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Neutrophil Tethering to and Rolling on E-selectin Are Separable by Requirement for L-selectin

Michael B. Lawrence,* Dorothy F. Bainton,† and Timothy A. Springer*
*Center for Blood Research, Incorporated
and the Department of Pathology
Harvard Medical School
Boston, Massachusetts 02115
†Department of Pathology
University of California, San Francisco
San Francisco, California 94143

Summary

Neutrophil tethering and rolling in shear flow are mediated by selectins and have been thought to be two indistinguishable manifestations of a single molecular interaction between selectin and ligand. However, we report that under physiologic flow conditions, tethering to E-selectin requires a ligand distinct from the one that supports neutrophil rolling. Tethering under shear to E-selectin requires a carbohydrate ligand that is closely associated with the lectin domain of L-selectin on the neutrophil surface, as enzymatic removal of L-selectin, chemotactic factor–induced shedding of L-selectin, and L-selectin MAbs effectively block tethering. In contrast, this ligand is dispensable for the ability to roll on E-selectin, since rolling adhesions formed after static incubations were not affected by the presence or absence of L-selectin. Thus, E-selectin interactions with ligands on neutrophils persist after L-selectin shedding. These findings add an additional step for regulation of leukocyte localization in inflammatory sites.

Introduction

Using intravital microscopy and in vitro flow chambers, selectin molecules have been shown to mediate tethering and rolling of leukocytes in shear flow. This is the first of at least three steps required for leukocyte localization in inflammatory sites (Lawrence and Springer, 1991; Ley et al., 1991; von Andrian et al., 1991; von Andrian et al., 1992; Kansas et al., 1993). Tethering through selectins is a prerequisite in physiologic shear flow for activation-induced adhesiveness through integrins, which results in the arrest of rolling cells. L-selectin is expressed on all circulating leukocytes and mediates adhesion under shear conditions to both the endothelium of postcapillary venules and the high endothelial venules of peripheral lymph nodes (Ley et al., 1991; von Andrian et al., 1991; Lewinsohn et al., 1987; Smith et al., 1991; Spertini et al., 1991d). Early in the inflammatory process, P-selectin, stored preformed in the Weibel–Palade bodies of endothelial cells and the α granules of platelets, can be mobilized to the plasma membrane to bind flowing neutrophils and monocytes (Larsen et al., 1989; Geng et al., 1990; McEver et al., 1989; Mayadas et al., 1993; Buttrum et al., 1993). A few hours later, E-selectin is induced on vascular endothelial cells by cytokines, requiring de novo protein synthesis, and has recently been shown to mediate neutrophil rolling at venular shear stresses in vitro (Bevilacqua et al., 1987; Lawrence and Springer, 1993).

All three selectins recognize sialylated carbohydrate determinants on their counterreceptors (Lasky, 1992) that are closely related to sialyl Lewis^x; removal of sialic acid greatly diminishes ligand activity (Larsen et al., 1989; Polley et al., 1991). L-selectin binds to at least two sialylated glycoproteins: one, the mucin-like GlyCAM-I on high endothelial venules (Lasky et al., 1992), and a more broadly distributed glycoprotein, sgp 90, now known to be CD34 (Baumhueter et al., 1993). Both E-selectin and P-selectin coupled to affinity matrices have allowed isolation of specific glycoproteins from the neutrophil surface that appear to be counterreceptors (Levinovitz et al., 1993; Moore et al., 1992; Sako et al., 1993). In the mouse, a neutrophil glycoprotein that binds to E-selectin–immunoglobulin chimeras has been characterized as to M_r and is distinct from L-selectin; but has not been studied in cell adhesion assays (Levinovitz et al., 1993). In the human, multiple glycoproteins, including the P-selectin glycoprotein ligand and L-selectin, can express E-selectin ligands (Moore et al., 1992; Sako et al., 1993; Picker et al., 1991).

There is some controversy about the identity of the E-selectin ligand on neutrophils. Based on nonadditive inhibition by E-selectin and L-selectin of neutrophil binding to cytokine-stimulated endothelium (Kishimoto et al., 1991; Picker et al., 1991), it has been suggested that L-selectin binds to E-selectin. Picker et al. have proposed that L-selectin presents sialyl Lewis^x to E-selectin by virtue of its preferential localization on the tip of microvilli of neutrophils, thereby mediating adhesion. In support of this, they showed that neuraminidase treatment destroys the E-selectin ligand activity of neutrophils (Picker et al., 1991). Nevertheless, neutrophil binding to E-selectin transfectants is only inhibited 50% by L-selectin MAbs, implying the existence of other ligands on the neutrophil surface (Kishimoto et al., 1991). Other investigators have found additive inhibition by L-selectin and E-selectin MAbs of neutrophil binding to cytokine-stimulated endothelium (Spertini et al., 1991d), which suggests that E-selectin and L-selectin do not bind to each other.

We hypothesized that the inconsistencies in the literature may be due to differences in shear conditions in the adhesion assays, particularly if there were multiple E-selectin ligands on the neutrophil surface. Both tethering and rolling involve selectin-carbohydrate ligand interactions, and have been assumed to involve the same ligand on the neutrophil surface. We demonstrate here that tethering and rolling on E-selectin are separable steps and involve distinct ligands on the neutrophil surface as assayed in a controlled shear adhesion assay. Expression of L-selectin on the neutrophil surface is required for efficient tethering to E-selectin, and this interaction is only detectable in the dynamic setting of a shear flow. Rolling on

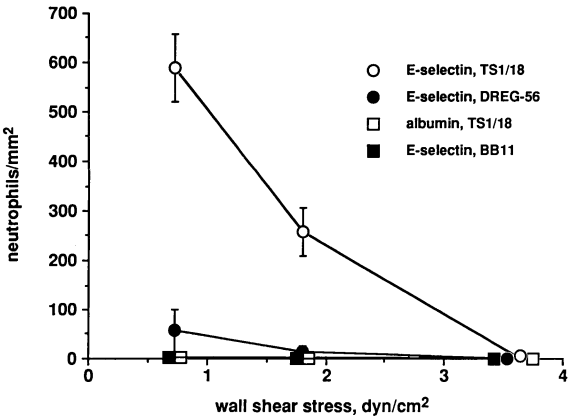

Figure 1. Effect of L-Selectin MAb on Neutrophil Tethering in Shear Flow to E-Selectin

A neutrophil suspension (10^6/ml) containing and pretreated 10 min with L-selectin MAb DREG-56 (10 μg/ml) or binding control CD18 MAb TS1/18 (10 μg/ml) was perfused through a flow chamber containing immobilized E-selectin or albumin. Results in presence of CD18 MAb or buffer alone were identical (data not shown). After 3 min of flow at the indicated shear stress, the number of adherent neutrophils was quantitated. Bars show SEM of two experiments.

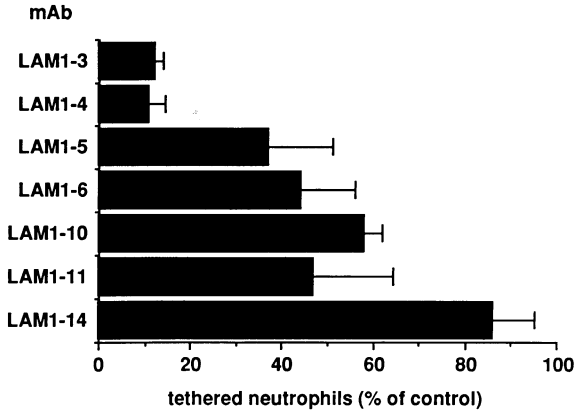

Figure 2. Effect of a Panel of L-Selectin MAb on Neutrophil Tethering to E-Selectin in Flow

Neutrophils treated with the LAM series of L-selectin MAb (2.5 μg/ml) for 10 min (Kishimoto et al., 1991; Picker et al., 1991) were perfused through the chamber at a wall shear stress of 1.8 dyn/cm². Site density of E-selectin was 335/μm², and tethered cells were counted as described in the legend to Figure 1. Control binding was 197 ± 25 neutrophils per mm². Bars show the SEM of two experiments.

E-selectin is mediated by a distinct receptor that is sialylated and protease resistant.

Results

L-Selectin Expression Is Required for Neutrophil Tethering to E-Selectin in Flow

To study neutrophil interactions under flow conditions with E-selectin in the absence of influences from other components of the endothelial cell surface, neutrophil adhesion to purified E-selectin was quantified at defined wall shear stresses (1–30 dyn/cm²) within the range found in postcapillary venules, the site of leukocyte tethering and emigration in vivo (Lawrence and Springer, 1991, 1993). We observed that neutrophils tethered to E-selectin at venular shear stresses (Figure 1) and rolled in response to fluid drag forces (Lawrence and Springer, 1993). Thus E-selectin, as well as P-selectin (Lawrence and Springer, 1991; Doré et al., 1993; Mayadas et al., 1993) and L-selectin (Ley et al., 1991; von Andrian et al., 1991; Smith et al., 1991) can mediate neutrophil tethering in shear, and form labile adhesions that permit rolling.

L-selectin or a carbohydrate presented by L-selectin on neutrophils has been variously reported to contribute or not to contribute to adhesive interactions with E-selectin. We wished to ascertain the physiologic significance of this interaction by examining it under physiologic shear conditions. At venular levels of wall shear stress, we found that tethering of flowing neutrophils to purified E-selectin was inhibited more than 90% by L-selectin MAb DREG-56, whereas a binding control CD18 MAb had no effect (Figure 1). A blocking E-selectin MAb was completely inhibitory, indicating that the interaction observed was E-selectin dependent. Neutrophils did not tether to albumin adsorbed to polystyrene, also indicating that tethering under flow conditions depends on the presence of E-selectin. Reactivity of DREG-56 and TS1/18 was confirmed by flow cytometry (data not shown).

Effects of a Panel of L-Selectin MAb on Neutrophil Tethering to E-Selectin

To ascertain what epitopes of L-selectin were important for tethering to E-selectin, we tested a panel of purified L-selectin MAbs (provided by Dr. T. Tedder). Of a panel of MAbs to distinct epitopes on L-selectin (Spertini et al., 1991c, 1991d; Kansas et al., 1991), the LAM1-3 and LAM1-4 MAbs were effective inhibitors of tethering (Figure 2). These MAbs map to the same epitope in the lectin domain as DREG-56 and also inhibit lymphocyte binding to high endothelial venules and PPME. MAbs to other epitopes on the lectin or EGF domains of L-selectin gave intermediate levels of inhibition, whereas a MAb to the short consensus repeats, LAM1–14, was without effect. Based on these inhibitory effects, the epitope of L-selectin that mediates tethering to E-selectin under flow conditions resides in the N-terminal lectin domain.

Inhibition by MAbs to L-selectin was not related to an effect on neutrophil shape. No shape change was observed with either LAM1-3, LAM1-4, or DREG-56 (Figures 3a–3b; data not shown). Some neutrophil shape change was observed after incubations with the noninhibitory MAb LAM1-14 and the partially inhibitory MAb LAM1-5 (data not shown). As previously observed (Picker et al., 1991; Erlandsen et al., 1993), we confirmed the localization of L-selectin mainly on the tips of microvilli of neutrophils treated with MAb to L-selectin under the same conditions as used in flow assays (Figure 4).

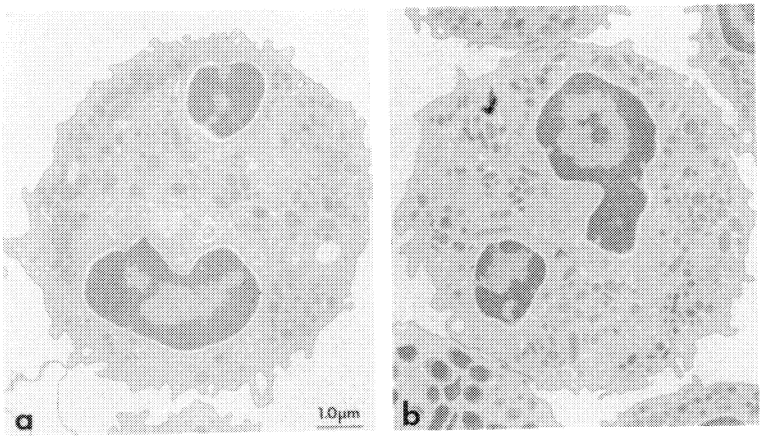

Figure 3. Shape of Neutrophils Treated with L-Selectin MAb

Neutrophils were incubated in MAb for L-selectin for 10 min at 22°C and then fixed in 1.5% glutaraldehyde and processed for electron microscopic examination as described in Experimental Procedures.

in (a), the control in which no antibody was added, the shape of the cell is illustrated and demonstrates that the plasma membrane is covered with numerous microvilli of varying lengths.

(b) MAb DREG-56, resulted in morphology similar to the control. Only minimal changes were noted using MAb LAM 1-4. The most severe shape changes were seen with MAb LAM 1-14 in which 20%–30% of the cells had polarized and had longer cytoplasmic extensions (data not shown). Magnification, 5600×.

L-Selectin MAbs Do not Inhibit Neutrophil Rolling on E-Selectin

To characterize further the role of L-selectin in neutrophil adhesion to E-selectin, we assayed the strength of neutrophil rolling adhesions in a detachment assay (Chan et al., 1991; Lawrence and Springer, 1991, 1993). The number of neutrophils that remained adherent following a static incubation was quantitated as the shear stress was increased, giving a measure of the strength of adhesion to E-selectin. Under these conditions, all of the adherent neutrophils rolled. By contrast with the results of the assay of tethering in flow, MAbs to L-selectin did not inhibit rolling, and had no significant effect on the percentage of neutrophils bound or their strength of adhesion (Figure 5A). A blocking MAb to E-selectin (BB11) was completely inhibitory, as no adhesion or rolling of neutrophils was detectable on E-selectin substrates pretreated with the MAb, nor was there any adhesion to the albumin-blocked substrate. As a further control on the specificity of the interaction with E-selectin, Hank's balanced salt solution (HBSS) plus EDTA (5 mM) was infused after neutrophils had adhered and begun rolling on E-selectin. All bound

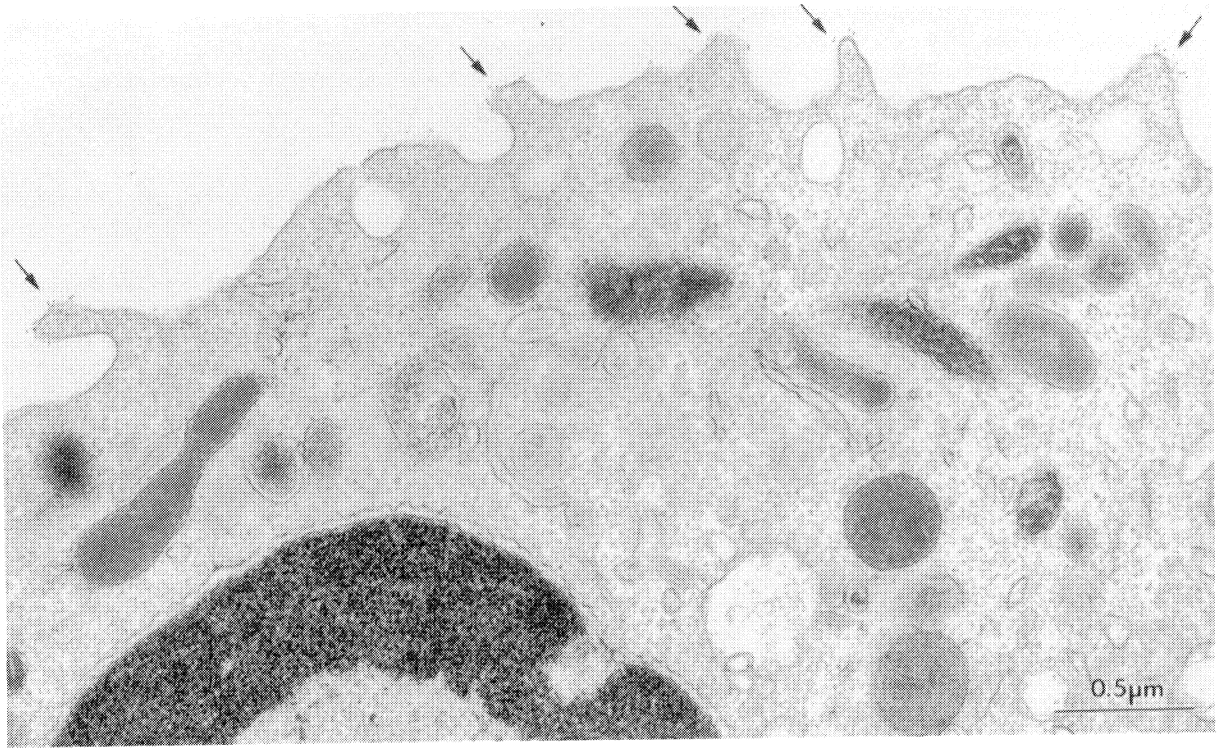

Figure 4. Immunocytochemical Labeling of L-Selectin Using MAb DREG-200 on Human Neutrophils

Note that the majority of gold label is on the tips of microvilli (arrows). Magnification, 35,200×.

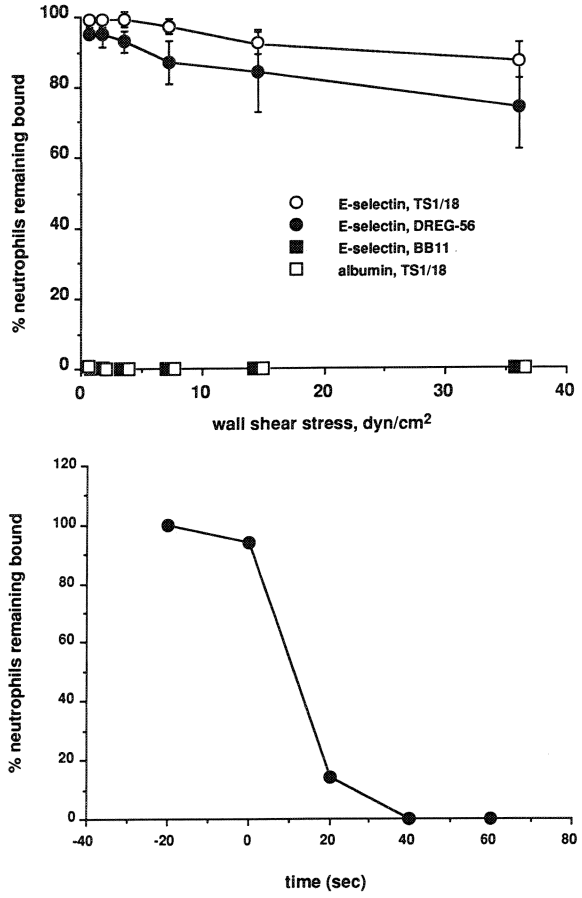

Figure 5. L-Selectin MAb DREG-56 Fails to Inhibit Neutrophil Adhesion and Rolling on E-Selectin following a Static Incubation

(A) Neutrophils (2×10^6/ml) pretreated 10 min with 10 μg/ml MAb were injected through a port in the side of the flow chamber and allowed to settle onto E-selectin under quiescent conditions for 2 min. L-selectin MAb DREG-56 was present continuously during the experiment. Pretreatment of the substrate with E-selectin MAb BB11 (10 μg/ml) was for 5 min before neutrophil addition. Flow with buffer containing the same MAb used for pretreatment was initiated and increased every 20 s; the number of neutrophils remaining tethered at the end of each interval was quantitated. Bars show SEM of three independent experiments.

(B) Kinetics of EDTA mediated release of neutrophils rolling on E-selectin. Assay media containing 5 mM EDTA mixed with a dilute suspension of erythrocytes to mark the arrival of the EDTA solution was perfused through the flow chamber after neutrophils had adhered and had been rolling for 5 min on E-selectin. Complete removal of bound neutrophils indicates that the interaction was completely calcium-dependent. Specificity of neutrophil adhesion to and rolling on E-selectin was confirmed by infusion of EDTA in every measurement of adhesion. Neutrophil adhesion to incompletely blocked plasitic was not dependent on calcium. Data shown representative of 50 determinations.

neutrophils were released from the substrate within approximately 10 s of exposure to HBSS plus EDTA (Figure 5B).

Shedding and Enzymatic Removal of L-Selectin also Inhibits Tethering to E-Selectin, but Does not Weaken Rolling Adhesions Formed on E-Selectin under Static Conditions

Shedding of L-selectin from the neutrophil surface following chemotactic activation may be important in releasing

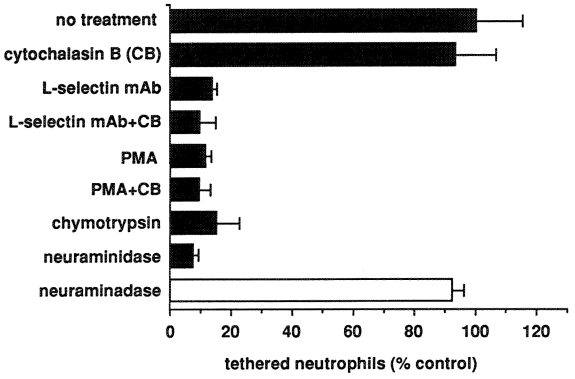

Figure 6. Inhibition by PMA Activation, Chymotrypsin Treatment, L-Selectin MAb, and Neuraminidase Treatment of Neutrophil Tethering to E-Selectin

Neutrophils (closed bars) were untreated or treated with L-selectin MAb DREG-56 as described in Figure 1: PMA (30 ng/ml) for 10 min at 21°C, chymotrypsin (4 U/ml) for 10 min in HBSS, 0.5% HSA at 21°C, Vibrio cholerae neuraminidase (0.1 U/ml) in HBSS, 2 mM Ca^{2+} for 30 min at 21°C, and washed twice. Some treatments were followed by a wash with 5 mM EDTA in HBSS, and resuspension in 5 mM EDTA, 20 μM cytochalasin B, in HBSS at 4°C for 20 min, and two washes before the flow assay. The E-selectin substrate (open bar) was treated with Vibrio cholerae neuraminidase (0.1 U/ml) in HBSS, 2 mM Ca^{2+} for 30 min at 21°C. Other methods were as in Figure 2A. Control binding of untreated neutrophils was 223 ± 34/mm² after 3 min of flow. Bars are SEM of three to five experiments.

selectin–ligand interactions so that transendothelial migration can occur (Kishimoto et al., 1989). Therefore, we examined the effect of activation-induced L-selectin shedding, or alternatively, enzymatic release of L-selectin, on neutrophil interactions with E-selectin (von Andrian et al., 1992; Picker et al., 1991). Chymotrypsin treatment of neutrophils strongly inhibited tethering at a venular shear stress of 1.8 dyn/cm² (Figure 6), similar to the effects of L-selectin MAbs on tethering. However, if neutrophils were allowed to tether to E-selectin under static conditions, and were subsequently subjected to flow, chymotrypsin-mediated removal of L-selectin did not weaken adhesion, similar to the lack of effect of L-selectin MAbs on strength of adhesion (Figure 7). These effects correlated with 89% release by chymotrypsin of L-selectin and little or no release of structures bearing sialyl Le^x (Table 1).

Phorbol myristate acetate (PMA) stimulation of neutrophils resulted in L-selectin shedding (Kishimoto et al., 1989) (Table 1), inhibition of neutrophil tethering in flow, and reduction of the strength of rolling adhesions (see Figure 6, Figure 7). The effects of PMA on E-selectin interactions may be influenced by the change in shape of neutrophils from round to bipolar (Figure 8D). To reduce the possible influence of neutrophil shape change, PMA-treated neutrophils were washed, incubated with EDTA and cytochalasin B (CB), and washed to restore them to a spherical morphology (Figure 8E). Following CB and PMA treatment, neutrophils were still unable to tether (see Figure 6) but in contrast with neutrophils only treated with PMA, they rolled normally and bound almost as strongly as resting neutrophils (see Figure 7). Neither treatment with chymotrypsin nor the blocking L-selectin MAb used here had an effect on neutrophil shape, as verified by light

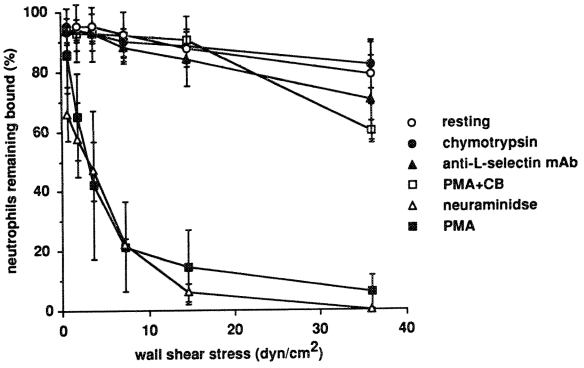

Figure 7. Effect of Treatments on Strength of Rolling Adhesions on E-Selectin

Neutrophils treated as in Figure 6 were allowed to settle onto an E-selectin substrate (335 sites/μm^2) under quiescent conditions and, after 2 min, were exposed to increasing levels of shear stress and the percentage of remaining bound cells was quantified as in Figure 5A. Bars show SEM of three to six experiments.

microscopy (see Figures 6A, 6B, and 6C) and transmission electron microscopy (see Figure 3). Further, tethering to E-selectin had no effect on neutrophil shape (Figures 8A and 8B).

Requirement for Sialic Acid Residues for Neutrophil Tethering to and Rolling on E-Selectin

Neuraminidase abolishes the activity of ligands for both L-selectin (Lasky et al., 1992; Spertini et al., 1991d) and E-selectin (Lasky, 1992) and was used to test whether L-selectin was presenting a carbohydrate ligand to E-selectin, or acting as a receptor by binding a carbohydrate ligand on E-selectin. Expression of sialyl Lewis^x epitopes on neutrophils was almost completely eliminated by neuraminidase treatment based on reactivity of MAb CSLEX1 (Table 1). Consistent with the importance of sialyl Lewis^x in E-selectin ligand recognition, neuraminidase treatment greatly diminished neutrophil adhesion to E-selectin (see Figure 7), even after contact under static conditions. Rolling on E-selectin apparently requires a sialylated ligand. Neuraminidase treatment of neutrophils also inhibited tethering to E-selectin under flow conditions, while treatment of E-selectin with neuraminidase had no effect on tethering (see Figure 6). Thus, L-selectin or an associated molecule displays a sialylated carbohydrate ligand that is important in tethering to E-selectin.

Discussion

We have found that neutrophil adhesion to E-selectin in shear flow can be separated into two steps. The first step, the initial tethering event in flow, and the second step, rolling, are mediated by distinct ligands on the neutrophil surface. L-selectin expression is required for neutrophils to tether to purified E-selectin at venular shear stresses. However, we were able to separate the tethering in flow from rolling by allowing neutrophils to interact with E-selectin under static conditions, then assaying neutrophil rolling on E-selectin by imposing controlled fluid shear stress. L-selectin was not required for rolling on E-selectin. The receptor that supports rolling is distinct from L-selectin, as inhibition with MAb, enzymatic removal, or shedding of L-selectin virtually abolishes tethering in flow, while failing to affect rolling on E-selectin. Neuraminidase treatment to remove sialic acid, a key part of the carbohydrate structures E-selectin recognizes, not only inhibited tethering, but also virtually eliminated neutrophil rolling on E-selectin, even after contact under static conditions, confirming the carbohydrate specificity of E-selectin recognition.

The importance of a sialylated carbohydrate associated with L-selectin in tethering to E-selectin may be related to the advantageous location of L-selectin on the tips of microvilli (Picker et al., 1991; Erlandsen et al., 1993). This first interaction serves to retard the flowing neutrophil enough such that a second receptor, which is chymotrypsin-resistant and sialylated, can then interact and mediate rolling on E-selectin. L-selectin bears a negligible fraction of neutrophil cell surface sialyl Lewis^x, which is a recognition site for E-selectin, as shown by shedding or chymotrypsin treatment (Table 1) (Picker et al., 1991; Erlandsen et al., 1993), consistent with its minimal contribution to adhesion strength. The identity of the sialylated protease-insensitive receptor(s) is currently unknown, but may be related to or identical with a murine glycoprotein that interacts with immobilized E-selectin immunoglobulin chimera (Levinovitz et al., 1993), or to glycolipids that bear sialyl Lewis^x. The velocity of a rolling neutrophil, as opposed to a flowing neutrophil, is slowed such that ligands that

Table 1. Expression of Neutrophil Adhesion Receptors[a]

Antigen	MAb	Resting	PMA[b]	PMA + CB[c]	Chymotrypsin[d]	Neuraminidase[e]
			Linear fluorescence intensity			
sialyl Le^x	CSLEX1	1369	1345	1273	1528	3
L-selectin	DREG-56	296	21	27	34	318
CD18	TS1/18	223	883	999	302	505
Mac-1, CD11b	LM2/1	119	693	616	310	292

[a] After the treatments, neutrophils were stained with MAb and subjected to immunofluorescence flow cytometry. Fluorescence intensity is the average of two experiments.
[b] 30 ng/ml for 10 min at room temperature in HBSS, 2 mM Ca^{2+}, 0.5% HSA.
[c] Cytochalasin B (2×10^{-5} M) was added to neutrophils at the same time as PMA.
[d] 4 U/ml in HBSS plus 0.5% HSA for 10 min at room temperature.
[e] 0.1 U/ml in HBSS plus 2 mM Ca^{2+} for 30 min at room temperature.

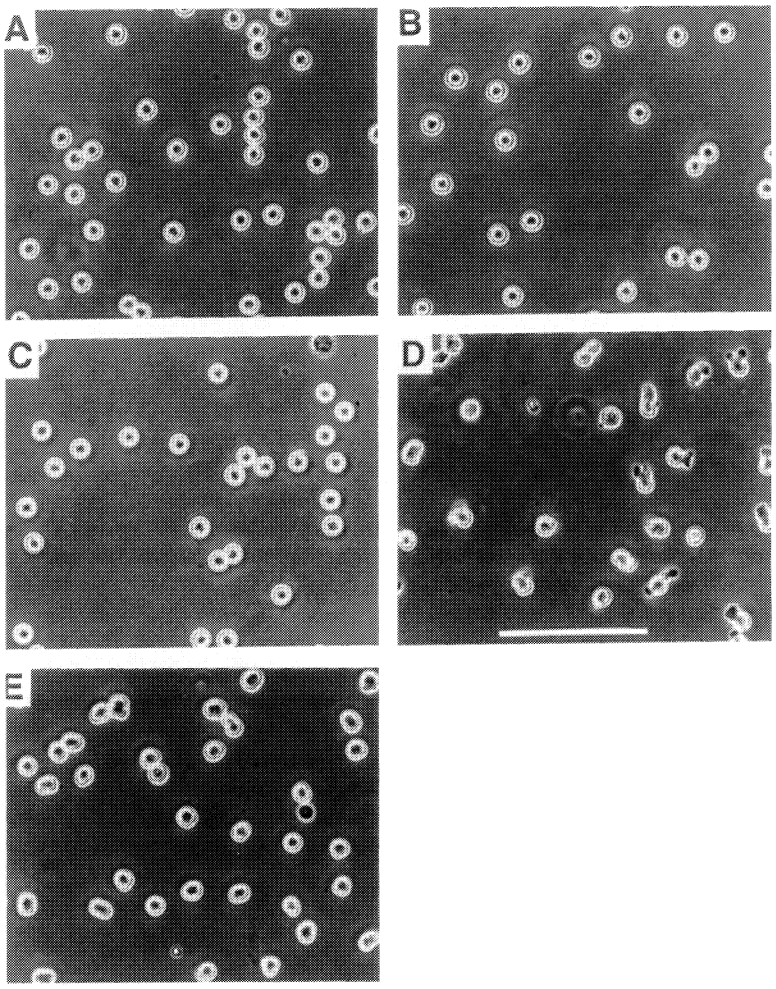

Figure 8. Morphology of Neutrophils Bound to E-Selectin

(A) Neutrophils incubated with control CD18 MAb, TS1/18 for 10 min.

(B) Neutrophils incubated with L-selectin MAb DREG-56 for 10 min.

(C) Chymotrypsin treated neutrophils as described in Experimental Procedures.

(D) PMA-treated neutrophils.

(E) PMA-treated neutrophils after washout of cytochalasin B after 30 min incubation as described in Experimental Procedures. E-selectin site density was 335 sites/μm^2. Scale bar is 100 μm.

maintain rolling adhesions could have association rate constants two to three orders of magnitude lower than those required for the initial tethering. Thus, soon after the initial tethering of a flowing neutrophil to E-selectin substrates, presumably through a single ligand–E-selectin bond, other ligand–E-selectin bonds can form, facilitating the rolling interaction and strengthening adhesion to the E-selectin substrate, as reflected in resistance to detachment at higher shear stresses. We hypothesize that rolling requires one or more bonds with the substrate at all times, so that bond dissociation does not result in release from the substrate, but allows the cell to be pushed forward by flow to a position where it is held by another bond (Lawrence and Springer, 1991, 1993).

How is the carbohydrate important in attachment associated with L-selectin? The blocking studies with a series of seven MAbs, the epitopes of which have been functionally and structurally mapped on L-selectin (Spertini et al., 1991b; Kansas et al., 1991), strongly suggest that the carbohydrate is associated with the N-terminal lectin-like domain. The best blockers of neutrophil attachment to E-selectin, DREG-56, LAM1-3, and LAM1-4, are also effective in blocking binding of carbohydrate ligands to L-selectin, and map to the lectin-like domain (Spertini et al., 1991b; Saunders and Tedder, 1994). The intermediate blockers map to distinct epitopes on the lectin and EGF domains, and the nonblocker, LAM1-14, maps to the short consensus repeats.

There are two ways in which a carbohydrate ligand important in tethering to E-selectin could associate with L-selectin. First, it could be covalently linked. There are two N-linked glycosylation sites in the lectin domain of L-selectin (Tedder et al., 1989; Siegelman and Weissman, 1989). Based on the crystal structure of the homologous mannose-binding protein complexed with an oligosaccharide ligand (Weis et al., 1992), the N-linked site at residue 66 is predicted to lie close to the ligand binding site in the lectin domain. Since MAbs LAM1-3, LAM1-4, and DREG-56 are predicted to bind close to this N-linked site, our data could be compatible with blocking accessibility of this carbohydrate to E-selectin. This N-linked carbohydrate might be processed differently than that attached to other N-linked sites, because processing differs for N-linked sites on the same glycoprotein, and is thought to be influenced by interactions of the carbohydrate with local amino acid residues on the protein surface (Ashford et al., 1993). L-selectin isolated from neutrophils bears covalently attached sialylated Lewis^x and binds E-selectin in static assays (Picker et al., 1991); however, it is not known whether this covalently attached carbohydrate is important in attachment in flow. The second possibility is that a molecule distinct from L-selectin could bear the car-

bohydrate ligand important in tethering in flow. There could be a cis interaction on the neutrophil surface, between this distinct molecule and the carbohydrate-binding site in the lectin domain of L-selectin. This is feasible, because L-selectin can bind sialyl Lewis^x (Berg et al., 1992; Foxall et al., 1992), and because this E-selectin ligand might have multiple carbohydrate side chains, one of which could bind L-selectin while others were free to bind E-selectin. Further, L-selectin might act to concentrate such molecules on the tips of microvilli and make them more accessible for interactions in flow.

Our findings help to resolve conflicting reports concerning whether L-selectin and E-selectin interact (Kishimoto et al., 1991; Picker et al., 1991; Spertini et al., 1991d) and concerning the protease sensitivity of the ligand for E-selectin (Picker et al., 1991; Larsen et al., 1992). The differences between reports may reflect differences in shear conditions in various adhesion assays and, consequently, differences in cell–cell contact times. In support of this, other laboratories have recently observed significant inhibitory effects of L-selectin MAbs in neutrophil adhesion to cytokine-stimulated endothelium (Abbassi et al., 1993) and platelet monolayers (Buttrum et al., 1993) under defined flow conditions similar to ours. We see greater inhibitory effects, presumably because contributions of other adhesion mechanisms, e.g., integrins, are eliminated in our purified system.

Adhesiveness of L-selectin for its ligands can be regulated by cell activation (Spertini et al., 1991b), and studies on transfected cells have shown that truncation of the cytoplasmic domain or treatment with cytochalasin B inhibits binding to ligands on high endothelial venules and rolling in vivo (Kansas et al., 1993). We have also found inhibition of tethering in flow in the continued presence of cytochalasin B (E. Finger and T. A. S., unpublished data). In the present study, cytochalasin B treatment had no effect on tethering of resting neutrophils to E-selectin, and enabled PMA-activated neutrophils to roll on E-selectin. It is important to note that our cytochalasin B treatment was designed to return neutrophils to a round shape and that the cytochalasin was washed out before tethering or rolling assays were performed.

Rolling experiments in vivo strongly suggest cooperation between L-selectin and E-selectin and between L-selectin and P-selectin. L-selectin antagonists inhibit by 80%–90% neutrophil rolling interactions during early stages of inflammation (such as occurs during tissue preparation for intravital microscopy), when P-selectin would be predicted to be expressed on endothelium, as well as at later stages, when E-selectin would be expressed on endothelium (Ley et al., 1991; von Andrian et al., 1991, 1992, 1993). Similarly, in mice genetically deficient in P-selectin, no rolling is observed during tissue preparation but, after several hours, rolling begins, suggesting that L-selectin and P-selectin participate in a common adhesive pathway (Mayadas et al., 1993). The existence of sialyl Lewis^x-dependent and independent rolling interactions observed by intravital microscopy (von Andrian et al., 1993) provides in vivo evidence that L-selectin interacts with either E-selectin or P-selectin, along with another as yet uncharacterized ligand on endothelium. Recent reports that L-selectin mediates rolling independent of E-selectin and P-selectin does not contradict our observations, because the transfectants lacked the appropriate glycosylation to allow interaction with E-selectin, and were much less efficient in interacting with the vessel wall than neutrophils that bear sialyl Lewis^x (Kansas et al., 1993; Ley et al., 1993).

Sequential steps in leukocyte interaction with endothelium provide great combinatorial diversity, since multiple receptors may be used in each step, and may explain the selectivity in the leukocyte subpopulations that emigrate in different inflammatory reactions (Butcher, 1991; Springer, 1994). The finding that selectins mediate two distinct steps in this process provides greater combinatorial diversity, because distinct selectins could be dominant in the tethering and rolling steps. Our finding that shedding of L-selectin has little effect on the strength of neutrophil adhesion to E-selectin suggests a neutrophil tethered to the vessel wall would remain adherent to E-selectin after neutrophil activation and shedding of L-selectin. The ability of fluid shear to peel neutrophils continuously along an E-selectin substrate suggests that a tenacious pull exerted by the leukocyte integrins Mac-1 and LFA-1 could similarly overcome interactions through E-selectin, and disengage the neutrophil from the luminal surface of the endothelium during transendothelial migration.

Experimental Procedures

Monoclonal Antibodies

The following murine MAbs against human antigens were used at 10 μg/ml as purified immunoglobulin: BB11 (anti-E-selectin [IgG2b]) (Lobb et al., 1991); HECA-452 (anti-cutaneous lymphocyte antigen and anti-sialyl Lewis^a and sialyl Lewis^x, [IgM]) (Picker et al., 1990; Berg et al., 1991); and DREG-56 and DREG-200 (anti-L-selectin [IgG1]) (Kishimoto et al., 1990). We used as purified immunoglobulin (5 μg/ml) a panel of anti-L-selectin MAbs consisting of the following: LAM1-3 (IgG1); LAM1-4 (IgG1); LAM1-5 (IgG1); LAM1-6 (IgG1); LAM1-10 (IgG1); LAM1-11 (IgG1); LAM1-14 (IgG1) (Spertini et al., 1991a, 1991c; Kansas et al., 1991). CSLEX-1 (anti-sialyl Lewis^x [IgM]) (Fukushima et al., 1984) and X63 (nonbinding antibody [IgG1]) were used as 1:4 dilutions of culture supernatants. TS1/18 (anti-CD18 [IgG1]) (Sanchez-Madrid et al., 1983) and LM2/1 (anti-CD11b [IgG1]) (Miller et al., 1986) were used at a 1:400 dilution of ascites fluid.

Purification of Soluble E-Selectin and Adsorption to Polystyrene

Recombinant soluble E-selectin was a gift of Dr. R. Lobb and was purified from supernatants of transfected CHO cells as described (Lobb et al., 1991). Soluble E-selectin (820 μg/ml) was diluted 1:1000 or 1:2000 into binding buffer (0.1 M $NaHCO_3$ [pH 9.2] before being adsorbed on a polystyrene slide (Becton Dickinson Labware, Lincoln Park, New Jersey) as described elsewhere (Lawrence and Springer, 1993). HBSS plus 1% human serum albumin (HSA) was added for 30 min at 37°C to block nonspecific binding sites. Immediately before an experiment, the immobilized protein was treated with phosphate-buffered saline (PBS) (pH 7.4) plus 1% Tween 20 for 2 min and washed with PBS to reduce nonspecific adhesive interactions between neutrophils and the protein-coated polystyrene surface (Geng et al., 1990).

Determination of E-Selectin and ICAM-1 Site Densities

Soluble E-selectin was adsorbed as described above onto polystyrene microtiter plates (Flow Laboratories, Incorporated, McLean, Virginia) for determination of site densities. MAb BB11 to E-selectin (Lobb et al., 1991) was iodinated to a specific activity of 1.9 μCi/μg and site density was determined by saturation binding, assuming binding of one IgG molecule per antigen molecule as previously described (Chan et al., 1991; Lawrence and Springer, 1993). The site density was determined three times in duplicate for adsorbed E-selectin and expressed in sites/μm^2: 334 ± 29.

Neutrophil Isolation and Treatments

Neutrophils were isolated from citrate anticoagulated whole blood following dextran-sedimentation and density separation over Ficoll–Hypaque (Miller et al., 1987). Following isolation, neutrophils were stored in modified HBSS, Ca^{2+} and Mg^{2+} free (pH 7.3) (GIBCO, New York); supplemented with 0.5% HSA. Before use in experiments, the neutrophils were washed into HBSS supplemented with 2 mM Ca^{2+}. In some experiments, neutrophils were treated with PMA (30 ng/ml, Calbiochem Corporation, La Jolla, California) for 10 min before perfusing through the flow chamber. Enzyme treatments were performed before the flow assays were begun. Neutrophils (0.5–1.0 × 10^7/ml) were incubated with chymotrypsin (4 U/ml, Calbiochem Corporation) for 10 min at room temperature in HBSS plus 0.5% HSA and then washed twice before being used. To convert sialyl Lewis^x to Lewis^x, neutrophils (0.5–1.0 × 10^7/ml) were incubated with neuraminidase (0.1 U/ml, Calbiochem Corporation) in HBSS plus 2 mM Ca^{2+} for 30 min at room temperature and then washed twice before use. Some treatments were followed by a wash with 5 mM EDTA in HBSS, and resuspension in 5 mM EDTA, 20 μM cytochalasin B, in HBSS at 4°C for 20 min, and two washes before the flow assay. Flow cytometry was used to examine antigen expression and the degree of removal of sialyl Lewis^x. For MAb inhibition assays, neutrophils were incubated with either purified antibody or dilutions of ascites fluid 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.

Flow Assays

Polystyrene slides on which E-selectin or HSA was adsorbed were 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 Incorporated, Garden City, New York). Tethering during continuous flow was assayed as previously described (Lawrence and Springer, 1991). The number of adherent neutrophils/U 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 the viscosity of assay buffer equal to that 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 under quiescent conditions as previously described (Chan et al., 1991; Lawrence and Springer, 1993). The syringe pump was started to initiate flow two minutes after all the neutrophils had settled onto the E-selectin-coated substrate. The flowrate was raised in staged increments every 20 s. Multiple fields of view were scanned during the intervals between each increase in flow rate to quantitate the percentage of rolling neutrophils remaining bound to E-selectin. The percentage bound was based on counting the number of neutrophils that initially settled onto the substrate before flow was started.

Electron Microscopy

To evaluate the effect of the addition of the MAb on shape of the cells before being placed in the flow chamber, as described above, a portion of the cells were immediately fixed in 1.5% glutaraldehyde for 1 hr at 22°C, postfixed in 1% O_sO_4 for 1 hr at 4°C, stained with aqueous uranyl acetate for 15 min at 22°C, dehydrated, embedded in Epon, and examined by electron microscopy. In addition, immunogold labeling for L-selectin was also performed before fixation using MAb DREG-200. The unfixed cells suspended in the HBSS supplemented with 2 mM Ca^{2+} were incubated in DREG-200 (1/200 dilution) for 10 min at 22°C, washed and fixed in 2% paraformaldehyde, 0.05% glutaraldehyde for 30 min at 22°C. The cells were washed in HBSS, and rabbit anti-mouse antibody (Zymed Laboratories, Incorporated, South San Francisco, California) diluted 1:200 was then added. After further washing, protein A–gold (5 nm) was incubated for 1 hr at 22°C. After washing, the cells were refixed in 1.5% glutaraldehyde and subsequently processed as described above.

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