

Effect of *Mycobacterium leprae*'s Phenolic Glycolipid-I on Interferon-gamma Augmentation of Monocyte Oxidative Responses¹

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Mycobacterium leprae synthesizes a unique phenolic glycolipid (PGL-I). It is a phenol-phthiocerol triglycoside with an antigenically distinct trisaccharide, 3,6-di-*O*-methyl- β -D-glucopyranosyl-(1 \rightarrow 4)-2,3-di-*O*-methyl- α -L-rhamnopyranosyl-(1 \rightarrow 2)-2,3-di-*O*-methyl- α -L-rhamnopyranose that constitutes 2% of the mass of isolated *M. leprae* (3). It has been detected in the sera of leprosy patients (1,17) as well as in skin biopsies of lepromatous leprosy patients (15,16). PGL-I is structurally similar to mycoside A of *M. kansasii*, differing in the trisaccharide moiety. PGL-I and the related non-glycosylated, non-phenylated dimycocerosyl phthiocerol (DIM) are found in large amounts in the tissues of experimentally infected armadillos (3,4). *M. leprae* releases the lipids into its milieu since the lipids are found in tissues freed of organisms.

The specific immunological unresponsiveness seen in lepromatous leprosy pa-

tients may be due in part to the accumulation of large amounts of PGL-I in tissues infected with *M. leprae*. It has been reported that PGL-I suppresses mitogenic responses of lymphocytes from lepromatous leprosy patients (7). In addition, these patients have been shown to have defective interferon-gamma (IFN- γ) production (9). Mononuclear phagocytes have been shown to serve as host cells for *M. leprae*. We have previously shown (14) that peripheral blood monocytes pretreated with PGL-I release less superoxide anion (O₂⁻) when stimulated with *M. leprae* than do control monocytes. On the other hand, monocytes pretreated with dimycocerosyl phthiocerol (DIM), mycoside A of *M. kansasii*, or mycoside B of *M. microti*, release O₂⁻ in quantities comparable to control monocytes in response to *M. leprae* stimulation. Monocyte O₂⁻ release in response to other stimuli of the oxidative metabolic burst, such as phorbol myristate acetate (PMA), zymosan, *M. bovis* BCG, or *M. kansasii*, was unaffected by lipid pretreatment. These data demonstrate that PGL-I affects monocyte O₂⁻ generation specifically when the challenging organism is *M. leprae*, and suggest that PGL-I plays an important role in the pathogenesis of leprosy. We undertook this study to determine if PGL-I affects the IFN- γ activation of monocytes and thus contributes further to the intracellular survival of *M. leprae*.

MATERIALS AND METHODS

Lipids. The lipids used in this study, PGL-I, DIM, and mycoside A, were kindly supplied by Dr. Patrick J. Brennan (Colorado State University, Fort Collins, Colorado, U.S.A.) through National Institutes of Health contract no. AI-52582. The lipids were purified as described previously (3,4). To assure stability, lipid sonicates were pre-

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pared immediately prior to monocyte exposure as previously outlined (¹⁴).

Cells. Peripheral blood was drawn from consenting healthy volunteers into heparinized (10 IU/ml) syringes. Monocytes were isolated as described (¹⁴). Briefly, mononuclear cells were obtained following centrifugation of the diluted blood over Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Missouri, U.S.A. and Winthrop-Breon, New York, New York, U.S.A.). The monocytes were then obtained by elutriation using a Beckman model J2-21 centrifuge with a JE-6B rotor. A flow rate of 21 ml/min and rotor speed of $600 \times g$ were used to exclude the lymphocytes, contaminating platelets, and erythrocytes. The flow rate was then increased to 28 ml/min and 200 ml of the elutriation buffer was collected to obtain the monocytes. After washing, the cells were resuspended in RPMI 1640 (Whittaker Bio-products, Inc., Walkersville, Maryland, U.S.A.) medium containing 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah, U.S.A.) and 50 $\mu\text{g/ml}$ gentamicin, and then counted. Viability was always greater than 98% as determined by trypan blue dye exclusion. Latex ingestion was used to determine the percentage of phagocytic monocytes (²), and this was consistently greater than 85%.

Culture conditions. Sonicates of either PGL-I, DIM, mycoside A, or buffer alone were added to the monocytes in Teflon vials (Savillex, Minnetonka, Minnesota, U.S.A.) at 10 μg lipid/ 2×10^6 cells. The vials were tumbled end over end at 12 rotations/min for 2 hr at 37°C. We have previously determined that under these conditions an average of 15 μg PGL-I/ 2×10^6 cells was taken up by the monocytes as determined by dot-blot analysis following the extraction of the PGL-I from the monocytes (¹⁴). We have also shown that comparable amounts of DIM and mycoside A were taken up by the monocytes, and that the cells were viable after treatment with the various lipids (¹⁴). The cells were washed by centrifugation and then incubated 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 medium for 2 hr at 37°C in 5% CO₂ in humidified air to allow adherence. After washing, the cells were incubated overnight in 0.2 ml/well of medium alone

or medium containing 500 U/ml IFN- γ (purified native human IFN- γ ; Sigma) at 37°C in 5% CO₂ in humidified air.

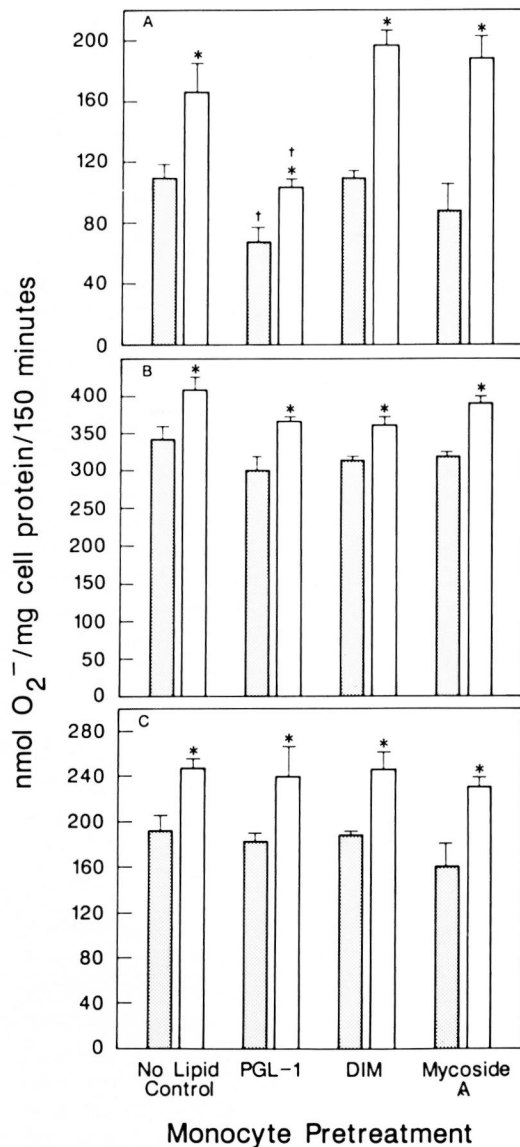
Superoxide anion assay. After washing the wells, O₂⁻ release by the adherent monocytes was determined by measuring the superoxide dismutase (SOD)-inhibitable cytochrome C (Sigma) reduction as described previously (^{2, 10, 14}). The stimuli used in the assay included PMA (500 ng/ml; Sigma), opsonized zymosan (1:1 v/v with fresh serum at 37°C for 20 min), and killed *M. leprae* (50:1 particle-to-monocyte ratio). The preparation of the bacteria for use in the O₂⁻ assay has been described (²). The amount of cell protein, as determined by the Lowry method (⁶), did not differ significantly between the experimental and control groups.

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

Statistical analysis. The differences between control and experimental groups were evaluated for statistical significance by Student's *t* test.

RESULTS

The O₂⁻ release by untreated control cells was about twice that of PGL-I-pretreated monocytes when *M. leprae* was used as the stimulus (Fig. 1A). Pretreatment with mycoside A of *M. kansasii* or the non-glycosylated non-phenylated dimycocerosyl phthiocerol (DIM) did not significantly alter the O₂⁻ release in response to *M. leprae*. IFN- γ augmented the O₂⁻ release by all cells regardless of lipid pretreatment. However, the O₂⁻ release by PGL-I-pretreated monocytes when activated by IFN- γ was significantly less than that of untreated monocytes activated by IFN- γ . IFN- γ was only able to enhance the O₂⁻ release of the PGL-I-treated cells to the same level as the preactivated state of the untreated monocytes. When the monocytes were pretreated with PGL-I, DIM, mycoside A, or buffer alone and then stimulated with PMA (Fig. 1B) or with opsonized zymosan (Fig. 1C), they released comparable amounts of O₂⁻. IFN- γ was able to augment the O₂⁻ generation by all of the cell groups equally well.



THE FIGURE. Effect of mycobacterial lipids on IFN- γ augmentation of monocyte O_2^- release. Monocytes were pretreated with indicated lipids and then cultured in the presence (\square) or absence (\square) of IFN- γ overnight. The cells were then stimulated with (A) *M. leprae*, (B) PMA, or (C) opsonized zymosan, and O_2^- release measured. Results are means \pm S.E. of 8 experiments in A, 6 experiments in B, and 4 experiments in C. * = $p < 0.05$ by Student's *t* test when compared with no IFN- γ treatment; † = $p < 0.05$ by Student's *t* test when compared with no lipid control pretreatment.

DISCUSSION

Our data indicate that, even though PGL-I suppresses O_2^- release by monocytes stimulated with *M. leprae*, these cells can be

activated by IFN- γ . However, the degree of activation was significantly less than that observed with control monocytes. It has been shown by us and by others (7, 14) that PGL-I can suppress T cell and monocyte function. It has also been reported that IFN- γ production is decreased in lepromatous leprosy patients (9), but that monocytes from those patients can respond to IFN- γ with augmented oxygen radical production (5). However, in studies using mouse macrophages, it was shown that foot pad macrophages gorged with *M. leprae* are refractory to activation by IFN- γ (12), and peritoneal macrophages infected with *M. leprae* respond to IFN- γ if it is administered within 24 hr of infection but not if administered 3 to 5 days postinfection (13). In our studies with human monocytes, we attempted to use experimental conditions that resemble those found *in vivo* in lepromatous leprosy (14), where the cells are exposed to products of *M. leprae*, such as PGL-I. We have reported that the various lipids were taken up by the monocytes in comparable amounts (14). We have shown that PGL-I-pretreated monocytes released less O_2^- , only when stimulated with *M. leprae*, than did control cells or cells pretreated with lipids structurally similar to PGL-I. Here, we have also shown that cells pretreated with PGL-I respond to IFN- γ with an increased oxidative potential but to a lesser degree than control cells when the stimulus is *M. leprae*. PGL-I may contribute further to the intracellular survival of *M. leprae* by down-regulating the IFN- γ enhancement of monocyte/macrophage oxidative responses. In ongoing clinical trials using intradermal administration of IFN- γ (8, 11), the suppressive effects of accumulated PGL-I in the macrophages of leprosy lesions should be considered.

SUMMARY

Peripheral blood monocytes were pretreated with phenolic glycolipid-I (PGL-I), dimycocerosyl phthiocerol (DIM), or mycoside A, then cultured in the presence or absence of interferon-gamma (IFN- γ). Their oxidative responses to *Mycobacterium leprae*, phorbol myristate acetate (PMA), and opsonized zymosan were evaluated. In response to *M. leprae*, monocytes pretreated with PGL-I released less O_2^- than nonlip-

id-treated control cells. The IFN- γ augmentation of oxidative responses was suppressed only in PGL-I-pretreated monocytes and only when the stimulus was *M. leprae*. This suggests that PGL-I, by affecting the IFN- γ enhancement of phagocytic cell oxidative responses, aids further the intracellular survival of *M. leprae*.

RESUMEN

Se pretrataron monocitos de sangre periférica con glicolípido fenólico-1 (PGL-1), con dimicocerosil phtiocerol (DIM), o con micósido A y después se cultivaron en presencia o ausencia de interferón gamma (IFN- γ). Enseguida se evaluaron sus respuestas oxidativas hacia el *Mycobacterium leprae*, hacia el acetato de forbol miristato (PMA) y hacia levaduras opsonizadas. Los monocitos pretratados con PGL-1 liberaron menos O_2^- en respuesta al *M. leprae* que los controles no tratados con el lípido. El aumento de la respuesta oxidativa inducido por el IFN- γ , fue suprimido sólo en los monocitos pretratados con PGL-1 y sólo cuando el estímulo fue *M. leprae*. Esto sugiere que el PGL-1, al afectar el incremento en la respuesta oxidativa de la célula fagocítica inducido por el IFN- γ , ayuda a la supervivencia intracelular del *M. leprae*.

RÉSUMÉ

Après avoir été prétraités par l'antigène phénoglycolipidique-1 (PGL-1), le dimycocerosyl phtiocerol (DIM), ou le mycoside A, des monocytes du sang périphérique ont été cultivés en présence ou en absence d'interferon-gamma (IFN- γ). On a alors évalué leur réponse oxydative au *Mycobacterium leprae*, au myristate acétate de phorbol (PMA), et au zymosan opsonisé. En présence de *M. leprae*, les monocytes prétraités avec le PGL-I ont libéré moins d'oxygène que les cellules témoins non traitées par des lipides. L'augmentation de la réponse oxydative en présence d'IFN- γ a été supprimée uniquement dans les monocytes prétraités par le PGL-I, mais ceci seulement lorsqu'ils étaient stimulés par *M. leprae*. Ces observations suggèrent que le PGL-I, en entraînant un renforcement de la réponse oxydative des cellules phagocytaires en présence d'IFN- γ , contribue à assurer la survie intracellulaire de *M. leprae*.

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