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Ultrastructure of *Legionella pneumophila*

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SUMMARY Eleven lung samples positive for Legionnaires' disease, 12 strains of *Legionella pneumophila* cultured on various bacteriological media, and one strain grown in the yolk sac of fertile hens' eggs were examined by negative staining, thin sectioning, and scanning electron microscopy. All organisms studied were ultrastructurally similar irrespective of strain, source, or method of cultivation, presenting mainly as short rods, 0·6 × 1·5 μm, with tapered ends, though long forms and filaments were also evident. In this they resembled typical Gram-negative organisms. Division was by non-septate binary fission, and the cell wall was composed of two triple-unit membranes with morphological evidence of a peptidoglycan layer. The bacterial cytoplasm was rich in ribosomes and nuclear elements and often contained vacuoles. No acid polysaccharides or bacterial appendages were detected surrounding the organisms. In lung tissue and yolk sac membranes, the organisms replicated within the cytoplasm of infected cells and in the intercellular spaces and were specifically identified in thin sections by immunoferritin techniques.

Legionnaires' disease (LD), predominantly a severe form of acute lobar pneumonia, is now known also as legionellosis. It was widely publicised because of the outbreak that followed an American Legion convention in Philadelphia in July 1976 (Fraser *et al.*, 1977).

Extensive investigations at the Center for Disease Control, Atlanta, and elsewhere yielded the aetiological agent of the disease: a fastidious, slow-growing bacterium cultivated with difficulty from postmortem lung tissue by guinea-pig and yolk-sac inoculation and on supplemented Mueller-Hinton agar (McDade *et al.*, 1977). The organism was a small Gram-negative rod, 0·3 to 0·5 μm wide and 1-2 μm long, though longer and even filamentous forms could be found on occasion. From its properties, the organism was thought to constitute a new bacterial species and was provisionally named *Legionella pneumophila* (Brenner *et al.*, 1979). Its presence in sections of lung tissue has been shown by stains such as Dieterle silver impregnation (Chandler *et al.*, 1977), more specifically by immunofluorescence (Cherry *et al.*, 1978), and by immunoferritin electron microscopy (IFEM) (Rodgers and Macrae, 1979). Electron microscopic observations of negatively stained lung suspension and of thin sections of lung tissue have shown a distinctive morphology for the organism (Rodgers *et al.*, 1978).

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The ultrastructure of the organism in pathological tissues, infected yolk-sac membranes, and when cultured bacteriologically is now presented.

Material and methods

LUNG TISSUE

Postmortem lung tissue from 50 patients with fatal lobar pneumonia was received for examination; in no case was any other recognised pathogen found by the sending laboratory. The lungs were examined by negative staining electron microscopy for *L. pneumophila*, 11 having been previously found positive by specific immunofluorescent antibody test. By the IFEM method legionella organisms only were present. This technique not only specifically identified *L. pneumophila* but can demonstrate the presence of non-LD organisms in mixed cultures. Portions of lung tissue were emulsified in distilled water, and a drop of each suspension was mixed with an equal volume of 1% phosphotungstic acid (PTA) negative stain, pH 6·8.

Aliquots of each mixture applied to formvar-carbon-coated EM grids were examined in either an AEI Corinth 500 or Jeol 100C electron microscope. Six positive specimens fixed in formol-saline were further prepared for thin sectioning electron microscopy. These tissues were cut into 0·5 mm cubes, each rinsed for 5 minutes in phosphate buffered saline (PBS-A) at room temperature 10

times, and post-fixed in 1% osmium tetroxide in cacodylate buffer (CB) for 30 minutes. They were then dehydrated in a graded ethanol series, embedded in epon-araldite resin mixture, and polymerised for 24 hours at 60°C. Sections 1 μm thick cut on an LKB Ultratome III with glass knives were stained at 60°C for 1 minute with 1% toluidine blue or 0·1% azure II in 1% sodium borate solution to locate organisms by optical microscopy (Rodgers, 1979). Thin sections, cut with diamond knives, were stained for 1 minute with 5% uranyl acetate and for 20 seconds with 0·4% lead citrate.

CULTURES

Twelve strains of *L. pneumophila* were examined by negative staining, seven additionally by thin sectioning. The strains of serogroup 1 examined were: Pontiac, Philadelphia 1 and 2, Bloomington 1, Washington 1, Cambridge 1, and Nottingham N/P/1 and N/M/2; serogroup 2, Togus 1; serogroup 3, Bloomington 2, Oxford 1; and serogroup 4, Los Angeles 1. All strains were passaged on Mueller-Hinton, F-G, CYE (Feeley *et al.*, 1978) and modified blood agar (Greaves *et al.*, 1979) media and were incubated at 37°C in a 5% CO_2 atmosphere. Samples were removed and suspensions were made in PBS-A for examination by negative staining. For thin sectioning, culture suspensions from Mueller-Hinton agar were fixed overnight in 3% glutaraldehyde in CB and washed by centrifugation, and concentrated suspensions were then layered onto 1% molten agarose at 60°C and centrifuged in pointed tubes at 5000 rpm for 10 minutes, and the solidified agar culture pellets were cut into 0·5 mm cubes. These were processed as described for lung tissues. Yolk sac membranes from fertile hens' eggs inoculated at seven days of incubation with the Nottingham N/P/1 strain and incubated for a further five days were harvested, and samples were examined by both negative staining and thin sectioning.

FERRITIN ANTIBODY LABELLING

After primary fixation in either formol-saline or glutaraldehyde, pieces of positive lung tissue from two patients, culture pellets from Pontiac and Philadelphia 2 strains, and yolk-sac membrane infected with the N/P/1 strain were rinsed with PBS-A, placed in 10% dimethyl sulphoxide in PBS-A for 30 minutes at room temperature, and freeze-thawed at −70°C. Samples were washed in buffer and incubated with continuous rotation at room temperature for 2 hours in a 1/20 dilution of anti-Philadelphia 2 rabbit antiserum. They were then rinsed in PBS-A and re-incubated in a 1/200 dilution of goat anti-rabbit ferritin conjugated antiserum (Dynatech Laboratories). After a final rinsing in PBS-A they were embedded. In the control experiments normal rabbit serum was used.

RUTHENIUM RED STAINING

Samples of the culture pellets and lung material were also stained for the presence of polyanions such as acid polysaccharide by ruthenium red (Luft, 1971). These specimens were fixed for 1 hour in a solution containing equal volumes of 6% glutaraldehyde and 0·15% ruthenium red. After rinsing in PBS-A, they were post-fixed for 30 minutes in a solution containing 2% osmium tetroxide and 0·15% ruthenium red. These procedures were done in the dark, and, after embedding, sections were examined without further staining.

SCANNING ELECTRON MICROSCOPY

Glutaraldehyde fixed samples of Pontiac, Philadelphia 2, Los Angeles 1, and Bloomington 2 strains and formol-saline fixed suspensions from three positive lungs were critical point dried in CO_2, placed on specimen holders, coated with gold for 90 seconds at 30 mA in an EMScope sputter coater, and examined by scanning electron microscopy in a Jeol 100C Temscan at an accelerating voltage of 40 kV.

Results

MORPHOLOGY BY NEGATIVE STAINING AND SCANNING

The morphology of Legionnaires' disease organisms was basically similar whether from postmortem lung, yolk-sac membrane, or bacteriological culture. The organisms appeared as short coccobacillary rods, 0·3-1·0 μm in width, averaging 0·6 μm and 1-2 μm in length (Fig. 1—top left). Long filamentous forms were seen occasionally in lung material, 10-20 μm in length. The bacteria had non-parallel sides, wider in the centre, and tapered to the ends, while the surfaces appeared smooth with stain-filled vacuoles present. A loose outer membrane was evident, but some organisms had irregular feathery or convoluted surfaces and were often curved with no vacuoles (Fig. 1—top centre). Division of the organisms was by a process of pinching binary fission within the outer lamella of the bacterial membrane (Fig. 1—top right). This was particularly clear when organisms were viewed in the scanning electron microscope. By this method both the smooth and wrinkled surface organisms were seen (Fig. 1—middle left). Less morphological variation in surface details was observed in organisms from bacteriological media; the coccobacillary form was rarely seen. The bacteria were 0·5 μm in width and 2 μm or more in length, with long filamentous

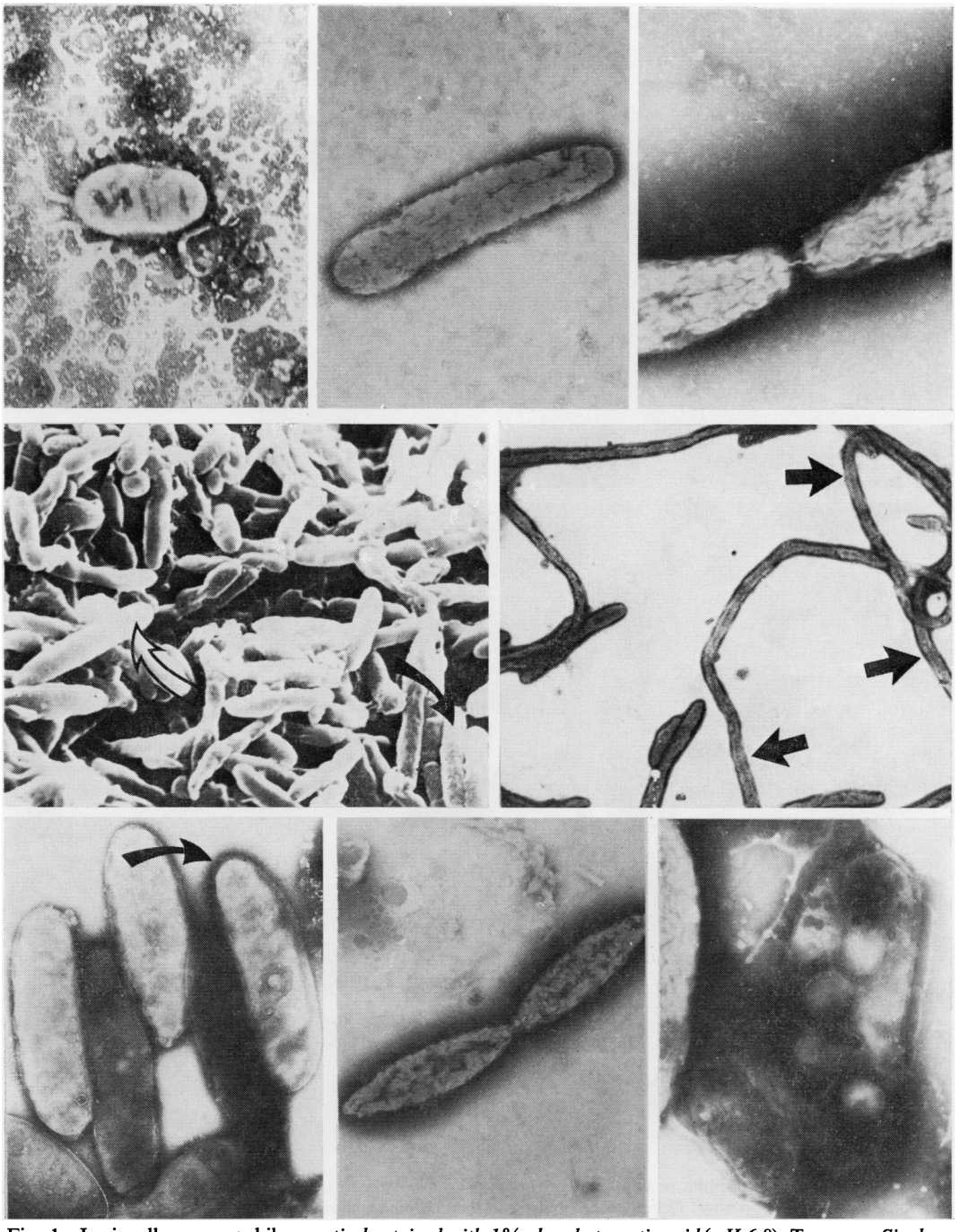

Fig. 1 Legionella pneumophila *negatively stained with 1% phosphotungstic acid (pH 6·8).* Top row: *Single coccobacillus from postmortem lung tissue. Note stain-filled vacuoles* (× *16 000*); *Los Angeles 1 organism with convoluted surface details and rounded ends from bacteriological media* (× *20 000*); *Pair of organisms in the final stage of pinching binary fission (postmortem lung)* (× *32 000*). Middle row: *Scanning electron micrograph of Pontiac strain showing tapered morphology and progressive stages of cell division. Smooth surface organisms are evident* (open arrow) *and others with feathery surface details* (solid arrow) (× *10 800*); *Filamentous forms of Philadelphia 2 strain. Arrowed organism is in excess of 50 μm in length* (× *4000*). Bottom row: *Pontiac organisms with smooth, fine-grained surfaces, rounded ends, and nonparallel sides. Note lack of vacuoles; the outer membrane is evident* (arrow) (× *20 000*); *Bloomington 2 organisms with convoluted surface details showing highly pointed forms* (× *16 000*); *Electron-lucent organisms with internal globular masses (postmortem lung)* (× *24 000*).

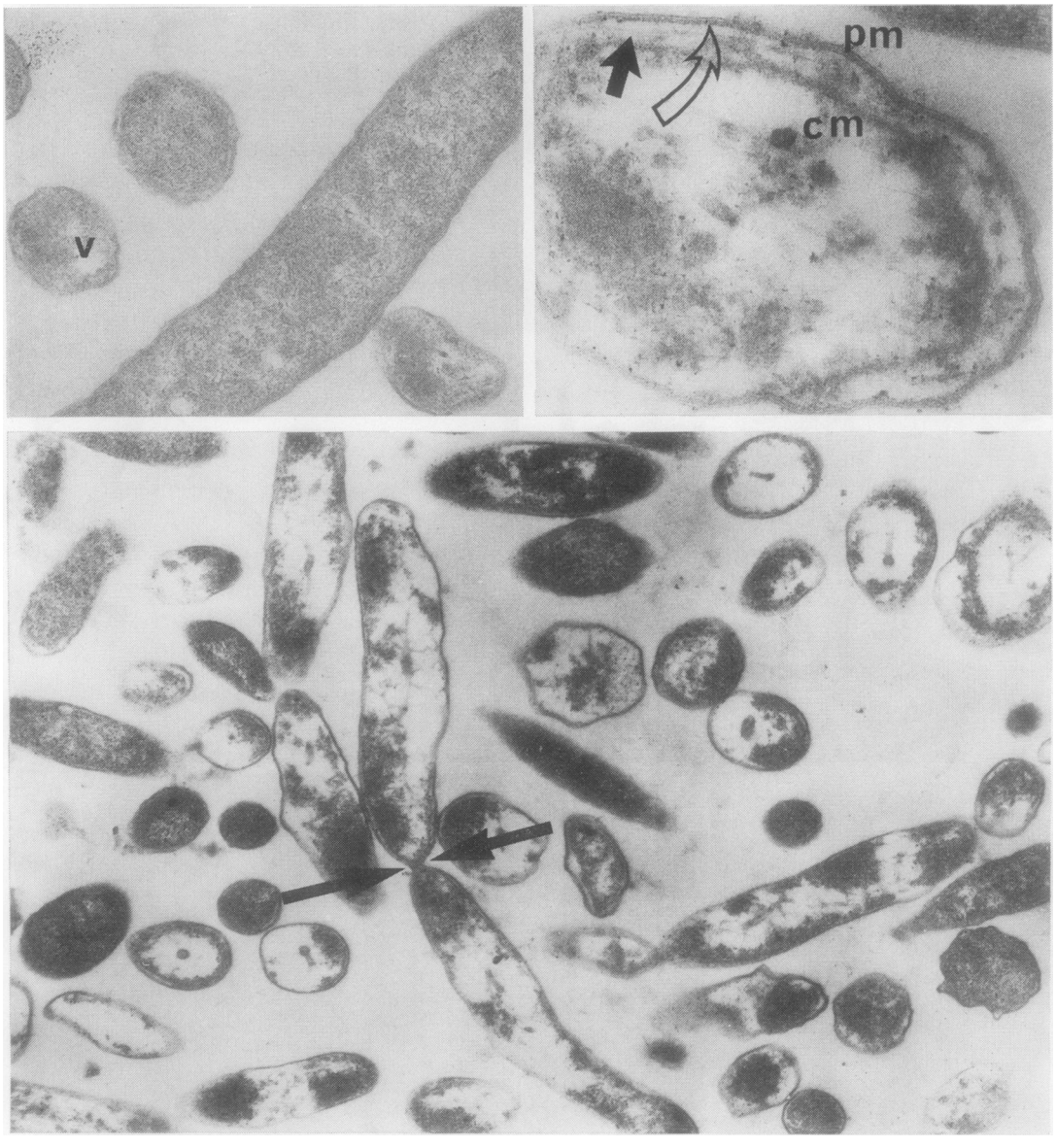

Fig. 2 Legionella pneumophila *in thin sections stained with uranyl acetate and lead citrate. From bacteriological media.* Top row: *Organisms in cross and longitudinal section. These show properties consistent with those of other Gram-negative bacteria—double membrane cell envelope, ribosomes, and dispersed nuclear material. Apart from small vacuoles* (*v*) *in the cytoplasm no other organelles are evident* (× *40 000*); *High-power micrograph of a legionella showing plasma membrane* (*pm*), *cytoplasmic membrane* (*cm*), *and electron dense, peptidoglycan-like layer* (arrow) *in the periplasmic space. The bacterial cytoplasm has retracted from the plasma membrane, which remains rigid. Note electron dense layer* (open arrow) *subjacent to the plasma membrane* (× *125 000*). Bottom row: *Low-power micrograph showing organisms of various width, length, and degree of cytoplasmic vacuolation. Note organisms in final stages of pinching binary fission* (arrows) (× *25 000*).

forms, often in excess of 50 μm in length, a common feature (Fig. 1—middle right). Surfaces were smooth and rarely vacuolated, while the outer membrane was often visible; some strains showed organisms with rounded (Fig. 1—bottom left) or pointed (Fig. 1—bottom middle) ends. Globular material of variable size was often observed distributed within electron-lucent bacteria (Fig. 1—bottom right), but this was less after repeated subculture. Appendages resembling flagella or pili have not been observed on these organisms. There were no consistent morphological features distinguishing serogroups or strains, but the filamentous form was often seen with the Philadelphia 1 and 2 and N/M/2 strains.

MORPHOLOGY BY THIN SECTIONING

All preparations examined from lung, yolk sac, or bacteriological media showed organisms of prokaryotic type with features consistent with the known properties of Gram-negative rods (Fig. 2—top left). They measured 0·3-0·5 μm in diameter and, apart from scattered ribosomes, discrete electron-lucent vacuoles, and fine thread-like nuclear elements, the bacterial cytoplasm had no other inclusions or organelles as observed in Gram-positive organisms. The bacilli were enclosed within a thin-walled, complex, double-cell envelope of variable thickness, which at its narrowest measured 25 nm. The outer unit membrane, approximately 10 nm wide, appeared electron dense, was loose and undulating, and corresponded to the loose outer membrane seen by negative staining and scanning electron microscopy. Between this outer plasma membrane and the less electron dense, 10 nm thick, cytoplasmic membrane in contact with the bacterial cytoplasm was a discontinuous electron dense layer resembling the peptidoglycan band of other Gram-negative bacteria. This was more often seen when the cytoplasmic contents had retracted from the outer cell envelope (Fig. 2—top right). The cytoplasmic membrane was less obvious in organisms from lung tissues while those grown on bacteriological media showed variation in length and frequency of vacuoles (Fig. 2—bottom). No structures resembling the septa of Gram-positive organisms were found, confirming the negative stain and scanning observations that this agent divides by pinching binary fission within the plasma membrane. In postmortem lung tissues the organisms were found replicating within the intercellular spaces of the alveoli and singly and in small groups in degenerating lung cells and alveolar macrophages, both scattered in the cytoplasm (Fig. 3—top left) and in membrane-bound vacuoles (Fig. 3—top right). Organisms were also found in the lumen of intact blood vessels but were not seen infecting the cells of the bloodstream (Fig. 3—bottom left).

Positive specific immunoferritin reactions showed that the organisms present in lung tissue (Fig. 3—bottom right), yolk-sac membrane, and cultures were legionella. No intranuclear organisms or antigens were detected. By ruthenium red staining acid polysaccharides were not detected surrounding organisms in lung or from culture.

The properties of *L. pneumophila* are listed in the Table.

Table *Properties of* Legionella pneumophila

Coccobacillary 0·6 × 1-2 μm
Tapered ends
Vacuolated surface (stain filled)
Convoluted surface details
Long forms >10 μm in length
Internal globular masses
Division by non-septate binary fission
Non-motile (no appendages)
Unit membrane limiting layer 10 nm thick
Peptidoglycan-like band present
Unit membrane cytoplasmic layer 10 nm thick
Rich in ribosomes
Fine thread-like nuclear elements
Extra- and intracellular replication in vacuoles and scattered in cytoplasm
No intranuclear replication
Released into the blood vessels
No evidence of polysaccharide capsule

Discussion

Ultrastructural examination of lung tissue from patients with Legionnaires' disease and *L. pneumophila* strains grown in yolk-sac membrane or on bacteriological media showed organisms with structural properties characteristic of Gram-negative rods. These were approximately 0·6 μm wide and of variable length with specific antigens detectable in thin sections by immunoferritin techniques.

Earlier reports (Chandler *et al.*, 1978; Rodgers *et al.*, 1978) agree, but those of Katz and Nash (1978) differ significantly. These authors reported three morphologically distinct organisms in yolk-sac membrane in addition to shadow forms and spore-like bodies, probably derived from contaminants and normal membrane structures.

The stain-filled vacuolated surfaces on organisms from postmortem lung tissues may be artefactual due to formalin fixation; they were rarely present in glutaraldehyde-fixed cultures of negatively stained legionella or in the surface details seen by scanning electron microscopy. The significance of the electron-lucent vacuoles in negative stained preparations and in thin sections is not clear. They may be poly-β-

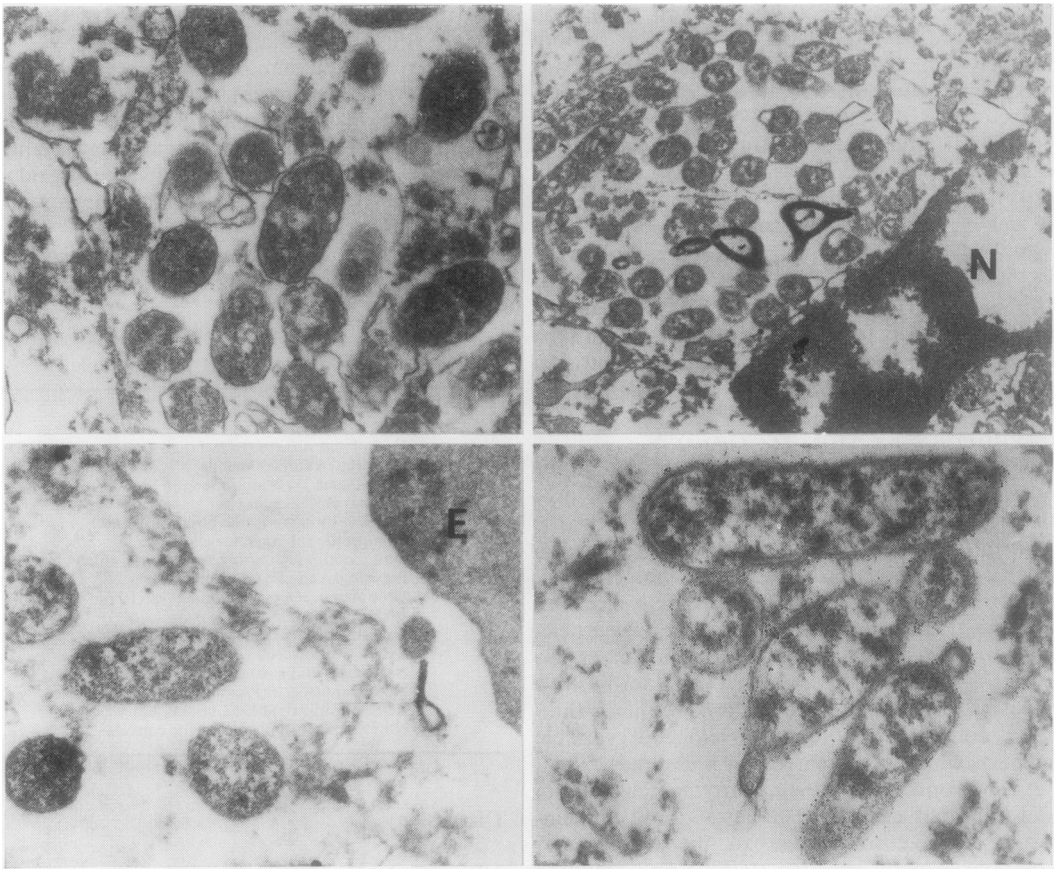

Fig. 3 Legionella pneumophila *in thin sections, stained with uranyl acetate and lead citrate. From postmortem human lung tissue.* Top row: *Organisms scattered in the cytoplasm of infected lung cell. Small vacuoles are present in the bacteria* (× *21 500*); *Degenerating lung cell with large numbers of organisms in a well-defined vacuole alongside the nucleus* (*N*) (× *11 250*). Bottom row: *Organisms surrounded by serum proteins in the lumen of a blood vessel. E, erythrocyte* (× *30 000*); *Legionella labelled with immunoferritin in the cytoplasm of a lung cell. Note ferritin molecules specifically attached to the organisms* (× *25 000*).

hydroxybutarate granules, as suggested by Chandler *et al.* (1978), or lipid droplets deposited under adverse conditions as an energy store. Such a requirement could decline with passage on an enriched bacteriological medium. The lack of flagella on an organism recovered from environmental sources is of interest. The possibility that these organisms lose flagella on *in vivo* or *in vitro* multiplication, although unlikely, cannot be excluded. This could be resolved by immune electron microscopy of primary source material, such as contaminated cooling waters, by looking for the presence of flagella on organisms specifically labelled as legionella with ferritin (Rodgers and Macrae, 1979).

Organisms were found in degenerating lung cells, alveolar macrophages, and the alveolar extracellular spaces associated with inflammatory exudate and collagen fibres. When Chandler *et al.* (1979a) examined lung tissues from peritoneally infected guinea-pigs they suggested that legionella do not infect cells *per se* but become phagocytosed by neutrophils. That organisms were dispersed in the cytoplasm of human lung cells in addition to being present in membrane-bound phagocytic vacuoles is against this. In thin sections of lung, legionella were seen in the lumen of blood vessels, supporting the existence of a bacteraemic phase of infection put forward by Boyd *et al.* (1978). Organisms have been recovered from blood (Edelstein *et al.*, 1979), but

whether they actually replicate there remains to be determined.

Because of an electron-lucent gap surrounding organisms in thin sections, Glavin *et al.* (1979) suggested the possible existence of a microcapsule, but no mucopolysaccharides have been found surrounding organisms when ruthenium red staining methods were applied.

The presence of a peptidoglycan cell wall in the envelope of *L. pneumophila* has been disputed. The electron dense layer found in the periplasmic space between the plasma and cytoplasmic membranes resembles the peptidoglycan region described for other Gram-negative bacteria (Thornley and Glauert, 1968). These workers suggested that the wrinkled surface of Gram-negative bacteria corresponds to material in the periplasmic space, and the observation that some legionella are electron-lucent and others wrinkled and electron-opaque suggests variation in the cell wall constituents. A peptidoglycan cell wall has been observed after plasmolysis of legionella (Flesher *et al.*, 1979) and components detected biochemically (Keel *et al.*, 1979), though these workers were unable to identify diaminopimelic acid, the cross-linking material in the peptidoglycan of most Gram-negative organisms. Chandler *et al.* (1979b) did, however, detect this substance. There is therefore now considerable support for the presence of a peptidoglycan layer in legionella.

Legionella appears to constitute a new group of human bacterial pathogens ultrastructurally resembling other Gram-negative organisms. Histochemical, biochemical, and cultural studies are in progress to characterise these organisms further.

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Addendum

Since this paper was submitted for publication, polar flagella and fimbriae have been detected on legionella organisms cultured on enriched bacteriological media inducing rapid growth of the bacteria by negative stain electron microscopy (Rodgers *et al.*, 1979) and flagella by optical and fluorescence microscopy (Thomason *et al.*, 1979).

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