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α4 Integrins Mediate Lymphocyte Attachment and Rolling under Physiologic Flow

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Summary

Of the several families of adhesion receptors involved in leukocyte-endothelial cell interactions, only the selectins have been shown to initiate leukocyte interaction under physiologic shear; indeed, β2 (CD18) integrins responsible for neutrophil arrest are unable to engage without prior selectin-mediated rolling. In contrast, α4 (CD49d) integrins are shown here to initiate lymphocyte contact ("tethering") in vitro under shear and in the absence of a selectin contribution. The α4 integrin ligands MAdCAM-1 and VCAM-1 support loose reversible interactions including rolling, as well as rapid sticking and arrest that is favored following integrin activation. Moreover, α4β7 mediates L-selectin (CD62L)-independent attachment of blood-borne lymphocytes to lamina propria venules in situ. Scanning electron microscopy of α4β7^{hi} lymphoid cells reveals that, like L-selectin, α4β7 is highly concentrated on microvillous sites of initial cellular contact, whereas the β2 integrin LFA-1 is excluded from villi. Thus, α4 but not β2 integrins can initiate leukocyte adhesion under flow, a capacity that may be in part a function of topographic presentation on microvilli.

Introduction

The interaction of blood-borne leukocytes with venular endothelial cells (ECs) represents a key control point in leukocyte recruitment at sites of trafficking and inflammation and, thus, is central to the regulation and maintenance of local immune and inflammatory reactions (Picker and Butcher, 1992; Mackay, 1993; Springer, 1994). An understanding of the mechanisms involved may lead to means of suppressing and/or manipulating local immune responses, for example, in autoimmune and other inflammatory disorders. Blood-borne leukocytes pass through venules at high shear rates (Atherton and Born, 1972), so that their initial interaction with the blood vessel wall requires specialized adhesive mechanisms permitting rapid association with vascular ligands. Initial functional "contact" is often followed by transient, reversible "rolling" along the vascular endothelium, a process that may facilitate sampling of the local microenvironment for activating factors that then trigger the leukocyte to activation-dependent sticking and arrest (Kishimoto et al., 1989; von Andrian et al., 1991; Bargatze and Butcher, 1993). Leukocyte adhesion can be regulated at any or all of these sequential steps (contact/rolling, activation, sticking), a fact that may provide for combinatorial diversity and specificity in leukocyte–EC interactions and leukocyte recruitment (Butcher, 1991; Shimizu et al., 1992).

Previous studies focusing on neutrophil interactions with inflamed venules, or with isolated vascular ligands have implicated the selectin family of adhesion receptors in initiating contact and supporting the loose interactions required for rolling, whereas neutrophil β2 integrins are unable to initiate interactions under shear and instead appear specialized to support adhesion strengthening and sticking in response to activation of rolling cells (von Andrian and Arfors, 1993; Abassi et al., 1993; Springer, 1994). These observations have been interpreted to imply that selectins are uniquely specialized for initiating contact and mediating rolling under flow. Lymphocytes, however, express α4 integrins that are not shared by neutrophils. α4β7 (lymphocyte–Peyer's patch adhesion molecule 1 [LPAM-1]) has been implicated as a lymphocyte homing receptor for the mucosal addressin cell adhesion molecule 1 (MAdCAM-1), a vascular ligand selectively expressed in gut-associated lymphoid tissues and in the intestinal lamina propria (LP) (Streeter et al., 1988a; Hu et al., 1992; Berlin et al., 1993; Briskin et al., 1993; Hamann et al., 1994). α4β1 (very late antigen 4 [VLA-4]) is thought to participate in mononuclear cell trafficking to sites of inflammation through interaction with an inducible vascular ligand, the vascular cell adhesion molecule 1 (VCAM-1) (reviewed by Postigo et al., 1993). α4β7 can also bind VCAM-1. Here, we explore the ability of α4 integrins, especially α4β7, to mediate selectin-independent interaction and rolling of lymphocytes under physiologic flow in vitro and in vivo.

Results

α4β7-Mediated Attachment of TK1 Lymphoma Cells under Flow

Mouse TK1 lymphoma cells, which express high levels of α4β7 (Berlin et al., 1993), were assessed for their capacity

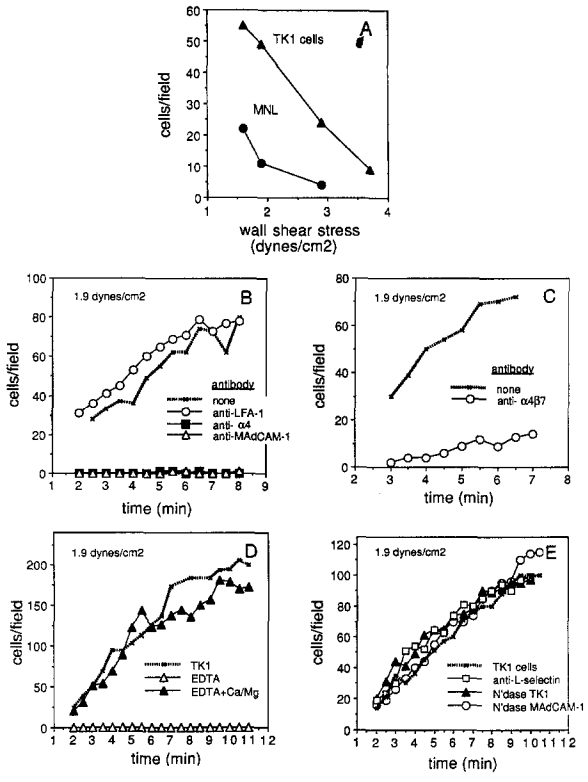

Figure 1. TK1 Lymphoma Cells Use α4β7 to Bind rMAdCAM-1 under Flow

(A) Number of TK1 cells bound per 0.2 mm^2 field, measured 4 min after initiation of flow at the indicated WSSs (dynes/cm^2). LNCs, which express lower levels of α4β7, are shown for comparison. Both cell types were at 1.5 × 10^6 ml. Coated tubes were treated with neuraminidase in this experiment to exclude any contribution of L-selectin (see below), but similar results were observed on untreated rMAdCAM-1 (data not shown). Results of a single representative experiment are shown, but the results of binding under flow were surprisingly consistent from day to day (see for comparison Figures 2 and 3). Estimated rMAdCAM-1 site density is 200/μ^2.

(B and C) TK1 binding to rMAdCAM-1 under shear is blocked by antibodies to α4 and MAdCAM-1, but not LFA1 (B); and by antibodies the α4β7 heterodimer (C). Background binding to serum-coated portions of the tubes was assessed in each experiment and was negligible (<2–3 cells/field at 1.9 dynes/cm^2, 0–1 at higher WSS).

(D) Effect of EDTA on TK1 binding. TK1 cells washed one time in Ca^{2+}/Mg^{2+}-free (CMF) Hank's balanced salt solution (HBSS), incubated in 2 mM EDTA in HBSS, and resuspended in CMF–HBSS failed to interact with rMAdCAM-1 (EDTA). EDTA-treated cells resuspended in HBSS with 2 mM $CaCl_2$ and $MgCl_2$ (EDTA+Ca/Mg) bound nearly as well as control untreated cells.

(E) Lack of significant effect of anti-L-selectin MAb MEL-14, or of neuraminidase treatment of TK1 cells or of the rMAdCAM-1-coated tube, on binding under shear.

to interact with mouse recombinant MAdCAM-1 (rMAdCAM-1) under laminar flow conditions. rMAdCAM-1 was isolated by antibody affinity chromatography from a permanently transfected mouse L1-2 pre-B cell line and was used to coat the interior surface of glass capillary tubes at an estimated density of ~200 sites/μ^2. Freshly cultured TK1 cells were capable of interacting with rMAdCAM-1-coated surfaces, but not with control serum-coated portions of the tube, up to wall shear stresses (WSSs) of >3 dynes/cm^2 (Figure 1A), within the physiologic range of venular shear stresses in vivo (Perry and Granger, 1991).

Binding at 1.9 dynes/cm^2 was highly efficient, leading to rapid stable adhesion of almost all cells coming into close contact with the wall. This was well illustrated in experiments in which the frequency and velocity of noninteracting cells adjacent to the wall was determined. These cells, which display "tumbling" due to shear stresses near the wall, were identified by plane of focus and by lack of visible interaction with the surface. On an anti-MAdCAM-1 blocked tube (experiment illustrated in Figure 1B) at 1.8 × 10^6 TK1 cells/ml and 1.9 dynes/cm^2, ~11 tumbling cells/s passed through a 100 μm bar perpendicular to the plane of flow (as determined by videotape analysis); their mean velocity was 750 ± 106 μ/s (SD), slightly higher than that reported for human neutrophils at similar shear stress (Lawrence and Springer, 1991), as predicted from the larger size of TK1 cells. On rMAdCAM-1-coated surfaces in the same experiment, no tumbling cells were seen over a 2 min observation period, indicating that almost all cells coming in close proximity to rMAdCAM-1 bound rapidly.

Binding was blocked by anti-MAdCAM-1 monoclonal antibody (MAb) MECA-367 and anti-α4 integrin MAb PS2/1 (Figure 1B), by anti-α4β7 MAb DATK32 (Figure 1C), and by EDTA chelation of cations (Figure 1D): under these conditions, only rare cells interacted detectably, indicating that the treatments prevented functional contact required for local accumulation. We attempted to exclude involvement of selectins in the interaction. TK1 cells display little L-selectin (mean ~1% of that expressed by normal lymph node cells [LNCs]) (Berlin et al., 1993), and anti-L-selectin MAb MEL-14 had no effect on adhesion (Figure 1E). Neuraminidase treatment effectively destroys physiologically relevant selectin-binding carbohydrates (reviewed by Lasky, 1992): neuraminidase treatment of TK1 cells or of the rMAdCAM-1-coated tube, alone (Figure 1E) or in combination (data not shown), had no significant effect on TK1 adhesion under flow.

Normal Lymphocyte Attachment through α4β7

Having established the capacity of α4β7 to mediate selectin-independent adhesion under flow, we next assessed the role of this integrin on LNCs, which express much lower levels of α4β7 than TK1 cells but higher levels of L-selectin. L-selectin can support binding to lymph node–derived MAdCAM-1 modified in vivo by L-selectin-binding carbohydrate determinants (Berg et al., 1993), but in earlier studies was unable to support interaction of α4β7$^-$ lymphoid cells (L1-2 pre-B cells expressing transfected L-selectin) with the rMAdCAM-1 employed here (Berg et al., 1993). Nonetheless, to exclude rigorously a contribution of L-selectin to LNC adhesion in the current studies, assays were carried out on rMAdCAM-1-coated tubes pretreated with neuraminidase (NM–rMAdCAM-1). Additionally, in many experiments, LNCs were treated with low concentrations of chymotrypsin (Cx), which cleaves off >95% of L-selectin while leaving integrin-mediated adhesion intact (von Andrian et al., 1992; Jutila et al., 1989; confirmed for the present studies, data not shown). LNCs

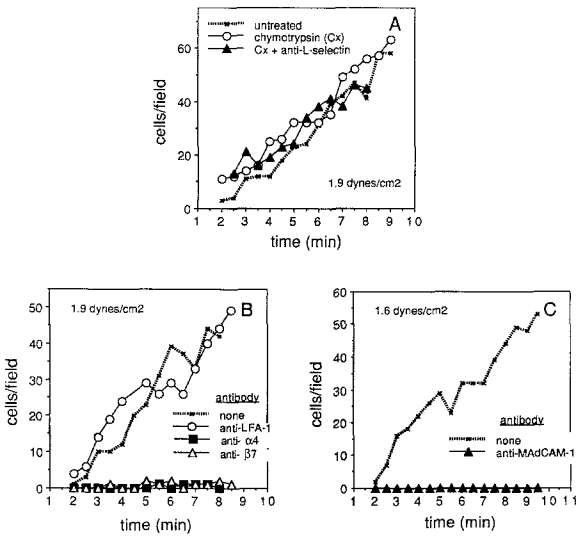

Figure 2. α4β7-Dependent Binding of LNCs to rMAdCAM-1 under Shear

LNCs were assayed for interaction with NM–rMAdCAM-1-coated tubes under shear.

(A) Pretreatment of LNCs with Cx to cleave off L-selectin did not significantly reduce interaction. Anti-L-selectin had no effect on binding of Cx-treated cells.

(B and C) Binding of Cx-treated LNCs is blocked by MAbs to α4, β7 (B), and MAdCAM-1 (C), but not to LFA1 (B). The tumbling velocity of noninteracting LNCs, determined for anti-α4 blocked lymphocytes (B), was ~560 ± 143 μ/s (SD) at 1.9 dynes/cm².

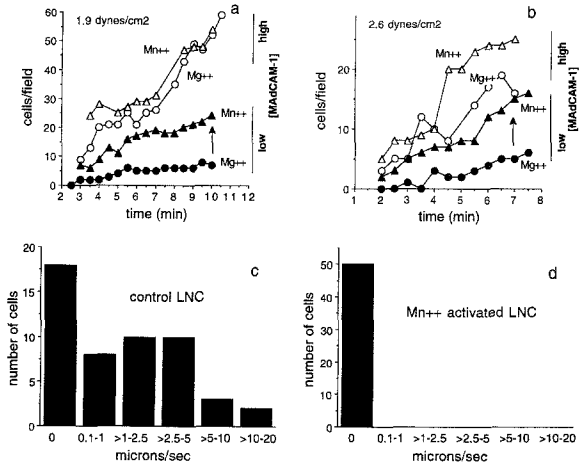

Figure 3. Effect of Mn^{2+}-Induced Integrin Activation on LNC Binding to rMAdCAM-1 under Shear

(a and b) Comparison of the effect of Mn^{2+} activation on Cx–LNC binding to high versus low density NM–rMAdCAM-1 at 1.9 (a) or 2.6 (b) dynes/cm². Enhancement of binding by activation is most evident at lower MAdCAM-1 densities (arrows). Tubes were coated with rMAdCAM-1 as for Figures 1 and 2 (1:10 from stock eluate; high density [~200 sites/μ²]), or at a 10-fold dilution (1:100 from stock eluate; low density [<5 sites/μ²]). LNCs were preincubated with Cx as for Figure 2. Following neuraminidase treatment of the coated tubes, the binding of control versus Mn^{2+}-treated Cx–LNCs was assayed.

(c and d) Effect of Mn^{2+} activation on the behavior of interacting cells. Many control untreated Cx–LNCs roll on rMAdCAM-1 (c), whereas activated cells stick without noticeable rolling (d). The rolling velocities of individual interacting cells were determined from videotapes by measuring the distance moved over 20–60 s. All interacting cells in a field were included, unless they rolled off the field or the field was moved before they could be observed for at least 20 s. 1.9 dynes/cm², high (normal) concentration MAdCAM-1 (~200 sites/μ²).

bound well to rMAdCAM-1 under shear, although their adhesion was less efficient than that of TK1 cells (Figure 1A), and Cx had no significant effect on the extent of LNC interaction (Figure 2A). As expected, anti-L-selectin MAb had no effect on adhesion of Cx-treated cells to NM–rMAdCAM-1 (Figure 2A), nor did anti–lymphocyte function–associated antigen 1 (anti-LFA-1) (Figure 2B). In contrast, as with TK1 cells, anti-α4, anti-β7, and anti-MAdCAM-1 MAbs blocked binding (Figures 2B and 2C), abrogating visible interaction with the tube. These studies demonstrate that α4β7 can initiate lymphocyte contact and support adhesion to its vascular ligand MAdCAM-1 under physiologic shear and in the absence of a selectin contribution.

α4β7 Supports Rolling as Well as Activation-Enhanced Sticking

In nonflow assays, we have shown that α4β7-mediated lymphocyte adhesion to MAdCAM-1 can be enhanced by integrin activation (Berlin et al., 1993), either by treatment of lymphocytes with phorbol esters or by replacement of magnesium (Mg^{2+}) in the medium with manganese (Mn^{2+}). Mn^{2+}-treated LNCs displayed increased adhesion to NM–rMAdCAM-1 under flow as well (Figures 3a and 3b). The effect of integrin activation was most pronounced at lower rMAdCAM-1 densities (coating tubes at a 10-fold greater dilution of rMAdCAM-1, resulting in an estimated site density of <5 sites/μ²), and reflected at least in part an increase compared with unactivated cells in the stability and duration of adhesion events. Not surprisingly, where adhesion was already near optimal (e.g., LNC and, especially, TK1 binding to the normal high density of rMAdCAM-1 employed here), activation had less or no measurable effect (Figures 3a and 3b; data not shown). The results suggest that lymphocytes expressing high levels of α4β7 or displaying preactivated integrins may be particularly suited for selectin-independent trafficking in sites of relatively low MAdCAM-1 density, for example, in small intestinal LP venules (see below and Discussion).

Rolling of leukocytes is a characteristic of selectin-mediated interactions under shear. In contrast, engagement of neutrophil β2 integrins is associated with rapid sticking and arrest. We found that α4β7 could support both of these behaviors. At 1.9 dynes/cm² on high density MAdCAM-1, many Cx–LNCs (Figure 3) or native LNCs (data not shown) displayed irregular rolling with velocities ranging up to 20 μ/s, but with many cells rolling between 1–5 μ/s (Figure 3c). These values are comparable to reported rolling velocities of human neutrophils on P-selectin in vitro (Lawrence and Springer, 1991). Rolling behavior was lost upon integrin activation; Mn^{2+}-treated cells instead displayed rapid stable sticking and arrest (Figure 3d). At the lower MAdCAM-1 density, lymphocytes often displayed very brief "stop-and-go" interactions or irregular

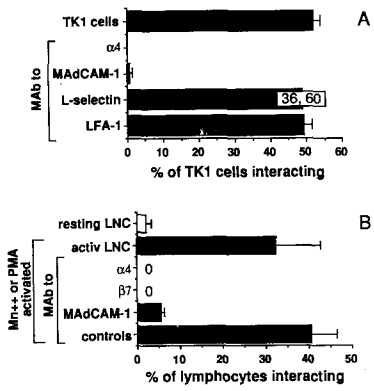

Figure 4. α4β7-Dependent Lymphocyte Attachment to LP Venules In Situ

Fluorescence-labeled TK1 cells (A) or lymph node cells (B) were injected intravenously into anesthetized recipients. Cells entering LP venules were observed in the exteriorized small intestine under epifluorescence microscopy, and their behavior was recorded for subsequent video analysis. The effect of MAbs on the frequency of interaction was assessed either by preincubating and injecting lymphocytes with an excess of MAb (generally 0.25–0.5 mg), or by injecting MAbs after the cells, which allowed assessment of the behavior of the lymphocytes before and after MAb blockade in the same venules. The mean frequency of interaction in three to six experiments is presented with SEM. In cases in which one or two experiments were performed, the mean is illustrated and presented with individual values. In (B), control MAbs included anti-L-selectin (40% of cells interacting), anti-LFA1 (46%), combined treatment with anti-L-selectin and then to LFA-1 (50%), and neuraminidase pretreatment combined with anti-L-selectin (26%).

skipping, whereas Mn^{2+}-activated cells again displayed more frequent sticking. Thus, α4β7 can (like the selectins) support transient interactions, which seem to be favored at lower receptor densities and which can involve either rolling or rapid stop-and-go events, as well as shear-resistant sticking favored at high receptor densities and following integrin activation.

α4β7 Mediates Lymphocyte Contact and Adhesion in LP Venules In Situ

To determine whether α4β7 could support lymphocyte attachment to ECs under physiologic conditions in vivo, we carried out in situ videomicroscopic studies of lymphocyte interactions with venules in the small intestinal LP. These vessels were selected because they express MAdCAM-1, yet are thought to lack L-selectin-binding carbohydrates (reviewed by Picker and Butcher, 1992). The intestine was exteriorized and positioned for epifluorescence videomicroscopic analyses. Sample lymphocytes were labeled with fluorescent dyes and injected intravenously, and the behavior of cells entering LP venules was recorded for subsequent video analyses. As we were interested in assessing the ability of α4β7 to contribute to cell contact and attachment in this physiologic setting, all vessel wall interactions of at least 1 s duration were scored, whether transient or leading to stable arrest.

$\alpha4\beta7^{hi}$ TK1 cells displayed a significant frequency of interaction in LP venules (Figure 4A), and this was completely abrogated by anti-α4 MAbs, which prevented all visible interactions with the vessel wall. Anti-MAdCAM-1 MAb MECA-367 also blocked functional contact but, as expected from the lack of significant L-selectin expression by TK1 cells, anti-L-selectin MAb had no effect. Anti-LFA-1 MAb also failed to prevent attachment (Figure 4A).

In contrast with TK1 cells, with rare exceptions, resting LNCs failed to interact detectably with the venular wall, confirming the inability of L-selectin to mediate attachment in this site. Integrin activation with Mn^{2+}, or by brief pretreatment with phorbol myristate acetate (PMA), however, led to a substantial frequency of attachment, nearly comparable in some experiments to that of TK1 cells; and this was also inhibited by anti-α4, anti-β7, and anti-MAdCAM-1 MAbs (Figure 4B). In contrast, in separate individual experiments, activated lymphocytes retained the ability to interact well in the presence of MAbs to L-selectin, LFA-1, combined L-selectin plus LFA-1, or following neuraminidase pretreatment plus anti-L-selectin (data pooled as control MAb treatments in Figure 4B).

Although our focus in these in situ studies is on the ability of α4β7 to initiate functional interactions, whether transient or stable, it is also of interest that the behavior of lymphocytes during contact with the venular wall was variable: some TK1 or activated LNCs displayed very brief (<1 s) glancing or stop-and-go contact (not scored for data analysis, but also inhibited by anti-α4β7 and anti-MAdCAM-1 MAbs). Most cells, however, interacted for ⩾1 s, and of these, the majority displayed rapid sticking (as seen most often for activated lymphocytes and TK1 cells in vitro), although in vivo many arrested cells subsequently released back to the circulation within a few seconds. Rolling and mixed behaviors were also common, however, with 15%–25% of interacting TK1 cells rolling for 1 s or longer in different experiments.

The WSS in LP venules was calculated using the maximal velocity of noninteracting cells as an estimate of centerline blood velocity and determining mean venule diameters from videotape analyses. Calculated values ranged from 11–34 dynes/cm^2 (mean 18 ± 7 SD, n = 10 venules from four animals).

These studies strongly support the ability of α4β7 to mediate selectin-independent contact and attachment of lymphocytes to MAdCAM-1 in LP venules in situ. They also confirm the importance of integrin activation in regulating lymphocyte interactions with ECs under physiologic conditions.

α4β7 Is Presented on Microvilli

Leukocytes in the blood are characterized by a remarkable display of cell surface microvilli, which are thought to represent the principal sites of initial contact with the vascular endothelium under flow (van Ewijk, 1980). L-selectin, which can initiate leukocyte interactions with its vascular ligands under shear (von Andrian et al., 1991; Berg et al., 1993; Lawrence et al., submitted), is displayed selectively on these microvillous processes (Picker et al., 1991; Erlandsen et al., 1993). Scanning electron microscopy of TK1 cells reveals the typical topography of an unactivated lymphocyte with numerous microvilli extending out from the

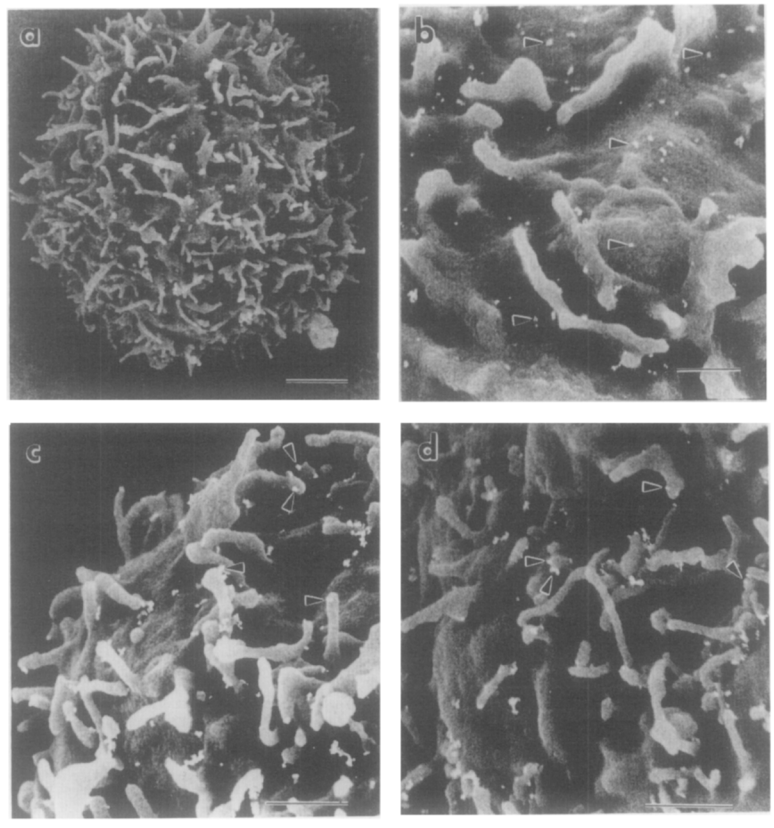

Figure 5. Immunolocalization of Cell Adhesion Molecules on Unstimulated TK1 Cells by Low Voltage Scanning Electron Microscopy

(a) Low magnification image illustrating the surface morphology of TK1 cells (bar = 1.0 μm). (b) High magnification image of cells stained for the β2 (CD18) integrin LFA1 (bar = 200 nm). Note immunogold staining on membrane surface of cell body (arrowheads). (c and d) immunogold staining for (c) α4 and (d) β7 (bar = 400 nm). Both α4 and β7 are predominantly detected on microvilli and membrane ruffles (arrowheads).

cell body (Figure 5a), and immunogold staining for α4 or β7 (Figures 5c and 5d) demonstrates that, like L-selectin, the α4 and β7 integrin chains are localized primarily on these processes, with many of the gold particles at or near the tips of the microvilli. Analyses by stereoscopic examination reveals that ~80% of gold particles labeling α4 or β7 were detected on microvilli (α4: 79% on villi, n = 5 cells, 938 gold particles analyzed; β7: 79%, n = 6, 1063 gold particles). This distribution is in sharp contrast to that of LFA-1 (a_Lβ2), which, as shown previously for the β2 integrin Mac-1 on unactivated neutrophils (Erlandsen et al., 1993), is largely excluded from TK1 microvilli: (β2: 88% on planar cell body, n = 5 cells, 1135 gold particles) (Figure 5b).

Lymphocyte Attachment through α4 Integrin-VCAM-1 but Not LFA-1 ($\alpha_L\beta2$)-ICAM-1 Pathways under Shear

To determine whether the capacity to initiate functional contact is unique to MAdCAM-1, or is shared with the related α4 integrin ligand VCAM-1, we assessed lymphocyte interactions with VCAM-1 under flow. We also compared interactions with intercellular adhesion molecule 1 (ICAM-1), an LFA-1 ligand. In the representative experiment illustrated in Figure 6, VCAM-1 was coated at a density of ~37 sites/μ^2; MAdCAM-1 was titred to yield similar densities of ~20 sites/μ^2; and ICAM-1 was used at ~400 or 1000 sites/μ^2. The ICAM-1 was functional in static assays (data not shown), binding activated LNCs as well as or better than MAdCAM-1. In contrast, MAdCAM-1 and VCAM-1 but not ICAM-1 supported attachment and accumulation of Mn^{2+}-activated LNCs at 1.9 dynes/cm^2. Detectable interactions with ICAM-1-coated segments of the tube were extremely infrequent.

Interactions of LNCs with VCAM-1-coated tubes were greatly enhanced by Mn^{2+} pretreatment (Figure 7a), indicating that integrin activation is important for lymphocyte contact and adhesion through VCAM-1 as well as through MAdCAM-1 under shear at these relatively low site densities. As on MAdCAM-1, resting LNCs interacting with VCAM-1 displayed transient stop-and-go and irregular rolling behaviors, whereas rapid arrest was favored following integrin activation. As shown in Figure 7b, treatment with anti-α4 or anti-VCAM-1 abrogated all visible interaction. Anti-β7 MAbs, which completely blocked interaction with MAdCAM-1 assayed in parallel (data not shown), reduced binding to VCAM-1 only minimally. The efficent interaction of anti-β7-treated LNCs with VCAM-1 is consistent with participation of α4β1.

To confirm the ability of α4β1 to mediate binding to VCAM-1 under shear, we assessed interaction of the L1-2 lymphoma cell line, which expresses α4β1 but not β7 or L-selectin (Andrew et al., 1994). Although L1-2 cells are considerably larger than LNCs or TK1 cells and tumble at significantly higher velocity (940 ± 160 μ/s), they nonetheless interacted well, and this was inhibited by anti-α4 and by anti-VCAM-1 MAbs (Figure 7c). TK1 cells, which express α4β7 but not α4β1, also interact with VCAM-1,

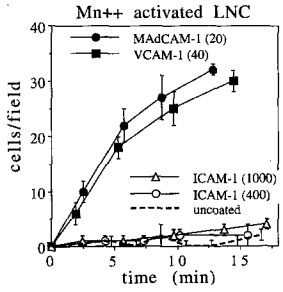

Figure 6. Mn^{2+}-Activated LNCs Interact with rMAdCAM-1 and VCAM-1 but Not ICAM-1-Coated or Control Uncoated Tubes under Shear

In these experiments, rMAdCAM-1 was coated at ~20 sites/m^2, VCAM-1 at ~40 sites/m^2, and ICAM-1 at either ~400 or ~1,000 sites/μ^2 as indicated in parentheses. All assays at 1.9 dynes/cm^2.

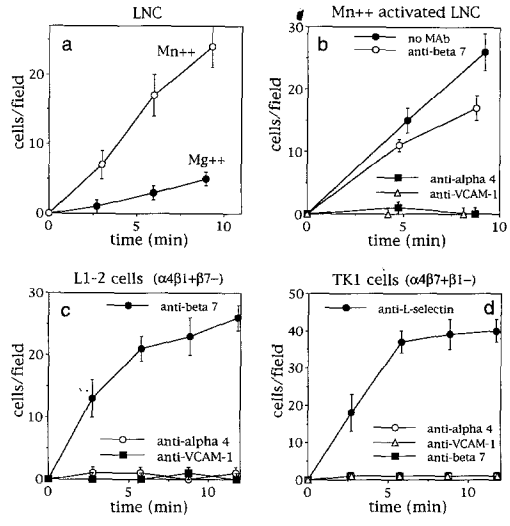

Figure 7. Effects of Activation and of Antibodies on Interactions with VCAM-1 under Flow

All assays are at 1.9 dynes/cm^2.

(a) Comparison of binding of Mn^{2+}-activated versus control unactivated LNCs to VCAM-1 under shear (~50 sites/μ^2).

(b) Effects of anti-α4, anti-β7, and anti-VCAM-1 MAbs on activated LNC binding to VCAM-1 under shear (VCAM-1 ~50 sites/μ^2).

(c) Binding of $\alpha4\beta1^+\beta7^-$ L1-2 lymphoma cell line to VCAM-1 under shear is inhibited by antibodies to α4 and to VCAM-1. Anti-β7 MAb pool served as the nonbinding antibody control in this experiment. (VCAM-1 ~100 sites/μ^2).

(d) Binding of $\alpha4\beta7^{hi}\beta1^-$ TK1 cells to VCAM-1 under shear. Interactions are inhibited by MAbs to α4, β7, and VCAM-1 (VCAM-1 ~100 sites/μ^2). Note that TK1 cells express high levels of α4β7, substantially greater than the levels of α4β1 expressed by L1-2 cells (as indicated by comparisons of binding of anti-α4 MAb PS2/1). L1-2 cells are also larger and, thus, experience greater forces under comparable shear. Therefore, no conclusions should be drawn from the results presented regarding the relative abilities of α4β7 versus α4β1 to initiate contact with VCAM-1 under flow.

and this is blocked by anti-β7 as well as by anti-VCAM-1 and anti-α4 MAbs (Figure 7d), demonstrating that α4β7 can also recognize VCAM-1 under shear.

Discussion

Recent models suggest that the specificity of lymphocyte homing is determined by a multistep process (Butcher, 1991; Shimizu et al., 1992; Picker and Butcher, 1992; Springer, 1994) encompassing a primary transient contact/adhesion event allowing sampling of the vessel wall, a rapid activating event that in high endothelial venules (HEVs) involves G protein–linked signaling receptors (Bargatze and Butcher, 1993), activation-triggered sticking and arrest, and subsequent chemotaxis and extravasation. Although originally framed as a general model in which adhesion molecules of different classes might participate in both primary and activation-dependent interactions (Butcher, 1991), it has subsequently become widely assumed based on studies of neutrophils that initiation of contact and rolling under flow is the exclusive province of selectins. Our in vitro results indicate, however, that the integrins α4β7 and α4β1 can also initiate primary contact and can support subsequent transient interactions including rolling as well as activation-enhanced firm adhesion and arrest. The ability of α4 integrins to initiate functional contact and to mediate attachment under physiologic conditions is confirmed by the observation of α4β7-dependent interactions of TK1 cells and of activated LNCs with LP venules in situ. These findings require revision of the dogma that the "adhesion cascade" leading to leukocyte recruitment must be initiated by selectins.

The ability of α4β7 to mediate adhesion to MAdCAM-1 under flow is enhanced by integrin activation. In vitro, this effect was minimal at the highest MAdCAM-1 densities employed (~200 sites/μ^2), but dramatic when MAdCAM-1 levels were limiting. The effect was even more striking in vivo, as only rare LNCs interacted at all with the LP venules in the small intestine in the absence of integrin activation. LP venules display relatively low levels of MAdCAM-1 compared with Peyer's patch HEV, and they also fail to stain detectably with soluble L-selectin constructs or with MECA-79, an antibody whose epitope is associated with HEV ligands for the L-selectin (Streeter et al., 1988b; Berg et al., 1991, 1993): consistent with this, constitutive L-selectin expression on resting LNCs was unable to initiate lymphocyte contact in this site. Our observations suggest that lymphocytes displaying preactivated integrins (e.g., gut-homing immunoblasts) may be particularly suited for trafficking to the small intestinal LP. In fact, this pattern of selective trafficking is well documented: ^{125}IUdR-labeled mesenteric lymph node (MLN) or thoracic duct lymphoblasts home much more efficiently than resting (primarily naive) LNCs to the small intestine (reviewed by Picker and Butcher, 1992), and this homing is inhibited by anti-α4 or β7 MAbs (Hamann et al., 1994). $\alpha4\beta7^{hi}$ memory cells (Schweighoffer et al., 1993; Erle et al., 1994), many of which are L-selectin$^-$ (L. Rott and E. C. B., unpublished data), may also be able to traffic via MAdCAM-1^{lo} LP venules. Indeed, a specialized subset of memory T cells recirculates through the intestines, and recent studies confirm that these gut-recirculating T cells are predominantly $\alpha4\beta7^{hi}$ and L-selectin$^-$, whereas memory T cells recirculating through cutaneous sites are predominantly $\alpha4\beta7^-\beta1^{hi}$

(Mackay, 1993; C. Mackay and E. C. B., unpublished data). It is important to note that our results suggest that lymphoblasts expressing preactivated integrins, and possibly memory cells displaying very high levels of α4β1 or α4β7, may have an ability to attach and arrest on ECs without any requirement for local, vessel-associated activating signals, thus bypassing step 2 (activation) in the general multistep paradigm of leukocyte–EC recognition mentioned above. It will be important to assess the requirement for G protein signaling in lymphoblast interactions with LP venules in situ and in subsequent diapedesis.

HEVs in the mucosal Peyer's patches and MLNs express much higher levels of MAdCAM-1 than LP venules. Furthermore, these HEVs can modify MAdCAM-1 as well as other glycoproteins with L-selectin-binding carbohydrate ligands (Berg et al., 1993), allowing participation of both L-selectin and α4β7 in lymphocyte homing to these mucosal lymphoid organs (Hamann et al., 1994). This combination of ligands may help explain the remarkably efficient homing of naive lymphocytes (which are uniformly L-selectin^{hi}, α4β7^{lo}) to Peyer's patches: L-selectin would facilitate initial attachment, and the expression of high levels of MAdCAM-1 should support efficient binding even of naive α4β7^{lo} lymphocytes. Consistent with this model, recent in vivo homing studies demonstrate that both anti-L-selectin and anti-α4 or β7 MAbs display significant inhibition of short-term homing of LNCs to Peyer's patches (Hamann et al., 1994).

It is of interest that WSSs that support selectin and α4 integrin-mediated leukocyte contact in vitro (generally ≤4 dynes/cm^2) are at the very low end of physiologic shear stresses reported for postcapillary venules that support leukocyte rolling in vivo. In the present studies, for example, TK1 cells and activated LNCs interacted reasonably efficiently with LP venules in situ, in spite of physiologic shear rates in the range of 11–34 dynes/cm^2. In contrast, under the controlled laminar flow conditions in vitro, few if any interactions were detectable above 4 dynes/cm^2. A variety of rheological and physical parameters may operate in vivo to enhance the efficiency of interactions under flow, including displacement of leukocytes from the midline by erythrocytes (von Andrian and Arfors, 1993), nonlaminar flow conditions associated with branch points or turns in vessels and irregularities in the EC lining, and turbulence produced by previously adherent leukocytes.

Microvillous projections represent the initial site of cell–cell contact under flow, and we have shown here that α4β7 is concentrated on these projections. L-selectin, which is also highly effective at initiating contact under shear, is similarly concentrated on microvilli (Picker et al., 1991; Erlandsen et al., 1993), suggesting that this may be a common feature of molecules specialized for initiating leukocyte–EC interactions under physiologic conditions. The microvillous distribution of L-selectin and α4β7 is in contrast with the β2 integrins, which are concentrated on the nonvillous planar cell body (Erlandsen et al., 1993). Our present results as well as others demonstrate that β2 integrins are largely unable to initiate functional contact in vitro or in vivo under physiologic shear (von Andrian and Arfors, 1993; Springer, 1994). We propose that the exclusion of β2 integrins from the tips of microvilli may in fact ensure that they participate selectively in events that follow initial cellular contact in vivo, possibly including slowing of rolling as well as activation-dependent sticking. It will be important to determine the topographic distribution of other integrins implicated in EC interactions, including α4β1 but also a6β1 (Dunon and Imhof, 1993), to define the molecular signals that target α4β7 and L-selectin to microvilli, and to ask whether the topographic distribution of receptors can be regulated independently of overall levels of expression. Such regulation could allow an additional level of control of the ability of adhesion receptors to initiate leukocyte attachment under flow.

The ligand densities that support α4 integrin-mediated lymphocyte contact and rolling or adhesion are similar to those reported to support rolling of neutrophils on P-selectin (Lawrence and Springer, 1991). Interaction of α4β7^{hi} TK1 cells is extremely efficient on rMAdCAM-1 at ~200 sites/μ^2, with almost all cells in the focal plane of the capillary wall attaching rapidly. At lower ligand densities, contact is less efficient and, when initiated, often leads to irregular jerky rolling and skipping behaviors. Transient irregular interactions were exaggerated in experiments at the lowest MAdCAM-1 densities (e.g., <5 sites/μ^2 in Figure 3). It is likely that such irregular behavior reflects the making and breaking of individual microvillous contacts, perhaps involving only one or a few molecular bonds. In scanning electron microscopic images of glutaraldehyde-fixed TK1 cells, the diameters of microvilli range from 60–100 nm, and the area of a microvillous tip can be roughly approximated as ≤0.01 μ^2. The area available for interaction under flow is likely to be in this range as well (although a somewhat larger area could be available if microvilli are flexed). Moreover, it appears that no more that 50–100 microvilli could reasonably contact a surface at a given instant in the absence of extensive cellular deformation. Thus at 50 sites/μ^2, each microvillus is likely to contact 0 or 1 sites on average; and as a rough approximation, the complement of microvilli available for interaction at any instant could potentially contact 25–50 sites. The ability of cells to initiate functional contact and roll even if unevenly under these circumstances suggests that lymphocytes must be quite efficient at taking advantage of these potential interactions. The high density of α4 integrins and L-selectin receptors on the tips of microvilli may be critically important in this regard.

We have focused here primarily on interactions of α4β7 with its vascular ligand MAdCAM-1. MAdCAM-1 is a mucosal vascular addressin, selectively expressed by venules involved in lymphocyte trafficking to mucosal tissues (Streeter et al., 1988a). The α4 integrins can also bind to VCAM-1, however, a related EC immunoglobulin family member that is induced on ECs in sites of tissue inflammation and that serves as a ligand for α4β1 and for activated α4β7, and we have shown that LNCs and α4β1$^+$β7$^-$ L1-2 lymphoid cells can make contact and roll on VCAM-1 and that attachment and accumulation on VCAM-1 are enhanced by integrin activation. α4β7 on α4β7^{hi} TK1 cells can also initiate adhesion to VCAM-1 but anti-β7 MAbs reduce LNC interaction with VCAM-1 only slightly, sug-

gesting that α4β1 may predominate in LNC interactions with VCAM-1 under shear (as reported previously based on nonflow assays; Berlin et al., 1993). Our results indicate that the α4 integrins are highly versatile, with the potential to play a critical role in initiating physiologic interactions and rolling of lymphocytes under flow in vivo, as well as in secondary, activation-dependent sticking and arrest. This conclusion is supported by the recent demonstration that α4β1 can participate in rolling of eosinophils in situ in interleukin-1-activated rabbit mesenteric venules (Sriramarao et al., 1994) and by independent studies of α4β1-dependent lymphocyte attachment to VCAM-1 transfectants or protein or to ECs under flow (Wolber et al., 1993; Jones et al., 1995; Alon et al., 1995; Stoolman et al., unpublished data). In the case of lymphocytes, the contribution of the α4 integrin:VCAM-1 pathway to primary attachment is likely to be especially important for trafficking of immunoblasts and/or L-selectin$^-$ memory lymphocyte subsets to sites of tissue inflammation.

In conclusion, the α4 but not β2 integrins can mediate lymphocyte contact and adhesion under physiologic shear and in the absence of selectin involvement. Adhesion is sensitive to and can therefore be regulated physiologically by multiple parameters, including the following: the level of integrin expression, the extent of integrin activation, the density of ligand decorating target surfaces, and the local microvascular hemodynamics. These parameters likely work in concert with engagement of selectins and of other adhesion and activating factors, to fine-tune the homing of lymphocytes to mucosal lymphoid tissues and to the intestinal wall, and to tissue sites of inflammation. Finally, our results suggest that topographic presentation 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.

Experimental Procedures

Antibodies

MAbs utilized were the following: anti-α4 PS/2, anti-VCAM-1 MK2.7, anti-LFA-1 α chain FD441.8, anti-L-selectin MEL-14, anti-ICAM-1 YN1/1.7 (ATCC); anti-α4β7 heterodimer DATK32 and anti-β7 MAbs FIB22, FIB21, FIB30, and FIB504 (Andrew et al., 1994); anti-CD44 MAb MJ64 (M. Jutila and E. C. B., unpublished data); and anti-MAdCAM-1 MECA-367 (Streeter et al., 1988a).

Lymph Node Lymphocytes and Cell Lines

The AKR/Cum TK1 cell line and the C57L pre-B lymphoma line L1-2 have been described (Butcher et al., 1980; Hu et al., 1992; Berlin et al., 1993) and were cultured in RPMI 1640 with 5% iron supplemented bovine calf serum (BCS) (Hyclone Labs, Logan, UT). TK1 variants exhibiting plastic adherence were excluded from these assays. LNCs were isolated from 6- to 8-week-old BALB/c mice, resuspended in RPMI plus 5% BCS 20 mM HEPES (pH 7.0) medium at room temperature unless otherwise specified, and then used within 45–60 min.

Assay of Adhesion under Flow

rMAdCAM-1 was isolated by MECA-367 antibody affinity chromatography as described (Berlin et al., 1993; Nakache et al., 1989; purity of isolated MAdCAM-1 is illustrated for example in the latter reference) from lysates of mouse L1-2 pre-B lymphoid cells stably transfected with pMAd7 cDNA (Briskin et al., 1993) in pMRB101 (L1-2$^{MAdCAM-1}$ cells; see Berg et al., 1993 for transfection protocols). Isolated rMAdCAM-1 in elution buffer containing 1% β-octylglucoside was diluted below the critical micelle concentration with phosphate buffered saline (PBS), ~50 μl was taken up into one half of a glass capillary tube (100 μl disposable "microcaps", 1.025 mm I.D., Drummond Scientific Company, Broomall, PA), and the tubes were incubated in humidity at 4°C overnight. After coating, tubes were blocked with 100% BCS. Cells (1×10^6–2×10^6/ml in different experiments) were transfused at uniform flow rate through the capillary tubes using a syringe pump (Model 33, Harvard Instruments). Except as indicated, cells were only used for a single passage. After a 1–2 min stabilization period, the number of cells binding per randomly selected 0.2 mm^2 microscope field was determined at 30 s intervals by computerized image analysis of videotapes. WSSs were calculated from Poiseuille's law for Newtonian fluids, with viscosity 0.01 P. [WSS in dynes/cm^2 = mean flow velocity (mm/s) × (8 ÷ tube diameter (mm)) × viscosity (P)].

ICAM-1 and VCAM-1 were isolated from mouse spleen lysates by affinity chromatography on YN1/1.7- or MK2.7-conjugated Sepharose columns, and flow assays were performed as above except that for data collection six 0.2 mm^2 microscope fields were recorded as a group in rapid succession (one each 15 s) from one coated region of the tube. The microscope was then shifted to an uncoated region for two control fields and then to another coated area for six. The number of cells binding was determined in each field recorded, and the mean for each group calculated.

Ligand densities were determined by saturation binding of radiolabeled MAbs as described by Lawrence and Springer (1991). MAbs against MAdCAM-1 (MECA-367), ICAM-1 (YN1/1.7), or VCAM-1 (MK2.7) were iodinated (Iodo-Beads, Pierce, Rockford, IL) to known specific activity (1×10^{17}–5×10^{17} CPM/M in different experiments) and incubated above saturation in coated capillary tubes or in control mock-coated or, in some experiments, irrelevant ligand-coated capillary tubes. After extensive washing, the tubes were counted for calculation of site densities assuming monomeric binding (Lawrence and Springer, 1991). Site densities for a given ligand preparation showed variation from day to day within a 2-fold range. Densities for rMAdCAM-1 as used in Figures 1–4 were determined after the experiments, using the same fraction of purified rMAdCAM-1 coated under identical conditions. Densities for Figures 6 and 7 were determined concurrently with the adhesion assays presented, on tubes coated in parallel.

Antibody, Enzyme, and Ion Treatments

For in vitro antibody inhibition studies, all antibodies were used at 5- to 10-fold over saturating levels as determined by flow cytometry (10–25 μg/ml). Anti-lymphocyte antibodies were preincubated with test cells for 20–30 min at room temperature and were not washed away prior to assay. FIB30 was used for β7 inhibition in Figures 1, 2, and 4; a pool of FIB30, FIB504, and DATK32 MAbs was employed for Figures 6 and 7. Anti-MAdCAM-1 MAb MECA-367 was incubated at 50 μg/ml in the tube for 20 min at room temperature.

For neuraminidase treatment, lymphocytes at 10^7 cells/ml or the coated tubes were treated with neuraminidase (sialidase) from Arthrobacter ureafaciens (Boehringer, Mannheim, Germany) at 40 U/ml in medium for 30 min at 37°C; this treatment effectively cleaved the sLe^x-associated HECA-452 epitope from U937 cells (>98% reduction in specific staining).

For chymotrypsin treatment, LNCs were incubated 5 min at 37°C in HBSS plus 10 mM HEPES (pH 7.0) containing 0.1 U/10^6 cells α-chymotrypsin (Fraction IV-S from bovine pancreas, Sigma, St. Louis), washed, and resuspended in medium for assay. Cx treatment reduced staining by anti-L-selectin MAb MEL-14 by >97%.

For activation, LNCs were incubated at room temperature in Ca^{2+}/Mg^{2+}-free HBSS plus 10 mM HEPES (pH 7.0) (HBSS/HEPES) containing 2 mM EDTA and were then diluted 3-fold with Ca^{2+}/Mg^{2+}-free HBSS/HEPES, pelleted, and resuspended in HBSS/HEPES containing either 2 mM $CaCl_2$ and $MgCl_2$ (control cells), or 2 mM $CaCl_2$ and 1 or 2 mM $MnCl_2$ (Mn^{2+}-activated cells).

In Situ Videomicroscopic Analyses of Lymphocyte Interactions with LP Venules

In situ videomicroscopic analyses were carried out as described (Bargatze and Butcher, 1993) except that the exteriorized bowel segment was positioned for epifluorescence microscopy and video recording of LP venules in the small intestine. Approximately 2.5×10^7 fluorochrome-labeled cells (either TK1 or LNC) in 0.5 ml DMEM were injected

intravenously into the tail vein. In some experiments, TK1 cells or lymphocytes were pretreated with anti-lymphocyte MAbs (100 μg/ml) at room temperature for 20 min (not washed) before injection with an excess of MAb (250–500 μg). In others, lymphocytes were injected and observed for 3–7 min and then 250 or 500 μg of MAb was infused intravenously with or without additional cells, allowing visualization of the effect of MAb in the same vessels. Analyses were routinely initiated immediately after infusion of sample cells. When antibody was administered after initial cell infusion, the antibodies were allowed to circulate and achieve saturation for 2 min (for anti-lymphocyte antibodies) or ~5 min (for anti-MAdCAM-1 MAb) prior to further analysis of cell behavior. All cells entering the observed venules were analyzed for each treatment (range 25–100 cells per experiment). Interactions of ⩾1 s were considered significant and were scored. For determination of WSS in vivo, ten venules from four different animals were analyzed. In each venule, the velocity of ten noninteracting lymphocytes was assessed. The highest velocity observed was taken as a conservative estimate of centerline blood velocities (range 2.2 to 5.0 mm/s). The mean diameter of vessels was measured by videotape analysis (range 12–42 μ). WSSs were calculated from these parameters as described (von Andrian et al., 1992).

Scanning Electronmicroscopy

TK1 cells were stained with primary MAbs against LFA1 (FD448.1), α4 (PS2/1), and β7 (FIB21), incubated with secondary goat anti-mouse immunoglobulin 12 nm colloidal gold, and prepared for imaging as described (Erlandsen et al., 1993). No significant staining was seen with second stage only. Samples were examined using a Hitachi S-900 field emission SEM equipped with a YAG crystal for high resolution backscatter electron detection at an accelerating voltage of 3.5 kV.

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