A Direct Comparison of Selectin-Mediated Transient, Adhesive Events Using High Temporal Resolution

McRae J. Smith,* Ellen L. Berg,# and Michael B. Lawrence*

*Department of Biomedical Engineering, University of Virginia School of Medicine and School of Engineering and Applied Science, Charlottesville, Virginia 22908; and #Protein Design Laboratories, Inc., Fremont, California 94555 USA

ABSTRACT Leukocyte capture and rolling on the vascular endothelium is mediated principally by the selectin family of cell adhesion receptors. In a parallel plate flow chamber, neutrophil rolling on purified selectins or a selectin-ligand substrate was resolved by high speed videomicroscopy as a series of ratchet-like steps with a characteristic time constant (Kaplanski, G., C. Farnarier, O. Tissot, A. Pierres, A.-M. Benoliel, M. C. Alessi, S. Kaplanski, and P. Bongrand. 1993. *Biophys. J.* 64:1922–1933; Alon, R., D. A. Hammer, and T. A. Springer. 1995. *Nature (Lond.).* 374:539–542). Under shear, neutrophil arrests due to bond formation events were as brief as 4 ms. Pause time distributions for neutrophils tethering on P-, E-, L-selectin, or peripheral node addressin (PNAd) were compared at estimated single bond forces ranging from 37 to 250 pN. Distributions of selectin mediated pause times were fit to a first order exponential, resulting in a molecular dissociation constant (k_{off}) for the respective selectin as a function of force. At estimated single bond forces of 125 pN and below, all three selectin dissociation constants fit the Bell and Hookean spring models of force-driven bond breakage equivalently. Unstressed k_{off} values based on the Bell model were 2.4, 2.6, 2.8, 3.8 s^{-1} for P-selectin, E-selectin, L-selectin, and PNAd, respectively. Bond separation distances (reactive compliance) were 0.39, 0.18, 1.11, 0.59 Å for P-selectin, E-selectin, L-selectin, and PNAd, respectively. Dissociation constants for L-selectin and P-selectin at single bond forces above 125 pN were considerably lower than either Bell or Hookean spring model predictions, suggesting the existence of two regimes of reactive compliance. Additionally, interactions between L-selectin and its leukocyte ligand(s) were more labile in the presence of flow than the L-selectin endothelial ligand, PNAd, suggesting that L-selectin ligands may have different molecular and mechanical properties. Both types of L-selectin bonds had a higher reactive compliance than P-selectin or E-selectin bonds.

INTRODUCTION

Under flow conditions, neutrophil tethering to the endothelial lining of the blood vessel is mediated by various combinations of L-, P-, and E-selectin, depending on the nature of the inflammatory stimulus (Ley and Tedder, 1995). When ordered in close succession, the formation of transient selectin bonds by neutrophils results in rolling on the endothelium, ultimately leading to β_2 integrin (CD18) mediated arrest and firm adhesion. It is hypothesized that in order to support tethering under shear, selectins have high rates of bond formation and dissociation relative to β_2 integrins. While ineffective under venular flow conditions, β_2 integrins nevertheless have bond formation rates sufficient to ligate co-receptors expressed on inflamed endothelium once selectins have initiated rolling (von Andrian et al., 1991; Lawrence and Springer, 1991). It may be hypothesized that the effects of shear forces on molecular kinetics may control the outcome of adhesive interactions (Bell, 1978; Hammer and Apte, 1992; Tozeren and Ley, 1992).

All three selectins share identical tandem domain arrangements and have very high levels of sequence homology. Additionally, their ligands require a highly specific arrangement of sialic acid and fucose, called sialyl Lewis^x, for molecular recognition. While sharing many common elements, the tissue distribution and regulation of the three selectins are quite different, possibly reflecting their critical involvement in a number of immunological processes (Kansas, 1996). P-selectin is induced on endothelium and platelets by inflammatory mediators (McEver et al., 1989) and its leukocyte ligand, P-selectin glycoprotein ligand-1 (PSGL-1), is preferentially located on the tips of leukocyte microvilli (Moore et al., 1995). E-selectin is expressed on the surface of cytokine-stimulated endothelial cells and binds to carbohydrate ligands on leukocytes (Lenter et al., 1994; Knibbs et al., 1996; Wagers et al., 1997). L-selectin, like PSGL-1, is constitutively expressed on the tips of leukocyte microvilli. L-selectin can bind PSGL-1 (Guyer et al., 1996; Walcheck et al., 1996b; Spertini et al., 1996) and other unknown ligands (Fuhlbrigge et al., 1996; Ramos et al., 1998) on leukocytes to mediate leukocyte-leukocyte interactions in addition to recognizing carbohydrate ligands on endothelium (Zakrzewicz et al., 1997). Peripheral node addressin (PNAd, also known as MECA-79 antigen) is a conglomerate of mucin-like sialoglycoproteins expressed on high endothelial venules (HEV) that mediates L-selectin dependent rolling (Lawrence et al., 1995) and directs lymphocytes to peripheral lymph nodes (Berg et al., 1991, 1998). PNAd contains at least four HEV L-selectin ligands (Sassetti et al., 1998; Rosen and Bertozzi, 1994).

A number of methods have been applied to measure the effect of forces on biologic adhesive bonds, including ap-

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Address reprint requests to Michael B. Lawrence, Ph.D., University of Virginia, Department of Biomedical Engineering, Health Sciences Center, Box 377, Charlottesville, VA 22908. Tel.: 804-982-4641 (office), 804-924-9085 (lab); Fax: 804-982-3770; E-mail: mbl2a@virginia.edu.

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proaches such as micromanipulation (Evans et al., 1995), shear flow chambers and centrifugation assays (Piper et al., 1998; Kaplanski et al., 1993), optical trapping (Schmidt et al., 1993), atomic force microscopy (Fritz et al., 1998), surface force apparatus (Leckband et al., 1994), and more recently, dynamic force probes (Merkel et al., 1999). In contrast to micromechanical approaches, direct measures of molecular equilibrium and kinetics by methods such as surface plasmon resonance cannot readily include the effect of blood flow-induced forces on bond lifetimes (van der Merwe and Barclay, 1996). Fluid shear in a parallel plate flow chamber generates forces and loading rates on cell adhesive bonds not unlike the situation in vivo when leukocytes encounter the inflamed vessel wall or each other. Leukocyte rolling on purified selectins or cultured endothelial cells in vitro has been resolved by videomicroscopy as a series of discrete steps, or pauses, hypothesized to be due to bonds that temporarily prevent further downstream motion (Alon et al., 1995; Goetz et al., 1994; Lawrence et al., 1997). The insight that adhesive bond dissociation constants can be estimated from the distribution of pause times observed during singular leukocyte adhesive interactions has been used to quantify the effect of force on bond lifetimes (Alon et al., 1995; Kaplanski et al., 1993) and determine molecular mechanical properties based on the Bell and Hookean spring models of force-driven molecular dissociation (Bell, 1978; Dembo et al., 1988; Kuo et al., 1997).

Recent reports and biophysical modeling studies have hypothesized that selectin-mediated leukocyte rolling requires a relatively high level of bond stiffness (or low reactive compliance) compared to monoclonal antibody-antigen interactions (Tempelman and Hammer, 1994; Alon et al., 1995; Dembo et al., 1988). P-selectin bonds have been estimated to have a bond separation distance of $\sigma = 0.30$ Å, as defined in Bell's model (Bell, 1978), a value that is considerably lower (producing a stiffer bond) than the only estimate of σ that exists for a monoclonal antibody-antigen interaction, that of CD15, where $\sigma = 0.8$ Å (Chen et al., 1997). Mathematical models of leukocyte rolling adhesion (Hammer and Apte, 1992; Tozeren and Ley, 1992) have suggested that the reactive compliance of a selectin bond would correlate positively with rolling velocity, i.e., a selectin with higher reactive compliance would mediate faster rolling. However, whether or not the reactive compliance is an important determinant of rolling velocity has been unclear for the special case of L-selectin bonds (Alon et al., 1997, 1998). Under increasing levels of force, it has been reported that L-selectin dissociation constants do not increase nearly as rapidly as E-selectin or P-selectin dissociation constants, suggesting that L-selectin has a much lower bond-reactive compliance than the two other selectins, yet supports much faster rolling interactions (Alon et al., 1997). This observation suggested that bond compliance may not correlate with characteristic leukocyte rolling velocities as previously hypothesized (Hammer and Apte, 1992; Tozeren and Ley, 1992). Because the duration of L-selectin bonds may be on the order of standard video capture rates (Taylor et al., 1996; Alon et al., 1997; Lawrence et al., 1997), we hypothesized that improved temporal and spatial resolution would allow direct measurement of adhesive interactions too brief to have been previously detected, therefore clarifying the role of reactive bond compliance in L-selectin mediated rolling.

MATERIALS AND METHODS

Isolation of P-selectin, E-selectin, L-selectin, and PNAd substrates

Human P-selectin was purified from out-dated platelet lysates as previously described (Lawrence et al., 1997). Human E-selectin was purified from Chinese Hamster Ovary (CHO) cell lysates transfected with wild-type human E-selectin cDNA as previously described (Lobb et al., 1991; Lawrence et al., 1997). L-selectin was purified from human tonsil (provided by Dr. R. S. Larson, University of New Mexico, Albuquerque, NM) lysates by DREG-56 monoclonal antibody (mAb) affinity chromatography (Kishimoto et al., 1991). The human tonsil was homogenized in 20 mM Tris, pH 8.0; 140 mM NaCl; and 0.025% azide (TSA, pH 8.0) with 5 mM EDTA, 10 mM leupeptin (Sigma, St. Louis, MO), 0.1 U/ml aprotinin (Sigma), and 1% Triton X-100. The lysate was then centrifuged at 2000 rpm and 33,000 rpm for 15 min and 1 h, respectively. After centrifugation the lysate was passed over a column of CNBr-activated Sepharose 4B (Pharmacia Biotech, Piscataway, NJ) coupled to DREG-56 (2 mg/ml) twice. The column was washed with TSA (20× bed volume), pH 8.0, containing 1% octylglucopranoside (OG; Sigma Chemical Co.). L-selectin was eluted with 50 mM acetate (5× the bed volume), pH 3.0, containing 1% OG; and neutralized with 1 M Tris, pH 9.0, 1% OG (15% vol/vol). PNAd was purified from human tonsil lysates with MECA-79 mAb affinity chromatography as previously described (Berg et al., 1991).

Site densities of adsorbed selectin proteins were determined by saturation binding radioimmunoassay using mAbs DREG-56 for L-selectin (Kishimoto et al., 1991), G1 for P-selectin (gift from Dr. R. P. McEver, University of Oklahoma, Oklahoma City, OK) (Geng et al., 1990), and BB11 for E-selectin (a gift from Dr. R. Lobb, Biogen, Inc., Cambridge, MA) (Lobb et al., 1991). The mAbs were iodinated with Iodobeads (Pierce, Rockford, IL) to a specific activity of 10 μCi/μg for DREG-56, 7 μCi/μg for G1, and 5 μCi/μg for BB11.

Antibodies

The mAb against human purified P-selectin, G1 (IgG1, 10 μg/ml), was used to block P-selectin-dependent adhesion of neutrophils to immobilized P-selectin. The mAb against human purified E-selectin, BB11 (IgG1, 10 μg/ml), was used to block E-selectin-dependent adhesion of neutrophils to immobilized E-selectin. DREG-56 (IgG1, 10 μg/ml), a mAb against human purified L-selectin, was used to block adhesion between neutrophils and immobilized L-selectin. Plates with P-, E-, or L-selectin were incubated with 10 μg/ml of blocking mAbs (G1, BB11, DREG-56, respectively) for 15 min after assembly into the flow chamber. Plates were then rinsed with assay media for 3 min at 1.25 ml/min (1 dyn/cm^2) in preparation for cell perfusion.

Neutrophil isolation and cell lines

Forty to eighty million human neutrophils were obtained from 60 ml heparin (10,000 U/ml) anti-coagulated whole blood. Neutrophils were isolated with a one-step density 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, 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.

For studies on the effect of L-selectin shedding on transient neutrophil tether lifetimes, a murine pre-B lymphocytic cell line, 300.19, was used (a gift of Dr. T. F. Tedder, Duke University, NC). One group of 300.19 cells was transfected with L-selectin (300.19L), while the other group was a mutated variant with L-selectin that could not be shed (300.19Lns) (Chen et al., 1995). Both shedding and nonshedding L-selectin transfected 300.19 cells were cultured in RPMI Medium 1640 with 10% fetal bovine serum (FBS) and 10 mM 2-mercaptoethanol (GIBCO-BRL, Grand Island, NY).

Laminar flow adhesion assay

Polystyrene slides were cut from bacteriological 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 3% HSA in TSA overnight at 4°C. The site densities of the adhesion molecules used as a substrate were determined by radioimmunoassay to a limit of 50 sites/μm^2, the lower limit of detection in our binding assay. Site densities for lower amounts of immobilized selectin were then estimated by proportional dilution. The P-selectin site densities used were 25 sites/μm^2 (1:650 dilution), 12 sites/μm^2 (1:1350 dilution), and 9 sites/μm^2 (1:1800 dilution). The E-selectin site densities used were 25 sites/μm^2 (1:800 dilution), 12 sites/μm^2 (1:1667 dilution), and 9 sites/μm^2 (1:2222 dilution). The L-selectin site densities used were 170 sites/μm^2 (1:5 dilution), 85 sites/μm^2 (1:10 dilution), and 50 sites/μm^2 (1:17 dilution). The PNAd dilutions (from a stock at 100 μg/ml) used were 1:3, 1:6, and 1:15, which gave approximately the same level of adhesion as the L-selectin dilutions used in the bond lifetime estimate studies. The purified human adhesion molecules were diluted in 50 mM Tris, pH 8, and 0.025% azide. In many cases, immobilization of adhesion receptors on polystyrene creates significant background due to interactions of the neutrophil CD18b/CD18 (Mac-1) integrin. Firm adhesion observed in our system was comparable to that observed on lipid bilayers and was ~0.1% or less of the total number of interactions.

The chamber was mounted over an inverted phase-contrast microscope (Diaphot-TMD; Nikon, Garden City, NY) at 20× magnification. For each substrate slide, 5 ml of a 0.5% Tween-20 in TSA solution was perfused over the substrate and incubated for 5 min to aid in the blocking of nonspecific adhesion.

Data acquisition and cell tracking

A Kodak MotionCorder Analyzer, Model 1000 camera (Eastman Kodak Co., Motion Analysis System Division, San Diego, CA) was used for high temporal resolution of neutrophil adhesive events with the substrates. Neutrophils perfused over L-selectin and PNAd were viewed at a frame rate of 240 frames/s (fps). Cells on P-selectin and E-selectin were viewed at 48 fps. Images were recorded on VCR tapes for cell tracking analyses at a later time.

Pause times for neutrophils interacting with L-selectin, P-selectin, E-selectin, and PNAd were acquired using a computer tracking program coded in MATLAB 5 (Dr. W. F. Walker, University of Virginia), which uses a sum-of-absolute-difference algorithm to identify the cell in consecutive image frames. Video memory from the high-speed camera was played back at standard video rates for archiving on VHS tapes. Images from the VCR playback were then captured onto a computer (Apple, Inc., Cupertino, CA) with NIH Image v.1.62. The amount of time a cell remained bound was determined by counting the number of image frames in which it remained stationary. Assuming the selectin bonding event can be modeled as a reversible, bimolecular reaction such that $A + B = C$, the rate of bond formation can be described by $dC/dt = k_{on}(A)(B) - k_{off}(C)$. When the probability of a second bond formation during the lifetime of the first bond is low due to the sparse substrate density of adhesion receptors, the product $k_{on}(A)(B)$ is negligible. The expression for dC/dt simplifies to $k_{off}C = -(dC/dt)$ which can be integrated, giving the equation $\ln(C) = -k_{off}t + \text{const}$. This allows the determination of k_{off} from the slope of the natural log of number of interactions versus the duration of each event, or pause. A high degree of fit of the linear regression suggests that a quantal unit of bond(s), either a single bond or a threshold number of uniform bonds (bond cluster), mediates the detected pauses in the motion of neutrophils. In the case of neutrophil-endothelial cell interactions, the condition of low site density has been achieved by infusion of partially saturating concentrations of blocking antibodies to reduce effective site density (Kaplanski et al., 1993). One important qualification should be noted regarding the existence of multiple bonds in the contact patch on the neutrophil's motion under flow conditions. If additional bonds form and their location is such that they are not loaded by the fluid stresses, then they are not influencing the duration of the pause. Unloaded bonds are nevertheless positioned to bear loads once the bond at the trailing edge releases, and this is how rolling is likely supported.

RESULTS

Relative capture rates and rolling velocities for neutrophils interacting with P-selectin or L-selectin

P-selectin or L-selectin was adsorbed to the lower wall of a flow chamber at the same site density to compare neutrophil tethering (capture) rates under flow conditions. In this system, selectin presentation, density, and anchorage are identical so that direct comparisons may be made at comparable site densities. Therefore, immobilization of P-selectin and L-selectin in a flow chamber serves to simulate the conditions in which primary and secondary neutrophil capture mechanisms can be compared (Bargatze et al., 1994).

Neutrophils were perfused over immobilized L-selectin adsorbed at a density of 170 sites/μm^2, at wall shear stresses ranging from 0.5 dyn/cm^2 to 5.0 dyn/cm^2 for 2 min at each flow condition to determine the tethering rate (Fig. 1 *A*). Neutrophil accumulation on L-selectin was dominated by primary tethers that were detected at wall shear stresses as high as 5.0 dyn/cm^2, significantly higher than previous observations of P-selectin and E-selectin tethering in vitro in flow chamber assays. Neutrophils perfused over immobilized P-selectin at the same site density tethered over a lower range of shear forces than L-selectin, and with a much lower rate of accumulation at wall shear stresses of 1.0 dyn/cm^2 and greater (Fig. 1 *A*). Only at wall shear stresses below 1.0 dyn/cm^2, near the shear threshold for L-selectin mediated rolling (Finger et al., 1996; Lawrence et al., 1997), was P-selectin capture efficiency greater than through L-selectin. Neutrophil rolling on either purified L-selectin or purified P-selectin was completely blocked by the appropriate antibodies (DREG-56 or G1, respectively, data not shown).

In order to analyze neutrophil rolling for evidence of discrete, quantal patterns of momentary arrest, which have been hypothesized to depend on selectin bond lifetime (Kaplanski et al., 1993; Alon et al., 1995), neutrophils rolling over immobilized L-selectin, P-selectin, E-selectin, or PNAd were tracked by high-resolution videomicroscopy (240 fps) at low site densities. Representative position traces of individual neutrophils are shown. At the low site densi-

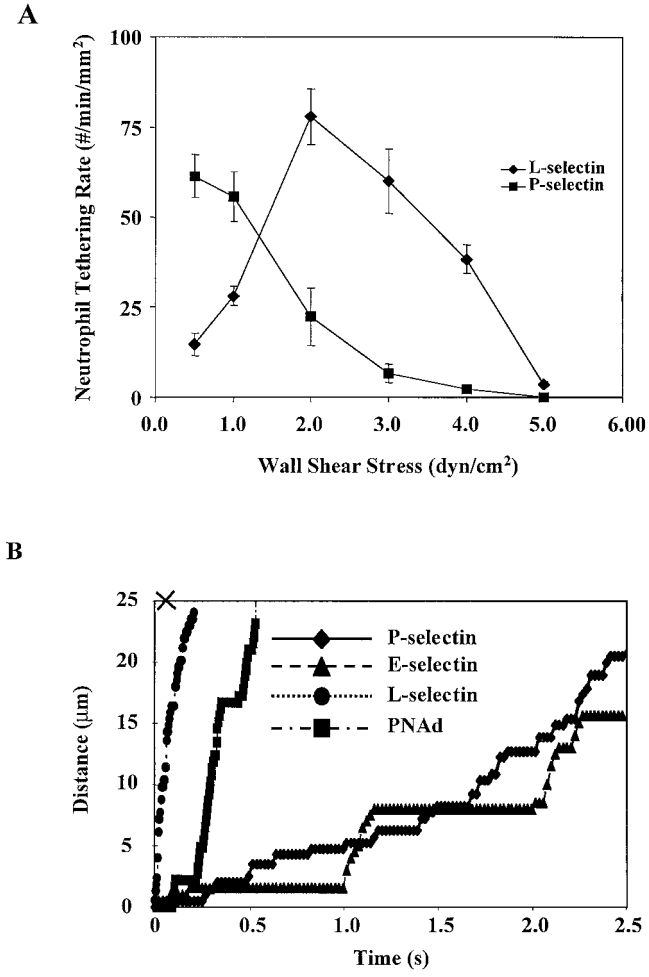

FIGURE 1 Relative neutrophil capture rates of immobilized L-selectin and P-selectin at comparable site densities contrasted with rolling velocities. (*A*) Neutrophils were perfused over substrates expressing either immobilized L-selectin (170 sites/μm^2) or P-selectin (150 sites/μm^2) for 3 min at the indicated wall shear stresses. (*B*) Distance-time plot of individual neutrophils rolling on immobilized L-selectin (170 sites/μm^2), P-selectin (25 sites/μm^2), E-selectin (25 sites/μm^2), and PNAd (7 μg/ml concentration, 1:15 dilution) at 2.0 dyn/cm^2 wall shear stress and resolved at 20× magnification (smallest resolvable step size was 0.5 μm). The symbol (X) at the top and left in the plot indicates the time required to travel 25 μm at critical velocity. The pauses occurring for the neutrophil interacting with the immobilized L-selectin last only a few video frames and are therefore difficult to see in the distance-time tracking at the scale used. Data in (*A*) represent the mean of three independent experiments at each flow rate. Data in (*B*) represent individual tracks of representative rolling neutrophils on the indicated substrates.

ties of selectins and selectin ligands examined, the stochastic nature of rolling was readily evident, consisting of skips punctuated by pauses, even at image acquisition rates of 240 fps (4 ms/frame). The pause times for neutrophils interacting with E-selectin or P-selectin were significantly longer than those mediated by L-selectin, even though the site densities were considerably lower (Fig 1 *B*). As a result The PNAd site density is unknown because it consists of several glycoproteins with L-selectin ligand activity, though CD34 (a component of PNAd) concentration has been shown to correlate with overall PNAd activity (Purl et al., 1995, 1997). Neutrophil translocation velocities over immobilized L-selectin ranged from 50 to 125 μm/s, while P-selectin and E-selectin translocation velocities were 8 μm/s and 6 μm/s, respectively. The order of magnitude differences in translocation velocity at a wall shear stress of 2.0 dyn/cm^2 suggests that pause times of L-selectin and the vascular selectins may be significantly different at venular levels of fluid shear.

Pause times for selectin-mediated rolling as a function of wall shear stress

Inasmuch as neutrophil rolling on low site densities of selectins and selectin ligands was characterized by discrete ratchet-like steps resolvable by videomicroscopy, the magnitude of pause times was analyzed to identify a characteristic time of interaction (Fig. 2) (Kaplanski et al., 1993; Alon et al., 1995). The pause time for neutrophils interacting with P-selectin, E-selectin, L-selectin, or PNAd at 0.5–2.0 dyn/cm^2 wall shear stress was calculated from the distance-time trajectories of tracked neutrophils. This allowed estimation of the intrinsic time constant of the selectin bond. Based on previous observations of bond lifetimes for P-selectin and E-selectin (Alon et al., 1995; Kaplanski et al., 1993), an image frame capture rate of 48 fps was chosen, 60% faster than standard video rates of 30 fps.

Neutrophils interacting with P-selectin and E-selectin substrates at densities of 9 sites/μm^2 had very similar declines in pause time variation with increasing wall shear stress. The pause times for the P-selectin substrate fell 31% as wall shear stress was increased fourfold (Fig. 2 *A*), while the pause times for the E-selectin substrate fell 49% for the same fourfold increase in flow (Fig. 2 *B*). For the range of site densities examined (9–25 sites/μm^2) the mean pause time did not increase significantly, suggesting that within the range of site densities tested, bond cluster lifetimes were not strongly dependent on site density (data not shown). Mean pause times were briefer than previously reported (Alon et al., 1995), possibly due to the use of higher temporal and spatial resolution that allowed quantification of a population of selectin-mediated pauses previously undetected.

The pause times for neutrophil adhesive events with the L-selectin substrate were approximately half the value of those for P- and E-selectin at a wall shear stress of 0.5 dyn/cm^2. However, as flow was increased to 1.0 dyn/cm^2 wall shear stress, L-selectin-mediated pauses became almost an order of magnitude briefer than those of P- and E-selectin, necessitating the use of frame capture rates of 240 fps, eightfold faster than standard video rates (Fig. 2). The pause times for the L-selectin substrate at 50 sites/μm^2 dropped from 0.074 to 0.012 s as wall shear stress increased from 0.5 to 2.0 dyn/cm^2 (Fig. 2 *C*). Data were pooled for the L-selectin site densities of 50 and 85 sites/μm^2 at a wall shear stress of 0.8 dyn/cm^2 in order to increase the number

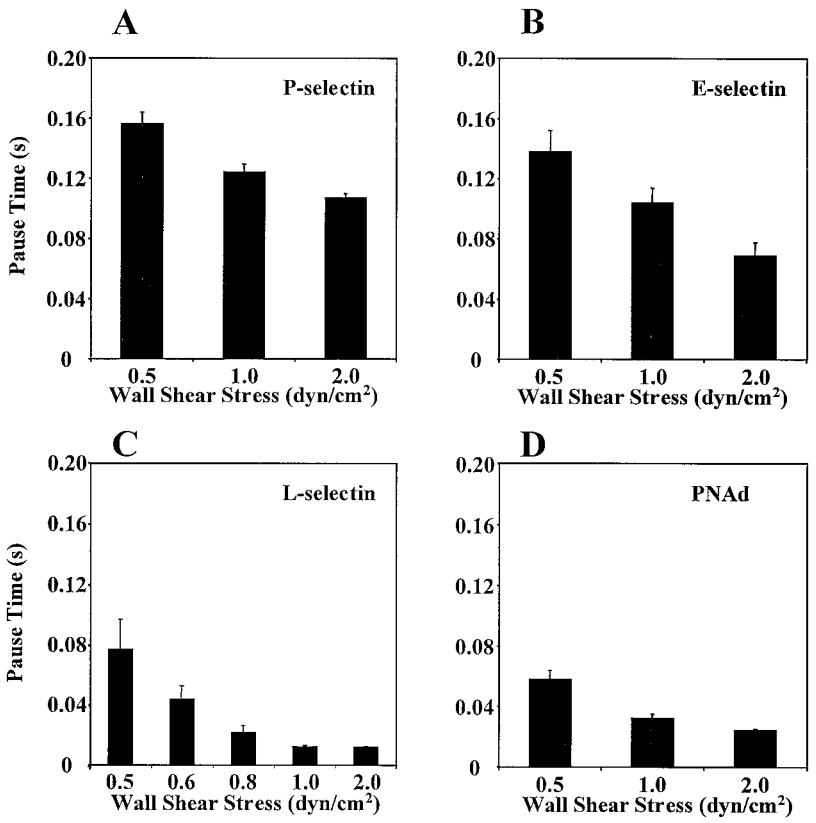

FIGURE 2 Pause times (mean ± SE) of neutrophil tethers on selectins or PNAd as a function of wall shear stress. (*A*) Neutrophil pause times on P-selectin at 9 sites/μm^2. (*B*) Neutrophil pause times on E-selectin at a site density of 12 sites/μm^2. (*C*) Neutrophil pause times on L-selectin at 50 sites/μm^2. (*D*) Neutrophil pause times on PNAd (1:15 dilution). A minimum of 23 cells with 35 interactions (PNAd substrates) were averaged at each condition, with most averaging 40 cells and ~120 interactions (pauses). Rolling neutrophils would have multiple pauses while rolling through the microscope field of view. Image acquisition rate for neutrophils rolling on P-selectin and E-selectin was 48 fps, while for neutrophils interacting with L-selectin or PNAd the acquisition rate was 240 fps.

of interactions analyzed, as the pause time did not vary significantly with site density. The pause times for the PNAd substrate were longer than those for L-selectin substrates, dropping from 0.061 to 0.024 s as wall shear stress increased from 0.5 to 2.0 dyn/cm^2 (Fig. 2 *D*).

Comparison of pause times for selectin-mediated neutrophil rolling: effect of L-selectin shedding on pause time

The contribution of L-selectin proteolytic cleavage (shedding) to the unusual brevity of L-selectin-mediated interactions was examined by quantifying the mean pause times of a murine B cell line transfected with a mutated form of L-selectin resistant to shedding (Chen et al., 1995). Inhibition of shedding has been demonstrated to lower the rolling velocity of neutrophils on isolated PNAd and on venules in the mouse cremaster muscle, possibly because L-selectin proteolysis is required for rapid bond dissociation (Walcheck et al., 1996a; Hafezi-Moghadam and Ley, 1999).

300.19 cells were transfected with either wild-type L-selectin (300.19L) or a mutated form of L-selectin lacking the protease recognition site (300.19Lns). The pause time for neutrophil tethers on immobilized L-selectin at a wall shear stress of 2.0 dyn/cm^2 was 0.013 ± 0.001 s (mean ± SE), while for tethering on PNAd at 2.0 dyn/cm^2 the pause time was 0.024 ± 0.001 s (mean ± SE) (Fig. 3). The cell lines 300.19L and 300.19Lns had pause times of 0.025 ± 0.003 s and 0.024 ± 0.003 s (mean ± SE), respectively, for tethering on PNAd at a wall shear stress of 2.0 dyn/cm^2 (Fig. 3). There was no statistical difference in the mean pause times of the 300.19L and 300.19Lns cell lines tethering on PNAd, suggesting that L-selectin shedding did not modulate pause times. Rather, it appears that the duration of L-selectin-mediated pauses likely represents an intrinsic biomechanical property.

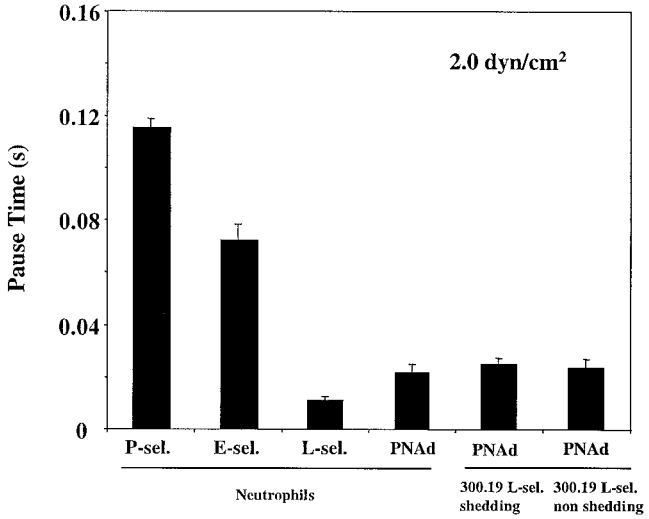

FIGURE 3 Pause times (mean ± SE) of neutrophils and 300.19 L-selectin transfectants interacting with substrates at 2.0 dyn/cm^2 wall shear stress. Pause times for 300.19L transfectants and 300.19Lns (nonshedding) transfectant interactions with PNAd substrate at a dilution of 1:3.

Distribution of pause times for selectin-mediated neutrophil adhesive interactions allows determination of selectin dissociation constants under force

To estimate the dissociation constants (k_{off}) of selectins, the duration of individual neutrophil tethers with purified selectins and selectin ligands was determined by automatic tracking. P-selectin k_{off} values increased with increasing wall shear stress (Fig. 4 *A*), consistent with previous observations (Alon et al., 1995). Dissociation constants for P-selectin at 9 sites/μm^2 varied from 5.73 to 10.22 s^{-1} for wall shear stresses of 0.5–2.0 dyn/cm^2 (Fig. 4 *A*). Maximum wall shear stresses during selectin tethering were twice as high as in previous studies so bond lifetimes could be quantified over a broader range of forces. The highest k_{off} estimated for P-selectin was twice that reported using P-selectin immobilized in a lipid bilayer (Alon et al., 1995). This finding indicates that neutrophil tethers were not influenced by longer duration β_2 integrin-mediated interactions and that the biomechanical responses of the receptor was not altered significantly by immobilization onto polystyrene.

Dissociation constants for neutrophils interacting with E-selectin were similar to those for P-selectin (Fig. 4 *B*) with the values for k_{off} increasing with increases in wall shear stress. At an E-selectin site density of 9 sites/μm^2, dissociation constants ranged from 3.60 to 7.86 s^{-1} as wall shear stresses were increased from 0.5 to 2.0 dyn/cm^2 (Fig. 4 *B*). R^2 values for the interactions with E-selectin were significantly lower than for the other substrates examined and may reflect the effect of pooling multiple ligands for E-selectin on the neutrophil surface that may have different biophysical properties. At this time, a specific glycoprotein ligand has not been described for E-selectin analogous to the PSGL-1-P-selectin interaction. Nevertheless, despite the possible existence of multiple E-selectin ligands, E-selectin dissociation constants scale to similar magnitudes as P-selectin dissociation constants (Fig. 4).

Dissociation constants for neutrophils tethering on L-selectin were strikingly different from those derived for P- and E-selectin (Fig. 4 *C*), increasing ninefold with only a doubling of wall shear stress. The neutrophil tethers to L-selectin at 50 sites/μm^2 produced k_{off} values that ranged from 10.2 to 101.5 s^{-1} as wall shear stresses were increased from 0.5 to 2.0 dyn/cm^2 (Fig. 4 *C*). The dissociation constant for 0.8 dyn/cm^2 was calculated from data pooled from 50 and 85 sites/μm^2 of L-selectin to give a greater number of interactions. Assuming a monoexponential decay of bonds, the probability of a bond lasting longer than a given period of time (Δt) can be estimated by $P_b = 1 - \exp(-k_{off}*\Delta t)$ (Hammer and Apte, 1992; Tees and Goldsmith, 1996). In the case of L-selectin interactions with the neutrophil L-selectin ligand, ~80% of the pauses detected are

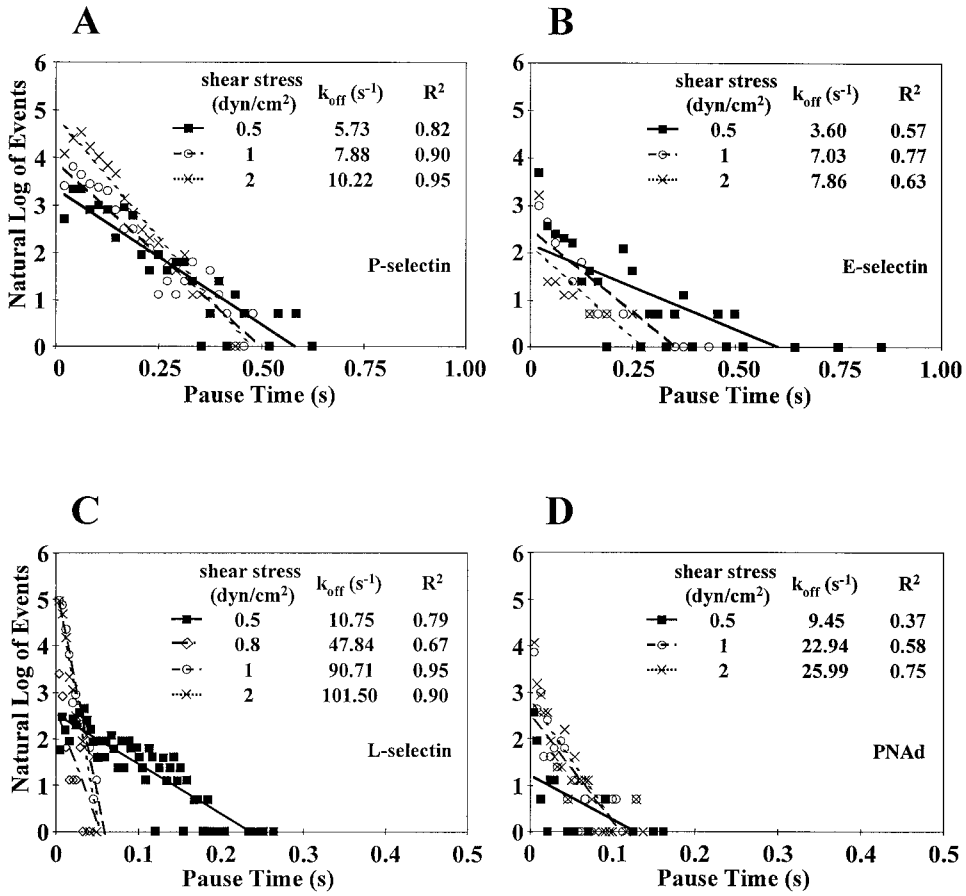

FIGURE 4 The distribution of pause times for neutrophils interacting in a transient manner with four substrates at the lowest site densities evaluated. The dissociation rate constant, k_{off}, is equal to the negative slope of the linear regression for the corresponding distributions. (*A*) Dissociation kinetics for P-selectin substrate at 9 sites/μm^2 at indicated wall shear stresses. (*B*) Dissociation kinetics for E-selectin substrate at 9 sites/μm^2 at indicated wall shear stresses. (*C*) Dissociation kinetics for L-selectin substrate at 50 sites/μm^2 at indicated wall shear stresses. (*D*) Dissociation kinetics for PNAd substrate at a dilution of 1:15 and indicated wall shear stresses.

briefer than one standard video frame of 33 ms at 0.8 dyn/cm^2 wall shear stress. Compared to the severalfold increase in k_{off} at flows up to 1.0 dyn/cm^2 wall shear stress, there was a near-plateau in k_{off} values between 1.0 and 2.0 dyn/cm^2.

While the dissociation constants for the PNAd substrate increased more with the increase in wall shear stress than P- and E-selectin substrates, the proportional change in k_{off} values was less than that observed for the L-selectin substrate. At a 1:15 dilution of PNAd, the dissociation constants increased from 9.45 to 25.99 s^{-1} as wall shear stresses increased from 0.5 to 2.0 dyn/cm^2 (Fig. 4 *D*). L-selectin bonds with PNAd were less susceptible to force than the interaction of L-selectin with its ligand on neutrophils, but more susceptible to force than P-selectin and E-selectin. An apparent plateau in k_{off} was evident for neutrophil interactions with L-selectin, PNAd, and P-selectin. Spatial aliasing does not appear to account for the plateau effect inasmuch as it was observed for both PNAd and immobilized L-selectin, and P-selectin, all with widely differing dissociation constants.

Variation of measured selectin dissociation rate constants with site density and wall shear stress

To further characterize the dependence of selectin dissociation constants with force, we measured the selectin k_{off} values for three site densities where quantal patterns of pauses were observed. For neutrophil adhesive events with the P-selectin substrates, dissociation constants varied by at most a factor of 2 between P-selectin site densities 9 and 25 sites/μm^2 (Fig. 5 *A*); with the lower site densities producing distributions of pauses with higher estimated dissociation constants. The slowest dissociation constant (at 25 sites/μm^2) was similar to those derived from neutrophil interactions on lipid bilayer immobilized P-selectin (at 5 sites/μm^2) (Alon et al., 1995). Increasing wall shear stress produced an increase in the P-selectin dissociation constant for each site density. Even at 25 sites/μm^2, neutrophil rolling may therefore be mediated by a significant population of single selectin bonds at wall shear stresses as high as 2.0 dyn/cm^2. The dependence of k_{off} on site density likely reflects a combination of two types of effects. One, there are increased numbers of multiple bond events. Two, with higher site densities, the average ratchet step becomes smaller and in some cases more difficult to detect—leading to a perceived longer pause as two shorter events are observed as one longer event. This results in a slight decrease in the estimated k_{off} values. These two effects nevertheless appear to be small as shown by the relatively small changes in estimated k_{off} values.

E-selectin dissociation constants (Fig. 5 *B*) were similar in magnitude to the P-selectin dissociation constants, although the trend between site densities was not as clear, possibly due to a smaller number of interactions that were analyzed. Dissociation constants for neutrophils interacting with E-selectin in general increased with increasing wall shear stress. One observed decrease in k_{off} as wall shear stress increased appeared to be a consequence of small sample size. E-selectin mean k_{off} values as a function of wall shear stress were very similar to mean k_{off} values for the P-selectin substrates.

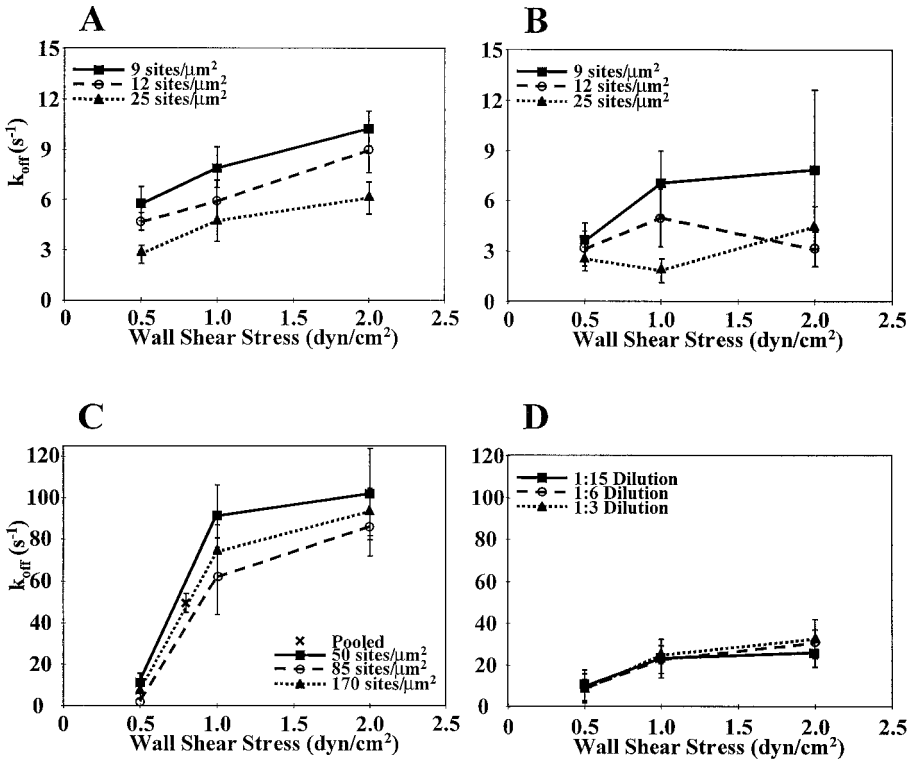

FIGURE 5 The effect of wall shear stress on the dissociation kinetics of neutrophils interacting with four substrates at various site densities. (*A*) Dissociation kinetics for P-selectin substrate at 9, 12, and 25 sites/μm^2 for increasing wall shear stress (0.5, 1.0, and 2.0 dyn/cm^2). (*B*) Dissociation kinetics for E-selectin substrate at 9, 12, and 25 sites/μm^2 for increasing wall shear stress (0.5, 1.0, and 2.0 dyn/cm^2). (*C*) Dissociation kinetics for L-selectin substrate at 50, 85, and 170 sites/μm^2 for increasing wall shear stress (0.5, 1.0, and 2.0 dyn/cm^2). (*D*) Dissociation kinetics for PNAd substrate at dilutions of 1:15, 1:6, and 1:3 for increasing wall shear stress (0.5, 1.0, and 2.0 dyn/cm^2). The error bars represent a 95% confidence level on the slope of the linear regression for each distribution of adhesive events.

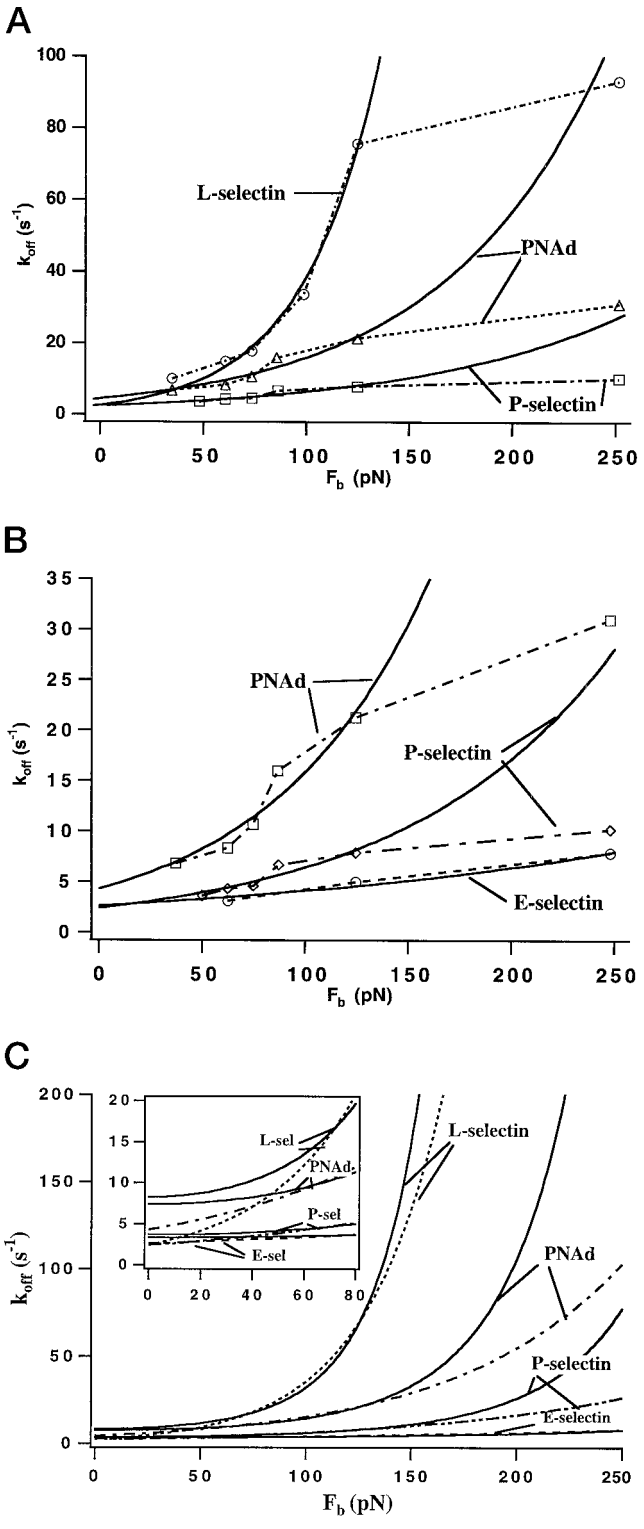

FIGURE 6 Comparison of selectin dissociation constants as a function of force. (*A*) Dissociation constants of selectin and selectin ligand substrates for neutrophils tethering on L-selectin, PNAd, and P-selectin are compared. Over the range of bond forces from 37 to 125 pN, the dissociation constants were fit by the Bell model (*solid lines*) using the Levenberg-Marquardt algorithm (see Methods and Results). (*B*) Dissociation constants for neutrophils tethering on E-selectin, P-selectin (repeated from panel *A* at different scale), and PNAd (repeated from panel *A*) are compared. The Bell model predictions are indicated by solid lines. (*C*) Comparison of the Bell (*dashed* and *dotted lines*) and Hookean spring (*solid lines*) models for all four substrates. The inset plot is scaled to show unstressed k_{off} estimates. Dissociation constants were estimated from pause time distributions for flow rates corresponding to 37–250 pN of force on the adhesive bond/crossbridge. For conversion of the wall shear stress to force in the parallel plate flow chamber, F_b was calculated to be equal to 125 pN/dyn/cm^2. The Bell model, in which k_{off} is modulated by the force on the bond, is described by the relationship $k_{off} = k^0_{off}\exp(\sigma F_b/k_BT)$, was fitted to experimentally determined dissociation constants. k^0_{off} is the zero-stress dissociation constant for the selectin bond, k_B is the Boltzmann constant, and T is absolute temperature. The Hookean spring model is described by the relationship $k_{off} = k^0_{off}\exp(f_\kappa F_b^2/2\kappa k_BT)$, where f_k is the fractional spring slippage, and κ is the bond spring constant.

The dissociation constant ranged from 101.50 s^{-1} for the L-selectin substrate at 50 sites/μm^2 to 80 s^{-1} at 170 sites/μm^2 at a wall shear stress of 2.0 dyn/cm^2 (Fig. 5 *C*). Distributions of pause times did not vary as much with site density as P- or E-selectin bonds, possibly due to the relative brevity of L-selectin bonds. Dissociation constants for neutrophil adhesive events with PNAd substrates were independent of PNAd surface concentration.

Analysis of response of selectin dissociation constants to force based on Bell and Hookean spring models of reactive compliance

Determinations of L-selectin, PNAd, and P-selectin dissociation constants at intermediate levels of force were undertaken to generate estimates of bond reactive compliance based on Bell and Hookean spring molecular models of force driven dissociation (Bell, 1978; Dembo et al., 1988). Over the range of estimated single bond forces from 37 to 125 pN (see Fig. 7 for F_b calculation), the dissociation constants for all four substrates were fitted to the Bell model using the Levenberg-Marquardt algorithm, a nonlinear, least-squares fitting routine (Fig. 6, *A* and *B*). The algorithm searches for the coefficients σ/k_BT and k^0_{off} that minimize chi-square (χ^2) in the expression $k_{off} = k^0_{off}\exp(\sigma F_b/k_BT)$. The parameter σ characterizes the bond interaction distance or distance between receptor ligand interfaces at which the bond rapidly dissociates. A larger σ correlates positively with a higher reactive compliance and a bond's susceptibility to force-driven dissociation. The product of the Boltzmann constant (k_B) and absolute temperature (T) scales the exponential to the thermal energy. Over the range of 37 pN/bond to 125 pN/bond, the χ^2 values for the fits of the Bell equation to the values of k_{off} were good (Table 1). However, including the value of k_{off} at 250 pN resulted in a highly significant deterioration in the χ^2 values of all the substrates except E-selectin, which had the smallest variation in k_{off} with force. The large deterioration in χ^2 values suggested that at least two regimes of force driven dissociation existed (Table 1 legend), hence only the values of dissociation constants at 125 pN/bond and lower were used to establish the parameters bond reactive compliance and k^0_{off} (Fig. 6, *A* and *C*; Table 1).

TABLE 1 Effect of sampling rate and magnification on estimates of selectin bond lifetimes

			Bell Model		Hookean Spring Model	
Substrate	Frame Rate (fps)	Stressed (100 pN) k_{off} (s^{-1})	Unstressed k^0_{off} (s^{-1})	Bond Separation Length (σ) (Å)	Unstressed k^0_{off} (s^{-1})	Spring Constant/ Fractional Slippage κ/f_κ (N/m)
P-selectin	**48**	**6.3**	**2.4 ± 0.47**	**0.39 ± 0.08**	**3.70 ± 0.51**	**2.50 ± 0.61**
	30	2.4*	0.93[#,*]	0.40 ± 0.08[#,*]	ND	ND
E-selectin	**48**	**5.0**	**2.6 ± 0.45**	**0.18 ± 0.03**	**3.35 ± 0.47**	**9.12 ± 1.9**
	30	2.1*	0.5–0.7[§,*]	0.31 ± 0.02*	ND	ND
L-selectin	**240**	**36**	**2.8 ± 0.72**	**1.11 ± 0.12**	**8.20 ± 0.17**	**0.90 ± 0.1**
	30	10[¶]	7.0–9.7[¶]	0.24 ± 0.02[¶]	9.7 ± 0.66[¶]	6.31 ± 0.96[¶]
PNAd	**240**	**16**	**3.8 ± .097**	**0.59 ± 0.10**	**7.42 ± 1.27**	**1.80 ± 0.37**
	30	10.5[¶,*]	6.6[¶,*]	0.20 ± 0.01[¶,*]	8.8 ± 0.2*	7.1 ± 0.4*

Indicated substrates were adsorbed to polystyrene slides fitted to the wall of a parallel plate flow chamber. Neutrophil interactions were tracked by computer software using a digital camera at the indicated frame rates (fps) and observed at 20× magnification (values in bold). Measures of reactive bond compliance (bond separation distance) as defined in the Bell model and f_k/κ (fractional slippage divided by the bond spring constant) in the Hookean spring model were derived by a nonlinear least-squares fit of the dependence of k_{off} on the force/bond over the range of 37–125 pN. Both the Bell model and the Hookean spring model estimates deviated considerably from measured dissociation constants at forces above 125 pN. Chi-squared values (χ^2) for a Levenberg-Marquardt fit of the Bell model increased with the inclusion of the 250 pN data point for P-selectin from 1.43 to 6.7; for E-selectin from 0.32 to 2.63; for PNAd from 9.93 to 55.6; and for L-selectin (interacting with the neutrophil L-selectin ligand) from 4.9 at 88 pN to 27.4 at 125 pN to 1624 at 250 pN. As with the Bell model, the χ^2 for the Hookean spring model deteriorated significantly with the inclusion of the k_{off} value at 250 pN. The χ^2 for E-selectin increased from 0.2 to 1.23, for P-selectin increased from 2.11 to 8.84, for PNAd-increased from 8.65 to 94.2, and for the L-selectin substrate increased from 0.69 to 2162. For the calculation of the force on the bond, the lever arm was assumed to be 3 μm, with the neutrophil radius set at 4.25 μm, resulting in an estimate of F_b as 125 pN per 1 dyn/cm^2 wall shear stress.

ND, Not determined.

*Alon et al., 1997.

[#]Alon et al., 1995.

[§]Kaplanski et al., 1993.

[¶]Alon et al., 1998.

Curves of k_{off} versus force (F_b) on the selectin bond based on the tabulated parameters of the Bell model are plotted in Fig. 6, *A* and *B* to illustrate the deviation of the Bell model from measured k_{off} as forces increase past 125 pN per bond. Fig. 6 *C* plots a comparison of the Bell model and the Hookean spring model (see Table 1 for bond mechanics). Comparison of the models in Fig. 6 *C* illustrates that in the force range for which the parameters were derived, both models fit equivalently, with the Hookean spring model having slightly lower χ^2 values (Table 1).

As shown in Table 1, the estimates of σ for P-selectin, E-selectin, and the two classes of L-selectin ligands, PNAd and the neutrophil L-selectin ligand(s), are compared with previous determinations at 30 fps and 10× microscopy. The most significant difference observed, attributable to the higher temporal and spatial resolution analysis, was for the neutrophil tethers on immobilized L-selectin, which has a bond interaction distance (reactive compliance) value for σ that is fivefold greater than previously reported (Alon et al., 1998). Similarly, the L-selectin-PNAd bond interaction distance (σ) is approximately threefold greater than previously reported (Alon et al., 1997). At the lowest forces and flow rates examined, the values for k_{off} were fairly close to previous estimates for L-selectin. The response of L-selectin bonds to force indicated that tensile stresses shorten bond lifetimes far more than predicted by the effects of thermal forces (Brownian motion) exclusively, as illustrated in Fig. 6, *A* and *B* and in Table 1, where k_{off} at 100 pN/bond is tabulated and compared to estimated zero shear dissociation, k^0_{off}.

Analysis of P-selectin at slightly higher frame rates (1.6-fold) and higher microscope magnification (2-fold) resulted in higher values for the dissociation constants, but the derived bond reactive compliance was very close in magnitude to previous estimates (Alon et al., 1995). In the case of E-selectin, overall k_{off} values were also higher, but the estimated reactive compliance (σ) for E-selectin was slightly smaller than previously reported. Analysis of the change in L-selectin k_{off} with increasing force between the range of 37–125 pN suggests that L-selectin bonds are more susceptible to force-driven dissociation than P-selectin or E-selectin bonds. Estimates of k^0_{off} for all four substrates were surprisingly close to each other based on fitting the Bell model to the data.

Table 1 contains a comparison of the Bell model and the Hookean spring model (Dembo et al., 1988) for bond mechanics. The Hookean spring model for force-driven bond dissociation was developed to introduce a bond potential based on the distance between the recognition interfaces with an assumed functionality of a spring: $k_{off} = k^0_{off}\exp(f_\kappa F_b^2/2\kappa k_B T)$. The parameter $f_\kappa = [(\kappa - \kappa_{ts})/\kappa]$ is the fractional spring slippage, or the component of bond stretching energy that goes into increasing the rate of bond dissociation (Kuo et al., 1997). The spring constant for the bond is κ and the spring constant for the transition state of the bond is κ_{ts}. Over the estimated bond forces ranging from of 37 to 125 pN/bond, the dissociation constants for all four substrates were fitted to the Hookean spring model using the Levenberg-Marquardt algorithm.

DISCUSSION

One of the most visually distinctive interactions between leukocytes and endothelium is the series of transient selectin-mediated bond formation events that results in leukocyte rolling. The focus of this study was to determine the effect of force on the duration of L-, P-, and E-selectin bonds, a critical parameter in the biophysical characterization leukocyte rolling. Using high-speed video microscopy (8× standard, 240 fps), we made a direct comparison of dissociation rates of bonds formed by neutrophils with four different substrates: P-selectin, E-selectin, L-selectin, and PNAd, all receptors specialized for initiating leukocyte capture in a variety of immune responses. Previous studies of E-selectin (Kaplanski et al., 1993) and P-selectin bond cluster lifetimes (Alon et al., 1995) were substantially confirmed using the higher-resolution video. In contrast, L-selectin bond with neutrophil L-selectin ligands were observed to be significantly more labile in the presence of shear forces than either of the two vascular selectins. Additionally, bond lifetimes of two classes of L-selectin ligands—one expressed on leukocytes and one expressed on HEV of peripheral lymph nodes—were found to have significant differences in reactive compliance, demonstrating that selectin ligands may control certain biophysical properties of the interaction (Puri et al., 1998).

Presentation of P-selectin and L-selectin on a substrate in shear flow simulates two types of adhesive interactions that are hypothesized to regulate leukocyte accumulation: P-selectin-mediated primary capture to endothelium and L-selectin-mediated secondary capture of flowing leukocytes by previously adherent leukocytes (Bargatze et al., 1994; Kunkel et al., 1997; Walcheck et al., 1996b; Alon et al., 1996). Based on a comparison of neutrophil capture rates in shear flow, it may be hypothesized that the L-selectin bond formation rate with its neutrophil ligand under flow conditions are faster than that of P-selectin with PSGL-1. In contrast to the faster tethering rate of the neutrophil L-selectin ligand relative to the PSGL-1-P-selectin interaction, L-selectin tethering of neutrophils to CD34 (a component of PNAd/MECA-79 antigen) has comparable tethering rates to P-selectin and E-selectin (Puri et al., 1997). However, the non-CD34 PNAd component of L-selectin ligand activity (Alon et al., 1997) supports neutrophil tethering at a higher level wall shear stress similar to the neutrophil L-selectin ligand(s). Certain L-selectin ligands therefore appear to support higher tethering rates than P-selectin interactions while others are indistinguishable from E- and P-selectin. No data are available at this time on L-selectin-MAdCAM-1 tethering rates or bond lifetimes to compare to either the vascular selectins or other L-selectin ligands to establish the full range of cellular tethering rates that different L-selectin ligands might support (Berg et al., 1993).

Differences in tethering rates between L-selectin ligands are suggestive of differences in k_{on} based on the hypothesis that forward bond formation rates strongly control attachment of cells under conditions of flow (Tempelman and Hammer, 1994; Swift et al., 4998). Likewise, differences between dissociation constants of two L-selectin ligands also appear to exist and may have functional consequences regarding maintenance of stable rolling interactions. However, the role of L-selectin ligands in controlling the kinetic and biophysical properties of L-selectin bonds has been unclear, in particular the role of reactive compliance in the prediction of rolling velocities. Due to the brevity of L-selectin bonds relative to standard video rates, a significant population of adhesive events may have been undetected in previous analyses of the force response of L-selectin bonds (Alon et al., 1997, 1998; Puri et al., 1998), particularly at flow rates over 0.8 dyn/cm^2 wall shear stress. Inclusion of the population of previously undetected pauses briefer than 33 ms and higher temporal resolution of pause events at longer bond lifetimes results in significant differences in estimates of the response to force of L-selectin bonds in particular (Alon et al., 1997, 1998). Over the range of 37–125 pN/bond, the L-selectin bond with its neutrophil ligand has an estimated bond separation distance (σ) more than fivefold greater than previous determinations. Rather than being a bond less responsive to stress, L-selectin interactions with its neutrophil ligand appears to have much more of a "slip" bond characteristic than either P-selectin or E-selectin bonds. In contrast to L-selectin interactions with its neutrophil ligand, L-selectin interactions with HEV (PNAd) endothelial ligands had significantly greater bond lifetimes and a lower reactive compliance, in contrast to previous results (Alon et al., 1997, 1998). Both P-selectin and E-selectin have a lower bond reactive compliance than the L-selectin neutrophil ligand and PNAd, suggesting that they formed stiffer bonds that were less sensitive to breakage by applied force. It has been shown that L-selectin interactions with CD34 modified by borohydride treatment has radically different kinetic and biomechanical properties (Puri et al., 1998). While L-selectin-CD34 k_{off}^0 was not altered, several biophysical properties, notably the requirement for shear flow for L-selectin rolling, were absent and the reactive compliance was increased, suggesting that biochemical equilibrium properties were independent of biomechanical properties. Consistent with this interpretation, the k_{off}^0 values extrapolated from both the Bell model and Hookean spring model for all three selectins were remarkably close together, while large differences in k_{off} were observed to arise once the bonds were under force. It also appears that selectin ligands may control or influence characteristic bond lifetimes, as is suggested by the observed differences in reactive compliance between PNAd and the leukocyte L-selectin ligand.

Measured dissociation constants from neutrophil tethering events at 250 pN/bond were considerably lower than predictions of k_{off} derived from both Bell and Hookean spring models. The apparent plateau in k_{off} values above 125 pN/bond forces levels suggest distinct force domains might exist where the Bell and Hookean spring models are inappropriate (Alon et al., 1997). Whether this is a molecular, mechanical effect or one that is influenced by leukocyte deformability is unclear at this time. It has been hypoth-

esized (Shao et al., 1998) that as a selectin bond experiences a mechanical load above 64 pN, its microvillus anchorage point will stretch, changing the moment arm acting on the bond (see Fig. 7). The estimated force acting on a selectin bond is very

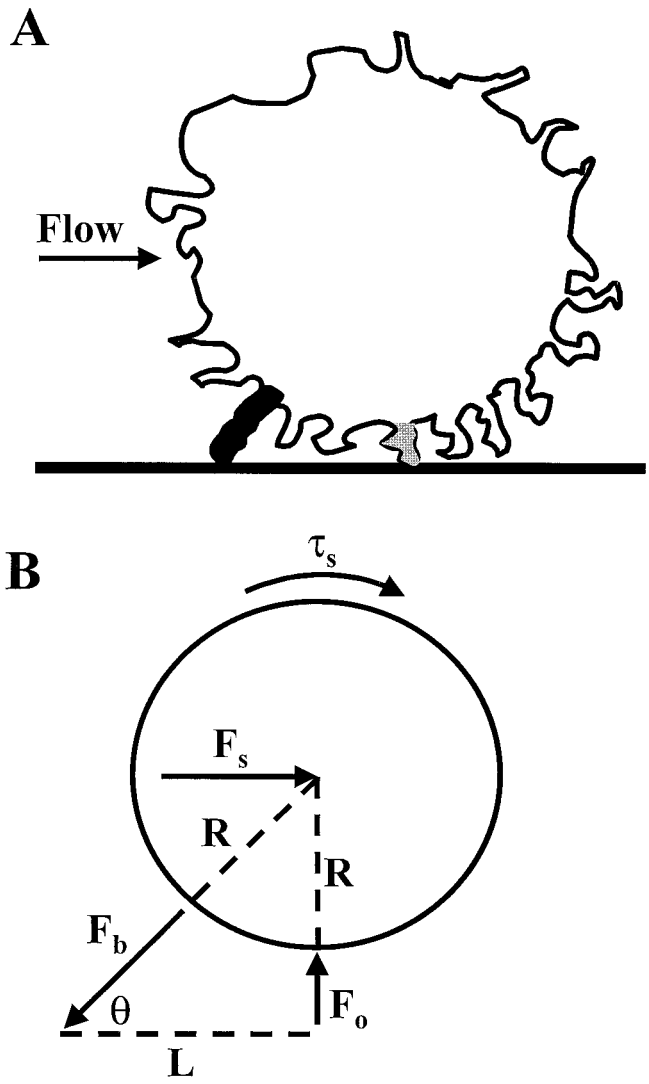

FIGURE 7 Free body diagram of neutrophil in static equilibrium and the estimation of force on a single bond. (*A*) A leukocyte in momentary, static equilibrium during rolling. The black microvillus a forms bond with its ligand on the substrate, which is solely responsible for the complete cessation of the leukocyte's motion. The gray microvillus is a second point of contact that balances the forces and moments, bringing the cell to a state of static equilibrium. (*B*) The free body diagram shows forces and moments acting on a rolling leukocyte as it pauses in static equilibrium when the adhesive bond momentarily arrests the cell. Major assumptions in this statically determinate problem are that the cell is a spherical object, there are only two points of contact, and a single bond momentarily arrests the cell. In static equilibrium, the sum of the forces in each direction must equal zero and the sum of the moments must equal zero. The sum of the forces in the *x*-direction gives $F_s = F_b\cos\theta$. The sum of the forces in the *y*-direction gives $F_o = F_b\sin\theta$. The sum of the moments about the point of contact at F_o gives $\tau_s + F_sR = (F_b\sin\theta)L$. For a direct comparison of dissociation constants with values previously reported in the literature, we used values of $R = 4.25\ \mu$m, $L = 3\ \mu$m, $\theta = 62.3°$, $F_s = 58.9$ pN per dyn/cm^2 wall shear stress so that $F_b = 124.4$ pN per dyn/cm^2 wall shear stress (Alon et al., 1997; Goldman et al., 1967).

dependent on distance between the bond and the forward point at which the neutrophil is balanced against the substrate, which in turn is dependent on microvillus length. In effect, the bond is somewhat shielded from the full effect of the increased shear force and would be able to support adhesion at higher flow rates. The resultant force on the bond as the microvillus stretches would be predicted to increase less than expected for a stiff cell or microvillus.

An alternative explanation of the plateau in k_{off} values with increasing force is that the mechanical loading rate may influence apparent bond strength, as suggested by both theoretical and experimental analysis of biotin-streptavidin bonds (Izrailev et al., 1997; Merkel et al., 1999). By varying molecular force loading rates over several orders of magnitude, the rupture force of the biotin-streptavidin bond was observed to increase with loading rate, ranging up to 170 pN at a loading rate of 60,000 pN/s. It was hypothesized that rapid loading allowed detection of short-lived, but relatively strong interactions that must be overcome in order for a receptor to dissociate under force (Evans and Ritchie, 1997; Merkel et al., 1999). At a wall shear stress of 2.0 dyn/cm^2, the loading rate on a selectin bond may be high enough, on the order of 10^5 pN/s (200 pN/0.002 s), to sample a population of the transition states with deeper potential wells. The bond rupture forces sampled in the rolling assay appear as strong as the biotin-streptavidin bond despite equilibrium affinities at least four orders of magnitude lower. Interestingly, when the biotin-streptavidin bond is loaded at 60,000 pN/s, its lifetime varies between 1 and 3 ms, slightly below the median L-selectin bond lifetime of 10 ms with its neutrophil ligand, but is nevertheless considerably briefer than the other selectin interactions analyzed in this report. Unlike the biotin-streptavidin bond, the selectin binding site is not buried within a cleft of the receptor, but instead is situated on a relatively small patch on the top of the N-terminal lectin domain (Graves et al., 1994). It is not known how the lectin domain of a selectin deforms under mechanical stress, but its two α-helixes are located on opposite faces of the domain and the anti-parallel β-sheets are aligned somewhat vertically on the third face, leaving the binding site located within a series of loops lacking a defined secondary structure. The loops associated with ligand recognition may be able to adopt a number of different conformations, which based on entropic arguments would favor high bond formation rates characteristic of selectins compared to more rigid structures, such as the immunoglobulin superfamily integrin ligands (Wang and Springer, 1998). A less ordered domain structure may also allow more deformation under stress before the selectin bonding interface is perturbed, thereby maintaining a stress response closer to that of much higher-affinity interactions such as the biotin-strepavidin bond.

The differences between L-selectin ligands and their characteristic bond lifetimes under stress may be extremely significant in the modulation of leukocyte accumulation under flow conditions. For example, the neutrophil L-selectin ligand's relatively brief bond lifetime under shear and its

shear threshold property (Finger et al., 1996) may limit the opportunity for irreversible neutrophil aggregation in the blood stream, yet may last long enough to move neutrophils into fluid streamlines closer to the vessel wall. An interesting outcome may be predicted between a neutrophil rolling on PNAd as it interacts with a flowing neutrophil presenting L-selectin ligands. Because of the relative differences in bond lifetime under shear, neutrophils rolling on PNAd may be more likely to capture a flowing neutrophil than to be captured by it (M. B. Lawrence, unpublished observations). The longer duration of an L-selectin-PNAd bond may therefore result in the adherent neutrophil winning the pulling contest. Meanwhile, the flowing neutrophil following its brief interaction is torqued closer to the wall, favoring subsequent adhesion downstream. Since radial diffusion of cells in the blood stream is so low, the effect of several L-selectin-mediated interactions between flowing and adherent neutrophils might contribute to populating near-wall streamlines with neutrophils. Because lifetimes for the neutrophil L-selectin ligand(s) are significantly more stress-sensitive than those with L-selectin endothelial cell ligands, the short bond lifetime under stress may reduce opportunities for aggregation in the bloodstream of resting neutrophils, as suggested by recent modeling studies that probed the effect of increased L-selectin bond lifetimes on neutrophil aggregation (Tandon and Diamond, 1999). A similar hypothesis regarding capture from flow may be put forward for neutrophils rolling on P-selectin and E-selectin where it would be predicted that a flowing neutrophil would rarely succeed in pulling an adherent neutrophil off the wall.

Among the unique aspects of biologic adhesion is a requirement for reversibility of molecular interactions to allow tissue remodeling, cell signaling and migration, and other processes in immune surveillance. In the case of leukocyte interactions within the vasculature, in which adhesive bonds are subject to widely varying levels of force, a bond's response to mechanical stress may have as much effect on function as its intrinsic kinetic properties. This study showed that neutrophil tethers with immobilized L-selectin have a higher bond-reactive compliance than for tethers with immobilized P-selectin, E-selectin, or PNAd, all endothelium-derived substrates.

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