Activation of LFA-1 through a Ca^{2+}-dependent Epitope Stimulates Lymphocyte Adhesion

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Abstract. The leukocyte function–associated molecule-1 (LFA-1) plays a key role in cell adhesion processes between cells of the immune system. We investigated the mechanism that may regulate LFA-1–ligand interactions, which result in cell–cell adhesion. To this end we employed an intriguing anti-LFA-1 α mAb (NKI-L16), capable of inducing rather than inhibiting cell adhesion. Aggregation induced by NKI-L16 or Fab fragments thereof is not the result of signals transmitted through LFA-1. The antibody was found to recognize a unique Ca^{2+}-dependent activation epitope of LFA-1, which is essentially absent on resting lymphocytes, but becomes induced upon in vitro culture. Expression of this epitope correlates well with the capacity of cells to rapidly aggregate upon stimulation by PMA or through the TCR/CD3 complex, in-

dicating that expression of the NKI-L16 epitope is essential for LFA-1 to mediate adhesion. However, expression of the NKI-L16 epitope in itself is not sufficient for cell binding since cloned T lymphocytes express the NKI-L16 epitope constitutively at high levels, but do not aggregate spontaneously. Based on these observations we propose the existence of three distinct forms of LFA-1: (*a*) an inactive form, which does not, or only partially exposes the NKI-L16 epitope, found on resting cells; (*b*) an intermediate, NKI-L16⁺ form, expressed by mature or previously activated cells; and (*c*) an active (NKI-L16⁺) form of LFA-1, capable of high affinity ligand binding, obtained after specific triggering of a lymphocyte through the TCR/CD3 complex, by PMA, or by binding of NKI-L16 antibodies.

T HE lymphocyte function–associated antigen-1 (LFA-1)[1] is a member of the leukocyte cellular adhesion molecule (LeuCAM) group, which belongs to the integrin family of adhesion receptors (Hynes, 1987). The LeuCAM group consists of three structurally related heterodimeric molecules: LFA-1, CR3 (Mo-1), and p150,95 (Sanchez-Madrid et al., 1983). These molecules share a common beta subunit with a molecular mass of 95 kD (CD18), but differ in their alpha subunits with a molecular mass of 170, 165, and 150 kD (for CD11a, b, and c), respectively (Keizer et al., 1985).

LFA-1 is expressed on the cell surface of most leukocytes and plays an important immunoregulatory role by mediating adhesion among leukocytes or between leukocytes and other cell types (Springer et al., 1982, 1987; Martz, 1987). The

function of LFA-1 as an intercellular adhesion molecule has been defined by the ability of anti-LFA-1 mAbs to inhibit adhesion-dependent leukocyte functions, such as cytotoxic T lymphocyte (CTL)–target cell interaction (Sanchez-Madrid et al., 1982; Spits et al., 1986) and many other homotypic and heterotypic cell–cell interactions (Haskard et al., 1986; Mentzer et al., 1986; Rothlein and Springer, 1986). Cell adhesion mediated by LFA-1 is established by binding of LFA-1 to its ligands on the opposing cell. Recently two ligands were defined, intercellular adhesion molecule (ICAM)-1 and -2 (Marlin and Springer, 1987; Staunton et al., 1989). LFA-1–mediated binding is energy and temperature dependent and requires an intact cytoskeleton (Rothlein and Springer, 1986). Furthermore, LFA-1–mediated cell–cell interactions strictly depend on the presence of Mg^{2+} ions, and at suboptimal concentrations also require Ca^{2+} ions (Rothlein and Springer, 1986; Martz, 1980; Dustin and Springer, 1989). Ca^{2+} and Mg^{2+} may contribute to the stabilization of the LFA-1 α,β subunit interaction. Structural studies of the LFA-1 α chain have revealed three cation binding repeats in proximity of the I ("inserted/interactive") domain (Larson et al., 1988). Expression of a Mg^{2+}-dependent epitope located on

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1. *Abbreviations used in this paper:* AIM, activation inducer molecule; CTL, cytotoxic T lymphocyte; ICAM, intercellular adhesion molecule; LFA-1, lymphocyte function-associated antigen-1; PDBU, phorbol-12,13 dibutyrate; PKC, protein kinase C.

the leukocyte integrin α subunits, possibly associated with functionally active molecules (Dransfield and Hogg, 1989), further illustrates the importance of cations in regulation of leukocyte adhesion.

The precise mechanism by which LFA-1 promotes intercellular adhesion has not yet been elucidated. Stimuli that lead to activation of PKC, such as antigen, lectins, or phorbol esters have been shown to induce LFA-1-dependent aggregation of B and T lymphocytes (Rothlein and Springer, 1986; Patarroyo et al., 1985, 1986). Recently, antibodies directed to other cell surface molecules, such as CD2, CD3, or CD43, and antibodies directed against LFA-1 itself, were found to induce LFA-1-dependent aggregation and adhesion (Axelsson et al., 1988; Keizer et al., 1988; Dustin and Springer, 1989; Van Kooyk et al., 1989). These observations suggest that activation of LFA-1 may be required for stable cell adhesion.

In this study we investigated the mode of action of LFA-1 employing a unique mAb, directed against the α chain of LFA-1, that is capable of inducing LFA-1-dependent cell adhesion (Keizer et al., 1988). The results demonstrate that this antibody recognizes a Ca^{2+}-dependent epitope on LFA-1 that is generated upon cell activation. Binding of the antibody stimulates lymphocyte adhesion, possibly by intramolecular changes in LFA-1.

Materials and Methods

Cell Lines and Cell Culture

The human cytolytic, alloreactive T cell clone JS-136 ($CD3^+CD4^+CD16^-$) used in this study is directed against a HLA-DRw6 determinant (Borst et al., 1986). Cloned T cells were cultured in Iscove's medium supplemented with 5% human serum and stimulated weekly with irradiated allogeneic peripheral blood mononuclear cells (PBMC) and cells of the EBV transformed B cell line JY, phytohaemagglutinin (PHA, 0.2 μg/ml) and IL-2 (50 U/ml) (Proleukin, batch LAP-704) kindly provided by Cetus Corporation (Emeryville, CA). JY cells were maintained in Iscove's medium containing 10% FCS. A homogenous population of highly purified T lymphocytes were isolated from buffy coats of healthy donors by centrifugal elutriation, as described previously (Figdor et al., 1981), and were cultured in Iscove's medium containing 10% FCS.

Antibodies

The mAbs SPV-L7 (IgG1), NKI-L15 (IgG1), and NKI-L16 (IgG2a) reactive with the α chain of human LFA-1 (CD11a) and CLB LFA-1/1 (IgG1), reactive with the β chain of LFA-1 (CD18), were raised as described previously (Keizer et al., 1985, 1988; Miedema et al., 1984). mAb RR1/1, directed against ICAM-1 (CD54) was kindly provided by Dr. T. Springer (Center for Blood Research, Boston, MA) (Marlin and Springer, 1987). Direct FITC-conjugated mAb TB30 reactive with the IL-2R α-chain (CD25), was kindly provided by Dr. R. van Lier (Central Laboratory of Blood Transfusion Service, Amsterdam, The Netherlands). Direct FITC-conjugated anti-AIM mAb was kindly provided by Dr. F. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain) (Cebrian et al., 1988). mAbs SPV-T3b (IgG2a), reactive with CD3 and anti-CD71 are described previously (Spits et al., 1983; Van de Rijn, 1983). mAb CEM (anti-KLH, IgG1) was used as control antibody for negative antibody binding. The reaction pattern of a control antibody of the IgG2A isotype did not differ from that of the IgG1 control antibodies (data not shown). Fab fragments of NKI-L16 were generated by papain digestion as described by Mather et al. (1987) and checked for purity by SDS-PAGE, followed by silver staining.

Immunofluorescence

Cells were incubated (30 min, 0°C) in PBS, containing 1% wt/vol BSA (Sigma Chemical Co., St. Louis, MO) and 0.01% wt/vol sodium azide, with appropriate dilutions of the different mAbs, followed by incubation with

FITC-labeled goat F(ab')$_2$ anti-mouse IgG antibody (Nordic, Tilburg, The Netherlands) for 30 min at 0°C. The relative fluorescence intensity was measured by FACScan analysis (Becton Dickinson & Co., Oxnard, CA). To determine the cation-dependent expression of the NKI-L16 epitope on lymphocytes, JS-136 T cells were incubated for 1 h at 37°C with 0.9% NaCl containing 1% wt/vol BSA and 5 mM EDTA or 5 mM EGTA to remove all bound cations. After incubation with EDTA or EGTA the viability of the cells was determined by trypan blue exclusion, and was always >95%. Subsequently, cells were washed twice in cation-depleted PBS (dPBS) to remove the excess of EDTA or EGTA present. Cation-depleted PBS was obtained by rotary mixing PBS with 1% wt/vol Chelex 100 microspheres (Bio-rad Laboratories, Cambridge, MA) for 4 h at 4°C. Reconstitution of cations was followed through the addition of 1 mM $MgCl_2$ or 1 mM $CaCl_2$. Cations were incubated for 30 min at 4°C, after which the fluorescence assay was performed as described, using mAbs that were also depleted for cations, by 4 h incubation with Chelex 100 microspheres.

Qualitative Aggregation Assay

Homotypic aggregation of cells was measured in a qualitative manner by a modification of the method described by Rothlein and Springer (1986). Cells were resuspended in Iscove's medium containing 10% FCS and seeded in 96-well microtiter plates (no. 3595; Costar Data Packaging Corp., Cambridge, MA) at 2 × 10⁵ cells per well in 50 μl. mAbs (0.1-10 μg/ml) or PMA (1-10 ng/ml) were added in a volume of 50 μl. Cells were incubated for various periods (see Results) at 37°C and aggregate formation was determined by at least two investigators using a light microscope. Scores ranged from <1 to 100, where <1 indicated that essentially no cells were aggregated in clusters and 10, 20, 40, 60, 80, and 100 corresponded with cell aggregations of: <10%, <30%, <50%, <70%, >80% in compact clusters, and >90% in large compact clusters, respectively. Cell aggregation was induced by the addition of NKI-L16 mAb, anti-CD3 mAb, PMA, or phorbol-12,13 dibutyrate (PDBU) in concentrations as indicated.

To inhibit cell aggregation, various agents known to inhibit signal transduction were added: Staurosporine (200 nM; Kyowa Hakko, Europe GMBH, Düsseldorf, FRG); 1-0-alkyl-2-0-methyl-glycerol (AMG, 60 mM; Bachem AG Bubendorf, Switzerland); and sodium azide (0.2% wt/vol).

Cation dependence was determined by preincubation of cells with 5 mM EDTA or 5 mM EGTA for 30 min at 4°C, after which the aggregation assay was performed at 37°C.

Quantitative Aggregation Assay

Homotypic aggregation was quantitatively determined by double fluorescence (Kuijpers et al., 1990). Cells (1 × 10⁶ cells/ml in Iscove's medium) were stained separately with the red dye Hydroethidine (HE; Polyscience, Inc., Warrington, PA; 80 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 and 10% FCS and seeded in flatbottom plates, 1 × 10⁵ HE-labeled cells per well. Subsequently, 1 × 10⁵ SFDA-labeled cells were added. Aggregation took place at 37°C, and at different time points cells were fixed with 0.5% paraformaldehyde (PFA) in PBS. The fixed samples were measured in a FACScan. Double-colored aggregates were calculated as a percentage of the total colored events counted. Cell aggregation was induced by the addition of NKI-L16 mAb, anti-CD3 mAb, PMA in concentrations as indicated. Cell aggregation was inhibited by the addition of anti-LFA-1 mAb (LFA-1/1) or anti-ICAM-1 mAb (RR1/1).

Binding of JS-136 T Cells to Purified ICAM-1

JS-136 T cells were labeled with ⁵¹Cr by incubation of cells at 4 × 10⁶ cells/ml with 200 μCi/ml $Na_2^{51}CrO_4$ for 1.5 h at 37°C in complete media. Purified ICAM-1, kindly provided by Dr. S. Shaw (National Institutes of Health, Bethesda, MD), was obtained by immunoaffinity chromatography from a Triton X-100 lysate of cells from the Reed-Sternberg line L428, (Marlin et al., 1987; Makgoba et al., 1988). A 1:100 dilution of purified ICAM-1 was incubated in microtiter wells (Sterilin) in 50 μl PBS overnight at 4°C. These and control wells were blocked with 100 μl of 1% BSA in PBS for 1 h at 37°C after which they were washed twice with PBS. [⁵¹Cr]JS-136 cells were preincubated for 30 min at 4°C with or without mAb NKI-L16 10 μg/ml in Iscove's medium and added (50 μl) to the coated wells. The cells were allowed to settle during a 1-h incubation at 4°C, followed by an incubation at 37°C for 30 min. Nonadherent cells were removed

by washing with warm Iscove's medium. Subsequently, cells were lysed with 100 μl of 1% Triton X-100 in PBS and radioactivity was quantified. Results are expressed as the mean percent of cells binding from three replicate wells.

Radiolabeling and Immunoprecipitation

Cells were surface labeled with $Na^{125}I$ (Amersham International, Amersham, UK) through the lactoperoxidase method (Pink and Ziegler, 1979). For immunoprecipitation, peripheral blood mononuclear cells (PBMCs) (30×10^6) were lysed for 1 h at 4°C in immunoprecipitation buffer (IPB), which contained 1% NP-40, 50 mM Triethanolamine (pH 7.4), 150 mM NaCl and as protease inhibitors, 1 mM PMSF, ovomucoid trypsin inhibitor (0.02 mg/ml, Sigma Chemical Co.; 0.01 M TEA/150 mM NaCl buffer), 0.02 mg/ml leupeptin, and 1 mM Nd-P-tosyl-L-lysine chloromethyl ketone were added. The IPB was depleted for Ca^{2+} and Mg^{2+} ions, or Ca^{2+} ions only, by the addition of 5 mM EDTA or 5 mM EGTA, respectively. Immunoprecipitation studies in the presence of cations were performed through the addition of 2 mM $CaCl_2$ and 2 mM $MgCl_2$, or by the addition of 2 mM $CaCl_2$ only. Nuclear debris was removed from the lysates by centrifugation at 13,000 g for 15 min at 4°C. Lysates were precleared by successive incubations with mouse IgG covalently coupled to protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals, Piscataway, NJ). Precleared lysates were incubated for 3–4 h with a specific mAb coupled to protein A-Sepharose. The immunoprecipitates were removed from the lysates by centrifugation at 13,000 g. Subsequently, immunoprecipitates were washed extensively in 0.01 M triethanolamine/150 mM NaCl buffer, pH 7.8, 1% NP-40, in the presence or absence of 1 mM $CaCl_2$ and/or 1 mM $MgCl_2$.

Electrophoresis and Autoradiography

SDS-PAGE was carried out on vertical slab gels (5–15%) according to a modification of the Laemmli procedure (Laemmli, 1970). Samples were analyzed under reducing conditions, with 5% 2-mercaptoethanol in SDS sample buffer. Kodak XAR-film was used in combination with intensifier screens (Cronex Lightning Plus; Dupont Chemical Co., Newtown, CT) for autoradiography of ^{125}I-labeled materials.

Analysis of Cytosolic Free Calcium ($[Ca^{2+}]i$)

Cells loaded with the fluorescent calcium-sensitive dye indo-1, were used to analyze changes in $[Ca^{2+}]i$. 10^7 cells/ml were incubated in medium containing 1 mM indo-1/AM (Molecular Probes) for 15 min, followed by fivefold dilution with fresh medium and incubation for another 30 min. Subsequently, cells were washed and $[Ca^{2+}]i$ changes were measured upon the addition of mAb, in a spectrofluorimeter (λ excitation = 340 nm and λ emission = 390 nm) after 15 min preincubation at 37°C. Calibration of the fluorescence signal in terms of $[Ca^{2+}]i$ was carried out according to Bijsterbosch et al. (1986).

Results

Expression of the NKI-L16 Epitope of LFA-1 on Resting Versus Activated Lymphocytes

In a previous report (Keizer et al., 1988) we have described a mAb, NKI-L16, that reacts with the α chain of LFA-1, and induces LFA-1-dependent cell aggregation. Here we show that the epitope recognized by NKI-L16 is differently expressed on resting and activated cells. A homogeneous population of resting lymphocytes, isolated from peripheral blood, shows low expression of the NKI-L16 epitope as compared to other LFA-1 epitopes as detected by SPV-L7 and NKI-L15 mAbs (Table I) (Keizer et al., 1988; Larson et al., 1990). Culture of resting lymphocytes in Iscove's medium containing 10% FCS for 24 h resulted in a significant (twofold) increase in expression of the NKI-L16 epitope. This was not accompanied by a concomitant increase in expression of other LFA-1 epitopes, since no enhanced binding of other anti-LFA-1 α (Table I) or β (CLB LFA 1/1; not shown) antibodies was observed. An even greater increase (300%) in expression of the NKI-L16 epitope was found when resting lymphocytes were stimulated with 10 ng/ml PMA (24 h) or with 100 U/ml IL-2 (48 h) (Table I). From these results we conclude that activation of resting cells by in vitro culture, as detected by increased expression of CD25 and activation inducer molecule (AIM), induces expression of the NKI-L16 epitope of LFA-1, whereas the number of LFA-1 molecules expressed at the cell surface is not significantly altered. Expression of ICAM-1, a counter-structure of LFA-1, remained equally expressed during culture, whereas culture in the presence of IL-2 leads to a small (50%) increase in expression. Cloned $CD3^+CD8^+$ or $CD3^+CD4^+$ CTL cells, or cloned $CD3^-$ NK cells, show a twofold higher expression of LFA-1 and ICAM-1 as compared to resting or short term cultured lymphocytes (Table I). However, these cloned T cells express 10-fold higher levels of the NKI-L16 epitope compared to resting cells, compatible with the notion that NKI-L16 recognizes an activation epitope on the LFA-1 molecule.

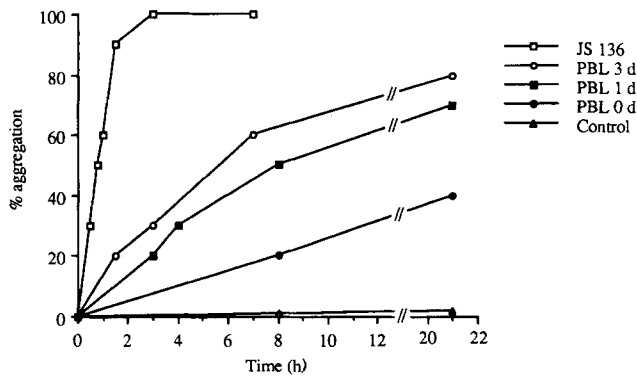*Table I. Effect of Activation of Lymphocytes on the NKI-L16 Expression*

Cell type	Culture period	Relative fluorescence intensity						
		Control	CD25	RR 1/1	SPV-L7	NKI-L15	NKI-L16	NKI-L16/SPV-L7
	d							
*Lymphocytes**								
Medium	0	4	18 (23)‡	41	165	153	32 (19)‡	0.19
	1	4	61 (49)	45	178	150	69 (95)	0.38
	3	3	100 (81)	53	197	172	73	0.37
PMA	1	4	148	75	195	174	85	0.44
IL-2	2	4	113	61	188	167	109	0.58
T cell clone JS-136§								
Medium	5	6	75	81	303	280	248	0.81

* Lymphocytes were cultured for various days in Iscove's medium containing 10% FCS, with or without PMA (10 ng/ml), or IL-2 (100 U/ml).
‡ Percentage positive cells (if less than 100%).
§ JS-136 T cells were used 5 d after the addition of allogeneic feeder cells, PHA and IL-2.
A typical experiment out of four is shown.

Figure 1. Effect of the activation state of lymphocytes on the kinetics of aggregation. Aggregation was induced with optimal concentration PMA (10 ng/ml) and followed in time (scored under microscope). Aggregation of resting PBL, day 0 (●), PBL cultured for 1 (■) and 3 (○) d in Iscove's medium containing 10% FCS, and JS-136 T cells (□) was compared. The spontaneous aggregation of PBL (△) was always <10%. JS-136 cells were used after 5 d of culture in the presence of PHA, IL-2, and allogeneic feeder cells, when spontaneous aggregation was <20%. One representative experiment out of four is shown (SD <10%).

LFA-1-mediated Adhesion Depends on Expression of the NKI-L16 Epitope

mAb NKI-L16 induces homotypic cell aggregation of different lymphoid cells, such as T and B lymphocytes, NK cells (Keizer et al., 1988; Van Kooyk et al., 1989) and monocytes (data not shown), which can be completely bocked by anti-LFA-1 or anti-ICAM-1 mAbs, indicating that the induced aggregation primarily involves the LFA-1/ICAM-1 adhesion pathway. Depending on the activation state of the cells, aggregation is induced within 1-21 h of incubation (Fig. 1). Induction of adhesion by phorbol ester PMA (Fig. 1) or NKI-L16 antibodies (not shown) of freshly isolated resting peripheral blood lymphocytes is slow and takes 21 h of incubation to obtain 40% of the cells in aggregates. However, culture of these resting cells in Iscove's medium containing 10% FCS for 1 or 3 d, leads to activation of the cells, allowing the lymphocytes to respond much faster to PMA or NKI-L16 antibodies, and aggregate (30%) within 2 h after stimulation. Cells of a human allogeneic T cell clone (JS-136), cultured for 5 d in the presence of alloreactive stimulator cells, representing highly activated lymphocytes, as determined by high CD25 and AIM expression, are able to aggregate (40%) within 1 h after stimulation with PMA (Fig. 1), anti-CD3-, or NKI-L16 antibodies (not shown). These results show that NKI-L16 epitope expression clearly correlates with the kinetics of aggregation induced by PMA or other stimuli (data not shown). Low NKI-L16 expression, only found on resting lymphocytes, corresponds with a slow induction of homotypic cell aggregation, whereas preactivated cells with high NKI-L16 expression can be induced to aggregate almost immediately.

These results suggest that the NKI-L16 epitope is an activation epitope and that expression of this epitope is associated with LFA-1-mediated adhesion. However, it should be noted that expression of this epitope in itself is not sufficient to induce aggregation, since CTL cannot aggregate spontaneously, despite high NKI-L16 expression (Fig. 1).

Clustering of integrin receptors has been shown to be strongly associated with adhesion promoting activity (Detmers et al., 1987). To exclude the possibility that the NKI-L16–induced aggregation is the result of crosslinking of LFA-1 molecules or of crosslinking of cells by binding to Fc receptors, Fab fragments of NKI-L16 were tested on their ability to induce cluster formation. Fab fragments were free of any contamination of intact IgG or F(ab')₂ fragments, as judged by SDS-PAGE after silverstaining, which allowed detection of contaminating total IgG or F(ab')₂ at levels as low as 0.2% (Fig. 2, *A* and *B*). In addition, a sensitive ELISA was performed, (detection limit 0.01 ng/ml) in which the absence of intact Fc fragments was confirmed (data not shown). Addition of 1 μg/ml NKI-L16 Fab fragments to T-cell clone JS-136 induced cluster formation (Fig. 2 *C*). Aggregation induced by Fab NKI-L16 was comparable to that induced by purified total IgG, although the Fab fragments were somewhat less effective, possibly due to a lower binding affinity. From these results we conclude that the cluster formation induced by NKI-L16 is the result of specific binding to LFA-1, and is not caused by crosslinking of LFA-1 molecules or by binding to FcR.
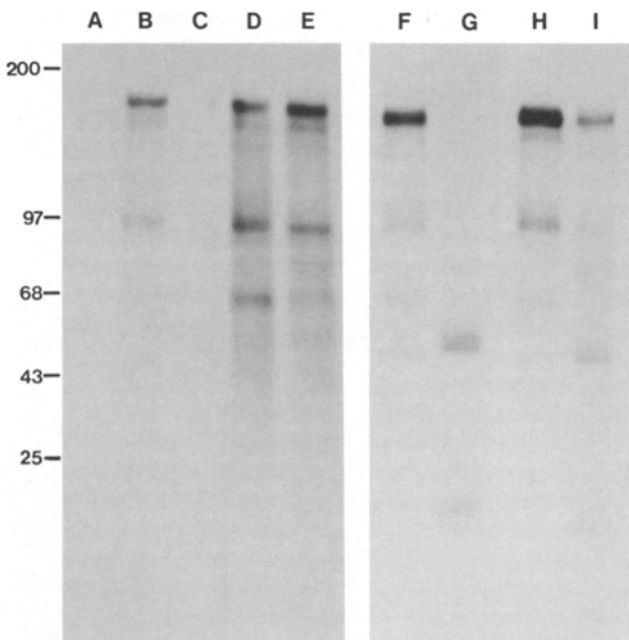
Involvement of Cations in the Formation of the NKI-L16 Epitope

Since Ca²⁺ and Mg²⁺ ions are important for functional activity of the LFA-1 molecule (Rothlein and Springer, 1986), we investigated the role of these cations in the formation of the NKI-L16 epitope. Immunoprecipitation studies revealed that mAb NKI-L16 weakly precipitated LFA-1 when chelating agents such as EDTA or EGTA were present in the lysis buffer, whereas other LFA-1 mAbs, such as NKI-L15, readily precipitated LFA-1 under these conditions (Fig. 3, *B*, *C*, *F*, and *G*). However, in the presence of Ca²⁺ and Mg²⁺ ions (Fig. 3, *D* and *E*), or in the presence of Ca²⁺ alone (Fig. 3, *H* and *I*), similar quantities of LFA-1 were precipitated from peripheral blood mononuclear cells, with NKI-L16 compared to NKI-L15. The fact that the presence of EGTA, which specifically removes Ca²⁺ cations, abrogates LFA-1 precipitation by mAb NKI-L16, indicates that the presence of Ca²⁺ cations is essential for the expression of the NKI-L16 epitope. Although expression of NKI-L16 on resting lymphocytes is low in the presence of cations, NKI-L16 could precipitate equal amounts of LFA-1 compared to other anti-LFA-1 mAbs. It can not be excluded that solubilization of the cell membrane alters the tertiary structure of LFA-1, causing exposure of the NKI-L16 epitope. This notion is supported by the finding that isolation of the LFA-1 molecule yields only the activated form of LFA-1 (Dustin and Springer, 1988).

Cation dependency of the expression of the NKI-L16 epitope was further substantiated by immunofluorescence studies carried out with JS-136 T cells. The NKI-L16 epitope was completely lost upon incubation with EDTA or EGTA (Table II), whereas expression of other epitopes on LFA-1 recognized by the mAbs SPV-L7 or NKI-L15 remained unaffected. Interestingly, addition of CaCl₂ to the EDTA-treated cells completely restored expression of the NKI-L16 epitope, whereas the addition of MgCl₂ could not restore NKI-L16 expression. Addition of Ca²⁺ ions in combination with Mg²⁺ ions resulted in a restored NKI-L16 expression,
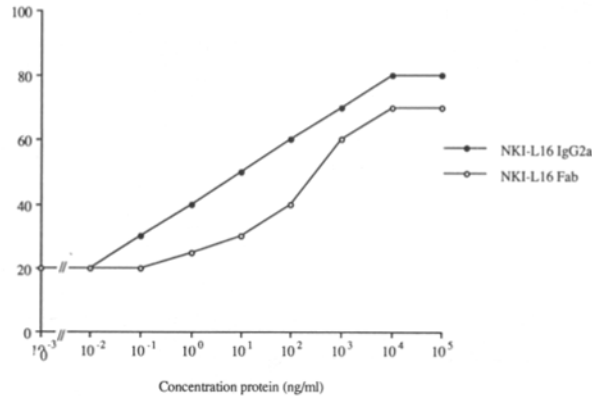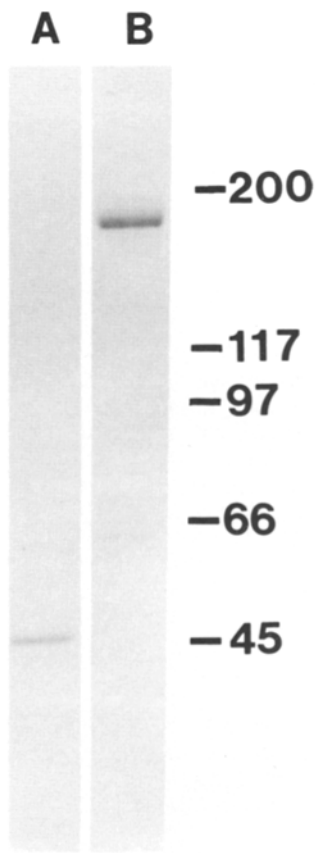
Figure 2. SDS 10% PAGE under nonreducing conditions of 10 μg of NKI-L16 Fab (lane A) and 10 μg of NKI-L16 IgG2a (lane B). (C) Comparison of purified NKI-L16 IgG and NKI-L16-Fab fragments in their capacity to induce homotypic aggregation of JS-136 T cells. The percentage of aggregation was measured qualitatively after 45 min incubation at 37°C. Protein concentrations ranged from 0.1 ng/ml to 10^5 ng/ml total IgG (●), or Fab fragment (○). One experiment out of three is shown (SD <10%).

although expression was somewhat lower compared to addition of only Ca^{2+} ions alone.

These results indicate that the expression of the unique epitope recognized by NKI-L16 depends on the presence of Ca^{2+} ions, which are required to create the NKI-L16 epitope.

Are Signal Transduction Pathways Involved in the NKI-L16–induced Cell Aggregation?

In an attempt to find out how NKI-L16 induces LFA-1–mediated cell adhesion, we compared different stimuli that are able to induce homotypic cell aggregation. The protein kinase C (PKC) activator PMA as well as the functionally important CD3 receptor, expressed on T lymphocytes, are able to induce homotypic cell aggregation (Patarroyo et al., 1985;

Figure 3. The effect of the presence of cations on the immunoprecipitation of LFA-1 from ^{125}I-labeled lysate of peripheral blood mononuclear cells with two different anti-LFA-1 mAbs, NKI-L15 (B, D, F, and H) and NKI-L16 (C, E, G, and I). Immunoprecipitation in the absence of Ca^{2+} and Mg^{2+} ions through addition of 5 mM EDTA (B and C) or in the presence of 2 mM $CaCl_2$ and 2 mM $MgCl_2$ (D and E) and in the absence of Ca^{2+} ions through addition of 5 mM EGTA (F and G) or the presence of 2 mM $CaCl_2$ (H and I). Lane A shows normal mouse control antibody. Bars indicate relative molecular mass (kD) as defined by prestained markers. A representative experiment out of three is shown.

Table II. Effect of Cations on NKI-L16 Epitope Expression

Treatment	Relative fluorescence intensity			
	Control	SPV-L7	NKI-L15	NKI-L16
PBS	5	51	71	67
EGTA	5	76	68	5
EDTA	5	75	55	6
+ Mg^{2+}	7	70	78	11
+ Ca^{2+}	5	79	59	85
+ Mg^{2+} + Ca^{2+}	7	77	55	63

JS-136 T lymphocytes were pretreated with 0.9% NaCl containing 5 mM EDTA or 5 mM EGTA for 1 h at 37°C, to remove all bound cations. Subsequently, cells were washed twice with cation-depleted PBS (dPBS), followed by the addition of 1 mM $MgCl_2$ and/or 1 mM $CaCl_2$ to the EDTA-treated cells, and incubated for 30 min at 4°C. Expression of different epitopes of LFA-1 was determined by immunofluorescence. A typical experiment out of four is shown.

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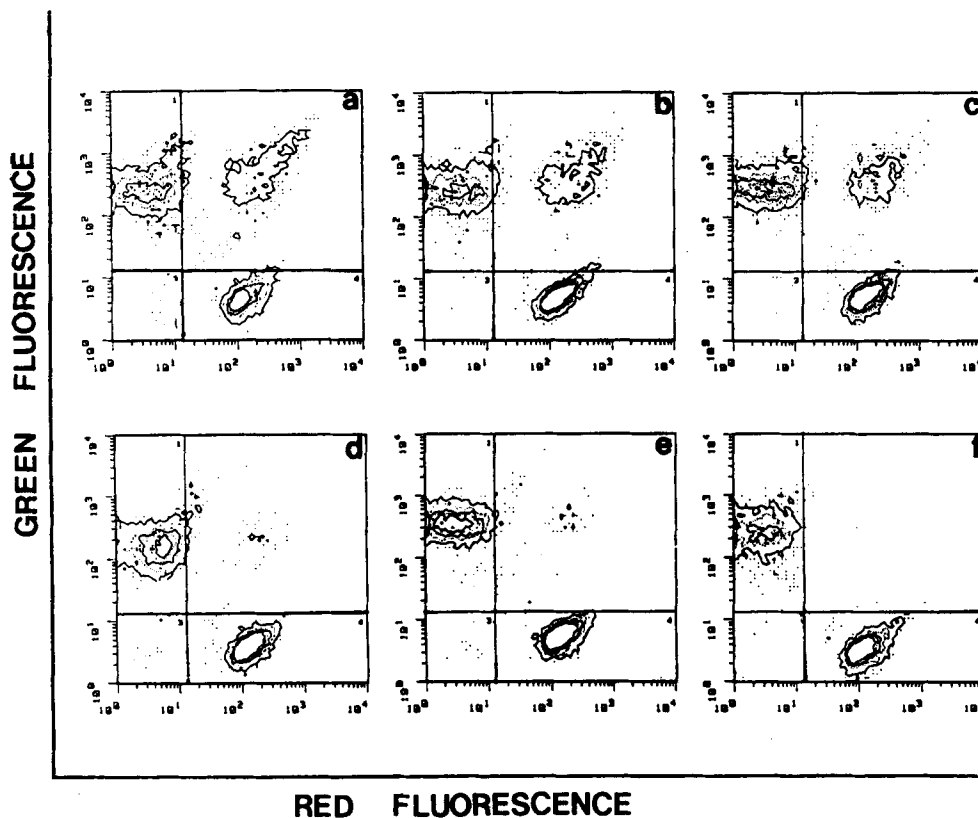
RED FLUORESCENCE

Figure 4. Comparison of the capacity of mAb NKI-L16 (*a*) 0.5 mg/ml (20% aggregation); PMA (*b*), 20 ng/ml (17.4%); and mAb T3b anti-CD3 (*c*), 5 µg/ml (14.7%) to induce aggregation of JS-136 T cells, quantitatively determined by double fluorescence. The NKI-L16–induced aggregation was blocked with anti-LFA-1 mAb CLB-LFA-1/1 (*d*) (1.8%) or anti-ICAM-1 mAb RR1/1 (*e*) (1.3%). Control (*f*) stimulation with anti-CD71 (transferrin receptor). The percentage of aggregates of JS-136 T cells are the percentage of double fluorescent events, after 45 min incubation at 37°C. A typical experiment out of four is shown (SD = 4%).

Rothlein and Springer, 1986; Dustin and Springer, 1989; Van Kooyk et al., 1989). Stimulation of the CD3 molecule by mAbs causes activation of PKC (Imboden and Stobo, 1985), suggesting that activation of PKC directly affects LFA-1–mediated adhesion. Homotypic cell aggregations induced by NKI-L16, PMA, or anti-CD3 antibodies, were

⊢⊣ 1 min
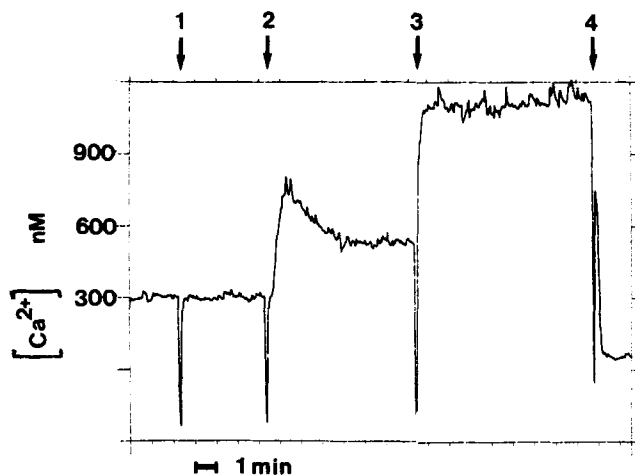
Figure 5. Measurement of [Ca²⁺]i in JS-136 T cells. Addition of 50 µg/ml NKI-L16 (↓1) to 3 × 10⁶ Indo-1–loaded cells was unable to increase (Ca²⁺]i, whereas stimulation with anti-CD3 antibodies (↓2) resulted in an immediate response. Saturation of Indo-1 with extracellular Ca²⁺ was obtained after addition of 0.1% (wt/vol) Triton-X (↓3), and baseline levels of fluorescence were achieved upon addition of MnSO₄ solution (↓4). Similar results were obtained in two other experiments.

compared in a quantitative manner. Purified NKI-L16 mAb or PMA induce aggregation of JS-136 T cells (Fig. 4, *a* and *b*, respectively). Similarly, addition of anti-CD3 antibodies also leads to induction of aggregation of JS-136 cells (Fig. 4 *c*), but not of CD3 negative NK-clone cells, demonstrating the specificity of the response (not shown). Isotype-matched control mAbs did not induce aggregation (Fig. 4 *f*). Cell aggregation induced by NKI-L16 and the two other stimuli (anti-CD3 and PMA, data not shown) was LFA-1 dependent, since it was completely inhibited by anti-LFA-1 antibodies (Fig. 4 *d*), or anti-ICAM-1 antibodies (Fig. 4 *e*). These results demonstrate that these three stimuli are equally capable of inducing cell aggregation.

We next explored the possibility that these stimuli employ a similar activation pathway. PMA activates PKC (Nishizuka, 1984), and has been shown to phosphorylate the β chain of LFA-1 (Hara and Fu, 1986; Chatila and Geha, 1988; Chatila et al., 1989). Thus, phosphorylation catalysed by PKC might activate LFA-1. Alternatively, it has been reported that LFA-1 itself may transduce signals into the interior of the cell (Carrera et al., 1988; Van Noesel et al., 1988). We therefore investigated whether binding of mAb NKI-L16 to LFA-1 leads to signal transduction. The possible involvement of PIP₂ hydrolysis resulting in the production of inositol 1,4,5-triphosphate (IP₃) and diacylglycerol, the physiological activator of PKC (Isakov et al., 1987), was studied indirectly by measuring the intracellular free Ca²⁺ concentration in JS-136 T cells, which would change due to IP₃ production (Isakov et al., 1987). Addition of 50 µg/ml NKI-L16 had no effect on the cytosolic free Ca²⁺ concentration (Fig. 5), whereas subsequent addition of anti-CD3 antibody (5 µg/ml), which enhances the intracellular free Ca²⁺

Table III. Effect of Different Signal Transduction Blockers, Temperature, and Cations on the PMA, NKI-L16 and Anti-CD3 Induced Homotypic Aggregation of T Cell Clone JS-136

	Aggregation of T cell clone JS-136 (%)			
Treatment	Control	PMA	NKI-L16	Anti-CD3
–	10	70	80	60
Staurosporine	10	10	80	20
AMG	10	10	80	10
Sodium azide 1:10	10	40	80	10
1:1	10	10	60	10
4°C	0	0	0	0
37°C + Medium	10	70	80	60
+ EDTA	0	0	0	0
+ EGTA	0	0	0	0

Cells were stimulated with PMA (10 ng/ml) NKI-L16 (10 μg/ml), or anti-CD3 antibodies (10 μg/ml) in the presence or absence of various inhibitors. All inhibitors were added in optimal concentrations (Staurosporine, 200 nM; AMG, 60 μM), and remained present during the test. After 45 min at 37°C, aggregate formation was scored visually. For inhibition with sodium azide (0.2% wt/vol 1:1) cells were preincubated for 1 h at 4°C followed by addition of PMA, NKI-L16, or anti-CD3 antibodies. To determine aggregation in the absence of NKI-L16 epitope expression, cells were preincubated for 30 min at 4°C with 5 mM EDTA or 5 mM EGTA, followed by the aggregtion assay also performed in the presence of 5 mM EDTA or 5 mM EGTA for 45 min at 37°C. Experiments were carried out in triplicate (SD <10%); the mean out of four experiments is shown.

Table IV. Expression of IL-2R and AIM on Lymphocytes Cultured in the Presence of NKI-L16

|	Relative fluorescence intensity		
Lymphocytes cultured in	Control	CD25	AIM
Medium	4 (2)*	45 (43)*	4 (2)*
NKI-L16	3 (2)	30 (24)	4 (2)
IL-2	4 (2)	85 (100)	4 (2)
PMA	4 (2)	49 (73)	127 (100)
NKI-L15	4 (2)	36 (27)	4 (2)

* Percentage positive cells.

Lymphocytes were cultured for 24 h in Iscove's medium containing 10% FCS, in the presence of different activation stimuli (100 U/ml rIL-2 or 10 ng/ml PMA). Antibodies NKI-L16 (10 μg/ml) or NKI-L15 (10 μg/ml) were added to lymphocytes and cultured for 24 h. IL-2R and AIM expression were determined by direct immunofluorescence. A representative experiment out of three is shown.

concentration in T cells (Truneh et al., 1985), led to a significant response.

The possibility that NKI-L16 activates PKC via diacylglycerol, derived from other cellular lipids, was examined by means of different inhibitors (Table III). Two potent PKC blockers, AMG (Van Blitterswijk et al., 1987) and staurosporine (Tamaoki et al., 1986), completely abrogated PMA- and anti-CD3–induced aggregation but were unable to block NKI-L16–induced aggregation. Furthermore, cells in which PKC activity was down-regulated by incubation for 72 h in the presence of PDBU, were unable to aggregate in response to PMA, but were still responsive to NKI-L16 mAb (not shown). These data indicate that NKI-L16 indeed does not induce aggregation by activation of PKC. Addition of azide, an inhibitor of mitochondrial ATP production, abolished PMA- and anti-CD3–induced adhesion but not NKI-L16–induced adhesion (Table III), suggesting that cell adhesion induced by NKI-L16 is exclusively an extracellular phenomenon. Only addition of EDTA, or EGTA, which completely removes NKI-L16 expression, or incubation of cells at 4°C inhibited NKI-L16–induced adhesion, showing that cations, temperature and NKI-L16 expression are both important for LFA-1-mediated adhesion. Moreover, the expression of the NKI-L16 epitope in itself is not sufficient to induce aggregation, since JS-136 T cells do not spontaneously aggregate, but require stimuli to induce aggregation.

To determine whether binding of NKI-L16 indirectly stimulates other cellular processes, through at presently unknown signaling routes, we investigated induction of early activation markers by direct fluorescence studies. We measured expression of the AIM and IL-2R (CD25) that appear early (20 h) on membranes of T cells upon stimulation with PMA or after triggering of TCR/CD3 (Cebrián et al., 1988). Culture of resting lymphocytes for 24 h in the presence of

mAb NKI-L16 neither increased AIM nor IL-2R expression above background levels induced by culturing in Iscove's containing 10% FCS. Culturing of resting lymphocytes with 10 ng/ml PMA, however, resulted in increased expression of AIM as well as IL-2R (Table IV). These results indicate that mAb NKI-L16 solely activates the LFA-1 molecule and does not cause cell activation.

mAb NKI-L16 Induces Adhesion of JS-136 T Cells to ICAM-1

To demonstrate that the induced cell adhesion by NKI-L16 antibodies involves the LFA-1/ICAM-1 interaction, and to prove that LFA-1 activation is involved rather than ICAM-1 activation, we performed adhesion experiments with JS-136 T cells to purified ICAM-1. The finding that mAb NKI-L16 recognizes LFA-1-transfected COS cells (data not shown; Larson et al., 1990) indicates that NKI-L16 recognizes specifically LFA-1 and not possible LFA-1-associated molecules. JS-136 cells are highly activated cells that bind aspecific to BSA for 4% of total cells, but show an increased specific binding to ICAM-1 of 29% (Fig. 6). Incubation of JS-136 T cells with mAb NKI-L16 or Fab fragments thereof for 30 min at 4°C, leads to an enhanced adhesion to ICAM-1 up to 73 and 57%, respectively. Anti-LFA-1 antibodies could block the adhesion to ICAM-1 completely (data not shown). The finding that the Fab fragments are slightly less effective
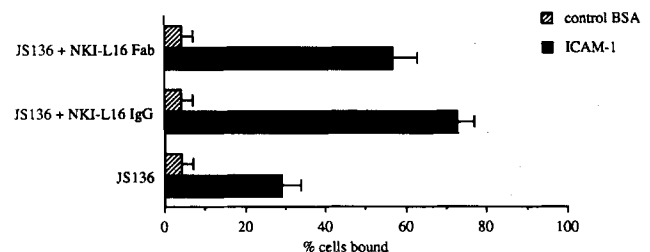
Figure 6. Binding of JS-136 T cells to purified ICAM-1 after 30 min incubation at 37°C. ^{51}Cr-labeled JS-136 T cells were incubated in microtiter wells coated with BSA (▨) or with purified ICAM-1 (■). Separate parts of the cells were incubated for 30 min at 4°C with 50 μg/ml mAb NKI-L16 IgG, or 50 μg/ml NKI-L16 Fab, before the adhesion assay.

in enhancing binding to purified ICAM-1 is in line with the findings of the aggregation assay where the Fab fragments also show lower binding affinity. These data indicate that mAb NKI-L16 or Fab fragments, can specifically activate the LFA-1 molecule thus increasing ligand binding. Furthermore, the increased adhesion through activation of LFA-1, induced by NKI-L16, excludes the involvement of conformational changes of its ligand ICAM-1 or involvement of increased expression of ICAM-1.

Taken together, these experiments indicate that cell aggregation induced by NKI-L16 antibodies is an exclusively extracellular event leading to an increased binding to ICAM-1, which is not due to crosslinking phenomena or to demonstrable induction of signaling pathways that stimulate other cellular processes. We therefore hypothesize that binding of NKI-L16 to the extracellular portion of the LFA-1 molecule leads to a conformational change in LFA-1, resulting in a high-affinity state of the LFA-1 molecule.

Discussion

In this study we show that the LFA-1 α chain antibody NKI-L16 defines a Ca^{2+}-dependent activation epitope. The epitope is essentially absent from resting lymphocytes (Table I) and monocytes (our unpublished results), but becomes expressed upon in vitro culture, whereas expression of other LFA-1 epitopes remains unchanged. This suggests that lymphocytes must reach a certain activation stage to express this epitope. At present it is not known whether the increased epitope expression upon cell activation is the result of more binding sites that become available or of a greater affinity of the NKI-L16 mAb for the epitope (under investigation). LFA-1-tranfected COS cells express the NKI-L16 epitope (Larson et al., 1990), demonstrating that the epitope is expressed by LFA-1. However we can not rule out the possibility that other molecules interacting with LFA-1 affect NKI-L16 expression, when resting lymphocytes are cultured in vitro.

We observed that the expression of the NKI-L16 epitope correlates with the capacity of lymphocytes to aggregate after triggering with PMA, anti-CD3 antibodies, or NKI-L16. The enhanced capacity of the cells to aggregate could not be attributed to increased LFA-1 expression, which did not alter significantly (Table I). In addition, induction of expression of ICAM-1 after culture with IL-2 (Table I and Dustin and Springer, 1988) is low (50%) compared to the increased NKI-L16 epitope expression (300%). The NKI-L16 epitope thus may distinguish resting lymphocytes from (recently) activated lymphocytes. However, it must be emphasized that expression of the NKI-L16 epitope, although essential, is not sufficient to induce cell adhesion. This is best illustrated by T cell clones (CD4+ or CD8+), or NK cell clones (CD3−), that express constitutively high levels of this epitope, but do not spontaneously aggregate, unless specifically stimulated (by antigen, anti-CD3 antibodies, PMA, or by extracellular binding of NKI-L16 antibodies).

Therefore, we propose that LFA-1 can be expressed on the cell membrane in three distinct forms (Figdor et al., 1990). The first is an inactive form of LFA-1 in which the NKI-L16 epitope is absent or only partially exposed. This form is primarily expressed by resting lymphocytes. The second is an intermediate form that expresses the Ca^{2+}-dependent NKI-

L16 epitope. This intermediate form is expressed by activated PBL and mature lymphocytes, and can be rapidly converted by specific triggering of the TCR/CD3 complex, PMA, or by binding of NKI-L16 antibodies, into the third form, activated LFA-1, which is possibly created by a conformational change of LFA-1. It should be noted that transition of the second (intermediate) form to the third (active) form is not associated with a further increase in expression of the NKI-L16 epitope.

LFA-1-mediated cell adhesion requires the presence of Mg^{2+} and at suboptimal Mg^{2+} concentration also Ca^{2+} ions (Rothlein and Springer, 1986; Marlin and Springer, 1987). They are thought to stabilize the interaction between the α and β subunits of LFA-1 (Corbi et al., 1987) and the LFA-1-ligand complex (Larson et al., 1988). We found that expression of the NKI-L16 epitope is absolutely dependent on the presence of Ca^{2+} cations; binding of Ca^{2+} presumably alters the tertiary structure of LFA-1. Possibly, expression of the NKI-L16 epitope is in some way associated with the formation of clusters of LFA-1 (Figdor et al., 1990) stabilized by Ca^{2+} ions. Preliminary experiments indicate that LFA-1 is clustered on CTL expressing high levels of NKI-L16 compared to dispersed distribution on resting lymphocytes. The fact that NKI-L16 epitope expression is low on resting lymphocytes, in spite of Ca^{2+} presence in serum, indicates that Ca^{2+} binding to LFA-1 is an active process. It is tempting to speculate that the NKI-L16 antibody binds to a site on the LFA-1 molecule that is in close proximity to the cation-binding region.

Recently, Dransfield and Hogg (1989) defined an antibody (mAb 24) that recognizes a strictly Mg^{2+}-dependent epitope on all three leukocyte integrin α subunits, possibly associated with functionally active molecules. Expression of this epitope is temperature and energy dependent and inhibits adhesion-dependent processes. In contrast, mAb NKI-L16 induces LFA-1-dependent adhesion and recognizes an activation epitope restricted to the α chain of LFA-1, which is Ca^{2+} dependent and temperature independent. Both observations suggest that binding of different cations may alter the conformation of LFA-1, and thereby the functional status of the receptor. The importance of cation-binding domains on the functional state of the integrins is also demonstrated by a recently described mAb that reacts with a Ca^{2+}-binding site of GPIIb and induces platelet aggregation (Gulino et al., 1990). Platelet activation is associated with changes in the GPIIb/IIIa complex, most likely by a conformational change.

Indirect evidence suggests that LFA-1 is capable of transmitting signals into the interior of the cell, since anti-LFA-1 antibodies have been demonstrated to enhance anti-CD3-induced cell proliferation (Carrera et al., 1988; Van Noesel et al., 1988). However, based on recent observations (Dustin and Springer, 1989; Van Kooyk et al., 1989) it cannot be excluded that enhanced proliferation is the result of enhanced cell-cell interactions by activation of LFA-1 through CD3. Cell aggregation induced by NKI-L16, however, is not likely to be mediated by signals generated through LFA-1, because blocking of signal transduction pathways does not affect induction of cell aggregation by NKI-L16, whereas it completely abrogates PMA-induced cell aggregation. Further evidence shows that PKC down-regulation, by prolonged incubation of cells with PDBU, does not influence the NKI-

L16–induced aggregation (not shown). Furthermore, induction of aggregation by NKI-L16 was not accompanied by a concomitant induction of early activation markers, such as IL-2R and AIM, which are readily induced by PMA, suggesting that NKI-L16 does not lead to cell activation through unknown signaling routes. Finally, the observation that Fab fragments of NKI-L16 are also capable of inducing cell aggregation excludes the involvement of crosslinking phenomena. We therefore hypothesize that binding of the antibody causes a conformational change of the LFA-1 molecule, converting it from an inactive (second form) into an active state (third form). This would allow binding of its ligands with high affinity, resulting in stable cell adhesion. Although NKI-L16 and PMA act quite differently on LFA-1, the kinetics of the aggregation response to both stimuli are remarkably similar (Keizer et al., 1988). We therefore assume that stimulation with PMA might, via PKC (Nishizuka, 1984), lead to a similar intramolecular change of LFA-1.

Activation of LFA-1 is a dynamic process. Recent observations showed that activation of LFA-1 can be differentially regulated. Anti-CD3 antibodies induce LFA-1–dependent aggregation by transient activation of the LFA-1 molecule (Dusting and Springer, 1989; Van Kooyk et al., 1989), whereas anti-CD2 antibodies persistently activate LFA-1 (Van Kooyk et al., 1989). Activation of LFA-1 via CD2 or CD3 probably occurs by the generation of second messengers leading to PKC activation (Imboden and Stobo, 1985; Pantaleo et al., 1987). Whether triggering of CD43, which has also been shown to induce LFA-1–dependent adhesion (Axelsson et al., 1988), results in activation of PKC remains to be determined.

Activation of PKC by PMA may lead to phosphorylation of the LFA-1 β subunit (Hara and Fu, 1986; Chatila and Geha, 1988; Chatila et al., 1989). We speculate that PKC-dependent phosphorylation processes induce intramolecular changes in LFA-1 and thus control activation of LFA-1 (Figdor et al., 1990). Alternatively, activation of PKC can lead to the phosphorylation of cytoskeletal components, which then might interact with LFA-1 thus leading to enhanced cell adhesion.

We provided evidence that LFA-1–mediated lymphocyte adhesion is regulated by PKC-dependent mechanisms, when triggered by anti-CD3 mAb or PMA, presumably giving rise to a conformational change of the LFA-1 molecule. Ca^{2+}/Mg^{2+} cations play a central role in this process. Recently similar mechanisms are employed by other members of the integrin family (Gulino et al., 1990). Both GPIIb/IIIa and CR3 (CD11b/CD18) have been shown to express neo-epitopes upon activation, indicating that activation causes intramolecular modifications which may alter ligand affinity (Wright and Meyer, 1986; Ding et al., 1987; Altieri and Edgington, 1988; Philips et al., 1988; Danilov and Juliano, 1989).

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