

# Platelet Activation by *Streptococcus pyogenes* Leads to Entrapment in Platelet Aggregates, from Which Bacteria Subsequently Escape

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**Platelet activation and aggregation have been reported to occur in response to a number of Gram-positive pathogens. Here, we show that platelet aggregates induced by *Streptococcus pyogenes* were unstable and that viable bacteria escaped from the aggregates over time. This was not due to differential activation in response to the bacteria compared with physiological activators. All the bacterial isolates induced significant platelet activation, including integrin activation and alpha and dense-granule release, at levels equivalent to those induced by potent physiological platelet activators that induced stable aggregates. The ability to escape the aggregates and to resist the antibacterial effects of platelets was dependent on active protein synthesis by the bacteria within the aggregate. We conclude that *S. pyogenes* bacteria can temporarily cover themselves with activated platelets, and we propose that this may facilitate survival of the bacteria in the presence of platelets.**

During sepsis, the coagulation system has been reported to become deregulated, and this contributes to the pathogenesis of the sepsis syndrome (1, 2). Platelets are the major regulators of coagulation and have been ascribed an emerging role in the immune response (2). Thrombocytopenia can occur during severe infection, and the decrease in the platelet count has been reported to correlate with the severity of the disease (3, 4). The genesis of this thrombocytopenia is not clear, and it may reflect decreased production, increased destruction, or *in vivo* platelet activation and consumption in thrombi. A number of significant Gram-positive pathogens have been shown to stimulate platelet activation *in vitro* (5), including *Streptococcus pyogenes* (6); however, the significance for the pathogenesis of infection has not been elucidated. Furthermore, a recent study has shown that bacteria isolated from patients presenting with Gram-positive bacteremia can activate platelets from the infected individual *ex vivo*, implying that bacterium-mediated platelet activation may occur during bloodstream infection and sepsis (7). *S. pyogenes* is a common cause of mild maladies, such as pharyngitis and impetigo, but can also cause severe infection. *S. pyogenes* has been estimated to be responsible for a half million deaths worldwide each year, mainly due to complications of untreated *S. pyogenes* infections, such as rheumatic heart disease (8). Another important cause of morbidity and mortality is invasive infection caused by the bacteria. Streptococcal toxic shock syndrome and necrotizing fasciitis have a higher mortality rate than invasive disease caused by other Gram-positive bacteria (9). *S. pyogenes* can be classified into different serotypes based on variations in the cell surface M protein, an important virulence factor for the bacteria (10). Certain M protein serotypes, including the M1 serotype, are more frequently isolated from patients with invasive disease (11, 12). M protein is a cell wall-associated protein; however, it can also be released from the bacterial surface in a functionally active, soluble form (13). Soluble M1 protein and M5 protein isolated from *S. pyogenes* have previously been shown to mediate platelet activation and aggregation (14, 15). M1 protein released from the bacterial surface forms aggregates with plasma fibrinogen and specific anti-M1 IgG antibodies that simultaneously engage the fibrinogen receptor and the Fc receptor on the platelet surface to elicit activation (15). In the present study, we investigated the

consequences of platelet activation and aggregation for *S. pyogenes* bacteria.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacteria were isolated and identified at the Clinical Microbiology Laboratory, Skåne University Hospital, Lund, Sweden. *S. pyogenes* was isolated from blood cultures (designated BB5 and BB7) or throat cultures (designated BT1 and BT7). *emm* typing was carried out as previously described (16), with some adjustments. The bacteria were transferred from freeze stocks onto blood agar plates and grown overnight. DNA was isolated from fresh colonies, and PCR was performed using TrueStart polymerase (Fermentas) with primer *emm*1 (TATT[C/G]GCTTAGAAAATTAA) and primer *emm*2 (GCAAGTTCTT CAGCTTGTTT). PCR cleanup was carried out using a GenElute PCR-Clean Up Kit (Sigma) according to the manufacturer's instructions, and sequencing was performed by GATC Biotech.

For platelet activation studies, bacteria were grown overnight in Todd-Hewitt broth (Difco/BD) supplemented with 0.2% yeast extract (Oxoid) at 37°C in the presence of 5% CO<sub>2</sub>. All experiments were carried out with overnight bacterial cultures at an optical density at 620 nm (OD<sub>620</sub>) of approximately 0.7.

**Preparation of platelet-rich plasma and platelet-poor plasma.** Human platelets from four healthy donors, two males and two females between 25 and 30 years of age, were used throughout the study. The donors had not taken any antiplatelet medication for at least 10 days. The regional Ethical Review Board in Lund, Sweden, approved the study (reference no. 657/2008), and informed consent was obtained. Citrated blood was centrifuged for 15 min at 150 × *g* to yield platelet-rich plasma (PRP). The PRP was removed, and the remaining blood was centrifuged again for 10 min at 2,000 × *g* to yield platelet-poor plasma (PPP).

Received 7 May 2014 Returned for modification 7 June 2014

Accepted 23 July 2014

Published ahead of print 28 July 2014

Editor: A. Camilli

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/IAI.02020-14>.

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doi:10.1128/IAI.02020-14

**Platelet aggregation in plasma.** A platelet aggregometer (ChronoLog 490) was used to assess platelet aggregation in response to *S. pyogenes*. A sample of 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 was representative of platelet aggregation. Twenty microliters of washed bacteria ( $1 \times 10^8$  CFU/ml) was added to 450  $\mu$ l of PRP. Collagen I (Chrono-Log Corp.) was used as a positive control at a final concentration of 0.005 mg/ml. The aggregation curves were analyzed using the AggroLink software.

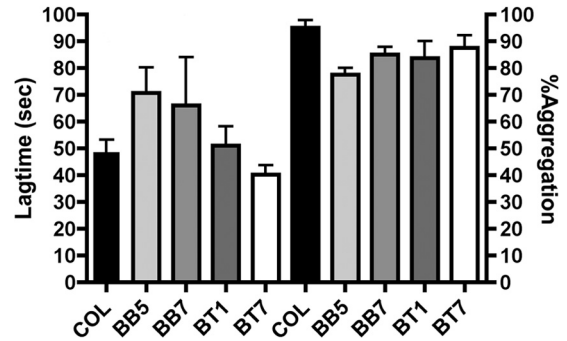
**Scanning electron microscopy.** Scanning electron microscopy was used to study the morphology of the platelet aggregates formed in response to collagen I (0.005 mg/ml), ADP (10  $\mu$ M), or *S. pyogenes* isolates ( $1 \times 10^8$  CFU/ml). Following incubation for 5, 60, or 120 min, platelet aggregates were pelleted by centrifugation for 5 min in a microcentrifuge at maximum speed. Plasma was removed and replaced with 20  $\mu$ l of fixation fluid (2.5% [vol/vol] glutaraldehyde in 0.15 M sodium cacodylate buffer). After 24 h, the samples were pelleted and immobilized on poly-L-lysine coverslips; fixed in 2.5% (vol/vol) glutaraldehyde-0.15 M sodium cacodylate, pH 7.4; and prepared for scanning electron microscopy as previously described (17). Specimens were examined in a Philips XL 30 FEG scanning electron microscope at the Core Facility for Integrated Microscopy (CFIM), Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark.

**Recovery of bacteria from platelet-rich plasma.** Bacteria were washed once with phosphate-buffered saline (PBS) and diluted to  $2 \times 10^9$  CFU/ml. Twenty microliters of bacteria was added to 450  $\mu$ l PRP in a microcentrifuge tube and incubated at 37°C with stirring. At time zero ( $t_0$ ) and at 20-min intervals, 10- $\mu$ l samples were taken from the plasma, diluted, and plated on Todd-Hewitt broth supplemented with yeast extract (THY) agar plates for viable-count determination. A negative-control sample for autoaggregation of platelets in the absence of bacteria was performed in parallel. Two positive-control samples with either collagen (0.005 mg/ml)- or ADP (10  $\mu$ M)-induced aggregation were also performed in parallel. All samples were macroscopically monitored for the integrity of the platelet aggregates. In order to determine the bacterial load within platelet aggregates, sonication of aggregates induced by *S. pyogenes* BB5 was performed after 20 min and compared to the bacterial load at  $t_0$ .

In order to determine the importance of platelet activation for aggregation and survival of *S. pyogenes* in PRP, assays were performed in PPP alone or in PRP in the presence of a biochemical platelet blocker (prostaglandin E<sub>1</sub> [PGE<sub>1</sub>] (0.001 mM; Sigma). In order to determine the importance of the platelet Fc receptor for platelet aggregation in response to *S. pyogenes*, the bacterial recovery assays were performed in PRP pretreated for 30 min with a monoclonal antibody against platelet FcR $\gamma$ IIA (AT10; Serotec; 100  $\mu$ g/ml).

To determine the role of active protein synthesis by the bacteria in platelet disaggregation, *S. pyogenes* isolates were pretreated with kanamycin (1 mg/ml) for 50 min at 37°C with stirring. Following kanamycin treatment, 20  $\mu$ l of bacteria was added to 450  $\mu$ l of PRP in microcentrifuge tubes. At  $t_0$  and at 20-min intervals, 10- $\mu$ l samples were taken from the plasma, diluted, and plated on THY agar plates for viable-count determination. In some experiments, the bacterial load within aggregates induced by kanamycin-treated *S. pyogenes* isolates was determined by using sonication to manually disaggregate the samples, followed by viable-count determination.

**Flow cytometry of platelet activation.** Platelet activation was measured using flow cytometry on a FACSCalibur flow cytometer (Becton Dickinson). Twenty microliters of PRP was added to 40  $\mu$ l HEPES buffer, pH 7.4. As a positive control for platelet activation, either thrombin receptor-activating peptide 6 (TRAP-6) (0.01 mg/ml [Sigma]) or ADP (5  $\mu$ M [Sigma]) was used. For platelet activation, 15  $\mu$ l of washed bacteria ( $5 \times 10^8$  CFU/ml 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% formalde-



**FIG 1** *S. pyogenes* isolates mediate rapid platelet aggregation. Platelet aggregation of PRP was determined in a platelet aggregometer on addition of collagen (COL), *S. pyogenes* strains isolated from blood (BB5 and BB7), or *S. pyogenes* strains isolated from throat infections (BT1 and BT7). Platelet aggregation is defined as the percent change in light transmission of the PRP (right axis). Lag time to aggregation is defined as the time in seconds between addition of an agonist and the onset of aggregation (left axis). The data are shown as means plus standard deviations (SD) for two independent experiments, each with four donors ( $n = 8$ ). Statistical analyses determined that there was no significant variation in lag time or percent aggregation between agonists.

hyde. CD42a peridinin chlorophyll protein (PerCP) (Becton Dickinson) antibody was used as a marker for both resting and activated platelets. CD62P phycoerythrin (PE) and PAC-1 fluorescein isothiocyanate (FITC) (Becton Dickinson) were used as markers for activated platelets that had released CD62P from the alpha granules and that had undergone activation of the GPIIb/IIIa integrin, respectively.

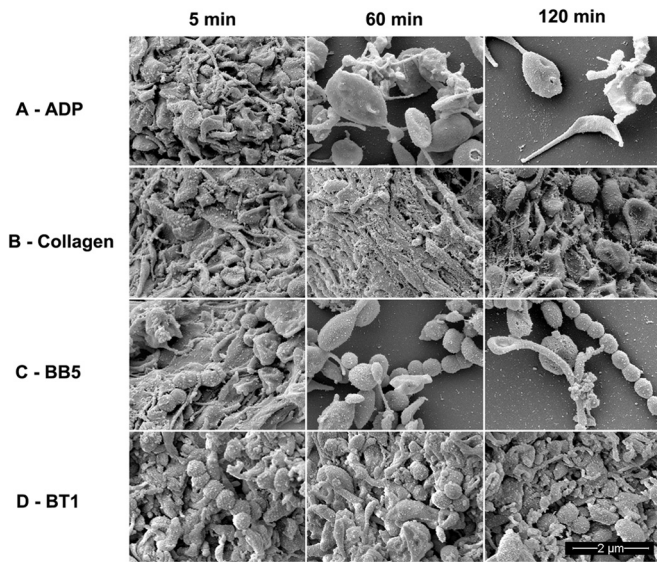
In order to determine the importance of the platelet Fc receptor for activation by *S. pyogenes*, flow cytometry was performed with PRP pretreated for 30 min with a monoclonal antibody that blocks engagement of platelet FcR $\gamma$ IIA (AT10; Serotec; 100  $\mu$ g/ml). In order to determine the role of fibrinogen in platelet activation by bacteria, flow cytometry was performed with PRP pretreated with a monoclonal antibody that blocks the binding of fibrinogen to the platelet surface (ReoPro [Abciximab]; Centcor; 10  $\mu$ g/ml). As a negative control for the monoclonal antibodies, PRP was preincubated with mouse anti-human CD64 (Serotec; 100  $\mu$ g/ml) for 30 min at 37°C. In order to determine the role of plasma IgG, platelet activation was performed in PRP following incubation with an IgG-specific bacterial proteinase (IdeS; 100  $\mu$ g/ml; 1 h at 37°C) that cleaves plasma IgG only and has no other known targets in plasma (18).

**Release of serotonin from platelet dense granules.** Platelet aggregation was induced by collagen, ADP, and *S. pyogenes* as described above. The 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 enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturers' instructions (Labor Diagnostic Nord GmbH and Co).

**Statistics.** All statistical analyses were performed using GraphPad v4.0.

## RESULTS

***S. pyogenes* isolates induce rapid platelet aggregation, but the aggregates are unstable.** The *emm* types of the four *S. pyogenes* isolates were determined. BB5 and BB7, isolated from blood, were both *emm* 28.0. *S. pyogenes* BT1 and BT7, isolated from the throat, were *emm* 4.14 and *emm* 89.0, respectively. A platelet aggregometer was used to assess platelet aggregation. All agonists tested caused maximum aggregation of the platelets, with levels of >80% aggregation for the physiological platelet agonist, collagen, and all *S. pyogenes* isolates (Fig. 1). The lag time to induction of aggregation was short for all *S. pyogenes* isolates and was equivalent to that

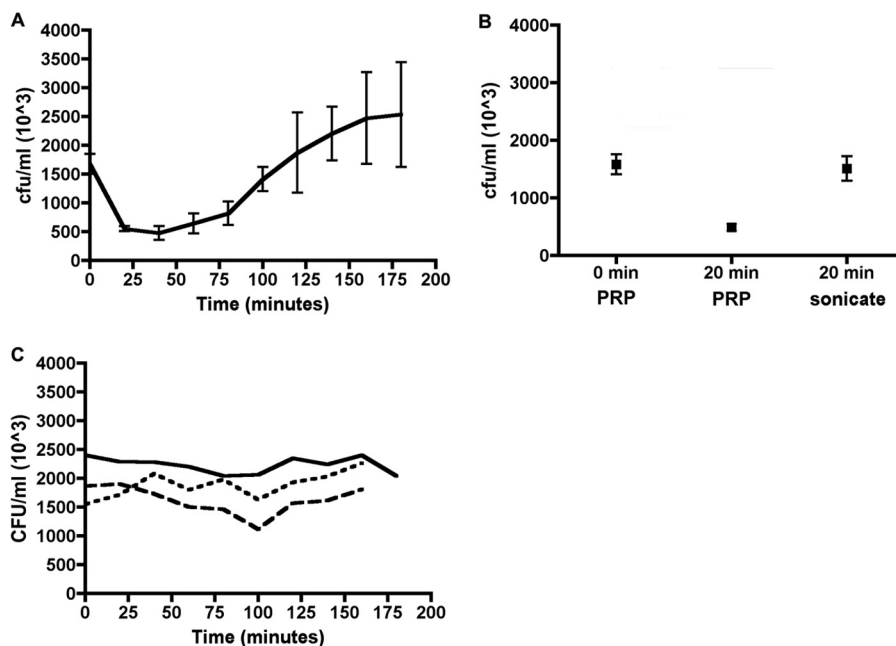


**FIG 2** Morphology and stability of platelet aggregates formed in response to different agonists. Scanning electron microscopy was used to assess the morphology of the platelet aggregates formed in PRP on addition of ADP (A), collagen (B), *S. pyogenes* BB5 (C), or *S. pyogenes* BT1 (D) at 5, 60, and 120 min postaggregation. Representative images from one of three independent experiments are shown for each time point.

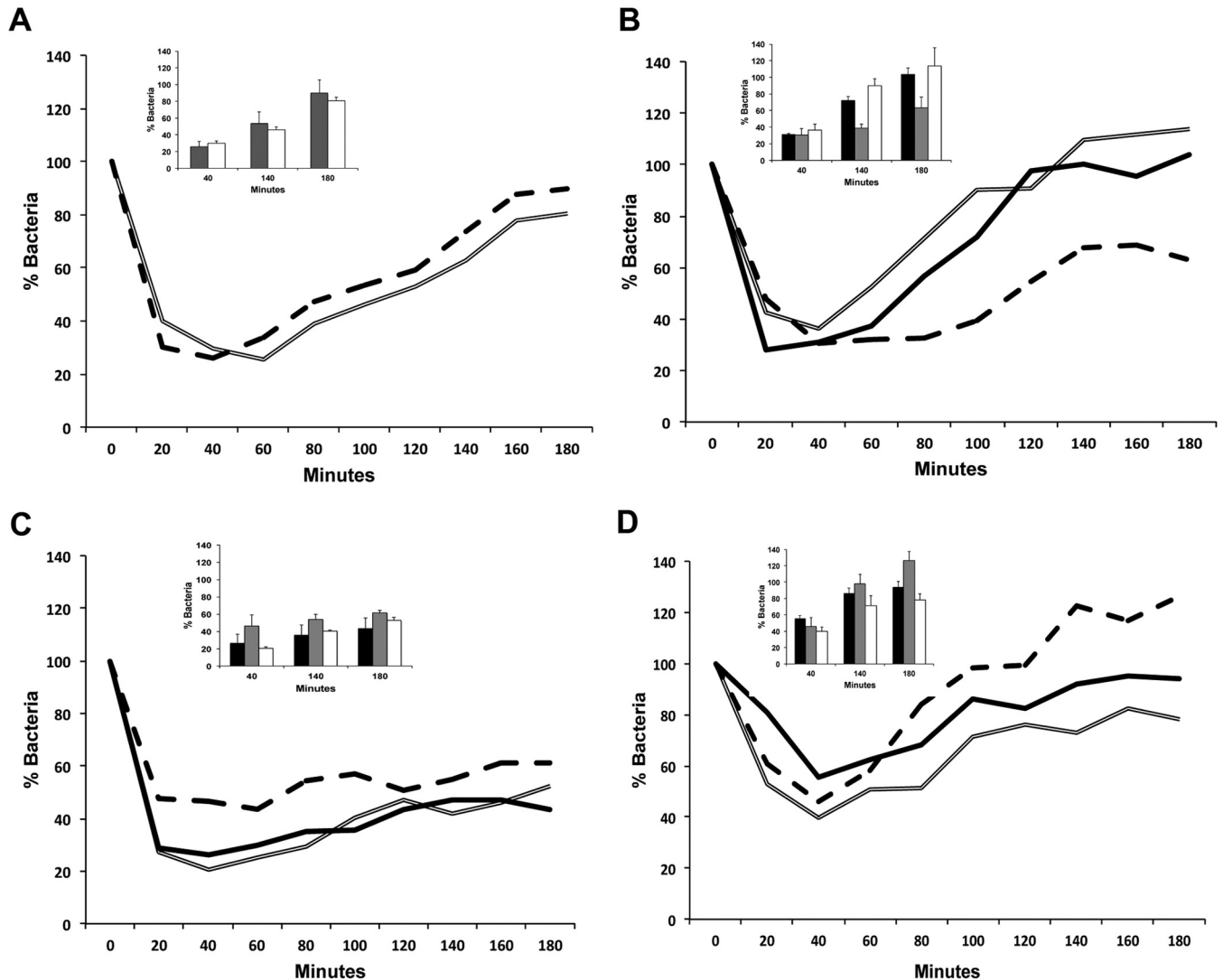
induced by the physiological platelet agonist, collagen (Fig. 1). There was no significant variation among agonists for either lag time ( $P = 0.2248$ ; one-way analysis of variance [ANOVA]) or aggregation ( $P = 0.0987$ ; one-way ANOVA).

Scanning electron microscopy was used to study the morphology of the aggregates generated on addition of a physiological platelet agonist (collagen or ADP) or all four *S. pyogenes* isolates. Collagen, ADP, and the bacteria induced large stable aggregates within 5 min (Fig. 2). The bacteria were trapped in these aggregates, and few bacteria were visible at the surface of the aggregate after 5 min (Fig. 2C and D). The aggregate induced by the potent platelet agonist collagen remained stable over time (Fig. 2B), while the aggregate induced by the weak platelet agonist ADP disaggregated over time (Fig. 2A). The majority of *S. pyogenes* isolates (*S. pyogenes* BB5, BT7, and BB7) induced aggregates that had disaggregated within 1 h (Fig. 2C; representative images of *S. pyogenes* BB5). *S. pyogenes* escaped from these platelet aggregates, and chains of viable streptococci were observed. *S. pyogenes* BT1 induced platelet aggregates that were stable for the duration of the experiment (Fig. 2D).

**Viable *S. pyogenes* BB5 bacteria are maintained within platelet aggregates, from which bacteria subsequently escape.** The fate of the bacteria after induction of platelet aggregation was investigated for *S. pyogenes* BB5 in one donor tested in 5 independent experiments. Platelet aggregation was mediated by *S. pyogenes* BB5, and the amount of viable bacteria in the supernatant was determined at 20-min intervals (Fig. 3A). Platelet aggregation in response to *S. pyogenes* BB5 coincided with a rapid decrease of bacteria from the PRP after 20 min. Within 1 h, the bacterial load in the PRP had again increased (Fig. 3A). In parallel to this increase in bacterial recovery, macroscopic dissolution of the aggregate was observed, suggesting that the bacteria recovered from the plasma escaped from the platelet aggregate. At 20 min postaggre-



**FIG 3** *S. pyogenes* BB5 disaggregates platelets to escape and grow in PRP. (A) *S. pyogenes* BB5 was added to PRP, and the bacterial load of the plasma was determined by plating of 10- $\mu$ l samples at 20-min intervals. The data are shown as mean CFU/ml  $\pm$  standard errors for 5 independent experiments in one donor ( $n = 5$ ). (B) *S. pyogenes* BB5 was added to PRP, and the bacterial load in plasma was determined immediately (0 min PRP), in plasma at 20 min postaggregation (20 min PRP), and in plasma 20 min after sonication of the aggregates (20 min sonicate). The data are shown as means  $\pm$  standard errors for 5 independent experiments in one donor ( $n = 5$ ). (C) *S. pyogenes* BB5 was added to PPP (solid line;  $n = 3$ ) or to PRP in the presence of a biochemical blocker, PGE<sub>1</sub> (dashed line with long dashes;  $n = 3$ ), or a monoclonal antibody that blocks platelet Fc $\gamma$ RIIA (dashed line with short dashes;  $n = 3$ ), and the bacterial load of the plasma was determined at 20-min intervals. The data are shown as means for 3 independent experiments in one donor ( $n = 3$ ).

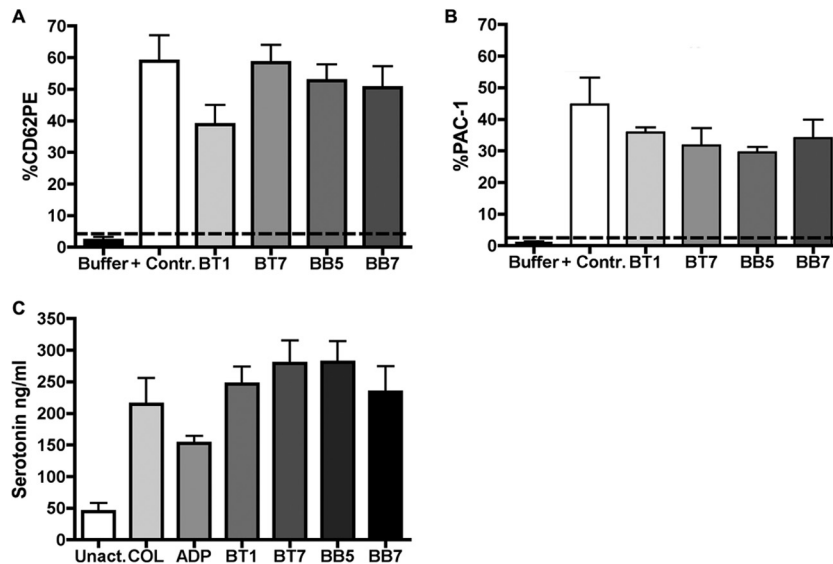


**FIG 4** Diverse *S. pyogenes* isolates escape from platelet aggregates. *S. pyogenes* BB5 (A), *S. pyogenes* BB7 (B), *S. pyogenes* BT1 (C), or *S. pyogenes* BT7 (D) was added to PRP from donor I (solid lines), donor II (dashed lines), or donor III (hollow lines), and the bacterial load of the plasma was determined at 20-min intervals. The initial bacterial load determined at  $t_0$  was set as 100%, and all subsequent values were expressed as a percentage of this value. The mean of three independent experiments for each donor is shown in each line graph ( $n = 3$ /strain/donor). The insets demonstrate the experimental variation of the data at representative time points as means plus standard errors.

gation, the bacterial load of the PRP had decreased to approximately 33% of the original inoculum. After this time point, the bacterial numbers steadily increased over time, and at least 100% of the bacteria were again present in the PRP after 120 to 180 min (Fig. 3A). We determined that the rapid decrease of bacteria in the supernatant was due to aggregate formation and not a bactericidal effect, since 100% of the bacteria added at  $t_0$  were recovered from samples that were manually disaggregated by sonication after 20 min (Fig. 3B). The bacterial load did not decrease when *S. pyogenes* BB5 was incubated in PPP, demonstrating that the decrease was dependent on platelets (Fig. 3C, solid line). Furthermore, the bacterial load did not decrease when *S. pyogenes* BB5 was incubated in PRP pretreated with a biochemical blocker of platelet activation ( $PGE_1$ ) (Fig. 3C, dashed line with long dashes). Platelet activation in response to the *S. pyogenes* isolates was determined to be dependent on fibrinogen and IgG binding to the platelet receptors GPIIb/IIIa and FcR $\gamma$ IIA, respec-

tively (see Fig. S1 in the supplemental material). Importantly, the bacterial load in PRP did not decrease when *S. pyogenes*-mediated platelet aggregation was inhibited using anti-FcR $\gamma$ IIA (Fig. 3C, dashed line with short dashes). The results demonstrate that IgG-dependent platelet activation and aggregation occur and that the bacteria are incorporated into platelet aggregates from which they later escape.

**The ability of viable *S. pyogenes* bacteria to escape from the platelet aggregate is strain dependent.** The ability of *S. pyogenes* BB5 to mediate platelet aggregation and disaggregation was confirmed for two additional platelet donors tested in three independent experiments per donor (Fig. 4A). The abilities of *S. pyogenes* BB7, BT1, and BT7 to mediate platelet aggregation and disaggregation were investigated in three platelet donors tested in three independent experiments per donor (Fig. 4B to D). The amount of viable bacteria in the supernatant was determined immediately after addition of bacteria ( $t_0$ ) and at 20-min intervals thereafter.



**FIG 5** *S. pyogenes* isolates mediate platelet integrin activation and alpha and dense-granule release. Flow cytometry was used to assess platelet activation in response to a negative-control buffer; a positive-control platelet agonist (+ Contr; ADP or TRAP-6); or *S. pyogenes* isolate BT1, BT7, BB5, or BB7. The percentage of the platelet population that was positive for CD62P PE (A) or PAC-1 FITC (B) is shown on the y axis. The data are shown as means plus SD for three independent experiments each in three donors ( $n = 9$ ). Statistical analyses determined that there was no significant difference in the levels of activation between agonists (one-way ANOVA). (C) ELISA was used to determine serotonin release in the plasma of PRP in response to a positive-control platelet agonist (collagen or ADP) or *S. pyogenes* isolate BT1, BT7, BB5, or BB7. The data are shown as means plus SD for three independent experiments each in three donors ( $n = 9$ ). Statistical analyses determined that there was no significant difference in the amounts of serotonin release between agonists (one-way ANOVA).

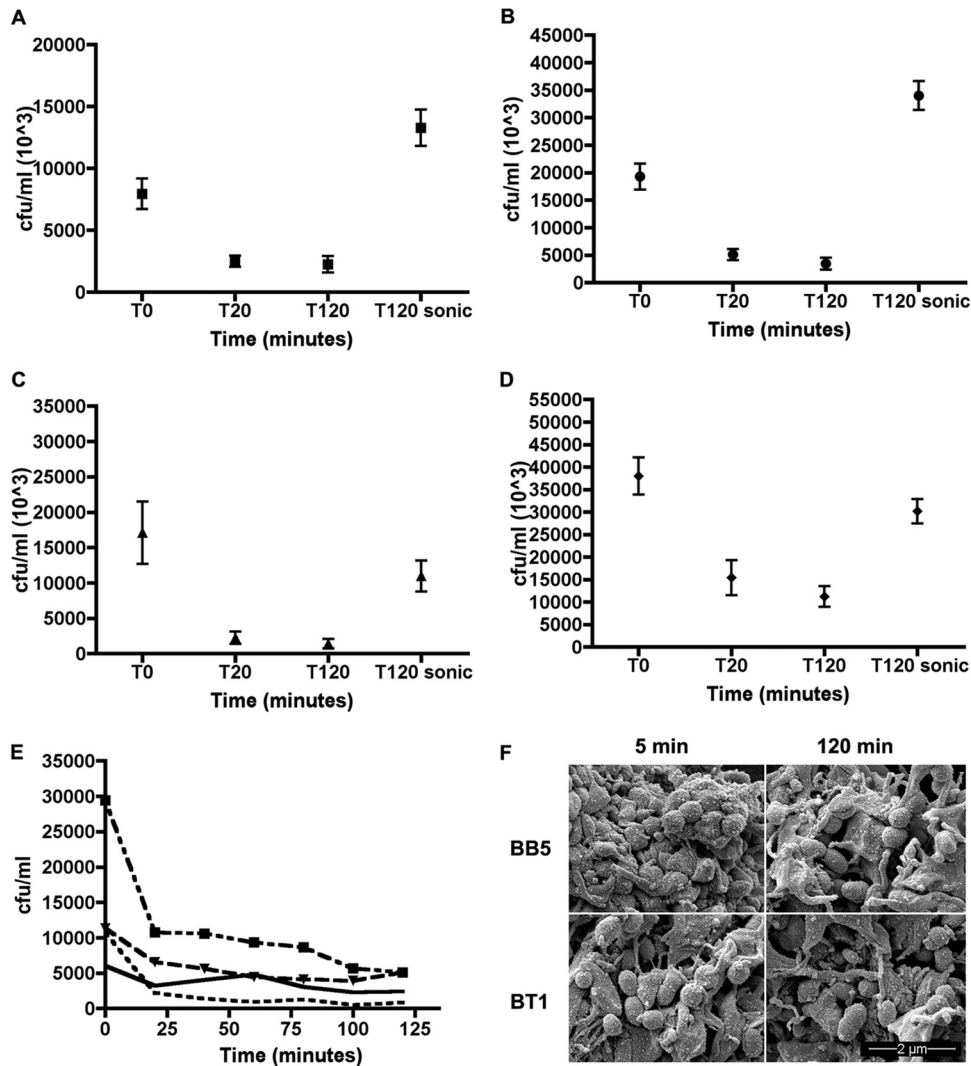
The initial inoculum (CFU/ml at  $t_0$ ) was set as 100%, and all subsequent bacterial counts were expressed as a percentage of this value. The means of three independent experiments for each donor are presented in Fig. 4A to D, and the experimental variation of the data is illustrated for representative time points in the insets. *S. pyogenes* isolates rapidly aggregated platelets in all three donors, and this was associated with a decrease in the bacterial load in the PRP when the bacteria became incorporated into the aggregate (Fig. 4B to D). At 20 min postaggregation, the bacterial load of the PRP had decreased to at least 40% of the original inoculum for *S. pyogenes* BB5, BB7, BT1, and BT7 in the majority of donors and to 55% for *S. pyogenes* BT7 in donor 1. After this time point, the bacterial load of the PRP inoculated with either *S. pyogenes* BB7 or BT7 steadily increased over time, indicating that both isolates escaped from the aggregates formed in all three donors (Fig. 4B and D). Disaggregation of the platelet aggregates formed in response to *S. pyogenes* BB7 or BT7 was macroscopically evident in all three donors. The bacterial load of the PRP inoculated with *S. pyogenes* BT1 was relatively stable between 20 and 180 min in all donors (Fig. 4C), and stable macroscopic aggregates were visible in all of the samples after 180 min. This indicates that this isolate was unable to escape from the aggregates formed. The reduced recovery of *S. pyogenes* BT1 from the supernatant of the aggregate was not due to a bactericidal effect, since 100% of the bacteria could be recovered from samples that were manually disaggregated by sonication after 20 min and 120 min (data not shown).

**Platelet activation by *S. pyogenes* mediates platelet integrin activation and alpha and dense-granule release.** In order to assess if differential activation of the platelets by the bacteria was responsible for the instability of the platelet aggregates, platelet activation in response to physiological activators and bacteria was determined. Activation was determined by flow cytometry, using antibodies directed at epitopes present on resting and activated

platelets. CD42a was used to identify the platelet population, and activation was defined as the amount of platelets positive for the granule-released protein CD62P and the active GPIIb/IIIa integrin (PAC-1). There were no significant differences between the three donors with regard to background activation or platelet activation in response to a physiological agonist (TRAP-6 or ADP) or individual *S. pyogenes* isolates; therefore, pooled data for all donors tested in triplicate are presented in Fig. 5. The four *S. pyogenes* isolates mediated both alpha granule release (CD62P) (Fig. 5A) and activation of the GPIIb/IIIa integrin (PAC-1) (Fig. 5B), and there was no significant difference in the levels of activation mediated by the physiological control agonist or bacterial isolates (one-way ANOVA).

Serotonin release from platelet dense granules in response to physiological agonists (ADP and collagen) and *S. pyogenes* BB5, BB7, BT1, and BT7 was measured using an ELISA. Serotonin release was mediated in response to all agonists (Fig. 5C). All four isolates of *S. pyogenes* generated significant serotonin release at levels equivalent to collagen-activated platelets. There was a tendency for serotonin release to be higher in response to collagen and bacterial isolates than ADP; however, this variance was not statistically significant (one-way ANOVA).

**Active protein synthesis by bacteria within aggregates is required for bacterial escape.** In order to assess the role of active protein synthesis by the bacteria within the platelet aggregates for the subsequent disaggregation and escape, bacteria were treated with kanamycin and incubated in PRP. Kanamycin had no effect on the ability of platelets in PRP to aggregate in response to the physiological activator, collagen, and all four *S. pyogenes* isolates (data not shown). Kanamycin was bacteriostatic at the concentration used, since viable bacteria could be recovered at 120 min. In response to all four *S. pyogenes* isolates, platelet aggregation occurred and was associated with a decrease in the bacterial load at



**FIG 6** Active protein synthesis by *S. pyogenes* is required for platelet disaggregation. (A to D) Kanamycin-treated *S. pyogenes* BB5 (A), *S. pyogenes* BB7 (B), *S. pyogenes* BT1 (C), or *S. pyogenes* BT7 (D) was added to PRP. The bacterial load of the samples was determined immediately (T0), at 20 min postaggregation (T20), at 120 min postaggregation (T120), and after sonication of the aggregates at 120 min (T120 sonic). The error bars represent standard error of the mean. (E) In a parallel experiment, kanamycin-treated bacteria were added to PRP, and the bacterial load was determined immediately and at 20-min intervals up to 120 min for *S. pyogenes* BB5 (solid line), *S. pyogenes* BB7 (triangles), *S. pyogenes* BT1 (dashed line), or *S. pyogenes* BT7 (squares). (F) Scanning electron microscopy was used to assess the morphology of the platelet aggregates formed in PRP on addition of *S. pyogenes* BB5 or *S. pyogenes* BT1 at 5 min and 120 min postaggregation. Representative images are shown for each time point from one of two independent experiments.

20 min in all three donors (Fig. 6A to D). In the presence of kanamycin, the bacterial load in PRP remained unchanged after 120 min (Fig. 6A to D). Manual disaggregation of the samples by sonication after 120 min resulted in recovery of viable bacteria at levels equivalent to or higher than the original inoculum (Fig. 6A to D). In a parallel experiment, these results were confirmed by determination of viable bacteria in PRP after addition of kanamycin-treated bacteria ( $t_0$ ) and at 20-min intervals thereafter (Fig. 6E). Once again, the bacterial load in PRP decreased after 20 min for all isolates, remained low, and was stable for 120 min (Fig. 6E). Importantly, macroscopically visible aggregates were present after 120 min, and this was confirmed by scanning electron microscopy. Bacteria-platelet aggregates were visualized after 5 min, and intact aggregates were still evident after 120 min in response to *S. pyogenes* BB5 and BT1 (Fig. 6F). Taken together, the results demonstrate that active protein synthesis by the bacte-

ria within the aggregate was required for disaggregation in response to *S. pyogenes* BB5, BB7, and BT7, while active protein synthesis was not required to mediate stable aggregates in response to *S. pyogenes* BT1.

## DISCUSSION

Platelet activation and aggregation occur in response to pathogenic bacteria; however, the consequences of this aggregate formation for the bacteria have not been elucidated. We report that four clinical isolates of *S. pyogenes* mediate rapid platelet aggregation. The lag time to aggregation for bacteria has previously been reported to be shorter than for physiological activators; however, the clinical isolates of *S. pyogenes* used here exhibited lag times equivalent to those of collagen-activated platelets. Significantly, aggregate formation was not a dead end for the bacteria, and 3 of 4 bacterial isolates could disaggregate the platelets over time. This

confirms early reports, which showed that viable bacteria could be retrieved from platelet aggregates (19). Our data demonstrate for the first time that bacteria within the aggregates can disaggregate the platelets to escape into plasma. The relative instability of bacterium-induced aggregates was not due to incomplete platelet activation, since there was no significant difference in the levels of activation in response to escaping and nonescaping *S. pyogenes* isolates. All four *S. pyogenes* isolates mediate platelet activation that is dependent on fibrinogen and IgG binding to the platelet receptors GPIIb/IIIa and FcγIIA, respectively (see Fig. S1 in the supplemental material); therefore, a different activation pathway does not explain the ability of certain bacterial isolates to escape from the aggregates.

Assays performed in the presence of the bacteriostatic antibiotic kanamycin prevented disaggregation, demonstrating that it was dependent on growth of the bacteria and *de novo* synthesis of proteins within the aggregate. The inability of *S. pyogenes* BT1 to escape the platelet aggregates suggests that any bacterial factor that is required for escape may not be conserved by all strains of *S. pyogenes*. Recovery of viable bacteria from platelet aggregates demonstrates that platelet activation and aggregation do not have significant antibacterial effects on *S. pyogenes*. It has been reported that platelets harbor bactericidal (20, 21) and parasiticidal (22) properties. We failed to detect an antibacterial effect of platelets on *S. pyogenes*, and the bacteria may be resistant to platelet-derived products, as has previously been reported for isolates of *Staphylococcus aureus* (23). Alternatively, activated platelets may provide a growth advantage to *S. pyogenes*, as has previously been reported for other pathogenic bacteria (24).

*S. pyogenes* is a pathogen that is well known to circumvent components of the host immune response (25). A role for platelets as immune cells is currently emerging. The novel finding that *S. pyogenes* can mediate unstable platelet aggregates may reflect a counterstrategy by certain isolates of *S. pyogenes* against entrapment and the bactericidal effects of platelets. Activated and aggregated platelets facilitate metastasis of tumor cells, in part by providing a physical cloak against immune cells (26). Interestingly, fibrinogen and platelets have been demonstrated to be important for immune evasion by tumor cells in animal models (27). We propose that *S. pyogenes* may use a cloak of fibrinogen and activated platelets to evade the immune response and disseminate through the bloodstream during invasive infection. The ability of *S. pyogenes* to bind fibrinogen is associated with enhanced bacterial survival in blood (28, 29). Importantly, we have recently demonstrated that platelets contribute to the dissemination of *S. pyogenes* in a mouse model of streptococcal sepsis (30); however, the direct role of bacterium-mediated platelet activation cannot be addressed in this murine model, since mouse platelets lack the Fc receptor required to mediate platelet aggregation in response to the bacteria.

Microthrombi containing platelets and fibrin have previously been reported to occlude the microcirculation in patients (31) and animals (32) suffering from streptococcal sepsis. In tissue biopsy specimens from patients with invasive *S. pyogenes* infection, we have previously demonstrated that bacteria and platelets are colocalized in microthrombi at the site of infection (15), implying that platelet aggregation mediated by bacteria may occur during infection. Furthermore, this may explain in part the genesis of thrombocytopenia during *S. pyogenes* sepsis, where platelets are consumed within bacteria-platelet aggregates. Interestingly, it has

been reported that a low-dose antiplatelet treatment during sepsis may reduce the risk of multiorgan failure (33, 34), implying that platelet activation may directly contribute to the pathogenesis of bloodstream infections. Taken together, the results of our study support the important role of platelets for the pathophysiology of invasive *S. pyogenes* infection.

## ACKNOWLEDGMENTS

We express our gratitude to Magnus Rasmussen and Kehinde Isola for donating the clinical isolates used in this work.

This work was supported in part by the Swedish Research Council (projects 21112 and 21587); the Royal Physiographic Society in Lund; and the foundations of Crafoord, Åke-Wiberg, Harald and Greta Jeansson, and Österlund.

We have no competing financial interests.

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