Activation of Phospholipase D Is Tightly Coupled to the Phagocytosis of *Mycobacterium tuberculosis* or Opsonized Zymosan by Human Macrophages

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Summary

Phagocytosis of *Mycobacterium tuberculosis* by human mononuclear phagocytes is mediated primarily by complement receptors (CRs) but the transmembrane signaling mechanisms that regulate phagocytosis of the bacterium are unknown. We have analyzed the activation of phospholipase D (PLD) during phagocytosis of the virulent Erdman and attenuated H37Ra strains of *M. tuberculosis* by human monocyte-derived macrophages (MDMs), radiolabeled with [^{3}H]-lyso-phosphatidylcholine. Phagocytosis of either Erdman or H37Ra *M. tuberculosis* in the presence of autologous non-immune serum was associated with a 2.5–3-fold increase in phosphatidic acid (PA). Definitive evidence for activation of PLD by *M. tuberculosis* was provided by markedly increased generation of the PLD-specific product phosphatidylethanol (PEt)(9.9-fold increases in [^{3}H]-PEt for both Erdman and H37Ra strains compared to control, $P < 0.001$, $n = 12$), in the presence of 0.5% ethanol. Phagocytosis of opsonized zymosan (OZ), which is also mediated by CRs, was similarly associated with activation of PLD (12.2-fold increase in PEt, $P < 0.001$, $n = 12$). The competitive PLD inhibitor 2,3-diphosphoglycerate (2,3-DPG) produced concentration-dependent inhibition of PLD activity stimulated by either *M. tuberculosis* ($-78 \pm 8\%$) or OZ ($-73 \pm 6\%$). Inhibition of PLD by 2,3-DPG was associated with concentration-dependent reductions in phagocytosis of *M. tuberculosis* ($-74 \pm 4\%$) and OZ ($-68 \pm 5\%$). Addition of purified PLD from *Streptomyces chromofuscus* to 2,3-DPG-treated macrophages restored phagocytosis of *M. tuberculosis* to control levels. Inhibition of *M. tuberculosis*- or OZ-stimulated PA generation by ethanol was associated with concentration-dependent reductions in phagocytosis of both particles.

Incubation of MDMs with either Erdman or H37Ra *M. tuberculosis*, or OZ, resulted in rapid (onset 1 min) and sustained (60 min) increases in the tyrosine phosphorylation (Tyr-P) of multiple MDM proteins. Prominent Tyr-P was noted in proteins of 150, 95, 72, 56, and 42 kD. The protein tyrosine kinase (PTK) inhibitors genistein and herbimycin A reduced *M. tuberculosis*-stimulated PLD activity by 66–84%. Inhibition of PLD activity by genistein or herbimycin A was associated with inhibition of phagocytosis of *M. tuberculosis* and OZ. These data demonstrate that PLD is activated during macrophage phagocytosis of *M. tuberculosis* or OZ, that PTKs are involved in this stimulation of PLD, and that the extent of phagocytosis of these particles is tightly coupled to activation of PLD.

Tuberculosis is an infectious disease of global importance, whose morbidity and mortality have recently increased (1). The worsening health burden of tuberculosis is due, in part, to increased antibiotic resistance in the causative organism, *Mycobacterium tuberculosis*, as well as to coinfection of patients with HIV (1, 2). *M. tuberculosis* is a facultative intracellular pathogen of human mononuclear phagocytes. After phagocytosis by monocytes and macrophages, *M. tuberculosis* survives and replicates within these cells, despite the presence of multiple host antimicrobial effector mechanisms (2). These observations suggest that a central feature of the pathogenesis of tuberculosis involves mycobacterial interference with, or evasion of, pathways of mononuclear phagocyte activation which normally lead to the killing of ingested organisms. The signal transduction mechanisms activated during phagocytosis of *M. tuberculosis* are unknown, both in regard to those transmembrane signaling pathways required for progression of the phagocytic process itself, as

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well as those which may be associated with more distal macrophage microbicidal functions.

Complement receptors (CR)[1] CR1 and CR3 mediate phagocytosis of *M. tuberculosis* by human monocytes, whereas CR4 and the mannose receptor are additionally involved in uptake by macrophages (3–5). Most analyses of signal transduction during complement receptor (CR)-mediated phagocytosis have used nonmicrobial ligands (e.g., latex beads, erythrocytes, or zymosan coated with C3b/C3bi)(6–9), and the validity of extrapolations to the uptake of bacteria is not known. Detailed analysis of phagocyte transmembrane signaling mechanisms activated by specific organisms, such as *M. tuberculosis,* is indicated, as the nonmicrobial models of CR-dependent phagocytosis differ from each other in several important respects, most notably, in the involvement of additional receptor classes, the dependence of ingestion on the "activation state" of the phagocyte, and the initiation of antimicrobial responses (6–11). Nevertheless, previous work suggests that diglycerides and protein kinase C (PKC) regulate the CR-dependent phagocytosis of specific particles (7–9, 12–14). Andersson and coworkers have recently demonstrated that phospholipase D (PLD) is activated during phagocytosis of opsonized zymosan (OZ) by neutrophils, and that the PLD/phosphatidic acid phosphohydrolase pathway is the major source of diglycerides for activation of PKC during this event (8). The PLD-initiated pathway is also the primary route of diglyceride generation in phagocytes stimulated by soluble agonists such as formylated peptides and complement component C5a (15, 16).

The aims of the present study were to determine whether PLD is activated during phagocytosis of *M. tuberculosis* or OZ by human macrophages, and to analyze the relation between activation of PLD and the extent of particle phagocytosis.

Materials and Methods

Chemicals. Hepes, zymosan, 2,3-diphosphoglycerate (2,3-DPG), trypan blue, leupeptin, PMSF, aprotinin, bovine pancreatic trypsin, phosphatidylethanol (PEt), and phosphatidic acid (PA) were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 and PBS were from GIBCO BRL (Gaithersburg, MD), Middlebrook 7H9 broth was from BBL Microbiology Systems (Cockeysville, MD), and 7H11 agar, oleic acid-albumin-dextrose-catalase enrichment medium, and auramine-rhodamine stain were from Difco Laboratories (Detroit, MI). Teflon wells were obtained from Savillex Corp. (Minnetonka, MN). All organic solvents (HPLC grade) were obtained from Fisher (Fairlawn, NJ). [^{3}H]-1-O-alkyl-lyso-PC, [^{3}H]-dipalmitoylphosphatidylcholine (DPPC) and the reagents for Western blot detection by ECL were from Amersham Corp. (Arlington Heights, IL). HSA and genistein were purchased from Calbiochem Novabiochem (San Diego, CA). *S. chromofuscus* PLD and herbimycin A were from Biomol (Plymouth Meeting, PA). PY-20 α-P-Tyr mAb was from ICN ImmunoBiologicals (Costa Mesa, CA).

Preparation of Macrophage Monolayers. Peripheral blood mononuclear cells were isolated from healthy, purified protein derivative (PPD)-negative, adult volunteers, cultured in Teflon wells, and monocyte-derived macrophages (MDM) were purified as previously described (4). Monolayers were washed and incubated in RPMI-Hepes with 2.5% serum for use in experiments. MDM viability was assessed by exclusion of trypan blue, and monolayer density by nuclei counting with napthol blue-black (17).

Bacteria. The virulent Erdman (35801; American Type Culture Collection [ATCC], Rockville, MD) and attenuated H37Ra (ATCC 25177) strains of *M. tuberculosis* were cultured and prepared for use in experiments as previously described (3, 4). The final preparation of *M. tuberculosis* contained >90% single bacteria, with ≥75% viability by determination of colony forming units (CFU). For experiments in which killed *M. tuberculosis* were used, bacteria were heated to 80°C for 1 h, and the efficiency of heat killing was verified by absence of growth on 7H11 agar.

Analysis of PLD Activity. MDMs were cultured in 6-well tissue culture plates at ~2.0 × 10^6 MDMs/well, and radioisotopically labeled with [^{3}H]-1-O-alkyl-lyso-PC (5 uCi/well) for 90 min at 37°C in RPMI-20 mM Hepes with 2.5% serum. After repeated washings, MDMs were incubated with *M. tuberculosis* or serum-OZ at a multiplicity of infection (MOI) of 10:1. In certain assays, 0.5% ethanol was added 5 min before *M. tuberculosis* or OZ to permit detection of the specific transphosphatidylation product, [^{3}H]-PEt, as a metabolically stable index of PLD activity (15, 16). After a 30-min incubation, reactions were terminated with 1.7 volumes of ice-cold methanol, MDMs were scraped, placed in polypropylene tubes, and 3.3 volumes of $CHCl_3$ added. No viable mycobacteria were present after $CHCl_3$/MeOH extraction, as determined by culture on 7H11 agar. After phase separation, the $CHCl_3$ layer was dried, and [^{3}H]-PA and [^{3}H]-PEt were isolated by TLC in an ethyl acetate/iso-octane/acetic acid (9:5:2) solvent system (23), by co-migration with pure phospholipid standards. Quantitation of [^{3}H]-cpm in PA and PEt were performed by liquid scintillation spectrophotometry, and counts were normalized for total CPM in phospholipid to correct for minor differences in labeling between experiments. Neither 2,3-DPG, ethanol, genistein, nor herbimycin A affected the viability of MDM or *M. tuberculosis*. Only herbimycin A caused a decrease in monolayer density, which was dose-dependent (maximal reduction of 18 ± 5% at 0.6 μg/ml). For the PLD assay, [^{3}H]-PEt cpm were normalized to total [^{3}H]-cpm in phospholipid to correct for the herbimycin A–induced reduction in monolayer density.

Exogenous Substrate Assay of PLD Activity. PLD activity of intact *M. tuberculosis* was analyzed using an exogenous substrate assay developed by Brown et al. (18), with the following modifications. [^{3}H]-DPPC was used as the PLD substrate and presented as mixed vesicles containing phosphatidylethanolamine/phosphatidylinositol-4,5-bisphosphate/DPPC at molar ratios of 16:1.4:1, with 0.4 uCi [^{3}H]-DPPC/sample. Lipids were dissolved in $CHCl_3$/MeOH (2:1), dried under N_2 and resuspended in 50 mM Hepes (pH 7.5), 3 mM EGTA, 8 mM KCl, 1 mM DTT, by sonication for 10 min at 25°C. 10 μl of the lipid substrate was incubated with *M. tuberculosis*, buffer, or purified PLD from *S. chromofuscus* in RPMI, 20 mM Hepes, 2.5% serum, for 30 min at 37°C, in a reaction volume of 100 μl. Reactions were terminated with five volumes of $CHCl_3$/MeOH (2:1), and samples analyzed for [^{3}H]-PA.

[1] *Abbreviations used in this paper:* 2,3-DPG, 2,3-diphosphoglycerate; CR, complement receptor; DPPC, dipalmitoylphosphatidylcholine; MDM, monocyte-derived macrophage; MOI, multiplicity of infection; MR, mannose receptor; OZ, opsonized zymosan; PA, phosphatidic acid; PEt, phosphatidylethanol; PKC, protein kinase C; PLD, phospholipase D; PPD, purified protein derivative; PTK, protein tyrosine kinase; Tyr-P, tyrosine phosphorylation.

Table 1. *Phagocytic Index for Opsonized Zymosan*

	Method*		
	Phase-contrast microscopy	Trypsin proteolysis	Trypan blue
Control	808 ± 33	792 ± 41	822 ± 46
2,3-DPG (10 mM)	388 ± 17	409 ± 22	393 ± 9
Ethanol (0.75%)	313 ± 16	289 ± 20	297 ± 17
Genistein (300 μM)	145 ± 9	155 ± 11	140 ± 6
Herbimycin A (0.6 μg/ml)	259 ± 14	244 ± 13	266 ± 8

*Mean ± SEM of triplicate determinations from two experiments; 50–200 MDMs were analyzed for each condition.

Analysis of Phagocytosis. Phagocytosis of *M. tuberculosis* was determined as previously described (3, 4). Briefly, MDMs adherent to glass coverslips (~2×10^5 MDMs/coverslip) in 24-well plates were incubated with *M. tuberculosis* (MOI 10:1) in RPMI-20 mM Hepes with 2.5% autologous non-immune serum. After incubation for 30–120 min, MDMs were washed repeatedly to remove nonadherent bacteria, fixed in 10% formalin and stained with auramine-rhodamine. Previous electron microscopic studies of this assay have indicated that all adherent (total cell-associated) mycobacteria are phagocytosed, both under control conditions, as well as in experiments in which phagocytosis is inhibited or augmented (3, 4, 19). Adherent bacteria were quantitated by fluorescence microscopy of triplicate coverslips for each experimental condition (50–200 MDMs/coverslip), and results of a set of experiments expressed as the mean (± SEM) number of adherent *M. tuberculosis*/MDM.

Phagocytosis of OZ was analyzed by phase-contrast microscopy, as noted previously (20, 21). Zymosan particles were opsonized with 25% fresh human serum for 30 min at 37°C, washed and presented to MDMs at a particle to MDM ratio of 10:1. After a 30-min incubation, monolayers were washed repeatedly and fixed in 2.5% glutaraldehyde, before microscopy. In select experiments, the accuracy of phase-contrast determinations of zymosan phagocytosis were confirmed by two additional methods; (*a*) limited tryptic digestion and (*b*) staining with trypan blue. (*a*) 30 min after the addition of OZ, MDM monolayers were washed and incubated with 2.5% trypsin for 10 min at 37°C, before fixation. Since controlled exposure to trypsin results in proteolysis of CR3 and mannose receptors (MRs), attached zymosan particles are removed whereas ingested particles are unaffected, and can be enumerated by phase-contrast microscopy (21, 22). (*b*) MDM monolayers were prepared and incubated with OZ for 30 min, as described above. 0.01% trypan blue was added, and coverslips analyzed by phase-contrast microscopy. Attached particles are stained with the dye, whereas phagocytosed zymosan retains its clear appearance (23). Triplicate coverslips were analyzed for each condition. Results of the analysis of OZ phagocytosis by these three methods were in close agreement (Table 1).

Analysis of Protein Tyrosine Phosphorylation. MDMs were adhered to 12-well tissue culture plates (~1.0×10^6 cells/well) and cultured in RPMI (pH 7.4), 20 mM Hepes, 2.5% serum. *M. tuberculosis* was added to MDM monolayers at a MOI of 10:1, and incubated for various times, at 37°C in 5% CO_2. At the end of the incubation, monolayers were washed with ice-cold PBS, 1 mM vanadate and then lysed in 100 μl of 1% Triton X-100, 20 mM Tris (pH 8.0), 140 mM NaCl, 1 mM Na_3VO_4, 2 mM EDTA, 10% glycerol, 1 mM PMSF, 20 μM leupeptin, 0.15 μ/ml aprotinin. After incubation on ice for 20 min, lysates were centrifuged at 14,000 *g* for 15 min at 4°C, and supernatants transfered to separate tubes. Supernatants contained no viable mycobacteria, as determined by plating on 7H11 agar. Supernatants were boiled in an equal volume of electrophoresis sample buffer, subjected to SDS-PAGE electrophoresis, transferred to PVDF membrane, and Western blotted with PY-20 α-P-Tyr mAb, with detection by enhanced chemiluminescence (ECL)(24, 25). To correct for the effects of herbimycin A on density of the MDM monolayer, protein concentrations of Triton X-100 extracts were determined by the bicinchoninic acid method (33), and equal amounts of protein were used for the analysis of tyrosine phosphorylation (Tyr-P).

Analysis of Data. Data from each experimental group were subjected to an analysis of normality and variance. Differences between experimental groups composed of normally distributed data were analyzed for statistical significance using Student's *t* test. Non-parametric evaluation of other data sets was performed with the Mann-Whitney Rank Sum test. Analysis of correlation was performed with the Spearman Rank Order test (27).

Results

Phagocytosis of M. tuberculosis or Opsonized Zymosan by Human Macrophages Is Accompanied by Activation of Phospholipase D. The PLD activity of human macrophages was quantitated by accumulation of the PLD-specific transphosphatidylation product, PEt, in the presence of 0.5% ethanol. MDMs were radiolabeled with [^{3}H]-1-O-alkyl-lyso-PC, and [^{3}H]-PEt generation was determined by thin layer chromatography. As shown in Fig. 1 *A*, resting macrophages exhibited very little PLD activity, as PEt accumulation in the 30 min buffer control was not significantly different than the basal value at time = 0. In contrast, MDMs incubated with either the virulent Erdman or the attenuated H37Ra strains of *M. tuberculosis* showed significant increases in PLD activity (9.9-fold over control for each, $P < 0.001$, $n = 12$). The two strains of *M. tuberculosis* were phagocytosed to a similar extent by MDMs; at a multiplicity of infection (MOI) of 10:1, the mean (± SEM) number of ingested bacilli per macrophage was 5.36 ± 0.58 for Erdman and 5.84 ± 0.51 for H37Ra ($n = 3$). Incubation of macrophages with OZ also resulted in increased PLD activity (12.2-fold over control, $P < 0.001$, $n = 12$). 8.08 ± 0.63 zymosan particles were ingested/MDM, and the number of attached OZ per MDM was 1.61 ± 0.23 (Table 2). Treatment of MDMs with 100 nM PMA served as the positive control, with generation of 647 ± 96 cpm [^{3}H]-PEt ($P < 0.001$, $n = 10$).

Phagocytosis of *M. tuberculosis* or OZ in the absence of ethanol was accompanied by increased production of PA, the physiologic product of PLD catalysis (Fig. 1 *B*). The mean (± SEM) [^{3}H]-PA cpm in resting MDMs was 177 ± 24, whereas it was 498 ± 76 or 461 ± 59 cpm after addition of H37Ra or Erdman *M. tuberculosis*, respectively, and 688 ± 65 cpm in macrophages phagocytosing OZ ($P < 0.001$ for each, $n = 5$). Incubation with increasing concen-

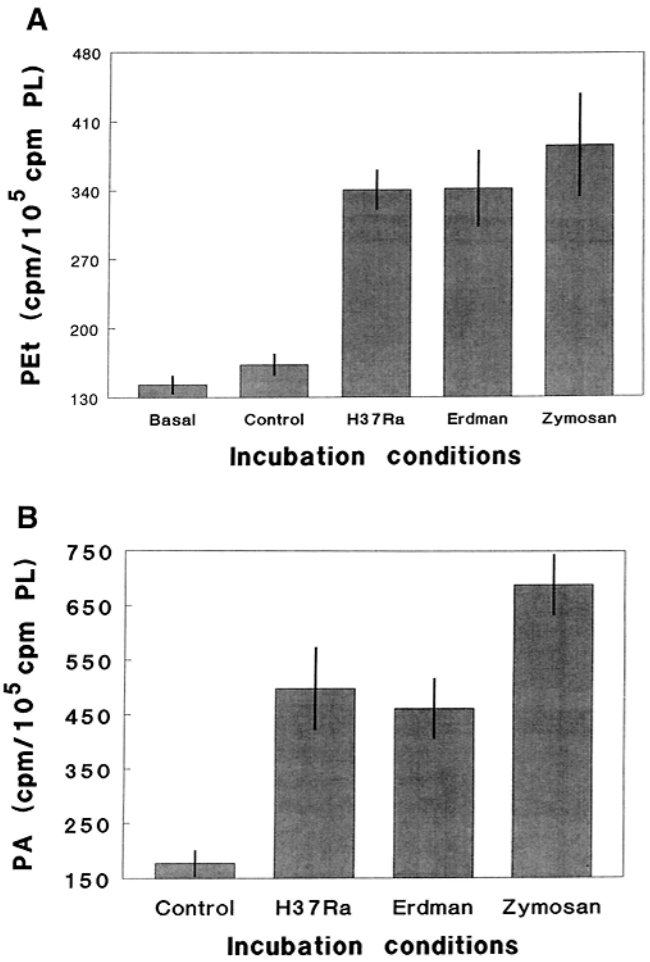


Figure 1. Activation of macrophage phospholipase D accompanies the phagocytosis of *M. tuberculosis* or opsonized zymosan. (*A*) MDM monolayers radiolabeled with [^{3}H]-1-O-alkyl-lyso-PC were incubated under the indicated conditions. At 30 min, lipids were extracted and PLD activity assayed as [^{3}H]-PEt accumulation. Basal value represents PEt at time = 0, while control is PEt at 30 min in the presence of media alone. [^{3}H]-cpm in PEt were normalized for total cpm in phospholipid (*PL*). Data represent mean ± SEM of duplicate samples from 12 determinations. (*B*) Experiments were conducted as in *A*, except for the absence of ethanol. Results are from five experiments.

trations of ethanol (0–0.75% vol/vol) resulted in decreased accumulation of PA and increased production of PEt (data not shown). This inverse relationship of PA and PEt generation to ethanol concentration is characteristic of the unique transphosphatidylation capacity of PLD enzymes (16). As with *M. tuberculosis*, PA and PEt generation during phagocytosis of OZ were inversely related, dependent on ethanol concentration (data not shown). Fig. 2 shows the kinetics of MDM PEt accumulation in response to *M. tuberculosis* or OZ. An increase in PLD activity was noted 1 min after the addition of either particle, and PEt levels reached a maximum between 30–60 min. PA generation was also detectable within 1 min of the addition of *M. tuberculosis* or OZ, and peaked at 30–60 min (data not shown). The maximal rates of PEt and PA generation occurred within the first 15 min after particle addition (Fig. 2).

Table 2. *Effects of Inhibition of PLD on the Phagocytosis and Attachment of Opsonized Zymosan by Human Macrophages*

	Opsonized zymosan		
	Phagocytic* index	Attachment index	Total cell-association
Control	808 ± 33	161 ± 14	969 ± 48
2,3-DPG (10 mM)	388 ± 17	187 ± 11	575 ± 32
Ethanol (0.75%)	313 ± 16	191 ± 10	514 ± 21
Genistein (300 μM)	145 ± 9	212 ± 13	357 ± 10
Herbimycin A (0.6 μg/ml)	259 ± 14	205 ± 10	464 ± 21

Serom-OZ particles were incubated with MDM monolayers at a particle/MDM ratio of 10:1. At 30 min, ingested and attached zymosan were enumerated by phase-contrast microscopy. In selected experiments, samples were subjected to limited tryptic digestion or staining with trypan blue to verify the accuracy of phase-contrast determinations of zymosan phagocytosis (Table 1).
*Phagocytic index: number of particles ingested by 100 MDMs. Attachment index: number of particles attached (extracellularly) to 100 MDMs. Total cell-association: the sum of the phagocytic and attachment indices.

Activation of PLD during macrophage phagocytosis of *M. tuberculosis* did not require viability of the organisms, since heat-killed *M. tuberculosis* also stimulated significant PLD activity (live: 252 ± 13 [^{3}H]-PEt cpm; dead: 202 ± 24 cpm, n = 3). Additional evidence that the PLD activity accompanying phagocytosis of *M. tuberculosis* was not of mycobacterial origin was obtained with an exogenous substrate assay which has been used to measure PLD activities from a wide variety of sources, including mammalian cells,

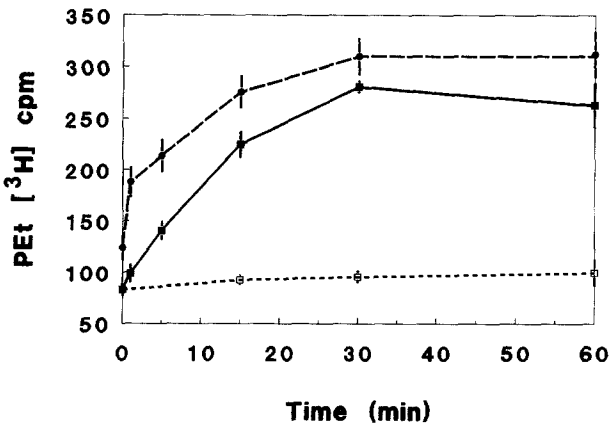


Figure 2. Time-dependence of PLD activation during macrophage phagocytosis of *M. tuberculosis* or OZ. MDMs were incubated with H37Ra *M. tuberculosis* (*solid squares*) or OZ (*solid circles*) at a particle to MDM ratio of 10:1. At the indicated times, [^{3}H]-PEt was measured as an index of PLD activity. Open squares represent PEt accumulation in control MDMs, in the absence of *M. tuberculosis* or OZ. Results are mean ± SD of duplicate determinations from a single experiment, representative of five. Four experiments with Erdman *M. tuberculosis* yielded very similar results.

bacteria, and plants (18). *M. tuberculosis*, buffer, or purified PLD from *S. chromofuscus* was added to vesicles composed of [^{3}H]-DPPC, phosphatidylethanolamine, and phosphatidylinositol-4,5-bisphosphate, and [^{3}H]-PA was determined as a measure of PLD activity. Incubation of live *M. tuberculosis* with the vesicle substrate for 30 min at 37°C did not result in increased production of PA when compared with buffer-treated samples (603 ± 36 [^{3}H]-PA cpm for *M. tuberculosis* vs. 598 ± 22 cpm for buffer control, $n = 3$). In contrast, addition of *S. chromofuscus* PLD to [^{3}H]-DPPC-labeled vesicles resulted in marked PA accumulation (32,858 ± 1692 cpm).

Previous work has demonstrated that in the absence of serum, or in the presence of heat-inactivated serum (HI-serum, 56°C, 30 min), phagocytosis of *M. tuberculosis* by human macrophages and monocytes is reduced approximately 70–80%, which correlates with the absence of C3 on the bacterial surface (3, 4). To further examine the association between stimulation of macrophage PLD activity and phagocytosis of *M. tuberculosis*, we studied the serum-dependence of phagocytosis-associated PLD activation. The level of PLD activity stimulated by *M. tuberculosis* was reduced 70 ± 8% in the absence of serum and 61 ± 7% in the presence of HI-serum ($n = 3$). Correspondingly, phagocytosis of *M. tuberculosis* was reduced 76 ± 7% in the absence of serum and 69 ± 6% in the presence of HI-serum ($n = 3$). Thus, both *M. tuberculosis*-stimulated PLD activity and the degree of mycobacterial phagocytosis exhibit similar serum-dependence.

The Competitive PLD Inhibitor 2,3-Diphosphoglycerate Inhibits M. tuberculosis-stimulated PLD Activity and Phagocytosis of the Bacterium. To further evaluate the role of PLD in phagocytosis of *M. tuberculosis*, we studied the effects of the competitive PLD inhibitor 2,3-diphosphoglycerate (2,3-DPG) on PEt accumulation and mycobacterial ingestion (28). 2,3-DPG (2–10 mM) inhibited *M. tuberculosis*-stimulated PLD activity in a concentration-dependent manner, with a maximal reduction of 78 ± 9% (Fig. 3). These levels of 2,3-DPG did not affect the viability or density of the macrophage monolayer, or the viability of *M. tuberculosis* (data not shown). MDMs incubated with 2,3-DPG (2–10 mM) also exhibited decreased phagocytosis of *M. tuberculosis* (Fig. 3), with a maximal inhibition of 74 ± 5%. Treatment with 2,3-DPG resulted in decreases in both the phagocytic index (Fig. 3, Table 1), and the number of macrophages phagocytosing at least one tubercle bacillus (data not shown). The results in Fig. 3 represent the inhibition of phagocytosis at 30 min after the addition of *M. tuberculosis*. Similar inhibition of the uptake of *M. tuberculosis* by 2,3-DPG was noted at both earlier (15 min) and later (120 min) time points (data not shown). The effects of 2,3-DPG on macrophage PLD activity and phagocytosis were not limited to the ingestion of *M. tuberculosis*, since 5 mM 2,3-DPG produced a 54 ± 6% reduction in OZ-stimulated PLD activity ($n = 6$), and a 52 ± 5% inhibition of the phagocytosis of OZ (Table 2). Treatment with 2,3-DPG resulted in a small increase in the attachment index of OZ; from 161 ± 14 OZ/100 MDMs to 187 ± 11.

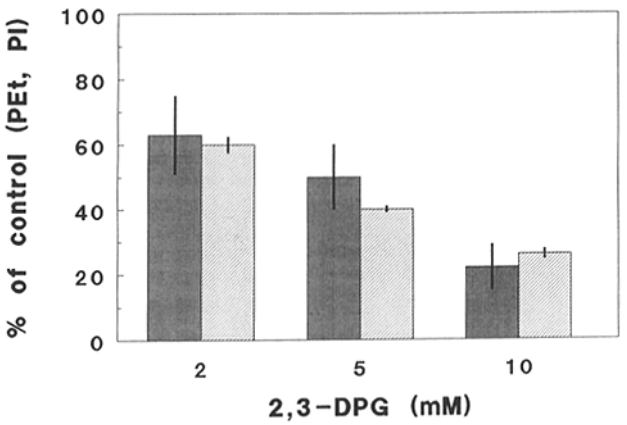


Figure 3. The PLD inhibitor 2,3-diphosphoglycerate reduces macrophage PLD activity and phagocytosis of *M. tuberculosis*. Macrophages were incubated with the indicated concentrations of 2,3-DPG or buffer control for 15 min at 37°C. H37Ra *M. tuberculosis* was added to MDMs and accumulation of PEt (*filled bars*) and phagocytosis (*striped bars*) were determined at 30 min. Results (mean ± SEM, $n = 4$) are expressed as the percentage of PLD activity and phagocytosis of control samples. Control macrophages phagocytosed 5.26 ± 0.37 mycobacteria per cell.

Purified Bacterial PLD Restores PEt Accumulation and Phagocytosis of M. tuberculosis in 2,3-DPG-treated Macrophages. The effects of 2,3-DPG suggest an association between the level of macrophage PLD activity stimulated by *M. tuberculosis*, and the degree of phagocytosis of the bacterium. 2,3-DPG is a selective inhibitor of PLD since it does not block several other activation-dependent signal transduction mechanisms in phagocytes (28), and inhibits purified PLD in a competitive manner. However, as with most inhibitors, conclusions derived from the use of 2,3-DPG are complicated by the potential for nonspecific effects which could decrease phagocytosis by a mechanism other than inhibition of PA production. To address these limitations, we studied the effects of purified bacterial PLD from *S. chromofuscus* on PEt accumulation and phagocytosis of *M. tuberculosis*. We reasoned that if activation of macrophage PLD by *M. tuberculosis* is tightly coupled to phagocytosis, then exogenously added PLD might restore phagocytic capacity to MDMs in which endogenous PLD had been inhibited by 2,3-DPG. This approach of adding purified phospholipases, including PLD, of bacterial or plant origin to mammalian cells has been used successfully to analyze the effects of their eukaryotic homologues on cellular activation (29, 30). Initial experiments, in the absence of *M. tuberculosis*, demonstrated that the addition of *S. chromofuscus* PLD to [^{3}H]-lyso-PC–labeled macrophages resulted in concentration-dependent accumulation of [^{3}H]-PEt (results not shown). Bacterial PLD (2–50 μ/ml) did not affect MDM viability or the density of the cell monolayer. PEt levels were elevated within 1 min of addition of *S. chromofuscus* PLD, and continued to rise over the 30 min assay (results not shown).

Fig. 4 *A* shows the effect of bacterial PLD on PEt accumulation by 2,3-DPG-treated macrophages incubated with *M. tuberculosis*. 5 mM 2,3-DPG inhibited *M. tuberculosis*-stimulated PLD activity by approximately 50%, which is in

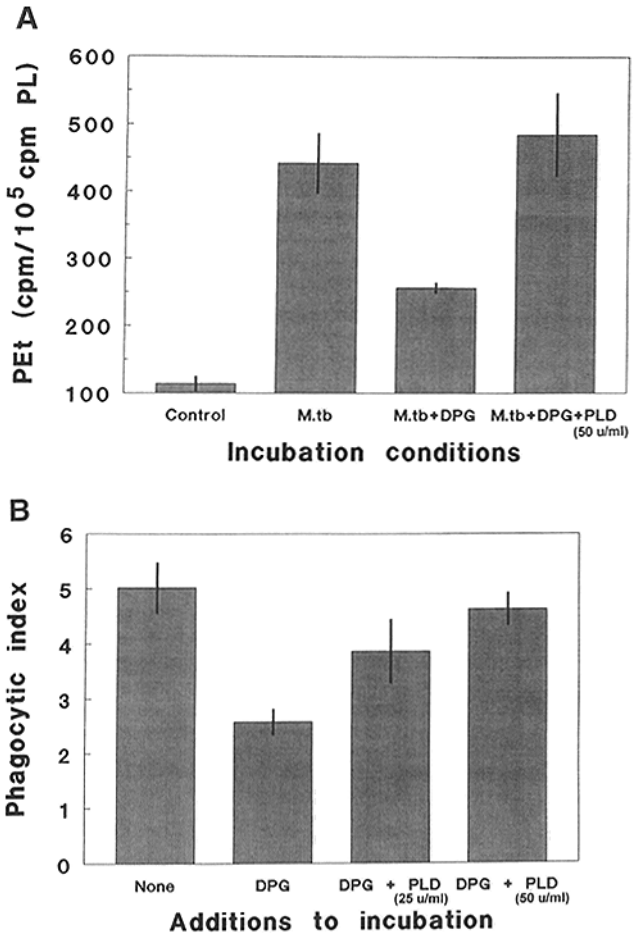


Figure 4. Effect of 2,3-DPG and bacterial PLD on PEt generation and macrophage phagocytosis of *M. tuberculosis*. (*B*) MDMs, labeled with [^{3}H]-lyso-PC, were incubated with 5 mM 2,3-DPG or buffer control for 15 min at 37°C. H37Ra *M. tuberculosis* was added to all samples except control MDMs. *S. chromofuscus* PLD (50 μ/ml) was added to the indicated samples concurrent with *M. tuberculosis*. (*B*) MDMs were incubated with 5 mM 2,3-DPG or buffer control for 15 min before exposure to *M. tuberculosis*. Bacterial PLD was added to the indicated samples concurrent with mycobacteria and phagocytosis was assayed at 30 min. Results are from three experiments performed in triplicate.

close agreement with the results presented in Fig. 3. Addition of bacterial PLD (50 μ/ml) restored PEt accumulation in these 2,3-DPG-treated macrophages to approximately the level found in untreated MDMs after incubation with *M. tuberculosis*. In separate experiments, *S. chromofuscus* PLD did not affect the viability of *M. tuberculosis* (data not shown). The effects of 2,3-DPG and bacterial PLD on phagocytosis of *M. tuberculosis* are demonstrated in Fig. 4 *B*. Incubation of macrophage monolayers with 5 mM 2,3-DPG resulted in an ~50% decrease in mycobacterial ingestion, comparable to that shown in Fig. 3. *S. chromofuscus* PLD produced a concentration-dependent increase in the level of phagocytosis of *M. tuberculosis* by 2,3-DPG-treated macrophages. Incubation of macrophages with 50 μ/ml bacterial PLD resulted in essentially complete recovery of control levels of mycobacterial phagocytosis.

Table 3. *Effect of Ethanol on Phosphatidic Acid Generation and Phagocytosis of M. tuberculosis*

	Phosphatidic acid production		Phagocytosis	
Ethanol	[^{3}H]-PA cpm	% change	Phagocytic index	% change
% vol/vol				
0	554 ± 77	—	504 ± 41	—
0.10	471 ± 24	−15 ± 5	449 ± 32	−11 ± 4
0.25	377 ± 22	−32 ± 6	323 ± 19	−36 ± 7
0.50	305 ± 12	−45 ± 11	207 ± 13	−59 ± 10
0.75	116 ± 17	−79 ± 11	176 ± 15	−65 ± 7

Macrophages were incubated with buffer or the indicated concentrations of ethanol for 5 min at 37°C, prior to the addition of H37Ra *M. tuberculosis*. PA generation and phagocytosis were determined at 30 min, and expressed as the percentage change from control values. Results are from three experiments performed in triplicate.

Inhibition of M. tuberculosis- or OZ-stimulated PA Generation by Ethanol Is Accompanied by Inhibition of Phagocytosis. Incubation of cells with low concentrations of ethanol has been used to study the involvement of PLD in various physiologic processes (16, 31). By serving as a nucleophilic substrate for the transphosphatidylation reaction, ethanol diverts PA generation to the production of PEt, thereby effectively inhibiting the generation of the product of the PLD-catalyzed reaction without inhibiting enzyme turnover. Incubation of macrophages with ethanol (0.1–0.75% vol/vol) for 5 min before addition of *M. tuberculosis* produced a concentration-dependent inhibition of PA generation, with a maximal reduction of 79 ± 11% (Table 3). Ethanol also inhibited macrophage phagocytosis of *M. tuberculosis* in a concentration-dependent manner, with a maximal reduction of 65 ± 7% (Table 3). These concentrations of ethanol did not affect macrophage viability, density of the MDM monolayer, or the viability of *M. tuberculosis* (data not shown). Similarly, ethanol-treatment of macrophages resulted in dose-dependent inhibition of OZ-stimulated PA generation, with a maximal reduction of 71 ± 8% at 0.75% ethanol. The degree of phagocytosis of OZ was reduced in a parallel manner by exposure to ethanol, with a maximal inhibition of 60 ± 6% (n = 4). As with 2,3-DPG, incubation of MDMs with ethanol resulted in a small increase in the attachment index for OZ; from 167 ± 10 to 191 ± 9 (Table 2).

Macrophage Phagocytosis of M. tuberculosis Is Accompanied by Increased Protein Tyrosine Phosphorylation. To study the mechanism of phagocytosis-associated activation of PLD, we analyzed levels of protein Tyr-P in resting MDMs and those undergoing phagocytosis of *M. tuberculosis*. Protein tyrosine kinases (PTKs) activate PLD in many cell types, including phagocytic leukocytes (24, 25, 32, 33). Recent evidence indicates that PTKs function in transmembrane signaling after the ligation of β2 integrins (34, 35) and Fcγ receptors

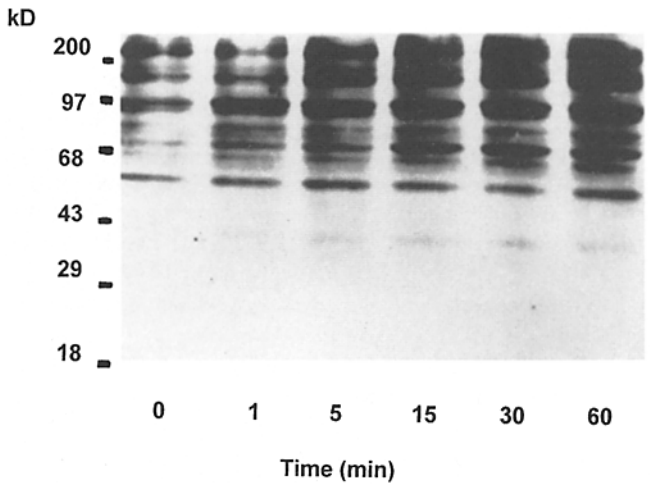
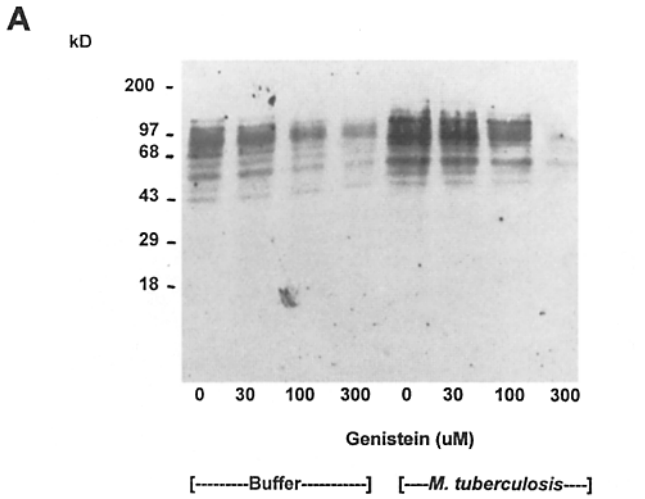


Figure 5. *M. tuberculosis* induces an increase in macrophage protein tyrosine phosphorylation. MDM monolayers were incubated at 37°C with buffer or H37Ra *M. tuberculosis* for the indicated times. 1% Triton X-100 lysates were subjected to SDS-PAGE, Western blotted with PY-20 mAb to P-Tyr, followed by HRP-conjugated α-mouse IgG, with detection by ECL. Results are representative of data from five identical experiments.



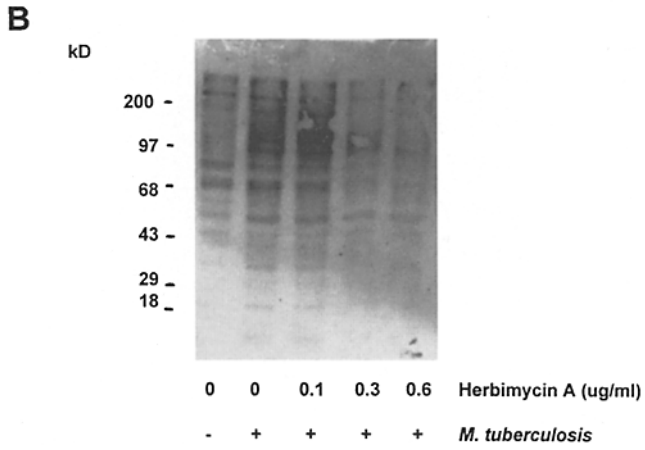


Figure 6. Genistein and herbimycin A inhibit *M. tuberculosis*-induced protein tyrosine phosphorylation. (*A*) MDMs were incubated with the indicated concentrations of genistein or 0.1% DMSO solvent control for 15 min at 37°C. H37Ra *M. tuberculosis* or buffer was added for an additional 15 min, and P-tyr proteins analyzed as described in the legend to Fig. 5. (*B*) Macrophages incubated with 0.1% DMSO or the indicated concentrations of herbimycin A for 18 h, were incubated with *M. tuberculosis* or buffer for 15 min. Protein Tyr-P was analyzed as noted above. Results represent data from one of five experiments for each inhibitor.

(FcγRs)(37, 38). MDMs were incubated with *M. tuberculosis* or buffer control, under conditions identical to those used in assays of PLD activity and phagocytosis, and Tyr-P proteins were analyzed in Triton X-100 extracts by Western blotting with α-P-Tyr mAb. Resting MDMs contained several Tyr-P proteins (Fig. 5). Incubation of MDMs with *M. tuberculosis* was associated with a significant increase in protein Tyr-P, particularly in proteins of ~150, 95, 72, 56, and 42 kD. Increased levels of protein Tyr-P were detected within 1 min of the addition of *M. tuberculosis*, and persisted for 60 min. *M. tuberculosis* alone contained no detectable tyrosine phosphoproteins (data not shown). Incubation of MDMs with OZ also resulted in rapid and sustained increases in protein Tyr-P (not shown).

Tyrosine Kinase Inhibitors Reduce the Accumulation of Tyr-P Proteins Induced by M. tuberculosis. To study the relation between *M. tuberculosis*-induced increases in Tyr-P and (*a*) stimulation of PLD and (*b*) phagocytosis, we studied the effect of two highly specific PTK inhibitor, genistein and herbimycin A, on these responses (39, 40). Incubation of MDMs with either genistein or herbimycin A produced concentration-dependent inhibition of *M. tuberculosis*-induced protein Tyr-P (Fig. 6). The PTK inhibitors also reduced basal levels of Tyr-P proteins in resting MDMs, suggesting that a balance of tyrosine kinase and phosphatase activities exists in unstimulated macrophages.

Tyrosine Kinase Inhibitors Block the Activation of PLD During Phagocytosis of M. tuberculosis or OZ and Reduce the Extent of Particle Ingestion. Incubation of MDMs with genistein resulted in concentration-dependent inhibition of *M. tuberculosis*-stimulated PLD activity, with a maximal decrease of 66 ± 15% (Fig. 7 *A*). Genistein also produced a dose-dependent inhibition of OZ-stimulated PLD activity, with a maximal 74 ± 9% reduction (n = 3). Inhibition of PLD activity by genistein was accompanied by concentration-dependent reductions in phagocytosis of *M. tuberculosis* (−62 ± 8%, Fig. 7 *A*) and OZ (−82 ± 7%, Table 2). Treatment of MDMs with 0.1–0.6 μg/ml herbimycin A resulted in concentration-dependent inhibition of *M. tuberculosis*-stimulated PLD activity, with a maximal reduction of 87 ± 5% (Fig. 7 *B*). 0.6 μg/ml herbimycin A reduced PEt accumulation secondary to OZ by 73 ± 9%. Herbimycin A also produced dose-dependent inhibition of the phagocytosis of *M. tuberculosis* and OZ, with maximal reductions of 78 ± 9% (Fig. 7 *B*) and 68 ± 7% (Table 2), respectively. These data indicate that macrophage phagocyto-

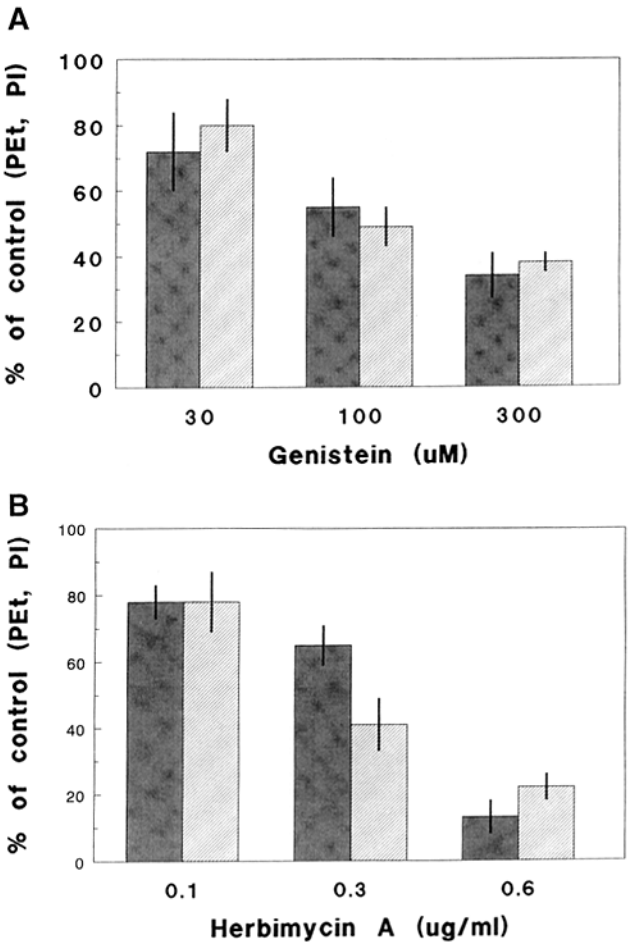


Figure 7. Tyrosine kinase inhibitors reduce *M. tuberculosis*-stimulated PLD activity and mycobacterial phagocytosis. (*A*) MDMs were incubated with genistein, or solvent control, as in Fig. 6 *A*. H37Ra *M. tuberculosis* was added and PLD activity (*filled bars*) and phagocytosis (*striped bars*) were determined at 30 min and expressed as the percentage of control values. (*B*) MDMs were pretreated with herbimycin A or solvent control as in Fig. 6 *B*. PLD activity and phagocytosis were determined as in *A*. Control macrophages phagocytosed 5.14 ± 0.28 mycobacteria per cell. Results are mean ± SEM of four determinations for each assay.

sis of two complement-opsonized particles is accompanied by prominent increases in protein Tyr-P, and that PTK inhibitors block the ingestion of *M. tuberculosis* and OZ.

The Level of M. tuberculosis-stimulated PLD Activity Is Highly Correlated with the Extent of Mycobacterial Phagocytosis by Human Macrophages. An attempt was made to quantify the relation between the degree of PLD activity stimulated by *M. tuberculosis* and the extent of phagocytosis of the bacterium. The results of all experiments in which PLD activity was modified (negatively by the exclusion of serum, use of HI-serum, treatment with 2,3-DPG, ethanol, genistein or herbimycin A, and positively, by addition of bacterial PLD) were expressed as a percentage of control values (PLD activity and phagocytosis) for a given experiment. The correlation coefficient for the association between PLD activity and phagocytosis of *M. tuberculosis* was 0.949, which was highly significant ($P < 0.005$, $n = 15$).

Discussion

The present studies indicate that phagocytosis of virulent or attenuated strains of *M. tuberculosis* by human macrophages is accompanied by activation of PLD. This is the first documentation that PLD is activated during phagocytosis of a microorganism, as well as the first report of this signal transduction pathway accompanying ingestion of any particle by mononuclear phagocytes. That this PLD activity is of macrophage rather than mycobacterial origin is supported by several lines of evidence. First, macrophage membrane lipids were radiolabeled in phosphatidylcholine substrate (with [^{3}H]-lyso-PC) before the addition of *M. tuberculosis*. Second, phagocytosis of heat-killed *M. tuberculosis* was associated with a level of PLD activity that was comparable to that elicited by live bacteria. Third, live *M. tuberculosis* alone exhibited no detectable PLD activity against an exogenous PC substrate. Fourth, macrophage phagocytosis of OZ was also accompanied by an increase in PLD activity.

The level of macrophage PLD activity correlated closely with the extent of phagocytosis of *M. tuberculosis* or OZ under a variety of experimental conditions. However, our limited understanding of the cellular localization of PLD and the likelihood that several isoforms of this enzyme exist in mammalian cells (16), preclude more detailed conclusions from the inhibitor studies. For example, although 2,3-DPG is known to be a competitive inhibitor of purified bacterial PLD (28), the mechanism by which it inhibits mammalian PLD is unknown. The high charge-density of 2,3-DPG makes passive diffusion through the lipid bilayer unlikely. However, metabolism of 2,3-DPG (e.g., acylation-deacylation) may significantly affect its partitioning between extracellular and intracellular compartments. It is of interest that the concentration-dependence of 2,3-DPG-mediated inhibition of PLD is very similar in macrophages and neutrophils (28).

The strong correlation between the level of macrophage PLD activity and the degree of phagocytosis suggests, but does not prove, a causal linkage between these processes. This hypothesis is strengthened by the restoration of control levels of phagocytosis in 2,3-DPG-treated macrophages by addition of bacterial PLD (at a concentration which resulted in restoration of control levels of PEt accumulation). Although the mechanism by which exogenously added phospholipases mimic their endogenous homologues is incompletely understood, this method has been applied sucessfully to PLD, PLC, PLA_2, and sphingomyelinase (29, 30). It is clear that exogenous phospholipases can use plasma membrane lipids as substrates to catalyze the formation of bioactive lipid products. However, questions remain regarding lipase access to specific leaflets of the membrane, and the localization of products to membrane microdomains containing distal effector molecules. In fact, these issues of interfacial catalysis and intramembrane localization remain largely unsolved for endogenous phospholipases as well (16).

Our results and the work of Andersson and coworkers (8, 12) indicate that the association of PLD activity and

phagocytosis is not limited to either a single particle or phagocytic cell type. Concerning the initiation of these responses, the primary phagocyte surface receptors which mediate the phagocytosis of *M. tuberculosis* (3–5), and OZ (23, 41) are CRs. Macrophage MRs also contribute to the phagocytosis of virulent *M. tuberculosis* (4) and OZ (20, 42). Because of their prominent role in the ingestion of these particles, CRs are also likely to function in the accompanying activation of PLD. Such a role for CRs is supported by the serum-dependence of PLD stimulation in macrophages and the activation of PLD after cross-linking of CRs in neutrophils (16).

Since there are significant differences in certain of the biochemical requirements and functional consequences of phagocytosis of EC3b/bi, OZ, and *M. tuberculosis*, comparative studies of signal transduction during uptake of these particles will be informative (6, 7). It is likely that these differences involve the activation, and perhaps deactivation, of distinct host biochemical signaling pathways by each of these particles. Since virulent and attenuated strains of *M. tuberculosis* stimulated similar levels of macrophage PLD activity (in the 60 min after addition to MDMs), we hypothesize that this early activation of PLD is a relatively proximal step in the interaction between *M. tuberculosis* and macrophages, and that it is more likely to be involved in the regulation of mycobacterial phagocytosis than to function as a biochemical determinant of virulence.

Phagocytosis requires attachment of the extracellular particle to plasma membrane receptors on the phagocytic cell, followed by ingestion of the particle within a membrane-bound phagosome. The data in Table 1 indicate that inhibition of OZ-stimulated PLD activity and phagocytosis was not accompanied by inhibition of the adherence of OZ to the macrophage surface. In fact, inhibition of PLD by different mechanisms was associated with a small increase in the attachment index for OZ. These considerations support the following model of signal transduction during CR-mediated phagocytosis: (*a*) ligation of complement receptors by the phagocytic particle results in, (*b*) activation of PLD, which is involved in the regulation of (*c*) particle ingestion. The effects of ethanol, which diverts the generation of the physiologic product, PA, to the metabolically inactive PEt, support this hypothesis, since the catalytic activity of PLD is preserved, yet phagocytosis is markedly inhibited. Thus, we propose that PLD inhibitors dissociate the attachment of a particle from its phagocytosis. In support of this model, incubation of electroporated polymorphonuclear leukocyte/neutrophil (PMN)(which were not competent for phagocytosis) with OZ resulted in stimulation of PLD in the absence of particle ingestion (8).

The effects of genistein and herbimycin A suggest that PTKs function in both stimulation of PLD and the regulation of CR-mediated phagocytosis. Consistent with this hypothesis, *M. tuberculosis* and OZ stimulate significant increases in macrophage protein Tyr-P. Although it is recognized that the use of PTK inhibitors may reduce the degree of phagocytosis by mechanisms other than inhibition of PLD activity, this hypothesis is consistent with the established roles of PTKs in: (*a*) β2 integrin-dependent signaling (34, 35), (*b*) the activation of PLD in many cell types, including phagocytic leukocytes (24, 25, 32, 33), and (*c*) phagocytosis mediated by FcγRs (37, 38). In addition, Mege and coworkers have demonstrated that phagocytosis of unopsonized zymosan is associated with activation of PTKs, although the specific receptors responsible for this response are unknown (36).

The mechanism by which stimulation of PLD is coupled to the progression of phagocytosis is unknown. PA activates several lipid-modifying enzymes, such as PI-PLC$_{\gamma 1}$, phospholipase A$_1$, and phosphoinositide-4- and 5-kinases (44), which may participate in the membrane remodeling and/or biochemical signaling which occur during phagocytosis. PA also activates protein kinases that likely function as effectors in PLD-initiated signaling pathways (45). Finally, the conversion of PA to DG, and the resultant activation of PKC, could promote CR-mediated phagocytosis by multiple mechanisms, including phosphorylation of CR1, MARCKS, and other, as yet undefined, substrates (8, 13, 14, 32). In addition to its proposed role in the regulation of receptor-mediated phagocytosis, the activation of PLD which accompanies particle uptake may modify subsequent phagocyte functions, including antimicrobial responses, via generation of PA and DG (31, 45, 46). Molecular definition of the relevant signaling components (PLD isoforms, PTKs), their intracellular loci, and detailed quantitation of their respective activities will be required to further our understanding of the role of PLD in receptor-mediated phagocytosis.

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