J. Biosci., Vol. 6, Number 2; June 1984, pp. 221–231.

An *in vitro* test using cholesterol metabolism of macrophages to determine drug sensitivity and resistance of *Mycobacterium leprae*

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MS received 20 January 1984; revised 9 April 1984

Abstract. Macrophages that have ingested live *Mycobacterium leprae* show a preferential accumulation of cholesterol ester. Such an accumulation is not seen, on the ingestion of dead bacteria. Among the macrophages that ingest live *Mycobacterium leprae*, the presence of dapsone or rifampicin prevents largely the alteration in the anticipated increase in the cholesterol ester indicating the sensitivity of the bacteria to the drug. In the small number of relapsed patients, the presence of dapsone did not reduce the cholesterol ester increase, suggesting that the *Mycobacterium leprae* present are either resistant or escaped detection. The method provides a rapid drug screening system for *anti-Mycobacterium leprae* activity of known and unknown compounds.

Keywords. *Mycobacterium leprae*; colesterol; cholesterol ester; macrophage; drug sensitivity/resistance.

Introduction

The screening of antimicrobials for anti leprosy activity has not been extensively undertaken. The mouse foot-pad growth technique with *Mycobacterium leprae* is claimed to be a well defined and reliable technique to test the viability and growth of *M. leprae* (Shepard, 1960). The same technique can be used to find out whether the *M. leprae* are susceptible to a drug or not by determining the growth in the foot-pad of mice, fed on a diet containing the drug (Shepard, 1967). This method has been refined and made more sensitive using thymectomised mice (Rees, 1966) or genetically bred nude mice (nu/nu) (Kohsaka *et al.*, 1978). However all these test systems need a minimum of 6-8 months even before preliminary assessment could be made. In view of this there is an urgent need for a rapid test system to determine drug sensitivity/resistance of *M. leprae*. The major limitation that has to be overcome with *M. leprae* is our inability to grow it *in vitro*, on any of the currently available bacteriological media in a reasonably short time.

We describe here a method for testing viability that could also be used for drug sensitivity testing of *M. leprae*, when the bacteria are resident in macrophages. The basic reaction monitored in this test system, is the influence that only live *M. leprae* exert on the cholesterol metabolism of the macrophages (Ishwari Kurup and

Abbreviations used: DDS, Dapsone (di-(4-aminophenyl)-sulphone; DOPA, dihydroxyphenylalanine.

Mahadevan, 1982) leading to the formation of cholesterol ester from cholesterol taken up, as compared with macrophages with no live *M. leprae* inside. The present paper describes the adaptation of this observation for the development of a suitable and rapid drug sensitivity test that can even be used in an anti-leprosy drug screening programme.

Materials and methods

Mycobacterium leprae were obtained from lepromatous tissue of bacillary positive, untreated patients or those under varying periods of chemotherapy. Bacillary suspensions were prepared (Ambrose *et al*., 1974) and shown to be acid-fast and free from other contaminating bacteria. These fail to grow in normal mycobacteriological media. The bacilli were counted and 5×10^6 bacilli were added to each Leighton tube containing cultured macrophages. *M. leprae* obtained from infected armadillo tissue (supplied by Dr. E. Storrs, Florida, USA) were also used in some of the experiments.

Macrophage cultures

Macrophages from Swiss white mice were obtained from the peritoneal cavity following injection of 5 ml Eagle's minimal essential medium +20% inactivated human serum (AB blood group) into the cavity, after killing the animal by cervical dislocation immediately after the injection of the medium. The peritoneal fluid was collected after agitating the cavity, and 0·7 ml of the fluid was added to each Leighton tube. The macrophages obtained from the peritoneal fluid adhered to the Leighton tubes. The medium was changed every 24 h; to remove non adherent cells. After 3 days of such culturing, esterase positive phagocytic cells, were predominantly distributed as an uniform layer at the flat bottom of Leighton tubes. There were no contaminating neutrophils, and non-adherent lymphocytes were not present in any significant numbers. Such tubes were divided into 5 sets. One set received viable *M. leprae* (5×10^6/Leighton tube) either from human biopsies or armadillo and another heat-killed *M. leprae* (5×10^6/Leighton tube). The control sets did not receive the *M. leprae* inoculum. The level of *M. leprae* to be added had earlier been confirmed as a good dose to elicit clear response from the macrophages (Ishwari Kurup and Mahadevan 1982). After 24 h of phagocytosis of the bacteria, the excess bacilli were removed and the macrophages were incubated with [^{3}H]-cholesterol. The experiments were also carried using [^{14}C]-acetate as precursor. Some of the cultured macrophages were exposed to drugs like dapsone (di(4-aminophenyl)-sulphone (DDS) (Burroughs Wellcome Co., Bombay) at a concentration of 1 μg/Leighton tube (1 μg/0·7 ml of medium) or rifampicin (Sigma Chemicals Co., St. Louis, Missouri, USA) (10 $\mu g/ml$) for 72 h before *M. leprae* were added. Some of the control macrophages were not infected with *M. leprae* and used to study the effect of the drug alone and cholesterol uptake on lipid synthesis. In the drug treated cultures after 24 h of phagocytosis excess bacilli was washed off and the macrophages were labelled with [^{3}H]-cholesterol/[^{14}C]-acetate and left for 4 days for studying the uptake and synthesis of the lipids in the presence of the drug.

[^{3}H]-Cholesterol was obtained as crude tritiated product from Bhabha Atomic Research Centre, Bombay and purified by repeated thin layer chromatography and

0·16 μ Ci was used for the uptake studies in each Leighton tube. [^{14}C]-Acetate (with sp. activity of 56·7 μCi/m mol from Bhabha Atomic Research Centre, Bombay) was added in each Leighton tube at a level of 0·5 μCi. Many of the other parameters have been optimised during our earlier work, that has been reported (Iswari Kurup and Mahadevan 1982).

Extraction and separation of lipids

Following incubation, the macrophages were scrapped off the surface of the glass, the cells were microscopically counted and the lipids were extracted according to Dole's method (Dole, 1956). Lipids were separated by thin-layer chromatography on 20 × 20 cm plates coated with silica gel (Chemical division, Glaxo laboratory, Bombay), at room temperature using hexane: ether:acetic acid (80:20:1) as development solvent. Identification of the lipid spots was made by staining with iodine vapours, using for reference, standard lipids (Sigma Chemicals Co., St. Louis, Missouri, USA).

Determination of incorporation into lipids

After identification of the lipid spots, the silica gel corresponding to the position of each standard, from the experimental sample, was carefully scrapped into scintillation vials. This was done after the evaporation of iodine and full decolourization. Bray's scintillation fluid (10 ml) was added to each vial and its radioactivity determined by using Kontron MR-300 automatic scintillation counter.

Since each experiment is done with separate crop of macrophages from mice and bacteria derived from different patient materials, variability is a problem. This has been partially overcome with averaging the experimental values with indication of standard deviation and *P* values.

Results

We had shown earlier that macrophages with or without *M. leprae* were able to incorporate [^{14}C]-acetate into lipids and also take up cholesterol and convert a portion of it to cholesterol ester (Ishwari Kurup and Mahadevan, 1982).

When labelled cholesterol was used for uptake studies it was observed that the total uptake of cholesterol inside the macrophages was comparatively less when they were infected with *M. leprae.* Nevertheless, presence of *M. leprae*, induced increased level of cholesterol ester as seen by the higher ratio of labelled cholesterol ester to cholesterol in the culture, as compared with macrophages with no *M. leprae* (table 1). No such increase in the ester level was seen in macrophages exposed to autoclaved *M. leprae* (table 1). But if the macrophages had been exposed to the drug earlier and then *M. leprae* were added and the bacteria were exposed to such intracellular drug for 24 h before the cholesterol uptake during the next 4 days, then the results would be as shown in table 1. This showed that DDS was able to inactivate *M. leprae* from both armadillo as well as those derived from human leproma tissues. Due to this inactivation the cholesterol ester level was not increased and the ratio of cholesterol ester to cholesterol (as radioactivity associated with ester to that associated with cholesterol) was

Table 1. Cholesterol and cholesterol ester level in macrophages after incubation with [^{3}H]-cholesterol in the presence and absence of *M. leprae* (as cpm/10^6 macrophages).

Culture	Armadillo *M. leprae* Cholesterol	Armadillo *M. leprae* Cholesterol ester	Armadillo *M. leprae* Ratio*	Human *M. leprae* (Biopsy) 411 Cholesterol	Human *M. leprae* (Biopsy) 411 Cholesterol ester	Human *M. leprae* (Biopsy) 411 Ratio*	Human *M. leprae* (Biopsy) 414 Cholesterol	Human *M. leprae* (Biopsy) 414 Cholesterol ester	Human *M. leprae* (Biopsy) 414 Ratio*
Macrophage only	35782	3741	0·10	11331	304	0·027	15420	1600	0·10
Macrophages + heat killed *M. leprae*	37071	5189	0·14	10501	422	0·04	13886	670	0·05
Macrophage + live *M. leprae*	22344	5347	0·24	8320	980	0·12	9562	1912	0·20
Macrophages + DDS only	38125	4685	0·12	11680	350	0·03	13467	537	0·04
Macrophages + DDS + live *M. leprae*	36475	5280	0·14	11830	621	0·052	16700	665	0·04

DDS–Diphenyl diaminosulphone (dapsone).

* Ratio of cpm in cholesterol ester/cpm in cholesterol.

statistically similar to the control and/or heat killed *M. leprae* added macrophages. There was a lower level of cholesterol in macrophages, in live *M. leprae* added cultures as compared with others. This could be due to a decreased uptake of cholesterol since label is added after *M. leprae* addition or due to conversion of cholesterol to some other components, besides the ester. This is a noteworthy feature for future studies.

Table 2 presents data from a typical experiment using [^{14}C]-acetate as a precursor to determine the level of cholesterol ester in the macrophages. Results are similar as seen in table 1, indicating the sensitivity of *M. leprae* to the drug DDS. The consolidated data of 9 such experiments are presented in table 3 and the statistically significant difference

Table 2. Incorporation of [^{14}C]-acetate into cholesterol and cholesterol ester in macrophages (cpm/10^6 macrophages) with and without human *M. leprae* and drug (biopsy No. 483).

	Macrophage	Macrophage + heat killed *M. leprae*	Macrophage + live *M. leprae*	Macrophage + DDS*	Macrophage + DDS* *M. leprae*
Cholesterol	3812	3516	1811	3350	3190
Cholesterol ester	1075	970	897	1250	977
Ratio of ester to cholesterol**	0·28	0·275	0·495	0·373	0·30

* Dapsone (DDS).

** Ratio of radioactivity (cpm) in cholesterol ester to that in cholesterol/10_6 macrophages.

Table 3. Ratio of cholesterol ester/cholesterol during different treatments with different preparation of *M. leprae* (ratio of radioactivity (cpm) in cholesterol ester to that in cholesterol/10^6 macrophages).

M. leprae isolated from	Macrophage	Macrophage + heat killed *M. leprae*	Macrophage + live *M. leprae*	Macrophage + DDS (dapsone)	Macrophage + DDS + *M. leprae*
	a	*b*	*c*	*d*	*e*
Armadillo tissue*	0·10	0·14	0·24	0·12	0·14
Armadillo tissue	0·03	0·01	0·19	0·02	0·07
Armadillo tissue	0·04	0·03	0·12	0·04	0·05
Human M. leprae					
Biopsy 411	0·03	0·04	0·12	0·03	0·05
414	0·10	0·05	0·20	0·04	0·04
417	0·18	0·19	0·32	0·18	0·14
426	0·27	0·12	0·67	0·22	0·20
483**	0·28	0·20	0·50	0·37	0·31
497**	0·18	0·14	0·39	0·19	0·19
Mean with S.D.	0·133 ± 0·09	0·102 ± 0·06	0·30 ± 0·17	0·123 ± 0·11	0·132 ± 0·08

* Using labelled cholesterol.

** Using labelled [^{14}C]-acetate.

a – *b* *P* – (n.s.)

c – *a* $P < 0.025$

c – *b* *P* – (*n.s.*)

e – *a* *P* – (n.s.)

d – *e* *P* value > 0.5 (n.s.)

(by student '*t*' test) of the cholesterol ester level in macrophage containing live *M. leprae* and drug treated *M. leprae* is clear. It is noteworthy that the human biopsy derived *M. leprae* were from six different patients and the three armadillo derived *M. leprae* are from one infected tissue of armadillo. The value of ratio for each of the experimental conditions varied from experiment to experiment probably due to variability in the macrophage population. So a larger number of experiments will be needed in fully standardised conditions to narrow the difference and also do an analysis of variance. However, the same population of macrophage has consistent behaviour, as expected by the condition of experimentation. Thus, the data on the ratio is represented as an average of all experiments with the standard deviation under each category and *P* values determined for these average values for each comparable categories.

One basic requirement in any method that indicates the viability of *M. leprae* is that the parameter tested should vary with difference in the viability of the test bacterium. We could clearly demonstrate this using varying levels of *M. leprae* with constant amount of macrophages (table 4). This showed that, as viable *M. leprae* increased, the level of cholesterol ester was also augmented with increasing level of the ratio of cholesterol to its ester. This indicated that the phenomenon depended on the presence of viable bacteria and thus would reflect the viability of the test *M. leprae* as well. No linear relationship is seen, because, there could not be an uniform ratio of live to dead *M. leprae* in all aliquots, since *M. leprae* basically came from human tissues.

Table 4. Effect of increasing amount of *M. leprae* in the cholesterol metabolism of macrophages (ratio of radioactivity (cpm) in cholesterol ester to that in cholesterol/10^6 macrophages).

Amount of *M. leprae* added to 1×10^6 macrophages	Ratio of cholesterol ester to cholesterol
No. *M. leprae* added	0·10
$2{\cdot}5 \times 10^6$ *M. leprae*	0·10
$3{\cdot}0 \times 10^6$ *M. leprae*	0·11
8×10^6 *M. leprae*	0·25
10×10^6 *M. leprae*	0·50
15×10^6 *M. leprae*	0·53

Recently the concentration of DDS within 10^6 macrophages when the cells were exposed to 1 μg of DDS has been determined by Jagannathan and Mahadevan (personal communication). It is estimated as 25ng/10^6 macrophages and each macrophage is expected to have DDS to a level of 1·7–12·5 μg/ml. The estimation was done by them using fluorometric measurement which has a sensitivity upto 4 ng level of DDS. The concentration obtained in the macrophages is much higher than the minimum inhibition concentration 0·003 μg/ml needed to function as bacteriostatic to *M. leprae* as reported by Ellard *et al.* (1971) and Peters *et al.* (1975).

Experiments were also done using *M. leprae* derived from clinically relapsed patients and those suspected to harbour drug resistant bacteria in their bodies. In such experiments it was clear that *M. leprae* showed no sensitivity to the drug, dapsone, since

even in the presence of dapsone inside the macrophages, they behaved as if they were alive, by increasing the cholesterol ester level more than the control. The data in table 5 presents one such isolate (biopsy No. 499 using [^{14}C]-acetate as labelled precursors) and table 6 presents a consolidated data on all the five separately isolated bacteria.

Table 5. Cholesterol and the ester level in macrophages exposed to "resistant" bacilli isolated from clinically relapsed patient during dapsone (DDS) treatment (cpm/10^6 macrophages).

	Cholesterol	Cholesterol ester	Ratio*
Macrophage	2308	313	0·136
Macrophage + heat killed *M. leprae*	2541	432	0·17
Macrophage + DDS	2020	303	0·15
Macrophage + DDS + *M. leprae*	1550	687	0·44
Macrophage + *M. leprae*	2034	799	0·39

*Ratio of radioactivity (cpm) is cholesterol ester to that in cholesterol/10^6 macrophages.

Table 6. Ratio of cholesterol ester/cholesterol in macrophages exposed "resistant" bacteria during different conditions (ratio of radioactivity (cpm) in cholesterol ester to that in cholesterol/10^6 macrophages).

M. leprae from	Macrophage	Macrophage + heat killed *M. leprae*	Macrophage + live *M. leprae*	Macrophage + DDS (dapsone)	Macrophage + DDS + *M. leprae* (dapsone)
	a	*b*	*c*	*d*	*e*
423*	0·14	0·11	0·35	0·12	0·57
435*	0·09	0·03	0·55	0·06	0·54
495**	0·18	0·16	0·49	0·19	0·57
499**	0·14	0·17	0·39	0·15	0·44
277**	0·14	0·12	0·30	0·15	0·61
Mean with S.D.	0·138 ± 0·029	0·118 ± 0·05	0·416 ± 0·091	0·134 ± 0·043	0·546 ± 0·057

* Using labelled cholesterol.
** Using labelled acetate.
a – *e*; $P < 0·025$
c – *e*; $P < 0·05$
(no. of experiments = 5).

Similar results were obtained with another drug rifampicin and with [^{14}C]-acetate as precursors as shown in table 7 and it could be seen that of the 3 isolates tested all the 3 were sensitive to rifampicin.

Table 7. Ratio of cholesterol ester/cholesterol in macrophages during different conditions with rifampicin (ratio of radioactivity (cpm) in cholesterol ester to that in cholesterol/10^6 macrophages).

Type of *M. leprae*	Macrophage	Macrophage heat killed *M. leprae*	Macrophage + live *M. leprae*	Macrophage + Rifampicin	Macrophage + Rifampicin + live *M. leprae*
Armadillo Bacilli	0·30	0·44	0·93	0·47	0·45
Armadillo Bacilli	0·30	0·44	0·93	0·47	0·47
FMR 142 (original human biopsy bacteria)	0·28	0·20	0·65	0·22	0·32

Confirmation by mouse foot-pad

Since the mouse foot-pad test is generally accepted as a reliable procedure for viability (Shepard, 1960, 1967) we confirmed the drug sensitivity/resistance of some isolates from the human biopsies by both mouse foot-pad method and the method described in this paper. Such isolates are FMR 423,435,277. The mouse foot-pad tests were carried out as per the procedures of Rees (1964) and Shepard (1967). All the three isolates tested and shown to be resistant to dapsone by the cholesterol uptake method were found to be resistant to the drug by growing in mice fed with varying concentration of the drug (tables 8 and 9).

Table 8. Correlation of resistance of *M. leprae* to drug (dapsone) by the *in vitro* method and mouse foot-pad experiment.

Bacteria isolated from biopsies	Present *in vitro* test resistance to DDS	Experiment No.	Resistance to drug DDS (mouse foot-pad growth)
FMR 423	Resistant	AH 227	Resistant (as indicated by growth at 9th month in drug fed animals)
FMR 435	Resistant	231	Resistant (see table 9)
FMR 277	Resistant	143	Resistant (as indicated by growth of *M. leprae* seen at 9th month drug fed animals)

Table 9. Mouse foot-pad growth of *M. leprae* from patient biopsy FMR 435 suspected to have drug resistant *M. leprae.*

Clinical assessment:- Treated case, possibly relapsed.		Inoculum: 10^4 AFB/0·03 ml/H.F.P. Swiss white random bred mice BI = 5+ : MI = 3%
	Number of bacteria 7th month post inoculation	Number of bacteria 9th month post inoculation
Untreated controls	7×10^5 AFB/FP	2×10^5 AFB/FP
0·0001 mg% DDS*	10×10^5 AFB/FP	$1{\cdot}5 \times 10^5$ AFB/FP
0·001 mg% DDS*	$12{\cdot}5 \times 10^5$ AFB/FP	$1{\cdot}5 \times 10^5$ AFB/FP
0·01 mg% DDS*	24×10^5 AFB/FP	$1{\cdot}3 \times 10^5$ AFB/FP

BI–Bacteriological index–total bacterial load.
MI–Morphological index–viable bacteria load.
AFB/FP–Acid fast bacilli per foot-pad.
* Mice fed with indicated concentration of DDS.
PP–Foot-pad.

Discussion

It is clear from our earlier data (Ishwari Kurup and Mahadevan, 1982) and from the present experiments that heat killed *M. leprae* have no ability to interact with macrophages of mice to allow increased accumulation of cholesterol ester. Using this information the viability of *M. leprae* in the presence of drug inside the macrophages could be assessed. This has been possible to do so, as illustrated from the data provided in tables 1 and 6 when DDS and rifampicin, respectively, were used as the drugs. Viability or otherwise in the presence of drug would be indicated by cholesterol ester accumulation and this in turn would indicate sensitivity/resistance of the *M. leprae* to the drug tested. This method is reliable and consistent as has also been shown by correlative information of the drug sensitivity resistance of the same *M. leprae* preparation as tested by mouse foot-pad technique.

A few other *in vitro* techniques have been described using uptake of radioactive dihydroxyphenylalanine (DOPA) by *M. leprae* as an indicator of viability (Ambrose *et al*., 1978); incorporation of [^{3}H]-thymidine by *M. leprae*, phagocytosed inside macrophages (Nath *et al*., 1982 and Prasad *et al*., 1981) or incorporation of [^{14}C]-acetate into lipids of *M. leprae* while the bacteria are still in isolated human tissues (Lakshmi *et al*., 1983). All these have been used with reasonable success for screening the viability and drug sensitivity of *M. leprae* from human biopsies. A major problem that has to be overcome with *M. leprae* is their inability to grow *in vitro* in any bacteriological medium in a short time. Thus one has to use some metabolic activity as an indicator of the non dividing bacteria for viability testing.

The advantages of the drug screening system described here is that it can use macrophages derived from mice and the readily available labelled compound [^{14}C]-acetate. The test can be completed in 9 days time and the results are consistent thus far. Since metabolic compounds are isolated and the radioactivity (at appreciable levels) are estimated in these compounds, so greater confidence exists. By a clear demonstration of a metabolic trigger in the host cell by the live bacteria, we are able to assay the viability of non-dividing *M. leprae* in the *in vitro* system which is probably closer to what happened in an *in vivo* situation. As indicated in our earlier paper cholesterol ester accumulation has a significance to the foamy nature of host cells infected with *M. leprae.* Thus the process monitored is one that could be occurring in the pathological state of leprosy in the patient. These advantages clearly put this technique, perhaps, above all the other methods described so far.

Experiments for further standardization of the technique and adoption of the method to determine minimum inhibition concentration of the known drugs are in hand.

Acknowledgements

The authors express their gratitude to Acworth Leprosy Hospital, Bombay for human biopsies and to Dr. E. Storrs (Florida, USA) for armadillo material (aided by Lepra, UK).

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