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The kinetics and shear threshold of transient and rolling interactions of L-selectin with its ligand on leukocytes

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ABSTRACT **The kinetics of rolling and transient adhesions through selectins may depend on the kinetics and mechanical properties of the selectin:ligand bond, as well as on cellular properties including receptor-anchoring to the cell membrane and cytoskeleton. Kinetics are known to depend on the selectin and may also be ligand dependent. Here, we study the kinetics of transient and rolling interactions of leukocytes with L-selectin immobilized on a substrate. Remarkably, all properties examined are similar to those seen when the sidedness is opposite, i.e., when the L-selectin ligand is on the substrate and when the ligand is isolated from HEV rather than present on leukocytes. The similar properties include rolling velocity, a threshold shear stress above 0.4 dyn/cm^2 required to support rolling, a k°_{off} of 7.0 to 6.8 s^{-1} for the L-selectin tether bond, and a mechanical bond length of 0.24 to 0.20 Å. Our results argue against a model in which L-selectin shedding mediates rolling. Furthermore, the fast and force-resistant kinetic properties suggest that L-selectin is specialized dynamically for tethering leukocytes to vessel walls and adherent leukocytes.**

L-selectin is an adhesive glycoprotein on the surface of leukocytes that is well known to bind to carbohydrate ligands expressed on high endothelial venules (HEV) and to function in lymphocyte homing (1–3). The ligand on HEV has been termed peripheral node addressin (PNAd). More recently, L-selectin has been shown to function in interactions between resting leukocytes in shear flow and leukocytes adherent to a variety of substrates (4–8). These interleukocyte interactions can mediate leukocyte-on-leukocyte rolling (4–6, 9) and can augment leukocyte accumulation on substrates bearing purified adhesion molecules (7, 8). The ligand for L-selectin on leukocytes is sialylated and fucosylated (4, 10) and can mediate rolling of leukocytes on substrates bearing purified L-selectin (10). Counterintuitively, shear forces above a certain level are required for stabilizing rolling of L-selectin-bearing leukocytes on substrates bearing PNAd, leukocyte-on-leukocyte rolling, and accumulation in strings (6, 7, 11).

The velocity of rolling cells, and the kinetics of transient tether dissociation, have been compared on substrates bearing E-selectin, P-selectin, and PNAd (11–14). When these are present on a substrate at concentrations too low to support rolling, they support transient cellular binding to the substrate, i.e., transient tethers that have kinetic properties suggestive of single receptor-ligand bonds. Leukocytes roll with a jerky motion, and evidence suggests that multiple tethers to the substrate are present at any one time and that tether dissociation corresponds to a jerky step in rolling. Measurements on L-selectin binding to a purified ligand from HEV show a k_{off} of ≥ 10 s^{-1} (15), which is comparable to the k_{off} in the absence of applied force, i.e., k°_{off} of 6.8 s^{-1}, estimated from transient tethers (13, 16).

The rapidity of leukocyte rolling correlates with the kinetics of transient tether dissociation; both are markedly faster on PNAd than on E-selectin or P-selectin, suggesting that bond dissociation kinetics may be an important determinant of rolling velocity (13). Could the geometry of the system, i.e., the presence of L-selectin on the rolling leukocyte and E-selectin and P-selectin on the substrate, contribute to these differences? Here, comparisons between L-selectin and P-selectin when both are present on the substrate address this question. Evidence has been presented that the kinetics of transient tethers correspond to receptor:ligand dissociation rather than to alternative mechanisms such as receptor or ligand uprooting from cells (13, 14, 16). However, further evidence to distinguish receptor-ligand dissociation from uprooting would be desirable. In our system, purified molecules are adsorbed irreversibly to one wall of a flow chamber, and, thus, uprooting can only occur from the surface of the rolling cell. Uprooting would depend on the mechanical strength of linkages to the membrane and cytoskeleton and hence should vary for different cell surface molecules. Thus, the uprooting hypothesis would suggest that kinetics of rolling and transient tethers could vary depending on whether PNAd or L-selectin was present on the substrate and thus whether L-selectin or the ligand for L-selectin on leukocytes, respectively, was being uprooted. PNAd also differs from the leukocyte ligand for L-selectin in sulfation, as shown by reactivity only with PNAd of mAb MECA-79, and the different structure of the carbohydrate ligand also could alter kinetics. An alternative mechanism to bond dissociation has been proposed to explain the rapid kinetics of L-selectin rolling: proteolytic shedding or release of L-selectin from the cell surface (17). L-selectin has a well defined cleavage site in the extracellular linker to the membrane and is cleaved by a membrane-associated metalloproteinase (17–20). Cleavage occurs in cis but not in trans; thus, this mechanism would not be operable for rolling of leukocytes on L-selectin adsorbed to a substrate (19, 20). Here, we examine kinetics and mechanical properties for leukocyte rolling and transient tethers on L-selectin substrates. These properties differ from those on P-selectin substrates examined in parallel but are remarkably similar to those previously measured on PNAd (13, 16).

MATERIALS AND METHODS

Antibodies and Reagents. Purified L-selectin mAbs DREG-56 and DREG-200 (21) were provided by T. K. Kishimoto (Boehringer Ingelheim). Fucoidin (22), Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution, and n-octyl-β-D-

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Abbreviations: HEV, high endothelial venules; PNAd, peripheral node addressin.

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glucopyranoside (octyl glucoside) were from Sigma. Human serum albumin (Fraction V) was from Calbiochem.

Blood Cell Isolation. Peripheral blood granulocytes were isolated as described (13). Cells were maintained at 4°C at 10^7 cells/ml in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution, supplemented with 10 mM Hepes (pH 7.4) (H/H medium) and diluted into binding medium at 2×10^6 cells/ml at 24°C immediately before use in flow assays. Binding medium was H/H medium containing 2 mM Ca^{2+} and 2 mg/ml human serum albumin.

Purification of Native L-Selectin and Preparation of Selectin Substrates. Human L-selectin was immunoaffinity purified from detergent lysates of peripheral blood lymphocytes by using DREG200 Sepharose (23). Purity was confirmed by SDS/PAGE and silver stain (not shown). Concentrated L-selectin was stored in PBS containing 1% octyl-glucoside at 4°C. For coating onto a substrate, selectin concentrate was diluted to a final concentration of 0.15 to 2 μg/ml in Tris-saline-azide (20 mM Tris·NaCl/0.03% NaN3, pH 8.0) and immediately was adsorbed to a polystyrene dish overnight at 4°C. Immunoaffinity purified platelet P-selectin (24) solubilized in octyl-glucoside (a generous gift of R. McEver, University of Oklahoma) was diluted in PBS buffered with 10 mM bicarbonate (pH 8.0) at 0.5 μg/ml and was similarity spotted on the polystyrene plate. Plates were blocked with 2% human serum albumin for 2 hr at room temperature before use.

Cell or Substrate Treatments. For blocking L-selectin, mAb DREG-56 was perfused at 10 μg/ml in binding medium into the flow chamber and was allowed to interact for 5 min at room temperature with the L-selectin substrate. For partial blocking of L-selectin adsorbed on the substrate, the soluble ligand fucoidin was perfused at 5 μg/ml shortly before perfusion of cells. Fucoidin was also present in the leukocyte suspension during the entire experiment. To remove L-selectin ligands from the leukocyte surface, cells were incubated with 0.1 units/ml Vibrio cholera neuraminidase (Calbiochem) for 30 min at 25°C in H/H medium with 2 mM Ca^{2+}. Cleavage was terminated by washing the cells twice with H/H medium with 5 mM EDTA, and cells were resuspended in binding medium and immediately were perfused into the chamber. For comparisons of effects of L-selectin blocking or shear stresses, identical fields of view were used to ensure that the results reflected uniform site density and distribution of the immobilized selectins.

Cell Accumulation in Shear Flow. The polystyrene slide on which adhesion molecules were adsorbed was incorporated as the lower wall in a parallel wall flow chamber and was mounted on the stage of an inverted phase-contrast microscope (25, 26). A 1-ml volume of cell suspension (2×10^6 cells/ml) in binding medium was perfused through the flow chamber with an automated syringe pump (Harvard Apparatus) attached to the outlet side, and the cells interacting with the field of view (with a ×20 and ×40 objective the field of view was 0.1 and 0.025 mm^2, respectively) during flow were quantitated by analysis of images videotaped with a TEC-470 charge-coupled device video camera (Optronics International, Goleta, CA) and Hi 8 Sony CVD-1000 recorder. To avoid modulation of tethering events by adherent cells upstream from the field of view, the observation field was located at the upstream edge of the spot of adsorbed protein and included a portion of the unadsorbed surface to which no cell binding occurred. At the end of each observation period, residual adherent cells were removed by perfusion with H/H medium with 5 mM EDTA.

Shear Resistance and Rolling Velocity Measurements. Detachment assays were performed on cells after they had bound to the substrate for 60 sec at 1.0 dyn/cm^2. The shear flow was increased every 10 sec to a maximum of 35 dyn/cm^2, in 1.5- to 2-fold increments, and the number of cells remaining bound at the end of each 10-sec interval was determined. Rolling velocities were measured by following cell displacements over 5- to 10-sec intervals as described (25).

Neutrophil Micromotion on Selectin Substrates. Motions of neutrophils were analyzed by determination of cell center positions in successive video frames. Coordinates of cell centers were determined within 0.7 μm for images from a 40× objective, and the instantaneous velocities between each frame were calculated for individual cells.

Determination of Rolling, Stationary, and Free-Flowing Neutrophils. Cells were allowed to sediment in the flowstream at 0.20 or 0.25 dyn/cm^2 for 10 sec and were subjected to a shear increase to 2 or 5 dyn/cm^2. A cell was defined as freely moving if it moved in the focal plane at the hydrodynamic velocity, i.e., a velocity identical to that of a cell perfused in the presence of EDTA. A rolling leukocyte was defined as a cell moving on the substrate for a period of at least 5 sec at a velocity of at least 1 μm/sec but not higher than one-fifth of the hydrodynamic velocity. A leukocyte was defined as stationary or slowly rolling if it remained bound to the substrate for at least 5 sec at its initial contact point or within a distance of <3 μm from it.

Determination of Dissociation Rate Constants from Transient Tethering Events. Transient tethers were defined as cell attachment events separated by at least 50 μm of motion at the hydrodynamic velocity and when no cell motion (<1 μm displacement) occurred while the cell was tethered to the immobilized ligand (14).

The duration of transient tethers on L-selectin was calculated by a computerized imaging system developed for quantitative tethering analysis to determine the integral number of frames during which cells were motionless (27). The imaging system has the resolution of 0.033 sec and $\approx$0.2-μm position resolution for cell images from a ×20 objective. Sufficient videotape was analyzed (30 to 90 sec) to obtain 30 to 200 tethering events, and the natural log of the number of cells that remained bound as a function of time after initiation of tethering was plotted.

RESULTS

Neutrophils Roll on L-Selectin 5- to 10-Fold Faster than on P-Selectin. Neutrophil rolling was compared on lymphocyte-derived L-selectin and platelet-derived P-selectin coated on polystyrene substrates (Fig. 1). Optimal levels of rolling adhesions were obtained on substrates coated with input concentrations of 2 μg/ml of purified L-selectin or 0.5 μg/ml of purified P-selectin. On both substrates, essentially all neutrophils established continuous rolling immediately after tethering in physiological shear stresses of 1–3 dyn/cm^2 (not shown). Tethered neutrophils also nucleated formation of strings of rolling cells on both substrates (7, 8). The extent of primary tethers on the 2-μg/ml L-selectin substrate (i.e., tethers mediated by direct leukocyte binding to the substrate, excluding cells tethered downstream of previously adherent cells) was $\approx$2-fold higher than on the 0.5-μg/ml P-selectin substrate at 1–3 dyn/cm^2 (data not shown). The 2-μg/ml L-selectin substrate supported a higher strength of polymorphonuclear leukocyte adhesion than the 0.5 μg/ml P-selectin substrate as shown by the higher fraction of cells resisting detachment or rapid clearance from the field of view at increased shear flows (Fig. 1*A*). Despite this greater shear resistance, leukocytes rolled on L-selectin at 8- to 10-fold faster velocities than on P-selectin (Fig. 1*B*). As with other selectins or ligands, reducing the density of immobilized L-selectin was associated with a reduction of shear resistance and concomitant increase of rolling velocity. Cells rolled 2.5- to 3-fold faster on substrates coated with 2 μg/ml than 5 μg/ml L-selectin (not shown). L-selectin coated at 0.3 μg/ml supported low efficiency tethering followed by very jerky, fast rolling that was poorly shear-resistant (Fig. 1). Neutrophils completely failed to establish rolling after tethering at input concentrations of 0.15

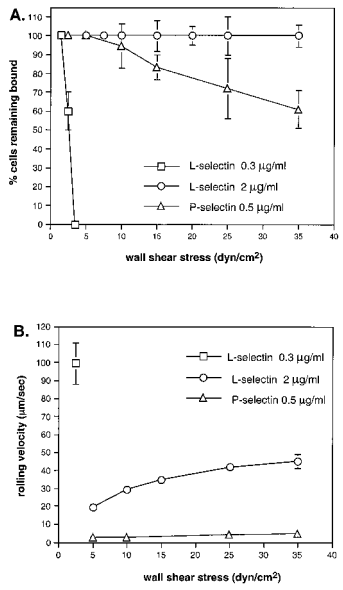

FIG. 1. Fast neutrophil rolling on L-selectin compared with P-selectin. (*A*) Resistance to detachment by increasing wall shear stress. (*B*) Rolling velocity. Selectins were coated at the indicated concentrations directly on polystyrene. Leukocytes were allowed to accumulate at 1.5 dyn/cm^2, shear then was increased every 10 sec as indicated, and the number of cells remaining bound was determined at the end of each interval. Mean rolling velocities (±SEM) of 10–15 neutrophils were determined at each wall shear stress. Error bars smaller than 10% of mean values were not plotted.

μg/ml or lower, and all tethering events on these substrates were transient. Neutrophil transient tethering to low density L-selectin was highly specific because it was inhibited completely by pretreatment of the substrates with L-selectin mAb DREG-56 and by inclusion of EDTA or soluble fucoidan ($\geq$10 μg/ml) in the perfusion medium. Neuraminidase treatment of neutrophils abolished all rolling adhesions on both selectin substrates (not shown).

The Jerkiness of Rolling on L-Selectin. Frame-by-frame analysis of neutrophil micromotions on selectin substrates at medium shear stresses showed that rolling on L-selectin is very jerky (Fig. 2*A*). Rolling consisted of multiple pauses separated by short fast forward motions, i.e., jerks, in the direction of flow. By contrast, pauses between jerks are prolonged markedly more on P-selectin (Fig. 2*B*). The average pause time on L-selectin was 8-fold shorter than on P-selectin (0.052 vs. 0.43 sec at 5 dyn/cm^2). Correspondingly, the frequency of pauses during rolling was higher on L-selectin than on P-selectin. In contrast, the average time spent by neutrophils per movement between successive pauses on L- and P-selectin was similar (Fig. 2). The major difference between L- and P-selectin-mediated rolling was, therefore, the shorter pause duration. Thus, although the strength of adhesion and the frequency of pauses supported by L-selectin was higher than by P-selectin, L-selectin tethers supported order of magnitude shorter pauses than P-selectin tethers during rolling. The direct outcome of these shorter pauses was markedly faster rolling velocities on L-selectin.

Neutrophil Adhesion to Purified L-Selectin Requires a Shear Threshold. Neutrophils failed to roll on L-selectin-coated substrates below a shear stress threshold of 0.3 dyn/cm^2 (Fig. 3*A*). Below 0.3 dyn/cm^2, neutrophils moved over the L-selectin substrate at near the hydrodynamic velocity, measured on identical substrates in the presence of EDTA. A sharp increase in the number of stably rolling cells was observed above 0.4 dyn/cm^2 (Fig. 3*A*) in striking similarity to the shear dependence of L-selectin-dependent rolling of neutrophils on

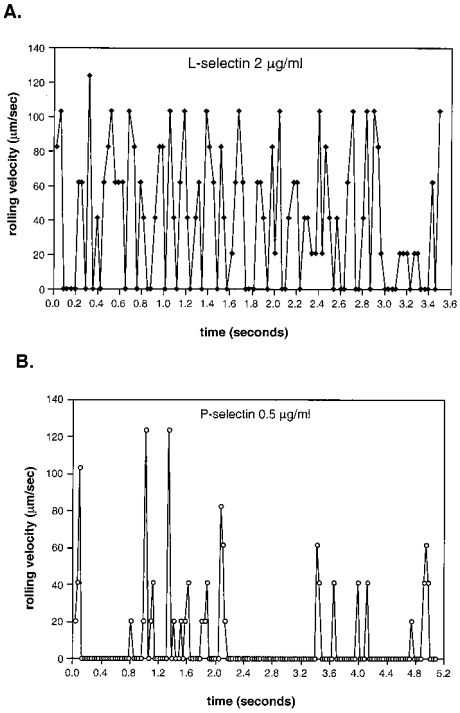

FIG. 2. Rolling on L-selectin is jerkier than on P-selectin and consists of frequent short pauses. Velocities were determined between each video frame for representative neutrophils rolling at 5 dyn/cm^2 on L-selectin (*A*) or P-selectin (*B*) from the same experiment as shown in Fig. 1. Velocities were calculated from cell displacement between each video frame; a ×40 microscope objective was used. The hydrodynamic velocity was 1,500 ± 120 μm/sec.

PNAd (6). When cells were settled at stasis for 10 sec and then were subjected to low shear stresses ranging between 0.15 and 0.25 dyn/cm^2, none of the settled cells remained bound to the L-selectin substrate. Essentially all of the cells similarly settled on the P-selectin substrate remained bound when subjected to low shear flow.

We studied the micromotion of neutrophils that were moving on L-selectin at a shear stress just below the threshold required for rolling and then were subjected to an abrupt increase in shear. Cells were allowed to sediment in the flow stream at 0.20 or 0.25 dyn/cm^2 so that they were close to the substrate. On the L-selectin substrate, cells moved at the hydrodynamic velocity at these subthreshold shear stresses (Fig. 3*B*). Within 0.2 sec of an increase in shear to 5 dyn/cm^2, >95% of the cells near the substrate tethered and rolled (Fig. 3*B*). They rolled at the high shear stress with an average velocity slower than their velocity at the low shear stress (Fig. 3*C*). In contrast, cells were rollingly adherent on P-selectin at 0.25 dyn/cm^2 (Fig. 3*B*). They exhibited only an increase in rolling velocity when shear was increased.

The Kinetics of Dissociation of Transient Tethers on L-Selectin. We measured the duration of transient tethers on two sets of substrates. The first substrate bearing L-selectin coated at 0.3 μg/ml was able to support jerky rolling of a minority of the tethered neutrophils within a narrow shear range (0.75–2.5 dyn/cm^2), but most neutrophils were tethered transiently. A set of measurements was performed on this substrate in the presence of subsaturating levels (5 μg/ml) of the soluble L-selectin inhibitor, fucoidin, which blocked 60–70% of all transient tethering events and all of the rolling interactions on this substrate. On a second, lower-density substrate coated at 0.15 μg/ml, all neutrophils were tethered transiently at all shears tested; i.e., there were no rolling interactions in the presence or absence of fucoidin. Tethering

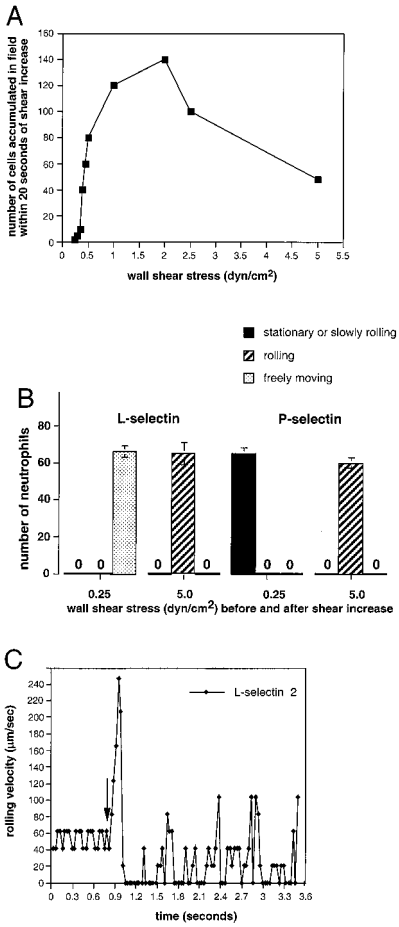

FIG. 3. Neutrophils fail to accumulate or roll on L-selectin at low shear. (*A*) Neutrophil accumulation on L-selectin at different shear stresses. Cells were allowed to sediment in the flow stream for 10 sec at 0.25 dyn/cm^2 on a substrate coated with L-selectin at 2 μg/ml to allow similar number of cells to come into direct contact with the substrate. Shear then was increased abruptly to the indicated values, cells were allowed to accumulate for 20 sec, and the number of adherent cells that accumulated was counted. (*B*) Tethering of neutrophils after an abrupt increase in shear. Neutrophils were allowed to sediment during flow at 0.25 dyn/cm^2 on substrates coated with 2 μg/ml L-selectin or 0.5 μg/ml P-selectin and then were subjected to a sharp increase in wall shear stress to 5 dyn/cm^2. The number of cells in each of three classes of interaction with the substrate were scored as described in *Materials and Methods* at the low and high shear stresses immediately before and after the increase in shear flow, respectively. New cells arriving from outside the field of view upstream at the high shear stress were ignored. Values are mean of two fields of view. Results in *A* and *B* are representative of five experiments. (*C*) Velocity between each video frame of a representative neutrophil perfused at 0.2 dyn/cm^2 over the same substrate used in *B* and then subjected to an increase in shear to 5 dyn/cm^2 at $t = 0.8$ sec, marked by the arrow. The cell is nonadherent from 0 to 1 sec and is rollingly adherent after 1 sec. Instantaneous velocities were calculated as in Fig. 2.

events in both experimental conditions were specific, because they were abolished entirely by including EDTA in the binding medium or by pretreatment of the substrate with function-blocking L-selectin mAb DREG 56. The durations of transient tethers were measured for large numbers of individual cells by digitized image analysis with a single frame resolution limit of 0.033 sec. Synchronizing the initiation of tethering to $t = 0$, >90% of transiently tethered neutrophils in the two experimental conditions dissociated from the substrates with first-order kinetics (Fig. 4). A variable, small proportion of cells (3 of 90 cells in Fig. 4) dissociated more slowly, perhaps because of multivalent or nonspecific interactions. Consistently, brief treatment of neutrophils with a low concentration of neuraminidase to desialylate partially functional L-selectin ligands reduced the frequency of tethering to L-selectin substrates by 60% but had no significant effect on k_{off} (23.1 vs. 21.1 s^{-1} at 1.5 dyn/cm^2).

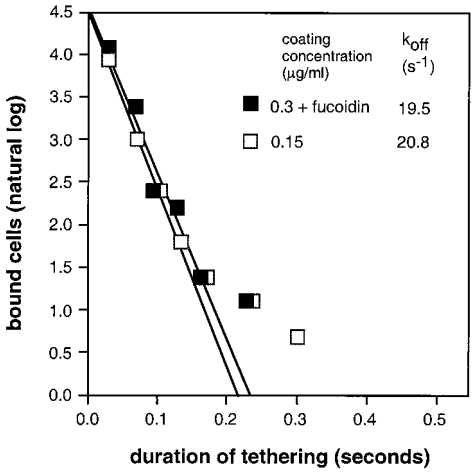

FIG. 4. Transiently tethered polymorphonuclear leukocytes dissociate from low-density L-selectin substrates with first order kinetics. Representative data at 1.5 dyn/cm^2 from the two types of experimental conditions was used for collection of transient tether data, i.e., with a coating concentration of L-selectin at 0.15 μg/ml or with a coating concentration of L-selectin of 0.3 μg/ml and with tethers measured in the presence of 5 μg/ml of fucoidin. Frequency of tethering at 1.5 dyn/cm^2 was 0.056 and 0.017 events cell^{-1}·mm^{-1} on the L-selectin substrates coated with 0.3 μg/ml and 0.15 μg/ml, respectively.

The Mechanical Stability of the L-Selectin Tether. We examined the effect of increasing force on the tether bond, which is proportional to wall shear stress, on the kinetics of tether dissociation. Kinetics were measured at different shear stresses on the substrate with L-selectin coated at 0.15 μg/ml or on the substrate with L-selectin coated at 0.3 μg/ml in the presence of partially blocking fucoidin. In both cases, transient tethering but no rolling was observed.

The rate constant for tether dissociation, k_{off}, increased with increasing wall shear stress, and there was no systematic difference between k_{off} measurements in the two different experimental conditions (Fig. 5). The susceptibility to force of the tether dissociation reaction is termed reactive compliance and is the inverse of mechanical stability. To obtain quantitative estimates of reactive compliance and k°_{off}, i.e., k_{off} in the absence of applied force, the data were fit to two theoretical relationships between k_{off} and force on the tether bond, F_b (lines in Fig. 5). The relationship between wall shear stress and force on the cell was estimated from Goldman's equation (28), and the relationship between force on the cell and force on the tether bond was estimated based on measurements on tethered cells (13, 14). The data fit the Bell equation (29) ($\chi^2 = 1.19$ with 9 degrees of freedom) better than a Hookean spring model (30) ($\chi^2 = 3.0$ with 9 degrees of freedom). The fit to the Bell model yields $k^\circ_{off} = 7.0 \pm 0.49$ s^{-1}, and $\sigma = 0.24 \pm 0.2$ Å. The σ is an estimate of the separation distance over which the receptor:ligand bond weakens but is not yet broken and may be termed the mechanical bond length.

DISCUSSION

We have examined the kinetics and other characteristics of the interaction between the carbohydrate ligand for L-selectin expressed on leukocytes and purified L-selectin adsorbed to the wall of a flow chamber. This interaction is opposite in directionality to that between L-selectin on leukocytes and peripheral node addressin adsorbed to a substrate (31–33). The MECA-79 antibody used to define PNAd recognizes a

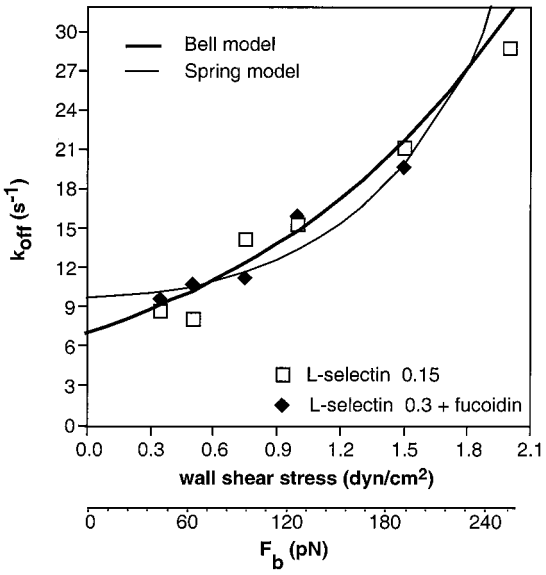

FIG. 5. The increased kinetics of L-selectin transient tether dissociation at increased wall shear stress and force on the tether bond. The k_{off} values at different shear stresses were determined from neutrophil first order dissociation rate constants measured as in Fig. 4. The thick line is the fit of Bell's equation to all experimental points: $k_{off} = k^\circ_{off} \exp(\sigma F_b/kT)$, where k°_{off} is the unstressed k_{off}, σ is the separation between receptor and ligand that weakens the bond enough to increase k_{off} by e, k is Boltzmann's constant, and T is the absolute temperature (29). The thin line is the fit to an Hookean spring model (13, 30): $k_{off} = k^\circ_{off} \exp(f_\kappa F_b/2\kappa kT)$, where κ is the spring constant for the tether bond and f_κ is the fraction of the bond spring constant devoted to bond dissociation, also known as the fractional spring slippage (30). This fit yields $k^\circ_{off} = 9.7 \pm 0.66$ sec^{-1}, $\kappa/f_\kappa = 6.31 \pm 0.96$ N/m. Data was fit by using the program IGOR (WaveMetrics, Lake Oswego, OR).

sulfation-dependent epitope that is expressed on HEV but not on leukocytes (34). Thus, the carbohydrate ligand for L-selectin expressed on leukocytes lacks this sulfation-dependent epitope. Despite these differences in the directionality of the interaction and sulfation of the carbohydrate ligand, we find that the characteristics of these two types of L-selectin interactions are quite similar and are markedly distinct from those through E-selectin and P-selectin. A portion of the carbohydrate ligand for L-selectin on neutrophils is expressed on PSGL-1, the P-selectin glycoprotein ligand (8), as is a portion of the carbohydrate ligand for E-selectin (35). Despite this similarity in recognition of carbohydrate ligands on neutrophils by all three selectins, and in the directionality of the interaction, the characteristics of rolling and transient tethers on L-selectin differed dramatically from those on E-selectin and P-selectin.

Rolling of leukocytes on L-selectin substrates is fast, similar to that of L-selectin-dependent leukocyte rolling on PNAd (31, 32), which is faster than rolling on P-selectin and E-selectin (12). Direct comparisons here showed that neutrophil rolling on L-selectin was faster than on P-selectin substrates, which supported rolling adhesions of comparable shear-resistance. The micromotions of neutrophils rolling on L-selectin substrates were strikingly different from those observed on P-selectin. On L-selectin there were frequent short pauses, separated by short movements of the tethered leukocyte forward in the direction of flow. Even on a P-selectin substrate supporting weaker adhesion than an L-selectin substrate and formed by using a lower concentration of selectin, pause durations were markedly longer. Pause times of leukocytes rolling at a representative shear stress of 5 dyn/cm^2 were 0.053 sec on L-selectin and 0.25 sec on P-selectin, corresponding to the faster rolling velocity on L-selectin of 19.5 ± 1.2 μm/sec than on P-selectin of 2.6 ± 0.3 μm/sec. Both velocities and jerkiness of rolling on L-selectin were reminiscent of L-selectin-mediated rolling of leukocytes on the endothelial L-selectin ligand PNAd (13). A shear stress above a threshold level of $\approx$0.4 dyn/cm^2 was required for stable rolling on L-selectin. This is similar to the threshold of $\approx$0.5 dyn/cm^2 required for rolling on PNAd (6, 11). We found that, when leukocytes were subjected to a rapid increase in shear from below to above the threshold, the transition from nonadherence to rolling on L-selectin substrates occurred within 0.2 s.

Transient tethers were observed on substrates where L-selectin was too sparse to support rolling, and >90% of these tethers dissociated with first order kinetics. These properties suggest that transient tethers on L-selectin may represent a quantal unit that underlies rolling and that this smallest detectable unit may represent a univalent bond, although this has not been proven directly. The properties of transient tethers have been discussed and examined in greater detail on P-selectin (7), E-selectin (13), and PNAd substrates (13), and transient tether kinetics measured on E-selectin substrates are within 2-fold of estimates based on micromotions of neutrophils rolling on activated endothelial monolayers (36) or E-selectin (37). Furthermore, transient tether dissociation kinetics for L-selectin binding to PNAd substrates are within 1.5-fold of those measured for L-selectin binding to GlyCAM-1 in BIAcore (15). The force on a cell near a wall in shear flow, and the relationship between the force on the cell and the force on the tether bond (7, 13), have been estimated and are proportional to wall shear stress. We found that, as force increased, the rate constant for transient tether dissociation increased. To estimate k_{off} in the absence of force, i.e., k°_{off}, and the mechanical property of how greatly force increases k_{off}, we fit the data to two theoretical predictions. The better fit was to the Bell model, in which k_{off} increases exponentially with force on the tether bond. We obtained $k^\circ_{off} = 7.0 \pm 0.49$ sec^{-1} for the dissociation of neutrophils bound through their carbohydrate ligand to L-selectin on the substrate. This is within experimental error of our estimate of $k^\circ_{off} = 6.8 \pm 0.2$ sec^{-1} for the dissociation of neutrophils bound through L-selectin to PNAd on a substrate (13). The similarities in k°_{off} are remarkable, given the difference in sulfation of the carbohydrate ligand and the opposite directionalities of the interactions. The similarities in k°_{off} suggest that, despite sulfation differences, the binding interface between L-selectin and the carbohydrate ligands on neutrophils and on HEV may be similar. Structural similarities between the neutrophil and endothelial ligands would be in agreement with the ability of L-selectin to recognize ligands on HEV that both express and lack the MECA-79 sulfation-dependent epitope (38). Furthermore, the similarities in k°_{off} strengthen the evidence that we are measuring properties intrinsic to ligand:receptor bonds because directionality would be expected to influence other properties; e.g., the carbohydrate ligands and L-selectin on neutrophils would be expected to differ in attachment to the cytoskeleton and membrane and hence in resistance to uprooting from the cell surface. By contrast to L-selectin, k°_{off} for P-selectin and E-selectin are 0.93 ± 0.15 sec^{-1} (13, 14) and 0.70 ± 0.05 sec^{-1} (13). The slower k°_{off} of P-selectin and E-selectin correlates with the slower rolling on these selectins and the longer duration of pauses during rolling. The faster k°_{off} of interactions through L-selectin in both directions correlates with the similarity in the fast speed of rolling on L-selectin and PNAd substrates and the considerably shorter duration of pauses on both substrates. This strengthens evidence that k°_{off} is an important determinant of rolling velocity (13).

The susceptibility of the k_{off} reaction to applied force was estimated with the exponential constant σ, which may be termed the mechanical bond length. Smaller σ corresponds to greater mechanical stability of the bond. The value of σ determined here for the interaction of the neutrophil ligand with L-selectin on a substrate is 0.24 ± 0.02 Å and is similar

to the σ for the interaction of neutrophil L-selectin with PNAd on the substrate of 0.20 ± 0.01 Å (13). By contrast, these values are considerably lower than the σ values for P-selectin and E-selectin of 0.40 ± 0.08 Å and 0.31 ± 0.02 Å, respectively (13, 14). Furthermore, transient tethers through a mAb to Lewis^x (CD15) have a $\sigma = 0.86 \pm 0.06$ Å (27). Therefore, of all tether bonds thus far examined, those through L-selectin have the shortest mechanical bond length, i.e., are the most resistant to applied force. The similarities in mechanical bond length for the interaction of L-selectin with two different types of ligands suggest that high mechanical strength may be a general feature of L-selectin interaction with its biological ligands. High resistance to force is biologically important to the ability of L-selectin to initiate interactions of cells in flow to the vessel wall or adherent leukocytes because the force applied per bond will be highest on the first bond to form.

The present study shows that the transient nature of L-selectin-mediated rolling and the kinetics and mechanical properties of transient tethers are conserved whether L-selectin is present on the rolling leukocyte or on the substrate. Furthermore, cell-free L-selectin interacting with cell-surface ligands requires a shear threshold for rolling nearly identical to that of L-selectin on leukocytes interacting with cell-free ligands. This shows that properties of L-selectin rolling adhesions are independent of the sidedness or distribution of the selectin. Recently, fast rolling through L-selectin on PNAd on a substrate was attributed to proteolytic shedding by a cell-surface metalloprotease because an inhibitor of L-selectin shedding was shown to slow neutrophil rolling on PNAd but not on P-selectin (17). L-selectin is cleaved by a cell-associated protease at a specific site close to its transmembrane domain (18). It has been shown that this protease can act in cis on L-selectin on the same cell but not in trans on L-selectin on other cells (19, 20). Therefore, the finding that purified L-selectin on a substrate can mediate rolling adhesions as fast as those mediated by cell-based L-selectin supports the argument that inherent properties of L-selectin tethers to glycoprotein ligands, rather than a cellular shedding machinery, account for its ability to mediate fast rolling.

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