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A Comparison of Monocyte Oxidative Responses in Leprosy Patients and Healthy Subjects as Influenced by Mycobacterial Lipid Pretreatment[1]

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Phenolic glycolipid-I (PGL-I) is unique to *Mycobacterium leprae* and makes up 2% of the mass of the bacterium ([7]). It has been detected in the sera, urine, and skin nodules of leprosy patients ([2, 10, 20, 21]). PGL-I and the related nonglycosylated, nonphenylated dimycocerosyl phthiocerol (DIM) are found in large amounts in experimentally infected armadillos, even in tissues free of *M. leprae* ([3, 7, 8]), indicating that *M. leprae* releases the lipids into its milieu. The presence of large amounts of PGL-I in tissues infected with *M. leprae* may be a factor associated with the specific immunological unresponsiveness seen in lepromatous leprosy patients. PGL-I has been shown to induce suppression of mitogenic responses of leprosy patients' lymphocytes *in vitro* ([14, 16]). We have previously shown ([19]) that healthy donors' monocytes pretreated with PGL-I release less superoxide anion (O_2^-) when stimulated with *M. leprae* than nonlipid-treated monocytes. However, monocytes pretreated with DIM, mycoside A of *M. kansasii*, or mycoside B of *M. microti*, release O_2^- in quantities comparable to nonlipid-treated monocytes in response to *M. leprae* stimulation. In response to other stimuli of the oxidative metabolic burst, such as phorbol myristate acetate (PMA), zymosan, *M. bovis* BCG, or *M. kansasii*, monocyte O_2^- release was unaffected by lipid pretreatment.

In this study, we examined the effect of PGL-I on leprosy patients' monocytes and assessed the ability of these patients' monocytes to respond oxidatively to various stimuli including *M. leprae*.

MATERIALS AND METHODS

Patients. Informed consent for a blood donation was given by healthy laboratory personnel and University of Illinois students, and by patients over 18 years of age with a confirmed diagnosis of leprosy in the Hansen's Disease Clinic at the University of Illinois at Chicago. Disease classification was according to the criteria of Ridley and Jopling ([17]). The characteristics of the patient population are shown in Table 1. To enable us to do paired statistical analysis, blood from a healthy donor within the same age group as the patient was drawn at the same time as the patient. The monocytes of each pair were then analyzed in the same manner.

Monocytes. Peripheral blood was drawn into heparinized (10 IU/ml) syringes. Monocytes were isolated by a modification of the method of Boyum ([1]). Briefly, the blood was diluted with an equal volume of Hanks' balanced salt solution (HBSS) without calcium or magnesium, layered over Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Missouri, and Winthrop-Breon, New York, New York, U.S.A.), and centrifuged at 400 × *g* for 25 min. The mononuclear layer was collected and washed with HBSS without calcium or magnesium. The monocytes were then isolated by elutriation using a Beckman Model J2-21 centrifuge with a JE-6B rotor. The elutriation buffer was HBSS without calcium or magnesium sup-

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TABLE 1. *Characteristics of patients studied.*

Patient	Sex/age	Leprosy type	Bacterial index	Therapy (duration)
1	M/38	BL	0	Dapsone (6 yr)
2	M/46	BT	0	Dapsone (2 yr); rifampin (2 yr); clofazimine (2 yr)
3	M/28	BL	0	Dapsone (5 yr)
4	M/34	LL	5+	Dapsone (21 mo); clofazimine (21 mo); thalidomide (16 mo); prednisone (21 mo)
5	M/38	BL	5+	None on day drawn
6	F/30	BT	0	Dapsone (14 mo); rifampin (6 mo)
7	M/32	LL	4+	Dapsone (6 yr); rifampin (6 yr); thalidomide (5 yr)
8	F/35	LL	0	Dapsone (12 yr)
9	M/46	BT	0	Dapsone (3 yr)
10	M/44	BT	0	Dapsone (10 mo); rifampin (9 mo); clofazimine (9 mo); prednisone (9 mo)
11	M/48	LL	0	Dapsone (12 yr)
12	F/25	LL	0	Dapsone (10 yr)
13	M/32	BT	0	Dapsone (8 yr)
14	M/35	LL	6+	Dapsone (18 mo); clofazimine (18 mo); thalidomide (18 mo)

plemented with EDTA (100 mg/l) and 2% fetal bovine serum (Hyclone, Logan, Utah, U.S.A.). A flow rate of 21 ml/min and rotor speed of 600 × *g* were used to exclude the lymphocytes and contaminating platelets and erythrocytes. The flow rate was then increased to 28 ml/min and 200 ml of elutriation buffer was collected to obtain the monocytes. After washing, the cells were resuspended in RPMI 1640 (Whittaker Bioproducts, Inc., Walkersville, Maryland, U.S.A.) medium containing 10% heat-inactivated fetal bovine serum (Hyclone) and 50 μg/ml gentamicin. The cells were counted, and viability as determined by trypan blue dye exclusion was always greater than 98%. Latex ingestion was used to determine the percentage of phagocytic monocytes ([6]), and it was consistently greater than 85%.

Lipids. The lipids used in this study, PGL-I, DIM, and mycoside A, were generously supplied by Dr. Patrick J. Brennan (Colorado State University, Fort Collins, Colorado, U.S.A.) through National Institutes of Health Contract no. AI-52582. PGL-I and DIM were purified as previously described ([8, 9]). Mycoside A was isolated from *M. kansasii* as described previously ([4, 7]). The lipids were each stored in chloroform:methanol (2:1), and sonicates were prepared immediately prior to monocyte exposure. A portion of the stock lipids was dried under a stream of nitrogen. HBSS, 1 ml, (Whittaker) was added to the vial and directly sonicated for 30–40 sec with a 3-mm probe of a Fisher Sonic Dismembrator Model 300 at 70% relative output.

Culture conditions. Buffer or sonicates of either PGL-I, DIM, or mycoside A were added to monocytes in teflon vials (Savillex, Minnetonka, Minnesota, U.S.A.) at 100 μg lipid/2 × 10^6 cells. The vials were tumbled end over end at 12 rotations/min for 2 hr at 37°C. The cells were then transferred to centrifuge tubes and washed. We have previously determined that under these conditions an average of 15 μg PGL-I is taken up by the monocytes as determined by dot-blot analysis following the extraction of the PGL-I from the monocytes ([19]). We have also shown that DIM and mycoside A were taken up in comparable amounts by the monocytes ([19]). The cells were counted and their viability, after treatment with the various lipids, was greater than 98%. The cells were placed in 96-well, flat-bottom microtiter plates (Corning Inc., Corning, New York, U.S.A.) at 2 × 10^5 cells/well in a 0.2 ml volume of media and incubated overnight at 37°C in 5% CO_2 in humidified air. After washing the wells, O_2^- release by the adherent monocytes was determined.

Superoxide anion assay. O_2^- release was determined by measuring the superoxide dismutase (SOD)-inhibitable cytochrome c (Sigma) reduction as described previously ([5, 15]). Briefly, cytochrome c and stimulus in HBSS without phenol red were added to triplicate wells which were blanked against triplicate wells containing SOD in addition

TABLE 2. *Monocyte O_2^- generation.*

Stimuli	Donors	Pretreatment			
		Buffer	PGL-I	DIM	Mycoside A
M. leprae	Healthy subjects	130 ± 10 (14)[a]	79 ± 10 (14)[b]	158 ± 22 (8)	156 ± 22 (8)
	Patients	107 ± 9 (14)[c]	93 ± 11 (14)	109 ± 18 (8)	112 ± 19 (8)
M. kansasii	Healthy subjects	178 ± 9 (8)	132 ± 15 (8)	186 ± 20 (8)	183 ± 17 (8)
	Patients	141 ± 16 (8)[c]	156 ± 30 (8)	148 ± 27 (8)	155 ± 14 (8)
Zymosan	Healthy subjects	196 ± 12 (12)	177 ± 16 (11)	209 ± 30 (8)	106 ± 17 (8)
	Patients	158 ± 13 (12)[c]	169 ± 18 (11)	198 ± 29 (8)	186 ± 29 (8)
PMA	Healthy subjects	310 ± 22 (14)	289 ± 32 (14)	329 ± 36 (8)	309 ± 30 (8)
	Patients	268 ± 20 (14)	307 ± 31 (14)	324 ± 31 (8)	332 ± 41 (8)

[a] Mean nmol O_2^-/mg cell protein/150 min ± S.E.; figures in parentheses are numbers of donors.
[b] $p < 0.05$ versus Buffer group.
[c] $p < 0.05$ versus Healthy subjects.

to the cytochrome c and stimulus. The absorbance was measured at 550 nm using an EIA Reader (Biotek, Burlington, Vermont, U.S.A.) immediately after the addition of reaction mixtures (0 time) and 150 min later. The amount of O_2^- reported is that released by the cells in the 150 min time interval. We chose to read the plates after 150 min instead of the conventional 90 min because we had previously shown ([5]) that *M. leprae* is a weak and slow stimulus of the metabolic burst of monocytes. Between readings, the plates were incubated at 37°C on a microtiter plate shaker (Dynatech Laboratories, Alexandria, Virginia, U.S.A.) to facilitate the interaction of the particulate stimuli with the monocytes. The stimuli used in the assay included opsonized zymosan (1:1 v/v with fresh serum at 37°C for 20 min), irradiated *M. leprae,* and *M. kansasii* (all at 50:1 particle-to-monocyte ratio) and PMA (Sigma; 500 ng/ml). The preparation of the bacteria for use in the O_2^- assay has been previously described in detail ([5,6]). Protein determinations were performed on the washed adherent cells by the Lowry, *et al.* method ([11]) with bovine serum albumin (Sigma) used for the standard curve. The amount of cell protein did not differ significantly between the lipid-treated and the nonlipid-treated control groups. The amount of O_2^- released by nonstimulated monocytes was always substantially lower than that released by stimulated cells regardless of the origin of the cells.

Endotoxin contamination. Endotoxin was not detected in the lipid preparations, buffers or media as determined by a quantitative chromogenic limulus amebocyte lysate assay (Whittaker) with a sensitivity of 10 pg/ml.

Statistics. Differences between control and experimental groups were evaluated for statistical significance by Student's *t* test.

RESULTS

As shown in Table 2, buffer-pretreated monocytes from leprosy patients released significantly ($p < 0.05$) less O_2^- than monocytes from healthy donors in response to the particulate stimuli, *M. leprae, M. kansasii,* and zymosan. However, patient monocytes pretreated with PGL-I, DIM, or mycoside A did not differ from monocytes of the healthy donors in the amount of O_2^- released (Table 2). We had previously shown ([19]), and it was observed in this study, that healthy donors' monocytes pretreated with PGL-I released less O_2^- than buffer-treated cells when the stimulus was *M. leprae* (Table 2). Interestingly, monocytes from tuberculoid patients responded to *M. leprae* with significantly ($p < 0.05$) less O_2^- release when pretreated with PGL-I than when buffer-treated (71 ± 16 vs 121 ± 14 nmole O_2^-/mg cell protein/150 min ± S.E., respectively; N = 5). In contrast, lepromatous patients' monocytes stimulated with *M. leprae* released similar amounts of O_2^- whether or not they had been pretreated with PGL-I (98 ± 11 vs 100 ± 20 nmole O_2^-/mg cell protein/150 min ± S.E.; N = 9).

When the data from the patient population were further analyzed, it was observed that the lepromatous patients' monocytes not treated with any lipids released significantly ($p < 0.05$) less O_2^- than monocytes from healthy subjects in response to *M. lep-*

TABLE 3. *Monocyte O_2^- release in response to* M. leprae.

Patients		Normals	*t* Test
Lepromatous	98 ± 11 (9)[a]	121 ± 14 (9)	p < 0.05
Tuberculoid	121 ± 14 (5)	135 ± 18 (5)	NS[b]
BI > 0	106 ± 12 (4)	125 ± 14 (4)	p < 0.05
BI = 0	113 ± 12 (10)	126 ± 13 (10)	NS
Rifampin therapy	122 ± 15 (4)	126 ± 24 (4)	NS
No rifampin	109 ± 12 (10)	126 ± 12 (10)	p < 0.05
Clofazimine therapy	106 ± 13 (4)	116 ± 12 (4)	NS
No clofazimine	107 ± 13 (10)	130 ± 15 (10)	p < 0.05
Thalidomide therapy	93 ± 8 (3)	110 ± 16 (3)	NS
No thalidomide	113 ± 11 (11)	128 ± 13 (11)	NS
Prednisone therapy	91 ± 12 (2)	117 ± 16 (2)	NS
No prednisone	109 ± 11 (12)	127 ± 13 (12)	NS

[a] Mean nmol O_2^-/mg cell protein/150 min ± S.E.; figures in parentheses are numbers of donors.
[b] NS = Not significant.

rae; whereas monocytes from the tuberculoid patients generated amounts of O_2^- similar to those generated by monocytes from the healthy donors (Table 3). Cells from patients with a high bacterial index released significantly ($p < 0.05$) less O_2^- in response to *M. leprae* than did the cells from the corresponding healthy individuals. Monocytes from patients with a negative bacterial index released similar amounts of O_2^- when challenged with *M. leprae* as did cells from the paired healthy subjects (Table 3). O_2^- release by monocytes from patients on the various therapies (dapsone, rifampin, clofazimine, thalidomide, or prednisone) did not differ from that released by monocytes from healthy donors (Table 3). Monocytes from patients not receiving rifampin or clofazimine released significantly less O_2^- than did monocytes from the healthy donors paired with those patients. Although the number of donors studied is small, and we cannot exclude a selection bias in the type of patients receiving these drugs, these data suggest that rifampin or clofazimine therapy can help patients in terms of their monocyte oxidative responses.

The data were analyzed by a paired Student's *t* test, where appropriate, since a healthy donor was drawn the same day as each patient and the monocytes from both donors were treated in a similar manner.

DISCUSSION

Monocytes from leprosy patients released less O_2^- than monocytes from healthy subjects when stimulated with *M. leprae, M. kansasii,* or zymosan. There was no difference in the O_2^- release when the stimulus was PMA, perhaps because PMA is such a potent stimulus of the oxidative burst of phagocytes. We had previously shown ([19]) that PGL-I-pretreated monocytes from healthy donors, when stimulated with *M. leprae,* released less O_2^- than did buffer-pretreated cells or cells pretreated with lipids structurally similar to PGL-I. We suspect that we did not observe depressed O_2^- release by PGL-I-pretreated patient monocytes compared to buffer-pretreated patient monocytes because the latter group of cells released less O_2^- than buffer-pretreated monocytes from the healthy donors. On further analysis of the data, the following observations were made. Monocytes from lepromatous patients released less O_2^- than monocytes from tuberculoid patients or from healthy donors. The monocytes from patients with a high bacterial index had depressed O_2^- production compared to monocytes from healthy donors. O_2^- release by monocytes from patients with a negative bacterial index did not differ from that released by monocytes from healthy subjects. In addition, monocytes from patients on rifampin or clofazimine therapy did not differ from monocytes of healthy subjects in their oxidative responses. It appears that therapy may restore the ability of leprosy patients' monocytes to respond oxidatively.

The results of previous studies measuring oxidative product release by leprosy patients' monocytes are not in clear agreement. The spontaneous and stimulated nitroblue tetrazolium (NBT) reduction as a measure of monocyte O_2^- release has been

reported to be elevated in leprosy patients when compared to healthy individuals [13]. In contrast, monocyte O_2^- release, as measured by cytochrome c reduction, in response to *M. leprae* and PMA was reportedly similar whether the cells were from leprosy patients or from healthy subjects [18]. Most recently, it was reported that monocyte-derived macrophages from leprosy patients were unable to produce O_2^- (as measured by NBT) in response to live *M. leprae*, while heat-killed *M. leprae* induced O_2^- release by cells from tuberculoid and bacteriologically negative LL patients but not bacteriologically positive LL patients [12].

None of the studies mentioned above have been paired studies. Ours took into account assay variability, and we performed the experiments on one patient's and one healthy donor's monocytes at the same time. The cytochrome c reduction method we used is the more generally used method to determine O_2^- release. In addition, the microassay we utilized is ideal for continuous measurements of O_2^- generation, and allows for monitoring simultaneous replicates for each stimulus desired. Moreover, to better standardize the O_2^- assay and to assure that the number of cells remaining in the wells were the same for the various donor populations, we performed protein determinations on the monocyte-containing wells. We have reported our data as nmol O_2^- per mg cell protein per unit of time. None of the above studies took the protein content of the wells into account. The results of Mariola and Mahadevan [12] are in closest agreement with our data. However, the *M. leprae* used in our studies were irradiated, our assay measured monocyte O_2^- release by the cytochrome c reduction method, and ours was a paired study.

Monocytes/macrophages are preferential host cells for parasitization by *M. leprae*. Modulation of oxidative responses, such as O_2^- release of these cells, may contribute to the ability of *M. leprae* to survive intracellularly. A better understanding of the interaction of *M. leprae* with these cells is necessary to further our understanding of the pathogenesis of leprosy.

SUMMARY

Superoxide anion (O_2^-) release by monocytes from leprosy patients in a paired study was lower than that released by monocytes from healthy controls. Pretreatment of healthy control monocytes with phenolic glycolipid-I (PGL-I) of *Mycobacterium leprae* resulted in the release of less O_2^- than released by buffer-treated cells or cells pretreated with structurally similar lipids. However, pretreatment of patient monocytes with PGL-I did not affect the O_2^- generation, perhaps because the cells already had a lower capacity to produce O_2^-. Upon further examination of the data from the patient population, monocytes from lepromatous patients released significantly less O_2^- than cells from normal controls, while tuberculoid patient cells released O_2^- in amounts similar to that generated by cells from normal controls. In addition, monocytes from patients with a high bacterial index had a lower capacity to generate O_2^- when compared to cells from healthy individuals.

RESUMEN

La liberación del anión superóxido (O_2^-) por monocitos de pacientes con lepra fue menor que la liberación de O_2^- por monocitos de controles sanos. El pretratamiento de los monocitos de controles sanos con el glicolípido fenólico I (PGL-I) de *Mycobacterium leprae*, dió como resultado la liberación de menos O_2^- que el liberado por células tratadas con regulador o por células pretratadas con lípidos estructuralmente similares. Sin embargo, el pretratamiento de los monocitos de los pacientes con PGL-I no afectó la generación de O_2^-, quizá porque estas células ya mostraban una menor capacidad para producir O_2^-. Comparados con los controles sanos, los monocitos de los pacientes con lepra tuberculoide produjeron cantidades semejantes de O_2^-, los monocitos de los pacientes lepromatosos liberaron cantidades significativamente menores del anión y esta producción de O_2^- fue todavía menor en los pacientes lepromatosos con índices bacteriológicos altos.

RÉSUMÉ

La libération d'anions superoxydes ($O\bar{2}$) par des monocytes de patients lépreux dans une étude appariée était plus basse que celle de monocytes en provenance de témoins sains. Le traitement préalable des monocytes de témoins sains avec du glycolipide phénolique-I (GPL-I) de *Mycobacterium leprae* résulta en la libération de moins d'$O\bar{2}$ que des cellules traitées par des tampons ou des cellules traitées au préalable avec des lipides de structure similaire. Cependant, le traitement préalable des monocytes des patients avec GPL-I ne modifia pas la production de $O\bar{2}$, peut-être parce que les cellules avaient déjà une capacité amoindrie de pro-

duire de l'oxygène O_2^-. Après examen plus approfondi des données en provenance de la population de patients, les monocytes de patients lépromateux libéraient significativement moins d'O_2^- que les cellules des témoins normaux, tandis que les cellules des patients tuberculoïdes libéraient des O_2^- en quantités semblables à celles produites par les cellules de témoins normaux. De plus, les monocytes des patients avec un indice bactériologique élevé avaient une capacité moindre de produire des O_2^-, en comparaison aux cellules d'individus en bonne santé.

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