$, none; ∇ , 0.01; $\blacklozenge$, 0.1; $\blacktriangle$, 1; $\blacksquare$, 10; $\bullet$, 2000; (b) $\circ$, none; $\blacktriangle$, 1; $\blacksquare$, 100; ∇ , 1000; $\bullet$, 2000; $\square$, benzylpenicillin only, 0.1. Growth was followed by nephelometry (see Methods).

resuspension in detergent-containing buffer, and the samples were subjected to SDS-PAGE immediately. On the corresponding Western blots, aztreonam-PBP and ceftazidime-PBP complexes could be visualized with the help of anti-aztreonam antibodies since the antibodies are directed against the side chain R1 which is identical in both compounds (Fig. 3). With both antibiotics, binding to PBPs occurred far below the respective MIC. This was especially striking with aztreonam, where even at $0.01 \mu\text{g ml}^{-1}$ (10^{-4} times the MIC), binding to PBP 3 became apparent, followed at higher concentrations by binding to PBP 1b (10^{-3} times the MIC). Almost all PBPs had reacted with the exception of PBP 2b at $100 \mu\text{g aztreonam ml}^{-1}$ and $10 \mu\text{g ceftazidime ml}^{-1}$. For unknown reasons, the ceftazidime-PBP 3 complex was less reactive on the Western blot with the antiserum than was the corresponding aztreonam complex.

We also determined the antibiotic binding in the 'indirect competition' assay, i.e. after labelling intact cells with the nonradioactive antibiotic, and postincubation of the cell lysates with radioactive penicillin. Fluorograms (Fig. 4) confirmed the results obtained with the immune staining. Again, PBP 2b appeared to have very low affinity for both the monobactam and the cephalosporin. Only at concentrations above $800 \mu\text{g ml}^{-1}$ was some binding to PBP 2b apparent. Incubation at 37°C of the cell lysates before addition of the radioactive β -lactam did not increase the label on PBP 2b, indicating low affinity for both antibiotics. However, some turnover of aztreonam occurred under these conditions on PBP 1a (not shown).

Morphological alterations during antibiotic treatment

The morphological response was followed by phase-contrast microscopy of Gram-stained pneumococci. Cells were grown in medium with or without addition of 2% (w/v) choline. At 2% (w/v) choline, the autolytic system is repressed and the cells grow in chains, which facilitates the observation of morphological alterations (Briese & Hakenbeck, 1983). Fig. 5 shows cells after exposure to aztreonam ($100 \mu\text{g ml}^{-1}$) for 105 min (b) and 305 min (f), and ceftazidime ($1 \mu\text{g ml}^{-1}$) for 105 min (c, d). At both concentrations, the antibiotics showed interaction with all PBPs except PBP 2b (see Fig. 3). The cells appeared more elongated with aztreonam, and more lemon shaped with ceftazidime. Below the MIC, at $0.1 \mu\text{g aztreonam ml}^{-1}$ and $0.01 \mu\text{g ceftazidime ml}^{-1}$, the morphology was affected and could be followed especially in the 2% choline grown cultures. Rather than growing in straight chains, as seen in Fig. 5(e), the cells appeared zig-zagging as if the division plane could not be centred correctly (not shown). At very high concentrations, where an almost immediate growth inhibition was observed, hardly any defects in cell shape could be seen as if cell wall biosynthesis had stopped too rapidly to allow any manifestation of abnormal wall growth.

Fig. 3. Binding of aztreonam (a) and ceftazidime (b) to PBP in intact cells revealed on Western blots with anti-aztreonam antiserum. Samples (1 ml) of drug-treated cells were centrifuged and prepared for SDS-PAGE as described in the text. After Western blotting, antibiotic-PBP complexes were stained with anti-aztreonam antiserum and alkaline-phosphatase conjugated anti-rabbit IgG antibodies. The antibiotic concentrations ($\mu\text{g ml}^{-1}$) are indicated.

Fig. 4. Fluorogram of PBP from drug-treated cells after post-labelling with $[^3\text{H}]$ propionylampicillin. Lysates of drug-treated cells (a, aztreonam; b, ceftazidime) were incubated with $1 \mu\text{l}$ $[^3\text{H}]$ propionylampicillin and proteins were separated on SDS gels (see text for details). Shown are the fluorograms of the gels after 3 d exposure.

DISCUSSION

The results presented here suggest that the lytic effect of β -lactam antibiotics in pneumococci is correlated with binding of these compounds to PBP 2b. The antibiotics used, aztreonam and ceftazidime, showed almost no interaction with PBP 2b and no lysis occurred at concentrations as high as 2 mg ml^{-1} (16 and 4000 times the MIC, respectively).

One aspect concerns the binding specificity of the two compounds: they share a common side chain but have a different ring system, aztreonam being a monobactam, and ceftazidime a cephalosporin. We have recently extended the investigations using another three related cephalosporins (cefotaxime, ceftixozime, and cefmenoxime) in which the R1 side chain carries only a methyl group instead of the 2-methylpropanoic acid residue as in aztreonam (see Fig. 1). All three drugs did not bind to PBP 2b, and did not induce cellular lysis (unpublished observations). Another common feature of all the five β -lactams was an extremely high affinity to PBP 3, the pneumococcal DD-carboxypeptidase (Hakenbeck & Kohiyama, 1982b). The

Fig. 5. Morphological response of pneumococci to aztreonam and ceftazidime. Exponentially growing cultures were treated as follows: (a-d) No choline added, incubation for 105 min: (a) untreated control; (b) aztreonam ($100 \mu\text{g ml}^{-1}$); (c, d) ceftazidime ($1 \mu\text{g ml}^{-1}$). (e, f) Choline (2%) added, incubation for 305 min: (e) control; (f) aztreonam ($100 \mu\text{g ml}^{-1}$). Photographs were taken in a phase contrast microscope, after Gram staining of the cells. The bar represents $5 \mu\text{m}$ (the magnification is the same for all the photographs).

similarity of their interaction with pneumococcal PBP suggests that it is only the R1 side chain that directs the specificity of these compounds.

Also of great importance is the fact that the β -lactams used here failed to induce lysis and killing in *S. pneumoniae*, producing only growth inhibition and morphological alterations. Pneumococci belong to the most penicillin-sensitive group of organisms. Usually, β -lactam treatment leads to rapid lysis after an initial short period of growth inhibition. A tolerant response of wild-type *S. pneumoniae* is unusual and, to our knowledge, has not been described before. In pneumococci it has been well established that lysis is due to the activity of the autolytic enzyme, an amidase, and thus tolerance has been found to be associated with autolysin-deficiency (Tomasz *et al.*, 1971; Tomasz & Waks, 1975). Nevertheless, the relation between the first measurable effect after penicillin addition, binding to PBPs, and the final lytic event is far from clear (for a review see Tomasz, 1979). In two recent reports, tolerant behaviour in pneumococci has been described which did not involve alterations in either PBP or the autolytic enzyme, indicating some defect in the pathway of autolysin triggering (Williamson & Tomasz, 1980; Liu & Tomasz, 1985).

It is clear that binding of a β -lactam to only one PBP does not necessarily induce lysis; for instance, inhibition of PBP 3 or 2 in *Escherichia coli*, although these are essential proteins, does not result in lysis for several generations (Spratt, 1977). Acylation of only PBP 3 in pneumococci, which occurs at low concentrations of, for example, aztreonam or ceftazidime, caused neither lysis nor inhibition of growth, although irregular cell shapes were observed under these conditions. It is amazing that acylation of even five out of six PBPs, which occurs at concentrations higher than the MIC values of the antibiotics used here, resulted in inhibition of growth and an abnormal cell morphology, but not lysis. Inhibition of PBP 2b, and only of this PBP, seems to be the necessary first event in the still unknown chain of reactions which finally leads to cellular lysis.

Thus, PBP 2b could be addressed as the lethal target in *S. pneumoniae*, confirming the vital biological role of this protein, as has been suggested in recent reports (Williamson *et al.*, 1980; Williamson & Tomasz, 1985). High- M_r PBPs in several other Gram-positive organisms have

been identified as killing targets on the basis of their high and exclusive sensitivity towards β -lactams at the corresponding MICs (Chase *et al.*, 1978; Reynolds *et al.*, 1978; Fontana *et al.*, 1980). The converse situation, namely saturation of all but one PBP far below the MIC, has been observed in natural and acquired penicillin-resistant staphylococci and enterococci. It has been concluded that this PBP with the lowest antibiotic affinity is the biologically important enzyme (Brown & Reynolds, 1980; Fontana *et al.*, 1983). The situation with PBP 2b in pneumococci is different again: this protein is neither especially sensitive to the β -lactams investigated here, nor does it bind at the MIC values of these compounds. In fact, it does not interact at all and the typical effects of penicillin treatment, like lysis and killing, are not evident. This protein might play a role similar to PBPs 1a and 1b in *E. coli*, where acylation of these proteins is correlated with the rapid lytic response.

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