Induction of homotypic lymphocyte aggregation: evidence for a novel activation state of the β_1 integrin

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Abstract: Intercellular adhesion of Jurkat lymphocytic cells was investigated by use of monoclonal antibodies 33B6 and 18D3, which bind to the β_1 integrin receptor. 33B6 induced homotypic aggregation of Jurkat cells, whereas 18D3 inhibited this aggregation. Jurkat cells could be induced to aggregate at low 33B6 concentrations corresponding to 5% β_1 integrin site occupancy, and the rate of aggregation was maximum at 30% occupancy. Simultaneous addition of mAb 18D3 and 33B6 demonstrated that the two antibodies mediate changes in the β_1 integrin activation state that are competitive in nature. Aggregation through β_1 integrin induced by 33B6 was reversed by subsequent addition of 18D3. To further examine the mechanism by which 33B6 and 18D3 affect cell adhesion function, we explored the binding of monoclonal antibody (mAb) 15/7. This mAb recognizes an activation epitope of the β_1 integrin and has been shown to sustain cell adhesion to vascular cell adhesion molecule 1 (VCAM-1) and fibronectin. Activation of Jurkat cells with Mn^{2+} caused a 2.5-fold increase in 15/7 binding but did not increase binding of 33B6. 33B6 partially blocked 15/7 binding to β_1 integrin on unstimulated and Mn^{2+}-activated Jurkat cells. 18D3 did not affect mAb 15/7 binding. These results indicate that 33B6 and 18D3 modulated homotypic aggregation by inducing a novel activation state of the very late activation integrin distinct from the state recognized by 15/7, which supports cell binding to VCAM-1 and fibronectin. *J. Leukoc. Biol.* 59: 872–882; 1996.

***Key Words:** very late activation integrin · adhesion · mechanism*

INTRODUCTION

Integrin proteins play an important role as receptors mediating the recognition and adhesion of lymphocytes. These molecules are transmembrane $\alpha\beta$ heterodimeric glycoproteins with two distinct domains [1–4]. Recent studies indicate that ligand binding to the extracellular domain of the β_1 integrin receptor is associated with a change in its conformation and topography [5, 6]. This may result in membrane signal transduction and cell activation. Cell signaling through integrin receptors is associated with tyrosine phosphorylation, cytoplasmic alkalization, and a subsequent increase in cell proliferation rates and cytoskeletal organization [7–9].

The very late activation (VLA) sub-family of integrins is composed of heterodimeric glycoproteins where the common β_1 subunit is noncovalently associated with one of the α chains (α_{1-9}, α_v). It is expressed on most leukocytes [10, 11] and mediates a diverse set of functions including the adhesion of leukocytes to the extracellular matrix [12–15], endothelial cells [16, 17], and to other leukocytes [18, 19]. The VLA proteins interact with extracellular matrix components such as fibronectin, laminin, and collagen, thereby mediating a diverse set of adhesion-dependent functions such as embryogenesis and lymphocyte homing [11]. β_1 interactions with the endothelium play a role in lymphocyte homing, cell migration, and in the pathogenesis of diseases such as arthritis, atherosclerosis, and encephalomyelitis [20–23]. Cell-cell interactions involving the integrin are important during cytotoxic T lymphocyte activity [24] and during homotypic aggregation [18, 25]. Thus, it is essential to study the role of the β_1 chain in regulating ligand binding affinity and in mediating the attachment of cells expressing VLA integrins. We have focused our studies on the role of anti-β_1 antibodies in the homotypic aggregation of lymphocytes with other lymphocytes. This system is used as a model to study integrin activation and function.

Antibodies to both the α and β chain of the VLA integrin have been shown to cause changes in integrin activity including ligand recognition. This point was demonstrated by monoclonal antibodies (mAbs) to the VLA-4 integrin, which activated the integrin and induced homotypic lymphocyte aggregation by an adhesion mechanism independent and distinct from the LFA-1/ICAM-1 pathway [18, 19]. Homotypic aggregation has been shown to be induced by antibodies to various other VLA proteins including

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Abbreviations: mAb, monoclonal antibody; VLA, very late activation subfamily of integrins; VCAM-1, vascular cell adhesion molecule 1; IgG, immunoglobulin G; FITC, fluorescein isothiocyanate; FBS, fetal bovine serum.

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VLA-3 [26] and VLA-6 [27]. Certain antibodies that bind to the β_1 integrin induce cell activation and homotypic aggregation through a mechanism other than cross-linking by bivalent antibodies [15, 18, 28, 29]. β_1-integrin stimulated aggregation is a heterophilic molecular event in which the ligand and receptor mediating cell adhesion are different [9, 18, 29]. Heterotypic aggregation studies between Jurkat lymphocytic cells and B cells demonstrated that, whereas Jurkats have both the receptor and the ligand necessary for aggregation, B cell lines such as JR2B10 only present the ligand [29]. A recent work suggests that the α_4 subunit on adjacent cells may itself act as a ligand for binding $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins [30].

Homotypic aggregation induced by activation of the VLA integrin has been shown to be epitope specific and independent of activation through Fc receptors [25]. It required an active metabolism, cytoskeletal integrity, physiological levels of intracellular calcium, and a functional Na^+/H^+ antiport [14, 15]. Upon activation, VLA binding activity in T cells was rapidly augmented without changes in the level of expression of the VLA molecule [10]. Several mechanisms that may contribute in modulating cell adhesivity include clustering of receptors at the cell-cell contact interface and cell spreading on the substrate, both of which may increase the number of receptor-ligand bonds [9, 31, 32].

β_1 integrins may be stimulated to bind a variety of substrates depending on the particular activating antibody. It has been proposed that β_1 integrins may adopt an active conformation capable of expressing a range of affinity states that can be modulated at the cell ligand-binding surface [5]. mAb binding to neoepitopes provides further evidence that integrin activation may be accomplished through changes in conformation. mAb 15/7 has been shown to recognize an activation epitope on the β_1 integrin and identify a subset of the VLA-4 integrin that mediates cell binding to fibronectin and vascular cell adhesion molecule 1 (VCAM-1) [6]. Another mAb 9EG7 binds to a β_1 integrin epitope only when it is induced by soluble ligand and Mn^{2+}. This conformation may be reversed by Ca^{2+} [33].

In the current study, we quantify the role of mAbs to the β_1 integrin in modulating Jurkat cell avidity and the mechanism by which these mAbs affect integrin activation during homotypic aggregation. We have recently developed a quantitative assay based on video microscopy and image analysis to measure the kinetics of homotypic aggregation [34, 35]. In this study, we apply this technique along with flow cytometry and radiolabeling to quantitate the relationship between β_1 integrin occupancy and aggregation kinetics. Jurkat cells (a lymphoblastoid T cell line) were incubated with two anti-β_1 antibodies, 33B6 and 18D3. 33B6 has been shown to promote aggregation of peripheral blood T cells, various T cell lines, B cell lines, and myelomonocytic lines [36]. 18D3 inhibits this aggregation [36]. Our findings indicate that the rate and extent of adhesion in Jurkat cells may be modulated by the number of integrins occupied by 33B6. This aggregation may be reversed by addition of 18D3. We explored the stoichiometry and kinetics of this competition in terms of changes in aggregate morphology. The mechanism by which 33B6 and 18D3 affect changes in VLA ligand recognition was assessed by using 15/7, an antibody that reports on an activation epitope on the β_1 integrin.

MATERIALS AND METHODS

Monoclonal antibodies

We explored the homotypic aggregation of cells of the Jurkat cell line (a human lymphoblastoid T cell line) induced by addition of the following antibodies to the β_1 integrin: 33B6 and 18D3. 33B6, an immunoglobulin IgG_{2b} monoclonal antibody to the β1.2 epitope [36], and the anti-CD2 control antibody, OKT11, were maintained in our laboratory.

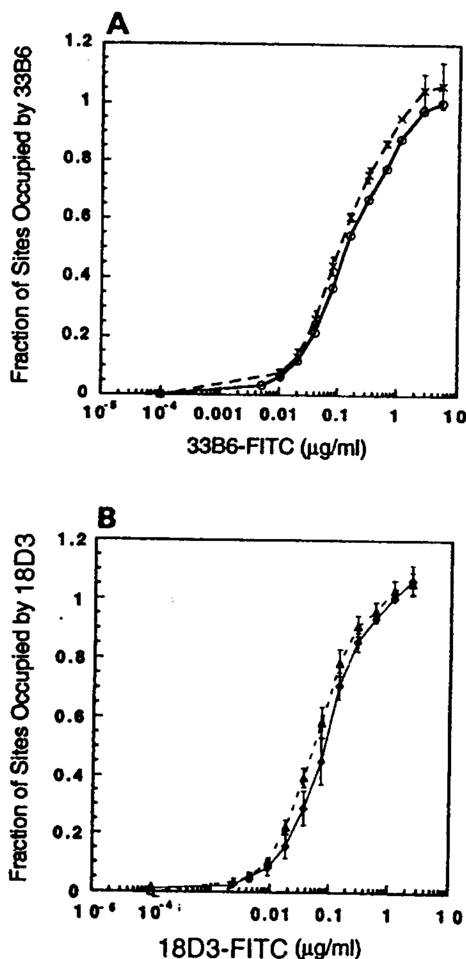

Fig. 1. Binding of 33B6 and 18D3. (A) Pre-incubation with 100% 18D3. Jurkat cells (5×10^5 cells/mL) were pre-incubated with saturation levels of 18D3 for 30 min before addition of various dosages of FITC-conjugated 33B6(○). There was no significant difference between the binding curve of 33B6 in the presence or absence (×) of 18D3. The K_d value for 33B6 was 0.11 μg/mL. Error bars show SE for 3 independent experiments. (B) Pre-incubation with 100% 33B6. All 33B6 sites were covered before addition of FITC-conjugated 18D3 (◇). There is only a slight difference in the binding curves in the presence or absence (△) of excess 33B6. The K_d value for 18D3 was 0.05 μg/mL. Error bars show SE for 5 independent experiments.

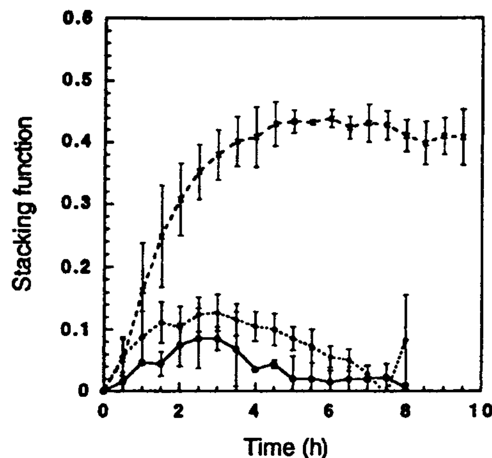

Fig. 2. Kinetics of mAb stimulated aggregation. Jurkat cells were placed in a 96-well tissue culture plate and activated with 33B6 corresponding to 30% site occupancy (×), 18D3 corresponding to 100% site occupancy (◆), and 1:500 OKT11 ascites (○). 33B6 induced vigorous homotypic aggregation while 18D3 and OKT11 solution did not form large aggregates. Error bars show SE for 3 independent experiments.

OKT11 bound to the Jurkat cell receptor but did not affect VLA-mediated aggregation. 18D3, an IgG_1 antibody to the β1.1 epitope, was both maintained in our laboratory and purchased from Endogen, Inc. (Boston, MA) [36]. Cell activation by these antibodies is Fc independent, temperature dependent, and requires divalent cation and an intact cytoskeleton [15, 18, 25]. mAb 15/7, an IgG_1 antibody to the β_1 integrin, was generously provided by Dr. Ted Yednock (Athena Neuroscience, S. San Francisco, CA) 15/7 recognizes an activation epitope on β_1 integrin and identifies the subset of $\alpha_4\beta_1$ integrin molecules that mediate cell adhesion to fibronectin and VCAM-1 [6]. Control for the flow cytometry experiments was an IgG_1-fluorescein isothiocyanate (FITC) mouse antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA). Anti-CD45 (Becton Dickinson) was used as a control for the radiolabeled binding experiments.

Flow cytometry

The effect of mAb dosage on integrin occupancy was performed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Purified antibodies, 33B6 and 18D3, were labeled with FITC by use of a commercial kit (QuickTag FITC Conjugation Kit, Boehringer-Mannheim, Indianapolis, IN). The labeled antibodies at various concentrations were added to the Jurkat cells at a concentration of 5×10^5 cells/mL. The mixture was then incubated at 37°C for 30 min before being read on the flow cytometer as previously described [37]. Twenty micrograms per milliliter IgG_1-FITC was used as control.

The number of cell-surface antibody binding sites was determined using Quantum Simply Cellular microbead standards (Flow Cytometry Standards Corp., Research Triangle Park, NC). These uniform microbeads have a calibrated number of goat-anti-mouse IgG sites on their surface. Both the calibrated microbeads and the Jurkat cells were directly labeled with 33B6 and 18D3 at saturating concentrations. The number of binding sites per cell was then determined by quantifying the fluorescence intensity of the labeled cells and translating this value into the number of bound antibodies using the microbead standards and the F/P ratio as previously described [38].

Radiolabeling with 15/7-^{125}I

Experiments were performed with 15/7 to correlate the effect of anti-β_1 mAbs (33B6 and 18D3) and Mn^{2+} on the expression of the β_1 activation epitope. The mAb 15/7 was radiolabeled with $Na^{125}I$ as previously described [39]. 5×10^6 Jurkat cells were first incubated for 20 min at 37°C in 500 μL of RPMI 1640 supplemented with 10% fetal bovine serum (FBS). During this incubation period, 5 mM Mn^{2+} was added to the appropriate vials. Following this, the mAbs 33B6 and 18D3 at a concentration corresponding to 90% site occupancy were added to the vials and the cells were incubated for an additional 30 min at 37°C. ^{125}I-labeled mAb 15/7 (5×10^6 cpm) was then added and the cells were incubated for another 30-min period. The samples were washed four times with the incubation medium and bound antibody was assayed on a Gamma counter (model A5650, Packard Instrument Company, Inc., Downers Grove, IL). Anti-CD45 mAb was used as a binding control antibody.

Homotypic aggregation

Cells of the Jurkat cell line were maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS (Sigma). During experimentation, these cells were washed and made into suspensions with a cell concentration of 5.4×10^5 cells/mL. They were then activated with prescribed concentrations of mAbs to the β_1 integrin. One hundred microliters of the activated cell suspension was placed in a flat-bottom 96-well tissue culture-treated plate (Corning Glass Works, Corning, NY). This corresponded to a cell seeding density of 1700 cells/mm^2. The suspension was allowed to settle for 30 min at room temperature at which time the cells were observed to be motile at the bottom of the well. The well was then placed in a micro-incubator under a light microscope for 10 h. The conditions in the incubator were maintained at 37°C with 5% CO_2. Data were obtained by digitizing images at 0.5 h intervals and by time-lapse video recording [34, 35].

Digital image analysis

The experimental setup consists of a custom-built micro-incubator placed on the motorized stage of an inverted light microscope (Sedival ausJena, Seiler Instruments, St. Louis, MO). The incubator was maintained at 37°C and 5% CO_2. The motion of the motorized stage was controlled by a computer (Macintosh Plus, Apple Computer Inc., Cupertino, CA) using software written in LabVIEW 2.1 (National Instruments, Austin, TX). At 0.5 h intervals, four adjacent images of the cells on the 96-well plate were digitized by an image acquisition computer (Macintosh II, Apple Computer) and stored on an optical laser disk (Model 600X, Third Wave Computing Inc.). The software used for image acquisition and processing was NIH-Image 1.51 (written by Wayne Rasband at the U. S. National Institutes of Health and available electronically via Internet by anonymous ftp from zippy.nimh.nih.gov). The data from the experiment was also recorded by a time-lapse video recorder (model 6750A, Panasonic) in the 120 h mode, i.e., 120 h of experiment was stored on 2 h of video tape. The details of the image acquisition setup have been described in previous publications [34, 35].

Homotypic aggregation assay

At the start of aggregation experiments, the cells were found to be mainly singlets along with a few small aggregates. There were no large cell clumps at this point and all the cells were in contact with the plate surface. During the experiment, the activated Jurkat cells and cell aggregates migrated on the surface of the tissue culture plate and collided with other cells to form larger aggregates. It appears that the strength of the cell-cell bonds was stronger than the cell-substrate interactions, and thus vertical stacking of cells was observed in the aggregates formed during these experiments. The aggregation kinetics were characterized by following the extent of vertical cell stacking during the course of the experiment by use of the stacking function [34, 35]. The advantages of this index over other qualitative and semi-quantitative parameters have been outlined earlier [35]. The temporal evolution of the stacking function over the course of an aggregation experiment was obtained by use of time-resolved measurements of the projected areas of cells and aggregates observed under the video microscope.

The aggregation assay used to quantify this phenomenon consists of the following steps. First, image analysis techniques were used to identify all the cells and aggregates for each digital image acquired, and to measure their projected areas. At the beginning of an aggregation experiment all the cells are in contact with the culture plate and there is

no vertical stacking. The total projected area of all the aggregates at this time, S_0, was used to estimate the total number of cells in the experiment. During the course of the experiment multilayer aggregates form and the total projected area $S(t)$ of cells and aggregates decreased with time t. $S(t)$ gives a measure of the number of cells that touch the plate at that instant. Because the number of cells is constant throughout the experiment, the difference $S_0 - S(t)$ can be used to estimate the number of cells that are stacked on top of other cells in an aggregate and therefore, not in contact with the culture plate. This procedure provides for each time point, the stacking function defined by the ratio $[S_0 - S(t)]/S_0$.

The stacking function will be equal to zero if no multilayer aggregates are formed. Low values of the function in the 0.1–0.2 range indicate the formation of aggregates with some vertical stacking, whereas higher values in the 0.4–0.6 range indicate the presence of aggregates where 40–60% of the cells are stacked on top of other cells forming large multilayered clumps.

Experimental results obtained for several lymphocyte aggregation systems showed that stacking function only increased during the first 4 h of an experiment before reaching a maximal value. The ultimate extent of cell stacking depended on the nature of treatment of the lymphocytes. The size of the aggregates, however, continuously increases with time as the motile aggregates continue to combine. Even a day after the start of an experiment we continue to observe the formation of larger aggregates. Although the aggregate size may continue to increase, the stacking function remained virtually unchanged after the first 4 h. Thus the stacking function appears to be a better estimate of aggregation kinetics than the aggregate size. The extent of aggregation observed for different protocols was evaluated by comparing the stacking function at the 8-h time point for our experiments, whereby the stacking function reached it saturation value. The stacking index is the value of the stacking function 8 h after addition of an antibody.

RESULTS

Flow cytometry confirms that 33B6 and 18D3 bind to different epitopes

In order to study the binding characteristics when both the antibodies were present, 18D3 binding sites were saturated on the Jurkat cells by addition of excess unlabeled mAb. Subsequently, 33B6-FITC was titrated at dilutions ranging from 0.005 to 5 μg/mL and the level of binding was measured (**Fig. 1A**). There was no significant difference between the binding curve of 33B6 in the presence or absence of 18D3. This indicates that 18D3 binding to its site on the β_1 integrin does not prevent the binding of 33B6. The K_d (dissociation constant) value of 33B6 was calculated to be 0.11μg/mL.

We next assessed the ability of 33B6 to block 18D3 binding by pre-incubating the Jurkat cells with saturation amounts of unlabeled 33B6 for 30 min before the addition of 18D3-FITC (Fig. 1B). As seen, 33B6 caused a slight decrease in 18D3 binding. The blocking effect was maximal at an 18D3 concentration of 0.1 μg/mL, where 33B6 caused a 12% decrease in the amount of 18D3 bound. Although we observed some mAb blocking, 18D3 and 33B6 appear to bind to different sites on the β_1 integrin. MAb 18D3 has a K_d of 0.05 μg/mL.

Using the calibrated microbead standards we found that the average Jurkat cell expressed ~50,000 binding sites for either 33B6 or 18D3 (data not shown). For the purposes of correlating receptor occupancy and adhesion function we will quantify mAb binding in terms of the fraction of β_1 integrin sites that are occupied by the antibody.

33B6 induces homotypic aggregation of Jurkat cells

The ability of the two mAbs to mediate adhesion function upon binding was studied over a 10-h time course. **Figure 2** demonstrates the kinetics of aggregation when Jurkat cells were incubated with antibodies to the β_1 integrin at concentrations corresponding to 30% occupancy of 33B6 sites or 100% 18D3. Control cells were treated with 1:500 OKT11 ascites solution. When 33B6 was added to the

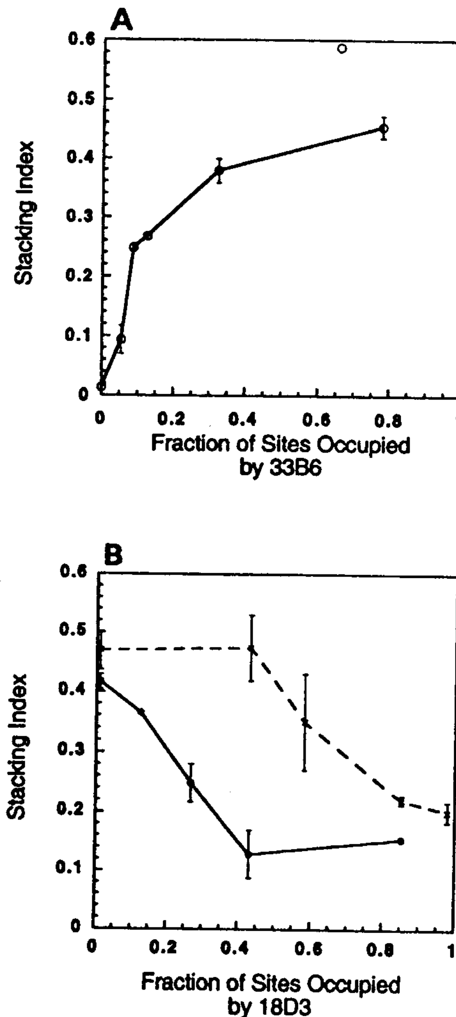

Fig. 3. Effect of β_1 integrin occupancy on the extent of aggregation. (A) Dosage studies with 33B6. Jurkat cells were incubated with 33B6 at concentrations varying from 0 to 80% site occupancy. At low 33B6 mAb concentrations, stacking index (stacking function at 8 h time point) increased rapidly with antibody concentration. However, this curve leveled off above a 33B6 concentration corresponding to 30% site occupancy. Error bars show SE for 3 independent experiments. (B) Simultaneous incubation with 33B6 and 18D3. Jurkat cells were activated with varying concentrations of 18D3 (corresponding to coverage of 0–100% of the 18D3 sites) and a fixed concentration of 33B6 [corresponding to either 30% (O) or 80% (x) 33B6 site occupancy]. The stacking index was monitored. Error bars show SE for 2–4 independent experiments.

Jurkat cells, the stacking function increased sharply during the first 4 h, leveling off at a value of 0.4 after 5 h, at which time large three-dimensional clumps were formed. Jurkat cells treated with the 18D3 antibody exhibited a small amount of vertical stacking in the first 4 h. However, aggregation was transient with 18D3 and the clumps eventually collapsed to yield flat (planar) aggregates after ~6 h. Similar trends in the stacking function were observed when the control mAb was added to the culture media. Results indicate that the final morphology and extent of stacking of the aggregates was specific to 33B6 activation.

Aggregation increases with the fraction of integrin sites occupied by 33B6

To find the relationship between β_1 receptor occupancy and aggregation kinetics, we measured Jurkat cell aggregation rates using video microscopy and image analysis at concentrations of 33B6 mAb ranging from 0 to 80% β_1 integrin site occupancy. **Figure 3A** shows the onset of aggregation with as little as 5% occupancy of the β_1 integrin sites. At low concentrations of 33B6 (corresponding to 20% 33B6 site occupancy or less), the stacking index increased rapidly with the fraction of integrin sites occupied by 33B6. The stacking index leveled off after 33B6 concentrations corresponding to 33B6 site occupancy >30%.

Time-lapse video recordings confirmed that the efficiency of binding leveled off at 30% antibody occupancy, whereby nearly all collisions between the cells and cell aggregates resulted in adhesion. We have also observed under time-lapse recording that, when the concentration of 33B6 was below 30% site occupancy, larger aggregates were weakly adherent and dissociated into singlets and smaller aggregates. At lower antibody concentrations the

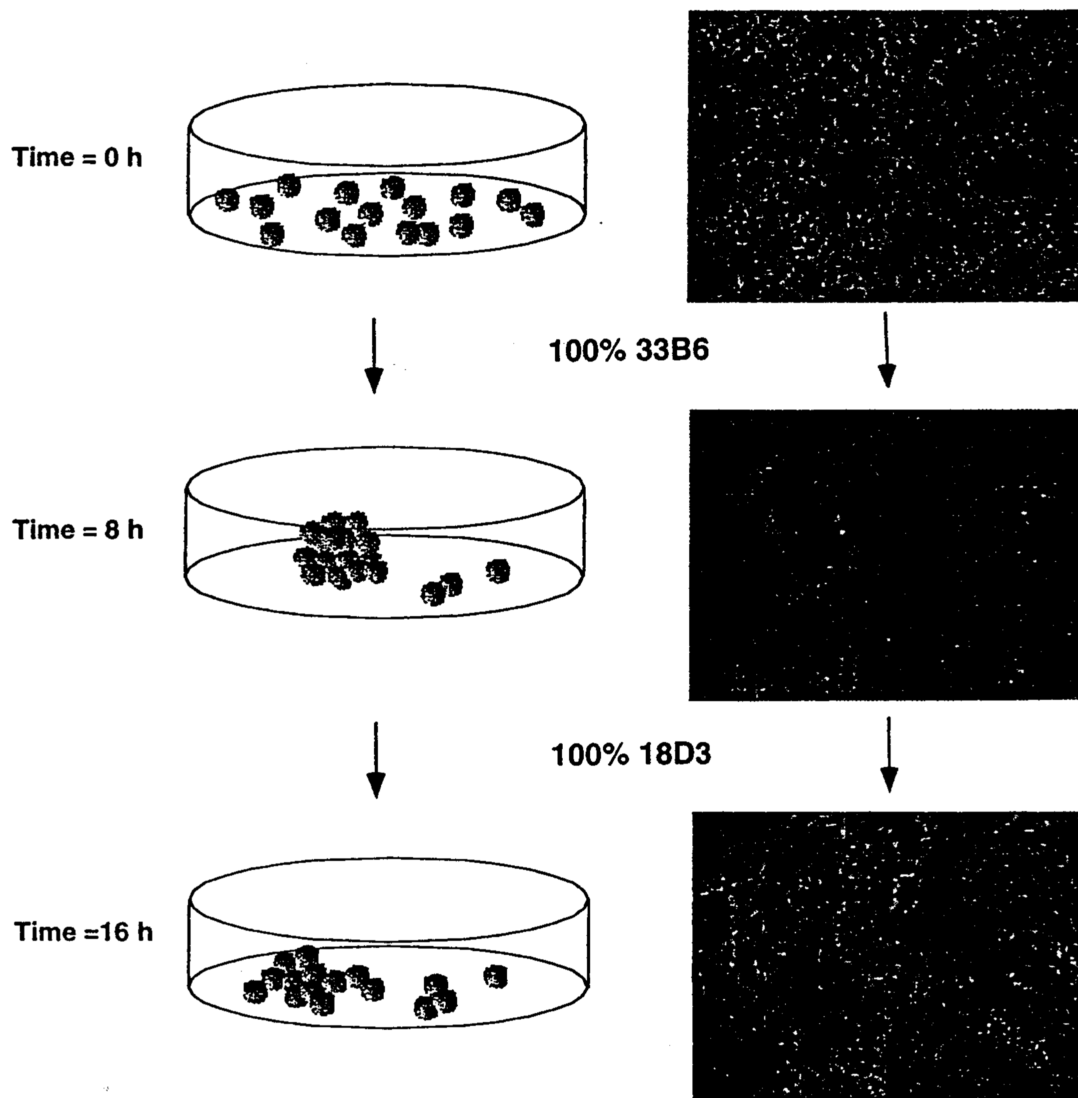

Fig. 4. Schematic of the break-up of homotypic aggregates on delayed addition of 18D3. At t = 0 h, the cells were predominantly singlets. On addition of 33B6 the cells aggregated homotypically and formed large vertical aggregates. At t = 8 h on addition of 18D3, the avidity between the Jurkat cells decreased and the multilayer stacks collapsed. However, the aggregates did not breakup into small aggregates and singlets because there were some weak bonds that held the cells together.

aggregates remained smaller, suggesting a direct correlation between the bond strength, the fraction of sites occupied, and the stability of aggregates formed.

Addition of 18D3 modulates the extent of 33B6-induced aggregation

We next explored the effect of simultaneous addition of 18D3 and 33B6 in modulating the adhesion kinetics of Jurkat cells. In these experiments cells were activated with a low and high concentration of 33B6 corresponding to occupancy of ~30% and ~80% of the 33B6 binding sites. 18D3 was titrated to this suspension so that the percentage of sites occupied by 18D3 varied between 0 and 100%. On addition of increasing amounts of 18D3 to a fixed concentration of 33B6, a threshold concentration of 18D3 was observed above which cell aggregation was inhibited (Fig. 3B). On increasing the concentration of 33B6 from 30 to 80% site occupancy, more 18D3 was required to inhibit aggregation. At a level of 30% 33B6 site occupancy, even low concentrations of 18D3 caused a drastic reduction in stacking index. When ~80% of 33B6 sites were occupied, however, inhibition of aggregation required that at least 60% of the 18D3 sites be occupied. These data seem to indicate that the inhibitory effects of 18D3 could reverse the stimulation with 33B6. In order to confirm this, we simultaneously added supersaturating concentrations of both 33B6 and 18D3 to Jurkat cells and studied the aggregation rates. The kinetics of aggregation in this experiment very closely resembled those found when saturating amounts of 18D3 were added in the absence of 33B6 (data not shown). Thus, the inhibitory effect of 18D3 addition superseded the aggregation inducing effect of 33B6.

Figure 3B demonstrates the competitive effect of mAbs 33B6 and 18D3 on adhesive function. Because these two antibodies bind to different epitopes of the β_1 integrin (Fig. 1) and do not significantly block respective binding, they seem to either generate signals to the cell or mediate changes in the β_1 integrin activation state at the cell membrane, which are competitive in nature.

Reversible kinetics of homotypic aggregation induced by 33B6 and 18D3

The competitive effects of binding 18D3 and 33B6 were explored to determine whether the adhesion induced in lymphocytes was a dynamic process. **Figure 4** illustrates the growth and collapse of multilayered Jurkat cell aggregates that were incubated with 33B6 for 8 h before addition of 18D3. Initially, cells were distributed mostly as singlets on the tissue culture surface. On addition of 33B6, the cells formed large multilayer aggregates by the 8-h time point. Digital images taken from the light microscope as shown in Figure 4 do not provide information on the vertical stacking of the cell clumps. A schematic that illustrates the aggregate morphology (Fig. 4) was produced by measuring the height of the aggregates. We analyzed the three-dimensional structure of the aggregates by use of confocal microscopy (data not shown). Addition of 33B6 led to the formation of aggregates having two to three layers of stacked cells. Following addition of 18D3 at the 8 h time point, we observed a collapse of the multilayer aggregate structure as indicated by the rapid increase in the total projected area of the cells and aggregates on the well surface. There was, however, only a small decrease in the size of the planar aggregates on the substrate. Large aggregates did not dissociate into smaller aggregates and singlets even 8 h after exposure to 18D3 for 8 h (Fig. 4). Apparently, bond strength was sufficient to hold these aggregates together on the substrate surface, but insufficient to maintain cell stacking.

The kinetics of the process described above are shown in **Figure 5A**. Stable aggregates formed in the presence of

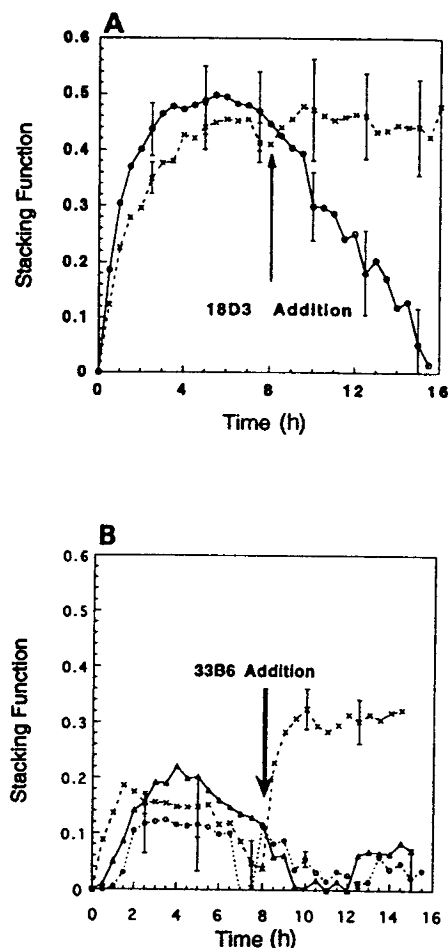

Fig. 5. Dynamics of homotypic aggregation mediated by 33B6 and 18D3. (A) Effect of delayed addition of 18D3 on aggregation rates. Jurkat cells were incubated with 33B6 (at 100% site occupancy) for 8 h before addition of saturation amounts of 18D3 (○). Aggregates formed in the first 8 h can be broken up by subsequent addition of 18D3. The control experiment (×) consists of Jurkat cells incubated with 100% 33B6 for the entire 16-h duration of the experiment. In this case there was no break-up of aggregates. Error bars show SE for 3 independent experiments. (B) Effect of delayed addition of 33B6 on aggregation rates. Jurkat cells incubated with 18D3 at 60% site occupancy for 8 h could be induced to aggregate on subsequent addition of saturation amounts of 33B6 (×). Preincubation with 100% 18D3 for 8 h before addition of 100% 33B6 did not induce aggregation (△). Control sample was incubated with 60% coverage of 18D3 (○). Error bars show SE for 3 independent experiments.

33B6 (100% site occupancy) in the first 8 h collapsed upon subsequent addition of saturation amounts of 18D3 as indicated by the rapid reduction in the stacking function. In contrast, Jurkat cells, which were incubated with a saturation concentration of 33B6, did not demonstrate disaggregation up to 16 h.

Inhibition of aggregation by 18D3 can be reversed by saturation levels of 33B6

In order to determine whether the inhibition induced by 18D3 can be reversed by binding of 33B6, we performed the inverse of the previous break-up experiment. Jurkat cells were first incubated with 18D3 at 60% site occupancy for 8 h before addition of saturation amounts of 33B6 (Fig. 5B). 33B6 addition was able to trigger the formation of large homotypic aggregates at this level of 18D3 inhibition. However, 33B6 added to Jurkat cells that were preincubated with saturation concentrations of 18D3 did not stimulate aggregate formation. In the control experiment we confirmed that Jurkat cells incubated with 18D3 (60% site occupancy) over 16 h did not aggregate beyond the background level. This experiment demonstrated that the cells became refractory to 33B6 stimulus only when 18D3 was bound at saturation. The inhibition of aggregation by 18D3 was a function of the 18D3 dose applied.

Binding studies with mAb 15/7 to an activation epitope on the β_1 integrin

To explore the mechanism by which 33B6 and 18D3 induce changes in the activation state of the β_1 integrin, we tested their ability to affect binding of 15/7, an antibody that recognizes and stabilizes an active conformation of the VLA protein [6]. Radiolabeling and flow cytometry were used to quantify antibody binding as described in Materials and Methods. 15/7 was found to bind constitutively to Jurkat cells both in the presence of the control binding anti-CD45 antibody (1μg/mL) and in the absence of any antibody (**Fig. 6**). Preincubation with 5 mM Mn^{2+} caused a 2.5-fold increase in 15/7 binding. This is consistent with previous observations that 15/7 recognized an activation epitope induced by Mn^{2+} addition [6]. On preincubating with 33B6 corresponding to 90% occupancy, there was a 50% decrease in 15/7 binding compared with the CD-45 control (Fig. 6). This decrease in 15/7 binding was dose-dependent and an increase in the concentration of 33B6 decreased 15/7 binding proportionally (data not shown). Under conditions of epitope up-regulation with Mn^{2+}, 33B6 also blocked 15/7 binding by 30%. Incubation with 18D3 corresponding to 90% occupancy did not alter the binding of 15/7. In the presence of 5 mM Mn^{2+}, 18D3 appeared to stabilize the binding of 15/7, causing a slight increase (≈15%) in binding. Simultaneous addition of Jurkat cells with 33B6 and 18D3 did not augment the decrease in 15/7 binding found with 33B6 alone. This data is consistent with a mechanism where 33B6 decreased

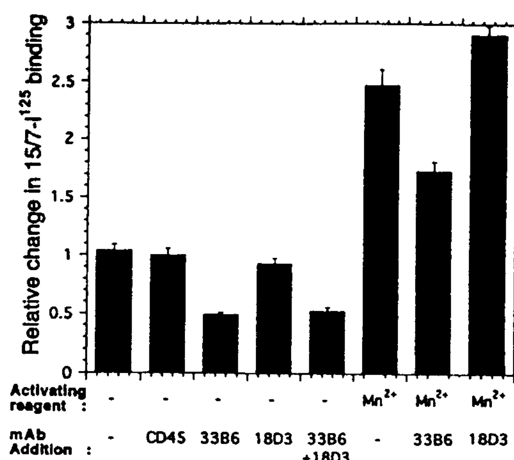

Fig. 6. 15/7 Binding in the presence of 33B6, 18D3, and Mn^{2+}. Jurkat cells (5×10^6) in a 500-μL volume were pretreated for 20 min at 37°C in presence or absence of 5 mM Mn^{2+} as shown. Samples were then incubated with mAbs, 33B6, or 18D3, at concentrations corresponding to 90% occupancy for a 30-min interval at 37°C. Subsequently 5×10^6 cpm 15/7-^{125}I was added and its binding was quantified in a gamma counter after incubation for 30 min at 37°C. Data was normalized with respect to the count in the presence of 1 μg/mL CD45 control and plotted in terms of relative change in 15/7-^{125}I binding. Error bars show SE for 3 independent experiments performed in duplicate.

15/7 binding under constitutive and Mn^{2+} activating conditions, whereas 18D3 did not significantly affect it.

We next used flow cytometric analysis to explore the effect of 15/7 and Mn^{2+} on the binding of FITC-labeled 33B6 and 18D3 (**Fig. 7**). Although Mn^{2+} treatment induced more than a two-fold increase in 15/7 binding, it did not potentiate the number of sites recognized by either 33B6 or 18D3. Pretreatment with 15/7 alone caused only a slight decrease in 33B6 binding. However, in the presence of both 5 mM Mn^{2+} and 15/7, 33B6 binding was decreased by 40%. Apparently Mn^{2+} addition augmented the binding of 15/7, which in turn blocked a portion of 33B6 binding. Under the same conditions, Mn^{2+} and 15/7 did not cause changes in 18D3 binding. In summary, 33B6 and 15/7 appeared to partially block each other under conditions where 15/7 binding was induced with Mn^{2+}. 18D3 did not appear to affect 15/7 binding.

Because 15/7 only affected 33B6 binding in the presence of an activation state induced by Mn^{2+}, homotypic aggregation studies were performed as described in Materials and Methods to determine the ability of Mn^{2+} or 15/7 to effect aggregation function of Jurkat cells. Neither 5 mM Mn^{2+} nor Mn^{2+} in the presence of 15/7 (5 μg/mL) induced aggregation of Jurkat cells beyond control levels observed with OKT11 (data not shown). Upon addition of 33B6 to cells pretreated with Mn^{2+} and 15/7, aggregation of Jurkat cells was observed. However, the aggregate size and stacking was less than that demonstrated by 33B6 activation at saturation. Apparently, the decrease in the fraction of 33B6 sites bound in the presence of 15/7 and Mn^{2+} resulted in a consequential decrease in the extent of aggregation.

DISCUSSION

In this study, we used a novel image analysis assay [34, 35] and flow cytometric detection of integrin surface expression to explore the mechanism of homotypic lymphocyte aggregation induced by anti-β_1 antibodies. The method proved to be sensitive to subtle changes in cell adhesivity over time in a reproducible fashion. The stacking index provided a measure for quantifying homotypic aggregation and the relative strength of cell attachment. The standard error in estimating the stacking function was < 15% at all time points (Figs. 2, 3, and 5).

The adhesion-inducing signal of 33B6 was proportional to the number of β_1 integrin sites occupied by 33B6. The adhesion inhibiting effect of 18D3 was also proportional to the fraction of β_1 integrin sites bound by this antibody. We have also demonstrated that the mAb 15/7 binding was not induced by, nor did it recognize, the activation epitope that supports homotypic aggregation. Based on these results, we speculate that homotypic aggregation is a receptor-mediated adhesion event regulated at the cell surface by a novel activation state of the β_1 integrin.

The homotypic aggregation of Jurkat lymphocytic cells is not induced by the cross-linking of the receptor and ligand by the IgG antibody. Previous studies with other activation-inducing antibodies present evidence that this aggregation is Fc independent [25]. Moreover, Jurkat cells do not express Fc receptors on their cell surface. Although there is increasing evidence that the VLA receptor itself participates as the receptor in intercellular adhesion [9, 18], the counter-ligand has yet to be firmly established [18, 29, 30]. The mechanisms by which 33B6 and 18D3 mediate their effects on homotypic aggregation may be due to: (1) changes in the external conformation or topography of the receptors; (2) transmembrane signal transduction and mobilization of secondary messengers; or (3) some combination of both mechanisms.

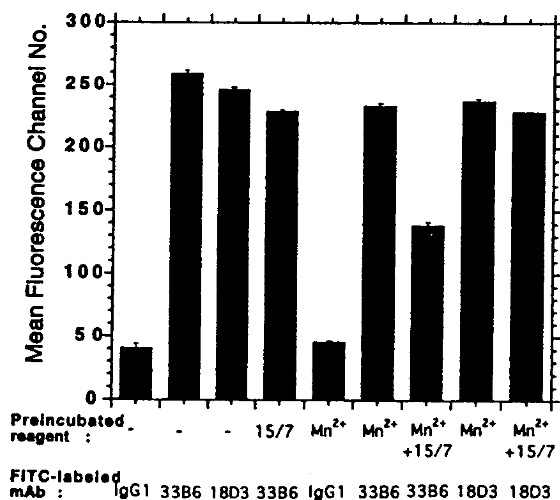

Fig. 7. Binding of mAbs 33B6 and 18D3 in the presence of Mn^{2+} and 15/7 Jurkat cells at 5×10^5 cells/mL were pretreated with 5 mM Mn^{2+} and 5 µg/mL 15/7 for 30 min at 37°C. Following this, FITC-labeled 33B6 and 18D3 were added at concentrations corresponding to 90% site occupancy for 30 min at 37°C and binding was quantified by flow cytometry. Error bars show SE for 3 independent experiments.

Homotypic aggregation stimulated through mAb binding to β_1 integrin

Dosage studies showed that a significant fraction of the 33B6 sites (30%) were required to be occupied to induce the maximum extent of aggregation (Fig. 3A). In this non-agitated static assay, the rate of aggregation is not only governed by the frequency of cell collisions but also by the efficiency and the contact time of the cell-cell collisions. On activation with 33B6, the motility characteristics of the cells changed. Activation by 33B6 decreased the frequency of collision of Jurkat cells in our static aggregation assay [unpublished observations]. However, the extent of aggregation observed was higher with 33B6 addition, suggesting that 33B6 resulted in an increase in bond avidity and adhesive efficiency. These experiments also indicate that there is a strong correlation between the fraction of integrins bound by 33B6 and the strength of intercellular adhesion. The increased adhesivity caused the cells to overcome the gravitational force and form multilayered aggregates. Stacking index is thus an indirect measure of cell-cell bond strength.

Addition of 18D3 to cells preactivated with 33B6 showed that homotypic aggregation is a reversible and dynamic process (Figs. 4 and 5A). Here, the multilayer aggregates formed by addition of saturating amounts of 33B6 collapsed onto the plate surface upon subsequent addition of 18D3. We have confirmed by cell motility experiments that addition of 18D3 did not change the motility characteristics of the cell [unpublished observations]. Hence, the decrease in the stacking function was not likely due to the disruption and loss of integrity of the cytoskeleton upon addition of 18D3. The collapse of vertical cell stacking and the loss of adhesiveness between Jurkat cells appears to be due to a decrease in the number or strength of bonds mediating adhesion. A second possibility is that a change in receptor topography occurred as a result of 18D3 binding. This may act to decrease the availability of contacts through the capping or patching of receptors, thus decreasing their surface density. We are currently exploring these possibilities.

Aggregation and receptor binding experiments performed by simultaneous addition of 33B6 and 18D3 show that, although the two antibodies bind different epitopes on the integrin, they affect adhesive avidity along a similar pathway (Figs. 1 and 3B). A large fraction of the 18D3 sites were required to be occupied to inhibit 33B6-induced aggregation (Fig. 3B). The stoichiometry was such that the aggregation induced by the occupation of ~80% of the 33B6 sites required >60% occupancy of the 18D3 sites for inhibition. Assuming that these mAbs bind simultaneously to the same β_1 integrins, it may be estimated that only 33% of the integrins are solely occupied by 33B6 under these conditions [i.e., $0.8 \times (1-0.6)$]. The rest of the integrins would then be bound by 18D3 alone, or simulta-

neously by 18D3 and 33B6. The aggregation kinetics under these conditions were comparable to the single antibody experiments where 30% of the 33B6 sites were occupied (Fig. 3A). Together, these results suggest that the strength of the adhesion-inducing signal is proportional to the number of β_1 integrins bound by 33B6 alone. Because 18D3 blocks the aggregation-inducing effect of 33B6, a simplistic model is that any integrin which has both the antibodies attached to it becomes refractory to stimulation. Similarly, integrins with no antibody bound or with only 18D3 do not promote cell adhesion along this pathway. If F(33B6) and F(18D3) are the fraction of sites bound by the 33B6 and 18D3, respectively, then the extent of homotypic aggregation process should be proportional to $F(33B6)*[1-F(18D3)]$.

The β_1 integrin has been shown to play an important role for several disease models, including encephalomyelitis and vascular inflammation [23, 40]. A quantitative understanding of the relationship between the number of activated receptors and the subsequent adhesive function may be a useful tool for rational development of novel therapeutic strategies to control the abnormal adhesion of lymphocytes. In these cases, the simple analytical expression above may be a very useful tool.

A novel activation state associated with homotypic aggregation

MAb 15/7 specifically recognizes an activation epitope on the human β_1 integrin and identifies a subset of the VLA-4 molecule, which mediates cell adhesion to VCAM-1 and fibronectin [6]. 33B6 is known to induce homotypic aggregation while simultaneously blocking cell adhesion to fibronectin and VCAM-1 [36]. In this study, we provide evidence that pretreatment of Jurkat cells by mAb 33B6 or 15/7 partially inhibits binding of the other (Figs. 6 and 7). The mechanism may involve steric hindrance, in which occupancy of 33B6 and 15/7 mutually inhibit the binding of the other by recognizing neighboring or overlapping binding sites on the β_1 integrin. While Mn^{2+} up-regulates the expression of 15/7 binding sites, it does not affect 33B6 binding. It has been shown that the region of the β_1 integrin critical for the recognition of functional antibodies lies between two putative ligand binding sites [41]. Because these ligand binding epitopes lie in close proximity on the β_1 integrin, it is possible that 33B6 occupancy sterically inhibits the binding site for 15/7 and also reduces cell adhesion to VCAM-1 and fibronectin in a similar fashion.

Another possibility for the binding inhibition between these antibodies is that a conformational change is induced by 33B6, which occludes the expression of the 15/7 binding site. Similarly, the enhanced binding of 15/7 in response to Mn^{2+} may alter the conformation of the β_1 integrin and hence down-regulate 33B6 binding sites. Irrespective of whether the mechanism is due to steric hindrance between the antibodies or due to a conformational change that mutually occludes recognition, their effect on

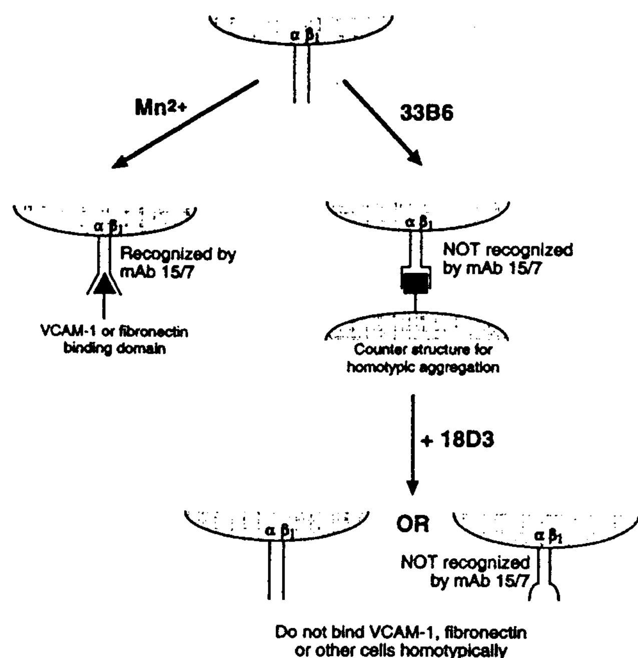

Fig. 8. Activation states of the β_1 integrin. Addition of Mn^{2+} induces cell binding to VCAM-1 and up-regulates mAb 15/7 binding sites without inducing homotypic aggregation. Addition of mAb 33B6 induces an activation state that causes homotypic lymphocyte aggregation without inducing cell binding to VCAM-1 and without up-regulating 15/7 binding. Addition of 18D3 to cells activated with 33B6 does not increase the expression of the 15/7 binding epitope. It prevents both the homotypic aggregation of lymphocytes and cell binding to VCAM-1.

the adhesion function is identical. Aggregation rates remained solely dependent on the stoichiometry of sites bound by 33B6.

Our results indicate that neither 33B6 nor 18D3 increase expression of the binding site recognized by 15/7. Homotypic aggregation of Jurkat cells was not induced by Mn^{2+} nor by 15/7. Hence, we propose that 33B6 mediates homotypic aggregation by inducing a novel activation state of the β_1 integrin, distinct from that induced by Mn^{2+} and recognized by 15/7. A model illustrating the main conclusions of this study is presented in **Figure 8**. It is based on the assumption that the β_1 integrin itself acts as the receptor that recognizes the counter-ligand during lymphocyte adhesion. This assumption is supported by recent experimental evidence [18, 28, 30]. Addition of 33B6 induces an active state of the integrin that is distinct from the constitutively expressed unactivated β_1 integrin and the active state, which is recognized by 15/7 and which enables cell adhesion to VCAM-1 and fibronectin (Fig. 8). 18D3 binding to lymphocytes activated by 33B6 either induces a third activation state that does not recognize any of the above-mentioned ligands or it returns the integrin to its resting state. Under these conditions the integrin does not recognize the counter-structure for homotypic aggregation nor does it bind VCAM-1 or increase recognition by mAb 15/7.

Our current experimental results, demonstrating the role of antibody binding stoichiometry and reversibility in modulating adhesion events, support a model in which the

binding of 33B6 regulates adhesion through changes at the cell surface possibly by inducing conformational changes to the receptor. 18D3 binding was able to override this change in conformation by down-regulating the expression of the ligand binding domain in a dynamic fashion. 18D3 may down-regulate adhesion by sterically hindering the binding site on the VLA integrin, which mediates homotypic aggregation, or it may induce a conformational change in the β_1 integrin, thereby hindering the expression of the ligand binding epitope induced by 33B6. These results and those of others indicate that β_1 integrins can adopt a range of affinity states that can be modulated at the cell surface [5]. Supporting this concept, another antibody TS2/16 to the β_1 integrin has also been shown to induce a conformational change during the adhesion of myelomonocytic cells (U-937) to RGD-containing fragments of fibronectin [28].

The experimental evidence provided in this study does not rule out the role of signaling in modulating cell adhesion kinetics. We have previously demonstrated that binding of the two antibodies 18D3 and 33B6 inhibits pseudopodial extension of lymphoid cells (HPB-ALL) on a fibronectin substrate by engaging a signaling cascade [31]. Recent reports also indicate that antibody binding to the VLA proteins causes increased tyrosine phosphorylation [7], actin reorganization [9], and cell spreading [31]. These studies indicate that the VLA receptor may trigger signal transduction pathways that directly affect cell behavior. The adhesion measured in the current study, however, appears to be mediated primarily by events at the receptor level, possibly involving a novel conformation of the β_1 receptor.

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REFERENCES

1. Albelda, S. M., Buck, C. A. (1990) Integrins and other cell adhesion molecules. *FASEB J.* **4**, 2868–2880.
2. Shimizu, Y., Van Seventer, G. A., Horgan, K. J., Shaw, S. (1990) Roles of adhesion molecules in T-cell recognition: fundamental similarities between four integrins on resting human T cells (LFA-1, VLA-4, VLA-5, VLA-6) in expression, binding, and costimulation. *Immunol. Rev.* **114**, 109–143.
3. Springer, T. A. (1990) Adhesion receptors of the immune system. *Nature* **346**, 425–433.
4. Hynes, R. O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11–25.
5. Neugebauer, K. M., Reichardt, L. F. (1991) Cell-surface regulation of β_1-integrin activity on developing retinal neurons. *Nature* **350**, 68–71.
6. Yednock, T. A., Cannon, C., Vandevert, C., Goldbach, E. G., Shaw, G., Ellis, D. K., Liaw, C., Fritz, L. C., Tanner, L. I. $\alpha_4\beta_1$ integrin-dependent cell adhesion is regulated by a low affinity pool that is conformationally responsive to ligand. *J. Biol. Chem.* **270**, 28740–28750.
7. Kornberg, L. J., Earp, H. S., Turner, C. E., Prockop, C., Juliano, R. L. (1991) Signal transduction by integrins: increased protein tyrosine phosporylation caused by clustering of β_1 integrins. *Proc. Natl. Acad. Sci. USA* **88**, 8392–8396.
8. Teague, T. K., McIntyre, B. W. (1994) MAb 18D3 triggering of integrin β_1 will prevent but not terminate proliferation of human T cells. *Cell Adhesion Commun.* **2**, 169–184.
9. Sánchez-Mateos, P., Campanero, M. R., Balboa, M. A., Sánchez-Madrid, F. (1993) Co-clustering of β_1 integrins, cytoskeletal proteins, and tyrosine-phosphorylated substrates during integrin-mediated leukocyte aggregation. *J. Immunol.* **151**, 3817–3828.
10. Shimizu, Y., Van Seventer, G. A., Horgan, K. J., Shaw, S. (1990) Regulated expression and binding of three VLA (β_1 integrin receptors on T cells. *Nature* **345**, 250–253.
11. Hemler, M. E. (1990) VLA proteins in the integrin family: structures, functions and their role on leukocytes. *Annu. Rev. Immunol.* **8**, 365–400.
12. Wayner, E. A., Garcia-Pardo, A., Humphries, M. J., McDonald, J. A., Carter, W. G. (1989) Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J. Cell Biol.* **109**, 1321–1330.
13. Darribère, D., Guida, K., Larjava, H., Johnson, K. E., Yamada, K. M., Thiery, J., Boucaut, J. (1990) In vivo analyses of integrin β_1 subunit function in fibronectin matrix assembly. *J. Cell Biol.* **110**, 1813–1823.
14. Arroyo, A. G., Sánchez-Mateos, P., Campanero, M. R., Martín-Padura, I., Dejana, E., Sánchez-Madrid, F. (1992) Regulation of the VLA integrin-ligand interactions through the β_1 subunit. *J. Cell Biol.* **117**, 659–670.
15. van de Wiel-van Kemenade, E., van Kooyk, Y., de Boer, A. J., Huijbens, R. J. F., Weder, P., van de Kasteele, W., Melief, C. J. M., Figdor, C. G. (1992) Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the β subunit of VLA. *J. Cell Biol.* **117**, 461–470.
16. Elices, M. J., Osborn, L., Takada, Y., Crouse, C., Luhowskyj, S., Hemler, M. E., Lobb, R. R. (1990) VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell* **60**, 577–584.
17. Kovach, N. L., Carlos, T. M., Yee, E., Harlan, J. M. (1992) A monoclonal antibody to β_1 integrin (CD29) stimulates VLA-dependent adherence of leukocytes to human umbilical vein endothelial cells and matrix components. *J. Cell Biol.* **116**, 499–509.
18. Bednarczyk, J. L., McIntyre, B. W. (1990) A monoclonal antibody to VLA-4 α-chain (CDw49d) induces homotypic lymphocyte aggregation. *J. Immunol.* **144**, 777–784.
19. Campanero, M. R., Pulido, R., Ursa, M. A., Rodríguez-Moya, M., de Landázuri, M. O., Sánchez-Madrid, F. (1990) An alternative leukocyte homotypic adhesion mechanism, LFA-1/ICAM-1-independent, triggered through the human VLA-4 integrin. *J. Cell Biol.* **110**, 2157–2165.
20. Picker, L. J., Butcher, E. C. (1992) Physiological and molecular mechanisms of lymphocyte homing. *Annu. Rev. Immunol.* **10**, 561–591.
21. Cybulsky, M. I., Gimbrone, M. A., Jr. (1991) Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* **251**, 788–791.
22. Masumoto, A., Hemler, M. E. (1993) Multiple activation states of VLA-4. *J. Biol. Chem.* **268**, 228–234.
23. Yednock, T. A., Cannon, C., Fritz, L. C., Sanchez-Madrid, F., Steinmann, L., Karin, N. (1992) Prevention of experimental autoimmune encephalomyelitis by antibodies against $\alpha_4\beta_1$ integrin. *Nature* **356**, 63–66.
24. Clayberger, C., Krensky, A. M., McIntyre, B. W., Koller, T. D., Parham, P., Brodsky, F., Linn, D. J., Evans, E. L. (1987) Identification and characterization of two novel lymphocyte function-associated antigens, L24 and L25. *J. Immunol.* **138**, 1510–1514.
25. Campanero, M. R., Arroyo, A. G., Pulido, R., Ursa, A., de Matías, M. S., Sánchez-Mateos, P., Kassner, P. D., Chan, B. M. C., Hemler, M. E., Corbí, A. L., de Landázuri, M. O.,Sánchez-Madrid, F. (1992) Functional role of α_2/β_1 and α_4/β_1 integrins in leukocyte intercellular adhesion induced through the common β_1 subunit. *Eur. J. Immunol.* **22**, 3111–3119.
26. Weitzman, J. B., Pasqualini, R., Takada, Y., Hemler, M. E. (1993) The function and distinctive regulation of the integrin VLA-3 in cell adhesion, spreading, and homotypic cell aggregation. *J. Biol. Chem.* **268**, 8651–8657.
27. Wuthrich, R. P. (1992) Monoclonal antibodies to the murine VLA-6 α-chain trigger homotypic lymphocyte aggregation. *Immunol.* **77**, 214–218.
28. Arroyo, A. G., García-Pardo, A., Sánchez-Madrid, F. (1993) A high affinity conformational state on VLA integrin heterodimers induced by an anti-β_1 chain monoclonal antibody. *J. Biol. Chem.* **268**, 9863–9868.
29. Caixia, S., Stewart, S., Wayner, E., Carter, W., Wilkins, J. (1991) Antibodies to different members of the β_1 CD29 integrins induce homotypic and heterotypic cellular aggregation. *Cell. Immunol.* **138**, 216–228.
30. Altevogt, P., Hubbe, M., Ruppert, M., Lohr, J., von Hoegen, P., Sammar, M., Andrew, D. P., McEvoy, L., Humphries, M. J., Butcher, E. C. (1995) The α_4 integrin chain is a ligand for $\alpha_4\beta_7$ and $\alpha_4\beta_1$. *J. Exp. Med.* **182**, 345–355.
31. Szabo, M. C., Teague, T. K., McIntyre, B. W. (1995) Regulation of lymphocyte pseudopodia formation by triggering the integrin $\alpha_4\beta_1$. *J. Immunol.* **154**, 2112–2124.
32. Sánchez-Mateos, P., Arroyo, A. G., Balboa, M. A., Sánchez-Madrid, F. (1993) Post-receptor occupancy events in leukocytes during β_1 integrin-ligand interactions. *Eur. J. Immunol.* **23**, 2642–2648.

33. Bazzoni, G., Shih, D., Buck, C. A., Hemler, M. E. (1995) Monoclonal antibody 9EG7 defines a novel β_1 integrin epitope induced by soluble ligand and manganese, but inhibited by calcium. *J. Biol. Chem.* **270**, 25570–25577.
34. Munn, L. L., Clacken, M. W., McIntyre, B. W., Zygourakis, K. (1993) Analysis of lymphocyte aggregation using digital image analysis. *J. Immunol. Meth.* **166**, 11–25.
35. Nellamegham, S. Zygourakis, K. A quantitative assay for intercellular aggregation. *Ann. Biomed. Eng.* In press.
36. Bednarczyk, J. L., Wygant, J. N., Szabo, M. C., Molinari-Storey, L., Renz, M., Fong, S.,McIntyre, B. W. (1993) Homotypic leukocyte aggregation triggered by a monoclonal antibody specific for a novel epitope expressed by the integrin β_1 subunit: conversion of nonresponsive cells by transfecting human integrin α_4 subunit cDNA. *J. Cell. Biochem.* **51**, 465–478.
37. Simon, S. I., Chambers, J. D., Butcher, E., Sklar, L. A. (1992) Neutrophil aggregation is β_2-integrin and L-selectin-dependent in blood and isolated cells. *J. Immunol.* **149**, 2765–2771.
38. Simon, S. I., Rochon, Y. P., Lynam, E. B., Smith, C. W., Anderson, D. C., Sklar, L. A. (1993) Beta-2 integrin and L-selectin are obligatory receptors in neutrophil aggregation. *Blood* **82**, 1097–1106.
39. McIntyre, B. W., Evans, E. L., Bednarczyk, J. L. (1989) Lymphocyte surface antigen L25 is a member of the integrin receptor superfamily. *J. Biol. Chem.* **264**, 13745–13750.
40. Bochner, B. S., Luscinskas, F. W., Gimbrone, M. A., Newman, W., Sterbinsky, S. A., Derse-Anthony, C. P., Klunk, D., Schleimer, R. P. (1991) Adhesion of human basophils, eosinophils, and neutrophils to interleukin 1-activated human vascular endothelial cells: contributions of endothelial cell adhesion molecules. *J. Exp. Med.* **173**, 1553–1556.
41. Takada, Y., Puzon, W. (1993) Identification of a regulatory region of integrin β_1 subunit using activating and inhibiting antibodies. *J. Biol. Chem.* **268**, 17597–17601.