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Homotypic Leukocyte Aggregation Triggered by a Monoclonal Antibody Specific for a Novel Epitope Expressed by the Integrin β1 Subunit: Conversion of Nonresponsive Cells by Transfecting Human Integrin α4 Subunit cDNA

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Abstract The monoclonal antibody 33B6 was found to be specific for the β1 integrin subunit. Treatment of leukocytes with this antibody induced a vigorous homotypic aggregation that had similar physiologic conditions as aggregation induced by a monoclonal antibody specific for the α4 subunit. Expression of a β1 subunit on the cell surface was not sufficient for mAb 33B6-mediated aggregation to occur, since cells of the K562 erythroleukemia line failed to respond even though they expressed the β1 subunit and the 33B6 epitope. However, after transfection with cDNA encoding the α4 subunit, K562 cells acquired the ability to aggregate in response to mAb 33B6 binding. By contrast, mAb 33B6 blocked cell binding to the endothelial surface protein vascular cell adhesion molecule-1 and the extracellular matrix protein fibronectin. These results suggest that the β1 epitope defined by mAb 33B6 may play a novel role in regulating leukocyte adhesive interactions.

Key words: adhesion, fibronectin, VCAM-1, VLA-4, cDNA

Leukocytes express an array of β1 integrins which can influence cellular function [Hemler, 1990; Springer, 1990; Hynes, 1992]. These receptor molecules can bind to extracellular matrix (ECM) proteins, and in the case of α4β1 (VLA-4, CD49d/CD29), to the ECM protein fibronectin (FN) and the endothelial adhesion protein vascular cell adhesion molecule-1 (VCAM-1), with subsequent transduction of activation signals [Matsuyama et al., 1989; Osborn et al., 1989; Wayner et al., 1989; Davis et al., 1990; Garcia-Pardo, et al., 1990; Guan and Hynes, 1990; Nojima et al., 1990; Shimizu et al., 1990b; Burkly et al., 1991; Damle et al., 1991; van Seventer et al., 1991; Yamada et al., 1991; Nojima et al., 1992]. In addition, α4β1 has been implicated in cytotoxic T-lymphocyte cytolysis [Clayberger et al., 1987; Takada et al., 1989], leukocyte adhesion to endothelial cells (through binding to VCAM-1) [Elices et al., 1990; Schwartz et al., 1990; Jonjic et al., 1992; Schleimer et al., 1992], B-cell interactions with germinal centers [Freedman et al., 1990, 1992; Koopman et al., 1991] and leukocyte precursor interactions with both bone marrow and thymic stromal cells [Kina et al., 1991; Miyake et al., 1991; Ryan et al., 1991; Utsumi et al., 1991; Williams et al., 1991]. The α4β1 complex has also been found on melanoma tumor lines and functions in adhesion to fibronectin (FN) and endothelium [Rice and Bevilacqua, 1989; Mould et al., 1990; Hart et al., 1991]. In addition to melanoma, other tumor lines of neural crest lineage, such as rhabdomyosarcoma, neuroblastoma, glioblastoma, and astrocytoma

Abbreviations used: VLA-4, very late activation antigen-4; ECM, extracellular matrix; FN, fibronectin; LFA-1, lymphocyte function associated antigen-1; VCAM-1, vascular cell adhesion molecule-1; LAD, leukocyte adhesion deficiency; MFI, mean fluoresence intensity.

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can express functional α4β1 [Hemler et al., 1987; Yoshihara et al., 1991; Bednarczyk and McIntyre, 1992].

Certain anti-α4 mAbs have been shown to induce homotypic aggregation of lymphocytes and lymphoid cell lines [Bednarczyk and McIntyre, 1990; Campanero et al., 1990]. Serologic and functional analyses of anti-α4 mAbs have been used to map four epitopes (A, B1, B2, and C) on the α4 subunit, and only mAbs reactive with the A and B2 epitopes induced aggregation [Pulido et al., 1991]. Recently, it has been reported that mAbs which react with the integrin β1 subunit can induce functional events such as homotypic aggregation [Caixia et al., 1991] and increased binding to umbilical vein endothelial cells, VCAM-1 transfected cells, or the ECM proteins laminin and fibronectin [Arroyo et al., 1992; Kovach et al., 1992; van de Wiel-van Kemenade et al., 1992; Wayner and Kovach, 1992].

Unlike the homotypic aggregation mediated by lymphocyte function associated antigen-1 (LFA-1) [Springer, 1990], the counter-receptor(s) of α4- and β1-mediated aggregation has not been defined. Although anti-α4 mAb-induced aggregation does not require the involvement of LFA-1 [Bednarczyk and McIntyre, 1990; Campanero et al., 1990], it is unclear whether α4β1 is directly involved in the adhesion event and, if so, what counter-receptor is used.

In the present study we identified the subunit specificity of mAb 33B6 and have analyzed some of the physiologic parameters of mAb 33B6-induced aggregation. In addition, we have determined the effect of mAb 33B6 treatment on cellular binding to VCAM-1 and FN. Finally, we have assessed the influence of transfected α4 subunit cDNA on mAB 33B6-induced aggregation.

MATERIALS AND METHODS

Cell Lines and Antibodies

The cell lines used in this study were maintained in complete RPMI-1640 medium (containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.0 mM sodium pyruvate and 2 mM L-glutamine [Hazelton Biologics, Lenexa, KS]). Epstein Barr virus-transformed B cells from a patient with leukocyte adhesion deficiency (LAD) were the generous gift of Dr. Donald Anderson, (Upjohn Co., Kalamazoo, MI). Human peripheral-blood lymphocytes were obtained by venipuncture and isolated by centrifugation on a cushion of Lymphocyte Separation Medium (Organon Teknika, Durham, NC). T lymphocytes were enriched using a T Cell Recovery Kit (Biotex Laboratories, Inc., Edmonton, Alberta, Canada) according to the supplier's instructions. After recovery, lymphocytes were routinely >95% positive for the T-cell receptor complex (CD3) as determined by fluorescence-activated cell sorting analysis. Activated T lymphocytes were produced by treating purified T cells with 10 μg/ml phytohemagglutinin in the presence of lethally irradiated peripheral-blood mononuclear cells and the addition of 100 U/ml interleukin-2 twice a week.

Ascites containing the α4-specific (B2 epitope) mAb L25 [Clayberger et al., 1987; McIntyre et al., 1989; Pulido et al., 1991] was the gift of Drs. Elizabeth Evans and Pila Estes (Becton Dickinson, Mountain View, CA). The α4-specific mAbs HP1/1, HP1/7 (A epitope), and HP2/1 (B1 epitope) [Sánchez-Madrid et al., 1986; Pulido et al., 1991] were the gift of Dr. Francisco Sánchez-Madrid (Universidad Autónoma de Madrid, Madrid, Spain). The anti-CD18 mAb H52 [Hildreth and August, 1985] was the gift of Dr. J.E.K. Hildreth (Johns Hopkins University, Baltimore, MD). The anti-HPB-ALL TCR_{id} mAb T40/25 hybridoma [Kappler et al., 1983], the anti-β1 mAb 18D3 hybridoma [Bednarczyk and McIntyre, 1992; Bednarczyk et al., 1992; Schleimer et al., 1992] and the P3 myeloma, were maintained in this laboratory. The mAb 33B6 hybridoma arose from a hybridoma fusion using draining lymph node lymphocytes from a BALB/c mouse immunized with activated human T cells.

Cloning of Murine VCAM-1 cDNA and Construction of VCAM-1-IgG

A mouse cDNA library was constructed using an InVitroGen λgt10 cDNA library kit and poly (A)+ RNA isolated from popliteal lymph node cells. The popliteal lymph node cells (the gift of Dr. S. Watson, Genentech, Inc., San Francisco, CA) were prepared by immunizing mice with sheep red blood cells in Ribi adjuvant and harvesting the cells 7 days later. A full-length human VCAM-1 cDNA (the gift of Dr. D. Dowbenko, Genentech, Inc.) was used to probe the λgt10 cDNA library. A full-length VCAM-1 cDNA was identified and subcloned into pBluescript (Stratagene, Inc., San Diego, CA) for sequence confirmation (Sequenase version 2.0, United States Biochemical, Cleveland, OH). A VCAM-1 cDNA lacking the transmembrane and cytoplas-

mic regions was generated by the polymerase chain reaction using the following primers: Primer #1: 5′-GGAAGAATTCGCGGCCGCTT-CACGTGGGGC-3′ and Primer #2: 5′-GGAAG-ATATCATGTTCTTTTCCTTTTAC-3′. The resulting 2.2-kb product was digested with *Eco*R1 and *Eco*RV and ligated to an *Eco*RV–*Sal*1 cDNA fragment encoding the hinge, CH2 and CH3 domains of murine IgG1. The VCAM-1-IgG chimera was subcloned into an eukaryotic expression vector and stably transfected into cells of the human embryonic kidney cell line 293.

Purification of Murine VCAM-1-IgG

VCAM-1-IgG was isolated from the culture supernatants of transfected 293 cells grown in serum-free medium by affinity absorption on protein A–Sepharose (Pharmacia LKB, Uppsala, Sweden) and elution with 100 mM acetic acid, pH 3.0, 500 mM NaCl. The eluent fractions were neutralized by dripping into a one tenth volume of 1 M Tris/HCl, pH 7.5 and then dialyzed in PBS. The purity of the VCAM-1-IgG preparation was assessed by SDS–PAGE.

Construction of Full-Length α4 cDNA and Transfection of K562 Cells

A cDNA library of the human T cell tumor line Jurkat (λ ZAP, Stratagene, San Diego, CA) was initially screened with a mixture of three synthetic oligonucleotides. Oligo #1: 5′-ATAAT-GAAAAAAACAATAAAC-3′, Oligo #2: 5′-ATATTTTACATAAAGAATGAA-3′, Oligo #3: 5′-GGCTTCTTTAAAAGA-3′. The oligonucleotides were constructed based on the published nucleotide sequence of α4 and comparison with the human α subunit sequences from α2, α5, αIIb, αV, αM, and αX [Takada et al., 1989]. After two further rounds of screening and selection, phage DNA was extracted from clones by the liquid lysate method [Ausubel et al., 1987]. The inserts were excised using *Eco*RI, purified by electrophoresis on agarose, and subcloned into the Bluescript plasmid (Stratagene). The nucleotide sequence was confirmed by the double-stranded ^{35}S-dATP dideoxynucleotide chain termination method of Sanger et al. [1977]. One clone contained the entire coding region of α4, but lacked the transcription start site and subsequent 83 nucleotides. Additional plaques were screened in a similar manner using a 433-bp fragment of the 5′ end of α4 obtained by amplification of HPB-ALL mRNA by the polymerase chain reaction using the following primers: Primer #1: 5′-AATAATCTAGACCGCCATGTC-CCCACCGAGAG-3′ and Primer #2: 5′-CAT-GATCCATTTTCTCCTG-3′. Sequence analysis indicated that one of the clones isolated encoded 409 bp of the untranslated sequence and the missing 5′ region. A full-length α4 cDNA was obtained by ligation of both fragments at the *Bam*HI restriction site located 433 bp downstream from the ATG initiation site [Takada et al., 1989]. The intact α4 cDNA was subcloned into the eucaryotic expression vector pRc/CMV which was then transfected into the erythroleukemia cell line K562 using the LIPOFECTIN reagent as recommended by the manufacturer (Gibco BRL, Gaithersburg, MD). The resulting transfected cells were cloned and the clones analyzed for the expression of cell surface α4. To control for the effects of the transfection protocol itself, K562 cells were transfected with the pRc/CMV vector alone (K562.CMV).

Lymphocyte Aggregation

Aggregation experiments were done essentially as previously described [Bednarczyk and McIntyre, 1990]. Briefly, 1.5×10^5 cells were placed in 96-well microtiter plates in the presence of the indicated dilutions of mAb ascites or culture supernatant. Aggregation was scored visually and photomicrography (20×) done at the indicated times. Relative levels of aggregation were scored using the 1+ to 5+ system of Rothlein and Springer [1986].

Binding to Murine VCAM-1-IgG and ECM Molecules

Recombinant murine VCAM-1-IgG was precoated onto polyvinyl microtiter wells at 0.1 μg/well in 100 μl of PBS for 1 h at 4°C. Ramos cells were labeled with 2′,7′-bis(2-carboxyethyl)-5 (and 6)-carboxyfluorescein, acetoxymethyl ester (BCECF) (Molecular Probes, Eugene, OR) at 37°C for 30 min, according to the method of Gimbrone et al. [1989]. The labeled cells were resuspended at 4×10^6 cells/ml in RPMI-1640 containing 5 mg/ml BSA. The cells were pretreated at room temperature for 30 min with test antibodies or medium alone. The final concentrations of mAbs were 10 μg/ml for H52, HP 2/1, and L25 and 1:10 dilutions of mAb 18D3 or 33B6 ascites. Antibody treated cells were added to the VCAM-1-IgG coated microtiter wells (50 μl/well) and incubated at 37°C for 15 min. To remove nonadherent cells, 200 μl of medium was added to each well and then the plates were

sealed and centrifuged in an inverted position at 400*g* for 5 min. The supernatants were then aspirated from the wells. The cells that remained bound to the wells were solubilized with 0.1% SDS in 50 mM Tris/HCl, pH 8.5. The proportion of adherent cells was determined by the degree of fluorescence per well as detected by a Pandex Fluorescence Concentration Analyzer (Pandex, Mundelein, IL) at a test wavelength of 485 nm and a reference wavelength of 535 nm.

Purified human fibronectin (Sigma Chemical Co., St. Louis, MO) was suspended in PBS at 10 μg/ml and 100-μl aliquots added to Covalink (Nunc Inc., Naperville, IL) microtiter wells. After overnight incubation at room temperature the wells were washed twice with PBS, followed by the addition of 200 μl of PBS containing 2% BSA (Sigma Chemical Co.) and a further overnight incubation. Before use, the wells were washed twice with complete RPMI-1640 medium. Cells (1×10^6/ml) were preincubated for 45 min at room temperature in complete RPMI-1640 containing 1:200 dilutions of mAb ascites or 15 ng/ml phorbol myristic acetate. Aliquots (100 μl) of pretreated cells were added to the FN-treated microtiter wells and incubated another 45 min at 37°C. Nonadherent cells were removed by gently filling each well twice with complete RPMI-1640 and aspirating the wells each time. The number of bound cells was determined by the method of Mosmann [1983]. Briefly, 100 μl of complete RPMI-1640 containing 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Chemical Co.) was added to each well, and the plates were incubated at 37°C for 4 h. The dark-blue formazan crystals, resulting from the cleavage of MTT by metabolically active cells, were solubilized by adding 100 μl of 0.04 N HCl in isopropanol. After 15 min, the optical density was measured using a Dynatech MR700 plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm. To determine the number of cells bound, a standard curve was generated using known numbers of each cell type.

Radiolabeling

Labeling of cell-surface proteins with ^{125}I has been described previously [McIntyre et al., 1989]. To biosynthetically label α4β1 [Bednarczyk et al., 1992], cells of the T-cell tumor line HPB-ALL were washed three times in HBSS and resuspended at 1×10^8 cells/ml in cysteine- and methionine-free RPMI labeling medium (GIBCO BRL, Grand Island, NY) containing 5% FBS and 600 μCi/ml each of ^{35}S-cysteine and ^{35}S-methionine (NEN/DuPont, Boston, MA). The cells were labeled with the radioactive amino acids for 10 min, washed once, and resuspended at 5×10^7 cells/ml in complete RPMI-1640. Equal aliquots of cells were harvested at the indicated times. The cells were lysed at a concentration of 5×10^7 cells/ml in 10 mM Tris–HCl, pH 8.0, with 1% Triton X-100, 150 mM NaCl, 2% BSA and 1 mM each of $CaCl_2$, $MgCl_2$, and $MnCl_2$. In some experiments, the lysis buffer lacked divalent cations.

Immunoprecipitation and SDS–PAGE

The immunoprecipitation procedure has been described previously [Bednarczyk et al., 1991]. The immunoprecipitated material was analyzed by SDS–PAGE according to the method of Laemmli [1970]. Molecular weight markers were obtained from Bio-Rad Laboratories (Richmond, CA). Prior to autoradiography, the gels were fixed and stained with 0.2% Coomassie brilliant blue (Sigma Chemical Co.) in 40:10:50 (methanol: acetic acid: H_2O), washed once with H_2O, soaked for 30 min in 1% glycerol (for radioiodinated proteins) or in 1 M sodium salicylate (for biosynthetically labeled proteins [Chamberlain, 1979]), and then dried for 60 min at 80°C under vacuum. Autoradiography was carried out at −80°C using intensifying screens (Cronex Lightning Plus; DuPont, Boston, MA) and Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY).

RESULTS

Induction of Homotypic Aggregation by mAb 33B6

The mAb 33B6 was initially identified during hybridoma screening through its ability to induce homotypic aggregation among leukocyte cell lines. Normally, cells of the human T-cell tumor line HPB-ALL grew as a single cell suspension (Fig. 1A, 0 aggregation). HPB-ALL cells aggregated in response to both the α4-specific mAb L25 (Fig. 1B, 5+ aggregation) and mAb 33B6 (Fig. 1C, 5+). The anti-β1 mAb 18D3 blocked the aggregation induced by mAb L25 (Fig. 1D, 1+) and mAb 33B6 (Fig. 1E, 1+). By itself, mAb 18D3 did not induce aggregation (0

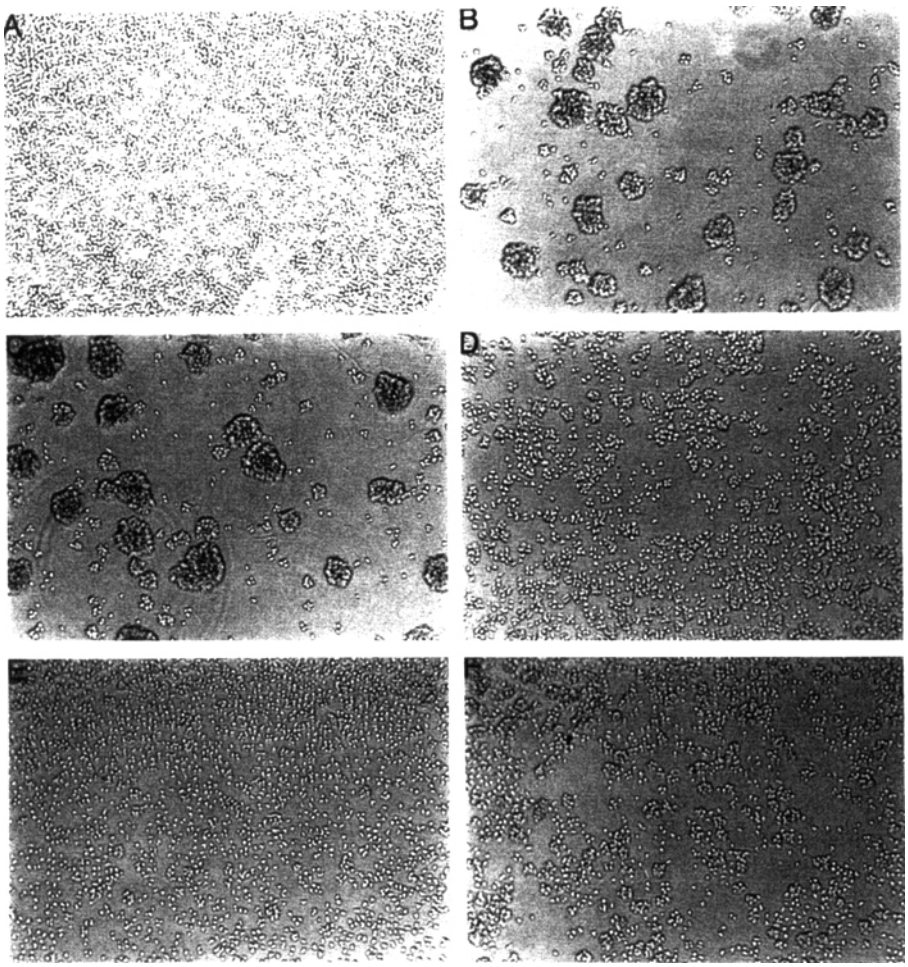

Fig. 1. Induction of homotypic aggregation by mAb 33B6 and mAb L25. Approximately 150,000 HPB-ALL cells, in 100 μl of complete RPMI-1640 medium, were added to wells of a microtiter plate. To each well, 100 μl of 1:200 dilutions of ascites were added and the volume of the wells equalized at 300 μl by adding medium where necessary. The cells were incubated at 37°C and photomicrographs taken after 4 h. **A**: no mAb. **B**: mAb L25. **C**: mAb 33B6. **D**: mAb 18D3 and mAb L25. **E**: mAb 18D3 and mAb 33B6. **F**: mAb 33B6 and mAb L25.

aggregation, data not shown). When used separately, either mAb 33B6 or mAb L25 induced vigorous aggregation, but when the cells were treated with a combination of these mAbs aggregation was minimal (Fig. 1F, 1+). Aggregation could be induced among Epstein Barr virus-transformed B cells from a patient with LAD [Springer et al., 1984] by both mAb L25 (4+) and mAb 33B6 (4+), indicating that LFA-1 and T-cell-specific surface proteins were not required to mediate aggregation. The physiologic parameters of mAb 33B6-induced aggregation were analyzed and compared with the aggregation induced by mAb L25 (Table I). The aggregation induced by both mAb L25 and mAb 33B6 required ATP (inhibited by NaN_3 and 2-deoxyglucose), divalent cations (inhibited by EDTA), and an intact cytoskeleton (inhibited by cytochalasin B). The mAb-treated cells did not aggregate at 4°C but aggregated in a normal manner when warmed to 37°C.

Most of the cell types tested aggregated in response to treatment with mAb 33B6, including peripheral blood T cells; the T-cell lines Jurkat, CCRF-HSB-2, and Molt-4; the B cell lines, Ramos, Daudi, Pally, JY and Raji; and the myelomonocytic line U937 (data not shown). By contrast, cells of the erythroleukemia K562 failed to aggregate. This absence of aggregation did not seem to be due to a lack of aggregation ability, since K562 cells would aggregate in response to anti-CD43 mAbs (5+ aggregation).

TABLE I. Comparison of the Physiologic Parameters of mAb L25- and mAb 33B6-Induced Homotypic Aggregation*

Treatment	Aggregation	
	L25	33B6
Control (37°C)	5+	5+
4°C	0	0
4°C → 37°C	5+	5+
5.0 mM EDTA	2+	1+
5.0 mM 2-deoxyglucose + 0.1% NAN_3	0	0
1.0 mM cytochalasin B	1+	1+

*HPB-ALL cells were added to microtiter wells at 150,000 cells/well in 100 μl of complete RPMI-1640 containing the indicated concentrations of metabolic perturbants and were pretreated as indicated for 2 h prior to the addition of a 1:300 dilution of mAb ascites. The concentration of metabolic perturbants remained constant throughout the experiment. The cells were then incubated for 3 h and aggregation scored visually. In the absence of mAb treatment, the HPB-ALL existed as single cell suspensions (0 aggregation) under all conditions throughout the experiment.

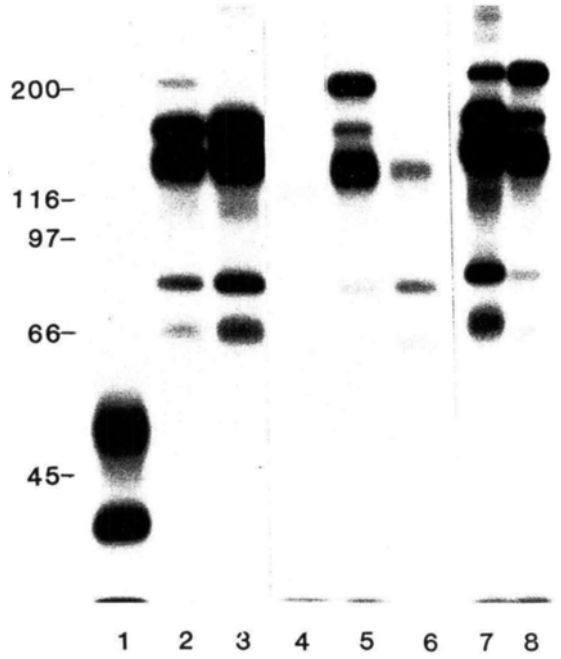

Fig. 2. Identification of cell-surface molecules immunoprecipitated by mAb 33B6. The cell-surface proteins of HPB-ALL cells (**lanes 1–3,7**) and activated T lymphocytes (**lanes 4–6,8**) were labeled with ^{125}I and the cells lysed in Triton X-100 containing divalent cations as described in Materials and Methods. Immunoprecipitations were done using mAb T40/25 (**lanes 1,4**), mAb 18D3 (**lanes 2,5**), mAb L25 (**lanes 3,6**), and mAb 33B6 (**lanes 7,8**). The immunoprecipitates were analyzed by SDS–PAGE on a 6.25% acrylamide gel under reducing conditions.

mAb 33B6 Binds to the Integrin β1 Subunit

Immunoprecipitates from ^{125}I-cell surface radiolabeled HPB-ALL cells and activated peripheral blood T lymphocytes were analyzed by SDS–PAGE (Fig. 2). The mAb T40/25 reacts with a clonally expressed epitope on the T cell antigen receptor (TCR) from HPB-ALL cells [Kappler et al., 1983] and immunoprecipitated the 46-kD α and 40 kD β subunits of the TCR from HPB-ALL cells (Fig. 2, lane 1) but not activated T lymphocytes (Fig. 2, lane 4). The α4-specific mAb L25 immunoprecipitated a complex of polypeptides from HPB-ALL cells consisting of the 150 kD α4 subunit, 120 kD β1 subunit, and two 75 kD and 65 kD α4 fragments (Fig. 2, lane 3). Activated T lymphocytes expressed a similar pattern of bands immunoprecipitated by mAb L25, however the ratio of intact to fragmented α4 was significantly less (Fig. 2, lane 6). The β1-specific mAb 18D3 immunoprecipitated intact and fragmented α4 in addition to the 120-kD β1 subunit, but also detected other β1-associated α polypeptides such as the 200 kD α1 subunit (Fig. 2, lanes 2 and 5). The mAb 33B6 immunoprecipitated polypeptides from HPB-ALL and activated T cells identical in SDS–PAGE mobility to those seen using mAb 18D3 (Fig. 2, compare lanes 7 and 8 with lanes 2 and 5).

The previous results suggested that mAb 33B6 bound to an epitope expressed by the β1 subunit but did not rule out the possibility that the mAb 33B6 epitope required the presence of an associated α subunit. Therefore, biosynthetic analysis of the α4 and β1 subunits produced by HPB-ALL cells was undertaken (Fig. 3). After 10-min labeling with ^{35}S-methionine and ^{35}S-cysteine (pulse), the cells were harvested at the indicated times (chase) then lysed in Triton X-100 in either the absence (Fig. 3A) or presence (Fig. 3B) of divalent cations. We have found that lysing cells in 1% Triton X-100 in the absence of divalent cations can disrupt the association of the α4 and β1 subunits, allowing the two subunits to be analyzed separately. The addition of 1 mM divalent cations to the lysis buffer can stabilize the association of the two subunits. At the end of the pulse period, the β1-specific mAb 18D3 immunoprecipitated a diffuse β1 precursor of 90–100 kD, regardless of the presence of divalent cations (Fig. 3A,B, lane 1). Twenty min later, a 110-kD band appeared (Fig. 3A,B, lane 2), and by 60 min of chase, the mature 120-kD β1 subunit could be detected (Fig. 3A,B, lane 4). In the absence of cations, at 20 min of chase, the α4-specific mAb L25 immunoprecipitated a faint

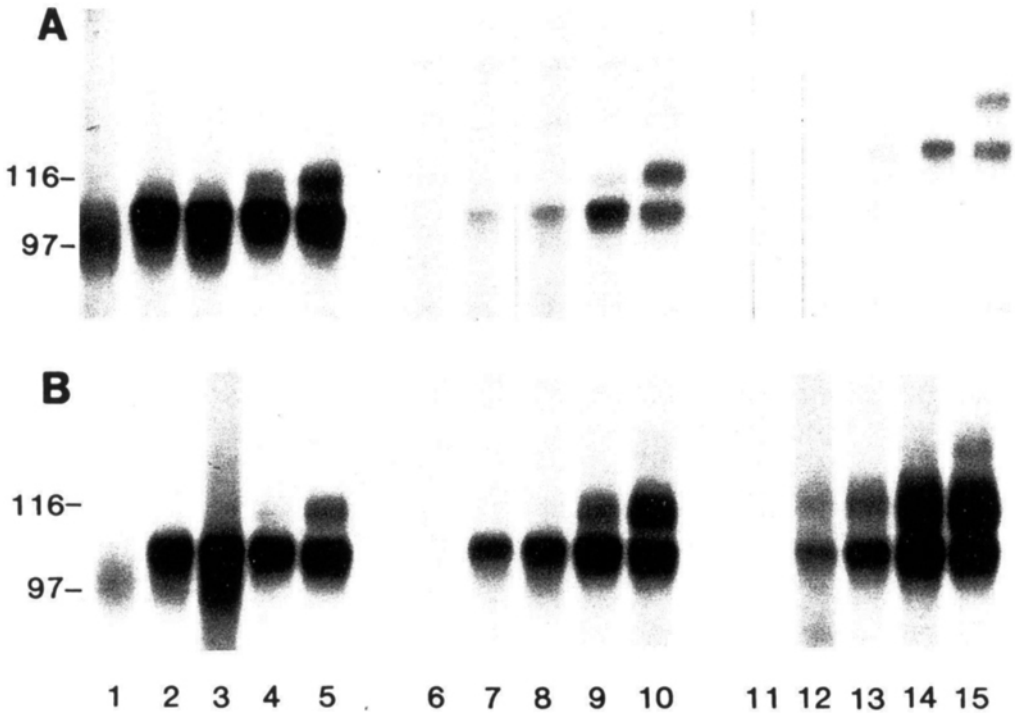

Fig. 3. Biosynthetic analysis of α4 and β1 subunits. HPB-ALL cells were biosynthetically labeled as described in Materials and Methods. Aliquots of cells were harvested at 0 min (**lanes 1,6,11**), 20 min (**lanes 2,7,12**), 40 min (**lanes 3,8,13**), 60 min (**lanes 4,9,14**), and 120 min (**lanes 5,10,15**) after the end of the pulse-labeling period. The cells were lysed in Triton X-100 in the absence (**A**) or presence (**B**) of divalent cations. Immunoprecipitations were done using mAb 18D3 (**lanes 1–5**), mAb 33B6 (**lanes 6–10**), and mAb L25 (**lanes 11–15**). The immunoprecipitates were analyzed by SDS–PAGE on a 6.25% acrylamide gel under nonreducing conditions.

band of 125 kD, which increased in intensity over time (Fig. 3A, lane 12). At 120 min of chase, the mature 140-kD α4 polypeptide was detected (Fig. 3A, lane 15), as were trace amounts of the α4 fragments (data not shown). In the presence of divalent cations, mAb L25 immunoprecipitated a heterodimer consisting of the α4 and β1 subunits (Fig. 3B, lanes 12–15). mAb 33B6 did not immunoprecipitate the 90–100 kD β1 precursor (Fig. 3A,B, lane 6) but did react with the 110-kD and 120-kD forms of the molecule (Fig. 3A,B, lanes 7–10). The pattern of bands immunoprecipitated by mAb 33B6 was distinct from that immunoprecipitated by mAb L25 but closely paralleled that of mAb 18D3, supporting the idea that the mAb 33B6 binding epitope is expressed on the β1 subunit, independent of specific α-subunit association.

Crossblocking of mAb 33B6-Induced Aggregation by anti-α4 mAb

Since mAb 33B6 reacted with the β1 subunit and yet crossblocked aggregation with mAb L25, we next analyzed the aggregation responses of HPB-ALL cells after treatment with mAb 33B6 and the α4-specific mAbs L25, HP1/1, and HP1/7, alone or in various combinations (Table II). The HP 1/1 and HP 1/7 mAbs react with the A epitope expressed on the α4 polypeptide, whereas mAb L25 binds to the B2 epitope [Pulido et al., 1991]. The ability of α4-specific mAbs to induce homotypic aggregation is restricted to those that react with the A and B2 binding epitopes [Pulido et al., 1991]. The HPB-ALL cells aggregated in response to all four mAbs

TABLE II. Monoclonal Antibody Crossblocking of Aggregation*

	Control	L25	33B6	HP1/1	HP1/7
Control	1+	4+	4+	4+	4+
L25		4+	1+	1+	1+
33B6			4+	2+	2+
HP1/1				4+	4+
HP1/7					4+

*HPB-ALL cells were added to microtiter wells at 150,000 cells/well in 100 μl of complete RPMI-1640. To each well was added 100 μl of 1:200 diluted mAb ascites, or 1:20 diluted mAb HP1/1 culture supernatant, as indicated at the top of the panel and at the side of the panel. Aggregation was scored visually after 4-h incubation.

used individually (33B6, L25, HP1/1, and HP1/7). When an A epitope-specific mAb is mixed with a B2 epitope-specific mAb, little or no aggregation occurs [Pulido et al., 1991]. As expected, mixtures of mAb L25 with mAb HP1/1, or HP1/7, resulted in minimal aggregation among HPB-ALL cells. When mAb 33B6 was mixed with either HP 1/1 or HP 1/7 aggregation was also inhibited, however, the level of crossblocking was not as complete when mixed with mAb L25.

Aggregation of K562 Cells Transfected With α4 cDNA in Response to Treatment With mAb 33B6

Flow cytofluorometry indicated that K562 cells expressed the β1 subunit (>99% positive, mean fluoresence intensity (MFI) = 16.1) and the α5 subunit (>99% positive, MFI = 13.5), but little or none of the other β1-associated α subunits α1, α2, α3, α4, α6, or αV (average MFI = 0.5; MFI of control = 0.5). K562 cells also expressed the mAb 33B6 binding site (>99% positive, MFI = 22.2) but did not aggregate in response to treatment with mAb 33B6. Since α4-mediated leukocyte aggregation had been described [Bednarczyk and McIntyre, 1990; Campanero et al., 1990, Pulido et al., 1991], we analyzed whether or not transfection of α4 cDNA into K562 cells would lead to the development of mAb 33B6-triggered aggregation. A full-length α4 cDNA was ligated into the eucaryotic expression vector pRc/CMV for transfection into K562 cells. The resulting transfectants were cloned and analyzed for the expression of α4. Cells of the clone K562.E5 expressed high levels of surface α4 and were used to study α4 structure and determine if it associated with β1 after transfection. Radioiodinated cell surface proteins of K562.E5, and of K562 cells transfected with pRc/CMV alone (K562.CMV), were immunoprecipitated with mAb L25, mAb 18D3 or mAb 33B6 (Fig. 4). Both mAb 18D3 and mAb 33B6 immunoprecipitated bands of 150 kD and 115 kD from K562.CMV lysates (Fig. 4, lanes 2 and 4). Based on the flow cytometry data, the 150 kD/110 kD complex immunoprecipitated by both mAb 18D3 and mAb 33B6 was probably the α5β1 heterodimer. No mAb L25-reactive material could be detected in the lysates of the K562.CMV cells (Fig. 4, lane 3) consistent with the results of flow cytofluorometry. These results confirmed the observation that mAb 33B6 did not react with the α4 subunit and showed that the expression of the 33B6 epitope was

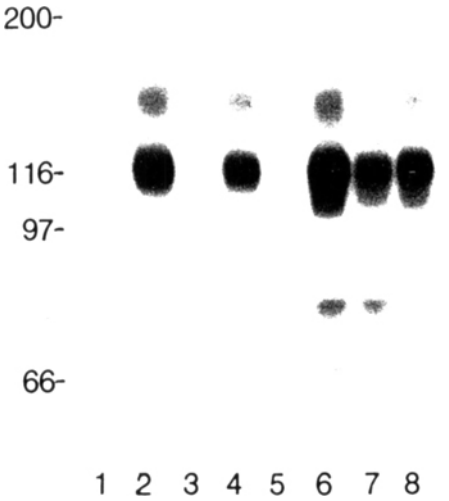

Fig. 4. Integrin α4 subunit is expressed on the cell surface of K562 cells transfected with α4 cDNA. The cell surface proteins of K562.CMV (pRc/CMV transfected, **lanes 1–4**) and K562.E5 (α4 transfected, **lanes 5–8**) were labeled with ^{125}I as described in Materials and Methods. Labeled cells were lysed in 1% Triton X-100 (containing divalent cations) and the lysates immunoprecipitated with the negative control P3 ascites (**lanes 1,5**), mAb 18D3 (**lanes 2,6**), mAb L25 (**lanes 3,7**), and mAb 33B6 (**lanes 4,8**). The immunoprecipitates were analyzed by SDS–PAGE on a 6.25% acrylamide gel under nonreducing conditions.

independent of the presence of the α4 subunit. After transfection with α4 cDNA K562 cells expressed cell surface α4 primarily in the form of the 85 and 70 kD fragments (Fig. 4, lane 7). A small amount of intact 140 kD α4 subunit was detectable in the mAb L25 immunoprecipitates after longer autoradiographic exposure times. The presence of a 115 kD band in the mAb L25 immunoprecipitate (Fig. 4, lane 7) suggested that the α4 subunit became associated with the endogenous β1 subunit in the transfected cells. This hypothesis was confirmed by the ability of the β1-specific mAbs 18D3 and 33B6 to immunoprecipitate the α4 fragments as well as the α5 subunit (Fig. 4, lanes 6 and 8).

The K562.E5 and K562.CMV cells were tested for the ability to aggregate in response to treatment with the inducing mAbs HP1/1 (A epitope of α4; [Pulido et al., 1991]), L25 (B2 epitope of α4; [Pulido et al., 1991]) and 33B6. Both cell lines grew as single cell suspensions in the absence of any treatment (Fig. 5A,B). The α4-negative K562.CMV cells failed to aggregate in response to any of the mAbs (Fig. 5C,E,G). The α4-transfected K562.E5 cells aggregated in response to both mAb L25 (Fig. 5D) and mAb HP1/1 (Fig. 5F). However, mAb L25 aggregation (2+) was significantly less than that in-

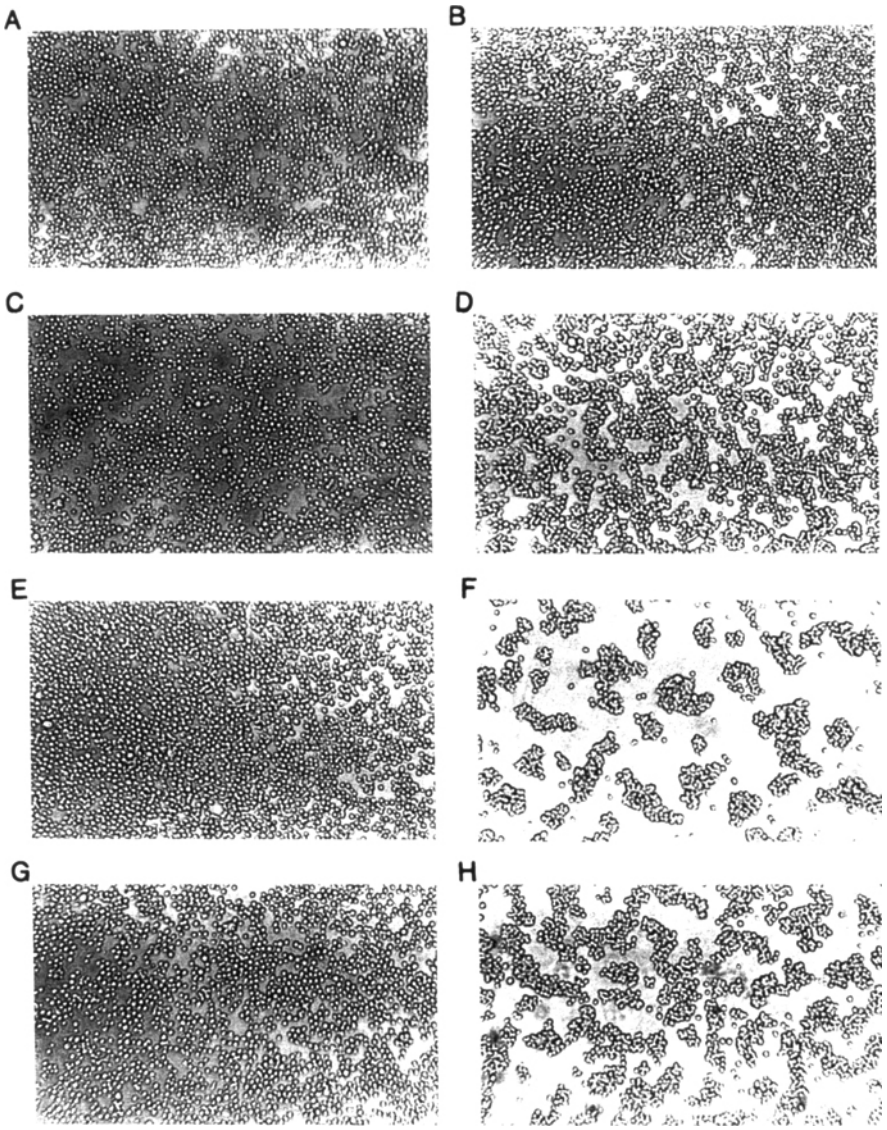

Fig. 5. Induction of aggregation among K562 cells transfected with α4 cDNA. Aggregation analysis was done at approximately 150,000 cells/well in a microtiter plate. The cells were left untreated (**A,B**) or treated with a 1:300 dilution of mAb L25 ascites (**C,D**), a 1:30 dilution of mAb HP 1/1 culture supernatant C (**E,F**), or a 1:300 dilution of mAb 33B6 ascites (**G,H**). The cells were incubated at 37°C and photomicrographs taken after 4 h. A,C,E,G: K562.CMV cells; B,D,F,H: K562.E5 cells.

duced by mAb HP1/1 (4+). Significantly, K562 cells transfected with α4 cDNA developed the ability to aggregate in response to treatment with mAb 33B6 (Fig. 5H, 3+).

mAb 33B6 Did Not Induce Adhesion to Murine VCAM-1-IgG or FN

Recently, it has been reported that the β1-specific mAbs TS2/16, A-1A5 and 8A2 have the ability to enhance the binding of leukocytes to ECM proteins and VCAM-1 [Kovach et al., 1992; van de Wiel-van Kemenade et al., 1992; Wayner and Kovach, 1992], however they do not induce homotypic aggregation [Bednarczyk and McIntyre, 1992; Campanero et al., 1990; Kovach et al., 1992]. Unlike these other β1-specific mAbs, mAb 33B6 did not potentiate the binding of cells of the B lymphoblastoid line Ramos to recombinant murine VCAM-1 (Fig. 6). In fact, mAb 33B6 and the anti-α4 (B1 epitope) mAb HP2/1 completely inhibited adhesion. The anti-α4 (B2 epitope) mAb L25 and the anti-β1 mAb 18D3 also inhibited Ramos adhesion to VCAM-1, although at significantly lower levels than mAb 33B6.

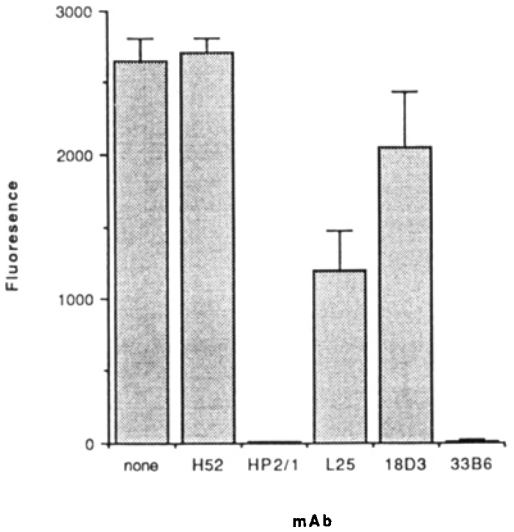

Fig. 6. Inhibition of Ramos cell binding to VCAM-1-IgG by anti-α4 and anti-β1 antibodies. BCECF-labeled Ramos cells were preincubated with mAb and added to the VCAM-1-IgG coated microtiter wells at 2 × 10^5 cells/well. Incubation and washing was done as described in Materials and Methods. The degree of adherence is reported as relative fluorescence intensity.

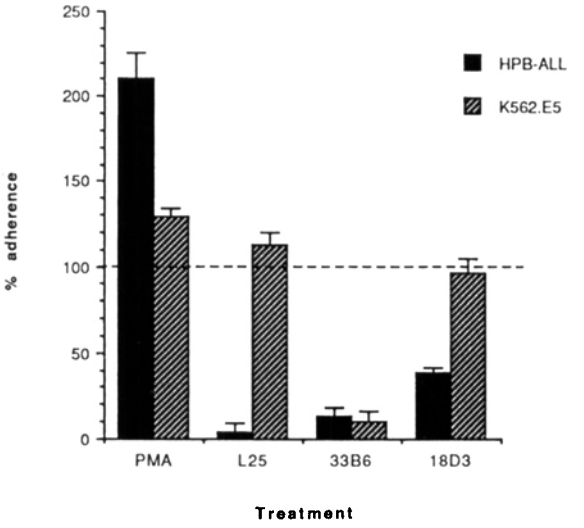

Fig. 7. Binding of HPB-ALL and K562.E5 cells to the extracellular matrix protein fibronectin. Cells were pretreated in complete RPMI-1640 at 1 × 10^6 cells/ml containing 15 ng/ml PMA or 1:200 dilutions of mAb ascites as described in Materials and Methods. Aliquots (100 μl) containing 1 × 10^5 pretreated cells were added to each well and incubated as described in Materials and Methods. Cell binding to BSA-coated wells was less than 1% of untreated cells to FN. The binding of the treated cells to FN is presented as a percentage of the binding of untreated cells to FN.

Next we analyzed the ability of mAb 33B6 to influence the binding of HPB-ALL and K562.E5 cells to the ECM protein FN (Fig. 7). Treating HPB-ALL or K562.E5 cells with mAb 33B6 almost totally blocked the binding of both cell lines to FN. The binding of HPB-ALL cells was almost totally blocked by mAb L25 and mAb 18D3 blocked by more than 60%. By contrast, neither mAb L25 nor mAb 18D3 significantly influenced the binding of the transfected K562.E5 cells. Treating the cells with phorbol ester increased cell binding to FN; by twofold for HPB-ALL cells and by approximately 30% for the K562.E5 cells suggesting that the β1 integrins on these cells could be induced to higher adhesive states [Shimizu et al., 1990a].

DISCUSSION

The level of mAb 33B6-induced aggregation, in terms of rate, size of aggregate formation, and physiologic parameters was nearly identical to that seen for the α4-specific mAb L25 (Fig. 1 and Table I). However, immunochemical analysis of cell surface and biosynthetically labeled proteins indicated that mAb 33B6 reacted with an epitope expressed on the β1 subunit (Figs. 2, 3 and 4). Unlike the β1-specific mAb 18D3, mAb 33B6 did not react with the 90–110 kD β1 precursor at 0 min of chase but did react with the 110 kD precursor and 120 kD mature forms of β1. This result suggests that mAb 33B6 recognized an epitope on the β1 subunit that developed later in post-translational processing than did the mAb 18D3 binding epitope. This result is similar to the differential epitope development found during biosynthesis of the αVβ3 complex and the α4 subunit [Cheresh and Spiro, 1987; Bednarczyk et al., 1992].

There is growing precedence for regulation of integrin function without alteration in levels of expression. The adhesive capacity of both β1 and β2 integrins can be regulated by signals mediated through the T-cell receptor complex, or the CD2, CD4, and CD31 accessory molecules [Dustin and Springer, 1989; Hershkoviz et al., 1992; Tanaka et al., 1992; van Kooyk et al., 1989]. In addition, treating leukocytes with phorbol esters can activate β1- and β2-mediated adhesion [Rothlein and Springer, 1986; Dransfield and Hogg, 1989; Shimizu et al., 1990a]. While it has not been ruled out that mAb reactive with the α4β1 complex regulate adhesion through other adhesion proteins, it is possible that the binding of certain anti-α4 or anti-β1 mAbs can induce conformational changes in the receptor that mimic activated adhesive states. Support for such a conformational hypothesis comes from

multiple sources: Figdor and colleagues have described a Ca^{2+}-dependent epitope on the α subunit of the leukocyte integrin LFA-1 (αLβ2) [Keizer et al., 1988; van Kooyk et al., 1991]. Binding of the mAb NKI-L16 to this epitope induced an interaction, that was not due to intracellular signaling, of LFA-1 with its counter-receptor ICAM-1 and resulted in homotypic lymphocyte aggregation [Keizer et al., 1988; van Kooyk et al., 1991]. In contrast to the Ca^{2+}-dependent NKI-L16 binding epitope, an epitope requiring the presence of Mg^{2+} or Mn^{2+}, but down-regulated by the presence of Ca^{2+}, has been shown to be expressed by all three of the β2-associated α subunits [Dransfield and Hogg, 1989; Dransfield et al., 1992]. The expression of this epitope correlated with enhanced ligand binding function for αLβ2 (LFA-1) and it was proposed that cation binding could alter the conformation and potentially the function of the receptor [Dransfield et al., 1992]. In addition, a similar epitope has been described for the platelet glycoprotein IIb/IIIa (αIIbβ3), which could be induced by treating platelets with EDTA or when the receptor was bound to ligand [Ginsberg et al., 1986; Frelinger et al., 1988]. Finally, an epitope has been described that was expressed by both the β2 integrin Mac-1 (αMβ2) and αIIbβ3, which appeared only when the receptors were in their activated and adhesive states [Altieri and Edgington, 1988; Coller, 1985].

A unique aspect of the anti-α4 mAb specific for the A and B2 epitopes is their ability to crossblock aggregation. Individually, mAb reactive with either epitope induce aggregation, however, treating cells with a mixture of mAb specific for both epitopes results in minimal aggregation (Table II) [Pulido et al., 1991]. The same situation was observed when a mixture of mAb 33B6 and aggregation inducing anti-α4 mAb were added to cells (Fig. 1 and Table II). The problem lies in rationalizing the ability of two positive adhesion signals to result, in conjunction, in a nonsignaling event. One possibility is that, individually, mAb binding to any of the aggregation inducing epitopes (A, B2, and 33B6) results in subtly different types of conformational changes, each of which is capable of mediating aggregation. The binding of mAbs concurrently to two of the epitopes might result in offsetting sets of conformational changes and paralysis of the receptor activation event.

The aggregation induced by mAb 33B6 correlated with the cell surface expression of the α4 subunit. However, the presence of the mAb 33B6 epitope was independent of the presence of α4 (Figs. 3, 4). Cells of the erythroleukemia cell line K562 expressed the β1 subunit and the 33B6 epitope, but did not aggregate in response to mAb 33B6 (Fig. 5). Only after transfection with α4 cDNA did the K562 cells develop the ability to aggregate in response to treatment with mAb 33B6. Since untransfected K562 cells expressed the α5 subunit but did not aggregate it is probable that α5 did not mediate this event. The mechanism involved in the influence of α4 expression on mAb 33B6-mediated aggregation is unknown, however, it has been proposed that the cytoplasmic regions of the integrin α subunits may influence post-ligand binding functional events [Chan et al., 1992]. During that study, α2 chimeric proteins were constructed that differed only in whether they contained the α2, α4 or α5 cytoplasmic sequences. These constructs were transfected into the rhabdomyosarcoma line RD. Functionally, only those cells transfected with the chimeras containing the α2 and α5 cytoplasmic regions could mediate collagen gel contraction and, reciprocally, only the α4 chimeras could enhance cell migration on collagen- or laminin-coated surfaces. One possibility discussed was that the intracellular machinery involved in these two events differed [Chan et al., 1992]. A similar situation might occur in mAb 33B6-induced aggregation, with the cytoplasmic region of the α5 polypeptide unable to interact with the cytoplasmic components involved in mediating aggregation. Alternatively, mAb 33B6-induced aggregation may involve a counter-receptor that, on K562 cells, is recognized by the α4β1 complex but not by α5β1. The ability of α4 to reconstitute K562 aggregation indicated that the other β1-associated α subunits (α1, α2, α3, α6, and αV) were not necessary for mAb 33B6-mediated aggregation. However, we cannot rule out the possibility that other α subunits can also mediate aggregation.

Evidence for multiple regulatory sites on the extracellular domains of α4 has been established [Pulido et al., 1991] and there is now growing evidence that a multiregulatory view of the β1 subunit can be taken. In the present report, we present evidence for two functionally discrete epitopes expressed by the β1 subunit, which we shall term epitopes β1.1 (mAb 18D3) and β1.2 (mAb 33B6). Binding of mAb 18D3 to epitope β1.1 did not induce an adhesive event but did block leukocyte aggregation (Fig. 1) and the

binding of cells to VCAM-1 (Fig. 6 and Schliemer, et al., 1992) and FN (Fig. 7). By contrast, binding of mAb 33B6 to epitope β1.2 resulted in the induction of aggregation (Figs. 1 and 5). However, mAb 33B6 was similar to mAb 18D3 in the ability block the binding of cells to VCAM-1 (Fig. 6) and FN (Fig. 7). The recent work of several laboratories [Arroyo et al., 1992; Kovach et al., 1992; van de Wiel-van Kemenade et al., 1992; Wayner and Kovach, 1992] suggests that there might be at least one other functionally distinct epitope expressed by the β1 subunit. In those studies, the β1-specific mAbs 8A2, A-1A5 and TS2/16 were found to potentiate cell binding to ECM proteins and VCAM-1, but not to induce aggregation. In addition, mAb 8A2 enhanced the binding of T-cell lines to synthetic peptide ligands specific for the α4β1 receptor complex [Wayner and Kovach, 1992]. Both mAb A-1A5 and TS2/16 have been shown to block leukocyte aggregation [Bednarczyk and McIntyre, 1990; Campanero et al., 1990] indicating that the epitope bound by these two mAb is functionally distinct from the β1.1 and β1.2 epitopes and, for the sake of nomenclature, we will name it β1.3. If the binding of mAb to functionally relevant epitopes causes conformational changes in the receptor leading to regulation of function it would appear that the three putative β1 epitopes mediate different types of conformational changes. A comprehensive analysis of β1-specific mAbs should provide useful insight into further definition of functionally relevant regions of the β1 subunit.

In summary, we have identified two functionally discrete epitopes expressed by the integrin β1 subunit. The β1.2 epitope-specific mAb 33B6 induced leukocyte homotypic aggregation, and the conversion of K562 cells into mAb 33B6 responders after transfection with α4 subunit cDNA suggests that the α4β1 complex could mediate this adhesive event. The multiple epitopes expressed by the subunits of the α4β1 receptor, that differentially influence adhesive interactions, reveal a complexity in the regulation of integrin receptor function, making this molecule an attractive model for analyses of subtle relationships between integrin structure and function.

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