An Alternative Leukocyte Homotypic Adhesion Mechanism, LFA-1/ICAM-1-independent, Triggered through the Human VLA-4 Integrin

M. R. Campanero, R. Pulido, M. A. Ursa, M. Rodríguez-Moya, M. O. de Landázuri, and F. Sánchez-Madrid

Servicio de Inmunología, Hospital de la Princesa, Universidad Autónoma de Madrid, Diego de Leon, 62.28006 Madrid, Spain

Abstract. The VLA-4 (CD49d/CD29) integrin is the only member of the VLA family expressed by resting lymphoid cells that has been involved in cell–cell adhesive interactions. We here describe the triggering of homotypic cell aggregation of peripheral blood T lymphocytes and myelomonocytic cells by mAbs specific for certain epitopes of the human VLAα4 subunit. This anti-VLA-4-induced cell adhesion is isotype and Fc independent. Similar to phorbol ester–induced homotypic adhesion, cell aggregation triggered through VLA-4 requires the presence of divalent cations, integrity of cytoskeleton and active metabolism. However, both adhesion phenomena differed at their kinetics and temperature requirements. Moreover, cell adhesion triggered through VLA-4 cannot be inhibited by cell preincubation with anti-LFA-1α (CD11a), LFA-1β (CD18), or ICAM-1 (CD54) mAb as opposed to that mediated by phorbol esters, indicating that it is a LFA-1/ICAM-1 independent process. Antibodies specific for CD2 or LFA-3 (CD58) did not affect the VLA-4-mediated cell adhesion. The ability to inhibit this aggregation by other anti-VLA-4-specific antibodies recognizing epitopes on either the VLAα4 (CD49d) or β (CD29) chains suggests that VLA-4 is directly involved in the adhesion process. Furthermore, the simultaneous binding of a pair of aggregation-inducing mAbs specific for distinct antigenic sites on the α4 chain resulted in the abrogation of cell aggregation. These results indicate that VLA-4-mediated aggregation may constitute a novel leukocyte adhesion pathway.

THE integrin superfamily includes receptors for extracellular matrix (ECM)[1] components as well as receptors involved in cell–cell adhesive interactions (19, 38). Structurally, the integrin molecules are α_1:β_1 heterodimers, in which the α subunit is noncovalently associated with the β chain. Three subfamilies of integrins have been classically described, each one defined by a common β subunit (designated as β1, β2, and β3) that shares multiple α subunits (19, 38).

The β2 integrins make up the leukocyte LFA-1, Mac-1, and p150,95 family (23, 34). Mac-1 and p150,95 function as complement receptors and they are also involved in the adhesion of monocytes and granulocytes to endothelial cells and other substrates. The LFA-1 member is implicated in cell–cell interactions, thus being essential in the adhesive function of all leukocyte cell types (23, 39). The β3 integrins are ECM protein receptors and include the vitronectin receptor and platelet glycoprotein IIb/IIIa (20, 29).

Finally, the β1 integrin subfamily, also known as VLA proteins, contains at least six different α chains (VLA-1–VLA-6). Among the VLA proteins, VLA-2, VLA-5, and VLA-6 function as receptors for ECM proteins such as collagen, fibronectin, and laminin, respectively, whereas VLA-3 binds both fibronectin and laminin (5, 12, 13, 32, 38). Recently, a role has been suggested for the VLA-4 integrin in intercellular leukocyte interactions (16, 40). Alternatively, VLA-4 can also function as a receptor for ECM components and has recently been shown to mediate T lymphocyte attachment to human fibronectin (43).

The VLA-4 integrin is expressed on thymocytes, resting and activated peripheral blood lymphocytes, monocytes, T and B cell lines and myelomonocytic cell lines (9, 13, 15, 35). The association between α4 and β chains is weaker than other VLA α/β heterodimers and it can be readily broken by detergents or high ionic concentrations (15, 35). Recently, cDNA clones for the α4 subunit have been isolated and sequenced (40).

Within the VLA family, VLA-4 is atypical because it is the only VLA heterodimer reported to participate in cell–cell interactions. Thus, VLA-4 has been previously shown to be involved in effector–target cell association during cytolytic T lymphocyte-mediated killing based on the inhibitory effect exerted by anti-VLA-4 mAb on this activity (7, 40). Similarly, an anti-VLA-4 antibody was also suggested to block heterotypic interaction between helper and suppressor cells (21). The mouse homologue of VLA-4, designated as

1. *Abbreviations used in this paper*: ECM, extracellular matrix; ICAM-1, intracellular adhesion molecule-1; LFA, lymphocyte function–associated antigen; VLA, very late activation antigen.

LPAM-1, has been recently identified as a Peyer's patch homing receptor (17). Moreover, the LPAM-1 α chain can be found as two heterodimeric forms, one in association with β1 (LPAM-1), and another associated with a novel β chain (βp) (LPAM-2) (18). Both VLA-4 molecular complexes function as accessory molecules in organ-specific adhesion to Peyer's patch high endothelial venules (HEV) (16). LFA-1, a member of the β2 family of integrin, is also involved in both leukocyte intercellular adhesive interactions and lymphocyte binding to endothelium. Recently, an mAb specific for the LFA-1 α subunit has been shown to trigger homotypic adhesion (22).

To examine the possible role of VLA-4 in homotypic leukocyte interactions, we have assayed a wide panel of anti-VLA-4 mAb for both induction and inhibition of homotypic cell aggregation. The anti-VLA-4 HP antibodies used in this study were originally reported to recognize distinct epitopes of a novel association within VLA (35), now designated as VLA-4 (12, 15). Further detailed comparative biochemical and cell distribution studies of HP mAb with respect to other anti-VLA-4 reagents of the IV Leukocyte Differentiation Antigen Workshop have demonstrated their specificity for the VLA-4 (CD49d) integrin (24, 36).

The present report demonstrates that homotypic cell aggregation can be triggered by mAb specific for two different epitopes on VLAα4 chain. The study of the characteristics of this cell aggregation suggests that VLA-4 may constitute a cell adhesion pathway independent from that mediated through LFA-1/ICAM-1 molecules.

Materials and Methods

Cells

Peripheral blood lymphocytes (PBL) were obtained from the heparinized venous blood of normal volunteers by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) centrifugation. T cells were purified from peripheral blood mononuclear cells by removal of adherent cells on plastic petri dishes followed by passage through a nylon wool column. U-937 myelomonocytic cell line and Jurkat and JM leukemic T cell lines were grown in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% FCS (Flow Laboratories), 2 mM L-glutamine, and 50 μg/ml gentamicine (complete medium).

Monoclonal Antibodies, F(ab')$_2$ and Fab Fragments

mAb HP1/1, HP1/3, HP1/7, HP1/2, HP2/1, HP2/4, HP2/7, and B-5G10, are all directed against the α chain of VLA-4 (CD49d) (15, 35). mAb TS2/16 and K20 recognize the β chain of VLA (CD29) (3, 14). mAb B-5G10 and K20 were obtained from the VLA panel of the IV Leukocyte Differentiation Antigen Workshop. mAb TS1/11 and TS1/18 are specific for the α (CD11a) and β (CD18) chains of LFA-1, respectively (34). mAb RR1/1 recognizing ICAM-1 (CD54) (31) was kindly provided by Dr. T. A. Springer (Harvard Medical School, Boston, MA). mAb TS2/18 and TS2/9 recognize CD2 and LFA-3 (CD58), respectively (33). mAb W6/32 is specific for a monomorphic determinant on HLA-A,B molecules (4). F(ab')$_2$ and Fab fragments of anti-VLAα4 HP2/4 mAb were prepared as previously described (28).

Aggregation Assays

Cells were washed twice with RPMI 1640 medium and resuspended to a concentration of 1×10^6 cells/ml for cell lines or 2×10^6 cells/ml for T lymphocytes. Aliquots of 50 μl of cell suspensions were added to each well of flat-bottomed, 96-well microtiter plates (Costar Corp., Cambridge, MA). Then 10 μl of appropriate mAb culture supernatants, purified mAb or its corresponding F(ab')$_2$ and Fab fragments, or phorbol myristate acetate (Sigma Chemical Co., U.K.) were added to give a final concentration as indicated under each Figure. Finally, wells were filled with complete medium to a final volume of 100 μl. Cells were allowed to settle into a cell incubator at 37°C and 5% CO_2 atmosphere. Then, cell aggregate formation was determined at different periods of time by at least two independent observers by direct visualization of the plate with an inverted microscope. Photomicrographs were taken at varying times with a photo camera coupled to the microscope. We considered that aggregation induction assay was positive when more than 50% of the cells were aggregated.

For quantitative measurement of cell aggregation, a modification of the method described by Keizer et al. (21) was used. Cells were seeded in flat-bottomed microtiter plates as described above. The number of free cells was counted by using a special mask, consisting of squares (0.5 mm), under the plate. Within each well, at least five randomly chosen areas were counted, after which the mean and the total number of free cells by well was calculated. Percent aggregation was determined by the following equation: percent aggregation = 100 × (1−[number of free cells])/(total number of cells). The experiments were carried out in duplicate and the SD within each experiment was always <10%.

Table I. Effect on Leukocyte Homotypic Aggregation of Different Anti-VLAα4 (CD49d) and VLA-β (CD29) mAb

mAb	CD	Isotype	Inhibition of PMA-induced cell aggregation*	Induction of cell aggregation‡	VLA-4 epitope§
HP1/1	CD49d	IgG1	−	+	A
HP1/3	CD49d	IgG3	−	+	A
HP1/7	CD49d	IgG1	−	+	A
HP2/7	CD49d	IgG2a	−	+	A
HP1/2	CD49d	IgG1	−	−	B
HP2/1	CD49d	IgG1	−	−	B
HP2/4	CD49d	IgG1	−	+	B
B-5G10	CD49d	IgG1	−	−	
TS2/16	CD29	IgG1	−	−	
K20	CD29	IgG2a	−	−	
TS1/18	CD18	IgG1	+	−	
TS1/11	CD11a	IgG1	+	−	
RR1	CD54	IgG1	+	−	

* U-937 cells were preincubated for 30 min at 37°C with a 1:10 dilution of mAb culture supernatant before treatment with 20 ng/ml of PMA.
‡ U-937 cells were treated with 1:10 dilution of mAb culture supernatant.
§ VLAα4 epitopes are as previously described (16), based on cross-blocking cell binding assays.
Inhibition or induction of cell aggregation was estimated after 20 h of culture at 37°C as described in Materials and Methods.

Table II. Induction of Homotypic Cell Aggregation by Purified Ig, F(ab')$_2$ and Fab Fragments of Anti-VLAα4 mAb

mAb	CD	Antibody preparation	Induction of cell aggregation	
			Peripheral blood T lymphocytes	U-937 cells
HP2/4	CD49d	Total Ig	+	+
HP2/4	CD49d	F(ab')$_2$	+	+
HP2/4	CD49d	Fab	+	+
HP2/1	CD49d	Total Ig	−	−

T lymphocytes and U-937 cells were treated either with 10 μg/ml of HP2/1 purified Ig, or with 1 μg/ml of purified Ig, F(ab')$_2$, or Fab fragments of HP2/4 mAb. Induction of cell aggregation was estimated after 4 h of culture at 37°C as described in Materials and Methods.

Results

Cell Adhesion Induced through Epitopes of VLA-4 Integrin

To evaluate the possible role of VLA-4 integrin in homotypic cell adhesion, we tested the functional effects of mAb against VLA-4 either on the induction or blockade of cell aggregation, using the U-937 myelomonocytic cell line as a model. The results of this assay are summarized in Table I. Four anti-α4 (CD49d) (HP1/1, HP1/3, HP1/7 and HP2/7) mAbs, recognizing an identical or closely related antigenic site (epitope A), previously defined by cross-blocking binding experiments (35), were able to induce U-937 homotypic cell aggregation. The same result was obtained with one anti-α4 (HP2/4) mAb specific for other different antigenic sites (epitope B). By contrast, no inductive effect on adhesion was observed with two anti-α4 (HP1/2 and HP2/1) mAb specific for site B, another anti-α4 (B-5G10) mAb, and two anti-β chain (CD29) mAb (Table I). Homotypic cell adhesion triggered through the VLA-4 integrin was also operative in resting peripheral blood lymphocytes and purified T cells, as well as on leukemic T cell lines Jurkat and JM (Tables II and III and data not shown).

Purified anti-VLA-4 HP2/4 mAb as well as the corresponding F(ab')$_2$ and Fab fragments triggered strong homotypic cell adhesion (Table II). The effect was dose-dependent and could be observed at IgG and F(ab')$_2$ fragment concentrations as low as 50 ng/ml. However, higher concentrations of monovalent antibody Fab fragments (0.5 μg/ml) were

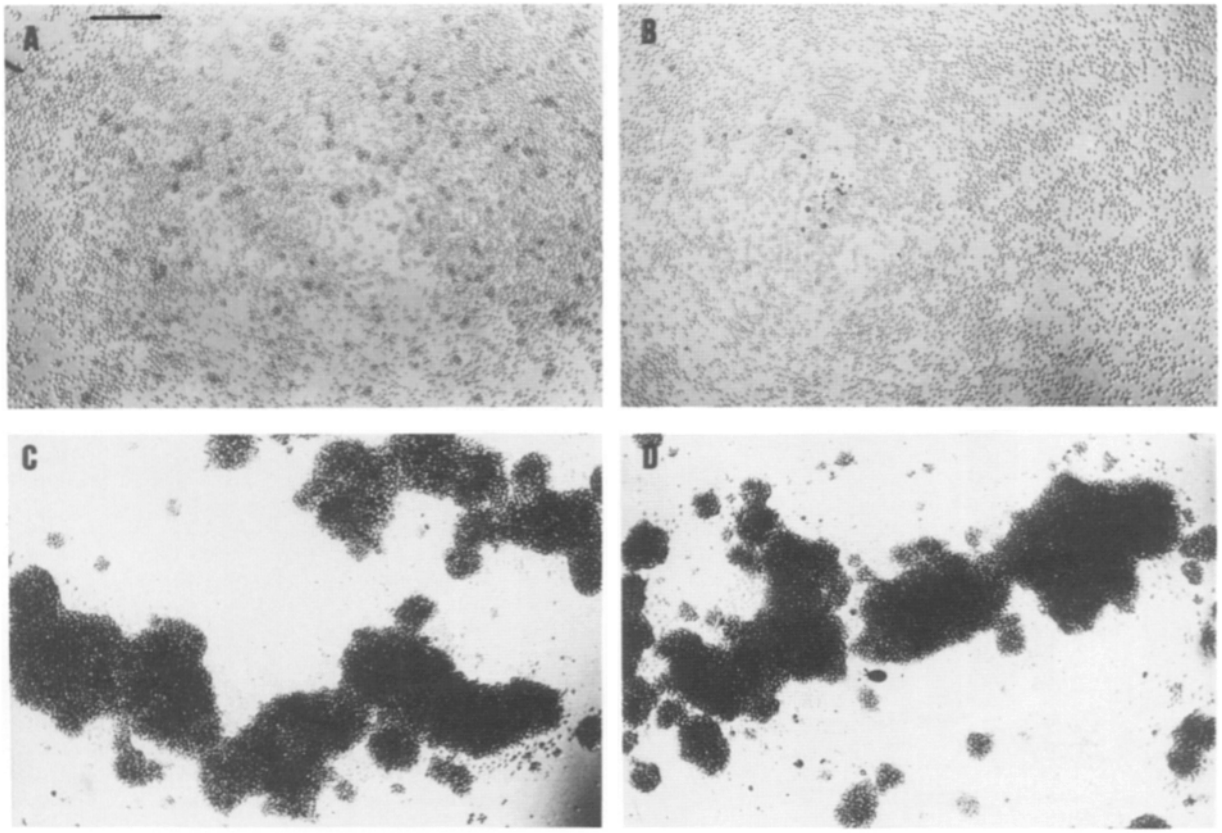

Figure 1. Homotypic adhesion induced by anti-VLA-4 mAb. U-937 cells were incubated for 20 h at 37°C in the presence of: (*A*) 10 μg/ml of the noninducer aggregation purified anti-VLAα4 HP2/1 mAb; (*B*) anti-VLA β chain TS2/16 mAb; (*C*) 1 μg/ml of the aggregation inducer anti-VLAα4 HP2/4 mAb; (*D*) 1 μg/ml of the F(ab')$_2$ fragment of HP2/4 mAb. Photomicrographs were taken after 20 h from the beginning of the assay. Bar, 250 μm.

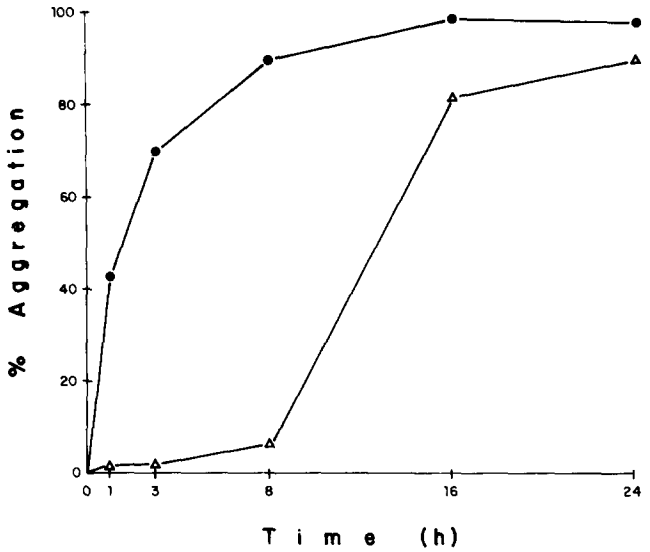

Figure 2. Kinetics of aggregation of U-937 cells induced by anti-VLA-4 mAb. U-937 cells were incubated at 37°C either with 1 μg/ml of HP2/4 mAb (●) or with 20 ng/ml of PMA (△). Aggregation was quantitatively determined as described in Materials and Methods.

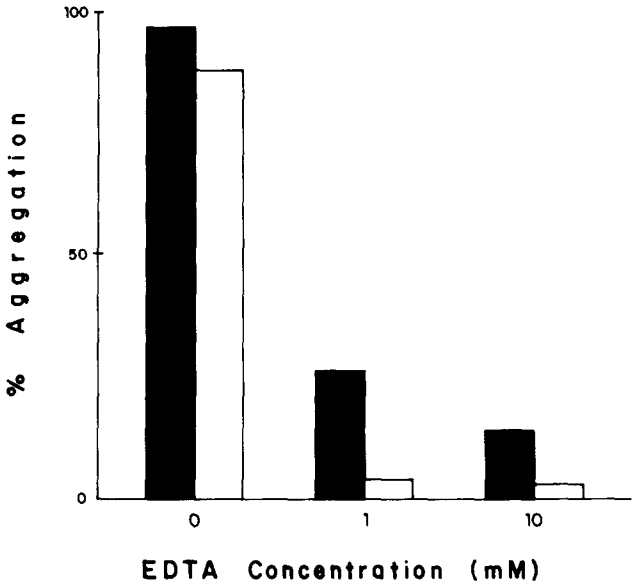

Figure 4. Cell aggregation through VLA-4 requires the presence of divalent cations. U-937 cells were preincubated with 0, 1, or 10 mM EDTA for 30 min and then, either 1 μg/ml of HP2/4 mAb (*shaded bars*) or 20 ng/ml of PMA (*open bars*) were added for the following 20 h of culture. Then, percent of aggregation was determined.

necessary to induce cell aggregation (data not shown). A representative experiment that demonstrates the specificity of the VLA-4-mediated cell adhesion by HP2/4 mAb is visualized in Fig. 1 (*C* and *D*). The lack of induction of cell aggregation in the presence of either anti-VLA-4 HP2/1 or anti-VLA-β TS2/16 mAb is also shown (Fig. 1, *A* and *B*, respectively).

Thus, the effect on cell aggregation of anti-VLA-4 mAb is isotype and Fc independent as well as epitope specific, since it is selectively induced by mAb to specific antigenic sites. Moreover, the cell aggregation assay has allowed the distinction of two sites with different functional behavior within epitope B, previously defined by cross-blocking binding experiments (35).

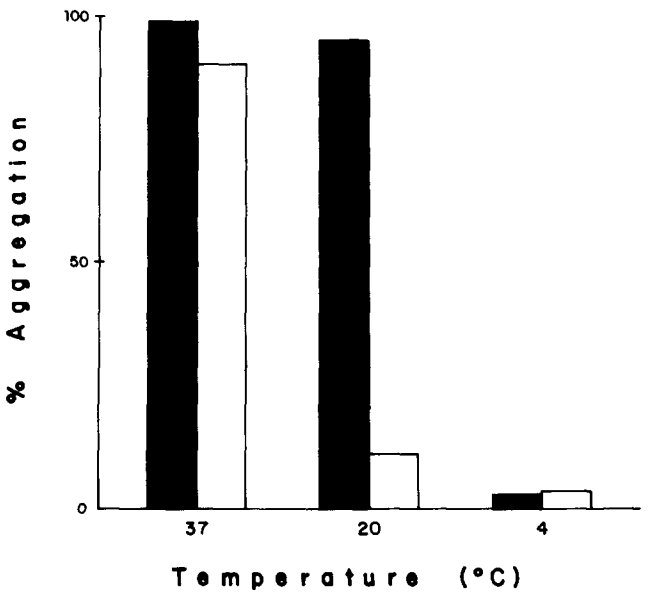

Figure 3. Temperature dependence of the VLA-4-mediated cell aggregation. U-937 cells were incubated at 37, 20, or 4°C either with 1 μg/ml of HP2/4 mAb (*shaded bars*) or with 20 ng/ml of PMA (*open bars*). Percent of cell-cell homotypic adhesion was measured after 20 h of culture as described in Materials and Methods.

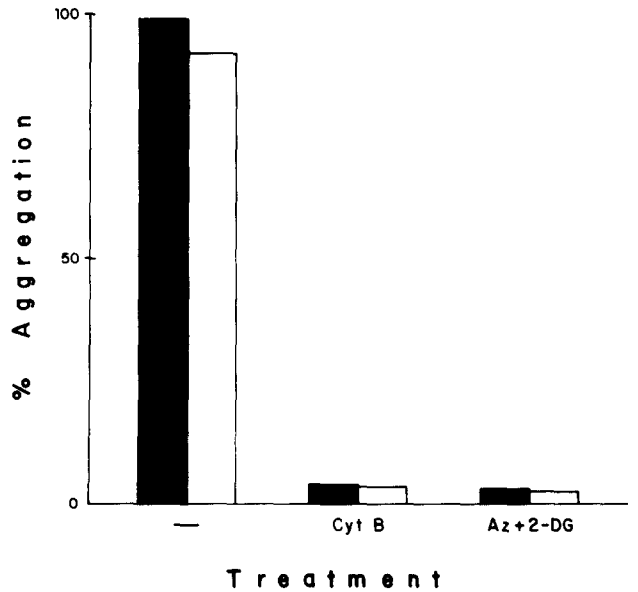

Figure 5. VLA-4 induced cell aggregation requires cytoskeleton integrity and active metabolism. U-937 cells were pretreated for 30 min at 37°C with the following reagents: no treatment; 20 μM cytochalasin B; or a mixture of 50 mM 2-deoxy-D-glucose plus 10 mM sodium azide. Then, cells were treated either with 1 μg/ml HP2/4 mAb (*shaded bars*) or with 20 ng/ml PMA (*open bars*) for the following 20 h of culture, and the aggregation was quantified.

Requirements of VLA-4-mediated Cell Aggregation

Next, we studied the characteristics of VLA-4-mediated cell adhesion compared with that induced by phorbol esters. The kinetics of the homotypic adhesion were clearly different in both systems (Fig. 2). Cell adhesion could be rapidly induced in the presence of anti-VLA-4 mAb and was observed after 1 h of mAb treatment, reaching the plateau at 8 h, when the PMA-induced aggregation had not yet taken place. Therefore, both stimuli appear to be using different pathways for triggering cell aggregation.

Differences between both adhesion systems were also observed by studying the temperature requirements (Fig. 3). Although no aggregation was found with both stimuli at 4°C, the anti-VLA-4 mAb was able to exert its effect at 20°C, whereas no cell aggregation was observed with PMA under these conditions. Both anti-VLA-4 and PMA induced cell adhesion at 37°C.

Similarly to cell adhesion triggered by PMA, VLA-4-mediated cell aggregation required the presence of divalent cations as demonstrated by the dose-dependent inhibitory effect of the Ca^{2+}/Mg^{2+} chelating agent EDTA (Fig. 4). Moreover, the integrity of cytoskeleton as well as an active metabolism were also necessary in both anti-VLA-4 and PMA induced cell adhesions since inhibitors of both processes totally abrogated the adhesion inductive effects (Fig. 5).

VLA-4-induced Cell Aggregation Is Independent of LFA-1/ICAM-1 Adhesion Pathway

Homotypic adhesion can also be induced by mAb against such molecules as syalophorin (26). In this case, the aggregation appears to be LFA-1/ICAM-1 mediated. To ascertain whether VLA-4-mediated cell aggregation was functionally related to the LFA-1/ICAM-1 molecular adhesion pathway, cross-blocking cell adhesion experiments were carried out by using as inhibitors mAb specific for the LFA-1 complex (CD11a, CD18) and the ICAM-1 (CD54) molecule.

U-937 cells or peripheral blood T lymphocytes were preincubated with anti-CD18 mAb, and then, either PMA or the adhesion inducer anti-α4 HP1/7 mAb were added as inductive stimuli. The anti-CD18 mAb virtually abolished the PMA-induced adhesion, but did not affect the VLA-4 mediated cell aggregation (Table III and Fig. 6, *A* and *B*, respectively). The same results were obtained by using as inhibitors mAb specific for CD11a and ICAM-1 molecules (Tables I and III). Furthermore, when anti-LFA-1 mAb were added after disruption of VLA-4-induced cell aggregates no inhibitory effects were observed (data not shown). These results indicate that VLA-4-mediated cell adhesion is functionally independent of LFA-1/ICAM-1 adhesion pathway.

Similar studies of cross-blocking cell adhesion performed on purified T cells with anti-VLA-4 as adhesion inducer mAb and anti-LFA-3 (CD58) and anti-CD2 as inhibitor mAb also indicated that both VLA-4 and CD2/LFA-3 adhesion mechanisms are functionally unrelated and independent (Table III).

Blockade of Cell Aggregation by Simultaneous Binding of Antibodies Specific for Different Sites on VLAα4 and β Chains

To determine whether the VLA-4 molecule itself was mediating the U-937 homotypic adhesion triggered by anti-VLAα4 mAb, we analyzed the influence on cell aggregation of the simultaneous binding of pairs of different mAbs specific for different antigenic sites on α4 and β VLA chains. Preincubation of U-937 cells with anti-VLA β or the anti-VLAα4 HP2/1, a non-adhesion-inducing mAb specific for epitope B, resulted in a profound inhibitory effect of VLA-4 mediated adhesion through α4 epitope A (Fig. 6, *E* and *F*, respectively). By contrast, none of them affected PMA-mediated adhesion (Fig. 6, *B* and *C* and Table I).

The functional interferences in cell aggregation of mAb-binding to distinct epitopes localized in both α4 and β chains of the VLA-4 complex are summarized in Table IV. All antibodies specific for α4 site B, including the adhesion-inducing (HP2/4) and noninducing (HP2/1 and HP1/2) mAb, were able to virtually abrogate cell aggregation triggered by mAb directed to α4 site A (HP1/1 and HP1/7). Similar inhibitory effects were observed in reciprocal experiments using as adhesion-inducer the mAb specific for site B (HP2/4). Moreover, the non-adhesion-inducing anti-VLAα4 HP2/1 mAb was able to disperse cell aggregates preformed by treatment with adhesion inducers anti-VLAα4 mAb (data not shown). On the other hand, the anti-VLA β TS2/16 mAb blocked cell aggregation induced by anti-VLA-4 mAb specific either for site A or site B. No inhibition of aggregation

Table III. VLA-4-mediated Homotypic Cell Adhesion Is LFA-1/ICAM-1 and CD2/LFA-3 Independent

mAb inhibitor	Specificity (CD)	Cell aggregation induced by anti-VLA-4	
		Peripheral blood T lymphocytes	U-937 cells
		%	
None		67	96
TS1/11	LFA-1α (CD11a)	62	96
TS1/18	LFA-β (CD18)	64	93
RR1/1	ICAM-1 (CD54)	70	85
TS2/18	LFA-2 (CD2)	69	95
TS2/9	LFA-3 (CD58)	73	98
TS2/16	VLA-β (CD29)	28	37
HP2/1	VLA-α4 (CD49d)	10	20
W6/32	HLA-A,B	55	100

T lymphocytes and U-937 cells were preincubated for 30 min at 37°C with 10 μl of inhibitor culture supernatant mAb before treatment with 1 μg/ml of adhesion inducer anti-VLA-4 HP1/7 mAb. Percentages of aggregation were calculated after 20 h of culture as described in Materials and Methods. Similar results were obtained when cell aggregation was monitored at 4 h.

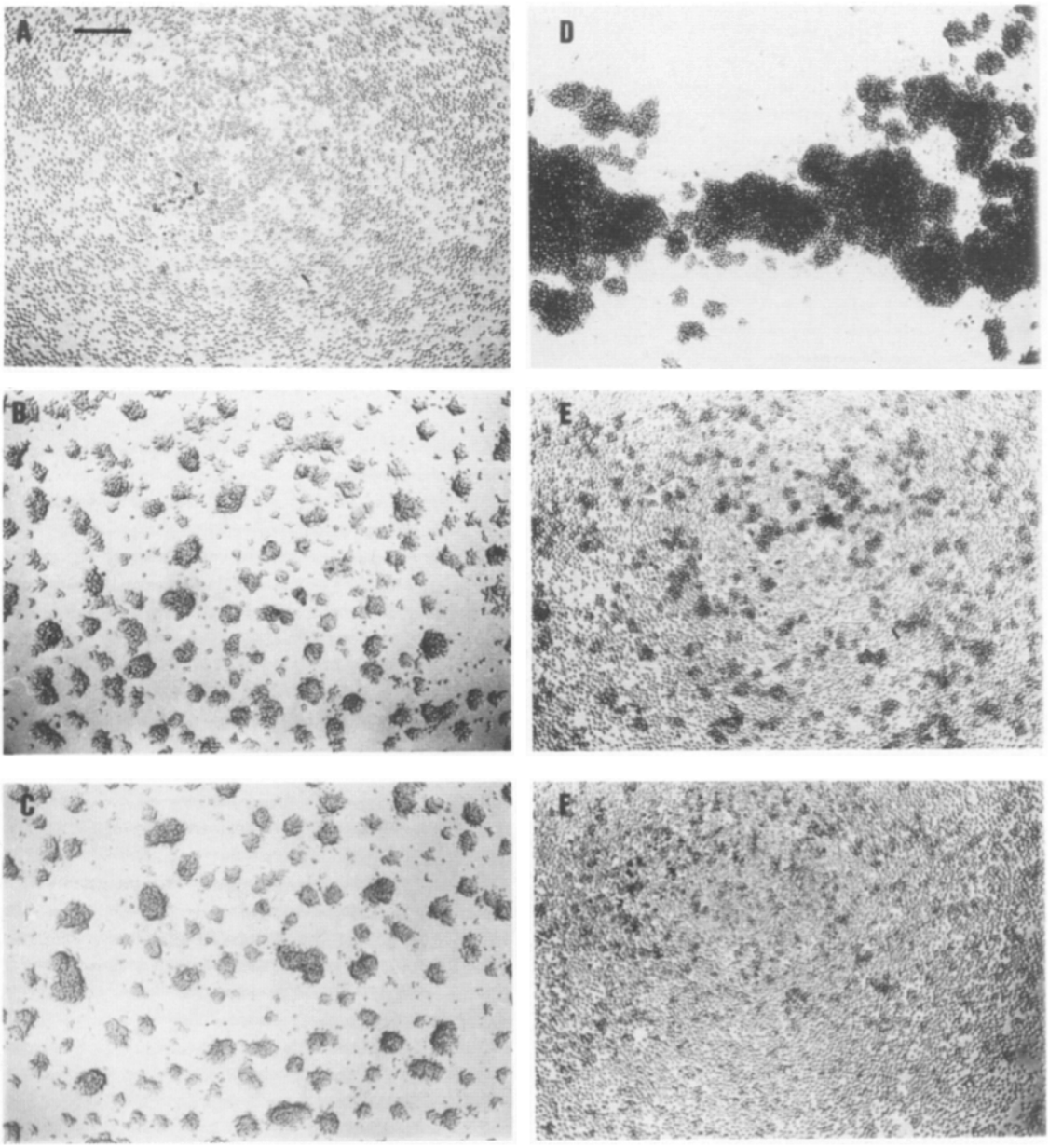

Figure 6. Cell aggregation induced by anti-VLA-4 mAb is LFA-1/ICAM-1 independent, but is interfered by other anti-VLA-4 mAb. U-937 cells were pretreated for 30 min at 37°C with 10 μl of culture supernatant containing the following mAbs: anti-LFA-β TS1/18 (*A* and *D*); anti-VLA-β TS2/16 (*B* and *E*); anti-VLA-4 HP2/1 (*C* and *F*). Then, cells were treated either with 20 ng/ml PMA (*A–C*) or with 1 μg/ml HP1/7 mAb (*D–F*) and photomicrographs were taken after 20 h of culture. It is important to note that HP1/7 mAb recognizes an antigenic site (epitope A) on VLAα4 topographically unrelated to the one defined by HP2/1 mAb (epitope B). Bar, 250 μm.

Table IV. Cross-Neutralizing Effect of Leukocyte Adhesions by Simultaneous Binding of Anti-VLA-4 mAbs Specific for Distinct Epitopes

	Inhibitors (mAbs)						
Adhesion inducing mAb	W6/32 (HLA-A,B)	TS1/18 (CD18)	HP1/1 (CD49d A)	HP1/7 (CD49d A)	HP2/1 (CD49d B)	HP2/4 (CD49d B)	TS2/16 (CD29)
	Cell aggregation (%)						
HP1/1 (CD49d A)	82	80	79	85	11	9	28
HP1/7 (CD49d A)	85	82	75	87	10	12	25
HP2/4 (CD49d B)	73	75	17	15	9	70	22

U-937 cells were preincubated for 30 min at 37°C with 10 μl of inhibitor culture supernatant mAb before treatment for the next 20 h at 37°C with 1 μg/ml of the adhesion-inducing mAb. Afterwards, the percentage of cell aggregation was quantitatively estimated as described in Materials and Methods. Similar results were obtained when cell aggregation was monitored at 4 h.

was observed in the presence of anti-HLA-A or B, or anti-CD18 mAb.

The inhibitory effects of anti-VLA-4 mAb indicate that VLA-4 may be directly involved in the adhesion mechanism, although signaling through VLA-4 cannot be ruled out. In addition, these results demonstrate the existence of at least two different sites on the $\alpha 4$ chain involved in the induction of homotypic cell adhesion. Moreover, cell adhesion triggered by mAb binding to one site can be affected by mAb binding to another site on the same chain or on the associated β chain.

Discussion

In this study, we report data indicating that VLA-4 integrin can mediate leukocyte cell homotypic adhesions. We have found that antibodies recognizing at least two topographically distinct epitopes on the VLA$\alpha 4$ chain are capable of specifically inducing leukocyte cell clustering formation, and that this cell aggregation can be inhibited by other anti-VLA-4 mAb.

Our results indicate that leukocyte cell adhesion triggered through VLA-4 is a process that is Fc independent and site specific, because anti-VLA$\alpha 4$ antibodies of identical isotype but directed to other distinct epitopes on the same $\alpha 4$ chain did not induce cell aggregation. Moreover, anti-VLA$\alpha 4$ F(ab')$_2$ and Fab fragments were able to trigger cell–cell adhesion. However, since higher concentrations of Fab fragments were necessary to trigger the cell aggregation, the possibility cannot be ruled out that cross-linking of VLA-4 molecules on individual cells could also contribute in part to the homotypic adhesion phenomena. Similar results have been recently described for LFA-1, another receptor molecule of the integrin family involved in leukocyte cell–cell adhesive interactions. An antibody to a novel and unique activation epitope on LFA-1 α chain was able to induce homotypic cell adhesion, which could be blocked by other anti-LFA-1 mAb specific for other distinct epitopes (22). Similarly, simultaneous binding of anti-VLA-4 antibodies specific for distinct epitopes localized either on the $\alpha 4$ or on the β chains resulted in the abrogation of VLA-4-mediated cell clustering formation. Hence, VLA-4-mediated cell adhesion, as well as that described for LFA-1, appears to be epitope dependent. In a previous study, we identified two distinct antigenic sites (A and B) on the VLA$\alpha 4$ chain defined by cross-blocking cell binding assays (35). The cell aggregation assay has now allowed the further definition of two sites displaying different functional behavior within epitope B. The relation between these antigen epitopes and putative functional domains awaits further studies.

Comparison of requirements of VLA-4-mediated adhesion with cell clustering formation induced by phorbol esters (LFA-1 mediated) (30) reflects similarities and differences. Both adhesion phenomena require divalent cations, integrity of the cytoskeleton, and active metabolism. However, cell aggregation triggered by anti-VLA-4 mAb takes place more rapidly than that induced by PMA. Most importantly, phorbol ester–induced adhesion is LFA-1/ICAM-1 dependent, whereas VLA-4 mediated seems to be functionally independent because antibodies specific for both LFA-1 and ICAM-1 molecules did not interfere with the anti-VLA-4 triggering of cell aggregation. Likewise, the VLA-4 adhesion pathway is also functionally independent of the CD2/LFA-3 adhesion mechanism (37).

There are two possible alternative explanations for the adhesive effect induced by anti-VLA-4 mAb. First, conformational changes on the VLA-4 integrin mediated by anti-$\alpha 4$ mAb binding may result in a functionally active form of the molecule enabling the interaction with a ligand(s) present either on the cell membrane or in the extracellular environment. In this sense, conformational changes taking place during cell activation have been detected in gpIIb/IIIa, Mac-1, and LFA-1 integrins resulting in active forms with higher efficiencies in their respective receptor–ligand interactions (1, 2, 8, 10, 20, 29, 44).

Second, triggering of intracellular signals by anti-integrin antibodies could also play a role in cell adhesion cluster formation (10). Hence, anti-LFA-1 mAb have been reported both to influence intracellular Ca^{2+} levels and to induce T cell mitogenesis in conjunction with submitogenic concentrations of anti-CD3 mAb (6, 41, 42). Recently, the induction of CD4+ T cell proliferation which is inhibitable by an anti-VLA-β (CD29) antibody has been observed by interaction of fibronectin with the VLA-5 receptor in a serum-free medium culture system (25). Similar inhibitory effects by anti-CD29 mAb have been recently reported in CD4+ T cell proliferation triggered by CD2 or CD3 (21). These authors also detected an increase in cyclic AMP levels induced on activated T cells by anti-CD29 antibody, probably playing a role in these negative signaling effects (21). It will be of interest to measure cAMP concentration in cells treated with either inducer or noninducer adhesion anti-VLA-4 antibodies to learn whether a relation between induction of cell adhesion and levels of this second messenger could be established.

Recently, a function has been ascribed to VLA-4 as an ECM protein receptor, similarly to that described for other VLA members (12, 13). The attachment of T lymphocytes to a 38-kD fragment, containing the Hep II domain and the type III connecting segment of human plasma fibronectin has been demonstrated to be mediated through the VLA-4 integrin (43). In addition, VLA-4 has also been found to interact with a novel cytokine-inducible adhesion molecule on endothelial cells, designated as VCAM-1 (11). This endothelial molecule functions as an adhesion ligand that binds lymphocytes and, like ICAM-1 and ICAM-2, the two ligands of LFA-1 (37), is a member of the immunoglobulin gene superfamily (27). Thus, VLA-4 constitutes a singular example of a $\beta 1$ integrin having a role in both cell–ECM and cell–cell adhesion functions through two identified ligands, fibronectin and VCAM-1. Furthermore, the sites of interaction of VLA-4 with either VCAM-1 on endothelial cells or fibronectin mapped on distinct domains of $\alpha 4$ by means of two antibodies here studied (HP1/3 and HP2/1), representing epitopes A and B, respectively.

Therefore, it will be of interest to study the possible relationship of the inductive effect on cell aggregation of different mAbs directed to VLA$\alpha 4$ with their effects on these two novel receptor functions described for VLA-4 (11, 43). Lymphocyte binding to either fibronectin or endothelial cells provides good models to test whether VLA-4 function could be modulated by mAb binding to different antigenic sites.

The biological significance of the homotypic adhesion triggered by anti-VLA-4 mAb remains unknown. It is con-

ceivable to speculate whether the induction of cell adhesion resembles the effect of the interaction of VLA-4 receptor with one or more physiological ligands. In this sense, we have observed that homotypic cell adhesion triggered through VLA-4 requires the presence of certain amounts of serum (Campanero, M. R., et al., unpublished observations). Whether fibronectin or other cell interacting serum proteins are components necessary for the induction of cell adhesion needs to be elucidated.

On the other hand, we have found that cell adhesion triggered by mAb binding to one epitope on $\alpha 4$ can be blocked by mAb binding to another epitope on the same chain or on the associated β chain. These results may indicate that cell adhesion triggered through VLA-4, similar to that which is LFA-1/ICAM-1 mediated (10), is a dynamic and reversible process, where adhesion–deadhesion could be regulated by binding of either specific antibodies or multiple ligands to different sites of the receptor molecule. Thus, our finding of at least two sites on the $\alpha 4$ chain involved in triggering of cell adhesion, which are topographically separated but functionally interdependent, may provide a suitable, simple, and rapid system to test the influence of multiple ligand interactions.

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