Effect of Mycobacterial Phospholipids on Interaction of Mycobacterium tuberculosis with Macrophages

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This study demonstrates that pretreatment of macrophages with phosphatidylinositol, of either soya bean or mycobacterial origin, results in a down-regulation of the binding and uptake of Mycobacterium tuberculosis by the phagocytes. We also describe the novel observation that cardiolipin induces an increase in the binding and uptake of M. tuberculosis by macrophages. Neither phospholipid interacts with macrophages via the 2F8 epitope of scavenger receptor A, and treatment of macrophages with either phospholipid results in a down-regulation of CR3 function and tumor necrosis factor alpha production by the phagocyte. We have also shown that the ability of macrophages to interact with mycobacteria is greatly affected by an as yet unidentified product from the interaction of chloroform and polypropylene tubes.

Mycobacterium tuberculosis is a major human pathogen that resides in the host lung as a facultative intracellular pathogen and is found primarily in mononuclear phagocytic cells. Interactions between M. tuberculosis and host macrophages (Mφ) will therefore be of paramount importance in defining the pathogenesis of the bacterium. Since bronchoalveolar fluid is considered to contain insufficient amounts of serum opsonins to mediate phagocytosis (34, 38) and alveolar macrophages (Mφ) do not express significant levels of receptors for serum opsonins (2, 7, 10, 33, 48), serum-independent (nonopsonic) ingestion by Mφ is considered to be essential in the host defense of the lung. We have shown previously that nonopsonic binding of M. tuberculosis to mouse Mφ is partly mediated via an epitope within CR3 (CD11b/CD18, Mac-1) that is distinct from that which binds iC3b (46).

In contrast to our improved understanding of the receptors involved in the nonopsonic uptake of mycobacteria, little is known of the mycobacterial molecules involved in nonopsonic interactions with Mφ. The mycobacterial lipopolysaccharides (LAM), lipomannan (LM), and phosphatidylinositol mannosides (PIMs) are abundant molecules in the cell envelope of mycobacteria and have been proposed as having a role in the receptor-mediated uptake of mycobacteria (28, 42), even though it is uncertain whether these molecules are exposed at the surface of the bacteria and can therefore act as ligands. However, these molecules are thought to be released into the extracellular environment (15, 49), where they may act as such ligands. However, these molecules are thought to be released into the extracellular environment (15, 49), where they may have numerous effects on the host’s immune system (15), including the inhibition of mycobacterial uptake by Mφ (47). LAM is a large molecule with extensively branched arabinan and mannan chains, PIMs refer to molecules with 2 to 6 mannose residues, while LM is essentially a long PIM, with about 20 mannose residues, and may be regarded as a precursor of LAM, lacking the branched arabinan (9, 12, 15). All of these molecules possess a common phosphatidylinositol (PI) anchor which could be inserted in the plasma membrane (15, 29) or an outer lipid bilayer shown to exist in mycobacteria (32). A plasma membrane location would allow the glycosylated portion of LAM and LM, but not the shorter PIMs, to be exposed on the outer surface of the envelope. However, the observation that PIMs can be released preferentially from the mycobacterial surface by gentle mechanical treatments indicates that such molecules may be located on the outside of the envelope and thus exposed at the surface of the mycobacterial cell (36). Although LAM, LM, and PIM have widely different glycosylation patterns, all three molecules have been found to inhibit binding of M. tuberculosis to Mφ when added in a cell-free form (47). This, along with the observation that decylation of LAM abrogates the capacity for LAM to down-regulate binding of M. tuberculosis to Mφ (47), indicates that the common PI anchor is the inhibitory component. In support of this contention, commercially prepared PI from soybean was shown to inhibit binding of M. tuberculosis to Mφ when added in a dose-dependent manner (47). However, the fatty acyl groups of soya PI are palmitoyl and linoleoyl, whereas those of mycobacterial PI are palmitoyl and tuberculostearoyl. Thus, it is possible that PI from mycobacteria would not have the same inhibitory property that soya PI has. The purpose of the present study was, therefore, first to investigate whether mycobacterial PI acts on Mφ in a similar fashion as does soya PI and second to further elucidate the role of mycobacterial lipidated moieties in the interaction of mycobacteria with Mφ.

MATERIALS AND METHODS

Mycobacteria. Mycobacterium tuberculosis strain Erdman (Trudeau Mycobacterial Collection 107, American Type Culture Collection [Manassas, Va.] 35801) was grown and stored as previously described (46). Mycobacterium smegmatis 607 was grown in Sauton’s medium in a fermentor.

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Commercial Phospholipids. Diphosphatidyl glycerol (cardiolipin; CL) and soya PI were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada).

Preparation of phospholipids of mycobacterial origin. *M. smegmatis* 607 was grown on sheep blood with 0.05% Tween 80 and distilled water to give a pellet of 550 g (wet weight). Phospholipids were prepared as acetone-ethanol-insoluble material (3 g) and further purified by chromatography on a DEAE-cellulose DE52 (acetate form) column (3). In a scaled-up version of the chromatography method, the DE52 (Whatman, Clifton, N.J.) was packed as a 4-cm-diameter by 32-cm (400-ml) column. The void volume, determined with azulene (Aldrich, Gillingham, Dorset, United Kingdom), was 417 ml. Thus, the 3 g of glycerolipids was applied to the column (50 ml of elution buffer; 100 ml of elution buffer per 209/1 by volume). First, 600 ml of elution solvent was applied, then a 0 to 0.1 M ammonium gradient in elution solvent was applied over 1.5 liter, and then the remaining phospholipids were eluted in 0.1 M ammonium acetate in elution solvent. Fractions (30 ml) were monitored for their lipid content by thin-layer chromatography (TLC). The material that eluted at 750 to 900 ml (0.025 to 0.05 M ammonium acetate) from the DE52 column contained all of the PI. However, it also included some PIM and CL about equal in quantity to PI. This single batch of material was used throughout this study and is referred to as pool 2.

Purification of PI by TLC fractionation of pool 2. Up to 12 mg of pool 2 was applied as a 15-cm streak to a 20- by 20-cm silica gel 60 TLC plate (Merck, Darmstadt, Germany), placed in a nitrogen-filled tank, and developed twice in chloroform-methanol-water (65/25/4 by volume). The plate was air dried and then sprayed with MilliQ water to visualize opalescent bands of lipid. The band corresponding with PI was scraped, recovered in chloroform-methanol (2:1 by volume), filtered through a 4-mm PTFE filter (Whatman) high-pressure liquid chromatography (HPLC) filter, and partitioned into the upper phase of a butan-1-ol-water-two-phase system (1 ml) in which any residual, colloidal silica collected on the interface. About 0.6 mg of pure (purity and quantity estimated on high-performance TLC [HPTLC] plates [Merck 13727]) was obtained from 12 mg of pool 2. Plate controls were obtained by scraping an area of the plate developed with solvent but containing no lipid.

Two directional TLC (2D-TLC; see below) was not suitable for monitoring preparative chromatography; for this purpose, fractions were applied to the concentration zone of 10- by 10-cm silica gel HPTLC plates and developed once in chloroform-methanol-water (65/25/4 by volume). Plates were air dried, then sprayed with 5% (wt/vol) molybdophosphoric acid in ethanol-water (95/5 by volume), and charred at 200°C for up to 10 min, until deep blue spots representing all lipids appeared.

Purification of PI by HPLC fractionation of pool 2. Normal-phase HPLC of pool 2 was performed using a Partisil 5 WCS (Whatman) 4.6- by 250-ml column. Quantities injected were 2.4 mg in 50 ml, followed by isocratic elution at 0.4 ml/min with chloroform-methanol-water-glacial acetic acid (65/25/4/0.4 by volume). Fractions (0.25 ml) were collected, and 25-ml portions were analyzed by HPTLC (UV absorption was not a reliable way of monitoring eluant). PI eluted between 12.5 and 16 min, but from 14.5 min PIMs were also eluted. The 12.5- to 16-min concentration zone of 10- by 10-cm silica gel HPTLC plates and developed once in chloroform-methanol-water (65/25/4 by volume). The plate was air dried and then sprayed with 5% (wt/vol) molybdophosphoric acid in ethanol-water (95/5 by volume), and charred at 200°C for up to 10 min, until deep blue spots representing all lipids appeared.

Preparation of PI by HPLC and negatively charged phospholipids. Phospholipids were prepared for 2D-TLC by resuspension in 2:1 chloroform-methanol (Omnisolve, Ems Science, Gibbstown, N.J.). Approximately 40 μg of material was spotted onto the lower left corner of a 6.6-cm-square aluminum-backed TLC plate (Merck), 1 cm in from both edges. The plate was set at a 90° angle in chloroform-methanol-distilled water (dH2O) (60:30:6) for the first direction. When the solvent front reached the top of the plate, it was air dried, rotated another 90°, and placed in chloroform-acetic acid-methanol-dH2O (80:15:12:4) for the second direction. The plate was then air dried, sprayed with 5% molybdophosphoric acid in 70% ethanol, and air dried at 200°C for 10 min.

Preparation of lipids for Mø studies. Initially, experiments were done using phospholipids that had been resuspended in chloroform and stored long-term in propylene microcentrifuge tubes (Sarstedt, Numbrecht, Germany) at −20°C. On the day of the experiment, these phospholipids were dried under N2, resuspended in binding medium (138 mM NaCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KC1, 0.6 mM CaCl2, 1 mM MgCl2, 5.5 mM D-glucose [45]), and dispersed by horn sonication for 90 s, using a micro-cup horn and Vibra-cell ultrasonic processor (Sonics & Materials, Danbury, Conn.). A procedure was developed for the preparation of commercially prepared lipids that would simulate the preparation of the purified mycobacterial phospholipids described above. All purchased phospholipids were first prepared in 2:1 (vol/vol) chloroform-methanol (Omnisolve) and dried under N2, resuspended in 1:1 (vol/vol) ether (ACS grade; Fisher Scientific, Nepean, Ontario, Canada)-ethanol (LC grade; Sigma-Aldrich, St. Louis, Mo.), aliquoted into glass vials with Tecoflipid fish (Fisher Scientific), dried again under N2, and stored at −20°C. For preparation of solvent controls, equivalent amounts of solvents with no phospholipids were dried in glass vials. On the day of the experiment, phospholipids and control vials were reconstituted with binding medium and horn sonicated for 90 s as described above. Microscopic observation of these lipid preparations revealed that both phospholipids were insoluble and formed a mixture of small (0.5- to 2-μm) and large (2- to 6-μm) lamellar vesicles.

Interactions with Macrophages. Fractions (0.25 ml) were collected, and 25-μl portions were analyzed by HPLC.

PI, Mø, and PI/Mø interactions. In all experiments, Møs were incubated for 10 min at 37°C in 5% CO2 (control cells received binding medium alone). Mycobacteria were pelleted and resuspended in binding medium by passage through a 25-gauge needle 10 times to break up clumps, and 250 μl of the suspension was added to each well to give a multiplicity of infection of approximately 50 bacteria:1 Mø. PI was added reagent. In all groups some aggregates formed, but these did not appear to be ingested by Møs. We have previously investigated whether pretreatment of Møs with PI preparations affects the ability of the Mø to interact with M. tuberculosis. In these experiments, Møs were incubated with 40 μg of soya PI per ml overnight, washed, and tested for the ability to associate with M. tuberculosis as described here. The association of the mycobacteria with the pretreated Møs was inhibited, but only at 50% of the levels of inhibition seen when PI was present during the interaction of Møs and mycobacteria (unpublished observations). This led us to conduct the current series of experiments with the phospholipids present during the assay.

Following incubation, the monolayers were washed gently three times with binding medium and then fixed and stained. The distribution of acid-fast bacilli, latex, or ELgMC within the Mø population was estimated as previously described (46). Although in this study we made no attempt to diferentiate attachment from ingestion, previous studies from our laboratory, in which the association of M. tuberculosis with Møs was assessed after 3 h of incubation at either 37°C (attatched and ingested) or 4°C (attached only) in 5% CO2, demonstrated that >90% of the bacteria associated with Møs at 3 h were ingested (unpublished observations).

To test the effect of solvents on Møs, 100 μl of chloroform or methanol was added to polypropylene microcentrifuge tubes, which were then dried under N2. After addition of 1 ml of binding medium to each tube, the tubes were horn sonicated for 90 s. Either 250 μl of binding medium from these solvent-treated tubes (plus 1 μl of binding medium containing 5% PI added) was added to each well. The Møs were incubated for 10 min in 37°C in 5% CO2 before addition of bacteria. To test whether CL or PI acted on Mø via scavenger receptor A, (SR-A), Møs were treated with PI (40 μg/ml) or CL (80 μg/ml) alone or preceded by a 10-min incubation with 2F8 (20 μg/ml), a monoclonal antibody (MAb) that recognizes SR-A (25). The Møs were incubated for a further 10 min in 37°C in 5% CO2 before addition of bacteria. To test whether CL or PI acted on Mø via scavenger receptor A, (SR-A), Møs were treated with PI (40 μg/ml) or CL (80 μg/ml) alone or preceded by a 10-min incubation with 2F8 (20 μg/ml), a monoclonal antibody (MAb) that recognizes SR-A (25). The Møs were incubated for a further 10 min in 37°C in 5% CO2 before addition of bacteria.
factor alpha (TNF-α) content of each sample was measured using an enzyme-linked immunosorbent assay (ELISA)-based kit (R&D Systems, Minneapolis, Minn.) as instructed by the manufacturer. Control experiments demonstrated that CL and PI did not interfere with the detection of TNF-α in this assay. Mφ were incubated with M. tuberculosis for 3 h and the supernatants were collected and filter sterilized. Then CL or PI in 10 μl of binding medium was added to replicate samples to give a final concentration of 60 or 40 μg/ml, respectively. Untreated supernatants received 10 μl of binding medium alone. The TNF-α in these samples was then assessed using the ELISA kit. The TNF-α present in CL- and PI-treated samples did not differ significantly from that in the untreated controls (P = 0.4 and 0.6, respectively).

Statistical analysis. Data are expressed as means ± standard errors of the means (SEM). When applicable, Student’s t test for independent means was used to evaluate binding data. Differences were considered significant at P < 0.05.

RESULTS

Effect of phospholipid storage protocols on their subsequent effect on the binding of M. tuberculosis to Mφ. Soya PI and TLC-purified mycobacterial PI that had been stored in chloroform in polypropylene tubes were added to Mφ at 40 μg/ml to assess their effect on binding of M. tuberculosis (Fig. 1A). Both phospholipids inhibited binding significantly (P < 0.05) compared to the control, for both percentage of Mφ binding one or more bacteria and percentage of Mφ binding more than five bacteria. However, the plate control also inhibited significantly (P < 0.05) compared to the control. Since this was an unexpected result, we suspected the integrity of the materials used and therefore investigated our storage methods, in particular, the use of chloroform and methanol with polypropylene tubes.

To test if chloroform or methanol alone could account for the inhibition, they were added directly to Mφ at a final concentration of 1% to assess the effect of possible residual contamination in lipid preparations (Fig. 1B). There was no inhibition of binding of M. tuberculosis to Mφ in the presence of either solvent. When binding medium that had been horn sonicated in solvent-treated polypropylene tubes was added to Mφ, there was no inhibition mediated by medium from methanol-treated tubes, but there was significant inhibition mediated by medium from chloroform-treated tubes (P < 0.05 compared to the control). This indicated the presence of an unknown contaminant resulting from the interaction of chloroform and polypropylene.

From this information we developed a storage system whereby all phospholipids, including commercial material, were reconstituted, dried under N2, and stored in glass vials with Teflon-lined lids as described in Materials and Methods.

Effect of CL and PI on binding of Mφ to M. tuberculosis. A subsequent source of mycobacterial PI, pool 2, was prepared and stored according to our new methods. TLC analysis of pool 2 (Fig. 2) indicated the presence of CL and PIMs as well as PI.

Solvent controls prepared using procedures identical to those used for the phospholipids had no effect on Mφ binding of M. tuberculosis compared to controls with binding medium alone (data not shown), and so we were confident that data obtained with phospholipids stored using the new methods were meaningful.

We tested the effect of pool 2 on the interaction of M. tuberculosis with Mφ (Fig. 3A). Pool 2 contained approximately 60% CL and PIMs along with 40% PI. As previous studies (47) had shown that 40 μg of soya PI per ml resulted in strong inhibition of the association of M. tuberculosis with Mφ, pool 2 was added at a concentration of 100 μg/ml to give 40 μg of mycobacterial PI per ml. Therefore, soya bean PI was added at 40 μg/ml and CL was added at 60 μg/ml for comparison. Pool 2 significantly inhibited the interaction of M. tuberculosis with Mφ compared to controls (P < 0.05), though not to the same extent as did soya PI which, when added to Mφ at 40 μg/ml, was inhibitory compared with both the control and pool 2 (P < 0.05). Pure CL enhanced the binding of M. tuberculosis to Mφ, with more than twice the number of Mφ binding >5 bacteria compared to either the control or pool 2 (P < 0.05). We had determined that both CL and PI form lamellar vesicles in aqueous media, which indicated that differences in the physical presentation of the lipids to Mφ could not explain the

![FIG. 1. Effect of phospholipid preparation and solvents on the interaction of M. tuberculosis with Mφ. (A) Effect of soya PI and mycobacterial (myco)PI at 40 μg/ml on binding of M. tuberculosis to Mφ compared to binding medium alone. Plate Control refers to material from blank plate scrapings that had been processed identically to mycobacterial PI. The percentage of Mφ binding ≥1 and >5 M. tuberculosis is shown. The mean ± SEM is shown for four experiments, each with triplicate coverslips. *, P < 0.05 compared with control containing binding medium only. (B) The binding of M. tuberculosis to Mφ in the presence of 1% chloroform (CHCl3 added) or 1% methanol (MeOH added) compared to that in the presence of binding medium sonicated in polypropylene tubes in which 100 μl of either chloroform (CHCl3 evaporated) or methanol (MeOH evaporated) had been added and then evaporated under N2. Control wells contained binding medium only. The percentage of the Mφ population binding ≥1 and >5 bacteria is shown. The mean ± SEM is shown for one experiment done in triplicate. *, P < 0.05 compared with control.](http://iai.asm.org/ on October 14, 2017 by guest)
different effects of CL and PI on the interaction of M. tuberculosis and Mφ.

The observation that pool 2 did not inhibit the interaction of M. tuberculosis with Mφ as much as did PI alone suggested that two opposing effects may be present in pool 2: a stimulatory effect of the CL portion and an inhibitory effect of the PI portion. We tested this by adding CL and PI sequentially to effect of the CL portion and an inhibitory effect of the PI two opposing effects may be present in pool 2: a stimulatory pool 2 also contained PIM and other lipids (Fig. 2), which may result did not explain the inhibitory effect of pool 2. However, pool 2 also contained PIM and other lipids (Fig. 2), which may have contributed to its overall inhibitory action.

**Effect of PI from different sources.** A subsequent source of PI purified from pool 2 by HPLC (containing no CL) was added to Mφ at 40 μg/ml (Fig. 3C) and compared with purified PIM, also from pool 2, and with soya PI, both at 40 μg/ml. All three phospholipids inhibited the association of M. tuberculosis with Mφ. The mycobacterial PI and soya PI (P = 0.042 and 0.00007, respectively), but not the PIM (P = 0.058), inhibited binding of M. tuberculosis to Mφ (P < 0.05), whereas CL significantly enhanced binding of bacteria to Mφ (P < 0.05). The effect of CL was dominant over that of PI, as the addition of CL, either before or after PI, always resulted in enhanced binding. This result did not explain the inhibitory effect of pool 2. However, pool 2 also contained PIM and other lipids (Fig. 2), which may have contributed to its overall inhibitory action.

**Investigations into the mode of action of CL and PI on Mφ.** SR-A has been implicated in binding a wide range of lipids, including lipoteichoic acid of gram-positive bacteria (20) and lipopolysaccharide (LPS) from gram-negative bacteria (26). We considered the possibility that either PI or CL was acting via binding to SR-A. A MAb (2F8) recognizing SR-A had no effect on the binding of M. tuberculosis to Mφ when added alone (Fig. 4) and did not affect the modulation of M. tuberculosis binding to Mφ mediated by PI or CL (Fig. 4), demonstrating that neither PI or CL acts via binding to the 2F8 epitope of SR-A.

The binding of mycobacteria and mycobacterial lipoglycans to Mφ is known to stimulate TNF-α production (11, 16, 23, 35). TNF-α is also known to modulate the expression and function of CR3 (17, 31), which is a major receptor involved in the binding of M. tuberculosis (18, 41, 46). Thus, we investigated the possibility that CL or PI affected Mφ TNF-α production, thereby affecting the association of Mφ with M. tuberculosis (Fig. 5). As expected, M. tuberculosis binding induced TNF-α production. However, pretreatment of the Mφ with either CL or PI inhibited the subsequent TNF-α production in response to the bacteria, suggesting that the different effects of CL and PI on M. tuberculosis binding and uptake by Mφ could not be attributed to different effects on TNF-α production.

We next investigated the effect of CL or PI on the association of latex beads or ElgMC′ with Mφ. Latex beads are comparable in size to mycobacteria and were used to identify effects on particle uptake. ElgMC′ are used to identify CR3 function. This is a major receptor for M. tuberculosis, even in the absence of complement (18, 46), and its function has been shown to be affected by lipoglycans (47). The association of latex with Mφ was unaffected by PI (Table 1), whereas CL treatment resulted in a significant reduction in both the percentage of Mφ associated with latex and the number of beads associated with each Mφ (Table 1). Neither PI nor CL had any notable effect on the percentage of Mφ able to bind at least one ElgMC′ (Fig. 6A). However, both PI and CL treatment reduced the number of ElgMC′ binding to the Mφ by about one-third (Fig. 6B).

**DISCUSSION**

The results of this study demonstrate that mycobacterial PI, like soya PI, inhibits the binding and uptake of M. tuberculosis by Mφ. We also describe, for the first time, how CL can increase the binding and uptake of M. tuberculosis by Mφ. In addition, we show that the association of Mφ with mycobacteria (and probably other particles) is greatly affected by an as yet unidentified product from the interaction of chloroform and polypropylene.

When we initially tested phospholipids that had been stored in chloroform, it was clear that there was a component present in both our phospholipids and our plate controls that inhibited...
binding. Since we saw evidence of the inhibitor only following the interaction of chloroform with polypropylene, it may be derived from the plastic, possibly a plastene. However, we did not pursue its identity and thus cannot rule out a volatile contaminant such as phosgene, an extremely toxic breakdown product of chloroform which condenses at 0°C and is soluble in most hydrocarbons, though its volatility suggests that it would dissipate from the phospholipids. A further caution is that contaminants such as those mentioned above might not appear on a TLC plate, commonly used as proof of purity of phospholipids.

Regardless of what the unknown component was, there are likely many areas of scientific investigation where the effect of using lipids stored in this manner would not be apparent. Mφ, in particular, are extremely sensitive to small amounts of potential stimulators or inhibitors of cell-signaling systems, and care should be taken to ensure purity of reagents used in all Mφ handling. We show in this work that lipids to be used in experiments with Mφ, should be dried under N₂ immediately upon reconstitution with solvents, glass vials with Teflon-lined caps.

**FIG. 3.** Effect of phospholipids on the binding and uptake of *M. tuberculosis* by Mφ. (A) Pool 2 added to Mφ at a final concentration of 100 μg/ml was compared with CL at 60 μg/ml and soya PI at 40 μg/ml. Control wells contained binding medium only (Control). The percentage of the Mφ population binding ≥1 and >5 bacteria is shown. The mean ± SEM is shown for five experiments, each with triplicate coverslips. *, *P < 0.05 compared with control; **, *P < 0.05 compared with control or pool 2. (B) PI and CL were added to Mφ at 40 μg/ml (PI) or 60 μg/ml (CL). In other groups, Mφ received PI first and then 10 min later received CL (PI + CL) or received CL first followed by PI (CL + PI). Control wells contained binding medium only (Control). All groups were then assessed for binding of *M. tuberculosis*. The percentage of the Mφ population binding ≥1 and >5 bacteria is shown. The mean ± SEM is shown for two experiments, each with triplicate coverslips. *, *P < 0.05 compared with control; **, *P < 0.05 compared with control or pool 2. (C) PI from *M. smegmatis* (mycobacterial [myco] PI) or soya PI and PIM were added at 40 μg/ml to Mφ, which were then assessed for binding of *M. tuberculosis*. Control wells contained binding medium only (Control). The percentages of the Mφ population binding ≥1 and >5 bacteria are shown. The mean ± SEM is shown for three experiments, each with triplicate coverslips. *, *P < 0.05 compared with control.

**FIG. 4.** Effect of an antibody recognizing SR-A on the binding of *M. tuberculosis* to Mφ treated with PI or CL. PI and CL were added to Mφ at 40 μg/ml (PI) or 60 μg/ml (CL). In addition, the anti-SR-A MAb 2F8 was added to Mφ at 20 μg/ml either alone (a-SR) or 10 min before the addition of PI (a-SR + PI) or CL (a-SR + CL). Control wells contained binding medium only (Control). Following a further 10-min incubation, all Mφ were assessed for the ability to bind *M. tuberculosis*. The percentages of the Mφ population binding ≥1 and >5 bacteria are shown. The mean ± SEM is shown for three experiments, each with triplicate coverslips. *, *P < 0.05 compared with control; **, *P < 0.05 compared with PI; **, *P < 0.05 compared with CL.

**FIG. 5.** TNF-α production by Mφ in response to *M. tuberculosis* in the absence (TB) and presence of PI (40 μg/ml; + PI) or CL (60 μg/ml; + CL), measured by ELISA and quantitated by reference to a standard curve. Background levels of TNF-α production by unstimulated cells is between 35 and 100 pg/ml. The mean ± SEM is shown for two experiments, each with triplicate wells. *P < 0.05 compared with TB group.
TABLE 1. Effect of CL and PI on the association of latex beads with Mφ*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Mφ with ≥1 latex bead</th>
<th>Mean no. of latex beads/Mφ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>97.2 ± 1.1</td>
<td>18.0 ± 1.4</td>
</tr>
<tr>
<td>CL</td>
<td>88.3 ± 3.6*</td>
<td>6.0 ± 0.1**</td>
</tr>
<tr>
<td>PI</td>
<td>99.0 ± 0.6</td>
<td>14.6 ± 0.7</td>
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* Mφ were incubated with 0.8-µm diameter latex beads in the presence of binding medium alone (control), CL (60 µg/ml), or PI (40 µg/ml). After 3 h, the monolayers were processed and the values shown (means ± SEM from two experiments, each with triplicate coverslips) were determined. * P < 0.05. ** P < 0.001.

We here demonstrate that mycobacterial PI is able to inhibit the association of M. tuberculosis with Mφ as had been previously shown for soya PI (47). However, it appears that mycobacterial PI is not as potent an inhibitor as is soya PI, probably due to the different acyl groups in the two molecules; both have palmitoyl but mycobacteria have tuberculostearoyl instead of linoleoyl moieties. This finding confirms the importance of PI as a potential regulator of Mφ interactions with mycobacteria and probably explains much of the activity of mycobacterial lipoglycans. LAM and, to a lesser extent LM and PIM have been implicated as having numerous biological activities, which has led to the suggestion that LAM is a mycobacterial virulence factor (1, 4, 13, 14, 16, 19, 35, 39, 43). Whenever it has been tested, the majority of studies have shown that deacetylation of LAM destroys its biological activity (14, 16, 35, 43, 44, 47), suggesting that the PI end of the molecule is essential for its activity. Using PI as a paradigm for PI and PI-based lipoglycans, our demonstration of the differential effects dependent on the acyl groups of the PI provides further evidence for the importance of the hydrophobic termini of these molecules in their interaction with Mφ. Nevertheless, it should be remembered that when the acyl groups are intact, the biological activity of LAM is affected by the nature of the glycosylation of the branched carbohydrate portion of the molecule (13, 16, 35).

The mode of action of cell-free PI upon Mφ-mycobacterium interactions is unknown. It is tempting to think that the action is simple competitive inhibition for a receptor recognizing PI. The current model for the mycobacterial cell wall places the PI component of LAM as anchored in the plasma membrane or an outer lipid bilayer, where it would not be available to bind to receptors for PI. However, the precise location of PIMs is still uncertain. They could be buried under a glycan matrix or exposed at the surface (21), but PIMs, like LAM, are likely to escape from the mycobacterial envelope; indeed, their recognition by antibodies and presentation by CD1 indicates that the host immune system sees all of these molecules (22, 27, 44). As free phospholipids and lipophosphoglycans, they would be able to interact with Mφ.

While the pretreatment of Mφ with PI results in a 50% inhibition of the subsequent interaction of the Mφ with M. tuberculosis, maximal inhibition occurs only when the PI is present during the interaction of Mφ with mycobacteria. This suggests that at least two mechanisms of inhibition are acting: one long-term effect on the Mφ, and one more transient effect on the Mφ or on the mycobacteria. The fact that PI inhibits the interaction of Mφ with mycobacteria, ElgMC', ElgG, and zymosan but not latex (reference 47 and this report) suggests an effect on several receptors but not a general inhibition of Mφ phagocytic function.

Cell-free LAM can bind to the Mφ mannose receptor (42) or CD14 (8, 37, 52), presumably by virtue of the glycosylated portion of the molecule. However, LAM can also integrate via its PI anchor into specialized plasma membrane domains (30). We also considered the possibility that SR-A could bind PI but did not obtain evidence to support this theory. Thus, while free PI would not be expected to bind to either the mannose receptor or CD14, current evidence suggests that it would integrate into the plasma membrane. How the PI then acts on the Mφ to inhibit the binding and uptake of M. tuberculosis and...
other particles remains unclear. Studies by Bate et al. (5, 6) indicate that the PI component of exoantigens from *Plasmodium yoelii*, a malarial parasite, is necessary for induction of TNF-α by murine peritoneal Mφ. As TNF-α can induce CR3 expression (17, 24), which in turn is important for binding and uptake of mycobacteria in a serum-free environment (18, 46), we investigated the activity of PI on TNF-α production and CR3 function and found it to be inhibitory of both parameters. Thus, we have evidence suggesting that PI may inhibit the interaction of Mφ with mycobacteria by inhibiting CR3 function, either independently or through the inhibition of TNF-α production.

The treatment of Mφ with CL resulted in a significant increase in the binding and uptake of *M. tuberculosis*. This novel finding was unexpected and explained why pool 2 did not inhibit mycobacterium-Mφ interactions as strongly as did PI, as this mixture of mycobacterial lipids contained the inhibitory molecules PI and PIM and the stimulatory molecule CL. CL, also known as diphosphatidyl glycerol, is commonly found in microorganisms, and current literature reports many and diverse effects of this negatively charged phospholipid, especially in patients with antiphospholipid syndrome. However, its effect on Mφ is less well studied. We found that CL did not bind to Mφ via the 2F8 epitope of SR-A; perhaps, like PI, it integrates on Mφ verse effects of this negatively charged phospholipid, especially microorganisms, and current literature reports many and diverse effects of this negatively charged phospholipid, especially in patients with antiphospholipid syndrome. However, its effect on Mφ is less well studied. We found that CL did not bind to Mφ via the 2F8 epitope of SR-A; perhaps, like PI, it integrates with the plasma membrane. CL has been shown to induce Mφ growth in vitro (50) and, at low doses, to augment TNF-α production by Mφ stimulated with LPS (51). More pertinent to our study, pretreatment with CL at high doses (40 μg/ml or more) has been shown to inhibit TNF-α production by Mφ stimulated with LPS (40, 51). Our observations are comparable in that treatment with CL inhibited the induction of TNF-α production by Mφ stimulated with *M. tuberculosis*. We also found that CL inhibited CR3 function. These two observations do not concur with our contention that decreased CR3 function will lead to decreased uptake of *M. tuberculosis* (as seen for PI). However, CR3 is a promiscuous receptor with more than one binding site, such that it is possible to inhibit the nonopsonic binding of *M. tuberculosis* with a MAb recognizing an epitope distal from the iC3b binding site, whereas a MAb recognizing the iC3b site has little effect (46). In addition, treatment of Mφ with phorbol myristate acetate will increase binding of ELgMC but not *M. tuberculosis* to CR3 (46), again demonstrating the diverse activity of CR3. It is therefore possible that CL, while inhibiting the iC3b binding activity of CR3, enhances the *M. tuberculosis* binding epitope. It is equally possible that CL acts to inhibit CR3 and to enhance another, unidentified receptor that mediates the uptake of mycobacteria. These possibilities await further investigation.

It was unlikely that either PI or CL had a global affect on the ability of Mφ to associate with particles, as PI inhibits the interaction of Mφ with *M. tuberculosis*, ELgMC, ELgG, and zymosan but not latex particles, whereas CL inhibits Mφ interactions with latex and ELgMC but enhances the uptake of *M. tuberculosis*. Both phospholipids appear to act separately on specific receptor-ligand interactions by acting on either the Mφ or the particle.

In conclusion, we have demonstrated that the two phospholipids PI and CL have significant effects on the binding and uptake of *M. tuberculosis*; PI is inhibitory, whereas CL is stimulatory. Neither molecule interacts with Mφ via the 2F8 epitope of SR-A, and treatment of Mφ with either phospholipid results in a down-regulation of CR3 function and TNF-α production by the phagocyte. Definition of the mode of action of PI and CL on Mφ awaits further investigation.

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REFERENCES


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