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ULTRASTRUCTURE OF L-PHASE VARIANTS ISOLATED FROM A CULTURE OF *MYCOBACTERIUM PHLEI*

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PLATES XXI-XXVII

L-PHASE variants of mycobacteria have been isolated from the blood of patients with tuberculosis and have been induced *in vitro* by treatment with various antibiotics (Mattman *et al.*, 1958; Mattman, 1970). Lysozyme treatment also results in the formation of L colonies of *Mycobacterium tuberculosis* (Willett and Thacore, 1966, 1967). Treatment with a mycobacteriophage or glycine reportedly produces protoplasts or cell-wall-deficient forms of mycobacteria *in vitro* (Millman, 1958; Sato, Diena and Greenberg, 1965; Thacore and Willett, 1966; Adámek *et al.*, 1969).

Studies of L-phase variants of other bacterial genera have made it clear that the ultrastructure of these cell surfaces depends on different stages of development or different types of L variants (Weibull, 1965; Dienes and Bullivant, 1968; Hofschneider and Martin, 1968; Gumpert, Schuhmann and Taubeneck, 1971; Fass, 1973; Schonfeld and de Bruijn, 1973).

During studies on mycobacterial mutants, relatively stable L colonies were isolated from old cultures of *M. phlei* without any previous treatment. The purpose of the present study is to clarify the ultrastructure of this mycobacterial L variant. Ultrastructure of walled revertants found during subsequent transfers of L-phase colonies are also described.

MATERIALS AND METHODS

Isolation of L variant and walled revertants

M. phlei strain N756 (originally no. ATCC11758) was maintained in Penassay agar medium composed of 1% (w/v) Penassay Broth (Difco) containing glycerol 2% (v/v) and agar (Difco) 1.5% (w/v). After more than 10 successive single-colony transfers at 52°C, a

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single colony was incubated in 1% (w/v) Penassay Broth containing glycerol 2% (v/v) and glucose 2% (w/v) at 37°C for 2 months without shaking. The viscous mass that settled was spread on Penassay agar medium and incubated at 37°C for 1 week. Very small colonies with the characteristic fried-egg appearance of L colonies were found among the abundant yellowish rough surface colonies of the wild type *M. phlei*. These L colonies were successively transferred on to Penassay agar medium and maintained at 37°C or 52°C. They also grew on Lowenstein-Jensen medium or in 1% (w/v) Penassay Broth containing glycerol 2% (v/v) and sucrose 10% (w/v) at 37°C.

Colonial morphology did not change during five successive single-colony transfers. However, the colony-forming ability of these L colonies decreased after subsequent cultures, although the entire colony was re-streaked at each transfer. After the eighth transfer, no further cultures of L colonies could be obtained in any media used in the present study.

On rare occasions, yellowish rough surface colonies ("revertants") consisting of acid-fast rods, developed after transfers on Penassay agar medium. In addition, opaque, smooth-surfaced colonies composed of short rods that were not acid-fast ("atypical-cell-wall variants") were occasionally found during successive subcultures of L colonies. Both types of colonies grew at 37°C and 52°C, and maintained their morphology during 10 successive transfers on the Penassay agar or Lowenstein-Jensen medium.

Mycobacteriophage assay

Cells from the wild-type, L variant, revertant and atypical-cell-wall variant were incubated in 1% Penassay Broth containing glycerol and sucrose at 37°C for 48 h with constant shaking. Mitomycin C was added at a final concentration of 1 µg per ml and cultures were kept at 37°C for 24 h in a dark room with shaking. Cultures grown for 24 h in Penassay Broth were subjected to ultraviolet irradiation with a General Electric Germicidal Lamp (15 W for 2 min. at a distance of 72 cm) followed by further incubation at 37°C for 24 h.

After treatment with mitomycin C or ultraviolet light, broth cultures were centrifuged at 10,000 *g* for 15 min. at 4°C. Supernates were filtered through sterile 0.45-µm membrane filters. Filtrates were spotted on lawns of *M. smegmatis* no. ATCC607, *M. smegmatis* no. ATCC14468, *M. bovis* strain BCG, *M. tuberculosis* no. H37Ra, *M. phlei* no. ATCC11758, *M. phlei* no. SN109, *M. fortuitum* and *M. sp.* strain 521.

Lytic areas were extracted with 0.01M Tris-HCl buffer at pH 7.2 containing 0.001M CaCl₂. Various dilutions of extracts in the same buffered salt solution were again spotted on the lawn of *M. smegmatis* no. ATCC607 to confirm the presence of infective phage particles.

Adsorption of mycobacteriophage DS6A (supplied by Dr W. Redmond, Atlanta, Ga, USA), which infects the wild type, was examined by spotting drops (c. 0.04 ml) of 10-fold dilutions of the phage stock (10⁸ plaque-forming units per ml) on lawns of the wild type, L variant, revertant and atypical-cell-wall variant. Failure of plaque formation in low dilutions was considered to be evidence that cells tested were resistant to the phage. Plaque-forming units of the phage suspension after mixing with cells under examination were also assayed by a modification of a previously described procedure (Imaeda and San Blas, 1969). Briefly, the cell-bacteriophage mixture (approximate ratio 1:1) in 1% Penassay Broth containing 0.001M CaCl₂ was incubated at 37°C for 20 min. The mixture was then filtered through a sterile 0.45-µm membrane and the resultant filtrate was assayed for non-adsorbed phage particles.

Anti-bacteriophage serum was prepared in rabbits by the use of the bacteriophage released from the wild-type *M. phlei* strain N756 after propagation on *M. smegmatis* no. ATCC607.

Electronmicroscopy

The wild-type *M. phlei* and its L variant, revertant and atypical-cell-wall variant were incubated separately on Penassay agar medium or Lowenstein-Jensen medium at 37°C. Colonies were removed after 48 h and 5–7 days, and immersed in 4% formaldehyde fixative

containing (w/v) CaCl_2 0.2% and NaCl 1% in 0.2M Tris-HCl buffer at pH 7.2, for 5 h at 4°C. After washing with the same buffered salt solution, cell pellets were post-fixed with osmium tetroxide 2% (w/v) in the buffered salt solution for 4–5 h at 4°C, then treated with a 2% aqueous solution of uranyl nitrate for 20 min. at room temperature. Cell pellets were then dehydrated with acetone and embedded in Araldite. Ultrathin sections were stained with lead citrate and examined with Hitachi HU-12 and JEOL JEM-7A electron microscopes.

For negative staining, unfixed cell pellets were resuspended in 0.1M ammonium acetate solution and treated with 2% sodium silicotungstate or phosphotungstate at pH 7.6.

RESULTS AND DISCUSSION

Ultrastructure of L variant

When suspensions of cells obtained from a formaldehyde-fixed single L-phase colony were observed by phase-contrast microscopy, the majority of cells appeared to be rod-shaped. Very large spherical bodies were occasionally found among the rods. After Ziehl-Neelsen staining, acid-fast granules were sometimes present within non-acid-fast rods or spheres.

In ultrathin section, regardless of growth media, L-variant colonies consisted of pleomorphic cells, such as long rods with occasional branching or budding processes, oval or round cells and large amoeba-like bodies (figs. 1–3). The cell surface of rods was composed of a double-layered membrane with two dense layers 4-nm thick separated by a less dense layer about 3-nm thick. Since this membrane system was directly attached to the granular cytoplasmic substance, it may represent the cytoplasmic membrane of the rod-shaped L variant. In negative-stained preparations, the surface layer appeared to be a homogeneous membrane (fig. 4), thus differing from that of the wild type (fig. 5) in which fibrous components of cell walls were characteristically present (Imaeda, Kanetsuna and Galindo, 1968).

Under the experimental conditions, cell-wall components were not visible outside the cytoplasmic membrane. In addition, mycobacteriophage DS6A was neither adsorbed on to L-variant cells nor formed plaques in a spot test. Therefore, this type of L variant may correspond to the protoplast L form (Hofschneider and Martin, 1968) or the type A L form (Dienes and Bullivant, 1967), which is believed to lack any cell-wall component.

Many of the cells from L variants were rod-shaped (figs. 1, 2 and 6). Van Iterson and op den Kamp (1969) reported that protoplasts derived from *Bacillus subtilis* and *B. megaterium* grown in acidic media maintained their rod shape in spite of the absence of the cell wall. In contrast, protoplasts derived from these micro-organisms grown in a neutral culture medium were round. This suggests that the former protoplasts possess more rigid membranes than the latter. Since the pH of old culture media from which the L variant had been isolated was less than 6, their findings might explain the present observation that L-variant cells can grow as a rod, even without a rigid cell wall.

The cytoplasm of rod-shaped L-variant cells contained abundant ribosomes, which sometimes formed polysomes, and dense threadlike nuclear substance (fig. 2). These cytoplasmic components were separated from the peripheral

membrane by a fine granular substance (fig. 2), in contrast to the wild type in which the cytoplasm showed a close proximity to the cytoplasmic membrane (Imaeda *et al.*, 1969). Neither small bodies nor mesosomes were found; in this respect the cytoplasmic components differed from those of L variants from other genera (Weibull, 1965; Dienes and Bullivant, 1968; Gumpert, Schuhmann and Taubeneck, 1971; Gilpin, Young and Chatterjee, 1973; Wyrick and Rogers, 1973).

In the large amoeba-like bodies, a second double-layered membrane system composed of dense layers about 2.5-nm thick and 2.5-nm apart was observed inside the outermost double-layered membrane. The latter consisted of two dense layers about 4-nm thick and 3-nm apart. These two double-layered membrane systems were especially noticeable at protruding regions (fig. 3). A fine granular substance was present between the two membrane systems, as reported in L variants of *Escherichia coli* and *Proteus* (Dienes and Bullivant, 1967; Gumpert *et al.*, 1971).

This type of membrane structure has been observed at the surface of large bodies contained in L colonies of *Proteus*, *Staphylococcus* and *Corynebacterium* (Weibull, 1965; Dienes and Bullivant, 1967, 1968). Dienes and Bullivant (1967, 1968) suggested that the outer membrane may correspond to the altered cell wall. In contrast, Weibull (1965) observed that the peripheral membrane development occurred inside a large L-form cytoplasmic membrane, thus suggesting that both membrane systems may represent L-form membranes.

In the present study, very small numbers of amoeba-like bodies were found among the rod-shaped L-variant cells and mycobacteriophage adsorption tests to determine the presence of cell-wall components as receptor sites were not practicable. The interpretation of the two double-layered membrane systems of these amoeba-like bodies therefore remains unsolved.

It was observed that in some populations the rod-shaped L-variant cells lacked the distinct limiting double-layered cytoplasmic membrane (figs. 2 and 6). In these cells, the cytoplasm appeared to be limited by a single-layered membrane (fig. 6). There was another type of cell lacking the double-layered structure, characterised by a ruffled cytoplasmic membrane (fig. 7). In these cells, granular substance was not observed between the cytoplasmic membrane and the cytoplasm, which was delimited by a single-layered membranous structure. It is improbable that these structures occur as a result of artifacts formed during preparation, because all types of cells described above have been encountered in the same specimen.

Based on these observations, it seems reasonable to suggest that these different stages represent initial plasmolysis of the L-variant cells, followed by degradation of both pericytoplasmic granular substance and cytoplasmic membrane with the formation of apparently denuded cytoplasmic matrices. It is also interesting to note that the resulting cytoplasmic matrices still maintain their rod shape, even without a typical cytoplasmic membrane. The fate of these denuded cytoplasmic matrices was not clarified in the present study. They may undergo further degradation or may synthesise new cytoplasmic membranes leading to the formation of new cells.

Ultrastructure of walled revertants

Rough colonies isolated during the successive transfer of the L-phase colony consisted of rod-shaped cells that show ultrastructures identical to those of the original wild-type *M. phlei* (fig. 8). The dense cell wall 5–10 nm in thickness was attached to the cytoplasmic membrane which was composed of two dense layers about 3-nm thick and 3-nm apart. The cytoplasm contained abundant ribosomes and nuclear substance. Invagination of the cytoplasmic membrane resulted in the formation of mesosomes. In addition, these walled cells possessed a unique characteristic of the wild type *M. phlei*, namely, ability to grow at 52°C. For these reasons, designation of the rough colony clone as the “revertant” would appear justified.

Smooth colonies isolated during the successive transfer of the L-phase colony were composed of rod-shaped cells that exhibited bizarre structures (fig. 9). Cell walls were very thick, varying from 30 to 40 nm. These cells were further characterised by the presence of disordered septation. Ribosomes were not readily revealed in the moderately dense cytoplasmic matrix. Vesicular mesosomes derived from the cytoplasmic membrane showed the same dimensions as the wild-type revertant. In addition, these cells possessed characteristics common to wild-type *M. phlei*, such as growth at 52°C and release of infective mycobacteriophage particles that were almost identical to those from the wild type, as described later. However, these cells failed to adsorb mycobacteriophage DS6A, and this suggests that their cell walls are structurally different from those of the wild type. Based on these findings, this type of cells has been designated as the “atypical-cell-wall variant”. A similar cell type has been reported in a cycloserine-mediated mutant of *M. smegmatis* (Imaeda, Kanetsuna and Rieber, 1968).

Origin of the L variant, revertant and atypical-cell-wall variant

Bacteriophage particles released from the wild-type *M. phlei*, L variant, revertant and atypical-cell-wall variant by mitomycin C treatment or by ultraviolet irradiation possessed very similar characteristics, namely, morphology (fig. 10), resistance to heat treatment at 52°C for 18 h, and neutralisation by antiserum prepared against bacteriophage particles released from the wild type. The host ranges of these bacteriophages were identical, i.e. *M. smegmatis* no. ATCC607, *M. tuberculosis* no. H37Ra, *M. bovis* strain BCG and *M. sp.* strain 521 were susceptible, whereas *M. phlei* no. ATCC11758, *M. phlei* no. SN109, *M. smegmatis* no. ATCC14468 were insusceptible. It appears, therefore, that all cell types examined are lysogenised with the same mycobacteriophage. These findings suggest that both the L variant and atypical-cell-wall variant are of mycobacterial origin although devoid of typical mycobacterial ultrastructures.

In addition, all cell types shared a unique characteristic of *M. phlei*, namely the ability to grow at 52°C (Gordon and Smith, 1953). Since the starting colony was selected after more than 10 successive single-colony transfers at 52°C, it is unlikely that all cell types examined were derived from contaminating mycobacteria, other than *M. phlei*, that were unable to survive during the selection

procedure. It seems, therefore, that the L variant and atypical-cell-wall variant as well as the revertant are derived from *M. phlei*.

SUMMARY

Relatively stable L-phase colonies were isolated from old cultures of a selected clone of *Mycobacterium phlei*. The colonies grew at 52°C and were composed of rod-shaped, oval or spherical cells. Large amoeba-like cells were occasionally present. These were usually limited by a double-layered membrane and devoid of normal cell-wall components such as bacteriophage receptors. The large amoeba-like bodies sometimes showed both outer and inner double-layered membranes, especially in pseudopodium-like cellular extensions. An unusual feature of rod-shaped cells was retention of the original shape despite the loss of their cell walls.

Two types of walled cells occurred during successive transfers of L colonies. One was the true revertant which had characteristics in common with the wild-type *M. phlei*, such as growth at 52°C and ultrastructural organisation. The other, designated as the "atypical-cell-wall variant", was characterised by growth at 52°C, thick cell walls, and disordered septation. Wild-type *M. phlei*, L variants, revertants and atypical-cell-wall variants released mycobacteriophage particles. These bacteriophages were almost identical in respect to morphology, host range, and neutralisation by antiserum. The results obtained suggest strongly that all types of cells examined were derived from *M. phlei*.

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L-PHASE VARIANTS OF *MYCOBACTERIUM PHLEI*

FIG. 1.—Ultrathin section of an L colony showing pleomorphism and possible branching (arrow) of organisms. EM. $\times 33,000$.

FIG. 2.—An L-variant cell enveloped by a double-layered membrane (LM). Granular substance (G) present within the peripheral membrane with invaginations (arrow) into the cytoplasm. Cytoplasm contains nuclear substance (N) and ribosomes (R). Note the cytoplasmic matrix lacking the double-layered membrane (upper left). EM. $\times 80,000$.

L-PHASE VARIANTS OF *MYCOBACTERIUM PHLEI*

FIG. 3.—Large amoeba-like body within L colony. Surface is composed of two double-layered membrane systems (inset) which are most noticeable at the protruding regions (arrows). Cytoplasm consists of scattered dense masses, vacuoles (V) and fine granular substance. EM. $\times 62,500$. (Inset $\times 125,000$.)

L-PHASE VARIANTS OF *MYCOBACTERIUM PHLEI*

FIG. 4.—Negatively stained L-variant cell in 6-day-old colony on Penassay agar medium. Surface composed of a homogeneous membranous matrix. EM. $\times 132,000$.

FIG. 5.—Wild-type cell of *M. phlei* stained with sodium phosphotungstate. Note ramified fibres in cell wall. EM. $\times 55,200$.

L-PHASE VARIANTS OF *MYCOBACTERIUM PHLEI*

FIG. 6.—Apparently denuded cytoplasms of rod-shaped L-variant cells, limited by a single-layered membrane (arrows). Note the double-layered membrane (LM) in the cell containing pericytoplasmic granular substance (G). R = Ribosomes; N = nuclear substance. EM. $\times 120,000$.

FIG. 7.—L-variant cell surrounded by a ruffled membranous structure (LMD). Pericytoplasmic granular substance not visible on this cell but can be seen (G) in an apparently intact cell (lower left) underneath the double-layered cytoplasmic membrane (LM). Note the presence of a single-layered membrane (arrow) on the surface of the cytoplasm. R = Ribosomes; N = nuclear substance. EM. $\times 132,000$.

L-PHASE VARIANTS OF *MYCOBACTERIUM PHLEI*

FIG. 8.—Cells in true revertant colony. The cell wall (CW) and cytoplasmic membrane (CM) surrounds the cytoplasm which contains nuclear substance (N) and ribosomes (R). A mesosome (M) shows the association with the cytoplasmic membrane at the septum. Note the close proximity of ribosomes to the cytoplasmic membrane. EM. $\times 84,000$.

L-PHASE VARIANTS OF *MYCOBACTERIUM PHLEI*

FIG. 9.—Atypical-cell-wall variants in smooth colony isolated during successive culturing of L-phase colony. Note the thick cell wall (CW) and septa. M = Mesosome, N = nuclear substance, CM = cytoplasmic membrane. EM. $\times 87,000$.

L-PHASE VARIANTS OF *MYCOBACTERIUM PHLEI*

FIG. 10.—Negatively stained L-variant cell after treatment with mitomycin C. The cell surface appears to be homogeneous. Note the abundant bacteriophage particles. EM. $\times 143,000$.