

ICAM-3 Regulates Lymphocyte Morphology and Integrin-mediated T Cell Interaction with Endothelial Cell and Extracellular Matrix Ligands

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Abstract. Leukocyte activation is a complex process that involves multiple cross-regulated cell adhesion events. In this report, we investigated the role of intercellular adhesion molecule-3 (ICAM-3), the third identified ligand for the $\beta 2$ integrin leukocyte function-associated antigen-1 (LFA-1), in the regulation of leukocyte adhesion to ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and the 38- and 80-kD fragments of fibronectin (FN40 and FN80). The activating anti-ICAM-3 HP2/19, but not other anti-ICAM-3 mAb, was able to enhance T lymphoblast adhesion to these proteins when combined with very low doses of anti-CD3 mAb, which were unable by themselves to induce this phenomenon. In contrast, anti-ICAM-1 mAb did not enhance T cell attachment to these substrata. T cell adhesion to ICAM-1, VCAM-1, FN40, and FN80 was specifically blocked by anti-LFA-1, anti-VLA $\alpha 4$, and anti-VLA $\alpha 5$ mAb, respectively. The activating anti-ICAM-3 HP2/19 was also able to specifically enhance the VLA-4- and VLA-5-mediated binding of leukemic T Jurkat cells to VCAM-1, FN40, and FN80, even in the absence of cooccupancy of the CD3-TcR complex. We also studied the localization of ICAM-3, LFA-1, and the VLA $\beta 1$ integrin, by immunofluorescence microscopy, on cells interacting with ICAM-1, VCAM-1 and FN80. We found that the anti-ICAM-3 HP2/19 mAb specifically promoted a dramatic change on the morphology of T lymphoblasts when these cells were

allowed to interact with those adhesion ligands. Under these conditions, it was observed that a large cell contact area from which an uropod-like structure (heading uropod) was projected toward the outer milieu. However, when T blasts were stimulated with other adhesion promoting agents as the activating anti-VLA $\beta 1$ TS2/16 mAb or phorbol esters, this structure was not detected. The anti-ICAM-3 TP1/24 mAb was also unable to induce this phenomenon. Notably, a striking cell redistribution of ICAM-3 was induced specifically by the HP2/19 mAb, but not by the other anti-ICAM-3 mAb or the other adhesion promoting agents. Thus, ICAM-3 was almost exclusively concentrated in the most distal portion of the heading uropod whereas either LFA-1 or the VLA $\beta 1$ integrin were uniformly distributed all over the large contact area. Moreover, this phenomenon was also observed when T cells were specifically stimulated with the HP2/19 mAb to interact with TNF α -activated endothelial cells. We found the localization of linear arrays of myosin within the heading uropod. In contrast, actin-based cytoskeleton presented a uniform distribution over the broad contact area with the substrate. In addition, butanedione monoxime, a myosin-disrupting drug, abolished both the morphological cell change and ICAM-3 clustering. Altogether, these results demonstrate that ICAM-3 has a regulatory role on multiple pathways of T cell adhesion and morphology.

THE interactions of cells with either other cells or with the extracellular matrix, have been involved in the regulation of multiple physiologic processes. On leukocytes, the members of the Integrin, Immunoglobulin, and Selectin families of cell adhesion receptors appear to be the

major molecules involved in those interactions (Springer, 1990; Hynes, 1992). Interestingly, the adhesion capability of Integrins can be regulated. Most of the members of the integrin family which are expressed by leukocytes require an activation step to acquire the ability to interact with their ligands. This activation process can be accomplished by cellular activation, as described for the members of the $\beta 1$, $\beta 2$, and $\beta 3$ integrins (Dustin and Springer, 1989; Shimizu et al., 1990; Springer, 1990; Postigo et al., 1991), or induced by the ligands themselves, as it occurs with the $\beta 3$ integrin $\alpha_{\text{IIb}}\beta 3$ (Du et al., 1991).

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T and B lymphocytes and monocytes interact through the very late activation antigen (VLA)-4¹ ($\alpha 4\beta 1$) and VLA-5 ($\alpha 5\beta 1$) integrins with several distinct sequences of human plasma fibronectin. Thus, VLA-5 recognizes the Arg-Gly-Asp sequence, while VLA-4 recognizes the CS1, CS5, and HI peptides of fibronectin (FN) (Wayner et al., 1989; García-Pardo et al., 1990; Mould and Humphries, 1991; Mould et al., 1991). Several proteolytic fragments can be obtained by trypsin digestion of FN. Among them, the 38-kD (FN40) and 80-kD (FN80) fragments are recognized by the VLA-4 and VLA-5 integrins, respectively (Wayner et al., 1989; García-Pardo et al., 1990; Yamada, 1991). In addition, VLA-4 interacts with a cell surface ligand termed vascular cell adhesion molecule (VCAM-1) (Elices et al., 1990), which belongs to the Immunoglobulin gene superfamily and has been shown to be expressed by cytokine-stimulated endothelial cells (Osborn et al., 1989). VLA-4 and VLA-5 interaction with fibronectin as well as VLA-4/VCAM-1 interaction appear to be essential for several physiologic processes such as B lymphocyte maturation (Roldán et al., 1992), lymphopoiesis (Williams et al., 1991), myogenesis (Rosen et al., 1992), leukocyte extravasation to the sites of inflammation (Laffón et al., 1991; Postigo et al., 1992; Yednock et al., 1992), and T lymphocyte activation (Matsuyama et al., 1989; Nojima et al., 1990; Burkly et al., 1991).

Three members of the Immunoglobulin superfamily, the intercellular adhesion molecule-1, -2, and -3 (ICAM-1, ICAM-2, and ICAM-3) have been described as counter-receptors for the lymphocyte function-associated antigen-1 (LFA-1) integrin (Springer, 1990; Fawcett et al., 1992; Vazeux et al., 1992; Campanero et al., 1993; De Fougerolles et al., 1993). In contrast to ICAM-1 and ICAM-2, ICAM-3 is exclusively expressed on cells of leukocyte lineage (De Fougerolles and Springer, 1992; Acevedo et al., 1993). Moreover, ICAM-3 is the most abundantly expressed LFA-1 counter-receptor on leukocytes (De Fougerolles et al., 1993).

We have recently shown that ICAM-3 participates in T cell activation and regulates the LFA-1/ICAM-1 adhesion pathway in these cells (Campanero et al., 1993; Hernández-Caselles et al., 1993). We have herein studied the functional role of ICAM-3 on T cell adhesion. We have found that this molecule is also involved in the regulation of T cell binding to ICAM-1, VCAM-1, and fibronectin, as well as in the control of cellular morphology. The relevance of these findings is discussed.

Materials and Methods

Antibodies, Protein Substrata, and Reagents

The anti-ICAM-3 HP2/19 and TP1/24, anti-CD11a TS1/11 and TP1/40, anti-ICAM-1 RRI/1 and R6.5D6, anti-CD29 TS2/16, anti-CD49d HP2/1, anti-CD49e SAM-1, and anti-CD3 SPV-T3b mAb have been described (Sánchez-Madrid et al., 1983, 1986; Hemler et al., 1984; Spits et al., 1985; Rothlein et al., 1986; Smith et al., 1988; Te Velde et al., 1988; Campanero et al., 1993). SAM-1 and RRI/1 and R6.5D6 mAbs were a generous gift of Dr. C. Figdor (Netherlands Cancer Institute, Amsterdam, The

Netherlands) and R. Rothlein (Boehringer Ingelheim, Ridgefield, CT), respectively. MAb were purified from ascites fluid with protein A coupled to Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). Anti-talin and anti- α -actinin rabbit sera were a generous gift of Dr. K. Burridge (University of North Carolina, Chapel Hill, NC). Anti-myosin rabbit polyclonal serum was purchased from Sigma Chem. Co. (St. Louis, MO). Recombinant chimeric ICAM-1-Fc and recombinant soluble (rs) VCAM-1 and endothelial leukocyte adhesion molecule-1 (rsELAM-1) were kindly provided by Dr. A. Craig (John Radcliffe Hospital, Oxford, Great Britain) and R. Lobb (Biogen, Cambridge, MA), respectively. The tryptic 38- and 80-kD fibronectin fragments were kindly provided by Dr. A. García-Pardo (Centro de Investigaciones Biológicas, Madrid, Spain).

FITC-conjugated phalloidin, cytochalasin D, colchicine, and butanedione monoxime were purchased from Sigma Chem. Co.

Cells and Cell Lines

Human T lymphoblasts were prepared from peripheral blood mononuclear cells by treatment with phytohemagglutinin (PHA, 0.5%; Pharmacia Fine Chemicals) for 48 h. Cells were washed and cultured in RPMI 1640 (Flow Lab., Irvine, Scotland) containing 10% FCS (Flow Lab.) and 20 U/ml IL-2. T lymphoblasts cultured by 7–12 d were typically used in all experiments. T lymphoblasts and T cell clones have been extensively used to study both LFA-1-mediated cell adhesion and T cell activation (Cantrell et al., 1984; van Kooyk et al., 1991; Dransfield et al., 1992; Campanero et al., 1993). The Jurkat T cell line was maintained in RPMI 1640 plus 10% FCS.

Human umbilical vein endothelial cells (HUVEC) were obtained as described (Dejana et al., 1987). Briefly, umbilical vein was cannulated, washed, and incubated with 0.1% collagenase P (Boehringer Mannheim GmbH, Mannheim, Germany) for 20 min at 37°C. Cells were seeded into flasks and cultured in M199 medium (Flow Lab.) supplemented with 20% FCS, 50 μ g/ml endothelial cell growth supplement, and 100 μ g/ml heparin (Sigma Chem. Co.). Cells within two passages were used. Cells were split 1:3 and detached with a solution of 0.05% Trypsin and 0.02% EDTA (Flow Lab.) before using.

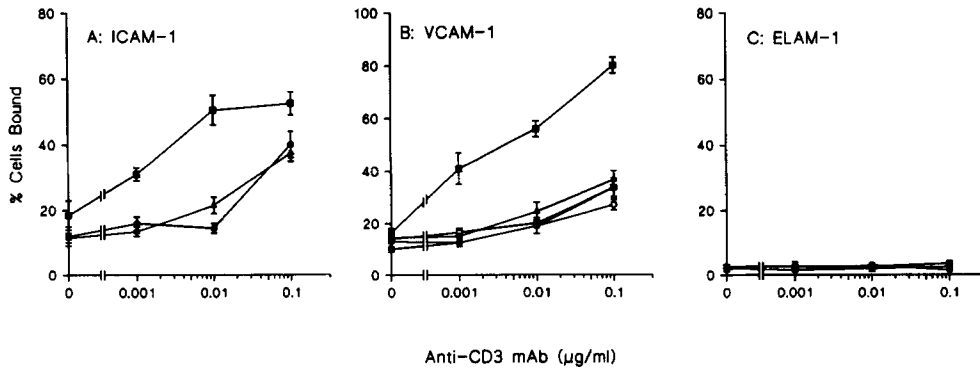
Cell Adhesion Assays

Adhesion assays were essentially performed as previously described (Arroyo et al., 1992). Briefly, 50 μ l/well of 20 mM Tris-HCl, pH 8.0, containing FN fragments, recombinant chimeric ICAM-1-Fc, rsVCAM-1, or rsELAM-1, were used to coat 96-well microtiter EIA II-Linbro plates (Costar, Cambridge, MA) for 2 h at 37°C and then saturated with PBS containing 1% HSA for 30 min at 37°C. Thereafter, plates were washed with PBS and either 2–3 $\times 10^5$ (T lymphoblasts) or 1 $\times 10^5$ (Jurkat cells) cells/well in 100 μ l were added and centrifuged for 5 min at 10 g before an incubation at 37°C for 10–20 min. To quantify cell attachment, the plate was washed with RPMI containing 0.5% HSA, 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 reader (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.

Immunofluorescence

Immunofluorescence experiments were performed as previously described (Sánchez-Mateos et al., 1993). Briefly, 2 $\times 10^6$ T lymphoblasts were incubated in flat-bottomed, 24-well plates (Costar Corp., Cambridge, MA) in a final volume of 500 μ l of complete medium on coverslips coated with 40 μ g/ml ICAM-1-Fc, 20 μ g/ml FN80, or 10 μ g/ml VCAM-1-FN-80. For some experiments, HUVEC were grown on gelatin-coated coverslips and stimulated with 10 ng/ml TNF α for 16 h at 37°C; then, T lymphoblasts were incubated on these coverslips. MAb or their F(ab)₂ fragments were added at a final concentration of 1 μ g/ml and cells were allowed to settle in a cell incubator at 37°C and 5% CO₂ atmosphere; after 30 min 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% NaN₃). To directly visualize the stimulatory mAb, cells were stained with a 1:50 dilution of a FITC-labeled rabbit F(ab)₂ anti-mouse IgG (Pierce, Rockford, IL) as a secondary antibody. For double label studies, after directly staining the stimulatory mAb with FITC-secondary antibody, cells were saturated with 10% nonspecific mouse serum in TBS. Then, cells were incubated with 5 μ g/ml biotinylated mAb to other proteins, followed by washing and

1. *Abbreviations used in this paper:* FN, fibronectin; HUVEC, human umbilical vein endothelial cells; ICAM, intercellular adhesion molecule; LFA, lymphocyte function-associated antigen; VCAM, vascular cell adhesion molecule; VLA, very late activation antigen.



Adhesion to microtiter wells coated with 20 $\mu\text{g/ml}$ ICAM-1-Fc (A), 5 $\mu\text{g/ml}$ rVCAM-1 (B), or 20 $\mu\text{g/ml}$ rELAM-1 (C) was quantified after 10 min of incubation at 37°C. Arithmetic mean \pm SD of five experiments run by duplicate are shown. Adhesion to albumin-coated wells was always lower than 5%.

labeling with TRITC-avidin D (Vector Labs., Inc., Burlingame, CA) for 30 min, washed with TBS and incubated with a biotinylated anti-avidin (Vector Labs., Inc.) for 30 min. Finally, a fourth incubation with TRITC-avidin D for 30 min was done. For staining of cytoskeletal proteins, cells were permeabilized with 0.2% Triton X-100 in TBS for 3 min. The secondary antibodies used were: RITC-goat anti-mouse IgG, affinity-purified and cross-species adsorbed, and FITC-donkey anti-rabbit IgG (Chemicon Intl. Inc., Terucula, CA). Polymerized actin was stained with 6.6 μM FITC-phalloidin. The cells were observed using a Nikon Labophot-2 photomicroscope with a 100 \times oil immersion objective and photographed on either Ektachrome 400 (color pictures) or TMAX 400 (black and white) film (Kodak). The latter was processed to 800–1600 ASA with TMAX developer (Kodak). Where indicated, red and green fluorescence was photographed on the same frame and, in some cases, we were compelled to move the focus in order to show both colors in focus.

Results

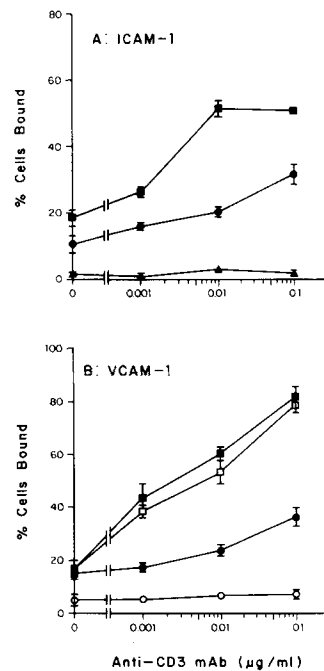
Regulation of $\beta 1$ and $\beta 2$ Integrin-mediated Cellular Interactions through ICAM-3

We have recently shown that LFA-1/ICAM-1 interaction can be regulated through ICAM-3 (Campanero et al., 1993). We have now tested the possibility that ICAM-3 could also regulate the T cell binding to other endothelial cell adhesion molecules as VCAM-1 and ELAM-1. As shown in Fig. 1, the anti-ICAM-3 HP2/19 mAb was able to enhance T lymphoblast attachment to both ICAM-1 and VCAM-1, but not to ELAM-1; this effect was observed only when the anti-ICAM-3 mAb was combined with very low doses of anti-CD3 mAb, which were unable by themselves to induce this effect. When we used an anti-ICAM-3 mAb of identical Ig subclass and comparable affinity but directed against another different epitope (TPI/24), or anti-ICAM-1 mAbs, no effect was detected (Fig. 1). ICAM-3-mediated increased cell binding to ICAM-1 and VCAM-1 was specifically inhibited by mAbs to the LFA-1 and VLA-4 integrins, respectively (Fig. 2). Therefore, these results show that ICAM-3 is capable to regulate the interaction of LFA-1 and VLA-4 with their respective endothelial cell counter-receptors.

Since it has been described that VLA-4 interacts with the 38-kD fibronectin fragment containing CS-1 (Wayner et al., 1989), we tested the possibility that ICAM-3 could also regulate this interaction. As shown in Fig. 3, the anti-ICAM-3 HP2/19 mAb enhanced the VLA-4-mediated T lymphoblast adhesion to this fragment. Moreover, we have obtained evidence suggesting that ICAM-3 is also capable to regulate

the VLA-5-mediated cell adhesion to its ligand. Thus, the HP2/19 mAb was able to enhance T lymphoblast adhesion to the 80-kD FN fragment containing the Arg-Gly-Asp peptide, and this event was specifically blocked by the anti-VLA $\alpha 5$ SAM-1 mAb (Fig. 3). However, the anti-ICAM-3 TPI/24, as well as the anti-ICAM-1 RRI/1 and R6.5D6 mAb failed to enhance cell adhesion to both FN fragments (Fig. 3). In addition, cell binding to fibrinogen was not enhanced by the anti-ICAM-3 HP2/19 mAb (data not shown).

When the Jurkat T cell line was stimulated with the anti-ICAM-3 HP2/19 mAb in the absence of anti-CD3 mAb, a dramatic increase in cell adhesion to either VCAM-1, FN40, or FN80, but not to ELAM-1 or fibrinogen, was observed (Fig. 4, and data not shown). Moreover, these effects



the ICAM-3-induced T cell binding to ICAM-1 and VCAM-1 is mediated by $\beta 1$ and $\beta 2$ integrins. T lymphoblasts were pretreated for 30 min at 4°C with a combination of different doses of the anti-CD3 T3b mAb with either RPMI (●) or HP2/19 mAb (■). Next, 10 $\mu\text{g/ml}$ sheep anti-mouse IgG was added, as cross-linker, for 10 min at 4°C. Cells activated with the ICAM-3 antibody were subjected to integrin blockade. To this end, after cell treatment with the cross-linker agent, cells were incubated for additional 15 min at 4°C with the inhibitors mAb: the anti-LFA-1 TPI/40 mAb (▲), the anti-VLA $\alpha 4$ HP2/1 (○), and the anti-VLA $\alpha 5$ SAM-1 mAb (□) were used. Adhesion to microtiter wells coated with either 20 $\mu\text{g/ml}$ ICAM-1-Fc (A) or 5 $\mu\text{g/ml}$ rVCAM-1 (B) was quantified after 10 min of incubation at 37°C. Arithmetic mean \pm SD of four separate experiments run by duplicate are shown. Adhesion to albumin-coated wells was always lower than 5%.

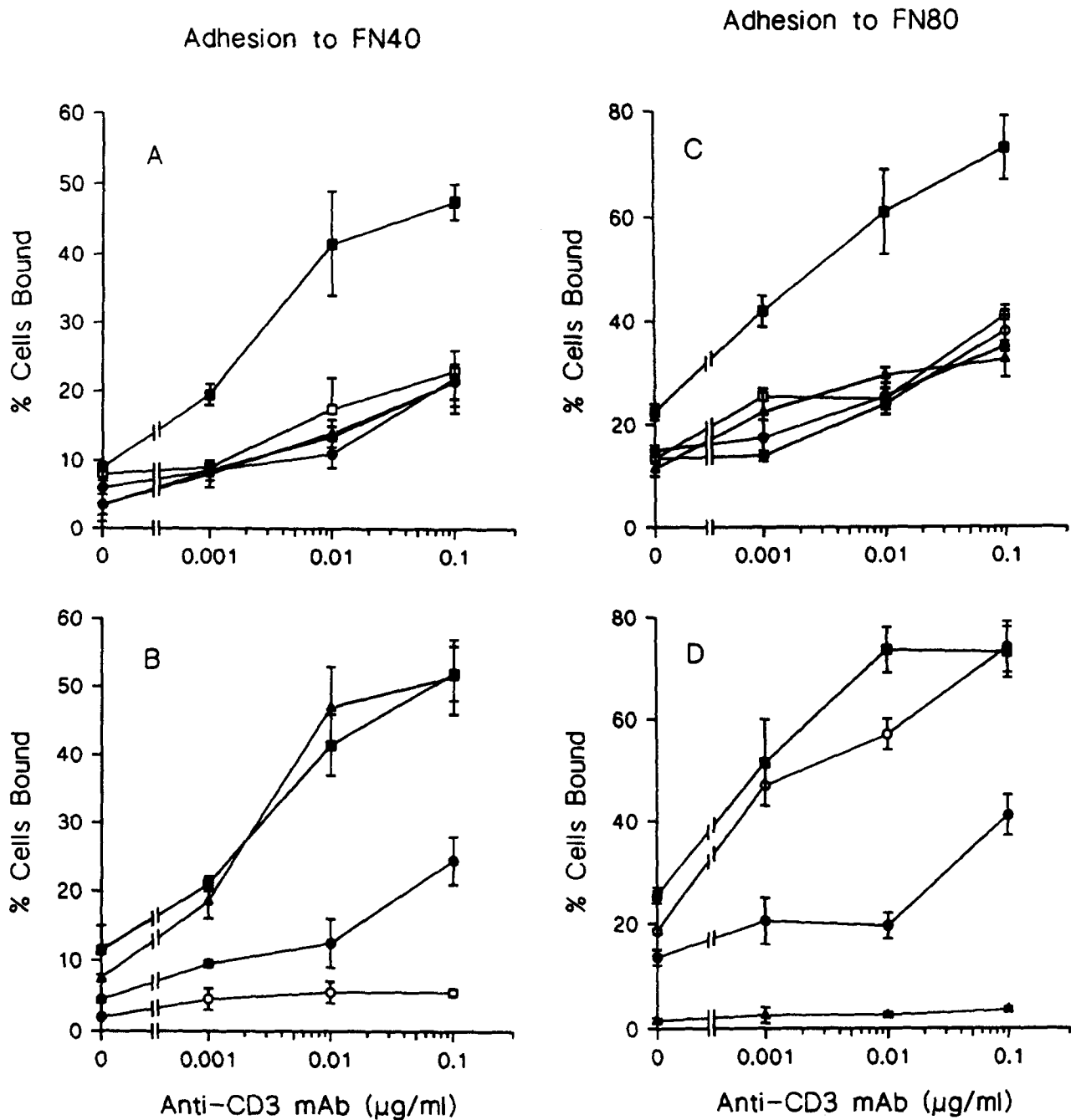


Figure 3. Induction of T cell adhesion to fibronectin fragments by ICAM-3. T lymphoblasts were pretreated for 30 min at 4°C with a combination of different doses of the anti-CD3 T3b mAb with either RPMI (●), HP2/19 (■), TP1/24 (▲), RR1/1 (○), or R6.5D6 (□) mAbs (A and C). Next, 10 µg/ml sheep anti-mouse IgG was added, as cross-linker, for 10 min at 4°C. Cells activated with the anti-ICAM-3 antibody HP2/19 were subjected to integrin blockade (B and D). To this end, after cell treatment with the cross-linker agent, cells were incubated for additional 15 min at 4°C with either RPMI (■), HP2/1 (○), or SAM-1 (▲) mAbs. In B and D cell treatment with no additional stimulus to anti-CD3 mAb is also shown (●). Adhesion to microtiter wells coated with either 5 µg/ml FN40 (A and B) or FN80 (C and D) was quantified after 10 min of incubation at 37°C. Arithmetic mean ±SD of four separate experiments run by duplicate are shown. Adhesion to albumin- or fibrinogen-coated wells was always lower than 5%.

were specifically blocked by either anti-VLA α 4 HP2/1 (VCAM-1 and FN40) or the anti-VLA α 5 SAM-1 (FN80) mAb (Fig. 4). These results strongly suggest that ICAM-3 is able to transduce stimulatory signals in T cells even in the absence of CD3-TcR occupancy.

ICAM-3 Redistribution During T Cell Attachment to Different Adhesion Ligands

We have performed double immunofluorescence analysis to investigate the distribution of ICAM-3 when cells were in-

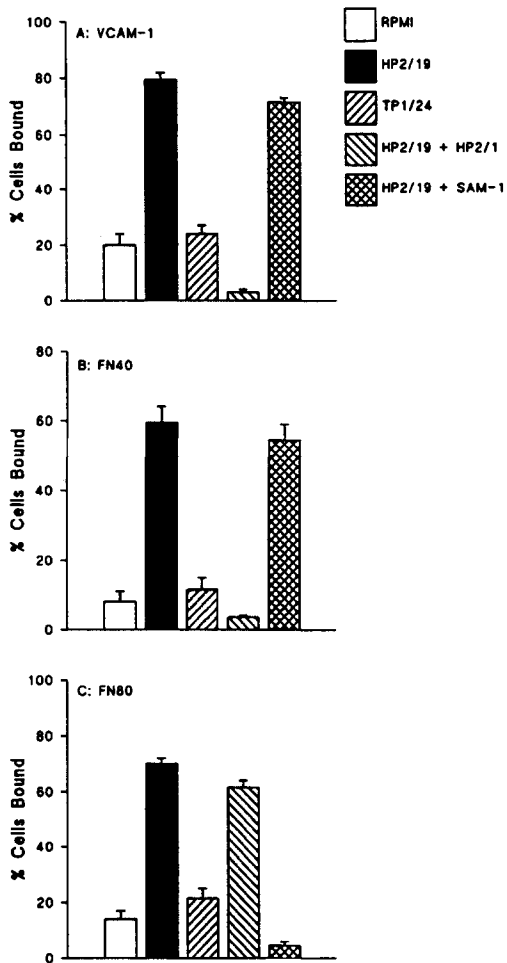


Figure 4. ICAM-3-mediated induction of Jurkat cell adhesion to VCAM-1 and fibronectin fragments in the absence of CD3-TcR stimulation. Jurkat cells were pretreated for 30 min at 4°C with different mAbs. Adhesion to microtiter wells coated with 5 µg/ml of either rVCAM-1, FN40, or FN80 was quantified after 7 min of incubation at 37°C. Arithmetic mean ±SD of five separate experiments run by duplicate are shown. Adhesion to albumin- or fibrinogen-coated wells was always lower than 5%.

duced to interact with immobilized ligands for $\beta 1$ and $\beta 2$ integrins. Very interestingly, when T lymphoblasts were stimulated with the anti-ICAM-3 HP2/19 mAb and allowed to interact with ICAM-1-coated coverslips, a dramatic change on T cell morphology was observed: most of the mAb-treated cells formed a large flattened contact area from which an uropod-like structure (hereafter designated as heading uropod), where ICAM-3 was located, was projected towards the medium (Fig. 5 A). The ICAM-3 redistribution to the uropod was even more pronounced when activating conditions of coengagement of ICAM-3 and CD3-TcR were used (data not shown). However, this structure was not detected on mAb-untreated cells, and ICAM-3 was found uniformly distributed on the cell surface (Fig. 5 J). Staining of the LFA-1 integrin on both conditions showed a uniform cell distribution on the large contact area with the ICAM-1-coated surface (Fig. 5, B and K). A completely rounded morphology was observed when T blasts stimulated with the

HP2/19 mAb were settled on BSA instead of ICAM-1 (data not shown).

Double exposure for ICAM-3 and LFA-1 fluorescence on the same cells was taken in the same photographs in order to compare the distribution of LFA-1 and ICAM-3 on cells interacting with ICAM-1. Fig. 5 C clearly shows that LFA-1 (red fluorescence) and ICAM-3 (green fluorescence) localized to different areas on the same cells upon T lymphoblast stimulation with the anti-ICAM-3 HP2/19 mAb: LFA-1 uniformly distributed all over the large flattened contact area whereas ICAM-3 selectively decorated the heading uropod. In contrast, on unstimulated cells, LFA-1 and ICAM-3 displayed a completely overlapping pattern of staining (orange color) (Fig. 5 L). Next, the distribution of ICAM-3 and the $\beta 1$ integrin was analyzed when T blasts were allowed to settle on either VCAM-1 or FN80. The appearance of the heading uropod and ICAM-3 redistribution to this projection was observed in HP2/19 mAb-treated cells (Fig. 5, D and G), but not on unstimulated T blasts (data not shown). In contrast, the $\beta 1$ integrin was uniformly distributed over the large contact area with either the VCAM-1- or the FN80-coated surfaces on both treated and mAb untreated T blasts (Fig. 5, E and H, and data not shown). Double exposure for ICAM-3 and $\beta 1$ integrin fluorescence on the same cells clearly demonstrated that both molecules localized to different areas when these cells were interacting with VCAM-1 or FN80 (Fig. 5, F and I).

Other stimuli, as phorbol esters or the regulatory anti-VLA $\beta 1$ TS2/16 mAb, have previously been described to enhance integrin-mediated cell adhesion to ICAM-1, VCAM-1, and FN (Dustin and Springer, 1989; Arroyo et al., 1992). However, the appearance of the heading uropod was specifically induced by the anti-ICAM-3 HP2/19. When T blasts were stimulated to interact with FN80 with the anti-VLA $\beta 1$ TS2/16 mAb or PMA, the heading uropod and the ICAM-3 redistribution were not observed (Fig. 6, C and D). Moreover, ICAM-3-mediated change of morphology is epitope-specific since the other anti-ICAM-3 TP1/24 mAb, which is directed against a different epitope, failed to enhance T cell adhesion, and it had no effect on these events (Fig. 6 B). Moreover, the latter mAb also failed to induce cell clustering of ICAM-3 (Fig. 6 B). Virtually all ICAM-3 molecules appeared to be sequestered in the uropod, as demonstrated with cells treated with the activating HP2/19 mAb, and then stained with the TP1/24 mAb (data not shown).

ICAM-3 Redistribution During T Cell Interaction with Endothelial Cells

To explore the possible in vivo relevance of ICAM-3-mediated induction of the heading uropod, we tested the ability of ICAM-3 to stimulate the appearance of this structure when T cells interact with endothelial cells. Human umbilical vein endothelial cells stimulated for 16 h with TNF α express both ICAM-1 and VCAM-1. When T lymphoblasts were stimulated to interact with these endothelial cells with the anti-ICAM-3 HP2/19 mAb, the heading uropod and the ICAM-3 redistribution were clearly observed (Fig. 7 A). In contrast, the anti-ICAM-3 TP1/24 mAb was unable to induce the appearance of the heading uropod or ICAM-3 clustering (Fig. 7 B). Moreover, phorbol esters also failed to induce both phenomena (Fig. 7 C).

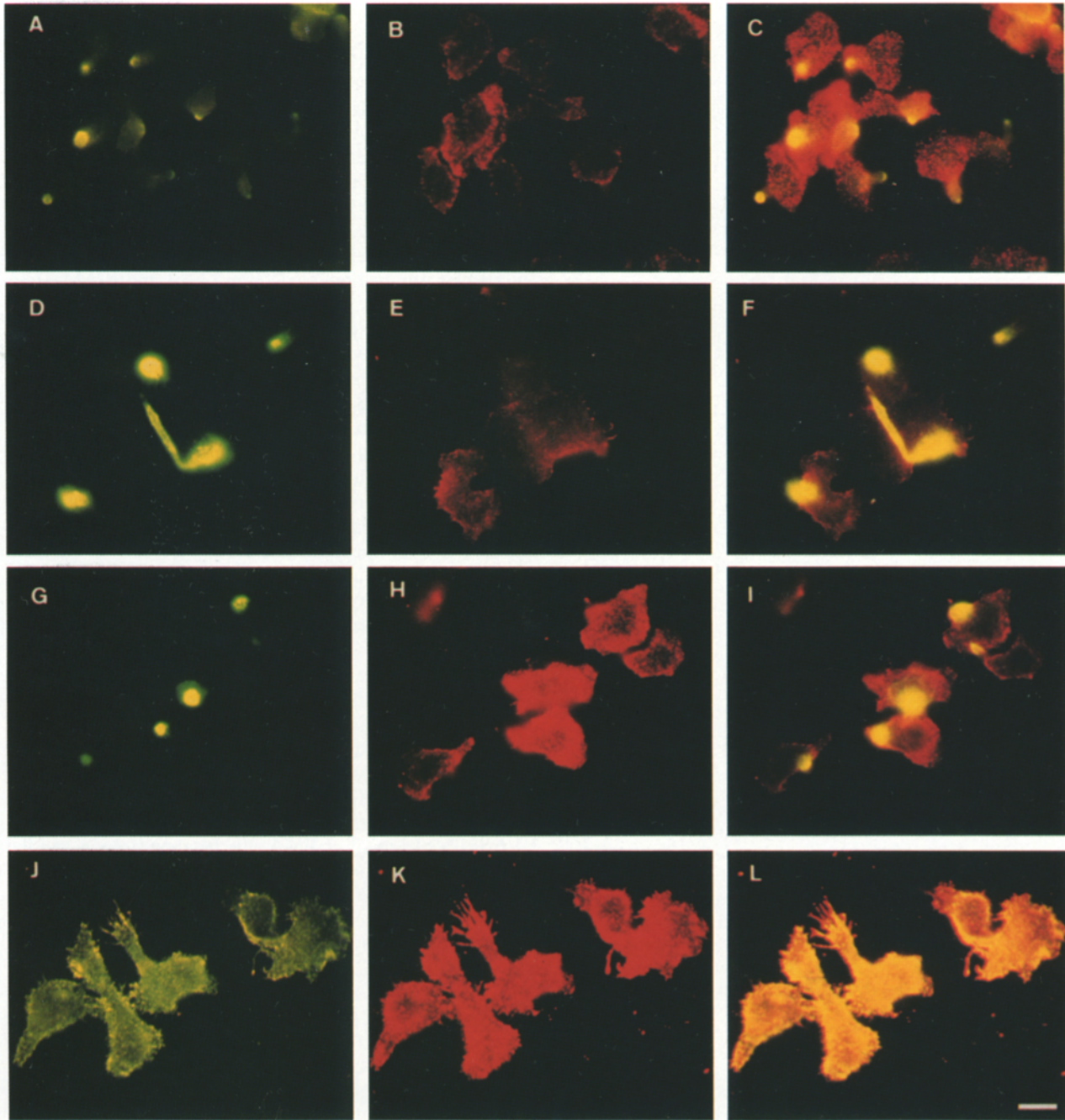


Figure 5. Cellular localization of ICAM-3 and LFA-1 during T lymphoblast attachment to ICAM-1, VCAM-1, and FN80. T lymphoblasts were allowed to bind to coverslips coated with either 40/ $\mu\text{g}/\text{ml}$ ICAM-1-Fc (A-C and J-L), 10 $\mu\text{g}/\text{ml}$ rsVCAM-1 (D-F), or 20 $\mu\text{g}/\text{ml}$ FN80 (G-I) for 30 min at 37°C either in the presence (A-I) or absence (J-L) of the anti-ICAM-3 HP2/19 mAb. Then, fixed cells were double stained for ICAM-3 (A, D, G, and J) and LFA-1 (B and K) or VLA β 1 (E and H) as described in Materials and Methods. In C, F, I, and L, red and green fluorescence was photographed on the same frame by double exposure. The heading uropod and the cell contact area with the substratum were indeed in a distinct plane of focus. Bar, 10 μm .

Cytoskeletal Reorganization during ICAM-3 Regulated T Cell Morphological Changes

To investigate the nature of the cytoplasmic interactions that drive ICAM-3 into the uropod region, the localization of f-actin and various cytoskeletal proteins was observed by fluorescence microscopy on ICAM-3-induced T cells adhering to FN80 (Fig. 8). F-actin was present through the

cytoplasm of the cells, but brighter fluorescence was detected in the area of contact with the substrate (Fig. 8 A). Three other cytoskeletal proteins, talin, α -actinin (Fig. 8, B and C), and vinculin (not shown) demonstrated no preferential distribution to the cell uropod, where ICAM-3 was specifically concentrated (Fig. 8, E, F, and G), and similarly to f-actin, they displayed more pronounced fluorescence at the trailing membrane.

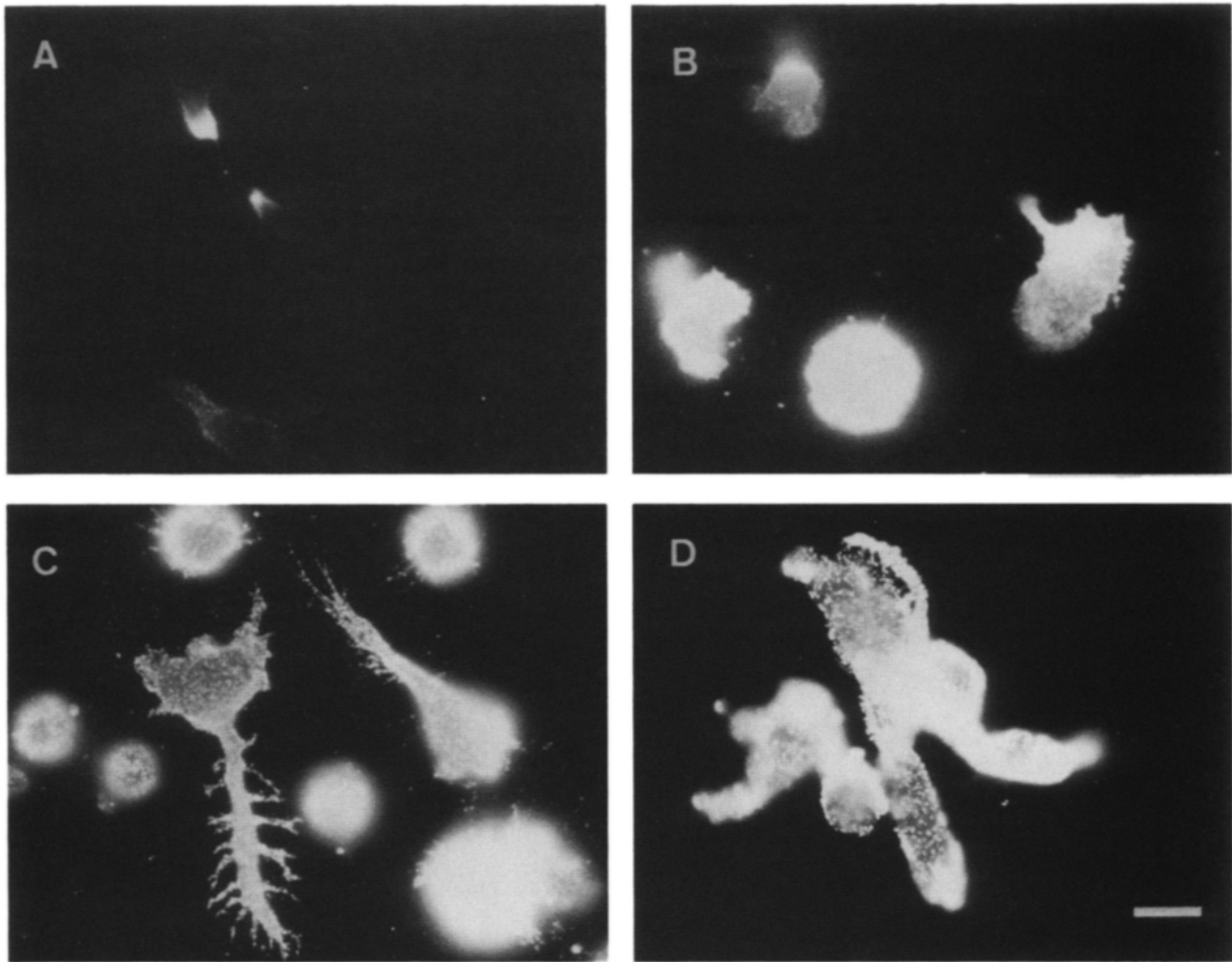


Figure 6. ICAM-3 but not other pro-adhesive stimuli specifically induces ICAM-3 relocation and the raising of the heading uropod. T lymphoblasts were allowed to bind to FN80-coated coverslips (20 $\mu\text{g/ml}$) for 30 min at 37°C in the presence of 5 $\mu\text{g/ml}$ HP2/19 mAb (A), TPI/24 mAb (B), TS2/16 mAb (C), or 20 ng/ml PMA (D). Then fixed cells were stained for ICAM-3, as described in Materials and Methods, by using biotinylated anti-ICAM-3 TPI/24 mAb. Bar, 10 μm .

Actin polymerization contributes to the protrusion of cell margins such as lamellipodia, but recent reports have described forms of cell protrusion that may instead be driven by myosin (Conrad et al., 1993; Cramer et al., 1994). Fig. 8 D shows the asymmetric distribution of myosin during cell adhesion to FN80 induced with the activating anti-ICAM-3 HP2/19 mAb. Cell uropods contained linear arrays of myosin, while a punctate distribution was observed uniformly through the rest of the cytoplasm.

To further investigate the involvement of the myosin motor in the generation of the cell uropod and ICAM-3 reorganization, we used the myosin-disrupting drug butanedione monoxime (Cramer et al., 1994). All cells treated with butanedione monoxime acquired a spherical morphology with a uniform distribution of ICAM-3 (Fig. 9 B). However, cell attachment to FN80 was not affected by this treatment (Fig. 9 B, and data not shown). In contrast, disruption of microtubules with colchicine did not affect the polarization of ICAM-3 (Fig. 9 C). As expected, treatment of the cells with a microfilament disrupting element, cytochalasin D,

resulted in a complete inhibition of cell adhesion (not shown).

Discussion

In this study, we have shown that ICAM-3 is a regulatory molecule that modulates $\beta 1$ and $\beta 2$ integrin-mediated T cell binding to endothelial cell surface molecules, as ICAM-1 and VCAM-1, and to ECM components as fibronectin. In addition, ICAM-3 distribution on the cell surface and T lymphoblast morphology were dramatically modified upon cell stimulation through ICAM-3.

It has been considered that integrins have an active functional role in cell activation, whereas their ligands do not. Thus, integrin ligands apparently only support cell attachment, whereas integrins display a more dynamic role. As a consequence of the interaction with their ligands, integrins transduce signals to the inside of cells, leading to changes in the cellular behavior. For instance, costimulatory signals are generated in T cells after LFA-1- and VLA-4-mediated

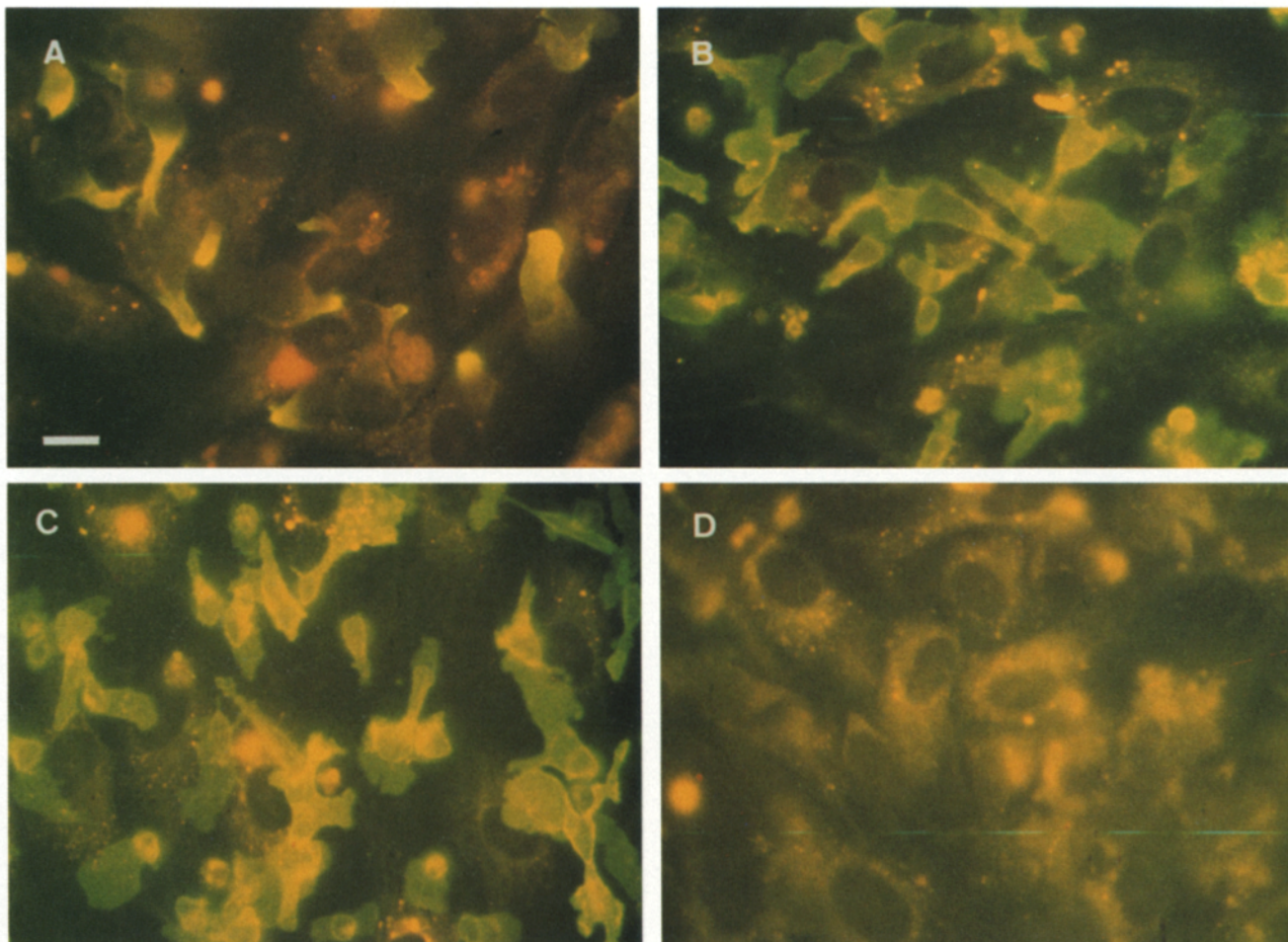


Figure 7. Regulation of ICAM-3 relocation and raising of the heading uropod on T cells that interact with endothelial cells. Human umbilical vein endothelial cells were stimulated with 10 ng/ml $\text{TNF}\alpha$ for 16 h at 37°C. Then, T lymphoblasts were allowed to bind to coverslips coated with these cells for 30 min at 37°C in the presence of either medium alone (D) or any of the following stimuli: 5 $\mu\text{g}/\text{ml}$ anti-ICAM-3 HP2/19 (A), TPI/24 mAb (B), or 20 ng/ml PMA (C). Then, fixed cells were stained for ICAM-3, as described in Materials and Methods, either by addition of FITC-labeled anti-mouse IgG (A and B) or by using biotinylated anti-ICAM-3 mAb (C and D). Endothelial cells displayed a basal fluorescence, even though they were not specifically stained, that allowed us to photograph them (F). Bar, 10 μm .

interactions with ICAM-1 and VCAM-1 (Burkly et al., 1991; van Seventer et al., 1991; Hernández-Caselles et al., 1993). In contrast, no evidence exists regarding ICAM-1- and VCAM-1-mediated stimulation of endothelial cells upon this interaction. Interestingly, we have herewith demonstrated that ICAM-3, the third identified counter-receptor for the leukocyte integrin LFA-1, is also a regulatory molecule able to modulate both cell binding to different proteins and cell morphology. These results suggest that other integrin counter-receptors could also have an active functional role. In this regard, the absence of a regulatory role for ICAM-1 in our system might be due to the particular ICAM-1 mAbs employed.

Stimuli that activate T lymphocytes, such as phorbol esters or antibodies against the CD3-TcR complex or the CD2 antigen, also activate the integrins expressed on the cell surface (Springer, 1990). In addition, the $\beta 1$, $\beta 2$, and $\beta 3$ integrins can be activated upon binding of regulatory mAbs to these integrins (Gulino et al., 1990; van Kooyk et al., 1991; Arroyo et al., 1992; Robinson et al., 1992). The mechanism

responsible for the regulation of integrins by ICAM-3 likely involves cell activation since this phenomenon has been observed upon cell treatment with the stimulatory anti-ICAM-3 HP2/19 mAb (Campanero et al., 1993; Hernández-Caselles et al., 1993). In addition, since regulation through ICAM-3 of LFA-1, VLA-4, and VLA-5 activity seems to be due to T cell activation, we can expect that additional integrins on these cells are also regulated through this LFA-1 counter-receptor.

Activation of T lymphocytes with a wide number of stimuli involves the rise of intracellular calcium levels and/or PKC activation. It has recently been reported that mAb directed to certain epitopes of ICAM-3, when crosslinked with second anti-Ig antibodies, induce a rise in intracellular calcium concentrations (Juan et al., 1994). However, when we analyzed the possibility that mAb engagement of ICAM-3 could induce these intracellular events, no increase of intracellular calcium levels was observed upon anti-ICAM-3 cell treatment (our unpublished observations). Our previous results showing that the expression of the AIM/CD69 activation an-

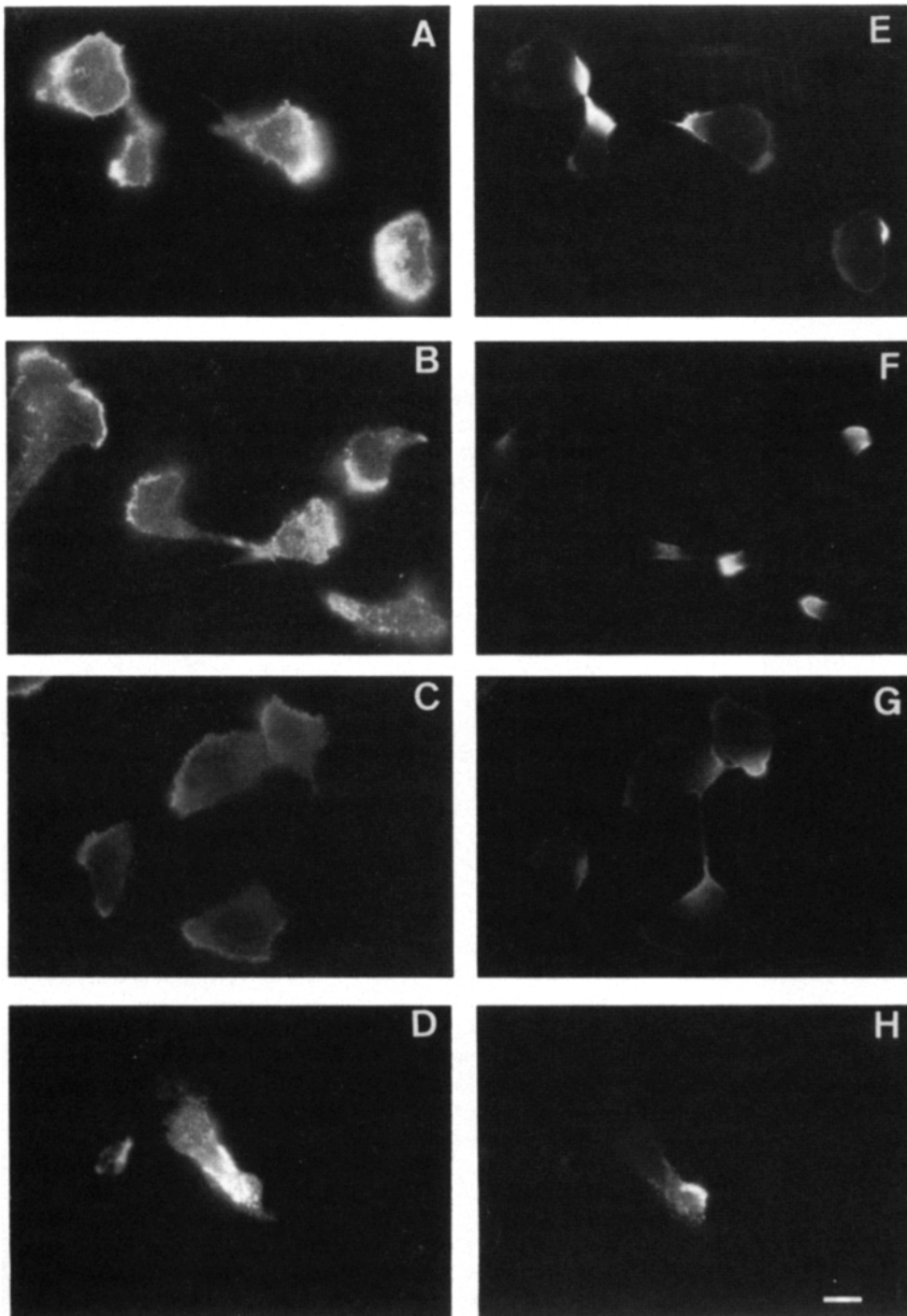


Figure 8. Double immunofluorescence staining of ICAM-3 and cytoskeletal proteins on T lymphoblasts. Cells were induced to adhere on FN80 for 30 min at 37°C by using the activating anti-ICAM-3 HP2/19 mAb. After fixation, ICAM-3 was detected directly with rhodamine-conjugated goat F(ab')₂ anti-mouse Ig (E-H). Cytoskeletal proteins in the same cells were detected after permeabilization with FITC-conjugated phalloidin to stain filamentous actin (A), and with rabbit antisera against α -actinin (B), talin (C), and myosin (D) followed by FITC-conjugated donkey F(ab')₂ anti-rabbit Ig. Bar, 10 μ m.

tigen on T lymphocytes could be induced upon cell treatment with the anti-ICAM-3 HP2/19 mAb (Hernández-Caselles et al., 1993) strongly suggest that PKC activation could be involved in ICAM-3-mediated cellular responses. AIM/CD69 is an inducible cell surface molecule whose expression on T lymphocytes is dependent on the activation of PKC (Cebrián et al., 1989). However, no conclusive results could be obtained on the putative involvement of this intracellular signaling pathway during ICAM-3-mediated regulation of cell binding to fibronectin by using PKC inhibitors as Staurosporine and H7 (our unpublished observations). Interestingly

enough, recent evidence demonstrate the induction of tyrosine phosphorylation of different protein substrates in T cells by antibody engagement of ICAM-3 molecule (Arroyo et al., 1994; Juan et al., 1994). Moreover, the CD45 leukocyte tyrosine phosphatase appears to be involved in the regulation of ICAM-3-mediated signaling and cell-cell interactions (Arroyo et al., 1994).

We have reported that phorbol esters as well as the activating anti-ICAM-3 HP2/19 and anti-VLA- β 1 TS2/16 mAb, regulate leukocyte binding to endothelial cells and ECM components (Arroyo et al., 1992; and this report). Interest-

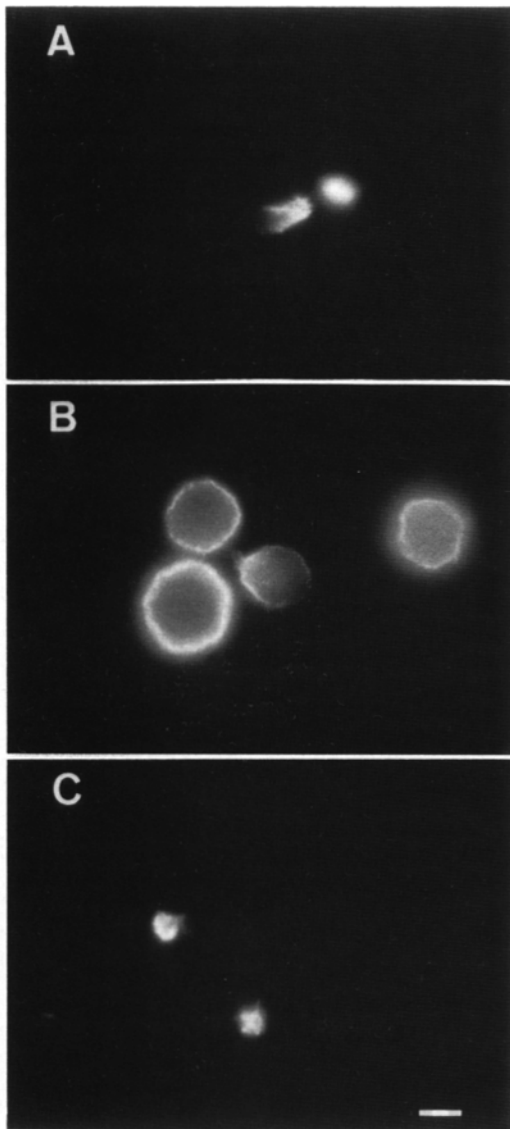


Figure 9. Effect of the myosin-disrupting drug butanedione monoxime on cell distribution of ICAM-3. Cells were induced to adhere on FN80 with the activating anti-ICAM-3 HP2/19 mAb, in the absence (A) or in the presence of 10 mM butanedione monoxime (B), and 10 μ M colchicine (C). Cells were fixed and ICAM-3 was directly visualized by rhodamine-conjugated F(ab')₂ anti-mouse Ig. Bar, 10 μ m.

ingly, these stimuli differ in their ability to promote changes on the cellular morphology. ICAM-3 seems to be unique for stimulating the generation of the heading uropod. Both phorbol esters and the anti-VLA- β 1 TS2/16 mAb also induced cell spreading on ICAM-1 and VCAM-1 (Arroyo et al., 1992; Sánchez-Mataeos et al., 1993), but the heading uropod was not detected. The recruitment of ICAM-3 to the uropod was observed on both T blast and Jurkat cells by engagement of ICAM-3 with the activating antibody without further cross-linking, as well as by coengagement of ICAM-3 and CD3-TcR (unpublished observations). Hence, both the raising of the heading uropod and ICAM-3 redistribution might be early events in the cellular activation cascade since both phenomena are observed under activating as well as under

nonactivating conditions. In addition, clustering of ICAM-3 and uropod generation are epitope-specific since an anti-ICAM-3 mAb of identical Ig isotype and comparable affinity, but directed against a different epitope, was not able to induce any of either events. Interestingly, this phenomenon also took place when T cells were stimulated through ICAM-3 to interact with endothelial cells instead of purified proteins. In this regard, it has been reported that lymphocyte interactions with endothelial cells induced pseudopod formation; the adhesion molecules CD2, CD44, and L-selectin, but not LFA-1, were redistributed to this structure (Rosenman et al., 1993).

The heading uropod may have a functional role and might be essential for the recruitment of other leukocytes to inflammatory foci because: (a) it is very well exposed since it is projected towards the outer milieu outgoing from the flattened cellular area in contact with the substratum; (b) ICAM-3 is highly concentrated there, thus facilitating LFA-1-mediated interaction of other leukocytes; and (c) we have previously shown that on small cell aggregates, T lymphocytes come in contact through a uropod where ICAM-3 is accumulated (Campanero et al., 1993). In good agreement, it has been postulated that a good exposure of the L-Selectin on neutrophils facilitates its interaction with its counter-receptor on endothelial cells (Picker et al., 1991). In addition, the local clustering of the leukocyte integrin Mac-1 or its ligand iC3b has been shown to facilitate their mutual interaction (Detmers et al., 1987; Hermanowski-Vosatka et al., 1988).

Another LFA-1 counter-receptor, ICAM-1, has previously been reported to be redistributed on the cell surface of JY cells to an uropod-like structure that apparently interacts with LFA-1-coated surfaces (Dustin et al., 1992). Therefore, relocation of ICAMs to adhesive cellular areas seems to be a common characteristic of ICAMs. This similarity suggests that ICAM-1 and ICAM-3 might share common features on their cytoplasmic tails that would enable them to interact with the same set of cytoskeletal proteins. However, this does not appear to be the case because we have previously shown that ICAM-3 and ICAM-1 localized to different cell areas on cell aggregates induced through ICAM-3 (Campanero et al., 1993).

In the same regard, it has been previously reported that the cytoplasmic tail of ICAM-1 interacts with the actin-containing cytoskeleton and α -actinin (Carpén et al., 1992). In contrast, ICAM-3 colocalizes neither with major actin concentrations in the cell nor with three other cytoskeletal focal adhesion proteins, talin, α -actinin, and vinculin. Actin-based cytoskeleton appeared to be more concentrated in the broad area of contact with the substrate most distant from the cell uropod. Interestingly enough, the presence of linear arrays of myosin within the uropod region supports a role for myosin motor in both driving ICAM-3 towards the uropod and in the generation of this cellular structure. Accordingly, a myosin-disrupting drug completely abolished both events. These findings are in good agreement with previous reports suggesting that myosin mediates membrane motility in *Drosophila*, Swiss 3T3 fibroblasts and the dorsal lip in *Drosophila* (Cramer et al., 1994; Fath and Burgess, 1994).

There is "cross-talk" between leukocyte and integrin activation, since each process is involved in the regulation of the other. Thus, leukocyte activation leads to integrin molecules

activation enabling them to interact with their ligands and, on the other hand, ligand binding by integrins contributes to leukocyte activation. Our results suggest that ICAM-3 has an essential role in this cross-talk. In this regard, we have previously shown that T cell activation enhances LFA-1-mediated T cell binding to ICAM-3 (Campanero et al., 1993). Herewith, we have shown that engagement of ICAM-3 with mAb enhances the integrin-mediated binding to components of endothelial cells (ICAM-1 and VCAM-1) and extracellular matrix (fibronectin). In addition, it has been reported that activation of T lymphocytes through the CD3-TcR complex is markedly enhanced by integrin-mediated adhesion events (Matsuyama et al., 1989; Burkly et al., 1991; van Seventer et al., 1991; Hernández-Caselles et al., 1993), as well as by ICAM-3 itself (Hernández-Caselles et al., 1993).

We propose a model for the participation of ICAM-3 in leukocyte migration to inflammatory foci. LFA-1-activated bearing cells (e.g., antigen-presenting cells) could bind to resting T lymphocytes through LFA-1/ICAM-3 interactions that could induce mutual cocapping of both molecules that, in turn, would reinforce cell stimulation on both cells (Campanero et al., 1993; Hernández-Caselles et al., 1993). This interaction might be mimicked by the ICAM-3 HP2/19 mAb in our *in vitro* system. Next, activated leukocytes would bind to endothelial cells through LFA-1/ICAM-1 and VLA-4/VCAM-1 interactions. Leukocytes would spread out on the endothelium and the heading uropod, where ICAM-3 would be concentrated, would raise from the flattened area of the cell, in close contact with the endothelial cell, towards the lumen of the vessel. The enormous density of ICAM-3 on this structure together with its location in the lumen of the vessel, would facilitate the interaction with the LFA-1 on circulating leukocytes which would be recruited and possibly stimulated.

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