J. Biosci., Vol. 10, Number 1, March 1986, pp. 127-135. © Printed in India.

In vitro tests for screening of immuno-modulating mycobacterial strains in leprosy

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MS received 25 March 1985; revised 7 November 1985

Abstract. There is an urgent need for the development of an *in vitro* assay for the initial screening of a large number of organisms from which potential candidates as vaccines can be identified. Our previous studies have demonstrated a crucial defect in the lepromatous macrophage. In this study by monitoring this defective macrophage response we have screened various mycobacteria for their ability to reverse the alterations induced by *Mycobacterium leprae.* Among the limited Mycobacteria tested *Mycobacterium vaccae* appears to be the most promising as an immunomodulator. Our results also indicate the need for caution in using the mouse model for this purpose.

Keywords. Leprosy; immuno-rnodulating strains; *in vitro* screening.

Introduction

The production of vaccine is a subject of high priority in the field of leprosy at present especially due to the emergence of drug resistant strains of *Mycobacterium leprae.* The organisms used for the production and testing of a vaccine at present consist of *M. leprae* (WHO 1983). *M. leprae* and BCG (Convit *et al.,* 1982), ICRC (Deo *et al.,* 1981), Mycobacterium W (Chaudhury *et al.,* 1983) and *M.vaccae* (Stanford *et al.,* 1981).

The absence of a quick, inexpensive method for testing potential vaccine candidates, has severely restricted the screening of organisms as suitable vaccine candidates. There seems to be a pressing need for the development of a quick *in vitro* assay system for the initial screening of a large number of organisms from which the most promising ones could be subjected to the much more expensive field trials.

Our previous studies have demonstrated a crucial defect in the response of the macrophage from lepromatous patients to *M. leprae* (Birdi *et al.,* 1983, 1984). The monitoring of this defective macrophage response might therefore prove to be a specific assay system.

The present study is an attempt to develop an *in vitro* assay using macrophages from lepromatous patients for screening of potential immuno-modulating mycobacteria, by checking the capacity of these mycobacteria to reverse the immune depression induced by *M. leprae.*

Abbreviations used: Bl + ve, Bacteriologically positive; BI —ve, bacteriologically negative; MEM, minimal essential medium; EA rosetting, erythrocyte rosetting; SRBC, sheep red blood cells; MAIS, *Mycobacterium avium intracellular scrofulaceum.*

Materials and methods

Choice of patients

Leprosy patients were classified according to the Ridley and Jopling (1966) classification. Lepromatous patients were further subdivided into bacteriologically positive (BI + ve) *i. e.* those who demonstrated acid fast bacilli in skin smears and bacteriologically negative (BI — ve) *i.e.* those who did not demonstrate acid fast bacilli in skin smears.

Macrophages

Human: Mononuclear cells were isolated from heparinized peripheral blood by sedimentation in 6 % Dextran, and freed from most of the lymphocytes by adherence to glass. The macrophages thus obtained were maintained for 7 days in minimal essential medium (MEM) containing 40 % human AB serum. The culture medium was changed after 24 h and subsequency after every 48 h. After 7 days in culture 95 % of the cells stained positive for non-specific esterase.

Mouse: The cells were obtained by irrigating the peritoneal cavity with MEM containing 20 % human AB serum. 0·7 ml of the cell suspension was dispensed into each leighton tube containing a coverslip. The culture was incubated at 37°C in 5% CO_2 atmosphere. The cultures were maintained for 7 days during which time the medium was changed after the first 24 h, and subsequently after every 48 h.

Source of M. leprae

Biopsies of nodules from untreated lepromatous patients were homogenised and then trypsinized. The *M. leprae* obtained after differential centrifugation was washed with saline, stored at 4°C and used within a week.

F_c mediated erythrocyte rosetting

The erythrocyte rosetting (EA rosetting) was done 72 h after a 24 h infection of the macrophage cultures with 5×10^6 *M. leprae*. Sheep erythrocytes (SRBC) in a 2% suspension in MEM were coated with an equal volume of goat anti SRBC antibody. A suspension of 1% coated SRBC was overlayed on the macrophage culture and incubated at 37°C for 30 min. The monolayers were washed free of unbound SRBC, fixed in 2·5% gluteraldehyde and stained with Ziehl-Neelsen acid fast stain. The percentage of cells with 2 or more SRBC attached was determined, after counting a total of 200 macrophages.

Antigen specific macrophage-lymphocyte physical interaction

Human: Mononuclear cells from peripheral blood of lepromatous (BI — ve) patients was isolated on a Ficoll-Triosil gradient.

The cells so obtained consisted of 80–90 % lymphocytes and 10–20% macrophages. The cells were then resuspended in MAM containing 20% human AB serum at a concentration of 4×10^6 cells/ml, and distributed into Leighton tubes containing

coverslips. The higher cell number was to allow for the varying numbers of monocytes finally obtained. After the appropriate mycobacterium (3×10^6 bacilli/tube) alongwith *M. leprae* (3×10^6/tube) was added the cells were incubated at 37°C for 18 h. The mycobacteria used were ICRC, BCG, *M. delhi, M. vaccae, M. avium, M. intracellulare* and *M. scrofulaceum.* The non-rosetted lymphocytes were washed off and the cells were fixed in 2·5% glutaraldehyde and stained with Ziehl–Neelsen acid fast stain. The percentage of macrophages with two or more lymphocytes adhering to it was calculated after counting a total of 200 macrophages.

Mouse: The mouse spleen was teased gently and the cells layered over Ficoll-Triosil. The cells were then washed and resuspended in 20 % human AB serum in MEM at a concentration of 1×10^6 cells/ml. 0·7 ml was distributed into each Leighton tube. The appropriate antigen was added and the culture incubated for 18 h at 37°C in 5% CO_2 atmosphere. The termination and the staining procedures were similar to the human cell system.

In vivo mouse studies

The mice were infected subcutaneously with viable *M. vaccae* (1×10^6/mouse). At intervals of 6 months and 18 months the peritoneal macrophages were harvested and checked for EA rosetting. Simultaneously the spleen was collected for interaction.

Preparation of macrophage lysate

Macrophages were cultivated *in vitro* from the blood of BI – ve lepromatous leprosy patients. After 5 days in culture the macrophages were exposed to the following mycobacteria: *M. vaccae, M. intracellulare, M. scrofulaceum* and *M. avium.* After 48 h of exposure to the bacilli the macrophages were washed in saline. The cells were scraped off the glass with a rubber policeman and suspended in a known volume of saline and the cell count was determined. The cells were exposed to 6 cycles of freezing and thawing. The intracellular material thus released is referred to as the lysate. The lysate thus obtained was passed through a sterile millipore filter (0·22 μ), to remove cell debris. Lysate was stored frozen till use. The lysate prepared in a similar manner from macrophages of normals and tuberculoid patient infected with *M. leprae* were termed as N-lysate and T-lysate respectively. The lysate of BI + ve lepromatous patients was termed as L-lysate.

Lymphocyte proliferation assay

Mononuclear cells from healthy individuals were separated over Ficoll-Triosil gradient. Cell count was taken and also checked for viability using trypan blue. The cell suspension was adjusted to 1×10^6 cells/ml in culture medium (MEM + 20 % AB serum). Aliquots of 100 μl were distributed into each well of a microtitre plate. Fifty μl of antigen was distributed into appropriate wells. *M, leprae* was used at a dose of 3×10^6 bacilli/ml. Lysate prepared from 2×10^5 cells in 50 μl volume was also added to appropriate culture wells. Cultures were harvested on the 6th day. Each culture combination was set up in triplicate. Eighteen hours prior to harvesting the cultures 0·5 μCi of [^{3}H] -thymidine (specific activity 9·8 curies/mM) was added to each well.

Cells were harvested, processed and total thymidine incorporation was determined using the liquid scintillation counting system.

Results

Production of the suppressor factor

Our earlier studies have demonstrated that L-lysate was capable of inhibiting a normal lymphocyte proliferation to *M. leprae* antigen. However no suppressive activity was observed with N-lysate and T-lysate. These results suggest that an active interaction between lepromatous macrophages and *M. leprae* is required for production of the suppressor factor (Salgame *et al.*, 1984).

In this study other mycobacteria were tested for their ability to induce the production of the suppressor factor in lepromatous macrophages. Lysate prepared from macrophages infected with *M. vaccae, M. scrofulaceum* and *M. kansasii,* did not show any inhibitory activity (figure 1). Out ofthe 3 experiments performed with *M. intracellulare* and *M. avium* in two experiments no suppressive activity was observed. However, in one set of cultures some inhibitory activity was seen (figure 1).

Mycobacterial induced lymphocyte proliferation in the presence of L-lysate

M. leprae induced normal mononuclear cell proliferation was significantly reduced in the presence of L-lysate. Proliferation to mycobacterial antigens, *M. scrofulaceum,*

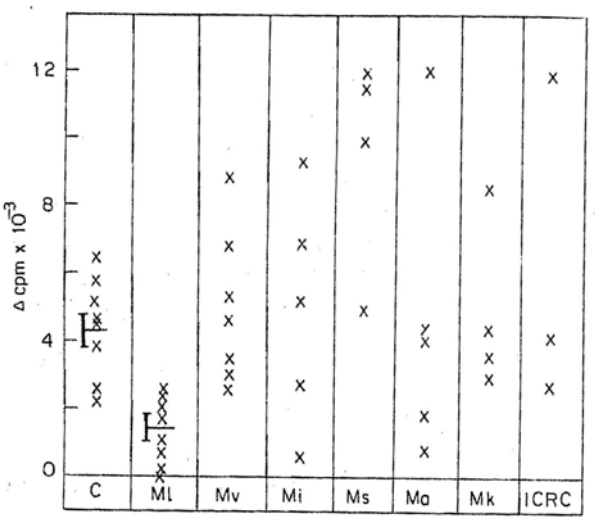

Figure 1. Effect of mycobacteria other than *M.leprae* on production of suppressor factor(s) from lepromatous bacillary negative macrophages. Each cross represents the normal mononuclear proliferation to *M. leprae* expressed as average counts per min of triplicate cultures of the same experiment. C, Lysate of macrophages not exposed to any bacilli, ML, Lysate of macrophages exposed to *M. leprae.* Lysate prepared in the presence of other mycobacteria. M.v, *M. vaccae.* M.i, *M. intracellulare.* M.s, *M. scrofulaceum.* M.a, *M. avium.* M.k, *M. kansasii* and ICRC.

Table 1. Lymphocyte proliferation of normal mononuclear cells to *M. leprae* and other mycobacteria in the presence of L-lysate as measured by [^{3}H]-thymidine incorporation.

	c.p.m.						
Exp. No.	1	2	3	4	5	6	7
Control	1608	859	395	3210	380	3488	289
+ *M. leprae*	3969	2328	507	6409	1825	15223	1244
+ *M. leprae* + L-ly	2313	1008	363	3200	1010	3059	525
+ *M. vaccae*	2073	2880		6671	2214	5436	
+ *M. vaccae* + L-ly.	2909*	2296*		6203*	2369*	5211*	
+ *M. scrofulaceum*	7137	8009		8887	16764		
+ *M. scrofulaceum* + L-ly.	3783	3813		2563	4438		
+ *M. intracellulare*	9217	9660		5873	6063	5749	
+ *M. intracellulare* + L-ly.	3620	2394		4385	4951	3904	
+ *M. avium*	6211	6419	4625	14752	2136		
+ *M. avium* + L-ly.	3096	1440	2306	4210	1832		
+ BCG	10956		1468			37807	9400
+ BCG + L-ly.	13615*		1220*			33631*	7535*
+ ICRC	13921			4546	577	33599	857
+ ICRC + L-ly.	3722			5336*	1586*	25696	912*
+ *M. delhi*	2894				1557	19643	
+ *M. delhi* + L-ly.	6258*				2370*	18208*	

* No significant suppression.

M. avium and *M. intracellular* was also blocked by L-lysate. However, blastogenesis induced by *M. vaccae* and BCG was not inhibited by L-lysate. Out of 5 experiments with ICRC only in two cases stimulation was significantly lowered by L-lysate (table 1).

Lepromatous macrophage F_c receptor expression

In an earlier paper (Birdi *et al.*, 1983) we have already described the effect of *M. leprae* infection of lepromatous (BI – ve) macrophage on EA rosetting. The percentage of EA rosetting was shown to be decreased in macrophage cultures infected *in vitro* with *M. leprae* as compared to the control monolayers of the same patient. From figure 2 it is evident that when the negative percentage difference was calculated between control cultures and *M. leprae* infected cultures a negative difference was obtained indicating a decreased 'EA' rosetting in the presence of *M. leprae.* Next the effect of the addition of a second mycobacterium was monitored. The mycobacteria used were those belonging to the *Mycobacterium avium intracellulare scrofulaceum* (MAIS) complex and the potential vaccine candidates enumerated to date. It appeared that *M. vaccae* was the only one able to totally abolish the efect of *M. leprae* and reverse the decrease in EA rosetting.

Macrophage-lymphocyte interaction in lepromatous patients

In the absence of any antigen in the system a baseline of less than 10 % interaction was obtained. In response to *M. leprae* macrophage-lymphocyte interaction in lepromatous patients was also below 10%, as reported earlier (Birdi *et al.*, 1984). Among the other mycobacteria tested, *M. vaccae* appeared to consistently enhance the interaction as evident from data presented in figure 3, while with the ICRC strains the results were

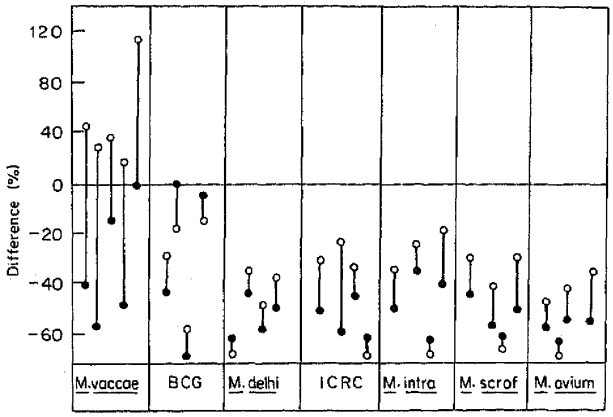

Figure 2. The effect of other mycobacteria on the decrease in EA rosetting in *M. leprae* infected lepromatous macrophages. The percentage difference was calculated taking the uninfected cultures as the baseline, *M. scrof., M. scrofulaceum. M. intra., M. intracellulare.* (●), *M,.leprae.* (O), *M. leprae* + Mycobacterium.

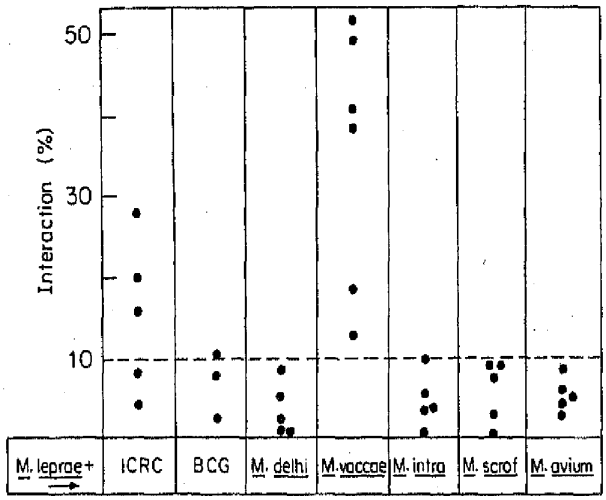

Figure 3. Macrophage-lymphocyte interaction in lepromatous patients in the presence of *M. leprae* (3×10^6) and the appropriate mycobacterium (3×10^6).

variable. Mycobacteria from the MAIS complex were not capable of inducing any increase in the interaction.

From these systems it was evident that among the mycobacteria tested only *M. vaccae* was consistency effective. Therefore, further experiments were done only with *M.vaccae.*

F_c receptor expression by peritoneal macrophages from Swiss white and C5 7B1/6 *mice*

In Swiss white mice there was a decrease in E A rosetting values in cultures infected *in vitro* with *M. leprae* when compared with control cultures. This was not observed in

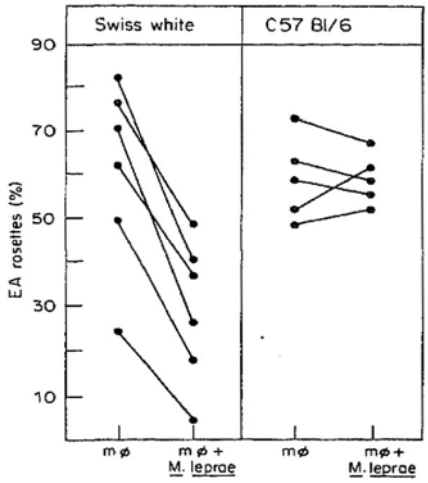

Figure 4. EA roesetting of peritoneal macrophages from Swiss white mice and C57 Bl/6 mice after *in vitro* infection with viable *M. leprae.*

infected cultures of C57B1/6 (figure 4). Thus, peritoneal macrophages from Swiss white and C57B1/6 mice behave similarly to macrophages from lepromatous and tuberculoid patients respectively (Birdi *et al.*, 1983).

Since the *in vitro* tests reported may be used only as indicator systems for effective immunotherapy but not immunoprophylaxis, an attempt was made to study the memory component using the mouse as a model. However our attempts failed since the Swiss white mouse responds to *M. leprae* and *M. vaccae* in the same manner *in vitro* (table 2).

This was further confirmed in the *in vivo* experiments where the Swiss white mice infected subcutaneously with *M. vaccae* showed a dramatic reduction in F_c receptor expression by their peritoneal macrophages even after an extended period of 6 months (table 3).

Table 2. *Fc* receptor expression in macrophage cultures infected *in vitro* with *M. leprae* or *M. vaccae*

	EA rosetting (%)		
	1	2	3
Control	53	95	85
+ *M. leprae* (5×10^6/culture)	38	56	60
+ *M. vaccae* (5×10^6/culture)	39	60	36
+ *M. leprae* (3×10^6/culture) + *M. vaccae* (3×10^6/culture)	23	25	56

Table 3. F_c receptor expression of mice sensitized with *M. vaccae in vitro*.

	EA rosetting (%)					
	1	2	3	4	5	6
Control	8	10	5	13	65	93
+ *M. leprae*	8	12	7	14	48	70

Mice 1-4 sacrificed after 6months (mice 5 and 6 sacrificed after $1\frac{1}{2}$ year).

Table 4. Macrophage-lymphocyte interaction of mice sensitized with *M. vaccae in vivo*.

		1	2	3	4	5
% Interaction	No. antigen	25	20	19	3	5
	M. leprae	38	54	31	18	15
	M. vaccae	30	33	27	19	12
% Macrophage with AFB	*M. leprae*	38	40	26	50	30
	M. vaccae	0	2	4	4	0

Mice 1-3 sacrificed after 6 months (mice 4-5 sacrificed after $1\frac{1}{2}$ years).

Macrophage-lymphocyte interaction in Swiss white and C57B1/6 mice

The interaction in response to *M. leprae* in spleen and lymph node was positive. However most striking was the clearance of *M. vaccae* in the interaction system. In contrast, the percentage of cells harbouring *M. leprae* was much higher (table 4).

Discussion

An analysis of the BCG field trials in Uganda and Burma by Stanford and his colleagues (Stanford *et al.,* 1981; Rook *et al.,* 1981) concluded that one of the factors influencing the protective efficacy of BCG was environmental mycobacteria. Sensitization to fast growing mycobacteria (*M. nonchromogenicum, M. vaccae*) was found to provide protection against leprosy. While *M. scrofulaceum* and possible other slow growers are antagonists of the protective effect of BCG.

On the basis of these observations they hypothesize that contact with environmental mycobacteria will induce the Listeria type or the Koch-type response and BCG vaccination will enhance it. Thus in those places where environmental species prime for the Listeria type of response subsequent BCG vaccination will afford good protection.

Our results of F_c receptor activity, macrophage-lymphocyte interaction and lymphocyte proliferation in the presence of L-lysate show that among the mycobacteria *tested in vitro, M. vaccae* appears to be the most successful as it consistently reverses all 3 parameters. These results support the observation of Rook *et al.* (1981) that exposure to *M. vaccae* or *M. nonchromogenicum* could lead to protection, while contact with *M. scrofulaceum* or *M. kansasii* did not result in protection.

An interesting outcome of our results is that BCG did not demonstrate any enhancement in EA rosetting or interaction values, however in the lymphocyte proliferation assays in addition to *M. vaccae,* BCG responses were also not inhibited by L-lysate and no suppressor factor(s) production was also noted.

It is important to note that in the interaction system 2 out of 5 of lepromatous patients could not be converted to positivity with ICRC and in the lymphocyte proliferative assay suppression in the presence of ICRC and L-lysate was evident also in 2 out of the 5 cases though in one case it was marginal. These figures are comparable to those obtained in clinical trials using ICRC (Deo *et al.,* 1981). Nevertheless F_c continued to remain negative. In contrast, *M. delhi* did not show any enhancement of reactivity both by the F_c and interaction systems though clinical trials appear promising (Chaudhury *et al.,* 1983). The existence of alternative mechanisms of immune activation cannot be overlooked.

Our results supply evidence that the mouse cannot be used as a model for vaccine studies though it may help in elucidating other aspects of the immune response to *M. leprae.* This is evident from the prolonged decrease of F_c receptor expression in *M. vaccae* infected mice and the inability of the splenic macrophages to clear *M. leprae,* while showing a concomitent clearance of *M. vaccae.* The observations put together highlight the potential use of *M. vaccae* as an immunomodulator and the report also describes 3 protocols that could be further developed as useful *in vitro* tests for screening of immunomodulating mycobacteria.

Acknowledgements

We thank the Acworth Leprosy Hospital for their cooperation in supplying the patient material. This work was supported by a grant from the Indian Council of Medical Research, New Delhi. ICRC bacillus and *M. delhi* were supplied by Dr. C. V. Bapat and Dr. G. P. Talwar respectively.

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