Protease inhibitors decrease IgG shedding from *Staphylococcus aureus*, increasing complement activation and phagocytosis efficiency

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*Staphylococcus aureus* is a major pathogen for immunologically intact humans and its pathogenesis is a model system for evasion of host defences. Antibodies and complement are essential elements of the humoral immune system for prevention and control of *S. aureus* infections. The specific hypothesis for the proposed research is that *S. aureus* modifies humoral host defences by cleaving IgG that has bound to the bacterial surface, thereby inhibiting opsonophagocytosis. *S. aureus* was coated with pooled, purified human IgG and assayed for the shedding of cleaved IgG fragments using ELISA and Western blot analysis. Surface-bound IgG was shed efficiently from *S. aureus* in the absence of host blood proteins. Broad-spectrum protease inhibitors prevented cleavage of IgG from the *S. aureus* surface, suggesting that staphylococcal proteases are responsible for IgG cleavage. Serine protease inhibitors and cysteine protease inhibitors decreased the cleavage of surface-bound IgG; however, a metalloprotease inhibitor had no effect. Using protease inhibitors to prevent the cleavage of surface-bound IgG increased the binding of complement C3 fragments on the surface of *S. aureus*, increased the association with human neutrophils and increased phagocytosis by human neutrophils.

INTRODUCTION

*Staphylococcus aureus* is responsible for an array of infections including those of skin and soft tissue, as well as life-threatening invasive infections in normal hosts (El-Gilany & Fathy, 2009; Rogers et al., 2009). Antibiotic treatment of *S. aureus* infections is becoming ever more challenging with the recent emergence of community-associated meticillin-resistant *S. aureus* (MRSA) (Klein et al., 2007). Understanding *S. aureus* evasion of host defences may prove critical in preventing and treating *S. aureus* infections in the future.

IgG plays an essential role in the humoral immune system control of bacterial infections (Brown et al., 1982). One of the important functions of this antibody is opsonizing bacteria and improving their recognition by phagocytic leukocytes (Leijh et al., 1981). IgG can also activate the classical complement pathway leading to additional opsonization by the complement C3b/iC3b fragments and the generation of anaphylatoxins (Joiner et al., 1983). *S. aureus* utilizes multiple strategies to evade IgG-mediated immune functions. *S. aureus* protein A is a cell-wall protein that binds IgG via the Fc region, thereby affecting its ability to act as an opsonin (Peterson et al., 1977). Another evasion mechanism utilizes staphylokinase, a bacteriophage-encoded protein that activates the serine protease plasmin, enabling the cleavage of IgG (Rooijakkers et al., 2005).

*S. aureus* encodes proteases in three categories: metallo-protease (aureolysin), serine proteases (SspA and Sla, SlaB, SplC, SplD, SplE and SplF) and cysteine proteases (SspB and ScpA) (Boles & Horswill, 2008). Prokesova et al. (1992) purified the serine protease V8 (SvpA) from *S. aureus* growth medium and demonstrated that it could cleave human immunoglobulins floating free in solution. They observed that V8 cleaved soluble IgG not only in the hinge region, as described for other bacterial proteases, but also at multiple sites on the antibody molecule. These studies only tested unbound IgG in solution and required...
high enzyme:substrate ratios (1:5–1:20) and lengthy incubation times (4–24 h). At enzyme:substrate ratios of 1:100 IgG, cleavage effects were minimal.

To our knowledge, the experiments described in the current study are the first to elucidate the shedding of *S. aureus* surface-bound IgG. These studies measured the cleavage of surface-bound IgG from *S. aureus* and evaluated classes of protease inhibitor that could modify IgG cleavage. These studies assayed whether protease inhibition of IgG shedding modulates IgG-mediated immune functions.

**METHODS**

**Ethics statement.** Healthy human volunteers donated blood under an Institutional Review Board-approved protocol (Eastern Virginia Medical School IRB: 02-06-EX-0216). This blood was used to generate serum and leukocytes for use as reagents. These products were used as reagents only and not to derive information about the donors. All donors provided written informed consent.

**Bacterial strains and growth.** A well-characterized *S. aureus* laboratory strain, Newman, was used in all experiments, unless otherwise noted. In some experiments, a clinical MRSA strain, MRSA7, was used that was obtained as a discarded de-identified isolate from a clinical microbiology laboratory (Eastern Virginia Medical School IRB protocol: 06-04-WC-0040). M RSA7 is a community-associated MRSA with a PFGE pattern consistent with strain USA300. Bacteria were grown in Columbia/2 % NaCl broth and incubated at 37 °C for 2 h with agitation to mid-exponential phase, unless otherwise stated. For some experiments, S. aureus was gently heat-killed at 70 °C for 12 min.

**Buffers.** Buffers used were GVBS2+ (Veronal-buffered saline, 0.1 % gelatin, 0.15 mM CaCl2, 1.0 mM MgCl2), EDTA-GVBS2− (Veronal-buffered saline with 0.1 % gelatin, 0.01 M EDTA) and raffinose buffer [15 g raffinose in 44 ml distilled water, 5 ml 50 mM Tris/HCl (pH 7.5), 1 ml 1 M MgCl2].

**Preparation of serum, IgG, complement proteins and protease inhibitors.** Normal human serum was prepared from the blood of healthy human volunteers and pooled as described previously (Cunnion et al., 2001). IgG-depleted serum was generated by ProteoPrep spin columns (Sigma-Aldrich) following the manufacturer’s protocol.

Purified pooled human IgG (Gammmagard; Baxter) was reconstituted at the manufacturer’s recommended concentration of 50 mg ml−1. In experiments involving complement or phagocytosis, the IgG was further diluted 1:50 (1 mg ml−1). Purified human complement protein C3 and polyclonal antibodies directed against human C3 were purchased from CompTech. Complete Mini protease inhibitor cocktail (Roche) was used for broad-spectrum inhibition. The Complete Mini tablet was dissolved in 1.5 ml distilled water. Individual protease inhibitors (PMSF, Pefabloc, aprotinin, E64, N-ethylmaleimide and EDTA) were used from the Protease Inhibitors Set (Roche); starting concentrations and buffer dilutions used for each protease inhibitor were as recommended by the manufacturer.

**Human IgG binding to and release from *S. aureus*.** Washed bacteria (1 ml containing 1×10⁸ cells) were combined with 100 μl purified IgG (1 mg ml−1) and incubated at room temperature for 5 min. After five washes with EDTA-GVBS2− and centrifugation, the pellets were resuspended in 50 μl PBS and incubated in a water bath at 37 °C for 30 min, unless otherwise indicated. Supernatants were collected for measurement of IgG fragment release by ELISA and Western blotting. Cell-wall preparations were generated from the bacterial pellets, which were washed twice in PBS and then resuspended in 300 μl raffinose buffer, 50 μl protease inhibitor cocktail, 5 μl lysostaphin (10 mg ml−1) and 5 μl DNase (1 μg ml−1) as described previously (Hair et al., 2008).

**IgG shed from *S. aureus* in the presence of protease inhibitors.** Washed mid-exponential-phase S. aureus was coated with IgG as described above, washed and incubated in 0.5 ml PBS (control) or 0.5 ml protease inhibitor (cocktail or individual protease inhibitor) at 37 °C for various lengths of time. IgG spontaneously shed from the surface was recovered in the supernatant and assayed by ELISA and Western blot.

**IgG-mediated complement activation.** Bacteria coated with IgG were prepared as described above. After centrifugation, pellets were resuspended in 1 ml GVBS2+. Of this suspension, 200 μl was combined with GVBS2+ and serum to achieve a volume of 1 ml with a 2 % final concentration of serum. Samples were incubated at 37 °C for 10 min, washed and centrifuged, and the pellets were stripped of C3 fragments, as described previously (Cunnion et al., 2001). Supernatants were analysed by C3 ELISA.

**ELISA.** Flat-bottomed Immulon 2 plates were coated with 50 μl chicken anti-human IgG (GenEx), diluted 1:10 000 in a carbonate coating buffer, for 4 h. Plates were washed three times with PBST (PBS with 0.05 % Tween 20) and blocked with 3 % BSA in PBST overnight at 4 °C. At the time of use, plates were washed three times with PBST, and the samples were diluted in 3 % BSA in PBST and allowed to incubate in the plates for 1 h at room temperature. Plates were washed three times with PBST and then probed with horseradish peroxidase-labelled rabbit anti-human IgG antibody (Dako) diluted 1:10 000, for 1 h at 25 °C. Plates were washed three times with PBST and developed with TMB Plus (Accurate Chemical); reactions were stopped with 0.5 M H2SO4 and the A450 was read. Purified IgG was used to generate standard curves. C3 ELISAs were performed as described previously (Cunnion et al., 2004).

**IgG assays by Western blotting.** Samples were reduced with SDS sample buffer containing 5 % 2-mercaptoethanol, unless otherwise stated, and boiled for 5 min before loading onto 4–20 % gradient SDS-PAGE mini-gels (Bio-Rad). Semi-dry transfer was performed onto a PVDF membrane. The membrane was blocked with 3 % BSA in Tris-buffered saline with 0.1 % Tween 20 and the A600 was read. Purified IgG was used to generate standard curves. C3 ELISAs were performed as described previously (Cunnion et al., 2004).

**Phagocytosis assay.** Human neutrophils were prepared using heparinized human blood from healthy human volunteers by Hypaque-Ficoll step gradient centrifugation, dextran sedimentation and hypotonic lysis, as described previously (Cunnion et al., 2005). One millilitre of washed exponential-phase bacteria (1×10⁹ cells ml−1) was combined with 100 μl purified IgG (1 mg ml−1) and incubated at room temperature for 5 min and then washed twice with EDTA-GVBS2−. Pellets were resuspended in either 500 μl protease inhibitor cocktail or buffer control at 37 °C for 30 min. After centrifugation, pellets were resuspended in 1 ml GVBS2+. To 100 μl of the bacterial suspension, GVBS2− and IgG-depleted serum were added to achieve a final volume of 500 μl at 2 % serum. After incubation at 37 °C for 10 min, 100 μl of this suspension was combined with 800 μl Hanks’ balanced salt solution and 100 μl purified human neutrophils to achieve a bacteria:neutrophil ratio of 20:1. This suspension was incubated in a rotator at 37 °C for 45 min.
After the final incubation, a 100 µl aliquot of the mixture was removed, stained with 5 µl acridine orange (0.01% final concentration) for 2 min, quenched with 10 µl crystal violet (0.03% final concentration) for 10 min and then fixed to a microscope slide using a cytospin. Slides were blinded to the reader and evaluated by fluorescence microscopy. For each slide, 100 neutrophils were counted to determine the percentage of neutrophils phagocytosing bacteria and the total number of S. aureus ingested.

**RESULTS**

**Bound IgG is shed from the S. aureus surface over time**

We first tested whether IgG bound to the S. aureus surface remained constant or decreased over time. After purified IgG was allowed to bind to the staphylococcal surface, the opsonized bacteria were washed and incubated in buffer. Bacteria were recovered and cell-wall preparations were generated and analysed under non-reducing conditions by Western blotting (non-reducing conditions), and without protease inhibitor cocktail. The amount of IgG and washing, bacteria were incubated in buffer with spectrum protease inhibitors. After coating with human IgG, washed and assayed for IgG fragment shedding by Western blotting, we tested whether IgG shedding from the S. aureus surface over time by ELISA. After an initial 17% decrease in the first minute, we found a further slower decrease resulting in a cumulative loss of 38% of bound IgG over 30 min (P=0.02) (Fig. 1b). These experiments suggested that human IgG is efficiently removed from the S. aureus surface. We then tested IgG release from the S. aureus surface into the surrounding buffer by ELISA (Fig. 1c) and demonstrated an initial shedding of 90 ng ml⁻¹ in the first minute, followed by a slower shedding of up to 262 ng ml⁻¹ by 30 min (P=0.05). Western blot analysis (non-reducing conditions) of IgG shed into the surrounding buffer (Fig. 1d) confirmed the ELISA findings. Thus, increasing amounts of immunoglobulin were shed from the bacterial surface over 30 min in the absence of other serum proteins.

We then compared live S. aureus with gently heat-killed bacteria (70°C for 12 min). Both were coated with IgG, washed and assayed for IgG fragment shedding by Western blotting. Interestingly, when analysed under reducing conditions (Fig. 1e), the IgG shed from live bacteria revealed novel sizes that were not evident for the dead bacteria. These novel bands were not present for control blots stained with secondary antibody alone. Some of these IgG-containing bands, at approximately 90 and 130 kDa, were larger than the IgG heavy chain. Additional fragments of approximately 18 and 20 kDa were also identified that were smaller than the IgG light chain. IgG fragments shed from the heat-killed bacteria did not include these novel fragment sizes. These findings suggest that, unlike dead S. aureus, live S. aureus can cleave surface-bound IgG into uniquely sized fragments, possibly mediated by staphylococcal proteases.

In order to confirm that the shed 130 kDa band identified in the Western blot contained IgG fragments, we performed MS. The 130 kDa band was excised from total protein-stained SDS-polyacrylamide gels and processed for analysis by liquid chromatography-MS/MS. The peptides identified are shown in Table 1. Nine distinct peptide sequences from IgG were identified, confirming that unique-sized IgG-containing complexes were shed from the S. aureus surface.

**Protease inhibitors block IgG cleavage from S. aureus**

We tested whether IgG shedding from the S. aureus surface could be inhibited by using a mixture of broad-spectrum protease inhibitors. After coating with human IgG and washing, bacteria were incubated in buffer with and without protease inhibitor cocktail. The amount of IgG cleaved and shed into the surrounding buffer, analysed by Western blotting (non-reducing conditions), appeared markedly decreased in the presence of protease inhibitors (Fig. 2a). We then performed ELISA quantification of IgG fragments shed from the S. aureus surface (Fig. 2b), which showed that, by 5 min, the amount of IgG released from the S. aureus surface was...
17-fold greater for the control compared with those treated with protease inhibitors (P<0.01). These findings showed that the shedding of IgG from the S. aureus surface could be blocked by protease inhibitors and suggested that the shedding of IgG is mediated by staphylococcal proteases.

Serine and cysteine protease inhibitors decrease the cleavage of bound IgG

S. aureus proteases belong to three broad categories: metalloproteases, serine proteases and cysteine proteases. After coating with human IgG and washing, S. aureus was
incubated with specific protease inhibitors for each protease group at concentrations recommended by the manufacturer (Roche). Shed IgG fragments were processed under reducing conditions and analysed by Western blotting to identify changes in the different sizes of IgG fragments. Testing of the serine protease inhibitors PMSF (Fig. 3a), Pefabloc (results not shown) and aprotinin (Fig. 3b) showed partial inhibition of IgG shedding for each as the concentrations of the protease inhibitor increased. Testing of the cysteine protease inhibitors E-64 (Fig. 3c) and N-ethylmaleimide (results not shown) also showed partial inhibition of IgG fragment shedding for each as the concentrations of the protease inhibitors increased. Testing of metalloprotease inhibition was performed with EDTA (Fig. 3d) and no inhibition of IgG shedding was demonstrated. These findings suggested that staphylococcal serine proteases or cysteine proteases, or both, may contribute to IgG cleavage from the S. aureus surface.

Blocking IgG cleavage with protease inhibitors increases C3-fragment deposition on S. aureus

To determine whether the cleavage of S. aureus-bound IgG by staphylococcal proteases would affect complement activation and opsonization by C3b/iC3b, we coated bacteria with IgG and then allowed them to incubate in the presence or absence of a protease inhibitor cocktail. After washing, S. aureus was incubated with normal human serum, washed and stripped of bound C3 fragments, which were measured by ELISA. Under both conditions, bound C3 fragments were noted to become saturated at the same value by 120 min. However, at 30 min, a 49.4 % increase in C3-fragment binding was noted for bacteria treated with protease inhibitors compared with buffer control (P<0.03) (Fig. 4). Because normal human serum contains IgG, we repeated these experiments using IgG-depleted serum instead of normal human serum. IgG-coated S. aureus incubated with protease inhibitor cocktail, washed and then incubated with IgG-depleted serum bound 492 ± 99 ng C3 ml⁻¹ compared with binding of 267 ± 40 ng C3 ml⁻¹ with no protease inhibitors. Thus, using protease inhibitors to inhibit the cleavage of IgG resulted in an 84.3 % increase in C3-fragment binding to bacteria compared with buffer control (P=0.04). These

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### Table 1. MS of the ~130 kDa IgG fragment shed from the S. aureus surface

<table>
<thead>
<tr>
<th>Expected molecular mass (Da)</th>
<th>Calculated molecular mass (Da)</th>
<th>Peptide score</th>
<th>Expect score</th>
<th>Peptide sequence*</th>
<th>IgG chain region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1671.5738</td>
<td>1670.8192</td>
<td>102</td>
<td>4 × 10⁻⁷</td>
<td>T.PPVLDSDGSFFLYSK.L</td>
<td>Heavy – C_H3</td>
</tr>
<tr>
<td>2138.6674</td>
<td>2138.0202</td>
<td>110</td>
<td>6.6 × 10⁻⁸</td>
<td>R.TPEVTVVVDVSHEDPEVK.F</td>
<td>Heavy – C_H2</td>
</tr>
<tr>
<td>2543.6954</td>
<td>2543.1241</td>
<td>122</td>
<td>4.7 × 10⁻⁹</td>
<td>K.GFYPSDIAVEWESNGQPENNYK.T</td>
<td>Heavy – C_H3</td>
</tr>
<tr>
<td>3815.5432</td>
<td>3814.8148</td>
<td>76</td>
<td>0.00012</td>
<td>T.KNQVSLTCLVKGYPQDIAYEWSNGQPENNYK.T</td>
<td>Heavy – C_H3</td>
</tr>
<tr>
<td>1794.5738</td>
<td>1793.9676</td>
<td>53</td>
<td>0.034</td>
<td>R.VVSVLTVHQDVLNGKE.E</td>
<td>Heavy – C_H2</td>
</tr>
<tr>
<td>3620.9002</td>
<td>3620.6701</td>
<td>121</td>
<td>3.5 × 10⁻⁹</td>
<td>K.VDNLQLQSNQESVTEQDSKDTYSSTSLTL.A</td>
<td>Light – C region</td>
</tr>
<tr>
<td>1743.1218</td>
<td>1742.8515</td>
<td>73</td>
<td>0.00034</td>
<td>K.YAASSYLSLTPQWK.S</td>
<td>Light – C region</td>
</tr>
<tr>
<td>2139.6274</td>
<td>2138.9678</td>
<td>52</td>
<td>0.048</td>
<td>R.TPEVTVVVDVSHEDPEVK.Q.F</td>
<td>Heavy – C_H2</td>
</tr>
<tr>
<td>2555.8897</td>
<td>2555.1414</td>
<td>67</td>
<td>0.0014</td>
<td>P.ENNYKTTPMLDSDGFFILYK.L</td>
<td>Heavy – C_H3</td>
</tr>
</tbody>
</table>

*The amino acid before and after the dot indicates the amino acid that precedes or follows that of the detected peptide in the normal full sequence of the protein.
findings suggested that cleavage of surface-bound IgG by staphylococcal proteases reduces C3-fragment opsonization by IgG-mediated complement activation.

**Blocking IgG cleavage increases *S. aureus* binding to neutrophils and phagocytosis**

To determine whether the cleavage of IgG on the *S. aureus* surface affects binding to neutrophils and phagocytosis, we performed the following experiments. *S. aureus* was coated with IgG as described above, incubated in the presence or absence of protease inhibitors, washed and incubated with purified human neutrophils. Bacteria were stained with acridine orange and adhered to slides using a cytospin. The number of adherent bacteria per 100 neutrophils was counted by fluorescence microscopy. After subtracting the background non-IgG-mediated binding, treatment with protease inhibitors led to 114 ± 14 bacteria associated per neutrophil compared with binding of 64 ± 22 bacteria with no protease inhibitors. Thus, using protease inhibitors to inhibit IgG cleavage increased *S. aureus* binding to neutrophils by 1.8-fold (*P* = 0.04). In order to better understand the full impact of IgG cleavage on *S. aureus* phagocytosis, we next coated bacteria with IgG as described above, incubated them in the presence or absence of protease inhibitors, and washed and incubated them with IgG-depleted serum (2%). IgG-depleted serum contains active complement components, therefore permitting complement-mediated opsonization to occur, as well as the generation of complement anaphylatoxins (e.g. C5a). To these suspensions, purified human neutrophils were
added. IgG-coated *S. aureus* incubated with protease inhibitors was phagocytosed at a ratio of 78±15 bacteria per 100 neutrophils compared with phagocytosis of 20±5 bacteria per 100 neutrophils with no protease inhibitors. Thus, phagocytosis by neutrophils increased fourfold for IgG-coated bacteria treated with protease inhibitors compared with the control (*P*=0.02). Measuring the number of neutrophils phagocytosing bacteria per 100 neutrophils showed that, for IgG-coated bacteria incubated with protease inhibitors, 41±5 neutrophils phagocytosed bacteria compared with 15±2 with no protease inhibitors. Thus, using protease inhibitors to limit IgG cleavage increased the number of neutrophils phagocytosing bacteria by 2.7-fold compared with the control (*P*=0.01). It is possible that residual protease inhibitors present on addition of serum could contribute to the differences in phagocytosis efficiency. However, such a contribution is likely to be minimal due to the large amount of protein in the serum, which should overwhelm minute amounts of residual protease inhibitors. Overall, these findings suggested that blocking the cleavage of surface-bound IgG using protease inhibitors increased binding to neutrophils and dramatically improved phagocytosis efficiency.

**DISCUSSION**

Previous studies have shown that human immunoglobulins can be cleaved by several bacterial proteases. Fick *et al.* (1985) published a study that showed the IgG proteolytic activity of *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis, whilst Molla *et al.* (1988) demonstrated cleavage of IgG and IgA by proteases from *Serratia marcescens* (Molla *et al.*, 1988). Later, Prokesová *et al.* (1992, 1995) showed that a serine protease from *S. aureus* was able to cleave human IgG, IgM and IgA in solution. Loomes *et al.* (1993) also demonstrated IgG cleavage *in vitro* and *in vivo* by *Proteus mirabilis* proteases. More recently, several studies identified the cysteine proteases IdeS and SpeB from *Streptococcus pyogenes*, which cleave host IgG (von Pawel-Rammingen & Björck, 2003). To our knowledge, the current study is unique in evaluating the shedding of human IgG bound to the *S. aureus* surface.

The experiments in this study showed that significant amounts of IgG are shed from the surface of *S. aureus* over 30 min. As noted above, previous investigators have shown that an *S. aureus* protease can cleave human immunoglobulins in solution; however, our results suggested that staphylococcal proteases are also active in cleaving *S. aureus*-bound IgG. The efficient cleavage of *S. aureus*-bound IgG occurred in the absence of serum proteins and therefore could not have been due to staphylokinase interaction with plasminogen. It is also possible that the protease activity may cleave the *S. aureus* surface components to which the IgG attaches. However, it seems likely that the majority of IgG shedding results from activity towards the IgG itself, given that the IgG is shed in fragments and it has been shown by other investigators that unbound IgG can be cleaved by staphylococcal protease.

Of the classes of protease known to be secreted by *S. aureus*, serine and cysteine protease inhibitors appeared to decrease IgG cleavage. Metalloprotease inhibitor did not appear to have any effect. None of the individual protease inhibitors appeared to inhibit IgG cleavage as efficiently as the broad-spectrum protease inhibitor cocktail. These findings suggest that serine proteases probably play a role in the cleavage of surface-bound IgG and raise the possibility that cysteine proteases may also contribute to this process.

Our most important goal in these studies was to evaluate whether blocking the shedding of *S. aureus*-bound IgG would alter the host defence functions of immunoglobulin. Using a cocktail of protease inhibitors to decrease the cleavage of surface-bound IgG, these studies showed that complement activation and C3b/iC3b deposition was increased, binding to neutrophils was increased and phagocytosis efficiency was increased fourfold. These studies suggest that the shedding of *S. aureus*-bound immunoglobulin inhibits normal immunoglobulin-mediated host defence functions and thus probably contributes to host defence evasion. These studies suggest that inhibition of staphylococcal protease-mediated IgG cleavage could be a viable strategy to improve host control of *S. aureus*.

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