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Superoxide production from macrophages of leprosy patients after stimulation with *Mycobacterium leprae*

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Abstract. The macrophages from peripheral blood of normal healthy individuals respond to live or killed *Mycobacterium leprae* by producing superoxide. On the other hand, the macrophages from bacteriologically positive (**B** + **LL**) or long term treated bacteriologically negative (**B** – **LL**) and tuberculoid leprosy patients are unable to produce superoxide when stimulated with live *Mycobacterium leprae.* While killed *Mycobacterium leprae* induce superoxide with the cells from tuberculoid and **B**(–)**LL** patients, cells from **B**(+)**LL** patients fail to respond. The deficiency in **B**(–)**LL** patients to produce superoxide appears to be specific with *Mycobacterium leprae* and the defect can be counteracted by the addition of colchicine. These observations indicate a preexisting membrane disposition which does not favour superoxide production. A similar situation is seen in the cells from tuberculoid leprosy patients. Thus it appears that both cured and active lepromatous leprosy patients have defective macrophages, unable to respond to live *Mycobacterium leprae* to produce superoxide anion, in contrast to the situation with the cells from normal healthy individuals.

Keywords. Superoxide; macrophages; leprosy patients.

Introduction

Phagocytic cells in tissues and blood take up *Mycobacterium leprae.* Such cells on contact with other microbes phagocytose the bacteria and produce reactive oxygen species such as hydrogen peroxide, superoxide(O_2^-) and hydroxyl radical (OH); which are microbicidal (Nathan and Nakagawara 1981). Several Mycobacteria, including *M. leprae* are shown to be susceptible to killing by hydrogen peroxide (Ando *et al.,* 1979; Walker and Lowrie, 1981; Jackett *et al.,* 1978; Klebnoff and Shepard, 1984).

Although entry of *M. leprae* into the phagocytes has been demonstrated, there is no evidence to indicate killing of the bacteria during the natural course of events. There are several reports which relate very low levels of reactive oxygen species such as hydrogen peroxide and O^-_2 being induced in the presence of *M. leprae* in the macrophages or monocytes from normal individuals and leprosy patients (Ohkawa 1985; Holzer *et al.,* 1986; Sharp and Banerjee, 1985). The report of Holzer *et al.* (1986), demonstrating that oxygen intermediates play very little role against *M. leprae,* thereby emphasizing role of activated T-cells and the accompanying immune reaction, is noteworthy. However, there is no information on the actual concentration of H_2O_2 and O^-_2that is needed to kill *M. leprae.* It is possible that both immunostimulation products as well as H_2O_2, O_2^- and OH are necessary for the successful killing of *M. leprae* by the phagocytes. Experimental evidences on this

Abbreviations used: O_2^-, Superoxide; MEM, fnimum essential medium; NBT, nitroblue tetrazolium; SOD, superoxide dismutase.

aspect are being obtained. The role of interferon-γ, in initiating such an event, has been strongly implicated by the observation of Nathan *et al.* (1986). However their evidence for the killing of *M. leprae* is equivocal and not foully supported.

In this communication, we report the level of O_2^- produced by the macrophages from normal individuals and leprosy patients and their possible role in the inactivation of *M. leprae.*

Materials and methods

Patients

Leprosy patients attending some of the clinics in the city specially Acworth Leprosy Hospital, Bombay, donated blood for our studies. Informed consent was obtained where blood was not taken as sample for routine clinical examination. The patients were classified based on the procedure described by Ridley and Jopling (1986). The lepromatous type was primarily, border line type (BL) and some full lepromatous (LL) and tuberculoid category were of borderline and polar types (BT, TT). Among the lepromatous type we studied two groups (i) long term treated (5 years), bacteriologically (smear) negative patients (B(–)LL) and (ii) short term treated or untreated bacteriologically (smear) positive patients (B(+)LL). In the tuberculoid group both treated and untreated were studied. The normal controls, were those healthy individuals in the city of Bombay who had exposure of *M. leprae* from the environment. These healthy individuals were neither close contacts, nor had regular contacts with leprosy patients. A minimum of 5 individuals were studied in each category. Each experiment was carried out with one individual's blood sample and more than 5 such individuals were studied under each type.

M. leprae used in these studies, were obtained from infected tissues of armadillo (supplied to us by Dr. E. Storrs, Florida, USA). Bacteria were removed from the tissue by repeated rinsing of infected armadillo tissues with sterile saline (1 N) and after centrifugation of rinsed liquid at 4500 *g* for 15 min, significant amount of acid-fast stainable bacteria were obtained. Such bacilli were largely free of tissue contaminants as judged by microscopic analysis. The presence of viable bacteria were determined in each preparation before use, by determining Fluorescein-di-acetate positivity by the method of Kvach *et al.* (1982). *M. leprae* were heat killed by autoclaving at 15 lbs/sq inch for 30 min.

Preparation of macrophage cultures

Macrophages from peripheral blood obtained from all types of subjects described above, were prepared as follows. Blood (150 ml) was collected in a sterile bottle containing 10 ml of a mixture of heparin (25 units/ml) and 6% dextran. The blood was allowed to settle at 37°C for 45 min. Plasma along with the buffy coat was transferred to a sterile tube and centrifuged at 800 *g* for 5 min. The sedimented leucocyte pack was washed once in minimum essential medium (MEM) and cells were suspended in a culture medium containing MEM (GIBCA, UK) and human AB type serum (added to 40% concentration). Five ml of the above suspension was distributed into 35 mm sterile Falcon petridishes. In control experiments it was

observed that 5 ml suspension of the cells contained on an average 0·8 –1 × 10^6 cells. After 24 h of incubation at 37°C in 5% CO_2 atmosphere, the non-adherent cells were removed by draining the liquid. The culture medium was changed thereafter every 48 h. This resulted in a fairly uniform layer of adherent, esterase positive phagocytic macrophages after 8 days of culture maintenance.

Stimulation and estimation of superoxide

To the mature macrophage cultures, 1 ml of 0·3 % nitroblue tetrazolium (NBT) was added in each petriplate along with 40 × 10^6 *M. leprae* (live or autoclaved). This normally gave a ratio of 50:1 for *M. leprae* to macrophages per culture dish. At the same time to one set of petriplates superoxide dismutase (SOD) (from canine blood, Sigma Chemical Co., USA) was also added at a concentration of 100μg/ml. The cultures were incubated at 37°C for 3 h. It has been observed that maximum release of O_2^- was obtained by 3 h exposure of *M. leprae* to the cells. After incubation, all cells and the extracellularly reduced NBT formazan were removed with a rubber policeman and collected in tubes containing 1 ml of 1 N HCl. The precipitated formazan was pelleted by centrifugation at 1000 *g* for 10 min and the residue was dissolved in 2·5ml of pyridine (British Drug House, Bombay) and the absorbance was determined at 515 nm. The method was essentially the same as used by Sugimoto *et al.* (1982). The amount of NBT reduced was converted into O_2^- concentration using an extinction coefficients 2·6 × 10^4 m^{-1} cm^{-1}. The level of O_2^- was expressed as nmol/h/ 10^6 cells.

Experiments with membrane modulators

To one set of macrophage cultures, colchicine at a concentration of 10^{-5} M was added along with 1 ml of 0·3% NBT, live *M. leprae* (40 × 10^6) and with or without SOD (100μg/ml). To another set cytochalasin B at a concentration of 10μg/ml along with all other components for assaying O_2^- released as stated above were added. Incubation of cultures were carried out at 37°C for 3 h and the level of O_2^- was estimated.

The specificity of low response in B(+) LL macrophages to *M. leprae* as compared to B (–) LL was tested using several other mycobacteria in place of *M. leprae* (table 2). The assay system was similar to the one described for *M. leprae.*

The effect of phagocytosed live *M. leprae* on the stimulatory ability with the B(–)LL macrophages was also determined. This was done by stimulating the cells with autoclaved *M. leprae* after phagocytosis of live *M. leprae* for 48 h.

Results

The level of O_2^- (susceptible to dismutation by SAD) induced on exposure of macrophages to *M. leprae* is presented in table 1. The macrophages from normal individuals was able to release 2·2 nmol/h of O_2^- on exposure to live *M. leprae* and 4·3 nmol/h on exposure to heat killed *M. leprae.* In contrast to this, the macrophages from tuberculoid leprosy, B (–) lepromatous and B(+) lepromatous patients showed very poor stimulation, 0·24 nmol, 0·3 nmol and 0·3 nmol/h respectively. However, it

Table 1. Level of SOD removable O^{-}_2 anion production on stimulation with normal and heat killed *M. leprae.*

Source of Mø	Mø only	Mø +SOD*	O^{-}_2 production: Mø + live *M. leprae*	O^{-}_2 production: Mø + *M. leprae* +SOD*	Mø + heat killed *M. leprae*	Mø + heat killed *M. leprae* +SOD*
Normal (8)	4·9 ± 0·39	4·3 (0·6)	8·7 ± 0·94	6·5 (2·2)	9·5 ± 0·95	5·2 (4·3)
Tuberculoid (8)	2·21 ± 0·38	2·10 (0·1)	2·32 ± 0·51	2·08 (0·24)	5·76 ± 0·54	2·8 (3·0)
B(−)LL (8)	3·9 ± 0·17	3·5 (0·4)	4·1 ± 0·34	3·8 (0·3)	6·8 ± 0·77	4·2 (2·6)
B(+)LL (8)	3·9 ± 0·35	3·4 (0·5)	3·8 ± 0·4	3·5 (0·3)	4·3 ± 0·27	3·9 (0·4)

* Value is average of 8 experiments.

The SOD removable O^{-}_2production (nmol/n/10^6 macrophages) is given in parentheses as difference between columns 2 and 3.

Mø, Macrophage.

is interesting to note that heat killed *M. leprae* was able to induce $O^{-}_2$to 3·0 nmol and 2·6 nmol in macrophages from tuberculoid and B(–)LL patients respectively. B(+)LL macrophages showed no response even for heat killed *M. leprae.*

The level of O^{-}_2produced by the macrophages from the two groups of patients was also tested with other mycobacteria. It was observed (table 2) that O^{-}_2

Table 2. Stimulation of macrophages for the release of O^{-}_2anion with other mycobacteria.

Treatment	O^{-}_2 production: Macrophages from B(+)LL patients*		O^{-}_2 production: Macrophages from B(−)LL patients*	
Control Mø	4·4	(0·3)	3·6	(0·1)
Mø + SOD	4·1		3·5	
Mø + *M. leprae*	4·3	(0·2)	3·6	(0·0)
Mø + *M. leprae* (live) + SOD	4·1		3·6	
Mø + *M. avium*	4·0	(0·2)	6·5	(2·0)
Mø + *M. avium* + SOD	3·8		4·5	
Mø + *M. intracellulare*	4·1		4·0	
Mø + *M. scrofulaceum*	4·1		5·1	
Mø + BCG	5·3		7·8	
Mø + *M. Vaccae*	4·0	(0·0)	6·2	(2·5)
Mø + *M. vaccae* + SOD	4·0		3·7	

* values are average of 3experiments.

The SOD removable O^{-}_2 production (nmol / n 10^6 macrophages) is given in Parentheses..

Mø, Macrophage.

production was uniformly low with all mycobacteria in cells from B(+)LL patients. However cells from B(–)LL patients showed that the ability to produce low levels of O^{-}_{2} with live *M. leprae* was quite specific and other mycobacteria could induce disernable levels of O^{-}_{2} from these cells.

The role of cell membrane modulators such as colchicine was studied to find out if the low stimulatory level with B(–)LL and tuberculoid macrophages with live *M. leprae* could be reversed. The data are presented in table 3. It was observed that after treatment with colchicine the cells were able to respond better to live *M. leprae* in both cases. However cytochalasin did not alter the level of response after treatment of cells from B(+)LL patients. The level of stimulation with cytochalasin was 0·3 nmol/h/10^6 cells before and 0·5 nmol/h/10^6 after treatment (data not presented).

Table 3. Effect of membrane modulators in release of O_2^- anion by macrophages in the presence of *M. leprae.*

	O^{-}_{2} production			
	Macrophages from B(−)LL patient		Macrophages from tuberculoid leprosy patient	
Control Mø	3·6*		2·8*	
Mø + H. K. *M. leprae*	7·1	(3·1)	5·5	(2·2)
Mø + H. K. *M. leprae* + SOD	4·0		3·3	
Mø + live *M. leprae*	4·2	(0·4)	3·0	(0·5)
Mø + live *M. leprae* + SOD	3·8		2·5	
Mø + colchicine	2·0		2·0	
Mø + colchicine + live *M. leprae*	5·0	(1·3)	4·3	(1·7)
Mø + colchicine + live *M. leprae* + SOD	3·7		2·6	
Mø + colchicine + H. K. + *M. leprae*	8·2		6·5	

Same notation as in table 2.

Table 4 shows the level of O^{-}_{2} produced by stimulation of B (–) LL macrophages with autoclaved *M. leprae* with or without prior phagocytosis of live *M. leprae.* It was clear that phagocytosis of live *M. leprae* and their presence did modify the cells

Table 4. Influence of exposure of macrophages from B(–)LL patients to live bacilli and later stimulated with heat killed *M. leprae* on O^{-}_{2}, anion production.

	O^{-}_{2} production (nmol/n/10^6 macrophages)	
Restimulation	I	II
Control Mø	3·8	2·8
Mø + H. K. *M. leprae*	8·4	6·9
Mø + live *M. leprae* (48 h) + heat killed *M. leprae*	5·1	3·4

to respond poorly to autoclaved *M. leprae.* There was 40-50% reduction in the level of stimulation by autoclaved *M. leprae,* once the cells had taken up live *M. leprae.*

Discussion

It was our idea to compare the behaviour of macrophages from bacteriological positive lepromatous patients with those of long term treated bacteriologically negative patients, to live *M. leprae.* The latter could be expected to respond like normal healthy individuals if the reported defective stimulating ability of B(+)LL patients with *M. leprae* was due to prevailing infection only.

It is clear from the results related here that given the same ratio of *M. leprae* to the cells, macrophages from normal subjects are stimulated by live *M. leprae* to a greater extent than those from B(–)LL or from tuberculoid patients. Macrophages from B(+)LL patients did not respond to both live or heat killed *M. leprae,* even though cells from B(–)LL and tuberculoid patients did. The presence of significant levels of SOD had been reported in *M. leprae* (Wheeler and Gregory, 1980). Thus, better stimulation by killed *M. leprae* in the above two groups of cells could be interpreted as due to the presence of SOD in live *M. leprae* degrading the O_2^- released. But this appears to be not the situation in B(+)LL cells, since the cells do not respond to heat killed *M. leprae.* The low stimulatory character of cells from both B(–)LL and tuberculoid patients was reversed by colchicine, which further indicated that the SOD associated with *M. leprae* may play very little role. Further it is also indicated that the receptors responsible for stimulation in these macrophages, are perhaps not fully expressed and colchicine, allows their expression so that live *M. leprae* could induce the cells to produce O_2^-.

The low stimulation $O_2^-$for production from cells of B(–)LL is specific to M. *leprae,* since these cells are stimulated quite well by other mycobacteria, However, cells from B(+)LL patients are poorly stimulated by all mycobacteria. Thus a clear specific low response of B(–)LL macrophages to live *M. leprae* is very much indicated, attaching significance to this deficiency in terms to tolerance of *M. leprae* by the cells. The defect seen in B(+)LL cells is induced by *M. leprae.* This was demonstrated by the fact that the cells from B(–)LL, allowed to phagocytose live *M. leprae* for 48 h, showed reduced stimulation with heat killed *M. leprae.* Thus the defects seen in B (–) LL and B(+)LL could be due to different reasons. Earlier, our observations had shown several changes in B(–)LL macrophages after entry of *M. leprae,* that affected both metabolic and membrane structures (Birdi *et al.,* 1983).

It may be noted that the level of O_2^- produced even from normal healthy individuals on exposure to *M. leprae* could be considered low, as compared to the reported level with agents like phorbolmyristate acetate, BCG or zymosan granules (Holzer *et al.,* 1986). Nevertheless, one can only judge the effective level after understanding what exactly the O_2^-does, in relation to viability of *M. leprae.*

Recently, we have reported several methods to determine viability of *M. leprae* inside human macrophages (Birdi *et al.,* 1983; Mankar *et al.,* 1984; Ramasesh *et al.,* 1984; Bhagria and Mahadevan, 1987; Vejare and Mahadevan, 1987). By use of these methods and an immunomodulator 'delipidified component of cell wall' of *M. leprae* (Vermani and Mahadevan, 1987) it has been observed that the modulator stimulates the macrophages of B(+)LL and B(–)LL patients to produce 4–5 nmol/h/10^6 of O_2^-

cells and this level could cause killing of *M. leprae* (unpublished data). This is also true for cells from normal healthy individuals (unpublished data). If in tuberculoid leprosy patients, *M. leprae* are tolerated due to poor induction, then the role of immuno protection is extremely important. Tuberculoid leprosy patients do have good cell mediated immunity. Thus the protection could be mainly by the immuno mechanism at least to start with.

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