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Electron Microscopic Study of *Mycobacterium leprae* Membrane[1]

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In recent publications ([11,12]) we presented preliminary results pointing to the conclusion that the membrane of normal *Mycobacterium leprae* cells present in the skin of lepromatous (LL) patients appears to be ultrastructurally different from that of all other Gram-positives (including acid-fasts) studied so far. The difference, not previously recognized by other workers studying the micromorphology of *Mycobacterium* cells, lies in the profile of the membrane as seen with the transmission electron microscope in lead-stained, ultrathin sections of chemically fixed, plastic-embedded samples. Such a profile was found to be asymmetric (with the outer layer thicker and denser than the inner layer) in several acid-fast ([11]) and non-acid-fast ([7,8,9]) Gram-positive bacteria grown *in vitro*, while it is symmetric in the case of *M. leprae* ([11,12]).

Since the electron microscopic profile of biological membranes reflects their native architecture in terms of distribution of chemical components between the two halves of the membrane bilayer (see refs. 9, 13, 17 for discussion of this point), the reported difference in membrane profile between cultivable mycobacteria and *M. leprae*, if confirmed, would have interesting implications.

In the present communication we present new data obtained by the study at high magnification of ultrathin sections of *M. leprae* cells in experimentally infected armadillo tissues which confirm our previous results.

MATERIALS AND METHODS

We studied *M. leprae* cells *in situ* in liver and skin lepromas of experimentally infected armadillos. The infected tissues were stored frozen until processed for electron microscopy. *M. leprae* cells isolated from the liver of infected armadillos were also used. Both the infected tissues and the isolated bacilli were kindly supplied by Dr. Hugo L. David (Institut Pasteur, Paris, France). *M. tuberculosis* (strain H37Ra from the Institut Pasteur) and *M. aurum* (strain A^+ from the Institut Pasteur) were grown in TB Broth (Difco Laboratories, Detroit, Michigan, U.S.A.) supplemented with 5% glycerol, at 37°C, with occasional shaking (twice a day) for 6 days and 2 days, respectively. The bacteria in the cultures or in the suspensions of isolated *M. leprae* were collected by centrifugation (2000 × *g*, 10 min).

Several fixation schedules were tested in the present study. In all cases large amounts of fixatives were used to ensure that an excess of fixative was always present. In the case of liver tissue, we fixed duplicate samples: in addition to small fragments (not more than 1 mm^3), we used tissue fragments rapidly homogenized in the fixative to facilitate the access of the fixative components to the bacteria. For isolated *M. leprae* and for *M. tuberculosis* and *M. aurum* cultures, the pelleted bacteria were quickly mixed with the fixative by vortexing. The following procedures were used for the fixation: a) Formaldehyde(4%)+glutaraldehyde(1.25%)+ Ca^{++}(10 mM) followed by 1% OsO_4 + Ca^{++}, followed by uranyl acetate (0.5–1.0%), as described ([11,12]). b) The same as in a), but with 10% or 20% dimethyl sulfoxide (DMSO) in the aldehyde mixture. c) One percent OsO_4 supplemented with 10 mM Ca^{++} ([5]). d) As in c), but OsO_4 supplemented with 30 mM Mg^{++}. e) As in c), but OsO_4 supplemented with 10 mM uranyl acetate. Fixations a) to e) were followed by a postfixation with 1.0% uranyl acetate in water. Fixation in the aldehyde mixture was carried out for 24–48 hr in the refrigerator or at room temperature. OsO_4 fixatives were used for 16–24 hr at room temperature. Uranyl postfixation was for 30–60 min at

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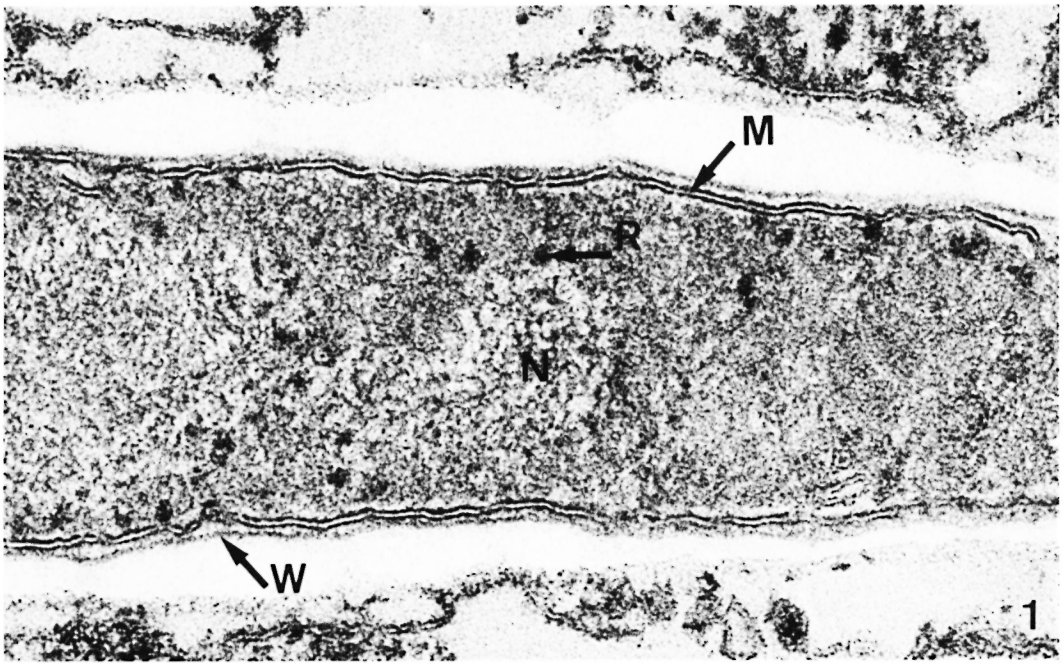

FIG. 1. Normal *M. leprae* in armadillo liver. Fixation with formaldehyde+glutaraldehyde+Ca^{++}-OsO_4+Ca^{++}-uranyl acetate (procedure a). Section stained with uranyl and lead. Notice the symmetric membrane (M) (×146,000).

W = dense layer of cell wall N = nucleoid R = ribosome

room temperature. A wash with cacodylate buffer (50 mM, pH 7.0, supplemented with 10 mM Ca^{++}) was done between aldehyde fixations and OsO_4. The fixed samples were dehydrated in ethanol and embedded in Epon ([3]) using only mixture B. Ultrathin sections were cut with an LKB Ultratome III, stained with lead citrate ([19]) or with uranyl acetate ([14]) and lead citrate, and observed with Siemens Elmiskopes (I A and 102).

RESULTS

Our observations show that the ultrastructural characteristics of *M. leprae* in experimentally infected armadillos are identical to those exhibited by *M. leprae* in LL patients. Figure 1 is a representative example of normal *M. leprae* in armadillo liver fixed by procedure a) which was extensively used in our previous study of skin biopsies from leprosy patients ([11,12]). Figure 2 shows the micromorphological aspect at high magnification of the membrane of *M. leprae* in armadillo liver after several fixations. In all cases symmetric membranes are present in bacilli with ultrastructural characteristics of normal mycobacteria (see Discussion). Bacteria with the same micromorphological characteristics as those shown in Figures 1 and 2 were present in the skin lepromas of infected armadillos and in the suspensions of isolated *M. leprae*.

Normal *M. tuberculosis* and *M. aurum* cells fixed by the procedures indicated in the Materials and Methods section exhibited asymmetric membranes identical to those shown in our previous report (see Figs. 2 and 3 in ref. 11).

DISCUSSION

An essential aspect to be considered when studying bacterial membranes by transmission electron microscopy is that particular requirements must be fulfilled by the fixation procedure in order to achieve good preservation of those structures ([9,14,15,16]). This is particularly important in the present situation: We found symmetric membranes in *M. leprae* cells and symmetric membranes may result from the inadequate fixation of membranes which, otherwise, will exhibit asymmetric profiles ([8,9,11]). In other

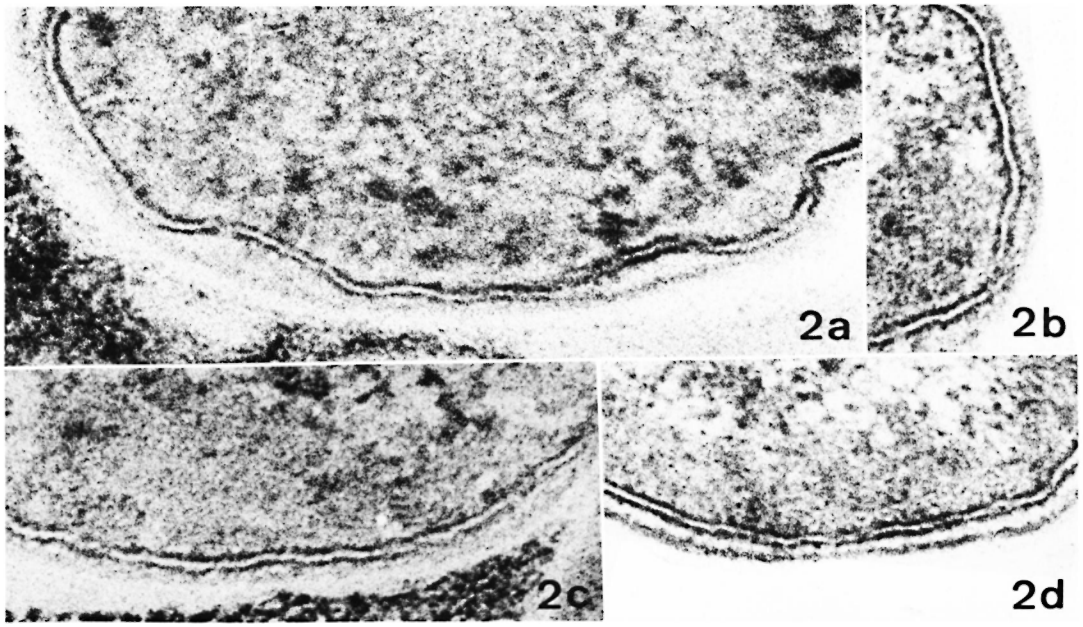

FIG. 2. High magnification (×226,000) of the membrane of normal *M. leprae* in armadillo liver. Sections stained with lead.

a = fixation as in Figure 1.

b = fixation with OsO_4+ uranyl acetate (procedure e), sample homogenized in the fixative.

c = as in 2b, but liver fragment fixed without previous homogenization.

d = fixation with OsO_4+Mg^{++} (procedure d).

Notice the symmetric profiles in all membranes.

words, to correctly characterize the ultrastructural pattern of *M. leprae* membranes we have to be sure that the fixation is adequate. The fixation conditions used in our preliminary study on the ultrastructure of *M. leprae* ([11,12]) took into consideration several aspects already discussed as essential for good preservation of bacterial membranes ([8,9,16]). We considered, however, that *M. leprae* might have special requirements regarding the fixation of their membranes in comparison with the more "conventional" mycobacteria. We therefore tested additional fixation methods in *M. leprae* in experimentally infected armadillo tissues in comparison with the procedures used in our previous study. The methods now used were based on relevant aspects concerning fixation and stabilization of bacterial membranes not included in the other study. The use of Mg^{++} at a relatively high concentration as a supplement for the OsO_4 fixative was based on the observations that this cation can replace Ca^{++} in its stabilizing action during the fixation of bacterial cells ([4]). Also Mg^{++} but not Ca^{++} was found to stabilize labile components of the membranes of Gram-positives like lipoteichoic acids ([1]). Uranyl ions were found to have a strong, membrane-stabilizing activity ([9,10,14,16]) and to preserve well bacterial membranes when used as a primary fixative ([16]). The inclusion of DMSO in the aldehyde fixative was prompted by the observation that it increases the penetration rate of the fixative components ([6]). As described in the Results section, all procedures now tested preserved the membrane of *M. leprae* in armadillo tissues as a symmetric structure, while the same methods revealed asymmetric membranes in *M. tuberculosis* and *M. aurum*.

Another important aspect has to be considered in the present discussion. To confirm that the membrane of normal *M. leprae* cells is symmetric, we have to be sure that we are, indeed, looking at normal bacilli. This is a crucial point since as previously shown ([7,9,11]) the membranes of lysing Gram-positives, including acid-fasts, are symmetric even when preserved by the fix-

ation procedures known to correctly fix bacterial membranes and to reveal asymmetric membranes in normal bacteria. In other words, we have to prove that the *M. leprae* cells which we label as normal are, in fact, normal and not lysing bacilli. This is important because it is known ([2,12]) that hosts infected with *M. leprae,* even when not treated with antileprosy drugs, have a bacterial population made up of varying proportions of normal and degenerating bacilli. To get the confirmation that we were seeing symmetric membranes in normal *M. leprae* cells we took into consideration the ultrastructural aspect of the whole mycobacterial cell, that is, we classify as normal the bacilli which have continuous cell wall and membranes, distinct ribosomes, and fibrilar DNA (Fig. 1; see also Figs. 4b and 5 in ref. 11 and Figs. 2a, b, c in ref. 12). We also compared the data obtained with the electron microscope with that given by light microscopy. Although it is not entirely safe to assume that all solidly staining (Ziehl-Neelsen) bacilli seen in skin smears represent bacteria which were alive in the samples, as is usually admitted ([2]), the fact that no *M. leprae* cells with asymmetric membranes have been found in dozens of electronmicrographs of bacilli in samples from all eight patients with LL leprosy with a high Morphological Index (MI) and solid, fragmented, and granular (SFG) index we have studied until now, is a significant support to our contention that the characteristic membrane profile of normal *M. leprae* is a symmetric one.

In conclusion, this work shows that the ultrastructural characteristics of *M. leprae* in experimentally infected armadillos are identical to those described for *M. leprae* in LL patients, exhibiting a pattern that, with the exception of the membrane profile, is typical of *Mycobacterium* cells. The micromorphological aspect of the *M. leprae* membrane found in both studies is identical and is characterized by a symmetric, triple-layered profile. Moreover, in the present study we tested additional fixation procedures which used conditions known to be efficient in the preservation of bacterial membrane components. Consequently, our present results strengthen the previous conclusion that the membrane of *M. leprae* is peculiar in having a profile not shared by any of the Gram-positives studied so far, including the cultivable acid-fasts *M. tuberculosis* ([11]), *M. aurum* ([11]), *M. phlei* ([8,11]), and *Nocardia asteroides* ([8,9,11]). Work in progress has shown that the membrane profiles of another 11 species of easily cultivable mycobacteria are also asymmetric (M. T. Silva and Paula M. Macedo, in preparation).

In the published reports from other laboratories on the ultrastructure of *M. leprae,* no reference is given about its membrane profile although in the pictures that show clear profiles these appear symmetric. We can advance at least two reasons for the fact that such a peculiar profile of *M. leprae* membrane has not been recognized before. First are technical aspects, either in the fixation, sectioning, or microscopy steps, which in several cases have prevented obtaining high resolution or high magnification images. Secondly, many electron microscopists working with bacteria are not aware of the significance of the membrane profiles and of the dependence of these profiles on the fixation conditions.

Since the electron microscopic profile of biomembranes is the morphological counterpart of the molecular architecture of the membrane components ([9,13,17]), the reported difference in the membrane profile between *M. leprae* and the other mycobacteria most probably results from not yet elucidated differences in the molecular organization of the respective membranes. This point is under current study in our laboratory and some data has been obtained indicating the participation of polysaccharide residues in the unique membrane pattern of *M. leprae* (M. T. Silva and Paula M. Macedo, in preparation). Whether such a peculiar characteristic of *M. leprae* has something to do with its noncultivability remains to be elucidated. A comparative ultrastructural study between *M. leprae* and *M. lepraemurium,* a related species that only recently could be cultivated in bacteriological media, is pertinent and is being carried out in our laboratory.

SUMMARY

We report the results of the study by transmission electron microscopy of normal *Mycobacterium leprae* in the tissues of experimentally infected armadillos. Several fixation procedures were used and com-

pared to those previously employed in the study of *M. leprae* in lepromatous leprosy patients. The results show that the ultrastructure of *M. leprae* is identical in both hosts. The demonstration of a symmetric membrane profile in *M. leprae* in armadillos confirms our previous results. This characteristic of the *M. leprae* membrane is peculiar in that it is not shared by any of the easily cultivable species of mycobacteria we have studied so far.

RESUMEN

Presentamos los resultados de un estudio por microscopía electrónica de transmisión del *Mycobacterium leprae* encontrado en los tejidos de armadillos infectados experimentalmente. Se usaron varios procedimientos de fijación y se compararon con los usados previamente en el estudio del *Mycobacterium leprae* de pacientes con lepra lepromatosa. Los resultados muestran que la ultraestructura del *M. leprae* as idéntica en ambos huéspedes. La demostración de un perfil membranal simétrico en el *M. leprae* de armadillos confirma nuestros resultados previos. Esta característica de la membrana del *M. leprae* es peculiar en el sentido de que no está presente en ninguna otra de las especies micobacterianas facilmente cultivables estudiadas hasta ahora.

RÉSUMÉ

On relate les résultats d'une étude par microscopie électronique de *Mycobacterium leprae* normaux dans les tissus de tatous infectés expérimentalement. Plusieurs procédés de fixation ont été utilisés; ils sont comparés à ceux qui ont été antérieurement employés pour l'étude. *M. leprae* chez des malades souffrant de lèpre lépromateuse. Les résultats montrent que l'ultrastructure de *M. leprae* est identique chez les deux hôtes. La démonstration d'un profil de membrane symétrique chez *M. leprae* de tatous confirme les résultats précédents. Cette caractéristique de la membrane de *M. leprae* est particulière car on ne la retrouve dans aucune espèce de mycobactéries facilement cultivables qui ont été étudiées jusqu'à présent.

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