Appearance of a Methoxy Mycolate-like Component by the Acid Methanolysis of *Mycobacterium leprae*[1,2]

Arun K. Datta, Vishwa M. Katoch, Kiran Katoch,
Vishnu D. Sharma, and Channappa T. Shivannavar[3]

Mycolic acids are important constituents of the mycobacterial cell wall structure, and have been found very useful as taxonomical markers for the identification and classification of mycobacteria. It has been reported that *Mycobacterium leprae* contains alpha- and keto-mycolic acids ([12]), a property which is shared only by *M. bovis* BCG ([17]). The presence of alpha- and keto-mycolic acids was further demonstrated by various workers using materials derived from both human sources ([7, 8, 12, 14, 24]) and from infected armadillos ([9, 11, 15, 22, 23]). However, Asselineau and her groups found evidence of the presence of a very small amount of methoxy mycolate in one sample of *M. leprae* isolated from the tissues of an experimentally infected (W45) armadillo ([1, 5]). Subsequent studies using bacilli from tissues of other experimentally infected armadillos did not show the presence of methoxy mycolates ([2]). In our previous study, we also could not find evidence for the presence of methoxy mycolic acids in *M. leprae* when these acids were extracted from infected human tissues by alkaline methanolysis ([7]). In the present study, however, we have observed that a methoxy mycolate-like component appeared in *M. leprae* only when the acid methanolysis procedure is followed for the release of mycolic acids from these bacilli.

MATERIALS AND METHODS

Materials. Skin-biopsy material from three highly bacillary-positive, untreated, lepromatous leprosy patients (The Table) have been studied. Normal skin tissue from healthy volunteers was collected as control. Purified *M. leprae* (total count = 2.3×10^{10} bacilli) was also used in this study. The organisms from spleen tissue of leprosy-infected armadillos (IMMLEP Bank Nos. 165, 194, 197, 1116) were purified following the method of Draper ([10]).

Extraction of mycolates and their analysis. For the extraction of mycolates as their methyl esters, both acid and alkaline methanolysis procedures were followed. Each of the tissue samples was divided into two pieces. Each piece was first delipidated by extracting the unbound lipids using a chloroform–methanol mixture following the method of Bligh and Dyer's monophasic system ([3]). After delipidation, one half of each delipidated tissue sample was subjected to alkaline methanolysis with 0.5% KOH in methanol as described elsewhere ([7]). The other half was subjected to acid methanolysis using methanol–toluene–sulfuric acid (30:15:1) following the method of Minnikin, *et al.* ([16]). Purified *M. leprae* were also delipidated and then subjected to acid methanolysis for the extraction of their mycolic acids as methyl esters. Methyl mycolates were first purified by washing with methanol ([13]). They were then analyzed by uni-dimensional thin-layer chromatography using aluminum-backed, high-performance thin-layer chromatography (HPTLC) plates (5×5 cm^2) of Silica gel $60F_{254}$ (Art. No. 5548, E. Merck, Germany), the separated bands being detected with dichromate-sulfuric acid spray as described earlier ([7]). Authentic samples of methyl mycolates

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[3] A. K. Datta, M.Sc., Ph.D., Assistant Research Officer; V. M. Katoch, M.D., Senior Research Officer; V. D. Sharma, M.Sc., Assistant Research Officer; C. T. Shivannavar, M.Sc., Research Assistant, Department of Microbiology; K. Katoch, M.D., Senior Research Officer, Department of Medicine, Central JALMA Institute for Leprosy (ICMR), P.O. Box 31, Taj Ganj, Agra 282001, India.

Reprint requests to: Dr. A. K. Datta, Department of Bacteriology, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706, U.S.A.

THE TABLE. *Acid methanolysis[16] products of the samples used in the study.*

Samples	Wet weight of tissue (g)	No. bacilli[a]	Types of mycolic acids present		
			Alpha-	Methoxy-[b]	Keto-
Patients					
H	03.7751	1.4×10^8/g	+[c]	+	+
VS	0.40218	2.5×10^8/g	+	+	+
NK	0.6268	ND[d]	+	+	+
M. leprae (purified from armadillo tissue)	—	2.3×10^{10}	+	+	+
Normal skin tissue (control)	1.0935	—	—	—	—

[a] The bacilli were counted according to the method of Shepard and McRae.[21].
[b] Chromatographic evidence.
[c] + = presence.
[d] ND = not done.

of *M. tuberculosis* (kindly provided by Dr. P. Draper, National Institute for Medical Research, London) were used as standards.

Derivatization of mycolic acid methyl esters. For further verification of the types of mycolic acids present in *M. leprae*, methyl mycolates extracted from purified bacilli and from infected skin tissues were converted to tert-butyldimethylsilyl (TBDMS) ether derivatives and analyzed by thin-layer chromatography using plastic-backed sheets of Silica gel $60F_{254}$ (Art. No. 5753, Merk). After multiple development in single dimension with the petroleum ether-diethyl ether (9:1) solvent system [7], the spots were stained with iodine vapor and the area was marked. The area containing methyl mycolates was cut from the sheets, and the components were eluted by diethyl ether. This ether extract was derivatized using tert-butyldimethylsilyl chloride (Sigma Chemcial Co., St. Louis, Missouri, U.S.A.) following a scaled-down procedure of Corey and Venkateswarlu [4]. In brief, methyl mycolates dissolved in ether extract were taken in a reacti-vial with a PTFE-lined screw cap (Pierce Chemical Co., Rockford, Illinois, U.S.A.) and dried under N_2. This was dissolved in toluene (15 μl) and 15 μl of a solution prepared by dissolving t-butyldimethylsilyl chloride (Sigma; 0.15 g) and imidazole (Sigma; 0.17 g) in dimethyl formamide (Spectrochem Pvt. Ltd., India; 1 ml) was added. The solution was heated in an oven at 75°C overnight. The cooled mixture was extracted with petroleum ether and the upper light petroleum ether layer was pipetted onto a 1-cm column of diethyl-ether-washed neutral alumina (Koso Chemical Co. Ltd., Japan) in a cotton-wool plugged Pasteur-pipette. The eluent was collected in a separate vial. The reaction mixture was again washed with petroleum ether and the upper layer was pipetted onto the column. The procedure was repeated twice more. The pipette used for the transfer was placed into the column and was washed with 1 ml of diethyl ether. The combined eluents were evaporated to dryness under N_2 and used for thin-layer chromatography. The methyl mycolates from purified *M. leprae* and *M. tuberculosis* were also derivatized following the same procedure. These derivatives were analyzed by thin-layer chromatography using petroleum-toluene (7:3 v/v) as described by Minnikin, *et al.* [20].

RESULTS AND DISCUSSION

The present study showed that the acid methanolysate products of purified *M. leprae* gave three spots by uni-dimensional (1D-) thin-layer chromatography (Fig. 1), and these spots were co-chromatographed with the authentic samples of alpha-, keto- and methoxy mycolates of *M. tuberculosis*. Acid methonolysate products of all leprosy-infected skin biopsies also gave three spots on the chromatogram co-chromatographed with the authentic alpha-, keto- and methoxy mycolates as shown in Figure 1. However, no such spots were observed when the acid or alkaline methanolysis products of normal skin tissue were run by thin-layer chromatography as was found in our earlier study [7]. The tert-butyldimethylsilyl ether derivatives of methoxy mycolates from pure

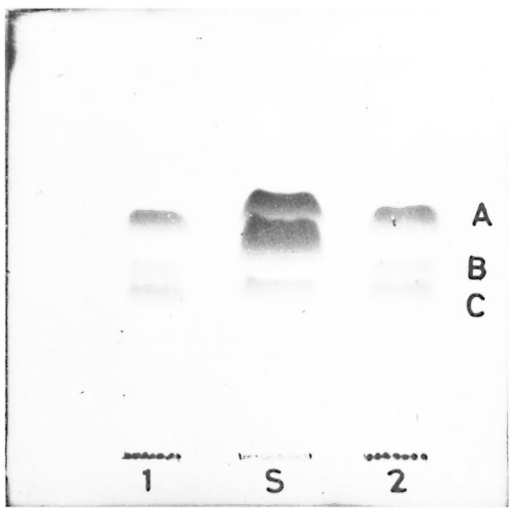

FIG. 1. Uni-dimensional thin-layer chromatography of methyl mycolates run five times in petroleum ether (bp 60–80°C)–ether (9:1 v/v) and once in methanol in the same direction. Spots were developed by heating the chromatogram at 120°C for 10–15 min after staining with $K_2Cr_2O_7/H_2SO_4$ reagent (7). In the chromatogram, the samples used were: S = authentic samples of methyl mycolates of *M. tuberculosis;* 1 = acid methanolysates of leprosy-infected skin tissue; 2 = acid methanolysates of purified *M. leprae.* A = alpha-mycolates; B = methoxy mycolate; C = keto-mycolate.

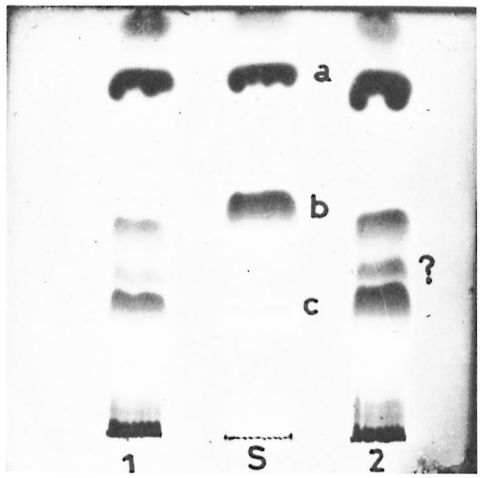

FIG. 2. Thin-layer chromatography of TBDMS derivatives of methyl mycolates run in petroleum ether (bp 60–80°C)–toluene (7:3 v/v). Spots were developed by heating the chromatogram at 120°C for 10–15 min after spraying with dichromate-sulfuric acid solution. In the chromatogram, the samples used were from leprosy-infected skin tissue (lane 1) and purified bacilli (lane 2). TBDMS derivatives of authentic samples of methyl mycolates of *M. tuberculosis* (S) were used as standard. a = derivatized alpha-mycolate; b = derivatized methoxy mycolate; c = derivatized keto-mycolate; ? = unknown derivatized product.

M. leprae and leprosy-infected skin-biopsy tissue also co-chromatographed with that of the authentic methoxy mycolates of *M. tuberculosis* (Fig. 2). Thus, the results showed the appearance of a "methoxy"-like component in *M. leprae* along with alpha- and keto-mycolates when the acid-methanolysis procedure was followed. However, as seen in Figures 1 and 2, the Rf values of this component from both infected skin extracts and purified *M. leprae* were marginally different from the authentic sample of methoxy mycolate from *M. tuberculosis,* suggesting some minor structural differences. Alkaline methanolysis, on the other hand, showed only two spots for infected skin tissue. These spots were co-chromatographed with the alpha- and keto-mycolates of *M. tuberculosis,* confirming our earlier report (7). The presence of methoxy mycolates could not be demonstrated in the leprosy-infected skin tissue when the alkaline methanolysis was followed.

The thin-layer chromatographic patterns of mycolates by alkaline methanolysis of mycobacteria may sometimes differ from those obtained by acid methanolysis as was earlier observed in the case of *M. fortuitum* and *M. smegmatis* (16, 19). In the case of *M. fortuitum* that difference was found to be due to the acid degradation of its mycolic acid components containing epoxy rings (6, 17). A similar situation in the chromatographic pattern has been observed in this study where the acid methanolysis products of *M. leprae* differ from those obtained by alkaline methanolysis producing an extra component. Therefore, further structural analyses are needed to find out the exact chemical nature of this component of *M. leprae* which co-chromatographed with the methoxy mycolate of *M. tuberculosis.* Nevertheless the consistent presence of this "methoxy"-like spot on TLC by acid methanolysis is an important observation which has to be kept in mind while taxonomically identifying this organism using chromatographic techniques.

SUMMARY

It has been reported that *Mycobacterium leprae* contains two types of mycolic acid,

namely, alpha- and keto-mycolic acids, thus it is taxonomically similar to *M. bovis* BCG. However, there was some controversy about the presence of methoxy mycolic acid which was observed in small amounts only in the case of experimentally infected (W45) armadillo-derived *M. leprae*. To investigate this fact, mycolic acids were extracted from the cell-wall structure of *M. leprae* and characterized using chromatographic techniques. The results showed the appearance of a methoxy mycolate-like component for both purified bacilli and infected human skin tissue materials. However, this appearance occurred only when the acid methanolysis procedure was followed for the release of mycolic acids from these bacilli. No such component appeared on the chromatogram when the alkaline methanolysis procedure was followed. Nevertheless, the consistent presence of this methoxy mycolate-like component by acid methanolysis is an important finding which has to be kept in mind while identifying this pathogen when using chromatographic techniques.

RESUMEN

Se ha publicado que el *Mycobacterium leprae* contiene 2 tipos de ácidos micólicos: alfa- y ceto-micólicos, y que por ésto, el *M. leprae* es taxonomicamente similar al *M. bovis*, BCG. Sin embargo, hubo cierta controversia sobre la presencia de ácido metoxi-micólico el cual se observó en pequeñas cantidades sólo en el caso del *M. leprae* derivado del armadillo (W45) infectado en forma experimental. Para investigar este hecho, se extrajeron los ácidos micólicos de la pared celular del *M. leprae* y se caracterizaron cromatograficamente. Los resultados mostraron la aparición de un componente similar al metoxi-micolato tanto en los bacilos purificados como en el material extraído del tejido infectado de piel humana. Sin embargo, ésto solo ocurrió cuando se siguió el procedimiento de metanolisis ácida para la liberación de los ácidos micólicos bacilares. El compuesto no se observó en los extractos obtenidos por metanolisis alcalina. La presencia consistente de este compuesto con las características del metoxi-micolato entre los productos de la metanolisis ácida, es un hallazgo importante que debe considerarse cuando se intenta identificar al *M. leprae* usando técnicas cromatográficas.

RÉSUMÉ

On a rapporté que *Mycobacterium leprae* contient deux types d'acides mycoliques, à savoir, l'acide alpha-mycolique et l'acide ceto-mycolique, ce qui le rend semblable sur le plan taxonomique à *M. bovis* BCG. Néanmoins, l'accord n'est pas fait concernant la présence d'acides méthoxymycoliques, qui n'ont été mis en évidence, et seulement en petites quantités, qu'après infection expérimentale par du *M. leprae* (W45) dérivé du tatou. Afin d'étudier le problème, on a procédé à l'extraction des acides mycoliques de la paroi cellulaire de *M. leprae*; ces acides ont alors été caractérisés par des techniques chromotographiques. Les résultats ont révélé l'apparition d'un constituant semblable au méthoxy-mycolate, tant dans les bacilles purifiés que dans des échantillons de tissus cutanés humains infectés. Ceci n'a cependant été observé que lorsqu'on a utilisé une procédure de méthanolyse acide pour extraire les acides mycoliques des bacilles. Aucun constituant de ce type n'est apparu à la chromatographie, lorsqu'on adaptait une procédure de méthanolyse alcaline. Il n'en reste pas moins que la présence constante de ce constituant, semblable au méthoxy-mycolate, mis en évidence par la méthanolyse acide, est une observation importante qui doit être gardée à l'esprit lorsque l'on identifie ce pathogène par des techniques chromatographiques.

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