

The Integrin VLA-4 Supports Tethering and Rolling in Flow on VCAM-1

Ronen Alon,* Paul D. Kassner,‡ Michelle Woldemar Carr,* Erik B. Finger,* Martin E. Hemler,‡ and Timothy A. Springer*

Harvard Medical School, Department of Pathology; and *The Center for Blood Research, Boston, Massachusetts 02115; and ‡Dana-Farber Cancer Institute, Boston, Massachusetts 02115

Abstract. Selectins have previously been shown to tether a flowing leukocyte to a vessel wall and mediate rolling. Here, we report that an integrin, VLA-4, can also support tethering and rolling. Blood T lymphocytes and $\alpha 4$ integrin-transfected cells can tether in shear flow, and then roll, through binding of the integrin VLA-4 to purified VCAM-1 on the wall of a flow chamber. VLA-4 transfectants showed similar tethering and rolling on TNF-stimulated endothelium. Tethering efficiency, rolling velocity, and resistance to detachment are related to VCAM-1 density. Tethering and

rolling did not occur on ICAM-1, fibronectin, or fibronectin fragments, and tethering did not require integrin activation or the presence of an $\alpha 4$ cytoplasmic domain. Arrest of rolling cells on VCAM-1 occurred spontaneously, and/or was triggered by integrin activating agents Mn^{2+} , phorbol ester, and mAb TS2/16. These agents, and the $\alpha 4$ cytoplasmic domain, promoted increased resistance to detachment. Together the results show that VLA-4 is a versatile integrin that can mediate tethering, rolling, and firm arrest on VCAM-1.

IMMIGRATION of leukocytes at inflammatory sites is regulated by traffic signal molecules that are displayed together on endothelium. These signals are widely held to act on leukocytes in a sequence, as first shown for neutrophils and hypothesized for monocytes and lymphocytes as well (11, 14, 41, 42, 62, 65, 66, 72, 75). In this model, selectins that bind to carbohydrate ligands allow flowing cells to tether and subsequently roll along the vessel wall. This labile adhesion to the vessel wall brings the cell into proximity with chemoattractants that are released from tissue or displayed on the surface of endothelium. Chemoattractants bind to receptors that couple to G proteins, which transduce signals that activate integrin adhesiveness. Integrins on leukocytes can then bind to Ig superfamily members on endothelium, mediating firm adhesion, and causing arrest of the rolling leukocyte. After directional cues from chemoattractants and using integrins for traction, leukocytes then cross the endothelial lining of the blood vessel and enter the tissue. These three steps, with multiple molecular choices at each step, provide great combinatorial diversity in signals and may explain the selective localization of leukocyte classes and subsets in different inflammatory states. While this paradigm is widely accepted for neutrophils, much remains to be worked out for monocytes, eosinophils, and in particular, for lymphocytes. These latter cells, but not neutrophils, express the integrin VLA-4. VLA-4 ($\alpha 4\beta 1$, CD49d/CD29) is an un-

usual $\beta 1$ integrin that functions as both a matrix and cell receptor (27, 32, 48, 66). As a matrix receptor, it binds to an alternatively spliced domain of fibronectin distinct from the classical cell binding site recognized by VLA-5. As a cell receptor, it binds to domains 1 and 4 of VCAM-1, an Ig superfamily member induced by inflammatory mediators on endothelium. Both VLA-4 and the integrin LFA-1 are important in vivo for immigration of T lymphocytes into inflammatory sites.

Current models suggest that the first step in accumulation of leukocytes at inflammatory sites is mediated by selectin molecules (11, 66). The L-selectin molecule expressed on leukocytes and the E-selectin and P-selectin molecules expressed on vascular endothelial cells and platelets have been shown to mediate tethering and rolling of leukocytes in shear flow. This has been demonstrated in vitro using purified proteins, and endothelial cell or platelet monolayers; in vivo with inhibitory mAb, or fusion proteins; and genetically in inherited deficiency diseases or gene knockout mice (1, 2, 8, 12, 36, 42, 43, 45, 46, 47, 54, 70, 72). In almost all cases, these studies have focused on neutrophils. Much less is known about how lymphocytes tether and roll, particularly in the case of the memory subset of lymphocytes. The majority of memory lymphocytes lack expression of L-selectin (10, 38, 51, 69). A subpopulation of lymphocytes that expresses the cutaneous lymphocyte-associated (CLA)¹ carbohydrate antigen can bind to E-selectin (57, 63). This CLA⁺

Please address all correspondence to Dr. Timothy A. Springer, The Center for Blood Research, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115. Tel.: (617) 278-3200; Fax: (617) 278-3232.

1. *Abbreviations used in this paper:* CB, cytochalasin B; CLA, cutaneous lymphocyte-associated.

subset of 10–15% of peripheral blood T cells is in the L-selectin⁺ lymphocyte subset (58). Thus, a major population of memory lymphocytes lacks both L-selectin and the ligand for E-selectin. A subset of lymphocytes can also bind to P-selectin (19, 55, 59). The subset of lymphocytes or lymphocyte clones that can tether and roll on purified E-selectin and P-selectin do so considerably less efficiently than neutrophils (4) (Alon, R., and T. Springer, unpublished).

We report here that an integrin, VLA-4, can mediate tethering and rolling of lymphocytes or transfected K562 cells in shear flow. Tethering and rolling occur on VCAM-1 but not on fibronectin. Integrin activation is not required for tethering and rolling, showing that interaction between VLA-4 and VCAM-1 can precede activation events that are required for development of firm adhesion through other integrins such as LFA-1 and Mac-1. Additional experiments using integrin-modulating agents, cytochalasin B treatment, and truncation of the $\alpha 4$ subunit provide several key insights into VLA-4 function, not previously observable in static adhesion assays.

Materials and Methods

Monoclonal Antibodies

Monoclonal antibodies used in this study as purified Ig's were TS2/16 (anti- $\beta 1$), (28), and HP 1/2 (anti- $\alpha 4$) (60). The A4-PUJ1 (anti- $\alpha 4$) mAb (Pujades, C., unpublished observation) and mAb 13 (anti- $\beta 1$) (3) and mAb CBRM1/34 (CD11b) (20) were used as ascites.

Preparation of Substrates

Affinity purified, recombinant 7 domain VCAM-1 (56), a generous gift of Dr. R. Lobb, Biogen, Cambridge, ICAM-1 (52), human fibronectin (FN, GIBCO BRL, Gaithersburg, MD) or its 40-kD CS-1-containing fragment (GIBCO BRL) were dissolved in PBS buffered with 10 mM bicarbonate, pH 9.1, at the indicated concentrations and coated onto a polystyrene plate for 1 h, washed three times with PBS, and blocked with 20 mg/ml HSA (Fraction V, Sigma Chem. Co., St. Louis, MO) in PBS for 2 h (43). Before the experiment, the substrate was incubated for 20 min with 0.2% Tween-20 in PBS to further block nonspecific binding sites, and washed with PBS. All steps were performed at 24°C.

Cells

The K562 erythroleukemia cell line was transfected with a wild-type human integrin $\alpha 4$ chain (KA4 cells) or with an integrin $\alpha 4$ chain which had been deleted of its cytoplasmic domain by truncation at residue 974 (X4CO cells) as previously described (39). These K562 cells as well as the T lymphoblastoid cell lines Jurkat and SKW3 were maintained in RPMI 1640 supplemented with 10% FCS and gentamicin. Human umbilical vein endothelial cells were cultured as previously described (24), and stimulated with 200 U/ml of TNF- α (Genzyme, Boston, MA).

Peripheral Blood T Lymphocytes

Peripheral blood mononuclear cells were isolated by dextran sedimentation and Ficoll-Hypaque centrifugation, and T cells were purified using mAb to CD14, CD11b, CD20, and CD16 and rat anti-mouse Ig coupled-magnetic beads to deplete monocytes, B cells, and natural killer cells, exactly as previously described (15). Purified T cells contained less than 1% monocytes, as shown by flow cytometry with CD14 mAb.

Laminar Flow Assays

The plastic slide on which adhesion molecules were adsorbed or the 150-mm tissue culture dish on which endothelial cells were cultured, was assembled as the lower wall in a parallel wall flow chamber and mounted on the

stage of an inverted phase-contrast microscope (Diaphot-TMD, Nikon Inc., Garden City, NY) as previously described (42, 43). Cells that tethered or arrested were quantitated by analysis of images videotaped with a TEC-470 CCD video camera (Optronics, Goleta, CA) and Hi 8 Sony CVD-1000 recorder. Lymphocytes (0.5×10^6 /ml) or K562 cells (0.4×10^6 /ml) washed twice with Hank's balanced salt solution/10 mM Hepes, pH 7.4 (H/H medium), and resuspended in binding medium (H/H medium containing 1 mM Mg²⁺ and 2 mM Ca²⁺ and 2 mg/ml human serum albumin) were perfused through the chamber at different flow rates to obtain the indicated shear stresses at the chamber wall.

For inhibition studies, T lymphocytes or KA4 cells (10^7 /ml or 8×10^6), respectively, were preincubated for 5 min in binding medium with either 30 μ g/ml HPI/2 or a 1:30 dilution of PUJ4, mAb 13 or control CD11b ascites. The cell suspension was diluted in a 20-fold vol of binding medium and immediately perfused into the flow chamber. To test the effect of cytochalasin B (CB) on tethering and rolling, KA4 cells were pretreated with 25 μ M cytochalasin B (Calbiochem Corp., La Jolla, CA), diluted from a 50-mM stock in DMSO, in binding medium for 10 min at room temperature, and perfused without dilution into the flow chamber. CB was present in the binding medium throughout the tethering and detachment assays.

For comparisons of mAb blocking, shear stresses, or cell types, identical fields of view were used for tethering experiments in order to ensure that the results reflected uniform site density and distribution of the immobilized adhesive proteins.

To assess the effects of activation on cell binding to the different substrates, cells were preincubated for 3–5 min at 24°C in binding medium in the presence of either 50 ng/ml PMA (Calbiochem, San Diego, CA), anti- $\beta 1$ mAb TS2/16 (3 μ g/ml), or Mn²⁺ (0.5 mM). A 3-ml vol of cell suspension was perfused through the flow chamber at a shear stress of 0.36 dyn/cm² for 1 min followed by perfusion of binding medium at increasing flow rates to test cell resistance to shear stress. To assess the effect of Mn²⁺ on adhesion stabilization, both tethering and detachment assays were performed in the presence of Mn²⁺.

Tethering was determined by counting the number of cells that tethered over the first 20–30 s of continuous shear flow to a given field of view (0.43 mm²) and remained tethered for at least 3 s.

Detachment assays were performed on cells after they had tethered at low shear flow to allow accumulation of tethered cells, or after they had bound to the substrate in stasis for 60 s. The shear flow was increased every 10 s to a maximum of 14.6 dyn/cm², in 2–2.5-fold increments and the number of cells remaining bound at the end of each 10-s interval was determined.

To measure cell displacement on VCAM-1, frames recorded on a Hi 8 Sony CVD-1000 were digitized using a SCION LG-3 frame grabber on a Macintosh 650. A routine developed with NIH Image 1.55 was used to follow the motion of individual cells as they tethered and rolled. Coordinates of cell centers were determined within one pixel accuracy ($\pm 0.7 \mu$ m).

Rolling velocities were measured for cells that were bound during low or high shear flow (0.36 or 0.73 dyn/cm², respectively). Cell displacements were measured over 5–10-s intervals. Velocities were measured only for cells that remained tethered for the 5–10-s interval. For comparisons between cells treated with different reagents, rolling velocities were measured in identical fields of view on the substrate, to ensure that the results were not affected by inhomogeneity of the substrate.

Results

VLA-4-dependent Tethering on VCAM-1

Tethering, rolling, and resistance to detachment are three distinct measures of leukocyte interactions with ligands on a vessel wall in shear flow. Tethering is the initial adhesive interaction between a cell in hydrodynamic flow and the wall of the flow cell or vessel, and is measurable as the arrest of the motion of the cell, or for a rolling cell, as a reduction in its velocity. Although previously demonstrated only for selectins, we found that the integrin VLA-4 could also mediate tethering of freshly isolated peripheral blood T lymphocytes to VCAM-1 (Fig. 1 A). This tethering varied with the concentration of VCAM-1, and was completely inhibited by mAb to either the $\alpha 4$ integrin subunit (Fig. 1 A) or to the $\beta 1$ integrin subunit (data not shown). The complete blocking

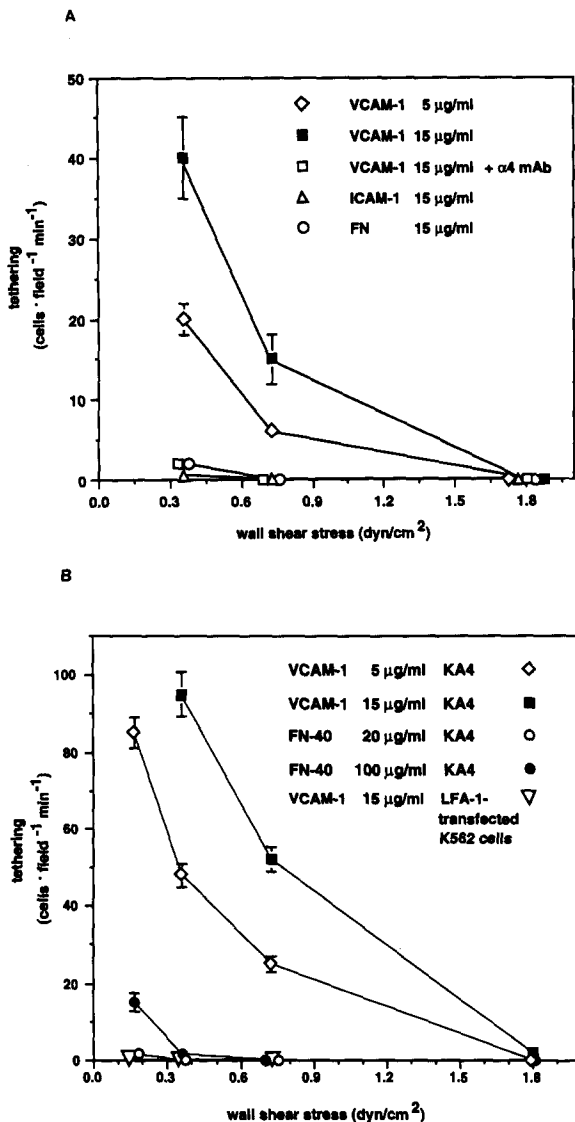


Figure 1. VLA-4-dependent tethering in shear flow of T lymphocytes and KA4 cells to VCAM-1 but not fibronectin. (A) Tethering of purified peripheral blood T lymphocytes to VCAM-1, ICAM-1, or FN adsorbed on plastic at the indicated concentrations. Pretreatment with A4-PUJ1 mAb to $\alpha 4$ was as described in Materials and Methods. (B) Tethering of KA4 cells to VCAM-1 and FN-40. Results are representative of four independent experiments. Each point represents the mean \pm range of two experiments performed on identical fields.

with $\beta 1$ mAb excludes a role for $\alpha 4\beta 7$, another integrin receptor for VCAM-1, in peripheral blood T cell tethering to VCAM-1. Although VLA-4 also binds to fibronectin in static assays, lymphocytes did not tether to fibronectin in flow (Fig. 1 A). Furthermore, there was no tethering to ICAM-1 (Fig. 1 A), even though its receptor, the integrin LFA-1, is present on T lymphocytes at levels comparable to VLA-4. Labeling with mAb to CD3 and fluoresceinated-anti-IgG showed that >90% of the lymphocytes that tethered to VCAM-1 were CD3⁺. Peripheral blood T cells, T cell clones, and the lymphoblastoid cell lines, Jurkat and SKW3, all expressed

VLA-4 at comparable levels and tethered with comparable efficiency (data not shown).

To examine VLA-4 in isolation from VLA-5 and LFA-1, we used K562 cells transfected with the $\alpha 4$ integrin subunit cDNA, termed KA4 cells (39). The KA4 cells were subjected to fluorescence-activated cell sorting to obtain a subline that expressed levels of VLA-4 comparable to peripheral blood T lymphocytes (data not shown). As for T cells, KA4 cell tethering to VCAM-1 was efficient and related to the concentration of VCAM-1 adsorbed to the substrate (Fig. 1 B). In contrast, control K562 transfectants expressing LFA-1 did not tether to VCAM-1. KA4 cells also did not tether to the 40-kD fibronectin fragment (FN40) that contains the CS-1 region to which VLA-4 binds (25, 31, 73), except for a small amount of tethering at a high concentration of FN40 and at the lowest shear rate tested.

VLA-4-dependent Rolling on VCAM-1

Just as selectins can mediate rolling subsequent to tethering, VLA-4 likewise mediated tethering to VCAM-1 in flow, and then rolling. The displacement in the direction of flow measured every 0.25 s is shown for representative lymphocytes in Fig. 2, A and B. The first one (Fig. 2 A) or three (Fig. 2 B) points represent motion at the hydrodynamic velocity of $\sim 280 \mu\text{m/s}$ at the wall shear stress of 0.73 dyn/cm^2 . The cells then tethered as shown by a dramatic decrease in displacement with time (velocity), and began to roll. Cells could be clearly seen to roll, rotating with a non-uniform velocity in response to the hydrodynamic drag force. In Fig. 2 A, the cell rotated forward ~ 2 cell diameters the first 10 s. The wall shear stress was then increased to 1.8, 3.6, and 7.3 dyn/cm^2 , which induced an increase in the average rolling velocity, with typical random variation in velocity and occasional pauses at any given wall shear stress. The cell shown in Fig. 2 A rolled a total of $180 \mu\text{m}$ or ~ 30 cell diameters, over a period of 60 s, before detaching after the last point shown at 7.3 dyn/cm^2 .

After tethering, several different patterns of T lymphocyte behavior were seen. Most of the tethered cells rolled (e.g. Fig. 2, A and B, lower curve). Some cells rolled throughout the observation period (Fig. 2 A), or until they detached when the shear stress was subsequently increased (40–50% of tethered cells). Other cells rolled for various distances, and then spontaneously arrested (15–20% of the cells [Fig. 2 B, lower curve]). Some cells arrested immediately after tethering (15–20%) (Fig. 2 B, upper curve). The remaining cells rolled short distances after tethering (2–5 cell diam), and then spontaneously detached.

Rolling velocity was related to the hydrodynamic force acting on the cell and the concentration of VCAM-1. When shear stress was increased, cells rolled faster, as shown for an individual cell (Fig. 2 A), and for the population of T cells as a whole (Fig. 2 C). T lymphocytes rolled more slowly when higher concentrations of VCAM-1 were adsorbed to the substrate (Fig. 2 C). The rolling velocity of the T cells was dependent on the applied shear stress, and was largely independent of the shear stress at which they had initially tethered (Fig. 2 C).

KA4 cells that tethered on VCAM-1 subsequently rolled (Fig. 2 D). KA4 cells were less likely than peripheral blood T lymphocytes to spontaneously arrest during rolling, and

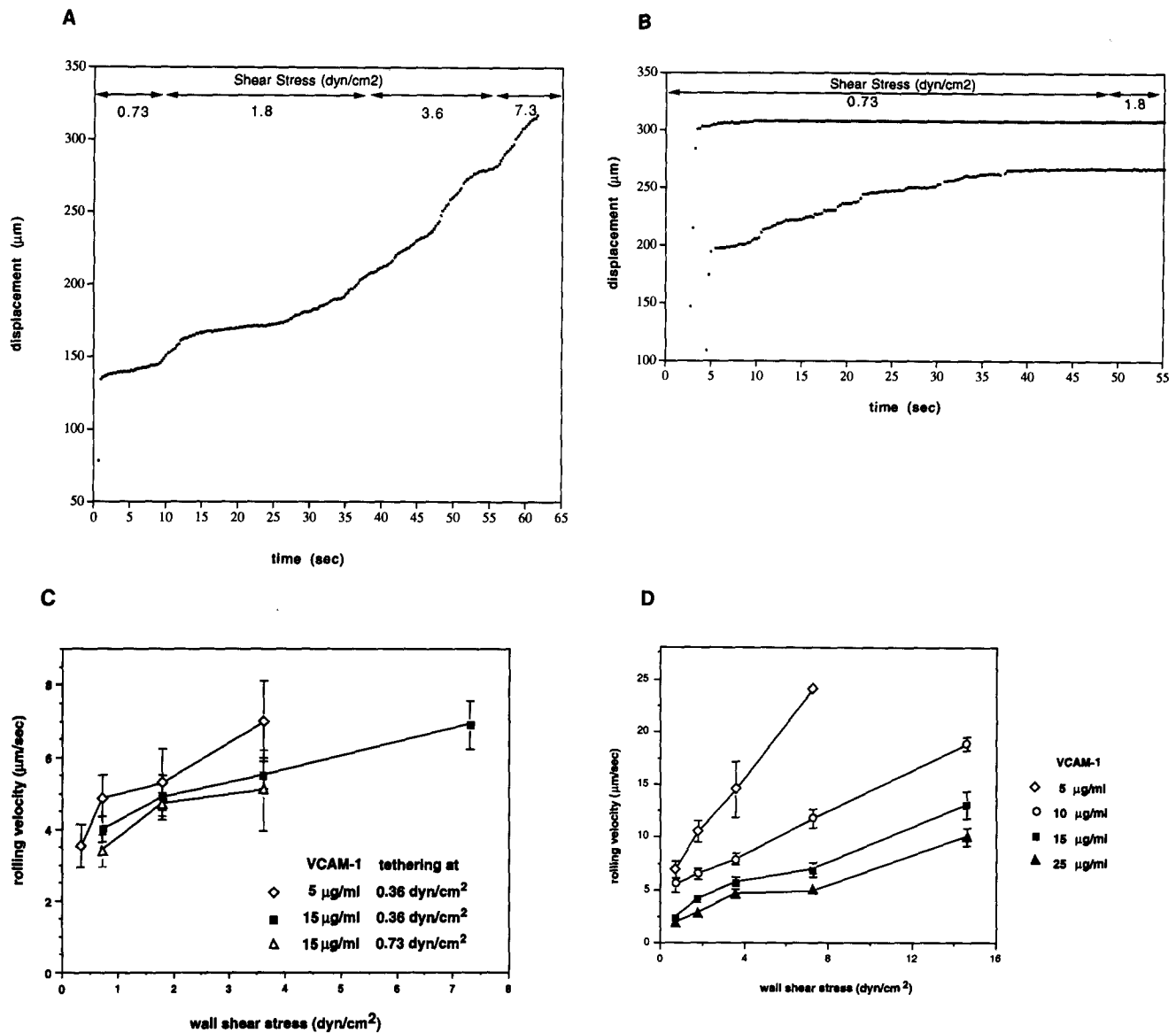


Figure 2. Rolling of T lymphocytes and KA4 cells on VCAM-1 in shear flow. (*A* and *B*) Displacement in the direction of flow of representative blood T lymphocytes interacting with purified VCAM-1 at the indicated wall shear stress. Positions of cells recorded on videotape were determined every 0.25 s. The first point (*A*) or first three points for each cell (*B*) show positions before tethering to VCAM-1. The cell in *A* rolled throughout the period shown whereas in *B* the upper curve shows a cell that arrested almost immediately whereas the lower curve shows a cell that rolled, and then arrested between 35 and 40 s. The wall shear stress was increased during the observation period and is shown in dyn/cm^2 . VCAM-1 was adsorbed at 15 $\mu\text{g}/\text{ml}$. (*C* and *D*) Rolling velocity of purified T lymphocytes (*C*) or KA4 cells (*D*) on VCAM-1 adsorbed at the indicated concentrations. Cells were tethered to the substrate at a shear stress of 0.36 or 0.73 dyn/cm^2 for 30 s and shear stress was then increased every 10–15 s. Each point shows the mean and SEM for 20 cells. Results are representative of four independent experiments.

the vast majority of KA4 cells continued to roll throughout the observation period. The velocity of KA4 cells rolling on VCAM-1 was related to the shear stress and inversely related to the concentration of VCAM-1 used to coat the substrate (Fig. 2 *D*). KA4 cells were more homogenous in rolling velocity than peripheral blood T lymphocytes as indicated by the smaller error bars in Fig. 2 *D* compared to Fig. 2 *C*.

VLA-4-dependent Tethering and Rolling on Endothelium

We examined KA4 cell interaction with TNF-stimulated hu-

man umbilical vein endothelial cells to study VLA-4-dependent interaction in isolation from selectin-dependent interactions. KA4 cells (and peripheral blood T lymphocytes, data not shown) tethered to TNF-stimulated endothelial cell monolayers in the flow chamber with a similar shear dependence as seen for tethering to purified VCAM-1 (Fig. 3 *A*). Tethering of KA4 cells was completely blocked by mAb to $\alpha 4$, and thus was dependent on VLA-4. All the KA4 cells that tethered to the endothelial monolayer subsequently rolled. The rolling velocity was dependent on the applied shear stress, and ranged from 13 to 30 $\mu\text{m}/\text{s}$ (Fig. 3 *B*). This

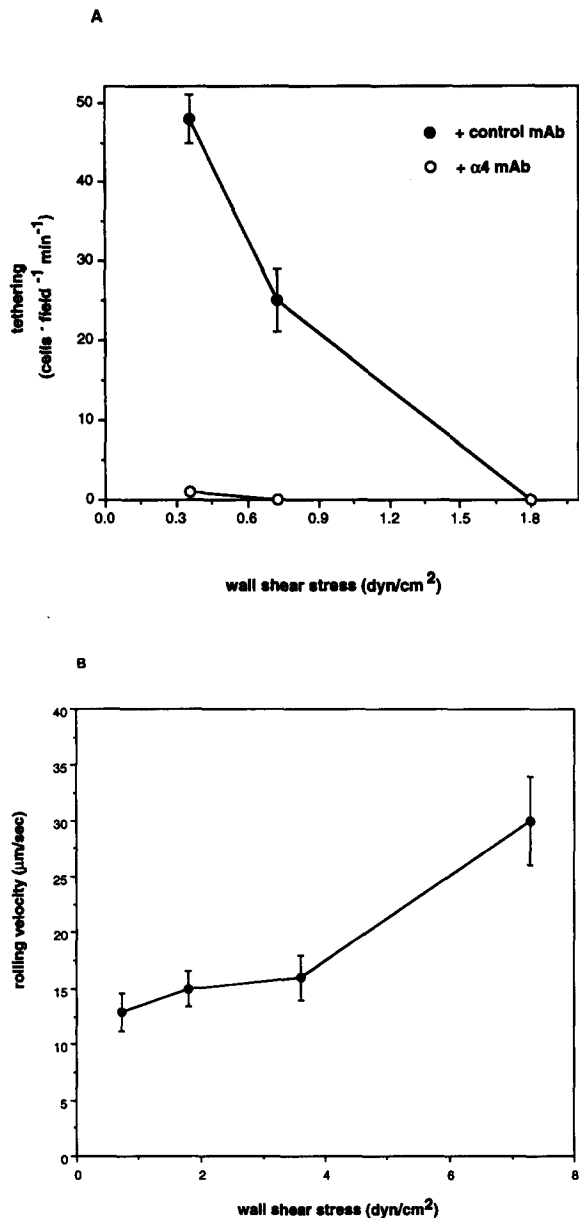


Figure 3. VLA-4-dependent tethering and rolling of KA4 cells on endothelial monolayers. Tethering (A) and rolling velocity (B) of KA4 cells was measured on human umbilical vein endothelial cell monolayers that had been stimulated for 7 h with TNF- α , and then assembled in the parallel wall flow chamber. In A, KA4 cells were pretreated with A4-PUJ1 mAb to VLA-4 α or with control mAb. Tethering and rolling of KA4 cells was not affected by pretreatment of endothelial monolayers with mAb to E-selectin (not shown). Results in A are an average and range of two experiments and in B are mean and SEM of 15–10 cells.

was similar to the range of velocities seen for rolling on purified VCAM-1 (Fig. 2 D).

Modulation of Tethering and Rolling

Agents known to modulate the strength of VLA-4 adhesive interactions in static assays, PMA, the anti- β 1 integrin activating mAb TS2/16, and Mn²⁺ ion, were examined for their effects on tethering and rolling interactions. As ex-

pected for integrin-ligand interactions, tethering was completely dependent on the presence of divalent cations (not shown). PMA and TS2/16 augmented tethering of T lymphocytes to VCAM-1 (Fig. 4 A). KA4 cells never showed more than 50% stimulation of tethering to VCAM-1 by PMA or TS2/16 across a wide range of shear stress conditions (0.3–1.8 dyn/cm²) and minimal stimulation of tethering was observed at higher VCAM-1 site densities (not shown). The addition of 1 mM Mn²⁺ (to binding medium that already contained 1 mM Mg²⁺ and 2 mM Ca²⁺) had only a moderate effect on tethering, and this effect decreased at higher VCAM site densities or lower shear stresses (not shown).

In contrast to their weaker effects on tethering, TS2/16 mAb, PMA, and Mn²⁺ had dramatic effects in converting rolling cells to firmly adherent cells over the entire range of VCAM-1 site densities. When the TS2/16 mAb was added to the perfusate, peripheral blood T cells that had been rolling at 1.8 dyn/cm² immediately arrested (Fig. 4 B). Furthermore, activation of protein kinase C by addition of PMA caused immediate arrest of rolling cells (Fig. 4 B). Arrest was induced even at low coating concentrations of VCAM-1 (5 μ g/ml) and at high shear stresses (3.6 dyn/cm²) as shown in Fig. 4 B. TS2/16 mAb, and to a lesser extent, PMA, also induced the arrest of KA4 cells rolling on VCAM-1, although at coating concentrations of 1 μ g/ml or less of VCAM-1, the ability of these agents to induce firm arrest was greatly reduced (data not shown).

Mn²⁺ both slowed rolling cells, and enhanced their arrest. When high concentrations of Mn²⁺ (2 mM or above) were added to T lymphocytes rolling on VCAM-1, all the rolling T lymphocytes immediately arrested (not shown). When 0.5 mM Mn²⁺ was added to T lymphocytes rolling on VCAM-1, some of the cells arrested (Fig. 4 B), and the remainder continued to roll at a dramatically slower velocity as shown in Fig. 4 C, for three representative cells. When the Mn²⁺ was subsequently diluted out by addition of buffer lacking Mn²⁺ to the perfusate, the rolling velocity increased back to its former value (Fig. 4 C). On the KA4 cells, Mn²⁺ at 0.1 or 0.2 mM showed similar reversible effects on rolling (data not shown).

Modulation of Resistance to Detachment (Adhesion Strengthening)

Modulating agents that had a strong effect on cell rolling also dramatically affected the resistance of cells to detachment under flow, a measure of the strength of adhesion to a substrate. Cells were allowed to tether to substrates either in shear flow or in stasis, and then shear was initiated or increased in 2 or 2.5-fold steps and the number of cells that remained bound was determined at each shear stress. Examination of resting T cells on VCAM-1 at 5 and 15 μ g/ml showed that coating concentration was related to resistance to detachment (Fig. 5 A). The same agents that increased arrest of rolling cells, TS2/16 and PMA, also dramatically increased shear resistance and thus increased the strength of adhesion of the T lymphocytes (Fig. 5 A); this was associated with essentially complete arrest of the treated cells (Fig. 4 B). Likewise, Mn²⁺ (0.5 mM) increased T lymphocyte adhesiveness for VCAM-1 as shown by a substantially greater resistance to detachment (Fig. 5 A).

To allow direct comparison between VLA-4-dependent

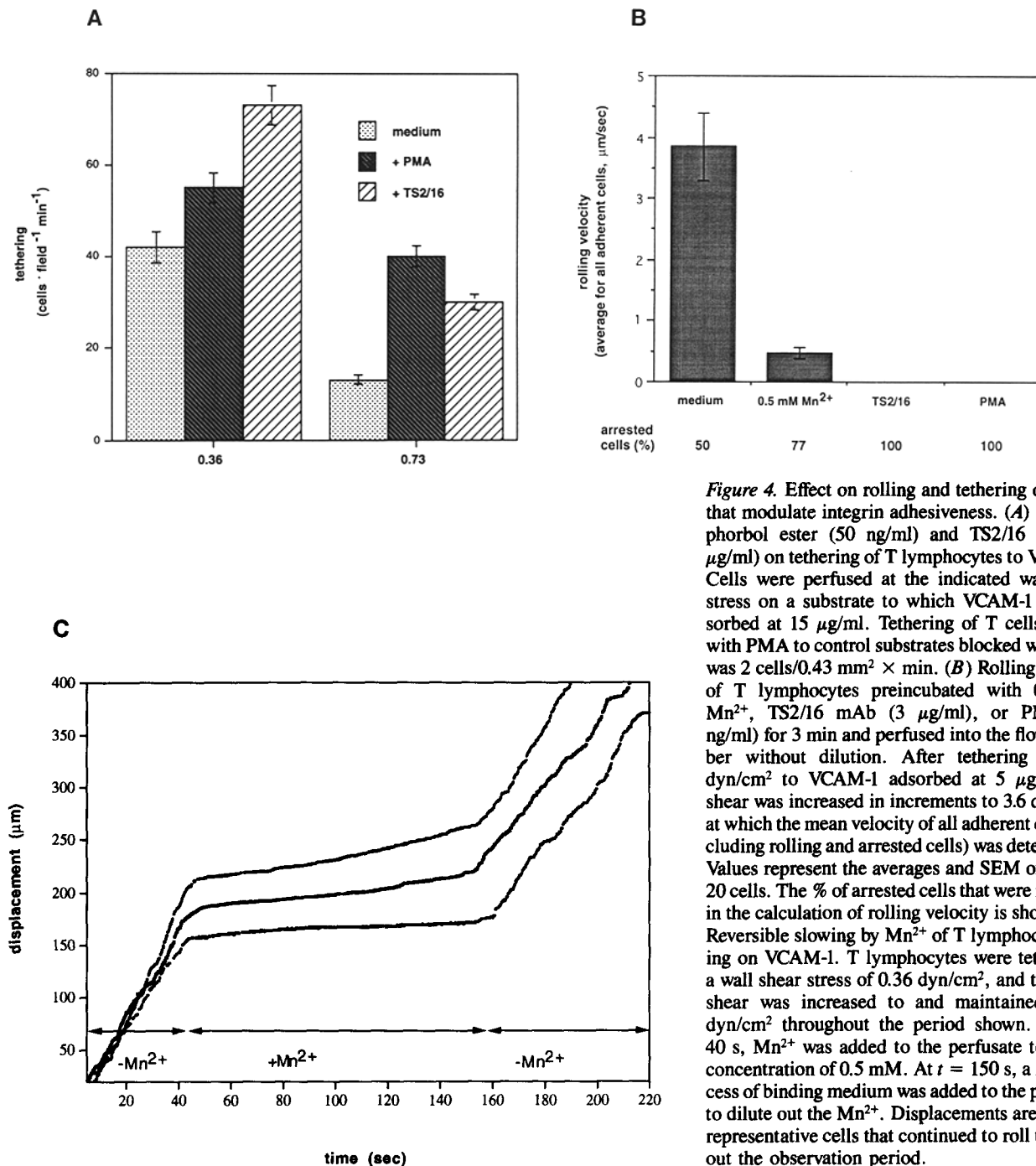


Figure 4. Effect on rolling and tethering of agents that modulate integrin adhesiveness. (A) Effect of phorbol ester (50 ng/ml) and TS2/16 mAb (3 μg/ml) on tethering of T lymphocytes to VCAM-1. Cells were perfused at the indicated wall shear stress on a substrate to which VCAM-1 was adsorbed at 15 μg/ml. Tethering of T cells treated with PMA to control substrates blocked with HSA was 2 cells/0.43 mm² × min. (B) Rolling velocity of T lymphocytes preincubated with 0.5 mM Mn²⁺, TS2/16 mAb (3 μg/ml), or PMA (50 ng/ml) for 3 min and perfused into the flow chamber without dilution. After tethering at 0.36 dyn/cm² to VCAM-1 adsorbed at 5 μg/ml, the shear was increased in increments to 3.6 dyn/cm², at which the mean velocity of all adherent cells (including rolling and arrested cells) was determined. Values represent the averages and SEM of at least 20 cells. The % of arrested cells that were included in the calculation of rolling velocity is shown. (C) Reversible slowing by Mn²⁺ of T lymphocyte rolling on VCAM-1. T lymphocytes were tethered at a wall shear stress of 0.36 dyn/cm², and then wall shear was increased to and maintained at 1.8 dyn/cm² throughout the period shown. At *t* = 40 s, Mn²⁺ was added to the perfusate to a final concentration of 0.5 mM. At *t* = 150 s, a large excess of binding medium was added to the perfusate to dilute out the Mn²⁺. Displacements are of three representative cells that continued to roll throughout the observation period.

interactions with fibronectin and VCAM-1, KA4 cells were allowed to bind for 1 min in stasis to FN40 or VCAM-1 in the presence or absence of the activating TS1/16 mAb, followed by the initiation of shear flow and measurement of the resistance to detachment (Fig. 5 B). Unstimulated KA4 cells adhered weakly to FN40, but TS2/16 mAb stimulated substantially greater resistance to detachment. However, neither mAb-treated or untreated KA4 cells rolled on FN40. The TS2/16 mAb also stimulated substantial adhesion strengthening on VCAM-1 as shown by greater resistance to detachment. The KA4 cells treated with TS2/16 mAb did not roll on VCAM-1, whereas all untreated cells rolled on

VCAM-1. The finding that in the absence of TS1/16 mAb treatment, cells rolling on VCAM-1 were more strongly adherent than cells arrested on FN40, clearly shows that rolling does not reflect a weaker strength of adhesion to the substrate, but rather may result from intrinsic kinetic properties of the VLA-4/VCAM-1 interaction.

Effects of Cytoskeletal Interactions on Tethering, Rolling, and Detachment

The cytoplasmic domain of the α4 integrin subunit plays a key role in cell adhesion (39, 40) and other cytoskeletal-

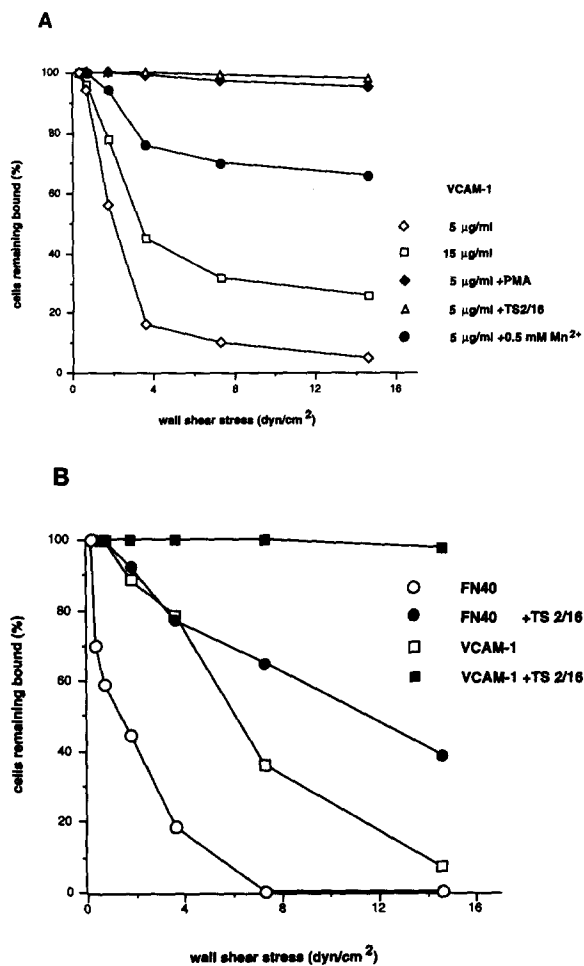


Figure 5. Resistance to detachment of T lymphocytes or KA4 cells from VCAM-1 and FN40 is increased by treatment with phorbol ester or TS2/16 mAb. (A) T lymphocytes. Cells were tethered at 0.36 dyn/cm² for 2 min in binding medium in the absence or presence of TS2/16 mAb or PMA. The shear stress was then increased in 2 or 2.5-fold increments with binding medium as the perfusate. In experiments with Mn²⁺, cells were tethered at 0.36 dyn/cm² and subjected to increasing shear stress in the continued presence of 0.5 mM Mn²⁺. The fraction of the initial population of cells that tethered at 0.36 dyn/cm² that remained bound after 10 s periods at each shear was determined. Representative of three experiments. (B) KA4 cells. KA4 cells were allowed to bind at stasis to VCAM-1 coated at 10 µg/ml or to FN40 coated at 20 µg/ml for 1 min in binding medium in the absence or presence of TS2/16 mAb. Perfusion with binding medium was initiated and the fraction of cells that remained bound was determined as in A. Cells adherent on FN-40 detached in response to the increased shear without rolling. Representative of three experiments.

dependent functions such as cell migration (17). However, VLA-4-dependent tethering was unaltered by deletion of the $\alpha 4$ cytoplasmic domain. As shown at two different levels of shear stress, cells containing truncated $\alpha 4$ subunits (X4CO cells) tethered as efficiently as cells expressing the intact VLA-4 integrin (Fig. 6 A). In contrast to the tethering results, measurement of shear resistance exposed a notable difference between cells expressing the wild-type and truncated $\alpha 4$ subunit (Fig. 6 B), with the latter being less resis-

tant to elevated shear stress. Upon TS2/16 stimulation, cells with both wild-type and truncated $\alpha 4$ subunits showed almost total resistance to elevated shear stress, again pointing out the strong adhesion-strengthening effect of TS2/16 mAb.

Disruption of actin microfilaments in KA4 cells with cytochalasin B dramatically inhibited tethering of KA4 cells at 0.73 dyn/cm², but had no effect on tethering at 0.17 dyn/cm² (Fig. 6 A). Similar results were observed with T lymphocytes (data not shown). This may be due to a requirement for interaction of integrins with the actin cytoskeleton for efficient tethering, or may be caused by indirect effects of disruption of the actin cytoskeleton. Both cytochalasin B-treated and untreated cells rolled on VCAM-1 (Fig. 6 C) and had similar resistance to detachment in shear flow (Fig. 6 D), demonstrating a lack of requirement for the actin cytoskeleton for these functions, although cells rolled more slowly on VCAM-1 after treatment with cytochalasin B (Fig. 6 C). Activation with TS1/16 mAb induced arrest and adhesion strengthening in cells treated with or without cytochalasin B, further demonstrating a lack of requirement for the actin cytoskeleton (Fig. 6 D).

Discussion

Here we show that the integrin VLA-4, on blood T lymphocytes and on K562 transfectants, can interact with VCAM-1 but not with fibronectin on a vessel wall to tether cells in hydrodynamic flow and to mediate rolling. Previous studies on Mac-1 and LFA-1 had shown that these integrins did not tether resting or activated cells to ICAM-1 in physiologically significant hydrodynamic flow (42). In agreement, our finding of significant tethering in hydrodynamic flow of lymphocytes to VCAM-1 contrasted with the absence of tethering to ICAM-1. For some T lymphocytes, VLA-4 mediated tethering and rolling was followed by firm adhesion to VCAM-1, even in the absence of external activating stimuli. The interaction of VLA-4 with VCAM-1 can thus support all the types of adhesive steps that are thought to be required for lymphocyte arrest on vascular endothelia at sites of inflammation.

VLA-4 was constitutively active in tethering and rolling. This is likely to be important in vivo, because activating molecules such as chemoattractants are likely to be rapidly swept away by blood flow, and tethering to the vessel wall is thought to facilitate and precede exposure to chemoattractants or other substances that activate firm adhesion of leukocytes (11, 62, 66). It has been hypothesized that fast kinetic rate constants for the association of a selectin with its carbohydrate ligand and subsequent dissociation are important for tethering and rolling (42). It is likely that the VLA-4 interaction with VCAM-1 may also have fast association and dissociation kinetic rate constants, and may differ from VLA-4 interaction with fibronectin and from LFA-1 and Mac-1 interaction with ICAM-1 in this respect. It is particularly interesting that the dynamic properties of tethering in flow and rolling are restricted to the endothelial ligand and do not occur with the extracellular matrix ligand of VLA-4.

Subsequent to tethering, resting peripheral blood T lymphocytes and to a much lesser extent K562 transfectants that express VLA-4 could spontaneously arrest. Arrest of unactivated T lymphocytes appeared to be random, because it

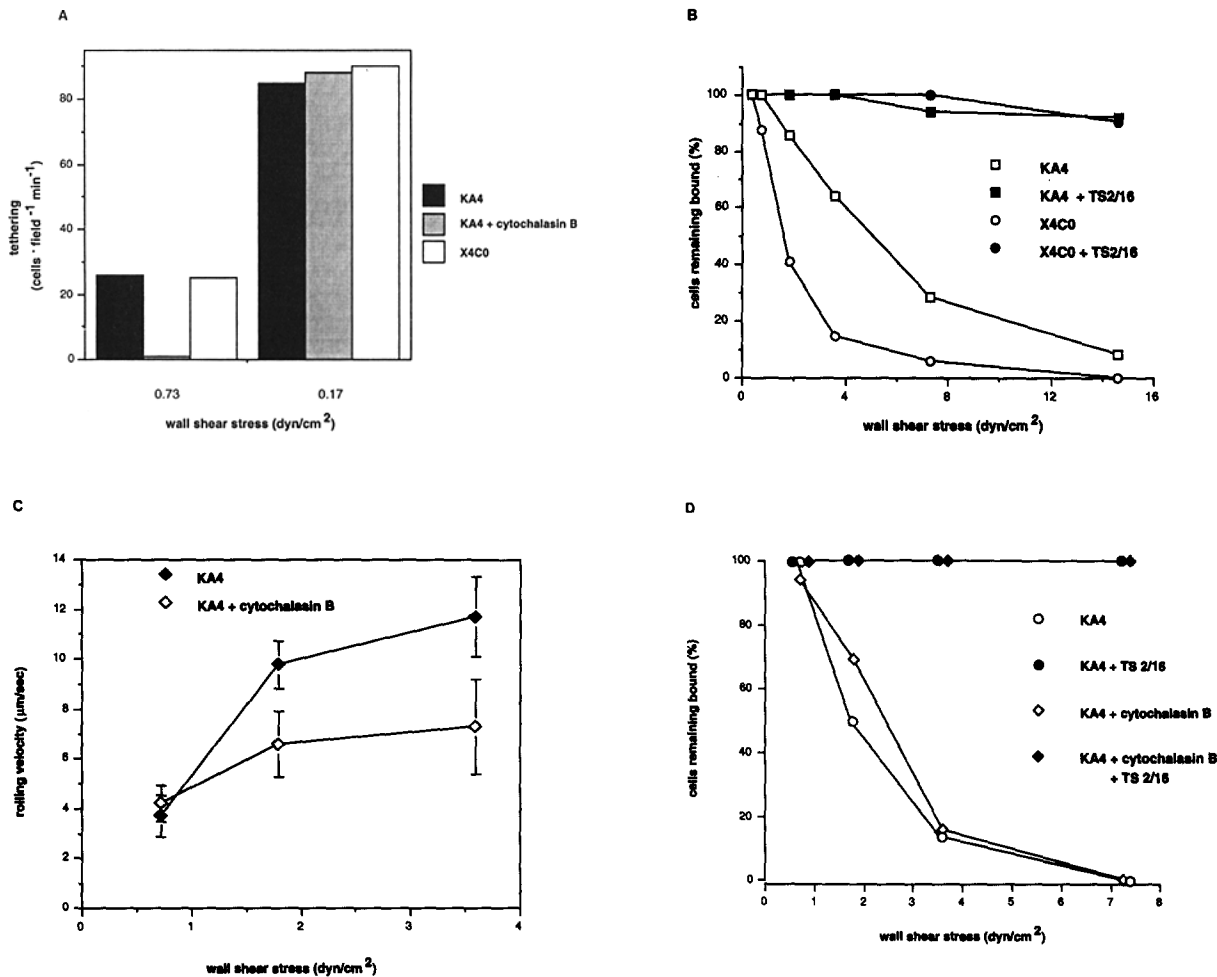


Figure 6. Effect of cytochalasin B (CB) treatment of KA4 cells and of $\alpha 4$ cytoplasmic tail truncation on tethering, rolling, and resistance to detachment on VCAM-1. (A) KA4 cells with or without pretreatment with CB, or K562 cells transfected with a truncated $\alpha 4$ subunit (X4CO) and selected by immunofluorescence flow cytometry to express a similar amount of VLA-4 as KA4 cells were tethered at low or high shear flow to VCAM-1 coated at 15 $\mu\text{g}/\text{ml}$ (B) Effect of $\alpha 4$ subunit cytoplasmic domain truncation on resistance to detachment. KA4 or X4CO cells treated with or without TS2/16 mAb were tethered for 1 min at 0.73 dyn/cm^2 to VCAM-1 adsorbed at 10 $\mu\text{g}/\text{ml}$, and resistance to detachment was measured as in C. Representative of three experiments. (C) Rolling velocity of intact and CB-treated KA4 cells tethered at 0.17 dyn/cm^2 to VCAM-1. Cells were perfused for 2 min at 0.17 dyn/cm^2 , in the absence or presence of CB, and allowed to tether to VCAM-1 adsorbed at 5 $\mu\text{g}/\text{ml}$. The rolling velocities at the indicated shear stresses were determined as in Fig. 2. Each value represents the mean velocity \pm SEM of 10 cells. (D) Resistance to detachment of KA4 cells. Cells pretreated with CB or control medium for 10 min were incubated for 3 min with or without mAb TS2/16, tethered to VCAM-1 adsorbed at 5 $\mu\text{g}/\text{ml}$ as in B, and then subjected to incremented shear stress and the fraction of cells remaining bound at each shear was determined as in Fig. 4. Representative of three experiments.

could occur immediately upon tethering or after rolling for varying durations of time. Almost all resting T lymphocytes have the potential to develop firm adhesion on VCAM-1, as shown by incubation in stasis followed by initiation of shear flow. After 10 min in stasis, 76% of T lymphocytes bound to VCAM-1 and almost all were arrested; whereas after 2 min in stasis 57% of the cells bound and these consisted of both rollingly adherent and arrested cells (data not shown). Interactions between VLA-4 and VCAM-1 have previously been shown to mediate cell attachment followed by immediate arrest on cytokine-stimulated endothelial monolayers in shear flow of sickle cell reticulocytes at 1 dyn/cm^2 (68) and melanoma cell lines at 3 dyn/cm^2 (23). Similarly, we found that certain cell lines that expressed VLA-4, i.e., Jurkat and SKW3 T lymphoblast and transfected CHO cells, tethered in

flow, and then immediately arrested on purified VCAM-1 or cytokine-stimulated endothelial monolayers at 0.73 or 1.8 dyn/cm^2 (data not shown). By contrast, peripheral blood T lymphocytes and transfected K562 cells rolled after tethering. Whether cells arrest or roll after tethering may reflect differences in avidity of VLA-4 on different cell types (53), as well as the densities of VCAM-1 and VLA-4.

Tethering of lymphocytes to VCAM-1 took place at shear stresses that are physiologically significant, since 0.73 dyn/cm^2 is close to the lower end of shear stresses seen in vivo in post capillary venules of 1 or 1.5 dyn/cm^2 (6, 26). The tethering efficiencies of CD3⁺ T lymphocytes on VCAM-1 reported here are close to those seen at 0.73 dyn/cm^2 on high densities of ~ 500 sites/ μm^2 of selectins; the percentage of lymphocytes near the wall that tethered within the

field of view was 4% on E-selectin, 10% on P-selectin, and 5% on VCAM-1 (Alon, R., T. Diacovo, and T. Springer, unpublished). It should be emphasized in comparisons to previously published data on neutrophils and monocytes that lymphocytes are generally less efficient in tethering in flow. On E-selectin and P-selectin at 0.73 dyn/cm², lymphocytes tether 5-fold and 1.2-fold less efficiently than neutrophils, respectively; and are 12-fold and 8-fold less efficient at 1.8 dyn/cm² (Alon, R., T. Diacovo, and T. Springer, unpublished). No in vitro system to date has given tethering at the higher range of shear stresses seen in vivo, of 4, 10, or 36 dynes/cm² (6, 26, 29). Accumulation of neutrophils and monocytes on cytokine-stimulated endothelium occurs at 2 but not 3 dyn/cm² (44), and at 1.8 but not 4.4 dyn/cm² (50), respectively. In these systems, multiple selectin:carbohydrate as well as integrin:CAM interactions can occur, and it is not surprising that tethering is seen at 2.5-fold higher shear stress than with purified VCAM-1. Indeed, cooperative interactions between L-selectin and VLA-4 were seen for monocytes on stimulated endothelium at 1.8 dyn/cm², since accumulation was inhibited almost completely by mAb to either adhesion pathway (50). In this system, VLA-4 was important in firm arrest of the monocytes and L-selectin in rolling. A role for VLA-4 in rolling interactions of eosinophils in vivo has been suggested by recent intravital microscopic studies in IL-1-stimulated mesenteric venules; mAb to VLA-4 inhibited the percent of rolling cells by ~50% and mAb to L-selectin gave similar inhibition (67).

The integrin $\alpha 4\beta 7$ is closely related to the integrin $\alpha 4\beta 1$ (VLA-4) and has been shown to function as a lymphocyte-homing receptor for mucosal lymphoid tissues and in this respect has a function parallel to that of L-selectin for peripheral lymphoid tissues (30). $\alpha 4\beta 7$ mediates binding to the Ig superfamily member MAdCAM-1, which is expressed on high endothelial venules of Peyer's patches (9). When lymphocytes are pretreated with pertussis toxin, they roll through high endothelial venules in Peyer's patches, suggesting the possibility of a rolling interaction through $\alpha 4\beta 7$ (7). Indeed, recent studies suggest that the $\alpha 4\beta 7$ interaction with MAdCAM-1, like the VLA-4 interaction with VCAM-1 studied here, can mediate rolling in shear flow (8a).

Binding of VLA-4 to VCAM-1 during tethering and rolling might be involved in signaling arrest by stimulating stronger adhesion through VLA-4. Ligand-induced activation has been documented for several integrins (13, 21), including VLA-4 (Bazzoni, G., and M Hemler, manuscript submitted for publication). It is also possible that the transition from rolling to arrest on VCAM-1 may be related to fluctuation in the number of VLA-4:VCAM-1 receptor-ligand bonds that are present at any one moment between the rolling cell and the substrate; the random occurrence of a particularly large number of receptor-ligand bonds may allow the rolling adhesion to be converted to a firm adhesion. Agents (PMA, TS2/16 mAb, or higher concentrations of Mn²⁺) that increase the adhesiveness of integrins had marginal or up to a twofold effect on tethering, but had dramatic effects on postligand-binding events. These agents caused immediate arrest of rolling cells and greatly increased their resistance to detachment by shear. This result may suggest that these agents do not modulate the initial phase of integrin-ligand interaction, but rather affect a later adhesion-strengthening phase. This insight was not obvious in earlier static adhesion

assays, or in simple ligand-integrin affinity determinations. Studies at lower concentrations of Mn²⁺ were particularly interesting, as the reversible slowing of rolling suggests that Mn²⁺ either increased the association rate constant or decreased the dissociation rate constant, either of which would increase the affinity.

The affinity of integrins for their ligands is increased by agents such as TS2/16 mAb and Mn²⁺ (5, 16, 22), and perhaps also PMA (49). It is possible that these agents, by increasing the affinity of VLA-4 for VCAM-1 resulted in a dramatic increase in the number of receptor: ligand bonds, and thus caused the more rapid arrest of rolling cells. Alternatively, agents such as PMA, and possibly the others, may enable VLA-4 molecules to assemble into higher order structures in which neighboring VLA-4 molecules cooperate with one another and with the cytoskeleton such that receptor: ligand bonds are not independent but must be broken all at once in order for dissociation to occur, resulting in adhesion strengthening. In notable contrast to rolling adhesions on VCAM-1, firm adhesions were much more shear resistant, and once cells arrested, they did not resume rolling even when the shear stress was increased severalfold. Arrest of both peripheral blood T lymphocytes and K562 transfectants was associated with spreading on VCAM-1 (data not shown).

Examination of a potential requirement for the actin cytoskeleton revealed that tethering at higher but not lower shear stresses was inhibited by cytochalasin B. Tethering through L-selectin is also cytochalasin-sensitive (37). However, cytochalasin B had only a minor effect on rolling, and no effect on the development of shear resistance.

Whereas, deletion of the cytoplasmic domain of L-selectin had a major effect on cell tethering and rolling (37), the VLA-4 $\alpha 4$ subunit cytoplasmic domain was not required for cell tethering to VCAM-1. Deletion of the $\alpha 4$ tail did, however, weaken VLA-4 interaction with VCAM-1 as shown by lower resistance to detachment by shear stress. From these results, we can now better interpret results from previous static adhesion assays (39, 40). Most likely, the diminished static adhesion previously seen upon deletion of the $\alpha 4$ tail occurred at the level of adhesion strengthening, rather than initial ligand binding.

The finding that the integrin VLA-4 can mediate tethering and rolling as well as firm adhesion shows that integrins, a versatile family of cell adhesion molecules, are even more versatile than previously thought. VLA-4 is absent from neutrophils and is expressed on lymphocytes, monocytes, and eosinophils, and thus may play an important role in the selective recruitment of these cells to sites of chronic inflammation. Accumulation of lymphocytes in vivo induced by a specific antigen, or by injection of IFN- γ or TNF- α , is significantly inhibited by mAb to either the LFA-1 α or the VLA-4 α subunit (18, 34, 35, 61, 74). Also, mAb to VCAM-1 inhibits lymphocyte accumulation in delayed type hypersensitivity (64). A combination of mAb to LFA-1 and VLA-4 α gives the most complete inhibition of lymphocyte immigration and of induration and plasma leakage in delayed type hypersensitivity (33). Tethering and rolling interactions of VLA-4 with VCAM-1 may be particularly important in vivo for trafficking of memory lymphocytes, because a substantial proportion of memory lymphocytes lack expression of L-selectin and CLA, the ligand for E-selectin (58). This subset can tether to P-selectin or VCAM-1 in flow; tethering of

CD3⁺ peripheral blood T lymphocytes to P-selectin is two-fold more efficient than to VCAM-1 at 0.73 dyn/ μm^2 (Alon, R., T. Diacovo, and T. Springer, unpublished). The relevance of our findings to the vascular endothelium was demonstrated by the finding that transfectants tethered and rolled through VLA-4 on TNF-stimulated venular endothelial cells. Peripheral blood lymphocytes showed the same dependence on shear stress for tethering to stimulated endothelial monolayers and this was partially dependent on VLA-4 and VCAM-1 (unpublished); since three different selectins contribute to lymphocyte-endothelial interactions, and expression of L-selectin and the ligands for E-selectin and P-selectin varies on lymphocyte subpopulations, dissecting the relative contributions of the selectins and VLA-4 in tethering and rolling, and of LFA-1 and VLA-4 in firm adhesion, requires considerable further work both in vitro on endothelial cell monolayers, and in vivo in the microvasculature.

One of the most provocative findings in this study is that tethering in shear flow of lymphocytes through interaction of VLA-4 with VCAM-1 can be followed immediately, or after a period of rolling, by development of firm adhesion to VCAM-1. In contrast, lymphocyte tethering to E-selectin, P-selectin, or peripheral node addressin in the same flow system, immobilized on the identical substrate, is never followed by development of firm adhesion (4) (4a) (Alon, R., T. Diacovo, T. A. Springer, unpublished). Thus, the VLA-4:VCAM-1 interaction may short-circuit the step of stimulation by chemoattractants of firm adhesion through integrins. An alternative pathway to the three-step model outlined in the introduction may exist, in which VCAM-1 can mediate both tethering and arrest of lymphocytes, perhaps in cooperation with other endothelial adhesion molecules, before stimulation by chemoattractants. In this alternative pathway, chemoattractants might be more important for providing directional cues during transendothelial migration than for upregulating integrin adhesiveness, and it is unclear whether upregulation of adhesiveness of other integrins such as LFA-1 would occur concomitantly with adhesion strengthening through VLA-4, or would require separate stimuli.

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