Competency of human-derived *Mycobacterium leprae* to use palmitic acid in the synthesis of phenolic glycolipid-I and phthiocerol dimycocerosate and to release CO_2 in axenic culture

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Summary Insufficient numbers of viable *Mycobacterium leprae* have hampered metabolic studies using human-derived *M. leprae*. In this study, sufficient numbers of *M. leprae* were obtained from an untreated lepromatous patient to titrate the effects of pH on the metabolism of ^{14}C-palmitic acid by *M. leprae*.

Catabolic metabolism (oxidation of ^{14}C-palmitic acid and release of $^{14}CO_2$) was maximal when *M. leprae* were incubated at 33°C and suspended in Middlebrook 7H9, ADC supplemented medium that had been buffered to maintain a pH of 4·8. Anabolic metabolism (synthesis of ^{14}C-phenolic glycolipid-I and its precursor, ^{14}C-phthiocerol dimycocerosate) was maximal when the pH was maintained at 6·8.

Introduction

Insufficient quantities of viable *Mycobacterium leprae* extracted from human tissues have severely hampered metabolic studies using human-derived *M. leprae*. Nevertheless, mycobacteria separated from human leprosy nodules have been shown to possess glutamic acid decarboxylase activity,[1] phenoloxidase activity,[2] and gamma glutamyl transpeptidase activity.[3]

In this study, we obtained *M. leprae* in sufficient numbers from an untreated human leproma to titrate the effects of low pH on the capacity of *M. leprae* to (a) oxidize ^{14}C-palmitic acid and release $^{14}CO_2$ and (b) assimilate ^{14}C-palmitic acid into ^{14}C-phenolic glycolipid-I (PGL-I) and its lipid precursor, phthiocerol dimycocerosate (PDIM).

Materials and methods

SOURCE AND CHARACTERIZATION OF *M. LEPRAE*

A leproma weighing 52 mg was removed from the eyelid of an untreated, 18-year-old male Ethiopian. The patient has been histologically classified as BL/LL. The acid-fast bacteria (AFB) extracted from the leproma was not only able to synthesize PGL-I, but the DNA derived from the AFB was also reactive in an *M. leprae*-specific polymerase chain reaction assay.[4]

PREPARATION OF INOCULUM

The leproma was ground using a mortar in a pestle containing Middlebrook 7H9 medium (DIFCO, Detroit, MI, USA). The large tissue debris was allowed to settle for 10 min and the supernatant centrifuged at 10,000 $\times g$. The pellet was treated for 5 min with a 10% v/v solution of 0·25 N NaOH and washed with Middlebrook 7H9 medium. The processed leproma yielded $7·8 \times 10^9$ AFB with a morphological index of 8%.

INCUBATION MEDIA AND CULTURE VESSELS

The pH of Middlebrook 7H9 medium was adjusted to 4·8, 5·8, 6·2, or 6·8 using citrate buffer.[5] We prepared suspensions containing 2×10^8 *M. leprae*/ml in citrate-buffered, Middlebrook 7H9 medium enriched with a 10% v/v solution containing albumin, dextrose and catalase (ADC, DIFCO) and supplemented with ampicillin (50 μg/ml), amphotericin B (2·5 μg/ml), and 1 (μCi/ml of universally labelled palmitic acid (NEC-534, palmitic acid [^{14}C(U)], 800 mCi/mmole; Dupont, Boston, MA, USA). In order to control for nonspecific, pH-influenced oxidation of ^{14}C-palmitic acid, the control cultures contained the 7H9 medium, ADC and antibiotic supplements and ^{14}C-palmitic acid. The above suspensions were placed in 10-ml glass serum vials (Wheaton Scientific, Vineland, NJ, USA). A plastic cup (Kontes, Scientific Glassware/Instruments, Vineland, NJ, USA) containing 400 μl of 4·0 N NaOH was suspended from a rubber stopper sealing the vials. These reaction vessels were incubated at 33°C for 7 days.

^{14}CO$_2$ ASSAY

The ^{14}CO$_2$ that evolved from the oxidation of [U-^{14}C] palmitic acid was trapped in the solution of NaOH. After 7 days' incubation at 33°C, the cup containing the NaOH was removed and 100 μl was added to 6·0 ml of Aquasol-2 (NEN Research Products, Boston, MA, USA). After adding 1·0 ml of glacial acetic acid to clarify the scintillation fluid, the amount of ^{14}CO$_2$ was assessed using a Model LS-5801 Beckman Liquid Scintillation Spectrophotometer.

LIPID EXTRACTION, ANALYSIS AND QUANTITATION OF PGL-I AND PDIM

The contents of the culture vessels were lyophilized and the lipids extracted overnight using 10 ml of chloroform:methanol, 2:1 v/v, in a 50°C water bath. The extract was filtered through cotton-plugged glass funnels and evaporated to dryness under a stream of nitrogen in a 50°C water bath. The dried extracts were redissolved in chloroflorm:methanol, 2:1 v/v, and partitioned by adding 0·2 volumes of distilled water.[6] The lower organic

phase containing the lipid fraction was removed and dried using nitrogen. The dried material was dissolved in chloroform and applied to a florisil:silicic acid (2:1 w/w) column. The neutral lipid fraction containing PDIM was eluted with two volumes of chloroform and taken to dryness under nitrogen. The PGL-I fraction was eluted by first passing two volumes of 2% methanol in chloroform through the column, followed by two volumes of 5% methanol in chloroform. The 2% and 5% eluates were combined and dried using nitrogen.

The neutral lipid fraction containing PDIM and the glycolipid fraction containing PGL-I, along with authentic standards of PGL-I or PDIM which served as carrier lipids and Rf markers, were applied to thin-layer chromatographic plates (Silica Gel 60, E. Merck AG, Darmstadt, Germany). Migration of the samples was induced in the appropriate solvent (PDIM: hexane:ether, 95:5 v/v; PGL-I ether:acetone, 80:20 v/v). The plates were air-dried and sprayed with a solution of 0·1% orcinol in 40% sulfuric acid. The lipid fractions were located by heating the plate in an oven at 110°C for 3–5 min or until the spots became visible. An area approximately 1 cm × 1 cm corresponding to the spotted PDIM or PGL-I standards for migration was scraped from the plate. Additional samples approximately 1 cm × 1 cm above and below the standard regions were also removed from the plates. After transferring the samples to glass vials, 10 ml of scintillation fluid (Econofluor, New England Nuclear, Boston, MA, USA), was added to each vial and the radioactivity was measured.

Results

OXIDATION OF PALMITIC ACID

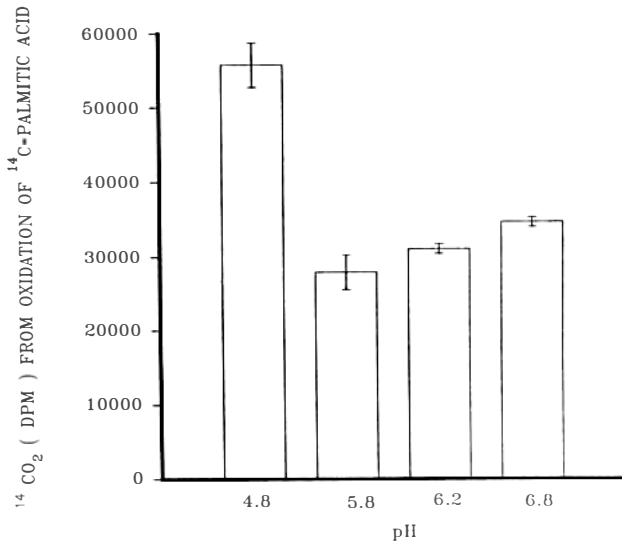The maximum release of $^{14}CO_2$ from the oxidation of ^{14}C-palmitic acid by *M. leprae*

Figure 1. $^{14}CO_2$ trapped in NaOH from the oxidation of ^{14}C-palmitic acid by 2×10^8 human-derived *M. leprae*. ^{14}C activity in control cultures ranged from 140 to 97 disintegrations per minute (N = 8, two values per pH assayed).
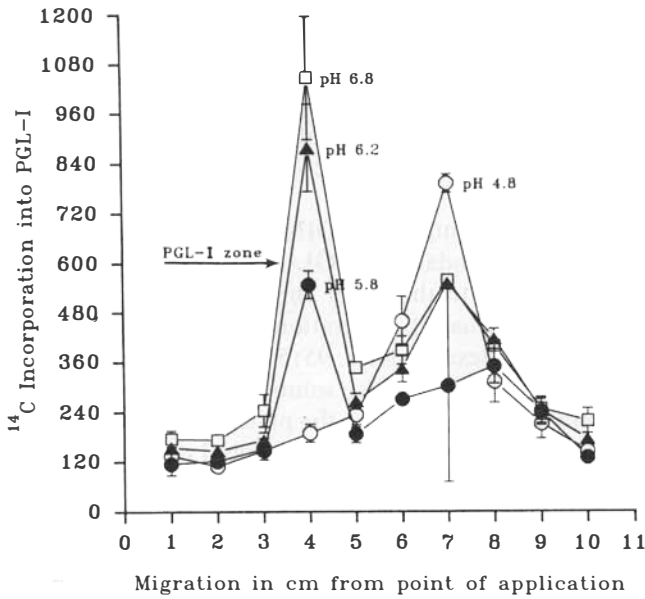
Figure 2. The effect of pH on the capacity of human-derived *M. leprae* to assimilate ^{14}C-palmitic acid into phenolic glycolipid-1 (PGL-I). Mean ± SD, N = 4.
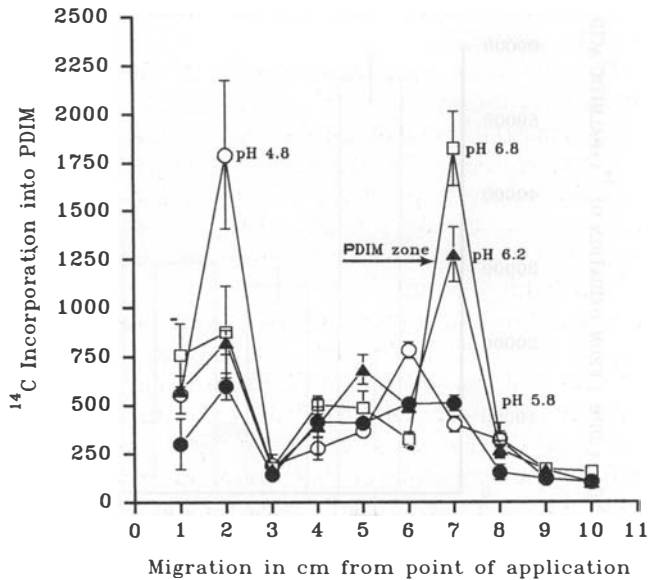

Figure 3. The effect of pH on the capacity of human-derived *M. leprae* to assimilate ^{14}C-palmitic acid into phthiocerol dimycocerosate (PDIM). Mean ± SD, N = 4.

occurred at a pH of 4·8. With an increase of 1 pH log unit to a pH of 5·8, the activity was reduced to approximately 50% (Figure 1).

SYNTHESIS OF PGL-I AND PDIM

The synthesis of PGL-I (Figure 2) and PDIM (Figure 3) increased progressively and significantly as the pH increased in the cultures. Maximum synthesis of ^{14}C-PGL-I and ^{14}C-PDIM occurred when the medium was buffered to maintain the highest pH (pH 6·8). A decrease by 1 pH log unit to a pH of 5·8 resulted in a reduction of greater than 50% in the synthesis of both ^{14}C-PGL-I and ^{14}C-PDIM. The synthesis of an unidentified lipid occurred at a pH of 4·8 (Figures 2 and 3).

Discussion

Utilization of radioactive palmitic acid as a substrate for metabolic activities of armadillo-or nude mouse-derived *M. leprae* has been demonstrated in axenic cultures[7–12] as well as a cell-culture system.[13] In the axenic culture systems, Franzblau[7] demonstrated the capacity of nude mouse-derived *M. leprae* to oxidize palmitic acid and release CO_2. The formation of ^{14}C-CO_2 from ^{14}C-palmitate has been described as the most easily detectable metabolic activity of *M. leprae*.[8] This activity is inhibited by the established antileprosy drugs and several other selected antimicrobial agents.[7,11] A limitation ascribed to the radiorespirometric procedure is the uncertainty about the enzymatic activity due to *M. leprae* rather than possible microbial contaminants or host tissues. This limitation has partially been overcome by the development of a radiometric procedure which measures the amount of ^{14}C-palmitate incorporated into the species-specific, phenolic glycolipid (PGL-I) unique to *M. leprae*.[8,9]

   The ability of armadillo- or nude mouse-derived *M. leprae* to catabolize (^{14}C-CO_2 release) or to anabolize (assimilation of ^{14}C-palmitate into PGL-I) in axenic medium is routinely employed in metabolic and drug-sensitivity experiments conducted at the Gillis W Long Hansen's Disease Center, Carville, LA, USA. Depending upon the nature of the study and the procedure used, the pH of the incubation mixture is adjusted accordingly. For maximum oxidation of ^{14}C-palmitate and subsequent release of ^{14}C-CO_2, a pH of 6·2 has been described.[7] Additionally, a pH of 5·1 has been determined to be optimal for the assimilation of ^{14}C-palmitic acid into PGL-I.[12]

   In this study, we employed a unique incubation system to assess the effect of pH on the utilization of ^{14}C-palmitate substrate for metabolic activities of human-derived *M. leprae*. A pH of 4·8 was optimal for the oxidation of ^{14}C-palmitic acid releasing ^{14}C-CO_2, whereas maximum assimilation of ^{14}C-palmitate into PGL-I and PDIM of *M. leprae* occurred at pH 6·8. When compared to nude mouse-derived *M. leprae*, there are discrepancies in the pH requirements for optimal metabolism of ^{14}C-palmitate by human-derived *M. leprae*. It is recognized that further experiments using human-derived *M. leprae* are needed to verify these results.

   Our findings demonstrate that human-derived *M. leprae* is competent to use ^{14}C-palmitic acid as a substrate for metabolic activity. Furthermore, a 7-day incubation period is ample for the distribution of radioactivity from palmitate into CO_2 and the

major lipid and glycolipid fractions of *M. leprae*. Although 2×10^8 AFB were used in each reaction vessel, the appreciable amount of radioactivity measured in each fraction suggests that lower numbers of *M. leprae* might also yield satisfactory results.

Drug effectiveness and the emergence of resistant organisms are two important factors that must be confronted in the treatment of leprosy. Until a medium capable of inducing multiplication and sustaining growth of *M. leprae* in axenic culture is developed, studies related to metabolism and drug sensitivity will of necessity depend upon tissue-derived organisms. The ease in detectability of the oxidation of palmitic acid to CO_2 and the specificity ascribed to the assimilation of palmitate into the complex lipids of *M. leprae* provide assays for the assessment of the metabolic integrity of the bacillus in a given culture environment. The ability to integrate both of these assays in a single incubation procedure provides a valuable tool for the investigator.

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Compétence de *Mycobacterium leprae* d'origine humaine pour utiliser l'acide palmitique dans la synthèse de phénol-glycolipide-I et de phthiocérol dimycocérosate et pour dégager du CO_2 en culture axénique

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Résumé Jusqu'à présent *Mycobacterium leprae* viable n'a pas été disponible en quantité suffisante, ce qui a entravé les études de métabolisme utilisant des souches de *M. leprae* d'origine humaine. Dans cette étude, nous avons obtenu des quantités suffisantes de *M. leprae* sur un patient lépromateux non traité pour titrer les effets du pH sur le métabolisme de l'acide ^{14}C-palmitique par *M. leprae*.

Le métabolisme catabolique (oxydation de l'acide ^{14}C-palmitique et dégagement de [14]CO_2) atteint son maximum lorsque *M. leprae* a été incubé à 33°C et mis en suspension dans le milieu de Middlebrook 7H9 additionné d'ADC et tamponné pour maintenir un pH de 4,8. Le métabolisme anabolique (synthèse de ^{14}C-phénol glycolipide-I et de son précurseur, ^{14}C-phthiocérol dimycocérosate) a atteint son maximum lorsque le pH a été maintenu à 6,8.

Competencia de *Mycobacterium leprae* de origen humano en el uso del ácido palmítico en la síntesis de glicolípido-I fenólico y dimicocerosato de ftiocerol y la liberación de CO_2 en un cultivo axénico

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Resumen Hasta ahora, han habido insuficientes cantidades de *Mycobacterium leprae* viables, lo cual ha impedido los estudios metabólicos con *M. leprae* de origen humano. En este estudio, se obtuvieron suficiente cantidad de un paciente lepromatoso sin tratar para titular los efectos del pH sobre el metabolismo del ácido ^{14}C-palmítico por *M. leprae*.

El metabolismo catabólico (oxidación del ácido ^{14}C-palmítico y la liberación de [14]CO_2) alcanzó un maximo cuando se incubó *M. leprae* a 33°C y se suspendió en un medio Middlebrook 7H9 suplementado con ADC y tamponado para mantener un pH de 4,8. El metabolismo anabólico (síntesis de glicolípido-I ^{14}C-fenólico y su precursor, dimicocerosato de ^{14}C-ftiocerol) llegó a un máximo cuando se mantuvo el pH en 6,8.