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Isolation of Characteristic Glycolipids Possibly Included in Spherical Droplets Around *M. Leprae*[1]

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It is well known that around *Mycobacterium lepraemurium* growing in murine lepra cells, or in standard cultures of *M. lepraemurium,* a peculiar material is accumulated. The material is usually observed as crystalline structures by electron microscopy using negative staining of the freeze-etching technique ([2, 3, 7, 14, 16]). Draper, *et al.* identified this material as of the mycoside C glycolipid family ([3]).

In the case of *M. leprae* growing in phagolysosomes of human, nude mouse, and armadillo lepra cells, material different from that of *M. lepraemurium* always accumulates around bacilli. By the freeze-etching technique, the material around *M. leprae* (peribacillary substance) is observed as small spherical droplets (Fig. 1) ([5, 6, 7, 14]). It is thought that the substance in these small spherical droplets is composed of glycolipids because histochemical stains for glycolipids are positive ([8]).

Young reported that *M. leprae* did not contain mycoside C ([17]). Young's quantitative analysis of mycobacterial lipids in lepromatous skin biopsies indicated that the concentrations of lipids were much higher than would be predicted from the number of acid-fast bacilli present ([17]).

Hunter, *et al.* described a phenolic glycolipid that was structurally isolated from *M. leprae,* as shown in Figure 2 ([10, 11]). They considered that these lipids were located in the peribacillary substance of *M. leprae.*

The main purpose of this work was to isolate acetone soluble lipids of a leproma of an armadillo inoculated with *M. leprae* from a mangabey monkey with naturally acquired leprosy by high-performance liquid chromatography (HPLC), and then to examine the mass spectrometric characteristics of the two peaks (molecular weights 2000 daltons [D] and 1600 D) found in the HPLC analysis system.

MATERIALS AND METHODS

Lepromas (15 g) were obtained from a nine-banded armadillo inoculated with *M. leprae* isolated from the mangabey monkey with naturally acquired leprosy ([5, 12, 13]). The armadillo had been screened repeatedly prior to infection and had no evidence of naturally acquired leprosy. Purified phenolic glycolipid ([11]) and phthiocerol dimycocerosate ([10]) from *M. leprae* and mycoside A from *M. kansasii* were kindly supplied by Dr. P. Brennan of Colorado State University. Polystylene calibration standards of known molecular weights were obtained from Waters Associates, Milford, Massachusetts, U.S.A.

The samples were minced and extracted with a series of chloroform/methanol solvents (1:1 followed by 2:1). The extracts were taken to dryness in a rotary evaporator. Acetone soluble lipids were obtained from the resulting residue, and the solvent was removed under reduced pressure. The lipids were taken up in tetrahydrofuran (THF) for further analysis. The procedure is outlined in Figure 3.

Hydrolysis and purification were in accordance with the methods of Hunter, *et al.* ([11]). Fatty acid methylation was accomplished with diazomethane. Alditol acetate formation was performed in accordance with standard methods ([15]).

Spectrophotometric analysis was made with a Perkin-Elmer model 283 infrared spectrophotometer and a Hewlett-Packard 8450A UV/Vis spectrophotometer.

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FIG. 1. Freeze-etching findings of nude mouse lepra cells. Small spherical droplets are observed around leprosy bacilli in phagolysosomes of macrophage (original magnification ×30,000). L = leprosy bacilli, S = small spherical droplets, Arrows = lysosomal membranes.

HPLC studies were made as follows: Solvent was delivered by Waters' 6000X system and injection was made by a Waters' U6K injector. The columns employed were: a) gel permeation—Shimadzu Ltd. HSG 15 (75 cm) and HSG 20 (25 cm), b) normal phase—DuPont Zorbax SIL (25 cm), and c) reverse phase—DuPont Zorbax ODS (25 cm) or Zorbax C8 (10 cm). Detection was with a Perkin-Elmer model LC 15 refractive index detector or Waters' R401 differential refractometer and Waters' model 440 dual wavelength absorbance detector. Integration was accomplished with a Hewlett-Packard 3390A digital integrator.

Gas liquid chromatographic analysis was performed with a Hewlett-Packard 5880A microprocessor controlled instrument equipped with a FID detector. Mass spectral analysis was done on a Hewlett-Packard 5985 GC/MS. Chromatography was on a 3% OV-17 column, and chromatographic conditions were modified from those of Fiereck and Tietz ([4]).

RESULTS

Chromatographic fraction

Gel permeation chromatography (GPC). Chromatography of acetone soluble lipids with detection by refractive index yielded 2 peaks peculiar to the THF solvent of the infected tissues, as shown in Figure 4A. The peaks were of relatively high molecular weights; GPC peak I at 2000 D and GPC peak II at 1600 D. The GPC peak I showed strong absorbance by the spectrophotometric detector at 254 nm and 280 nm, while the GPC peak II was not detected spectrophotometrically.

Purified phenolic glycolipid and mycoside A were analyzed under identical conditions, and each molecule yielded a single peak at 2000 D. Co-injection of the acetone soluble lipids and purified phenolic glycolipid from *M. leprae* was done and detected by refractive index. Under these circumstances, the GPC peak I at 2000 D was higher than that of the acetone soluble lipids alone, as shown in Figure 4B. With co-injection of the acetone soluble lipids and purified mycoside A from *M. kansasii,* the GPC analysis pattern was the same as that obtained

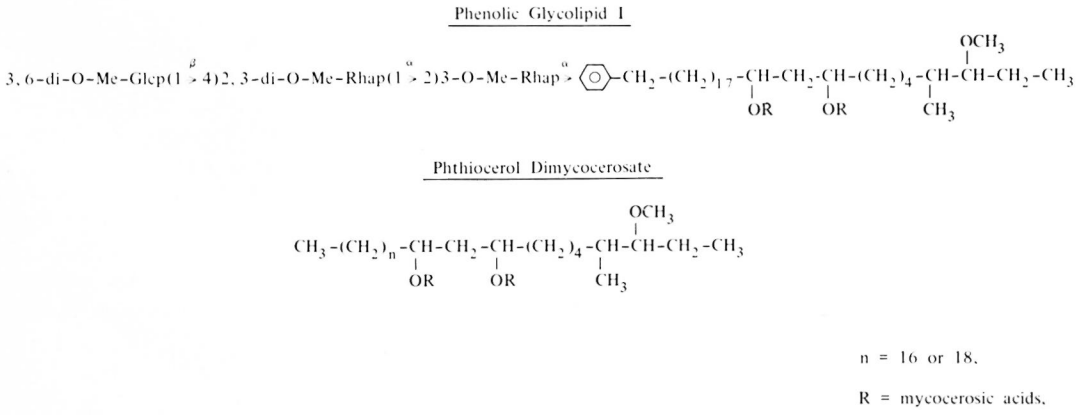

FIG. 2. Phenolic glycolipid I and phthiocerol dimycocerosate.

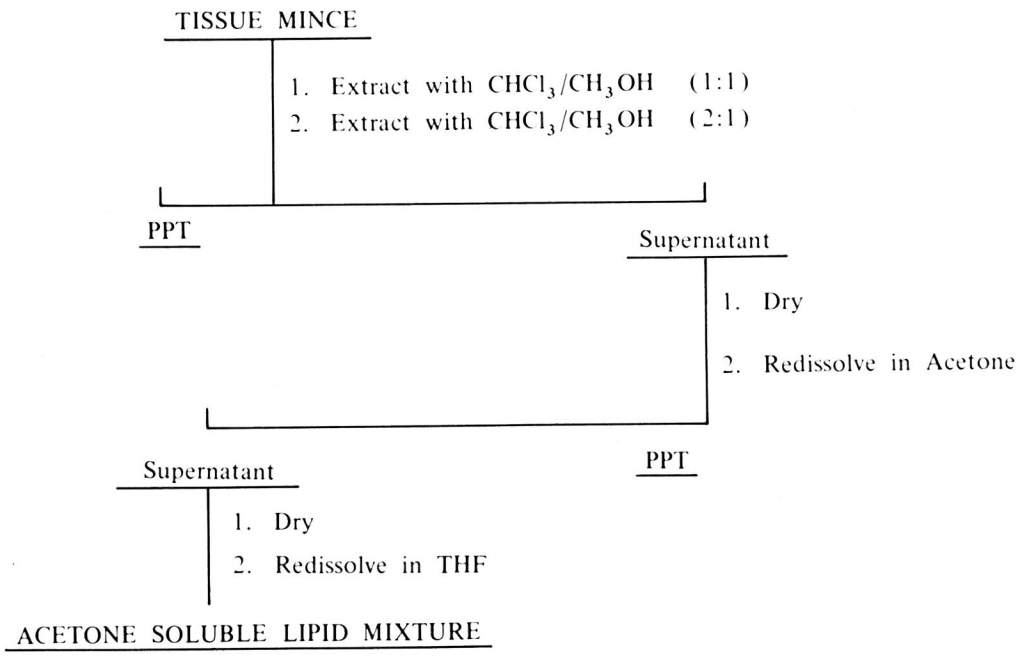

FIG. 3. Procedure for extraction of acetone soluble lipids.

with the co-injection of the acetone soluble lipids and purified phenolic glycolipid from *M. leprae*. Thus, judging from the results using the GPC analysis system, it could not be determined whether the GPC peak I lipid was made up of phenolic glycolipid or mycoside A. On the other hand, co-injection of the acetone soluble lipids and purified phthiocerol dimycocerosate from *M. leprae* revealed that the GPC peak II at 1600 D was higher than that of the acetone soluble lipids alone, as shown in Figure 4C. From these findings it was therefore thought that the component of the GPC peak II lipid was phthiocerol dimycocerosate.

Reverse-phase (C8) mode. Reverse-phase chromatography of the GPC peak I lipid, with detection by a spectrophotometric detector at 254 nm and 280 nm, yielded 2 sharp peaks in the THF/acetonitrile (65:35) solvent. Purified phenolic glycolipid and mycoside A were eluted most closely with the peak of lowest retention volume. Co-injection of the GPC peak I lipid and purified phenolic glycolipid showed that the peak of lowest retention volume was higher than that of the GPC peak I lipid alone. Co-injection of the GPC peak I lipid and mycoside A gave the same pattern as that of co-injection of the GPC peak I lipid and purified phenolic glycolipid.

Normal-phase (SILICA) mode. Normal-phase chromatography of the GPC peak I lipid, with detection by a spectrophotometric detector at 254 nm and 280 nm, revealed several contaminants of low retention volume (low polarity). The main peak, eluted with a retention volume of about 17 ml in hexane/THF (60:40) solvent, was separated clearly from the other lipids, as shown in Figure 5A. Purified phenolic glycolipid from *M. leprae* showed the same elution pattern and peak shape as the GPC peak I lipid, as shown in Figure 5B. The major peak in the phenolic glycolipid preparation had an elution volume of about 18 ml.

On the other hand, purified mycoside A presented a different elution pattern from phenolic glycolipid. The major peak on the mycoside A preparation had an elution volume of about 12 ml.

From the results of the SILICA analysis system, it was concluded that the polarities of the phenolic glycolipid and mycoside A were different from each other.

Spectroscope analysis of purified lipid

The infrared absorption spectrum of the purified GPC peak I lipid by SILICA column showed the same pattern as that of the phenolic glycolipid already described by Hunter, *et al.* (9).

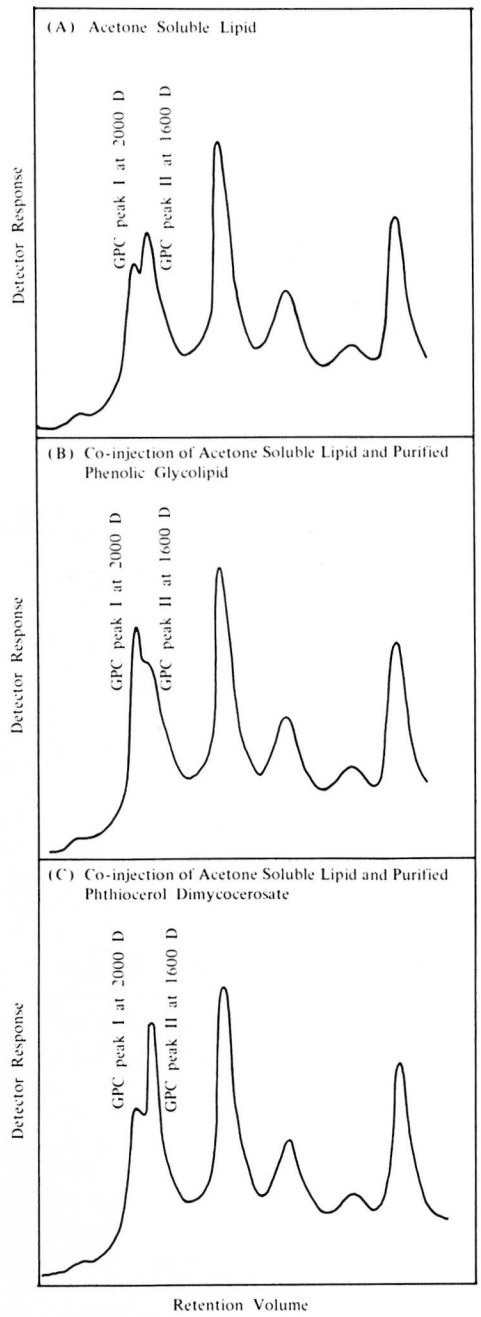

FIG. 4. Gel permeation chromatography (GPC) (Shimadzu-HSG) analysis with RI detector.

(A) GPC of acetone soluble lipids. Peculiar 2 peaks, GPC peak I (molecular weight about 2000) and GPC peak II (molecular weight about 1600) are detected in the THF solvent.

(B) GPC of co-injection of the acetone soluble lipids and purified phenolic glycolipid from *M. leprae*. GPC peak I at 2000 D is higher than that of the acetone soluble lipids alone.

(C) GPC of co-injection of the acetone soluble lipids and phthiocerol dimycocerosate. GPC peak II at 1600 D is higher than that of the acetone soluble lipids alone.

Hydrolysis and product analysis

Gas liquid chromatography (GLC) of fatty acid methyl esters produced by alkaline hydrolysis of purified GPC peak I lipid yielded 2 peaks, as shown in Figure 6. Those peaks represented 2 kinds of fatty acid branches. The mass spectral fragmentation patterns of the fatty acid methyl esters showed 2 significant peaks, consistent with mycocerosic acid. The fragmentation patterns of those 2 peaks, internal branchings and possible unsaturation are shown in (A) and (B) of Figure 7.

Alditol acetates were prepared from the monosaccharide released from the purified GPC peak I lipid by methanolysis and 3 peaks were detected in GLC. Retention times on GLC indicated that those peaks correspond to methylated derivatives of rhamnose and glucose. The relative abundance was 2:1. The mass spectral fragmentation patterns of the alditol acetates were the same as those of the phenolic glycolipid already described by Hunter, *et al.* ([11]).

The final core molecule after hydrolysis and methanolysis showed strong absorbance in the 240–280 nm wavelength spectrophotometrically. It is thought that the core molecule of purified GPC peak I lipid is phenolphthiocerol.

DISCUSSION

The GPC peak I lipid that had been extracted from a leproma of a nine-banded armadillo was studied. The armadillo had been infected with *M. leprae* isolated from tissues of a mangabey monkey with naturally acquired leprosy ([12]). The characteristics of the major component of the examined GPC peak I were in general agreement with those of the phenolic glycolipid of *M. leprae* described by Hunter, *et al.* ([11]). The relevant findings were based on: infrared spectra of intact molecules, spectral properties of the carbohydrate component and core molecule, total molecular weight by the GPC column system, and polarity by the SILICA column system.

The inconsistency noted between our data

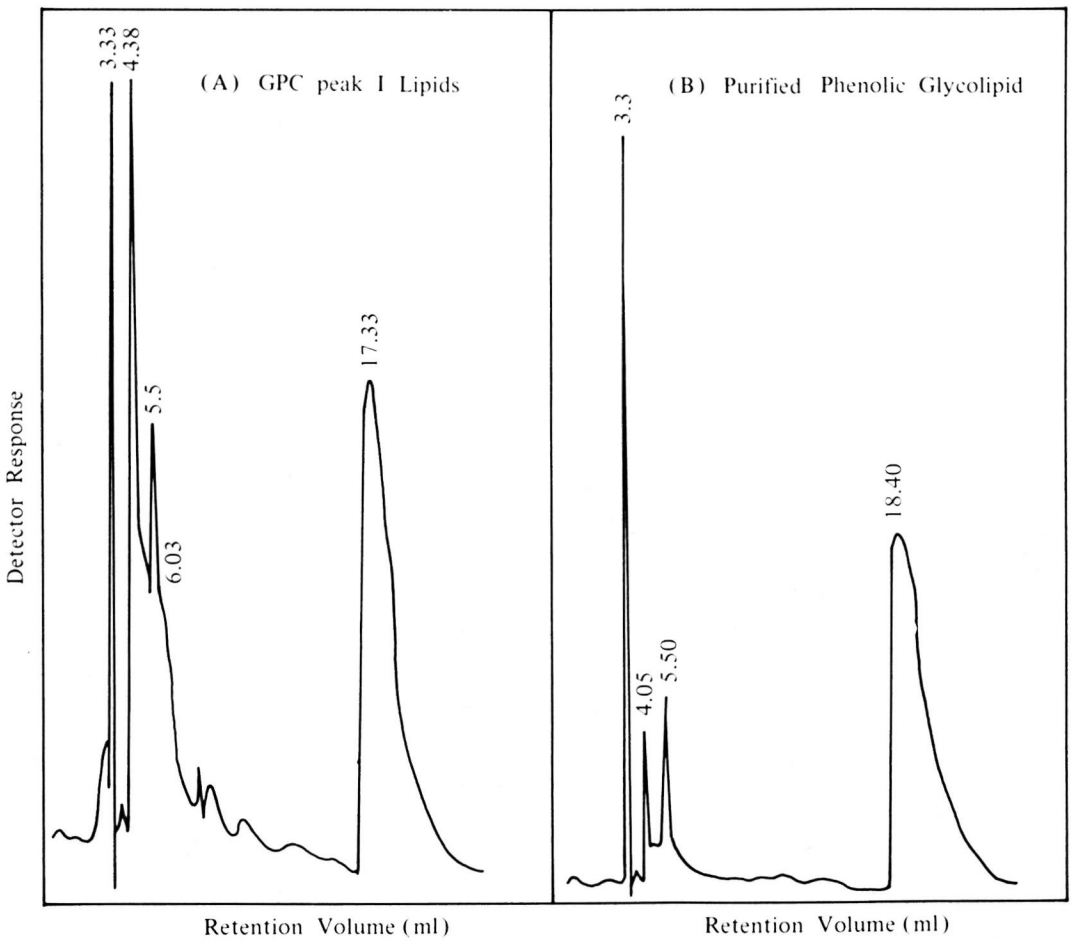

FIG. 5. Normal-phase (SILICA) analysis with spectrophotometric detector at 280 nm wavelength.
(A) Chromatography of GPC peak I lipid. The main peak, eluted with a retention volume of about 17 ml in the hexane/THF solvent, is clearly separated from the other lipids.
(B) Chromatography of purified phenolic glycolipid from *M. leprae.* Phenolic glycolipid showed the same elution pattern and peak shape as the GPC peak I lipid. The major peak in the phenolic glycolipid preparation has an elution volume of about 18 ml.

and that of Hunter, *et al.* ([11]) was only in the area of fatty acid composition. It should be noted that the starting materials were quite different. The local thermal and chemical environment (leproma versus liver) of the bacterium might influence the spectrum of elongated fatty acid produced. Another possible explanation of the difference may be that the fractionating procedure employed may have selected a subfraction or minor peak for analysis. A final possibility is the source of infection for the starting material. The organs used by Hunter, *et al.* were from an armadillo infected directly with human-derived *M. leprae.* Changes in

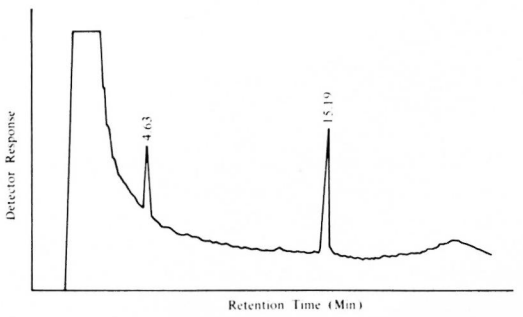

FIG. 6. Gas liquid chromatography. Fatty acid methyl esters produced by alkaline hydrolysis of purified GPC peak I lipid by SILICA column yield 2 peaks which represent 2 kinds of fatty acid branches.

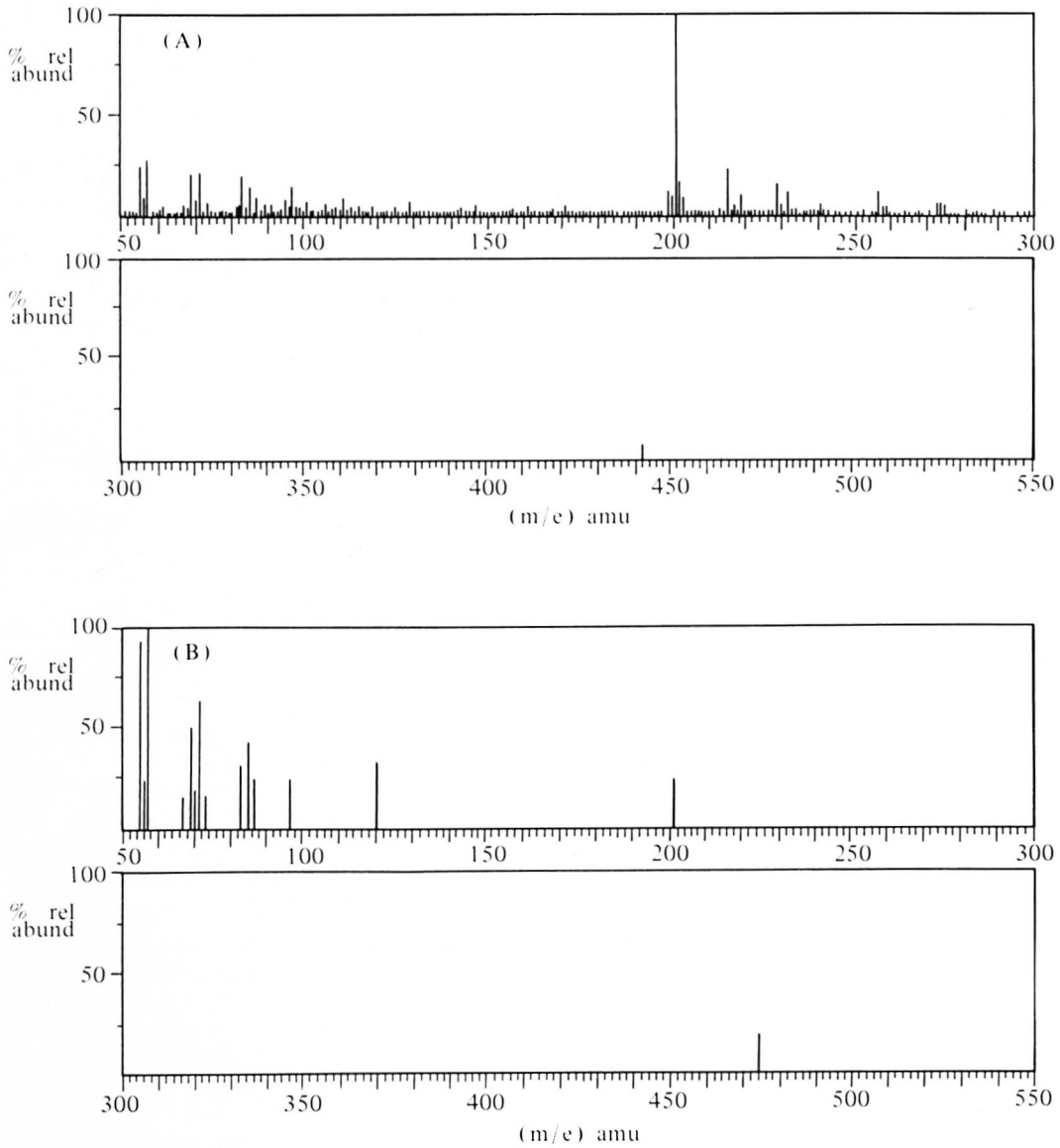

FIG. 7. Mass spectrometric analysis. Mass spectral fragmentation patterns of the fatty acid methyl esters show 2 significant peaks. These 2 peaks are consistent with mycocerosic acid. (A) and (B) show the fragmentation patterns of the 2 peaks; internal branchings and possible unsaturation are indicated.

the metabolism of *M. leprae* induced by passage through the mangabey monkey cannot be ruled out.

In 1983, phthiocerol dimycocerosate from *M. leprae* was described by Hunter, *et al.* ([10]) and Draper, *et al.* ([1]). There were some resemblances between phthiocerol dimycocerosate and the GPC peak II lipid. Both lipids could not be detected by spectrophotometry at 254 nm and 280 nm wavelengths which assessed aromatic content. Furthermore, molecular weights of the phthiocerol dimycocerosate and the GPC peak II lipid were the same, 1600 D. From these data, it was thought that the GPC peak II lipid was phthiocerol dimycocerosate.

Takeo, *et al.* studied 19 species of mycobacteria grown *in vitro* by the freeze-etching technique and spherical droplet-like structures were never observed around any

of the mycobacteria they examined, with the exception of *M. leprae* ([16]). In 1981, Fukunishi, *et al.* reported that high concentrations of the GPC peak I and GPC peak II lipids were extracted from bacteria-free precipitates and supernatants of homogenated nude mouse lepromas infected with *M. leprae.* These lipids were never found in extracts of normal mammalian tissues or murine lepromas ([8]). From these results, it was postulated that the phenolic glycolipid (GPC peak I lipid) and the phthiocerol dimycocerosate (GPC peak II lipid) were contained in the spherical droplets around *M. leprae.*

Lipids obtained from lepromas caused by the acid-fast bacillus obtained from lesions of naturally acquired leprosy in a mangabey monkey were found to be analogous to those of lesions provoked by *M. leprae* of human origin. It is thus concluded that the two organisms are identical or very closely related.

SUMMARY

The main purpose of this work was to isolate the components in acetone soluble lipids of lepromas of the nine-banded armadillo by high performance liquid chromatography (HPLC), and then to examine the mass spectrometric characteristics of the two peaks (molecular weights 2000 and 1600) found by HPLC. The armadillo had been inoculated with *Mycobacterium leprae* isolated from a mangabey monkey with naturally acquired leprosy.

According to the results of HPLC, gas liquid chromatographic and mass spectral analyses, the GPC peak I lipid at 2000 D was identified as phenolic glycolipid and the GPC peak II lipid at 1600 D, as phthiocerol dimycocerosate. It was thought that the GPC peak I lipid and the GPC peak II lipid were included in the spherical droplets (peribacillary substance) around *M. leprae.* It was concluded that the microorganisms causing leprosy-like changes in the mangabey monkey were either *M. leprae* or a very closely related bacillus.

RESUMEN

El principal propósito de este trabajo fue el aislamiento de los componentes de la fracción lipídica soluble en acetona derivada de lepromas de armadillos de 9 bandas. El fraccionamiento de los lípidos se hizo por cromatografía líquida de alta resolución (HPLC) y el examen de los picos por espectrometría de masas. El armadillo había sido inoculado con *Mycobacterium leprae* aislado de un mono mangabey con lepra adquirida naturalmente.

Se obtuvieron 2 picos cuyos pesos moleculares fueron 2000 y 6000 respectivamente. El pico I (2000 D) se identificó como glicolípido fenólico y el pico II (1600 D) como phthiocerol dimicocerostato. Pensamos que los lípidos de los picos I y II estuvieron incluídos en las gotillas esféricas (substancia peribacilar) que rodean al *M. leprae.* Se concluyó que los microorganismos causantes de los cambios parecidos a los de la lepra en el mono mangabey, fueron *M. leprae* o un bacilo muy estrechamente relacionado.

RÉSUMÉ

Le but principal de cette étude a été d'isoler les constituants lipidiques solubles dans l'acétone au niveau des lépromes de l'armadillo à neuf bandes. On a utilisé pour ce faire une méthode de chromatographie liquide très performante (HPLC), suivie d'un examen au spectromètre de masse des caractéristiques des deux pics, de poids moléculaire 2000 et 1600, relevés lors de la chromatographie. L'armadillo a été inoculé avec *Mycobacterium leprae* isolée à partir d'un singe mangabée atteint de lèpre acquise naturellement.

D'après les résultats obtenus par la chromatographie liquide à haute performance, la chromatographie gazeuse, et les analyses au spectromètre de masse, le pic I lipidique GPC observé à 2000 D a été identifié comme un glycolipide phénolyque; le pic lipidique GTC II à 1600 D comme un phthiocerol dimycocerosate. Le pic lipidique GTC I et le pic lipidique GTC II sont inclus dans les gouttelettes sphériques constituant la substance péribacillaire qui entoure *M. leprae.* On en conclut que les microorganismes qui produisent des modifications semblables à la lèpre chez le singe mangabée sont soit *M. leprae* ou un bacille qui lui est très proche.

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