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Synergy between Extracellular Group IIA Phospholipase A<sub>2</sub> and Phagocyte NADPH Oxidase in Digestion of Phospholipids of *Staphylococcus aureus* Ingested by Human Neutrophils<sup>1</sup>

Jon K. Femling,*† William M. Nauseef,*‡ and Jerrold P. Weiss<sup>2,*†‡</sup>

Acute inflammatory responses to invading bacteria such as *Staphylococcus aureus* include mobilization of polymorphonuclear leukocytes (PMN) and extracellular group IIA phospholipase A<sub>2</sub> (gIIA-PLA<sub>2</sub>). Although accumulating coincidentally, the in vitro anti-staphylococcal activities of PMN and gIIA-PLA<sub>2</sub> have thus far been studied separately. We now show that degradation of *S. aureus* phospholipids during and after phagocytosis by human PMN requires the presence of extracellular gIIA-PLA<sub>2</sub>. The concentration of extracellular gIIA-PLA<sub>2</sub> required to produce bacterial digestion was reduced 10-fold by PMN. The effects of added gIIA-PLA<sub>2</sub> were greater when present before phagocytosis but even apparent when added after *S. aureus* were ingested by PMN. Related group V and X PLA<sub>2</sub>, which are present within PMN granules, do not contribute to bacterial phospholipid degradation during and after phagocytosis even when added at concentrations 30-fold higher than that needed for action of the gIIA-PLA<sub>2</sub>. The action of added gIIA-PLA<sub>2</sub> required catalytically active gIIA-PLA<sub>2</sub> and, in PMN, a functional NADPH oxidase but not myeloperoxidase. These findings reveal a novel collaboration between cellular oxygen-dependent and extracellular oxygen-independent host defense systems that may be important in the ultimate resolution of *S. aureus* infections. The Journal of Immunology, 2005, 175: 4653–4661.

*S. aureus* is a frequent cause of human disease capable of infecting a broad range of tissues including bone, muscle, and skin (1). Despite its pathogenic potential and broad array of virulence factors, most *S. aureus*-human encounters do not result in clinically recognized disease. In fact, *S. aureus* may colonize upwards of 30–50% of humans at any given time, with >80% of all humans eventually carrying or being infected with *S. aureus* during their lifetime (1, 2). The host factors that normally prevent *S. aureus* from causing serious infections after incidental bacterial invasion include the professional phagocytes polymorphonuclear leukocytes (PMN)<sup>3</sup> and the secretory group IIA phospholipase A<sub>2</sub> (gIIA-PLA<sub>2</sub>). As important elements of innate host defense, both neutrophils and extracellular gIIA-PLA<sub>2</sub> are rapidly mobilized to sites of bacterial invasion to exert their protective host defense effects (3, 4).

PMN are normally the most abundant leukocytes in blood and respond quickly to a variety of chemotactic signals, thereby migrating to and accumulating at sites of bacterial invasion (4). Interaction of mobilized PMN with invading bacteria often requires the help of humoral components of the acute inflammatory response (5). In the case of *S. aureus*, complement-dependent modifications of the bacterial surface are usually most important for phagocytosis of *S. aureus* by PMN (6, 7). Engagement of PMN receptors (e.g., CR3, FcR) initiates several antimicrobial responses. Cytoplasmic granules laden with antimicrobial peptides and proteins are mobilized to and fuse with the nascent phagosome, as is needed to deliver their contents onto the ingested organism (8). Concomitantly, the respiratory burst NADPH oxidase is assembled and activated in close proximity to the attached and ingested bacterium (9). Both numerical and functional deficiencies of neutrophils are associated with increased risk of *S. aureus* infections (1, 4). Genetic or pharmacological alterations that preclude oxidase activation significantly reduce PMN cytotoxicity toward *S. aureus* (1, 4, 10–12). Reactive oxygen species (ROS) formed secondary to oxidase activation that are cytotoxic toward *S. aureus* have been identified (e.g., HOCl; Ref. 12), but controversy remains concerning their precise targets and roles during phagocytosis (12–15). Effects of oxidase activation on intracellular mobilization and activation of granule-derived enzymes have also been described (13, 16), suggesting that oxidase activation could increase PMN antibacterial action directly by generating cytotoxic ROS and indirectly by enhancing the activity of other, nonoxidative, cytotoxic products of the neutrophil.

The gIIA-PLA<sub>2</sub> is a member of a family of low *M<sub>r</sub>* (~14,000) secretory PLA<sub>2</sub> (sPLA<sub>2</sub>) with close overall structural and functional similarity to related enzymes in the venoms of snakes and insects (17, 18). Among the 10 sPLA<sub>2</sub> in humans (17, 18), the gIIA-PLA<sub>2</sub> is unique in its high net positive charge (+15) and antibacterial potency (19, 20). This enzyme can attack, at nanomolar concentrations, both Gram-negative and Gram-positive bacteria, although the former action generally requires the assistance of other host defense proteins to facilitate access of the PLA<sub>2</sub> to phospholipids (PL) in the Gram-negative bacterial envelope (21, 22). Extracellular levels of the gIIA-PLA<sub>2</sub> vary widely in time and

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<sup>2</sup>Address correspondence and reprint requests to Dr. Jerrold P. Weiss, Inflammation Program, 2501 Crosspark Road, Coralville, IA 52241. E-mail address: jerrold-weiss@uiowa.edu

<sup>3</sup>Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; gIIA-PLA<sub>2</sub>, group IIA phospholipase A<sub>2</sub>; ROS, reactive oxygen species; sPLA<sub>2</sub>, secretory PLA<sub>2</sub>;<br>PL, phospholipid; DPI, diphenylene iodonium; PG, phosphatidylglycerol; CL, cardiolipin; HSA, human serum albumin; MPO, myeloperoxidase; CGD, chronic granulomatous disease; TLC, thin-layer chromatography.

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space. In plasma and presumably many other tissue fluids, gIIA-PLA₂ contributions under resting conditions are subnanomolar but can increase 100- to 1000-fold during inflammation as part of the acute phase response (3, 23). In contrast, at certain sites (e.g., tears, seminal fluid), gIIA-PLA₂ levels are constitutively high and may exceed micromolar concentrations (23, 24). These gIIA-PLA₂-rich cell-free fluids display potent antibacterial activity against S. aureus and many other Gram-positive bacteria that is largely due to the presence of the gIIA-PLA₂ (3, 20, 23, 24). Transgenic mice overexpressing human gIIA-PLA₂ show increased host resistance to Escherichia coli (25) and S. aureus (26) infections, demonstrating protective effects of gIIA-PLA₂ mobilization in vivo, although under somewhat nonphysiological conditions.

Surprisingly, given their prominent role in host defense, human PMN contain little or no gIIA-PLA₂ (27). Thus, at an acute inflammatory site, mobilized antibacterial gIIA-PLA₂ is mainly found in the extracellular environment. Purified gIIA-PLA₂ and gIIA-PLA₂-rich inflammatory fluids can attack encapsulated (28) and clumped S. aureus (29), suggesting that the major target of this antibacterial enzyme may be invading bacteria that resist or exceed the phagocytic capacity of PMN. Two other sPLA₂ (group V and X) are present in granules of human PMN (27). However, these enzymes have much lower intrinsic anti-staphylococcal activity (19, 27, 30) and are present in PMN in low abundance (27). Therefore, the extent of bacterial PL degradation that accompanies phagocytosis of S. aureus by human neutrophils is uncertain. Extracellular gIIA-PLA₂ may bind to bacteria, including S. aureus, and thus be delivered to phagocytosing PMN and contribute to intracellular bacterial digestion by synergy with agents in the phagosome (30, 31). Therefore, in this study we examined the ability of phagocytosis of S. aureus by PMN to produce bacterial PL degradation in the absence and presence of extracellular gIIA-PLA₂. We show that appreciable bacterial PL degradation occurs only in the presence of catalytically active gIIA-PLA₂ and a functional respiratory burst NADPH oxidase in PMN. These findings reveal a novel collaboration between cellular O₂-dependent and extracellular O₂-independent host defense systems in bacterial digestion that may be important in the ultimate resolution of S. aureus infections.

Materials and Methods

Materials

Tryptic Soy Broth and Bacto Bacto Agar were purchased from BD Biosciences, and chloramphenicol was purchased from Research Products. HEPES, HBSS with or without divalent cations, and PBS were obtained from Mediatech CellGro. Clinical grade dextran was purchased from MP Biomedicines, Ficol-Paque PLUS was purchased from Amersham Biosciences, and sterile endotoxin-free 0.9% saline, H₂O, and HSA were purchased from Baxter Healthcare. Diphenhydramine iodidum (DPI), Biophenika Saponin, and poly-L-lysine-coated slides (Poly-Prep slides) were purchased from Sigma-Aldrich. 1-14C-oleic acid (50 mCi/mmol) was purchased from PerkinElmer. Chloroform and methanol were purchased from Fisher Scientific. Purified lipid standards for phosphatidylglycerol (PG) (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phospho-rac-(1-glycerol), cardiolipin (CL) (tetraeoyloyl cardiolipin), lysophosphatidylglycerol (1-Palmitoyl-2-Hydroxy-sn-Glycero-3-Phospho-rac-(1-glycerol)] monosycolcardiolipin (purified from bovine heart, ~92% linoleic), and diylsoycolcardiolipin (purified from bovine heart, 96% linoleic) were purchased from Avanti Polar Lipids. Saponin was obtained from Fluka and had an effective sapogenin content of 11.5%.

Bacteria

For the data presented, we used S. aureus ALC1435 (kindly provided by Ambrose Cheung, Department of Microbiology, Dartmouth Medical School, Hanover, NH). This strain is a derivative of RN6390 containing the plasmid pALC1420 encoding GFPuv preceded by the sar P1 promoter permitting essentially constitutive production of the GFP (32). Selected findings were reproduced with S. aureus RN450, SA113, and a clinical isolate from a patient with osteomyelitis.

All of the bacterial strains were grown in tryptic soy broth overnight at 37°C, diluted to an OD550 of 0.05 in fresh medium, and subcultured for 2–5 h to midlogarithmic phase. S. aureus ALC1435 was grown with 10 µg/ml chloramphenicol. Subcultured bacteria were washed once, resuspended in 20 mM HEPES-buffered HBSS (with divalent cations; pH 7.4), and used immediately or placed on ice (for <1 h) until opsonization (see below).

Bacterial lipid labeling with 14C-oleic acid

To metabolically label bacterial lipids during growth, subcultures were supplemented with 1 µCi/ml 1-14C-oleic acid and 0.01% (wt/vol) human serum albumin (HSA). After 2 h of incubation, S. aureus were pelleted by centrifugation for 5 min at ~5000 × g, resuspended in 0.5 volume of fresh medium without radiolabel, and incubated an additional 30 min at 37°C. HSA was then added to a final concentration of 0.5%, and the bacteria were pelleted by centrifugation to remove any remaining unincorporated 14C-oleic acid in the supernatant as complexes with albumin. The sedimented bacteria were resuspended in 20 mM HEPES-buffered HBSS (with divalent cations) and placed on ice (as above) until opsonization.

PLA₂

Purified recombinant human gIIA-PLA₂, and the D49S mutant were expressed and purified as described previously (31). Purified recombinant human group V and X PLA₂ were kindly provided by (27) Dr. Michael Gelb (Department of Chemistry and Biochemistry, University of Washington, Seattle, WA).

PMN

PMN from myeloperoxidase (MPO)-deficient, NADPH oxidase-deficient (chronic granulomatous disease; CGD), or normal healthy volunteers were purified from peripheral blood as described (33), after obtaining informed consent in accordance with a protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa. The recovered granulocytes were resuspended in HBSS without divalent cations, counted, and further diluted so that cell density was no greater than 30 × 10⁶/ml. Cell purity was determined by microscopy after staining of cells with HEMA 3 (Fisher Scientific) and was typically >95% PMN (the remainder being eosinophils). The purified PMN were stored at room temperature for up to 1 hour until use.

Ten minutes before addition of PMN to bacterial suspensions, purified PMN were diluted to 10⁴/ml in 20 mM HEPES-buffered HBSS with divalent cations supplemented with 1% HSA and 10% pooled human serum. These cells were then preincubated at 37°C for 10 min before addition to bacterial suspensions.

To inhibit the NADPH oxidase of normal PMN, preincubations were supplemented with 10 µM DPI. Diminished NADPH oxidase activity was confirmed by lucigenin-enhanced chemiluminescence detection of ROS (34).

Pooled human serum

Serum was freshly isolated from at least nine different donors, pooled, sterile filtered, aliquotted, and frozen at ~80°C until use. Thawed serum was from 1× frozen aliquots and used the day of thawing.

Oxpsorption of bacteria and incubation with PMN

Subcultured bacteria on ice were diluted to 10⁶ CFU/ml and incubated for 20 min at 37°C in 20 mM HEPES-buffered HBSS supplemented with 1% HSA and 10% pooled human serum. After preopsonization of bacteria and prewarming of PMN, these cells were mixed 1:1 (vol/vol) to yield final cell concentrations of 5 × 10⁶ PMN/ml and 5 × 10⁶ bacterial CFU/ml. The bacteria were mainly organized in groups of 2, 4, or 8; hence, 1 CFU corresponded to ~5 cocci on average, and incubations therefore contained ~5 cocci/PMN. Incubations were conducted in 5-ml round-bottom polypropylene tubes swirling at 120 rpm in a water bath at 37°C for up to 240 min.

Assays of bacterial uptake by PMN

Uptake of S. aureus by PMN was assayed in three different ways. 1) Differential centrifugation of extracellular and PMN-associated bacteria: After incubation of PMN and opsonized bacteria, the cell suspensions were spun at 100 × g for 5 min to sediment PMN and PMN-associated bacteria. The supernatant, containing extracellular bacteria, was removed, and the cell
pellet was resuspended in an equal volume of incubation medium. Aliquots of the supernatant and resuspended cell pellet were taken to measure 14C-bacteria by liquid scintillation spectroscopy. In the absence of added gIIA-PLA2, essentially all 14C-cpm remained associated with bacteria and thus provided an accurate marker of bacterial distribution and recovery. 2) Counting of cell-associated (ingested) GFP-expressing bacteria by epifluorescent and confocal microscopy: After incubation at 37°C, ~75,000 PMN and/or S. aureus CFU were sedimented using a cytocentrifuge (Shandon Cytospin 2; Shandon Southern Products) at 18 × g for 10 min onto polyt-l-lysine-coated slides that had been primed with HEPES-buffered HBSS supplemented with 0.2% HSA. Cell smears were fixed in 10% buffered formalin for 15 min at room temperature, washed with PBS, and covered with mounting medium (polyvinyl alcohol, Tris-base, glycerol, and 1,4-diazobicyclo-[2.2.2]-octane; Sigma-Aldrich) and a coverslip. S. aureus cocci/PMN were quantified during visualization with a Zeiss Axioplan2 photomicroscope counting bacteria in over 100 PMN per sample. 3) Transmission electron microscopy: After incubation for 30 and 120 min at 37°C, samples were fixed with 2.5% glutaraldehyde/0.1 M sodium cacodylate buffer, then with 1% osmium tetroxide, sequentially dehydrated with ethanol, embedded in Epon, and sectioned. Micrometre sections were stained with uranyl acetate and lead citrate. Stained sections were examined with a Hitachi H-7000 transmission electron microscope in the University of Iowa Central Microscopy Research Facility.

**Assay of metabolism of 14C-bacterial PL.**

Cell suspensions containing 14C-S. aureus were extracted via a modified Bligh-Dyer method (35). The combined chloroform phases were dried under nitrogen and resuspended in 20 μl of chloroform:methanol (2:1; v/v) and applied to a high-performance, thin-layer chromatography (TLC) plate (Silica gel 60; produced by Merck, distributed by EMD Chemicals). Lipids were resolved using a solvent system composed of chloroform, methanol, water, and acetic acid (65:25:4:1; v:v:v:v). Resolved 14C-lipids were visualized by exposure of TLC plates to tritium storage phosphor screens analyzed by an Amersham Typhoon 9410 Variable Mode Imager (Amersham Biosciences). Quantification was done using ImageQuant software from Molecular Dynamics. Major 14C-lipids and metabolites were identified by comigration with purified lipid standards (Avanti Polar Lipids). To verify the identity of PG and CL, two other solvent systems were used, which yielded relative migration of 0.4 and 0.8 (chloroform, methanol, acetic acid, 30:42:1; v:v:v) and 0.4 and 0.3 (chloroform, methanol, methyl acetate, 1-propanol, 0.25% KCl, 20:10:25:35:10, v:v:v:v:v) for PG and CL, respectively.

**Assay of bacterial viability**

A 10-μl aliquot of each cell suspension was added to 490 μl of 1% saponin in H2O. Samples were vortexed vigorously, incubated for 15 min at room temperature to lyse the PMN, and then serially diluted in PBS. Aliquots (10–20 μl) representing ~200 CFU were added to a 50-mm petri dish, followed by ~5 ml of molten agar (50°C), and stirred briefly. The agar was then allowed to harden, and CFU were counted after overnight incubation at 37°C.

**Results**

**Effects on bacterial PL during phagocytosis of S. aureus by PMN.**

We began by examining effects on PL of S. aureus incubated with or without PMN. Bacterial PL were biosynthetically prelabeled with 14C-oleate to facilitate assay of metabolic changes. As shown previously (20), bacterial PL were quantitatively recovered in the chloroform phase after Bligh/Dyer extraction of mid-late log phase bacteria; ~60% of the 14C-oleate-labeled lipids recovered in this fraction were PL (Fig. 1). By far, the most abundant 14C-PL was S. aureus PL (Fig. 1; Refs. 20, 36). Incubation of S. aureus with PMN resulted in little or no accumulation of 14C-lyso-PL and/or free fatty acid (Fig. 1). Thus, minimal bacterial PL degradation occurred despite rapid and nearly quantitative bacterial uptake by PMN, as assessed by cell association of 14C-bacteria (Fig. 2A) and epifluorescent microscopy of smears of PMN incubated with GFP-expressing S. aureus (at 30 min, 5.3 ± 0.4 cocci/PMN). Similar results were obtained with several different strains of S. aureus and each of >10 different donors of PMN (data not shown). The intracellular location of cell-associated S. aureus was confirmed by confocal microscopy (data not shown) and transmission electron microscopy (Fig. 2B). In contrast to the virtual absence of PL degradation, phagocytosis of S. aureus by PMN was accompanied by a marked decrease in PG and a reciprocal increase in CL (Fig. 1), suggesting bacterial conversion of PG to CL (37). In parallel with phagocytosis, most of the PG to CL conversion occurred within 30 min of exposure of S. aureus to PMN (Fig. 3). PMN and added gIIA-PLA2 act in synergy to degrade S. aureus PL

The absence of bacterial PL degradation during phagocytosis of S. aureus by PMN prompted examination of the effect of adding gIIA-PLA2, as would be present in PMN-rich inflammatory exudates. GIIA-PLA2 alone produced a dose-dependent degradation of S. aureus PL (Fig. 4), as described previously (20, 31). In the absence of PMN, PL degradation was manifest as the loss of prelabeled PG and the generation of 14C-lys-PL as the principal labeled degradation product. In the presence of PMN, bacterial PL degradation was seen at ~10-fold lower gIIA-PLA2 doses and concentrations after addition of PMN, or both for 2 h at 37°C were extracted, and recovered 14C-lipids were analyzed by TLC/image analysis as described in Materials and Methods. The major 14C-bacterial PL and breakdown products are indicated. Incubation of bacteria alone caused little or no change in 14C-lipid profile. These samples are representative of >10 independent experiments.

**FIGURE 1.** Effects of PMN and/or gIIA-PLA2 on 14C-PL of S. aureus. S. aureus incubated alone or with PMN, gIIA-PLA2 (150 ng/ml, final concentration after addition of PMN), or both for 2 h at 37°C were extracted, and recovered 14C-lipids were analyzed by TLC/image analysis as described in Materials and Methods. The major 14C-bacterial PL and breakdown products are indicated. Incubation of bacteria alone caused little or no change in 14C-lipid profile. These samples are representative of >10 independent experiments.

**FIGURE 2.** Uptake of opsonized S. aureus by PMN. A. Sedimentation of 14C-S. aureus after preopsonization and incubation ± PMN for 30 min. Data represent the amount of radioactivity (hence, bacteria) that were sedimented by a 100 × g centrifugation, (See Material and Methods for further experimental details.) B. Transmission electron microscopy of a representative PMN containing several ingested S. aureus after incubation at 37°C for 120 min.
resulted in roughly equal accumulation of dilyso-CL and lyso-PG, especially at the lower PLA₂ concentrations (Figs. 1 and 4). The reduced gIIA-PLA₂ dose requirements indicate that PMN and the gIIA-PLA₂ acted in synergy to produce degradation of PL of *S. aureus*. Similar effects were seen in several laboratory strains and clinical isolates of *S. aureus* including ALC1435, RN450, UAMS-1, and SA113 (data not shown) and with PMN from each of >10 donors (data not shown).

**Kinetics of bacterial PL degradation**

The generation of dilyso-CL indicates that PL degradation triggered by added gIIA-PLA₂ occurred after PMN-induced conversion of PG to CL; i.e., after phagocytosis of *S. aureus*. This was confirmed by examining the kinetics of *S. aureus* PL degradation induced by a relatively low concentration of gIIA-PLA₂ (150 ng/ml) in the presence of PMN. This dose was chosen to minimize the amount of degradation that occurred before addition of PMN. Whereas phagocytosis was complete by 15–30 min, PL degradation progressed for up to 180 min (Fig. 5). Thus, under these conditions, most gIIA-PLA₂-mediated degradation of bacterial PL occurred after ingestion of *S. aureus* by PMN.

**gIIA-PLA₂ catalytic activity is required for bacterial PL degradation in concert with PMN**

The generation of radiolabeled lyso-PG and dilyso-CL from ¹⁴C-oleate-labeled PG and CL, respectively, is indicative of the action of a PLA₂ (20). This could reflect the direct action of the added gIIA-PLA₂ on the target PL, the activation of endogenous PLA₂ by the exogenously added gIIA-PLA₂, or both (38–40). To test whether the catalytic activity of the added gIIA-PLA₂ was necessary for the bacterial PL degradation observed, we compared the effects of the wild-type recombinant enzyme and a catalytically inactive gIIA-PLA₂ mutant (D49S) (31). In contrast to the wild-type enzyme, the mutant PLA₂ caused no PL degradation either alone (Fig. 6A) or in the presence of PMN (Fig. 6B). Thus, the effect of added gIIA-PLA₂ required catalytically active enzyme, suggesting a direct action of the added gIIA-PLA₂ on ingested bacteria.

**Effects of gIIA-PLA₂ are not produced under same conditions by group V or X PLA₂**

The requirement of added catalytically active gIIA-PLA₂ for *S. aureus* PL degradation during phagocytosis by PMN suggests that group V and X PLA₂, present in PMN granules, did not contribute to bacterial digestion. To determine whether limiting amounts of the group V and X PLA₂ accounted for their inactivity against ingested *S. aureus*, we tested the effect of adding group V or X PLA₂ to suspensions of opsonized *S. aureus* in the absence or presence of PMN (Fig. 6B). This was true even at 10- to 30-fold higher doses than that needed for the gIIA-PLA₂ to trigger significant degradation of *S. aureus* PL in the presence of PMN (compare Figs. 4 and 6B).

**Synergy between PMN and added gIIA-PLA₂ against *S. aureus* requires a functional NADPH oxidase of PMN**

Optimal cytotoxicity of PMN against *S. aureus* requires a functional respiratory burst NADPH oxidase (11–13, 20). We therefore
sought to determine the role of the oxidative burst in the increased bacterial PL degradation seen when gIIA-PLA₂ and PMN were present together. To examine the role of the NADPH oxidase, we compared normal PMN to PMN pretreated with DPI, a flavoprotein inhibitor that blocks phagocyte NADPH oxidase activation (12, 41). Pretreatment of PMN with 10 μM DPI inhibited S. aureus-triggered oxidase activation by >95%. DPI treatment had no effect on bacterial uptake by PMN (at 30 min, 5.3 ± 0.4 cocci/PMN (no DPI); 5.1 ± 0.2 cocci/PMN (+DPI); n = 6) but significantly reduced killing of S. aureus by PMN (Fig. 7A). DPI treatment also eliminated the enhanced bacterial PL degradation seen when S. aureus were exposed to normal PMN (no DPI) and gIIA-PLA₂ (Fig. 7B). In fact, degradation of PL of S. aureus exposed to DPI-treated PMN and gIIA-PLA₂ was less than that caused by addition of gIIA-PLA₂ alone (Fig. 7B), indicating that in the absence of oxidase activation, phagocytosis protected S. aureus from the extracellular PLA₂. DPI had no effect on the action of gIIA-PLA₂ against S. aureus in the absence of PMN (data not shown). Similar findings were obtained using PMN that lack a functional NADPH oxidase from a patient with CGD (Refs. 4, 10; Fig. 7B), confirming the importance of a functional NADPH oxidase in the action of gIIA-PLA₂ against ingested S. aureus.

Effects of gIIA-PLA₂ on ingested S. aureus do not require MPO

Activation of the NADPH oxidase generates superoxide anion and, after dismutation, hydrogen peroxide (10). The latter is further metabolized with chloride by granule-associated MPO of PMN to form potently cytotoxic hypochlorous acid (10, 12). To test the possible role of MPO in the action of extracellular gIIA-PLA₂ against ingested S. aureus, we compared effects of normal and MPO-deficient PMN. As expected (12, 42), phagocytosis of S. aureus by normal and MPO-deficient PMN were closely similar (data not shown), but bacterial killing was slightly reduced in MPO-deficient PMN (Fig. 7A). In contrast to CGD or DPI-treated normal PMN, MPO-deficient PMN enhanced gIIA-PLA₂-mediated bacterial PL degradation in a manner that resembled the effects of normal PMN (Fig. 7B). Thus, whereas a functional NADPH oxidase was required for gIIA-PLA₂ action on ingested S. aureus, MPO activity and generation of MPO-derived ROS were not.

Conversion of bacterial PG to CL during phagocytosis does not require a functional NADPH oxidase but is reduced without MPO

Assay of bacterial PL degradation during phagocytosis of S. aureus by normal vs oxidase- or MPO-deficient PMN also permitted assay of the bacterial PG to CL conversion under these conditions.
As shown in Fig. 8, CGD and DPI-treated PMN induced conversion of PG to CL to a similar extent as did normal PMN. In contrast, conversion of PG to CL was somewhat reduced in MPO-deficient PMN. Taken together, these findings indicate that the requirements for enhancement of gIIA-PLA₂-mediated PL degradation and bacterial PG to CL conversion were strikingly different (compare Figs. 7 and 8). A functional respiratory burst NADPH oxidase was required for bacterial PL degradation but not for the PG to CL conversion. Conversely, MPO was not needed for increased PLA₂ action but was required for maximum induction of the PG to CL conversion during phagocytosis. Pretreatment of PMN with sodium azide, a heme protein inhibitor, blocking both MPO and hydrogen peroxide-catalyzing catalase (43), also reduced the extent of bacterial PG to CL conversion during phagocytosis of S. aureus (data not shown), suggesting that the greater accumulation of hydrogen peroxide in MPO-deficient PMN during phagocytosis (42) may dampen the signal(s) triggering the bacterial PL conversion.

GIIA-PLA₂ added after phagocytosis also causes increased bacterial PL degradation

During the acute inflammatory response, mobilization of extracellular gIIA-PLA₂ continues after the initial influx of PMN (44). This raises the question of what effect extracellular gIIA-PLA₂ could have against S. aureus already ingested by PMN. To address this question, S. aureus were incubated with PMN for 30 min, and any remaining extracellular bacteria were removed by differential centrifugation. PMN-associated S. aureus were incubated an additional 30 min to maximize intracellular sequestration of the bacteria before addition of gIIA-PLA₂. Remarkably, gIIA-PLA₂ added after phagocytosis by normal PMN induced bacterial PL degradation (Fig. 9). The PL degradation triggered by gIIA-PLA₂ added after phagocytosis was less extensive than that observed with pretreatment of bacteria with gIIA-PLA₂ (compare Figs. 7 and 9) and not further increased by adding higher concentrations of extracellular PLA₂ (Fig. 9). Bacteria ingested by DPI-treated PMN were resistant to the added PLA₂, demonstrating again the requirement of a functional NADPH oxidase for PLA₂ action against ingested S. aureus and confirming the intracellular sequestration of essentially all bacteria and bacterial PL.

Discussion

The cytotoxic effects of PMN and of extracellular gIIA-PLA₂ against S. aureus have been studied extensively (3, 11–13, 20, 26, 31, 45–47). Although both PMN and extracellular gIIA-PLA₂ are mobilized to sites of bacterial invasion concomitantly, their in vitro actions against S. aureus have been examined in isolation of one another. The facts that human PMN apparently lack gIIA-PLA₂ (27) and may proteolytically inactivate this enzyme upon cell activation (45) (N. S. Liang and J. P. Weiss, unpublished observations) and that extracellular gIIA-PLA₂ can act against strains and forms of S. aureus that are resistant to phagocytosis (28, 29) have raised the possibility that PMN and the extracellular gIIA-PLA₂ function in a complementary but separate manner against ingestible and noningestible bacteria, respectively. By testing in this study the possible combined effects of PMN and gIIA-PLA₂ on S. aureus PL, we have discovered that PMN and extracellular gIIA-PLA₂ acted synergistically against S. aureus (see Figs. 1, 4, 5, and 7). We have shown (Figs. 1, 4, and 5) that phagocytosis of serum-opsonized S. aureus by human PMN was accompanied by little bacterial PL degradation, thus necessitating the presence of extracellular gIIA-PLA₂ for greater digestion of this major bacterial envelope component. Remarkably, although virtually all of the bacteria were ingested within 15–30 min of addition of PMN and thereby sequestered from extracellular gIIA-PLA₂, PLA₂-induced bacterial PL degradation in the presence of PMN occurred at extracellular PLA₂ concentrations ~10-fold lower than those needed for action of PLA₂ alone against extracellular bacteria.

The absence of appreciable bacterial PL degradation during and following phagocytosis of S. aureus by PMN in the absence of extracellular gIIA-PLA₂ means that neither PMN nor bacterial phospholipases act on bacterial PL under these conditions. This observation differs from those in earlier studies with rabbit peritoneal exudate PMN and E. coli (48). In those studies, there was degradation of ~20–30% of prelabeled bacterial PL during phagocytosis, most due to activation of the bacterial outer membrane phospholipase A (48). In S. aureus, metabolic cycling of the major PL, PG, through release and resynthesis of the glycerol-phosphate headgroup is robust (35, 49). However, there is little evidence, as yet, of bacterial deacylases that could contribute to bacterial PL autodigestion or remodeling (31, 35).

Rabbit peritoneal exudate PMN contain small amounts of gIIA-PLA₂ (50). The source of this enzyme, either from endogenous synthesis or uptake from much more abundant pools of enzyme in
enzymes are low, but detectable (cytoplasmic granules that are mobilized during phagocytosis to the phagocytic vacuole (27). The levels of these granule-associated enzymes are low, but detectable (<1.5 ng/10^6 PMN; Ref. 27). These PLA2 are in comparison to gIIA-PLA2, are much less active as antibacterial enzymes against either E. coli or S. aureus (27, 52). In contrast to the gIIA-PLA2, the groups V and X PLAs have significantly higher activity against phosphatidylcholine-containing membranes (17). This activity is consistent with the observations that these PLAs play important roles in host lipid metabolism (53, 54). Whether or not these PLAs may also play a role in host defense against those bacteria capable of synthesizing phosphatidylcholine such as Pseudomonas aeruginosa, Borrelia burgdorferi, Legionella pneumophila, Streptococcus pneumoniae, and Haemophilus influenzae (55) has not yet been studied. The limited amounts and low intrinsic antistaphylococcal potency of the group V and X PLAs, and the apparent absence of the gIIA-PLA2 presumably account for the absence of host-mediated bacterial PL degradation during phagocytosis of S. aureus by PMN in the absence of extracellular gIIA-PLA2.

Our findings indicate that the extracellular gIIA-PLA2 contributes to digestion of S. aureus not only before but also during and after phagocytosis by PMN. Especially at low PLA2 concentrations, the combined effects of PMN and the gIIA-PLA2 were synergistic, not additive (Figs. 1, 4, and 5). The major labeled PLA2 breakdown products that accumulated ([3H]-lysyl-PG and [3H]-dilysyl-CL) indicate both direct PLA2 action (20, 35) and action after exposure of bacteria to PMN and induction of the conversion of bacterial PG to CL (Figs. 1, 3, and 4). Although less direct effects of the added gIIA-PLA2 are possible, such as extracellular PLA2-induced activation of intracellular PLA2 (17, 38), the much greater antistaphylococcal potency of the gIIA-PLA2 as compared with that of endogenous PMN PLAs (Fig. 4 vs 6B) and the requirement for catalytically active gIIA-PLA2 (Fig. 6A) are much more compatible with a direct action of the added gIIA-PLA2 on bacteria ingested by PMN.

We have not yet shown translocation of the extracellular PLA2 to the phagocytic vacuole but have verified (N. S. Liang and J. P. Weiss, unpublished observations) that the PLAs can associate with both extracellular bacteria and PMN before phagocytosis as demonstrated previously (31, 56, 57). How addition of extracellular gIIA-PLA2 after ingestion of bacteria by PMN increased bacterial PL degradation (Fig. 9) is less clear. Uptake of sPLA2, including gIIA-PLA2, by murine bone marrow-derived mast cells and delivery of the enzyme to a digestive (phagolysosomal) compartment have been demonstrated (58), possibly providing a mechanism by which the extracellular enzyme could access the phagosome even after prior phagocytosis of bacteria.

However PLA2 accesses ingested bacterial PL, our findings imply that conditions within the phagocytic vacuole are more favorable than are extracellular conditions for gIIA-PLA2 action. The precise nature of the conditions favoring PLA2 action is not known, but our findings clearly demonstrate that enhanced PL degradation depended on activation of the respiratory burst oxidase. Precluding activation of the oxidase, either by pharmacological (i.e., DPI) or genetic (i.e., CGD) means, prevented the synergy seen between normal PMN and extracellular gIIA-PLA2 (Figs. 7 and 9). Under these conditions, the PMN did not potentiate PLA2 action but, instead, protected the bacteria from the extracellular enzyme. These findings clearly demonstrate an essential role of the respiratory burst NADPH oxidase in PLA2 action against ingested S. aureus. The absence of bacterial PL degradation when oxidase activation is precluded, even at relatively high extracellular gIIA-PLA2 concentrations (Fig. 9), indicates the complete sequestration of bacteria and bacterial PL under these conditions.

It has been previously shown that oxidase activation and elaboration of ROS can increase protease activity against both ingested bacteria and extracellular substrates (13, 16, 59). These effects may be due to oxidative alterations of substrate, modifications of endogenous inhibitors that normally constrain enzyme activity, or both. The extent to which PMN potentiated PLA2 activity paralleled the bactericidal effects of the PMN against S. aureus, independent of PLA2 (Fig. 7), strongly suggesting that oxidase-dependent alterations of the bacteria by PMN enhanced bacterial sensitivity to the gIIA-PLA2. Our studies suggest no essential role for MPO-dependent oxidants in increased PLA2 action but do not exclude a possible role for MPO in normal PMN. Whether the observed MPO-independent effects reflect the action of ROS that are more abundant in activated MPO-deficient PMN (42) or ROS-independent effects of oxidase activation (13) requires further study.

The kinetics of PL degradation during and after phagocytosis of S. aureus by PMN, when extracellular concentrations of the gIIA-PLA2 were limiting, also suggest that PL degradation followed rather than led, to bacterial killing. However, when concentrations of the PLA2 were sufficient for the enzyme to initiate bacterial PL degradation before phagocytosis, contribution of the PLA2 to bacterial killing in the presence of PMN was also apparent, even in the absence of a functional NADPH oxidase (Fig. 7). We cannot judge from our data whether this increase in bacterial killing occurred before or after phagocytosis. In either case, however, the number of viable staphylococci within PMN was diminished.

These data suggest that, in an acute inflammatory exudate, maximal degradation of PLs of invading S. aureus may require both extracellular and intraphagocytic action of the gIIA-PLA2. Amounts of PLA2 gaining access to the phagocytic vacuole may be limited, and activation of PMN proteases during PMN activation and phagocytosis (13, 16, 60) may reduce the half-life of mobilized sPLA2. The intact appearance of ingested S. aureus observed in this (Fig. 2) and previous studies (46, 61) suggests that the bacterial cell wall remains largely intact, conferring an impediment to endogenous (group V and X) and exogenous (gIIA) PLAs action (31, 62). The conversion of bacterial PG to CL and accompanying release of glycerol (2 PG → CL + glycerol) soon after exposure of S. aureus to PMN indicate that the bacteria make an adaptive “stress response” (37, 63). Although we did not observe dramatic differences in bacterial membrane PG vs CL sensitivity to PLA2 attack (Figs. 1 and 4), this metabolic switch could have many other structural and physiological effects, including reducing membrane fluidity and ATP-consuming metabolic cycling between PG and bacterial glycolipids and increasing availability of glycerol as a potential carbon source and osmoprotectant (37, 63, 64). These changes could help ingested bacteria resist or tolerate PMN-derived cytoxins and, secondarily, PLA2 attack. It is also likely that the PG to CL conversion is just one of many stress responses that bacteria make in response to PMN (65).

Whatever the precise mechanisms by which bacterial PL degradation is enhanced or limited during phagocytosis, our findings have broader functional implications. Our results extend earlier observations (13, 16, 20) showing collaboration between O2-dependent and O2-independent antibacterial systems in phagocyte-dependent cytoxicity against prominent bacterial invaders. As our study reveals, this collaboration includes both PMN-derived

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and soluble extracellular agents, further emphasizing the complex integration of elements of innate immunity that is likely to occur in PMN-rich inflammatory exudates (22). The synergy between extracellular gIIA-PLA2 and PMN means that levels of PLA2 suffice to cause bacterial PL degradation are achieved sooner during the inflammatory response, when levels of extracellular gIIA-PLA2 are otherwise limiting. Conversely, ingestion by PMN of S. aureus before sufficient mobilization of the gIIA-PLA2 may provide a protective niche that increases the probability of persistence of bacteria and bacterial remnants (46). The relative amounts of extracellular S. aureus and gIIA-PLA2 may not only affect extracellular PLA2 action but also the extent of PMN and gIIA-PLA2 synergy and the subsequent fate of the ingested organism and its products. Our findings, together with those of others (13, 16), strongly suggest that host defense defects in CGD include both reduced elaboration of cytotoxic ROS and reduced activity of O2-, independent agents whose antibacterial activity is somehow increased by oxidative activation. The dependence of several antibacterial enzymes on oxidative activation predicts, as shown (Refs. 13, 16; this study), a defect in bacterial digestion as well as killing that may contribute to the chronic inflammation that is a hallmark of CGD (4, 10). Even in normal individuals, oxygen limitation in deep-seated infections, abscesses, and necrotic tissue could, in a similar manner, create circumstances less favorable for elimination of S. aureus and their remnants. The conversion of PG to CL, with or without oxidative activation (Fig. 8), may mean that incomplete resolution of S. aureus infections is accompanied by accumulation of CL and induction of immunological responses that can have adverse consequences, including autoimmunity (66–68).

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Disclosures

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