Michael B. Lawrence[1], Ellen L. Berg[2], Eugene C. Butcher[2] and Timothy A. Springer[1]

[1] The Center for Blood Research, Department of Pathology, Harvard Medical School, Boston, USA,
[2] Stanford University School of Medicine, Department of Pathology, Stanford, USA

Rolling of lymphocytes and neutrophils on peripheral node addressin and subsequent arrest on ICAM-1 in shear flow

We studied leukocyte interactions in shear flow with peripheral lymph node addressin (PNAd), a mixture of glycoproteins expressed on high endothelial venules (HEV) that is required for lymphocyte homing and has been shown to contain a ligand for L-selectin. T lymphocytes and neutrophils tether and roll on plastic-immobilized PNAd and E-selectin at 1.8 dyn/cm^2 wall shear stress, but fail to interact with immobilized ICAM-1, a ligand for LFA-1 and Mac-1, at the same flow rate. Cells roll faster on PNAd than on P-selectin or E-selectin. L-selectin mAb inhibit T lymphocyte and neutrophil tethering to PNAd, but do not inhibit T lymphocyte tethering to purified E-selectin. If allowed to interact with ICAM-1 under static conditions, phorbol ester-treated T lymphocytes, but not resting T lymphocytes, are able to form stationary adhesions that withstand the detachment force generated by 36 dyn/cm^2 wall shear stress. In contrast, a wall shear stress of 7.3 dyn/cm^2 detaches 50% of resting T lymphocytes bound to PNAd. Incubating T lymphocytes on PNAd and ICAM-1 does not result in adhesion strengthening, suggesting that adhesion through PNAd by L-selectin does not stimulate lymphocyte LFA-1 avidity for ICAM-1. Chemoattractant stimulation of neutrophils or phorbol ester stimulation of lymphoblasts rolling on co-immobilized PNAd and ICAM-1 results in rapid arrest and firm sticking, extending the model of sequential selectin-mediated rolling and subsequent integrin-mediated firm arrest to lymphocytes and ligands expressed on HEV.

1 Introduction

Immune surveillance in host defense depends on the trafficking of lymphocytes between the bloodstream, the extravascular space where pathogens are encountered, and lymphoid organs where humoral responses are mounted. Lymphocyte subsets differ in their traffic routes and organ-specific preference for emigration. Adhesion receptors expressed on lymphocytes and their counter-receptors expressed on vascular endothelium help determine the specificity for emigration in different vascular beds [1–5].

T lymphocytes specifically emigrate, or home, to peripheral lymph nodes, in part, by means of adhesive interactions through L-selectin, an adhesion receptor expressed on circulating lymphocytes and other leukocytes [6]. L-selectin mediates adhesion by binding to a component of peripheral node addressin (PNAd), a mixture of glycoproteins expressed on peripheral HEV that are immunoprecipitated by mAb MECA-79 [7]. MECA-79 recognizes a carbohydrate epitope on PNAd that is similar to that recognized by L-selectin. Recognition depends on sialylation, sulfation, and possibly fucosylation [8–10]. Like mAb to L-selectin, MECA-79 mAb blocks T lymphocyte adhesion to peripheral lymph node HEV in the Stamper-Woodruff assay of binding to tissue sections, and homing to peripheral nodes *in vivo* [7]. Using recombinant L-selectin coupled to Sepharose, an L-selectin ligand GlyCAM-1 was isolated from peripheral lymph node tissue [11]. GlyCAM-1 is a mucin-like glycoprotein that contains the MECA-79 epitope and is among the glycoproteins precipitated by MECA-79 [8]. Additionally, the mucin-like molecule CD34 has been identified as a L-selectin ligand by the same method [12]. However, neither CD34 or GlyCAM-1 have been shown to support or inhibit lymphocyte adhesion to date.

Intravital microscopic observations of individual blood vessels indicate that L-selectin mediates a significant portion of neutrophil rolling in post-capillary venules, an adhesive interaction postulated to be critical to localize neutrophils at sites of inflammation [13, 14]. Thus, L-selectin is critical for extravasation of neutrophils as well as being required for lymphocyte homing to peripheral lymph nodes. L-selectin transfectants roll on purified MAdCAM-1 *in vitro* [15] which is expressed on mucosal HEV but not on peripheral lymph node HEV, and roll *in vivo* on uncharacterized ligands expressed on postcapillary venules in non-lymphoid tissue in the mesentery [16, 17]. Peripheral node HEV have not been studied by intravital microscopy, and whether PNAd mediates rolling *in vivo* or *in vitro* has not previously been reported. It is unknown whether L-selectin on T lymphocytes supports rolling adhesions on PNAd.

[I 13838]

Present addresses: M. B. Lawrence, University of Virginia, Department of Biomedical Engineering, 1105 West Main Street, Stacy Hall, Charlottesville, VA 22903, USA
E. L. Berg, Protein Design Labs, 2375 Garcia Avenue, Mountain View, CA 94043, USA.

Correspondence: Timothy A. Springer, Center for Blood Research, 200 Longwood Ave., Boston, MA 02115, USA (Fax: 617-278-3232)

Abbreviations: **PNAd:** Peripheral nerve node addressin **HEV:** High endothelial venules

Key words: L-selectin / CD62L / Rolling ICAM-1 / CD54

0014-2980/95/0404-1025$10.00+.25/0

Intravital microscopy of Peyer's patch HEV has shown that lymphocytes roll, then arrest on the HEV prior to emigration, and that treatment with pertussis toxin, which blocks emigration, blocks arrest and prolongs rolling [18]. Homing of radiolabeled lymphocytes to Peyer's patch has been reported to be unaffected by MECA-79 mAb to PNAd and to be partially inhibited by mAb to L-selectin, MAdCAM-1, α4β7, and LFA-1 [2, 5]. The role of these receptors in lymphocyte rolling and arrest *in situ* as studied by intravital microscopy has not been reported.

Selectin-mediated tethering and rolling effectively retard the motion of leukocytes in blood flow, presumably so that leukocytes may then interact with the vessel wall through β_2 or β_1 integrin-dependent mechanisms. In this model, selectin-mediated adhesion provides one of several levels of specificity to regulate leukocyte emigration, and in some instances may be a prerequisite for adhesiveness through integrins, which has been reported to be activated both by chemoattractants and by ligation of selectins or their ligands [2, 4, 5, 19]. To extend this model, we wished to determine whether isolated PNAd, containing an HEV ligand for L-selectin, would support L-selectin-dependent tethering and rolling under physiological shear of T lymphocytes and neutrophils. We further wished to determine whether, once rolling on PNAd, β2 integrins on T lymphocytes and neutrophils could mediate arrest by binding to ICAM-1, a step required before transendothelial migration is possible [20], and whether PNAd itself or additional stimuli were required to activate β2 integrin adhesiveness.

2 Materials and methods

2.1 Monoclonal antibodies

DREG 56 and DREG 200 mAb to L-selectin [21] were used at 10 μg/ml as purified IgG1 in adhesion assays, as was BB11 to E-selectin [22].

2.2 Preparation of receptor coated surface

Recombinant soluble E-selectin, purified from supernatants of transfected CHO cells [22] was a generous gift of Dr. Roy Lobb (Biogen, Inc., Cambridge, MA). Soluble E-selectin (820 μg/ml) was diluted 1 : 1000 into binding buffer (0.1 M $NaHCO_3$, pH 9.2) and adsorbed to polystyrene slides cut from bacteriological petri dishes (Falcon Labware, no. 1058). E-selectin substrate density of 334 ± 29 sites/μm^2 was determined by a quantitative radioimmunoassay [23]. To purify ICAM-1, human tonsil (12 g) was homogenized in 200 ml Tris-0.01 M 0.14 M NaCl-0.025% azide (TSA, pH 8.0) and Triton X-100 detergent was added to a final volume of 1%. After 1 h, the lysate was centrifuged (10 min, 400 × g, 4 °C) and then ultracentrifuged (30 000 × g, 1 h, 4 °C) and the supernatant was applied to a column of Sepharose CL-4B conjugated with R6.5 (3.5 ml of 2.4 mg/ml of bead volume). ICAM-1 was eluted following a wash with TSA, pH 8.0, 1% octyl glucoside (OG, 30 column volumes) and eluted with TSA (pH 11.0, 1% OG). The elutant was neutralized with 1 M Tris-HCl, pH 7.0, 1% OG (15% v/v). Material was 85% pure by examination of a silver stained SDS-PAGE gel. Peripheral addressin was purified from human tonsil lysates with MECA-79 mAb-Sepharose as previously described [9]. PNAd or ICAM-1 in 1% OG were diluted 1 : 20 into TSA (pH 8.0) at room temperature (22 °C), and a 50 μl drop was adsorbed to plastic slides for 2 h. ICAM-1 was adsorbed at a density of 470 sites/μm^2 as determined by saturation binding [24]. It was not possible to ascertain the site density of PNAd because the active ligand is a carbohydrate that may represent only a subset of the carbohydrate determinants recognized by MECA-79 mAb, and the quantities of PNAd obtained were insufficient for saturation binding studies. In some experiments, PNAd and ICAM-1 were mixed together in equal volumes before dilution into TSA (1 : 10 of total protein solution to TSA, 1 : 20 for each protein respectively) to adsorb both molecules onto plastic. After adsorption, nonspecific adhesion of T lymphocytes and neutrophils was blocked by incubating the plate with Hanks' balanced salt solution (HBSS; Gibco Laboratories, Grand Island, NY), supplemented with 1% human serum albumin (HSA) for 30 min at 37 °C.

2.3 T lymphocytes and neutrophils

T lymphocytes and neutrophils were isolated from citrate anti-coagulated whole blood by dextran sedimentation followed by density separation over Ficoll-Hypaque [25]. T lymphocytes were further purified by sequential plastic and nylon wool absorbance as previously described [26]. T lymphocytes were stored in RPMI 1640 (Gibco, Long Island, NY) containing 2% FCS (Hyclone, Logan, UT) at room temperature following nylon wool purification. In some experiments, T lymphocytes were treated with phorbol 12-myristate 13-acetate (PMA, 30 ng/ml, Calbiochem Corp., La Jolla, CA) for 10 min before injection into the flow chamber. Following isolation, neutrophils were stored in Ca^{2+} and Mg^{2+}-free HBSS containing 10 mM Hepes pH 7.3, 0.5% HSA at room temperature for up to 4 h. Before use in experiments, neutrophils were washed into HBSS supplemented with 1 mM Mg^{2+} and 1 mM Ca^{2+}. Neutrophils were stimulated with formyl-L-methionine-L-leucine-L-phenylalanine (fMLP) (10^{-8} M, Calbiochem) in selected experiments. For mAb inhibition assays, T lymphocytes and neutrophils were incubated with purified antibody for 10 min at room temperature in their respective assay medium. The antibody was present at all times during the flow experiments. Jurkat T lymphoma line was cultured in RPMI 1640 with 10% FCS (Hyclone, Logan, UT) and antibiotics in 5% CO_2 in air.

2.4 Flow assays

The polystyrene slide with adsorbed glycoproteins was assembled in a parallel plate laminar flow chamber (260 μm gap) and mounted on the stage of an inverted phase-contrast microscope (Diaphot-TMD, Nikon Inc., Garden City, NY). Tethering during continuous flow was assayed as described [23, 27]. T lymphocytes and Jurkat cells were at 0.5 × 10^6/ml and neutrophils at 1 × 10^6/ml for flow assays. Multiple fields of view were videotaped, while scanning the lower plate of the flow chamber with the microscope, and the number of rolling or stationary leukocytes were counted. The hydrodynamic velocity was calculated based

cooperative effect under flow conditions of the two adhesion pathways [24].

E-selectin also supports tethering of peripheral blood lymphocytes in flow (Fig. 1). All tethered lymphocytes roll (data not shown). Lymphocytes tether on E-selectin less efficiently than on PNAd, consistent with E-selectin recognition by a subset of circulating T lymphocytes [32, 33] that is smaller than the subset that expresses L-selectin [34]. L-selectin mAb DREG-56 does not inhibit T lymphocyte interactions with E-selectin (Fig. 1). In contrast, neutrophil tethering in flow to E-selectin is inhibited by mAb to L-selectin [27, 35]. Tethering and rolling on E-selectin is specific, as shown by inhibition with BB11 mAb to E-selectin and with EDTA (Fig. 1).

T lymphocytes roll on purified PNAd at 20 μm/sec at a wall shear stress of 1.8 dyn/cm^2 (Fig. 2 and see Fig. 4 A). As the shear stress increases, rolling velocity increases, reaching 60 μm/s at 14.6 dyn/cm^2. Rolling on PNAd is jerky, and appears to involve intermittent dissociation and facile retethering to the PNAd substrate. In contrast, T lymphocytes roll steadily on E-selectin, with a velocity of 3 μm/s at 1.8 dyn/cm^2, as previously reported for neutrophils at the corresponding site density [24]. Neutrophils roll on PNAd at 40 μm/s at a wall shear stress of 1.8 dyn/cm^2, twice the velocity of T lymphocytes (Fig. 4 A).

3.2 Comparison of the strength of T lymphocyte binding to PNAd and ICAM-1

The resistance to detachment by fluid shear forces is one measure of the strength of adhesion between a leukocyte and a surface [36, 37]. T lymphocytes were allowed to settle on PNAd or ICAM-1 under quiescent conditions, and after a 6-min incubation, flow was initiated at 0.73 dyn/cm^2, and then increased in 2–2.5-fold increments, and the percentage of lymphocytes that remained bound at each level of shear stress was determined to measure resistance to detachment. The static incubation and flow at a wall shear stress of 0.73 dyn/cm^2 allows initiation of LFA-1-dependent and L-selectin-dependent adhesion, respectively. On PNAd, 83% of the resting T lymphocytes tether and roll as flow was started at 0.7 dyn/cm^2 (Fig. 3 A). As shear was increased in increments of 2- or 2.5-fold to 36 dyn/cm^2 wall shear stress, rolling velocity increased and the number of T lymphocytes that remained bound decreased (Fig. 3 A). Almost 50% were detached by a wall shear stress of 7.3 dyn/cm^2 and more than 90% by 36 dyn/cm^2. Resting T lymphocytes do not bind to purified ICAM-1 after settling under quiescent conditions onto purified ICAM-1, except for a small subpopulation of approximately 10% that may have been activated (Fig. 3 A). PMA-treatment of T lymphocytes, which increases avidity of LFA-1 for ICAM-1 [26], results in adhesion to the ICAM-1 coated substrate that resists detachment at the highest shear generated in the flow cell. No rolling is observed on ICAM-1 coated substrates, and the PMA-treated T lymphocytes are highly spread. In contrast to what was observed on purified ICAM-1, PMA treatment weakens the ability of T lymphocytes to remain adherent to PNAd (Fig. 3 A), and increases rolling velocities of those lymphocytes that remain adherent (47 ± 16 μm/s, (n = 23) for PMA-treated *vs.* 24 ± 7 μm/s for resting T lymphocytes (n = 15). The weakening of adhesion and

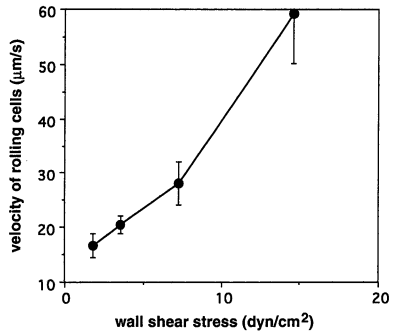

Figure 2. Rolling velocity of T lymphocytes on PNAd. Lymphocytes were tethered to PNAd at 0.73 dyn/cm^2 and the shear stress was increased in 2–2.5-fold steps every 10 s to 14.6 dyn/cm^2. The average rolling velocity of tethered cells and SD is shown.

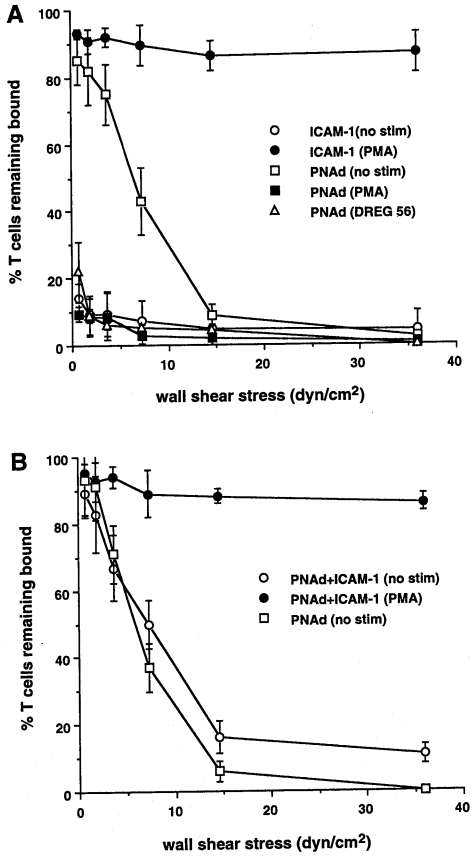

Figure 3. Detachment by shear of T lymphocytes following static incubation on PNAd, ICAM-1, or PNAd co-immobilized with ICAM-1. Resting T lymphocytes were injected through a port of the flow chamber and allowed to settle onto the substrate under static conditions in the presence or absence of 50 ng/ml PMA. After 6 min of contact, flow was initiated and increased in stages up to 36 dyn/cm^2. The number of adherent (and in some cases rolling) T lymphocytes were counted and compared to the number that initially settled onto the substrate to give the percentage remaining bound. (A) Comparison of T lymphocytes interacting either with ICAM-1 (470 sites/μm^2) or PNAd (1 : 20 dilution in TSA). Data represent the mean and SD or range of two or three independent experiments. (B) T lymphocytes interacting with PNAd + ICAM-1 compared to interactions with PNAd alone. Data are from the same experiments as described as in (A). Data represent the mean and range of two independent experiments.

velocity in shear flow of a hard sphere (7 μm in diameter) near a planar surface with which it does not interact [28]. Leukocytes moving more slowly than the hydrodynamic velocity are defined as adherent [17, 24]. 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 or PMA-stimulated T lymphocytes (1×10^6/ml) were injected into the chamber through a port and allowed to settle as described [24]. Once the population of T lymphocytes settled (2 min), flow was started 6 min later and wall shear stress was increased in increments to generate a detachment force. Comparison of the number of T lymphocytes remaining bound with the number initially interacting with the surface allows the percentage remaining adherent to be calculated. In the detachment assays, flow was increased every 20 s in 2–2.5-fold increments from 0.73 dyn/cm^2 to 36 dyn/cm^2. The number of T lymphocytes remaining bound was counted by scanning 10–20 fields of view at each flow rate. Scans typically took 15 s.

2.5 Integrin-mediated arrest of rolling neutrophils and the T lymphocyte line Jurkat

Resting neutrophils were perfused through the flow chamber on which the lower face of the chamber had ICAM-1 and PNAd co-immobilized. After 2 min of flow at 1.8 dyn/cm^2, fMLP (10^{-8} M), a potent chemoattractant for neutrophils, was added to the neutrophil suspension. Arrival of the fMLP in the field of view was estimated by the arrival of erythrocytes (10^5/ml) added to the fMLP solution. The motion of rolling neutrophils was tracked as a population, *i.e.* the velocities of all neutrophils in the field of view were averaged together at any given time. The same procedure was followed for Jurkat, except that PMA (50 ng/ml) was added to the perfusate instead of fMLP. All experiments were performed at room temperature.

3 Results

3.1 Comparison of T lymphocyte and neutrophil tethering to purified adhesion receptors PNAd, E-selectin, and ICAM-1

Tethering, rolling, and resistance to detachment are three distinct measures of leukocyte interactions with ligands on a vessel wall in shear flow. Tethering is the initial adhesive interaction between a cell and the wall of the vessel in hydrodynamic flow. A tethered cell may continue to move through rolling interactions. A cell is defined as tethered when its velocity drops below that of the hydrodynamic velocity, *i.e.* the velocity of a cell in hydrodynamic shear flow that is near to but not adhesively interacting with the wall. The hydrodynamic velocity can be calculated from the Goldman equation [28], or measured in control experiments. In hydrodynamic flow at a wall shear stress of 1.8 dyn/cm^2, some T lymphocytes that enter the field of view tether to PNAd (112 ± 16 cells/mm^2 of PNAd surface area over 3 min), whereas the other cells continue to move at the hydrodynamic velocity (Fig. 1). All tethered lymphocytes roll, rotating forward in response to hydrodynamic drag forces after they tether to the wall. Tethering is apparent as

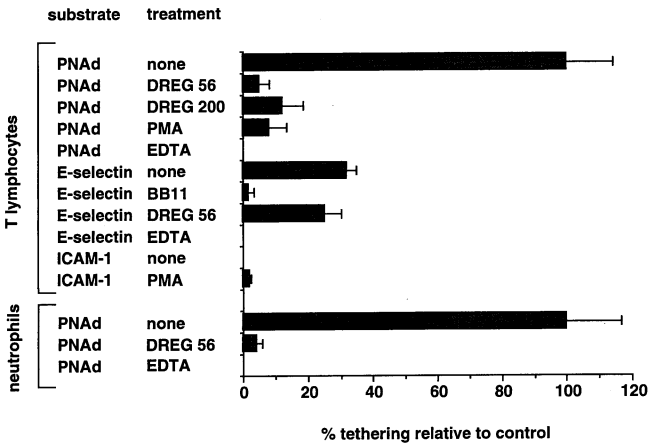

Figure 1. Tethering of leukocytes to purified adhesion receptors in hydrodynamic flow. Peripheral blood T lymphocytes or neutrophils were perfused through the flow chamber at a constant wall shear stress of 1.8 dyn/cm^2, and the number that tethered after 3 min was counted. ICAM-1, E-selectin, or PNAd were adsorbed to the lower wall of the flow chamber at a density of 470 sites/μm^2, 330 sites/μm^2, or at unknown site density, respectively. Leukocytes were treated with L-selectin mAb DREG-56 or DREG-200, PMA (50 ng/ml), or EDTA (5 mM) for 10 min at room temperature as indicated. Control binding to the PNAd substrate for resting T lymphocytes was 112 ± 16/mm^2, and for resting neutrophils, 185 ± 31/mm^2. Data represent means of two to three independent experiments normalized to control binding. Error bars show range or SD of independent experiments.

a rapid decrease (from one video frame to the next) from the hydrodynamic velocity of untethered cells of 430 μm/s, to a rolling velocity of 20 μm/s. In the presence of EDTA, which inhibits Ca^{2+}-dependent interactions by selectins, tethering to PNAd is completely inhibited (Fig. 1), and all cells move at the hydrodynamic velocity. Jurkat, a T lymphoma line that expresses L-selectin, also tethers to and rolls on purified PNAd (see Fig. 4). mAb to L-selectin, DREG 56 and DREG 200, potently inhibit T lymphocyte tethering to PNAd in shear flow (Fig. 1). L-selectin has been shown to mediate T lymphocyte adhesion to PNAd under non-flow conditions based on mAb blockage of binding to isolated PNAd [9]. This is, thus, the first demonstration of T lymphocyte tethering and rolling on isolated PNAd under physiological flow conditions.

Phorbol ester treatment of T lymphocytes triggers L-selectin shedding [29, 30]. Following treatment of resting T lymphocytes with PMA, 70% of the L-selectin is shed as determined by flow cytometry (data not shown). PMA treatment almost completely inhibits T lymphocyte tethering to PNAd (Fig. 1).

Resting and PMA-treated T lymphocytes are unable to tether to ICAM-1 at 1.8 dyn/cm^2 wall shear stress (Fig. 1), consistent with previous observations that β2 integrins such as LFA-1 are relatively ineffective at mediating neutrophil adhesion under conditions of venous shear stress [13, 24, 31]. A mixture of PNAd and ICAM-1 is similar to PNAd alone as a substrate for T lymphocyte tethering in flow (89 cells/mm^2 on a mixture of PNAd and ICAM-1 compared to 74 cells/mm^2 on PNAd alone), consistent with previous observations of resting neutrophil tethering to lipid bilayers containing P-selectin and ICAM-1, in which there was no

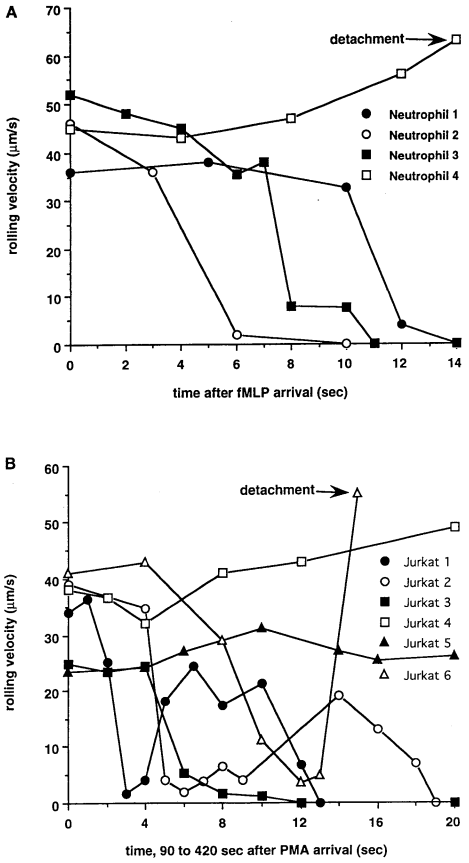

Figure 5. Velocities of individual leukocytes rolling on PNAd co-immobilized with ICAM-1 at 1.8 dyn/cm^2 wall shear stress. (A) Individual neutrophils. Rolling velocity as a function of time after arrival of 10^{-8} M fMLP is shown for four neutrophils. (B) Individual Jurkat cells. Rolling velocity as a function of time beginning 90–420 s after arrival of PMA is shown for six Jurkat cells. The time segments shown were selected to begin a few seconds before slowing of rolling was first detected, except for cells 4 and 5 that never slowed. Measurements were made by tracking the center of the rolling neutrophil or Jurkat cell over 0.5-s intervals. The cells that arrested (velocity = 0) continued to be arrested, and the subsequent velocity = 0 points are not plotted.

delayed. To show some examples of behavior around the time of arrest, Fig. 5 B shows the velocity of individual Jurkat cells, beginning at an interval of 90–420 s after the arrival of PMA in the field of view. More brief arrests or transient stops were observed with Jurkat cells than with neutrophils, as illustrated by Jurkat cells 1 and 2, which slowed down, then resumed rolling at nearly their initial velocity, before finally coming to a complete arrest. In the case of Jurkat cell 6, there was a brief arrest, then adhesion failed and the cell detached completely. Jurkat cells 4 and 5 never slowed down or arrested, and were eventually lost from the field of view.

4 Discussion

We show that isolated PNAd, an HEV ligand for L-selectin, supports lymphocyte and neutrophil tethering and rolling at venous levels of shear stress. We also show that an endothelial cell selectin, E-selectin, supports T lymphocyte tethering in flow and rolling. Interactions of lymphocytes with PNAd [9] and E-selectin [32, 33, 38] had previously been shown in static or shaking assays with a complex and undefined mixture of shear stresses; in one recent report, E-selectin-transfected L cells have been shown to support tethering and rolling of T lymphocytes [39]. Our studies show that T lymphocytes can tether in flow and roll through at least two selectin-dependent mechanisms, supporting the concept that selectins are specialized to mediate the initial adhesion of leukocytes to the vessel wall [2, 5]. Furthermore, we have examined how interactions of L-selectin with PNAd relate to those of integrins with ICAM-1, with regard to signaling influences of one interaction on the other, and within a 3-step model of leukocyte rolling, activation, and arrest.

Rolling interactions on PNAd are extremely rapid and loose compared to those studied previously for neutrophils on P-selectin [24] and E-selectin [23], as well as those studied here for lymphocytes on E-selectin. Lymphocytes and neutrophils rolling on PNAd appear momentarily to dissociate and reassociate with great facility, with the rolling velocity varying considerably if measured on a time scale of 0.1 s or less, but much more uniform if measured over 0.5–2 s time scales as reported here. Despite the appearance of dissociation and reassociation, rolling cells are tethered to PNAd $\geq 95\%$ of the time they are rolling, as shown by velocities that are 20–60-fold slower than for nonadherent cells at 1.8–14.6 dyn/cm^2, respectively. Rolling of L-selectin transfectants on appropriately glycosylated MAdCAM-1 is also quite fast, at 141 ± 68 μm/s [15]. The rolling velocity of neutrophils on PNAd of 40 μm/s at 1.8 dyn/cm^2 is higher than the highest velocity seen previously at the same wall shear stress for both P-selectin (10 μm/s) and E-selectin (2 μm/s). This is true even though rolling velocity is dependent on ligand density, and a wide range of ligand densities were tested in previous studies on neutrophils.

Emigration of lymphocytes from the bloodstream into peripheral lymph nodes can be almost completely inhibited by mAb to L-selectin, PNAd, or LFA-1; or pertussis toxin [2, 3, 5]. Two ligands for LFA-1, ICAM-1 and ICAM-2, are both strongly expressed on HEV [40]. It has been hypothesized that at least three sequential steps, including interaction of L-selectin with PNAd, the pertussis toxin-sensitive step, and interaction of LFA-1 with ICAM-1 and -2 contribute to selectivity in lymphocyte emigration in peripheral lymph nodes. Lymphocytes, but not neutrophils, emigrate into peripheral lymph nodes. Both cell types bind to HEV in the Woodruff-Stamper assay [41], to isolated PNAd as shown here, and when activated, to ICAM-1. We observed that rolling of T lymphocytes or neutrophils through interaction of L-selectin with PNAd does not stimulate β2 integrin-dependent binding to ICAM-1 of either cell type. Rather, we find that an additional signal is required to up-regulate β2 integrin adhesiveness. Our data support a model in which at least three steps are required for lymphocyte homing or neutrophil localization in inflammation when the first step involves L-selectin, and support the concept that a signal distinct from L-selectin ligation, such as a chemoattractant that binds to a pertussis toxin-sensitive G protein-coupled receptor, may be responsible for selective up-regulation of integrin adhesiveness on lymphocytes that emigrate into peripheral lymph nodes [2, 5].

the reduced number of T lymphocytes able to tether on PNAd following PMA-treatment is consistent with the loss of L-selectin following treatment with phorbol esters [29].

3.3 Lack of triggering by PNAd of adhesion to ICAM-1

To test for triggering by PNAd of adhesion to ICAM-1, binding of resting T lymphocytes to a mixture of PNAd and ICAM-1 was compared to binding to PNAd or ICAM-1 alone (Fig. 3 A, B). Resting T lymphocytes form rolling adhesions on PNAd co-immobilized with ICAM-1 that are similar in strength to those formed on PNAd alone: about 50% of T lymphocytes detach at 7.3 dyn/cm^2 (Fig. 3 B). The percentage of T lymphocytes remaining bound on PNAd co-immobilized with ICAM-1 at 36 dyn/cm^2 wall shear stress is similar to that for T lymphocytes bound to ICAM-1 alone (Fig. 3 A), and these cells do not roll, suggesting that there is a subpopulation of activated T lymphocytes. The majority (87%) of the T lymphocytes adherent on PNAd and ICAM-1 rolled and can be detached by the application of flow (Fig. 3 B). The similar susceptibility of unstimulated T lymphocytes to detachment from PNAd co-immobilized with ICAM-1 compared to PNAd and ICAM-1 immobilized separately suggests that L-selectin ligation to PNAd does not enhance LFA-1 avidity for ICAM-1.

The rolling velocity of T lymphocytes on PNAd was compared to that on PNAd plus ICAM-1 to further test for synergy or triggering. The rolling velocity of T lymphocytes on PNAd is not significantly different whether or not ICAM-1 is co-immobilized (Fig. 4 A).

3.4 Effect of activation of neutrophils or T lymphoma cells rolling on PNAd co-immobilized with ICAM-1

We examined whether leukocyte rolling, activation, and arrest could be demonstrated with PNAd, ICAM-1, and an activating stimulus. We utilized the Jurkat T lymphoblast cell line in these experiments rather than peripheral blood T lymphocytes. Both cell types show rolling and PMA-stimulated arrest on substrates containing PNAd and ICAM-1; however, a higher percentage of Jurkat T lymphoblasts remain adherent to the substrate and arrested. Neutrophils were examined in parallel, using the formyl peptide chemoattractant fMLP as the activating stimulus. Tethering and rolling of Jurkat T lymphoblasts and neutrophils on PNAd plus ICAM-1 was initiated at 1.8 dyn/cm^2 and subsequently, activating agents were added to the chamber perfusate. Rolling velocity and arrest of rolling cells were recorded as a function of time after arrival of the activating agent in the field of view (t = 0 in Fig. 4 A). Neutrophil β2 integrin activation was achieved with 10^{-8} M fMLP. The addition of fMLP rapidly triggers arrest of the rolling neutrophils, as indicated by the drop in the average rolling velocity (Fig. 4 A). Within a period of approximately 40 s of the arrival of fMLP, 69% of the rolling neutrophils arrest and begin to spread (Fig. 4 B). No significant decrease in neutrophil rolling velocity occurs in the absence of fMLP.

PMA infusion triggers the arrest of Jurkat T lymphoblasts rolling on PNAd co-immobilized with ICAM-1. Average

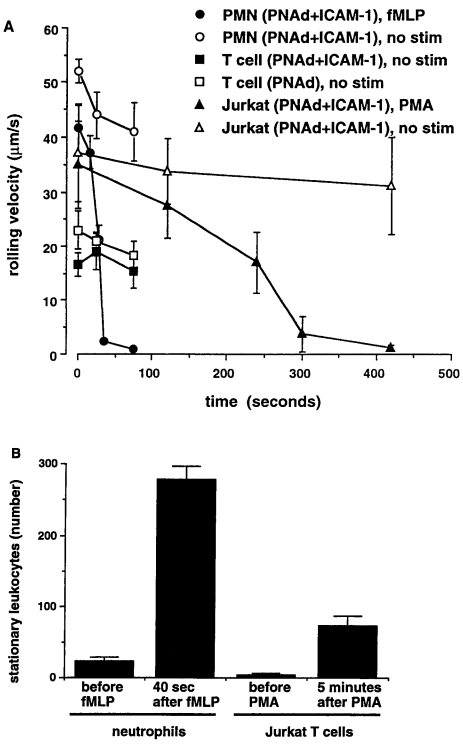

Figure 4. Increase of β_2 integrin avidity for ICAM-1 arrests neutrophils and Jurkat T lymphoblasts rolling on PNAd co-immobilized with ICAM-1, and lack of effect of ICAM-1 on rolling velocity of T lymphocytes on PNAd. (A) Cells (10^6/ml) were perfused over a substrate containing PNAd alone or PNAd + ICAM-1 at a wall shear stress of 1.8 dyn/cm^2, and after 3 min, rolling velocities were measured in several fields of view. T lymphocyte rolling velocities were compared on PNAd alone and on PNAd + ICAM-1. In experiments with neutrophils or Jurkat cells, 10^{-8} M fMLP or 50 ng/ml PMA, respectively, was added to the perfusate and rolling velocities of these cells were measured at various times after the arrival of the activating substances in the field of view. Error bars represent the SEM of rolling velocities of individual leukocytes in a field of view (110–300 neutrophils, 64 Jurkat cells, 91 peripheral blood T lymphocytes). (B) The number of stationary neutrophils and Jurkat cells on a mixture of PNAd + ICAM-1 in a flow chamber at a wall shear stress of 1.8 dyn/cm^2 is shown before and after activation of β2 avidity for ICAM-1 by arrival of the indicated stimulus in the field of view within the flow apparatus.

rolling velocities drop from approximately 35 μm/s to less than 2 μm/s within 400 s of the addition of PMA. Arrest of PMA-stimulated T lymphoblasts is slower than that of neutrophils following fMLP exposure. PMA dramatically increases the number of firmly adherent Jurkat cells (Fig. 4 B).

The transition from a rolling adhesion to stationary adhesion is typically very rapid for neutrophils (Fig. 5 A). The rolling velocities of four individual neutrophils tracked after exposure to fMLP at t = 0 are shown in Fig. 5 A. Neutrophils 1 and 2 arrested abruptly. Neutrophil 3 almost stopped, then rolled slowly for 2 s before coming to a complete stop. Neutrophil 4 never slowed and eventually detached.

The transition of individual Jurkat cells from rolling to firm arrest is almost as rapid as for neutrophils, although more

The ability of stimulated neutrophils and lymphocytes to arrest and to develop firm adhesion when rolling on selectins illustrates the crucial role that selectins play in slowing leukocytes enough to allow β2 integrins to interact with ligands such as ICAM-1. At the wall shear stress used in the flow assay, neutrophils and Jurkat cells were unable to tether to ICAM-1. However, immobilization of PNAd with ICAM-1 permitted tethering and rolling, which then permitted the β2 integrin-mediated arrest of the rolling lymphocytes and neutrophils. Neutrophils or Jurkat cells that had tethered and were rolling on PNAd co-immobilized with ICAM-1, were triggered to arrest when stimulated with fMLP or PMA-1 to activate Mac-1 and LFA-1 [24, 42] or LFA-1 [26] adhesiveness for ICAM-1, respectively. Tracking of individual rolling leukocytes indicated that the transition from selectin-mediated rolling to integrin-mediated arrest, or firm adhesion, is rapid, with a time-scale of seconds. Once the integrin is in a high-avidity state, arrest is possible if rolling is maintained. Some cells detached from the substrate before arrest occurred, possibly due to shedding of L-selectin. Our finding that PNAd and ICAM-1 are recognized in series, rather than in parallel, means that leukocyte specificity for adhesive ligands that are recognized in the rolling and firm adhesion steps can generate combinatorial diversity in leukocyte receptivity to area code signals displayed on endothelium. Our findings suggest that this model, based on studies with neutrophils, can be extended to lymphocytes.

Supported by NIH grants HL48675, CA31799, CA31798 and GM37734.

Received November 23, 1994; in revised form January 30, 1995; accepted February 3, 1995.

5 References

1 Springer, T. A., *Nature* 1990. *346:* 425.
2 Butcher, E. C., *Cell* 1991. *67:* 1033.
3 Picker, L. J. and Butcher, E. C., *Annu. Rev. Immunol.* 1992. *10:* 561.
4 Shimizu, Y., Newman, W., Tanaka, Y. and Shaw, S., *Immunol. Today* 1992. *13:* 106.
5 Springer, T. A., *Cell* 1994. *76:* 301.
6 Gallatin, W. M., Weissman, I. L. and Butcher, E. C., *Nature* 1983. *304:* 30.
7 Streeter, P. R., Rouse, B. T. N. and Butcher, E. C., *J. Cell Biol.* 1988. *107:* 1853.
8 Imai, Y., Singer, M. S., Fennie, C., Lasky, L. A. and Rosen, S. D., *J. Cell Biol.* 1991. *113:* 1213.
9 Berg, E. L., Robinson, M. K., Warnock, R. A. and Butcher, E. C., *J. Cell Biol.* 1991. *114:* 343.
10 Hemmerich, S. and Rosen, S. D., *Biochemistry* 1994. *33:* 4830.
11 Lasky, L. A., Singer, M. S., Dowbenko, D., Imai, Y., Henzel, W. J., Grimley, C., Fennie, C., Gillett, N., Watson, S. R. and Rosen, S. D., *Cell* 1992. *69:* 927.
12 Baumhueter, S., Singer, M. S., Henzel, W., Hemmerich, S., Renz, M., Rosen, S. D. and Lasky, L. A., *Science* 1993. *262:* 436.
13 von Andrian, U. H., Chambers, J. D., McEvoy, L. M., Bargatze, R. F., Arfors, K. E. and Butcher, E. C., *Proc. Natl. Acad. Sci. USA* 1991. *88:* 7538.
14 Ley, K., Gaehtgens, P., Fennie, C., Singer, M. S., Lasky, L. A. and Rosen, S. D., *Blood* 1991. *77:* 2553.
15 Berg, E. L., McEvoy, L. M., Berlin, C., Bargatze, R. F. and Butcher, E. C., *Nature* 1993. *366:* 695.
16 von Andrian, U. H., Chambers, J. D., Berg, E. L., Michie, S. A., Brown, D. A., Karolak, D., Ramezani, L., Berger, E. M., Arfors, K. E. and Butcher, E. C., *Blood* 1993. *82:* 182.
17 Ley, K., Tedder, T. F. and Kansas, G. S., *Blood* 1993. *82:* 1632.
18 Bargatze, R. F. and Butcher, E. C., *J. Exp. Med.* 1993. *178:* 367.
19 Lo, S. K., Lee, S., Ramos, R. A., Lobb, R., Rosa, M., Chi-Rosso, G. and Wright, S. D., *J. Exp. Med.* 1991. *173:* 1493.
20 Smith, C. W., Rothlein, R., Hughes, B. J., Mariscalco, M. M., Schmalstieg, F. C. and Anderson, D. C., *J. Clin. Invest.* 1988. *82:* 1746.
21 Kishimoto, T. K., Jutila, M. A. and Butcher, E. C., *Proc. Natl. Acad. Sci. USA* 1990. *87:* 2244.
22 Lobb, R. R., Chi-Rosso, G., Leone, D. R., Rosa, M. D., Bixler, S., Newman, B. M., Luhowskyj, S., Benjamin, C. D., Dougas, I. R., Goelz, S. E., Hession, C. and Chow, E. P., *J. Immunol.* 1991. *147:* 124.
23 Lawrence, M. B. and Springer, T. A., *J. Immunol.* 1993. *151:* 6338.
24 Lawrence, M. B. and Springer, T. A., *Cell* 1991. *65:* 859.
25 Miller, L. J., Bainton, D. F., Borregaard, N. and Springer, T. A., *J. Clin. Invest.* 1987. *80:* 535.
26 Dustin, M. L. and Springer, T. A., *Nature* 1989. *341:* 619.
27 Lawrence, M. B., Bainton, D. F. and Springer, T. A., *Immunity* 1994. *1:* 137.
28 Goldman, A. J., Cox, R. G. and Brenner, H., *Chem. Eng. Sci.* 1967. *22:* 653.
29 Jung, T. M., Gallatin, W. M., Weissman, I. L. and Dailey, M. O., *J. Immunol.* 1988. *141:* 4110.
30 Tedder, T. F., Penta, A. C., Levine, H. B. and Freedman, A. S., *J. Immunol.* 1990. *144:* 532.
31 Lawrence, M. B., Smith, C. W., Eskin, S. G. and McIntire, L. V., *Blood* 1990. *75:* 227.
32 Picker, L. J., Kishimoto, T. K., Smith, C. W., Warnock, R. A. and Butcher, E. C., *Nature* 1991. *349:* 796.
33 Shimizu, Y., Shaw, S., Graber, N., Gopal, T. V., Horgan, K. J., Van Seventer, G. A. and Newman, W., *Nature* 1991. *349:* 799.
34 Picker, L. J., Terstappen, L. W. M. M., Rott, L. S., Streeter, P. R., Stein, H. and Butcher, E. C., *J. Immunol.* 1990. *145:* 3247.
35 Abbassi, O., Kishimoto, T. K., McIntire, L. V., Anderson, D. C. and Smith, C. W., *J. Clin. Invest.* 1993. *92:* 2719.
36 Chan, P.-Y., Lawrence, M. B., Dustin, M. L., Ferguson, L. M., Golan, D. E. and Springer, T. A., *J. Cell Biol.* 1991. *115:* 245.
37 Tözeren, A., Sung, K.-L. P., Sung, L. A., Dustin, M. L., Chan, P.-Y., Springer, T. A. and Chien, S., *J. Cell Biol.* 1992. *116:* 997.
38 Walcheck, B., Watts, G. and Jutila, M. A., *J. Exp. Med.* 1993. *178:* 853.
39 Jones, D. A., McIntire, L. V., Smith, C. W. and Picker, L. J., *J. Clin. Invest.* 1994. *94:* 2443.
40 deFougerolles, A. R., Stacker, S. A., Schwarting, R. and Springer, T. A., *J. Exp. Med.* 1991. *174:* 253.
41 Lewinsohn, D. M., Bargatze, R. F. and Butcher, E. C., *J. Immunol.* 1987. *138:* 4313.
42 Diamond, M. S., Staunton, D. E., Marlin, S. D. and Springer, T. A., *Cell* 1991. *65:* 961.