THE MORPHOLOGICAL RESPONSE OF *PSEUDOMONAS AERUGINOSA* TO AZTHREONAM, CEFOPERAZONE, CEFTAZIDIME AND N-FORMIMIDOYL THIENAMYCIN

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SUMMARY. The morphological responses of *Pseudomonas aeruginosa* to N-formimidoyl thienamycin, azthreonam, ceftazidime and cefoperazone were studied by transmission electron microscopy. They were correlated with the results of viable counts and continuous turbidimetric monitoring. N-formimidoyl thienamycin initially caused the formation of abnormally shaped cells which developed into spheroplasts. The other antibiotics caused filamentation of the bacteria, which subsequently underwent lysis. The degree and mechanism of lysis varied between the antibiotics. Exposure to azthreonam also resulted in the deposition of electron-dense intracellular material. As judged by conventional minimum-inhibitory-concentration tests, all the agents exhibited similar activity against the strains of *P. aeruginosa* tested, with ceftazidime displaying the highest activity. However, N-formimidoyl thienamycin caused the most extensive morphological damage and resulted in the most rapid fall in viable count.

INTRODUCTION

In the presence of β -lactam antibiotics, *Pseudomonas aeruginosa* undergoes several morphological changes. These include filamentation, spheroplast formation and cell lysis (Greenwood and Eley, 1982). The changes vary with the bacterial strain, the concentration of antibiotic, the length of exposure to the antibiotic and the inoculum size (Greenwood and Eley, 1982; Elliott and Greenwood, 1983).

In the present study, the effect of several recently developed β -lactam antibiotics on *P. aeruginosa* was investigated. The antibiotics were azthreonam, a monocyclic β -lactam (Livermore and Williams, 1981; Jacobus, Ferreira and Barza, 1982). N-formimidoyl thienamycin, a β lactam that does not have a sulphur atom in the thiazolidine ring (Wise, Andrews and Patel, 1981) and two cephalosporins, cefoperazone (Kayser, Huf and Homberger, 1981) and ceftazidime (Phillips *et al.*, 1981). To elucidate further the action of these antibiotics on *P. aeruginosa*, the morphological effects were studied by transmission electron microscopy. The findings were correlated with conventional

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estimations of minimum inhibitory concentration (MIC), viable counts and continuous turbidimetric monitoring.

MATERIALS AND METHODS

Antibiotics. Azthreonam was provided by E. R. Squibb and Sons, Hounslow, London TW3 3JA, ceftazidime by Glaxo Group Research, Greenford, Middlesex UB6 0HE, cefoperazone by Pfizer Ltd, Sandwich, Kent T13 9NJ and N-formimidoyl thienamycin by Merck Sharp and Dohme, Hoddesdon, Hertfordshire EN11 9BU. Appropriate dilutions of antibiotic were prepared immediately before use in complete medium (Greenwood and O'Grady, 1973) supplemented with KNO_3 1%.

Bacterial strains. Two strains of *P. aeruginosa*, designated A1 and A2, were clinical isolates used in previous studies (Elliott and Greenwood, 1983).

Antibiotic titration. Minimum inhibitory concentrations of the antibiotics were determined by the broth-dilution method. Serial twofold dilutions of antibiotic were prepared in 1-ml volumes of broth which were inoculated with an equal volume of broth containing bacteria from culture in the logarithmic phase of growth, to achieve a final concentration of *c.* 10^5 organisms/ml. The broths were incubated for 16 h at 37°C and the lowest concentration of antibiotic preventing development of visible turbidity was taken as the MIC.

Turbidimetric studies. Ten-ml volumes of complete medium supplemented with KNO_3 were inoculated with bacteria from a 16-h broth culture to achieve a final concentration of 10^6 bacteria/ml. The cultures were incubated at 37°C in a modified version of a 12-channel continuous opacity-monitoring device (Mackintosh *et al.*, 1973). Antibiotic was added when the cultures were in the logarithmic phase of growth at an opacity of 30% of maximum. Approximately 10^8 bacteria/ml were present at this opacity level.

Viable counts. At various time intervals after addition of antibiotic, 0.1-ml samples were taken from the broth cultures in the turbidimetric experiments. From these, serial tenfold dilutions were made in complete medium and duplicate 0.1-ml portions of the diluted broth were plated on to blood-agar plates. The plates were incubated at 37°C for 16 h and the number of colonies counted.

Electron microscopy. Two-ml portions were taken from the broth cultures in the turbidimetric experiments at various time intervals for examination by transmission electron microscopy.

The bacteria were fixed for 16 h at 20°C in glutaraldehyde 5% v/v in 0.05M sodium cacodylate (pH 7.2) containing 10 mM MgSO_4 . The samples were then embedded in agarose 2% w/v, which was subsequently cut into 2–3 mm cubes. The cubes were washed three times in the sodium cacodylate buffer and post fixed in osmium tetroxide 1% w/v for 4 h at room temperature. The samples were washed again in buffer and transferred to uranyl acetate 0.5% w/v for 1 h at room temperature. After another washing for 5 min in buffer, the blocks were dehydrated in ethanol and then in propylene oxide. The dehydrated blocks were 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 ultra microtome (Ultratome III, LKB Instruments Ltd, Sweden) and mounted on formvar carbon-coated grids. Sections were stained for 1 min in the dark with uranyl acetate 1% (Elliott and Greenwood, 1983), washed in distilled water and air dried. They were post stained with lead citrate for 20 s, washed first in 0.02M NaOH, then twice in water and finally air dried. The specimens were examined in a Jeol 100C electron microscope at an accelerating voltage of 100 kV.

RESULTS

Minimum inhibitory concentrations

In broth MIC tests with an inoculum of *c.* 10^5 bacteria/ml, both strains were

FIG. 1.—Thin-section electronmicrograph of *P. aeruginosa* strain A1 showing outer (OM) and inner membrane (IM) of cell wall, with ribosomes and nuclear material dispersed through cytoplasm. Bar = 0.5 μ m.

inhibited by azthreonam 2 μ g/ml, ceftazidime 0.5 μ g/ml, cefoperazone 4 μ g/ml and N-formimidoyl thienamycin 2 μ g/ml.

Morphological response

The structure of both strains of *P. aeruginosa* was typical of gram-negative bacilli. Thin sectioning demonstrated the double membrane of the cell wall, and the presence of ribosomes and nuclear material in the cytoplasm (fig. 1). The morphological response to the antibiotics of both strains were similar and are described below.

Azthreonam. Within 2 h of exposure to azthreonam at concentrations of 4, 32, 64 and 128 μ g/ml, the bacteria became filamentous with no cross walls. After exposure

FIG. 2.—Thin-section electronmicrograph of *P. aeruginosa* strain A1 treated with azthreonam 32 μ g/ml for 6 h, demonstrating filamentation and swellings (S) and deposition of dense intracellular material (IM) along inner surface of cell wall and at a midzone area. Bar = 0.5 μ m.

for another 1–2 h, the filaments became swollen at intervals along their length but the cell wall remained intact. Dense intracellular material was also present at intervals along the filaments (fig. 2) as well as being evident along the cytoplasmic surface of the inner membrane. This material was not found in the presence of any of the other antibiotics. After incubation for a further 2 h, there was breakage of the cell wall at a restricted number of sites resulting in lysis of the filaments. After incubation for 16 h, there was more lysis, with collapse of the filaments. However, a few abnormally shaped cells and apparently viable filaments persisted throughout.

FIG. 3.—Thin-section electronmicrographs of *P. aeruginosa* strain A2 treated with ceftazidime 64 $\mu\text{g/ml}$ for 6 h (A) and for 16 h (B), showing breakage of cell wall (arrowed), and eventual disruption of the filament after prolonged incubation. Bar = 0.5 μm .

FIG. 4.—Thin-section electronmicrographs of *P. aeruginosa* strain A1 treated with cefoperazone 16 µg/ml for 6 h, showing filamentation with multiple breakage points (arrowed). Bar = 0.5 µm.

FIG. 5.—Thin-section electronmicrograph of *P. aeruginosa* strain A1 treated with N-formimidoyl thienamycin 16 µg/ml for 4 h, showing abnormally shaped cells with complete cell walls and aberrant division sites (arrowed). Bar = 0.5 µm.

Ceftazidime. After exposure to ceftazidime for 2 h at concentrations of 4, 32, 64 and 128 $\mu\text{g/ml}$, filaments were again formed; after incubation for 4 h breakage of the cell wall began at many sites along these cells (fig. 3A). Lysis subsequently occurred, eventually resulting in disintegration of the filaments (fig. 3B).

Cefoperazone. The morphological response was similar to that seen with ceftazidime. At concentrations of 4, 32, 64 and 128 $\mu\text{g/ml}$ filamentation occurred within 2 h. After 4 h, small breakage points started to appear along the walls of the

FIG. 6.—Thin-section electronmicrographs of *P. aeruginosa* strain A2 treated with N-formimidoyl thienamycin 32 $\mu\text{g/ml}$ for 6 h, showing a spherical cell emerging from the side of a filament (A) and a spherical cell attached to the end of the rod-shaped cell (B). Bars = 0.5 μm .

FIG. 7.—Thin-section electronmicrographs of *P. aeruginosa* strain A1 (A) treated with N-formimidoyl thienamycin 64 $\mu\text{g/ml}$ for 16 h, demonstrating a single breakage point (arrowed) in the cell wall of a spheroplast, and (B) treated with N-formimidoyl thienamycin 128 $\mu\text{g/ml}$ for 16 h, showing remains of cell wall with only a few breakage sites (arrowed). Bar = 0.5 μm .

filaments (fig. 4). These filaments retained their basic shape although some were collapsed. With ceftazidime and with cefoperazone, filaments, which appeared to be viable, were present even after incubation for 16 h in all concentrations of antibiotics tested. There were, however, more of these filaments when the lower concentrations of antibiotic were used.

N-formimidoyl thienamycin. The initial response to *N*-formimidoyl thienamycin after exposure for 2 h to 4, 32, 64 and 128 $\mu\text{g/ml}$ was the formation of spheroplasts and cells of grossly abnormal morphology (fig. 5). Some of the cells demonstrated evidence of cross-wall formation (fig. 5). After further incubation in all concentrations of *N*-formimidoyl thienamycin tested, spheroplast formation predominated. Most of the spheroplasts emerged at the mid zone of cells (fig. 6A) but a few were formed at the ends (fig. 6B). Lysis of these spheroplasts began after incubation for 4 h, during which time they had swollen to several times their initial diameter. Lysis appeared to occur through a single break in the cell wall (fig. 7A). After exposure for 16 h, most of the cells had lost their cytoplasmic contents and lengths of unbroken cell wall remained (fig. 7B) together with some spheroplasts.

A summary of all these morphological responses is shown in fig. 8.

Turbidimetric studies

The opacity of cultures of *P. aeruginosa* exposed to each of the antibiotics continued to rise for several hours (fig. 9). This initial increase in opacity was observed even at the highest drug concentrations tested and was least marked with *N*-formimi-

FIG. 8.—Diagrammatic representation of the time scale of morphological changes observed in *P. aeruginosa* strain A2 exposed to each antibiotic at a concentration of 64 $\mu\text{g/ml}$. The times represent the earliest period when the various morphological changes were first observed.

FIG. 9.—Continuous opacity records of *P. aeruginosa* strain A2. Antibiotics, as indicated, were added at arrow to achieve a concentration of 64 µg/ml. NFT = N-formimidoyl thienamycin.

FIG. 10.—Viable counts of *P. aeruginosa* strain A2 cultures exposed to 64 µg/ml of each antibiotic for various times.

doyl thienamycin. The continued rise in opacity corresponded to the period during which filaments developed after exposure to ceftazidime, cefoperazone and azthreonam and to the development of cells with aberrant morphology and spheroplasts in the presence of N-formimidoyl thienamycin. On further incubation a fall in opacity was detected corresponding to the appearance of the destructive lesions observed by electron microscopy.

Viable counts on these cultures showed that N-formimidoyl thienamycin was the only agent to cause a fall in viable count during the first 4 h of exposure (fig. 10). In the presence of the other antibiotics the number of colony-forming units continued to increase slightly during the first 4 h. During overnight incubation the viable count decreased corresponding to the decline in opacity observed turbidimetrically.

DISCUSSION

In a previous study (Elliott and Greenwood, 1983) we found that the primary response of *P. aeruginosa* to azlocillin, ticarcillin and cefsulodin was failure of the division process, giving rise to the formation of long filamentous bacteria. Ultrastructural studies revealed that the filaments subsequently underwent further morphological changes, the nature of which varied with the antibiotic used.

We have now extended these studies to include some newer antipseudomonal β -lactam antibiotics, including the structurally novel compounds azthreonam (a monocyclic β -lactam) and N-formimidoyl thienamycin (a carbapenem). The present investigation showed that the filamentation response previously observed also followed exposure to ceftazidime, cefoperazone and azthreonam, but that N-formimidoyl thienamycin evoked the formation of spheroplasts similar to those induced by suitable concentrations of most β -lactam agents on *Escherichia coli* and other enterobacteria (Greenwood and O'Grady, 1973).

As in the previous investigation, the damage subsequently incurred by filaments on continued exposure to antibiotic varied with the agent: azthreonam-treated bacteria exhibited large deposits of electron-dense intracellular material and a few punctate lesions of the cell wall, effects similar to those previously observed with azlocillin; cefoperazone-treated cells displayed multiple point lesions, similar to those seen with ticarcillin; ceftazidime-treated cells underwent lysis through large breakage points, as previously observed with cefsulodin. The spheroplast response elicited by N-formimidoyl thienamycin in *P. aeruginosa* is unique among the agents that we have examined.

Morphological changes in gram-negative bacilli exposed to β -lactam antibiotics are thought to be consequences of antibiotic interaction with penicillin-binding proteins (PBPs) located on the inner membrane of the cell (Spratt, 1975). In *P. aeruginosa*, the situation is complicated by the relative impermeability of the cell envelope (Richmond, 1980). The filamentation response is thought to arise from binding of the antibiotic to PBP 3 which appears to be the primary target in *P. aeruginosa*. Subsequent morphological damage may depend upon the degree of binding to other PBPs, which may proceed in a sequential manner (Chase, Fuller and Reynolds, 1981). Thienamycin, of which N-formimidoyl thienamycin is a more stable derivative, exhibits preferential affinity for PBP 2 and PBP 1, but does not bind to any significant extent to PBP 3, at least in *E. coli* (Spratt, Jobanputra and Zimmermann,

1977) and this may explain the different response to this agent seen in this present study.

The relationship of PBPs to the sequential morphological changes in *P. aeruginosa* observed in this study can be fully elucidated only by appropriate biochemical investigations.

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