

# THE EFFECT OF PHENOLIC GLYCOLIPID-1 FROM *MYCOBACTERIUM LEPRAE* ON THE ANTIMICROBIAL ACTIVITY OF HUMAN MACROPHAGES

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Leprosy is a chronic disease resulting from symptomatic infection with *Mycobacterium leprae*, which currently affects nearly 12 million persons worldwide (1). Although most prevalent in tropical areas, indigenous cases continue to occur in the United States (2). Leprosy is currently regarded as representing a spectrum of disease states in which the host response to the organism governs clinical manifestations. At one end of the spectrum is tuberculoid leprosy, in which skin lesions are paucibacillary and parameters of cell-mediated immunity (CMI)<sup>1</sup> are intact, and at the opposite end is lepromatous leprosy, in which macrophages and Schwann cells in skin lesions are packed with viable bacilli and *M. leprae*-specific CMI is poor.

Recently a phenolic glycolipid, PGL-1, unique to *M. leprae*, was isolated and characterized (3, 4). PGL-1 contain an antigenically distinct trisaccharide consisting of 3,6-di-*O*-methyl-glucose-linked  $\alpha 1 \rightarrow 4$  to 2,3-di-*O*-methyl-rhamnose-linked  $\beta 1 \rightarrow 2$  to 3-*O*-methyl rhamnose. This trisaccharide portion is glycosidically linked to a phenol that is linked to a 29-carbon phthiocerol to which are attached two tetra-methyl branched mycoserosic acids. Lesser amounts of phenolic glycolipid II, which contains 3-*O*-methyl rhamnose rather than 2,3-di-*O*-methyl rhamnose as the middle sugar, and phenolic glycolipid III, which contains 6-*O*-methyl glucose rather than 3,6-di-*O*-methyl glucose as the terminal sugar, also are present. PGL-1 is present in large amounts, up to 2% of the mass of the bacilli (3), and antibodies directed against it are detected in the sera of affected individuals (4-6). These antibodies are predominantly of the IgM class (7) and increase along the leprosy spectrum, with the highest titers in lepromatous cases (7). Antigenic specificity resides in the terminal sugars, and artificial antigens have been synthesized containing the terminal sugars for use in serodiagnosis and evaluation of cases of leprosy (8, 9). PGL-1 has also been directly detected in the sera and urine of leprosy patients (10, 11). It has been suggested that PGL-1 may function as part of a lipid capsule surrounding the organism which

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<sup>1</sup>Abbreviations used in this paper: CMI, cell-mediated immunity; dPGL, deacylated phenolic glycolipid-1; EPO, eosinophil peroxidase; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; MPO, myeloperoxidase; <sup>1</sup>O<sub>2</sub>, singlet oxygen; O<sub>2</sub><sup>-</sup>, superoxide anion; OH<sup>•</sup>, hydroxyl radical; PD, calcium- and magnesium-free PBS; PGL-1, phenolic glycolipid-1; SOD, superoxide dismutase; XO, xanthine oxidase.

protects it from the host environment (12, 13). We posed the question: does the PGL-1 surrounding the *M. leprae* scavenge the toxic oxygen metabolites formed by macrophages and in this way allow the organisms to survive and replicate?

Phagocytes contain a variety of antimicrobial systems, some which are dependent on oxygen and others which are not. The oxygen-dependent antimicrobial systems use toxic oxygen metabolites formed by the phagocytosis-induced respiratory burst (14). These include the superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $OH^\cdot$ ), and possibly singlet oxygen ( $^1O_2$ ), with the toxicity of  $H_2O_2$  being greatly potentiated by peroxidase (myeloperoxidase [MPO], eosinophil peroxidase [EPO]) and a halide. MPO is present in cytoplasmic granules of neutrophils and monocytes and is released into the phagosome after particle ingestion. When monocytes transform into macrophages, either in vitro or in vivo, their granule peroxidase is lost. In addition, there is a marked decrease in the respiratory burst and in part as a result, the microbicidal potency of the cells is decreased and certain pathogens can survive and replicate intracellularly. Activation of macrophages by  $IFN-\gamma$  potentiates their microbicidal activity due in part to an increase in the respiratory burst.

The xanthine oxidase (XO) system has been used as a model of the oxygen-dependent antimicrobial systems of phagocytes (15, 16). In the course of the oxidation of its substrate (xanthine, hypoxanthine, or acetaldehyde), XO reduces oxygen to  $O_2^-$ ,  $H_2O_2$  is formed either directly from oxygen or by dismutation of  $O_2^-$ , and the  $O_2^-$  and  $H_2O_2$  interact in an iron-catalyzed reaction to form  $OH^\cdot$  as follows:  $O_2^- + H_2O_2 \xrightarrow{Fe^{2+}} O_2 + OH^- + OH^\cdot$  (Haber-Weiss reaction). When a reaction initiated by the XO system is inhibited by catalase which degrades  $H_2O_2$ , superoxide dismutase which scavenges  $O_2^-$ , and by  $OH^\cdot$  scavengers such as mannitol or ethanol, this has been taken as presumptive evidence for the involvement of  $OH^\cdot$  (or a closely related species) generated by the Haber-Weiss reaction.

In this study we have examined the role of PGL-1 in the protection of *M. leprae* from the antimicrobial systems of human mononuclear phagocytes. Because studies of the susceptibility of *M. leprae* to the various antimicrobial systems of phagocytes, both in cell-free form and in intact cells, are complicated by the inability to grow the organism in vitro, thus making measurement of viability difficult, we have used fast-growing *Staphylococcus aureus* as the test organism. The staphylococci were exposed to the cell-free MPO and XO systems and to human monocyte-derived macrophages either untreated or activated by  $IFN-\gamma$  and the effect of the purified PGL-1 on viability was determined. The PGL-1 was either added as such or bound to the surface of the staphylococci by an immunologic technique. We found that PGL-1 is a weak scavenger of the toxic products of the peroxidase- $H_2O_2$ -halide system and that it prevents bacterial killing by  $OH^\cdot$  generated by the XO system and by  $IFN-\gamma$ -activated macrophages. These results suggest a role for PGL-1 in the intracellular persistence of *M. leprae* in certain cell types.

## Materials and Methods

*Special Reagents.* Histopaque 1077 was obtained from Sigma Chemical Co. (St. Louis, MO), sodium chloride (Suprapur) was obtained from Matheson-Coleman-Bell Manufac-

turing (Cincinnati, OH), and superoxide dismutase (SOD) was obtained from Miles Scientific Div. (Naperville, IL). RPMI 1640 was supplemented with Hepes buffer (10 mM), L-glutamine (0.2 mM), penicillin (25 U/ml), and streptomycin (25 µg/ml); all components were obtained from Whittaker M. A. Bioproducts (Walkersville, MD). Autologous or AB serum was prepared by allowing whole blood to clot for 30–60 min at room temperature, and the supernatant was collected after centrifugation and frozen at –70°C until use. MPO was purified from canine pyometrial pus by the method of Agner (17) to the end of step 6, and assayed by guaiacol oxidation (18). XO obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN), and catalase, obtained from Worthington Biochemical Corp. (Malvern, PA), were dialyzed against sterile nonpyrogenic water before use. Rabbit anti-mouse IgM and FITC-conjugated F(ab')<sub>2</sub> goat anti-rabbit IgG were obtained from Zymed Laboratories (San Francisco, CA) and FITC F(ab')<sub>2</sub> goat anti-mouse IgM was obtained from Tago Inc. (Burlingame, CA). Purified PGL and its deacylated derivative (dPGL) were kindly provided by P. Brennan, Colorado State University (Fort Collins, CO). mAb B8F (12) directed against the terminal glycoside moiety of PGL-1 was from T. Buchanan and R. Miller, University of Washington (Seattle, WA) and the human rIFN-γ was from P. Trown, Hoffman-La Roche (Nutley, NJ). IFN-γ activity was determined by the supplier in a WISH/VZV cytopathic effect microtiter assay standardized with NIH human IFN-γ (reference standard Gg 23-901-530). The 502A and Cowan strains of *S. aureus*, kindly provided by R. E. Bryant, Oregon Health Sciences University (Portland, OR), were maintained on blood agar plates and grown at 37°C in trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, MD) for the periods indicated.

Glassware was heated at 170°C for 24 h to destroy endotoxin. All media and buffers were prepared in sterile pyrogen-free water from Travenol Laboratories Inc. (Deerfield, IL) and were filtered before use with 0.22-µm filter units from Corning Glass Works (Corning, NY).

**Preparation of Phenolic Glycolipid.** Known amounts of PGL-1 and dPGL were dissolved in a mixture of chloroform/methanol 2:1 [vol/vol] and aliquots were blown to dryness under nitrogen in glass tubes. Suspensions of PGL-1 and dPGL were made by sonicating the lipids in either pyrogen-free water or PBS for two 30-s bursts at setting 6 of a model 200 Sonifier (Branson Sonic Power Co., Danbury CT). Lipid suspensions were kept at 37°C until use.

**Microbicidal Activity.** The components indicated in the legends to Tables I and II were incubated in 12 × 75-mm plastic tubes in a shaking water bath oscillating 80 times/min. At the times indicated, 0.1-ml aliquots were removed, serially diluted in 0.1 M sodium sulfate, and the viable cell count was determined by the pour-plate method using trypticase soy agar. The absence of colonies on plating 0.1 ml of the undiluted incubation mixture is designated 0 organisms/ml (actually <10).

**Preparation of PGL-coated Staphylococci.** The Cowan strain of *S. aureus*, which is known to be rich in protein A, was grown to mid-log phase for 4 h at 37°C in trypticase soy broth. After three washes in PBS with 1% BSA, the bacteria were resuspended to an OD of 0.18 at 540 nm (model M15 spectrophotometer; Cary Instruments, Monrovia, CA) and then 1 ml of this suspension was incubated with rabbit IgG anti-mouse IgM for 30 min in a shaking water bath at 37°C. The bacteria were then washed twice to remove unbound antibody. The staphylococci, bearing rabbit anti-mouse IgM bound to surface protein A, next were incubated with the IgM murine mAb B8F for 30 min in a shaking water bath at 37°C. The bacteria were washed twice to remove unbound antibody, resuspended, and divided into two equal aliquots. Aliquot A was incubated with PBS and aliquot B was incubated at 37°C in a shaking water bath with a suspension of dPGL at 1 mg/ml.

**Chromatography.** HPLC of dPGL was performed as described (10) using a µPorasil Column (0.39 × 30-cm, 10 µm), an automated gradient controller with model 501 HPLC pump, an automatic injector (model 710B), a data module (model 740) and a Lambda-Max spectrophotometer (model 481 LC) (Waters Associates, Milford, MA). The eluant was 4% methanol in chloroform at a flow rate of 1 ml/min.

*Monocyte Culture.* Venous blood was obtained after informed consent from healthy human volunteers and the monocytes were isolated as previously described (19). Briefly, after two washes with cold calcium- and magnesium-free PBS (PD), the cell suspension was underlayered with Histopaque 1077 and centrifuged at 800 *g* for 20 min at 23°C. Mononuclear cells, after removal from the PD–Histopaque interface, were washed twice in PD and resuspended in RPMI 1640 with 15% autologous or AB serum. Monocytes were added to 35-mm-diam tissue culture plates (Primaria type; Falcon Labware, Becton, Dickinson & Co., Oxnard CA) in 2-ml vol containing 2.5–3.0 million monocytes and incubated for 2 h at 37°C in humidified 5% CO<sub>2</sub>/95% air. Plates were then washed six to eight times with warm PBS to remove nonadherent cells, 2 ml of fresh media were added to each plate, and monolayers were maintained at 37°C in humidified 5% CO<sub>2</sub>/95% air. Monolayers supplemented with IFN- $\gamma$  had fresh medium containing 200 U/ml of the cytokine added on days 1–3. Control monolayers had fresh medium alone added at similar times.

On day 4, control monolayers and those supplemented with IFN- $\gamma$  were washed four to six times with warm HBSS. Reaction mixtures of antibody-coated staphylococci either with or without dPGL were added to macrophage monolayers. The tissue culture plates were centrifuged at 250 *g* for 2 min at room temperature and then incubated for 3 min at 37°C in humidified 5% CO<sub>2</sub>/95% air. The reaction mixtures were then removed and the monolayers washed twice with HBSS. To determine the number of cell-associated bacteria at this time point (designated T<sub>0</sub>), monolayers were incubated in water for 5–10 min, scraped with a sterile transfer pipette to lyse the cells, and the number of viable bacteria enumerated by a pour-plate method using trypticase soy agar. Remaining monolayers were incubated in RPMI 1640 with 15% AB serum without added antibiotics for 90 min in humidified 5% CO<sub>2</sub>/95% air. To determine the number of surviving bacteria at this time point (designated T<sub>90</sub>), the reaction mixtures were removed and the monolayers lysed as described above. Lysostaphin was not used because of evidence suggesting its uptake by phagocytes (20).

*Electron Microscopy.* Incubation of staphylococci with the mononuclear phagocytes was carried out as described above except that monolayers were fixed rather than lysed at the designated time points. Cells were fixed for 60 min in half-strength Karnovsky's fixative (21) and then washed twice with 0.1 M cacodylate buffer. Fixed monolayers were dehydrated with increasing concentrations of ethanol and gradually infiltrated with Poly/Bed 812 (Polysciences, Inc., Warrington, PA). Thin sections were stained with uranyl acetate and lead citrate, and examined with a Phillips 410 transmission electron microscope (Phillips Electronic Instruments, Inc., Mahwah, NJ).

## Results

*Effect of PGL-1 and dPGL on the Bactericidal Activity of the Peroxidase System.* The effect of PGL-1 and dPGL on the microbicidal activity of the MPO–H<sub>2</sub>O<sub>2</sub>–halide system is shown in Table I. Two experimental conditions were used. In system A, the H<sub>2</sub>O<sub>2</sub> concentration was 10<sup>-4</sup> M and the halide was either 10<sup>-1</sup> M chloride or 10<sup>-4</sup> M iodide. Under these conditions, the complete system produced a >3-log fall in viable cell count after a 30-min incubation whereas none of the individual components of the system was toxic. The addition of either PGL or dPGL at a concentration of 100  $\mu$ g/ml had no effect on the bactericidal activity of the peroxidase system under these conditions (Table I). The lipids also were ineffective when preincubated with the bacteria for 15 min before the addition of the components of the complete system. Neither lipid was toxic at these concentrations when incubated with the bacteria alone. The concentrations of H<sub>2</sub>O<sub>2</sub> and the individual halides were decreased to the lowest levels at which a >2-log fall in viable organisms were consistently observed after a 30-min incubation and these conditions (2.5  $\times$  10<sup>-6</sup> M H<sub>2</sub>O<sub>2</sub>, 10<sup>-2</sup> M chloride,

TABLE I  
Effect of *M. leprae* Phenolic Glycolipid on the MPO-H<sub>2</sub>O<sub>2</sub>-Halide System

Supplements	Viable cell count			
	System A	<i>p</i> <sup>*</sup>	System B	<i>p</i> <sup>‡</sup>
		<i>cfu</i> × 10 <sup>-6</sup> /ml		
None	3.55 (3) <sup>§</sup>	<0.001	3.52 (4)	<0.001
MPO + H <sub>2</sub> O <sub>2</sub> + Cl <sup>-</sup>	0.001 (3)	—	0.02 (3)	—
+ PGL (100 μg/ml)	0.00003 (3)	NS	3.60 (2)	<0.001
+ dPGL (100 μg/ml)	0.00001 (3)	NS	4.13 (2)	<0.001
MPO + H <sub>2</sub> O <sub>2</sub> + I <sup>-</sup>	0.00008 (3)	NS	0.002 (3)	NS
+ PGL (100 μg/ml)	0.00006 (3)	NS	0.06 (3)	NS
+ dPGL (100 μg/ml)	0.00005 (3)	NS	0.03 (3)	NS

Both systems A and B contained 0.01 M sodium phosphate buffer (pH 7.0), 0.067 M sodium sulfate, 5 × 10<sup>6</sup> *S. aureus* 502a, and 225 mU/ml MPO. System A contained 10<sup>-4</sup> M H<sub>2</sub>O<sub>2</sub> and where indicated 10<sup>-1</sup> M NaCl or 10<sup>-4</sup> M NaI. System B contained 2.5 × 10<sup>-6</sup> M H<sub>2</sub>O<sub>2</sub> and where indicated 10<sup>-2</sup> M NaCl or 5 × 10<sup>-6</sup> M NaI. Final volumes, 0.5 ml. Incubations were carried out at 37°C for 30 min.

\* Significance (*p*) vs. complete system A.

‡ Significance (*p*) vs. complete system B.

§ Mean of (*n*) experiments.

or 5 × 10<sup>-6</sup> M iodide) were designated system B. The addition of PGL and dPGL completely blocked the microbicidal effect of system B with chloride as the halide but did not completely prevent killing when iodide was used (Table I).

*Effect of PGL-1 and dPGL on the Bactericidal Activity of XO System.* The incubation of acetaldehyde, XO, and Fe<sup>2+</sup> with *S. aureus* produced a >2-log fall in the viable cell count after a 30-min incubation under the conditions used in Table II. As previously reported (15, 16), this microbicidal effect was abolished by SOD, catalase, mannitol, or ethanol, but not by heat-inactivated SOD or catalase, thus implicating OH<sup>·</sup> generated by the Haber-Weiss reaction. The addition of 100 μg/ml of PGL-1 or dPGL to the reaction mixture completely abolished the antimicrobial effect suggesting that the lipid can scavenge OH<sup>·</sup> generated by the XO system. The addition of either glycolipid had no effect on the activity of XO as measured by xanthine oxidation.

*Binding of dPGL to S. aureus.* A "surrogate *M. leprae*" was prepared by binding the phenolic glycolipid of *M. leprae* to the surface of *S. aureus* through the use of linking antibodies. The Cowan strain of *S. aureus* is rich in protein A which binds to the Fc region of IgG molecules. Because the murine mAb B8F, which is specific for the terminal trisaccharide of PGL-1, is an IgM, an antibody class that does not bind staphylococcal protein A, and IgG antibody directed against murine IgM was used to bind the PGL-1-IgM complex to surface protein A. The optimum conditions for antibody binding were established by fluorescence microscopy of bacteria coated with rabbit IgG anti-mouse IgM and subsequently stained with FITC-conjugated F(ab')<sub>2</sub> goat anti-rabbit IgG. No staining of uncoated bacteria occurred and maximum fluorescence was observed on incubation of the staphylococci with a 1:50 dilution of the rabbit IgG anti-mouse IgM. Optimum conditions were similarly established for binding of the monoclonal IgM antibody to the IgG-coated staphylococci by fluorescence microscopy of

TABLE II  
*Effect of M. leprae Phenolic Glycolipid on the Xanthine Oxidase System*

Supplements	Viable cell count	<i>p</i> *
	<i>cfu</i> × 10 <sup>-6</sup> /ml	
None	4.49 (9) <sup>‡</sup>	<0.001
Acet + XO + Fe <sup>2+</sup>	0.014 (9)	—
+ SOD (5 μg/ml)	4.39 (7)	<0.001
+ heated SOD (5 μg/ml)	0.054 (5)	NS
+ catalase (60 μg/ml)	5.96 (8)	<0.001
+ heated catalase (60 μg/ml)	0.032 (4)	NS
+ mannitol (10 <sup>-1</sup> M)	2.60 (6)	<0.001
+ ethanol (10 <sup>-1</sup> M)	3.16 (6)	<0.001
+ PGL (100 μg/ml)	3.52 (3)	<0.001
+ dPGL (100 μg/ml)	3.48 (3)	<0.001

The reaction mixture contained 0.01 M sodium phosphate buffer (pH 7.0), 0.067 M sodium sulfate, 5 × 10<sup>6</sup> *S. aureus* 502a, 1.4 × 10<sup>-5</sup> M acetaldehyde, 30 μg xanthine oxidase, and 10<sup>-6</sup> M ferrous sulfate. Where indicated, the SOD was autoclaved at 120°C for 30 min and the catalase was heated at 100°C for 15 min.

\* Significance (*p*) vs. complete system.

<sup>‡</sup> Mean of (*n*) experiments.

IgM-IgG-coated bacteria stained with FITC-conjugated F(ab')<sub>2</sub> goat anti-mouse IgM. Maximum binding of IgM anti-PGL-1 mAb occurred at a dilution of 1:50. The fluorescence with both antibodies was evenly distributed on all of the organisms. Staphylococci with surface-bound IgG-IgM were then incubated with dPGL. To determine that dPGL was bound to the staphylococci, bound lipid was extracted and dPGL demonstrated by HPLC. After washing four times, bacteria were extracted with chloroform/methanol (2:1 [vol/vol]), and the chloroform layer was removed and evaporated to dryness under nitrogen. Samples were taken up in 4% methanol in chloroform and subjected to HPLC. Representative chromatographs are shown in Fig. 1. HPLC of the chloroform/methanol extract of dPGL-coated staphylococci (Fig. 1B) yielded a compound with a retention time identical to that of authentic dPGL standard (Fig. 1A) (*n* = 3). The standard and extracted lipid both had equal changes in retention time when rechromatographed using a different solvent system (chloroform with 3% methanol) (data not shown). No dPGL peak was observed by HPLC of a chloroform/methanol extract of control staphylococci coated with the IgG and IgM antibodies but not with dPGL (Fig. 1C).

*Effect of dPGL on the Antimicrobial Activity of Monocyte-derived Macrophages.* Human blood monocytes were maintained as adherent monolayer cultures for 4 d with the culture medium supplemented with 200 U/ml of IFN-γ for the final 3 d in half of the cultures. When nonactivated macrophages (without IFN-γ treatment) were exposed to staphylococci coated with the IgG and IgM antibodies, but without PGL for 90 min, 9% of the cell-associated bacteria were killed (Table III). Activation of the macrophages with IFN-γ for 3 d resulted in a marked increase in antimicrobial activity with 74% of cell-associated bacteria

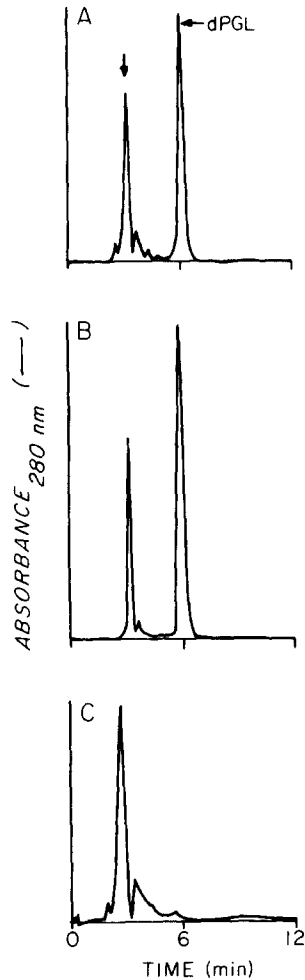


FIGURE 1. HPLC of (A) pure dPGL standard; (B) chloroform/methanol extraction of staphylococci coated with dPGL, and IgG and IgM antibodies; (C) chloroform/methanol extraction of control staphylococci coated with antibodies alone. HPLC conditions are described under Materials and Methods. Arrow denotes elution of void volume.

killed at the end of the 90-min incubation period. When dPGL-coated staphylococci were used, not only was killing by both the control and IFN- $\gamma$ -supplemented macrophages completely blocked but these log-phase organisms continued to replicate, resulting in an 84 and 39% increase in the viable cell count at the end of the 90 min (Table III).

**Electron Microscopy.** Nonactivated and IFN- $\gamma$ -activated human monocyte-derived macrophages incubated with *S. aureus* with or without dPGL on their surface were examined by transmission EM. The findings with IFN- $\gamma$ -treated macrophages are shown in Fig. 2. Macrophages, incubated either with staphylococci coated with the IgG and IgM antibodies but without dPGL (Fig. 2a), or with staphylococci coated with antibodies and dPGL (Fig. 2b), contained intraphagosomal organisms, indicating that coating with dPGL does not prevent phagocytosis by human monocyte-derived macrophages. Several of the staphylococci are dividing, which reflects the use of log-phase organisms for these

TABLE III  
*Effect of PGL-1 on S. aureus Phagocytosed by Human  
 Monocyte-derived Macrophages*

Presence of dPGL on S. aureus	Percent change in viable <i>S. aureus</i>	
	Macrophage monolayers	
	-IFN	+IFN
-dPGL	-9 (3)*	-74 (3)
+dPGL	+84 (3)	+39 (3)

Human blood monocytes were maintained as adherent monolayers for 4 d with some of the monolayers supplemented with 200 U/ml IFN- $\gamma$  on days 1-4. Monolayers were exposed to log-phase staphylococci coated with either IgG and IgM antibodies alone or with both antibodies plus dPGL. The number of viable bacteria was determined after lysis of the monolayer at the beginning and end of the 90 min incubation period, and the percent change in viable bacteria calculated.

\* Mean of (*n*) experiments.

studies. The findings were similar when nonactivated monocyte-derived macrophages were used.

### Discussion

We report that the major glycolipid antigen of *M. leprae*, PGL-1, and its deacylated derivative, can scavenge the reactive oxygen species generated by stimulated phagocytes, and that this may protect the organism from the antimicrobial activity of activated human macrophages. These conclusions are based on the following lines of evidence.

First, both PGL-1 and dPGL abolished the antimicrobial effect of the acetaldehyde-XO-Fe<sup>2+</sup> system on *S. aureus*. This system generates O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, and OH<sup>·</sup>, all of which may be produced by stimulated phagocytes and thus has been used as a model for the oxygen-dependent antimicrobial systems of these cells (15, 16). That the observed cytotoxicity of the XO system is due to OH<sup>·</sup> (or an oxidant with similar properties) generated by the Haber-Weiss reaction, is suggested by the inhibition of toxicity by catalase (which degrades H<sub>2</sub>O<sub>2</sub>) and SOD (which scavenges O<sub>2</sub><sup>-</sup>) but not by heated catalase or SOD, and by the OH<sup>·</sup> scavengers, mannitol and ethanol. These findings suggest that PGL-1 (or dPGL) can act as an OH<sup>·</sup> scavenger and thus protect the target organism from the harmful effects of this toxic oxygen species.

Second, both PGL-1 and dPGL exhibited a modest protective effect against the cytotoxicity of the peroxidase-H<sub>2</sub>O<sub>2</sub>-halide system. When the individual components of the MPO system were titrated to produce at least a 2-log decrease in the viable cell count, both PGL-1 and dPGL, at the concentrations used, completely inhibited toxicity with chloride, but not iodide, as the halide. It is possible that higher concentrations of the glycolipids would completely inhibit peroxidase-mediated toxicity with both halides. In patients with leprosy, large numbers of acid-fast bacilli have been detected in circulating polymorphonuclear leukocytes and monocytes (22), both of which contain myeloperoxidase in cytoplasmic granules. Presumably a proportion of these bacilli are viable, as organisms



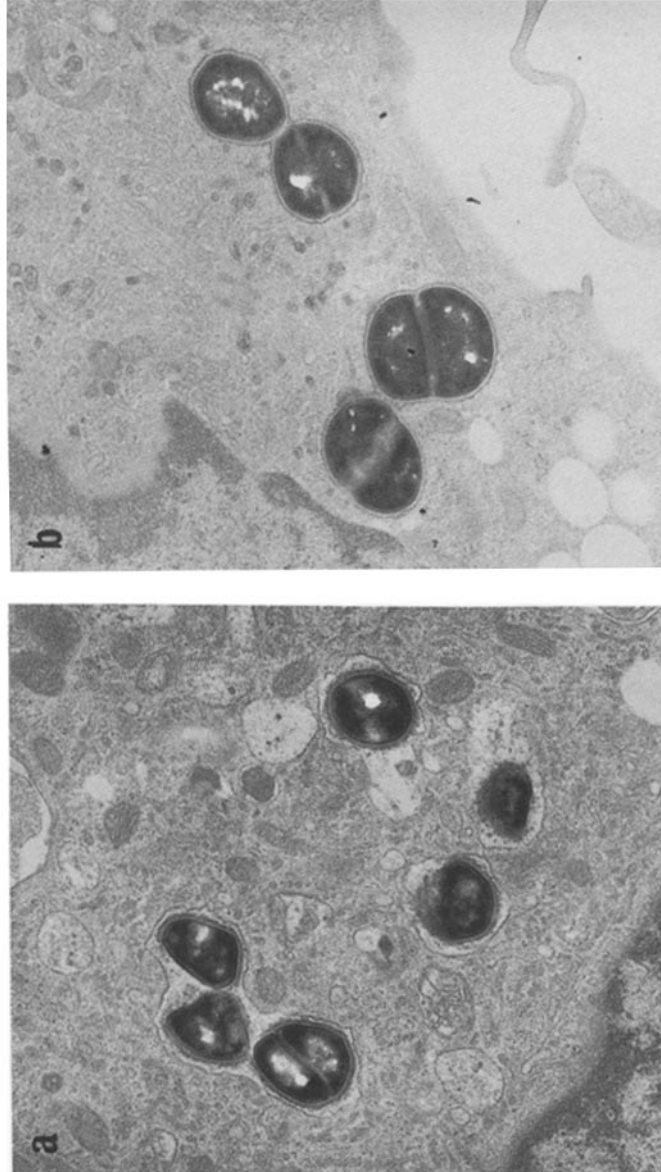


FIGURE 2. Transmission electron micrographs of IFN- $\gamma$ -activated macrophage monolayers with intracellular staphylococci. (a) Human monocyte-derived macrophages have ingested staphylococci coated with IgG and IgM antibodies. (b) Macrophages have ingested staphylococci coated with both IgG and IgM antibodies and dPGL. All staphylococci (a-b) are within phagosomes.  $\times 38,000$ .

recovered from blood in bacteremic leprosy patients have been shown to be infective in the mouse footpad (23). It is possible that when large amounts of the glycolipid are present, as in heavily parasitized blood phagocytes, PGL-1 may prevent peroxidase-mediated toxicity.

Lastly, dPGL prevented killing of staphylococci by human monocyte-derived macrophages activated by IFN- $\gamma$ . The dPGL was bound to the surface of the staphylococci by a double-antibody technique using a specific IgM mAb directed against the trisaccharide portion of PGL-1, and an IgG anti-IgM antibody which bound both to the PGL-1-IgM complex via its antibody combining site and to the staphylococci via the interaction of its Fc portion with the protein A of the organism. Human blood monocytes, when maintained as adherent monolayers, undergo striking biochemical and morphologic changes as they differentiate into macrophages, among which are the loss of granule peroxidase (24–26) and a marked decrease in their respiratory burst in response to stimulation (26). Supplementation of the culture medium with IFN- $\gamma$  results in activation of the macrophages as indicated by a marked increase in respiratory burst (27), antimicrobial (28, 29), and antiparasitic (30, 31) activities. Human monocyte-derived macrophages demonstrated considerably greater staphylocidal activity when cultured with IFN- $\gamma$ . The presence of the *M. leprae*-derived glycolipid on the surface of the staphylococci prevented killing by the IFN- $\gamma$ -activated macrophages, despite the presence of the microorganisms in phagosomes.

The mechanisms by which *M. leprae* survive and replicate in phagocytes are not well understood. The organisms are readily killed by the cell-free MPO-H<sub>2</sub>O<sub>2</sub> system (32) although it is not known whether this system is effective in intact neutrophils or monocytes. Exposure of *M. leprae* to 0.08% H<sub>2</sub>O<sub>2</sub> resulted in a marked loss of viability, as judged by loss of infectivity in the mouse footpad (33). The failure of *M. leprae* to induce a respiratory burst, as measured by O<sub>2</sub><sup>-</sup> production in murine (BALB/c) peritoneal macrophages, or in human peripheral blood neutrophils or monocytes has been reported (34), and *M. leprae* contains SOD (35, 36) which may protect the organisms by scavenging O<sub>2</sub><sup>-</sup>.

It has been suggested that PGL-1, being a surface component, may form a capsule around *M. leprae* (13). The presence of a capsule in certain microorganisms may be a mechanism for avoidance of host-defense systems by preventing phagocytosis (37). However, PGL-1 does not appear to be antiphagocytic as both *M. leprae* (38) and dPGL-coated staphylococci are ingested by human macrophages. Evidence has been presented here suggesting that a key contribution of PGL-1 to the intracellular survival of *M. leprae* may be the ability of the glycolipid to scavenge reactive oxygen species and thereby prevent microbial death within the phagosome.

### Summary

Purified PGL-1 and dPGL from *M. leprae* can prevent bacterial killing by intact phagocytes and cell-free antimicrobial systems. Both glycolipids completely abolished the antimicrobial effect of the acetaldehyde-XO-Fe<sup>2+</sup> system. Because the cytotoxicity of this system is inhibited by catalase, SOD, mannitol, and ethanol, but not by heated SOD or catalase, these data suggest that toxicity is due to OH<sup>·</sup> generated by the Haber-Weiss reaction. That the antimicrobial

killing in the XO system is completely blocked by the addition of PGL-1 or dPGL suggests that these glycolipids can act as OH<sup>·</sup> scavengers. A modest protective effect against the cytotoxicity of the MPO-H<sub>2</sub>O<sub>2</sub>-halide system by both PGL-1 and dPGL was also observed. The antimicrobial activity of the MPO system was abolished with chloride, but not iodide, as the halide. The effect of the *M. leprae*-derived glycolipid on bacterial killing by intact phagocytes was examined. Two linking antibodies were used to bind the dPGL to a rapidly growing test organism, *S. aureus*, a murine IgM mAb specific for the terminal glycoside of PGL-1, and a rabbit IgG anti-mouse IgM which bound the staphylococcal protein A via its Fc region. Examination by transmission EM of human monocyte-derived macrophages which had ingested staphylococci either coated with both antibodies and dPGL, or coated only with the IgG and IgM antibodies, demonstrated the presence of bacteria in phagosomes of control and IFN- $\gamma$ -activated macrophages. Activation of the macrophage monolayers by pretreatment with IFN- $\gamma$  markedly increased their staphylocidal activity. When dPGL coated staphylococci were ingested, killing by both control and IFN- $\gamma$ -activated macrophages was completely blocked. These results, suggesting that PGL-1 can scavenge reactive oxygen species and prevent microbial death within the phagosome, may in part explain the intracellular survival of *M. leprae* in certain cell types.

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