Determinants of Activation by Complement of Group II Phospholipase A2 Acting against *Escherichia coli*

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Prompt killing of many strains of *Escherichia coli* during phagocytosis in vitro by isolated polymorphonuclear leukocytes (PMN) requires the presence of nonlethal doses of nonimmune serum (B. A. Mannon, J. Weiss, and P. Elsbach, J. Clin. Invest. 86:631–641, 1990). Because this requirement is bypassed in a phospholipase A (PLA)-rich mutant (*pldA*⁺⁺⁺) of *E. coli*, we have examined the effect of serum on bacterial phospholipid (PL) degradation during phagocytosis of wild-type (*pldA*⁺) and PLA-deficient (*pldA*) *E. coli*. In parallel with increased killing, nonlethal doses of serum increased the degradation of prelabeled bacterial PL during phagocytosis by two- to fivefold, to nearly the same levels (ca. 50 to 60%) as those produced during phagocytosis of *E. coli pldA*⁺⁺⁺ in the absence of serum. The effects on the *E. coli pldA* mutant imply that there is a serum-mediated enhancement of granule-associated group II PMN PLA2 activity. At the same doses, serum promoted action against *E. coli* in the presence of purified rabbit and human group II PLA2 but did not activate bacterial PLA. Related PLA2s that lack specific structural determinants needed for optimal activity against *E. coli* treated with the bactericidal/permeability-increasing protein (BPI) of PMN are also less active than wild-type group II PLA2 against serum-treated *E. coli*. Treatment of *E. coli* with C7- or C9-depleted serum did not enhance bacterial killing or PL degradation during phagocytosis or the action of purified PLA2. In summary, these findings suggest that (i) nonlethal assemblies of the membrane attack complex promote intracellular killing and destruction of *E. coli* ingested by PMN, in part by promoting the action of granule-associated PLA2 against ingested bacteria, and (ii) structural determinants first implicated in PLA2 action against BPI-treated *E. coli* are also important in PLA2 action in concert with other host defense systems, such as complement.

In higher organisms, defense against bacterial invaders depends on the prompt mobilization of specialized cells (e.g., polymorphonuclear leukocytes [PMN]) and extracellular proteins (e.g., complement proteins and antibodies) from the blood to extravascular sites of invasion (3). A major function of the PMN is the arrest and elimination of proliferating extracellular microorganisms, which is accomplished in large part by phagocytosis and the intracellular destruction of the ingested microbes (11, 21, 37). Generally, phagocytosis by PMN requires the help of extracellular opsonins, such as antibodies and complement [e.g., C3b(i)] (3, 26). The intracellular killing and disassembly of ingested bacteria may also be promoted by extracellular factors that attach to the surface of the bacterium before phagocytosis. We have previously shown that the rate and extent of intracellular killing and overall destruction of many strains of *Escherichia coli* (and other species of gram-negative bacteria) ingested by PMN are markedly enhanced by preexposure of the bacteria to nonlethal doses of nonimmune serum (12, 23). This effect of serum requires the presence of late components of the complement system, suggesting that it is mediated by nonlethal assemblies of the membrane attack complex (MAC) that form on the surface of the bacterium during opsonization. In the absence of serum (or the presence of C7-depleted serum), damage to ingested bacteria, including degradation of envelope phospholipids (PL), appears limited mainly to the outer envelope, and the effects on viability are (initially) bacteriostatic. In contrast, after bacteria are exposed

to nonlethal doses of serum before phagocytosis, ingestion is followed by rapid killing coincident with disruption of the metabolic machinery associated with the inner (cytoplasmic) membrane.

How serum (i.e., nonlethal assemblies of MAC) promotes intracellular destruction of gram-negative bacteria is unknown. Because PL are essential structural elements in the bacterial envelope, we have speculated that factors controlling phospholipase action may play an important role in determining the rate and extent of bacterial damage during phagocytosis. In support of this hypothesis, we have previously shown that in a strain (*pldA*⁺⁺⁺) of *E. coli* containing 20 times the normal amount of the principal bacterial envelope phospholipase, phospholipase A (PLA), bacterial PL degradation during phagocytosis in the absence of serum is increased to levels that may indicate that the degradation of inner membrane PL is included, and bacterial killing and overall disassembly are markedly enhanced (23, 35). We now show that serum (complement)-dependent killing during phagocytosis of wild-type (*pldA*⁺) and PLA-deficient (*pldA*) cells is paralleled by marked enhancement of bacterial PL degradation, providing evidence suggesting that the increase in PL degradation reflects the activation of granule-associated PMN PLA2 by nonlethal assemblies of MAC. The combined action of complement and PLA2 under these conditions depends on the same structural determinants within a variable surface region of PLA2 that have been identified as being important in PLA2 action against *E. coli* exposed to the bactericidal/permeability-increasing protein (BPI) of PMN (32, 33). The ability of PLA2 to act in concert with BPI protein and/or complement may enable it to play an important role in the intracellular and extracellular destruction of *E. coli* and other gram-negative bacteria.

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MATERIALS AND METHODS

Preparation of PMN. Sterile inflammatory exudates from New Zealand White rabbits were collected 16 h after intraperitoneal injection of 300 ml of sterile physiological saline containing 2.5 mg of oyster glycogen per ml. PMN were sedimented at $100 \times g$ for 10 min and resuspended to a concentration of 10^8 per ml in Hanks' balanced salt solution (Whittaker). The cell concentration was determined by counting cells in a hemacytometer after dilution with 3% glacial acetic acid.

Preparation of serum. Venous blood was collected from healthy donors after informed consent. Rabbit blood was collected by puncture of the central auricular artery. After clot formation (1 h at 37°C and 1 h at 4°C), serum was separated by centrifugation at $10,000 \times g$ for 20 min and stored at -70°C . Serum which contained full complement activity but from which group II PLA2 had been depleted was obtained by adsorption of PLA2 to carboxymethyl-Sephadex (27, 36). C7- and C9-depleted human sera as well as purified C7 and C9 were purchased from Quidel. Rabbit C6-deficient serum was collected from C6-deficient rabbits (National Institutes of Health stock). All complement-depleted and -deficient sera that were used lacked bactericidal activity toward serum-sensitive strains of *E. coli*. This activity was fully restored by the addition of the missing complement protein in purified form to the serum (to 100 $\mu\text{g}/\text{ml}$).

Bacterial strains and growth conditions. *E. coli* PC1602 (*pldA*⁺) and CE1303 (*pldA* mutant) (35) were grown overnight in nutrient broth and then in subculture (diluted 25-fold in fresh medium) at 37°C for 2.5 to 3 h to mid-log phase. Bacteria were harvested by sedimentation at $14,000 \times g$ for 4 min and resuspended in saline. The bacterial concentration was determined by measuring A_{550} .

Radiolabeling of bacterial PL. Bacterial PL were labeled during growth by supplementing the growth medium with 0.2 μCi of [$1\text{-}^{14}\text{C}$]oleic acid per ml (56 mCi/mmol; DuPont/New England Nuclear, Boston, Mass.) as previously described (35). After being harvested, the bacteria were washed once with 1% (wt/vol) bovine serum albumin (BSA) and resuspended in saline to the desired concentration.

Purification of PLA2. Recombinant human group II and pig pancreatic group I PLA2s were purified as described previously (32, 33). Rabbit group II PLA2 was purified from inflammatory ascitic fluid as previously described (27). The PLA2 concentration was determined by measuring enzyme activity toward autoclaved *E. coli* (36).

Incubation of bacteria with PMN. *E. coli* cells (2.5×10^8 per ml) were resuspended in Hanks' balanced salt solution supplemented with 25 mM Tris-HCl or 10 mM HEPES (*N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid) buffer (pH 7.4) and 1.5% (wt/vol) BSA and incubated in the presence or absence of serum for 15 min at 37°C before the addition of buffered Hanks' balanced salt solution with or without PMN to give final concentrations of 2×10^8 bacteria per ml and 2×10^7 PMN per ml. Samples were incubated with shaking for various times before assays of bacterial viability and PL degradation.

Assay of bacterial viability. Aliquots of bacterial samples were vortexed and serially diluted in saline. Aliquots of the diluted samples were plated in nutrient agar with or without 0.1% BSA and incubated overnight at 37°C . Bacterial viability was determined by counting colonies and dividing the number of CFU in the experimental sample by the number of CFU in the bacterial inoculum (i.e., at time 0).

Assay of bacterial PL degradation. Samples of [$1\text{-}^{14}\text{C}$]oleate-labeled *E. coli* incubated with or without serum with or without PMN were vortexed and sedimented at $14,000 \times g$ for 4 min in an Eppendorf microcentrifuge to separate undigested PL cosedimenting with the bacteria from PL breakdown products that are quantitatively recovered in the supernatant (35). Radioactivity in the recovered supernatants was measured by liquid scintillation counting (35). Percent PL degradation was calculated by subtracting the count for the supernatant of the untreated sample from the count for the supernatant of the experimental sample, dividing that by the total count for the bacterial suspension, and multiplying that by 100. Treatment of bacteria with lethal doses of serum (complement) causes release of undegraded PL (13, 18, 34). Therefore, in these samples, recovered supernatants were extracted and analyzed by thin-layer chromatography to quantitate PL degradation (30). Bacterial PL degradation in the absence of serum and PMN was $<3\%$.

RESULTS

Pretreatment of *E. coli* with serum enhances bacterial PL degradation during phagocytosis by rabbit PMN. To determine whether the enhancement of the killing of *E. coli* by PMN with serum is accompanied by increased PL degradation, [$1\text{-}^{14}\text{C}$]oleate-labeled *E. coli* PC1602 (*pldA*⁺) was preincubated with or without nonlethal doses of serum (ca. 1 to 2%, vol/vol) and then incubated with PMN. As was shown before (8, 23), in the absence of serum, nearly all of the added bacteria were rapidly (≤ 15 min) ingested by PMN, suffered degradation of the prelabeled envelope PL of up to 30% (Fig. 1D), and lost their ability to replicate and form colonies in nutrient agar

FIG. 1. Pretreatment of *E. coli pldA*⁺ with nonlethal doses of serum enhances bacterial killing and PL degradation during phagocytosis by rabbit PMN. Radiolabeled *E. coli* PC1602 was incubated for 15 min with (+) or without (−) 2% (vol/vol) pooled normal human serum before further incubation with or without rabbit PMN as described in Materials and Methods. At the times indicated (relative to the addition of PMN), aliquots were taken for measurements of bacterial viability (A and B) and PL degradation (C and D) as described in Materials and Methods. Open symbols denote results without serum, and closed symbols indicate results with serum. Note that serum was added to some serum-free mixtures 30 min after the addition of PMN. Bacterial viability was measured in nutrient agar without (solid lines) and with (broken lines) 0.1% (wt/vol) BSA and was calculated as percent viability of the initial bacterial inoculum. Bacterial PL degradation was calculated as percent total bacterial radioactivity converted to PL breakdown products, with the percentage present in the initial inoculum ($<3\%$) being subtracted. All results shown represent the means $\pm$ standard errors of the means (where indicated) of three or more independent determinations.

(Fig. 1B). However, even after a 60-min incubation with PMN, $\geq 50\%$ of the bacteria could still form colonies after they were plated in nutrient agar supplemented with albumin, demonstrating that most of the bacteria were not dead (Fig. 1B). In contrast, after the bacteria were first pretreated with nonlethal doses of serum, the bacteria ingested by PMN were rapidly killed (Fig. 1B) and up to 50% of the prelabeled bacterial PL were degraded (Fig. 1D). At the doses used, serum alone had little or no effect on bacterial viability (Fig. 1A) and PL (Fig. 1C) and caused little increase in bacterial killing after being added 30 min after the exposure of *E. coli* to PMN (Fig. 1B and D). Thus, pretreatment of *E. coli* with nonlethal doses of serum before phagocytosis by PMN promotes both intracellular bacterial killing and degradation of bacterial PL.

Serum enhances PMN PLA action during phagocytosis of *E. coli*. Bacterial PL degradation during phagocytosis of *E. coli pldA*⁺ by PMN apparently reflects the combined action of the bacterial *pldA* gene product and granule-associated PMN PLA2 (35). To determine more directly the effect of serum treatment on the action of PMN PLA2 toward ingested *E. coli*, we carried out similar experiments with a *pldA* mutant strain of *E. coli* (CE1303) derived from *E. coli* PC1602. Again, uptake and inhibition of bacterial colony-forming ability by peritoneal exudate PMN occurred as readily in the absence of serum as in its presence. Bacterial killing during phagocytosis, however, required that *E. coli* be pretreated with nonlethal doses of serum (Fig. 2A) and was accompanied by a marked increase (up to fivefold) in bacterial PL degradation (Fig. 2B). In the presence of serum, bacterial PL degradation during phagocy-

FIG. 2. Nonlethal doses of serum enhance the action of PMN toward *E. coli pldA* mutants. Radiolabeled *E. coli* CE1303 was incubated with 0 to 2% (vol/vol) pooled normal human serum for 15 min before further incubation with rabbit PMN. The effects of PMN on bacterial viability (A) and PL degradation (B) were measured as described in Materials and Methods and the legend to Fig. 1. Note that only bacterial viability measured in nutrient agar supplemented with BSA is shown. The viability in nutrient agar without BSA of all samples incubated with PMN was <10%. Treatment of bacteria with serum alone caused a $\leq 20\%$ reduction in bacterial viability and no PL degradation. The results shown represent the means $\pm$ standard errors of the means of three or more independent determinations.

tosis was essentially the same in *pldA*⁺ and *pldA* mutant *E. coli* (compare Fig. 1D and 2B), suggesting a major role for granule-associated PMN (group II) PLA2 under these conditions.

Serum enhancement of bacterial killing and PMN PLA activity during phagocytosis requires components of the MAC of complement. In contrast to pretreatment with normal serum, pretreatment of *E. coli* with C7-depleted serum does not significantly increase bacterial killing (23) (Fig. 3A) or PL degradation (Fig. 3B) during phagocytosis. Pretreatment with C9-depleted serum slightly increased both bacterial killing and PL degradation during phagocytosis. Repletion of C7-depleted serum with purified C7 and C9-depleted serum with C9 (data not shown) fully restored the ability of these sera to promote

FIG. 3. Serum-mediated enhancement of bacterial killing and PL degradation by PMN requires MAC. Radiolabeled *E. coli* CE1303 was preincubated for 5 min with or without normal serum or with C7- or C9-depleted 1% (vol/vol) serum equivalent or with C7-depleted serum to which purified C7 (1 μ g/mL) had been added, as indicated, before the addition of PMN. Bacterial viability (measured in nutrient agar with BSA) (A) and PL degradation (B) were measured as described in Materials and Methods and the legend to Fig. 1. Sera alone had no effect on bacterial viability or PL. The results shown represent the means $\pm$ standard errors of the means of three or more independent determinations.

FIG. 4. Nonlethal doses of serum promote the enzymatic action of purified rabbit and human group II PLA2. Radiolabeled *E. coli* CE1303 cells (2.5×10^8 cells per ml) were incubated with or without serum before the addition of purified PLA2. PL degradation was measured, as described in Materials and Methods, before and after 60-min incubations with PLA2. The results represent the means $\pm$ standard errors of the means of three or more independent determinations.

bacterial killing and PL degradation during phagocytosis. These findings suggest that the enhancement of intracellular bacterial killing and phospholipolysis by serum requires the assembly of (nonlethal) MAC.

Nonlethal doses of serum promote the action of purified group II PLA2 against *E. coli*: requirement of late components of complement. The preceding experiments suggest that pretreatment of *E. coli* with serum increases bacterial sensitivity to PMN PLA. The most likely enzyme contributing to bacterial PL degradation during phagocytosis is granule-associated, 14-kDa (group II) PLA2 (32, 35, 36). Figure 4 shows that the same concentrations of serum that enhance bacterial PL degradation during phagocytosis also promote the action of purified group II rabbit (Fig. 4A) and human (Fig. 4B) PLA2. These effects of serum are absent in C7- and C9-depleted sera (Fig. 5), suggesting that the enhancement of group II PLA2 action against *E. coli* by serum is mediated by nonlethal MAC.

Structural determinants of type II PLA2 action against serum-treated *E. coli*. We have previously shown that the ability of group II PLA2 to act against *E. coli* either during phagocytosis by PMN (32) or in concert with BPI purified from PMN (29, 32, 33) depends on the presence of a cluster of basic amino acids in a surface region near the N terminus of the enzyme

FIG. 5. Serum-mediated PLA2 action requires MAC. Radiolabeled *E. coli* CE1303 *pldA* mutants were preincubated with or without 1% sera as indicated for 15 min before the addition of wild-type (WT) group II PLA2. PL degradation was determined as described in the legend to Fig. 4. The results shown represent the means $\pm$ standard errors of the means of three or more independent determinations.

FIG. 6. Comparison of the activities of various 14-kDa PLA2s toward the PL of serum-treated *E. coli*. Radiolabeled *E. coli* CE1303 cells (2×10^8 cells per ml) were preincubated for 15 min with and without PLA2-depleted serum before the addition of PLA2 and further incubation for 60 min at 37°C. Panels A to C show the effects of added PLA2 on bacteria treated with 1, 2, and 10% serum, respectively. These doses of serum produced <10, 20 to 50, and >99% bacterial killing during the 75-min incubation without PLA2. Panel D shows the effects of added PLA2 on PL recovered from extracellular medium after the treatment of *E. coli* with 90% PLA2-depleted serum (see Materials and Methods). The results shown represent the means $\pm$ standard errors of the means of three or more independent determinations.

that is topologically and functionally distinct from the catalytic site. Figure 6 shows that a double mutant (R7S.K15Q) of group II human PLA2 that retains normal catalytic activity toward dispersions of extracted PL and toward autoclaved *E. coli* but that displays lower activity toward BPI-treated *E. coli* (17, 32) also exhibits less activity than the wild-type enzyme toward *E. coli* treated with nonlethal doses of serum (Fig. 6). Moreover, group I pig pancreatic PLA2, which is as active toward autoclaved *E. coli* as the group II enzymes but virtually inactive toward BPI-treated *E. coli* (33), also exhibits much less activity than human group II PLA2 toward *E. coli* treated with either nonlethal (Fig. 6A and B) or lethal (Fig. 6C) doses of serum and toward PL released from *E. coli* treated with supralethal doses of serum (Fig. 6D). Thus, structural determinants first identified in the context of PLA2 action against *E. coli* in concert with BPI also appear to be important for PLA2 action against complement-treated bacteria.

DISCUSSION

The findings presented in this study demonstrate significant amplification of bacterial PL degradation concomitant with increased intracellular bacterial killing after the pretreatment of *E. coli* with nonlethal doses of serum prior to phagocytosis (Fig. 1 to 3). Among the multiple phospholipolytic activities present in *E. coli* (6, 7), the predominant enzyme activated under many adverse conditions, including phagocytosis by PMN and treatment with purified BPI, is the *pldA* gene product—a PLA localized in the bacterial outer membrane (4, 24, 35). The fact that serum produces virtually identical effects on isogenic *pldA*⁺ and *pldA* mutant strains strongly suggests that this pretreatment affects phospholipase activity of host, not bacterial, origin. Only deacylation of bacterial PL is evident during phagocytosis by PMN (35), and the products released

from *E. coli pldA* mutants under these conditions are indicative of PLA2 action (28a, 35). Therefore, it seems most likely that the increased *E. coli* PL degradation observed is due to a host PLA2 mobilized to the phagolysosome during phagocytosis.

PMN contain multiple PLA2 activities (5, 36), including a 14-kDa group II (36) enzyme localized mainly in cytoplasmic granules that fuse with phagocytic vacuoles during phagocytosis (15, 36). This enzyme's action against *E. coli* during phagocytosis has been demonstrated previously (32, 35). The ability of this (and other) PLAs to degrade *E. coli* PL depends on perturbations of the bacterial envelope that is otherwise highly resistant to PLA action (1, 9, 10, 29). Our studies have strongly suggested that among the multiple cytotoxic weapons of the PMN, the granule-associated BPI plays a major role in the intracellular action of PMN against *E. coli* (12, 23, 32). BPI action, either alone or in concert with the actions of certain other PMN granule proteins, includes activation of PLA(2) action against target bacteria (10, 22, 29).

In the absence of serum (or in the presence of serum from which one of the late components of complement has been depleted), the extent of bacterial PL degradation during phagocytosis is quite limited (Fig. 1 to 3) (8, 35), apparently reflecting the limited access of the small amounts of PLA to pools of envelope PL beyond the outer membrane (35). Either overexpression of the bacterial *pldA* gene product (35) or pretreatment of *E. coli* with serum (Fig. 1 and 2) partially surmounts these limitations, resulting in substantially greater bacterial PL degradation during phagocytosis and, in parallel, much more rapid bacterial killing and structural disassembly (12, 23) (Fig. 1 and 2). Because the intracellular fates of *E. coli* under these two conditions are essentially the same, we speculate that the more rapid and extensive intracellular lethal damage inflicted on *E. coli* after prior exposure to complement is due to enhanced phospholipolytic activity. The close similarity in the serum (complement) requirements for bacteria killing and phospholipolysis and in the kinetics of these effects (Fig. 1 to 3) supports such a relationship.

In the absence of PMN, killing of gram-negative bacteria by serum is mediated by the assembly of MACs (C5b-9_n) (2, 20, 25) and accompanied by envelope alterations that render membrane PL susceptible to attack by both bacterial and serum PLA (19, 29). The promotion of intracellular bacterial killing and phospholipolysis within PMN by nonlethal doses of serum is also dependent on the late components of complement (Fig. 3) but is associated with assembly of far fewer complexes (≤ 200 per cell) (2a) than are needed to induce lethal membrane injury (2, 20). The activity of purified group II PLA2 against *E. coli* treated with nonlethal doses of serum is remarkable, because these treated bacteria otherwise display little or no envelope alterations (2, 20) and little or no susceptibility to either endogenous bacterial PLA (Fig. 1C) or certain closely related, exogenous 14-kDa PLA2s (Fig. 6). The ability of native group II (PMN) PLA2 to act under these circumstances favors a primary role for this enzyme in the intracellular destruction of the bacteria. Extracellular (e.g., serum) group II PLA2 can bind to *E. coli* before phagocytosis and further contribute to intracellular bacterial PL degradation after ingestion (32, 35). However, in our experiments, the amount of PLA2 added in serum is only 1% of the amount present in PMN (3), making it unlikely that extracellular PLA2 contributes significantly to intracellular digestion under these conditions. It should be noted that the amount of purified PLA2 needed to produce appreciable PL degradation of *E. coli* treated with nonlethal doses of serum is 10 to 100 times greater per bacterium than can be mobilized from intracellular stores during phagocytosis (36). Even at these relatively high PLA2

doses, little bacterial killing is produced. Thus, intracellular bacterial killing and phospholipolysis likely reflect the concerted action of not only nonlethal assemblies of MAC and group II PLA2 but other antibacterial systems as well. A likely contributor is BPI, which acts synergistically with both complement and PLA2 against *E. coli* (12, 23, 31).

Virtually all members of the conserved 14-kDa family of PLA2 (including group I and II enzymes) display similar catalytic activities toward the PL of *E. coli* (10, 29). However, these enzymes differ greatly in their activities toward BPI-treated *E. coli* (10, 29). The latter activity depends on not only the highly conserved catalytic machinery of 14-kDa PLA2 but also a cluster of basic residues within a discrete variable surface region that promotes PLA2 interaction with BPI-treated *E. coli* (32, 33). The demonstration in this study that similar structural properties are important for PLA2 action against bacteria treated with nonlethal doses of serum suggests that these basic residues facilitate the interaction of PLA2 with discretely altered surfaces of gram-negative bacteria (created either by complement or by BPI) and not with BPI itself. The ability of mammalian group II PLA2 to act in concert with prominent host defenses against gram-negative bacteria in addition to its potent independent action against many gram-positive bacteria (16, 27) further supports the notion that participation in antibacterial defenses is an important function of this enzyme. This role may not be limited to attack against ingested bacteria. Complement, BPI, and PLA2 are all mobilized in the extracellular fluid of PMN-rich inflammatory exudates in which potent bactericidal activity against complement- and phagocytosis-resistant gram-negative bacteria is manifest (28). Group II PLA2 levels in inflammatory fluid are up to 50 times greater than the amount of intracellular (PMN) enzyme within the exudate (14, 36). Hence, this may be a setting particularly favorable for an important contribution by group II PLA2 to extracellular as well as intracellular antibacterial defenses.

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