THE RESPONSE OF *PSEUDOMONAS AERUGINOSA* TO AZLOCILLIN, TICARCILLIN AND CEFSULODIN

T. S. J. ELLIOTT AND D. GREENWOOD

*Department of Microbiology and Public Health Laboratory,
University Hospital, Queen's Medical Centre, Nottingham NG7 2UH*

SUMMARY. The morphological response of *Pseudomonas aeruginosa* to azlocillin, ticarcillin and cefsulodin was investigated by electron microscopy. Each antibiotic initially caused the formation of filaments. On further incubation in the presence of azlocillin, deposits of dense intracellular material were observed; these were absent from cells exposed to the other two antibiotics. On continued incubation, lysis of the filaments occurred, but the mode of lysis differed between the antibiotics: azlocillin caused breakage at a restricted number of sites in the cell wall, ticarcillin produced breakage at many points and cefsulodin caused extensive cell-wall damage. In addition, ticarcillin and cefsulodin appeared to cause more lysis and spheroplast formation than did azlocillin.

The morphological changes correlated with turbidimetric measurements of bacterial response to the three antibiotics, which showed ticarcillin and cefsulodin to act more rapidly than azlocillin.

INTRODUCTION

Beta-lactam compounds that exhibit activity against *Pseudomonas aeruginosa* are now available. Among them are ticarcillin (Sutherland, Burnett and Rolinson, 1971), an α -carboxypenicillin, azlocillin (König *et al.*, 1977), an acylureidopenicillin, and cefsulodin (Tsuchiya, Kondo and Nagatomo, 1978), a cephalosporin structurally related to sulbenicillin. Previous studies have shown differences between these agents with regard to their antibacterial effects on *P. aeruginosa*. Azlocillin has been reported as being more active than ticarcillin (Coppens and Klastersky, 1979), although other findings suggest the converse (White, Comber and Sutherland, 1980). Cefsulodin has been shown to be more active than either azlocillin or ticarcillin (Zak *et al.*, 1979).

In the presence of β -lactam antibiotics *P. aeruginosa* undergoes various morphological alterations, resulting in the formation of filamentous forms or spheroplasts, with subsequent cell lysis (White *et al.*, 1980; Greenwood and Eley, 1982a). The effect varies with the strain (Greenwood and Eley, 1982a) and may also depend on the length of exposure, inoculum size and on the amount of antibiotic used.

Received 11 Oct. 1982; accepted 3 Nov. 1982.

To elucidate other differences in the effect of anti-pseudomonal β -lactam antibiotics, we have used transmission and scanning electron microscopy to investigate the effects of azlocillin, ticarcillin and cefsulodin on two strains of *P. aeruginosa*. Electron microscopic findings were correlated with results obtained by continuous turbidimetric monitoring and with conventional estimations of minimum inhibitory concentrations.

MATERIALS AND METHODS

Antibiotics. Azlocillin was provided by Bayer UK Ltd, Haywards Heath, West Sussex RH16 1TP, ticarcillin by Beecham Pharmaceuticals Research Division, Betchworth, Surrey RH3 7AJ, and cefsulodin by CIBA-Geigy AG, Basel, Switzerland. Appropriate dilutions of antibiotic were prepared immediately before use in "complete medium" (Greenwood and O'Grady, 1973) supplemented with KNO_3 1% (w/v).

Bacterial strains. Two strains of *P. aeruginosa*, designated A1 and A2, were clinical isolates selected from those investigated in previous studies (Greenwood and Eley, 1982a and b).

Titration of antibiotic. Minimum inhibitory concentrations (MICs) were determined by the broth dilution method. Serial twofold dilutions of antibiotic were prepared in 1-ml volumes of broth. These were inoculated with an equal volume of broth containing bacteria from a culture in the logarithmic phase of growth, to give a final concentration of approximately 10^7 organisms/ml. The broths were incubated for 16 h and the lowest concentration of antibiotic inhibiting growth was taken as the MIC.

Turbidimetric studies. Ten-ml volumes of KNO_3 -supplemented complete medium were inoculated with bacteria from a 16-h broth culture to give a final concentration of 10^6 – 10^7 bacteria/ml. The cultures were incubated in a modified version of a twelve-channel continuous-opacity monitoring device described by Mackintosh *et al.* (1973). Antibiotic was added when the cultures were in the logarithmic growth phase at an opacity level of 30% of maximum, equivalent to a viable count of *c.* 10^8 bacteria/ml.

Electron microscopy. Samples were taken from the broth cultures of the turbidimetric experiments at various time intervals for scanning electron microscopy and for ultra-thin sectioning.

Thin sectioning. The bacteria were fixed for up to 16 h at room temperature in glutaraldehyde 5% in 0.05M sodium cacodylate (pH 7.2) containing 10mM MgSO_4 . The samples were then embedded by centrifugation (2500 rpm for 5 min) in 2% agarose, which was subsequently cut into 2–3 mm cubes. The cubes were washed three times in the sodium cacodylate buffer and then fixed in osmium tetroxide 1% (w/v) for 16 h at room temperature. The samples were washed again in buffer and then transferred to uranyl acetate 0.5% (w/v) for 2 h at room temperature. After another washing for 5 min in buffer, the blocks were dehydrated in ethanol and finally embedded in a mixture of Epon 812 and Araldite CY212 (Mollenhauer, 1964), which was polymerised at 60°C for 24 h. Sections were cut on an ultramicrotome (Ultratome III, LKB Instruments Ltd, S-161 25, Bromma, Sweden) and mounted on formvar-carbon coated grids. Sections were stained for 1 min in the dark with uranyl acetate 1% (Reynolds, 1963), washed in distilled water and dried on filter paper. They were then stained with lead citrate for 20 s, washed first in 0.02M NaOH, then twice in water and finally dried in air. The specimens were examined in a Jeol 100C electron microscope at an accelerating voltage of 100 kV.

Scanning electron microscopy. Samples were fixed in glutaraldehyde 5% in 0.05M sodium cacodylate containing 10mM MgSO_4 for 1 h and then dehydrated in alcohol. After centrifugation at 3000 rpm for 5 min, they were suspended in acetone, placed on scanning stubs and dried in a critical-point drier (Polaron Equipment Ltd, 60 Greenhill Crescent, Holywell Industrial Estate, Watford, Herts WD1 8XG). The stubs were then coated with gold for 2 min in a sputter coater (E.M. Scope, Kingsnorth Industrial Estate, Wotton Road, Ashford, Kent TN23 2LN). Electron micrographs were obtained by the scanning facility of the Jeol 100C microscope.

RESULTS

Minimum inhibitory concentrations

In broth MIC tests with an inoculum of *c.* 10^5 bacteria/ml, both strains were inhibited by azlocillin 2 $\mu\text{g/ml}$, ticarcillin 8 $\mu\text{g/ml}$ or cefsulodin 1 $\mu\text{g/ml}$.

Morphological response

The structure of both strains of *P. aeruginosa* as observed by thin section or scanning electron microscopy was similar. The outer and inner membranes were closely adherent to the intervening wall and nuclear material was dispersed throughout the cytoplasm, which contained numerous ribosomes (fig. 1A). The outer membranes were smooth and intact (fig. 1A) and septum formation was evident (figs. 1A and 1B).

Azlocillin. After exposure to azlocillin for 2 h at concentrations of 16, 32, 64 and 128 $\mu\text{g/ml}$, the bacteria became filamentous. After exposure for a further 1–2 h, swollen areas appeared at intervals along the cells, but the cell wall remained intact (fig. 2) and there was still no evidence of cell division. During the next 2 h, electron-dense material was deposited at various intracellular sites including the inner edge of the cytoplasmic membrane, the terminal part of filaments (fig. 3A) and areas of swellings. This material was distinct from the cytoplasm and the nuclear material and occasionally appeared in layers that were parallel to the long axis of the filament (fig. 3B). After exposure to azlocillin for 8 h the outer membrane of some of the filaments became disrupted at a restricted number of sites and breaks subsequently appeared in the cell wall, through which intracellular contents eventually extruded. The swollen areas, however, remained intact and the filaments retained their basic shape, although they appeared somewhat collapsed. After exposure for 16 h, further lysis was evident, although many bacteria with normal morphology were also present.

Ticarcillin. The morphological response to ticarcillin (32, 64 and 128 $\mu\text{g/ml}$) differed from that seen with azlocillin in that intracellular material was not formed and there was increased disruption (fig. 4A) and breakage (fig. 4B) of the outer membrane, with subsequent cell lysis (fig. 4C). The breakage sites were randomly placed and were of variable size (fig. 4B). These morphological changes occurred during the first 6 h of exposure to ticarcillin. In addition, exposure of the cells to ticarcillin 64 and 128 $\mu\text{g/ml}$ caused spheroplast formation in a proportion of the bacterial population.

Cefsulodin. In the presence of cefsulodin at concentrations of 16, 64 and 128 $\mu\text{g/ml}$, filamentation was also observed (fig. 5). However, bacteria exposed to cefsulodin displayed fewer but more extensive breakage points along the outer membrane (fig. 5A) through which extrusion of the cytoplasmic contents occurred (fig. 5B). In addition, spheroplasts were formed at all drug concentrations tested and these also showed breakage of the cell wall at only a few sites (fig. 6). Destructive changes in bacteria exposed to cefsulodin were detected about 1 h earlier than in the presence of ticarcillin. After overnight incubation in the presence of cefsulodin, most of the bacteria had been lysed, but some cells with apparently normal morphology persisted (fig. 7).

The relative times at which the various morphological events were observed to occur with the three antibiotics is shown diagrammatically in fig. 8.

FIG. 1.—(A) Scanning electron micrograph of *P. aeruginosa* strain A1 showing smooth surfaces and septation (s); (B) thin section of the same strain showing septation (s), complete outer (om) and inner (im) membranes, nuclear material (n) and numerous ribosomes. Bar = 0.5 μ m.

FIG. 2.—(A) thin section and (B) scanning electron micrographs of *P. aeruginosa* strain A1 exposed to azlocillin 64 $\mu\text{g/ml}$ for 4 h, showing filaments with intact cell surfaces and swollen areas (sw). Bar = 0.5 μm .

FIG. 3.—Thin-section electron micrographs of *P. aeruginosa* strain A1 exposed to azlocillin 32 $\mu\text{g/ml}$ for 6 h. showing dense intracellular material arranged in layers (A) terminally and (B) along the axis of the filament. Bar = 0.5 μm .

Turbidimetric studies

After exposure to each of the antibiotics at concentrations of 32, 64 and 128 $\mu\text{g/ml}$, the opacity of the cultures continued to rise for several hours, corresponding with the period during which filamentation was observed as the predominant morphological

FIG. 4.—Electron micrographs of *P. aeruginosa* strain A1 exposed to ticarcillin $64\text{ }\mu\text{g/ml}$ for 6 h. Thin-section micrographs showing (A) disrupted outer membrane (om) and (B) multiple breakage points (arrowed); (C) scanning electron micrograph showing lysis and collapse of filaments. Bar = $0.5\text{ }\mu\text{m}$.

FIG. 5.—Electron micrographs of *P. aeruginosa* strain A1 exposed to cefsulodin 64 $\mu\text{g}/\text{ml}$ for 16 h. (A) Thin section showing extensive breakage in outer membrane (arrowed); (B) scanning electron micrograph demonstrating extrusion of intracellular material from breakage points in the cell wall. Bar = 0.5 μm .

FIG. 6.—Thin section electron micrograph of *P. aeruginosa* strain A2 exposed to cefsulodin 32 $\mu\text{g/ml}$ for 16 h, showing fully developed spheroplast with relatively intact but disorganised cell wall. Bar = 0.5 μm .

FIG. 7.—Thin-section electron micrograph of *P. aeruginosa* strain A1 exposed to cefsulodin 32 $\mu\text{g/ml}$ for 16 h showing the presence of cells with apparently normal morphology among much lysed cell debris. Bar = 0.5 μm .

FIG. 8.—Diagrammatic representation of the time scale of morphological changes observed in *P. aeruginosa* exposed to azlocillin, ticarcillin or cefsulodin, each at a concentration of $64 \mu\text{g/ml}$.

response. The period during which the opacity continued to increase was longer for azlocillin than for the other two antibiotics. On subsequent incubation a decrease in opacity occurred, due to cell lysis. During incubation for 16 h, regrowth of cultures exposed to azlocillin occurred, but such regrowth was not observed after exposure to ticarcillin or cefsulodin.

Fig. 9 shows representative continuous opacity records obtained with one of the strains after exposure to each antibiotic at a concentration of $64 \mu\text{g/ml}$.

FIG. 9.—Continuous opacity records of *P. aeruginosa* strain A1. Antibiotics, as indicated, were added at arrow to achieve a concentration of $64 \mu\text{g/ml}$.

DISCUSSION

Previous studies by turbidimetric methods supported by light microscopy revealed differences in response of *P. aeruginosa* strains exposed to antipseudomonal β -lactam agents (Greenwood and Eley, 1982*a* and *b*). The present study extends these findings by demonstrating differences in the ultrastructural response of *P. aeruginosa* to three representative compounds: the α -carboxypenicillin, ticarcillin, the acylureidopenicillin, azlocillin and the cephalosporin, cefsulodin.

Although the predominant response of susceptible *P. aeruginosa* strains to all three antibiotics was the formation of long, non-septate, filaments, electron microscopy revealed differences in the extent of the cell-wall damage induced in the filamentous bacteria by the three agents. Furthermore, microscopy and turbidimetry indicated differences in the rapidity with which the antibiotics achieved a destructive effect. Breaks in the cell wall of filaments induced by azlocillin were evident only after exposure for 6–8 h, whereas such morphological changes were detected after exposure to ticarcillin or cefsulodin for 3–4 h. With ticarcillin, the integrity of the cell wall was breached through small point lesions, whereas cefsulodin produced fewer, but much more extensive cell-wall lesions.

One of the most striking and unexpected observations made in this study was that of the deposition of regular arrays of electron-dense material within bacteria exposed to azlocillin. It is tempting to speculate that this material represents excess peptidoglycan laid down aberrantly in the absence of septation. These inclusions were not observed in *P. aeruginosa* exposed to ticarcillin or cefsulodin and this may indicate a more nearly complete inhibition of peptidoglycan synthesis by these agents, corresponding with their more rapid action revealed by turbidimetric monitoring.

These and previous findings (Greenwood and Eley, 1982*a* and *b*) suggest that the activity of antipseudomonal β -lactam agents may differ in ways not indicated by conventional tests of antimicrobial susceptibility. The explanation of these differences and their therapeutic implications remain to be elucidated.

REFERENCES

- COPPENS, L. AND KLASTERSKY, J. 1979. Comparative study of anti-pseudomonas activity of azlocillin, mezlocillin and ticarcillin. *Antimicrobial Agents and Chemotherapy*, **15**, 396–399.
- GREENWOOD, D. AND ELEY, A. 1982*a*. A turbidimetric study of the responses of selected strains of *Pseudomonas aeruginosa* to eight antipseudomonal β -lactam antibiotics. *Journal of Infectious Diseases*, **145**, 110–117.
- GREENWOOD, D. AND ELEY, A. 1982*b*. Comparative antipseudomonal activity of some newer β -lactam agents. *Antimicrobial Agents and Chemotherapy*, **21**, 204–209.
- GREENWOOD, D. AND O'GRADY, F. 1973. Comparison of the responses of *Escherichia coli* and *Proteus mirabilis* to seven β -lactam antibiotics. *Journal of Infectious Diseases*, **128**, 211–222.
- KÖNIG, H. B., METZGER, K. G., MÜRMANN, R. P., OFFE, H. A., SCHACHT, P. AND SHRÖCK, W. 1977. Azlocillin – ein neues Penicillin gegen *Pseudomonas aeruginosa* und andere gram-negative Bakterien. *Infection*, **5**, 170–182.
- MACKINTOSH, I. P., O'GRADY, F., GREENWOOD, D., WATSON, B. W., CRICHTON, T. C., PIPER, R. AND FERRER, A. 1973. A twelve channel bacterial growth monitoring system. *Biomedical Engineering*, **8**, 514–515, 526.
- MOLLENHAUER, H. H. 1964. Plastic embedding mixtures for use in electron microscopy. *Stain Technology*, **39**, 111–114.

- REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology*, **17**, 208–213.
- SUTHERLAND, R., BURNETT, J. AND ROLINSON, G. N. 1971. α -carboxy-3-thienyl-methylpenicillin (BRL 2288) a new semi synthetic penicillin: *in vitro* evaluation. *Antimicrobial Agents and Chemotherapy*, **1970**, 390–395.
- TSUCHIYA, K., KONDO, M. AND NAGATOMO, H. 1978. SCE-129, antipseudomonal cephalosporin: *in vitro* and *in vivo* antibacterial activities. *Antimicrobial Agents and Chemotherapy*, **13**, 137–145.
- WHITE, A. R., COMBER, K. R. AND SUTHERLAND, R. 1980. Comparative bactericidal effects of azlocillin and ticarcillin against *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **18**, 182–189.
- ZAK, O., KONOPKA, E. A., TOSCH, W., AHRENS, T., ZIMMERMAN, W. AND KRADOLFER, F. 1979. Experimental evaluation of CGP 7174/E (SCE 129), a new injectable cephalosporin antibiotic active against *Pseudomonas aeruginosa*. *Drugs under Experimental and Clinical Research*, **5** (2-3), 45–59.