Phenolic Glycolipid-1 of *Mycobacterium leprae* Binds Complement Component C3 in Serum and Mediates Phagocytosis by Human Monocytes

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Summary

Previous studies from this laboratory have demonstrated that *Mycobacterium leprae*, an obligate intracellular bacterial parasite, enters human mononuclear phagocytes via complement receptors on these host cells and bacterium-bound C3. The present study investigates the role of *M. leprae* surface molecules in C3 fixation and phagocytosis. By enzyme-linked immunosorbent assay, C3 binds selectively to phenolic glycolipid-1 (PGL-1), a major surface molecule of the leprosy bacillus. C3 fixation to PGL-1 is serum concentration dependent and is abolished in heat-inactivated serum or serum containing ethylenediaminetetraacetic acid. C3 fixation is also abolished in serum containing ethyleneglycol-bis (β-aminoethyl ether)*N,N,N'*-tetraacetic acid and $MgCl_2$ indicating that isolated PGL-1 fixes C3 via the classical complement pathway. The capacity of PGL-1 to fix C3 is dependent upon its terminal trisaccharide since sequential removal of monosaccharide units of the trisaccharide results in a stepwise reduction in C3 fixation. Deacylation of PGL-1 also abolishes C3 fixation. C3 fixes to the trisaccharide of PGL-1 that is chemically linked to bovine serum albumin via the chemical carrier, 8-methoxycarbonyloctanol. PGL-1 mediates C3 fixation to polystyrene microspheres, and PGL-1 and C3 together mediate ingestion of polystyrene microspheres by human monocytes, wherein these inert test particles reside in membrane-bound phagosomes. Thus, complement receptors on mononuclear phagocytes, complement component C3, and PGL-1 comprise a three-component receptor-ligand-acceptor molecule system for mediating phagocytosis of *M. leprae*.

Mycobacterium leprae is an obligate intracellular bacterium that is phagocytized by and resides within mononuclear phagocytes. Previously we have demonstrated that complement receptors—CR1 and CR3 on monocytes and CR1, CR3, and CR4 on monocyte-derived macrophages—and fragments of complement C3 on the bacterial surface mediate phagocytosis of *M. leprae* (1, 2).

The surface structure of *M. leprae* has been studied in detail and the major surface molecules have been purified (3–6). Ultrastructural studies have suggested that the cell wall of *M. leprae* has three layers—an electron dense innermost layer containing peptidoglycan (5); an electron-transparent middle layer composed of long chain mycocerosyl fatty acyl groups and arabinogalactan, an abundant polysaccharide of *M. leprae* (4); and an outer layer composed largely of carbohydrates (6). These carbohydrates include the terminal saccharides of the phenolic glycolipids, the major glycolipid moieties of *M. leprae*; the terminal sugars of lipoarabinomannan (LAM)[1], the major complex lipopolysaccharide of *M. leprae*; and lipomannan (LM), a molecule resembling LAM but devoid of arabinose moieties (4). Of the three closely related *M. leprae* phenolic glycolipids phenolic glycolipid-1 (PGL-1) a molecule found in enormous quantities in *M. leprae*-infected tissues, is by far the most abundant (7–9). The molecules in the outer layer of *M. leprae* are believed to be arrayed such that their carbohydrate moieties form the outermost coat of the bacterium. In this study, we have explored the capacity of molecules on the *M. leprae* surface to serve as acceptor molecules for the covalent linkage of C3 activation products.

[1] *Abbreviations used in this paper:* DIM, dimycocerosyl-phthiocerol; HI, heat inactivated; LAM, lipoarabinomannan; LM, lipomannan; MOMP, major outer membrane protein; PGL-1, phenolic glycolipid-1.

Materials and Methods

Mycobacterium leprae Components and mAbs Against them. PGL-1 (8); LAM (10); arabinogalactan (11); monodeglycosylated PGL-1 (8); Mycoside B from *Mycobacterium* bovis (12); dimycocerosyl-phthiocerol (DIM) (8); deacylated PGL-1 (8); native trisaccharide -0- BSA (NT-O-BSA) (13); mouse mAb (ascites) against PGL-1 (ML 8B2) (14); and mouse mAb (ascites) against LAM (ML 9D3) (15) were generously provided by Dr. Patrick Brennan and colleagues (Colorado State University, Fort Collins, CO) through the Na-

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Sera. Serum from four healthy adult volunteers who were PPD negative and had no known exposure to patients with leprosy was used in all experiments and obtained as described (16).

Enzyme-linked Immunosorbent Assay (ELISA) of C3 Fixation to Mycobacterium leprae Components. Stock PGL-1, monodeglycosylated PGL-1, Mycoside B, DIM, and deacylated PGL-1 (in chloroform/methanol 2:1 v/v) were aliquoted in various amounts into borosilicate tubes and dried under N_2. The molecules were then resuspended in 100% ethyl alcohol by sonication in a sonic bath until clear. 50 μl of each solution, or ethyl alcohol only as a control, was aliquoted into ELISA plate wells (Costar Corp., Cambridge, MA) and evaporated to dryness (2 h). Stock solutions of LAM, LM, arabinogalactan, and NT-O-BSA were further diluted to various concentrations in carbonate-bicarbonate buffer (0.05M, pH 9.6). 50 μl of each solution, or carbonate-bicarbonate buffer with or without BSA (ICN Immunobiologicals, Lisle, IL) as controls, was aliquoted in duplicate into ELISA plate wells, incubated at 37°C overnight on a shaking platform, and washed three times with PBS. From this point on, all ELISA plates were handled identically.

Nonspecific protein binding sites were blocked with PBS containing 5% HSA (Cat. no. 126658; Calbiochem Corp., La Jolla, CA) for 2 h at 37°C (shaking platform at 40 rpm). The blocked wells were washed with PBS and incubated with various preparations of 2.5% human serum or with PBS containing 0.5% HSA for 30 min at 37°C. The wells were then washed five times in PBS (4°C) and incubated with a primary antibody in 0.5% HSA or with 0.5% HSA alone as a control (overnight, 4°C). Primary antibodies were chicken anti-human C3 (Accurate Chemical and Scientific Co., Westbury, NY) diluted 1:10,000; mAb to C3bi (Cytotech, San Diego, CA) diluted 1:150,000, and mAbs to PGL-1 and LAM diluted 1:800. The wells were washed three times in PBS, incubated with a HRP-conjugated secondary antibody (goat anti-mouse IgG [Biorad, Richmond, CA] diluted 1:3000 or human absorbed goat anti-chicken IgG [Bethyl Labs, Inc., Montgomery, TX] diluted 1:5000) in 0.5% HSA for 2 h at room temperature, washed again, and incubated with HRP-substrate (Bio-Rad) for 10 min at room temperature. The reaction was stopped with 1% (final concentration) oxalic acid. Absorbance at 405 nm was measured. The mean ± SD of the absorbance for duplicate wells of each type was calculated. The optical density of control wells was subtracted out and was typically ≤ 0.10.

Preparation of PGL-1-coated Microspheres. 2.0×10^8 Polybead polystyrene microspheres (Cat. number 07310; Polysciences, Inc., Warrington, PA), 1 μm in diameter, were washed two times in carbonate-bicarbonate buffer by centrifugation at 350 *g* for 5 min in presiliconized polypropylene tubes (Research Products, Mount Prospect, IL). The supernate was removed and the beads were incubated for 2 h at 37°C on a Adams Nutator (Clay Adams, Parsippany, NJ) with PGL-1 (100 μg) that had been suspended in 1 ml of carbonate-bicarbonate buffer by sonication for 10 min with a 3-mm sonicator probe. Control microspheres were incubated with carbonate-bicarbonate buffer alone. The microspheres were washed twice and then incubated in PBS containing 5% HSA for 2 h at 37°C to block nonspecific protein binding sites. The beads were then washed in PBS containing 0.5% HSA and incubated for 30 min at 37°C in 500 μl of 2.5% fresh serum, 2.5% heat-inactivated (HI) serum (heated at 56°C for 30 min), or 0.5% HSA. Finally, the microspheres were washed, resuspended in 500 μl of 0.5% HSA, and adjusted to 10^8/ml.

Immunofluorescence Assay for Detection of C3 on Opsonized PGL-1-coated Microspheres. PGL-1 coated microspheres (10^7), incubated in either 2.5% fresh serum or 0.5% HSA as above, were incubated with fluorescein-conjugated rabbit anti-human C3c (Dako Corp., Carpinteria, CA) (1:20 dilution) for 30 min at 37°C on a nutator. The beads were then washed in 0.5% HSA, resuspended in 50 μl of the same buffer, and examined on glass slides by fluorescence microscopy.

Assays of Phagocytosis of PGL-1-coated Microspheres. Human mononuclear cells were isolated and plated on glass coverslips in monolayer culture as previously described (1, 17). The monocyte monolayers were then cultured in RPMI containing 10% autologous serum for 24 h, washed vigorously in RPMI, resuspended in RPMI-20 mM HEPES (Sigma Chemical Co., St. Louis, MO), and incubated with variously prepared microspheres (10^7) at 37°C in 5% CO_2-95% air on a shaking platform (100 rpm). After 60 min, the monocytes were washed to remove nonadherent microspheres and fixed in 10% formalin. The percentage of monocytes with ≥1 adherent microsphere and the mean number (± SD) of microspheres per monocyte on each of triplicate coverslips were determined by counting a minimum of 100 consecutive monocytes per coverslip by phase contrast microscopy.

In studies to determine if polystyrene microspheres that adhere to monocytes are ingested, monocytes were plated on plastic coverslips as previously described (1). The adherence assay was performed as described above except that the number of microspheres added was increased to 2.5×10^7 to optimize conditions for enumerating ingested microspheres. In addition, after the 60 min incubation period, the monocytes were washed and incubated for an additional 30 min before fixation and preparation for electron microscopy (1). Greater than 50 consecutive monocyte cross-sections were examined for each experimental condition.

Results

PGL-1 Fixes Complement Component C3. To determine if any of *M. leprae's* outermost surface molecules are capable of fixing C3, we developed a sensitive ELISA to assess C3 fixation to purified *M. leprae* surface carbohydrates. This ELISA enabled us to quantify C3 fixation to *M. leprae* molecules in low concentrations of serum. Maximal phagocytosis of *M. leprae* by monocytes occurs in ≥2.5% serum (1). The choice of HSA as a blocking agent was critical in this ELISA as it decreased nonspecific binding of native C3 to negligible levels.

PGL-1 fixed C3 avidly, and it did so in a dose-dependent manner (Fig. 1). In contrast, LAM, LM, and arabinogalactan did not fix appreciable C3 in ELISA wells either at the doses shown in Fig. 1 or at higher or lower doses. All carbohydrates were efficiently adsorbed to the ELISA plate wells as determined by using mAbs against these molecules. Therefore, C3 fixation was specific for PGL-1 in the ELISA.

C3 fixation to PGL-1 was strictly dependent upon serum concentration; maximal amounts of C3 were deposited in ≥2.5% fresh serum (Fig. 2). In these studies, in addition to using a polyclonal anti-C3 antibody, we used a mAb specific for the C3 activation product C3bi. This latter antibody does not recognize native C3. The results with the two primary antibodies were similar, i.e., both antibodies revealed a similar

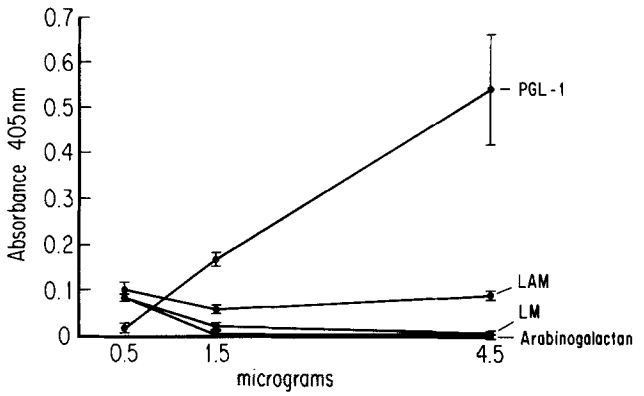

Figure 1. PGL-1 fixes complement component C3 to its surface whereas LAM, LM, and arabinogalactan do not. Purified *M. leprae* surface molecules in the amounts indicated were adhered to wells of an ELISA plate. The wells were blocked with 5% HSA in PBS, washed, incubated with 2.5% fresh serum at 37°C for 30 min, washed again, and examined for C3 deposition using a primary polyclonal antibody against human C3 and an HRP-conjugated secondary antibody. The wells were incubated for 10 min with HRP substrate, the reaction was terminated with 1% oxalic acid, and absorbance at 405 nm was measured. Data are the mean ± SD of duplicate measurements in one representative experiment. Absorbance in wells devoid of antigen was subtracted out in each case and was always ≤0.10 OD.

dependence of C3 fixation to PGL-1 on serum concentration. This result provided further evidence for the specificity of our assay in detecting C3 activation products. It also demonstrated that at least a portion of C3 bound to PGL-1 is in the form of C3bi. To confirm that C3 fixation to PGL-1 was dependent upon heat-labile complement components in serum, we compared C3 fixation in fresh and HI serum. In 2.5% fresh serum, significant amounts of C3 were fixed. In contrast, in 2.5% HI serum, C3 fixation was abolished.

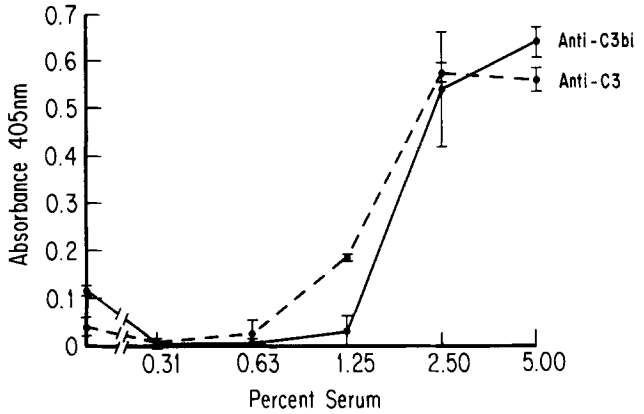

Figure 2. C3 fixation to PGL-1 is serum-dependent. PGL-1 (4.5 μg) was adhered to ELISA plate wells. The wells were blocked with 5% HSA and incubated with various amounts of fresh nonimmune serum as indicated for 30 min at 37°C. Fixed C3 in the wells was probed with either a polyclonal antibody against C3 (*dotted line*) or a mAb against C3bi (*solid line*) as the primary antibody and detected as in the previous figure. Data are the mean ± SD absorbance for duplicate wells in one representative experiment. The absorbance in wells devoid of antigen was subtracted out in each case.

We next examined the roles of the classical and alternative complement pathways in mediating C3 fixation to PGL-1. Compared with the level in fresh serum, C3 fixation was abolished both in serum containing EGTA and $MgCl_2$, a condition in which the classical pathway but not the alternative pathway is inhibited (18), and in serum containing EDTA, a condition in which both pathways are inhibited (Fig. 3). The amount of PGL-1 in ELISA plate wells, assayed with a primary mAb against PGL-1, was not affected by the presence of EDTA or EGTA-$MgCl_2$. These results indicate that C3 fixation to isolated PGL-1 takes place by the classical complement pathway.

C3 Fixation to PGL-1 Is Dependent Upon PGL-1's Terminal Trisaccharide and Long-chain Fatty Acids. PGL-1 consists of a phenolic phthiocerol core linked to a unique trisaccharide at one end (3-*O*-methylrhamnose, 2,3-di-*O*-methylrhamnose, and 3,6 di-*O*-methylglucose at the terminal position) and a mixture of long-chain mycocerosyl fatty acyl groups at the other (Fig. 4).

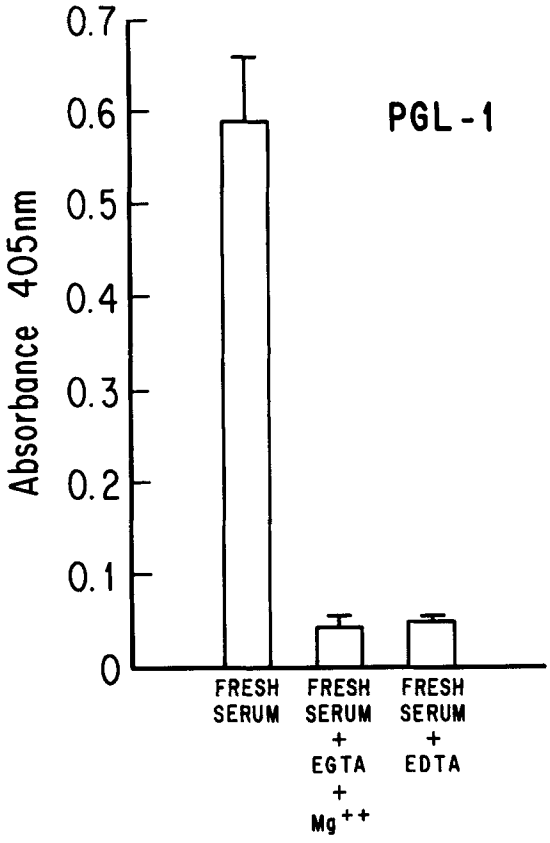

Figure 3. C3 fixation to PGL-1 is mediated by the classical pathway of complement activation. PGL-1 (4.5 μg) was adhered to ELISA plate wells and then incubated at 37°C for 30 min in 2.5% fresh serum, 2.5% fresh serum containing 10 mM EGTA and 7 mM $MgCl_2$, or 2.5% fresh serum containing 10 mM EDTA. C3 fixation was detected using a polyclonal antibody against C3. Data are the mean ± SD absorbance for duplicate wells in the one representative experiment.

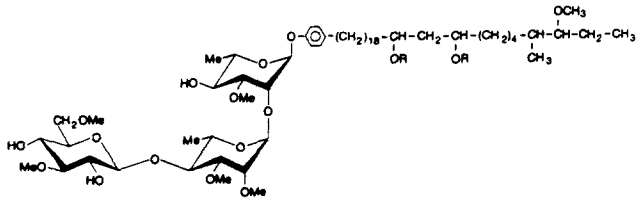

Figure 4. Structure of PGL-1. "R" represents a mixture of 2 of 3 long chain mycocerosyl fatty acyl groups. (Reproduced, with permission, from the *Annu. Rev. Microbiol.*; Vol. 44, 1987, by Annual Reviews Inc.).

C3 forms a covalent linkage to either hydroxyl or amino groups on appropriate acceptor molecules. As PGL-1 contains only hydroxyl groups, located on the carbohydrate moieties, we hypothesized that these hydroxyl groups were the acceptor sites for C3 on PGL-1 and that the carbohydrates were therefore required for C3 fixation. To test this hypothesis, we studied C3 fixation by PGL-1 molecules whose terminal sugars were, in essence, sequentially removed (Fig. 5). Intact PGL-1 fixed significant amounts of C3. In contrast, when the terminal glucose of PGL-1 was removed (monodeglycosylated PGL-1), C3 fixation was markedly reduced (57 ± 4% SE, n=2); control studies using a primary mAb against PGL-1 indicated that comparable amounts of PGL-1 and monodeglycosylated PGL-1 were bound to ELISA plate wells. When the 2 distal sugars were removed from a surrogate PGL-1 molecule, Mycoside B, whose core structure and mycocerosyl acyl groups are identical to PGL-1, C3 fixation was reduced further (67 ± 5%, SE, n=2). Finally, when all 3 terminal sugars were removed from PGL-1, C3 fixation to the remaining molecule, dimycocerosal pthiocerol (DIM) was reduced to the background level obtained with HI serum. Thus, the terminal trisaccharide of PGL-1 appears to play a critical role in C3 fixation.

In the ELISA, isolated PGL-1 was capable of both activating the complement cascade and fixing C3. We hypothesized that the long chain mycocerosyl acyl groups, the major lipid portion of the molecule, may be involved in C3 activation. To test this hypothesis, we studied C3 fixation to deacylated PGL-1; a molecule in which the long chain mycocerosyl acyl groups have been removed but the terminal trisaccharide remains intact (8). Deacylated PGL-1 fixed C3 poorly despite having all 3 sugars present (Fig. 5). That deacylated PGL-1 bound efficiently to ELISA plate wells was confirmed with a primary mAb directed against the terminal trisaccharide of PGL-1. This result indicates that the lipid portion of PGL-1 plays a major role in influencing complement activation.

We also evaluated the capacity of a synthetic glycolipoprotein, NT-O-BSA, to fix C3. This glycoprotein contains the native terminal trisaccharide of PGL-1 linked to BSA via a 8-methoxycarbonyloctyl chemical linker. As little as 0.5 μg of NT-O-BSA fixed significant C3. The mean optical density reading for C3 with 0.5 μg of NT-O-BSA was 0.42 ± 0.01 (SD,n=2), 2.5-fold the level obtained with 0.5 μg of BSA alone (0.17 ± 0.02). Thus, the combination of BSA and the chemical linker appears to provide a suitable environment for both complement activation and C3 deposition to the trisaccharide moiety of PGL-1.

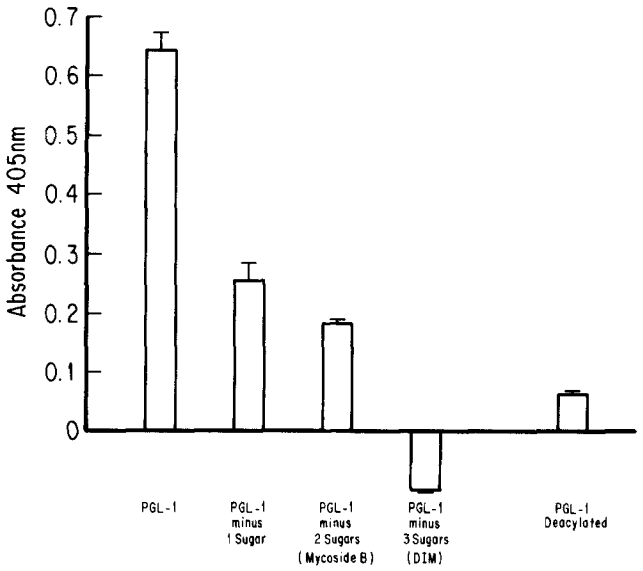

Figure 5. C3 fixation to PGL-1 requires the presence of its terminal trisaccharide and long chain fatty acids. Intact PGL-1, monodeglycosylated PGL-1 (PGL-1 lacking the terminal sugar of the trisaccharide, 3,6 di-O-methylglucose), Mycoside B (a surrogate PGL-1 molecule lacking the two terminal sugars of the trisaccharide), DIM (PGL-1 lacking its terminal trisaccharide), or deacylated PGL-1 (PGL-1 minus its mycocerosyl fatty acyl groups) was adhered to ELISA plate wells (4.5 μg of each molecule). The wells were blocked with 5% HSA in PBS and incubated at 37°C for 30 min in either 2.5% fresh serum or 2.5% HI serum as a control. C3 fixation was detected using a polyclonal antibody against C3. Data are the mean ± SD absorbance for duplicate wells in one representative experiment. Absorbance in control wells incubated in HI serum was subtracted out in each case.

PGL-1 and C3 Mediate Ingestion of Polystyrene Microspheres by Human Monocytes. To determine if C3 activation products bound to PGL-1 can mediate phagocytosis, we studied the influence of PGL-1 and C3 on phagocytosis of inert particles - polystyrene microspheres. We preincubated microspheres with HSA before incubating them in serum to inhibit "background" opsonization in nonimmune serum. This allowed us specifically to evaluate the role of PGL-1 in mediating C3 fixation to microspheres and adherence of opsonized microspheres to human monocytes.

To confirm that PGL-1 mediates binding of C3 on microspheres, we examined the microspheres for C3 by immunofluorescence microscopy. Whereas PGL-1 coated microspheres incubated in fresh serum bound readily detectable amounts of C3, PGL-1 coated microspheres incubated in buffer had no detectable C3.

Precoating microspheres with PGL-1 before incubating them in serum significantly increased their adherence to monocytes. Whereas, 34 ± 1% (mean ± SE, n=2) of monocytes adhered ≥1 PGL-1 coated microsphere, only 8 ± 1% adhered plain microspheres (Table 1). Even more striking, monocytes bound 10 ± 2-fold (mean ± SE, n=2) more PGL-1 coated microspheres than plain microspheres (Table 1). Thus, PGL-1

mediates adherence of polystyrene microspheres to human monocytes.

To determine if adherence of PGL-1 coated microspheres by monocytes requires C3 deposition onto their surface, we compared adherence of PGL-1 coated microspheres incubated in fresh serum, conditions in which C3 is deposited, with adherence of PGL-1 coated microspheres incubated in HI serum or no serum, conditions under which C3 is not deposited. Compared with the level after incubation in fresh serum, adherence to monocytes of microspheres incubated in HI serum or no serum was dramatically reduced (84 ± 6% reduction for HI serum and 83 ± 9% reduction for no serum, mean ± SE, n=2; Table 2). Thus, monocytes adhere PGL-1 coated microspheres only under conditions in which C3 is deposited.

To determine if PGL-1 and C3 mediate ingestion of polystyrene microspheres by monocytes, we studied uptake of the microspheres by electron microscopy. Monocytes avidly ingested PGL-1 coated microspheres that we preincubated in fresh serum (Fig. 6); 47% of monocyte cross-sections contained ≥1 microsphere in intracellular vacuoles (mean of 0.90 microspheres per cross-section). In contrast, monocytes ingested few PGL-1 coated microspheres that were preincubated in HI serum; only 6% of monocyte cross-sections contained ≥1 intracellular microsphere (mean of 0.07 microspheres per cross-section). Similarly, monocytes ingested few uncoated microspheres that were preincubated in fresh serum; only 10% of monocyte cross-sections contained ≥1 intracellular microspheres (mean of 0.12 microspheres per cross-section). Thus, PGL-1 and C3 mediate ingestion as well as attachment of polystyrene microspheres by human monocytes.

Table 1. *PGL-1 Mediates Adherence of Opsonized Microspheres to Human Monocytes*

Exp.	Microsphere treatment	Monocytes with ≥1 adherent microsphere	Microspheres/ monocyte	Fold increase in adherence*
		%	*mean ± SD*	
A	PGL-1	33	1.47 ± 0.26	8
	None (Control)	7	0.18 ± 0.18	
B	PGL-1	35	1.71 ± 0.47	11
	None (Control)	9	0.15 ± 0.09	

In two independent experiments, PGL-1 coated or plain microspheres were incubated in 2.5% fresh serum for 30 min at 37°C, washed, and incubated with monocytes for 1 h at 37°C. The monocytes were fixed and examined by phase contrast microscopy, and the percentage of monocytes with ≥1 adherent microsphere and the number of adherent microspheres per monocyte were enumerated. A minimum of 100 consecutive monocytes on triplicate coverslips was counted for each treatment condition.

* Fold increase in the mean number of microspheres/monocyte for PGL-1 coated microspheres compared with plain microspheres.

Table 2. *Adherence of PGL-1–Coated Microspheres to Human Monocytes Is Dependent upon Heat-labile Serum Components*

Exp.	Serum	Monocytes with ≥1 adherent microsphere	Microspheres/ monocyte	Decrease in adherence*
		%	*mean ± SD*	*%*
A	Fresh	64	5.04 ± 0.69	
	HI	15	0.50 ± 0.07	90
	None	15	0.42 ± 0.02	92
B	Fresh	63	4.20 ± 0.40	
	HI	26	0.91 ± 0.33	78
	None	36	1.10 ± 0.19	74

In two independent experiments, PGL coated microspheres were incubated in 2.5% fresh serum, 2.5% HI serum, or no serum for 30 min at 37°C, vigorously washed, and incubated with monocytes for 1 h at 37°C. The monocytes were fixed and examined by phase contrast microscopy, and monocyte-adherent microspheres enumerated. A minimum of 100 consecutive monocytes on triplicate coverslips was counted for each serum condition.

* Percent decrease in the mean number of microspheres/monocyte compared with the level in fresh serum.

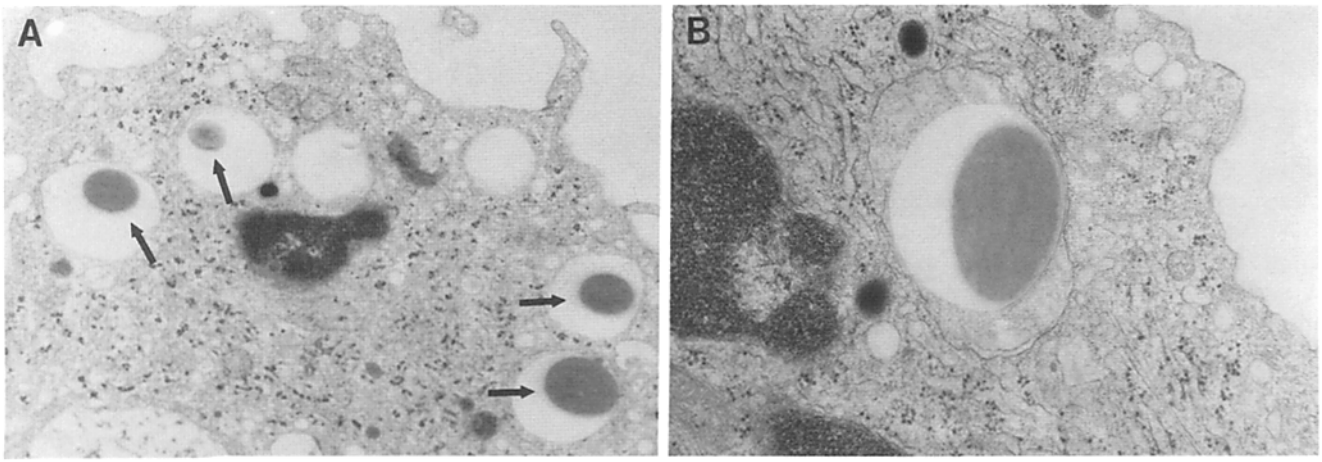

Figure 6. PGL-1 and C3 mediate ingestion of polystyrene microspheres by human monocytes. Monocytes in monolayer culture were incubated with polystyrene microspheres that had been precoated with PGL-1 and incubated in fresh serum as described in the text. The monocytes were then washed, fixed, and prepared for electron microscopy. (*A*) The monocyte has ingested at least 4 microspheres (*arrows*) (×9,700). The clear areas are artifacts due to contraction of the microspheres during processing. (*B*) At higher magnification, a representative microsphere is found in a membrane-bound vacuole (×36,000).

Discussion

Our studies have identified a three-component receptor-ligand-acceptor molecule system for mediating phagocytosis of *M. leprae*. This system consists of complement receptors (CR1 and CR3 on monocytes, and CR1, CR3, and CR4 on monocyte-derived macrophages), fragments of complement component C3, and PGL-1.

Complement receptors play a general role in mediating phagocytosis of a diverse group of intracellular pathogens. Ligation of complement receptors by C3 does not trigger an appreciable oxidative burst, and thus these receptors may provide these intracellular pathogens safe passage into host cells. In this regard, ingestion of *M. leprae* by mononuclear phagocytes is not accompanied by a significant oxidative burst (19). Interestingly, PGL-1, adhered diffusely to the extracellular bacterium *Staphylococcus aureus*, inhibited killing of this organism by macrophages in a previous study (20). While other attributes of PGL-1, such as a capacity to scavenge free radicals, may have contributed to its protective effect, it is also possible that the PGL-1 coat on *S. aureus* directed entry of the bacterium into macrophages via complement receptors and allowed the bacterium to escape the toxic consequences of the oxidative burst.

PGL-1 is found in abundance on *M. leprae* (9, 21, 22), and it is arrayed such that its terminal trisaccharide, the portion of the molecule available for covalent linkage to C3, is exposed at the outermost surface. Thus, PGL-1 is situated optimally for mediating C3 fixation and phagocytosis of *M. leprae*.

PGL-1, and not the other major carbohydrates of *M. leprae*, was found to deposit significant C3 on its surface. It is noteworthy that LAM did not fix C3 in our assay, as it is the major lipopolysaccharide of *M. leprae* and is also located on the outer surface of the bacterium. This finding is reminiscent of previous work demonstrating that the major outer membrane protein (MOMP) but not the LPS of *Legionella pneumophila* fixes C3 to this intracellular bacterium (23). PGL-1 and MOMP have in common that they are major constituents of the outer surface of these microbes.

Our study demonstrates that C3 fixation to PGL-1 is dependent upon PGL-1's terminal trisaccharide. Complement activation by lipids in liposome models is also enhanced by short chain saccharide moieties (24). Schultz et al. (25), studying the role of saccharide chain length in complement activation, determined that 3 saccharide moieties, but not 1, 2, or 4, were required for efficient C1 activation and C4 consumption. Thus, the capacity for PGL-1 to fix C3 may derive in part from the fact that its distal oligosaccharide is a trisaccharide.

Deacylated PGL-1 fixed C3 poorly in our ELISA. As the lipid portion of PGL-1 possesses no suitable acceptor region for covalent linkage to the reactive thiolester of C3, this portion of the molecule may serve to orient the trisaccharide on the ELISA plate (or on a lipid membrane) so as to maximize complement activation and C3 fixation. Along these lines, in this study, the trisaccharide of PGL-1 also fixed C3 when linked via a chemical carrier to BSA (NT-O-BSA). Schultz et al. studied complement activation by a different trisaccharide linked to BSA via the same chemical carrier (25). These authors showed that the trisaccharide activated the complement cascade when linked to BSA via the chemical carrier but not when linked directly to BSA. Thus, the chemical carrier may allow for proper orientation of the trisaccharide for complement activation.

Our studies demonstrate that optimal C3 fixation to PGL-1 requires both the terminal trisaccharide and the mycocerosyl fatty acyl side chains. That different portions of a molecule influence complement activation has been extensively studied with LPS molecules from gram-negative bacteria (26, 27). These studies have demonstrated that complement activation is dependent upon both the carbohydrate portion of the mol-

ecule (O-antigens) and Lipid A. Moreover, the length of the O-antigens influences the pathway of complement activation and can modify the complement activity of Lipid A.

Our studies demonstrate that activation of C3 in nonimmune serum by isolated PGL-1 occurs by the classical complement pathway. In ongoing experiments, we have found that, at low serum concentrations, comparable to those used in this study, a substantial proportion of C3 fixation to whole *M. leprae* occurs by the classical pathway. Consistent with this, these studies indicate that Clq binds avidly to *M. leprae* and PGL-1. Interestingly, these studies also indicate that C3 fixation and Clq binding to *M. leprae* and PGL-1 are triggered by extremely small amounts of cross-reactive (natural) antibody (either IgM or IgG) in nonimmune serum. Importantly, although these studies have determined that relatively more antibody in nonimmune serum binds to LAM, LM, and arabinogalactan than to PGL-1, only PGL-1 fixes appreciable amounts of C3, underscoring the selectivity of C3 fixation to PGL-1.

Understanding the molecular basis for phagocytosis of intracellular parasites may not only yield new insights into the pathogenesis of intracellular parasitism but also point to new strategies for interfering with the life cycle of these organisms. This in turn may lead to the development of new modalities for preventing and treating diseases caused by this important group of pathogens.

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