

Bacterial Phospholipid Hydrolysis Enhances the Destruction of *Escherichia coli* Ingested by Rabbit Neutrophils

Role of Cellular and Extracellular Phospholipases

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Abstract

Escherichia coli ingested by PMN are promptly growth arrested but undergo limited destruction. We have studied bacterial phospholipid hydrolysis as a possible limiting factor in the disassembly of ingested *E. coli*, comparing the fates, during phagocytosis by rabbit peritoneal exudate PMN, of three isogenic strains, differing in their content of the *pldA* gene encoding the principal *E. coli* phospholipase A (PLA), i.e., *pldA*–, *pldA*+, *pldA*+++ (the latter strain bearing the *pldA* gene in a multicopy plasmid resulting in a 20-fold increase in PLA content). Ingestion and growth inhibition (> 99% within 15 min) were the same for the three strains, but phospholipid degradation differed according to bacterial PLA content: *pldA*+++ up to 60%, *pldA*+ up to 30%, and *pldA*– up to 20%. Since the *pldA*– strain has no activatable PLA, phospholipid degradation in this strain demonstrates the action of a PMN PLA. Added PLA₂-rich ascitic fluid (AF) or purified AF PLA₂ increased the rate and extent of degradation of the *pldA*– strain, provided the enzyme was added before ingestion was complete. ¹²⁵I-AF-PLA₂ binds to both *E. coli* and PMN and thus can enter the vacuole during phagocytosis. Although up to 50-fold more AF-PLA₂ than the PLA₂ content of the PMN could be loaded into the PMN in this way, degradation of *pldA*– *E. coli* did not exceed 30%. Increased phospholipid degradation had no effect on the degradation of bacterial macromolecules. In contrast, bacterial disassembly manifest as structural disorganization, release of bacterial protein derived material, and inhibition of protein synthesis were markedly enhanced when > 50% of prelabelled bacterial phospholipids were degraded. These findings reveal a link between envelope phospholipid degradation and overall bacterial destruction, suggesting therefore that factors limiting PLA action limit the destruction of *E. coli* ingested by PMN. (*J. Clin. Invest.* 1990. 85:1925–1935.) *pldA* gene • plasmid • mutant *Escherichia coli* strains • ascitic fluid • phospholipases A₂

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Introduction

Ingestion of microorganisms by PMN provides an essential first-line defense against infection and sets the stage for the digestive process that accompanies phagocytosis. However, bacterial macromolecular degradation of organisms such as *Escherichia coli* and *Staphylococcus aureus* tends to be only partial (1–4). That effective growth arrest by PMN of *E. coli* occurs with little initial structural and functional disintegration is also indicated by morphological observations (5) and metabolic studies (1, 6), showing continued macromolecular synthesis despite growth arrest. What prevents more extensive destruction and digestion of ingested bacteria is not clear, because PMN contain in their granules a broad-spectrum digestive apparatus, that is delivered early into the phagocytic vacuole during degranulation (7–9). Moreover, under adverse conditions bacteria activate autolytic enzymes (10).

Phospholipids are among the constituents of *E. coli* that undergo limited degradation during phagocytosis (1, 11, 12), raising the possibility that the rate and extent of breakdown of these structural envelope elements determine in part the effectiveness of bacterial destruction.

We have shown before, by comparing phospholipid hydrolysis in wild-type *E. coli* and in a mutant strain lacking activatable phospholipases, that the phospholipolytic enzymes of the PMN as well as of *E. coli* itself can degrade the bacterial phospholipids (11, 13, 14). Isolated bactericidal agents such as the bactericidal permeability-increasing protein (BPI)¹ and the antibiotic polymyxin B, also trigger bacterial phospholipid degradation by both endogenous and exogenous phospholipases (15, 16). The more envelope disruptive the agent the more extensive the hydrolysis, presumably because of greater phospholipase access to various bacterial phospholipid pools (15).

In this study we aimed at (a) identifying the phospholipases participating in breakdown of the phospholipids of *E. coli* ingested and killed by rabbit peritoneal exudate PMN, (b) determining the factors that limit their action, and (c) establishing by morphologic and biochemical criteria the relationship between varying levels of phospholipid hydrolysis and the rate and extent of destruction of ingested *E. coli*.

We have taken advantage of the availability of three isogenic *E. coli* strains differing in their content of the *pldA* gene encoding the principal deacylating phospholipase (A) (PLA) of *E. coli* (17).

1. Abbreviations used in this paper: AF, ascitic fluid; BPI, bactericidal permeability-increasing protein; PLA, phospholipase A.

We show the participation of three defined PLA in the attack on the phospholipids of the ingested *E. coli*: the pldA gene product of *E. coli*, a PMN PLA₂, and a PLA₂ present in the cell-free (ascitic) fluid of the inflammatory exudate (18). We also show that the speed and extent of disassembly of ingested *E. coli* are increased when bacterial phospholipid degradation during phagocytosis is amplified by increasing the bacterial phospholipase content. These findings suggest therefore that the more limited phospholipid degradation produced under normal circumstances limits destruction of ingested *E. coli* by PMN.

Methods

Collection of ascitic fluid (AF) and PMN. Sterile inflammatory exudates were collected from New Zealand White rabbits 14–16 h after intraperitoneal injection with of 250–300 ml of glycogen-saturated saline (1). Cells were sedimented by centrifugation at 100 g for 10 min and resuspended in Hanks' buffered salt solution (HBSS, without phenol red, Microbiological Associates, Bethesda, MD) to a final concentration of 10⁸/ml. The cell-free supernatant was spun at 20,000 g for 30 min to yield AF. Cell suspensions contained > 90% PMN as judged by differential counting.

Preparation of PMN sonicates. PMN were disrupted by sonication (60 W, 15 s, 0–4°C, cell disrupter, Branson Sonic Power Co., Danbury, CT). The efficiency of sonication was monitored by light microscopy.

Growth of *E. coli*. The *E. coli* strains used were 1303 (pldA–, lacking the principal envelope phospholipase), 1602 (pldA+, wild-type strain), and 1303pPI232 (pldA+++), a phospholipase-rich strain containing the pldA gene product in 20-fold excess in a multicopy plasmid (17). *E. coli* were cultured in phosphate-buffered yeast broth (Difco Laboratories, Detroit, MI) (pH 7.3), or in nutrient broth (Difco Laboratories) (pH 7.3). Growth media were supplemented with 50 µg/ml chloramphenicol (Parke-Davis, Morris Plains, NJ) for growth of *E. coli* 1303 pPI232 to select for bacteria containing the plasmid encoding the pld A gene product and chloramphenicol resistance. Bacterial cultures were grown overnight to stationary phase and were then subcultured for 2–3 h in fresh growth medium (diluted 1:10), at 37°C. Bacterial concentrations were determined by measuring absorbance at 550 nm with a Coleman junior spectrophotometer. The bacteria were sedimented by centrifugation at 6,000 g for 10 min and resuspended in sterile physiological saline to a final concentration of 10⁹/ml.

Labeling of *E. coli*. Bacterial constituents were labeled by adding the appropriate radiolabeled precursors to the growth medium as follows: phospholipids, with 0.5 µCi/ml [1-¹⁴C]oleic acid or [1-¹⁴C]palmitic acid (Dupont-New England Nuclear, Boston, MA) as described previously (15); proteins with 0.5 µCi/ml L-¹⁴C amino acids (Dupont-New England Nuclear) or 5 µCi/ml [³⁵S]methionine (Dupont-New England Nuclear); RNA and DNA with 0.5 µCi/ml [¹⁴C]uracil and 1 µCi/ml [¹⁴C]thymidine + 2 mM uridine; peptidoglycan with 1 µCi/ml [¹⁴C]diaminopimelic acid (DAP) + 100 µg/ml lysine (to minimize labeling of protein). After incubation for 2 h at 37°C, the bacteria were sedimented by centrifugation at 3,000 g for 8 min, resuspended in fresh growth medium without added precursors, and further cultured for 30 min at 37°C. The bacteria were washed once with 1% bovine serum albumin in normal saline to remove unincorporated radiolabeled precursors, and resuspended in sterile normal saline to a final concentration of 10⁹/ml. In all cases, ≥ 95% of the incorporated precursor was present in the target macromolecule.

Assay of PLA₂ activity. PLA₂ activity was determined by incubation with 2.5 × 10⁸ autoclaved [1-¹⁴C]oleic acid-labeled *E. coli* (~ 5 nmol phospholipid) (12, 15) at 37°C for 10 min. Incubation mixtures contained 40 mM Tris-HCl (pH 7.5), 10 mM CaCl₂, in a total volume of 250 µl. Reactions were terminated by the addition of an equal volume of ice-cold 0.5% (wt/vol) bovine serum albumin (BSA), and samples were spun in an Eppendorf microfuge for 2 min to sediment *E. coli*. The radioactive products of hydrolysis (free fatty acid and

lysocompounds) released into the supernatant and trapped by association with BSA (19) were quantitated by liquid scintillation counting. One arbitrary unit of phospholipase activity has been defined as 1% hydrolysis per hour.

Standard incubation conditions for intact *E. coli* and PMN. Typical incubation mixtures contained 40% (vol/vol) HBSS, 1.5% (wt/vol) BSA, 2% (vol/vol) human serum, 20 mM Hepes (Sigma Chemical Co., St. Louis, MO; pH 7.4) *E. coli* (2.5 × 10⁷), and rabbit PMN (5 × 10⁶) in a total volume of 250 µl. Serum stored at –20°C before use provided opsonins but had no effect on bacterial viability or phospholipid degradation at the concentration used. Incubations were carried out at 37°C in a shaking (~ 100 rpm) water bath for the indicated times.

Assay of bacterial viability. At the indicated time, 5-µl samples were taken from the incubation mixtures, serially diluted in sterile physiological saline, and plated on nutrient agar plates. The number of colony-forming units was determined after 24 h at 37°C.

Assay of phospholipid degradation in intact *E. coli*. *E. coli* prelabeled with [¹⁴C]oleate or [¹⁴C]palmitate were incubated at 37°C in the presence and absence of PMN for the indicated times. Incubations were terminated by spinning in an Eppendorf microfuge for 2 min to pellet PMN and *E. coli*, and the supernatant was removed. The radiolabeled products of hydrolysis (free fatty acid and/or lysocompounds) released into the supernatant were determined by liquid scintillation counting. The complete recovery of the radiolabeled products of hydrolysis in the supernatant and retention of undegraded phospholipids in the cell pellet was verified by thin-layer chromatography (TLC) (13). Phospholipid degradation is expressed as the percentage of total radiolabel that is recovered as the products of hydrolysis.

Purification of AF PLA₂. The enzyme was purified from rabbit AF as described before (18).

Radiolabeling of AF PLA₂. Radioiodination (20) was carried out for 10 min at 4°C with occasional shaking, in glass tubes precoated with 60 µg of iodogen (Pierce Chemical Co., Rockford, IL), in a reaction mixture containing 35 µg of PLA₂, 20 µCi of Na ¹²⁵I (Dupont-New England Nuclear), 10 mM Tris-HCl (pH 7.5), and 250 µl of saline. The solution was carefully removed without disturbing the iodogen coat, the reaction tube was rinsed with an equal volume of 0.4 N sodium acetate buffer (pH 4.0), and concentrated KI and BSA were added to obtain final concentrations of 0.1 N KI, 1 mg/ml BSA, and 0.2 N sodium acetate buffer (pH 4.0). Radiolabeled phospholipase was separated from free Na ¹²⁵I by chromatography on Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 200 mM sodium acetate buffer (pH 4.0). The recovered PLA₂ was further purified by reverse-phase HPLC on a C4 (Vydac, The Separations Group, Hesperica, CA) column, as described (18). Recovery of PLA₂ activity was > 90%.

Measurement of PLA₂ binding to *E. coli* and PMN. *E. coli* pldA– (2.5 × 10⁷) or PMN (5 × 10⁶) were incubated for 15 min at 37°C with trace ¹²⁵I-labeled PLA₂ (~ 3,000 cpm) and increasing amounts of unlabeled AF PLA₂. Typical incubation mixtures contained 40 mM Tris-HCl (pH 7.4), 10% HBSS, and 0.25% BSA. PMN or *E. coli* were pelleted by centrifugation and washed with buffer to remove all unbound phospholipase, and both pellet and supernatant-associated radioactivity were determined in a γ-counter (Hewlett-Packard Co., Palo Alto, CA). The presence of bound PLA₂ on the *E. coli* in the absence of PMN did not result in any appreciable degradation of bacterial phospholipids (< 5%). Addition of the BPI, purified from PMN (14), to the PLA₂-coated bacteria triggered phospholipid degradation in a manner corresponding to the estimated amount of bound PLA₂, indicating that the radiolabeled tracer PLA₂ and bulk unlabeled PLA₂ have comparable binding properties.

Measurement of bacterial uptake by PMN. *E. coli* prelabeled with [¹⁴C]thymidine were incubated with rabbit PMN in the standard incubation mixtures. At various times (0–60 min), 250 µl of ice-cold heparin (Lyphomed, Inc., Melrose Park, IL) diluted to 200 U/ml in normal saline was added to the incubation mixtures to inhibit phagocytosis (21), and the samples were spun at 100 g for 7 min to separate ingested and extracellular bacteria. The pellet was washed twice with 1% BSA in

HBSS to remove trapped and loosely adherent bacteria. The distribution of the radiolabel was determined by measuring aliquots of the recovered supernatants and the final resuspended cell pellet by liquid scintillation counting. There was no detectable conversion of radiolabeled material from trichloroacetic acid (TCA)-precipitable to a TCA-soluble form during the course of the incubation. Uptake (ingestion) is expressed as the percentage of the total recovered radioactivity that is associated with the cell pellet. Recovery of total radioactivity was $\geq 90\%$.

Assay of bacterial protein synthesis. To measure bacterial protein synthesis, the standard incubation mixture was supplemented with 0.5 mM cycloheximide (to inhibit protein synthesis by the PMN) and 0.1% casamino acids (Difco Laboratories) (1). At various times, L- ^{14}C amino acids (20 $\mu\text{Ci}/\text{ml}$ final concentration) were added and the mixtures were incubated for an additional 30 min. Reactions were terminated by the addition of 250 μl of ice-cold 20% TCA. TCA-precipitable material was collected by filtration through a 0.45- μm Millipore HAWP membrane, washed three times with 1 ml 10% ice-cold TCA, and measured by liquid scintillation counting. Protein synthesis is expressed as the percentage of the amino acid incorporated into TCA-precipitable material by untreated *E. coli* in the first 30 min.

Assay of degradation of bacterial macromolecules. Prelabeled bacteria (5×10^7) were incubated with or without PMN (5×10^6) (or whole PMN sonicate where indicated) in standard incubation mixtures. At the times indicated, reactions were terminated by the addition of 250 μl of ice-cold TCA and placed at 4°C for at least 30 min. TCA-precipitable material was collected and counted as described above. Degradation is expressed as a percent of the initial TCA-precipitable radioactivity converted to TCA-soluble form.

Assay of release of bacterial protein-derived material. *E. coli* prelabeled with ^{14}C - or ^{35}S -amino acids were incubated with either PMN or whole PMN sonicate in standard incubation mixtures. At various times incubations were terminated by spinning in an Eppendorf microfuge for 2 min to pellet *E. coli*. Radiolabel released into the supernatant was quantitated by liquid scintillation counting before and after precipitation with TCA.

Electron microscopy. *E. coli* prelabeled with [^{14}C]oleate were incubated at 37°C for up to 3 h in standard incubation mixtures with a bacteria/PMN ratio of 50:1. Bacterial viability and phospholipid degradation at this ratio were the same as at a ratio of 10:1. For electron microscopy cells were sedimented by centrifugation and the pellets were resuspended in 2.5% glutaraldehyde in Kellenberger's veronal acetate buffer (22), pH 6.1 and fixed for 2 h at 10°C . The cells were then washed three times with the veronal acetate buffer and postfixed with 1% osmium tetroxide in the same buffer for 16 h at 10°C . Cells were stained with uranyl magnesium acetate (0.5%) for 1 h at 20°C and embedded in 1% noble agar. The agar-embedded blocks were dehydrated in increasing concentrations of ethyl alcohol, which was replaced with propylene oxide as a last step. Specimens were infiltrated and embedded in a low-viscosity spurr epoxy resin (Polysciences Inc., Warrington, PA), and thin sections were obtained with an ultratome (using a diamond knife; LKB Instruments, Inc., Gaithersburg, MD). Sections were mounted on Formvar carbon-coated grids and double-stained with uranyl acetate and lead citrate by the method of Frasca and Parks (23). Stained samples were viewed in an Elmiskop 1A (Siemens-Allis, Inc., Cherry Hill, NJ) and images were recorded on electron microscope film 4489 (Eastman Kodak Co., Rochester, NY).

Results

Contribution of cellular (bacterial and PMN) phospholipases to the degradation of the phospholipids of ingested *E. coli*. *E. coli* possess a complex phospholipid-degrading apparatus, but under adverse conditions, the major PLA activated is the pldA gene product, the principal PLA of *E. coli* which is located in the outer envelope of the bacteria (11, 17). To determine the role of this enzyme in the degradation of bacterial phospho-

lipids during phagocytosis by PMN, we compared the fate of three isogenic *E. coli* strains differing in their content of the pldA gene: (a) a wild-type K12 strain (pldA+) containing from 200–500 PLA molecules per bacterium; (b) a mutant strain with a defective pldA gene (pldA-); and (c) this mutant infected with a multicopy plasmid encoding the wild-type pldA gene (pldA+++), raising the bacterial PLA content approximately 20-fold. In the absence of PMN, there was normal bacterial growth and no detectable degradation of bacterial phospholipids. In the presence of PMN, (at bacteria/PMN ratios ranging from 2:1 to 50:1) all three strains were ingested and lost their colony-forming ability within 15–30 min, but the rate and extent of degradation of bacterial phospholipids differed according to bacterial PLA content (Fig. 1). Ingestion of the pldA- strain resulted in a slowly progressive hydrolysis that reached a plateau of $\sim 20\%$ after 3 h of incubation. In contrast, up to 30% and 60% respectively of the prelabeled phospholipids of the wild-type (pldA+) and PLA-rich plasmid-bearing (pldA+++) strains were degraded within 30 min of incubation, indicating that the bacterial PLA is promptly activated by the PMN and that the maximal levels of degradation achieved depend on the amount of available PLA. The radiolabeled products recovered from the pldA- mutant were almost exclusively ($> 95\%$) free fatty acids with [^{14}C]oleate-labeled bacteria and mainly ($\sim 70\%$) lysocompounds with [^{14}C]palmitate-labeled bacteria, consistent with the action of a PMN PLA₂ and, to a lesser extent, a lysophospholipase activity acting before sequestration of the products by BSA. Thus, both the bacterial outer envelope PLA and the PMN PLA₂ contrib-

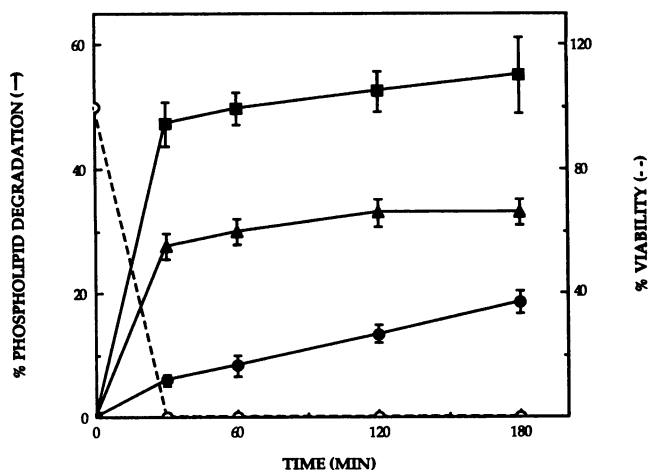


Figure 1. Contribution of *E. coli* and PMN phospholipases to the degradation of bacterial phospholipids during incubation with PMN. Each of the three isogenic *E. coli* strains (2.5×10^7), pldA- (●), pldA+ (▲), pldA+++ (■) prelabeled with [^{14}C]oleic acid during growth were incubated with rabbit PMN (5×10^6) in the standard incubation mixture (250 μl) at 37°C as described in Methods. At the indicated times, phospholipid degradation (solid lines) and bacterial viability (dashed lines) were measured as described in Methods. Each point represents the mean \pm SE of at least five independent determinations. Phospholipid degradation, measured as the accumulation of ^{14}C -labeled hydrolytic products, is expressed as the percent of total *E. coli* radioactivity. Viability of bacteria incubated with PMN is expressed as the percentage of viability of untreated *E. coli*. In the absence of PMN, there is no detectable degradation of bacterial phospholipids.

ute to the degradation of bacterial phospholipids during phagocytosis.

Action of AF PLA₂ on *E. coli* pldA- during phagocytosis by PMN. PMN-rich inflammatory peritoneal exudates also contain large amounts of an extracellular (AF) PLA₂ (18) that is closely similar to a PMN PLA₂ that has been isolated recently in this laboratory (24). To determine whether this PLA₂ could also contribute to bacterial phospholipolysis during phagocytosis, we compared phospholipid degradation in the pldA- strain during incubation with PMN in the presence and absence of added AF PLA₂. AF PLA₂ added either as the purified enzyme or as whole AF caused a dose-dependent increase in the degradation of bacterial phospholipids by PMN (Fig. 2). In the absence of added PLA₂ only 7% of the bacterial phospholipids were degraded during 60-min incubations, compared with 30% in the presence of added enzyme. The effect of the purified enzyme was less than that of the crude AF indicating that other factors in the inflammatory exudate contribute to the PLA₂ action. In the absence of PMN, purified enzyme alone had no detectable effect on either bacterial viability or phospholipid degradation, showing that the action of this extracellular enzyme depended on the presence of PMN.

Evidence that extracellular AF PLA₂ is acting on intracellular *E. coli*. The PMN-dependent action of the AF PLA₂ raised the possibility that the extracellular enzyme acted on ingested bacteria. Parallel assays of bacterial colony-forming ability and uptake demonstrated that inhibition of bacterial multiplication coincided with bacterial uptake by the PMN (correlation coefficient = 0.96) (Fig. 3). Whereas inhibition of bacterial colony formation, and hence ingestion, were complete within 15–30 min, degradation of bacterial phospholipids continued for up to 60 min (Fig. 4) consistent with the

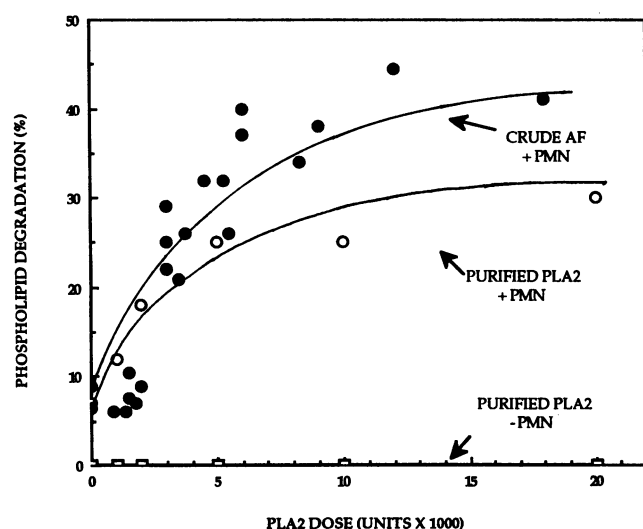


Figure 2. Effect of extracellular AF PLA₂ on degradation of bacterial phospholipids in the presence and absence of PMN. [¹⁴C]Oleate-labeled *E. coli* pldA- (2.5×10^7) were incubated at 37°C for 60 min in standard incubation mixtures containing increasing amounts of either crude AF (solid symbols) or purified AF PLA₂ (open symbols), in the presence (circles) or absence (squares) of PMN (5×10^6). Bacterial phospholipid degradation was measured as described in Methods and is expressed as the percent of total *E. coli* radioactivity. Bacterial viability was not affected by incubation with purified PLA₂ and was reduced to < 1% of control after incubation with PMN.

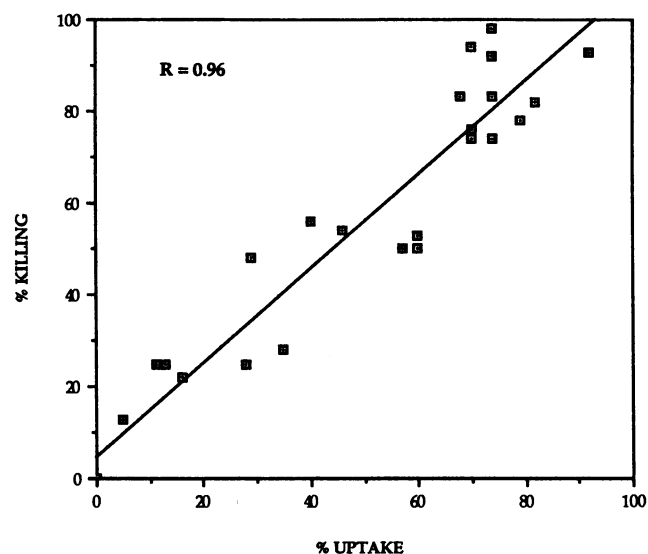


Figure 3. Correlation between uptake and viability of bacteria incubated with PMN. [³H]Thymidine-labeled *E. coli* pldA- (2.5×10^7) and rabbit PMN (5×10^6) were incubated at 37°C as described in Methods, and at various times uptake and bacterial viability were determined. Uptake is expressed as the percentage of total bacterial radioactivity which is associated with the PMN; there was no degradation of bacterial DNA as judged by the recovery of acid precipitable radioactivity. The line drawn represents the best fit determined by linear regression analysis ($r = 0.96$).

action of the extracellular enzyme on intracellular bacteria. Addition of the AF PLA₂ after all *E. coli* (pldA-) had been ingested (≥ 30 min) caused little or no increase in bacterial phospholipid degradation. This protection against the action of the added extracellular PLA₂ further confirms that the

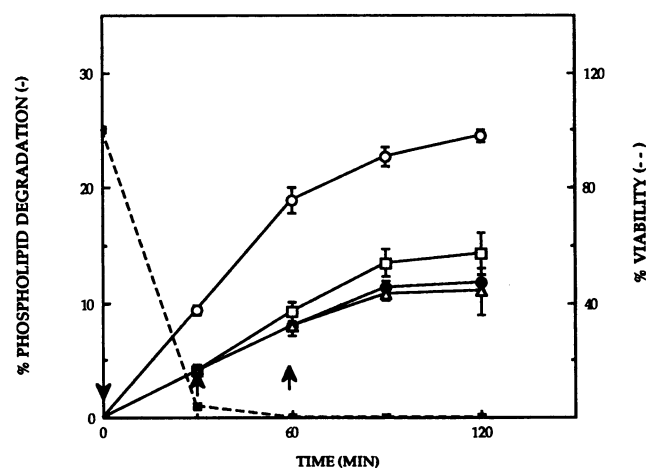


Figure 4. Effect of the time of addition of extracellular AF PLA₂ on the degradation of bacterial phospholipid during incubation with PMN. [¹⁴C]Oleate-labeled *E. coli* pldA- (2.5×10^7) were incubated with rabbit PMN (5×10^6) in standard incubation mixtures, in the absence (solid symbols) and presence (open symbols) of purified AF PLA₂ added at 0 (circles), 30 (squares), and 60 (triangles) min. Addition of AF PLA₂ (5,000 U) is denoted by solid arrows. Phospholipid degradation (solid lines) and bacterial viability (dashed lines) were measured as described in Methods. Each point represents the mean \pm SE of at least three determinations.

PMN-associated bacteria were indeed intracellular and suggests that the action of the AF PLA₂ may require co-internalization of the enzyme with the bacteria.

Mechanism of internalization of an extracellular enzyme. The PLA₂ could be internalized as a component of the ingested extracellular fluid, or if bound to the *E. coli* and/or PMN. Incubation of increasing amounts of ¹²⁵I-AF PLA₂ with *E. coli* pldA⁻ for 15 min at 37°C resulted in binding of nearly 50% of the added PLA₂ over the entire PLA₂ dose range tested (up to 60,000 U (~ 300 ng)/2.5 × 10⁷ *E. coli*). Binding was maximal within 15 min and did not increase during longer incubations. PLA₂ binding was the same when either whole AF or purified PLA₂ was added (Fig. 5) indicating that no other exudate proteins were required to promote PLA₂ binding. Precoating of *E. coli* pldA⁻ with increasing amounts of PLA₂ enhanced bacterial phospholipid degradation during phagocytosis by PMN in a manner similar to that seen when purified enzyme or whole AF was added directly to the incubation mixture (Fig. 5; compare with Fig. 2), indicating that the bound PLA₂ contributed to the digestion of ingested bacteria. Note that despite the delivery of increasing amounts of bound PLA₂ on the ingested *E. coli*, phospholipid degradation did not exceed 35% indicating additional constraints on phospholipase action.

Incubation of PMN with ¹²⁵I-AF PLA₂ and purified unlabeled PLA₂ or whole AF resulted in binding of substantial amounts of the added PLA₂ (data not shown). Precoating of PMN with PLA₂ also enhanced phospholipolysis of ingested *E. coli* pldA⁻ from 7.0 ± 0.5% (*n* = 12) to 35% ± 1.0 (*n* = 7). Precoating of PMN in addition to precoating of *E. coli* did not further increase bacterial phospholipid degradation. It appears therefore that the extracellular phospholipase can enter the phagocytic vacuole either "piggy-back" on the *E. coli* or on invaginated PMN plasma membrane and, once inside, can contribute to the hydrolysis of the bacterial phospholipids.

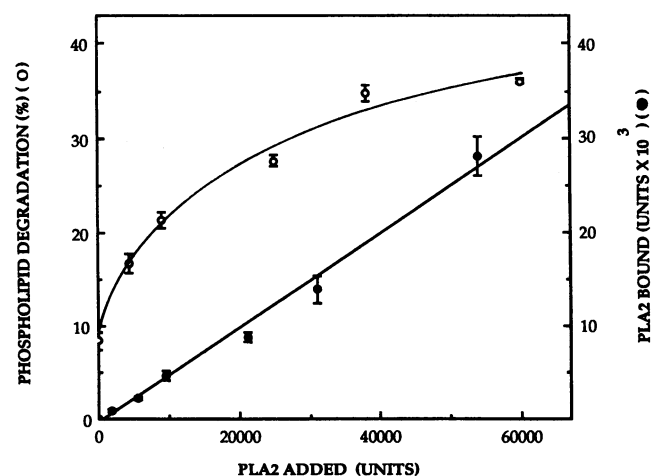


Figure 5. Binding of AF PLA₂ to intact *E. coli* pldA⁻. *E. coli* pldA⁻ (2.5 × 10⁷) prelabeled with [¹⁴C]oleate, were incubated with trace ¹²⁵I-AF PLA₂ and increasing amounts of purified AF PLA₂. *E. coli* were pelleted by centrifugation and binding of PLA₂ (●) was determined (a) as described in Methods and (b) by measuring the residual phospholipase activity in the unbound supernatant. The "precoated" *E. coli* were incubated with PMN (5 × 10⁶) for 60 min at 37°C and phospholipid degradation (○) was measured as described in Methods.

Additive effects of cellular and extracellular phospholipases on ingested *E. coli*. Phospholipid degradation in the pldA⁻ strain was enhanced by the addition of AF PLA₂ but reached a plateau at ~ 30% hydrolysis. With both the pldA⁺ and the pldA⁺⁺⁺ strains, phospholipid degradation was not increased by the addition of extracellular PLA₂ and again leveled off at ~ 30%, and 50%, respectively (Fig. 6). Thus pools of bacterial phospholipid not degraded by the bacterial PLA are also refractory to the added AF PLA₂.

Effect of bacterial phospholipid degradation on the degradation of bacterial macromolecules. To determine the effect of bacterial phospholipid degradation on the breakdown of other bacterial constituents, we compared the degradation of pre-labeled protein, RNA and peptidoglycan in *E. coli* strains pldA⁻, pldA⁺, pldA⁺⁺⁺ during the course of incubation. *E. coli* incubated alone showed no net degradation (loss of acid-precipitable radioactivity) of these three radiolabeled macromolecules. However, as shown before, during phagocytosis all these bacterial constituents underwent prompt and similar degradation that reached a plateau after 30 mins at ~ 50% (1). Degradation was the same in the three strains indicating that increased phospholipid degradation had no noticeable effect on bacterial macromolecular degradation (Table I).

Effect of phospholipid degradation on bacterial protein synthesis. The effect of enhanced phospholipid degradation on bacterial metabolism was assessed by measuring protein synthesis in the three strains during phagocytosis by PMN (Fig. 7). Although ingestion and growth arrest of the three strains were complete within 30 mins (Fig. 1), protein synthesis by the pldA⁻ and pldA⁺ strains was only modestly inhibited even after incubation for up to 3 h. In contrast, protein synthesis by the pldA⁺⁺⁺ strain was inhibited more promptly and markedly, both by about fourfold (Fig. 7).

Effect of phospholipid degradation on the release of bacterial protein during killing by intact PMN and whole PMN sonicates. Effects on amino acid uptake and incorporation into protein, a process which requires an intact cytoplasmic membrane implies that increased phospholipid degradation may result in disruption of the energy-producing apparatus and the overall structural integrity of the bacteria. As a crude index of bacterial disintegration we measured the release of prelabeled bacterial protein-derived material. Fig. 8 A shows the time course of phospholipid degradation and release of protein-derived material from the three *E. coli* strains during incubation with intact PMN. In the absence of PMN there was minimal release of labeled material (≤ 10%) which was mostly acid soluble. During phagocytosis protein release from the pldA⁺⁺⁺ strain was markedly stimulated, but very limited from the pldA⁻ or pldA⁺ strains. Up to 50% of the released material was acid precipitable, i.e., as intact protein or as large fragments.

Estimates of protein release from ingested *E. coli* are complicated because protein released from the bacteria may also have to traverse PMN phagolysosomal and plasma membrane barriers to reach the extracellular medium. To measure more directly release of protein-derived material from *E. coli* further studies were carried out with whole PMN sonicates that are as growth inhibitory against *E. coli* as intact PMN (1, 25, 26).

When incubated with fully growth inhibitory concentrations of whole PMN sonicate the three *E. coli* strains exhibited levels of phospholipid degradation equal to those seen with intact PMN. The levels of release of protein-derived material

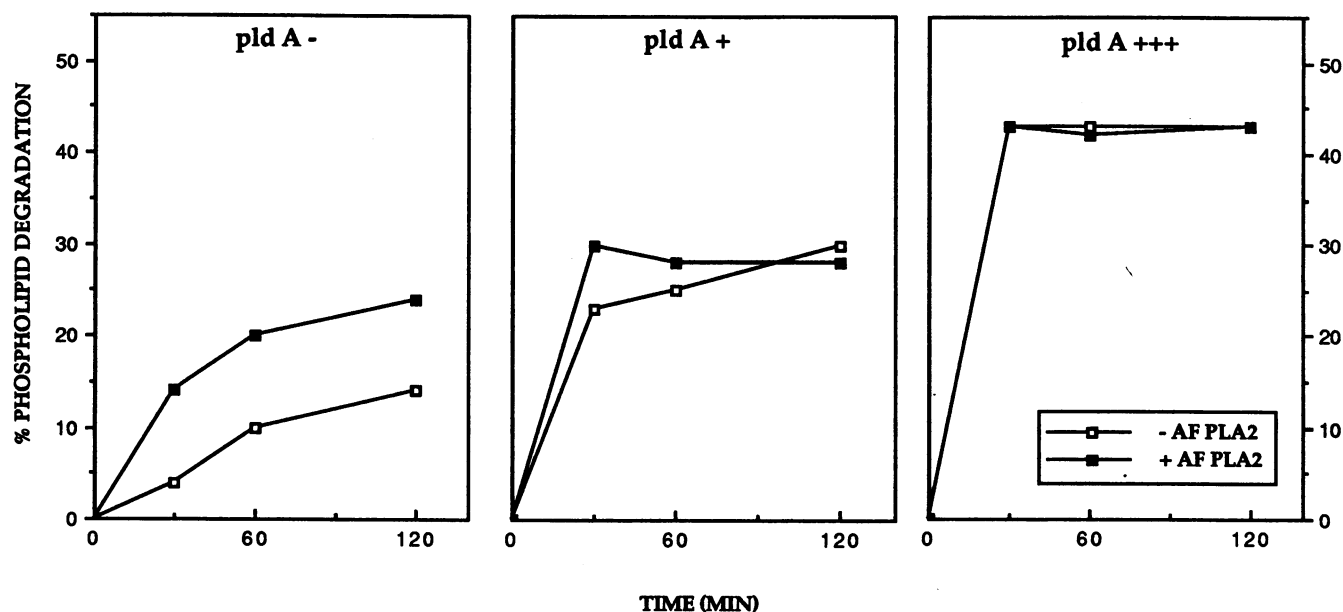


Figure 6. Additive effect of extracellular and cellular phospholipases on ingested *E. coli*. Each of the three isogenic *E. coli* strains (2.5×10^7) (pldA⁻, pldA⁺, pldA⁺⁺⁺), prelabeled with [¹⁴C]oleate, were incubated with rabbit PMN (5×10^6) in standard incubation mixtures, in the presence (■) and absence (□) of added AF PLA₂ (4,000 U) and phospholipid degradation was measured. Each point represents the mean of at least two determinations. Bacterial viability was < 1% during incubation with PMN.

were also similar to those obtained with intact cells; 40% in the pldA⁺⁺⁺ strain compared to ≤ 4% in both the pldA⁻ and pldA⁺ strains (Fig. 8 B). Release was maximal within 30–60 min in contrast to the 6–8 h seen with intact PMN. All of the released material was acid precipitable, reflecting the lack of protein-degradative activity of PMN sonicate toward *E. coli* (27).

Effect of increased phospholipid degradation on the fine structure of ingested E. coli. To monitor more directly the structural integrity of ingested *E. coli* in relation to bacterial phospholipid degradation, thin sections of PMN-*E. coli* suspensions after incubation for up to 3 h were prepared for elec-

Table I. Effect of Bacterial Phospholipid Hydrolysis on Degradation of Other Bacterial Components during Phagocytosis

Strain	Degradation of		
	Protein	RNA	Peptidoglycan
	% remaining		
pldA ⁻	59.6±2.2	42.6±2.9	45.4±1.3
pldA ⁺	77.0±2.2	43.4±4.5	51.0±2.7
pldA ⁺⁺⁺	65.0±4.4	49.4±5.1	44.6±1.2

E. coli strains pldA⁻, pldA⁺, pldA⁺⁺⁺, prelabeled during growth, were incubated with rabbit PMN in standard incubation mixtures and degradation of protein, RNA, and peptidoglycan was measured as described in Methods. Degradation is expressed as the percentage of remaining TCA-precipitable radioactivity.

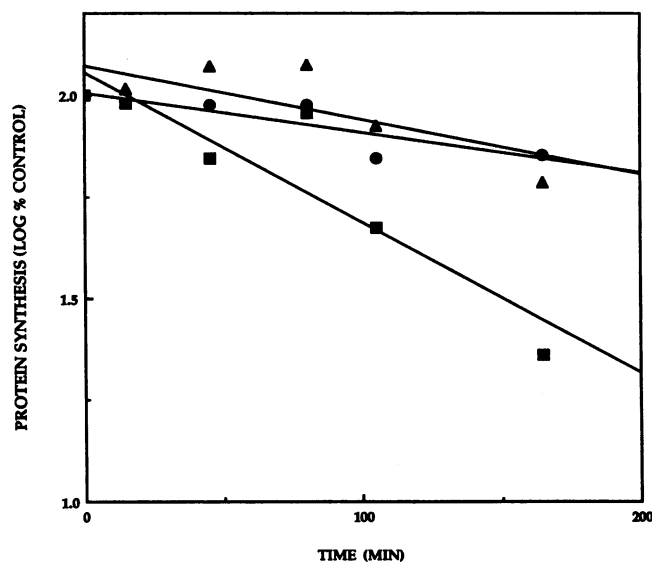


Figure 7. Protein synthesis by *E. coli* during incubation with PMN. Each of the three isogenic *E. coli* strains (2.5×10^7) pldA⁻ (●), pldA⁺ (▲), pldA⁺⁺⁺ (■) were incubated with PMN (5×10^6) in standard incubation mixtures (250 μ l) at 37°C as described in Methods. At the indicated times, [¹⁴C]-amino acids were added and bacterial protein synthesis was measured as described in Methods. Protein synthesis is expressed as the log of the percent of total [¹⁴C]-amino acids incorporated into TCA-precipitable material in the first 30 min by *E. coli* incubated alone. Lines drawn represent the best fit determined by linear regression analysis ($r > 0.8$). The slopes for the pldA⁻, pldA⁺, pldA⁺⁺⁺ strains were respectively 0.98, 1.32, and 3.78. Viability of *E. coli* incubated with PMN for 30 min was < 1% of untreated bacteria.

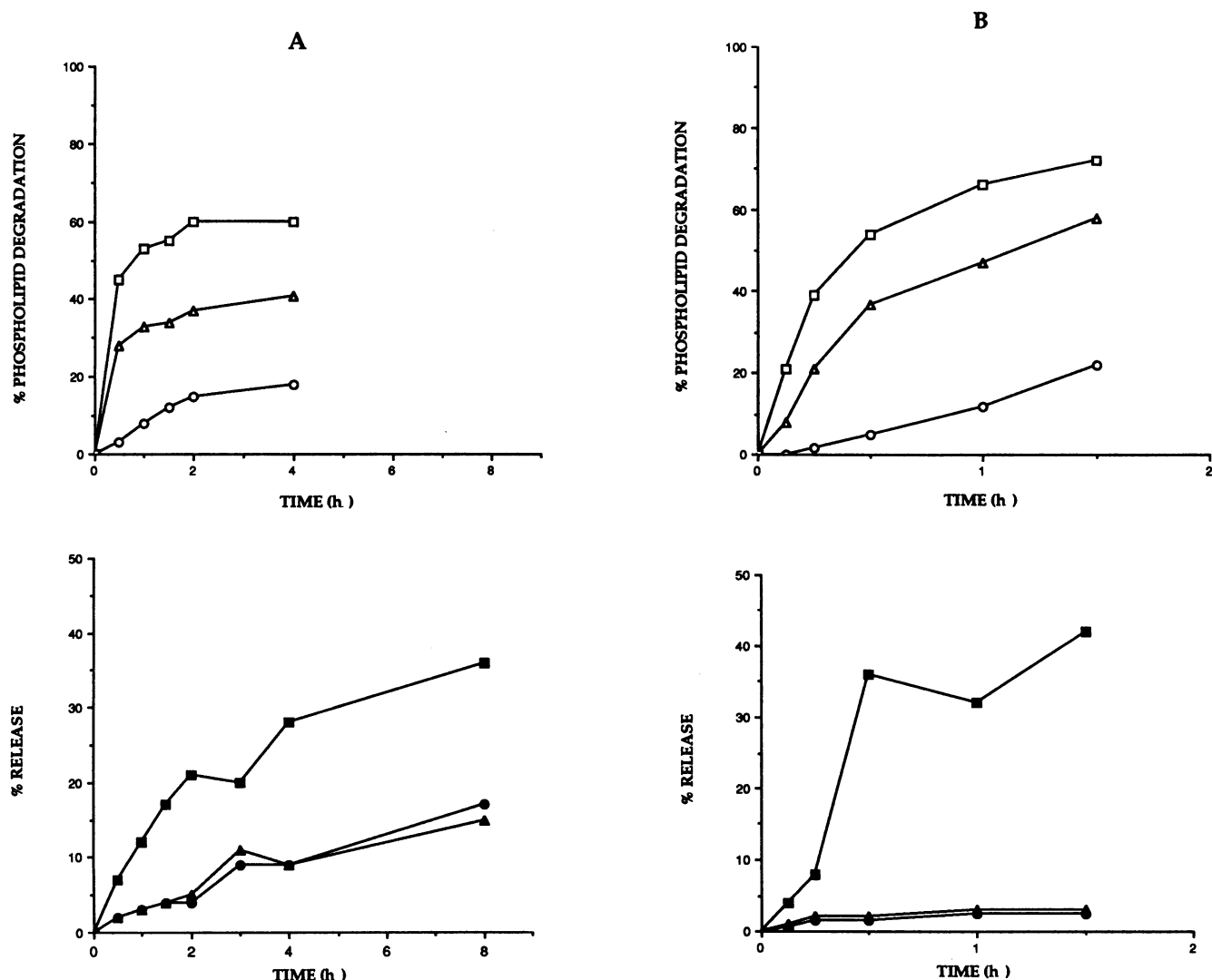


Figure 8. Effect of phospholipid degradation on the release of bacterial protein derived-material during incubation with (A) intact PMN and (B) whole PMN sonicates. [^{14}C]Oleate or [^{14}C]amino acid-labeled *E. coli* strains pldA $^-$ (circles), pldA $^+$ (triangles), pldA $^{+++}$ (squares) were incubated with rabbit PMN (5×10^7 *E. coli*/ 5×10^6 PMN) or whole PMN sonicates (10^7 *E. coli*/ 10^6 PMN equivalents) in standard incubation mixtures. Phospholipid degradation (open symbols, top panels) and release of bacterial protein-derived material (solid symbols, bottom panels) were measured as described in Methods. Viability of *E. coli* treated with either intact PMN or PMN sonicates was < 1% of untreated *E. coli*.

tron microscopy. After 30 min of incubation 84% of the ingested *E. coli* pldA $^-$ showed little or no detectable ultrastructural alteration (Table II). Only after 3 h of incubation was more extensive damage (consisting of gross plasmolysis with condensation of cytoplasmic contents and ghost formation and/or lysis) apparent in the majority of the bacterial population (compare Fig. 9, A and B; Table II). Addition of AF PLA $_2$ during ingestion of *E. coli* pldA $^-$ did not markedly accelerate or amplify the ultrastructural disintegration. In contrast, ~ 70% of ingested *E. coli* pldA $^{+++}$ showed signs of extensive damage after only 30 min of incubation which progressed over the course of 3 h (Fig. 9, C and D; Table II). Thus, when > 50% of the bacterial phospholipids are degraded, overall destruction is more rapid and pronounced. The morphology of the PMN is also dramatically altered; evident as giant phagolysosomes containing multiple bacteria or bacterial remnants and near complete degranulation (Fig. 9, C and D).

Discussion

These studies of bacterial phospholipid degradation during the interaction of rabbit PMN with three isogenic *E. coli* strains, genetically manipulated to vary their content of the principal *E. coli* PLA, provide new insights into (a) the determinants of the phospholipid degradation that is part of the post-phagocytic process and (b) its role in the overall destruction of ingested *E. coli*.

Identification of PLA participating in bacterial phospholipolysis. In the inflammatory environment, phospholipid-degrading enzymes are present in the cellular and extracellular elements mobilized by the host in response to microbial invasion (28, 29), as well as in the target bacteria (11). Among the many classes of phospholipases present in prokaryotic and eukaryotic cells and fluids (30, 31), we have identified three deacylating phospholipases (PLA) that participate in the hydroly-

Table II. Degree of Bacterial Damage

Time min	pIdA-				pIdA-(+)				pIdA+++			
	0	1+	2+	3+	0	1+	2+	3+	0	1+	2+	3+
	% of total											
30	60	24	9	7	62	23	13	2	12	19	30	39
60	56	19	13	12	56	27	13	4	9	7	41	33
120	40	35	11	14	27	16	27	30	4	12	27	57
180	17	22	23	38	25	15	30	30	3	5	34	58

The experiment was carried out as described in Methods. Intracellular bacteria were counted and evaluated for structural alterations: 0, intact bacteria; 1+, plasmolysis; 2+, gross plasmolysis with condensation of cytoplasmic contents; 3+, ghost formation and/or lysis. The data in the table indicate the percentage of bacteria displaying these structural changes. At least 100 bacteria of each strain were evaluated for each time point and results are presented as the percentage of total bacteria evaluated at each time point. Sections were evaluated by three independent observers and the standard error of these determinations was < 2% for each time point.

sis of the phospholipids of ingested *E. coli*: the pIdA gene product encoding an *E. coli* PLA with limited substrate specificity (17, 32, 33), and two PLA₂ that are structurally and functionally closely similar—(a) a well-defined protein present in the cell-free fluid of sterile inflammatory exudates produced in the peritoneal cavity of rabbits (34), and (b) a granule associated PMN PLA₂ that has been purified recently to homogeneity (24). Under the conditions of these experiments the contribution of lysophospholipases has been minimized by the use of albumin in the incubation mixture. Albumin is an effective trapping agent of both free fatty acids and lysocompounds and thereby removes the lysophospholipase substrate (19). Since no labeled diglyceride or phosphatidic acid accumulates, neither phospholipase C (PLC) nor phospholipase D (PLD) appears to be involved in the attack on the *E. coli* phospholipids.

The bacterial PLA plays the dominant role in the hydrolysis of the phospholipids of ingested *E. coli*. The comparison of the three isogenic *E. coli* strains establishes that among the several phospholipases that have been identified in *E. coli*, it is the pIdA gene product that is activated during phagocytosis. Two PLA genes have been identified by Nojima and Doi and their co-workers (35, 36) in *E. coli*, one encoding a detergent-sensitive PLA (dsPLA), and the other a detergent-resistant PLA (drPLA). The *E. coli* mutant (1303, pIdA-) used in this study only lacks the gene for the drPLA (17). However, since this strain shows no hydrolysis when exposed to bactericidal agents such as BPI and polymyxin B (unpublished observations), it follows that the drPLA is the enzyme that is activated under adverse conditions. Moreover, the insertion of multiple copies of the pIdA gene in this mutant, resulting in a 20-fold increase in PLA content relative to the wild type-parent strain (1602, pIdA+), increased bacterial phospholipid degradation in response to either PMN (Fig. 1), BPI, or polymyxin B (Weiss et al., manuscript in preparation). It should be noted that under physiological conditions, the presence of a large excess of this gene product has no effect on the phospholipids nor on the growth of the organism, indicating that the characteristic tight control of this enzyme remains intact in the absence of major perturbations.

The use of the pIdA- strain and analysis of the radioactive products of hydrolysis of both ¹⁴C-16:0 and ¹⁴C-18:1 labeled *E. coli* phospholipids in this and our earlier studies (13) also implicate two host PLA₂ in the observed degradation. One of these is a PMN PLA₂, acting on the ingested *E. coli* when no other PLA are in evidence. A novel finding is that the PLA₂ present in the cell-free fluid of the peritoneal exudate also contributes to the phospholipolysis of ingested *E. coli*. Addition of this PLA₂ produced a dose-dependent increase in the phospholipid degradation of ingested *E. coli* pIdA- (Figs. 2 and 4). This is remarkable because it demonstrates that a component present in the fluid phase of the inflammatory environment can be translocated into the phagolysosome via the ingested *E. coli* and/or invaginated PMN membrane, and once internalized can contribute to bacterial degradation.

These findings indicate that the extent of bacterial phospholipid degraded during phagocytosis is a function of the amount of available bacterial PLA or host PLA₂. Apparently the closely related AF PLA₂ (24) can supplement limiting amounts of cellular PLA₂. However, other factors also limit bacterial phospholipid hydrolysis. Thus, (a) a 20-fold increase in bacterial PLA only increases phospholipid degradation twofold, to a maximum of 60% (Fig. 1); (b) addition of large amounts of AF PLA₂ caused proportionally increased enzyme binding, but no further increase in hydrolysis (Fig. 5); (c) addition of large amounts of AF PLA₂ to *E. coli* pIdA+ and pIdA+++ also did not further increase phospholipolysis (Fig. 6). These results imply that substantial pools of the bacterial phospholipids remain inaccessible to PLA action, even in the pIdA+++ strain. In both the wild-type and in the pIdA+++ strains the pIdA gene product is tightly and exclusively associated with the outer membrane (17, 33), suggesting that the accessible phospholipid substrate is mainly limited to that portion of the envelope lipid that is located in the outer membrane (about half) (37). Because bacterial and host PLA actions are not additive, the phospholipids in the inner membrane may also be less accessible to the PMN and AF PLA₂.

What triggers bacterial phospholipolysis during phagocytosis by PMN? Neither endogenous nor exogenous phospholipases cause detectable phospholipid degradation in unperturbed *E. coli* (11, 38, 39). The action of all three phospholipases on *E. coli* is triggered by agents and conditions associated with membrane perturbation or bacterial trauma (40). A major component of the complex antimicrobial arsenal of the PMN is the BPI (11). Effects of BPI on gram-negative bacteria include prompt growth inhibition, an immediate breakdown of the outer membrane permeability barrier, and activation of bacterial phospholipases and of a few selected exogenous PLA₂ (e.g., of PMN and AF) (14, 19, 39–41). The prolonged preservation of bacterial protein synthesis and incomplete phospholipid degradation seen when *E. coli* are ingested by PMN are also typical of BPI action (25). These remarkable similarities between the initial injuries inflicted by isolated BPI and by intact PMN, together with the evidence that BPI binds to ingested *E. coli* (42), are compatible with the hypothesis that BPI accounts for PLA activation during phagocytosis.

Effect of phospholipid degradation on the overall fate of ingested E. coli. We have previously proposed that limited phospholipid hydrolysis may be a factor in limiting overall digestion and destruction of ingested *E. coli* (1, 10). This study, using three *E. coli* strains that were equally effectively ingested and growth arrested, but that underwent different levels of

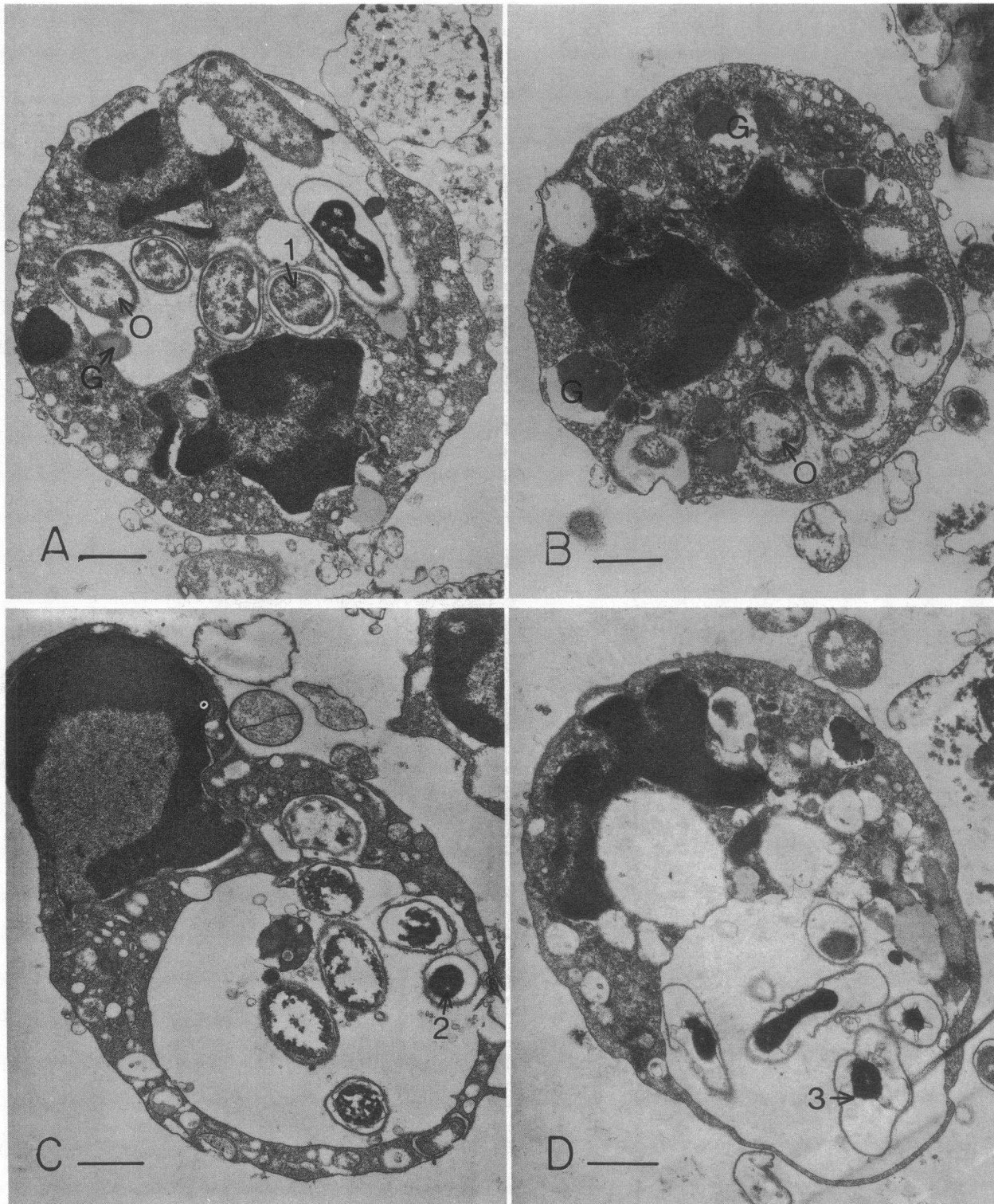


Figure 9. Effect of phospholipid degradation on the morphology of ingested bacteria. Transmission electron micrographs of ingested *E. coli* pldA⁻ after (A) 60 min and (B) 180 min of incubation with PMN, and of *E. coli* pldA⁺⁺⁺ after (C) 30 min and (D) 120 min of incubation with PMN. 0, 1, 2, and 3, bacteria at different stages of destruction (see legend to Table II); G cytoplasmic granules. The experiment was carried out as described in Methods.

phospholipolysis, supports this contention. When phospholipid hydrolysis is increased beyond the level that can be reached during phagocytosis of the wild-type strain, by raising

the bacterial PLA content through genetic manipulation, overall destruction is markedly enhanced. This shows for the first time that phospholipid degradation can play a major role

in subsequent disassembly of the ingested *E. coli*, thereby contributing to the ultimate disposal of the killed bacteria. This is best illustrated by comparison of the ultrastructural appearance of ingested *E. coli* pldA⁻ and of *E. coli* pldA⁺⁺⁺ (Fig. 9, B and C). The release of bacterial protein-derived material and the more rapid inhibition of protein synthesis by the ingested *E. coli* pldA⁺⁺⁺ also provides biochemical evidence that more extensive bacterial phospholipid hydrolysis during phagocytosis is accompanied by gross envelope alterations that are not apparent at lower levels of phospholipolysis. The substantial extracellular release of TCA-precipitable radiolabeled material from ingested ¹⁴C-amino acid-labeled *E. coli* pldA⁺⁺⁺ (Fig. 8) may also point to structural alterations in the PMN membranes. Profound morphologic changes are indeed seen in the PMN that ingested pldA⁺⁺⁺ *E. coli*, consisting of giant vacuoles, presumably formed by fusion of multiple smaller phagosomes, and the absence of recognizable granules at 30 min (compare Fig. 9, A and C). These observations suggest that the activation of the increased bacterial PLA content of the plasmid-carrying *E. coli* has deleterious effects not only on the ingested bacteria, but also on the phagocyte. How the effect on the PMN is mediated is not yet clear.

Release of ¹⁴C-amino acid-labeled material from the pldA⁺ strain during exposure to either intact PMN or broken PMN was no greater than from the pldA⁻ strain, despite a substantial difference in phospholipid degradation (Fig. 8). This suggests that there is not simply a graded effect of phospholipid degradation on the extent of bacterial destruction, but rather that major structural alterations do not become apparent until a critical threshold of phospholipid hydrolysis is reached or certain pools are degraded. Further, bacterial phospholipid synthesis continues and may be stimulated, when phospholipolysis is triggered in *E. coli* growth-inhibited by cell-free PMN preparations. However, when hydrolysis exceeds 50% phospholipid biosynthesis is inhibited (Weiss et al., manuscript in preparation). Therefore, the ability of the bacteria to compensate for phospholipid breakdown by synthesis may temper the structural consequences of phospholipase action.

Two sets of observations (not shown) establish that the more pronounced envelope alterations during phagocytosis of the pldA⁺⁺⁺ strain are linked to increased phospholipid degradation and not to the presence of a high copy plasmid: First, during phagocytosis, two pldA⁻ *E. coli* strains containing a high-copy plasmid (a) *E. coli* pPI2342, bearing the same plasmid as *E. coli* pPI232 but with an insertion mutation resulting in an inactive truncated gene product, and (b) *E. coli* pBR322 carrying an unrelated plasmid, are as resistant to destruction as are the pldA⁻ and the pldA⁺ strains. Secondly, the structural alterations observed in the pldA⁺⁺⁺ strain can be reproduced in the pldA⁻ strain treated with bactericidal concentrations of cell-free PMN fractions supplemented with purified AF PLA₂ in amounts that cause the same high level of phospholipolysis.

In striking contrast to the enhanced hydrolysis of phospholipid and the release of more bacterial protein-derived material during phagocytosis of the pldA⁺⁺⁺ *E. coli*, compared with the pldA⁺ and pldA⁻ strains, degradation of specifically labeled bacterial protein, RNA, and peptidoglycan of the three strains is not appreciably different. Apparently, the more extensive bacterial destruction does not imply greater effectiveness of the degradative enzymes of bacteria and host acting on these macromolecules. However, we cannot yet exclude subtle

differences in the degree of fragmentation of the macromolecules of the three strains during phagocytosis.

In conclusion, these studies support our previous contention that in the interaction between PMN and bacteria, phospholipid hydrolysis may be a limiting factor in the rate and extent of bacterial destruction. Hence, the finding that a PLA₂ in the cell-free fluid of the inflammatory exudate enhances the degradation of the phospholipids of the internalized *E. coli* points to an integrated host-defense system in which disassembly of invading microorganisms while initiated, but only partly executed, by the short-lived PMN is amplified by extracellular factors and may be continued by longer-lived scavenger cells that appear on the inflammatory scene later. Alternatively, bacterial remnants remain in the host environment with potentially pathologic consequences, or to maintain immunological responses. These concepts are now ready for further exploration.

Acknowledgments

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