ICAM-3 Interacts with LFA-1 and Regulates the LFA-1/ICAM-1 Cell Adhesion Pathway

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Abstract. The interaction of lymphocyte function-associated antigen-1 (LFA-1) with its ligands mediates multiple cell adhesion processes of capital importance during immune responses. We have obtained three anti-ICAM-3 mAbs which recognize two different epitopes (A and B) on the intercellular adhesion molecule-3 (ICAM-3) as demonstrated by sequential immunoprecipitation and cross-competitive mAb-binding experiments. Immunoaffinity purified ICAM-3-coated surfaces were able to support T lymphoblast attachment upon cell stimulation with both phorbol esters and cross-linked CD3, as well as by mAb engagement of the LFA-1 molecule with the activating anti-LFA-1 NKI-L16 mAb. T cell adhesion to purified ICAM-3 was completely inhibited by cell pretreatment with mAbs to the LFA-1α (CD11a) or the LFA-β (CD18) integrin chains. Anti-ICAM-3 mAbs specific for epitope A, but not those specific for epitope B, were able to trigger T lymphoblast homotypic aggregation. ICAM-3-mediated cell aggregation was dependent on the LFA-1/ICAM-1 pathway as demonstrated by blocking experiments with mAbs specific for the LFA-1 and ICAM-1 molecules. Furthermore, immunofluorescence studies on ICAM-3-induced cell aggregates revealed that both LFA-1 and ICAM-1 were mainly located at intercellular boundaries. ICAM-3 was located at cellular uropods, which in small aggregates appeared to be implicated in cell–cell contacts, whereas in large aggregates it appeared to be excluded from cell–cell contact areas. Experiments of T cell adhesion to a chimeric ICAM-1-Fc molecule revealed that the proaggregatory anti-ICAM-3 HP2/19 mAb was able to increase T lymphoblast attachment to ICAM-1, suggesting that T cell aggregation induced by this mAb could be mediated by increasing the avidity of LFA-1 for ICAM-1. Moreover, the HP2/19 mAb was costimulatory with anti-CD3 mAb for T lymphocyte proliferation, indicating that enhancement of T cell activation could be involved in ICAM-3-mediated adhesive phenomena. Altogether, our results indicate that ICAM-3 has a regulatory role on the LFA-1/ICAM-1 pathway of intercellular adhesion.

A fine regulation of intercellular interactions is essential for the normal function of the immune system. The interaction of the leukocyte integrin lymphocyte function-associated antigen-1 (LFA-1,[1] CD11a/CD18) with its ligands is one of the several regulated pathways involved in leukocyte cell interactions (Springer, 1990). Two members of the immunoglobulin superfamily, the intercellular adhesion molecule-1 (ICAM-1) and -2 (ICAM-2), act as counter-receptors for the LFA-1 integrin (Marlin and Springer, 1987; Staunton et al., 1989). ICAM-1 is an inducible molecule which is highly expressed on the cell surface of cytokine-activated endothelial and epithelial cells, as well as on macrophages and activated lymphocytes (Dustin et al., 1986). In contrast, ICAM-2 is constitutively expressed on vascular endothelium and most leukocytes (De Fougerolles et al., 1991; Nortamo et al., 1991*a*,*b*). Recently, a third counter-receptor for LFA-1 (ICAM-3) has been identified by the ability of the CBR-IC3/1 mAb to inhibit, in conjunction with anti-ICAM-1 and anti-ICAM-2 mAb, cell attachment to purified LFA-1 (De Fougerolles and Springer, 1992). Cloning and sequencing studies revealed that ICAM-3 also belongs to the immunoglobulin superfamily (De Fougerolles et al., 1993; Fawcett et al., 1992; Vazeux et al., 1992). Interestingly, ICAM-3 is absent on endothelial cells, and its expression appears to be restricted to the leukocyte lineage, being highly expressed by monocytes, lymphocytes, and neutrophils (De Fougerolles and Springer, 1992). Whereas

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1. *Abbreviations used in this paper*: HSA, human serum albumin; ICAM, intercellular adhesion molecule; LFA-1, lymphocyte function-associated antigen 1.

ICAM-1 and ICAM-2 have been described to be involved in different immune processes, no functional role has so far been reported for ICAM-3.

We here describe the generation and epitope mapping of three novel anti-ICAM-3 mAb. We have used these mAb to purify ICAM-3, and to study the regulation of leukocyte adhesion to the purified molecule. We have also found a regulatory role of ICAM-3 on the LFA-1/ICAM-1 adhesion pathway.

Materials and Methods

Monoclonal Antibodies

Anti-ICAM-3 HP2/19, TP1/24, and TP1/25; anti-LFA-1α TP1/40 and TP1/32; and anti-LFAβ Lia 3/2 mAb were obtained from fusions with splenocytes from mice immunized with the human leukemic JM cell line (HP2/19 mAb), human T-lymphocytes activated for 24 h with a combination of PMA and anti-CD3 mAb (TP series), or the promyelomonocytic U937 cell line (Lia 3/2 mAb). The anti-ICAM-3 CBR-IC3/1, anti-CD11a TS1/11 and NKI-L16, anti-CD18 TS1/18, anti-ICAM-1 RR1/1 and LB-2, anti-CD29 Lia 1/2 and TS2/16, anti-CD45 D3/9, anti-CD43 TP1/36, anti-HLA-A,B W6/32, and anti-CD3 SPV-T3b mAb have been described (De Fougerolles and Springer, 1992; Sánchez-Madrid et al., 1983; Keizer et al., 1988; Rothlein et al., 1986; Clark et al., 1986; Campanero et al., 1991, 1992; Hemler et al., 1984; Pulido et al., 1988; Barnstable et al., 1978; Spits et al., 1985). The anti-CD31 TP1/15 mAb was generated in our laboratory, and will be described elsewhere. For cell adhesion and aggregation experiments as well as for mAb radiolabeling, mAbs were purified from ascites fluid with protein A coupled to Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). F(ab')2 fragment of HP2/19 mAb was prepared as previously reported (Parham et al., 1982). P3X63 myeloma culture supernatant was used as negative control.

Cells and Cell Lines

Neutrophils were isolated from fresh human blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia LKB Biotechnology, Piscataway, NJ), followed by sedimentation at 1 *g* in dextran (Sigma Chem. Co., St. Louis, MO) at room temperature. The neutrophil-enriched fraction was further purified by hypotonic lysis of erythrocytes, giving a purity higher than 98%.

T lymphoblasts were prepared from peripheral blood mononuclear cells by treatment with phytohaemagglutinin (0.5%; Pharmacia LKB Biotechnology) for 48 h. Cells were washed and cultured in RPMI 1640 (Flow Labs, Irvine, Scotland) containing 10% FCS (Flow Labs) and 20 U/ml IL-2. 7–12 d T lymphoblasts were typically used in all the experiments. T lymphoblasts and T cell clones have been extensively used to study both LFA-1-mediated cell adhesion and T cell activation (Cantrell and Smith, 1984; Van Kooyk et al., 1991; Dransfield et al., 1992). The Jurkat T cell line was maintained in RPMI 1640 plus 10% FCS.

Purification of ICAM-3 by mAb Affinity Chromatography

ICAM-3 was purified from detergent lysates of human neutrophils by affinity chromatography using the anti-ICAM-3 TP1/24 mAb coupled to CNBr-activated Sepharose CL-4B (Pharmacia LKB Biotechnology) at 2 mg/ml. All chromatography steps were performed at 4°C, and all buffers contained 5 mM iodoacetamide, 1 mM PMSF, 0.2 U/ml aprotinin, and 0.025% NaN_3. Human neutrophils were lysed in a 20 mM Tris-HCl, pH 8.0, 0.5% Triton X-100 (TX-100) buffer. Lysates were centrifuged at 2,500 *g* for 60 min and the supernatant was passed in series through columns of CNBr-activated glycine-quenched Sepharose CL-4B and anti-ICAM-3 TP1/24-Sepharose CL-4B. The TP1/24-Sepharose column was washed with 50 mM ethanolamine, pH 10, 0.5 M NaCl, 1% *n*-octyl-β-D-glucopyranoside (octylglucoside, OG). Then, proteins bound to the column were eluted with 50 mM ethanolamine, pH 11, 0.5 M NaCl, 1% octylglucoside. Eluates were immediately neutralized with 2 M Tris-HCl pH 6.7. Fractions containing the eluted ICAM-3 were identified by SDS-7% PAGE, and proteins were visualized by silver staining (Pulido et al., 1988). Fractions containing ICAM-3 were pooled and concentrated ~30-fold by using Centricon-100 microconcentrators (Amicon, Danvers, MA), and reanalyzed by SDS-7% PAGE. As estimated by silver staining and comparison to standard proteins, ICAM-3 was concentrated up to 40 μg/ml.

Radiolabeling and Cross-Competitive mAb Cell-Binding Assays

Both purified ICAM-3 and anti-ICAM-3 mAb, as well as neutrophils, were radioiodinated in solution with chloroglycoluril (IODO-GEN, Pierce Chem. Co., Rockford, IL), as described (Sánchez-Madrid et al., 1983; Pulido et al., 1991). For cross-competitive mAb cell-binding assays, Jurkat cells were preincubated in RPMI 1640 (5×10^6/ml) with an excess of unlabeled mAb for 30 min at 4°C. Then, ^{125}I-labeled mAb (2×10^5 cpm) were added and incubated for another 30 min at 4°C. Afterwards, unbound radioactivity was removed by washing with RPMI, and cell-bound radioactivity was measured in a gamma counter.

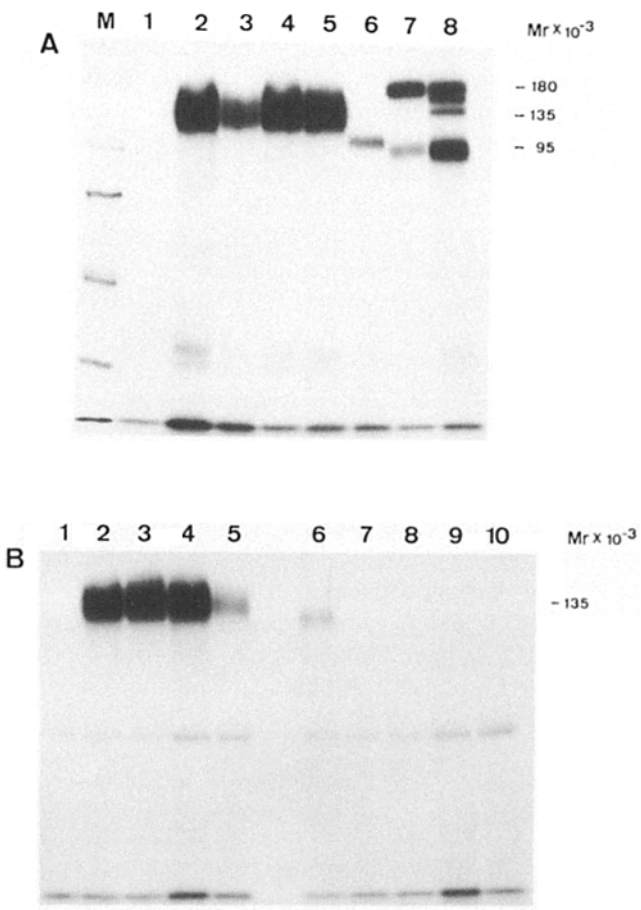

Figure 1. Characterization of three novel anti-ICAM-3 mAb. (*A*) ^{125}I-labeled cell lysates from neutrophils were immunoprecipitated with the following mAbs: control P3X63 (lane *1*), anti-ICAM-3 CBR-IC3/1 (lane *2*), HP2/19 (lane *3*), TP1/24 (lane *4*), and TP1/25 (lane *5*), anti-CD43 TP1/36 (lane *6*), anti-LFA-1 TP1/40 (lane *7*), and anti-LFA-β Lia 3/2 (lane *8*); *M*, M_r markers (200, 100, 95, 69, 46, and 30 kD). (*B*) ^{125}I-labeled neutrophil lysates were precleared with the anti-CD31 TP1/15 mAb (lanes *1–5*)or with the anti-ICAM-3 TP1/24 mAb (lanes *6–10*), and then were immunoprecipitated with the anti-CD31 TP1/15 (lanes *1* and *6*), anti-ICAM-3 TP1/24 (lanes *2* and *7*), TP1/25 (lanes *3* and *8*), CBR-IC3/1 (lanes *4* and *9*), and HP2/19 (lanes *5* and *10*) mAbs. Immune complexes were isolated, and reduced samples were subjected to SDS-10% PAGE and autoradiography.

Immunoprecipitation and Electrophoresis

Radiolabeled neutrophils were lysed in lysis buffer (PBS, pH 7.4 containing 1% Triton X-100, 1% hemoglobin and 1 mM PMSF). Equal amounts of ^{125}I-labeled proteins (either from neutrophil lysates or purified ICAM-3) were mixed with 100 μl of mAb-containing culture supernatant. To isolate immune complexes, 100 μl of 187.1 anti-mouse kappa chain mAb followed by 30 μl protein A coupled to Sepharose were added. For sequential immunoprecipitation, lysates were precleared by incubation with the first mAb coupled to CNBr-Sepharose, followed by centrifugation to pellet the immune complexes. Then, the supernatants were precipitated with the second mAb. Immunoprecipitates were processed as described (Sánchez-Madrid et al., 1983) and analyzed by SDS-PAGE and autoradiography.

Cell Adhesion Assays

Adhesion assays were essentially performed as previously described (Arroyo et al., 1992). Briefly, 40 μl/well of either purified ICAM-3 (0.1–4 μg/ml) or human serum albumin (HSA; 100 μg/ml) in TSM (washing buffer; 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM $MgCl_2$, 0.1% octylglucoside), were used to coat 96-well microtiter EIA II-Linbro plates (Costar Corp., Cambridge, MA) for 2 h at 37°C, and then saturated with washing buffer containing 1% HSA for 30 min at 37°C. Thereafter, plates were washed with washing buffer, and 2–3 × 10^5 cells/well in 100 μl were added and centrifuged for 5 min at 10 *g* before incubation at 37°C for 10–20 min. Cell adhesion to a chimeric recombinant soluble ICAM-1-Fc (Berendt et al., 1992) (40 μl/well at 10 μg/ml) was performed in a similar way except that 0.1% octylglucoside was excluded from all the steps. To quantify cell attachment, the plate was washed with washing buffer, cells were fixed with a mixture of acetone/methanol 1:1, and dyed with violet crystal 0.5%. Then, absorbance at 540 nm was measured in an ELISA detector (LP400, Kallestad, Chaska, MN) and optical density was found to be a linear function of number of cells by a calibration curve (optical density vs number of cells). Total cellular input was calculated by spinning wells with original number of cell aliquots, staining, and measuring optical density.

Aggregation Assays

Homotypic cell aggregation was performed as previously described (Campanero et al., 1990). Briefly, cells were incubated in flat-bottomed, 96-well microtiter plates (Costar Corp.) in duplicate at 1.5 × 10^6/ml in a final volume of 100 μl of complete medium. mAbs were added at 1 μg/ml and cells were allowed to settle in a cell incubator at 37°C and 5% CO_2 atmosphere. Aggregation was then determined at different periods of time by direct visualization of the plate with an inverted microscope and counting the free cells of at least five randomly chosen areas of 0.025 mm^2, delimited by a special mask consisting of squares, under the plate. Results were expressed as percent of aggregated cells.

Immunofluorescence

T lymphoblasts were incubated in flat-bottomed, 24-well microtiter plates (Costar Corp.) at 4 × 10^6 cells/ml in a final volume of 500 μl of complete medium. mAbs or their F(ab')$_2$ fragments were added at a final concentration of 1 μg/ml and cells were allowed to settle in a cell incubator at 37°C and 5% CO_2 atmosphere; after either 1 (small cell aggregates) or 2 h (large cell aggregates), cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature and rinsed in TBS (TBS: 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% NaN_3). To directly visualize the mAb that induced the process of aggregation, cells were stained with a 1:50 dilution of the FITC-labeled rabbit F(ab')$_2$ anti-mouse IgG (Pierce) as a secondary antibody. To visualize other membrane proteins, cell aggregation was induced with the F(ab')$_2$ fragment of anti-ICAM-3 HP2/19 mAb (1 μg/ml) and after fixation, the cells were incubated with another mAb followed by washing and labeling with a 1:50 dilution of the FITC-conjugated goat anti-mouse Fc fragment of IgG (Sigma Chem. Co.). The cells were observed using a Nikon Labophot-2 photomicroscope with a 60 × oil immersion objective and photographed on TMAX 400 film (Kodak) processed to 800–1600 ASA with TMAX developer (Eastman Kodak Co., Rochester, NY).

Proliferation Assays

50 μl F(ab')$_2$ sheep anti-mouse IgG at 7.5 μg/ml in 20 mM Tris-HCl, pH 8.0, was placed in each well of a 96-well flat-bottomed microtiter plate (Costar Corp.) overnight at 4°C. After washing twice with PBS, the indicated amounts of mAb directed against different lymphocyte surface antigens were placed in each well and incubated at 37°C for an additional hour. Then, wells were washed twice with RPMI 1640. Peripheral blood mononuclear cells were depleted of monocytes by adherence to plastic plates at 37°C for >3 h. 50,000 cells in 200 μl of RPMI 1640–10% FCS were added to each well and cultured in triplicate for 3 d. Cell proliferation was estimated by [^{3}H]dThd (New England Nuclear, Boston, MA) incorporation during the last 16 h of culture. Cells were harvested and the radioactivity measured in a liquid scintillation counter.

Results

Characterization of the Specificity of Three Novel Anti-ICAM-3 mAbs

Three mAbs (HP2/19, raised against the JM leukemic cell line; and TP1/24 and TP1/25, raised against human activated T lymphocytes) were selected. These mAbs immunoprecipitated from ^{125}I-cell surface labeled human neutrophils a 135-kD polypeptide (Fig. 1 *A*, lanes *3–5*) that resembled the immunoprecipitation pattern produced by the recently described anti-ICAM-3 CBR-IC3/1 mAb (De Fougerolles and Springer, 1992) (Fig. 1 *A*, lane *2*). To ascertain the specificity of these three novel mAbs, sequential immunoprecipitation experiments were carried out in conjunction with the anti-ICAM-3 CBR-IC3/1 mAb. The TP1/24 mAb precleared

Table I. Topographic Epitopes on the ICAM-3 Molecule

Unlabeled mAb	Specificity	^{125}I-labeled mAb			Epitope	Induction of cell aggregation[‡]
		CBR-IC3/1	HP2/19	TP1/24		
		Bound radioactivity[*]				
CBR-IC3/1	ICAM-3	1536	3238	17152	A	+
HP2/19	"	1552	2862	19345	A	+
TP1/24	"	6434	26614	3718	B	−
TP1/25	"	6188	26850	3531	B	−
Control mAb						
TS1/11	LFA-1	6642	31723	23611		
P3X63	−	6694	30769	19289		

* Binding of ^{125}I-labeled purified mAb (2 × 10^5 cpm) to Jurkat cells was inhibited with an excess of unlabeled competitor mAb, as described under Materials and Methods. Bound radioactivity was measured and data (cpm) are expressed as mean of two separate experiments.
‡ Cell aggregation of T lymphoblasts was quantified as described under Materials and Methods. + and − mean, respectively, over 50% and below 20% of cell aggregation.

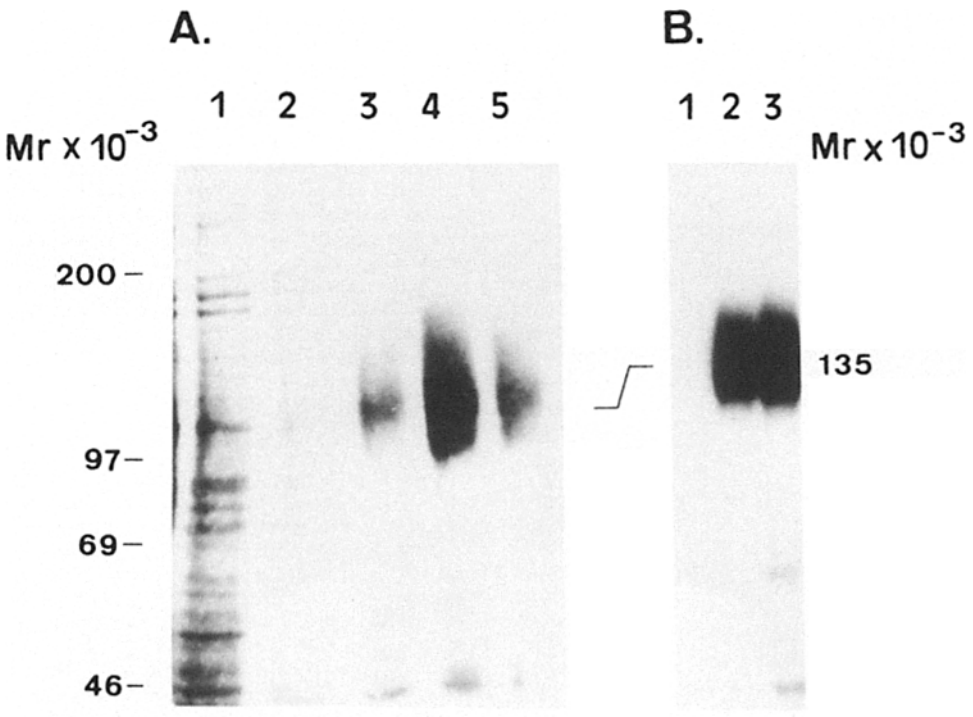

Figure 2. SDS-PAGE of immunoaffinity purified ICAM-3. (*A*) ICAM-3 from human neutrophils was purified by immunoaffinity chromatography. Samples of flow through (lane *1*), pH 10 washing (lane 2), and pH 11 eluates (lanes *3–5*) containing purified ICAM-3, were electrophoresed under reducing conditions on an SDS-7% PAGE and visualized by silver staining. (*B*) Purified ICAM-3 was radiolabeled and immunoprecipitated with the following mAbs: anti-LFA-1 TS1/11 (lane *1*), anti-ICAM-3 TP1/25 (lane 2), and TP1/24 (lane *3*) mAb. Immune complexes were isolated, and reduced samples were subjected to SDS-7% PAGE and autoradiography.

the antigen recognized by either TP1/25 and HP2/19 mAb, as well as that precipitated by the anti-ICAM-3 CBR-IC3/1 mAb, but not that recognized by the anti-CD31 TP1/15 mAb, included as a control (Fig. 1 *B*, lanes *6–10*). Conversely, the anti-CD31 TP1/15 mAb did not preclear the antigen recognized by CBR-IC3/1, HP2/19, TP1/24, or TP1/25 mAb (Fig. 1 *B*, lanes *1–5*). Thus, these results demonstrate that the novel HP2/19, TP1/24, and TP1/25 mAb recognize the ICAM-3 molecule.

Next, epitope mapping on the ICAM-3 molecule was per-

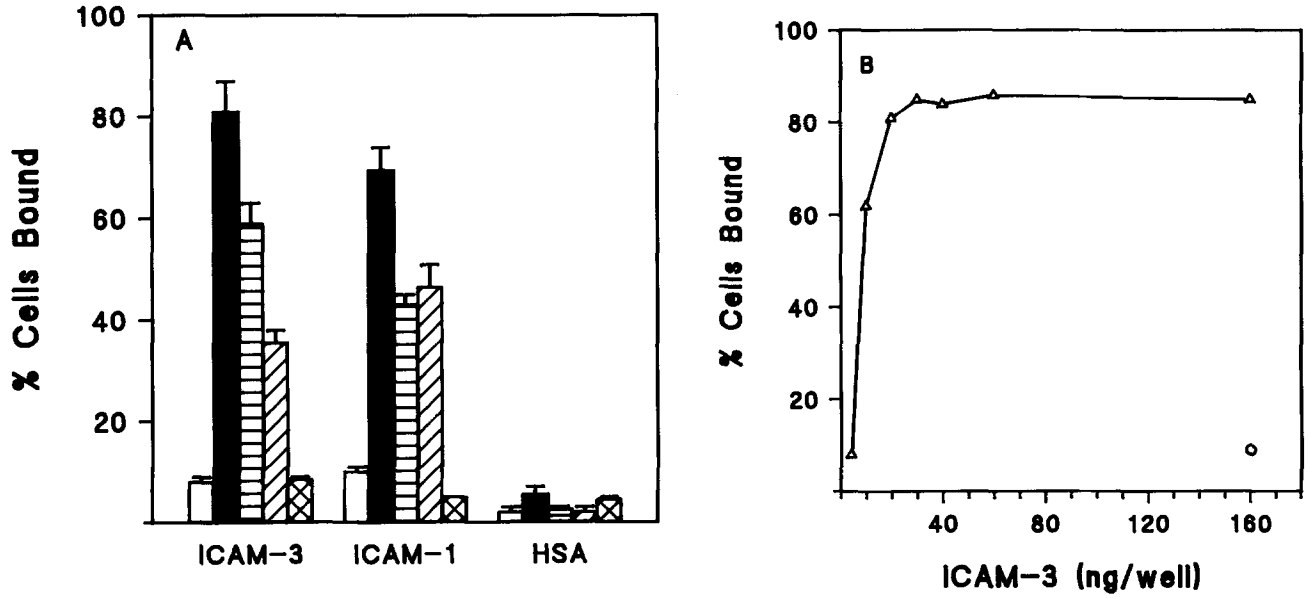

Figure 3. Regulated lymphocyte adhesion to purified ICAM-3. (*A*) 40 μl/well of ICAM-3 (0.4 μg/ml), ICAM-1-Fc (10 μg/ml), and HSA (100 μg/ml) were used to coat 96-well microtiter plates. T lymphoblasts were pretreated for 30 min at 4°C with the following stimuli: RPMI (□), 5 ng/ml PMA (■), 10 μg/ml anti-CD3 T3b cross-linked by using 10 μg/ml sheep anti-mouse IgG (⊟), 1:50 dilution of culture supernatant from the anti-LFA-1 NKI-L16 mAb (⧄), and 10 μg/ml of the anti-VLAβ1 Lia 1/2 mAb (⊠). Adhesion was quantified after 10 min of incubation at 37°C. Arithmetic mean and SD of duplicate wells is shown. Data are representative of three experiments. (*B*) Dose-dependence of cell adhesion of purified ICAM-3. 40 μl/well of ICAM-3 at doses ranging from 0.1 to 4 μg/ml were used to coat 96-well microtiter plates. T lymphoblasts were stimulated with either 5 ng/ml PMA (*triangles*) or RPMI (*circle*). Data are representative of two experiments. Cell adhesion to HSA was always lower than 5%.

formed by cross-competitive binding studies with unlabeled and labeled anti-ICAM-3 mAb (Table I). The HP2/19 and CBR-IC3/1 mAbs cross-blocked each other, but were not competed by the TP1/24 and TP1/25 mAbs. Conversely, TP1/24 mAb was competed by TP1/25 mAb, but not by HP2/19 and CBR-IC3/1 mAbs. Thus, two different topographic epitopes can be defined on the ICAM-3 molecule: epitope A, recognized by HP2/19 and CBR-IC3/1 mAb, and epitope B, recognized by TP1/24 and TP1/25 mAbs.

Regulation of T Cell Binding to Immunopurified ICAM-3

ICAM-3 was purified by mAb immunoaffinity chromatography from human neutrophils which exhibit a high membrane expression of ICAM-3 (De Fougerolles and Springer, 1992). Neutrophils were lysed with the detergent Triton X-100, and ICAM-3 was purified from the lysate by binding to a TP1/24 mAb Sepharose column. Immunopurified ICAM-3 appeared as a broad band around 135 kD upon SDS-PAGE (Fig. 2 *A*). The purified molecule was radiolabeled and subjected to immunoprecipitation with different mAbs. Both anti-ICAM-3 TP1/24 and TP1/25, but not the anti-LFA-1α TS1/11 mAb, were able to precipitate the 135-kD purified ICAM-3 protein (Fig. 2 *B*) consistent with previous observations from immunoprecipitation experiments (Fig. 1).

To determine whether ICAM-3 could mediate cellular adhesion, a solution containing the purified glycoprotein was used to coat plastic dishes on which cell binding could be analyzed. When untreated T lymphoblasts were added to ICAM-3 coated wells, a small percentage of cells ($\pm$10%) was specifically bound (Fig. 3 *A*). However, phorbol ester treatment of T lymphoblasts greatly enhanced cell binding to ICAM-3 (Fig. 3, *A* and *B*).

To test whether other stimuli, besides phorbol esters, could be able to enhance cell binding to ICAM-3, T lymphoblasts were treated with different mAbs against functionally relevant molecules on T cells. Engagement of either CD3-TcR by cross-linked anti-CD3 mAb, or the LFA-1 integrin by the proaggregatory and activating anti-LFA-1 NKI-L16 mAb (Keizer et al., 1988) greatly enhanced T lymphoblast adhesion to purified ICAM-3 (Fig. 3 *A*). In contrast, cell treatment with the proaggregatory anti-VLAβ1 (CD29) Lia 1/2 mAb (Campanero et al., 1992) did not modify cell attachment to purified ICAM-3 (Fig. 3 *A*). These results indicate that cellular avidity for ICAM-3 can be specifically regulated upon cell activation and suggest that the LFA-1 integrin can be directly involved in this process. A similar profile of cell adhesion was obtained with ICAM-1-coated surfaces, while no binding was observed with purified HSA (Fig. 3 *A*).

A dose-response curve (Fig. 3 *B*) showed that, upon T lymphoblast activation with PMA, significant cell binding could be supported with ICAM-3 amounts as low as 10 ng per well and reached maximal binding with 20 ng per well. Dose dependence of cell adhesion to the purified glycoprotein suggested the specificity of the assay that was further confirmed in mAb blocking experiments (see below).

To determine the direct involvement of the LFA-1 integrin in cell binding to purified ICAM-3, inhibition studies with specific mAb were carried out. Three different anti-LFA1α (CD11a) mAbs (TP1/40, TS1/11, and TP1/32), as well as two different anti-β2 (CD18) mAbs (Lia 3/2 and TS1/18) completely blocked cell attachment to purified ICAM-3, whereas the anti-CD45 D3/9 mAb did not affect cell adhesion (Fig. 4, *upper panel*). Neither anti-ICAM-1 nor anti-ICAM-3 mAb directed to epitope A or B were able to ablate cell binding to the ICAM-3 molecule (Fig. 4, *upper panel*, and data not shown). In contrast, the anti-ICAM-1 RR1/1 mAb almost completely blocked cell adhesion to recombinant ICAM-1-Fc (Fig. 4, *lower panel*). The anti-LFA1α and anti-LFA-β mAbs also inhibited cell binding to ICAM-1 (Fig. 4, *lower panel*). Altogether, these data demonstrate that T lymphoblasts are able to specifically interact with purified ICAM-3 through the LFA-1 integrin.

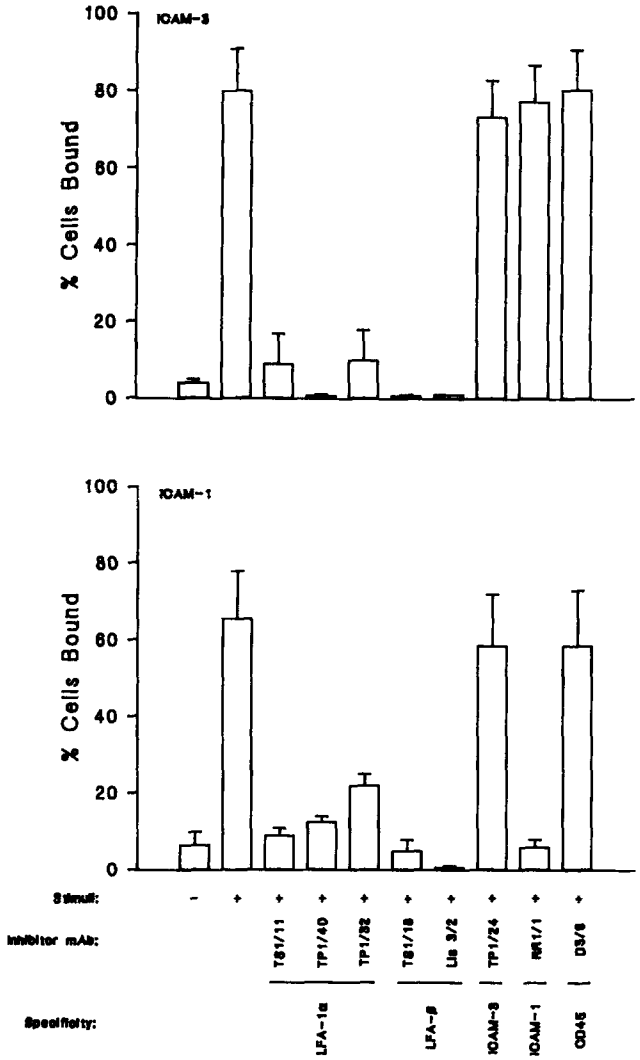

Figure 4. Inhibition of T-cell adhesion to purified ICAM-3 by anti-LFA-1α and β mAb. Unstimulated and PMA-treated (5 ng/ml) T lymphoblasts were allowed to bind to either (*A*) purified ICAM-3- (0.4 μg/ml) or (*B*) ICAM-1-Fc-coated (10 μg/ml) microtiter wells in the presence of blocking mAb. 1 μg/ml inhibitor mAbs were used to pretreat for 30 min at 4°C either the cells (anti-LFA-1α, anti-LFA-β, and anti-CD45) or the wells (anti-ICAM-1 and anti-ICAM-3). Adhesion was quantified after 10 min of incubation at 37°C. Arithmetic mean and SD of duplicate wells is shown. Data are representative of three experiments. Cell adhesion to HSA was always lower than 5%.

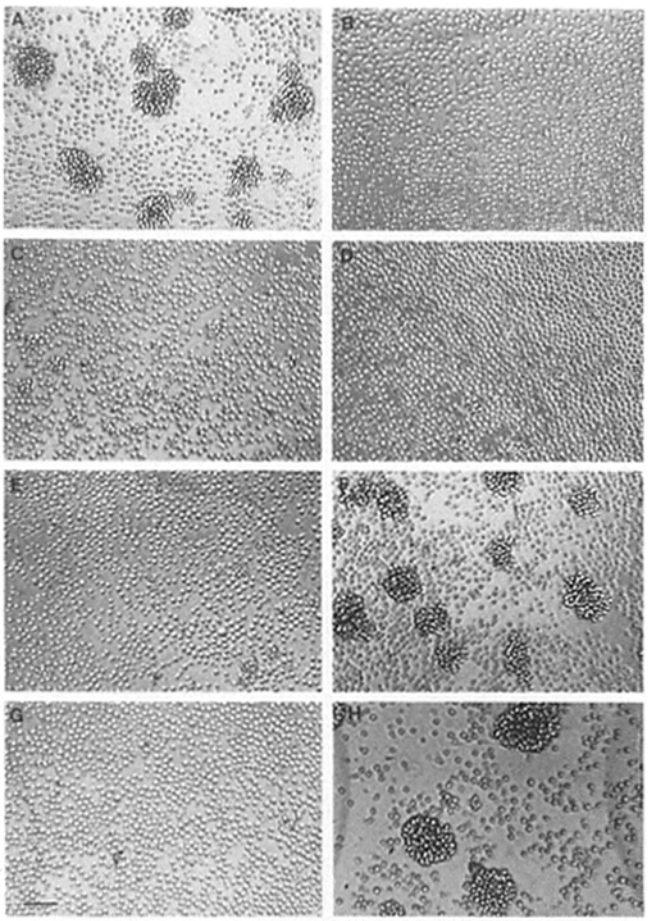

Figure 5. ICAM-3-mediated leukocyte intercellular interactions are LFA-1/ICAM-1 dependent. T lymphoblasts were incubated in the presence of either anti-ICAM-3 HP2/19 (*A*) and TP1/24 mAb (*B*), RPMI (*G*), or anti-LFA-1 NKI-L16 mAbs (*H*). In *C–F*, cells were preincubated for 30 min at RT with anti-ICAM-3 TP1/24 (*C*), anti-LFA-1 TP1/40 (*D*), anti-ICAM-1 RR1/1 (*E*), and anti-VLAβ TS2/16 (*F*) mAbs before the addition of anti-ICAM-3 HP2/19 mAb. Photomicrographs were taken after 2 h from the beginning of the assay. Bar, 150 μm.

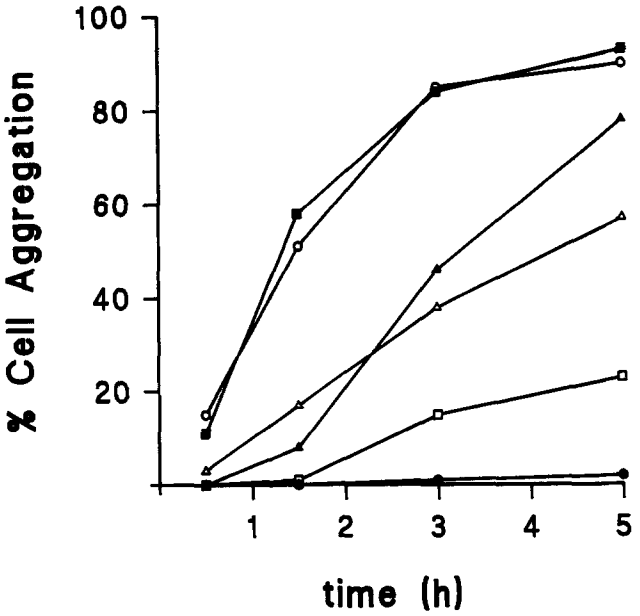

Figure 6. Kinetics of ICAM-3-mediated leukocyte intercellular interactions. Inhibitory effects of anti-LFA-1 and anti-ICAM-1 mAb. T lymphoblasts were pretreated for 30 min at RT with RPMI (○), anti-ICAM-3 TP1/24 (△), anti-ICAM-1 RR1/1 (□), anti-LFA-1 TP1/40 (▲), a combination of TP1/24 plus RR1/1 (●), and anti-VLAβ TS2/16 mAb (■), before the addition of anti-ICAM-3 HP2/19 mAb. Percent of cell aggregation was calculated, as described under Materials and Methods, at different times from the beginning of the assay. Untreated cells showed always less than 10% of aggregation. Data are representative of five experiments.

LFA-1/ICAM-1-dependent Leukocyte Intercellular Interactions Induced through ICAM-3

To explore the functional role of ICAM-3 in leukocyte interactions, we have examined the effect of our panel of anti-ICAM-3 mAbs in different cell adhesion assays. Incubation of T lymphoblasts in the presence of purified anti-ICAM-3 epitope A HP2/19 (Table I), as well as with its F(ab')$_2$ fragment, resulted in the rapid induction of strong homotypic cell aggregation (Figs. 5 *A* and 8). Similar effects were observed with the CBR-IC3/1 mAb specific for epitope A (Table I). However, the anti-ICAM-3 TP1/24 and TP1/25 mAbs, specific for epitope B, did not induce cell clustering (Fig. 5 *B*, and Table I). Induction of cell aggregation through the ICAM-3 molecule required both an intact metabolism and a physiological temperature (data not shown), therefore ruling out the possibility that intercellular adhesion could be due to cell agglutination. Moreover, cell aggregation triggered by the anti-ICAM-3 epitope A HP2/19 mAb could be blocked by mAb directed against other distinct epitopes on the ICAM-3 molecule, as well as by mAb specific for the LFA-1α (CD11a) integrin (Fig. 5, *C* and *D*, respectively). Interestingly, the anti-ICAM-1 RR1/1 mAb also inhibited ICAM-3-induced cell aggregation (Fig. 5 *E*). By contrast, the anti-VLAβ1 TS2/16 mAb did not affect the ICAM-3-mediated cell aggregation (Fig. 5 *F*). T lymphoblast aggregation triggered by the anti-LFA-1 NKI-L16 mAb is also shown for comparison (Fig. 5 *H*).

Kinetic experiments revealed that maximal ICAM-3-induced cell aggregation was reached upon 3 h of T lymphoblast incubation with the HP2/19 mAb (Fig. 6). Cell preincubation with mAb against LFA-1α (TP1/40), ICAM-1 (RR1/1 and LB-2), and ICAM-3 (TP1/24 and TP1/25) partially inhibited cell aggregation triggered by the anti-ICAM-3 HP2/19 mAb, whereas the anti-VLAβ1 TS2/16 mAb showed no inhibitory effect at all (Fig. 6, and data not shown). Interestingly, complete inhibition of cell aggregation could be achieved by combination of the anti-ICAM-1 RR1/1 and anti-ICAM-3 TP1/24 mAb (Fig. 6).

LFA-1 and ICAM-1 Cluster at Sites of Cell–Cell Contacts during ICAM-3 Mediated Leukocyte Aggregation

The results shown above strongly suggested that the LFA-1/ICAM-1 interaction could be involved in ICAM-3-mediated cell aggregation. To further explore the role of the LFA-1/ICAM-1 adhesion pathway, we studied the localization of all of these adhesion molecules on ICAM-3-induced cell aggregates. ICAM-3 displayed a striking distribution, with a

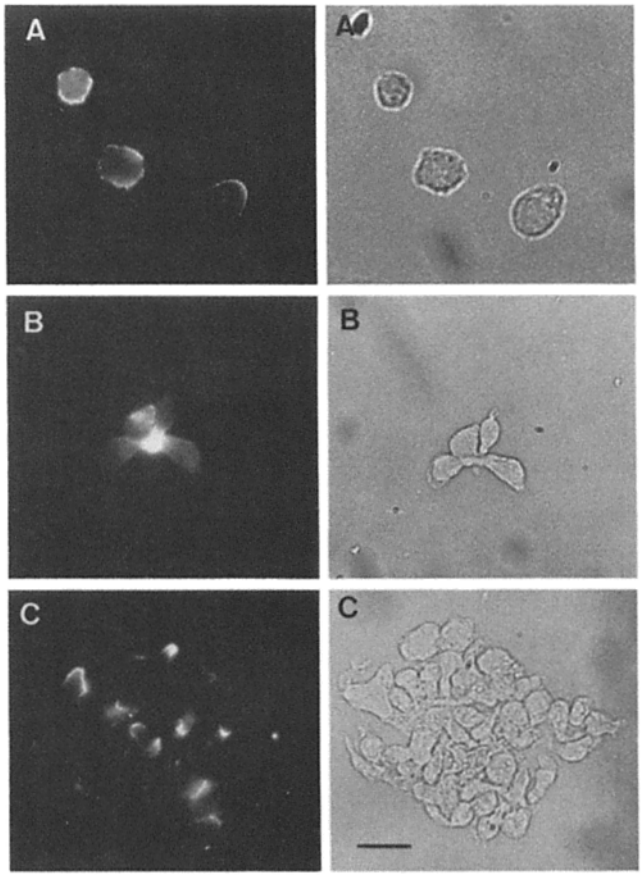

Figure 7. Immunofluorescence localization of ICAM-3 in cell aggregates. Untreated T lymphoblasts were fixed and stained for ICAM-3 (*A*). T lymphoblast aggregation was induced by using the anti-ICAM-3 HP2/19 mAb, and upon cell incubation at 37°C for 1 (*B*) or 2 h (*C*), the cells were fixed and stained for ICAM-3. Same fields were photographed under epifluorescent (*left panels*) and bright field (*right panels*) conditions. Bar, 60 μm.

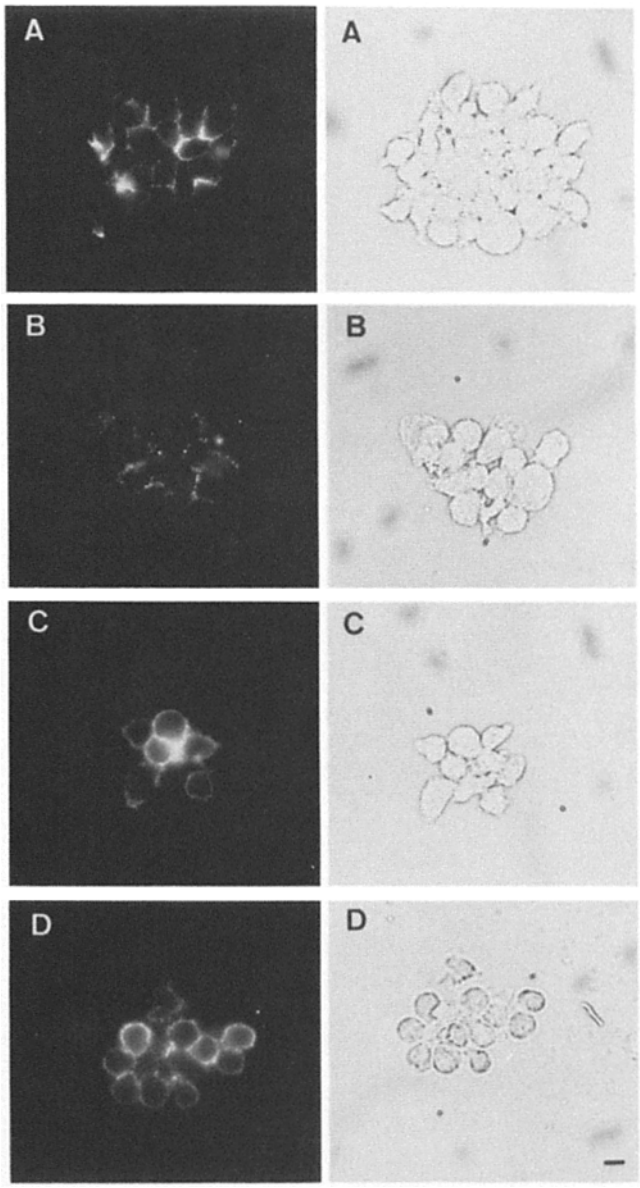

Figure 8. Immunofluorescence localization of LFA-1 and ICAM-1 in cell aggregates. Aggregation of T lymphoblasts was induced for 2 h at 37°C by using the proaggregatory anti-ICAM-3 HP2/19 mAb. Then cells were fixed and stained with either anti-ICAM-1 RR1/1 (*A*), anti-LFA-1 NKI-L16 (*B*) and TS1/11 (*C*), or anti-VLAβ TS2/16 (*D*) mAbs. Same fields were photographed under epifluorescent (*left panels*) and bright field (*right panels*) conditions. Bar, 30 μm.

stalk-like structure or uropod where ICAM-3 was preferentially located (Fig. 7, *B* and *C*). In contrast, most of mAb-untreated cells (over 80%) showed a rounded morphology, and ICAM-3 displayed a more diffuse pattern of staining (Fig. 7 *A*). In small aggregates, cells appeared to contact each other through their uropods (Fig. 7 *B*), whereas in large aggregates, the uropods where ICAM-3 was located appeared to be excluded from the areas of cell–cell contact (Fig. 7 *C*). Interestingly, the anti-ICAM-1 RR1/1 mAb, as well as the NKI-L16 mAb, which recognizes an activation-related epitope on the leukocyte integrin LFA-1 (Van Kooyk et al., 1991), preferentially reacted with cell–cell boundaries (Fig. 8, *A* and *B*). By contrast, the anti-LFA-1 TS1/11 mAb, which recognizes a constitutively expressed epitope, as well as the anti-VLAβ1 TS2/16 mAb showed a more diffuse pattern of cell surface staining (Fig. 8, *C* and *D*). Altogether, these results suggest that ICAM-3 could be mediating cellular events involved in the initiation phase of cell aggregation likely by enhancing the avidity of LFA-1 for ICAM-1.

Increased Cell Binding to ICAM-1 Induced through ICAM-3

To study the regulation of cell binding to ICAM-1, we have used a chimeric recombinant soluble ICAM-1-Fc molecule (Berendt et al., 1992). Adhesion of untreated T lymphoblast to plastic well coated with recombinant ICAM-1-Fc was completely blocked by both the anti-LFA-1 TP1/40 and the anti-ICAM-1 RR1/1 and LB-2 mAb (data not shown), thus indicating that T lymphoblast adhesion is specifically mediated by the leukocyte integrin LFA-1. Cell pretreatment with the proaggregatory anti-ICAM-3 HP2/19 mAb provoked about a twofold increase of T lymphoblast adhesion to recombinant ICAM-1-Fc, whereas no effect was observed in cells treated either with the anti-VLAβ1 Lia 1/2 mAb, which also induced cell aggregation (Campanero et al., 1992), or

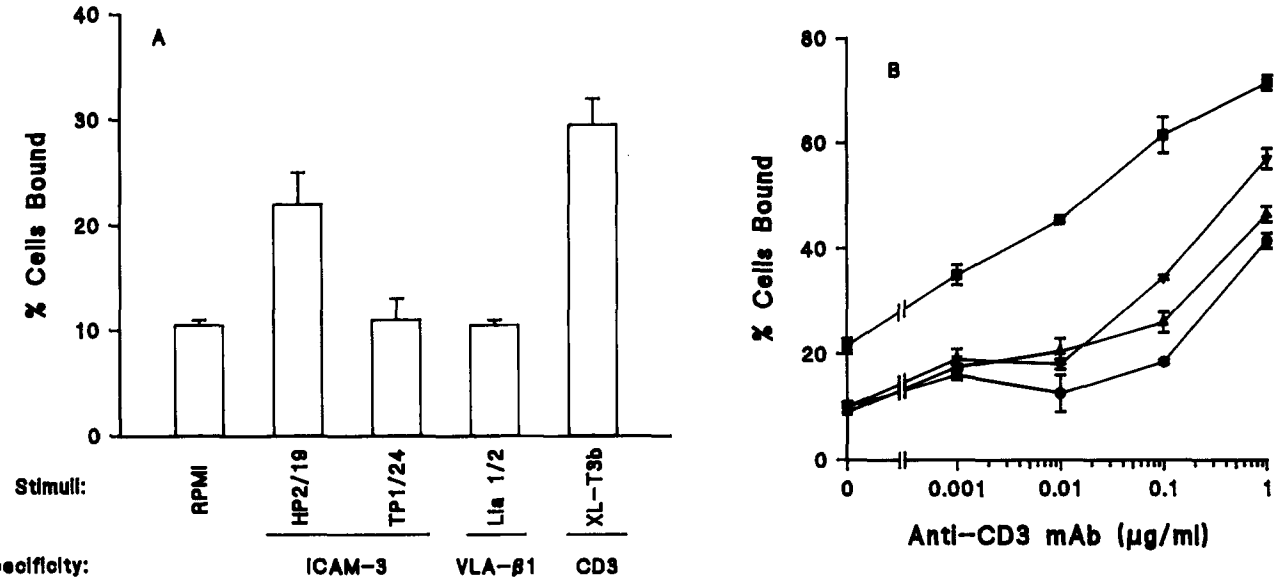

Figure 9. ICAM-3-mediated induction of cell adhesion to ICAM-1. (*A*) T lymphoblasts were pretreated at 4°C with the following stimuli: RPMI, 10 μg/ml of HP2/19, Lia 1/2, TP1/24, and anti-CD3 T3b. CD3 was cross-linked by using 10 μg/ml sheep anti-mouse IgG (*XL-CD3*). (*B*) T lymphoblasts were pretreated for 30 min at 4°C with a combination of either RPMI (●), HP2/19 (■), TP1/24 (▲), or Lia 1/2 (▼) mAbs with different doses of the anti-CD3 T3b mAb. Next, 10 μg/ml sheep anti-mouse IgG was added, as cross-linker, for 15 min at 4°C. Adhesion to ICAM-1-Fc-coated (20 μg/ml) microtiter wells was quantified after 20 min of incubation at 37°C. Specific adherence of stimulated T lymphoblasts was blocked by anti-LFA-1α TP1/40 mAb (data not shown) which reduced adherence below the level of untreated samples. Arithmetic mean and SD of duplicate wells are shown. Data are representative of six experiments.

with the nonaggregatory anti-ICAM-3 TP1/24 mAb (Fig. 9 *A*). As expected, cross-linked anti-CD3 mAb also enhanced cell adhesion to ICAM-1 (Fig. 9 *A*). Very interestingly, the anti-ICAM-3 HP2/19 but not the TP1/24 mAb was able to strongly enhance the LFA-1-mediated cell adhesion to ICAM-1-Fc when these stimuli were combined with very low doses of cross-linked anti-CD3 which were unable to trigger cell adhesion by themselves (Fig. 9 *B*). Moreover, this effect was abolished when cells were simultaneously incubated in the presence of the TP1/24 mAb (data not shown). These results suggest that ICAM-3-induced cell aggregation could be mediated by increasing the affinity of LFA-1 for ICAM-1.

Induction of Cell Activation through ICAM-3

To further investigate the mechanism involved in the ICAM-3-mediated regulation of cell binding to ICAM-1, we tested the possibility that cell activation could be achieved by mAb engagement of ICAM-3. As shown in Fig. 10, the anti-ICAM-3 HP2/19 mAb (epitope A) was able to greatly stimulate peripheral blood T cell proliferation when coimmobilized with suboptimal doses of the anti-CD3 T3b mAb. By contrast, the anti-HLA-A,B W6/32 mAb was not costimulatory. These data indicate that ICAM-3 is able to mediate peripheral blood T cell activation.

Discussion

In this report, we show that purified ICAM-3 can directly support LFA-1-dependent lymphocyte adhesion. The results suggest that ICAM-3 has a role in the initial phases of leukocyte cell-cell contacts and that ICAM-3 is involved in the regulation of LFA-1/ICAM-1-dependent leukocyte intercellular interactions.

A previous report suggested that ICAM-3 could be a ligand for LFA-1 supported by the fact that the anti-ICAM-3 CBR-IC3/1 mAb was able, in conjunction with both anti-ICAM-1 and anti-ICAM-2 mAb, to block cell attachment to purified LFA-1 (De Fougerolles and Springer, 1992). Recent studies have shown that transfected cells with cDNAs encoding the ICAM-3 molecule were able to support leukocyte attachment in a CD11a/CD18-dependent manner (De Fougerolles et al., 1993; Fawcett et al., 1992; Vazeux et al., 1992). We demonstrate here that T lymphoblasts are able to bind directly to purified ICAM-3 through the LFA-1 integrin. Moreover, we have shown that LFA-1-dependent cell binding to purified ICAM-3 can be regulated either by cell activation (with phorbol esters or by cross-linking CD3-TcR) or by direct LFA-1 stimulation with the activating anti-LFA-1 NKI-L16 mAb. Taking into account that anti-LFA-1 mAb completely blocked cell adhesion to ICAM-3, it appears that additional receptors, besides LFA-1, are not involved.

Epitope mapping on the ICAM-3 molecule revealed the existence of two distinct topographic epitopes, A and B, that showed distinct functional behavior. mAbs against epitope A, but not those against epitope B, induced a very rapid aggregation of T lymphoblasts that could be inhibited by anti-ICAM-3 (epitope B), anti-LFA-1, and surprisingly, by anti-ICAM-1 mAbs. Immunofluorescence studies supported the involvement of LFA-1 and ICAM-1 in ICAM-3-induced T cell aggregation. Both ICAM-1 and activated LFA-1, but not ICAM-3, were almost exclusively localized in cell-cell boundaries. Notably, two different anti-LFA-1 mAbs displayed a very different pattern of cellular staining: NKI-L16 mAb, recognizing an activation-related epitope on this integrin (Van Kooyk et al., 1991), was mainly located at intercellular contact sites, whereas the regular anti-LFA-1 TS1/11

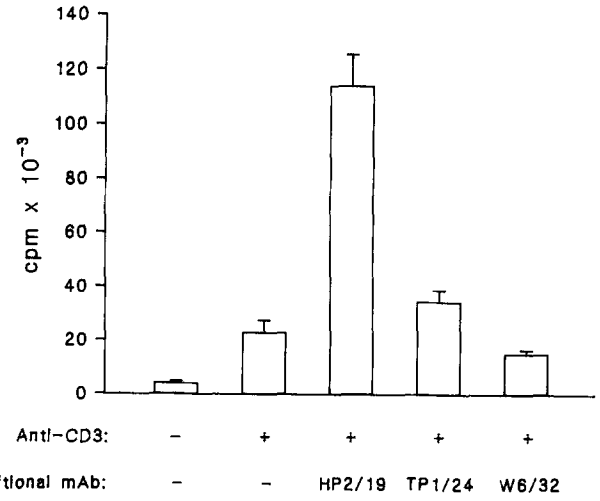

Figure 10. ICAM-3-mediated enhancement of peripheral blood T cell proliferation. Flat-bottomed plates coated with F(ab')$_2$ sheep anti-mouse IgG were incubated, as indicated under Materials and Methods, with 50 μl anti-CD3 T3b mAb at 50 ng/ml plus either medium alone or 50 μl at 2 μg/ml of each of the following mAb: anti-ICAM-3 HP2/19 and TP1/24, and anti-HLA-A,B W6/32. Monocyte-depleted peripheral blood mononuclear cells were cultured in these plates and proliferation was measured at day 3 by [^{3}H]dThd uptake. When flat-bottomed plates were coated in the absence of anti-CD3 mAb, [^{3}H]dThd incorporation was always less than 6,000 cpm. Arithmetic mean and SD of triplicate wells are shown. Data are representative of four experiments.

mAb, showed a diffuse pattern of staining. The presence of inductive and inhibitory sites for intercellular adhesion has been also described in members of the integrin superfamily of adhesion molecules such as LFA-1α (Keizer et al., 1988), VLA-α4 (Campanero et al., 1990), and VLA-β1 (Campanero et al., 1992).

Since the inhibitory anti-ICAM-3 mAb (epitope B) did not ablate mAb binding of proaggregatory (epitope A) mAb, nor blocked LFA-1 recognition of ICAM-3, we postulate that its inhibitory effect is due to interference with the mechanism of induction of cell aggregation by the anti-ICAM-3 HP2/19 mAb. In this regard, we have observed that the increased LFA-1-mediated cell binding to ICAM-1 observed upon cell treatment with the proaggregatory anti-ICAM-3 HP2/19 mAb was blocked by anti-ICAM-3 mAb specific for epitope B. These results point to the possibility that enhancement of LFA-1 affinity for ICAM-1 could be one of the mechanisms of ICAM-3-mediated induction of cell aggregation. Likely, additional mechanisms could be acting; for instance, LFA-1/ICAM-3 interaction promoted by treatment with the proaggregatory anti-ICAM-3 HP2/19 mAb could induce the switch of LFA-1 to its activated state at the intercellular contact sites. Subsequently, redistribution of these adhesion molecules on the cell surface membrane could facilitate LFA-1 interaction with ICAM-1.

Other functionally relevant cell surface molecules such as CD2, CD3, and CD11a have been shown to enhance both cell aggregation and LFA-1-mediated cell binding to ICAM-1 (Van Kooyk et al., 1989, 1991; Dustin and Springer, 1989). However, this is the first evidence indicating that a counter-receptor for LFA-1 is able to mediate induction of LFA-1-mediated cell aggregation, as well as to increase the avidity of this molecule for a second counter-receptor, ICAM-1. In this regard, integrin activation by ligand, has been recently described for the $\alpha_{IIb}\beta_3$ integrin (Du et al., 1991). The mechanism involved in ICAM-3-mediated regulation of LFA-1 avidity for ICAM-1 is presently unknown. However, we have obtained evidence suggesting that T cell activation could be involved in this process, since the proaggregatory anti-ICAM-3 HP2/19 mAb was also costimulatory for peripheral blood T cells.

We have shown that cell treatment with the anti-ICAM-3 HP2/19 mAb induced changes in cell morphology, as well as relocation of the ICAM-3 molecule on the cell surface. Cells moved from rounded to elongated morphology, and an evident structure similar to an uropod, where ICAM-3 molecules were accumulated, was induced with this proaggregatory mAb. Previous studies have shown that ICAM-1 can also preferentially localize to the uropod of both T and B cell lines (Dougherty et al., 1988; Dustin et al., 1992).

A striking difference in the distribution of the ICAM-3$^+$ uropod between small and large aggregates was seen. In small aggregates, cells appear to contact each other through the uropods, whereas in large aggregates, uropods appear to be excluded from areas of cell–cell contact. Interestingly, cell boundaries were specifically stained by the anti-ICAM-1 RR1/1 and the anti-LFA-1 NKI-L16 mAbs. The location of the uropod and, therefore, that of ICAM-3, would be compatible with its role as a mediator of the initial interactions between contacting leukocytes as well as in the recruitment of additional cells.

Our data concerning the ability of ICAM-3 to support LFA-1-mediated cell attachment and to mediate induction of cell aggregation, confer to ICAM-3 a pivotal role in the regulation of leukocyte intercellular interactions. It could be postulated that a physiologic role for ICAM-3 during antigen-triggered intercellular lymphocyte interactions is to act as the first counter-receptor initiating the interaction with the preactivated LFA-1 integrin. The affinity of LFA-1 for ICAM-3 could be lower than for ICAM-1, but, due to its much higher expression (De Fougerolles and Springer, 1992), ICAM-3 would be the initial counter-receptor for LFA-1. Accordingly, it has been shown that adhesion of resting T lymphocytes to LFA-1 occurs primarily via ICAM-3 (De Fougerolles and Springer, 1992). In a second step, the initial interaction of ICAM-3 with LFA-1 on the opposite cell could increase the degree of activation of LFA-1 on both cells, thus facilitating the LFA-1-mediated cell binding to ICAM-1, and the establishment of a more stable cell–cell interaction. In this regard, the existence of intermediate degrees of activation on the LFA-1 integrin has been previously reported (Van Kooyk et al., 1991). Later, ICAM-3 molecules would be displaced from sites of intercellular contact, thus becoming able to reinitiate additional interactions with other LFA-1 molecules on newly recruited cells. This putative role of ICAM-3 could be crucial during the initiation phase of important immune processes such as allogeneic and autologous mixed lymphocyte reactions and lysis by T cells of certain target cells, which involve other LFA-1 ligands distinct from ICAM-1 (Bagnasco et al., 1990; Makgoba et al., 1988).

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