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Ultrastructural Characteristics of *Mycobacterium bovis* BCG and *Mycobacterium leprae*[1]

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Mycobacteria are considered to be Gram-positive organisms, although they lack teichoic acids in the cell wall ([2]). Among them there are strains pathogenic for humans, such as *Mycobacterium leprae* (ML) and *Mycobacterium tuberculosis*, well adapted to survive and grow within the phagocytic cells ([9]). The striking features of the immunopathology caused by the mycobacteria, especially ML, are the varied symptoms manifested clinically ([19]). The symptoms, nature, and intensity of this disease are to a greater extent due to the individual character of host immune reactions ([8]), which probably depends on the presentation of antigenic components of this intracellular parasite. An essential prerequisite to understanding the immunological mechanisms underlying leprosy is to gain an insight into the organelle antigenic organization of the bacterium at the ultrastructural level.

Although various investigators have previously reported the characteristics of the genus *Mycobacterium*, at an ultrastructural level ([2, 11, 13, 17]), the results are not always without ambiguity.

Recently, however, Nguyen, *et al.* ([17]) observed by the freeze-fracture method that ML and *Mycobacterium lepraemurium* (MLm) differed considerably in the organization of the cell envelope. This communication reports the results of ultrastructural studies on *M. bovis* (BCG), antigenically closely related to ML ([5, 10]), and then compares these findings with those on ML isolated from the infected armadillo (*Dasypus novemcinctus*).

MATERIALS AND METHODS

Culture conditions. *M. bovis* BCG was a gift from the National Institute of Public Health (RIV, Bilthoven, Holland). BCG were cultured in Ungar's medium ([21]) under constant stirring and vigorous aeration at 37°C. The BCG were harvested at the beginning of the late exponential growth phase. *M. leprae* were a gift from Drs. R. J. W. Rees and P. Draper, National Institute for Medical Research, London, England, and were isolated from heavily infected armadillo liver, which had been irradiated and stored at −70°C before use, according to Draper's purification procedure ([27]). Such an *M. leprae* preparation was suspended in distilled water.

Electron microscopy. For freeze-fracture studies glycerol was added as a cryoprotectant. In the case of BCG, a final concentration of 15, 20, 25, 30, or 35% (by volume) was added to the bacterial suspension in Ungar's medium 15 min before harvesting ([22]). In the case of ML, a final concentration of 25% was added to the bacterial suspension. The bacteria were then pelleted and the pellets were placed onto the specimen holder. The BCG pellet was equilibrated for 10 min at various temperatures (20–40°C); the ML pellet was equilibrated only at room temperature, after which both specimens were quenched in liquid Freon and kept in liquid nitrogen until further use.

Freeze-fracture was performed in Balzers 300, Balzers 301, and a Denton freeze-fracture apparatus. The stage temperature was −120°C. The replicas obtained by platinum and carbon evaporation were cleaned by repeated washings with sodium hypochlorite (commercial bleach) and then with

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30% sodium hydroxide and finally twice with distilled water. Freeze-fracturing nomenclature is according to Branton, *et al.* [4]. For ultrathin sectioning OsO_4 was added to the bacterial suspension to a final concentration of 0.1% and the cells were fixed for 60 min. The bacteria were then pelleted and resuspended in 2% agar (45°C). The agar was cut into small blocks, which were treated overnight with 1% OsO_4 in acetate-veronal buffer (pH 6.0) and for 30 min with uranyl acetate in distilled water. The blocks were then dehydrated in acetone and embedded in Epon (Merck, Darmstadt, Germany). Gold to silver sections were cut with glass knives on an LKB ultratome III and they were post-stained with uranyl acetate and occasionally with lead citrate. Electron microscopic observations were carried out with a Philips EM 300 electron microscope operated at 80 KV.

RESULTS

Freeze-fracturing. Freeze-cleavage of *M. bovis* BCG indicated the presence of two kinds of cell populations in the following respects. Some produced four fracture faces (Figs. 1a, 1b) in concordance with the findings of Nguyen, *et al.* [17] for ML and MLm. However, the majority of the bacteria were preferentially cleaved along a plane across the cell wall (CW), which was devoid of particles and which had a smooth appearance, both for the protoplasmic (PF) and exoplasmic fracture faces (EF). The plasma membrane was rarely cleaved under the conditions used. Furthermore, the PF and EF faces of the cell wall of some cells showed linear depressions and linear ridges (Fig. 2).

Neither varying the temperature before quenching (20°C, 30°C, 35°C, or 40°C) nor the concentration of glycerol produced differences in the morphology of the CWPF and CWEF.

The PF face of the plasma membrane showed large smooth areas and a small number of aggregated particles (Fig. 3) when the bacteria were quenched from room temperature. On the other hand, freeze-fracturing of *M. leprae* showed a rather smooth CWPF and CWEF, the PF face of the plasma membrane showed large smooth areas with particles present in clusters (Fig. 4a). The EF face also showed smooth areas with fewer particles than the PF face (Fig. 4b). These findings are in concordance with those of Nguyen, *et al.* [17]. Linear depressions and ridges were also observed on the CWPF and CEWF of some cells, although not in all.

Ultrathin sectioning. Ultrathin sectioning of BCG showed at least two populations of cells. In most cells the mycobacterial cell envelope consisted of three substructures: a) an electron-transparent zone, b) a more dense layer containing the murein layer, and c) the triple layer of the plasma membrane (Fig. 5). However, in some cells the electron-transparent zone could not be observed. Ultrathin sections of *M. leprae* showed similar substructures of the cell envelope as those of BCG, except that the electron-transparent zone appeared to be more translucent in the case of *M. leprae* than in BCG (Fig. 6).

DISCUSSION

We found that the BCG cell envelope contained two sites of cleavage, in agreement with the findings for other mycobacterial species[25]. However, in most cells, the cell wall was the preferential site of cleavage as compared to the plasma membrane, which was rarely split to show its apolar sites [3, 16, 18, 20]. No particles could be detected on the CWPF and CWEF under the various conditions used. The absence of particles would suggest that the cell wall consists of pure (phospho) lipids (and perhaps some lipid-polysaccharide complexes) as inferred for *E. coli* [26].

The chemical composition of the outer lipid layer is not comparable with that of the plasma membrane lipid bilayer, as can be seen from Figure 6, since the layers differ in their reactivity towards osmium. The PF face of the plasma membrane resembles that of a gram-negative bacterium such as *E. coli*, in which segregation of lipid and protein upon solidification of membrane lipids takes place [12, 15, 24], although the number of membranous particles on the PF face of the BCG plasma membrane appears to be far smaller.

It is well known that growth conditions markedly influence the chemical composition of the bacterial cell envelope [7], as well as the morphology of the cell envelope

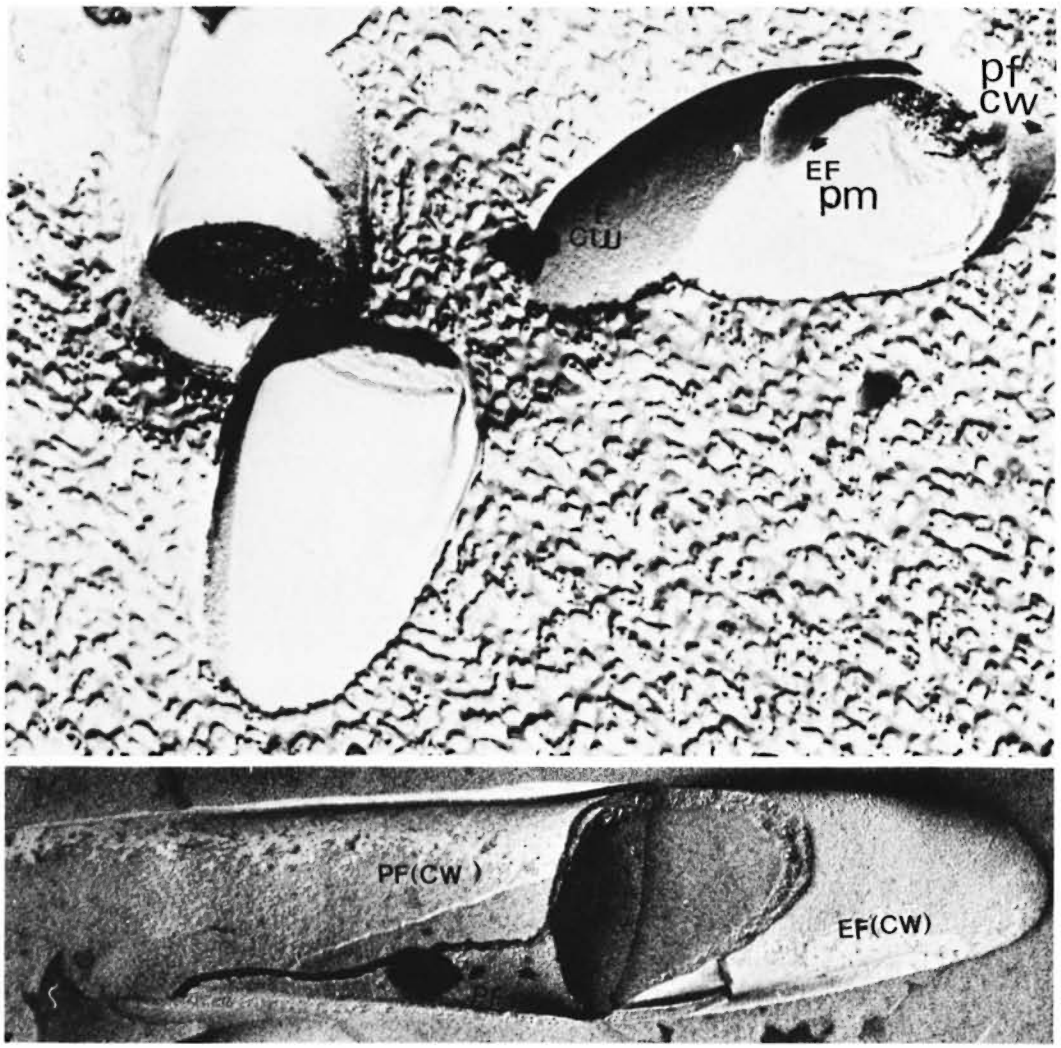

FIG. 1a. Freeze-fracture faces of *Mycobacterium bovis* BCG. The protoplasmic fracture faces (PF) and exoplasmic fracture faces (EF) of the cell wall (CW) have a smooth appearance. Large smooth areas are discernible on the PF face of the plasma membrane (PM) (original ×80,000).

FIG. 1b. Freeze-fracture faces of *Mycobacterium bovis* BCG. The protoplasmic fracture faces (PF) and exoplasmic fracture faces (EF) of the cell wall (CW) have a smooth appearance. Large smooth areas are discernible on the PF face of the plasma membrane (PM) (original ×80,000).

observed in ultrathin sections ([6]). This may account for the different appearances of the electron-transparent zones in BCG and *M. leprae* (Figs. 5 and 6) since BCG had been grown under conditions completely different from those of *M. leprae*. It is also known that the freeze-fracture characteristics of the bacterial cell envelope (e.g., outer membrane) may alter due to the changes in the chemical composition of the envelope as has been demonstrated for *E. coli* mutants ([23]).

Freeze-fracturing of BCG and *M. leprae* showed a heterogeneity in that in both cases we could observe cells with smooth CWPF and CWEF faces and cells with linear ridges (CWEF) or depressions (CWPF) (Figs. 1 and 2). However, very few differences between BCG and *M. leprae* after freeze-fracturing could be observed; only the number of cells with smooth CWPF and CWEF faces was higher for *M. leprae* than for BCG. Nguyen, *et al.* ([17]) correlated the linear structures on the CW fracture planes

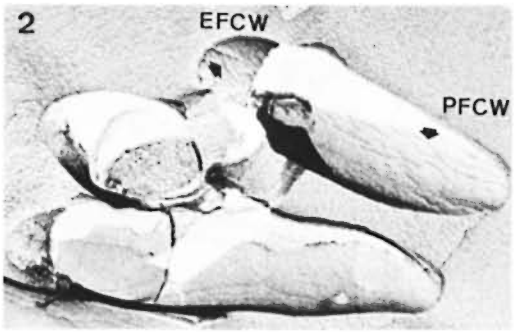

FIG. 2. Freeze-fracture faces of *M. bovis* BCG. The protoplasmic fracture faces (PF) and exoplasmic fracture faces (EF) of the cell wall (CW) sometimes show linear depressions and ridges, respectively (original ×45,000).

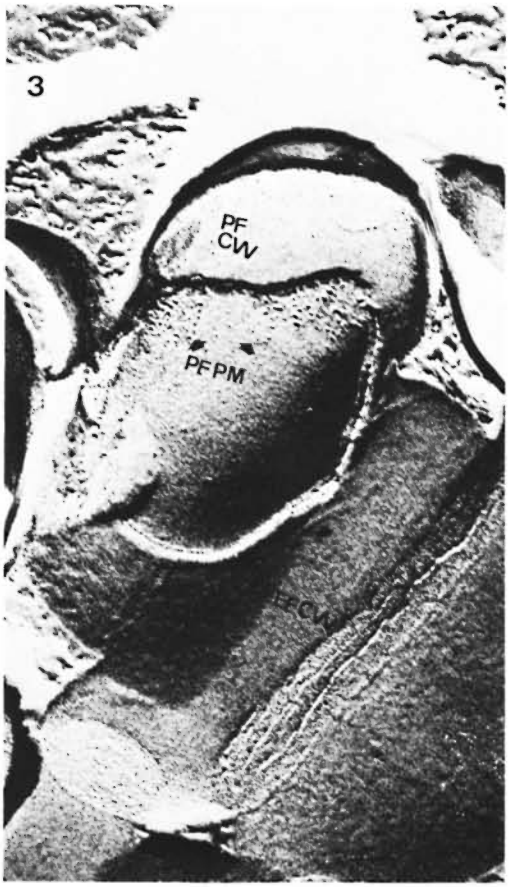

FIG. 3. Convex fracture face (PF) of *M. bovis* BCG cell wall (CW) and plasma membrane (PM). Large smooth areas and clusters of membrane particles (arrows) are present on the PF face of the plasma membrane (original ×10,000).

with the presence of external peptidoglycolipids and mycosides. We do not, however, exclude the possibility that the presence of the ridges and depressions could be due to the methodology, or perhaps due to the presence of lysed and/or old cells. The latter possibility is more likely in *M. leprae* than in BCG, these characteristics showing on the CW fracture faces. A difference in morphology could be observed between BCG and *M. leprae* in ultrathin sections with respect to the electron-transparent zone, but this was not clearly distinct in the morphology of the cell wall fracture faces. However, one should be cautious in such interpretations since at present the interrelationships between the electron-transparent zone, and the CW fracture faces has not yet been established. We had the impression that the plasma membrane of *M. leprae* was more easily split than that of BCG. The *M. leprae* plasma membrane fracture faces resembled those of Gram-negative bacteria freeze-fractured after quenching from a temperature below the temperature of the transition phase, i.e., large smooth areas and clusters of particles on the PF face ([23]).

Attempts to observe a PF face of the plasma membrane with a homogenous or netlike distribution of particles by raising the temperature of the BCG suspension before quenching was not successful, since in this case only the cell wall was fractured. Thus, in which temperature range the phase transition of the membrane lipids took place could not be visualized by freeze-fracturing. Estimation of the phase transition temperature must be attempted by physical methods such as differential scanning calorimetry.

Experiments with glutaraldehyde fixation of BCG prior to freezing did not produce "satisfactory" freeze-fracture faces of the plasma membrane because the plasma membrane was again very rarely split (unpublished results). Thus, whether glutaraldehyde did affect the plasma membrane of BCG similarly as it has been reported to affect the plasma membrane of *E. coli* as visualized by freeze-fracturing ([1, 12]) is uncertain. It appears that mycobacteria are unique among bacteria with respect to their freeze-fracture characteristics, which could reflect the complicated chemistry of their cell walls ([14]).

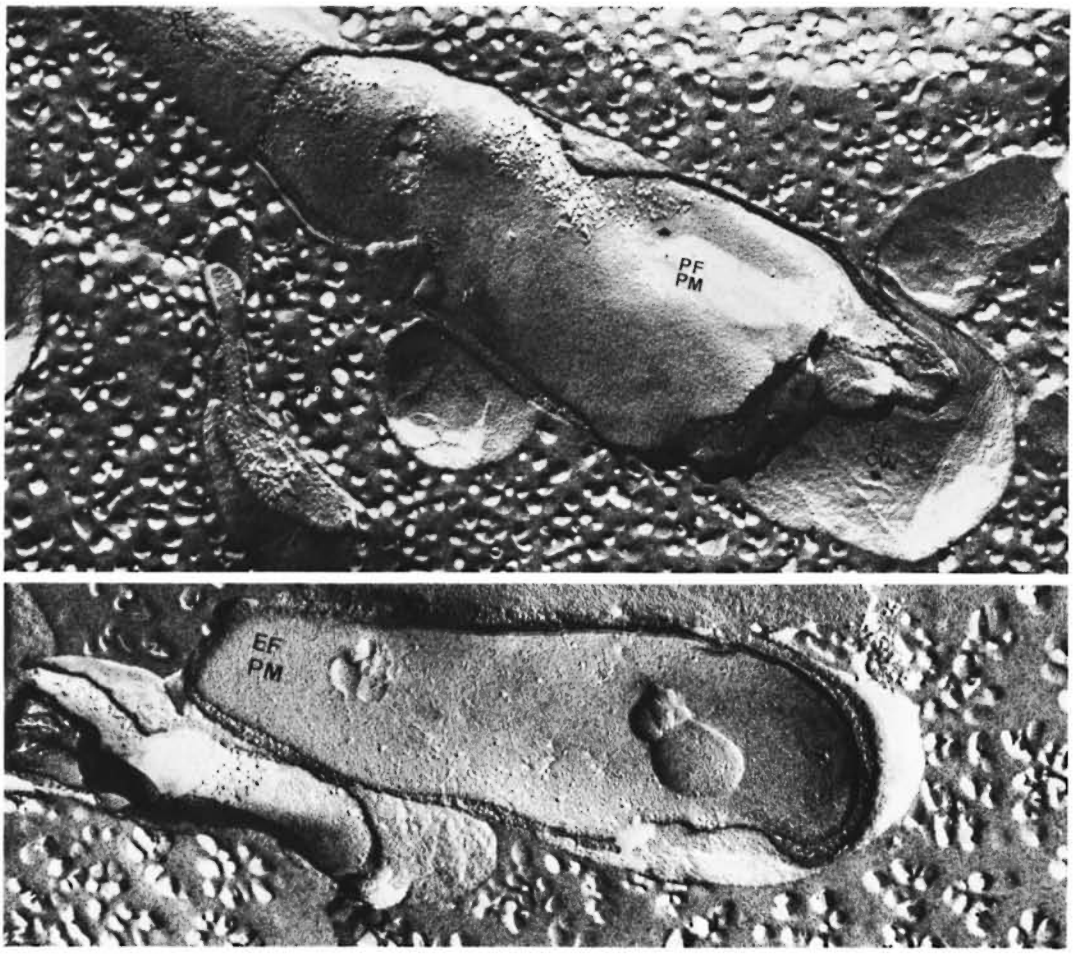

FIG. 4a. Freeze-fracture faces of *M. leprae* (ML) cell wall (CW) and plasma membrane (PM). The protoplasmic fraction (PF) face of the cell wall is rather smooth, a depression is discernible, however (arrow). The exoplasmic fracture (EF) face of the cell wall shows some ridges, but not abundantly. Large smooth areas and regions with clusters or particles are present on the PF face of the plasma membrane (arrows) (original ×120,000).

FIG. 4b. Exoplasmic fracture (EF) face of the plasma membrane (PM) of *M. leprae* (original ×120,000).

In spite of the earlier reports regarding the existence of species specific variations among mycobacteria at the ultrastructural level ([2, 11, 13]), we observed few differences (with some exceptions as stated earlier) between BCG and *M. leprae* in both freeze-fracturing and ultrathin sectioning.

SUMMARY

Freeze-fracture studies (FF) and ultrathin sectioning (UTS) have been performed on *Mycobacterium bovis* (BCG) and *Mycobacterium leprae* (ML). FF of BCG revealed the presence of two populations of cells: one group having four fracture faces and the other showing only two; the latter showed a preferential cleavage along a plane across the cell wall (CW) having a smooth appearance devoid of particles, on both protoplasmic (PF) and exoplasmic (EF) fracture faces. On the other hand, FF of the plasma membrane of ML often showed rather large smooth areas with particles present in clusters. Linear depressions and ridges were more frequently observed in CWEF and CWPF faces of ML as compared to those of BCG.

Similarly, UTS of BCG revealed two populations of cells: the majority with cell envelopes consisting of three substructures

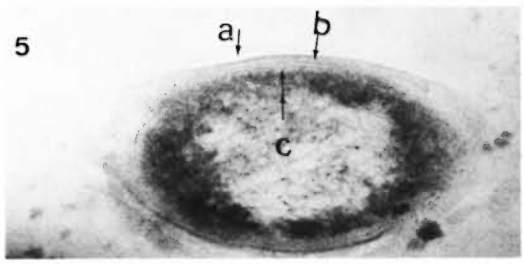

FIG. 5. Ultrathin section of BCG, showing layers a) electron-transparent zone, b) a more dense zone, and c) the plasma membrane (original ×80,000).

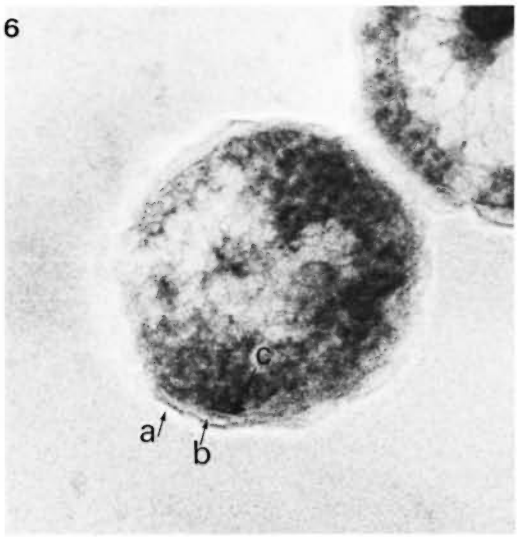

FIG. 6. Ultrathin section of *M. leprae*. The same layers are discernible as in BCG (Fig. 5), only layer a) is more translucent (original ×80,000).

and the rest with only two. UTS of ML showed similar substructures as for BCG, except that the electron-dense zone appeared more translucent. We postulate that mycobacteria of the same genus may exist in heterogeneous forms.

RESUMEN

Se hicieron estudios de "fracturas por congelación" (FC) y estudios en cortes ultradelgados (CUD) en *Mycobacterium bovis* (BCG) y en *Mycobacterium leprae* (ML). Los estudios de FC en BCG revelaron la presencia de 2 poblaciones celulares; una con cuatro planos de fractura y otra con sólo dos. Esta última mostró un rompimiento preferencial a través de la pared celular (PC) y el plano de rompimiento tuvo una apariencia lisa carente de partículas tanto en la cara de fractura protoplásmica (PP) como en la exoplásmica (EP). Por otro lado, los estudios de FC de la membrana plasmática del ML mostraron a menudo grandes áreas lisas con algunas partículas agrupadas en racimos. Comparando con el BCG, las depresiones lineares y la crestas o rebordes fueron más frecuentes en las caras PCEP y PCPP del ML.

Similarmente, los CUD del BCG revelaron 2 poblaciones de células: la mayoría con envolturas celulares consistentes de tres subestructuras y el resto con sólo dos. Los CUD del ML mostraron subestructuras similares a las del BCG, excepto que la zona electrodensa apareció más translucente.

Postulamos que las micobacterias del mismo género pueden existir en formas heterogéneas.

RÉSUMÉ

On a mené des études par cryofractures (FF) et par coupes ultra-minces (UTS) sur *Mycobacterium bovis* (BCG) et *Mycobacterium leprae* (ML). La première méthode a révélé chez les *Mycobacterium bovis* la présence de deux populations de cellules dont l'une a quatre faces de fractures, et dont l'autre ne présente que deux surfaces; ce dernier groupe de cellules montre un plan de clivage préférentiel selon un plan qui traverse la paroi cellulaire (CW) et présente un aspect lisse dépourvu de particules, tant sur les faces protoplasmiques (PF) qu'exoplasmiques (EF) de la fracture. D'autre-part, cette même méthode (FF) appliquée à la membrane plasmatique de ML montre souvent des régions lisses assez étendues, assorties de particules agglomérées. Des dépressions linéaires et des crêtes ont été plus fréquemment observées dans les parois cellulaires tant protoplasmiques qu'exoplasmiques de *M. leprae*, par comparaison avec ces parois chez *Mycobacterium bovis*. De même les coupes ultra-minces de *Mycobacterium bovis* ont révélé deux populations de cellules dont la majorité présentait des enveloppes cellulaires constituées par trois sous-structures, alors que les autres n'en présentaient que deux. Les coupes ultra-minces de *Mycobacterium leprae* ont montré des sous-structures semblables à celles de *Mycobacterium bovis*, à ceci près que les régions denses en électrons apparaissaient moins translucides. On postule dès lors que les mycobactéries de la même espèce peuvent exister sous des formes hétérogènes.

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