Comparison of PSGL-1 Microbead and Neutrophil Rolling: Microvillus Elongation Stabilizes P-Selectin Bond Clusters

Eric Y. H. Park,* McRae J. Smith,* Emily S. Stropp,* Karen R. Snapp,† Jeffrey A. DiVietro,* William F. Walker,* David W. Schmidtke,‡ Scott L. Diamond,‡ and Michael B. Lawrence*

*Department of Biomedical Engineering, University of Virginia, Charlottesville, Virginia 22908; †Department of Microbiology and Immunology, Northwestern University Medical School, Northwestern University, Chicago, Illinois 60611; and ‡Department of Chemical Engineering, Institute for Engineering in Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104 USA

ABSTRACT A cell-scaled microbead system was used to analyze the force-dependent kinetics of P-selectin adhesive bonds independent of micromechanical properties of the neutrophil's surface microvilli, an elastic structure on which P-selectin ligand glycoprotein-1 (PSGL-1) is localized. Microvillus extension has been hypothesized in contributing to the dynamic range of leukocyte rolling observed in vivo during inflammatory processes. To evaluate PSGL-1/P-selectin bond kinetics of microbeads and neutrophils, rolling and tethering on P-selectin-coated substrates were compared in a parallel-plate flow chamber. The dissociation rates for PSGL-1 microbeads on P-selectin were briefer than those of neutrophils for any wall shear stress, and increased more rapidly with increasing flow. The microvillus length necessary to reconcile dissociation constants of PSGL-1 microbeads and neutrophils on P-selectin was 0.21 μm at 0.4 dyn/cm^2, and increased to 1.58 μm at 2 dyn/cm^2. The apparent elastic spring constant of the microvillus ranged from 1340 to 152 pN/μm at 0.4 and 2.0 dyn/cm^2 wall shear stress. Scanning electron micrographs of neutrophils rolling on P-selectin confirmed the existence of micrometer-scaled tethers. Fixation of neutrophils to abrogate microvillus elasticity resulted in rolling behavior similar to PSGL-1 microbeads. Our results suggest that microvillus extension during transient PSGL-1/P-selectin bonding may enhance the robustness of neutrophil rolling interactions.

INTRODUCTION

During an inflammatory response, leukocytes are recruited to the vascular endothelium through a series of sequential adhesive events that consists of tethering, rolling, and firm adhesion. The first two steps typically require engagement of selectin receptors, a family of structurally related, membrane-anchored C-type lectins that bind to carbohydrate epitopes on apposing membranes (Somers et al., 2000; Weis et al., 1998). Due to the favorable binding kinetics of selectins, circulating leukocytes are able to form bonds with the endothelium under flow conditions, resulting in leukocyte rolling along the vessel wall. The selectin family of receptors consists of three members, L-, P-, and E-selectin, all specialized for mediating rolling interactions among platelets, leukocytes, and endothelium. P-selectin in particular is necessary for the recruitment of neutrophils in response to the release of mediators of acute inflammation such as thrombin or histamine. The storage of P-selectin preformed in endothelial cell secretory granules permits rapid upregulation of P-selectin on the endothelium, resulting in the accumulation of rolling neutrophils. Two ligands with mucin-like characteristics have been identified for P-selectin: P-selectin glycoprotein ligand-1 (PSGL-1) and CD24. PSGL-1 supports neutrophil rolling through P-selectin in vitro (Moore et al., 1995) and also mediates neutrophil rolling in vivo (Norman et al., 1995, 2000; Yang et al., 2001). The physiologic role for CD24 is unknown, as it does not appear to support leukocyte rolling in vivo. Nevertheless, CD24 supports P-selectin-dependent rolling in flow chamber assays and may play a role in tumor clearance (Aigner et al., 1997, 1998).

PSGL-1 has an unusual surface distribution on neutrophils in that it is found concentrated on the tips of microvilli that extend from the cell body (Moore et al., 1995). Two other adhesion receptors, L-selectin and very late antigen-4 (VLA-4), are also localized on the microvilli tips of leukocytes (Berlin et al., 1995; Picker et al., 1991). The location of PSGL-1 on microvilli tips (which extend 0.2–0.3 μm from the leukocyte surface) may enhance association with P-selectin on apposing cell surfaces, as has been suggested for L-selectin and VLA-4 with their respective ligands (Berlin et al., 1995; Picker et al., 1991; von Andrian et al., 1995). In contrast to the surface distribution of selectins, the β2 integrins on neutrophils are only found on the planar regions of the membrane between the microvilli, a location that may limit interactions with endothelial cell ligands during rolling (Bruehl et al., 1996; Erlandsen et al., 1993).

As shear forces are increased, leukocyte rolling velocities on P-selectin typically increase in a nonlinear fashion, such that a plateau in velocity is evident at physiological flow rates (Atherton and Born, 1973; Lawrence and Springer, 1993). In contrast, physical models of bond dissociation predict an exponential decrease in selectin

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Address reprint requests to Dr. Michael B. Lawrence, Department of Biomedical Engineering, 415 Lane Rd., University of Virginia, Charlottesville, VA 22908. Tel.: 434-982-4269; Fax: 434-982-3870; E-mail: mlawrence@virginia.edu.

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bond lifetime with force (Bell, 1978). As predicted by such models, P-selectin bond lifetimes decrease exponentially with force in the 0–100 pN/bond range (Smith et al., 1999). However, above an estimated 100 pN/adhesive bond cluster, neutrophil P-selectin dissociation constants increase only linearly with additional force (Smith et al., 1999). Because both the cellular dissociation constant (k_{off}) and rolling velocity plateau with increasing force, it may be hypothesized that mechanisms exist at the cellular level to control rolling.

Because PSGL-1 bonds are localized to microvilli that may stretch (Shao et al., 1998), it is possible that the geometrical relationship between the anchorage point and the rolling neutrophil may change significantly under stress. Indeed, individual microvilli on neutrophils stretch into surprisingly long tethers if the anchorage point lasts long enough (Schmidtke and Diamond, 2000), suggesting that microvilli may elongate at forces a neutrophil might encounter on the blood vessel wall. Additionally, the contribution of cellular deformation to the stability of neutrophil rolling may also become significant at in vivo wall shear stresses if the contact area available for receptor ligation increases (Cao et al., 1998; Dong et al., 1999; Firrell and Lipowsky, 1989; Lei et al., 1999). Both microvillus stretching and increases in the leukocyte-endothelium membrane contact area may therefore contribute to the ability of leukocytes to roll at in vivo flow rates.

In this study we compared the rolling interactions of PSGL-1-coated microbeads on P-selectin to those of neutrophils to examine the hypothesis that microvillus elongation may regulate P-selectin bond lifetimes. Similar approaches using microbeads as model leukocytes have been used successfully to elucidate molecular features that influence selectin mediated rolling (Goetz et al., 1997; Patil et al., 2001; Rodgers et al., 2000, 2001). Comparison of the dynamics of PSGL-1 microbeads with neutrophils suggests P-selectin bond lifetimes may be significantly increased by microvillus extension during rolling.

MATERIALS AND METHODS

Antibodies and protein substrates

KPL1, a P-selectin and L-selectin blocking monoclonal antibody (mAb) to PSGL-1, was developed as previously described (Snapp et al., 1998). The mAb against LFA-1, TS1/22, was purified from hybridoma supernatant as described (Sanchez-Madrid et al., 1982). FITC-conjugated goat anti-mouse secondary antibody was purchased from Pharmingen (San Diego, CA). Human P-selectin was purified from platelet lysates as described previously (Lawrence et al., 1997). PSGL-1 was purified from harvested HL-60 cells homogenized in 20 mM Tris, 140 mM NaCl, 0.025% sodium azide (TSA buffer) with 5 mM EDTA, and 1% Triton X-100 in the presence of protease inhibitors 10 μM leupeptin and 0.1 U/ml aprotinin, adjusted to pH 8.0. The cell lysate was passed twice over a column of CNBr-activated Sepharose 4B (Pharmacia Biotech AB, Uppsala, Sweden) coupled to KPL1 (1.3 mg/ml). The column was washed (30 column volumes) with TSA adjusted to pH 7.6 and containing 1% octylglucopyranoside (OG; Sigma, St. Louis, MO). PSGL-1 was eluted from the column using TSA, pH 12.0, with 1% OG, and neutralized with ~10% (v/v) 1 M Tris, pH 4.0, 1% OG. PSGL-1 purification was verified by Western Blot analysis using KPL1.

Neutrophil isolation

Neutrophils were isolated from whole blood with a one-step separation consisting of 94% mono-poly resolving medium (MPRM, ICN Biochemicals, Aurora, OH) and 6% sterile water (Taylor et al., 1996). After isolation, the neutrophils were suspended in Hanks' balanced salt solution (HBSS) without calcium and magnesium, that was supplemented with 10 mM HEPES, pH 7.4, and 0.1% human serum albumin (HSA), and placed on ice. For flow assays, the neutrophils were taken as needed from this reserve and washed into HBSS with 2 mM $CaCl_2$, 10 mM HEPES, pH 7.4, and 0.1% HSA at room temperature.

Preparation of microbeads

Polystyrene microbeads (9.76 $\pm$ 0.86 μm) were purchased from Polysciences, Inc. (Warrington, PA). After washing with alternating steps of water and ethanol (2×) the microbeads (5×10^7 beads/ml) were incubated with PSGL-1 in 0.1 M borate buffer, pH 8.5, for 4 h at room temperature under end-to-end rotation. To facilitate adsorption, PSGL-1 (solubilized in 1% OG) was diluted to well below the critical micelle concentration of 0.7% in buffer, and was then added to the bead suspension. After washing with HBSS, the microbeads were incubated in 0.5% Tween-20 solution for 30 min with agitation to block the remaining surface against nonspecific adhesion. The microbeads were stored in HBSS containing 0.5% Tween-20. Before use with the laminar flow chamber, the microbeads were resuspended in assay media (HBSS, 10 mM HEPES, pH 7.4, and 2 mM $CaCl_2$) at a concentration of 5×10^5 beads/ml.

To address concerns regarding receptor uprooting from the microbead surface, PSGL-1 was also coupled to the microbead surface using carbodiimide chemistry. Briefly, 10-μm-diameter carboxylate-modified polystyrene microbeads (Polysciences) were washed in alternating stages of carbonate (pH 9.6) and phosphate buffer (pH 4.0) following the manufacturer's instructions. The washing phase was followed by incubation of the microbeads in a 2% carbodiimide solution in phosphate buffer for 3 h at room temperature on a rotary shaker. The microbeads were then washed repeatedly with borate buffer and incubated in a 1 μg/ml concentration of PSGL-1 in borate buffer overnight at room temperature with end-to-end rotational mixing. The PSGL-1 microbeads were then blocked with a Tween-20 solution as described above.

In experiments to remove sialic acid residues, PSGL-1 microbeads were incubated with 0.1 U/ml neuraminidase (Calbiochem, La Jolla, CA) for 1 h at room temperature. Microbeads were then washed twice with HBSS and stored in assay media before use.

Laminar flow assay

Polystyrene slides were cut from petri dishes (Falcon 1058) and the diluted adhesion molecules were applied to the plates and allowed to adsorb for 2 h at room temperature. The slides were then blocked for nonspecific adhesion with 0.5% Tween-20 in HBSS for 1 h at room temperature. The site densities of the adsorbed purified adhesion molecules were determined by a quantitative radioimmunoassay. The P-selectin site densities used were 100 sites/μm^2 (1:200 dilution) and 25 sites/μm^2 (1:650 dilution). The wall shear stress of the parallel plate flow chamber (GlycoTech, Rockville, MD) was regulated by the gasket dimensions, which were 0.01 inches thick with a channel width of 1 cm. Cell suspensions were drawn into the chamber at room temperature using a syringe pump (Harvard Apparatus, South Natick, MA). The chamber was mounted over an inverted phase-contrast micro-

scope (Diaphot-TMD; Nikon, Garden City, NY) at 20× magnification. High-resolution differential interference contrast microcopy of neutrophils rolling on P-selectin was conducted as previously described (Schmidtke and Diamond, 2000).

Flow cytometry

Bead or neutrophil preparations (1×10^6 particles/ml) were incubated in 2 μg/ml of primary mAb in HBSS containing 1% HSA for 30 min. The microbeads or neutrophils were washed three times and then incubated for 30 min with fluorescein isothiocyanate (FITC)-conjugated, goat anti-mouse secondary antibody specific for the Fc region of the primary mAb. Samples were washed and resuspended in 400 μl of 0.2% paraformaldehyde. Fluorescence was detected with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using incorporated CellQuest software.

Scanning electron microscopy

Neutrophils were fixed in 2.5% glutaraldehyde followed by incubation in 1% osmium tetroxide. The cells were dehydrated through a series of graded ethanol solutions and critical-point dried in liquid CO_2. The slides were placed on aluminum stubs with silver paste, coated with gold-palladium, and observed in a JOEL 6400 scanning electron microscope.

Data analysis

Images from the VCR playback were digitized on an AG-5 frame acquisition card (Scion Corp., Frederick, MD) installed in a G3 Macintosh (Apple, Inc., Cupertino, CA) with NIH Image v.1.62. Cell position and velocity measurements of individual particles were acquired using an automated tracking program coded in MATLAB 5, which used a sum-of-absolute-difference algorithm to identify the cell in consecutive image frames generated through NIH Image 1.62. Velocity tracings and pause time distribution data were collected using a VC2410 charged-coupled device camera (Vicon Industries, Melville, NY). To resolve microbead and neutrophil motion from high-speed video images (240 frames/s), an interpolation routine was incorporated into the tracking program that allowed sub-image pixel resolution of changes in position (manuscript in preparation). To determine the lifetime of a PSGL-1/P-selectin tether, the amount of time a cell remained bound was determined by counting the number of image frames in which it remained stationary. To determine the bond or cellular dissociation constant, the total number of interactions was treated as a population in which all interactions had the same start time. The decline in the number of events (bond clusters) versus time was fitted to an exponential curve and a dissociation constant was calculated (Alon et al., 1995).

Cell flux measurements were obtained by counting the number of microbeads that transiently attached in a given area for 1 min. The number of interacting particles per area per observation time determined the cell flux. Microbeads were considered firmly adherent if they remained bound for >20 s.

Geometrical parameters of the system (Smith et al., 1999) and hydrodynamic force equations (Goldman et al., 1967) were used to calculate the specific bond lengths necessary to reconcile the cellular dissociation rates of the microbeads and neutrophils. The torque and force equations used were as follows:

$$T_s = 4\pi\mu R^2\delta\gamma(0.944) \quad (1)$$

$$F_s = 6\pi\mu R\delta\gamma(1.7) \quad (2)$$

The neutrophil P-selectin bond lifetime versus force relationship was matched to the curve derived from the Bell model fit of PSGL-1 microbead interactions with P-selectin (see Fig. 6) to determine the force acting on the ligand-microvillus complex. In brief, the magnitude of microbead bond lifetimes was used to estimate the actual forces acting on the bond. The force estimated from the bond dissociation constant was inserted in Eq. 3 to determine the microvillus plus bond length.

$$F_b = \frac{T_s + R\cdot F_s}{l\cdot \sin\theta} = \frac{T_s + R\cdot F_s}{\sqrt{(R+b)^2 - R^2}(R/(R+b))} \quad (3)$$

With the microbead, the parameters l, θ, b (the microvillus tether length, including the PSGL-1/P-selectin bond), and R are set by the geometry, so the flow rate can be directly related to the force on the bond, F_b. Comparison to the neutrophil k_{off} then allows us to back out the actual F_b on the neutrophil bond, and the microvillus plus bond length is solved for directly. Values of R for the neutrophil were 4.25 μm, and for the microbead the value of R was 4.9 μm.

RESULTS

PSGL-1 presence on microbeads

PSGL-1-coated microbeads were developed to explore the molecular dynamics of leukocyte rolling on P-selectin in isolation from cellular factors. PSGL-1 levels on the 10-μm beads were assessed through flow cytometry for two different concentrations of PSGL-1: a 1:100 dilution (0.01 μg/ml) and 1:25 dilution (0.04 μg/ml) of the protein in detergent-free buffer. Flow cytometry using monoclonal antibody KPL1 (Snapp et al., 1998) confirmed the adsorption of PSGL-1 on the microbeads (Fig. 1, *A--C*). A monoclonal antibody to the I-domain of the β2 integrin LFA-1, TS1/22 (Knorr and Dustin, 1997), did not recognize the PSGL-1-coated microbeads (Fig. 1 *D*). Flow cytometry demonstrated comparable levels of PSGL-1 expression on human neutrophils and the 1:100 adsorbed PSGL-1 beads (Fig. 1 *E*). Based on a comparison to neutrophils with similar mean fluorescence values and 30,000 PSGL-1 receptors/neutrophil (Moore et al., 1992), the 1:100 dilution of PSGL-1 was estimated to adsorb onto the polystyrene microbeads at a site density of ~95 sites/μm^2.

To match the PSGL-1 surface density of the microbeads with that of neutrophils as closely as possible, the 1:100 concentration of adsorbed PSGL-1 was used in the reported experiments. Binding of KPL-1 antibody to PSGL-1 microbeads and other surfaces suggested an intact antigen epitope still existed subsequent to immobilization (Ramos et al., 1998; Snapp et al., 1998).

Specificity of PSGL-1 interactions with immobilized P-selectin

PSGL-1 microbead flux was quantified to assess the tethering frequency to purified P-selectin under shear and to establish the specificity of interaction (Fig. 2). Rolling has been previously demonstrated using both an N-terminal 19-amino acid sequence of PSGL-1 or sialyl Lewis^x glyco

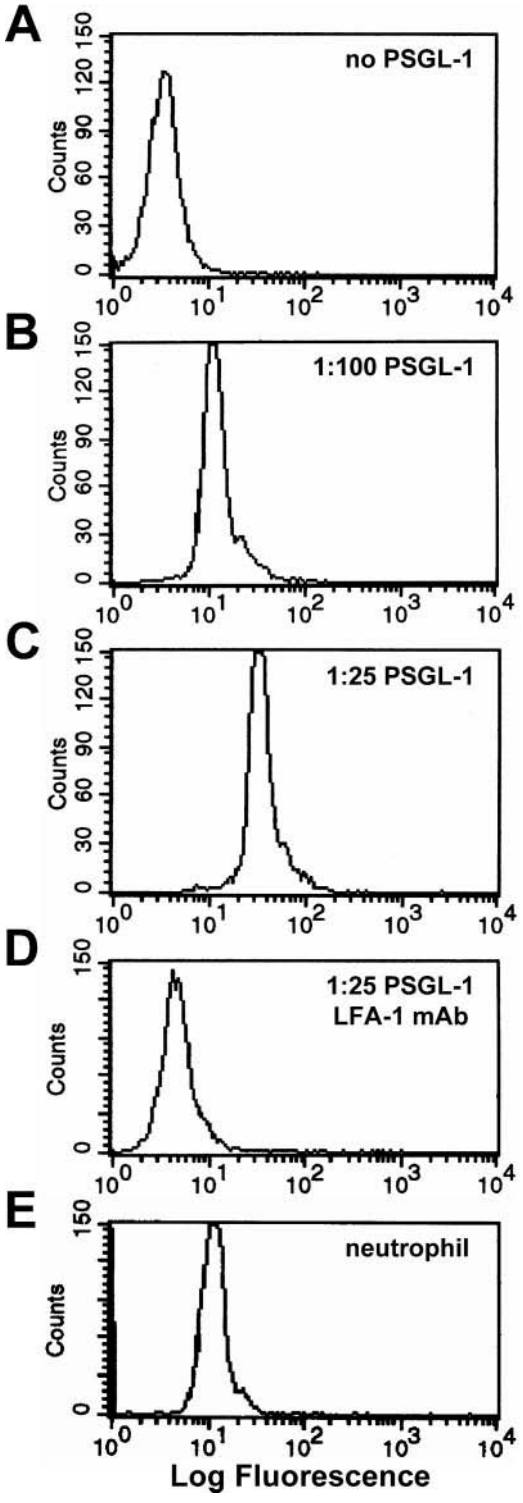

FIGURE 1 Flow cytometry results displaying the presence of PSGL-1 on the microbeads. (*A–C*) Varying concentrations of PSGL-1 were adsorbed to 10-μm microbeads. The microbeads were then incubated with mAb KPL-1, specific for PSGL-1, followed by an FITC-conjugated goat anti-mouse secondary antibody. (*D*) TS1/22, antibody to LFA-1, control experiment on the PSGL-1 coated microbeads. (*E*) KPL-1 binding to PSGL-1 on human neutrophils.

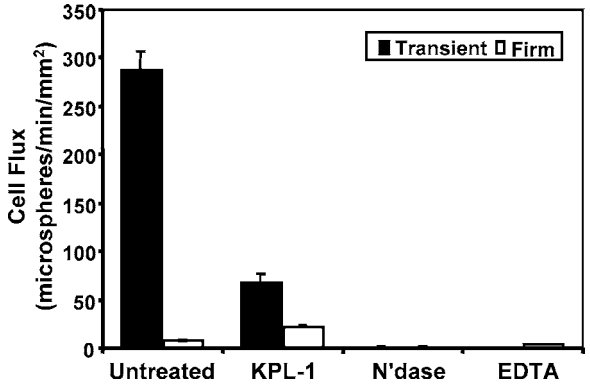

FIGURE 2 Cell flux interactions of the adsorbed PSGL-1 beads flowing at a wall shear stress of 0.7 dyn/cm^2 over 90 sites/μm^2 P-selectin substrate. The shaded bars represent transiently adhered microbeads, while the open bars represent beads that were firmly adherent for >20 s. Microbeads treated with neuraminidase or KPL-1 were shown to inhibit attachment to the substrate. Addition of EDTA was shown to eliminate transient interactions. Error bars represent one standard deviation.

conjugates immobilized on a microbead surface (Brunk et al., 1996; Brunk and Hammer, 1997; Goetz et al., 1997; Rodgers et al., 2000). We used surface-immobilized purified P-selectin as the substrate rather than CHO cells transfected with P-selectin (Goetz et al., 1997; Ramachandran et al., 1999) to minimize potential effects of membrane deformation of cellular substrates.

PSGL-1 microbeads bound to purified P-selectin-coated substrates in flow and were detected by videomicroscopy. Most PSGL-1 microbeads formed transient interactions lasting <2 s before continuing on to form more adhesive events. Transient interactions appeared to be similar in qualitative terms to neutrophil rolling on sparsely coated selectin substrates. Nonspecific, firm adhesion events were infrequently observed. On average, <0.3% of the microbeads contacting the surface formed firm adhesions as defined by pauses lasting longer than 20 s. The firm adhesion events were EDTA-insensitive and tended to not occur after several minutes of flow.

To determine the role of sialic acid residues of PSGL-1 in P-selectin recognition independent of cellular membrane carbohydrate, the PSGL-1 beads were treated with 0.1 U/ml neuraminidase. Enzymatic reduction of sialic acid of PSGL-1 on microbeads eliminated (>98%) interactions with the P-selectin substrate (Fig. 2), suggesting that tyrosine sulfation of PSGL-1 is by itself insufficient to mediate microbead rolling at forces associated with 0.7 dyn/cm^2 wall shear stress (estimated 340 pN/bond for a 92-nm bond length and lever arm of 0.95 μm). Interactions between de-sialylated PSGL-1 with P-selectin appear to be detectable at lower forces, as recently reported (Rodgers et al., 2001). The PSGL-1 microbead rolling was inhibited by KPL1, a monoclonal antibody to PSGL-1, confirming receptor specificity (Fig. 2). The presence of 5 mM EDTA in

the media completely abolished adhesion by chelating calcium cations (Fig. 2).

PSGL-1 or P-selectin receptor uprooting may be a possible mechanism of cell dissociation during rolling interactions. To address this concern, purified PSGL-1 was coupled to carboxylate microbeads through carbodiimide chemistry. Microbeads thus prepared supported rolling with pause times similar, if slightly briefer, than microbeads with adsorbed PSGL-1 (data not shown). Adsorption of PSGL-1 appeared to have created an adequate anchorage for microbead rolling at the shear forces tested.

Rolling comparisons of PSGL-1 microbeads with fixed and resting neutrophils

A representative PSGL-1 microbead velocity profile shown indicated that rolling on P-selectin consisted of distinct start and stop motions (Fig. 3 *A*). Resolvable pauses (stops) of microbeads were separated by motion at or near the estimated hydrodynamic velocity. At the same wall shear stress, neutrophils displayed a pattern of longer pause events interspersed with smaller steps and lower velocities between pauses (Fig. 3 *B*). A comparison of Fig. 3, *A* and *B* illustrates a striking difference in rolling dynamics between beads and neutrophils despite both having similar PSGL-1 surface densities.

To explore the role of microvillus extension as a source of the significant difference between the rolling dynamics of microbeads and neutrophils, glutaraldehyde-fixed neutrophils were compared to the PSGL-1 microbeads. Fixation alters the viscoelastic properties of the neutrophil by chemically cross-linking proteins (including the cytoskeleton) with each other, effectively "freezing" the neutrophil into a significantly more rigid and less deformable shape. The fixation process preserves antigenicity of PSGL-1 and L-selectin antibody binding epitopes and does not rearrange their surface distribution (Hasslen et al., 1996). The possibility that glutaraldehyde fixation may alter selectin biorecognition and related molecular properties, such as the receptor's bond compliance, cannot be ruled out at this time.

Fixed neutrophils (Fig. 3 *C*) had dynamic characteristics that were much closer to those of the coated microbeads than to the untreated neutrophils. In particular, they possessed a distinctive stop-and-go activity similar to the PSGL-1 microbeads. The behavior of the PSGL-1 microbeads and the fixed and nonfixed neutrophil populations were further contrasted by determining their average rolling velocities. The PSGL-1 microbeads, with PSGL-1 surface densities approximating those of neutrophils, rolled faster than the fixed or untreated neutrophils despite significantly higher P-selectin substrate densities (Fig. 4). Neutrophils traveled only 7.4% of the speed of the PSGL-1 beads, while fixed neutrophils were found to roll at 31.3% of the velocity of the microbeads.

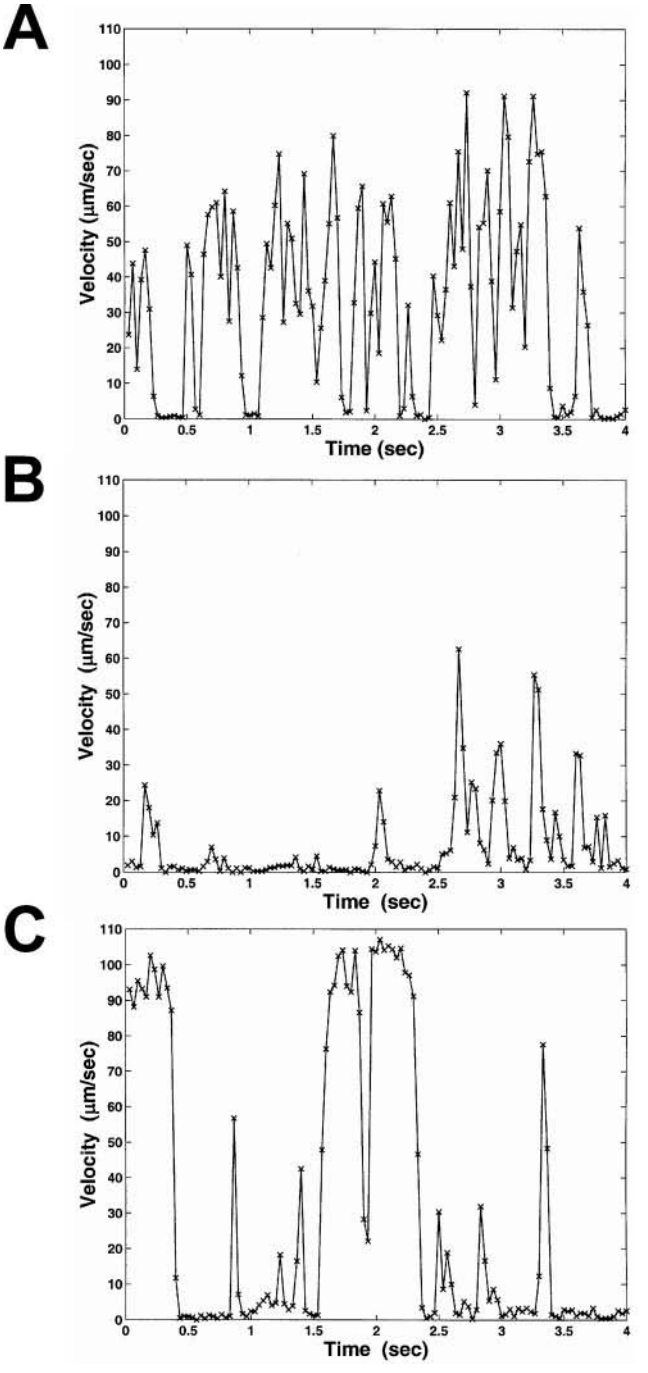

FIGURE 3 Instantaneous velocity profiles of cell and microbead rolling at 0.5 dyn/cm^2 wall shear stress. Free-flow velocity near the plate surface was ~120 μm/s. (*A*) PSGL-1 microbead rolling over 90 sites/μm^2 P-selectin. (*B*) Neutrophil rolling over 12 sites/μm^2 P-selectin. (*C*) Fixed neutrophil rolling over 12 sites/μm^2 P-selectin.

Pause time distributions reveal kinetic properties of selectin bonds and bond clusters

Transient interactions between PSGL-1 microbeads and the P-selectin substrate averaged ~0.076 s in duration at a wall shear stress of 0.5 dyn/cm^2 (Fig. 5), while the average of

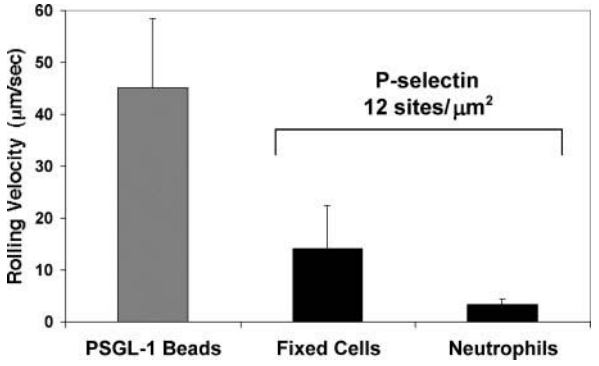

FIGURE 4 Comparison of the average rolling velocities of PSGL-1 microbeads, fixed neutrophils, and untreated neutrophils rolling at 0.5 dyn/cm^2 wall shear stress. The hatched bar designates the interactions over a P-selectin site density of 90 sites/μm^2 and the solid bar represents 12 sites/μm^2 P-selectin. Error bars represent one standard deviation.

neutrophil pauses was much longer, at 0.317 s. Glutaraldehyde-fixed neutrophils were observed to exhibit an intermediate mean pause lifetime of 0.215 s.

Selectin bond dissociation can be described through first-order reaction kinetics when the interactions are dominated by single bond events or bond clusters that behave functionally like single bonds (Tees et al., 2001). The P-selectin k_{off} value was calculated from the cumulative pause time distributions of neutrophils and PSGL-1 microbeads. In all cases the k_{off} value increased with increasing wall shear stress (Fig. 5, *A--D*), consistent with previous observations of all three selectins (Smith et al., 1999). The k_{off} values for the PSGL-1 microbead system on P-selectin ranged from 3.08 to 14.72 s^{-1} at wall shear stresses of 0.109–0.5 dyn/cm^2 (Fig. 5 *A*). The dissociation rate constants of the PSGL-1 microbeads were significantly higher than that of neutrophils when compared at the same wall shear stress (Fig. 5 *B*). By comparison, neutrophil dissociation constants ranged from 3.6 to 10.2 s^{-1} on P-selectin as the wall shear stress increased from 0.4 to 2.0 dyn/cm^2.

The force on a bond cluster of the microbead was calculated from the relationship $F_b = F_s/\cos(\theta)$, where θ is evaluated for a bond length of 92 nm and F_s represents the

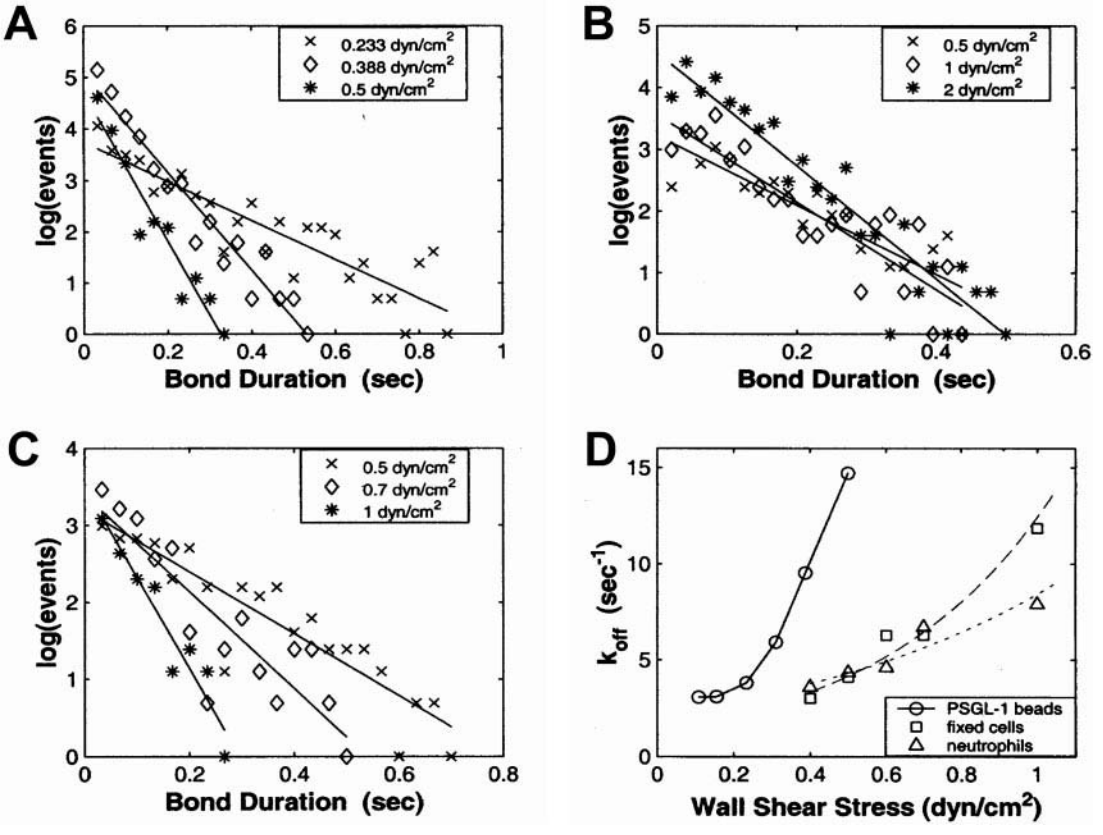

FIGURE 5 Pause time distributions used to calculate the bond dissociation constants of the PSGL-1 microbeads interacting with the P-selectin substrate. The dissociation constant values, k_{off}, correspond to the negative slope of the logarithmic plots of the cumulative distributions. Dissociation constant (k_{off}) values were calculated for the (*A*) PSGL-1 microbeads, (*B*) untreated neutrophils, and (*C*) fixed neutrophils. (*D*) Relationship between the off-rate of PSGL-1 and P-selectin relative to the wall shear stress. Data from the PSGL-1 microbeads rolling on 90 sites/μm^2 P-selectin, along with that of the fixed neutrophils and untreated neutrophils rolling on 12 sites/μm^2 P-selectin.

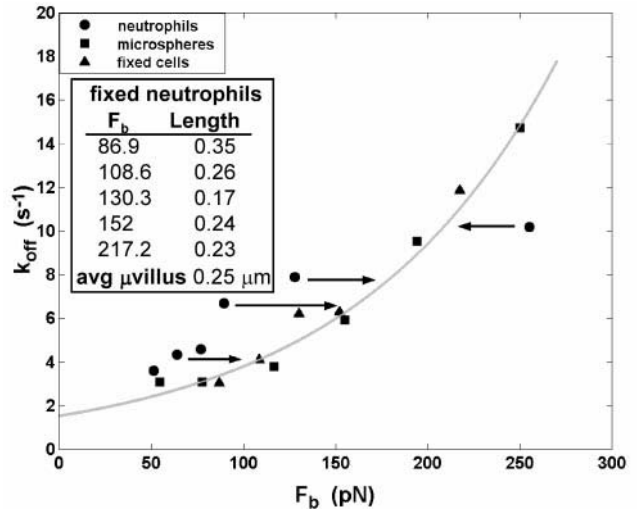

FIGURE 6 Off-rate of the PSGL-1/P-selectin bond as a function of the force exerted on the linkage. Comparison of the k_{off} of neutrophils, glutaraldehyde-fixed neutrophils, and PSGL-1-coated microbeads. Data from the ligand-coated microbeads were fitted to Bell's equation. The unstressed dissociation constant (k^o_{off}) was 1.54 s^{-1} with a bond compliance (σ) of 0.37 Å. The arrows indicate the direction of the force change necessary to equate the molecular dissociation properties of PSGL-1 on the beads with PSGL-1 expressed on neutrophils. Initial lever arm length predictions for the untreated neutrophils were overestimated for lower shear force ranges and underestimated at higher ranges. Fixed neutrophils were analyzed with the same data from the PSGL-1 microbeads, and microvilli lengths at each force measurement were calculated to extract an average microvillus length of 0.25 μm (*inset*). The points representing the fixed neutrophils were then re-plotted using the 0.25 μm microvillus length.

shear force from flow (Fig. 6). Nonlinear fitting of the microbead dissociation kinetic constants to the Bell model produced a bond separation distance (σ) (also described as reactive bond compliance) of 0.37 Å and an unstressed dissociation constant (k^o_{off}) of 1.54 s^{-1}, similar to previous observations (Alon et al., 1995; Smith et al., 1999). The nonlinear fit of the Bell equation to the k_{off} data of the microbeads was better ($\chi^2 = 1.19$) than the fit of data from a similar analysis of rolling neutrophils ($\chi^2 = 6.7$) (Smith et al., 1999), suggesting that PSGL-1/P-selectin interactions mediated through microbeads fit the Bell model better than in a cellular context.

In contrast to observations with PSGL-1 microbeads, the k_{off} values of neutrophils tethering on P-selectin exhibited a plateau effect as the wall shear stress was increased up to and above 1 dyn/cm^2, as previously observed (Smith et al., 1999). There was no apparent plateau in dissociation constants observed with the PSGL-1 microbeads at estimated forces of the same or greater magnitude (Fig. 5, *A* and *D*). It is possible that at higher flow rates a plateau in PSGL-1 microbead k_{off} might exist; however, the low frequency of microbead tethering and the brevity of rolling interactions precluded reliable statistical sampling. Fixed neutrophils appear to dissociate with relatively similar off-rates as resting neutrophils at low wall shear stresses (0.4–0.7 dyn/cm^2), but their dissociation constants increased exponentially with wall shear stress (Fig. 5, *C* and *D*).

Microvillus extension increases lever arm of a transiently tethered neutrophil and lowers resultant force on the bond

Definition of a lever arm length allowed the calculation of force exerted on the bond cluster under various wall shear stresses. The lever arm calculated for a microbead was 0.95 μm with a bond angle of 79°, based on the geometrical constraints of a sphere in contact with a flat surface and 92 nm for the length of the P-selectin/PSGL-1 bond (Li et al., 1996; Ushiyama et al., 1993). Bonds formed between PSGL-1 microbeads and P-selectin at 1 dyn/cm^2 wall shear stress were therefore subjected to a minimum of 480 pN of force. At such levels of force a stressed P-selectin bond cluster would be expected to last only a millisecond. In comparison, the previously estimated lever arm of a neutrophil resulted in a force per bond estimate of 124.4 pN at 1 dyn/cm^2 wall shear stress (Alon et al., 1997).

We hypothesized that the differences in the off-rate constants between PSGL-1 microbeads and neutrophils were due primarily to microvillus extension at the relatively low wall shear stress used in the flow chamber assay. Consequently, an appropriate microvillus length was calculated for each wall shear stress to reconcile the neutrophil's dissociation constant with that of the PSGL-1 microbead's k_{off} versus force curve (Fig. 6). By using the bond lifetime as a reporter of the force, the lever arm (and microvillus length) was calculated for the rolling neutrophil as a function of force.

A comparison of the fixed neutrophil's k_{off} versus force curve with that of PSGL-1 microbeads (Fig. 6) suggested the average microvillus length of the fixed neutrophils was 0.25 $\pm$ 0.07 μm throughout the range of shear forces investigated. The microvillus length on fixed neutrophils appeared therefore to be constant. The estimate of microvillus length of 0.25 μm scaled closely to values from electron microscope measurements of 0.3 μm for resting neutrophils (Bruehl et al., 1996; Hasslen et al., 1996; Shao et al., 1998).

By mapping the non-fixed (resting) neutrophil data onto those of the rigid beads and fixed neutrophils, the microvillus length was calculated to range from 0.21 μm at 0.4 dyn/cm^2 wall shear stress to 1.58 μm at 2 dyn/cm^2 (Table 1). A spring constant was then calculated from the relationship between the F_b at rupture and the calculated change in length of the microvillus (Table 1). For microvillus extensions estimated to be on the order of 0.1 μm (30% strain), the spring appeared to be very stiff. As force was increased, however, it appeared that the mi-

TABLE 1 Microvillus lengths and associated spring constants

Wall Shear Stress (dyn/cm^2)	Force on Bond (pN)	k_{off} (s^{-1})	Microvillus Length (μm)	Δl (μm)	Strain (%)	Spring Constant (pN/μm)
0.4	94	3.6	0.21	—	—	—
0.6	121	4.6	0.3	0.09	43	1342
1	180	7.9	0.38	0.17	81	1059
2	209	10.2	1.58	1.31	624	152

A spring constant is calculated by dividing the force on a single tether/bond anchorage point (pN) by the change in neutrophil microvillus length (μm) that was determined by comparing neutrophil and microbead k_{off} constants. Microvillus extension is modeled as a Hookean spring, so that $F = k*L$, with the spring constant k (in pN/μm) and the length L (in μm), i.e., the extension of the microvilus. Strain calculated by dividing ΔL by the resting microvillus length.

crovillus became progressively easier to extend. At a wall shear stress of 2 dyn/cm^2 and an estimated 200 pN at the anchorage point, the microvillus spring constant appeared to have fallen almost ninefold in stiffness, from 1342 pN/μm to 152 pN/μm. Varying average microvilli lengths from a resting value of ~0.21 μm to 1.6 μm completely reconciled the k_{off} versus F_b relationship obtained with microbeads and neutrophils (Fig. 6 and Table 1). Similarly, the use of noncompliant 0.25-μm microvilli on fixed neutrophils completely reconciled the k_{off} versus F_b relationship obtained with PSGL-1 microbeads.

High-speed video analysis of neutrophil tethering events and microscopic visualization of long membrane tether formation in shear

Using high-temporal-resolution video microscopy, microvillus extension was estimated from the deceleration of the neutrophil subsequent to forming a bond with P-selectin (Fig. 7, *A* and *C*). Immediately following tethering, the "creeping" displacement of the neutrophil centroid measured using high-speed video was <4% of the hydrodynamic velocity. Consequently, it was hypothesized that the submicron displacement of the neutrophil's centroid was a consequence of stretching of the anchored microvillus following a bond formation event.

Neutrophil and PSGL-1 microbead skipping motions on P-selectin were obtained with a temporal resolution of 240 frames/s (4 ms/frame). A representative trajectory showing a bond formation event (neutrophil tethering) followed by a bond dissociation event is shown in Fig. 7 *A*. The time duration between motion of a PSGL-1 microbead in flow and a detectable pause on the substrate was <4 ms on average (Fig. 7, *A* and *B*). For the last time point before neutrophil detachment, the lifetime of the PSGL-1/P-selectin bond corresponded to a 42% bond breakage probability.

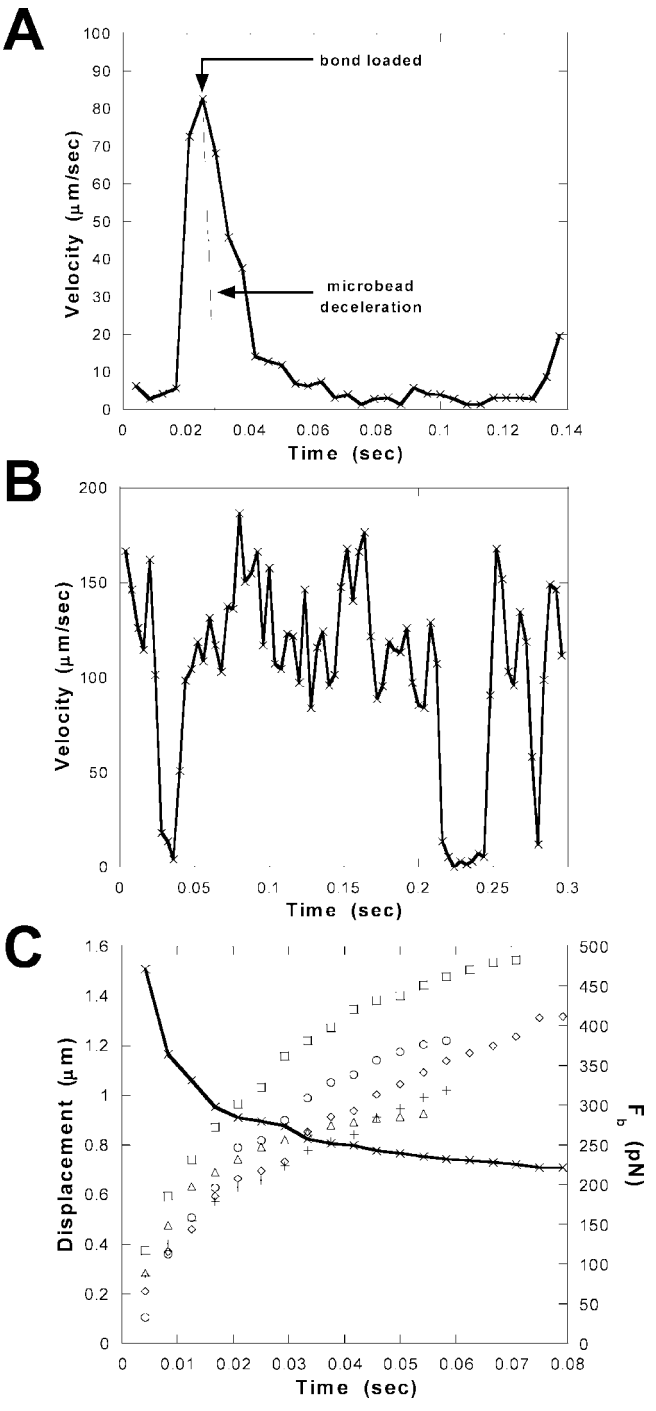

FIGURE 7 Measurement of dynamic microvillous extension directly from velocity profile. (*A*) High temporal resolution of 240 frames/s (4 ms/frame) can discern the minuscule motions of the neutrophil after an attachment has formed with the substrate. (*B*) High-speed resolution of PSGL-1 microbead tethering at 1.0 dyn/cm^2 displayed a lack of slow deformation. (*C*) Neutrophil displacement data (+, □, ○, ◇, △) after initial attachment under 2.0 dyn/cm^2 wall shear stress. The predicted force loaded on the selectin bond (*) during the elongation process was calculated for the cell labeled ◇.

The deceleration of the neutrophil was considerably slower than that of a representative PSGL-1 microbead

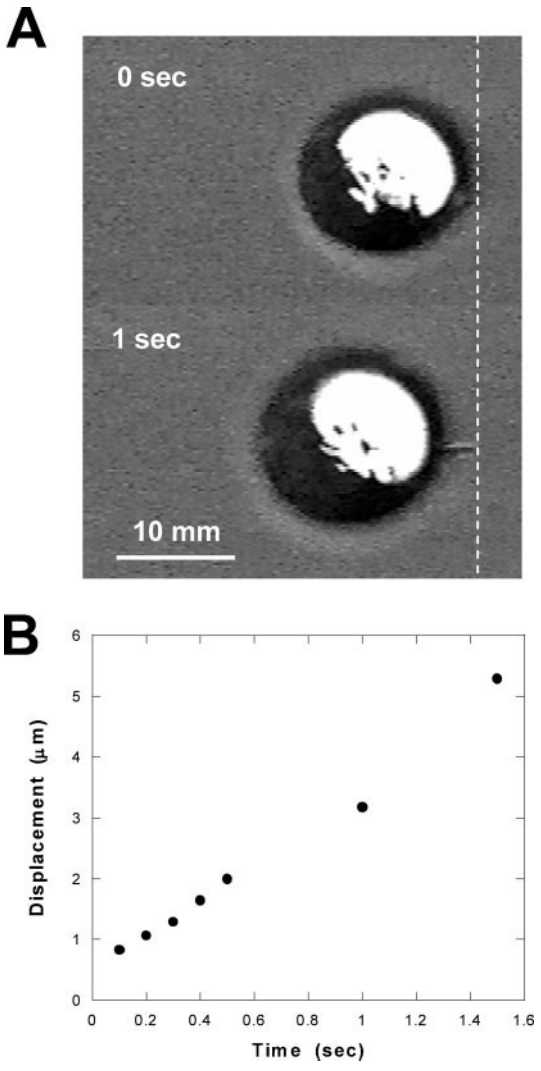

FIGURE 8 Neutrophil tether elongation seen with differential interference contrast microscopy. (*A*) Visual evidence of tether formation of a neutrophil attaching at 1.5 dyn/cm^2 on 10 sites/μm^2 P-selectin. (*B*) The extent of elongation with the formation of long-lived tethers as seen with the extension measurements from visualized microvilli tethers at 1.5 dyn/cm^2. Bar, 10 μm.

under the same flow conditions (Fig. 7, *A* and *B*). Creeping-like motions associated with neutrophils were absent during PSGL-1 microsphere rolling. Distances moved between formation of an adhesive tether and release and return to hydrodynamic flow velocity were used to generate a displacement (stretch) versus time curve for five neutrophils (Fig. 7 *C*). Changes in the estimated force on the bond for a representative microvillus extension (Fig. 7 *C*) are concentrated in the first 15% of the median lifetime of the tether. As the tether linkage (bond plus stretched microvillus) lengthened, the resultant force changed progressively less for each unit of length extension (see Materials and Methods).

Some neutrophils formed membrane tethers long enough to be detected by differential interference contrast (DIC) microscopy (Fig. 8 *A*). A representative time-stretch curve of a long tether was plotted to compare with both the short-time displacement curves generated by neutrophil motion (Fig. 8 *B*) and previous measures of microvilli mechanical properties (Shao et al., 1998). Long-duration (>1 s) of creeping-like motions (Schmidtke and Diamond, 2000; Shao et al., 1998) were not observed in any of the PSGL-1 microbead interactions with P-selectin, but were occasionally observed when neutrophils rolled on P-selectin. Tether extensions visualized by DIC microscopy at 0.2 s matched well with estimates of microvillus extension determined from tracking neutrophil displacement subsequent to tether formation (Figs. 7 *C* and 8 *B*). At longer times (>0.1 s) the elastic structure of the microvillus appeared to break down, as indicated by the steady extension of the tether with time at constant force (Fig. 8 *B*).

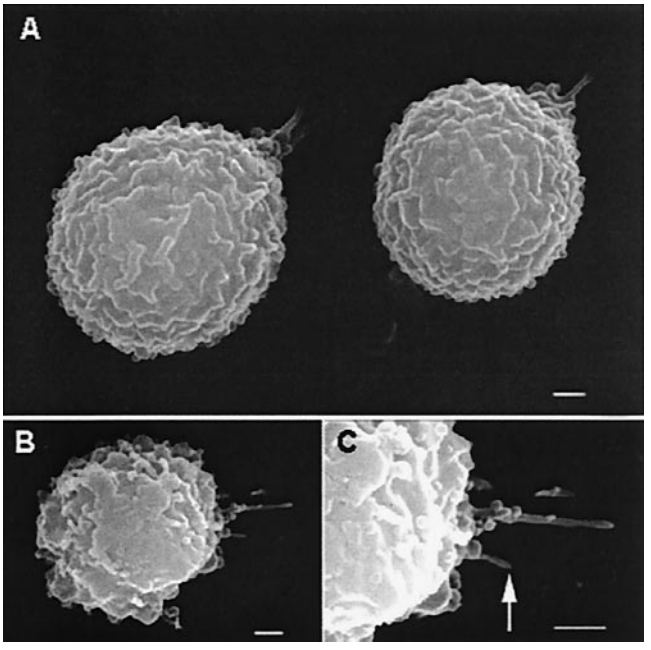

FIGURE 9 Surface topography of rolling neutrophils on 200 sites/μm^2 P-selectin. Neutrophils were subjected to 2 dyn/cm^2 wall shear stress in assay media before infusion of 2.5% glutaraldehyde at the same flow rate. (*A*) Low magnification images of two adjacent rolling neutrophils showing a singlet and doublet tether restraining the cells. (*B*) Low-magnification and (*C*) high-magnification image of a tethered neutrophil. The arrow shows a possible tether just beginning to form. Bars, 1 μm.

Microvilli tethers were apparent in the scanning electron micrographs (SEM) made from neutrophils that were glutaraldehyde-fixed while rolling (Fig. 9). The neutrophils' exteriors maintained their characteristic ruffled appearance during rolling on P-selectin, even with the microvilli trailing the cell body. A few of the adherent cells displayed two or three tethers. The presence of these multiple tethers may be a result of the high P-selectin site density (200 sites/μm^2) of the substrate. A majority of the microvilli extensions from the neutrophil membrane examined under SEM were either

single or double tethers, with lengths ranging from 1 to 3 μm. This measurement correlates well with the dynamic analysis of microvillus extension at 2 dyn/cm^2. The calculated estimate of 1.58 μm from the pause-time distribution data (Table 1) and the visual surveillance of tethers 1–3 μm in length are consistent in scale.

DISCUSSION

Comparison of the dynamics of rolling neutrophils with PSGL-1 microbeads suggested that the elasticity of structures such as microvilli that display PSGL-1 significantly lowered the stresses on PSGL-1 bonds with P-selectin during rolling. The elasticity of microvilli appeared to be much greater than that of the PSGL-1/P-selectin molecular linkage (Alon et al., 1997; Howard, 2001) and may therefore serve to stabilize neutrophil rolling interactions with vascular endothelium in vivo. PSGL-1, like L-selectin, is localized on the tips of microvilli (Bruehl et al., 1996; Moore et al., 1995) or more precisely, on microridges formed by the neutrophil's excess membrane area (Schmid-Schonbein et al., 1980). Presentation of PSGL-1 on the tips of leukocyte or neutrophil microvilli has been hypothesized to facilitate capture or tethering under flow conditions, presumably by concentrating binding sites on the point first in contact with an apposing cell surface (Moore et al., 1995; Picker et al., 1991; von Andrian et al., 1995). In addition, it has been recently hypothesized that the position of PSGL-1 on microvilli tips may facilitate neutrophil rolling on P-selectin, particularly if microvilli can stretch under physiologically relevant forces (Schmidtke and Diamond, 2000; Shao et al., 1998).

The effect of force on noncovalent selectin bond lifetimes has been quantified by analysis of the time-varying motions of leukocytes as they roll in shear (Alon et al., 1995; Kaplanski et al., 1993; Smith et al., 1999). In parallel, studies of leukocyte rolling using microbead systems have demonstrated that the physical chemistry of selectins imparts the ability to support rolling interactions in the absence of cellular factors (Goetz et al., 1997; Rodgers et al., 2000, 2001). A significant advantage of the rigid microbead system exploited in this study is that bond formation is limited to a small area within reach of the adhesive receptors on the respective surfaces of the bead and substrate. For instance, the force on a selectin anchorage point is constrained to a minimum 480 pN/dyn/cm^2 wall shear stress by the length of the stressed PSGL-1/P-selectin bond (Fritz et al., 1998; Li et al., 1996; Ushiyama et al., 1993). Unlike the case with a microbead, however, the force on a neutrophil's PSGL-1/P-selectin bond cluster additionally depends on the microvillus length and any other structure that contributes to the tether arm. Analysis of neutrophil motion during rapid flow reversal has previously suggested microvilli are $\sim$1 μm in length, a value threefold greater than estimates based on electron micrographs (Alon et al., 1997).

In contrast to the model of a rigid microvillus, recent micropipette measurements indicate that neutrophil microvilli might be elastic enough to extend in response to forces that neutrophils might encounter in vivo (Shao et al., 1998). Pulling of neutrophil membrane tethers by micropipette aspiration suggests that under a stress of 34 pN or less, microvilli exhibit a spring constant of 43 pN/μm (Shao et al., 1998). With forces in this range the microvillus eventually reaches an extension of $\sim$1 μm, resulting in a lower force on the bond due to the movement of the anchorage point relative to the neutrophil during rolling. A transition regime above 34 pN and below 61 pN also exists in which both elastic behavior and membrane "tether" formation can be observed. At stresses above 61 pN, most microvilli convert to tethers (Shao et al., 1998) and extend steadily with time. Membrane tethers pulled from microvilli therefore appear to have substantially different viscoelastic properties, depending on the force applied and duration of pulling.

The ability of microvilli to stretch and form membrane tethers as neutrophils roll on immobilized platelets and P-selectin substrates has been directly observed by high-resolution microscopy (Schmidtke and Diamond, 2000) and takes place at forces considerably below that necessary to significantly deform a rolling neutrophil (Cao et al., 1998; Dong et al., 1999; Firrell and Lipowsky, 1989; Lei et al., 1999). Consequently, in this study we focused our comparison of microbeads and neutrophils at forces where the influence of microvilli elongation on rolling dynamics would be predicted to be most apparent. Critical to the comparison of rolling dynamics of microbeads and neutrophils was the assumption that PSGL-1 bonds with P-selectin shared the same mechanical properties in the two systems. In support of this assumption, even the N-terminal 19-amino acid truncated form of PSGL-1 embodies sufficient structure to support microbead rolling on P-selectin (Goetz et al., 1997; Rodgers et al., 2000). The significantly longer native form of PSGL-1 used in this study is likely to be even less affected by the surface properties of the microbead. Additionally, purified PSGL-1 immobilized on surfaces has also been shown to support L-selectin-dependent neutrophil rolling (Ramos et al., 1998; Walcheck et al., 1996), supporting the hypothesis that purified and cellular PSGL-1 have similar functionality. Therefore, with the assumption that PSGL-1 receptors on microbeads and neutrophils share equivalent molecular mechanics, it became possible to exploit the stochastic properties of P-selectin bonds as a force reporter.

A second major assumption underlying the comparison of PSGL-1 microbead rolling dynamics to those of neutrophils is that the same numbers of PSGL-1/P-selectin bond clusters are being compared in the two systems to determine the dissociation constant. If the bond clusters mediating microbead and neutrophil were significantly different in number or size, then the conclusion that microvillus extension

imparts greater dynamic stability on a rolling neutrophil would be subject to uncertainty.

The effect of multiple bonds has been recently examined in the special case of the influence of dimerization on PSGL-1 and P-selectin interactions (Ramachandran et al., 2001). When dimeric P-selectin is compared to monomeric P-selectin, the apparent k_{off} value of neutrophils is lowered, presumably because at least two bonds are supporting the load. As a result, the entire force versus dissociation constant curve is flattened and the bond's apparent reactive compliance is lowered. By contrast, the dissociation constants of neutrophils rolling on P-selectin shift from an exponential dependence on force to an almost linear dependence at 1 dyn/cm^2 wall shear stress (Smith et al., 1999). For such a shift to be a consequence of a shear-induced increase in bond number the effect would have to be extremely abrupt, almost as if a switch were flipped and the neutrophil interactions became mediated by multiple rather than single bonds. An alternative explanation is that the shift to a linear k_{off} increase may indeed reflect the effect of microvillous extension. At this time it is clear that further modeling analysis, perhaps using adhesive dynamics simulations (King and Hammer, 2001), will be necessary to fully explore the consequences of the potentially complex force distributions that might exist during multibond rolling interactions.

While it is difficult at this time to resolve the question of whether multiple or single bonds are controlling PSGL-1 microbead and neutrophil rolling, experimental evidence offered some support for the validity of the quantal bond cluster hypothesis. The strongest evidence was the apparent absence of small bond breakage events leading to steps during the adhesive interactions of both microbeads and neutrophils while tethering (or skipping) on P-selectin. In particular, PSGL-1 microbeads decelerated within an average of 4 ms from hydrodynamic velocity and became stationary until release from the surface. To escape detection, the P-selectin bond with PSGL-1 would have to be briefer by almost an order of magnitude than previously supposed (Alon et al., 1995; Smith et al., 1999; Tees et al., 2001) and be closer to a sub-millisecond lifetime as recently proposed for L-selectin interactions with PSGL-1 (Evans et al., 2001). In the case of neutrophils, motion subsequent to tethering was very slow and appeared to be dominated by viscoelastic extension of a putative microvillus structure. Neutrophil steps between adjacent bonds stressed in series would have to be smaller than 20 nm and considerably briefer than 4 ms to escape detection. A further point to be weighed in the question of possible differences in the number of bonds being compared between microbeads and neutrophils is that k_{off} estimates matched most closely at low forces, where the effect of microvillus stretching would be expected to be least significant.

Consistent with the estimates derived from the PSGL-1 microbead and neutrophil bond cluster lifetime comparisons, scanning electron microscopy of neutrophils rolling on P-selectin indicated that microvilli tether lengths of 1–3 μm were frequent at the wall shear stresses predicted by the bond lifetime analysis. While the apparent stiffness of the microvillus spring constant decreased eightfold between 0.4 and 2.0 dyn/cm^2 wall shear stress, its lowest value was still at least fourfold higher (stiffer) than that estimated using monoclonal antibody anchored tethers (Shao et al., 1998). One explanation for the discrepancy between the two sets of observations may be the significant difference in the time scale of the measurements. It is important to note that only a few of the neutrophil pauses analyzed in this study lasted as long as the shortest-lived membrane tether pulled by micropipette aspiration. Over the broad range of pulling times examined in this study and that of Shao and coworkers (1998), microvillus mechanical stiffness might vary significantly as cytoskeletal elements disassemble or otherwise rearrange under the applied loads. For instance, the long membrane tethers occasionally formed by neutrophils rolling on P-selectin extended steadily, much like those detected during micropipette pulling experiments.

Another important consideration in the interpretation of the PSGL-1 microbead data is the potential contribution of cell body deformation to increasing the neutrophil contact area with a P-selectin expressing surface. The moment the neutrophil is anchored by a bond it experiences a force perpendicular to the plane of the substrate from the fluid shear-induced torque. If the neutrophil were flattened to any significant degree its contact area with the substrate would be expected to increase, enhancing the likelihood of additional bond formation (Lawrence et al., 1997). In fact, blood flow in vivo can visibly deform a rolling neutrophil into a more hydrodynamic profile, which likely increases the contact area with the vascular endothelium (Dong et al., 1999; Firrell and Lipowsky, 1989). In vitro flow chamber studies indicate that an increase from 4 to 20 dyn/cm^2 wall shear stress can compress the height of an HL-60 cell by as much as 2 μm and double the contact area with the substrate (Lei et al., 1999). Under the flow conditions ($<$2 dyn/cm^2 wall shear stress) at which we examined neutrophils, the reduction in height would be calculated to be $<$0.2 μm, significantly constraining the potential for contact area increase and bond formation at the low site densities examined. At this time, however, the effect of neutrophil flattening as a possible explanation for the differences in behavior compared to microbeads and fixed neutrophils in this study cannot be rigorously excluded.

The highly elastic nature of microvilli in conjunction with whole cell deformation would appear to have a significant influence on the strength of neutrophil rolling interactions in vivo, particularly when coupled with the effects of force-sensitive adhesive bonds. Mathematical modeling efforts have suggested that selectin bonds must be very close to "ideal" bonds, in that their lifetimes must be nearly force independent to support rolling interactions over the wide

range of fluid shear forces that exist in vivo (Chang et al., 2000; Hammer and Apte, 1992). However, direct measures of selectin bond lifetimes under stress (Alon et al., 1995; Smith et al., 1999) indicate that breakage increases more rapidly with force, i.e., selectins appear to be "slip" bonds (in the Bell model, the bond separation distance (σ) is greater than 0) rather than "ideal" ($\sigma = 0$) or "catch" bonds ($\sigma < 0$) to use the terminology of Dembo and colleagues (Dembo et al., 1988). Microvillus extension, by shielding the selectin bond from the torque it would experience if anchored on a nonextensible surface, could contribute to creating an ideal bond, or more properly, an "ideal linkage" that would facilitate rolling in shear over a wide range of fluid forces. Both the positioning of PSGL-1 on extensible microvilli and the unique physical chemistry of its bond with P-selectin may therefore contribute the complex mechanical and molecular process that control leukocyte trafficking and homing to inflammatory sites. Neutrophil microvillus extension subsequent to selectin tether formation may therefore have important physiological implications on neutrophil recruitment in vivo through the modulation of selectin bond lifetimes.

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