INTERNATIONAL JOURNAL OF LEPROSY
Volume 57, Number 2
Printed in the U.S.A.

Reactive Oxygen Intermediates Inactivate *Mycobacterium leprae* in the Phagocytes from Human Peripheral Blood[1]

Jolly Marolia and P. R. Mahadevan[2]

The microbicidal ability of phagocytes, through reactive oxygen intermediates (ROI), such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical (OH·), is a basic defense mechanism of the human host against microbial infection ([1, 5, 11]). Some mycobacteria are also identified as susceptible to H_2O_2 ([4, 22]). There are reports that claim to have demonstrated an inability of the phagocytes from normal healthy individuals to release O_2^- on stimulation with *Mycobacterium leprae* ([3]), yet others reported that H_2O_2 and O_2^- are produced by the macrophages of leprosy patients in response to agents like phorbol myristate (PMA) ([16]). This was thus interpreted that the survival of *M. leprae* in such patients was due to failure of cell-mediated immunity.

It may be recalled that in the absence of a rapid *in vitro* test to determine the viability of *M. leprae*, the role of ROI in killing the pathogen inside the host phagocytes could be uncertain. The only report of susceptibility of *M. leprae* to H_2O_2 has been by demonstration of the loss of viability of *in vitro* H_2O_2 treated *M. leprae*, by their failure to grow in the mouse foot pad ([6, 17]). Thus, it appears that the reports on the role of ROI in relation to *M. leprae* inside the phagocytes are incomplete and speculative.

Only a few rapid *in vitro* tests are available to determine the viability of phagocytosed *M. leprae* inside macrophages from human peripheral blood. Some of these are adopted and applied in this report. Further, we have earlier established that these *in vitro* tests are reliable by proving that those samples of *M. leprae* identified as nonviable by these tests were also found to be nonviable in the classical mouse foot pad tests carried out in parallel experiments ([8]).

We have determined that H_2O_2 is induced well in the phagocytes of lepromatous leprosy patients in the presence of live *M. leprae* but that superoxide ions are not produced ([9]). However, phagocytes from normal healthy individuals produce both H_2O_2 and O_2^- ([10]). Thus, if H_2O_2 should induce killing of *M. leprae*, then we should see a loss of viability by our tests in the cells of both normal and leprosy patients. But if both O_2^- and H_2O_2 are needed, then we would see killing only in the macrophages of the normal healthy individuals and not in those from lepromatous leprosy patients. We were able to test this concept, and this paper reports the results.

If we can modify the macrophages from lepromatous leprosy patients to respond to *M. leprae* by producing O_2^-, then the role of O_2^- in relation to *M. leprae* can be further clarified. This has been possible due to the following reasons: We have been successful in isolating and identifying a component of *M. leprae*, the delipidified portion of the cell wall (DCW), as an immunomodulating agent. The potentiality of DCW has been reported in detail ([14, 21]). If the deficiency of superoxide production in leprosy patients is rectified by DCW, then its role in relation to the inhibition or killing of *M. leprae* could be determined. We report the results of experiments using DCW stimulation.

MATERIALS AND METHODS

Patients. Leprosy patients reporting to the Acworth Leprosy Hospital, Bombay, India, formed part of the study. The patients were classified on the basis of the Ridley and Jopling system ([13]). The lepromatous group that formed one part of our study was of

[1] Received for publication on 20 April 1988; accepted for publication in revised form on 20 January 1989.

[2] J. Marolia, Ph.D., and P. R. Mahadevan, Ph.D., The Foundation for Medical Research, 84-A R.G. Thadani Marg, Worli, Bombay 400018, India.
Reprint requests to Dr. Mahadevan.

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 they had had a record of regular treatment in the clinic. These patients were not on treatment at the time of this study.

The normal 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 neither close contacts nor had regular closeness with leprosy patients.

M. leprae were obtained from tissues, such as the spleen and liver, from infected armadillos obtained from Dr. E. Storrs, Melbourne, Florida, U.S.A. The tissues were collected under aseptic conditions and transported under dry ice to Bombay within a period of 1 week. The tissues were aliquoted and stored at −90°C until needed.

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

Macrophage culture from peripheral blood was prepared as follows. Blood (150 ml) was collected in sterile bottles containing 10 ml of a mixture of 20 units/ml of heparin (Biological Evans, India) and 6% dextran (Rallis India, Bombay). 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* × 5 min. The cells were suspended in culture medium after washing them once with minimum essential medium (MEM; Gibco, U.K.). The culture medium consisted of MEM containing human AB-type serum (added at 40% concentration). An aliquot of 5 ml was added to each 55-mm diameter sterile Falcon petri dish. After a 24-hr incubation of the cells at 37°C in a humidified 5% CO_2 atmosphere, the non-adherent cells were removed by draining the liquid. The culture medium was changed thereafter every 48 hr. This resulted in a fairly uniform layer of adherent, esterase-positive phagocytic macrophages after 8 days of culture maintenance. In the above culture conditions we obtained 0.8–1.0 × 10^6 macrophages per culture dish. In cases where the cells were harvested for an experiment, the cell count was determined before use and the results were expressed as product obtained per 10^6 macrophages.

H_2O_2 was estimated following the method of Pick and Mizel ([12]) and adopted to mature macrophage cultures. Phenol red solution (Phenol red; Sigma Chemical Co., St. Louis, Missouri, U.S.A.) 0.2 mg/ml and horseradish peroxidase (Sigma) at 2 units/ml in Eagle's balanced salt solution (EBSS) along with 50 × 10^6 *M. leprae* (live or heat killed) were added to mature macrophage culture dishes. These were incubated for 3 hr at 37°C. The reaction was stopped by the addition of 20 μl of 1 N NaoH, and the color intensity was recorded as the optical density (OD) in a spectrophotometer at 600 nm. The quantity of H_2O_2 released was expressed as nmol/hr/10^6 cells using a standard curve for H_2O_2 and determining the number of cells in each dish after the experiment.

Superoxide was estimated by the method reported by Sugimoto, *et al.* ([19]). To well-matured macrophages 1 ml of 0.3% nitroblue tetrazolium (Sigma) was added to each Falcon dish along with 50 × 10^6 *M. leprae* (live or heat killed). To one set of cultures, superoxide dismutase (SOD) (Canine blood; Sigma) at a concentration of 100 μg/ml was added and incubated at 37°C for 3 hr. The cells were scraped off, counted, and together with extracellularly reduced formazan were pelleted at 1000 × *g*. The pellet was dissolved in 2.5 ml of pyridine and the OD was recorded at 515 nm. The amount of SOD-removable O_2^- was determined by using an extinction coefficient of 2.6 × 10^4 M^{-1} cm^{-1} and expressed as nmol/hr/10^6 cells.

The scavengers used to remove various oxygen intermediates were 2000 units of catalase (Sigma), 100 μg of SOD (Sigma), 20 mM of sodium benzoate (Ranbaxy Laboratories, India), and 1 mM thiourea (Sisco

Research Lab., Bombay). Since sufficient concentrations of these chemicals have been used, it is presumed that effective levels were achieved both inside and outside the phagocytes.

***In vitro* viability inside phagocytes.** *M. leprae* (25 × 10^6) were added to well-matured macrophage cultures maintained in sterile Falcon dishes. After 48-hr maintenance of the bacteria inside the phagocytes, their viability was determined by various methods.

One method used to determine the viability of *M. leprae* inside the macrophages was the fluorescein diacetate (FDA) staining method described earlier ([8]), which was a modification of that of Kvach and Veras ([7]). The cells with phagocytosed *M. leprae* were scraped off the culture dish and lysed with 10 freezing-thawing cycles to release the bacilli. A suspension of the *M. leprae* (0.001 ml) was placed on a slide and incubated with FDA (4 μg/ml) and ethidium bromide (EB) (4 μg/ml) in the dark for 30 min. The suspension was then examined with a fluorescence microscope under ultraviolet (Fluoval II). A parallel sample from the same preparation was smeared on a slide and stained for acid-fast bacilli (AFB). The number of AFB gave the number of total bacilli and the green fluorescing bacilli in the suspension, as indicated by FDA staining, gave the proportion of bacilli that showed the breakdown of FDA as an indication of viability.

For the labeled uracil incorporation assay, ^{3}H-uracil (Amersham, U.K.; sp. act. 35 Ci/nmol) at a concentration of 5 μCi/dish was added 24 hr after the addition of *M. leprae* and maintained for 6 days at 37°C in a humidified 5% CO_2 atmosphere. On the sixth day, the medium was removed, the cells were scraped off the petri dishes, and suspended in 1 ml of saline. Cell lysis was carried out by 10 freezing-thawing cycles. *M. leprae* were released from the macrophage lysate by centrifugation at 4500 × *g* × 30 min. The bacteria were then washed with 20% trichloroacetic acid to remove the acid-soluble radioactivity. The precipitated material was washed with methanol, suspended in Bray's fluid, and the level of incorporation was determined in a Kontron MR 300 scintillation counter. Heat-killed *M. leprae* were used as controls. Since there was a wide variation in the incorporation between experiments, the data from individual experiments are presented. Comparison of the results obtained in any one experiment appeared more useful than comparisons of mean values. The method followed here was developed by Vejare and Mahadevan ([20]). A similar technique was used by Rook ([15]) to study the viability of BCG inside phagocytes.

Loss of viability of *M. leprae* phagocytosed by mature macrophages was also determined by recovering the bacilli after the necessary incubations inside the cells and testing their viability in the foot pads of mice. The recovery of the bacilli was as done after 10 freezing-thawing cycles and centrifugation. The bacterial suspension of 1 × 10^4 bacilli was used, and the growth obtained at 8 months and 12 months postinoculation was determined. The method of counting the bacillary load in the foot pad was that of Shepard and McRae ([18]). This test was specifically done to correlate the loss of viability with the production of superoxide ions.

In all experiments where bacteria were obtained by freezing-thawing, the loss of viability due to this technique itself could be determined by comparing the viability of the original bacillary suspension used for phagocytosis.

Preparation of DCW-stimulated culture supernatants. DCW of *M. leprae* as prepared by the method reported by Robinson and Mahadevan ([14]) was used from the stock suspension that was available in our laboratory. Briefly, DCW was prepared by sonication (55 watts power) of *M. leprae* from armadillo tissues for a period of 2–2½ hr with pulse sonication of 5 min while the bacteria were kept in an ice bath. The residue obtained after sonication was delipidified by repeated treatment (3 times) with chloroform methanol (2:1) followed by acetone and either (1:1) at room temperature. The residue was centrifuged, homogenized, and used after determining the level of protein in the suspension. The material was kept sterile during the processing and recovery.

Mononuclear cells were separated over ficoll triosil (Lymphoprep-Nyegaard & Co.,

TABLE 1. *Levels of ROI (H_2O_2 and O_2^-) released by macrophages on encounter with live* M. leprae.

Sample	Normal healthy individuals		Lepromatous leprosy patients[a]	
	H_2O_2[b]	O_2^-[b] (SOD-removable)	H_2O_2[b]	O_2^-[b] (SOD-removable)
	(nmol/hr/10^6 cells)		(nmol/hr/10^6 cells)	
Macrophages only	16.0 ± 5.0 (A)	0.6 ± 0.2 (C)	15.0 ± 1.4 (E)	0.5 ± 0.3 (G)
Macrophages with live *M. leprae*	111.2 ± 10.5 (B)	2.2 ± 0.6 (D)	89.0 ± 2.6 (F)	0.3 ± 0.2 (H)
Phagocytic index[c]	16.0 ± 2.9		60.0 ± 3.5	

[a] Long-term-treated, bacteriologically negative.
[b] Mean ± S.D., N = 8. A–B = $p < 0.001$; C–D = $p < 0.001$; E–F = $p < 0.001$; G–H = $p > 0.01$.
[c] Percent phagocyting cells times average number of bacteria/cell.

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-type serum) and distributed in petri dishes. One set of cultures was stimulated with DCW (400 μg as protein for 1 × 10^6 cells). Incubation was carried out for 96 hr at 37°C in 5% CO_2 atmosphere. The culture supernatant was obtained by filtration (Millex filter; 0.22 μ). The supernatant was stored at −20°C until further use but used within 4 days to avoid possible loss of stimulatory activity.

Loss of *M. leprae* viability in macrophage cultures exposed to DCW-stimulated culture filtrate. To the mature macrophage culture, the full 1 ml of DCW-stimulated culture supernatant (from the same patient) was added and incubated for 24 hr 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, SOD, benzoate, etc.) were carried out, and phagocytosis was allowed for 48 hr at 37°C in a humidified 5% CO_2 atmosphere. The cultures were terminated, the bacilli were released by freezing-thawing 10 times as described earlier, and the viability determined by the methods mentioned before. In a parallel culture, the ROI released were also determined with special reference to superoxide.

Results are expressed as average values with standard deviations (± S.D.). Statistical significance was determined by Student's *t* test.

RESULTS

Mature macrophages from the peripheral blood of normal healthy individuals are able to produce notable levels of H_2O_2 (111.2 ± 10.5 nmol/hr/10^6 cells) and O_2^- (2.2 ± 0.6 nmol/hr/10^6 cells) when challenged with live *M. leprae.* On the other hand, macrophages from long-term-treated, bacteriologically negative patients (B(−)LL) responded to *M. leprae* by producing H_2O_2 (89 ± 2.6 nmol/hr/10^6 cells), but produced very low levels of superoxide (0.3 ± 0.2 nmol/hr/10^6 cells). The lower threshold level of sensitivity of superoxide estimation in our assay was 0.4 nmol/10^6 macrophages. The superoxide level in Table 1 is expressed as SOD-removable O_2^-, and the levels obtained in eight separate experiments have been expressed as mean values by subtracting the level of O_2^- with SOD from that obtained without SOD. The phagocytic activity of the macrophages from the patients was much higher than that of the cells from normal individuals (Table 1).

Table 2 shows that along with phagocytosis of the bacteria by the cells there was a loss of viability as measured by FDA-EB staining. It had been observed that there was a significant loss of viability as measured by FDA-EB staining of the bacteria inside the macrophages by just freezing and thawing, as is seen in Table 5. In this experiment (Table 2), phagocytosis was associated with H_2O_2 and O_2^- production and, specifically, the level of O_2^- released averaged 1.8 nmol/hr/10^6 cells. There was a reduced loss of viability after phagocytosis when either SOD

TABLE 2. *Viability of* M. leprae *(green staining by FDA-EB) inside macrophages from normal healthy individuals.*

Sample[a]		% Green staining *M. leprae* by FDA-EB Experiment no. 1	2	3	4	5	6	Mean ± S.D.	Superoxide production (nmol/hr/10^6 cells)
Macrophages only		—	—	—	—	—	—	—	5.2 ± 0.8
Macrophages with live *M. leprae*	(A)	9.8	29	16	16	22	33	21.0 ± 8.8	7.8 ± 1.3 } 1.8[b]
Macrophages with live *M. leprae* + SOD	(B)	18.0	40	34	35	42	38	34.0 ± 8.6	6.0 ± 1.0
Macrophages with *M. leprae* + catalase	(C)	14.0	66	36	38	45	53	42.0 ± 17.6	—
Macrophages with *M. leprae* + SOD + catalase	(D)	29.0	77	40	40	56	82	54.0 ± 21.6	—
Macrophages + *M. leprae* + sodium benzoate	(E)	—	57	44	65	—	—	55.3 ± 10.6	—
Original *M. leprae* used before adding to macrophages	(F)	42.0	70	50	60	50	65	56.2 ± 10.6	—

[a] A–B = $p < 0.01$; A–C = $p < 0.03$; A–F = $p < 0.001$; A–E = not calculated due to three experiments with sample E.
[b] In three out of the total six experiments, SOD-removable superoxide was also determined and mean value is given.

or catalase, or both of the scavengers of ROI, were added. Since both catalase and SOD maintained good viability as measured by FDA-EB staining, it was appropriate to determine the role of OH· radicals by using an OH· antagonist such as sodium benzoate. The addition of sodium benzoate appeared to block the killing of *M. leprae* in all three experiments in which it was tried, suggesting a probable role for OH·.

We also examined the ability of *M. leprae* to take up labeled uracil as an indicator of viability ([20]). Table 3 presents the data which indicate the level of incorporation of labeled uracil by *M. leprae* after phagocytosis. This level is not statistically different from that of the uptake of uracil by heat-killed *M. leprae* phagocytosed by the macrophages. However, if one compares the level of incorporation in the four separate experi-

TABLE 3. *Viability of* M. leprae *inside macrophages from normal healthy individuals as indicated by ability to incorporate ^{3}H-uracil (expressed as dpm/10^8* M. leprae*).*

Sample[a]		Level of ^{3}H-uracil incorporation (dpm/10^6 *M. leprae*) Experiment no. 1	2	3	4	Mean ± S.D.
Macrophages + heat-killed *M. leprae*	(A)	950	410	1803	810	993 ± 506
Macrophages + live *M. leprae*	(B)	1646	842	2522	1046	1514 ± 753
Macrophages + live *M. leprae* + SOD	(C)	1897	1640	2909	1359	1951 ± 675
Macrophages + live *M. leprae* + catalase	(D)	2807	2128	2970	1329	2308 ± 747
Macrophages + live *M. leprae* + SOD + catalase	(E)	—	3933	4936	—	4454 ± 737[b]
Macrophages + live *M. leprae* + sodium benzoate	(F)	4961	—	—	2899	3930 ± 1458[b]

[a] B–E/F = $p < 0.01$.
[b] Average value of 4182 ± 980.4 was obtained from data in E and F for calculation of p value.

TABLE 4. *Correlation of superoxide production and loss of viability of* M. leprae *by FDA method and ability to grow in foot pads of mice.*

	Superoxide production nmol/hr/10^6 cells		% viable *M. leprae* (FDA test)	Viability of *M. leprae* inoculated into mouse foot pad (avg. of four foot pads) at harvest (mos.) 6	8	10	12
			Experiment 1				
Control macrophages	5.0		—	—	—	—	—
Macrophages + live *M. leprae*	9.0	2.5[a]	6.0	No AFB	No AFB	No AFB	No AFB
Macrophages + live *M. leprae* + SOD	6.5		16.0	—	9.0×10^4	—	6.0×10^4
Macrophages + live *M. leprae* + catalase	—		16.6	—	2.25×10^5	—	2.1×10^5
			Experiment 2				
Control macrophages	4.5		—	—	—	—	—
Macrophages + live *M. leprae*	6.4	1.5[a]	7.3	No AFB	No AFB	No AFB	No AFB
Macrophages + live *M. leprae* + SOD	4.9		35.0	—	3.15×10^5	—	1.95×10^5
Macrophages + live *M. leprae* + catalase	—		54.0	—	2.7×10^5	—	2.25×10^5

[a] The difference is SOD-removable superoxide.

ments, in two of them SOD and catalase were used and in another two sodium benzoate was used, the level of incorporation appears to be higher compared to the control (with live *M. leprae*).

Lastly, we confirmed the loss of *M. leprae* viability inside the macrophages of healthy controls by recovering the phagocytosed bacteria 48 hr after phagocytosis and injecting 1×10^4 of these bacilli into the foot pads of at least 15 Swiss white mice. This was compared with the *M. leprae* obtained under various conditions of treatment of the macrophages. Data presented in Table 4 show that the *M. leprae* obtained after incubation inside the macrophages showed no multiplication. The minimum number of bacilli/foot pad that could be detected was 7.5×10^3 by our method; thus the 1×10^4 inoculum had not multiplied further. Those bacteria phagocytosed by macrophages and maintained in the presence of SOD or catalase, however, did show growth at the 8- and 12-month harvests, indicating survival of the phagocytosed *M. leprae*.

Since a low O_2^- level is demonstrable in the macrophages from leprosy patients, one could expect survival of *M. leprae* inside these cells. The data presented in Table 5 show that with a low level of SOD-removable O_2^- (0.2 nmol/hr/10^6 cells), the viability of *M. leprae* after phagocytosis was as good as the viability of bacilli used for the experiment. This was in spite of a good production of H_2O_2 as indicated by the data presented in Table 1. It thus appears that in spite of the ability to produce H_2O_2, the viability of *M. leprae* is maintained. This could be due to a low production of O_2^-. When the culture filtrate from DCW-stimulated leukocyte cultures from patients was added to the patients' own macrophages, the cells responded well to *M. leprae* and produced SOD-removable O_2^- (Table 5). Along with this, the viability of *M. leprae* was reduced from 50% to 14%, as measured by the FDA-EB test system. This correlated with a lowered incorporation of labeled uracil by *M. leprae* (Table 5). Such a loss of viability as measured by FDA-EB could be prevented by SOD and catalase as well as sodium benzoate or thiourea (Table 5).

DISCUSSION

The virulence of leprosy bacilli and the inherent microbicidal ability of the mac-

TABLE 5. *Superoxide production and viability of* M. leprae *after phagocytosis of bacteria by macrophages of lepromatous leprosy patients (B(−)LL).*

	Superoxide production (nmol/hr/10^6 cells)		FDA[a] method	Uracil incorporation (dpm/10^6 bacilli)	
				Exper. 1	Exper. 2
Controls (Macrophages (Mø) only)	3.9 ± 0.5				
Mø + heat killed *M. leprae*			—		
(ML) − Control	7.8 ± 1.1		—	314	540
Mø + live ML	3.7 ± 0.7	0.2[b]	50.4 ± 19 (A)	4823	1120
Mø + live ML + SOD	3.5 ± 0.6		—	—	—
Mø + DCW stimulated supernatant	4.1 ± 0.4		—	—	—
Mø + DCW stimulated supernatant + ML	6.7 ± 0.9	2.3[b]	14.6 ± 14 (B)	1493	750
Mø + DCW stimulated supernatant + ML + SOD	4.4 ± 1.0		29.0 ± 17 (C)	1559	995
Mø + DCW stimulated supernatant + ML + catalase	—		30.1 ± 19 (D)	2079	810
Mø + DCW stimulated supernatant + SOD + catalase + ML	—		55.0 ± 6[c]	—	—
Mø + DCW stimulated lysate + ML + sodium benzoate	—		47.0 ± 10[c]	—	—
Mø + DCW stimulated lysate + ML + thiourea	—		42.0 ± 11[c]	—	—
Smear viability of *M. leprae* used for phagocytosis	—		48.0 ± 15[c]	—	—

[a] Average of five experiments (A, B, C, D).
[b] The difference is SOD-removable superoxide: A–B = $p < 0.001$; B–C = $p < 0.001$; B–D = $p < 0.001$.
[c] Average of only three experiments.

rophages that ingest them are important parameters in establishing leprosy infection. Immune processes are also called into play soon afterward. A rapid accumulation and activation of macrophages occur in hosts possessing cell-mediated immunity. The rapid accumulation of the immune-activated macrophages usually inhibits the growth of the bacilli, thereby preventing clinical disease. If these macrophages do not acquire enough microbicidal abilities, the bacilli will grow intracellularly and soon the disease will express itself. This is what is observed in lepromatous leprosy.

The results in these studies indicate that given the same ratio of *M. leprae* to the cells, the macrophages from the normal individuals were stimulated by live *M. leprae* to produce O_2^- and H_2O_2; whereas the cells of B(−)LL patients have a very low superoxide stimulatory ability. Thus, in normal healthy individuals live *M. leprae* encountering host cells may be killed by ROI produced by macrophages when the bacteria are being phagocytosed. In a recent paper, Holzer, *et al.* ([2]) showed that *M. leprae* are able to induce a low but significant level of superoxide in the macrophages of normal healthy individuals and also phagocytose the bacteria slowly. This was a modification of their earlier report ([3]). Cell-mediated immunity may also play a role as a subsequent event. In the minority who express lepromatous leprosy, the bacteria survive after phagocytosis by the macrophages, perhaps due to poor O_2^- and $OH\cdot$ radicals. Thus, both cell-mediated immunity and ROI defects exist. This observation has been confirmed by the data presented here.

The survival and killing of *M. leprae* inside the phagocytes have been determined by two *in vitro* tests as well as in the mouse foot pad. Without relying on only any one test, if we generally compare all of the three test systems it appears that the observations of survival or killing have given us a true picture in relation to reactive oxygen intermediates. It appears most likely that the cells exposed to DCW are able to be activated through cell cooperation, and the ac-

tivated macrophages are then able to kill the *M. leprae* through reactive oxygen species.

The importance of ROI and the deficiency in these components in lepromatous leprosy patients were further confirmed. When such deficient cells were exposed to culture supernatants from DCW-stimulated leukocyte cultures from the same patient, the cells attained an ability to produce O_2^- while recognizing *M. leprae* and then to kill the bacteria.

Earlier, we have reported the immunomodulatory ability of DCW in mice and human cells tested *in vitro* ([14, 21]). In recent experiments (unpublished), we have shown that mice injected with DCW showed activated macrophages in the peritoneal cavity. Such macrophages were able to inactivate *M. leprae* in an *in vitro* culture, something not seen with the macrophages of nonimmunized mice. It appears clearer now how our earlier observation of the killing of *M. leprae* inside the macrophages of leprosy patients could have been mediated.

By relating all these observations, it is possible to predict that DCW is recognized as an antigen by the immune-deficient cells of leprosy patients, and the cells can then develop the ability to induce cell-mediated immune reactions leading to the production of active lymphokines which could, in turn, activate the deficient macrophages. Such activated macrophages are able to handle *M. leprae* as the macrophages from normal healthy individuals do through reactive oxygen intermediates. Several recent observations (unpublished) also support this prediction.

Such an event and process, if well documented, could then add immense potentiality to DCW as a powerful immunomodulator with the possibility for use in leprosy patients.

SUMMARY

Reactive oxygen intermediates such as hydrogen peroxide, superoxide, and hydroxyl radicals are important microbicidal components, and they could also play a role in an infection with *Mycobacterium leprae*. A comparative study of the level of hydrogen peroxide and superoxide produced by peripheral blood phagocytes from normal healthy individuals and lepromatous leprosy patients showed a deficiency in superoxide production in the patients. In the phagocytes from normal healthy individuals, there was good release of superoxide ions, and this mediated the killing of *M. leprae*. The lack of superoxide production allowed the viability of *M. leprae* inside the macrophages from leprosy patients. This deficiency could be rectified by the use of an immunomodulator, the delipidified cell wall of *M. leprae*. This modulation resulted in the ability of the patients' phagocytes to respond to *M. leprae*, to produce reactive oxygen intermediates such as superoxide, and also to kill the bacteria. These observations indicate that delipidified cell wall could have significant potential to positively modulate the immune-deficient cells of leprosy patients.

RESUMEN

Los intermediarios reactivos del oxígeno tales como el peróxido de hidrógeno, el superóxido, y los radicales hidroxilo, son agentes microbicidas importantes y pueden participar en la infección por el *Mycobacterium leprae*. Un estudio comparativo sobre los niveles de peróxido de hidrógeno y de superóxido producidos por los fagocitos periféricos de individuos normales y de pacientes con lepra, mostró una deficiencia en la producción de superóxido por los pacientes. En los fagocitos de los individuos sanos hubo una buena liberación de iones superóxido y éstos mediaron la muerte del *M. leprae*. La falta de producción de superóxido permitió la viabilidad del *M. leprae* dentro de los macrófagos de los pacientes con lepra. Esta deficiencia pudo ser rectificada por el uso de un modulador, la pared celular deslipidizada del *M. leprae*. Esta modulación capacitó a los fagocitos de los pacientes para responder al *M. leprae*, para producir intermediarios reactivos del oxígeno tales como superóxido, y también para matar a la bacteria. Estas observaciones indican que la pared celular deslipidizada puede tener el potencial de modular positivamente a las células inmunodeficientes de los pacientes con lepra.

RÉSUMÉ

Les intermédiaires oxygénés réactifs, tels que le peroxyde d'hydrogène, le superoxyde, et les radicaux hydroxylés, ont un pouvoir microbicide important; ils pourraient également jouer un rôle dans l'infection par *Mycobacterium leprae*. Une étude comparative des taux de peroxyde d'hydrogène et de superoxyde produits par les phagocytes du sang périphérique chez des individus normaux en bonne santé et chez des malades atteints de lèpre lépromateuse, a révélé un déficit dans la production de superoxyde chez les malades. Dans

les phagocytes provenant d'individus normaux en bonne santé, on a constaté une libération satisfaisante d'ions superoxydes, qui intervenaient dans la lyse de *M. leprae*. Vu l'absence de production de superoxyde, *M. leprae* peut dès lors survivre à l'intérieur des macrophages des malades de la lèpre. Ce déficit pourrait être corrigé par l'utilisation d'un immuno-modulateur, à savoir la paroi cellulaire délipidifiée de *M. leprae*. Cette modulation a conféré aux phagocytes des malades la capacité de répondre à *M. leprae*, entraînant la production d'intermédiaires oxygénés réactifs, tels que le superoxyde, ce qui entraîne les lyses des bactéries. Ces observations indiquent que la paroi pourrait être dotée du pouvoir de moduler, de manière positive, les cellules immunodéficientes des malades de la lèpre.

Acknowledgments. The authors wish to express their gratitude to Dr. E. Storrs, Melbourne, Florida, U.S.A., for the supply of infected armadillo tissues (aided by LEPRA, U.K.) and to Acworth Leprosy Hospital, Wadala, Bombay, India, for providing us with human materials. The work was carried out under grant no. D71/85 approved and financed by the Department of Science and Technology, Government of India.

REFERENCES

1. Babior, B. M. Oxygen-dependent microbial killing by phagocytes. N. Engl. J. Med. **298** (1978) 659–668.
2. Holzer, T. J., Kizlaitis, L., Vachula, M., Weaver, C. W. and Andersen, B. R. Human phagocytic cell responses to *Mycobacterium leprae* and *Mycobacterium bovis* bacillus Calmette-Guérin; an *in vitro* comparison of leprosy vaccine components. J. Immunol. **141** (1988) 1701–1708.
3. Holzer, T. J., Nelson, K. E., Schauf, E., Crispen, R. G. and Andersen, B. R. *Mycobacterium leprae* fails to stimulate phagocytic cell superoxide anion generation. Infect. Immun. **51** (1986) 514–526.
4. Jackett, P. S., Aber, V. R. and Lowrie, D. B. Virulence and resistance to superoxide, low pH and hydrogen peroxide among strains of *Mycobacterium tuberculosis*. J. Gen. Microbiol. **104** (1978) 37–45.
5. Klebanoff, S. J. Oxygen-dependent cytotoxic mechanisms of phagocytes. Adv. Host Def. Mech. **1** (1982) 111–162.
6. Klebanoff, S. J. and Shepard, C. C. Toxic effect of the peroxidase-hydrogen peroxide-halide antimicrobial system on *Mycobacterium leprae*. Infect. Immun. **44** (1984) 534–536.
7. Kvach, J. T. and Veras, J. R. A fluorescent staining procedure for determining the viability of mycobacterial cells. Int. J. Lepr. **50** (1982) 183–192.
8. Mahadevan, P. R., Jagannathan, R., Bhagria, A., Vejare, S. and Agarwal, S. Host-pathogen interaction—new *in vitro* drug test systems against *Mycobacterium leprae*—possibilities and limitations. Lepr. Rev. **57** Suppl. 3 (1986) 182–200.
9. Marolia, J. and Mahadevan, P. R. Superoxide production from macrophages of leprosy patients after stimulation with *Mycobacterium leprae*. J. Biosci. **12** (1987) 273–279.
10. Marolia, J. and Mahadevan, P. R. *Mycobacterium leprae* mediated stimulation of macrophages from leprosy patients and hydrogen peroxide production. J. Biosci. **13** (1988) 295–303.
11. Nathan, C. F., Nogueira, N., Juangbhanich, C., Ellis, J. and Cohn, Z. A. Activation of macrophages *in vivo* and *in vitro*. Correlation between hydrogen peroxide release and killing of *Trypanosoma cruzi*. J. Exp. Med. **149** (1979) 1056–1068.
12. Pick, E. and Mizel, D. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in cultures using an automatic enzyme immunoassay reader. J. Immunol. Methods **46** (1981) 211–226.
13. Ridley, D. S. and Jopling, W. H. Classification of leprosy according to immunity; a five-group system. Int. J. Lepr. **34** (1966) 255–273.
14. Robinson, P. and Mahadevan, P. R. A component of *Mycobacterium leprae* as immunomodulating agent for immune deficient cells of leprosy patients. J. Clin. Lab. Immunol. **24** (1987) 171–176.
15. Rook, G. A. W. An isotope incorporation assay for the antibacterial effects of human monocytes. Ann. Immunol. **132D** (1981) 281–289.
16. Sharp, A. K. and Banerjee, D. K. Hydrogen peroxide and superoxide production by peripheral blood monocytes in leprosy. Clin. Exp. Immunol. **60** (1985) 203–206.
17. Sharp, A. K., Colston, M. J. and Banerjee, D. K. Susceptibility of *Mycobacterium leprae* to the bactericidal activity of mouse peritoneal macrophages and to hydrogen peroxide. J. Med. Microbiol. **19** (1985) 77–84.
18. Shepard, C. C. and McRae, D. H. A method for counting acid-fast bacteria. Int. J. Lepr. **36** (1968) 78–82.
19. Sugimoto, M., Higuchi, S., Ando, M., Horio, S. and Takuomi, H. The effect of cytochalasin B on the superoxide production by alveolar macrophages obtained from normal rabbit lungs. J. Reticuloendothel. Soc. **31** (1982) 117–130.
20. Vejare, S. and Mahadevan, P. R. Importance of determining viability of *Mycobacterium leprae* inside macrophages—an *in vitro* method using uracil. J. Biosci. **11** (1987) 455–463.
21. Vermani, M. and Mahadevan, P. R. A component of *Mycobacterium leprae* as immunomodulating agent—activation of macrophages to contain viability of *M. leprae in vivo* and *in vitro*. Health Coop. Papers **7** (1987) 153–161.
22. Walker, L. and Lowrie, D. B. Killing of *Mycobacterium microti* by immunologically activated macrophages. Nature (Lond.) **293** (1981) 69–70.