Effect of d-Alanylation of (Lipo)Teichoic Acids of *Staphylococcus aureus* on Host Secretory Phospholipase A2 Action before and after Phagocytosis by Human Neutrophils

Catherine L. Hunt, William M. Nauseef and Jerrold P. Weiss

*J Immunol* 2006; 176:4987-4994; doi: 10.4049/jimmunol.176.8.4987

http://www.jimmunol.org/content/176/8/4987

---

**References**

This article cites 45 articles, 23 of which you can access for free at:

http://www.jimmunol.org/content/176/8/4987.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Effect of $\alpha$-Alanylation of (Lipo)Teichoic Acids of \textit{Staphylococcus aureus} on Host Secretory Phospholipase A$_2$ (gIIA PLA$_2$) Action before and after Phagocytosis by Human Neutrophils

Catherine L. Hunt, William M. Nauseef, and Jerrold P. Weiss

Intracellular antibacterial proteins (e.g., group IIa phospholipase A$_2$ (gIIA PLA$_2$)). Accumulation of gIIA PLA$_2$ in inflammatory fluids confers potent extracellular antistaphylococcal activity and at lower concentrations promotes bacterial phospholipid degradation during phagocytosis of \textit{S. aureus} by human neutrophils. $\alpha$-alanylation of (lipo)teichoic acids of \textit{S. aureus} increases bacterial resistance to gIIA PLA$_2$ ~100-fold, raising the possibility that the resistance of ingested \textit{S. aureus} to related gV and gX secretory PLA$_2$ present in human neutrophil granules depends on $\alpha$-alanylation mediated by the \textit{dlt} operon. However, we show that isogenic wild-type and \textit{dltA} \textit{S. aureus} are equally resistant to gV/X PLA$_2$ during phagocytosis and when exposed to the purified enzymes. The fates of wild-type and \textit{dltA} \textit{S. aureus} exposed to serum and human neutrophils differed significantly only when extracellular gIIA PLA$_2$ was also present before phagocytosis. The extreme potency of the gIIA PLA$_2$ toward \textit{dltA} \textit{S. aureus} suggests that even small amounts of this extracellular enzyme mobilized early in inflammation could contribute substantially to the overall cytotoxicity of acute inflammatory exudates toward \textit{S. aureus} when $\alpha$-alanylation of (lipo)teichoic acids is limiting. The Journal of Immunology, 2006, 176: 4987–4994.

The Gram-positive bacteria \textit{Staphylococcus aureus} is part of the normal flora of many humans and frequently colonizes the anterior nares of many healthy individuals (1, 2). \textit{S. aureus} has the potential, however, to cause an array of localized and more systemic invasive diseases (3). The mobilization and action of specific innate immune systems, including professional phagocytes and soluble antimicrobial agents, at the site of bacterial invasion are crucial for control of staphylococcal infections and limitation of infection-induced host morbidity and mortality (4–7).

Polymorphonuclear leukocytes (PMN, neutrophils) are the most abundant circulating professional phagocyte and play an essential role in phagocytosis and killing of invading microorganisms such as \textit{S. aureus} (8, 9). Neutrophils respond to chemotactic signals originating from the site of infection and are rapidly mobilized from the bloodstream to arrest multiplication and dissemination of invading bacteria. During phagocytosis, bacteria are internalized within a phagocytic vacuole and exposed to membrane-bound, toxic, reactive oxygen species and granule-derived, oxygen-independent antimicrobial proteins delivered by granule-phagosome fusion (6, 10–12).

Despite the high concentrations of antimicrobial compounds within the phagocytic vacuoles of neutrophils, not all ingested \textit{S. aureus} are killed, and most, if not all, ingested cocci remain grossly intact even after many hours within the neutrophil (13–15). Appreciable degradation of bacterial phospholipids (PL) during and after phagocytosis of \textit{S. aureus} by human neutrophils in vitro requires the added presence of group IIA (gIIA) phospholipase A$_2$ (PLA$_2$) (13), a potent extracellular antistaphylococcal enzyme that is mobilized in addition to neutrophils during acute inflammation (4, 16–19). Human neutrophils lack gIIA PLA$_2$ (20), but contain two structurally related PLA$_2$, gV and gX (20, 21). To date, the gV and gX PLA$_2$ are not known to contribute to digestion of ingested \textit{S. aureus} (13), presumably because of their relatively low intrinsic antistaphylococcal activity and low abundance within human neutrophils (20, 22, 23).

$\alpha$-alanylation of cell wall and membrane (lipo)teichoic acids, mediated by the \textit{dlt} operon, increases bacterial resistance to gIIA PLA$_2$ ~100-fold (24). Reported effects of $\alpha$-alanylation on the ability of \textit{S. aureus} to form biofilms, express autolysin activity, and resist oxygen-independent neutrophil cytotoxicity suggest pleiotropic effects of this envelope modification (24–29). Most focus has been on the effect of $\alpha$-alanylation on the charge properties of these abundant polyanions and on interactions of environmental cations, including cationic antimicrobial compounds, with the bacterial envelope (24, 25, 28). In the case of gIIA PLA$_2$, the effect of $\alpha$-alanylation is not on initial protein binding to the bacterial surface, but, rather, on the ability of the bound enzyme to penetrate the cell wall and degrade membrane PL (24). This effect of $\alpha$-alanylation is the same for the highly cationic wild-type (wt) enzyme and less cationic and less active gIIA PLA$_2$ mutants, raising the possibility that the resistance of ingested \textit{S. aureus} to gV and gX PLA$_2$ in human neutrophils (13) might also depend on $\alpha$-alanylation of bacterial (lipo)teichoic acids.
Therefore, in this study we compared the sensitivity of ingested wt and dlt A. *S. aureus* to the cytotoxic effects of human neutrophils, including the actions of gV and gX PLAs. In contrast to effects of d-alanylation on the antistaphylococcal activity of gIIA PLA2, there was little or no effect of d-alanylation of *S. aureus* (lipo)teichoic acids on serum (component)-mediated opsonization of the bacteria, intracellular killing by neutrophils, or the actions of gV/X PLAs. However, substantial differences in the fate of wt or dlt A. *S. aureus* exposed to neutrophils were seen when the bacteria were exposed to extracellular gIIA PLA2 before phagocytosis. Thus, the effects of d-alanylation on resistance of *S. aureus* to innate human host defenses appear to principally reflect alterations in susceptibility to the action of gIIA PLA2. The extreme potency of this enzyme toward dlt A. *S. aureus* suggests that even small amounts of extracellular enzyme mobilized early in the host response could contribute substantially to the overall cytotoxicity of acute inflammatory exudates toward *S. aureus* when d-alanylation of (lipo)teichoic acids is limiting.

### Materials and Methods

**Materials**

Tryptic soy broth and Bacto Agar were purchased from BD Biosciences. HEPS and HBSS with (HBSS+) or without (HBSS−) divalent cations were obtained from Mediatech CellGro. Clinical grade dextran 500 was purchased from Sigma-Aldrich. [1-14C]oleic acid (50 mCi/mmol) was purchased from PerkinElmer. Chloroform, methanol, glacial acetic acid, and the HEMA 3 staining kit were purchased from Fisher Scientific. Silica gel 60 high performance TLC plates were purchased from EMD Chemicals. Purified lipid standards for phosphatidylglycerol (PG: 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-ra(c-acid)], cardiolipin (CL: tetraoleoyl cardiolipin), lysophosphatidylglycerol (1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-ra(c-acid)]), monolysocardiolipin (purified from bovine heart; ≥92% linoleic), and dilysocardiolipin (purified from bovine heart; ≥96% linoleic) were purchased from Avanti Polar Lipids.

**Bacteria**

Isogenic dlt+ (SA113) and dlt A. *S. aureus* were provided by A. Peschel (University of Tubingen, Tubingen, Germany) (28). The bacteria were grown overnight in tryptic soy broth at 37°C, diluted to an OD550 of 0.05 in fresh medium, and subcultured for 2–2.5 h to midlogarithmic phase. Subcultured bacteria were washed once, resuspended in 20 mM HEPES-in fresh medium, and subcultured for 2–2.5 h to midlogarithmic phase. These cells were then preincubated at 37°C for 10 min before addition to the bacterial suspension.

**Pooled human serum**

Serum was isolated from at least nine different healthy donors, pooled, filter-sterilized, aliquoted, and frozen at −80°C until use. Stored aliquots of serum were subjected to only one freeze/thaw cycle and used the day of thawing.

**Opsonization of bacteria and incubation with PMN**

Subcultured bacteria (10^5 CFU/ml) were incubated for 20 min at 37°C in 20 mM HEPES-buffered HBSS− supplemented with 1% HSA and 10% pooled human serum. After this incubation, bacteria were mixed 1/1 (v/v) with prewarmed neutrophils to yield final cell concentrations of 5 × 10^6 PMN/ml and 5 × 10^5 CFU/ml. Incubations were conducted in 5-ml round-bottom polystyrene tubes swirling at 180 rpm in a water bath at 37°C for up to 5 h.

**Assays of bacterial uptake by neutrophils**

After incubation at 37°C, aliquots of cell suspensions containing 7.4 × 10^4 neutrophils were spun using a cytocentrifuge (Cytospin 2; Shandon Southern Products) at 18 × g for 10 min to sediment the cells onto poly-L-lysine-coated slides that had been primed with HEPES-buffered HBSS− supplemented with 0.2% HSA. Cell smears were fixed and stained using the HEMA 3 stain kit according to the manufacturer’s protocol, then rinsed gently with deionized H2O. The smears were allowed to dry at room temperature and were subsequently covered with mounting medium (polyvinyl alcohol, Tris-base, glycerol, and 1,4-diazobicyclo[2.2.2]octane; Sigma-Aldrich) and a coverslip. The number of bacterial cocci per PMN was enumerated by visualization of the samples with a Zeiss Axioplan2 photomicroscope. At least 100 neutrophils were examined per sample.

**Assay of degradation of 14C-labeled bacterial PL by purified PLA2**

Subcultured [14C]oleate-labeled *S. aureus* (10^6 CFU/ml) were incubated for 20 min at 37°C in 20 mM HEPES-buffered HBSS− supplemented with 1% HSA, 10% pooled human serum, and purified gV, gX, or gIIA PLA2 in concentration ranging from 0–1,000 ng/ml. After this incubation, bacteria were mixed 1/1 (v/v) with a buffer solution containing 20 mM HEPES-buffered HBSS− supplemented with 1% HSA and 10% pooled human serum to yield final cell concentrations of 5 × 10^6 CFU/ml. After incubation at 37°C for 1 h, the cell suspensions were mixed with HSA to bring the final concentration of HSA to 2%. The samples were vortexed for <5 s, then spun for 5 min at ~10,000 × g to separate undigested [14C]PL that cosedimented with the bacteria from [14C]PL breakdown products ([14C]free fatty acids and [14C]lyso-PL), which were quantitatively recovered as complexes with albumin (31). Aliquots of the whole suspension, before sedimentation of bacteria and the recovered supernatant were analyzed by liquid scintillation spectroscopy to determine the percentage of [14C]lipid released by the bacteria, i.e., as a quantitation of degraded [14C]labeled bacterial PL. (18, 31).

**Assay of metabolism of 14C-labeled bacterial PL after incubation of [14C]oleate-labeled S. aureus with neutrophils**

After incubation of [14C]oleate-labeled S. aureus with neutrophils, cell suspensions containing 14C-labeled *S. aureus* and neutrophils were extracted via a modified Bligh-Dyer method (13, 32). Briefly, whole cell suspensions (1 vol) were mixed with 6 vol of chloroform-methanol (1/2; v/v) and stored at 4°C until analyzed. Samples were spun at 1900 × g for 10 min, and the fluid was removed and transferred to a fresh glass tube, leaving the pellet behind. Three volumes of 50 mM potassium chloride and 2 vol of chloroform were then added, and the samples were vortexed. The samples were spun for 10 min at 1900 × g to separate the water-methanol phase from the chloroform phase (bottom phase). The chloroform phase was removed and saved. The remaining aqueous phase was mixed with 4
vol of chloroform, vortexed, and spun for 10 min at 1900 × g. The chloroform phase was again removed and combined with the previously removed chloroform phase. The combined chloroform phases were dried under a stream of nitrogen, resuspended in 10 ml of chloroform/methanol (2/1, v/v), and applied to a high performance TLC plate. 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-labeled lipids were visualized by exposure of TLC plates to tritium storage phosphor screens analyzed by a Typhoon 9410 variable mode imager (Amersham Biosciences). Quantification was performed using ImageQuant software (Molecular Dynamics).

Assay of bacterial viability

After incubation at 37°C, aliquots (10 μl) of the cell suspension were added to 490 μl of 1% saponin in H2O. Samples were vortexed vigorously, incubated for 15 min at room temperature to lyse the neutrophils, and then serially diluted in HBSS supplemented with 10% pooled human serum and 1% HSA (incubation medium). At the times indicated, uptake of S. aureus by neutrophils was measured as described in Materials and Methods. The results represent the mean of three experiments ± SE.

Assay of bacterial lysis

Subcultured bacteria (10^8 CFU/ml) were incubated at 37°C in 20 mM HEPES-buffered HBSS supplemented with 1% HSA, 10% pooled human serum, and 1000 ng/ml recombinant gIIA PLA2. At various times, the bacterial suspension was measured using a Bio-Rad SmartSpec3000 to monitor bacterial lysis, i.e., reduced A_550.

Results

Comparison of serum requirements for uptake of wt and dltA S. aureus by human neutrophils

To facilitate comparison of bacterial PL degradation and, by implication, intracellular gV/X PLA2 action against ingested wt and dltA S. aureus, we preincubated S. aureus (10^7 CFU/ml) with or without the indicated amount of pooled human serum and then mixed (1/1, v/v) with human neutrophils (10^7 cells/ml) in HBSS supplemented with 20 mM HEPES, pooled human serum, and 1% HSA (incubation medium). At the times indicated, uptake of S. aureus by neutrophils was measured as described in Materials and Methods. The results represent the mean of three experiments ± SE.

Comparison of serum requirements for uptake of wt or dltA S. aureus by human neutrophils.

S. aureus (10^7 CFU/ml) were preincubated with or without the indicated amount of pooled human serum and then mixed (1/1, v/v) with human neutrophils (10^7 cells/ml) in HBSS supplemented with 20 mM HEPES, pooled human serum, and 1% HSA (incubation medium). At the times indicated, uptake of S. aureus by neutrophils was measured as described in Materials and Methods. The results represent the mean of three experiments ± SE.
**FIGURE 3.** Similar microscopic appearance of wt and dltA *S. aureus* after ingestion by human neutrophils. *S. aureus* (10^7 CFU/ml) were opsonized with serum and incubated with human neutrophils (10^7 cells/ml) for 10 min (A and B) or 5 h (C and D). Smears of cell suspensions were prepared and stained as described in Materials and Methods. Cocci showing normal staining properties are noted by arrows; cocci exhibiting less intense staining are noted by arrowheads. The micrographs shown are representative of six or more experiments.

*dltA S. aureus*, we chose experimental conditions that permitted measurement of bacterial uptake, killing, and digestion in the same samples. After preincubation with serum to opsonize the bacteria, we incubated equal numbers of bacterial CFU and neutrophils (5 × 10^7/ml, each). Because *d*-alanylation of (lipo)teichoic acids might affect the efficiency and/or consequences of important interactions of serum components with the bacterial envelope, we first compared the dose requirements for serum-dependent opsonophagocytosis by human neutrophils (Fig. 1). Optimal uptake of wt or *dltA S. aureus* required ≥4% pooled human serum; at serum concentrations ≥4%, uptake of nearly all bacteria from each strain occurred within 30 min. At lower serum concentrations, bacterial uptake at 30 min was less than that at ≥4% serum, but increased progressively over time to levels greater than those in the initial inoculum, reflecting the gradual uptake of some of the remaining extracellular bacteria that had multiplied. In the absence of neutrophils, both strains grew exponentially in the presence or the absence of serum (Fig. 2B; data not shown). Heat inactivation of serum greatly reduced bacterial uptake, consistent with an essential role of complement in normal serum-dependent uptake of *S. aureus* by human neutrophils (data not shown) (33, 34). Taken together, these data demonstrate that the serum requirements were essentially the same for opsonophagocytosis of wt or *dltA S. aureus* by human neutrophils.

**Effect of *d*-alanylation of (lipo)teichoic acids on sensitivity of *S. aureus* to PL degradation by human neutrophils**

To facilitate assay of bacterial PL degradation during and after phagocytosis, the membrane PL of wt or *dltA S. aureus* were metabolically labeled with [14C]oleic acid during bacterial growth. There was rapid and nearly complete uptake (Fig. 2B) and ≥1 log killing (Fig. 2A) of both bacterial strains. Each strain grew exponentially in the presence of serum but the absence of neutrophils (Fig. 2A). The nearly constant number of cocci (either wt or *dltA*) observed with neutrophils after 10 min to 5 h of incubation indicated rapid bacterial uptake and little or no subsequent bacterial lysis (Fig. 2B). Both strains showed a neutrophil-induced conversion of much of the major bacterial PL, PG, to CL (Fig. 2, A and D). Smears of bacterial suspensions were prepared and stained as described in Materials and Methods. The results shown are the mean of two or more experiments ± SE.

**FIGURE 4.** Comparison of the sensitivity of wt or *dltA S. aureus* to gIIA, gV, and gX PLA2. [14C]oleate-labeled *S. aureus* (5 × 10^7 CFU/ml) (A, wt; B, *dltA*) were incubated with the indicated concentrations of gIIA, gV, or gX PLA2. Bacterial PL degradation was measured as described in Materials and Methods. The results shown are the mean of two or more experiments ± SE.

Comparison of the sensitivities of wt and *dltA S. aureus* to human gIIA, V, and X PL-2

The absence of neutrophil-induced bacterial PL degradation during phagocytosis could reflect limiting amounts of granule-derived gV and X PL-2 as well as to gIIA PL-2 for comparison. The much greater sensitivity to gIIA PL-2 of the *dltA* strain compared with that of wt *S. aureus* was confirmed (Fig. 4). In contrast, neither gV nor gX PL-2 was active against the wt or *dltA* strain, even when added at concentrations 100- to 1000-fold greater than that needed for gIIA PL-2 action against the *dltA* strain (Fig. 4B). These findings suggest that the low intrinsic antistaphylococcal activity of gV
Effect of combined presence of human gIIA PLA2 and neutrophils on fate of wt or dltA S. aureus

Neutrophil-rich, acute inflammatory exudates contain extracellular gIIA PLA2 (17) (N. S. Liang, J. Lee, and J. Weiss, unpublished observation), thus exposing invading bacteria to both neutrophils and gIIA PLA2 at the same time. Therefore, we examined the fate of wt or dltA S. aureus exposed simultaneously to neutrophils and gIIA PLA2, as well as to serum factors needed for opsonization. GIIA PLA2 at low concentrations (100 ng/ml; Fig. 5) resulted in dramatically greater cytotoxicity against the mutant vs the parent strain, as manifested by degradation of phospholipids, loss of CFU, and apparent lysis of the bacteria (Fig. 5). At these enzyme concentrations, gIIA PLA2-dependent bacterial phospholipid degradation and killing of the dltA strain occurred with or without neutrophils present (Fig. 5, D and E). However, phagocytosis was nearly complete within 10 min (Fig. 2A) and thus was much faster than the rate of extracellular PLA2 action, which required 30–60 min (24) (data not shown). In light of these differential kinetics, the data suggest that most degradation and killing seen in the presence of neutrophils occurred after bacterial ingestion. In addition, apparent lysis of the bacteria (i.e., disappearance of bacterial cocci) occurred only when the bacteria were simultaneously exposed to neutrophils and gIIA PLA2 (Figs. 5F and 6F) and not to either neutrophils (Figs. 3D and 5F) or PLA2 (Figs. 5F and 6D) alone. Addition of PLA2 after the dltA bacteria had been ingested by neutrophils had much less effect than when it was added simultaneously (Fig. 5, D–F), indicating that under these circumstances, phagocytosis by neutrophils could provide a protective haven for S. aureus.

Discussion

In the present study we have reproduced the observation that there is a marked difference in the sensitivities of wt and dltA S. aureus to gIIA PLA2 (24). The sensitivity of the dltA strain is so great that even small amounts of the extracellular PLA2 (∼10 ng/ml) are sufficient to dramatically increase the antibacterial cytotoxicity effect in human neutrophil-bacterial suspensions as judged by bacterial phospholipid degradation, CFU, and number of detectable cocci. Even without neutrophils, gIIA PLA2 at these low doses can produce nearly complete phospholipid degradation and killing of dltA S. aureus (Figs. 5, D and E). However, the rapidity of bacterial ingestion by neutrophils (Fig. 2A) relative to the rate of the extracellular action of PLA2 (18, 24, 35) makes it very likely that most of the observed cytotoxic effects occur within neutrophils, amplified by PLA2 bound to the bacteria before phagocytosis (13, 24, 25). The virtual disappearance of visible cocci after simultaneous exposure of dltA S. aureus to gIIA PLA2 and neutrophils (Figs. 5F and 6F) suggests that extensive bacterial phospholipid degradation mediated by gIIA PLA2 potentiates bacterial lysis within neutrophils, possibly by facilitating the action of neutrophil as well as bacterial cell wall-degrading enzymes (35).

The sensitivity of wt strains of S. aureus to gIIA PLA2 can be significantly enhanced by exposure of the bacteria to neutrophils and requires a functional neutrophil NADPH oxidase to permit PLA2 action at extracellular concentrations that are otherwise limiting (Fig. 5A) (13). Synergy between neutrophils and gIIA PLA2 was not observed with the dltA mutant (data not shown), probably because this mutant strain is already so sensitive to gIIA PLA2. Despite the exquisite intrinsic sensitivity of dltA S. aureus to gIIA PLA2, they are virtually resistant to the added extracellular enzyme once they have been ingested by neutrophils (Fig. 5, D–F). Whether this simply reflects too little added enzyme gaining access
either as purified lipid dispersions or as part of cell wall-depleted protoplasts (22–24) (N. S. Liang and J. Weiss, unpublished observations). Thus, the relative inactivity of gV and gX PLAs compared with gIIA PLA2 toward intact bacteria probably reflects their inability to efficiently bind to and penetrate the cell wall as needed to access PL in the cell membrane (23, 24, 35). The apparent retention of the overall structural integrity of the cell wall of the ingested bacteria during and after phagocytosis by human neutrophils in the absence of gIIA PLA2 may thus be important in maintaining bacterial resistance to neutrophil PLA2.

We cannot exclude the possibility that higher concentrations (>1 μg/ml) of gV and/or gX PLAs would have shown some activity. This may be most likely for the gV enzyme, whose cationic properties (net charge, +6) (42) would be expected to facilitate its action against dltA S. aureus (22–24). However, no circumstances are known in which either gV or gX PLA2 accumulates to such high extracellular levels. In neutrophil-rich inflammatory exudates, the much more potent gIIA PLA2 is also the more abundant enzyme (4, 18, 19, 40). Our experiments clearly show that the levels of gV and gX PLA2 in human neutrophils are insufficient to permit these enzymes to contribute to antibacterial cytotoxicity, even of the dltA mutant.

In contrast to the remarkable differences in sensitivities of wt and dltA S. aureus to gIIA PLA2, we did not observe appreciable differences in either human serum-dependent opsonization or subsequent neutrophil-dependent cytotoxicity toward the two strains in the absence of extracellular gIIA PLA2. As expected, the serum effects were heat sensitive, implying complement-dependent opsonization, and thus indicate that there was little or no effect of α-alanylation of (lipo)teichoic acids on complement fixation on the bacterial surface or subsequent engagement of neutrophil complement receptors (33, 34). The similarity of neutrophil-dependent cytotoxicity toward wt and dltA S. aureus was apparent by three independent assays conducted in the same neutrophil-bacterial suspension: bacterial phospholipid degradation, CFU, and microscopic appearance (Figs. 2 and 3). Our findings differ from those reported previously by Collins et al. (26), who showed significantly greater serum- and human neutrophil-dependent killing of dltA than of wt S. aureus using the same strains we used in this study. Attempts to date to identify which of the many differences in experimental design could account for the different observations have been unsuccessful.

In summary, our findings strongly suggest that despite the pleiotropic effects of α-alanylation of (lipo)teichoic acids on bacterial envelope properties, the absence of α-alanylation has a much greater effect on bacterial sensitivity to gIIA PLA2 than on other neutrophil antimicrobial effector systems. Extracellular gIIA PLA2 is required for bacterial PL degradation during and after phagocytosis by human neutrophils of dltA as well as dltA S. aureus (Fig. 5, A and D). During local and/or systemic acute inflammation, extracellular gIIA PLA2 is mobilized in a time-dependent fashion by a variety of acute inflammatory stimuli, including, but not limited to, infection by S. aureus (4, 16, 18, 19, 40) (N. S. Liang, I. Lee, and J. Weiss, unpublished observation). Inducers of gIIA PLA2 synthesis and secretion include specific bacterial products (43, 44) and host cytokines (e.g., IL-1, IL-6, TNF-α, and IFN-γ) (40, 45) that can be induced by a variety of microbial and/or host products (39), including Gram-positive bacterial products. Extracellular gIIA PLA2 under these conditions may reach concentrations as high as 1–2 μg/ml (100–1000 times above resting levels) and are sufficient for direct extracellular antibacterial action (4, 18, 19). However, early in the acute inflammatory response, when

to the PL of the ingested bacteria and/or adaptive changes that render ingested bacteria and their PL less sensitive to gIIA PLA2 remains to be determined. Phagocytosis of wt or dltA S. aureus is accompanied by conversion of much of the bacterial PG to CL (Fig. 2, C and D), probably reflecting a bacterial stress response that could increase the resistance of the bacterial envelope to gIIA PLA2 (100 ng/ml) for 60 min (A–D) or opsonized with serum without PLA2 for 20 min, then incubated for 60 min with human neutrophils (10^7 cells/ml) and gIIA PLA2 (100 ng/ml). After 60 min, smears were prepared by cytospin and stained as described in Materials and Methods. A–D, Representative fields of wt or dltA S. aureus after 60 min without (A and B) and with (C and D) gIIA PLA2. E and F, Representative fields of wt or dltA S. aureus after 60-min incubation with neutrophils and gIIA PLA2, showing normal staining properties are noted by arrows; cocci exhibiting less intense staining are noted by arrowheads. The micrographs shown are representative of three or more experiments.

FIGURE 6. Different microscopic appearances of wt and dltA S. aureus exposed to gIIA PLA2, and human neutrophils simultaneously. S. aureus (10^7 CFU/ml) were incubated with 10% pooled human serum containing gIIA PLA2 (100 ng/ml) for 60 min (A–D) or opsonized with serum without PLA2 for 20 min, then incubated for 60 min with human neutrophils (10^7 cells/ml) and gIIA PLA2 (100 ng/ml). After 60 min, smears were prepared by cytospin and stained as described in Materials and Methods. A–D, Representative fields of wt or dltA S. aureus after 60 min without (A and B) and with (C and D) gIIA PLA2. E and F, Representative fields of wt or dltA S. aureus after 60-min incubation with neutrophils and gIIA PLA2, showing normal staining properties are noted by arrows; cocci exhibiting less intense staining are noted by arrowheads. The micrographs shown are representative of three or more experiments.
extracellular gIIA PLA2 concentrations may be limiting, the concerted actions of extracellular gIIA PLA2 and neutrophils (13) may be critical. 

Although initial gIIA PLA2 binding to wt or dltA S. aureus is the same, the much greater sensitivity of dltA S. aureus to bound PLA2 permits initiation of bacterial PL degradation at low PLA2 concentrations and additional PLA2 binding before phagocytosis (24). The greater binding and activity of bound PLA2 toward dltA S. aureus result in much more rapid and extensive bacterial membrane PL degradation before, during, and after phagocytosis and, in parallel, diminished intracellular persistence of viable bacteria or their visible remnants (Figs. 5, D and F, and 6F). These findings strongly suggest that factors regulating the mobilization and action of PLA2 at infectious sites are important determinants of the ultimate fate of S. aureus ingested by neutrophils. The extraordinary sensitivity of dltA S. aureus to gIIA PLA2 means that effective extracellular enzyme concentrations will be achieved earlier during the inflammatory response if d-lalanation of the invading bacteria is limiting, promoting gIIA PLA2 interaction with S. aureus before the bacteria are sequestered by neutrophils, thereby enhancing bacterial digestion and disassembly during and after phagocytosis and increasing the generation of potentially bioactive lipid metabolites. Conversely, the resistance of d-lalanation confers to extracellular gIIA PLA2, makes it more likely that S. aureus will be ingested by neutrophils under conditions that favor the survival of some of the ingested bacteria and persistence of potentially proinflammatory bacterial remnants (13–15). The fact that levels of d-lalanation can be transcriptionally (T. Koprivnjak and J. Weiss, submitted for publication) and posttranslationally (46) regulated may mean that there are circumstances in which the levels of d-lalanation of dltA S. aureus are reduced. These regulatory systems may offer novel targets for intervention that could significantly increase the sensitivity of invading S. aureus to host defenses.

Acknowledgments
We thank Dr. Michael Gelb for providing purified recombinant gV and gX PLA2, and Dr. Andreas Peschel for supplying the bacterial strains that we used. We also thank members of the Inflammation Program for their technical assistance and critiques of these studies.

Disclosures
The authors have no financial conflict of interest.

References

Downloaded from http://www.jimmunol.org/ by guest on April 13, 2017


