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Detection of Mycobacterial Lipids in Skin Biopsies from Leprosy Patients[1]

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A high lipid content and the presence of a variety of unusual lipids are characteristic features of mycobacteria. Various mycobacterial lipid fractions have been shown to possess activity as immunological adjuvants, to act as antigens, to exert toxic effects on mammalian cells, and to regulate the surface properties of the bacteria ([2, 21]). The mechanisms involved in synthesis of some of these lipids (particularly mycolic acids) may be important sites of action for anti-mycobacterial drugs ([28, 30]).

Almost all of our knowledge of mycobacterial lipids is, however, based on experiments involving cultures grown in cell free media and it is not always clear to what extent properties observed *in vitro* are modified when mycobacteria are growing as intracellular parasites *in vivo* ([14]). Attempts to study properties of tissue-grown mycobacteria ([5, 6, 7, 8, 9, 12, 16, 19, 25]) are of particular importance with regard to *Mycobacterium leprae*, for which accepted *in vitro* grown conditions have not been established ([23]).

In this paper the distinctive features of various mycobacterial lipids have been exploited in order to permit their detection even in the presence of large amounts of host tissue lipids. The lipids extracted from skin biopsies of patients with lepromatous leprosy have been compared with those from normal skin and also from a sample of *M. leprae* purified from infected armadillo spleen. Several lipids were found in infected skin which were absent from normal skin but corresponded to lipids present in the armadillo *M. leprae*. Comparison of the *M. leprae* lipids with those present in several other mycobacteria indicated some interesting distinguishing features of the leprosy bacillus.

Sakurai and Skinsnes ([24]) have previously reported differences in the lipid content of leprosy-infected and normal skin biopsies. These authors attributed the increased polar lipid content in lepromatous skin samples to the presence of *M. leprae* lipids but did not identify any mycobacteria-specific phospholipids or glycolipids.

Data are also presented on the quantitative determination of the amounts of some *M. leprae* lipids in skin biopsies. It is clear that the acid-fast bacilli represent only a small percentage of the total amount of *M. leprae*-derived material present in a lepromatous lesion.

MATERIALS AND METHODS

Bacteria. *Mycobacterium tuberculosis* $H_{37}Ra$, *Mycobacterium smegmatis* NCTC 10265, and *Mycobacterium phlei* NCTC 8151 were obtained from the Haffkine Institute, Bombay, India. *Mycobacterium kansasii* 8 was obtained from Dr. J. Stanford, Middlesex Hospital, London, U.K. *Mycobacterium lepraemurium* was originally obtained from Dr. M. Nishiura, Kyoto, Japan, and is maintained in our laboratory by passaging in Swiss white mice. A sample of *M. leprae*-infected armadillo spleen was obtained from Dr. R. J. W. Rees, National Institute of Medical Research, London, U.K. Biopsy material from lepromatous leprosy patients was provided by the Acworth Leprosy Hospital, Bombay, India. Normal skin biopsy material was provided by the Tata Department of Plastic Surgery, J. J. Hospital, Bombay, India.

Bacteria were grown in Sauton's medium and harvested during the early stationary phase of growth.

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M. leprae were isolated from a gamma-irradiated sample of armadillo spleen, and *M. lepraemurium* was from infected mouse spleen. Bacilli were purified from homogenized tissue samples (0.4–0.5 g wet weight) by density gradient centrifugation ([31]).

Biopsy material from three highly bacillary positive lepromatous leprosy patients was combined to form the "infected skin" sample (wet weight 1.5 g). A sample of "normal skin" (1.7 g wet weight) was obtained from the abdomen of a patient undergoing plastic surgery.

Mycobacterial pellets and homogenized tissue samples were washed with saline and then processed for lipid extraction.

Lipid extraction. Wet bacterial pellets were extracted with chloroform: methanol (2:1) (3 × 5 ml) for 24 hr at room temperature. Tissue samples were extracted overnight at room temperature with chloroform: methanol (1:2) (3 × 30 ml) and then with chloroform: methanol (2:1) (3 × 30 ml) for a further 48 hr. Chloroform-methanol extracts were combined and evaporated to dryness under reduced pressure. Neutral lipids were separated by extraction of the dried lipids with acetone for 30 min at room temperature, and polar lipids were then re-extracted with chloroform: methanol (2:1). Water-soluble material was removed from the polar lipid fraction by the Folch procedure ([13]).

Material remaining after extraction of "unbound" lipids with chloroform-methanol was saponified by incubation in 0.5% (w/v) KOH/methanol for 3 days at 37°C ([31]). Pellets were washed twice with methanol and ether-soluble ("bound") lipid was then collected.

Thin-layer chromatography. Thin-layer plates were routinely prepared using Silica Gel G at a thickness of 0.25 mm for analytical plates and 0.5 mm for preparative plates. For thin-layer chromatography (TLC) of phosphatidyl inositol mannosides the system described by Banerjee, *et al.* ([1]) was adopted: plates were prepared using Silica Gel H impregnated with ammonium sulfate. TLC analysis of A1 fractions was done on pre-coated Silica Gel 60 plates (E. Merck, Darmstadt).

Total lipids were detected by charring after spraying plates with 50% (v/v) sulfuric acid. Glycolipids were detected using α-naphthol spray as described by Jacin and Mishkin ([18]) or by spraying with diphenylamine reagent (diphenylamine 0.5 g, aniline 0.5 ml, acetone 50 ml, H_3PO_4 5 ml), and heating at 110°C for 30 min.

TLC solvents (composition in volume ratios); A, hexane : ether : acetic acid (80:20:1); B, chloroform : methanol (93:7); C, petroleum spirit (b.p. 40 to 60°C) : ether (97:3); D, chloroform : methanol : water (10:5:1); E, petroleum spirit (b.p. 40 to 60°C) : ether (90:10); F, hexane : ether (1:4).

Analysis of A1 glycolipids. Carbohydrate was released from glycolipid samples by incubation with 1 M H_2SO_4 for two hr at 105°C ([7]). Lipid was extracted with chloroform, and aqueous fractions were assayed for 6-deoxyhexoses by the method of Dische and Shettles as described by Herbert, *et al.* ([17]) except that reagent volumes were scaled down for use with 0.2 ml sample volumes. Standard samples were prepared by dissolving L-rhamnose in 1 M H_2SO_4, and the difference in optical density at 396 and 427 nm was proportional to the 6-deoxyhexose concentration. The assay could be used to detect 0.5 μg of 6-deoxyhexose even in the presence of a twenty-fold excess of glucose. The remaining lipid was incubated with 4 M HCl for 16 hr at 105°C, and acid was then removed over NaOH *in vacuo*. Propanol : water (1:1, v/v) extracts were analyzed on Silica Gel G plates developed with butanol : acetic acid : water (4:1:1, by volume) and sprayed with ninhydrin reagent ([4]).

Quantitative analysis of mycobacterial lipids in skin biopsies. Quantitative analysis was carried out using a single very large skin biopsy removed from the elbow of a patient who had been undergoing dapsone therapy for three years. The BI was 5+ and the MI 4%. The total number of acid-fast bacilli was estimated by processing part of the biopsy using the method developed for mouse foot pad analysis ([26]). The remainder of the biopsy was processed for lipid extraction.

M. leprae component **w** was purified from the acetone-soluble A6 fraction by preparative TLC using solvent C. Glycolipid **I** was estimated by measuring the amount of 6-deoxyhexose in fraction A1. Methyl mycolates were purified by prepa-

rative TLC of methanol-insoluble bound lipids in solvent E.

Total carbohydrate was assayed by the method of Dubois, *et al.* ([11]) with L-rhamnose as standard.

Total lipid concentrations were estimated by the phosphovanillin method ([29]) with stearic acid as standard.

RESULTS

Fractionation of acetone-soluble lipids. Acetone-soluble lipids were separated into six fractions in order of migration in solvent A. Fraction A1 consisted of material remaining at the origin during TLC. Fractions A2 to A5 contained mainly mono- and diglycerides, cholesterol, free fatty acids, and triglycerides (these fractions were not subjected to detailed analysis). Fraction A6 was made up of very low polarity lipids which migrated with the solvent front during TLC.

TLC of fraction A1. A1 fractions were analysed by TLC on precoated Merck plates using solvent B; plates were sprayed with diphenylanine reagent for detection of glycolipids (Fig. 1).

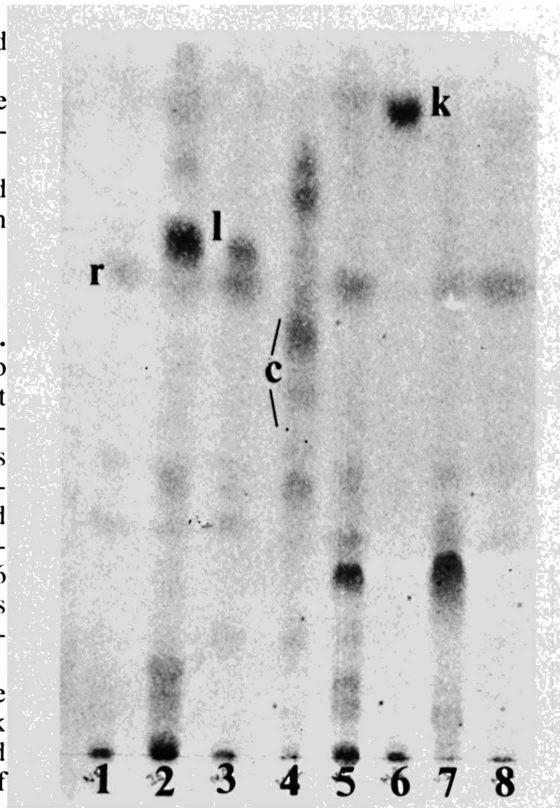

FIG. 1. TLC of A1 fractions in solvent B. Lipids visualized with diphenylamine reagent. Samples: 1, normal skin (60 μg total lipid). 2, infected skin (60 μg). 3, *M. leprae* (40 μg). 4, *M. lepraemurium* (40 μg). 5, *M. tuberculosis* (40 μg). 6, *M. kansasii* (40 μg). 7, *M. smegmatis* (40 μg). 8, *M. phlei* (40 μg). **l**—glycolipid present in extracts from *M. leprae* and infected skin. **c**—set of glycolipids present in *M. lepraemurium*. **k**—glycolipid present in *M. kansasii*. **r**—component present in mycobacterial and skin extracts giving a red color with diphenylamine.

A component present in both the skin biopsy samples and the mycobacterial extracts had an Rf of 0.66 and produced a brick red color with the diphenylamine reagent (Fig. 1, **r**). A glycolipid (Fig. 1, **l**) giving a blue-green spot with diphenylamine was found running slightly ahead of this compound in the infected skin sample. This lipid was absent from normal skin but was seen as a major component in the lipid extract from the purified armadillo *M. leprae*. It seems probable, therefore, that this lipid originates from the mycobacteria, and glycolipid **l** can be considered as a characteristic *M. leprae* lipid. The A1 fraction of *M. leprae* was clearly different from that of *M. lepraemurium* (another mycobacterium isolated from spleen tissue). *M. lepraemurium* was characterized by a set of glycolipids (Fig. 1, **c**) with Rf between 0.37 and 0.85 (3 major components had Rf 0.37, 0.60 and 0.79)—these lipids gave a green color with diphenylamine.

A sample of *M. leprae* glycolipid **l** was partially purified from infected skin lipid extracts by TLC using solvents A, B, and F and compared with partially purified A1 glycolipids from *M. lepraemurium* (the lipid content of both samples was estimated as 150–200 μg). Both samples were found to contain 6-deoxyhexoses, but while the *M. lepraemurium* sample showed the presence of at least 4 amino acids, glycolipid **l** contained no ninhydrin-positive material.

The characteristics of the *M. lepraemurium* A1 glycolipids suggest that they may correspond to the set of non-polar C-type mycosides characteristic of the *M. avium-M. intracellulare-M. scrofulaceum* group of organisms ([3]). The lack of amino acids in the *M. leprae* lipid suggests that it is a glycolipid rather than a peptidoglycolipid as in the case of the C-mycosides ([3]).

TLC of fraction A6. A6 fractions were

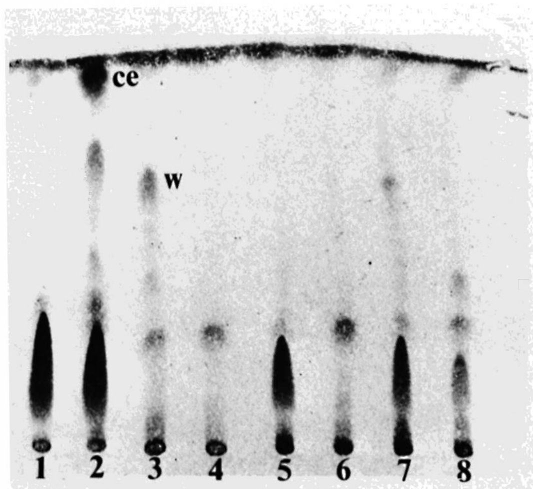

FIG. 2. TLC of A6 fractions in solvent C. Lipids visualized by charring with H_2SO_4. Samples: 1, normal skin (200 μg total lipid). 2, infected skin (200 μg). 3, *M. leprae* (50 μg). 4, *M. lepraemurium* (50 μg). 5, *M. smegmatis* (50 μg). 6, *M. phlei* (50 μg). 7, *M. tuberculosis* (50 μg). 8, *M. kansasii* (50 μg). **w**—wax ester present in *M. leprae* and infected skin samples—similar to a component of *M. tuberculosis*. **ce**—cholesterol esters.

analysed by TLC using solvent C—spots were visualized by charring with H_2SO_4 (Fig. 2). At least two additional spots were seen in the infected skin sample, the major component having an Rf of 0.7–0.75. Once again this spot corresponded to one of the lipids extracted from the purified *M. leprae* (Fig. 2, **w**) and would thus appear to be of mycobacterial origin.

Component **w** was extracted from *M. leprae* isolated from armadillo spleen and purified by preparative TLC in solvent C. Infra-red spectroscopy showed an absorption band at 1730 cm^{-1} indicative of ester functions. There was no absorption in the hydroxyl group region (3300 cm^{-1}). Purified component **w** had the same Rf as an authentic sample of phthiocerol dimycocerosate from *M. tuberculosis* ([15]) when run in solvent systems consisting of chloroform : methanol (9:1, v/v) and petroleum ether : ether (97:3, v/v).

TLC of phosphatidyl inositol mannosides (PIM) ([22]). Acetone-insoluble lipid fractions were analyzed for the presence of PIM using the system described by Banerjee, *et al.* ([1]). Although lipid extracts from purified armadillo bacilli contained components with Rf and staining properties consistent with their preliminary identification as phosphatidyl inositol mannosides, similar compounds were not clearly visible in the infected skin samples. Difficulty in detecting PIM in lepromatous biopsies may be due to their relatively low concentration in comparison to the host phospholipids present in the acetone-insoluble fraction. The number of major sugar-positive spots detected with the tissue-isolated bacilli (*M. leprae*, *M. lepraemurium*, two major spots) was less than with *in vitro* grown bacilli (*M. tuberculosis*, *M. smegmatis*, *M. phlei*, *M. kansasii*, four major spots).

TLC of mycolic acids. The ether-soluble fraction extracted after saponification of mycobacteria is rich in cell-wall mycolic acids released in the form of their methyl esters ([31]). A small amount of lipid (<0.5% of total lipid) was present in this fraction from the infected and normal skin biopsies, and this material was analyzed on TLC plates developed first with solvent E and then with methanol (Fig. 3). Two spots were visualized in the infected skin sample after charring while all the material in the normal skin sample migrated with the solvent front.

The two lipids present in the infected biopsy had the same mobility as the two compounds extracted from the armadillo *M. leprae* previously identified as mycolic acid methyl esters after comparison of Rf values with those of authentic methyl mycolates from *M. tuberculosis* ([31]).

Quantitative analysis of mycobacterial lipids in skin biopsies. Data from the quantitative analysis of a single large biopsy sample is shown in the Table. The total carbohydrate in fraction A1 was 97.5 μg of which 37.5 μg was found to be 6-deoxyhexose. 6-Deoxyhexoses are not generally found in mammalian glycolipids, and none were detected in the acetone-soluble fraction from normal skin samples. It seems reasonable, therefore, to conclude that the 6-deoxyhexose present in the lepromatous biopsy originates from *M. leprae* glycolipid I.

From the total number of acid-fast bacilli, the mass of the mycobacteria present in the biopsy sample can be estimated to be approximately 40 μg ([10]). The mass of the methyl mycolates alone comes to 800 μg. Clearly the observed acid-fast bacilli rep-

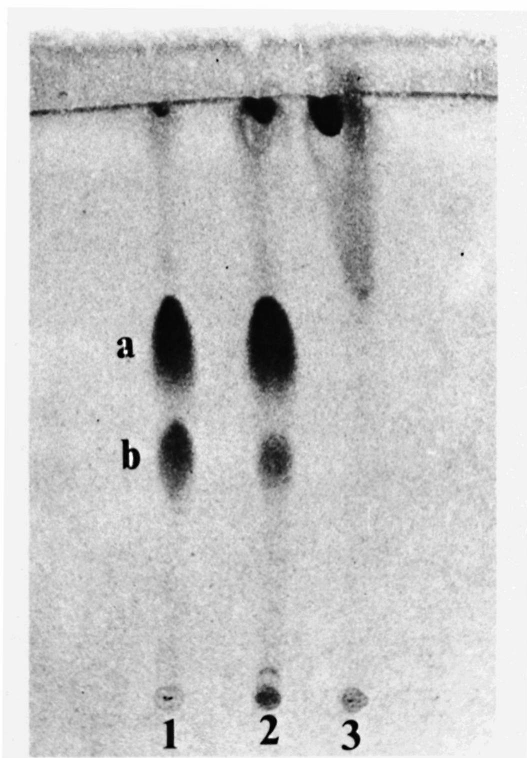

FIG. 3. TLC of ether extracts after saponification. Plate developed with solvent E and then with methanol. Lipids visualized by charring with H_2SO_4. Samples: 1, *M. leprae* (15 μg total lipid). 2, infected skin (25 μg). 3, Normal skin (50 μg). **a** and **b**—*M. leprae* methyl mycolates.

THE TABLE. *Quantitative analysis of a lepromatous skin biopsy.*

Wet weight of tissue	5.84 g
Dry weight after lipid extraction	911 mg
Acetone-soluble lipids	286 mg
Acetone-insoluble lipids	71 mg
Component **w**	0.9 mg
Glycolipid I	36.5 μg of 6-deoxyhexose
Methyl mycolates	0.8 mg
Acid-fast bacilli	9.3×10^8

resent only a small proportion of the total amount of mycobacterial material present in the biopsy.

The methods developed using the large tissue sample were also applied to a smaller lepromatous biopsy (0.4 g wet weight of tissue). The total amount of 6-deoxyhexose was determined using material released by incubation of the whole acetone-soluble lipid extract with 0.5 ml of H_2SO_4 and was found to be 3.25 μg (total carbohydrate 5.5 μg). The methyl mycolate fraction contained 48 μg of lipid. *M. leprae* component **w** was not estimated.

DISCUSSION

The results reported in this paper demonstrate the possibility of carrying out qualitative and quantitative analysis of the lipids of *Mycobacterium leprae* in skin biopsy samples from lepromatous patients. While the amount of material available from such clinical specimens is insufficient for detailed chemical analysis of the characteristic *M. leprae* lipids, several potentially interesting observations have been made.

Unlike *M. lepraemurium*, *M. leprae* does not synthesize the family of C-type mycosides characteristic of the *M. avium-M. intracellulare-M. scrofulaceum* group of organisms ([3]). *M. leprae* from armadillo and human sources does synthesize a 6-deoxyhexose-containing glycolipid, but the absence of a peptide component in this lipid suggests that it may be related to the glycolipid mycosides A and B from *M. kansasii* and *M. bovis* ([14]) rather than the peptidoglycolipid type-C mycosides.

M. leprae synthesizes a wax ester compound which, on preliminary analysis, shows some resemblance to phthiocerol dimycocerosate, a lipid previously thought to be specific to *M. tuberculosis* and *M. bovis* ([15, 19, 27]).

M. leprae from human and armadillo sources has a characteristic methyl mycolate profile which differs from those found in other mycobacteria ([31]).

Further analysis of these characteristic *M. leprae* lipids will be required in order to determine whether or not they play a significant role in the pathology of leprosy (for example, in the formation of a protective capsule around the bacilli ([6, 8, 20]) and to determine their usefulness in taxonomic identification of *M. leprae*.

It is interesting to note that, as judged by the characteristics analyzed in this study, the features of *M. leprae* grown in the spleen of an infected armadillo are the same as those of *M. leprae* present in the skin of a human patient. The large amount of bacilli available from a systemically-infected armadillo make this an attractive source of material for biochemical analysis, and it is

reassuring to note that these bacilli (at least in the respects analyzed so far) do appear to be identical to those involved in human pathogenesis.

Data from quantitative analysis indicates that the amount of mycobacterial lipids present in a lepromatous skin biopsy is far in excess of that predicted on the basis of the number of acid-fast bacilli. An explanation for this observation would be that while the patient's immune system (with the help of dapsone therapy) is capable of killing and partially degrading the bacilli, clearance of the *M. leprae* lipids is not efficiently accomplished. The accumulated lipid debris may be providing a sheltered environment for the remaining bacilli.

These results are consistent with previous reports ([24]) which suggest that the accumulated lipid material found in "lepra" or "foam" cells is derived in part from degraded mycobacteria.

It may be interesting to compare the relative amounts of acid-fast bacilli and of *M. leprae* lipids present in skin biopsies during different stages of treatment. A method of enhancing the rate of removal of *M. leprae* lipids from the site of infection may stimulate killing of remaining bacilli.

SUMMARY

Thin-layer chromatography was used to compare lipid extracts from lepromatous skin biopsies with those from normal skin and from *Mycobacterium leprae* purified from armadillo spleen. Several lipids were found in infected skin which were absent from normal skin but corresponded to lipids present in the purified *M. leprae*. These included mycolic acids, a 6-deoxyhexose-containing lipid (glycolipid I) and a wax ester (possibly related to the *Mycobacterium tuberculosis* wax, phthiocerol dimycocerosate). Unlike *Mycobacterium lepraemurium, M. leprae* contained no C-type mycosides. In terms of lipid profile, *M. leprae* from armadillo spleen showed the same characteristics as bacilli from human skin samples.

Quantitative analysis of mycobacterial lipids in lepromatous skin biopsies indicated that their concentrations were much higher than would be predicted from the number of acid-fast bacilli present. Accumulation of lipid debris from dead *M. leprae* could provide a protective environment in infected cells for remaining viable bacilli.

RESUMEN

Se usó la cromatografía en capa fina para comparar los extractos lipídicos de biopsias de piel lepromatosa con aquellos de piel normal y de *M. leprae* pruificado a partir del bazo de armadillo. Se encontraron varios lípidos en la piel infectada que no existieron en la piel normal pero que correspondieron a lípidos presentes en el *M. leprae* purificado. Estos incluyen ácidos micólicos, un lípido conteniendo 6-desoxihexosa (glicolípido "I") y una cera esterificada (posiblemente idéntica a la cera del *Mycobacterium tuberculosis*, phthiocerol dimycocerostato). A diferencia del *Mycobacterium lepraemurium*, el *M. leprae* no contuvo micosidos tipo C. El *M. leprae* aislado del bazo de armadillo presentó el mismo perfil de lípidos que los bacilos de las biopsias de piel humana.

El análisis cuantitativo de los lípidos micobacterianos en las biopsias de piel lepromatosa reveló una concentración lipídica mayor de lo que podría esperarse a partir del número de bacilos ácido resistentes presentes. La acumulación de residuos lipídicos derivados de *M. leprae* muertos podría proporcionar, en las células infectadas, un microambiente protectivo para los bacilos viables remanentes.

RÉSUMÉ

On a utilisé la chromatographie en couches minces pour comparer les extraits lipidiques obtenus de biopsies cutanées de malades lépromateux, avec ceux obtenus dans des peaux normales, ainsi qu'à partir de *Mycobacterium leprae* purifié provenant de rates de tatou. Dans la peau infectée on a trouvé plusieurs lipides qui étaient absents dans la peau normale, mais qui correspondaient aux lipides présents dans les bacilles purifiés. Ces lipides comprenaient des acides mycoliques, un lipide contenant du 6-deoxyhexose (glycolipide I), de même qu'un ester cireux, qui pourraient être identiques à la cire de *Mycobacterium tuberculosis*, le phthiocerol dimycocerosate. Contrairement à *Mycobacterium lepraemurium*, *M. leprae* ne contennait aucun mycosides de type C. En termes de profil lipidique, *M. leprae* obtenu à partir de rates de tatou, a présenté les mêmes caractéristiques que les bacilles provenant d'échantillons de peau humaine.

L'analyse quantitative des lipides mycobactériens dans les biopsies cutanées de malades lépromateux indique que la concentration de ces lipides est beaucoup plus élevée qu'on aurait pu le prédire à partir du nombre de bacilles acido-résistants présents. L'accumulation des débris lipidiques de *M. leprae* morts pourrait fournir un environnement protectif pour les bacilles viables qui persistent dans les cellules infectées.

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