**Microcirculation (2002) 9,** 523–536

www.nature.com/mn

# Viscosity-Independent Velocity of Neutrophils Rolling on P-Selectin *In Vitro* or *In Vivo*

**MICHAEL L. SMITH,* McRAE J. SMITH,* MICHAEL B. LAWRENCE,† AND KLAUS LEY†**

*Department of Biomedical Engineering and †Cardiovascular Research Center, University of Virginia, Health Science Center, Charlottesville, VA, USA

ABSTRACT

**Objective:** To determine whether selectin-mediated leukocyte-rolling velocity in inflamed venules *in vivo* is determined by wall shear rate (WSR) or by wall shear stress (WSS).

**Methods:** WSS was manipulated independently of WSR by altering the viscosity of blood plasma in mice with an isovolemic exchange of blood for low- or high-viscosity dextran solutions. Rolling of neutrophils or beads coated with P-selectin glycoprotein ligand-1 (PSGL-1) was reconstituted on P-selectin immobilized on the wall of a parallel plate flow chamber at two different viscosities of the perfusion medium.

**Results:** Leukocytes *in vivo* showed no increase in rolling velocity when shear stress was doubled by doubling viscosity. Neutrophils in the parallel-plate flow chamber *in vitro* showed the same dependence on WSR as leukocytes *in vivo*, but bead-rolling velocities correlated best with WSS. Rolling leukocytes, but not beads, deformed significantly in shear flow, and deformation correlated better with WSS.

**Conclusion:** These data suggest leukocyte deformation during rolling offsets increased bond breakage at higher shear stress. The stable rolling velocity allows sufficient surveillance of the endothelial surface, even in venules with high WSS. *Microcirculation* (2002) **9,** 523–536. doi:10.1038/sj.mm.7800165

KEY WORDS: leukocyte rolling, wall shear rate, wall shear stress, P-selectin

## INTRODUCTION

Leukocyte rolling along the inflamed endothelium is an important step in inflammatory cell recruitment (24). Most leukocyte rolling is mediated by the selectin class of adhesion molecules and their ligands, as shown by severe defects of leukocyte rolling in selectin-deficient mice (25). The rolling motion is driven by shear stress and hydrostatic pressure distributed during the exposed surface area of the rolling leukocyte (5,18,36). Schmid-Schoenbein et al. used a kinematically and dynamically similar model of the leukocyte-venule system and determined that fluid drag depended on the leukocyte to vessel diameter ratio, hematocrit, and blood flow rate (33). Fluid drag is balanced by adhesive bonds between the rolling leukocyte and the endothelium, and a resultant normal force (34). As a result, leukocytes move downstream at a velocity less than that of a fluid element or an unattached cell in the same position (39). The rolling velocity is controlled in part by the lifetime of the bond(s) at the trailing edge of the leukocyte and the time the leukocyte spends without intimate molecular contact with the endothelium. Leukocyte rolling has been described as a sequence of steps, during which the leukocyte may

*Supported by National Institutes of Health Grant HL64381 to K.L. M.L.S. supported by T32 GM 08715-01A1 to Gordon Laurie and K.L.*
*For reprints of this article, contact Klaus Ley, M.D., Director, Cardiovascular Research Center, Professor of Biomedical Engineering, Molecular Physiology and Biological Physics, University of Virginia Health Science Center, PO Box 801394, Charlottesville, VA 22908, USA; e-mail: e-mail:klausley@virginia.edu*
*Received 8 May 2002; accepted 15 August 2002*

coast at hydrodynamic velocity and may show pauses, during which it does not move (7,39).

The lifetime of the load-bearing bond(s) is inversely related to the off-rate, or reverse reaction rate of the selectin-ligand interaction (20). This off-rate can be measured in flow chamber systems with low densities of selectins and/or selectin ligands (8). At very low densities of selectins, leukocytes do not show smooth rolling but a series of transient attachments. Leukocytes seem to skip from one adhesive contact to another flowing at hydrodynamic velocity. From the distribution of the durations of these attachments or "pauses," the off-rate can be determined. This off-rate has been shown to increase systematically with increased wall shear stress (WSS) and hence increased force per bond (34). WSS is equal to the product of wall shear rate (WSR) and fluid viscosity. Therefore, increasing viscosity or increasing WSR are both expected to decrease the lifetime of bonds formed between a transiently tethering cell and the substrate. Chen and Springer recently reported that the off-rate of PSGL-1 on neutrophils transiently interacting with P-selectin in a parallel wall-flow chamber increased with increasing suspending medium viscosity at constant WSR (8).

*In vivo*, the site density of selectins and their ligands, although not exactly known, seems to be high enough to support smooth rolling, where the leukocyte rarely reaches critical velocity (16). In addition, leukocyte-rolling velocity has been shown to be relatively invariant with increasing WSS in venules *in vivo* (2,13) and in flow chamber experiments *in vitro* (22). It is of interest that no plateau was observed when rolling was reconstituted *in vitro* by using beads coated with sialyl Lewis$^x$ in a flow chamber on which L-, E-, or P-selectin was adsorbed (6,15). The apparent off-rate of leukocytes on sparse P-selectin substrates increases exponentially at low WSSs, as predicted by theoretical analysis (8) but fails to increase much at higher shear stresses including those observed in venules *in vivo* (34). Possible mechanisms for a less than proportional increase in rolling velocity with increasing WSS include an automatic breaking system, through which more bonds may be formed at elevated shear stress (7), or cellular deformation (23). Leukocytes deform considerably while rolling *in vivo*, flattening against the endothelial wall and reaching aspect ratios (length/height) of up to 1.4 (9,13). Theoretical modeling studies suggest that cellular deformation decreases the drag on the cell and may account for a plateau of rolling velocity (5,23).

If bond dissociation was the major determinant of rolling velocity, one might predict velocity increases with the force on the cell and hence WSS. By contrast, if increased leukocyte deformation at higher shear stress provided more adhesion molecules in the contact zone, rolling velocity would be predicted to increase less than proportional to WSS. We designed experiments in which WSS was manipulated independent of WSR by changing the viscosity of the suspending medium. Both WSS and WSR were determined in the absence of a leukocyte to serve as an indicator of the forces and velocity gradients a cell may experience.

In venules of the untreated mouse cremaster muscle, leukocyte rolling is mediated by P- and L-selectins (25,26), and E-selectin is not or marginally expressed (19). In L-selectin-deficient mice, rolling is entirely P-selectin dependent (25). We compare the results with those for isolated human leukocytes or P-selectin ligand (PSGL-1)-coated beads on P-selectin in a flow chamber system at two different viscosities of the perfusion medium. Finally, to test whether our findings were applicable *in vivo*, we investigated leukocyte rolling at two different plasma viscosities in wild-type mice that express both P- and L-selectin.

## MATERIALS AND METHODS

### Animals

All animal experiments were conducted under a protocol approved by the University of Virginia institutional animal care and use committee (protocol 2474). L-selectin-deficient mice were obtained from established colonies derived from gene-targeted founders as described and back-crossed to C57BL/6 for at least eight generations (1). Wild-type mice (C57Bl/6) were obtained from Hilltop Labs (Scottsdale, PA). All mice seemed healthy and were between 8 and 14 weeks of age.

### Intravital Microscopy

Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (125 mg/g body weight, Ketalar; Parke-Davis, Morris Plains, NJ), xylazine (12.5 mg/g body weight; Phoenix Scientific, Inc., St. Joseph, MO), and atropin sulfate (0.025 mg/g body weight; Elkins-Sinn, Inc., Cherry Hill, NJ) and placed on a 38 °C heating pad. Each mouse received a 1.0-mL i.p. injection of isotonic saline to prevent fluid imbalance. The trachea was intubated by using polyethylene (PE) 90 tubing (Becton Dickinson and Company, Sparks, MD) to allow spontaneous respiration. The left jugular vein and left carotid artery were cannulated by using PE10 tubing. The jugular

cannula was used to administer additional anesthesia and the dextran solution. The carotid cannula was needed for blood pressure monitoring, blood samples, and withdrawal of blood during hemodilution.

The cremaster muscle was prepared for intravital microscopy as described (3,25). The cremaster was exteriorized, pinned to the stage, and superfused with thermocontrolled (37 °C) bicarbonate-buffered saline (131.9 mM NaCl, 18 mM $NaHCO_3$, 4.7 mM KCl, 2.0 mM $CaCl_2 \bullet 2H_20$, and 1.2 mM $MgCl_2$) equilibrated with 5% $CO_2$ in $N_2$. This procedure was completed in 6–10 minutes.

Mice received an isovolemic blood exchange with H-(500,000) or LMWD (40,000) solutions (10). Dextrans were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Each dextran was dissolved in isotonic saline (5 g/L dextran, 0.9% NaCl, osmolarity 308 mOsmol/L; Baxter Healthcare Corp., Deerfield, IL) and filtered through a 0.45-μm filter (Osmonics Laboratory Products, San Francisco, CA). The mice were bled via the carotid cannula into a syringe tube for volume measurements. Blood was exchanged during a 15-minute period by infusing the dextran solution into the jugular vein and matching the rate of infusion to the carotid bleed rate. Systemic erythrocyte counts and blood pressure measurements (model AH 60-3002; Harvard Apparatus, Inc., Holliston, MA) were taken before and after each exchange. A 10-μL carotid blood sample was obtained in a capillary tube and placed in 2.0-mL isotonic saline for analysis with a hemocytometer to obtain numbers of erythrocytes/μL.

Microscopic observations were made on a Zeiss intravital microscope (Axioskop, Carl Zeiss, Inc., Thornwood, NY) with a saline immersion objective (SW 40/0.75). Venules at least 5 diameters from the nearest upstream bifurcation and between 15- and 60-μm diameter were selected for intravital microscopy, and recordings were made through a CCD camera (model VE-1000CD, Dage-MTI, Michigan City, IN) on a Panasonic S-VHS recorder. Vessels were aligned on the video screen so that blood flowed across the screen from left to right. Centerline erythrocyte velocity ($v_{cl}$) was measured in each venule by using a dual photodiode and digital online cross-correlation program (Circusoft Instrumentation, Hockessin, DE). At the end of each experiment, blood samples were taken for measurement of plasma viscosity (μ, Cannon-Manning Semi-micro Viscometer, Cannon Instrument Co., State College, PA).

**Wall Shear Rate and Wall Shear Stress**

WSR is a critical parameter in these experiments. For this reason, independent WSR measurements were made in some venules by using FITC-labeled microspheres to validate calculations made with measured centerline velocity. Fluoresbrite YG Microspheres (0.48 ± 0.01 μm, ρ = 1.05 g/cm$^3$; Polysciences, Inc., Warrington, PA) were washed twice in isotonic saline with 1% BSA and resuspended in saline. A small volume of microspheres (<0.03 mL) was injected through the carotid cannula until 5–10 beads per second passed through the vessel. The microspheres were visualized by using stoboscopic double-flash (1–8 ms apart; Strobex 236, Chadwick Helmuth, Mountain View, CA) epi-illumination. This technique yields in one picture two images of the same microsphere displaced during a certain distance for the given time interval. Some transillumination was maintained to keep the vessel wall clearly visible. The time interval was chosen so that a microsphere's two images were 3–10 μm apart. The distances between the images and from the microsphere to the wall of the vessel were measured for at least five microspheres within the erythrocyte-free plasma zone at the top and bottom of each horizontally aligned vessel. The two-dimensional image of the three-dimensional vessel allows two plasma width measurements to be made. Velocity, the distance between images divided by the double-flash time interval, was determined as a function of relative radial position. Velocity gradient was estimated by assuming a linear velocity profile from $V_x$, the average velocity and position of the two closest microspheres at the top and bottom of the vessel (four measurements total), to zero at the wall. WSR can thus be estimated

$$\left.\frac{\partial v}{\partial r}\right|_w = \frac{V_x}{R - x} \tag{1}$$

where

$$\left.\frac{\partial v}{\partial r}\right|_w$$

is WSR, $R$ is the vessel radius, and $x$ is the distance from the center of the vessel to the average position of the two closest microspheres. Mean blood velocity ($\bar{v}_b$) was determined from the centerline velocity using the empirically derived relationship $\bar{v}_b = 0.625 v_{cl}$ (28). WSR was estimated by using mean blood velocity and vessel diameter ($D$) as follows:

$$\left.\frac{\partial v}{\partial r}\right|_w = 2.1 \left(8 \frac{\bar{v}_b}{D}\right) \tag{2}$$

The factor 2.1 appearing in Eq. (1) is the median ratio of the measured WSR obtained in rabbit mesenteric microvessels to the corresponding Poiseuille value (37). This factor accounts for velocity gradients at the wall that are steeper than in a Poiseuille flow due to axial concentration of red blood cells (31). WSS was calculated as the product of WSR and plasma viscosity measured for each mouse.

**Neutrophil Isolation**

Forty to eighty million human neutrophils were obtained from 60 mL of heparin (35 U/mL) anticoagulated whole blood. Neutrophils were isolated as described (38). H- or LMWD (2 g/L) was added to the assay medium before the addition of isolated neutrophils, and the viscosity of the resultant medium was measured.

**PSGL-1 Beads**

Human PSGL-1 was purified from harvested HL-60 cells as described (30). Polystyrene microspheres (9.76 ± 0.86 μm, $\rho = 1.05$ g/cm$^3$, Polysciences, Inc.) were washed twice with alternating steps of water and ethanol and incubated with PSGL-1 in PBS ($5 \times 10^7$ beads/mL, pH 7.4) for 2 hours at room temperature under end-to-end rotation. After washing with HBSS, the microspheres were incubated in 0.5% Tween-20 for 30 minutes to block nonspecific adhesion. The microspheres were stored in HBSS containing 0.5% Tween-20 for same-day use. The microspheres were suspended in assay media (HBSS, 10 mM HEPES (pH 7.4), 1 mM $CaCl_2$, and 2 g/l H- or LMWD) at a concentration of $5 \times 10^5$ beads/mL. Flow cytometry using KPL1 confirmed comparable levels of PSGL-1 expression on human neutrophils and adsorbed PSGL-1 microspheres. The PSGL-1 was estimated to adsorb onto the polystyrene microbeads at a site density of 50 sites/μm$^2$ based on a comparison to neutrophils with similar mean fluorescence values and estimated PSGL-1 site densities of 30,000/neutrophil (29).

**Laminar Flow Adhesion Assay**

Polystyrene slides were cut from bacteriological petri dishes (Falcon 1058). Human P-selectin was purified from platelet lysates as described (21). Purified human P-selectin was applied to the plates and allowed to adsorb for 2 hours at room temperature. The slides were blocked to prevent nonspecific adhesion with 0.5% Tween-20 in PBS 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$^2$. Site densities for lower amounts of immobilized selectin were estimated by proportional dilution in PBS. The mAb against human P-selectin, G1 (IgG1, 10 μg/mL), was used to block P-selectin-dependent adhesion of neutrophils to immobilized P-selectin. Neutrophil and PSGL-1 microsphere-rolling adhesion to P-selectin was also specifically blocked by infusing 10 μg/mL KPL1.

The chamber was mounted during an inverted phase-contrast microscope (Diaphot-TMD; Nikon, Garden City, NY) at ×20 magnification. Recordings were made through a CCD camera system (model VC2410; Vicon, Melville, NY) on a Panasonic S-VHS recorder. For each substrate slide, 5 mL of a 0.5% Tween-20 in PBS solution was perfused during the substrate and incubated for 5 minutes to block nonspecific adhesion.

**Data Analysis**

Data obtained on videotapes were analyzed off-line on a Macintosh computer using the public domain National Institutes of Health's Image program (http://rsb.info.nih.gov/nih-image/). Vessel diameter was determined as the average of three measurements for each vessel. The thickness of the erythrocyte-free zone at two sides of each vessel was found by averaging three measurements obtained in one video frame. Only leukocytes rolling along lateral vessel walls in the central axial plane of each microvessel were used for analysis. Rolling velocities of leukocytes *in vivo*, neutrophils *in vitro*, and PSGL-1-coated microspheres *in vitro* were calculated by measuring the distance that cells or microspheres rolled during a two-second interval. Ten leukocytes were measured per venule, and at least 35 neutrophils or microspheres were measured at each WSR *in vitro*. The length and height of rolling leukocytes *in vivo* were also collected in these experiments (9). The deformation index was defined as the ratio $l/h$, where $h$ is the maximum height of the cell and $l$ is the length of the cell measured at height $h/2$ above the endothelium. This ratio was calculated for 10 leukocytes per venule.

Least-squares linear regression was performed by using commercially available software (Microsoft Excel, Santa Rosa, CA). For velocity data divided into high-or low-WSR or WSS groups, statistical significance between groups was determined by using a two-tailed $t$-test with $p = 0.05$ for all raw velocity data using Microsoft Excel. To avoid binning and show all data, rolling velocity data are shown as cumulative distributions for high-or low-WSR or WSS groups. Tests for the coincidence of two regression lines were also performed (14). The improvement

obtained by fitting the two data sets with separate regression lines, as opposed to a single line through both sets, was computed by using an F-test statistic. An observed value of F, which exceeds the critical value ($p < 0.05$), is found when a significantly better fit to the data occurs with separate regression lines for each of the two samples. In addition, it was determined whether rolling velocity correlated better with WSR or WSS. Correlation coefficients were determined for least-squares linear regression fits to all of the data in both L- and HMWD groups versus WSR or WSS using Microsoft Excel.

## RESULTS

### Hemodilution

Hemodilution in L-selectin−/− mice with H- or LMWD resulted in reductions in systemic erythrocyte counts by 44 ± 8% and 50 ± 6%, respectively, and plasma viscosities of 2.2 ± 0.1 cP and 1.1 ± 0.1 cP, respectively (Fig. 1A). Hemodilution with H- or LMWD increased plasma viscosity over control (0.9 cP). All mice survived the hemodilution procedure without apparent stress, and blood pressure remained in the normal range for anesthetized mice (mean 76.9 ± 9.4 mm Hg, 76.2 ± 11.4 mm Hg after hemodilution).

### Hemodynamics

Plasma layer thickness was measured in 19 venules before hemodilution, 7 venules after hemodilution with HMWD, and 15 venules after hemodilution with LMWD in L-selectin−/− mice. The erythrocyte-free zone is on average 1.5-μm thick and increases to 1.8 or 1.9 μm after hemodilution with H- or LMWD, respectively, although this difference was not statistically significant (Fig. 1B). Dual-slit WSR calculations, using Eq. (2), were independently verified by measuring the velocity of two microspheres flowing closest to the venular wall after hemodilution with dextran (Fig. 1C). Dual-slit calculations were similar to the direct measure of WSR using 0.48-μm microspheres; the slope of a linear regression through all of the data in Fig. 1C forced through a y-intercept of zero (m = 1.06) was not significantly different from unity (data not shown).

### P-Selectin-Dependent Leukocyte Rolling Velocities *In Vivo*

We measured the velocities of 320 leukocytes in 32 venules of 8 low-viscosity L-selectin−/− mice and of 270 leukocytes in 27 venules of 6 high-viscosity L-selectin−/− mice. The average rolling velocity of 10 leukocytes in each venule is shown as a function of WSR or WSS in the same venule (Fig. 2A and B). Rolling velocities in low-viscosity and high-viscosity mice seem to form a single population when plotted as a function of WSR but two different populations ($p < 0.05$) when plotted as a function of WSS. Linear regression of all data in both low- and high-viscosity groups confirmed that rolling velocity correlates much better with WSR ($R^2 = 0.65$) than WSS ($R^2 = 0.36$).

We constructed cumulative histograms of leukocyte-rolling velocities for all leukocytes measured in venules with low WSR (200–700 $s^{-1}$) or high WSR (700–1500 $s^{-1}$). This technique displays all data because no binning is used. In both cases, the rolling velocity histograms of leukocytes rolling in high-viscosity L−/− mice and in low-viscosity L−/− mice were very similar (Fig. 2C and E). By contrast, when we did the same analysis for venules with low WSS (4–12 dyn/$cm^2$) or high WSS (12–22 dyn/$cm^2$), the leukocytes in low-viscosity mice rolled significantly faster ($p < 0.05$) than those in high-viscosity mice (Fig. 2D and F).

### Leukocyte Rolling Velocities *In Vitro*

To test whether we could recapitulate this behavior in a simplified *in vitro* model, we infused isolated human neutrophils into a flow chamber whose lower wall was coated with purified human P-selectin at a nominal site density of 20 sites/$\mu m^2$. We analyzed rolling velocities over a WSR range of 50–300 $s^{-1}$. This corresponded to wall shear stresses of 1.1–6.6 dyn/$cm^2$ in high-viscosity (2.2 cP) tissue culture suspension media and 0.6–3.6 dyn/$cm^2$ in low-viscosity (1.2 cP) tissue culture media. When rolling velocities were plotted as a function of WSR, they formed one population (Fig. 3A, $R^2 = 0.97$). By contrast, the same WSS resulted in two groups of rolling velocities (Fig. 3B, $R^2 = 0.69$). The same conclusion can be drawn from inspecting the cumulative rolling velocity histograms for low- and high-WSR groups (Fig. 3C and E) and low- and high-WSS groups (Fig. 3D and F). This finding confirmed our *in vivo* observation in a well-controlled system.

### Polystyrene Bead Rolling Velocities *In Vitro*

To address whether cellular properties were involved in the observed dependence of rolling velocity on WSR rather than WSS, we investigated the rolling of polystyrene beads coated with purified PSGL-1 on P-selectin. We initially conducted experiments with

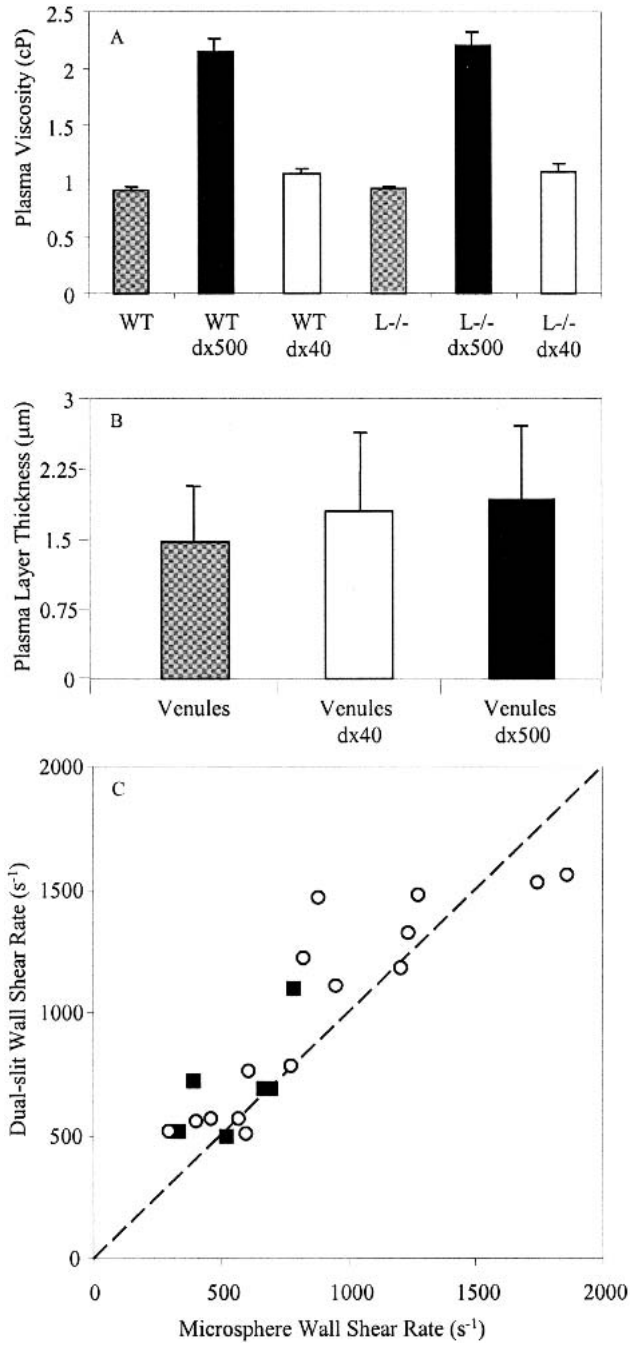


**Figure 1.** Plasma viscosity (A) and erythrocyte-free plasma layer thickness (L–/–, B) before (cross-hatched bars) or after hemodilution with high- (H-, black bars) or low-molecular weight dextran (LMWD, open bars) in wild-type (WT) and L-selectin–/– (L–/–) mice. Viscosity measured in plasma from 1.0 mL of whole blood withdrawn from the carotid catheter and centrifuged to remove erythrocytes and the buffy coat in a capillary viscometer at 37 °C. Relationship between wall shear rate measured with microspheres or dual-slit (C) in venules after hemodilution with H- (black squares) or LMWD (open circles). Line of unity shown (dashed line) for comparative purposes.

beads rolling on the same site density as that used for neutrophil-rolling experiments, but this resulted in unsteady bead rolling and skipping. We increased the site density of P-selectin to 170 sites/$\mu m^2$ and achieved a bead-rolling velocity comparable to the rolling velocity of leukocytes. In contrast to leukocytes, bead-rolling velocity correlated better with WSS ($R^2$ = 0.97) than WSR ($R^2$ = 0.65; Fig. 4A and B). This finding was confirmed by inspecting the cumulative rolling velocity histograms constructed for low- and high-WSR (Fig. 4C and E) and low- and high-WSS (Fig. 4D and F). These data suggest that a cellular property is required for leukocyte-rolling velocity to increase with WSR rather than WSS. Apparently, beads did not possess this property.

### Leukocyte Deformation

Leukocytes, but not beads, can deform while rolling (9). This is easier to observe *in vivo* than in a parallel-plate flow chamber, because each venule provides a side view of the leukocytes rolling along its lateral wall (Fig. 5A). We analyzed the deformation of 570 rolling leukocytes in 57 venules of 14 hemodiluted L-selectin–/– mice and found an average deformation index (length/height) of 1.17 ± 0.09. The deformation index increased with WSR (Fig. 5B) and WSS (Fig. 5C). When analyzing leukocyte deformation indices separately for high-viscosity and low-viscosity L-selectin–/– mice, it becomes apparent that leukocyte deformation correlates better with WSS ($R^2$ = 0.51) than WSR ($R^2$ = 0.39). At any given WSR, leukocytes rolling in venules of mice with high-plasma viscosity were more deformed than those rolling in mice with low-plasma viscosity under otherwise identical conditions. In addition, the degree of leukocyte deformation increases with increasing rolling velocity (Fig. 5D). Although leukocytes in low-viscosity venules seem to show a stronger relationship between deformation and velocity, this difference was not statistically significant. This finding suggests that leukocyte deformation is an important parameter influencing rolling velocity.

### Leukocyte Rolling Velocity and Deformation in Wild-Type Mice

To test whether the findings obtained in simplified model systems were applicable to the *in vivo* situation in normal mice, we conducted experiments in wild-type mice in which rolling is mediated by P- and L-selectin (25). Hemodilution with H- or LMWD resulted in plasma viscosities of 2.2 ± 0.1 cP

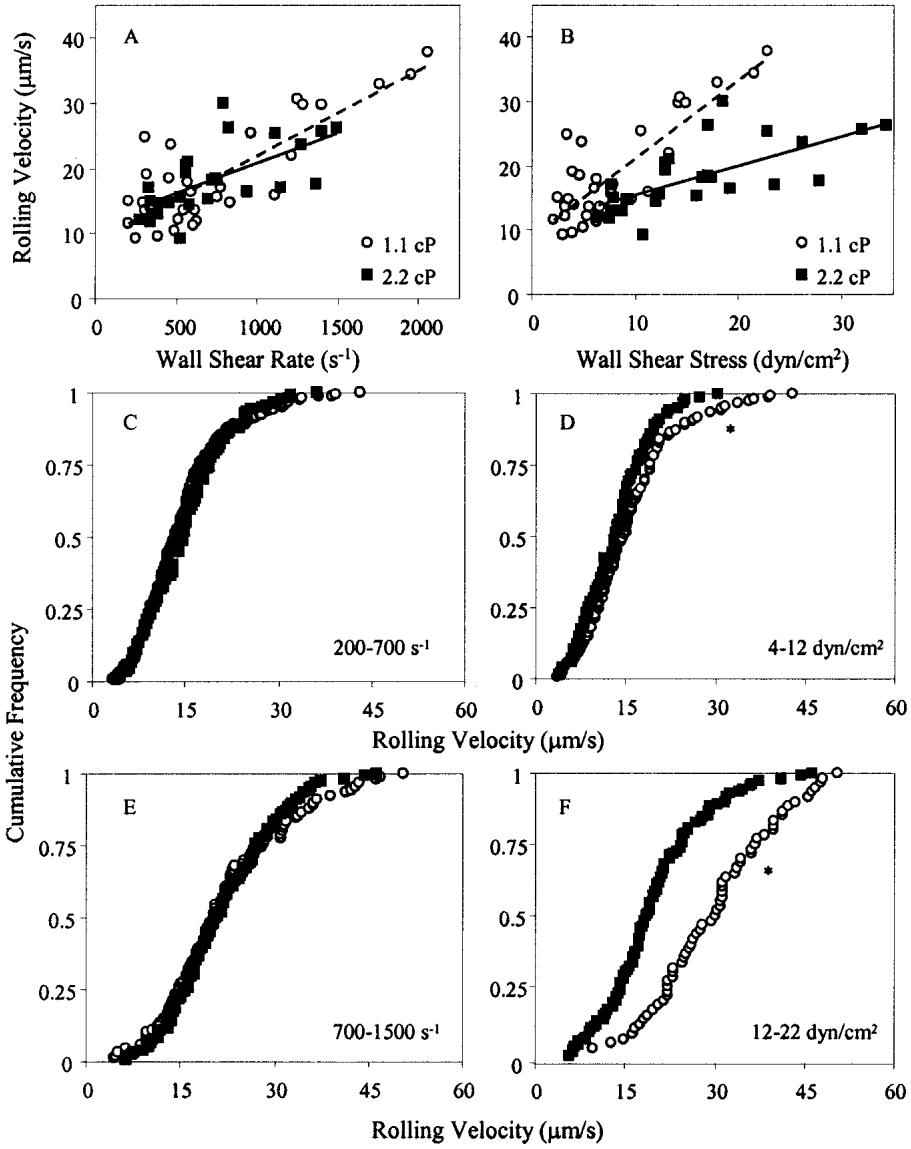


**Figure 2.** Leukocyte-rolling velocity as a function of wall shear rate (A) or stress (B) in L–/– mice after a 1.0-mL hemodilution with H- (black squares) or LMWD (open circles). Each data point represents mean rolling velocity of 10 cells per vessel measured during 2 seconds. Linear regression fit to all the data in both groups (line not shown) revealed rolling velocity correlated better with WSR ($R^2$ = 0.65) than WSS ($R^2$ = 0.36). Cumulative histograms constructed from all measured rolling velocities for wall shear rates of 200–700 $s^{-1}$ (C) and 700–1500 $s^{-1}$ (E) or grouped by wall shear stresses of 4–12 dyn/$cm^2$ (D) and 12–22 dyn/$cm^2$ (F). Asterisk (*) indicates statistically significant difference ($p < 0.05$) by two-tailed $t$- test.

($n$ = 6) and 1.1 ± 0.1 cP ($n$ = 7), respectively. In these mice, our findings were completely recapitulated (Fig. 6A and B). Leukocyte-rolling velocity correlated better with WSR ($R^2$ = 0.62) than WSS ($R^2$ = 0.26). The deformation indices of rolling leukocytes were consistently higher in high-viscosity venules at a given WSR (Fig. 6C). Unlike rolling velocity, leukocytes were deformed to a certain extent at a given WSS independent of the contribution of WSR versus plasma viscosity. These data suggest that leukocyte deformation is a physiologically relevant parameter influencing rolling velocity *in vivo*.

## DISCUSSION

We have studied how alterations in fluid viscosity affect selectin-mediated leukocyte rolling *in vivo* and neutrophil and PSGL-1-coated microsphere rolling on adsorbed P-selectin *in vitro*. Plasma viscosity was altered in L-selectin–/– and wild-type mice by ex-

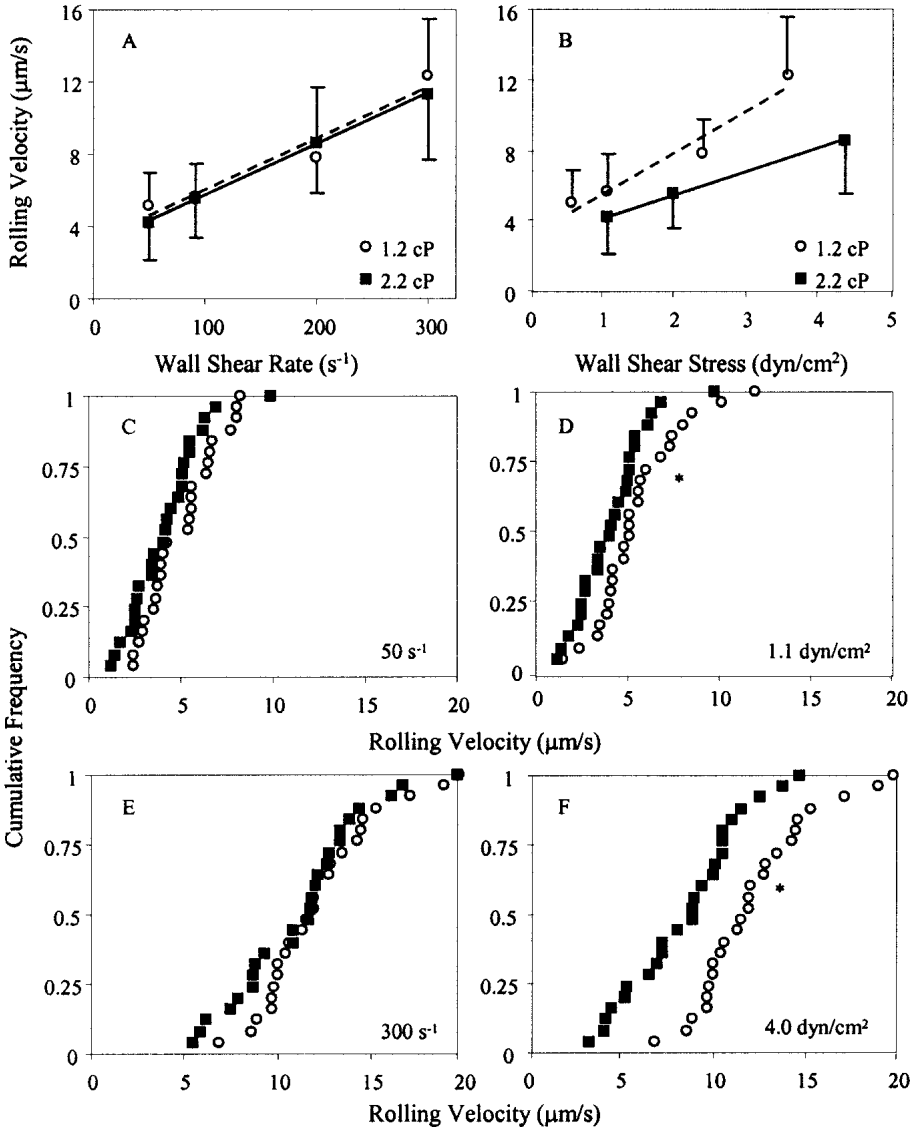


**Figure 3.** Rolling velocity of human neutrophils on human P-selectin (20 sites/$\mu m^2$) in tissue culture media containing 2 g/L H- (black squares) or LMWD (open circles) as a function of wall shear rate (A, $R^2 = 0.97$) or wall shear stress (B, $R^2 = 0.69$). Each data point was determined by the distance that at least 35 cells rolled during 2 seconds. Data for each wall shear rate and wall shear stress group was obtained from at least two experiments performed on separate days. Cumulative histograms are shown by using velocities from all measured cells for wall shear rates of 50 $s^{-1}$ (C) and 300 $s^{-1}$ (E) and for wall shear stresses of 1.1 dyn/$cm^2$ (D) and 4.0 dyn/$cm^2$ (F). Asterisk (*) indicates statistically significant difference ($p < 0.05$) by two-tailed $t$-test.

changing blood for H- or LMWD in physiological saline. Analysis of mean leukocyte-rolling velocities in venules of these mice revealed that rolling velocity correlated better with WSR than WSS during the range of plasma viscosities investigated. This behavior was recapitulated in an *in vitro* flow chamber with isolated human neutrophils rolling on purified P-selectin. In contrast, PSGL-1-coated microsphere-rolling velocity on P-selectin in a parallel plate flow chamber correlated better with WSS than WSR.

WSS in the absence of a leukocyte was used as an indirect indicator of the fluid drag force acting on rolling leukocytes because it is impossible to obtain direct drag force measurements. Leukocytes rolling in different venules with the same WSS could experience different drag forces. This could, for instance,

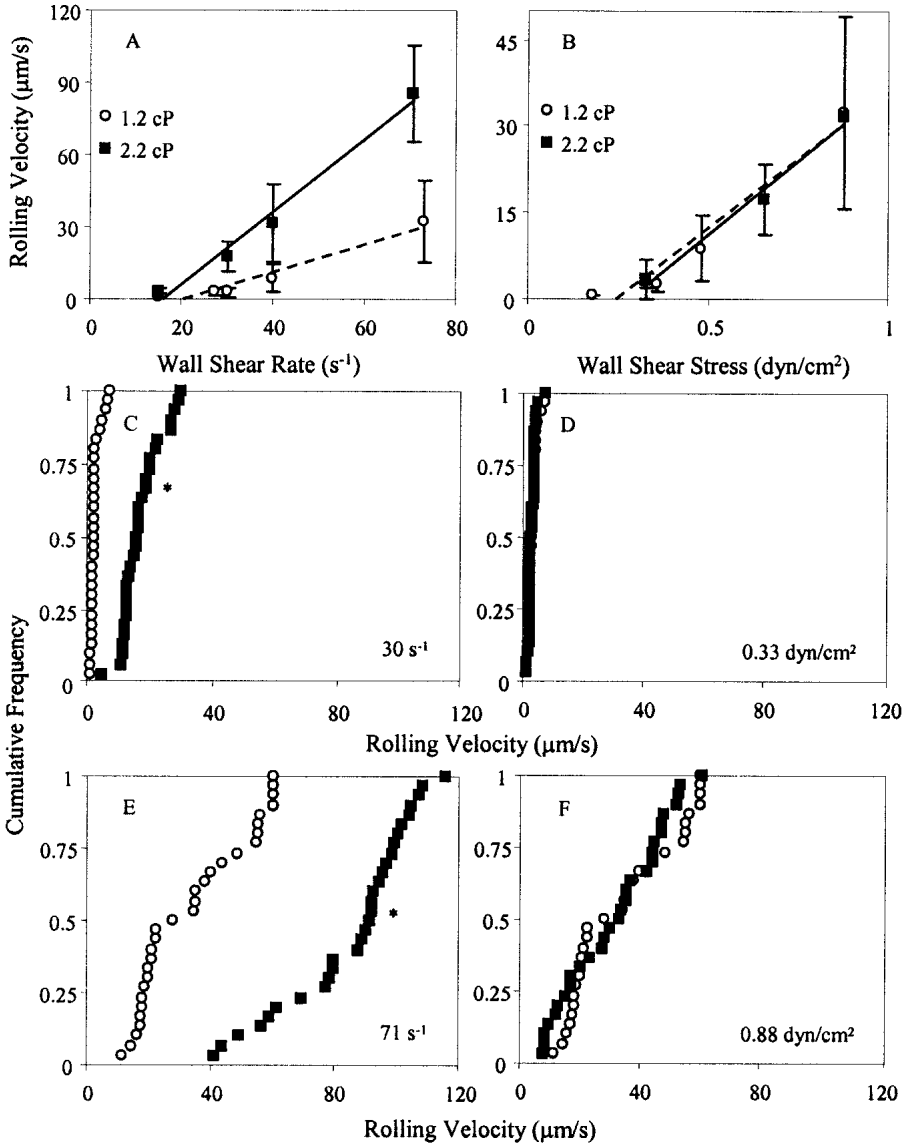


**Figure 4.** PSGL-1-coated microsphere (~50 sites/$\mu m^2$) rolling on P-selectin substrate (170 sites/$\mu m^2$) in tissue culture media containing 2 g/L H- (black squares) or LMWD (open circles) as a function of wall shear rate (A, $R^2 = 0.65$) or stress (B, $R^2 = 0.97$). Each data point was determined by the distance that 35 microspheres rolled during 2 seconds. Cumulative histograms are shown by using velocities from all measured microspheres for wall shear rates of 30 $s^{-1}$ (C) and 71 $s^{-1}$ (E) and for wall shear stresses of 0.33 dyn/$cm^2$ (D) and 0.88 dyn/$cm^2$ (F). Asterisk (*) indicates statistically significant difference ($p < 0.05$) by two-tailed $t$-test.

occur in geometrically similar venules with the same blood volume flow rate but different tube hematocrits (33). To mitigate the influence of such variations, rolling velocities were determined for leukocytes in many vessels over similar diameter ranges in both low- and high-viscosity groups.

The relationship between shear rate and shear stress is complicated for a particulate solution. Microvessels consistently show a layer of 1–3 μm at the vessel wall in which no red cells enter. To confirm previous estimates of the width of the erythrocyte-free plasma zone (35) and investigate the effect, if any, of dextran on the width of this zone, we measured plasma zone thickness before and after hemodilution with dextrans (Fig. 1B). As expected, the width of this zone increased after hemodilution. The presence of a red cell-free plasma layer allows the use of plasma viscosity in the calculation of WSS in cremaster muscle venules.

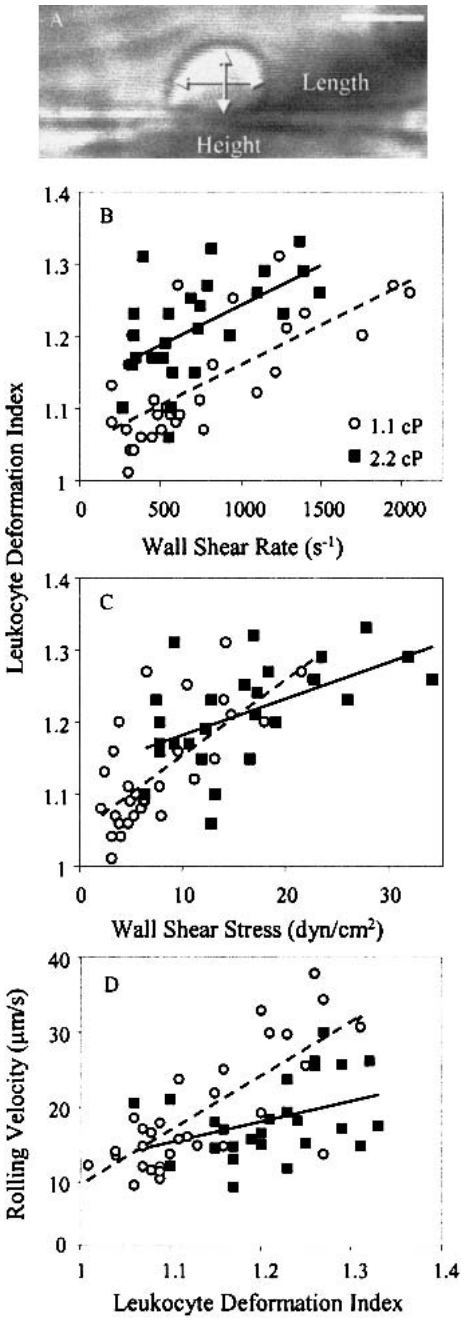


**Figure 5.** Videomicrograph frame showing a leukocyte rolling along a postcapillary venule (flow from left; wall shear rate, 900 $s^{-1}$). Bar = 10 μm. Leukocyte deformation indices (axial length/radial height) for L-selectin–/– mice hemodiluted with H- (black squares) or LMWD (open circles) as a function of wall shear rate (B, $R^2$ = 0.39) or wall shear stress (C, $R^2$ = 0.51). Each data point from frame-captured video images of 10 cells per vessel.

An empirical relation exists, which was used to convert dual-slit centerline erythrocyte velocity into WSR. To validate these WSR calculations, WSR was measured directly in some venules with velocity measurements of 0.47-μm microspheres flowing in close proximity to the vessel wall. The slope of a linear fit from the microspheres to zero at the vessel wall (no-slip condition) provided direct WSR measurements in venules for which the dual-slit centerline velocity was also measured. Despite the reduced systemic hematocrit after hemodilution and an increased tendency for erythrocyte aggregation after dextran administration (4), the slope of a linear regression through all of the data relating dual-slit WSR to microsphere WSR was not significantly different from unity. Therefore, the use of dual-slit measurements in the calculation of WSR is valid for these experimental conditions.

Leukocyte deformation, adhesion molecule dissociation, and hence rolling velocity are each a function of the fluid drag force experienced by the rolling cell. This drag force, in turn, is proportional to the shear stress on the surface of the leukocyte. Because shear stress is the product of the velocity gradient and local viscosity, increasing shear rate or viscosity would increase the force on the rolling cell. Fluid velocity and its gradient, however, determine the speed at which a leukocyte moves between adhesive zones without contact with the endothelium. We show here that leukocyte-rolling velocity correlates better with WSR than WSS. This is likely caused by increased deformation of rolling leukocytes, offsetting increased drag force, and increasing the number of adhesive bonds mediating endothelial attachment. Indeed, leukocyte deformation correlated better with WSS.

At any given WSR, flow-independent increases in WSS (through increases in fluid viscosity) increase the force on cells interacting with adhesion molecules in flow. A recent report showed that dissociation kinetics were a function of WSS and not WSR and, hence, of force on the bond (8). Bond lifetimes of these transient interactions were shown to decrease with increases in assay medium viscosity from 1.0 to 2.6 cP, but rolling velocities were not investigated in this study. Assuming the number of bonds mediating rolling remains constant at increasing flow rate, the velocity of a rolling leukocyte would be predicted to increase with the force acting on the cell and, hence, WSS because of the decreased time spent motionless during bond events.

Although it may be possible that dextrans adversely affect endothelial cells or leukocytes, this study uses dextrans at the same concentration in both high- and low-viscosity groups, eliminating the possibility that the behavior seen in the high-viscosity group is a result of the dextran and not a cellular property such

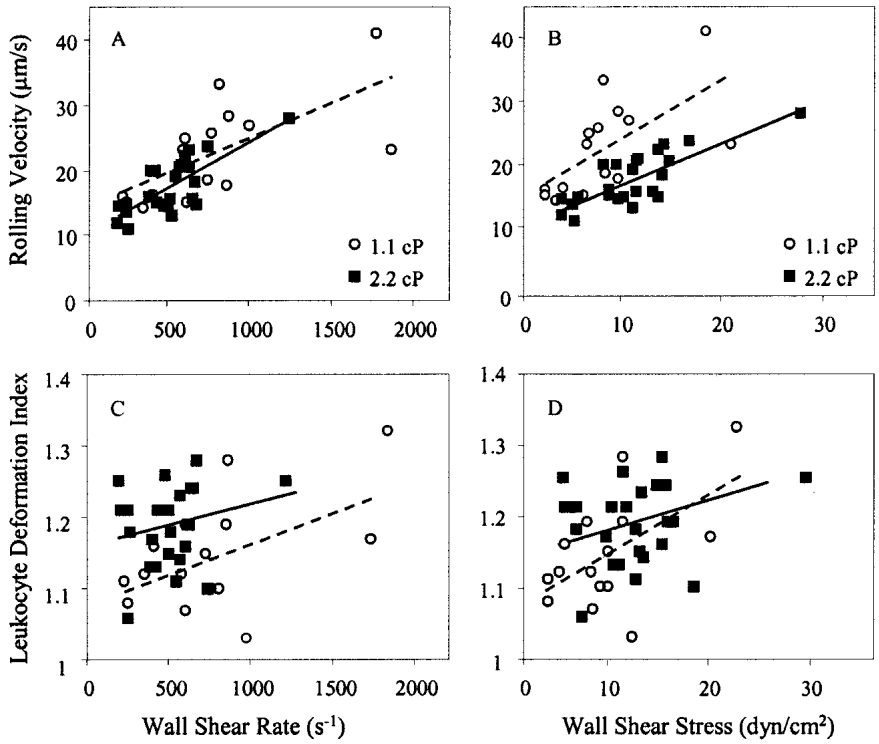


**Figure 6.** Leukocyte-rolling velocity (top) and deformation index (bottom) in wild-type mice after hemodilution with H- (black squares) or LMWD (open circles) versus wall shear rate (A and C, respectively) or wall shear stress (B and D, respectively).

as deformability. Long-chain dextrans do not adversely affect the strength of adhesion between P-selectin and PSGL-1. Leukocyte-rolling velocities measured *in vivo* in L–/– mice hemodiluted with LMWD are not significantly different from rolling velocities in L–/– mice with no hemodilution versus WSR or WSS (data not shown). Similar findings exist between leukocytes rolling in wild-type mice with or without hemodilution with LMWD (data not shown). In addition, previously published data show that neutrophils rolling on adsorbed P-selectin in standard tissue culture media ($\mu$~1.0 cP) *in vitro* maintain similar rolling velocities during the range of shear rates investigated here to those found after the addition of LMWD (12). Velocities were reported between 2 and 5 $\mu$m/s from 0.5 to 2.0 dyn/cm$^2$ (see Fig. 6) versus 4–7 $\mu$m/s during the same shear stress range measured in this report. If dextran affected bond strength or lifetime, one would hypothesize that rolling velocity would increase in the LMWD-treated group.

Because the presence of HMWD increases the viscosity of the suspending fluid, increased hydrodynamic drag on the cell and increased rolling velocity would be expected if HMWD interfered with selectin bonds. However, the rolling velocity, as a function of WSR, of leukocytes in high-viscosity venules *in vivo* or neutrophils in high-viscosity suspending medium *in vitro* was not significantly different from the rolling velocity of leukocytes or neutrophils, respectively, in the low-viscosity group (Figs. 2A, 3A, and 6A).

It is well known that isolated neutrophils rolling *in vitro* cannot sustain interactions at the same wall shear rates or stresses as in microvessels *in vivo*, but the exact reason is not known. Possible explanations include the shedding of L-selectin or rearrangement or loss of P-selectin ligands during the neutrophil isolation procedure. The percentage of P-selectin that remains functional after adsorbing to the flow chamber wall is not known and could account for decreased bond numbers *in vitro*. The present study does not address the causes for this difference between *in vivo* and *in vitro* experiments; instead, both approaches in parallel are used.

The data presented here show that rolling velocity does not increase when plasma viscosity is elevated, suggesting the presence of a mechanism that regulates velocity despite increased viscous drag forces. This mechanism likely involves deformation of roll-

ing cells. Although deformation of rolling leukocytes was observed in earlier studies (9,13), its impact on the force exerted on the molecular bonds impeding the downstream movement of rolling cells was not clear. An initial analysis suggested that the major effect of cell deformation was a reduction of force on the rolling cell (13,27). However, theoretical analysis predicts that cellular alignment and elongation in the direction of flow could reduce the fluid force acting on a cell in two-dimensional flow by not more than 25% (5). A later study confirmed that deformation from a sphere into an ellipsoid results in a small decrease of drag force in a cylindrical vessel (36). Cells in venules with high-viscosity plasma had deformation indices that were on average 9% greater than cells in venules with the same wall shear rate but decreased viscosity, suggesting that although the viscosity of the plasma was doubled, the force on the cells was not.

A recent two-dimensional model helps understand how viscous and elastic membrane forces mediate the adhesion of cells to a planar adhesive surface in the presence of shear flow (17). Hodges and Jensen used lubrication theory to describe the thin-film flow between the cell and the plane along with a Hookean spring model of bond mechanics (11). At the spreading edge of the cell, fluid was predicted to be trapped in the closing gap, resulting in elevated pressure. However, fluid is sucked into the opening gap at the peeling edge resulting in low pressure. The resultant predicted rolling velocity was independent of viscosity because increased drag on the cell is offset by an increasing resistance to flow at the spreading and peeling contact lines. This predicted independence of rolling velocity from viscosity is remarkably similar to the results presented here.

Extension of microvilli may also contribute to the independence of leukocyte-rolling velocity on drag force. Park et al. showed that microbeads or fixed neutrophils had similar dissociation rates that were briefer than those of neutrophils and increased faster with increasing flow (30). An evaluation of geometric parameters and hydrodynamic force equations showed the presence of a 1.6-μm microvillus with spring constant 152 pN/μm (at 2.0 dyn/cm$^2$) was required to reconcile these differences. Although this study evaluated transient adhesive interactions, as opposed to robust rolling here, it is likely that microvillus extension contributes to whole cell deformation.

Rinker et al. recently investigated rolling velocities of Mono Mac 6 cells, a monocyte-like cell line, during TNF-α–stimulated human umbilical vein endothelial cells and found that an increase in medium viscosity from 0.8 to 7.3 cP by addition of 5% high-molecular weight dextran decreased rolling velocity during the range of 40–120 $s^{-1}$ (32). The median arrest duration increased for firmly adherent cells (defined as cells that arrested for at least 0.2 s) as WSS increased at constant WSR. The authors suggested that hydrodynamic forces might augment the probability of bond formation during rolling and arrest. This report seems to confirm Chen and Springer's automatic braking system in which the number of bonds mediating cell rolling is increased at higher shear stress (7). In neither of these reports, however, was the impact of cellular deformation explored by comparison to a bead-based system.

One proposed hypothesis to explain the mechanism behind the automatic braking system was that a repulsive barrier exists, which must be overcome, through force, for the formation of adhesive bonds. It was further postulated that the force driving these molecular contacts is related to the translational veloctiy of the cell relative to the substrate (7). Microspheres do not show a plateau of rolling veloctiy, even at high site densities (Fig. 4A and B), suggesting that the automatic braking system is the result of a cellular property and not an inherent molecular property of the selectin-ligand interaction. In addition, the velocity of cells relative to the substrate is likely not important for an increase in bond number with shear, because leukocytes *in vivo* roll at a relatively constant velocity during a wide range of shear rates above ~1000 $s^{-1}$ and rarely reach critical velocity (2). A reexamination of the automatic braking system with microspheres might elucidate whether the increase in bond number with shear is the result of cellular deformability. Although the data presented here do not directly address bond formation rate, they suggest that cell deformation is a major determinant of rolling velocity under shear.

Although rolling velocity correlates better with WSR than WSS, this does not mean that rolling velocity is determined by free flow at hydrodynamic velocity between bonding events. Neither in the present study nor in earlier studies (9,16) did we find evidence for cells approaching hydrodynamic velocity at the time resolution used (30 frames/s). Observations of rolling leukocytes *in vivo* suggests that cells are in continuous adhesive contact with the underlying endothelium at all times. Instead, the dependence of rolling velocity on WSR independent of viscosity is likely caused by two effects: increased bond forma-

tion and breakage and decreased fluid drag force caused by increased cell deformation.

The present study does not explore the impact of adhesion receptor presentation, cytoskeletal anchoring, proteolytic adhesion molecule removal, or integrins on leukocyte rolling. These phenomena are likely to further modulate rolling velocity. However, our observations help explain why rolling velocities *in vivo* are relatively constant, even at high WSS (2,13), despite an exponential increase in bond dissociation rate with increasing WSS (8). Cell deformation is essential for stabilizing rolling velocities, as shown by the inability of rolling beads to maintain a constant rolling velocity at elevated WSS, thus identifying a major mechanism by which rolling velocity is kept almost constant *in vivo*.

## ACKNOWLEDGMENTS

We thank Michele Kirkpatrick for animal husbandry.

## REFERENCES

1. Arbones ML, Ord DC, Ley K, Ratech H, Maynard-Curry C, Otten G, Capon DJ, Tedder TF. (1994). Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity* 1:247–260.
2. Atherton A, Born GV. (1973). Relationship between the velocity of rolling granulocytes and that of the blood flow in venules. *J Physiol* 233:157–165.
3. Baez S. (1973). An open cremaster muscle preparation for the study of blood vessels by in vivo microscopy. *Microvasc Res* 5:384–394.
4. Bishop JJ, Popel AS, Intaglietta M, Johnson PC. (2001). Effects of erythrocyte aggregation and venous network geometry on red blood cell axial migration. *Am J Physiol* 281:H939–H950.
5. Brooks SB, Tozeren A. (1996). Flow past an array of cells that are adherent to the bottom plate of a flow channel. *Computers Fluids* 25:741–757.
6. Brunk DK, Hammer DA. (1997). Quantifying rolling adhesion with a cell-free assay: E-selectin and its carbohydrate ligands. *Biophys J* 72:2820–2833.
7. Chen S, Springer TA. (1999). An automatic braking system that stabilizes leukocyte rolling by an increase in selectin bond number with shear. *J Cell Biol* 144: 185–200.
8. Chen S, Springer TA. (2001). Selectin receptor-ligand bonds: formation limited by shear rate and dissociation governed by the Bell model. *Proc Natl Acad Sci USA* 98:950–955.
9. Damiano ER, Westheider J, Tozeren A, Ley K. (1996). Variation in the velocity, deformation, and adhesion energy density of leukocytes rolling within venules. *Circ Res* 79:1122–1130.
10. de Wit C, Schafer C, von Bismarck P, Bolz S, Pohl U. (1997). Elevation of plasma viscosity induces sustained NO-mediated dilation in the hamster cremaster microcirculation in vivo. *Proc Natl Acad Sci USA* 434:354–361.
11. Dembo M, Torney DC, Saxman K, Hammer DA. (1988). The reaction-limited kinetics of membrane-to-surface adhesion and detachment. *Proc R Soc Lond Biol Sci* 234:55–83.
12. DiVietro JA, Smith MJ, Smith BR, Petruzzelli L, Larson RS, Lawrence MB. (2001). Immobilized IL-8 triggers progressive activation of neutrophils rolling in vitro on P-selectin and intercellular adhesion molecule-1. *J Immunol* 167:4017–4025.
13. Firrell JC, Lipowsky HH. (1989). Leukocyte margination and deformation in mesenteric venules of rat. *Am J Physiol* 256:H1667–H1674.
14. Glantz SA. (1997). How to test for trends. In: *Primer of Bio-Statistics*. McGraw-Hill, New York. 213–281.
15. Greenberg AW, Brunk DK, Hammer DA. (2000). Cell-free rolling mediated by L-selectin and sialyl lewis(x) reveals the shear threshold effect. *Biophys J* 79:2391–2402.
16. Hafezi-Moghadam A, Thomas KL, Prorock AJ, Huo Y, Ley K. (2001). L-selectin shedding regulates leukocyte recruitment. *J Exp Med* 193:863–872.
17. Hodges SR, Jensen OE. (2002). Spreading and peeling dynamics in a model of cell adhesion. *J Fluid Mech* 460:381–409.
18. House SD, Lipowsky HH. (1988). In vivo determination of the force of leukocyte-endothelium adhesion in the mesenteric microvasculature of the cat. *Circ Res* 63:658–668.
19. Jung U, Ley K. (1997). Regulation of E-Selectin, P-Selectin, and Intercellular Adhesion Molecule 1 expression in mouse cremaster muscle vasculature. *Microcirculation* 4:311–319.
20. Kaplanski G, Farnarier C, Tissot O, Pierres A, Benoliel AM, Alessi MC, Kaplanski S, Bongrand P. (1993). Granulocyte-endothelium initial adhesion. Analysis of transient binding events mediated by E-selectin in a laminar shear flow. *Biophys J* 64:1922–1933.
21. Lawrence MB, Kansas GS, Kunkel EJ, Ley K. (1997). Threshold levels of fluid shear promote leukocyte adhesion through selectins (CD62L,P,E). *J Cell Biol* 136:717–727.
22. Lawrence MB, Springer TA. (1993). Neutrophils roll on E-selectin. *J Immunol* 151:6338–6346.
23. Lei X, Lawrence MB, Dong C. (1999). Influence of cell deformation on leukocyte rolling adhesion in shear flow. *J Biomech Eng* 121:636–643.
24. Ley K. (2001). Pathways and bottlenecks in the web of inflammatory adhesion molecules and chemoattractants. *Immunol Res* 24:87–95.
25. Ley K, Bullard DC, Arbones ML, Bosse R, Vestweber D, Tedder TF, Beaudet AL. (1995). Sequential contribution of L- and P-selectin to leukocyte rolling in vivo. *J Exp Med* 181:669–675.

26. Ley K, Gaehtgens P. (1991). Endothelial, not hemodynamic, differences are responsible for preferential leukocyte rolling in rat mesenteric venules. *Circ Res* 69:1034–1041.
27. Lipowsky HH, Scott DA, Cartmell JS. (1996). Leukocyte rolling velocity and its relation to leukocyte-endothelium adhesion and cell deformability. *Am J Physiol* 270:H1371–H1380.
28. Lipowsky HH, Zweifach BW. (1978). Application of the "two-slit" photometric technique to the measurement of microvascular volumetric flow rates. *Microvasc Res* 15:93–101.
29. Moore KL, Stults NL, Diaz S, Smith DF, Cummings RD, Varki A, McEver RP. (1992). Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. *J Cell Biol* 118:445–456.
30. Park EY, Smith MJ, Stropp ES, Snapp KR, DiVietro JA, Walker WF, Schmidtke DW, Diamond SL, Lawrence MB. (2002). Comparison of PSGL-1 microbead and neutrophil rolling: microvillus elongation stabilizes P-selectin bond clusters. *Biophys J* 82: 1835–1847.
31. Pries AR, Secomb TW, Gaehtgens P. (1996). Biophysical aspects of blood flow in the microvasculature. *Cardiovasc Res* 32:654–667.
32. Rinker KD, Prabhakar V, Truskey GA. (2001). Effect of contact time and force on monocyte adhesion to vascular endothelium. *Biophys J* 80:1722–1732.
33. Schmid-Schoenbein GW, Fung YC, Zweifach BW. (1975). Vascular endothelium-leukocyte interaction; sticking shear force in venules. *Circ Res* 36:173–184.
34. Smith MJ, Berg EL, Lawrence MB. (1999). A direct comparison of selectin-mediated transient, adhesive events using high temporal resolution. *Biophys J* 77: 3371–3383.
35. Soutani M, Suzuki Y, Tateishi N, Maeda N. (1995). Quantitative evaluation of flow dynamics of erythrocytes in microvessels: influence of erythrocyte aggregation. *Am J Physiol* 268:H1959–H1965.
36. Sugihara-Seki M. (2000). Flow around cells adhered to a microvessel wall. I. Fluid stresses and forces acting on the cells. *Biorheology* 37:341–359.
37. Tangelder GJ, Slaaf DW, Arts T, Reneman RS. (1988). Wall shear rate in arterioles in vivo: least estimates from platelet velocity profiles. *Am J Physiol* 254:H1059–H1064.
38. Taylor AD, Neelamegham S, Hellums JD, Smith CW, Simon SI. (1996). Molecular dynamics of the transition from L-selectin- to β2-integrin-dependent neutrophil adhesion under defined hydrodynamic shear. *Biophys J* 71:3488–3500.
39. Zhao Y, Chien S, Skalak R. (1995). A stochastic model of leukocyte rolling. *Biophys J* 69:1309–1320.