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# Neutrophil String Formation: Hydrodynamic Thresholding and Cellular Deformation during Cell Collisions

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## Abstract

Neutrophils unexpectedly display flow-enhanced adhesion (hydrodynamic thresholding) to L-selectin in rolling or aggregation assays. We report that the primary collision efficiency ( $\epsilon$ ) of flowing neutrophils with preadhered neutrophils on intercellular adhesion molecule-1 (ICAM-1) or fibrinogen also displayed a maximum of  $\epsilon \sim 0.4\text{--}0.45$  at a wall shear rate of  $100\text{ s}^{-1}$ , an example of thresholding. Primary collision lifetime with no detectable bonding decreased from 130 to 10 ms as wall shear rate increased from 30 to  $300\text{ s}^{-1}$ , whereas collision lifetimes with bonding decreased from 300 to 100 ms over this shear range using preadhered neutrophils on ICAM-1, with similar results for fibrinogen. Antibodies against L-selectin, but not against CD11a, CD11b, or CD18, reduced  $\epsilon$  at  $100\text{ s}^{-1}$  by  $>85\%$ . High resolution imaging detected large scale deformation of the flowing neutrophil during the collision at  $100\text{ s}^{-1}$  with the apparent contact area increasing up to  $\sim 40\text{ }\mu\text{m}^2$ . We observed the formation of long linear string assemblies of neutrophils downstream of neutrophils preadhered to ICAM-1, but not fibrinogen, with a maximum in string formation at  $100\text{ s}^{-1}$ . Secondary capture events to the ICAM-1 or fibrinogen coated surfaces after primary collisions were infrequent and short lived, typically lasting from 500 to 3500 ms. Between 5 and 20% of neutrophil interactions with ICAM-1 substrate converted to firm arrest ( $>3500\text{ ms}$ ) and greatly exceeded that observed for fibrinogen, thus defining the root cause of poor string formation on fibrinogen at all shear rates. Additionally, neutrophils mobilized calcium after incorporation into strings. Static adhesion also caused calcium mobilization, as did the subsequent onset of flow. To our knowledge, this is the first report of 1), hydrodynamic thresholding in neutrophil string formation; 2), string formation on ICAM-1 but not on fibrinogen; 3), large cellular deformation due to collisions at a venous shear rate; and 4), mechanosensing through neutrophil  $\beta_2$ -integrin/adhesion. The increased contact area during deformation was likely responsible for the hydrodynamic threshold observed in the primary collision efficiency since no increase in primary collision lifetime was detected as shear forces were increased (for either surface coating).

## Comments

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# Neutrophil String Formation: Hydrodynamic Thresholding and Cellular Deformation during Cell Collisions

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**ABSTRACT** Neutrophils unexpectedly display flow-enhanced adhesion (hydrodynamic thresholding) to L-selectin in rolling or aggregation assays. We report that the primary collision efficiency ( $\epsilon$ ) of flowing neutrophils with preadhered neutrophils on intercellular adhesion molecule-1 (ICAM-1) or fibrinogen also displayed a maximum of  $\epsilon \sim 0.4\text{--}0.45$  at a wall shear rate of  $100\text{ s}^{-1}$ , an example of thresholding. Primary collision lifetime with no detectable bonding decreased from 130 to 10 ms as wall shear rate increased from 30 to  $300\text{ s}^{-1}$ , whereas collision lifetimes with bonding decreased from 300 to 100 ms over this shear range using preadhered neutrophils on ICAM-1, with similar results for fibrinogen. Antibodies against L-selectin, but not against CD11a, CD11b, or CD18, reduced  $\epsilon$  at  $100\text{ s}^{-1}$  by  $>85\%$ . High resolution imaging detected large scale deformation of the flowing neutrophil during the collision at  $100\text{ s}^{-1}$  with the apparent contact area increasing up to  $\sim 40\text{ }\mu\text{m}^2$ . We observed the formation of long linear string assemblies of neutrophils downstream of neutrophils preadhered to ICAM-1, but not fibrinogen, with a maximum in string formation at  $100\text{ s}^{-1}$ . Secondary capture events to the ICAM-1 or fibrinogen coated surfaces after primary collisions were infrequent and short lived, typically lasting from 500 to 3500 ms. Between 5 and 20% of neutrophil interactions with ICAM-1 substrate converted to firm arrest ( $>3500\text{ ms}$ ) and greatly exceeded that observed for fibrinogen, thus defining the root cause of poor string formation on fibrinogen at all shear rates. Additionally, neutrophils mobilized calcium after incorporation into strings. Static adhesion also caused calcium mobilization, as did the subsequent onset of flow. To our knowledge, this is the first report of 1), hydrodynamic thresholding in neutrophil string formation; 2), string formation on ICAM-1 but not on fibrinogen; 3), large cellular deformation due to collisions at a venous shear rate; and 4), mechanosensing through neutrophil  $\beta_2$ -integrin/adhesion. The increased contact area during deformation was likely responsible for the hydrodynamic threshold observed in the primary collision efficiency since no increase in primary collision lifetime was detected as shear forces were increased (for either surface coating).

## INTRODUCTION

During an inflammatory response, neutrophils interact with the endothelium via specific cell adhesion molecules and their ligands. These interactions can be broken down into transient tethering (capture), rolling, and firm adhesion steps. Selectin interactions mediate capture and rolling of neutrophils (Guyer et al., 1996), whereas the  $\beta_2$ -integrins CD11a/CD18 (leukocyte function-associated antigen-1, LFA-1) and CD11b/CD18 (Mac-1) mediate firm adhesion to endothelial cells, neutrophils, or platelets. Adherent neutrophils can form long linear assemblies (strings) in the direction of flow under in vitro conditions (Lawrence and Springer, 1991; Alon et al., 1996) or on atherosclerotic plaques in vivo (Eriksson et al., 2001).

During neutrophil homotypic interactions, rapid P-selectin ligand glycoprotein-1 (PSGL-1)/L-selectin bond formation mediates capture followed by binding via LFA-1/intercellular adhesion molecule-3 (ICAM-3) and Mac-1/Mac-1 ligand(s) (Taylor et al., 1996; Lynam et al., 1998).

Neutrophil LFA-1 also binds ICAM-1 on inflamed endothelium. Mac-1 is a more promiscuous integrin; known ligands include fibrinogen, as well as other less defined ligands on the platelet, neutrophil, and endothelium. The PSGL-1/L-selectin interaction obeys the Bell model for rigid beads  $\{k_{\text{off}} = 1.54\text{ s}^{-1} \exp(0.037\text{ nm } F_B/k_B T)\text{ for } 50 < F_B < 250\text{ pN}\}$  and fixed neutrophils with  $0.25\text{ }\mu\text{m}$  nonextending microvilli (Park et al., 2002). However, deformable unfixed neutrophils deviate from Bell model behavior at  $F_B > 100\text{--}150\text{ pN}$  (Park et al., 2002) most likely due to microvilli extension (Shao et al., 1998; Schmidtke and Diamond, 2000).

In several circumstances, neutrophil adhesion can display unexpectedly increased adhesiveness in the presence of increasing force loading. This diverse set of observations with neutrophils include single cell and population measurements: 1), a threshold requirement for shear stress to facilitate rolling on selectins (Lawrence et al., 1997; Finger et al., 1996); 2), increase in rolling flux as shear rate is increased in vivo (Lawrence et al., 1997) and in vitro (Greenberg et al., 2000); 3), stable rolling of individual cells under flow that destabilizes when flow stops (Lawrence et al., 1997); 4), increased collision efficiency during homotypic aggregation of *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-activated neutrophils (Taylor et al., 1996);

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5) flow-induced aggregation of resting neutrophils in suspension that deaggregate upon cessation of flow (Goldsmith et al., 2001); and 6), a plateauing in rolling velocity as wall shear rate is continually increased (Lawrence and Springer, 1993; Chen and Springer, 1999). Multiple mechanisms at the cellular and/or molecular level may underlie these distinct observations. In general, hydrodynamic thresholding refers to a biphasic adhesion phenomenon that displays an increase in adhesion with force loading followed by a decrease in adhesion as forces become exceedingly high. In this work, we reserve the term thresholding as a description of macroscopic systems that display force-enhanced adhesion. Relating macroscopic cellular observations to underlying molecular phenomena often requires the titration of receptor densities to limiting dilutions and various consistency tests of the discrete event data sets (Zhu et al., 2002). Thresholding is also seen for the platelet glycoproteinIb/von Willebrand factor (GPIb/vWF) A1 interaction, a bond with a high zero-stress off-rate ( $k_{\text{off}}(0) = 3.45 \text{ s}^{-1}$ ) with a reactive compliance ( $\sigma = 0.06 \text{ nm}$ ) that is strictly positive for  $36 < F_B < 145 \text{ pN}$  (Doggett et al., 2002), as it is for selectins.

Hydrodynamic thresholding can be viewed as the net effect of force loading and convection on processes that modulate bond formation and processes that modulate bond disruption over a regime of increasing hydrodynamic flow. The rolling dynamics of neutrophils can also be influenced by membrane tether pulling that shields bonds from force loading (Schmidtke and Diamond, 2000; Park et al., 2002) or by cellular deformation that enhances the rolling contact area (Lei et al., 1999). Interestingly, membrane tether formation on P-selectin (Schmidtke and Diamond, 2000) occurs over the same shear rate regime as hydrodynamic thresholding (Lawrence et al., 1997). Although tangential convection of surfaces relative to each other (Chang and Hammer, 1999) may enhance the overall on-rate ( $k_{\text{on}}$ ) during capture of a flowing cell or bead, it remains unstudied the contribution of this mechanism during stable rolling after the velocity of the neutrophil has decreased considerably from its free stream velocity. Normal convective effects may also occur as the bonds at the trailing edge of the contact area facilitate compression at the leading edge of new membrane normal to the wall. Although living neutrophils are very soft and deformable in rolling assays, thresholding has also been observed in a bead system (Greenberg et al., 2000). Laboratory observations of polystyrene bead deformation at forces as low as 40 pN preclude strict conclusions about the role of deformation during bead thresholding. Even the flow-enhanced angular rotation of a cell or rigid bead around a single bond of finite length may facilitate the formation of the next bond, via a normal convective mechanism as a receptor moves closer to the surface. Chen and Springer (1999) developed a Monte Carlo simulation that predicted an increase in bond number with an increase in shear and also calculated the shear threshold below which rolling would not

be seen in the system. This shear threshold was calculated to be 0.4–0.8 dyne/cm<sup>2</sup> for neutrophil rolling on peripheral lymph node addressin (PNAd), which compares well with the observed value of 0.4–0.5 dyne/cm<sup>2</sup>.

Another hallmark of neutrophil rolling interactions in parallel-plate chambers is the formation of string assemblies of neutrophils. Linear strings of adherent neutrophils in the direction of shear flow have been visualized for neutrophil rolling on P-selectin, E-selectin, and human umbilical vein endothelial cells (HUVECs), as well as in vivo on mouse atherosclerotic lesions. Treatment of neutrophils with antibodies blocking either L-selectin or PSGL-1 block the formation of strings in vitro, indicating that the formation of strings depends upon homotypic neutrophil interactions.

Since the collision of flowing neutrophils with adherent neutrophils provides experimental advantages unique to both rolling assays (good visualization) and aggregation assays (discrete collisions), we used high speed, high resolution videomicroscopy to probe these events. This assay may help in the determination of receptor dynamics and capture probabilities during assembly processes in inflammation and thrombosis.

## EXPERIMENTAL PROCEDURES

### Materials

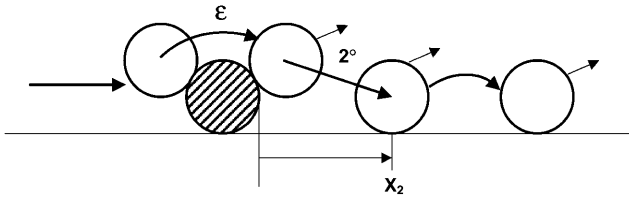
Recombinant human ICAM-1 (rhICAM-1) immunoglobulin G (IgG) chimera was purchased from R&D Systems (Minneapolis, MN) and diluted with Hanks' buffered saline (HBS). The cell-permeant calcium dye Fluo-4 acetoxymethyl ester (AM) (Molecular Probes, Eugene, OR), human serum albumin (Golden West Biologicals, Temecula, CA), and fibrinogen (Enzyme Research Labs, South Bend, IN) were stored according to manufacturers' instructions. Blocking monoclonal antibodies (mAb) against CD11a (38), CD11b (ICRF44), CD18 (IB4), and CD62L (LAMI-116) were from Ancell (Bayport, MN).

### Cell isolation

Human blood was obtained via venipuncture from healthy adult donors and was mixed with anticoagulant sodium citrate (nine parts blood/one part citrate). Neutrophils were isolated by centrifugation with a separation medium (Robbins Scientific, Sunnyvale, CA) as previously described (Schmidtke and Diamond, 2000; Goel and Diamond, 2001). Neutrophils were counted and diluted with a 2% solution of human serum albumin in HBS to a final concentration of  $10^6$  cells/ml. The suspension buffer of sterile HBS (Invitrogen, Carlsbad, CA; manufacturer specification of low endotoxin, <10 endotoxin units/ml) was used for all perfusion studies. For cells labeled with calcium-sensitive dye, cells were incubated for 20 min with Fluo-4 (9.1  $\mu\text{M}$ ) before dilution. In some experiments, cells were preincubated for 20 min with 20  $\mu\text{g/ml}$  mAb before perfusion.

### Microcapillary flow chambers and imaging

Rectangular glass capillaries (Vitrocom, Mountain Lakes, NJ) having a cross section of  $0.2 \times 2.0 \text{ mm}$ , a length of 7 cm, and a wall thickness of 0.15 mm were used as flow chambers, as described previously (Schmidtke and Diamond, 2000; Goel and Diamond, 2001). Chambers were incubated with recombinant human ICAM-1 (1  $\mu\text{g/ml}$ ) or fibrinogen (100  $\mu\text{g/ml}$ ) for 120

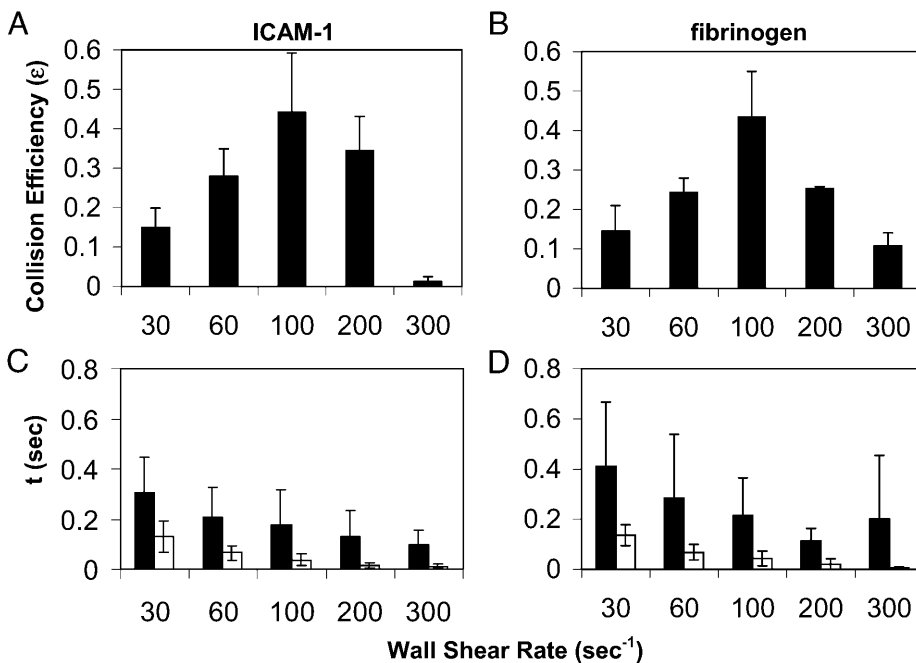


**FIGURE 1** Neutrophil-neutrophil interactions during string formation. Individual cell-cell interactions were visualized using videomicroscopy. A free-flowing neutrophil (*open circle*) collides with a preadhered neutrophil (*dashed circle*). The primary collision efficiency ( $\epsilon$ ) is the ratio of collisions with bonding to the total number of collisions. The flowing cell may either release from the primary cell or form a secondary adhesive event with the ICAM-1 or fibrinogen coated on the surface of the chamber.  $X_2$  refers to the position, measured from the outer edge of the primary cell to the center of the flowing cell, where the flowing cell makes its first adhesive interaction with the surface. This rolling cell may then release from this position into the free-flowing stream, or may reattach further downstream from its initial point of interaction.

min at room temperature and rinsed with HBS for 10 min. Neutrophils were perfused into the chambers using a syringe pump (Harvard Apparatus, South Natick, MA) and then preadhered for 10 min under static conditions. The wall shear stress ( $\tau_w$ ) upon the surface of the chamber was calculated from the solution of the Navier-Stokes equation for laminar flow of a Newtonian fluid:  $\tau_w = (6Q\mu)/(B^2W)$ , where  $Q$  represents the flow rate ( $\text{cm}^3/\text{s}$ ),  $\mu$  is the viscosity (0.01 Poise at room temperature),  $B$  is the total plate separation (0.02 cm), and  $W$  is the width (0.2 cm). The wall shear rate,  $\gamma_w$ , is calculated from  $\gamma_w = 6Q/B^2W$ . For differential interference contrast (DIC) microscopy, a Zeiss Axiovert 135 microscope (Thornwood, NY) with a  $63\times$  (NA 1.40) oil immersion objective lens (Plan Apochromate) was used. For experiments where fluorescence imaging was needed, a GenIISys focused image intensifier charge-coupled device (DAGE-MTI, Michigan City, IN) was attached to the microscope. For high speed, high resolution studies, images were captured using a MotionCorder Analyzer high speed digital camera (Eastman Kodak, Rochester, NY) at an imaging rate of 240 frames per second (fps). The images were played back at 5 fps or frame-by-frame to videotape for analysis.

## Analysis of neutrophil rolling and adhesion

When unstimulated neutrophils were perfused over neutrophils adherent to ICAM-1 or fibrinogen-coated surfaces, two different types of interactions occurred: collisions with the attached cells and subsequent collision with the surface (Fig. 1). In a primary collision, the flowing neutrophil collided with and rolled over an adherent neutrophil. In the absence of observable binding (acute reductions in motion or a pause before escape), the interaction time from arrival to departure fell within a narrow, hydrodynamically expected range with the approach and escape velocity being essentially the same. Cells that had collisions lasting longer than this range that also had detectable pauses before escape were scored as adhesive interactions. The ratio of adhesive collisions to total collisions was defined as the primary collision efficiency. This efficiency is not the same as used in bulk aggregation studies analyzed with population balances (Taylor et al., 1996; Tandon and Diamond, 1998). Binding events lasting  $<5$  ms cannot be distinguished from hydrodynamic interactions at 240 fps and therefore were not binned as adhesive interactions. Thus, the measured value of  $\epsilon$  represents a minimal estimate of bonding at  $63\times$  and 240 fps. For flowing cells that made bonds during the primary collision (detectable pause before escape) and had a detectable secondary interaction with the surface ( $2^\circ$ ), we then measured the lifetime of this secondary interaction (Fig. 1). Cells with no detectable bonding during the primary collision had exceedingly low capture probability downstream of the primary target cell, as expected from L-selectin and PSGL-1 antibody experiments that greatly reduce string formation (Alon et al., 1996). Of cells with a primary collision (with or without binding), the secondary capture efficiency ( $2^\circ\epsilon$ ) was rather small ( $2^\circ\epsilon < 0.032$  at  $100 \text{ s}^{-1}$ ), as expected for a surface lacking a selectin. Since  $2^\circ\epsilon$  was so small in the absence of a selectin on the surface, it was not practical to measure this parameter. This low value of  $2^\circ\epsilon$  was also consistent with poor cell capture by surfaces (ICAM-1 or fibrinogen) lacking preadherent neutrophils. Based on the histogram of  $2^\circ$  adhesion times, any neutrophil adhering to the surface for longer than 3.5 s was considered to be firmly adherent, and all such interactions were binned together. Likewise, interactions that lasted from 500 to 3500 ms were binned together as transient adhesions. Adhesions between 0 and 500 ms were binned as highly transient. A time of 500 ms was chosen as the cutoff between transient and highly transient events because 75% of the interactions observed lasted less than 500 ms, on average, over all the shear rates and surface coatings.



**FIGURE 2** Primary collision efficiencies ( $\epsilon$ ) and interaction times for neutrophil collisions. Neutrophils were perfused at different shear rates over neutrophils adherent to ICAM-1 or fibrinogen for measurement of  $\epsilon$  (A and B) and interaction times (C and D). Interaction times for collision with bonding (*solid bar*) or without detectable bonding (*open bar*) represent interaction times calculated for at least 30 interactions, with the exception of the fibrinogen at  $300 \text{ s}^{-1}$  for which only 17 interactions were observed.

## RESULTS

### Shear threshold for neutrophil-neutrophil collisions

Primary collision efficiencies ( $\epsilon$ ) were calculated for interactions between flowing neutrophils and those adherent to ICAM-1 or fibrinogen for a range of wall shear rates ( $30\text{--}300\text{ s}^{-1}$ ). In addition, the average interaction times were measured for both primary and secondary interactions. A maximum in  $\epsilon = 0.45$  for ICAM-1 coated surfaces or  $\epsilon = 0.40$  for fibrinogen coated surfaces occurred for collisions with neutrophils at  $100\text{ s}^{-1}$  (Fig. 2, A and B). More than 30 collisions were analyzed at each shear rate to allow a reasonable sampling of the upstream cross sectional area except on fibrinogen at  $300\text{ s}^{-1}$ . No successful collisions with binding were detectable at  $\gamma_w = 400\text{ s}^{-1}$  (not shown), indicating that hydrodynamic interactions lasting under 5 ms produce no binding lasting longer than 5 ms. A cluster of several L-selectin/PSGL-1 bonds would be required for detection at 240 fps imaging at  $400\text{ s}^{-1}$  when shear forces exceed 400 pN.

The adhesion lifetime for bonding decreased from 0.300 to 0.100 s as shear forces increased 10-fold as  $\gamma_w$  increased from 30 to  $300\text{ s}^{-1}$  (Fig. 2, C and D). These values are considerably shorter than observed for transient capture of flowing neutrophils to adherent platelets via PSGL-1/P-selectin bonding (Schmidtke and Diamond, 2000). Multiple bonds are likely detected in neutrophil-neutrophil or neutrophil-platelet collisions observed under physiological conditions due to the abundance of the receptors on the cells, based on comparison with assays in which single bond interactions dominate.

To identify receptors that contribute to the primary collision efficiencies, monoclonal antibodies against CD11a, CD11b, and CD18, as well as against CD62L (L-

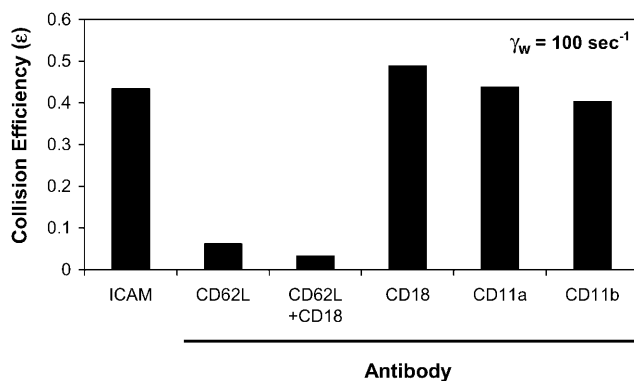


FIGURE 3 Primary collision efficiency for neutrophils treated with various monoclonal antibodies. Neutrophils were incubated with  $20\text{ }\mu\text{g/ml}$  mAb against known surface receptors for 20 min at room temperature. The treated neutrophils were then perfused over nontreated, ICAM-1 adherent neutrophils. In the case of treatment with anti-L-selectin, both ICAM-1 adherent and perfused neutrophils were treated with mAb. The primary collision efficiency was calculated for neutrophils treated with each of the antibodies tested.

### ICAM-1, $\gamma_w = 100\text{ sec}^{-1}$

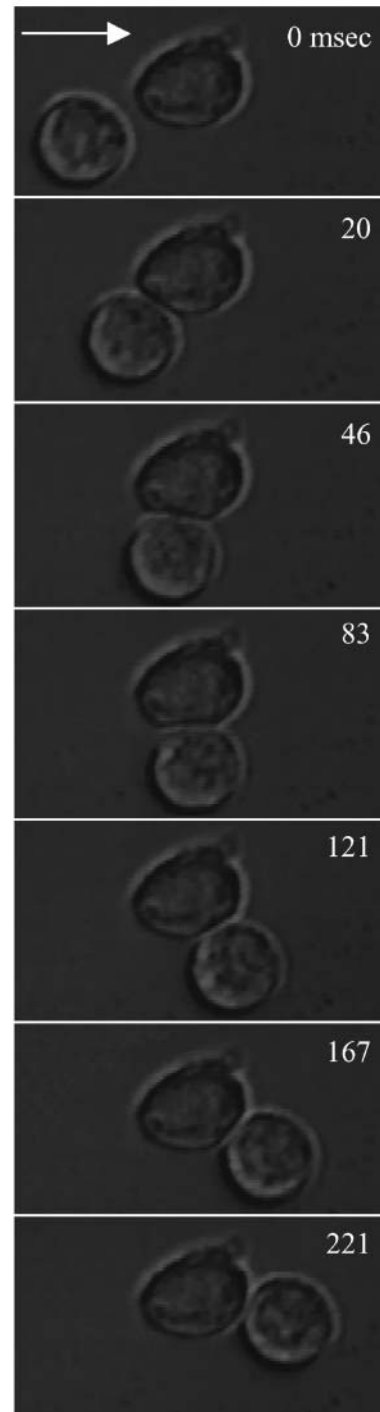


FIGURE 4 Neutrophils deform during primary collision at  $100\text{ s}^{-1}$ . Neutrophils were perfused at  $100\text{ s}^{-1}$  over neutrophils preadhered to an ICAM-1 surface, and primary adhesive interactions were imaged using a high speed digital camera at 240 fps.

selectin), were incubated with neutrophils before efficiency studies (Fig. 3). The anti-CD18, CD11a, and CD11b antibodies had no significant effect on the primary collision efficiency. The mAb against CD62L significantly reduced the collision efficiency of the cells. These results indicate that L-selectin, but not the  $\beta_2$ -integrins, mediated the 100–300-ms bonding during collision of neutrophils where both PSGL-1 and L-selectin were presented in their native membranes.

### Neutrophil deformation during collision may contribute to the shear thresholding effect

The biphasic behavior seen in Fig. 2, *A* and *B*, is an example of hydrodynamic thresholding for PSGL-1/L-selectin that was detected in single cell observations. As the shear rate increased, the force with which one neutrophil collided with another also increased. The likelihood of neutrophil deformation becomes more significant at higher shear rates, and some authors (Taylor et al., 1996) have pointed to the competing effects of deformation and increased shear as a reason for the shear thresholding effects seen in the neutrophil homotypic collision efficiency. Using high speed imaging at  $100\text{ s}^{-1}$ , the deformation of a flowing neutrophil during a collision with an ICAM-1 anchored neutrophil was clearly observed (Fig. 4). Within the first 20 ms of the collision, compression was detected. By 46 ms, the full development of an adhesive contact area had grown to  $\sim 40\ \mu\text{m}^2$  as the rolling cell experienced maximal shear. By the conclusion of the interaction at 221 ms, the contact area was reduced to less than  $\sim 12\ \mu\text{m}^2$  and the cell rebounded to a near spherical shape. For this off-center collision with both cells in a focal plane near the surface, the combination of adhesion and hydrodynamics caused large changes in cell shape even at  $100\text{ s}^{-1}$ . The rate of bond formation may increase due to a growth of the contact area and enhanced compression of the opposing membranes together.

### Linear strings of neutrophils form on ICAM-1 coated surfaces

Neutrophils were perfused into ICAM-1 and fibrinogen-coated flow chambers and allowed to incubate for 10 min. Neutrophils were then perfused into the chambers at a specified shear rate (10, 100, 200, or  $300\text{ s}^{-1}$ ) for 10 min, and images of the neutrophil aggregates were taken (Fig. 5). Linear strings of neutrophils in the direction of flow formed on the ICAM-1 coated surfaces, but not on the fibrinogen-coated surfaces. These strings formed at shear rates between  $60\text{ s}^{-1}$  (data not shown) and  $200\text{ s}^{-1}$ , with optimal formation of strings at  $100\text{ s}^{-1}$ . Without preadhered neutrophils, the ICAM-1 surface did not yield strings after 10 min since selectins were not present for capture. Even with preadherent neutrophils, fibrinogen-coated surfaces did not support string formation, suggesting a deficiency in secondary processes ( $2^\circ$  in Fig. 1) since the primary collision efficiency ( $\varepsilon$ ) was essentially the same for the two surfaces (Fig. 2, *A* and *B*).

At  $10\text{ s}^{-1}$ , shear forces were insufficient to drive secondary adhesion on either ICAM-1 or fibrinogen to lifetimes exceeding 500 ms (Fig. 6). Transient interactions (500–3500 ms) and a few firm adhesions ( $>3500\text{ ms}$ ) were only detected at  $\gamma_w \geq 30\text{ s}^{-1}$ , indicating a hydrodynamic shear threshold for secondary capture of neutrophils to ICAM-1 and fibrinogen-coated surfaces lacking selectins. At  $\gamma_w = 100\text{ s}^{-1}$ , the average secondary interaction time was greater on ICAM-1 than fibrinogen ( $\bar{t} = 850\text{ ms}$  for ICAM-1 vs.  $330\text{ ms}$  for fibrinogen). Of those cells having any detectable secondary bonding interaction with ICAM-1, an increasing number from  $\sim 5$  to  $\sim 20\%$  achieved firm adhesion ( $>3500\text{ ms}$ ) as  $\gamma_w$  increased from 30 to  $200\text{ s}^{-1}$ , which is an additional instance of thresholding. Firm arrest to fibrinogen after primary collision was only sporadically detected and represented the root cause of poor string formation on fibrinogen. This suggests that LFA-1 and Mac-1 bind ICAM-1 faster than Mac-1 can bind fibrinogen. No firm adhesion lasting  $>3500\text{ s}$  was detected on either surface at  $\gamma_w = 300\text{ s}^{-1}$ .

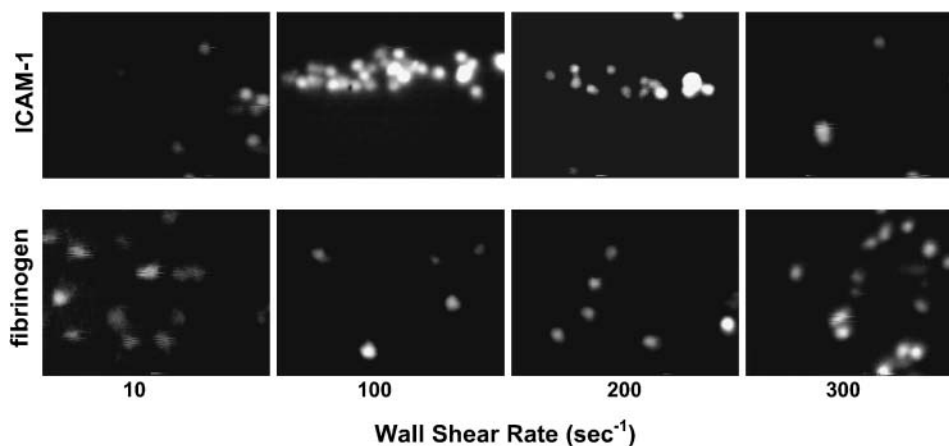
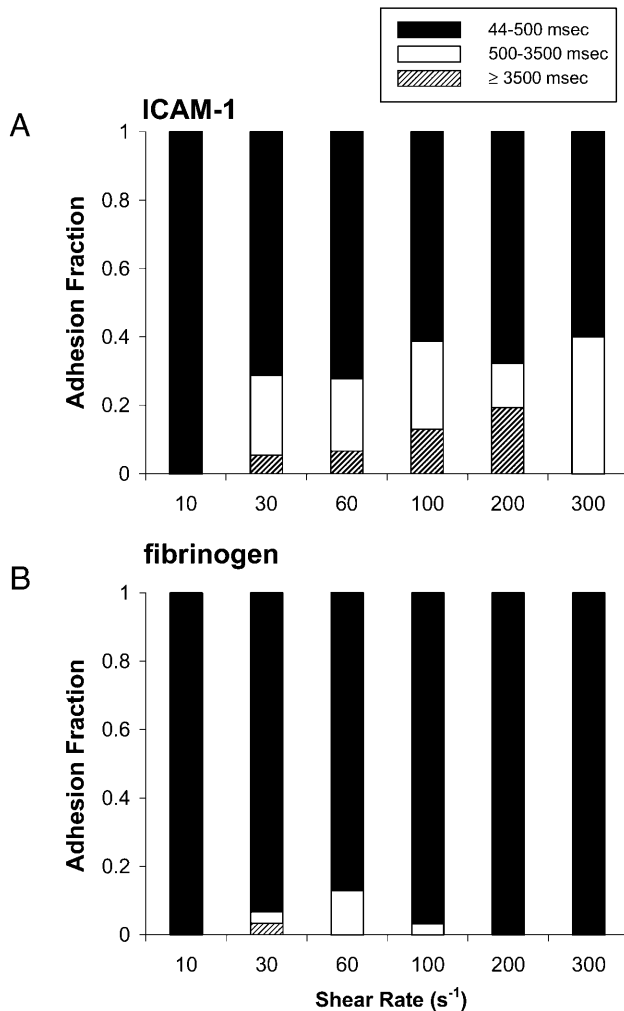


FIGURE 5 Strings of neutrophils form on ICAM-1 but not fibrinogen-coated surfaces. Fluo-4 treated neutrophils were perfused at various shear rates over ICAM-1 adherent (*top*) or fibrinogen-adherent (*bottom*) neutrophils. Cells formed linear string-like structures on preseeded ICAM-1 surfaces at shears between 60 and  $200\text{ s}^{-1}$ , with maximal string formation occurring at  $100\text{ s}^{-1}$ . Strings failed to form on fibrinogen-coated surfaces for any of the shear stresses tested.



**FIGURE 6** Secondary adhesion for neutrophils on ICAM-1 and fibrinogen have very different lifetimes. Neutrophils were perfused at shear rates of 10–300 s<sup>-1</sup> over ICAM-1 or fibrinogen-adherent cells, and the adhesive fraction of the cells was determined. Interactions were binned based upon the duration of the adhesive event: highly transient (0–500 ms), transient (500–3500 ms), and firmly adherent ( $\geq 3500$  ms). The contribution of each interaction type to the overall adhesive fraction was calculated for all shear rates tested.

### Calcium mobilization in neutrophils

As neutrophils adhered to the ICAM-1 surface, they became activated, as evidenced by mobilization of their calcium stores. Neutrophils were loaded with calcium-binding dye Fluo-4, and then observed during string formation. Fig. 7 shows calcium traces for cells adhering to a surface of ICAM-1 within a growing string. Intracellular calcium peaked at between 30 and 120 s after adhesion of each neutrophil to the surface. On occasion, oscillations of calcium were seen after adhesion (not shown). Adhesion of neutrophils to ICAM-1 under static conditions (Fig. 8) also caused calcium mobilization. In these experiments, Fluo-4 labeled neutrophils were imaged during adhesion for 3 min

without flow. The excitation was shuttered off to minimize photobleaching. At 9 min after adhesion, fluorescence imaging was resumed, and at 10 min, shear stress exposure was resumed. When a wall shear stress of 2 dyne/cm<sup>2</sup> was applied at 10 min, the neutrophils demonstrated calcium mobilization that was about half that of the initial adhesion activation state. This signaling was observed for neutrophil adhesion to both ICAM-1 and fibrinogen-coated surfaces, but was more prevalent on the ICAM-1 surface. Rapid calcium mobilization resulting from shear stress onset was seen for ICAM-1 at 10, 30, 60, and 400 s<sup>-1</sup> and for fibrinogen at 400 s<sup>-1</sup>.

### Trajectory profiles for flowing neutrophils

The trajectories of flowing cells colliding with adherent cells were determined by monitoring the position of the flowing cell's centroid relative to the centroid of the adherent cell for collisions with and without bonding (Fig. 9 A). A reduction in velocity is apparent in the trajectory profile when the cell forms bonds with the adherent cell. Due to bonding, the  $y$  position of the average release trajectory ( $\bar{y} = 2.68 \pm 0.77$ ) was closer to the center line ( $y = 0$ ) of the adherent cell, whereas there was no migration toward the center line after release for nonbonding cells ( $\bar{y} = 6.10 \pm 1.24$ ). This difference in release trajectories due to bonding may focus and facilitate string formation into linear chains as opposed to a more branched structure. Also, an asymmetry of approach and release trajectories was detected, potentially influenced by deformation in the cell membranes, especially in the case where bonds form between the interacting cells.

Both the adhesive and nonadhesive cases exhibit a fore-aft asymmetry. In the case of the adhesive collisions, the free stream cell is irreversibly brought from a farther streamline (higher  $y$ ) to closer streamline (lower  $y$ ) during collision. Conversely, in the nonadhesive cases the free stream cell is irreversibly brought from a closer streamline (lower  $|y|$ ) to a farther streamline (higher  $|y|$ ). The asymmetry of the latter case was pointed out by King and Hammer (2001) in simulations of hard sphere collisions. The former case of asymmetry between adhesive cells appears to be a novel observation in this context. Also, for both adhesive and nonadhesive collisions the distribution of  $y$  values appears to be narrower after collision than before collision.

In a new observation, membrane tether formation was discovered in trajectory data at the termination of the collision where a cell translates without rotation at a steady and reduced velocity. Membrane tether formation between L-selectin and PSGL-1 was more pronounced at 200 than at 100 s<sup>-1</sup>. A tether forming event at a wall shear rate of 200 s<sup>-1</sup> (Fig. 9 B) had a membrane extension rate of 11.7  $\mu\text{m/s}$  for a 1.46- $\mu\text{m}$  tether that grew for 100 ms. Tether initiation, elongation, and cell release is shown below the graph. The



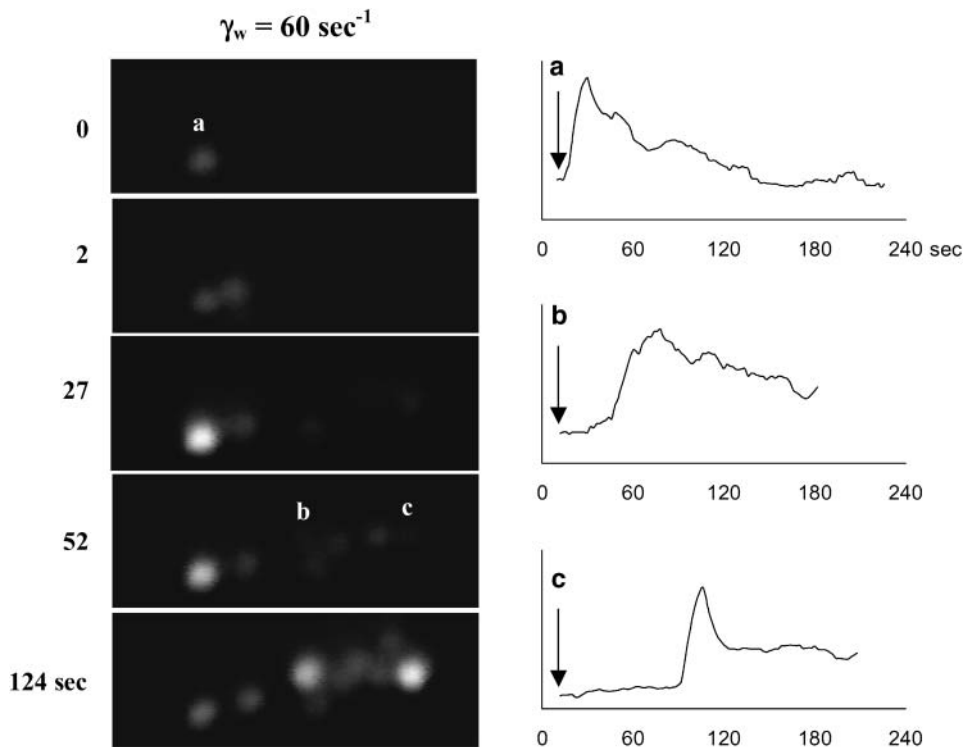


FIGURE 7 Activation profiles for neutrophils in ICAM-1 during string formation. Activation of neutrophils as they formed strings on an ICAM-1 coated surface was monitored by loading the cells with Fluo-4 calcium indicator. The fluorescence of the neutrophils is directly proportional to the activation state of the cell. A trace of fluorescence intensity versus time was generated for adhering cells in the string of neutrophils.

average tether life decreased as the wall shear rate was increased from 100 to 200  $\text{s}^{-1}$  ( $\bar{t} = 487$  and 250 ms,  $p < 0.05$ ). The average tether length was 1.1 and 2.7  $\mu\text{m}$  at 100 and 200  $\text{s}^{-1}$ , considerable shorter than those observed between PSGL-1 and P-selectin (Schmidtke and Diamond, 2000).

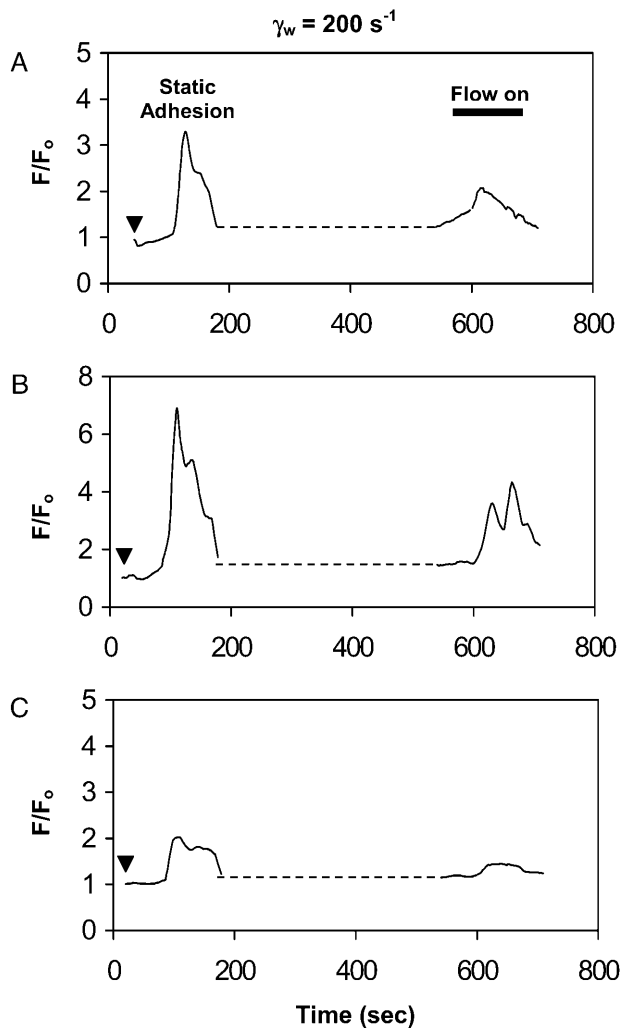
## DISCUSSION

Using high speed, high resolution imaging and fluorescence imaging, we have analyzed the differences in neutrophil adhesion to ICAM-1 or fibrinogen surfaces. Fig. 5 shows the difference in the geometry of cell adhesion to ICAM-1 or to fibrinogen surfaces. Whereas long, linear strings of neutrophils form on the  $\beta_2$ -integrin receptor ICAM-1, these structures are not present on fibrinogen. The primary collision efficiencies and lifetimes of these primary collisions were similar for shear stresses in the range of 30–300  $\text{s}^{-1}$  regardless of the surface coating (Fig. 2). The efficiencies reach a maximum at 100  $\text{s}^{-1}$ . The biphasic distribution indicated the presence of shear thresholding as shear forces increased. High speed analysis revealed the large-scale deformation of neutrophils undergoing primary collision at 100  $\text{s}^{-1}$  (Fig. 4), which may explain the shear threshold observed. Incubation with antibodies against the cell-surface molecule CD62L nearly abolished these adhesive events (Fig. 3), whereas mAb against  $\beta_2$ -integrins had no effect, indicating that the primary collision was dependent upon L-selectin and PSGL-1.

Further analysis of secondary capture on ICAM-1 or fibrinogen demonstrated radically different dynamics (Fig. 6). The fraction of cells that exhibited either transient or firm adhesion to ICAM-1 was constant at  $\sim 30\%$  for shear rates between 30 and 300  $\text{s}^{-1}$ . In contrast, transient adhesion to the fibrinogen surface was only seen for limited shear rates (30–100  $\text{s}^{-1}$ ), and the fraction adhering to fibrinogen was much lower than that seen on ICAM-1. Firm adhesive events (defined as events lasting longer than 3500 ms) on fibrinogen were only sporadic. Thus, the overall adhesiveness of neutrophils to a fibrinogen-coated surface is much less than that of an ICAM-1 coated surface. These results indicate that the strings of neutrophils seen on ICAM-1 are most likely the result of secondary interactions between  $\beta_2$ -integrins and ICAM-1.

An analysis of calcium activation profiles revealed that neutrophils activate during string formation and adhesion of cells to the surface (Fig. 7). Further analysis of calcium mobilization in the neutrophils revealed that cells activate upon application of a shear stress to adherent cells (Fig. 8). This activation is rapid upon initiation of fluid shear, and always less than the calcium response seen when the cells initially adhere to the surface.

Clearly, thresholding is most easily detected in systems with high off-rates ( $k_{\text{off}}$ ) such as detected for selectins and the GPIb/vWF A1 domain (Doggett et al., 2002) and in systems of low ligand density. It remains unclear to what extent hydrodynamic thresholding requires catch bonding, a positive reactive compliance in the Bell model over a portion of



**FIGURE 8** Activation profiles for shear-induced neutrophil activation. Activation of neutrophils was monitored through fluorescence via Fluo-4 calcium indicator. Cells were loaded with the dye and then perfused into the ICAM-1-coated chamber, at which point the flow was turned off. The cells were monitored for 3 min in static environment during cell adhesion to the surface, and then the fluorescent lamp was turned off to avoid photobleaching. The lamp was turned on again after 9 min, and then flow at  $200 \text{ s}^{-1}$  was restarted at  $t = 10 \text{ min}$ . Activation of the cells was monitored by fluorescence again from 9 to 12 min. Three representative responses from individual cells are shown in A–C.

the force regime, as was recently detected by atomic force microscopy for monomeric and dimeric P-selectin/PSGL-1 bonds (Marshall et al., 2003). Neutrophil thresholding is best observed with rolling on L-selectin. Neutrophil rolling fluxes on L-selectin or PSGL-1 peak at  $100 \text{ s}^{-1}$  ( $F_{\text{bond}} \sim 100 \text{ pN}$ ), whereas the peak bond lifetime for P-selectin/PSGL-1 detectable with atomic force microscopy occurs at a very lower force of  $25 \text{ pN}$  (Zhu et al., 2002) that already approaches hemodynamic conditions of stasis ( $<0.25 \text{ s}^{-1}$ ) where rouleaux formation would dominate any pathophysiology. The  $k_{\text{on}}$  and  $k_{\text{off}}$  values for L-selectin are generally

viewed as being higher than those for P-selectin. This translates into better capture and faster rolling, in addition to a higher reactive compliance in rolling assays (Smith et al., 1999; Zhang and Neelamegham, 2002).

The two- to threefold increase in  $\varepsilon$  to a maximal value of 0.4 as  $\gamma_w$  increased from 30 to  $100 \text{ s}^{-1}$  was consistent with the fourfold increase to 0.4 seen in bulk aggregation assays as  $\gamma$  increased from 100 to  $400 \text{ s}^{-1}$  (Taylor et al., 1996) since peak forces in our assay are greater and sustained in comparison to those for rotating doublets in a linear shear field (Tandon and Diamond, 1998). From flow chamber data and calculated cell fluxes to the adherent cell, Zhang and Neelamegham (2002) derived a cell-cell capture efficiency to be 0.2 at low shear rates ( $50$  and  $100 \text{ s}^{-1}$ ), and 0.07 at  $200 \text{ s}^{-1}$ , reasonable estimates for an ensemble average.

We found in our system that most neutrophils that experienced a secondary interaction attached to the ICAM-1 surface  $\sim 1.5$ – $2$  cell diameters downstream from the initial adherent cell (data not shown) at  $100 \text{ s}^{-1}$ . These results were obtained by measuring the distance between the center of the adherent cell and the outer edge of the rolling cell when it first forms an adhesive interaction with the chamber surface. This result differs from the results found by King et al. (2001), where they determined that sialyl Lewis<sup>x</sup> coated beads tended to attach to a P-selectin surface from four to five cell diameters downstream from an adherent sphere. However, there are some differences between the two systems. First, flowing sialyl Lewis<sup>x</sup> coated beads do not experience adhesive interactions with adhered beads. In our system, flowing neutrophils bond via PSGL-1/L-selectin during primary collisions. Second, in our system the neutrophils were captured to a surface that was essentially free of selectins, aside from any putative neutrophil microparticles that may be present on the surface. This low or near-zero concentration of surface-presented selectin reduces the overall adhesiveness of these surfaces to neutrophil adhesion.

In summary, we provide insight into the dynamics of neutrophil adhesion under various shear flow conditions. We have shown how  $\beta_2$ -integrins affect the formation of neutrophil strings, which have been observed in vivo and are therefore not an in vitro artifact as previously hypothesized (Kunkel et al., 1998). We also have demonstrated a shear threshold in neutrophil-neutrophil primary collisions, which may be caused by the subsecond deformation of neutrophils upon collision after L-selectin bond formation. Finally, calcium activation upon onset of shear stress for neutrophils adhering to an ICAM-1 surface indicates a role of  $\beta_2$ -integrins as mechanosensors. With a better understanding of neutrophil dynamics of adhesion and activation, and clarification of the roles of LFA-1, Mac-1, and L-selectin in these dynamics, both drug development and computer modeling for thrombosis and inflammation may be improved.

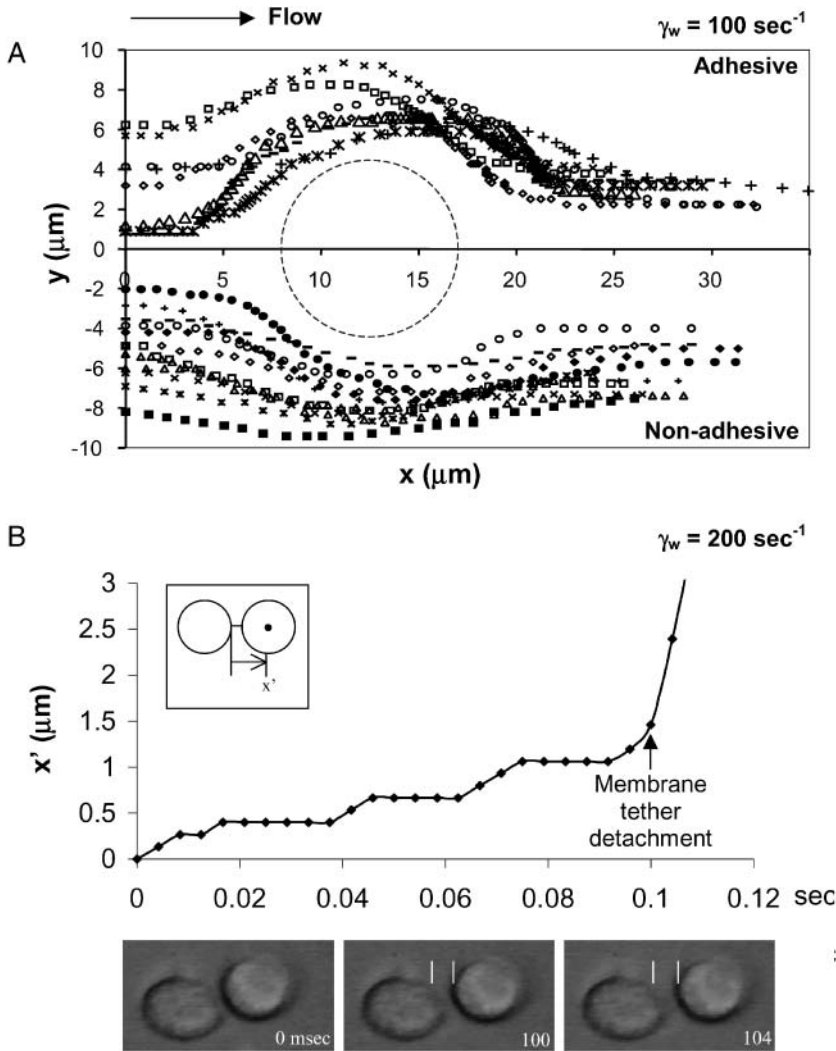


FIGURE 9 Top view of relative trajectories of off-center binary collisions. The position of the centroid of a flowing cell was tracked relative to an adherent cell for collisions with bonding (A, top) or without bonding (A, bottom) at  $100 \text{ s}^{-1}$ . Detection of the growth of a membrane tether at  $200 \text{ s}^{-1}$  (B) at the conclusion of a bonding collision is also presented. Images of a cell during membrane tether formation at the initiation, elongation, and detachment are presented.

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