JOURNAL OF BACTERIOLOGY, May 1979, p. 552-558
0021-9193/79/05-0552/07$02.00/0

Vol. 138, No. 2

Comparative Ultrastructure of *Mycobacterium leprae* and *Mycobacterium lepraemurium* Cell Envelopes

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Received for publication 12 February 1979

The structural properties of the cell envelopes of *Mycobacterium leprae* and *Mycobacterium lepraemurium* were investigated by freeze-fracture, freeze-etching, and negative-staining techniques. Freeze-fracture split the cell wall and exposed the internal features of the peptidoglycolipid mycosidic filamentous network. The cell membrane was also split into two asymmetric faces. The external fracture face was characterized by linear arrays of intramembranous particles, whereas the protoplasmic fracture face showed randomly distributed clusters of particulate entities. Comparative analysis of the ultrastructural features observed in *M. leprae* and *M. lepraemurium* indicated that the organization of the cell envelope in these two species differed particularly with respect to the amount and complexity of the superficial peptidoglycolipid and mycosidic integument, which is poorly developed in the mycobacterium responsible for human disease.

Mycobacterium leprae and *Mycobacterium lepraemurium* are obligate parasites well adapted to survival within the phagocyte cells of human and rodent tissues (11). In these microorganisms the envelope consists of a basal structure of mucopeptides covalently linked to arabinogalactane mycolates (2, 17). Beyond this rigid polymeric layer the microorganism is further protected by an elaborated integument which has been said to be of peptidoglycolipids and mycosidic in nature (2, 6, 14, 17). The morphological definition of these superficial layers has been accomplished mainly by electron microscopy of negatively stained preparations (2, 10). The results of these studies lead to the conclusion that the peptidoglycolipid mycosidic components form a superficial complex filamentous network which is believed to be a characteristic property of the genus *Mycobacterium* (2, 14). However, differences between the organization of the surface network in *M. leprae* and in other *Mycobacterium* species growing in artificial cultures have been described by Gordon and White (10).

It is the purpose of this report to present the comparative surface ultrastructure of two species of mycobacterium which have not previously been extensively studied by freeze-fracture and by freeze-etching. The advantage of these methods compared with other techniques of specimen preparations is that from the study of fractured and etched exposed surfaces the three-dimensional organization of the cell envelopes can be characterized. It is shown that the superficial tubular network exposed by deep etching and negative staining correlates and reflects the complexity of the internal organization of the cell envelopes revealed by the fracture. Moreover, some evidence is provided that the ultrastructural features of the cell envelopes in *M. leprae* differ from those in *M. lepraemurium* in regard to the extension and assembly of the peptidoglycolipid and mycosidic pattern.

MATERIALS AND METHODS

Human material was obtained from several biopsies of 10 patients presenting the lepromatous type of the disease and under chemotherapy treatment. Swiss female mice, 3 weeks old, were injected in the footpad with 10 μl of a suspension of *M. lepraemurium* Hawaii containing about 2.5×10^7 bacilli per ml. Human tissue, and mouse nodular lepromas, which developed 5 to 6 months after injection, were homogenized in phosphate-buffered saline at pH 7.2 in a tight glass tissue grinder. The crude suspension was centrifuged at 1,000 × g for 10 min at 4°C. The supernatants were sedimented at 40,000 × g for 30 min. The pellets were either suspended in phosphate-buffered saline or cut into small fragments. Some of these fragments were placed on gold-coated specimen holders, rapidly frozen in liquid freon 22, and subsequently stored in liquid nitrogen. Other pellet fragments were fixed in 2% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4) for

30 min and progressively impregnated with a 25% glycerol-water solution. Small drops of glycerol-impregnated samples were rapidly frozen as described above. Freeze-fracture and freeze-etching were carried out in Balzers 360 and 301 apparatuses. The stage temperature was −140°C for freeze-fracture experiments and −100°C when etching was desired. Etching was carried out for 1 min 45 s. The replicas obtained by carbon and platinum evaporation were cleaned by repeated washings with sodium hypochlorite and 70% sulfuric acid and, if necessary, with 1 M sodium hydroxide, and finally with twice-distilled water. For negative staining, 1% uranyl acetate or ammonium molybdate was applied to microorganism suspensions spread on carbon-coated grids. Some bacterial samples were sonicated for 1 min in an MSE ultrasonic disintegrator at maximum frequency prior to negative staining. Electron microscopic observations were carried out with a Philips 400 electron microscope operating at 80 kV. Electron micrographs were taken at various magnifications (6,000 to 64,000).

RESULTS

Freeze-etching applied to *M. leprae* shows that the outer cell surface has a rather smooth appearance (Fig. 1). Conversely, most of the *M. lepraemurium* studied by freeze-etching were characterized by an elaborated superficial integument. Figure 7 shows that the outer cell surface is wrapped round by ribbon- and rodlike forms.

Negative staining of *M. lepraemurium* clearly reveals that the outer surface of the cell wall comprises ribbon- and rodlike projections which after sonication appear to consist of fragmented tubular elements (Fig. 13). The negative staining of *M. leprae* shows instead that the ribbons and tubules are undetectable even after sonication and only small patches of a filamentous network are visible (Fig. 6).

Freeze-fracture provides further evidence concerning the tubular nature of the material accumulated at the surface of *M. lepraemurium*. Figure 12 shows that ribbon- and rodlike projections are cleaved along their major axis, and convex and concave elongated surfaces are exposed. Adjacent tubules are also seen to be twisted about each other.

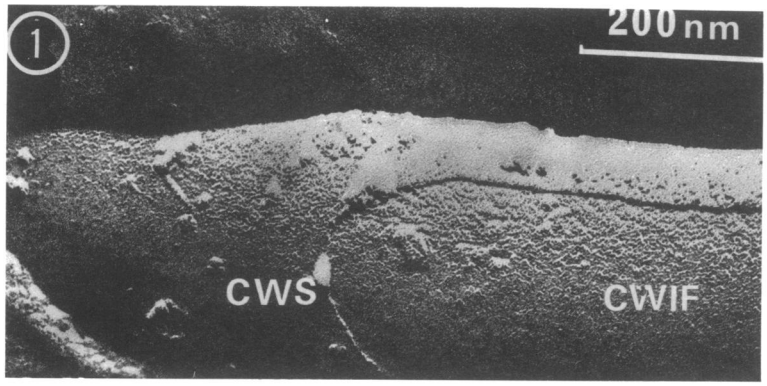
Freeze-cleavage occurs at different levels of the cell envelopes and produces four fracture faces. The inner cleavage plane splits the cell membrane. In *M. lepraemurium* the convex fracture face outwardly directed and close to the cytoplasm (PF) contains clusters of intramembranous particles (Fig. 9 and 10). The concave, inwardly directed fracture face (EF) is studded with linear arrays and small clusters of particles (Fig. 8). In *M. leprae* the PF (Fig. 5) and EF fracture faces are characterized by the presence of very few particulate clusters.

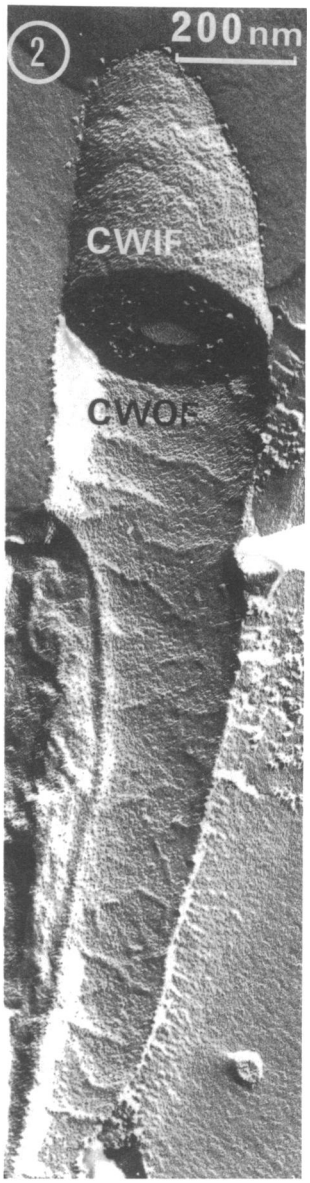
The outer cleavage plane splits the cell wall also into two halves. The convex inner fracture face, outwardly directed and close to the mycobacterium body (CWIF), is characterized by the presence of linear depressions and a few ridges (Fig. 2 and 11). Most of the ridges remain associated with the concave, inwardly directed, outer fracture face (CWOF) (Fig. 2 and 12). The ridges exist as discontinuous but aligned segments (Fig. 2 and 12). The average diameter of the depressions is 10 nm, and that of the ridges is 15 nm.

In most of the *M. leprae* the cell envelope shows a loss of the linear depressions and ridges. Hence, the convex and concave fracture faces have a rather smooth appearance (Fig. 3 and 4). Occasionally, individual mycobacteria possess a rather elaborate filamentous network although never as developed as in *M. lepraemurium* (Fig. 2).

DISCUSSION

In gram-positive microorganisms, the localization of the freeze-cleavage plane within the cell wall is still a matter of controversy (2, 14, 16, 18). If, as is believed, the cell wall constituents form a network through covalent and hydrogen bonds (9), then the site of the fracture within the cell is not easily identifiable, since at low temperature these bonds may still be sufficiently strong to hinder cleavage (4). However, this assumption suffers from uncertainties related to possible differences in cell wall configuration and molecular organization among various species and types of gram-positive bacteria, in particular mycobacteria. Actually, analytical data bring the true gram-positive state of mycobacteria into question and indicate a striking chemical and structural complexity in the cell wall of these microorganisms (1, 2, 6, 7, 9, 14, 17). From these interesting studies a multilayered model for the structure of the mycobacterial cell wall has been proposed (2, 14). In agreement with the observations of Kim et al. (14) and Barksdale and Kim (2), our results on *M. leprae* and *M. lepraemurium* give a strong indication that the cell wall and the cell membrane both contain a preferential site of freeze-cleavage, producing convex and concave fracture faces. Hence, the cell wall in mycobacteria could comprise a planar hydrophobic continuum where peptidoglycolipid and mycosidic filaments are assembled. Furthermore, these components could accumulate at the outermost cell surface and form the ribbon-like and tubular elements revealed by negative staining, freeze-fracture, and freeze-etching. This assumption is supported by the morphological evidence that the filamentous network visualized on outer and in-


1
200 nm
CWS
CWIF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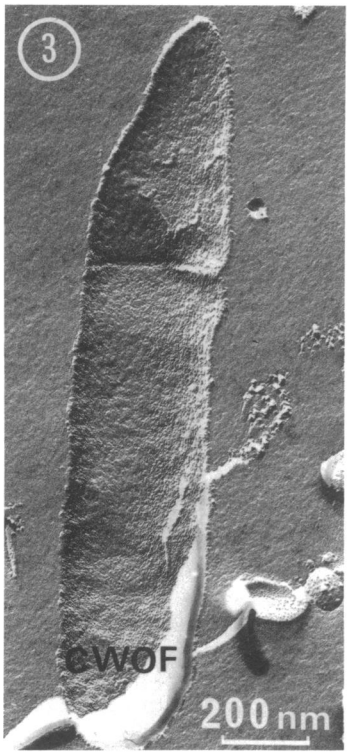
200 nm
CWIF
CWOF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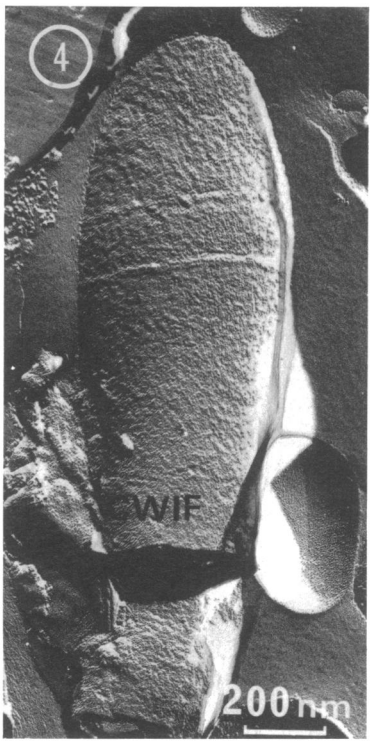
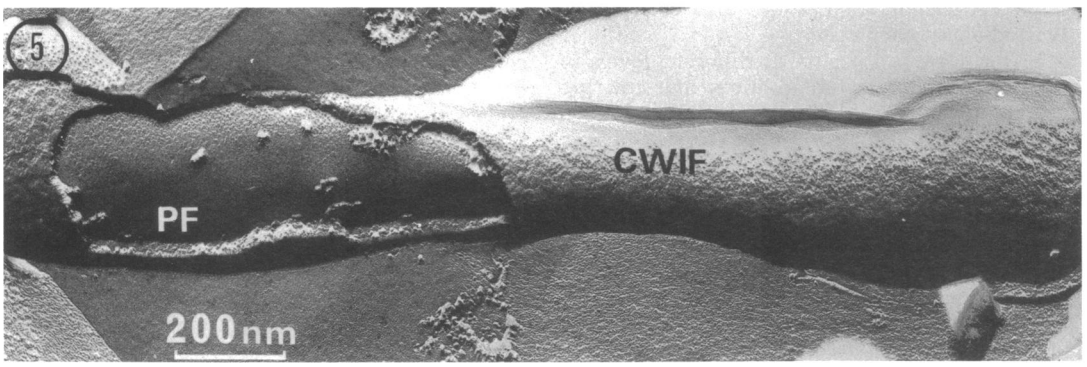
CWOF
200 nm


4
CWIF
200 nm


5
CWIF
PF
200 nm

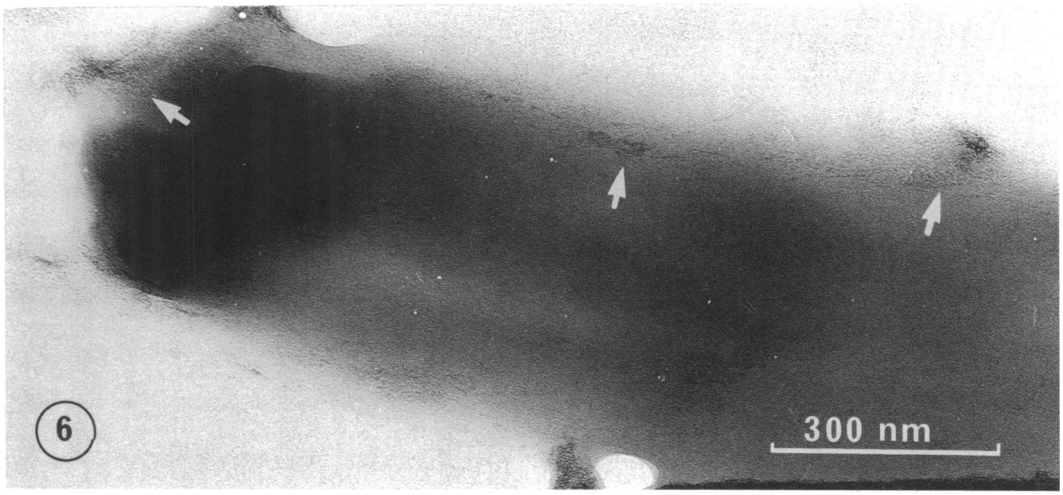


FIG. 6. *Negative staining of M. leprae after sonication. Small patches of ramified fibrous projections are visualized at the outer cell surface (arrows).*

ner fracture faces, respectively, as linear ridges and depressions is decreased in *M. leprae* to the same extent as the reduction of the external peptidoglycolipid and mycosidic accumulation. It is striking that these cell wall components have some structural resemblance to the tubular forms of Gaucher's disease cerebrosides (15) and also to the peptidoglycolipid complexes extracted from the myelin (21). We have also observed that the particulate entities visualized on the EF fracture face of the cell membrane, particularly in *M. lepraemurium*, form rows and arrays extending along linear patterns allusive to those of the fibrillar network found within the outer cell wall. The interpretation of these structural features probably resides in the knowledge that, in other types of biological membranes and envelopes, linear arrays of particulate entities, ridges, and complementary depressions exposed on fracture faces are usually markers for membrane assembly and differentiation (cf. ref. 3, 13, and 19). For instance, particulate arrays at fracture faces of the outer membrane of *Acetobacter xylinum* are believed to be the synthetic sites of cellulose microfibrils organized at the surface of the microorganism (5). Evidence has been provided that in the gram-positive *Streptococcus faecalis* the cell wall is cleaved and rows of globules are visualized on the freeze-fracture faces. Tentatively, the latter particulate entities have been identified with assembly units of the outer cell envelope (20). It is therefore tempting to set side by side the observation that the filamentous and tubular network is poorly developed in *M. leprae* and the finding that the intramembranous particles are represented only by a few clusters.

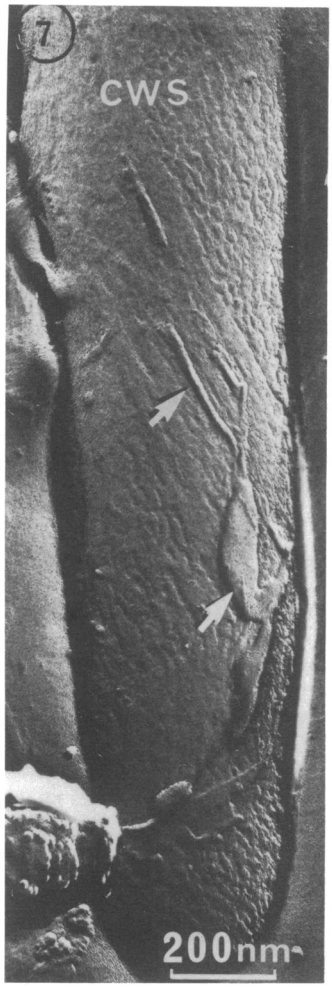
There is probably more than one alternative to explain the differences between the features of the cell envelopes of *M. leprae* and *M. lepraemurium*. The superficial mycosidic integument and peptidoglycolipid filamentous network are shown to occur in a wide variety of mycobacterial species (2, 14). It seems that these components of the cell wall display very few differences among the various species of myco-

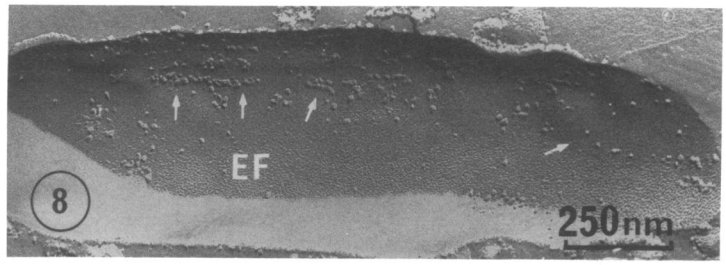
FIG. 1. *Freeze-etching and fracture view of M. leprae. The cell wall surface (CWS) exposed by deep etching is smooth. Note the lack of ribbon-like projections. The convex cell wall inner face (CWIF) exposed by the fracture is also amooth.*

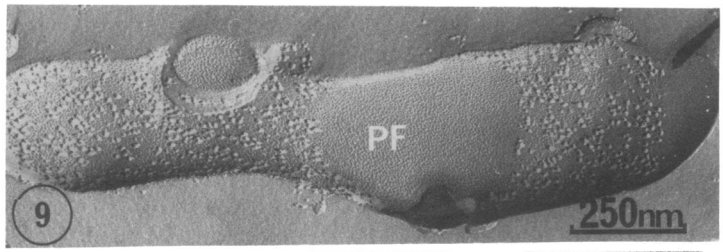
FIG. 2. *Freeze-fracture of M. leprae. The cleavage exposes both the cell wall outer and inner fracture faces (CWOF and CWIF). Note that only a few ridges are visible on the concave fracture face (CWOF) and correspondingly very few linear depressions are present on the convex fracture face (CWIF).*

FIG. 3 and 4. *Concave (CWOF) and convex (CWIF) fracture faces of the M. leprae cell wall. Note that both faces are smooth. The perpendicular beltlike ridges in Fig. 4 and the grooves in Fig. 3 correspond to septa.*

FIG. 5. *Freeze-fracture of M. leprae. Two fracture planes are visible. The inner one, characterized by smooth areas with a few particulate clusters, corresponds to the cell membrane inner fracture face (PF). The outer cleavage plane exposes the convex fracture face of the cell wall (CWIF). Note the deep cleavage step between the two fracture planes.*







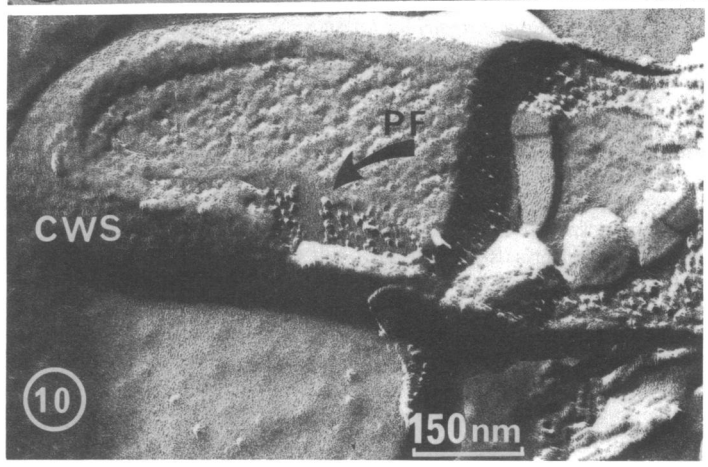


FIG. 7. *Freeze-etching of the outer cell wall surface of M. lepraemurium. The etched surface shows the accumulation of ribbon-like and filamentous projections (arrows).*

FIG. 8. *Outer fracture face (EF) of M. lepraemurium. The intramembranous particles form linear arrays and small clusters (arrows).*

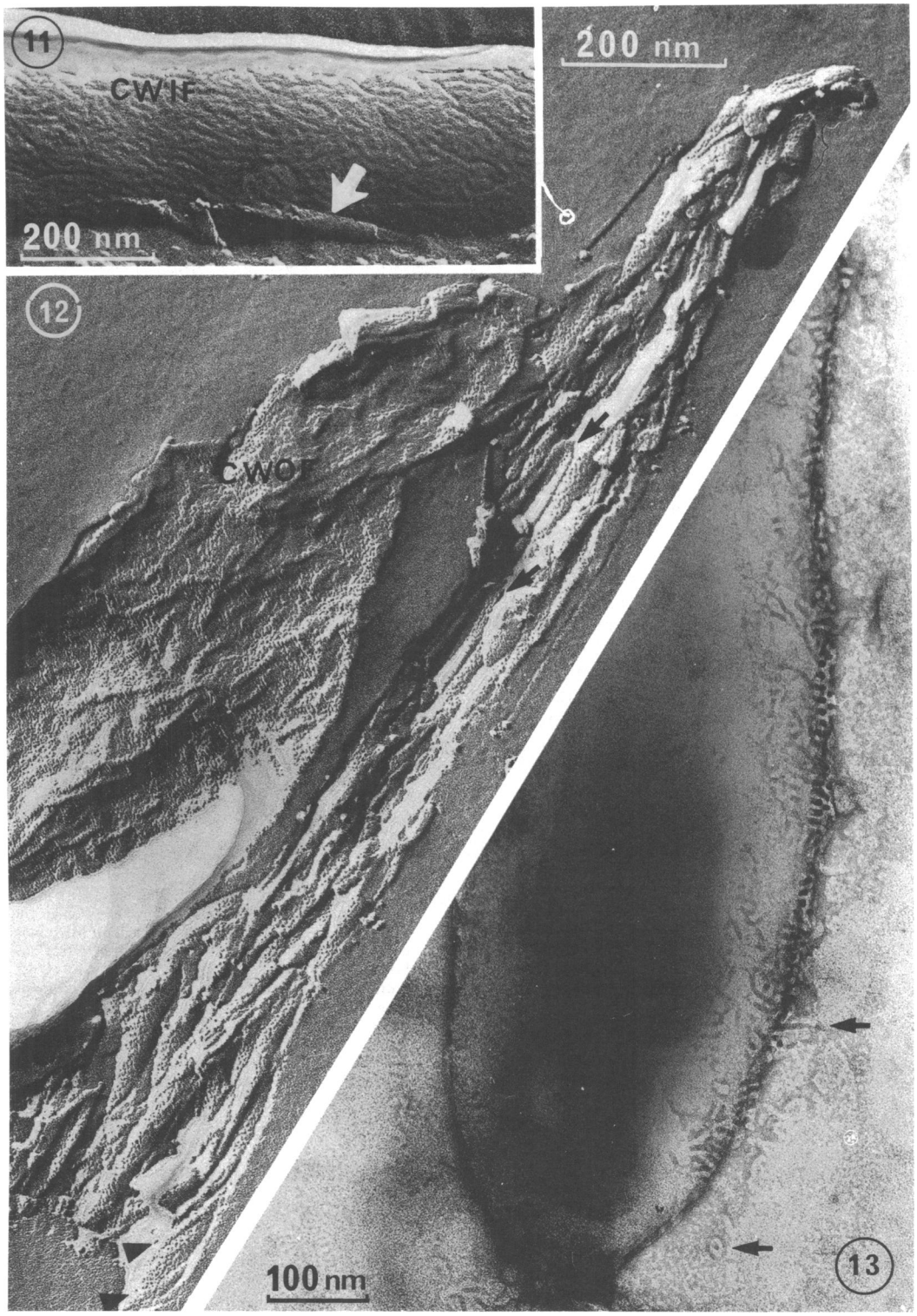
FIG. 9. *Inner fracture face (PF) of M. lepraemurium. The cleavage has exposed numerous intramembranous particles forming large clusters interspaced by smooth areas.*

FIG. 10. *Freeze-fracture and etching of M. lepraemurium. The etching has exposed the outermost surface of the microorganism (CWS), while a fortunate cleavage event has exposed the inner fracture face of the cytoplasmic membrane (PF) (arrow). This latter surface shows clusters and rows of intramembranous particles. The cleavage step across the cell wall required to expose the inner plasma membrane core is remarkably deep.*

FIG. 11. *Inner cell wall fracture face (CWIF) of M. lepraemurium. The surface is characterized by the presence of several linear depressions gently twisted around the bacterial body. Arrow points to ribbon-like projections exposed by the etching.*

FIG. 12. *Freeze-fracture of M. lepraemurium. Multiple segmented ridges are visualized on the cell wall concave fracture face (CWOF). The cleavage has exposed tubular structures twisted along each other (arrow heads). Tubular elements have also been fractured along their major axis (arrows).*

FIG. 13. *Negative staining of M. lepraemurium after sonication. The outermost surface components appear as fragmented tubular and ramified projections (arrows).*


11
CWIF
200 nm
200 nm
12
CWOT
100 nm
13

bacteria so far investigated (2, 14). The variations that have been detected are believed to correlate with the mycobacterium cell cycle. The accumulation both of peptidoglycolipids and mycosidic components would be an inherent feature of actively growing cells (2). One possible explanation for the fact that the cell wall features of *M. leprae* differ from those of *M. lepraemurium* could therefore be that the adaptive processes of mycobacterium phenotypes living in different environments lead to alterations in cell wall morphopoiesis.

It should also be recalled that all of the *M. leprae* used in the present investigation originated from lesions in patients receiving chemotherapy. A study is in progress on the *M. leprae* found in human lesions before and after treatment with antibiotics. Similar comparative investigations will also be carried out using *M. lepraemurium*.

On the other hand, we cannot rule out that the different surface ultrastructure of these two species of mycobacteria could rely upon the existence of species-specific variations among mycobacteria. Striking differences in the composition of the mycosidic integument of *Mycobacterium smegmatis* mutants compared with that of the wild type have been already found by Furuchi and Tokunaga (8) and by Goren et al. (12). To our knowledge these interesting data have not been supplemented with ultrastructural studies, which could be highly significant to a better interpretation of the results here reported.

ACKNOWLEDGMENTS

We extend our deep appreciation and thanks to François Gros, Director of Institut Pasteur, who helped make this work possible by his encouragement and criticism.

This work has been aided by the International Cell Research Organization and UNESCO. E.L.B. expresses his deep appreciation for the generous support received from the State Committee for Sciences and Techniques of the Republic of Viet Nam and from the Service de Coopération Culturelle et Technique du Ministère des Affaires Etrangères.

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