VLA-4 Integrin Mediates Lymphocyte Migration on the Inducible Endothelial Cell Ligand VCAM-1 and the Extracellular Matrix Ligand Fibronectin*

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We herein tested the ability of lymphocytes to utilize the β4 integrin VLA-4 to mediate cell migration when adhering to its cytokine-inducible endothelial cell ligand VCAM-1 or its extracellular matrix ligand fibronectin. We used an in vitro system consisting of purified VCAM-1/Fc fusion protein or fibronectin immobilized on porous polycarbonate membranes to quantitatively measure the migration efficiency of an Epstein-Barr virus-transformed B cell line (SLA) and T lymphoblasts derived from normal donors. We found that both SLA cells and T lymphoblasts migrated across membranes coated with VCAM-1/Fc or fibronectin in a site density-dependent manner. Above and below an optimal site density of VCAM-1/Fc or fibronectin, the migration efficiency decreased. A 6-20-fold higher number of lymphocytes migrated across membranes coated with VCAM-1/Fc than with fibronectin. The differential migration efficiency is consistent with a higher number of adherent lymphocytes and a higher avidity of adhesion for VCAM-1/Fc than for fibronectin when the ligands were immobilized on plastic, and is independent of the activation state of the cells. These results demonstrated a stringent regulation of migratory response by cell adhesion strength and a delicate balance between stationary and migratory behaviors of a cell on the adhesive substrates. Like the β2 integrin LFA-1, VLA-4 may be a locomotive adhesion receptor which is involved in the transendothelial migration of lymphocytes and the infiltration of lymphocytes into lymphoid or peripheral tissues by binding to VCAM-1 and fibronectin.

The exit of lymphocytes from circulation and their migration to lymphoid tissues or inflammatory sites are prerequisites for eliciting an immune response. This recirculation involves specific adhesion of lymphocytes to the vascular endothelium, and transmigration through the intercellular junctions, the basement membrane, and the extracellular matrix in the connective tissues. Recirculation of lymphocytes through specific lymph nodes generally referred as “lymphocyte homing,” involves lymphocytes transmigrating through the specialized high endothelial venules (1-3). At sites of inflammation where the endothelium of the microvascular venules is activated by locally released cytokines, specific adhesion molecules are induced on the endothelial cells which mediate lymphocyte binding and subsequent transmigration (4, 5).

The β2 integrin LFA-1, which is expressed on all leukocytes and binds ICAM-1 and ICAM-2 on endothelial cells, has been shown by both in vitro and in vivo monoclonal antibody (mAb)1 inhibition studies to be crucial in mediating lymphocyte adhesion and subsequent transendothelial migration (6-10). Lymphocytes from patients suffering from leukocyte adhesion deficiency (LAD) which do not express LFA-1 are, however, able to transmigrate and these patients can elicit relatively normal immune responses (11). Integrins are heterodimers of noncovalently associated α and β subunits. They are categorized into subfamilies with members sharing a common β subunit pairing with a unique α subunit. The β2 integrin VLA-4, which is structurally related to the β2 integrin (12-14) and binds VCAM-1 on cytokine-activated endothelium (15-17), is thus a likely candidate adhesion receptor which participates in the transendothelial migration of LAD lymphocytes. VLA-4 is expressed on most peripheral blood leukocytes except neutrophils, as well as on melanomas and neural crest-derived tumor cells (18-20). VCAM-1 is also expressed on dendritic cells, cultured mesothelial cells, neural cells, synovocytes, synovial endothelium, and inflamed cutaneous venules (21-25). Like LFA-1, the avidity of VLA-4-mediated binding is up-regulated upon lymphocyte activation (26, 27). VLA-4-mediated lymphocyte adhesion to VCAM-1 has been described in vitro in rheumatoid synovium (28), and in vivo in subcutaneous delayed type hypersensitivity and autoimmune encephalomyelitis (29-32). The transendothelial migration and subsequent infiltration of lymphocytes could be inhibited by anti-VLA-4 in some in vivo models of inflammation (29, 30, 32) with some exceptions (33, 34). Furthermore, the migration of eosinophils from allergic individuals through IL-4-activated endothelium could be inhibited by anti-VLA-4 in vitro (35). However, VLA-4/VCAM-1-mediated transmigration has not been established in vitro for lymphocytes (8, 36). The recent observation that rhabdomyosarcoma transfecants expressing a fusion protein containing the extracellular and transmembrane domains of the VLA-2 α subunit and the cytoplasmic domain of the VLA-4 α subunit exhibited enhanced migratory behavior on the laminin and collagen substrates for VLA-2, nevertheless, suggests a possible role for VLA-4 in cell locomotion (37).

VLA-4 also binds fibronectin, an extracellular matrix component, by interacting with at least three fibronectin sites, CS-1 and REDV in the IIICS region, and H1 in the HepII region. These regions are distinct from the Arg-Gly-Asp (RGD) motifs.

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1The abbreviations used are: mAb, monoclonal antibody; LAD, leukocyte adhesion deficiency; EBV, Epstein-Barr virus; PHA, phytohemagglutinin; HUVEC; human umbilical cord vein endothelial cells; PBS, phosphate-buffered saline; BCECF, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; FMA, phorbol 12-myristate 13-acetate.
tripeptide used by another β1 integrin VLA-5, the classical fibronectin receptor (38–42). The role of VLA-4/fibronectin adhesion in lymphocyte migration has not been established. Lymphocyte adhesion to high endothelial venule cells of cervical lymph nodes has been shown to involve both VLA-4 and VLA-5 binding to fibronectin, but only antibodies against VLA-5 could block the subcutaneous transmigration (43).

Another integrin αβ2 (also known as αLαM-1 or ααβ2) was recently shown to mediate lymphocyte adhesion to VCAM-1 and fibronectin (44). The β2 subunit is most homologous to β1 and β2 by primary sequence (45–48). αβ2 is expressed on all peripheral blood lymphocytes (45, 49) and was first described as a specific lymphocyte homing receptor for high endothelial venules in Peyer’s patches by interacting with an undefined ligand (50, 51). Its role in lymphocyte migration has not been extensively studied.

In this report, we examined the ability of lymphocytes to utilize VLA-4 in cell migration. An in vitro system to quantitatively measure lymphocyte migration on the purified ligands, VCAM-1 and fibronectin, was used to eliminate the contribution of other adhesion mechanisms. We showed that the VLA-4-mediated lymphocyte migration on surfaces coated with either recombinant VCAM-1 or fibronectin is site density-dependent, and that the adhesion efficiency is higher for VCAM-1 than for fibronectin.

MATERIALS AND METHODS

Monoclonal Antibodies—All mAbs used are of mouse anti-human origin. Anti-VLA-4 antibodies HP2/1 (IgG3) and 4B4 (IgG1) recognize the α4 and β1 subunits, respectively. Purified antibody HP2/1 was purchased from AMAC (Westbrook, ME) and 4B4 was purchased from Coulter (Hialeah, FL). Antibodies against the α subunits of VLA-3 (3B4, IgG1), VLA-5 (PlD6, IgG2a), VLA-4 (PlB5, IgG1) and VLA-5 (PlD6, IgG2a) were purchased as ascites from L. Grosmaire and M. Diegel (Bristol-Myers Squibb, Seattle, WA). Anti-VLA-4 antibodies HPW1 (IgG1) were a generous gift from J. Ledbetter (Bristol-Myers Squibb, Seattle, WA). Purified anti-VCAM-1 and fibronectin was purchased from L. Harlan (University of Washington, Seattle, WA). Anti-CD2 (9.6, IgG1) was a generous gift from J. Ledbetter (Bristol-Myers Squibb, Seattle, WA). Anti-CD8 (V85) was purchased from L. Harlan (University of Washington, Seattle, WA). Anti-CD3 (Ab3, IgG1) was purchased from Harlan (University of Washington, Seattle, WA).

All purified mAbs were used at 10 pg/ml and ascites were used at 100 pg/ml. VCA"1 and fibronectin, was used to eliminate the contribution of other adhesion mechanisms. We showed that the VLA-4-mediated lymphocyte migration on surfaces coated with either recombinant VCAM-1 or fibronectin is site density-dependent, and that the adhesion efficiency is higher for VCAM-1 than for fibronectin.

Cell Preparations and Cell Lines—SLA is an Epstein-Barr virus (EBV)-transformed B lymphoblastoid line derived from a LAD patient (44). It was maintained in RPMI medium containing 10% fetal calf serum (Hyclone, Logan, UT), 50 mg/ml gentamicin, 100 unit/ml penicillin, and 100 pg/ml streptomycin. T lymphoblasts were prepared by culturing peripheral blood mononuclear cells from normal donors in RPMI medium containing 10% fetal calf serum and 25 mm Hepes (pH 7.4). Polycarbonate membranes at the bottom of the cell culture inserts (Transwell, 8.0-μm pore size, 6.5-mm diameter, Costar, Cambridge, MA) in 24-well tissue culture plates were coated on both surfaces with VCAM-1/Fc and fibronectin. The proteins were added to the upper chamber (25 μg/ml) and the lower chambers (24-well) (150 μl) at 25 °C for 1.5 h. After rinsing with PBS, nonspecific binding sites on the plastic were saturated by incubation with Tris-buffered saline at pH 8.0 containing 1% heat-treated (80 °C, 20 min) bovine serum albumin at 25 °C for 1 h. The microtitre wells were then washed extensively with radioimmunoassay medium. RPMI containing 10% fetal calf serum and 25 mm Hepes (pH 7.4). The VCAM-1/Fc fusion protein was purified as previously described (53). The affinity-purified Cos cells (cat no. 697785, Costar, Cambridge, MA) were transiently transfected with the VCA"1/Fc plasmid by the DEAE-dextran method and cultured in serum-free Dulbecco’s modified Eagle’s medium on the following day for 6–9 days. The culture supernatant was used to pass through a protein A–agarose (Repligen, Cambridge, MA) affinity column. After extensive washing with phosphate-buffered saline (PBS) at pH 7.4, the VCA"1/Fc fusion protein was eluted at pH 3 in 0.1 M citrate (pH 5.0) and dialyzed against PBS. Purified fusion protein was quantified by Bradford’s reagents (Bio-Rad) using bovine γ-globulin for calibration. The fusion protein was essentially pure as visualized by SDS-polyacrylamide gel electrophoresis and silver staining.

Determination of Site Densities by Radioimmunoassays—Radioimmunoassays were used to determine the actual amount of VCAM-1/Fc and fibronectin immobilized on plastic or polycarbonate membranes. Purified VCAM-1/Fc fusion protein or human plasma fibronectin (Collaborative Biomedical Products) at various concentrations were immobilized on 96-well polystyrene plates (FALCON 3915, Becton Dickinson) at 25 °C for 1 h. After rinsing with PBS, nonspecific binding sites on the plastic were saturated by incubation with Tris-buffered saline at pH 8.0 containing 1% heat-treated (80 °C, 20 min) bovine serum albumin at 25 °C for 1 h.

Polycarbonate membranes at the bottom of the cell culture inserts (Transwell, 8.0-μm pore size, 6.5-mm diameter, Costar, Cambridge, MA) in 24-well tissue culture plates were coated on both surfaces with VCAM-1/Fc and fibronectin. The proteins were added to the upper chamber (25 μg/ml) and the lower chambers (24-well) (150 μl) at 25 °C for 1.5 h. After rinsing with PBS, nonspecific binding sites were saturated as mentioned above at 25 °C for 1.5 h, followed by extensive washing with radioimmunoassay medium.125I-Labeled BBA6 (anti-VCAM-1) or MAB122 (anti-fibronectin) at 3 μCi/μg specific activity and 100 μg/ml was added to the 96-well plates or Transwell membranes at a final concentration of 10 μg/ml and a total volume of 50 μl/96-well, or 150 μl/upper chamber and 700 μl/lower chamber. After incubation at 25 °C for 30 min, excess antibodies were removed by washing four times with medium. Bound antibodies in 96-well plates were removed for counting in 0.1 M NaOH and those in Transwell membranes were removed by 0.1 M NaOH at 25 °C for 1 h. Bound antibodies were counted by gamma counting.

Adhesion Assays—VCAM-1/Fc and fibronectin were immobilized in duplicates on 96-well plates as described above. Lymphocytes were fluorescently labeled with 17 μg/ml 2',7'-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein (acetoxymethyl) ester (BCECF) (Molecular Probes, Eugene, OR) in binding medium (same as radioimmunoassay medium above) at 25 °C for 20 min and washed. For inhibition studies, the cells were pretreated with 10 μg/ml 2'-deoxy-DTDA, HP2/1, PlD6, 4B4, 100 μg/ml EDTA, or 50 μg/ml anti-VCAM-1 (Staph-109) at 25 °C for 30 min or with 100 μg/ml cytochalasin B (Calbiochem) at 37 °C for 1 h before use. Uncoated wells or wells coated with VCAM-1/Fc were pretreated with or without 10 μg/ml 4B9 before use. Where indicated, SLA cells were pretreated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Calbiochem) at 25 or 37 °C for 30 min before use. The mAbs, inhibitors, or PMA were present at the same concentrations throughout the adhesion assays. The cells

TCGAGATCTTACGAGAGTAG and 5'-GCCATGCTATGATGATC.
were vortexed, dispensed at $5 \times 10^4$ cells/well in 100 μl, and allowed to settle under gravity at 37°C, 5% CO₂ for 30 min. At this input density, the SLA cells and the PHA blasts were not in close contact, minimizing homotypic aggregation. Nonadherent cells were removed sequentially by aspirating four times with hypodermic needles, starting from 25-gauge (smaller bore size) to 16-gauge (bigger bore size), with the bevel directed against the side of the well. The aspiration created a shear force along the bottom of the well which detached the nonadherent cells and cells which adhered with lower avidity. After each cycle of needle aspiration, adherent cells were measured using a fluorescence plate reader (Pandex Division, Baxter Healthcare Corp., Mundelein, IL) at 485 nm excitation and 535 nm emission. The percentage of cells bound was determined by dividing the fluorescence of adherent cells by that of total input cells.

Migration Assays—VCAM-1/Fc or fibronectin was immobilized on both surfaces of the polycarbonate membrane in Transwell cell culture inserts as described above. Lymphocytes labeled with BCECF were vortexed, dispensed in binding medium at $10^6$ cells/upper chamber, and allowed to migrate across the membrane at 37°C, 5% CO₂ for 4 h. Where indicated, mAbs, inhibitors, or PMA were used throughout the assays as described above for the adhesion assays. Nonadherent cells were removed by rinsing the upper chamber with the binding medium. Cells adherent to the upper surface of the membrane were removed by twice scraping gently with cotton-tip applicators, followed by washing. Migrating cells which stayed adherent to the lower surface of the membrane were fixed in PBS containing 1% formaldehyde, 1 mM MgCl₂, and 1 mM CaCl₂. The migrating cells in three low power fields (40x magnification) were counted using epifluorescence microscopy (Nikon Diaphot) using a reticule (1 x 1 cm square) in the eyepiece.

Flow Cytometry—Lymphocytes were analyzed by flow cytometry for the expression of VLA-3, VLA-4, VLA-5, FcR, type I, II, and III, and CD2. They were stained with the corresponding mAbs in RPMI containing 1% fetal calf serum and 25 μM Hepes (pH 7.4) at 4°C for 30 min, followed by fluorescein isothiocyanate-conjugated goat F(ab')₂ anti-mouse Ig (Tago, Burlingame, CA). A nonbinding mAb X63 was used as the negative control antibody.

RESULTS
SLA Cells Adhered Specifically to Immobilized VCAM-1/Fc Fusion Protein—Soluble recombinant VCAM-1 was used to study VLA-4-mediated lymphocyte adhesion and migration. We have previously constructed a fusion protein consisting of the first three amino-terminal Ig-like domains of VCAM-1 and the hinge, CH2, and CH3 regions of Ig Fc (designated VCAM-Rg). VCAM-Rg has been shown to bind VLA-4 on lymphocytes, melanomas, and other carcinomas (64, 55). Two recent reports, however, have suggested that both the first and the fourth amino-terminal Ig-like domains of VCAM-1 are involved in VLA-4-mediated lymphocyte adhesion (56, 57). In an effort to study VLA-4-mediated migration on its physiologic ligands, we constructed a similar fusion protein which contains the central extracellular domain of VCAM-1 (hereafter referred as VCAM-1/Fc). As predicted from the primary sequence of the construct, the VCAM-1/Fc fusion protein purified from transfected COS cells was a disulfide-linked homodimer of approximately 240 kDa in size by SDS-polyacrylamide gel electrophoresis analysis (data not shown). The EBV-transformed B cell line SLA was used to study VLA-4-mediated lymphocyte adhesion and migration. EBV-transformed cells are more motile than other immortalized lymphoid cell lines (58) and migrate on surfaces coated with ICAM-1 in the absence of chemoattractants by binding to LFA-1 (28). SLA cells were derived from a patient suffering from LAD. They do not express the β₂ integrin LFA-1 which often causes strong homotypic aggregation of lymphocytes expressing high levels of ICAM-1 (28). In our in vitro system, which utilizes immobilized adhesive ligands (as described below), strong homotypic aggregation of cells may render quantitative analysis of adhesion and migration difficult to interpret when the cells preferentially cluster to each other rather than adhere to or migrate on the adhesive substrates. Although a portion of SLA cells exhibit weak homotypic aggregation in culture via an unknown adhesion pathway, visual examination under phase-contrast microscopy confirmed that SLA cells adhered to immobilized VCAM-1/Fc or fibronectin without aggregation. In addition to VLA-4, two other β₂ integrins, ICAM-3 and VLA-5, also bind fibronectin. Immunofluorescence staining followed by flow cytometric analysis showed that SLA cells expressed a moderate level of VLA-4 (HP2/1, anti-α₄ and 4B4, anti-β₂), but negligible levels of VLA-3 (P1B5, anti-α₃) and VLA-5 (P1D6, anti-α₅) (Fig. 1). It should be noted that the mean fluorescence level obtained with HP2/1 (anti-α₄) was slightly higher than that obtained with 4B4 (anti-β₂), suggesting the surface expression of the α₄β₇ heterodimer. The SLA cells did not express FcR, type I (197), type II (IV.3), or type III (FC-2) at any significant level (Fig. 1). Hence, the VLA-3- and VLA-5-mediated fibronectin adhesion and migration of SLA cells was significantly minimized and the effect of FcR interaction via the Fc portion with the VCAM-1/Fc fusion protein was eliminated (see results below).

SLA cells binding to VCAM-1/Fc immobilized on plastic exhibited characteristics of VLA-4-mediated adhesion (Fig. 2). The adhesion was completely abolished by pretreating the cells with HP2/1 (anti-α₄), which blocks both VLA-4 and α₄β₇ binding to VCAM-1, and by pretreating the substrate with 4B4, a mAb against VCAM-1 which blocks VLA-4 binding. The anti-β₂ mAb 4B3 inhibited over 80% of SLA cell adhesion to VCAM-1/Fc, suggesting that α₄β₇ might be used as a minor adhesion pathway, accounting for less than 20% of the cell adhesion. No inhibition was observed with an isotype-matched mAb against ICAM-1 (84H10). The adhesion was sensitive to EDTA and EGTA which are known to abolish all integrin-mediated adhesion. Pretreatment with cytochalasin B, which inhibits actin polymerization, diminished SLA adhesion to VCAM-1/Fc significantly, suggesting that disruption of cytoskeleton reduces the avidity of VLA-4 adhesion to VCAM-1. SLA cells did not adhere at all to uncoated plastic or plastic coated with an irrelevant fusion protein CD7/Fc (55).

SLA Cells Migrated Specifically Across Polycarbonate Membranes Coated with VCAM-1/Fc or Fibronectin—A cell culture system (Transwell, Costar) consisting of an upper and a lower chamber separated by a 10-μm thick tissue culture-treated porous polycarbonate membrane (0.8-μm pore diameter) was used to study SLA migration. VCAM-1/Fc or fibronectin was immobilized on both the upper and the lower surfaces of the membrane. Fluorescently labeled SLA cells were allowed to bind to the upper surface of the membrane, randomly migrate in the absence of chemoattractants through the pores, and adhere to the lower surface of the membrane. The number of adherent cells on the lower surface of the membrane in three low power fields were counted under epifluorescence microscopy, and used to quantify the relative migration efficiency of cells on the immobilized substrate. Examination of the upper and lower surfaces of the membrane showed that the number of cells which migrated across the membrane usually constituted a low percentage (less than 20%) of the cells bound to the upper
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**Fig. 2.** Specificity of SLA adhesion to VCAM-1/Fc immobilized on plastic. VCAM-1/Fc was used to coat 96-well flat-bottom plates at 20 μg/ml. Fluorescently labeled SLA cells pretreated with medium, 84H10 (anti-ICAM-1), HP2/1 (anti-α4), 4B4 (anti-β1), EDTA, EGTA, or cytochalasin B were allowed to adhere to VCAM-1/Fc with or without pretreatment with 4B9 (anti-VCAM-1) at 37 °C, 5% CO_2 for 30 min. Nonadherent cells were removed by aspiration using a 25-gauge needle. Error bars represent standard deviations of adherent cells in duplicate wells. Results are representative of two separate experiments.

The migration of SLA cells across membranes coated with VCAM-1/Fc was specifically mediated by VLA-4 as it could be completely abolished by pretreating the cells with HP2/1 (anti-α4) or 4B4 (anti-β1), or pretreating the membranes with 4B9 (anti-VCAM-1) (Fig. 3A). Pretreatment of the cells with 84H10 (anti-ICAM-1) did not inhibit SLA cell migration but enhanced it. Since ICAM-1 triggered signal transduction has not been characterized, further studies on the effects of ICAM-1 perturbation on lymphocyte motility may yield interesting insight. Similar to VCAM-1/Fc, the migration of SLA cells across membranes coated with fibronectin was also mediated by VLA-4 (Fig. 3B). The migration was, however, equally inhibited by HP2/1 (anti-α4), CS-1 peptide, and P1D6 (anti-α5), and to a lesser extent by P1B5 (anti-α4), suggesting the contribution of VLA-3 and VLA-5 in SLA cell migration on fibronectin. Since SLA cells express very low levels of VLA-3 and VLA-5 (Fig. 1), VLA-5 appeared to be a more effective migratory pathway than fibronectin than VLA-4 (see inhibition of adhesion below). None of the mAbs alone inhibited migration completely. As predicted, cytochalasin B, which inhibits actin polymerization and thus cell motility, completely abolished the SLA cell migration on either substrates (data not shown). The significantly higher number of migrating cells across membranes coated with VCAM-1/Fc than with fibronectin prompted us to quantitatively compare the migration efficiency of SLA cells on the two substrates.

**SLA Cells Migrated Across Polycarbonate Membranes Coated with VCAM-1/Fc or Fibronectin in a Site Density-dependent Manner and More SLA Cells Migrated on VCAM-1/Fc than on Fibronectin**—In order to compare the migration efficiencies of SLA cells on VCAM-1/Fc and fibronectin, the Transwell membranes were coated with either substrate on both surfaces and the total site densities of each substrate on the membrane were determined by radioimmunoassays. Iodinated anti-VCAM-1 mAb BBAG, an adhesion-blocking antibody, was used to determine the number of active VLA-4 binding sites available for cell adhesion. The adhesion-blocking status of the iodinated anti-fibronectin MAB122 is unknown and the site densities determined using this mAb represent an estimate of the immobilized fibronectin molecules containing the MAB122 epitope. The immobilization efficiencies of VCAM-1/Fc and fibronectin were comparable. The site densities were expressed in molecules/membrane because the actual area of the membrane including the pores could not be accurately calculated.

SLA cells migrated across membranes coated with either VCAM-1/Fc or fibronectin in a site density-dependent manner (Fig. 4). The number of migrating cells decreased above and below an optimal site density which was comparable for both VCAM-1/Fc and fibronectin. The number of migrating SLA cells varied significantly between different experiments. In general, a higher migration efficiency of SLA cells was observed.
in experiments using cells at an earlier stage of log phase growth. These cells expressed slightly higher levels of VLA-4 (data not shown). It is also possible that SLA cells exhibited a higher random motility under these culture conditions. Nevertheless, the number of SLA cells which migrated on VCAM-1/Fc-coated membranes was always 10-14-fold higher than on fibronectin-coated membranes at the optimal site density regardless of the cell culture density.

SLA Cells Adhered to VCAM-1/Fc Immobilized on Plastic More Efficiently than to Fibronectin—The higher number of migrating SLA cells on VCAM-1/Fc than on fibronectin-coated membranes may be attributable to a higher percentage of cells which adhered to VCAM-1 than to fibronectin. In order to test this hypothesis, the adhesion efficiency of SLA cells to VCAM-1/Fc and fibronectin immobilized on plastic was characterized. Site density determination by radioimmunoassays revealed that, in contrast to immobilization on polycarbonate membranes, fibronectin was immobilized on plastic more efficiently than VCAM-1/Fc. However, SLA cells adhered more readily to lower site densities of VCAM-1/Fc than to fibronectin (Fig. 5A). For 15% of the input SLA cells to be bound, 20-fold higher fibronectin site density (800 versus 40 sites/square μm) was required than VCAM-1/Fc. At a site density (200 sites/square μm) of VCAM-1/Fc where 35% of SLA cells were adherent, only 2% of cells adhered to fibronectin. The avidity of SLA cell adhesion for the two ligands was also compared by detaching adherent cells remaining bound when various shear forces (25 to 16-gauge aspirating needles) were used for cell detachment (Fig. 5B). Inhibition studies using mAbs showed that the SLA cell adhesion to fibronectin was mediated primarily by VLA-4 and was inhibited equally well by HP2/1 (anti-a4) and 4B4 (anti-b1) (Fig. 7A) and one-third less well by CS-1 peptide (data not shown). The adhesion was also to a lesser extent mediated by VLA-5. Similarly, the up-regulated adhesion of SLA cells to fibronectin following PMA pretreatment involved primarily VLA-4 and to a lesser extent VLA-3 (Fig. 7B). Since SLA cells express very low levels of VLA-3 and VLA-5 (Fig. 1), the avidity of VLA-3 and VLA-5 for fibronectin appeared to be significantly higher than that of VLA-4.

Phorbol Ester Enhanced SLA Cell Migration Across Polycarbonate Membranes Coated with Either VCAM-1/Fc or Fibronectin—PMA pretreatment of SLA cells enhanced cell migration across membranes coated with VCAM-1/Fc and fibronectin (Fig. 8). The optimal site densities for maximal SLA cell migration on both VCAM-1/Fc and fibronectin decreased, and the overall number of migrating cells on either substrates increased at all site densities (Fig. 8). These results suggest an overall increase in random motility of the cells and an increased adhesion avidity at higher site densities which may

**Fig. 4. Migration of fluorescently labeled SLA cells across Transwell membranes coated with VCAM-1/Fc or fibronectin at different site densities.** Migrating cells were counted under microscopy after 4 h of incubation at 37 °C, 5% CO2. Error bars represent standard deviations of migrating cells in three low power fields. Results are representative of five separate experiments.

**Fig. 5. Adhesion of SLA cells to VCAM-1/Fc or fibronectin immobilized on plastic at different site densities.** A, fluorescently labeled SLA cells were allowed to adhere in precoated 96-well flat-bottom plates at 37 °C, 5% CO2 for 30 min and nonadherent cells were removed by aspiration using a 25-gauge needle. B, aspiration was repeated using 23 to 16-gauge needles and the percentage of adherent cells on VCAM-1/Fc or fibronectin was determined after each washing cycle. The percentage of adherent cells which remained bound was calculated based on the number of adherent cells following the initial 25-gauge needle aspiration (100%). Error bars represent standard deviations in duplicate wells. Results are representative of three separate experiments.
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A

Fig. 6. Adhesion of PMA-pretreated SLA cells to VCAM-1/Fc or fibronectin immobilized on plastic at different site densities. A, fluorescently labeled cells were pretreated with PMA and allowed to adhere in precoated 96-well flat-bottom plates at 37 °C, 5% CO₂ for 30 min. Nonadherent cells were removed by aspiration using a 25-gauge needle. B, aspiration was repeated using 23 to 16-gauge needles and the percentage of adherent cells on VCAM-1/Fc or fibronectin was determined after each washing cycle. The percentage of adherent cells which remained bound was calculated based on the number of adherent cells following the initial 25-gauge needle aspiration (100%). Error bars represent standard deviations in duplicate wells. Results are representative of two separate experiments.

B

restrict cell locomotion. The maximal number of PMA-pretreated cells migrating on VCAM-1/Fc was only 3-fold higher than that on fibronectin at the optimal site density.

Adhesion and Migration of PHA Blasts on Immobilized VCAM-1/Fc or Fibronectin—Peripheral blood lymphocytes from normal donors were tested for migration on VCAM-1/Fc or fibronectin. Resting peripheral blood lymphocytes have low avidity for VCAM-1 and fibronectin and are relatively less motile compared to activated lymphocytes (27, 59). They did not migrate on either VCAM-1/Fc- or fibronectin-coated membranes after 4 h of incubation in our assays, and PMA pretreatment induced nonspecific migration across uncoated membranes (data not shown). T lymphoblasts generated by stimulating peripheral blood mononuclear cells from normal donors with PHA for 5 days were then used to study VLA-4-mediated adhesion and migration. The lymphoblast preparations were depleted of cells expressing VLA-3, VLA-5, FcγR, type II and III by negative selection in order to minimize cell adhesion and migration mediated by other fibronectin receptors or the Fc portion of the VCAM-1/Fc fusion protein. Immunofluorescence staining followed by flow cytometric analysis showed that the PHA lymphoblasts derived from different donors expressed variable levels of VLA-4, but negligible levels of the negatively selected markers. Similar to SLA cells, the T lymphoblasts adhered more efficiently to VCAM-1/Fc than to fibronectin, and a higher number of cells migrated on VCAM-1/Fc than on fibronectin (data not shown). The site density-dependence of T lymphoblast migration also resembled that of SLA cells.

DISCUSSION

Lymphocyte migration is an important adhesion-triggered cellular response that allows cell trafficking between the circulation and lymphoid tissues and extravasation at sites of inflammation. It is well established that the β₂ integrin LFA-1 expressed on all leukocytes is crucial in mediating adhesion to endothelial cells and transendothelial migration by binding to ICAM-1 and possibly ICAM-2. The β₂ integrin VLA-4 resembles LFA-1 in that it is also expressed on most leukocytes except neutrophils, and it mediates cell adhesion by binding to VCAM-1 on cytokine-activated endothelial cells and to fibronectin in the extracellular matrix. We herein presented evidence that VLA-4 is specifically involved in the locomotion of lymphocytes through porous polycarbonate membranes coated with its purified ligands, VCAM-1 (recombinant VCAM-1/Fc) and fibronectin. The EBV-transformed B cell line SLA migrated specifically on both VCAM-1/Fc and fibronectin in a site density-dependent manner. Above and below an optimal site density of VCAM-1/Fc or fibronectin, the migration efficiency de-
Increased. Inhibition studies using mAbs showed that the migration of SLA cells was mediated by VLA-4 with no contribution from α5β1.

We have compared the SLA cell migration efficiency for VCAM-1/Fc and fibronectin by normalizing the site densities of each ligand immobilized on the membrane. An adhesion blocking antibody was used in the radioimmunoassay of immobilized VCAM-1/Fc to quantify the amount of VCAM-1/Fc binding sites available for cell adhesion and subsequent migration. Since an antibody of underdetermined binding epitope or adhesion blocking status was used in the radioimmunoassay of immobilized plasma fibronectin, the actual amount of VLA-4 binding sites, CS-1 and REDV (38, 41, 42). Another VLA-4 binding site H1 resides in the heparin-binding region (HepII) NH2-terminal to the IIICS region (39). In addition, two other segments in the proximity have also been identified to bind chondroitin sulfate proteoglycan and yet be sensitive to anti-α4 (62). Considering the various regions which may be involved in VLA-4 binding, our experiments using immobilized intact fibronectin and anti-α4 inhibition studies are probably more appropriate than using immobilized fibronectin peptides in assessing the role of VLA-4 in cell migration.

Although the optimal site densities for migration for VCAM-1/Fc and fibronectin are comparable, over 10-fold higher numbers of SLA cells migrated across membranes coated with VCAM-1/Fc than with fibronectin. The difference in the number of SLA cells migrating on VCAM-1/Fc and fibronectin is consistent with the higher percentage of SLA cells which adhered to VCAM-1/Fc as compared to fibronectin. Furthermore, SLA cells adhered to VCAM-1/Fc with a stronger avidity than to fibronectin, as demonstrated by using various shear forces for detachment of bound cells. PMA pretreatment up-regulated the avidity of SLA cell adhesion to VCAM-1/Fc and fibronectin. Nevertheless, the PMA-pretreated cells still migrated more efficiently on VCAM-1/Fc than on fibronectin, suggesting that the different migration efficiencies do not depend on the activation state of the cells. T lymphoblasts derived from normal donors also exhibited the same pattern of differential adhesion and migration efficiency on VCAM-1/Fc and fibronectin.

Our observation that the SLA cells and T lymphoblasts from normal donors can migrate on VCAM-1/Fc suggests that, like LFA-1, VLA-4 may be an adhesion receptor which mediates transendothelial migration of lymphocytes. VCAM-1, ICAM-1, and ICAM-2 are expressed on cytokine-activated vascular endothelium (21, 23, 63). The LFA-1/ICAM-1 (and possibly LFA-1/ICAM-2) interaction constituted a major adhesion pathway for resting and activated T lymphocytes binding to unstimulated HUVEC which did not express VCAM-1 (38, 63). Anti-LFA-1 and anti-ICAM-1 mAbs significantly blocked the transmigration of T lymphocytes across unstimulated HUVEC (6, 10, 36). In contrast, the VLA-4/VCAM-1 interaction accounted for a significant portion of the resting T lymphocyte adhesion to interleukin-1-activated HUVEC where the LFA-1/ICAM-1 and LFA-1/ICAM-2 adhesion pathways were not detected (36). MAbs against VLA-4 and VCAM-1, however, did not significantly inhibit the transendothelial migration of either resting or activated T lymphocytes, or T cell clones derived from LAD patients, suggesting the presence of as yet unidentified adhesion/migration pathways (8, 36). It should be emphasized that in these in vitro systems, the endothelial cells were presenting multiple adhesive ligands to the interacting lymphocytes, and that the behavior of cell adhesion and transendothelial migration are related but yet distinct properties which probably involve more than one receptor/ligand pair. The presence of a strong adhesive pathway would render other weaker pathways difficult to study. For example, the involvement of VLA-4 in lymphocyte adhesion to activated HUVEC was only apparent in the presence of blocking anti-LFA-1 (17). Furthermore, mAb inhibition of lymphocyte migration may be attributable to three nonexclusive causes: disruption of the adhesion contact mediated by adhesion receptors; blocking of the interaction of locomotive receptors different from the adhesion receptors which initiate the adhesion contact; or triggering of an inhibitory signal for the adhesive or migratory responses. Therefore, results from mAb inhibition studies should be interpreted with caution.

Our in vitro system utilizing purified adhesive ligands to study adhesion-triggered migration was designed to delineate the function of selected receptor/ligand pairs. Our results showed that lymphocytes migrated specifically on surfaces coated with purified VCAM-1/Fc or fibronectin, and the migration could be blocked by antibodies against VLA-4, suggesting that VLA-4 is a bona fide locomotive adhesion receptor. Our data, however, do not exclude the possibility that anti-VLA-4 provided a negative signal which inhibited cell motility, but we considered this unlikely. Our observation that SLA cells did not express significant levels of VCAM-1 (data not shown) and that pretreatment of the VCAM-1/Fc substrate with anti-VCAM-1 completely inhibited SLA cell migration supported this contention and the hypothesis that cell migration is adhesion-triggered.

There are several possible explanations for the lack of VLA-4-mediated transendothelial migration of lymphocytes in vitro using HUVEC which expressed VCAM-1 (8, 36). First, the site density of VCAM-1 on interleukin-1-activated HUVEC is much lower than that of ICAM-1 (8). Second, the expression level of LFA-1 on the peripheral blood lymphocytes is higher than that
of VLA-4 (36). Third, the LFA-1/ICAM-1 interaction or as yet unidentified adhesion/migration pathways may have a higher avidity than that of VLA-4/VCAM-1 as suggested by mAb inhibition studies (17). These factors alone or in combination may render the VLA-4/VCAM-1 interaction a less efficient migration pathway than LFA-1/ICAM-1 in the migration of lymphocytes across HUVEC monolayers in vitro. The role of VLA-4/VCAM-1 interaction in lymphocyte migration to inflammatory sites in vivo has been established by mAb inhibition studies (29, 30, 32), despite the lack of evidence in vitro using interleukin-1-stimulated HUVEC (8, 36). These disparate observations may reflect the differences in physiologic responses between HUVEC, which are derived from large vessel endothelium, and microvascular endothelial cells, with which lymphocytes interact during extravasation from the circulation. For instance, dermal microvascular and mesenteric lymph node endothelial cells differ from HUVEC in the induction of VCAM-1 and up-regulation of ICAM-1 expression following cytokine stimulation (64, 65). Furthermore, endothelial cells express different combinations of adhesion molecules depending on the proinflammatory cytokine stimuli. For example, in contrast to interleukin-1 and tumor necrosis factor-alpha, interleukin-4 induces VCAM-1 expression without up-regulating ICAM-1 on both HUVEC and microvascular endothelial cells (64, 66). Therefore, it is conceivable that the VLA-4/VCAM-1 interaction may play an important role in transendothelial migration of lymphocytes, depending on the location of the microvasculature and the combination of the locally released cytokines. Further studies are required to establish the VLA-4/VCAM-1-mediated lymphocyte migration under physiologic conditions. The role of VLA-4/fibronectin interaction in lymphocyte migration has not been established previously. Fibronectin synthesized by endothelial cells is organized in strands marking the endothelial cell-cell border and deposited as a network on the basolateral surface (67). It is possible that this unique distribution of fibronectin on endothelial cells, in conjunction with the abundance of fibronectin and other extracellular matrix components in connective tissues and the stroma of lymphoid tissues, may provide an effective migratory substrate for lymphocyte trafficking. A distinct example is the reticular fibers of fibronectin, collagen, and laminin oriented parallel to the migratory paths of lymphocytes from the high endothelial venules to the antigen-presenting dome areas in lymph nodes. These structures may provide crucial routes for lymphocyte localization and functional interactions in lymph nodes (68). It has been reported that the migration of memory T cells to cutaneous inflammatory sites in mice could be inhibited by synthetic peptides of fibronectin which contain binding sites for VLA-4 and VLA-5 (69). Hence, it is likely that VLA-4 plays a role in lymphocyte migration and cell trafficking on fibronectin.

The avidities of VLA-4-mediated lymphocyte adhesion to VCAM-1 and fibronectin have not been compared quantitatively in previous studies because the actual amount of purified substrates immobilized on plastic was not determined. Depending on the average hydrophobicity and the steric structure, protein molecules vary in their efficiency of immobilization on plastic surfaces and in their accessibility for ligand binding. As a result, incubation of plastic surfaces with equal concentrations of different proteins may not result in comparable site densities as with VCAM-1/Fc and fibronectin (data not shown). Our observation that lymphocytes, whether or not pretreated with PMA, adhered more avidly and in higher numbers to VCAM-1/Fc than to fibronectin at equivalent site densities suggests that the affinity of VLA-4 for VCAM-1 is higher than that for fibronectin, independent of the activation state of the cell. It is also possible that the binding of VLA-4 by VCAM-1, but not by fibronectin, may further strengthen the adhesion by specifically inducing conformational changes in the integrin molecules, or by redistribution of the adhesion receptor to the substrate contact area. Such ligand-triggered adhesion strengthening and receptor redistribution have been previously described in other integrin-mediated adhesion (70-77).

Recently, Masumoto and Hemler (78) reported that stimulating antibodies against the beta-1 integrin subunit or including Mn2+ in adhesion assays could significantly enhance the VLA-4-mediated cell adhesion to either VCAM-1 or fibronectin, and that the requirements for Mg2+ and Ca2+ varied depending on the cell lines and ligand specificity. Although no effective comparison of the cell adhesion efficiency for either ligands was available because the actual amount of ligands immobilized on plastic was not determined, their data suggest that VLA-4 has variable activation states and different VLA-4 activation states on different cell lines affect the cell adhesion efficiency and cation dependence for VCAM-1 and fibronectin. While the VLA-4 activation state on SLA cells was not determined and its effects on migration were not tested in our in vitro system by using stimulating anti-beta-1 or including Mn2+, we found that PHA blasts which represent a heterogeneous population of activated T cells exhibited differences in cell adhesion and migration efficiency for VCAM-1 and fibronectin. It remains to be tested if a certain activation state of VLA-4 is preferentially induced under physiologic conditions such that the cell adhesion or migration efficiency for fibronectin may approach that for VCAM-1.

That VLA-4 is a bona fide locomotive adhesion receptor suggests a functional and physical association of VLA-4 with the cytoskeletal network in the cytosol. Cell locomotion is a concerted cellular behavior which involves changes in cell shape normally manifested as an initial spreading of the cell on an adhesive substrate, formation of a migratory front, dynamic rearrangement of actin-associated cytoskeletal elements partly responsible for the tensile force developing at the leading edge, movement of the cytosolic mass in the direction of migration, and detachment from the substrate at the trailing end of the cell (79-82). This concerted cellular response is triggered partly by the adhesion of cells via adhesion receptors. It is thought that these receptors are either directly associated to the cytoskeletal machinery or to signal transduction pathways which modulate cytoskeletal rearrangement and promote cell migration. The physical association of integrins to the cytoskeleton has been described previously. When T lymphocytes were activated via T cell receptor, LFA-1 has been localized with talin and F-actin (77, 83). Likewise, VLA-4 has been shown to colocalize with F-actin in activated LAD T cells (83). It has also been shown that LFA-1 mediates lymphocyte migration upon adherence to purified ICAM-1 (26). Our observation that VLA-4-mediated adhesion-triggered migratory response of lymphocytes resembles the response mediated by LFA-1 and corroborates the significance of the localization of both integrins with the cytoskeleton. The beta-1 subunit has been shown to directly associate with talin and alpha-actinin in fibroblasts (84-86), and natural killer cells and fibroblasts utilize the classical fibronectin receptor VLA-5 to migrate on fibronectin (7, 87-89). Recently, Chan et al. (37) reported that transfection of the cytoplasmic domain of the alpha-4 subunit fused to the extracellular and transmembrane domains of alpha-2 into rhabdomyosarcoma cells rendered the cells migratory on the VLA-2 ligands laminin and collagen. Their results suggest that alpha-4, in addition to beta-1, may also be associated with cytoskeletal elements and contributes to the migratory behavior. Alternatively, the alpha-4 subunit may promote migration by association with signal transduction mechanisms which regulate cytoskeletal rearrangements.
Since the migration of both SLA cells and T lymphocytes occurred in the absence of exogenous chemokinetic factors in our assays, we postulate that VLA-4 is physically associated with cytoskeletal elements as well as functionally associated with signal transduction mechanisms which regulate cytoskeletal rearrangements. The role of α4β1 in SLA cell migration was not observed in further studies. The site density dependence of VLA-4-mediated lymphocyte migration on immobilized VCAM-1/Fc and fibronectin substrates suggests a delicate balance between the stationary and motile behavior of a cell on an adhesive substrate. This contention is supported by at least two recent reports. First, neural crest cells migrated more efficiently on immobilized antibodies against β1 integrins of lower affinities than higher affinities. The migration efficiency was also higher if the antibodies were immobilized at lower input concentrations than higher input concentrations. Since no direct comparison has been reported so far of the site density dependence of VLA-4-mediated lymphocyte adhesion and migration observation with fibronectin within the range of ligand site densities tested. Alternatively, the reduction in migration efficiency may reflect a diminished percentage of SLA cells which adhered too avidly are unable to migrate. In our system, the migration of SLA cells decreased with increasing VCAM-1/Fc or fibronectin site densities above the optimal site density, suggesting that the more avidly the cells adhered to the higher site densities, the less efficiently they could migrate. Below the optimal site density, the number of migrated cells decreased with decreasing VCAM-1/Fc or fibronectin site densities, probably because the adhesion avidity was too weak to provide anchorage required to support efficient cell motility. Alternatively, the reduction in migration efficiency may reflect a diminished percentage of SLA cells which bound to the substrates. Consistent with this avidity-dependent regulation of cell locomotion, PMA pretreatment up-regulated the adhesion avidity and lowered the optimal site density required for SLA cell migration on either substrates. In contrast to the neural crest cell model of cell migration, SLA cells were unable to migrate across membranes coated with various amounts of HP2/1 (anti-α4). This may reflect a specific property of VLA-4 which is distinct from the β1 integrins used by the neural crest cells, or a locomotive behavior unique for neural crest cells.

We have demonstrated that VLA-4-mediated lymphocyte adhesion and migration with VCAM-1/Fc is more efficient than with fibronectin within the range of ligand site densities tested. Since no direct comparison has been reported so far of the quantity of VCAM-1 expressed on the surfaces of cytokine-activated endothelium or that of fibronectin localized in the cell-cell border and basal surface of endothelium, it may be difficult to directly derive physiologic significance from our observation in vitro. It is conceivable that the distinct localization of VCAM-1 and fibronectin in the microvasculature environment, in conjunction with the higher adhesion strength for VCAM-1 than fibronectin, may allow efficient lymphocyte transmigration when the cells adhere to VCAM-1 on the luminal surface of the endothelium under constant fluid shear stress in the circulation, actively crawl to the intercellular endothelial junctions, then migrate underneath the endothelium and through the connective tissue stroma where fibronectin is abundant.
VLA-4-mediated Lymphocyte Migration