Group G streptococci mediate fibrinogen-dependent platelet aggregation leading to transient entrapment in platelet aggregates

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Platelets have been reported to become activated in response to bacteria and this is proposed to contribute to the acute response to bacterial infection. In the present study, we investigated platelet aggregation in response to group G streptococci (GGS) in vitro in healthy human donors and in vivo in a mouse model of streptococcal sepsis. Platelet aggregation by GGS was dependent on the bacterial surface protein FOG and engagement of the platelet fibrinogen receptor; however, it was independent of IgG and the platelet Fc receptor. Platelets exerted no antibacterial effects on the bacteria, and aggregates formed were markedly unstable, allowing bacteria to rapidly return to the plasma and grow post-aggregation. Thrombocytopenia and platelet activation occurred during invasive infection with GGS, and platelets were demonstrated to contribute to bacterial dissemination during infection. These findings reveal an important role for bacteria–platelet interactions during the pathogenesis of streptococcal infection.

INTRODUCTION

The family of Gram-positive streptococci can be defined according to variations in the carbohydrate antigen on the surface of the bacteria (Lancefield, 1933). Group A streptococci (GAS) are strict human pathogens that have been extensively investigated for their interactions with and manipulation of the human host (Cunningham, 2000; Carapetis et al., 2005). Group B streptococci (GBS) are also well-appreciated human pathogens, and studies have been conducted focusing on neonatal infections (Heath & Jardine, 2014; Bekker et al., 2014). Group G and group C streptococci (GGS and GCS) are associated with other animal species and animal infections, and have previously been considered to rarely cause human infection. This has resulted in a relative paucity of studies focussed on interactions between these species and the human host. However, both GGS and GCS are now recognized as important emerging human pathogens (Brandt & Spellerberg, 2009; Bergmann & Nitsche-Schmitz, 2015). The repertoire of human infections caused by GGS mimics that of GAS, ranging from mild infection, such as pharyngitis, to severe infection, such as sepsis and necrotizing fasciitis. As the incidence of GGS infections increases, it is important to elucidate the interactions between GGS and the human host.

Two main proteins found on the surface of GGS, protein G and FOG, have been the primary focus of studies regarding bacterial interactions with the human host (Johansson et al., 2004; Nitsche-Schmitz et al., 2007; Egesten et al., 2011). Protein G is the main IgG-binding protein of GGS and exhibits high affinity for IgG from different species (Björck & Kronvall, 1984). FOG is the main fibrinogen-binding protein of GGS, a member of the M protein family and an important virulence factor (Johansson et al., 2004). The ability of bacteria to bind fibrinogen and bacteria-specific IgG, and the subsequent engagement of their counter receptors on the platelet surface, results in platelet activation in response to distinct bacterial species (Kerrigan & Cox, 2010; Arman & Krauel, 2015).

Platelets isolated from patients presenting with Gram-positive bacteraemia can activate platelets from the infected individual, ex vivo, indicating that bacteria-mediated platelet activation may occur during bloodstream infection and sepsis (Johansson et al., 2011). Furthermore, platelets may play a significant role in the pathogenesis and prognosis of sepsis (Levi, 2005; Winning et al., 2009; Sharron et al., 2012). Therefore, in this study we investigated the interaction between platelets and GGS, with particular emphasis on the role of the fibrinogen- and IgG-binding surface proteins, FOG and protein G. Platelet aggregation and disaggregation in response to GGS were described in vitro in healthy human donors. Furthermore, platelet activation occurred in vivo in a mouse model of invasive GGS.
infection and platelets contributed to bacterial dissemination in the same model.

**METHODS**

**Bacterial strains and growth conditions.** GGS wild-type strain G45 was isolated at the Royal Brisbane Hospital in Brisbane, Australia. Two isogenic mutant strains G45ΔPG and G45ΔFOG (Nitsche-Schmitz et al., 2007), lacking protein G or protein FOG, respectively, were also used. Bacteria were grown overnight in Todd–HeWitt broth (Difco/BDF) supplemented with 0.2 % yeast extract (Oxoid) (THY) at 37 °C, in the presence of 5 % CO2. For both mutant strains erythromycin (25 μg ml–1) was added to the culture media. Overnight cultures were inoculated in fresh THY medium, grown to OD620 0.4 and subsequently used in experiments.

**Bacterial proteins.** Recombinant protein FOG was purified as previously described (Johansson et al., 2004). A Coomassie stained SDS-PAGE gel of the purified protein FOG is shown in Fig. S1 (available in the online Supplementary Material). The mature protein G (aa 34–562) was cloned and expressed in E. coli using the GST (glutathione S-transferase) gene fusion system (Amersham Biosciences). The protein was amplified by PCR using the 5′ primer 5′-GCAAGATCCGTT GACTCCAACTCGAAGA-3′, containing a BamHI site, and the 3′ reverse primer, 5′-ATAGTCGACTTAGCTTTAGATGGTTTGG-3′, containing a SalI site. Following digestion with the indicated restriction enzymes, the PCR product was cloned into the pGEX-6P-1 vector as described for recombinant expression of FOG (Johansson et al., 2004). The protein was analysed for purity by SDS-PAGE (10 % gel) (Fig. S1) and the protein concentration was determined by using the Coomassie (Bradford) protein assay reagent (Thermo Scientific).

**Preparation of platelet-rich plasma (PRP) and platelet-poor plasma (PPP).** Human platelets were obtained from healthy donors who had not taken any anti-platelet medication for at least 10 days prior to the experiments. The regional Ethical Review Board in Lund approved the recruitment of healthy blood donors to the study (reference no. 657/2008). PRP was prepared by centrifugation for 15 min at 150 g. The PRP was transferred to fresh tubes and the remaining blood was centrifuged again for 10 min at 2000 g to yield PPP.

**Platelet aggregation in PRP.** A platelet aggregometer (Chrono–Log 490; Chrono–Log) was used to assess platelet aggregation in response to GGS strains G45, G45ΔPG and G45ΔFOG, as well as purified FOG and protein G. PPP was used in the reference well to set the baseline for transmission at 100 %. PRP was added to the sample wells and the change in light transmission is representative of platelet aggregation. Twenty microlitres of washed bacteria (1 × 109 c.f.u. ml–1) was added to 450 μl PRP. Collagen I (Chrono–Log) was used as a positive control, at a final concentration of 0.005 mg ml–1. Purified protein G and FOG were titrated to final concentrations of 0.0055–0.0165 mg ml–1 and assessed for their ability to induce platelet aggregation (Fig. S1). The aggregation curves were analysed using AggroLink software. To assess the mechanism responsible for GGS-induced platelet aggregation, PRP was pre-incubated with Ides (40 μg ml–1), AT10 (50 μg ml–1) or PGE2 (3.5 μg ml–1) prior to induction of platelet aggregation with GGS G45.

**Release of serotonin from platelet-dense granules.** Platelet aggregation was induced by collagen and GGS strains G45 as described for platelet aggregation. Platelet aggregates were pelleted by centrifugation for 5 min in a microcentrifuge at maximum speed. The supernatant was transferred into new tubes and the release of serotonin was measured using a Serotonin ELISA kit according to the manufacturer’s instructions (Labor Diagnostika Nord).

**Platelet aggregation in whole blood.** Aggregation in whole blood was determined using an impedence aggregometer (Multiplate; Roche), where the increase of impedance between the electrodes is representative of the amount of platelet aggregation occurring. Fresh citrated human blood was tested for aggregation in response to GGS G45. Collagen was used as a positive control of normal platelet function, at a final concentration of 0.0025 mg ml–1. To further test the mechanism by which the bacteria induce aggregation, whole blood was pre-incubated with Ides (40 μg ml–1), AT10 (50 μg ml–1; Serotec) or ReoPro (Abciximab) (10 μg ml–1; Centocor) prior to induction of platelet aggregation with GGS G45.

**Platelet activation in PRP.** To further platelet activation in response to the bacteria, PRP was pre-incubated with AT10 (50 μg ml–1) or ReoPro (10 μg ml–1). Platelet activation was assessed using flow cytometry. Twenty microlitres of PRP was added to 40 μl HEPES buffer, pH 7.4. As a positive control for platelet activation ADP (5 μM; Sigma) was used. For platelet activation, 15 μl washed bacteria (5 × 108 c.f.u. ml–1 in PBS) was added to PRP. After incubation for 15 min at room temperature, antibodies were added and incubated for 10 min prior to fixation with 0.5 % formaldehyde. Anti-CD42a–PerCP (Becton Dickinson) antibody was used as a marker for both resting and activated platelets. CD62P PE was used as marker for activated platelets that have released P-selectin/CD62P from the alpha granules.

**Recovery of bacteria from PRP.** GGS was grown to mid-exponential phase (OD620 ≤ 0.4), washed once with PBS and diluted to 2 × 107 c.f.u. ml–1. Twenty microlitres of bacteria was added to 450 μl PRP in a microcentrifuge tube and incubated at 37 °C with stirring. At h and at 10 min intervals for the first hour, and subsequently at 30 min intervals, 10 μl samples were taken from the plasma, diluted and plated on THY agar plates for viable count determination. All samples were monitored macroscopically for the integrity of the aggregates for the duration of the experiment. The bacterial load in PPP was tested in parallel. Bacterial recovery from aggregates formed in mouse PRP was also investigated; however, pooled blood from five individual animals was used to yield enough PRP for individual experiments and three individual experiments were performed.

**Animal experiments.** BALB/c mice were purchased from Charles River Laboratories. The animals were housed under standard conditions of light and temperature, and fed laboratory chow and water ad libitum. Experiments were carried out when the mice were 9–13 weeks old and all procedures were approved by the local ethics committee (M115/13; Lund University, Sweden). GGS G45 was grown overnight, inoculated into fresh THY broth and grown to mid-exponential phase (OD620 ≤ 0.4). Bacteria were washed with PBS, and mice were infected by intraperitoneal injection with bacteria (2 × 107 c.f.u. per animal). Blood from healthy and infected animals was harvested at 24 h post-infection. Platelet function was assessed by determination of platelet count using flow cytometry. The remaining blood was centrifuged at maximum speed for 5 min in a microcentrifuge to yield PPP. CD62P P-selectin levels in plasma were subsequently determined with a CD62 P ELISA kit (Abcam) as a secondary marker of platelet activation in vivo during infection.

In order to determine the role of platelet activation for pathogenesis of infection, mice were rendered thrombocytopenic. A total of 4 μg purified hamster anti-mouse anti-CD42b antibody (Emefret Technologies) was administrated by intraperitoneal injection 24 h prior to infection. This diminished the circulating platelet count to 20–30 % of normal within 12 h and the platelet count remained low for 3 days (Fig. S2). In order to determine bacterial dissemination from the local site of administration, two groups of animals (controls and platelet-depleted) were sacrificed 24 h after initiation of infection. The kidney was removed and homogenized in 5 ml sterile Dulbecco's PBS, and
Fig. 1. GGS G45 induces platelet aggregation and escapes entrapment. (a) Platelet aggregation in PRP was determined in a platelet aggregometer on addition of GGS G45. The percentage change in light transmission of the PRP indicates platelet aggregation (right axis). Lag time to aggregation is defined as the time in minutes between addition of agonist and onset of aggregation (left axis). (b) Release of serotonin from the granules of platelets was also determined in PRP on addition of buffer (unactivated), collagen (**P=0.0021) and GGS G45 (*P=0.017). (d) Platelet aggregation and disaggregation was determined in PRP as OD450 measurements at 0, 10 and 60 min post-addition of collagen and GGS G45. For collagen, a significant decrease in OD450 occurred at 10 min (****P<0.0001) and 60 min (**P<0.0001). For GGS G45, a significant decrease in OD450 nm occurred at 10 min (****P<0.0001); however, after 60 min there was no significant decrease (ns) compared with the initial time point. (c, e) In parallel experiments, the bacterial load of the plasma in PRP (c) or PPP (e) was determined by the plating of 10 μl samples over time. Data are shown as the mean ± SD for two independent experiments, each in three donors (n=6). COL, Collagen.
bacterial load was determined by viable count determination on THY plates incubated overnight. Prior to homogenization the kidneys were weighed and the bacterial count was adjusted to the weight.

**Statistical analysis.** Statistical analyses were performed using Prism 6. Significance was determined using the Mann–Whitney test and/or Student’s t-test.

**RESULTS**

**GGS induce platelet aggregation and escape entrapment within the aggregates**

GGS G45 was added to PRP from healthy human donors and aggregation was determined using the Chrono-Log 490 aggregometer. G45 stimulated platelet aggregation with a lag time of 8 ± 2 min and a maximum aggregation of 80% (Fig. 1a). Pretreatment of PRP with the biochemical platelet inhibitor PGE₁ abolished aggregation in response to GGS G45 (data not shown), confirming that platelet activation is a pre-requisite for aggregation to occur. Furthermore, the bacteria generated release of serotonin from the platelet granules, at levels equivalent to collagen-activated platelets (Fig. 1b). Viable count determination was used to monitor bacterial recovery from the supernatant of PRP. Platelet aggregation coincided with a decrease in bacterial recovery from the supernatant of PRP at 10 min (Fig. 1c). After 20 min the bacterial load in PRP increased (Fig. 1c) and this coincided with a macroscopic disaggregation of the previously formed aggregates. The bacteria grew during the remaining 2 h to generate a 204% increase in bacterial load (Fig. 1c).

![Fig. 2](https://www.microbiologyresearch.org/fig2.png)

Fig. 2. Platelet aggregation by GGS G45 in PRP is dependent on fibrinogen binding and protein FOG. Platelet aggregation of PRP was determined in a platelet aggregometer. The percentage change in light transmission of the PRP indicates platelet aggregation (right axis). Lag time to aggregation is defined as the time in minutes between addition of agonist and onset of aggregation, and after 25 min the experiment was terminated as a failure to aggregate (left axis). (a) Platelet aggregation occurred in response to GGS G45ΔPG (black bars) or purified protein FOG (0.0055 mg ml⁻¹, white bars), but failed to occur in response to GGS G45ΔFOG (striped bar) or purified protein G (0.0055 mg ml⁻¹, grey bar). (b, c) Platelet aggregation in response to GGS G45 (black bars) or G45ΔPG (grey bars) was determined in PRP pretreated with mAbs against platelet Fc-receptor (b), or with the IgG protease IdeS (c). Data are shown as the mean ± SD for two independent experiments, each in three donors (n=6).
aggregates formed in response to collagen were stable over time and the OD$_{50}$ remained low after 60 min (Fig. 1d). Disaggregation of platelet aggregates, and the subsequent recovery of bacteria, was a platelet-dependent event, since incubation of GGS G45 in PPP did not result in a decrease in bacterial load after 10 min (Fig. 1e). The bacterial load in PPP remained stable for the 60 min, and after this time the bacteria grew in this plasma environment to yield a 300% increase in bacterial load (Fig. 1e).

**Platelet aggregation by GGS is dependent on protein FOG**

The mechanism by which GGS G45 induced platelet aggregation was investigated further. To this end, the isogenic mutants G45ΔPG and G45ΔFOG, and purified protein G (0.0055 mg ml$^{-1}$) or FOG (0.0055 mg ml$^{-1}$), were utilized in the aggregation assays. G45ΔFOG and purified protein G failed to induce platelet aggregation, whereas both G45ΔPG and purified protein FOG induced platelet aggregation (Fig. 2a). A dose-dependent ability to stimulate platelet aggregation was observed when multiple concentrations of FOG were used (Fig. S1). Samples were run for a maximum of 25 min or until a baseline was achieved. Samples that failed to mediate platelet aggregation within 25 min were defined as negative. Pre-incubation of PRP with AT10 to block the platelet IgG receptor, Fc$\gamma$RIIA (CD32) or cleavage of plasma IgG with IdeS (Johansson et al., 2008) revealed that platelet aggregation in response to GGS G45 is not significantly affected by complementary strategies of IgG blockade (Fig. 2b, c). We conclude that platelet aggregation in response to GGS G45 can occur in...
the absence of IgG. As a positive control for IgG cleavage with IdeS, a GAS strain that had previously been demonstrated to mediate IgG-dependent platelet aggregation was studied in parallel (Svensson et al., 2014). Platelet aggregation was completely abolished by pretreatment of Streptococcus pyogenes BB5 with IdeS (a representative experiment of six performed is shown in Fig. S3), in agreement with previously published findings (Svensson et al., 2014).

Platelet adhesion and aggregation is mediated by GGS in whole blood

Platelet adhesion and aggregation were studied in human whole blood using an impedance aggregometer. Light transmission aggregometry reflects platelet–platelet aggregation and cross-linking in solution, while impedance aggregometry involves adhesion and aggregation of platelets on the surface of electrodes. GGS G45 induced aggregation in human whole blood at equivalent levels to that of the positive control, collagen (Fig. 3a). Platelet aggregation in response to GGS G45 was significantly decreased to background levels after pre-incubation of blood with ReoPro for 30 min (Fig. 3b), while aggregation by the platelet agonist collagen was retained. ReoPro is a mAb that binds to GPIIb/IIIa and irreversibly prevents fibrinogen binding on the platelet surface (Coller, 1995). Furthermore, pre-incubation of whole blood with IdeS and AT10 failed to diminish platelet aggregation significantly in response to GGS G45 (Fig. 3b). Platelet activation in PRP was assessed using flow cytometry for P-selectin presentation at the platelet surface after pre-incubation with buffer, AT10 or ReoPro and subsequent addition of GGS G45 or ADP. Platelet activation occurred in response to both ADP and GGS G45. ADP activation was retained in the presence of both platelet receptor blockers, while activation induced by GGS G45 was inhibited by ReoPro but not significantly affected by pre-incubation with AT10 (Fig. 3c).

GGS stimulate aggregation of murine platelets and transient entrapment of bacteria occurs

In order to further investigate the role of bacteria-mediated platelet aggregation during infection, we sought to establish a mouse model of infection. We initially investigated platelet aggregation in blood and PRP from healthy mice exposed to GGS G45 ex vivo. In a pilot experiment, a

![Fig. 4. GGS G45 induces aggregation of murine platelets and escapes entrapment. (a) Platelet aggregation in whole blood was determined in a Multiplate analyser on addition of GGS G45 or collagen (0.0005 mg ml⁻¹), and the AUC was calculated. Each symbol represents an individual mouse. (b) Platelet aggregation of PRP was determined in a platelet aggregometer on addition of GGS G45. The percentage change in light transmission of the PRP indicates platelet aggregation (right axis). Lag time to aggregation is defined as the time in minutes between addition of agonist and onset of aggregation (left axis). (c) In parallel experiments, the bacterial load of the plasma in PRP was determined by the plating of 10 μl samples at 10 min intervals for the first hour and subsequently at 30 min intervals. PRP was prepared from pooled whole blood from five mice in three independent experiments. COL, Collagen.](image-url)
bacterial dose of GGS G45 stimulated aggregation of mouse platelets at levels equivalent to the positive control, collagen (Fig. 4a). Inter-individual variability was higher for mouse samples as compared with human donors (Fig. 3a) and this likely reflects the inherent variation in coagulation status of terminal blood samples from mice. The inter-individual variation was similar in the paired collagen and GGS G45 treated samples.

GGS G45 mediated aggregation of mouse platelets in pooled PRP with a lag time of 8 min and a maximum aggregation of 60 % (Fig. 4b). In parallel experiments, the bacterial load in PRP was determined. After 20 min, reduced numbers of bacteria were recovered from the supernatant of the PRP, indicating that bacteria were entrapped (Fig. 4c). After 40 and 60 min the numbers of bacteria in the supernatant increased once again (Fig. 4c), and this coincided with a macroscopic disaggregation of the previously formed aggregates.

Platelets are activated during infection and facilitate bacterial dissemination

Female BALB/c mice were infected by the intraperitoneal route with GGS G45. In a pilot experiment using four different bacterial doses, it was determined that $2 \times 10^7$ c.f.u. per animal generated significant weight loss and bacterial dissemination to the organs within 24 h. Bacteria were detected in the blood, liver and spleen of infected animals; however, the highest bacterial load was detected in the kidney. All subsequent experiments were performed with $2 \times 10^7$ c.f.u. per animal. After 24 h, the animals were sacrificed, and citrated blood samples were analysed for platelet count and platelet activation. The kidneys were also harvested and bacterial load was determined by viable count determination (d). Horizontal lines represent the median c.f.u. (g organ)$^{-1}$ for 10 mice per group; the Mann–Whitney test was applied (**P=0.0004).

Fig. 5. Platelet activation occurs during murine infection with GGS G45 and platelets contribute to bacterial dissemination. (a, b) Mice were infected with GGS G45 by the intraperitoneal route and blood samples were taken at 24 h post-infection. The platelet count (a) and plasma P-selectin levels (b) were determined. Healthy untreated mice served as controls. Data are expressed as mean ± so for 15 mice (**P=0.0023, ***P=0.0007). (c, d) In order to further assess the role of platelets during infection with GGS G45, one group of animals were rendered thrombocytopenic with an anti-CD42b antibody prior to infection (treated) and compared to control infected animals (controls). The animals were weighed prior to infection and at 24 h post-infection, and the weight loss was determined (c). At 24 h post-infection, the kidneys were harvested and the bacterial load was determined by viable count determination (d). Horizontal lines represent the median c.f.u. (g organ)$^{-1}$ for 10 mice per group; the Mann–Whitney test was applied (**P=0.0004).
CD62P is primarily derived from the alpha granules of activated platelets; therefore, we investigated CD62P levels in plasma as a secondary indicator of platelet activation in vivo. Increased levels of CD62P were detected in infected animals as compared with healthy controls, indicating that platelet activation occurred during GGS infection (Fig. 5b).

Since platelet activation occurred during infection, we decided to investigate the role of platelets during the initial pathogenesis of this infection by rendering animals thrombocytopenic prior to initiation of infection. There was no significant difference in weight loss between the two groups, although control mice exhibited a slightly enhanced weight loss (Fig. 5c). Bacterial dissemination from the local site of administration to the kidneys was significantly increased in control animals as compared to animals rendered thrombocytopenic prior to infection (Fig. 5d). This demonstrates that platelets and platelet activation contribute to the dissemination of GGS during invasive infection.

**DISCUSSION**

Platelets become activated and aggregate in response to diverse inflammatory stimuli, including bacterial pathogens; however, the consequences of these interactions for bacterial pathogenesis have not been elucidated. Herein, we describe a novel mechanism of platelet aggregation by GGS that is independent of plasma IgG and the platelet Fc-receptor and dependent on acquisition of fibrinogen by the bacterial surface protein, FOG, and engagement of the platelet receptor for fibrinogen. Furthermore, we have demonstrated that platelet activation occurs in vivo in GGS-infected animals and determined that platelets contribute to bacterial dissemination during the pathogenesis of invasive GGS infection.

GAS have previously been demonstrated to mediate platelet activation and aggregation that is dependent on both fibrinogen and IgG bridging between bacterial surface proteins and platelet receptors (Svensson et al., 2014). Interestingly, despite the high homology between GGS and GAS (Watanabe et al., 2013; Takahashi et al., 2010), we report that GGS utilize a distinct mechanism to mediate platelet aggregation, whereby acquisition of fibrinogen alone is sufficient. We demonstrate that the platelet receptor for IgG is not required since platelet activation and aggregation occur despite blockade of this receptor in human blood, and in the absence of this receptor in mouse blood. Furthermore, cleavage and inactivation of plasma IgG with IdeS do not significantly diminish platelet aggregation in response to the bacteria. This confirms that although closely related, these streptococcal species are distinct pathogens that have evolved different mechanisms to interact with the same host targets. This may to some extent influence the pathogenesis of infection or treatment strategies and is an important factor to consider. Our results confirm previous reports that have established FOG as a significant virulence factor for GGS (Johansson et al., 2004; Nitsche-Schmitz et al., 2007; Wollein Waldetoft et al., 2012), and adds platelets to the repertoire of interacting partners for FOG at the host–pathogen interface.

It is particularly intriguing that GGS do not require IgG to mediate platelet aggregation. Aggregation occurs after a lag time of 8 min, which is longer than the lag time of <2 min reported for GAS (Svensson et al., 2014). This may reflect a more efficient activation mediated by GAS due to the presence of both a primary fibrinogen-mediated and a secondary IgG-mediated interaction. The non-requirement of IgG may reflect distinct interactions of GGS with IgG. Protein G has a high affinity for the IgG of several species (Björck & Kronvall, 1984), and it is tempting to speculate that binding of IgG via the Fc region to protein G on the GGS surface leaves it unavailable for binding via the Fab region to other bacterial proteins that would facilitate engagement of the Fc receptor on platelets. This is perhaps unlikely since we failed to observe an enhanced lag time for the isogenic protein G mutant; however, platelet aggregation in response to this isogenic mutant does show a tendency, although not significant, to become decreased in the presence of IgG blockade (Fig. 2b, c). This suggests a partially IgG-dependent mechanism of platelet aggregation for G45 in the absence of protein G.

Platelet aggregation induced by GGS G45 in human and mouse PRP resulted in a transient entrapment of the bacteria within highly unstable aggregates. The fact that both human and mouse platelets exhibit the same phenotype further confirms that an IgG-independent platelet aggregation occurs, since mouse platelets lack FcγRIIA (Kato et al., 1998; McKenzie et al., 1999). This provided us with a unique opportunity to study the role of direct platelet–bacteria interactions during invasive infection. Mouse platelets will not become aggregated in response to bacterial pathogens that utilize an IgG-dependent mechanism of platelet activation, such as GAS (Kahn et al., 2013). Importantly, we confirmed that platelet activation and thrombocytopenia occurred during invasive GGS infection, verifying that our model exhibits important hallmarks of invasive human infection. Thrombocytopenia is an important prognostic marker in human invasive infections (Vandijck et al., 2010; Hui et al., 2011). It has been speculated that thrombocytopenia during infection reflects in vivo platelet activation, and by analysing plasma from healthy and infected mice we were able to demonstrate that platelet activation does occur in vivo. Based on our ex vivo findings with GGS G45, we propose that direct activation of platelets by bacteria may contribute to this platelet activation in vivo.

The ability of platelets to respond to bacterial pathogens may represent an important antibacterial strategy to combat the pathogen and/or certain pathogens may manipulate this host response. We determined that dissemination of GGS to the kidneys was significantly increased in the infected control mice as compared with mice rendered thrombocytopenic prior to infection.
contribute to multiple aspects of the immune response to infection (Semple et al., 2011). Our findings may reflect an indirect role of platelets on bacterial dissemination due to platelet-dependent release of pro-inflammatory substances that modulate recruitment and activation of other immune cells, such as monocytes or neutrophils. Platelets may also have a direct role in either survival of bacteria in the blood or trafficking of the bacteria to distant sites. We propose that transient entrapment of bacteria in fibrinogen-mediated platelet aggregates, as demonstrated ex vivo, may shield bacteria from the immune system during passage through the blood in vivo. Fibrinogen binding has also been reported to facilitate complement evasion and phagocytosis resistance of GAS in whole blood (Carlsson et al., 2005). FOG from GGS has previously been reported to utilize fibrinogen to evade neutrophil killing (Johansson et al., 2004), and FOG modulates the pro-inflammatory and antibacterial effects of the contact system (Wollein Waldetoft et al., 2012). The direct contribution of FOG to bacterial dissemination and pathogenesis of infection was not investigated in our animal model, since the focus of our hypothesis was the contribution of platelet activation and aggregation to the pathogenesis of GAS infection. Platelets have been reported to contribute to the pathogenesis of GAS infection in an animal model (Kahn et al., 2013); however, platelets have also been proposed to protect in an animal model of sepsis (de Stoppelaar et al., 2014; Xiang et al., 2013). Interestingly, studies of human sepsis patients indicate that antiplatelet therapy is beneficial for the outcome of invasive infection (Winning et al., 2009; Otto et al., 2013; Akinosoglou & Alexopoulos, 2014). Sepsis is a complex clinical syndrome and antiplatelet therapy may only be relevant at a particular stage of sepsis or for sepsis caused by specific bacterial pathogens; therefore, robust investigations are required to assess this possibility.

ACKNOWLEDGEMENTS

We are indebted to Gisela Håkansson for excellent technical support. This study was supported in part by the Swedish Research Council (projects 21112, 21587 and 7480), the Royal Physiographic Society in Lund, the Åke-Wiberg Foundation, the Crafoord Foundation and the Österlund Foundation.

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Edited by: F. Sargent