Activation of Lymphocyte Function-Associated Molecule-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) Mimicked by an Antibody Directed Against CD18[1]

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The β_2-integrin (CD18) family members bind to their ligands subsequent to activation of a number of well defined and diverse signal transduction pathways. The precise molecular changes associated with activation of the integrin family members have remained elusive. Here, we characterize a monoclonal, CBR LFA-1/2, that binds to the β_2-subunit and is able to mimic activation induced upon stimulation by phorbol esters. The Ab induces binding of the LFA-1-expressing cell line, JY, to ICAM-1 (CD54) and ICAM-3 (CD50). Activation of binding by this Ab is independent of Fc interactions and does not occur through cross-linking at the cell surface, because the Fab fragment of the Ab is able to modulate the same effect. Stimulation of neutrophils with CBR LFA-1/2 induces binding to ICAM-1 through activation of both LFA-1 and Mac-1. Activation of Mac-1 by CBR LFA-1/2 was further confirmed by stimulation of neutrophil binding to fibrinogen, a ligand for Mac-1. CBR LFA-1/2 lowers by 10-fold the concentration of Mg^{2+} required to achieve maximal binding of LFA-1 to ICAM-1. It therefore appears that CBR LFA-1/2 induces a conformational change that directly increases the avidity of β_2-integrins for ligands. *The Journal of Immunology,* 1995, 155: 854–866.

The β_2-integrins play an essential role in the adhesion events of the immune and inflammatory responses (1). They are members of a larger family of integrin proteins that are structurally related and that mediate cell-cell and cell-extracellular matrix interactions (2). The leukocyte integrins share the common β_2 (CD18)-subunit noncovalently associated with one of three α-subunits, α_L (CD11a), α_M (CD11b), and α_X (CD11c), to form LFA-1, Mac-1, and p150,95, respectively (3). The β-subunit contains an extracellular region that is highly conserved in other integrins, followed by a cysteine-rich region, a transmembrane domain, and a short cytoplasmic tail (4). Structural features of the α-subunits include an extracellular region with an inserted or I domain and three EF hand-like putative divalent cation-binding repeats, a transmembrane region, and a short cytoplasmic tail (5).

LFA-1 is expressed on all leukocytes and is the receptor for three Ig superfamily members, ICAM-1, ICAM-2, and ICAM-3 (6–8). Mac-1 and p150,95 are expressed primarily on myeloid cells and bind to ICAM-1 and iC3b; Mac-1 is also the receptor for fibrinogen and factor Xa (9–13). Patients with leukocyte adhesion deficiency have an underlying defect in the common β-subunit and fail to express all three of the leukocyte integrins on their cell surface (14). Cell surface expression of leukocyte integrins is essential for the inflammatory response to bacterial infections (14, 15). Furthermore, they play a critical role in adhesive events that are important in T and B cell function as well as the interaction of monocytes, neutrophils, and lymphocytes with endothelial cells and fibroblasts (16–19).

In their resting state, leukocytes exhibit little homotypic aggregation or interaction with endothelial cells (1). However, activation of a number of signal transduction pathways results in an increase in the adhesive properties of the cell through an increase in avidity of LFA-1 and Mac-1 for their ligands (2, 20). Leukocyte integrins may be activated by cell surface receptors known to be important in lymphocyte interactions, including CD2, CD3, Ig, and MHC class II; by stimulation of neutrophils with chemoattractants; and by activation of protein kinase C with phorbol esters (2, 20). Activation of integrins is transient and independent of a change in cell surface density (21–23).

Received for publication February 17, 1995. Accepted for publication May 5, 1995.

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[1] This work was supported by National Institutes of Health Grants CA31798 and CA31799.

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0022-1767/95/$02.00

Structural features of the LFA-1 molecule that are essential for its activation include the presence of a threonine-rich stretch within the cytoplasmic domain of the β-subunit (amino acids 758–760) as well as phenylalanine at position 766 (24, 25). Although protein kinases play a role in the activation of LFA-1 (21), the exact mechanism by which the change in avidity occurs has not been elucidated. Phorbol ester-stimulated phosphorylation occurs on serine-756 of the β-subunit, but has been dissociated from activation of adhesiveness (25).

Activation of β_1, β_2, and β_3-integrins can be mimicked by Ab that bind to the α- or β-subunits of these molecules (26–40). Several Ab have been described against the α_L- and α_M-subunits of the leukocyte integrin family that mimic the activation induced by intracellular signaling pathways or tag their activation state (26, 40–43). Recently, β_2-subunit-specific Ab, designated KIM127 and KIM185, were isolated that can modulate LFA-1-dependent homotypic aggregation and binding to ICAM-1, Mac-1-dependent binding of neutrophils to glass, and increase binding to iC3b by twofold (34, 35). Here we describe and characterize a β_2-subunit-specific mAb, CBR LFA-1/2, that activates the function of both LFA-1 and Mac-1. We extend previous work with studies on binding to multiple purified ligands. We demonstrate that CBR LFA-1/2 activates binding through LFA-1 to purified ICAM-1 and ICAM-3, and through Mac-1 to fibrinogen. Activation is independent of receptor clustering or Fc receptor interactions. Furthermore, CBR LFA-1/2 mAb appears to induce a conformational change in LFA-1 that alters its requirement for the divalent cation, Mg^{2+}.

Materials and Methods

Cell lines

The B-lymphoblastoid cell line, JY, and the T cell line, Jurkat, were grown in RPMI containing 5% FCS. The erythroleukemia cell line, K562, transfected with Mac-1, was a gift from Michael Diamond. To prepare K562 cells expressing LFA-1, 20 μg of CDM8 containing the β-subunit (44) and α_L-subunit (5) were linearized with *Nhe*I, extracted once with phenol-chloroform (1:1), precipitated with ethanol, and resuspended in 20 mM Tris (pH 8.0) and 5 mM EDTA. Plasmid (100 ng) containing the hygromycin-resistant gene, pBShyg (obtained from L. Klickstein), was digested with *Xmn*I. The DNA was introduced by electroporation into 10^7 cells in 800 μl of 20 mM HEPES (pH 7.05), 5 mM KCl, 0.7 mM Na_2HPO_4, 5 mM dextrose, and 137 mM NaCl at 1000 μF and 280 V (0.4-cm path length cells) (44). After 48 h in RPMI containing 10% FCS, the cells were transferred to selection medium (RPMI 1640 and 10% FCS containing 200 μg/ml hygromycin; Calbiochem, La Jolla, CA). The medium was replaced every 3 days, and cells expressing LFA-1 were further subcloned by limiting dilution and maintained in hygromycin selection.

PBL were isolated by centrifugation through Ficoll-Hypaque (density = 1.077) and depletion of monocytes by adherence to plastic (8). T Cells were further purified on nylon wool (8). The neutrophils, collected as the pellet from the sedimentation, were depleted of erythrocytes by hypotonic lysis for 10 s and resuspended in HBSS (Life Technologies, Grand Island, NY) containing 2 mM $MgCl_2$ and 10 mM HEPES, pH 7.6.

Generation of monoclonal antibodies

LFA-1 was purified by affinity chromatography on TS2/4-Sepharose (45). LFA-1 (5 μg/immunization) was combined with trehalose dimycolate from *Mycobacterium phlei*, monophosphoryl lipid A from *Salmonella minnesota* R695, 0.2% Tween 80, and squalene according to the manufacturer's directions (RIBI Immunochemical Research, Inc., Hamilton, MT) and was injected i.p. into female BALB/c mice on day −28 (13). On day −3, i.v. (50 μl) and i.p. (250 μl) injections of LFA-1 (0.02 mg/ml) were administered, and fusion was conducted on day 0, using the nonsecreting murine myeloma cell line P3X62Ag8.653 (CRL 1580; American Type Culture Collection, Rockville, MD). The protocol for the fusion, maintenance, and subcloning of the cells has previously been described (13).

Other Ab used within this study have been previously described and include TS1/22 (46), TS1/18 (46), TS2/4 (46), R6.5 (47), CBRM1/29 (48), and CBR-LFA-1/6 (49).

ELISA (13)

Detergent-solubilized purified LFA-1 was adsorbed to polystyrene 96-well plates (Linbro/Titertek, Flow Laboratories, Rockville, MD) by diluting 5 μl of LFA-1 containing 1% octylglucoside in 50 μl of buffer A (50 mM Tris (pH 8.0), 0.15 M NaCl, and 2 mM $MgCl_2$) for 2 h at room temperature. After rinsing the plates six times with buffer A, they were blocked with 1% BSA in buffer A. Ab supernatants (50 μl) were incubated with the immobilized LFA-1 for 1 h at 0°C. After rinsing the plates four times with buffer A containing 1% BSA, horseradish peroxidase-conjugated rabbit anti-mouse IgG (Zymed Laboratories, San Francisco, CA) was added at a 0.5 μg/ml final concentration. After 30 min at 0°C, the unbound second Ab was removed, and the bound Ab was detected with 2,2-azino-di[3-ethylbenthio-zoline]sulfonate substrate (Zymed) and 0.05% hydrogen peroxide in 100 mM sodium citrate, pH 4.0, at 414 nm in a multiwell plate reader.

Flow cytometry

Flow cytometry was performed as previously described (13).

Cell binding to immobilized ICAM-1

ICAM-1 was purified and adsorbed to plastic (Linbro/Titertek 96-well plates) as previously described (50). Cells (2 ml of 2×10^7/ml) were labeled with 3 μg of 2′,7′-bis-(2-carboxyethyl)-5-(and -6)carboxyfluorescein acetoxymethyl ester for 20 min at 37°C in the presence of 4 μg/ml R6.5 mAb to block cell aggregation. Cells were washed three times in buffer B (L15 medium and 2.5% FCS) and resuspended at 2×10^6 cells/ml. Cells (50 μl) were mixed with an equal volume of hybridoma supernatant in microtiter wells, and the fluorescence content of the cells in each well was quantitated on a Fluorescent Concentration Analyzer (IDEXX, Westbrook, ME), after which the 96-well plate was centrifuged at 200 × *g* for 2 min and incubated for 1 h at 0°C. The plate was transferred to a 37°C water bath to submerge the bottom for 10 min. The unbound cells were removed by aspiration six times at 90° intervals around the well at room temperature with a 20-gauge needle and resuspended in buffer B. Bound cells were quantitated in the Fluorescent Concentration Analyzer and expressed as a percentage of total input cells per sample well. Assays using T cells were performed as described; however, the washing was conducted with six manual flicking steps rather than aspiration.

Cell binding to immobilized ICAM-3

ICAM-3 was purified (51) and adsorbed to polystyrene 96-well plates (Linbro/Titertek). After 1.5 h, the unbound protein was removed with PBS, and nonspecific binding was blocked by the addition of 1% BSA in PBS for 30 min at room temperature. Cells were labeled with 2′,7′-bis-(2-carboxyethyl)-5-(and -6)carboxyfluorescein acetoxymethyl ester as described above and incubated with the stimulus indicated in the figure legends. After 1 h at 37°C, unbound cells were removed by aspiration three times with a 27-gauge needle, and bound cells were quantitated in the Fluorescent Concentration Analyzer.

Binding of neutrophils to ICAM-1 and fibrinogen

Assays were performed as previously described (48). Briefly, 25 μl of ICAM-1 (200 μg/ml), fibrinogen (2 mg/ml), or BSA (2 mg/ml) in PBS

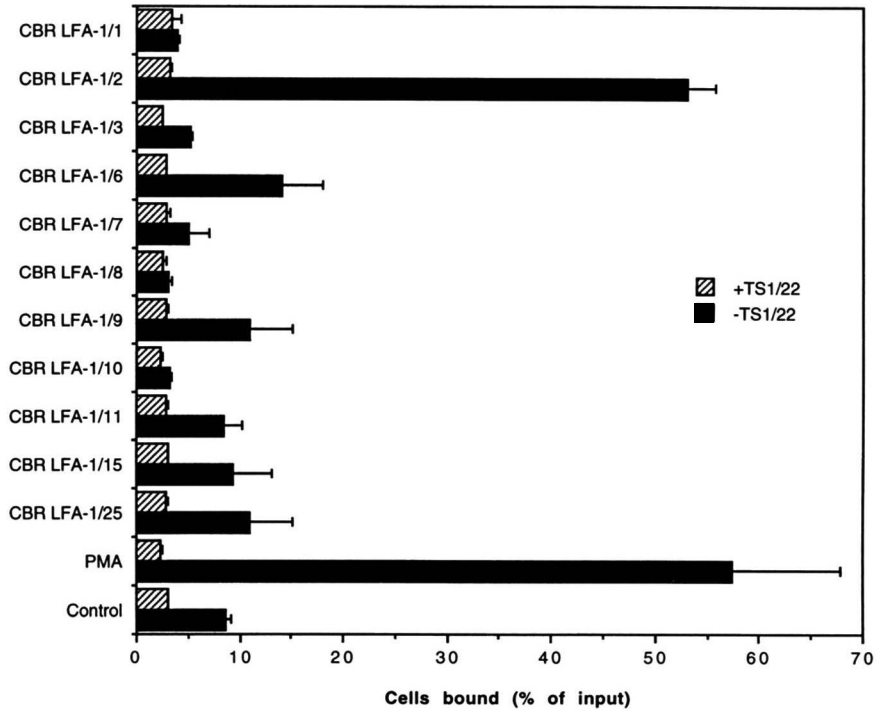


FIGURE 1. Effect of hybridoma supernatants on binding to ICAM-1. Supernatants of anti-LFA-1 hybridomas or X63 myeloma as the control were incubated with 2′,7′-bis-(2-carboxyethyl)-5-(and -6)carboxyfluorescein acetoxymethyl ester-labeled JY cells as described in *Materials and Methods*. Binding to purified ICAM-1 adsorbed to microtiter wells was performed at 37°C for 10 min. Binding was conducted in the presence of supernatant alone (solid bars) or with the LFA-1 blocking Ab, TS1/22 (hatched bars).

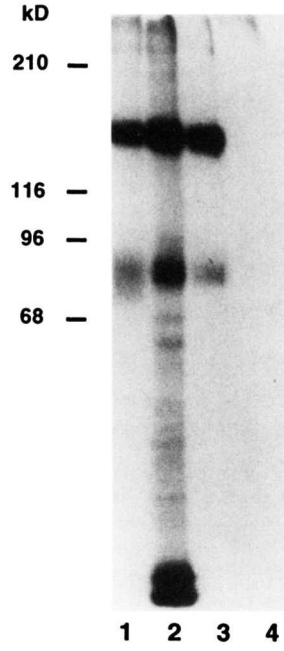


FIGURE 2. Immunoprecipitation of cell surface labeled proteins. Lysates of ^{125}I surface-labeled JY cells were immunoprecipitated with CBR LFA-1/2 (*lane 1*), TS1/22 (*lane 2*), TS1/18 (*lane 3*), or X63 as the control (*lane 4*) and subjected to reducing SDS-7.5% PAGE. Radiography with enhancing screen was performed overnight at −70°C, and m.w. standards are indicated at the *left*.

was spotted on a 5-mm circle in a 60-mm polystyrene Petri dish (Falcon 1007, Becton Dickinson, Lincoln Park, NJ) for 1.5 h at room temperature. The unbound proteins were aspirated, and the plate was rinsed with PBS containing 1% Tween-20. After 2 min, the plates were washed six times with PBS and covered with 2 ml of HBSS containing 2 mM $MgCl_2$ and 10 mM HEPES, pH 7.6. Neutrophils were incubated in the presence or absence of the indicated Ab (10 μg/ml). After 20 min at room temperature, the neutrophils (2×10^6/ml; 1 ml/plate) were added to the plates containing the bound proteins in the presence of FMLP, CBR LFA-1/2, or CBR LFA-1/6. After 10 min at room temperature, the plates were washed 15 times by withdrawing the medium with a transfer pipette and gentle replacement with HBSS-Mg^{2+}-HEPES. Bound cells were quantitated by counting four or five high powered fields per dish.

Aggregation assay

Cells cultured to a density of 5×10^5/ml were washed with buffer B and incubated with Ab (ZP0–1 control or TS1/22; 10 μg/ml) for 20 min at room temperature. The cells were washed three times with buffer B and resuspended at 2×10^6 cells/ml. The cells were then incubated in the presence of TS2/4 (10 μg/ml), CBR LFA-1/2 (10 μg/ml), or phorbol esters (50 ng/ml). Aggregation was monitored at 10, 30, 60, and 2 h and scored as previously described (52).

Preparation of Fab fragments (53)

CBR LFA-1/2 in 2 l of spent medium containing 5% FCS was collected after precipitation with 45% saturated ammonium sulfate buffered with 100 mM HEPES, pH 7.6, for 16 h at 4°C. After dialysis against 100 mM Tris, pH 8.3, the Ab was further purified on protein A-Sepharose (20 mg/ml; 6 ml) and eluted with 0.1 M citric acid, pH 3.0. The peak of protein, as measured by OD 280, was collected. To prepare the $F(ab)_2$ fragments, 10 mg of Ab was digested with 0.5 mg of papain that had been preactivated in 3 mM EDTA, 0.1 M sodium acetate (pH 5.5), and 50 mM cysteine for 30 min and separated from cysteine by gel filtration (Sephadex G-25 Superfine, Pharmacia, Piscataway, NJ). The optimal time for digestion at pH 5.5 was 6 h at 37°C. Digestion was stopped by the addition of iodoacetamide (0.025 M, final concentration). The $F(ab)_2$ fragment was separated from intact Ab and enzyme by size exclusion chromatography (Sephacryl S200, Pharmacia) in PBS. The $F(ab)_2$ was further purified on DEAE-cellulose (Whatman, Clifton, NJ) in 5 mM Tris, pH 7.5, using a linear salt gradient from 0 to 0.1 M NaCl. The $F(ab)_2$

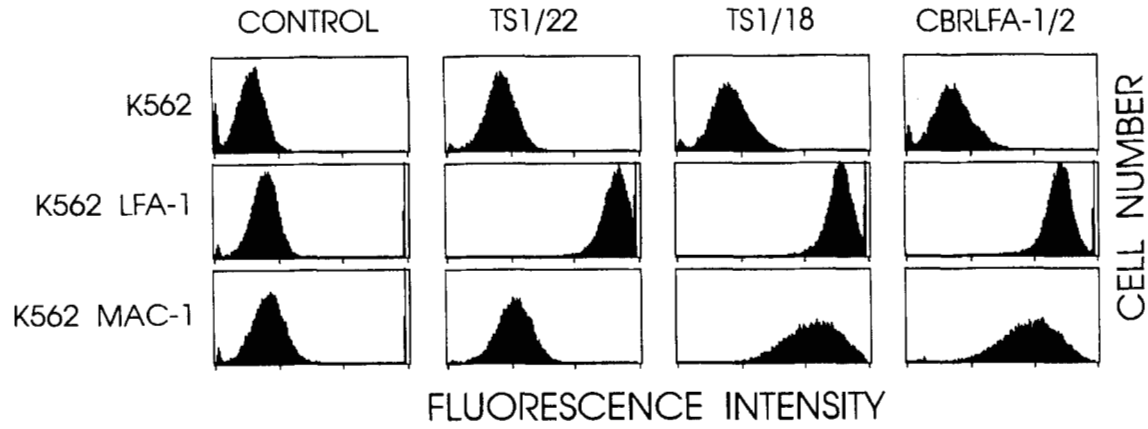


FIGURE 3. Immunofluorescence flow cytometry of transfected K562 cells. Untransfected K562 cells or cells transfected with the α_L- and β_2-subunits (K562 LFA-1) or with the α_M- and β_2-subunits (K562 Mac-1) were incubated with control Ab (ZP0–1), the α_L-subunit Ab TS1/22, the β_2-subunit Ab, TS1/18, or CBR LFA-1/2. Bound Ab was detected by FITC-conjugated goat anti-mouse IgG, and the cells were subjected to flow cytometry. Fluorescence intensity is on a 3-decade log scale.

fragment was reduced with 0.005 M Tris-HCl (pH 7.5) and 0.01 M cysteine for 2 h at 37°C and alkylated with iodoacetamide at a final concentration of 0.15 M for 30 min at room temperature. The F(ab') fragment was separated from the $F(ab)_2$ fragment by gel filtration chromatography (Sephadex G-75 Superfine, Pharmacia) in PBS. The purity of each step was monitored with SDS-PAGE and silver stain (54, 55).

Immunoprecipitation (44)

Briefly, 1×10^7 cells were washed twice in 10 ml of PBS, resuspended in 1 ml of PBS, and placed in a vial coated with 250 μg of Iodogen. One microcurie of $Na^{125}I$ was added for 10 min, and the reaction was quenched by the addition of RPMI 1640 medium. The cells were washed three times in medium and resuspended in 1 ml of 25 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 1% Triton X-100, 0.02 mg/ml aprotinin, 5 mM iodoacetamide, and 1 mM PMSF. After 5 min at 0°C, the sample was centrifuged at $15,000 \times g$ for 20 min. The cell lysate was precleared with bovine Ig conjugated to Sepharose CL-4B overnight and incubated with Ab supernatant for 2 h at 4°C (1:1). Protein A conjugated to Sepharose was preincubated with rabbit anti-mouse IgG and added for 30 min at 4°C. The beads were washed twice with 25 mM Tris (pH 8.0), 0.15 M NaCl, and 1% Triton X-100; twice with 25 mM Tris (pH 8.0), 2 M NaCl, and 0.1% Triton X-100; and once with 50 mM Tris (pH 8.0). The beads were resuspended in 50 μl of 50 mM Tris (pH 6.8), 20% glycerol, 2% SDS, and 1% β-ME and heated to 100°C for 5 min, and the eluate was subjected to SDS-PAGE.

Immunoblotting

Purified LFA-1 (45) was subjected to SDS-8% PAGE in 50 mM Tris (pH 6.8), 20% glycerol, and 2% SDS with and without 1% β-ME. The samples were transferred to nitrocellulose (Schleicher and Schuell, Keene, NH), and the blot was treated as previously described (56). CBR LFA-1/2 was used at 2 μg/ml, and the bound Ab was detected with alkaline phosphatase-conjugated second Ab according to manufacturer's directions (Promega Corp., Madison, WI).

Results

Isolation of a mAb that stimulates binding of cells expressing LFA-1 to purified ICAM-1

Of 1920 hybridomas obtained from mice immunized with purified LFA-1, 235 reacted with purified LFA-1 by ELISA. Of these, 64 were positive by flow cytometry on human peripheral blood T cells, the B-lymphoblastoid cell line JY, and the T cell line SKW3. Of 64 supernatants, one, CBR LFA-1/2, induced marked enhancement of binding of cells to immobilized purified ICAM-1 (Fig. 1). The increase in binding was comparable to the binding achieved with phorbol ester stimulation. With the other mAb, binding to ICAM-1 was comparable to that seen with the X63 control or inhibited. CBR LFA-1/2-stimulated binding to immobilized ICAM-1 was completely inhibited by the blocking Ab TS1/22 to LFA-1 α (Fig. 1) and blocking mAb to the β-subunit, TS1/18 (not shown). The binding to ICAM-1 was specific, since it was also blocked by treatment of the wells with an anti-ICAM-1 Ab (R6.5; not shown).

Characterization of CBR LFA1/2

CBR LFA1/2 specifically immunoprecipitated a surface iodinated protein from JY cells that migrated as a heterodimer with subunits of 180,000 and 95,000 M_r on reducing SDS-PAGE (Fig. 2, *lane 1*). The pattern was identical with that seen in immunoprecipitates with mAb to the LFA-1 α-subunit (Fig. 2, *lane 2*) and β-subunit (Fig. 2, *lane 3*).

The subunit specificity of CBR LFA-1/2 was examined by immunofluorescent staining of transfected K562 cells (Fig. 3). CBR LFA-1/2 stained K562 cells transfected with the α_L- and β_2-integrin subunits (K562 LFA-1) and with the α_M- and β_2-subunits (K562 Mac-1), but not untransfected K562 cells. The same pattern was obtained with TS1/18 mAb to the β_2-subunit, whereas TS1/22 mAb to the α_L-subunit stained only K562 LFA-1 transfectants. The epitope recognized by CBR LFA-1/2 Ab is distinct from that recognized by TS1/18, because they do not compete for binding to the β-subunit (data not shown). Immunoblotting of purified LFA-1 subjected to nonreducing SDS-PAGE (Fig. 4) demonstrated that the Ab recognizes an epitope within the β_2-subunit (M_r 95,000). This epitope was lost if the sample

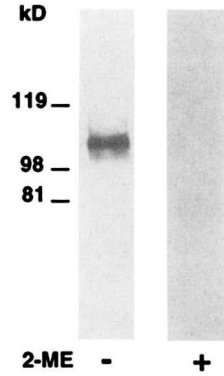


FIGURE 4. Immunoblotting of purified LFA-1. LFA-1 (2 μg) treated with SDS sample buffer in the absence (−) or presence (+) of 2-ME was subjected to SDS-8% PAGE. The proteins were transferred to nitrocellulose and incubated with CBR LFA-1/2. The bound Ab was detected with alkaline phosphatase-conjugated second Ab.

was reduced before SDS-PAGE (Fig. 4). Control supernatant, X63, and mAb TS1/18 to β_2 did not react with nonreduced or reduced material (data not shown).

The epitope recognized by CBR LFA-1/2 is present on cells at both 4 and 37°C. A previously described activating mAb, KIM127, recognizes a temperature-dependent epitope (34). In contrast to these results, the epitope recognized by CBR LFA-1/2 is present on cells both at 4 and 37°C (Fig. 5). Thus, the epitopes recognized by these mAb are distinct.

Effects of divalent cations on CBR LFA-1/2-mediated cell binding to ICAM-1

Divalent cations play a critical role in the ability of integrins to bind to their ligands. The presence of divalent cations, in particular Mg^{2+}, is essential for binding of LFA-1 to ICAM-1 (6). We first investigated whether divalent cations were necessary for recognition of LFA-1 by CBR LFA-1/2 using flow cytometry. CBR LFA-1/2 bound to cells in the absence of divalent cations in the medium (Fig. 6*A*). Furthermore, once bound, CBR LFA-1/2 binding was not diminished by the addition of the chelating agents EDTA or EGTA (Fig. 6*A*). We then compared the requirements for the divalent cations Ca^{2+}, Mg^{2+}, and Mn^{2+} in activation of LFA-1 by phorbol esters and CBR LFA-1/2. We saw no difference in the pattern of binding induced by CBR LFA-1/2 compared with that caused by PMA (Fig. 6*B*) in the presence of Ca^{2+}, Mg^{2+}, or Mn^{2+}. Magnesium was required to achieve activation by either stimulus, calcium in the absence of magnesium was not sufficient for activation, and manganese ions activated binding in the absence of added stimuli.

We examined whether CBR LFA-1/2 altered the requirement for Mg^{2+} (Fig. 6*C*). As in earlier experiments, PMA and CBR LFA-1/2 stimulated comparable binding to ICAM-1 of JY cells in 1 and 2 mM Mg^{2+} (Fig. 6*C*). This binding was about fivefold greater than that with unstimulated cells. However, binding was stimulated by CBR LFA-1/2 mAb at considerably lower Mg^{2+} concentrations than by phorbol ester. Half-maximal phorbol ester-stimulated binding required 0.3 mM Mg^{2+} compared with 0.03 mM for half-maximal CBR LFA-1/2-stimulated binding. Binding of resting JY cells was half-maximal at 0.7 mM Mg^{2+}.

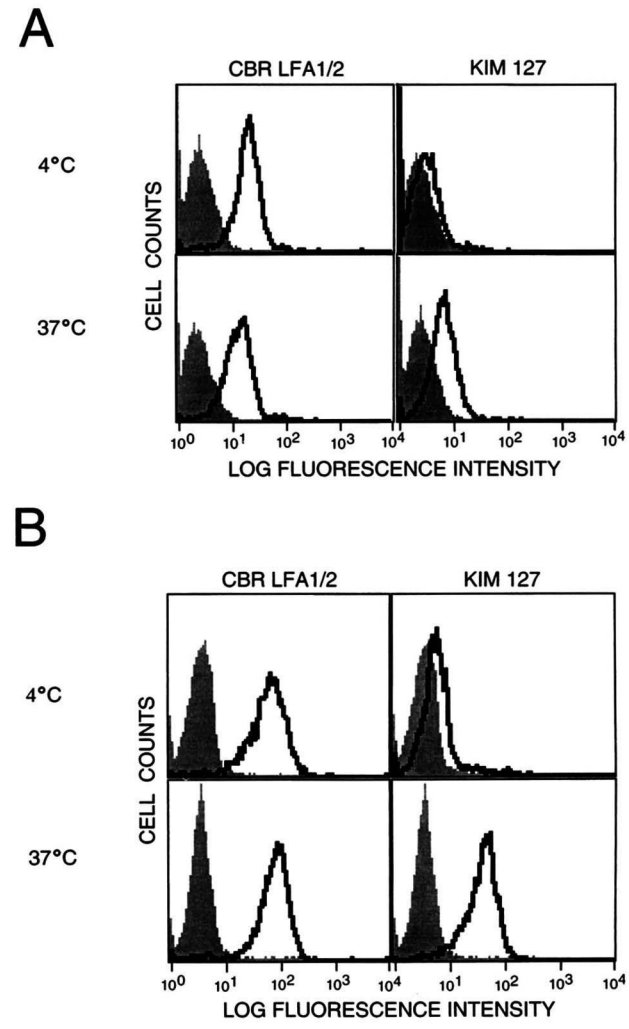
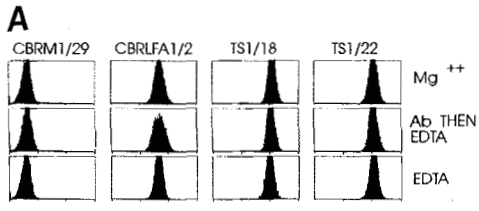
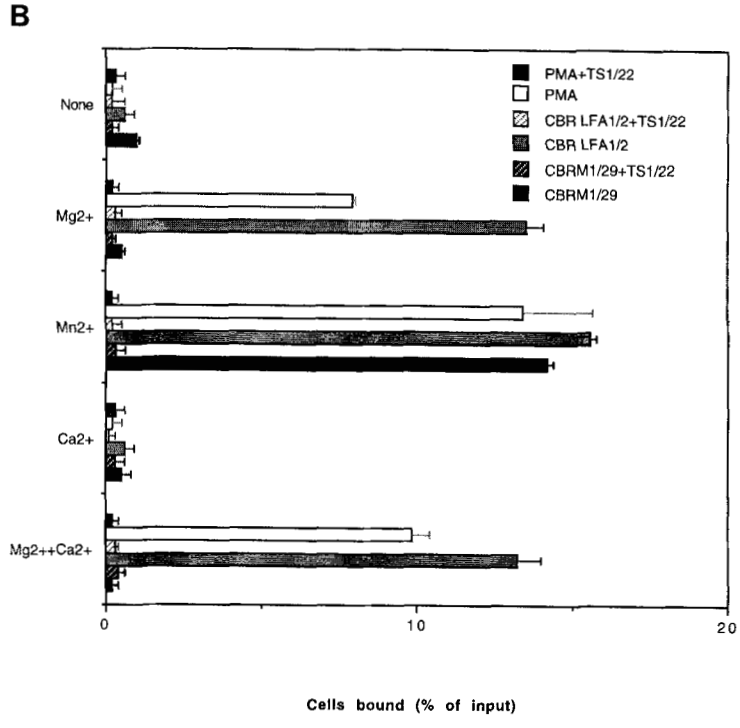


FIGURE 5. The epitopes of CBR LFA-1/2 and KIM127 differ in temperature dependence. JY cells (*A*) or SKW3 cells (*B*) were incubated with CBR LFA-1/2 or KIM127 mAb (open curves) or control mAb (shaded curves) at 10 μg/ml for 30 min on ice (4°C) or at 37°C. Cells were washed with cold medium, bound mAb was detected with FITC-conjugated goat anti-mouse IgG (4°C, 30 min), and cells were subjected to flow cytometry.

Activation of adhesiveness of LFA-1 for ICAM-1 and ICAM-3

To test for up-regulation of adhesiveness of LFA-1 not only for ICAM-1, but also for ICAM-3, JY cells were incubated with phorbol esters, CBR LFA-1/2, or control Ab, and binding was assessed. CBR LFA-1/2 markedly





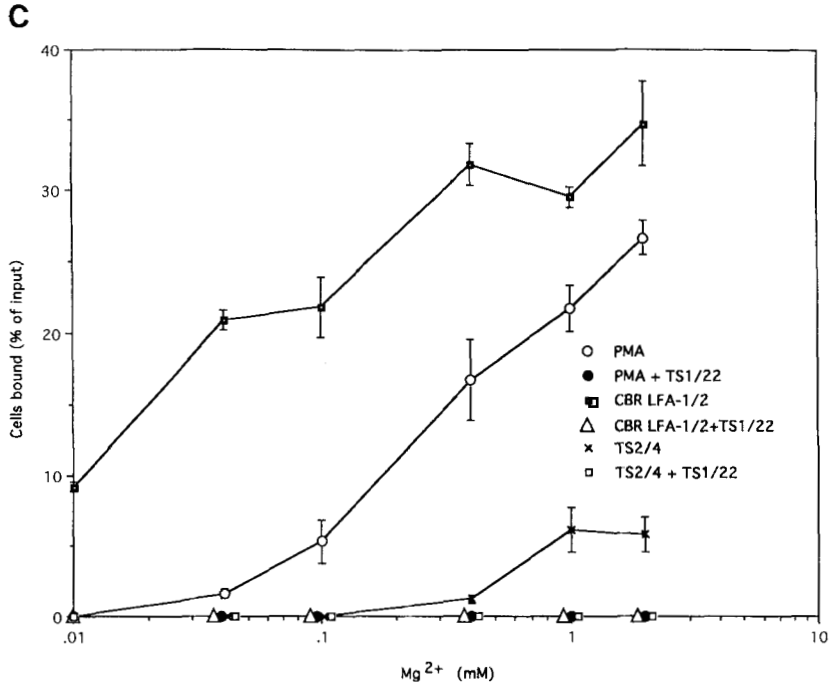


FIGURE 6. Effect of divalent cations on modulation of binding through CBR LFA-1/2. *A*, Flow cytometry was performed after cells were incubated in HEPES-buffered saline containing 2 mM $MgCl_2$ or EDTA. Ab was also prebound in 2 mM $MgCl_2$, and EDTA was added subsequent to binding. In addition to CBR LFA-1/2, the Mac-1-specific Ab CBRM1/29, the β-specific Ab TS1/18, and the α-specific Ab TS 1/22 were used. *B*, JY cell binding to ICAM-1 was examined in buffered saline alone or containing Mn^{2+} (0.5 mM), Mg^{2+} (2 mM), Ca^{2+} (1 mM), or Mg^{2+} and Ca^{2+} (2 and 1 mM, respectively, in the presence of the indicated mAb). Data are the mean and SE of quadruplicate determinations. *C*, JY cell binding was assessed in the presence of increasing concentrations of $MgCl_2$. Binding to ICAM-1 was performed in the presence of CBR LFA-1/2, PMA, or the absence of stimulus. Binding in the presence of TS1/22 was determined for all three groups, and in all cases was zero.

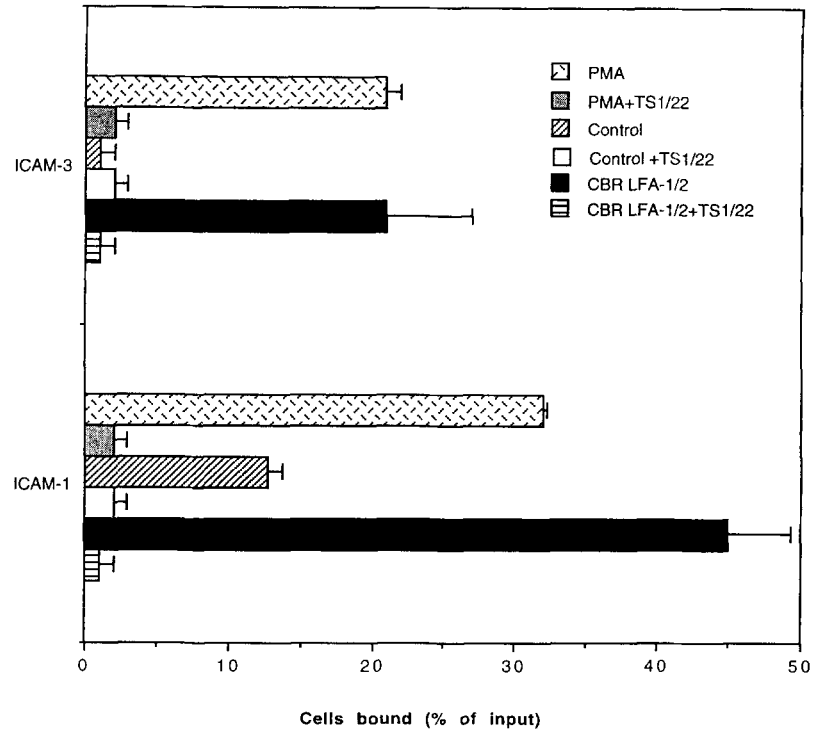


FIGURE 7. Stimulation of binding of JY cells to ICAM-1 and ICAM-3. Binding of JY cells was assessed in the presence of the indicated additions. After incubation with Ab for 1 h at 4°C, the cells were incubated for 10 min with ICAM-1 substrates or for 1 h with ICAM-3 substrates at 37°C.

up-regulated binding to both ICAM-1 and ICAM-3 (Fig. 7). The binding was specific for LFA-1, as the blocking Ab TS1/22 reduced binding to background.

Effect of CBR LFA-1/2 on Mac-1 function

Since CBR LFA-1/2 detects an epitope on the β_2-integrin subunit, we studied whether it was able to enhance the function of other β_2-integrins. We examined the ability of CBR LFA-1/2 to modulate binding of neutrophils to ICAM-1, a function that depends on both LFA-1 and Mac-1 (10, 57). CBR LFA-1/2 enhanced binding of neutrophils to ICAM-1 (Fig. 8*A*). The chemoattractant FMLP also greatly enhanced binding, as previously reported (43). The binding was largely blocked by an Ab directed against LFA-1 (TS1/22) or partially by an Ab directed against Mac-1 (CBRM1/29). The combination of both Ab inhibited binding completely. These findings suggested that CBR LFA-1/2 was able to modulate the function of two distinct integrin molecules, Mac-1 and LFA-1. We further examined neutrophil binding to fibrinogen, which occurs through Mac-1, but not LFA-1. CBR LFA1/2 was comparable to FMLP in its ability to increase binding of neutrophils to fibrinogen (Fig. 8*B*). Binding was inhibited by mAb to Mac-1, but not mAb to LFA-1, showing that binding was dependent on Mac-1 activation.

CBR LFA-1/2-induced aggregation of JY cells

To assess whether CBR LFA-1/2 activated cell-cell adhesion through LFA-1, we examined the effect of this Ab on homotypic adhesion of JY cells. The Ab modulates homotypic adhesion in a time-dependent manner comparable to that of phorbol ester stimulation. By 30 min, 2+ to 3+ aggregation was evident, and by 1 h, the aggregation was enhanced to 3 to 4+ (Fig. 9). Aggregation was completely blocked by preincubation with the blocking Ab TS1/22, but not by control Ab, demonstrating that aggregation occurred through an LFA-1-dependent pathway.

Effect of CBR LFA-1/2 Fab' fragment on activation of LFA-1-bearing cells

To determine whether the Ab-induced activation of LFA-1 required cross-linking of LFA-1 or Fc receptor interactions, we generated Fab' fragments of CBR LFA-1/2. SDS-PAGE under reducing and nonreducing conditions followed by silver staining revealed no evidence of intact Ab or $F(ab')_2$ in the preparation (Fig. 10*A*). The Fab' runs as a single protein of 50,000 daltons under nonreducing conditions and is composed of two species after treatment with 2-ME. Both the intact Ab and the Fab' fragment were able to activate binding of purified T cells to immobilized ICAM-1 (Fig. 10*B*). A titration curve of the Fab' fragment revealed that a slightly higher concentration of Fab' fragment was needed to achieve equivalent stimulation of binding, reflecting a lower affinity of the Fab' fragment for LFA-1. The Fab' fragment was similar to the intact Ab in its ability to up-regulate JY and Jurkat cell binding to immobilized ICAM-1 (Fig. 10*C*). The level of binding was comparable to that seen with phorbol ester stimulation, and in

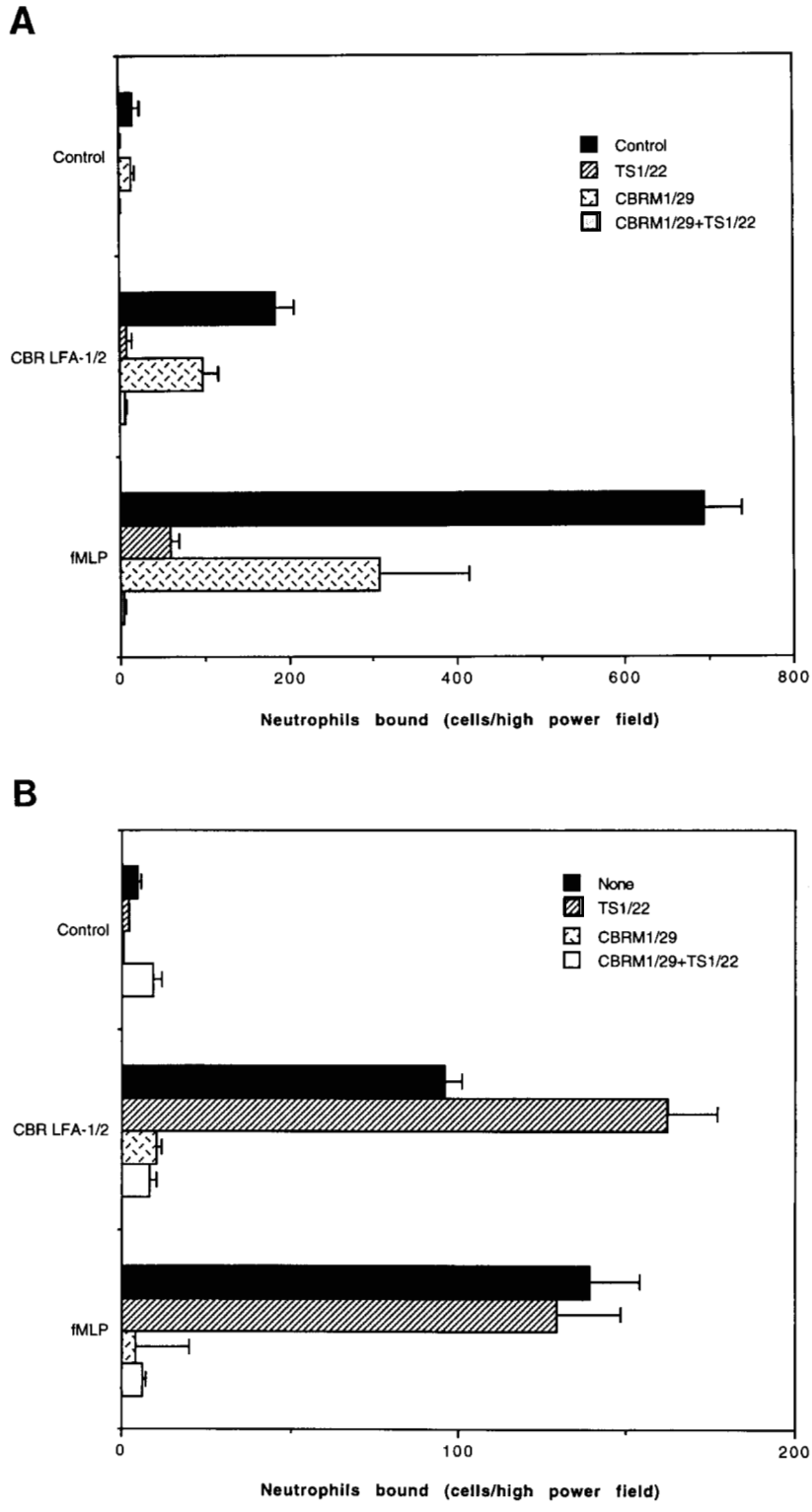


FIGURE 8. Effect of CBR LFA-1/2 on binding of neutrophils to ICAM-1 and fibrinogen. *A*, Purified ICAM-1 was immobilized on Petri dishes as described in *Materials and Methods*. Neutrophils were incubated with control Ab (CBR LFA-1/6), FMLP, or CBR LFA-1/2 at room temperature in the absence of blocking Ab or in the presence of the anti-LFA-1 Ab TS1/22, the anti-Mac-1 Ab CBRM1/29, or both TS1/22 and CBRM1/29, as indicated. After washing 15 times, cell binding to ICAM-1 in five high powered fields (hpf) was assessed, and the average and standard error are reported. Background, as assessed by binding to immobilized BSA, was 5 cells/hpf. *B*, Binding of neutrophils to fibrinogen in the presence of control Ab (CBR LFA-1/6), FMLP, or CBR LFA-1/2. The assay was performed and scored as described above. Binding to BSA under the same conditions was 5 cells/hpf.

all cases, binding was completely inhibited by TS1/22. The Fab′ fragment also induced cell aggregation (Fig. 9).

Discussion

Members of the β_1-, β_2-, and β_3-integrin families share the common property of undergoing activation through a number of known signal transduction pathways (1, 2). This process occurs through the stimulation of an array of signal transduction pathways and is transient (21, 22, 58, 59). The mechanism by which signals inside the cell are translated into a change in adhesiveness of the extracellular domain of integrin molecules has remained elusive. We have generated a

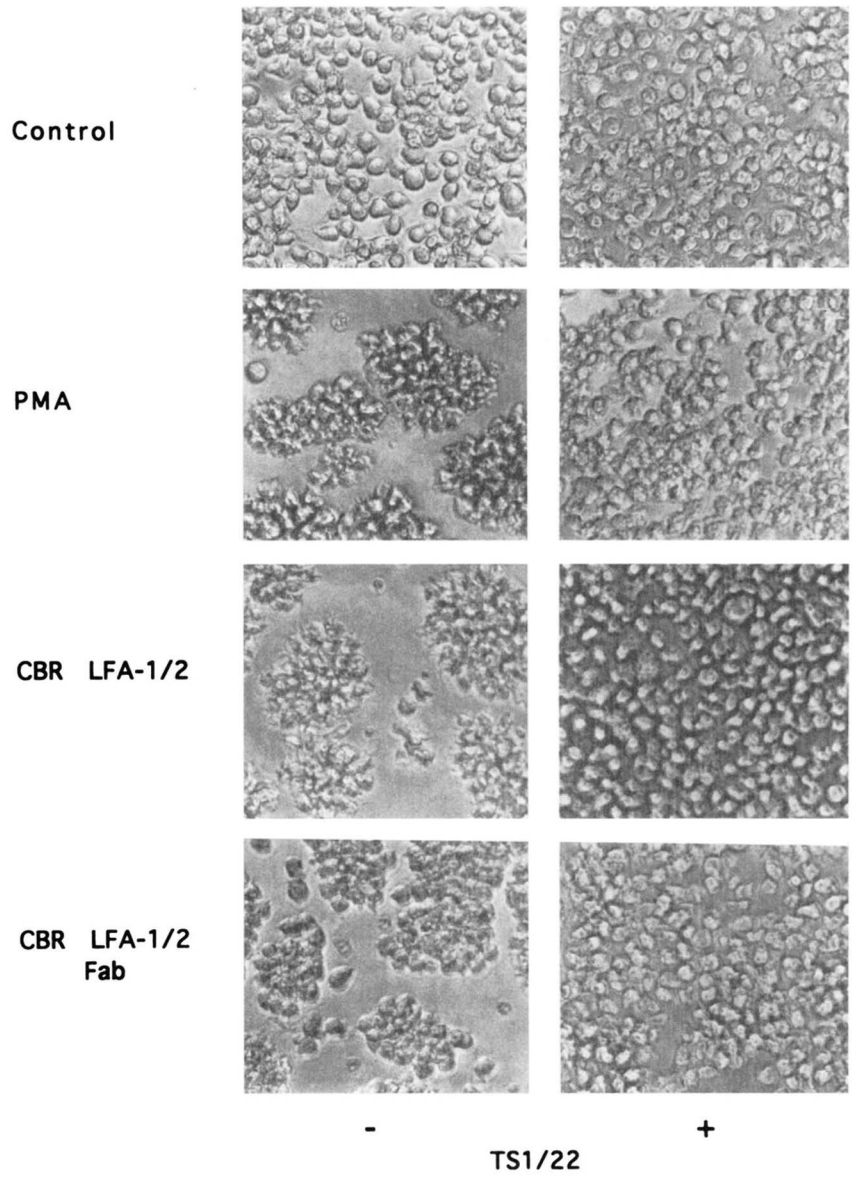


FIGURE 9. Stimulation of aggregation of JY cells by CBR LFA-1/2 and CBR LFA-1/2 Fab′ fragment. JY cells were incubated with (+) and without (−) TS1/22 for 20 min at room temperature. After removing the unbound Ab, aggregation was assessed in the presence of control Ab (TS2/4), phorbol ester (PMA) stimulation, and CBR LFA-1/2. Aggregation was induced by the latter two treatments as early as 10 min. The picture is representative of an average field after 30 min of stimulation.

mAb, CBR LFA-1/2, that is able to activate the leukocyte integrin, LFA-1, to probe the structural and functional changes that are associated with activation.

Several properties of the interaction of the CBR LFA-1/2 Ab with LFA-1 suggest that it activates by direct modulation of LFA-1 avidity. It induces binding of resting LFA-1-bearing cells to purified ICAM-1 or purified ICAM-3 immobilized on a substrate. Indeed, induction of binding to purified ICAM-1, rather than induction of homotypic aggregation, was selected as our screening assay because a number of investigators found that a majority of activating Ab induce aggregation by cross-bridging of cell surface receptors rather than by directly activating surface receptors (42). The effects of CBR LFA-1/2 are specific, as shown by inhibition with mAb to LFA-1. CBR LFA-1/2 acts independently of cell surface cross-linking, since the Fab′ fragment is fully active. Finally, CBR LFA-1/2 induces activation of LFA-1 rapidly and at a level comparable to that seen with phorbol esters. It remains to be determined whether CBR LFA-1/2 modulates the affinity of the binding site on LFA-1 and Mac-1 for ligand (43, 60) or promotes post-ligand binding events that modulate avidity through interaction with the cytoskeleton (61).

CBR LFA-1/2 mAb recognizes the β_2-subunit and activates receptors that have different α-subunits. Both LFA-1 and Mac-1 share a common substrate, ICAM-1.

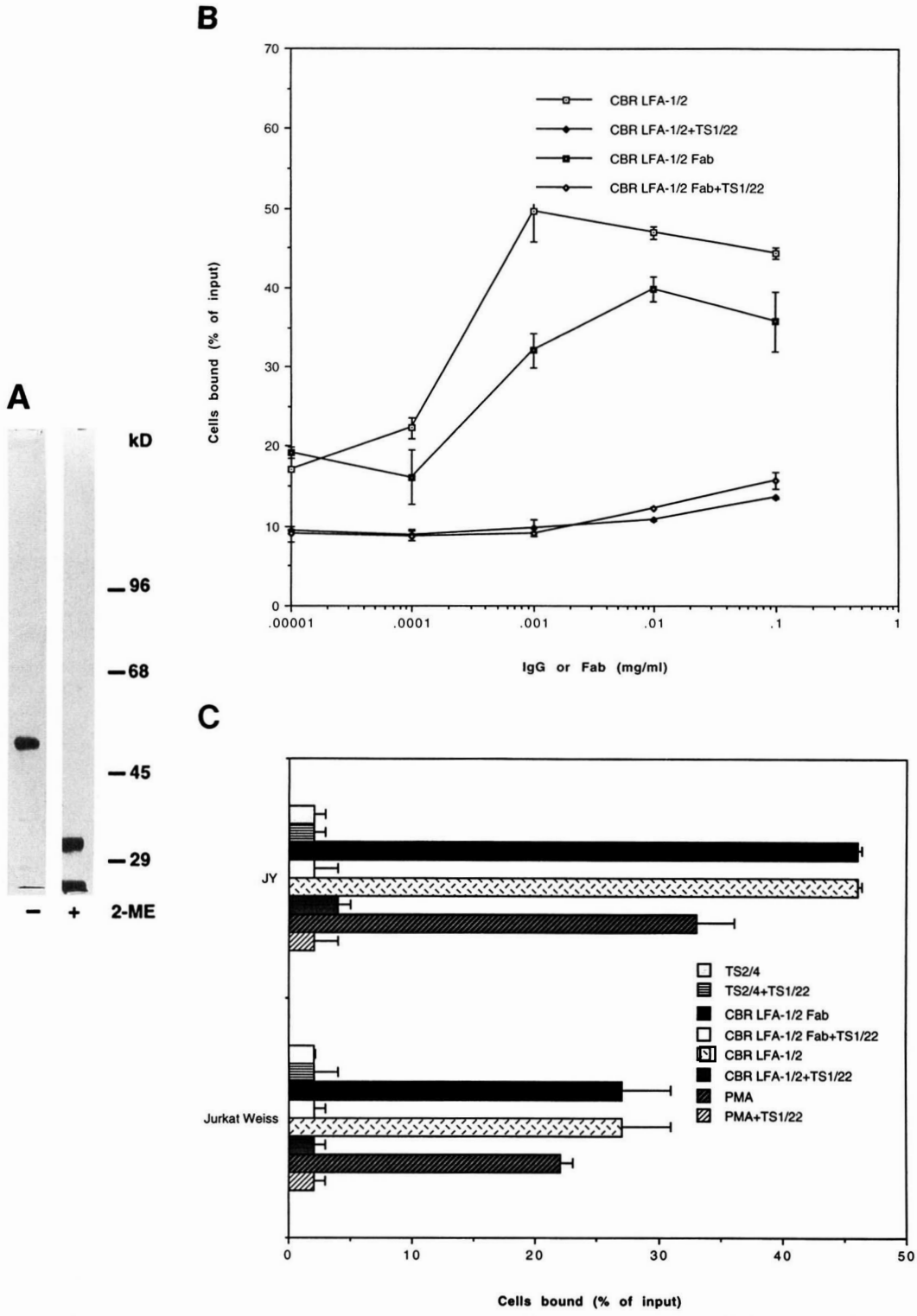


FIGURE 10. Stimulation of cell binding to ICAM-1 by CBR LFA-1/2 Fab' fragments. *A*, The Fab' fragment of CBR LFA-1/2 was treated with SDS sample buffer with or without 2-ME and subjected to SDS-10% PAGE and silver staining. The m.w. standards are indicated. *B*, Effect of intact CBR LFA-1/2 and its Fab' fragment on peripheral blood T lymphocyte binding to ICAM-1. The Ab and the Fab' fragment were incubated with T lymphocytes at increasing concentrations in the presence or absence of TS1/22. Data are the average and SE of quadruplicate determinations. *C*, Effect of CBR LFA-1/2 Fab fragment on JY and Jurkat cell binding to ICAM-1. JY and Jurkat cells were incubated with CBR LFA-1/2 IgG (10 μg/ml), CBR LFA-1/2 Fab' fragment (10 μg/ml), PMA, or control Ab (CBRM1/29) in the presence of the blocking Ab TS1/22 and tested for binding to purified ICAM-2 in microtiter wells. Data are the average and SE of triplicate determinations.

CBR LFA-1/2 activates binding of neutrophils to ICAM-1 through both Mac-1- and LFA-1-dependent pathways. Both LFA-1 and Mac-1 blocking Ab were needed to completely inhibit CBR LFA-1/2-stimulated binding to ICAM-1. By examining the effect of CBR LFA-1/2 on neutrophil binding to fibrinogen, we were able to show that it activates binding to fibrinogen through a Mac-1-dependent pathway. These findings indicate that an Ab directed against the β_2-subunit is able to activate functions associated with at least two distinct α-subunits. This has been previously seen with activating β_1-subunit Ab (31, 32) and β_2-specific Ab (34, 35). Our experiments extended previous work by showing that CBR LFA-1/2 would activate binding to multiple specific integrin ligands, including ICAM-1, ICAM-3, and fibrinogen.

We have extended previous studies with mAb that have been used to tag the activation state of LFA-1 (41, 42) or induce activation of LFA-1 through either its α- or β-subunits (26, 34, 35, 40, 62, 63). These Ab differ in requirement for divalent cations for binding to LFA-1. CBR LFA-1/2, like the previously described β_2-subunit-specific Ab KIM127 and KIM185 (34, 35) and the α_L-subunit-specific Ab MEM 83 (40), recognizes an epitope that is not sensitive to chelation of divalent cations. This is in contrast to the α_L-specific activating Ab NKI-L16; recognition of its epitope requires the presence of the divalent cation Ca^{2+} in the medium (64).

Since CBR LFA-1/2 does not require divalent cations for binding to its epitope, we were able to study its effect on the requirement for divalent cations for ligand binding. Recent studies have suggested divalent cation binding sites are present on both the α- and β-subunits of integrins (65), and a Mg^{2+} binding site has been identified in an integrin I domain (66). We found that CBR LFA-1/2 mAb markedly lowered the concentration of Mg^{2+} required for binding to ICAM-1. The Mg^{2+} ED_{50} values required for half-maximal adhesion were 0.6 mM for resting lymphocytes, 0.2 mM for PMA-stimulated cells, and 0.03 mM for CBR LFA-1/2-stimulated cells. It has been proposed that divalent cations coordinate between integrins and their ligands (67). According to this model, ligand binding requires a trimolecular interaction between LFA-1, ICAM-1, and Mg^{2+}; an increased concentration of any of the reactants or an increase in affinity of any of the individual bimolecular interactions is predicted to increase adhesion. Thus, the approximately 10-fold lower requirement for Mg^{2+} in the presence of CBR LFA-1/2 mAb is predicted to result from increased affinity of LFA-1 for Mg^{2+} or ICAM-1.

Further studies on the mechanism by which CBR LFA-1/2 modulates binding of ligands by $\alpha_L\beta_2$ and $\alpha_M\beta_2$ should reveal similarities and differences with binding modulated by activation of cellular signaling pathways and provide insights into the molecular steps involved in activation of LFA-1 and Mac-1 and the events that take place after engagement of ligands.

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