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Electron Microscopic Observations of Cell Wall and Cytoplasmic Membrane in Murine and Human Leprosy Bacilli[1]

Tsunehiko Hirata[2]

Electron micrographs of sectioned microorganisms indicate most bacterial cell envelopes have a multilayered structure. In high resolution micrographs the wall of Gram-positive organisms appears to consist of two thin, electron-dense layers separated by a wider layer of slightly less electron-dense materials ([2]). The cytoplasmic membrane appears in high resolution electron micrographs of sectioned bacteria as a "unit" membrane ([12]) consisting of two electron-dense strata separated by a less electron-dense layer in general. Several electron micrographs have been published showing invaginations and/or foldings in the cytoplasmic membrane. It is said that these invaginations and/or foldings become deep and wide, giving rise to intracytoplasmic membranous structures, namely mesosomes, connected with the cytoplasmic membrane ([4, 5]).

Electron micrographs of actively growing mycobacteria, negatively stained, freeze fractured, metal shadowed, or ultrathin sectioned and stained, indicate that beyond the cytoplasmic membrane there is a rigid layer, the cell wall, which has three superposed discernible layers ([1]).

The ultrastructure of the cell wall of *Mycobacterium leprae* has been found to be similar to that of other mycobacteria. However, there are different points of view concerning the electron microscopic observation of the cytoplasmic membranes of leprosy bacilli; for example, Edwards ([3]) discussed how the membrane of *M. leprae* did not differ from several observations of other workers who had studied mycobacteria. On the other hand, Silva, *et al.* ([14–16]) have recently described how the cytoplasmic membrane of all otherwise normal-looking *M. leprae* cells found in the skin biopsies of leprosy patients exhibited a symmetric profile.

The fine structures of the cell wall and the cytoplasmic membrane of *M. lepraemurium* in murine lepromas and *M. leprae* in human lepromas have been observed convincingly in the electron microscope. These ultrastructural studies are reported here.

MATERIALS AND METHODS

Murine lepromas. *M. lepraemurium,* Hawaiian strain, have been maintained in mice for more than 20 years in our institute by serial transmission at five- to six-month intervals.

Female mice (18–20 g) of the ddY strain were given intraperitoneal injections of 0.25 ml (containing approximately 10^6 acid-fast bacilli) of a partially purified suspension of *M. lepraemurium* prepared from the homogenized liver of a mouse injected 4–6 months previously.

In the present studies, the mice were sacrificed 6–8 months after injection. *M. lepraemurium* cells *in situ* in liver of infected mice were observed in the electron microscope (EM).

Human lepromas. Skin biopsies of thigh lesions from newly diagnosed patients with progressive lepromatous leprosy were examined. The biopsies were collected at the National Leprosarium of Tama Zensho-en, Tokyo. *M. leprae* cells *in situ* in skin lepromas were observed electron microscopically.

Preparation of tissues for electron microscopy. The murine and human lepromas were cut into 1 mm cubes. The cubes were immersed in 2% agar aqueous solution and separately put as a drop of the solution on a microscope slide. These manipulations with the agar must be made at 45°C. After

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[2] T. Hirata, B.Sc., M.D., Chief, Electron Microscopic Laboratory, National Institute for Leprosy Research, 2-1, 4-Chome, Aobacho, Higashimurayamashi, Tokyo 189, Japan.

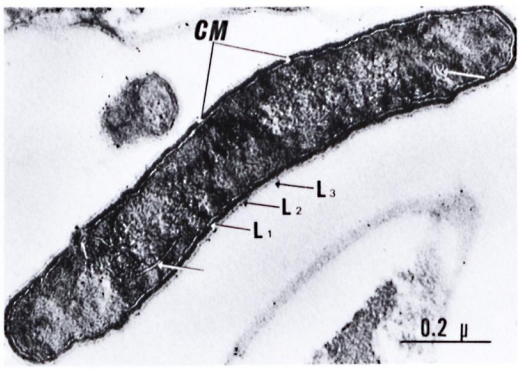

FIG. 1. Longitudinal thin section of *M. lepraemurium*. The cell wall is composed of three layers: an innermost electron-dense layer (L_1), an intermediate electron-transparent zone (L_2), and a thin, outermost electron-dense layer (L_3). Arrows show the intracytoplasmic membrane systems; CM = cytoplasmic membrane.

cooling and gelation, the agar drop was cut into small blocks. These blocks were fixed by immersion in osmium tetroxide buffered to pH 6.4–6.6 with Ca^{++} (0.01 M as $CaCl_2$) according to the technique of Kellenberger, *et al.* ([7]), dehydrated in a graded ethanol series, and embedded in methacrylate resins by means of ultraviolet polymerization ([9, 18]). The ultrathin sections were processed serially on a LKB Ultratome, picked up on Formvar-covered grids, and contrasted with saturated uranyl acetate aqueous solution. After contrasting, the sections on grids were rinsed rapidly in distilled water.

The material was examined on a JEOL-100 and/or a Hitachi 500 electron microscope operated at 50–100 kV.

RESULTS

A stratified cell wall and a cytoplasmic membrane were discernibly observed in the bacillary cells of *M. lepraemurium* (Figs. 1–4). The cell wall, which was occasionally seen as a moderately dense layer, seemed to be composed of three layers: an innermost electron-dense layer, an intermediate electron-transparent zone, and a thin, outermost electron-dense layer (L_1, L_2 and L_3 in Fig. 1). The innermost layer seemed not to be distinct from the outer leaflet of the cy-

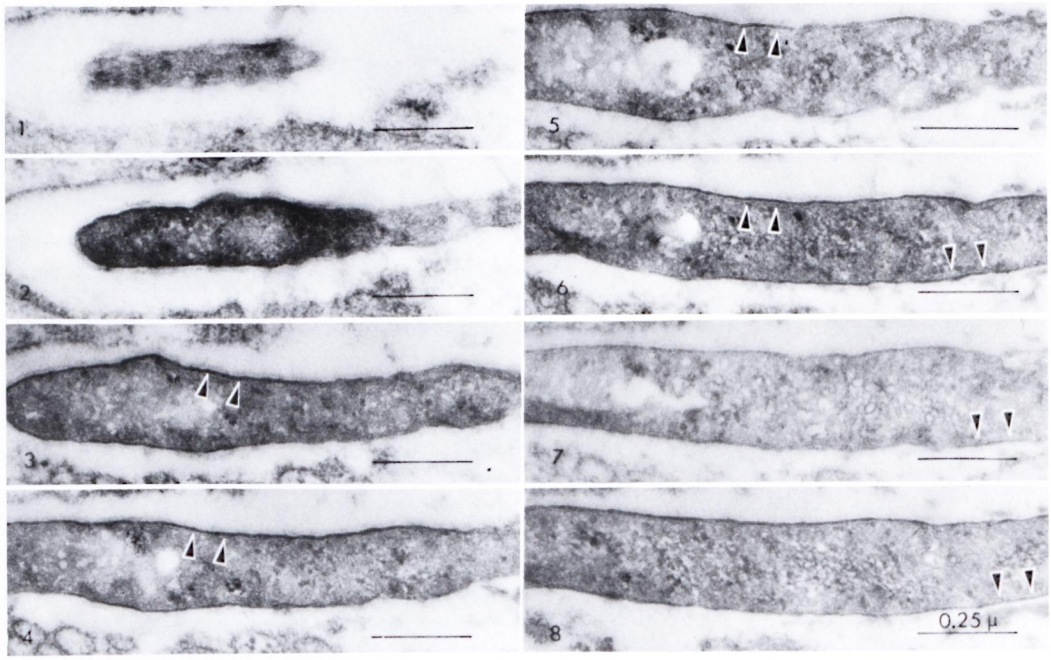

FIG. 2. Longitudinal serial thin sections of *M. lepraemurium*. The innermost layer of the cell wall and the outer leaflet of the cytoplasmic membrane are firmly pegged together and no space is apparent between them (▲ indicate the cytoplasmic membrane).

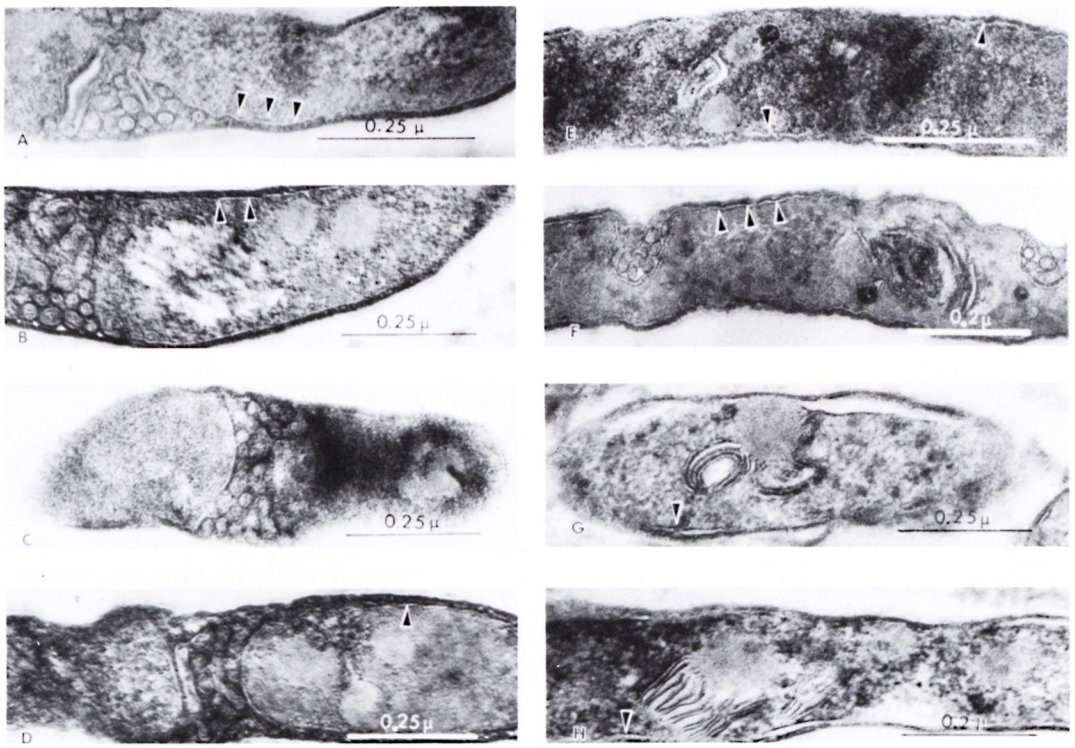

FIG. 3. Thin sections of *M. lepraemurium*. A variety of intracytoplasmic membrane systems is observed. From these photos, it is clear that the cytoplasmic membrane is connected to the intracytoplasmic membrane systems (▲ indicate the cytoplasmic membrane).

toplasmic membrane (Fig. 2). The cytoplasmic membrane appeared to explain the triple-layered, dense-light-dense strata, the so-called unit membrane. This membrane was connected to complicated intracytoplasmic membrane systems, to form the mesosomes, as unique membranous structures which appeared to be the result of folding and/or invagination and subsequent bifurcation of the cytoplasmic membrane.

In *M. leprae,* the ultrastructural features of the cell wall were much less clear than those observed in *M. lepraemurium,* and the typical features of the cell wall were not observed in some serial sections of the organisms (Figs. 5–8). The cytoplasmic membrane of *M. leprae* was composed of two leaflets having about the same thickness. And there seemed faintly to be some structures which adhered to the outermost superficial part of the cytoplasmic membrane (Figs. 5 and 6). The membrane also was continuous with the mesosomes in the cytoplasm (high power magnification D in Fig. 8).

DISCUSSION

Serious efforts and attempts are still being made by many workers to find much better fixing and embedding methods for preserving the cell constituents, especially in bacterial cells ([11]), for electron microscopy. Kellenberger, *et al.* ([7]), for example, have discussed the nature of the bacterial nuclear apparatus based on the understanding that this represented the real nuclear structure, independent of the physiological state of bacteria.

In a detailed study on the micromorphology of leprosy bacilli, Edwards ([3]) reported that glutaraldehyde fixation followed by post-fixation with osmium tetroxide buffered either at pH 7.2 or at pH 6.1, according to Kellenberger, *et al.,* gave satisfactory pictures of both the bacteria and the cells in which they were situated. Silva, *et*

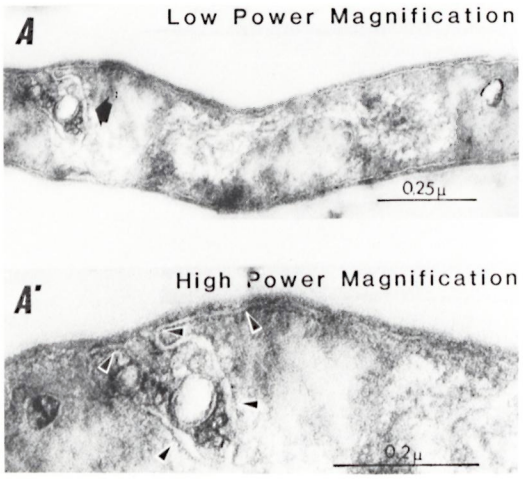

FIG. 4. Longitudinal thin section of *M. lepraemurium.* Continuity of the cytoplasmic membrane with the intracytoplasmic membranous organelles is clearly observed. A′ shows high-power magnification of the arrow mark in A.

al. ([14]) described that the presence of Ca^{++} in the fixatives was found to be essential for the satisfactory preservation of bacterial membranes and ribosomes.

In the present study, 2% agar aqueous solution was used to consolidate the materials before fixation and osmium tetroxide solution with Ca^{++}, according to Kellenberger, *et al.,* was used for fixation after gelation of the agar. After ultrathin sectionings, saturated uranyl acetate aqueous solution was used to obtain high contrast in electron microscopy.

Ordinarily, it is said that the peripheral part of the bacterial cell may be divided into three layers: a) the capsule or slime layer, b) the cell wall, and c) the cytoplasmic membrane. These descriptions of fine structures in the bacterial cell may also be followed in describing the electron microscopic observations of leprosy bacilli. In *M. lepraemurium,* there are, in fact, no arguments that can be adduced for or against the above description of the fine structure, except that a capsular or a slime-like structure is not apparent. It was not possible to clarify the capsular or slime-like structure of *M. lepraemurium in vivo* in the present electron micrographs. Even so, the cell wall and the cytoplasmic membrane in murine leprosy bacilli were distinctly resolved by electron microscopy (Figs. 1, 2 and 4). There seems no reasonable doubt that they are firmly pegged together and consequently no space is apparent between them ([6, 13]), and the fine structure of the membrane appears to be similar to that of other mycobacterial cells ([1]).

The cell wall and the cytoplasmic membrane in *M. leprae in vivo* have been considered similar to those of other mycobacteria, but the present electron microscopic observations of *M. leprae* in human skin lepromas showed slightly different features (Figs. 5–8). The typical fine structures of the cell wall were not observed in careful comparison with *M. lepraemurium* through several serial thin sections of the organisms (Fig. 6), and the cytoplasmic membrane composed of two leaflets having about the same

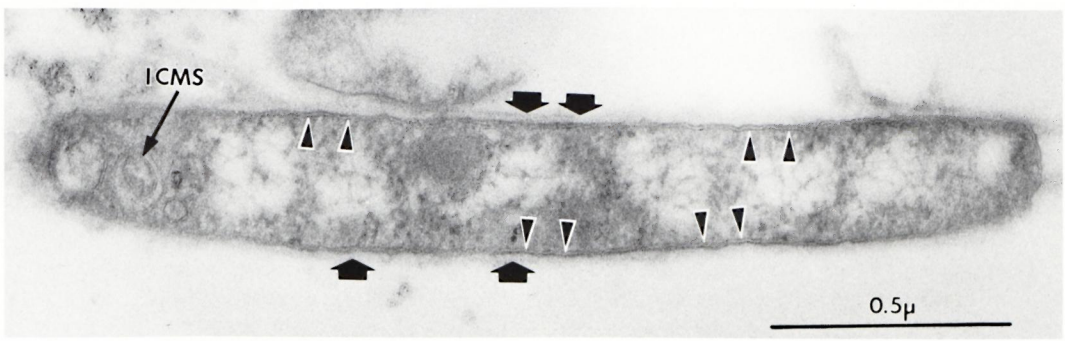

FIG. 5. Longitudinal thin section of *M. leprae.* The bacillary cell envelope is clearly demonstrated as a symmetric profile (▲). The arrows show some indefinite structures which adhere to the outermost superficial part of the cytoplasmic membrane; ICMS = intracytoplasmic membranous structure.

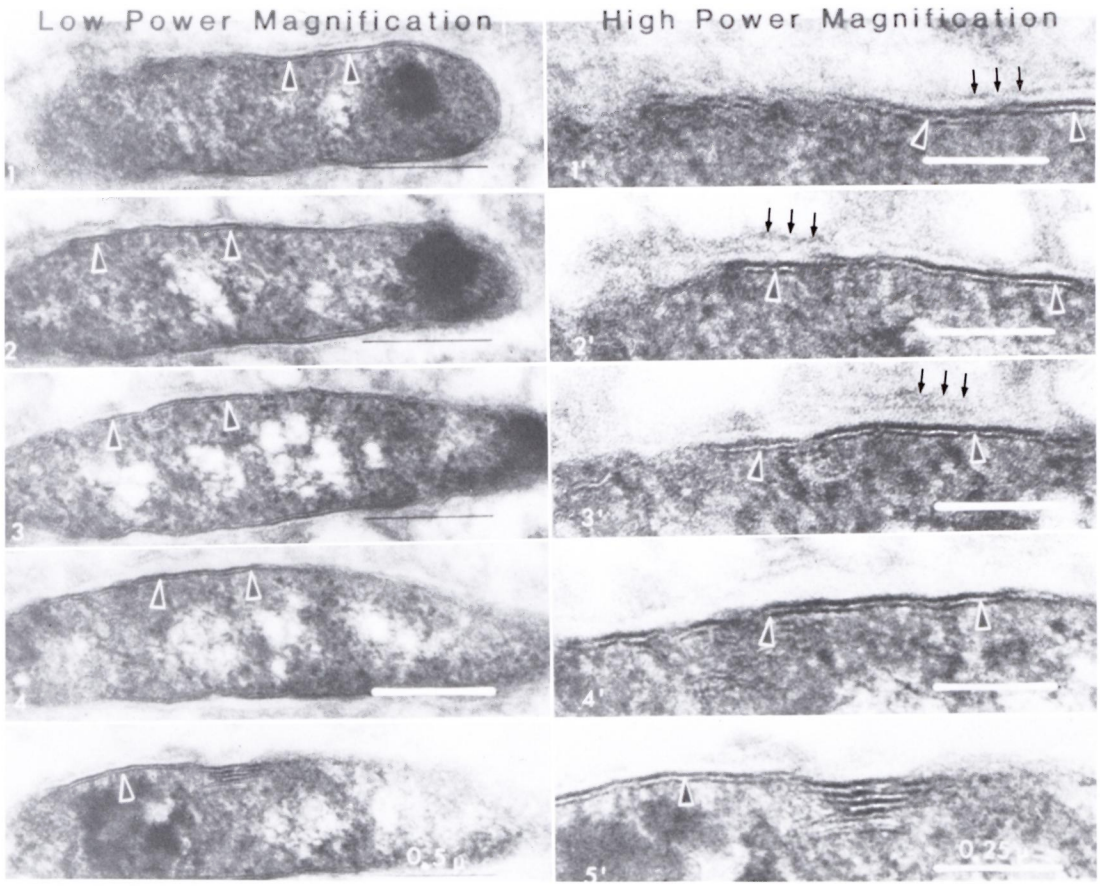

FIG. 6. Longitudinal serial thin sections of *M. leprae* (▲ show the cytoplasmic membrane). Small arrows point out some indefinite structures around the bacillary cell envelope. From these serial thin sections, it is presumed that the symmetric profile is apparent only when the membrane is detached from the innermost cell wall layer.

thickness was different from the membrane profile in murine leprosy bacilli. There were some indefinite structures which adhered to the outermost superficial part of the cytoplasmic membrane as shown in Figures 5 and 6. It is tempting to look at these structures as the cell wall and/or to say that there is some cell wall but only very little.

Since microbial surface components are often major determinants of pathogenicity, affecting all stages and aspects of disease production ([17]), the cell surfaces of the leprosy bacilli must also be important with respect to the host-cell reactions. Therefore, the existence of a cell wall in human leprosy bacilli seems to be presupposed as a matter of course at the level of electron microscopic thin section, and a number of different sorts of electron microscopic studies were consequently done. However, Nguyen, *et al.* ([10]) explained the differences in the cell envelope in *M. lepraemurium* and *M. leprae* by proposing that the adaptive processes of mycobacterium phenotypes living in different environments could lead to alterations in cell wall morphopoiesis.

The cytoplasmic membrane, as a rule of electron microscopic observations, has been regarded as the triple-layered "unit membrane," which is the fundamental unit of all living cell membranes and membranous cell organelles ([12]). In recent publications ([14–16]), the cytoplasmic membrane in *M. leprae* was presented to have a symmetric profile. This profile might as well be said to bear such a construction as the unit membrane. The

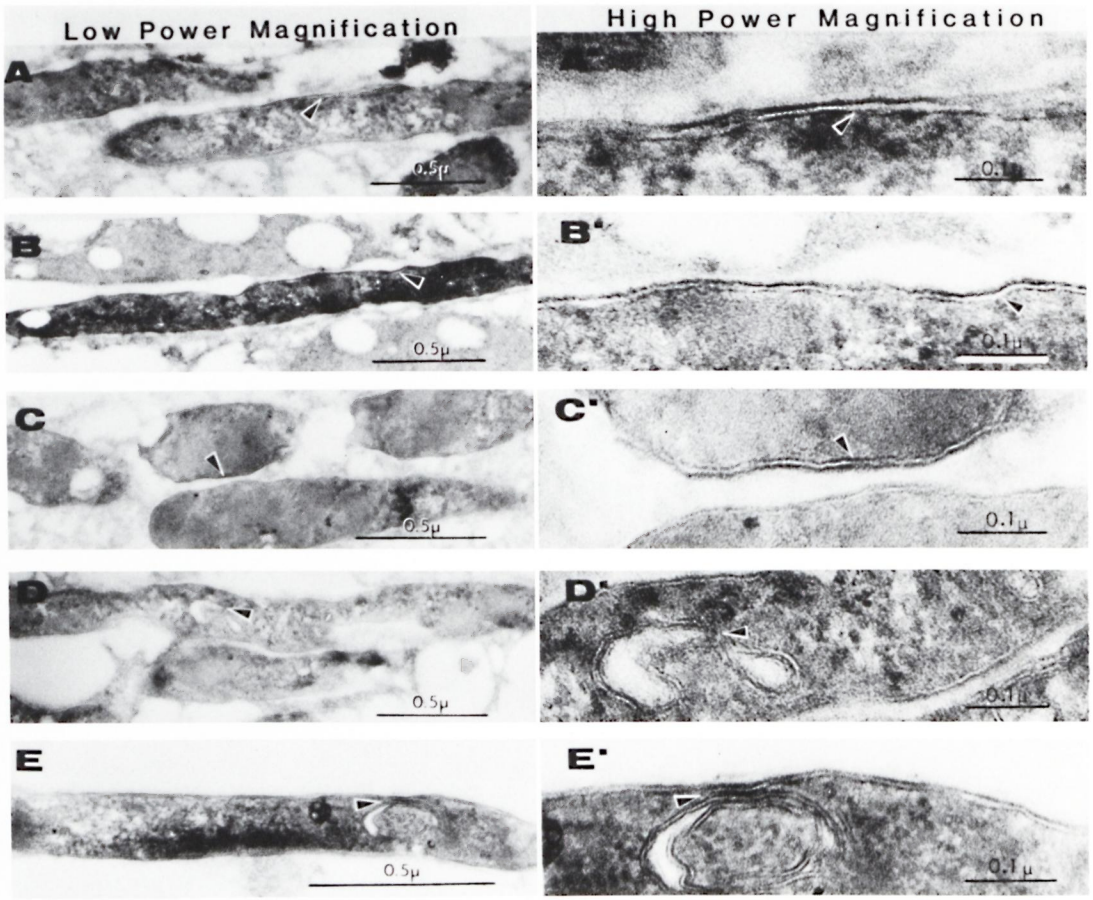

FIG. 7. Thin sections of *M. leprae*. The ▲ show the cytoplasmic membrane (A and A', B and B', C and C') and/or the intracytoplasmic membranous structure (D and D', E and E'). In D' and E', the intracytoplasmic membranous structure is clearly visible.

membranous fine structure of *M. leprae* depicted in Figures 5–8 appears to show a similar symmetric membrane profile.

It is presumed from the serial thin sections (Fig. 6) that the profile described by Silva is apparent only when the membrane is detached from the innermost cell wall layer, as L_1 layer in the case of *M. lepraemurium* (Fig. 1). Otherwise, it may be said that the cytoplasmic membrane as the unit membrane is identical to *M. lepraemurium* and other mycobacteria.

The dissimilar profile of the cell envelope in *M. lepraemurium* and *M. leprae* requires further careful examination in micro-cytological respects, especially when studying the leprosy bacilli in different host cells such as the nude mouse and/or armadillo. Also, comparative micro-morphological observations made *in vivo* and *in vitro* may reveal the true biological properties of the cell envelope in leprosy bacilli. At this time, it may be premature and unsatisfactory to attempt to infer the true picture of the envelope structure of leprosy bacilli on the basis of electron microscopic observations only.

SUMMARY

The cell wall and the cytoplasmic membrane of *Mycobacterium lepraemurium* in murine lepromas and *M. leprae* in human skin lepromas were studied in ultrathin serial sections at the electron microscopic level. The cell wall in *M. lepraemurium* was composed of three layers: an innermost electron-dense layer, an intermediate elec-

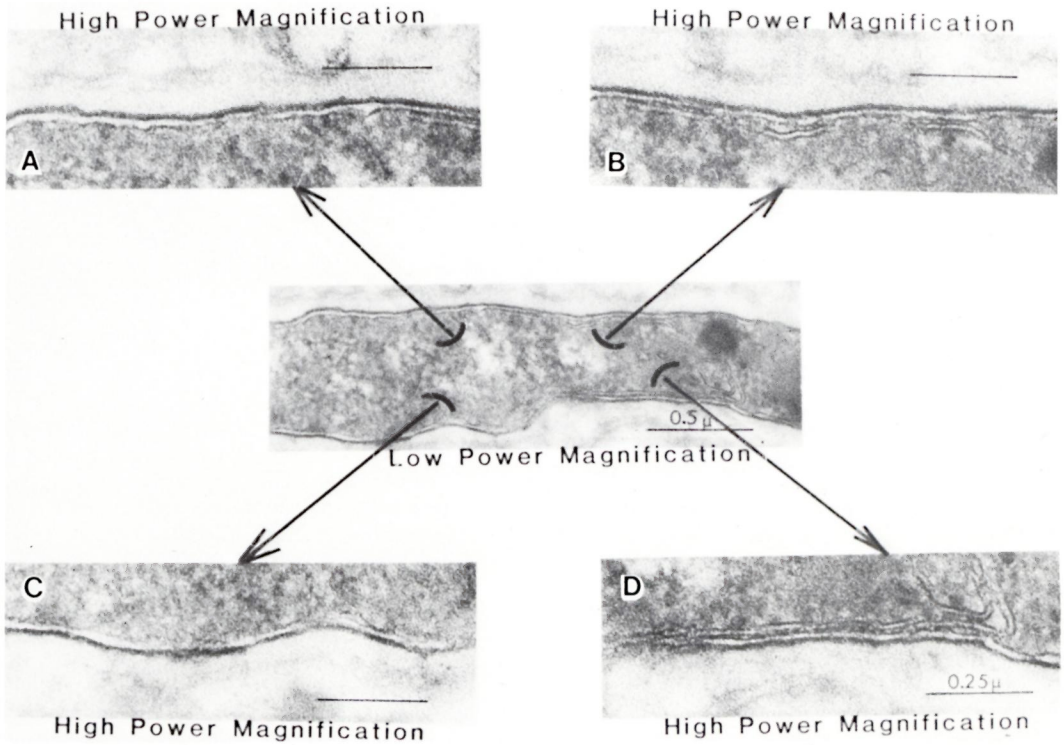

FIG. 8. Longitudinal thin section of *M. leprae*. The cell envelope is shown as a symmetric profile. The profile may be regarded as the unit membrane.

tron-transparent zone, and a thin outermost electron-dense layer. The fine structure of the cell wall in *M. leprae* was slightly different. In general, the cytoplasmic membrane of *M. lepraemurium* and *M. leprae* seemed to have a similar structure.

RESUMEN

Usando microscopía electrónica en cortes seriados ultradelgados se estudiaron la pared celular y la membrana citoplásmica del *Mycobacterium lepraemurium* en lepromas murinos y del *M. leprae* en lepromas humanos. La pared celular en el *M. lepraemurium* estuvo compuesta de 3 capas: la más interna, densa a los electrones, la zona intermedia transparente a los mismos, y la más externa, también electrodensa. La estructura fina de la pared celular en *M. leprae* fue ligeramente diferente. En general, las membranas citoplásmicas del *M. lepraemurium* y del *M. leprae* parecieron tener una estructura similar.

RÉSUMÉ

La paroi cellulaire et la membrane cytoplasmique de *Mycobacterium lepraemurium* et de *M. Leprae* ont été étudiées respectivement dans les lépromes murins et dans des lépromes cutanés chez l'homme, sur des coupes en séries ultrafines observées au microscope électronique. La paroi cellulaire de *M. lepraemurium* se compose de 3 couches: une couche interne, opaque aux électrons; une couche intermédiaire, transparente aux électrons; et une couche externe, mince, opaque aux électrons. Les détails de la structure de la paroi cellulaire de *M. leprae* étaient légèrement différents. En général, la membrane cytoplasmique de *M. lepraemurium* et de *M. leprae* semblent dotées d'une structure similaire.

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