Clin. exp. Immunol. (1990) **79**, 7–14

A complex component modulating immune-deficient cells in leprosy patients leading to loss of viability of *Mycobacterium leprae*— a possible vaccine

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(Accepted for publication 7 August 1989)

SUMMARY

Macrophages from peripheral blood of leprosy patients, both multi-bacillary and paucibacillary are unable to kill phagocytosed *Mycobacterium leprae* due to their inability to produce superoxide (O_2^-) and hydroxyl radicals (OH·). The macrophages from healthy individuals are able to kill *M. leprae* along with release of O_2^- and OH· radicals. The deficiency in the macrophages of both types of leprosy patients is removed by activation of these cells when exposed to a culture supernatant obtained after stimulation of peripheral blood mononuclear cells from the same patients with delipidified cell components of *M. leprae* which are most likely cell wall proteins. The activation of macrophages also leads to recognition of whole live *M. leprae* as an antigen by cells from lepromatous patients. This activation of the phagocytes by delipidified cell components is blocked by cyclosporin A, indicating the possible role of several steps involved in immune activation of cells. The observations thus indicate the significant ability of delipidified cell components to eliminate the deficiencies in the macrophages from leprosy patients and restore them to behave like the cells from healthy individuals. Considering all these, it is suggested that delipidified cell components could be potential modulators, and are probably capable of functioning as a vaccine for leprosy.

Keywords immunomodulation delipidified cell components leprosy killing *Mycobacterium leprae*

INTRODUCTION

The importance of cell-mediated immunity in resistance to infection caused by intracellular pathogens has been well established. Mononuclear phagocytes clearly play primary roles in the efferent limb of cellular control of such infections. The microbicidal ability of phagocytes through reactive oxygen intermediates (ROI) like hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radicals (OH·) is also a basic defence mechanism of the human body against microbial infections (Babior, Curnutte & Kipnes, 1975; Nathan *et al.*, 1979; Klebanoff, 1982).

Mycobacteria like *M. bovis* BCG (Ando *et al.*, 1979), *M. microti* (Walker & Lowrie, 1981), *M. tuberculosis* (Jackett, Aber & Lowrie, 1978) and *M. intracellulare* (Gangadharam & Pratt, 1984) as well as *M. leprae* (Klebanoff & Shepard, 1984; Sharp, Colston & Banerjee, 1985) have been shown to be susceptible to killing by H_2O_2. There are reports that claim an inability of the phagocytes from healthy individuals to release O_2^- on stimulation with *M. leprae* (Holzer *et al.* 1986), yet others have reported that H_2O_2 and O_2^- are produced by macrophages of leprosy patients in response to agents such as phorbol myristate acetate (PMA) (Sharp & Banerjee, 1985). Nevertheless, a recent report (Holzer *et al.*, 1988) indicated that *M. leprae* are able to induce low but significant levels of O_2^- in the macrophages of healthy individuals and phagocytose the bacteria slowly.

Recently, we have reported that along with an ability to produce O_2^- and H_2O_2, phagocytes from healthy individuals are able to kill *M. leprae*, unlike from lepromatous leprosy patients (Marolia & Mahadevan, 1987, 1988, 1989). The inability of *M. leprae* to be killed by macrophages from lepromatous leprosy patients was then found to be due to lack of O_2^- production. Further observations made in our laboratory (Desai, Birdi & Antia, 1989) have also confirmed *M. leprae* being killed by macrophages from healthy individuals. These experiments were independently carried out in connection with the role of interferon-gamma (IFN-γ). As well as recognizing ROI like H_2O_2, O_2^- and OH· as basic defence mechanism, the role of IFN-γ to stimulate macrophages to produce ROI on challenge with infection of mycobacteria is also substantiated (Nathan *et al.*, 1979; Nogueria *et al.*, 1983; Kaplan & Cohn, 1985).

We have been successful in isolating and identifying a component of *M. leprae*, the delipidified portion of the insoluble cell components of *M. leprae* (DCC) as an immunomodulating

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agent. The potentialities of DCC which was obtained as an insoluble complex have been reported in detail (Vermani & Mahadevan, 1986; Robinson & Mahadevan, 1987).

Here we report on the comparative ability of peripheral blood macrophages from healthy individual and different types of leprosy patient to respond to *M. leprae* in relation to production of ROI and, further, the relative ability of the cells to kill *M. leprae*. Additional results are also presented to show that stimulation of immune-deficient cells of leprosy patients by DCC leads to activation of macrophages to kill *M. leprae* through ROI. The results reported here identify the basic deficiency leading to the tolerance of *M. leprae* by the cells of all types of leprosy patients (paucibacillary and multi-bacillary) and further indicate a method for intervention to rectify the deficiency. The experiments also indicate the potential immunomodulation nature of the insoluble DCC of *M. leprae*.

MATERIALS AND METHODS

Patient

Leprosy patients reporting at the Acworth Leprosy Hospital, Bombay, formed the major part of the study. The patients were classified on the basis of the Ridley & Jopling (1966) system. The lepromatous group was of the long-term treated (>4 years) bacillary-negative type (B^-LL). They were identified as bacteriologically negative, when bacilli were undetectable at multiple skin sites and in the nose, and having had a record of regular treatment in the clinic. The second group is the bacteriologically positive lepromatous type untreated or undergoing treatment (B^+LL). The other group of patients were paucibacillary, classified under the group of BT, BB.

The healthy subjects were volunteers living in the same environment, who may have had varied exposure to *M. leprae* in the city of Bombay. These healthy individuals were not close contacts and had no regular closeness with leprosy patients.

M. leprae were obtained from infected tissues such as spleen and liver from infected armadillo, kindly provided by Dr E. Storrs, Florida, USA. The tissues were collected under aseptic conditions and transported under dry ice to Bombay within a period of 1 week. The tissues were stored at −90°C and pieces were taken out as needed.

For obtaining host tissue-free *M. leprae* suspension, the infected pieces were repeatedly rinsed in sterile normal saline, to elute the bacilli. The suspension was centrifuged first at 500 ***g*** for 10 min to remove any larger debris and later the supernatant was centrifuged at 4500 ***g*** for 30 min. This procedure sedimented the acid-fast stainable bacteria. The bacterial suspension showed no catalase activity and was thus considered as free from tissues contaminants. Catalase is a component of host tissue and not produced by *M. leprae*.

Macrophage culture from the peripheral blood was prepared as reported earlier (Marolia & Mahadevan, 1989).

H_2O_2 was estimated following the method of Pick & Keisari (1980) and adopted to mature macrophage cultures. The method has been described earlier in detail (Marolia & Mahadevan, 1988).

O_2^- was estimated by the method of Sugimoto *et al.* (1982) as detailed previously (Marolia & Mahadevan, 1987).

The scavengers used to remove various oxygen intermediates, were 2000 units of catalase (Sigma Chemical Co., St Louis, MO) 100 μg of O_2^- dismutase (Sigma), 20 mM of sodium benzoate (Ranbaxy Laboratories, India) and 1 mM thiourea (Sisco Research Lab., Bombay, India). Since sufficient concentration of these chemicals has been used, it is presumed that effective levels were expected inside and outside the phagocytes.

Viability of M. Leprae after phagocytosis

M. leprae (25×10^6) were added to well-matured macrophage cultures maintained in sterile Falcon dishes. After 48 h maintenance of the bacteria inside the phagocytes, their viability was determined by *in vitro* and *in vivo* methods. In 48 h more than 75% of the cells from leprosy patients have *M. leprae* inside, and on an average 50–60% of the cells have more than 10 bacilli. In the phagocytes of healthy individuals more than 50% of the cells showed phagocytosis with an average of 5–20 bacilli in majority of them.

Viability of *M. leprae* inside the macrophages was determined using fluorescein diacetate (FDA) staining method described earlier (Mahadevan *et al.*, 1986; Bhagaria & Mahadevan, 1987; Marolia & Mahadevan, 1989), a modification of that described by Kvach, Mungia & Strand (1984). The cells containing *M. leprae* were scraped off the Petri dishes and suspended in 1 ml of 1 M saline. The suspension was subjected to 12 cycles of freeze–thawing. The lysate so obtained was centrifuged at 500 rev/min to pellet the cell debris. The supernatant containing *M. leprae* was centrifuged at 5000 ***g*** for 30 min. The *M. leprae* pellet was resuspended in 1 ml of saline. Three loopfuls each of 0·001 ml were spread on a glass slide within a diameter of 16 mm. The suspension was air-dried and a drop of the staining solution was placed on it. The staining solution consisted of 4 μg/ml FDA and 4 μg/ml ethiolium bromide in phosphate-buffered saline (PBS). A cover-slip was placed on it and the edges were sealed with nail polish to avoid drying of the solution. The preparation was incubated for 40 min in the dark. The slide was then observed under incident u.v. light using a blue excitation filter on a Fluoval II Fluorescence microscope at a magnification of ×400. By using this modified method, the entire population of the bacteria phagocytosed could be stained by FDA and also as AFB by Ziehl Neelson's method. The quantum of viability was expressed as percent green fluorescing bacilli in a total AFB population. The entire slide spot is scanned and all bacilli counted; this could yield 800–1000 bacilli. We have not considered those bacilli with indeterminate staining.

Loss of viability of *M. leprae* phagocytosed by mature macrophages was also determined by recovering the bacilli after the necessary incubation inside the cells and testing their ability to grow in the foot-pads of mice. The recovery of the bacilli was done after 10 cycles of freeze–thawing and centrifugation. The bacterial suspension of 1×10^4 bacilli was used and the growth obtained at 8 months and 12 months post-inoculation was determined. Counting bacillary load in the foot-pad was performed according to Shepard & McRae (1965). This test was specifically done to correlate the loss of viability along with the production of superoxide ions. We did not follow the method to determine the maximum viable number of bacilli in the test samples, but determined the growth at various harvest periods.

In all experiments where bacteria were obtained by freeze–thawing, loss of viability due to this technique itself could be determined by comparing the viability of original bacillary suspension used for phagocytosis. This is determined in a smear preparation and data are reported along with other results.

Preparation of DCC-stimulated culture supernatants

DCC of *M. leprae* was prepared by the method reported by Robinson & Mahadevan (1987) and used from the stock suspension available in the laboratory. Basically, it was the insoluble residue obtained after prolonged sonication of *M. leprae* and delipidified repeatedly to remove the wall-associated lipids. It was prepared as follows: *M. leprae* were separated from infected armadillo tissues, washed with saline twice, and 10^{10}–10^{11} cells/5 ml suspension of the bacilli were prepared in saline. The cells were sonicated in a soniprep (MSE) ultrasonicator at 4°C with 5-min pulse followed by 5-min rest for 5 h at a power of 55 W using a 9·5-mm probe. The sonicate was subjected to differential centrifugation as described by Rastogi & David (1981), with modifications. The crude cell wall was then delipidified by extraction with C1:M (2:1) (three times) for 15 min at room temperature (Folch, Lees & Stanley, 1957) (DCC-I). This fraction was further delipidified by extraction with acetone (three times) and ethanol:ether (1:1) three times for 15 min at room temperature (Robinson & Mahadevan, 1987). The resulting insoluble and sedimented DCC (DCC-II) obtained as a pellet was then suspended in saline and sonicated for 5 min at an amplitude of 14 in order to get a homogenous suspension. The protein content of the DCC was estimated by the method of Lowry *et al.* (1951). The material was kept sterile during the processing and recovery. Since some essential features typical of cell wall components of *M. leprae* have not been tested in this insoluble preparation, it is referred to as DCC but was earlier referred to as delipidified cell wall (DCW).

Mononuclear cells were separated over Ficoll–Triosil (Lymphoprep-Nyegaard, Oslo, Norway) density gradient and the cell count was determined. The cell suspension was adjusted to 1×10^6 cells/ml in culture medium (MEM + 20% human **AB** serum) and distributed in Petri dishes. One set of culture was stimulated with DCC (400 μg as protein for 1×10^6 cells). Incubation was carried out for 96 h at 37°C in 5% CO_2 atmosphere. The culture supernatant was obtained by filtration (Millex filter 0·22 μm). The supernatant referred to as 'DCC-stimulated supernatant,' was stored at −20°C till further use; it used within 4 days, to avoid possible loss of stimulatory activity. Such preparation could be made by using the peripheral blood mononuclear cells from any type of leprosy patients.

Lymphocyte proliferation assay

Blood collected under sterile conditions from the subjects was used to prepare mononuclear cells. These cells were separated by centrifuging the blood over Ficol–Triosil (Lymphoprep) gradient. Lymphocyte proliferation using ^{3}H-thymidine (specific activity 9·8 μCi/mmole) was performed as previously reported (Robinson & Mahadevan, 1987).

Loss of viability of M. leprae *in macrophage cultures exposed to DCC-stimulated culture filtrate*

To the mature macrophage culture, 1 ml of DCC-stimulated culture supernatant (from the same patient) was added and incubated for 24 h at 37°C. At the end of the incubation period, the medium was aspirated and fresh medium along with *M. leprae* (25×10^6) was added. At that time different treatments (addition of catalase, O_2^- dismutase, sodium benzoate, thiourea, etc.) were carried out and phagocytosis was allowed for 48 h at 37°C in 5% CO_2 atmosphere. The cultures were terminated, bacilli were released by freeze–thawing as described earlier and viability determined by the methods mentioned before. In parallel cultures, ROI released were also determined with special reference to O_2^- after exposure of *M. leprae* to the cells for 3 h.

Role of cyclosporin A

Macrophage cultures were set up as indicated earlier from a patient. At the same time, DCC-stimulated supernatant was produced from the same patient in presence and absence of cyclosporin A (5 μg/1 $\times$ 10^6 cells). The supernatant was collected after 4 days of incubation, filtered and stored. Seven-day-old macrophages from the same patient were exposed to the above culture supernatant for 24 h, and the supernatant was then removed. *M. leprae* was added and after 3 h stimulation O_2^- was estimated. In separate experiments the role of the DCC-stimulated supernatants as prepared above were also tested on the killing of *M. leprae*.

Statistical analysis

Results are expressed as mean ± s.d. and statistical significance was determined by Student's *t*-test.

RESULTS

The mature macrophages from the peripheral blood of healthy individuals are able to produce substantial levels of H_2O_2 (111 nmoles/h per 10^6 cells) and O_2^- (2·2 nmoles/h per 10^6 cells), when

Table 1. Correlation between reactive oxygen intermediates and loss of viability of *Mycobacterium leprae* inside the macrophages of leprosy patients and healthy individuals

	B^+LL	B^-LL	Tuberculoid leprosy	Healthy
O_2^- dismutase removable superoxide (nmol/h per 10^6 cells)	0·3 ± 0·3	0·3 ± 0·2	0·2 ± 0·3	2·2 ± 0·5
H_2O_2 (nmol/h per 10^6 cells)	25·0 ± 1·5	89·0 ± 2·6	62·9 ± 10·1	111·2 ± 10·5
Original smear viability (% viability of *M. leprae* in suspension)	53·0 ± 9·7	48·0 ± 16·3	55·5 ± 9·0	56·2 ± 10·6
Viability of *M. leprae* inside the macrophage (%)	62·0 ± 16·6	50·5 ± 19·0	59·2 ± 9·6	21·0 ± 8·5*

All values are mean of five separate experiments.

* $P < 0.05$

B^+LL, bacillary-negative lepromatous leprosy; B^-LL, bacillary-negative lepromatous leprosy.

Table 2. Superoxide production (nmol/h per 10^6 cells) by macrophages from various types of individuals exposed to *Mycobacterium leprae* and influence of DCC-stimulated supernatant (mean ± s.d.)

	B^-LL ($n=7$)	Difference	B^+LL ($n=7$)	Difference	Tuberculoid ($n=3$)	Difference	Healthy ($n=3$)	Difference
Control MΦ (no *M. leprae* added)	4·0 ± 0·5		3·4 ± 0·4		2·4 ± 0·6		5·2 ± 0·8	
MΦ heat-killed *M. leprae*	7·6 ± 1·0		3·8 ± 0·2		4·4 ± 0·6		9·5 ± 1·6	
		3·5		0·3		2·1		2·2
MΦ + heat-killed *M. leprae* + O_2^- dismutase	4·1 ± 0·3		3·5 ± 0·3		2·3 ± 0·6		7·3 ± 1·4	
MΦ + live *M. leprae*	4·1 ± 0·4		3·5 ± 0·3		2·3 ± 0·8		7·8 ± 1·3	
		0·4 (A)		0·1 (C)		0·1		1·7
MΦ + live *M. leprae* + O_2^- dismutase	3·7 ± 0·6		3·4 ± 0·3		2·2 ± 0·7		6·1 ± 1·0	
MΦ + DCC-stimulated supernatant	5·0 ± 0·8		3·4 ± 0·7		2·8 ± 0·8		6·1 ± 0·8	
MΦ + DCC-stimulated supernatant + live *M. leprae*	7·0 ± 0·8		5·0 ± 0·4		4·2 ± 0·6		10·1 ± 2·6	
		2·8 (B)		2·0 (D)		1·7		2·4 (E)
MΦ + DCC-stimulated supernatant + live *M. leprae* + O_2^--dismutase	4·2 ± 0·8		3·0 ± 0·4		2·5 ± 0·7		7·7 ± 1·7	

* The differences indicate superoxide dismutase removable superoxide.
A *versus* B, $P<0·05$; C *versus* D, $P<0·05$; B *versus* E, $P=0·05$ not significant.
D *versus* E, $P=0·05$ not significant.
DCC, delipidified cell components; MΦ, macrophage.

Table 3. Viability of *Mycobacterium leprae* as determined by FDA staining method inside the macrophages from various individuals on exposure to DCC-stimulated supernatant and in relation to production of reactive oxygen intermediates (mean ± s.d.)

	B^-LL ($n=7$)	Tuberculoid ($n=6$)	B^+LL ($n=6$)	Healthy ($n=4$)
MΦ + live *M. leprae* in culture	(A) 50·5 ± 19·0	(B) 54·2 ± 9·6	(C) 62·0 ± 16·6	(D) 8·2 ± 1·8
MΦ + DCC supernatant *M. leprae*	(E) 14·6 ± 14·5	(F) 19·1 ± 8·4	(G) 23·3 ± 8·6	3·6 ± 1·7
MΦ + DCC supernatant + *M. leprae* + O_2^- dismutase	29·0 ± 16·0	27·3 ± 8·8	25·5 ± 2·1	27·7 ± 13·3
MΦ + DCC supernatant + *M. leprae* + catalase	30·0 ± 18·7	34·5 ± 15·6	25·0 ± 4·2	29·3 ± 21·4
MΦ + DCC supernatant + *M. leprae* + O_2^- dismutase + Catalase	(H) 56·5 ± 3·5	(I) 42·3 ± 6·7	32·0	—
Original viability of *M. leprae* before phagocytosis	48·0 ± 6·3	55·5 ± 9·0	53·0 ± 9·7	(K) 33·8 ± 13·2

B^-LL, bacillary-negative lepromatous leprosy; B^+LL, bacillary-positive lepromatous leprosy; MΦ, macrophage; DCC, delipidified cell component; A *versus* D, $P<0·05$; B *versus* D, $P<0·05$; C *versus* D, $P<0·05$; A *versus* E, $P<0·05$; B *versus* F, $P<0·05$; C *versus* G, $P<0·05$; A *versus* H, $P<0·05$ (NS); B *versus* I, $P=0·05$ (NS); D *versus* K, $P<0·05$.

Table 4. Correlation of superoxide production and loss of viability of *Mycobacterium leprae* inside macrophages of normal healthy individuals by FDA method and correlated with the ability of the phagocytosed bacteria (1×10^4) to grow in the foot-pads of mice

	Superoxide production (nmol/h per 10^6 cells)	Viable *M. leprae* (FDA test) (%)	Viability of *M. leprae* inoculated in the foot-pad of mice (average of four foot-pads) at harvest (months)			
			6	8	10	12
Control macrophage	5·0	—	—	—	—	—
Macrophage + live *M. leprae*	9·0 *	6·0	no AFB	no AFB	no AFB	no AFB
Macrophage + live *M. leprae* + O_2^- dismutase	6·5 *	16·0	—	$9·0 \times 10^4$		$6·0 \times 10^4$
Macrophage + live *M. leprae* + Catalase	—	16·6	—	$2·25 \times 10^5$	—	$2·1 \times 10^5$

FDA, fluorescein diacetate; AFB, acid fast bacilli.
* Difference, O_2^- dismutase removable superoxide.

Table 5. Viability of *Mycobacterium leprae* in the foot-pad of mice after phagocytosis by macrophages from paucibacillary leprosy patients in the presence of DCC-stimulated lymphokines with or without cyclosporin A

	Month 6	Month 7	Month 8
M. leprae from control macrophages (no other treatment)	$1·5 \times 10^5$	$1·57 \times 10^5$	$2·1 \times 10^5$
M. leprae from macrophages exposed to DCC stimulated from same patient	No AFB seen	No AFB seen	No AFB seen
M. leprae from macrophages exposed to DCC stimulated from same patient and prepared in the presence of cyclosporin A	6×10^4	$1·35 \times 10^5$	$4·5 \times 10^5$

Only two growth and harvest periods were obtained.
DCC, delipidified cell component.

challenged with live *M. leprae*. However, the phagocytes from patients could also respond to *M. leprae* by producing H_2O_2, although to a lesser extent, compared with individuals. However, these cells produced negligible levels of O_2^- dismutase removable O_2^-.Table 1 shows a loss of viability of *M. leprae* inside the cells from healthy individuals, associated with O_2^- and H_2O_2. The *M. leprae* used for the experiments showed an average viability of 56% in smear preparation by FDA method. However, phagocytosis of the same by the cells resulted in a viability of 21% (on an average) inside the mature macrophages. This indicated a statistically significant loss in viability ($P < 0·05$).

The viability of *M. leprae* after phagocytosis by the cells of all types of leprosy patients was as good as the viability of bacilli used for the experiment with the cells from such leprosy patients (smear preparation, 48–55%, compared with 50·5–62% after phagocytosis).

The results presented in Table 2 indicate that in the presence of DCC-stimulated culture supernatant the macrophages from all three groups of patients could get stimulated to release O_2^-. In the case of healthy individuals the culture supernatant could only further enhance the production of O_2^-, which was already induced in any case by live *M. leprae* itself. Thus it was observed that when the supernatant of culture fluid in which the deficient cells from patients were exposed to DCC was added to the same patient's phagocytes, the cells responded well to live *M. leprae* by producing O_2^-.

It was also demonstrable (Table 3) that along with the O_2^- produced in the deficient cells on exposure to the culture supernatant, there was also a reduction in viability of *M. leprae* phagocytosed by the macrophages. The viability of *M. leprae* inside the macrophages was reduced from 50·5% to 14·6% in the macrophages from B^-LL patients. The viability of *M. leprae* in the macrophages of tuberculoid leprosy or paucibacillary patients was high (54·2%) compared with the original smear viability of 55·5% indicating a lack of killing ability of these cells. In presence of the DCC-stimulated culture supernatant, a drop in viability was seen (19·1%). The importance of O_2^- and H_2O_2 was further indicated, since treatment with O_2^- dismutase along with catalase prevented the loss of viability of *M. leprae* inside the macrophages. Similar type of observations were seen with macrophages from B^+LL patients as well. The macrophages of healthy individuals have an in-built defence mechanism, since they show a normal respiratory burst activity. So the

Table 6. The ability of lymphocytes from B^+LL patients to respond to *Mycobacterium leprae* as an antigen in the presence of lymphokines generated by the same cells on stimulation with DCC

Antigen used in presence of PBMC	Stimulation index* Experiment 1	Experiment 2	Experiment 3
Live *M. leprae*	1·13	1·0	1·0
DCC of *M. leprae*	2·57	2·36	1·68
Control lymphocyte supernatant + live *M. leprae*	< 1·0	1·06	< 1·0
Lymphokines from DCC exposure + live *M. leprae*	2·12	2·0	1·98
Lymphokines from DCC exposure with live *M. leprae* and cyclosporin	< 1·0	1·0	< 1·0

B^+LL, bacillary-negative lepromatous leprosy; DCC, delipidified cell components; PBMC, peripheral blood mononuclear cells.

*Calculated as:

$$\frac{\text{average d/min (triplicates) of experimental value}}{\text{average d/min (triplicates) of control values}}$$

Table 7. Ability of macrophages from B^-LL patients to release O_2^- in response to *Mycobacterium leprae* after treatment with DCC-stimulated supernatant prepared in presence and absence of cyclosporin A

	Amount of superoxide released n mol/h 10^6 cells)				Mean*	Viability (%)†
Control MΦ (no *M. leprae* added)	2·5	3·7	2·5	3·1	2·95	
MΦ + live *M. leprae*	3·1	3·7	2·8	4·5	3·50	61·3
MΦ + heat-killed *M. leprae*	5·9	6·0	6·9	7·1	6·47	—
MΦ + DCC-stimulated supernatant + live *M. leprae*	6·4	5·7	7·1	6·4	6·4	16·3
MΦ + DCC-stimulated supernatant + live *M. leprae* + O_2^- dismutase	3·3	3·2	2·3	3·2	3·0	
Difference‡	3·1	2·5	4·8	3·2	3·4	
MΦ + DCC-stimulated supernatant prepared in presence of cyclosporin A + live *M. leprae*	3·4	3·2	2·3	3·2	3·02	59·3
MΦ + DCC-stimulated supernatant prepared in presence of cyclosporin A + live *M. leprae* + O_2^- dismutase	3·1	3·2	—	—	3·15	
Difference	0·3	0·0				

B^-LL, bacillary-negative lepromatous leprosy; DCC, delipidified cell components; MΦ, macrophage.

*Average of four experiments

†Average value of three separate experiments.

‡O_2^- dismutase removable O_2^-.

role of DCC stimulated supernatant was not of further significant effect, even though there was further reduction in viability.

The loss of viability of *M. leprae* inside the macrophages of healthy individuals and the B^-LL patients and paucibacillary individuals was also tested by recovering the phagocytosed bacteria 48 h after phagocytosis and injecting 1×10^4 of these bacilli in the foot-pads of Swiss white mice. This was compared with *M. leprae* obtained under various conditions of treatment of the macrophages.

Table 4 shows that *M. leprae* obtained after incubation inside the macrophages showed no growth in the harvest periods of 6, 8, 10 and 12 months. However, those bacteria phagocytosed by macrophages and maintained in presence of O_2^- dismutase or catalase did show on harvest at month 8 and 12, indicating survival of the phagocytosed *M. leprae* and thus viability of the infected bacilli. Since we found no growth in any harvest periods in the control group, it was likely that there was total loss of viability after phagocytosis of *M. leprae*. Yet, *M.*

leprae derived from cells of leprosy patients after phagocytosis showed no loss of viability as tested in the foot-pad (data not presented).

In a similar experiment, using macrophages from paucibacillary patients, it was demonstrated (Table 5) that macrophages on their own allow viability of *M. leprae*, but those cells exposed to DCC-stimulated supernatant showed loss of viability of *M. leprae* phagocytosed by them. This loss of viability was not seen when the cultures were exposed to DCC-stimulated supernatant prepared in presence of cyclosporin A and the *M. leprae* phagocytosed under that condition did not lose viability.

In the B^+LL patients, when the DCC-stimulated supernatant was added to the same patients' lymphocytes, these cells which do not normally respond to *M. leprae* antigen responded very well to *M. leprae* by showing a lymphocyte proliferation (Table 6). This lymphocyte proliferation indicated a modulation of the B^+LL cells for LTT after the addition of the supernatant.

Table 7 shows that cyclosporin A appears by its presence to block the production of an active DCC-stimulated supernatant. The supernatants produced in presence of cyclosporin A and DCC were not capable of modifying the phagocytes to recognize *M. leprae* and produce O_2^-, and also to kill the phagocytosed bacteria. In the absence of cyclosporin A, DCC supernatant enabled the cells to produce 3·1 nmol/h per 10^6 cells O_2^- and to induce killing from 61·3% viability to 16·3%. However, in presence of cyclosporin A, no O_2^- was produced, and viability was not reduced.

The activation of deficient macrophages by DCC-stimulated cell supernatant was tested after preparation of supernatants in the presence of cyclosporin A. Data presented in Table 7 shows supernatant prepared in presence of cyclosporin A had no ability to activate the macrophages to respond to live *M. leprae* with production of O_2^-, nor the ability to kill the phagocytosed *M. leprae*.

DISCUSSION

Our own results reported earlier (Marolia & Mahadevan, 1987, 1988, 1989) and the results of others (Desai *et al.*, 1989) have now indicated that healthy individuals from a leprosy-endemic city like Bombay are capable of killing *M. leprae* through ROI. Since this process is blocked by sodium benzoate or thiourea, as well as by O_2^- dismutase along with catalase, one could implicate OH radicals as most effective ones. We have not assessed as yet role of metal ions like iron in this function. It is also indicated that H_2O_2 on its own is least effective, since in leprosy patients H_2O_2 production does not lead to loss of viability. Thus H_2O_2 needs some other component to be effective. O_2^- fits in well with this requirement, as discussed earlier (Marolia & Mahadevan, 1987, 1988).

The tolerance of *M. leprae* by the macrophages of all types of leprosy patients is quite similar to what is observed *in vivo*. One could appreciate this feature in lepromatous type, but in paucibacillary cases it has been repeatedly mentioned that the bacilli are killed. It was obvious from our data presented here and from our earlier observations (Marolia & Mahadevan, 1987, 1988), that in paucibacillary patients, killing mechanisms was not through ROI as seen in healthy individuals. The protective mechanism then could be due to cell-mediated immunity leading to activation of macrophages. This point needs further elucidation in paucibacillary cases. Nevertheless, our data clearly indicated the reason for transitory survival of *M. leprae* in paucibacillary leprosy patients. In lepromatous patients the cells showed no ability to produce ROI or kill *M. leprae*. It is noteworthy that long term treated lepromatous patients do not recover the ability to produce O_2^- and kill *M. leprae* even after prolonged treatment. This could thus indicate a pre-existing inefficient nature in the patient in handling *M. leprae*. When the individual is carrying bacteria as in B^+LL, the cells exhibit still more deficiency parameters, that lead to no response at all to produce even sufficient H_2O_2 to *M. leprae* and also O_2^- even to killed *M. leprae*.

The deficiency noted in the leprosy patients in relation to ROI was further confirmed by modifying the deficiency using the earlier identified (Vermani & Mahadevan, 1986; Robinson & Mahadevan, 1987) immunomodulator, DCC. We had reported that DCC-stimulated supernatant obtained from peripheral blood mononuclear cells of a patient could induce killing of *M. leprae* in the phagocytes of the same patient. Our present results clearly indicate that DCC-stimulated supernatant are able to modify the phagocytes to respond to *M. leprae* in several ways. Some of them are documented by our observations. The DCC-stimulated supernatants alter the phagocytes to recognize *M. leprae* as an antigen and initiated *in vitro* lymphocyte proliferation. The phagocytes were also made to recognize *M. leprae* to produce O_2^- which in turn inactivated the phagocytosed *M. leprae*. Both these features which were not demonstrable in the cells of lepromatous leprosy patients, bacteriologically positive or negative, are thus exhibited as in the healthy individuals. In the case of paucibacillary patients, ability to kill *M. leprae* was restored as in the healthy individuals. It is interesting that production of an active DCC-stimulated supernatant and lymphocyte proliferation by cells of leprosy patients are blocked by cyclosporin A. Cyclosporin A is reported to block antigen presentation, lymphocyte activation and lymphokine production (Palay *et al.*, 1986; Varey, Champion & Cooke 1986). From our observations it can be assumed that DCC could be presented as antigen, and that following lymphocyte activation there could be release of useful lymphokines. Recent observations of Robinson & Mahadevan (personal communication) have confirmed this assumption.

Thus, our very early observation regarding the importance of delipidified cell wall (Khandke, Salgame & Mahadevan, 1984) and other reported data (Vermani & Mahadevan, 1986; Robinson & Mahadevan, 1987) as well as the recently reported results of Kaplan *et al.* (1988) indicating a role for cell wall proteins as good T cell activating antigens, all point to the importance of delipidified cell wall components.

It seemed that the *in vitro* stimulation by the complex insoluble antigen from *M. leprae* (probably as a subunit) endowed a status to the immune-deficient cells of both paucibacillary and multi-bacillary leprosy patients to behave as cells from healthy individuals. Such a modification leads to recognition of live *M. leprae*, followed by phagocytosis and killing. It may be suggested that this could be the mechanism to induce modulation by any vaccine to be effective in both types of leprosy. If this is so, the DCC has a definite potential for testing as a vaccine in humans. In experiments with mice vaccinated with DCC, activation of the peritoneal macrophages to kill *M. leprae* through increased ROI was observed. Such a mechanism was absent in non-immunized mice (unpublished data). Taken together, these observations and those of our earlier reports

(Vermani & Mahadevan, 1986; Robinson & Mahadevan, 1987; Marolia & Mahadevan, 1989), seem to indicate that DCC of *M. leprae*, which is a complex set of proteins probably from the cell wall, has definite potential as a potent modulator for multibacillary as well as paucibacillary leprosy.

ACKNOWLEDGMENTS

The authors are thankful to Acworth Leprosy Hospital, Bombay, for patient materials. The research work was supported by a grant No. D71/85 from the Department of Science and Technology, Government of India and by the Indian Council of Medical Research.

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