Adhesion of T and B Lymphocytes to Extracellular Matrix and Endothelial Cells Can Be Regulated through the β Subunit of VLA

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Abstract. Investigating the regulation of very late antigen (VLA)-mediated functions, we found that TS2/16, a mAb directed against the β chain of the VLA group of integrins, can induce binding of resting peripheral blood lymphocytes, cloned T lymphocytes, and Epstein Barr virus–transformed B cells to extracellular matrix components, fibronectin, laminin, and collagen, but not to fibrinogen. The antibody stimulates VLA-4-, VLA-5-, and VLA-6-mediated binding. Furthermore, it induces VLA-4–mediated binding to vascular cell adhesion molecule-1 expressed by rTNF-α-stimulated endothelial cells, but it does not stimulate homotypic aggregation of cells as described for a number of anti-VLA-4α antibodies (Bednarczyk, J. L., and B. W. McIntyre. 1990. *J. Immunol.* 144: 777–784; Campanero, M. R., R. Pulido, M. A. Ursa, M. Rodríguez-Moya, M. O. de Landázuri, and F. Sánchez-Madrid. 1990. *J. Cell Biol.* 110:2157–

2165). Therefore, the stimulating activity of this anti-β1 antibody clearly contrasts with that of the anti-VLA-4α antibodies, which induce homotypic cell aggregation, but not binding of cells to extracellular matrix components or endothelial cells, indicating that TS2/16 may generate different signals.

The observation that also F(ab')₂ or Fab fragments of this anti-β1 antibody stimulate binding to extracellular matrix components and endothelial cells excludes the possibility that binding requires receptor crosslinking, or is Fc receptor mediated. Induction of this adhesion is cation and energy dependent and requires an intact cytoskeleton. Although changes in the conformation of VLA integrins induced by this antibody may regulate their functional activity, the dependence on metabolic energy indicates that intracellular processes may also play a role.

T HE very late antigens (VLAs)[1] are expressed on both hematopoietic and nonhematopoietic cells and form the β1 group of the integrin superfamily of adhesion receptors (Hynes, 1987). Other groups of this still expanding family comprise the β2, or leukocyte integrin group (Sanchez-Madrid et al., 1983), and the β3 group, which consists of the platelet antigen IIb/IIIa (Phillips et al., 1988) and the vitronectin receptor (Pytela et al., 1985). The VLA molecules mediate adhesion of cells with components of the extracellular matrix (ECM) (Hemler, 1990). Recently, it has been observed that VLA-4 is also able to interact with glyco-proteins expressed at the surface of various types of cells including endothelium. One of these ligands has been identified as the vascular cellular adhesion molecule-1 (VCAM-1) (Elices et al., 1990; Taichman et al., 1991).

High affinity interaction of integrins with their respective ligands, requires activation of the integrin receptor. This has now been demonstrated for the β1 (Shimizu et al., 1990), β2 (Van Kooyk et al., 1989; Dustin and Springer, 1989), and β3 (O'Toole et al., 1990) group of this adhesion receptor family. Phorbol esters, such as PMA, are able to induce a high affinity state of the β1, β2, and β3 integrins (Patarroyo et al., 1985; Phillips et al., 1988; Shimizu, 1990; Springer, 1990; Wilkins et al., 1991). In addition, a number of more physiological stimulatory pathways have been recognized to stimulate integrin-mediated adhesion. Among others, signals mediated through CD2 and CD3, which are expressed by T lymphocytes, can stimulate β1- and β2- mediated interactions (Dustin and Springer, 1989; Van Kooyk et al., 1989; Nojima et al., 1990), and thrombin is able to induce adhesion of platelets, through IIb/IIIa, a β3 integrin (Ginsberg et al., 1980).

We previously showed that an antibody directed against a unique epitope located on the α chain of lymphocyte function–associated antigen-1 (LFA-1), is capable of inducing a high affinity state of LFA-1, a β2 integrin. Similarly, specific antibodies have been recognized that stimulate β3-mediated functions (Gulino et al., 1990). Recently, Campanero et al.

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1. *Abbreviations used in this paper*: Coll, collagen type I; EBV, Epstein Barr virus; ECM, extracellular matrix; FG, fibrinogen; FN, fibronectin; HE, hydroethidine; LAD, leukocyte adhesion deficiency; LFA-1, lymphocyte function–associated antigen-1; LM, laminin; PBL, peripheral blood lymphocyte; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1; VLA, very late activation antigen.

(1990) and Bednarczyk and McIntyre (1990) observed that certain antibodies directed against VLA-4 α subunit are capable of inducing homotypic aggregation of leukocytes suggesting that antibodies against VLA-4 can also induce a high affinity state of this receptor.

In the present study, we demonstrate that an anti-β1 antibody is also capable of inducing a high affinity state of VLA-4 and stimulates VLA-4-mediated adhesion. In addition we show that several other VLA antigens that comprise the β1 family can also be stimulated. Interestingly, a clear distinction can be made between the VLA-mediated functions stimulated by anti-VLAα antibodies and those stimulated by the anti-β1 antibody used in this study.

Materials and Methods

Cell Lines and Cell Culture

Peripheral blood lymphocytes (PBLs) were isolated from mononuclear cells by centrifugal elutriation as described previously (Figdor et al., 1984). The human Tetanus toxoid specific T helper clone HY 827 was described by Yssel et al. (1986); the cytotoxic, alloreactive T cell clone JS 136, directed against an HLA-DRw6 determinant by Borst et al. (1986). The LFA-1 negative T cell clones, LAD 4, LAD 6.1, and LAD 6.6, were raised by limiting dilution of peripheral blood lymphocytes of a patient (VA) with leukocyte adhesion deficiency (LAD) lacking mRNA expression for the CD18 protein (Van Kooyk et al., 1989). T cell clones, KZ 21.1 and KZ 25.7 were established from LFA-1 positive lymphocytes. The B cell lines were established by Epstein Barr virus (EBV) transfection of B cells from patient VA and B cells from donor JS. Cloned T cells were cultured in Iscove's medium supplemented with 5% human serum and stimulated weekly with irradiated allogeneic peripheral blood mononuclear cells (PBMCs) and EBV-transformed B cells (JY), 0.2 μg/ml phytohaemagglutinin, and 100 U/ml rIL-2, kindly provided by Eurocetus (Amsterdam, The Netherlands). EBV-transformed B cells were maintained in Iscove's medium containing 10% FCS.

Antibodies

The following VLAα mAbs were used. HP1/2 and HP1/3 (anti-VLA-4, CD49d), (Campanero et al., 1990); B-5G10 (anti-VLA-4, CD49d) (Hemler et al., 1987); SAM-1 (anti-VLA-5, CD49e) (Keizer et al., 1987); and GoH3 (anti-VLA-6, CD49f) (Sonnenberg et al., 1988) are directed against α subunits of the VLAs. The antibodies TS2/16 and A-1A5 (Hemler et al., 1984), K20 (Amiot et al., 1988), and AIIB2 (Hall et al., 1989) are directed against the β1 subunit of the integrins (CD29). CLB-LFA-1/1 (CD18) (Miedema et al., 1984), C17 (CD41) (Tetteroo et al., 1983), and 439.9B (CD61) (Falconi et al., 1988) are reactive with other β subunits of the integrins. The antibodies 32.2 and IV.3 are directed against FcγRI (CD64) and FcγRII (CD32) (Anderson et al., 1986), respectively, and antibody CLB-FcγR gran 1 against FcγRIII (CD16) (Klaassen et al., 1990). mAb 4B9 recognizes VCAM-1 (Schwartz et al., 1990) and W6/32, which is used as control antibody reacts with MHC class I (Barnstable et al., 1978).

Fab and F(ab')2 fragments of TS2/16 were generated by papain and pepsin digestion as described by Mather et al. (1987) and checked for purity with an Fc-specific ELISA and by SDS-PAGE, followed by Coomassie blue staining. SDS-PAGE was carried out on vertical slab gels (5–15%) according to a modification of the Laemmli procedure (Laemmli, 1970).

Cell Binding to ECM Components

For cell attachment to ECM, flat bottom 96-well microtiter plates (Costar, Cambridge, MA) were coated with 100 μl of 20 μg/ml fibronectin (FN), 20 μg/ml laminin (LM), 40 μg/ml collagen type I (ColI), or 40 μg/ml fibrinogen (FG), (Sigma Chemical Co., St. Louis, MO), for 16 h at 4°C, washed, and subsequently incubated with 1% wt/vol BSA for 1 h by 37°C. T cell clones and EBV-transformed B cells were labeled with ^{51}Cr for 1.5–2 h, washed, and resuspended in Iscove's medium containing 0.25% wt/vol BSA. 1 × 10⁴ radiolabeled cells were plated in triplicate (50 μl final volume), centrifuged for 1 min at 1,000 rpm and incubated for 30 min at 37°C and 5% CO_2. Unbound cells were removed by washing with PBS supplemented with 0.25% wt/vol BSA, 1 mM Mg^{2+}, and 1 mM Ca^{2+}.

Bound cells were lysed by detergent and the radioactivity of the lysate was counted in a gamma counter. Results were expressed as the mean percentage of cell binding from triplicate wells. For induction or enhancement of binding to ECM, 2 × 10⁵ cells/ml were preincubated with 10 μg/ml purified TS2/16, or 50 nM PMA for 10 min. For antibody inhibition, 25 μl with 1:100 dilution or 10 μg/ml purified mAb was added to 25 μl cell suspension and preincubated for 20 min at 4°C. Sodium azide (2% wt/vol) and deoxyglucose (50 mM, SERVA, Heidelberg, FRG) were added to inhibit cell metabolism. Staurosporin (200 nM; Kyowa Hakko, Europe GMBH, Düsseldorf, FRG) was added to inhibit protein kinase C activity. Involvement of cytoskeleton was determined by adding Cytochalasin B (20 μM; Sigma Chemical Co.). Cation dependence was determined by preincubation of cells with 5 mM EDTA or 5 mM EGTA for 30 min at 37°C.

Cross-Competitive mAb Cell Binding Assay

For cross-competitive mAb cell binding assays, cells were preincubated (30 min, 0°C) with different dilutions of unlabeled mAb in Iscove's medium containing 0.25% wt/vol BSA. Then, purified TS2/16 mAb (10 μg/ml) labeled with ^{125}I (Amersham International, Amersham, UK), as described by Greenwood et al. (1963), was added and incubated for another 30 min on ice. Unbound radioactivity was removed by washing four times and bound labeled antibody was counted in a gamma counter and expressed as the mean cpm of triplicate tests.

Immunofluorescence

Cells were incubated (30 min, 0°C) in PBS containing 1% wt/vol BSA (Sigma Chemical Co.), and 0.02% wt/vol sodium azide, with appropriate dilutions of the different mAb, followed by incubation with FITC-labeled goat F(ab')2 anti-mouse IgG antibody (GAM-FITC; Nordic, Tilburg, The Netherlands) or by FITC-labeled mouse anti-rat kappa antibody (MARK-FITC, Sanbio, Uden, The Netherlands) for 30 min at 0°C. The relative fluorescence intensity was measured on a FACScan (Becton Dickinson, Mountain View, CA).

Binding of Cells to Endothelium

Endothelial cells (2 × 10⁵ cells/ml), isolated from human umbilical cord veins and cultured two or three passages as described by Te Velde et al. (1987), were grown in 100 μl RPMI/well containing 10% human serum, in FN-coated (2 μg/100 μl), flat bottom 96-well plates (Costar, Cambridge, MA). After 2 d of culturing, the cells were stimulated, for 24 h, with 100 U/ml rTNF-α (supernatant of cDNA-transfected COS cells). Before initiation of the adhesion experiment the wells with endothelial cells were washed with RPMI. T cells were labeled with ^{51}Cr for 1.5–2 h, washed, and resuspended in Iscove's medium with 0.25% wt/vol BSA. 1 × 10⁴ radiolabeled T cells were plated in triplicate (100 μl final volume), and incubated for 30 min at 37°C and 5% CO_2. Unbound cells were removed by washing with PBS supplied with 0.25% wt/vol BSA, 1 mM Mg^{2+}, and 1 mM Ca^{2+}. Bound cells were lysed by detergent and the radioactivity of the cell lysate was counted in a gamma counter. Results were expressed as the mean percentage of cell binding from triplicate wells. For induction or enhancement of binding to ECM, cells were preincubated with 1:100 ascites dilution of mAb or 10 μg/ml purified TS2/16 for 30 min at 4°C. For antibody inhibition studies, cells were preincubated (30 min, 4°C) with 1:100 ascites dilution or 10 μg/ml purified mAb.

Quantitative Aggregation Assay

Homotypic aggregation was quantitatively determined by double fluorescence (Kuijpers et al., 1990). Cells (2 × 10⁶ cells/ml in Iscove's medium) were stained with the red dye Hydroethidine (HE; Polyscience, Inc., Warrington, PA; 40 mg/ml in N,N-dimethylacetonide) at concentrations of 3 ng/ml, or with the green dye sulfofluorescein diacetate (SFDA; Molecular Probes, Junction City, OR) at a concentration of 5 μg/ml. After 1 h of incubation at 37°C, cells were washed twice with Iscove's medium with 10% FCS. Cells were seeded in round bottom wells (5 × 10⁴ HE labeled and 5 × 10⁴ SFDA labeled cells) in 100 μl. After 2 h of incubation at room temperature, cells were fixed with 0.5% wt/vol paraformaldehyde, and aggregation was counted in a FACScan. Double-colored aggregates were calculated as a percentage of the total HE colored events counted from duplicate wells.

Results

Induction of β1-mediated Adhesion of T and B Lymphocytes to ECM

LFA-1 positive (JS 136) and LFA-1 negative (LAD 6.1) cloned T cells and LFA-1 positive (JS EBV) and LFA-1 negative (LAD EBV) EBV-transformed B cells were studied with respect to VLA-mediated binding to ECM. Table I shows that the cloned T cells hardly bind to FN, LM, Coll type I, or

Table I. Induction of T and B Cell Adhesion to ECM Components

Treated with		BSA	FN	LM	Coll(I)	FG
		Adhesion*				
		%	%	%	%	%
Medium	JS 136	<‡	<	<	<	<
	LAD 6.1	<	<	<	<	<
	JS EBV	<	10	<	<	<
	LAD EBV	<	9	<	<	<
PMA	JS 136	<	37	8	12	<
	LAD 6.1	<	19	18	26	<
	JS EBV	<	22	<	<	<
	LAD EBV	<	19	<	<	<
TS2/16	JS 136	<	36	14	17	<
anti-VLA-β	LAD 6.1	<	31	28	31	<
	JS EBV	<	43	<	<	<
	LAD EBV	<	37	<	<	<
A-1A5	JS 136	<	19	<	<	<
anti-VLA-β	LAD 6.1	<	11	<	<	<
	JS EBV	<	25	<	<	<
	LAD EBV	<	19	<	<	<
AIIB2	JS 136	<	<	<	<	<
anti-VLA-β	LAD 6.1	<	<	<	<	<
	JS EBV	<	<	<	<	<
	LAD EBV	<	<	<	<	<
K20	JS 136	<	<	<	<	<
anti-VLA-β	LAD 6.1	<	<	<	<	<
	JS EBV	<	14	<	<	<
	LAD EBV	<	11	<	<	<
HP 1/3	JS 136	<	<	<	<	<
anti-VLA-4	LAD 6.1	<	<	<	<	<
	JS EBV	<	14	<	<	<
	LAD EBV	<	12	<	<	<
HP 1/2	JS 136	<	<	<	<	<
anti-VLA-4	LAD 6.1	<	<	<	<	<
	JS EBV	<	<	<	<	<
	LAD EBV	<	<	<	<	<
B-5G10	JS 136	<	<	<	<	<
anti-VLA-4	LAD 6.1	<	<	<	<	<
	JS EBV	<	12	<	<	<
	LAD EBV	<	11	<	<	<
SAM-1	JS 136	<	<	<	<	<
anti-VLA-5	LAD 6.1	<	<	<	<	<
	JS EBV	<	12	<	<	<
	LAD EBV	<	13	<	<	<

* SD <3%
‡ < = <5% adhesion.

FG, while the EBV transformed B cells showed significant binding to FN. We observed that, TS2/16, an anti-β1 antibody, stimulated adhesion to FN, LM, and Coll but not to FG (Table I). To extend this observation, we tested a large panel of anti-β1 and anti-VLA-4 antibodies, as well as a number of antibodies directed against other β integrin subunits, for their capacity to induce binding of cloned T cells or EBV-transformed B cells to ECM (Table I). None of the other anti-β or anti-α antibodies induced strong ECM binding, except mAb A-1A5, which stimulated moderate binding of lymphocytes to FN. Furthermore, antibodies directed against other anti-β integrin subunits did not induce ECM binding (not shown), which is not surprising since expression of these integrins is low or absent on these T and B cells (Table II). Treatment with the phorbol ester PMA also stimulated binding of both T cell clones and EBV-transformed B cells to FN, had a moderate effect on cloned T cell binding to LM and Coll, but did not stimulate binding of B cells to LM and Coll (Table I).

From these results we conclude that TS2/16 has a unique property to induce T and B cell binding to FN, and T cell binding to LM and Coll type I.

To exclude that the TS2/16 induction of adhesion to FN was restricted to the two T cell clones and B cell lines used, we tested a number of T cell clones and B cell lines with comparable expression of integrin molecules on the cell surface. Furthermore, resting lymphocytes were tested on their capacity to bind to ECM after induction with TS2/16. Only a low number of resting PBLs spontaneously adhered to FN and LM (17 and 8%, respectively). After incubation with TS2/16 adhesion of PBL to FN and LM was enhanced to 39 and 35%, respectively. No significant binding was observed to Coll I and FG after TS2/16 inducement (data not shown). Table III shows that for all T cell clones, B cells, and resting PBL tested, adhesion to FN was induced by TS2/16 and inhibited by mAb HP1/2, an anti-VLA-4 antibody, and not by mAb against β2.

Intact Anti-β1 Antibodies, but also F(ab')₂ and Fab Fragments Induce β1-mediated Adhesion

To determine whether TS2/16-induced adhesion required receptor crosslinking, or depended on the Fc part of the molecule, we prepared F(ab')₂ and Fab fragments. The purity of the preparations was checked with an Fc-specific ELISA (detection level ≥ 0.001% intact IgG) and by SDS-PAGE under nonreducing conditions (Fig. 1 A). Both assays showed no detectable level of IgG. The results in Fig. 1 B show that F(ab')₂ and Fab fragments are as effective in inducing binding of cloned T cells to FN as intact IgG.

These results demonstrate that crosslinking of VLA molecules is not required to induce adhesion, and that the Fc portion of IgG plays no role. Moreover, the T cells used in this study are Fc receptor negative (Table II).

Anti-β1-stimulated Adhesion Is Mediated by Different VLA Molecules

The results shown in Table I suggest that TS2/16 stimulates binding through various VLA integrins, because adhesion was induced to FN, LM, and Coll. We performed blocking studies with different anti-α antibodies to determine which adhesion molecules mediated induced adhesion by TS2/16

Table II. Surface Expression of Integrins on T and B Cells

mAb	Antigen	Relative fluorescence intensity				
		HY 827	LAD 6.1	JS EBV	LAD EBV	PBL
GAM FITC	Control	4	4	5	4	3
MARK FITC	Control	4	3	4	3	5
CLB-10G11	VLA-2	10	10	6	5	4
J143	VLA-3	12	13	13	10	3
HP1/2	VLA-4	152	128	67	92	20
HP1/3	VLA-4	145	128	63	88	19
B-5G10	VLA-4	136	113	58	84	16
SAM-1	VLA-5	7	7	8	6	6
GoH3*	VLA-6	8	7	5	4	25
TS2/16	β1	75	68	55	59	41
A-1A5	β1	73	68	60	58	37
AIIB2*	β1	70	80	45	66	51
K20	β1	61	50	57	47	46
CLB-LFA-1/1	β2	81	6	21	5	23
C17	β3	4	4	6	5	6
439.9B	β4	5	5	5	5	4
Control mAb						
4B9	VCAM-1	5	4	5	5	4
32.2	FcγRI	6	5	5	4	3
IV.3	FcγRII	4	5	5	7	4
CLB-FcγR gran1	FcγRIII	3	4	5	4	3

* Rat antibody.

Fab fragments. The results in Fig. 2 show that TS2/16-induced binding of cloned T cells to FN was completely mediated by VLA-4 and VLA-5, whereas binding of resting PBL to FN was only partially dependent on VLA-4 and VLA-5 (Fig. 2, A and C). Furthermore, binding to LM of cloned T cells after TS2/16 induction, was partially mediated by VLA-6, whereas binding of resting PBL seemed to be completely dependent on VLA-6 (Fig. 2, B and D), apparently due to the high expression of VLA-6 and the absence of VLA-3 on resting PBL (Table II). Inhibition of PMA-induced adhesion to ECM showed similar results (Fig. 2). mAb against β2, β3, or β4 integrins did not affect TS2/16-induced cell adhesion (not shown). No differences were obtained between LFA-1 positive and LFA-1 negative T and B cells (not shown).

From these data we conclude that TS2/16 induces T and B cell adhesion to ECM by activation of various VLA integrins.

Blocking of TS2/16 Stimulated Adhesion by Pretreatment with Other Anti-β1 Antibodies

We repeatedly found that other anti-β1 antibodies, AIIB2 and K20, were not capable of inducing adhesion, and that A-1A5 induced only moderate binding to FN (Table I). These three anti-β1 antibodies could not inhibit TS2/16-induced adhesion when added simultaneously or after TS2/16 to the cells (not shown). Therefore, we tested their capacity to prevent TS2/16-induced adhesion by incubating the cells with the antibodies before addition of TS2/16. The antibodies AIIB2 and A-1A5, but not K20, prevented induction of adhesion of cloned T cells (Fig. 3 A) and EBV-transformed B cells (not shown) by TS2/16, indicating that AIIB2 and A-1A5 recognize an epitope close to that of TS2/16. Crossblocking experiments (Fig. 3 B) confirmed that AIIB2 and A-1A5 indeed prevented binding of 125I-labeled TS2/16. Consistent with reports in the literature (Hemler et al., 1984; Hall et al., 1989), incubation of the cells with AIIB2, but not A-1A5, inhibited spontaneous adhesion to ECM (Fig. 3 A).

From these data we conclude that TS2/16, A-1A5, and AIIB2 recognize closely located epitopes on the β1 subunit. However, these antibodies have entirely different effects on VLA-mediated adhesion.

Anti-β1– and PMA-induced Adhesion Have Similar Kinetics Characteristic for Integrin Molecules

Fig. 4 shows that TS2/16-induced adhesion of cloned T cells

Table III. Lymphocyte Adhesion to FN after Induction with TS2/16

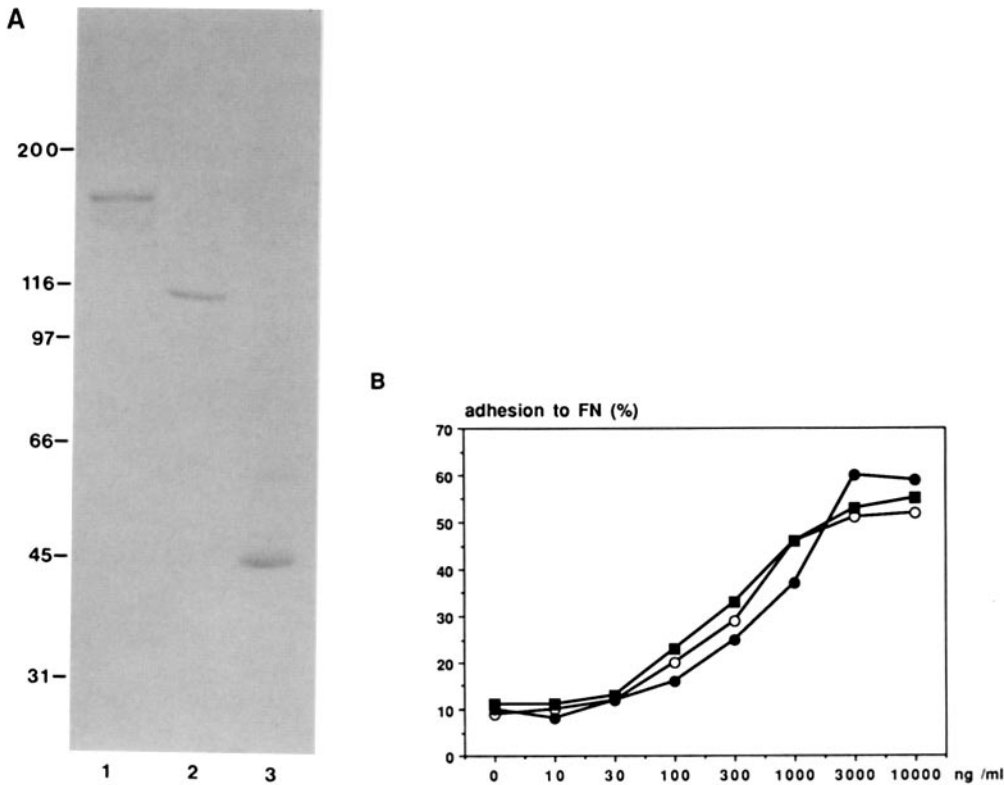|	Adhesion (%)*		
		TS2/16 induced	
	Without activation	mAb HP1/2 (anti-VLA-4)	mAb CLB-LFA1/1 (anti-β2)
T cell clones			
LAD 4	14	38	10	39
LAD 6.1	4	29	6	31
LAD 6.6	9	29	12	29
JS 136	3	19	7	17
HY 827	14	44	18	46
KZ 25.7	3	18	8	21
B cell lines			
JY EBV	12	25	4	22
JS EBV	15	32	5	34
LAD EBV	9	25	2	26
Resting PBL	17	39	21	37

* SD <3%.

Figure 1. (*A*) SDS 5–15% PAGE under nonreducing conditions of 10 μg of TS2/16 IgG1 (lane *1*), 10 μg TS2/16 F(ab′)₂ fragments (lane *2*), and 10 μg TS2/16 Fab fragments (lane *3*). (*B*) Comparison of purified TS2/16 IgG (-○-), TS2/16 F(ab′)₂ (-●-), and TS2/16 Fab fragments (-■-) in their capacity to induce binding of cloned T cells HY 827 to FN. One representative experiment out of two is shown.

to FN, LM, and Coll occurred more rapidly compared to adhesion induced by PMA. In addition, a higher percentage of the cells adhered to LM and Coll after incubation with TS2/16 in comparison with PMA. Adhesion is initiated after 5 min of incubation with TS2/16 and a maximum of adhesion is reached after 40 min. LFA-1 positive and LFA-1 negative T cell clones showed the same kinetics (not shown).

Furthermore, we observed that TS2/16- and PMA-induced adhesion is cation and temperature dependent, and requires an intact cytoskeleton, since EDTA, incubation at 0°C, and cytochalasin B completely inhibited adhesion of LFA-1 negative cloned T cell to FN (Fig. 5) LM and Coll (not shown). Induction of adhesion by TS2/16 or PMA could not be inhibited by deoxyglucose or sodium azide, but a combination
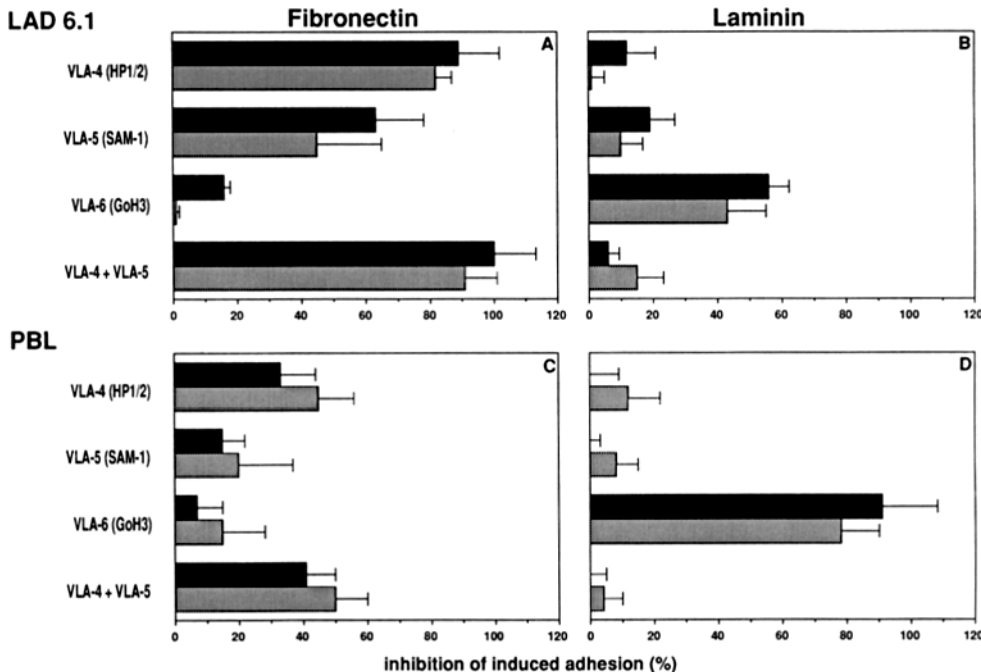
Figure 2. Inhibition of cell binding to FN and LM. Cells were stimulated with TS2/16 (■) or PMA (▨) for 10 min. Subsequently adhesion was followed for 30 min with an inhibitory antibody as indicated. Binding of LFA-1 negative cloned T cells to FN (*A*), and LM (*B*), and resting PBL to FN (*C*), and LM (*D*). One representative experiment out of three is shown.

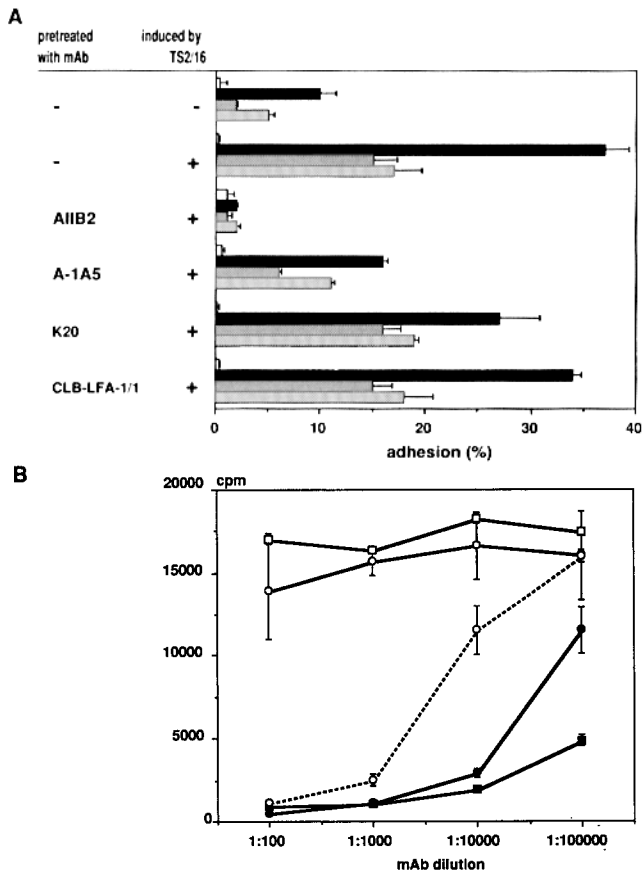*Figure 3.* (*A*) Effects of other β1 antibodies on TS2/16 induced binding to ECM of T cell clone HY 827. T cells were pretreated with AIIB2, A-1A5, K20, and CLB-LFA1/1 as control, for 10 min, before activation with TS2/16. Adhesion to BSA (□), FN (■), LM (▦), and Coll (▨) was measured after 30 min at 37°C. One representative experiment out of two is shown. (*B*) Cross-competition of anti-β1 mAb. Cells were incubated with unlabeled mAb, before the incubation with 10 μg/ml ^{125}I-labeled TS2/16. Unlabeled TS2/16 (1 mg/ml --○--), A-1A5 (ascites -■-), AIIB2 (ascites -●-), K20 (1 mg/ml -○-), and HP1/3 (ascites -□-) were used in a dilution of 1:100–1:100,000.

Figure 4. Kinetics of TS2/16 and PMA induced binding to ECM to: (*A*) FN, (*B*) LM, (*C*) Coll type I. HY 827 cells stimulated with PMA (-●-), or TS2/16 (-■-), or without stimulation (-○-).

of both components completely blocked adhesion (Fig. 5), suggesting that active cell metabolism is important. Staurosporin, a protein kinase C inhibitor, inhibited PMA-induced adhesion (Fig. 5 *B*) but had no effect, or only a moderate effect on TS2/16-induced adhesion (Fig. 5 *A*). Using these agents to inhibit TS 2/16 and PMA-induced LFA-1 positive T and B cell adhesion to FN showed similar results (not shown).

We conclude from these data that TS2/16-induced adhesion is characteristic for integrin molecules, and that it may require intact signalling functions of the T and B cells.

Anti-β1 Induces Adhesion to Endothelial Cells

Since TS2/16 antibodies induce VLA-4–mediated adhesion to ECM we investigated the capacity of this antibody to stimulate VLA-4–mediated binding of cloned T and EBV-transformed B lymphocytes to endothelium. We and others (Schwartz et al., 1990; Vennegoor et al., 1992) showed that binding of lymphocytes to endothelium was mediated by LFA-1/ICAM-1 or -2 and the VLA-4/VCAM-1 interactions.
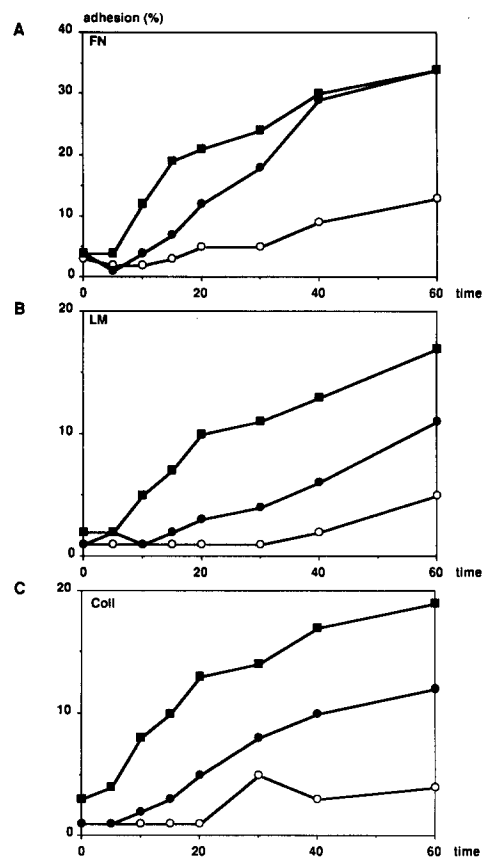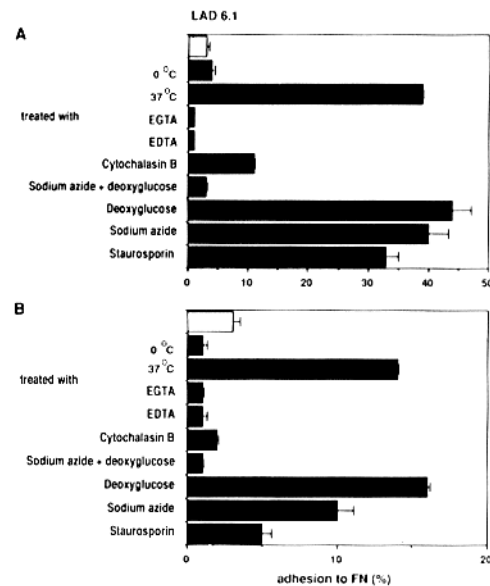

Figure 5. Effect of temperature, depletion of cation, cytochalasin B, sodium azide, deoxyglucose and staurosporin on T cell binding to FN. (□) control cells (without activation). (■) T cell clone LAD 6.1 after activation with (*A*) TS2/16 and (*B*) PMA. One representative experiment out of four is shown.
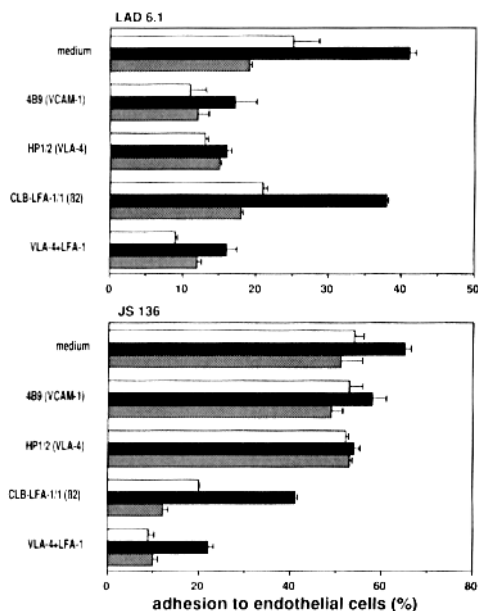
Figure 6. TS2/16 enhanced binding of cloned T cells to rTNF-α–activated cultured human endothelial cells. Adhesion was measured after treatment of (A) LAD 6.1 (LFA-1 negative) and (B) JS 136 (LFA-1 positive) cells with medium (□), TS2/16 (■) and HP1/3 (▦). Anti-VLA-4, anti-VCAM-1 and anti-LFA-1 antibodies were tested on their capacity to inhibit adhesion. One representative experiment out of five is shown.

VLA-4–mediated adhesion depended on the expression of VCAM-1, which was best visualized with LFA-1 negative (LAD) cells and after treatment of the endothelial cells with cytokines like rTNF-α (Fig. 6 A). TS2/16 was capable to enhance binding to cloned LAD T lymphocytes to rTNF-

α–treated (24 h), VCAM-1 positive endothelial cells (Fig. 6 A) but not to untreated endothelium (not shown). TS2/16 enhancement of adhesion to rTNF-α–stimulated endothelium of LFA-1 positive cells (JS 136) was hardly possible since these cells utilize primarily the LFA-1/ICAM pathway (Fig. 6 B). Both anti-VCAM-1 and anti-VLA-4 antibodies inhibited TS2/16-induced adhesion of LFA-1 negative cloned T lymphocytes, demonstrating that TS2/16 specifically stimulated the VLA-4/VCAM-1 adhesion pathway (Fig. 6 A), whereas LFA-1 positive cloned T cells (Fig. 6 B) were inhibited by antibodies against the LFA-1 β chain. However, after induction with TS2/16 the inhibitory capacity of the LFA-1 mAb decreased, suggesting a more dominant role for the VLA-4/VCAM-1 adhesion route (Fig. 6 B). Pretreatment of the cells with mAb AIIB2, a distinct anti-β1 antibody, completely abrogated TS2/16-induced adhesion (not shown), comparable to the TS2/16-induced adhesion to ECM (Fig. 3 A), confirming that the epitope recognized by AIIB2 prevented binding of TS2/16.

From these results we conclude that β1-induced adhesion via VLA-4 implicates both adhesion to FN and to VCAM-1.

TS2/16 Does Not Stimulate Homotypic Cell Aggregation

None of the anti-VLA α subunit antibodies used in this study were capable of inducing adhesion to ECM or endothelial cells (Table I, Fig. 6). Recently it has been reported that certain anti-VLA-4 antibodies were capable of inducing adhesion (Campanero et al., 1990; Bednarczyk and McIntyre, 1990). These observations were confirmed by the results in Fig. 7, which show that mAb HP1/3 directed against VLA-4 induced homotypic aggregation of both LFA-1 positive and LFA-1 negative cloned T cells and EBV-transformed B cells. In contrast, we never observed homotypic aggregation in-
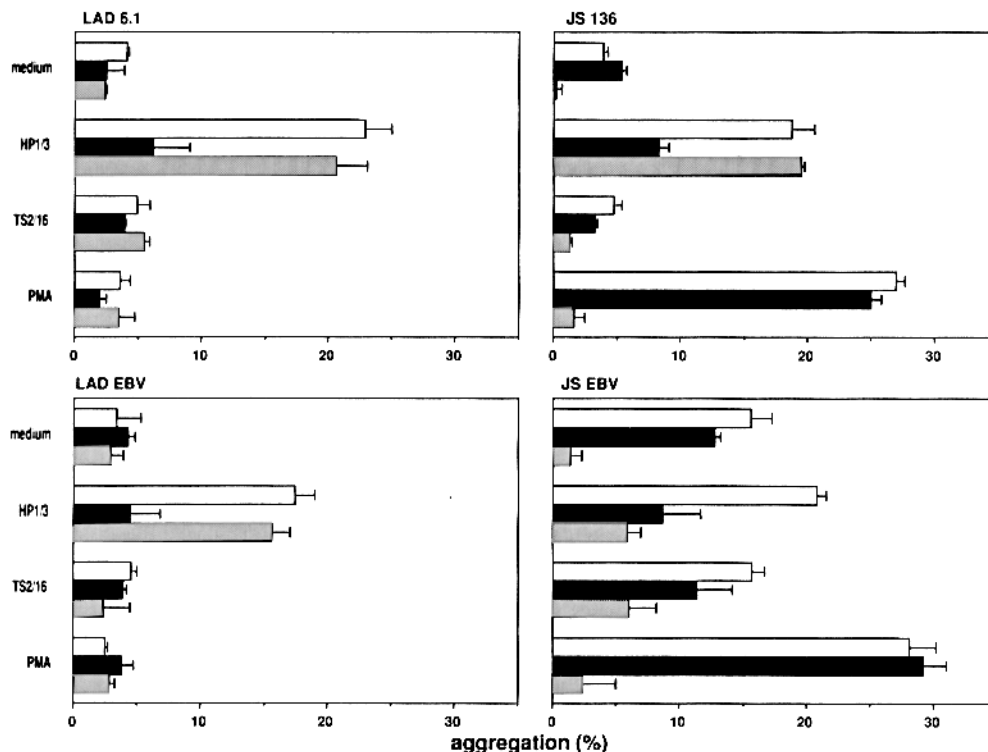

Figure 7. Homotypic aggregation of LFA-1 positive (JS), and LFA-1 negative (LAD) cloned T and EBV transformed B cells. Cells were stimulated with medium, HP1/3 (anti-VLA-4), TS2/16 (anti-β1), and PMA. Antibodies HP1/2, (■, anti-VLA-4), CLB-LFA-1/1 (▦, anti-β2) and W6/32 (□, control mAb) were tested on their capacity to inhibit adhesion. One representative experiment out of four is shown.

duced by TS2/16. HP1/3-induced aggregation was inhibited by anti-VLA-4 antibody (HP1/2) and not by mAb against $\beta2$ (CLB-LFA-1/1), demonstrating the specificity of the response. Furthermore, PMA readily induced cell aggregation of LFA-1 positive cells, which could be inhibited by anti-$\beta2$ antibody, but hardly by blocking anti-VLA-4 antibody (HP1/2).

These results indicate that TS2/16 and the stimulating anti-VLA-4α antibodies (Campanero et al., 1990; Bednarczyk and McIntyre, 1990) may distinctly regulate VLA-mediated adhesion. PMA mimics the effects induced by both anti-α or -β antibodies.

Discussion

High affinity interactions of integrins with their respective ligands require activation of the integrin receptor. This has now been demonstrated for the $\beta1$ (Nojima et al., 1990), $\beta2$ (Van Kooyk et al., 1989; Dustin and Springer, 1989), and $\beta3$ (O'Toole et al., 1990) group of this adhesion receptor family. PMA has been recognized to induce $\beta2$-mediated adhesion (Patarroyo et al., 1985; Springer, 1990), $\beta3$-mediated adhesion (Phillips et al., 1988), and more recently also $\beta1$-mediated adhesion (Shimizu et al., 1990).

We have previously observed that $\beta2$-mediated adhesion can be induced by an antibody, termed NKI-L16, (Keizer et al., 1988) directed against a Ca^{2+}-dependent epitope located on the α subunit of LFA-1 (Van Kooyk et al., 1991). The antibody induces homotypic lymphocyte aggregation, as well as binding to purified ICAM-1 (Van Kooyk et al., 1991). In addition, the antibody enhances LFA-1–dependent adhesion to endothelium (our unpublished results). As expected, expression of the epitope recognized by TS2/16 is not dependent on the presence of divalent cations (not shown), since the cation binding domains are located on the α subunit of integrins. In this respect, TS2/16 contrasts with NKI-L16 or antibody 24, both of which recognize α subunits of the $\beta2$ group of integrins (Dransfield and Hogg, 1989). Nevertheless, a striking parallel exists between the earlier observations with NKI-L16 and those reported with TS2/16 in this paper (a) Both antibodies induce adhesion to their respective ligands. (b) Induction of adhesion is not dependent on the Fc portion of the antibody. (c) Stimulation of adhesion is also not dependent on receptor crosslinking, since Fab fragments of both antibodies are as effective as IgG. (d) The kinetics of the induced adhesion by both antibodies parallels that of PMA. (e) Induction of adhesion requires an intact cytoskeleton and the presence of divalent cations, all characteristics of integrin-mediated interactions. (f) The induction of VLA-mediated cell adhesion by TS2/16 is dependent on metabolic energy, since a combination of deoxyglucose and sodium azide prevents induction of adhesion. We have previously reported that induction of LFA-1–mediated cell adhesion is not affected by sodium azide (Van Kooyk et al., 1991). Similarly deoxyglucose alone does not affect adhesion (our unpublished results). However, a more detailed analysis shows that a combination of azide and deoxyglucose is inhibitory (our unpublished results), similar to what we observe for TS2/16.

One explanation for the capacity of these antibodies to induce cell adhesion is that they affect the tertiary structure of integrins, and that they induce a high affinity state of the

receptor by conformational changes. Gulino et al. (1990) elegantly demonstrated that this can indeed be the case. They have shown that a high affinity state of IIb/IIIa can be induced by anti-IIb antibodies in isolated IIb/IIIa molecules bound to a cell-free substrate (Gulino et al., 1990). These results also demonstrate that high avidity interactions can be obtained in the absence of associated molecules, for instance, cytoskeletal elements. Induction of $\beta1$- (this study) and $\beta2$- (Van Kooyk et al., 1991) mediated interactions by the antibodies TS2/16 and NKI-L16, respectively, may also involve conformational changes, but their dependence on metabolic activity of the cell suggests that intracellular processes play a role. At present it is not known which steps in the adhesion process are affected by these inhibitors of cell metabolism. It is tempting to speculate that direct conformational changes induced by these antibodies affect intracellular changes, for example the association of the intracellular domains of these integrin molecules with the cytoskeletal elements, and that these latter interactions are disturbed by metabolic inhibitors. This hypothesis is supported by the observation that induction of a high affinity state of LFA-1 induced by CD2 and CD3 triggering, or PMA is completely abrogated by the protein kinase inhibitor staurosporin or by azide alone, whereas this does not affect NKI-L16–induced adhesion (Van Kooyk et al., 1991). Similarly, staurosporin inhibits PMA-induced adhesion (Fig. 5) but not TS2/16-induced adhesion via $\beta1$.

TS2/16 specifically induces binding to different ligands of VLA integrins used in this study, including extracellular matrix components (FN, LM, Coll type I) and cell surface molecules (VCAM-1). It is of interest to note that, on cloned T cells but not on EBV-transformed B cells, expression of the α subunit of VLA-4 is much higher than that of the $\beta1$ subunit (Table II), suggesting a possible association of the VLA α subunit with a β subunit on cloned T cells other than the ones we have tested. Furthermore, it is noteworthy that binding to LM can be readily induced despite the low expression of VLA-6 on the T cell clones used in this study. In addition, the mAb blocking studies indicate that binding of cloned T cells to LM is not exclusively mediated by VLA-6, suggesting that other LM receptors (VLA-2 and VLA-3) are involved. In contrast, binding of resting PBL to LM is completely VLA-6 dependent, according to the high expression of VLA-6. Binding to FN implicated both the so called CS-1 site (Wayner et al., 1989) and the RGD site (Ruoslahti and Pierschbacher, 1987) since both VLA-4– and VLA-5–mediated FN binding has been observed, respectively. This suggests that the activity of TS2/16 is not restricted to certain VLA molecules but probably involves all members of this integrin group. In addition, the results emphasize a major role for the $\beta1$ subunit in the regulation of VLA-mediated adhesion. Similarly, anti-$\beta3$ antibodies have been demonstrated to stimulate $\beta3$-mediated adhesion (Kouns et al., 1990). This function is however not restricted to anti-β antibodies, since stimulatory anti-α antibodies have also been described (Gulino et al., 1990).

A clear distinction can be made between the stimulatory activities of certain antibodies against the VLA-4α subunit and the anti-$\beta1$ antibody used in this study. Previous reports show that anti-VLA-4α antibodies can induce homotypic cell aggregation (Campanero et al., 1990; Bednarczyk and MacIntyre, 1990). Our present observations are in line with these studies and indicate that anti-VLA-4α antibodies

stimulate homotypic cell aggregation but are unable to induce binding to isolated ECM components or to VCAM-1, as measured by binding to rTNF-α-stimulated endothelium. Similar observations have been made in a study in which a large panel of anti-VLA-4α antibodies has been tested (Pulido et al., 1991). In contrast to these anti-α antibodies, TS2/16 does not induce homotypic aggregation but stimulates all other VLA-mediated interactions tested so far. Furthermore, the study of Neugebauer et al. (1991) indicates that various VLA-mediated functions can be distinctly regulated through the $\beta 1$ subunit. They have observed that an anti-$\beta 1$ antibody inhibits adhesion to vitronectin but at the same time stimulates adhesion to LM and Coll. From these results two conclusions may be drawn: (a) VLA-mediated functions can be differentially regulated at the cell surface, depending on the epitope that is triggered; and (b) VLA-mediated homotypic cell aggregation may require other signals than interactions of VLA molecules with ECM or VCAM-1. This latter conclusion is supported by the observation that VLA-4-mediated homotypic adhesion is not mediated through VCAM-1 (Pulido et al., 1991). Moreover, the T and B cells used in this study do not express VCAM-1 (Table III).

Only a limited number of antibodies is capable of stimulating VLA integrin-mediated functions. Merely anti-α antibodies directed against certain epitopes of VLA-4 induce aggregation (Pulido et al., 1991), whereas of all the antibodies tested in this study, TS2/16 is the only anti-$\beta 1$ antibody capable of inducing strong binding of cloned T lymphocytes and EBV-transformed B lymphocytes to ECM and VCAM-1. This observation suggests that TS2/16 recognizes a unique epitope on the $\beta 1$ subunit despite the fact that other antibodies, A-1A5 and AIIB2, have been allocated to the same or a closely related site (Fig. 3 B; Hemler et al., 1984). Cross-blocking and adhesion inhibition studies (Fig. 3) suggest that A-1A5, AIIB2, and TS2/16 recognize partially overlapping, but functionally different epitopes. The differences observed between cloned T and EBV-transformed B cells in VLA-mediated adhesion can be due to variable cleavage of the α-4 molecule (Hemler et al., 1990), or to differences in glycosylation, which can directly alter the binding properties of antibodies to VLA-4. Similar to these findings, induction of $\beta 2$ or $\beta 3$ integrin-mediated adhesion is also limited to antibodies directed against specific epitopes (Van Kooyk et al., 1991; Gulino et al., 1990; Kouns et al., 1990). Little is known about the intracellular events stimulated by TS2/16 or by the stimulating anti-VLA4α antibodies that induce aggregation. The functional data suggest different signalling pathways depending on the epitope against which the antibody is directed. So far we can not dissect differences in signal transduction. PMA, which stimulates both homotypic adhesion and binding to ECM or VCAM-1 and thus behaves like a stimulating anti-VLA-4α and anti-β antibody, is sensitive to PKC inhibitors, whereas TS2/16-stimulated adhesion is insensitive. These findings suggest that two or three distinct intracellular events may be operative in VLA-4-mediated adhesion. Additional evidence supporting the concept that intracellular signals are generated by VLA integrins and regulate leukocyte functions, comes from observations that VLA-ligand interactions alone, or in conjunction with anti-CD3 antibodies, stimulate serine esterase release by cytotoxic T cells, T lymphocyte proliferation, and cytokine production by T helper cells or hybridoma cells (Davis et al.,

1990; Nojima et al., 1990; Yamada et al., 1991). Alternatively, binding of antibodies against VLA-4 or $\beta 1$ may distinctly affect the tertiary structure of the molecules, thus, resulting in different binding properties of VLA-4.

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