Lymphocyte Adhesion through Very Late Antigen 4: Evidence for a Novel Binding Site in the Alternatively Spliced Domain of Vascular Cell Adhesion Molecule 1 and an Additional α4 Integrin Counter-Receptor on Stimulated Endothelium

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Summary

Recent studies demonstrate that alternative splicing of mRNA from a single gene can produce two forms of vascular cell adhesion molecule 1 (VCAM-1): a six-immunoglobulin (Ig) domain form (VCAM-6D) and a seven-Ig domain form (VCAM-7D). Using a COS cell transient expression assay, we investigated whether VCAM-6D and VCAM-7D differ functionally in adhesion to the integrin VLA-4 (CD49d/CD29) on lymphoid cells. Binding of lymphoid cell lines and peripheral blood lymphocytes was completely blocked by VLA-4 monoclonal antibody (mAb) and one VCAM-1 mAb (4B9) to both VCAM-6D and VCAM-7D, whereas one VCAM-1 mAb (E1/6) completely blocked binding to VCAM-6D but only partially inhibited binding to VCAM-7D. We conclude that there is one VLA-4 binding site in the six Ig domains shared between VCAM-6D and VCAM-7D, and that the alternatively spliced domain 4 present in VCAM-7D provides a second VLA-4 binding site that is blocked by 4B9 but not the E1/6 mAb. We compared the inhibitory effects of anti-VCAM-1 and anti-VLA-4 mAbs on lymphoid cell adhesion to cultured human umbilical vein endothelial cells (HUVEC). The anti-VCAM-1 mAb 4B9 blocked the binding of PBL and lymphoid tumor cells to stimulated HUVEC better than the anti-VCAM-1 mAb E1/6. Because VCAM-7D is the predominant form of VCAM-1 expressed by stimulated endothelial cells, this difference in VCAM-1 mAb inhibition is attributed to lymphoid cell binding to VCAM-7D on stimulated HUVEC. Although the anti-VLA-4 mAb and anti-VCAM-1 mAb 4B9 equally inhibited PBL binding to stimulated HUVEC, mAb 4B9 inhibited the binding of two lymphoid cell lines significantly less than anti-VLA-4 mAb. Combination of 4B9 mAb with function-blocking antiserum to human fibronectin, a second known ligand for VLA-4, also failed to inhibit as much as anti-VLA-4 mAb. These findings suggest that adhesion of lymphoid cell lines through VLA-4 or other α4 integrins may involve inducible counter-receptor(s) on endothelium distinct from either VCAM-1 or fibronectin. Time course experiments indicate that the fraction of α4 integrin–dependent binding that can be blocked by anti-VCAM-1 mAb E1/6 rises and peaks within 2 h of tumor necrosis factor (TNF) stimulation. We suggest that the binding site shared between VCAM-6D and -7D is important soon after TNF stimulation, but that domain 4 of VCAM-7D or potentially other non-VCAM-1 counter-receptors are sufficient to mediate most lymphoid cell adhesion at later time points.

Molecular interactions between the surfaces of lymphocytes and endothelial cells play a critical role in the extravasation of lymphocytes from the blood stream (1, 2). Studies from this (3, 4) and other laboratories (5, 6) demonstrate that two members of the integrin family of cell surface heterodimers (LFA-1 and very late activation antigen 4 [VLA-4])[1] mediate distinct mechanisms for lymphocyte–endothelial cell adhesion. LFA-1, whose expression is limited to leu-

[1] *Abbreviations used in this paper:* HUVEC, human umbilical vein cord endothelial cells; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; VLA, very late antigen.

kocytes, can bind to intercellular adhesion molecule 1 (ICAM-1) or to ICAM-2 on the surface of stimulated or unstimulated endothelial cells (1). ICAM-1 expression by endothelial cells in culture is substantially upregulated after stimulation by proinflammatory cytokines such as TNF, IL-1, or INF-γ (3, 7). ICAM-2 expression is constitutively high in vitro and not upregulated by cytokines (4, 8). ICAM-1 and ICAM-2 are also constitutively expressed by PBL, with ICAM-1 showing a significant increase in expression after cell activation (4). Interactions between LFA-1 and its counter-receptors have been implicated in a number of lymphocyte functions, including CTL killing, delivery of T cell help, B lymphocyte responses, and graft rejection, as well as the adherence of lymphocytes and neutrophils to endothelial cells, fibroblasts, or epithelial cells (1, 9–11).

The integrin VLA-4, which contains the α4 (CD49d) subunit noncovalently associated with the β1 (CD29) subunit, is expressed by lymphocytes, monocytes, and neural crest–derived cells, and can interact with vascular cell adhesion molecule 1 (VCAM-1) (5). Like ICAM-1 and ICAM-2, VCAM-1 is a member of the Ig superfamily (12), but unlike the ICAMs, VCAM-1 is not expressed by lymphocytes (13, 14). VCAM-1 expression is very low or absent on resting endothelial cells in culture but can be induced by cytokines such as TNF or IL-1 with kinetics of induction similar but not identical to that of ICAM-1 (13, 15). Peak expression of VCAM-1 after continuous treatment of endothelial cells with TNF in culture occurs somewhat earlier than the peak expression of ICAM-1, but both persist at levels substantially higher than basal expression for at least 48 h (15). Unlike LFA-1, however, VLA-4 can also interact with fibronectin, binding to the alternatively spliced CS-1 region located COOH terminal to the RGD site of fibronectin recognized by the integrin VLA-5 (16–18). VLA-4 and its counter-receptors have been implicated in a number of physiologic and pathophysiologic processes in addition to lymphocyte–endothelial cell adhesion, including cytotoxic T cell killing (19), lymphopoiesis (20–23), germinal center development (24), tumor metastasis (25, 26), atherogenesis (27), and acute graft rejection (28).

Recent studies have demonstrated that two different VCAM-1 precursors can be produced by alternative mRNA splicing (29–32). The original VCAM-1 cDNA clone, identified from IL-1-stimulated human umbilical vein cord endothelial cells (HUVEC) by a functional expression cloning strategy, encodes a transmembrane glycoprotein with six predicted Ig-like domains (VCAM-6D) (12). Several subsequently identified VCAM-1 cDNA clones, which were produced from stimulated HUVEC using PCR, differ from the original clone by containing a 276-bp insert at nucleotide 1034 of the originally published sequence (30–32). This insert is predicted to encode an additional Ig-like domain after the first three domains of VCAM-1, suggesting a seven-domain form of VCAM-1 (VCAM-7D). The predicted amino acid sequence of the inserted domain (designated domain 4, with the following domains redesignated domains 5, 6, and 7) is 73% identical with that of the NH_2-terminal domain 1. There is also high sequence identity between domains 2 and 5 (60%) and 3 and 6 (58%), but other pairwise comparisons show much less identity, suggesting that a three-domain module was tandemly duplicated to give rise to domains 1–3 and 4–6. Messenger RNA for both VCAM-6D and VCAM-7D have been identified in stimulated HUVEC, although the seven-domain form has been found to be predominant (31, 32). The two forms of VCAM-1 mRNA most likely represent alternatively spliced products of the same precursor mRNA. Sequence of a genomic VCAM-1 clone indicates that domain 4 corresponds to a single exon located between the exons encoding domains 3 and 5 (29).

There is increasing evidence for multiple ligand recognition by integrins. LFA-1, for example, interacts with ICAM-1 and ICAM-2 (1). Recently, a functional comparison of anti-LFA-1 mAb vs. a cocktail of anti-ICAM-1 and anti-ICAM-2 mAbs has led to the identification of a third LFA-1 ligand, ICAM-3 (4, 33). Although VLA-4 has been shown to bind to fibronectin and VCAM-1, it is not known whether VLA-4 interacts differentially with the two alternatively spliced forms of VCAM-1 or whether VLA-4 interacts with other ligand(s) completely distinct from VCAM-1. In experiments reported here, we compared the adhesion of PBL and lymphoid tumor lines to VCAM-6D vs. VCAM-7D. Our results show that both VCAM-6D and VCAM-7D support VLA-4-dependent adhesion of lymphoid cells, but that based on mAb blocking studies, adhesion to VCAM-7D involves one epitope shared with VCAM-6D and another epitope involving the alternatively spliced domain 4 found in VCAM-7D. We also assessed adhesion of PBL and lymphoid cell lines to stimulated and unstimulated HUVEC and compared the inhibitory effects of anti-VLA-4 and anti-VCAM-1 mAbs. Antifibronectin antiserum was also studied to assess the role of fibronectin in lymphocyte–endothelial cell interactions. Our findings in the HUVEC system are consistent not only with the adhesion of lymphoid cells via two distinct VLA-4 binding sites on VCAM-1, but in the case of cell line adhesion to HUVEC, also provide evidence for an inducible α4 integrin counter-receptor distinct from VCAM-7D, VCAM-6D, or fibronectin.

Materials and Methods

Cell Culture. HUVEC were purchased from Clonetics Corp. (San Diego, CA) and maintained for up to six doublings in M199 media with 20% fetal bovine low endotoxin–defined serum (HyClone Laboratories, Logan, UT), 100 μg/ml bovine endothelial cell growth supplement (Biomedical Technologies, Stoughton, MA), 100 μg/ml heparin, 20 mM Hepes, 5 mM glutamine, and 50 μg/ml gentamicin. Tissue culture surfaces were pretreated with 1 μg/cm^2 of human plasma fibronectin in HBSS for 30 min at 37°C to promote endothelial cell attachment. Human lymphocytic cell lines and SV-40-transformed African green money kidney cells (COS) were maintained in complete media (RPMI 1640 with 10% FCS, 5 mM glutamine, and 50 μg/ml gentamicin). Peripheral mononuclear cells were obtained by dextran sedimentation and Ficoll-Hypaque (1.077) centrifugation. PBL were enriched by incubating mononuclear cells in complete media on tissue culture plastic twice for 45 min at 37°C.

Antibodies. Mouse anti-human mAbs used were TS1/22 (anti-LFA-1) (34), HP2/1 (anti-VLA-4) (35), 4B9 (anti-VCAM-1) (15),

and E1/6 (anti-VCAM-1) (26). Antibodies were titrated and used at concentrations 10-fold greater than required to give maximal inhibition of function: 1:250 dilution of TS1/22 ascites, 40 μg/ml of purified HP2/1, 40 μg/ml of purified 4B9, and neat tissue culture supernatant of E1/6. The concentration of E1/6 was both 10-fold higher than the saturation concentration determined from flow cytometry of labeled stimulated HUVEC as well as 10-fold higher than that required to achieve maximum inhibition of Ramos cell adhesion to TNF-stimulated HUVEC. More than 75% of HUVEC stimulated with 25 ng/ml TNF for 24 h were positive for E1/6. Affinity-purified goat antiserum to human fibronectin was from Sigma Chemical Co. (St. Louis, MO).

Transfected COS Cell Adhesion Assay. Purified cDNA of VCAM-1 clones 41 (VCAM-6D) and 1E11 (VCAM-7D) in the transient expression vector CDM8 (32) were digested with each of the restriction enzymes Xho I, Bsu36 I, and Afl III (New England Biolabs, Beverly, MA) to confirm that the two clones corresponded to six- and seven-domain forms of VCAM-1. Immunoprecipitation of transfected cell lysates with the anti-VCAM-1 mAb 4B9 confirms that these cDNAs correspond to VCAM-6D and VCAM-7D (32). Purified cDNA of VCAM-6D or VCAM-7D in CDM8, or CDM8 vector alone, were transfected into COS cells using DEAE-dextran (4–5 μg plasmid per 10-cm plate of COS cells at 50%–60% confluence) (36). COS cells were suspended using trypsin-EDTA 2 d after infection and reseeded at ~10 COS cells/mm^2. 3 d after transfection, COS cells were washed three times with 1% FCS/RPMI at 25°C, and in some cases, preincubated for 15 min with mAb 4B9 or E1/6. PBL or lymphoid tumor cells in 1% FCS/RPMI were labeled with the carboxyfluorescein compound 2′-7′-bis-(-2-carboxyethyl)-5(and-6) carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Molecular Probes Inc., Eugene, OR) (10 μg/ml, diluted from stock in DMSO; 20 min at 37°C), resuspended in 1% FCS/RPMI, and in some cases, preincubated with mAb HP2/1 for 15 min at 25°C. Labeled lymphoid cells were added at 2 × 10^3/mm^2 and allowed to settle and adhere for 10 min at 25°C. Nonadherent cells were removed by five washes using 1% FCS/RPMI.

For each experiment, bound cells were quantified using a fluorescence microscope to score the number of lymphoid cells in 5–10 100× fields (12–24 mm^2). COS cells were removed from each plate using 5 mM EDTA in HBSS to calculate the number of COS cells/mm^2. The numbers of VCAM-1-transfected and vector only–transfected COS cells/mm^2 were within <10% of each other. COS cell transfection efficiency was determined as described (4) by flow cytometric analysis of COS cells stained with the anti-VCAM-1 mAb E1/6 or the nonbinding mAb X63. Transfection efficiency ranged from 20 to 53% (mean 32%). Binding was quantified as: cells bound/transfected COS cell = (cells bound to VCAM-1-transfectants/mm^2 − cells bound to CDM8-transfectants/mm^2)/[(COS cells/mm^2) × (VCAM-1 transfection efficiency)].

HUVEC Adhesion Assay. PBL or lymphoid tumor cells in complete media/20 mM Hepes were labeled with BCECF-AM and resuspended in complete media/20 mM Hepes. Lymphoid cells were preincubated with one or more mAbs for 30 min at 37°C, or with medium alone. HUVEC were grown to confluence in 96-well tissue culture plates and incubated for 0–24 h at 37°C with 25 ng/ml of recombinant human TNFα (Genzyme, Boston, MA). Before testing cell line adhesion, HUVEC monolayers were washed three times with complete media/20 mM Hepes, and to some wells, mAbs and/or diluted antiserum were added for 30 min at 37°C, 5% CO_2. Lymphoid cells (10^5/well) were overlayed on HUVEC and allowed to settle and adhere for 30 min at 37°C, 5% CO_2. To remove nonadherent cells, wells were washed five times by aspirating with a 21-gauge needle and adding 100 μl of complete media/20 mM Hepes. Percent adherence was determined using a fluorescence concentration analyzer (Pandex Laboratories, Inc., Mundelein, IL) by comparing the residual fluorescence concentrations in each well to the input fluorescence concentration.

Fibronectin Adhesion Assay. Nontissue culture 96-well plates were incubated with 5 μg/ml of human plasma fibronectin (New York Blood Center, New York) in 100 mM $NaHCO_3$ for 2 h at 37°C, and then with 1% heat-treated (30 min, 56°C) BSA in RPMI 1640 for 1 h at 37°C. Diluted antiserum or mAbs were added to some wells for 30 min at 37°C. Lymphoid cells were labeled with BCECF-AM, resuspended in 1% BSA/RPMI, and in some cases, preincubated with a mAb for 30 min at 37°C. Labeled lymphoid cells were added at 10^5 cells/well and allowed to settle and adhere for 30 min at 37°C, 5% CO_2. Unbound cells were removed by washing five times with 1% BSA/RPMI, and percent adherence was determined using a fluorescence concentration analyzer.

Results

Lymphoid Cell Binding to COS Cells Expressing VCAM-6D or VCAM-7D. To test whether the two alternatively spliced forms of VCAM-1 differ functionally, we assayed the adhesion of PBL and two lymphoid cells lines to COS cells transfected with either VCAM-6D or VCAM-7D cDNA. The inhibitory effects of the anti-VLA-4 mAb HP2/1 and the anti-VCAM-1 mAbs 4B9 and E1/6 were compared. Transfected COS cells expressed VCAM-6D and VCAM-7D at roughly the same level, as demonstrated by flow cytometric analysis using the anti-VCAM-1 mAb E1/6 (Fig. 1). PBL bound well to COS cells expressing either form of VCAM-1, forming rosettes of at least 20 cells per transfected COS cell (Fig. 2 *A*). Two lymphocytic cells lines (Ramos [B cell origin] and SKW3 [T cell origin] also bound well to VCAM-1 transfectants, forming rosettes of at least 10 cells per transfected COS cell (Fig. 2, *B* and *C*). Binding of PBL or either of the cell lines to COS cells transfected with the CDM8 vector alone was 5–10-fold less than binding to VCAM-6D or VCAM-7D transfectants.

PBL or cell line binding to VCAM-6D- or VCAM-7D-transfected COS cells was strictly VLA-4 dependent, as demon-

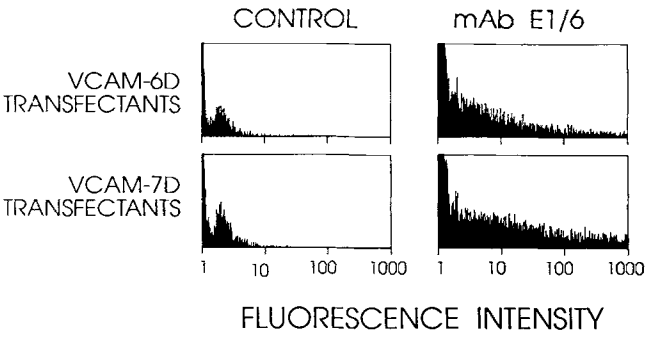


Figure 1. Flow cytometric analysis of COS cells transfected with either VCAM-7D or VCAM-6D. Transfected COS cells were labeled with nonbinding control mAb X63 or the anti-VCAM-1 mAb E1/6.

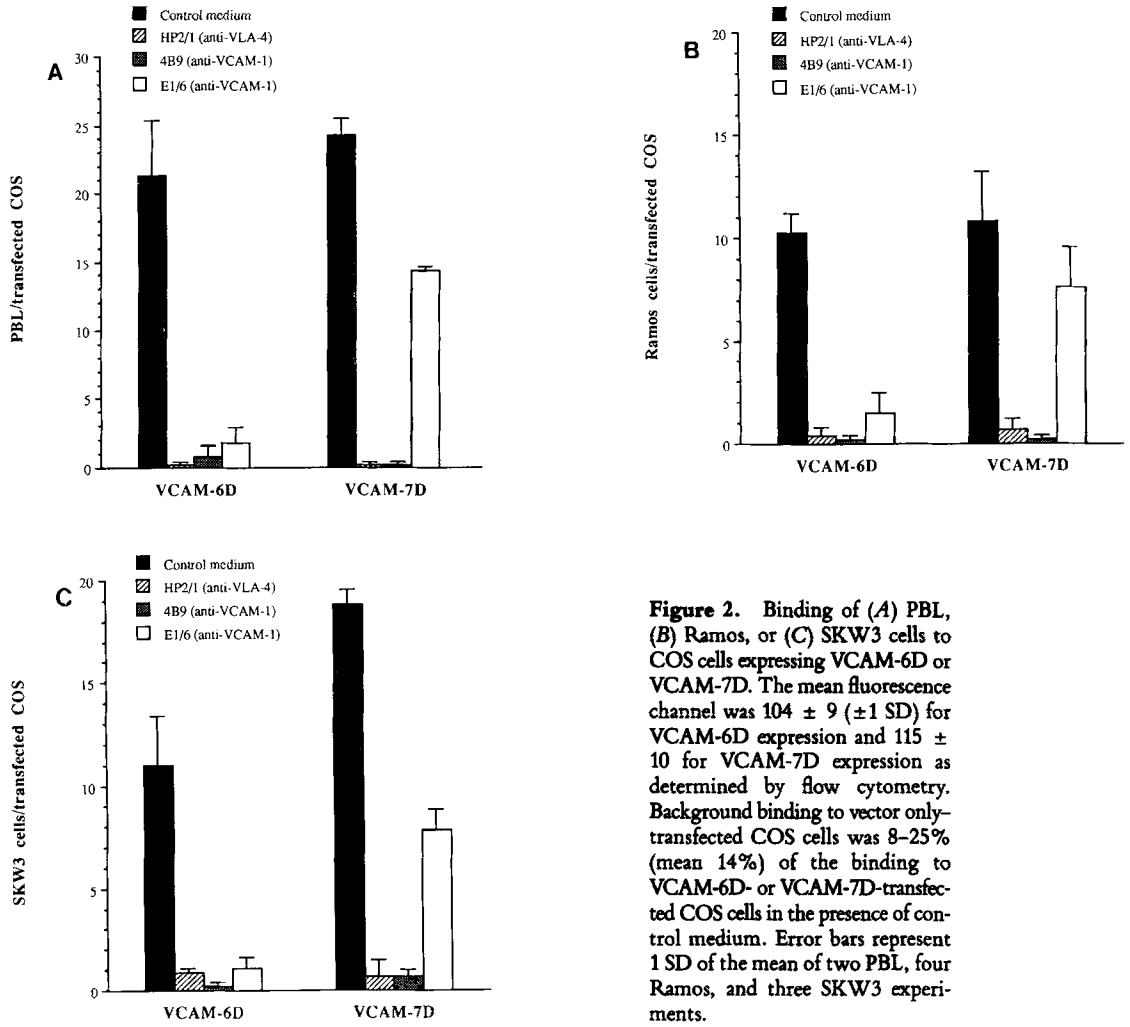


Figure 2. Binding of (*A*) PBL, (*B*) Ramos, or (*C*) SKW3 cells to COS cells expressing VCAM-6D or VCAM-7D. The mean fluorescence channel was 104 ± 9 (±1 SD) for VCAM-6D expression and 115 ± 10 for VCAM-7D expression as determined by flow cytometry. Background binding to vector only–transfected COS cells was 8–25% (mean 14%) of the binding to VCAM-6D- or VCAM-7D-transfected COS cells in the presence of control medium. Error bars represent 1 SD of the mean of two PBL, four Ramos, and three SKW3 experiments.

strated by the abolition of binding after preincubation with the anti-VLA-4 mAb HP2/1 (Fig. 2). The anti-VCAM-1 mAb 4B9 also completely blocked PBL or cell line binding to VCAM-6D or VCAM-7D transfectants. In contrast, preincubation with the anti-VCAM-1 mAb E1/6 completely blocked PBL or cell line binding to VCAM-6D-transfected COS cells, but only partially blocked binding to VCAM-7D-transfected COS cells (PBL by an average of 40%, Ramos by 30%, and SKW3 by 60%). Thus, VLA-4-dependent adhesion to VCAM-6D involved one binding site dependent on the epitope recognized by mAb E1/6; VCAM-7D expressed a second VLA-4 binding site that neither was shared with VCAM-6D nor functionally inhibited by mAb E1/6.

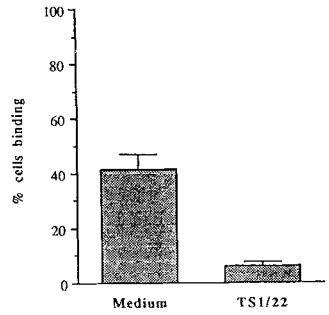


Figure 3. JY cell binding to 24-h TNF-stimulated HUVEC. Before binding, JY cells were incubated with control medium or with mAb TS1/22 (anti-LFA-1). Results are from one experiment with error bars indicating 1 SD of four replicates.

VLA-4-dependent Adhesion of Lymphocytic Cell Lines to TNF-stimulated and Unstimulated HUVEC. The anti-VLA-4 mAb HP2/1 and the anti-VCAM-1 mAbs 4B9 and E1/6 were compared for their abilities to inhibit the adhesion of PBL, Ramos, or SKW3 cells to unstimulated HUVEC or to HUVEC stimulated for 24 h with TNF. To block interactions between LFA-1 and endothelial ICAM-1 or ICAM-2, lymphoid cells were preincubated with the anti-LFA-1 mAb TS1/22. This mAb was confirmed as a functional inhibitor of LFA-1-dependent adhesion by preincubating the B lymphocytic cell line JY with mAb TS1/22 and demonstrating a >85% inhibition of JY cells binding to 24-h TNF-stimulated HUVEC (Fig. 3 and reference 3). JY cells express LFA-1 but little if any of the VLA-4 CD29 β subunit (37).

Preincubation with TS1/22 alone inhibited the binding of Ramos or SKW3 cells to TNF-stimulated HUVEC by <10% compared with preincubation with medium alone; TS1/22 inhibited PBL binding by <20% compared with

medium alone (Fig. 4). When PBL, Ramos, or SKW3 cells were preincubated with the anti-VLA-4 mAb HP2/1 in addition to TS1/22, binding to TNF-stimulated HUVEC was significantly inhibited compared with binding after incubation with medium alone (Fig. 4). Adhesion of PBL to TNF-stimulated HUVEC was blocked by 55%, Ramos cells by 85%, and SKW3 cells by 60%.

Preincubation of TNF-stimulated HUVEC with the anti-VCAM-1 4B9 (in addition to lymphoid cell preincubation with TS1/22) blocked binding of PBL equally as well as preincubation with HP2/1 and TS1/22 (Fig. 4 *A*). In contrast, the anti-VCAM-1 mAb E1/6 blocked binding of PBL to TNF-stimulated HUVEC by only a fraction of that observed after preincubation with HP2/1 and TS1/22, or 4B9 and TS1/22. For Ramos or SKW3 cells (Fig. 4, *B* and *C*), preincubation of TNF-stimulated HUVEC with either 4B9 or E1/6 (in addition to cell line preincubation with TS1/22) failed to block binding as well as HP2/1 and TS1/22. For each cell line, however, inhibition with 4B9 was significantly greater than that with E1/6.

For each cell type tested, adhesion to unstimulated HUVEC was substantially less than adhesion to TNF-stimulated HUVEC (Fig. 4). Preincubation with TS1/22 alone modestly but significantly inhibited adhesion of PBL and SKW3 cells to unstimulated HUVEC; TS1/22 did not inhibit binding of Ramos cells to unstimulated HUVEC. When PBL, Ramos, or SKW3 cells were preincubated with HP2/1 in addition to TS1/22, only slight further inhibition in binding was observed, indicating that VLA-4 counter-receptor(s) on endothelium are cytokine inducible. Basal cell line adhesion to unstimulated HUVEC was not affected by additionally preincubating HUVEC with anti-VCAM-1 mAb 4B9 or E1/6 (Fig. 4).

A Ligand on Stimulated Endothelium Distinct from VCAM-1 and Fibronectin. The two cell lines we examined, but not PBL, bound to stimulated endothelium through a pathway that was blocked by mAb to the VLA-4 α subunit but not by 4B9 mAb to VCAM-1. These mAbs completely blocked binding of the same cells to COS cells expressing VCAM-1. Because VLA-4 can bind to an alternatively spliced form of fibronectin (16, 17), we assessed whether antifibronectin antiserum could block cell line binding to TNF-stimulated HUVEC. To demonstrate antifibronectin antiserum as an inhibitor of lymphocyte-fibronectin adhesion, we determined the binding of Ramos and SKW3 cells to purified plasma fibronectin absorbed onto plastic microtiter plates at 5 μg/ml. For both cell lines, binding to purified fibronectin was completely inhibited by preincubation of the plates with antiserum to human fibronectin (Fig. 5 *A*). Cell line preincubation with the anti-VLA-4 mAb HP2/1 also completely blocked binding to fibronectin, whereas preincubation with the anti-LFA-1 mAb TS1/22 had no effect. Cell line binding to BSA was minimal (Fig. 5 *A*). Neither Ramos nor SKW3 express VLA-5, as determined by flow cytometric analysis (data not

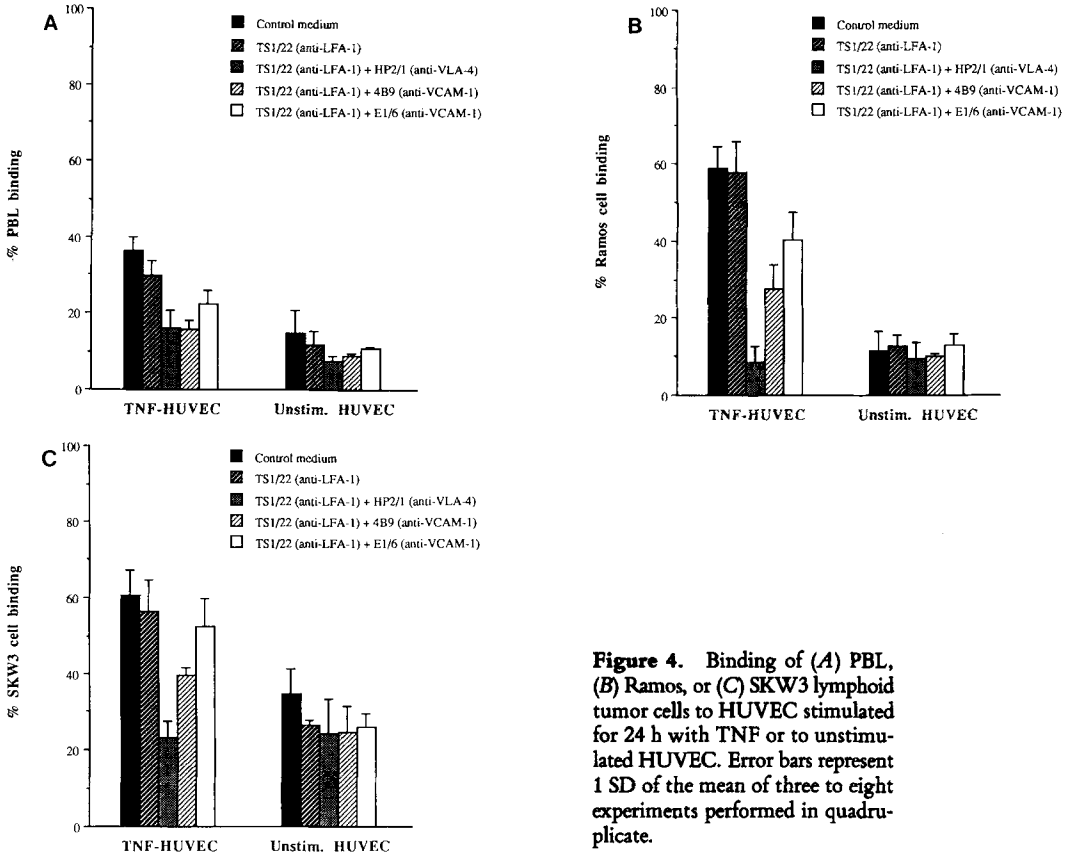


Figure 4. Binding of (*A*) PBL, (*B*) Ramos, or (*C*) SKW3 lymphoid tumor cells to HUVEC stimulated for 24 h with TNF or to unstimulated HUVEC. Error bars represent 1 SD of the mean of three to eight experiments performed in quadruplicate.

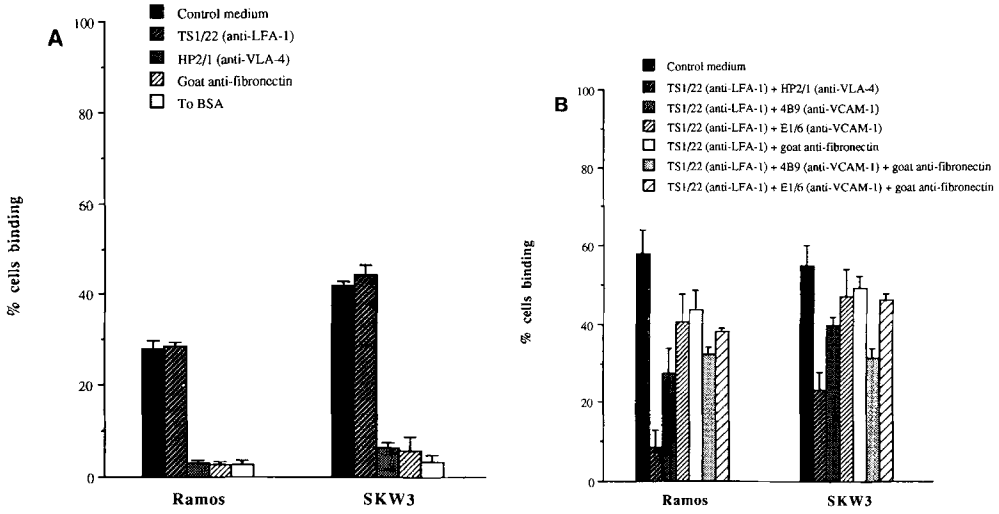


Figure 5. Ramos and SKW3 cell binding to (*A*) fibronectin coated on plastic or (*B*) 24-h TNF-stimulated HUVEC. Error bars represent 1 SD of the mean of three experiments performed in quadruplicate.

shown). This result therefore confirms previous reports of VLA-4-dependent/VLA-5-independent adhesion of lymphocytes to fibronectin (16, 17).

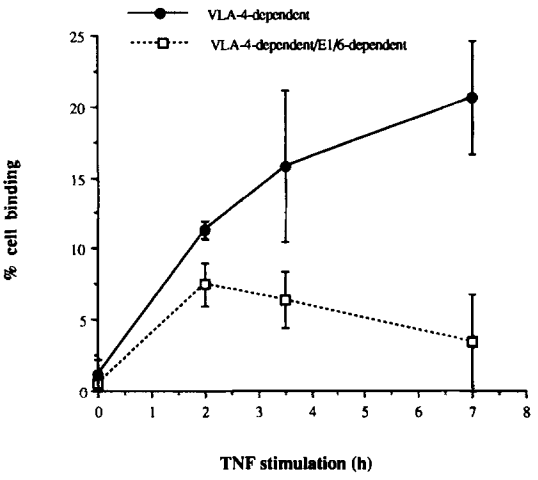
Preincubation of 24-h TNF-stimulated HUVEC with antifibronectin antiserum (in addition to cell line preincubation with the anti-LFA-1 mAb TS1/22) inhibited Ramos cell adhesion by <20% and SKW3 cell adhesion by <12% (Fig. 5 *B*). When antifibronectin antiserum was used in combination with anti-VCAM-1 mAb 4B9 or E1/6, no or only a moderate additive effect on the inhibition of adhesion to TNF-stimulated HUVEC was observed (additional inhibition <8%) (Fig. 5 *B*). Inhibition remained substantially less than that obtained with VLA-4 mAb. These results suggest that cell lines express an integrin containing the VLA-4 α subunit that can recognize a ligand on stimulated endothelium that is distinct from VCAM-1 and fibronectin.

Induction of E1/6-dependent/VLA-4-dependent Ramos Cell Adhesion to TNF-stimulated HUVEC. To determine the induction of E1/6-dependent adhesion of lymphoid cells to HUVEC, we assessed Ramos cell binding to HUVEC after 0, 2, 3.5, or 7 h of TNF stimulation. HUVEC used for any one experiment were from a single umbilical cord. VLA-4-dependent adhesion was calculated as the percentage of Ramos cell binding that was blocked by anti-VLA-4 mAb HP2/1 in the presence of anti-LFA-1 mAb TS1/22. For five experiments, VLA-4-dependent adhesion increased as the time of TNF stimulation increased (Fig. 6), consistent with the cytokine inducibility of VLA-4 counter-receptor(s) on endothelium. There was no significant VLA-4-dependent adhesion to unstimulated HUVEC.

VLA-4-dependent adhesion that could be blocked by E1/6 in the presence of anti-LFA-1 mAb TS1/22 was characterized as E1/6 dependent. Results from five experiments showed that after 2 h of TNF stimulation, the majority of VLA-4-dependent binding of Ramos cells was E1/6 dependent, but after 7 h of stimulation, the majority of VLA-4-dependent adhesion was not blocked by E1/6 (Fig. 6).

Discussion

The integrin VLA-4 mediates lymphocyte adhesion to stimulated endothelium by binding to VCAM-1, a member of the Ig superfamily whose expression is induced on endothelium by proinflammatory cytokines (5, 12, 26). At least



mAb	% cells binding			
	0 h	2 h	3.5 h	7 h
TS1/22	8.2 ± 1.3	17.7 ± 2.3	22.7 ± 5.6	26.5 ± 4.9
TS1/22 + HP2/1	7.1 ± 2.3	6.4 ± 1.8	6.9 ± 2.3	6.0 ± 1.7
TS1/22 + E1/6	9.5 ± 3.0	10.2 ± 1.8	16.2 ± 4.0	23.8 ± 3.2

Figure 6. Comparison of VLA-4-dependent vs. VLA-4-dependent/E1/6-dependent adhesion of Ramos cells to HUVEC as functions of time of TNF stimulation. Error bars represent 1 SD of the mean of five experimental calculations. The mean of the raw data on which these calculations were based are also shown ± 1 SD.

two different precursors for VCAM-1 can be generated from the human VCAM-1 gene as a result of alternative mRNA splicing (29). The resulting proteins correspond to a six-Ig domain form of VCAM-1 (VCAM-6D) and a seven-Ig domain form (VCAM-7D). To test whether VCAM-6D and VCAM-7D differ functionally in mediating VLA-4-dependent adhesion of lymphoid cells, we transiently expressed VCAM-6D or VCAM-7D in COS cells and assayed the adhesion of PBL or lymphoid tumor cells. Both PBL and lymphoid tumor cells bound well to each form of VCAM-1 in a strict VLA-4-dependent fashion. One anti-VCAM-1 mAb, 4B9, abrogated binding of lymphoid cells to either VCAM-6D or VCAM-7D, as expected from a previous study (32) using this mAb that reported no functional difference between VCAM-6D and VCAM-7D. In contrast, we found that a second anti-VCAM-1 mAb, E1/6, completely blocked binding to VCAM-6D but only partially inhibited binding to VCAM-7D. Several previous reports (29, 31, 32) confirm that VCAM-6D and VCAM-7D are identical in primary sequence except for an inserted Ig domain in VCAM-7D (domain 4). Our findings suggest, therefore, that one VLA-4 binding site, which can be blocked by mAb E1/6, involves only the six Ig domains shared between VCAM-6D and VCAM-7D; the addition of domain 4 in VCAM-7D provides a second VLA-4 binding site that is not blocked by mAb E1/6.

It is most likely that this second VLA-4 binding site localizes specifically to the primary protein sequence of domain 4, although it remains a formal possibility that the additional expression of domain 4 confers a change in conformation that reveals a second binding site elsewhere in VCAM-1. Previous studies of ICAM-1 argue against this latter explanation (38, 39); multiple integrin binding sites on ICAM-1 that localize to distinct Ig domains were clearly suggested by results from experiments using domain deletion mutants and subsequently confirmed by experiments using amino acid substitution mutants of ICAM-1. Two lines of evidence allow us to predict that the VLA-4 binding site blocked by mAb E1/6 and shared between VCAM-6D and VCAM-7D localizes to domain 1. First, the epitope recognized by E1/6 has recently been localized to the three NH_2-terminal domains of VCAM-1 (domains 1–3), based on immunostaining of a protein for which domains 1–3 were fused to the Fc portion of human IgG1 (25). mAb E1/6 completely blocks VLA-4-dependent adhesion of melanoma cells to this fusion protein. Second, domain 4 and domain 1 have a much higher degree of amino acid identity with each other (73%) than they have with any other domain of VCAM-1 (8–23%).

Previous studies of ICAM-1 (38–40) provide precedence for our findings here that mAbs can differentially inhibit multiple integrin binding sites on an Ig-like cellular adhesion molecule. ICAM-1 expresses two integrin binding sites: one in domain 1 for LFA-1 (38) and another in domain 3 for Mac-1 (40), a sister integrin of LFA-1 that shares the same $\beta 2$ subunit. Several mAbs whose epitopes map to domain 1 of ICAM-1 inhibit ICAM-1 binding to LFA-1 but not Mac-1 (38, 40). In contrast, one mAb (R6.5) completely blocks LFA-1 binding to domain 1 as well as Mac-1 binding to domain 3 (40). Interestingly, the epitope of mAb R6.5 maps to domain 2 of ICAM-1 (38).

In parallel to studies on transfected COS cells, we examined the inhibitory effects of anti-VCAM-1 and anti-VLA-4 mAb on PBL and lymphoid tumor cell adhesion to TNF-stimulated or unstimulated HUVEC. The function blocking anti-LFA-1 mAb TS1/22 was included in each experiment to prevent interactions with endothelial ICAM-1 or ICAM-2. For each cell type, the anti-VCAM-1 mAb 4B9 inhibited adhesion significantly better than the anti-VCAM-1 mAb E1/6, similar to the pattern of mAbs 4B9 and E1/6 inhibition observed with lymphoid cell adhesion to VCAM-7D-transfected COS cells. Because VCAM-7D is the predominant form of VCAM-1 expressed on well-stimulated HUVEC (such as 24-h TNF-stimulated HUVEC) (31, 32), the observed difference in inhibition between mAbs 4B9 and E1/6 in the HUVEC system most likely reflects lymphoid cell adhesion to VCAM-7D. Preincubation of HUVEC with mAb E1/6 blocks the VLA-4 binding site shared between VCAM-6D and VCAM-7D but does not block the second binding site on VCAM-7D involving domain 4. The mAb 4B9 blocks both sites on VCAM-7D.

Using the HUVEC system, we also directly compared the inhibitory effects of the anti-VLA-4 mAb HP2/1 and the anti-VCAM-1 mAb 4B9 to determine whether there might exist VLA-4 counter-receptors distinct from VCAM-1. A similar strategy of comparing the inhibitory effects of receptor and counter-receptor mAbs has been previously used to provide evidence for LFA-1 counter-receptors distinct from ICAM-1 (3, 41, 42), and led to the subsequent identification of ICAM-2 and ICAM-3 (4, 8, 33). Here, we chose mAb HP2/1 to VLA-4α and 4B9 to VCAM-1 for comparison because our COS cells experiments showed that either mAb used alone completely blocks lymphoid cell binding to either form of VCAM-1. It has also been shown that mAb HP2/1 completely blocks two other adhesive functions of VLA-4; namely, interactions with fibronectin and lymphocyte homotypic aggregation (43). In experiments with PBL, mAbs HP2/1 and 4B9 blocked adhesion to stimulated HUVEC equally well, suggesting no use of alternative VLA-4 counter-receptors; however, for the two cell lines tested, mAb HP2/1 blocked adhesion to stimulated HUVEC significantly better than mAb 4B9. When function-blocking antiserum to human fibronectin was used in combination with mAb 4B9, inhibition was still significantly less than that observed with mAb HP2/1. These results provide evidence for the existence of counter-receptor(s) for an $\alpha 4$ integrin that are inducible on the surface of endothelium and are distinct from VCAM-6D, VCAM-7D, and fibronectin. Our functional evidence for a pathway of adhesion of lymphocytic cell lines to stimulated endothelium that is blocked by VLA-4 α subunit mAb but not VCAM-1 mAb is particularly strong because of our demonstration that these mAbs equally block adhesion of the same cell lines to transfected COS cells, and adhesion of PBL to stimulated endothelium. It was similar evidence that ICAM-1 and LFA-1 mAbs would equally block adhesion of some cell types, but that ICAM-1 mAb was less effective than LFA-1 mAb in

blocking other adhesion assays (41), which subsequently led to the identification of ICAM-2 and ICAM-3 (4, 33). The current studies should stimulate similar efforts, by production of function-blocking mAbs, or functional clone screening assays, to identify and definitively characterize alternative $\alpha 4$ integrin ligand(s).

It is not known why the two lymphocytic cell lines were found to bind novel $\alpha 4$ integrin counter-receptor(s) but resting PBL were not. There may be differences in the activation of $\alpha_4\beta_1$, by resting PBL vs. lymphoid tumor cells that confer variations in function, such as by proteolytic cleavage of the $\alpha 4$ subunit or cellular signals that regulate $\alpha_4\beta_1$ avidity (18, 37, 44, 45). Alternatively, the $\alpha 4$ subunit may associate with distinct β subunits, such as $\beta 7$ (46, 47), and function with a unique ligand specificity. Curiously, mRNA for $\beta 7$ has been easily detected in some lymphocytic cell lines but not in resting peripheral T cells (47).

Adhesion to endothelium that is dependent on $\alpha 4$ integrin(s) and that is not ascribable to VCAM-1 or fibronectin has not been previously suggested. One previous study of resting T cell adhesion to stimulated HUVEC found no difference in inhibition between the anti-VCAM-1 mAb 4B9 and a function-blocking anti-VLA-4 mAb (48), consistent with our results using PBL. In a study (6) of LFA-1-negative B cells obtained from a patient with leukocyte adhesion deficiency (49), mAb 4B9 failed to inhibit binding to stimulated HUVEC as well as a function-blocking anti-VLA-4 mAb; the difference was attributed to lymphocyte interactions with fibronectin on HUVEC, but this was not tested with antiserum to fibronectin as we have done here. In studies (50, 51) of other anti-VCAM-1 mAbs used with various mAbs that block LFA-1 function, binding of resting T cells to stimulated HUVEC was found to be inhibited but not to the same level as binding to unstimulated HUVEC in the presence of the same mAbs. We obtained similar results using PBL and the anti-VCAM-1 mAb E1/6. In one study of a lymphocytic cell line (43), mAbs 4B9 and HP2/1 inhibited binding to stimulated HUVEC equally well. In another study of cell lines (15), however, binding to stimulated HUVEC after preincubation with 4B9 and a mAb to LFA-1 β chain was greater than binding to unstimulated HUVEC after preincubation with the same mAb.

The studies comparing Ramos cell adhesion to HUVEC stimulated for various times with TNF provide functional evidence that the binding site shared by VCAM-6D and -7D is important soon after TNF stimulation. At 2 h of TNF stimulation, E1/6-dependent adhesion reached its maximum, constituting the majority of the VLA-4-dependent binding. VLA-4-dependent binding, however, continued to increase with time of stimulation of HUVEC by TNF. This may be either because VCAM-6D is expressed earlier than VCAM-7D, or if VCAM-6D and VCAM-7D are expressed with similar kinetics, because two-site binding is important early on at low VCAM-1 cell surface density, whereas later on at higher density the alternatively spliced domain 4 is sufficient to give efficient binding. VCAM-7D mRNA is present in much higher amounts than VCAM-6D mRNA at all time points examined, but VCAM-6D is present in higher amounts at 2.5 h than at subsequent time points (32). Although endothelial expression of VCAM-6D at the protein level has not yet been directly demonstrated, there is good reason to expect its expression based on cDNA cloning (12), isolation of VCAM-6D mRNA from stimulated HUVEC (31, 32), and the expression of VCAM-6D as a mature glycoprotein in transfected cells (32).

The generation of one vs. two VLA-4 binding sites on VCAM-1 represents an alternative mechanism for regulating the density of integrin binding sites on the surface of endothelium. Assuming that like ICAM-1, VCAM-7D is an unpaired molecule with Ig domains arranged end to end, size considerations suggest that it should be possible for two VLA-4 molecules to bind to the same VCAM-7D molecule (1). By alternatively splicing VCAM-1, endothelial cells would be able to control the number of VLA-4 binding sites expressed. ICAM-1 also expresses two integrin binding sites (one for LFA-1 [38] and a second for Mac-1 [39]), but these sites are not alternatively spliced. Binding of a single vs. multiple VLA-4 molecules to VCAM-7D may affect the strength of adhesiveness, and may also transmit distinct signals for lymphocyte adhesion to endothelium or for transmigration across it. Furthermore, because lymphocytes can modulate the adhesiveness of VLA-4 for its counter-receptors (44, 45), it may be possible for such modulation to affect the avidity of VLA-4 for its two VCAM-1 binding sites differentially. Understanding the interaction of $\alpha 4$ integrin(s) with counter-receptors and the regulation of this interaction by both endothelial cells and lymphocytes will be important for future endeavors aimed at disrupting these adhesion pathways in the clinical management of chronic inflammatory diseases or graft rejection.

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