Ultrastructural Characterization of Normal and Damaged Membranes of *Mycobacterium leprae* and of Cultivable Mycobacteria

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Microdensitometry showed that the membrane profiles of normal cultivable mycobacteria were very asymmetric (outer layer denser and thicker than the inner layer), while the profiles of normal-looking *M. leprae* in lepromatous patients, in experimentally infected armadillos and in nude mice were approximately symmetric; moreover, the membrane of *M. leprae* was thicker than that of cultivable species. Using two cytochemical methods for the ultrastructural detection of periodic acid–Schiff (PAS)-positive molecules (the Thiéry procedure, and staining with phosphotungstic acid at low pH) we found that the membrane of cultivable mycobacteria, growing *in vitro* or *in vivo*, had PAS-positive components exclusively in the outer layer, while the normal-looking *M. leprae* in patients and in armadillos had membranes with PAS-positive components in both layers. The membranes of damaged cultivable mycobacteria, *in vivo* or *in vitro*, and of damaged *M. leprae*, in patients or armadillos, were PAS-negative.

INTRODUCTION

In previous publications (Silva & Macedo, 1982*a*, 1983*a*; Silva *et al.*, 1982*a*) we reported that the ultrastructural profile seen in lead-stained ultrathin sections of the membrane of ultrastructurally normal *M. leprae*, in patients with lepromatous leprosy (LL) and in experimentally infected armadillos, was symmetric. This pattern contrasted with that found in all normal Gram-positive bacteria, including acid-fast bacteria, studied so far (Silva, 1967, 1971, 1975, 1984; Silva & Macedo, 1982*a*, 1983*b*), and, consequently, represented a rather peculiar characteristic of the leprosy bacillus. We recently found that the symmetric membrane of normal-looking *M. leprae* had PAS-positive components in both layers, while the cultivable mycobacteria studied had such components only in the outer layer (Silva & Macedo, 1983*c*). In the present paper we describe the results of a more detailed study of the membranes of normal and degenerating *M. leprae* in comparison with the membranes of normal and degenerating cultivable mycobacteria, using cytochemical methods for the detection of PAS-positive molecules and microdensitometry. Some of these results have been presented in abstract form (Silva & Macedo, 1982*b*).

METHODS

Bacterial strains and growth conditions. For the study of *in vitro* cultures, the strains listed in Table 1 were grown in TB or 7H9 broth (Difco) to late-exponential phase, at 37 °C (except for *M. marinum*, which was incubated at 30 °C), with shaking twice a day. *Mycobacterium lepraemurium* was grown in Ogawa's egg yolk medium at 35 °C for 5 weeks (Portaels & Pattyn, 1981).

Mycobacterium lepraemurium (Douglas strain) was studied (*a*) in homogenates from rat lymph nodes (kindly supplied by Dr P. Lagrange, Institut Pasteur, Paris); and (*b*) after inoculation in the peritoneal cavity of mice

Abbreviations: LL, lepromatous leprosy; PAS, periodic acid–Schiff; PTA, phosphotungstic acid; MI, morphological index (Jopling, 1978); BI, bacteriological index (Jopling, 1978).

Table 1. *Cultivable Mycobacterium strains used*

<i>M. smegmatis</i> (ATCC 14468; IPP 141330001)	<i>M. marinum</i> (ATCC 927; IPP 140120001)
<i>M. aurum</i> (strain A ⁺ ; IPP 141210005)	<i>M. gordonae</i> (ATCC 14470; IPP 140210001)
<i>M. scrofulaceum</i> (strain HB1565; IPP 140220031)	<i>M. flavescens</i> (ATCC 14474; IPP 140230001)
<i>M. fortuitum</i> (strain HB1792; IPP 140410002)	<i>M. tuberculosis</i> (strain H37Rv; IPP 140010001)
<i>M. szulgai</i> (strain HB5728; IPP 140220031)	<i>M. tuberculosis</i> (strain H37Ra NCTC 7417; M6710, IMT)
<i>M. chelonae</i> subsp. <i>chelonae</i> (strain 810099; IPP 140420019)	<i>M. terrae</i> (strain W45; IPP 140320001)
<i>M. chelonae</i> subsp. <i>abscessus</i> (strain 810192; IPP 140420020)	<i>M. lepraemurium</i> (strain Douglas, Dr P. Lagrange, IPP)
<i>M. triviale</i> (strain T-254-3; IPP 140330002)	<i>M. lepraemurium</i> (strain 'cat leprosy' 406, IMT)
<i>M. simiae</i> (ATCC 25275; IPP 141020001 bis)	

IPP, Institut Pasteur, Paris; IMT, Institut de Médecine Tropicale, Antwerpen.

Table 2. *Samples from experimentally infected armadillos used*

Sample	Sample preparation	Origin	Armadillo	Supplied by
Liver	Homogenate	Dr G. P. Walsh*	no. 6	Dr F. Portaels, IMT
Liver	Homogenate	Dr Y. Robin†	AJ	
Liver	Homogenate	Dr C. C. Shepard‡	no. 31	
Liver	Homogenate	Dr R. J. W. Rees§	2457/10	
Liver	Isolated bacilli	Dr Y. Robin	AU	Dr H. L. David, IPP
Liver	Tissue fragments	Dr Y. Robin	AU	
Skin leproma	Tissue fragments	Dr Y. Robin	AU	

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(strain Charles River CD-1); samples of peritoneal exudate taken after 5 months contained many macrophages with mostly normal bacilli. *Mycobacterium tuberculosis* H37Rv was studied after intraperitoneal inoculation in mice (same strain as above); after 8 d the peritoneal exudate contained mostly normal bacilli inside macrophages. *Mycobacterium marinum* was inoculated in the hind foot pads of mice (same strain as above) where a self-limiting infection develops; multiplying bacilli are present during the initial phase of the infection (up to about 7 d) (Colston & Hilson, 1976).

Mycobacterium leprae cells were studied in the following. (a) Skin biopsies from 15 patients with lepromatous leprosy (Ridley & Jopling, 1964), including 7 without anti-leprosy treatment; this group of untreated patients includes the 4 patients already described in a previous publication (Silva *et al.*, 1982a). Patients nos. 2, 4, 5 and 7 were selected for detailed ultrastructural study because they had great numbers of acid-fast bacilli in skin smears (BI 5+ or 6+) and a significant proportion of the bacilli stained solidly by the Ziehl-Neelsen technique (MI between 13% and 37%). (b) Biopsies from experimentally infected armadillos (Table 2). (c) Homogenates of nude mouse foot pads (kindly supplied by Dr Françoise Portaels, Institut de Médecine Tropicale, Antwerpen, Belgium).

Study of damaged membranes of cultivable mycobacteria. *Mycobacterium tuberculosis* H37Rv, *M. marinum* and *M. aurum* cultures in TB broth (Difco) were incubated long enough to enter the decline phase during which increasing numbers of autolysing cells occur. Exponentially growing *M. tuberculosis* H37Ra in TB broth (Difco) was exposed to rifampin (10 µg ml⁻¹) or to sodium azide (75 mM) and samples were taken at intervals. *Mycobacterium tuberculosis* H37Ra, grown exponentially as above, was broken by sonication in a Branson sonifier model W185D operated at 100 W; the samples were kept in ice during sonication cycles.

Degenerating *M. tuberculosis* H37Ra and *M. marinum*, in mouse peritoneal macrophages and in mouse foot pads, respectively, were obtained from samples collected in the regressive phase of the infection; lysing bacilli were largely predominant 4 d after inoculation with *M. tuberculosis* H37Ra and 9 d after inoculation with *M. marinum*.

Electron microscopy. Bacteria from broth cultures were collected by centrifugation (2500 g for 10 min). *Mycobacterium lepraemurium* was scraped from the surface of Ogawa's medium. Macrophages with phagocytosed mycobacteria were collected from the peritoneal cavity using previously described methods (Cohn & Benson, 1965). *Mycobacterium marinum* in the mouse foot pads were taken by scraping the purulent exudate underneath the

skin. Skin biopsies from LL patients were fixed immediately after being taken. Samples from experimentally infected armadillos were either kept at -70°C until fixation or fixed immediately. Homogenates of nude mouse foot pads were kept at -70°C until fixation.

All samples were fixed with 4% (w/v) formaldehyde/1.25% (w/v) glutaraldehyde/10 mM- Ca^{2+} , followed by 1% (w/v) OsO_4 /10 mM- Ca^{2+} , followed by 0.5% (w/v) uranyl acetate in veronal/acetate buffer or in water (Silva & Macedo, 1982a). After dehydration in ethanol, the samples were embedded in Epon (Luft, 1961). Ultrathin sections were stained with lead citrate (Venable & Coggeshall, 1965) for 5 min or stained for PAS-positive molecules. For this purpose, we used two different procedures. (a) The Thiéry technique (Thiéry, 1967; Thiéry & Rambourg, 1974) done as follows: 1% (w/v) periodic acid, 30 min; 1% (w/v) thiosemicarbazide, 60 min; 1% (w/v) silver proteinate or 1% (w/v) silver vitelinate (Silva *et al.*, 1982b), 30 min. The washings were done as described by Thiéry (1967). In all experiments duplicate grids were pretreated with 3% (w/v) hydrogen peroxide before the Thiéry procedure to ensure complete removal of bound osmium (Robertson *et al.*, 1975). Control tests were performed by treating sections with 0.1% (w/v) sodium borohydride (Robertson *et al.*, 1975) between periodic acid oxidation and thiosemicarbazide treatment and by omitting periodic acid or thiosemicarbazide treatments. (b) PTA at low pH (Rambourg, 1971; Rosseau & Hermier, 1975) done as follows: 1% (w/v) periodic acid, 15 min, followed by 1% (w/v) phosphotungstic acid in 1 M-HCl, 15 min, without subsequent wash; as control, sections were treated with 1% (w/v) phosphotungstic acid at pH 7.0. Observations were made with Siemens electron microscopes (Elmiskop IA and 102).

Tracings of membrane profiles were made on photographic negatives of lead-stained sections with a Joyce-Loebl MK III CS microdensitometer, set to an arm ratio of 50 $\times$ and a slit width of 0.5 nm. The microscopes were calibrated with a grating grid.

Definition of normal and altered mycobacterial cells. One of the essential aspects of the present work deals with the comparison of the membrane ultrastructure in normal and damaged mycobacteria. The ultrastructural criteria, as seen in lead-stained sections, used to label the bacilli as normal or degenerating were the following, as previously discussed (Silva & Macedo, 1983b): normal mycobacterial cells have continuous cell wall and cytoplasmic membrane, distinct ribosomes and fibrillar nucleoids (see Fig. 1 in Silva & Macedo, 1983a; Fig. 1 in Silva & Macedo, 1983b); degenerating cells are characterized by an intracellular compartment without ribosomes, with blocks of compact, homogeneous material, which is progressively degraded as cell lysis proceeds, and with dispersed DNA fibrils; this compartment is enveloped by a membrane that may exhibit partial solubilization (at the final stages of lysis it is completely solubilized); in cells with advanced degenerative alterations, the cell wall is distorted or discontinuous (see Figs 2 and 3 in Silva & Macedo, 1983b; Figs 4a, 5a, 5b in Silva *et al.*, 1982a). The characterization of mycobacterial cells as normal or degenerating in sections stained by the Thiéry or PTA procedures is more difficult because less morphological detail is visible; however, the distinction is still possible in most cases by using some of the criteria indicated for lead-stained sections, namely: continuity of membranes and cell wall and compactness of cytoplasm. Moreover, the proportion of normal versus damaged bacilli in lead-stained preparations is a useful indication of the proportion of such bacillary forms in sections stained for PAS-positive molecules in the same samples.

RESULTS

Densitometry of mycobacterial membrane profiles

In order to get quantitative information on mycobacterial membrane geometry we compared the densitometric tracings of membrane profiles in lead-stained sections of normal and degenerating bacilli. The results (Table 3, Fig. 1) showed that: (a) all normal-looking cells of the cultivable species studied (Table 1) had membranes with very asymmetric profiles, both *in vitro* and *in vivo*; (b) normal-looking *M. leprae* cells, in armadillos, nude mice or patients had membranes with approximately symmetric profiles, with peak-to-peak distances higher than those of the cultivable species; (c) lysing cells of the cultivable species, both *in vitro* and *in vivo*, had approximately symmetric profiles.

Ultrastructural detection of PAS-positive components in normal mycobacterial membranes

Since the profile of the membrane of normal-looking *M. leprae* was symmetric because of the increase in density and thickness of its inner layer compared to that of the cultivable species (Fig. 1) the *M. leprae* membrane might have some additional components in its inner layer. The observation by Petitprez & Derieux (1970) that the cultivable *M. phlei* has membranes with an asymmetric distribution of PAS-positive components, revealed by the Thiéry procedure, which parallels the asymmetry seen in lead-stained sections, prompted us to study the membranes of

Table 3. *Peak-to-peak distances from microdensitometric tracings of mycobacterial membranes in lead-stained Epon sections*

The samples were fixed as described in Methods. Statistical analysis (Student's *t* test) of the values for the peak-to-peak distances in the strains studied showed that: (a) the difference between the thickness of the membrane of *M. leprae* and those of all the cultivable species is highly significant ($P < 0.001$); (b) there is no significant difference in membrane thickness between the cultivable species ($P > 0.05$).

Bacterium	No. of measurements	Peak-to-peak distance and SD (nm)
<i>M. leprae</i> *		
(in LL patients and armadillos)	90	7.05 $\pm$ 0.50
<i>M. tuberculosis</i> H37Ra		
<i>in vitro</i> (TB broth)*	13	6.36 $\pm$ 0.22
<i>in vivo</i> (mouse macrophages)	12	6.48 $\pm$ 0.45
<i>M. lepraemurium</i>		
<i>in vivo</i> (mouse macrophages)	33	6.45 $\pm$ 0.23
<i>M. aurum</i>		
<i>in vitro</i> (TB broth)*	16	6.37 $\pm$ 0.37
Average values for the cultivable species	74	6.42 $\pm$ 0.31

* Values taken from Silva & Macedo (1983c).

Fig. 1. Microdensitometric tracings of mycobacterium membranes. The peaks corresponding to the outer layer are on the right side of each tracing. (a) Normal *M. tuberculosis* H37Ra, *in vitro* (TB broth); (b) normal *M. tuberculosis* H37Ra, *in vivo* (mouse macrophage); (c) normal *M. lepraemurium* (Douglas strain), *in vivo* (mouse macrophage); (d) lysed *M. lepraemurium* (Douglas strain), *in vivo* (mouse macrophage); (e) normal *M. leprae*, in patient no. 2; (f) lysed *M. leprae*, in patient no. 2.

normal mycobacteria by the same procedure. We found that both layers of the membrane of normal-looking *M. leprae* in patients and in armadillos (Silva & Macedo, 1983c) stained strongly, irrespective of pretreatment with hydrogen peroxide (Fig. 2a, b), while only the outer layer of the membrane was stained in all the cultivable strains studied, namely *M. tuberculosis* H37Rv and H37Ra, *M. marinum*, *M. lepraemurium*, *M. aurum*, *M. terrae*, *M. smegmatis*, grown *in vitro* (Silva & Macedo, 1983c) (Figs 3a, 3c, 5b, 5c) and *M. chelonae* subsp. *chelonae*, *M. szulgai*, *M. simiae*, *M. fortuitum* and *M. flavescens*, grown *in vitro*.

A similar staining pattern was observed with the PTA technique (Figs 2c, 3b), although the interpretation of the results with that method is sometimes more difficult than with the Thiéry procedure, because the PTA staining is more diffuse; moreover, since this technique stains the cell wall, the membrane may be difficult to resolve whenever it is closely apposed to the wall (Fig. 2c).

We considered the possibility that the symmetric profile of *M. leprae* membrane might be due to the particular environment in which the bacilli grow, that is, inside macrophages of the naturally or experimentally infected hosts. We therefore studied the membrane profiles of *M. tuberculosis* H37Ra and H37Rv and *M. lepraemurium* multiplying inside mouse peritoneal macrophages and those of *M. marinum* in mouse foot pads. Again, we found in bacilli with normal ultrastructure an asymmetric staining of the membrane by the Thiéry and PTA procedures (Fig. 4a-d).

Fig. 2. *Mycobacterium leprae* cells stained by the Thiéry procedure, showing PAS-positive symmetric staining of membrane profiles. (a) Skin biopsy from patient no. 7. (b) Same sample as in (a), but section treated with hydrogen peroxide before use of the Thiéry procedure. (c) Same sample as above, stained by PTA at low pH; note that the wall is densely contrasted and that the symmetric staining of the membrane is visible only in the part of the membrane that is separated from the wall. In this and in all other figures the bar markers represent 100 nm.

We found that the positive Thiéry staining exhibited by the membranes of the mycobacteria studied, including *M. leprae* and the cultivable species, had the following characteristics: (a) it was abolished by the reduction of aldehydes with sodium borohydride; (b) it was observed in sections pretreated with hydrogen peroxide (Fig. 2b); (c) it was not observed when the periodic acid or the thiosemicarbazide treatments were omitted. These results indicated that the silver staining was probably revealing polysaccharide residues (Robertson *et al.*, 1975; Thiéry, 1967; Thiéry & Rambourg, 1974). PTA at pH 7.0 did not stain the membranes of the mycobacteria studied. Although the mechanism of PTA staining at low pH is not well established, it had been proposed that such staining reveals polysaccharides (Fléchon & Huneau, 1974; Rambourg, 1971; Roland, 1974).

Fig. 3. Ultrastructural staining for PAS-positive molecules of *in vitro* grown mycobacteria. (a) *M. terrae* stained by the Thiéry procedure; (b) same sample stained by PTA at low pH; (c) *M. lepraemurium* stained by the Thiéry procedure. PI, PAS-positive inclusions; M, membrane with the outer layer preferentially stained; W, cell wall.

Fig. 4. Asymmetric Thiéry staining of the membrane of *in vivo* grown *M. tuberculosis* H37Rv (a), *M. tuberculosis* H37Ra (b), *M. lepraemurium* (Douglas strain) (c), and *M. marinum* (d). PI, PAS-positive inclusions.

Fig. 5. *In vitro* autolysing mycobacterial cells. (a) *M. tuberculosis* H37Rv, section stained with lead; the membrane is symmetric. (b) Same sample stained by the Thiéry procedure; the cell on the left is normal and has a membrane with the outer layer Thiéry positive; on the right side the membrane remnants of a lysed cell are Thiéry-negative. (c) *M. marinum*, section stained by the Thiéry procedure; the cell at the top is normal and its membrane has a Thiéry-positive outer layer; the lower two cells are lysed and their membranes (M) are Thiéry-negative. (d) *M. aurum* under advanced lysis; the membrane (M) is Thiéry-negative. W, cell wall; PI, PAS-positive inclusions.

A systematic survey of samples from the 15 LL patients, including 7 not subjected to anti-leprosy treatment, never showed bacilli with an asymmetric staining of membranes. Careful study of samples from armadillos experimentally infected with *M. leprae* showed Thiéry-symmetric membranes, except for a few bacilli in the liver of armadillo AU (Table 2) which contained Thiéry-asymmetric membranes.

Fig. 6. Cultivable mycobacterial cells damaged by different treatments. (a, b) *M. tuberculosis* H37Ra, broken by sonication; the membrane (M) is approximately symmetric after lead staining (a) and Thiéry-negative (b). (c, d) *M. tuberculosis* H37Ra exposed to 75 mM-sodium azide in TB broth for 4 d; the membrane (M) is symmetric after lead staining (c) and Thiéry-negative (d). PI, PAS-positive inclusions; W, cell wall.

Ultrastructure and cytochemistry of damaged mycobacterial membranes

In lead-stained sections, the membranes of cultivable species undergoing degenerative processes, either *in vitro* or *in vivo*, or subjected to membrane-damaging treatments, as indicated in Methods, exhibited symmetric profiles (Figs 5a, 6a, 6c), as briefly reported elsewhere (Silva & Macedo, 1983b). Such symmetric membranes were Thiéry-negative (Figs 5b–d, 6b, 6d, 7a, 7b), indicating that the PAS-positive residues, which in normal membranes are present in the outer layer, had been lost or modified.

Mycobacterium leprae cells, in patients or in armadillos, with ultrastructural signs of advanced degenerative alterations also had Thiéry-negative, symmetric membranes (Fig. 7c, d).

DISCUSSION

As previously discussed (Silva, 1971, 1975, 1984; Silva & Macedo, 1982a, 1983a, b), in order to define the membrane ultrastructural profile of Gram-positive bacteria, including mycobacteria, it is essential to consider two aspects: (a) the fixation conditions used to prepare the samples for electron microscopy, because such conditions markedly influence the micromorphological pattern of membranous structures, and (b) the condition of the bacterial cells, because in properly fixed samples normal and altered membranes have different profiles. Regarding point (a), we have investigated the influence of several fixation parameters on the final electron microscopic image of bacterial membranes and have developed a fixation procedure that, on the basis of morphological, chemical and functional grounds, we consider to preserve bacterial membranes satisfactorily (Silva & Macedo, 1982a, 1983b); such a fixation procedure was used in the present study. Regarding point (b), we had observed that the membranes of all normal Gram-positive bacteria studied, including members of the genera *Bacillus*, *Streptococcus*, *Micrococcus*, *Sarcina*, *Sporosarcina*, *Listeria*, *Streptomyces*, *Nocardia* and cultivable *Mycobacterium*, when fixed according to the conditions previously found to be

Fig. 7. Thiéry staining of mycobacterial cells under lysis in infected hosts. (a) *M. marinum* in mouse foot pads after 15 d infection; the membrane (M) is Thiéry-negative. (b) *M. tuberculosis* H37Ra in mouse peritoneal macrophages after 8 d infection; the membrane is Thiéry-negative. (c, d) *M. leprae* in a skin biopsy from patient no. 2; the bacilli are under advanced lysis and the membranes (M) are Thiéry-negative. PI, PAS-positive inclusions.

appropriate, had an asymmetric profile, with the outer layer (facing the cell wall) thicker and more electron-dense than the inner layer (facing the cytoplasm); we further found that such a profile changed to a symmetric one when the membrane was affected by diverse situations like bacteriolysis (either autolysis or heterolysis), or treatments with membrane-active agents (Silva,

1967, 1975, 1984; Silva & Macedo, 1982*a*, 1983*b*; Silva & Sousa, 1972; Silva *et al.*, 1976, 1978, 1979*a, b*). In other words, in properly fixed Gram-positive bacteria, the presence of symmetric membranes in lead-stained sections indicates damaged membranes.

Consequently, our observation that ultrastructurally normal *M. leprae* in patients (Silva & Macedo, 1982*a*; Silva *et al.*, 1982*a*) and in experimentally infected armadillos (Silva & Macedo, 1983*a*) had membranes with symmetric profiles in lead-stained sections, a result now confirmed and extended to the bacilli in nude mice, is quite surprising. The results presented in this paper make it necessary to re-evaluate our previous interpretation that the membrane of normal *M. leprae* is symmetric. First, the results of the Thiéry and PTA staining of mycobacterial membranes have to be considered. Although we do not know, at present, which molecules are responsible for the positive reaction observed with those procedures in the outer layer of all the cultivable species studied, it is likely that they could be equivalents of the surface amphiphiles found in the outer layer of the cytoplasmic membranes of Gram-positive bacteria (Wicken & Knox, 1980). Such amphiphiles have sugar residues in the hydrophilic portion of the molecule (Wicken & Knox, 1980) and it is possible that those carbohydrates are responsible for the Thiéry and PTA staining of the outer layer of the cytoplasmic membrane of all Gram-positive bacteria studied up to now: *Mycobacterium* (Petitprez & Derieux, 1970; Silva, 1984; Silva & Macedo, 1983*c*; the present work), *Nocardia* (Silva, 1984), *Streptomyces* (Silva, 1984), *Bacillus* (Rosseau & Hermier, 1975; Silva, 1984) and *Micrococcus* (Silva, 1984; Silva *et al.*, 1977). Consequently, our result showing that the membrane of *M. leprae* that looks ultrastructurally normal has PAS-positive components in both layers is striking, because it represents the only exception to the absolute asymmetry of PAS-positive molecules in bacterial surface membranes. Therefore we have now to consider alternative interpretations for the symmetric profile of *M. leprae* membrane. The possibility that the symmetric membranes we have seen in all normal-looking leprosy bacilli represent altered membranes deserves consideration because, as discussed above, damaged membranes of Gram-positive bacteria, including cultivable acid-fast bacteria, exhibit symmetric profiles. However, Thiéry staining showed that the symmetric membranes of affected cultivable mycobacteria were Thiéry-negative, and the symmetric membranes of normal-looking *M. leprae* were Thiéry-positive. Moreover, *M. leprae* cells with ultrastructural signs of bacteriolysis had Thiéry-negative membranes. Therefore, if the symmetric, Thiéry-positive membranes we have found in all *M. leprae* cells with the ultrastructural characteristics of normal mycobacteria were not the normal membranes of the leprosy bacillus, then they would represent a peculiar form of abnormal membrane not found in the cultivable species. Furthermore, if this were the case it would indicate that all the intact *M. leprae* we found by electron microscopy were abnormal. This would be a surprising conclusion mainly in the case of the untreated patients with high BI and MI. The finding that the liver of armadillo AU contained a few bacilli with Thiéry-asymmetric membranes can be explained by the recent observation by Portaels & Pattyn (1982), who isolated unidentified cultivable mycobacteria from the livers of some experimentally infected armadillos.

At present it is difficult to decide whether the symmetric PAS-positive membranes seen in *M. leprae* cells with ultrastructural characteristics of normal mycobacteria represent normal or altered membranes, since both alternatives raise puzzling questions. This adds new facets to the peculiarity of the leprosy bacillus and implies that more work is necessary to clarify that point. Our results indicate, however, that there are some interesting differences in the ultrastructure of the membranes of the two most important human pathogenic mycobacteria: *M. tuberculosis* and *M. leprae*. To what extent such differences relate to physiology and pathology remains to be elucidated.

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