MORPHOLOGICAL RESPONSE AND GROWTH CHARACTERISTICS OF *LEGIONELLA PNEUMOPHILA* EXPOSED TO AMPICILLIN AND ERYTHROMYCIN

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SUMMARY. The morphological response of two strains of *Legionella pneumophila* to ampicillin 10 µg/ml and erythromycin 10 µg/ml *in vitro* was studied by electronmicroscopy, MIC estimations and viable counts. In the presence of ampicillin, discrete lesions appeared in the bacterial cell walls through which cytoplasmic contents extruded and lysis occurred. A few spheroplasts, together with minicells of 0.15-µm diameter, and apparently normal cells were present after exposure to ampicillin for several hours. Conversely, erythromycin initially resulted in inhibition of division and the formation of filamentous organisms. The cell walls of these filaments were eventually disrupted with numerous small membranous vesicles appearing on their surfaces. On further erythromycin treatment, breakage of the cell wall at a restricted number of sites occurred, leading to cell lysis. In the presence of erythromycin, a few morphologically normal cells were present but no spheroplasts or minicells were observed. Viable counts demonstrated that ampicillin killed the bacteria faster than erythromycin. Regrowth did not occur in the continued presence of either antibiotic, but after their removal regrowth was observed.

INTRODUCTION

Legionella pneumophila is the major bacterial pathogen responsible for legionellosis in man. The morphology of this gram-negative organism has been extensively studied by negative stain, thin section, freeze-etching and scanning electronmicroscopy (Rodgers, 1979; Rodgers and Davey, 1982). In conditions of active replication in lung tissues *in vivo* and on bacteriological media, the bacteria are rod shaped, 0.5 µm wide and 1–2 µm long. Although several antibiotics are active against *L. pneumophila in vitro*, including ampicillin and erythromycin (Edelstein and Meyer, 1980), the efficacy of these drugs in the therapy of legionellosis remains uncertain (Miller, 1981; Meyer, 1983). Nonetheless, some structural properties attributed to legionellae seen in lung specimens obtained at autopsy (Rodgers *et al.*, 1978) undoubtedly reflect damage to

Received 4 Jun. 1984; accepted 22 Aug. 1984.

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organisms by antibiotics administered in life. In order to investigate further the activity of ampicillin and erythromycin, both of which have been used in the treatment of pneumonia (Macfarlane *et al.*, 1983), the morphological response of *L. pneumophila* to these antibiotics *in vitro* was studied by electronmicroscopy. The findings were correlated with estimations of the MICs and viable counts, performed simultaneously on the antibiotic-treated cultures. Additionally, the regrowth kinetics of *L. pneumophila* after removal of the antimicrobial drugs were determined.

MATERIALS AND METHODS

Strains and cultivation. *L. pneumophila* serogroup 1, strain Nottingham N₇, a clinical isolate from sputum passed once on bacteriological media, and serogroup 3, strain Bloomington 2, an environmental isolate, were examined by scanning electronmicroscopy (SEM) and transmission electronmicroscopy (TEM) after negative staining and thin sectioning. The strains were grown on buffered enriched blood agar containing low concentrations of sodium chloride (Dennis *et al.*, 1981) at 37°C for 48 h. Growth from the plates was harvested and used to inoculate enriched broth medium (Rodger *et al.*, 1980) to give an initial concentration of approximately 10⁵ organisms/ml. These cultures were incubated at 37°C for 24 h.

Antibiotics. Appropriate dilutions of ampicillin (sodium salt) supplied by Beecham Pharmaceuticals, and erythromycin lactobionate from Abbott Laboratories, were prepared in enriched broth medium (Rodgers *et al.*, 1980) immediately before use. The antibiotics were added to the 24-h broth cultures in the mid-logarithmic phase of growth when 10⁷–10⁸ organisms/ml were present. Final concentrations of 10 µg/ml were selected for both antibiotics as these were considered to be clinically achievable levels. Portions (5 ml) were taken after incubation for 6, 24 and 48 h in the presence of the antibiotics. The bacteria were harvested by centrifugation at 600 *g* for 10 min, washed gently twice in broth and viable counts and electronmicroscopy studies performed. Similar samples were taken from control cultures without antibiotic and treated in the same way.

Viable counts. Portions of cultures removed for viable counts were serially diluted in broth and inoculated on to enriched blood agar (Dennis *et al.*, 1981). The number of colonies on each plate was determined every 24 h for 5 days.

Electronmicroscopy. Bacteria were fixed in glutaraldehyde 3% v/v in 0.1 M sodium cacodylate containing 10 mM MgSO₄. For SEM, organisms were attached to specimen stubs, critical-point dried and coated with gold by a Balzers sputter coater as previously described (Rodgers, 1979). A target-to-specimen distance of 5 cm, and a current of 40 mA for 2 min were used. For thin-section electronmicroscopy, the organisms were dehydrated in an ethanol series and embedded in an epon-araldite mixture as previously described (Elliott and Greenwood, 1983). For negative-staining electronmicroscopy of the bacteria, 25 µl of each sample was mixed with an equal volume of phosphotungstic acid, pH 6.7, 1% w/v and applied to 400 mesh formvar-carbon-coated copper specimen grids. All samples were examined in a Jeol 100C Temscan electron microscope used at 40 kV in the SEM mode and at 100 kV for TEM.

Titration of antibiotics. MICs were determined by the broth dilution method. Serial twofold dilutions of each antibiotic were prepared in 1-ml volumes of enriched broth (Rodgers *et al.*, 1980). These were inoculated with an equal volume of broth containing organisms of each strain from cultures in the logarithmic phase of growth, to give final concentrations of 10⁵ bacteria/ml. The broths were incubated for 16 h and the lowest concentration of antibiotic that inhibited growth was taken as the MIC.

RESULTS

Morphological studies. The electronmicroscopy studies demonstrated that both strains had the typical appearance of *Legionella*. The bacterial cells, which divided symmetrically by pinching division (fig. 1) had smooth or gently ruffled surfaces (fig. 2).

FIGS. 1-3.—Electronmicrographs of untreated *L. pneumophila*.

1. Serogroup-1 organism (negative stain); note initiation of division (arrow).

2. SEM of a serogroup-3 organism with smooth bacterial surfaces.

3. Serogroup-1 organisms in thin-sections showing the typical features of gram-negative bacteria with ribosomes and nuclear matrix dispersed in the cytoplasm limited by double-track membranes. Inset: bacterial cell wall at high magnification; outer membrane (solid arrow), inner membrane (open arrow) and the intra-membraneous space, this latter showing little evidence of an electron-dense peptidoglycan layer. Bars = 0.5 μ m.

The cell wall was a double-membrane structure with little evidence of electron-dense peptidoglycan, whilst the internal constituents consisted of ribosomes and nuclear material dispersed throughout the bacterial cytoplasm (fig. 3).

Exposure to ampicillin induced changes in the shape and size of the bacteria as well as disrupting the outer cell envelope. After incubation for 6 h in the presence of ampicillin 10 μ g/ml, discrete vacuole-like lesions appeared in the cell-wall structure (figs. 4 and 5). Extrusion of the cytoplasmic contents from organisms also occurred at lytic points in the cell membrane (fig. 6). Further incubation for 24 h resulted in the formation of spheroplasts (fig. 7), disruption of the cell wall with bacterial lysis (fig. 8) and eventual collapse (fig. 9). Some of the lysed cells had numerous membrane vesicles distributed over the entire surface (figs. 10 and 11). Some apparently unaffected organisms together with a few spherical minicells of 0.15- μ m diameter with normal cell-wall structure (fig. 12) were also present.

In comparison to the results with ampicillin, incubation of bacteria in the presence of erythromycin 10 μ g/ml caused relatively less damage to microbial structure. After exposure to erythromycin for 6 h, occasional breakage points were seen in the bacterial cell wall (fig. 13). The ribosomes of these cells were more prominent both in size and in electron density. Filamentous organisms, with numerous membrane vesicles on their surfaces (fig. 14), were also formed, often with circumscribed zones of cytoplasmic clearing (figs. 15 and 16) and bulbous distortions in cell shape (fig. 17). Incubation with erythromycin for 24 h resulted in an increase in the number of lysed cells. However, these lysed cells retained their bacillary shape despite numerous small breakage points in the cell wall (figs. 18 and 19). Some cells with an apparently normal morphology were present, but no spheroplasts or minicells were seen in the presence of this antibiotic.

FIGS. 4-12.—Electronmicrographs of *L. pneumophila* exposed to ampicillin (10 $\mu\text{g/ml}$).

4. Negatively stained organism showing lesions on the outer surface appearing as stain-filled vacuoles (arrows).
5. SEM of organism with bulbous lesions on the outer membranes (arrows).
6. Thin section of organism extruding cytoplasmic contents through a break in the cell wall.
7. Thin section of a swollen spheroplast.
8. Thin section showing cell lysis with retention of shape of organism. Note apparently normal cell (arrow).
9. SEM of bacterium which, having lost its cytoplasmic contents, has collapsed.
10. Thin section of organism with cytoplasmic vesicles lining the inner membrane.
11. SEM of two distorted bacteria. Surfaces are covered with vesicle-like extrusions.
12. Thin section of morphologically normal minicells of 0.15 μm diameter. Outer (solid arrow) and inner (open arrow) membranes are evident. Bars = 0.5 μm . Serogroup 1: fig. 5, 7, 10 and 12. Serogroup 3: fig. 4, 6, 8, 9 and 11.

FIGS. 13–19.—Electronmicrographs of *L. pneumophila* exposed to erythromycin 10 $\mu\text{g/ml}$.

13. Thin section of organism with a break in the outer membrane and swollen, electron-dense ribosomes.

14. SEM of a filamentous bacterium, the surface of which is covered with tiny vesicle-like eruptions.

15. Negatively stained organism showing electron-lucent zones of cytoplasmic clearing.

16. Thin section of an elongated organism with circumscribed regions of cytoplasmic clearing associated with bulging of the bacterial membranes (arrow).

17. Thin section of a much distorted, partially lysed organism. Note vigorously convoluted outer membrane (arrows).

18. SEM of an organism showing lytic extrusions, but no collapse of the membranes.

19. Thin section of lysed bacterium with numerous pin-point breakages in the cell membranes (arrows).

Bacillary shape is retained. Bars = 0.5 μm . Serogroup 1: fig. 14, 15, 16, 17, 18. Serogroup 3: fig. 13, 19.

FIG. 20.—Proportion of bacterial cells showing normal morphology (□), those partially damaged and lysed (▨), spheroplast formation (▩) and mini-cells (■) after exposure for 6 and 24 h to either ampicillin or erythromycin (10 $\mu\text{g/ml}$). Percentages were calculated from counts of 250 cells for each treatment.

The relative proportion of lysed and partially damaged cells to those morphologically normal following treatment with each antibiotic are shown in fig. 20. However, it was difficult to determine on morphological grounds alone whether the partially damaged organisms in fig. 20 were viable. Although spheroplasts and mini-cells were observed after treatment with ampicillin for 6 h, these were few in number compared with the population of organisms present after 24 h.

Viability studies. The MIC of ampicillin for both strains was 0.5 $\mu\text{g/ml}$ and that of erythromycin was 0.4 $\mu\text{g/ml}$. Growth kinetics for both strains of *L. pneumophila* were also similar. At the time of addition of the antibiotics, the serogroup-1 strain had a mean generation time of 2 h and that for the serogroup-3 strain was 5 h (fig. 21). Despite this greater than two-fold difference in growth rates, there were no qualitative or quantitative differences in the morphological response of the two strains to either antibiotic. Addition of either antibiotic produced a rapid decline in viable counts; ampicillin resulted in a more dramatic reduction than erythromycin (fig. 21). From this it would appear that the proportion of viable organisms within the partially damaged group of cells (fig. 20) was greater following erythromycin treatment. No regrowth occurred in the continued presence of either antibiotic (fig. 21). However, on removal of the antimicrobial agents, a proportion of the organisms remained viable even after exposure for 48 h to either ampicillin or erythromycin at 20 and 25 times the MIC respectively.

DISCUSSION

The MIC results suggested that there was little difference in the antimicrobial activity of ampicillin and erythromycin against the strains of *L. pneumophila* tested *in vitro*. However, the viable count estimations demonstrated that ampicillin had greater bactericidal activity resulting in a faster decline in numbers of viable organisms

FIG. 21. Growth of *L. pneumophila* serogroup 1 (circles) and serogroup 3 (squares) in broth. Cultures had a mean generation time of 2 h for the serogroup-1 strain and 5 h for the serogroup-3 strain. Ampicillin or erythromycin was added to cultures to give final concentrations of 10 $\mu\text{g/ml}$ during the logarithmic phase of growth after incubation for 24 h. No regrowth occurred in the presence of either antibiotic.

compared with erythromycin. This was confirmed by the morphological studies in which lysed cells were more evident in the presence of ampicillin than erythromycin. Conversely it has been reported that the aminoglycosides, cephalosporins and penicillins including ampicillin are clinically ineffective in the treatment of legionellosis (Kirby *et al.*, 1980). Indeed, these workers described the development of Legionnaires' disease in patients receiving ampicillin therapy at the onset. *L. pneumophila* is a facultative intracellular pathogen, replicating in alveolar macrophages. Erythromycin enters and is concentrated in macrophages (Johnson *et al.*, 1980) and this may offer an explanation for the greater activity of this drug *in vivo*, as compared with ampicillin. Similar intracellular protection of legionellae would account for the discrepant results with the aminoglycosides, active *in vitro*, but clinically ineffective.

The presence of minicells as well as apparently normal cells in the presence of ampicillin suggests a mechanism for protracted infection in patients undergoing

therapy with this agent. Furthermore, spheroplasts were formed only in the presence of ampicillin; these could survive, osmotically protected, intracellularly in macrophages and continue infection following reversion to the vegetative form on discontinuation of therapy. The presence of bacteria with apparently normal morphology after exposure to ampicillin or erythromycin may further explain treatment failures with these agents. Moreover, despite treatment with high concentrations of antibiotics, the persistence of viable organisms, although fewer in number with ampicillin compared with erythromycin (Rodgers and Elliott, 1984), further support this suggestion.

The morphological response of the two strains of *L. pneumophila* to ampicillin and erythromycin was different. Ampicillin acted primarily on the cell wall resulting in the formation of spheroplasts, breakage of the bacterial cell membranes and eventual lysis, whereas erythromycin inhibited cell division, inducing filament formation, and cell lysis, and also prevented the formation of minicells and spheroplasts. Recently it has been proposed that a combination of ampicillin and erythromycin should be used in the treatment of atypical pneumonia (Macfarlane *et al.*, 1983). Perhaps the different modes of action of these antibiotics, as seen in the morphological studies, would enhance the antibacterial activity *in vivo*. Further work is in progress to characterise the effects on morphology and viability of *L. pneumophila* due to other antimicrobial agents and, in particular, the response of intracellular organisms.

We thank Mrs N. Douglass for help in printing the electronmicrographs and Mrs S. Brailsford for typing the manuscript. This study was presented in part at the 2nd International Symposium on *Legionella*, Atlanta, GA, June, 1983.

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