

# Antibacterial Action of Extracellular Mammalian Group IIA Phospholipase A2 against Grossly Clumped *Staphylococcus aureus*

MARY E. DOMINIECKI<sup>1</sup> AND JERROLD WEISS<sup>2\*</sup>

Department of Microbiology, New York University School of Medicine, New York, New York 10016,<sup>1</sup>  
and Inflammation Program, Departments of Internal Medicine and Microbiology,  
University of Iowa College of Medicine, Iowa City, Iowa 52242<sup>2</sup>

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**Fibrinogen-dependent interactions of *Staphylococcus aureus* are believed to contribute to bacterial virulence by promoting bacterial attachment to fibrinogen-coated surfaces and inducing the formation of bacterial clumps that are likely resistant to phagocytosis. Although *S. aureus* produces several fibrinogen-binding proteins, the cell wall-associated protein clumping factor (encoded by *clfA*) appears to be most important in bacterial interactions with immobilized or soluble purified fibrinogen. We have compared bacterial clumping in several strains of *S. aureus*, including isogenic ClfA<sup>+</sup> and ClfA<sup>-</sup> Newman strains, in the presence of purified rabbit fibrinogen, human plasma, and inflammatory fluid and examined the effect of clumping on bacterial sensitivity to mammalian group IIA phospholipase A2 (PLA2). This enzyme is the major extracellular bactericidal agent in inflammatory fluid active against *S. aureus*. Both ClfA-dependent and ClfA-independent bacterial clumping was observed, depending on the source and fibrinogen content of the biological fluid. In each case, clumping only partially reduced the antibacterial activity of PLA2, suggesting that this extracellular enzyme can substantially penetrate dense bacterial clumps. Bacterial clumps could be dispersed by added proteases, restoring full antibacterial activity to PLA2. Thus, the extracellular mobilization of group IIA PLA2 during inflammation may provide a mechanism by which the host can control the proliferation and survival of *S. aureus* even after bacterial clumping.**

*Staphylococcus aureus* is an opportunistic pathogen that causes many different infections, including endocarditis, septicemia, abscesses, and catheter-related infections. A major characteristic of *S. aureus* is its ability to form bacterial aggregates (clumps) while adherent to host tissue or even in suspension. Bacteria are often found in clumps within abscesses and endocarditis vegetations (16, 17). Most clumping is mediated through fibrinogen-binding proteins that allow bacteria to bind to fibrinogen at these sites (4, 24, 25). *S. aureus* contains several fibrinogen-binding proteins, including coagulase (Coa) (25, 34), Map (26), Efb (2, 3), fibrinogen-binding protein (Fbp) (5), and clumping factors A and B (ClfA and ClfB) (24, 30). Data currently available suggest that, of the various surface-associated fibrinogen-binding proteins, ClfA has the highest affinity for fibrinogen ( $K_d = 9.9 \times 10^{-9}$  [7, 13, 24]) and therefore may be expected to play a prominent role in fibrinogen-dependent clumping at low fibrinogen concentrations. Most clinical isolates of *S. aureus* are clumping factor positive (18, 23, 36, 37, 40, 41). Clumping may be advantageous to the bacteria by reducing susceptibility to host defense mechanisms such as phagocytosis by polymorphonuclear leukocytes (16). Because clumped bacteria are likely too large to be phagocytosed, the host may depend more on extracellular defenses to eliminate clumped bacteria.

Group IIA phospholipase A2 (PLA2) is an important component of extracellular defenses (11, 35, 47–49). PLA2s are ubiquitous enzymes that catalyze the hydrolysis of the *sn*-2

fatty acyl bonds of phospholipids, liberating free fatty acids and lysophospholipids. The mammalian secretory 14-kDa group IIA PLA2 is mobilized at sites of inflammation and exerts potent antibacterial activity against gram-positive bacteria, including *S. aureus* (47, 48), which is not exhibited by other, closely related PLA2s. The potent extracellular antibacterial activity of inflammatory fluid against *S. aureus* is accounted for almost entirely by the presence of group IIA PLA2 (100 to 1,000 ng/ml) (47, 48).

In this study, we have examined the clumping of *S. aureus* in the presence of purified fibrinogen, plasma, and a cell-free inflammatory fluid and the effects of clumping on bacterial susceptibility to extracellular group IIA PLA2. We show that clumped bacteria remain susceptible to the bactericidal action of PLA2, suggesting that PLA2 may aid in the destruction of clumped bacteria.

## MATERIALS AND METHODS

**Bacteria.** The *S. aureus* strains used in this study, Newman ClfA<sup>+</sup> and Newman ClfA<sup>-</sup>, were obtained from Timothy Foster (Trinity College, Dublin, Ireland) and have been described elsewhere (8, 25). Strains 3, 5A, and 18 are clinical isolates from Tisch Hospital Clinical Microbiology Laboratory (New York, N.Y.). RN450 (8325-4) (31) was obtained from Barry Kreiswirth (Public Health Research Institute, New York, N.Y.). Bacteria were grown overnight at 37°C in Trypticase soy broth (TSB; Difco, Detroit, Mich.), washed in sterile physiological saline, and diluted to  $1.5 \times 10^7$  bacteria/ml in fresh medium for subculture. Bacteria were subcultured to mid-log phase, washed, and resuspended in saline to a final concentration of  $10^9$ /ml.

**Materials.** Purified rabbit fibrinogen, proteinase K, plasmin, plasminogen, V8 protease, and pronase were obtained from Sigma Chemical Co. (St. Louis, Mo.). Sheep anti-rabbit fibrinogen was obtained from Enzyme Research Laboratories, Inc., South Bend, Ind. RPMI was obtained from BioWhittaker, Walkersville, Md. Blood was collected from healthy donors, with informed consent, into citrated, siliconized tubes and centrifuged to obtain human plasma. PLA2 was purified from the cell-free (ascitic) fluid (AF) of glycogen-elicited inflammatory rabbit peritoneal exudates as previously described (48). PLA2 and other cationic

\* Corresponding author. Mailing address: Departments of Internal Medicine and Microbiology, University of Iowa College of Medicine, 200 Hawkins Dr., Iowa City, IA 52242. Phone: (319) 384-8622. Fax: (319) 356-4600. E-mail: jerrold-weiss@uiowa.edu.

proteins were quantitatively removed by adsorption of AF to CM-Sephadex as previously described (48). The resulting "PLA2-depleted AF" contains >99% of the total AF protein. AF filtrate (<1% of the protein content of AF), which has the same electrolyte and small-molecule content as AF, was prepared by ultrafiltration of PLA2-depleted AF through Centriprep-10 filters (Amicon, Beverly, Mass.). Filtrate was supplemented with 1% albumin (wt/vol) before use to match the overall protein concentration of AF.

**Assay of bacterial clumping.** The ability of certain fluids to produce bacterial clumping was assessed by microscopic examination and by assay of bacterial CFU. Assay mixtures typically contained  $10^8$  bacteria/ml and AF filtrate or RPMI supplemented with 1% albumin and 20 mM HEPES (pH 7.4). Various amounts of purified fibrinogen, PLA2-depleted AF, or human plasma were added to individual assay mixtures. Mixtures were incubated at 37°C. CFU was measured by diluting samples in sterile saline and plating in Trypticase soy agar (Difco). Bacterial colonies were enumerated after incubation at 37°C for 18 to 24 h.

**Immunoelectrophoresis.** Laurell Rocket immunoelectrophoresis was performed as previously described (21). In brief, twofold serial dilutions of rabbit fibrinogen (starting concentration, 5 mg/ml) and PLA2-depleted AF in saline were electrophoresed overnight at 70 V in a 1% agarose gel containing 90 µg of sheep anti-rabbit fibrinogen/ml. The running buffer was 7 mM Tris-Tricine buffer (pH 8.6). The gel was washed twice in saline and once in distilled water. The gel was pressed, dried, and stained with Coomassie blue. The peak heights obtained with purified rabbit fibrinogen were used to create a standard curve, and the amount of fibrinogen in PLA2-depleted AF was determined by comparison to the standard curve. In agreement with results obtained by Hawiger et al. (12), the amount of fibrinogen in PLA2-depleted AF was determined to be ~300 µg/ml.

**Assay of effect of added protease(s) on clumped bacteria.** Bacteria ( $10^8$ /ml) were incubated with purified rabbit fibrinogen (12 µg/ml) in AF filtrate for 60 min to allow clumping to occur. Various concentrations (0 to 100 µg/ml) of different proteases (proteinase K, pronase, plasmin, and plasminogen) were added to clumped bacteria and incubated at 37°C for 15 min, followed by an assay of bacterial colony-forming capabilities as described above.

**Radiolabeling of lipids of *S. aureus*.** *S. aureus* phospholipids were radiolabeled by growth in TSB supplemented with 1 µCi of  $^{14}$ C-oleate/ml and 0.1% albumin as previously described (48). In brief, washed bacteria were subcultured ( $1.5 \times 10^7$ /ml) in 2 ml of TSB with 2 µCi of  $^{14}$ C-oleate for 2 1/2 h at 37°C. Bacteria were washed and resuspended in fresh TSB and further incubated for 20 min. Bacteria were then washed in TSB containing 0.5% albumin (to remove unesterified free fatty acids) and resuspended in saline to a final concentration of  $10^9$ /ml.

**Measurement of degradation of labeled phospholipids from *S. aureus*.** Labeled bacteria (Newman ClfA<sup>+</sup> or ClfA<sup>-</sup>) were incubated at 37°C for 60 min in the typical assay mixtures, supplemented as indicated with purified rabbit fibrinogen or with PLA2-depleted AF containing fibrinogen (0 to 270 µg/ml) or 90% human plasma, to allow clumping to occur. Purified rabbit group IIA PLA2 was then added, and bacteria were further incubated at 37°C for 60 min. The action of PLA2 against *S. aureus* was assayed as the enzyme-triggered release of  $^{14}$ C-oleate-labeled material, which reflects the breakdown of membrane phospholipids and the capture of phospholipid breakdown products by extracellular albumin.

**Visualization of clumped bacteria by light microscopy.** Aliquots of assay mixtures (10 µl) were routinely Gram stained (Bacto 3 step kit; Difco), and clumping was assessed microscopically.

**Statistics.** One-tailed *t* tests were performed where indicated. *P* values of <0.05 were considered statistically significant.

## RESULTS

**Clumping of ClfA<sup>+</sup> and ClfA<sup>-</sup> *S. aureus* in biological fluids: role of bacterial and fibrinogen concentrations.** In previous studies, fibrinogen-*S. aureus* interactions were assessed by measuring bacterial adherence to fibrinogen-coated surfaces (1, 6, 25, 27, 43, 44), but few studies have analyzed bacterial clumping in solution. Therefore, to define experimental conditions in which clumping of *S. aureus* occurred, initial experiments were carried out in which the clumping of isogenic strains of ClfA<sup>+</sup> and ClfA<sup>-</sup> *S. aureus* was measured in the presence of purified fibrinogen, PLA2-depleted inflammatory AF, and human plasma by using different starting bacterial concentrations. Because clumping reflects (fibrinogen-mediated) cross-linking of bacteria, it likely depends on bacterial as well as fibrinogen concentrations. Figure 1 shows the fibrinogen and bacterial concentration dependence of clumping of the Newman ClfA<sup>+</sup> and ClfA<sup>-</sup> strains. Both the rate (data not shown) and the extent of bacterial clumping in the presence of purified rabbit fibrinogen or more complex biological fluids were greater at higher bacterial concentrations (greatest at  $10^8$ /ml, intermediate at  $10^7$ /ml, and least at  $10^6$ /ml) and higher fibrinogen con-

centrations (Fig. 1). No clumping occurred in the absence of fibrinogen (i.e., in filtrate alone). Differences in clumping between the ClfA<sup>+</sup> and ClfA<sup>-</sup> strains were most pronounced at lower fibrinogen concentrations. Visualization of the bacteria by light microscopy confirmed the differences in clumping between ClfA<sup>+</sup> and ClfA<sup>-</sup> bacteria at low-intermediate concentrations of fibrinogen and revealed a remarkably dense three-dimensional network of aggregated bacteria at higher fibrinogen concentrations, again more pronounced in ClfA<sup>+</sup> bacteria (Fig. 2).

Comparison of bacterial clumping induced by purified fibrinogen, PLA2-depleted AF, or plasma showed that bacterial clumping was similar in all three media when similar amounts of fibrinogen were present. Clumping of ClfA<sup>+</sup> bacteria (as assessed by reduced bacterial CFU and visual inspection) was induced at concentrations of fibrinogen 25 to 100 times lower (Fig. 1) than those required for clumping of the ClfA<sup>-</sup> strain.

**Clumping of other strains of *S. aureus*.** The Newman strain has been used in many studies of fibrinogen-dependent adherence and clumping of *S. aureus* because it expresses high levels of clumping factor (8). Therefore, we examined the clumping of a few other strains of *S. aureus*, including three randomly chosen clinical isolates (Table 1). The extent of bacterial clumping in media containing purified fibrinogen or in more complex biological fluids varied depending on the bacterial strain. One clinical strain (strain 18) exhibited clumping properties, in each of the media tested, similar to those of the ClfA<sup>+</sup> Newman strain.

**Susceptibility of clumped bacteria to PLA2.** Since clumped bacteria are likely to be refractory to phagocytosis (16), we sought to test the susceptibility of clumped bacteria to group IIA PLA2, a potent member of the extracellular host defense arsenal. Figure 3 shows that fibrinogen produces dose-dependent inhibition of PLA2 activity against both ClfA<sup>+</sup> and ClfA<sup>-</sup> Newman strains of *S. aureus*. At lower fibrinogen concentrations, PLA2 activity was reduced against the ClfA<sup>+</sup> strain only (Fig. 3). However, at high concentrations of purified fibrinogen or in 90% human plasma ( $\geq 270$  µg/ml), when clumping of both ClfA<sup>+</sup> and ClfA<sup>-</sup> bacteria is similar (Fig. 1), the activity of PLA2 against both strains was significantly reduced. Little or no inhibitory effect of fibrinogen (or PLA2-depleted AF) on PLA2 activity was seen when incubations were carried out with bacterial concentrations too low for clumping (Fig. 4). Thus, the reduced sensitivity of bacteria to PLA2 reflects bacterial clumping and not simply the effect of fibrinogen binding to the bacterial surface. It should be noted that PLA2 retains appreciable activity even against densely clumped bacteria and that degradation of  $\geq 50\%$  of the membrane phospholipids of the entire bacterial population can be produced when higher concentrations of PLA2 are added (Fig. 4) (i.e., within the range of PLA2 concentrations mobilized at inflamed sites [19, 28, 29, 47, 48]).

**Reversal of fibrinogen-induced bacterial clumping by the addition of proteases.** The ability of PLA2 added to the medium to degrade the membrane phospholipids of densely clumped *S. aureus* suggested that the fibrinogen attached to *S. aureus* could be susceptible to added proteases and hence that added proteases could reverse bacterial clumping. The addition of proteinase K, pronase, or plasmin to clumped bacteria promptly reversed bacterial clumping in a dose-dependent fashion (data not shown). Reversal of clumping was manifest by visual appraisal (Fig. 2G) and restoration of CFU (Table 2). Maximum effects occurred with  $\geq 10$  µg of protease/ml. Plasminogen at 10-fold-higher concentrations also reversed clumping. After protease treatment (Table 2; Fig. 5), the CFU of previously clumped bacterial suspensions was as great as

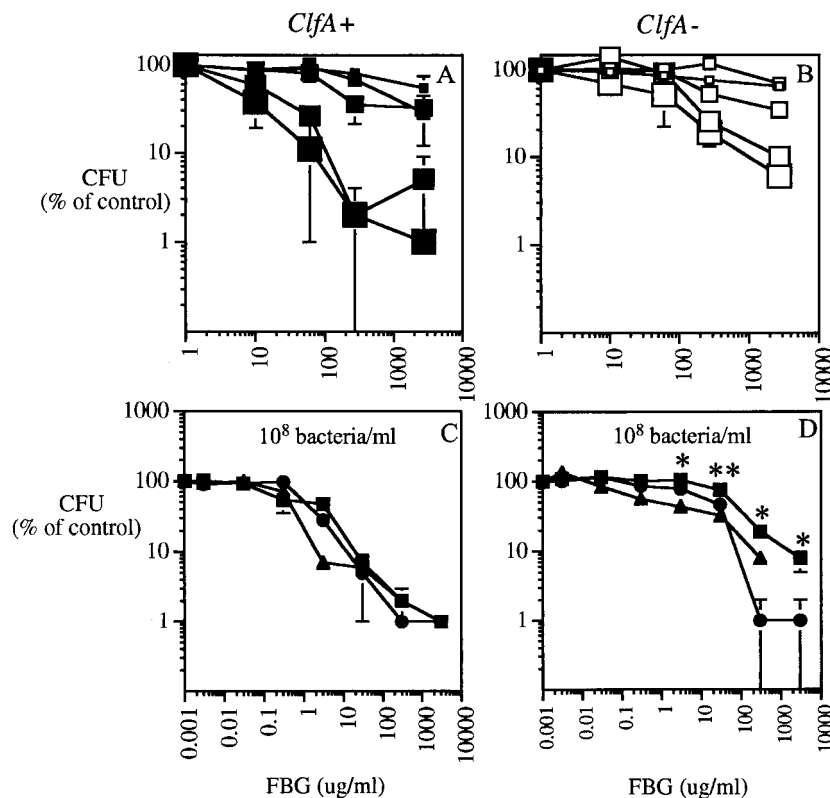


FIG. 1. Comparison of bacterial clumping in purified rabbit fibrinogen, PLA2-depleted AF, and human plasma. Bacteria (*ClfA*<sup>+</sup> [A and C] or *ClfA*<sup>-</sup> [B and D]) were incubated at 37°C for 60 min with increasing doses of purified rabbit fibrinogen (FBG) in buffered AF filtrate (A and B) or with increasing doses of purified fibrinogen (squares), PLA2-depleted AF (triangles), or human plasma (circles) in buffered AF filtrate (C and D). (A and B) Sizes of squares, from smallest to largest, correspond to increasing bacterial concentrations of 10<sup>6</sup>, 3 × 10<sup>6</sup>, 10<sup>7</sup>, 3 × 10<sup>7</sup>, and 10<sup>8</sup>/ml. (C and D) The concentration of bacteria was 10<sup>8</sup>/ml. Doses of purified fibrinogen, PLA2-depleted AF, and plasma added are represented according to the amount of fibrinogen added. After incubation, bacterial CFU were measured as described in Materials and Methods; bacterial clumping is manifest as reduced CFU. Each result is expressed as the percentage of the CFU of bacteria incubated in AF filtrate without added fibrinogen and represents the mean ± standard error of the mean (SEM) of at least three independent determinations. Asterisks highlight experimental conditions in which clumping of the *ClfA*<sup>+</sup> strain is significantly greater than that of the *ClfA*<sup>-</sup> strain (\*, *P* < 0.05; \*\*, *P* < 0.0005).

that of bacteria not exposed to clumping conditions. Thus, bacterial growth continued at a normal rate during bacterial clumping. This was true even when bacteria were clumped for as long as 3 h (data not shown).

**Reversal of bacterial clumping by plasmin restores sensitivity to PLA2.** Since the proteases were able to dissolve bacterial clumps, we tested whether protease treatment could also increase bacterial susceptibility to PLA2. As shown in Fig. 5, plasmin rendered previously clumped bacteria as susceptible to the bactericidal (Fig. 5A) and phospholipolytic (Fig. 5B) activities of PLA2 as bacteria not previously exposed to fibrinogen. Plasmin had no effect on PLA2 activity toward bacteria normally dispersed in suspension (Fig. 5B). Thus, the effect of plasmin on PLA2 activity apparently reflects its effect on bacterial clumping.

## DISCUSSION

The principal goal of this study was to determine the effect of clumping on bacterial sensitivity to the antibacterial action of group IIA PLA2. This study was made possible by our ability to evaluate bacterial clumping in a semiquantitative fashion by measuring decreases in CFU and by microscopic analysis.

Clumping of *S. aureus* has long been studied as a potential virulence factor. Numerous studies have shown that *S. aureus* can bind to several extracellular matrix proteins (laminin, collagen, fibrinogen, and fibronectin) through microbial sur-

face components recognizing adherent matrix molecules (MSCRAMMs) (9, 10, 14, 15, 33, 42, 46). Fibrinogen appears to be the predominant host matrix protein involved in clumping and adherence to surfaces such as catheters and prosthetic devices (e.g., heart valves) (1, 6, 25, 27, 43, 44). Clumping and adherence to surfaces containing bound fibrinogen are chiefly mediated by clumping factor (*ClfA*), apparently the highest-affinity fibrinogen-binding protein of *S. aureus* (7, 24, 25, 27, 44). Our results indicate an important role of fibrinogen and *ClfA* in clumping of the Newman strain in biological fluids. In isogenic strains differing only in *ClfA*, clumping of the *ClfA*<sup>+</sup> strain was greater. These differences were observed under all conditions but especially at low fibrinogen concentrations (Fig. 1; Table 1), when the high-affinity fibrinogen-binding properties of *ClfA* are likely most important. In media containing higher levels of fibrinogen, such as undiluted biological fluids, extensive clumping of the *ClfA*<sup>-</sup> strain was also observed. Clumping of this strain was similar in medium containing added purified fibrinogen and in more complex biological fluids, provided that similar amounts of fibrinogen were added (Fig. 1; Table 1). These results suggest that *ClfA*-independent clumping of the Newman strain in biological fluids is also largely fibrinogen dependent and thus is likely mediated by *ClfA*-independent cell wall-associated fibrinogen-binding proteins such as *ClfB* (30), *Fbp* (5), and *Map* (26).

Examination of several other strains of *S. aureus* revealed a wide spectrum of clumping behaviors (Table 1). The molecular



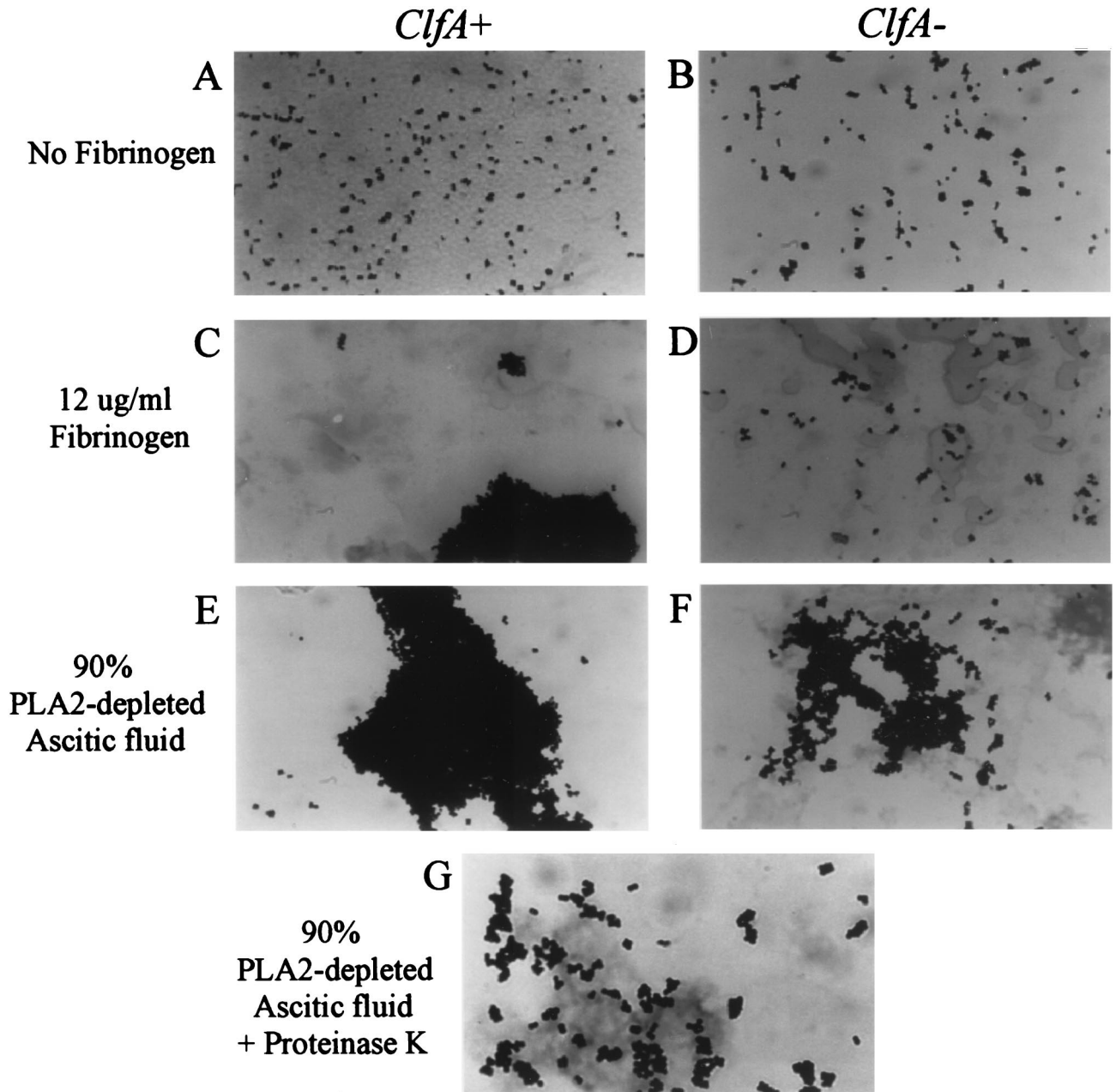


FIG. 2. Visual appraisal of clumping. Aliquots ( $10\ \mu\text{l}$ ) of assay mixtures were Gram stained and visualized by light microscopy as described in Materials and Methods. The Newman *ClfA*<sup>+</sup> (A, C, and E) and *ClfA*<sup>-</sup> (B, D, and F) strains ( $10^8$  bacteria/ml) are shown after 60 min of incubation in AF filtrate, AF filtrate plus  $12\ \mu\text{g}$  of fibrinogen/ml, or 90% PLA2-depleted AF, respectively. (G) Newman *ClfA*<sup>+</sup> strain incubated for 60 min in 90% PLA2-depleted AF and then for 15 min with proteinase K ( $10\ \mu\text{g}/\text{ml}$ ).

bases of these differences are unknown but presumably reflect variable expression of MSCRAMMs. In strains 5A and RN450, clumping was greater in biological fluids than in defined medium containing equivalent amounts of fibrinogen (Table 1), suggesting that clumping of these strains in biological fluids may include fibrinogen-independent interactions. Despite the presence of *ClfA* in strain RN450 (24), the clumping properties of this strain more closely resembled those of the *ClfA*<sup>-</sup> mutant derivative of the Newman strain (Table 1). Whether the more potent clumping properties of the wild-type Newman strain reflect higher levels of surface expression of *ClfA* or other surface properties of the Newman strain that act in

concert with *ClfA* to increase bacterial clumping is unknown and requires further study.

The *in vivo* importance of adherence and clumping is still in question, but several studies have been performed to address this issue. Recent studies have shown that the lack of certain MSCRAMMs (*Cna*, *FnbA* and *FnbB*, *Efb*, and *ClfA*) decreases virulence in certain animal models (27, 32, 33, 38), implying that the presence of MSCRAMMs may increase virulence, perhaps in part by impairing the ability of host defenses to deal effectively with these strains. *S. aureus* bacteria adherent to polymethyl methacrylate coverslips are more resistant to phagocytosis than bacteria in suspension (45). Bacteria in large

TABLE 1. Clumping of several *S. aureus* strains in various media<sup>a</sup>

Strain	Result <sup>b</sup> in:					
	Purified rabbit fibrinogen in AF filtrate at:				90% PLA2-depleted AF	90% Human plasma
	0 µg/ml	10 µg/ml	270 µg/ml	2.7 mg/ml		
3	156 ± 29	55 ± 12	31 ± 7	37 ± 31	51 ± 10	39 ± 13
5A	202 ± 58	181 ± 36	82 ± 20	36 ± 12	38 ± 13	47 ± 19
18	257 ± 60	31 ± 3	16 ± 6	9	22 ± 2	2.5
RN450	165 ± 9	141 ± 35	58 ± 24	18	24 ± 12	13.5
Newman <i>ClfA</i> <sup>-</sup>	173 ± 35	135 ± 27	17 ± 4	7 ± 2	34 ± 8	1
Newman <i>ClfA</i> <sup>+</sup>	194 ± 54	27 ± 8	5 ± 2	2 ± 1	23 ± 7	0

<sup>a</sup> Several strains of *S. aureus* at 10<sup>8</sup> bacteria/ml were incubated in the media indicated for 60 min, and CFU were assessed.

<sup>b</sup> Expressed as the percentage of the CFU of the initial inoculum. Each result is the mean ± SEM of at least three independent determinations.

clumps also appear to be refractory to ingestion by phagocytes (16, 17). When phagocytosis is impaired, the action of extracellular host defenses may be more critical. Mammalian group IIA PLA2 appears to be the most prominent and potent component of the extracellular host defense arsenal against *S. aureus* (11, 35, 47, 48). PLA2 is present in inflammatory settings and accumulates in response to bacterial infection (19, 28, 29, 47, 48).

This is the first study to examine the susceptibility of clumped bacteria to an extracellular host defense (e.g., PLA2). Our findings demonstrate that the ability of group IIA PLA2 to cause bacterial phospholipid hydrolysis and killing is diminished by bacterial clumping. This is true both for *ClfA*-dependent and -independent clumping and when clumping occurs either with purified fibrinogen or in biological fluids (Fig. 3 and 4). Thus, whatever the molecular mechanism, clumping

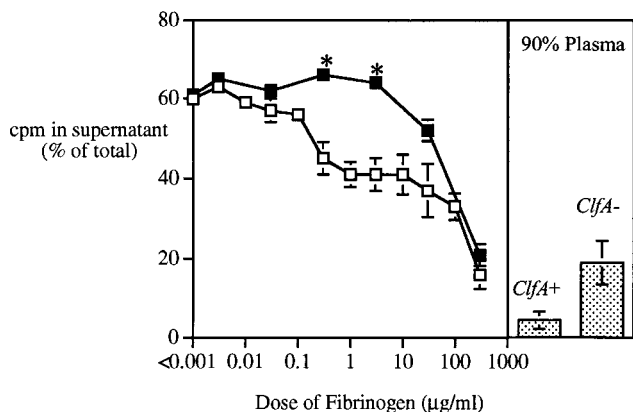


FIG. 3. Effect of bacterial clumping on susceptibility of *S. aureus* to rabbit group IIA PLA2. <sup>14</sup>C-oleate-labeled bacteria (10<sup>8</sup> bacteria/ml; Newman *ClfA*<sup>+</sup> [open squares] or *ClfA*<sup>-</sup> [solid squares]) were incubated in AF filtrate with increasing amounts of rabbit fibrinogen added in purified form or as part of PLA2-depleted AF (12.5 ng/ml to 270 µg/ml) or with 90% plasma for 60 min. PLA2 (500 ng/ml) was then added, and bacteria were further incubated for 60 min. The level of phospholipid degradation was assessed by measuring the release of labeled material into the supernatant after the bacteria had been pelleted. Results shown are means ± SEMs of at least three independent determinations. Results obtained in AF filtrate plus purified fibrinogen or PLA2-depleted AF were essentially the same and are therefore combined to simplify the presentation. Note that phospholipids represent about 70% of bacterial <sup>14</sup>C-oleate-labeled material (48). Hence, release of ca. 60% of total counts per minute into the supernatant corresponds to nearly quantitative degradation of bacterial phospholipids. Asterisks highlight conditions under which phospholipid hydrolysis of the *ClfA*<sup>-</sup> strain is significantly greater than that of the *ClfA*<sup>+</sup> strain (*P* < 0.007).

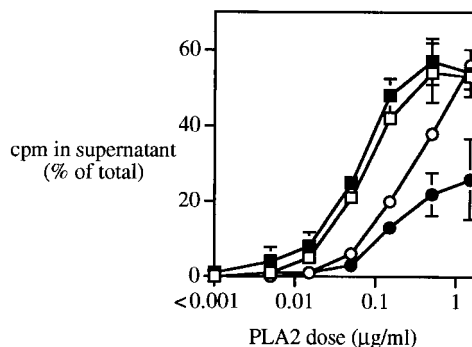


FIG. 4. The Newman *ClfA*<sup>+</sup> strain (<sup>14</sup>C-oleate labeled; 1 × 10<sup>6</sup> [squares] or 3 × 10<sup>7</sup> [circles] bacteria/ml) was incubated with AF filtrate alone (open symbols) or AF filtrate plus 270 µg of fibrinogen/ml (solid symbols) for 60 min. Increasing amounts of PLA2 were then added, and bacteria were further incubated for 60 min. The level of phospholipid degradation was measured as described in Materials and Methods and in the legend to Fig. 3. Results shown are means ± SEMs of at least three independent determinations. Note that fibrinogen caused extensive clumping of bacteria at the higher but not at the lower bacterial concentration (see Fig. 1).

of *S. aureus* at sites of infection may promote bacterial survival and persistence by reducing the efficacy of both cellular (phagocytosis) and extracellular host defenses. Fibrinogen induced clumping of growing and nongrowing bacteria (Fig. 3 and 4; also data not shown), suggesting that clumping may have protective effects both early in abscess formation and during later stages of persistent infections.

It should be noted, however, that the greater resistance of bacterial clumps to PLA2 can be (partially) overcome when higher concentrations of enzyme are added. The ability of PLA2 to produce extensive bacterial phospholipid degradation under these conditions implies that even dense bacterial clumps are sufficiently porous to allow diffusion of PLA2 to many bacteria within the clump. The protective effects of PLA2 may extend beyond the direct cytotoxic effects of this enzyme. Extensive phospholipid degradation caused by PLA2 may activate autolysins (8a), disrupting the cell wall integrity needed to maintain bacteria-fibrinogen interactions and thereby increasing the likelihood of phagocytosis.

Group IIA PLA2 is not unique in its ability to penetrate bacterial clumps. Proteases gain access to bacterial cross-links and, in so doing, disperse bacterial clumps and render the bacteria more sensitive to extracellular PLA2 and perhaps also to phagocytes (Table 2; Fig. 5). Since proteases (e.g., plasmin) are mobilized during inflammation, they may synergize with PLA2 in this setting, enabling efficient killing and digestion of previously clumped bacteria. Mice lacking urokinase, a plasminogen activator, are more susceptible to local *S. aureus* infections,

TABLE 2. Reversal of clumping by protease treatment<sup>a</sup>

Additive (concn; incubation time)	Bacterial CFU (%) <sup>b</sup>
Filtrate (no fibrinogen; 75 min)	246 ± 38
Fibrinogen (12 µg/ml; 60 min)	12 ± 4
+ pronase (10 µg/ml; 15 min)	271
+ proteinase K (10 µg/ml; 15 min)	281 ± 53
+ plasmin (10 µg/ml; 15 min)	194 ± 20
+ plasminogen (100 µg/ml; 15 min)	169 ± 64

<sup>a</sup> Incubations were carried out with 10<sup>8</sup> bacteria (*ClfA*<sup>+</sup> Newman strain)/ml. In the absence of fibrinogen, added proteases had no effect on bacterial CFU.

<sup>b</sup> Data are expressed as percentages of the CFU of the initial inoculum and are means of at least two determinations.

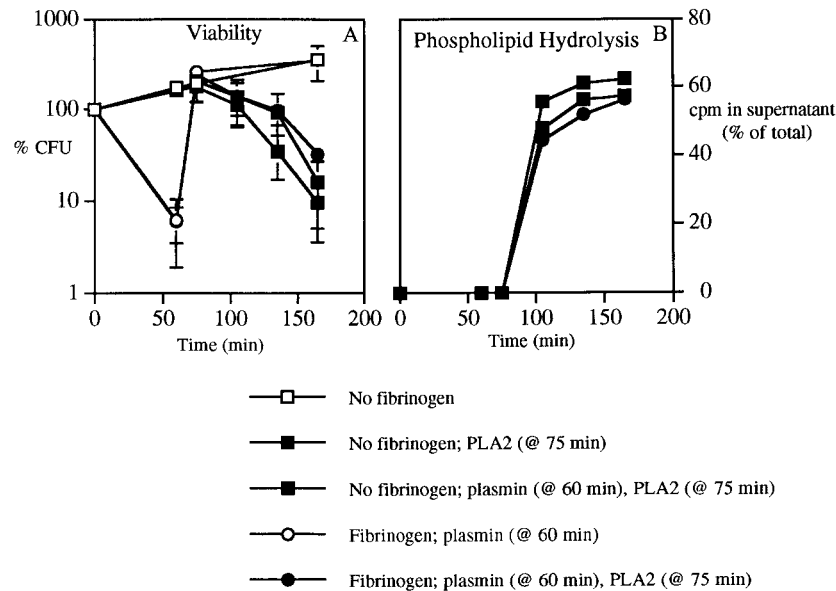


FIG. 5. Effects of protease treatment on susceptibility to PLA2. Bacteria ( $\text{ClfA}^+$ ;  $10^8$  bacteria/ml) were incubated with AF filtrate or AF filtrate plus 270  $\mu\text{g}$  of rabbit fibrinogen/ml for 60 min. Then plasmin (100  $\mu\text{g}/\text{ml}$ ) was added, and after further incubation for 15 min, PLA2 (1  $\mu\text{g}/\text{ml}$ ) was added and incubations were continued for an additional 30, 60, or 90 min. CFU and phospholipid hydrolysis were assessed as described in Materials and Methods. Results shown are means  $\pm$  SEMs of at least three independent determinations. CFU are expressed as percentages of the CFU in the initial inoculum.

consistent with a possible role for plasmin in host defense against *S. aureus* (39). We demonstrate (Table 2) that, in vitro, bacterial clumps could also be dispersed by added plasminogen, suggesting that the bacteria are secreting a plasminogen activator, such as staphylokinase (20, 22). It would be advantageous to the host to produce plasmin locally, where it could be less susceptible to circulating host plasmin inhibitors. Conversely, the secretion of a plasminogen activator could be advantageous to the bacteria by providing a mechanism for bacterial dissemination after host defenses, mobilized during inflammation, have waned.

In summary, the outcome of *S. aureus* infection is the result of a race between the rate and extent of bacterial multiplication and the rate of mobilization and potency of host antibacterial defenses. Clumping reduces the efficiency of action of some host defenses, and  $\text{ClfA}$  accelerates the onset of clumping by reducing fibrinogen and bacterial concentration requirements. Conversely, mobilization of extracellular defenses, including PLA2 together with systems that reduce clumping (e.g., plasmin), permits host antibacterial action even when phagocytes may be less effective. It should be noted that interactions among bacteria are likely superimposed on interactions between bacteria and the host (tissue, matrix, foreign bodies). The effects of such interactions on extracellularly mobilized host defenses will require further study.

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