Biomechanics of P-Selectin PSGL-1 Bonds: Shear Threshold and Integrin-Independent Cell Adhesion

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ABSTRACT Platelet-leukocyte adhesion may contribute to thrombosis and inflammation. We examined the heterotypic interaction between unactivated neutrophils and either thrombin receptor activating peptide (TRAP)-stimulated platelets or P-selectin-bearing beads (Ps-beads) in suspension. Cone-plate viscometers were used to apply controlled shear rates from 14 to 3000/s. Platelet-neutrophil and bead-neutrophil adhesion analysis was performed using both flow cytometry and high-speed video-microscopy. We observed that although blocking antibodies against either P-selectin or P-selectin glycoprotein ligand-1 (PSGL-1) alone inhibited platelet-neutrophil adhesion by ~60% at 140/s, these reagents completely blocked adhesion at 3000/s. Anti-Mac-1 alone did not alter platelet-neutrophil adhesion rates at any shear rate, though in synergy with selectin antagonists it abrogated cell binding. Unstimulated neutrophils avidly bound Ps-beads and activated platelets in an integrin-independent manner, suggesting that purely selectin-dependent cell adhesion is possible. In support of this, antagonists against P-selectin or PSGL-1 caused dissociation of previously formed platelet-neutrophil and Ps-bead neutrophil aggregates under shear in a variety of experimental systems, including in assays performed with whole blood. In studies where medium viscosity and shear rate were varied, a shear threshold for P-selectin PSGL-1 binding was also noted at shear rates <100/s when Ps-beads collided with isolated neutrophils. Results are discussed in light of biophysical computations that characterize the collision between unequal-size particles in linear shear flow. Overall, our studies reveal an integrin-independent regime for cell adhesion and weak shear threshold for P-selectin PSGL-1 interactions that may be physiologically relevant.

INTRODUCTION

Diverse clinical and animal studies support the proposition that cross talk between leukocytes (specifically monocytes and polymorphonuclear leukocytes) and platelets contributes to inflammatory and thrombotic processes. Platelet-leukocyte adhesive interactions in the systemic circulation are enhanced after percutaneous coronary interventions (1), unstable angina (2), myocardial infarction (3), and sepsis (4). Studies with animals also suggest that the interaction between these cell types can contribute to both inflammatory and thrombotic events during atherosclerosis (5), leukocyte recruitment at sites of vascular injury (6), and microvascular occlusion after ischemia-reperfusion injury (7). Besides the physical association between leukocytes and platelets, bioactive peptides including cytokines and growth factors secreted by these cells also influence both cells and the vascular endothelium.

The adhesion molecules involved in platelet-leukocyte interactions have been identified. The recognition of P-selectin expressed on activated platelets (8,9) by its neutrophil counter-ligand P-selectin glycoprotein ligand-1 (PSGL-1) (10,11) has been shown to mediate the rolling of neutrophils on stimulated, immobilized platelet monolayers and to facilitate platelet-neutrophil adhesion in suspension. The neutrophil CD18 integrin subunit Mac-1 (CD11b/CD18) mediates the firm arrest of neutrophils on platelet monolayers (12,13). This molecule also contributes to platelet-neutrophil heterotypic cellular aggregation in suspension (10,14,15). Putative ligands for integrins on platelets are numerous and their contribution to platelet-neutrophil adhesion appears to depend on the nature of the experimental system used. These ligands include ICAM-2 (16,17), junctional adhesion molecule-3 (JAM-3) (18), GpIbα (19), and $\alpha_{IIb}\beta_3$ (16). Among these, $\alpha_{IIb}\beta_3$ is bridged to Mac-1 via plasma fibrinogen.

Besides the functional importance of platelet-leukocyte interactions in human physiology, there is also considerable interest in understanding the biophysical parameters regulating selectin and integrin binding at both the cellular and molecular levels. Such studies have quantified the on- and off-rates of selectins and integrins, and reported on a ''shear-threshold'' phenomenon for selectins (20).

In this article, we examined the dynamic contributions of selectins and integrins to heterotypic platelet-neutrophil aggregation under controlled fluid shear, with focus on the contrast between the roles of L-selectin in homotypic neutrophil adhesion and those of P-selectin in neutrophil-platelet attachment. The contrast between the contributions of P-selectin PSGL-1 bonds to neutrophil-platelet adhesion in suspension and its role in mediating leukocyte localization on immobilized platelet substrates is also emphasized. In this regard, the molecular requirements for cell adhesion in suspension can be different from those required for localization on ligand-bearing substrate. There are several reasons for this. 1), When neutrophil PSGL-1 binds P-selectin on immobilized platelets, the neutrophil cell membrane is

Submitted May 4, 2005, and accepted for publication December 5, 2005.

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0006-3495/06/03/2221/14 $2.00

doi: 10.1529/biophysj.105.065789

stretched and forms tethers that shield the application of high fluid tensile forces on the P-selectin bond (21). Leukocyte tethers, however, are not formed when cells adhere in suspension due to the rapid rotation of aggregates (22,23). 2), At a given shear rate, the maximum force applied on platelet-neutrophil aggregates in suspension is expected to be ~20-fold lower than the force applied when neutrophils tether onto platelet-bearing substrates (22). 3), The nature of cell-cell contact and the frequency of receptor-ligand encounter on apposing cells is also very different when two cells bind in suspension compared to cell-substrate attachment.

Two specific questions are addressed in this manuscript: 1), Are functional integrins necessary for stable platelet-neutrophil adhesion? 2), Do P-selectin PSGL-1 interactions exhibit a shear threshold in suspension assays? To address these questions, we performed cell-adhesion experiments with isolated blood cells, reconstituted systems with P-selectin bearing beads, and whole blood. Cone-plate rheometers were used to apply defined shear rates to the cell and bead suspensions. Flow cytometry-based analysis of aggregate distribution was noted to be reliable only at the higher shear rates >100/s. Thus, high-speed videomicroscopy coupled with a rheometer was applied to directly visualize, in real-time, shear-induced cell and bead collisions at low shear rates <100/s. These studies at low shear rates (<100/s) distinguish this article in relation to previous rheometer studies (24,25), since we were able to study P-selectin PSGL-1 interactions at low force-loading rates that are of interest to the biophysics community (26,27). Our experiments suggest a weak shear-threshold behavior for P-selectin PSGL-1 interaction at shear rate <100/s. Although previous studies have demonstrated a dominant role of selectins in mediating platelet-leukocyte binding (10,24,25,28,29), they were unable to unambiguously rule out the possibility that other molecular pairs like integrins are necessary for this cellular aggregation process. This is because data from cellular systems are complex to interpret since activated platelets can secrete (or express on their surface) an array of cytokines and proteases that can affect neutrophil function. To overcome this limitation, we developed a simpler reconstituted system with P-selectin-bearing beads. These beads were observed to adhere to quiescent human neutrophils efficiently, in the absence of functional CD18-integrins. Antagonists against P-selectin PSGL-1 interactions were also able to dynamically dissociate previously formed platelet-neutrophil aggregates under fluid shear in a range of experimental systems, including in studies conducted using whole blood. Together these experiments reveal an integrin-independent regime for cell adhesion that may be physiologically important.

METHODS

Materials

All antibodies were from mouse hosts unless otherwise stated, and were used at saturating concentrations (~15–20 μg/mL) for blocking studies. Monoclonal antibody (mAb) against human P-selectin/CD62P G1 was from Ancell (Bayport, MN), Ep5C7 (humanized IgG2) was a gift from Protein Design Laboratory (Fremont, CA), and phycoerythrin-labeled AK-4 was from Becton Dickinson (San Diego, CA). Function-blocking mAb against PSGL-1/CD162 KPL-1 was from Pharmingen (San Diego, CA), anti-ICAM-2/CD102 B-T1 from Serotec (Raleigh, NC), anti-GpIbα/CD42b VMD16d from Cell Sciences (Norwood, MA), and anti-$\alpha_{IIb}\beta_3$ 7E3 was kindly provided by Dr. J. Balthasar (SUNY-Buffalo, Buffalo, NY). Blocking mAbs against human β_2-integrin/CD18 IB4, α_L-chain/CD11a TS1/22, α_M-chain/CD11b 44, and L-selectin/CD62L DREG-56 were produced from hybridomas obtained from ATCC (Manassas, VA). F(ab′)$_2$ fragments of IB4 were purchased from Ancell. Nonfunction-blocking, fluorescein isothiocyanate (FITC)-conjugated anti-β_3/CD61 mAb Y2/51 was from Dako (Carpinteria, CA). FITC-conjugated CBRM1/5, an mAb that recognizes an activation-dependent epitope on Mac-1 (CD11b/CD18), was from eBiosciences (San Diego, CA). All isotype-matched control mAbs were from Becton Dickinson. Sialyl Lewis-X analog TBC1269 (1,6-bis [3-(3carboxymethylphenyl)-4-(2-α-D-mannopyrano-syloxy)phenyl]hexane) was generously provided by Kurt Berens (Encysive Pharmaceuticals, Houston, TX). Thrombin receptor-activating hexapeptide (TRAP) was from Bachem Bioscience (King of Prussia, PA) and nuclear stain LDS-751 from Molecular Probes (Eugene, OR). P-selectin fusion protein (P-selectin-IgG) containing the human P-selectin lectin and EGF domains fused to a mouse IgG2a Fc portion was produced using the baculovirus expression system (Invitrogen, Carlsbad, CA) (30). When required, FITC conjugation of mAbs was performed by addition of 50-fold molar excess fluorescein-5-isothiocyanate (Molecular Probes) to protein in 0.1 M sodium bicarbonate buffer at pH 8.6 for 1 h, followed by quenching of reaction with 50 mM Tris, and separation of labeled product from unconjugated FITC using PD-10 desalting column (Amersham, Piscataway, NJ).

Neutrophil and platelet isolation

Human blood was obtained by venipuncture from healthy, nonsmoking adult volunteers. d-Phe-Pro-Arg-chloromethyl ketone (PPACK, Bachem; 100 μM) was used as an anticoagulant for platelet isolation, and 10 U/mL heparin was used for neutrophil isolation. Blood was drawn into 30 U/mL heparin for whole-blood studies. Platelet-rich plasma (PRP) was obtained from blood supernatant after first-step centrifugation at 135 $\times$ g for 15 min (31). Platelet-poor plasma (PPP) was obtained after a second centrifugation step at 2000 $\times$ g for 30 min. For viscometer studies, polymorphonuclear cells (PMNs) were isolated using PMN isolation media (Robbins Scientific, Sunnyvale, CA), as described elsewhere (32). Isolated cells were maintained in calcium-free HEPES buffer containing 0.1% human serum albumin at 4°C until use. For rheoscope experiments, PMNs were isolated from blood using a two-step centrifugation procedure (23), suspended in Ca^{2+}, Mg^{2+}-free Tyrodes containing human serum albumin, and kept on ice until use. In all cases, neutrophils constituted >90% of the PMNs. Thus, we refer to these isolated PMNs as neutrophils. Experiments were completed within 2 h of neutrophil isolation. Whole-blood studies were completed within 1 h of drawing blood.

P-selectin-bearing bead (Ps-bead) preparation

Fluoresbrite YO carboxylate microspheres (YO beads) 3.0 and 5.7 μm in diameter and nonfluorescent polybead carboxylate microspheres (white beads) of the same sizes were purchased from Polysciences (Warrington, PA). Primary amine groups on (Fab′)$_2$ fragments of polyclonal goat antimouse IgG (Jackson ImmunoResearch, West Grove, PA) were covalently coupled to these microspheres using carbodiimide chemistry (Application Notes 238C, Polysciences). To couple P-selectin to goat antimouse-bearing microspheres, soluble P-selectin-IgG was incubated with beads overnight at 4°C. Selectin site density on beads was varied by serial dilution of soluble protein during this step. Before experimentation, beads were washed twice and resuspended in HEPES buffer containing either 0.03 w/v % Pluronic

P105 or 1% BSA to block nonspecific adhesion. Before each experiment, a small aliquot of the Ps-beads (P-selectin beads) was incubated with humanized Ep5C7-FITC to quantify the selectin site density. Since the antibody is humanized, it binds minimally to free goat antimouse sites on the beads. Also, it is possible to quantify the selectin density on YO beads since these beads fluoresce in the FL2 (yellow-orange) channel of the FACSCalibur instrument (Becton Dickinson) and their effect on the FITC channel (FL1) can be readily accounted for by cytometry compensation. Quantum Simply Cellular microspheres (Bangs Laboratories, Fishers, IN) were used to determine the absolute P-selectin density (sites/μm^2) on Ps-beads and platelets. YO beads were used for flow-cytometry-based experiments and white beads for videomicroscopy runs (see below).

Flow-cytometry analysis of platelet-neutrophil and bead-neutrophil adhesion

All runs of platelet-neutrophil adhesion and some runs of bead-neutrophil binding were performed using a VT550 cone-plate viscometer (Haake, Paramus, NJ) to apply defined shear rates. This instrument consists of a 1° rotating cone placed over a stationary plate. Both the cone and plate were machined in plexiglass. Rotation of the cone at fixed angular velocity produces an approximately linear shear field (33).

Before platelet-neutrophil binding runs, three separate samples were prepared and incubated at room temperature for 5 min followed by 5 min incubation at 37°C, either in the presence or absence of function-blocking reagents. The three samples included platelets in PRP incubated with 1:15 CD61-FITC mAb, neutrophils with 4 μM LDS-751, and a third vial with PPP. HEPES buffer containing 1.5 mM calcium was used as the diluent in the above step. After the incubation, PRP, neutrophils, and PPP were placed as separate drops on the viscometer plate surface along with a fourth drop of TRAP stimulus. The plate was then quickly raised to the cone via a spring-mounted mechanism and shear was initiated. Thus, shear and stimulus were applied simultaneously. Unless otherwise stated, the final neutrophil concentration was $1 \times 10_6$ cells/ml, there were 8.12 ± 0.16 platelets per neutrophil, and plasma constituted 40% of the sheared sample volume. Physiological concentrations of platelets were not included in the study since this results in excessive platelet-platelet aggregation and precludes the accurate quantitation of cellular adhesion efficiency (discussed below). Samples withdrawn from the viscometer at fixed time intervals during experimentation were fixed in 1% paraformaldehyde at 4°C. In controls, aggregating samples were fixed with paraformaldehyde on the viscometer plate surface while shear was being applied. No difference was observed with runs where samples were withdrawn before fixation.

After overnight fixation at 4°C, samples were read in the flow cytometer. The neutrophil population was gated based on forward versus side scatter. Single neutrophils [N_1] and percent platelet-neutrophil adhesion ($100 \times \Sigma PN_i/\Sigma(N_i + PN_i)$; $i = 1$–4) were quantified (Fig. 1 *A*). Here, N_i denotes the number of homotypic neutrophil aggregates with i cells and PN_i is the number of aggregates with i neutrophils and at least one attached platelet. Percent platelet-neutrophil adhesion is a measure of the fraction of total cytometry events detected that consist of heterotypic events. The percent of neutrophils in homotypic and heterotypic aggregates was measured using two additional parameters, percent neutrophil homotypic adhesion ($100 \times (\Sigma iN_i - N_1)/\Sigma(N_i + PN_i)i$; $i = 1$–4) and percent neutrophil heterotypic adhesion ($100 \times (\Sigma iPN_i)/\Sigma(N_i + PN_i)i$; $i = 1$–4), respectively. Platelet aggregation was also monitored in all runs using the fluorescence due to CD61-FITC, but this was negligible due to the low concentration of platelets. Platelet microparticle formation was not observed. Representative cell-aggregation samples were observed under the microscope to confirm the validity of our cytometry measurements. Specific antibody-blocking studies described in this article provide additional validation for the cytometry detection methods employed.

The procedure for bead-neutrophil binding studies was similar to that for platelet-neutrophil runs, described above, except that PPP and TRAP were absent. Here, samples were read in the flow cytometer immediately (within 5–10 s) after sample collection, without fixation. Neutrophil concentration was 1×10^6 cells/ml. Bead concentration exceeded that of neutrophils by 13.09 ± 0.63-fold for runs with 3.0-μm beads and 2.14 ± 0.22-fold for those with 5.7-μm beads. Percent of bead-neutrophil adhesion was quantified as number of neutrophil events with bound beads/number of total neutrophil events ($100 \times (\Sigma NB_i)/(N_1 + \Sigma NB_i)$; $i = 1$–4), where NB_i is the number of aggregates with i beads and at least one neutrophil.

Cell- and bead-adhesion data were also analyzed in terms of ''adhesion efficiency'', as we have described elsewhere (34,35). Briefly, adhesion efficiency is defined as the fraction of cell-cell or bead-cell rectilinear collisions that result in cell adhesion. This was calculated for neutrophil-neutrophil, platelet-neutrophil, and bead-neutrophil collision events. In performing adhesion-efficiency computations for neutrophil-platelet adhesion runs, it is noted that the exact number of platelets bound to each neutrophil is not available from the cytometry data, though an approximate idea can be obtained based on the fluorescence of single platelets and that of aggregates with bound platelets. The exact computation of adhesion efficiency thus involved the fitting of percent platelet-neutrophil adhesion, neutrophil homotypic adhesion, and neutrophil heterotypic adhesion data from experiments using the model predictions while accounting for the approximate number of platelets bound to each neutrophil.

High-speed imaging of bead-neutrophil adhesion

Two-body bead-neutrophil collisions in linear shear flow were visualized in a rheoscope, which consists of an MR-1 transparent counter-rotating cone-plate rheometer (Myrenne, Fremont, CA) with a 2° cone maintained at 22 ± 1°C, mounted on a Zeiss Axiovert inverted microscope (Montreal, Quebec, Canada) (36). Data were acquired using a high-speed digital camera (Kodak Motion-Corder SR-500c, Roper Scientific, San Diego, CA) connected to a PowerMac G4 computer. Frame-by-frame analysis was performed using NIH Image1.62. In runs with 5.7-μm beads, equal concentrations of neutrophils and beads (5.0×10^6/ml) were mixed in Tyrodes buffer containing Mg^{2+} and Ca^{2+} with either 5 or 10% Ficoll added to increase media viscosity to 2.7 or 5.5 mPa·s, respectively. In 3.0-μm bead runs, 1.25×10^7/ml beads were mixed with 5.0×10^6/ml neutrophils. Two types of experiments were performed:

Adhesion-efficiency experiments

Detailed cell/bead trajectories before and after collision were recorded at shear rates of 14, 66, and 110/s at 60, 125, and 250 frames/s, respectively (23). Cells and beads traveling along adjacent streamlines in the flow field came together, and upon collision were subject to compressive forces between particles (Supplemental Materials, Fig. S1 and Movies). Doublets thus formed orbited about the center of free rotation, which lies along the axis joining the centers of the two colliding species. Beyond the point where the axis was oriented normal to the direction of flow, the forces became tensile for one-quarter orbit, and they attempted to break the doublet apart. ''Nonseparating doublets'' (Supplemental Materials, Movie A) were defined as those in which selectin bonds withstood the tensile force during this quarter orbit. These were followed until they broke-up (Movie B) or disappeared from view. ''Transient doublets'' were defined as those that broke up within this quarter-orbit of tensile force-loading. Bead-neutrophil adhesion efficiency was quantified by dividing the number of nonseparating doublets by the total number of collisions analyzed.

Doublet lifetime is reported in a normalized form as $t_{normalized} = \langle t_{meas} \rangle / \langle t_{theor} \rangle$, for all nonseparating doublets. Here, mean doublet lifetime $\langle t_{meas} \rangle$ (lifetime of aggregate) corresponds to the period between the time of first apparent contact of neutrophil with bead either to the time of doublet separation/break-up or the time when the doublet leaves the field of view. $\langle t_{theor} \rangle$ is the predicted theoretical average lifetime of the transient doublet formed without any adhesive contacts between the colliding species (i.e., in the absence of intercellular selectin bond formation). The calculation of $\langle t_{theor} \rangle \left(= \pi\left(r_e^D + 1/r_e^D\right)/G) r_e^D + 1\right))$ assumes that the rectilinear approaches between the colliding spheres along all angles of approach result

in the formation of a rigid doublet with an equivalent ellipsoidal axis ratio, r_e^D (37). Values of r_e^D for rotating rigid dumbbells of unequal diameter have been tabulated (38). Thus, $r_e^D = 1.72$ and 1.36 for the 3.0- and 5.7-μm bead-neutrophil doublets, respectively. $\langle t_{theor} \rangle$ is 2.79/G and 2.66/G for the two cases above.

Aggregation-kinetics experiments

These were obtained by recording bead-cell collisions at a lower framing rate (60 frames/s) at all shear rates. Single frames at intervals of 0.8 s were analyzed. Neutrophils in this frame were binned into single cells, homotypic or heterotypic aggregates. The time and shear-rate-dependent increase in the number of heterotypic aggregates was then computed and this was termed ''percent bead-neutrophil adhesion''.

Overall, percent bead-neutrophil adhesion and bead-neutrophil adhesion efficiency quantified using both the rheoscope (this section) and cytometry (previous section) methods have very similar physical meaning, even though they were determined using different experimental methods. Both methods offer distinct advantages: 1), The rheoscope is superior in that it allows direct visualization of collisions, whereas cell adhesion rates in the cytometer-based assay must be indirectly inferred after sample fixation in most cases. Although indirect inferences are reasonably reliable above shear rates of 100/s, they are not reliable at lower shear rates (see Results). 2), The statistics of data obtained from the cytometer, on the other hand, are superior to the rheoscope measurements since several thousand particles are analyzed at each time point in this device. The effect of the stochastic nature of bond formation on cell adhesion and its implications on the rheoscope experiments are discussed elsewhere (39). 3), In another distinction, the rheoscope follows the detailed trajectory of beads and cells before and after collision. Thus, this method accounts for the curvilinear trajectory of particles (36,37), whereas the modeling of cytometry data assumes that all linear trajectories result in cell collision (34). This may result in slightly lower estimates of ''adhesion efficiency'' using the cytometry method when the particle sizes are unequal, and when curvilinear trajectories are marked. As noted in Results, both systems yield adhesion efficiencies in the same range at ~100/s.

Platelet and neutrophil activation

Platelet activation was quantified by measuring cell-surface P-selectin expression using flow cytometry and labeled mAbs, AK-4, and Ep5C7. Two indices were measured (10): 1), the percent of platelets with greater-than-baseline P-selectin expression (percent P-selectin positive); and 2), the expression of P-selectin on activated cells based on the geometric mean fluorescence intensity of bound mAbs. Experimental conditions for these runs were designed to exactly mimic conditions in corresponding adhesion runs. We observed that platelet P-selectin expression was dependent on TRAP stimulus dose, and independent of applied shear and the presence of neutrophils.

Neutrophil activation was quantified by monitoring the binding of CBRM1/5-FITC to activated Mac-1 on the neutrophil surface, after 5 min incubation at 4°C.

Statistics

All data are presented as mean ± SE. Statistical significance was assessed using Students' t-test with unequal variance for dual comparisons, and ANOVA along with Student-Newman-Keuls test for multiple comparisons. $p < 0.05$ was considered significant.

RESULTS

TRAP-stimulated platelet-neutrophil adhesion under shear

Platelet-neutrophil adhesion was studied in the presence of serum proteins and physiological calcium levels using a cone-plate viscometer to apply fluid shear and flow cytometry to monitor cell adhesion (Fig. 1). Fig. 1 *A* shows representative cytometry data at a shear rate of 650/s 60 s

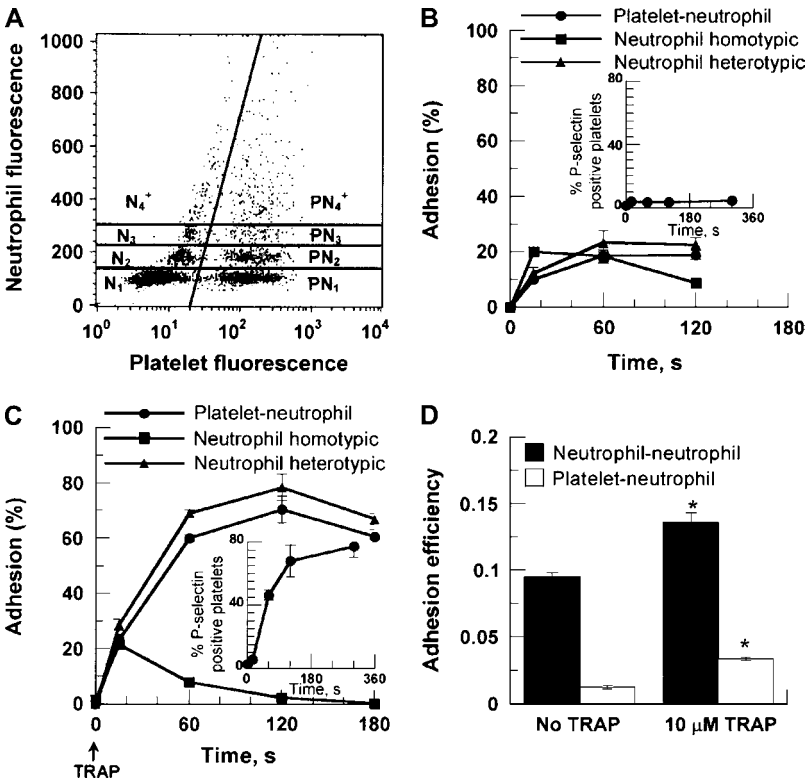

FIGURE 1 TRAP augments platelet-neutrophil adhesion. Neutrophils labeled with LDS-751, PRP labeled with CD61-FITC antibody, and PPP were mixed in a 1° cone and plate viscometer at 37°C in the presence or absence of TRAP stimulus. Samples obtained during the run were fixed in buffer containing 1% paraformaldehyde and read using a flow cytometer. (*A*) Neutrophil populations were gated based on their characteristic forward- versus side-scatter profile. Single neutrophils (N_1), platelet-neutrophil (PN_i, i = 1–4), and neutrophil homotypic (N_i, $i > 1$) aggregates were identified based on the red fluorescence of LDS-751 (neutrophil marker) and green fluorescence due to CD61-FITC (platelet marker). Data are representative of shear runs at 650/s, where cells were stimulated with 10 μM TRAP at 0 s, and samples were collected at 60 s after shear. Upon comparison of the mean fluorescence of individual platelets with that of singlet neutrophils with bound platelets (PN_1 population), we estimate that on average ~2.2 ± 0.1 platelets were bound to each neutrophil. (*B*) Cell adhesion kinetics in the absence of stimulus. (*C*) Cell adhesion kinetics upon addition of 10 μM TRAP at 0 s. Shear rate is 650/s in panels *B* and *C*. Insets show platelet P-selectin expression in these runs. Platelet-neutrophil adhesion was more pronounced in the presence of TRAP. (*D*) Adhesion efficiency for platelet-neutrophil and neutrophil-neutrophil adhesion estimated by modeling aggregation data at 650/s over the first 60 s. Error bars represent the standard error for four to seven independent experiments. *$p < 0.05$ with respect to no TRAP runs.

after application of shear and stimulus. In the absence of stimulus, ~20% of neutrophils formed aggregates with platelets in 2 min (Fig. 1 *B*). Platelet stimulation with 10 μM TRAP at zero time markedly augmented cell adhesion rates, and ~80% of neutrophils were bound to platelets within 2 min (Fig. 1 *C*). The extent of heterotypic cell adhesion correlated with the expression of P-selectin on the platelet surface (Fig. 1, *B* and *C*, *insets*). In both cases, within 15 s, ~20% of neutrophils were observed to rapidly form homotypic aggregates. This homotypic cell binding became reversible within 2 min. The more rapid decrease in neutrophil homotypic adhesion in the presence of TRAP suggests that some of the homotypic aggregates also bound activated platelets to form heterotypic aggregates. The homotypic neutrophil aggregation process could be blocked by antagonizing the Src tyrosine kinase signaling pathways in neutrophils (Xiao and Neelamegham, State University of New York at Buffalo, unpublished). Blocking antibodies against L-selectin and CD18 also abrogated this homotypic adhesion process in a manner similar to that described previously (32,34). Adhesion efficiency (fraction of intercellular collisions resulting in adhesion) was quantified for neutrophil-neutrophil and platelet-neutrophil collisions (Fig. 1 *D*). Platelet-neutrophil and neutrophil homotypic adhesion were higher in the presence of TRAP. Approximately 4% of platelet-neutrophil collisions resulted in adhesion upon TRAP addition.

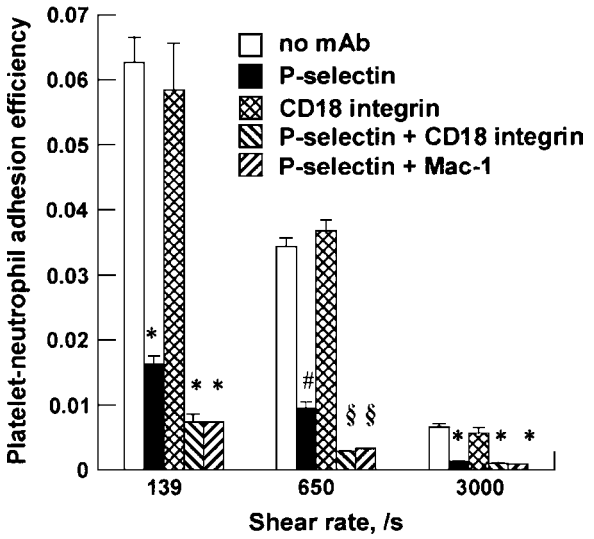

FIGURE 2 Contribution of P-selectin and Mac-1 as a function of shear rate. Effect of blocking antibodies against P-selectin (G1), CD18 integrin (IB4), and Mac-1 (mAb44) on heterotypic cell adhesion efficiency was studied over a range of shear rates. A 10-μM TRAP stimulus was added at 0 s in these runs. Anti-P-selectin antibody acted in cooperation with anti-Mac-1 to abrogate adhesion at all shear rates. Error bars represent the standard error for three to six experiments. $p < 0.05$ with respect to: *, no mAb and CD18 integrin; $^{\#}$, all other treatments; and §, no mAb, P-selectin, and CD18 integrin.

Cooperativity between platelet P-selectin and neutrophil Mac-1 over a range of shear rates

We next examined the contribution of P-selectin and Mac-1 to platelet-neutrophil adhesion over a range of shear rates (Fig. 2). Function-blocking antibodies to P-selectin partially inhibited cell adhesion at low shear rates and completely abrogated platelet-neutrophil binding at the higher shear rate, 3000/s (shear stress = 2.15 Pa or 21.5 dyn/cm^2). Antibodies to CD18-integrin or Mac-1 alone did not markedly alter cell binding at any shear rate, though these reagents abrogated cell adhesion when added with P-selectin antibodies. Blocking antibody against LFA-1 did not alter heterotypic adhesion when added alone or in combination with P-selectin (data not shown). The observation that neither P-selectin nor CD18-integrin mAbs completely blocked heterotypic cell adhesion at low shear rates suggests that either adhesion molecule may be capable of individually mediating cell binding under these conditions.

Unactivated neutrophils avidly bind P-selectin-bearing beads under shear

Blocking studies in Fig. 2 suggest that P-selectin bonds alone, without CD18-integrin participation, may be capable of mediating platelet-neutrophil adhesion in a purely selectin-dependent fashion. However, in the above studies, it is also possible that cytokines and proteases secreted or borne on the activated platelet surface may affect the function of either the CD18-integrins or their ligands. To clearly distinguish between these two features and to study the biomechanics of P-selectin PSGL-1 interactions under flow, we developed a reconstituted system where the adhesion of 3.0- and 5.7-μm Ps-beads to isolated human neutrophils was studied. Experiments were conducted using both flow cytometry to detect bead-cell adhesion (Fig. 3, *A–C*) and the rheoscope to visually observe this phenomenon (Fig. 3 *D*, Supplemental Movies). Flow cytometry was suited for studies conducted at higher shear rates (100-3000/s), whereas the rheoscope was better at lower shear (14–110/s).

Fig. 3 *A* presents representative cytometry histograms that quantify the expression of P-selectin on microspheres compared to activated human platelets. Platelets stimulated with 25 μM TRAP for 3 min expressed 16,000 $\pm$ 1170 P-selectins/cell, which is in reasonable agreement with published data (40,41). In comparison, 3.0- and 5.7-μm Ps-beads expressed selectins in the range of 37,000–83,000 and 64,000–190,000 sites/bead, respectively. After accounting for platelet size and contours of its cell membrane, selectin densities on our beads are ~2.5–10 times greater than that on platelets. As discussed later, such high densities are necessary in bead experiments, due to the pronounced curvilinear trajectory and reduced receptor-ligand encounter frequency as smooth particles approach cells. Such trajec-

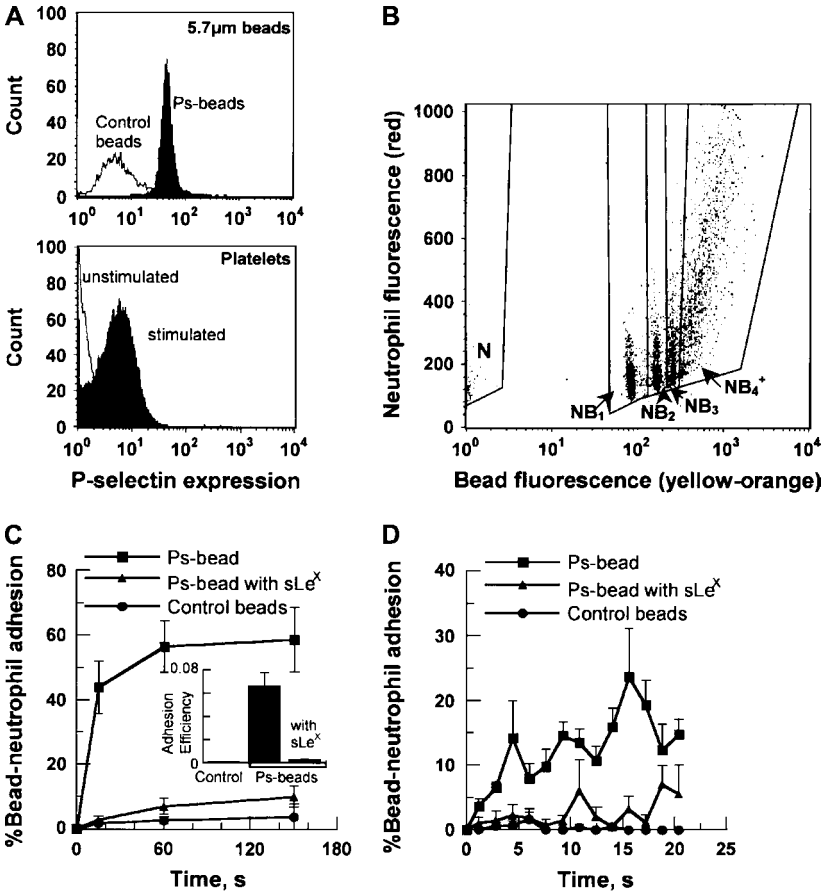

FIGURE 3 Neutrophil binding to Ps-beads. (*A*) P-selectin expression detected on 5.7 μm white Ps-beads using FITC-conjugated humanized antibody Ep5C7 (*upper*) compared to platelets activated with 25 μM TRAP for 3 min (*lower*). Flow cytometry settings are the same in both panels. Control beads (goat anti-mouse IgG-bearing beads without recombinant P-selectin) incubated with Ep5C7-FITC was the negative control for the upper panel. Unstimulated platelets were used as the negative control for the lower panel. (*B*) Binding of 5.7-μm Ps-beads to neutrophils was detected using yellow-orange fluorescence of beads and red fluorescence of LDS 751-labeled neutrophils. (*C*) Binding of 5.7-μm Ps-beads (1400 $\pm$ 103 P-selectin/μm^2) to neutrophils at 650/s is completely blocked by 2 mM soluble sLe^X analog TBC1269 in flow cytometry runs. Inset presents calculated adhesion efficiency. (*D*) Binding of 5.7-μm Ps-beads (650 P-selectin/μm^2) to neutrophils at 110/s (shear stress 0.6 Pa) is also blocked by 2 mM TBC1269 in rheoscope runs. Error bars represent the standard error for three or more experiments in panels *A–C* and five or more experiments in panel *D*.

tories dramatically reduce the frequency and duration of bead-cell interactions in comparison with platelet-neutrophil collisions.

When Ps-beads were mixed with unactivated neutrophils, we observed the rapid formation of bead-neutrophil aggregates suggesting that exogenous leukocyte activation is not necessary for cell adhesion (Fig. 3 *B*). Consistent with other reports (42), Ps-bead binding to neutrophils also did not augment activation of neutrophils as measured using mAb CBRM1/5 binding to these cells (data not shown). At 120 s, ~60% of neutrophils formed aggregates with 5.7-μm Ps-beads at 650/s (Fig. 3 *C*). Adhesion of Ps-beads to neutrophils was abrogated by TBC1269 (sLe^X analog) to levels comparable to control goat anti-mouse beads without P-selectin. Similar binding specificity was observed in rheoscope studies (Fig. 3 *D*).

Dependence of P-selectin-mediated adhesion on shear rate, shear stress, receptor density, and cell size

We measured neutrophil adhesion to platelets and Ps-beads over a range of shear rates to determine if P selectin-mediated adhesive interactions exhibit a shear-threshold phenomenon similar to L-selectin-mediated homotypic neutrophil aggregation (23,32). In platelet-neutrophil studies, two different TRAP doses were applied (Fig. 4 *A*). P-selectin receptor expression at 15 s at the higher TRAP dose was ~25 times that at the lower dose. Under both stimulus conditions, platelet-neutrophil adhesion efficiency was observed to decrease continuously with increasing shear rate from 79 to 3000/s. The efficiency of Ps-bead adhesion to neutrophils also decreased with increasing shear from 139/s to 1077/s (Fig. 4 *B*), and the shear-threshold phenomenon was not observed. The efficiency of 5.7-μm Ps-bead adhesion to neutrophils was similar to that of platelet-neutrophil binding after 10-μM TRAP stimulus. Overall, heterotypic aggregates were observed over the range of shear rates tested (shear stress = 0.035–2.15 Pa). This suggests that platelet-neutrophil aggregation is viable in both the venous and arterial circulation.

Rheoscope experiments were performed to contrast the dependence of adhesion rate on applied shear rate versus shear stress, and to study cell adhesion at low shear rates. We observed that bead-neutrophil adhesion increased with shear rate (Fig. 4 *C*). Approximately 50% of the neutrophils bound at least one 5.7-μm Ps-bead after 22 s when the shear rate

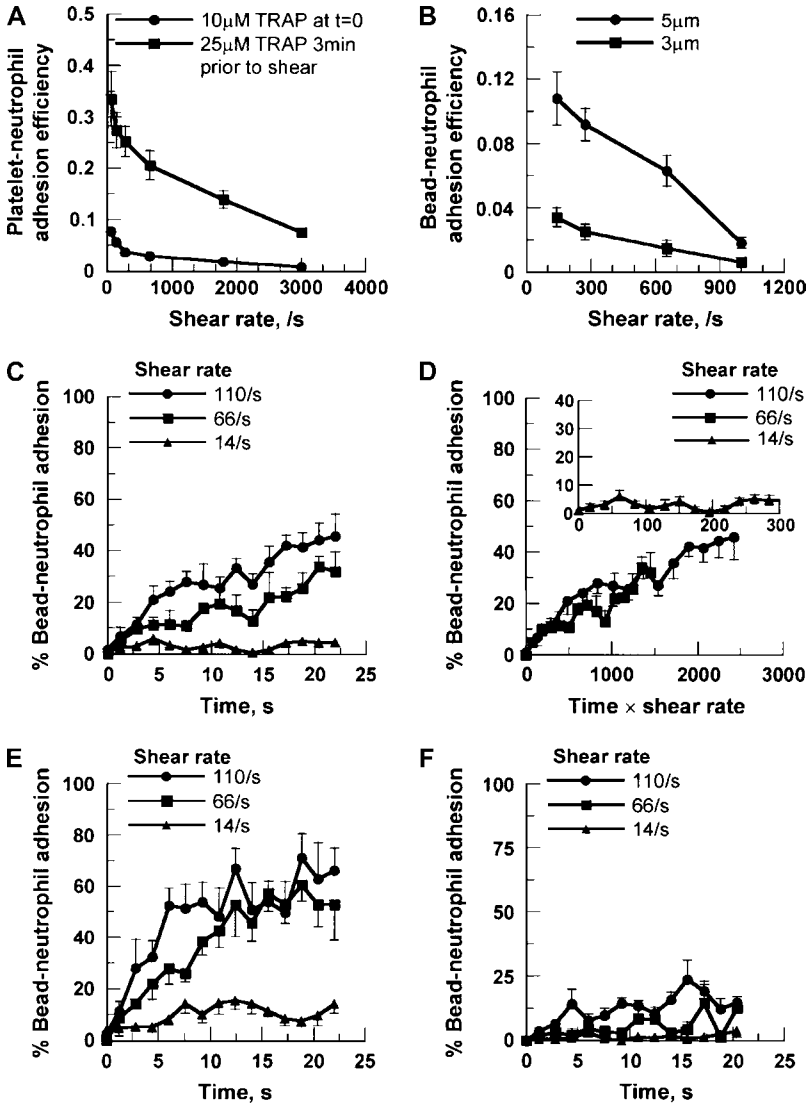

FIGURE 4 Shear dependence of P-selectin PSGL-1 binding. (*A*) Platelet-neutrophil adhesion experiments were conducted from shear rates of 79/s to 3000/s using flow cytometry to detect adhesion rates. Adhesion efficiency was quantified at low (10 μM TRAP at 0 s) and high (25 μM TRAP added to PRP 3 min before shear) stimulus conditions. Platelet-neutrophil adhesion efficiency decreased monotonically with shear from 79 to 3000/s. (*B*) Neutrophil adhesion to Ps-beads was examined for both 3.0- and 5.7-μm beads over the shear-rate range from 139/s to 1077/s using flow cytometry to quantify cell-bead adhesion. P-selectin density was 1300 $\pm$ 136 sites/μm^2 for 3.0-μm beads and 1400 $\pm$ 103 sites/μm^2 for 5.7-μm beads. Bead-neutrophil adhesion efficiency was quantified. It displayed a decrease with increasing shear. (*C–F*) Adhesion of 5.7-μm Ps-beads to neutrophils was measured using the rheoscope. In panels *C* and *E*, the P-selectin density was kept constant at 1899 sites/μm^2 while suspending fluid viscosity was increased from 2.76 mPa·s (*C*) to 5.49 mPa·s (*E*) by addition of ficoll. Data in panel *D* are identical to those in panel *C*, except that the *x* axis is normalized to account for the linear increase in bead-cell collision number with increasing shear rate. Conditions in panel *F* are identical to those in panel *E*, except that P-selectin density was reduced to 650 sites/μm^2. Error bars represent the standard error for three or more experiments in the first two panels, and five or more experiments in panels *C–F*.

was 110/s. Aggregation kinetics were low at 14/s. Increasing shear rate proportionally increases the frequency of particle collisions (34,43); thus, we normalized the adhesion kinetics by collision number by changing the *x* axis in Fig. 4 *D* from time to time $\times$ shear rate. After normalization, we still observed lower aggregation kinetics at 14/s compared to higher shear rates. When the suspending-medium viscosity was doubled by the addition of 10% Ficoll (Fig. 4 *E*), thereby doubling the applied shear stress without altering the cell collision frequency (23,32), we noted a marked increase in bead-neutrophil adhesion rate upon increasing medium viscosity (Fig. 4, *C* versus *E*). Similar observations as in Fig. 4, *C–E*, were made during studies where 3.0-μm Ps-beads collided and aggregated with human neutrophils in the rheoscope apparatus (Fig. 5).

With the goal of more carefully examining the above observations, we acquired rheoscope data at higher framing rates and analyzed individual collisions (Tables 1 and 2). Such studies complement data in Figs. 4 and 5, since in addition to quantifying stable bead-cell aggregates after multiple rotations, they also account for nonseparating doublets that form for a few orbits before breaking up. These experiments revealed that the efficiency of 3.0-μm Ps-bead adhesion to neutrophils increased with shear rate from 66/s to 110/s. In another set of experiments (Table 2) performed with 5.7-μm beads at a shear rate of 14/s and two different medium viscosities, we observed a statistically lower level of adhesion efficiency in runs performed at the lower shear stress (lower medium viscosity) compared to runs where the applied shear stress was higher. Studies performed at the lower shear stress also exhibited a greater ($\sim$52%) rate of breakage of nonseparating Ps bead-neutrophil doublets compared to runs at the higher shear stress ($\sim$15%). The above observations are consistent with the proposition that P selectin-PSGL 1 interactions in suspension exhibit a shear threshold at low shear stresses.

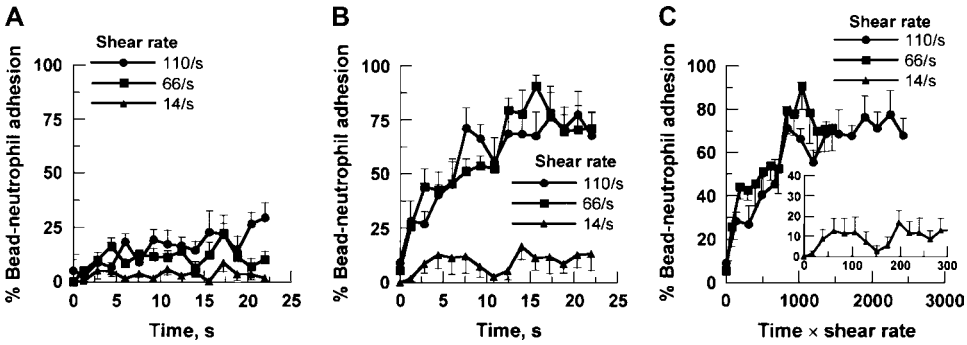

FIGURE 5 Shear threshold during the interaction between 3-μm Ps-beads and neutrophils. Neutrophil adhesion to 3.0-μm Ps-beads was measured using the rheoscope and methods identical to those described in Fig. 4, *C–E*, legend. P-selectin density was 2938 sites/μm^2 in all cases. Suspending fluid viscosity was increased from 2.76 mPa·s (*A*) to 5.49 mPa·s (*B*) by addition of ficoll. Data in panel *C* are identical to those in panel *B*, except that the *x* axis is normalized to account for the linear increase in bead-cell collision number with increasing shear rate. Error bars represent the standard error for at least five experiments.

To determine whether the shear-dependent selectin-binding interaction observed in the rheoscope runs also took place when activated platelets collided with neutrophils, studies were undertaken at low shear rates (15–180/s) using the viscometer to apply defined shear and flow cytometry to detect cell adhesion rates. These studies, which were conducted in the presence of an $F(ab')_2$ blocking mAb against CD18, were, however, inconclusive since the level of shear and the number of cell-cell collisions taking place during preparation for these low-shear runs and during the sampling step were comparable to the shear rates studied in the individual runs. Neutrophil-platelet adhesion studies could also not be performed using the rheoscope due to limitations of the microscope's optics. Taken together, the above studies provide evidence of a weak shear-dependent process controlling P-selectin-mediated neutrophil adhesion to Ps-beads at low shear rates. This is unlike L selectin-mediated neutrophil-neutrophil binding, where the shear-threshold phenomenon is very prominent (23,32,34).

Platelet-neutrophil (Fig. 4 *A*) and Ps bead-neutrophil (Fig. 4, *E* versus *F*) adhesion rates decrease when P-selectin coverage is low. Collision analysis (Table 1) revealed that although decreasing P-selectin density on Ps-beads did not affect the adhesion efficiency, the fraction of nonseparating doublets that broke up before leaving the field of view was greater at the lower densities. Thus, high selectin densities likely favor the sustenance of platelet-neutrophil aggregates in circulation.

P-selectin PSGL-1 bonds alone can hold platelet-neutrophil aggregates together over multiple rotations

We examined whether P-selectin PSGL-1 bonds alone can stably form platelet-neutrophil aggregates under hydrodynamic shear. To test this, PRP was first stimulated with 25 μM TRAP for 3 min to achieve high platelet P-selectin expression. Stimulated platelets were then mixed with unactivated neutrophils at a shear rate of 650/s for 60 s before addition of blocking mAbs. Anti-P-selectin or anti-PSGL-1 mAbs added at 60 s, but not anti-CD18-integrin antagonists, rapidly dissociated platelet-neutrophil aggregates formed in the first 60 s (Fig. 6, *A* and *B*). Break-up of previously formed

TABLE 1 Frame-by-frame analysis of P-selectin PSGL-1 interactions in rheoscope

Site density, selectin (μm^2)	Shear rate (/s)	Shear stress (Pa)	No. of doublets analyzed	Bead-neutrophil adhesion efficiency*	% Nonseparating doublets that break up†	$t_{normalized}$§
			5.7-μm diameter Ps-beads			
650	66	0.36	347	0.150 ± 0.025 ($n = 32$)	6.8 (3/44)	9.69 ± 0.83
650	110	0.60	222	0.133 ± 0.025 ($n = 24$)	6.3 (2/32)	11.72 ± 2.45
1899	66	0.36	200	0.141 ± 0.018 ($n = 12$)	0 (0/27)	16.02 ± 4.63
1899	110	0.60	216	0.124 ± 0.025 ($n = 26$)	0 (0/28)	12.14 ± 1.63
			3.0-μm diameter Ps-beads			
2938	66	0.36	202	0.023 ± 0.011 ($n = 18$)	0 (0/4)	9.75 ± 2.36
2938	110	0.60	139	0.099 ± 0.029 ($n = 34$)‡	0 (0/12)	19.3 ± 6.32

Medium viscosity was 5.49 mPa·s in all runs.

*Data are presented as mean ± SE. Numbers in parentheses denote the number of sequences for each condition. Mean ± SE was calculated based on the adhesion efficiency measured in each sequence.

†Percentage of all nonseparating doublets that were seen to break up after surviving tensile forces in alternate quarter orbits before being lost from the field of view.

‡$p < 0.05$ with respect to 66/s run with 3.0-μm Ps-beads.

§Doublet lifetime [$t_{normalized} = \langle t_{meas} \rangle / \langle t_{theor} \rangle$] ± SE is reported for nonseparating doublets as defined in Methods.

TABLE 2 Rheoscope studies with 5.7-μm-diameter Ps-beads at shear rate of 14/s

Shear stress (Pa)	No. of doublets analyzed	Bead-neutrophil adhesion efficiency*	% Nonseparating doublets that break up†
0.039	221	0.092 ± 027 ($n = 15$)	52.1 (12/23)
0.077	241	0.225 ± 024 ($n = 15$)‡	18.4 (9/49)

Medium viscosity was 2.76 mPa·s at the lower shear stress and 5.49 mPa·s at the higher stress. Selectin site density was ~2000/μm^2.

*Data are presented as mean ± SE. Numbers in parentheses denote the number of sequences for each condition. Mean ± SE was calculated based on the adhesion efficiency measured in each sequence.

†Percentage of all nonseparating doublets that were seen to break up after surviving tensile forces in alternate quarter orbits before being lost from the field of view.

‡$p < 0.05$ with respect to a shear stress of 0.039 Pa.

aggregates was also observed when 10 μM TRAP was applied as a stimulus at 0 s.

We distinguished between the effects of the P-selectin/PSGL-1 antibodies above in preventing the formation of new platelet-neutrophil aggregates and in breaking up previously formed aggregates. To this end, we performed experiments where sheared samples were diluted with sevenfold excess buffer after 60 s of shear (Fig. 6 *C*). Sevenfold dilution effectively reduces the rate of aggregate formation by 49-fold without altering the break-up kinetics (34). If platelet-neutrophil binding were indeed spontaneously reversible, such treatment would be expected to lead to a rapid decrease in percent of platelet-neutrophil adhesion. In our runs, we did not observe such break-up of heterotypic aggregates after dilution. Even reduction of the shear rate to 49/s along with sample dilution did not promote break-up. Thus, platelet-neutrophil aggregates once formed via selectin bonds alone appear stable over multiple rotations, and addition of P-selectin PSGL-1 antagonists at 60 s (Fig. 6, *A* and *B*) breaks up previously formed heterotypic aggregates.

The dynamic break-up phenomenon could also be observed in whole-blood experiments (Fig. 6 *D*) and in studies where neutrophils bound Ps-beads (Fig. 6 *E*). In whole-blood studies, the break-up of platelet-neutrophil aggregates was observed when P-selectin antagonist was added at either 30 s (Fig. 6 *D*) or 150 s (data not shown). This suggests that the contribution of CD18 integrins to platelet-leukocyte adhesion is low even at longer times. In bead experiments, dilution after 60 s of shear did not break up previously formed bead-neutrophil aggregates. Similarly, Ps-bead neutrophil aggregates formed at 110/s in the rheoscope did not break up when shear rate was suddenly dropped to 14/s. Moreover, as shown by the values of $t_{\text{normalized}}$ in Table 1, most nonseparating platelet-bead doublets had long lifetimes and were seen to rotate over multiple orbits. Overall, our results demonstrate an integrin-independent regime for cell adhesion. Results of our studies with whole blood suggest that such a phenomenon may be physiologically relevant.

DISCUSSION

The biophysics of P-selectin interaction with neutrophil PSGL-1 was studied using a variety of experimental systems. To facilitate the Discussion, Table 3 compares the intercellular hydrodynamic forces, collision frequency, and receptor-ligand encounter frequencies for neutrophil binding in different experimental systems. These results are in part based on an earlier publication (22). Table 3 provides theoretical estimates of the degree to which results from the Ps-bead-neutrophil adhesion experiments mimic platelet-neutrophil adhesion runs.

Platelet-neutrophil aggregation in suspension was observed over both venous and arterial shear rates from 50 to 3000/s and shear stresses from 0.04 to 2.15 Pa. In contrast, neutrophil tethering on immobilized platelets occurs only below a shear stress of 0.4 Pa (9,12,44). This difference is likely due to the ~23-fold higher magnitude of fluid force applied on selectin bonds at a given shear rate, in the parallel-plate flow-chamber experiments compared to the suspension assay (Table 3).

The important role of P-selectin PSGL-1 binding, and CD18-integrin subunit (Mac-1) recognition by its platelet ligand(s) during cell adhesion was noted. Although formyl peptide and other chemokine-stimulated homotypic neutrophil aggregation is completely blocked at all shear rates by anti-CD18-integrin mAbs alone (32), in agreement with other published reports (24,25,28,29), CD18 antagonists were only marginally effective at blocking platelet-neutrophil binding when added alone. These reagents acted in synergy with P-selectin blockers to abrogate cell adhesion. This feature is likely due to the low off-rates of P-selectin PSGL-1 bonds, as discussed in detail below. Detailed studies performed using a panel of blocking mAbs against putative Mac-1 ligands (ICAM-2, $\alpha_{\text{IIb}}\beta_3$ and GpIbα) did not reveal any potent inhibitor over the range of shear rates tested when these reagents were added alone (Supplemental Material, Fig. S2). When added in combination with an anti-P-selectin blocking antibody, this panel of molecules also did not block cell adhesion more than the P-selectin antagonist alone. Blocking antibodies to JAM-3, which has also been implicated in platelet-neutrophil adhesion (18), were not available. As Santoso et al. (18) suggest, it is possible that multiple ligands have to be blocked along with JAM-3 to observe marked inhibition of heterotypic adhesion.

Observations of leukocytes rolling on substrates has firmly established the idea that selectin-mediated cell tethering/rolling is only the first step in a multistep process that is required for cell adhesion on vessel walls in vivo (45). Our studies extend this notion. They demonstrate that leukocyte-platelet aggregation in suspension is possible via both selectin-independent and integrin-independent mechanisms (Fig. 7). This is supported by observations that P-selectin PSGL-1 antagonists, but not CD18-integrin blockers, can break up

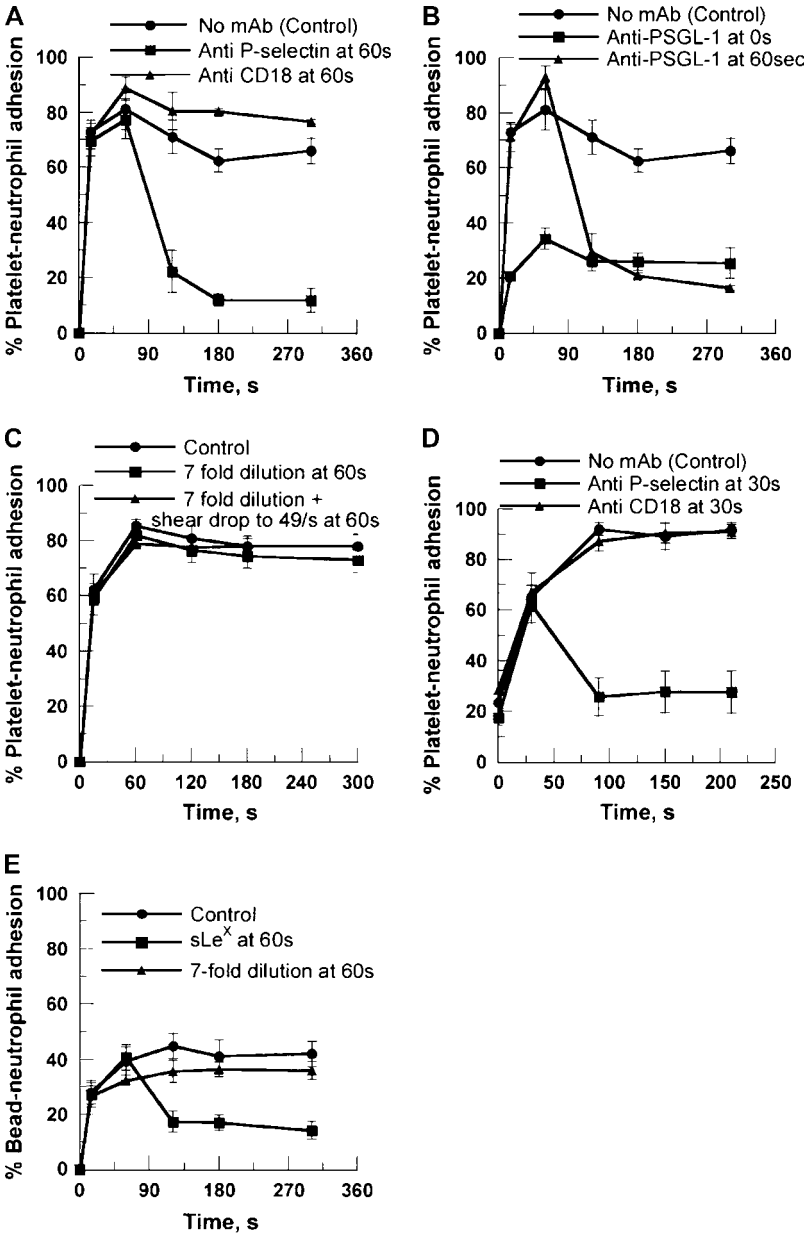

FIGURE 6 Antagonists against P-selectin PSGL-1 binding dynamically break up previously formed platelet-neutrophil aggregates. (*A* and *B*) PRP was incubated with 25 μM TRAP for 3 min before being sheared with PPP and unactivated neutrophils in the viscometer. Anti-P-selectin mAb G1, anti-CD18 integrin mAb IB4, or anti-PSGL 1 mAb KPL-1 were added to the cell suspension at 60 s in some runs and shear was continued. mAbs to P-selectin and PSGL-1, but not CD18 integrin, broke up previously formed platelet-neutrophil aggregates. (*C*) The control sample (same as no mAb treatment in *A*) was diluted sevenfold at 60 s with excess HEPES buffer containing calcium. Shear was also dropped to 49/s at the time of dilution in some cases. Neither sample dilution nor reduction in shear caused previously formed aggregates to dissociate. (*D*) Undiluted heparinized blood was stimulated with 2 μM TRAP at 0 s in the presence of GpIIb/IIIa blocking antibody 7E3 and sheared. Anti-P selectin mAb G1 or CD18-integrin mAb IB4 was added at 30 s in some cases. P-selectin antagonist broke up previously formed heterotypic aggregates. (*E*) Adhesion of 3-μm Ps-beads to neutrophils was studied. Addition of 2 mM TBC1269 (sLe^X analog) at 60 s caused previously formed cell-bead aggregates to break up. Applied shear rate was 650/s for all runs. Error bars represent the standard error for three or more experiments.

platelet-neutrophil doublets formed in both isolated cell systems and in whole blood. Our observations are reasonable given the following. 1), P-selectin PSGL-1 bonds have high tensile strengths that can withstand mechanical forces up to 165 pN (46). 2), Such interactions also exhibit lower off-rates in leukocyte-rolling experiments compared to L-selectin (47). Surface plasmon resonance measurements also show that L-selectin-GlyCAM-1 bonds are short-lived ($k_{off} > 10$/s) (48) compared to P-selectin PSGL-1 interactions ($k_{off} = 1.4 \pm 0.1$/s) (49). 3), The hydrodynamic forces that attempt to separate platelets from neutrophils are also approximately sixfold smaller than those that attempt to break neutrophil-neutrophil doublets at any given shear rate (Table 3). This is due to the small size of platelets, which shield the intercellular bonds from fluid forces. 4), Finally, P-selectin is present at high densities on both activated platelets and beads, and thus multiple bonds can engage as the aggregates rotate in shear flow. Real-time videomicroscopy observations of Ps-bead-neutrophil collisions reveal that although the initial efficiency of transient nonseparating Ps bead-neutrophil doublet formation was independent of P-selectin density, bead-cell doublets with higher P-selectin density were more stable after multiple orbits. Thus, P-selectin is important not only for the formation of nonseparating heterotypic aggregates but also for their sustenance over multiple rotations. Such sustenance extends the time available for neutrophil Mac-1 activation and thus enhances leukocyte-platelet adhesion rates.

TABLE 3 Biophysical parameters regulating neutrophil adhesion in a variety of experimental systems

	Cell adhesion in suspension*					Cell attachment to substrate†
Cell parameter	Neutrophil-neutrophil	Platelet-neutrophil	5.7-μm Ps-bead neutrophil adhesion		3.0-μm Ps-bead neutrophil adhesion	Neutrophil binding to immobilized P-selectin
Radius of second species (μm)	4.5	1.5	2.85	2.85	1.5	–
Separation distance (μm)	0.63	2.33	0.315	0.315	0.315	0.315
Ligand density on second species (sites/μm^2)	235.8	246.0	650.0	1899.0	2938.0	250.0
Maximum intercellular force (pN)‡	267.3	48.6	164.0	164.0	66.7	1139
Collision frequency (normalized)§	0.423	0.637	0.302	0.302	0.186	–
Receptor-ligand encounter frequency (number/collision)¶	7.36	1.72	2.95	8.62	7.79	–

All calculations were performed assuming that the fluid is Newtonian with a viscosity (μ) of 0.001 Pa·s, and applied shear rate (G) is 600/s. Cells were approximated to be equivalent to spheres with surface protrusions dictated by their microvilli length. Neutrophils were designated to be the ''first species'' and these were modeled as spheres of radius 4.5 μm and 0.315-μm-length microvilli bearing PSGL-1 at 235.8 sites/μm^2. The ''second species'' refers to the cells or particles that interact with neutrophils. This may be another neutrophil with L-selectin density at 235.8 sites/μm^2, an activated platelet of size 1.5 μm with surface extensions up to 2.01 μm or smooth P-selectin-bearing beads of varying size and ligand density.

*Such attachment may occur in suspension either in viscometer studies or in vivo.

†Such attachment may occur in flow-chamber experiments or on the injured blood vessel walls. Force calculations are based on previously published work (52,57), without accounting for neutrophil tether formation/extension.

‡Calculations, performed as described elsewhere (22), were used to determine maximum intercellular force. Force varies linearly with μ and G.

§To calculate cell collision frequency, particle trajectories were simulated by solving the creeping-flow equation (22) assuming that there are no attractive/repulsive forces acting between the colliding species. Cell-cell collision was defined as occurring when the separation distance between the first and second species was less than the sum of the surface microvilli/protrusions. Collision-frequency data are presented as collision number/time/volume normalized by collision frequency predicted by two-body linear collision theory (43).

¶Receptor-ligand encounter frequency was calculated by determining the surface area on the two colliding species that came in contact with each other during the intercellular collision simulations described above. A contact zone size of 50 nm was assumed to form between the two colliding species. The number of receptors and ligands that entered this contact zone during a single collision was then defined to contribute to receptor-ligand encounter frequency. This parameter varies linearly with G, receptor density, and ligand density.

We tested whether P selectin-mediated platelet-neutrophil adhesion in suspension exhibits the shear-threshold phenomenon. This phenomenon has been observed during L-selectin-mediated neutrophil homotypic aggregation (23,32) and during leukocyte rolling on peripheral node addressin (50). Leukocyte rolling on P-selectin substrates also exhibits the shear-threshold phenomenon, though this is only observed in a narrow range of selectin densities ($\sim$150 sites/μm^2) and when the applied shear stress is low (26,51). Shear threshold during leukocyte rolling occurs at $\sim$0.025–0.05 Pa for P-selectin versus 0.1 Pa for L-selectin (50,51). Shear-threshold phenomena in suspension assays have shown that 1), homotypic neutrophil aggregation is low at $<$250/s and $>$1200/s, and it peaks at intermediate shear rates (32); 2), unstimulated neutrophils spontaneously form nonseparating doublets at 110/s, but these doublets dissociate when shear is reduced to 14/s (23); and 3), increasing shear stress by altering suspending medium viscosity also augments neutrophil-neutrophil binding rates below 250/s (23,32). In contrast to these observations, we did not observe the break-up of heterotypic doublets upon abrupt reduction in shear. We did, however, visualize augmented Ps bead-neutrophil binding upon doubling suspending medium viscosity at low shear rates. An increase in Ps bead-neutrophil aggregation rates was observed upon increasing shear rate from 14/s to 66/s in the case of 5.7 μm bead-neutrophil aggregates (site density of 1899 sites/μm^2), and between 14 and 110/s in the case of 3.0-μm bead-neutrophil aggregates. As seen in Table 3, the estimated receptor-ligand encounter frequency for both cases is very comparable and it coincides closely with the encounter rates in the cellular systems. Further, the force loading rates on single selectin-ligand bonds is estimated to be $\sim$287 pN/s at 30/s for 5.7 μm bead-neutrophil doublets, and 325 pN/s at 60/s for 3.0-μm-bead-neutrophil doublets. This estimate of force loading rate observed in our experiments matches remarkably well with the estimates of Evans et al. (27). Using the biomembrane force probe, these authors suggest that the strength and lifetime of PSGL-1 P-selectin bonds drops anomalously when loaded below 300 pN/s. Taken together, our observations suggest a shear-dependent regulation of P-selectin PSGL-1 bonding at low shear rates.

Although the extent of platelet-neutrophil adhesion was lower than that of neutrophil-neutrophil adhesion when 10-μM TRAP was used as a stimulus, the efficiency of the homotypic and heterotypic adhesion processes was comparable when 25 μM TRAP was the stimulus. This is a little surprising given the low on-rate of P-selectin bonds compared to L-selectin bonds (52). Although the off-rate and cell-surface densities of this molecule undoubtedly contribute to these efficiencies, our data also highlight an important role of platelet surface roughness in augmenting adhesion rates. In this context, fluid mechanical theory (53) shows that smooth unequal-sized spheres/discs with diameters equivalent to those of platelets and neutrophils cannot collide and adhere with the high efficiencies noted in our work ($\sim$0.42 at

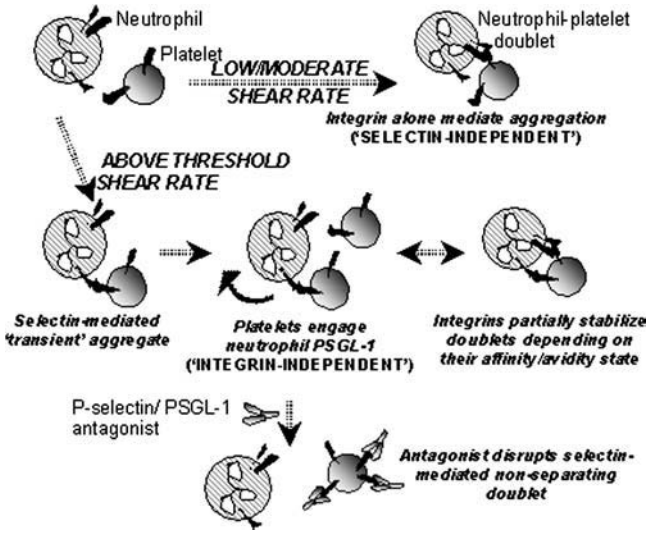

FIGURE 7 Model for platelet-neutrophil adhesion. Purely Mac-1-dependent (selectin-independent) heterotypic cell adhesion can take place at low/moderate shear rates (<650/s) as seen in Fig. 2. This process is driven by the modest activation of neutrophils when mixed with platelets, PPP, and TRAP. TRAP-stimulated platelets also upregulate their P-selectin expression. Due to the low off-rate and high tensile strength of P-selectin PSGL-1 bonds, this molecular interaction alone can mediate the formation of platelet-neutrophil aggregates over most shear conditions in an integrin-independent manner. CD18 integrins may help further stabilize these interactions, though the contribution of these molecules varies with time after stimulation (58). Thus, the contribution of CD18 integrins in forming heterotypic cellular aggregates depends on their affinity and avidity state. Addition of antagonists to P-selectin PSGL-1 bonds can dynamically break up previously formed platelet-neutrophil aggregates, provided integrins are not engaged.

49/s). Due to their smaller size, upon approach, these smooth spheres tend to follow a pronounced curvilinear trajectory, which reduces the number of collisions from that predicted by Smoluchowski's linear two-body collision theory (43). Such a feature is also evident in Table 3, which predicts that only ~42% of linear neutrophil-neutrophil trajectories are expected to result in collision events. In the case of platelets, however, the roughness of the cell allows ~64% of trajectories to result in collision events. This fraction is dramatically lower in the case of encounters between 3.0-μm beads and neutrophils, where only ~18% of the rectilinear trajectories result in collision. Due to the curvilinear trajectory of cells/particles and the absence of surface roughness on beads, the P-selectin density was kept high on the Ps-beads used in this study.

Overall, our study highlights the important biophysical features that contribute to platelet-leukocyte interactions. This understanding and the model of cell adhesion (Fig. 7) are likely to be important in the context of various therapeutic studies that suggest that inhibition of platelet-leukocyte binding with anti-P-selectin or recombinant PSGL-1 can be beneficial in treating vascular diseases including venous (54) and arterial thrombosis (55) and in-stent restenosis (56).

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

We acknowledge grant support from the National Institutes of Health (HL63014 and HL76211) and the Quebec Heart and Stroke Foundation.

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