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Lymphocyte–fibroblast adhesion

A useful model for analysis of the interaction of the leucocyte integrin LFA-1 with ICAM-1

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The adhesion of human T lymphoblasts to ICAM-1-expressing normal dermal fibroblasts has been assessed as a sensitive model system for the analysis of the interaction of the leucocyte integrin LFA-1 with its counter-receptor ICAM-1. Using this model system, the effects of factors known to regulate the activity of LFA-1 have been quantitated: temperature; concentration of divalent cations; and exposure to phorbol esters. We show here that under the appropriate assay conditions, this model system represents a useful and simple alternative to the detection of leucocyte binding to purified ICAM-1 and also has the additional advantage of permitting more sensitive quantification than is possible using the homotypic adhesion assay.

LFA-1 activation; ICAM-1; Leucocyte adhesion; Intercellular adhesion

1. INTRODUCTION

The LFA-1 (CD11a/CD18) molecule is a member of the leucocyte integrin family of adhesion receptors [1–6]. The expression of this adhesion receptor, which is restricted to white blood cells, is required for many leucocyte functions including T cell-mediated killing, T-helper and B lymphocyte responses, natural killing, antibody-dependent cellular cytotoxicity mediated by monocytes and granulocytes, and adherence of leucocytes to endothelial and epithelial cells and to fibroblasts. These leucocyte functions are known to require an adhesion step which is mediated by the interaction of LFA-1 molecules expressed on leucocytes with counter-receptor or ligand molecules expressed on target cells. ICAM-1, the principal ligand for LFA-1, was identified using as a model system, formation of intercellular homotypic aggregates induced after treatment of several lymphoid and myeloid cell lines with phorbol esters [6–8]. Monoclonal antibodies (mAb) specific for ICAM-1 have been selected by testing for ability to inhibit such LFA-1-dependent aggregates [9].

The fact that circulating and freshly isolated resting leucocytes express significant levels of both LFA-1 and ICAM-1 but do not aggregate strongly suggests the existence of mechanisms that regulate the interaction of LFA-1 with ICAM-1. Although these mechanisms are not yet fully understood, it is known that they are dependent on temperature and metabolic energy, and require the presence of divalent cations, particularly Mg^{2+} [8,10–11]. Dependence on all these factors demonstrates that the LFA-1/ICAM-1-mediated cellular adhesion is an active process that requires more than the mere presence of receptor and counter-receptor molecules on opposing cell membranes [10,12,13]. Moreover, this regulatory effect is mediated via LFA-1 with ICAM-1 molecules seemingly being constitutively avid for LFA-1 [4,5,10,11]. The transition of LFA-1 into an active form which readily binds to ICAM-1 can be induced by intracellular signals generated through a number of leucocyte surface molecules including TcR/CD3 [12], CD2 [12,14], CD43, CD44, CD14 and MHC class II (for review see [15]). As phorbol esters are potent activators of protein kinase C, their LFA-1 activating effect can also be explained by the involvement of an intracellular signalling mechanism.

In order to characterize the mechanisms involved in the activation of LFA-1, sensitive quantification of the LFA-1/ICAM-1 binding is required. So far, the two most frequently used model systems for studying this interaction have been the induction of leucocyte homotypic aggregation [8,16–18] and binding of leucocytes to purified ICAM-1 [10–12]. In this paper we have characterized the adhesion of human T lymphoblasts to monolayers of ICAM-1-expressing fibroblasts under different

Abbreviations: mAb, monoclonal antibody(ies); PdBu, phorbol-12,13-dibutyrate; LFA-1, lymphocyte-function-associated antigen-1; ICAM-1, intercellular-adhesion-molecule-1.

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experimental conditions and describe this technique as a highly useful model system for the analysis of intracellular as well as extracellular factors affecting the activation of LFA-1 molecules resulting in enhanced interaction with ICAM-1.

2. MATERIALS AND METHODS

2.1. *Reagents*

$MgCl_2$, $CaCl_2$ and ethyleneglycol-*bis*-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) were obtained from Fisons (Loughborough, UK); ^{51}Cr (10–35 mCi/ml) was from Amersham International; (*N*-[2-hydroxyethyl]-piperazine-*N'*-[2-ethanesulfonic acid]) (HEPES), phorbol-12,13-dibutyrate (PdBu), cytochalasin D, and fluorescein isothiocynate (FITC)-goat anti-mouse IgG were from Sigma (UK). All tissue culture reagents were obtained from Gibco (UK) and all plasticware from Becton Dickinson (UK).

2.2. *Monoclonal antobodies*

The monoclonal antibodies 15.2 (anti-ICAM-1, CD54), 38 (anti-LFA-1, CD11a), 44 (anti-CR3, CD11b) and 3.9 (anti-p150,95, CD11c) have been described elsewhere [19–22]. The anti-ICAM-1 mAb 8.4A2 is a generous gift from Dr. Dorian Haskard (London, UK). The following monoclonal antibodies were generous gifts: P1E6 (anti-VLA-2, CD49b), P1B5 (anti-VLA-3, CD49c), P1D6 (anti-VLA-5, CD49e) and P4C10 (anti-VLA β_1, CD29) (Dr. Elizabeth Wayner, Seattle, USA); HP2/1 (anti-VLA-4, CD49d) (Dr. Francisco Sanchez-Madrid, Madrid, Spain); GT2 (anti-LFA-2, CD2) (Dr. Alain Bernard, Villejuif, France); 4B2 (anti VCAM-1) (Dr. David Simmons, Oxford, UK); UCHT1 (anti-CD3) (Dr. Peter Beverly, London, UK); TS2/9 (anti-LFA-3, CD58) (Dr. Timothy Springer, Boston, USA). The null mAb MOPC 21 (generous gift from Dr. Gordon Ross) has been used as a control in all experiments.

2.3. *Flow cytometric analysis*

Cells (5×10^5/test) were pelleted in flexible 96-well microtitre plates, resuspended in 50 μl of mAb tissue culture supernatant and incubated for 30 min on ice. After three washes with RPMI-1640 medium, cells were resuspended in 50 μl of FITC-goat anti-mouse IgG (Cappel, 1:400 in medium) and incubated for 30 min on ice. Finally, cells were washed twice with RPMI-1640, resuspended in 300 μl of RPMI-1640 and analyzed using a Becton Dickinson FACScan flow cytometer.

2.4. *Cells and cell culture*

Human foreskin dermal fibroblasts, obtained from the ICRF Cell Production Department and cultured in MEM medium supplemented with 10% FCS, were used between passages 6 and 10. Peripheral blood mononuclear cells (PBMC) were obtained from freshly-drawn heparinised blood by centrifugation over Ficoll/Hypaque (Pharmacia, Uppsala, Sweden). Human T lymphoblasts were prepared by stimulating PBMC with 5 μg of phytohaemagglutinin/ml and 50 nM phorbol-12,13-dibutyrate (PdBu) for 48 h in RPMI-1640 medium supplemented with 10% FCS, and then culturing for up to 14 days in RPMI-1640 medium containing 10% FCS and 5% culture supernatant from the IL-2-producing cell line MLA [23].

2.5. *Binding assay*

Human fibroblasts were transferred from tissue culture flasks into 96-well plates and grown to confluence. Culture supernatants were then discarded and fibroblasts were fixed with 2% formaldehyde in PBS (150 μl/well) for 1 h at room temperature. Using this protocol, the fibroblasts remained firmly adherent to the wells during all the described assay conditions. Wells were then washed six times for 10 min with 200 μl of PBS without Ca^{2+} or Mg^{2+}. At this stage, plates could be stored at 4°C for several days. 5×10^7 T lymphoblasts were labelled with 200 μCi $^{51}CrO_4^{-2}$ in 1 ml of RPMI/2% FCS for 90 min at 37°C, washed and incubated in RPMI/10% FCS for 40 min at 37°C. Finally, T lymphoblasts were washed three times in assay buffer consisting of 20 mM HEPES, 140 mM NaCl, 2 mg/ml glucose, pH 7.4, and resuspended in this buffer at 4×10^6 cells/ml. Fifty μl of assay buffer containing the appropriate concentrations of reagents (mAbs, divalent cations, PdBu, cytochalasin D) and 50 μl of T lymphoblasts were added to each well on ice. Plates were then centrifuged at 30 × *g* for 1 min in order to gently pellet the T cells onto the fibroblast monolayer, and incubated at the temperature required (4°C or 37°C) for 35 min. After incubation, wells were washed three times with 200 μl of warm RPMI-1640 (37°C) and finally the T lymphoblasts were lysed with 40 μl of 1% Triton X-100 in water for determination of incorporated radioactivity. All binding assays were carried out in triplicate and the standard errors were always less than 10%.

3. RESULTS AND DISCUSSION

The model systems most generally used to analyze the activation of LFA-1 have been the homotypic leucocyte aggregation assay and leucocyte binding to purified ICAM-1. Although determination of leucocyte binding to immobilized ICAM-1 is the best defined system in molecular terms, purification of ICAM-1 is a tedious, expensive and time consuming procedure which involves growing large amounts of ICAM-1-expressing cells and yields only small amounts of purified ICAM-1. On the other hand, the induction of leucocyte homotypic aggregates is a very simple qualitative assay but has the drawback of being difficult to precisely quantitate. These difficulties arise from the subjectivity implicit in the counting of large numbers of aggregates and free cells. In this paper the adhesion of human T lymphoblasts to monolayers of ICAM-1-expressing normal skin fibroblasts has been used as a simple alternative model system giving highly specific and sensitive quantification of the interaction of LFA-1 with ICAM-1 and allowing analysis of the requirements for the activation

Table I

Expression of adhesion receptors and counter-receptors on human T lymphoblasts and skin fibroblasts

mAb	T lymphoblasts	Fibroblasts
–	2.97 (2.1)	4.93 (3.5)
38 (CD11a, LFA-1)	94.79 (100.0)	ND
44 (CD11b, CR3)	3.85 (4.2)	ND
3.9 (CD11c, p150-95)	3.94 (6.9)	ND
15.2 (CD54, ICAM-1)	15.06 (84.1)	109.64 (99.9)
UCHT1 (CD3)	50.58 (97.5)	ND
TS2/9 (CD58, LFA-3)	17.97 (93.4)	33.3 (99.7)
GT2 (CD2, LFA-2)	15.03 (95.0)	ND
P1E6 (CD49b, VLA-2)	7.61 (37.0)	71.34 (100.0)
P1B5 (CD49c, VLA-3)	8.05 (49.3)	53.69 (100.0)
HP2/1 (CD49d, VLA-4)	32.34 (97.2)	45.19 (100.0)
P1D6 (CD49e, VLA-5)	13.16 (77.4)	136.88 (100.0)
P4C10 (CD29, VLA β_1)	36.60 (99.7)	280.02 (100.0)
4B2 (VCAM-1)	ND	6.15 (11.4)
W6/32 (HLA Class I)	ND	240.77 (100.0)

Binding of mAbs was assayed by flow cytometry as described in Materials and Methods. Values represent mean peak fluorescence (MFI) and numbers in parentheses represent percentage of positive cells. ND, not determined.

of LFA-1 molecules which results in binding to ICAM-1. Other studies have already described this adhesion between human or murine T cells and fibroblasts but none of these studies have characterized the LFA-1/ICAM-1 interaction and focussed on the factors that control LFA-1 activity [24,25].

The expression of adhesion receptors and counter-receptors on both the human T lymphoblasts and fibroblasts used in this work is shown in Table I. It is interesting that these T lymphoblasts express significant levels of both LFA-1 and ICAM-1 but do not spontaneously form homotypic intercellular clusters after two weeks in culture. This fact indicates that LFA-1 is in an inactive form and has to be activated in order to readily interact with ICAM-1. In addition, these T lymphoblasts also express the adhesion pair CD2/CD58 and several members of the β_1 integrin family of VLA receptors specific for extracellular matrix proteins. On the other hand, the dermal fibroblasts express ICAM-1, VLA and LFA-3 (CD58) but show very little expression of VCAM-1, a ligand for VLA-4.

In order to restrict effects to the activity of LFA-1 on T cells it was decided to fix the ICAM-1-expressing fibroblasts prior to the addition of T lymphoblasts. This fixation step adds the additional advantage of permitting the study of LFA-1-dependent T cell adhesion under conditions such as the absence of divalent cations or low temperature that would lead to the detachment of many fibroblasts from plastic, with the subsequent loss of sensitivity and accuracy of the assay. Since formaldehyde, as well as many other cross-linking fixatives, is known to alter or even destroy some epitopes on a number of cell surface antigens [26] the effect of the fixation protocol was tested on the expression of two different functional epitopes expressed on ICAM-1 molecules on fibroblasts. The expression of ICAM-1 epitopes recognized by mAb 15.2 and 8.4A2 remained unaffected after fixation (Fig. 1) suggesting that the conformation of the ICAM-1-molecules on fibroblasts is preserved after a rigorous fixation protocol and that functional activity might also be unaffected. Fig. 2 shows that this is indeed the case since T lymphoblast adhesion to the ICAM-1-expressing fibroblasts was achieved when the binding assay was done in RPMI-1640 at 37°C in the presence of phorbol ester PdBu (50 nM). The LFA-1/ICAM-1 specificity of this interaction is demonstrated by the inhibitory effect of both anti-LFA-1 or anti-ICAM-1 mAb as compared to the control mAb MOPC 21. In a previous report [24], Abraham et al. found that the spontaneous binding of T lymphoblasts to monolayers of human fibroblasts in normal medium and in the absence of any stimulation was almost completely lost after fixation of the fibroblasts with paraformaldehyde but that such adhesion seemed to be LFA-1/ICAM-1 independent. This finding is consistent with our results since the percentage of spontane-

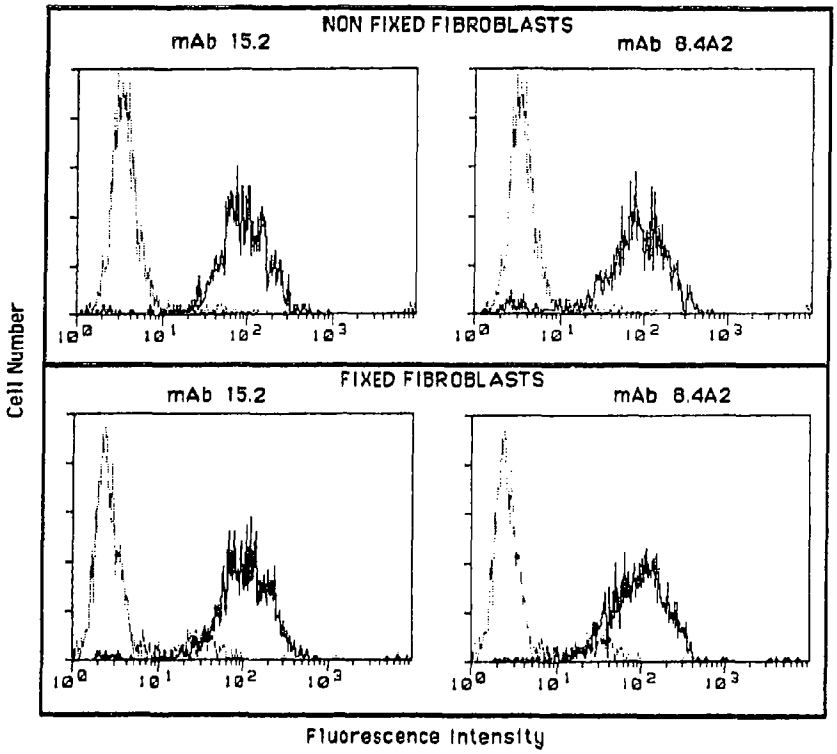

Fig. 1. Effect of formaldehyde fixation on ICAM-1 expressed on human dermal fibroblasts. The expression of two functionally different epitopes detected by mAbs 15.2 and 8.4A2 on ICAM-1 remains unaffected after fixation of the fibroblasts with 2% formaldehyde for 1 h. Staining with mAb MOPC 21 is also included as a negative control.

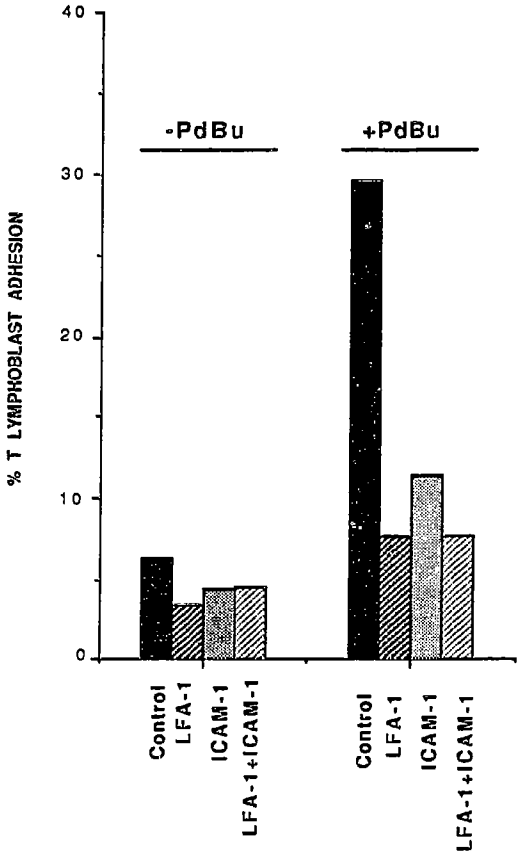

Fig. 2. Effect of phorbol ester treatment (50 nM) on the adhesion of T lymphoblasts to ICAM-1-expressing human dermal fibroblasts. The LFA-1/ICAM-1 specificity of this adhesion is demonstrated by the strong inhibitory effect of anti-LFA-1 (mAb 38) or anti-ICAM-1 (mAb 15.2) mAbs. The null mAb MOPC 21 was used as a control. All mAbs were used at 10 μg/ml. The binding assay was carried out in RPMI-1640 at 37°C for 35 min.

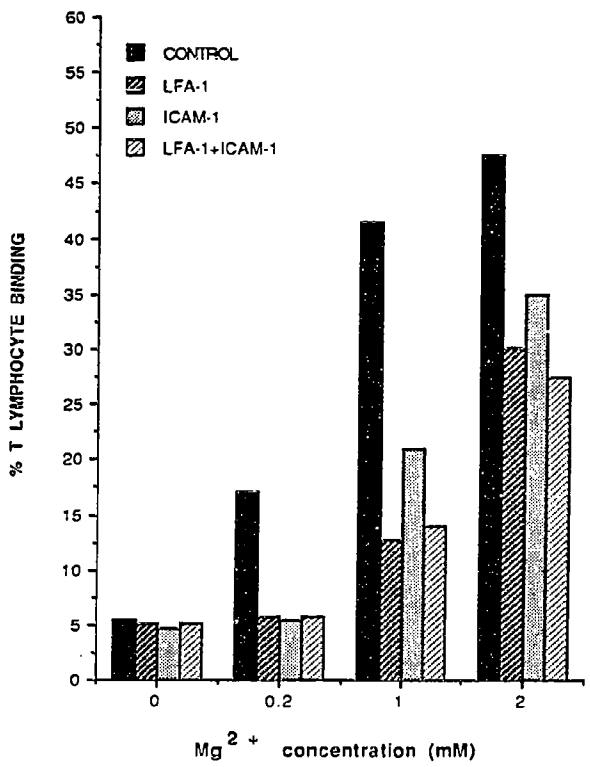

Fig. 3. Phorbol ester-induced adhesion of T lymphoblasts to ICAM-1-expressing fibroblasts is Mg^{2+}-dependent. Concentrations of phorbol ester PdBu and mAbs are the same as in Fig. 2. The assay was done in assay buffer containing 20 mM HEPES, 140 mM NaCl, 1 mM EGTA, 2 mg/ml glucose and the indicated concentrations of Mg^{2+} at 37°C for 35 min.

ous T lymphoblast binding to the fixed fibroblasts in the absence of phorbol ester stimulation is low (Fig. 2), which clearly indicates a requirement for LFA-1 activation. Also, very little binding of T lymphoblasts (always below 5%) could be observed at 4°C (data not shown) which is also in agreement with the known temperature dependence of LFA-1 activation [12].

This adhesion model system was next applied to the analysis of the Mg^{2+} dependence of LFA-1 activation. In the absence of any divalent cation, PdBu did not induce T lymphoblast binding to the fixed fibroblasts, which is in accordance with the known Mg^{2+} requirement for LFA-1/ICAM-1 interaction [8,10,11]. The percentage of T lymphoblasts that bind to ICAM-1-expressing fibroblasts increases with the concentration of Mg^{2+} (Fig. 3). At a concentration of 1 mM Mg^{2+} approximately 45% of T lymphoblasts adhere to the fibroblasts and this adhesion is LFA-1/ ICAM-1-dependent as demonstrated by inhibition with anti-LFA-1 or anti-ICAM-1 mAbs. It is worth noting that the normal extracellular concentration of Mg^{2+} is in the range of 1–2 mM, which suggests that the amount of this cation required for LFA-1 activation is consistent with physiological levels. It is interesting that the percentage of inhibition of T cell adhesion that can be obtained with anti-LFA-1 or anti-ICAM-1 mAb in the presence of PdBu is greatly decreased when the concentration of Mg^{2+} is higher than 1 mM. This finding suggests that at high concentrations of Mg^{2+}, phorbol esters preferentially activate other lymphocyte-fibroblast adhesion pathways. It seems unlikely that this effect of Mg^{2+} and phorbol esters is mediated by the CD2/LFA-3 pair as this adhesion pathway is neither regulated by divalent cations nor by phorbol esters [27,28]. Another possibility is that it is mediated by members of the β_1 integrin family since the activity of various fibronectin receptors has been reported to be regulated by divalent cations and phorbol esters [29–31].

Using this model system, the synergistic effect of Ca^{2+} on the activation of LFA-1 in the presence of low concentrations of Mg^{2+} (below 1 mM) and PdBu has been quantitated (Fig. 4). The results are in accordance with those described by other groups using as model systems the phorbol ester-induced homotypic aggregation of B and T lymphocytes or detection of B cell binding to

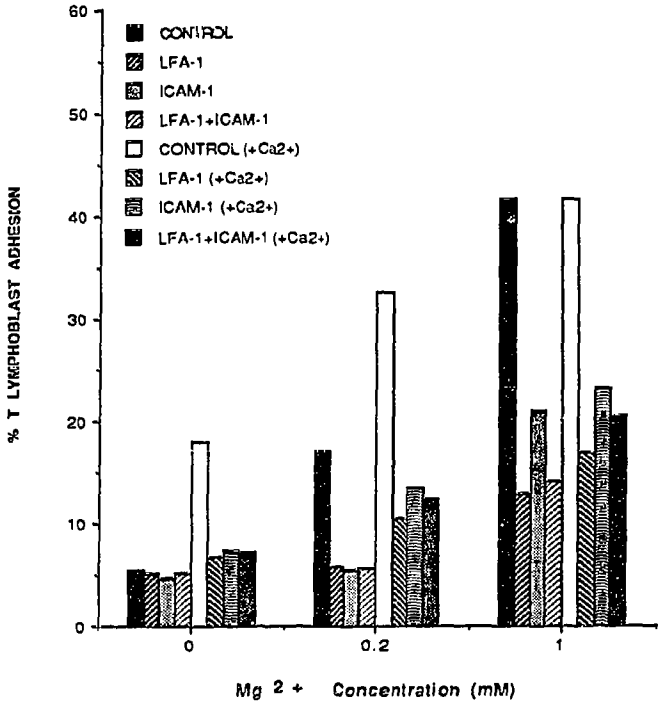

Fig. 4. Ca^{2+} (1 mM) has a potentiating effect on the PdBu-induced adhesion of T lymphoblasts to ICAM-1-expressing fibroblasts. Concentrations of PdBu and mAbs are the same as in Figs. 2 and 3. The assay was done in assay buffer containing 20 mM HEPES, 140 mM NaCl, 2 mg/ml glucose plus either EGTA (1 mM) or Ca^{2+} (1 mM) and the indicated concentrations of Mg^{2+} at 37°C for 35 min.

purified ICAM-1 [8,11]. This synergistic effect of Ca^{2+} and Mg^{2+} on phorbol ester-induced activation may explain the strong potentiating effect of phorbol esters on LFA-1/ICAM-1 interaction when the assay is done in normal culture medium RPMI-1640 (where the concentrations of Mg^{2+} and Ca^{2+} are both 0.4 mM) (see Fig. 2). Under these conditions, a large proportion of the binding of T lymphoblasts to the fibroblasts is LFA-1/ICAM-1 dependent.

In summary, using the adhesion of human T lymphoblasts to monolayers of normal dermal ICAM-1-expressing fibroblasts as a model system for analysis of the LFA-1/ICAM-1 interaction, we have been able to quantitate all the previously known requirements for the activation of LFA-1 molecules. The results show that the percentage of the adhesion of T lymphoblasts to the fibroblasts that is LFA-1/ICAM-dependent can be regulated by selecting the assay conditions, i.e. the concentrations of Mg^{2+} and Ca^{2+} and presence or absence of phorbol esters. Also, under the appropriate conditions this model system could be applied to large-scale screening for mAbs specific for ICAM-1 or for LFA-1 based on their ability to inhibit this interaction. Finally, this model system also permits an easy quantification of the relative importance of defined LFA-1 or ICAM-1 epitopes on this interaction simply by revealing the inhibitory effect of mAbs directed to any such epitopes.

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