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Mycobacterium leprae mediated stimulation of macrophages from leprosy patients and hydrogen peroxide production

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Abstract. Macrophages cultured from the peripheral blood of normal individuals, tuberculoid leprosy patients and long-term-treated, bacteriologically negative lepromatous leprosy patients are able to release hydrogen peroxide on stimulation with *Mycobacterium leprae.* Macrophages from lepromatous leprosy patients who are bacteriologically positive produce considerably lower levels of hydrogen peroxide, even though stimulation of these cells with *Mycobacterium leprae* is definitely demonstrable. This differential stimulation of macrophages appears to be largely specific to *Mycobacterium leprae.* There is also a good indication that decreased stimulation of macrophages from positive patients could be due to an after-effect of infection. It is possible that while other factors aid survival of *Mycobacterium leprae* in the macrophages, hydrogen peroxide may not be as effective in the killing of the bacteria in infected patients as it would be, perhaps, in other infections.

Keywords. Hydrogen peroxide; normal persons; leprosy patients; deficiency; role in production.

Introduction

Macrophages are credited with the ability to inactivate and kill bacteria that have been phagocytosed. The ability of macrophages in this regard is a consequence of their activation through immune processes mediated by T-lymphocytes and their products (Mackaness, 1969). Such activation of macrophages results in the release of reactive chemical species such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) anions and hydroxyl radicals (OH). This has been explained as the principal *in vivo* process of killing intracellular pathogens (Jackett *et al.,* 1978; Klebanoff, 1982; Nathan *et al.,* 1979; Walker and Lowrie, 1981).

Mycobacterium leprae, the causative organism in leprosy, has been shown to be susceptible to hydrogen peroxide in the presence of myeloperoxidase' and halides (Klebanoff and Shepard, 1984), and direct treatment and incubation with 0·08% of H_2O_2 (Sharp *et al.,* 1985). Even though both these reports show susceptibility of *M. leprae* to H_2O_2, they are under two different conditions. Recently, Sharp and Banerjee (1985) reported that hydrogen peroxide is produced by monocytes from all types of leprosy patients, and suggested further that macrophages from leprosy patients are competent to inactivate *M. leprae.* According to them, a T-lymphocyte defect probably contributes to the susceptibility of individuals to *M. leprae.*

This paper reports studies on the effect of addition of *M. leprae* to well-matured macrophages from various types of leprosy patients and normal healthy persons on the *in vitro* production of H_2O_2 by the macrophages. These studies were carried out

Abbreviations used: $^{\cdot}$, O_2^- Superoxide; OH, hydroxyl radicals; MEM, minimum essential medium; EBSS, Eagle's balanced salt solution; PMA, Phorbal myristate acetate; SOD, superoxide dismutase.

with a view of find out the survival of *M. leprae* in relation to production of H_2O_2 by macrophages.

We recently reported that production of O_2^- is defective in macrophages of all types of leprosy patients on contact with live *M. leprae* in contrast to what was observed with macrophages from normal healthy individuals (Marolia and Mahadevan, 1987). Some recent observations have also helped us to elucidate the role of H_2O_2 O_2^- and OH in the inactivation of *M. leprae* inside the phagocytes of both normal and leprosy patients (Jolly Marolia and P. R. Mahadevan, unpublished results).

Materials and methods

Patients

Leprosy patients attending some of the clinics in Bombay, specially Acworth Leprosy Hospital, donated blood voluntarily for our studies. They were typed according to the classification of Ridley and Jopling (1966). The lepromatous type were primarily BL and some, LL; the tuberculoid type were primarily BT and a few, TT. Among the lepromatous type we studied two groups: (i) long-term-treated (> 5 years treatment) bacteriologically negative (smear-negative, B(–)LL) patients and (ii) short-term-treated or untreated bacteriologically positive (smear-positive, B(+)LL) patients. In the tuberculoid leprosy group both untreated and treated patients were studied and treated as a single category. The normal controls were those healthy individuals in Bombay who had various degrees of exposure to *M. leprae* from the environment. These healthy controls were neither close contacts, nor people who had regular contacts with leprosy patients. A minimum of 5 individuals have been studied in each category, and these are considered as 5 different experiments,

M. leprae bacilli were obtained from infected tissues of armadillo (supplied by Dr E. Storrs, Florida, USA). Bacteria were removed from infected tissue by repeated rinsing of the tissue in sterile saline (1 N). After centrifugation of the rinsing liquid at 4500 *g* for 15 min, a significant number of acid-fast-staining bacteria was obtained. Such bacilli have been found to be largely free from tissue contaminants by microscopic analysis. Further, incubation of such bacilli with concentrated peroxide solution released no visible bubbles of H_2O_2. Lastly, such bacteria, as the data would indicate, did not show differences in macrophage-stimulating ability when used live or as heat-killed cells. If host catalase was present as a contaminant, one would expect a lower stimulation (H_2O_2 measured) with live *M. leprae* than with an equal number of heat-killed *M. leprae,* because in a heat-killed preparation, the enzyme would have been inactivated. The presence of viable bacteria was confirmed in each preparation before use by using fluorescein diacetate according to the method of Kvach *et al.* (1984).

M. leprae cells were heat-killed by autoclaving at 121°C and 15 lbs/sq inch for 30 min.

Preparation of macrophage cultures

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 and buffy coat were transferred to a sterile tube and centrifuged at 800 *g* for 5 min. The sedimented pellet of leucocytes was washed once with minimum essential medium (MEM) (Gibco, UK) and the cells were then suspended in MEM supplemented with human AB-type serum (added to 40% concentration). Aliquots of 5 ml of the suspension were transferred to 35 mm sterile Falcon Petri dishes. In control experiments it was observed that 5 ml of the cell suspension contained, on an average, 0·8-1 × 10^6 macrophages. After 24 h of incubation at 37°C in 5% CO_2 atmosphere, non-adherent cells were removed by draining the liquid. The culture medium was changed every 48 h thereafter and the culture was maintained for 5 days. This resulted in a fairly uniform layer of adherent, esterase-positive, phagocytic macrophages.

Assay for H_2O_2

The mature macrophage cultures were washed thrice with Eagle's balanced salt solution (EBSS). To the cultures were added phenol red (0·2 mg/ml, 1 ml), horseradish peroxidase (Sigma Chemical Co., USA) (2 units/ml) and *M. leprae* live or autoclaved (50 × 10^6/dish). Since we had earlier determined that the culture contained 0·8 – 1·0 × 10^6 macrophages, bacteria and macrophages were in a ratio of about 50:1. All solutions were prepared in EBSS. The total volume of the incubation mixture was 2 ml. To control Petri dishes 20 μl of 1 N NaOH was added before the incubation in order to kill the cells and block H_2O_2 production. The cultures were incubated at 37°C for 3 h after the addition of *M. leprae* and the reaction was stopped by adding 20 μl of 1 N NaOH. The colour developed at the end of the reaction was measured as absorbance at 610 nm and H_2O_2 was quantitated. The number of macrophages in each culture dish was counted microscopically after scraping and resuspending them. The assay used here is essentially the same as the one described by Pick and Mizel (1981). The level of H_2O_2 is expressed as nmol/h/10^6 macrophages. This was determined from the extinction coefficient calculated from a standard curve. The mean of values from 5 different experiments and the standard deviation of the mean were calculated and the statistical significance of difference between values for different samples was determined by the Student's '*t*' test.

The specificity of the low response to *M. leprae* in B(+)LL macrophages compared to B(–)LL macrophages was tested using several other mycobacteria in place of *M. leprae* (listed in table 5). The assay system was similar to the one described for *M. leprae.*

The effect of already phagocytosed live *M. leprae* on H_2O_2 production by the macrophages carrying the bacilli upon addition of heat-killed *M. leprae* was also determined. This was done by stimulating B(–)LL macrophages with heat-killed *M. leprae* after they had phagocytosed live or heat-killed *M. leprae* for various periods.

Results

The H_2O_2 released by macrophages from healthy individuals after 3 h stimulation with *M. leprae* is shown in table 1 along with similar data for macrophages from

Table 1. H_2O_2 release by macrophages from tuberculoid leprosy patients and normal individuals on exposure to *M. leprae.*

	Tuberculoid leprosy			Normal		
	Control	Macrophage + heat-killed *M. leprae*	Macrophage + live *M. leprae*	Control	Macrophage + heat-killed *M. leprae*	Macrophage + live *M. leprae*
	(A)	(B)	(C)	(D)	(E)	(F)
	10·7	50·7	60·0	16·0	66·7	113·3
	10·7	29·3	46·7	24·0	57·3	106·6
	16·0	48·0	72·0	10·7	80·0	124·0
	21·3	53·3	66·7	16·0	72·0	116·0
	10·7	48·0	69·3	13·3	76·0	96·0
Mean ± SD	13·88 ± 4·7	45·9 ± 9·5	62·9 ± 10·1	16 ± 5	70·4 ± 8·8	111·18 ± 10·5
M. leprae preparation with tissue contamination (catalase)						
	12·0	50·0	8·0*			

*Average values of results of 4 separate experiments, nmol/h/10^6 macrophages.
Significance of difference: A–C, $P < 0{\cdot}001$; D–F, $P < 0{\cdot}001$; A–B, $P < 0{\cdot}001$; D–E, $P < 0{\cdot}001$; A–D, not significant.

tuberculoid leprosy patients. The data show that there is very good stimulation by *M. leprae,* as indicated by the increase in the level of H_2O_2. However, H_2O_2 production by macrophages from tuberculoid leprosy patients was considerably less than that by macrophages from healthy individuals with both live *M. leprae* (62·9 nmol compared with 111·18 nmol) and heat-killed *M. leprae* (45·9 nmol compared with 70·4 nmol). Further, killed *M. leprae* caused less stimulation than live *M. leprae* in both types of macrophages. The killed *M. leprae* preparation was used to check for contaminating catalase as well as to determine the ability of such *M. leprae* to stimulate the macrophages. The results clearly indicate that there was no contaminating catalase in the live *M. leprae* preparation because if catalase was present, the H_2O_2 measured would be less than in the control. The effect of contaminating catalase is clearly seen in the H_2O_2 levels when a contaminated preparation is used (table 1).

Data from similar experiments with macrophages from the two types of lepromatous leprosy patients are presented in table 2. From a comparison of data in tables 1 and 2, it is clear that macrophages from bacteriologically positive lepromatous leprosy patients are stimulated much less by *M. leprae* compared to macrophages from normal individuals or tuberculoid leprosy patients ($P < 0{\cdot}001$). On the other hand, macrophages from long-term-treated bacteriologically negative patients were capable of being stimulated very well by both live and heat-killed *M. leprae* (89 and 83 nmol H_2O_2 respectively). This result is thus similar to what was observed with macrophages from normal individuals or tuberculoid leprosy patients. However, the B(–)LL macrophages differ from the normal and tuberculoid leprosy in another aspect: the former were stimulated to similar extents by live and heat-killed bacilli whereas the latter types were stimulated to a greater extent by live bacilli. The B(+)LL macrophages produced low levels of H_2O_2 even though phagocytosis by these cells was not different from that by normal and tuberculoid leprosy macrophages (table 3).

Table 2. H_2O_2 release by macrophages from B(+)LL and B(–)LL lepromatous leprosy patients on exposure to *M. leprae*.

	B(+)LL			B(−)LL		
	Control	Macrophage + heat-killed *M. leprae*	Macrophage + live *M. leprae*	Control	Macrophage + heat-killed *M. leprae*	Macrophage + live *M. leprae*
	(A)	(B)	(C)	(D)	(E)	(F)
	10·7	16·0	23·9	16·0	88·0	93·3
	10·7	13·3	23·9	13·3	80·0	88·0
	10·7	23·9	26·7	16·0	73·3	86·6
	12·0	18·7	23·9	16·0	89·3	88·0
	13·3	23·9	26·7	13·3	85·3	89·3
Mean ± SD	11·48 ± 1·16	19·16 ± 4·73	25·02 ± 1·53	14·9 ± 1·98	83·18 ± 6·6	89·04 ± 2·6
M. leprae preparation with tissue contamination (catalase)						
	12·0	20·0	5·0	15·0	75·0	07·0*

*Average values of results of 4 separate experiments, nmol/h/10^6 macrophages.
Significance of difference: A–B, $P > 0{\cdot}02$ (not significant); A–C, $P < 0{\cdot}001$ (significant); D–E, $P < 0{\cdot}001$; D–F, $P < 0{\cdot}001$; A–D, not significant.

Table 3. Phagocytic indices for macrophages at various times after infection*.

Macrophages from	Phagocytic index (h)				
	1	3	5	7	24
Normal	8 ± 1·01	16 ± 2·90	96 ± 04·3	150 ± 10·60	720 ± 27·50
Tuberculoid lerrosy	8 ± 1·02	20 ± 2·99	100 ± 05·0	140 ± 10·00	900 ± 28·70
B(+)LL	6 ± 1·00	40 ± 5·00	95 ± 03·5	100 ± 05·00	840 ± 24·49
B(−)LL	20 ± 3·00	60 ± 3·50	200 ± 10·7	240 ± 10·19	1800 ± 86·02

*5×10^6 *M. leprae* per Leighton tube culture of macrophages.

$$\text{Phagocytic index} = \frac{\text{Average number of bacilli per macrophage}}{\text{Total number of macrophages with phagocytosed } M.\ leprae}$$

Values are means of results of 5 experiments ± SD. P (B(–)LL–Normal)< 0·005.

To confirm that the levels of released H_2O_2 recorded were realistic, some control experiments were also carried out. The levels of H_2O_2 released by macrophages from leprosy patients after stimulation by phorbol myristate acetate (PMA) were determined. It is clear from the data in table 4 that PMA stimulated the macrophages of B(–)LL and tuberculoid leprosy patients (55 and 58 nmol) and that this stimulation is blocked in the presence of either added catalase or contaminating catalase.

Four other species of mycobacteria could not cause differential stimulation of macrophages from B(–)LL and B(+)LL patients (table 5). *M. vaccae* showed a tendency to discriminate between these macrophages. In all these cases phagocytosis of the bacteria was quite satisfactory and comparable to each other (data not shown).

The ability of B(–)LL macrophages to be stimulated by heat-killed *M. leprae is* definitely reduced after exposure to phagocytosed live *M. leprae* (table 6, compare means of rows A, B, C, D). There is no total inhibition. That this is an effect due to only live phagocytosed *M. leprae* is indicated by the fact that macrophages that had

Table 4. H_2O_2 release by macrophages from leprosy patients on stimulation by various agents.

Stimulating agent	Macrophages from B(−)LL	Macrophages from Tuberculoid leprosy
None (control)	13·55	13·90
PMA (1 μg/ml)	55·00	58·20
PMA (1 μg/ml) + catalase (100 μg/ml)	00·00	13·30
Heat-killed *M. leprae*	56·00	48·50
Heat-killed *M. leprae* + catalase (100 μg/ml)	15·90	00·00
Live *M. leprae* (with tissue contamination)	00·00	26·50
Live *M. leprae* (normal exptl. sample)	00·00	58·23

Values are averages of results of 3 experiments, nmol/h/10^6 macrophages.

Table 5. H_2O_2 release by macrophages from leprosy patients on stimulation by various species of mycobacteria.

Stimulating bacteria	Macrophages from B(+)LL	Macrophages from B(−)LL	Macrophages from Tuberculoid leprosy
None	13·30 ± 2·4	15·2 ± 01·3	12·60 ± 02·8
Heat-killed *M. leprae*	23·90 ± 0·0	72·8 ± 17·2	38·50 ± 13·3
Live *M. leprae*	26·50 ± 0·0	80·0 ± 18·8	56·60 ± 14·0
M. avium	19·41 ± 0·6	26·0 ± 06·8	18·80 ± 11·2
M. intracellulare	18·80 ± 2·8	29·1 ± 08·3	17·14 ± 05·9
M. scrofulaceum	18·40 ± 1·3	29·0 ± 06·1	16·80 ± 06·6
M. vaccae	22·30 ± 1·1	62·8 ± 02·1	32·80 ± 01·3
M. bovis (BCG)	22·60 ± 5·5	38·2 + 04·1	22·70 ± 05·5

Values are means of results of 3 experiments ± SD nmol/h/10^6 macrophages.

Table 6. H_2O_2 release by B(–)LL macrophages carrying phagocytosed live or heat-killed *M. leprae* on stimulation with heat-killed *M. leprae.*

Treatment	1	2	3	4	Mean ± SD
None (control)	12·9	13·3	13·3	13·3	13·3 ± 00·2
Heat-killed *M. leprae* (A)	90·6	90·0	84·8	79·6	86·4 ± 05·3
Live *M. leprae* (24 h) + heat-killed *M. leprae* (3 h) (B)	69·2	—	36·9	—	53·1 ± 22·0
Live *M. leprae* (48 h) + heat-killed *M. leprae* (3 h) (C)	64·1	74·4	68·6	—	69·0 ± 05·2
Live *M. leprae* (96 h) + heat-killed *M. leprae* (3 h) (D)	—	—	—	53·1	53·1
Heat-killed *M. leprae* (24 h) + heat-killed *M. leprae* (3 h) (E)	80·0	—	77·3	—	78·6 ± 01·9

Values are nmol/h/ 10^6 macrophages.

Phagocytosed heat-killed *M. leprae* responded as well as macrophages that had not phagocytosed and bacilli (table 6, compare means of rows A and E). The presence of phagocytosed *M. leprae* could be shown by staining the intracellular bacteria.

Discussion

The striking observation is that macrophages from healthy controls, tuberculoid leprosy patients and long-term-treated, bacteriologically free, 'cured' lepromatous leprosy patients have the ability to produce H_2O_2 on encountering *M. leprae* in *in vitro* culture. In contrast, macrophages from bacteriologically positive lepromatous leprosy patients show poor ability to respond to *M. leprae* by producing H_2O_2.It appears that the poor response to *M. leprae* in these macrophages is an after-effect of infection. The other interesting feature is that macrophages from tuberculoid leprosy patients are comparatively less responsive than macrophages from healthy individuals or 'cured' lepromatous leprosy patients. We are unable to identify the reason for this.

In macrophages from normal individuals, live *M. leprae* induced 60% more H_2O_2 than heat-killed bacilli whereas in tuberculoid patients live *M. leprae* induced only 37% more H_2O_2 than heat-killed bacilli. The greter stimulating ability of live bacilli could be interpreted as a result of the conversion of induced O_2^- to H_2O_2 by superoxide dismutase (SOD) present in live *M. leprae.* However, in B(–)LL patients, live *M. leprae* induce levels of H_2O_2 that are similar to those induced by heat-killed *M. leprae*. Since SOD of *M. leprae* can convert O_2^- to H_2O_2, high levels of H_2O_2 indicate high levels of O_2^- production in macrophages from normal individuals; correspondingly, there may be much less O_2^- in macrophages from tuberculoid leprosy patients and possibly very little or nil in macrophages from B(–)LL patients when stimulated with live *M. leprae.* Recent observations by us have shown that more O_2^- is produced by macrophages from normal individuals on encountering live *M. leprae* than by the other types of macrophages (Marolia and Mahadevan, 1987).

The levels measured by us appear to be realistic and comparable to other reported values (Nathan and Root 1977; Nathan *et al.,* 1983; Kaplan *et al.,* 1980). Stimulation with PMA also resulted in similar levels of H_2O_2 and added catalase or contaminating tissue catalase reduced the H_2O_2 to very low levels, indicating a true production of H_2O_2. This was the case with macrophages from both B(–)LL and tuberculoid leprosy patients. In several samples studied by Sharp and Banerjee (1985) in all categories of patients, there was no stimulation at all. We have used 5-day-matured macrophages and 3 h exposure to *M. leprae.* Our results clearly point out the inability of macrophages of infected patients to produce as much hydrogen peroxide as macrophages from the other types of individuals. Most of the studies reported in the literature, except the recent report of Kaplan *et al.* (1986), have not distinguished LL patients as we have done. In the study of Kaplan *et al.* (1986), one patient had zero bacterial load and the macrophages from this patient showed higher stimulation than macrophages from patients with 3+ or 4+ bacteriological load.

The phagocytic abilities of macrophages from healthy individuals and those from B(+)LL patients are similar to each other. The ratio of *M. leprae* cells to macrophages in the cultures was approximately 50:1 in all the experiments. Thus the difference in H_2O_2 levels is due to an inability of the macrophages to respond to *M. leprae* after the infection sets in. We are not commenting on the phagocytic index of B(–)LL cells in this context.

The results have also revealed that preincubation of B(–)LL macrophages with live *M. leprae* for 24–96 h results in the macrophages becoming less responsive to heat-killed *M. leprae.* This is indicative of some changes induced by the phagocytosed live *M. leprae* in the B(–)LL macrophages. The 20–40% reduction in the response of such macrophages to heat-killed *M. leprae* could be due to these changes. A possible reason for the poor H_2O_2 production *in vivo* in macrophages of B(+)LL patients is indicated by this *in vitro* experiment.

It appears that the low level of stimulation of B(+)LL macrophages is largely specific for *M. leprae.* Other mycobacteria are not able to distinguish between macrophages from the different groups of patients, although *M. vaccae* showed some discrimination between B(+)LL and B(–)LL macrophages.

It is possible that H_2O_2 by itself may not be playing significant role in inactivating *M. leprae* inside macrophages in leprosy because of two observations:

(i) In long-term-treated patients where H_2O_2 production is high, *M. leprae* are phagocytosed and remain metabolically active.
(ii) Changes caused by phagocytosed live *M. leprae* eventually reduce the ability of macrophages to be stimulated by fresh *M. leprae* to produce sufficient H_2O_2 even if it were to kill or inactivate the bacteria.

Such events may take place during *in vivo* infection also. Thus O_2^- and concomitant immune stimulation may be the basic requirements. Recent data (Marolia and Mahadevan, 1987) point to the importance of O_2^- as a critical component of reactive oxygen species involved in bacterial killing. O_2^- was not produced in all leprosy patients on encountering live *M. leprae.* Additional information regarding the role of hydroxyl radicals and the loss of viability of *M. leprae* in macrophages has also been obtained. This has clearly indicated that O_2^- as well as OH radicals are much more important than H_2O_2 in killing *M. leprae* inside the macrophages (Jolly Marolia and P. R. Mahadevan, unpublished results).

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References

Jackett, P. S., Aber, V. R. and Lowrie, D. B. (1978) *J. Gen. Microbiol.,* **104,** 37.
Klebanoff, S. J. and Shepard, C. C. (1984) *Infect. Immun.,* **44,** 534.
Klebanoff, S. J. (1982) *Adv. Host. Def. Mech.,* **1,** 111.
Kaplan, G., Nathan, C. F., Gandhi, R., Horwitz, M. A., Levis, W. R. and Cohn, Z. A. (1980) *J. Immunol.,* **137,** 983.
Kvach, J. T., Mungia, G. and Strand, S. H. (1984) *Int. J. Lepr.,* **52,** 176.
Marolia Jolly and Mahadevan, P. R. (1987) *J. Biosci.,* **12,** 273.
Mackaness, G. B. (1969) *J. Exp. Med.,* **129,** 973.
Nathan, C. F. and Root, R. K. (1977) *J. Exp. Med.,* **146,** 1648.
Nathan, C. F., Noguera, N., Juangbhanich, C., Ellis, J. and Cohn, Z. (1979) *J. Exp. Med.,* **149,** 1056.
Nathan, C. F., Murray, H. W., Michael Dicke and Rubin, B. (1983) *J. Exp. Med.,* **158,** 670.

Pick, E. and Mizel, E. (1981) *J. Immun. Methods,* **46,** 211.
Ridley, D. S. and Jopling, W. H. (1966) *Int. J. Lepr.,* **34,** 255.
Sharp, A. K., Colston, M. J. and Banerjee, D. K. (1985) *J. Med. Microbiol.,* **19,** 77.
Sharp, A. K. and Banerjee, D. K. (1985) *Clin. Exp. Immunol.,* **60,** 203.
Walker, L. and Lowrie, D. B. (1981) *Nature* (*London*)*,* **293,** 69.