J. Biosci., Vol. 14, Number 1, March 1989, pp. 37–45. © Printed in India.

Correlation of bacterial viability with uptake of [^{14}C] acetate into phenolic glycolipid-1 of *Mycobacterium leprae* within Schwannoma cells

Y. MISTRY, N. H. ANTIA† and R. MUKHERJEE*
The Foundation for Medical Research, 84-A, R. G. Thadani Marg, Worli, Bombay 400 018, India
*Present address: Department of Microbiology, National Institute of Immunology, J. N. U. Complex, Shahid Jeet Singh Marg, New Delhi 110 067, India

MS received 23 August 1988; revised 8 February 1989

Abstracts. The viability of *Mycobacterium leprae,* maintained within 33B Schwannoma cells, was estimated in terms of incorporation of [^{14}C] acetate into its specific phenolic glycolipid-1. This measure of viability was correlated with two other assays, *viz.,* fluorescein diacetate/ethidium bromide staining and mouse footpad growth. Observation of a 2-fold increase in the number of intracellular *Mycobacterium leprae* over an experimental period of 12 days also corroborated this contention. Furthermore, on addition of anti-leprosy drugs to these intracellular *Mycobacterium leprae* there was significant decrease in phenolic glycolipid-1 synthesis indicative of loss of viability of the organisms. This study also established the importance of the host cell for active bacillary metabolism, as *Mycobacterium leprae* maintained in cell-free conditions showed no incorporation into phenolic glycolipid-1. Moreover, compromising the host's protein synthesis capacity with cycloheximide, also led to reduction in bacillary metabolism. As this system measures the metabolic synthesis of a unique *Mycobacterium leprae* component, it would be useful for development and screening of compounds acting against specific bacillary targets.

Keywords. *Mycobacterium leprae*; *Schwannoma* cell line; lipid metabolism.

Introduction

Mycobacterium leprae, an intracellular parasite, displays a unique affinity for the Schwann cells of the peripheral nerves (Iyer, 1965; Job, 1979) which they infect and in which they multiply. *In vitro* studies have been successful in demonstrating the limited multiplication of *M. leprae* within murine Schwann cells (Mukherjee and Antia, 1985). Within such host cells, *M. leprae* synthesize a specific component, phenolic glycolipid-1 (PGL-1), a dimycocerosyl phthiocerol with a unique trisaccharide component (Hunter and Brennan, 1981). This has been demonstrated in macrophages (Ramasesh *et al.,* 1987) and in a glial cell line (Mukherjee *et al.,* 1985). A distinct advantage of monitoring PGL-1 synthesis is that it is unique to the bacillus and can be readily distinguished from contaminating host components. However, it was imperative to first determine whether the synthesis of PGL-1 as measured by incorporation of radioactive acetate into the molecule reflects viability of the intracellular bacilli.

†To whom all correspondence should be addressed.
Abbreviations used: PGL-1, Phenolic glycolipid-1; DDS, 4,4′-diaminodiphenyl sulphone; RFP, rifampicin; FDA, fluorescein diacetate; EB, ethidium bromide; MFP, mouse footpad; DMEM, Dulbecco's minimum essential medium; FCS, foetal calf serum; dpm, disintegration per minute; ELISA, enzyme-linked immunosorbent assay; GFB, green fluorescing bacilli; AFB, acid fast bacilli.

In the present study, therefore, incorporation of [^{14}C]acetate into PGL-1 of intracellular *M. leprae* has been assessed as a measure of bacterial viability. Schwannoma cells were selected as hosts in preference to normal Schwann cells, for the availability of bulk material. Synthesis of PGL-1 was also studied in bacilli whose viability was compromised by addition of both bacteriostatic and cidal drugs, *viz.,* 4,4'-diaminodiphenyl sulphone (DDS) and rifampicin (RFP). The viability of *M. leprae* as estimated by incorporation of ^{14}C-acetate into PGL-1 was correlated with other tests established for bacterial viability *viz.,* fluorescein diacetate/ethidium bromide (FDA/EB) staining and mouse footpad (MFP) growth. Further, the role played by the host cells in the maintenance of activity of these intracellular pathogens was examined by inhibiting their protein metabolism using cycloheximide. Results indicated that incorporation of ^{14}C-acetate into PGL-1 correlated well with viability of the organism; this phenomenon being depressed in nonviable, drug-treated bacilli.

Materials and methods

Chemicals.

The culture medium, Dulbecco's minimum essential medium (DMEM) and foetal calf serum (FCS) were purchased from GIBCO Laboratories, USA, while nutrient agar and Lowenstein-Jensen media were obtained from Hi-media, Bombay. Silicic acid was from Loba-Chemie Indoaustranal Co., Bombay, and silica gel from Acme Synthetic Chemicals, Bombay. Florisil (100-200 mesh), RFP and cycloheximide were purchased from Sigma Chemical Co, St. Louis, Missouri, USA. DDS was obtained from Burroughs Wellcome Co., Bombay. All the solvents used were of Analar grade obtained from Glaxo Laboratories, Bombay.

Host cells

The 33B glial cell line originally derived from an ethylnitrosurea-induced tumour in Wistar-Furth rats (Fields *et al.,* 1975) maintained in our laboratory was used as the host cell. These cells were maintained in a growth medium consisting of DMEM containing 10% FCS and antibiotics. When these cells formed a confluent sheet, the cultures were irradiated at 4000 rads to stop further multiplication.

Inoculation of M. leprae

A suspension of *M. leprae* was prepared from infected armadillo tissue by homogenization and sequential centrifugation to minimize tissue debris (Ambrose *et al.,* 1978). Bacillary count was determined using the method described by Hart and Rees (1960). The presence of other mycobacterial contaminants was checked by inoculation onto nutrient agar and Lowenstein-Jensen media.

Irradiated Schwannoma cultures were inoculated with freshly isolated *M. leprae* (5×10^6/ml) and incubated at 37°C for 72h which was found optimal for phagocytosis. The unphagocytosed *M. leprae* were then removed by repeated

washing. One set was inoculated with bacilli which were killed by formalin-fixation for 24 h.

Another control used was freshly harvested *M. leprae* maintained in the above mentioned culture medium consisting of FCS-enriched DMEM in the absence of host cells. These bacilli were incubated with 0·5 μCi/ml [^{14}C] acetate for the same duration of 12 days at 37°C.

Radioactive counts from cultures not infected with *M. leprae* were subtracted from those of experimental cultures, to minimize host contamination.

Acetate incorporation

After removal of non-phagocytosed *M. leprae,* [1,2-C-14] acetate (0·5 μCi/ml) (LCC-161, sodium acetate, sp. act. 60·3 mCi/mmol, Bhabha Atomic Research Centre, Bombay) was added to all cultures. The culture medium containing radiolabelled acetate was replaced twice a week and cultures were maintained for a total period of 12 days.

Addition of drugs

Prior to the addition of radiolabelled precursor to the *M. leprae* infected 33B cell cultures, anti-leprosy drugs, *viz.,* DDS (5 μg/ml) and RFP (5 μg/ml) were added to their respective cultures which were then incubated at 37°C for 12 days in the presence of [^{14}C] acetate.

Effect of cycloheximide

Cultures infected with *M. leprae* were treated with 3 μg/ml cycloheximide along with [^{14}C] acetate. Cultures not infected with M. *leprae* served as controls. All cultures were fed twice a week, each time with fresh drug and label and maintained for 12 days.

Incorporation into PGL-1 was compared between untreated and drug or cyclo-heximide-treated cultures.

Termination and processing of cultures

For termination of the cultures, the cells were detached from the culture surface using 0·25% trypsin. The bacilli were released by sonication at 23 KHz in a Soniprep 150 ultrasonic disintegrator for 2 min. The host cell debris was removed by centri-fugation at 220 g for 10 min. The supernatant obtained was centrifuged at 10,000 *g* for 45 min to obtain the *M. leprae* in an enriched form, relatively free from tissue contamination. This bacillary pellet was washed twice with phosphate-buffered saline before estimating the bacillary count. Control cultures were processed identically.

Lipid extraction and estimation

Total lipids were extracted by the method of Bligh and Dyer (1959). These extracts

were dried, suspended in a minimal volume of chloroform and loaded onto a silicic acid:florisil (2:1) column. The column was eluted with 2 bed volumes each of chloroform to remove out the neutral lipids and 2 and 5% methanol in chloroform, to obtain the glycolipids· These latter fractions contained the PGL-1 component. This eluant was dried and resolved by thin-layer chromatography (TLC) on silica gel G coated plates (thickness 0·5 mm) using chlorofom: methanol: water (90: 10:1, v/v/v) as the solvent system. Incorporation into PGL-1 was estimated from the spot with an R_f value corresponding to standard PGL-1 on a Kontron 300 Scintillation counter. Counts were expressed as disintegration per minute (dpm) for 10^6 bacilli.

Purification and characterization of PGL-1

PGL-1 was extracted in larger quantities by preparative TLC. Its presence was confirmed using α-naphthol spray reagent following TLC. Authenticity of this component was determined by (i) indirect immunofluorescence staining using the monoclonal antibody raised against the specific trisaccharide moiety (antibody 46·7 from Dr. Bloom, Albert Einstein, USA), (ii) enzyme-linked immunosorbent assay (ELISA) using the above mentioned antibody and (iii) comparison of IR absorbtion spectra, carried out on a Perkin-Elmer 684 spectrophotometer using methylene chloride as the solvent (Hunter and Brennan, 1981).

Visual quantitation

33B cultures on coverslips were infected with $0{\cdot}5\times10^6$ *M. leprae/*ml for 24h to permit minimal phagocytosis. Cultures were terminated at various time intervals up to 12 days. These cultures were stained by the Ziehl-Neelsen method and the number of bacilli per cell estimated.

M. leprae viability assays

FDA/EB staining: The protocol followed was similar to that adapted by Ramasesh *et al.* (1984) from the original method of Kvach *et al.* (1984), for intracellular bacilli. The viable bacilli which are able to convert the FDA to fluorescent fluorescein by the action of active esterases appear green when viewed under UV light. Staining was carried out on *M. leprae* within 33B cell cultures terminated at various time intervals. Per cent viability was calculated by comparison of 33B cells containing green fluorescing bacilli (GFB) to those containing acid fast bacilli (AFB) from identical cultures stained by the Ziehl-Neelsen method.

M. leprae growth in MFP: The procedure described by Rees (1964) was followed. *M. leprae* released from 33B cells after 12 days in culture were inoculated into MFP to check for growth. Freshly harvested armadillo *M. leprae,* inoculated directly into MFPs served as control.

Statistical analysis: For tabular representations, dpm values or the percentages are expressed as mean ±SE. The paired Student's *t* test has been employed for assessment of significance values.

Results

Characterization of PGL- 1

PGL-1 extracted from 33B cell-resident *M. leprae* was characterized by comparison of its IR spectrum with that of Standard PGL-1. An ELISA using the extracted PGL-1 as antigen and the monoclonal antibody raised against the specific trisaccharide, established its identity. Further, immunofluorescence staining of intracellular *M. leprae* using the above-mentioned monoclonal antibody also confirmed the presence of PGL-1.

Incorporation of [^{14}C]acetate into PGL-1 fraction of intracellular M. leprae

Increased incorporation of [^{14}C]acetate into the PGL-1 fraction of intracellular *M. leprae* was noted over a period of 15 days (table 1). This demonstrated the active synthesis of PGL-1 in this system.

Table 1. Relationship between duration of [^{14}C] acetate labelling and its incorporation into PGL-1 of *M· leprae·*

Days of incubation	Radioactivity in PGL-1 (dpm)
5	3,648 ± 256
10	6,913 ± 717
15	10,919 ± 669

Data are the mean of 3 sets of cultures expressed for 10^6 *M. leprae.*

Determination of viability of M. leprae within 33B cells

The experiments mentioned below were designed to correlate uptake of [^{14}C]acetate with bacterial viability.

The number of M. *leprae* within 33B cell cultures was visually counted after Ziehl-Neelsen staining over a period of time. As observed from table 2, the number

Table 2. Determination of viability of intracellular *M. leprae* by visual quantitation following Ziehl-Neelsen staining.

Days post-infection	AFB/cell
0	8·8 ± 1·1
6	11·4 ± 2·0
12	19·1 ± 0·5

Results are expressed as the mean ± SE of duplicates from 3 sets of experiments.

of AFB/cell increased 2-fold after 12 days of incubation. This indicated that intracellular *M. leprae* are maintained in a structurally integral form during the course of the experiment. The fact that these organisms were viable was obtained from the following two assays.

(i) Cultures of *M. leprae* within 33B cells were also stained using the FDA/EB assay to determine their viability. Results showed that per cent viability of these bacilli increased up to 12 days in culture, (table 3a) confirming that the 33B cells were suitable for the maintenance of viable and metabolically active *M. leprae.*

Table 3a. Viability of intracellular *M. leprae* ascertained over a period of 12 days using the FDA/EB technique.

Days post infection	Viability (%)
0	64·1 ± 3·9
6	66·4 ± 2·4
12	83·3 ± 2·5

$$\% \text{ viability} \frac{\text{no. of 33B cells containing GFB}}{\text{no. of 33B cells containing AFB}} \times 100.$$

(ii) Bacilli inoculated into MFPs, after incubation within 33B cells showed the characteristic growth curve of *M. leprae* (table 3b), confirming the viability of these intracellular organisms.

Table 3b. The MFP harvest counts obtained for *M. leprae* released from 33B cells after 12 days of incubation.

Duration of food-pad inoculation (months)	Bacillary count		
	Control	Set I	Set II
0	$1·0 \times 10^4$	$1·0 \times 10^4$	$1·0 \times 10^4$
6	$1·3 \times 10^5$	$2·4 \times 10^5$	$1·2 \times 10^5$
7	$1·5 \times 10^5$	—	$1·1 \times 10^5$
8	$4·5 \times 10^4$	$3·0 \times 10^4$	—
10	—	—	$1·8 \times 10^5$
12	$2·7 \times 10^5$	$1·8 \times 10^5$	—

Each valu is the mean from 4 smears.
(—), No bacilli observed.
Control, *M. leprae* isolated from armadillo.
Sets I and II, *M. leprae* derived from 33B cells.

Effect of drugs on PGL-1 synthesis

Earlier studies using both MFP and FDA/EB have demonstrated that drug concentrations equivalent to 5 μg/ml or even less, are effective in decreasing the viability of intracellular *M. leprae* (Holmes and Hilson, 1972; Bhagria and Mahadevan, 1987). Incubation of *M. leprae* infected 33B cell cultures in the presence of 5 μg/ml of anti-leprosy drugs DDS and RFP caused a significant

decrease in the incorporation of [^{14}C]acetate into PGL-1, implying its lowered synthesis and a decrease in viability (table 4).

Table 4. [^{14}C] Acetate incorporation into PGL-1 of *M. leprae.*

ML		FF		DDS		RIF	
X	%	X	%	X	%	X	%
4,128	100	586	14·20	—	—	283	6·83
235	100	131	55·75	—	—	—	—
618	100	138	22·33	—	—	183	29·61
2,550	100	—	—	1,089	42·71	980	38·43
51,602	100	23,765	46·05	33,993	65·88	2,635	5·12
458	100	—	—	212	46·29	—	—
458	100	—	—	368	80·35	—	—
Mean %	100	34·58 ± 9·77		58·81 ± 6·68		20·01 ± 8·30	
t value		$P < 0·01$		$P < 0·02$		$P < 0·005$	

(–), Not done.
X, dpm obtained for 10^6 *M. leprae* after subtraction of counts for control cultures.
%, Calculated as experimental value divided by control value (for *M. leprae)* multiplied by 100.
ML, *M. leprae* (control).
FF, Formalin-fixed *M. leprae.*
DDS, *M. leprae* in the presence of DDS.
RFP, *M. leprae* in the presence of RFP.

Control cultures utilising formalin-fixed *M. leprae* were simultaneously maintained to correlate incorporation of radiolabel with viability. Significantly reduced, dpm counts were obtained in the PGL-1 fraction (table 4).

Role of host cells

The incorporation of radiolabelled acetate into PGL-1 maintained within Schwannoma cells was compared with that of *M. leprae* maintained in growth medium in the absence of host cells. In the latter case, no radiolabel was detected even though a spot corresponding to PGL-1 was observed following TLC, indicating a requirement of the host cell for the recording of active bacterial lipid metabolism. These results were corroborated by compromising the protein synthesis capacity of the 33B cells, using cycloheximide. A significant decrease was obtained in PGL-1 synthesis of the cycloheximide treated cultures (table 5), indicating that a metabolically competent host cell is essential for the survival of these bacilli.

Discussion

The highlight of this present study is the observation that incorporation of [^{14}C] acetate into PGL-1 of *M. leprae* maintained within 33B cells is a reliable indication of its viability. This was ascertained through the following observations (i) a 2-fold increase in the number of *M. leprae* per cell over time, (ii) FDA/EB staining which showed a marginal increase in the number of viable bacilli over the experimental period, (iii) correlation of radiolabel incorporation with establishment of growth in MFP and (iv) decreased incorporation obtained in the presence of anti-myco-

Table 5. Role of host cells—incorporation of [^{14}C]-acetate into PGL-1 in the presence of cycloheximide.

ML		CY	
X	%	X	%
412	100	233	5·64
235	100	38	16·17
618	100	41	6·63
2550	100	114	0·05
Mean %	100		7·12 ± 2·23
t value			$P < 0·005$

X, dpm counts for 10^6 bacilli in experimental cultures after subtraction of counts for control cultures.
%, Per cent incorporation as compared with control Cultures.
ML, Control, *M. leprae* infected cultures:
CY, *M. leprae* infected cultures in the presence of cycloheximide.

bacterial drugs. Of the two anti-leprosy drugs, the reduction in incorporation was noted to be greater with RFP than with DDS, probably because RFP is bactericidal while DDS being bacteriostatic may not affect lipid synthesis to the same magnitude. Hence, the sensitivity of this system as a drug-screening assay may be less for static drugs. As a control, *M. leprae* killed by formalin-fixation were added to 33B cells. In this set, significantly lowered incorporation was observed, however, detectable radioactivity was recovered from the PGL-1 spot. This residual activity could be attributed to non-specific acetylation of long chain portions of this lipid, as formalinization may not be sufficient to inactivate all the enzymes. Uptake into PGL-1 of formalin-killed *M. leprae* has also been reported by others (Ramasesh *et al.,* 1987). Earlier studies by us were carried out using heat-killed bacilli as control, however, these gave extremely variable counts due to non-specific adsorption of radiolabel, and hence were discontinued.

An important observation of this study was the inability to incorporate [^{14}C] acetate into PGL-1 by *M. leprae* maintained in cell-free conditions· Incorporation of [^{14}C] palmitic acid into this component has nevertheless, been reported for *M. leprae* in axenic medium (Franzblau *et al.,* 1987). This may be indicative that a host cell milieu is necessary for the initial synthesis of fatty acids from free acetate. The importance of the host cells was further supported by observations on addition of the protein synthesis inhibitor, cycloheximide to the Schwannoma cells. Results demonstrated that incapacitating the host cell protein synthesis significantly decreased *M. leprae* metabolism, as reflected in reduction in incorporation of [^{14}C] acetate into bacterial PGL-1.

The host cells used in the present study were the 33B Schwannoma cells. These cells had to be irradiated to prevent their rapid multiplication, in order to provide sufficiency time for a slow-growing organism like *M. leprae* to adapt to the host environment. The easy availability of these cells was an advantage, as it eliminated the dependence on other less accessible tissues like human and mouse macrophages, as utilised in other radiolabel assays (Prasad and Nath, 1981; Mittal *et al.,* 1983; Prasad and Hastings, 1985; Ramasesh *et al.,* 1987). Moreover, the use of a cell line

permitted the maintenance of simultaneous, identical, uninfected controls, whose radioactive counts were subtracted from those of experimental infected cultures, thus minimizing even the residual contamination by the host cells.

Besides its specificity, monitoring of the synthesis of PGL-1 has some advantages in the application of this system for screening of compounds that could act on the novel intermediates in the PGL-1 synthesis pathway. More generally, this system also appears to be capable of evaluating drugs that do not interfere directly with bacterial lipid metabolism.

Acknowledgements

We thank LEPRA and Dr. E. Storrs for providing the armadillo material and Dr. P. J. Brennan for the Standard PGL-1. We also wish to thank the Radiation and Oncology Department, Tata Memorial Hospital, Bombay for providing facilities for regular irradiation of 33B cell cultures. We gratefully acknowledge Dr. Nalini Vemuri and Dr. Nerges Mistry for their assistance and helpful suggestions throughout the course of the study and preparation of the manuscript. This investigation received financial support from the UNDP/World Bank/WHO special programme for Research and Training in Tropical Diseases.

References

Ambrose, E. J., Khanolkar, S. R. and Chulawalla, R. G. (1978) *Lepr. India,* **50,** 131.
Bhagria, A. and Mahadevan, P. R. (1987) *Indian J. Lepr.,* **59,** 9.
Bligh, E. G. and Dyer, W. J. (1959) *Can· J. Biochem. Physiol.,* **37,** 911.
Fields, K. L., Gosling, G, Megsom, M. and Stearn, P. L. (1975) *Proc. Natl. Acad. Sci. USA,* **72,** 1296.
Franzblau, S. G., Harris, E. B. and Hastings, R. C. (1987) *FEMS Microbiol. Lett.,* **48,** 407.
Hart, P. D. and Rees, R. J. W. (1960) *Br. J. Exp· Pathol.,* **41,** 414.
Holmes, I. B. and Hilson, G. R. F. (1972) *J. Med. Microbiol,* **5,** 251.
Hunter, S. W. and Brennan, P. J. (1981) *J. Bacteriol.,* **147,** 728.
Iyer, C. G. S. (1965) *Int. J. Lepr.,* **33,** 634.
Job, C. K. (1979) *Arch. Pathol.,* **89,** 195.
Kvach, J. T., Munguia, G. and Strand,. S. H. (1984) *Int. J. Lepr.,* **52,** 176.
Mittal, A., Sathish, M., Seshadri, P. S. and Nath, I. (1983) *J. Clin. Microbiol.,* **21,** 704.
Mukherjee, R. and Antia, N. H. (1985) *J. Clin. Microbiol.,* **21,** 808.
Mukherjee, R., Mistry, Y., Antia, N. H., Klein, N. and Vemuri, N. (1985) *IRCS Med· Sci.,* **13,** 203.
Prasad, H. K. and Nath, I. (1981) *J. Med. Microbiol.,* **14,** 279.
Prasad, H. K. and Hastings, R. C. (1985) *J. Clin. Microbiol.,* **21,** 861.
Ramasesh, N., Bhagria, A. and Mahadevan, P. R. (1984) *IRCS Med. Sci.,* **12,** 1014.
Ramasesh, N., Hastings, R. C. and Krahenbuhl, J. L. (1987) *Infect. Immun.,* **55,** 1203.
Rees, R. J. W. (1964) *Br. J. Exp. Pathol.,* **45,** 207.