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BRIEF REPORT

Cathepsin G Degrades *Staphylococcus aureus* Biofilms

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Polymorphonuclear leukocytes (PMN) phagocytose and kill individual bacteria but are far less efficient when challenged with bacterial aggregates. Consequently, growth within a biofilm affords *Staphylococcus aureus* some protection but PMN penetrate *S. aureus* biofilms and phagocytose bacteria, suggesting that enzymes released through neutrophil degranulation degrade biofilms into fragments small enough for phagocytosis. Here we show that the capacity of PMN to invade biofilms depended largely on the activity of secreted cathepsin G.

Keywords. *Staphylococcus aureus*; biofilm; polymorphonuclear leukocytes; cathepsin G.

Staphylococcus aureus biofilms are communities of bacteria encased in a complex extracellular matrix. *S. aureus* biofilm infections are often chronic and reoccurring because they are recalcitrant to antibiotic therapy as well as the host immune response [1]. Given this, effective therapies require an understanding of the matrix, as well as the interactions between the immune system and the biofilm. While the composition of the matrix is not fully defined and varies with growth conditions, it consists largely of positively charged proteins, either secreted or membrane anchored, and extracellular DNA [1]. Together, the DNA and proteins, along with teichoic acids on the bacterial cell surface, form an electrostatic net capable of maintaining the integrity of very large biofilms on both host tissues and medical implants.

The size of biofilms relative to that of polymorphonuclear leukocytes (PMN) affords *S. aureus* protection [2, 3], because PMN can phagocytose individual bacteria or bacterial aggregates smaller than 11 µm in diameter [4, 5]. Therefore, given a *S. aureus* cell diameter of approximately 1 µm, PMN can phagocytose only relatively small aggregates of bacteria. Nevertheless, PMN migrate towards *S. aureus* biofilms where they degranulate, penetrate the biofilm surface, and successfully phagocytose bacteria [6–9]. This suggests that enzymes released by degranulation degrade biofilms to allow PMN entry. By isolating azurophilic granules from human PMN and fractionating their contents by ion-exchange chromatography, we show that the capacity to degrade biofilm resides primarily with the protease cathepsin G (CG).

METHODS

Microtiter Plate Biofilm Assay

Antibiofilm activity against *S. aureus* US300 isolate LAC [10] was tested using a static microtiter plate assay [11] in which plates are precoated with human plasma and biomass is quantified spectrophotometrically using crystal violet staining.

PMN Granule Purification

Blood was collected from healthy donors, in accordance with protocols approved by the University of Iowa Institutional Review Board, and PMN were isolated from venous blood using dextran sedimentation and Hypaque-Ficoll density gradient separation, and granules were isolated from Percoll gradients of nitrogen-cavitated PMN, as previously described [12]. A portion of the total granule contents was treated with 2 mM of serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) diluted from a 20 mM stock in dimethyl sulfoxide (DMSO). At the same time, control granule contents were mock treated with DMSO alone. Before use in biofilm assays, the treated granule contents were incubated for 6 half-lives of PMSF so that no active PMSF would be added to the biofilm assay [13].

Cation-Exchange Chromatography of Alpha Granule Contents

Lysed azurophilic granules were dialyzed against equilibration buffer, consisting of 10 mM sodium phosphate pH 6.5, and absorbed to a 1 × 15 cm column containing Toyopearl CM650M resin equilibrated with the same buffer. Following extensive washing with equilibration buffer, proteins were eluted with a 500 mL linear gradient of 0–500 mM NaCl in 10 mM sodium phosphate pH 6.5. Protein content of elution fractions was determined by Bradford, the locations of CG and elastase activities were determined using protease-specific *p*-nitroanilide peptide substrates [14], and antibiofilm activity was measured in duplicate using the microtiter plate biofilm assay. The identities of CG and elastase were confirmed by mass spectrophotometric analysis of tryptic digests conducted at the University of Iowa Protein Structure Core.

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Protease Dose-Response Curves

Purified CG and elastase (Lee Biosolutions) were dialyzed against phosphate-buffered saline (PBS) and then 2-fold serial dilutions were added to microtiter plates at the time the biofilm plates were set up. Mock PBS dilution series were included as a control. Following 18 hours of incubation, biomass was quantified by crystal violet staining.

Liberation of Planktonic Bacteria From Biofilms

Following dialysis against PBS, purified CG (Lee Biosolutions) solution was split in 2, and half was inactivated using PMSF in DMSO and half underwent mock treatment with DMSO alone. Enzyme inactivation was confirmed using *p*-nitroanilide peptide substrate. Two-fold dilution series of inactivated and mock treated CG were added to microtiter biofilm plates either at the time the plates were set up or after biofilms were allowed to grow statically at 37°C for 15 hours. Buffer control wells were also included in the biofilm microtiter plate. Following incubation, 20 µL of biofilm supernatant was removed from each well, being careful to not disrupt biofilms attached to the well bottoms and sides, and added to 180 µL of fresh tryptic soy broth in another 96-well plate. This plate was incubated for 3 hours in a Stuart microtiter plate incubator at 37°C and shaking at 1000 rpm. The relative outgrowth in these wells, measured by optical density 600 using a Tecan M Plex plate reader, was used as a gauge of the planktonic cell density in the biofilm supernatants. For post treatment with active and inactive GC, biofilms were grown without any enzyme for 15 hours and then 90 µL of 2-fold dilution series of enzyme was added to the wells. The plate was incubated statically at 37°C for 1 hour before quantifying planktonic cell densities as described above.

RESULTS

Formation of *S. aureus* biofilms in plasma-coated microtiter plates [11] (Figure 1A) was prevented by total pooled PMN granule contents [12] (Figure 1B), and comparison of the antibiofilm activity of the various granule types shows that azurophilic granules had the greatest activity (Figure 1C). This finding and the observation that serine protease inhibition [13] reduces the antibiofilm activity of pooled granule contents (Figure 1D), suggest that one or more of the neutrophil serine proteases (NSP) in azurophilic granules might be responsible for the antibiofilm activity. Azurophilic granule contents were therefore further fractionated by cation-exchange chromatography, and elution fractions were assayed for both protease activity, using protease-specific substrates [14], and antibiofilm activity, using the microtiter plate biofilm assay (Figure 2A). Protease assays showed that CG and neutrophil elastase (NE) activities were well-resolved, with the proteases eluting at positions within the elution salt gradient consistent with their relative isoelectric points (pIs). The profile of the biofilm biomass curve suggests a normal distribution, with just 7 of the 96 fractions having a biomass that deviated by more than 2 standard deviations from the mean biofilm biomass. Four of these outliers (fractions 5, 38, 45, and 48) were isolated individual fractions, suggesting they arose from random experimental error, whereas 3 of the outliers, fractions 54–56, were contiguous, suggesting that the reduced biomass associated with these fractions was due to genuine antibiofilm activity. The antibiofilm activity in fractions 54–56 coincided with CG activity (Figure 2A) which, in conjunction with the observation that serine protease inhibition reduced antibiofilm activity of pooled granule contents (Figure 1D), suggests CG was responsible for antibiofilm activity. The fact that CG in fractions 54–56 was highly purified, as indicated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Figure 2B), suggests that CG was sufficient for preventing *S. aureus* biofilm formation. In contrast, the absence of antibiofilm activity in NE-containing fractions indicates that NE either cannot digest the matrix proteins that maintain biofilm integrity or is alone unable to compromise biofilm formation. To distinguish between these possibilities, serial 2-fold dilution series of purified CG and NE were tested for their ability to prevent biofilm formation (Figure 2C). NE was able to reduce biofilm formation on its own, but in comparison to CG approximately 20-fold more activity was required, suggesting that the proteins stabilizing *S. aureus* biofilms are significantly more sensitive to cleavage by CG. To further characterize the antibiofilm activity of CG, the ability of CG to liberate planktonic bacteria from biofilms was assessed. Addition of active CG, added either at the time biofilm microtiter plates were set up or to established biofilms, was able to release planktonic bacteria from the biofilm in a dose-dependent manner, whereas chemically inactivated CG could not release planktonic bacteria regardless of when it was added (Figure 2D).

DISCUSSION

Given that biofilms have been associated with chronic and reoccurring infections, the adeptness of *S. aureus* at forming biofilms likely contributes to the fact that *S. aureus* is a leading cause of many infection types [1]. *S. aureus* biofilm infections are particularly difficult to treat because when encased within the biofilm matrix, bacteria are protected from both antibiotic therapy and the innate immune response. Protection from the immune response is due, at least in part, to the fact that PMN can only phagocytose individual bacteria or very small bacterial aggregates [4, 5]. Although this suggests *S. aureus* biofilms should not be susceptible to killing by PMN [2, 3], numerous studies have shown that PMN are able to degrade and penetrate *S. aureus* biofilms and successfully phagocytose bacteria [6–9]. Collectively, the studies reported here suggest the ability of PMN to degrade *S. aureus* biofilms is largely dependent on the proteolytic activity of CG. We found that, amongst PMN granule types, the azurophilic granules had the greatest ability to prevent biofilm formation (Figure 1C), and when their

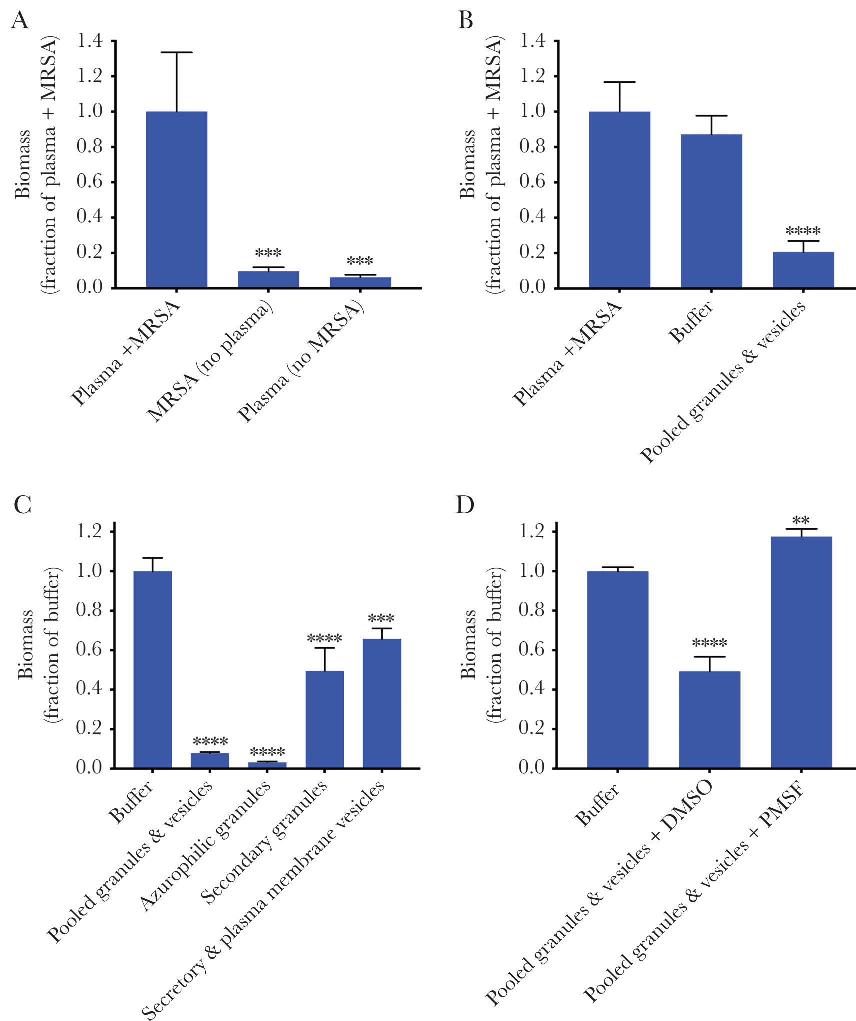

Figure 1. Biofilm quantification by crystal violet staining demonstrates *Staphylococcus aureus* biofilm formation was dependent on human plasma (*A*) and was reduced by the contents of lysed pooled PMN granules (*B*) but not by the relaxation buffer used during granule purification. Comparison of the antibiofilm activity of various granule types shows azurophilic granules have the highest activity (*C*), and antibiofilm activity of pooled granules was reduced by serine protease inhibition, in comparison to mock treatment with DMSO (*D*). In all cases biofilms were treated with 1×10^6 PMN equivalents of granule contents. Statistics are from 1-way ANOVA with *P* values **** <.0001, *** <.0005, ** <.005, with n varying between 3 and 7. Abbreviations: ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; MRSA, methicillin-resistant *Staphylococcus aureus*; PMN, polymorphonuclear leukocyte; PMSF, phenylmethylsulfonyl fluoride.

contents were fractionated this antibiofilm activity was associated with CG (Figure 2A), suggesting that the matrix proteins responsible for maintaining *S. aureus* biofilm integrity are particularly sensitive to cleavage by CG. The enhanced sensitivity of the biofilms to CG could be due in part to CG cleaving a specific matrix protein, or a small number of proteins, that other NSP cannot cleave. For example, it has been shown that CG, but not the other NSP, can cleave the active domain from the adhesion protein clumping factor A (see [15] and reference 28 therein). However, based on their broad sequence specificities all of the NSP should be able to cleave many, if not the majority of, proteins found within the biofilm matrix. This assumption, in combination with the multitude of proteins found in the biofilm matrix (see [1] and references therein) and the numerous adhesions found on the surface of *S. aureus,* makes it unlikely that a single protein would be solely responsible for biofilm stability. Consistent with this conjecture is the observation that NE alone also was able to reduce biofilm biomass (Figure 2C), but only at higher levels of activity. The enhanced sensitivity to CG may also be due in part to the differential ability of *S. aureus* to inhibit each of the NSP. It has been shown that *S. aureus* secretes 3 proteins, extracellular adherence protein (Eap) and its homologues EapH1 and EapH2, that bind NSP in one-to-one complexes and thereby inhibit NSP activity [15]. Given that Eap is abundant in biofilms (see [1] and references therein) it therefore seems likely that NSP will be at least partially inhibited when they encounter the biofilm matrix. Because the individual NSP have roughly equal abundance in granules, the finding that the K_i of Eap proteins toward CG are higher than (ie, less potent) the K_i toward the other NSP

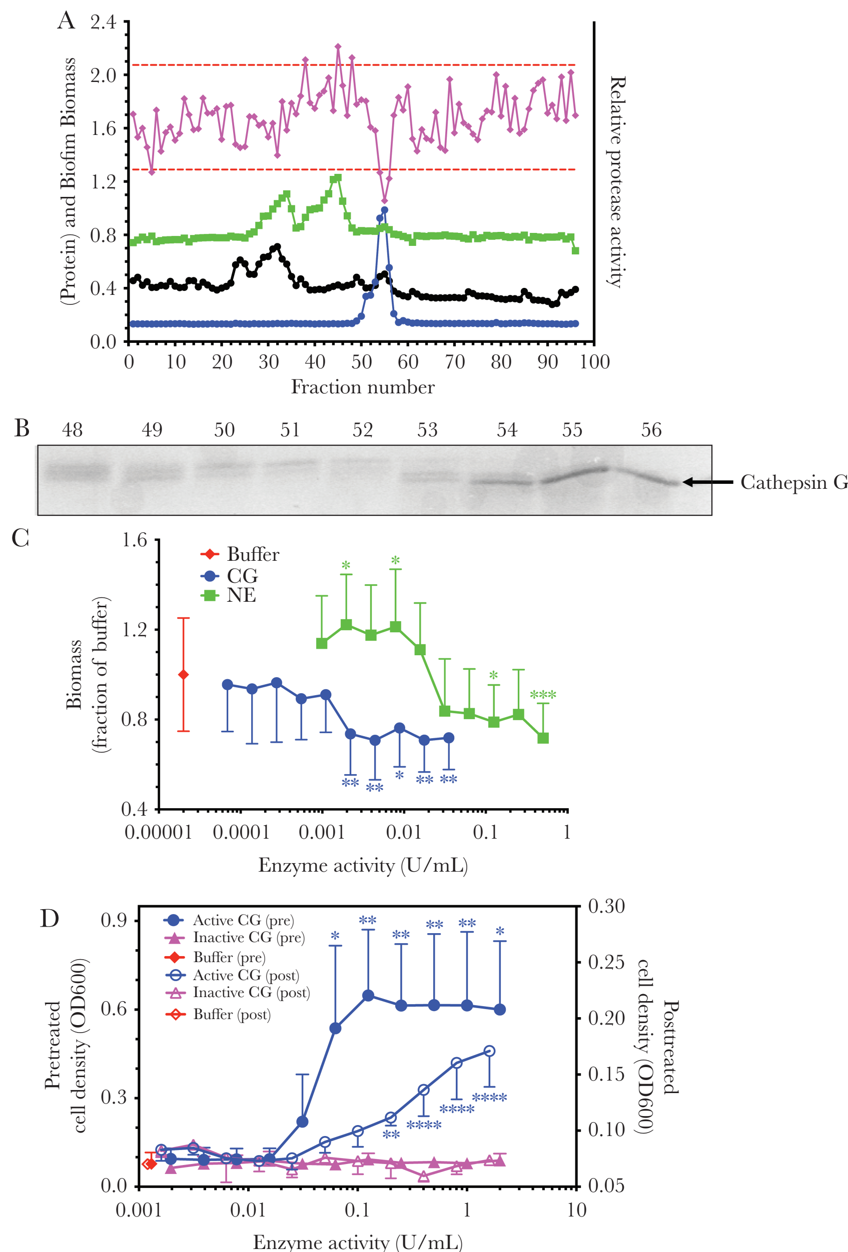

Figure 2. *A*, Characterization of cation-exchange elution fractions shows reduction in biofilm biomass (magenta diamonds) coincided with CG activity (blue circles), whereas no reduction in biomass was associated with elastase activity (green squares). The bimodal distribution of elastase activity is due to a C-terminal processing event that impacts pI but not enzyme activity. Protein content of fractions (black circles) was quantified by Bradford. *B*, SDS-PAGE of fractions 48–56 showed that CG in fractions 54–56 was highly purified. *C*, Impact of 2-fold dilutions series of CG (blue circles), elastase (green squares), and phosphate-buffered saline control (red diamond) on biofilm biomass shows biofilms were more sensitive to cleavage by CG. Error bars are plus 1 standard deviation for NE, minus 1 standard deviation for CG, and plus/minus 1 standard deviation for buffer. *D*, Enumeration of planktonic bacteria in biofilm supernatants following pretreatment (filled symbols) or posttreatment (open symbols) with active (blue circles) or inactive (magenta triangles) CG, as measured by bacterial outgrowth in tryptic soy broth inoculated with biofilm supernatants, shows CG must be enzymatically active to release bacteria from biofilms. Error bars are plus 1 standard deviation for pretreatment and minus 1 standard deviation for posttreatment. Statistics are from 1-way ANOVA referenced to buffer control (red diamonds) with *P* values *** <.0005, ** <.005, and * <.05, with n values of 19 for buffer and 5 for CG and NE for (*C*), and n values of 3 and 4 for pretreatment and posttreatment, respectively, in (*D*). Abbreviations: ANOVA, analysis of variance; CG, cathepsin G; NE, neutrophil elastase; pI, isoelectric point; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

(see [15] and reference 46 therein) means that CG would be most likely to elude inhibition, and thereby the most effective at disrupting biofilms. Regardless of the exact mechanism underlying the sensitivity of *S. aureus* biofilms to CG, we found that addition of active CG, but not chemically inactivated CG, to established biofilms (Figure 2D) released planktonic bacteria, which should be susceptible to both antibiotics as well as phagocytosis and killing by PMN. This finding suggests adjunct

treatment of *S. aureus* biofilm infections with active CG, either by topical application or administration via catheter, may facilitate infection clearance by enhancing both antibiotic efficacy and killing by PMN.

Notes

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