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Immunology

Ligand intercellular adhesion molecule 1 has a necessary role in activation of integrin lymphocyte function-associated molecule 1

(adhesion/activation epitope/induced fit)

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ABSTRACT **The signaling that causes the leukocyte integrin lymphocyte function-associated molecule (LFA-1) to bind firmly to its ligand intercellular adhesion molecule 1 (ICAM-1) is transduced indirectly through other T-cell receptors and is termed inside-out signaling. We show here that the high-affinity state of LFA-1 is characterized by expression of the LFA-1 epitope detected by monoclonal antibody 24. This epitope is expressed not in response to the initial agonist-mediated signal but when LFA-1 binds to ICAM-1, indicating that ligand binding induces an alteration in LFA-1. As would be predicted, the monoclonal antibody 24 epitope is confined to the LFA-1, which is located at the site of contact between T cells and ICAM-1-expressing transfectants. When a fixation protocol for "freezing" receptors is used, only T cells that are fixed after prior exposure to ICAM-1 bind firmly to ICAM-1 a second time. This suggests that, in addition to the inside-out signaling, a previously unrecognized requirement for full activation of the leukocyte integrin LFA-1 is the initial interaction with its ligand ICAM-1. Thus, activation of LFA-1 is in part achieved by an induced fit imposed from without by interaction with ligand.**

The integrin lymphocyte function-associated molecule 1 (LFA-1) and immunoglobulin-like intercellular adhesion molecule 1 (ICAM-1) form a receptor/ligand pair of adhesion molecules required for many leukocyte functions (1, 2). Regulation of LFA-1/ICAM-1 adhesion resides with LFA-1, which, under normal circumstances, binds only weakly to ICAM-1 (3). On T cells, the affinity of LFA-1 for ICAM-1 can be increased transiently by cross-linking other membrane receptors such as CD3 (XL-CD3) associated with the T-cell receptor for longer periods of time by treatment with phorbol esters such as phorbol dibutyrate (PdBu) (3, 4) or by incubating T cells with the divalent cations required by LFA-1 for function (5). We have described an epitope recognized by monoclonal antibody (mAb) 24 that is present on the CD11 α subunit and is expressed in a manner that correlates with Mg^{2+} (and Mn^{2+}) binding (5–7). This epitope has been thought to be a "reporter" of the activated state of LFA-1 in that its expression parallels receptor activity (5–8). A role for the mAb 24 epitope in LFA-1 activation was further suggested by the ability of mAb 24 to inhibit some LFA-1 mediated functions, possibly by locking the integrin in activated form and preventing a return to the low binding state (8). In the present study, we show that in addition to signals such as XL-CD3, another necessary step in the induction of fully active LFA-1 is fulfilled by the initial binding of ligand ICAM-1 to LFA-1. This causes exposure of the activation marker or epitope detected by mAb 24, which is characteristic of LFA-1 when it is able to complex with ICAM-1 in a stable manner.

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MATERIALS AND METHODS

mAbs and ICAM-1. Production, isolation, and characterization of mAb 24 has been described (6). Other mAbs used were mAb 38 (6) and MHM24 (9), both recognizing the LFA-1 α subunit (CD11a); RR1/1, recognizing ICAM-1 (CD54) (10); CD3 mAb UCHT1 (11); and control mAbs 52U (IgG1) and 4U (IgG2a) (unpublished data). mAbs 24, 38, RR1/1, 52U, and 4U were used as purified immunoglobulin and the other mAbs were used as tissue culture supernatant. Fab fragments of mAb 24 were prepared by pepsin digestion (1%; wt/wt) of intact IgG1 in order to generate $F(ab')_2$ fragments, which were subsequently reduced with 0.01 M cysteine for 1 h at room temperature and blocked for a further hour with 0.03 M iodoacetamide (8). Any intact IgG and $F(ab')_2$ fragments were separated from Fab 24 by extensive adsorption with protein A-coupled Sepharose followed by fractionation using gel-filtration chromatography. Analysis of 10 μg of Fab 24 by SDS/PAGE showed it to be free of any such contaminants (results not shown). Direct fluorescein isothiocyanate (FITC) conjugates of mAb 24 were prepared as described and were used at 10 μg/ml (8, 12).

Recombinant ICAM-1-Fc protein consisted of the first three domains of ICAM-1 and the hinge, CH2, and CH3 domains of IgG1 (Fc) (13). The expression plasmid containing this construct was a gift from A. Craig and A. Berendt and was used to produce ICAM-1-Fc protein by transient expression in COS-1 cells and protein A chromatography. The ICAM-1-CHO transfectant was a gift from J. Trowsdale and D. Sansom and was maintained in selection medium containing geneticin (0.5 mg/ml).

T Cells. Peripheral blood mononuclear cells (PBMCs) were prepared from freshly drawn heparinized blood by centrifugation over Ficoll/Hypaque (Pharmacia). As previously reported, activated T cells were expanded from unstimulated PBMCs by culture in RPMI 1640 medium containing 10% fetal calf serum (FCS) plus treatment with phytohemagglutinin (10 μg/ml) for 48 h (5, 8). Cells were then washed and cultured in 10% FCS plus recombinant interleukin 2 (20 ng/ml) (Cetus).

T-Cell Binding Assay. *Standard T-cell assay.* The LFA-1-dependent adhesion of cultured T cells to immobilized ICAM-1-Fc has been described (5, 8). Briefly, 40 μl of ICAM-1-Fc per well in phosphate-buffered saline (PBS) (5 μg/ml) was incubated overnight at 4°C, followed by saturation of any remaining plastic sites with 2% bovine serum albumin in PBS. T cells ($2–5 \times 10^7$ cells per ml) were labeled with 200 μCi (1 Ci = 37 GBq) of ^{51}Cr for 1 h at 37°C. Cross-linking of CD3 was accomplished by incubating an aliquot of the T cells on ice for 20 min with UCHT1 mAb (1:5

Abbreviations: FITC, fluorescein isothiocyanate; ICAM-1, intercellular adhesion molecule 1; LIBS, ligand-induced binding site(s); LFA-1, lymphocyte function-associated molecule-1; mAb, monoclonal antibody; PdBu, phorbol dibutyrate.

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dilution of culture supernatant; IgG2a isotype), followed by a second incubation period of 20 min on ice with goat anti-mouse IgG2a in RPMI 1640 medium (1:100 dilution; Nordic, Maidenhead, U.K.). Either the XL-CD3- or PdBu (50 nM)-treated or control T cells (2×10^5 cells per well) were added to the ICAM-1-Fc-precoated wells, centrifuged at $75 \times g$ for 2 min, and incubated at 37°C for the periods of time indicated. After washing four times with warm RPMI 1640 medium to remove unbound T cells, the radioactivity of the bound T cells was assayed with a Betaplate counter (LKB).

Fixed T-cell assay. Briefly, [^{3}H]thymidine-labeled T cells were incubated at 2.5×10^5 cells per well in flexiwell plates for 20 min at 37°C in 40 nM PdBu/RPMI 1640 medium in either the presence or absence of anti-ICAM-1 mAb RR1/1 at 40 μg/ml. After two washes, cells were fixed with the indicated percentages of freshly prepared paraformaldehyde in Hepes buffer for 10 min at 37°C and the fixation reaction was terminated with 0.1% glycine for 10 min at room temperature. Because the T cells were now fixed, Mg^{2+}/EGTA was used as a means of activating receptors from outside the cell. After three washes, the fixed T cells were transferred into ICAM-1-Fc-containing wells precoated at 60 μg/ml and incubated a second time in Hepes buffer containing 1 mM $MgCl_2$ and 1 mM EGTA for 30 min at 37°C. Finally, the T cells were washed three times with warm Hepes buffer and the total radioactivity per well was assessed. LFA-1-specific adhesion to ICAM-1 was calculated by treating samples with CD11a mAb 38 at 10 μg/ml and determining the difference between this value and the total counts per well. The level of mAb 24 epitope expression was not significantly affected by the fixation procedure (data not shown).

Flow Cytometric Analysis. Flow cytometric determination of the mAb 24 epitope was performed as described (5–8). To measure the mAb 24 epitope on adherent cells, 2×10^5 T cells in 50 μl were aliquoted into 96-well plates that had been coated with recombinant ICAM-1-Fc, centrifuged, and incubated at 37°C. At the appropriate time, 10 μl of FITC–mAb 24 (10 μg/ml in RPMI 1640 medium) was added to each well for 20 min. The ICAM-1-Fc-bound T cells were then removed from the wells by vigorous pipetting. Specifically for determining expression of the mAb 24 epitope in the presence of specific divalent cations, the T cells (2×10^5 cells per well) were washed three times in 20 mM Hepes/140 mM NaCl/2 mg of glucose per ml, pH 7.4 (Hepes/NaCl buffer) in flexiwell plates (Dynatech). Dilutions of either Mg^{2+} (in the presence of 1 mM EGTA) or Mn^{2+} chlorides were made in Hepes/NaCl buffer and 50 μl of the appropriate divalent cation dilution was added to each well containing the cells (5, 8). Cells were appropriately washed and fixed with 5% formaldehyde in PBS, and their fluorescence intensity was determined with a FACScan flow cytometer (Becton Dickinson). Expression of the mAb 24 epitope detected with FITC–mAb 24 is represented as the mean fluorescence intensity ± SD. As a control, the basal level of expression of the mAb 24 epitope on T cells kept at 4°C was determined at the initial time point.

Analysis of Adherent Cells by Immunofluorescence. ICAM-1-CHO transfectants were cultured in minimal essential medium plus 10% FCS and 0.5 mg of geneticin per ml on round 13-mm coverslips. T cells (10^5 cells per well in RPMI 1640 medium) pretreated with either anti-CD3 mAb UCHT1 (IgG2a isotype) followed by crosslinking with goat anti-mouse IgG2a antiserum (Nordic) or 50 nM PdBu were added to each coverslip and allowed to bind to the ICAM-1-CHO transfectants for 20 min at 37°C. The unbound T cells were then removed by gently washing the wells three times with RPMI 1640 medium. For single antibody staining, XL-CD3 T cells were incubated for 20 min at 37°C with 25 μl of either directly conjugated FITC mAb 24 (10 μg/ml) or anti-CD11a mAb MHM24 (IgG1; 1:3 dilution of culture supernatant) with the latter samples undergoing an additional incubation at 0°C with tetramethylrhodamine B isothiocyanate (TRITC)-conjugated goat anti-mouse IgG1 antiserum (1:50 dilution). The cells were then fixed with 5% formaldehyde in PBS for 20 min at room temperature. For double-antibody staining, PdBu-treated T cells were incubated with 25 μl of directly conjugated FITC-mAb 24 (IgG1) at 10 μg/ml in RPMI 1640 medium followed by a second incubation with the anti-CD11a mAb 38 (IgG2a) at 10 μg/ml and a final incubation with TRITC-conjugated goat anti-mouse IgG2a antiserum (1:50 dilution in RPMI 1640 medium). Incubations with various control mAbs showed the observed staining patterns to be specific.

RESULTS

Expression of mAb 24 Epitope Correlates with Activation of LFA-1. Although previous experiments suggested that the presence of the mAb 24 epitope paralleled LFA-1 activity, its expression had never been assessed in a system that mimicked physiological T-cell activation. Therefore, we triggered cultured T cells in several ways and tested the activation status of LFA-1 by its ability to bind to ICAM-1 using the following two assays. (*i*) Cultured T cells express low levels of ICAM-1 (14) and when activated by treatment with certain agonists will form LFA-1/ICAM-1-dependent aggregates in suspension. (*ii*) Activated T cells will bind via LFA-1 to ICAM-1 immobilized on plastic, which acts as the preferred substrate in this form, particularly when T cells are spun onto it at the beginning of the incubation procedure. Therefore, using this latter protocol, we followed the time course of LFA-1 activation, which was brought about indirectly through stimulation of the T-cell receptor/CD3 complex (3, 4). By cross-linking CD3, LFA-1 became transiently avid for ICAM-1, reaching maximum levels of ligand binding by ≈30 min and returning to the nonavid state by ≈2 h (Fig. 1 *Inset*). Expression of the mAb 24 epitope followed the same time course (Fig. 1). Second, we investigated the increase in

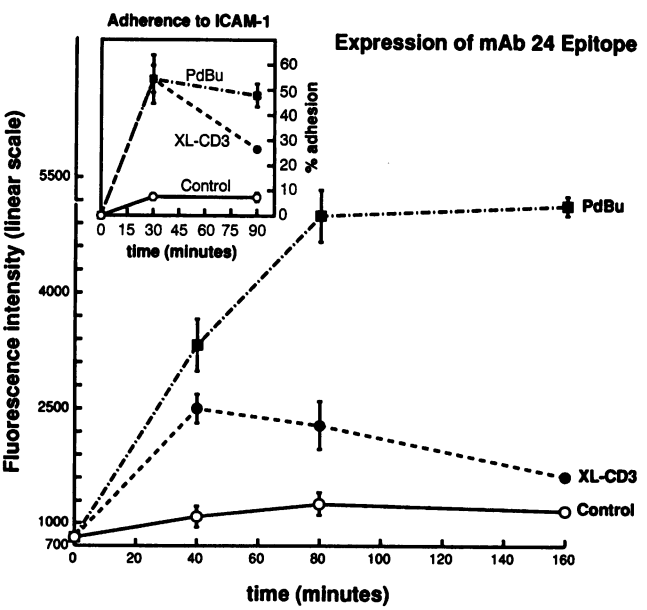

FIG. 1. Expression of mAb 24 epitope, induced by cross-linking of CD3 or treatment with the phorbol ester PdBu, parallels binding activity of T-cell LFA-1 for ICAM-1 immobilized on plastic. Transient induction of both mAb 24 epitope expression and LFA-1 binding to ICAM-1-Fc (13) (*Inset*; a representative experiment) is observed upon cross-linking of CD3. Similarly, more prolonged induction of mAb 24 epitope expression and LFA-1 activation is induced by PdBu treatment.

adhesion between LFA-1 and ICAM-1 mediated by PdBu, which had similar "on" kinetics but was not transient and persisted over a 2-h period. The expression of the mAb 24 epitope again reflected the more prolonged state of PdBu-induced LFA-1 activation. Previous work had shown that the mAb 24 epitope was expressed when LFA-1 was artificially activated by using divalent cations Mn^{2+} and Mg^{2+} with EGTA (5, 6). The results described here show that that expression of the mAb 24 epitope mirrored the activation status of LFA-1 in model conditions that more closely resemble physiological T-cell activation.

The mAb 24 Epitope Is Expressed by LFA-1 Following Binding to ICAM-1. Next it was of interest to explore the factors that contribute to the expression of the mAb 24 epitope. For example, the epitope might be induced as a result of the initial XL-CD3 or PdBu treatment or at a later stage when binding of LFA-1 to ICAM-1 has occurred. To separately assess these factors (particularly the role of ICAM-1), T cells were treated with the activating protocols in suspension, using the assay of homotypic aggregation (10, 15). Thus, cultured T cells expressing ICAM-1 formed LFA-1/ICAM-1-dependent aggregates either transiently when exposed to XL-CD3 conditions or for a longer time when treated with PdBu with a time course similar to that shown in Fig. 1 (*Inset*) (data not shown). Using this protocol, expression of the mAb 24 epitope followed the same time kinetics (Fig. 2). However, in the presence of the anti-ICAM-1 mAb RR1/1, the mAb 24 epitope was not expressed in spite of treatment with XL-CD3 or PdBu over a time course of ≈3 h. Thus, expression of the mAb 24 epitope on LFA-1 depended on exposure to ICAM-1. As mAb RR1/1 blocks the binding between LFA-1 and ICAM-1 (10), the expression of the mAb 24 epitope must depend on physical interaction between this receptor/ligand pair. This is further confirmed by the fact that nonblocking anti-ICAM-1 mAb 8F5 (13) allowed mAb 24 epitope expression (data not shown). The result is not explained by competitive antibody inhibition between anti-ICAM-1 mAb and mAb 24 on individual T cells, as mAb RR1/1 bound equally to T cells in the presence or absence of mAb 24 (data not shown). The conclusion is that the mAb 24 epitope is induced not by the agonists XL-CD3 or PdBu but by the interaction of ICAM-1 with LFA-1. Therefore, the mAb 24 epitope represents a ligand-induced binding site (LIBS) (16) expressed by LFA-1 following exposure to ICAM-1.

Expression of mAb 24 Epitope Requires ICAM-1 in the Presence of Mg^{2+} or Mn^{2+}. The divalent cation Mg^{2+} or Mn^{2+} is required for LFA-1 activity (5, 17). Previously, we have shown that the level of LFA-1 activity and expression of the mAb 24 epitope can be artificially manipulated from outside the T cell by increasing the concentration of these divalent cations, suggesting an association with expression of the mAb 24 epitope (5, 6). However, using LFA-1-activating concentrations of Mg^{2+} and Mn^{2+}, mAb 24 epitope expression is prevented by anti-ICAM-1 mAb (Fig. 3). This further strengthens the argument that the mAb 24 epitope is a LIBS, as exposure to ICAM-1 is required for its expression even when LFA-1 is activated from outside the cell. These experiments also suggest that the relationship between mAb 24 epitope expression and divalent cation binding may only be an indirect one insofar as both events are part of the LFA-1 activation process.

ICAM-1 Is Required for Activation of LFA-1. The implication of previously published studies has been that signals delivered by agonists such as XL-CD3 or PdBu were all that was required for the observed increase in the affinity of LFA-1 for ICAM-1 (3, 4). However, as ICAM-1 is widely expressed on leukocytes and here shown to induce a conformational change in LFA-1 associated with its high-affinity state, the question is raised as to whether ICAM-1 itself participates in the activation of LFA-1. To investigate this question, it was necessary to distinguish the role of ICAM-1 as ligand from its possible role as LFA-1 activator. Anti-ICAM-1 mAb was used to block the possible activation of LFA-1 by cell-associated ICAM-1 when the T cells were treated with agonist. Thus, ICAM-1-expressing cultured T cells were treated with agonist PdBu in the presence or absence of anti-ICAM-1 mAb followed by fixation with increasing amounts of paraformaldehyde (18, 19). Both sets of T cells were then assessed for their ability to bind to plastic-immobilized ICAM-1. Anti-ICAM-1-treated T cells were diminished in their ability to bind to ICAM-1 on plastic,

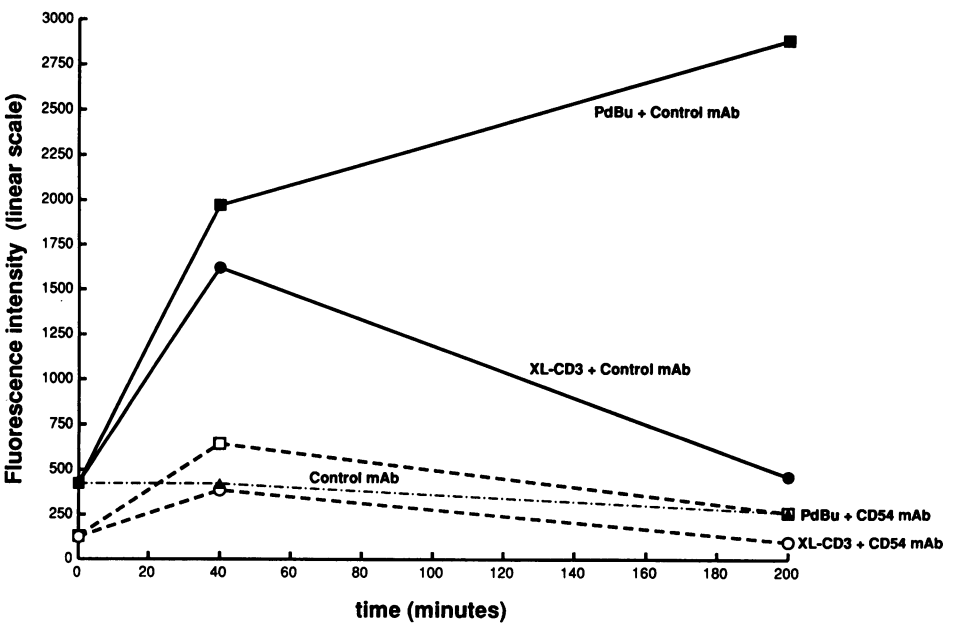

FIG. 2. Induction of mAb 24 epitope expression on T cells is prevented by anti-ICAM-1 mAb. Flow cytometric determination of expression of the mAb 24 epitope on T cells forming homotypic LFA-1/ICAM-1-dependent aggregates in response to treatment with PdBu or by cross-linking CD3 as in Fig. 1. Purified anti-CD54 mAb RR1/1 (10) was added to each sample at the beginning of the time course at a final concentration of 10 μg/ml.

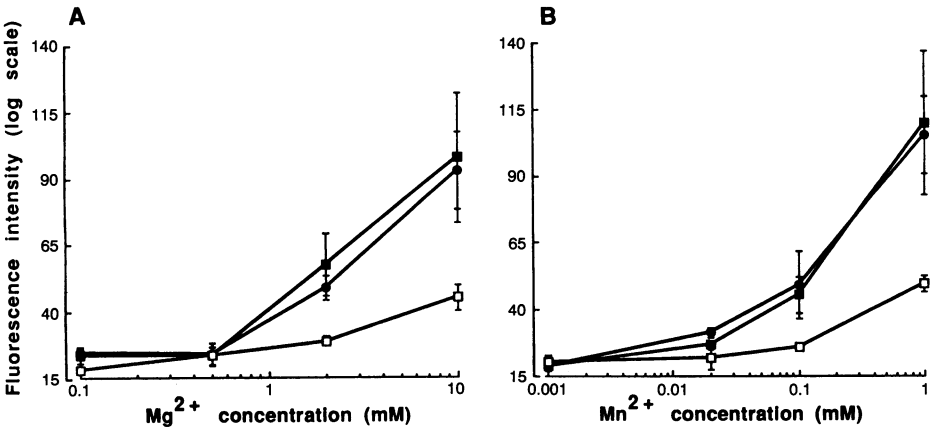

FIG. 3. Mg^{2+} and Mn^{2+} induction of mAb 24 epitope expression is inhibited by anti-ICAM-1 mAb. Dilutions of either Mg^{2+} in the presence of 1 mM EGTA (*A*) or Mn^{2+} chlorides (*B*) were made in Hepes/NaCl buffer and 50 μl of the appropriate divalent cation dilution was added to each well containing the T cells (5, 6). Where appropriate, purified anti-CD54 mAb RR1/1 (□), control anti-CD8 mAb 14 (●), or no mAb (■) was added at a final concentration of 10 μg/ml. At the same time, FITC-mAb 24 (10 μg/ml) was added to each well and the plate was incubated for 30 min at 37°C.

suggesting either that LFA-1 had not been fully activated in the first part of the experiment or that it had been damaged by the fixation process (Fig. 4*A*). However, the T cells that had prior exposure to cell-bound ICAM-1 were able to bind normally to ICAM-1 on plastic except at the highest fixative levels, indicating that their receptors had been fixed in an activated state (Fig. 4*B*). This result suggested that exposure to ICAM-1 (and agonist) was necessary for generating fully active LFA-1. However, to show that the LFA-1 on the anti-ICAM-1-treated cells was capable of being activated (i.e., that anti-ICAM-1 treatment had not indirectly affected the LFA-1), the necessary control for the experiment was to omit the fixing step (Fig. 4, see 0% paraformaldehyde). Now LFA-1 present on the anti-ICAM-1-treated T cells was able to be activated via plastic immobilized ICAM-1 in a manner analogous to T cells whose LFA-1 had prior exposure to cell-bound ICAM-1. Finally, there was no significant binding to ICAM-1 in the absence of PdBu treatment (data not shown). This experiment shows that ICAM-1 has a necessary role in agonist-mediated LFA-1 activation.

The mAb 24 Epitope Is Expressed at Sites of Contact Between LFA-1 and ICAM-1. If ICAM-1 contributes to the induction of the fully active form of LFA-1, the mAb 24 epitope should be expressed on T cells where they are in physical contact with ligand. To test this hypothesis, we used fluorescence microscopy to compare the surface distribution of the mAb 24 epitope with that of LFA-1 on T cells adherent to ICAM-1-transfected CHO cells. For the single PdBu-activated T cell shown, the distribution of LFA-1 is unclustered and in an even distribution around the cell membrane (Fig. 5*A*, red fluorescence). In contrast, the same T cell, double-labeled with mAb 24, shows this epitope to be localized to a discrete area of the cell membrane directly in contact with the ICAM-1 transfectant (green fluorescence). CD3-cross-linked T cells exhibited an identical result, showing an even distribution of LFA-1 around the cell membrane (Fig. 5*B*, red fluorescence) but expression of the mAb 24 epitope only at the area of cell contact with the ICAM-1 transfectant (green fluorescence). Control untreated T cells did not adhere to the ICAM-1 transfectant, were unlabeled by mAb 24, and

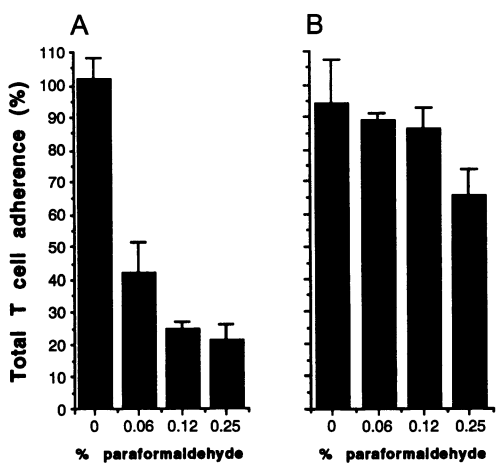

FIG. 4. Anti-ICAM-1 mAb prevents agonist-mediated activation of LFA-1. ICAM-1-expressing T cells were activated with 40 nM PdBu in the presence (*A*) or absence (*B*) of anti-ICAM-1 mAb followed by fixation with increasing amounts of paraformaldehyde. The T cells were then exposed to ICAM-1 a second time in the form of plastic immobilized ICAM-1-Fc and their adherence levels were measured.

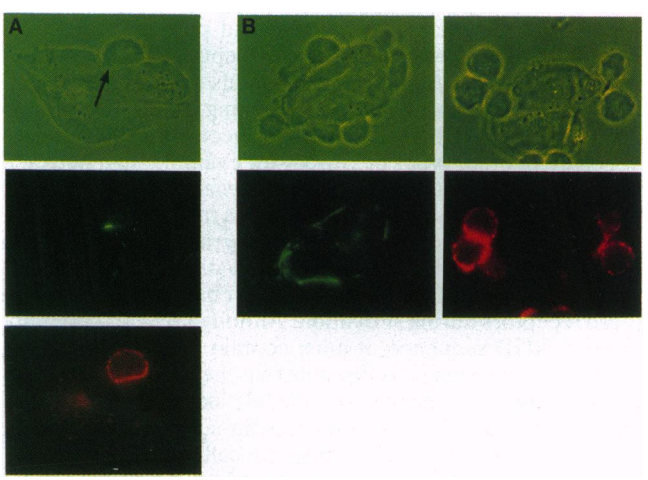

FIG. 5. Fluorescence micrographs of T cells bound to ICAM-1-CHO transfectants showing that distribution of staining with mAb 24 is restricted to T-cell contact areas with ICAM-1, whereas LFA-1 shows an even distribution around the T-cell membrane. (*A*) T cell after stimulation with PdBu adhering to an ICAM-1-CHO transfectant (phase) labeled by double mAb immunofluorescence to show T-cell distribution of both mAb 24 epitope (green; for localization see arrow in phase photo) and LFA-1 (CD11a mAb 38; red.) (*B*) CD3-cross-linked T cells adhering to an ICAM-1-CHO transfectant (phase) labeled by single mAb immunofluorescence to show either mAb 24 epitope (green) or LFA-1 (red).

had an even membrane distribution of LFA-1 (data not shown). Thus, expression of the mAb 24 epitope is confined to the sites of cell–cell contact between LFA-1 and ICAM-1, consistent with the interpretation that ligand is required to induce a conformational alteration in LFA-1 locally that is coincident with its ability to bind firmly to ligand.

DISCUSSION

Previous studies have implied that the signals delivered by agents such as XL-CD3 or phorbol ester were all that was required for the increase in affinity of LFA-1 for its ligand ICAM-1 (3, 4). The work described here supports the idea that ICAM-1 itself also has an essential role in the activation of LFA-1. Thus, anti-ICAM-1 mAb inhibits expression of the activation reporter mAb 24 epitope on agonist-treated T cells, expression of the mAb 24 epitope is confined on the T-cell membrane to areas where LFA-1/ICAM-1 ligation causes cell–cell contact, and, most critically, T cells that have their LFA-1 receptors frozen by chemical fixation after agonist treatment will not bind to ICAM-1 unless they have had prior exposure to this ligand.

The speculation is that signaling via other surface receptors together with an initial weak interaction with ligand ICAM-1 together produce a conformationally altered high-affinity form of LFA-1, which is now capable of forming a stable LFA-1/ICAM-1 complex, characterized by the mAb 24 epitope. This sequence of events suggests the means by which the adhesive strength provided by LFA-1/ICAM-1 pairing can be restricted to situations where a productive but low avidity interaction of cells has been stimulated by more specific receptors. As would be predicted, activated LFA-1 is induced only at sites of cell contact, which contradicts a study showing gross clustering of LFA-1 following activation of a T-cell line (20).

The mAb 24 epitope represents the only LIBS described so far for the β_2 integrins and is unique in characterizing the LFA-1/ICAM-1 complex. Other LIBS are hidden when ligand binds to integrin (21). It is speculated that upon initial interaction with intact ligand ICAM-1, unfolding (or other alterations) in the LFA-1 leads to exposure of additional sites to which ICAM-1 itself can now bind with higher affinity leading to more stable receptor/ligand pairing. This effect resembles that observed with the peptide RGD, a motif recognized by the β_3 integrins in ligands such as fibrinogen, vitronectin, and fibronectin. The binding of RGD peptide to the β_3 integrins GPIIbIIIa and vitronectin receptor causes exposure of other types of LIBS—for example, that detected by mAb PAC-1 on GPIIbIIIa (21) and enables these integrins to bind intact soluble ligand (18, 19). As in the present study, chemical fixation of the two β_3 integrins has allowed an intermediate RGD-bound stage of activation to be identified, further confirming that conformational changes must occur in these receptors during activation. Although ICAM-1 does not have an RGD sequence, it must contain a similar triggering sequence. However, it is possible that the activating features of an RGD-containing matrix molecule like fibronectin might differ from that of an immunoglobulin superfamily member like ICAM-1 located on an opposing cell surface.

The process of ligand-induced integrin activation is similar to the induced-fit model of interaction between substrate and enzyme, whereby the active site of the enzyme assumes a shape complementary to substrate only after it is bound (22). Induced fit has recently been shown to have a key role in the structural alterations leading to antigen/antibody binding (23). It remains to be seen how general a mechanism it is for receptor activation.

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