

Demonstration of the leukocyte-endothelial cell adhesion cascade involved in lymphocyte recirculation and neutrophil and lymphocyte localization to sites of inflammation
by Robert Frost Bargatze

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Molecular Biology
Montana State University
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Abstract:
Experimental evidence derived from multiple experimental systems, presented in this thesis, support the hypothesis that tight leukocyte adhesion to endothelial cells (EC) is regulated by a multi-step process that includes rolling, up-regulation of secondary adhesion molecule affinity through signaling, and permanent arrest. Developing an in vivo video microscopy analysis system, I have shown that mouse lymphocytes exhibit a multi-step behavior of binding when interacting with Peyers patch (PP) high endothelial venules (HEV). Also using this system, I have identified the molecules responsible for the multi-step lymphocyte PP-HEV rolling and adhesion behaviors: L-selectin mediates rolling, α4β7 integrin mediates slowing of rolling and activation dependent adhesion, LFA-1 may play a role in α4β7 activation, and MadCAM on the HEV acts as a ligand for both L-selectin and α4β7. Through developing an in vitro shear system to study human peripheral blood lymphocytes (PEL) interactions with E-selectin transfectants and human umbilical vein endothelial cells (HUVECs), I have demonstrated that E-selectin can support L-selectin-independent lymphocyte rolling. HUVECs in this system can support rolling through E-selectin and all aspects of the in vivo three-step binding process. Also using this system I examined the effects of chemokine signaling on lymphocyte adhesion and defined a new mechanism where MIP-lβ and IP-10, in minutes, induce increased avidity through selectins, slowing lymphocyte rolling. Further, I have shown in vitro that human neutrophils roll on E-selectin transfectants and HUVEC and defined the molecular basis through functional blocking with a unique mAb, EL-246, that acts on both L- and E-selectin impeding their adhesive functions. Finally, in vitro I have demonstrated a new mechanism for neutrophil localization where neutrophils roll on pre-adhering neutrophils augmenting their continued recruitment.

DEMONSTRATION OF THE LEUKOCYTE-ENDOTHELIAL CELL ADHESION CASCADE INVOLVED IN LYMPHOCYTE RECIRCULATION AND NEUTROPHIL AND LYMPHOCYTE LOCALIZATION TO SITES OF INFLAMMATION

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MONTANA STATE UNIVERSITY
Bozeman, Montana

December 1994

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I dedicate this thesis to my grandmothers and grandfathers:
Anna Frost and Alex C. Frost and Katie Bargatze and Robert C. Bargatze;
for imparting to me the gift of curiosity for exploring the natural universe
and an unending desire to understand its depths.

ACKNOWLEDGMENTS

I thank my thesis committee Mark A. Jutila, Michael W. White, Bruce L. Granger, Jim E. Cutler, Norman D. Reed, and Eugene C. Butcher for their support and direction.

For all of the help, support, and inspiration over all the years
I respectfully thank:

Barbara Jean Frost

Fred Orval Bargatze

Lee Frost Bargatze

Dawn Frost McCormick

Holly Frost Martinez

Ivy Merriot

Rebecca Joyce Mead

Sandy Kurk

Gayle Watts

Kathy Jutila

Dana Hoover

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ABSTRACT

Experimental evidence derived from multiple experimental systems, presented in this thesis, support the hypothesis that tight leukocyte adhesion to endothelial cells (EC) is regulated by a multi-step process that includes rolling, up-regulation of secondary adhesion molecule affinity through signaling, and permanent arrest. Developing an *in vivo* video microscopy analysis system, I have shown that mouse lymphocytes exhibit a multi-step behavior of binding when interacting with Peyers patch (PP) high endothelial venules (HEV). Also using this system, I have identified the molecules responsible for the multi-step lymphocyte PP-HEV rolling and adhesion behaviors: L-selectin mediates rolling, $\alpha 4\beta 7$ integrin mediates slowing of rolling and activation dependent adhesion, LFA-1 may play a role in $\alpha 4\beta 7$ activation, and MadCAM on the HEV acts as a ligand for both L-selectin and $\alpha 4\beta 7$. Through developing an *in vitro* shear system to study human peripheral blood lymphocytes (PBL) interactions with E-selectin transfectants and human umbilical vein endothelial cells (HUVECs), I have demonstrated that E-selectin can support L-selectin-independent lymphocyte rolling. HUVECs in this system can support rolling through E-selectin and all aspects of the *in vivo* three-step binding process. Also using this system I examined the effects of chemokine signaling on lymphocyte adhesion and defined a new mechanism where MIP-1ß and IP-10, in minutes, induce increased avidity through selectins, slowing lymphocyte rolling. Further, I have shown *in vitro* that human neutrophils roll on E-selectin transfectants and HUVEC and defined the molecular basis through functional blocking with a unique mAb, EL-246, that acts on both L- and E-selectin impeding their adhesive functions. Finally, *in vitro* I have demonstrated a new mechanism for neutrophil localization where neutrophils roll on pre-adhering neutrophils augmenting their continued recruitment.

CHAPTER 1

INTRODUCTION

The Leukocyte-Endothelial Cell Adhesion Cascade

The interactions of leukocytes with endothelium, which facilitate their blood-borne recruitment to sites of inflammation and lymphoid organs, are a critical step in regulating the distinct but closely related processes of inflammation and immune host surveillance. These defensive processes have evolved in parallel to provide an effective means for overlapping host protection. Leukocyte surveillance can protect against infection, act as a vigil for neoplastic conversions, employ mechanisms to recognize and reject foreign tissues, and, unfortunately in some cases, result in overt pathology. The unravelling of the molecular mechanisms of endothelial-leukocyte recruitment is brief, spanning only one-and-a-half decades. Even so, these recent advances in understanding the processes and molecular basis of leukocyte-endothelial cell recruitment have spurred vigorous inquiry into developing novel therapeutics and approaches for treating many age-old immune/inflammatory diseases.

The first event required for leukocyte entry into the body's tissues from the circulating blood, is recognition and adhesion to the surface of the blood vessel wall. A strong functional paradigm has been developed to describe leukocyte trafficking and consists of a three step process for

leukocyte-endothelial cell binding. Work conducted for this thesis has led directly to the generally accepted theory that during homing and inflammatory recruitment, lymphocytes, like neutrophils, employ this three-step process of recognition leading to strong endothelial cell adhesion. The three step model requires the leukocyte to make a very rapid (1.5-2.0 sec) and accurate assessment as to whether it should bind to the endothelium and transmigrate into a tissue after only a brief encounter with the endothelial-cell surface on the vessel wall. This assessment is based on endothelial-cell presented leukocyte adhesion molecules and molecular signaling information in either soluble or attached form [Tanaka et al., (1); van Kooyk et al., (2)]. In both cases, the molecules reveal critical information on the condition of the underlying tissue.

The experimental evidence supporting the multi-step hypothesis is significant and has been endorsed in many independent studies [reviewed in (3-8)]. The first event to occur in the temporal sequence is the collision of leukocytes with the blood-vessel wall. These collisions, a consequence of blood-vessel morphology and hemodynamic forces, can productively lead to leukocyte-endothelial cell rolling or in the return of the cell to the circulation. Rolling occurs only after tethering (contact) of leukocyte shear-capable adhesion molecules with their counter ligands on the endothelium, as illustrated in Figure 1, and can be mediated by separate leukocyte molecules. These tethering and rolling interactions can be mediated by both adhesive molecules identified as "selectins" as well as the $\alpha 4\beta 7$ and $\alpha 4\beta 1$ integrins. These molecules slow the cell from the blood flow allowing an "adhesokine" (an adhesion molecule signaling factor that induces increased adhesion molecule avidity and/or numbers) to bind its leukocyte receptor.

The binding of this effector can trigger a transmembrane signal, the second step, resulting in the induction of adhesion activity by an additional class of adhesion receptor, the leukocyte integrins, and in some cases increases their numbers on the leukocyte surface (Figure 1). If the endothelium presents a counter ligand for the signal-induced integrin adhesion molecules, the cell may be further slowed to where it arrests on the endothelial surface, completing the third step. The leukocyte may at this point go on to strengthen its adhesive hold on the endothelium via morphology changes and engagement of low-to-non-shear-acting integrin adhesion receptor pairs that promote strong adhesion, motility, and transmigration into the underlying tissue to follow chemotactic signals (Figure 1). Alternatively, the leukocyte may choose to release from the endothelium and return to the blood. This three step model of cell adhesion can be descriptively termed a "leukocyte-endothelial cell adhesion cascade."

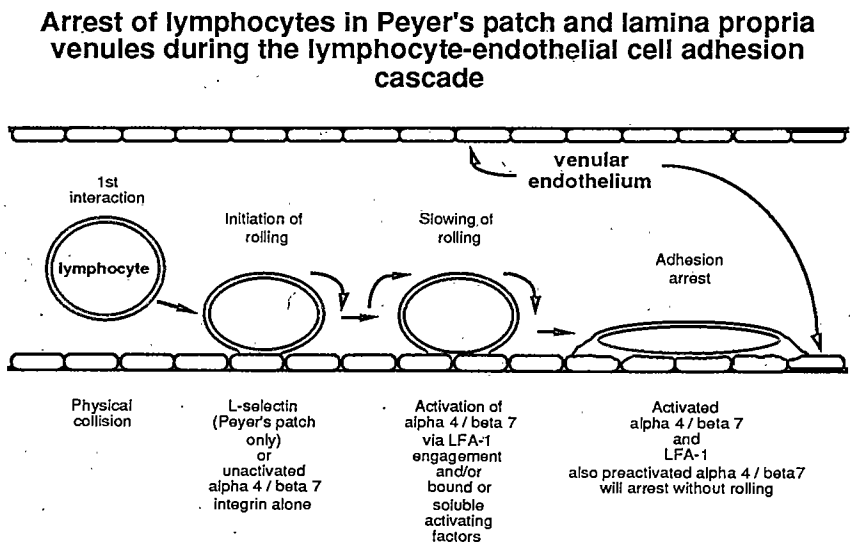


Figure 1. Events of the leukocyte-adhesion cascade, shown specifically for

mucosal binding lymphocytes. The lymphocyte enters from the blood flow, colliding with the blood-vessel wall and initiating rolling via L-selectin mediated-tethering to MadCAM. During rolling, $\alpha 4\beta 7$ integrin slows rolling, again via MadCAM ligation, as it becomes activated via signaling interactions of LFA-1 or endothelial cell-presented "adhesokines" that engage their leukocyte cell-surface receptors. As the full potential of adhesion avidity is reached by greater numbers and/or higher affinity of $\alpha 4\beta 7$ interactions, the lymphocyte is brought to a full arrest. Cells possessing preactivated $\alpha 4\beta 7$ can arrest on mucosal venules without L-selectin participation with little rolling before arrest.

Completion of each stage of the three-step process is required for successful strong adhesion of the leukocyte to the endothelium. Since each step is a unique recognition event, specificity can reside at any or all points in the cascade. Furthermore, each step may be mediated by many receptor-ligand pairs; hence, the number of potential leukocyte-endothelial cell specificities is the product of diversity at each of these recognition steps. Thus, selective recruitment can be very specific and plastic, recognizing the unique tissue-associated aspects of the vessel beds of lymphoid organs, or alternatively, remarkably responsive to inflammatory-induced molecular changes on the endothelium occurring to combat infection, disease, or trauma.

Constitutive lymphocyte recirculation and tissue-selective localization.

The first leukocyte tissue-selective adhesion system to be studied was the homing of lymphocytes to lymph nodes and Peyers patches which

occurs during constitutive recirculation and immune surveillance. In the early 80's, leukocyte adhesion receptors for the endothelium were theorized to have tissue-specific binding properties. This hypothesis was first based on lymphocyte recirculation data generated in rat [Gowans and Knight, (9)] showing that mucosal and peripheral-derived lymphocytes, labeled with radioactive tracers, followed distinct recirculation patterns that generally returned them to their lymphoid tissues of origin. In the late seventies and early eighties, studies showed that there are distinct lymphocyte subsets that have unique lymph-node high-endothelial venule (HEV) binding properties.

HEV are specialized postcapillary venules, comprised of rounded "high" or plump endothelial cells, that are present in lymph nodes and at sites of chronic inflammation. Lymphocyte-HEV binding competence correlates well with lymphocyte development and differentiation. Germinal center B cells and cortical thymocytes are sessile immature cells that do not bind HEV in vitro [Reichert et al., (10); Butcher et al., (11)]. Alternatively, recirculation competent, mature resting lymphocytes are capable of HEV binding in all lymphoid tissues; however they are composed of subsets, dependent on tissue origin, that show a general preferential binding for peripheral lymph nodes (PLN) or Peyer's patch (PP) HEV [Stevens et al., (12)]. The tissue-selective hypothesis was more directly shown in work by Butcher, Scollay and Weissman (13), where mouse lymphoma cell lines were isolated and shown to preferentially adhere to the HEV of PLN or PP. This was later shown to be the case for human

lymphomas, with the addition of the identification of lymphomas specific for inflamed joint synovial venules [Jalkanen et al., (14)]. These studies employed the ex vivo lymphocyte-HEV binding assay developed by Stamper and Woodruff (15) and for the first time demonstrated endothelial-selective binding. Lymphoma phenotypes were characterized that showed peripheral, mucosal, dual, and non-binding phenotypes. The mouse-derived lymphomas, exhibiting each of the specific binding phenotypes from these studies, were tested in vivo. We found that HEV-binding, but not HEV-non-binding, lymphomas spread metastically via the blood, suggesting that homing receptors are a key component in lymphoid metastasis [Bargatze et al., (16)]. Collectively, these findings strongly support the hypothesis for "tissue-specific" lymphocyte adhesion receptors for HEV and intensified interest in their identification and characterization.

L-selectin: The lymphocyte PLN homing receptor.

The first "tissue-specific" lymphocyte adhesion receptor to be characterized was phenotypically and functionally identified by the monoclonal antibody (mAb) MEL-14. Gallatin et al. (17) demonstrated that MEL-14 recognizes a lymphocyte surface glycoprotein, now known as L-selectin. In this first paper describing the physical and functional properties of L-selectin, it was clearly demonstrated that MEL-14, both in vitro and in vivo, almost completely inhibits lymphocyte binding or homing to PLN HEV. (This paper also represented my first effort in leukocyte adhesion biology; the in vivo data coming from my experiments.) Rasmussen et al.

(18) produced a mAb against rat PLN homing receptors that has function-blocking properties similar to MEL-14, but the molecule(s) it sees was never fully characterized. Also in the mid 80's, L-selectin was found to be expressed on the surface of other leukocytes, including neutrophils, monocytes, and eosinophils. In the study by Lewinsohn et al. (19), we demonstrated that MEL-14 would block neutrophil binding to PLN HEV and their recruitment to acute inflammatory sites in a mouse model. It is now known that L-selectin is expressed on all leukocytes except for a population of lymphocyte memory cells [Lewinsohn, (19); Stoolman, (20)].

The 80-95 kD MEL-14-defined L-selectin molecule functions as an animal lectin, blockable by phosphomannan competitors, capable of binding unique carbohydrate determinants on PLN HEV [Yednock et al., (21)]. Cloning of L-selectin by Siegelman et al. (22) and Lasky et al. (23) revealed striking homology to the C-type mammalian lectins in the L-selectin N-terminal domain. The L-selectin "Drickamer motif" lectin domain [(23); Watson et al., (24)] exhibits a typical strict calcium dependence for carbohydrate recognition, which is the precise cation requirement for lymphocyte-PLN HEV binding. Thus, the rolling/binding specificity of L-selectin for endothelial ligands is believed to reside largely in this domain; however, an epidermal growth factor (EGF) domain that is contiguous with the lectin domain expresses a mAb blockable epitope that contributes to the rolling/binding function of the molecule. The remaining structure of L-selectin is composed of two identical repeats of a complement binding protein-like domain (SCRs), a transmembrane domain, and a short cytoplasmic tail containing one potential phosphorylation site. The same

general domain structures are present in the other members of the selectin family: E-selectin on endothelium and P-selectin on endothelium and platelets [Bevilacqua et al., (25); Johnston et al., (26)]. These molecules are specifically involved with inflammation-driven, blood-borne recruitment of leukocytes to acute and chronic inflammatory sites of the body.

Evaluating the blood-borne spread of murine lymphomas again led us to a new finding; L-selectin could support adhesion to PP HEV [Bargatze et al., (27)]. This seemed to conflict with earlier findings that L-selectin mediated binding to PLN but not PP HEV. Now, in the context of a three-step adhesion model, it is clear that L-selectin mediates only the tethering/rolling interaction with the mucosal endothelium that leads to specific integrin-mediated tight adhesion. Hamann et al. (28) confirmed these observations in vivo showing a 40-50% reduction of homing to mouse PP when lymphocytes were treated with the anti-L-selectin mAb MEL-14.

The peripheral node addressin is a functional receptor for lymphocytes on HEV.

Identification of the PLN ligand for L-selectin on the HEV was the focus of intense effort soon after L-selectin and its specificity for binding were identified. It was reasoned that if a specific leukocyte counter-receptor for PLN HEV was required for binding then an equally specific receptor expressed on the endothelium must exist. Using the mAb MECA-79, Streeter et al. (29) were rewarded with the identification of a group of PLN HEV-expressed glycoproteins which they named peripheral lymph node

addressin (PNAd). Functional characterization of the MECA-79 mAb using the HEV binding assay and homing of radiolabeled lymphocytes in vivo showed that it blocks HEV binding and PLN localization of lymphocytes in a manner identical to MEL-14, though less efficiently. Work done by Berg et al. (30) showed that the MECA-79 epitope is apparently a carbohydrate antigen presented on the PNAd glycoprotein and that this epitope is critical for supporting lymphocyte adhesive interactions.

More recently, MECA-79 was used to purify the PNAd for in vitro assays. Berg et al. (31) demonstrated that the isolated PNAd supports binding of lymphocytes which is L-selectin dependent. Also, the ability of MECA-79 isolated PNAd to support lymphocyte and neutrophil rolling under shear was tested in vitro. Glass capillary tubes were coated with isolated PNAd and individually integrated into a closed silicone tubing loop attached to a peristaltic pump. Neutrophils or lymphocytes in tissue culture media were infused into the loop, flow was started to simulate blood flow, and events occurring in the capillary tube were recorded by video microscopy (Figure 2). This system demonstrated that lymphocytes and neutrophils roll on the isolated PNAd molecules exhibiting behaviors very similar to those observed for these cell types in vivo (Bargatze, Berg, Palecanda, Butcher, and Jutila, unpublished observations).

Subsequent studies have demonstrated that the PLN addressin is induced in postcapillary venules at diverse sites of the body and is found in association with chronic inflammation [Michie et al., (32); Hanninen et al., (33)], and autoimmune disease [Berg et al., (34)]. In fact, using the L-selectin-positive PLN HEV-binding lymphoid tumors, I found that MECA-79 defined

an L-selectin ligand expressed on PP HEV at low but functional levels (27). Berg et al. (35) confirmed that the MECA-79 epitope, expressed on in vivo isolated mucosal addressin (MadCAM, which supports lymphocyte trafficking to PP) supports L-selectin-dependent rolling in the capillary tube shear system. These reports have led directly to the studies reported in Chapter 4, in which L-selectin-dependent rolling on MadCAM in vivo is demonstrated to support the tethering of lymphocytes to PP HEV, the first step in the recruitment process for lymphocyte recirculation in mucosal immune surveillance.

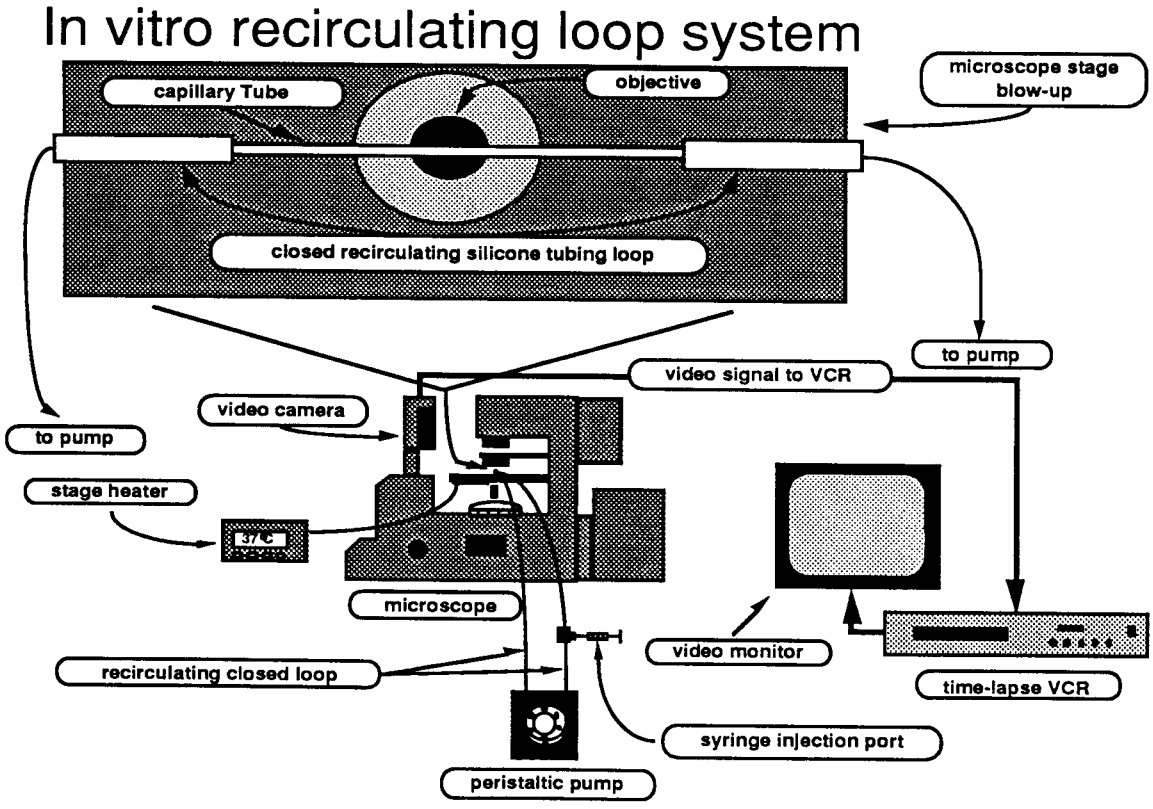


Figure 2 To examine leukocyte adhesion (tethering-rolling, slowing, and

permanent arrest) in a controlled in vitro setting I developed the novel capillary tube, closed-loop, shear assay system as depicted above. This system provides the means to make measurements of the interaction of suspended cells with immobilized substrates, such as endothelial cells, transfected fibroblasts, marginating leukocytes, and purified adhesion proteins, under conditions reflective of those seen under blood flow.

Identification of a mucosal-specific lymphocyte homing receptor.

The recognition of PP-specific HEV-binding lymphomas suggested that, as with the peripheral HEV system, mucosal-specific homing receptors for MadCAM must exist. Again, several groups reported early findings suggesting the identification of such a receptor for lymphocytes. Chin et al. (36) produced a mAb that blocks rat lymphocyte binding to PP HEV, as did Jalkanen et al. (37). The molecule identified by Chin et al. was never fully characterized; the molecule identified by Jalkanen has been shown by cDNA cloning [Golstein et al., (38)] to be a lymphocyte-specific form of CD44 involved with adhesion, but not to be the central player in mucosal lymphocyte trafficking. In the first study to examine the cell-surface phenotype of gut intraepithelial leukocytes (IEL), Schmitz et al. (39) reported that these cells lacked expression of L-selectin, as assessed by MEL-14 mAb staining. They also discovered that these cells avidly bound PP HEV in contrast to PLN HEV, where IEL showed a near background level of binding. These experiments clearly demonstrated that a population of leukocytes, IEL, could demonstrate exclusive tissue selectivity for their PP versus PLN homing phenotype.

A mucosal homing receptor breakthrough occurred in 1989 with the production of the R1-2 monoclonal antibody [Holtzmann et al., (40)]. This antibody recognizes the integrin heterodimer α4β7 expressed on mouse lymphocytes with function distinct from the previously characterized VCAM-1 binding integrin α4β1 (40). Holtzmann et al. demonstrated that lymphocyte binding to PP HEV in vitro could be specifically blocked by R1-2, showing this interaction to be distinct from that occurring in PLN. Unfortunately, this mAb was not effective in blocking lymphocyte HEV interactions in vivo, so confirmation of this interaction under physiological conditions waited until Issekutz (41) showed that α4 in the rat and later Hamann et al. (42) showed that α4 as well as β7 in the mouse was required for in vivo homing to mucosal lymphoid tissues.

Interestingly, α4β7 has also recently been demonstrated to bind VCAM-1 and fibronectin in static assays [Postigo et al., (43)]. We have confirmed VCAM-1 binding under physiological shear (Berlin et al. in press, Cell), with data strongly suggesting that α4 is the primary chain of the heterodimer supporting the specificity.

The mucosal addressin directs lymphocyte traffic to gut HEV and lamina propria venules.

During our initial quest for tissue-specific ligands expressed on HEV for lymphocytes, coinciding with the characterization of the PNAD mAb MECA-79 we found a new mAb that specifically stained PP versus PLN HEV. This antibody, MECA-367, blocks binding in vitro and homing in vivo of

mucosal-specific lymphomas and normal lymphocytes Berg et al. (30) and was used to characterize the mucosal addressin glycoprotein (MadCAM). Using a non-blocking mAb to MadCAM (MECA-89) in an immunoaffinity column, the MadCAM molecule was isolated from mucosal lymphoid vessels and immobilized in planar lipid membranes. Both mucosal-specific lymphomas and normal lymphocytes bound the isolated MadCAM in static assays, and binding was blocked by treatment with MECA-367.

MadCAM was recently cloned by Briskin et al. (44), revealing an unexpected and unique domain structure. The N-terminus of MadCAM presents a domain with a structure and sequence similar to both ICAM-1 and VCAM-1 which is followed by a VCAM-1 like domain. It is likely these domains interact with $\alpha 4\beta 7$ integrin on lymphocytes, facilitating adhesion (44). These regions are followed by a mucin-like domain which may facilitate lymphocyte rolling in PP HEV (44). The final domains, include an IgA-like domain, a single membrane-spanning segment and cytoplasmic tail. Much of the behavior observed for lymphocyte-HEV interactions can be extrapolated from the properties of MadCAM based upon this diverse presentation of defined adhesion molecule-related structures. It is interesting to note that MadCAM can be induced on endothelial cells in culture [Sikorski et al., (45)], while PNAD has not yet been successfully expressed in vitro.

Most recently, we have shown that MadCAM, when isolated from mucosal lymphoid venules, supports lymphocyte rolling adhesion in the in vitro capillary tube (shown in Figure 2) to examine leukocyte shear-dependent interactions [Berg et al., (46)]. These results provide a specific

identification for the molecular basis of the L-selectin binding to PP HEV which we reported earlier (27). Interestingly, MECA-79, the mAb recognizing PNAD, identifies a subset of MadCAM that supports L-selectin-dependent rolling (46). This carbohydrate epitope, which can decorate MadCAM, appears to be the critical adhesive binding/recognition element for L-selectin. Thus, expression of this epitope can be induced in non-peripheral locations directing peripheral lymphocyte trafficking to mucosal sites.

In a manuscript just accepted for publication (Berlin et al., Cell, in press), we have again used the in vitro shear system to show that MadCAM, under shear, can support the binding of lymphocytes and lymphomas requiring only activated $\alpha4\beta7$. We also confirmed these observations in vivo in gut lamina propria venules where there is no L-selectin component of adhesion. There again, $\alpha4\beta7$-MadCAM interactions are primarily responsible for supporting the lymphocyte/lymphoma blood-borne localization (Berlin et al., Cell, in press). Thus, it appears that on the HEV of the Peyer's patch, MadCAM acts as a constitutive ligand that mediates lymphocyte-selectin rolling and facilitates strong integrin adhesion to support the lymphocyte homing process. In gut lamina propria venules, MadCAM and activated $\alpha4\beta7$ can arrest circulating lymphocytes in the absence L-selectin, stopping them after a very short $\alpha4\beta7$ mediated rolling interaction with the endothelium. This is of particular interest since it demonstrates that a single integrin-mediated adhesive step can compress the multistep process into one event leading to lymphocyte permanent arrest on the endothelium.

LFA-1 is a non-tissue-specific participant in lymphocyte binding to HEV.

Lymphocyte interactions with HEV during static assay conditions have been known since the late 1980s to be supported by the integrin LFA-1 [Hamann et al., (47)]. These adhesive interactions, however, are not tissue selective, and mAbs only block lymphocyte HEV binding approximately 40 percent on either PP and PLN HEV. Interestingly, LFA-1 also represents a molecule involved in both receiving and delivering activating signals [Binnerts et al., (48)]. LFA-1 is a versatile adhesion molecule capable of interacting with ICAM-1 on lymphocytes, epithelial cells, and inflamed endothelium; ICAM-2 on the vascular endothelium and some lymphoid cells; and ICAM-3 only on leukocytes (48). ICAM-2 is believed most likely to be involved in the recirculation of resting lymphocytes as it is the predominant ICAM form expressed on resting endothelium [de Fougerolles and Springer, (49)]. Crosslinking of T-cell surface receptors, such as CD2 and CD3, or addition of the phorbol ester PMA generates intracellular signals that lead to increased avidity of LFA-1 for ICAM-1, resulting in stronger lymphocyte adhesion to the endothelium [Dustin and Springer, (50); van Kooyk et al., (51); , Rothlein and Springer, (52)]. It also was discovered that a mAb-binding LFA-1 could similarly alter its adhesive properties inducing tighter LFA-1-ICAM-1 binding (48). Interestingly, engagement of LFA-1 and its ligand ICAM-3 can also act to deliver a co-stimulatory signal for both resting and activated T-cells [Hernandez-Casselles et al., (53); Campanero et al., (54)]. These diverse adhesion and signaling properties suggest that LFA-1

on lymphocytes may play a multifunctional role in the lymphocyte-endothelial cell adhesion cascade that involves regulation of other adhesion receptors through receiving and delivering intracellular signals, as well as directly supporting HEV binding.

Lymphocytes, signal transduction, and the three-step model of leukocyte binding.

When I started my thesis work in 1990, a muti-step hypothesis for localization of leukocytes to endothelium during lymphocyte recirculation or to sites of inflammation had yet to be formulated. The adhesion receptors that support the process had been largely identified and characterized, but their physiological function and temporal role in the blood-borne adhesion process had not been accurately determined. The interactions of lymphocytes with the PP HEV of mice [Bjerknes et al., (55); (16)] and neutrophils in mesenteric venules of rabbits [Arfors et al., (56)] had been observed and described in detail in vivo to show a diversity of rolling and sticking behaviors. Dillon et al., (57) had demonstrated that platelet activating factor (PAF) could promote tight adherence of leukocytes to endothelium. LFA-1 was shown to be capable of activation to support tight adhesion to endothelium possibly through factors, such as PAF. All of these were critical elements in the multi-step process yet to be discovered. In 1989, a key observation by Huang et al. (58) demonstrated that pertussis toxin (Ptx) treatment, which interrupts transmembrane signaling, is effective in preventing lymphocyte localization to HEV-bearing lymphoid organs. This

finding suggested a requirement for signal transduction in the homing process. The stage was set for unravelling the intricate details of the leukocyte-endothelial cell adhesion cascade. The model was first presented in a two-step form by our group at Stanford in 1991 [von Andrian et al. (59)].

With these initial observations, Kishimoto (60) proposed a dynamic three-step model for neutrophil localization to inflammatory sites and soon after Butcher (61) proposed a generalized model for leukocytes predicting that a three-step model would also hold for lymphocyte localization to HEV during constitutive recirculation. It was at this point in early 1991 that we began to evaluate the in vivo interaction of lymphocytes in PP HEV (Figure 3). We used Ptx and PMA to determine if lymphocytes required a functional Ptx-sensitive adhesion-strengthening signaling pathway, as suggested by Huang et al. (58) to arrest on PP HEV. The results of these experiments were recently published (16) and are described in detail in Chapter 2 of this thesis. The findings showed that lymphocytes, like neutrophils, required multiple adhesion steps to arrest on the endothelium.

The data describing the identification of the adhesion receptor pairs responsible for the behavior of lymphocyte binding to PP HEV are contained in Chapter 3 of this thesis and has recently been submitted for publication. In this extensive in vivo study, we evaluated each step in the rolling, slowing, and tight adhesion steps of lymphocyte binding to HEV with adhesion receptor-specific function-blocking mAbs. Our evaluation showed that lymphocyte-HEV tight adhesion could be blocked by mAbs directed against LFA-1.

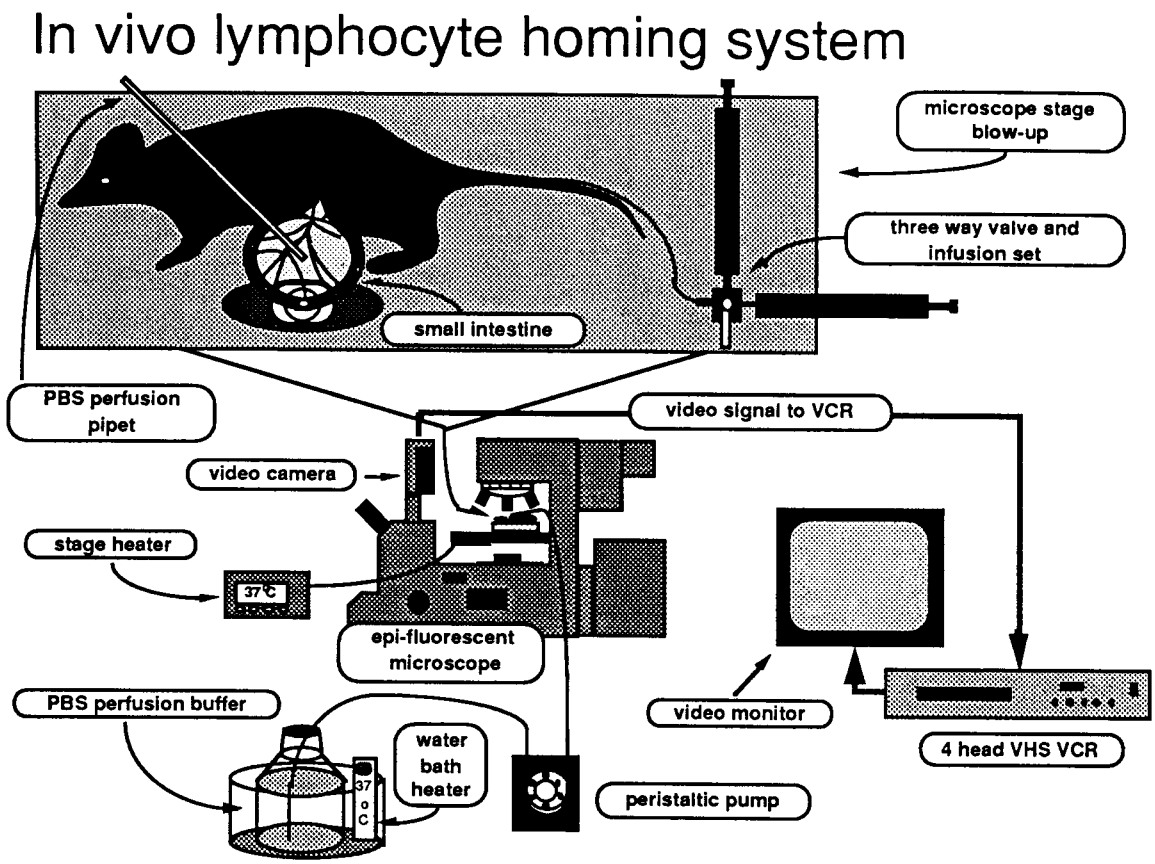


Figure 3. The in vivo video-microscopy recording system was developed to analyze leukocyte-endothelial-cell interactions in the animal. This method makes detailed examination of blood-infused FITC-labeled lymphocyte interactions with high endothelial venules (HEVS) in exteriorized mouse Peyer's patches possible.

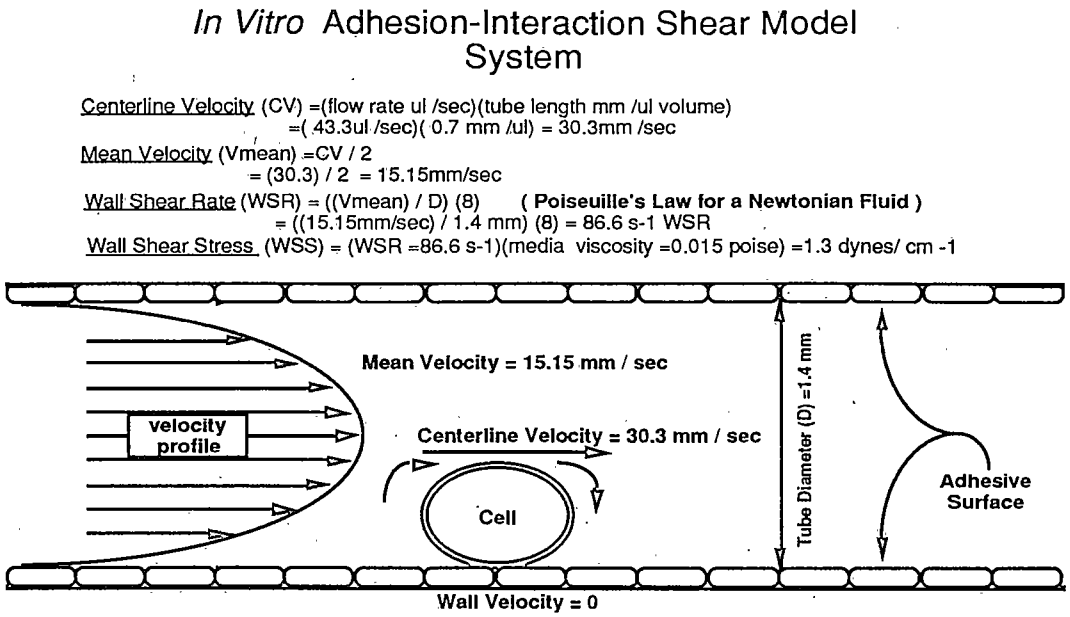
What are the "adhesokine" factors responsible for signaling lymphocyte strong adhesion to endothelium during inflammatory recruitment?

It is now generally assumed that IL-8 treatment of neutrophils promotes neutrophil strong adhesion under flow to endothelium by induction of CD11-CD18 activation [reviewed in Kelvin et al., (62); Shaw and

Adams, (63)]. In human skin, presentation of IL-8 by postcapillary venular endothelial cells has been demonstrated. IL-8 can also induce neutrophils to migrate along a gradient in vitro, suggesting it is likely to cause neutrophils to emigrate and migrate into inflamed tissues [Rot, (64)]. Other factors that possess similar properties and protein structures have been identified for neutrophils (MGSA) and monocytes (MCAF). This family of peptides has been termed "chemokines," with each member having proinflammatory activity, and belonging to one of two subfamilies: the "α" C-X-C or the "β" C-C. The C-X-C and C-C nomenclature refers to presence or absence of an intervening amino acid between the first two of four conserved cysteines in the peptide sequence. Chemokines act on leukocytes through the serpentine family of the seven transmembrane-spanning G-protein-coupled receptors and are involved in rapid signaling events. This receptor class is responsible for signal transduction exemplified by the rodopsin receptor in visual perception, where high speed signaling is an absolute requirement. The pertussis toxin blocking of integrin-mediated lymphocyte strong adhesion, reported in Chapter 2 of this thesis, likely occurs through this class of coupled signaling pathway.

Chemokines have been identified that target lymphocytes, upregulating their static surface-binding capacities (reviewed in Miller and Krangel, (65)]. It has been hoped that the lymphocyte chemokines would exhibit the same rapid activation properties that IL-8 shows for neutrophils, inducing integrin activation to support slowing and tight adhesion during lymphocyte-endothelial cell rolling. The RANTES chemokine was the first shown to be active on T lymphocytes, stimulating chemotaxis and adhesion

[Oppenheim et al., (66); Schall, (67)]. Studies by Kelvin (62) and Shaw (63) have shown that chemokines will upregulate the adhesiveness of lymphocytes for isolated VCAM-1 or activated endothelium after 1-24 hr long preincubations in culture. Individual chemokines such as MIP-1α & β acting on CD8 and CD4 T cells respectively, appear to act most effectively on the memory T-cell populations. Vasoactive intestinal peptide (VIP) [Ottaway, (68)], a 28-amino acid peptide of the glucagon-secretin family, also interacts with a serpentine G-protein-coupled lymphocyte cell-surface receptor. VIP has the property of acting on unstimulated T cells, promoting increased binding to VCAM and ICAM in static assays. Based upon these observations of lymphocyte chemokine and VIP-induced adhesive activity, researchers have proposed that these small peptides may act to signal lymphocyte tight adhesion during rolling at sites of inflammation [Johnston et al., (69)]. Chapter 4 of this thesis reports data from an ongoing project for which a manuscript is currently in preparation. We examine the effect of chemokine treatment on lymphocytes in the in vitro loop HUVEC shear binding assay and plan to investigate the VIP effects in future studies. The effects of the chemokines have been examined in detail using the system described in Figure 4.



Reference: Perry, M. A. and Granger, D. N.. J. Clin. Invest. 87:1798-1804

Figure 4. Calculations for the typical in vitro shear system, shown in this figure, provide detailed information describing cell-cell/substrate interactions. These include rolling speed, rolling behavior, number of cells binding per unit area versus time, and the effects of modulators of these interactions allowing comparison of leukocyte subsets under a variety of experimental conditions. Either leukocytes and/or endothelial cells can be activated in this system using a spectrum of cytokines. Blocking of interactions by mAb, enzymatic treatment, or direct chemical inhibitors can be observed and accurately quantified.

Acute inflammation-driven neutrophil and lymphocyte localization and selective recruitment to inflammatory sites bear striking similarities and differences.

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Comparing the recruitment of lymphocytes and neutrophils to acute sites of inflammation suggests that both the myeloid and lymphoid subsets

appear to use a three-step process of recognition and binding to the endothelium at these sites [Shaw in (63)]. What is truly of interest is that these different cell types do not simultaneously compete for endothelial binding, but are recruited to the site of inflammation in a temporally distinct sequence. This was clearly demonstrated by Jutila et al. (70) where, in leukocyte recruitment to thioglycolate-inflammed peritoneum, they showed that the first cell population to be recruited was neutrophils, followed by monocytes, and finally lymphocytes. This work and the work of others provide a potentially clear explanation for this sequential regulation. That is, there is a temporally regulated expression of adhesion receptors and adhesokines on the endothelium specific for the leukocyte subset that is selected at any time point for recruitment. The temporal regulation of these molecules can be directly related, as will be discussed in Chapter 4, to the production of cytokines at the inflammatory site. Thus, an understanding of the nature of the molecular requirements for early neutrophil recruitment in Chapters 5 and 6 will provide a means of identifying those requirements that are unique for the recruitment of lymphocytes vs neutrophils to acute inflammatory sites.

The behavior of leukocytes in the microvascular circulation has been of interest to scientists for over a century [von Andrian et al., (3)]. The mechanisms and adhesion receptors regulating the recruitment process for leukocytes to endothelium were largely unknown until the 1980s. This knowledge has largely been gained through the use of mAbs selected to identify and block the function of the adhesive process. To the surprise of

many researchers, many unique adhesion-receptor pairs have been identified for leukocytes and endothelial cells. This is likely due to the versatility that has developed in host defense to select a specific leukocyte subpopulation to react at key times during the progression of an inflammatory episode. The endothelium during inflammation responds to pro-inflammatory cytokines, such as IL-1, IL-4, γ interferon, and TNF, as released by the inflamed tissue, each inducing differential expression of E-selectin, VCAM-1, and ICAM-1 and yet-to-be-defined receptors on the endothelial surface. These adhesion-supporting cell-surface glycoproteins provide the adhesive foothold needed to initiate rolling and subsequent leukocyte arrest, as I have previously described for the lymphocyte. If the correct receptors and counter ligands are present on the leukocyte and endothelium, the circulating cell, rolling slowly relative to the blood flow, will find accessible signaling molecules on the endothelium capable of altering the avidity of rolling adhesion. Gradients of these chemotactic/chemokine factors may be presented by endothelial surface proteoglycan molecules acting to induce strong adhesion of leukocytes. After stopping, these factors and/or others are likely required for subsequent transmigration [Tanaka et al., (71)].

A first critical observation by Kishimoto et al. (72) provided insight into the consequences of chemotactic factor action on neutrophil adhesion receptor regulation. They found that activation of the cells resulted in rapid cleavage of L-selectin form the surface and equally rapid upregulation of Mac-1 expression on the neutrophil surface. The consequences of this regulation clearly showed that if L-selectin were cleaved before firm

attachment to the endothelium, the neutrophil would return to the blood flow. However, if the upregulated Mac-1 had cemented the neutrophil to the blood-vessel wall, the loss of L-selectin might ease the process of transendothelial migration. This hypothesis was in fact confirmed by Jutila et al. (70) in vivo where neutrophils that had migrated into an inflammatory site lost their L-selectin and had greatly upregulated their expression of Mac-1.

As has been discussed, a number of adhesion receptors and their ligands, such as Mac-1 and ICAM-1, were identified for having adhesive function that could be blocked by mAbs under static assay conditions. In fact a leukocyte adhesion deficiency (LAD-1) was identified as a deficit in the expression of the Mac-1, LFA-1, p150,95 integrin glycoproteins. But, Arfors et al. (56) were the first to show that the ß2 integrin MAC-1, blocked by an anti-CD18 mAb, inhibited accumulation of neutrophils in a dynamic in vivo model. This work was elaborated on in a paper by Ley et al. (73) who first showed that L-selectin was responsible for mediating neutrophil rolling in the same in vivo rabbit mesenteric venule model used to describe CD18 function.

These in vivo observations set the stage for the first in vitro selectin rolling model which was presented by Lawrence and Springer (74). In their paper, where P-selectin was demonstrated to act as a ligand that supports neutrophil rolling, the authors also proposed that rolling interactions were a prerequisite for engagement of MAC-1 with ICAM-1. This paper was rapidly followed by the finding that E-selectin also supported neutrophil rolling under flow conditions Kishimoto et al., (75). Since P-selectin is rapidly

upregulated on endothelial cells and platelets, it was hypothesized that P-selectin would be rapidly downregulated after providing a rapid response mechanism for recruitment of neutrophils to an acute inflammatory site. E-selectin, requiring several hours for endothelial cell surface expression, would then support additional accumulation if the inflammatory site continued to mature. This may not hold true in vivo since the regulation of these molecules was extrapolated from in vitro data.

Kishimoto et al. (75) again presented new results demonstrating that L-selectin and E-selectin act in a common adhesion pathway which suggests these two selectins may be counter ligands in neutrophil rolling and recruitment. Picker et al. supported this observation and suggested it was also true for L-selectin and P-selectin. New ligands have been demonstrated for both E- and P-selectin on neutrophils [Levinovitz et al., (76); Moore et al., (77); Lenter et al., (78)] and the full characterization and identification of their role of participation in binding to endothelial cells is currently under way.

We have very recently published a paper in the Journal of Immunology confirming the participation of L- and E-selectin in human neutrophil binding to inflamed HUVECs. Chapter 5 of this thesis reports these findings in detail, describing the properties of a unique anti-human mAb EL-246 that binds and blocks the adhesive functions of both L- and E-selectin and has profound effects when used as a clinical therapeutic. This antibody possesses the biochemical characteristic of "jumping" to E-selectin from L-selectin when it is prebound by L-selectin on neutrophils and then co-incubated with E-selectin transfectants or stimulated HUVECs. Because of the EL-246 "jumping" property, the leukocyte can directly deliver this mAb

to the endothelium. Therefore, it may be possible to use smaller dosages than used for other anti-selectin mAbs to get the same adhesion blocking effect. This mAb binds and blocks L- and E-selectin from many species and, as we report, it is very effective in vivo, as seen in blocking bovine lymphocyte homing to mouse PLN. The wide effectiveness of this antibody may make it useful for treatment of animal inflammatory diseases, as well as for humans.

During the course of examining neutrophil-endothelial interactions with activated HUVEC, as reported in the November issue of The Journal of Experimental Medicine and Chapter 6 of this thesis, we observed a new previously unreported phenomenon of neutrophil rolling. These findings have provided evidence for a new theory describing the augmented recruitment of neutrophils to acute inflammatory sites which may be generalized to numerous leukocyte subsets in a variety of inflammatory settings.

Statement of the hypothesis of leukocyte recruitment to the vascular endothelium.

The global hypothesis to be tested is that leukocyte recruitment to the vascular endothelium is a rapid, multi-step process, regulated at each of three sequential events. This process can be accurately termed an "adhesion cascade." The intent of this thesis has been to define the "adhesion cascade" by 1) characterizing the active process of leukocyte-endothelial cell recruitment; 2) defining the individual roles of leukocyte-endothelial cell

adhesion receptors during recruitment; and 3) determining the means by which endothelial-leukocyte signaling can occur to induce leukocyte adhesion strengthening for arresting leukocytes from the blood flow.

First I have used direct observation of lymphocyte homing to Peyer's patch high endothelial venules in vivo as a means to formulate a molecular-based theory of lymphocyte homing. Second, by observing the rolling and attachment of human neutrophils and lymphocytes to the surface of activated HUVEC in an in vitro shear model, I have formed the basis for my theory of acute inflammatory site recruitment of leukocytes.

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CHAPTER 2

RAPID G PROTEIN-REGULATED ACTIVATION EVENT INVOLVED IN LYMPHOCYTE BINDING TO HIGH ENDOTHELIAL VENULES

Introduction

The traffic of lymphocyte subsets is directed by binding to specialized venules, especially the high endothelial venules (HEV) in lymphoid tissues and in sites of inflammation (1,2). These interactions display tissue selectivity that has been explained in part by the description of lymphocyte homing receptors capable of binding to tissue-selective endothelial cell adhesion receptors known as the vascular addressins (3). Additional complexity in the regulation of lymphocyte-endothelial cell recognition is implied, however, by the observation that adhesion pathways used by lymphocytes can also be employed in other physiologic settings, for example, by neutrophils interacting with acutely inflamed endothelium. Recently, we and others have proposed a model of leukocyte-EC interaction as an active, multi-step process in which stable binding to the endothelium involves at least 3 sequential events (4, 5). In this model, interaction is initiated by primary, activation-independent but unstable adhesion which serves to slow the passage of the leukocyte, allowing sampling of the EC surface and of any local soluble factors. If activating factors are present to which the leukocyte

can respond, activation (the second step) occurs leading to triggering of activation-dependent adhesion receptors. Binding of activation-dependent adhesion receptors to their EC ligands results in stable binding, resistant to physiologic shear, thus completing the process of "recognition." Additionally, the recruitment of the bound leukocyte from the vessel wall into the surrounding tissues may be regulated independently of leukocyte-EC recognition mechanisms; for example, recruitment could be regulated by tissue-derived chemoattractant or haptotactic signals. The requirement for sequential engagement of primary adhesion, activation, and secondary adhesion pathways allows the possibility of regulating leukocyte-EC recognition at any of the three steps involved.

However, the central features of the three-step model have been confirmed experimentally only in the case of neutrophil interactions with acutely inflamed venules in the rabbit, in which a primary "rolling" interaction involving the leukocyte selectin is followed by an activation-dependent "sticking" and arrest mediated by ß2 (CD18) integrins (6-8). Intravital microscopic studies, as well as *in vitro* models of neutrophil interactions with vascular adhesive ligands, demonstrate that under physiologic shear, each of the three steps is required for completion of the recognition process and for stable arrest of neutrophils (9, 10). We have suggested that a similar process might be used by other leukocytes, including lymphocytes (4). However, although *in vitro* studies demonstrate that lymphocytes can be triggered by activation to upregulate adhesion molecules, including integrins, the kinetics of such *in vitro* activation-dependent adhesion seem too slow (occurring over minutes) to explain the extremely

rapid interactions of circulating lymphocytes with HEV *in vivo*, which occur within seconds (4, 11).

The present study was designed to explore directly the relevance of the model to lymphocytes. To this end, we assessed the involvement of lymphocyte activation in lymphocyte-HEV interactions, employing pertussis toxin (PTX) as a tool. The rationale for this approach was based on two considerations. First, many chemoattractant factors that are capable of triggering activation-dependent adhesion of leukocytes act via PTX-sensitive G-protein-mediated intracellular signaling pathways. Second, Daynes and coworkers demonstrated that PTX-treated lymphocytes home poorly into lymph nodes from the blood in spite of retaining a capacity to bind in *in vitro* assays to lymph node HEV via the (activation-independent) lymph node homing receptor L-selectin (12). These considerations led us to hypothesize that a PTX-sensitive signal transduction pathway might play an essential role in lymphocyte-HEV recognition and binding *in vivo*.

Materials and Methods

Sample lymphocyte preparation

Peripheral and mesenteric lymph node lymphocytes from young adult BALB/C mice (Simonsen Labs, Gilroy, CA) were suspended at a concentration of $5x10^6$/ml in DMEM [without sodium bicarbonate (Sigma Chemicals, ST Louis, MO) supplemented with 20mM HEPES, pH 7.0 (Research Organics, Cleveland, OH)] and containing 1 or 4% iron-supplemented calf serum (Hyclone Labs, Logan, UT), and were incubated at

37°C with or without PTX (100ng/ml, List Biological Labs, Campbell, CA) for 2 hrs. In some experiments, a mutant pertussis holotoxin (PT9K 129G) was employed. (This mutant PTX retains the agglutinating, hemolytic and antigenic properties of native PTX, and gave indistinguishable subunit banding patterns with native PTX[13,14] when compared by SDS-PAGE and Coomassie staining.) During the last 30 min of incubation, the cells were labeled with either fluorescein isothiocyanate (FITC) at 60 mg/ml in DMEM 4% calf serum as described (15) or with 5-carboxyfluorescein diacetate (CFDA-AM, Molecular Probes, Eugene, OR, used at a 1:4,000 dilution from a 1 mg/ml stock in dimethylsulfoxide) in DMEM 1% serum. Alternatively, after 2 hrs at 37°C, cells were labeled for 20 mins with tetramethylrhodamine isothiocyanate (TRITC, Sigma, St. Louis, MO) at 1.7 mg/ml in 4% serum at room temperature. Labeled cells were washed through a calf serum cushion and resuspended at ~$5x10^7$/ml in DMEM for intravenous injection. In some instances, PMA (Sigma) was added to a 100 ng/ml final concentration, and cells were incubated an additional 5 mins at 37°C immediately before injection. (This brief treatment does not downregulate L-selectin homing receptor expression).

In situ video microscopic analyses of lymphocyte-HEV interactions

Syngeneic recipients were anesthetized, and an aseptic abdominal incision was made to exteriorize the small intestine and Peyer's patches. A selected patch was embedded in silicon high-vacuum gel (Dow Corning, Midland, MI) to provide a matrix allowing application of a coverslip and

positioning for epifluorescence microscopy and video recording using a silicon-intensified target video camera (Cohu, San Diego, CA). The small intestine was bathed in freshly made Dulbecco's PBS at 37°C. The recipient was maintained at 37°C by a stage mounted strip heater regulated via a temperature probe beneath the mouse.

Approximately 2.5×10^7 labeled cells in 0.5ml DMEM were injected i.v. into the recipient's lateral tail vein. Injected cells were then observed and recorded entering and interacting with Peyer's patch HEV for the duration of each experiment. Because there is a transient increase in blood flow immediately after injection, and because the Peyer's patch often requires minor repositioning to focus on HEV, data analyses were routinely initiated ~30 sec after infusion of sample cells and continued during the next 2.5 minutes. Evaluation of the interactions of individual cells was carried out by replaying the video recording on a 4-head VCR with shuttle wheel single frame forward and reverse control (JVC, Elwood Park, NJ). Initial control experiments showed no significant differences in the *in vivo* behavior of cells labeled with TRITC, FITC, or CFDA.

Analyses of lymphocyte behavior

The behavior traces are plots of distance cells moved along an HEV from the site of initial interaction vs. time in seconds after the initial interacting contact. The position of each selected cell was determined by analysis of the video image at 1/3 sec intervals from the time and site of initial interaction, until the cell either released to the circulation ("passed," with the cell trace exiting the plot vertically) or became stably attached at a

given site on the HEV wall ("arrested," with the trace exiting the plot horizontally). Note that a typical non-interacting cell would pass completely through an HEV in 2/3 seconds or less.

For frequency plots summarizing population behaviors, a rapid procedure for quantifying the motion of cells was used. Individual cells were analyzed on a second-by-second basis for 5 sec after initiation of interaction or until the cell passed from the HEV. Each one-second period of interaction was assigned a value of 0 for sticking behavior (defined arbitrarily as static binding to the HEV wall during at least 2 consecutive seconds) or a value of 1 to indicate a predominant rolling interaction, including "stop-and-go" behavior with static binding of <1 sec. If during an interaction sequence, a cell "skipped," releasing transiently from the vessel wall, only the time interval during which rolling or sticking took place was scored. For each cell, the values assigned to each second of interaction were averaged, yielding a time-averaged indicator of the cells' mean behavior. Inherent in this scoring procedure is a bias toward more interactive behaviors, so that the very brief <1 sec initial rolling, displayed by many PMA-preactivated lymphocytes (illustrated in Figure 5e), is not reflected in the mean score. In most experiments, at least 20 lymphocytes interacting with 2-3 or more independent distinct HEV were scored. The histograms present the relative distribution of behaviors among normal, PTX-treated, and PTX-plus PMA-treated lymphocyte populations from 3-5 separate animals for each treatment type.

Results and Discussion

Lymphocyte interactions with high endothelial venules (HEV), which are specialized vessels that support lymphocyte extravasation into lymphoid tissues, can be visualized in the mouse by intravital microscopy of exteriorized Peyer's patches (11). Fluorescent-labeled lymphocytes are injected intravenously, and their interaction with Peyer's patch HEV is observed and recorded by video epifluorescence microscopy. We analyzed the *in situ* behavior of lymph node lymphocytes between ~30 sec and 3 min following intravenous injection.

Individual lymphocytes displayed wide variability in behavior. Many interacted transiently with the HEV, then released only to interact again further downstream, as described by Bjerknes (11). During periods of interaction, some lymphocytes "rolled" along the venule without stopping, others (the majority) rolled a variable distance before stopping either briefly (<1 sec, termed "stop-and-go behavior") or longer (>1 sec, termed "sticking"). Overall, roughly 35% arrested stably, attaching statically to the vessel wall for at least 5 or more seconds (Table 1).

Table 1. Frequency and outcome of interactions between entering lymphocytes and HEV.

	Cell Treatment		
	Control	Pertussis Toxin (PTX)	PTX+PMA
Percent of cells entering HEV			
that interact	84 ± 3	88 ± 3	88 ± 4
Percent of interacting cells			
that return to the blood (pass)	66 ± 4	95 ± 2	28 ± 6
that arrest (> 5 sec.)	34 ± 4	5 ± 4	72 ± 4

Table 1. Percentage of lymphocytes entering HEV that interact with the vessel wall, and of interacting lymphocytes that return to the circulation (pass) or interact stably (>5 sec.). For the data in Table 1, all cells interacting with selected HEV during some or all of the observation period were analyzed. Similar results were obtained in 3-5 independent animals for each cell treatment, and the data are pooled and expressed as the percent of cells displaying the indicated behavior with the standard error of the mean.

The detailed behavior of several individual lymphocytes is displayed in Figure 5a as a plot of distance traveled from the site of initial interaction with the vessel wall, vs. the elapsed time. A histogram summarizing the frequency of lymphocytes displaying predominant rolling, mixed (rolling and sticking), or predominant sticking behaviors is presented in Figure 5b.

The data illustrate the variability of normal lymphocyte interactions.

To determine the effect of pertussis toxin (PTX), lymphocytes were incubated with PTX (100 nanograms/ml x 2 hrs at 37^{O}) prior to injection. PTX-treated lymphocytes interacted with the same frequency as normal cells, but demonstrated a striking reduction in stable arrest (Table 1). Most importantly, the character of interaction was altered dramatically, consisting almost exclusively of rolling with interspersed stop-and-go behavior (illustrated in Fig. 5c), with static interactions rarely exceeding 1 sec in duration. Figure 5d emphasizes the almost exclusive rolling behavior of PTX-treated lymphocytes. Thus PTX treatment appears to uncouple physiologically a primary rolling phenotype from a PTX-sensitive sticking and arrest.

To rule out nonspecific toxicity, we next asked if we could reverse the inhibitory effect of PTX with phorbol myristate acetate (PMA), which can directly stimulate protein kinase C (PKC) and trigger activation-dependent lymphocyte adhesion (16). PTX-treated lymphocytes pulsed with PMA for 5 min just prior to injection demonstrated restored and even enhanced sticking and arrest (Table 1 and Fig. 5e and 1f). Lymphocytes treated with PMA alone behaved similarly (not shown). Although PKC does not appear to play a critical role in physiologic lymphocyte-HEV interactions (12), the ability of PKC stimulation to mimic the effects of *in vivo* activation, and to circumvent the inhibition of sticking by PTX suggests that PTX does not interfere with sticking mechanisms directly, but rather it may block an activation pathway that triggers these mechanisms.

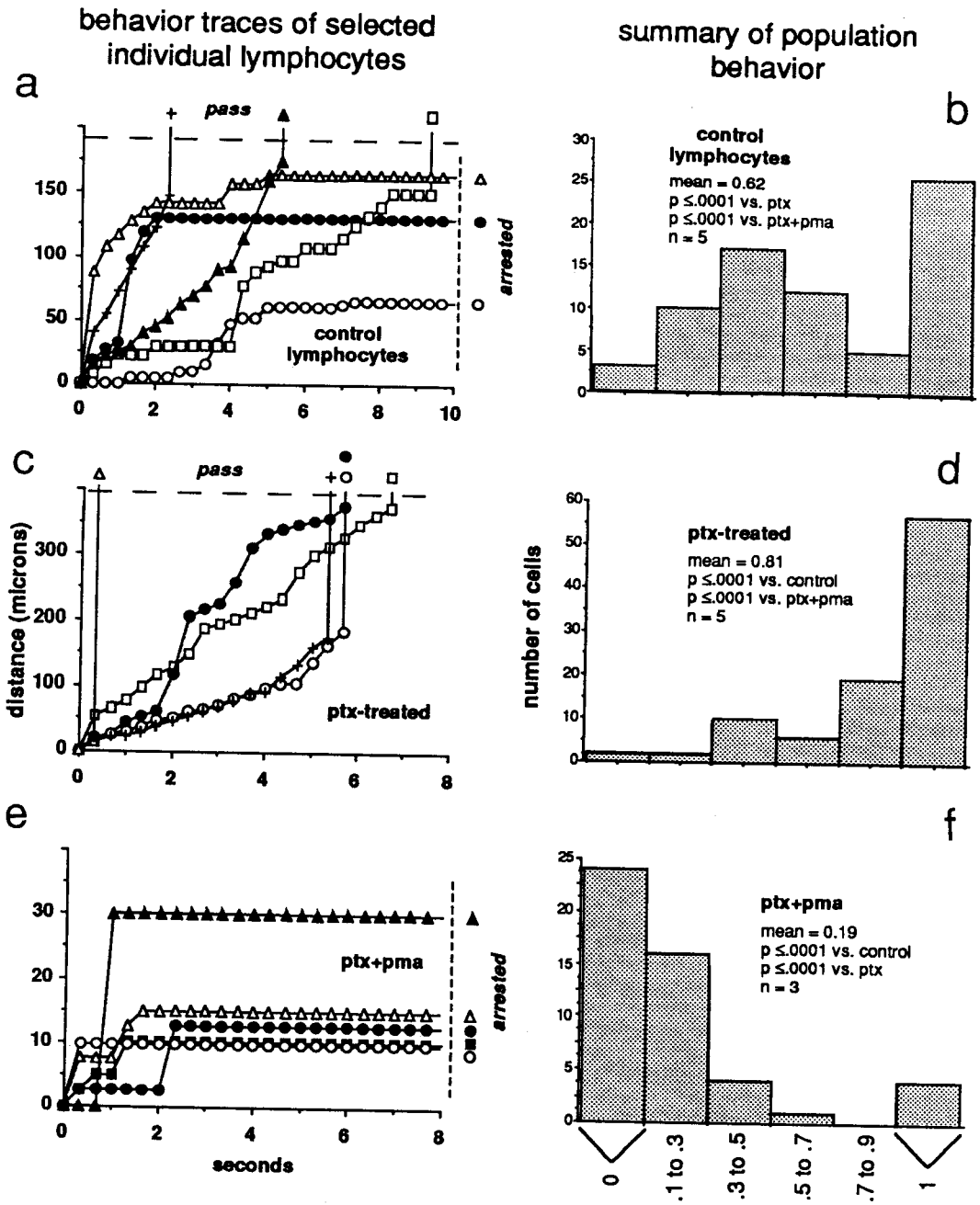


Figure 5. In situ behavior of injected, normal, PTX-treated, and PTX-plus PMA-treated lymphocytes during interaction with Peyer's patch HEV. Behavior traces of selected individual lymphocytes (left) and histograms of the frequency of time-averaged lymphocyte behaviors (right) illustrate the varied behavior of normal lymphocytes (a, b), the predominant rolling phenotype of PTX-treated lymphocytes (b, c) and the rapid sticking behavior

of PTX-plus PMA-treated cells (e, f). The histograms (right) present pooled analyses of 3-5 experiments for each treatment (see Materials and Methods).

PTX ADP-ribosylates sensitive G-proteins, interfering with G-protein-mediated signal transduction from cell-surface receptors (17). A mutant pertussis holotoxin, specifically lacking ADP-ribosyltransferase activity by virtue of two amino acid substitutions in the S1 subunit (13,14), had no effect on the *in vivo* behavior of lymphocytes (Fig. 6). The results suggest that lymphocyte sticking and arrest are triggered by an activation event mediated or regulated by PTX-sensitive G proteins.

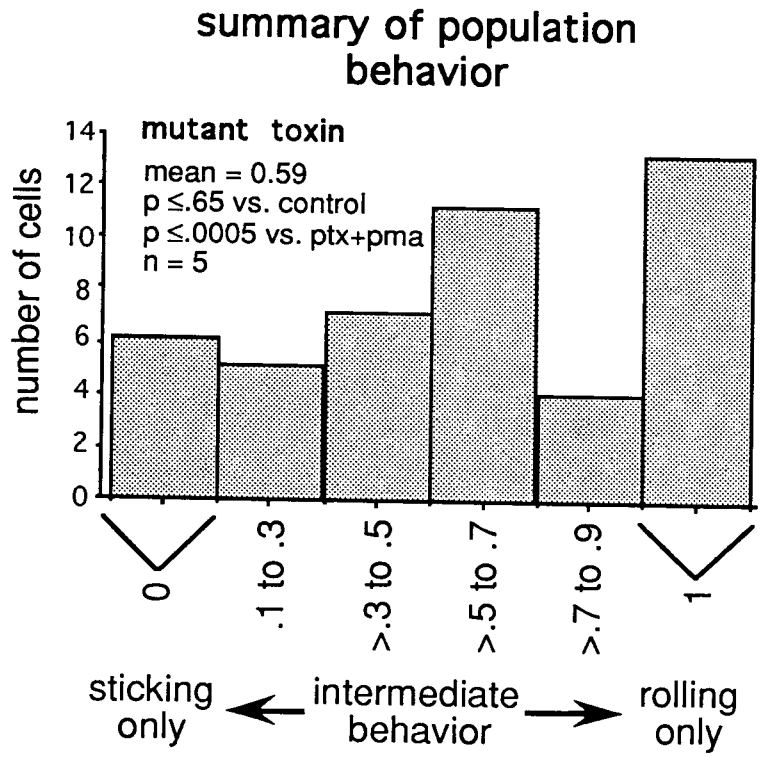


Figure 6. In situ behavior of lymphocytes pretreated with an enzymatically inactive pertussis holotoxin. The distribution of behaviors of lymphocytes

treated with the mutant toxin is not significantly different from that of untreated control lymphocytes (Fig. 5b). Procedures were as for Figure 5c and 5d (see Materials and Methods), except that a purified mutant holotoxin (PT9K 129G), specifically lacking ADP-ribosyltransferase activity, was employed. Results are from a representative experiment from a total of five performed with the mutant toxin.

Under physiologic conditions, this activation event may occur while lymphocytes are rolling along the endothelium. Consistent with this hypothesis, normal lymphocytes take longer to stick and arrest than PMA-preactivated cells (Fig. 5a and e). Physiologic activation and triggering of shear-resistant adhesion receptors must nonetheless occur with surprising speed: The mean interaction time prior to sticking of those normal lymphocytes that go on to arrest stably is only ~1-3 seconds (mean = 1.3; range 0.2 to 4 sec., N =58 lymphocytes from 5 experiments). In keeping with such a rapid change in adhesive state, Ottaway reported a fairly abrupt increase between 1-3 seconds in the probability that an interacting cell would bind stably (11).

It should be emphasized that the conclusions here are drawn from the behavior displayed by the majority of injected lymph node lymphocytes. The fact that most lymphocytes interact with PP-HEV and that a significant fraction of them adhere is consistent with previous studies demonstrating that the major populations of B and T lymph node lymphocytes home well to organized lymphoid tissues, including the Peyer's patches (18). Less abundant subsets of lymphocytes, particularly memory and blast- cell subsets which can display highly tissue-selective homing properties, may behave

differently (2). In particular, it will be important to determine if lymphoblasts returning to the circulation after local antigenic stimulation may (like PMA preactivated cells) be able to stick and arrest to HEV independent of the receptor-mediated, vessel-associated, activating signals implied by the current studies. Indeed, a small subset of normal lymphocytes observed bound almost immediately to HEV, especially during the initial 30 sec period of stabilization after i.v. injection (not included in the quantitative studies presented). These may represent such pre-activated lymphocytes among the mesenteric lymph node lymphocyte population.

G-protein signaling pathways appear well suited to the very rapid alterations in cellular behavior required for lymphocyte sticking to HEV under physiologic flow conditions. Cellular activation through G-protein-linked receptors can be extraordinarily rapid, with global effects on cellular activities. Examples include the rapid depolarization of retinal neurons by photo activation of rhodopsin; and, perhaps more relevantly, stimulation of the aggregation of neutrophils within seconds after triggering of the FMLP receptor. Lymphocytes are known to use PTX-sensitive G-proteins to transduce signals from a number of cell-surface receptors, notably including receptors for cytokines of the chemokine family (19-21) which have been hypothesized to play a role in regulation of lymphocyte-endothelial interactions (4). The hypothesized role of chemokines in lymphocyte-endothelial recognition has received additional support from the recent demonstration that members of the chemokine family can bind to and be displayed at the lumenal surface of venules involved in leukocyte or lymphocyte traffic (22,23). Chemokines are also thought to be important in

lymphocyte chemotaxis through tissues (19-21), and may be able to regulate diapedesis independently of the rapid activation of HEV-binding studied here. Crosslinking of some lymphocyte surface receptors can also trigger activation-dependent lymphocyte adhesion mechanisms (reviewed in 5), and it will be important to determine if these activating pathways also involve G-protein-mediated signaling.

In conclusion, our results demonstrate that a rapid activation-dependent adhesion event involving PTX-sensitive G-proteins can participate in lymphocyte-HEV interactions, leading to stable arrest of lymphocytes under shear flow. For the major population of unactivated lymph node lymphocytes studied here, this activation-dependent sticking appears to follow a primary "rolling" interaction that may allow these lymphocytes to receive stimulating signals by initiating contact with the endothelium and slowing their passage. Our findings are compatible with *in vitro* studies suggesting that PTX can inhibit activation-dependent participation of the integrin LFA-1 in lymphocyte binding to lymph node HEV in frozen sections (24). The observations establish a strong parallel with similar cellular events involved in neutrophil interactions with inflamed endothelium (4,6-10,25), and support a general model of leukocyte-endothelial cell interaction as a multi-step process (4,5). In this model, the local factors responsible for lymphocyte activation may assume equal importance with lymphocyte homing receptors and vascular addressins in determining the specificity of lymphocyte-HEV interactions and lymphocyte homing in organized lymphoid tissues and sites of inflammation.

Summary

The homing of lymphocytes into lymph nodes and Peyer's patches is mediated in part by recognition and binding to specialized HEV. We have used the toxin of Bordetella pertussis to demonstrate that a rapid receptor-mediated pertussis toxin-sensitive lymphocyte activation event can participate in lymphocyte recognition of HEV. *In situ* video microscopic analyses of lymphocyte interactions with HEV in exteriorized mouse Peyer's patches reveal that pertussis toxin has no effect on an initial "rolling" interaction displayed by many lymphocytes, but inhibits an activation-dependent "sticking" event required for lymphocyte arrest. The effect of the toxin is dependent on ADP ribosyltransferase activity, which can modify G-proteins involved in transmembrane signaling. Sticking behavior of toxin-treated lymphocytes is restored by phorbol myristic acetate, which bypasses cell-surface receptors to trigger activation-dependent adhesion. The time from initiation of interaction to "sticking" of normal lymphocytes can be very brief (1-3 sec.). We conclude that a rapid receptor-mediated activation event involving G-protein signaling pathways can trigger stable lymphocyte attachment to HEV *in vivo*, and may play a critical role in regulating lymphocyte homing.

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CHAPTER 3

DISTINCT ROLES FOR L-SELECTIN, α4β7 INTEGRIN, AND LFA-1 IN LYMPHOCYTE INTERACTIONS WITH HEV *IN SITU*: THE MULTI-STEP MODELCONFIRMED AND REFINED

Introduction

The recruitment of lymphocytes from the blood is an essential response to chronic tissue inflammation and is key to the regulation of physiologic lymphocyte trafficking and recirculation (1). Lymphocyte extravasation is directed *in vivo* by mechanisms of selective lymphocyte-endothelial cell (EC) recognition, mechanisms which facilitate immune surveillance by directing recirculation pathways of naive and memory lymphocytes, and which support regional immune responses by targeting lymphocyte effector cells to particular organs and tissues in the body as a function of the local microenvironment and inflammatory state (1,2). In an effort to explain the extraordinary specificity of lymphocyte (and other leukocyte) trafficking *in vivo*, we and others have proposed a general model of leukocyte-EC interactions as a multi-step process, involving primary adhesion in some instances separable into molecularly distinct attachment and rolling phases, lymphocyte activation through G-protein-linked receptors, subsequent activation-dependent sticking and arrest,

followed by diapedesis in response to chemoattractant or haptotactic signals (3,4,5). This hypothesis, in which lymphocyte recruitment results from an active decision process or algorithm requiring a yes/no decision at each step, has profound implications, providing a combinatorial mechanism for generating diversity and determining specificity in lymphocyte-EC interactions (3). The multi-step paradigm is well established for neutrophil interactions with inflamed venules in vivo (6), and has also received substantial support from *in vitro* modeling of leukocyte adhesive interactions (5). However, it remains to be verified as a general model *in vivo*, and in particular in the context of lymphocyte homing.

In earlier published work, we used *in situ* videomicroscopy to demonstrate the involvement of a rapid pertussis toxin (Ptx)-sensitive G-protein signaling/activation event in lymphocyte interactions with PP-HEV (7): Lymph node cells treated with pertussis toxin displayed efficient attachment and transient rolling in PP-HEV, but were unable to make the transition to sticking and stable arrest. This study demonstrated the involvement of sequential rolling, activation, and sticking steps in lymphocyte recognition of HEV in this site.

Independent investigations involving short-term *in vivo* homing studies have implicated several defined adhesion and homing receptors in lymphocyte trafficking to PP. Of particular importance is the lymphocyte "PP homing receptor," the integrin $\alpha 4\beta 7$, (8,9,10) which plays a critical role in the targeting of lymphocytes to mucosal PP and lamina propria through interaction with the mucosal vascular addressin MAdCAM-1 (11,12), an immunoglobulin family member that is selectively expressed by venules in

mucosa-associated lymphoid tissues and sites of inflammation (13,14). Antibodies to the lymphocyte L-selectin and to the ß2 (CD18) integrin LFA-1 also reduce the localization of injected lymph node cells in PP *in vivo* (10). However, the respective functions of L-selectin, α4β7, and LFA-1 and their vascular ligands in lymphocyte homing to PP has not been reported.

Here we have used *in situ* videomicroscopy, in conjunction with inhibitory antibodies to adhesion receptors, to dissect the molecular mechanisms of lymphocyte attachment, rolling, and activation-dependent arrest in PP-HEV. We demonstrate sequential and overlapping involvement of L-selectin, α4ß7, and LFA-1 in recognition of PP-HEV by resting lymph node cells, but show that α4ß7 is both necessary and sufficient for attachment and arrest of lymphocytes expressing preactivated or elevated α4ß7 levels. We also present evidence implying a critical role for the mucosal addressin MAdCAM-1 not only in α4ß7-mediated interactions but also in L-selectin-mediated attachment and rolling in this site. Our findings provide the first direct demonstration of a multi-step adhesion cascade leading to lymphocyte-EC recognition and arrest in a physiologic setting, as well as novel insights into the specialization and versatility of the adhesion receptors involved.

Materials and Methods

Lymphocyte and TK-1 cell preparation and labeling.

Lymphocytes from both peripheral and mesenteric lymph nodes were freshly isolated from 8-to-12-week-old adult BALB/c mice of either sex (Simonsen Labs, Gilroy, CA) and were resuspended at a concentration of $5x10^6$/ml in DMEM [without sodium bicarbonate (Sigma Chemicals, ST Louis, MO) supplemented with 20mM HEPES, pH 7.0 (Research Organics, Cleveland, OH)] and iron-supplemented calf serum at 1 or 4% (Hyclone Labs, Logan, UT). The TK-1 $\alpha4\beta7^{hi}$ lymphoma cell line was cultured in RPMI 1640 media with 5% iron supplemented bovine calf serum (Hyclone Labs, Logan, UT). These cells were maintained at an approximate density of $1x10^6$ cells/ml and at a pH of 6.8 where they grew non-adherently as single cells in suspension (TK-1 cells exhibiting adherence to plastic were excluded from these assays). TK-1 cells were resuspended in DMEM after centrifugation at a density of $1x10^7$/ml. Lymphocytes were labeled for 30 min at 37°C, TK-1 cells were labeled 20 min, with either fluorescein isothiocyanate (FITC) at 60 µg/ml in DMEM 4% calf serum or with 5-carboxyfluorescein diacetate (CFDA-AM, Molecular Probes, Eugene, OR, used at a 1:4,000 dilution from a 1 mg/ml stock in dimethylsulfoxide) in DMEM 1% serum. Alternatively, cells were labeled at 37°C for 20 mins with tetramethylrhodamine isothiocyanate (TRITC, Sigma, St. Louis, MO) at 1.7 µg/ml in 4% serum. Both lymphocytes and TK-1 cells were washed through a calf serum cushion and resuspended at ~$5x10^7$ cells/ml in DMEM for intravenous injection.

Lymphocyte Activation.

For Mn^{2+} activation, lymphocytes were incubated at RT in Ca^{++}/Mg^{++}-free Hanks balanced salt solution (HBSS) + 10mM HEPES pH 7.0 containing 2 mM EDTA, and were then diluted 3-fold with Ca^{++}/Mg^{++}-free HBSS/HEPES, pelleted, and resuspended in HBSS/HEPES containing either 2 mM $CaCl_2$ and 2 mM $MgCl_2$ (control cells), or 2 mM $CaCl_2$ and 2 mM $MnCl_2$ (Mn^{++}-activated cells). Cells were then injected. For activation with (PMA) phorbol myristate acetate (Sigma, St Louis) lymphocytes were incubated immediately before injection for 5 mins. at 37°C in DMEM with 1% serum containing PMA at a final concentration of 100 ng/ml.

In situ videomicroscopic analyses of lymphocyte interactions with Peyer's patch venules.

An aseptic abdominal incision was made in anesthetized syngeneic mice to exteriorize the small intestine and visualize the Peyer's patches. The small intestine was embedded in silicon high vacuum gel (Dow Corning, Midland, MI) to provide a matrix allowing application of a coverslip and positioning for epifluorescence microscopy and video recording using a silicon-intensified target video camera (Cohu, San Diego, CA). The exteriorized small intestine was bathed in freshly made Dulbecco's PBS at 37°C. The mouse was maintained at 37°C by a stage-mounted strip heater regulated via a temperature probe beneath the animal.

Approximately $2.5x10^7$ labeled cells (either TK-1 or lymphocytes) in 0.5ml DMEM were injected i.v. into the mouses' lateral tail vein. The TK-1

cells or lymphocytes were either pretreated with anti-lymphocyte mAbs (100ug/1ml) at room temperature for 20 min (not washed) before injection or treated after injection *in vivo*, in which case 250-500 μg of mAb or Fab was infused i.v.. Injected cells were then observed and recorded entering and interacting with Peyer's patch venules for the duration of each experiment. Video data collection (recording) was initiated before infusion of sample cells and continued during the course of the antibody treatments. Evaluation of the interactions of individual cells was carried out by playing the video recording on a 4-head VCR with shuttle-wheel single-frame forward and reverse control (JVC, Elwood Park, NJ; Sony). Initial control and subsequent experiments showed no significant differences in the *in vivo* behavior of cells labeled with TRITC, FITC, or CFDA.

Analyses of lymphocyte and TK-1 behavior.

Cell interaction data were collected as soon as fluorochrome labeled cells appeared in the sampled vessels after injection. Data were collected until a sufficient number of events (≥25) were recorded for each experimental condition. The percent of cells interacting with Peyer's patch venules was determined by video frame analysis for the presence or absence of individual cell rolling or sticking interactions. Individual cells were analyzed on a second-by-second basis for 10 seconds after initiation of a venular interaction or until the cell passed from the Peyer's patch venule. A cell was arbitrarily defined as having an interaction with the venule wall

if during at least 2 consecutive seconds it exhibited rolling, arrested or mixed behavior. Inherent in this scoring procedure is a bias toward more interactive behaviors, so that the very brief (<1 sec) rolling or sticking displayed by some lymphocytes and TK-1 cells is not reflected in the data. In all experiments, at least 25 lymphocytes were scored, generally from 2-3 independent HEV. In some experiments, however, sequential comparisons were made within the same vessel following different mAb treatments. Each one second period of interaction was assigned an integer value: 1 for rolling (including "stop and go" behavior with static times less than 1 second), and 0 for sticking as defined independently for each cell. All interaction values during the ten second sampling period were averaged to give a single value falling into the continuous range from 0 to 1 (or a non-interaction value of 2). Histograms are presented, displaying the frequency of cells with predominant sticking values of (0-0.2), intermediate or mixed, (>0.2-0.8), or predominant rolling (>0.8-1.0) behaviors, or of non-interacting cells (2.0). Statistical comparisons were made by the Student's t test for the rolling speed data (Fig 8) or in the case of the histogram data by Chi square contingency tables. Confidence intervals were calculated based on Student's t distribution (for $N \geq 3$) or the binomial distribution (1 or 2 independent experiments). Rolling velocities were determined by measuring the transit times of a minimum of 10 individual lymphocytes, from the site of original attachment in the HEV to the point of permanent release into the blood flow and dividing those times by the distance rolled in microns. Non-interacting lymphocyte velocities were variable from HEV to HEV ranging from 176 μM to 1130 μM/sec.

Video sequence depicting lymphocyte rolling velocities and calculation of shear rates in PP-HEV.

Multiple video frame images captured at one second intervals were overlapped to produce composite images illustrating the rolling behavior of individual representative lymphocytes (Figure 8). Video image processing and composite image generation was carried out using a Macintosh Quadra 660AV computer (Apple, Cupertino, CA) and Photoshop software (Adobe, Mt View, CA). Fluorescent intensity is depicted as apparent vertical relief in the processed image.

Calculations estimating shear rates in PP-HEV were made by measuring velocities of fluorescently labeled beads or cells as they passed through HEVs without interacting with the vessels walls. Values were generated from ten HEVs from five animals at times in experiments when normal binding interactions were occurring. The distance in microns each bead or cell traveled in 1/30 of a second (1 frame of NTSC standard SP video) was measured to determine centerline blood flow velocities. Velocities for seven to 25 cells were measured for each vessel; means and standard deviations were determined for each. Using the individual HEV velocity means and the corresponding internal diameters in the Hagen-Poisueille equation provided blood shear values for each individual HEV. These values provide our estimate of maximum and minimum HEV shear rates and pooled, estimate the average shear in mouse Peyer's patch HEV.

Results

Interactions of injected lymph node cells with PP-HEV: Sequential L-selectin, α4ß7, and LFA-1-mediated steps.

Lymph node cells from young Balb/c mice were injected i.v. and their behavior during passage through PP-HEV was observed by video epifluorescence microscopy. As reported previously (7) and reproduced here (Figure 7A) the majority of normal lymph node cells interacting with HEV displayed variable rolling and sticking behaviors (Figure 7A). In order to define the role(s) of individual adhesion receptors in the process, the effects of blocking anti-L-selectin, α4b7, or LFA-1 MAbs on the frequency and characteristics of interactions were explored.

L-selectin >α4ß7 mediates initial lymphocyte attachment.

Anti-L-selectin antibody significantly decreased the frequency of detectable interaction of injected lymph node cells (Figure 7B). Those cells which were capable of interacting in the presence of anti-L-selectin only rarely displayed residual rolling, instead arresting rapidly: these may represent preactivated cells among the lymph node lymphocytes (see below). Thus L-selectin appears to play a prominent role in initiating attachment of resting lymphocytes in PP-HEV. However, anti-L-selectin in combination with anti-α4 MAb or Fab fragments (Figure 7E) is even more effective at

abrogating interaction, suggesting that α4ß7 can also support lymphocyte attachment, albeit at a lower frequency. These findings are consistent with *in vitro* studies that have demonstrated efficient L-selectin-mediated lymphocyte attachment and rolling, but also α4ß7-mediated attachment of lymphocytes to MAdCAM-1 under flow, as well (15,16). The present results suggest that for most lymph node cells, the majority of which are naive lymphocytes expressing high levels of L-selectin and low/intermediate levels of α4ß7, L-selectin plays the predominant role in initiating physiologic interactions with PP-HEV.

Both α4ß7 and LFA-1 are required for lymphocyte arrest.

As shown in Figure 7C, anti-α4 MAb treatment had no significant effect on the frequency of interaction with HEV, but converted behavior to rolling with almost total abrogation of lymphocyte arrest. Similar results were obtained with anti-ß7 integrin MAb (Figure 7D). Thus inhibition of α4b7 prevents engagement of activation-dependent adhesion mechanisms. Surprisingly, anti-LFA-1 MAb also inhibited lymphocyte arrest almost completely, resulting in a predominant rolling phenotype as well (Figure 7F). Combined treatment with anti-α4 and anti-LFA-1 yielded a similar predominant rolling behaviour (Figure 7G). As expected, the combination of

all three antibodies completely abrogated detectable interaction (Figure 7H). Control anti-CD45 MAb had no significant effect on cell behavior (Figure 7I). These results suggest that both α4ß7 and LFA-1 are required for adhesion strengthening leading to sticking and stable arrest of lymphocytes in PP-HEV.

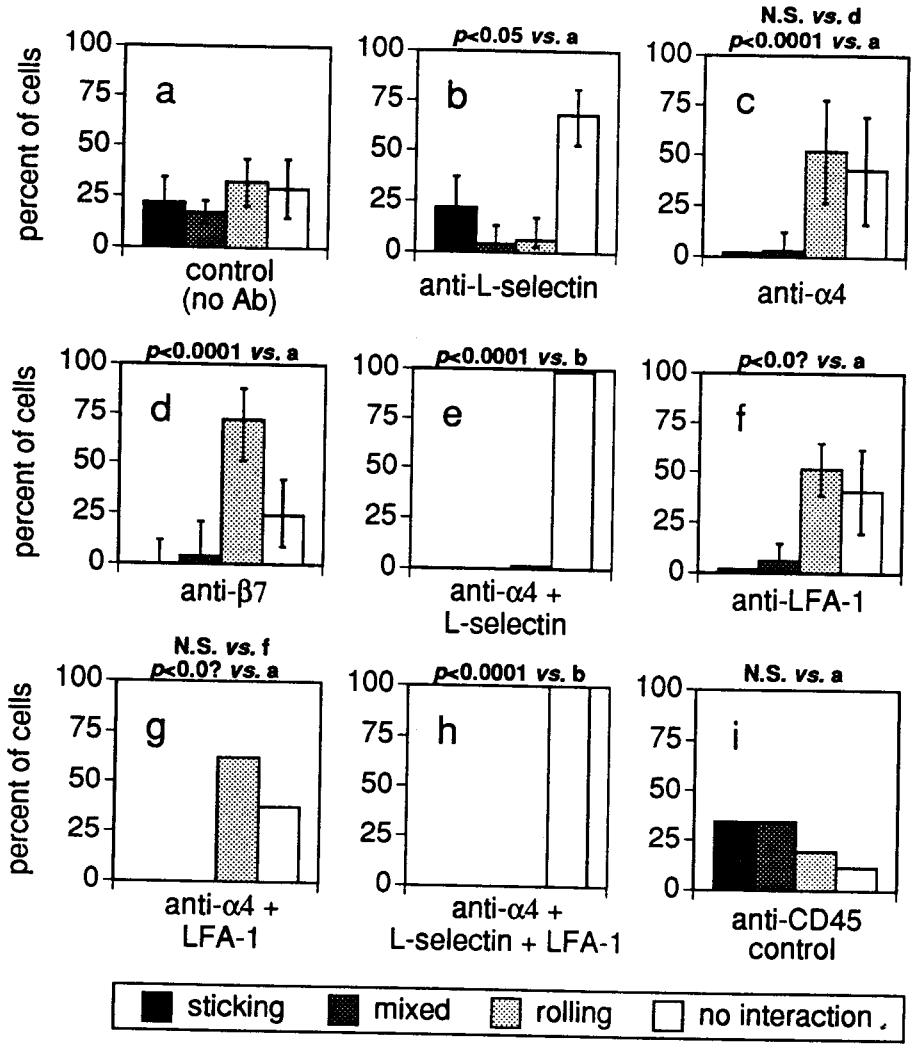


Figure 7: Effects of anti-L-selectin, α4, ß7, and LFA-1 MAbs on interactions of normal lymph node cells with PP-HEV in situ. Fluorescent lymph node cells were injected intravenously, and their behavior during passage through

HEV was recorded videomicroscopically for analysis. The mean behaviour of each cell was scored as predominant sticking, intermediate, rolling or non-interacting, and the population behaviour is summarized in the histograms presented. Pretreatment or injection of MAbs was carried out as described in the Materials and Methods section, and in Results. Histograms represent pooled analyses of 2-8 independent experiments, except for panels D and I which were from individual animals. The number of replicate experiments is given in the upper right hand corner of each panel. Error bars in this and subsequent figures represent 95% confidence intervals based on Student's t (for N>2) or binomial distributions (when N=1 or 2).

α4ß7 participates in lymphocyte rolling.

The velocity of lymphocyte rolling is a function of molecular interactions and densities, and of local hemodynamics, which vary significantly from vessel to vessel *in vivo*. Therefore, to examine this parameter, we compared the speed of rolling lymphocytes in the same HEV during sequential treatments. Representative experimental results are illustrated in Figure 8. Anti-LFA-1-treated cells displayed slow rolling, which increased substantially in velocity following subsequent injection of anti-α4 (Figure 8A). Thus, α4b7 participates with L-selectin in lymphocyte rolling in PP-HEV, resulting in an increase in the apparent avidity of interaction. To illustrate this visually, video images of rolling lymphocytes pretreated with anti-LFA-1 alone (Figure 8B), or following subsequent injection of anti-α4 (Figure 8C) are presented. Injection of anti-L-selectin MAb into the same animal resulted in complete abrogation of all visible interactions (not shown).

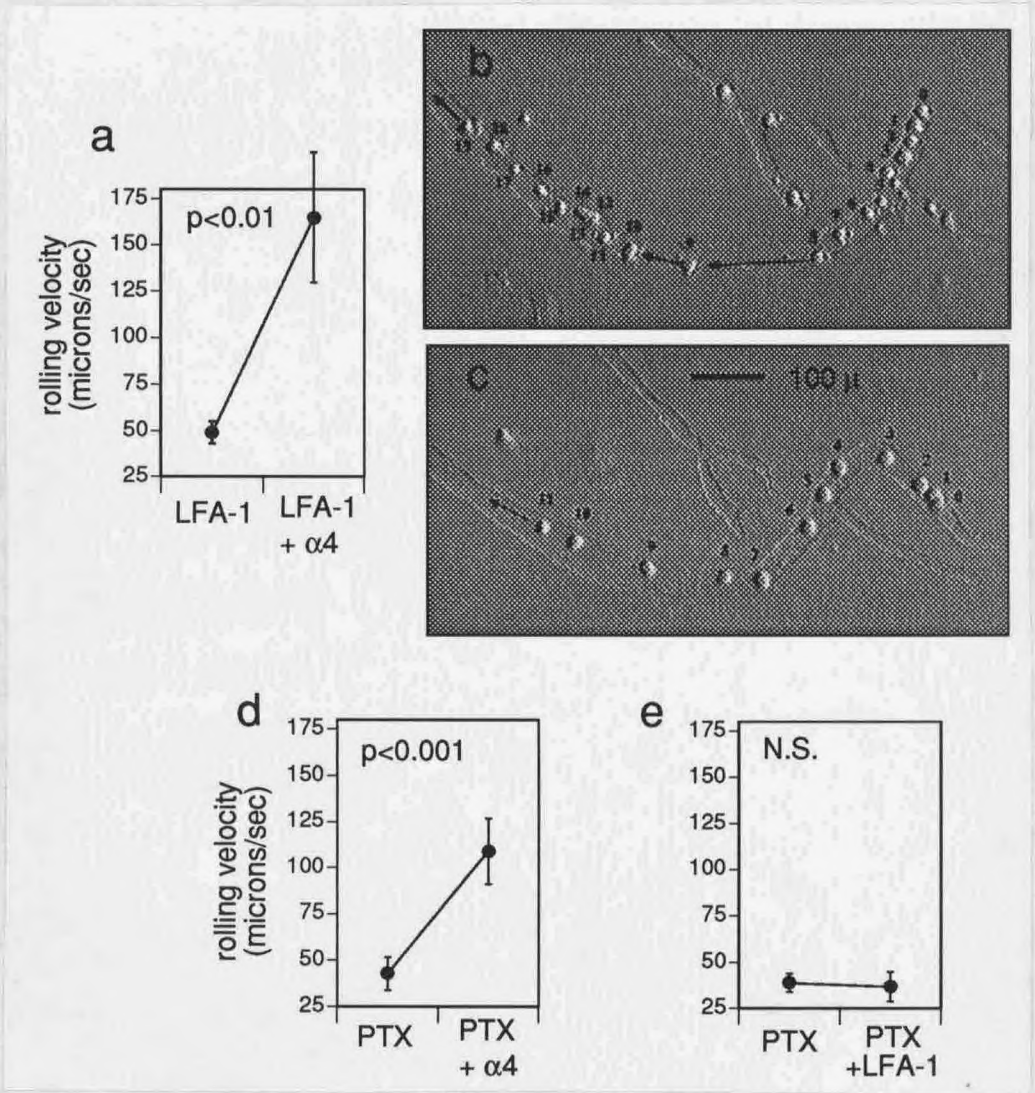


Figure 8. Effects of anti-α4 and anti-LFA-1 MAbs on the velocity of lymph node cell rolling in HEV. The presence of anti-LFA-1 vs. anti-α4 MAbs. Each panel presents paired data from a single HEV or set of HEV's in a given experiment. A) Results of a representative experiment in which the behavior of LNC was observed during sequential treatment with anti-LFA-1 (incubated with cells prior to injection, and coinjected in excess) and anti-α4 (subsequently injected intravenously). B & C) Composite photographs illustrating superimposed images of the position of individual representative cells from the experiment in A, with images captured at 1 sec intervals: the distance between images reflects the velocity of the cell. Rolling of an anti-LFA-1 treated cell is depicted in B. The cell "skipped" over the sections indicated by the arrows. More rapid rolling of a representative

lymphocyte following anti-α4 is shown in C. D) The slow rolling of Ptx pretreated cells is converted to rapid rolling following anti-α4 injection. E) Anti-LFA-1 MAb has no effect on the rolling velocity of Ptx-treated lymphocytes. The mean and standard error of rolling velocities determined from 10-20 individual cells per condition are presented. P values for comparisons of paired data within each experiment are given.

The participation of α4ß7 in lymphocyte rolling is independent of Gi-protein-mediated lymphocyte activation, as Ptx-pretreated lymphocytes display slow rolling that is dramatically increased in velocity upon injection of anti-α4 (Figure 8D). In contrast, anti-LFA-1 had no detectable effect on the rolling velocity of Ptx-treated cells (Figure 8E), suggesting that in this setting participation of LFA-1 may be activation-dependent and largely limited to adhesion strengthening. Together, these findings demonstrate that α4ß7 plays an important role in increasing the avidity and reducing the velocity of rolling lymphocytes in PP-HEV. This may dramatically increase the opportunity for lymphocyte activation at the EC surface, and may also facilitate subsequent engagement of LFA-1.

Interactions of TK1 lymphoma cells and preactivated lymphocytes with PP-HEV: α4ß7 is necessary and sufficient for binding.

To model interactions of activated lymphocytes or $\alpha4\beta7^{hi}$ memory cells, which are thought to display distinctive homing properties *in vivo*, we initially assessed the *in situ* behavior of TK1 lymphoma cells. TK1 is a $CD8^{+}$, L-$selectin^{lo\ to\ -}$, $\alpha4\beta7^{hi}$, $LFA\text{-}1^{+}$ lymphoma that binds avidly to MAdCAM-1 and to PP-HEV, and to isolated MAdCAM-1 in static assays and

under low physiologic shear *in vitro* (11,16). As shown in Fig. 9A, when injected i.v., TK1 cells stuck almost immediately to PP-HEV *in vivo*, often with little apparent rolling. Anti-α4 completely abrogated TK1 cell interactions (Figure 9B), whereas anti-L-selectin and anti-LFA-1 had no significant effect (Figure 9C,D). Figure 9E illustrates the inability of sequential anti-L-selectin and anti-LFA-1 to block TK1 cell adhesion and arrest, whereas subsequent injection of anti-α4 effectively inhibited interactions in the same HEV.

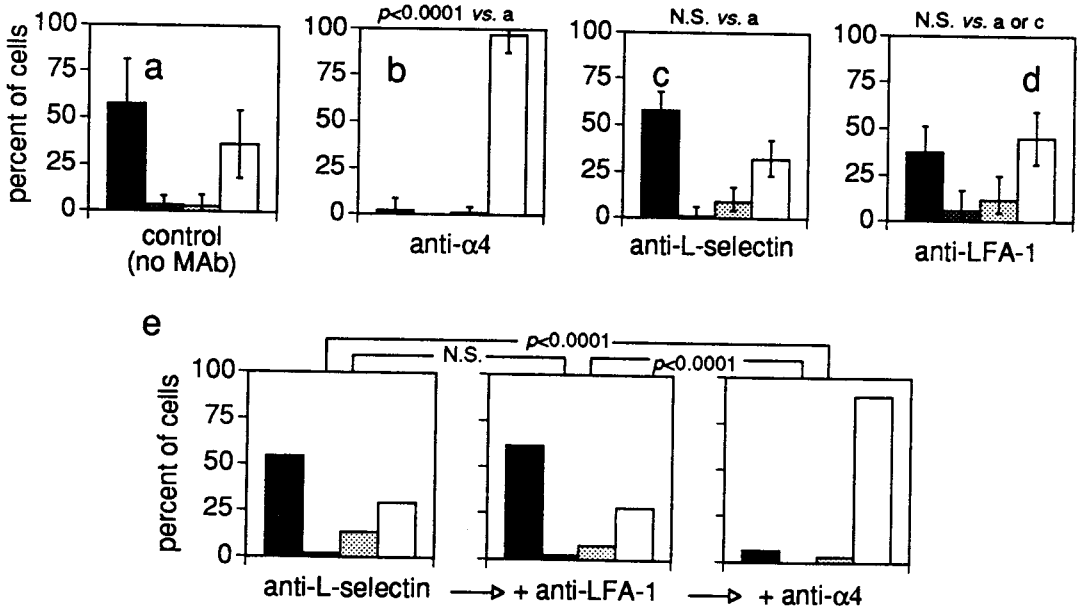


Figure 9. Anti-α4 but not anti-L-selectin or anti-LFA-1 inhibits TK1 cell interactions with Peyer's patch HEV. TK1 cells were labeled, injected iv., and visualized in PP-HEV in the presence of the indicated MAbs. Data analyses and presentation in A-D are as for Figure 7. In E, the behavior of TK1 cells was analyzed in a single HEV during sequential treatment with anti-L-selectin, anti-LFA-1, and finally anti-α4.

We also assessed the effects of experimental integrin activation on interactions of lymph node cells (Figure 10). Lymphocytes were activated either with PMA (100 ng/ml, 5 min at 37^{O}C), which triggers integrins while leaving substantial if variable levels of L-selectin on the cell surface; or by replacement of Mg^{++} with Mn^{++} in the medium. As reported previously (7), preactivated lymphocytes, like TK1 cells, tend to arrest rapidly in HEV with only very brief (<1 sec) or no initial rolling (Figure 10A). Intermediate/mixed and rolling behaviors are usually diminished, but in some experiments a significant if variable fraction of rolling cells was also observed: when present, these cells often rolled loosely and rarely arrested, which suggest that they may lack α4ß7 rendering them incapable of arrest in PP. Cells that display a non-interacting phenotype may lack L-selectin, reducing their ability to attach and establish rolling.

Anti-α4 or ß7 MAb blocked activated lymphocyte sticking and arrest, and dramatically if variably reduced the frequency of interaction (Figure 10B, C; and also 4D, middle panel). Residual interacting cells displayed rolling consistent with retention of cell-surface L-selectin, and were more apparent with Mn^{++} than with PMA-activated cells (Figure 10D, middle panel). In contrast, anti-LFA-1 MAb alone (Figure 10D, left panel) or in combination with anti-L-selectin (see Figure 10E, middle panel) had no substantial effect on the predominant sticking behavior of interacting activated cells. The predominant role of α4ß7 is illustrated in Figure 10D, which presents the results of sequential antibody treatments on the behaviour of Mn^{++}-activated lymphocytes in the same HEV. Lymphocytes pretreated and injected with anti-LFA-1 MAb retained the ability to arrest; subsequent injection of anti-α4 blocked sticking but left residual rolling that was in turn

abrogated by anti-L-selectin. The minimal effect of seqential anti-L-selectin and anti-LFA-1 treatments is illustrated in Figure 10E.

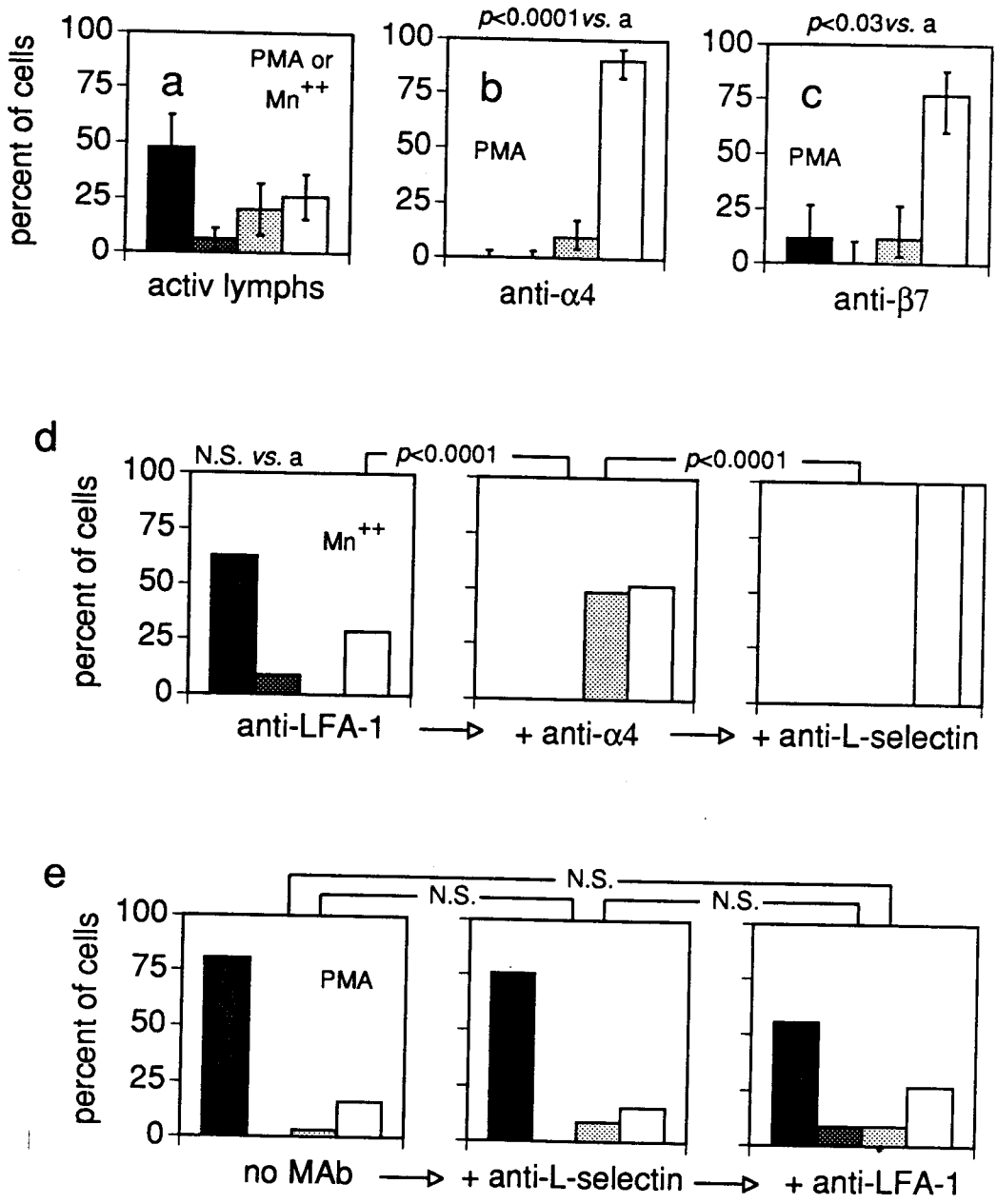


Figure 10. Effects of anti-L-selectin, α4/ß7, and LFA-1 Mabs on activated lymph node cell interactions with PP-HEV. Lymphocytes were labelled and

activated by brief pretreatment with PMA, or by replacement of Mg^{++} with Mn^{++} in the medium, and were then injected iv for analyses of in situ behavior as in Figures 7 and 9. A) Pooled data from 3 experiments with PMA-activated LNC, and two with Mn^{++}-activated cells. B and C) PMA-activated cells. D) Mn^{++}-activated LNC were preincubated and injected with anti-LFA-1 MAb, and analyzed in the same vessels following subsequent injection of anti-α4 and then anti-L-selectin MAbs. E) Mn^{++}-activated LNC were injected and analyzed following subsequent injection of anti-L-selectin and then anti-LFA-1 MAbs.

We conclude that when expressed at high functional levels (which can be influenced both by receptor density and activation state), α4ß7 can mediate lymphocyte recognition of PP-HEV with no requirement for L-selectin, LFA-1 participation, or probably for additional local activating signals (although under physiologic circumstances such signals may be required for maintenance of adhesion and/or for diapedesis). These findings suggest that lymphocytes expressing very high levels of α4ß7 (e.g., $\alpha 4/ß7^{hi}$ memory T cells) or preactivated α4ß7 gut-homing immunoblasts may be able to attach and stick to PP-HEV in a selectin- and LFA-1-independent manner, and that their arrest may also be independent of local activating signals (although activation and LFA-1 may still help support efficient diapedesis).

MAdCAM-1 is involved in both α4ß7 and L-selectin-dependent interactions.

In vitro studies have implicated MAdCAM-1 as a vascular receptor for α4ß7, and also (under certain circumstances) for lymphocyte L-selectin. When expressed by HEV, MAdCAM-1 can be modified by peripheral node

addressin-associated L-selectin-binding carbohydrate epitopes, which allows it to support L-selectin-mediated attachment and rolling under physiologic shear *in vitro* (15). Although PNAd-associated glycotopes can modify multiple mucin-like (and possibly other) HEV glycoproteins (17), the abundance of MAdCAM-1 on PP-HEV raised the possibility that MAdCAM-1 might play a prominent role as an L-selectin ligand in this site.

To address this question, we initially assessed the effect of anti-MAdCAM-1 MAb MECA-367 on attachment, rolling, and arrest of lymphocytes in PP-HEV. MECA-367 recognizes the N-terminal Ig domain of MAdCAM-1 (18) and inhibits α4ß7 binding (11). As shown in Figure 11A, i.v. injection of MECA-367 inhibited both rolling and sticking of subsequently injected lymphocytes, in a manner most comparable to anti-L-selectin or anti-L-selectin + anti-α4 treatments (compare with Figure 7B,E), suggesting inhibition of L-selectin-mediated attachment and rolling. Control class-matched anti-EC MAb MECA-32 had no effect on *in situ* interactions (Figure 11B).

Importantly, monovalent Fab fragments of MECA-367 prevented lymphocyte sticking and arrest much like anti-α4ß7 or anti-ß7 treatment, but had little or no effect on the frequency of rolling (Figure 11C and middle panel of 5E). The ability of whole but not monovalent Fab fragments of MECA-367 MAb to inhibit lymphocyte rolling suggested that the effect might require crosslinking of MAdCAM-1. Consistent with this, injection of whole MAb MECA-89 against an independent epitope on MAdCAM-1 [MECA-89 recognizes the second Ig domain, and has no effect on α4ß7 binding to MAdCAM-1 *in vitro* (19)] did effectively inhibit rolling in the animal

initially treated with MECA-367 Fab fragments (Figure 11C). MECA-89 injected alone inhibits only lymphocyte rolling having no effect on α4ß7 mediated sticking (data not shown).

In additional experiments designed to isolate the contribution of L-selectin, lymph node cells (LNC) were injected with an excess of anti-α4 MAb: as shown above, under these conditions, lymphocytes display L-selectin-dependent rolling in PP-HEV. Anti-MAdCAM-1 MAb MECA-367 was injected and, after several minutes, interactions with HEV were largely abrogated, confirming the inhibition of L-selectin interaction (Figure 11D). In a second experiment, Fab fragments of anti-α4 MAb were used to block α4 participation. As shown in Figure 11E, injection of MECA-367 Fab fragments had no additional effect on the residual L-selectin-dependent rolling, but whole MECA-89 again inhibited remaining interactions.

Thus, intact but not monovalent anti-MAdCAM-1 MAbs against two distinct epitopes inhibit L-selectin-dependent lymphocyte rolling in PP-HEV. The inhibitory effects of these anti-MAdCAM-1 MAbs on L-selectin-mediated attachment and rolling are unlikely to be due to direct steric blockade of access to the mucin domain, as neither MECA-367 nor MECA-89 significantly inhibits L-selectin-mediated rolling on purified MAdCAM-1 *in vitro* (Berg and Berlin, unpublished observations). One possibility was that crosslinking resulted in removal of MAdCAM-1 from the HEV surface. To address this, we assessed the effects of anti-MAdCAM-1 MAbs on binding of Mn^{++}-activated lymphocytes, whose interactions are mediated through α4ß7 without requirement for L-selectin (as demonstrated above).

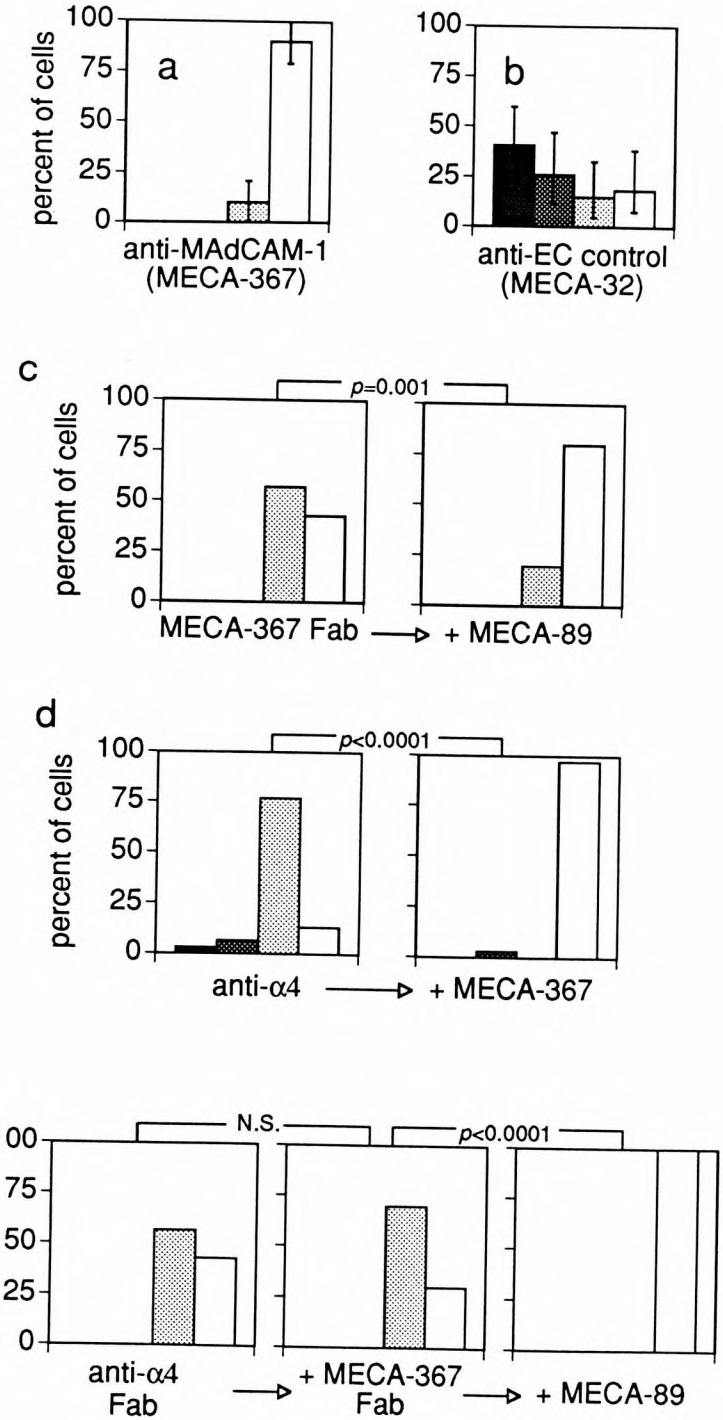


Figure 11. Effects of anti-MAdCAM-1 MAbs on interactions of lymph node

cells in PP-HEV. Analyses and data presentation as in Figures 7 and 9. A) Anti-MAdCAM-1 MAb MECA-367 prevents sticking and inhibits rolling of LNC. B) Control class-matched antibody MECA-32 against a pan-endothelial cell antigen has no significant effect on behavior. C) Monovalent Fab fragments of MECA-367 inhibited sticking, but left residual rolling that was inhibited by subsequent injection of whole anti-MAdCAM-1 MAb MECA-89 (against a second epitope). D) Lymphocytes pretreated with anti-α4 display rolling that is inhibited by subsequent injection of whole anti-MAdCAM-1 MAb MECA-367. E) Rolling of lymphocytes pretreated with anti-α4 Fab fragments was not blocked by monovalent Fab fragments of MECA-367, but was inhibited by subsequent injection of anti-MAdCAM-1 MAb MECA-89. Antibodies MECA-367 or MECA-89 (or Fab's) to MAdCAM-1, or control class-matched antibody MECA-32 against a pan-endothelial-cell antigen, were injected iv and allowed to saturate vessels for ~4-7 min prior to injection of labeled LNC (in B and C) or prior to analysis of previously injected lymphocyte behavior (in D and E).

As illustrated in Figure 12A, MECA-367 almost completely abrogated interactions of Mn^{++}-preactivated LNC, again consistent with the inhibition of L-selectin- as well as α4ß7-dependent events. However, MECA-89 had no significant inhibitory effect on activated LNC interactions (Figure 12B), consistent with its ability to block only L-selectin not α4 mediated adhesion as shown above. Subsequent injection of anti-α4 Fab (or MECA-367, data not shown) completely abrogated binding in the presence of MECA-89, demonstrating that the binding observed is mediated through α4 integrin-MAdCAM-1 interaction and confirming the continued presence of MAdCAM-1 on the HEV surface.

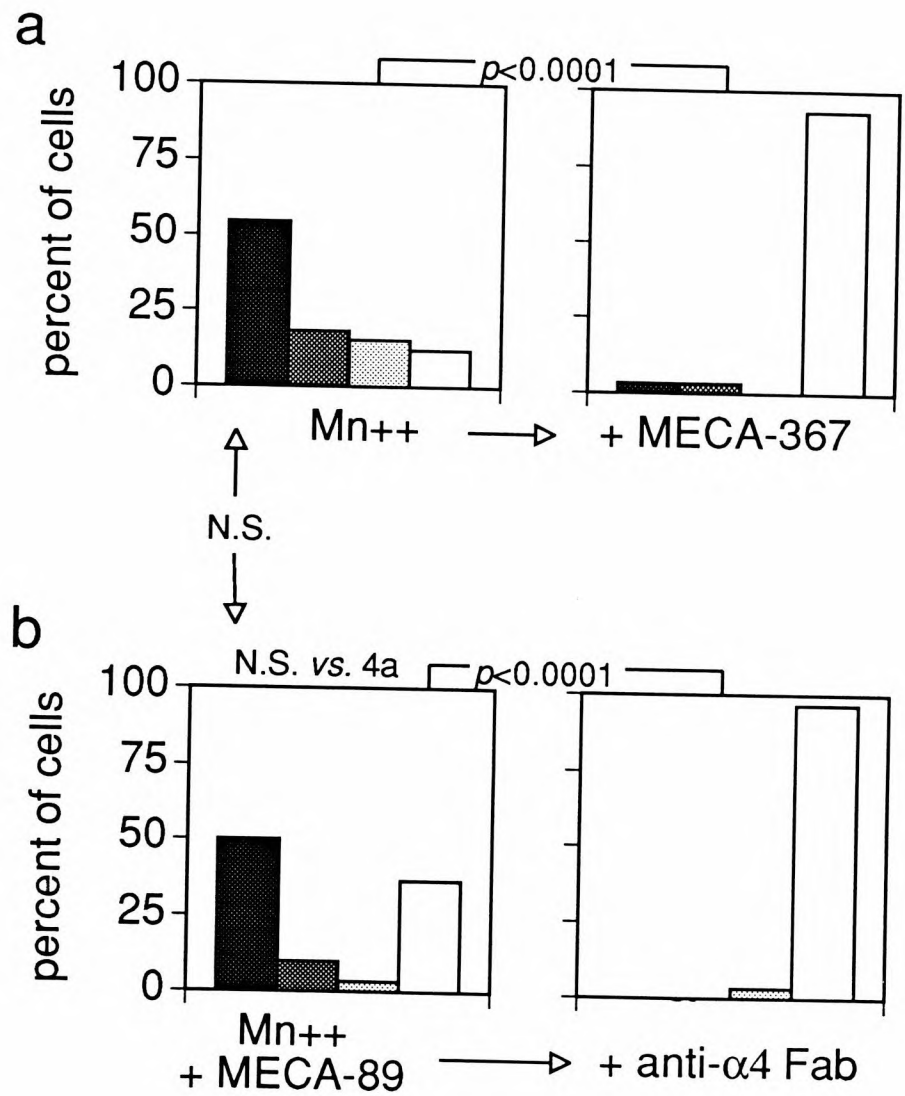


Figure 12. Effects of anti-MAdCAM-1 MAbs on interactions of Mn^{++}-activated lymph node cells in PP-HEV. Analyses and data presentation as in Figures 10 and 11. A) Anti-MAdCAM-1 MAb MECA-367 blocks both sticking and rolling of preactivated LNC. B) Anti-MAdCAM-1 MAb MECA-89 had no significant effect on the behavior of activated cells (N.S. vs. Fig 10A), but all interactions were inhibited following subsequent injection of anti-α4 Fab fragments.

Discussion

We have shown that the interaction of resting lymphocytes with PP-HEV *in vivo* involves sequential engagement of L-selectin, α4ß7, and LFA-1. Each component is required for efficient arrest, although overlapping roles appear to permit some residual interaction when any one of the components is blocked. L-selectin dominates primary contact point attachment, but α4ß7 can also initiate adhesion at lower efficiency. Both L-selectin and α4ß7 participate in activation-independent rolling, with α4ß7 playing an apparently essential role in increasing the avidity and reducing the velocity of rolling cells. Engagement of α4ß7 is required for activation-dependent adhesion strengthening and arrest, which also requires participation of the ß2 integrin LFA-1. Overlap in functions may explain, in part, the less than complete inhibition of lymph-node cell homing to PP by antibodies to any one of the receptors involved (although this may also reflect heterogeneity in lymphocyte subsets). These results define the specific molecular basis of resting lymphocyte interactions with PP-HEV. They represent the first demonstration of a multi-step adhesion cascade in physiologic lymphocyte-EC interactions, providing strong support for the multi-step paradigm of leukocyte-EC recognition.

It should be emphasized that the discussion here focuses on the behavior displayed by the majority of injected lymph node lymphocytes entering HEV. Lymph node lymphocytes are comprised predominantly of naive T and B cells that express uniform high levels of L-selectin and moderate levels of α4ß7 and LFA-1 and that home well to organized

lymphoid tissues, including Peyer's patches. Less abundant subsets of lymph node lymphocytes, including memory and recently activated cells, may behave differently: heterogeneity in homing receptor expression among these cells may help explain the less frequent extremes in behavior observed in the present experiments, as well as the consistent presence of non-interacting cells (which may lack L-selectin) (10).

α4ß7 occupies a unique intermediate "bridging" position in the process of lymphocyte homing. Its participation is apparently required for efficient conversion of L-selectin-mediated primary interaction to LFA-1-mediated arrest. This is in contrast to observations of neutrophils, which lack α4 integrins and, when rolling through selectins, can directly engage ß2 integrins in response to *in situ* activation. The ability of α4ß7 to participate in activation-independent rolling may relate to the expression of this integrin in a constitutively activated state: although α4ß7 binding to MAdCAM-1 can be dramatically enhanced by lymphocyte activation, even resting lymphocytes bind reasonably well *in vitro* without requirement for experimental stimulation. Relatively low avidity and rapid on-and-off rates of quiescent α4ß7 may also be required to permit rolling (vs. sticking) (20). It is attractive to hypothesize that α4ß7, as well as the related α4 integrin α4ß1 (VLA4), may be able to fulfill a similar role in diverse *in vivo* settings, whenever the avidity of primary adhesion initiated through selectins is limiting. Consistent with this hypothesis, recent studies have revealed that α4 integrins participate in eosinophil rolling in inflamed rabbit mesenteric venules (21), and that α4ß1 can mediate lymphocyte attachment and rolling on VCAM-1 transfectants (16).

Given the ability of experimentally activated α4ß7 to mediate lymphocyte arrest in the absence of LFA-1 participation (discussed below), the apparent requirement for LFA-1 during sticking of most lymphocytes is perhaps surprising. It is possible that on naive lymphocytes which express relatively low levels of α4ß7, physiologic levels of α4ß7 triggering are insufficient to mediate arrest under high shear. Kinetic aspects of integrin activation might also be important: i.e., LFA-1 may be more rapidly triggered in response to local activating signals than α4ß7. Alternatively, engagement of LFA-1 might be required for efficient triggering of α4ß7 to its activated state in this setting.

Our results suggest that α4 integrins may play a very different and more direct role in the homing of preactivated immunoblasts, and potentially of memory lymphocytes characterized by high levels of α4ß7 expression. α4ß7 was both necessary and sufficient for binding of $\alpha4\beta7^{hi}$ TK1 cells and of PMA or Mn^{++}-activated lymph node cells to PP-HEV *in situ*. α4ß7 can also mediate direct L-selectin-independent interactions of TK1 cells and of activated (but not resting) lymph node lymphocytes with venules in the small intestinal lamina propria: lamina propria venules express MAdCAM-1, but lack demonstrable L-selectin-binding peripheral node addressin-associated glycotopes and thus appear entirely dependent on the α4ß7-MAdCAM-1 pathway for initiation of adhesion by circulating cells (16). The dramatic effect of integrin triggering on the role of α4ß7 and on the requirement for L-selectin underscores the potential of lymphocyte activation to regulate both the mechanisms and sites of lymphocyte homing.

The ability of α4ß7 expressed at high functional levels to initiate and consummate adhesion may help explain the distinctive gut-homing properties of mucosal immunoblasts (1) and memory T-cell subsets, a hypothesis that is consistent with the lack of L-selectin expression by many gut-recirculating memory T cells in sheep [most of which are $\alpha4ß7^{hi}$; (2) and ECB, manuscript in preparation]. α4ß1 may have a similar capacity that could underlie the L-selectin-independent trafficking of inflammatory T cells to inflamed peripheral lymph nodes and to sites of tissue inflammation (22,23). The ability of α4 integrins to mediate primary attachment under physiologic shear has been modeled *in vitro* as well and requires revision of the dogma that selectins are required for initiation of leukocyte adhesion under physiologic flow (16,24,25).

One possible explanation for the ability of α4ß7 to initiate primary adhesion could be that it has a more rapid "on" rate than ß2 integrins, facilitating engagement of moving lymphocytes. Alternatively, we have recently shown that α4ß7 is concentrated on the tips of microvillous processes the sites of initial cell contact under flow (16). α4ß7 shares this topographic distribution with the L-selectin (26,27), and it is likely that microvillous presentation is a requirement for participation of adhesion receptors in primary contact-point attachment under physiologic conditions. In contrast to α4 integrins, the ß2 integrins are largely excluded from microvilli (27) and appear unable to initiate cellular attachment under flow: In the case of neutrophils, for example, engagement of selectins is a clear prerequisite for ß2 integrin-mediated arrest both *in vitro* and *in vivo* (28). Similarly, *in vitro* studies of human peripheral blood T cells show that

LFA-1 cannot engage ICAM-1 under physiologic shear rates (16,29).

The present studies confirm for the first time the inability of lymphocyte LFA-1 to mediate primary attachment to endothelium in a physiologic *in vivo* setting: Anti-α4ß7 treatment completely prevents engagement of lymphocyte or TK1 cell LFA-1 in PP-HEV. Particularly surprising is the inability of LFA-1 to participate even when lymphocyte attachment and rolling is initiated through L-selectin: As discussed above, resting lymphocytes roll but do not stick in the presence of anti-α4ß7 MAbs, indicating that slowed rolling by α4ß7 is a prerequisite for efficient LFA-1 engagement by resting lymphocytes. In this setting, the relatively high velocity of rolling via L-selectin alone may present reduced opportunities for *in situ* triggering of secondary adhesion mechanisms. It is also formally possible that binding of α4ß7 to MAdCAM-1 induces intracellular events required for subsequent triggering of LFA-1 by physiologic signals on HEV. However, in some experiments Mn^{++}-pretreated lymphocytes displayed loose L-selectin-dependent rolling without sticking in the presence of anti-α4 MAbs: thus, even when preactivated experimentally, LFA-1 may be inefficient at mediating binding in the absence of α4ß7 participation. This observation suggests that L-selectin-dependent rolling may simply be 1) too rapid to permit engagement of LFA-1 because of a slower "on" rate for ß2 than α4 integrins; or 2) too loose to enforce contact of the non-microvillous cell body bearing LFA-1 with the EC surface under the high shear in HEV. Thus, the differences in requirements for ß2 vs α4 integrin involvement in lymphocyte-EC interactions are striking.

L-selectin plays a much more prominent role in lymphocyte homing to peripheral lymph nodes than to Peyer's patches, leading to its characterization as a lymph node homing receptor (30). We predict that L-selectin will mediate a much more avid interaction of lymphocytes with lymph node HEV, which express extremely high levels of PNAd-associated L-selectin-binding glycotopes: α4 integrins do not appear to be required for lymphocyte homing to uninflamed lymph nodes (31,10), suggesting that interactions through L-selectin may be sufficiently avid to permit engagement of LFA-1 (or may be even to permit integrin-independent arrest) in lymph node HEV.

The PP-HEV ligand for α4ß7 is the mucosal vascular addressin MAdCAM-1, an Ig family member homologous to the α4 integrin ligand VCAM-1, and to the ß2 integrin ligand ICAM-1. α4ß7 binding to MAdCAM-1 involves the N-terminal Ig domain (18). MAdCAM-1 also contains a mucin-like domain which can be modified (in lymph node or PP-high endothelium) with the peripheral node addressin-associated MECA-79 epitope, associated with HEV ligands for L-selectin. Moreover, purified HEV MAdCAM-1 supports L-selectin-mediated lymphocyte attachment and rolling under shear *in vitro* (15). Many HEV glycoproteins can be so modified, however (17,32), and we have emphasized that only *in situ* analyses can reveal which of these is physiologically relevant in L-selectin-mediated attachment and rolling (17). Furthermore, the importance of particular ligands is likely to vary depending upon the organ or microenvironment involved.

Our present results, however, suggest that MAdCAM-1 dominates L-

selectin as well as α4ß7 recognition of PP-HEV in *situ*: whole anti-MAdCAM-1 MAbs dramatically inhibit L-selectin-dependent rolling on PP-HEV. This effect likely requires crosslinking leading to local redistribution or conformational changes in MAdCAM-1 (with altered carbohydrate presentation), as 1) Fab fragments of MAb MECA-367, which inhibit α4ß7-mediated interactions, had no effect on L-selectin-dependent rolling; 2) inhibition was conferred by MAbs to two distinct epitopes: MECA-367 and 89, which recognized the first and second Ig domains respectively, in MAdCAM-1. Neither of these MAbs significantly inhibit L-selectin mediated rolling on purified MAdCAM-1 *in vitro* (Berg, Berlin, and Butcher, unpublished observations), thus ruling out direct antibody blockade of the carbohydrate-presenting mucin domain. 3) MAdCAM-1 is not internalized or cleaved from the HEV surface as antibody MECA-89, against the second Ig domain, inhibits L-selectin-dependent rolling, but not the direct α4ß7-dependent attachment of PMA-activated lymphocytes. Although short of formal proof, these findings provide strong evidence that MAdCAM-1 plays a prominent role in presentation of L-selectin-binding glycotopes in PP-HEV.

Figure 13 presents a schematic summary of the adhesion/activation cascades involved in resting lymphocyte and preactivated lymphocyte homing to PP and in trafficking to lamina propria (16). The schematic emphasizes the sequential but overlapping roles of L-selectin, α4ß7, and LFA-1 in interactions of predominantly naive lymph node cells; and the self-sufficient involvement of α4ß7 in preactivated and potentially $\alpha4ß7^{hi}$ lymphocyte homing to PP-HEV and to lamina propria venules. We hypothesize that α4ß1 may be able to support roles similar to those revealed

for α4ß7 in the present study, but in the context of other lymphocyte subset trafficking in nonmucosal tissues in the body. For example, α4ß1/VCAM-1 interactions may play a bridging role between cutaneous lymphocyte antigen (CLA)/E-selectin-mediated primary attachment and ß2 integrin arrest in memory lymphocyte trafficking to inflamed skin (33,1), whereas preactivated $\alpha 4\beta 1^{hi}$ immunoblast may be able to traffic to sites of inflammation without selectin involvement. Thus the insights from the present *in situ* study provide an important context for considering the potential involvement of diverse adhesion receptors in the tissue- and inflammation-specific regulation of lymphocyte trafficking in general and, in particular, the tissue-selective homing of specialized memory and effector lymphocyte subsets

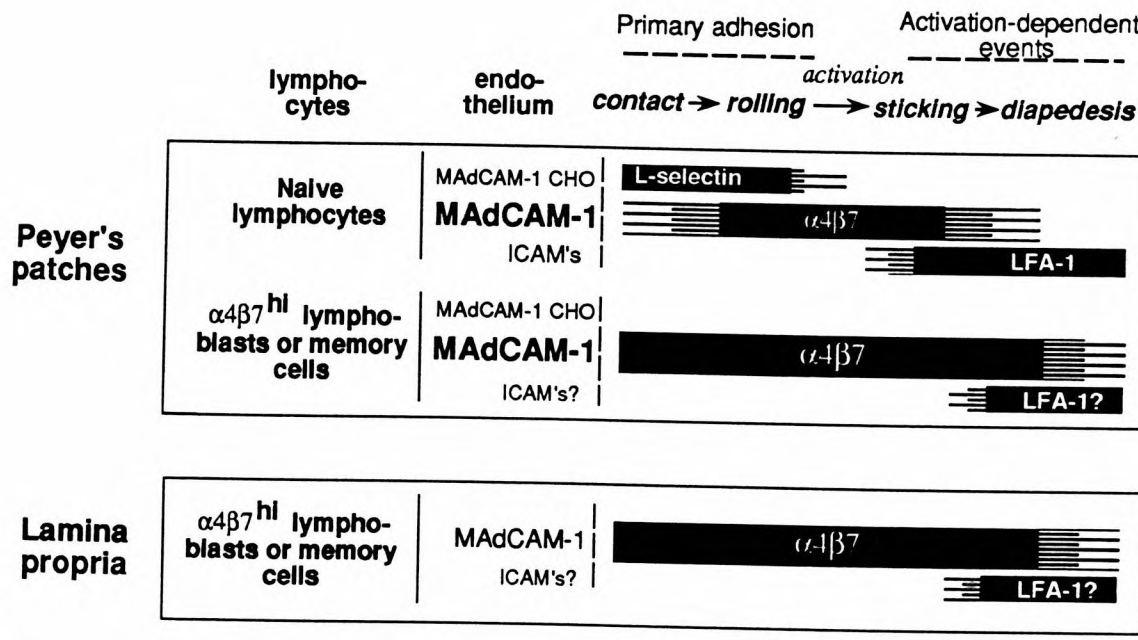


Figure 13. Schematic summary of the proposed sequential but overlapping roles of L-selectin, α4ß7 and LFA-1 in lymph node cell (primarily naive)

lymphocyte interactions with PP-HEV; and of the dominant involvement of α4ß7 in interactions of activated or $\alpha4\beta7^{hi}$ cells (mucosal memory cells and immunoblasts?) in PP and venules of the small intestinal lamina propria.

In conclusion, *in situ* analyses have defined distinct if overlapping roles for L-selectin, α4ß7, and LFA-1 in the attachment, rolling, and arrest of lymphocytes in PP-HEV and have revealed predominance of α4ß7 in interactions of preactivated or $\alpha4\beta7^{hi}$ lymphocytes. The studies have provided a number of novel insights into the molecular regulation of lymphocyte-EC recognition *in vivo*. α4 integrins, at least α4ß7, display surprising versatility and, in different situations, can dominate lymphocyte attachment, support lymphocyte rolling, and mediate lymphocyte adhesion strengthening and arrest. Quantitative as well as qualitative aspects of receptor regulation are shown to be critically important in determining the role of receptors in the multi-step process of EC recognition and, by extension, the patterns of lymphocyte subset homing. The lessons learned here may prove applicable not only to lymphocyte trafficking to diverse sites, but also to trafficking of eosinophils, mast cells, and monocytes, which, like lymphocytes, coordinately express selectin, α4 integrin, and ß2 integrin adhesion receptors. Finally, the findings validate and extend our understanding of the multi-step model of leukocyte-EC recognition, and demonstrate the operation of two extreme variants of the model: PP homing of resting lymph node cells, which involves sequential engagement of 3 adhesion receptors and at least one as-yet-unidentified activating signal; and the simpler interactions of $\alpha4\beta7^{hi}$ cells, which can be mediated by α4ß7 alone.

Summary

Circulating lymphocytes are recruited to the mucosal lymphoid organs, Peyer's patches, through specialized high endothelial venules. Here we have used video epifluorescence microscopy in combination with inhibitory antibodies to define the roles of adhesion receptors during physiologic lymphocyte interactions with PP-HEV *in situ*. The interaction of intravenously injected lymph node cells (primarily resting, naive lymphocytes) with PP-HEV involves a series of sequential but overlapping adhesion events: L-selectin much greater than α4ß7 initiates contact-point interaction, L-selectin can support but α4ß7 also participates in "rolling", and a G_i-protein-mediated activation event triggers sticking and arrest that requires the participation of both α4ß7 and LFA-1. Rolling via L-selectin alone does not permit efficient engagement of LFA-1 in PP, thus enforcing participation of α4ß7 (which dramatically reduces rolling velocity) as an intermediate or "bridging" element. In contrast, on preactivated or $\alpha4ß7^{hi}$ lymphoid cells, α4ß7 can directly mediate attachment and arrest with little rolling and no requirement for L-selectin or LFA-1 participation. Our findings reveal a surprising functional versatility for α4 integrins, at least α4ß7, which can mediate primary attachment, rolling, and arrest of lymphocytes under different physiologic conditions. Both L-selectin and α4ß7-dependent lymphocyte interactions are inhibited by antibodies to the mucosal vascular addressin MAdCAM-1, suggesting that MAdCAM-1 plays a prominent role in PP-HEV as a ligand for L-selectin as well as for α4ß7. The

results also demonstrate for the first time the involvement of a multi-step adhesion cascade during physiologic lymphocyte-endothelial cell (EC) recognition *in vivo*.

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CHAPTER 4

CHEMOKINES SLOW LYMPHOCYTE SELECTIN-MEDIATED ROLLING BUT DO NOT TRIGGER RAPID INTEGRIN-MEDIATED ARREST ON CYTOKINE-ACTIVATED ENDOTHELIUM

Introduction

The recruitment of leukocytes to acute inflammatory sites is a key initial response in the host defense for clearing an invading foreign organism from the body. The success of this process is due to a sequential attack on the organism in a progression that moves from being non specific, which can be highly destructive to the invaded host tissue, to a highly specific attack aimed at the invading organism (1). The responding cells are made up of leukocyte subsets; neutrophils, monocytes, and lymphocytes, and other cells (2).

The sequence for the defensive response can be simply generalized in the following scenario. Upon entry into a tissue neutrophils, the first blood cells recruited to the inflamed site, can degranulate and release a chemical assault that destroys many of the infectious organisms, rendering them into fragments. This chemical assault can be deleterious to the healthy tissues in

the local area of the lesion, as well. Cell-mediated responses in the tissue can further signal for the recruitment of monocytes to the site where they process fragments from the infectious agent for presentation to lymphocytes. Monocyte activity and induced cytokine production can signal the endothelium for recruitment of lymphocytes which, on arrival, may be activated by monocyte presentation of the processed antigen (2). Recognition of processed and unprocessed antigen can stimulate T-and B-cell-specific responses that can be either cellular or humoral and determine the nature of the ongoing response. The T cells, cytotoxic and helpers, may participate in specific infected host cell killing or may stimulate B cells to generate antibody responses, either or both of which may act to clear the pathogen.

Central in this directed progression of cellular and immune responses is leukocyte recruitment. How the temporal sequence of the arrival leukocyte subsets is regulated has been an open question posed in recent years while the molecular basis of inflammatory cell homing has been examined and defined (3,4).

It has recently been demonstrated that neutrophil recruitment is directed to the inflamed tissue vessel bed by both upregulation of counter ligands on the endothelium and molecular signals that instruct the leukocyte to roll and adhere (5,6). Several specific receptor-ligand pairs participate in this dynamic process. First, the selectins have been shown to facilitate the initial blood cell rolling. This is supported by any of three selectins, L- on leukocytes, E- on endothelium or P-selectin on both platelet and endothelial cell surfaces and their counter ligands (7,8,9,10). If rolling is established, the cells can undergo a signaling event through receptors of

chemokines, such as IL-8, that increase the adhesive potential for the CD11b/18 MAC-1 ß2 integrin and/or increase their numbers and distribution on the cell surface (3,4,11). The activated ß2 integrins then slow the cell arresting it on the endothelial cell surface. The leukocyte eventually undergoes shape changes required for motility and diapedisis.

Lymphocytes too, have the capacity to utilize the three-step "adhesion cascade" (12). During constitutive lymphocyte homing to PP via the high endothelial venules (HEV), L-selectin, as with neutrophils, promotes rolling which allows engagement of integrin molecules. But here, the integrin that directly mediates slowed rolling is an α4 integrin, which neutrophils lack (12). The CD11a/CD18 lymphocyte integrin LFA-1 does not appear to directly support slowing, but may play a prominent role in the signaling responsible for increased α4β7 participation-mediated slowing of the rolling speed (12). This difference is a significant clue as to how the endothelium distinguishes between lymphocytes and neutrophils during inflammatory recruitment. If there are not counter ligands for α4, then the lymphocyte will likely be recruited less efficiently than neutrophils. It is clear from this single example that if each of the three steps of this process exhibits high diversity, then very precise constraints on the specificity of the leukocyte subset recruited can be implemented.

A number of researchers have recently identified pro-inflammatory molecules, related to and including IL-8, that bind and regulate T-lymphocyte function (13,14,15). It is likely that these molecules may participate in the signaling that initiates integrin-mediated lymphocyte as well as neutrophil slowing or arrest. This class of molecules has been

termed "chemokines." These low molecular weight proteins are highly potent at physiological concentrations and bind G-protein-coupled serpentine receptors on the lymphocyte cell surface (13,14,15). These seven transmembrane-spanning receptors are characterized by the fMLP receptor (14), the receptors for neurotransmitters, and the prototypical rhodopsin receptor which signals light sensitivity in retinal cones. These molecules transduce signals by activating GTP/GDP-bound proteins. The intracellular loops of serpentine receptors interact with GDP-bound G-proteins, and upon ligand binding the receptor activates the G-protein, which exchanges GTP for GDP. The GTP-bound form of the G-protein then activates effector enzymes, GTP is hydrolyzed to GDP, and the GDP form of the G-protein completes the circuit by re-forming a complex with the unoccupied receptors. Many studies have implicated G-proteins in chemotaxis (16) and we have recently shown them to be involved in regulating integrin adhesion where they induce the slowing and arrest of lymphocytes on PP HEV (12).

Although the circumstantial evidence suggests that chemokines could signal lymphocytes to arrest from the blood flow, there is no direct proof. Experiments to date have examined lymphocyte-endothelial binding only under static conditions and after 1-24 hrs of chemokine preincubation with lymphocytes (17). The key focus of this study is to define, <u>in vitro</u>, the effects of chemokines on lymphocyte recruitment to cytokine-activated endothelium. First, we report the experimentally determined cytokine induced activation conditions required for stimulation of endothelial cells (HUVEC) to support human lymphocyte rolling in simulated blood flow.

Second, we examine if activation of lymphocytes with PMA or chemokines can induce behavior changes in lymphocyte rolling, slowing, or permanent endothelial arrest. Finally, we determine the molecular specificity of the lymphocyte-endothelial adhesion by function-blocking mAbs.

Materials and Methods

Lymphocyte cell suspensions.

Lymphocytes were isolated from human peripheral blood as previously described (18). Briefly, blood was collected into citrate anti-coagulant tubes (Becton Dickenson), diluted 1:2 at room temperature in sterile hepes (20mM) buffered HBSS (pH 7.0) (Fisher Scientific), underlayed with Histopaque 1077 (Sigma), and centrifuged at 2,300 RPM for 30 min at room temperature. Mononuclear cells were collected from the Histopaque/plasma interface. Monocyte contamination of the lymphocyte population was evaluated by staining with an anti-TcR pan T-cell marker, (Coulter) using flow cytometry and was found to be less than 22 percent.

Examination of the blood of normal donors for lymphocytes positive for the HECA 452 cutaneous lymphocyte-associated antigen (CLA) resulted in the selection of two donors. Each donor possessing 24-26% CLA positive cells vs the 5-8% normally observed for the average population (L. Rott, personal communication). CLA is a carbohydrate determinant expressed on skin-homing lymphocytes that have been demonstrated to have a high level binding capacity for E-selectin. (19). We reasoned that selecting for larger

numbers of E-selectin interacting cells would increase the number of lymphocyte-HUVEC interaction events observed in the shear system and this was the case (data not shown).

Monoclonal antibodies.

The DREG series of mAbs (DREG 56 and DREG 200), which are mouse IgGs, recognize human L-selectin (20,21), and were used to evaluate L-selectin participation in the shear flow experiments. EL-246, a unique mouse IgG_1 mAb, binds an epitope in the SCR domain of both L- and E-selectin, blocking their adhesive functions in many mammalian species (18). EL-112, produced in the same fusion as EL-246, specifically recognizes E-selectin and blocks E-selectin-dependent leukocyte binding (18). The EL and DREG mAbs were partially purified and concentrated by ammonium sulfate precipitation of serum-free supernatants. Other mAbs, 60.3 (a gift from J. Harlan), and R15.7 (a gift from R. Rothlein) both anti-human CD18 , WAPS 12.2 (anti P-selectin, a gift of R.A. Warnock and E.C. Butcher), anti-VCAM (Becton Dickinson), anti-ICAM (a gift of K. Bishop), were used either to assess integrin participation in binding or as negative controls in many of the experiments. All mAbs used for staining were unconjugated and followed by an appropriate second stage for visualization by fluorescence or immunoperoxidase.

Immunofluorescence staining and flow cytometric analysis.

Immunofluorescence staining of lymphocytes and HUVEC was carried out as described (14,22,23). The cells were washed and then incubated with primary antibody at 50 μg/ml (or undiluted culture supernatant) for 20 min on ice. After washing, bound antibodies were revealed by incubation with PE- or FITC-conjugated F(ab)'2 goat anti-mouse Ig (Tago) at a 1:80 dilution in 5% FBS in DMEM. Flow cytometric analysis was performed on a FACScan (Becton Dickinson) as described (14,22,23). Data were collected from 10,000-50,000 cells and are presented as histograms or contour plots.

Activation of HUVEC to induce expression of cell-surface adhesion molecules for in vitro shear assays and adhesion receptor flow-cytometry staining analysis.

HUVEC were cultured on bovine collagen type I (Collaborative Research), 10μg/ml and then grown in endothelial-cell growth media [Clonetecs, (EGM)] for 24 hr before a 4 hr pretreatment with human rIL-1 (1 unit/ml) (Genzyme). These treatments were done in parallel using Labtech slides and glass capillary tubes for simultaneous evaluation of cell surface expression of adhesion molecules, via flow cytometry, and determination of shear-dependent lymphocyte rolling and binding.

For flow cytometry, after cytokine pretreatment, the HUVEC were treated with EDTA in media and cells in each well were collected. Cells were then washed in media and incubated with anti-E-selectin, anti-VCAM, and

anti-ICAM or a negative control mAb (10-50 μg/ml) in HBSS plus 1 percent serum; the cells were then washed by centrifugation, fluorescent second stages were added, the cells were incubated, and washed again. The cells were then analyzed by flow cytometry. The potential for HUVEC supporting lymphocyte rolling in the glass capillary tubes under shear was evaluated, as described below.

Chemokine activation of human lymphocytes.

Chemokines (supplied and pretested for static binding activity by Dr. David Kelvin,) were analyzed at concentrations ranging from 8-100 ng/ml to establish maximal effects on induced lymphocyte rolling and binding. These concentrations were based on the ranges shown to be effective in static adhesion assays, as reported by Kelvin et al. (24). Chemokine pretreatment (8ng/ml) was used in some cases to induce higher initial affinities of lymphocyte-E-selectin transfectant or lymphocyte-HUVEC rolling and binding for parallel comparison with untreated lymphocytes. In other experiments, chemokines were directly infused into the circulating loop at 8-100ng/ml with pretreated or untreated lymphocytes and their effects recorded. Chemokines were tested for LPS at a 100-fold greater concentration than that used for lymphocyte treatments using the E-TOXATE® (Sigma, St. Louis) assay and were found to be below 0.015 EU/ml (2-3 EU/ng endotoxin).

Activated human umbilical-vein endothelial cell (HUVEC) and E-selectin cDNA transfectant-mediated lymphocyte rolling.

Freshly isolated human umbilical-cord endothelial cells, which were Factor VIII and LDL-receptor positive, were cultured in endothelial-cell growth media (Clonetecs, EGM) or E-selectin cDNA transfectants were grown to confluency in GPT media (RPMI, 10% fetal bovine serum, HT, Xanthine, mycophenolic acid) on the internal surface of sterile glass 1.36 mm diameter capillary tubes (Drummond Scientific, Broomall, Penn), coated with 10μg/ml bovine collagen type I for one or more hrs at RT (Collaborative Research) at least 24 hrs prior to shear experiments.

Tubing was attached to the ends of the glass capillary tube to form a closed loop in which media and cells could be recirculated; the tube was then mounted on a Nikon inverted microscope. Using a variable speed peristaltic pump, flow was regulated to simulate *in vivo* shear conditions. The circulation loop allowed multiple infusions, via an injection port, of various activating factors chemokines, or mAbs during the continuous recirculation of lymphocytes across the interactive surface of HUVEC or E-selectin cDNA transfectants. The inverted microscope video-capture system, employing a mechanical stage, allowed a survey of the entire length of the HUVEC-or E-selectin-transfectant monolayers and high resolution phase contrast recording of the interactive field for subsequent analysis.

The isolated human lymphocytes were infused into the system at a concentration of $1x10^7$ cell/ml in sterile hepes buffered (20mM) DMEM (pH 7.0) plus 2% FBS. The rolling interactions, which did not occur on unactivated endothelial cells or mock transfected L-cells, were observed at a

shear rate of 2 dynes/cm^2 (with a center line velocity of 10.1 mm/sec). Rolling was established and continuously monitored for the duration of the experiment while being videotaped for off-line analysis. After rolling was established PMA (10-100ng), or chemokines (8-100ng), and/or antibodies at a concentration of 50 μg/ml were infused into the recirculating loop. Both rolling cell numbers and numbers of bound cells were determined over time.

Analyses of lymphocyte rolling speed and enumeration of bound cells

The number of lymphocytes rolling on the activated endothelial cells were quantified at 1 min intervals, before and after the injection of PMA, chemokines and/or mAb, by individual frame analysis of the recording using NIH, IMAGE, software and an Apple Macintosh Quadra 660Av computer (14 inch diagonal Apple RGBAv monitor. Data were reported as the number of rolling cells in each field of view versus time in minutes. Lymphocyte rolling speed was measured, within the 350 μm (horizontal) by 250 μm (vertical) video-microscopic field, by following individual lymphocytes frame by frame (1/30th of a sec/frame), recording distance vs time (μm rolled/sec).

Results and Discussion

Human lymphocytes roll on E-selectin cDNA-transfected L-cells

In a study currently in press, we reported that bovine γ/δ T cells roll on human E-selectin transfectants in our *in vitro* shear system (25,26). Here, under the same assay conditions, we have demonstrated that human T lymphocytes exhibit similar rolling behavior The conditions of this assay simulate physiological blood flow conditions, over a range of 0.6 to 3.0 dynes/cm^2, and the level of expression of E-selectin is similar to that on cytokine activated endothelial cells (data not shown). The most efficient rolling was observed at approximately 1.8-2.0 dynes/cm^2 (Figure 14). Rolling did not occur on mock transfected L-cells and rolling did not lead to lymphocyte arrest on the E-selectin transfectant monolayer (data not shown). PMA-treated cells showed an accelerated rate of attachment and higher rolling numbers relative to control values.

We have shown that the three-step model of lymphocyte rolling and arrest *in vivo* is dependent on an intracellular signalling event that can be mimicked by a short (PMA) activation of lymphocytes [Chapter 1 (12)]. Upon infusion into the venus blood, these cells will home and rapidly arrest on the PP-HEV and lamina propria venules (Chapter 3 and Berlin et al., in press, Cell). We therefore treated human lymphocytes with PMA in the shear system and observed their ability to arrest on the E-selectin transfectants. PMA treatment of lymphocytes, in the E-selectin shear system loop, initially enhanced the numbers of lymphocytes rolling on the

transfectants by two fold, which was followed by a rapid decline of rolling cells to zero (Figure 14).

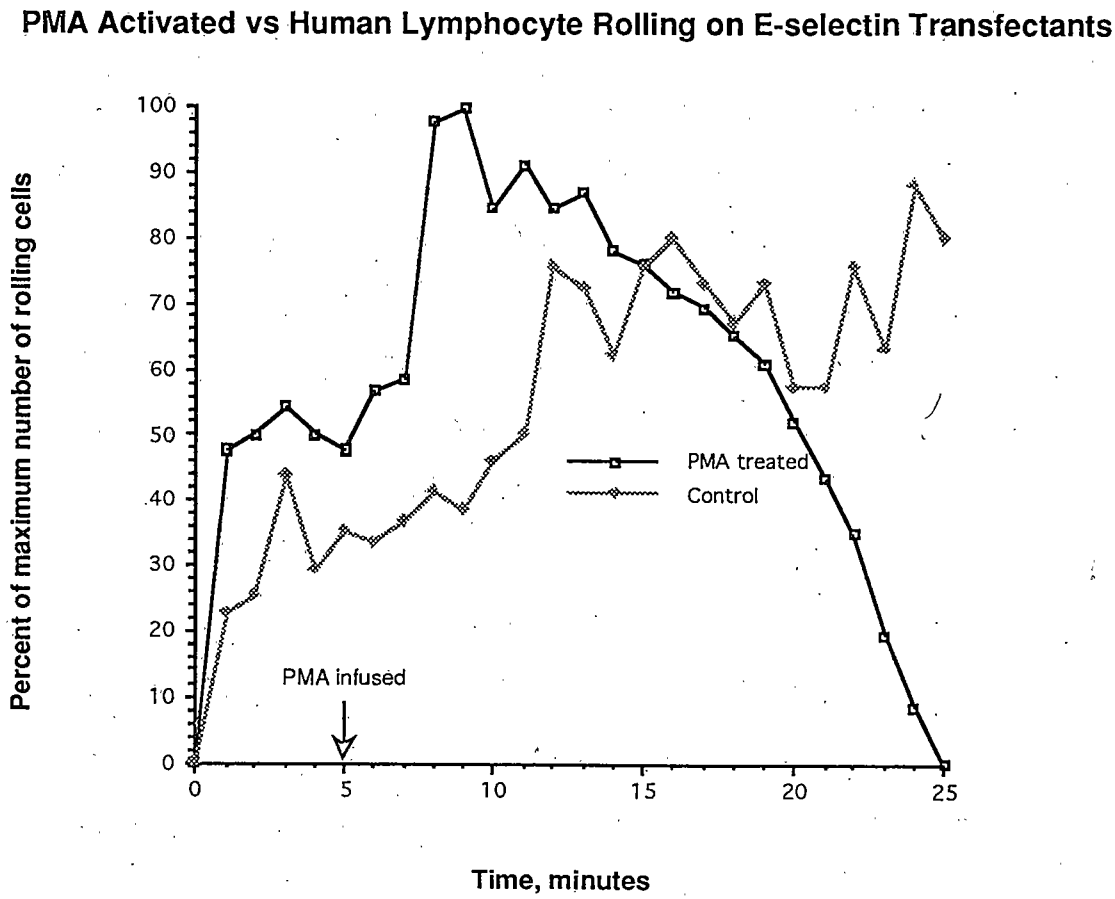


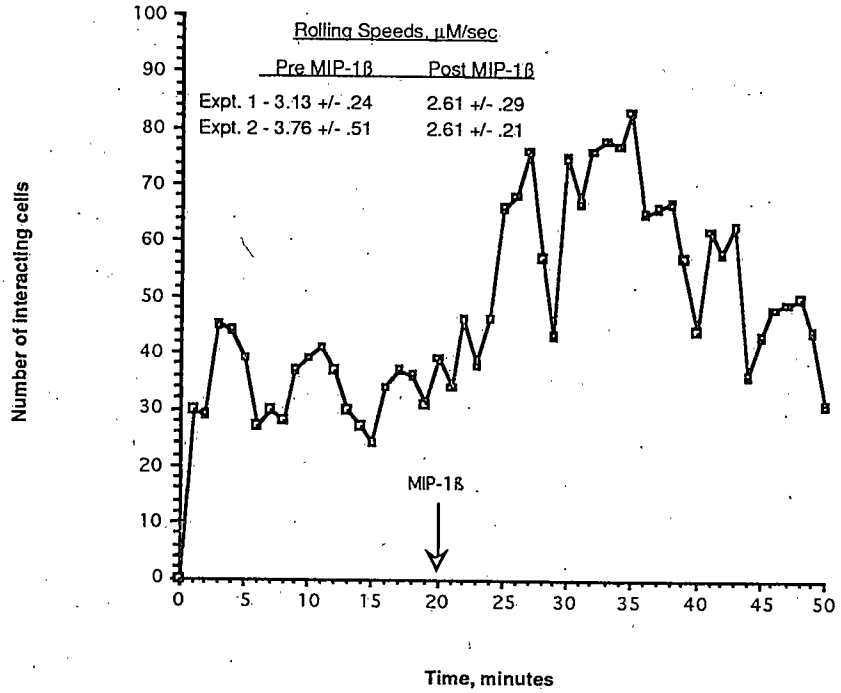
Figure 14. Human lymphocyte rolling on E-selectin transfectants (typical experiments of multiple repeats). Lymphocytes were infused into the loop system and interactions were observed and recorded by video microscopy. PMA (100ng/ml) was infused at 5 min, after rolling was well established. After ten minutes, the PMA-activated rolling cell numbers decreased to background while the control cells continued their rolling interactions. The observed reduction in rolling of the PMA-treated cells suggests that

activation may result in cell morphology changes and loss or modulation of E-selectin lymphocyte ligands. Control cells show a continuous rise over the same time period.

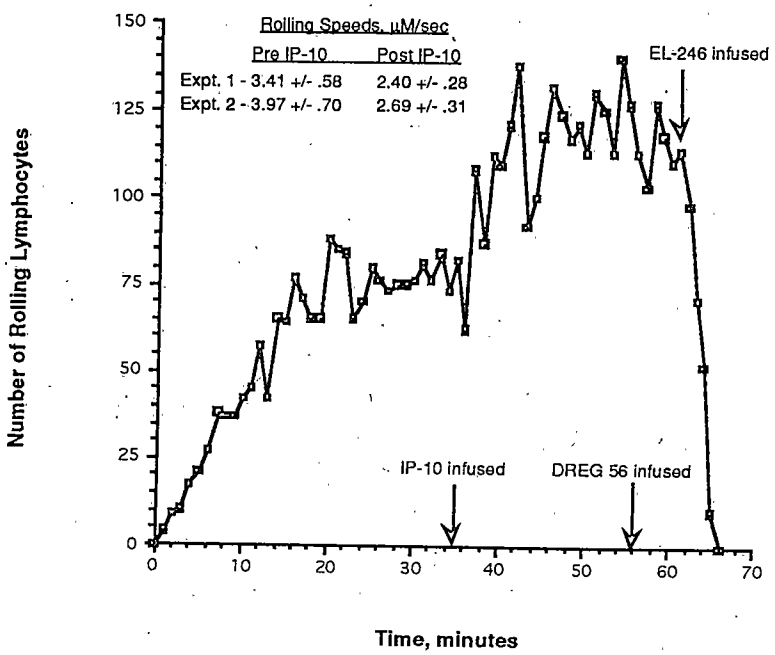
The loss of rolling follows the time course established for the loss of L-selectin and upregulation of CD18-MAC-1 expression, as shown for neutrophils (22), suggesting a similar regulatory system for human lymphocytes. However, as PMA activation of lymphocytes has been shown to greatly enhance the binding of lymphocytes to endothelium (12), the mouse E-selectin L-cell transfectant appears to lack an integrin ligand required to support lymphocyte arrest since no lymphocytes permanently bound.

As observed for PMA, treatment with the IP-10 and MIP-1ß (Figure 15A&B), but not MCAF or MIP-1α chemokines (Figure 15C), increased the number of rolling cells and decreased their rolling speeds on the E-selectin transfectants (Figure 15A&B). The observed slowing was completely blocked by EL-246, but DREG 56 had no effect (Figure 15B). Since DREG 56, a mAb directed against L-selectin, had no effect on human T-cell rolling, (as was seen with the bovine cells) it is unlikely that L-selectin participates in E-selectin-supported lymphocyte rolling in the L-cell system (Figure 15B). Antibodies directed against CD-44 and P-selectin had no significant effect on the rolling interaction (data not shown).

A MIP-1ß Effects on Human Lymphocyte Rolling on E-selectin Transfectants



B IP-10 treatment of Lymphocytes and Anti-L and E-selectin mAb Blocking Effects



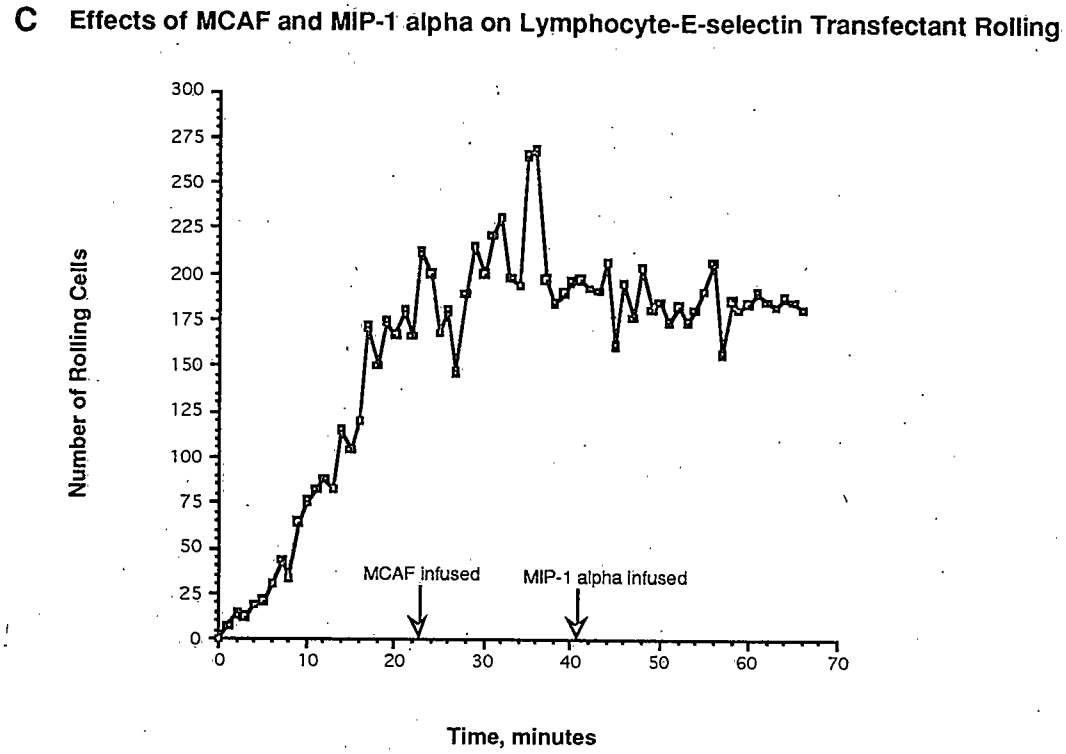


Figure 15 The effect of various chemokines on human lymphocyte rolling on E-selectin transfectants. In panel A, MIP-1ß was infused into the circulating loop after an initial plateau of lymphocyte rolling was established inducing a rapid increase in the rolling cell numbers. Rolling speeds are decreased by one third by this treatment. In panel B, very similar results are observed for the infusion of IP-10. In this experiment, both DREG 56 and EL-246 were infused after an IP-10 rolling cell plateau was reached. DREG 56 failed to block rolling, whereas EL-246 blocked all rolling in minutes. Panel C shows the rolling interaction curve for two chemokines not effecting lymphocyte rolling interactions with E-selectin transfectants.

Human peripheral blood lymphocytes roll and bind on IL-1-stimulated HUVEC.

In our recent studies examining neutrophil rolling and binding

interactions with IL-1-activated HUVEC, we observed that, in addition to supporting rolling, HUVEC also supported permanent neutrophil arrest. Rolling interactions were found to be sensitive to treatment with anti-L- and E-selectin mAb and arrest of neutrophils shown to be dependent on CD18. To better understand the surface expression of endothelial-cell adhesion molecules presented for lymphocyte interactions on IL-1-activated HUVEC, we fluorescently stained these cells with anti-E-selectin and anti-VCAM mAbs. IL-1-induced significant levels of both VCAM-1 and E-selectin (data not shown) which likely could support rolling and lymphocyte arrest. The shear system was used to directly test if IL-1-activated HUVEC could support the complete lymphocyte three-step adhesion cascade (26).

Human lymphocytes were tested in the closed loop HUVEC shear assay using the same shear condition used for the E-selectin transfectant system. The unactivated HUVEC supported no rolling under shear flow (data not shown). In contrast, the IL-1-activated HUVEC supported large numbers of rolling cells that increased and plateaued (Figure 16). A few lymphocytes arrested and accumulated on the monolayer (Figure 17&18). Sticking of low numbers of peripheral blood lymphocytes was expected, as 5-10 percent of the circulating lymphocyte pool has been reported to be preactivated (19). The adhesion molecule specificity for this rolling was examined by infusion of anti-L- and E-selectin mAbs directed against either endothelial or lymphocyte adhesion ligands. Unlike the E-selectin transfectant results, infusion of DREG mAbs blocked a significant amount of the rolling interaction (Table 2, Figure 16). As seen in the transfectant system, EL-246 completely blocked all interactions of lymphocytes with the

HUVEC monolayer (Figure 16). These results closely mirror those observed for neutrophils in the IL-1 HUVEC system (26). Control mAb directed against P-selectin and CD-44 had no effect on either rolling or binding interactions (Table 2).

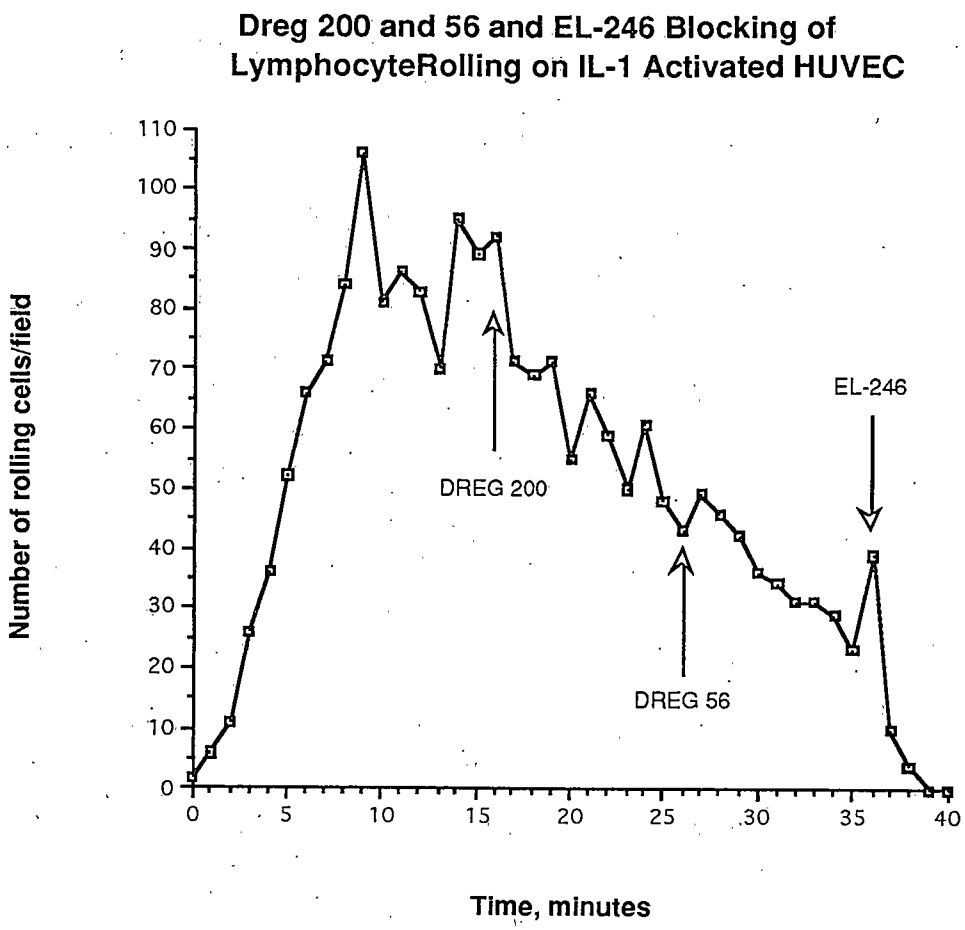


Figure 16. The blocking effects of anti-L-selectin and anti-E-selectin mAbs on human lymphocyte rolling on IL-1-activated HUVEC. DREG 200 and DREG 56 were added sequentially in this case, showing a potentiated combined blocking effect. The subsequent addition of EL-246 blocks all subsequent rolling.

Table 2. Percent blocking of lymphocyte rolling on IL-1-activated HUVEC by anti-adhesion molecule-specific monoclonal antibodies, at five minutes post infusion into the recirculating loop[1].

MAb :	HERMES-3	WAPS 12.2	DREG56	EL-112	EL-246
Percent Blocking :	0.0	0.0	33.8	61.7	100.0

[1] The results are data from sequential addition mAb blocking experiments where first the nonblocking control antibodies were infused. These were followed by infusion of either DREG 56, EL-112 or EL-246.

IP-10 treatment slows lymphocyte rolling speeds and increases their frequency of arrest on IL-1-treated HUVEC.

In the recent cytokine and chemokine literature, it has been shown that, in both experimental (27) and mycobacteria containing clinical cutaneous inflammatory sites, local rIL-2 injection is capable of eliciting expression of the IP-10 chemokine on keratinocytes and inflammatory cells. In both cases, a local accumulation of T cells at the inflammatory site was observed to peak 24 hrs after injection of the rIL-2. IP-10 has also been shown to mediate *in vivo* T-cell recruitment to tumors expressing this chemokine, resulting in a strong host-mediated anti-tumor effect (28). Based on these reports that suggest IP-10 involvement in T-lymphocyte skin accumulation, and the success of the IP-10 treatment on the increased avidity and speed of

lymphocyte rolling in the E-selectin transfectant shear system, we concentrated on the effects of this one chemokine.

IP-10 (10-100 ng/ml) was infused into the recirculating loop, after baseline rolling and sticking of human lymphocytes to IL-1-treated HUVEC were established (Figure 17). Alternatively, lymphocytes were pretreated (8-100 ng/ml IP-10) before infusion into the loop, with similar results (Figure 18). Within 7-10 min after infusion, IP-10 treatment increased lymphocyte rolling numbers by three-to-ten fold (Figure 17). In some cases, there were secondary increases of rolling cell numbers (Figure 17 after 28 min) which are, as of yet, unexplained. The rolling speeds for IP-10-treated cells were significantly altered as well. At a constant shear rate of 2.0 dynes/cm^2, IP-10 treated lymphocyte rolled an average of 70% slower on HUVEC than untreated lymphocytes (Table 3).

Table 3. Rolling Speeds For Control vs IP-10 (50ng/ml) Treated Lymphocytes on IL-1-Activated HUVEC.

	Lymphocyte Rolling Velocity, μm/second	
	Pre IP-10	Post IP-10
Experiment 1	5.44 +/- 0.42	1.89 +/- 0.16
Experiment 2	6.39 +/- 0.72	1.64 +/- 0.43

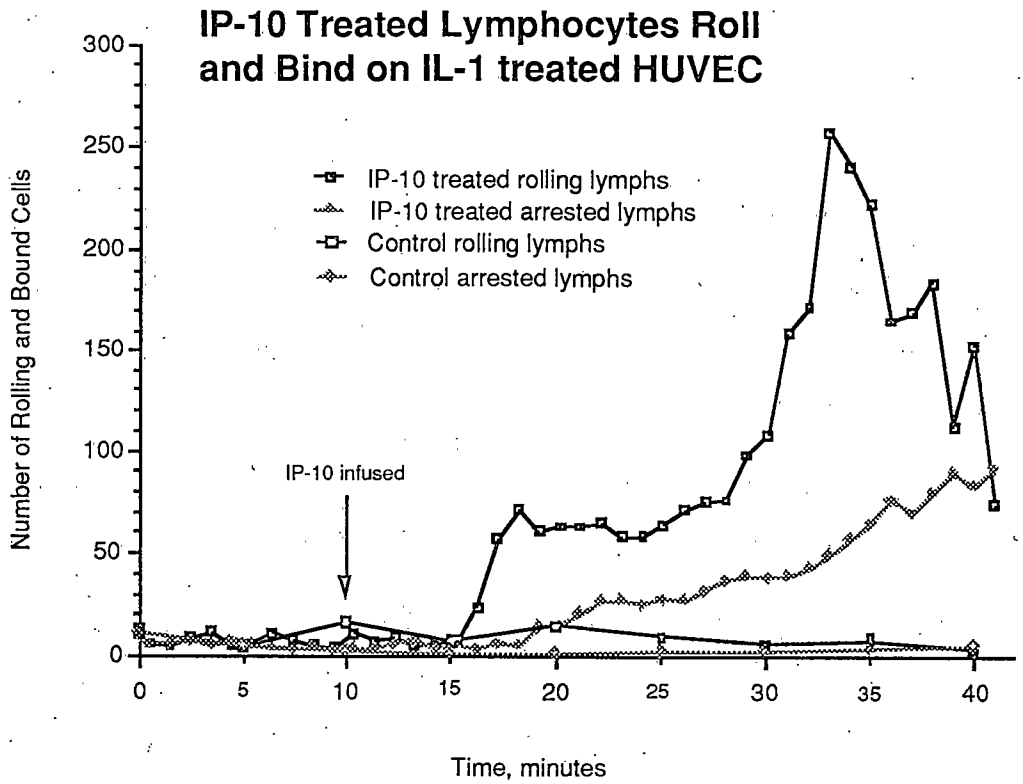


Figure 17. IP-10 infusion after establishment of baseline rolling and arrest. Addition of IP-10 at 10 min induces a dramatic rise in the number of rolling lymphocytes. Rising at a slower rate, IP-10-treated cells accumulate slowly over time. Control cells continued to interact at baseline levels during the same time course.

Interestingly, lymphocyte arrest also occurred, promoting accumulations of lymphocytes on the HUVEC surface that were greater than the numbers observed for control lymphocytes (Figure 18). In IP-10-lymphocyte preincubation assays, lymphocytes immediately rolled in numbers greater than controls; and the numbers of bound cells steadily

increased during the experiment, exceeding control values by two-to-five fold (Figure 18). Evaluation of the cells binding the HUVEC monolayer by immunoperoxidase staining with the pan T-cell marker (Coulter) showed that 79% of the interacting cells were T cells.

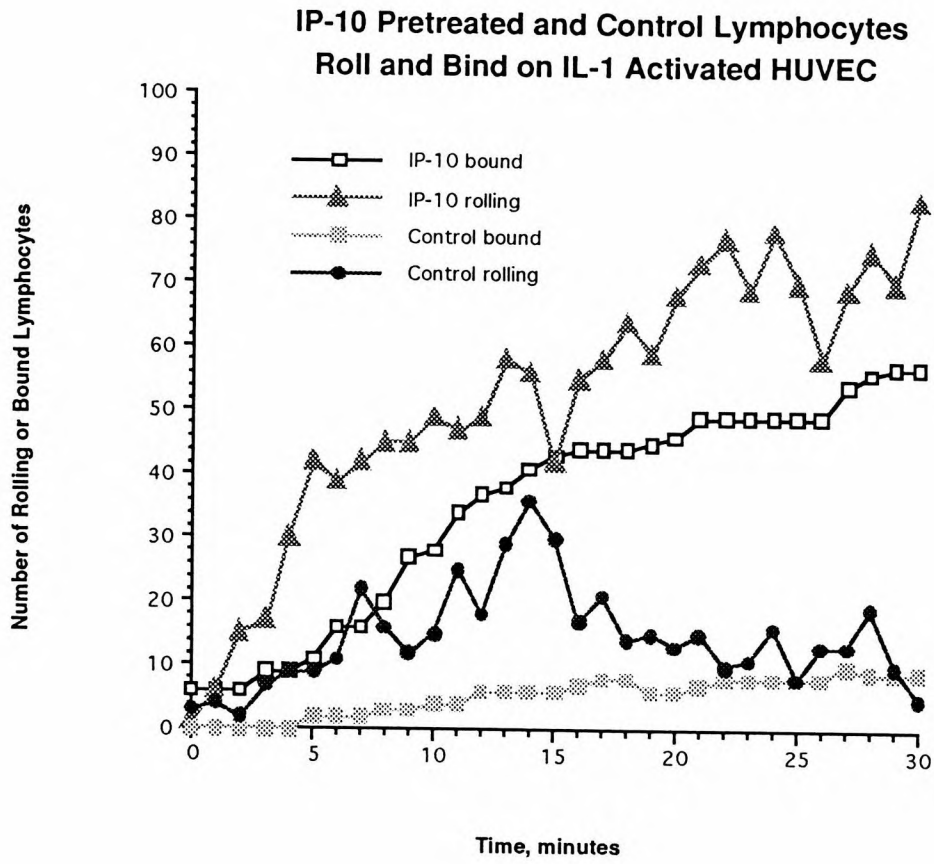


Figure 18 Lymphocyte IP-10-pretreatment effects on rolling and binding. Lymphocytes were either pretreated with 10ng/ml IP-10 before infusion or not. The IP-10-treated cells showed accelerated rates of accumulation for both rolling and binding relative to control cells. This was a consistent observation in multiple repeat experiments.

To provide a positive control for activation and a comparison for the

effects of IP-10 in a combined experiment, MCAF chemokine, IP-10, and PMA were sequentially introduced into the closed loop. As shown in Figure 19, MCAF failed to increase lymphocyte-activatedHUVEC rolling. The levels of rolling cells actually dropped and plateaued. Within 10 minutes of the addition of IP-10, the numbers of rolling cells increased four fold, after which PMA was infused into the recirculating loop. During the first ten minutes after the PMA infusion, the number of rolling cells decayed with most arresting on the HUVEC, while some released into the flow (Figure 19). No new lymphocyte rolling occurred after the ten-minute time point.

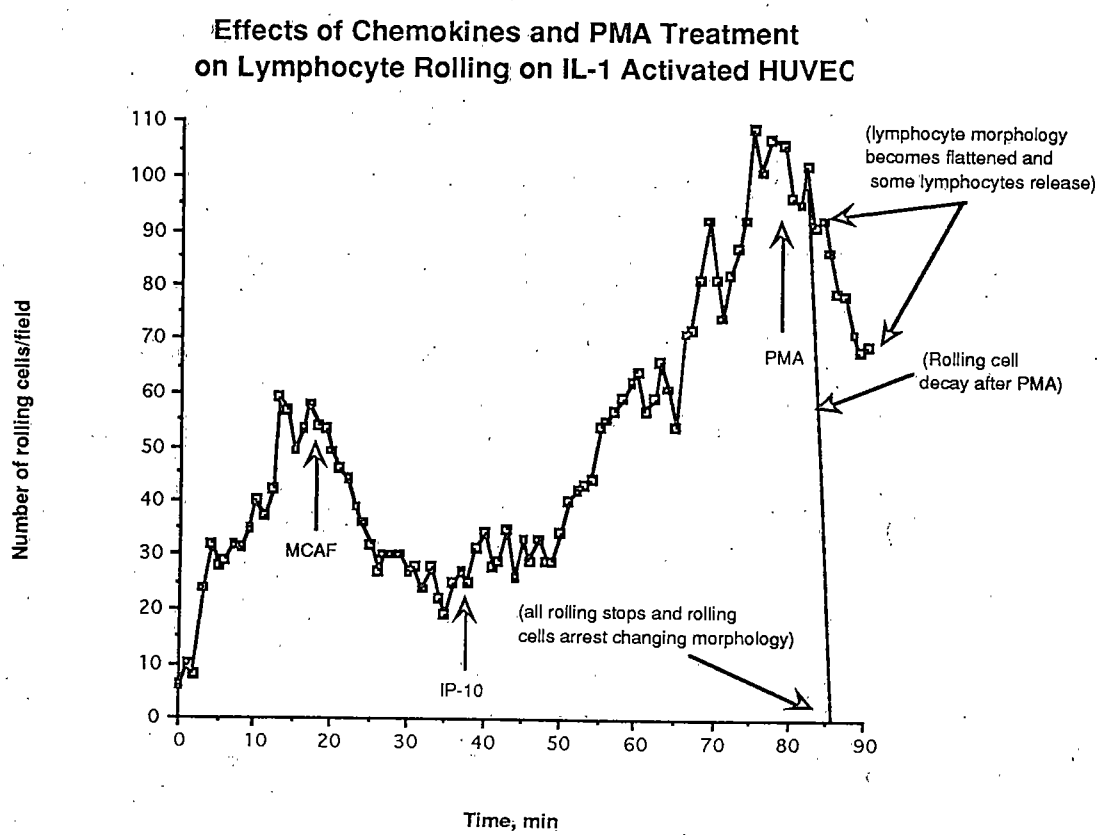


Figure 19 The sequential addition of MCAF (10 ng/ml), IP-10 (10ng/ml), and PMA (100ng/ml) to human lymphocytes rolling on IL-1 activated HUVEC.

MCAF shows an inhibitor effect on rolling cell numbers that is reversed and enhanced by addition of IP-10. Addition of PMA has an overt effect, inducing rolling lymphocytes to bind, and rapidly causes loss of lymphocyte-HUVEC rolling interactions.

Both DREG 56 and EL-112 infusion blocked much of the rolling of IP-10-treated lymphocytes on the IL-1-activated HUVEC (Figure 20). These results mirrored those seen for control lymphocytes (Figure 16), suggesting in this system that L- and E-selectin were primarily responsible for initiating lymphocyte rolling for both IP-10-treated and untreated lymphocytes.

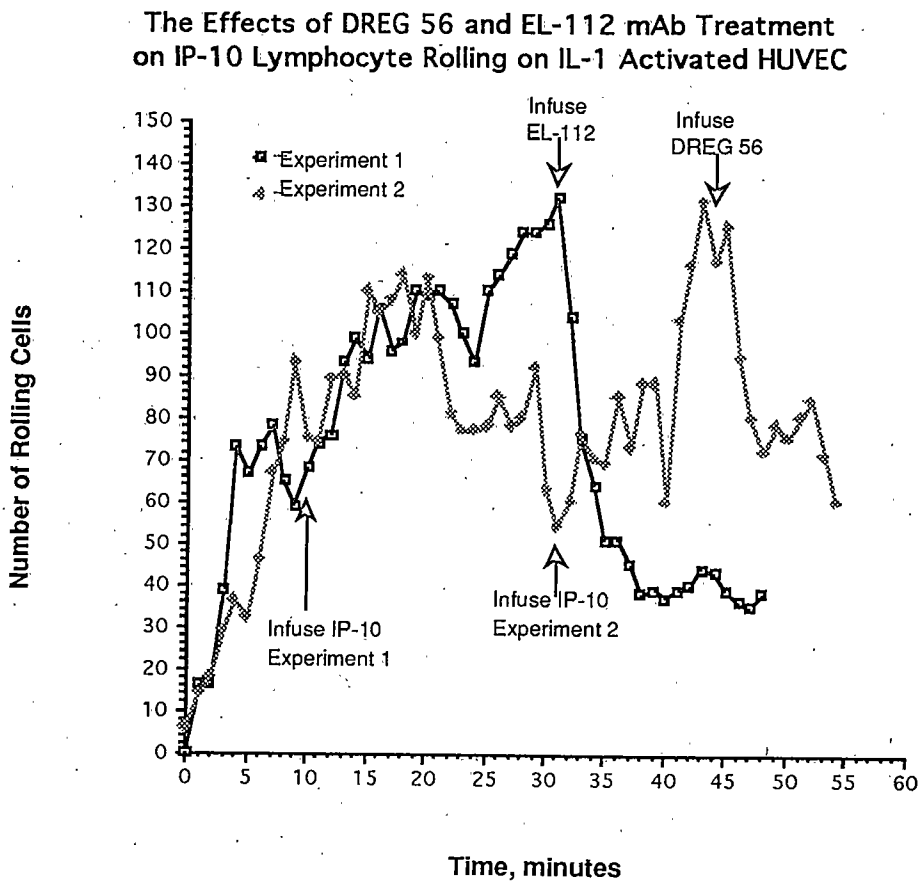


Figure 20. Both DREG 56 and EL-112 inhibit IP-10-enhanced rolling on IL-1-

activated HUVEC. IP-10 infusion in both experiments induces increased levels of lymphocyte rolling. Addition of either EL-112 or DREG 56 rapidly reduces rolling cell numbers, leaving residual rolling cells not blocked by either of these mAbs alone.

However, the differences seen in the frequency of arrest and rolling speed for IP-10-treated versus control cells suggested that an additional adhesion receptor may also be involved, possibly $\alpha 4\beta 1$ integrin (29). In fact, some IP-10-treated lymphocytes continue to roll after EL-112 treatment. These cells rolled at three-fold higher velocities, 5.9 μM/sec. +/- 0.6 after EL-112 treatment vs. 1.6 μM/sec. +/- 0.4 before, at 2 dynes/cm^2 shear force.

Discussion

Human lymphocytes are key participants in cell-mediated immune responses at sites of inflammation. The mechanisms of T-cell recruitment are currently the topic of intense investigation. In this study we show that lymphocytes roll on E-selectin transfectants via an L-selectin independent ligand, and that the IP-10 and MIP-1ß chemokines induce significantly slowed rolling speeds. Rolling of both control and chemokine-treated lymphocytes is sensitive to blocking by mAbs that block E-selectin adhesion. E-selectin transfectants in this system are not capable of supporting full arrest of lymphocytes.

As was seen with the bovine lymphocytes (25), E-selectin on the transfectants does not interact with human T-cell L-selectin, but likely with a ligand that has been recently identified on γ/δ T cells (30). Work continues in

our lab to fully characterize this new E-selectin receptor. Slowed rolling of lymphocytes may occur by mechanisms that increase affinity and/or avidity through redistribution of the adhesion receptors on the lymphocyte surface. This classically has been thought to occur through leukocyte integrin- mediated adhesion pathways (3,4). Now alternatively, or in concert, an induced increase in the affinity of the E-selectin lymphocyte ligands during rolling interactions appears also to play a key role. These increased affinities result in slower lymphocyte-endothelial cell rolling speeds (averaging 30% lower than controls), and slower rolling speeds may be required to maximally engage lymphocyte integrins to arrest the cells. Because the EL-246 anti-L- and E-selectin mAb blocks all rolling interactions, there is likely no significant integrin contribution to initial rolling or chemokine-reduced rolling speeds in the E-selectin transfectant system. The reduction of rolling speeds via alteration of selectin binding avidity represents a new mechanism for regulation of lymphocytes. This adds a new event to the already complex selectin, α4 integrin, ß2 integrin adhesion cascade.

IL-1-activated HUVEC also avidly support rolling and limited arrest of normal lymphocytes. Upon IP-10 chemokine infusion into the recirculating shear system, lymphocytes exhibit slowed rolling and greatly enhanced arrest. The rolling speeds of lymphocytes are reduced here by a factor of three indicating participation of an additional adhesive interaction and/or through increased selectin avidity upon IP-10 chemokine treatment. Lymphocytes in this system display the full lymphocyte three-step adhesion

cascade as demonstrated for lymphocytes homing to PP *in vivo*.

The effects observed for IP-10 infusion and PMA infusion, mirror those we have reported for *in vivo* lymphocyte binding to PP-HEV (Chapter 1). There, the normal lymphocyte population, similar to the effects of IP-10 treatment, show only a low level of efficiency (30%) for arrest (>5 sec) on the HEV. However, like PMA pretreatment of lymphocytes infused *in vivo*, the PMA-treated lymphocytes *in vitro* arrested far more efficiently on the endothelium suggesting a highly efficient activation of lymphocyte integrins is necessary to alter the bulk of the lymphocyte population. It may be that, under inflammatory conditions, lymphocytes need more than one chemokine signal or a solid state presentation of the chemokine to induce rapid arrest (17). In any case, the chemokines, as we have tested them, fail to induce the rapid transition from rolling to arrest induced by PMA treatment.

We plan to expand the scope of these observations in a new study to examine the effects of multiple cytokine combinations and varied time courses of *in vitro* endothelial cell activation. In initial experiments, we have examined the combined effects of IL-4 and IL-1 activation. Our initial observations show that twenty-four-hour IL-4, followed by four-hour IL-1, pretreatment of HUVEC results in control lymphocyte interactions that include both slow rolling and high levels of arrest. HUVEC treated in this regiment appear to not only present the necessary adhesion molecules for arrest, but also efficiently present "chemokine" signaling for the arrest of a large percentage of control lymphocytes.

This study shows that the adhesion cascade for lymphocyte-endothelial cell binding can be fully explored *in vitro*. Using the closed-

loop capillary tube recirculation system, we have demonstrated the lymphocyte three-step adhesion model. The further evaluation of the individual components of human lymphocyte-endothelial cell recruitment can now be examined under a variety of controlled inflammatory conditions. This model can be effectively used to examine the modification of lymphocyte-endothelial adhesive interactions by new chemical activating factors and inhibitors, chemokines, cytokines, peptide inhibitors, and mAbs at each step in the recruitment process. Such evaluations will likely lead to the development of new clinical approaches to treatments for T-cell sensitive disease pathology.

Summary

Lymphocytes participate in immune responses at acute inflammatory sites. The mechanisms regulating their recruitment are only partially understood. This study examines *in vitro* the participation of adhesion molecules and chemokine signaling in the recruitment process. Our examination of the IP-10 chemokine in this study shows that it alters the avidity of lymphocyte rolling. This, apparently occurs through alteration of selectin interactions, as mAbs against only E-selectin block both IP-10 treated and control lymphocyte rolling on E-selectin transfectants. The slowing of lymphocyte rolling via chemokine alteration of selectin-mediated avidity represents a new mechanism for slowing lymphocytes which may improve the efficiency of integrin binding. On IL-1-activated HUVEC, both anti-L-

and E-selectin mAbs contribute to blocking control and IP-10-slowed rolling. This blocking however is not complete since residual rolling still occurs. The residual rolling is likely mediated by lymphocyte α4 integrins. The L-selectin contribution to rolling seen on HUVEC suggests an additional adhesive interaction beyond that observed with E-selectin on the transfectants. E-selectin mediates a part of this interaction the remaining portion of the interaction has yet to be characterized. In addition to enhancing lymphocyte rolling on IL-1-activated HUVEC, IP-10 enhances the levels of lymphocyte arrest. The accumulation of arrested cells is gradual and falls far below the levels induced by PMA. PMA induces rapid lymphocyte arrest from rolling on IL-1-activated HUVEC, demonstrating that it is possible to observe the three-step adhesion cascade in the *in vitro* capillary flow system. Therefore, chemokines do not appear to be the key signaling molecules responsible for inducing rapid lymphocyte arrest at sites of inflammation. The search for rapidly acting mediators of lymphocyte arrest is still open.

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CHAPTER 5

IN VIVO AND IN VITRO FUNCTIONAL EXAMINATION OF A CONSERVED EPITOPE OF L- AND E-SELECTIN CRUCIAL FOR LEUKOCYTE–ENDOTHELIAL CELL INTERACTIONS

Introduction

Selectins are a family of cell surface adhesion proteins that regulate entry of leukocyte into lymphoid tissues and sites of inflammation (1, 2). Three members of this family have been identified: E-selectin and P-selectin (originally called ELAM-1 and GMP-140/PADGEM, respectively) are inducible endothelial cell-surface proteins that support adhesion of myeloid cells and certain subsets of lymphocytes (3-9). In contrast, the third member, L-selectin (also called MEL-14 antigen, gp^{90MEL}, LAM-1, LECAM-1, Leu-8, TQ-1, or peripheral lymph node homing receptor), is constitutively expressed by leukocytes, mediates lymphocyte adhesion to peripheral lymph-node high-endothelial venules by binding carbohydrate determinants on the peripheral vascular addressin (10, 11), and participates in neutrophil adhesion to cytokine-activated endothelial cells (12-14). In vivo, selectins appear to support the initial interaction of circulating leukocytes with endothelial cells

at sites of inflammation, as represented by the phenomenon of leukocyte rolling along the lumenal surface of the vasculature (15, 16). Several groups have presented evidence suggesting that leukocyte and vascular selectins may serve as receptor/counter-receptor pairs to mediate these interactions (17, 18); other evidence, including work from this lab, shows that additional endothelial selectin ligands are involved (19-21). In contrast, tight adhesion, aggregation, and transendothelial migration of the inflammatory leukocyte (all later steps in the extravasation process) are thought to be primarily mediated by another class of leukocyte adhesion proteins: the $ß_2$ integrins (22-30).

All three selectins consist of an N-terminal C-type lectin domain, an epidermal growth factor (EGF)-like domain, multiple short-consensus repeat (SCR) domains homologous to those found in complement regulatory proteins, a transmembrane segment, and a short cytoplasmic tail (4, 6, 31-34). The carbohydrate-binding lectin domains of each selectin are believed to be critical to the adhesive functions of these proteins. Other spatially distinct domains of the selectins may contribute to ligand recognition (31, 32). Antibodies against the mouse or human L-selectin EGF domain block lymphocyte adhesion to HEV, but have little effect on binding of the mannose-6-PO_4-rich mannan PPME, which is mediated by L-selectin (35, 36). Studies of chimeric L-selectin/immunoglobulin constructs suggest that the SCR domains provide structural conformation necessary for efficient adhesive function (37).

We have recently described the generation and characterization of a novel anti-selectin mAb that recognizes a unique epitope common to both

human L- and E-selectin which is also conserved in a number of animals, including goats, sheep, cattle, and pigs (38). Preliminary mapping of the EL-246 epitope suggests that it is encoded by or requires some portion of the SCR domain, since replacement of this domain in L-selectin with the SCR domain from P-selectin abrogates expression of the epitope while a variety of other epitopes are maintained. Our initial study of EL-246 suggested that it may be possible to simultaneously inhibit the function of two distinct adhesion proteins thought to be important in initiating inflammatory responses *in vivo*. Therefore, in this study we have extended our functional analysis and specifically evaluated the effects of EL-246 on 1) E-selectin-mediated neutrophil rolling on HUVECS stimulated with PMA or IL-1, 2) neutrophil L-selectin interactions with E-selectin cDNA transfectants, 3) lymphocyte homing to lymphoid tissues *in vivo*, and 4) binding of E-selectin cDNA transfectants to carbohydrate determinants on HEV. The binding of neutrophils to E-selectin cDNA transfectants can be blocked by treating either cell type alone. EL-246 completely inhibits the capacity of E-selectin to support neutrophil rolling, mediate lymphocyte adhesion, and bind ligands expressed on mouse peripheral lymph node HEV. EL-246 also inhibits the capacity of lymphocytes to enter peripheral lymph nodes *in vivo*. Thus, EL-246 is as effective as other anti-selectin mAbs in blocking cell-cell interactions mediated by E- or L-selectin; but, importantly, unlike any other blocking anti-selectin mAb, EL-246 recognizes and blocks both molecules.

Materials and Methods

Animals.

BALB/c mice, ranging in age from 6-12 weeks, were used as a source of cells and tissues. The mice were housed in the small animal facility at Montana State University. One- to three-month-old calves, which were housed in the MSU large animal facility, were used as sources of peripheral blood.

Leukocyte cell suspensions.

Leukocytes were harvested from the peripheral blood of humans and cattle as previously described (38). Briefly, blood was collected into citrate anti-coagulant tubes, diluted 1:2 with warm HBSS, underlayed with Histopaque 1077, and centrifuged at 2,300 RPM for 30 min at room temperature. Mononuclear cells were collected from the Histopaque/plasma interface. The pellet, which included RBCs and neutrophils, was resuspended to its original volume in HBSS, and the neutrophils were isolated by Dextran sedimentation. Residual RBCs in both the mononuclear cell and neutrophil preparations were lysed by hypotonic treatment.

Monoclonal antibodies.

EL-246, whose epitope is the subject of extensive characterization in this

paper, is a unique mouse IgG_1 mAb recognizing a xenogeneically expressed structure in the SCR domain of L- and E-selectin and blocks their adhesive functions in many mammalian species. Production and initial characterization of EL-246 was discussed in an earlier report (38). Leu-8 (purchased from Becton Dickinson) and the DREG series of mAbs (DREG 56, DREG 200, and DREG 152), which are mouse IgGs shown to recognize human L-selectin (22, 35), were used in the flow cytometric and Western blot analysis described below. Leu-8 was used as a phycoerythrin (PE) conjugate, and the DREG mAbs were used unconjugated, followed by an appropriate second stage, or used as FITC-conjugates. Other mAbs, 60.3 (a gift from J. Harlan), and R15.7 (a gift from R. Rothlein) both anti-human CD18, WAPS 12.2 (anti P-selectin, a gift of R.A. Warnock and E.C. Butcher), SH43 (mouse anti-sheep platelet IgG_1, M.A. Jutila, unpublished), and EL-81, a nonblocking mouse anti-E-selectin IgG_1, were used to assess integrin participation in binding or as negative controls in many of the experiments described below. The EL and DREG mAbs were partially purified by ammonium sulphate precipitation of serum-free supernatants.

Immunofluorescence staining and flow cytometric analysis.

Immunofluorescence staining of leukocytes was carried out as described (38-40). Briefly, $1x10^6$ cells were initially incubated in 2% rabbit serum for 10 min on ice to block Fc receptors. The cells were washed and then incubated with primary antibody at 50 μg/ml (or undiluted culture supernatant) for 20 min on ice. After washing, bound antibodies were revealed by incubation with

PE- or FITC-conjugated F(ab)'2 goat anti-mouse Ig (Tago, Burlingame, CA) at a 1:80 dilution in 5% FBS in DMEM. Flow cytometric analysis was performed on a FACScan (Becton and Dickinson, Mountain View, CA) as described (38-40). For two-color analysis, PE-conjugated Leu-8 (Becton Dickinson) or a FITC-conjugated DREG mAb were used in combination with EL-246. The cells stained with second stage were treated with 10% mouse serum to block any available anti-mouse Ig binding sites, and negative control mouse mAbs were used to evaluate the level of background staining. Data were collected from 10,000-50,000 cells and are presented as histograms or contour plots.

Peripheral lymph node HEV binding assay.

The *in vitro* assay of lymphocyte binding to HEVs in frozen sections (38) has been extensively described (recently reviewed in 10). Mouse pre-B L1/2 cells, transfected with E-selectin cDNA or vector cDNA, were the same as those used in our earlier report (38). E-selectin cDNA-transfected L1/2 cells have been shown to specifically bind mouse peripheral lymph-node HEV (40). That observation is repeated here. Mouse L1/2 cells expressing functional human E-selectin and the non transfected control parent line were resuspended in cRPMI at $1x10^7$ cells/ml and 100 µl added to 10 µm sections of mouse peripheral lymph node and the HEV binding assay was done as described (38). Cell binding was quantified by first identifying HEVs in each field by their characteristic autofluorescence or unique plump morphology and then counting cells bound to HEV, as described (38). Data were calculated as number of cells bound per individually scored HEV. The effect

of antibody treatment on the transfectants was compared to medium alone.

Neutrophil-E-selectin cDNA transfectant binding assay.

L-cells, stably expressing human E-selectin cDNA (80% E-selectin-positive determined by flow cytometry), as previously described (38), were grown on plastic 8-well Lab Tek slides (Miles Scientific). Neutrophils isolated from human peripheral blood were resuspended at $1x10^6$ cells/ml in cRPMI and 400 µl added to the wells of the transfected L-cell cultures. The neutrophils were allowed to adhere at room temperature for 15 min under constant rotation, as previously described (38). After the incubation, the medium in each well was aspirated, slide chambers were removed, and the slides placed in a coplin jar with 1.0% glutaraldehyde in HBSS. Adhesion was measured by counting the number of neutrophils/L-cell. The effect of mAb treatment on the neutrophil was determined as follows: In all experiments, the neutrophils were pre-coated with 10% rabbit serum to block available Fc binding sites and then treated for 20 min on ice with saturating concentrations of EL-246, DREG 56, or a negative control MAb (10-50 µg/ml); the cells were then washed and added to the L-cell transfectants, as described above.

Activated human umbilical-vein endothelial cell (HUVEC) and E-selectin cDNA transfectant-mediated neutrophil rolling.

Freshly isolated human umbilical-cord endothelial cells, which were Factor

VIII and LDL-receptor positive [cultured in endothelial-cell growth media (Clonetecs, EGM)] or E-selectin cDNA transfectants were grown to confluency on the internal surface of sterile glass 1.36 mm diameter capillary tubes (Drummond Scientific, Broomall, Penn) at least 24 hrs prior to shear experiments. Four hrs prior to the assay, the endothelial cells were treated with PMA (50 or 100 ng/ml) or IL-1 (1unit/ml), to induce E-selectin expression (determined by flow cytometry analysis, data not shown). Tubing was attached to the ends of the glass capillary tube to form a closed loop in which media and cells could be recirculated; the tube was then mounted on a Nikon inverted microscope. Using a variable speed peristaltic pump, flow was regulated to simulate *in vivo* shear conditions. The circulation loop allowed multiple infusions, via an injection port, of various mAbs during the continuous recirculation of leukocytes across the interactive surface of HUVECs or E-selectin cDNA transfectants. The inverted microscope-video capture system, employing a mechanical stage, allowed a survey of the entire length of the HUVEC or E-selectin transfectant monolayers and high resolution phase contrast recording of the interactive field for subsequent analysis. Human neutrophils were isolated from peripheral blood and separated on Ficoll in sterile Ca^{++}, Mg^{++}-free, hepes (20mM) buffered DMEM (pH 7.0) and placed on ice until the time of the experiment. Mouse neutrophils were isolated from BALB/c femurs using the same media and maintained under the same conditions as the human neutrophils. Both mouse and human neutrophils were infused into the system at a $1x10^7$ cell/ml concentration in sterile Ca^{++}, Mg^{++}-containing hepes buffered (20mM) DMEM (pH 7.0) plus 2% FBS. The rolling interaction, which did not

occur on unactivated endothelial cells or mock transfected L-cells, was observed at a shear rate of 2 dynes/cm^2 (with a center line velocity of 10.1 mm/sec). Rolling was established and continuously monitored for at least 5 min while being videotaped; during that time, either an isotype-matched control antibody, DREG 56, DREG 200, WAPS-12.2, or EL-246 at a concentration of 50 μg/ml was infused into the recirculating loop. The leukocyte/ endothelial-cell interactions were observed and videotaped for an additional 10 min. The number of neutrophils rolling on the activated endothelial cells at 10-30 sec intervals before and after the injection of mAb was determined by individual frame analysis of the recording. Data were recorded as the number of rolling cells within the field of view vs time.

Xenogeneic lymphocyte in vivo homing assay.

Bovine lymphocytes were isolated from peripheral blood, washed, suspended at $1x10^7$ cells/ml in HB101, and labeled with FITC as described (22, 24). The FITC-labeled lymphocytes were washed in HBSS, resuspended at $1x10^8$ cells/ml, and 0.5 ml of the cell preparation injected into the lateral tail vein of 6-to-12-week-old BALB/c mice. After 3-4 hrs, the animals were sacrificed and Peyer's patches, mesenteric lymph nodes, peripheral lymph nodes, spleen, and peripheral blood were collected. Single cell preparations were made from each tissue, RBCs in the peripheral blood were lysed by hypotonic shock, and then cells were analyzed by flow cytometry to quantify the capacity of the transferred lymphocytes to enter lymphoid tissues of the

mouse, as previously described (24). The percentage of transferred cells in each tissue was determined. Only experiments having control values of 0.2% or greater were used to quantify the effects of the mAb treatments. The effect of antibody treatment of the infused lymphocytes was then compared to medium and antibody controls.

Results

EL-246 blocks neutrophil "rolling" on activated human umbilical cord endothelial cells (HUVECs).

The adhesive rolling interactions of circulating leukocytes with endothelium are a critical step in the recruitment of immune cells during the inflammatory process, and are best studied under dynamic conditions simulating physiological shear stress of the vasculature. We tested whether EL-246 could inhibit the ability of activated endothelial cells to support neutrophil rolling under physiological shear forces. HUVECs were grown on the internal surface of sterile glass capillary tubes and induced to express E-selectin, as described in Materials and Methods. Using an assay that measures leukocyte interactions with ligands under constant shear, we examined 1) whether human and mouse neutrophils would roll on monolayers of activated human endothelial cells under conditions of this assay, 2) whether EL-246 could specifically block any rolling event as compared to antibodies directed against L-selectin, and 3) how the effects of EL-246 compared to the anti-P-selectin and anti-CD18 blocking mAbs WAPS 12.2, and 60.3, respectively.

Under controlled shear conditions, we found that activated HUVECs were quite effective at supporting both human and mouse neutrophil rolling. It appears that there has been significant conservation of E-selectin-dependent neutrophil adhesion across species, as mouse neutrophils roll in nearly equivalent numbers as human neutrophils on PMA-stimulated HUVECs (Figure 21). To test the effect of EL-246, a rolling interaction between isolated human or mouse neutrophils on PMA-activated HUVECs was established and then EL-246 was infused into the closed loop system. The number of neutrophils rolling on the endothelial cells was determined before and after the infusion of EL-246 by analyzing individual frames of the video-recording over a 10 min observation period. Figure 21 shows a plot of the number of cells rolling on the activated endothelial cells versus time. Within 90 seconds after the injection of EL-246, greater than 75% of the rolling interaction was blocked in both human and mouse systems, and by 4 min the blocking was complete. It should be noted that the EL-246 blocking of mouse-neutrophil rolling on HUVECs is completely due to its effect on E-selectin on the endothelial cells, since EL-246 does not interact with the mouse cells.

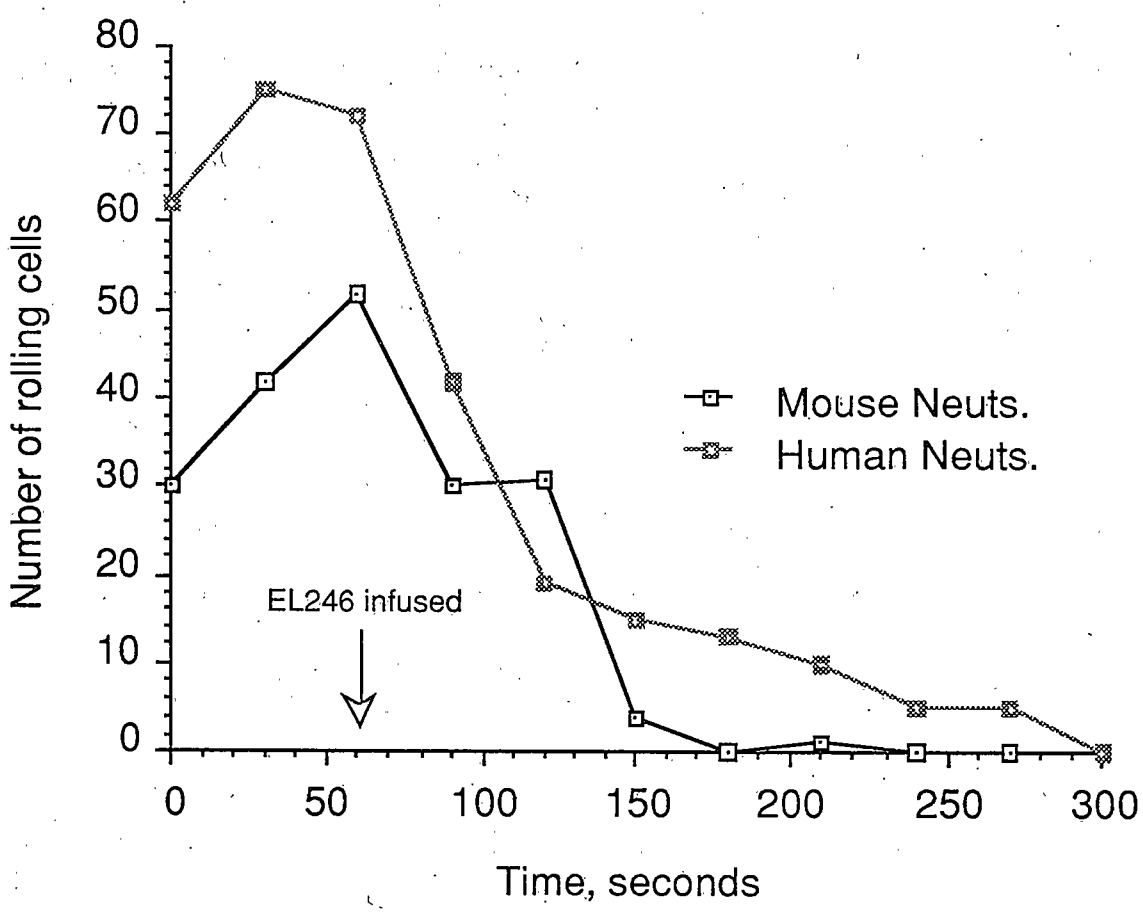


Figure 21. EL-246 blocks both human and mouse neutrophil rolling on activated endothelial cells, shown as data from a typical experiment which has been done in triplicate. The effect of EL-246 on the capacity of neutrophils to roll on activated endothelial cells was analyzed in the *in vitro* closed-loop assay as described in Materials and Methods. A rolling interaction was established and then EL-246 was injected into the system. The number of rolling neutrophils within the microscopic field of observation was quantified over time by analysis of individual frames from the videotape recording of the interaction. Similar loops injected with medium alone exhibited slight increases in the number of rolling neutrophils over the time period of the assay (data not shown).

An isotype-matched control mAb had no effect on neutrophil rolling behavior, whereas subsequent addition of EL-246 completely inhibited the rolling interaction (Figure 22a). Figure 22b follows the onset of neutrophil interactions from the initiation of rolling to several minutes after infusion of either a control isotype-matched mAb or the DREG 200 anti-L-selectin mAb. It was observed that in the control group, the number of rolling cells continued to increase, while neutrophil rolling declined in response to Dreg 200 infusion, though not as rapidly as that seen with EL-246. Thus, blocking of L-selectin alone can affect neutrophil rolling in this assay.

Examination of neutrophil rolling on 4hr IL-1-stimulated HUVECs in comparison to that observed on 4hr PMA-stimulated HUVECs showed a similar frequency in numbers of rolling cells (Figure 22a vs. Figure 23), suggesting a similar rolling mechanism. The effects of WAPS 12.2 (blocking anti-P-selectin mAb) and EL-246 were then tested. The results clearly showed that WAPS 12.2 had no effect on neutrophil rolling (Figure 23). This suggested that P-selectin did not contribute to leukocyte rolling under these conditions. In contrast, in the same assay, EL-246 completely blocked neutrophil rolling on IL-1-stimulated HUVECs within 90 seconds of the time of infusion in the closed loop.

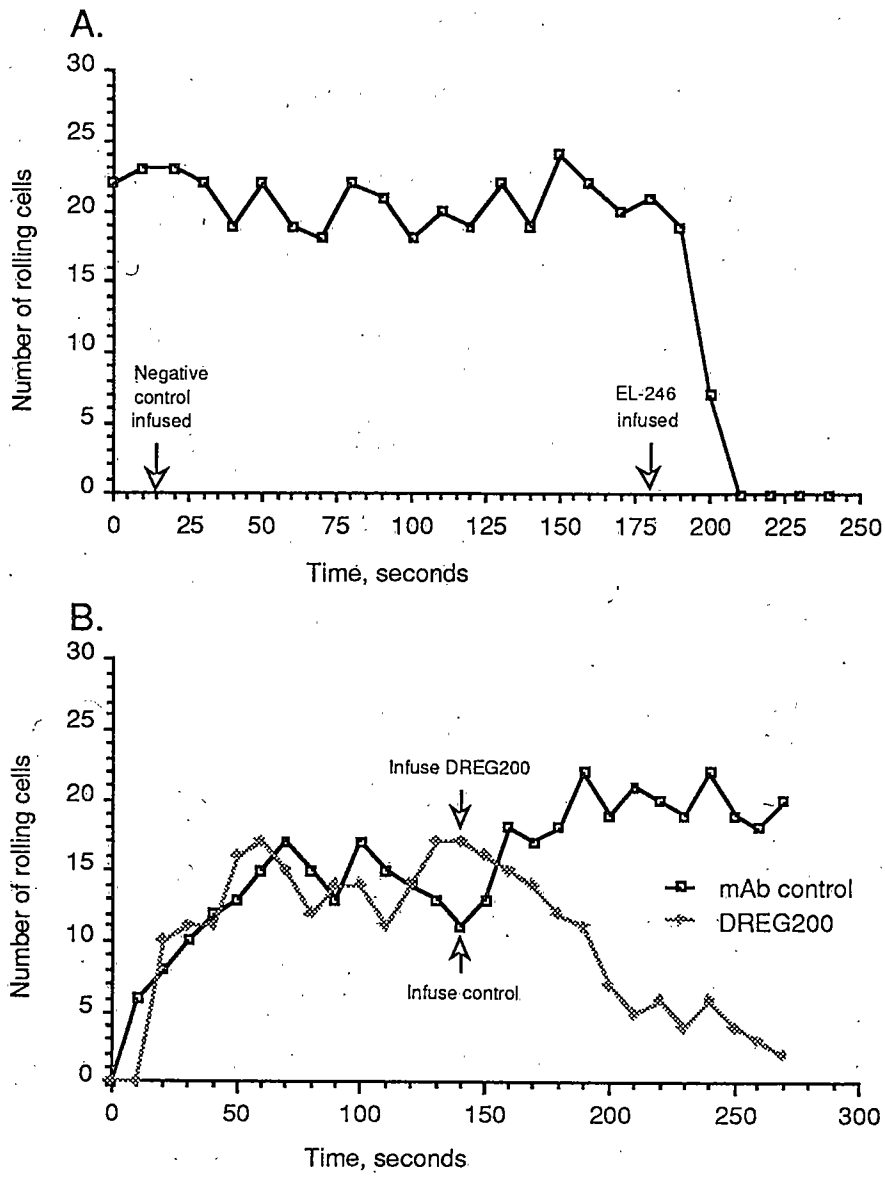


Figure 22. DREG 200 or a class-matched antibody control are compared to EL-246 for blocking of rolling on PMA-stimulated HUVECs, as demonstrated in these typical experimental repeats. As in Figure 21, human neutrophils were allowed to establish rolling interactions with PMA-stimulated HUVECs, except an isotype-matched control mAb was injected and its effect on rolling was analyzed over a 150-second time interval. After 180 seconds, EL-246 was infused in the same loop (2a). The negative-control mAb had no effect on

established rolling, but the same interaction was completely inhibited by EL-246. In separate experiments run over the same time course, the effect of DREG 200 was compared with control isotype-matched mAb (2b).

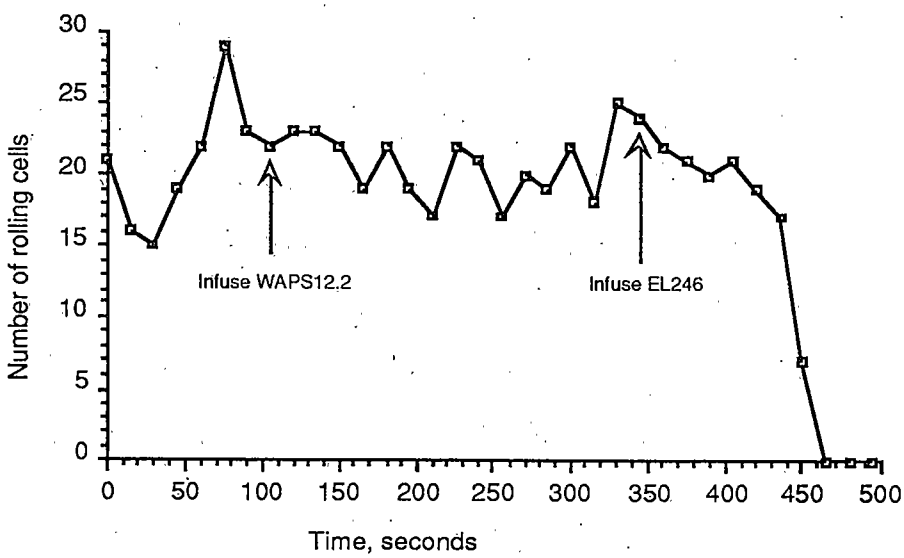


Figure 23. Human neutrophil rolling on IL-1-stimulated HUVECs is blocked by EL-246, but not by WAPS 12.2. HUVECs were stimulated with 1 unit/ml IL-1 for 4hrs before freshly isolated human neutrophils were infused into the closed loop in this typical experiment. Neutrophils rolled in numbers very similar to those observed for PMA-stimulated HUVECs. WAPS 12.2 and EL-246 were injected sequentially into the loop system and their effect on the rolling interaction was monitored over time.

It was reproducibly observed that by 15 min of neutrophil interactions on IL-1-treated HUVECs large numbers of these cells had completely arrested on the HUVEC monolayer. EL-246 alone, while effectively stopping rolling interactions (Figure 21, 22A, 23), had no effect on reversing the static adhesion of these bound neutrophils to HUVECs (Figure 24). However,

treatment of the HUVEC-adherent neutrophils with the anti-CD18 mAb 60.3 effectively released the bound neutrophils over a period of minutes. The presence of EL-246 and 60.3 in the closed loop prevented any additional neutrophil rolling or sticking (Figure 24). These results indicate that L-selectin, E-selectin, and CD-18 are responsible for the primary rolling and sticking interactions of neutrophils with activated HUVECs in this model.

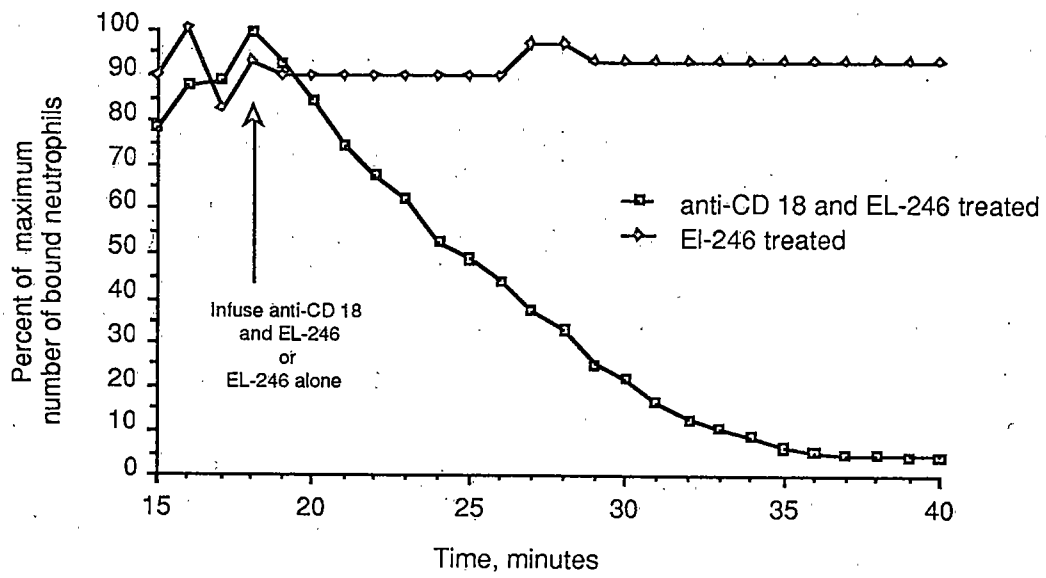


Figure 24. Reversal of neutrophil tight adherence to IL-1-stimulated HUVECs by infusion of the anti-CD18 mAb 60.3, but not by EL-246 under shear. Human neutrophils were allowed to roll and arrest on stimulated HUVECs for 18 minutes prior to infusion of 60.3 mAb and EL-246 or EL-246 alone. The data presented are from a typical representative experiment.

Pretreatment of neutrophils with EL-246 blocks their ability to bind E-selectin cDNA transfectants.

In a previous report (38), we showed that pretreatment of E-selectin cDNA-transfected fibroblasts with EL-246 blocks (>90%) the ability of the

transfectants to bind neutrophils, indicating that binding in this assay is completely dependent on E-selectin. Here we examined the issue of E-selectin and L-selectin serving in the same adhesion pathway (17, 18), by testing if neutrophil binding to E-selectin could be inhibited by only treating L-selectin on the neutrophil. Peripheral blood neutrophils were incubated with saturating levels of EL-246 on ice for 20 min, washed, and then added to cultures of mouse L-cells transfected with human E-selectin cDNA. The effect of EL-246 on binding was evaluated and compared with an anti-L-selectin mAb (DREG 56) and two negative isotype control mAbs (anti-CD45, which stains leukocytes, and EL-81 a nonblocking anti-E-selectin, mAb). As shown in Figure 25, EL-246 neutrophil pretreatment blocked adhesion by 64%, DREG 56 inhibited by 53%, and the negative-control mAbs had little effect.

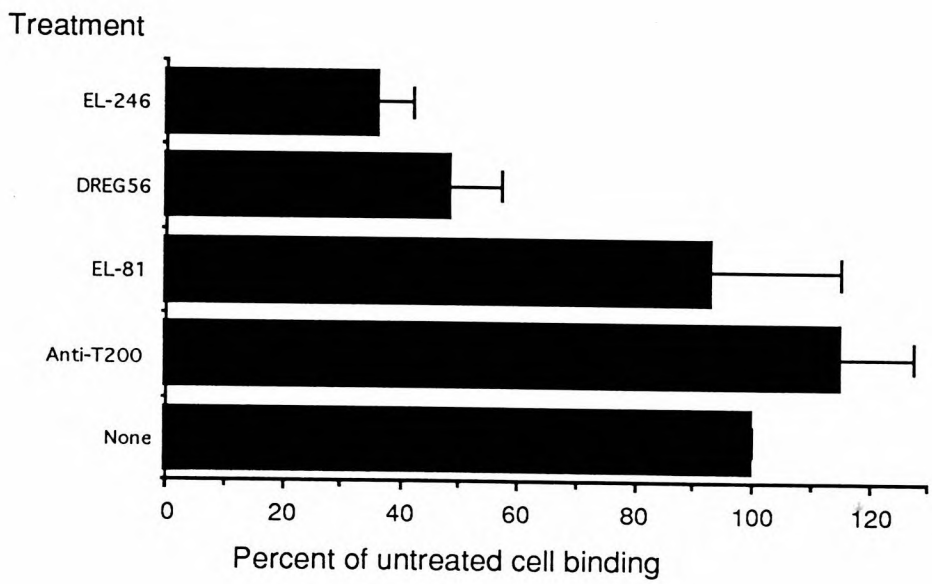


Figure 25. Pretreatment of neutrophils with EL-246 blocks their ability to

bind E-selectin transfectants. Neutrophils were pretreated with the indicated antibodies at 50 ug/ml concentrations for 20 min on ice, washed extensively to remove unbound mAb, and then added to the E-selectin transfectants. The binding assay was performed as described in Materials and Methods, and the effect of the antibody treatments quantified and recorded as percent of control (untreated) cell binding. Values represent means +/- S.D. of 8 values from 2 separate experiments.

During the course of these experiments, we observed that EL-246 was lost from the surface of pretreated neutrophils following exposure to E-selectin transfectants. To determine what happens to the antibody, neutrophils were saturated with EL-246 at the beginning of the assay (Figure 26A) and co-incubated with E-selectin transfectants for 15 min. After the incubation period, no EL-246 antibody could be detected on the neutrophil cell surface as visualized by addition of a FITC-labeled anti-mouse 2nd stage antibody (Figure 26B). Since L-selectin can be shed from the surface of leukocytes (39), we tested whether the loss of EL-246 antibody on the neutrophil during the binding assay was due to shedding of the molecule. Interestingly neutrophils that no longer had EL-246 on their cell surface after the assay (Figure 26B) still stained brightly with a second anti-L-selectin mAb (DREG56) directed against another L-selectin epitope, indicating that L-selectin was not shed from these cells (Figure 26C). To determine whether the EL-246 mAb was transferred from L-selectin on the neutrophils to E-selectin on the E-selectin transfectants, the transfectants were reacted with FITC-labeled anti-mouse IgG second-stage antibody to visualize the mouse EL-246 mAb. High levels of EL-246 were detected on the surface of the E-selectin transfectants following co-incubation with EL-246 pretreated

(Figure 26E). Transfectants that were not co-incubated with EL-246 pretreated neutrophils did not react with the FITC-labeled second-stage antibody (Figure 26D). The level of fluorescence intensity of the transfectants exposed to EL-246-treated neutrophils followed by second stage (Figure 26E) was comparable to the level of fluorescence obtained after a conventional indirect stain of the E-selectin transfectants using EL-246 (Figure 26F). These results showed that when neutrophils are pretreated with EL-246, the mAb apparently transfers from L-selectin on the neutrophil surface to E-selectin on the L-cell transfectants. We have recently shown a similar result with endothelial-cell E-selectin (data not shown) implying that EL-246 may have a higher affinity for E-selectin. Thus, in this assay EL-246 likely blocks at the level of E-selectin even though only neutrophil L-selectin is pretreated with EL-246.

EL-246 blocks neutrophil "rolling" on E-selectin cDNA transfectants more effectively under shear than either anti-L or E-selectin monoclonal antibodies alone.

Abbassi et al. (41) have recently shown that the E-selectin cDNA-transfected L-cells support shear-dependent rolling of neutrophils which is blocked by either anti-L- or E-selectin mAbs. We reproduced those findings in our capillary tube system and compared the relative blocking efficiencies of EL-246 versus anti-L- and -E-selectin specific mAbs.

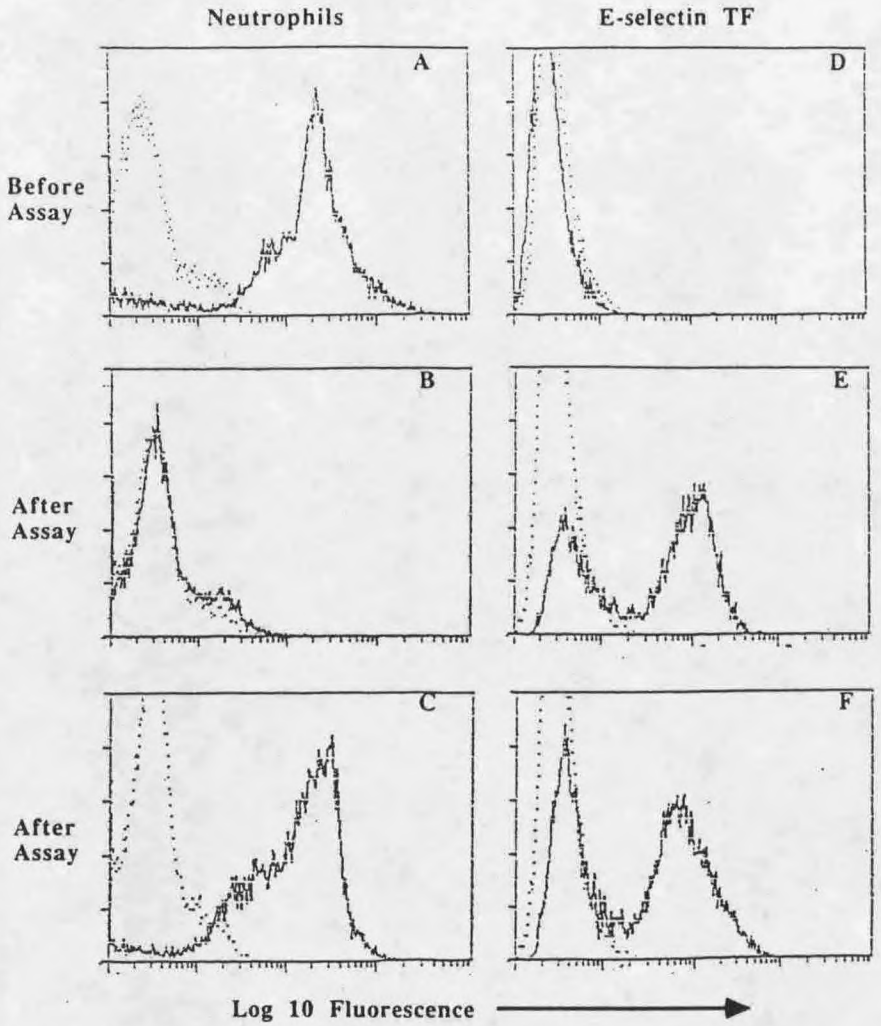


Figure 26. EL-246 transfers from the neutrophil to the E-selectin transfectants during the binding assay. The neutrophils used in the experiments for Figure 25 that were pretreated with EL-246 and then washed, were analyzed for the presence of EL-246 on their cell surface before (Figure 26A) and after the assay (Figure 26B) by addition of FITC-labeled anti-mouse Ig second stage followed by flow cytometric analysis. Cells depicted in Figure 26C were stained by conventional methods with a second anti-L-selectin mAb (DREG56) to test for the presence of L-selectin on their cell surface. E-selectin transfectants were also stained with the FITC-second-stage antibody before (Figure 26D) and after co-incubation with EL-246-treated neutrophils (Figure 26E), to test whether EL-246 was transferred from the neutrophil surface to the E-selectin transfectants. Figure 26F shows the results of a conventional

indirect stain of the transfectants for E-selectin. The dotted lines in each histogram represent background fluorescence after staining with negative control antibodies.

One anti-E-selectin (EL-112) and two anti-L-selectin (DREG 56 and DREG 200) mAbs were tested for the rapidity with which they could block neutrophil-E-selectin-transfectant rolling as compared with EL-246 and a non-blocking anti-E-selectin mAb (EL-81). In a series of experiments where individual mAbs were compared (all at 50μg/ml) (Figure 27A), none of the mAbs directed against either L- or E-selectin alone could block rolling completely by the two-minute time point where EL-246 consistently blocked 100%. The anti-E-selectin nonblocking control had no effect on rolling in these assays (Figure 27A).

Sequential addition of anti-L-selectin and anti-E-selectin mAbs in the presence or absence of anti-CD18 mAb resulted in the inhibition of neutrophil rolling over time which followed very similar decay curves (Figure 27B). It should be noted that residual rolling that was not blocked by the anti-L-selectin mAb treatments was readily blocked by both EL-112 and EL-246 (Figure 27B). These results demonstrate that EL-246 blocks neutrophil rolling on E-selectin under shear more rapidly than anti-L- or E-selectin mAb alone, and more completely than the anti-L-selectin mAb at their maximum level of effect.

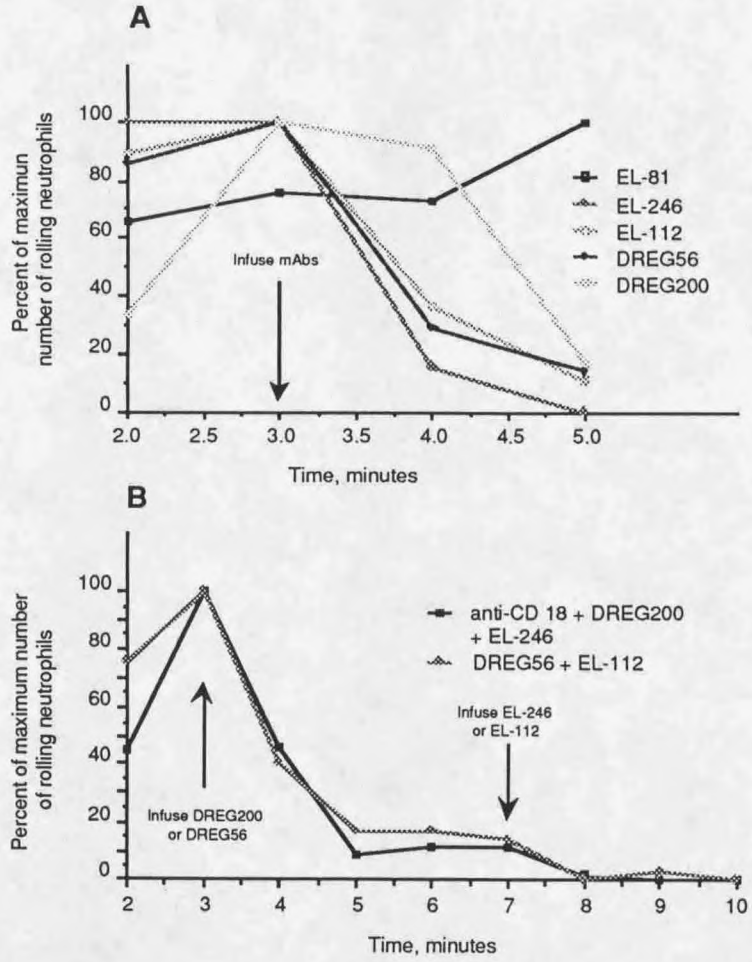


Figure 27. Infusion of anti-L- or anti-E-selectin mAbs alone blocks the rolling of neutrophils less efficiently than EL-246. Freshly isolated human neutrophils were infused into the recirculating loop and allowed to establish rolling interactions under flow. DREG 56 and DREG 200 (anti-L-selectin), EL-112 (anti-E-selectin) EL-81 (a nonblocking anti-E-selectin control) and EL-246 mAbs were infused at 50μg/ml (final concentration) in individual experiments after establishment of greater than 20 rolling neutrophils per field (panel A). The interactions were observed and recorded on videotape for off-line analysis. In panel B, rolling interactions were established as in panel A, either in the presence or absence of R15.7 (anti-CD18) which had no effect on the rolling interaction. Infusion of DREG 56 or DREG 200 partially

blocked the rolling interactions, which were allowed to stabilize for four minutes before the addition of EL-112 or EL-246.

EL-246 blocks the ability of E-selectin cDNA transfectants *in vitro* and lymphocytes *in vivo* to localize in peripheral lymph node HEV.

To test the ability of EL-246 to block binding of E-selectin transfectants to a ligand distinct in function and specificity to L-selectin and other possible E-selectin ligands expressed on neutrophils, we examined peripheral lymph node adhesive interactions. Berg, Robinson, and colleagues have recently shown that there are overlapping binding specificities between L- and E-selectin (40). Both molecules bind the same carbohydrates (42-46), and, interestingly, E-selectin transfectants avidly adhere to peripheral lymph node HEV--a molecular interaction originally thought to be unique for L-selectin (26). Blocking the interaction of the E-selectin transfectants with peripheral lymph node HEV would specifically determine if the EL-246 recognized epitope of E-selectin participated in this alternative ligand binding interaction, unique from E-selectin-neutrophil binding. Reproducibly, the mouse L1/2 lymphoma cell-line, transfected with human E-selectin cDNA, avidly bound to mouse peripheral lymph node HEV and in four experiments was completely blocked when the cells were pretreated with EL-246 (data not shown).

Based upon the success of the EL-246 blocking of E-selectin-transfectant binding to the HEV of frozen sections of peripheral lymph node, we used a xenogeneic homing model to test whether EL-246 was effective at blocking lymphocyte homing into peripheral lymph nodes in vivo. In other studies,

we have found that xenogeneic lymphocytes home with appropriate specificity to lymphoid tissues of mice in short-term homing assays. Earlier work has shown that selectin adhesion molecules and ligands participating in homing are highly conserved in several mammalian species (38, 42, 47, 48). As the assay is completed within 3-4 hrs, no complications related to xenogeneic responses are detected. This approach provides a powerful system to measure the homing capacity of lymphocytes from large animals in which homologous homing experiments are technically difficult due to the large number of cells required. We chose to examine bovine lymphocyte homing in the mouse because 1) EL-246 recognizes bovine L-selectin (38), 2) large numbers of cells can be easily obtained, 3) young animals (1-3 mo), in which virtually all circulating lymphocytes are L-selectin positive (as any animal matures the percentage of L-selectin-positive lymphocyte drops), can be readily used, and 4) the effects of medication and the health of the animals can be controlled.

FITC-labeled bovine lymphocytes treated with EL-246 or medium-alone were injected into identical mice and allowed to home for 3-4 hours (22). After the incubation, the animals were sacrificed and blood and various lymphoid organs collected. The percentage of FITC-labeled cells compared to unlabeled host cells was determined for each tissue and compared between the various treatments. Table 4 shows the combined data from 7 different experiments (except for the Peyer's patch, which was analyzed only 3 times). The percentage of FITC-labeled control lymphocytes found in the peripheral lymph node ranged from 0.2%-1.6%, comparing favorably with results obtained when the same number of homologous FITC-labeled mouse

lymphocytes were reinjected in the animal (data not shown). As expected, there was variability (indicated by high SEM) in the level of homing between experiments likely due to variability in cell preparations and experimental animals. However, EL-246 significantly blocked homing into the peripheral lymph node by 65%. EL-246-treated lymphocytes showed reduced trafficking to other tissues tested, including mesenteric lymph node, where homing is thought to be partially (≈50%) peripheral specific (49). The reduction of homing to other tissues that lack peripheral selectivity was not statistically significant, suggesting preferential, tissue-selective blocking by EL-246.

Table 4. EL-246 pretreatment of bovine lymphocytes blocks their ability to home to peripheral lymph nodes of the mouse.

Tissue	Percentage of host cells[a]		Percent blocking[b]	
	Control	EL-246		
PLN	0.40+/-0.14	0.14+/-0.05	65%	(n=7)
MLN	0.48+/-0.22	0.24+/-0.08	50%	(n=7)
PP	0.45+/-0.24	0.34+/-0.03	25%	(n=3)
Spleen	1.54+/-0.63	1.30+/-0.50	15%	(n=7)
Blood	0.66+/-0.32	1.10+/-0.55	No reduction	(n=7)

a. Values represent the percentage of FITC-labeled cells in 50,000 cells analyzed from each tissue and are means+SEM from the number of experiments indicated.

b. Percent blocking by EL-246, calculated as follows:100-(100x percentage of cells in tissues following EL-246 treatment/percentage of cells in tissues of controls). Controls were cells treated with medium alone and then injected into the mice.

The reduced homing after EL-246 treatment was not due to increased clearance of the treated cells from the circulation, since blood levels were the same in the two treatment groups (Table 4). We then examined the effect of an isotype-matched, negative-control antibody (DREG55), which does not cross react with bovine lymphocytes. Figure 28 shows representative flow-cytometric contour plots of the data collected from animals injected with medium-alone, DREG 55, and EL-246-treated FITC-labeled cells. Again, EL-246 blocked homing to the peripheral lymph node and slightly diminished accumulation in the spleen. DREG55 had no effect on the accumulation of cells in the peripheral lymph node; however, it affected accumulation in the spleen to the same extent as EL-246. Importantly, EL-246 blocked homing to peripheral lymph node by 70% in comparison to the effect of DREG55, even though there was twice the level of circulating EL-246-treated versus DREG55-treated cells in the test animals. These results show that EL-246 is an effective inhibitor of L-selectin in this *in vivo* model.

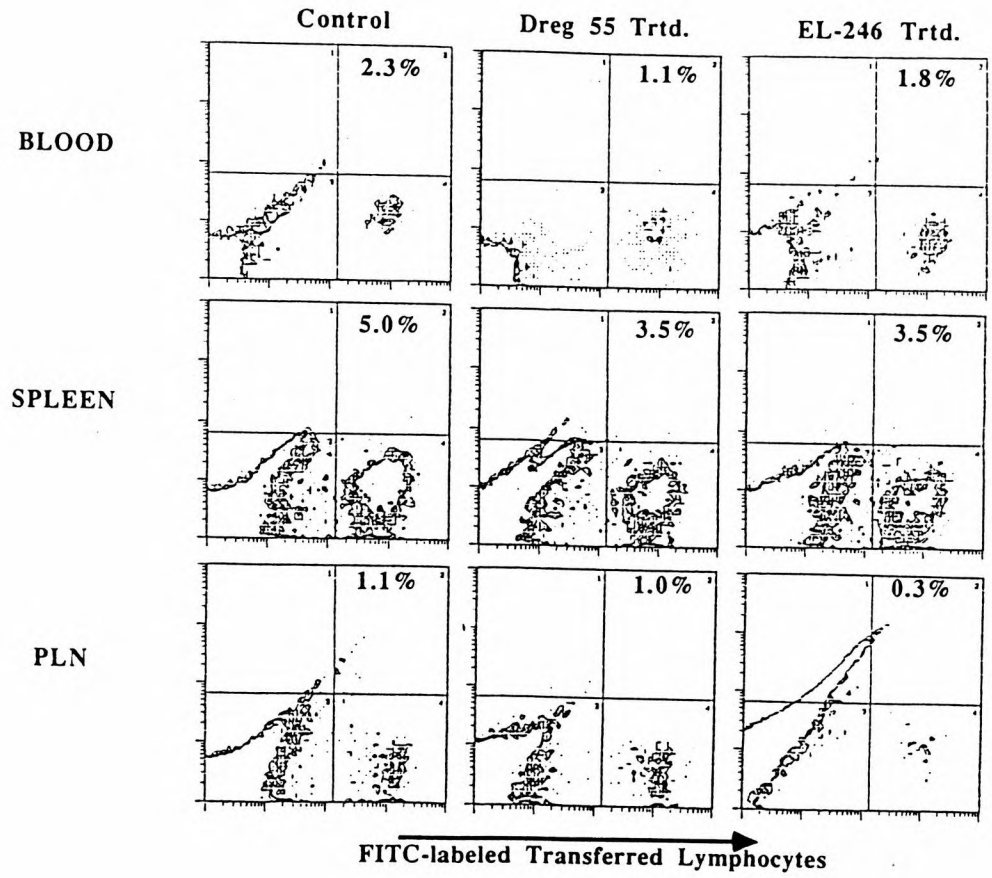


Figure 28. EL-246 specifically blocks the ability of bovine lymphocytes to home to mouse peripheral lymph nodes. The xenogeneic lymphocyte *in vivo* homing assay was done as described in Table 4, and the effects of EL-246 and a negative control antibody DREG 55 (DREG 55 is the same antibody isotype as EL-246, but does not recognize bovine lymphocytes) were evaluated by flow cytometry. The contour plots shown in Figure 28 represent the analyses of this experiments and report the percentage of FITC-labeled bovine lymphocytes that homed into spleen and peripheral lymph node following treatment with EL-246, DREG 55, or medium alone (control). 50,000 cells were analyzed for each time point and the threshold for the contour levels were the same in each plot. The quadrants were based on the upper limit of background fluorescence.

Discussion

The vascular and leukocyte selectins have been shown to mediate diverse cell/cell adhesive interactions thought to be important in regulating inflammatory responses and lymphocyte homing (reviewed in 50, 51). Some of these interactions include leukocyte rolling along vascular endothelium, lymphocyte adhesion to HEV (tissue-specific homing), neutrophil adhesion to cytokine-activated endothelial cells, and neutrophil and monocyte accumulation in sites of inflammation (51). Understanding the molecular basis for the diverse function of selectins is an important goal if novel inhibitors of these molecules are to be eventually exploited in the treatment of immune and inflammatory disease.

Antibodies are effective inhibitors of adhesion proteins and are currently being pursued as potential treatments for inflammation, such as transplant rejection (52, 53). Anti-selectin antibodies have only recently been produced and considered as novel therapeutics. Unlike other anti-selectin mAbs which recognize only one member of the family, EL-246 recognizes an unusual epitope common to two different selectins. Our preliminary analysis suggests that it effectively blocks the function of both L-and E-selectin. We extend those observations here and show that EL-246 is a powerful inhibitor of multiple cell/cell interactions mediated by either L- or E-selectin under shear conditions. EL-246 terminates both initial and subsequent continuous neutrophil rolling on both HUVECs activated with PMA or IL-1 and on E-selectin cDNA transfectants. EL-246, because it does not interact with mouse L-selectin, can block mouse neutrophil rolling on PMA-stimulated HUVECs in an E-selectin-dependent fashion, independent

of its L-selectin-blocking function. These results show the likelihood that complete and simultaneous inhibition of the function of two distinct and interacting selectin adhesion proteins important in regulating inflammation can potentially be attained by one reagent *in vivo*. Additionally, neutrophil L-selectin-mediated delivery of the EL-246 mAb to the endothelial cell surface, where it may be transferred to E-selectin, would facilitate blocking of other neutrophil E-selectin ligand interactions (20) by the use of this single mAb. The rapidity with which EL-246 inhibits rolling *in vitro* may, in fact, be a direct result of this simultaneous inhibition and/or the specific delivery of EL-246 from the neutrophil to the endothelial cell by L-selectin to E-selectin during the interaction, as discussed below.

Aside from being an effective blocker of selectin function *in vitro*, two other characteristics of EL-246 suggest that it may be uniquely potent *in vivo*. First, we recently found that high levels of shed L-selectin are found in the blood of normal, healthy adults (54). This result has been confirmed by Schleiffenbuam et al. (55) who have extended the observation and quantified the actual levels of soluble L-selectin at > 1μg/ml in normal serum. Therefore, most anti-L-selectin therapeutics will need to be given in sufficiently large quantities to saturate both circulating L-selectin and L-selectin on leukocytes to be effective at blocking inflammation *in vivo*. EL-246 does not readily recognize the shed form of L-selectin (T.K. Kishimoto and M.A. Jutila, unpublished observations); thus, only cell-surface antigen may need to be saturated *in vivo* for EL-246 to be effective. Second, is the finding that leukocytes may efficiently deliver EL-246 to sites that specifically express E-selectin. In assays where neutrophils were precoated with EL-246,

washed, and added to either E-selectin cDNA transfectants or cytokine-activated HUVECs, the mAbs were transferred from the leukocyte to E-selectin. Indeed, the amount of EL-246 on the E-selectin transfectants after transfer from the leukocyte, as measured by staining with FITC anti-mouse second stage followed by flow cytometry, was comparable to the amount after directly staining the transfectants with saturating levels of EL-246. This implies, not only that EL-246 may have a higher affinity for E-selectin, but also the existence of an intriguing and novel mechanism for targeting anti-E-selectin mAbs in vivo, bypassing the need to have high serum concentrations of the antibodies to saturate E-selectin expressed on endothelial cells. Thus, leukocytes may efficiently deliver EL-246 to inflamed endothelium *in vivo,* as saturating levels of EL-246 in circulation may not need to be maintained for the mAb to block *in vivo.*

In this report, we initiated our *in vivo* analysis of EL-246 by testing the effect of pretreating lymphocytes with EL-246 *in vitro* on their ability to home to lymphoid tissues after being reinjected into the animal. We used a xenogeneic *in vivo* homing model that we have recently developed for these experiments. We found that lymphocytes from humans, goats, sheep, and cows will home in a tissue-specific fashion to lymphoid tissues of the mouse after being injected intravenously. These results are not surprising, since earlier studies have shown that components of homing mechanisms can be highly conserved between mammals (38, 42, 47, 48). Human lymphocytes tested in this model, although showing tissue-specific lymphoid localization and apparent EL-246 blocking, accumulated in

inconsistent and small numbers making it not possible in this analysis to generate statistically significant data. Whether these low efficiencies reflect technical difficulties or actual differences in the efficiency of xenogeneic adhesion receptor-ligand interactions occurring after the initial selectin-mediated rolling has yet to be determined. However, this assay has proved to be a rapid efficient means of testing whether antibodies directed against selectins expressed on leukocytes from experimental animals can cause altered localization in vivo. In this assay, EL-246 blocks bovine lymphocyte entry into peripheral lymph nodes by 65%.

The most important test of the effectiveness of EL-246 *in vivo* will come from studies where the antibody is injected into animals, and the effect on either lymphocyte recirculation or, more importantly, inflammation is evaluated. In our earlier report, we showed that EL-246 cross-reacts in sheep, goats, cows, and pigs, but not smaller animals, such as rabbits (38). We have recently confirmed that activated endothelial cells (E-selectin) as well as leukocytes (L-selectin) are stained in pigs and sheep; and we are in the process of testing the effectiveness of EL-246 in preventing reperfusion injury and sepsis-induced inflammation in these animals. Preliminary experiments of reperfusion after ischemia in a sheep lung model show reproducible total protection (survival) of animals from neutrophil-induced inflammatory injury when EL-246 is introduced into the blood just prior to blood flow being reestablished into the occluded lung. Control animals receiving an isotope-matched non blocking mAb experienced severe lung necrosis and two thirds of animals failed to reach a six hour survival time that marked the end of the experiment.

To conclude, we have extended our analyses of this common L- and E-selectin functional epitope in several *in vivo* and *in vitro* assays of selectin function, examining adhesive interactions both statically and under physiological shear. We conclude that EL-246 is widely and uniquely effective in blocking E- and L-selectin-mediated cell/cell interactions. Additional characterization of the EL-246 antibody and its epitope may lead to important insight into the development of new pharmacologic agents that simultaneously inhibit the function of two different selectin adhesion protein pathways important in regulating inflammatory responses in vivo.

Summary

Selectins are a three-member gene family of carbohydrate-binding adhesion proteins found on the surface of leukocytes and endothelial cells central to inflammation-associated leukocyte recruitment and lymphocyte recirculation. E- and P-selectin are inducible and expressed on the surface of endothelial cells under inflammatory conditions while L-selectin is constitutively expressed on most circulating leukocytes. Previously, we have characterized a unique monoclonal antibody (EL-246) that recognizes a common epitope on both E- and L-selectin presented or determined by their short-consensus repeat domains. This report defines the functional properties of EL-246 and its' cognate epitope. In a novel, *in vitro*, physiological shear system, we show that neutrophil rolling on activated human umbilical-vein endothelial cells and E-selectin cDNA transfectants is blocked 45 to 120 sec after infusion of EL-246. The examination of the

binding of neutrophils to E-selectin cDNA transfectants reveals that their adhesion is blocked by EL-246 treatment of either cell type. A unique antibody transfer mechanism is demonstrated where El-246 is delivered uni-directionally from L- to E-selectin to surpass the adhesion blocked by mAbs recognizing either L- or E-selectin alone. Employing flow cytometry and in vivo homing techniques, we show that pretreating bovine lymphocytes with EL-246 blocks their ability to home to mouse peripheral lymph nodes by >65%. Cumulatively these results suggest that EL-246 is a uniquely potent pharmacologic inhibitor of leukocyte/endothelial-cell interactions mediated by either E- or L-selectin.

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CHAPTER 6

NEUTROPHILS ROLL ON ADHERENT NEUTROPHILS BOUND TO CYTOKINE-INDUCED ENDOTHELIAL CELLS VIA L-SELECTIN ON THE ROLLING CELLS.

Introduction

The recruitment of leukocytes to sites of acute inflammation is controlled by sequential interactions that eventually lead to migration of the cell from the blood into the affected tissue. Blood-borne neutrophils recognize the endothelium of the vascular bed within the inflamed tissue, stop on the lumenal vessel wall while within the blood flow, migrate between the endothelial cells lining the vessel, and accumulate in the extravascular tissue within approximately 10 min (1-7). Intravital microscopy shows that the initial contact between endothelial cells and leukocytes leads to a reversible adhesive interaction that results in rolling of leukocytes along the vessel wall. Members of the selectin family of C-type, lectin-like, adhesion proteins expressed on the plasma membrane of leukocytes (L-selectin) and inflamed endothelium (E- and P-selectin), help

regulate these rolling events and cause the leukocyte to slow below the rate of blood flow. If appropriate activating factors are presented on the endothelium or released from acute inflamed tissue during rolling, rapid signalling events will occur in neutrophils which lead to up-regulation of their adhesiveness. These events promote neutrophils to adhere tightly via activated integrins to the vascular endothelium, after which they migrate into the underlying tissue (8-12).

In vitro systems have been used to show that monolayers of activated endothelial cells, E- or P-selectin-transfected fibroblasts, or isolated vascular selectins support neutrophil rolling under shear (13-15). However, during the course of inflammation *in vivo,* inflammatory leukocytes rapidly accumulate on the vascular wall. This creates an irregular layer of adherent cells forming a barrier that prevents the interaction between newly arriving leukocytes and the inflamed endothelium. We demonstrate here that adherent neutrophils, like activated endothelial cells, can directly support neutrophil rolling, thus providing a mechanism for continued leukocyte recruitment throughout the inflammatory process.

Materials and Methods

Recirculating loop neutrophil/neutrophil HUVEC capillary-tube shear assay.

In experiments observing HUVEC-bound neutrophil/neutrophil rolling, freshly isolated or 2nd to 6th passage human umbilical-cord

endothelial cells (factor VIII and LDL-receptor positive) were grown to confluence (24-72 hrs) in endothelial cell growth media (EGM, Clonetics) on the lower interior wall of capillary tubes [1.36 mm internal diameter by 2 cm long, sterile, thin wall, borasilicate glass (Drummond Scientific)] after plating at $3x10^6$/ml The endothelial cells were then stimulated with IL-1 (10ng/ml)(Immunex) for 4 hrs at 37°C, followed by up to 2 hrs incubation in fresh IL-1-free EGM media (37°C). The IL-1-treated HUVECs expressed high levels of E-selectin [determined by flow cytometry (data not shown)] and were used in shear experiments at 4 to 6 hrs hrs post stimulation. Human blood leukocytes collected into sodium citrate-containing Vacutainers (Becton Dickinson) were separated on ficoll [Histopaque 1119, (Sigma)] at room temperature after dilution in DMEM [Ca^{++}, Mg^{++}-free (Irvine Scientific)]. Neutrophils from this isolation (a greater than 90% pure population by flow cytometry) were resuspended in Ca^{++}, Mg^{++}-containing DMEM [(Irvine Scientific) buffered with 20mM HEPES (Mediatech), (complete DMEM)] after centrifugation and infused via a sterile three way stopcock injection port at a concentration of $2x10^6$ cells/ml into a media prewashed recirculating silicone tubing loop [1.2mm internal diameter size 14, (#96400-14 Cole-Parmer)] in which the HUVEC-containing capillary tube was integrated. The capillary tube was then positioned upon an inverted microscope stage (Nikon Diaphot) maintained at 37°C and a variable speed peristaltic pump [with a Masterflex 6-roller pump head (model #7519-10 Cole-Parmer)]. The 6-roller pump head generated smooth physiological flow (2.0 dynes/cm^2 shear stress), which was immediately started. At 2 dynes/cm^2 shear stress the recirculation time for one circuit of the 3 ml loop was 44.9

sec. Neutrophil viability was determined by trypan blue exclusion before infusion and after termination of the assays and did not change.

Anti-selectin blocking mAbs [WAPS-12.2, anti-P-selectin (Warnock and Butcher, Stanford University), DREG 56 (16) and EL-246 (17), anti-L and E-selectin] were infused via a sterile T-extension (Abbott Hospitals, INC.) into the recirculation loop for a final concentration of 100 μg/ml to observe their blocking effect. LPS levels were determined to be < 2 picograms/ml (at 10μg/ml mAb concentration) for all mAbs tested. Continuous high resolution, phase contrast, video recordings [30 frames/sec, (SP) rate (NTSC standard) via a .7x video coupler and 20x phase contrast objective (Nikon) using a color CCD-TV camera (Sony) and a four-head, shuttle wheel, VHS video recorder (Sony)] of the flow-induced neutrophil/HUVEC rolling and subsequent neutrophil/neutrophil rolling were made for later analysis.

Recirculating loop, neutrophil/neutrophil, capillary tube shear assay.

For experiments examining neutrophil/neutrophil rolling on neutrophils bound to glass, borasilicate glass capillary tubes (identical to those described above) were precoated with freshly collected human serum at 37°C for 2-4 hrs before the addition of freshly ficoll-isolated (as above) human peripheral-blood neutrophils ($1x10^7$cells/ml). After allowing the neutrophils to statically adhere for 10 min at 37°C in complete DMEM, the capillary tube was integrated into the recirculating loop (as described above), and flow was initiated with freshly infused neutrophils from the same neutrophil cell isolate. The freshly infused neutrophils established a rolling

interaction with adherent neutrophils which was recorded for off-line analysis.

Anti-L-selectin mAbs DREG 56, DREG 110 (16), and EL-246 were infused into the recirculating loop for a final concentration of 100 μg/ml for mAb blocking experiments (concentrations of DREG 56 as low as 10μg/ml also completely blocked rolling). Similar concentrations of HERMES-3 (anti-CD44) (18), R15.7 (anti-CD18) (Kishimoto and Rothlein, Boehringer Ingelheim Pharmaceuticals Inc.), and HECA-452 (anti-CLA) (19) were also tested. Again, LPS levels were determined to be < 2 picograms/ml (at 10μg/ml mAb concentration) for mAbs used. No activation as a consequence of mAb treatment was observed as determined by measurement of both L-selectin and CD-18 level on the surface of pre- and post-mAb-treated cells (data not shown).

Neutrophils were exposed to chemical inhibitors of rolling, both in suspension and while bound, by treating with FMLP, $5x10^{-6}$ M (Sigma) in complete DMEM or with neuraminidase, 1 unit/ml/10^7 cells (Sigma) in acetate buffer (50 mM NaOAc, 154 mM NaCl pH 6.5, 4 mM $CaCl_2$) (final concentrations) by preincubation of capillary-tube neutrophil monolayers (20 min at 37°C for both FMLP and neuraminidase) or by direct infusion into the recirculating loop, treating neutrophil cell suspensions and neutrophil capillary monolayers during the course of the flow experiment. DMEM or acetate buffer (pH 6.8), respectively, were infused into the loop as controls. Neutrophils treated with neuraminidase for examination of rolling behavior on neutrophil monolayers (as above) showed unaltered L-selectin

and CD-18 levels by flow cytometry indicating no induced activation as a consequence of this treatment (data not shown).

Data analysis.

Rolling cells were quantified by manual counting from moving and frozen video-tape frames on the video monitor and reported as the number of neutrophils rolling on the bound neutrophil monolayer within the 350 μm by 250 μm video-microscopic field as a function of time. Initial measured rolling, reported in the figures, was defined as greater than 10 neutrophils/video field rolling on adherent neutrophils. Rolling speed was measured by following individual neutrophils frame by frame (1/30th of a sec/frame) and recording distance vs time rolling, and was reported as μm rolled/sec on the bound neutrophil monolayer within the 350 μm by 250 μm video-microscopic field using an Apple Macintosh Quadra 660Av computer.

Results and Discussion

Using a closed-loop shear assay, we have established that interleukin-1-treated human umbilical-vein endothelial cells (HUVECs) expressing E-selectin (20) strongly support neutrophil rolling that is blocked by anti-E-selectin mAbs (15). In these analyses, we noted that neutrophils avidly arrest on HUVECs, forming a secondary monolayer of neutrophils (Figure 29 A,B,C) that can be disrupted by anti-CD18 mAb treatment (15), and, subsequently, these bound neutrophils themselves support rolling of newly

arriving neutrophils (Figure 29 D,E,F; circles). Neutrophils rolling on HUVECs (Figure 29 G), when treated with a function-blocking anti-P-selectin mAb experienced no decrease in either neutrophil/HUVEC or neutrophil/neutrophil rolling indicating no role for P-selectin in either interaction Figure 29 H, circles). Formation of the bound neutrophil monolayer on HUVECs continued, as seen by the large number of uncircled, bound neutrophils (Figure 29 H). However, when treated with anti-L-selectin, all forms of rolling stopped (Figure 29 I). A similar treatment, using the function-blocking anti-L- and anti-E-selectin mAb (EL-246) prevented initial neutrophil rolling and formation of the bound neutrophil monolayer on HUVECs (data not shown).

The analysis of an individual neutrophil/neutrophil rolling sequence is demonstrated in Figure 30 (panel A) where overlapped video frames, five frames apart are displayed representing 3 seconds of real time in which a single neutrophil is shown highlighted, arrows indicating the direction of motion, and numbered at 0,1,2, and 3 sec. This neutrophil established a variable speed rolling interaction (demonstrated by the varing widths of each video frame displayed) initially on the underlying monolayer and subsequently on the bound neutrophils. It followed a nonlinear rolling path (Figure 30 panel B, in black) during which it jumped across several gaps between the bound neutrophils (Figure 30 white arrows, panel A; gaps in the black rolling track panel B) to eventually roll once again onto the underlying monolayer where it arrested. From these recorded sequences, neutrophil behavior, rolling speed, and numbers of rolling cells were easily determined and quantified.

Figure 29

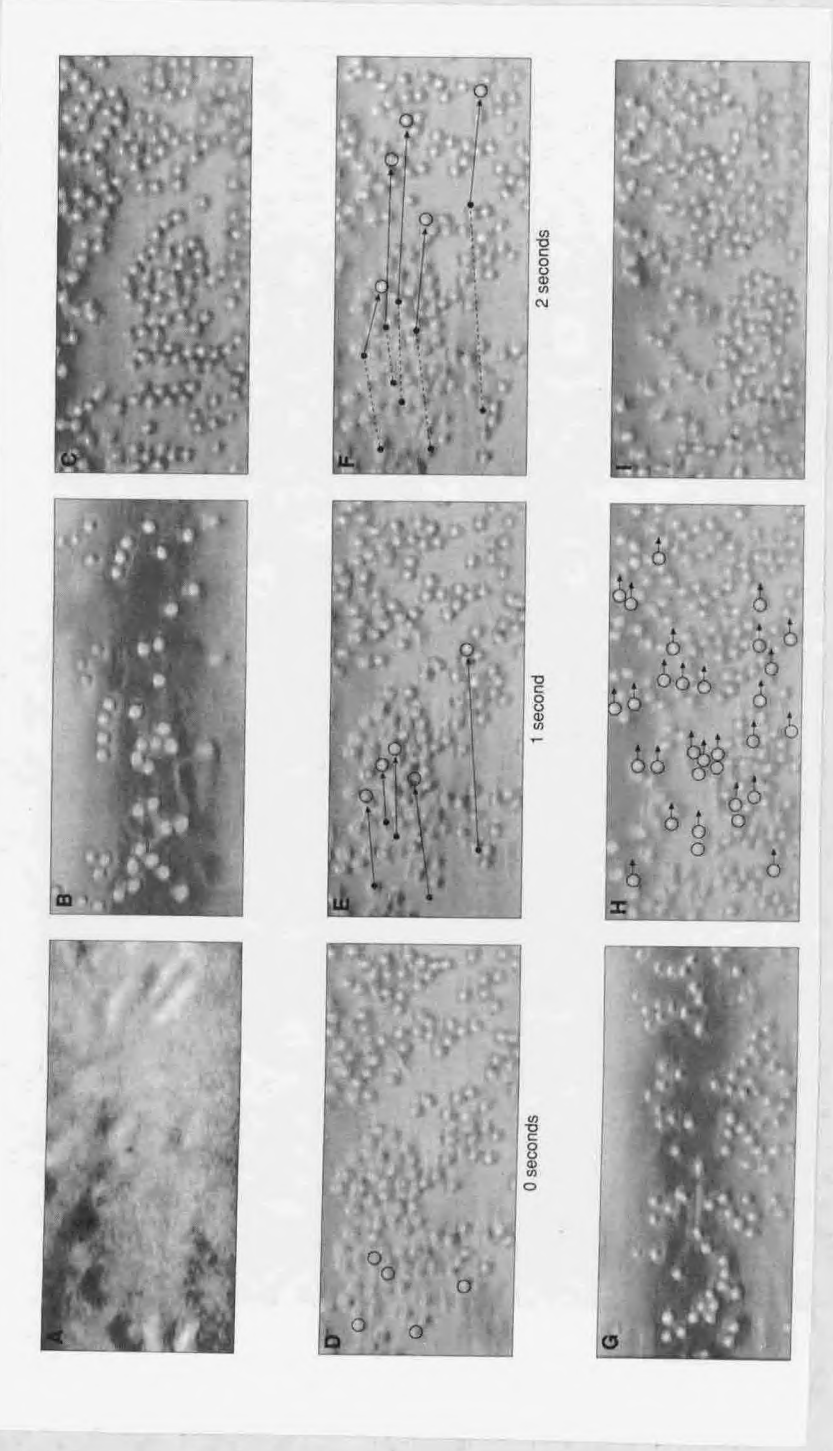


Figure 29. Video-frame stills of the shear-dependent accumulation of neutrophils on an IL-1-activated HUVEC monolayer and initiation of neutrophil/neutrophil rolling followed by antibody inhibition (12). The HUVEC monolayer (A) supported the shear-dependent onset of neutrophil-HUVEC rolling at 1 min after beginning flow (B), and under constant shear at 3 min, neutrophils arrested on the HUVECs to form a monolayer capable of supporting subsequent neutrophil rolling (C). Individual neutrophils, denoted by circles in (D), (E), and (F) (at the 3 min assay time point), rolled across the HUVEC-bound neutrophil monolayer; starting and intermediate positions are shown by black dots, 0 sec and 1 sec positions (E) and (F). Rolling neutrophils moved at velocities well below those observed for non-interacting cells; dashed lines and solid arrows represent the distance traveled in one second (in this experiment averaging 64 μm/sec at 2.0 dynes/cm^2) in (E) and (F) (noninteracting cells moved too rapidly to appear as clearly defined objects on the video-frame stills). In panels (G), (H), and (I), the effects of treatment with anti-selectin mAbs were examined during neutrophil/neutrophil rolling. Panel (G) represents untreated neutrophil/endothelial-cell and neutrophil/neutrophil interactions prior to infusion of anti-selectin mAbs. Panel (H) shows that the anti-P-selectin blocking mAb (WAPS 12.2, 100 μg/ml) had no effect on either neutrophil/endothelial-cell rolling or neutrophil/neutrophil rolling 2.5 min after injection (rolling cells denoted by circles with short arrows). This was in great contrast to the effect of the anti-L-selectin mAb (DREG 56 100 μg/ml) and the anti-L, anti-E-selectin mAb (EL-246 100μg/ml), both of which blocked, within 2 min, all neutrophil/endothelial-cell and neutrophil/neutrophil rolling interactions without release of adherent cells (I).

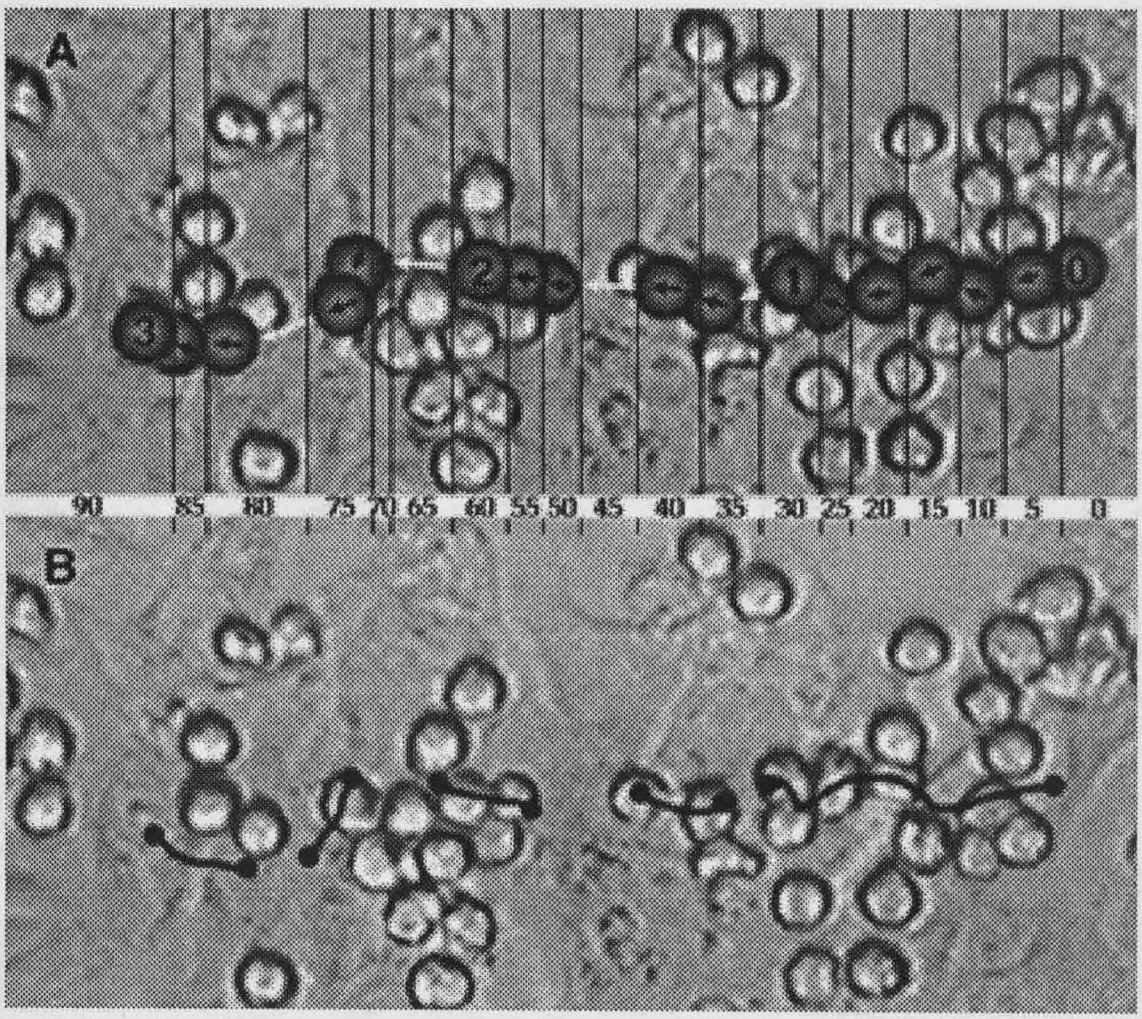


Figure 30. Behavior of an individual neutrophil, rolling on several groups of arrested neutrophils, which facilitated its eventual arrest on the underlying monolayer. Early in the establishment of the neutrophil monolayer, Panel A shows a single neutrophil rolling as the neutrophil interacts with bound neutrophils in 19 individual computer-digitized and overlapped video frames. Individual frames shown in panels A and B are demarcated by vertical black lines and labeled with the total number of elapsed frames from right to left (in the space between panel A and panel B). The total sequence shows a single highlighted neutrophil with arrows

indicating the direction of motion and represents 3 seconds of real time. The rolling cell is labeled at the 0,1,2, and 3 sec time points (panel A). White arrows indicate discontinuities in rolling where the neutrophil jumps from bound neutrophil to bound neutrophil along its track of motion. In panel B, the track of the rolling neutrophil across the field is shown as a discontinuous black line with gaps indicating jumps from one bound neutrophil to another. The image of the rolling neutrophil has been removed by computerized digital frame subtraction to allow visualization of the bound neutrophils with which the rolling cell was interacting.

As expected (and commonly seen during the isolation of neutrophils from the blood of human donors), neutrophil aggregation occurred in varying degrees which was observed in many of the video sequences. The aggregation was the result of the physiological state of the donor and/or caused by the isolation procedure. The neutrophil aggregates also showed the ability to interact and roll over the surface of the bound neutrophil monolayer. Sporadic interactions of these irregular aggregates, composed of highly variable numbers of neutrophils, with the neutrophil/endothelial cell monolayer, mirrored the behavior of individual neutrophils. Treatment with anti-L-selectin mAbs completely blocked this interaction, but failed to disrupt the neutrophil aggregates present and suspended in the flow (data not shown). Because the neutrophil aggregates exhibited behaviors similar to isolated cells but whose presence was variable from experiment to experiment, they were excluded from the quantitative analyses reported below.

To exclude the possibility of a contribution of the HUVECs in this neutrophil/neutrophil rolling interaction, we tested whether neutrophils

bound to serum-coated glass could support neutrophil rolling. In both systems (serum and endothelial-cell bound), rolling occurred under a shear stress of 0.5-2.0 dynes/cm^2 (determined by the Hagen-Poiseuille equation), levels that have been defined for leukocyte/endothelial-cell rolling *in vitro* and *in vivo* (13). Rolling velocities of neutrophils rolling on adherent neutrophils had a mean value of 88.4 μm/sec +/- 30.1 (S.D.) over the entire course of experiments, as compared to 16.6 μm/sec +/- 24.6 (S.D.) for neutrophils rolling directly on HUVECs. Both values were within the lower range of velocities reported for neutrophil rolling *in vivo* (10 - 840μm/sec) (21). The number of neutrophils rolling on the bound neutrophil monolayers initially increased over time (Figure 31 A). Maximal rolling occurred within 4-6 min after infusion of the neutrophil cell suspension into the recirculating loop (14-16 min after the onset of formation of the tube-bound neutrophil monolayer) and was maintained for approximately 8-10 min. After 10-15 min, the number of rolling cells decreased dramatically (Figure 31 A). Infusion of additional suspended neutrophils into the rolling assay after 9 min did not result in a renewed rolling increase (Figure 31 B), demonstrating that the decreased neutrophil/neutrophil rolling interaction was due to changes in the adherent neutrophil monolayer. Considerable shape change of the bound neutrophils occurred in correlation with the decreased rolling (data not shown). These results indicate that the bound neutrophils can directly support neutrophil rolling.

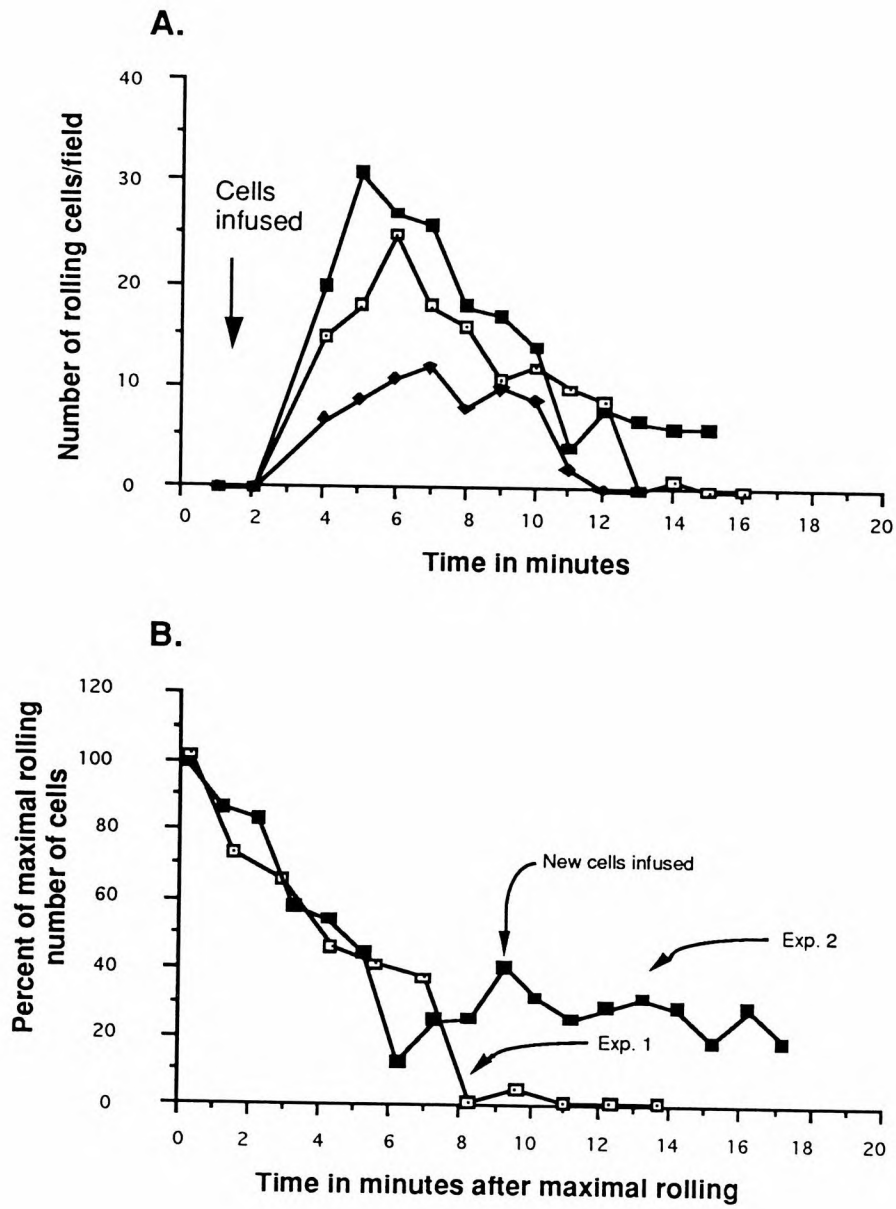


Figure 31. Numbers of neutrophils demonstrating rolling on bound neutrophil monolayers as a function of time. Neutrophils were allowed to bind firmly to serum-coated tubes (13) for 10 minutes at 37° C, forming an adherent monolayer; then, new neutrophils were passed over the

monolayer under flow, and the number of rolling cells were followed over time. Panel (A) presents results from three typical experiments. Neutrophil/neutrophil rolling, identical to that seen with HUVEC, was supported by neutrophil monolayers. Rolling was observed to have a finite time course after binding of the monolayer-forming neutrophils, rapidly increasing to peak at 4 to 6 min after infusion of cells (14-16 min after initiating the binding of adherent neutrophils) followed by a decline to baseline 7 min later. As demonstrated in panel (B), in two experiments duplicating the same protocol as in (A), fresh neutrophils (from the original isolation held at 15° C) were infused after the total decay of the initial neutrophil/neutrophil rolling interactions. No new peak in the number of rolling cells was observed, indicating that decreased rolling was due to secondary changes in the adherent cells.

To determine the receptor mechanisms of this interaction, we further tested the effects of inhibitors on neutrophil/neutrophil rolling. Rolling interactions were first established, then various antibody or chemical inhibitors were infused into the closed-loop system. Similar results were obtained with neutrophils bound to cytokine-activated endothelial cells or serum-coated tubes; more detailed analyses were conducted using serum-coated capillary tubes. The rolling cells were monitored for 4 min after injection into the loop--well before their normal decline in rolling described above. Within 1-2 min after the infusion of the anti-L-selectin mAbs DREG 56, DREG 110, and EL-246, rolling was completely inhibited (Figure 32 A). In separate experiments using the same anti-L-selectin mAbs, pretreatment of neutrophils prior to their infusion inhibited all rolling interactions (data not shown). Antibodies directed against CD44, CLA, or CD18 were infused without effect (Figure 32 B). Experiments examined for 10-20 min showed

the normal decline in the number of rolling cells, which was not accelerated by the CD44, CLA, or CD18 antibodies (data not shown). Treatment with R15.7 (anti-CD18 mAb) did cause a loss in the number of HUVEC and serum-coated tube adherent neutrophils by 20 min, demonstrating that the antibody was functional (data not shown). HECA-452 (anti-CLA mAb) was also tested at an increasing range up to 400 μg/ml in one experiment, where no effect on rolling was observed (data not shown).

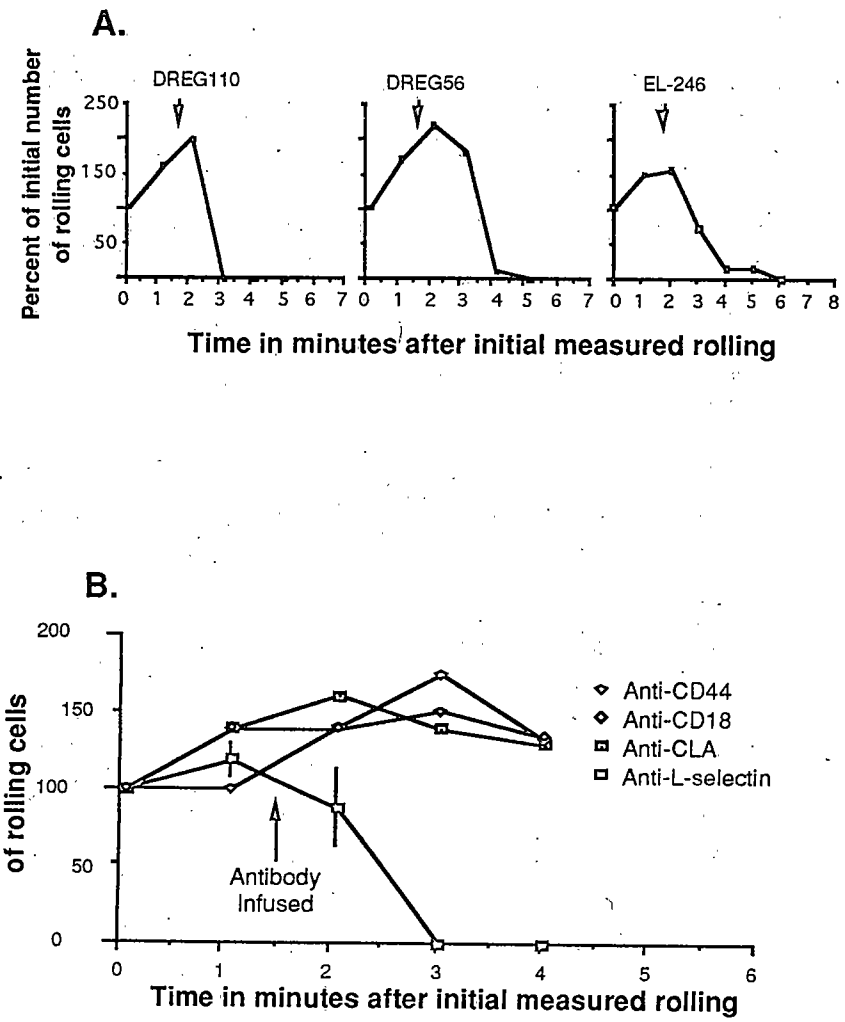


Figure 32. The effects of treatment with anti-adhesion molecule mAbs after

the onset of initial measured neutrophil/neutrophil rolling. Panel (A) shows the blocking effects of anti-L-selectin mAb (DREG 110, DREG 56, and EL-246) treatment when infused into the recirculating loop after initiation of neutrophil/neutrophil rolling. In all three experiments, mAbs were infused at the 3 min time point after neutrophil infusion during the increasing phase of neutrophil rolling interactions; the existing rolling and the initiation of new neutrophil/neutrophil rolling rapidly declined to baseline within 2 min in all cases. Panel (B) shows the results of three independent anti-adhesion molecule mAbs tested for their blocking effects on neutrophil/neutrophil rolling in parallel with anti-L-selectin mAb controls. As before, these mAbs were infused during the increasing phase of neutrophil/neutrophil rolling interaction. The data plots for anti-CD44, anti-CD18, and anti-CLA mAbs demonstrated that they had no effect on neutrophil/neutrophil rolling as shown by representative plots of 3 replicate experiments. The anti-L-selectin mAb results, shown as pooled data with standard error bars, demonstrated reproducibly complete blocking.

Inhibition of rolling by anti-L-selectin could have been due to an effect on the rolling neutrophils, the adherent neutrophils, or both. To distinguish between these possibilities, experiments were done to determine whether the adherent or rolling cells were inhibited by the anti-L-selectin mAbs. A rolling interaction was established and blocked 2 min later by the addition of DREG 56. The loop system was then flushed with media to remove the free DREG 56 mAb. New, untreated neutrophils were then added to the loop. As shown (Figure 33 A), the neutrophil monolayer, after DREG 56 treatment and washing, supported renewed rolling of non-antibody-treated neutrophils. These results show that the DREG 56 anti-L-selectin mAb inhibited rolling by blocking L-selectin function on the rolling cells. The same results were seen with other anti-L-selectin mAbs (data not shown). In separate experiments using DREG 56, pretreatment of the

suspension of neutrophils prior to their infusion inhibited all rolling interactions, where DREG 56 pretreatment of the neutrophil monolayer had no effect (Figure 33 B). These results show that the DREG 56 anti-L-selectin mAb inhibited rolling by blocking L-selectin function on the rolling cells.

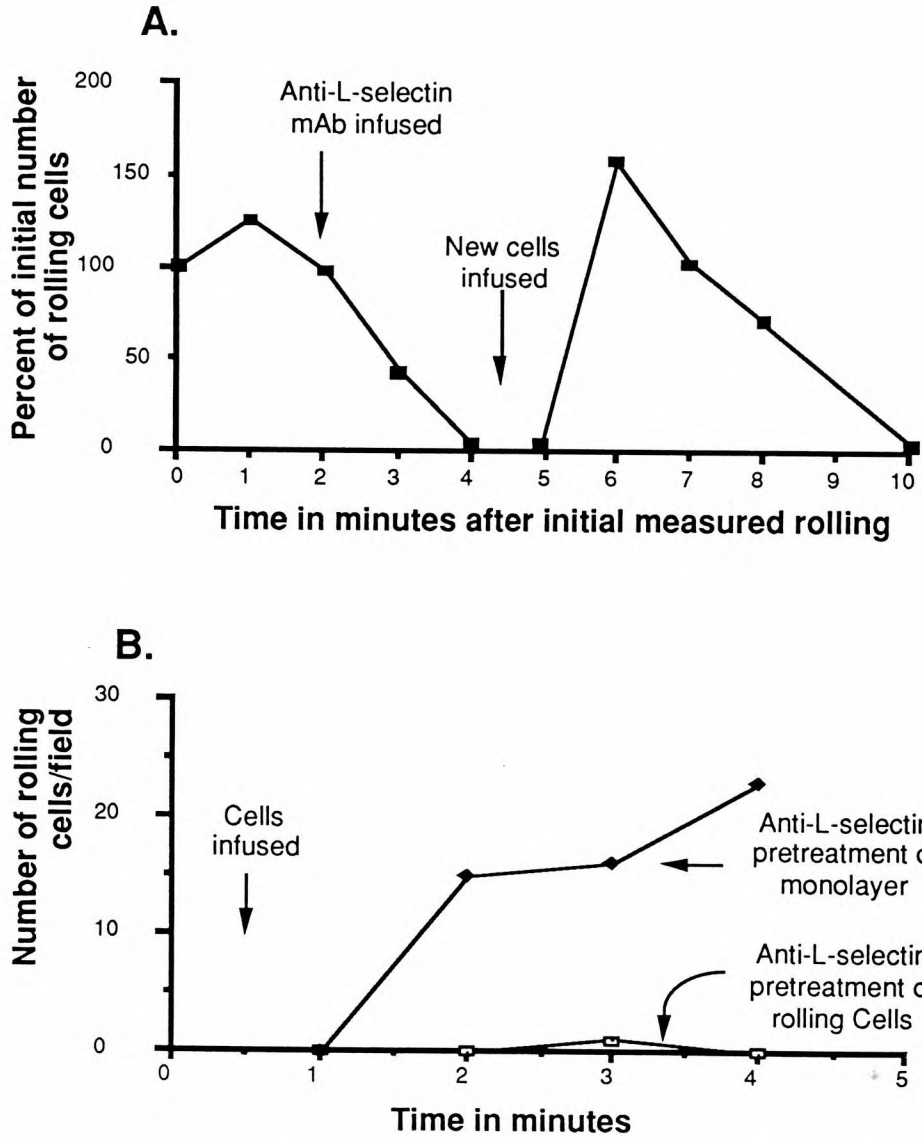


Figure 33. In (A), anti-L-selectin mAb (DREG56) was infused as in (Figure 31 B), blocking neutrophil rolling. At the 5-min time point, during the normal

increase in the rolling phase of the neutrophil/neutrophil interaction, the neutrophils and medium were replaced with fresh cells and medium without mAbs, placed under shear, and observed over time. The addition of fresh neutrophils at this time point resulted in rapid re-establishment of neutrophil/neutrophil rolling. After this second rapid onset of rolling, a rapid decline was also observed, following the normal time-related decay of rolling interactions. In panel (B), DREG 56 pretreatment of neutrophil suspensions or neutrophil monolayers was completed prior to the initiation of rolling interactions. DREG 56 pretreatment had a blocking effect on neutrophil/neutrophil rolling only after pretreatment of L-selectin on neutrophils in suspension.

Enzymatic and chemical treatments were tested to further characterize the neutrophil/neutrophil rolling interaction. Removal of terminal sialic acid residues by infusion of neuraminidase (1 unit/ml) into the loop completely inhibited rolling within 3 min (Figure 34 A). Infusion of $5x10^{-6}$ M FMLP, which causes rapid activation of neutrophils, also led to rapid inhibition of rolling (Figure 34 A). Although an increase in the number of aggregates of neutrophils was observed, large numbers of individual non-interacting neutrophils were seen and recorded after FMLP treatment. Neuraminidase, but not FMLP treatment of the bound neutrophils, blocked their ability to support rolling (Figure 34 B); however, neuraminidase treatment had no effect on rolling neutrophils (data not shown). The neuraminidase acetate buffer control had no effect on the rolling interaction. In contrast, FMLP treatment of the rolling cells alone blocked the interaction (data not shown). These results show that 1) activation of the rolling but not of the adherent cells leads to diminished rolling, consistent with shedding of L-selectin, and 2) terminal sialic acid residues on the adherent cell are

required for the interaction, consistent with the predicted role of the siaylated Lewis^X carbohydrate ligands (22).

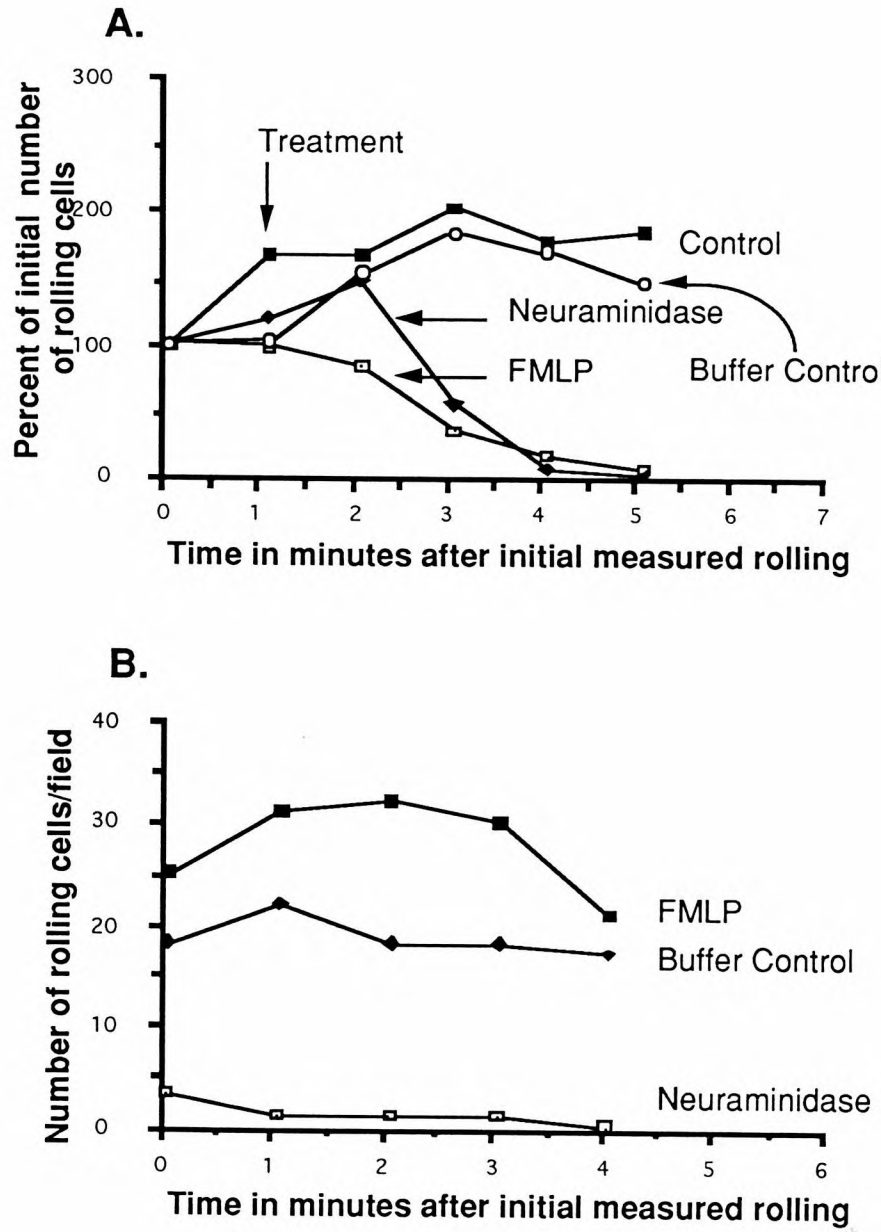


Figure 34. Demonstration of the effects of FMLP activation and neuraminidase treatment on neutrophil/neutrophil rolling in serum-coated glass capillary tubes. In panel (A), infusion of FMLP ($5x10^{-6}$ M/ml) or

neuraminidase (1 unit/ml/10^7 cells) into the recirculating loop resulted in a rapid decline of neutrophil rolling. In contrast, no effects were seen when appropriate buffer (acetate, pH 6.8) controls were infused into the loop. In panel (B), where only the neutrophil monolayer had been treated with FMLP, there was no effect on neutrophil/neutrophil rolling, indicating that activation affected only the rolling neutrophils. In contrast, panel (B) shows that neuraminidase [at the same concentration as in panel (A)], after a 20 min pretreatment of the tube-bound neutrophils, prevented establishment of neutrophil/neutrophil rolling with fresh neutrophils under flow. Again, buffer controls had no effect.

Our *in vitro* observations suggest that when neutrophils arrest on endothelium at sites of inflammation, they themselves can serve as adhesive substrates to support shear-dependent recruitment of additional neutrophils. Adherent neutrophils *in vivo* support neutrophil rolling which has been recently observed by intravital microscopy in both rabbit and mouse venules (von Andrian, Bargatze, and Butcher, unpublished observations). This interaction is likely important for the continued influx of inflammatory cells beyond the adhesive events that occur within the initial few min after induction of inflammation. Interestingly, neutrophil/neutrophil shear-dependent rolling like neutrophil/endothelial-cell rolling is dependent on L-selectin on the rolling cell. The data suggest that the ligand(s) for L-selectin, likely comprised in part by the sialylated Lewis^x carbohydrate moiety on adherent neutrophils, is exquisitely regulated as rolling only occurs for a short time (10-14 min) after neutrophil binding and initial spreading. The ligand could be shed after the short interaction time frame as previously shown for L-selectin (16,23), internalized, or during neutrophil adherence-associated shape changes, modulated in its surface expression making it unavailable to the rolling

neutrophil. These results greatly extend those of Simon et al. who showed that chemoattractant-induced neutrophil aggregation (examined only in stirred suspension) can be blocked by anti-L-selectin mAb (24). Uniquely, in the flow-induced shear experiments reported here, in vitro neutrophil/neutrophil rolling augmented recruitment to endothelium is only dependent on L-selectin expression on the rolling cell (not the bound neutrophil). In addition, anti-$ß_2$ integrin mAb antibodies which block aggregate formation in suspension (24) have no effect on neutrophil/neutrophil rolling at 2 dynes/cm^2. This is not surprising as the estimated shear in suspension experiments reported by Simon et al. was less than 1 dyne/cm^2, where integrin interactions may play a primary role in cell adhesion interactions. We are currently studying the nature of the L-selectin ligand expressed by bound neutrophils to better compare these two L-selectin-dependent neutrophil/neutrophil interactions.

Effective blocking of inflammatory reactions by anti-L-selectin mAbs occurs *in vivo* (9,25,26). Indeed, anti-L-selectin mAbs are the most consistent anti-selectin blocking reagents in models of peritonitis, and lung and dermal inflammation (27-31). Blocking by anti-L-selectin mAbs is thought to be due to inhibition of neutrophil/endothelial-cell interactions. However, in virtually all *in vitro* inflammation and L-selectin-dependent models of neutrophil/endothelial-cell adhesion, the blocking by anti-L-selectin mAbs only accounts for a portion of the observed adhesion (7,15,17,32-35). Since neutrophil/neutrophil rolling is consistently and completely blocked by anti-L-selectin mAbs in our experiments, the effectiveness of these mAbs in inhibiting inflammation *in vivo* may possibly result from blocking of this interaction. Thus, shear-dependent

neutrophil/neutrophil interactions may provide a new target in the design of therapeutic inhibitors of inflammation. It is probable that these inhibitors would be effective in diverse inflammatory settings, because neutrophil/neutrophil rolling may occur during many if not all types of inflammation.

In conclusion, adherent neutrophils, like activated endothelial cells, support continuous neutrophil rolling by presentation of a novel L-selectin ligand as demonstrated *in vitro* by the consistent anti-L-selectin mAb blocking of this interaction. The demonstration of L-selectin-mediated neutrophil/neutrophil rolling *in vitro* suggests a new mechanism for leukocyte recruitment *in vivo* which could support augmented accumulation of neutrophils during inflammation.

Summary

Specific arrest of neutrophils in venules is central to their rapid accumulation during local inflammatory responses. Initial neutrophil rolling on endothelium is mediated by leukocyte L-selectin and the inducible vascular adhesion proteins P- and E-selectin. This rolling is a prerequisite for endothelial-dependent neutrophil arrest. Here we describe rolling of neutrophils on the surface of previously arrested neutrophils and demonstrate that this interaction involves L-selectin exclusively on rolling cells. The adherent neutrophil support of L-selectin-dependent neutrophil rolling *in vivo* can promote continuous and augmented leukocyte recruitment at sites of previous neutrophil accumulation.

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CHAPTER 7

CONCLUSIONS

The general findings ; the test of the hypothesis.

The findings of the work presented in this thesis lead to strong support of the three-step model hypothesis proposed for blood-borne leukocyte recruitment of both lymphocytes and neutrophils. The detailed *in vitro* and *in vivo* analyses show in some cases a more simplistic orchestration of events than originally hypothesized, as with lymphocytes, where preactivated cells arriving at the endothelium may bind using a single integrin class and its counter ligand for arrest. Alternatively, I found the signalling step of the "adhesion cascade" to be complex, regulated by chemokines and LFA-1, but also through as-yet-undefined molecules that together regulate the affinity and/or avidity of selectins as well as leukocyte integrins. Chemokines do not provide an overt signal, as is delivered by PMA, leading to the rapid binding/arrest of lymphocytes (as was predicted by Steve Shaw and David Kelvin). Answering the question of what the critical players are in signaling lymphocyte tight adhesion will require a renewed examination. The extensive evaluation of the functional effects of the EL-246 mAb *in vitro* and *in vivo* has provided significant insight into the

potential of using this antibody clinically. The results demonstrate that the effectiveness of blocking of both L- and E-selectin-mediated leukocyte binding during inflammation, with this single mAb, makes EL-246 a potentially powerful therapeutic. Discovery of a new mechanism for neutrophil recruitment during this work has added a new level of complexity and insight to the localization process. The finding that endothelial cell-bound neutrophils provide an adhesive surface for the ongoing recruitment of newly arriving neutrophils from the blood was a serendipitous observation. This recruitment which occurs even when the endothelial surface has been blocked by the already adhering neutrophils, is a radical deviation from the accepted dogma and implies that there is a need to reevaluate and revise the earlier mAb blocking-data-derived models for the molecular basis of inflammatory blood-borne neutrophil recruitment. Discussion of the detailed finding of the individual chapters, the questions they raised, and the new directions they suggest follow.

As reported in the Journal of Experimental Medicine and Chapter 2, the use of a multistep endothelial-leukocyte cascade in lymphocyte homing to PP- HEV is confirmed. Viewed by intravital microscopy, fluorescently labeled normal lymphocytes demonstrate rolling, slowing, and arrest on PP-HEV. The initial interaction, a loose rolling of the lymphocyte on the HEV, occurs independent of a Ptx-sensitive intracellular signaling event. However, slowing and arrest of the rolling lymphocyte is sensitive to a 2 hr treatment with Ptx, indicating a probable G-protein-mediated signal transduction step in the tight adhesion pathway. Treatment of the

lymphocytes with a modified Ptx lacking the G-protein ribosylation property of the wild type toxin failed to have any inhibitory effect on adhesion. The tight adhesion of lymphocytes can be recovered, in the wild type Ptx treated lymphocyte, if treated for 5 min with PMA before being infused into the mouse. Lymphocytes treated in this way entered HEV and, more rapidly than normal, lymphocytes arrested on PP HEV. Thus, the PMA treatment appears to bypass the signaling blockade of Ptx, possibly stimulating activation of the downstream adhesion pathway through protein kinase C, as discussed in Chapter 2. This overt activation brings about a very large increase in the avidity of lymphocytes for binding to HEV as compared to the normal untreated lymphocytes. These findings are consistent with earlier *in vitro* studies showing that Ptx can inhibit activation-dependent participation of the integrins in lymphocyte binding to lymph node HEV in frozen sections. The findings of Chapter 2 establish a strong parallel with analogous cellular events involved in neutrophil interactions with inflamed endothelium, and support the general model of leukocyte-endothelial cell interaction as a multi-step adhesion cascade.

In Chapter 3 and in a manuscript recently submitted to the journal "Cell," we describe for the first time a temporal sequence of participation for L-selectin, α4ß7, and LFA-1 in regulating the PP lymphocyte-HEV adhesion cascade. Extensive intravital evaluation of processes defined in Chapter 2, using function blocking mAbs, have defined both distinct and overlapping roles for L-selectin, α4ß7, and LFA-1 in the attachment, rolling, and arrest of lymphocytes in PP-HEV. To summarize the lymphocyte component of the HEV binding process; 1) L-selectin initiates rolling/tethering (which is not

required if the α4β7 integrin is preactivated to support strong adhesion in which case the cell arrests in a single step), 2) α4β7 engages, slowing the rolling and allow LFA-1 to interact with its ligand signaling increased adhesion avidity, 3) as α4β7 reaches its fully activated state, the lymphocyte stops where LFA-1 can cement its tight adhesion to the HEV.

Signaling, LFA-1, and the lymphocyte-endothelial adhesion cascade. Anti-LFA-1 mAb treatment of lymphocytes mimics the effects Ptx treatment. The behavior of the cells, slow jerky rolling, was of the same character and speed as observed for Ptx-treated cells, as reported in Chapter 2. Interestingly, as seen with Ptx treatment, anti-LFA-1 mAb-treated lymphocytes, when treated with PMA, bind more avidly than normal lymphocytes. This suggests that LFA-1 may serve, both in a signaling capacity, delivering an activating signal increasing α4β7 avidity, and in an adhesion role, cementing the lymphocyte to HEV after arrest. In addition to the anti-LFA-1 mAb, both mAbs against α4 and/or β7 integrins block tight adhesion. The mAb blocking results also show that α4β7 participates in slowing rolling. When α4 and/or β7 function is blocked, the lymphocyte rolling speed on the HEV is reproducibly increased two fold. When α4β7 slow rolling has been abrogated with mAbs, the lymphocyte continues to exhibit a fast, tethering rolling behavior that, upon anti- L-selectin mAb (MEL-14) treatment, all lymphocyte-HEV interactions are ended.

PMA treatment has no effect on the lymphocytes blocked by anti-α4β7 mAbs, and both normal and PMA-treated lymphocytes exhibit only L-selectin-mediated rolling interactions and no slowing or arrest. In

experiments where lymphocytes are first treated with PMA and then blocked with MEL-14, an α4β7 blockable tight adhesion is observed, indicating that activated lymphocytes do not require a selectin-mediated rolling interaction to initiate the initial adhesive interaction. Arrest, therefore, requires α4β7 tight adhesion but not LFA-1 or L-selectin participation, when lymphocytes are PMA activated. These results together support that it is unlikely that LFA-1 significantly participates in slowing of rolling and arrest.

For the first time, the molecular basis for the two extreme variants of the lymphocyte homing model are defined; 1) PP homing of resting lymph-node cells, which involves sequential engagement of 3 adhesion receptors and at least one activating signal, and 2) the simpler binding interaction of α4ß7-activated cells, which can be mediated by α4ß7 alone.

Anti-MadCAM mAb MECA-367, when used to pretreat mice before the infusion of interacting lymphocytes, results in a near complete blocking of all lymphocyte-PP HEV interactions. These data strongly suggest that MadCAM represents the primary endothelial ligand on PP HEV for lymphocytes. It is of interest, however, that when FAbs of MECA-367 are used to pretreat mice, L-selectin-dependent rolling of lymphocytes still occurs on PP HEV. Another anti-MadCAM mAb MECA-89 recognizes an epitope unique from that seen by MECA-367 and blocks L-selectin-mediated rolling. Therefore, it appears that MadCAM, presenting muti-specific adhesion domains, may provide for all of the lymphocyte adhesion interactions required for homing to PP HEV.

The results of this study will likely prove applicable, not only to lymphocyte trafficking to discrete sites on the endothelium, but also to

trafficking of other leukocytes: eosinophils and monocytes which, like lymphocytes, co-express selectin, α4 integrin, and ß2 integrin adhesion receptors. The findings of Chapter 3 confirm and expand our understanding of the multi-step model of leukocyte-EC recognition.

Chapter 4 presents the work of a manuscript in preparation. Here we examined homing mechanisms of human lymphocyte participation in cell-mediated responses at sites of acute inflammation. The mechanisms of T-cell recruitment are currently the topic of intense investigation for regulating the progression and resolution of inflammatory lesions and in the treatment of inflammation-induced pathology. Chemokines, hopeful candidates for mediating leukocyte recruitment, are small, soluble peptides that have been shown to increase leukocyte binding in static adhesion assays and induce leukocyte chemotaxis. The study reported in Chapter 4 demonstrates that, *in vitro*, lymphocytes roll on E-selectin transfectants via an L-selectin-independent ligand and that the IP-10 and MIP-1ß chemokines induce slowed rolling speeds. Also, a key finding of this study is that *in vitro* IP-10-treatment of lymphocytes slows their L-selectin- dependent rolling and leads to modest levels of permanent arrest on activated endothelium. Lymphocytes in this system, upon PMA activation, overtly display the full lymphocyte three-step adhesion cascade, as demonstrated for lymphocytes homing to PP in vivo. This is not the case for the IP-10 chemokine. The slowing of their rolling speed across the HUVECs as compared to control lymphocytes is approximately 30% of their normal velocity. In addition, the number of cells achieving permanent arrest (relative to control) is increased greater than two fold. These results and

those from the HUVEC, imply that lymphocyte treatment with IP-10 may play a role in slowing rolling speeds by alteration of the affinity of L-selectin or the E-selectin ligand, suggesting a new mechanism augmenting lymphocyte recruitment. In addition, IP-10 may modestly increase integrin affinity and/or avidity since lymphocytes do accumulate over an extended number of minutes. Although the rapidity of binding is not as dramatic as that reported for fMLP-treated neutrophils, the total numbers of binding cells is significant over time.

This study is currently being expanded to examine the effects of multiple cytokine combinations and varied time courses of *in vitro* endothelial cell activation. In preliminary experiments, we have examined the combined effects of IL-4 and IL-1 activation. To summarize, twenty-four hr IL-4, followed by four hour IL-1 pretreatment of HUVEC results in control lymphocyte interactions that include both slow rolling and high levels of arrest. It appears these IL-4 + IL-1-treated HUVEC not only present the necessary adhesion molecules for arrest, but also efficiently present a "chemokine" activity signaling for the arrest of a large number of control lymphocytes. IP-10 chemokine pretreatment of lymphocytes does (N=2) enhance initial rates of accumulation, but not total numbers of lymphocytes arresting per unit area over time.

In experiments using γ interferon (24 hrs) followed by IL-1 (4 hrs) (N=2), we have observed the same accumulation and arrest rates as for IL-4 + IL-1-treated HUVEC. Interestingly, we also see more rapid lymphocyte flattening morphology changes, suggesting higher expression of ICAM on

the HUVEC. This is consistent with the observation that γ interferon treatment alone for 24 hrs results in no rolling interactions, but if allowed time (5 min) to bind under static conditions, lymphocytes avidly bind to these HUVEC. This suggests that IL-1 treatment is responsible for E-selectin and VCAM expression, whereas ICAM is upregulated by γ interferon. We are also currently examining the effects of TNF in this system. We find that 4 hr TNF treatment activates the endothelium to support both rolling and arrest, but after 24 hrs of treatment, no rolling interactions are preserved.

The HUVEC-lymphocyte *in vitro* capillary shear system is proving to be a very useful experimental system that has the capacity for evaluating many of the parameters of inflammatory recruitment seen in vivo. This, coupled with the ability to assess the effects of enhancers and suppressors of each of the adhesion cascade steps via video microscopy and computer analysis, should lead to important new findings defining the regulation of lymphocyte homing

The data presented in Chapter 5 were recently published in the Journal of Immunology. Here the dynamic adhesive interactions of neutrophils with HUVEC via a unique epitope of E- and L-selectins and also ß2 integrin were examined in simulated blood flow. An unusual monoclonal antibody, EL-246, recognizes a common epitope of the short-consensus repeat domains shared by both E- and L-selectin. The functional analysis of this epitope was performed in several *in vivo* and *in vitro* assays to define the adhesive interactions this epitope supports in both static assays and under physiological shear. The static binding of neutrophils to E-selectin cDNA transfectants shows that their adhesion is blocked by EL-246

treatment of both or either cell type. It was discovered during these assays that EL-246 posseses a unique antibody transfer mechanism, such that EL-246 can be unidirectionally transferred from leukocyte L- to endothelial E-selectin, as determined by flow cytometry. Likely due to this property, EL-246 surpasses the adhesion blocked by mAbs recognizing either L- or E-selectin alone. Developed for this study, the *in vitro* capillary shear system shows that neutrophil rolling on IL-1-activated HUVEC and E-selectin cDNA transfectants is rapidly blocked in 45 to 120 sec after infusion of EL-246 at rates that exceeded blocking by L-selectin or E-selectin mAbs alone. Employing flow cytometry and in vivo homing techniques, pretreating bovine lymphocytes with EL-246 blocks their ability to home to mouse peripheral lymph nodes by >65%. From these studies the conclusion can be drawn that the EL-246 mAb may be a potentially effective therapeutic agent in clinical applications where suppressive regulation of E- and L-selectin-mediated inflammation is required.

Chapter 6 recounts the findings of a very recent report in the Journal of Experimental Medicine. It demonstrates the experimental discovery of a new mechanism of neutrophil recruitment of acute inflammatory sites. The specific recruitment of neutrophils to venules is central to their rapid accumulation during local inflammatory responses. The initiation of neutrophil rolling on endothelium is supported by leukocyte L-selectin and the inducible vascular adhesion protein E-selectin in this system. The key finding of this study is that previously arrested adherent neutrophils support L-selectin-mediated non-adhered neutrophil rolling on their surface and that rolling is supported by L-selectin exclusively expressed on rolling cells.

Even when the endothelial-neutrophil recruitment is completely impeded by bound neutrophils, this mechanism provides a means of ongoing neutrophil accumulation. As neutrophils transmigrate, leaving the endothelial cell surface, areas previously occupied are liberated to bind new rolling neutrophils recruited by the adherent neutrophil monolayer. This behavior was observed *in vivo* during preliminary experiments evaluating homing of mouse neutrophils to cutaneous acute inflammatory sites. Von Andrian had observed similar events while examining neutrophil rolling and binding in rabbit mesenteric venules (personal communication). The requirements for the rolling event are the same as previously shown in other L-selectin interactions: calcium, lectin-carbohydrate interaction, and chymotrypsin-cleavable sensitivity. The counter ligand was found not to be a known selectin and not LFA-1, but a yet-to-be characterized molecule that facilitates the rolling interaction for only a brief few minutes as the bound neutrophil undergoes morphological flattening. The possibility exists that the L-selectin ligand is modulated in expression, making it unavailable for binding, or it may be shed from the bound neutrophil surface. To summarize simply, this newly described interaction provides a novel mechanism for the sustained recruitment of neutrophils to inflammatory sites.

As an extension of the *in vitro* lymphocyte-chemokine (Chapter 4) and neutrophil HUVEC shear interaction studies (Chapter 5 & 6), we conducted an examination of γ/δ T- cell inflammatory recruitment to endothelial and platelet surfaces. This paper, titled "Cell Surface P- and E-

Selectin Support Shear-Dependent Rolling of Bovine γ/δ T Cells," is currently in press at the "Journal of Immunology." Here we discovered, using a new, shear-dependent assay, that E- and P-selectin were responsible for γ/δ T-cell rolling interactions that lead to permanent binding. To summarize, this is one of the first studies to 1) clearly document a selectin-dependent lymphocyte rolling interaction, 2) show that P-selectin expressed by thrombin-activated platelets supports lymphocyte rolling, 3) demonstrate that bovine γ/δ T cells avidly bind P-selectin, and 4) analyze lymphocyte interactions in an animal model which allows for determination of the *in vivo* relevance of these events. These results define and provide a role for the first step of the multi-step model of extravasation defined for myeloid cells in the migration of certain lymphocyte subsets into sites of inflammation.

In a collaborative effort for which I developed the *in vitro* isolated MadCAM shear assay and conducted the *in vivo* lamina propria experiments, we describe the interactions of lymphocyte α4β7 with VCAM and MadCAM. The manuscript, entitled "Integrin-mediated Lymphocyte Attachment and Rolling Under Physiologic Flow," has recently been accepted at the journal "Cell" for publication. The conclusions of this study show that α4 but not β2 integrins can mediate lymphocyte contact and adhesion under physiologic shear and in the absence of selectin involvement. Rolling adhesion is sensitive to and can therefore be regulated physiologically by multiple parameters, including 1) the level of integrin expression; 2) the extent of integrin activation; 3) the density of ligand decorating target surfaces; and 4) the local microvascular

hemodynamics. These parameters likely work in concert with engagement of selectins and other adhesion and activating factors to fine-tune the homing of lymphocytes to mucosal lymphoid tissues, the intestinal wall, and to tissue sites of inflammation. Finally, our results suggest that topographic presentation α4 integrin on microvilli may be a critical determinant of adhesion under flow, potentially allowing diverse adhesion receptors to engage their vascular ligands under physiologic shear stresses *in vivo*.

To add a final note in conclusion, I believe the work of this thesis makes a significant contribution to the field of leukocyte-endothelial cell immunology, providing answers to several of the basic questions concerning the mechanisms of leukocyte tissue-specific targeting. The work presented, though it does not begin to touch on all the questions of leukocyte recruitment, has created novel empirical approaches to examine the process and has raised new questions. Through current and future collaborative efforts, I hope to have the opportunity to fully develop the models necessary to gain a full grasp of the intricacies of leukocyte-endothelial blood borne recruitment.

Some of the references for the work discussed in this conclusion are in part or whole from the the following papers:

1. Bargatze, R.F. and E.C. Butcher. 1993. Rapid G protein-regulated activation event involved in lymphocyte binding to high endothelial venules. *J. Exp. Med.* 178:367.

2. Bargatze, R.F., S. Kurk, G. Watts, T.K. Kishimoto, C.A. Speer, and M. A. Jutila. 1994. In vivo and in vitro functional examination of a conserved epitope of L- and E-selectin crucial for leukocyte-endothelial cellinteractions. J. Immunol. 152:5814.

3. Bargatze, R.F., S. Kurk, E.C. Butcher, and M.A. Jutila. 1994. Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells via L-selectin on the rolling cells. J. Exp. Med. in press.

4. Jutila, M. A., R. F. Bargatze, S. Kurk, R. A. Warnock, N. Ehsani, S. R. Watson, and B. Walcheck. 1994. Cell surface P- and E- selectin support shear-dependent rolling of bovine g/d T cells. J. Immunol. in press.

5. Berlin, C., R. F. Bargatze, J.J. Campbell, U. H. von Andrian, M.C. Szabo, S. R. Hasslen, R. D. Nelson, E.L. Berg, S. L. Erlandsen, and E.C. Butcher. 1995. Integrin-mediated Lymphocyte attachment and rolling under physiologic flow. Cell. in press.

APPENDIX

Additional scientific contributions:

A requested review article, for: "Transplantation Science":

"Lymphocyte Tissue-Specific Recruitment From The Blood: A Complex Multi-step Process"

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Summary

We present evidence from three experimental systems which support the hypothesis that tight lymphocyte adhesion to endothelial cells (EC) is regulated by a multi-step process that includes rolling, up-regulation of secondary adhesion molecule affinity through signaling, and permanent arrest. We show that bovine gamma/delta T cells specifically bind E-selectin via a newly described lymphocyte adhesion receptor. Also, bovine gamma/delta T cells *in vivo* home efficiently to 6hr TNF-alpha-inflamed skin which exhibits E-selectin positive venules but, these cells appear to lack the proper signaling or adhesion molecules to transmigrate into the surrounding TNF-inflamed tissue. In a second study employing *in vivo* video microscopy, we show that mouse lymphocytes exhibit a multi-step behavior of binding when interacting with Peyers patch high endothelial venules (HEV). *Bordetella pertussis* toxin (Ptx)-treated lymphocytes roll without arresting, an indication of a potential G-protein signaling event which regulates alterations in secondary-adhesion-molecule affinity during the arresting process. Phorbol myristic acetate (PMA) treatment of Ptx-treated lymphocytes restores the arresting function. The final study, using an *in vitro* shear system to study human peripheral blood lymphocytes (PBL) and bovine gamma/delta T-cell interactions with E-selectin transfectants and human umbilical vein endothelial cells (HUVECs), demonstrates that E-selectin can support L-selectin-independent lymphocyte

rolling. HUVECs, in this system, support all aspects of the *in vivo* three-step binding process, whereas E-selectin L-cell transfectants only support rolling, indicating deficiencies in subsequent steps needed for arrest. In summary, we present evidence from three novel *in vitro* and *in vivo* approaches that strongly support the multi-step hypothesis for lymphocyte binding to EC.

Research papers of key significance, to which the work of this thesis has made significant contributions:

1. A key paper describing bovine γ/δ T cell interactions with P- and E-selectin, is currently in press at the "Journal of Immunology."

"Cell Surface P- and E-Selectin Support Shear-Dependent Rolling of Bovine γ/δ T Cells"

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Summary

The vascular selectins, P- and E-selectin, are inducible adhesion proteins expressed by endothelial cells, which have been shown to support shear-dependent rolling of myeloid cells. This interaction is thought to be a prerequisite event for subsequent steps--tight adhesion/aggregation and transendothelial cell migration--involved in the accumulation of leukocytes into tissues. Certain lymphocyte subsets have also been shown to bind the vascular selectins, but the importance of this interaction in mediating shear-dependent rolling as described for myeloid cells has not been demonstrated. We expand upon our earlier observation that bovine g/d T cells bind E-selectin by showing that this interaction leads to a reproducible rolling event in assays done under shear forces which approximate those that occur in

vivo. E-selectin, expressed by L-cell transfectants or cytokine-stimulated human and bovine endothelial cells, equally supports the shear-dependent rolling interaction. The lymphocyte adhesion proteins L-selectin, CD44, and CD2 do not contribute to this event. Neuraminidase treatment of the g/d T cell or addition of EDTA to the assay completely blocks the rolling interaction. We further show for the first time that P-selectin expressed by thrombin-activated platelets or a soluble P-selectin/human Ig chimera specifically binds g/d T cells. The P-selectin interaction is similar to the rolling event mediated by E-selectin--it requires divalent cations and sialic acid on the lymphocyte, it lacks involvement of L-selectin and CD44, and rolling occurs under physiological shear conditions. These results provide the documentation that the vascular selectins can support shear-dependent rolling of a lymphocyte subset and that P-selectin mediates the adhesion of g/d T cells.

2. A key paper detailing the funtions of α4β7, MadCAM, and VCAM, recently accepted for publication in "Cell."

"Integrin-mediated Lymphocyte Attachment and Rolling Under Physiologic Flow."

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Summary

Of the several families of adhesion receptors involved in leukocyte-endothelial cell (EC) interactions, only the selectins have been shown to

initiate leukocyte interaction under physiologic shear; indeed, ß2 (CD18)-integrins responsible for neutrophil arrest are unable to engage without prior selectin-mediated rolling. In contrast, a4 (CD49d) integrins are shown here to initiate lymphocyte contact *in vitro* under shear and in the absence of a selectin contribution. The a4 integrin ligands MAdCAM-1 and VCAM-1 support loose reversible interactions including rolling, as well as rapid sticking and arrest which is favored following integrin activation. Moreover, a4b7 mediates L-selectin (CD62L)-independent attachment of blood-borne lymphocytes to lamina propria venules *in situ*. Scanning electron microscopy of $a4b7^{hi}$ lymphoid cells reveals that, like L-selectin, a4ß7 is highly concentrated on microvillous sites of initial cellular contact; whereas the b2 integrin LFA-1 is excluded from villi. Thus a4 but not b2 integrins can initiate leukocyte adhesion under flow, a capacity that may be in part a function of topographic presentation on microvilli.


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