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A MONOCLONAL ANTIBODY (NKI-L16) DIRECTED AGAINST A UNIQUE EPITOPE ON THE α-CHAIN OF HUMAN LEUKOCYTE FUNCTION-ASSOCIATED ANTIGEN 1 INDUCES HOMOTYPIC CELL-CELL INTERACTIONS[1]

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In the present study a unique antibody (NKI-L16) reacting with the α-chain of the human leukocyte function-associated Ag-1 (LFA-1) is described, which stimulates homotypic cell-cell interactions in a manner very similar to 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), in contrast to other anti-LFA-1 mAb which inhibit cell aggregation. The induction of aggregate formation of EBV-transformed B cells (JY) and CTL clones by TPA or NKI-L16 is not accompanied by an increase in the expression of LFA-1. Nevertheless, this cluster formation is LFA-1 dependent, inasmuch as anti-LFA-1 antibodies, other than NKI-L16, completely abrogate aggregation. Simultaneous addition of NKI-L16 and TPA did not result in a further increase of the speed of cluster formation, suggesting that a similar pathway is activated. Immunoprecipitation and enzyme digestion studies revealed that NKI-L16 recognizes a unique epitope on the α-chain of LFA-1, most likely situated close to the transmembrane segment of the molecule.

It is hypothesized that NKI-L16 or TPA can cause the LFA-1 molecule to convert from an inactive to an active configuration, thereby permitting binding of LFA-1 to its natural ligand.

LFA-1[3] belongs to a family of adhesion molecules consisting of LFA-1, CR3 (C3biR), and p150.95. All three Ag are bimolecular glycoprotein complexes with an α, β-stochiometry. They share a common β subunit with a M_r of 95 kDa but differ in their α subunits with M_r of 170, 165, and 150 kDa, respectively (1, 2).

LFA-1 was first identified by mAb that inhibit target cell lysis by cytotoxic T cell clones, both in mouse (3) and man (4, 5) by preventing conjugate formation (6, 7). Since then it has become clear that LFA-1 acts as a general cellular adhesion molecule mediating a variety of heterotypic and homotypic cell-cell interactions (2, 8–11).

Both B and T lymphocytes can be induced to form aggregates in vitro after stimulation with Ag, lectins, or phorbol esters (TPA) (12, 13). These cell-cell interactions can be blocked by anti-LFA-1 mAb, indicating an important role for this molecule in intercellular adhesion processes (11, 12).

In addition, recently three independent reports have been published which indicate that some anti-LFA-1 mAb are able to induce proliferation and cell aggregation in murine lymphocytes, which underlines the importance of LFA-1. The precise role of LFA-1 in this process is not clear, inasmuch as the proliferation occurred only after concomitant addition of Ag (14), Con A supernatant (15), or antibodies which cross-link the δ-chains of surface IgG molecules (16). However, the ability to use stimulatory and inhibitory mAb in immunologic processes may be very helpful in delineating the working mechanism of LFA-1.

We now present the characteristics of a mAb directed against the human LFA-1 α-chain, which, in contrast to other anti-LFA-1 antibodies, stimulates rather than inhibits homotypic cell aggregate formation, but does not affect cell proliferation (G. D. Keizer, unpublished results). The results obtained with this antibody are very similar to those obtained after treatment of cells with TPA. To the best of our knowledge, this is the first report of an antibody directed against human LFA-1 which stimulates cell-cell contact.

MATERIALS AND METHODS

Antibodies. The mAb SPV L1, SPV L7, and SPV L11 (further named L1, L7, and L11, respectively), and CLB 54 were raised as described previously (2, 17). The mAb SPV-L10, NKI-L15, and NKI-L16 (further named L10, L15, and L16) were obtained by immunizing BALB/c mice with a CTL clone, JY cells, and monocytes, respectively. Hybridoma supernatants were screened for inhibition of T cell-mediated killing or for inhibition or stimulation of the formation of homotypic aggregates between JY cells. The mAb MHM 23 and MHM 24 were a gift of Dr. A. McMichael (Nuffield Department, John Radcliffe Hospital, UK) (18). The purified mAb 60.3 was kindly provided by Dr. M. Harlan (Departments of Medicine, Pathology, and Pediatrics, University of Washington, Seattle) (19, 20). Two antibodies Klos and Rupi-1 were used as controls. Klos recognizes an idiotypic determinant on Ig. Rupi-1 recognizes an Ag with a M_r of 35 kDa on human monocytes. Both antibodies are nonreactive with the cells used, as detected by immunoprecipitation. All antibodies were purified on a Baker-Bond mAb HPLC column (J. T. Baker, Philipsburg, NJ).

Cells. The human cytotoxic T cell clone JS-136 was used in this study. This clone is directed against HLA-DR (21) and was cultured in serum-free medium (linolea) as described previously (22). Activated PBL were obtained by culturing PBL, isolated by density gradient centrifugation over Ficoll-Hypaque, in the presence of PHA (0.1 μg/ml) for 4 days. The human EBV-transformed B cell line JY was cultured in linolea supplemented with 2% human AB serum.

Labeling of mAb. mAb were directly labeled with FITC by using the following procedure. HPLC-purified mAb were dialyzed against 0.1 M

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[3] Abbreviations used in this paper: LFA-1, leukocyte function-associated antigen 1; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; PBL, peripheral blood leukocytes; RI, relative immunofluorescence intensity; TEA, triethanolamine.

Na_2CO_3 (pH 8.5) for 2 h. Subsequently, 37.5 μg FITC (Sigma Isomer 1, No. 7250) in 0.5 M Na_2HCO_3 (pH 9.6) were added to each mg of protein, followed by a 2-h incubation at room temperature. mAb-FITC conjugates were separated from free FITC by means of a Bio-Gel P-2 column (Bio-Rad, Richmond, CA).

Immunofluorescence. Cells were incubated for 30 min at 0°C in PBS containing 1% BSA (Sigma, St. Louis, MO), 0.01% sodium azide, and appropriate dilutions of the different mAb 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 FACS IV analysis, and was calculated as:

$$\frac{\text{Fluorescence intensity of a mAb reactive with the cells}}{\text{Fluorescence intensity of unlabeled cells.}}$$

Epitope determination. 1. Trypsin digestion: PHA-activated PBL (5×10^7) were washed twice and resuspended in PBS at a concentration of 2×10^6 cells/ml. Samples of 250 μl of this suspension (or equivalent amounts) were preincubated in 15-ml Falcon tubes at 37°C for 5 min followed by the addition of a similar volume of PBS containing trypsin (17.5 U/ml, Boehringer, Mannheim, FRG) and DNase-I (strain Cowan-I, Sigma). The reaction was stopped at various time points by the addition of 14 ml ice-cold DMEM supplemented with 10% BSA and trypsin inhibitor (5 mg/ml, Boehringer Mannheim). Subsequently the cells were washed twice in the same medium and kept on ice until use in the immunofluorescence assay. The viability of the cells was >85% as determined by trypan blue exclusion.

2. Cross-blocking experiments: To determine whether the various mAb were directed against shared or unique epitopes, two different methods were applied. In method A, cells were incubated with appropriate dilutions of a single mAb (mAb 1 or mAb 2) or with a mixture of two mAb (mAb 1 and mAb 2). Subsequently, cells were incubated with a FITC-labeled goat anti-mouse IgG antibody after which the RI was determined. mAb were judged to share common epitopes, i.e., to cross-block each other, when the RI of the cells treated with the mixture of mAb did not exceed the highest RI value of the cells treated with a single mAb (either mAb 1 or mAb 2). Furthermore, mAb were defined to recognize unique determinants if the addition sum of the RI values of cells treated with mAb 1 or with mAb 2 was equal (±20%) to the RI value of the cells treated with the mixture of both mAb 1 and 2. In method B, the mAb LI, L7, L11, L16, and CLB 54 were directly labeled with FITC as described above. Cells were incubated with unlabeled mAb 1 for 30 min at 4°C, followed by the addition of FITC-labeled mAb 2. After 30 min, cells were washed once with PBS and analyzed by FACS IV measurements of the RI value. Each measurement was accompanied by a control sample to which only the FITC-labeled mAb were added. Usage of method A or B led to identical results.

Radiolabeling and immunoprecipitation. Cell surface radioiodination with Na ^{125}I (Amersham, UK) was catalyzed by 1,3,4,6-tetrachloro-3α,6α diphenylglycoluril (Iodogen, Pierce Chemical Co., Rockford, IL) as described previously (23). For immunoprecipitation (24), radiolabeled cells were lysed with 1% Nonidet P-40 in 0.01 M triethanolamine-HCl (pH 7.8), 0.15 M NaCl, 1 mM PNSF, 0.02 mg/ml ovomucoid trypsin inhibitor (Sigma, TEA/NaCl buffer). Nuclear debris was removed from the lysates by centrifugation at 13,000 × *g* for 15 min at 4°C. Material insoluble in the lysis buffer was removed by centrifugation at 100,000 × *g* for 30 min in an air-driven centrifuge (Beckman Instruments, Palo Alto, CA). Lysates were precleared further by successive incubations with formalin-fixed *Staphylococcus aureus* (strain Cowan I) and mouse IgG coupled to protein A Sepharose. Precleared lysates were incubated for 3 to 4 h with a specific mAb coupled to protein A Sepharose. The immunoprecipitates were removed from the lysates by centrifugation at 13,000 × *g*. Precipitates were resuspended in 0.2 ml TEA/NaCl buffer with 0.5% sodium deoxycholate and centrifuged for 15 sec at 13,000 × *g*. Subsequently immunoprecipitates were washed extensively in 0.01 M TEA/HCl (pH 7.8), 0.2% Nonidet P-40.

Electrophoresis and autoradiography. SDS-PAGE was carried out on vertical slab gels (6 to 8%) according to a modification of the Laemmli procedure (25), as described previously (2). Kodak XAR-film was used in combination with intensifier screens (Cronex Lightning Plus, Dupont Chemical Co., Newton, CT) for autoradiography of ^{125}I-labeled materials.

Peptide mapping. To perform one-dimensional peptide mapping, specific bands were cut out of the gel that had previously been fixed, dried, and autoradiographed. The gel slices were soaked in a Tris-HCl buffer, containing 0.5% SDS, for 15 min, and were transferred to a second gel (stacking gel 5%; running gel 15 to 17%). The gel slices were overlayered with 10 μl 20% glycerol. Subsequently a second layer (10 μl) was administered containing 10% glycerol and V8 protease (0.1 mg/ml). Subsequently the gel was run (100 V) until the dye front of the sample buffer (of m.w. markers) reached the border between stacking and running gel. After an incubation period of 30 min (25°C) running of the gel was continued followed by fixation and autoradiography.

Qualitative aggregation assay. Homotypic aggregation of cells was measured in a qualitative manner by a modification of the method described by Rothlein and Springer (12). Cells were washed twice in linolea containing 1% pooled AB serum resuspended and seeded in 96-well microtiter plates (No. 3596, Costar, Cambridge). Each well contained 1×10^5 cells, mAb (0.1 to 10 μg/ml), and/or TPA (1 to 10 ng/ml). Cells were incubated for various periods of time (see *Results*) at 37°C, after which aggregate formation was determined by at least two investigators by light microscopy. Scores ranged from 0 to 5+ where 0 indicated that essentially no cells were aggregated in clusters; 1+ indicated that <10% of the cells were found in loose clusters; 2+ indicated that <50% of the cells formed loose clusters; 3+ indicated that <80% of the cells were found in loose clusters; 4+ indicated that >80% of the cells were found in small compact aggregates; 5+ indicates that >90% of the cells formed large compact clusters. It was our experience that the differences in the results obtained by two independent observers was negligible.

Quantitative aggregation assay. Cells were seeded in flat bottom microtiter plates as described above. The number of nonaggregating cells was counted by the use of a calibrated mask consisting of squares (0.46 mm) in the ocular (Zeiss CPL W10×/18) of an inverted microscope (Leitz Diavert). Within each well at least six randomly chosen areas were counted, followed by the calculation of the mean and the total number of nonaggregating cells. Percent aggregation was determined according to the formula:

$$\text{Percent aggregation} = 1 - \frac{\text{No. of free cells}}{\text{total No. of cells}} \times 100\%.$$

The experiments were carried out in triplicate. The SD within each experiment was usually less than 10%.

RESULTS

Immunoprecipitation. We previously described several antibodies reactive with LFA-1 (2). In a comparative immunoprecipitation study with ^{125}I-labeled PHA-activated PBL, two newly generated antibodies L15 and L16 showed the characteristic pattern of LFA-1 (Fig. 1). These mAb, like L1, L7, L10, L11, and MHM 24, precipitated an α-chain

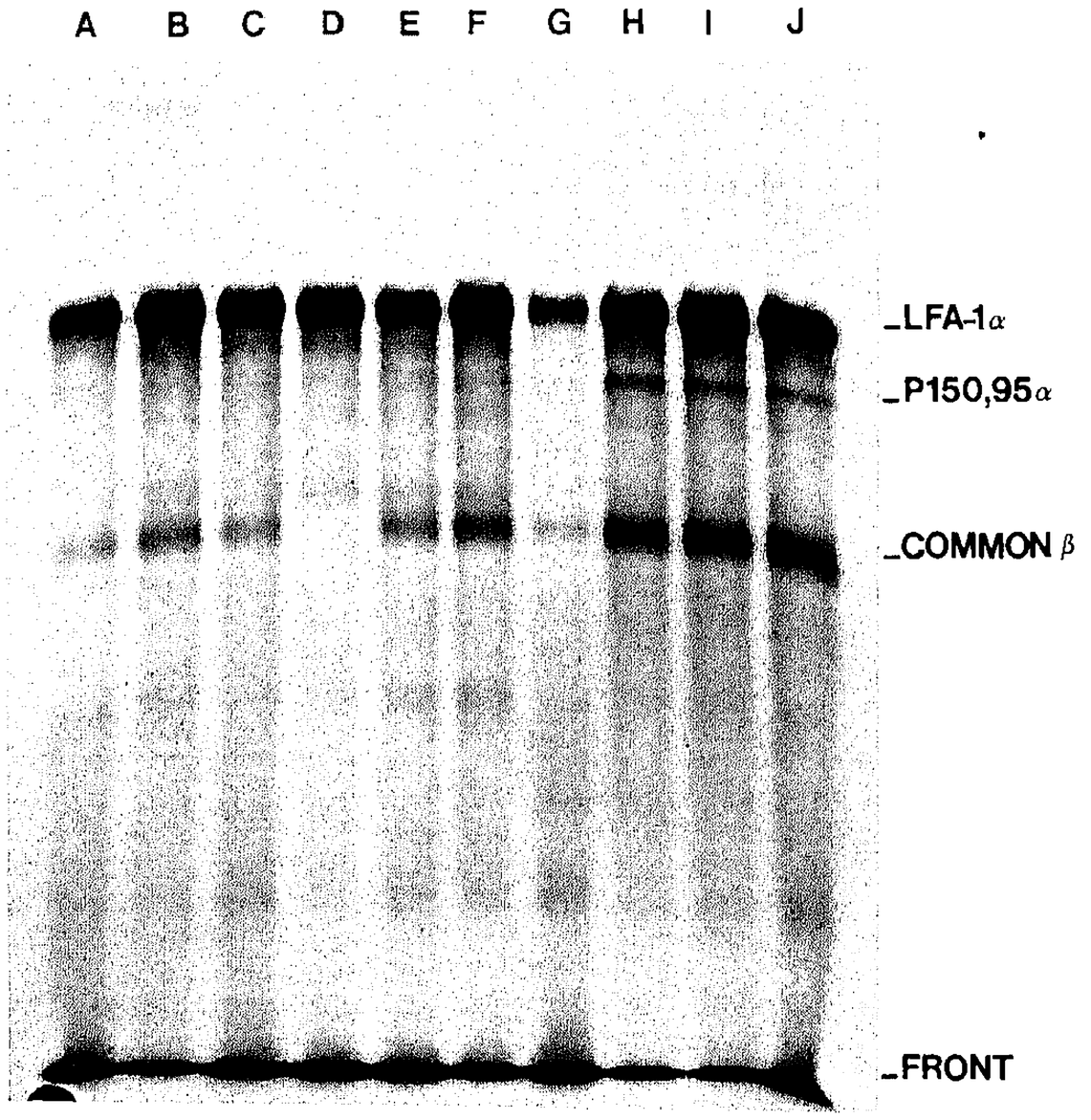

Figure 1. Immunoprecipitation of LFA-1 from a ^{125}I-labeled lysate of peripheral blood mononuclear cells, with various anti-LFA-1 mAb. Immunoprecipitates were subjected to SDS-PAGE and autoradiography. (*A*) SPV-L1, (*B*) SPV-L7, (*C*) SPV-L10, (*D*) MHM 24, (*E*) SPV-L11, (*F*) NKI-L15, (*G*) NKI-L16, (*H*) MHM 23, (*I*) 60.3, and (*J*) CLB 54.

with a M_r of 170 kDa and a β-chain of 95 kDa, respectively. Anti-β-chain mAb (CLB 54, MHM 23, 60.3) precipitate in addition to these two bands, the α-chain of the p150.95 molecule (Fig. 1). To prove that L15 and L16 reacted with LFA-1 but not with an LFA-1-like Ag, preclear and peptide mapping experiments were carried out. Repeated preclearing (4 times) of lysates of CTL with mAb 60.3 or L7, followed by immunoprecipitation with L15 or L16, resulted in a considerable reduction (>90%) of the immunoprecipitated material (results not shown). Similar observations were made in reciprocal experiments which indicates that mAb L15 and L16 both recognize LFA-1. Further evidence is provided by a peptide map which was made of immunoprecipitates obtained with the mAb L11, L15, L16, and MHM 24. The α- and/or β-chains were excised from the gel, digested by V8 protease, and subsequently subjected to 8 to 15% SDS-PAGE. As shown in Figure 2, identical patterns were observed, showing that all mAb recognize the LFA-1 Ag.

JY-JY interactions. Recently it has been reported that mAb directed against LFA-1 inhibit both spontaneous and TPA-induced homotypic cell-cell interactions of JY cells (12, 13). The increased adhesion of cells after TPA stimulation could not be attributed to a higher expression of LFA-1 or to an increased motility. These results were confirmed as shown in Tables I and II. All mAb directed against LFA-1, except L16, strongly inhibited the aggregate formation of JY cells (Table I), whereas the increased adhesiveness caused by addition of TPA was not accompanied by an increased expression of LFA-1 (Table II). Surprisingly, addition of mAb L16 strongly stimulated the adhesive capacity of spontaneously aggregating JY cells, quite similar to TPA (Table I). In some experiments, stimulation of aggregation by mAb L16 could already be observed at a concentration of 50 ng/ml (data not shown).

Both TPA induced JY cell clustering, and aggregation induced by mAb L16, could be inhibited by all other anti-LFA-1 mAb available (Table I). To study the process of aggregate formation in more detail and to compare the effects of TPA and mAb L16, kinetic experiments were performed. As shown in Figure 3, both mAb L16 and TPA induced a rapid cluster formation reaching a maximum (i.e., >90% of the cells in large compact clusters) after 3 h of incubation at 37°C. Simultaneous addition of TPA and mAb L16 did not further accelerate the aggregation process in comparison with the addition of either TPA or L16. The hyper-aggregation induced by these compounds lasted for more than 72 h for both stimuli. Inhibition of spontaneous or stimulated aggregate formation induced by other anti-LFA-1 mAb decreased after 24 h (Fig. 3). Taken together these data suggest that TPA or L16 may induce aggregate formation by a similar LFA-1-mediated mechanism.

We excluded the possibility that mAb L16 accidently had been contaminated with TPA, since both hybridoma supernatants and HPLC-purified antibodies were effective in inducing aggregation. Furthermore, addition of TPA to neutrophils caused a rapid oxidative burst, whereas in contrast addition of mAb L16 was found to be ineffective (results not shown).

CTL-CTL interactions. In contrast to JY cells, unstimulated cloned human CTL do not form spontaneous homotypic conjugates in vitro (Fig. 4). Nevertheless, the addition of small quantities of TPA (1 ng/ml) or mAb L16 (50 ng/ml) to a $CD3^+CD4^+CD16^-$ CTL clone (clone JS-136) induces rapid aggregation of the cells (Figs. 5 and 6, respectively). Similar results were obtained by using other CTL clones regardless of their phenotype. Similar to JY cells, the aggregate formation of JS-136 cells induced by TPA was not accompanied by an increase in expression of LFA-1 on the cell surface of CTL (Table II). Nevertheless, aggregation induced by mAb L16 or TPA seems to depend on the presence of LFA-1, since other anti-LFA-1 mAb were able to completely abrogate aggregation (Table I). Furthermore, anti-LFA-1 β-chain mAb were found to be slightly less inhibitory than anti LFA-1 α-chain mAb, both for JY-JY and CTL-CTL interactions.

We also carried out kinetic studies with JS-136 (Fig. 7). In spite of the 7- to 10-fold higher expression of LFA-1 on the CTL clones compared with JY cells (Table II), the onset of aggregation after addition of TPA or mAb L16 occurred after 30 to 60 min. After this period, cells aggregated in a similar fashion as JY cells. In general, no differences in kinetics were observed between TPA treatment or stimulation with L16. Again, these data suggest that TPA- or L16-induced cell aggregation is mediated by a similar mechanism.

No involvement of FcR. Inasmuch as mAb L16 has a different isotype (IgG2a) than most of the other anti-LFA-1 antibodies applied in this stujdy (Table I), induction of aggregate formation of JY cells or CTL might be due to an FcR-mediated process. However, strong evidence against this possibility comes from the following observations. (1) A control antibody, W6/32 (IgG2a) directed against HLA

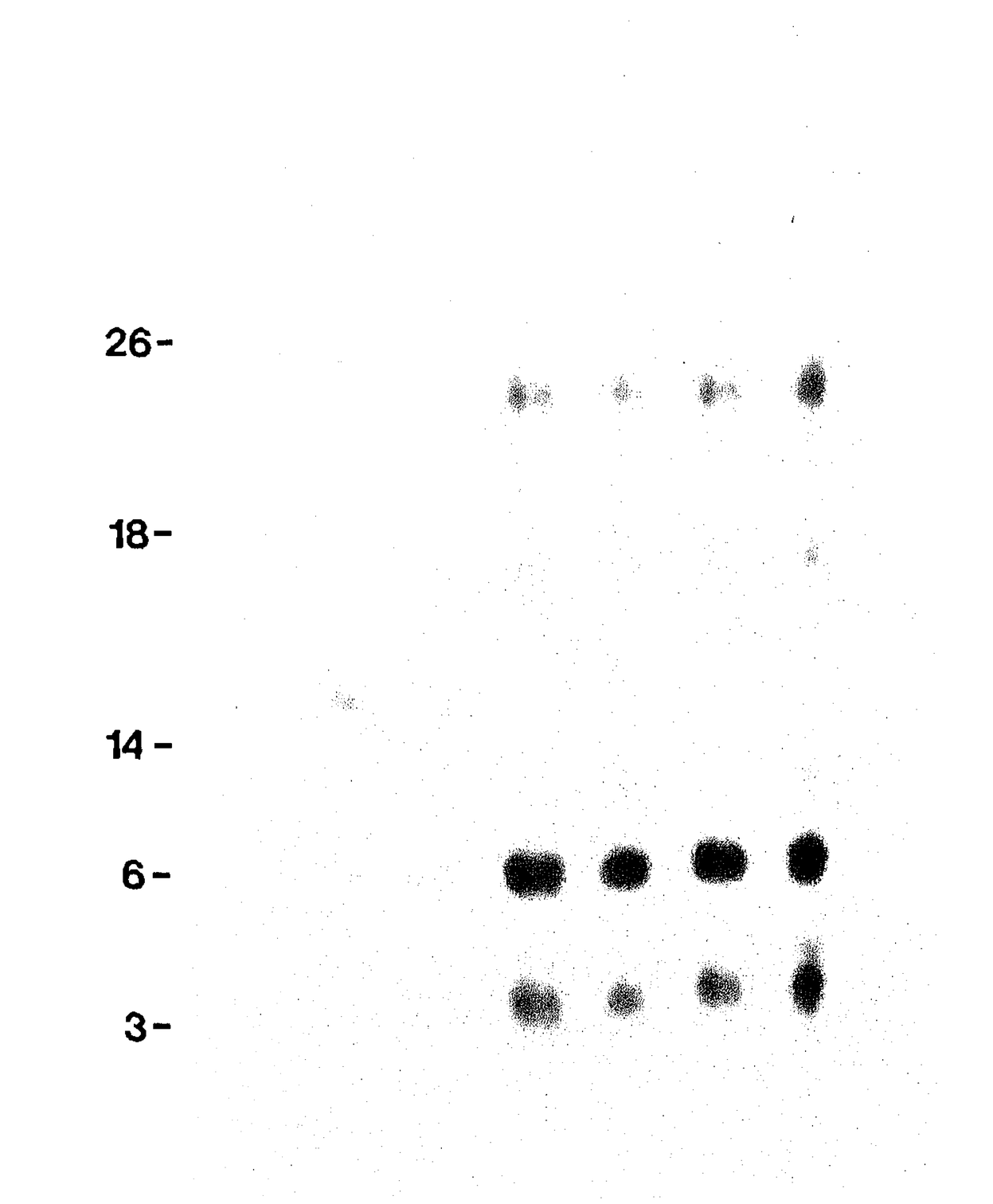

Figure 2. Treatment of the SDS-PAGE-purified α- and β-chains of LFA-1 with V8 protease. (*A*) L11 β-chain; (*B*) L16 β-chain; (C) MHM 24 α-chain; (*D*) L16 α-chain; (*E*) L15 α-chain; (*F*) L11 α-chain. *Bars* indicate relative M_r (kDa) as defined by prestained markers.

TABLE I

Ability of mAb to inhibit or stimulate formation of spontaneous or induced homotypic cell aggregates[a]

Ag	mAb	Isotype	JY			JS-136		
			Medium	TPA	L16	Medium	TPA	L16
			3+	5+	5+	0	4+	4+
LFA-1α	MHM 24	IgG1	0	1+	1+	0	0	0
LFA-1α	L1	IgG1	0	0	0	0	0	0
LFA-1α	L7	IgG1	0	0	1+	0	0	0
LFA-1α	L10	IgG1	0	0	0	0	0	0
LFA-1α	L11	IgG1	0	0	0	0	0	0
LFA-1α	L15	IgG1	0	1+	1+	0	0	0
LFA-1α	L16	IgG2a	5+	5+	5+	4+	4+	4+
HLA-ABC	W6/32	IgG2a	3+	5+	5+	0	4+	4+
LFA-1β	60.3	IgG2a	1+	2+	1+	0	1+	1+
LFA-1β	MHM 23	IgG1	1+	2+	2+	0	1+	1+
LFA-1β	CLB 54	IgG1	1+	1+	1+	0	1+	1+
	Rupi-1[b]	IgG1	3+	5+	5+	0	4+	4+
	Klos[b]	IgG2a	3+	5+	5+	0	4+	4+

[a] mAb (5 μg/ml) were added to unstimulated and TPA (10 ng/ml)- or L16 (0.5 μg/ml)-stimulated JY cells or a CTL clone (clone JS-136). After 4 h, the aggregate formation was scored visually as described in *Materials and Methods*.
[b] Antibodies nonreactive with the cells.

TABLE II

Expression of LFA-1, HLA class I, and CD16 antigens on JY cells and the CTL clone JS-136 as measured by an indirect immunofluorescence assay[a]

mAb	Relative Fluorescence Intensity			
	JY	JY (TPA)	JS-136	JS-136 (TPA)
Klos	0.4	0.3	0	0
Rupi-1	0	0.1	0	0
L10	3.7	3.5	23.7	26.0
L16	3.2	3.4	15.5	15.1
60.3	3.7	3.6	22.5	25.0
W6/32	57.5	56.2	43.7	43.7
α-CD16	ND	ND	0	0

[a] Cells were cultured at 37°C and treated with or without TPA (10 ng/ml) for 1 h. Subsequently an immunofluorescence assay was performed as described in *Materials and Methods*. The results of a typical experiment are shown ($n = 5$).

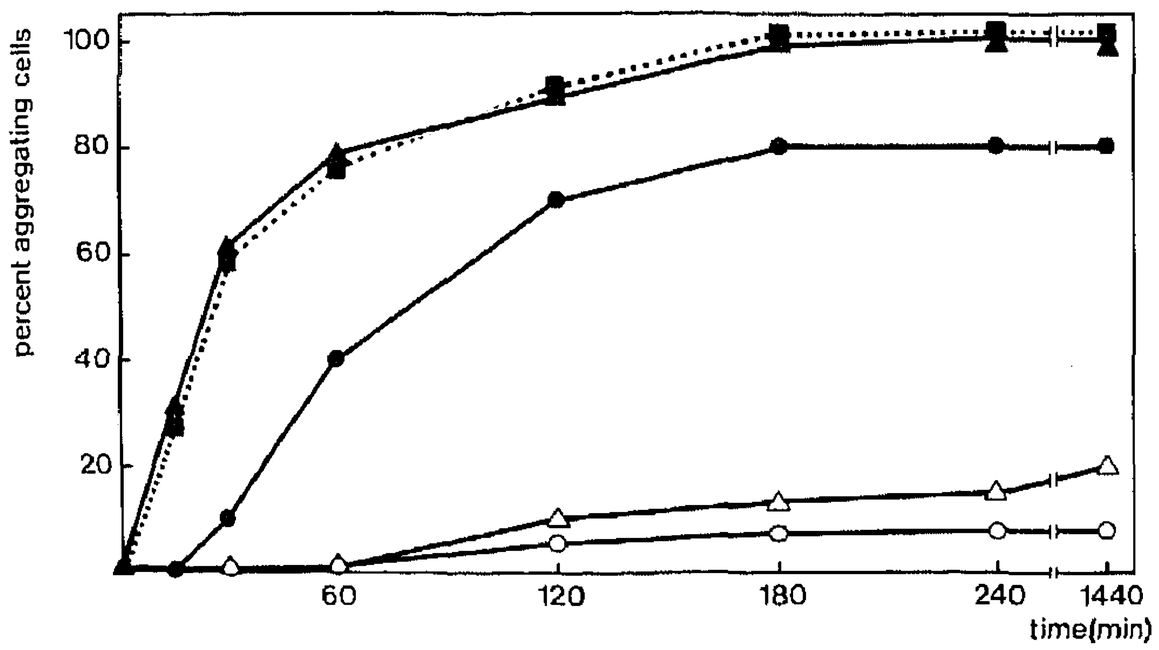

Figure 3. Kinetics of aggregation of JY cells. Aggregation in the presence of TPA (■), 5 ng/ml; L16 (▲), 0.1 μg/ml or medium alone (●). The *open symbols* represent the addition of mAb CLB 54 (1 μg/ml) to L16 stimulated (△) or unstimulated (○) cells.

class I Ag did not induce aggregate formation, despite the fact that these Ag are abundantly present on both CTL and JY cells (Table II); (2) the mAb 60.3 (IgG2a) which reacts with the β-chain of LFA-1 strongly inhibits aggregate formation (Table I); and (3) the CTL clone (JS-136) does not express FcR as judged by (a) nonreactivity with antibodies directed against CD16 and (b) nonreactivity with a control mAb (Klos) of the same isotype (Table II).

Epitope determination. As described above, mAb directed against LFA-1, with the exception of mAb L16, inhibited homotypic aggregate formation. Therefore it was of interest to determine whether L16 reacted with a thus far unknown epitope of the LFA-1 molecule. The finding that L16 induces aggregate formation indicates that L16 does not react with the binding site of LFA-1. To determine the epitopes of the various LFA-1 antibodies, PHA-stimulated PBL were treated with trypsin for various lengths of time, followed by immunofluorescence. As depicted in Figure 8*A*, mAb L16 recognizes an epitope on the LFA-1 α-chain, which is relatively insensitive to trypsin treatment. After 2.5 min of treatment, no determinants were lost, and after 40 min almost 50% of the determinants were still conserved. In contrast, the mAb MHM 24, L1, L7, L10, L11, and L15 were found to be reactive with an epitope which was already affected after 2.5 min of trypsin treatment. These mAb can be divided into two groups discernible after 40 min of exposure to trypsin. mAb belonging to the first group (MHM 24, L1, L7, and L10) lost all reactivity, whereas about 25% of the determinants recognized by the mAb of the second group (L11 and L15) were still present. Similar observations were made with anti-LFA-1 β-chain mAb (Fig. 8*B*). All epitopes were found to be trypsin sensitive, but a clear distinction could be made between the epitopes recognized by mAb CLB 54 and the other two mAb (MHM 23 and 60.3). After 5 min of trypsin treatment, more than 50% of the epitope recognized by CLB 54 was lost, whereas the greater part of the epitope recognized by the mAb MHM 23 and 60.3 was still present. It should be noted that, after 5 min of trypsin treatment, the β-chain of LFA-1 becomes relatively insensitive to further degradation for a period of approximately 15 min (Fig. 8*B*). These results indicate that the epitope recognized by mAb L16 is most likely located close to the transmembrane segment of the molecule successively followed by the epitopes recognized by the mAb L11, L15, and the epitopes of L7, L10, L12, and MHM 24. Similarly, the mAb MHM 23 and 60.3 might bind closer to the transmembrane of the molecule than mAb CLB 54.

To determine whether the mAb directed against the various trypsin-defined epitopes could inhibit each other's binding, cross-blocking experiments were carried out. Within each of the trypsin-defined epitopes all mAb cross-blocked each other (Fig. 9). Moreover, binding of a mAb to one of the trypsin-defined epitopes does not inhibit binding of mAb to other trypsin-defined epitopes (Fig. 9). These results indicate that none of the trypsin-defined epitopes is lost due to conformational changes within the LFA-1 molecule after binding of a single mAb. Furthermore, these results show that both methods (trypsin treatment and cross-blocking studies) yield identical results. It is concluded that mAb L16 and CLB 54 define unique epitopes on the α- and β-chain of LFA-1, respectively.

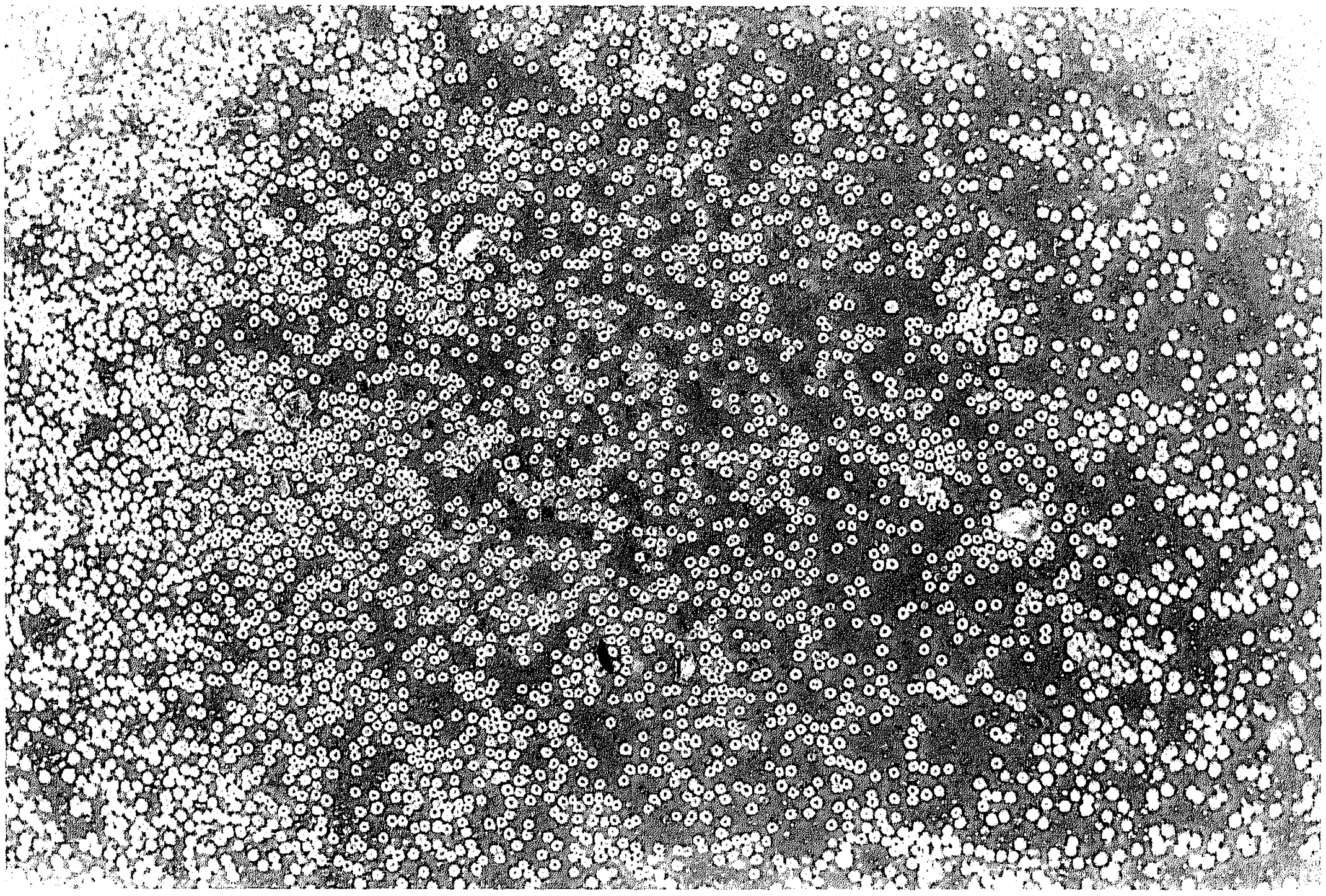

Figure 4. Photomicrograph of aggregation of CTL (clone JS-136), after a 3-h incubation period with medium.

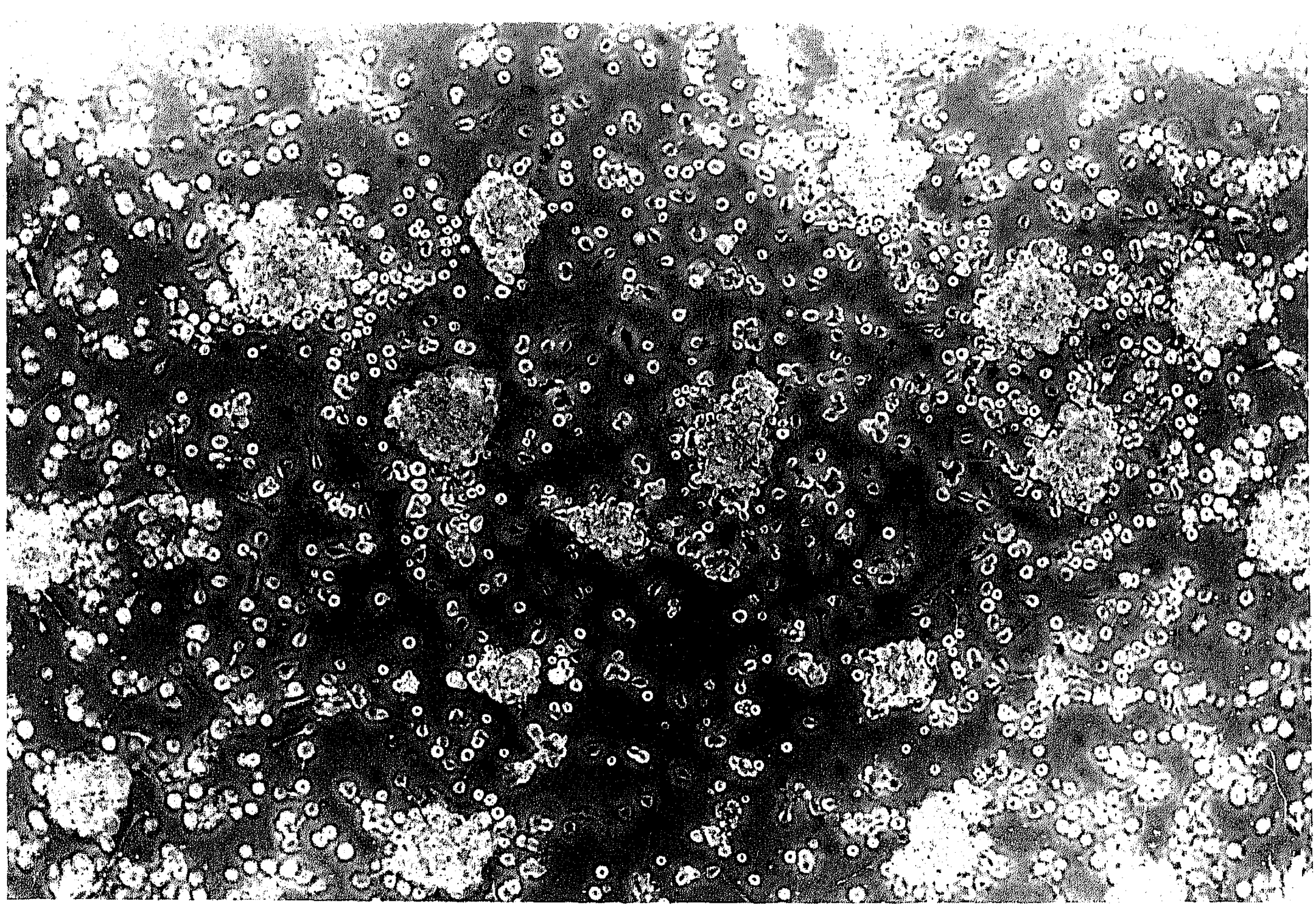

Figure 5. Photomicrograph of CTL clone JS-136 aggregation after a 3-h incubation with TPA, 1 ng/ml.

DISCUSSION

Homotypic cellular interactions have been shown to be mediated by an LFA-1-dependent mechanism since mAb directed against this Ag are able to inhibit cell clustering (10–12). From our findings it becomes clear that the

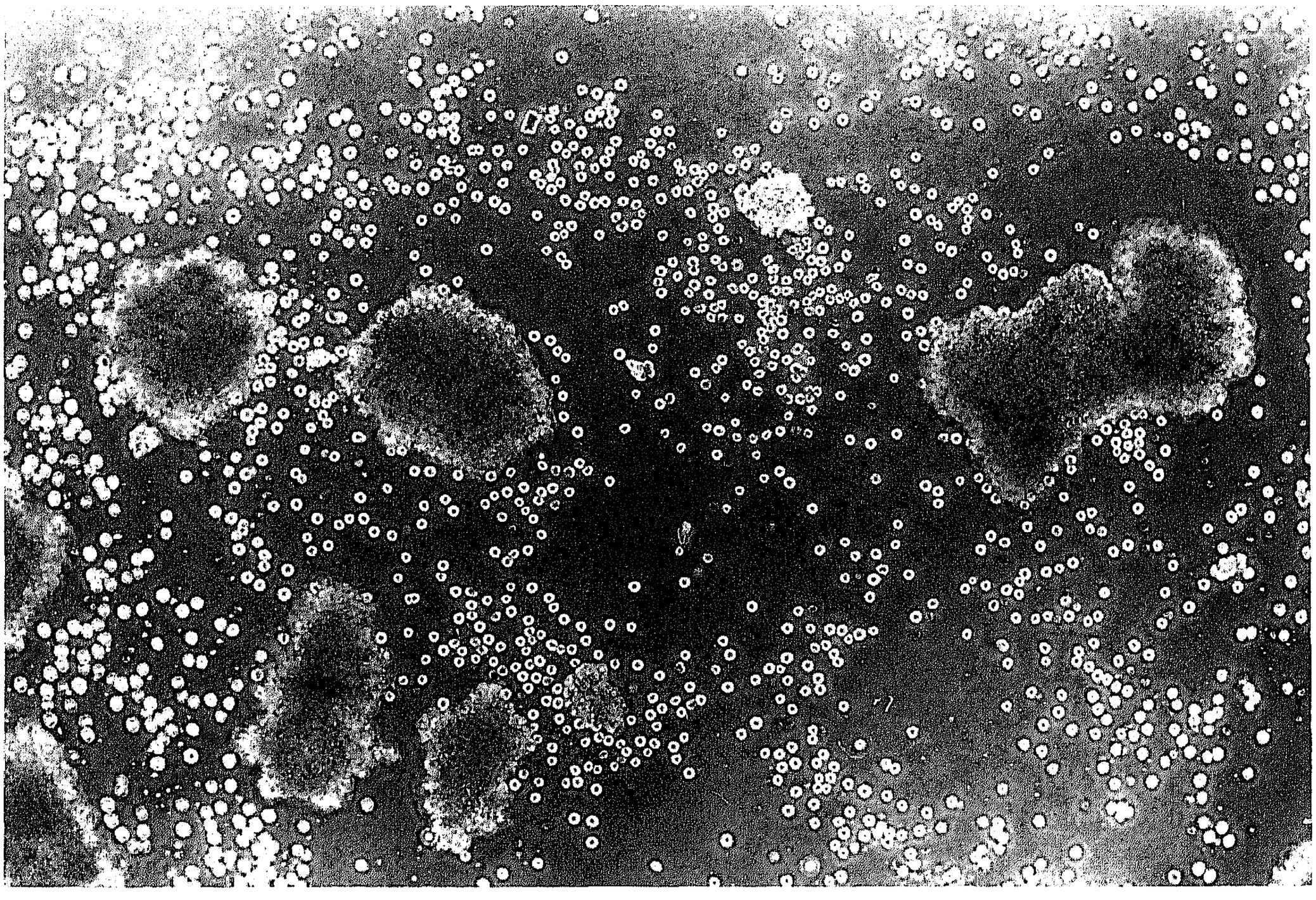

Figure 6. Photomicrograph of aggregation of CTL clone JS-136 after a 3-h incubation with L16, 0.1 μg/ml.

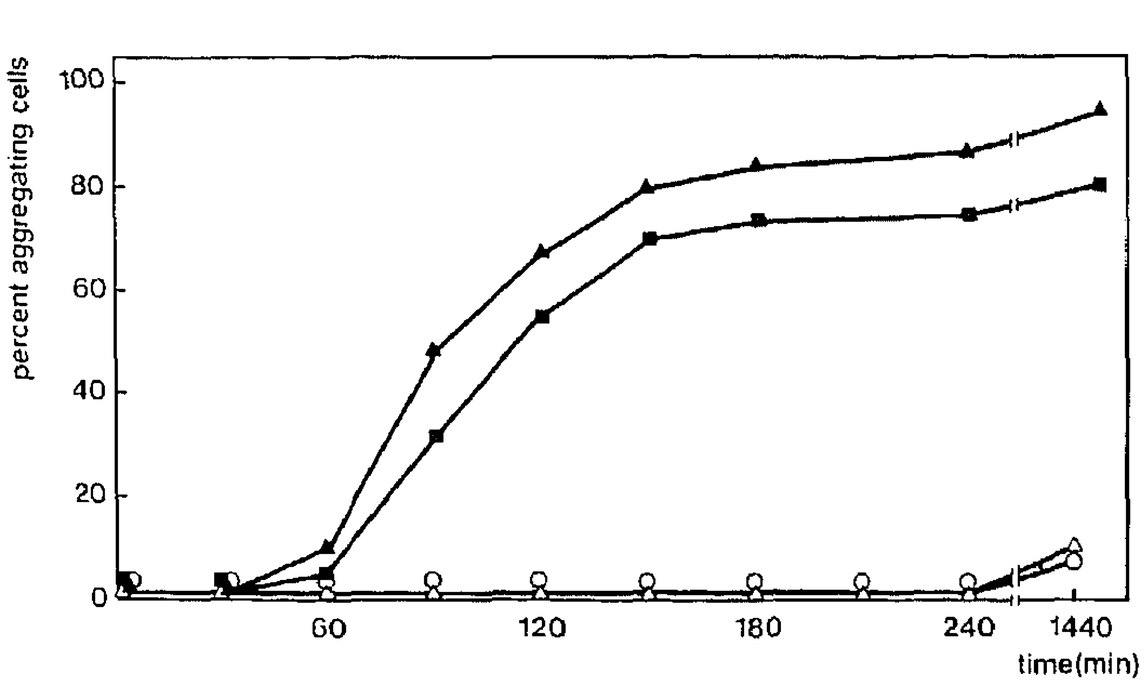

Figure 7. Kinetics of aggregation of CTL clone JS-136. Aggregation in the presence of TPA, 1 ng/ml (■) or L16, 0.1 μg/ml (▲). The *open symbols* represent unstimulated cells (○) and L16 stimulated cells in the presence of mAb CLB 54, 10 μg/ml (△).

expression of LFA-1 on the cell surface does not correlate with homotypic aggregate formation. First, weak LFA-1 positive JY cells form spontaneous cell clusters, whereas strong LFA-1-positive CTL clones were shown not to aggregate (Fig. 4). Second, induction of homotypic aggregate formation by means of TPA is not accompanied by an increase in LFA-1 expression (Table II). Similar findings were reported by Rothlein and Springer (12) who in addition showed that LFA-1 is requird for cell aggregation since EBV-transformed LFA-1-negative B cell lines could not be induced to form homotypic cell aggregates. However, these cells could bind to other LFA-1-positive EBV-transformed B cells. Furthermore, their results indicated that metabolic activity and cell motility are required for cell aggregation. The differences in kinetics of aggregation of CTL or JY cells (Figs. 3 and 7) might be due to these phenomena since these cells may differ in their state of activation.

The mechanism whereby TPA induces aggregate formation has not yet been elucidated. Via triggering of the protein kinase C pathway, TPA may have effects on the cell membrane, cytoskeleton, or motility (12). Alternatively, TPA may act on LFA-1 itself. In this respect the characterization of mAb NKI-L16 may be helpful to further delineate the role of TPA in cell aggregation. mAb L16 is directed against the α-chain of LFA-1 and stimulates cellular adhesion in a manner similar to TPA (Figs. 3 and 7). Simultaneous addition of TPA and mAb L16 did not further increase the speed of aggregation which suggests the involvement of a similar activation mechanism. From these data it can be hypothesized that TPA may activate the LFA-1 molecule, by inducing a conformation change possibly via protein kinase C and subsequent phosphorylation (26). Binding of mAb L16 to LFA-1 may induce a similar conformational change leading to activation of the LFA-1 molecule. This hypothesis is supported by the finding that the epitope recognized by mAb L16 is most likely situated close to the transmembrane segment of the molecule which is nearest to the site of phorphorylation.

Taken together our results strongly indicate the existence of two forms of LFA-1, an active configuration by means of which cells aggregate in an LFA-1 dependent fashion, and an inactive configuration in which cells express LFA-1 but do not aggregate. A similar change in configuration has been postulated for CD2 (T11, LFA-2) (6, 27–30). Like LFA-1, CD2 is involved in CTL- target cell adhesion (6, 31). In addition, CD2 appears to play an important role in T cell activation (32, 33). Upon cell activation, CD2 acquires a new epitope as recognized by a mAb termed anti-

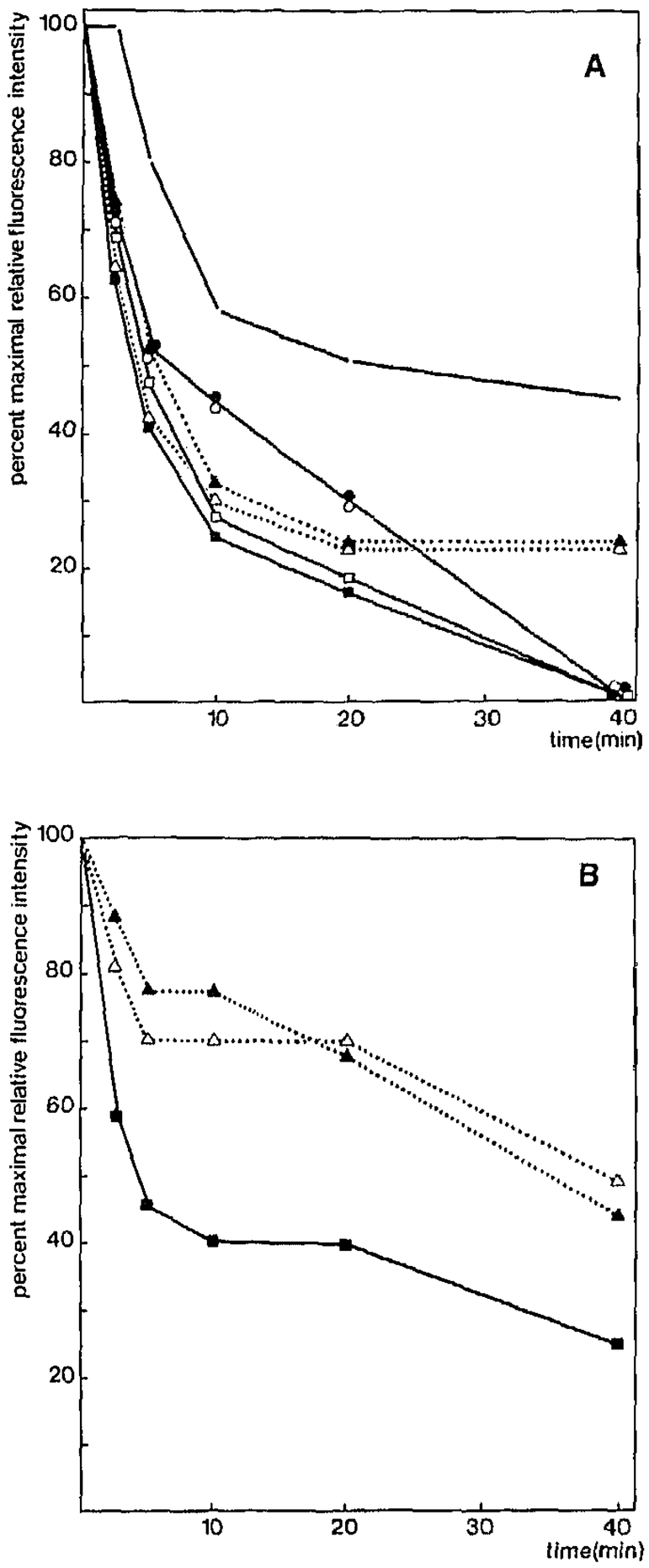

Figure 8. Epitope determination of the various anti-LFA-1 mAb by the use of trypsin. PHA-stimulated lymphocytes were treated with trypsin for various lengths of time after which an immunofluorescence assay was carried out. The relative immunofluorescence of untreated samples represented the 100% value. (*A*) Trypsin sensitivity of the epitopes located on the α-chain of LFA-1: L1 (●), L7 (○), L10 (□), L11 (△), L15 (▲), L16 (-----), MHM 24 (■). (*B*) Trypsin sensitivity of the epitopes located on the β-chain of LFA-1: CLB 54 (■), 60.3 (△), MHM 23 (▲).

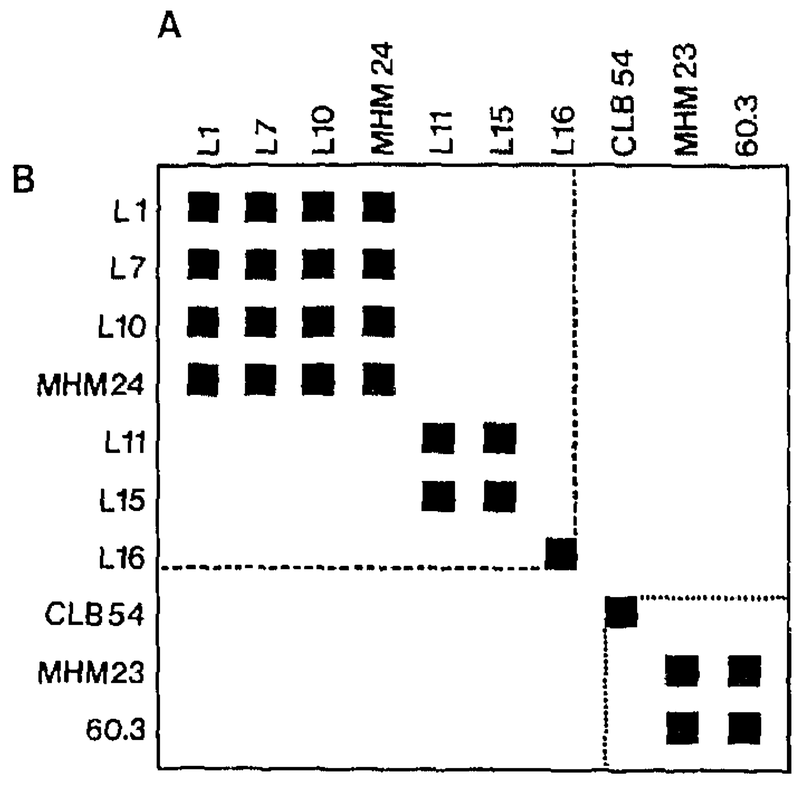

Figure 9. Cross-blocking (■) of the various anti-LFA-1 mAb as detected by direct and indirect immunofluorescence (see *Materials and Methods*). The *open bars* and *dotted lines* separate anti-LFA-1 α-chain and anti-LFA-1 β-chain mAb, respectively.

$T11_3$ (32). The induction of the expression of this epitope is most likely caused by a conformational change in the molecule, since binding of another anti-CD2 mAb (anti-$T11_2$) to resting T cells for 30 min at 0°C induces the appearance of the $T11_3$ epitope (32, 33). Interestingly, addition of anti-$T11_3$ mAb together with certain other anti-CD2 mAb stimulates rather than inhibits T cell functions (32–35). In this context it is noteworthy that the epitope recognized by mAb L16 is expressed only on activated cells, and is induced on resting PBL after mitogenic stimulation (G. D. Keizer, manuscript in preparation).

What is the natural signal that causes LFA-1 to switch from the resting to the activated state? The most simple explanation would be the presence of its counterstructure. Unfortunately, this model is too simple since CTL do not interact with each other spontaneously, whereas reasonable evidence can be provided that the counterstructure is present on CTL. First, it is difficult to assume that binding of mAb L16 will induce aggregate formation of cells by inducing the expression of a counterstructure of LFA-1. Moreover, the hypothetical counterstructure of LFA-1, the intercellular adhesion molecule 1 (ICAM-1) (36) appears to be present on CTL (37). These results indicate that LFA-1 becomes activated via a different mechanism. One explanation is that LFA-1 is not the adhesion-initiating molecule but may play a role in adhesion strengthening, which has been proposed by others (12, 38). In this regard, the existence of an LFA-1-independent adhesion pathway may be relevant as described both for CTL-target cell (31) and monocyte-endothelial cell interactions (39). In the latter case, Strassman et al. (40) elegantly demonstrated that weak LFA-1 independent adhesion preceded LFA-1 dependent cell-cell interaction which resulted in strong adhesion. Moreover, it was shown that only the strong interaction, which could be accelerated by the addition of TPA, was inhibitable by anti-LFA-1 mAb in contrast to the initial weak cell-cell interactions (40, 41). Whether the LFA-1-independent pathway activates LFA-1 or whether LFA-1 is activated by a different mechanism remains to be determined.

To the best of our knowledge, mAb L16 is the first antibody directed against human LFA-1 which stimulates cell-cell contact. Recently three antibodies (1-17, H155-78, and G-48) have been described, all reactive with LFA-1, which induce proliferation and/or aggregation of murine T and B cells (14–16). These effects cannot be easily explained by assuming that LFA-1 is just a general adhesion molecule without further function. It should be noted, however, that proliferation occurred only after a second signal, i.e., addition of Ag (14), Con A supernatant on (15), or cross-linking δ-chains of, surface Ig molecules (16). From those studies and from the results reported here, it becomes clear that antibodies like L16 will be of great help to delineate the precise role of LFA-1.

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