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In vitro drug screening system using membrane alteration in macrophages by *Mycobacterium leprae*

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Abstract. The observation that live *Mycobacterium leprae* on entry into macrophages from lepromatous leprosy patients reduced the number of EA rosetting macrophages, was extended to macrophages from Swiss white mice also. Further, the fact that dead *Mycobacterium leprae* do not bring about such a change in macrophages from mice, allowed us to develop this into a bacterial viability testing system. Thus drug treated macrophages in the presence of *Mycobacterium leprae* showed normal rosetting ability if *Mycobacterium leprae* are inactivated by the drug, but showed reduced level of rosetting when bacteria were not susceptible to the drug. It was shown that a drug like dapsone, does act on *Mycobacterium leprae* based on its permeability, quantity available inside the macrophages and inhibition of its action by Para amino benzoic acid. The inactivation of *Mycobacterium leprae* by sulphone and rifampicin was also proved by the flourescence diacetate method, which showed poorly viable bacteria after exposure to drugs. Thus it has been possible to develop a rapid drug screening method for testing the activity of unknown compound against *Mycobacterium leprae.*

Keywords. Macrophage; membrane changes; F_c receptors, *Mycobacterium leprae;* viability; drug screening.

Introduction

With the advent of drug resistance to sulphone (dapsone di-(4-aminophenyl)-sulphone, DDS) and recently to rifampicin, two accepted drugs for leprosy, there is a need for identifying new compounds with activity against *Mycobacterium leprae.* Rapid *in vitro* screening methods are basic requirement for expanded intense drug development work. Such methods are then supplemented with *in vivo* test systems. The efforts towards identifying new compounds with activity against *M. leprae* have been insignificant, partly due to lack of rapid drug screening systems. The mouse foot pad method developed by Shepard (1960) as being adopted for routine screening work (Shepard, 1976; Rees, 1964) is time consuming. There are few *in vitro* techniques that have been described recently (Ambrose *et al.,* 1978; Nath *et al.,* 1982; Prasad *et al.,* 1981; Vithala *et al.,* 1983; Nair and Mahadevan, 1984).

We describe here, the utility of a method which uses the membrane changes induced in mice macrophages by live *M. leprae,* which leads one to distinguish the dead or

Abbreviations used: DDS, Dapsone (di-4-aminophenyl)-sulphone; MEM, minimul essential medium; EA, erythrocyte-amboceptor; PABA, *p*-amino benzoic acid; FDA, fluorescein diacetate; EB, Ethidium Bromide.

inactivated *M. leprae* from live ones. Such an observation was made and was convincingly demonstrated by Birdi *et al.* (1983). Birdi *et al.* (1984) also described how this method could be adopted as a drug screening system using human macrophages. This communication provides further data for the usefulness of this method.

Materials and methods

Mycobacterium leprae was obtained from lepromatous tissue of bacillary positive, treated or untreated patients. Bacilli were prepared as per the method of Ambrose *et al.* (1974). Such isolated bacilli were acid-fast stainable and free from other contaminating bacteria. These did not grow in normal mycobacteriological media. The bacilli were counted and 5×10^6 bacilli were added to each Leighton tube containing macrophage cultures. *M. leprae* were also obtained from infected armadillo tissue, supplied by Dr. E. Storrs, Florida, USA. Such armadillo-derived bacteria were used as a source of drug sensitive bacteria to standardise the method of drug testing.

Macrophage cultures

Macrophages from Swiss white mice (random bred) were obtained from the peritoneal cavity following injection of 5 ml of Eagle's minimal essential medium + 20% inactivated human serum (AB blood group) into the peritoneal cavity, after killing the animal by cervical dislocation. The peritoneal fluid was collected after agitating the cavity and 0·7 ml of the fluid was added to each presterilised Leighton tube. The macrophages obtained from the peritoneal fluid adhered to the coverslip placed inside the Leighton tube. The medium was changed every 24 h and after 3 days of such culturing, esterase positive, phagocytic cells were predominantly distributed as a uniform layer in the coverslip inside the petridish. There were no contaminating neutrophils and non adherent lymphocytes were not present in any significant numbers. All these steps were done under sterile conditions.

Seventy two hours after distribution of macrophage, the drug dapsone (Burroughs Wellcome Co., India) or rifampicin (Sigma Chemical Co., USA) was added along with the medium. The drug was washed off after 72 h exposure, following which *M. leprae* were fed to the macrophage cultures. The *M. leprae* were phagocytosed by the macrophages and were allowed to interact with the drug inside the macrophage for 72 h.

In the final step the macrophages were washed with minimul essential medium (MEM) to remove any external bacilli and then were subjected to erythrocyte-amboceptor (EA) rosetting following the method described by Birdi *et al.* (1983).

p -Amino benzoic acid reversal of DDS effect

To show that DDS does enter the macrophages and its action is blocked by its well known modulator *p*-amino benzoic acid (PABA) the following experiment was done.

The macrophage culture from the peritoneum of Swiss white mice was cultured and after 72 h of culturing it was exposed to drug DDS and PABA. The compounds were allowed to enter the macrophage for another 72 h following which a wash with MEM

was given and *M. leprae* was added. The *M. leprae* inside the macrophage were allowed to interact with the compounds for further 72 h. Following the incubation period the macrophages were washed with MEM to remove excess bacilli and were then subjected to EA rosetting.

Estimation of DDS inside the macrophages

The macrophages grown *in vitro* for 72 h, were treated as described above with the drug dapsone (DDS) following which they were harvested by scarping them with a rubber policeman. The macrophages were then washed three times with saline so as to get rid off extra cellular DDS. The count of the macrophages was taken in a haemocytometer after suspending the macrophages in 1 ml saline.

The macrophages were then lysed by subjecting them to 8 cycles of freeze-thawing. Redistilled ethyl acetate (6 ml) (checked for fluorescence quenching) was added to the lysate in the presence of 0·5 ml 10 N NaOH. The mixture was vortexed to facilitate the extraction of DDS from lysate to ethylacetate. After separation of the solvent and aqueous phase (20 min), the ethylacetate phase was aspirated out into another tube with a pasteur pippette. To the ethylacetate phase 0·5 g of NaCl was added to remove off any water molecules carried during the extraction. The ethyl acetate phase was then allowed to separate from the NaCl which sedimented at the bottom. The ethylacetate phase was transferred to another tube and then checked for fluorescence using a spectroflourimeter (SFM23-Kontron) which had already been standardised to ng levels of DDS, with a sensitivity upto 2 ng/ml. The wave length settings were 285 nm for excitation and 350 nm for emmission. There was always a control culture of macrophages which had not been exposed to the drug to compare with the macrophage culture which was exposed to the drug. The results were computed as follows to eliminate the background fluorescence of macrophage itself (Peters *et al.,* 1981).

DDS (in ng)/million macrophages is concentration that causes an absorption level of 1 million macrophages exposed to drug minus absorption level of 1 million macrophages not exposed to drug. The quantitation of the drug was determined from a standard curve of fluorescence absorption determined for various DDS standard concentrations.

Determination of viability of M. leprae within macrophages

The method used was the fluorescein diacetate (FDA)/ethidium bromide (EB) (Both from Sigma Chemical Co., USA) staining of *M. leprae* (Kvach *et al.,* 1982 as modified in our laboratory by Nalini, R., Alaka, B. and Mahadevan, P. R., Personal communications).

One set of coverslips with adherent macrophages was processed in the manner described above with drug and bacilli following which they were checked for viability.

The coverslips on the day of termination were rinsed in MEM to wash off the excess of bacilli and placed over a drop of the staining solution (20 μg of FDA and 20 μg EB in 5 ml of phosphate buffered saline). This was incubated for 29 min in the dark. The coverslip was sealed with nail polish to prevent evaporation and observed under incident fluorescent ultra-violet light using Carl Zeiss fluorescence microscope at a magnification of 400.

A total number of 200 macrophages were counted and the number of macrophages with green bacilli (denoting live *M. leprae*) were calculated. The total number of macrophages with bacteria were estimated by determining their level with acid fast bacteria. The viability is then indicated in the number of macrophages showing green fluorescencing bacteria to the total number of acid fast bacteria containing the macrophages.

Results

The entry of the drug DDS into the macrophages was clearly demonstrable by an estimation of the level of DDS in purified macrophages. The internal concentration of DDS increased with increasing level of DDS outside the cells (table 1) and it has been possible to calculate the DDS concentration/10^6 macrophages.

Table 1. DDS uptake by macrophages when exposed to various levels of the drug .

DDS added in the medium		DDS incorporated ng/10^6 macrophages (three separate experiments)		
ng/tube	ng/ml	1	2	3
10	14·2	1·6	1·7	1·6
20	28·5	2·1	2·5	2·6
50	71·4	5·2	7·1	5·1
100	142·8	8·1	8·5	8·2

The ability of macrophages from the peritoneal cavity of mice to reveal the F_c receptors as monitored by EA rosetting is clearly reduced in the presence of phagocytosed live *M. leprae* (table 2). If however the bacteria were killed or inactivated before phagocytosis then the rosetting ability is the same as the control. Since *M. leprae* are susceptible to DDS, the drug treated macrophages show rosetting ability as good as the control, indicating that the drug has inactivated the phagocytosed *M. leprae* (table 2). The data presented in table 3 show a similar observations using rifampicin at a concentration of 5 μg/Leighton tube.

To demonstrate that the action of the drug on *M. leprae* is a true feature, an experiment was done with various concentrations of DDS so as to decide the level at which *M. leprae* was inactivated. Data presented on table 4 showed that at concentrations below 20 ng/ml in the medium, the bacteria inside the macrophages were not inactivated, and thus the control level of EA rosetting ability of the macrophages is not restored. Thus a clear concentration dependence was demonstrable.

PABA a known inhibitor of sulphone could reverse the effect of DDS on *M. leprae* in those macrophages that were given both DDS and PABA (table 5). A degree of antagonism has been thus demonstrated.

The drug exposed *M. leprae* that were inactivated and was responsible to show the normal level of EA rosetting of the macrophages, could also be shown to be nonviable

Table 2. Per cent EA rosetting macrophages in presence of armadillo derived *M. leprae* with and without exposure to dapsone.

No. of the Expt.	Control (Mθs only)	DDS only	Macrophages with Live *M. leprae* only	DDS and *M. leprae*	Heat killed *M. leprae* only
1	53	40	25	51	59
2	38	36	23	38	49
3	50	40	34	51	58
4	62	44	25	53	64
5	66	46	33	52	66
6	60	40	34	61	68
7	64	42	37	62	69
8	59	48	34	60	68
9	57	46	26	58	66
10	56	39	42	54	55
11	54	41	41	56	52
Mean ± S.D.	56 ± 2	42 ± 1	32 ± 2	54 ± 2	61 ± 2

Control: *M. leprae* added culture significant $P < 0{\cdot}05$ M. *leprae* only: Drug + *M. leprae* significant $P < 0{\cdot}05$.

Table 3. Per cent EA rosetting macrophages in presence of armadillo derived *M. leprae* with and without exposure to rifampicin.

No. of the Expt.	Control (only Mθs)	Rifampicin only	Macrophages with Live *M. leprae* only	Live *M. leprae* and rifampicin	Heat killed *M. leprae* only
	A	*B*	*C*	*D*	*E*
1	49	32	29	70	66
2	50	35	35	73	60
3	54	32	34	70	65
4	56	34	37	71	69
5	70	54	34	69	68
6	65	49	26	68	67
Mean	57 ± 4	39 ± 4	33 ± 2	70 ± 1	66 ± 2

P value A – C < 0·05 Significant
P value D – C < 0·05 Significant

by the fluorescent staining method. The data presented in table 6 clearly show that on exposure of *M. leprae* to a level 100 ng (0·1 μg/ml) of DDS caused the *M. leprae* to become nonviable; since such drug treated macrophages showed reduced number of macrophages with green fluorescent bacteria; but showed acid fast stainable bacteria in much great numbers. The drug untreated macrophages containing viable *M. Leprae* were seen as green bacteria fluorescing in higher number of macrophages and comparable well with macrophages containing acid fast bacteria. Similar results were

Table 4. Per cent of macrophage exhibiting EA rosetting in presence of *M. leprae* and exposed to various concentration of DDS.

DDS added per tube	Concentration of DDS (ng/ml medium)	Control macrophages only	Live *M. leprae* only	Macrophages containing DDS only	Macrophages containing DDS and live *M. leprae*	Macrophages containing Heat killed *M. leprae*
		A	*B*	*C*	*D*	*E*
10	14·2	49 ± 14	24 ± 6	30 ± 8	31 ± 9	55 ± 13
15	21·4	70 ± 7	46 ± 7	59 ± 3	59 ± 3	70 ± 0
20	28·5	75 ± 5	48 ± 6	65 ± 5	76 ± 5	75 ± 5
50	71·4	72 ± 2	46 ± 8	60 ± 2	71 ± 2	72 ± 2
100	142·8	68 ± 3	42 ± 3	56 ± 9	70 ± 3	69 ± 5

Each value is an average of 4 experiments with each concentration of the drug added.
A–B $P < 0{\cdot}05$ significant.
A–C $P < 0{\cdot}05$ –do–
A–D $P < 0{\cdot}05$ significant upto 15 ng only.
A –E $P > 0{\cdot}05$ not significant.
A –D $P < 0{\cdot}05$ significant above 15 ng only.

Table 5. Effect of PABA in relation to DDS action on *M. leprae.*

Per cent EA rosetting macrophages					
			Macrophage with		
Control	Live *M. leprae*	PABA	PABA + live *M. leprae*	DDS + live *M. leprae*	DDS + PABA + live *M. leprae*
60	33	45	51	59	28
85	48	66	50	84	68
88	58	74	62	84	74

Dosage DDS –1 μg/tube –1·42 μg/ml.
PABA – 0·5 mg/tube – 0·70 mg/ml.

obtained by using rifampicin as the drug (table 6). In such an experiment the EA rosetting ability was also well correlated. The viable bacteria (green fluorescent) containing macrophages in drug treated samples can be contrasted with number of macrophages containing acid fast bacteria to show the loss of viability in the presence of the drug since acid fast staining give total bacterial load and green fluorescent bacteria indicate only viable among them.

Discussion

The earlier observation by Birdi *et al.* (1983), that live *M. leprae,* on entry into the macrophages cultured *in vitro* from human peripheral blood, could reduce the number of EA rosetting macrophages, has been found to be true with the peritoneal macrophages from Swiss white mice. Like, in the human system, heat killed *M. leprae*

Table 6. Correlation of viability of *M. leprae* as determined by flourescent diacetate method (FDA/EB) with EA rosetting ability of macrophages and *M. leprae* in presence and absence of drugs.

Percent macrophages having phagocytosed *M. leprae* (acid fast staining)	Per cent EA rosetting macrophages			Viability by FDA/EB		
	Control	With live *M. leprae*	With DDS/ rifampicin and *M. leprae*	With live *M. leprae*	With DDS and *M. leprae*	
	DDS		0·1 μg/ tube	0·5 μg/ tube		0·1 μg/ tube	0·5 μg/ tube
78	80	41	78	81	61	36	31
50	74	38	73	75	47	29	21
	Rifampicin						
85	79	49	80*	88**	39	28*	16**
75	56	40	59	70	44	21	12

* 0·2μg/tube
** 0·5μg/tube rifampicin

could not do so. Birdi *et al.* (1984) had projected the usefulness of this system to screen drugs active against *M. leprae* by demonstrating the activity of rifampicin on *M. leprae* using EA rosetting method with human macrophages. The data presented in this paper show the adaptation of the method by a standardised procedure using macrophages from mice and armadillo derived *M. leprae.* Entry of DDS into macrophages has been clearly demonstrated and estimation of DDS inside the macrophages could also be done. The concentration effect of DDS to inactivate the *M. leprae* was also indicated by the fact that DDS treated *M. leprae* did loose their viability which was further proven by the loss of their ability to pick up fluorescent labelling after treatment with fluorescent diacetate (Kvach, 1982). That the DDS is acting through the recognised pathway of a sulpha drug against *M. leprae* (Seydel, 1980) was further demonstrated by the partial reversal of DDS activity by PABA. Besides DDS, rifampicin a known anti *M. leprae* agent could also be shown to be active against *M. leprae,* ingested inside the macrophages, by the loss of their ability to pick up fluorescent labelling after treatment with fluorescent diacetate (Kvach, 1982). This was also shown by monitoring the ability of the bacteria to reduce the number of EA rosetting macrophages in the presence of the drug.

Several clinically isolated *M. leprae* have been tested for drug sensitivity and resistance to DDS and rifampicin using this system. They are also being correlated with behaviour of the isolates in mice foot pad (data not given).

Thus it is clear that by using macrophages and armadillo derived *M. leprae,* the drug sensitivity of the bacteria to known and unknown drugs could be determined using the EA rosetting ability of the macrophages. The test system can be completed in a period of 8–9 days, the shortest time needed in any of the test systems described so far (Vithala *et al.,* 1983; Prasad *et al.,* 1981; Nath *et al.,* 1982) and quite similar to the one described from our earlier work using the cholesterol metabolism of the macrophages (Nair and Mahadevan, 1984).

Several derivatives of methoxyindole have been tested using the method described

here and some of them were found to be effective against *M. leprae* (data not given)

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