Editor’s Choice

Staphylococcus aureus-induced clotting of plasma is an immune evasion mechanism for persistence within the fibrin network

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Recent work has shown that coagulation and innate immunity are tightly interwoven host responses that help eradicate an invading pathogen. Some bacterial species, including Staphylococcus aureus, secrete pro-coagulant factors that, in turn, can modulate these immune reactions. Such mechanisms may not only protect the micro-organism from a lethal attack, but also promote bacterial proliferation and the establishment of infection. Our data showed that coagulase-positive S. aureus bacteria promoted clotting of plasma which was not seen when a coagulase-deficient mutant strain was used. Furthermore, in vitro studies showed that this ability constituted a mechanism that supported the aggregation, survival and persistence of the micro-organism within the fibrin network. These findings were also confirmed when agglutination and persistence of coagulase-positive S. aureus bacteria at the local focus of infection were studied in a subcutaneous murine infection model. In contrast, the coagulase-deficient S. aureus strain which was not able to induce clotting failed to aggregate and to persist in vivo. In conclusion, our data suggested that coagulase-positive S. aureus have evolved mechanisms that prevent their elimination within a fibrin clot.

INTRODUCTION

The innate immune system is our first line of defence against invading pathogens. Once alerted, it triggers inflammatory and antimicrobial reactions, which in a concert action constitute an important part of our host response, and can prevent otherwise systemic and life-threatening complications. Many invasive micro-organisms try to avoid detection and elimination by these innate immune reactions, and thus they have established strategies that counteract the host defence machinery. In such cases, the pathogen often specifically targets the weakest link in a chain of immune reactions, which then may render the immune response completely ineffective.

Coagulation is one of the first responses to an intruder upon vascular injury. It has recently attracted considerable attention and, because of its central role in intravascular immunity, a new term ‘immunothrombosis’ has been introduced (Engelmann & Massberg, 2013). When activated during infection, coagulation can acquire protective features, trigger pro- and anti-inflammatory reactions, and promote antimicrobial activity. As long as these responses are locally evoked and at an early stage of the infectious process, they are part of the immune response. However, under systemic conditions the pathological activation of coagulation is associated with high morbidity and mortality (Angus & van der Poll, 2013).

Abbreviations: FPA, fibrinopeptide A; HE, haematoxylin/eosin.
One supplementary figure is available with the online Supplementary Material.
Many clinical *Staphylococcus aureus* isolates secrete prothrombin-activating factors (also known as coagulases) and their interaction with prothrombin has been studied intensively. These investigations have shown that coagulases have an important role in the pathogenicity of this micro-organism (for a review, see McAdow et al., 2012). In addition, coagulases have also been reported to have immunoprotective functions. Cheng et al. (2010), for instance, noted that two coagulases of *S. aureus*, namely Coa (coagulase) and vWb (von Willebrand factor binding protein), promoted abscess formation which allowed the bacteria to reside and proliferate without being attacked by the host immune system. *S. aureus* was found to be surrounded by a fibrin-containing pseudocapsule (Cheng et al., 2010), which could also act as a mechanical barrier against neutrophils as demonstrated in a three-dimensional *in vitro* infection model (Guggenberger et al., 2012).

We recently reported that activation of the coagulation cascade led to an immobilization of *Streptococcus pyogenes* within a formed clot which was followed by their subsequent killing (Loof et al., 2011). Here, we report that *S. aureus* could circumvent this immune attack by releasing coagulase. Our data further showed that the staphylococcal-induced pro-coagulant activity enabled the microorganism to persist inside a fibrin clot and cause systemic complications.

**METHODS**

**Bacterial strains and culture conditions.** The *S. aureus* strains used in this study were 8325-4 (Novick, 1967) and its derivative SH1000 with a restored rsbU+ gene which activates sigma factor B (σB) (Horsburgh et al., 2002). The *S. aureus* mutant strain lacking coagulase (ΔCoa) was generated in the 8325-4 background as described previously (Phonimdaeng et al., 1990; McDevitt et al., 1992). *S. aureus* RN6390 is a laboratory strain related to 8325-4 with a truncated non-functional rsbU gene (Peng et al., 1988; Goerke et al., 2001). ALC1001 is a σB-deficient mutant strain as described previously (Cheung et al., 1999; Haslinger-Löffler et al., 2005). Bacteria were grown overnight at 37 °C in brain heart infusion (BHI; Becton Dickinson) with shaking (120 r.p.m.); the coagulase-deficient and σB-deficient mutant strains were grown in the presence of 3 μg tetracycline ml⁻¹ and 5 μg erythromycin ml⁻¹, respectively. For animal infection experiments, mid-exponential-phase bacteria were harvested from a subculture, washed three times with sterile PBS and the bacterial concentration was adjusted to 10⁶ c.f.u. ml⁻¹. Aliquots of *S. aureus* suspension were frozen at ~80 °C for further use.

**Mice.** CBA/CaOlAthsd mice were purchased from Harlan. Mice were housed in a pathogen-free animal facility at the Helmholtz Centre for Infection Research and maintained under standard conditions according to institutional guidelines. All experiments were approved by the appropriate ethical committee for animal experimentation (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany; permit 33.9-42502-04-10/0296).

**Determination of thrombin activity at the bacterial surface.** *S. aureus* were grown overnight as described above, washed with 12.9 mM sodium citrate and resuspended in the same buffer. *S. aureus* (2 × 10⁵ c.f.u.) were then mixed with 100 μl citrated normal human plasma (purchased from Skåne University Hospital, Lund, Sweden) or plasma prekallikrein-deficient human plasma (George King Bio-Med) and incubated at 37 °C. The tetrapeptide H-Gly-Pro-Arg-Pro-NH₂ (H-1998; Bachem) was added at a final concentration of 15 mg ml⁻¹ to prevent clotting. Bacteria incubated in sodium citrate buffer served as a negative control. At indicated time points, samples were washed once with sodium citrate and bacterial pellets were resuspended in 100 μl chromogenic substrate S-2238 (Chromogenix). After incubation for 1 h at 37 °C, samples were centrifuged, supernatants collected and A₄0₅ was measured.

**Fibrinopeptide A (FPA) release in coagulase-activated plasma.** Bacteria were grown overnight as described above and 5 × 10⁶ c.f.u. *S. aureus* in 12.9 mM sodium citrate buffer were mixed 1:1 with normal human plasma. The tetrapeptide H-1998 was added at a final concentration of 1.5 mg ml⁻¹ to prevent clotting and samples were incubated at 37 °C. Aliquots of 10 μl of each sample were removed at the indicated time points and, after a centrifugation step, supernatants were diluted 1:200 in sample diluent. The release of FPA was measured using a zymo-test FPA-kit (Hyphen Bio-Med) according to the manufacturer’s instructions.

**Clot induction and measurement of bacterial uptake.** For measurement of bacteria-induced clot formation, 5 × 10⁶ c.f.u. *S. aureus* in 12.9 mM sodium citrate buffer were mixed 1:1 with normal human plasma and incubated at 37 °C. Samples were monitored for clot formation and at the indicated time points aliquots of 50 μl supernatant were plated onto blood agar in 10-fold serial dilutions. The number of bacteria was determined by counting colonies after 18 h incubation. For scanning electron microscopy analysis, clots were fixed after 6 h incubation.

**Generation of thrombin-induced clots.** Bacteria were grown overnight as described above and 5 × 10⁶ c.f.u. *S. aureus* in 100 μl sodium citrate buffer were mixed with 100 μl normal human plasma. Haemoclot thrombin reagent (50 μl; Hyphen Bio-Med) was added to induce clotting. Samples were then fixed and prepared for scanning electron microscopy analysis.

**Scanning electron microscopy.** Specimens were fixed in 2.5% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.2), prepared for electron microscopic analysis and analysed with a Zeiss DSM 960A electron microscope as described previously (Nordin et al., 2013).

**Mouse infection model and histological examination of skin samples.** Mice were subcutaneously infected with 5 × 10⁶ c.f.u. *S. aureus* 8325-4 WT or the coagulase-deficient mutant in 100 μl PBS. After 48 h infection, mice were killed by CO₂ inhalation. Skin samples from the local focus of infection were surgically removed, fixed in 4% neutrally buffered formaldehyde and embedded in paraffin according to standard histological procedures. Sections (3 μm thick) were stained with haematoxylin/eosin (HE) and evaluated by light microscopy. Gram staining was performed according to the modified Brown–Brenn method (Bancroft & Gamble, 2002). In some experiments, skin samples and kidneys were removed 48 h after bacterial inoculation and homogenates were plated onto blood agar or BHI agar in 10-fold serial dilutions. The bacterial loads in skin and kidneys were determined by counting colonies after 18 h incubation. Samples obtained from animals infected with the coagulase-deficient mutant strain were plated onto BHI agar containing 3 μg tetracycline ml⁻¹.

**Statistical analysis.** Data were analysed using Prism 5 (GraphPad). Statistical significance was determined by using the unpaired Student’s *t*-test for the comparison of two groups; otherwise, ANOVA followed by Tukey’s test was used. In all analyses, *P*<0.05 was considered statistically significant.
RESULTS

Increased thrombin activity at the surface of coagulase-positive S. aureus triggers the release of FPA

In order to decipher the molecular mechanisms used by coagulase-positive S. aureus to evoke pro-coagulant activity, two S. aureus WT strains (SH1000 and 8325-4 WT) and one coagulase-deficient strain (8325-4 ΔCoa) were incubated with human plasma. After a washing step to remove unbound plasma proteins, bacteria were resuspended with a thrombin-specific chromogenic substrate and thrombin activity was monitored. Fig. 1(a) shows that already after 30 min, increased activity of the coagulation factor was seen at the surface of all three strains when incubated with normal, but not with plasma prekallikrein-deficient plasma. These findings suggested that the thrombin activity measured was caused by an activation of the contact system which occurred within minutes upon incubation of S. aureus with human plasma (Mattsson et al., 2001). Longer incubation of coagulase-deficient bacteria in normal human plasma did not promote further cleavage of the substrate, whereas a significant increase was noted when WT strains SH1000 or 8325-4 WT were used (Fig. 1b). Similar findings were recorded when S. aureus RN6390 or the σB-deficient mutant strain ALC1001 were used, implying that the effect seen was not dependent on σB (Fig. S1, available in the online Supplementary Material). Together, these findings suggested that most thrombin activity seen at the surface of WT bacteria was triggered by coagulase, whilst contact activation contributed only to a minor extent. To study whether the increase in thrombin activity had an impact on the ability of the bacteria to convert fibrinogen to fibrin, the generation of FPA was measured. To this end, bacteria and plasma were mixed, and the release of FPA was followed by indirect ELISA. Fig. 1(c) shows that significant levels of the peptide were generated when WT, but not coagulase-deficient bacteria were mixed with plasma, suggesting that fibrinogen conversion was dependent on coagulase and not under control of the contact system.

Coagulase-induced clotting of plasma promotes uptake of S. aureus into the fibrin network

We next investigated whether the rise in thrombin activity at the bacterial surface led to increased pro-coagulative activity. S. aureus were incubated with human plasma and clot formation was monitored. Fig. 2(a) shows that an incubation time of 3–4 h was sufficient for S. aureus WT strains SH1000 and 8325-4 WT to form a fibrin network, and similar clotting times were observed with RN6390 and ALC1001 (data not shown). This was in contrast to the coagulase-deficient S. aureus mutant strain 8325-4 ΔCoa, where no clot formation was detected even after longer incubation times (>36 h) (Fig. 2a).

Based on these findings, we wished to study whether fibrin clot generation could affect the number of bacteria in the supernatants. All three strains were therefore incubated with human plasma, and after different time points (0, 1.5, 3, 4.5 and 6 h) aliquots of the supernatants were collected and plated onto blood agar. We found that bacterial loads in the supernatants were significantly decreased when the two WT strains were mixed with human plasma followed by clot induction (Fig. 2b, c). However, this was not seen when the coagulase-deficient S. aureus strain was used (Fig. 2d). As control, all three strains were incubated with sodium citrate buffer instead of plasma. This treatment did not cause a decrease in the amount of bacteria (data not shown) and it suggested that the reduction of bacterial numbers in the supernatant was evoked by clot formation.

Fig. 1. Coagulase-dependent thrombin activity on the bacterial surface and FPA release. (a, b) S. aureus WT strains (SH1000 and 8325-4 WT) and the coagulase-deficient mutant (8325-4 ΔCoa) were incubated with normal or plasma prekallikrein (PK)-deficient human plasma. Samples were washed at the indicated time points and the chromogenic substrate S-2238 was added to measure thrombin activity. (c) Bacteria were incubated with normal human plasma and FPA release was determined in the supernatant at the indicated time points. Both graphs represent the mean ± SD obtained from three to five independent experiments. *P<0.05, **P<0.01, ***P<0.001.
This observation prompted us to study the fate of the bacteria inside the clot by scanning electron microscopy. For this purpose, all three S. aureus strains (SH1000, 8325-4 WT and 8325-4 ΔCoa) were grown overnight and incubated in a 1:1 ratio with human plasma for 6 h. As control, bacteria and plasma were mixed, and clotting was induced by the addition of thrombin. The resulting clots were then fixed and prepared for scanning electron microscopy. Fig. 3(a, c) depicts clots induced by SH1000 and 8325-4 WT in the absence of thrombin. It appeared that clot morphology did not significantly differ from the respective thrombin-induced clots (Fig. 3b, d), although the fibrin fibres tended to be smaller in diameter and the bacterial surface was covered with plasma proteins in coagulase-induced clots. When precipitates from the coagulase-deficient mutant strain were analysed after incubation with plasma in the absence of thrombin, no fibrin formation was detected (Fig. 3e), whilst thrombin-induced clots were almost indistinguishable from thrombin-induced clots in the presence of WT bacteria (Fig. 3f). These results showed that coagulase-deficient S. aureus could form small aggregates, but the bacteria failed to cover themselves with a fibrin network.

**Coagulase is required for persistence of S. aureus at the site of infection**

In the next series of experiments we wished to decipher the role of bacterial aggregation and clot induction *in vivo*. CBA mice were subcutaneously infected with 5 × 10^6 c.f.u. S. aureus 8325-4 (WT and ΔCoa). Skin biopsies were obtained from the site of infection 48 h after bacterial inoculation and examined histologically. Skin samples obtained from non-infected animals served as controls. As expected, tissue samples from non-infected mice failed to show any signs of inflammation after HE (Fig. 4a) or Gram staining (Fig. 4b), whilst animals challenged with S. aureus suffered from necrotic dermatitis (Fig. 4c–h). We noted, however, that when animals were infected with the S. aureus WT strain, bacteria were mostly detected in defined and large aggregates, as shown after HE (Fig. 4c, arrows) and Gram staining (Fig. 4d, e, arrows). When the coagulase-deficient S. aureus mutant strain was used, however, no aggregate formation was recorded (Fig. 4f, g) and only a few bacteria were found at the site of infection (Fig. 4h, arrowheads). Together, these results suggested that coagulase was involved in the encapsulation of the bacteria at the infected site.
In a second approach, we studied the role of coagulase in the persistence of bacteria at the infected site and their dissemination to other organs. To this end, mice were infected with *S. aureus* 8325-4 WT or coagulase-deficient bacteria. Skin samples from the site of infection and kidneys were homogenized 48 h after bacterial inoculation, and the c.f.u. count in these samples was determined. As shown in Fig. 5(a, b), when switching from the WT to the coagulase-deficient mutant strain, >100 times fewer bacteria were recovered from skin and kidneys. These data implied that coagulase was a virulence factor that not only allowed proliferation at the site of infection, but also the systemic spread of the bacteria.

**DISCUSSION**

Following the first description of an isolated microorganism, namely *Bacillus anthracis* by Robert Koch in 1876 (Blevins & Bronze, 2010), it took only a little longer than 30 years to discover that *S. aureus* releases procoagulant substances (Much, 1908). This knowledge was then later used in the 1940s to screen for staphylococcal infections in patients (Field & Smith, 1945) and today is still an important parameter used to classify clinical *S. aureus* strains. These reports exemplify that coagulases have been the subject of investigation from the very beginning of the history of modern microbiology and underline their important role in staphylococcal pathogenesis. Coagulases promote their pro-coagulant activity by targeting thrombin and converting the coagulation factor to its active form. Unlike the conversion of thrombin to thrombin by coagulation factor Xa, activation by coagulases does not require proteolytic processing of prothrombin (Friedrich et al., 2003). It is worth noting that a similar mechanism has been described for plasminogen activation by staphylokinase.
cations. This conclusion is in line with findings by Vanassche et al. (2011) showing that coagulase contributes to the development of skin abscesses after subcutaneous S. aureus injection. Mice inoculated with a coagulase-deficient S. aureus mutant strain or treated with a coagulase inhibitor developed smaller abscesses than animals infected with the S. aureus WT strain (Vanassche et al., 2011). Furthermore, it has been shown in other S. aureus infection models that this pathogen has the ability to persist in host tissues and at the site of infection in vivo (Cheng et al., 2010; Nippe et al., 2011). In addition, here we report for the first time, to the best of our knowledge, that subcutaneous injection of S. aureus lacking coagulase significantly reduces the persistence and dissemination of bacteria in a murine infection model. Our data further show that coagulase-deficient bacteria fail to survive in the skin of infected mice and already 48 h after bacterial inoculation they are more efficiently cleared by the immune system than WT bacteria which tend to persist at the site of infection. Consequently, we also find significantly lower amounts of coagulase-deficient S. aureus bacteria in the kidneys of the infected animals compared with kidneys of mice infected with the WT strain. These results reveal that S. aureus coagulase is required for persistence of the pathogen and establishment of a systemic infection. To conclude, here we show that S. aureus is able to take advantage of a host defence system to undergo an immune response and promote its proliferation.

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