of 10^5 Balb c/3T3, α-PKC/3T3 or 3T3 containing the pLTR expression vector alone did not form tumours even after two months (Table 2).

To investigate whether the transforming properties of the mutant PKC gene are due to the four point mutations in the coding region, we have back-mutated the transforming allele of the PKC gene. Oligonucleotide-directed mutagenesis was used to back-mutate the four point mutations in the mutated PKC cDNA and the region of each mutation was sequenced to verify the alteration. A back-mutated UV25 cDNA (B-UV25) clone was isolated and inserted into the pLTR expression vector. Balb c/3T3 cells were co-transfected with the back UV-25 cDNA and PSV-Neo plasmids. Transfected cells were selected for G418 antibiotic-resistance and we isolated one clone expressing the back-mutated PKC gene (B-UV25/3T3). The B-UV25/3T3 cell line contains about ten times the level of high-affinity phorbol-ester binding sites and its PKC activity is increased tenfod (Table 1). The revertant B-UV25/3T3 cell line, however, does not display features typical of malignant transformed cells (such as morphology, formation of dense foci and anchorage-independent growth) and fails to induce tumours *in vivo* when inoculated into nude mice (Table 2).

Our study establishes that the mutant PKC gene can be activated as a potent oncogene when its expression is driven by a strong promoter. Moreover, it is also evident that the mere expression, without overexpression, of the mutant α-PKC gene is sufficient to fully transform Balb c/3T3 fibroblasts. This mutated α-PKC gene differs from the α-, β- and γ-PKC-isoenzymes by four point mutations located in the regulatory and the catalytic domains. These results corroborate the findings of a number of distinct activating point mutations in the coding regions of the *ras*, *neu* and *fms* genes[18-20]. It may be that a subset of proto-oncogenes, including the PKC gene, are activated after exposure to carcinogens or ultraviolet light, which act as point mutagens[21]. The idea of the PKC gene belonging to such a family is strengthened by the finding of a tumour-specific microscopic substitution in the α-PKC gene in a malignant melanoma primary cell line[22]. Oncogenic conversion of PKC may shed light not only on the process of carcinogenesis but also on the critical aspects of signal transduction and the mitogenic response. □

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Enhancement of LFA-1-mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes

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THE lymphocyte function-associated molecule LFA-1 (CD11a/CD18) plays a key part in lymphocyte adhesion[1,2]. Lymphocytes do not adhere spontaneously; activation of protein kinase C (PKC)[3] by phorbol esters, however, gives rise to strong LFA-1-dependent adhesion[4], indicating that activation of LFA-1 is required to induce cell adhesion. We have now investigated whether the functionally important CD2 and CD3 surface structures on T lymphocytes[5-8] are involved in the activation of LFA-1. The stimulation of these molecules, which causes activation of PKC[9,10], strongly promoted LFA-1-dependent adhesion. Furthermore, we demonstrate by using cells from an LFA-1-deficient patient that this enhanced lymphocyte adhesion is caused by activation of the LFA-1 molecule and not by activation of its ligands. LFA-1 was persistently activated by triggering through CD2 but only transiently by triggering through CD3. We postulate that CD2 and CD3 can differentially regulate the affinity of LFA-1 for its ligands by modulating its molecular conformation through PKC-dependent mechanisms.

LFA-1, CR3 and p150, 95 comprise a group of heterodimeric adhesion receptors with a unique α-chain and a common β-chain. They mediate adhesion among leukocytes and of

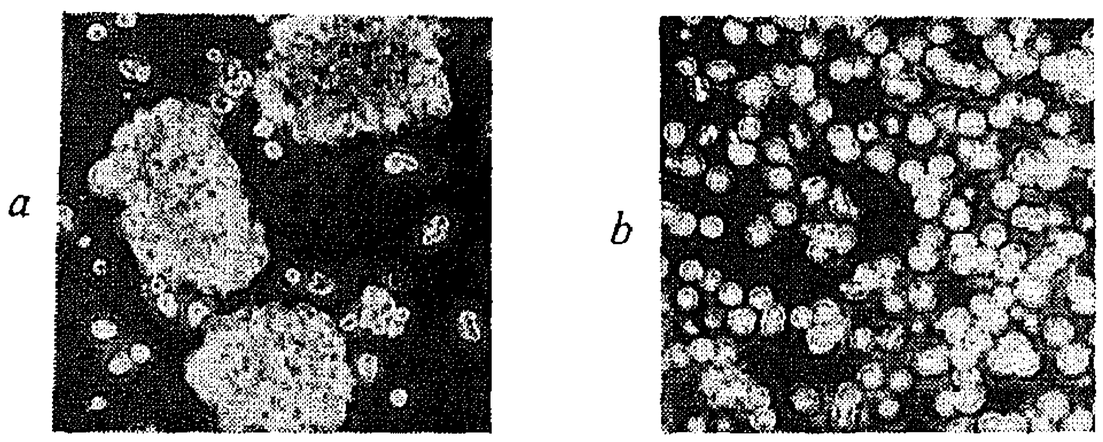

FIG. 1 Photomicrographs of homotypic cell adhesion and its inhibition by anti-LFA-1 antibodies. JS-136 cells were incubated with: *a*, NKI-L16 antibody (1 $\mu g\,ml^{-1}$); 80% adhesion; *b*, NKI-L16 (1 $\mu g\,ml^{-1}$) and anti-LFA-1 (5 $\mu g\,ml^{-1}$); <1% adhesion; or *c*, anti-CD3 (10 $\mu g\,ml^{-1}$; 60% adhesion), and scored in the qualitative assay (Table 1) for 40 min.

TABLE 1 Homotypic cell adhesion of JS-136 T cells and NL-3 NK cells

	Aggregation (%)					
	JS-136			NL-3		
	Medium	+Anti-LFA-1	+Anti-LFA-3	Medium	+Anti-LFA-1	+Anti-LFA-3
Medium	10	<1	10	10	<1	10
TPA	60	<1	60	80	<1	80
NKI-L16	80	<1	80	70	<1	70
Anti-CD3	60	<1	60	10	<1	10
Anti-CD2	70	<1	60	60	<1	60
Anti-CD4	10	<1	10	10	<1	10
Antl-CD71	10	<1	10	10	<1	10

Induction of homotypic cell adhesion (%) of JS-136 T cells and NL-3 NK cells after stimulation with TPA, mAbs NKI-L16, anti-CD3 and anti-CD2, and with isotype-matched control antibodies directed against CD4 and the transferrin receptor (CD71). Stimuli were added in the following concentrations: TPA (10 ng ml^{-1}), NKI-L16[15] (1 μg ml^{-1}), anti-CD3 antibodies[25] (SPV-T3b; 10 μg ml^{-1}), a combination of anti-CD2 antibodies[17] (CLB-T11.1/1 clone 6G4 and CLB- T11.2/1 clone 4B2; 10 μg ml^{-1}), anti-CD4 (CLB-T4/1; ref. 26) and anti-CD71 (661G10; 10 μg ml^{-1}; ref. 27). Cell adhesion was blocked after addition of anti-LFA-1 antibodies[19] (CLB LFA-1/1; 5 μg ml^{-1}) or anti-ICAM antibodies (RR 1/1; data not shown). Addition of anti-LFA-3 antibodies (TS 2/9; 5 μg ml^{-1}) did not inhibit cell adhesion. One representative experiment out of four performed is shown (s.d. < 10%). All experiments were carried out in triplicate (s.d. < 10%). JS-136 ($CD2^+$ $CD3^+$ $CD4^+$ LFA-1^+ LFA-3^+ ICAM-1^+) is a cytolytic T-cell clone, directed against HLA-DR[28]. The NK clone (NL-3) expressed the following surface molecules: $CD3^-$ $CD2^+$ LFA-1^+ LFA-3^+ ICAM-1^+. Cells were cultured as described previously[28] and tested 4 days after re-stimulation when spontaneous cell aggregation was ≤10%. Homotypic cell adhesion was measured as described previously[15]. Before the test, wells were washed twice in Iscove's medium containing 10% FCS, resuspended, and 2×10^5 cells seeded in 96-well microtitre plates in a volume of 50 μl. Monoclonal antibodies were added in a volume of 50 μl and the cells were incubated for 40 min at 37 °C, after which aggregate formation was determined through light microscopy by at least two investigators. Scores ranged from <1–100, in a scale of 10, where <1 indicated that essentially no cells were aggregated in clusters and 10, 20, 40, 60, 80 and 100 correspond with cell aggregations of <10%, <30%, <50%, <80%, >80% in compact clusters, and >90% large compact clusters, respectively. The percentage aggregation was determined by counting the number of free cells, using the equation: percentage of aggregation = 100 × [1 − (no. free cells/no. input cells)]; no. of input cells = no. cells per ml in control tube containing only cells and medium; no. free cells = no. nonaggregated cells per ml from experimental tube.

leukocytes with other cell types by binding to their respective ligands. Defined ligands of LFA-1 are the intercellular adhesion molecule-1 (ICAM-1, CD54)[11] and -2 (ICAM-2)[12]. Leukocytes do not spontaneously aggregate; activation of PKC by phorbol esters, such as TPA (12-*O*-tetradecanoylphorbol-13-acetate), however, can induce LFA-1- and CR3-dependent adhesion[4,13–14]. An anti-LFA-1 monoclonal antibody (NKI-L16) or its Fab fragments, reactive with a unique epitope on the α-chain, is also capable of inducing LFA-1-dependent adhesion with kinetics strikingly similar to those seen with TPA[15]. These observations indicate that LFA-1-dependent adhesion requires a change of the conformation of LFA-1.

This prompted us to investigate whether the T-cell receptor–CD3 complex, responsible for recognition of antigen[5], or CD2, which is believed to contribute to cell adhesion by binding to the LFA-3 molecule[6–8], can modulate LFA-1-mediated adhesion. The binding of ligands by these glycoproteins generates second messengers mediating the activation of PKC[9–10]. Assuming that PKC can activate LFA-1, triggering of the CD2 or T-cell receptor–CD3 pathways should therefore result in T-cell adhesion[16]. Anti-CD3 monoclonal antibodies (mAbs) or a combination of two anti-CD2 mAbs required for T-cell proliferation[17], added in soluble form or bound to plastic, indeed strongly enhanced homotypic aggregation of cells of an alloreactive human T-cell clone, JS-136 (Fig. 1; Table 1). Furthermore, anti-CD2 but not anti-CD3 mAbs promoted homotypic adhesion of $CD3^-$ NK clone (NL-3) cells, whereas TPA or NKI-L16 induced adhesion of both $CD3^+$ and $CD3^-$ cells, without an apparent increase in expression of LFA-1 or ICAM-1. Isotype-matched control antibodies directed against other surface molecules expressed by these clones were ineffective at inducing adhesion, indicating that the adhesion induced by anti-CD3 or anti-CD2 mAbs is not caused by agglutination. The induced adhesion is mediated by LFA-1 because it can be inhibited by anti-LFA-1 antibodies, but not by anti-LFA-3 antibodies. This notion is supported by the characteristic Mg^{2+} dependence and temperature dependence of LFA-1-mediated adhesion (data not shown).

The kinetics of aggregation were examined next (Fig. 2). Maximal aggregation of JS-136 cells was found within 20 min, regardless of whether the cells were stimulated with TPA, NKI-L16 or mAbs anti-CD3 or anti-CD2. To measure the time course of LFA-1 activation, aggregates were disrupted at various time-points after initiation of adhesion, and the ability of the suspended cells to re-form aggregates was determined. Cells stimulated by TPA, NKI-L16 and mAb anti-CD2 were able to re-aggregate without extra addition of stimuli, and remained maximally aggregated for >12 h. By contrast, the anti-CD3-induced aggregates were unable to re-form aggregates when the cells were resuspended later than 30 min after initial induction (Fig. 2), indicating that anti-CD3 mAb transiently activates

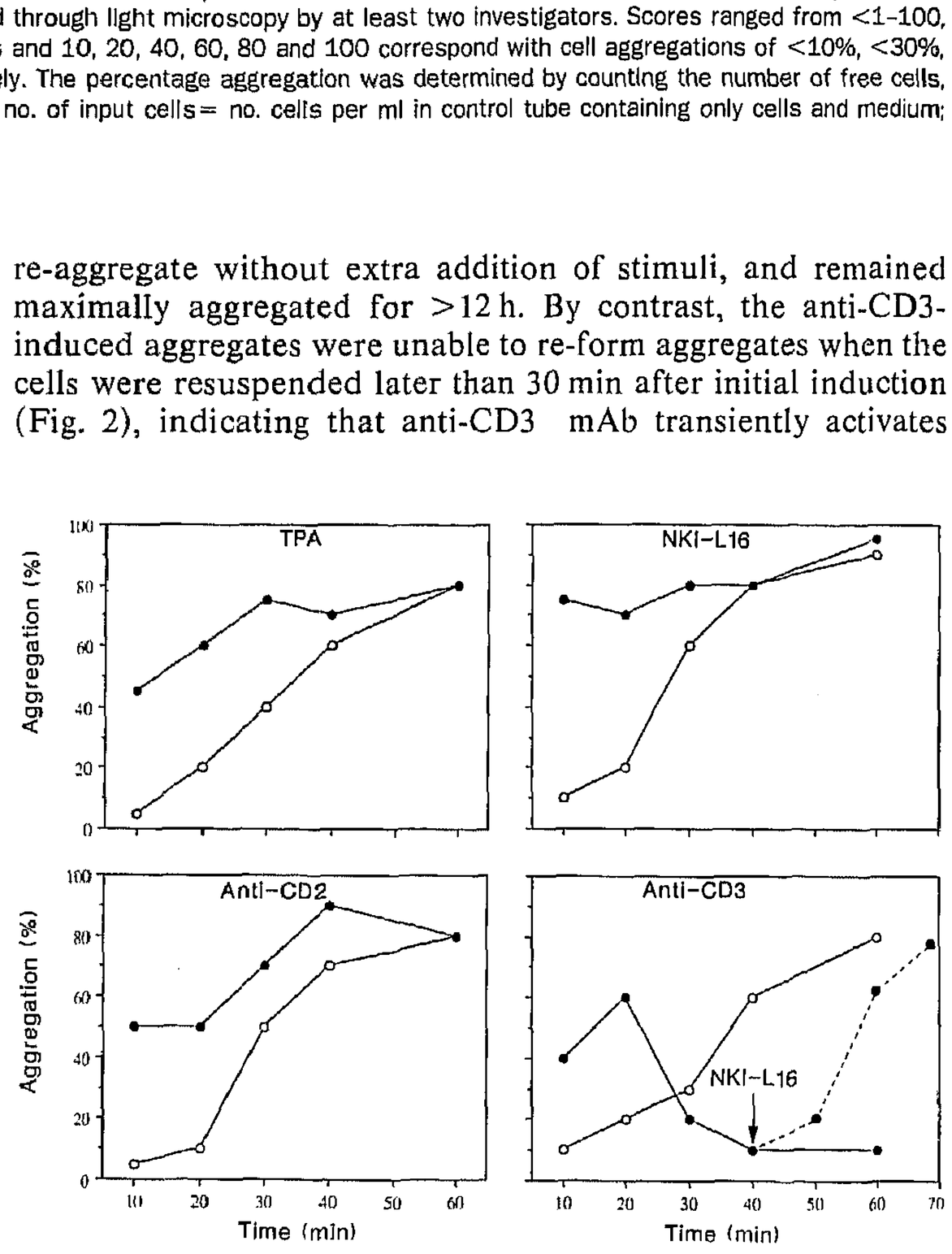

FIG. 2 Kinetics of homotypic cell adhesion of T-cell clone JS-136 (%) induced by TPA, or mAbs NKI-L16, anti-CD2 or anti-CD3. Cell adhesion was followed in time (—○—) and the capacity of cells to re-aggregate after disruption of cell clusters (—●—) is shown. Cells were stimulated for various periods of time. Subsequently, aggregates were disrupted, and the % aggregation was determined after an additional incubation period of 40 min without extra addition of stimuli (—●—). The inability of anti-CD3-treated cells to re-aggregate could be completely restored by the addition (arrow) of NKI-L16 antibody (---●---).
METHODS. Homotypic cell adhesion of cytotoxic T-lymphocyte clone JS-136 cells was performed as described in Table 1. The percentage of aggregated cells was determined in the course of time, and the concentrations of mAbs used (—○—) are given in Tables 1 and 2. Activation of LFA-1 was measured at various time intervals (—●—). Cell clusters were disrupted by vigorous pipetting to form single-cell suspensions. Subsequently the capacity of cells to re-aggregate was measured without the addition of extra stimuli (experiments were carried out in triplicate (s.d. < 10%; one representative experiment out of four performed is shown, s.d. ≤ 10%). Nonspecific aggregation (spontaneous aggregation and aggregation induced by control antibodies of IgG1 and IgG2a isotype) was always <10%.

TABLE 2 Heterotypic cell adhesion of LFA-1$^-$ with LFA-1$^+$ T-cell clones

T-cell clones			
LFA-1$^-$ LAD 6 pretreatment	LFA-1$^+$ JS-136 pretreatment	Stimulus	Heterotypic aggregation (%)
—	—	—	10
—	—	NKI-L16	70
—	—	TPA	40
—	—	Anti-CD3	40
—	—	Anti-CD2	50
NKI-L16	—	—	<1
TPA	—	—	<1
Anti-CD3	—	—	<1
Anti-CD2	—	—	<1
—	NKI-L16	—	60
—	TPA	—	40
—	Anti-CD3	—	40
—	Anti-CD2	—	40

Heterotypic cell adhesion of cloned LFA-1$^-$ T cells (LAD 6) with LFA-1$^+$ T cells (JS-136). Heterotypic cell adhesion was determined 90 min after addition of medium, NKI-L16 (1 μg ml^{-1}), TPA (10 ng ml^{-1}), anti-CD3 (SPV-T3b; 10 μg ml^{-1}) and anti-CD2 (T11$_1$ + T11$_2$, 10 μg ml^{-1}). Pre-incubation of LAD 6 with NKI-L16, TPA, mAbs Anti-CD3 or anti-CD2 did not result in the formation of cell clusters, whereas pre-incubation of JS-136 cells caused formation of heterotypic aggregates indicating that adhesion is induced through LFA-1. TPA, or mAbs NKI-L16, anti-CD3 or anti-CD2 did not induce homotypic aggregation of LAD 6 cells (data not shown). A representative experiment out of three is shown (s.d. < 10%); experiments were carried out in triplicate (s.d. < 10%). The LFA-1$^-$ T cell clone LAD 6 (CD2$^+$ CD3$^+$ CD4$^+$ CD8$^-$ LFA-3$^+$ ICAM$^+$) was raised by limiting dilution of perhipheral blood lymphocytes (PBL) from a LAD patient. Clones were grown weekly on stimulation with irradiated allogeneic PBL and cells of the B-cell line JY, supplemented with phytohaemagglutinin (PHA; 0.2 μg ml^{-1}) and interleukin 2 (IL-2; 100 U ml^{-1}). Heterotypic cell adhesion was determined by means of double fluorescence. LAD 6 cells (1 × 10^6 ml^{-1} were stained with red hydroethidine (HE; Polyscience, in *N,N*-dimethylacetonide) at a concentration of 3 mg ml^{-1} in Iscove's medium. JS-136 cells (1 × 10^6 ml^{-1}) were stained with the green dye (sulphofluorescein diacetate (SFDA) in dimethyl sulphoxide; Molecular Probe) at a concentration of 5 μg ml^{-1}. After 1 h incubation at 37 °C, cells were washed twice with Iscove's medium containing 10% FCS and seeded in flat-bottomed plates in a concentration of 1 × 10^5 HE-labelled cells together with 1 × 10^5 SFDA-labelled cells per well. Aggregation was performed at 37 °C, and after 90 min the cells were fixed in 0.5% paraformaldehyde followed by fluorescence microscope analysis to determine the percentage of heterotypic cell aggregates. The aggregates were checked to be composed of equal ratio of the two different cell types. In those cases where LAD 6 cells or JS-136 cells were pretreated with NKI-L16, TPA, mAbs anti-CD2 or anti-CD3 for 20 min at 37 °C, the cells were extensively washed to remove nonbound antibodies.

LFA-1, and that CD2 and CD3 could activate LFA-1 through different signalling pathways. The transient CD3-mediated response was not due to decreased expression of LFA-1 or its ligand(s), because these anti-CD3-treated cells could still be induced to aggregate by NKI-L16 antibodies (Fig. 2). When, instead of activated T cells, resting peripheral blood lymphocytes were used, a maximal aggregation was only observed after 6 h incubation. This seems to be directly correlated with the expression of the NKI-L16 epitope on the LFA-1 molecule (Y.v.K., unpublished results).

To prove that induction of adhesion is a consequence of activation of the LFA-1 molecule, rather than activation of its ligand(s) or other adhesion molecules (CD2–LFA-3), we performed heterotypic cell-binding studies with LFA-1-positive (LFA-1$^+$) and LFA-1-deficient (LFA-1$^-$) T cells of a patient suffering from leukocyte adhesion deficiency syndrome[1,2,18]. Leukocytes from these patients lack cell-surface expression of all CD11 and CD18 molecules, generally owing to a genetic defect in the common β-chain gene. A T-cell clone derived from this 'severe-type' leukocyte adhesion deficient patient completely lacked β-chain messenger RNA expression and LFA-1, CR3 and p150,95 surface expression. But T cells expressed normal levels of ICAM-1, CD2, CD3 and LFA-3 (data not shown). LFA-1$^-$ cells could not be induced to adhere among themselves, but were able to bind to JS-136 (CD3$^+$ LFA-1$^+$) cells after addition of TPA, or mAbs NKI-L16, anti-CD3 or anti-CD2 (Table 2). When the LFA-1$^-$ cells were incubated with the different stimuli, before mixing with the LFA-1$^+$ cells, neither homotypic (among LFA-1$^-$ or LFA-1$^+$ cells) nor heterotypic adhesion was observed. By contrast, pre-incubation of the LFA-1$^+$ cells with the same stimuli resulted in the formation of large clusters of cells containing both LFA-1$^+$ and LFA-1$^-$ cells in a ratio of 1:1 (Table 2). These results show that T-cell adhesion is induced by activation of LFA-1 and not by activation of its ligands. Induction of heterotypic T-cell adhesion by these stimuli exclusively activates the LFA-1-mediated adhesion pathway and not the CD2–LFA-3 adhesion pathway, despite the fact that the LFA-1$^-$ T cells express normal levels of LFA-3 and CD2.

Evidence is thus presented for the existence of an activated form of LFA-1. Binding of NKI-L16 to LFA-1, or stimulation of PKC by TPA, or mAbs anti-CD2 or anti-CD3, probably results in a conformational modification of the LFA-1 molecule, leading to high-affinity ligand binding. Although induction of adhesion by NKI-L16 is thought to be exclusively an extracellular event, the molecular alteration induced by the other stimuli would be catalysed by PKC. This notion is supported by the observation that PKC inhibitors completely block TPA- or anti-CD3-induced aggregation, but have no effect on induction of adhesion by NKI-L16 (Y.v.K. *et al.*, unpublished results). Alternatively, LFA-1 itself could be involved in signal transduction[19].

We propose that numerous LFA-1-dependent T-lymphocyte interactions occurring in an immune response are controlled by the T-cell preceptor–CD3 complex and by CD2. A case in point is the interaction between cytotoxic T lymphocytes and their target cells. It has been reported that LFA-1-dependent binding of the effector *T* cell to the target cell precedes recognition by the T-cell receptor–CD3 complex[20]. The results presented here indicate that this cell–cell interaction, although of low affinity, facilitates antigen recognition by the T-cell receptor–CD3 complex. On antigen recognition, PKC, activated through CD3, could activate LFA-1 by direct phosphorylation of the β-chain of LFA-1[21,22], or alternatively, by phosphorylation of a cytoskeletal protein such as talin[23], which then might bind and activate LFA-1. This strengthens the interaction between cytotoxic lymphocytes and target cells for efficient delivery of cytotoxic mediators causing target-cell lysis. Target-cell detachment could also be controlled by PKC. Phosphorylation and subsequent modulation of CD3[24] possibly results in downregulation of PKC, and could thus explain the inactivation of LFA-1 observed 20 min after triggering with anti-CD3 antibody. □

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