The Faster Kinetics of L-Selectin Than of E-Selectin and P-Selectin Rolling at Comparable Binding Strength[1]

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Selectins are a family of lectins that mediate tethering and rolling of leukocytes on endothelium in vascular shear flow. To test the hypothesis that the kinetics and the strength of rolling interactions can be independently varied for different selectin:ligand pairs, we have directly compared all three selectins with regard to distinct measures of selectin-mediated interactions in shear flow: tethering, rolling velocity, and strength of rolling adhesions. At comparable site densities of E-selectin, P-selectin, and the L-selectin counter-receptor CD34, neutrophils tethered with similar efficiency and developed rolling adhesions of similar strength as measured by resistance to detachment. Under the same conditions, neutrophils rolled 7.5- to 10.5-fold faster on CD34 than on E-selectin and P-selectin. These findings suggest that the kinetics of bond dissociation and bond formation are faster for L-selectin than for E- and P-selectin. We also compared the behavior of neutrophils and lymphocytes on the same selectin. Both cell types showed comparable strength of binding to CD34; however, neutrophils rolled with faster velocities than lymphocytes. ***The Journal of Immunology,*** **1997, 158: 405–413.**

The selectins are a family of Ca^{2+}-dependent membrane-bound lectins that initiate the adhesion of leukocytes to endothelial cells, platelets, or other leukocytes on the vessel wall under the shear forces found in postcapillary venules (1–3). L-selectin is expressed on leukocytes and binds to ligands expressed on high endothelial venules (HEV)[3] of secondary lymphoid tissues and on certain types of leukocytes (4). P-selectin and E-selectin can be induced on endothelial cells by inflammatory mediators, and both bind to carbohydrate ligands on myeloid cells and subsets of lymphocytes (1–3). The ligand for L-selectin on HEV is present on a mixture of mucin-like glycoproteins, including CD34 (5), that bear the MECA-79 epitope and are collectively referred to as peripheral node addressin (PNAd) (6–8). PNAd (9), appropriately glycosylated CD34 (10), and MAdCAM-1 (11) have been shown to support L-selectin-mediated tethering and rolling of leukocytes under shear flow conditions. P-selectin (12) and E-selectin (13–15) incorporated into planar lipid bilayers, coated onto plastic, or on the endothelial cell surface also support neutrophil rolling. P-selectin glycoprotein ligand (PSGL-1) (16–18) and E-selectin ligand (19) bear selectin ligands on myeloid cells. PSGL-1 is also the major counter-receptor for P-selectin on stimulated T cells (20, 21). PSGL-1 supports leukocyte attachment and rolling on P-selectin (22). Both L-selectin (23–26) and PSGL-1 (22) are concentrated on the tips of microvilli or microfolds on the neutrophil surface, and exclusion of L-selectin from these sites impairs initial cell tethering at high, but not at low, shear flow without affecting rolling (27).

Selectin-ligand bonds have been hypothesized to have rapid association and dissociation rate constants (12) by virtue of which they can mediate rolling interactions in the shear flow of the microcirculation. A rapid association rate of the selectins may facilitate the initial tethering of leukocytes in shear flow to the vessel wall, whereas both rapid dissociation and association rates are likely to be important for the maintenance of rolling interactions. Recent analysis of transient binding events on substrates with low densities of purified P-selectin in laminar shear flow or of pauses in rolling on endothelial monolayers expressing E-selectin have suggested selectin-ligand bond dissociation rates of 1 s^{-1} for P-selectin (28) and 0.5 s^{-1} for E-selectin (29).

Studies of the dynamics of selectins are important to test hypotheses about the molecular mechanisms that regulate rolling velocity, the strength of rolling adhesions, and the rate of tethering in hydrodynamic flow. Specifically, the hypothesis that rolling velocity and the strength of rolling adhesions are regulated by distinct properties of selectin:ligand bonds, such as kinetics and equilibria, respectively, would be supported if comparisons among selectins demonstrated that rolling velocities differed significantly when the strength of rolling adhesions was the same. Neutrophil interactions have been studied with defined densities of P-selectin (12) and E-selectin (13) under defined shear flow conditions. In a recent study, neutrophils were shown to bind more efficiently and roll at twofold lower velocities on P-selectin than on E-selectin when the proteins were immobilized on plastic or expressed on CHO cells (30). Previous studies on L-selectin have been limited to one rolling velocity measurement of L-selectin transfectants on one undefined density of MAdCAM-1 (11) and to rolling velocities and resistance to shear of neutrophils on one undefined density of PNAd (9). However, there were no comparisons to E-selectin and P-selectin in these studies and no studies on a range of defined densities of L-selectin ligands. These are important considerations, because all measures of selectin function are influenced by ligand density on the substrate. Increasing ligand density results in higher tethering rates, stronger rolling adhesions as measured by resistance to detachment, and slower rolling. In previous studies, binding to the substrate was defined as an overall accumulation of

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[3] Abbreviations used in this paper: HEV, high endothelial venules; PNAd, peripheral node addressin; MAdCAM-1, mucosal addressin cell adhesion molecule-1; PSGL-1, P-selectin glycoprotein ligand; CHO, Chinese hamster ovary; dyn, dyne; HSA, human serum albumin; TS, 25 mM Tris-HCl, 150 mM NaCl, pH 8.0; OG, octyl glucoside.

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neutrophils onto P-selectin or E-selectin under continuous shear flow conditions. However, the frequency of direct tethering to the substrate was not compared. Recently, neutrophils have been shown to express L-selectin ligands and to roll on adherent neutrophils on a substrate (4). Furthermore, the accumulation of neutrophils on selectin substrates is not a pure measure of tethering to the substrate as accumulation is facilitated by neutrophil-neutrophil interactions through L-selectin that nucleate subsequent rolling interactions on these substrates (31).

In this study, we directly compared the three selectins in tethering efficiency, velocity of rolling, and strength of rolling adhesions. These characteristics were compared at a range of densities of immobilized CD34 and E-selectin and at one comparable density of P-selectin. We show that neutrophils tether with similar efficiencies to CD34, E-selectin, and P-selectin when immobilized at comparable site densities, and that the strength of rolling adhesions is similar as measured by resistance to detachment. By contrast, rolling through L-selectin is markedly faster than that through P-selectin or E-selectin. We also compare the behaviors of neutrophils and lymphocytes on the three substrates and show some specific differences.

Materials and Methods

mAbs and cell lines

mAbs 581 (IgG1) and 547 (IgG2a, κ) to human CD34 (32, 33) were kindly provided by Dr. Gustav Gaudernack (Oslo, Norway). MECA-79 mAb (rat IgM, κ) (34) to PNAd and WAPS 12.2 mAb (35) to human P-selectin were gifts from Dr. Eugene Butcher (Stanford University, Stanford, CA). Purified Dreg-56 (IgG1) and Dreg-200 mAbs to L-selectin (36), BB11 (IgG2b) mAb to E-selectin (37), and G1 (IgG) mAb to P-selectin (38) were gifts from Drs. T. K. Kishimoto (Boehringer-Ingelheim, Ridgefield, CT), R. Lobb (Biogen, Cambridge, MA), and R. McEver (University of Oklahoma Health Sciences Center, Oklahoma City, OK), respectively, and were used for blocking selectin function. X63 (myeloma, IgG1) was used as a control at a 1/5 dilution of culture supernatant. CL-3 mAb to E-selectin (39) was purified using protein A-Sepharose from culture supernatants. SKW3 (acute T cell leukemia) cells and CHO cells stably transfected with full-length human E-selectin (37) (CHO-E) were maintained in RPMI 1640 and α-MEM medium, respectively, containing 10% fetal bovine serum.

Lymphocytes and neutrophils

Human neutrophils and lymphocytes were isolated from anticoagulated whole blood by centrifugation on Ficoll-Hypaque density gradient (40). The buoyant mononuclear cells were washed three times and incubated in 150-cm^2 culture flasks at 37°C twice for 30 min each time to allow monocytes to adhere firmly. Nonadherent lymphocytes (~90% T cells) were washed once and stored in HBSS (Ca^{2+}- and Mg^{2+}-free; Life Technologies, Grand Island, NY); 10 mM HEPES, pH 7.3; and 0.5% human serum albumin (HSA). Neutrophils were purified by hypotonic lysis of erythrocytes and stored in the above medium. Cells were washed with and suspended in H/H binding medium (HBSS, 2 mM Ca^{2+}, 0.2% HSA, and 10 mM HEPES, pH 7.3) before flow assays. Cells were treated for 30 min with or without *Vibrio cholera* neuraminidase (10 mU/ml) in the binding medium at room temperature, washed three times, and resuspended in the binding medium.

Preparation of substrates

PNAd (MECA-79 Ag) was purified from tonsil stromal lysates using sequential wheat-germ agglutinin-agarose (Sigma Chemical Co.) and immunoaffinity chromatography (6, 10). The CD34 component of PNAd was isolated with mAb-Sepharose, and its concentration was determined by capture ELISA (10). Recombinant full-length human E-selectin was purified from the detergent lysates of CHO cells transfected with full-length human E-selectin cDNA by immunoaffinity chromatography using E-selectin mAb BB11 coupled to Sepharose 4B (37), and purity was determined by SDS-PAGE. P-selectin purified from human platelets (41) was a gift from Dr. R. McEver. Liposomes containing 5 μg/ml of CD34; 0.5, 1, and 2 μg/ml of E-selectin; and 3 μg/ml of P-selectin were prepared by mixing 0.4 mM egg phosphatidylcholine (Avanti, Alabaster, AL) in 150 mM NaCl; 25 mM Tris-HCl, pH 8.0 (TS); and 2% (w/v) octyl glucoside (OG), with an equal volume of protein solution to give the indicated final concentration in TS and 1% OG; followed by extensive dialysis against TS at 4°C (12). Planar membranes containing CD34 and selectins were formed on cleaned glass slides (12), and nonspecific adhesion was blocked by incubating the plate with HBSS supplemented with 2% HSA for 30 min at room temperature. Incorporation of CD34 in vesicles appeared to be less efficient than that for P- and E-selectin, and CD34 in vesicles appeared unstable during storage at 4°C. Therefore, in most experiments CD34 was adsorbed to plastic, which required far less protein; the characteristics of interactions with leukocytes under flow appeared identical with CD34 in planar lipid bilayers or adsorbed to plastic.

Site density determination

mAbs WAPS 12.2 to P-selectin, CL-3 mAb to E-selectin, and 581 mAb to CD34 were iodinated to specific activities of 7.83, 6.84, and 7.5 μCi/μg, respectively, using Iodogen (Pierce Chemical Co., Rockford, IL). CD34 site densities at the concentrations used for flow experiments were determined as previously described (10). E-selectin and P-selectin bilayers were prepared in clean borosilicate glass culture tubes in a manner similar to that for those used on glass slides for adhesion assays. Following blocking with 1 mg/ml human Ig, 1% HSA in HBSS, and 10 mM HEPES, pH 7.4, the bilayers were incubated in saturating levels of iodinated P-selectin and E-selectin function-blocking Abs. Nonspecific binding was determined by inclusion of a 50-fold excess of unlabeled mAb. Unbound Ab was removed by extensive washing in HBSS/HEPES containing 1% HSA, and bound Ab was quantified by placing the glass tubes directly into a gamma counter. Site density was determined using the approximation of one mAb per selectin molecule. Site densities (mean sites per square micron ± SD for three measurements at each protein concentration) and the rounded values given in the text were: for E-selectin: 53.4 ± 6.8, 55; 78.7 ± 5.7, 80; and 182 ± 29.7, 180; for P-selectin: 87.8 ± 6.4, 90; and for CD34: 38 ± 7, 40; 50 ± 13, 50; 90 ± 12, 90; and 195 ± 27, 195.

Laminar flow assay

A glass slide with a supported phospholipid bilayer containing a selectin or polystyrene slide on which CD34 was adsorbed was assembled in a parallel plate laminar flow chamber (260-μm gap thickness) and mounted on the stage of an inverted phase contrast microscope (Diaphot-TMD, Nikon, Inc., Garden City, NY), as previously described (12). Cells resuspended at 5×10^5 cells/ml in H/H binding medium were perfused through the flow chamber at the desired shear stress. Tethering, resistance to detachment by shear forces, and rolling velocity were measured twice on different ×10 microscopic fields for each immobilized substrate. Cells were detached with 5 mM EDTA in H/H medium in between observations on different fields. Three or four independent substrates on different days were prepared; the data reported are the average and SD of results for three to four substrates unless stated otherwise.

Tethering in flow was defined as the number of nonadherent cells that tethered within the field of view and remained rollingly adherent for at least 5 s. Only those cells observed to tether directly to the substrate in the absence of prior contact with adherent cells were counted and defined here as primary tethers, and tethers that were formed after an interaction via adherent leukocytes were not scored. The number of cells that tethered in 1 min of continuous flow was measured at different wall shear stresses.

For detachment assays, cells were infused into the chamber at 0.84 dyn/cm^2 and allowed to tether for about 3 min until sufficient accumulation was reached. Nonadherent cells were cleared by perfusion with H/H binding medium at 0.84 dyn/cm^2 for 30 s. Subsequently, wall shear stress was increased every 10 s to a maximum of 36 dyn/cm^2 to generate a detachment force. The number of cells remaining bound was calculated as a percentage of the number of cells rolling on the substrate at 0.84 dyn/cm^2.

Rolling velocities were calculated as previously described (12) for 30 to 50 of the cells observed during detachment assays. Cell displacement was measured over 5- to 8-s intervals. Velocities were measured only for cells that remained adherent throughout the 10-s period during which a given shear was applied.

Inhibition with L-selectin mAb, X63 myeloma IgG control, fucoidan, and EDTA was determined as previously described (10). Inhibition with E-selectin and P-selectin or control mAbs was conducted by incubating selectin bilayers to which baseline tethering had already been measured with 10 μg/ml mAb for 20 min. All mAbs and inhibitors remained present during the adhesion assay. Identical fields of the immobilized substrate were monitored for the comparison of tethering, rolling velocity, and strength of rolling adhesions of different cell types.

Flow cytometric analysis

Flow cytometric analysis of human neutrophils and PBL was performed on a Becton-Dickinson FACScan (San Jose, CA) as previously described (42).

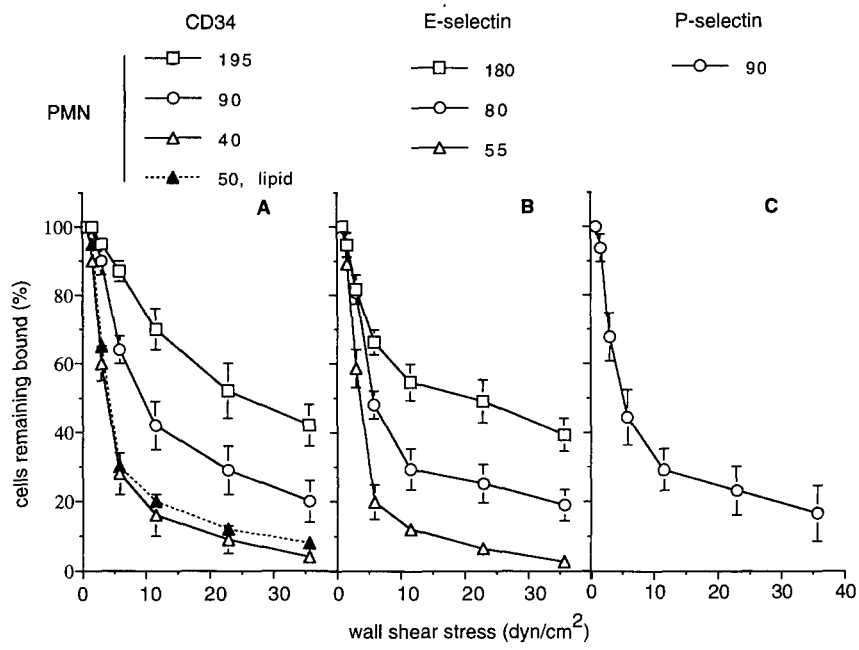

FIGURE 1. Resistance to detachment by shear of neutrophils on sialomucin CD34, E-selectin, and P-selectin. Neutrophils were allowed to tether at 0.84 dyn/cm^2 for 2 to 3 min at the indicated densities (sites per square microns) of CD34 (*A*), E-selectin (*B*), and P-selectin (*C*). The shear stress was then increased every 10 s to a maximum of 36 dyn/cm^2, and the percentage of cells remaining bound at each shear was determined. The data points represent the mean ± SD of the number of neutrophils that remained bound in a ×10 field in three experiments, each performed in duplicate, and one experiment performed in duplicate for CD34 in lipid monolayer.

Table I. *Comparisons of kinetics and strength of rolling adhesions*

Substrate	Site Density (sites/μm^2)	Rolling Velocity (μm/s) 0.84 dyn/cm^2	2.0 dyn/cm^2	36 dyn/cm^2	Tethered Cells Remaining Bound at 36 dyn/cm^2 (%)	Shear Stress for 50% Detachment (dyn/cm^2)	Tethering at 1.5 dyn/cm^2 (tethers/min/mm^2)
CD34	90	31 ± 4.2	38.2	99 ± 6	20 ± 6	9.65	66 ± 6
E-selectin	80	2.65 ± 1.2	3.85	12 ± 4	19 ± 4.5	5.62	40 ± 5
P-selectin	90	3.9 ± 2.4	4.85	12.73 ± 5	16.7 ± 8	5.15	56 ± 6

Cells (10^5) were stained with Dreg-56 (IgG) mAb against the lectin domain of human L-selectin. mAb X63 (myeloma, IgG) was used as the control. FITC-conjugated goat anti-mouse (IgG) was used as secondary Ab.

Results

Strength of neutrophil rolling adhesions on sialomucin CD34, E-selectin, and P-selectin

To determine the strength of selectin-mediated rolling adhesions, neutrophils were perfused on E-selectin-, P-selectin-, or CD34-containing substrates at 0.84 dyn/cm^2. After a sufficient number of cells had accumulated, they were subjected to progressively increasing wall shear stresses, and the percentage of rolling cells that remained adherent was determined (12). The hydrodynamic drag force on a leukocyte in shear flow near a wall can be calculated and is proportional to the wall shear stress (43). Several different site densities of CD34 and E-selectin and one of P-selectin were examined for neutrophil binding strength. Resistance to detachment is a measure of the strength of adhesion to a substrate (44). Resistance to detachment increased as a function of ligand density on the substrate (Fig. 1), suggesting that site density was related to the number of receptor-ligand bonds that formed and supported rolling interactions. At densities of 80 to 90 sites/μm^2 of CD34, E-selectin, and P-selectin, the strength of neutrophil rolling adhesions was comparable, with about 20% tethered cells remaining bound at a shear stress of 36 dyn/cm^2 (Table I). There was some difference in the shape of the detachment curves; detachment on E-selectin and P-selectin was slightly steeper than that on CD34 and required about 5.5 dyn/cm^2 for 50% detachment as opposed to 9.6 dyn/cm^2 on CD34. The resistance to detachment on CD34, E-selectin, and P-selectin was clearly comparable at 80 to 90 sites/μm^2, since the variation in the detachment profiles among the three selectins at this density was much less than the variation in detachment profiles when the density of a single ligand was changed by twofold, as shown by comparison among the three different densities of CD34 (Fig. 1*A*) or E-selectin (Fig. 1*B*). Furthermore, leukocyte interactions were not altered by CD34 immobilization procedure as resistance to detachment at 40 sites of CD34/μm^2 on polystyrene slides was in close agreement with 50 sites of CD34/μm^2 in lipid planar surfaces (Fig. 1*A*).

Neutrophil tethering to sialomucin CD34, E-selectin, and P-selectin

We compared the tethering efficiencies of neutrophils on the same substrates. Direct tethers to the immobilized substrate were scored, and tethers that followed interaction with previously bound cells were not included in the analysis. Under continuous flow conditions, neutrophils tethered and rolled on CD34, E-selectin, and P-selectin at a range of wall shear stresses as high as 4.4 dyn/cm^2 (Fig. 2). Neutrophil tethering efficiency was similar at comparable site densities of CD34, E-selectin, and P-selectin. At about 90 sites/μm^2 of CD34, E-selectin, and P-selectin and at 1.5 dyn/cm^2, neutrophil tethering to CD34 was 1.2- and 1.65-fold higher than that to P-selectin and E-selectin, respectively (Table I). At higher shear stresses, tethering to CD34 was only marginally higher than that to E- and P-selectin at 80 to 90 sites/μm^2, except at 3.7 dyn/cm^2, where no tethering to E-selectin was observed in comparison

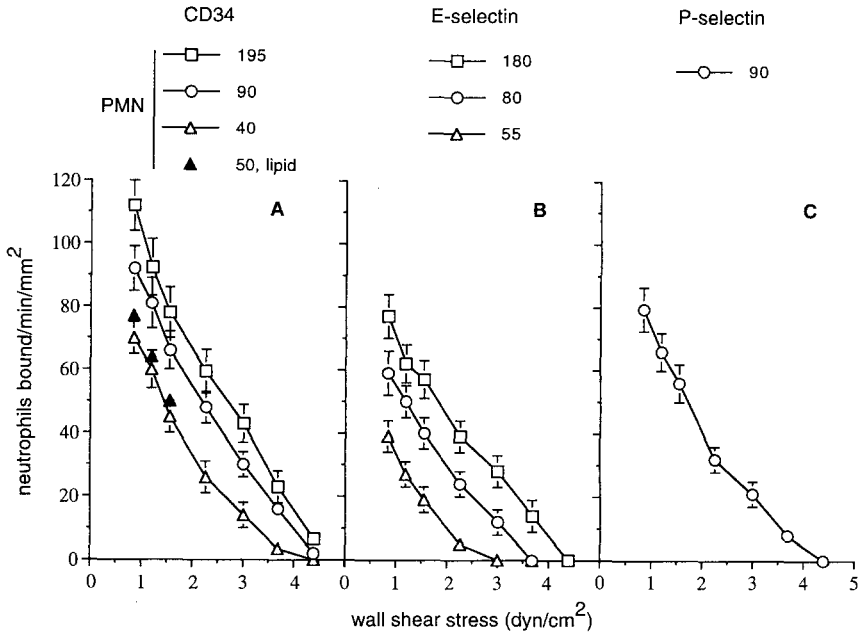

FIGURE 2. Comparison of neutrophil tethering in shear flow to sialomucin CD34, E-selectin, and P-selectin. Neutrophils (5×10^5 cells/ml) were infused at varying wall shear stresses through the parallel wall flow chamber. Sialomucin CD34 (*A*), E-selectin (*B*), and P-selectin (*C*) were immobilized at the indicated densities (sites per square micron). The number of tethers formed per minute at a given shear stress was quantitated. Only primary tethering events directly to the immobilized substrate were scored. The data points represent the mean ± SD of the number of tethered neutrophils in a ×10 field in three experiments, each performed in duplicate, and one experiment performed in duplicate for CD34 in lipid monolayer.

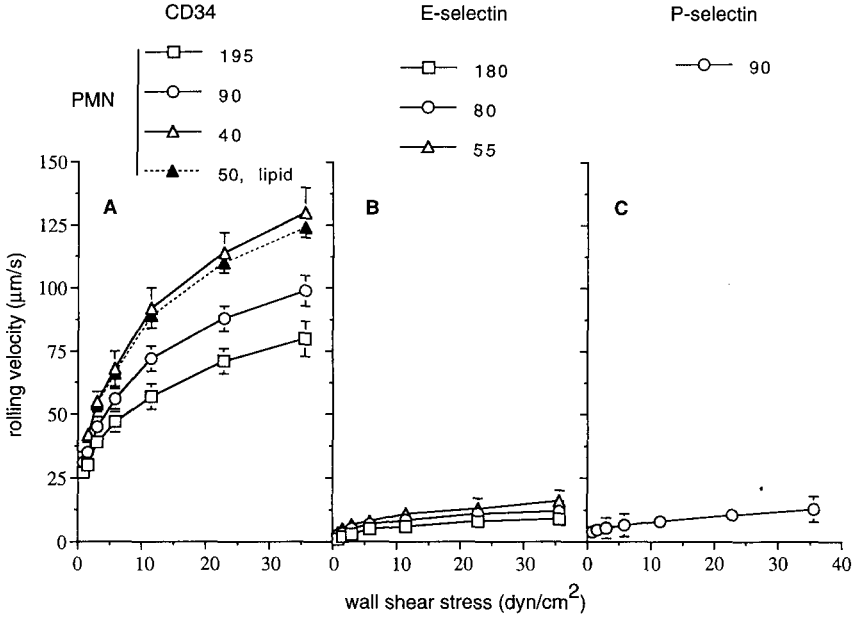

FIGURE 3. Comparison of neutrophil rolling velocities on CD34, E-selectin, and P-selectin. Neutrophils were allowed to tether at 0.84 dyn/cm^2 for 2 to 3 min at the indicated densities (sites per square micron) of CD34 (*A*), E-selectin (*B*), and P-selectin (*C*). Wall shear stress was then increased every 10 s to a maximum of 36 dyn/cm^2, and rolling velocities were measured for 15 to 20 cells. The data points represent the mean rolling velocity ± SD of four to six experiments and for one duplicated experiment for CD34 in lipid monolayer.

to CD34 and P-selectin. Tethering was site density dependent, with more efficient tethering occurring at higher site densities of immobilized substrates. Neutrophil tethering frequency on CD34 (50 sites/μm^2) in planar membrane and that on CD34 (40 sites/μm^2) on polystyrene slides were in close agreement. Tethering required Ca^{2+}, and tethering to CD34, E-selectin, and P-selectin was completely inhibited by mAb Dreg-56 to L-selectin, mAb BB11 to E-selectin, and mAb G1 to P-selectin, respectively (not shown).

Neutrophils roll dramatically faster through L-selectin than E-selectin and P-selectin

We next compared the rolling velocities of neutrophils on immobilized CD34, P-selectin, and E-selectin. The rolling velocity of neutrophils decreased as the site density of the immobilized substrate was increased, suggesting the presence of more selectin-ligand bonds, and increased as shear stress was increased (Fig. 3). At a site density of about 90 sites/μm^2 of the three substrates and at a wall shear stress of 2 dyn/cm^2, neutrophils rolled with more than 8-fold higher velocities on CD34 than on E-selectin and P-selectin (Table I). Rolling velocities at 80 to 90 sites/μm^2 over a wide range of shear stresses were 7.5- to 11.5-fold higher on CD34 than on E-selectin and P-selectin (Fig. 3). Furthermore, neutrophils also rolled with significantly higher rolling velocities on CD34 at 195 sites/μm^2 than on E-selectin at 180 sites/μm^2, despite greater resistance to detachment on the CD34 substrate. Neutrophils rolled with similar velocities on E-selectin and P-selectin at 80 to 90 sites/μm^2. Neutrophil rolling velocity at 40 sites of CD34/μm^2 on polystyrene surface was in agreement with that at 50 sites of CD34/μm^2 in planar membranes.

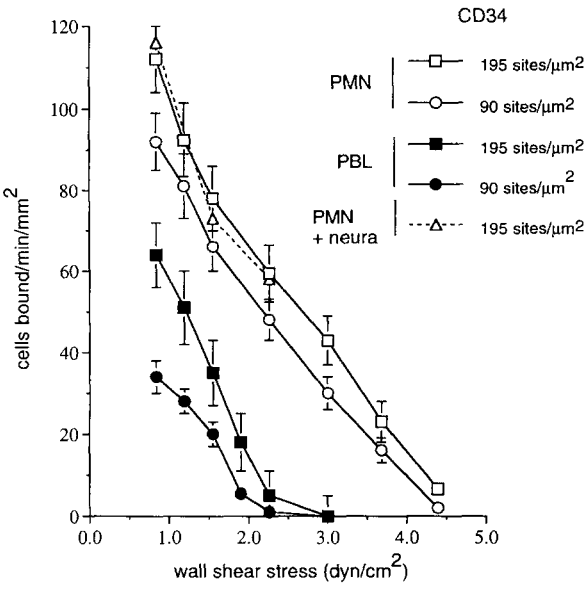

FIGURE 4. Neutrophils tether more efficiently than PBL to sialomucin CD34 in shear flow. Cells (5×10^5/ml) with or without neuraminidase treatment were infused at varying wall shear stresses through the parallel wall flow chamber at the indicated site densities of CD34. The number of tethers formed directly to CD34 per minute at a given shear stress was quantitated. The data points represent the mean ± range of the number of tethered cells in a ×10 field and are representative of two different independent experiments performed in duplicate.

Neutrophils tether more efficiently than peripheral blood lymphocytes on CD34

We extended our comparison between dynamic behavior in shear flow of a single cell type on different selectin ligands to a comparison of the behaviors of various cell types on the same selectin. Neutrophils or lymphocytes were perfused through a parallel wall flow chamber containing immobilized CD34, and the number of cells that tethered directly to the substrate per minute was counted (Fig. 4). At the identical site density of CD34, neutrophils tethered more efficiently than lymphocytes at all wall shear stress values. Neutrophil tethering was about two- to threefold higher than lymphocyte tethering at shear stresses <1.5 dyn/μm^2, and at shear stresses of 3 dyn/cm^2 and above, neutrophils, but not lymphocytes, tethered.

Neutrophils, but not lymphocytes, possess L-selectin ligands, and adherent cells assist tethering of cells in flow (4, 31, 45). These secondary tethers are particularly difficult to distinguish when the adherent cells are rolling fast, as on CD34. L-selectin ligands on neutrophils are sensitive to neuraminidase (4). Thus, to confirm that we were counting only primary tethering events and not tethers assisted by interaction with the L-selectin ligands on adherent cells, neuraminidase and sham-treated cells were compared for tethering to CD34 (Fig. 4). Neuraminidase treatment abolished neutrophil-neutrophil interactions and decreased the overall accumulation of cells (data not shown); however, the primary tethering rate was not affected. Neuraminidase treatment did not alter CD63 (46) or L-selectin expression (data not shown).

Neutrophils resist detachment comparably but roll faster than lymphocytes on CD34

The strength of neutrophil and lymphocyte rolling adhesions and the velocity of rolling on CD34 were compared. Resistance to detachment of the two cell types was essentially identical at both 90 and 195 sites of CD34/μm^2 (Fig. 5*A*). By contrast, neutrophils

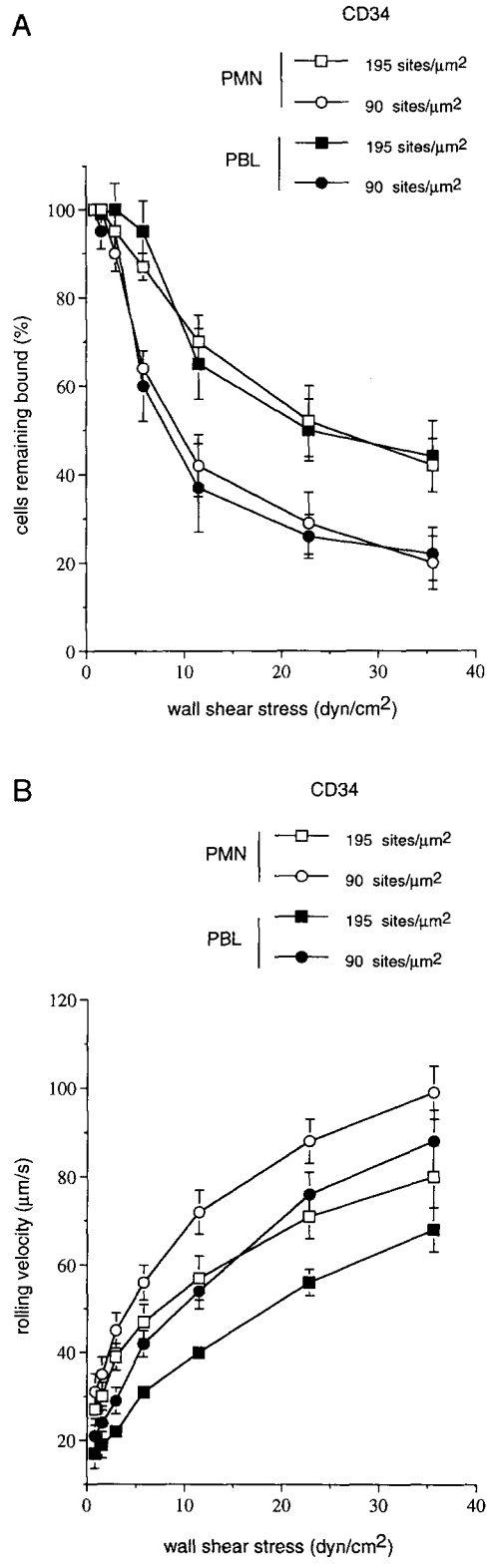

FIGURE 5. Neutrophils resist detachment equally but roll faster than PBL on sialomucin CD34. Cells were allowed to accumulate on CD34 for about 3 min at 0.84 dyn/cm^2. Wall shear stress was then increased every 10 s to a maximum of 36 dyn/cm^2, and the percentage of cells remaining bound and the rolling velocities for 30 to 50 of the cells at each shear were determined. *A*, Resistance to detachment by shear. The data points represent the mean ± range of the number of cells that remained bound in a ×10 field of CD34 and are representative of two independent experiments performed in duplicate. *B*, Rolling velocities. Data are the mean rolling velocity ± SEM and are representative of two independent experiments.

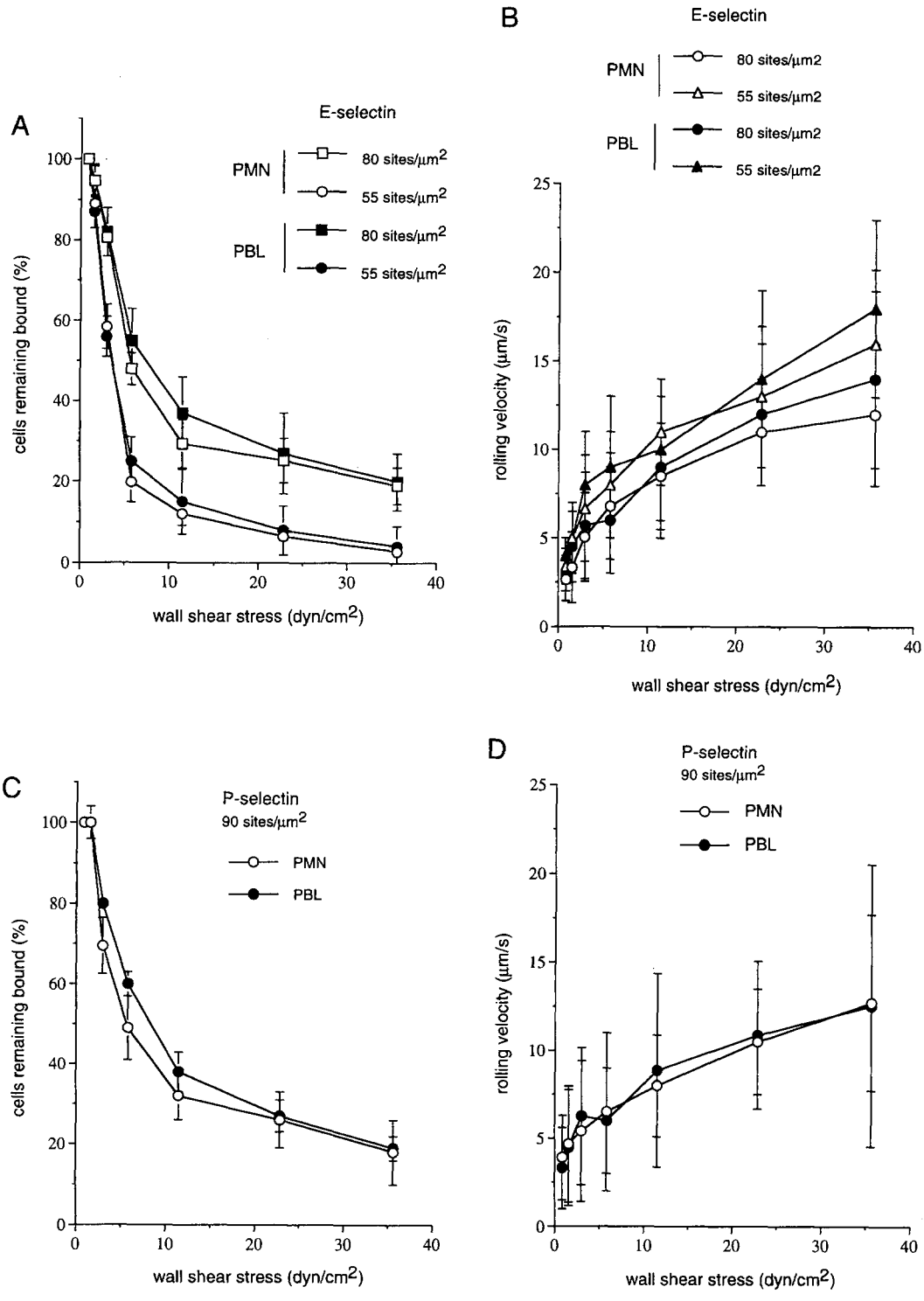

FIGURE 6. Neutrophils and PBL have comparable resistance to detachment and rolling velocities on E-selectin and P-selectin. Cells were allowed to accumulate on E-selectin or P-selectin for about 3 min at 0.84 dyn/cm^2. Wall shear stress was then increased every 10 s to a maximum of 36 dyn/cm^2, and the percentage of cells remaining bound and the rolling velocities of 30 to 50 cells at each shear were determined. The data points represent the mean ± range of the number of cells that remained bound in a ×10 field of E-selectin (*A*) or P-selectin (*C*) and are representative of two independent experiments, each performed in duplicate. The data points represent the mean rolling velocity ± SEM on E-selectin (*B*) or P-selectin (*D*) and are representative of two different experiments.

rolled faster than lymphocytes at all wall shear stresses and site densities tested (Fig. 5*B*).

Similar comparisons between neutrophils and lymphocytes were made with E-selectin and P-selectin (Fig. 6). Resistance to detachment and rolling velocity were essentially identical for neutrophils and lymphocytes on E-selectin (Fig. 6, *A* and *B*) and P-selectin (Fig. 6, *C* and *D*). These results contrast with the different rolling velocities of lymphocytes and neutrophils on CD34. Furthermore, at comparable densities of 80 to 90 sites/μm^2 of E-selectin, P-selectin, and CD34, lymphocytes demonstrated similar resistance

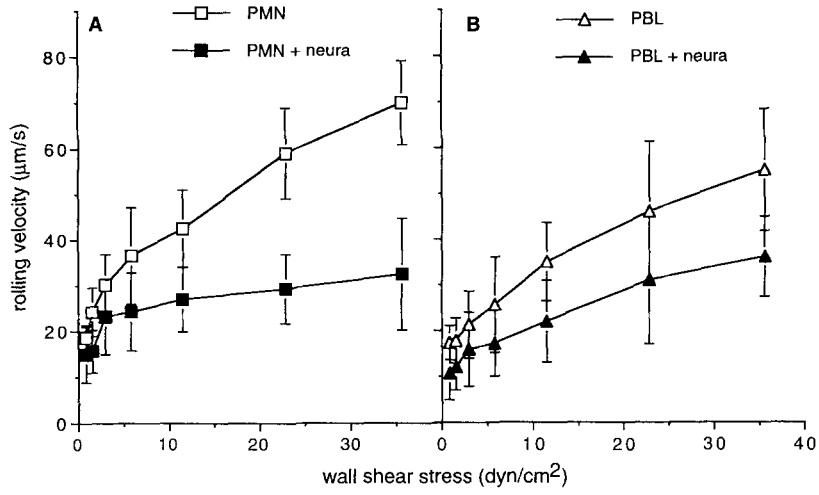

FIGURE 7. Effect of neuraminidase treatment on L-selectin-mediated leukocyte rolling on CD34. Untreated or neuraminidase-treated neutrophils (*A*) and lymphocytes (*B*) were allowed to accumulate on CD34 (287 sites/μm^2) for about 3 min at 0.84 dyn/cm^2. Wall shear stress was then increased every 10 s to a maximum of 36 dyn/cm^2, and rolling velocities were measured for 20 to 30 cells. Data are the mean rolling velocity ± SEM and are representative of two independent experiments.

to detachment, yet on CD34 rolled 5.3- to 7-fold faster than on E-selectin and rolled 4.6- to 7-fold faster than on P-selectin over a wide range of wall shear stresses. These results thus extend to lymphocytes the faster kinetics of rolling on CD34 compared with those on E-selectin and P-selectin.

Discussion

In the present study, we have directly compared E-selectin, P-selectin, and L-selectin in three distinct measures of selectin-mediated leukocyte interactions in shear flow: tethering, rolling velocity, and resistance to detachment by shear-induced forces. Rolling involves a continuous formation and dissociation of receptor-ligand bonds. It is thought that when one of the bonds between the cell and the substrate dissociates, this allows it to be rolled forward a small distance by the hydrodynamic drag force until it is held by the most upstream bond. Rolling velocity has been hypothesized to be limited by the rate at which receptor-ligand bonds dissociate (k_{off}) (12). The highest velocity at which cells can maintain rolling interactions will also be limited by k_{on}, because the rate of bond formation must keep pace with bond dissociation for a cell to remain rollingly adherent. However, unlike rolling velocity, resistance to detachment is predicted to depend on the number of receptor-ligand bonds at any one time, which depends on K_{eq}. In other words, the strength of rolling adhesions, as measured by resistance to detachment, should be more dependent on the ratio between k_{on} and k_{off} ($k_{on}/k_{off} = K_{eq}$) than on the absolute values of k_{on} and k_{off}. Tethering efficiency, measured as tethers that persist for at least 5 s, is likely to reflect both k_{on} for the initial tether interaction and also k_{off} for the cell to remain rollingly adherent long enough to be counted as a stably tethered cell. To test the idea that rolling velocity and the strength of rolling adhesions should be independently variable, we compared leukocyte interactions with the three selectins in shear flow.

We show that at comparable binding strength, neutrophils roll dramatically faster through L-selectin than through the vascular selectins, E-selectin and P-selectin. By contrast, resistance to detachment and tethering efficiency of neutrophils are comparable at similar site densities of CD34, E-selectin, and P-selectin. Over a range of wall shear stress values from 0.84 to 35.6 dyn/cm^2, neutrophil rolling through L-selectin on CD34 was 7.5- to 9.0-fold faster than that on P-selectin and 8- to 11.5-fold faster than that on E-selectin. Similar results were obtained with lymphocytes. These differences are not due to the presence on the substrate of the ligand in the case of L-selectin and the receptors in the case of E-selectin and P-selectin, since when L-selectin is on the substrate and interacts with its ligand on neutrophils (45), neutrophils also roll 5- to 8-fold faster when the densities are adjusted to give similar resistance to detachment (data not shown). Furthermore, leukocyte interactions with CD34 appeared to be unaffected by the immobilization procedure. Neutrophil tethering, rolling velocity, and binding strength were in agreement at similar site densities of CD34 on polystyrene or planar membranes. Neutrophil rolling velocity on purified E-selectin in planar membrane, as reported here, was also in agreement with rolling velocity on purified E-selectin adsorbed to plastic at similar densities (13). Recently, off-rates have been measured for transient tethers through selectins. The unstressed off-rate of L-selectin transient tethers is 7- and 9-fold faster than that of P-selectin and E-selectin tethers, respectively (28) (R. Alon, S. Chen, K. D. Puri, E. B. Finger, and T. A. Springer, manuscript in preparation). This bears striking similarity to the 7.5- to 9.0-fold and 8- to 11.5-fold faster rolling velocity through L-selectin, supporting the hypotheses that k_{off} is an important determinant of rolling velocity and that resistance to detachment can vary independently of the kinetics of receptor-ligand bonds.

Faster rolling through L-selectin than through E-selectin and P-selectin at similar strength of binding implies a faster cellular rate of association for L-selectin as well as k_{off}, since rapid dissociation of ligand-receptor bonds requires a compensating increase in bond association, governed by the product of k_{on} and ligand density on the substrate, to maintain stable rolling on the substrate. Increases in both k_{on} and k_{off} for L-selectin compared with E-selectin and P-selectin would not affect resistance to detachment as long as k_{on}/k_{off} was kept similar and a similar number of receptor: ligand bonds was formed with the substrate. We thus predict that a higher k_{off} of L-selectin-ligand bonds would be compensated with a higher k_{on}.

Neutrophils rolled with similar velocities on E- and P-selectin at comparable site densities. Our results on neutrophil rolling velocity on E- and P-selectin are in agreement with previous studies and show that the difference between the endothelial selectins (E and P) is insignificant with the difference in rolling velocity noticed here between endothelial and leukocyte (L) selectins. The rolling velocity in different studies at 2 dyn/cm^2 ranged from 1 to 6 μm/s on E-selectin (35–230 sites/μm^2) (13, 30) and from 1 to 9 μm/s on P-selectin (50–400 sites/μm^2) (12, 30). Neutrophils formed stable tethers with similar frequency to E- and P-selectin and with somewhat higher frequency to the L-selectin ligand CD34. These small

differences in the tethering efficiency among the three selectins could be related to differences in surface density or the topographic localization of the corresponding counter-receptor on the neutrophil surface. There is two- to threefold more L-selectin than PSGL-1 on the neutrophil surface (47). Considering the many potential causes of differences in tethering efficiency, the most remarkable finding was that it was so similar for all three selectin-ligand interactions. Indeed, it should be emphasized that for L-selectin we have measured the density of its counter-receptor CD34 and not the density of the carbohydrate ligand for L-selectin expressed on CD34, since methods for measuring the latter are not currently available. The material used was first isolated with MECA-79 mAb and then with CD34 mAb (10), so that there is at least one MECA-79 determinant per CD34 molecule. The MECA-79 mAb largely inhibits L-selectin-dependent binding to PNAd (6), showing that the MECA-79 determinant is closely physically associated with the L-selectin ligand; however, recent studies have emphasized differences between them. Whereas both the L-selectin ligand and the MECA-79 determinant in HEV are dependent on sulfation, neuraminidase destroys the L-selectin ligand but not the MECA-79 determinant (8). Furthermore, fucosyl transferase VII deficiency greatly diminishes L-selectin ligand expression on HEV, but has little effect on MECA-79 reactivity (48). Because of uncertainty in the actual density of selectin ligands on the substrate, we have emphasized comparisons at substrate densities where the strength of rolling adhesions is similar as measured by resistance to detachment.

We have also compared neutrophils and lymphocytes for differences in measures of selectin function. Neutrophils tether more efficiently than lymphocytes to each of the three substrates, sialomucin CD34, E-selectin, and P-selectin (49) (data not shown). These observations correlate well with the presence of functional E- and P-selectin ligands only on a subpopulation of lymphocytes (Refs. 49 and references therein). Similarly, L-selectin is expressed on a subpopulation of lymphocytes (50). Differences in the clustering of receptors or ligands on the cell surface may also contribute to the observed differences. However, once tethered to CD34, E-selectin, or P-selectin, neutrophils and lymphocytes showed similar resistance to detachment on the three substrates.

Studies of rolling velocity revealed interesting differences between neutrophils and lymphocytes. They rolled with similar velocities on E- and P-selectin. However, neutrophils rolled 10 to 20 μm/s faster than lymphocytes on CD34. At lower wall shear stresses, the neutrophils rolled 1.5-fold as fast as the lymphocytes. It was curious that whereas rolling velocity increased about 3-fold from 1.55 to 35.7 dyn/cm^2, there was no increase in the velocity of neutrophils relative to lymphocytes; the difference remained a fairly constant 10 to 20 μm/s. L-selectin is concentrated on microvilli on both lymphocytes and neutrophils, and there are no major differences in microvilli number or in distribution of L-selectin on the cell surface that appear to account for the observed differences (26). Differences in the glycocalyx or surface charge on the two cell types could contribute. Neuraminidase treatment to remove sialic acid, a major contributor to the net negative surface charge of leukocytes, enhanced the binding strength (data not shown) and reduced the rolling velocity of neutrophils as well as lymphocytes on CD34 substrates (Fig. 7). However, both measures were affected to a greater extent on neutrophils than on lymphocytes, suggesting that charge could contribute to the differences between the cell types.

In conclusion, we have demonstrated that the dynamics of leukocyte interactions through L-selectin are distinct from those through the vascular selectins, E- and P-selectin, and vary independently of the strength of rolling adhesions. Leukocytes roll dramatically faster through L-selectin compared with E-selectin and P-selectin, and this faster rolling correlates with the faster k_{off} of L-selectin tethers (R. Alon, S. Chen, K. D. Puri, E. B. Finger, and T. A. Springer, manuscript in preparation). Furthermore, fast interactions through L-selectin may correlate with its function in the capturing and rolling of leukocytes in the earlier steps of adhesion cascades. Interaction through E-selectin and P-selectin may then reduce the velocity of rolling cells, exposing them to chemoattractants and enhancing the interaction of leukocyte integrins with their ligands. Similarly, it appears that lymphocyte rolling through vascular cell adhesion molecule-1 (51) and MAdCAM-1 (52) is slower than that through L-selectin, and in the Peyer's patch, interactions through L-selectin may precede those through MAdCAM-1, which appear to be required for subsequent activation-dependent adhesion strengthening and arrest through the β_2 integrin LFA-1 (53). Thus, kinetic differences between adhesion molecules may correlate with their positions in adhesion cascades.

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