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Research Article

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The Potent Anti–*Staphylococcus aureus* Activity of a Sterile Rabbit Inflammatory Fluid Is Due to a 14-kD Phospholipase A2

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Abstract

The cell-free fluid (ascitic fluid, AF) of a sterile inflammatory peritoneal exudate elicited in rabbits is potently bactericidal for complement-resistant gram-negative as well as gram-positive bacterial species. This activity is absent in plasma. We now show that essentially all activity in AF against *Staphylococcus aureus* is attributable to a group II 14-kD phospholipase A2 (PLA2), previously purified from AF in this laboratory. Antistaphylococcal activity of purified PLA2 and of whole AF containing a corresponding amount of PLA2 was comparable and blocked by anti–AF-PLA2 serum. At concentrations present in AF ($\sim$ 10 nM), AF PLA2 kills $>$ 2 logs of 10^6 *S. aureus*/ml, including methicillin-resistant clinical isolates, and other species of gram-positive bacteria. Human group II PLA2 displays similar bactericidal activity toward *S. aureus* ($LD_{90} \sim$ 1–5 nM), whereas 14-kD PLA2 from pig pancreas and snake venom are inactive even at micromolar doses. Bacterial killing by PLA2 requires Ca^{2+} and catalytic activity and is accompanied by bacterial phospholipolysis and disruption of the bacterial cell membrane and cell wall. These findings reveal that group II extracellular PLA2, the function of which at inflammatory sites has been unclear, is an extraordinarily potent endogenous antibiotic against *S. aureus* and other gram-positive bacteria. (*J. Clin. Invest.* 1996. 97:250–257.)
Key words: inflammation • bacteria • ascitic fluid • phospholipid degradation

Introduction

Inflammatory exudates are a rich source of antimicrobial systems that are contributed by both the cells (mainly phagocytes) and the cell-free fluid. Whereas the components of cell-associated systems have been analyzed in great detail (1–3), much less is known about what constitutes the extracellular antimicrobial capability of inflammatory exudates (4–6). The introduction of a sterile irritant into the peritoneal cavity of experimental animals provides a useful in vivo and ex vivo experimental model for the study of inflammatory exudates. In rabbits, the injection of glycogen in sterile saline triggers the formation of a cell-rich exudate consisting almost exclusively of polymorphonuclear leukocytes (PMN) (6, 7). We have recently initiated studies of the factors that determine the antibacterial properties of the cell-free (ascitic) fluid (AF)[1] during the evolution of the inflammatory exudate. Over a 2–24-h period, potent antibacterial activity accumulates in AF against both gram-negative and gram-positive bacteria (6) and toward fungi (Weinrauch, Y., and A. Foreman, unpublished observations). The activity against gram-negative bacteria is mainly traceable to two PMN-derived antibacterial proteins acting synergistically, the bactericidal/permeability-increasing protein (BPI) and the p15s (6), and is abolished by anti-BPI serum, thus showing absolute dependence on BPI. By contrast, antibacterial activity in AF against *Staphylococci* is unaffected by anti-BPI serum indicating that another, as yet unidentified, agent (or agents) is responsible for this activity. For decades, investigators have recognized antibacterial activity against gram-positive bacteria in serum (5, 8, 9) and also in inflammatory fluids (4, 5). Attempts at purifying proteins thought to account for the activity have been largely unsuccessful (5, 10). However, circumstantial evidence was presented suggesting involvement of phospholipolytic activity (11). We now report that the potent antistaphylococcal activity in the cell-free fluid of a sterile inflammatory peritoneal exudate can be attributed entirely to the action of an $\sim$ 14-kD group II PLA2.

Methods

Bacteria. Bacteria used in this study (see Table IV) included random clinical isolates obtained either from the Department of Microbiology collection (New York University Medical School) or from Dr. Philip Tierno (Department of Microbiology, Tisch Hospital, New York University Medical Center). *Bacillus subtilis* IS 230 (*trp*C2-*phe*A1) was obtained from Dr. Issar Smith (Public Health Research Institute, New York). *Escherichia coli* strains used (J5, K1/r) have been described previously (6). Bacteria were grown at 37°C overnight, washed once, and diluted 1:50 or 1:100 in fresh media and subcultured to mid-to-late logarithmic phase. All bacteria were grown in trypticase soy or Luria broth except *Streptococcus pneumoniae* and *Neisseria meningitidis* which were grown in brain heart infusion broth supplemented with 0.5% (vol/vol) horse blood or on chocolate agar plates. After harvesting, the bacteria were sedimented by centrifugation at 14,000 *g* for 5 min and resuspended in sterile physiological saline at a concentration of 10^9 bacteria/ml.

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1. *Abbreviations used in this paper:* AF, ascitic fluid; BPI, bactericidal/permeability-increasing protein; HSE, high salt eluate; PG, phosphatidylglycerol; PLA2, phospholipase A2.

Collection of AF. Sterile inflammatory peritoneal exudates were elicited in New Zealand White rabbits (2–3 kg) by intraperitoneal injection of 300 ml of sterile physiological saline supplemented with oyster glycogen (2.5 mg/ml). At 16 h after injection, the inflammatory exudate was collected from the peritoneal cavity as previously described (6). The inflammatory AF was collected by sedimentation of the cells in the exudate (100–200 *g* for 5 min) followed by centrifugation of the recovered supernatant at 20,000 *g* for 10 min to remove particulate material before storage at 4°C.

Collection of plasma and serum. Rabbit peripheral blood was collected from the central auricular artery just before and 16 h after intraperitoneal inoculation of glycogen/saline. Blood was collected into a tube containing buffered citrate as an anticoagulant. Plasma was isolated by sedimentation of blood cells by centrifugation at 2,500 *g* for 10 min. Serum was isolated after collection of rabbit blood in tubes without anticoagulant. Both serum and plasma were stored at −70°C before use.

PLA2. Purified PLA2 isozymes from the snake venom *Naja mossambica mossambica* (CM-I and CM-III) (12, 13) were obtained from Sigma Immunochemicals (St. Louis, MO). Recombinant wild-type and mutant pig pancreas PLA2 and human secretory (group II) PLA2 were expressed and purified as described previously (14, 15). Protein concentrations of purified PLA2 preparations were determined by OD_{280} using the known extinction coefficients of these proteins.

Purification of AF PLA2. PLA2 was purified from AF by chromatography on CM-Sephadex and a C4 reversed-phase HPLC column (1 × 25 cm, 5 mM particle size; VYDAC, Hesperia, CA) as described previously (16, 17), with the following modifications. AF stored at 4°C was applied to CM-Sephadex (resin equaled 0.1 vol of AF) that was preequilibrated in sterile physiological saline buffered with 2.5 mM Tris-HCl, pH 7.5. After washing to remove unbound proteins, bound proteins were eluted with 1.5 M NaCl buffered with 20 mM sodium acetate/acetic acid (pH 4.0) and dialyzed against 20 mM acetate buffer, pH 4.0. Fractions containing bactericidal activity toward *S. aureus* were pooled (high salt elute, HSE) and further purified by HPLC, first using a gradient of acetonitrile (0–95%) in 0.1% trifluoroacetic acid developed over 30 min and then a gradient of 0–70% acetonitrile developed in 60 min, each at 2.5 ml/min. Eluted proteins were collected (0.2–0.4 min/fraction), dried, and resuspended in 20 mM acetate buffer before testing for antibacterial activity. Recovery from HPLC of antibacterial activity toward *S. aureus* was nearly 100%. Purity of the recovered fractions was analyzed by SDS-PAGE on a Phast Gel system (Pharmacia Biotech Inc., Piscataway, NJ) using Phast Gel 8–25% gradient polyacrylamide gels and Coomassie blue for detection of protein species and by NH_2-terminal amino acid sequence analysis (17) and laser desorption mass spectral analysis. Protein content of fractions containing purified AF PLA2 was deduced from the measured catalytic activity and known specific catalytic activity of AF PLA2 (16, 17) and confirmed by densitometric analysis of Coomassie blue–stained protein species after SDS-PAGE using pig pancreas and human group II PLA2s as standards.

Assay of bactericidal activity. An effect of various protein fractions on bacterial viability was determined by measuring bacterial colony-forming ability after incubation of the bacteria with or without the protein fraction at 37°C. Typical incubation mixtures contained 10^5 bacteria in a total volume of 100 μl also containing 10 mM Hepes buffer (pH 7.4), the protein fraction as indicated, and "AF filtrate" supplemented with 10 mg/ml of bovine serum albumin to mimic the electrolyte and total protein content of the inflammatory fluid. AF filtrate was prepared by ultrafiltration (Centricon-10; Amicon, Inc., Danvers, MA) of AF and sterile filtration of the resulting filtrate and contained < 0.3% of the total protein content by OD_{280} of AF and no detectable antibacterial activity. Similar results were obtained using AF filtrate or Hank's balanced salt solution (with divalent cations, $HBSS^+$; BioWhittaker, Inc., Walkersville, MD). (Partially) Purified protein fractions in acetate buffer were added in a maximum of 10% of the total incubation volume. LD_{90} values were derived by regression analysis of dose curves. To compare the antibacterial activities of plasma, serum, and AF, incubations were carried out in buffered citrate (6). The antistaphylococcal activity of AF and serum was reduced by ~ 50% in buffered citrate versus Hepes-buffered media. To determine the effects of Ca^{2+} and Mg^{2+} on antistaphylococcal activity of purified PLA2, incubations were carried out in $HBSS^-$ (without divalent cations) supplemented with divalent cations as indicated. At the end of the incubation, aliquots of the suspensions were taken, serially diluted in sterile physiological saline, and transferred to 5 ml of molten (50°C) 1.3% (wt/vol) Bacto-agar (Difco Laboratories Inc., Detroit, MI) containing 3% (wt/vol) trypticase soy broth. *Pneumococci* and *Meningococci* were plated in molten brain heart infusion agar supplemented with 0.5% defibrinated horse blood. Bacterial viability was measured as the number of colonies formed after incubation at 37°C for 18–48 h.

Assay of PLA2 activity. PLA2 activity of various protein fractions was measured by assay against [1-^{14}C]oleate-labeled autoclaved *E. coli* as described previously (16, 17). One arbitrary unit of PLA2 activity is defined as 1% hydrolysis of bacterial substrate/h, corresponding to 50 pmol of phospholipid degraded/h. Assays of plasma PLA2 activity included recombinant hirudin (1 U/ml) (Calbiochem-Novabiochem Corp., La Jolla, CA) to prevent Ca^{2+}-induced clotting. Hirudin had no effect on PLA2 activity of AF and serum tested under similar conditions.

Anti–AF PLA2 serum. Normal and immune (anti–AF PLA2) chicken sera were obtained as described previously (17) and pretreated before use with Liposorb (Calbiochem-Novabiochem Corp.) to deplete lipoproteins. Neutralizing effects of chicken (anti)serum on the antistaphylococcal and PLA2 activities of rabbit AF and serum were determined by preincubation of AF or serum with chicken serum for 30 min at room temperature in the standard incubation mixture before addition of *S. aureus* or PLA2 substrate (autoclaved *E. coli*). Chicken serum contained no detectable antistaphylococcal or PLA2 activities.

Radiolabeling of lipids of S. aureus during growth. To uniformly label the lipids (mainly phosphatidylglycerol, PG) (18) of *S. aureus* to high specific radioactivity, the bacteria were grown in subculture at 37°C in nutrient broth supplemented with 2.5 μCi/ml of [1-^{14}C]oleic acid (56 mCi/mmol) (DuPont-New England Nuclear, Boston, MA). Bacteria were grown to mid-log phase, washed, resuspended in fresh media without oleic acid, incubated at 37°C for 20–30 min, and washed in media supplemented with 0.5% (wt/vol) bovine serum albumin before use.

Extraction and chromatographic analysis of radiolabeled bacterial lipids. To identify the bacterial molecular species labeled with [1-^{14}C]oleic acid, the labeled bacteria were extracted according to the procedure of Bligh and Dyer (19) and the extracted labeled species resolved by TLC on silica gel G (Analtech Inc., Newark, DE) and measured by liquid scintillation counting (13, 20). To facilitate identification of the labeled species, samples were supplemented with the corresponding unlabeled species and stained with I_2 vapor after TLC to detect the lipid standards. Greater than 95% of the incorporated radioactivity was recovered in the $CHCl_3$ phase, ~ 90% of which remained at the origin along with PG during TLC in petroleum ether/diethyl ether/glacial acetic acid (80:20:1, vol/vol) (20), 10% comigrated with diglyceride, and < 2% with free fatty acid. The species at the origin were eluted with $CHCl_3/CH_3OH$/glacial acetic acid/dH_2O (50:30:8:4, vol/vol) and resolved by TLC in $CHCl_3/CH_3OH/dH_2O$/glacial acetic acid (65:25: 4:1, vol/vol) (20), revealing that ~ 80% of these labeled species comigrated with PG. The identity of these species as PG was confirmed by elution and TLC in $CHCl_3/CH_3OH$/glacial acetic acid (7:3:1, vol/vol) (21) and $CHCl_3/CH_3OH$/30% NH_4OH (65:35:5, vol/vol) (22). To determine the acyl chain position of [1-^{14}C]oleic acid incorporated in PG, eluted radiolabeled PG was dried, resuspended in physiological saline containing 10 mM $CaCl_2$, 50 mM Tris-HCl (pH 7.5), and 1 mg/ml albumin, sonicated for 10 min at 4°C in a water bath sonicator (40 W) to disperse the phospholipid (1–2 nmol/100 μl), and incubated for 60 min with 50 ng/ml of AF PLA2.

After the incubation, labeled lipids and products were extracted and resolved by TLC as described above. Nearly quantitative degradation by the added PLA2 of labeled PG was accompanied by a reciprocal accumulation of labeled lyso-PG distributed between the $CHCl_3$ and H_2O/CH_3OH phases after Bligh-Dyer extraction. There was little or no accumulation of labeled free fatty acid (< 5% of the total labeled product formed). Thus, [1-^{14}C]oleic acid was incorporated almost exclusively into the 1-acyl position of PG consistent with previous studies of acyl chain distribution in *S. aureus* lipids (18).

Measurement of bacterial phospholipid (PG) degradation during treatment of S. aureus with purified PLA2. [1-^{14}C]Oleate-prelabeled *S. aureus* (2,500 cpm/10^6 bacteria) were incubated with or without PLA2 in the standard incubation mixture except that 5×10^6 bacteria/ml were used. After the incubation, samples were extracted and resolved by TLC as described above. Radiolabeled species recovered in the H_2O/CH_3OH phase (e.g., lyso-PG) were dried, resuspended in CH_3OH, and resolved by TLC. The identity of the degraded lipid species and accumulating product(s) was determined by TLC in each of the solvent systems described above.

Radiolabeling of proteins of S. aureus during growth. To label the proteins of *S. aureus* to high specific radioactivity, the bacteria were grown in subculture at 37°C in methionine assay medium (Difco Laboratories Inc.) supplemented with 3–5 μCi/ml of [^{35}S]methionine (1,175 Ci/mmol) (DuPont-New England Nuclear) plus 600 ng/ml of unlabeled methionine. The bacteria were grown to mid-log phase, washed once, resuspended in fresh media with unlabeled methionine, and incubated an additional 20–30 min at 37°C before harvesting. The labeled bacteria contained 3,000 cpm/10^6 bacteria, > 90% of which were precipitable in 10% trichloroacetic acid (23).

Measurement of bacterial protein release. [^{35}S]Methionine-prelabeled *S. aureus* were incubated with or without PLA2 in the standard incubation mixture except that 10^7 bacteria/ml were used. After the incubation, the bacteria were sedimented at 14,000 *g* for 5 min and the radioactivity in an aliquot of the recovered supernatant was measured both directly and after precipitation with 10% trichloroacetic acid to monitor release and degradation of labeled bacterial proteins.

Results

Purification of antibacterial activity of AF versus S. aureus yields 14-kD PLA2. Fractionation by batch-wise adsorption of AF to CM-Sephadex and elution of bound proteins with 1.5 M NaCl yielded potent anti–*S. aureus* activity in the HSE. This fraction contains ~ 60% of the antibacterial activity of AF versus *S. aureus* but ≤ 0.5% of the total AF protein. In contrast, proteins recovered in the unbound fraction, representing > 95% of the total AF protein, alone exhibit little or no antistaphylococcal activity (data not shown) and do not modify the activity of the HSE (Fig. 1). Further fractionation of the HSE by reversed-phase HPLC on a C4 column gave a discrete peak of antistaphylococcal activity coincident with PLA2 activity, each in nearly quantitative yield. By using more shallow gradients of acetonitrile in 0.1% trifluoroacetic acid, a fraction was obtained that contained a single protein species of 14 kD, as judged by SDS-PAGE, and both antistaphylococcal and PLA2 activities. NH_2-terminal amino acid sequence analysis confirmed that the protein was the group II 14-kD PLA2 that we had purified previously from rabbit AF (16, 17).

The antistaphylococcal activity of whole AF, HSE, and purified PLA2 was nearly the same when normalized for the PLA2 activity of these protein fractions (Fig. 1). This was true for each of five (clinical and laboratory) strains of *S. aureus* tested, including methicillin-resistant clinical isolates. The LD_{90} of purified AF PLA2 toward these strains (10^6 bacteria/

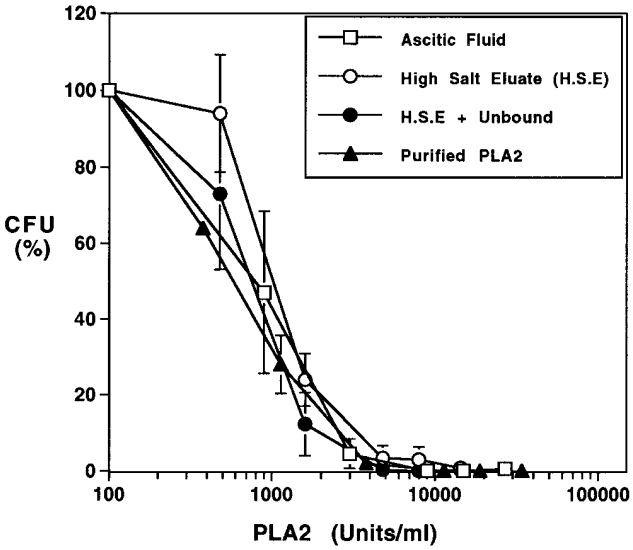

Figure 1. Effect of increasing concentrations of whole AF and of (partially) purified protein fractions of AF on viability of *S. aureus.* Incubation mixtures contained 10^6 bacteria/ml, 10 mM Hepes buffer (pH 7.4), and the protein fraction, as indicated, diluted either in the protein-rich fraction of AF that does not bind to CM-Sephadex (*H.S.E. + Unbound*) or in a protein-poor filtrate of AF supplemented with 10 mg/ml of bovine serum albumin to match the total protein concentration of AF. The PLA2 activity of each protein fraction added was measured as described in Methods. After incubation for 90 min at 37°C, bacterial viability was measured as described in Methods. The results shown represent the mean±SEM of three or more independent determinations.

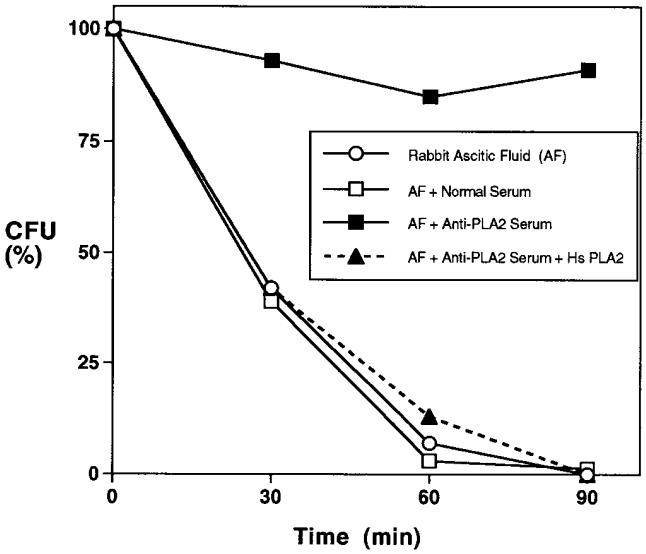

Figure 2. Bactericidal activity toward *S. aureus* of whole AF is blocked by neutralizing antibodies to AF PLA2. *S. aureus* (10^6 bacteria/ml) were incubated with Hepes-buffered (pH 7.4) AF (90%, vol/vol) after preincubation of AF with 10% (vol/vol) normal chicken serum, anti–AF PLA2 serum, antiserum + 30,000 U/ml of purified recombinant human group II PLA2, or AF filtrate supplemented with 10 mg/ml bovine serum albumin. Under these conditions, anti-PLA2 serum completely inhibited AF PLA2 activity but did not inhibit the activity of human group II PLA2. After the indicated times of incubation at 37°C, aliquots were taken for measurement of bacterial viability as described in Methods. The results shown represent the mean of at least two independent experiments.

Table I. Comparison of Antistaphylococcal and PLA2 Activities of Rabbit Plasma and Serum

Fraction	PLA2	CFU*
	U/ml	*%*
Plasma (0 h)‡	$4\pm1.0\times10^3$	93±11
Plasma (16 h)‡	$5\pm2.5\times10^3$	76
Plasma (16 h) + AF PLA2	3×10^4	0
Serum	3×10^4	0
Serum + anti-PLA2 serum§	3×10^3	155

*Effects of plasma and serum on viability of *S. aureus* (10^6 bacteria/ml) were measured as described in Methods. Results are expressed as percent viability of bacteria incubated alone. The data shown represent the mean±SEM of two or more determinations. ‡Plasma was collected just before (0 h) or 16 h after intraperitoneal inoculation of glycogen/saline. §Serum was preincubated for 30 min at room temperature with 10% (vol/vol) normal (not shown) or anti-PLA2 chicken serum before assay of PLA2 and antistaphylococcal activities as described in Methods.

ml) ranged from 1,000 to 20,000 U (5–100 ng/ml). At concentrations corresponding to those present in AF (~ 200 ng/ml) (16, 17) and tested under conditions that mimic the natural inflammatory fluid (see Methods), purified PLA2 could kill ≥ 2 logs of 10^7 *S. aureus*/ml. Under these conditions bacteria harvested in stationary phase were only slightly less sensitive than log phase bacteria to killing by either whole AF or purified AF PLA2 (data not shown).

Antibacterial activity of AF versus S. aureus is inhibited by anti-PLA2 serum. The remarkably similar antibacterial potency toward *S. aureus* of whole AF and purified AF PLA2 suggested that the activity of AF reflects the action of PLA2. This appears to be so because killing of *S. aureus* by AF was blocked by pretreatment of AF with anti-PLA2 (but not normal) chicken serum, using doses of immune serum that neutralized AF PLA2 activity (Fig. 2). Moreover, addition of functionally similar (15) (see below) but antigenically distinct recombinant human secretory (group II) PLA2 fully restored the antistaphylococcal activity of AF pretreated with anti-PLA2 serum (Fig. 2).

Relation of PLA2 activity to antistaphylococcal activity of plasma and serum. Serum also displays potent bactericidal activity toward *S. aureus* whereas plasma collected from either unchallenged animals or from rabbits at the time of collection of AF possesses little or no anti–*S. aureus* activity (5, 24) (Table I). These differences in antistaphylococcal activity parallel differences in PLA2 activity. The antibacterial activity toward *S. aureus* of rabbit serum, like that of AF, is blocked by anti-PLA2 chicken serum. Plasma supplemented with purified AF PLA2 displays the same bactericidal activity toward *S. aureus* as serum and AF (Table I). These findings imply that group II PLA2 accounts for the potent bactericidal activity against *S. aureus* of rabbit AF and serum that is normally absent in plasma.

Comparison of antibacterial activities of various 14-kD PLA2. The AF PLA2 is a member of a large family of 14-kD PLA2s, with a highly conserved overall structure and Ca^{2+}-dependent catalytic machinery, but widely variable activity toward specific biological targets (13, 20, 25–28). With our recent observations on the action of a range of 14-kD PLA2s against BPI-treated *E. coli* as reference, we tested the antibacterial activity of several PLA2s against *S. aureus* and *B. subtilis.* Human group II PLA2 (HsPLA2) is the counterpart of the rabbit AF PLA2 and also accumulates in inflammatory fluids (15, 29–31). The activity of the recombinant human enzyme toward *S. aureus* is closely similar to that of the rabbit AF PLA2, whereas the PLA2s from pig pancreas and both basic and acidic isozymes of *N. mossambica mossambica* snake venom are inactive even at doses 10,000× the LD_{90} of AF PLA2 (Table II). *B. subtilis* is 50-fold more sensitive than *S. aureus* to rabbit and human inflammatory fluid PLA2 (LD_{90} 10–50 pM) and killed by the basic venom isozyme at 100–1,000-fold higher concentrations, but is resistant to even 10 μM doses of the pig pancreas and acidic venom PLA2s.

BPI-treated *E. coli* are also sensitive to the inflammatory fluid PLA2s and resistant to the pancreatic and acidic venom PLA2s, but highly sensitive to the basic venom enzyme, showing that the structural determinants of PLA2 activity toward gram-positive and BPI-treated gram-negative bacteria overlap in part but are not identical. This conclusion is supported by the effects of site-specific mutations (R7S.K15Q) of the human

Table II. Comparison of Antibacterial Activities of Various 14-kD PLA2

PLA2	*S. aureus*	*B. subtilis*	BPI-treated *E. coli*
	LD_{90} *(ng/ml)*	LD_{90} *(ng/ml)*	*ng/10^7 bacteria**
Rabbit AF (II)‡	10	0.2	2
Human secretory (II)			
Wild-type	50	0.8	2
Wild-type: p-BPB§ treated	Not active (500)‖	NT¶	Not active (50)
R7S.K15Q	700	1.25	50
Pig pancreas (I)			
Wild-type	Not active (20,000)	Not active (10,000)	Not active (1,000)
S7R(K)	Not active (40,000)	Not active (10,000)	100
Naja mossambica mossambica (I)			
CMI (acidic)	Not active (80,000)	Not active (10,000)	Not active (1,000)
CMIII (basic)	Not active (100,000)	250	2.5

*PLA2 dose required to produce degradation of ≥ 10% of phospholipids of BPI-treated *E. coli.* ‡Number in parentheses refers to group of 14-kD PLA2. §*p*-bromophenacylbromide. ‖Highest dose tested. ¶*NT*, not tested.

Table III. Effect of Divalent Cations on Bactericidal Activity of Rabbit AF PLA2 toward S. aureus

Divalent cations added	CFU
	%
None	68
1 mM Ca^{2+}	6
1 mM Mg^{2+}	92
20 mM Ca^{2+}	85
1 mM Ca^{2+} + 20 mM Mg^{2+}	122

S. aureus (1×10^6 bacteria/ml) were incubated at 37°C with 80 ng/ml of AF PLA2 in 10 mM Hepes-buffered HBSS (without divalent cations), plus added divalent cations, as indicated. After 90 min, bacterial viability was measured as described in Methods. Results are expressed as percent viability of bacteria incubated alone.

PLA2 that markedly reduce enzyme activity toward BPI-treated *E. coli* (without affecting catalytic activity toward phospholipid dispersions) (13, 15) but have less pronounced effects on activity toward *S. aureus* and *B. subtilis.* Further, a site-specific substitution of pig pancreas PLA2 [S7R(K)] that results in an active enzyme toward BPI-treated *E. coli* does not elicit antibacterial activity toward *S. aureus* or *B. subtilis* (Table II).

Antistaphylococcal activity of PLA2 depends on catalytic activity and Ca^{2+}. The vast differences among the PLA2 tested for antistaphylococcal activity raised the possibility that the bactericidal effects of active PLA2 were unrelated to the catalytic activity of these proteins. However, covalent modification of the active site histidine of HsPLA2 by treatment with *p*-bromophenacylbromide inactivated both enzymatic (13) and antistaphylococcal activities (Table II). In addition, the bactericidal activity of AF and (Hs) PLA2 toward *S. aureus* is Ca^{2+} dependent (Table III). Half-maximal activity required $\sim$ 20 μM Ca^{2+} and full activity was expressed at Ca^{2+} concentrations ranging from 0.1 to 2 mM (data not shown). Mg^{2+} does not support the bactericidal activity of PLA2 (Table III). At supraphysiological concentrations (20 mM), both Ca^{2+} and Mg^{2+} inhibit the antistaphylococcal activity of PLA2.

Bactericidal action of PLA2 is accompanied by bacterial phospholipid degradation and other structural alterations. The Ca^{2+} dependence and requirement of catalytically active PLA2 for antistaphylococcal activity strongly suggest that bacterial phospholipid degradation is required for bactericidal activity. To document phospholipid degradation, *S. aureus* lipids were prelabeled during growth with radioactive fatty acid. Chromatographic and biochemical analyses (see Methods) showed that $\geq$ 70% of the labeled lipids were PG (the predominant phospholipid species in *S. aureus*) (18) with $\geq$ 90% of the radiolabel in PG in the 1-acyl position. Treatment of *S. aureus* with a lethal dose of PLA2 produced, in close parallel to bacterial killing, nearly quantitative degradation of PG (Fig. 3 *A*) and a reciprocal accumulation of labeled lyso-PG, consistent with the acyl position specificity of the added PLA2. Little or no degradation was produced by "inactive" (e.g., pig pancreas) PLA2 (data not shown).

The nearly complete degradation of bacterial envelope phospholipid produced by lethal doses of PLA2 suggested that PLA2 treatment could cause extensive overall envelope disruption. Indeed, bacterial killing by PLA2 was accompanied by release of $\sim$ 50% of (TCA-precipitable) [^{35}S]methionine-labeled bacterial material (Fig. 3 *B*). In addition, Gram stains revealed rapid loss of gram-positive staining (not shown), coincident with bacterial loss of viability, and progressive reduction of visible bacteria indicating massive destruction.

Comparison of antimicrobial spectra of whole AF and purified AF PLA2. Both whole AF and purified AF PLA2 displayed potent bactericidal activity against several different species of *Staphylococci* and *Streptococci* including group A *Streptococcus pyogenes* (Table IV). Toward several gram-positive bacterial species, the effects of AF and a corresponding amount of purified PLA2 were closely similar. In contrast, toward *Streptococcus salivarius* the activity of whole AF exceeded that of purified PLA2, implying that toward this organism another factor (or factors) in AF contributes to bacterial killing. Similarly, toward each of the gram-negative bacteria tested, purified AF PLA2 exhibited no antibacterial activity, whereas whole AF possessed potent bactericidal activity (Table IV) (6). Whether the presence of type III capsule by itself and/or other genetic determinants rendered this strain *S. pneu-*

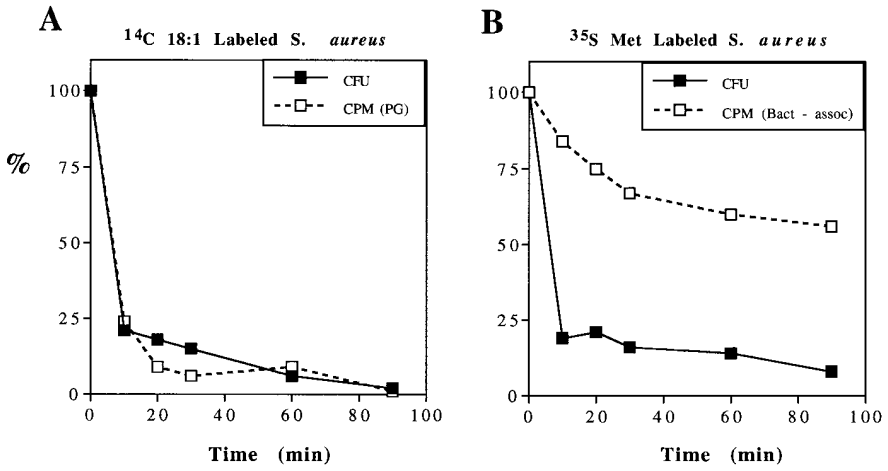

Figure 3. Killing of *S. aureus* by AF PLA2 is accompanied by nearly quantitative degradation of bacterial PG (*A*) and disruption of the bacterial cell membrane (*B*). (*A*) Bacteria (5×10^6/ml), prelabeled with [1-^{14}C]oleic acid, were incubated in the absence and presence of AF PLA2 (40,000 U/ml) in Hepes-buffered (pH 7.4) $HBSS^-$ supplemented with 1 mM $CaCl_2$. After the indicated time of incubation at 37°C, aliquots were taken for assay of bacterial viability and of radiolabeled bacterial PG as described in Methods. Loss of radiolabeled PG was accompanied by the reciprocal appearance of radiolabeled lyso-PG. In the absence of PLA2, there was little or no (< 5%) loss of radiolabeled bacterial PG. (*B*) *S. aureus* (10^7/ml), prelabeled with [^{35}S]methionine, were incubated with or without AF PLA2 (40,000 U/ml) at 37°C in Hepes-buffered $HBSS^-$ + 1 mM $CaCl_2$ (or $HBSS^+$). At the indicated times, aliquots were taken to measure bacterial viability and release of bacterial associated radioactivity into the extracellular medium as described in Methods. All results are expressed as a percentage of the values for bacteria before the start of the incubations and the results shown represent the mean of two closely similar experiments.

Table IV. Antibacterial Spectrum of AF and Purified AF PLA2

Organism	AF	PLA2
Gram-positive bacteria		
Staphylococcus aureus	+++	+++
Staphylococcus saprophyticus	+++	+++
Streptococcus pneumoniae nonencapsulated	+++	++
Streptococcus salivarius	+++	+
Staphylococcus epidermidis	++	++
Streptococcus pyogenes	++	++
Enterococcus faecalis	+	+
Streptococcus pneumoniae type III capsule	−	−
Gram-negative bacteria		
Escherichia coli	+++	−
Neisseria meningitidis	+++	−
Shigella sonnei	+++	−

Bacteria (10^6/ml) were incubated for 120 min in 90% (vol/vol) AF buffered with 10 mM Hepes, pH 7.4, or in Hepes-buffered AF filtrate (see Methods), with or without 150 ng/ml of purified AF PLA2. +++, Less than 10% survival when treated for 120 min with 90% AF or a corresponding amount of purified PLA2 as described in Methods. ++, 10–30% survival. +, 30–60% survival. −, No effect.

moniae resistant to both purified PLA2 and whole AF remains to be determined.

Discussion

Antimicrobial proteins and peptides have been detected in the body fluids of eukaryotic organisms from insects and Limulus to humans (3, 32). In mammals the complement system, immune globulins, and lysozyme were the first to be implicated in this activity (33), but evidence has also been presented that other agents (apparently proteins) contribute to the extracellular antimicrobial action of blood and inflammatory fluids (5, 6). Despite extensive efforts, purification and molecular characterization had not been achieved of such antimicrobial proteins that can act independently of the previously identified antimicrobial systems in body fluids. We now show that a group II 14-kD PLA2, mobilized during inflammation, by itself possesses remarkably potent antibacterial activity specific for gram-positive bacteria. The concentrations of this enzyme in the cell-free (ascitic) fluid of a sterile inflammatory exudate elicited in the peritoneal cavity of rabbits exceed the concentrations of the purified protein needed in vitro to kill as many as 10^7 *S. aureus*/ml, including multidrug resistant clinical isolates. A recombinant enzyme identical to human (group II) inflammatory fluid PLA2 displays similar bactericidal activity against *S. aureus* (LD_{90} 1–10 nM) and several other species of gram-positive bacteria (Tables II and IV). Previously, we have demonstrated an antibacterial role of these PLA2 and a closely similar enzyme in rabbit PMN toward gram-negative bacteria (15, 17, 23). However, this activity is manifest only in concert with other antibacterial agents, including BPI (15, 17, 34, 35) and the membrane attack complex of complement (34) (Madsen, L., and J. Weiss, unpublished observations). Thus, essential differences are apparent in the action of PLA2 toward gram-negative and gram-positive bacteria, presumably reflecting different determinants of activity in both the target bacteria and the enzyme and, in the case of gram-negative bacteria, a requirement for other (surface-perturbing) agents.

Three findings indicate that the antistaphylococcal activity of PLA2 is linked to catalysis. Antibacterial activity is: (*a*) calcium dependent (Table III); (*b*) parallels nearly quantitative envelope phospholipid degradation (Fig. 3 *A*); and (*c*) is abolished by pretreatment of PLA2 with *p*-bromophenacylbromide, a specific inhibitor of the catalytic activity of all family members of the 14-kD PLA2s (36). However, despite the very close similarity of the catalytic machinery of each of these enzymes, vast differences between individual PLA2s are apparent in antibacterial activity toward gram-positive bacteria (Table II). We have shown previously that PLA2 activity toward BPI-treated *E. coli* depends on the presence of a cluster of basic residues within a variable surface region near the NH_2 terminus, necessary for PLA2 binding to this biological target and, hence, indirectly, for catalytic action against the bacterial envelope phospholipids (14, 15, 37, 38). We do not know yet whether the differences among PLA2 in antistaphylococcal activity also reflect differences in noncatalytic interactions with the bacterial targets or rather differences in catalytic activity toward the phospholipids of these bacteria. Nevertheless, our findings suggest that the structural determinants for PLA2 action against BPI-treated gram-negative bacteria and against gram-positive bacteria are different. Thus, a basic venom PLA2 that is potently active against BPI-treated *E. coli* exhibits little or no activity against gram-positive bacteria, and mutant PLA2 with markedly diminished [R7S.K15Q mutant of human group II PLA2] or enhanced [S7R(K) mutants of pig pancreas PLA2] activity against BPI-treated *E. coli* shows little or no change in activity against *S. aureus* and *B. subtilis* (Table II).

The time-dependent loss of viability of *S. aureus* exposed to AF PLA2 closely parallels dramatic structural alterations (Fig. 3). Such extensive structural alterations that accompany loss of staphylococcal viability suggest that damage is not limited to envelope phospholipids but includes the cell wall and possibly activation of autolysins. How PLA2 reaches its substrate in the bacterial envelope and exerts its antimicrobial effect remains to be determined. The antagonistic effect of supraphysiological concentrations of Ca^{2+} and Mg^{2+} suggests that the initial targeting of PLA2 to *S. aureus* may involve electrostatic interactions between basic residues in the enzyme and anionic moieties in the bacterial envelope (e.g., lipoteichoic acids) (18, 39) that can also bind, with lower affinity, divalent cations. The remarkably low levels of ambient Ca^{2+} required for PLA2 action against *S. aureus* resemble the Ca^{2+} requirements for PLA2 action against BPI-treated *E. coli* and, as in that circumstance, may reflect a role for Ca^{2+} displaced in situ as cofactor for PLA2 action (35, 40, 41).

It has been proposed that the increased circulating levels of sPLA2 found in serious bacterial infections and septic states contribute to toxicity and organ damage, presumably by causing host phospholipid breakdown and formation of proinflammatory lipid metabolites (41, 42). However, it has not been shown that the elevated PLA2 levels are linked to increased degradation of host phospholipids, and natural endogenous substrate(s) of this and other mammalian extracellular group II PLA2s have not been identified. In contrast, our studies and recent observations of others (43) have established that invading bacteria are targets of extracellular group II PLA2, acting

both independently and in concert with other host defense systems (e.g., BPI, complement). In fact, against the important bacterial pathogen *S. aureus*, extracellular group II rabbit (and human) PLA2 display a bactericidal potency $\geq$ 100 times greater than any other known mammalian protein acting in an oxygen-independent manner (1, 3, 32) and fully account for the potent antistaphylococcal activity in AF (Figs. 1 and 2). Addition to plasma of purified PLA2 to levels reached during systemic inflammatory responses also elicits potent antistaphylococcal activity (Table I). Thus, elevated PLA2 levels during infections may be protective, particularly against pathogenic *S. aureus*, rather than harmful to the host.

Because many cell types (e.g., hepatocytes, platelets, phagocytes [PMN, macrophages], chondrocytes, Paneth cells, and lacrimal glands) can secrete group II PLA2 after cell activation (29, 41–45), the cellular origin of extracellular PLA2 during (local) inflammation is uncertain. Plasma collected both before glycogen challenge and also at the time of collection of the inflammatory exudate contains little or no PLA2 or antistaphylococcal activity suggesting that PLA2 is mobilized from local sites in this experimental model of inflammation. However, because this enzyme may be rapidly cleared from plasma (46, 47), it is possible that plasma-derived PLA2 is transferred to and trapped at the inflammatory site without noticeable accumulation in the circulating fluid. We have purified the dominant PLA2 in AF. Smaller amounts of PLA2 and antistaphylococcal activity are present in other highly purified fractions of both serum (17) and of AF (not shown), implying PLA2 heterogeneity that may be consistent with different cellular origins. The presence of high levels of PLA2 and antistaphylococcal activities in serum (but not in plasma) presumably reflects release from platelets during in vitro clotting (48). The fact that both activities are neutralized by antibodies to AF PLA2 (Table I) is consistent with the close structural relation of platelet and inflammatory fluid PLA2 (49) and indicates that serum antistaphylococcal activity, first described decades ago but not previously defined (5, 24), is also due to the action of group II PLA2.

The prominence of PLA2 activity in inflammatory fluids toward *S. aureus* (and certain other gram-positive bacteria, Table IV) suggests that resistance to PLA2 could be an important virulence trait of these bacteria after invasion. Among the $>$ 20 random clinical isolates of *S. aureus* tested to date, only moderate differences in resistance to PLA2 have been observed. Further investigation of possible genotypic and/or phenotypic determinants of bacterial resistance to PLA2 is needed. However, it is possible that successful invasion by *S. aureus* (and other bacteria) depends not on specific resistance but rather on inoculum size and bacterial multiplication to levels that exceed the rate of mobilization and antibacterial capacity of PLA2. Under these circumstances, the administration of exogenous PLA2 could provide a novel therapeutic approach to potentially life-threatening infectious agents that are increasingly resistant to conventional antibiotics (50–52).

In conclusion, the prominent role of the complement system in humoral defenses against gram-negative bacteria has long been recognized. In contrast, many attempts to identify specific humoral elements acting in body fluids against gram-positive bacteria have been unsuccessful. We have shown now that a PLA2 in AF is responsible for the killing by this inflammatory fluid of several gram-positive bacterial species, including clinical isolates of *S. aureus.* This is the first demonstration that a single protein in body fluids is acting as an independent antistaphylococcal agent. These findings add to our earlier evidence showing participation of PLA2 in the destruction of gram-negative bacteria (15, 23, 34, 53) and, taken together, strongly suggest that accumulation of extracellular PLA2 during inflammation contributes significantly to the mobilization of host defenses against invading microorganisms.

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