A Monoclonal Antibody to β_1 Integrin (CD29) Stimulates VLA-dependent Adherence of Leukocytes to Human Umbilical Vein Endothelial Cells and Matrix Components

Nicholas L. Kovach, Timothy M. Carlos, Esther Yee, and John M. Harlan

University of Washington, School of Medicine, Division of Hematology, Seattle, Washington 98195

Abstract. The leukocyte β_1 integrin receptor very late activation antigen-4 (VLA-4) ($\alpha_4\beta_1$, CD49d/CD29) binds to vascular cell adhesion molecule-1 (VCAM-1) expressed on cytokine-activated endothelium. A mAb designated 8A2 was identified that stimulated the binding of U937 cells to CHO cells transfected with VCAM-1 cDNA but not endothelial-leukocyte adhesion molecule or CD4 cDNA. mAb 8A2 also rapidly stimulated the adherence of peripheral blood lymphocytes (PBLs) to VCAM-1–transfected CHO cells or recombinant human tumor necrosis factor-treated human umbilical vein endothelial cells. mAb 8A2–stimulated binding of PBL was inhibited by mAbs to VLA-4 or VCAM-1. Surface expression of VLA-4 was not altered by mAb 8A2 treatment and monovalent Fab fragments of mAb 8A2 were active. Immunoprecipitation studies reveal that mAb 8A2 recognizes β_1-subunit (CD29) of integrin receptors. In contrast to mAbs directed to VLA-4 alpha-subunit (α_4, CD49d), mAb 8A2 did not induce homotypic aggregation of PBL. Additionally, mAb 8A2 stimulated adherence of PBL and hematopoietic cell lines to purified matrix components laminin and fibronectin. This binding was blocked by mAbs to the VLA alpha-subunits α_6 (CD49f), or α_5 (CD49e) and α_4 (CD49d), respectively. We conclude that mAb 8A2 modulates the affinity of VLA-4 and other leukocyte β_1 integrins, and should prove useful in studying the regulation of β_1 integrin function.

THE emigration of lymphocytes to extravascular sites of inflammation or immune reaction is mediated by the interaction of adhesive surface proteins or lymphocytes with ligands on endothelial cells and matrix components. The binding of lymphocyte function–associated antigen-1 (LFA-1)[1] to intercellular adhesion molecule-1 (ICAM-1) and very late activation antigen-4 (VLA-4) with vascular cell adhesion molecule-1 (VCAM-1, inducible cell adhesion molecule-110 [INCAM-110]) accounts for a major component of lymphocyte binding to cytokine-activated cultured endothelium (7, 16, 32, 34). LFA-1 (CD11a/CD18) is an integrin receptor of the β_1 subfamily that interacts with the endothelial cell ligands ICAM-1 (CD54) or ICAM-2, closely related members of the immunoglobulin superfamily (25, 40, 43, 44). VLA-4 (CD49d/CD29) is an integrin receptor of the β_1 subfamily that has recently been shown to bind to both the CS-1 fragment of fibronectin (18, 50) and to vascular cell adhesion molecule-1 (VCAM-1) (12), a cytokine-induced endothelial protein of the immunoglobulin superfamily (28). Migration of peripheral blood lymphocyte (PBL) through the subendothelial matrix is dependent at least in part upon other VLA antigens including VLA-5 (CD49e/CD29) and VLA-4 binding to fibronectin and VLA-6 (CD49f/CD29) to laminin (reviewed in 37).

The adhesive interactions of these receptors and ligands can be regulated at the level of the leukocyte or the endothelial cell. Expression of endothelial cell ICAM-1 is increased by interferon-gamma, lipopolysaccharide, interleukin-1, tumor necrosis factor (TNF) (11, 30), or phorbol esters (23), while VCAM-1 is induced by IL-1, TNF, lipopolysaccharide and interleukin-4 (7, 16, 26, 28, 32, 46). In the case of LFA-1, activation by phorbol ester treatment or cross-linking of T-cell receptor, CD2, or CD3 increases the binding of LFA-1 to ICAM-1 (10, 48). Phorbol esters also increase the avidity of β_1 integrins (4, 38, 39, 45, 51).

We now describe a mAb, 8A2, that is directed to an epitope on β_1 (CD29). mAb 8A2 rapidly induces enhanced binding of PBLs to cytokine-activated human umbilical endothelial cells (HUVEs). Stimulation of PBL adherence to HUVE by mAb 8A2 is dose dependent, requires energy, and is mediated primarily by binding of VLA-4 to VCAM-1. mAb 8A2 also stimulates binding of lymphocytes and hematopoietic cell lines to purified fibronectin via VLA-4 or VLA-5 and to purified laminin via VLA-6.

1. *Abbreviations used in this paper*: ELAM-1, endothelial leukocyte adhesion molecule-1; HUVE, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; LFA, lymphocyte function–associated antigen 1; NCS, normal calf serum; PBL, peripheral blood lymphocyte; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule-1; VLA, very late activation antigen.

Materials and Methods

Cell Culture

HUVEs were harvested by collagenase treatment of umbilical cord veins as previously described (31). Cells were grown and maintained in RPMI 1640 medium (M. A. Bioproducts, Walkersville, MD) supplemented with 10% adult bovine serum (Hyclone Sterile Systems, Logan, UT), 10% normal calf serum (Armour Pharmaceutical Co., Kanakee, IL), heparin 90 μg/ml (Sigma Chemical Co., St. Louis, MO), (47), and endothelial cell growth factor (50 μg/ml) prepared from bovine hypothalamus (24).

CHO cells stably transfected and expressing endothelial leukocyte adhesion molecule-1 (ELAM-1), VCAM-1, ICAM-1, and CD4 were the gift of Drs. Roy Lobb, Barbara Wallner, and Margaret Rosa, Biogen, Inc. (Cambridge, MA). Cells were grown and maintained in MEM-alpha medium without ribonucleosides or deoxyribonucleosides (Gibco Laboratories, Grand Island, NY), supplemented with 10% FBS (Hyclone Sterile Systems, Logan, UT), and 4 mM glutamine (Gibco Laboratories). Methotrexate (Sigma Chemical Co., St. Louis, MO), 200 mM, was added to the medium for the VCAM-1, ELAM-1, and ICAM-1 cells, while the CD4 transfectants were grown in the presence of 30 mM methotrexate.

Suspension cultures of the myelomonocytic cell line U937 (ATCC, Walkersville, MD), lymphoid cell lines Molt-4 and Ramos (ATCC), and erythroleukemic cell line K562 (ATCC) were maintained in MEM (M.A. Bioproducts) supplemented with 10% FBS. The lymphoblastoid cell line, JY, was a gift of Dr. Roy Lobb, Biogen, Inc. (Cambridge, MA), and was maintained in RPMI 1640 medium with 10% FBS.

The murine nonsecretory myeloma cell line, NS-1, was the gift of Dr. Charles Hart, Zymogenetics (Seattle, WA). The myeloma cells and subsequent hybridomas were maintained in RPMI 1640 supplemented with 15% heat-inactivated FBS, 4 mM glutamine, 0.1 mM nonessential amino acids (Gibco Laboratories), 1 mM sodium pyruvate (Gibco Laboratories) and 0.075% sodium bicarbonate (Gibco Laboratories).

The melanoma cell line, 2669, was the gift of Dr. Karl E. Hellstrom, Oncogene (Seattle, WA). The melanoma cells were maintained in Earles Modified Eagles Medium (M.A. Bioproducts) and supplemented with 10% FBS (Hyclone Sterile Systems), 2 mM glutamine (Gibco Laboratories), 1 mM sodium pyruvate (Gibco Laboratories) and 0.1 mM nonessential amino acids (Gibco Laboratories).

Antibodies and Reagents

Murine mAb 60.3 which recognizes the β_2 subunit (CD18) of the CD11/CD18 complex (1) was the gift of Dr. Patrick Beatty, Fred Hutchinson Cancer Research Center (Seattle, WA). Murine mAb 4B9 which recognizes VCAM-1 was generated as previously described (7). The α_4 (CD49d) mAb 163H was the gift of Dr. Michael Gallatin, ICOS Corporation (Seattle, WA) and the α_4 (CD49d) mAbs HP 1/1, HP 1/2 and HP 2/1 were the gifts of Dr. Francisco Sanchez-Madrid, Service Immunologia, Hospital de lao Princesa, (Madrid, Spain) (33). The rat α_6 (CD49f) mAb GoH3 was the gift of Drs. Taco Kuijpers and Arnoud Sonnenberg, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands) (41, 42). The anti-ELAM-1 mAb BB11 was the gift of Drs. Roy Lobb and Christopher Benjamin, Biogen, Inc. (Cambridge, MA) (3). The β_1 (CD29) murine mAb P4C10, the α_2 (CD49b) mAb P4B4, and α_3 (CD49c) mAb P1B4 were the gifts of Dr. Elizabeth Wayner, Cytel Corporation (La Jolla, CA) (8). Murine mAb 4B4 directed to β_1 (CD29) was purchased from Coulter Corp. (Hialeah, FL). Polyclonal antibody A108 which recognizes the VLA-5 fibronectin receptor ($\alpha_5\beta_1$, CD49e/CD29) and murine monoclonal P1D6 which recognizes α_5 (CD49e) were purchased from Telios Pharmaceuticals, Inc. (San Diego, CA).

Recombinant human TNF-alpha (rhTNF) was the gift of Dr. Roy Lobb, Biogen Corp. (Cambridge, MA). Sodium azide, 2-mercapto-ethanol and glutaraldehyde were purchased from Eastman Kodak Co. (Rochester, NY). Hydrogen peroxide, *o*-phenylenediamine, NP-40, PMSF, glucose, and 2-deoxyglucose were purchased from Sigma Chemical Co. (St. Louis, MO).

Leukocytes

Blood was obtained by venipuncture from normal healthy donors under approved protocol. Blood was drawn into polypropylene syringes containing heparin 10 U/ml. The blood was centrifugated over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) at 500 *g* for 40 min at 24°C. The mononuclear layer at the plasma-Ficoll interface was collected by aspiration. Monocytes were removed by two cycles of adherence to serum-coated tissue culture petri dishes (Becton Dickinson & Co., Lincoln Park, NJ). The suspension of nonadherent cells containing primarily lymphocytes was centrifuged for 6 min at 500 *g*, resuspended in RPMI 1640 medium with 2% bovine serum (Gibco Laboratories) for use in the adherence assays.

Polymorphonuclear leukocytes were isolated from the Ficoll-RBC interface of the above preparation by 3% dextran sedimentation and lysis of contaminating red blood cells by hypotonic saline.

Matrix-coated Plates

Purified human fibronectin and murine laminin were purchased from Collaborative Research, Inc. (Bedford, MA). The matrix components were resuspended in PBS at 15 μg/ml. 200 μl/well were added to 48-well nontreated styrene plates (Costar Corp., Cambridge, MA) and incubated overnight at 4°C. Wells were rinsed once with PBS, and then incubated for 2 h at room temperature with 0.5 ml of PBS/3% BSA to block nonspecific binding. Immediately before use, wells were rinsed three times with PBS and 0.2 ml of RPMI containing 5% normal calf serum (Gibco Laboratories) was added.

Adherence Assay

First- or second-passage HUVEs were plated onto gelatin-coated 48-well tissue culture plates (Costar Corp.) and grown to confluency. Designated wells were pretreated for 4 h at 37°C with rhTNF 10 ng/ml. CHO cell transfectants were plated in untreated 48-well tissue culture plates and grown to confluency. Wells were washed once with control medium, RPMI-5% normal calf serum (NCS) (Gibco Laboratories), and then 0.2 ml of control medium with or without various mAbs was added for 30 min at 37°C. Peripheral blood lymphocytes, U937 and K562 cells were isolated as previously described, suspended in 0.5 ml RPMI, and labeled with ^{51}Cr for 1 h at 37°C. Cells were washed twice with RPMI medium containing 5% NCS and resuspended in RPMI medium containing 5% NCS at a concentration of 1×10^6 cells/ml. Labeled cells were aliquoted and treated with mAb 8A2 at various concentrations for 30 min at 37°C. Labeled cells (0.2 ml/well) were then added to the wells containing monolayers of HUVE or CHO, or matrix components and incubated for 30 min at 37°C. Nonadherent cells were aspirated from each well and the wells were washed once with 0.5 ml/well PBS with 2% NCS and aspirated dry. Adherent cells were lysed with 0.5 ml/well of 1N NH_4OH, and the lysates were counted in a Micro-Medic Gamma Counter (Chicago, IL) for 1 min. Percent adherence was calculated as:

$$\% \text{ Adherence} = \frac{^{51}\text{CR cpm lysate}}{^{51}\text{CR cpm total added}} \times 100$$

Generation of mAb 8A2

BALB/c mice were immunized with hematopoietic cell line U937. Hybridomas were produced by fusion of immunized mouse splenocytes with NS-1 cell line using standard techniques (15) and grown in 96-well tissue culture plates (Costar Corp.). Hybridoma supernatants were screened by standard enzyme-linked immunoassay (7) for binding to the 2669 melanoma cells, known to express VLA-4 and to bind to VCAM-1 (N. Kovach and J. Harlan, unpublished observation). Positive hybridomas were then evaluated for effects on adherence of U937 cells to VCAM-1-transfected CHO cells. A hybridoma designated 8A2 was identified by stimulation rather than inhibition of adherence of U937 cells to VCAM-1-transfected CHO cells. It was then serially cloned three times by the method of limiting dilution. mAb 8A2 was determined to be IgG_1-kappa isotype using a mouse mAb isotyping kit (Amersham Corp., Arlington Heights, IL).

Ascites was produced by standard technique (21). Briefly, BALB/c mice were primed with 0.5 ml of pristane (2, 6, 10, 14-tetramethyl-pentadecane; Sigma Chemical Co.) intraperitoneally 1 wk before intraperitoneal injection of 3×10^6 8A2 hybridoma cells. Ascites was collected after 2–3 wk when significant accumulation was present. mAb 8A2 was purified from ascites by binding to immobilized protein A (Pharmacia Fine Chemicals) as previously described (13).

Monovalent Fab fragments were generated by incubation of purified mAb 8A2 with papain immobilized on sepharose bead (Pierce Chemical Co., Rockford, IL) for 5 h. Immobilized papain was removed by centrifugation, and the supernatant passed over an immobilized protein A column. The nonbinding fraction was subjected to 7% SDS-PAGE under nonreducing conditions to confirm purity of the preparation.

Radioimmunoprecipitation

U937 and K562 cells were surface-labeled with ^{125}I as previously de-

scribed (20). Briefly, 15 ml polypropylene tubes (Becton-Dickinson) were coated with 500 μg of Iodogen iodination reagent (Pierce Chemical Co.), dried under a stream of nitrogen, and stored dessicated until used. Tubes were rinsed with sample buffer at time of use, and 5×10^7 cells were added with 1 mCi of $Na^{125}I$, and incubated 20 min at room temperature. Cells were washed three times with PBS, and then lysed for 3 h at 4°C in PBS containing 0.5% NP-40 and 2 mM PMSF. Debris and unlysed cells were removed by centrifugation at 12,000 *g* for 15 min. Cell lysates were precleared by incubation for 1 h with 50 μg nonimmune mouse IgG (Cappel Research Products, Organon Teknika Corp., West Chester, PA) and 50 μl of rabbit serum, and subsequent incubation with Pansorbin, a 10% suspension of SACI cells coated with protein A (Calbiochem Corp., San Diego, CA). Lysates were then cleared by centrifugation. After preclearance, aliquots of U937 lysate were incubated for 1 h with 10 μg mAb 8A2, mAb P4C10, mAb 4B4, polyclonal antibody A108, or mAb 60.3. Aliquots of labeled precleared K562 lysate were incubated with 10 μg mAb P4C10, mAb P1D6, mAb 8A2, or polyclonal A108. Antibody-antigen complexes were removed from the lysates by incubation with 50 μl protein G immobilized to Sepharose beads (Gamma Bind Plus; Genex Corp., Gaitherburg, MD). Immobilized protein G-antibody-protein complexes were removed by centrifugation at 20,000 *g* for 15 min. The beads were washed three times with 0.1% NP-40 in PBS, and boiled in SDS-PAGE sample buffer for 10 min. Sepharose beads were pelleted by centrifugation and aliquots of supernatant treated with 5% mercaptoethanol. Immunodepletion was accomplished by three sequential precipitations of the lysates with designated antibodies. Final precipitation of the immunodepleted lysate with mAbs P4C10, 8A2 or 60.3 was performed as described. Samples were then analyzed by 7% SDS-PAGE and autoradiography.

Fluorescence-activated Cell Sorting

PBLs and neutrophils were isolated as described above. Aliquots of U937, Molt-4, Ramos, and JY were prepared from suspension cultures in exponential growth. Leukocytes, 1×10^6 total per tube, were placed in 5-ml polypropylene tubes (Becton-Dickinson), centrifuged 400 *g* for 6 min, washed once with HBSS (Gibco Laboratories) and kept at 4°C for all manipulations. 50 μl heat-inactivated adult bovine serum (Hyclone Sterile Systems) was added per tube for 20 min to reduce nonspecific binding. Cells were washed once with HBSS, 50 μl of the first mAb was added at final concentration of 1:50 for purified mAb and 1:5 for hybridoma supernatants, and the mixture was incubated for 30 min. In the same experiments, aliquots of U937 cells were pretreated for 30 min at 37°C with 1 μg/ml of mAb 8A2 Fab fragments and washed twice with HBSS at 4°C before addition of first intact mAb. After reaction with the first mAb, cells were washed three times with cold HBSS, and 50 μl of 1:25 dilution FITC-labeled goat antimouse Fc-specific second antibody (Tago Corp., Burlingame, CA) was added. In the case of the rat mAb GoH3 (α_6, CD49f), FITC-labeled goat anti-rat IgG was utilized as second antibody (Organon Teknika, Corp., West Chester, PA). After incubation for 30 min, cells were washed twice with cold HBSS, and fixed with 250 μl of 1% paraformaldehyde. Samples were analyzed on an Epics Cytofluorimeter (Coulter Corp., Hialeah, FL).

Aggregation Assay

50 μl of PBL or U937 cell suspensions, 2×10^6 cells/ml, were pipetted per well of a round-bottomed 96-well plate (Costar). 50 μl of the α_4 (CD49d) mAbs HP1/1, HP2/2, or HP2/1 cell supernatants at 1:100 dilution were added to designated wells. 50-μl dilutions of purified mAb 8A2 in RPMI with 2% NCS were added to designated wells with final concentrations ranging from 1 to 1,000 ng/ml. Plates were incubated in 5% CO_2 at 37°C for up to 18 h, and then visually scored for the presence of aggregation under an inverted microscope.

Results

mAb 8A2 Stimulates U937 Binding to VCAM-1–transfected CHO Cells

mAb 8A2 was identified by stimulation of U937 cell binding to VCAM-1–transfected but not CD4- or ICAM-1–transfected CHO cells (Fig. 1). mAb 8A2 induced a fourfold increase in binding of U937 to VCAM-1–transfected CHO cells, and this binding was inhibited by the anti-VCAM-1 mAb 4B9 (Fig. 1).

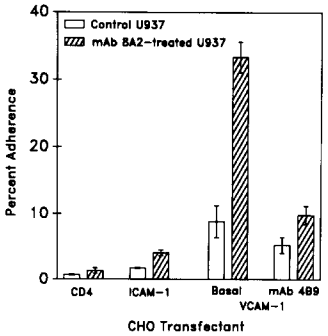

Figure 1. mAb 8A2 induces binding of U937 cells to VCAM-1–transfected CHO cells. ^{51}Cr-labeled U937 cells were pretreated for 30 min with medium alone or medium containing mAb 8A2 (1 μg/ml), and then added to confluent monolayers of CD4-, ELAM-1–, or VCAM-1–transfected CHO cells. VCAM-1 transfectants were pretreated for 30 min with medium alone or medium containing 5 μg/ml of the anti-VCAM-1 mAb 4B9. Percent adherence was determined after a 30-min incubation at 37°C. Values are the means ± SEM of three experiments.

mAb 8A2 Stimulates PBL Binding to VCAM-1

We next examined the effect of mAb 8A2 on adherence of PBL. Treatment of PBL with mAb 8A2 induced binding to the VCAM-1–transfected CHO cells, but not to ELAM-1- or CD4-transfectants (Fig. 2). The mAb 8A2–induced binding to PBL to VCAM-1 was inhibited by pretreatment of the transfectant with anti-VCAM-1 mAb 4B9 or pretreatment of PBLs with the α_4 (CD49d) mAb 163H (Fig. 2). Importantly, monovalent Fab fragments of mAb 8A2 were as active as intact IgG in stimulating PBL binding to VCAM-1 transfectants (Fig. 3).

mAb 8A2-induced Binding Is Energy Dependent

Sodium azide, NaN_3, binds tightly to cytochrome oxidase complex and blocks electron transport. 2-deoxyglucose (2-DOG), is a competitive inhibitor of glucose metabolism. The combination of 2-DOG and NaN_3 abolishes energy-dependent functions. Pretreatment of PBL with 2-DOG/NaN_3 inhibited basal binding of PBL to VCAM-1 transfectants, and abolished binding induced by mAb 8A2 (Table I). The inhibition of binding was partially reversed by addition of glucose to compete against the 2-DOG.

Actinomycin D, a potent inhibitor of RNA synthesis, slightly diminished binding of untreated PBLs to VCAM-1 transfectants, but had no effect on the mAb 8A2-stimulated increase in binding (Table I).

mAb 8A2 Stimulates PBL Binding to HUVE via VLA-4/VCAM-1

mAb 8A2 stimulated adherence of PBL to untreated and rhTNF-treated HUVE in a dose-dependent manner in the presence of the β_2 (CD18) mAb 60.3 (Fig. 4). A significant increase in adherence was observed at ~50 ng/ml, with maximal stimulation of adherence at 5 μg/ml. Dose-response curves were essentially parallel between untreated and rhTNF-treated HUVE, but with significantly greater binding to rhTNF-treated HUVE. Enhancement of binding occurred rapidly. Addition of mAb 8A2 to PBL at the beginning of the 30-min adherence assay resulted in maximal stimulation of adherence with no further enhancement when PBLs were pretreated with mAb 8A2 for up to 6 h (data not shown). All studies were subsequently performed with a 30-min coincubation of PBL and mAb 8A2 during the adherence assay.

Since PBL adherence to HUVE involves interaction of

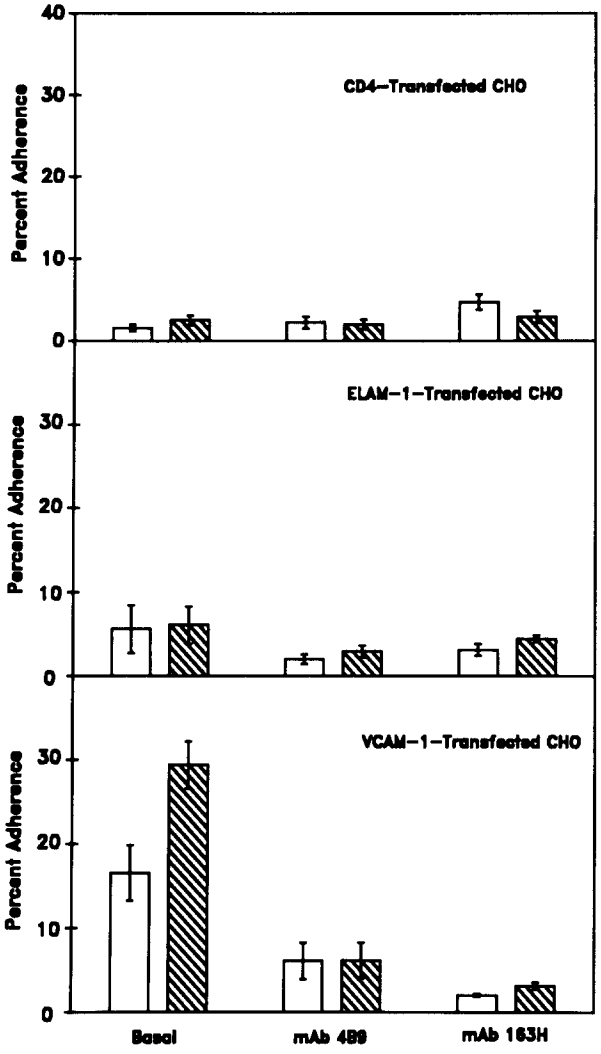

Figure 2. mAb 8A2 induces binding of PBL to VCAM-1-transfected CHO cells. ^{51}Cr-labeled PBLs were pretreated with mAb 8A2 (5 μg/ml) in the presence of the β_2 (CD18) mAb 60.3 (20 μg/ml) for 30 min at 37°C. An aliquot of PBL was also treated with 5 μg/ml of the α_4 (CD49d) mAb 163H. Confluent monolayers of CHO cells transfected with CD4, ELAM-1, or VCAM-1 were washed and pretreated with control medium with and without the anti-ELAM-1 mAb BB11 (5 μg/ml) or the anti-VCAM-1 mAb 4B9 (5 μg/ml) for 30 min. ^{51}Cr-labeled PBLs were added and percent adherence was determined after a 30-min incubation at 37°C. Values are means ± SEM of three experiments.

LFA-1 (CD11a/CD18) with ICAM-1 or ICAM-2 (25, 40, 43, 44), and VLA-4 with VCAM-1 (12, 34), we utilized blocking mAbs to these receptors and ligands to determine the effect of mAb 8A2 on PBL binding to HUVE. Results of three separate experiments are summarized in Table II. Pretreatment of HUVE with rhTNF for 4 h before addition of PBL significantly increased the adherence of PBL compared to untreated HUVE. The adherence of PBL to untreated and cytokine-activated HUVE was reduced by the β_2 (CD18) mAb 60.3. Adherence to rhTNF-treated HUVE, however, was significantly greater than adherence to untreated HUVE even in the presence of β_2 (CD18) mAb 60.3. This enhanced binding of PBL to rhTNF-activated HUVE in the presence of β_2 (CD18) mAb 60.3 has previously been shown to be due primarily to induction of VCAM-1 on HUVE which interacts with VLA-4 on PBL (7, 12, 16, 32, 34). In the presence of the CD18 mAb, the α_4 (CD49d) mAb 163H inhibited PBL binding to unstimulated and rhTNF-stimulated HUVE.

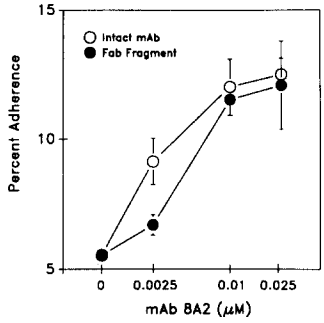

Figure 3. Monovalent Fab fragments of mAb 8A2 stimulate PBL adherence to VCAM-1 transfectants. Monovalent FAB fragments of mAb 8A2 were generated by papain digestion and affinity chromatography as described in Materials and Methods. ^{51}Cr-labeled PBL were pretreated for 30 min with the β_2 (CD18) mAb 60.3 (20 μg/ml) and varying concentrations of intact mAb 8A2 or Fab fragments. $_{51}$Cr-labeled PBL were added to confluent VCAM-1-transfected CHO cells and percent adherence was determined following a 30-min incubation at 37°C. Concentration of monovalent Fab fragments and intact mAb are expressed as micrometers for direct comparison. Values are means of four replicate wells in one representative experiment of four separate experiments.

mAb 8A2 enhanced adherence of PBL to unstimulated HUVE and slightly to rhTNF-stimulated HUVE. The mAb 8A2–induced adherence to unstimulated HUVE and rhTNF-stimulated HUVE was reduced by the β_2 (CD18) mAb 60.3, but the CD18-independent binding was significantly greater in the presence of mAb 8A2. The addition of the α_4 (CD49d) mAb 163H together with the β_2 (CD18) mAb 60.3 reduced mAb 8A2-stimulated binding to unstimulated HUVE and rhTNF-stimulated HUVE to values not significantly different from those observed in the absence of mAb 8A2.

mAb 8A2 Stimulates Binding by an Effect on PBL Not HUVE

Since HUVEs express the antigen recognized by mAb 8A2

Table I. Metabolic Inhibitors Prevent mAb 8A2-stimulated Binding of PBL to VCAM-1-transfected CHO Cells

	Control PBL	mAb 8A2-treated PBL
Basal	18.0 ± 2.2	28.7 ± 1.1
2-DOG/NaN_3-treated	2.1 ± 1.3	4.1 ± 3.5
2-DOG/NaN_3-treated with glucose	5.0 ± 3.9	18.6 ± 5.8
Actinomycin D	12.0 ± 4.4	21.0 ± 0.8

mAb 8A2-stimulated binding of PBl to VCAM-1-transfected CHO cells is inhibited by metabolic inhibitors. ^{51}Cr-labeled PBL were incubated in medium alone, medium containing 2-deoxyglucose (5 mM) and NaN_3 (0.1%) with or without 5 mM glucose, or actinomycin D (1 μg/ml) for 30 min at 37°C. Cells were washed once and treated with mAb 8A2 (1 μg/ml) for 30 min and then added to confluent VCAM-1-transfected CHO cells. Percent adherence was determined following a 30-min incubation at 37°C. Values are the means ± SEM of quadruplicate wells in a representative experiment of four separate experiments.

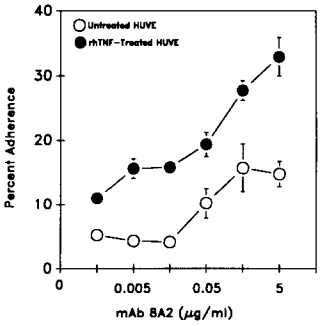

Figure 4. mAb 8A2 induces adherence of PBL to HUVE. ^{51}Cr-labeled PBL were pretreated with varying concentrations of mAb 8A2 for 30 min at 37°C in the presence of β_2 (CD18) mAb 60.3 (20 μg/ml). ^{51}Cr-labeled PBLs were then added to confluent monolayers of unstimulated HUVE or HUVE pretreated with rhTNF (10 ng/ml) for 4 h at 37°C. Percent adherence was determined after a 30-min incubation at 37°C. Values are the means ± SEM of triplicate wells in one representative experiment of four separate experiments.

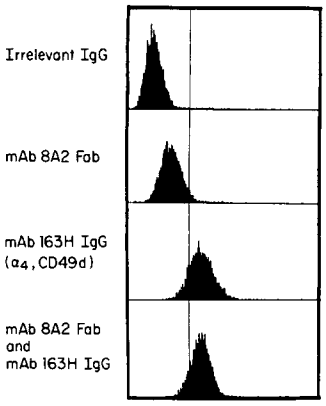

Figure 5. mAb 8A2 does not increase surface expression of α_4-subunit (*CD49d*) of VLA-4 (*CD49d*) on U937 cells. U937 cells were incubated with isotype-matched irrelevant IgG, mAb 8A2 Fab fragments alone, the α_4 (*CD49d*) mAb 163H alone, or mAb 163H after pretreatment with mAb 8A2 Fab fragments. Specific binding of mAb 163H was assessed by flow cytometry as described in Materials and Methods. Similar results were obtained in two additional experiments.

(data not shown), we determined whether the effect of mAb 8A2 was on the PBL or HUVE. Pretreatment of HUVE with mAb 8A2 followed by washing was without effect on adherence of PBL in the presence of β_2 (CD18) mAb 60.3 (6.7 ±

Table II. mAb 8A2 Stimulates PBL Binding to Untreated and rhTNF-treated HUVE via VLA-4/VCAM-1

	Experiment	Basal	mAb 8A2-treated
Untreated HUVE			
Basal	1	27.8 ± 2.3	33.9 ± 2.6
	2	19.9 ± 3.0	26.4 ± 2.2
	3	25.7 ± 4.1	33.0 ± 5.2
mAb 60.3	1	9.6 ± 1.5	16.6 ± 1.5*
	2	6.7 ± 0.8	16.8 ± 1.2*
	3	12.7 ± 1.1	20.3 ± 2.6*
mAb 163H	1	19.5 ± 2.7	27.4 ± 2.6
	2	17.3 ± 1.9	22.6 ± 3.1
	3	17.2 ± 0.8	20.4 ± 1.6
mAb 60.3/163H	1	4.3 ± 1.2	6.2 ± 1.8
	2	6.0 ± 0.8	5.1 ± 0.6
	3	5.1 ± 0.4	4.7 ± 0.2
rhTNF-treated HUVE			
Basal	1	52.1 ± 2.6	54.7 ± 5.3
	2	34.2 ± 3.6	34.6 ± 5.6
	3	32.2 ± 5.4	34.7 ± 6.5
mAb 60.3	1	25.5 ± 2.1	39.9 ± 4.9*
	2	16.6 ± 2.8	26.8 ± 1.9*
	3	19.9 ± 2.6	27.6 ± 6.3
mAb 163H	1	34.8 ± 5.1	46.5 ± 1.9
	2	31.2 ± 3.3	31.5 ± 1.8
	3	25.3 ± 2.9	26.7 ± 3.4
mAb 60.3/163H	1	7.9 ± 1.4	11.2 ± 0.6
	2	7.9 ± 0.7	7.5 ± 0.6
	3	8.0 ± 1.1	9.7 ± 2.0

mAb 8A2-stimulated PBL binding to untreated and rhTNF-treated HUVE is inhibited by blocking mAb to α_4 subunit (CD49d). HUVEs were pretreated with medium alone or medium containing rhTNF 10 ng/ml for 4 h at 37°C. ^{51}Cr-labeled PBLs were incubated in medium alone, medium containing the β_2 (CD18) mAb 60.3 (20 μg/ml), the α_4 (CD49d) mAb 163H (10 μg/ml), or in combination. Aliquots of the treated PBLs were treated with mAb 8A2 (1 μg/ml) for 30 min and then added to the HUVE cell monolayers. Percent adherence was determined following a 30-min incubation at 37°C. Values are the means ± SEM of triplicate wells in three separate experiments.
* Denotes statistically significant difference between untreated and mAb 8A2-treated binding in means by *t*-test ($P < 0.05$).

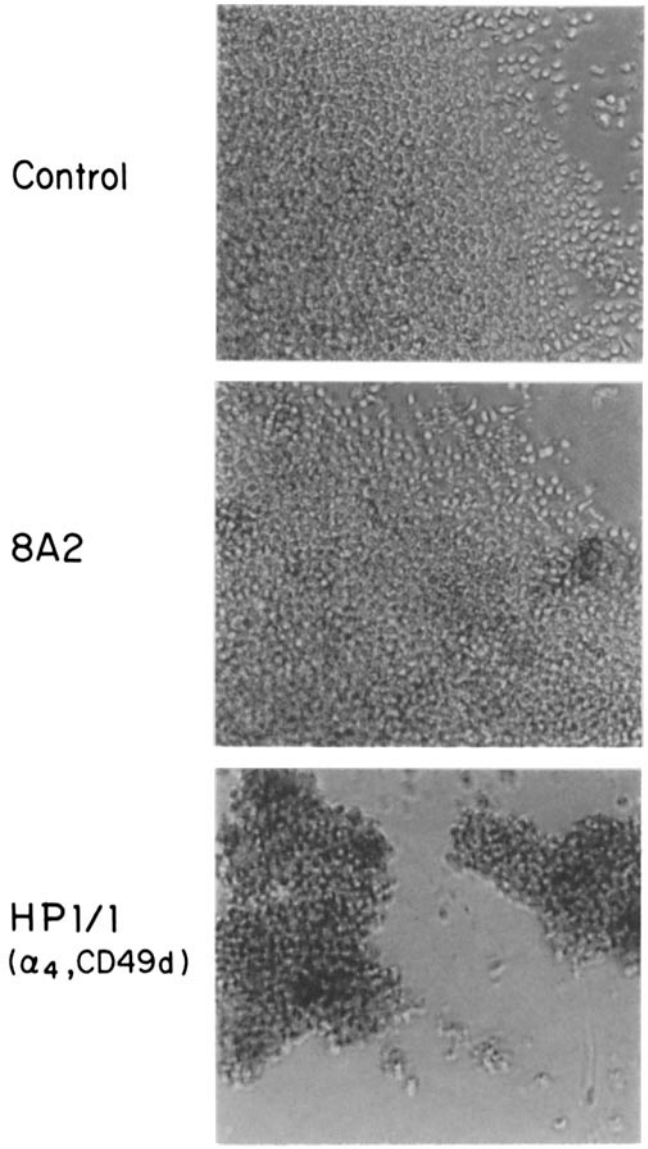

Figure 6. mAb 8A2 does not induce aggregation of U937 cells. Suspension cultures of U937 cells were incubated with isotype control mouse IgG (5 μg/ml), mAb 8A2 (5 μg/ml), or 1:100 dilution of the α_4 (*CD49d*) mAb HP 1/1 for 8 h at 37°C. Similar results were obtained in three separate experiments.

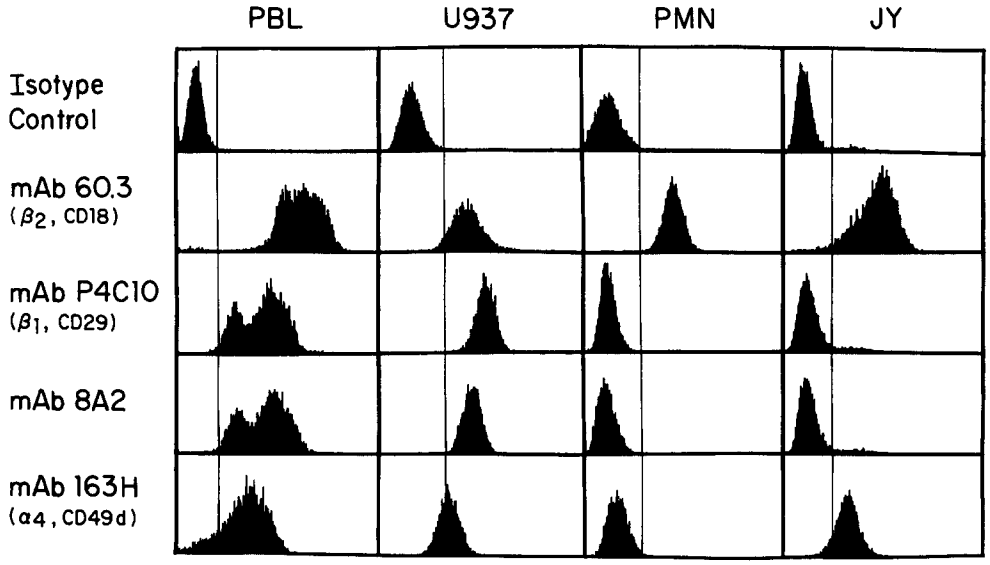

Figure 7. mAb 8A2 binds to U937 cells and PBL but not to PMN and JY cells. Binding of isotype-matched control IgG_1, the β_2 (*CD18*) mAb 60.3, the β_1 (*CD29*) mAb P4C10, the α_4 (*CD49d*) mAb 163H, and mAb 8A2 to JY, U937, PBL, and PMN was assessed by flow cytometry as described in Materials and Methods. Similar results were obtained in two additional experiments.

0.8% untreated and 7.2 ± 0.9% with mAb 8A2-treated), whereas pretreatment of PBL followed by washing produced stimulation of binding comparable to coincubation of the antibody (9.6 ± 0.6% untreated, 22.1 ± 2.1% with mAb 8A2 coincubation and 26.0 ± 2.5% with mAb 8A2 pretreatment).

mAb 8A2 Does Not Increase Surface Expression of VLA-4

To determine whether mAb 8A2 modulated VLA-4–dependent binding by altering the surface expression of VLA-4, we next determined whether surface expression of VLA-4 was increased by mAb 8A2. U937 cells were pretreated with Fab fragments of mAb 8A2, followed by saturating concentration of the α_4 (CD49d) mAb 163H. Secondary labeling was performed with FITC-labeled goat antimouse antibody specific for mouse heavy chain (Fc). On subsequent analysis by FACS mAb 8A2 did not induce any alteration of VLA-4 α-subunit expression (Fig. 5).

mAb 8A2 Does Not Stimulate Homotypic Adherence

Isolated PBL and U937 were aliquoted into individual wells in a 96-well plate. mAb 8A2 and the α_4 (CD49d) mAbs HP1/1, HP2/2, and HP2/1 were added at 1:100 dilution of hybridoma supernatants. Aggregation of PBL and U937 was observed in wells treated with HP1/1. No aggregation was observed in wells treated with HP2/2, HP2/1, dilutions of mAb 8A2 or with medium alone (Fig. 6).

mAb 8A2 Recognizes the β_1 Subunit (CD29)

PBLs, PMN, JY B-lymphoblastoid, cells and U937 cells were treated with mAbs P4C10 or 4B4 directed against β_1 subunit (CD29), the β_2 subunit (CD18) mAb 60.3, mAb 8A2, or isotype-matched control mAbs. After a second incubation with FITC-conjugated goat antimouse antibody, cells were analyzed by flow cytometry. Fig. 7 demonstrates that all cells bound the β_2 (CD18) mAb 60.3. U937, PBL, and JY cells expressed the α_4 subunit (CD49d) as demonstrated by binding of mAb 163H. Peripheral blood lymphocytes and U937 exhibited quantitatively similar binding of the two β_1 (CD29) mAbs and mAb 8A2, whereas PMN and JY cells did not bind the β_1 mAbs or mAb 8A2.

Detergent lysates from ^{125}I-labeled U937 cells were precipitated with polyclonal antibody (A108) directed against $\alpha_5\beta_1$ (CD49e/CD29), the β_1 (CD29) mAbs P4C10 or 4B4, the β_2 (CD18) mAb 60.3, or mAb 8A2. Lysates were precleared by three sequential precipitations with various antibodies and then precipitated with the antibody of interest. Immunoprecipitates were boiled in sample buffer, subjected to SDS-PAGE under nonreducing and reducing conditions, and then autoradiographed. Antibodies directed against β_1 (CD29) precipitated a 115-kD band under nonreducing conditions (Fig. 8 *A*) that migrated to 140 kD under reducing conditions (Fig. 8 *B*). mAb 8A2 precipitated a single band that comigrated with the band precipitated by β_1 antibodies under both reducing and nonreducing conditions. Preclearance of lysate with the β_1-directed antibodies abolished the precipitation of any band by mAb 8A2 (Fig. 8 *B*). Preclearance of lysate with mAb 8A2 ablated precipitation by β_1 (CD29)–specific antibodies. Precipitation with the β_2 (CD18) mAb 60.3 was not affected by preclearance with $\alpha_5\beta_1$-specific antibody A108 (Fig. 8 *B*). Two nonspecific bands in the 66–97-kD range were observed in all the precipitations including CD18 mAb 60.3. Similar results were obtained when lysates were subjected to SDS-PAGE under nonreducing conditions (data not shown).

Additional studies were performed using the erythroleukemic cell line K562 which expresses the VLA-5 fibronectin receptor ($\alpha_5\beta_1$) but not VLA-4 ($\alpha_4\beta_1$) (19). Detergent lysates from ^{125}I-labeled K562 cells were precipitated with polyclonal A108 directed against $\alpha_5\beta_1$ (CD49e/CD29), the α_5 (CD49e) mAb PlD6, the β_1 (CD29) P4C10, or mAb 8A2. Analysis by SDS-PAGE and autoradiography revealed that all four antibodies precipitated two bands under nonreducing conditions corresponding to α_5 and β_1 that comigrated as a single band under reducing conditions (Fig. 9).

mAb 8A2 Stimulates Binding of Hematopoietic Cell Lines and PBL to Purified Matrix Components Fibronectin and Laminin

Having established that mAb 8A2 bound to the common β_1 subunit of VLA (CD29), we next determined whether mAb 8A2 would modulate the affinity of VLA integrin receptors

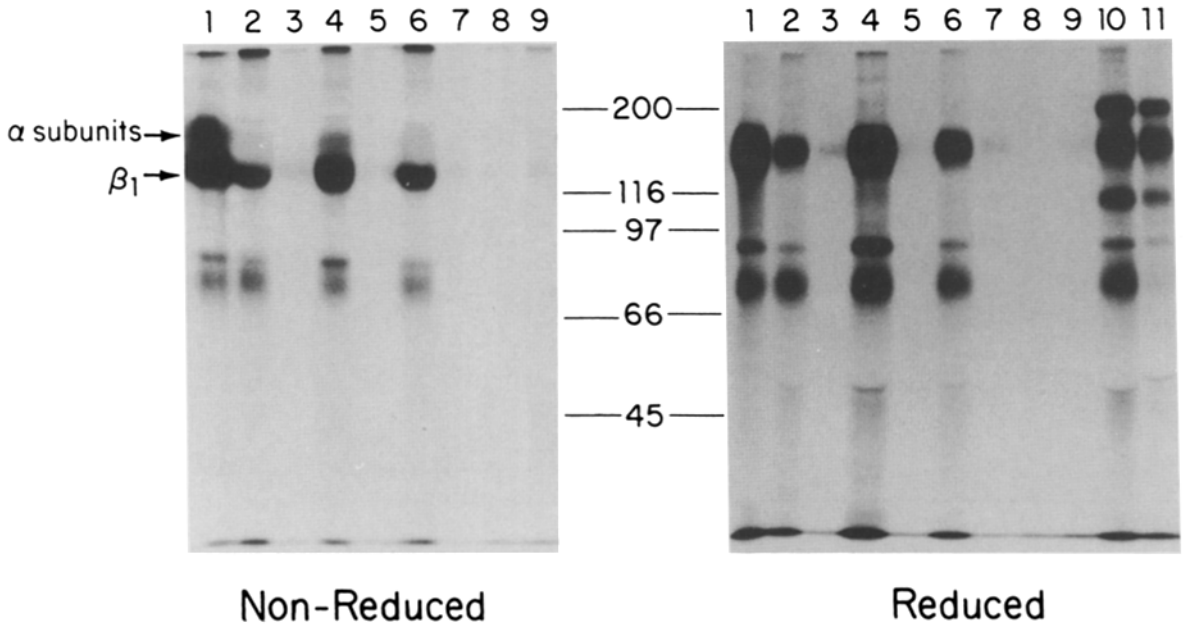

Figure 8. mAb 8A2 precipitates a band that co-migrates with a band precipitated by CD29 (β_1) antibodies. Detergent lysates of ^{125}I-labeled U937 cells were precipitated with polyclonal antibody A108 to VLA-5 fibronectin receptor ($\alpha_5\beta_1$) (lane *1*), the β_1 (CD29) mAbs P4C10 (lane *2*) or 4B4 (lane *4*), mAb 8A2 (lane *6*), or the β_2 (CD18) mAb 60.3 (lane *10*). Precleared samples were precipitated three times with the designated antibody, then precipitated with the final antibody. Samples were subjected to SDS-PAGE under nonreducing (*A*) and reducing (*B*) conditions and autoradiographed. Similar results were obtained in three experiments. The preclearing antibody and final precipitating antibody under reduced conditions are shown in the Fig. 8 as follows:

Lane	1	2	3	4	5	6	7	8	9	10	11
Clearing antibody	–	–	8A2	–	8A2	–	A108	P4C10	4B4	–	A108
Final mAb	A108	P4C10	P4C10	4B4	4B4	8A2	8A2	8A2	8A2	60.3	60.3

other than VLA-4. Binding to human fibronectin was evaluated for PBL and U937 cells which express both VLA-4 and VLA-5, K562 cells which express VLA-5 (19), and lymphoid lines Ramos and Molt-4 which express only VLA-4 (12, 19). Flow cytometry with alpha chain–specific mAbs confirmed the reported selective expression of VLA receptors. Both Molt-4 and Ramos cells expressed significant amounts of α_4 (CD49d) by flow cytometry analysis. Ramos cells exhibited no increase in mean cell fluorescence when treated with the α_5 (CD49e) mAb PlD6 when compared to isotype-matched irrelevant mAb, and there was only minimal binding of mAb PlD6 to Molt-4 cells (mean cell fluorescence 47 ± 22 mAb PlD6 compared to 34 ± 18 isotype control mAb). mAb 8A2 induced binding to fibronectin in all cell lines (Fig. 10) with the inhibition of binding by VLA-alpha chain-directed mAbs dependent upon expressed VLA. mAb 8A2–stimulated binding of PBL and U937 cells, which express VLA-4 and VLA-5, required both the α_4 (CD49d) mAb 163H and the α_5 (CD49e) mAb PlD6 to inhibit binding completely. Stimulated binding of K562 cells, which express only α_5 (CD49e), was inhibited by the α_5 mAb PlD6 but not the α_4 mAb 163H. Conversely, binding of Molt-4 cells which express predominately α_4 (CD49d) was inhibited by the α_4 mAb 163H but not by the α_5 mAb PlD6 (Fig. 10). Binding of Ramos cells which express only α_4 (CD49d) demonstrated an identical pattern to Molt-4, with basal binding of 8.3 ± 0.9% and mAb 8A2-induced binding of 33.3 ± 3.4%. mAb PlD6 to α_5 (CD49e) did not inhibit basal or stimulated binding (8.0 ± 1.1% and 30.2 ± 4.4%, respectively), while the α_4 mAb 163H inhibited basal and stimulated binding (0.9 ± 0.2% and 0.9 ± 0.1%, respectively).

PBL and U937 cells also express VLA-6 (CD49f/CD29) by flow cytometry (data not shown) (20), and bound to murine laminin (Fig. 11). mAb 8A2-stimulated binding to laminin was inhibited by α_6 (CD49f) mAb GoH3 but not α_4 (CD49d) mAb 163H (Fig. 11). Molt-4, K562, and Ramos cells do not express VLA-6 by flow cytometry (data not shown), and did not show significant basal binding or mAb 8A2-stimulated binding to laminin (Fig. 11).

Discussion

The β_1 integrin receptor VLA-4 ($\alpha_4\beta_1$, CD49d/CD29) functions in the binding of lymphocytes to extracellular matrix via the CS-1 domain of fibronectin (18, 50), homotypic aggregation of lymphocytes (2, 6), and lymphocyte adherence to endothelial cells via VCAM-1 (12, 34). The interaction of

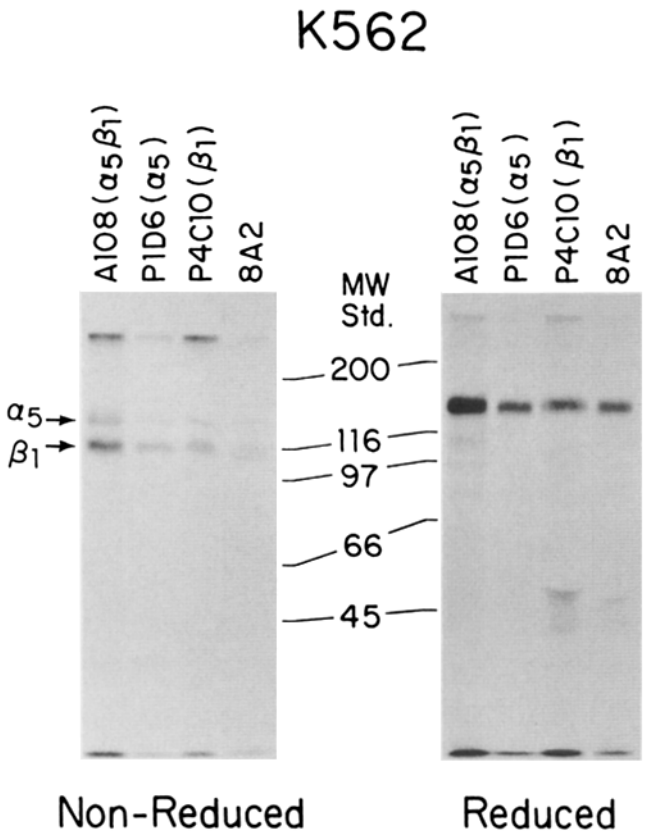

Figure 9. mAb 8A2 precipitates bands co-migrating with α_5 and β_1 in K562 cells. Detergent lysates of ^{125}I-labeled K562 cells were precipitated with the VLA-5 fibronectin receptor ($\alpha_5\beta_1$, CD49e/CD29) polyclonal antibody A108, the α_5 (CD49e) mAb P1D6, the β_1 (CD29) mAb P4C10, and mAb 8A2. Samples were subjected to 7% SDS-PAGE under nonreducing and reducing conditions and autoradiographed.

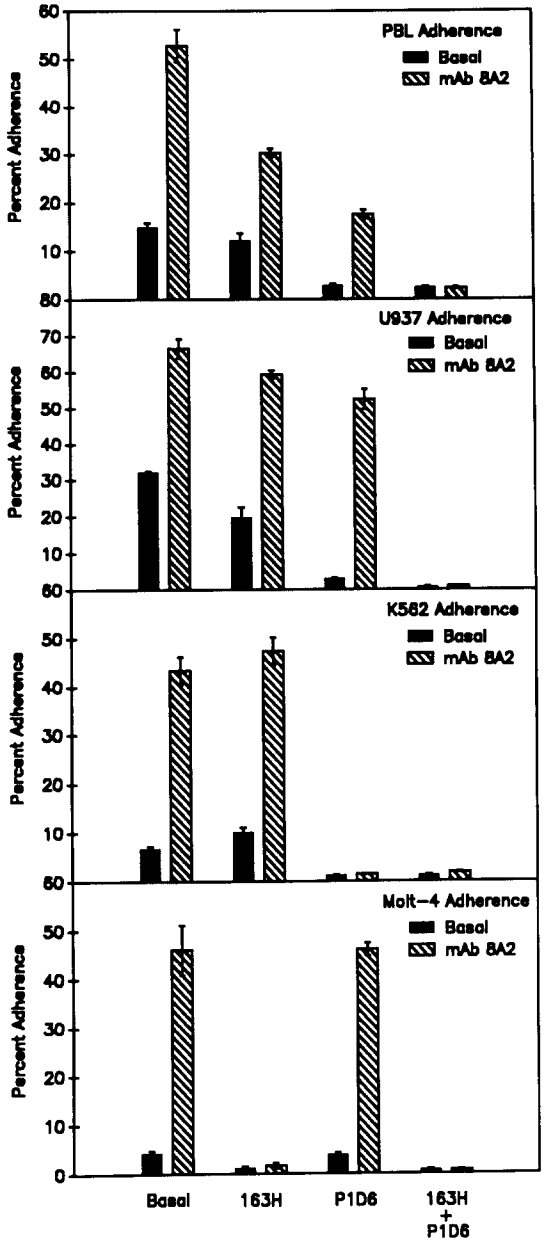

Figure 10. mAb 8A2 stimulates binding of U937, K562, and Molt-4 cells and PBL to purified fibronectin. ^{51}Cr-labeled cells were pretreated with medium alone or medium containing mAb 8A2 (5 μg/ml). Aliquots of ^{51}Cr-labeled cells were also treated with the α_4 (CD49d) mAb 163H, α_5 (CD49e) mAb P1D6 (5 μg/ml), or both mAbs. ^{51}Cr-labeled cells were added to wells containing purified fibronectin as described in Materials and Methods. Percent adherence was determined after a 30-min incubation at 37°C. Values represent means ± SEM of triplicate wells in one representative experiment of three separate experiments.

VLA-4 with VCAM-1 is likely an important component of the adherence of lymphocytes to endothelium at sites of inflammation or immune reaction. Modulation of the affinity of VLA-4 for VCAM-1 provides one mechanism for the control of lymphocyte emigration. In this paper we describe a mAb, 8A2, that rapidly induces VLA-4–dependent binding of peripheral blood lymphocytes or hematopoietic cells to VCAM-1–transfected CHO cells or to cytokine-activated HUVE.

mAb 8A2 was identified by stimulation of U937 cell binding to CHO cells transfected with VCAM-1 cDNA but not ELAM-1 or CD4 cDNA. Binding of U937 cells to VCAM-1–transfected CHO cells induced by mAb 8A2 was inhibited by blocking mAbs to VCAM-1 and α_4 (CD49d). mAb 8A2 also increased VLA-4–dependent binding of PBL to VCAM-1–transfected CHO cells and to rhTNF-treated HUVE. mAb 8A2-induced binding was dose-dependent and rapid with significant stimulation observed at 50 ng/ml and 10 min.

mAb 8A2 activates VLA receptors in addition to VLA-4. Treatment of K562 cells with mAb 8A2 increased binding to fibronectin. K562 cells express only VLA-5, and mAb 8A2-stimulated binding of K562 to fibronectin was completely inhibited by an α_5-subunit (CD49e) mAb. Molt-4 cells express primarily VLA-4 and trace VLA-5 and Ramos cells express only VLA-4. Binding of Molt-4 and Ramos cells to fibronectin was increased by mAb 8A2, and this stimulated binding was inhibited by the α_4 (CD49d) mAb 163H. Binding of PBL to fibronectin was also increased by mAb 8A2, and complete inhibition required both the α_5 (CD49e) mAb P1D6 and α_4 (CD49d) mAb 163H. Additionally, binding of PBL and U937 cells to laminin was stimulated by mAb 8A2 and inhibited by the α_6 (CD49f) mAb GoH3. These results show that mAb 8A2 also modulates function of VLA-5 and VLA-6 as well as VLA-4.

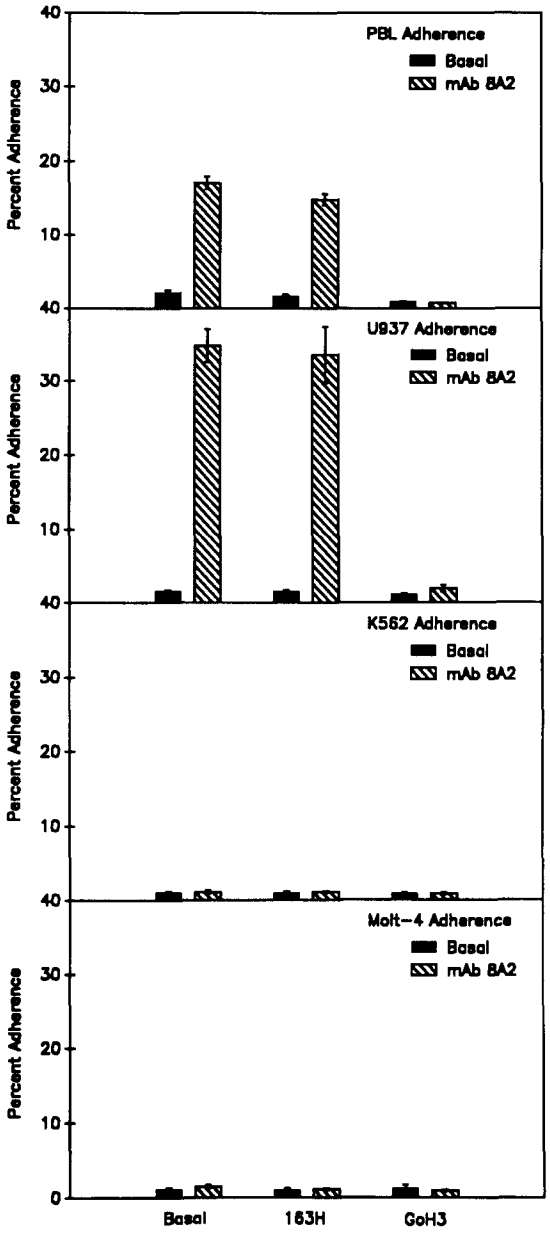

Figure 11. mAb 8A2 stimulates binding of PBL and U937 cells, but not K562 or Molt-4 cells, to purified murine laminin. ^{51}Cr-labeled U937, K562, and Molt-4 cells and PBL were pretreated with medium alone or medium containing mAb 8A2 (5 μg/ml). Aliquots of each cell type were also treated with the α_4 (CD49d) mAb 163H or α_6 (CD49f) mAb GoH3. ^{51}Cr-labeled cells were added to wells coated with purified laminin as described in Materials and Methods. Percent adherence was determined after a 30-min incubation at 37°C. Values represent means ± SEM of triplicate wells in one representative experiment of three separate experiments.

Our studies indicate that mAb 8A2 recognizes the common beta-subunit (β_1, CD29) of the VLA-4 integrin. Radioimmunoprecipitation studies revealed that a band precipitated by mAb 8A2 that comigrated under nonreducing and reducing conditions with a band precipitated by antibodies to β_1. Immunodepletion studies demonstrated that preclearance of labeled lysates with antibodies to β_1 ablated subsequent precipitation by mAb 8A2. Conversely, preclearance with mAb 8A2 ablated precipitation by antibodies to β_1. Furthermore, flow cytometry showed that mAb 8A2 bound to cell types known to express β_1 and that binding of mAb 8A2 was quantitatively similar to that observed with other β_1 (CD29) mAbs (P4C10, 4B4). Conversely, cells lacking β_1 (PMN, JY cells) failed to bind mAb 8A2. The possibility that mAb 8A2 recognized the α_4 subunit (CD49d) of VLA-4 was excluded by the failure of mAb 8A2 to bind to JY cells that express $\alpha 4$ but lack β_1. The beta-subunit which may associate with the α_4 subunit in JY cells is unknown. Additionally, mAb 8A2 precipitated bands from K562 cells which express only VLA-5 ($\alpha_5\beta_1$) that comigrated with bands precipitated by antibodies to α_5 or $\alpha_5\beta_1$. These initial studies do not exclude the possibility that binding of mAb 8A2 may require that the β_1 subunit be associated with an appropriate α-subunit.

mAb 8A2 could increase VLA-4-dependent binding of U937 or PBL to VCAM-1 by either increasing the surface expression or the avidity of VLA-4. The fact that monovalent Fab fragments of mAb 8A2 induced adherence, but did not increase surface expression of α_4 analysis by flow cytometry, indicates that increased binding is not due to increased surface expression of VLA-4 but rather to an increase in avidity.

mAb 8A2 could modulate the affinity of VLA-4 for VCAM-1 and fibronectin, VLA-5 for fibronectin, or VLA-6 for laminin either directly by inducing a conformational change in the receptor upon binding or indirectly by triggering cell activation. O'Toole et al. (29) have reported that monovalent Fab fragments of certain β_3-specific mAbs directly stimulated binding of fibrinogen to $\alpha_{IIb}\beta_3$ in fixed platelets, presumably lacking any intracellular signaling mechanisms, and to detergent-solubilized receptor in the absence of a membrane microenvironment. Figdor and colleagues have described a mAb (NK1-L16) that binds to the α-chain of LFA-1 (CD11a) and stimulates homotypic lymphocyte adhesion (14, 22). The activity of this mAb does not require intracellular signaling, but involves a conformational change in the LFA-1 receptor increasing its affinity for ligand.

Numerous studies have shown that the affinity of integrin receptors for their ligands can be modulated by cell activation. Stimulation of cells by phorbol esters or physiologic agonists have been shown to alter binding avidity of β_1 (4, 38, 39, 45, 51), β_2 (5, 49, 52), and β_3 (9, 35, 36) integrin receptors. Cross-linking of other cell surface receptors by mAbs has been shown to modulate avidity of β_2 (10, 48). In particular, mAbs to the α-subunit of VLA-4 (CD49d) have been shown to induce VLA-4–dependent homotypic lymphocyte adhesion (2, 6) by a mechanism requiring energy and intact cytoskeleton (6). Recently, binding of a mAb to the β_1-subunit (CD29) has been reported to transduce a signal to T cells resulting in elevation of cAMP (17), and neuron adhesion to laminin and collagen (27).

Our initial studies do not determine whether mAb 8A2 acts directly or indirectly. Monovalent Fab fragments of mAb 8A2 were active indicating that cross-linking of the receptor is not required. mAb 8A2-induced binding of PBL to VCAM-1 required a metabolically active cell, since pretreatment with sodium azide and deoxyglucose prevented the increase in binding. These latter results suggest that mAb 8A2 may increase the avidity of VLA-4 by an indirect mechanism requiring active cell metabolism. More detailed studies,

however, are required to determine the precise mechanism by which mAb 8A2 alters the affinity of VLA-4 and other leukocyte β_1 integrins.

In summary, we have described a mAb to the β_1 subunit that rapidly stimulates the binding of VLA-4 to VCAM-1 and fibronectin, as well as VLA-5 to fibronectin and VLA-6 to laminin. This mAb may prove to be an important tool in understanding the regulation of leukocyte β_1 integrin function.

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