Quantifying Rolling Adhesion with a Cell-Free Assay: E-Selectin and Its Carbohydrate Ligands

Debra K. Brunk and Daniel A. Hammer
School of Chemical Engineering, Cornell University, Ithaca, New York 14853 USA

ABSTRACT Rolling of neutrophils over stimulated endothelial cells is a prerequisite to firm attachment and subsequent transendothelial migration during the inflammatory response. The selectin family of adhesion molecules are thought to mediate rolling by binding counter-receptors that present carbohydrates, such as sialyl Lewis^x (sLe^x). Recently we described a cell-free system for rolling using sLe^x-coated microspheres and E-selectin molecules on inert substrates. We showed that sLe^x-coated microspheres rolled over E-selectin–IgG chimera substrates with dynamics that are similar to those of leukocytes rolling over stimulated endothelium. In this paper we provide a thorough quantitative description of the dynamics of adhesion for this system. We find that particle rolling velocity increases with increasing wall shear stress and decreases with increasing E-selectin or sLe^x surface densities. Large changes in the average rolling velocity can occur with small changes in sLe^x or E-selectin density; however, rolling velocity is more sensitive to E-selectin surface coverage than to the number of sLe^x molecules on the microspheres. Aided by dimensional analysis, we show that decreasing the wall shear stress or increasing either receptor (E-selectin) or ligand (sLe^x) surface coverage results in an equivalent decrease in particle rolling velocity. In addition, we find that different Lewis carbohydrates are more effective in mediating rolling on E-selectin, with effectiveness following the trend sialyl Lewis^a > sialyl Lewis^x ≫ sulfated Lewis^x ≫ Lewis^x. Rolling velocity fluctuated with time for all carbohydrate-selectin pairs tested, and the magnitude of the velocity fluctuations was linearly proportional to the mean rolling velocity for all combinations of E-selectin site density, sLe^x site density, wall shear stress, and carbohydrate chemistry tested.

INTRODUCTION

Neutrophil binding to endothelium is a two-step process in which reversible or rolling adhesion is followed by irreversible binding to the endothelium (Lawrence and Springer, 1991). Rolling occurs via the following steps: 1) a receptor-ligand bond forms, exerting an adhesive stress, which slows a cell's velocity; 2) the slower motion of the cell promotes additional bond formation; 3) bonds dissociate at the back edge of contact, causing the cell to tumble forward in the direction of flow. Rolling cells translate at a small fraction of the expected hydrodynamic velocity of an unencumbered particle. Interaction between selectin adhesion molecules and carbohydrate presenting counter-receptors is thought to induce rolling (Varki, 1994; Carlos and Harlan, 1994). Each member of the selectin family of adhesion receptors can support transient adhesion (Abbassi et al., 1993; Springer, 1994).

The selectin family of cell adhesion receptors consists of three molecules, L-selectin, P-selectin, and E-selectin. Each selectin molecule contains an amino-terminal calcium binding lectin-like domain, an epidermal growth factor (EGF)-like domain, a variable number of short consensus repeats (SCRs), a transmembrane domain, and a short cytoplasmic tail. The lectin-like domains of the selectins are highly homologous to each other and can bind specifically to various sialylated, fucosylated carbohydrates (Erbe et al., 1993). L-selectin is constitutively expressed on neutrophils and is found in clusters on the tips of neutrophil microvilli (Erlandsen et al., 1993; von Andrian et al., 1995). P-selectin is stored in Weibel-Palade bodies of endothelial cells and in α-granules of platelets (McEver et al., 1989; Bonfanti et al., 1989). Rapid expression of P-selectin on the endothelial surface occurs within minutes after stimulation by thrombin or histamine (Lasky, 1992). E-selectin is expressed on a subset of venules in sites of acute and chronic inflammation (Cotran et al., 1986). Inflammatory stimuli, such as interleukin-1β or tumor necrosis factor-α, induce maximum expression of E-selectin on the surface of endothelial cells in 4 h (Lasky, 1992).

All three selectins recognize the carbohydrate sialyl Lewis^x (sLe^x) under static conditions (Phillips et al., 1990; Polley et al., 1991; Foxall et al., 1992), with E-selectin showing the highest affinity. sLe^x is widely distributed on both glycoprotein and glycolipid components of the neutrophil (Fukuda et al., 1984; Symington et al., 1985). One hypothesis is that glycoproteins act as protein scaffolds for the presentation of sLe^x to the selectins (Varki, 1994). Variants of sLe^x, such as sialyl Lewis^a (sLe^a) and sulfated Lewis^x (sulfated Le^x), also bind to the selectins, specifically E-selectin, under static conditions (Varki, 1994; Nelson et al., 1992).

Most selectin ligands have been found with static assays. Although static assays may detect possible receptor-ligand pairs, they do not provide information on how ligands interact with receptors under more physiological, dynamic conditions. For cells to adhere under flow, the potential receptor-ligand pair must quickly react. Once a bond (or

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Address reprint requests to Dr. Daniel A. Hammer, Department of Chemical Engineering, University of Pennsylvania, 392 Towne Bldg., Philadelphia, PA 19104. Tel.: 215-573-6761; Fax: 215-573-2093; E-mail: hammer@seas.upenn.edu.

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bonds) is formed, it must be able to withstand the constant shearing (or drag) force exerted by the fluid. Because of these rigorous conditions, dynamic adhesion studies are more likely than static experiments to reveal receptor-ligand pairs that can mediate rolling (Varki, 1994).

Dynamic adhesion experiments using leukocytes and cultured endothelial cells in parallel-plate flow chambers have provided information on the rolling step observed in neutrophil/endothelial cell adhesion. It has been observed that rolling velocity varies with wall shear stress (Lawrence et al., 1987, 1990; Jones et al., 1993; Goetz et al., 1994) and that the velocity of rolling cells is not constant, but varies considerably with time (Kaplanski et al., 1993; Goetz et al., 1994). Although these experiments can be more easily controlled than in vivo studies of neutrophil rolling, there remain a large number of variables, such as endothelial cell surface heterogeneity and passage, adhesion molecule expression, neutrophil activation, and cell deformability, making it difficult to understand the biophysical basis of neutrophil/endothelial cell interaction.

Springer's laboratory has simplified the neutrophil/endothelial system by replacing cultured endothelial cells with purified P-selectin reconstituted in lipid bilayers (Lawrence and Springer, 1991; Alon et al., 1995b) and purified E-selectin adsorbed to polystyrene slides (Lawrence and Springer, 1993). Neutrophils rolled over both substrates with a velocity proportional to wall shear stress and inversely proportional to the selectin surface density. Although these experiments remove the variability contributed by the endothelial monolayer, neutrophil activation and deformability still present complications. Recently, Alon and co-workers developed a reconstituted system in which Chinese hamster ovary (CHO) cells expressing E-selectin roll over sLe^x- or sLe^a-glycolipid substrates (Alon et al., 1995a); however, the use of a cell as a carrier for molecules introduces confounding features, such as cell morphology and rheology, that obscure how carbohydrate/selectin interactions may lead to rolling.

Cell-free systems have been used to elucidate important information about the strength of antibody-antigen interactions (Cozens-Roberts et al., 1990; Kuo and Lauffenburger, 1993; Pierres et al., 1995); however, until recently, no one has attempted to study rolling interactions with a cell-free assay. Using a cell-free system, we have reported that sLe^x-coated microspheres interact specifically with and roll over E-selectin–IgG-coated substrates under flow conditions (Brunk et al., 1996). An E-selectin chimera was adsorbed to glass microscope slides, and biotinylated sLe^x was bound to avidin-coated 10-μm-diameter rigid polystyrene spheres. Spheres interacting with the selectin surface translated at an average velocity less than 10% of the free stream velocity at all shear stresses where adhesion was observed, which suggests that the particles were rolling on the surface. Nearly all adhesion was transient, with few microspheres firmly attaching to the E-selectin–IgG surface. Particle velocity did not gradually decrease before attachment, but decreased abruptly, followed by rolling. The specificity of the interaction was validated by adding an antibody to E-selectin, replacing the biotin-sLe^x on the particle surface with biotin, replacing the E-selectin–IgG on the glass substrate with human IgG_1, or adding EDTA. Each of these treatments reduced microsphere adhesion to the substrate to nearly zero. The rolling velocities we observed were quantitatively similar to those seen with neutrophil/endothelial cell and neutrophil/selectin systems at the same shear stresses, suggesting that we have created a cell-free system that closely mimics leukocyte rolling.

In this paper we provide a detailed quantification of rolling using our cell-free system and systematically varying the E-selectin and sLe^x concentrations on each substrate, varying the wall shear stress, and altering the Lewis carbohydrate on the microsphere surface (sLe^a, sulfated Le^x, Le^x, and sLe^x). We find that rolling velocity increases with increasing shear rate and decreases as the number of E-selectin or sLe^x molecules is increased. Furthermore, all particles that roll exhibit a variation in their rolling velocities with time. We characterize this fluctuation in rolling velocity and find that the magnitude of the velocity variation depends on wall shear stress and receptor/ligand site density, and that it scales with the mean velocity of motion.

MATERIALS AND METHODS

E-selectin substrates

Adsorption of E-selectin–IgG to glass substrates

The interior divisions of eight-well Flexiperm gaskets (Heraeus Instruments, South Plainfield, NJ) were removed to create one large well with a surface area of 9.5 cm^2. Modified Flexiperm gaskets were placed on Silane-Prep microscope slides (Sigma, St. Louis, MO), 2 ml of phosphate-buffered saline (PBS) (pH 7.4) was added, and the slides were incubated overnight at 4°C. Silanated slides were used because their hydrophobic surface should aid in adsorption and the free surface amines may correctly orient the chimera through the carboxyl terminus of the IgG. After incubation, the PBS was removed and 0.1, 0.2, 0.4, 0.8, or 1.6 μg/ml of an E-selectin–IgG chimeric molecule (E-selectin–IgG, gift from Dr. B. Brandley, Rush Medical Center, Chicago, IL), consisting of the lectin, EGF, and two SCR domains of human E-selectin linked to the hinge region of human IgG_1 (Watson et al., 1990; Goetz et al., 1996), was added to the slide surface. The chimera was diluted in PBS to a total incubation volume of 1.6 ml. Slides were gently mixed on a rocker for 2 h at room temperature and then washed twice with PBS, followed by two washes with PBS+ (PBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% bovine serum albumin (BSA), sterile filtered). Slides were then incubated with 2 ml PBS+ for 1 h at room temperature. Slides were always prepared the same day as experiments. For experiments using sLe^a, sulfated Le^x, and varying concentrations of sLe^x, an E-selectin–IgG incubation concentration of 1.6 μg/ml was used.

Determination of E-selectin site density

To determine the site density of E-selectin–IgG on the slide surface, the above procedure was followed, but unmodified eight-well Flexiperm gaskets were used so we could attach several different concentrations of E-selectin–IgG to one slide. In addition, a control well, with 1.6 μg/ml human IgG_1 (Sigma), was added to check for antibody specificity. Because the adsorption area decreased to 0.88 cm^2, the total incubation volume of E-selectin–IgG was decreased to 200 μl. After incubation with PBS+, a monoclonal antibody (mAb) to E-selectin (68–5H11, IgG_1; Pharmingen,

San Diego, CA) was added at 20 μg/ml to each well and allowed to incubate for 30 min at room temperature. After the primary antibody was removed, 30 μg/ml Fc specific fluorescein isothiocyanate (FITC) anti-mouse IgG (Sigma) was added to each well and allowed to incubate for 30 min at room temperature. Wells were washed several times with PBS+ and then PBS, and the site density was immediately determined. Fluorescence in counts per second was measured with a Photoscan (Nikon, Garden City, NJ) attached to a Nikon Diaphot inverted microscope equipped with a fluorescein cube.

We converted measured fluorescence to E-selectin site density by developing a calibration curve of counts per second as a function of FITC-conjugated protein concentration. To convert the protein concentration to a site density, an estimate of the height of fluid in the well was needed. This was determined by dividing the volume of fluid in each well by the well's surface area. In addition, we assumed 1:1 binding between the secondary and primary antibodies, and between the primary antibody and E-selectin.

Absolute measurements of E-selectin site density are difficult to achieve for several reasons. First, the assumption of 1:1 antibody binding is made for simplicity, and although it is most likely valid at the low chimera concentrations we are using, this assumption may overestimate the site density. Moreover, because we used a polyclonal secondary antibody, more than one secondary antibody could bind to a single primary antibody. In addition, the use of antibodies to measure site densities will inherently overestimate the number of E-selectin molecules available to bind ligand under flow, because these assays are carried out under static conditions and antibodies can bind to incorrectly oriented E-selectin. Finally, different antibody clones or even lots will likely bind differently to the E-selectin molecule. Given these obstacles to measuring absolute site densities, we have presented our data in terms of relative site density, using 3600 molecules E-selectin/μm^2, the maximum E-selectin site density obtained, as the basis (relative site density of 1 = 3600 molecules E-selectin/μm^2).

Covalent attachment of E-selectin to glass substrates

E-selectin–IgG and Tween 20 (Sigma) were added to activation buffer (0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.5 M NaCl, pH 6), followed by the simultaneous addition of 10 μl 0.06% water-soluble carbodiimide (Pierce, Rockford, IL) and 10 μl 0.18% sulfo-*N*-hydroxysuccinimide (Pierce), in activation buffer. The final concentration of E-selectin–IgG was 10 μg/ml in a total volume of 1.6 ml. This mixture was incubated with gentle mixing for 15 min at room temperature and then was pipetted directly onto a silanated glass slide (Sigma). Slides were incubated with the E-selectin–IgG mixture for 2 h at room temperature with gentle mixing, then 80 μl 10 mM ethanolamine was added, and the slides were mixed for another 30 min. After removing the E-selectin–IgG mixture, slides were washed three times with activation buffer and three times with PBS+, and then were incubated with PBS+ for 1 h at room temperature. E-selectin site densities were determined as described above. We were able to attain similar relative site densities of covalently attached E-selectin, using higher incubation concentrations. The molecule densities of E-selectin covalently attached to the surface will be presented relative to the maximum adsorbed E-selectin density (3600 molecules E-selectin/μm^2).

Carbohydrate-coated microspheres

Carbohydrate attachment

NeutrAvidin (Pierce) was covalently attached to 10-μm-diameter carboxylated latex microspheres (Polysciences, Warrington, PA), using a standard carbodiimide chemistry coupling technique (Goodfriend et al., 1964; Kuo and Lauffenburger, 1993). Briefly, microspheres were washed sequentially with carbonate (pH 9.6) and phosphate (pH 4.0) buffers. The microspheres were then incubated with a 2% solution of carbodiimide in phosphate buffer for 3.5 h at room temperature with gentle mixing. After removing the carbodiimide and washing repeatedly with borate buffer (pH 9), NeutrAvidin at 240 μg/ml in borate buffer with 0.5% Tween 20 (Sigma) was added to the beads, and this mixture was mixed overnight at room temperature. Microspheres were then incubated for 30 min with 0.2 M ethanolamine to block unreacted sites, followed by a 1 h incubation in PBS+ to prevent nonspecific adhesion. NeutrAvidin-coated beads could be stored at 4°C in PBS+ for at least a month with no loss of activity.

Synthetic carbohydrate probes were obtained from Syntesome (Munich, Germany) or GlycoTech (Rockville, MD). The carbohydrates are incorporated into a polyacrylamide matrix substituted with biotin. This creates a multivalent polymer of ~30 kDa, with a ratio of biotin molecules to carbohydrate molecules of 1:4. The biotin-conjugated carbohydrates we used in these experiments were biotin-sLe^x, biotin-sLe^a, biotin-Le^x, and biotin-sulfated Le^x. Biotin-*x* will be used when referring to all biotinylated carbohydrates. A schematic of the carbohydrate polymers used in this study is shown in Fig. 1.

For experiments using sLe^a, sulfated Le^x, Le^x, or sLe^x at a constant site density, 10^6 NeutrAvidin-coated microspheres were incubated in 100 μl of 0.2 μg/ml biotin-*x* in PBS+ for 45 min at room temperature with occasional mixing. This concentration of biotin-*x* resulted in a saturating number of carbohydrate molecules on the particle surface, because concentrations ranging from 0.1 μg/ml to 16 μg/ml biotin-sLe^x showed an equivalent fluorescence shift via flow cytometry (Brunk et al., 1996). Particles were then washed several times with PBS+ and incubated in 600 μl PBS+ for at least 1 h to block nonspecific adhesion. Immediately before flow chamber experiments, particles were resuspended in 600 μl PBS+(−BSA).

For experiments involving varying site densities of sLe^x on the particle surface, NeutrAvidin-coated microspheres were incubated with a mixture of 30% biotin-sLe^x/70% biotin-Le^x, 60% biotin-sLe^x/40% biotin-Le^x or 100% biotin-sLe^x, with a total protein concentration of 0.5 μg/ml. Biotin-Le^x was used as a competing molecule because its molecular weight is equal to that of biotin-sLe^x and because it did not interact with E-selectin–IgG under flow (see Results). A total protein concentration of 0.5 μg/ml was chosen, rather than the 0.2 μg/ml used above, to allow a greater range in which to vary molecule density. Particles were then prepared for flow chamber experiments exactly as described above.

Determination of sLe^x site density

To determine the site density of sLe^x on the microspheres, the desired concentration of biotin-sLe^x was incubated with NeutrAvidin-coated beads for 45 min as detailed above, but the number of particles was decreased to 5×10^5/ml, and the incubation volume was likewise decreased to 50 μl. Microspheres were prepared for flow cytometry using a mAb to sLe^x (SNH4, IgG_3; a gift from Dr. Anil Singhal, Biomembrane Institute, Seattle, WA) at a concentration of 30 μg/ml in MES+ (MES, pH 5.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% BSA, sterile filtered). Particles were incubated in this solution for 30 min at room temperature with occasional mixing. Flow cytometry preparations with this antibody (and other antibodies to sLe^x) were carried out using both PBS+ and MES+. Only preparations using

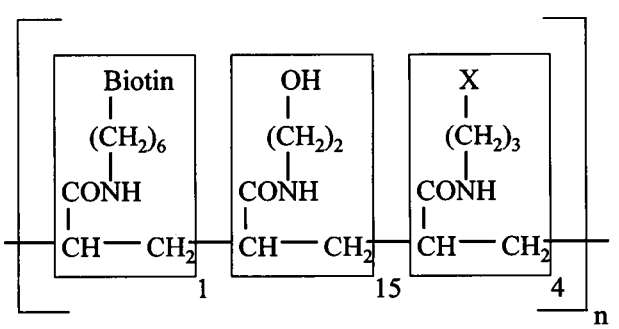

FIGURE 1 Diagram of biotin-*x* polymer. sLe^x, sLe^a, sulfated Le^x, or Le^x is found at the *x* position.

MES+ produced positive results; thus all flow cytometry experiments were carried out using MES+. We hypothesize that the antibodies to sLe^x need a weakly acidic environment to react with the negatively charged sLe^x. After the primary antibody was removed, particles were incubated in 50 μl of 60 μg/ml FITC anti-mouse IgG_3 (mAb, Pharmingen) for 30 min at room temperature with occasional mixing. Afterward, coated microspheres were washed once with MES+, once with PBS+, and once with PBS+(−BSA), and then were resuspended in 300 μg/ml PBS+(−BSA) containing 1% formaldehyde.

Molecule site density on beads was determined by flow cytometry. Fluorescence shifts were converted to site densities by developing a calibration curve relating mean peak fluorescence to the molecules of equivalent soluble fluorochrome (MESF), using Quantum 26 calibration beads from Flow Cytometry Standards Corporation (San Juan, PR). Once the MESF/protein ratio of the FITC-conjugated secondary antibody is known, the number of surface-bound molecules can be determined (Kuo and Lauffenburger, 1993), assuming a 1:1 relationship between sLe^x, the primary antibody, and the secondary antibody. By using this method, we determined that 90 molecules $sLe^x/\mu m^2$ is the maximum site density. All sLe^x site densities reported are relative to this number (relative sLe^x site density of 1 = 90 molecules $sLe^x/\mu m^2$). The value of 90 molecules $sLe^x/\mu m^2$ is different from the maximum sLe^x density we last reported (Brunk et al., 1996), because we are using an IgG_3 primary mAb to sLe^x for these site density estimates, whereas we previously used an IgM primary mAb to sLe^x.

Because biotin-sLe^a, biotin-sulfated Le^x, and biotin-Le^x are the same molecular weight as biotin-sLe^x and were added at the same incubation concentration (0.2 μg/ml), we infer that total carbohydrate surface density is 90 molecules/μm^2 when the total biotin-x concentration is 0.2 μg/ml. The surface density of any individual carbohydrate is dictated by its concentration in the reactive mixture.

Flow chamber

Theory

All adhesion experiments were done in a tapered-channel parallel-plate flow chamber similar to that described by Usami and co-workers (Usami et al., 1993). By allowing the sides of the channel to follow the streamlines formed by two-dimensional stagnation point flow, the wall shear stress (τ_w) varies linearly over the length of the channel:

$$\tau_w = \frac{6\mu Q}{h^2 w_1}\left(1 - \frac{z}{L}\right) \quad (1)$$

where μ is the fluid viscosity, Q is the volumetric flow rate, h is the channel height, w_1 is the channel entrance width, z is the axial position from the inlet, and L is the channel length. The tapered-channel design allows observations of particle-substrate interaction at various wall shear stresses during one experiment.

Design

The flow chamber consists of a bottom platform, a gasket that also acts as the flow channel, and a top platform. The height of the chamber is controlled by the gasket thickness and by six screw/wing nut combinations that hold the bottom and top platforms together. The bottom platform contains a well that holds a standard microscope slide (7.5 × 2.5 cm). The area beneath the slide is used to visualize the flow experiments with a Nikon Diaphot inverted microscope. The top platform has one entrance and three exit ports and fits snugly into the well of the bottom platform. The flow channel is cut into a 7.5 × 2.5 cm sheet of 250-μm-thick Duralastic sheeting (Allied Biomedical, Goose Creek, SC), where L = 6.6 cm and w_1 = 0.1 cm.

The flow chamber has been calibrated using 10-μm-diameter microspheres from Polysciences. The velocity of the beads near the surface was measured and found to be a linear function of wall shear stress, as expected (Goldman et al., 1967). Velocity results are consistent whether the wall shear stress is varied by changing the volumetric flow rate or by moving the microscope stage down the length of the flow channel, which shows that movement down the length of the chamber is accurate and reproducible.

Flow experiments

E-selectin–IgG-coated slides were placed in the well of the flow chamber, and the chamber was assembled in PBS+(−BSA) to keep air from getting trapped in the channel. The chamber was then placed on the stage of a Nikon Diaphot inverted phase-contrast microscope connected to a video camera (Dage-MTI, Michigan City, IN) and a S-VHS recorder (JVC, Elmwood Park, NJ). All flow chamber experiments were carried out at room temperature (23°C). Buffer and bead suspensions were drawn through the chamber by an infusion/withdrawal syringe pump (Harvard Apparatus, South Natick, MA). The height of the channel was measured at three locations before each experiment for an accurate determination of the wall shear stresses obtained. For instance, for a channel height of 175 μm (average channel height from day to day), the flow rate of the perfusion buffer or microsphere suspension must be 128 μl/min to obtain a range in wall shear stresses between 0 and 4 dynes/cm^2 over the length of the channel. The total volume of bead suspension required for one experiment (~15 min) was 2 ml. Before the microspheres were added, the chamber was perfused with PBS+(−BSA) at the desired flow rate for 15 min, during which the flow rate was measured. A suspension of carbohydrate-coated microspheres (5×10^5/ml in PBS+(−BSA)) was then introduced, and data were collected by stepping down the chamber from inlet to outlet in 0.5-cm steps, allowing about 1 min between steps. The channel was traversed at least twice during each experiment. Microsphere interaction with the E-selectin–IgG substrate was recorded at 200× for later analysis.

Data analysis

Particle flux was determined by manually counting the number of either rolling or firmly attached particles in a 0.03-mm^2 area as a function of time. Particles were considered firmly attached if they remained stationary for >10 s.

Velocity measurements were obtained from recorded data using a SCION-LG3 frame grabber card in a Power Macintosh 7100 running NIH Image, public domain image analysis software. Microsphere motion was captured from videotapes by creating a series of stacks, which allowed us to enter the desired time between captured frames (Δt). This time varied from 0.1 s to 5 s, depending on the rolling velocity. Particle coordinates were obtained with 1-μm (1-pixel) accuracy by clicking the mouse cursor at the center of a rolling microsphere over the total number of stacks collected or for as long as the particle was interacting with the surface.

Average rolling velocity (V_{avg}) was determined by dividing the total displacement of one particle by the total observation time ($\Delta t * n$), where n is the number of instantaneous velocity measurements. Velocities reported are the mean of at least five individual particle rolling velocities, obtained in at least two independent experiments. This is the minimum number of particles ever measured for a single set of conditions. As wall shear stress decreases or molecule density increases, the number of individual particles (and the number of independent experiments) included in the mean velocity calculation increases.

Instantaneous rolling velocities (V_{inst}) were determined by dividing the displacement of a rolling particle by the time between captured frames (Δt). The standard deviation of the instantaneous rolling velocity of each particle, or the rms rolling velocity (V_{rms}), was calculated using

$$\sigma_v = \left(\sum \frac{(V_{inst} - V_{avg})^2}{n-1}\right)^{1/2} = V_{rms} \quad (2)$$

The calculated V_{rms} for each particle was then averaged over all particles for all identical experiments to obtain the average rms velocity for the

system. The terms "rms velocity" and "velocity fluctuations," when used in the text, are V_{rms} (Eq. 2) averaged over all particles.

RESULTS

sLe^x-, sLe^a-, and sulfated Le^x-coated microspheres roll over E-selectin–IgG substrates

Several potential carbohydrate ligands for E-selectin (sLe^a, sLe^x, sulfated Le^x) were tested in our cell-free system. Le^x, which does not bind E-selectin under static conditions, was tested for completeness. All molecules were biotinylated and all were incubated with NeutrAvidin microspheres at a concentration of 0.2 μg/ml. Fig. 2 *A* compares rolling and firm attachment for sLe^x-, sLe^a-, sulfated Le^x-, and Le^x-coated microspheres at a relative E-selectin site density of 1, measured at 0.6 dyne/cm^2. Data are presented as the number of particles interacting with the E-selectin surface (rolling or firmly attached) per time per area (#/min/mm^2). sLe^x-, sLe^a-, and sulfated Le^x-coated beads roll over E-selectin–IgG at similar levels, whereas Le^x shows little interaction. Firm attachment is negligible. Fig. 2 *B* compares the rolling velocities obtained by sLe^a, sLe^x, and sulfated Le^x microspheres. The average rolling velocity varies with wall shear stress. Sulfated Le^x-coated beads roll most quickly, followed by sLe^x-coated beads, then by sLe^a-coated beads. Because sLe^a is probably not important in leukocyte trafficking (Carlos and Harlan, 1994) and sulfated Le^x-coated spheres do not interact strongly with E-selectin (faster rolling velocity), we used sLe^x to fully characterize our cell-free system. Because Le^x is not a ligand for E-selectin, Le^x was used to vary the number of sLe^x molecules per microsphere.

Effect of wall shear stress on rolling velocity

Average rolling velocities were determined at 0.6, 1.0, 1.4, 1.8, and 2.2 dynes/cm^2 for sLe^x-coated microspheres over surfaces to which E-selectin–IgG was adsorbed or covalently attached. In these experiments we define "rolling" as being in constant contact with the substrate, always moving at a velocity that is less than 10% of the hydrodynamic fluid velocity near the surface (Goetz et al., 1994). Although we tested wall shear stresses up to 3.3 dynes/cm^2, we did not observe any microsphere interaction with the surface at shear stresses greater than 2.2 dynes/cm^2. The number of rolling particles decreased as the wall shear stress increased; therefore there is more statistical uncertainty in the average rolling velocity at higher wall shear stresses. Fig. 3 illustrates how the average rolling velocity of sLe^x-coated microspheres over E-selectin–IgG substrates varies with wall shear stress. The relative site densities for sLe^x and adsorbed E-selectin are 1, whereas the covalently attached E-selectin has a relative density of 0.92. Each point is the mean ± standard deviation of average particle rolling

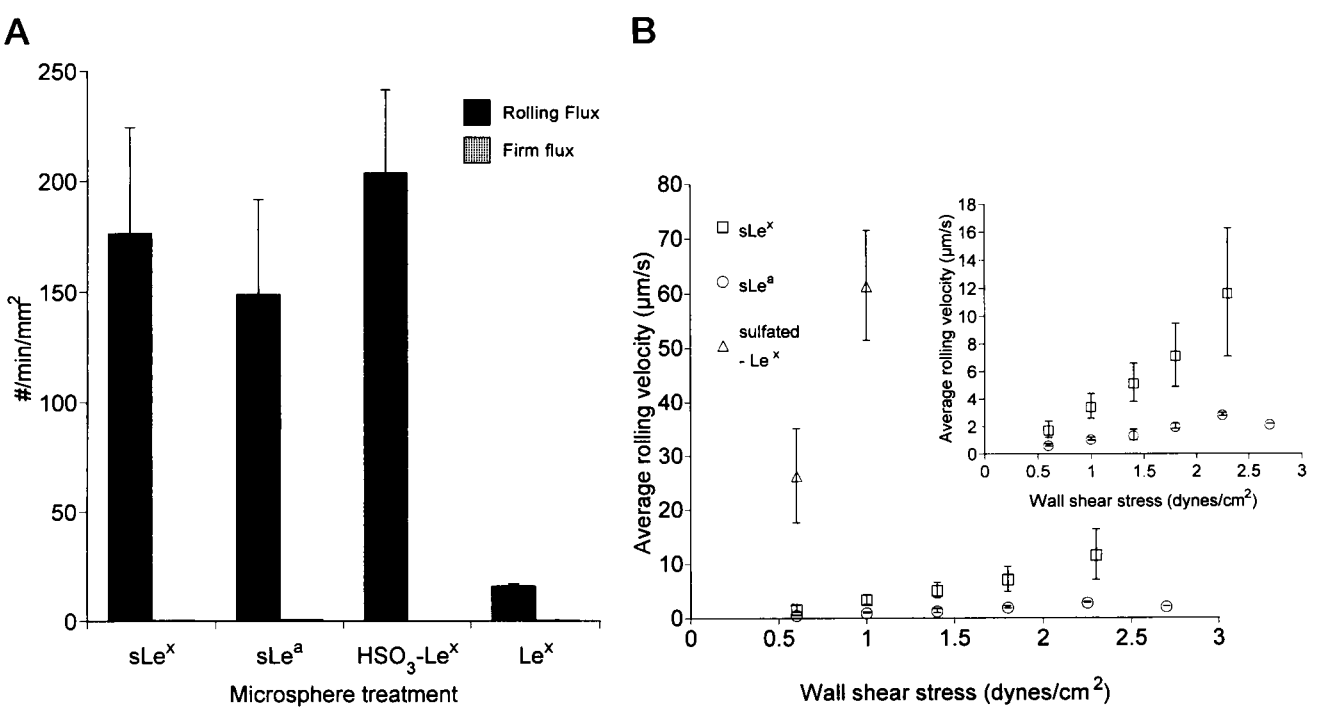

FIGURE 2 (*A*) Rolling and firm attachment flux measured for sLe^x-, sLe^a-, sulfated Le^x-, and Le^x-coated microspheres at a wall shear stress of 0.6 dyne/cm^2. Relative E-selectin and carbohydrate site densities are 1. Fluxes shown are the result of averaging at least four independent flux measurements. (*B*) Average rolling velocity as a function of wall shear stress for particles coated with biotin-sLe^x, biotin-sLe^a, and biotin-sulfated Le^x over E-selectin–IgG. Inset shows rolling velocity in greater detail for sLe^x- and sLe^a-coated spheres. The relative E-selectin site density was 1. Biotin-*x* concentration during NeutrAvidin microsphere incubation was 0.2 μg/ml, which confers a relative site density of 1.

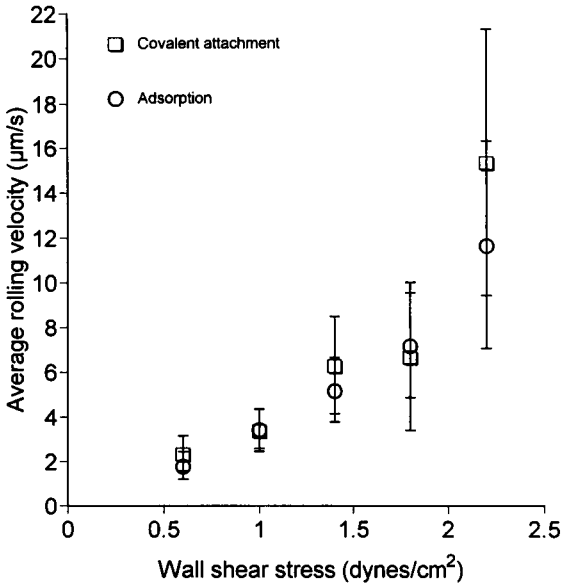

FIGURE 3 Average particle rolling velocity as a function of wall shear stress, where E-selectin–IgG was covalently attached or adsorbed. The relative site density of adsorbed E-selectin was 1. The relative site density of covalently attached E-selectin was 0.92. The relative site density of sLe^x was 1. The points reflect the mean ± SD for at least four independent experiments.

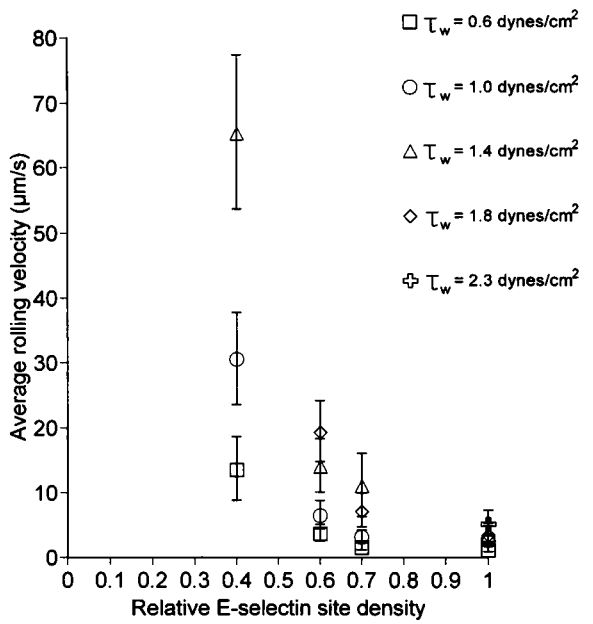

FIGURE 4 Average particle rolling velocity as a function of relative E-selectin site density measured at various wall shear stresses. The relative site density of sLe^x was 1. The points reflect the mean ± SD for at least three independent experiments.

velocities at each shear stress for at least four independent experiments. As wall shear stress is increased from 0.6 to 2.2 dynes/cm^2, the mean rolling velocity increases from 1.8 ± 0.6 μm/s to 11.7 ± 4.6 μm/s for adsorbed E-selectin–IgG and from 2.4 ± 0.8 μm/s to 15.4 ± 5.9 μm/s for covalently attached E-selectin–IgG. Because we observed similar rolling velocities for similar site densities of adsorbed and covalently attached E-selectin, we conclude that rolling is not caused by the detachment of adsorbed E-selectin–IgG from the surface. Further experiments were carried out by adsorbing E-selectin–IgG, because less of the chimera was required to obtain a specified site density.

Effect of E-selectin site density on rolling velocity

To determine the effect of receptor site density on rolling velocity, we looked at various E-selectin substrate densities (relative E-selectin densities = 0.2, 0.4, 0.6, 0.7, and 1) while keeping the relative sLe^x site density constant at 1. The dependence of average rolling velocity on E-selectin site density is illustrated in Fig. 4. At a fractional E-selectin coverage of 0.2, particles showed predominantly stop/start interactions rather than rolling. "Stop/start" means that the particle interacts with the substrate for a short time and then moves at the hydrodynamic velocity before it is caught again. The average velocities for these particles are ~50% of the hydrodynamic fluid velocity near the surface. Those particles that rolled did so for a very short time, making it difficult to obtain reproducible data on their velocities; therefore we show no velocity data at a relative E-selectin surface coverage of 0.2.

Average rolling velocity is a strong function of E-selectin site density for all shear stresses. For all wall shear stresses measured, the average particle rolling velocity decreases by 80–95% as the E-selectin site density is increased by a factor of 2.5.

Effect of sLe^x site density on rolling velocity

To determine the effect of ligand density on rolling velocity, we systematically varied the site density of sLe^x on the microsphere surface by incubating the particles in a 0.5 μg/ml solution containing 20% biotin-sLe^x/80% biotin-Le^x, 60% biotin-sLe^x/40% biotin-Le^x or 100% biotin-sLe^x. The relative site density of E-selectin was kept constant at 1 for each experiment. Fig. 5 illustrates how average particle rolling velocity varies with sLe^x site density. We show no rolling velocity data for wall shear stresses greater than 1.0 dyne/cm^2 for particles with a relative sLe^x site density of 0.02, because these particles exhibited largely stop/start, not rolling, behavior.

Average particle rolling velocity decreases with increasing sLe^x site density, similar to what is observed with increasing E-selectin density. However, increasing the sLe^x density by a factor of 5 decreases the average particle rolling velocity between 51% and 80%, depending on the wall shear stress. Fig. 6 shows a comparison of the average rolling velocities obtained at 0.6 dyne/cm^2 for relative E-selectin and relative sLe^x site densities. This plot clearly shows that the average particle rolling velocity is more

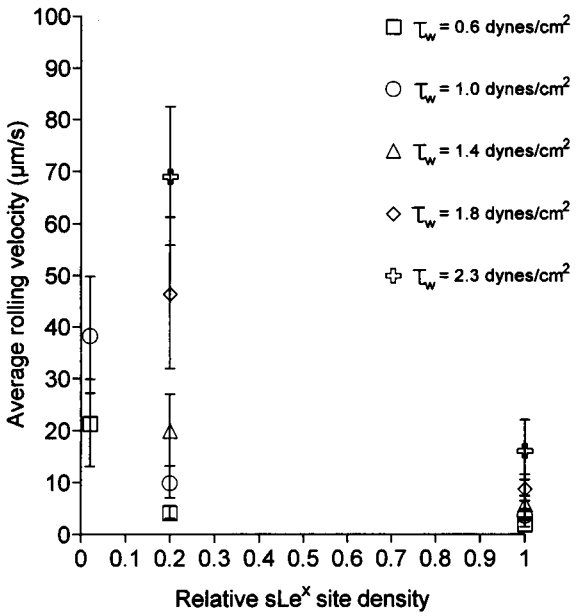

FIGURE 5 Average particle rolling velocity as a function of relative sLe^x surface density determined at various wall shear stresses. The relative E-selectin site density was 1. The points reflect the mean ± SD for three independent experiments.

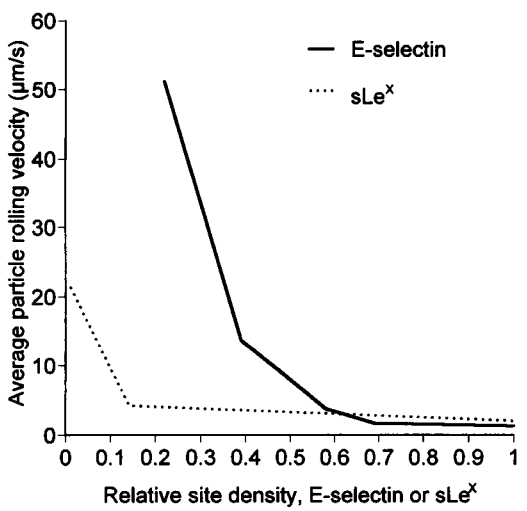

FIGURE 6 Average particle rolling velocity as a function of relative E-selectin and relative sLe^x site densities at a wall shear stress of 0.6 dyne/cm^2. The relative sLe^x site density was 1 when E-selectin density was varied. Likewise, when the sLe^x surface density was varied, the relative density of E-selectin was 1.

sensitive to changes in E-selectin substrate density than to changes in sLe^x density, because rolling velocity decreases more quickly with increasing E-selectin site density than with increasing sLe^x site density. The sensitivity of the rolling velocity occurs despite the fact that the site density of E-selectin on the substrate is always much greater than the site density of sLe^x on the sphere. This phenomenon is observed at each wall shear stress.

Dimensional analysis

The cell-free system allows us to examine transient adhesion in a well-defined system. A simple scaling analysis can be used to define dimensionless parameters that characterize the physics of motion and provide basic insight into the predominant mechanisms controlling adhesion. If the particle's velocity (V_{bead}) depends on the fluid velocity (V_{fluid}), fluid viscosity (μ), particle diameter (a), average dissociation force (strength) per bond (F_b), site density on the particle (M_{sLex}) and on the planar surface (M_{esel}), and the forward and reverse kinetic rate constants (k_f and k_r, respectively), we can obtain five dimensionless parameters by using the Buckingham-Pi theorem (Appendix). Of these five parameters, only three are relevant, because the others are not altered in our experiments. For our experiments, the relationship can be expressed in the form

$$\frac{V_{bead}}{V_{fluid}} = f\left(\frac{M_{sLex}}{M_{esel}}, \frac{V_{fluid}\mu}{aF_bM_i}\right) \quad (3)$$

where V_{bead}/V_{fluid} is the bead/free stream velocity ratio, M_{sLex}/M_{esel} is the sLe^x/E-selectin ratio, $V_{fluid}\mu/aF_bM_i$ is a ratio of the drag force to the bonding force, and M_i is the site density of either sLe^x or E-selectin, whichever is varying. The free stream velocity can be related to the wall shear stress ($V_{fluid} = a\tau_w/\mu$). Substituting for V_{fluid} yields

$$\frac{V_{bead}\mu}{a\tau_w} = f\left(\frac{M_{sLex}}{M_{esel}}, \frac{\tau_w}{F_bM_i}\right) \quad (4)$$

When we set the viscosity to 1 cP (the value for water at room temperature), use a particle radius of 5 μm, and set F_b equal to 1 μdyne (Lauffenburger and Linderman, 1993), all of our data obtained at different E-selectin site densities, while holding sLe^x site density constant, fall on a single curve, as predicted by Eq. 4 and as shown in Fig. 7 *A*. In addition, data taken at different sLe^x densities (at constant E-selectin density) collapse to a single curve, as illustrated in Fig. 7 *B*. The ratio of bead velocity to free stream velocity appears to be a linear function of the ratio of drag force to bonding force on a log-log plot, with different slopes for sLe^x and E-selectin. The solid line on these figures corresponds to F_b = 1 μdyne. Changing the value of F_b by an order of magnitude shifts the data, as illustrated by the dotted and dashed lines on Fig. 7, *A* and *B*, but the slope remains the same. Dimensional analysis suggests that we will obtain the same rolling velocity by either varying the wall shear stress or varying molecule site density, and our experiments confirm this.

Rolling velocity varies with time

Instantaneous velocity measurements were obtained by dividing the displacement of a rolling particle by the time between captured frames (Δt; see Materials and Methods). Fig. 8 shows typical particle trajectories as a function of time for different wall shear stresses (Fig. 8 *A*) and different

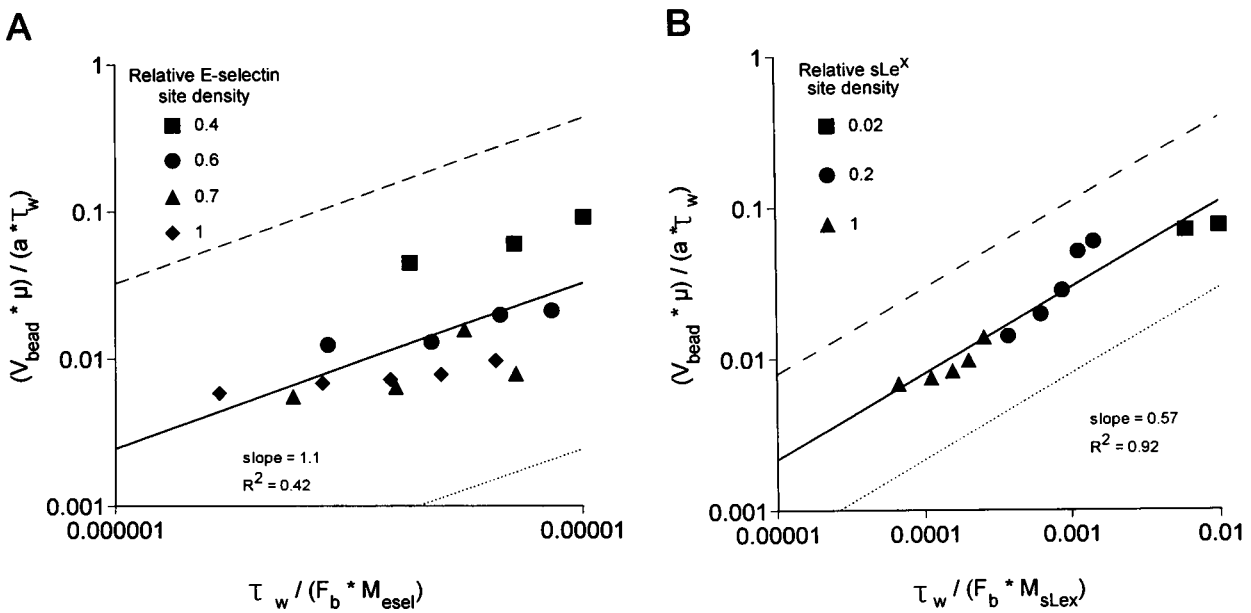

FIGURE 7 Particle rolling velocity nondimensionalized with the free stream velocity as a function of particle drag force nondimensionalized with the bond breakage force. Results are shown for varying E-selectin surface density (*A*) and varying sLe^x surface density (*B*). Points are plotted assuming F_b = 1 μdyne. The solid line is a best-fit linear regression of the data. Dashed and dotted lines are also best fits of the data (points not shown) when F_b = 10 or 0.1 μdynes, respectively.

densities of E-selectin (Fig. *B*) or sLe^x (Fig. *C*). These plots show that sLe^x-coated microspheres roll over E-selectin–IgG-coated substrates with a time-varying velocity.

To quantify the fluctuations in particle rolling velocity at different wall shear stresses and substrate preparations, we determined the root mean square (rms) rolling velocity for each particle, then averaged the rms velocity over all particles and plotted the average rms velocity as a function of wall shear stress (Goetz et al., 1994). Fig. 9 *A* shows the average rms velocity as a function of wall shear stress and E-selectin–IgG site density. At a single wall shear stress, the characteristic rms velocity significantly decreases with increasing E-selectin site density. The effect of wall shear stress on velocity fluctuations is more difficult to evaluate. The average rms velocity for a single E-selectin site density increases with increasing wall shear stress, but the standard deviation is large. Fig. 9 *B* illustrates how the average rms velocity varies with wall shear stress for different sLe^x site densities. The dependence on wall shear stress appears more distinct, with an increase in rms velocity with increasing wall shear stress (p always < 0.003). Velocity fluctuations also depend on the site density of sLe^x molecules, as the average rms velocity decreases as sLe^x site density increases. Rolling velocities also vary with time for both sLe^a- and sulfated Le^x-coated microspheres over E-selectin–IgG. An analysis of the average rms velocity shows that sulfated Le^x microspheres exhibit the largest characteristic rms velocity, whereas sLe^a microspheres exhibit a significantly smaller rms velocity than both sulfated Le^x and sLe^x microspheres (Fig. 9 *C*). Wall shear stress has a weak influence on the average rms velocity for all carbohydrates tested.

We also plotted the coefficient of variation (which scales the average rms velocity, and hence the magnitude of the velocity fluctuation, with the mean velocity) versus wall shear stress for all substrate preparations (not shown). These graphs suggested that the coefficient of variation was independent of site density and wall shear stress. By plotting the average rms velocity as a function of average particle rolling velocity for all experiments (sLe^x and E-selectin site density varied, sLe^a and sulfated Le^x microspheres), we find a linear relationship between rms velocity and mean velocity (Fig. 10). By carrying out linear regression on these data, we obtain a slope of 0.56 (a coefficient of variation of 56%), with an R^2 of 0.87. At velocities greater than 45 μm/s, there is some deviation from the fit; however, this deviation is probably due to the small number of data points in this region and the difficulty of measuring faster rolling velocities.

DISCUSSION

The goal of this paper was to quantitatively characterize the rolling interactions between E-selectin and its carbohydrate ligands, sLe^x, sLe^a, and sulfated Le^x, with particular emphasis on sLe^x. We show that sLe^x, sLe^a, and sulfated Le^x each mediate rolling with E-selectin–IgG substrates under flow. As expected, Le^x does not mediate rolling on E-selectin substrates. sLe^a appears to interact most strongly with E-selectin, followed by sLe^x, whereas sulfated Le^x interacts weakly with E-selectin. These experiments illustrate that our cell-free system is a useful tool for determining which ligands can support rolling adhesion with selectins. Because sLe^a is not important in leukocyte trafficking

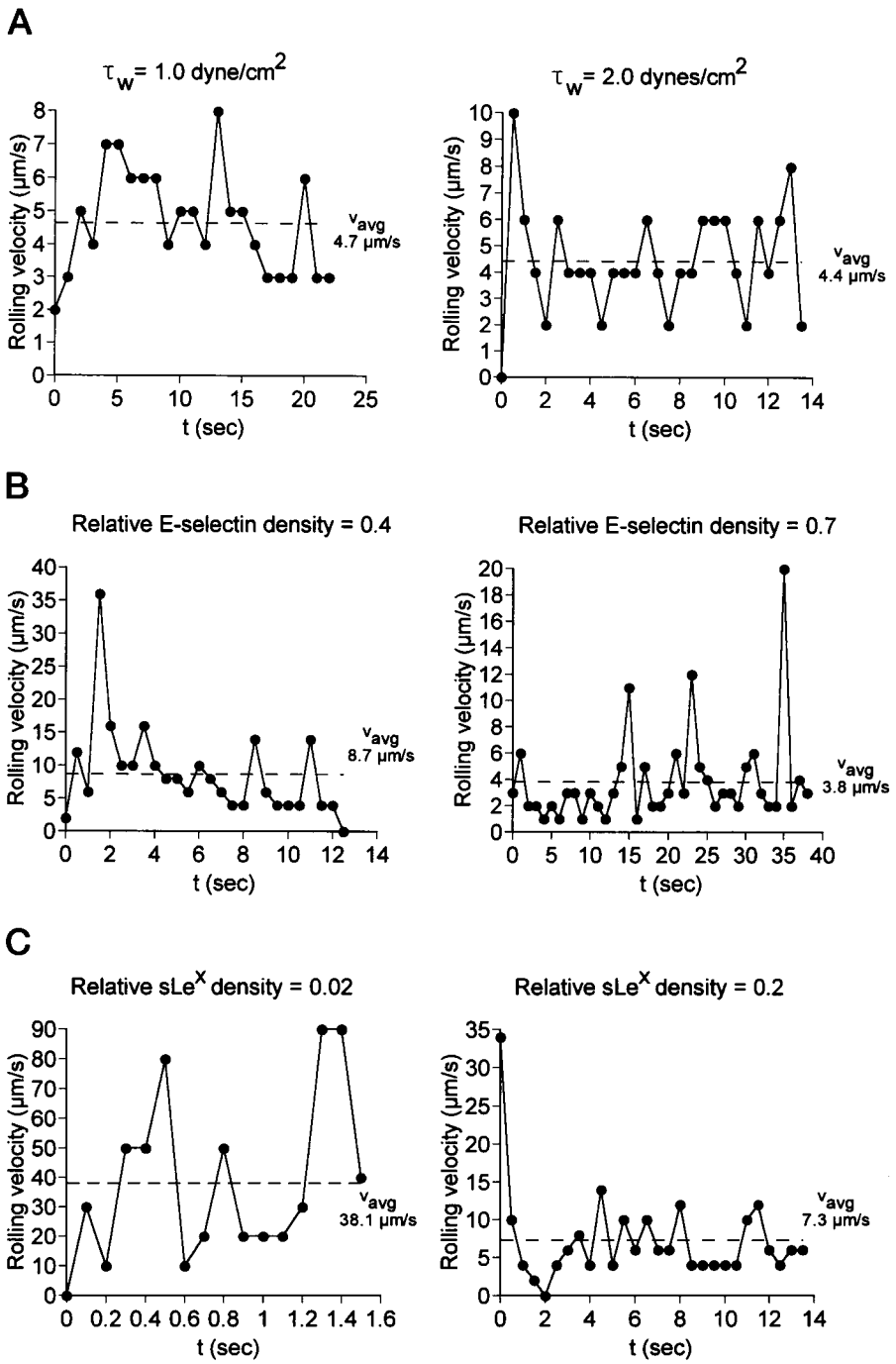

FIGURE 8 Instantaneous particle rolling velocities versus time for representative particles. (*A*) Wall shear stress is either 1 or 2 dynes/cm^2 and relative E-selectin and sLe^x densities are both 1. The time step, Δt, was 1 s for τ_w = 1 dyne/cm^2; Δt = 0.5 s for τ_w = 2 dynes/cm^2. (*B*) Site density of E-selectin is either 0.4 or 0.7 of the 3600 molecules/μm^2 maximum. Relative sLe^x density remains constant at 1. Wall shear stress is constant at 1 dyne/cm^2. Δt = 0.5 s for 40% E-selectin, Δt = 1 s for 70% E-selectin. (*C*) Relative sLe^x site densities of 0.02 and 0.2 keeping the relative E-selectin density constant at 1 and the wall shear stress is constant at 1 dyne/cm^2. Δt = 0.1 s and 0.5 s, for relative sLe^x site densities of 0.02 and 0.2, respectively.

and sulfated Le^x did not strongly interact with E-selectin, we used sLe^x as our model E-selectin ligand to fully characterize our cell-free system.

We were able to systematically vary wall shear stress and both E-selectin and sLe^x surface density. This is the first system in which both receptor and ligand densities have been varied to study rolling interactions. Despite the rather simple chemistry of our system (i.e., using sLe^x instead of a protein counter-receptor that presents sLe^x, such as P-selectin glycoprotein ligand-1), we can re-create the gross dynamic features of rolling. We show that the average rolling velocity increases with increasing wall shear stress up to 2.2 dynes/cm^2 for each relative sLe^x and E-selectin–IgG site density tested. This shear stress threshold dependence agrees with previous in vitro flow chamber experiments using cells (Lawrence et al., 1990; Alon et al., 1995a), which suggests that it is not an artifact of our cell-free system, but is rather controlled by the chemistry of the selectin-carbohydrate interaction (perhaps the on rate between selectin and ligand). Rolling velocities obtained at the highest site densities of sLe^x and E-selectin that we used are $\leq$10 μm/s, which quantitatively agrees with results

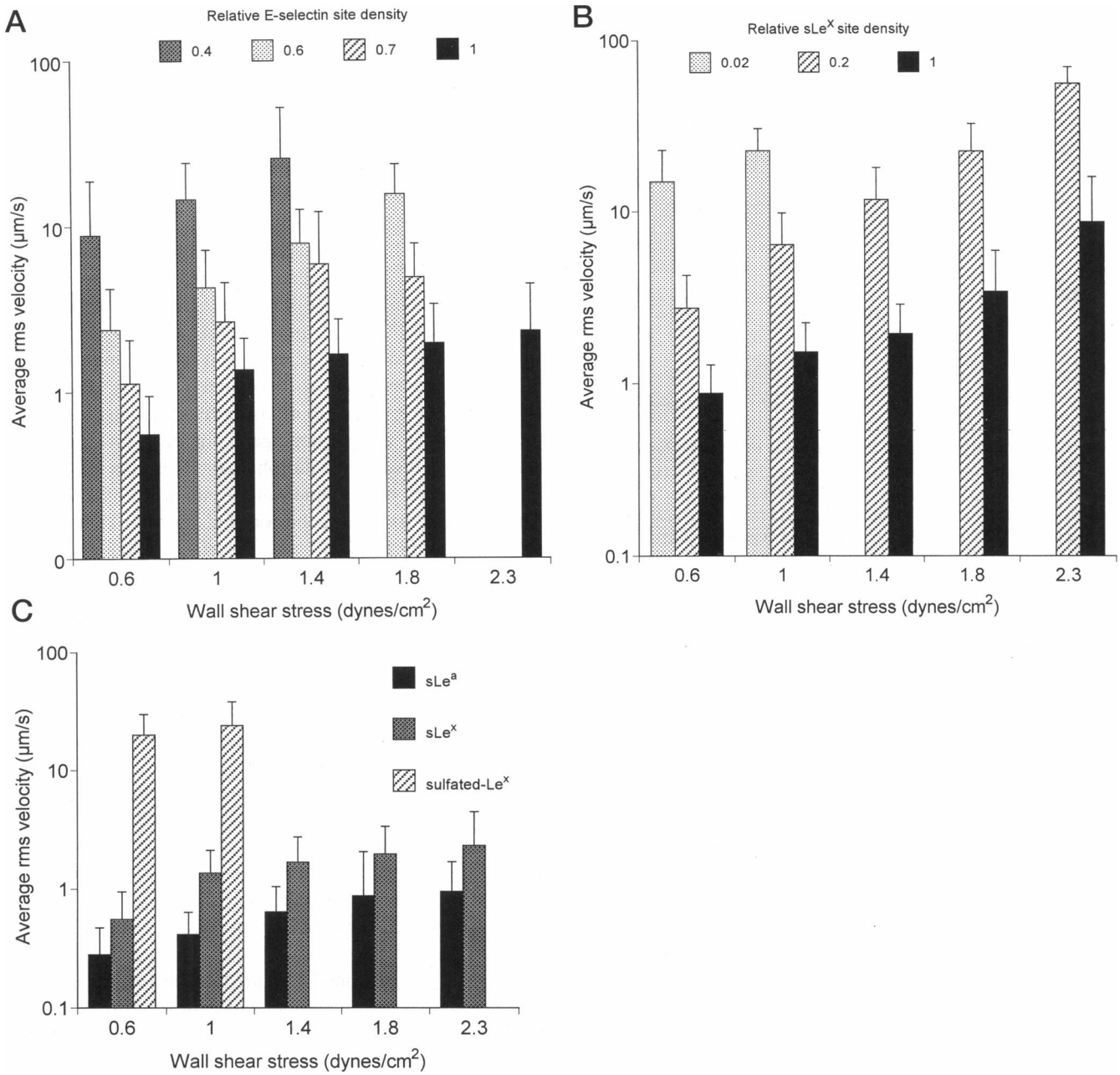

FIGURE 9 Average particle root mean square (rms) velocities as a function of wall shear stress for a population of sLe^x microspheres interacting with E-selectin–IgG. (*A*) Effect of varying E-selectin surface density. Relative sLe^x density remains constant at 1. Plotted on a log scale, because the velocity fluctuations fall within a large range. (*B*) Effect of varying sLe^x density. Relative E-selectin surface density is constant at 1. (*C*) Comparison between different E-selectin ligands: sLe^a, sLe^x, and sulfated Le^x. Relative site densities for E-selectin and each carbohydrate are 1.

reported by Goetz and co-workers for bovine neutrophils over lipopolysaccharide-stimulated bovine aortic endothelial cells (Goetz et al., 1994) and for carcinoma cells over E-selectin–IgG substrates (Goetz et al., 1996). Increasing the density of E-selectin–IgG on the planar surface or sLe^x on the microsphere surface resulted in a decreased particle rolling velocity. These results qualitatively agree with those of Lawrence and Springer, who showed that neutrophil rolling velocities varied with the site density of P-selectin reconstituted in lipid bilayers (Lawrence and Springer, 1991) or the site density of E-selectin adsorbed to plastic substrates (Lawrence and Springer, 1993). This is as expected, because increasing the number of molecules per area should increase the number of bonds forming within the contact region, which should slow rolling. Particle rolling velocity is most sensitive to wall shear stress at the lower site densities of sLe^x and E-selectin tested, most likely because there are fewer bonds within the contact region to counteract the disruptive shear force. Thus our system both captures the main dynamic features of cell rolling and

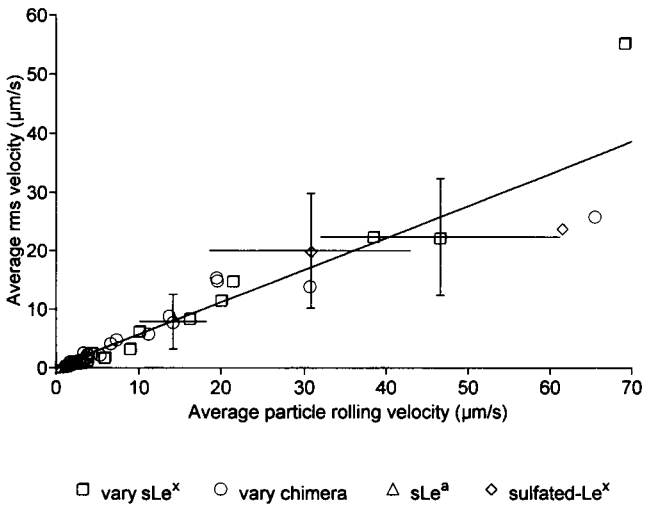

FIGURE 10 Average rms velocity (magnitude of velocity fluctuations) as a function of average particle rolling velocity for all molecules and all site densities tested. Plot includes data taken at many different wall shear stresses, all E-selectin and sLe^x site densities and for sLe^a and sulfated Le^x coated microspheres. Error bars represent one standard deviation. Three representative error bars are shown for illustration.

confirms the physical principles we expect, suggesting that our system is a useful tool for elucidating the molecular biophysics of cell rolling.

A surprising result comes from our measurements of rolling velocity as a function of E-selectin substrate density and sLe^x bead density. We find that rolling velocity is much more sensitive to E-selectin density than to sLe^x density, despite E-selectin being present in great excess to sLe^x. We do not have a clear biophysical understanding of why this is, but it suggests a mechanism by which the host surface (i.e., endothelial cells) can control leukocyte rolling, perhaps through changes in selectin or carbohydrate receptor expression.

Using dimensional analysis, we determined that the ratio of drag force to bonding force is a critical parameter that controls rolling. This suggests that the particle rolling velocity depends on how the receptor-ligand bonds can resist the shearing force exerted on the particle by the fluid. The ability of these bonds to balance the drag force is affected by the site densities of receptor and ligand, because these densities determine the number of bonds in the contact region. Our bead velocity data, obtained at different wall shear stresses and E-selectin/sLe^x site densities, falls entirely on a single curve when the ratio of bead to fluid velocity is plotted as a function of the ratio of drag to bonding forces. This means equal changes in the density of molecules or of wall shear stress will have an equivalent effect on rolling velocity. Our ability to systematically vary molecule density and wall shear stress in our cell-free system makes the development of such general rules governing rolling possible. Note that Fig. 7, *A* and *B*, applies to two different site density regimes, in which τ_w/F_bM_{esel} varies from 10^{-6} to 10^{-5} (Fig. 7 *A*) and τ_w/F_bM_{sLex} varies from 10^{-5} to 10^{-2} (Fig. 7 *B*). The slopes obtained by a linear fit of the data are different for E-selectin (Fig. 7 *A*) and sLe^x (Fig. 7 *B*). These different slopes are due to differences in the ratio between E-selectin and sLe^x (M_{esel}/M_{sLex}).

Our laboratory has previously shown that neutrophils roll over stimulated endothelium at a nonconstant velocity (Goetz et al., 1994). It was suggested that possible sources of variance in the cell motion might be caused by an inherent variability in binding between receptor and ligand, the heterogeneity of the endothelial cell surface or changes in the neutrophil surface structure. Because we observe these variations in a cell-free system, in which the microsphere and glass surfaces are relatively smooth and the molecules are assumed to be homogeneously distributed over the surface, time variations in velocity must be caused by fluctuations in receptor-ligand interaction, rather than cell/cell or cell/substrate morphology. This conclusion is in line with work by Hammer and Apte, who, by using dynamic simulations of adhesion for receptor-coated hard spheres binding to ligand-coated surfaces under flow, predicted that velocity fluctuations are controlled at the molecular level by fluctuations in binding (Hammer and Apte, 1992).

Further analysis of the velocity fluctuations suggests that the rms of the instantaneous rolling velocity increases as molecule site density is decreased, leading us to conclude that decreasing the number of receptor molecules results in larger velocity fluctuations. An increase in rms velocity with wall shear stress is also observed, although it is less pronounced. sLe^a- and sulfated Le^x-coated microspheres both display variations in rolling velocity as a function of time. The average fluctuations are also a weak function of wall shear stress for these carbohydrate ligands. Sulfated Le^x microspheres show the largest velocity fluctuations and sLe^a the least.

Interestingly, we found that the tendency of the velocity fluctuations to vary with molecule site density and wall shear stress for all molecules tested (sLe^x, sLe^a, sulfated Le^x) could be summarized by noting that the average particle rms velocity is a linear function of the average particle rolling velocity. This means that measurement of a particle's mean rolling velocity provides immediate information about the magnitude of the velocity fluctuations for that particle. Although different systems may have a different coefficient of variation, after a "calibration curve" is constructed for a particular system, information about the variance in particle motion under all conditions could be determined simply by measuring the particle's velocity. This also has important implications for computer modeling, because time-consuming stochastic simulations of particle motion might be replaced by this ad hoc rule governing the relationship between velocity fluctuation and mean rolling velocity.

In adhesion experiments there is always some question about the locus of bond failure. We believe that rolling

occurs in our system through the dissociation of the selectin-carbohydrate bond. NeutrAvidin dissociation from the microsphere surface is unlikely, as it has been covalently attached. The average force required to rupture a streptavidin-biotin bond is 100 μdynes, measured by atomic force microscopy (Lee et al., 1994). This is the same order of magnitude as the force required to break an ionic bond. It has been measured by Alon and co-workers that forces of tens of microdynes are sufficient to break selectin-ligand bonds (Alon et al., 1995b). Given that these bond dissociation forces are an order of magnitude less than that for the streptavidin-biotin bond and that NeutrAvidin binds biotin as well as streptavidin, it is unlikely that the biotin-NeutrAvidin bond will dissociate. Another possible node of breakage is that between the chimera and the microscope slide. Because the E-selectin–IgG is physisorbed to a hydrophobic glass surface, there are probably several points of molecule attachment to the surface. Simultaneous dissociation of all chimera contacts with the surface is highly unlikely (Horbett and Brash, 1987), suggesting that rolling is not caused by a rupture between the selectin chimera and the glass surface. To verify that rolling was not promoted by E-selectin desorption from the surface, we covalently attached E-selectin–IgG to glass microscope slides at site densities similar to those obtained with adsorption. Fig. 3 shows that the average rolling velocity obtained by sLe^x-coated microspheres is the same at similar densities of covalently attached or adsorbed E-selectin–IgG over the wall shear stresses measured. This strongly suggests that the rolling interaction is a result of the dissociation between selectin-carbohydrate bonds, rather than molecule desorption from the substrate.

We have characterized the effect of wall shear rate, receptor density, and ligand density on particle rolling velocity in a cell-free system. Our results qualitatively match those obtained with cellular systems, showing that we have created a cell-free assay that serves as a useful mimetic of leukocyte adhesion. A major advantage in using a cell-free system such as we have described is that it makes modeling of the rolling interaction more useful and meaningful, because it eliminates many uncertainties found in cellular assays, such as cell morphology, deformability, and activation. One question raised by our experiments is why a simple carbohydrate such as sLe^x can qualitatively mimic the dynamics of adhesion mediated by more complex molecules, such as P-selectin glycoprotein ligand-1, which possesses a protein backbone decorated by carbohydrates. What functional role do these other domains play in the dynamics of adhesion that is captured by our cell-free system? An interesting molecular characteristic of our current system that may influence this comparison between carbohydrate and physiological ligand is molecule valency; E-selectin–IgG is bivalent and the biotin-x analogs are each multivalent. It has been suggested that multivalency of the selectin and its oligosaccharide counter-receptor may enhance avidity (Varki, 1994). It would be interesting to further simplify this system so that both receptor and ligand are monovalent and to determine whether monovalent interactions are sufficient for rolling. The results obtained with such a system would provide important information about how, or if, molecule clustering mediates rolling. The system described in this paper is also particularly well adapted to studying the effects of inhibitors to adhesion, because an inhibitor's effect on molecular interaction (rather than membrane, cytoskeletal, or signaling processes) could be directly assessed.

APPENDIX: DIMENSIONAL ANALYSIS

We assume that particle velocity (V_{bead}) is a function of fluid velocity (V_{fluid}), fluid viscosity (μ), particle radius (a), average force required to break a bond (F_b), surface density of E-selectin molecules (M_{esel}), sLe^x molecules (M_{sLex}), and forward and reverse kinetic reaction rates for bond formation and breakage (k_f and k_r, respectively). These nine variables contain four dimensions: length, time, mass, and number. The Buckingham-Pi theorem tells us that the number of dimensionless parameters is the difference between the number of variables and the number of dimensions. Thus, for our analysis, we have five dimensionless parameters that describe the system. We can define three characteristic times in the system, the flow time, τ_{flow}, the forward reaction time, $\tau_{rxn,f}$, and the reverse reaction time $\tau_{rxn,r}$:

$$\tau_{flow} = \frac{a}{V_{fluid}} \quad \tau_{rxn,f} = \frac{1}{k_f M_i} \quad \tau_{rxn,r} = \frac{1}{k_r} \tag{5}$$

where M_i is the site density of either molecule (E-selectin or sLe^x). In addition, we can define two forces acting on the particle, a drag force, F_{drag}, and a bonding force, F_{bond}:

$$F_{drag} = \mu a V_{fluid} \quad F_{bond} = a^2 F_b M_i \tag{6}$$

Two independent parameters are obtained by taking the ratio of velocities, V_{bead}/V_{fluid}, and the ratio of the site densities, M_{sLex}/M_{esel}. The ratio of F_{drag} to F_{bond} is the third parameter. A fourth parameter is the ratio of forward and reverse reaction times. The fifth parameter is obtained by balancing the forward reaction time with the flow time. Table 1 lists these five nondimensional parameters and their definitions. The dimensionless rolling velocity, V_{bead}/V_{fluid}, can be written as a function of the other four dimen-

TABLE 1 Dimensionless parameters

Parameter	Description
$V_{bead}/V_{fluid} = V_{bead}\mu/a\tau_w$	Ratio of bead velocity to free-stream velocity
M_{sLex}/M_{esel}	Ratio of sLe^x to E-selectin surface density
$F_{drag}/F_{bond} = V_{fluid}\mu/aF_bM_i = \tau_w/F_bM_i$	Ratio of fluid drag or shear force to the force exerted by the bonds in the contact region
$\tau_{rxn,f}/\tau_{rxn,r} = k_r/k_fM_i$	Ratio of forward to reverse reaction times
$\tau_{rxn,f}/\tau_{flow} = V_{fluid}/ak_fM_i$	Ratio of forward reaction time to flow time

sionless parameters:

$$\frac{V_{bead}}{V_{fluid}} = f\left(\frac{M_{sLex}}{M_{esel}}, \frac{F_{drag}}{F_{bond}}, \frac{\tau_{rxn,f}}{\tau_{rxn,r}}, \frac{\tau_{rxn,f}}{\tau_{flow}}\right) \quad (7)$$

Because we are applying this analysis to a system that always involves the E-selectin/sLe^x pair, the reaction rates are always the same and the ratio of forward and reverse reaction times will be constant throughout our experiments. Because the rate of the forward reaction is fast, we expect the ratio of the forward reaction time to the flow time to be very small in our system, and roughly constant. This is tantamount to assuming that bond formation occurs readily at all densities. This leaves three meaningful parameters. We can represent the fluid velocity by the wall shear stress, using the relation $V_{fluid} = a\tau_w/\mu$, in which we have used the particle radius, a, as an approximation for the distance from the surface to the particle's center, because bond length is small compared to the sphere's radius. The relationship among the final three parameters, written in terms of wall shear stress, is

$$\frac{V_{bead}\mu}{a\tau_w} = f\left(\frac{M_{sLex}}{M_{esel}}, \frac{\tau_w}{F_b M_i}\right) \quad (8)$$

By plotting the left-hand side against the right-hand side, we expect to determine a functional dependence that should hold for all combinations of sLe^x and E-selectin site density and wall shear stress throughout our experiments. This functional dependence is illustrated in Fig. 7, *A* and *B*.

We thank Dr. Brian Brandley for the gift of the E-selectin chimera and Dr. Anil Singhal for the gift of the SNH4 antibody. We also thank Dr. Doug Goetz and Dr. Brett Brunk for helpful discussions.

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