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Titration of numbers of human-derived *Mycobacterium leprae* required to progressively oxidize ^{14}C-palmitic acid and release $^{14}CO_2$

E. J. SHANNON*, D. FROMMEL†,
M. GUEBRE-XABIER†‡ & H. S. HAILE-MARIAM‡
**Gillis W. Long Hansen's Disease Center, Carville, Louisiana; †Armauer Hansen Research Institute, Addis Ababa; and ‡Ali Africa Leprosy Rehabilitation and Training Center, Addis Ababa, Ethiopia*

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Summary *Mycobacterium leprae* was isolated from skin-punch biopsies of 2 untreated lepromatous leprosy patients. The bacteria were enumerated, diluted 10-fold and cultured in Middlebrook 7H9 medium supplemented with albumin, dextrose, catalase and ^{14}C-palmitic acid. The cultures were incubated at 33°C in a modified Buddemeyer radiorespiratory detection vessel. Those cultures containing at least 10^7 mycobacteria demonstrated a progressive evolution of $^{14}CO_2$.

Introduction

Until a medium capable of inducing multiplication and sustaining growth of *Mycobacterium leprae* in culture is developed, studies related to the metabolism and drug sensitivity will of necessity depend on tissue-derived organisms. The availability of *M. leprae* from the tissues of infected armadillos[1] or nude mice[2] and the competency of *M. leprae* to oxidize palmitic acid to carbon dioxide[3] has facilitated the development of *in vitro* drug sensitivity studies of established and new antileprosy drugs.[4,5]

We have shown that human-derived *M. leprae* is capable of utilizing palmitic acid as a substrate in catabolic metabolism (oxidation and release of CO_2) as well as anabolic metabolism (synthesis of ^{14}C-phenolic glycolipid).[6] In this study a large leproma was surgically removed from the eyelid of an 18-year-old male Ethiopian, enabling 2×10^8 *M. leprae* to be used in each culture. However, the appreciable amount of radioactivity recovered in the CO_2 and phenolic-glycolipid-I fractions suggested that fewer numbers of *M. leprae* might also yield satisfactory results.

After alterations of a $^{14}CO_2$ detecting Buddemeyer-type incubation vessel, we implemented the modified vessel to determine the number of human-derived-*M. leprae* capable of releasing detectable amounts of $^{14}CO_2$ from oxidation of ^{14}C-palmitic acid.

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Materials and methods

PREPARATION OF INOCULUM

The skin surfaces of 2 untreated, clinically diagnosed, lepromatous leprosy patients attending the clinic at ALERT were treated with a solution of 1% iodine in 70% ethanol. Usually 2, 4 or 6 mm punch biopsies were taken after the injection of local anaesthetic. After dissecting a small section for histological staining, the biopsies were weighed and the tissues separated at the dermal epidermal junction. Then, using a mortar and pestle, the dermis was homogenized in Middlebrook 7H9 broth (Difco, Detroit, Michigan, USA). The suspension was sonicated in an ice bath for 2 min (Sonifer, B-13 Branson Ultrasonics Corp., Danbury, Connecticut, USA). The large tissue debris was allowed to settle at $1 \times g$ for 3–5 min. The tissue remaining in suspension was centrifuged at $2000 \times g$. The pellet was suspended in 7H9 broth and the number of acid-fast bacilli (AFB) and the number of solid-staining bacteria (morphological index—MI) was determined.[7]

PREPARATION OF CULTURES AND CULTURE VESSELS

The enumerated *M. leprae* were diluted in 7H9 Middlebrook broth that had been supplemented with 10% v/v albumin-dextrose-catalase (ADC). The 7H9-ADC medium also contained 50 μg of ampicillin/ml and 2·5 μg amphotericin-B/ml. The AFB were diluted in 10-fold dilutions and placed in sterile Nunc tubes (Roskilde, Denmark). The

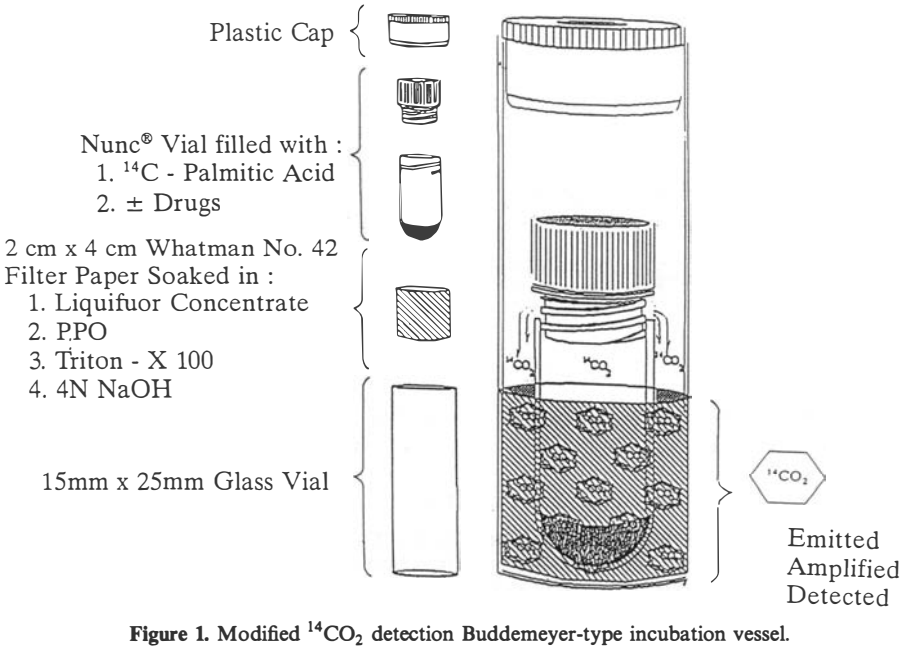

Figure 1. Modified $^{14}CO_2$ detection Buddemeyer-type incubation vessel.

tubes, with their caps tightened, were incubated at 33°C in an incubator with high humidity. On the 5th day we added 1·0 μCi of [1-^{14}C] palmitic acid (59 mCi/mmol; Amersham International plc, Buckinghamshire, UK) to each 1·0 ml of culture. The tubes with loosened caps were placed within 15×25 mm glass vials containing a dried strip of Whatman DE42 filter paper (Whatman, Inc., Clifton, New Jersey, USA). The paper had previously been dipped into a slurry of Liquifluor concentrate PPO-POPOP [2,5 diphenyloxazole-1,4-bis(5-phenyloxazoly) benzene] toluene concentrate (New England Nuclear, Boston, Massachusetts, USA); Triton X-100 and 4N NaOH.[3] The glass scintillation vials were sealed and the assembled culture vessels were returned to incubate at 33°C. The $^{14}CO_2$ captured on the filter paper was measured immediately after the addition of ^{14}C-palmitic acid and then daily using a RackBeta Liquid Scintillation Counter (LKB, Pharmacia, Uppsala, Sweden).

Results

Using the modified Buddemeyer-type detection system as illustrated in Figure 1 and in cultures containing 10^7 mycobacteria, the amount of $^{14}CO_2$ captured was proportional to the number of AFB/ml and to the time of incubation (Figures 2 and 3).

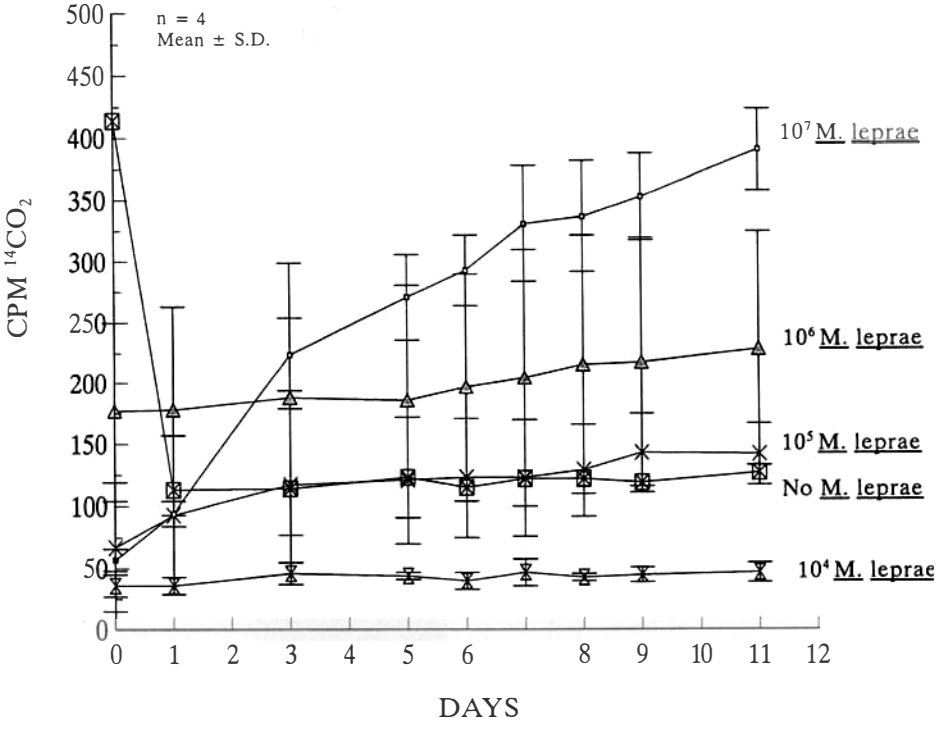

Figure 2. A 6 mm skin punch biopsy was obtained from a 35-year-old female, untreated, histologically confirmed BL/LL leprosy patient. The mean Bacterial Index from 6 sites was 5 and the Morphologic Index was 0·0; 2 × 10^8 acid-fast bacteria were recovered from the biopsy and the Morphologic Index was 0·0. On day 0, 1 μCi of ^{14}C-palmitic acid was added to each ml of Middlebrook 7H9 broth supplemented with albumin-dextrose catalase.

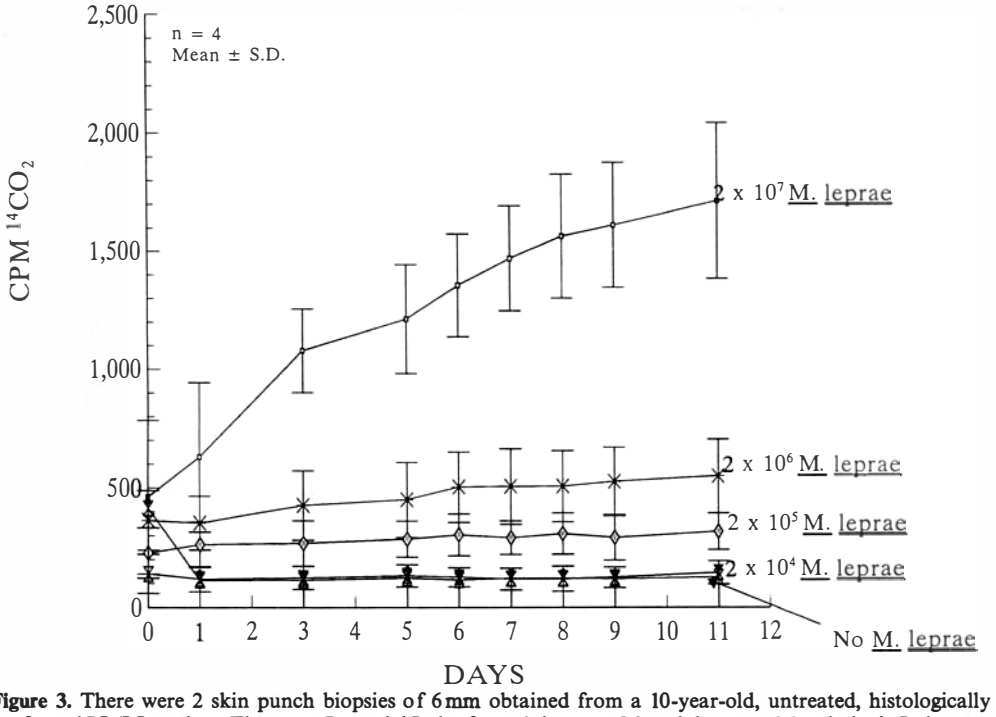

Figure 3. There were 2 skin punch biopsies of 6 mm obtained from a 10-year-old, untreated, histologically confirmed BL/LL patient. The mean Bacterial Index from 6 sites was 5·0 and the mean Morphologic Index was 0·6%; $3{\cdot}6 \times 10^8$ acid-fast bacteria with a Morphologic Index of 3% were recovered from the biopsy. There was 1 μCi of ^{14}C-palmitic acid added, as described in Figure 2.

Discussion

Inability to cultivate *M. leprae in vitro* continues to hinder leprosy research and leprosy control. A knowledge of the drug susceptibility of clinical isolates of *M. leprae* is vital to an effective control of leprosy. Clinical isolates of *M. leprae* are tested for drug sensitivity at AHRI and ALERT in a mouse footpad system. This technique is laborious and expensive, involving 6–12 months maintenance of mice as well as gram amounts of compounds. Reliable and rapid *in vitro* radiorespirometric methods (i.e. modified-Buddemeyer-type and BACTEC 640 systems) that measure, in the absence or presence of drugs, the catabolic (palmitate oxidation) capacity of *M. leprae* derived from nude mice have been developed by Franzblau *et al.*[8]

This study demonstrated that *M. leprae*, recovered from skin-punch biopsies of 2 untreated lepromatous patients, had sufficient numbers of bacteria to oxidize ^{14}C-palmitic acid in axenic culture. To detect progressive evolution of $^{14}CO_2$ the modified Buddemeyer-type assay required a minimum of 10^7 human-derived AFB. This is equal to the number of nu/nu-mouse-derived *M. leprae* used in the BACTEC system and 10-fold more than the 10^6/ml nu/nu-derived bacilli used in the Buddemeyer system.[8] To ensure sensitivity of the assay $2{\cdot}5 \times 10^7$ human-derived AFB are recommended. From a retrospective study, the number of *M. leprae* recovered from skin biopsies of clinically suspected untreated lepromatous patients attending the clinics at ALERT ranged from

Table 1. Recovery of human-derived AFB from skin biopsies of untreated multibacillary patients

AHRI Patient No.	Weight of punch biopsy in mg	Mean from 6 sites		MI of acid-fast bacilli in processed skin biopsy	No. of bacilli$\times 10^8$ recovered
		BI*	MI†		
739	ND	5·0	2·2	1·0	1·6
740	ND	2·7	0·0	1·0	5·7
752	120	4·2	0·5	1·2	6·2
783	170	3·0	0·0	6·0	4·4
853	330	3·8	0·0	3·0	5·2
873	420	4·2	0·3	4·0	0·6
881	111	4·0	0·3	4·0	6·8
817	360	5·0	1·0	6·0	13·0
917	160	4·2	0·0	3·0	0·5
919	280	4·0	0·3	8·0	68·0
924	230	4·0	1·0	4·0	4·7
959	260	3·0	0·0	0·0	1·1
960	170	4·3	0·0	0·0	6·9
962	ND	2·0	0·0	0·0	0·8
978	160	4·5	0·7	4·5	8·4
1022	70	4·3	0·0	3·0	1·8
Mean	219	3·9	0·39	3·0	8·5
SD	106	0·8	0·6	2·4	16·2

* BI, bacteriologic index.
† MI, morphologic index.

68×10^8 to $0{\cdot}6 \times 10^8$, with an average of $8{\cdot}5 \times 10^8$ AFB (Table 1). Based on these recovery data, the average radiorespirometric assay could employ 34 cultures of 1·0 ml, containing $2{\cdot}5 \times 10^7$ bacilli.

This type of assay would facilitate the rapid determination of the partial range of sensitivity of the *M. leprae* to established antileprosy drugs as well as the assessment of the efficacy of several promising new drugs.

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