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Neutrophils Roll on E-Selectin[1]

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Abstract. Using flow conditions that simulate those in post capillary venules, we have found that neutrophils attach and roll on a substrate bearing purified E-selectin. E-selectin resembles P-selectin (CD62) with regard to the dependence of attachment efficiency on wall shear stress and selectin density. In contrast, once attached, neutrophils form rolling adhesions on E-selectin that are much stronger than those on P-selectin. Rolling velocities on E-selectin are slower and have less variance than on P-selectin. With increasing shear stress, rolling velocities reach a plateau level that is dependent on E-selectin density, suggesting that the number of receptor-ligand bonds and the bond dissociation rate limit rolling velocity, and that the bonds are not broken by the applied force.

Three selectin molecules, characterized by homologous lectin-like N-terminal domains, an epidermal growth factor-like domain, and a variable number of short consensus repeats have been described on cells in the vasculature: leukocytes, platelets, and endothelium (1–3). L-selectin is expressed on all circulating leukocytes and has been shown to mediate adhesion by interacting with ligands on both the endothelium of post-capillary venules and the high endothelial venules of peripheral lymph nodes (4–8). P-selectin is stored preformed in the Weibel-Palade bodies of endothelial cells and the α-granules of platelets. In response to mediators of acute inflammation such as thrombin or histamine, P-selectin can be rapidly mobilized to the plasma membrane to bind neutrophils and monocytes (9–11). E-selectin is induced on vascular endothelial cells by cytokines such as IL-1, LPS, or TNF and requires de novo mRNA and protein synthesis (12).

After an inflammatory stimulus, neutrophils are observed by intravital microscopy to begin rolling along the walls of postcapillary venules (13, 14). As the inflammatory response progresses, rolling neutrophils are arrested and proceed to crawl through junctions between the endothelial cells lining the venule (15, 16). We recently reproduced this sequence of events in vitro in a parallel plate flow chamber (17). Purified P-selectin (CD62) and ICAM-1 (CD54), an Ig-family adhesion molecule that is induced on endothelium at inflammatory sites, were incorporated into supported planar lipid bilayers formed on one wall of the flow chamber. At wall shear stresses characteristic of those found in post-capillary venules, neutrophils attached to planar bilayers containing the purified P-selectin and rolled in response to fluid drag forces. Neutrophils rolled at velocities approximately 80-fold more slowly than when they were not adherent to the bilayer. Neutrophils did not attach at physiologic shear stresses to planar lipid bilayers containing ICAM-1. However, neutrophils rolling on a bilayer containing both P-selectin and ICAM-1 stopped and became strongly adherent when adhesiveness of LFA-1 and Mac-1, integrins that bind to ICAM-1, was activated by addition of a chemoattractant. Thus, rolling interactions through P-selectin were proposed to be a prerequisite for subsequent adhesion strengthening through integrins under physiologic flow conditions.

Experiments with L-selectin also suggest that it mediates leukocyte rolling. Intravascular infusion of a soluble IgG-L-selectin chimera inhibits neutrophil rolling attachments in vivo (5), as does infusion of anti-L-selectin mAb (6). mAb against L-selectin also inhibit neutrophil attachment to endothelial monolayers at venular levels of shear in controlled flow assays, indicating that L-selectin, like P-selectin, is specialized for the recruitment of neutrophils from the circulation (7).

mAb to leukocyte integrins do not inhibit rolling in vivo, but do inhibit their emigration into tissue (13). mAb to leukocyte integrins have been found to inhibit neutrophil adhesion to endothelial monolayers in vitro under static conditions but not in flow (18, 19), and to inhibit neutrophil transendothelial migration (18), Thus integrin/ICAM in-

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teractions function in a later step in leukocyte localization at inflammatory sites. By contrast, L-selectin was found to be rapidly shed by chemoattractant-stimulated neutrophils, suggesting action in an early step (7, 20).

Much remains to be learned about the function of E-selectin in leukocyte adhesion. It has not yet been determined whether E-selectin can support rolling adhesions or mediate attachment of neutrophils at physiologic shear stresses, properties it is predicted to have based on its structural homology with P- and L-selectin. In animal models there is evidence that E-selectin is involved in neutrophil emigration (21–23), and its delayed expression relative to that of P-selectin suggests that it might be important at later times. In this report, we describe studies of neutrophil interactions with purified E-selectin under controlled flow conditions. Our studies on the relationship between rolling velocity, shear stress, and E-selectin density have important implications for the biophysical characteristics of selectin-ligand interactions that permit rolling adhesions.

Materials and Methods

Monoclonal antibodies

The murine mAbs BB11 (anti-E-selectin (IgG2b) (24)) and R6.5 (anti-ICAM-1, IgG2a, (18)) were used at 10 μg/ml as purified Ig.

Purification of soluble E-selectin and soluble ICAM-1

Recombinant soluble E-selectin was a generous gift of Dr. Roy Lobb and was purified from supernatants of transfected Chinese hamster ovary cells as described (24). Recombinant soluble ICAM-1 was a generous gift of Dr. Steve Marlin (Boehringer Ingelheim Pharmaceuticals Inc.) and was purified from culture supernatants of transfected Chinese hamster ovary cell lines (25).

Preparation of E-selectin and ICAM-1 substrates

Soluble E-selectin (820 μg/ml) was diluted 1:500, 1:1000, 1:2000, 1:4000, 1:8000 into binding buffer (0.1 M $NaHCO_3$, pH 9.2). Once diluted, the E-selectin was pipetted onto a demarcated area of a polystyrene slide cut from a bacteriologic Petri dish (Becton Dickinson Labware, Lincoln Park, NJ) and allowed to incubate for 2 h at room temperature. HBSS+1% human serum albumin was added for 30 min at 37°C to block nonspecific binding sites. Immediately before an experiment, the immobilized protein was treated with phosphate buffer (PBS, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) plus 1% Tween 20 for 2 min and washed with PBS. Blocking with Tween 20 prevented nonspecific adhesive interactions between neutrophils and the protein-coated polystyrene surface (10). Soluble ICAM-1 was adsorbed as for E-selectin, except that it was diluted with Tris-saline-azide buffer (20 mM Tris, 150 mM NaCl, pH 8.0, 0.02% azide).

Determination of E-selectin and ICAM-1 site densities

Soluble ICAM-1 or soluble E-selectin was adsorbed as described above onto polystyrene microtiter plates (Flow Laboratories, Inc., McLean, VA) for determination of site densities. mAb R6.5 to ICAM-1 (18) and BB11 to E-selectin (24) were iodinated to specific activities of 2.3 and 1.9 μCi/μg mAb respectively, and site densities were determined by saturation binding, assuming binding of one IgG molecule per Ag molecule as previously described (26). The site densities were determined three times in duplicate for each concentration of adsorbed E-selectin or ICAM-1. E-selectin densities are expressed in sites/μm^2 and were rounded off as indicated: 35 = 37±18, 60 = 62±12, 200 = 194 ± 66, 335 = 334 ± 29, 885 = 885 ± 32. Soluble ICAM-1 at a concentration of 100 μg/ml produced a site density of 775 ± 82 sites/μm^2.

Isolation of polymorphonuclear leukocytes

Neutrophils were isolated from citrate anticoagulated whole blood after dextran-sedimentation and density separation over Ficoll-Hypaque (27). After isolation, neutrophils were stored in Modified HBSS (Ca^{2+} and Mg^{2+}-free, pH 7.3; GIBCO Laboratories, Grand Island, NY) supplemented with 10 mM HEPES and 0.5% human serum albumin at room temperature for up to 4 h. Before use in experiments, the neutrophils were washed into HBSS supplemented with 2 mM Ca^{2+}, because calcium is required for E-selectin interactions with leukocytes (24).

Neutrophil treatments

For mAb inhibition assays, neutrophils were incubated with purified antibody for 10 min at room temperature in HBSS plus 2 mM Ca^{2+} before the flow assay was begun. The antibody was continuously present in the assay media. Neutrophils in some experiments were fixed in 0.5% formaldehyde in HBSS at 20° for 20 min and then washed three times in HBSS. In some experiments, neutrophils were incubated in HBSS with 0.06% sodium azide and 50 mM 2-deoxy-D-glucose or with 20 μc dibutryl cyclic AMP (dbcAMP) for 10 min before beginning the flow assay.

Flow assays

A polystyrene slide on which E-selectin, ICAM-1, or irrelevant protein, HSA, 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). Attachment during continuous flow was assayed as previously described (17). The number of adherent neutrophils/

unit area was quantitated by a visual count of multiple fields of view videotaped while scanning the lower plate of the flow chamber. The wall shear stress was calculated assuming a viscosity of assay buffer equal to the viscosity of water at room temperature (1.0 centipoise, 24°C). For detachment assays, resting neutrophils (2×10^6/ml) were injected into the chamber through a port and allowed to settle as previously described. To determine the effect of chemotactic activation of neutrophils on interactions with E-selectin, PMA was added to the neutrophil suspension 10 min before injection into the flow chamber. Flow was initiated after a 2 min static incubation. All experiments were recorded on videotape and multiple fields of view were examined for each data point.

Analysis of neutrophil rolling

Rolling velocities were measured for all cells in two to five fields of view for each experiment at a given shear stress or ligand density. Results were presented as averages from three independent experiments. Images were recorded on a time-lapse video cassette recorder and analyzed as previously described (17) except that neutrophil displacement was measured over intervals of either 10 or 20 sec.

Results

Neutrophils attach to E-selectin at physiologic shear stresses

Neutrophil adhesion to E-selectin was examined in a flow assay designed to simulate the wall shear forces that exist in postcapillary venules (1 to 10 dyn/cm^2). Neutrophils in suspension were perfused through a parallel plate flow chamber containing a plastic substrate on which purified, soluble E-selectin was immobilized (17). Under continuous flow conditions, neutrophils bound to purified E-selectin at wall shear stresses up to 3.6 dyn/cm^2 (Fig. 1*A*). The formation of individual cell attachments was rapid, occurring between successive image frames of videotape (<33 ms). Attachment was always followed by rolling. Attachment was site density dependent, with more efficient attachment occurring at higher site densities of E-selectin. Below 60 sites/μm^2 of E-selectin, the number of successful neutrophil attachments dropped dramatically and those that did form were noticeably more transient.

Neutrophil binding to E-selectin under flow was completely inhibitable by anti-E-selectin mAb BB11 (24) (Fig. 1*B*). No attachment was observed when neutrophils were perfused over E-selectin with calcium-free media (10). Furthermore, all adherent neutrophils could be detached from E-selectin substrates within seconds after infusion of HBSS with 5 mM EDTA. To confirm routinely that interactions were E-selectin dependent, HBSS with 5 mM EDTA was infused after every binding experiment and the amount of EDTA-independent binding quantified. Experiments were discarded when EDTA-independent binding was more than 0.5% of the mean number of bound neutrophils before addition of EDTA, indicating some nonspecific binding because of incomplete blocking of the plastic substrate. In most experiments, 100% of the binding was EDTA-dependent.

Previously published data on purified P-selectin immobilized in a lipid bilayer (17) is included for comparison to E-selectin (Fig. 1*A*). Attachment of neutrophils to E-selectin under flow conditions was comparable to P-selectin at equivalent site densities (Fig. 1*A*) and see further data in (17). On both P and E-selectin, attachment efficiency appeared to reach a maximum at approximately 300 sites/μm^2.

PMA-stimulated neutrophils failed to attach to soluble ICAM-1 adsorbed to polystyrene at a density of 775 sites/μm^2 in the presence of venular levels of wall shear stress (Fig. 1*B*), consistent with previous observations of neutrophil interactions with intact ICAM-1 in lipid bilayers (17). At a sub-physiologic wall shear stress of 0.36 dyn/cm^2, attachment of PMA-stimulated neutrophils was 20-fold less than that observed on E-selectin at a lower site density of 335 sites/μm^2 (Fig. 1*B*). Resting neutrophils did not adhere to ICAM-1 at wall shear stresses of 0.36 dyn/cm^2 or above (Fig. 1*B*).

Neutrophils roll on purified E-selectin

At all site densities tested, neutrophils formed rolling adhesions after attachment to the E-selectin substrate. Neutrophils rolled because of the fluid drag forces acting on them in shear flow and would stop when the flow was stopped. Neutrophil rolling velocities varied inversely with E-selectin density (Fig. 2). Velocities on E-selectin were comparable whether neutrophils were bound during flow or bound at stasis and then subjected to flow. At all site densities of E-selectin, rolling velocities increased as a function of shear stress much more rapidly below 1 dyn/cm^2 than above this value. This was particularly evident at the highest site density examined, 885 sites/μm^2, where there was no increase in rolling velocity as wall shear stresses were increased above 3.6 dyn/cm^2 (Fig. 2). Up to 14.6 dyn/cm^2, neutrophil shape was unaffected by the fluid shear stress; thus a shape change cannot explain the plateau in rolling velocity. At 36 dyn/cm^2 wall shear stress, neutrophils visibly deformed, elongating slightly in the direction of flow. This was as expected for the behavior of a viscoelastic spherical body in shear flow and did not suggest any activation of the neutrophils. Furthermore, almost all the rolling neutrophils remained adherent (see Fig. 3 below), so the plateau effect was not because of skewing of the adherent population by detachment of a weakly bound subset of neutrophils. Compared with the velocity of a nonadherent cell in shear flow immediately adjacent to the wall (17, 28), the

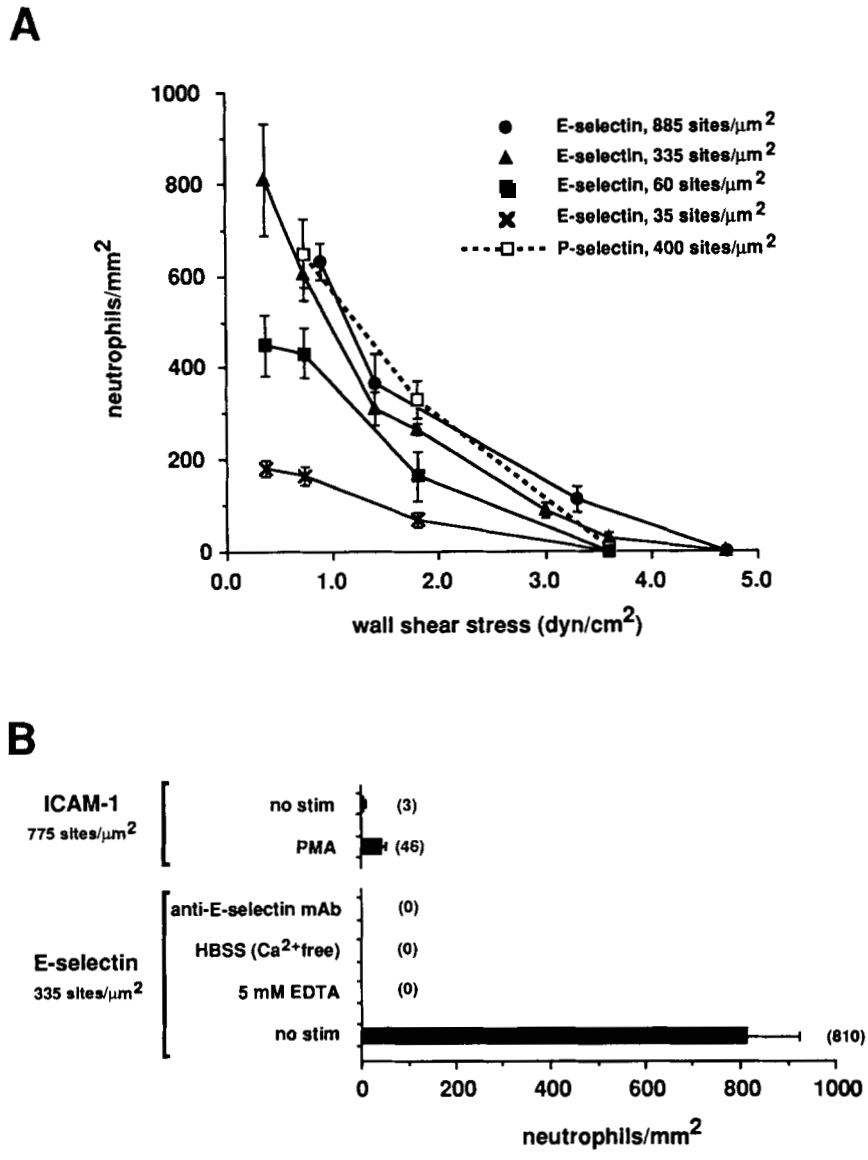

FIGURE 1. Attachment of neutrophils to E-selectin under flow conditions. (*A*) Resting neutrophils (1×10^6/ml) were infused at varying wall shear stresses through the parallel plate flow chamber. After 3 min of continuous flow at a given shear stress, the number of neutrophils binding to immobilized E-selectin was quantitated. (*B*) Neutrophil adhesion to E-selectin and ICAM-1 compared at 0.36 dyn/cm^2 wall shear stress. Recombinant soluble ICAM-1 and E-selectin was adsorbed onto the polystyrene plate to yield the indicated density. Neutrophils, untreated or treated with PMA (30 ng/ml) for 10 min at room temperature were perfused through the flow chamber and attachment after 3 min was quantitated. The number of attached neutrophils is shown in parentheses. For *A* and *B*, error bars represent the standard error for two to six independent experiments.

rolling velocities shown in Fig. 2 are 60 to 1,700-fold more slow, depending on the E-selectin density and shear stress.

Neutrophil-rolling velocities on E-selectin at a given shear stress were much lower than on P-selectin at comparable site densities (Fig. 2 and further data in (17)), suggesting either that the adhesive bonds dissociated more slowly or were more numerous. Neutrophil rolling was much steadier on E-selectin than on P-selectin, i.e., there was less variance over time in the velocity of individual cells. The lesser moment to moment variation in rolling velocity on E-selectin was suggestive of more adhesive bonds.

Strength of adhesion of neutrophils to E-selectin increases with site density and is greater than to P-selectin

A detachment assay that we have previously used to characterize neutrophil adherence to P-selectin and ICAM-1 (17) was used to examine the strength of neutrophil adhesion to E-selectin. Neutrophils were allowed to settle onto E-selectin substrates under static conditions. Neutrophils began to roll immediately after application of flow. The percentage of rolling neutrophils remaining adherent as a function of wall shear stress, i.e. the resistance to detachment is a measurement of the strength of adherence (26). Shear resistance was dependent on E-selectin density, suggesting that the density of E-selectin on the plate was related to the number of receptor-ligand bonds that could form (Fig. 3). On a site density basis, the rolling adhesions formed on E-selectin were much more resistant to detachment than rolling adhesions on purified P-selectin. The slower rolling velocities and the stronger adhesion of neutrophils on E-selectin than P-selectin contrasted with the equal efficiency of attachment in flow to E- and P-selectin.

Neutrophil adhesion to E-selectin was almost as strong as PMA- or fMLP-stimulated neutrophil adhesion to

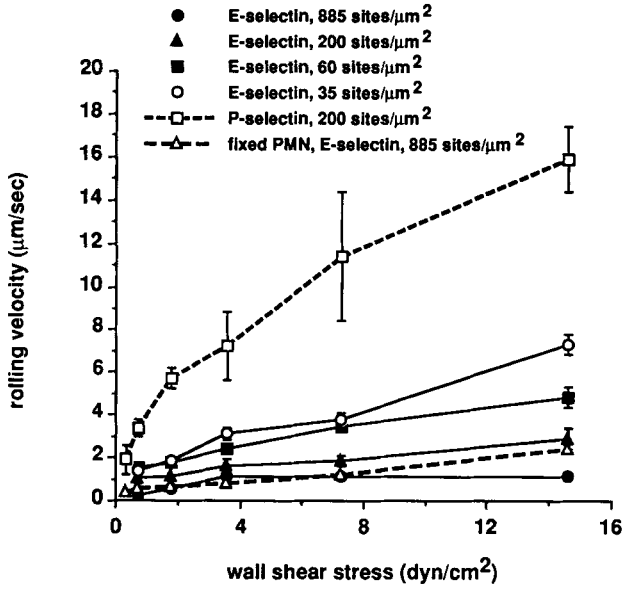

FIGURE 2. Rolling velocity as a function of E-selectin density and wall shear stress. Neutrophils which had either attached during flow or were bound during a static incubation were exposed to increasing levels of shear stress and their rolling velocities measured. Each point represents two to four independent experiments and a minimum of 180 bound neutrophils per data point. Broken lines with *open triangle* plot symbols represent rolling velocity of formaldehyde fixed neutrophils. *Broken lines with open squares* represent neutrophil rolling velocities on purified P-selectin included for comparison purposes. Bars show the standard error of the mean.

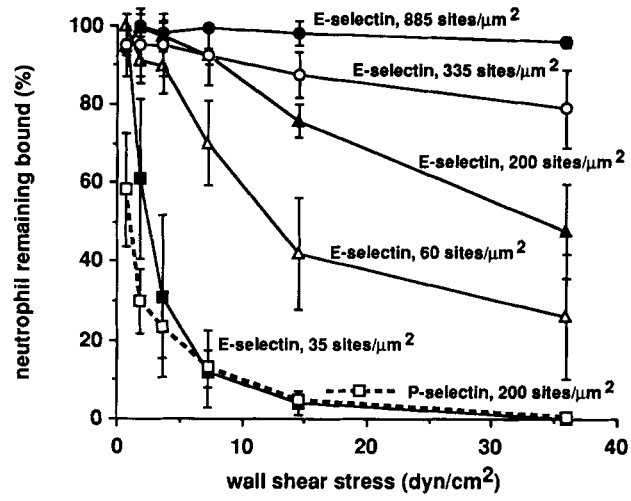

FIGURE 3. Detachment assay after static incubation of neutrophils on E-selectin at varying site densities. Neutrophils were injected through a port in the side of the flow chamber and allowed to settle onto E-selectin. After 2 min of contact under quiescent conditions, flow was started and increased in staged increments every 40 seconds. The percentage of neutrophils that remained bound and rolling on E-selectin was quantified after 20 seconds at each shear stress. *Broken lines* indicate shear resistance of neutrophils rolling on P-selectin (*open squares*) included for comparison purposes. Bars show the standard error for three to five independent experiments.

ICAM-1 at similar site densities (17). However, the arrest and spreading of PMA- and fMLP-treated neutrophils observed on ICAM-1 was in sharp contrast to rolling adhesions of resting neutrophils on E-selectin. Furthermore, resting neutrophils remained round while adherent to E-selectin (not shown), and showed no evidence of a shape change that would have been expected if they had become activated (29).

Lack of a requirement for cellular ATP in neutrophil rolling on E-selectin

Inhibition of energy metabolism by a combination of azide and deoxyglucose, which together potently inhibit leukocyte integrin function (30) had no effect on attachment efficiency or adhesion strength (Fig. 4*A,B*). Formaldehyde fixed neutrophils rolled on E-selectin at velocities comparable to untreated neutrophils, further indicating that cellular metabolism was not required for rolling (Fig. 2), though attachment in flow was partially inhibited (Fig. 4*A*). dbcAMP, which inhibits Ag receptor-triggered increase in LFA-1 avidity for ICAM-1 on lymphocytes (31), also had no effect on the interaction of resting neutrophils with E-selectin under flow conditions (Fig. 4*A*) or the detachment assay (Fig. 4*B*).

Effect of temperature on neutrophil rolling

The plateau effect on rolling velocity observed with increasing shear stress suggested that the dissociation rate of E-selectin with its neutrophil counter-receptor was controlling the rolling velocity. In bimolecular reactions, the forward rate typically drops less than the reverse rate as temperature is lowered, because the bound complex, having a lower Gibb's free energy than the unbound reactants, must adsorb more thermal energy than the unbound reactants to move into the transition state (32).

To examine the postulated dependence of bond formation and dissociation rates on rolling velocity, neutrophil rolling on E-selectin was measured as a function of temperature. To test this hypothesis, the temperature dependence of neutrophil rolling was measured by changing the temperature of the perfusion buffer. To minimize the effects of temperature on cellular processes, resting neutrophils were fixed in 0.5% paraformaldehyde at 20°C for 20 min. After fixation, neutrophils were able to roll at comparable velocities to resting neutrophils (Fig. 2) at 25°C. After rolling adhesions were initiated, the perfusion buffer at room temperature was changed to buffer chilled on ice at approximately 0°C. After perfusion of the cold buffer for 2 min (rinsing buffer exited the chamber at 3°C), rolling velocity dropped by 78% (Fig 5). Increasing the temperature of the perfusion buffer (to 17°C) resulted in an increase

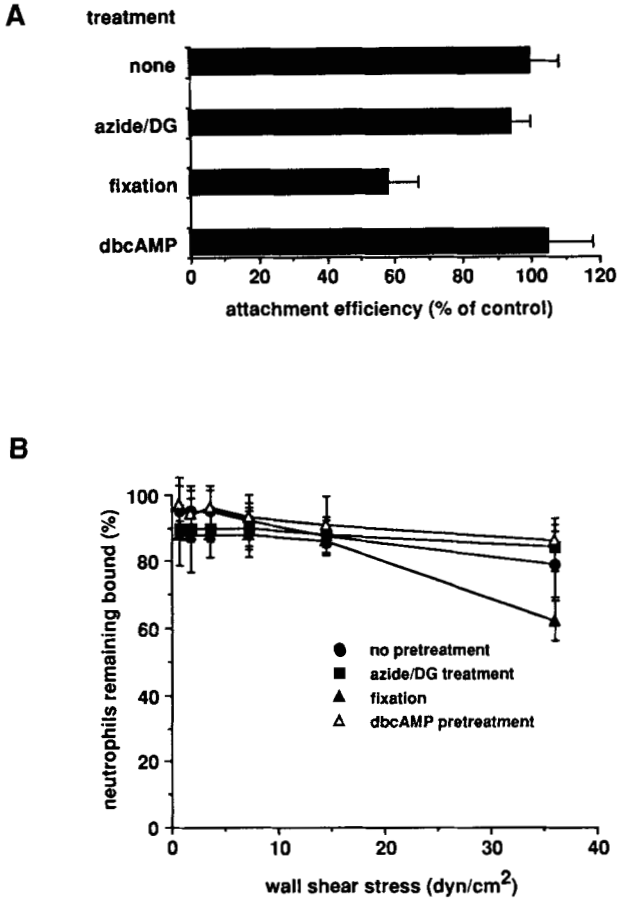

FIGURE 4. Effect of metabolic energy depletion and fixation on neutrophil attachment and rolling on E-selectin. Resting neutrophils were pretreated with either azide and deoxyglucose, dibutyryl cyclic AMP (dbcAMP), or fixed with formaldehyde as described in *Methods and Materials*. (*A*) Attachment of neutrophils (1×10^6/ml) infused at 1.8 dyn/cm^2 wall shear stress through the parallel plate flow chamber. After 3 min of continuous flow, the number of neutrophils binding to immobilized E-selectin (335 sites/μm^2) was determined, and the binding of treated neutrophils is presented as a percentage of untreated neutrophils. Control binding was 274 $\pm$ 37 neutrophils/mm^2. Bars represent the standard error of independent experiments. (*B*) Detachment assay after static incubation of neutrophils on E-selectin (335 sites/μm^2). After neutrophils contacted immobilized E-selectin for 2 min. under quiescent conditions, flow was started and increased in staged increments every 40 seconds. The percentage of neutrophils that remained bound and rolling on E-selectin was quantified after 20 seconds at shear stress. Bars represent the standard error of four independent experiments.

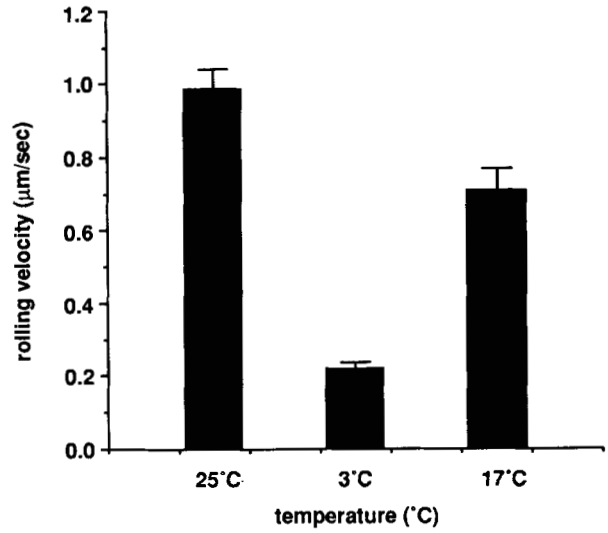

FIGURE 5. Effect of temperature on the rolling velocity of fixed neutrophils on E-selectin. Neutrophils were allowed to form rolling adhesions at 25°C on E-selectin at 885 sites/μm^2. Once rolling was established at a wall shear stress of 7.3 dyn/cm^2, flow was stopped momentarily and ice-cold buffer was substituted for room temperature buffer. After 3 min at 1.8 dyn/cm^2 wall shear stress, the temperature at the outlet was 3°C and the flow rate was raised to 7.3 dyn/cm^2 and neutrophil rolling velocity was measured. Rolling velocity was measured again as the temperature of the perfusion buffer approached room temperature. Error bars represent standard error of the mean for the two experiments.

in rolling velocity, recovering to 71% of the initial value. Rolling was completely calcium-dependent as infusion of EDTA (5 mM in the perfusion buffer) completely detached the bound neutrophils. This observation suggests that rolling velocity is not dependent on the rate of association of E-selectin with its counter-receptors, but instead dependent on the reverse rate of the reaction.

Discussion

We have demonstrated that under flow conditions, neutrophils attach to and form rolling adhesions on purified E-selectin. This observation allows some generalizations to be made about the adhesive characteristics of selectins. Previous studies with purified P-selectin in the same flow chamber as used here showed that it can mediate attachment and rolling at physiologic shear stress (17). Attachment and rolling of neutrophils on mesentery venules in vivo has been shown to be inhibited by mAb to L-selectin (6) and L-selectin immunoadhesins (5). Attachment at physiologic shear stress to stimulated endothelium has also been shown to be inhibited by L-selectin mAb in vitro (7). Thus, all three selectins can mediate attachment in flow and rolling.

Selectin expression is limited to cells that are found in the vasculature: leukocytes, platelets, and endothelial cells, suggesting that they are specialized for interactions within the confines of the lumen of the blood vessel. In particular, the ability of leukocytes to adhere to endothelium through selectins in contrast to the ineffectiveness of CD18 integrins under flow conditions confers an added degree of selectivity to leukocyte interactions within the vasculature. Selectivity is also conferred by differences in the cells on

which ligands for selectins are expressed, and the mechanisms that regulate selectin surface expression. For instance, E-selectin is absent from resting endothelium but is induced by inflammatory mediators such as TNF, IL-1, INF-γ, and LPS, both in vitro and in vivo (reviewed in (23)). Infiltration of neutrophils in acute inflammation in some animal models can be inhibited by E-selectin mAbs (21–23). E-selectin expression in models of delayed hypersensitivity has also been associated with the accumulation of a subset of T lymphocytes that bind to E-selectin (33, 34).

Neutrophil attachment to E-selectin was as rapid as that to P-selectin, suggesting the biophysical basis for attachment in flow is similar. The similar ability to attach to E- and P-selectin is consistent with the evidence that L-selectin on neutrophils presents carbohydrate to both E- and P-selectin (35). However, comparison of resistance to detachment of neutrophils on E- and P-selectin indicates significant differences in the strength of the rolling adhesions, suggesting differences between the neutrophil ligands for E- and P-selectin that mediate rolling. These differences are consistent with the report that the P-selectin ligand(s) on HL60 is protease sensitive, whereas the E-selectin ligand(s) is not (36).

Rolling adhesions are almost certainly explained by rapidly reversible receptor-ligand interactions. Cyclical activation and deactivation of ligand adhesiveness would require cellular ATP, and is ruled out by the lack of effect of energy deprivation on rolling and the ability of fixed neutrophils to roll. The decrease in rolling velocity with higher density of E-selectin, as with P-selectin, indicates that rolling is slowed by a higher number of receptor-ligand bonds.

The greater shear resistance and slower rolling velocities of neutrophils adherent to E-selectin than to comparable densities of P-selectin suggests either a greater number of receptor-ligand bonds or a slower bond dissociation rate. A greater number of receptor-ligand bonds is more compatible with our data on adhesion strengthening, and also the smoothness of the rolling, implying a shorter ratchet distance on E-selectin and the smoothing in statistical variations that occurs when the number of bonds is larger.

The much greater strength of rolling adhesions on E-selectin than P-selectin, despite similar attachment efficiencies, suggests that after attachment, a class of ligands comes into play for E-selectin that strengthens rolling adhesions. We have obtained evidence that E-selectin ligands for attachment and rolling are distinct. L-selectin on the neutrophil is required for attachment, but not for rolling adhesions, inasmuch as mAb to L-selectin, shedding of L-selectin stimulated by chemoattractant or phorbol esters, and release of L-selectin with chymotrypsin inhibits attachment but not the strength of rolling adhesions (Lawrence and Springer, submitted). L-selectin functions in attachment by presenting a carbohydrate ligand to E-selectin, consistent with a report that it bears sialyl Le^x and can function as an E-selectin ligand (35). Because neutrophils roll on varying densities of E-selectin at 1/60 to 1/1,700 of the velocity of nonadherent cells flowing immediately adjacent to the substrate, attachment increases the time available for interactions between E-selectin and ligands. Thus, interactions with kinetic rate constants that are 2 to 3 orders of magnitude slower than those required for attachment in flow can contribute to rolling adhesions, resulting in adhesion strengthening. Because of rolling, the spot of initial adhesion on the neutrophil surface will no longer be in contact with E-selectin after a time period of 0.1 to 1 s, and thus adhesion strengthening must occur on a time scale at least this rapid.

The nature of adhesion strengthening through E-selectin contrasts with that through other adhesion pathways. Adhesion of the Ig family CD2 molecule on T lymphocytes to the Ig family LFA-3 molecule requires 2 to 5 min for strengthening and is dependent on membrane fluidity (26). After addition of a chemoattractant to neutrophils rolling on a substrate containing P-selectin and ICAM-1, arrest of the rolling cells through integrin binding to ICAM-1 occurs after 2.5 to 5 min, reflecting the time required for the chemotactic receptor to trigger integrin avidity increase and for LFA-1 and Mac-1 to bind to ICAM-1 (17). The shear resistance of neutrophils adhering to E-selectin was almost as great as that of activated neutrophils adhering to ICAM-1 (17) and greater than that of T lymphocytes adhering to LFA-3 (26); however, at no site densities of ICAM-1 or LFA-3 was rolling ever observed, even during the process of detachment by shear of adherent cells. Thus, E selectin can develop adhesive strength as great as that of members of other protein families, yet differs in requiring far less time for adhesion strengthening and in the rolling nature of the adhesion. Furthermore, adhesion strengthening through E-selectin is not inhibited by azide +2-deoxyglucose or affected by fixation, and thus does not require active metabolism in contrast to β_2 integrin mediated adhesive interactions.

We found that rolling velocity plateaus on E-selectin at a value dependent on E-selectin density; a plateau in velocity was also reported for neutrophils rolling on rat mesenteric venules in vivo (37). One possible explanation for the plateau effect is that the normal force induced by the fluid shear enlarges the contact area with the substrate and allows more bonds to form, leading to a lower rolling velocity. However, fixed neutrophils, which are far more rigid than viable neutrophils, had an almost identical plateau in rolling velocity, suggesting that neutrophil deformation is not a major factor in this assay system. Another explanation is that the off rate of E-selectin with its counter receptor limits the rolling velocity. Lowering temperature slowed the rolling of fixed neutrophils in spite of the increase in fluid viscosity caused by the drop in temperature. These

findings suggest that at lower temperature k_{off} was lowered more than k_{on}. A drop in the off-rate, by preventing the neutrophil from ratcheting forward as quickly, would be expected to have a great impact on the rolling velocity. However, the total number of bonds would also be predicted to be greater when k_{off} is lowered more than k_{on}, and would also affect rolling velocity.

Previous studies in vitro and in vivo have suggested that selectins, chemoattractants, and integrins act sequentially in leukocyte interaction with the vessel wall and transendothelial migration (6, 17, 38). Our finding that E-selectin, like P-selectin supports attachment in flow and rolling generalizes this model to include all three selectins. E-selectin may play a unique part among the selectins because it mediates stronger adhesions and slower rolling than P-selectin or L-selectin. E-selectin's greater adhesion strength may compensate in part for its delayed surface expression compared with P-selectin during the inflammatory process. Furthermore, the slower rolling may lead to more tightly localized neutrophil accumulation as the inflammatory reaction progresses.

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References

1. Stoolman, L. M. 1989. Adhesion molecules controlling lymphocyte migration. *Cell 56:907.*
2. Springer, T. A. 1990. Adhesion receptors of the immune system. *Nature 346:425.*
3. Lasky, L. A., M. S. Singer, D. Dowbenko, Y. Imai, W. J. Henzel, C. Grimley, C. Fennie, N. Gillett, S. R. Watson, and S. D. Rosen. 1992. An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell 69:927.*
4. Lewinsohn, D. M., R. F. Bargatze, and E. C. Butcher. 1987. Leukocyte-endothelial cell recognition: Evidence of a common molecular mechanism shared by neutrophils, lymphocytes, and other leukocytes. *J. Immunol. 138:4313.*
5. Ley, K., P. Gaehtgens, C. Fennie, M. S. Singer, L. A. Lasky, and S. D. Rosen. 1991. Lectin-like cell adhesion molecule 1 mediates leukocyte rolling in mesenteric venules in vivo. *Blood 77:2553.*
6. von Andrian, U. H., J. D. Chambers, L. M. McEvoy, R. F. Bargatze, K-E. Arfors, and E. C. Butcher. 1991. Two-step model of leukocyte-endothelial cell interaction in inflammation: Distinct roles for LECAM-1 and the leukocyte b_2 integrins in vivo. *Proc. Natl. Acad. Sci. USA 88:7538.*
7. Smith, C. W., T. K. Kishimoto, O. Abbass, B. Hughes, R. Rothlein, L. V. McIntire, E. Butcher, and D. C. Anderson. 1991. Chemotactic factors regulate lectin adhesion molecule 1 (LECAM-1)-dependent neutrophil adhesion to cytokine-stimulated endothelial cells in vitro. *J. Clin. Invest. 87:609.*
8. Spertini, O., F. W. Luscinskas, G. S. Kansas, J. M. Munro, J. D. Griffin, M. A. Gimbrone, Jr., and T. F. Tedder. 1991. Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion. *J. Immunol. 147:2565.*
9. Larsen, E., A. Celi, G. E. Gilbert, B. C. Furie, J. K. Erban, R. Bonfanti, D. D. Wagner, and B. Furie. 1989. PADGEM protein: A receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell 59:305.*
10. Geng, J. G., M. P. Bevilacqua, K. L. Moore, T. M. McIntyre, S. M. Prescott, J. M. Kim, G. A. Bliss, G. A. Zimmerman, and R. P. McEver. 1990. Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. *Nature 343:757.*
11. McEver, R. P., J. H. Beckstead, K. L. Moore, L. Marshall-Carlson, and D. F. Bainton. 1989. GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J. Clin. Invest. 84:92.*
12. Bevilacqua, M. P., J. S. Pober, D. L. Mendrick, R. S. Cotran, and M. A. Gimbrone. 1987. Identification of an inducible endothelial-leukocyte adhesion molecule, E-LAM 1. *Proc. Natl. Acad. Sci. USA 84:9238.*
13. Arfors, K.-E., C. Lundberg, L. Lindborm, K. Lundberg, P. G. Beatty, and J. M. Harlan. 1987. A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage in vivo. *Blood 69:338.*
14. Atherton, A., and G. V. R. Born. 1972. Quantitative investigation of the adhesiveness of circulating polymorphonuclear leucocytes to blood vessel walls. *J. Physiol. 222:447.*
15. Cohnheim, J. 1889. *Lectures on General Pathology: A Handbook for Practitioners and Students.* The New Sydenham Society, London.
16. Marchesi, V. T. 1961. The site of leucocyte emigration during inflammation. *Q. J. Exp. Physiol. 46:115.*
17. Lawrence, M. B. and T. A. Springer. 1991. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell 65:859.*
18. Smith, C. W., R. Rothlein, B. J. Hughes, M. M. Mariscalco, F. C. Schmalstieg, and D. C. Anderson. 1988. Recognition of an endothelial determinant for CD18 - dependent neutrophil adherence and transendothelial migration. *J. Clin. Invest. 82:1746.*
19. Lawrence, M. B., C. W. Smith, S. G. Eskin, and L. V. McIntire. 1990. Effect of venous shear stress on CD18-mediated neutrophil adhesion to cultured endothelium. *Blood 75(1):227.*
20. Kishimoto, T. K., M. A. Jutila, E. L. Berg, and E. C. Butcher. 1989. Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. *Science 245:1238.*
21. Mulligan, M. S., J. Varani, M. K. Dame, C. L. Lane, C. W. Smith, D. C. Anderson, and P. A. Ward. 1991. Role of endothelial-leukocyte adhesion molecule 1 (ELAM-1) in neutrophil-mediated lung injury in rats. *J. Clin. Invest. 88:1396.*
22. Gundel, R. H., C. D. Wegner, C. A. Torcellini, C. C. Clarke, N. Haynes, R. Rothlein, C. W. Smith, and L. G. Letts. 1991. Endothelial leukocyte adhesion molecule-1 mediates antigen-induced acute airway inflammation and late-phase airway obstruction in monkeys. *J. Clin. Invest. 88:1407.*
23. Munro, J. M., J. S. Pober, and R. S. Cotran. 1991. Recruitment of neutrophils in the local endotoxin response: Association with de novo endothelial expression of endothelial leukocyte adhesion molecule-1. *Lab. Invest. 64:295.*

24. Lobb, R. R., G. Chi-Rosso, D. R. Leone, M. D. Rosa, S. Bixler, B. M. Newman, S. Luhowskyj, C. D. Benjamin, I. R. Dougas, S. E. Goelz, C. Hession, and E. P. Chow. 1991. Expression and functional characterization of a soluble form of endothelial-leukocyte adhesion molecule 1. *J. Immunol. 147:124.*
25. Marlin, S. D., D. E. Staunton, T. A. Springer, C. Stratowa, W. Sommergruber, and V. Merluzzi. 1990. A soluble form of intercellular adhesion molecule-1 inhibits rhinovirus infection. *Nature 344:70.*
26. Chan, P. -Y., M. L. Lawrence, M. L. Dustin, L. M. Ferguson, D. E. Golan, and T. A. Springer. 1991. Influence of receptor lateral mobility on adhesion strengthening between membranes containing LFA-3 and CD-2. *J. Cell Biol. 115:245.*
27. Miller, L. J., D. F. Bainton, N. Borregaard, and T. A. Springer. 1987. Stimulated mobilization of monocyte Mac-1 and p150,95 adhesion proteins from an intracellular vesicular compartment to the cell surface. *J. Clin. Invest. 80:535.*
28. Goldman, A. J., R. G. Cox, and H. Brenner. 1967. Slow viscous motion of a sphere parallel to a plane wall–II. Couette flow. *Chem. Engineer. Sci. 22:653.*
29. Lo, S. K., S. Lee, R. A. Ramos, R. Lobb, M. Rosa, G. Chi-Rosso, and S. D. Wright. 1991. Endothelial-leukocyte adhesion molecule 1 stimulates the adhesive activity of leukocyte integrin CR3 (CD11b/CD18, Mac-1, $a_m b_2$) on human neutrophils. *J. Exp. Med. 173:1493.*
30. Marlin, S. D., and T. A. Springer. 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell 51:813.*
31. Dustin, M. L., and T. A. Springer. 1989. T cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature 341:619.*
32. Mason, D. W. and A. F. Williams. 1986. Kinetics of antibody reactions and the analysis of cell surface antigens. In *Handbook of Experimental Immunology, 4th ed., Vol.I: Immunochemistry* . 4th ed. D.M. Weir, L.A. Herzenberg and C. Blackwell, eds. Blackwell, Oxford, p. 38.1.
33. Picker, L. J., S. A. Michie, L. S. Rott, and E. C. Butcher. 1990. A unique phenotype of skin-associated lymphocytes in humans: preferential expression of the HECA-452 epitope by benign and malignant T cells at cutaneous sites. *Am. J. Pathol. 136:1053.*
34. Picker, L. J., T. K. Kishimoto, C. W. Smith, R. A. Warnock, and E. C. Butcher. 1991. ELAM-1 is an adhesion molecule for skin-homing T cells. *Nature 349:796.*
35. Picker, L. J., R. A. Warnock, A. R. Burns, C. M. Doerschuk, E. L. Berg, and E. C. Butcher. 1991. The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. *Cell 66:921.*
36. Larsen, G. R., D. Sako, T. J. Ahern, M. Shaffer, J. Erban, S. A. Sajer, R. M. Gibson, D. D. Wagner, B. C. Furie, and B. Furie. 1992. P-selectin and E-selectin: distinct but overlapping leukocyte ligand specificities. *J. Biol. Chem. 267:11104.*
37. Atherton, A., and G. V. R. Born. 1973. Relationship between the velocity of rolling granulocytes and that of the blood flow in venules. *J. Physiol. 233:157.*
38. Butcher, E. C. 1991. Leukocyte-endothelial cell recognition: Three (or more) steps to specificity and diversity. *Cell 67:1033.*