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Fluid Shear Regulates the Kinetics and Receptor Specificity of *Staphylococcus aureus* Binding to Activated Platelets

Parag Pawar,* Pyong Kyun Shin,† Shaker A. Mousa,‡ Julia M. Ross,† and Konstantinos Konstantopoulos**

The interaction between surface components on the invading pathogen and host cells such as platelets plays a key role in the regulation of endovascular infections. However, the mechanisms mediating *Staphylococcus aureus* binding to platelets under shear remain largely unknown. This study was designed to investigate the kinetics and molecular requirements of platelet-*S. aureus* interactions in bulk suspensions subjected to a uniform shear field. Hydrodynamic shear-induced collisions augment platelet-*S. aureus* binding, which is further potentiated by platelet activation with stromal derived factor-1. Peak adhesion efficiency occurs at low shear (100 s⁻¹) and decreases with increasing shear. The molecular interaction of platelet εIIb, with bacterial clumping factor A through fibrinogen bridging is necessary for stable bacterial binding to activated platelets under shear. Although this pathway is sufficient at low shear (<400 s⁻¹), the involvement of platelet gpIb and staphylococcal protein A through von Willebrand factor bridging is essential for optimal recruitment of *S. aureus* cells by platelets in the high shear regime. IgG plays an inhibitory role in the adhesion process, presumably by interfering with the binding of von Willebrand factor to staphylococcal protein A. This study demonstrates that platelet activation and a fluid-mechanical environment representative of the vasculature affect platelet-*S. aureus* cell-adhesive interactions pertinent to the process of *S. aureus*-induced bloodstream infections. The Journal of Immunology, 2004, 173: 1258–1265.

*S. aureus* is a major pathogen involved in endovascular infections such as infective endocarditis (IE) (1, 2), suppurative thrombophlebitis (3), and vascular or heart valve prosthetic infection (4). The commencement and propagation of endovascular infections are regulated by complex interactions between surface components on the invading organism and host cells like platelets. Several lines of evidence suggest that platelets can serve as both positive and negative mediators of *S. aureus*-induced infections. On one hand, experimental and clinical observations suggest that the ability of *S. aureus* cells to bind to platelets correlates with the capacity of this organism to induce IE (5). It is believed that platelet aggregates may allow bacteria to settle and remain at the site of infection withstand the shear forces of flowing arterial blood. In fact, vegetations formed at the site of infection consist of *S. aureus* cells embedded in a meshwork of fibrin, platelets, and other cellular material similar to that used by the body to form blood clots (2, 5). In an animal model of endocarditis, it has been observed that rabbits given an inhibitor of platelet aggregation (aspirin) before inoculation with *S. aureus* had lower concentrations of bacteria (CFU per gram) within vegetations 24 h after infection (6), suggesting that platelets facilitate the initiation of *S. aureus*-induced IE pathogenesis. Use of aspirin also resulted in lower rates of embolic events, indicating that platelets aid bacterial cells in the metastasis process (6, 7). However, recent observations indicate that platelets are capable of phagocytosing *S. aureus* cells in a feedback-like mechanism, wherein the presence of bacterial cells activates platelets and activated platelets, in turn, engulf the bacterial cells (8). Platelets also contain small, cationic, staphylocidal peptides, termed thrombin-induced platelet-microbicidal proteins (tPMPs). tPMP-resistant strains of *S. aureus* show substantially lower levels of bacteremia compared with the tPMP-susceptible strains, suggesting that platelets may be involved in the host defense mechanism against *S. aureus* infections (9, 10).

Experimental studies involving static binding assays indicate that the molecular mechanisms of platelet-*S. aureus* cells' adhesive interactions are complex, involving a variety of bacterial surface adhesion molecules such as clumping factors (ClfA and ClfB), staphylococcal protein A (SPA), and serine-aspartate repeats, which interact with specific platelet receptors through plasma protein bridging. More specifically, ClfA and ClfB are known to bind to fibrinogen (11, 12), and SPA can bind to von Willebrand factor (vWF) and the Fc portion of IgG (13, 14) and serine-aspartate repeats to an unknown platelet receptor via a plasma protein bridge (11). However, a major limitation of our current knowledge stems from the fact that all previous studies aimed at investigating platelet-*S. aureus* cell interactions were performed under static conditions (8, 11), which neglect the rheological parameters of fluid flow in the vasculature. As has been appropriately argued in the literature, data obtained in vitro using static binding assays may not be relevant to the fluid dynamic environment encountered in the vasculature (15). It is now well established that the local fluid mechanical environment of the circulation critically affects the molecular pathways of cell-cell interactions (16–21). Consequently,
the relative contribution of each of the aforementioned molecular constituents in platelet-\textit{S. aureus} interactions under the action of hydrodynamic shear is unknown. Bacterial invasion is associated with the release of proinflammatory agents such as chemokines (22, 23), which activate platelets, and thus, can potentially affect the kinetics and receptor specificity of platelet-\textit{S. aureus} adhesion. To date, the effect of chemokines on platelet-\textit{S. aureus}-adhesive interactions in shear flow remains largely unknown.

In the present study, we used a rheometric-flow methodology in an effort to elucidate the molecular mechanisms of platelet-\textit{S. aureus} binding, and to understand the way it is affected by the hydrodynamic environment of the vasculature, platelet activation, and the presence or absence of plasma proteins such as fibronectin (11, 24), vWF (13, 25), and IgG (14). This study made use of the Newman strain of \textit{S. aureus} because it is well characterized and is known to express an array of cell surface receptors that bind to a variety of plasma proteins (26, 27). Mixtures of platelets and \textit{S. aureus} cells were subjected to a uniform shear field (100–5000 \textquotedbl}s\textsuperscript{−1}\textsuperscript{) in a cone-and-plate rheometer for prescribed periods of time (30–90 s), and the extent of aggregation was measured using a dual-color flow cytometric methodology. To quantify cell-cell interactions independent of experimental parameters such as shear rate, cell size, and initial cell concentration, we estimated the efficiency of platelet-\textit{S. aureus} heterotypic aggregation. We also report the increase in platelet-platelet aggregation after binding to \textit{S. aureus} cells, indicating quantitatively the measure of platelet activation induced by \textit{S. aureus} cells.

Materials and Methods

Reagents and mAbs

The IgG murine mAb HIP1 (function-blocking anti-gpIIb), 2F2-A9 (function-blocking anti-vWF), and Beb1 (anti-CD42a conjugated with FITC; anti-gpIX FITC) were purchased from BD Pharmingen (San Diego, CA). The IgG murine mAb HIP1 (function-blocking anti-gpIb), 2F2-A9 (function-blocking anti-vWF), and Beb1 (anti-CD42a conjugated with FITC; anti-gpIX FITC) were purchased from BD Pharmingen (San Diego, CA). Soluble vWF (factor VIII free) was from Hematologic Technologies (Essex Junction, VT). Trypsin soy broth (TSB; growth medium for \textit{S. aureus} cells) was purchased from BD-Microbiology Systems (Sparks, MD). Hexidium iodide (HI) was purchased from Molecular Probes (Eugene, OR). The chemokine stromal derived factor-1 (MOPC-21) was from Sigma-Aldrich (St. Louis, MO). Soluble vWF (factor VIII free) was from Hematologic Technologies (Essex Junction, VT). Trypsin soy broth (TSB; growth medium for \textit{S. aureus} cells) was purchased from BD-Microbiology Systems (Sparks, MD). Hexidium iodide (HI) was purchased from Molecular Probes (Eugene, OR). The chemokine stromal derived factor-1 (DUF-1β) was from R&D Systems (Minneapolis, MN).

Bacterial growth conditions and staining

Wild-type (WT) \textit{S. aureus} strain Newman and its clfA \textsuperscript{−} (DU 5876) and spa \textsuperscript{−} (DU 5971) strains were used in this study. Glycerol stocks were prepared from growing cell cultures in TSB and stored at −80 °C. Experimental cultures were started from glycerol stocks and grown in TSB at an OD of 6.7 (600 nm) at 37 °C with constant rotation for 16–18 h. Cells were harvested in Dulbecco’s PBS (D-PBS) with Ca\textsuperscript{2+}/Mg\textsuperscript{2+} containing 0.02% sodium azide to stop metabolic activities. Next, cells were washed three times with D-PBS containing 0.2% BSA to remove excess medium and sodium azide. \textit{S. aureus} cells were then stained with 60 \textmu g/ml HI for 1 h at room temperature with constant rotation, washed three times in D-PBS containing 0.2% BSA to remove excess dye, and resuspended in the same buffer.

Platelet preparation

Human blood was drawn by venipuncture from healthy volunteers into sodium citrate (0.38% w/v) anticoagulant. In selected experiments, citrated blood was treated with either 2 \textmu M PGE\textsubscript{2} or 10 mM EDTA immediately after venipuncture (30). Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 160 \times g for 15 min. Platelet-poor plasma was obtained by further centrifugation of the blood at 1900 \times g for 15 min. The final platelet count of the PRP was adjusted to the desired levels with platelet-poor plasma (21, 31). Specimens were stored at room temperature in capped polypropylene tubes and used within 3 h of isolation.

In selected experiments, platelets were washed with HEPEs-Tyrode-dextrose buffer (138 mM NaCl, 3.6 mM KCl, 10 mM NaHCO\textsubscript{3}, 0.4 mM Na\textsubscript{2}HPO\textsubscript{4}, 10 mM MgCl\textsubscript{2}, and 6 mM glucose) containing 5 mM EGTA and 2 \mu M PGE\textsubscript{2} to prevent platelet activation during the washing process (21, 31). After washing, platelets were resuspended in HEPEs-Tyrode-dextrose buffer without EGTA and PGE\textsubscript{2}, containing Ca\textsuperscript{2+} and Mg\textsuperscript{2+}.

Cone-and-plate rheometry assays

Platelets (1.25 \times 10\textsuperscript{9} cells/ml) and HI-stained \textit{S. aureus} cells (2.5 \times 10\textsuperscript{9} cells/ml) were mixed and then placed onto the stationary plate of a cone-and-plate rheometer (RS150; Haake, Paramus, NJ). Shear rates varied from 100 to 5000 \textsuperscript{−1} for prescribed periods of time ranging from 30 to 90 s. Static conditions were achieved by setting the shear rate to 0 \textsuperscript{−1}. Upon termination of shear or static incubation, samples (5 \mu l) were obtained and instantly fixed with 1% formaldehyde. Specimens were then allowed to incubate with a FITC-labeled platelet-specific mAb directed against either gpIX (0.9 \mu g/ml, anti-gpIX FITC) or \alpha\textsubscript{IIb}\textbeta\textsubscript{3} (5 \mu g/ml, C7E3 FITC) for 30 min in the dark at room temperature. The labeling reaction was then stopped by further dilution with 1% formaldehyde, and specimens were subsequently analyzed in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The 1° cone and plate of the rheometer were maintained at 37 °C during the entire experiment. The cone and plate were precoated with 0.2% BSA to prevent loss of cells due to sedimentation or nonspecific binding.

Cell treatment

To potentiate platelet activation, PRP specimens were incubated for 2 min before shear exposure with SDF-1β (10 and 50 ng/ml) at room temperature. For inhibition studies, platelets were pretreated at room temperature with function-blocking Abs specific for gpib (HIP1), vWF (2F2-A9), FCγR (AT10), or the isotype control Ab (MOPC-21) (all at 20 \mu g/ml) for 30 min, or the \alpha\textsubscript{IIb}\textbeta\textsubscript{3} blocking Ab (5G8) (50 ng/ml) for 10 min (21). To study the effect of exogenous plasma proteins such as fibronectin, vWF, and IgG on the adhesion process, washed platelet suspensions were mixed with soluble plasma proteins immediately before the initiation of the experiment.

Quantitation of aggregation

The particle distribution and cellular composition of stable aggregates generated in the rheometric assay were determined by a dual-color flow cytometric methodology (17, 21, 32). HI-stained \textit{S. aureus} cells and FITC-labeled platelets were identified on the basis of their characteristic forward scatter, side scatter, and fluorescence profiles in a FACSCalibur flow cytometer (Fig. 1, A and B). FITC and HI are excited efficiently at 490 nm by the argon laser of a flow cytometer, and their emission spectra are well separated (515 nm for FITC and 605 nm for HI), thereby allowing simultaneous two-color immunofluorescence measurements. Electronic compensation was used to remove spectral overlap between the two fluorescent populations. Acquisition of at least 3000 HI-stained \textit{S. aureus} events was then used to determine: 1) the percentage of bacterial cells bound to platelets, and 2) the population distribution of platelets and bacterial cells in the hetereotypic and homotypic aggregates.

The following strategy was used to determine the number of bacterial cells bound to platelets from the cytometric measurements. The mean and SD of a single \textit{S. aureus} cell HI fluorescence were computed. Six times the observed SD provides the range of a single \textit{S. aureus} cell fluorescence event with a 99% confidence (21). Superimposition of integral multiples of the fluorescence intensity range for single \textit{S. aureus} cells on the computed threshold value of platelet red fluorescence gives the fluorescence ranges for heteroaggregates containing progressively higher number of bacterial cells. A similar procedure was used to obtain the range of single platelet FITC fluorescence (21). Superimposition of integral multiples of this range to the computed threshold value of \textit{S. aureus} cell green fluorescence gives the fluorescence ranges for heteroaggregates containing progressively higher number of platelets. Using this methodology, heterotypic aggregates containing up to six platelets and/or six \textit{S. aureus} cells were detected and enumerated. Aggregates containing more than six platelets and/or \textit{S. aureus} cells were rare events (<1% of the total \textit{S. aureus} cell population). Under the experimental conditions of this study, pure homotypic platelet aggregation was negligible with <10% of platelets in homotypic aggregates even under the most extreme shear conditions reported in this study (data not shown).
Electron microscopy

Transmission electron microscopy was used to confirm the presence of heterotypic aggregates, especially those containing multiple platelets and S. aureus cells (Fig. 1C). Samples were prepared for electron microscopy by standard procedures (21). Briefly, stained specimens were fixed in 1.5% glutaraldehyde for 1 h at room temperature, and postfixed for 1 h in Kellenberger’s uranyl acetate solution overnight. After dehydration with a graded series of ethanol, cells were embedded in Epon. After polymerization, ultrathin sections were obtained on a Leica Ultracut UCT microtome (Leica Microsystems, Deerfield, IL) equipped with a diamond knife. Sections were then stained with uranyl acetate and lead curate before viewing with an electron microscope (Phillips 420 TEM).

### Determination of adhesion efficiency of platelet binding to S. aureus cells

Platelet-S. aureus cell adhesion efficiency is defined as the fraction of heterotypic shear-induced collisions that result in stable heteroaggregate formation. This index is a function of the intrinsic biological characteristics of the cells that are pertinent to their aggregation behavior, such as number and affinity of receptors and their response to applied shear. The percentage of platelets in pure homotypic aggregates was significantly lower than that in heteroaggregates, suggesting that platelets bound to S. aureus cells are more likely to bind homotypically to another platelet than platelets without adherent S. aureus cells. Moreover, more than three platelets were rarely encountered in any given purely homotypic platelet aggregate, whereas up to six platelets were present in platelet-S. aureus heteroaggregates. This is in agreement with previous studies that indicate that S. aureus cells induce platelet activation (8, 11), which accounts for the increased percentage of platelets in heteroaggregates and also the higher number of platelets in any given heteroaggregate. To account for this observation, we split the platelet efficiency into two components: 1) purely platelet-platelet adhesion efficiency, defined as the fraction of platelet-platelet collisions that result in stable pure homotypic platelet aggregates (EPB, i.e., aggregates not containing S. aureus cells, and 2) platelet-platelet/S. aureus adhesion efficiency, defined as the fraction of platelet-platelet collisions that result in stable larger heterotypic platelet-S. aureus aggregates (EPB, a). To more accurately predict the behavior of the system, S. aureus-aureus platelet adhesion efficiency (EPB, a) was also defined as the fraction of bacterial cells in aggregation that result in the formation of larger heteroaggregates. Thus, there are three types of collisions that can result in heterotypic platelet-S. aureus aggregate formation: 1) direct platelet-S. aureus collisions, which correspond to EPB, a 2) platelet-platelet collisions (wherein at least one of the colliding platelets is bound to one or more S. aureus cells), which correspond to EPB, a and 3) S. aureus-S. aureus collisions wherein at least one of the colliding bacterial cells is bound to one or more platelets), which correspond to EPB, a.

Formation of purely homotypic bacterial cell aggregates in response to shear exposure was not observed in any of our experiments, thus allowing us to neglect the purely homotypic bacterial adhesion efficiency (EPB, a = 0).

Platelet-S. aureus heteroaggregation in response to hydrodynamic shear exposure is determined by the intercellular collision frequency and the capture efficiency of these collisions (21, 32, 33). The two-body collision frequency per unit volume, \( f_{ij} = \frac{4G(r_{ij,i} + r_{ij,j})^3 C(i)C(j)(C^2 - 1)}{(1 + \delta_{ij})^2} \) (1)

in which \( \delta_{ij} \) is Kronecker δ function, \( r_{ij,i} \) and \( r_{ij,j} \) are radii of the two colliding particles, one composed of \( i \) platelet + \( j \) S. aureus singlets and the other of \( k \) platelet + \( l \) S. aureus singlets, \( C(i, j) \) and \( C(k, l) \) are their respective concentrations, and \( G \) is the shear rate. The radius of a single S. aureus cell was calculated to be 0.4 μm by image processing, whereas the radius for a platelet singlet was set to 1.34 μm (33, 34).

The adhesion efficiency of platelet binding to S. aureus cells is calculated by fitting the aggregation data over the first 30 s after application of shear with a mathematical model based on Smoluchowski’s two-body collision theory (21, 32). This model (equation 2) describes the temporal change of the concentrations of aggregates of \( C(k, l) \) composed of \( k \) platelet and \( l \) S. aureus cell singlets in the most general case (34):

\[
\frac{dC(k,l)}{dt} = \sum_{i=0}^{k} \sum_{j=0}^{l} \left[ 0.5(1 + \delta_{i-k,j-l}) K_{ij,i-k,j-l} \right] \times C(i,j)C(k-l,i-j, k-j) \times C(i-k,j-l) \times K_{ij,i-k,j-l} \times \left[ C(i-k,j-l) \times C(i,j) \times C(k,l) \right]
\] (2)
in which $K_{(i,j),(k,l)}$ is the aggregation rate coefficient, $k_{eq}$ and $I_{max}$ represent the maximal number of platelets, and $S. aureus$ cells in an aggregate, respectively, and were set equal to four each, because aggregates composed of more than four platelets and/or $S. aureus$ cells were rare events after 30 s of shearing. The first term in the right-hand side of equation 2 accounts for the formation of the combination from two smaller aggregates, whereas the second term represents the depletion of the combination due to the formation of a higher order aggregate. Because our goal is to measure the initial rate of recruitment of platelets and bacterial cells into heterotypic aggregates, the terms describing the fragmentation process have been neglected. The set of coupled differential equations represented by equation 2 was integrated using the fourth-order Runge-Kutta-Gill method, and the adhesion efficiencies ($E_{PP}$, $E_{PB}$, $E_{BB}$, and $E_{PB}$) were calculated using equation 3:

$$E_{(i,j),(k,l)} = \frac{K_{(i,j),(k,l)}C(i,j)C(k,l)}{I_{(i,j),(k,l)}} \quad (3)$$

An initial guess was made for the adhesion efficiencies, which was then used to calculate the particle size distribution. The calculated values were then compared with the experimental particle size distribution, and the differences were minimized using the Nelder-Mead Simplex method (35). In equation 3, if both $j$ and $l$ are zero, then $E_{(i,j),(k,l)}$ is the homotypic platelet adhesion efficiency, $E_{PP}$. If either $k$ or $l$ is not zero, then $E_{(i,j),(k,l)}$ corresponds to $E_{PB}$, $E_{BB}$, or $E_{PB}$. Under these conditions, an estimate is made, based on the contribution of surface area by platelets and $S. aureus$ cells, to the respective aggregates, whether the collision between the two aggregates will be: 1) two platelets, 2) two $S. aureus$ cells, or 3) a platelet from one aggregate and an $S. aureus$ cell from the other. The aforementioned model is based on the following key assumptions: 1) Single cells and aggregates have spherical geometries. The volume of any given aggregate is assumed to be equal to the sum of the volumes occupied by platelets and $S. aureus$ cells that constitute the aggregate. 2) Only single collisions between any two particles are considered at a given time. It is to be noted that before calculating the homotypic platelet adhesion efficiency, the homotypic platelet aggregates formed under static conditions in response to SDI-1β treatment (6.2% ± 2.5) were subtracted from those formed upon shearing. This was done to ensure that aggregates considered for calculating adhesion efficiencies were formed solely because of the shear-induced collisions.

**Statistics**

Data are represented as the mean ± SEM, unless otherwise stated. Statistical significance of differences between means was determined by ANOVA. If means were shown to be significantly different, multiple comparisons by pairs were performed by the Tukey test. Probability values of $p < 0.05$ were selected to be statistically significant.

**Results**

**Hydrodynamic shear-induced collisions support formation of platelet-$S. aureus$ cell aggregates**

Previous studies have shown that $S. aureus$ cells are capable of binding to platelets under static conditions (8, 11). To investigate how the hydrodynamic shear environment of the circulation modulates these heterotypic adhesive interactions, $S. aureus$ cells and platelets suspended in plasma were subjected to controlled levels of shear for prescribed periods of time in a cone-and-plate rheometer. A ratio of five platelets ($1.25 \times 10^8$ cells/ml) to one $S. aureus$ cell ($2.5 \times 10^7$ cells/ml) was maintained in all shearing experiments. This ratio resulted in higher levels of heteroaggregation compared with the other ratios that were experimented with (data not shown). Most importantly, under these conditions, platelet-platelet and bacteria-bacteria homotypic aggregation was minimal with <10% of platelets and <3% of all bacterial cells in homotypic aggregates (data not shown).

Adhesion of $S. aureus$ cells to platelets was minimal under static conditions (2.6% ± 0.1, $n = 5$). This level of heteroaggregation did not show any significant variation with the platelet-$S. aureus$ coinoculation time (30–90 s) at 0 s⁻¹ (Fig. 2). Application of shear in the absence of any exogenously added chemical agonist augmented platelet-$S. aureus$ heterotypic aggregation, as determined by the increase in platelet-bound $S. aureus$ cells (Fig. 1, A and B; Fig. 2). The extent of $S. aureus$ recruitment by platelets increased with increasing shear exposure time up to 60 s (Fig. 2). Moreover, the percentage of $S. aureus$ cells binding to platelets increased with increasing shear rate from baseline levels under static conditions (2.6% ± 0.1) to a maximum at 2000 s⁻¹ (31.9% ± 1.9) (Fig. 2). Further increase in shear rate did not appreciably affect the percentage of bacterial cells bound to platelets (29.2% ± 3.3 at 5000 s⁻¹) (Fig. 2). Increasing the shear exposure time to 90 s resulted in lower levels of heteroaggregation, presumably due to disintegration of aggregates (Fig. 2). Furthermore, platelet-$S. aureus$-adhesive interactions appear to have an absolute requirement for divalent cations (Ca²⁺/Mg²⁺), as evidenced by the complete abrogation of heteroaggregation upon platelet treatment with EDTA (10 mM) (Fig. 2).

**Characterization of molecular mechanisms mediating platelet-$S. aureus$ cell heteroaggregation**

We next investigated the roles played by various cell surface receptors expressed on platelets and $S. aureus$ cells in supporting platelet-$S. aureus$-adhesive interactions. As a first step, we examined the role of $S. aureus$ cell surface receptor ClfA by using a mutant strain of $S. aureus$ Newman, which lacks the fibrinogen-binding Cifa receptor. We found that the ClfA- mutant strain was unable to bind to platelets at both low and high shear rates (Fig. 3). Abrogation of heteroaggregation was also observed when platelets pretreated with the platelet $\alpha_{IIb} \beta_3$ antagonist XV454 were mixed with WT $S. aureus$ Newman in the shear field of a cone-and-plate rheometer, suggesting that fibrinogen acts as a bridge between platelet $\alpha_{IIb} \beta_3$ and ClfA (Fig. 3). Along these lines, inhibition of platelet activation using PGE, abolished platelet-$S. aureus$ heteroaggregation under all shear rates examined in this study (Fig. 3).

SPA is a bacterial cell surface receptor that is capable of binding to vWF (13) under static conditions. To assess the potential involvement of SPA in platelet-$S. aureus$ heteroaggregation in shear flow, platelets were mixed with a mutant strain of $S. aureus$ Newman lacking SPA and subjected to well-defined levels of shear. The extent of heteroaggregation between SPA-$S. aureus$ cells and platelets was similar to that observed for WT bacterial cells in the low shear regime (≤400 s⁻¹) (Fig. 4). However, a significant reduction in heteroaggregation was detected at higher shear rates.
We therefore wished to test whether the FcγRIIA receptor played a major role in mediating platelet-S. aureus interactions. We next assessed whether platelet gpIb-IX, a cell surface receptor complex known to bind to vWF, is involved in the platelet-S. aureus adhesion process. To this end, platelets were incubated with 2F2-A9, a function-blocking anti-vWF mAb, before being sheared with S. aureus cells. Blocking of vWF resulted in a partial inhibition of aggregation at high shear (Fig. 4). No inhibition was detected at low shear rates (≤400 s⁻¹), in accord with the results obtained with the SPA- mutant of S. aureus cells. These data suggest that vWF plays a major role in mediating platelet-S. aureus interactions at high shear rates.

We next assessed whether platelet gpIIb-IX, a cell surface receptor complex known to bind to vWF, is involved in the platelet-S. aureus-adhesive interactions. To this end, platelets were incubated with HIP1 (a function-blocking anti-gpIIb-IX mAb) before the shearing experiment. Partial inhibition of heteroaggregation was again observed only at high shear rates (from 14.0% to 1.9% at 4000 s⁻¹; from 28.9% to 15.4% at 5000 s⁻¹) (Fig. 4). Cumulatively, these data suggest that the molecular interaction between platelet gpIIb-IX and bacterial SPA through vWF bridging is essential for optimal recruitment of S. aureus cells by platelets in the high shear regime.

It was recently shown that the platelet FcγRIIA receptor played a key role in the thrombus formation induced by S. aureus cells (36). We therefore wished to test whether the FcγRIIA receptor was also involved in the direct recruitment of bacterial cells by platelets. Platelets, pretreated with AT10, a function-blocking anti-FcγRIIA mAb, before being sheared with S. aureus cells showed no significant reduction in their ability to bind to S. aureus cells at all shear rates studied (Fig. 4), suggesting that the platelet receptor FcγRII does not play a role in the adhesion process.

Effect of platelet activation by chemokines

To investigate the effect of exogenous platelet activation on the heteroaggregation process, platelets were incubated with the chemokine SDF-1β before shearing them with bacterial cells. The percentage of bacterial cells bound to platelets increased significantly, especially at high shear rates (Fig. 5). SDF-1β-activated platelets failed to bind to S. aureus cells if pretreated with XV454 or EDTA, indicating the absolute requirement of αIIbβ3 integrins and divalent cations in this process. Consistent with the data obtained in the absence of any exogenously added chemical agonist, partial inhibition of aggregation was observed in the presence of gpIIb-IX and vWF-blocking mAbs in the high shear regime (Fig. 5).

Role of exogenous plasma proteins

Various plasma and extracellular matrix proteins, such as fibrinogen, vWF, and IgG, have been found to be capable of binding to S. aureus (11, 13, 24, 25). We wished to investigate the relative roles played by exogenous plasma proteins as compared with those released upon platelet activation by using washed platelets in the presence or absence of exogenously added proteins. Washed platelets were capable of recruiting S. aureus cells in the absence of plasma proteins under hydrodynamic shear conditions. Addition of exogenous fibrinogen to washed platelets before their mixing with S. aureus cells in the linear shear field of a cone-and-plate rheometer did not alter the extent of heteroaggregation at all shear rates examined (Fig. 6). In distinct contrast, addition of purified vWF to washed platelet suspensions before shearing with S. aureus cells resulted in markedly enhanced levels of heterotypic aggregation only in the high shear regime (Fig. 6). These data further emphasize the critical involvement of vWF in platelet-S. aureus interactions at high shear.

It is noteworthy that at high shear rates, the recruitment of S. aureus cells by washed platelets in the absence of exogenous plasma proteins was consistently higher than platelets suspended in plasma (Fig. 6). This result may suggest that some plasma protein(s) may play an inhibitory role in platelet-S. aureus interactions. To identify the inhibitory plasma protein, we then reconstituted mixtures of washed platelets and S. aureus cell suspensions with purified, soluble plasma proteins. SPA is known to bind to the Fc portion of IgG (37). Because vWF binding to SPA is required for optimal platelet-S. aureus binding, we hypothesized that the presence of IgG in the plasma might interfere with SPA-vWF binding. Along these lines, washed platelets were reconstituted...
with physiological concentrations of human IgG (5 mg/ml) before shearing with S. aureus cells. Aggregation levels showed a significant reduction compared with washed platelet suspensions (Fig. 6). In contrast, reconstitution of washed platelets with HSA did not show any significant difference compared with the washed platelet control. The results clearly indicate that IgG plays an inhibitory role in platelet-S. aureus-adhesive interactions.

Adhesion efficiencies

The adhesion efficiency index provides a measure of the biological properties of the cells that control their aggregation behavior (21, 32). Maximal adhesion efficiency of platelet-S. aureus binding ($E_{PB}$) in the absence of any exogenously added chemical agonist was observed at the lowest shear (100 s$^{-1}$), at which ~1 of 100 collisions led to stable platelet-S. aureus aggregate formation (Fig. 7B), and decreased with increasing shear rate. This trend was observed for $E_{PB}$ (Fig. 7A) and $E_{P,B}$ (Fig. 7C). It is noteworthy that the pure homotypic platelet-platelet adhesion efficiency was found to be minimal at all shear rates (data not shown). However, platelet-platelet/S. aureus homotypic efficiency ($E_{PB}$) was markedly elevated with ~5 of 100 collisions resulting in stable aggregate formation at 100 s$^{-1}$ (Fig. 7A), demonstrating that platelets with adherent S. aureus cells get activated and can in turn interact with other platelets.

SDF-1β treatment significantly augmented $E_{PB}$ (Fig. 7A) and $E_{P,B}$ (Fig. 7B), whereas no appreciable change was observed in $E_{PB}$ (Fig. 7C). This result indicates that the increase in the extent of aggregation upon SDF-1β treatment of platelets results from platelet-platelet and platelet-S. aureus collisions, but not S. aureus-S. aureus collisions, consistent with the fact that SDF-1β treatment results in platelet activation, thereby enhancing the ability of platelets to recruit other platelets or S. aureus cells. SDF-1β treatment, however, does not affect the ability of S. aureus cells to recruit other bacterial cells, as a result of which values of $E_{PB}$ and $E_{P,B}$ are comparable for both cases. The SPA$^{-}$ strain showed lower values for $E_{PB}$ and $E_{P,B}$, consistent with the reduced levels of platelet-S. aureus heteroaggregation. Interestingly, for the SPA$^{-}$ strain, the value of $E_{PB}$ dropped to negligible values (Fig. 7C), indicating that SPA may play an important role in homotypic S. aureus binding. This is the quantitative outcome of our experimental observation that the presence of large heterotypic aggregates containing multiple S. aureus cells was nearly eliminated in the case of SPA-deficient strain as opposed to when the WT strain was subjected to shear with platelets.

Discussion

This is the first study to characterize the molecular interactions between platelets and S. aureus cells in free cell suspensions as a function of the dynamic shear environment. The major findings of this work are: 1) hydrodynamic shear-induced collisions augment platelet-S. aureus cell binding in the absence of any exogenously added chemical agonist; 2) the capture efficiency of these adhesive interactions is regulated by the state of platelet activation and decreases with increasing shear; 3) the molecular interaction of platelet $\alpha_{IIb}\beta_3$ and S. aureus ClfA through fibrinogen bridging is necessary for stable bacterial binding to activated platelets under all shear rates tested in this study; 4) hydrodynamic shear affects the receptor specificity of activation-dependent platelet binding to S. aureus cells, as evidenced by the transition from an SPA-independent/ClfA-dependent process at low shear rates ($\leq$400 s$^{-1}$) to an SPA/ClfA-dependent process at high shears; and 5) IgG inhibits platelet-S. aureus-adhesive interactions.

Hydrodynamic shear and platelet activation modulate the kinetics of platelet-S. aureus heteroaggregation

Our data indicate that platelets readily coaggregated with S. aureus cells when subjected to hydrodynamic shear conditions in the absence of any exogenous chemical stimulation. The efficiency of direct platelet-S. aureus heteroaggregation peaked at the low shear rate of 100 s$^{-1}$ (with ~1 of 100 collisions resulting in stable heteroaggregate formation), and decreased with increasing shear rate with ~3 of 10,000 collisions resulting in stable heteroaggregate formation at 5000 s$^{-1}$. The extent of heteroaggregation was significantly potentiated over a range of shear rates upon platelet treatment with the chemokine SDF-1β. SDF-1β activates platelets and thus elevates the values of $E_{PB}$ (corresponding to platelet binding to platelets with
prior studies have shown that ClfA is capable of binding to fibrinogen, which in turn can bind to platelet αIbβ3, thereby mediating platelet-S. aureus-adhesive interactions under static conditions (8, 11). Our data indicate that this molecular pathway is critical at both physiological and pathological shear stresses, as evidenced by abrogation of heteroaggregation upon platelet pretreatment with the platelet-αIbβ3 antagonist XV454, as well as by the use of the ClfA–mutant strain.

Treatment of platelets with mAbs against vWF or gpIb resulted in partial, but significant decrease in the percentage of S. aureus cells bound to platelets only at high shear rates. These molecular interventions did not affect platelet-S. aureus-adhesive interactions in the low shear regime (≤400 s⁻¹). Along these lines, the platelet-αIbβ3-deficient strain of S. aureus Newman was capable of binding to platelets, but the levels of heteroaggregation were significantly lower than those of the WT strain only at high shear. Previous work has shown that vWF-gpIb binding has high tensile strength and a relatively high off-rate, K_{off}, allowing vWF to function as a tethering molecule (16). In contrast, indirect evidence suggests that platelet-αIbβ3-fibrinogen molecular interaction displays low on-rate and off-rate constants and/or lower tensile strength (38). Based on this previous evidence and our data, we speculate that at high shear rates maximal platelet-S. aureus binding involves a sequential, two-step process, wherein vWF forms a bridge between gpIb and SPA, thereby increasing the contact time, and allowing fibrinogen to form a stable bridge between platelet-αIbβ3 and ClfA. As a result, levels of heteroaggregation decrease significantly when vWF binding to either platelet-gpIb-IX or SPA is blocked. The critical involvement of vWF is also demonstrated in studies in which washed platelets reconstituted with exogenous vWF were capable of recruiting S. aureus cells with a higher efficiency when exposed to high shear.

**IgG inhibits platelet-S. aureus-adhesive interactions in bulk suspensions**

Reconstitution of washed platelet-S. aureus suspensions with plasma resulted in lower levels of heteroaggregation, possibly suggesting that some plasma protein(s) may play an inhibitory role in the platelet-S. aureus-binding process. Addition of exogenous fibrinogen or vWF in washed platelet suspensions did not reduce the levels of aggregation. However, when washed platelet-S. aureus cell suspensions were reconstituted with IgG before shearing, significantly lower levels of heteroaggregation were observed, clearly indicating that IgG plays an inhibitory role in the adhesion process. Addition of exogenous IgG also negated the increase in heteroaggregation levels detected by the addition of exogenous vWF (data not shown). Based on the fact that SPA contains binding sites for the Fc portion of human IgG, we propose that one or both of the

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**FIGURE 7.** Adhesion efficiencies for platelet binding to WT S. aureus Newman (in the presence and absence of SDF-1β) or SPA–mutant as a function of hydrodynamic shear rate. A, Platelet-platelet/S. aureus adhesion efficiency corresponding to collisions between aggregates containing platelets with one or more adherent S. aureus cells (E_{P/P}); B, direct platelet-S. aureus heterotypic adhesion efficiency (E_{P/B}); and C, S. aureus/platelet adhesion efficiency (E_{PB}). Data represent mean ± SEM of three experiments.

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adherent S. aureus cells) and E_{P/B} (corresponding to direct platelet-S. aureus binding), but has negligible effect on E_{PB,B}, which is the result of S. aureus binding to S. aureus cells with adherent platelets. No detectable heteroaggregation occurred when platelets were pretreated with PGE1 before their mixing with S. aureus cells in a linear shear field. This compound has been shown to elevate cAMP levels in platelets and to inhibit platelet activation and aggregation (29).

The extent of platelet-S. aureus cell binding increases with increasing platelet concentration (13.9% ± 3.7% at 1.25 × 10⁷ platelets/ml to 31.9% ± 1.9% at 1.25 × 10⁸ platelets/ml at 2000 s⁻¹) at a constant S. aureus cell number density (2.5 × 10⁷ cells/ml), presumably due to the increase of the intercellular collision frequency. Maximal heteroaggregation was detected at the near physiological platelet concentration (1.25 × 10⁸ platelets/ml) used in this study in the near absence of pure homotypic platelet aggregation. However, further increase in platelet concentration resulted in substantial pure homotypic platelet aggregate formation (data not shown).

**Roles of surface receptors in platelet-S. aureus heteroaggregation under shear**

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following occurs: 1) vWF and IgG compete for binding spots on SPA; 2) binding of IgG results in steric hindrance, which prevents the binding of vWF to SPA.

In a recently published study (36), it was shown that thrombus formation in the presence of \textit{S. aureus} cells is a two-step process: 1) The process is initiated when the integrin \(\alpha_{\text{IIb}}\beta_3\) mediates platelet arrest onto immobilized bacterial constituents that have bound plasma fibrinogen; and 2) if blood contains Abs against bacterial cell surface components, IgG may cluster on the same surface and activate adherent platelets by binding to the platelet FcRIIA, ultimately leading to thrombus growth. The second step is a special case in which the donor contains IgG specific against bacterial cells. In this light, it is interesting to note that our study identifies a new area of involvement for plasma IgG: that of an inhibitory molecule, thereby defining its dual role. We now know that plasma IgG plays an inhibitory role in direct platelet-\textit{S. aureus} adhesion as opposed to its role in platelet activation in step 2. The key difference between the two roles played by IgG is that the inhibitory interaction in direct heterotypic adhesion does not require the IgG to be specific against CHO or any other bacterial cell surface component (because the Fc portion of IgG, and not F(ab')\(_2\), binds to SPA). In contrast, only IgG specific against bacterial cell surface components can induce platelet activation through FcRIIA.

Taken together, this work shows that the fluid mechanical environment of the circulatory system affects both the kinetics and receptor specificity of activation-dependent platelet binding to \textit{S. aureus} cells. Elucidation of the detailed physical and molecular basis underlying platelet-\textit{S. aureus} conjugate formation may provide insights for the rational development of novel therapeutic strategies aimed to alter these adhesive interactions.

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\section*{References}