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ULTRASTRUCTURAL CHARACTERISTICS OF MYCOBACTERIAL GROWTH

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PLATES XIII-XVIII

ULTRASTRUCTURAL studies have revealed various components in both the cell wall and the cytoplasm of mycobacterial cells (Yamaguchi, 1960; Koike and Takeya, 1961; Takeya *et al.*, 1961; Imaeda and Ogura, 1963; Imaeda, 1965; Petitprez, Roos and Taquet, 1967). These studies, however, are deficient in detailed information on cellular components because of inadequate magnification or resolution. In addition, these previous observations were not correlated with the growth stages of the organisms because a uniform population of cells of the same age was not obtained either on solid or in liquid media. On the other hand, when mycobacterial cultures are incubated with shaking, not only is there an acceleration of growth but also a more homogeneous distribution with respect to the age of the cells. Electron-microscope studies of these age-controlled cells clarify the morphological characteristics of mycobacteria during growth, and consequently facilitate the recognition of the growth stage of bacteria seen in the tissues in mycobacterial diseases such as tuberculosis and leprosy.

MATERIALS AND METHODS

The cultures examined in the present study were *Mycobacterium tuberculosis* H37Rv, H37Ra and BCG, and *Mycobacterium smegmatis* ATCC607 and ATCC14468. One-week-old (*Myco. smegmatis*), or one-month-old (*Myco. tuberculosis*) colonies grown on Middlebrook agar 7H10 (Difco) enriched with 0.25 per cent. bovine albumin fraction V and 0.75 per cent. dextrose were dispersed in Dubos broth (Difco) by means of a tissue homogeniser with teflon pestle, and diluted in culture medium to obtain an optical density of approximately 0.05 at 600 nm. The culture media used were Dubos broth enriched as mentioned above for *Myco. tuberculosis* H37Rv and H37Ra, and Dubos broth without enrichment for the other species.

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The cultures were incubated in a roller tube machine at 60 r.p.m. at 37°C. The effects obtained by this method are similar to those obtained in a shaking apparatus. Each tube was mechanically vibrated vigorously at least twice a day to homogenise the suspension further. The growth was measured turbidimetrically at 600 nm every 12 hr for *Myco. smegmatis* or 24 hr for *Myco. tuberculosis*. Some tubes were withdrawn from each group at intervals and used for studies of cellular morphology.

Fixation for electron microscopy was carried out by Stoeckenius and Rowen's method (1967) with slight modifications. Cells were fixed in 4 per cent. formaldehyde solution containing 1 per cent. NaCl and 0.2 per cent. CaCl₂ for 6–7 hr at 4°C, washed with the same salt solution overnight at 4°C, and embedded in 1.5 per cent. agar dissolved in the salt solution. The post-fixation was performed with 1 per cent. osmium tetroxide in the same salt solution for 6 hr at 4°C. After washing with distilled water for 10–15 min., agar blocks containing bacilli were immersed in 2 per cent. aqueous uranyl nitrate solution for 30 min. at ambient temperature, dehydrated in graded acetone and embedded in Araldite.

We also performed direct fixation with osmium tetroxide by the method of Ryter and Kellenberger (1958) in some experiments, and found no differences in mycobacterial ultrastructure from those observed after fixation by our routine method, despite the low pH (4.2–4.6) of the fixative used in the latter.

The ribosomal fraction was obtained from living cultures as follows: cells were washed with 0.8 per cent. NaCl solution, frozen with liquid nitrogen and ground in a mortar. Bacillary paste was extracted with approximately 3 volumes of 0.05M-Tris buffer at pH 7.2 containing 0.01M-MgCl₂. The resultant suspension was centrifuged at 20,000g for 20 min. at 4°C to remove cellular debris. The ribosomes were pelleted from the supernatant after 105,000g for 120 min. at 4°C. The pellet was resuspended in the same buffer and centrifuged again at 10,000g for 5 min. The supernatant was used as a ribosomal suspension.

Unfixed cells and ribosomes were stained negatively or positively with 2 per cent. sodium silicotungstate at pH 7.6 or with 0.2 per cent. uranyl acetate at pH 5.0. Electron microscopes used were Hitachi HU-11B and Jeol JEM-7A with direct magnification of 50,000–100,000 times.

RESULTS AND DISCUSSION

Growth characteristics. Fig. 1 shows the growth curve of 5 of the species of mycobacteria examined in the present study. According to morphological differences, growing cells are divided into three groups; the early log cells (up to two-thirds of exponential growth), the late log cells and the stationary-phase cells.

Morphology. Early log-phase cells of *Myco. smegmatis* are characterised by relatively thin cell walls, peripheral distribution of ribosomes and fine networks of nuclear filaments (figs. 2a and 2b). Negative staining reveals that the cell surface is covered with densely packed fibrils 50–120 AU (0.1 nm $\equiv$ 1 AU) in diameter (fig. 3). These fibrils are composed of lipopolysaccharide, the main constituent of which is glucan-mycolate, as described in detail by us elsewhere (Imaeda, Kanetsuna and Galindo, 1968).

The nuclear network consists of filaments approximately 30 AU across, which are uniformly distributed in the central portion of the cytoplasm.

At this stage, isolated ribosomes show various features. Small dense roughly oval particles measuring approximately 120 by 60 AU or 120 by 80 AU, which may represent the 30S and 50S ribosomal subunits obtained from BCG cells (Trnka, Wiegshauss and Smith, 1968), are adherent to each other to form a larger particle (fig. 4a). Rough spherical particles 160 AU in diameter, in which smaller subunits cannot be observed, possibly correspond to single 70S

ribosomes. Frequently, two or three 160 AU particles are clustered to form a polyribosome. These appearances are in concordance with the morphology of ribosomes in other bacterial genera (Borasky, Olenick and Hahn, 1966) and also in amoebae (Morgan, Slayter and Weller, 1968).

The ribosomes are interconnected by fibrillae measuring approximately 7 AU in diameter (fig. 4b), as observed in other bacterial ribosomes (Van Iterson, 1965; Schlessinger, Marchesi and Kwan, 1965; Okada and Nishiura, 1966). In chemical studies of *Escherichia coli* and *Micrococcus lysodeikticus*, Flessel, Ralph and Rich (1967) found that exponentially growing cells contain

FIG. 1.—Growth curves of *Mycobacterium tuberculosis* H37Rv, H37Ra and BCG, and *Mycobacterium smegmatis* ATCC607 and ATCC14468 at 37°C in shaken cultures. The media are described in the text.

ribosome fractions more than 80 per cent. of which are sedimented as polyribosomes, and that digestion with ribonuclease leads to the sedimentation of 70S ribosome particles only, whereas treatment with deoxyribonuclease does not dissociate polyribosomes into single particles. This suggests that the fibrillae between ribosomes are not filaments of nuclear DNA, but of RNA, possibly messenger RNA, which unite single ribosomes to form polyribosomes, as already observed in animal cells (Warner, Rich and Hall, 1962; Slayter *et al.*, 1963).

At the stage of late exponential growth, cell walls and cytoplasm exhibit significant morphological differences from those seen in early log cells. A homogeneous substance of arabinogalactan-rich lipopolysaccharide (Imaeda *et al.*, 1968) covers the outermost layer of cell walls (fig. 5). Single ribosomes and polyribosomes are distributed not only in the peripheral area, but also in the region previously occupied by nuclear filaments, which show a tendency to accumulate in the central portion of the cytoplasm (fig. 6).

Stationary-phase cells display condensation of nuclear filaments and decrease of ribosomal particles. After maximal growth, ribosomes disappear and the plasma membrane becomes detached from the inner surface of the cell wall, representing the initial stage of plasmolysis (figs. 7*a* and 7*b*). When the growth declines, nuclear filaments are barely recognisable and the cytoplasmic components become dense granular substances, indicating bacterial death.

Later in the stationary phase, network conglomerations of fibrils approximately 40 AU in diameter and bundles of thick fibrils about 100 AU in diameter become detached from the cell walls (figs. 8 and 9). These liberated materials are frequently encountered in culture media after maximal growth (fig. 10). Since they are constituents of the three main layers of cell wall (Imaeda *et al.*), their release from the cell surface reflects the disintegration of cell walls at this stage of growth.

Chemically, all three layers of the cell wall contain lipopolysaccharides with different sugar components: in the outermost layer are arabinogalactan-rich and glucan-rich polysaccharides; in the intermediate layer is a mannose-containing polysaccharide; and in the innermost layer is arabinogalactan mycolate (Imaeda *et al.*; Kanetsuna, 1968). The polysaccharides found in culture media correspond in chemical composition to similar components (Yamamura *et al.*, 1965; Azuma, Kimura and Yamamura, 1967) that are of cell-wall origin.

Comparison between Myco. tuberculosis and Myco. smegmatis. No significant morphological difference is noted between the cytoplasmic components of *Myco. tuberculosis* (H37Rv, H37Ra and BCG) and *Myco. smegmatis* (ATCC607 and ATCC14468) during their growth. On the other hand, the cell-wall structures revealed in ultrathin sections differ from each other. Cell walls of *Myco. smegmatis* near the maximal growth consist of an outer dense layer and an inner moderately dense layer. Both of them measure approximately 50 AU in thickness (figs. 2*a*, 2*b* and 6). *Myco. tuberculosis* at the similar growth stage is enclosed by a homogeneously dense cell wall 150–200 AU in thickness, which is attached directly to the plasma membrane (fig. 11). Furthermore, the dense cell wall is covered with a low-density layer which is difficult to find in *Myco. smegmatis*.

When formaldehyde-fixed cell walls of *Myco. tuberculosis* are successively extracted with neutral solvents, such as acetone, ethanol-ether (1:1, v/v) and chloroform, with reflux, their structures become very similar to those of *Myco. smegmatis* (fig. 12); a dense thin layer is separated from the plasma membrane by a moderately dense layer. This finding suggests that cell walls of *Myco. tuberculosis*, especially their inner layer, contain a larger amount of free low-molecular-weight lipids than do those of *Myco. smegmatis*.

SUMMARY

Changes in the morphology of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* were examined with the electron microscope during growth in liquid media in shaken cultures. Cells at the early stage of

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FIG. 2a.—Transverse section.

FIG. 2b.—Longitudinal section.

FIG. 2.—*Myco. smegmatis* after 9 hours' cultivation. Fine network of nuclear filaments (N) and peripheral distribution of ribosomes (R). Nuclear filaments attached to a mesosome (M); PM = plasma membrane. $\times 135,000$.

These and figures on subsequent plates are all from electron micrographs.

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FIG. 3.—Negatively stained cell of *Myco. smegmatis* after 15 hours' cultivation. The cell surface is covered with many fibrils (arrows). $\times 160,000$.

FIG. 4a.—Two subunits (arrows) form a larger ribosomal particle.

FIG. 4b.—Fibrilla (arrow) in close contact with ribosomal particles.

FIG. 4.—Positively stained ribosomal fraction from *Myco. smegmatis*. Uranyl acetate. $\times 540,000$.

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FIG. 5.—A negatively stained cell of *Myco. smegmatis* after 36 hours' growth. The cell surface is enclosed by a homogeneous substance. The fibrils shown in fig. 3 cannot be seen. $\times 160,000$.

FIG. 6.—Thirty-six-hour culture of *Myco. smegmatis*. Ribosomes (R) are distributed throughout the cytoplasm, including the region (N) where fine nuclear filaments are sparsely distributed. $\times 135,000$.

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FIG. 7a.—Transverse section. Circinate accumulation of nuclear filaments (N).

FIG. 7b.—Longitudinal section. Detachment of plasma membrane (PM) from cell wall.

FIG. 7.—*Myco. smegmatis* after 5 days' cultivation. $\times 135,000$

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FIG. 8.—Negatively stained BCG cell, with moderate positive stain effect, after 15 days' cultivation. Fine networks of fibrils (arrows) are released from the cell surface, where few single fibrils remain (double arrows). $\times 180,000$.

FIG. 9.—Negatively stained BCG cell after 20 days' cultivation. Release of fibrillar bundles (arrow) from cell wall. $\times 150,000$.

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FIG. 10.—Negative staining of fibrillar bundles in culture media of BCG after 10 days' culture.

FIG. 11.—Transverse section of *Myco. tuberculosis* H37Ra during late exponential growth. Compare the homogeneously dense, thick cell wall of this species with the thin cell wall of *Myco. smegmatis*. M=Mesosomes; R=ribosomes; PM=plasma membrane; LL=outermost low-density layer. $\times 135,000$.

FIG. 12.—Exponentially growing *Myco. tuberculosis* H37Ra after exhaustive extraction with neutral solvents. Cell walls composed of an outermost low-density layer (LL), an inner dense layer (DL) and an innermost moderately dense layer (ML). PM=Plasma membrane. $\times 150,000$.

exponential growth are characterised by a surface layer of fibrillar material consisting of cell-wall lipopolysaccharide, by the peripheral distribution of both single and clustered ribosomes, and by a fine network of nuclear filaments.

Late exponentially growing cells show cell walls overlaid with an amorphous lipopolysaccharide material which differs chemically from the fibrillar layer. The ribosomes at this stage are scattered throughout the cytoplasm.

Fibrillae, possibly ribonucleic acid in nature, interconnect single ribosomal particles during all stages of exponential growth.

In stationary-phase cells the nuclear filaments are concentrated in the central portion of the cytoplasm. Fine networks and bundles of fibrils, varying in size, become detached from the cell walls. After maximal growth, ribosomal structures disappear and the plasma membrane becomes detached from the inner surface of the cell wall, as an initial sign of plasmolysis.

Morphological differences between the cell-wall structure of *Myco. tuberculosis* and that of *Myco. smegmatis* are noted near the stage of maximal growth. In the former species, the cell wall consists of a dense thick layer, possibly containing large amounts of free lipid. Cell walls in the latter species are composed of a thin dense layer which is separated from the plasma membrane by a moderately dense layer.

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