Staphylococcus aureus α-Hemolysin Promotes Platelet-Neutrophil Aggregate Formation

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Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) causes severe hemorrhagic necrotizing pneumonia associated with high mortality. Exotoxins have been implicated in the pathogenesis of this infection; however, the cellular mechanisms responsible remain largely undefined. Because platelet-neutrophil aggregates (PNAs) can dysregulate inflammatory responses and contribute to tissue destruction, we investigated whether exotoxins from MRSA could stimulate formation of PNAs in human whole blood. Strong PNA formation was stimulated by toxins from stationary phase but not log phase CA-MRSA, and α-hemolysin was singularly identified as the mediator of this activity. MRSA exotoxins also caused neutrophil (polymorphonuclear leukocyte) activation, as measured by increased CD11b expression, although platelet binding was not driven by this mechanism; rather, α-hemolysin–induced PNA formation was solely platelet P-selectin dependent. These findings suggest a role for *S. aureus* α-hemolysin–induced PNA formation in alveolar capillary destruction in hemorrhagic/necrotizing pneumonia caused by CA-MRSA and offer novel targets for intervention.

Keywords. *Staphylococcus aureus*; α-hemolysin; platelet-neutrophil aggregates; necrotizing pneumonia.

Staphylococcus aureus has long been a major cause of healthcare-associated pneumonia. Recently, hemorrhagic necrotizing pneumonia caused by community-associated methicillin-resistant *S. aureus* (CA-MRSA) has emerged worldwide [1, 2] and carries a mortality rate of 50%–80%. Intra-alveolar hemorrhage and extensive lung necrosis characterize this infection, and when present, these features portend poor outcomes [3].

Massive polymorphonuclear leukocyte (PMNL) influx into lung parenchyma is also characteristic of *S. aureus* pneumonia [4]. Activated, hyperadherent PMNLs and inappropriate triggering of degranulation can damage or destroy the microvascular endothelium and adjacent tissues—a process mediated by the neutrophil complement receptor type III (CR3; CD_{11b}/CD_{18}) [5]. Platelets also mediate inflammatory reactions [6, 7], and platelet-neutrophil aggregates (PNAs) contribute to organ failure [8] and soft-tissue necrosis [9, 10] in some life-threatening infections by reducing microvascular perfusion and by direct injury to the endothelium. In addition, animal studies have shown that blockade of PNA formation prevented acid- and lipopolysaccharide-induced experimental lung injury [11]. Together, these findings suggest that toxin-mediated neutrophil activation and PNA-mediated disruption/occlusion of the pulmonary microcirculation may also contribute to the pathogenesis of MRSA necrotizing pneumonia.

In the present study, we demonstrate that exotoxins from stationary phase CA-MRSA induced both PNA formation and PMNL activation in human whole blood and that these activities are independent of Panton-Valentine leukocidin (PVL). Using both a classic biochemical approach and analysis of toxin-deficient isogenic mutants, we definitively demonstrate that *S. aureus* α-hemolysin is the sole exotoxin responsible for PNA formation. Finally, we show that α-hemolysin–induced

Table 1. Characteristics and Functional Activity of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Strains Used in This Study

Agonist	Site of Isolation	PVL Gene Probe	Hemolytic Activity, HU/mL	PNA Formation,[a]%, MFI ± SD	CD42b, MFI ± SD	CD11b, MFI ± SD
Media	NA	NA	0	8.3 ± 0.3	0.5 ± 0.01	37.9 ± 9.9
MRSA 3801	Blood	+	256	62.0 ± 4.4	3.9 ± 0.3	86.9 ± 2.7
FPR3757	Skin abscess	+	540	90.9 ± 2.9	2.9 ± 0.7	92.3 ± 2.3
MRSA 2349	Wound	+	128	38.2 ± 0.6	3.1 ± 0.2	77.6 ± 12.7
MRSA 2241	Wound	+	128	65.8 ± 3.0	2.9 ± 0.3	83.0 ± 17.6
MRSA 1740	Skin abscess	−	288	76.5 ± 5.7	2.9 ± 0.5	77.9 ± 7.4
LAC	Skin abscess	+	256	53.6 ± 2.6	1.71 ± 0.03	94.9 ± 2.3
LACΔ*pvl*	NA	−	256	57.4 ± 4.5	1.7 ± 0.3	88.7 ± 2.2
JE2	NA	+	160	46.7 ± 8.9	2.8 ± 0.02	124.9 ± 2.3
JE2Δ*hla*	NA	+	30	12.5 ± 1.3	2.3 ± 0.1	100.6 ± 4.1
JE2Δ*plc*	NA	+	160	51.9 ± 10.6	2.8 ± 0.6	117.1 ± 4.5

Stationary phase culture supernatants (40 µL) from the indicated strains were added to 100 µL of heparinized human blood for 10 minutes at 37°C. Hemolytic activity was determined by rabbit erythrocyte lysis assay. Platelet-neutrophil aggregate (PNA) formation and the mean fluorescence intensity (MFI) of the platelet CD42b and polymorphonuclear leukocyte CD11b markers were measured by flow cytometry, as described in Methods. All clinical isolates were kindly provided by Dr Francoise Perdreau-Remington (University of California, San Francisco) in 2003. The correlation coefficient (r^2) between hemolytic activity and PNA formation percentage, hemolytic activity and CD11b MFI, and CD11b and formation percentage were 0.75, 0.08, and 0.13, respectively. Data are mean ± SD, unless otherwise indicated, and represent duplicate experiments from 4 independent blood donors.

Abbreviations: HU, hemolytic units; NA, not applicable; PVL, Panton-Valentine leukocidin.

[a] Defined as the percentage of CD11b^{+} cells that were positive for CD42b.

PNA formation is mediated by platelet P-selectin. Together, these results suggest a unique mechanism by which *S. aureus* α-hemolysin may contribute to alveolar capillary leakage, hemorrhage, and lung destruction during MRSA necrotizing pneumonia and offer novel targets for intervention.

MATERIALS AND METHODS

Bacterial Strains and Exotoxin Preparation

Five clinical CA-MRSA strains, previously characterized by our laboratory [12] and others [13], were used (Table 1). These strains were obtained from patients with infections of varying severity and produced variable amounts of PVL [12]. In addition, a wild-type USA300 clinical MRSA isolate called "LAC" (for Los Angeles County) and its isogenic PVL-deficient mutant (LACΔ*pvl*) were kindly provided by Dr Frank R. DeLeo (Laboratory of Human Bacterial Pathogenesis, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT) [13]. Last, CA-MRSA USA300 JE2 strain, derived from USA300 LAC and cured of 3 small plasmids, was obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA; Chantilly, VA). All CA-MRSA isolates carried the SCC*mec* Type IV cassette and the genes for LukS and LukF but produced variable amounts of PVL [12]. To prepare exotoxins, overnight cultures of *S. aureus* in Mueller-Hinton II (MHII) broth were washed, diluted to 1–$3 × 10^6$ colony-forming units/mL in fresh MHII, and grown at 37°C in 5% CO_2 with shaking ($0.85 × g$) for 4 hours (mid-log phase) or 24 hours (stationary phase). Culture supernatants

(CSN) were cleared of bacteria by centrifugation and filter sterilization (0.22 µm) and stored frozen at −70°C.

PNA Formation, Platelets, and PMNL Activation

Work involving humans was approved by the Boise VAMC's institutional review board (IRB) of record (the VA Puget Sound Health Care System IRB; Seattle, WA), and the Boise VAMC's Research and Development Committee. All volunteers gave written informed consent prior to participation. Peripheral blood was drawn from healthy, nonsmoking adults (3 men and 5 women; age, 20–55 years) who had not taken any prescription or over-the-counter medication in the 10 days prior to participation and had abstained from caffeine consumption 24 hours prior to the blood donation. Female participants were not pregnant and were denied oral contraceptive use. Heparinized (10 U/mL) whole blood was collected without the aid of a tourniquet, and PNA formation was assessed as previously described [9]. Briefly, blood (100 µL) was incubated at 37°C for 10 minutes with 10 µL of fluorescein isothiocyanate–conjugated anti-human CD42b (clone HIP1, BD Biosciences, San Diego, CA) to identify platelets and phycoerythrin-conjugated anti-human CD11b (clone Bear1, Beckman Coulter Immunotech, Fullerton, CA) to identify PMNLs. Blood was stimulated for an additional 5 minutes with 10 µL of either (1) Dulbecco's phosphate-buffered saline (DPBS; Sigma) supplemented to contain 2 mM calcium and 100 µM zinc (negative control), (2) 0.124 U of recombinant phospholipase C (rPLC) from *Clostridium perfringens* (positive control) [14], (3) native toxins from log or stationary phase CA-MRSA [12], or (4) recombinant *S. aureus*

α-hemolysin or phosphatidylinositol-specific phospholipase C (PI-PLC; prepared as described below). It should be noted that *C. perfringens* PLC preferentially cleaves phosphatidylcholine moieties in cell membranes, whereas the MRSA PI-PLC targets phosphatidylinositol-containing lipids. The *C. perfringens* PLC is also a sphingomyelinase. After erythrocyte lysis, samples were fixed with paraformaldehyde and analyzed using a FACS Epic XL flow cytometer. Granulocytes were gated on the basis of forward- and side-scatter profiles. PNA formation was defined as the percentage of CD11b$^+$ cells that were positive for CD42b. The relative number of platelets/PMNL was given by the CD42b mean fluorescence intensity (MFI) of these dual-positive events. PMNL activation was indicated by an increased CD11b MFI. Some experiments used isolated human platelets and neutrophils obtained as we have previously described [15]. Tests were run in triplicate, and data are expressed as the mean ± SD for 4 independent experiments, with each experiment using blood from a different donor.

Isolation and Identification of Toxin(s) Mediating PNA Formation

Stationary phase culture supernatants from the LACΔ*pvl* was fractionated by 2 rounds of isoelectric focusing (pH 3–10). Sterile, uninoculated MHII media was similarly treated and used as a negative control. Resultant fractions were tested for their ability to induce PNA formation and/or PMNL activation as described above.

Proteins in fractions with the highest level of PNA activity were precipitated with trichloroacetic acid, resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and visualized with Bio-Safe Coomassie blue (Bio-Rad), and resultant bands were excised from the gel for identification by Orbitrap liquid chromatography tandem mass spectrometry (MS/MS) (Proteomics and Mass Spectrometry Core Facility, Oklahoma State University). MS/MS profiles were analyzed using Mascot (Matrix Science, London, UK) and X! Tandem (The GPM, version 2007.01.01.1). Scaffold Proteome Software 3.0 (Proteome Software, Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide and protein identifications were accepted if they could be established at >80% and >99% probability, respectively, as specified by the Peptide Prophet algorithm [16]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Plasmid Constructs

Work involving recombinant DNA was approved by the Boise VAMC's institutional biosafety committee of record (the VA Puget Sound Health Care System Recombinant DNA Committee). Cloning of the *S. aureus* α-hemolysin (*hla*) and phosphatidylinositol-specific phospholipase C (*plc*) genes was performed using standard molecular cloning procedures. Chromosomal DNA from *S. aureus* strain FPR3757 (USA 300) was used as a template for *hla* and *plc* gene amplification in a PerkinElmer GeneAmp polymerase chain reaction (PCR) System 2400 thermocycler. The *hla* forward (5′-CTCGAGGCAGATTCTGATA TTAATATTAAAACC-3′) and *hla* reverse (5′-CCCTTTTTCT TCTTTACTGTTTAATTCCTAGG-3′) primers were used to amplify mature α-hemolysin, as previously described by Eichstaedt et al [17], with appended *Xho*I and *Bam*HI restriction sites (underlined). The *plc* forward (5′-CATATGAGTGGT TGGTATCATTCAG-3′) and reverse (5′-GCATTCACTTTAA TATCTATCATTATTTATTGAGCTC-3′) primers were used to amplify the *plc* gene, with appended *Nde*I and *Xho*I restriction sites (underlined). Following purification, *hla* and *plc* PCR products were digested and cloned into the pET14b expression vector (Invitrogen, Carlsbad, CA), containing an N-terminal His tag. Plasmid sequences were verified through a commercial sequencing service (Gene Gateway, Hayward, CA).

Recombinant Protein Expression and Purification

BL21 (DE3) bacteria harboring either the pET14b-*hla* or pET14b-*plc* plasmid were grown in LB medium containing carbenicillin (75 µg/mL). Overnight cultures were diluted 1:100 in 300 mL of fresh LB medium and incubated at 37°C. At an OD$_{600}$ of 0.5, gene expression was induced with 1 mM IPTG, and cultures were grown for an additional 2.5 hours. Bacteria were harvested by centrifugation, and pellets were resuspended in BugBuster protein extraction reagent (Novagen, EMD Chemicals, Gibbstown, NJ) supplemented with protease inhibitors (1 mM AEBSF, 2.0 µg/mL aprotinin, 10 µM bestatin, 10 mM E-64, 100 µM leupeptin, and 1 µM pepstatin), benzonase nuclease (25 U/mL; Novagen), and lysozyme (1 kU/mL; Sigma). Lysates were cleared by centrifugation (13 000 × *g*; 20 minutes), and the resulting supernatant was loaded on a 5-mL HisTrap HP column (GE Healthcare), followed by 15 columnvolumes of wash buffer (20 mM sodium phosphate, 0.5 M sodium chloride, and 40 mM imidazole, pH 7.4), and bound proteins eluted with 2 column-volumes of elution buffer (20 mM sodium phosphate, 0.5 M sodium chloride, and 500 mM imidazole). Buffer exchange into DPBS was performed on a Sephadex G-25 column. Protein concentration was determined by use of a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL), and the purity of the proteins was assessed using SDS-PAGE analysis with silver staining. Recombinant proteins were filter sterilized and stored at −70°C.

Recombinant Protein Assays

α-hemolysin activity was assayed by a standard rabbit erythrocyte lysis assay [18]. Recombinant α-hemolysin contained 5120 HU/mL of activity and 0.125 mg/mL total protein. *S. aureus* phosphatidylinositol-specific phospholipase C (Sa-PI-PLC) activity was qualitatively assessed using a plate-based
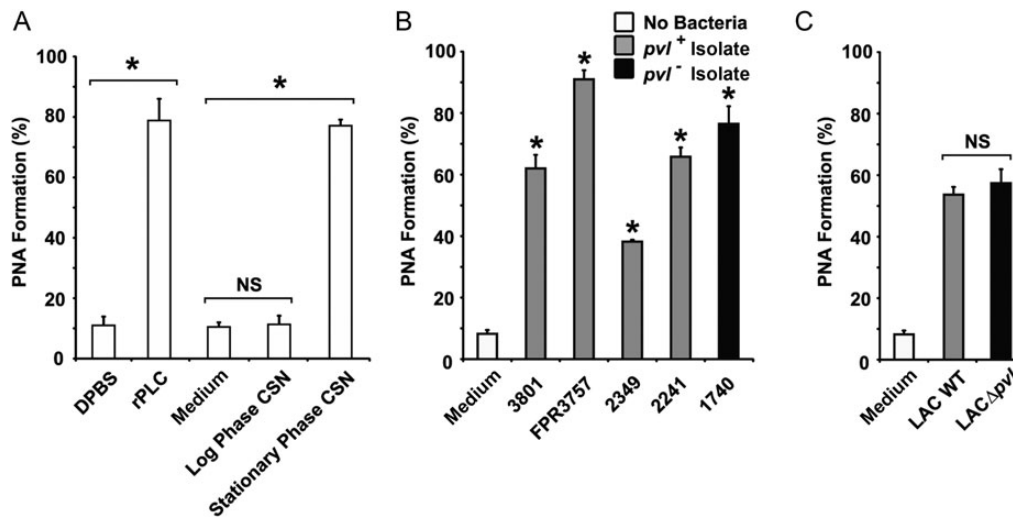
Figure 1. Methicillin-resistant *Staphylococcus aureus* (MRSA) exotoxin(s) promote platelet-neutrophil aggregate (PNA) formation independently of Panton-Valentine Leukocidin (PVL). Formation of PNA in human whole blood was quantitated by dual-color flow cytometry. *A*, Blood samples (100 μL) were preincubated with phycoerythrin-CD11b and fluorescein isothiocyanate–CD42b and then stimulated with 40 μL of bacteria-free log or stationary phase culture supernatant (CSN) from MRSA strain FPR3757 or an appropriate negative control (medium). *Clostridium perfringens* phospholipase C (r-PLC) served as a positive control. *B* and *C*, Stationary phase CSN from various *pvl* $^+$(grey bars) or a *pvl* $^-$ (black bar) community-acquired MRSA clinical isolates (*B*) or stationary phase CSN from wild-type (WT) MRSA strain LAC (grey bar) and its isogenic *pvl* deficient mutant (LAC*Δpvl*; black bar. The percentage of dual-positive CD11b/CD42b polymorphonuclear leukocytes was calculated using SYSTEM II flow cytometry software. Data represent the mean (±SD) of 4 samples from 2 independent blood donors. *P< .05, by the Student *t* test. Abbreviation: NS, not statistically significant.

enzymatic assay described by Wei et al [19] with minor modifications. Briefly, recombinant Sa-PI-PLC (0.005–1 μg in 20 μL of 10 mM HEPES) or PI-PLC from *Bacillus cereus* (Sigma) was added to individual wells punched in BBL CHROMagar Listeria plates (BD Biosciences, Franklin Lakes, NJ), which contain phosphatidylinositol bisphosphate (PIP$_2$) as substrate. After overnight incubation at 37°C, PI-PLC activity was detected as an opaque zone of insoluble diacylglycerol created by enzymatic cleavage of PIP$_2$.

Construction of *hla*- and *plc*-Deficient MRSA

CA-MRSA USA300 JE2 strain, cured of plasmids containing tetracycline and erythromycin resistance genes, was used for construction of toxin-deficient MRSA. Standard molecular procedures were used for DNA manipulation, plasmid isolation, purification, and transformation. An in-frame gene deletion procedure for gram-positive pathogens has previously been described by our laboratory [20]. Briefly, deletion constructs targeting replacement of the entire coding sequence of either the *hla* or *plc* gene with the kanamycin antibiotic selectable marker (*kan*) were constructed. Regions flanking the *hla* or *plc* targets were amplified using chromosomal DNA from *S. aureus* strain FPR3757 and ligated to the kanamycin gene in the pMAD vector, which contains an erythromycin marker, a thermosensitive replication origin, and constitutively expressed β-galactosidase [21]. Primers for amplifying the *hla* upstream/downstream

fragments were as follows: *hla*UPf1, 5′-GAGGATCCTCCATA CAAAATCCGCATCA-3′; *hla*UPr1, 5′-AAATGTAATTAAACA GTAAAGAAAACAGCTGAG-3′; *hla*DOWNf1, 5′-GAGTCGA CTGTTTTCATTTTCATCATCCT-3′; and *hla*DOWNr1, 5′-AC CATCATCAACTGGCTTACGACGTCAG-3′. Primers for amplifying the *plc* upstream/downstream fragments were as follows: *plc*UPf1, 5′-GAGGATCCAGGCTCAGAAGGGTGTATTTT GT-3′; *plc*UPr1, 5′-GTAAAATCATCACTACTCACCAACCCA GCTGAG -3′; *plc*DOWNf1, 5′-GAGTCGGACAGGATTCAA TAATGATATTAAGACGAG-3′; and *plc*DOWNr1, 5′-AGTTA GGGCCTTTTTCTCTCGACGTCAG-3′. The restriction sites were *Bam*HI, *Sal*I, and *Pst*I (underlined). Primers for *kan* were as follows: 5′-GAGTCGACCCCAGCGAACCATTTGAGGTG AT-3′ and 5′-GACCTACTTAACAAAATCQATGGATCTCAGC TGAG-3′, with appended *Sal*I as a restriction site (underlined). The resulting *hla* and *plc* targeting vectors were conditioned by passage through *S. aureus* strain RN4220 and introduced into *S. aureus* JE2 by phage transduction. Transductants were selected at 30°C on tryptic soy agar in the presence of erythromycin (10 μg/ mL), kanamycin 300 μg/mL, and X-Gal (50 μg/mL). Integration of the *kan* cassette into the JE2 chromosome at the *hla* or *plc* locus was achieved through temperature shift. Double-crossover events were identified by replica plating for susceptibility to erythromycin. PCR, restriction analysis, and Southern blot hybridization confirmed successful integration of a single targeting construct for *hla* and *plc*, yielding the mutant JE2*Δhla* and JE2*Δplc* strains. Growth
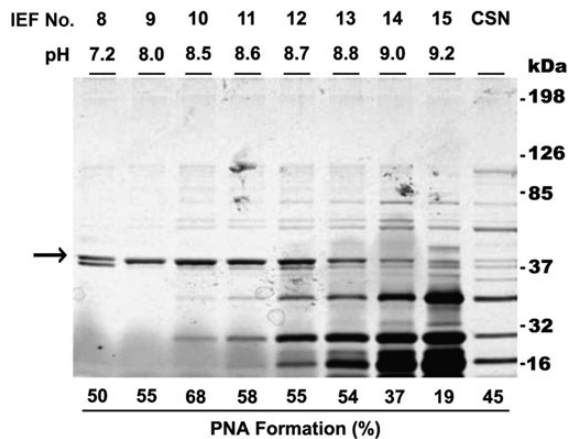
Figure 2. Presence of a 37-kDa protein band coincides with platelet-neutrophil aggregate (PNA) activity. Proteins from LACΔ*pvl* stationary phase culture supernatant (CSN) were fractionated by isoelectric focusing (IEF), resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and visualized by silver staining. Fraction numbers and their corresponding pHs are given above each lane. Unfractionated CSN from the original LACΔ*pvl* strain is also shown. PNA activity is given below each lane, and molecular weight markers are on the right. The intensity of an approximately 37-kDa protein band (arrow) appears to correlate with PNA-inducing activity. Data are representative of 2 independent experiments.

curves of the mutants were compared to that of the parental JE2 strain, and α-hemolysin activity and PI-PLC activity were assayed as described above. Total secretory proteins from the parental and mutant strains were resolved in 12% SDS-PAGE gel and visualized by silver staining.

Complementation Analysis

A genetic complementation method was designed for *hla* in strain JE2Δ*hla*. Briefly, the wild-type *hla* gene and endogenous promoter region were PCR amplified and cloned into the pMAD shuttle vector, using the following designated primers: 5′-GTCGACTAGCAATACTTTATTGTCCCATGA-3′ and (5′-CT TAAGGTACAACAGTTTGGGAAAGGTACC-3′, with appended *Sal*I and *Nco*I as restriction sites. The pMAD(*hla*) construct was introduced into *S. aureus* RN4220 prior to phage transduction into JE2Δ*hla*. PCR was used to detect *hla* expression in JE2Δ*hla* /*hla*⁺ complemented transductants. Stationary phase culture supernatants from these constructs were assayed for hemolytic activity by using the rabbit erythrocyte lysis assay to verify production of functional α-hemolysin by this construct.

Statistical Analysis

Data sets were analyzed using Stata/SE (StataCorp 2003, College Station, TX). Significant differences among groups were determined by implementing a 2-tailed, unequal variance Student t test. Statistical significance was defined as a P value of < .05.

RESULTS

MRSA Exotoxin(s) Promote PNA Formation

Exotoxins from log or stationary phase cultures of CA-MRSA strain FPR3757 were tested for their ability to induce PNA formation in human whole blood. The FPR3757 strain belongs to the most widespread CA-MRSA clone associated with invasive infections, USA300, and was the first such strain to undergo complete genome sequencing [22]. In negative control samples, few PMNLs had adherent platelets (mean [±SD], 9% ± 1.2% CD11b/CD42b dual-positive events; Figure 1*A*). A similar finding was observed in whole blood treated with log phase culture supernatants (Figure 1*A*). In contrast, stationary phase exotoxins induced high-level PNA aggregate formation (a mean [±SD] of 79% ± 3.2% of granulocytes were CD42b positive; Figure 1*A*) comparable to the known PNA agonist, *C. perfringens* PLC [23]. These findings demonstrate that at least 1 stationary phase–specific exotoxin from USA300 MRSA strongly elicits heterotypic platelet-neutrophil aggregation.

Formation of PNA Is Independent of PVL

The PNA activity of culture supernatants from 5 additional CA-MRSA clinical isolates was evaluated. These isolates produce widely variable amounts of PVL (0–796 ng/mL) [12]. All culture supernatants induced significant PNA formation, although no correlation was observed between the level of PNA activity and the presence or quantity of PVL produced (Figure 1*B*), suggesting that PVL is not involved in this process. This notion was confirmed by studies of stationary phase toxins from wild-type CA-MRSA strain LAC and its isogenic *pvl*-deficient mutant (LACΔ*pvl*), in which comparable PNA-inducing activity was observed (Figure 1*C*).

Identification of the *S. aureus* PNA–Inducing Factor

To identify the exotoxin(s) causing PNA formation, stationary phase culture supernatants from the LACΔ*pvl* strain were fractionated by isoelectric focusing, and fractions were tested for PNA-inducing activity. Eight fractions (pH range, 7.2–9.0) induced PNA formation, with maximal PNA activity (mean [±SD], 67.9% ± 7.2%) observed in fraction 10 (pH 8.5; Figure 2). PNA activity correlated with the presence of an 37-kDa protein band (Figure 2). Mass spectrometry analysis of this band identified 2 *S. aureus* proteins: α-hemolysin (Sa-AH; molecular weight, 36 kDa) and phosphatidylinositol-specific phospholipase C (Sa-PI-PLC; molecular weight, 35 kDa).

S. aureus α-Hemolysin Promotes PNA Formation

To elucidate which of these proteins stimulated PNA activity, recombinant proteins (Figure 3*A*) were generated. Both recombinant α-hemolysin (rSa-AH) and recombinant PI-PLC (rPI-PLC) were enzymatically active, and each was independently tested for the ability to promote PNA formation. Low amounts of rSa-AH (range, 0.015–0.125 µg) dose-dependently stimulated
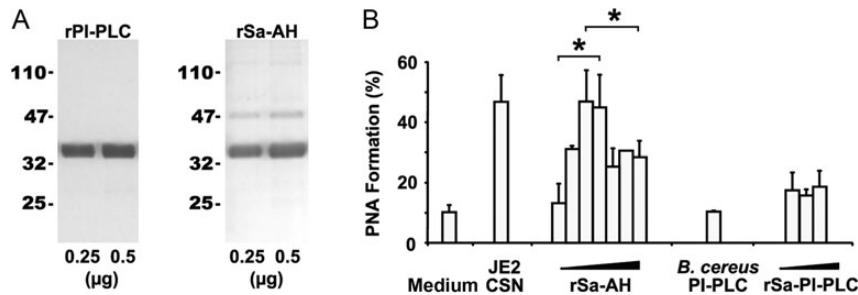
Figure 3. *Staphylococcus aureus* α-hemolysin, not phosphatidylinositol-specific phospholipase C (PI-PLC), promotes PNA formation. *A*, Purified recombinant *S. aureus* α-hemolysin (rSa-AH) and PI-PLC (rSa-PI-PLC) proteins were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized by silver staining. *B*, Increasing concentrations of rSa-AH (range, 0.015–5 μg) and rSa-PI-PLC (range, 0.5–10 μg) were assessed for PNA-inducing activity, using human blood as described in Figure 1. Data are representative of 2 independent experiments from 2 separate blood donors. *$P < .05$, by the Student *t* test. Abbreviation: *B. cereus, Bacillus cereus.*

PNA formation (Figure 3*B*); higher doses (range, 2.5–5 μg) were inhibitory (Figure 3*B*), possibly because of α-hemolysin–induced platelet lysis [24]. α-hemolysin is heat sensitive [25], and treatment of rSa-AH or culture supernatants at 60°C for 30 minutes ablated PNA activity (data not shown). In contrast to rSa-AH, neither rSa-PI-PLC (range, 0.5–10 μg) nor a commercial preparation of *B. cereus* PI-PLC (range, 0.5–1 U) promoted PNA formation (Figure 3*B*). Furthermore, a positive correlation was found between the amount of α-hemolysin activity in culture supernatants and the magnitude of the PNA response ($r^2 = 0.75$; Table 1).

As further confirmation, 2 toxin-deficient mutants (*hla* deficient or *plc* deficient) were created in the MRSA USA300 strain JE2 background. Southern blot analysis confirmed insertion of a kanamycin antibiotic gene cassette replacing either *hla* or *plc*

gene in the JE2 genome (data not shown). The *hla* (JE2Δ*hla*)– and *plc* (JE2Δ*plc*)–deficient mutant strains maintained growth kinetics similar to that of the parental JE2 strain. In both strains, protein banding patterns in stationary phase culture supernatants appeared to be identical to that for the parental JE2 strain, except for the marked absence of an approximately 37-kDa molecular weight band (Figure 4*A*). Unlike the *plc*-deficient mutant, the JE2Δ*hla* strain displayed a significant (81%) reduction in hemolytic activity (Table 1); residual hemolytic activity is likely attributable to γ-hemolysin and/or the LukED variant since both lyse rabbit erythrocytes [26, 27] and both are commonly found in *S. aureus* clinical isolates [26], including strain LAC (Dr Frank DeLeo, personal communication). Furthermore, only the stationary phase culture supernatants from the α-hemolysin–deficient JE2Δ*hla* strain failed to promote
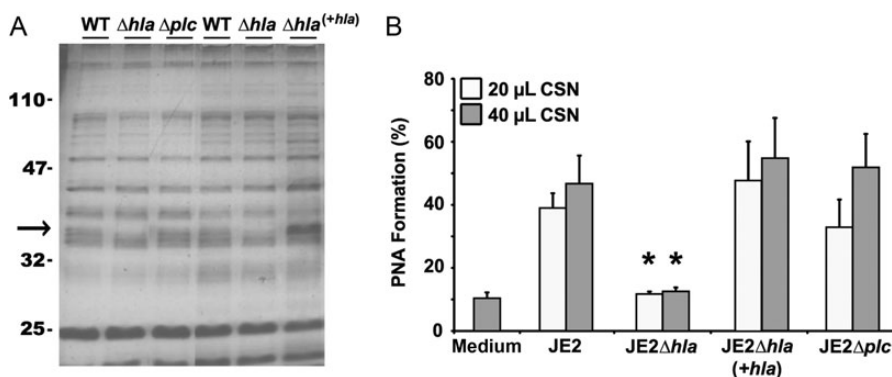
Figure 4. α-hemolysin is the sole mediator of exotoxin-induced platelet-neutrophil aggregate (PNA) formation. *A*, Proteins from stationary phase culture supernatant of the parental JE2 strain and its isogenic *hla* (JE2Δ*hla*)– and *plc* (JE2Δ*plc*)–deficient mutant strains were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized with silver staining. Both mutants lack an approximately 37-kDa band (arrow), which was restored only in the *hla*-complemented strain (JE2Δ*hla*/*hla+*). *B*, Stationary phase culture supernatant (CSN) from wild-type (WT) methicillin-resistant *Staphylococcus aureus* strain JE2, its isogenic *hla*-deficient and *hla*-complemented strains (JE2Δ*hla* and JE2Δ*hla*/*hla*+, respectively), and its isogenic *plc*-deletion mutant (JE2Δ*plc*) were assessed for PNA-inducing activity as described in Figure 1. Data are representative of 2 independent experiments from 2 blood donors. *$P < .05$, by the Student *t* test, versus JE2 wild-type CSN.
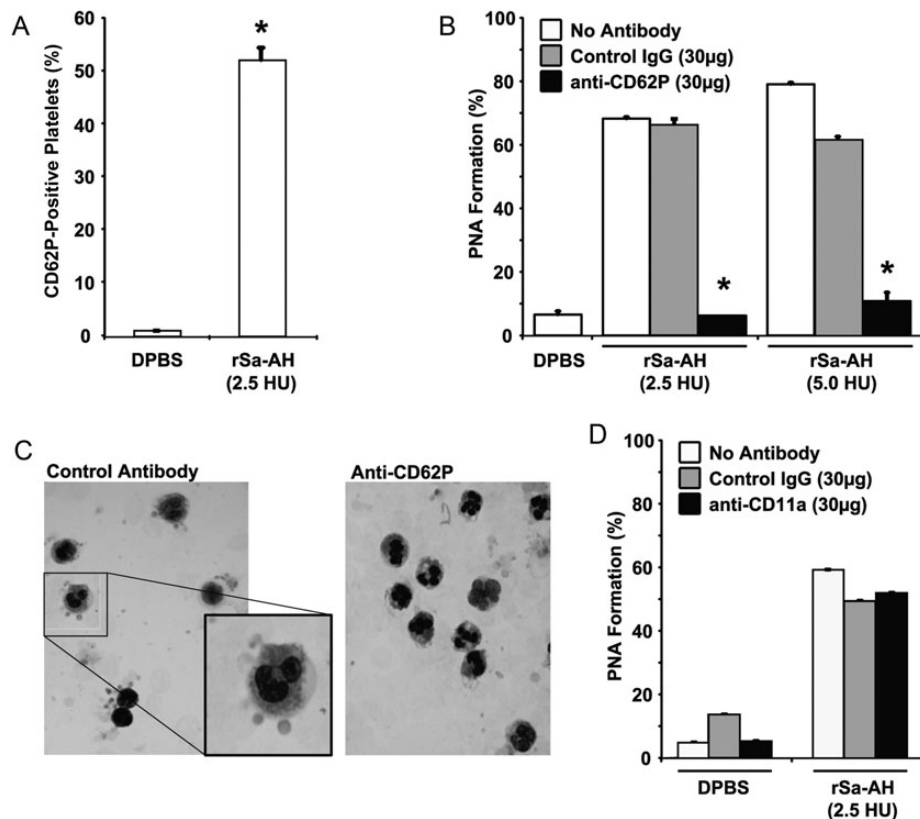
Figure 5. P-selectin mediates α-hemolysin–mediated platelet-neutrophil aggregate (PNA) formation. *A*, Recombinant *Staphylococcus aureus* α-hemolysin (rSa-AH)–induced P-selectin expression on isolated gel-filtered platelets was assessed by flow cytometry, using fluorescein isothiocyanate–CD62P antibody (Santa Cruz Biotechnology, Santa Cruz, CA). A total of 2.5 hemolytic units (HU) of rSa-AH equates to 0.06 μg of protein. Data are given as the percentage of platelets expressing CD62P. *B*, rSa-AH-induced PNA formation was assessed by flow cytometry in the presence of a blocking anti-CD62P antibody, an equal amount of isotype-matched control antibody, or vehicle control (ie, no antibody). *C*, A Wright's stained cytospin preparation of α-hemolysin–treated whole blood samples from panel *B* demonstrating a rosette-type pattern of platelet adherence to polymorphonuclear leukocytes in the presence of the control antibody (left; and enlarged inset); rosetting was prevented by anti-CD62P treatment (right). *D*, rSa-AH–induced PNA formation was assessed in the presence of 30 μg anti-LFA-1 blocking antibody, an isotype-matched control antibody (both from Santa Cruz Biotechnology, Santa Cruz, CA), or vehicle control (ie, no antibody). Data are given as the percentage of dual-positive events and are representative of 2 independent experiments from 2 blood donors. *$P < .05$, by the Student t test. Abbreviations: DPBS, Dulbecco's phosphate-buffered saline; IgG, immunoglobulin.

PNA formation (Figure 4*B*). Complementation of the *hla* gene in the JE2Δ*hla* mutant restored PNA activity (Figure 4*B*). Taken together, these data strongly suggest that α-hemolysin is the sole MRSA exotoxin that stimulates heterotypic platelet-neutrophil coaggregation in human whole blood.

Platelet P-selectin Mediates α-Hemolysin–Induced PNA Formation

The relative number of platelets bound per PMNL was assessed using the MFI of the CD42b signal. Despite the α-hemolysin–induced increase in the percentage of PMNLs that were positive for the platelet marker (Figure 1*A* and 1*B*), the number of platelets bound per PMNL was relatively low (MFI, 1.7–3.9; Table 1). This suggested that α-hemolysin causes platelets to bind PMNL individually or in a rosette-type pattern. Such a pattern is largely mediated by platelet P-selectin (CD62P) [28].

Since our data clearly show that rSa-AH directly increases CD62P on isolated platelets (mean CD62P positivity [±SD], 52.15% ± 2.4%; MFI [±SD], 2.07 ± 0.1; Figure 5*A*), we next tested whether α-hemolysin–induced PNA formation could be inhibited by blocking this adhesin. Anti-CD62P specifically reduced α-hemolysin–induced PNA formation by a mean (±SD) of 90% ± 2.5% (Figure 5*B*). This reduction was confirmed by light microscopy (Figure 5*C*). Similarly, anti-CD62P reduced PNA formation induced by culture supernatants from JE2 MRSA by 86% (data not shown). Antibody blockade of CD11a was without effect (Figure 5*D*).

To examine the relative contribution of each cell type to PNA formation, isolated, gel-filtered platelets were exposed to rSa-HA for 8 minutes and then combined with unstimulated isolated PMNLs for 2 minutes, or vice versa, and PNA formation was assessed as before. PNA formation only occurred
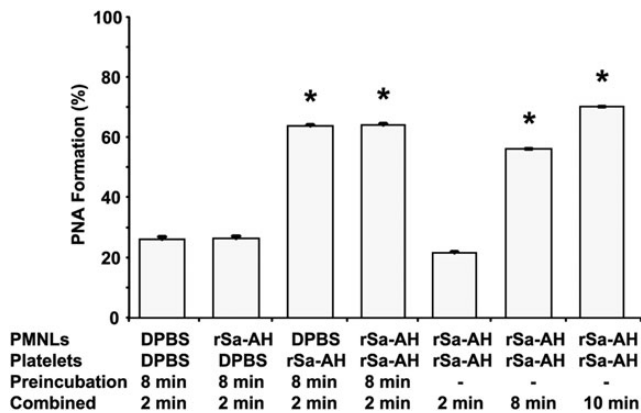
Figure 6. Activated platelets are essential for recombinant *Staphylococcus aureus* α-hemolysin (rSa-AH)–mediated platelet-neutrophil aggregate (PNA) formation. To examine the relative contribution of each cell type to PNA formation, isolated, gel-filtered platelets were exposed to rSa-HA (2.5 hemolytic units [HU]) for 8 minutes and then combined with unstimulated isolated polymorphonuclear leukocytes (PMNLs) for 2 min, or vice versa, and PNA formation was assessed as described in Figure 1. As controls, isolated cells were incubated together in the presence of rSa-HA for the indicated times. PNA formation above baseline only occurred when platelets were preactivated with α-hemolysin; preactivation of PMNLs with rSa-HA for 8 minutes did not stimulate aggregate formation. *$P < .05$, by the Student t test. Abbreviation: DPBS, Dulbecco's phosphate-buffered saline.

when platelets were preactivated with α-hemolysin (Figure 6); preactivation of PMNLs with rSa-HA for 8 minutes did not promote adherence of resting platelets (Figure 6). These findings are consistent with our data demonstrating that CD11b intensity did not correlate with α-hemolysin concentration ($r^2 = 0.08$; Table 1) and that increased CD11b intensity did not correlate with increased PNA formation ($r^2 = 0.13$; Table 1), and they agree with previous reports demonstrating that human PMNLs are largely resistant to α-hemolysin [29].

Taken together, these findings suggest that direct toxin-induced upregulation of platelet P-selectin, and not the gpIIbIIIa-fibrinogen-CD11b axis, plays the critical role in α-hemolysin–induced PNA formation.

DISCUSSION

The recent emergence of CA-MRSA hemorrhagic necrotizing pneumonia over the past decade is associated with a severe morbidity and high mortality [1]. Understanding the host response to bacterial toxins is crucial to devising strategies for therapeutic intervention, particularly when antibiotic selection may be limited due to bacterial resistance. Previous studies have shown that *S. aureus* exotoxins, including PVL and α-hemolysin, contribute to the pathology of CA-MRSA pneumonia through activation of alveolar macrophages/monocytes and by excessive PMNL recruitment [30–34]. Other studies have

shown that α-hemolysin enhances neutrophil adherence to endothelial cells [35, 36] and triggers NLRP3-inflammasome signaling in human monocytes, leading to the release of interleukin 1β and interleukin 18 [34]—processes that play major roles in the pathogenesis of acute respiratory distress syndrome [37]. Our findings extend these observations to include α-hemolysin–induced platelet-neutrophil coaggregation as another plausible cellular mechanism whereby the host response to toxin production could contribute to destruction of the lung microvasculature in hemorrhagic necrotizing MRSA pneumonia. Furthermore, we identify platelet P-selectin as the principal ligand supporting such coaggregation. Although controversy remains regarding the relative importance of individual staphylococcal exotoxins to the pathogenesis of necrotizing pneumonia, we conclusively demonstrate here that PVL has no role in this mechanism. Thus, our findings add to a growing body of evidence supporting a critical role for α-hemolysin in hemorrhagic necrotizing pneumonia [33, 34, 38].

We and others have clearly implicated a role for PNA formation in the pathogenesis of tissue destruction in other infectious diseases, including clostridial and streptococcal myonecrosis [9, 10, 15, 23], sepsis/septic shock [39, 40], hemolytic uremic syndrome [41], nephritis [42], and myocardial infarction [43]. P-selectin–dependent PNA formation has been shown to enhance neutrophil oxidative activity [44] and thereby cause increased vascular permeability and pulmonary dysfunction in mice with sickle cell disease [45]. Thus, targeting host response molecules for therapeutic intervention offers a novel alternative to traditional antimicrobial treatment strategies. Results presented here further demonstrate that platelet activation is essential for α-hemolysin–induced PNA formation and that heterotypic cellular aggregation is P-selectin dependent.

Although the signaling mechanism by which α-hemolysin activates platelets has yet to be identified, several possibilities exist. For instance, platelets express Toll-like receptors [46] and disintegrin/metalloproteinase (the ADAM family) receptors, of which Toll-like receptor 2 and ADAM10 recognize α-hemolysin directly [47, 48]. Engagement of these receptors could activate Src family kinases [48, 49], signaling molecules common in α-granule degranulation and P-selectin translocation in platelets. In addition, α-hemolysin could indirectly activate platelets through stimulation of leukocytes or endothelial cells, facilitating the release of platelet agonists such as platelet-activating factor [50], prostacyclin, collagen, adenosine diphosphate, or thromboxane-A_2. Last, changes in shear stress that may occur in the pulmonary microvasculature during CA-MRSA pneumonia may further stimulate platelet activation and augment α-hemolysin–induced PNA formation.

In summary, α-hemolysin, not PVL, mediates PNA formation in a P-selectin–dependent manner. This suggests that blockade of the P-selectin/PSGL-1 receptor-ligand interaction may limit α-hemolysin–induced lung destruction in MRSA pneumonia.

Notes

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