J. Biosci., Vol. 11, Numbers 1–4, March 1987, pp. 455–463. © Printed in India.

Importance of determining viability of *Mycobacterium leprae* inside macrophages—an *in vitro* method using uracil

SUNANDA VEJARE and P. R. MAHADEVAN
The Foundation for Medical Research, 84-A, R. G. Thadani Marg, Worli, Bombay 400 018, India

Abstract. It has been demonstrated that *Mycobacterium leprae,* are capable of taking up uracil and incorporating it into trichloroacetic acid-insoluble materials, both as free suspension of bacteria, as well as when they are inside the macrophages, a host cell for their *in vivo* survival. Same amount of bacteria show better incorporation inside macrophages than as free bacterial suspension. Both types of incorporation are inhibited by rifampicin an antileprosy drug and an RNA synthesis inhibitor. Thus uracil uptake by *Mycobacterium leprae* inside macrophages has been used for standardising a rapid *in vitro* viability assay for the leprosy causing bacteria.

Keywords. Uracil uptake; *Mycobacterium leprae;* macrophages; viability assay.

Introduction

Mycobacterium leprae, the causative organism for leprosy is an intracellular obligate pathogen. In patients suffering from lepromatous leprosy the bacteria are seen inside several host cells,but primarily macrophages in various tissues and schwann cells of peripheral nerves. These two cell types are important for immune functions and normal nerve functions, respectively. It is well documented that those susceptible to lepromatous leprosy, have poor cell mediated immunity (Bloom and Mehra, 1984) a function in which macrophages play an important role as antigen presenting cell. The patients also suffer from failure of nerve function leading to neuropathy, basically expressed as features related to damage in the myelin of myelinated nerves and the Schwann cells of unmyelinated peripheral nerves (Shetty *et al.,* 1980). Thus the understanding of host-pathogen interaction is basic necessity in explaining the pathogenesis in leprosy especially lepromatous leprosy.

The information available regarding metabolism of *M. leprae* was fully described recently by Wheeler (1984). The nucleic acid metabolism as studied by the uptake and incorporation of labelled pyrimidines and purines was also described by him. In analysing the reported ability of *M. leprae* from armadillo to incorporate the precursor of nucleic acid (Khanolkar *et al.,* 1978; Drutz and Cline, 1972; Prasad and Nath, 1981; Nath *et al.,* 1982; Khanolkar and Wheeler, 1983) it was concluded by Wheeler (1984) that purines like, hypoxanthine and adenosine were better precursors than pyrimidines like thymidine or uracil. However it is noteworthy that all experimental evidence by Nath and her colleagues, cited above, clearly indicate utility of thymidine as a metabolic precursor for determining viability and drug sensitivity of *M. leprae,* phagocytosed by macrophages. There are other *in vitro* assay systems for viability of *M. leprae* have also been described. They include the

Abbreviations used: DOPA, Dihydroxyphenylalamine; HEPES, N-2 hydroxypiperarine N-2 ethane sulphonic acid; TCA, trichloroacetic acid; DDS, dapsone; MEM, minimum essential medium.

dihydroxyphenylalanine (DOPA) uptake (Ambrose *et al.,* 1978) and ATP assay system (Dhople and Hanks, 1980).

Viability of *M. leprae* inside the host cells cannot be judged by size, shape, morphology or staining property of the bacteria. Such a procedure has always been arbitrary. Thus the dynamic metabolism expressed by *M. leprae* inside the host cell has to be used. Once such assay systems are well established, we could use them for determining the nature of *M. leprae* under various conditions that affect the viability. Such conditions involve exposure to antileprosy drugs, exposure to immune competent cells (lymphocytes) and cell products (lymphokines). These products could alter the viability of *M. leprae.* Thus it is essential to have such assay systems and some of them have already been identified (Nath *et al.,* 1982; Ambrose *et al.,* 1978; Vithala *et al.,* 1983; Mankar *et al.,* 1984).

In this paper we describe use of uracil as a precursor to assess the metabolism of *M. leprae,* inside macrophages. Potential use of uracil for determining viability of mycobacteria like BCG, was identified by Rook *et al.* (1981), who later adopted it to show killing of BCG inside macrophages by activated lymphocytes (Rook *et al.*, 1985). We have adopted a closely similar procedure with *M. leprae.*

This paper also presents detailed data to demonstrate that *M. leprae* are able to incorporate uracil even as free suspension and the two systems one as the free suspension and other inside host cells have been compared.

Materials and methods

Preparation of N-2 Hydroxypiperarine N-2 ethane sulphonic acid Buffer

N-2 Hydroxypiperarine N-2 ethane sulphonic acid (HEPES, Sigma Chemicals Co., USA) Powder—0·15 M, NaCl–0·15 M and $MgSO_4 7H_2O$–0·1 mM were dissolved to make a 100 ml solution with distilled water; and pH was adjusted to 7·2 with 1N HCl, autoclaved (15 lbs./121°C/30 min) and stored for use.

Preparation of M. leprae

M. leprae was obtained from tissue of leprosy patients as per the method of Ambrose *et al.* (1978). Bacteria obtained were acid fast stainable and were used only after ascertaining that they were free from other rapidly growing contaminating bacteria, including other mycobacterial strains. *M. leprae* normally does not grow in the common mycobacterial medium.

Assay of [^{3}H]-uracil incorporation in M. leprae

[3H]-Uracil labelled in positions 5–6 was obtained from Amersham Radiochemical Centre,London, (sp. act. 35 Ci/mmol). Reaction mixture containing 20×10^6 bacteria, HEPES Buffer, pH 7·2 and 1 μCi [^{3}H]-uracil in a final volume of 0·5 ml/ tube was incubated at 34°C for 6 days. Control tubes containing same materials, except that the bacilli pretreated with 8% formaldehyde for 24 h were used. After incubating the bacteria aseptically for 6 days, both the control and experimental

tubes were centrifuged at 5000*g*. To the residue, 20% cold trichloroacetic acid (TCA) was added. The precipitated material obtained after acid addition, was filtered through Millipore filter (0·22 μm pore size) which was then washed with methanol and dried. Use of methanol was just to remove the aqueous layer on the filter only. The filter paper was placed in vial containing 10 ml of Bray's scintillation fluid (Bray, 1960), and with the help of Kontron MR 300 scintillation counter the radioactivity as dpm of [^{3}H] -uracil in TCA insoluble material was reported.

Action of drugson incorporation

While the bacteria were being incubated with the labelled precursor [^{3}H] -uracil; they were exposed to Dapsone (DDS) or rifampicin at concentration ranging from 0·5 μg 10 μg/ml. This was to find out whether the known anti-. *leprae* drugs can block the metabolism of *M. leprae* and thus the incorporation of the precursor. DDS was obtained from Burroughs Wellcome, Bombay and rifampicin from Sigma Chemical Co., St. Louis, Missouri, USA.

Macrophage preparation

Macrophages were obtained from Swiss white mice. The mice were injected with 5 ml of minimum essential medium (MEM) containing inactivated human AB type serum (to 20% level) into peritoneal cavity, after killing the animal by cervical dislocation. The peritoneal fluid was collected from the animal by agitating the cavity with the medium and 0·7 ml of the collected fluid containing the cells was distributed in Leighton tubes. Tubes were then incubated at 37°C in 5% CO_2 atmosphere. The medium was changed every 24 h, so as to remove non adherent cells and the cell culture was thus matured for 3 days. This resulted in fairly good monolayers of adherent macrophages which were phagocytic and largely free from other types of cells.

[^{3}H]-Uracil incorporation studies with macrophages containing M. leprae

After getting the monolayer of adherent macrophages on the flat side of Leighton tubes (3 day old cultures), the tubes were divided in 3 sets. One set was infected with viable *M. leprae* and another with heat killed *M. leprae* (5×10^6 from human or armadillo tissue). The third set did not receive any *M. leprae* and was used as a control. After 24 h, all tubes were labelled with 1 μCi of [^{3}H]-uracil. After 6 days of incubation at 37°C in 5% CO_2 atmosphere, the cultures were terminated. Medium was drained from all tubes and one ml of fresh saline was added and tubes were kept in cold (4°C) for 15 min. The cells were scrapped from the surface of glass with silicone rubber policeman and using a small aliquot of the suspension, the macrophage cell count was obtained with a haemocytometer. After two washes with saline, macrophages were treated with 20 % cold TCA. The precipitated material was centrifuged at 2500 *g* and washed with methanol. The radioactivity was determined as described in the previous paragraph. The incorporation was represented as dpm/10^6 macrophages.

Incorporation associated with M. leprae alone using the macrophages containing M. leprae

In experiments similar to those described above, cultures exposed to *M. leprae* (5×10^6/tube heat killed and live) and the precursor ([^{3}H]-uracil) after incubation for 6 days were treated differently. The macrophages after recovery were lysed by freezing and thawing (8 cycles). The tissue debris was separated from bacteria by centrifugation at 1000 *g* for 15 min followed by bacterial separation by centrifuging at 5000 *g* for 30 min. Bacterial count was determined microscopically with acid fast staining by the Ziehl Neelsen method (Cruickshank, 1968). After treating the bacteria with 20% cold TCA, the incorporation associated with the precipitated bacteria was recorded as dpm/ 1×10^6 *M. leprae.*

Inhibition of incorporation by rifampicin

The *M. leprae* that were used to infect the macrophages were preincubated for 3 days with rifampicin (10 μg/ml) and added to macrophage cultures which were earlier exposed to rifampicin (10 μg/ml) for 24 h before the addition of *M. leprae.* The drug concentration was kept as 10 μg/ml since we wanted a definite action on the bacteria and the minimum inhibitory concentration with this type of assay system is yet to be worked out. The cultures were then exposed to precursor [^{3}H]-uracil and incubated in the presence of rifampicin (10 μg/ml). The control cultures had no drug treatment either to the bacteria or macrophages. The incorporation of the precursor was determined in the bacteria as described earlier.

Results

Six different *M. leprae* isolates from human leprosy nodules and two different *M. leprae* preparations from infected armadillo tissue showed incorporation of [^{3}H]-uracil in TCA insoluble product, when 20×10^6 bacteria were incubated with 1 μCi of labelled uracil. In all the cultures, the live *M. leprae* showed significantly higher incorporation than formalin treated *M. leprae* (table 1). However the formalin treated bacteria did not show low level of incorporation, consistently in all experiments, even though the level in each experiment was much less than that of the corresponding sample with live *M. leprae.*

In these experiments $20 \times 10_6$ bacteria were used. Before this was adopted, the level of incorporation when different number of bacteria exposed to same amount of uracil was also determined and this showed that increasing bacterial level resulted in higher incorporation, though not at a linear rate (data not presented). The non linearity may be due to different level of viability in each suspension since homogenous distribution of clumpy *M. leprae* is difficult.

Inhibition of uptake by drugs

The uracil uptake by the bacteria was indicated as an apparent metabolic function by the inhibition of uptake by two anti *M. leprae* drugs, DDS and rifampicin (table 2). In 5 different experiments (3 from spleen, and one each from nodule and leproma),

Table 1. *Incorporation* of [^{3}H]-uracil (as dpm) by *M. leprae* (armadillo and human).

Patient's name (Initials)	Formaldehyde killed *M. leprae* (F)	Live *M. leprae* (L)	Net uptake (L–F)	BI/MI
M.P.	466	3469	2997	6+, 3%
L.G.	181	6582	6401	6+, 1%
M.B.	1454	5463	4009	1+, 1%
M.P.	2051	4539	2488	6+, 1%
A.B.	2147	3627	1480	4+, –
M.G.	103	3786	3683	6+, 4%
Ar-1	661	4570	3909	6+, 10%
Ar-2	3804	6439	2635	6+, 35%
Mean ± S.D.	1358 ± 1278	5241 ± 549	3431 ± 1368	

All values are average of duplicates.
Radioactivity is expressed in dpm/20 × 10^6 *M. leprae.*
BI, Bacterial Index (Bacillary load).
MI, Morphological Index (live).
Difference between killed and live *M. leprae* is significant. P<0·001
S.D., Standard deviation.

Table 2. Incorporation of [3 H]-uracil by *M. leprae* (Armadillo) represented as dpm/20 × 10^6 *M. leprae* in the presence of antileprosy drugs (DDS and rifampicin).

	Ar–1 (Nodule)	Ar–2 (leproma)	Ar–3 (Spleen)*	Mean ± S.D.
Formaldehyde (8%) killed	1321	857	176	784 ± 575
Live *M. leprae*	8150	7215	1641	5668 ± 3507
Live *M. leprae* + DDS (0·1 μg/ml)	1066	914	447	809 ± 322
DDS (1 μg/ml)	1216	538	301	685 ± 474
DDS (10 μg/ml)	1843	536	219	866 ± 857
Rifampicin (0·5 μg/ml)	1268	434	241	647 ± 545
Rifampicin (2 μg/ml)	948	538	176	554 ± 386
Rifampicin (10 μg/ml)	780	437	233	483 ± 276

*The data with armadillo *M. leprae* from spleen (Ar–3 spleen) is an average value of 3 separate experiments from 3 separate spleen tissues.
All values are average of duplicates (in all other experiments) 20 × 10^6 *M. leprae* was used.
S.D., Standard deviation.

using armadillo derived *M. leprae,* the inhibitory activity on incorporation was demonstrable by 0·1–10 μg/ml of DDS and 0·5–10 μg/ml rifampicin added for the incubation mixture. The inhibition was revealed by lowered level of precursor incorporation in the drug treated *M. leprae* as compared to the live control *M. leprae.*

M. leprae from clinically relapsed and normal patients

M. leprae obtained from clinically relapsed or untreated patients (3) were also tested for ability to incorporate [^{3}H]-uracil in the presence and absence of rifampicin an

inhibitor of RNA synthesis. It was found that *M. leprae* from all the 3 patients showed no susceptibility to DDS and rifampicin. This was indicated by the level of incorporation by *M. leprae* in the presence of these drugs remaining similar to that of live *M. leprae* (table 3). On the other hand *M. leprae* from armadillo and some untreated patients were susceptible to DDS and rifampicin.

Table 3. Incorporation of [^{3}H]-uracil by *M. leprae* (human) in presence of antileprosy drugs (DDS and rifampicin).

BI/MI clinical assessment	FMR 738 6+, 1% relapsed	FMR 1431 54+, 4% untreated	FMR 722 6+, 1% 1y.treated	Mean ± S.D.
Formaldehyde (8%) killed	200	2241	382	940 ± 1128
Live *M. leprae*	4534	4173	12002	6902 ± 4418
Live *M. leprae* + DDS (0·1 μg/ml)	2732	3554	9913	5332 ± 3977
DDS (1 μg/ml)	3323	3971	10135	5808 ± 3758
DDS (10 μg/ml)	3639	3515	11846	6332 ± 4773
Rifampicin (0·5 μg/ml)	2570	3459	12144	6056 ± 5288
Rifampicin (2 μg/ml)	3000	4649	11579	6408 ± 5432
Rifampicin (10 μg/ml)	2990	4460	16700	8049 ± 7526

All values are average of duplicates.
Quantity of uracil incorporates is expressed as dpm/20 × 10^6 *M leprae*
S.D., Standard deviation.
BI/MI, Bacterial Index (load)/Morphological Index (live).

Uptake of uracil by M. leprae phagocytosed by macrophages

It was interesting to determine the level of uracil taken up by *M. leprae* phagocytosed by the macrophages, so as to compare with the ability of free *M. leprae.* The experiment with armadillo *M. leprae* phagocytosed by macrophages showing the level of incorporation in 1×10^6 macrophages indicated, that discernable level of incorporation was consistent in all the 8 experiments, even though the level of incorporation was different in each of the experiment. The level of *M. leprae* used was same in all cases (5×10^6 bacteria) and equal amount of phagocytosis by the macrophages is assumed to occur (table 4).

Increase in uptake of precursor by M. leprae inside the macrophages

Earlier, different concentration of *M. leprae* was used in free suspension with the precursors and we found, that the incorporation was insignificant and not discernable when the *M. leprae* used was less than 5×10^6 (data not presented). Thus it was necessary to compare the level of incorporation of uracil by *M. leprae* in a quantum less than 5×10^6 as free bacteria in suspension and same amount of *M. leprae* phagocytosed by macrophage cultures. Data provided (table 5) show level of incorporation by 1×10^6 *M. leprae* under the above two different conditions is significantly different. *M. leprae* (1×10^6) inside the macrophages showed several fold higher incorporation indicating a facilitation of increased uptake of uracil by *M. leprae* when they were inside the macrophages.

Table 4. Experimental data showing incorporation of [^{3}H]-uracil (as dpm) by *M. leprae* inside macrophages.

Experiment No. (armadillo)	Macrophage only	Macrophage + heat killed *M. leprae* (B)	Macrophage + live *M. leprae* (C)	(C–B)	
1	80	420	1600	1180	
2	80	160	700	540	
3	585	379	711	332	
4	148	294	451	157	$P<0.01$
5	—	331	445	114	
6	—	1384	2382	998	
7	—	1330	2480	1150	
8	—	2660	3080	420	

Radioactivity is expressed as dpm/1 × 10^6 macrophages. *M. leprae* 5 × 10^6/tube.

Table 5. Comparison of incorporation by *M. leprae* (armadillo or human) in buffer and inside macrophages as dpm/1 × 10^6 *M. leprae.*

Source of *M. leprae*	Incorporation in buffer by 1 × 10^6 *M. leprae* (L–F) (A)	Incorporation inside macrophage by 1 × 10^6 *M. leprae* (L–HK) (B)	Difference (inside Mϕ) (in buffer) (B − A)	
Ar (spleen)	174	511	337	
Ar (spleen)	0	538	538	
Ar (spleen)	20	1685	1665	$P<0.05$
Ar (leproma)	7	786	779	
Human	0	87	87	

L–F Live, formalin treated.
L–H.K.–Live, heat killed *M. leprae.*

Inhibition of such increased uptake by drugs

The increased incorporation of uracil by *M. leprae* while they were inside the macrophages was tested for inhibition with rifampicin. Data in table 6 show that in the presence of rifampicin (10 μg/ml) the increased incorporation of uracil is drastically reduced indicating that the uptake is a true metabolic process.

Discussion

It is very likely that uracil is not a preferred metabolite of *M. leprae* towards nucleic acid metabolism as suggested by Wheeler *et al.* (1984). However as compared to low incorporation obtained in armadillo derived bacteria by Khanolkar and Wheeler (1983), our experiments show that longer incubation time upto 6 days show

Table 6. Incorporation of [3H]-uracil by *M. leprae* (armadillo and human) inside macrophages (1×10^6) in presence of RFP).

	Ar_1	Ar_2	FMR 801*	FMR 805*	
Heat killed *M. leprae*	1660	496	840	140	$P<0{\cdot}01$ significant
Live *M. leprae* only	2950	1705	1140	994	$P<0{\cdot}025$ significant
+ Rifampicin (10 μg/ml) treated *M. leprae*	659	577	320	272	

*From patients.
Incorporation is expressed as dpm/ 1×10^6 *M. leprae.*

incorporation of radioactivity significant enough to measure as acid insoluble component. However as a quantity in p mol, the incorporation is very low. Several observations show that the uptake of uracil is a metabolic activity. The uptake is extremely low in formalin treated bacteria. The uptake is proportional to bacteria exposed to the metabolite and also dependent on period of incubation, and further it is blocked by anti *M. leprae* drugs like DDS and more particularly by rifampicin, an inhibitor of RNA synthesis. The incorporation of uracil was first demonstrated by *in vitro* grown acid fast bacteria derived from leprosy nodule (data not presented) and later tested with *M. leprae* from leprosy nodules as well as infected armadillo tissues. But results presented here are only with *M. leprae,* from the human and armadillo tissues because of obvious interest in this group of pathogenic mycobacteria.

The clinically relapsed patient who was diagnosed as having drug resistant bacteria, showed *M. leprae* in his tissue with resistance to the drug. Even an untreated patient showed primary resistance to the drug (table 3).

It is clear that *M. leprae* are capable of taking up uracil and using it as a meta bolite to some extent as indicated previously by Wheeler *et al.* (1984). The question that arose was, does this happen inside a host cell? If so, is there a modification in the level of incorporation? Thus comparison was made between the total uptake of uracil by free *M. leprae* in suspension and *M. leprae* phagocytosed by macrophages. The experimental data provided in table 5 clearly showed an equal amount of *M. leprae* could have higher level of incorporation inside the host cells than as a free suspension. This could be due to some modification or activation of the metabolism of *M. leprae* inside the cells which facilitated increased uptake. This could also account for the consistent uptake of thymidine with fewer bacteria inside the macrophages (Prasad *et al.,* 1981; Nath *et al.,* 1982) as compared to poor or no uptake of thymidine by greater number of bacteria as free cell suspension (Khanolkar *et al.,* 1978). Even though the conditions of treatment of free *M. leprae* and *M. leprae* inside macrophages appear different while studying the uptake of uracil; they are the most appropriate and optimum conditions for each of the type of the uptake system. Thus a comparison of the system appears justified.

From these observations and that of Nath *et al.* (1982), it is suggested that during early hours of incubation (about 120 h) of *M. leprae,* there is an activation of the metabolism of the bacteria inside the macrophages.

It is thus clear that uracil can be used as a metabolic marker for determining viability, monitor maintenance of the viability and drug sensitivity of *M. leprae* while they are inside the macrophages and also to some extent while they are in free

suspension. The advantage of determining viability inside the macrophages would be that one could determine the effect of immunomodulators as well. This is important in a disease like leprosy in which the patients have poor or no cell mediated immunity. We have recently used macrophages phagocytosed *M. leprae* and uracil uptake to show effect of immunomodulation by lymphokines, and immunosti mulants on the viability of *M. leprae* (data under publication).

Acknowledgements

The authors wish to acknowledge the help received from Acworth Leprosy Hospital, Bombay in the supply of human materials and Dr. E. Storrs and LEPRA, UK for help with the supply of infected tissues from armadillo. Financial support under PL-480 from National Institute of Health, USA is gratefully acknowledged.

References

Ambrose, E. J., Khanolkar, S. R. and Chullawalla, R. G. (1978) *Lep. India,* **50,** 131.
Bloom, B. R. and Mehra, V. (1984) *Immunol. Rev.,* **80,** 28.
Bray, G. A. (1960) *Anal. Biochem.,* **1,** 271.
Cruickshank, R. (1968) *Medical Microbiology,* (Living Stone: E and S).
Dhople, A. M. and Hanks, J. H. (1980) *Int. J. Lep.,* **49,** 59.
Drutz, D. J. and Cline, M. J. (1972) *J. Infect. Dis.,* **125,** 416.
Khanolkar, S. R., Ambrose, E. J., Chullawalla, R. J. and Bapat, C. V. (1978) *Lep. Rev.,* **49,** 187.
Khanolkar, S. R. and Wheeler, P. R. (1983) *F.E.M.S. Microbiol. Lett.,* **20,** 273.
Mankar, M. V., Jagannathan, R. and Mahadevan, P. R. (1984) *J. Biosci.,* **6,** 709.
Nath, I., Prasad, H. K., Sathish, M., Shreevastava, K. V., Sheshadri, P. S. and Iyer, C. G. S. (1982) *Antimicrob. Agric. Chemother.,* **21,** 26.
Prasad, H. K. and Nath, I. (1981) *J. Med. Microbiol.,* **14,** 279.
Rook, G. A. W. and Rainbow, S. (1981) *Ann. Immunol. (Inst. Pasteur).,* **132D,** 281.
Rook, G. A. W., Champion, B. R., Steele, J., Varey, A. M. and Stanford, J. L. (1985) *Clin. Exp. Immunol.,* **59,** 414.
Shetty, V. P., Mehta, L. N., Irani, P. R. and Antia, N. H. (1980) *Lep. India,* **52,** 5.
Vithala, L., Talati, S., Mahadevan, P. R. (1983) *J. Biosci.,* **5,** 235.
Wheeler, P. R. (1984) *Int. J. Lep.,* **52,** 208.