

Comparison of Radiometric Macrophage Assay and the Mouse Foot Pad Infection for the Evaluation of *Mycobacterium leprae* Sensitivity/Resistance to Dapsone¹

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The *in vitro* uptakes of various radiolabeled compounds by *Mycobacterium leprae* maintained alone (^{3,4,17}) or within cells (^{5,9,11,14}) have been reported in recent years. These studies, lasting 1–2 weeks, give precise measurements of *M. leprae* viability and have potential for screening antileprosy drugs and for diagnosing drug resistance. Our laboratory has developed a rapid *in vitro*, radiometric assay of *M. leprae* within resident macrophage (MØ) cultures for evaluating bacillary viability (^{6,11,14}), sensitivity to dapsone (DDS, 4,4'-diaminodiphenyl sulfone) (⁹), rifampin (⁷), and lymphokine activity (¹²). Over a two-week period of continual pulsing with high specific activity ³H-thymidine, human and murine MØ cultures containing freshly extracted ("live") *M. leprae* showed a significant uptake of ³H-thymidine ($p \leq 0.05$ to ≤ 0.001) as compared to control cultures containing heat-killed bacilli from the same skin biopsy. The specificity of the uptake and the location of the radiolabel in *M. leprae* and not in the host macrophage was shown by autoradiography (⁸), and DNase experiments (¹⁴).

We had earlier reported that seven *M. leprae* strains derived from lepromatous patients and suspected of dapsone resistance showed concordant results in the *in vitro*

assay and the conventional mouse foot pad infection (⁹). To evaluate the radiometric method further, a collaborative study was undertaken between three independent centers.

Skin biopsies from lepromatous patients clinically suspected of drug resistance at the Central Leprosy Training and Research Institute (CLTRI), Chingleput, India, were coded and shipped by air to the All India Institute of Medical Sciences (AIIMS), New Delhi, for testing in the MØ assay and the National Institute for Medical Research (NIMR), Mill Hill, London, for the mouse foot pad test. Results obtained in the *in vitro* assay were conveyed to the clinicians in 3–4 weeks time and compared with the mouse foot pad results a year later.

MATERIALS AND METHODS

Patients

Twenty-three lepromatous leprosy patients attending the clinics at the CLTRI from 1979 to 1981 were included in the study. They were clinically suspected of dapsone resistance on the basis of the development of new lesions and an increase in the bacterial index (BI) and/or the morphological index (MI) while on therapy. Excision biopsies of active skin lesions were taken at CLTRI on a Monday, coded and divided equally, and shipped by air on wet ice the same day. One half of each biopsy was sent to AIIMS and the other half to NIMR, reaching their destinations on Monday and Tuesday, respectively. Table 1 gives the clinical details of the 13 patients for whom results on both assays from the two centers are available.

Radiometric macrophage assay

Extraction of *M. leprae*. *M. leprae* were extracted from the biopsies within 24 hr of

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receipt of the biopsies, and the bacilli were inoculated into macrophages within 24 hr of extraction. After the removal of the epidermis and the adipose tissue, the bacilli were extracted in glass homogenizers in RPMI 1640 (GIBCO-Biotech, Irvine, Scotland), buffered with 20 mM N-2-hydroxyethyl piperazine-N'-2-ethane-sulfonic acid (HEPES, GIBCO) according to the method of Rees (¹³), and counted as described by Shepard and McRae (¹⁵).

Before inoculation into cultures, the extracted bacilli were screened for contaminating organisms by plating on Nutrient Agar and Löwenstein-Jensen (LJ) medium. The Nutrient Agar plates and LJ slants were incubated at 37°C for 48 hr and 8 weeks, respectively. None of the strains reported in the study grew on either medium.

Macrophage cultures. The details of the macrophage assay have been published earlier (^{9, 11, 14}). a) In brief, human macrophages were derived from glass-adherent monocytes of heparinized (preservative-free heparin, 10 u/ml; Upjohn and Co., Kalamazoo, Michigan, U.S.A.) peripheral blood of normal subjects. Equal volumes of leukocyte-rich plasma and RPMI 1640 were mixed and dispensed in 1.0 ml aliquots in Leighton tubes and incubated at 37°C for 16–18 hr. Subsequently, the nonadherent cells were removed by washing with prewarmed Hanks' balanced salt solution (HBSS, GIBCO). The adherent cells were cultured for 5–7 days in RPMI 1640 enriched with 50% AB serum to permit differentiation. b) Mouse macrophages were obtained from peritoneal resident cells of a close bred strain of Swiss white mice. Equal volumes of peritoneal resident cells and RPMI 1640 enriched with 30% fetal calf serum (FCS, GIBCO) were mixed, dispensed into Leighton tubes in 1 ml aliquots, and incubated at 37°C. After 16–18 hr, the nonadherent cells were removed and the adherent cells were cultured in 30% FCS-enriched RPMI for 48–72 hr to permit differentiation of macrophages.

One million *M. leprae* were inoculated into macrophage cultures and incubated at 37°C for 18 hr. The non-phagocytosed *M. leprae* were removed, and the medium replaced with 50% AB serum and 30% FCS-enriched RPMI for human and mouse macrophages, respectively. One μ Ci of ³H-

thymidine (methyl ³H-thymidine, specific activity 51 Ci/mmol; Amersham International, U.K.) and 3 ng/ml and 10 ng/ml of dapsone were added at this stage. The cultures were incubated for 14 days. The medium, drugs and the radiolabel were replaced every 7 days and 5 days for human and mouse macrophage cultures, respectively. The adherent macrophages were removed by incubating the cultures for 45–60 min at 37°C in the presence of 12 mM xylocaine (Astra-IDL, Bombay, India). The cells were transferred to glass-fiber paper discs (GF/C; Whatman Corp., Clifton, New Jersey, U.S.A.) using a vacuum filtration manifold (VFM-1; Amicon Corp., Lexington, Massachusetts, U.S.A.) and serially washed with cold saline containing 1 mg/ml of unlabeled thymidine (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), 5% trichloroacetic acid (BDH, Poole, London, U.K.) and cold methanol (Ranbaxy, New Delhi, India). The dried discs were counted for radioactivity in a Rackbeta II 1215 (LKB-Bromma, Finland) liquid scintillation β counter using a toluene-based scintillation fluid.

Five replicates each of macrophage cultures were put up with a) heat-killed *M. leprae*, b) freshly extracted ("live") *M. leprae*, c) "live" *M. leprae* + 3 ng/ml, and d) live *M. leprae* + 10 ng/ml of dapsone. Percent inhibition of ³H-thymidine uptake at each concentration of the drug was calculated as $(1 - \text{mean cpm of cultures in presence of "live" } M. leprae \text{ and drug} / \text{mean cpm of cultures in presence of live } M. leprae \text{ alone}) \times 100$.

Statistical analysis was done using Mann-Whitney's two-tailed U test (¹⁶)

Mouse foot pad assay

The biopsies still on wet ice reached NIMR, London, on Tuesday and *M. leprae* from the biopsies were inoculated into mice on Thursday or Friday (i.e., 3 or 4 days following excision from the patient, compared to 2 days for the radiometric macrophage assay).

M. leprae from each biopsy were extracted following homogenization and counted as described above for the radiometric assay, but in 0.1% w/v bovine serum albumin/saline instead of RPMI. For each assay, 20 female CBA mice were inoculated with

TABLE 1. Clinical details of lepromatous leprosy patients suspected of dapsone resistance.

No.	Age	Sex	Current therapy (dapsone daily)	Past therapy (dapsone 2-8 yrs)	ENL ^a	BI ^b	MI ^c
13	17	F	100 mg, 2 yr	Irregular, 10-25 mg	Nil	2.00	<1
16	50	M	Irregular, 50 mg, 10 yr	100-300 mg biweekly	Nil	3.00	<1
20	42	M	50 mg, 5 yr	1-5 mg daily	Nil	2.50	<1
26	32	M	50 mg + thiacetazone, 100 mg + INH, 200 mg, 2 yr	Irregular, 50-150 mg bi-weekly + hydnocarpus oil & Solapson	Frequent, thalido-mide & steroids	2.83	4
27	23	M	50 mg, 6 yr	Irregular, 1 biweekly-10 mg daily	Frequent, thalido-mide & steroids	3.33	<1
28	51	M	50 mg, 2 yr	Discharged as negative after Solapson; active 19 yrs later	Nil	3.00	1.6
29	30	F	100 mg, 5 yr	5 biweekly, 60 mg daily	Nil	3.50	<1
30	26	M	100 mg, 1 yr	Irregular	Nil	2.83	0
33	20	M	100 mg, 1 mo	Interrupted, 10-70 mg daily	Nil	3.00	22.1
37	24	M	50 mg, 2 yr	Irregular	Nil	3.50	1
38	32	M	100 mg, 1 yr	Very irregular, 10-75 mg daily	Frequent, thalido-mide	4.00	0.6
39	28	M	50 mg + thiacetazone	Irregular 3.75-50 mg daily	Nil	2.00	<1
40	37	F	100 mg, 2 yr	Irregular, 15-25 mg daily	Mild, steroids	2.33	0

^a ENL = erythema nodosum leprosum.^b BI = bacterial index.^c MI = morphological index.

1.0×10^4 acid-fast bacilli into both hind foot pads. The mice were randomized into groups of 5 mice each fed normal diet (group I), and diet mixed with dapsone $10^{-4}\%$ (group II), $10^{-3}\%$ (group III), and $10^{-2}\%$ w/w (group IV).

The results are expressed as the number of positive foot pads (i.e., a foot pad harvest ≥ 5 times the inoculum) per total number of foot pads harvested per group of mice. The schedule for harvesting of foot pads was modified to reduce the work load without affecting the results. At 6 months, a control mouse (untreated, group I) was harvested and if found to be positive for bacilli, 2 mice receiving the lowest concentration of dapsone ($10^{-4}\%$, group II) were harvested. If these mice were found to be negative, indicating the presence of dapsone-sensitive bacilli, all remaining group II mice were harvested, and all remaining groups of mice were discarded. If multiplication was observed, then 2 mice from the groups III and IV, receiving the next higher concentrations of dapsone, were serially harvested. If group IV, receiving the highest dose of dapsone ($10^{-2}\%$), had positive foot pads, indicating thereby the presence of fully resistant bacilli, then the groups receiving lower con-

centrations were not evaluated further. When control mice were negative at 6 months, further sampling of foot pads was done at 9 and 12 months in the same manner as described above.

RESULTS

Twenty-three lepromatous patients were clinically suspected of dapsone resistance based on the development of new lesions and an increase of BI and/or MI while on therapy. Excision skin biopsies from these patients were taken on Monday and concurrently tested in the macrophage (MØ) assay in New Delhi on Wednesday and in the mouse foot pad in London on Thursday or Friday. Of the 23 biopsies shared, 2 were discarded due to insufficient bacilli; 2×10^7 and 4×10^5 bacilli were required for the *in vitro* and *in vivo* assays, respectively. Four *M. leprae* strains failed to grow in the mouse foot pad although they showed incorporation of ^3H -thymidine in the MØ cultures. Four more strains showed multiplication in the mouse foot pad but failed to incorporate the radiolabel. It should be stressed that all of the biopsies were treated in a similar manner in both laboratories. Delays en route, insufficient bacilli, or experimental

TABLE 2. Criteria for assessing dapsone resistance and sensitivity.

Mouse foot pad					Macrophage assay		
Multiplication					% inhibition		
Control	% dapsone in diet			Conclusion ^a	Dapsone (ng/ml)		Conclusion ^a
	10 ⁻⁴	10 ⁻³	10 ⁻²		3	10	
+ ^b	— ^c	—	—	S	≥ 50 ^e	≥ 50 ^e	S
+	NH ^d	NH	+	R	< 50	< 50	R
+	NH	+	—	PR	< 50	≥ 50 ^e	R
+	+	—	NH	PR			

^a S = sensitive, R = resistant, PR = partially resistant.

^b + = evidence of multiplication.

^c - = no evidence of multiplication.

^d NH = not harvested.

^e p value ranging from ≤0.05 to ≤0.005 by Mann-Whitney two-tailed U test.

variables were not considered to be the causes for the above discrepancies.

Thus, *M. leprae* strains from 13 patients were available for comparison in both assays. Table 1 gives the details of therapy and the BI and MI of bacilli in smears of the skin scrapings from these patients. Following biopsy, all the patients were given rifampin. Some received an initial single dose of 1200 mg, whereas others received 600 mg of rifampin daily for 2 weeks followed subsequently by a monthly dose of 600 mg of rifampin. Daily dapsone (100 mg) was given throughout. In addition, patients 26, 27, and 38 received 100 mg of clofazamine daily. Follow up of one to three years was available on patients, 16, 20, 26, and 39, all of whom showed clinical improvement and reduction in BI and MI while on these regimens.

The criteria used for evaluating dapsone sensitivity/resistance are given in Table 2. The criteria for the MØ assay were based on an earlier study of 14 bacilliferous leprosy patients⁽⁹⁾ where >50% inhibition of radiolabel uptake in drug-treated cultures was found to be statistically significant by the Mann-Whitney two-tailed U test ($p \leq 0.05$ to ≤ 0.005). In the mouse assay, $\geq 5 \times 10^4$ bacilli harvested per foot pad was considered as positive multiplication. In subsequent tables, the results are expressed as number of positives over the total number of foot pads tested at each concentration of the drug.

Table 3 shows the data obtained in the MØ assay. Four and nine strains were tested in human and murine MØ, respectively. All

13 *M. leprae* strains showed significant levels of ³H-thymidine incorporation in cultures with freshly extracted "live" *M. leprae* as compared to parallel cultures with heat-killed bacilli ($p \leq 0.05$ to ≤ 0.005). The inhibition of ³H-thymidine uptake in the presence of 3 ng/ml and 10 ng/ml of dapsone is shown in terms of mean cpm and percent inhibition in Tables 3 and 4, respectively.

Concordant results were obtained for 12 *M. leprae* strains in the radiometric MØ and mouse foot pad assays (Table 4). Strains 26 and 33 were graded as sensitive since they did not multiply in the presence of the lowest concentration of dapsone (10⁻⁴%) and showed more than 50% inhibition of radiolabel uptake at both drug concentrations in the MØ assay. Eight strains showed growth in mice fed the highest concentration of dapsone (10⁻²%) and were considered fully resistant. Of these, seven showed <50% inhibition of the radiolabel uptake at 3 ng/ml dapsone concentration and variable levels of inhibition at the higher concentration of 10 ng/ml.

Strains 13, 28, and 30 were graded as partially resistant in the mouse foot pad infection because the bacilli multiplied at the lowest (10⁻⁴%) but not at the higher (10⁻³% and 10⁻²%) dapsone concentrations in the diet. These strains behaved as resistant strains in the MØ assay.

Strain 37 showed discordant results in the two assays, behaving as fully resistant in the mouse foot pad and showing sensitivity to both dapsone concentrations in the MØ assay.

TABLE 3. Incorporation of ^3H -thymidine in *M. leprae* strains derived from lepromatous patients and maintained in macrophage (MØ) cultures in the presence and absence of dapsone.

Strain no. ^a	MØ ^b	^3H -thymidine incorporation (mean cpm \pm S.D.)			
		Heat-killed <i>M. leprae</i>	Freshly extracted <i>M. leprae</i>		
			Alone ^c	+ Dapsone	
				3 ng/ml	10 ng/ml
13	H	524 \pm 60	1100 \pm 189	909 \pm 238	1183 \pm 505
16	H	89 \pm 10	618 \pm 456	418 \pm 176	116 \pm 14 ^d
20	H	161 \pm 10	340 \pm 141	278 \pm 139	281 \pm 99
26	H	272 \pm 73	926 \pm 233	356 \pm 78 ^e	340 \pm 57 ^e
27	M	395 \pm 303	859 \pm 469	512 \pm 364	47 \pm 7 ^e
28	M	58 \pm 18	528 \pm 274	364 \pm 331	310 \pm 74 ^e
29	M	120 \pm 68	395 \pm 214	493 \pm 217	371 \pm 134
30	M	312 \pm 199	1701 \pm 984	1534 \pm 623	150 \pm 123 ^e
33	M	1607 \pm 249	2610 \pm 1009	1171 \pm 737 ^d	1090 \pm 608 ^e
37	M	59 \pm 29	324 \pm 63	127 \pm 29 ^d	109 \pm 58 ^d
38	M	96 \pm 49	401 \pm 28	349 \pm 125	178 \pm 28 ^e
39	M	301 \pm 120	742 \pm 70	593 \pm 340	414 \pm 228 ^d
40	M	254 \pm 38	806 \pm 208	478 \pm 110 ^d	178 \pm 46 ^e

^a Strain numbers refer to patients whose clinical details are given in Table 1.

^b M = mouse macrophages, H = human macrophages.

^c All values significantly higher than heat-killed *M. leprae* controls, at most $p < 0.05$, Mann-Whitney U test.

^d Less than live *M. leprae* alone, $p < 0.05$, Mann-Whitney U test.

^e Less than live *M. leprae* alone, $p < 0.01$, Mann-Whitney U test.

Using the mouse foot pad infection as the reference point the present results a) confirm our earlier findings (⁹), and would indicate that b) $\geq 50\%$ inhibition of the radiolabel uptake at 3 ng/ml may be a realistic

criterion for the diagnosis of dapsone resistance since four strains considered fully resistant in the mouse showed significant inhibition of ^3H -thymidine incorporation at the higher 10 ng/ml concentration; c) by the

TABLE 4. Comparison of results obtained in mouse foot pad infection and the radio-metric macrophage (MØ) assay.

Strain no.	Mouse foot pad (no. positive ^a /total)					Macrophage assay (% inhibition)		
	Control	% dapsone in diet			Conclu-sion ^b	Dapsone ng/ml		Conclu-sion ^b
		10 ⁻⁴	10 ⁻³	10 ⁻²		3	10	
26	5/6	0/10	NH ^c	NH	S	62	63	S
33	6/8	0/10	NH	NH	S	55	58	S
37	10/10	NH	NH	8/10	R	61	66	S
20	6/6	NH	NH	6/6	R	18	17	R
29	6/6	NH	NH	6/6	R	-24 ^d	6	R
16	7/8	NH	NH	6/6	R	23	81	R
27	5/6	NH	NH	6/8	R	40	94	R
38	10/10	NH	NH	8/10	R	13	56	R
39	7/8	NH	NH	6/6	R	21	45	R
40	6/6	NH	NH	6/6	R	41	78	R
13	6/10	NH	5/10	0/10	PR	17	-7 ^d	R
28	6/8	2/10	0/8	NH	PR	31	41	R
30	8/10	NH	5/6	0/10	PR	10	92	R

^a Positive mouse foot pad indicates the presence of $\geq 5 \times 10^4$ acid-fast bacilli at the time of harvest.

^b S = sensitive, R = resistant, PR = partially resistant.

^c NH = not harvested.

^d Negative values indicate lack of inhibition.

present criteria, the partially resistant bacilli as tested in the murine model may not be identifiable in the *in vitro* MØ assay.

DISCUSSION

The present study undertaken in three independent centers was aimed at comparing the newer *in vitro*, macrophage-based radiometric assay with the conventional mouse foot pad infection for the diagnosis of dapsone resistance. The results on bacilli obtained from 13 lepromatous patients compared well in the two assays. Except for strain 37, which was sensitive to dapsone in the MØ assay but resistant in the mouse, all other strains showed concordance for sensitivity/resistance to dapsone. Taken together with our earlier collaborative study (where Central JALMA Institute, Agra, India, undertook mouse foot pad studies), only one of a total of 20 strains tested showed discordant results in the two assays.

The present investigation confirms the validity of using 50% inhibition of the radiolabel in the drug-treated cultures as an end point for the diagnosis of drug resistance. However, it would appear that 10 ng/ml dapsone concentration *in vitro* may be high. The lower concentrations had been selected earlier (⁹) because a) all sensitive *M. leprae* strains tested showed inhibition of ³H-thymidine uptake at these concentrations, b) 10 ng/ml correlated with plasma levels reported in mice fed 10⁻⁴% dapsone (^{1, 2, 10}), and c) one partially resistant strain showed differential uptake of the radiolabel at 3 ng/ml and 10 ng/ml.

It is evident from the present study that bacilli considered to be partially resistant to dapsone in the mouse cannot be identified reliably in the radiometric MØ assay using the present criteria. Whether or not a concentration lower than 3 ng/ml of dapsone in the MØ cultures would reveal these bacilli remains to be investigated. *M. leprae* resident in MØ cultures seem to be sensitive to lower concentrations of drugs than the same bacilli in the infected mouse (⁸).

It was not possible to correlate either assay with the clinical behavior of the patients since they had been subsequently treated with rifampin, clofazamine, or both. The follow-up data available for some patients show clinical improvement and reduction in BI 1–3 years after the institution of therapy. As expected, most of the patients who

developed dapsone resistance had taken dapsone irregularly or were given low doses for a prolonged period of time. It would appear that the level of dapsone resistance in bacilli from the patients seen at CLTRI is distressingly high, as evidenced by our earlier report (⁹), the present study, and our unpublished data on more than 100 patients tested by the MØ assay.

The radiometric MØ assay reported here has the advantage of early diagnosis of resistance, making it possible for the clinician to wait for the laboratory report before the introduction of new therapy. Moreover, within a 2–3-week period a full drug-sensitivity profile of the patient's bacilli can be made available with little extra work by the laboratory. Time kinetics and dose responses for rifampin (⁸) and clofazamine (⁷) have been defined on human- and armadillo-derived *M. leprae*. Moreover, the present constraint of the higher numbers of bacilli required in the MØ assay have been overcome by the development of a micro-culture method using 96-well, flat-bottom culture plates (⁶). As compared to the requirement of at least 20 mice over a 9–12-month period, macrophages derived from one mouse were found to be adequate for the screening of one patient. Thus, the need for large numbers of inbred mice may be avoided in leprosy-endemic countries where maintenance of animals in air-conditioned facilities is a major problem.

SUMMARY

Studies were undertaken in three independent centers to compare the newer, *in vitro* radiometric macrophage (MØ) assay with the conventional mouse foot pad infection for the diagnosis of dapsone resistance. Results obtained on 12 bacilliferous patients showed good concordance in both assays. One strain diagnosed as sensitive in the MØ assay was found to be resistant in the mouse foot pad. Three *Mycobacterium leprae* strains considered to be partially resistant in the mouse infection behaved as resistant strains in the MØ cultures. Attention is drawn to a rapid *in vitro* method for the identification of drug-resistant bacilli in leprosy patients.

RESUMEN

Se hicieron estudios en 3 centros independientes para comparar el recientemente diseñado ensayo radiométrico

trico con macrófagos (MØ), con el método convencional de infección en la almohadilla plantar del ratón usado para el diagnóstico de resistencia a la dapsona. Los resultados obtenidos en 12 pacientes bacilíferos, mostraron buena concordancia en ambos ensayos. Una cepa diagnosticada como sensible en el ensayo MØ resultó ser resistente en el ensayo de la almohadilla plantar. Tres cepas de *Mycobacterium leprae* consideradas como parcialmente resistentes por la infección en el ratón, se comportaron como resistentes en los ensayos MØ. Se enfatiza la utilidad del ensayo MØ *in vitro* para la rápida identificación de bacilos resistentes a drogas en pacientes con lepra.

RÉSUMÉ

Dans trois centres indépendants, on a comparé la valeur, pour le diagnostic de la résistance à la dapsona, de la méthode récemment mise au point pour le dosage radiométrique des macrophages *in vitro* (MØ), et l'inoculation classique du coussinet plantaire de la souris. Les résultats obtenus chez 12 malades bacillifères ont montré une bonne concordance des deux méthodes. Une souche qui avait été relevée comme sensible dans l'essai MØ, a été trouvée résistante par la méthode du coussinet plantaire. Trois souches de *Mycobacterium leprae*, considérées comme partiellement résistantes par l'inoculation à la souris, se sont révélées résistantes dans les cultures de macrophages. On attire l'attention sur l'intérêt d'une méthode *in vitro* rapide pour l'identification des bacilles résistant aux médicaments chez les malades de la lèpre.

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