Adhesion through L-selectin requires a threshold hydrodynamic shear

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SELECTINS are cell adhesion molecules that bind carbohydrate ligands and promote interaction between leukocytes and the vessel wall in vascular shear flow[1,2]. Selectin–ligand bonds have high mechanical strength, allowing initial tethering to the vessel wall through one or few bonds, and have fast on and off rates, permitting rolling in response to hydrodynamic drag[3]. The L-selectin molecule on leukocytes binds to peripheral node addressin on high endothelial venules of lymph nodes to mediate leukocyte rolling[4,5] and binds to a ligand on neutrophils to mediate rolling of leukocytes over one another[6]. Here we describe a surprising mechanism for regulation of these interactions, both *in vitro* and *in vivo*. Shear above a critical threshold is required to promote and maintain rolling interactions through L-selectin, but not through E-selectin, P-selectin or VCAM-1. The shear threshold requirement for L-selectin may be physiologically important in low shear to prevent inappropriate aggregation of leukocytes and interaction with the vessel wall.

When neutrophils or T lymphocytes were perfused through a flow chamber containing purified peripheral node addressin (PNAd) or the CD34 component of PNAd incorporated in a phosphatidylcholine bilayer on one wall, maximal accumulation of rolling cells was found at a wall shear stress of 1 dyn cm^{-2} (Fig. 1*a*). Accumulation dropped above 2 dyn cm^{-2}. However, there was also an unexpected decrease in accumulation of rolling cells below 0.6 dyn cm^{-2}. By contrast, efficient accumulation of rolling neutrophils was seen on E-selectin and P-selectin at the lowest shear stresses tested (Fig. 1*a*).

Shear above a threshold value was also required for formation of stable tethers, defined as newly initiated tethers to the PNAd substrate that persist as rolling adhesions for > 0.5 s (Fig. 1*b*). The number of stable tethers was divided by the number of cells that flowed through the field of view to correct for lower flux at lower shear; nonetheless, a threshold of about 0.4 dyn cm^{-2} was seen for stable tethering to PNAd and CD34 at all site densities tested (Fig. 1*b* and data not shown). By contrast, stable tethers on E-selectin, P-selectin, and VCAM-1 were most frequent at low shear (Fig. 1*c*). No stable tethers formed when leukocytes settled in stasis on PNAd substrates, because when flow was started at 1.8 dyn cm^{-2}, the cells travelled a few cell diameters at the hydrodynamic velocity (the velocity of a non-interacting cell near the wall[7]) before they tethered and slowed in motion as they began rolling (our unpublished results and E. Butcher, personal communication).

The shear dependence of rolling adhesions through L-selectin was dramatically illustrated by counterintuitive shifts in leukocyte velocity when the shear was shifted between values below and above the threshold. The hydrodynamic velocity of leukocytes was measured in EDTA, which inhibits Ca^{2+}-dependent selectin interactions[8]. The velocity in EDTA and Ca^{2+} was similar on PNAd below 0.4 dyn cm^{-2} (Fig. 2*a*). However, when shear stress was increased to 0.73 dyn cm^{-2}, most of the cells near the wall tethered and rolled at lower velocity (Fig. 2*a*). When lymphocytes rolling at 16.2 ± 1.0 μm s^{-1} on PNAd at 1.8 dyn cm^{-2} were subjected to a rapid reduction in shear stress to 0.18 dyn cm^{-2}, they detached from the PNAd substrate and moved at 41.3 ± 2.3 μm s^{-1} (the increase in velocity was significant, with $P < 0.001$ (Fig. 2*b*). The velocity at 0.18 dyn cm^{-2} matched that of lymphocytes in EDTA, showing no interaction with the substrate. When flow was resumed at a wall shear stress of 1.8 dyn cm^{-2}, the lymphocytes retethered and resumed rolling at lower velocity. Similar experiments with neutrophils are illustrated by superimposition of 10 images captured at 0.1-s intervals (Fig. 2*c*–*f*). In Ca^{2+}, neutrophils are rollingly adherent at 1.5 dyn cm^{-2} (Fig. 2*c*), but after shift to 0.22 dyn cm^{-2} the cells detach and move faster (Fig. 2*d*). The hydrodynamic velocities of neutrophils in EDTA at 1.5 dyn cm^{-2} (Fig. 2*e*; only 3 to 5 images of each cell are within the field of view) are much faster than in Ca^{2+} (Fig. 2*c*), but at 0.22 dyn the velocities of cells in EDTA (Fig. 2*f*) and in Ca^{2+} (Fig. 2*d*) are comparable. Reversible rolling at 1.5 dyn cm^{-2} and detachment at 0.22 dyn cm^{-2} was seen for at least 20 cycles of shifts in flow velocity, and behaviour of the cells at the new shear stress equilibrated in less than 1 s.

By contrast, when lymphocytes or neutrophils rolling on E-selectin or P-selectin, or VLA-4 transfectants rolling on VCAM-1 at ≥ 1.5 dyn cm^{-2} were subjected to a decrease in shear to 0.15 or 0.3 dyn cm^{-2}, they rolled more slowly, and none of the cells detached (Fig. 3). Similarly, L-selectin-dependent rolling on sialyl Lewis^a and fucoidan substrates did not exhibit a threshold; rolling slowed with no detachment when shear was reduced (Fig. 3). We suspect that this reflects the very high density of these ligands in the assay, that is, 12,000 sites μm^{-2} for sialyl Lewis^a; the density required to support rolling is about 100-fold higher for sialyl Lewis^a than for the CD34 component of PNAd[9,10].

As previously reported for neutrophils adherent to endothelial cells[6], we found that a layer of neutrophils adherent to and rolling slowly on E-selectin provided a substrate for tethering and rolling of further neutrophils. These neutrophil–neutrophil interactions were L-selectin-dependent (ref. 6 and data not shown). Furthermore, they were shear-dependent. Neutrophils rolled jerkily on other neutrophils at 1.5 dyn cm^{-2}, with a velocity of 127 ± 24 μm s^{-1} (Fig. 2*g*); however, when shear was reduced to 0.3 dyn cm^{-2} the cells detached and moved smoothly at increased velocity of 197 ± 44 μm s^{-1} ($P < 0.001$) (Figs 2*h*, 3).

Rolling in murine Peyer's patch HEV[11] was tested to determine whether a threshold shear was required for L-selectin-dependent interactions *in vivo*. Human neutrophils treated with neuraminidase to remove E- or P-selectin-dependent adhesion formed rolling adhesions in Peyer's patch HEV at physiological shear (Fig. 4). These interactions were $97 \pm 6.9\%$ inhibitable by L-selectin monoclonal antibody (not shown). To assess rolling at low shear, the vascular supply to the Peyer's patch was partially restricted and neutrophil behaviour in the same vessels was observed. The rolling fraction in the hypoperfusion state was decreased in 14/14 vessels examined, by a mean ± s.d. of $89 \pm 19\%$. The rolling fraction increased back to baseline levels when normal flow was reinstated (not shown). Plotting all the data (Fig. 4) revealed a bell-shaped dependence on venular shear rate with a threshold similar to that observed *in vitro*. Rolling *in vivo* compared to *in vitro* is favoured by more efficient leukocyte margination owing to contributions by red blood cells and other microhaemodynamic factors[12]. Thus, rolling was observed within a range of shear stresses from ~ 2.5 to 40 dyn cm^{-2}, assuming a blood viscosity of 0.025 poise.

Previously, cell adhesion has been reported to be upregulated by increased density on the cell surface, affinity, or clustering of adhesion molecules[13]. Increased shear is an unexpected and unprecedented mechanism for upregulating cell adhesion, and so far appears to be unique to L-selectin. A dramatic and counterintuitive illustration of the phenomenon we have described is that as the flow rate is increased to yield a wall shear stress above the

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threshold, the velocity of leukocytes slows as they tether and roll. Active regulation by cellular signalling appears not to be required, as the phenomenon occurs within less than 1 s, and is repeated through many cycles of shifts in shear. Shear above a threshold is required for both initiation and maintenance of rolling. Loss of rolling interactions within less than 1 s after a drop in shear below the threshold implies a lifetime for the L-selectin–carbohydrate bond of less than 1 s; indeed, recent measurements show a lifetime of 0.15 s (R.A. *et al.*, manuscript in preparation). 'Catch bonds' are theoretically possible that strengthen when tensile force is applied[14]; however, this is not the explanation, because tensile force shortens the lifetime of the L-selectin bond (R.A. *et al.*, in preparation). Transient tethers with a lifetime of 0.15 s occur below the shear threshold (R.A. *et al.*, in preparation) but do not progress to rolling adhesions or the stable tethers of duration > 0.5 s measured here. We suspect that once the first short-lived L-selectin bond forms between the cell and the substrate, hydrodynamic shear may provide what is known in engineering as transport, to promote the formation of additional L-selectin bonds with the substrate before the first bond dissociates. L-selectin is clustered on the tips of microvilli[15–17] and shear-driven transport of the cell may bring further microvilli in contact with the substrate. The short lifetime of L-selectin (R.A. *et al.*, in preparation) compared with P-selectin[3] and E-selectin[18] bonds is consistent with the uniqueness of the shear requirement to L-selectin and the postulated importance of short bond lifetime in the shear threshold phenomenon.

Regulation by hydrodynamic shear of adhesion through L-selectin is likely to be biologically important. Interactions through all other known adhesion molecules are enhanced by low shear; thus, loss of L-selectin interactions may provide a counterbalance to prevent inappropriate interactions with the vessel wall in low shear. This may be important in vessels with inherently low shear, such as sinusoids and large veins; in pathologies with hypoperfusion; and in the ascending aorta, which is prone to atherosclerosis and where flow reverses and shear goes briefly to

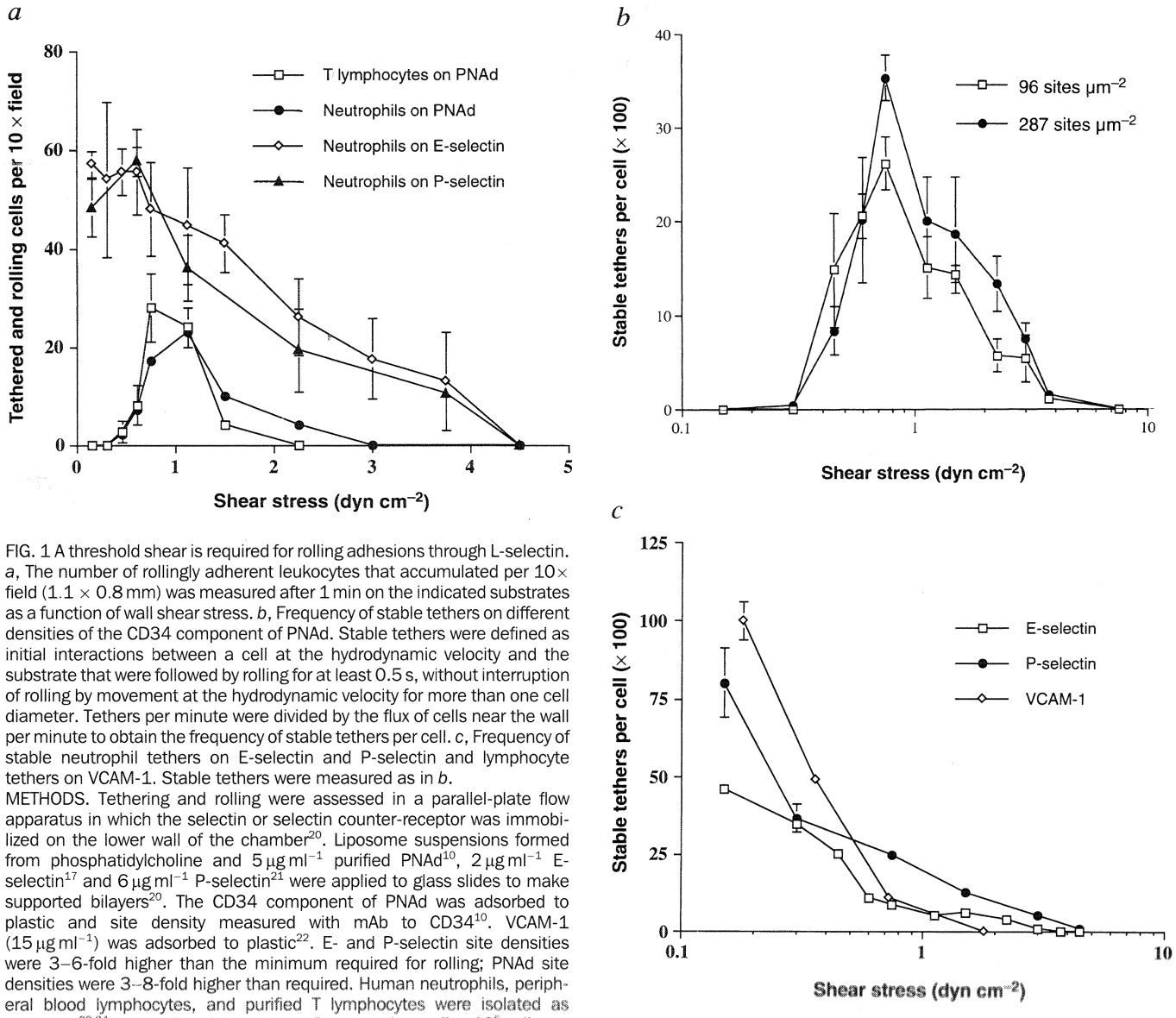
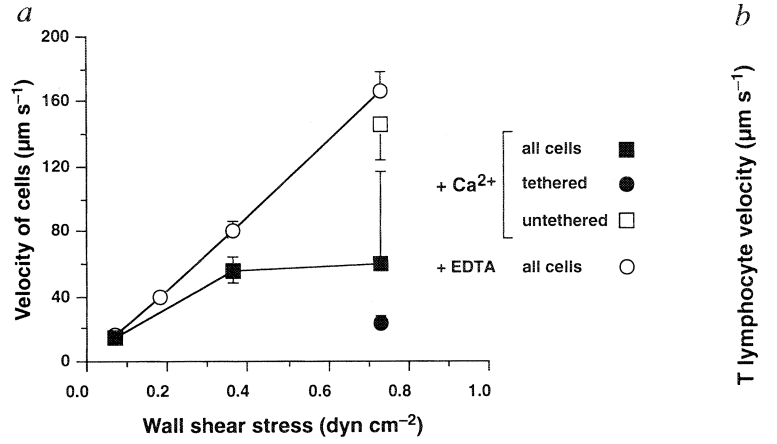
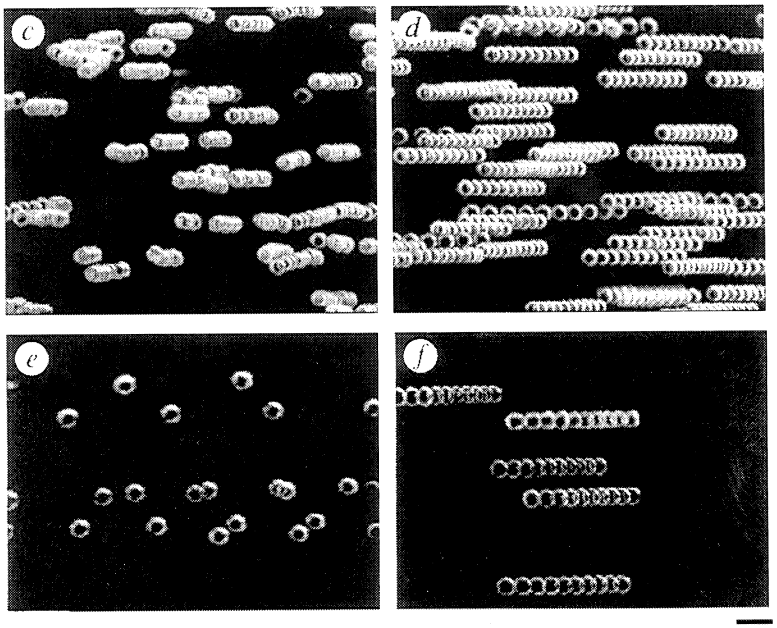


FIG. 1 A threshold shear is required for rolling adhesions through L-selectin. *a*, The number of rollingly adherent leukocytes that accumulated per 10× field (1.1 × 0.8 mm) was measured after 1 min on the indicated substrates as a function of wall shear stress. *b*, Frequency of stable tethers on different densities of the CD34 component of PNAd. Stable tethers were defined as initial interactions between a cell at the hydrodynamic velocity and the substrate that were followed by rolling for at least 0.5 s, without interruption of rolling by movement at the hydrodynamic velocity for more than one cell diameter. Tethers per minute were divided by the flux of cells near the wall per minute to obtain the frequency of stable tethers per cell. *c*, Frequency of stable neutrophil tethers on E-selectin and P-selectin and lymphocyte tethers on VCAM-1. Stable tethers were measured as in *b*.

METHODS. Tethering and rolling were assessed in a parallel-plate flow apparatus in which the selectin or selectin counter-receptor was immobilized on the lower wall of the chamber[20]. Liposome suspensions formed from phosphatidylcholine and 5 μg ml^{-1} purified PNAd[10], 2 μg ml^{-1} E-selectin[17] and 6 μg ml^{-1} P-selectin[21] were applied to glass slides to make supported bilayers[20]. The CD34 component of PNAd was adsorbed to plastic and site density measured with mAb to CD34[10]. VCAM-1 (15 μg ml^{-1}) was adsorbed to plastic[22]. E- and P-selectin site densities were 3–6-fold higher than the minimum required for rolling; PNAd site densities were 3–8-fold higher than required. Human neutrophils, peripheral blood lymphocytes, and purified T lymphocytes were isolated as described[23,24] and perfused through the flow chamber at 5×10^5 cells per ml in Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution supplemented with 10 mM HEPES, pH 7.4, 2 mM Ca^{2+}, 0.25% human serum albumin. Experiments were videotaped for later analysis. Specificity of interaction was confirmed and the hydrodynamic velocity was determined by addition of 5 mM EDTA; selectin interactions are Ca^{2+} dependent[6]. Cells accumulating on or forming stable tethers to E-selectin or P-selectin or forming stable tethers to PNAd after first interacting with a previously adherent cell were not counted. Error bars show s.d., $n = 3$–6.





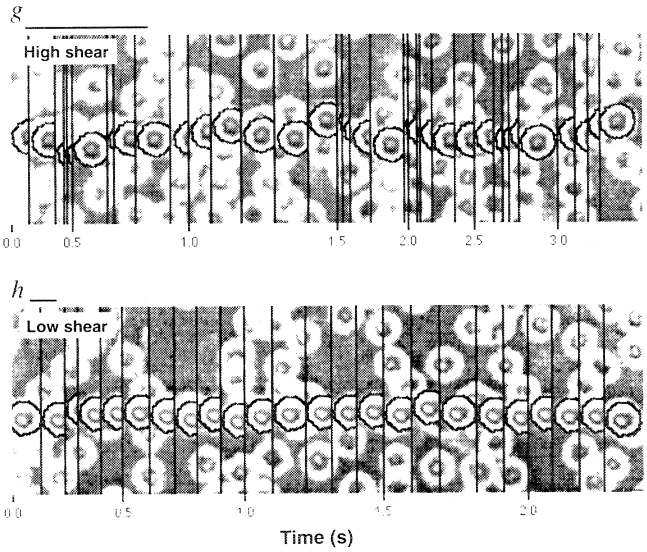


FIG. 2 Velocities of leukocytes on PNAd, and velocities of neutrophils interacting with adherent neutrophils, demonstrate rolling interactions only above a shear threshold, and that maintenance of rolling requires shear above the threshold. *a*, Following settling in stasis of T lymphocytes onto a PNAd-coated substrate, flow was initiated at 0.073 dyn cm^{-2} and increased in steps every 10 s to 0.73 dyn cm^{-2}. *b*, Reversible loss of lymphocyte rolling interactions on PNAd during temporary drop in shear stress from 1.8 to 0.18 dyn cm^{-2}. Measurements began after a period at a wall shear stress of 1.8 dyn cm^{-2} ($t = 0$). At 1.5 s, the shear stress was dropped to 0.18 dyn cm^{-2}. At 4.5 s, the flow was raised again to 1.8 dyn cm^{-2}. In the absence of EDTA, almost all the lymphocytes measured at 1.5 s (41 cells) were still visible at the 3 s mark. When flow was raised at 4.5 s, 80% of the lymphocytes (33 cells) tethered and rolled; the remainder moved at the hydrodynamic velocity and were swept out of the field of view too rapidly for inclusion in velocity measurements. In the presence of 5 mM EDTA, the cells moved much more rapidly. As some of the lymphocytes left the field of view and more entered, the average velocity is based on different cells. The velocity of all resolvable lymphocytes in the microscopic plane of focus on PNAd adsorbed to plastic[5] was measured as described[20]. *c–f*, Superposition of sequential images of neutrophils on PNAd substrates. Video images were captured at 0.1-s intervals for 1 s and 10 images were superimposed using routines created with NIH Image v.1.57. Bar, 25 μm. *c*, At 1.5 dyn cm^{-2} in Ca^{2+} cells are rolling. *d*, Soon after shift from 1.5 to 0.22 dyn cm^{-2} cells detach from the PNAd substrate as shown by more rapid and uniform movement than in *c*. *e*, At 1.5 dyn cm^{-2} in EDTA cells move rapidly at the hydrodynamic velocity (compare with *c*) and leave the field of view after capture of only 3 to 5 cell images. *f*, At 0.22 dyn cm^{-2} in EDTA, cells move with velocities within the range seen at 0.22 dyn cm^{-2} in Ca^{2+}. Velocities are on average slightly higher in *f* than in *d*, because in *d* cells have recently detached from and are on average closer to the wall, where fluid velocity is slower. *g* and *h*, Representative images of neutrophils on a layer of adherent neutrophils. A primary layer of neutrophils was allowed to accumulate on E-selectin in a phosphatidylcholine bilayer. Next, fluorescently labelled[24] or unlabelled 'secondary' neutrophils were infused and observed with combination of epifluorescent and phase illumination or phase alone. Some 'holes' were present in the layer of primary neutrophils, but after accumulation of the primary neutrophils to the density shown, secondary neutrophils rarely dipped into the holes and adhered to E-selectin. The primary layer rolled at < 2% of the velocity of the secondary neutrophils, and thus the two populations were readily distinguished. Images were captured every 0.1 s, an outlined image of a neutrophil was placed at the position of the neutrophil being tracked, and segments from each image corresponding in width to the distance the neutrophil moved in the 0.1 s interval (vertical lines) were pasted together. The running time is indicated below each panel. Bars show for comparison the distance traveled by a cell moving at the hydrodynamic velocity in a 0.1 s interval. *g*, At 1.5 dyn cm^{-2}, a representative neutrophil rolls on the layer of adherent neutrophils. Note the jerkiness of the rolling. *h*, After a shift from 1.5 to 0.3 dyn cm^{-2}, a representative neutrophil detaches and moves at the hydrodynamic velocity. Note the steadier velocity and direction than in *g*, and that it takes less time to traverse the same distance.

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