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Oxidation of Palmitic Acid by *Mycobacterium leprae* in an Axenic Medium

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The ability of *Mycobacterium leprae* to oxidize palmitic acid during incubation in an axenic medium was studied. By using a Buddemeyer-type detection system, partially purified nude-mouse-derived *M. leprae* was found to produce $^{14}CO_2$ from ^{14}C-labeled palmitic acid in a linear fashion for at least 1 week. Procedures known to remove residual host tissue did not diminish the rate of $^{14}CO_2$ evolution, indicating that bacterial metabolism was being measured. Palmitate oxidation was temperature sensitive, with an apparent optimum of 33°C, but pH insensitive. Bacilli exposed to a variety of antileprosy drugs for 1 or 2 weeks displayed significantly reduced rates of $^{14}CO_2$ evolution upon subsequent addition of ^{14}C-labeled palmitic acid. This activity could be readily detected with 10^6 bacilli, thus indicating its potential for use in clinical susceptibility testing.

The lipid-rich mycobacteria are known to readily metabolize a variety of fatty acids (3). Indeed, the oxidation of palmitic acid (PA) to carbon dioxide has become the basis of a rapid means of detection and drug susceptibility testing of *Mycobacterium avium-M. intracellulare* (19) and *Mycobacterium tuberculosis* by the BACTEC method (4, 5, 7, 10, 16). A number of studies have demonstrated a marked superiority of this radiometric assay over culture techniques, especially with regard to time.

For *M. leprae*, which has yet to be cultivated in vitro, growth in the mouse footpad remains the only accepted method for drug susceptibility testing, requiring 6 to 12 months to complete at considerable cost (14). In contrast to cultivable species, relatively few metabolic activities have been described in *M. leprae*, a task complicated by both the relative scarcity of viable bacilli and the need for differentiation between bacillary and host tissue-derived activity (17, 18).

Co-workers and I have recently described the effect of in vitro cultural conditions and antileprosy drugs on intracellular ATP levels (6) and on the incorporation of [U-^{14}C]PA into the unique phenolic glycolipid I of *M. leprae* (S. G. Franzblau, E. B. Harris, and R. C. Hastings, FEMS Microbiol. Lett., in press). While these metabolic assays appear to be useful for identifying conditions for metabolic stabilization and potential antileprosy compounds, these assays would probably not be readily adaptable for routine use in clinical susceptibility testing. In this paper, I describe the evolution of $^{14}CO_2$ from [^{14}C]PA by *M. leprae* in an axenic medium and the sensitivity of this activity to antileprosy drugs.

MATERIALS AND METHODS

Inoculum preparation. *M. leprae* suspensions were prepared as previously described (6). Briefly, footpads of athymic nude mice, infected 6 to 18 months previously, were surface decontaminated by using UV irradiation, ethyl ether, 1% iodine, and 70% ethanol. Footpads were then minced and manually homogenized in Dubos broth base without polysorbate 80 (GIBCO Diagnostics, Madison, Wis.), without pH adjustment containing 20% (vol/vol) Dubos medium albumin (Difco Laboratories, Detroit, Mich.), 50 μg of ampicillin per ml, and 2.5 μg of amphotericin B per ml. These antibiotics have shown no effect on ATP levels (6) and PA oxidation (unpublished observations) in *M. leprae*. Complete medium is referred to as Dubos albumin medium (DA). Bacilli were partially purified by slow-speed centrifugation (108 × *g*, 5 min, 10°C) to remove tissue debris, high-speed centrifugation of the supernatant (2,710 × *g*, 45 min, 10°C) to pellet bacilli, and resuspension of the pellet in 10 ml of DA. Subsamples were inoculated into a variety of culture media to confirm that contaminating bacteria were not present. In some cases, bacilli were further purified by a slight modification of a previously described procedure (15). Briefly, after slow-speed centrifugation of the homogenate, trypsin, collagenase, DNase, and elastase were added to the supernatant and incubated at 33°C for 1 h with intermittent mild agitation. Aliquots (1 ml) were then layered over two-step discontinuous Percoll gradients, consisting of a cushion of 2 ml of 100% Percoll and 8 ml of 50% Percoll in 0.2× phosphate-buffered saline. Gradients were centrifuged at 2,710 × *g* for 1.5 h at 15°C. The lower bands were removed from the interface and diluted with DA, and the bacilli were pelleted at 2,710 × *g* for 30 to 45 min at 10°C and washed two times in the same manner. Cell counts were determined by direct microscopy of Ziehl-Neelsen-stained preparations by the method of Shepard and McRae (14).

Incubation conditions and $^{14}CO_2$ assay. Inocula were diluted into DA or DA containing 50 μg of unlabeled PA per ml (DAP) as indicated below. Aliquots (1 ml) were distributed to 6-ml screw-cap vials (Wheaton Scientific, Millville, N.J.), and [U-^{14}C]PA (850 mCi/mmol; New England Nuclear Corp., Boston, Mass.) or [1-^{14}C]PA (5.7 mCi/mmol; ICN Pharmaceuticals, Inc., Irvine, Calif.) was added. Evolution of $^{14}CO_2$ was measured as described by Buddemeyer et al. (1, 2). Briefly, the vials, with caps loosened, were placed inside wide-mouthed scintillation vials (Poly-Q; Beckman Instruments, Brea, Calif.), where they were surrounded by a hollow cylinder of Whatman no. 1 filter paper (Whatman, Inc., Clifton, N.J.). The paper had been previously dipped into a concentrated liquid scintillation counting solution (Liquiflor PPO-POPOP [2,5-diphenyloxazole–1,4-bis(5-phenyloxazolyl)benzene] toluene concentrate; New England Nuclear), dried, and placed within the scintillation vial, and

then 100 μl of 2 NaOH was applied. The scintillation vial cap was then firmly tightened, and the assembly was incubated at 33°C, except as indicated. Cumulative $^{14}CO_2$ production was measured, usually at 24-h intervals, in a Beckman model LS-5801 liquid scintillation counter by using the ^{14}C window. In experiments to assess the effect of drugs or incubation parameters, the bacterial suspension was distributed into 6-ml vials or 1.5-ml screw-cap Microfuge tubes. Dapsone, ethionamide, rifampin, and erythromycin were obtained from the Sigma Chemical Co. (St. Louis, Mo.), and clofazimine was obtained from J. F. O'Sullivan, Trinity College, Dublin, Ireland. Drugs were added in a volume of 10 μl to achieve a final concentration of 2 μg/ml. Vessels were incubated with loosened caps at 33°C within a chamber receiving constant ventilation with an aquarium air pump. After an appropriate incubation interval, [^{14}C]PA was added, and the vials were transferred to the scintillation vials containing the pretreated filter paper.

ATP assay. ATP was determined by the firefly bioluminescence technique by a slight modification (6) of the method described by Kvach et al. (8).

Statistical evaluation. Student's *t* test was used to evaluate statistical significance. *P* values were calculated with mean counts per minute at the time intervals indicated.

RESULTS

Kinetics of $^{14}CO_2$ evolution and effect of host tissue. Preliminary experiments examined the effect of unlabeled PA on the evolution of $^{14}CO_2$ from 0.5 μCi of [U-^{14}C]PA and used sucrose gradient-purified (11), armadillo-derived *M. leprae* at a density of 1.3×10^9/2-ml sample in DA at pH 5.5. Evolution of $^{14}CO_2$ was roughly linear for at least 6 days, and the addition of unlabeled PA reduced $^{14}CO_2$ evolution in a concentration-dependent manner (Fig. 1).

All subsequent experiments used nude-mouse-derived suspensions of *M. leprae*, partially purified by differential centrifugation (DC) (6), and also produced $^{14}CO_2$ from [^{14}C]PA in a linear fashion for at least 6 days when incubated at 33°C. To determine whether residual footpad tissue debris contributed to this activity, the rate of $^{14}CO_2$ evolution of a DC preparation was compared with those of the following preparations: (i) the resuspended high-speed pellet of the original homogenate (insoluble tissue debris not removed by a prior slow-speed centrifugation); (ii) an identical preparation (as above) but treated with 1 N NaOH for 1 h at room temperature, followed by two washings and suspension in DA (a suspension of bacilli devoid of host tissue enzymatic activity); and (iii) a DC preparation which had been boiled for 10 min (i.e., a preparation devoid of all enzymatic activity). All suspensions were adjusted to yield 10^8 bacilli per ml. Although there was not a significant difference between the rate of $^{14}CO_2$ release in the DC and NaOH-treated preparations, both of these possessed significantly higher activity than the original crude homogenate (Fig. 2). The heated preparation was essentially devoid of activity. A subsequent experiment compared $^{14}CO_2$ evolution from [U-^{14}C]PA in a DC preparation, a DC preparation treated briefly with 0.1 N NaOH, and a Percoll-purified preparation, with means ± standard deviations of the counts per minute detected after 113 h of incubation of these preparations of 1,570 ± 125, 4,287 ± 356, and 16,649 ± 4,564, respectively.

Effect of biophysical parameters. Incubation at 37°C resulted in a highly significant reduction in the rate of $^{14}CO_2$ production from 0.25 μCi of [U-^{14}C]PA at pH values ranging from 5.2 to 7.3 (Table 1), whereas there was not a marked difference among cells incubated at 28 or 33°C over this pH range. A subsequent experiment (data not shown) in which cells were incubated for over 2 weeks at 33°C at a pH range of 5.1 to 6.8 confirmed the relative insensitivity of this activity to pH.

Effect of antileprosy drugs. Preliminary experiments with antileprosy drugs in DA at pH 5.5, in which [^{14}C]PA was added at the onset of the experiment, failed to show any effect on the rate of $^{14}CO_2$ release (data not shown). Subsequently, a 2-week preincubation of 2.4×10^7 Percoll-purified *M. leprae* with dapsone, rifampin, or clofazimine at 2 μg/ml in DAP was used. Suspensions were incubated in 1.5-ml screw-cap Microfuge tubes with caps loosened under constant humidified ventilation. After two washings and suspension in DAP without antileprosy drugs, the suspensions were transferred to 6-ml vials, 0.25 μCi of [U-^{14}C]PA was added, and the release of $^{14}CO_2$ was monitored as described above. Although the absolute rates of $^{14}CO_2$ evolution were low, due in part to a loss of cells during washings (unpublished observations), $^{14}CO_2$ evolution in all drug-treated suspensions was significantly lower ($P < 0.02$) than that of drug-free cells (Table 2). In addition, parallel responses were observed with respect to intracellular ATP content, with rifampin having the greatest effect.

A second experiment used DC-prepared *M. leprae* at 10^6/ml in DA with antimicrobial agents added at a final concentration of 2 μg/ml. Suspensions were incubated in 6-ml vials as described above. A 1-μCi amount of [1-^{14}C]PA was added directly to the vials after 6 days of incubation, and the vials were transferred to the counting system. All drugs effected a significant reduction ($P \leq 0.006$) in $^{14}CO_2$ evolution (Fig. 3). In addition, the drug-related reduction in the rate of $^{14}CO_2$ evolution was concentration dependent (Fig.

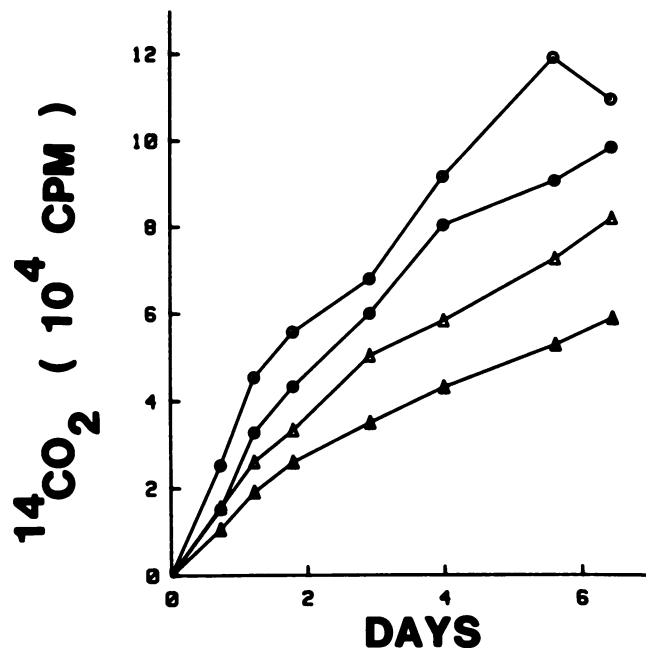

FIG. 1. Kinetics of $^{14}CO_2$ evolution from [U-^{14}C]PA by sucrose-KCl gradient-purified, armadillo-derived *M. leprae* (11), Competition by 0 (○), 5 (●), 25 (△), and 50 μg (▲) of unlabeled substrate per ml. Samples contained 1.3×10^9 *M. leprae* in 2 ml of DA at pH 5.5 containing 0.5 μCi of label. Data points represent means of triplicate samples.

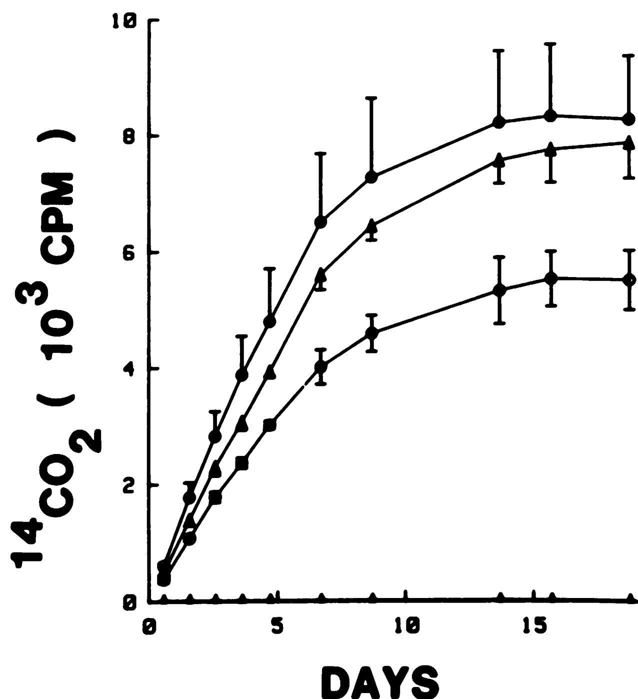

FIG. 2. Effect of inoculum purification method on rate of $^{14}CO_2$ evolution from [U-^{14}C]PA by nude-mouse-derived *M. leprae*. The cells were purified by DC (△), treatment with 1 N NaOH for 1 h (●), and heating at 100°C for 10 min (▲). ○, Crude homogenate. Samples contained 10^8 *M. leprae* in 1 ml of DAP containing 0.4 μCi of label. Data points represent means of triplicate samples.

4). In contrast to experiments with [U-^{14}C]PA, background activity of heat-killed bacilli was repeatedly observed when [1-^{14}C]PA was used. Activity was detectable immediately after addition of labeled substrate but did not increase during incubation. A subsequent experiment (data not shown) was performed in an identical manner, except that 1, 2, or 5 ml of BACTEC 12B medium (Johnston Laboratories, Towson, Md.) was added as a source of [1-^{14}C]PA after 6 days of incubation. Essentially identical results were obtained.

DISCUSSION

These studies clearly demonstrate that viable *M. leprae* is capable of oxidizing PA. Presumably, PA is first incorporated into triglycerides (9) but may ultimately be incorporated into a variety of other compounds (12). Carbon dioxide may be evolved in condensation reactions during the synthesis of longer-chain fatty acids, oxidation of compounds into which PA has been incorporated, or beta-oxidation of PA, yielding acetyl coenzyme A, which may then be oxidized in the tricarboxylic acid cycle (12). Alkaline treatment (5, 17, 18) and purification on Percoll gradients (8, 15) are both known to be effective in removing contaminating host tissue debris from homogenates of *M. leprae*. Both of these procedures, as well as DC, did not result in a loss of activity but in fact appeared stimulatory, possibly due to removal of competing tissue-associated fatty acids. In addition, studies in our laboratory (unpublished observations) indicate an insignificant level of activity associated with uninfected footpads processed by DC.

The sensitivity of PA oxidation to 37°C is consistent with in vivo observations (13) and our observations on the in vitro reduction of ATP content and phenolic glycolipid I synthesis in *M. leprae* at this temperature (compared with that found at 33°C). As such, it suggests the possibility of using this assay to assess viability in general. However, its relative insensitivity to medium pH is in sharp contrast to the pH sensitivity of in vitro phenolic glycolipid I synthesis and ATP content (S. G. Franzblau and E. B. Harris, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, U-7, p. 127).

TABLE 1. Effect of incubation temperature and medium pH on rate of $^{14}CO_2$ evolution from [U-^{14}C]PA

pH	Cumulative $^{14}CO_2$ evolution[a] (cpm) at:		
	28°C	33°C	37°C
5.2	1,772 ± 46	1,689 ± 107	327 ± 101
6.2	1,558 ± 40	1,947 ± 160	1,003 ± 71
7.3	1,253 ± 38	1,612 ± 231	846 ± 253

[a] Mean ± standard deviation of triplicate samples. Reading taken at 86 h postincubation.

TABLE 2. Effect on in vitro exposure to antileprosy drugs on PA oxidation and ATP in *M. leprae*[a]

Drug (2 μg/ml)	Palmitate oxidation[b] (cpm)	ATP content (pg/10^6 bacilli)
None	321 ± 97	423 ± 145
Dapsone	116 ± 9	181 ± 54
Clofazimine	43 ± 4	116 ± 57
Rifampin	18 ± 1	33 ± 8

[a] Cells were preincubated for 2 weeks in DAP with or without drugs.
[b] Mean ± standard deviation of triplicate or quadruplicate samples over 89 h of incubation.

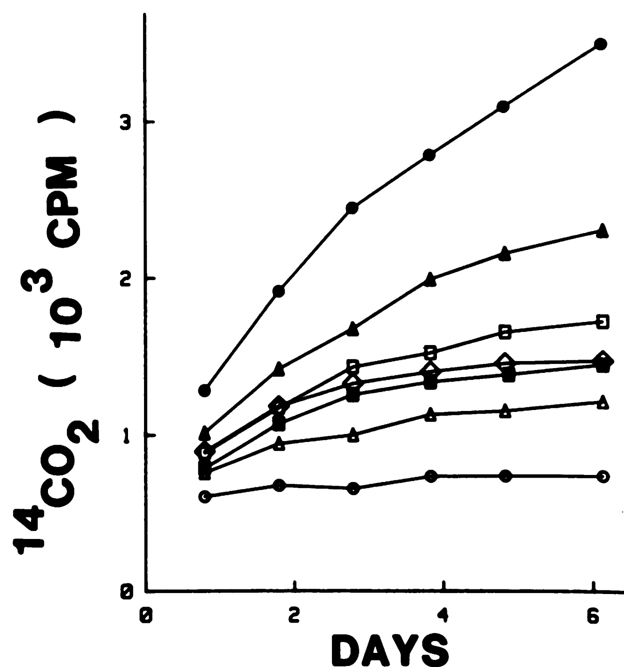

FIG. 3. Effect of antileprosy drugs on rate of $^{14}CO_2$ evolution from [1-^{14}C]PA. *M. leprae* cells (10^6) were preincubated for 6 days in 1 ml of DA without drugs (●) or with 2 μg of dapsone (▲), ethionamide (□), erythromycin (◇), rifampin (■), and clofazimine (△) per ml before addition of 1 μCi of substrate. ○, Heat-killed cells. Data points represent means of quadruplicate samples.

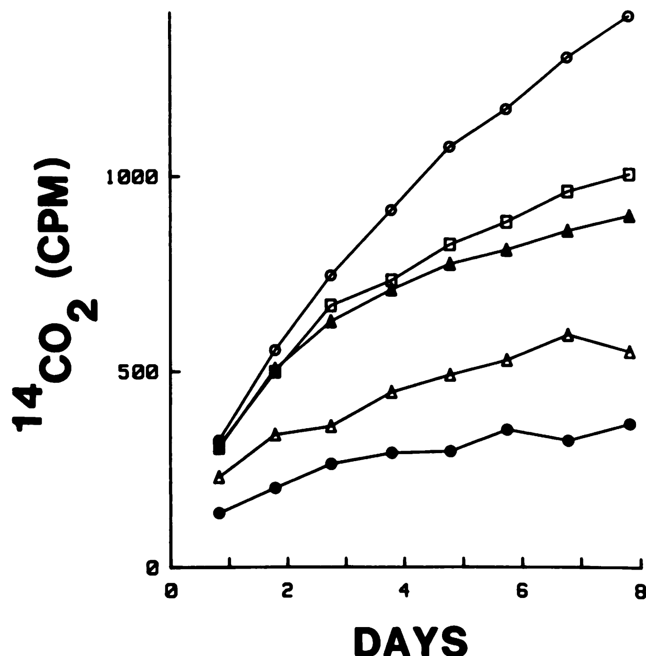

FIG. 4. Concentration-dependent reduction in $^{14}CO_2$ evolution from [1-^{14}C]PA by dapsone. *M. leprae* cells (10^6) were preincubated for 6 days in 1 ml of DA without drugs (○) or with 31 (□), 125 (▲), 500 (△), or 2,000 (●) ng of dapsone per ml before addition of 1 μCi (58 mCi/mmol) of substrate.

The sensitivity of PA oxidation to established antileprosy drugs and erythromycin is consistent with the in vitro effect of these compounds on ATP content in *M. leprae* (6; Table 2). This raises the prospect of using this assay, along with ATP measurement, as a simple, rapid method of identifying new antileprosy drugs. In addition, and perhaps more significantly, the ability to determine drug susceptibility within 7 to 10 days of 10^6 bacilli prepared by DC suggests the possibility of detecting secondary drug resistance in bacilli recovered from punch skin biopsies of lepromatous patients. This would represent a major clinical advance over the present lengthy and costly mouse footpad assays.

Use of the BACTEC ionization chamber for drug susceptibility testing of *M. leprae* would necessarily involve some procedural changes, as this system involves inoculation of cultivable mycobacteria into medium containing [1-^{14}C]PA at the onset of the incubation period and then relies on growth-dependent increases in the rate of PA oxidation (in controls and drug-resistant bacilli). A preliminary attempt to detect a drug-related reduction in $^{14}CO_2$ evolution by *M. leprae* in this system was unsuccessful, possibly due to the need for preincubation of bacilli and drugs before addition of labeled substrate. Further studies should determine the potential for using the BACTEC system.

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