Chemokines Regulate Cellular Polarization and Adhesion Receptor Redistribution during Lymphocyte Interaction with Endothelium and Extracellular Matrix. Involvement of cAMP Signaling Pathway

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Abstract. Leukocyte recruitment is a key step in the inflammatory reaction. Several changes in the cell morphology take place during lymphocyte activation and migration: spheric-shaped resting T cells become polarized during activation, developing a well defined cytoplasmic projection designated as cellular uropod. We found that the chemotactic and proinflammatory chemokines RANTES, MCP-1, and, to a lower extent, MIP-1α, MIP-1β, and IL-8, were able to induce uropod formation and ICAM-3 redistribution in T lymphoblasts adhered to ICAM-1 or VCAM-1. A similar chemokine-mediated effect was observed during T cells binding to the fibronectin fragments of 38- and 80-kD, that contain the binding sites for the integrins VLA-4 and VLA-5, respectively. The uropod structure concentrated the ICAM-3 adhesion molecule (a ligand for LFA-1), and emerged to the outer milieu from the area of contact between lymphocyte and protein ligands. In addition, we found that other adhesion molecules such as ICAM-1, CD43, and CD44, also redistributed to the lymphocyte uropod upon RANTES stimulation, whereas a wide number of other cell surface receptors did not redistribute. Chemokines displayed a selective effect among different T cell subsets; MIP-1β had more potent action on $CD8^+$ T cells and tumor infiltrating lymphocytes (TIL), whereas RANTES and MIP-1α targeted selectively $CD4^+$ T cells. We have also examined the involvement of cAMP signaling pathway in uropod formation. Interestingly, several cAMP agonists were able to induce uropod formation and ICAM-3 redistribution, whereas H-89, a specific inhibitor of the cAMP-dependent protein kinase, abrogated the chemokine-mediated uropod formation, thus pointing out a role for cAMP-dependent signaling in the development of this cytoplasmic projection. Since the lymphocyte uropod induced by chemokines was completely abrogated by *Bordetella pertussis* toxin, the formation of this membrane projection appears to be dependent on G proteins signaling pathways. In addition, the involvement of myosin-based cytoskeleton in uropod formation and ICAM-3 redistribution in response to chemokines was suggested by the prevention of this phenomenon with the myosin-disrupting agent butanedione monoxime. Interestingly, this agent also inhibited the ICAM-3–mediated cell aggregation, but not the cell adhesion to substrata. Altogether, these results demonstrate that uropod formation and adhesion receptor redistribution is a novel function mediated by chemokines; this phenomenon may represent a mechanism that significantly contributes to the recruitment of circulating leukocytes to inflammatory foci.

An important process in the inflammatory response is the recruitment of leukocytes into tissue. This phenomenon is regulated by a cascade of molecular events which involve the adhesion of leukocytes to endothelium followed by their migration into tissue (Butcher, 1991; Springer, 1994). Initially, selectin-mediated interactions cause leukocytes to roll over the endothelial cells, where they contact factors that, through the activation of leukocyte integrins, trigger strong adhesion (arrest) to endothelium. Thereafter, migration into tissue is directed by locally active promigratory factors. Although the adhesion molecules involved in the recruitment of leukocytes into tissues are well defined, less is known about the factors that trigger the adhesion and that direct extravasation of the inflammatory cells (Butcher, 1991; Springer, 1994).

It has recently been proposed that the chemokines or chemoattractive cytokines, trigger adhesion and migration of distinct leukocyte subsets either as soluble chemoattractants or as immobilized molecules bound to proteoglycans of the endothelial surface (Oppenheim et al., 1991; Schall, 1991; Miller and Krangel, 1992; Huber et al., 1991; and Tanaka et al., 1993*a*). Chemokines are a family of 70–80 aa

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(8–10 kD) polypeptides, characterized by the presence of four conserved cysteine residues. They have been grouped into two subfamilies, the α- or "C-X-C" subfamily that includes interleukin (IL)[1]-8/NAP-1, MGSA/gro, IP-10 and PF-4, and the β- or "C-C" subfamily that includes regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemotactic protein (MCP)-1, 2 and 3, macrophage inflammatory protein-1 (MIP)-1α and β, and I-309 among others (Schall and Bacon, 1994). Additionally, it has recently been discovered lymphotactin, a new chemokine lacking two of the four cysteine characteristic residues, that represents a new branch in the chemokine family (Kelner et al., 1994). Platelets and a broad spectrum of nucleated cell types produce chemokines. Endothelial cells are an important source of chemokines like IL-8, MGSA/gro, MCP-1, and IP-10. On the other hand, T lymphocytes are also able to secrete IL-8, RANTES, MIP-1α and β, and MCP-1 (Oppenheim et al., 1991; Woldemar Carr, 1994). With few exceptions, α-chemokines attract neutrophils, β-chemokines attract monocytes, eosinophils, and/or basophils, and the members of both subfamilies together with lymphotactin attract different subsets of lymphocytes. It has been described that MIP-1β enhances the adhesion of $CD8^+$ T cells to vascular cell adhesion molecule-1 (VCAM-1) and fibronectin, and that RANTES, IP-10, MIP-1α, and β increase the T cell binding to human umbilical vein EC (Tanaka et al., 1993*a*, *b*, *c*; Taub et al., 1993*a*, *b*; Gilat et al., 1994). Chemokines produce their biological effects by interacting with specific membrane receptors that belong to the seven transmembrane spanning family of G-protein coupled receptors (Murphy, 1994).

The interaction of lymphocyte function–associated antigen-1 (LFA-1) integrin with its ligands intercellular adhesion molecule (ICAM)-1, -2, and -3 is a regulated molecular pathway that plays a key role in T cell adhesion to endothelium (Springer, 1990; Fawcett et al., 1992; Vazeux et al., 1992; Campanero et al., 1993; de Fougerolles et al., 1992, 1993, 1994). ICAM-3 is the most abundant LFA-1 ligand on resting leukocytes, and it has been proposed as a pivotal molecule in the earliest phase of the immune response (Acevedo et al., 1993; Fawcett et al., 1992). The engagement of ICAM-3 triggers tyrosine phosphorylation of different substrates, induces T cell activation, and regulates the LFA-1/ICAM-1 and VLA-4/VCAM-1 adhesion pathways on T cells (Arroyo et al., 1994; Juan et al., 1994; Campanero et al., 1993, 1994; Hernández-Caselles et al., 1993). The ligation of ICAM-3 by specific antibodies induces the development of a uropod structure on lymphocytes, where ICAM-3 was almost exclusively concentrated (Campanero et al., 1994).

CD43 and CD44 are other adhesion molecules that have also been implicated in lymphocyte stimulation. The CD44 hyaluronate receptor is a broadly expressed glycoprotein involved in lymphocyte homing to peripheral lymph nodes, cell-ECM interactions, lymphocyte activation, and induction of homotypic cell aggregation (Aruffo et al., 1990; Koopman et al., 1990). Similarly, CD43 or sialophorin is a heavily sialylated protein expressed by all leukocytes that participates in leukocyte activation and aggregation (Nong et al., 1989).

In this report, we investigated the physiological molecules that are capable to trigger the formation of the cellular uropod. We found that chemokines that are released during lymphocyte-endothelial cells (EC) interaction are responsible for the induction of both uropod formation in T cells and adhesion receptors (ICAM-1, -3, CD43, and CD44) redistribution to this structure. The effect of each chemokine was selectively exerted on different T cell subsets, and appears to involve G proteins and a cAMP-dependent signal transduction pathway. The relevance of these chemokine-regulated functional effects on lymphocytes is discussed.

Materials and Methods

Antibodies, Cytokines and Reagents

The anti–ICAM-3 HP2/19 and TP1/24, anti-CD11a TP1/40, anti-VLA4 HP2/1, anti-CD43 TP1/36, anti-CD44 HP2/9, anti-CD45 D3/9, anti-CD5 TP1/21, anti-CD7 MAR21, anti-CD4 HP2/6, and anti-CD8 B9.4.2 mAb have been described (Campanero et al., 1991, 1993; Pulido et al., 1988, 1991; Carrera et al., 1989). The anti–ICAM-1 MEM 111 was a generous gift of Dr. V. Horejsi (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Videnska, Czech Republic). Recombinant human (rh) RANTES (specific activity $2–5 \times 10^3$ U/mg, purity >97%, endotoxin level <0.1 ng/μg cytokine) and rhMIP-1β (specific activity $1.6–2.5 \times 10^4$ U/mg, purity >97%, endotoxin level <0.1 ng/μg cytokine) were purchased from R&D Systems (Minneapolis, MN). rhIL-8 (purity >98%, endotoxin level <0.1 ng/μg cytokine), rhMCP-1 (purity >99%), rhMIP-1α (purity >99%, endotoxin level <0.1 ng/μg cytokine). rhTNF-α (spec. act. 5×10^7 U/mg, purity >95%) was provided by Genetech (San Francisco, CA), and IFN-γ (spec. act. 2×10^7 U/mg, purity >98%, LPS content <0.048 ng/ml) was a gift from Amgen Biologicals (Thousand Oaks, CA). Prostaglandin E_2, 8-Bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP), butanedione monoxime, colchicine, and cytochalasin D were purchased from Sigma Chemical Co. (St. Louis, MO). Forskolin, pertussis toxin, H-89 (N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquenilesulfonamide), and bisindolylmaleimide II (BIM II) were from Calbiochem (San Diego, CA).

Protein Substrata

Recombinant chimeric ICAM-1-Fc and VCAM-1-4D-Fc, consisting of the total extracellular domains fused to IgG1 Fc fragment were obtained as described (Berendt et al., 1992; Fawcett et al., 1992). Briefly, COS-7 cells were transiently transfected with pICAM-1-Fc and pVCAM-1-4D-Fc (ICAM-1 and VCAM-1-4D cDNAs cloned in pCD8IgG1). After 4 d, culture supernatants were precipitated with ammonium sulphate, and thereafter chimeric proteins were isolated by using protein A coupled to Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). Recombinant soluble E-selectin was kindly provided by Dr. R. Lobb (Biogen, Cambridge, MA). The tryptic 38-kD and 80-kD fibronectin fragments (FN40 and FN80) were a generous gift of Dr. A. García-Pardo (Centro de Investigaciones Biológicas, Madrid, Spain). Poly-L-lysine and fibrinogen were purchased from Sigma Chem. Co. (St. Louis, MO). Bovine serum albumin (BSA) was purchased from Boehringer Mannheim GmbH (Mannheim, Germany).

Cells

Human T lymphoblasts were prepared from peripheral blood mononuclear cells by treatment with phytohemagglutinin (PHA) 0.5% (Pharmacia) for 48 h. Cells were washed and cultured in RPMI 1640 (Flow Lab., Irvine, Scotland) containing 10% FCS (Flow Lab.) and 50 U/ml IL-2

1. *Abbreviations used in this paper*: BIM II, bisindolylmaleimide II; EC, endothelial cells; FN, fibronectin; FN40 and FN80, 38- and 80-kD fragments of fibronectin; ICAM, intercellular adhesion molecule; IL, interleukin; LFA-1, lymphocyte function–associated antigen-1; MCP, monocyte chemotactic protein; MIP-1, macrophage inflammatory protein-1; PT, pertussis toxin; RANTES, regulated on activation, normal T cell expressed and secreted; TIL, tumor infiltrating lymphocyte; VCAM-1, vascular cell adhesion molecule-1; VLA, very late activation antigen.

kindly provided by Eurocetus. 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 T cell adhesion and activation (Campanero et al., 1993).

HSB-2 T lymphoblastoid cell line was kindly provided by Dr. N. Hogg (Imperial Cancer Research Fund, London), and has previously been described (Dougherty et al., 1988).

$CD4^+$ and $CD8^+$ T cells were prepared by exhaustive negative selection from T lymphoblasts using anti-CD8 or anti-CD4 mAb, respectively, and immunomagnetic beads (Dynal, Oslo, Norway) as described (Vartdal et al., 1987). $CD8^+$ tumor infiltrating lymphocytes (TIL) were isolated from melanoma specimens obtained from the Department of Pathology, Hospital de la Princesa (Madrid, Spain). These cells were cultured in AIM-V medium (Flow Lab.) containing 10% HY-ultroser serum (Pharmacia) and 5,000 U/ml IL-2.

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) for 20 min at 37°C. Cells were seeded into flasks and cultured in M199 medium (Flow Lab.) supplemented with 20% FCS, 50 μg/ml endothelial cell growth supplement, and 100 μg/ml heparin (Sigma). Cells within two passages were used.

Immunofluorescence Analysis

Immunofluorescence experiments were performed essentially as described (Sánchez-Mateos et al., 1993). Briefly, 2×10^6 T lymphoblasts were incubated in flat-bottomed, 24-well plates (Costar Corp., Cambridge, MA) in a final volume of 500 μl complete medium on coverslips coated with different protein substrata. In some experiments, coverslips in that HUVEC were grown and stimulated with TNF-α (10 ng/ml for 16 h at 37°C) were used. Chemokines and other cytokines at different concentrations, or 5 μg/ml HP2/19 mAb were added and cells were allowed to settle in a cell incubator at 37°C and 5% CO_2 atmosphere. After different periods of time cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature and rinsed in TBS (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% NaN_3). To visualize different membrane adhesion molecules, cells were stained with specific mAb. After washing, cells were incubated with a 1:50 dilution of an FITC-labeled rabbit $F(ab')_2$ anti-mouse IgG (Pierce, Rockford, IL). For double-label studies in lymphocyte/EC cocultures, cells were saturated with 10% nonspecific mouse serum in TBS. Then, the coverslips were incubated with biotinylated mAb to other protein, followed by washing and labeling with TRITC-avidin D (Vector Laboratories, 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. The cells were observed using a Nikon Labophot-2 photomicroscope with 40, 60, and 100 × oil immersion objectives. The proportion of uropod-bearing cells was calculated by random choice of ten different fields (60 × objective) of each condition and direct counting of total cells (400–500) and uropod-bearing cells. Preparations were 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.

Cell Adhesion Assays

Adhesion assays were essentially performed as previously described (Arroyo et al., 1992). Briefly, 50 μl/well of 20 mM Tris-HCl, pH 8.0, containing FN80 or recombinant chimeric ICAM-1-Fc, were used to coat 96-well microtiter EIA II-Linbro plates (Costar Corp.) 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 3×10^5 T lymphoblasts per well in 100 μl were added and centrifuged for 5 min at 10 *g* before an incubation at 37°C for 20 min. To quantify cell attachment, the plates were washed with RPMI containing 0.5% HSA, and cells were fixed with a mixture of acetone/methanol 1:1 and stained with violet crystal 0.5%. Violet crystal was then extracted by the addition of a 1:1 mixture of sodium citrate 0.1 M, pH 4.2/ethanol, and absorbance at 540 nm was measured in an ELISA reader (LP400, Kallestad, Chaska, MN). For inhibition assays, cells were pretreated with different pharmacological agents for 30 min at 37°C.

Aggregation Assays

Homotypic cell aggregation was performed as previously described (Campanero et al., 1993). Briefly, T lymphoblasts were incubated by duplicate in flat-bottomed, 96-well microtiter plates (Costar Corp.) at 1.5×10^6/ml in a final volume of 100 μl of complete medium. Anti–ICAM-3 HP2/19 mAb was added at 1 μg/ml and cells were allowed to settle in an incubator at 37°C and 5% CO_2 atmosphere. Cell aggregation was 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 grid placed under the plate. Results were expressed as percent of aggregated cells. For inhibition assays, cells were pretreated with different pharmacological agents for 30 min at 37°C before the addition of the inducing mAb.

Results

Chemokines Induce Uropod Formation and ICAM-3 Clustering in This Structure

Cell polarization involves the development of a cytoplasmic projection which is termed uropod. This structure is characteristic of migrating lymphocytes and is displayed in the back, as a pseudopod-like projection. The induction of a cellular uropod has previously been described on T lymphoblasts adhering to either protein substrata or TNFα-activated HUVEC upon treatment with an activatory anti–ICAM-3 mAb; the ICAM-3 adhesion molecule was preferentially localized to the most distal portion of the uropod (Campanero et al., 1994). Nevertheless, a significant number of uropodia-bearing cells was observed in some experiments in the absence of the activatory anti–ICAM-3 mAb. To ascertain this issue, we thoroughly investigated the extent to which the interaction of T lymphoblasts with EC could elicit the formation of cell uropodia. To this end, HUVEC that were induced with TNF-α to express ICAM-1 and VCAM-1 were cocultured with T lymphoblasts during different periods of time. In these studies, we have confirmed that the anti–ICAM-3 HP2/19 mAb is a strong stimulus for uropod formation (Fig. 1 *B*); in addition, we have found that at early times of incubation (30 min), and in the absence of stimulatory antibody, uropod formation may be negligible or not, mainly depending upon cell (lymphoblast and HUVEC) donors. Thus, the generation of uropods where ICAM-3 was located could be observed as early as 30 min after culture (Fig. 1, *A a*, and *B*). However, the proportion of cells bearing uropodia significantly increased after 4 and 20 h of culture (Fig. 1 *B*). Freshly isolated peripheral blood T lymphocytes did not generally display cellular uropods at 30 min of culture, but comparable levels of cells bearing uropods than T lymphoblasts were observed after 20 h of incubation on EC (Fig. 1 *B*). When T lymphoblasts were simultaneously stained for ICAM-3 and LFA-1α, the latter molecule showed a punctate cell distribution along all the contact area of T cells with endothelial cells (Fig. 1 *A b*).

Numerous soluble mediators are released during the leukocyte-EC interaction. Chemokines are one of the important locally secreted peptides that are able to induce chemotaxis of specific leukocyte subsets (Oppenheim et al., 1991), and that play a key role in the regulation of the adhesive properties of leukocyte integrins (Tanaka et al., 1993*a*, *b*, *c*). To explore whether chemokines are involved in the uropod formation in T cells during their interaction with HUVEC, we assayed the ability of a large panel of these and other several cytokines to trigger the appearance of this cellular structure. Immunofluorescence analy-

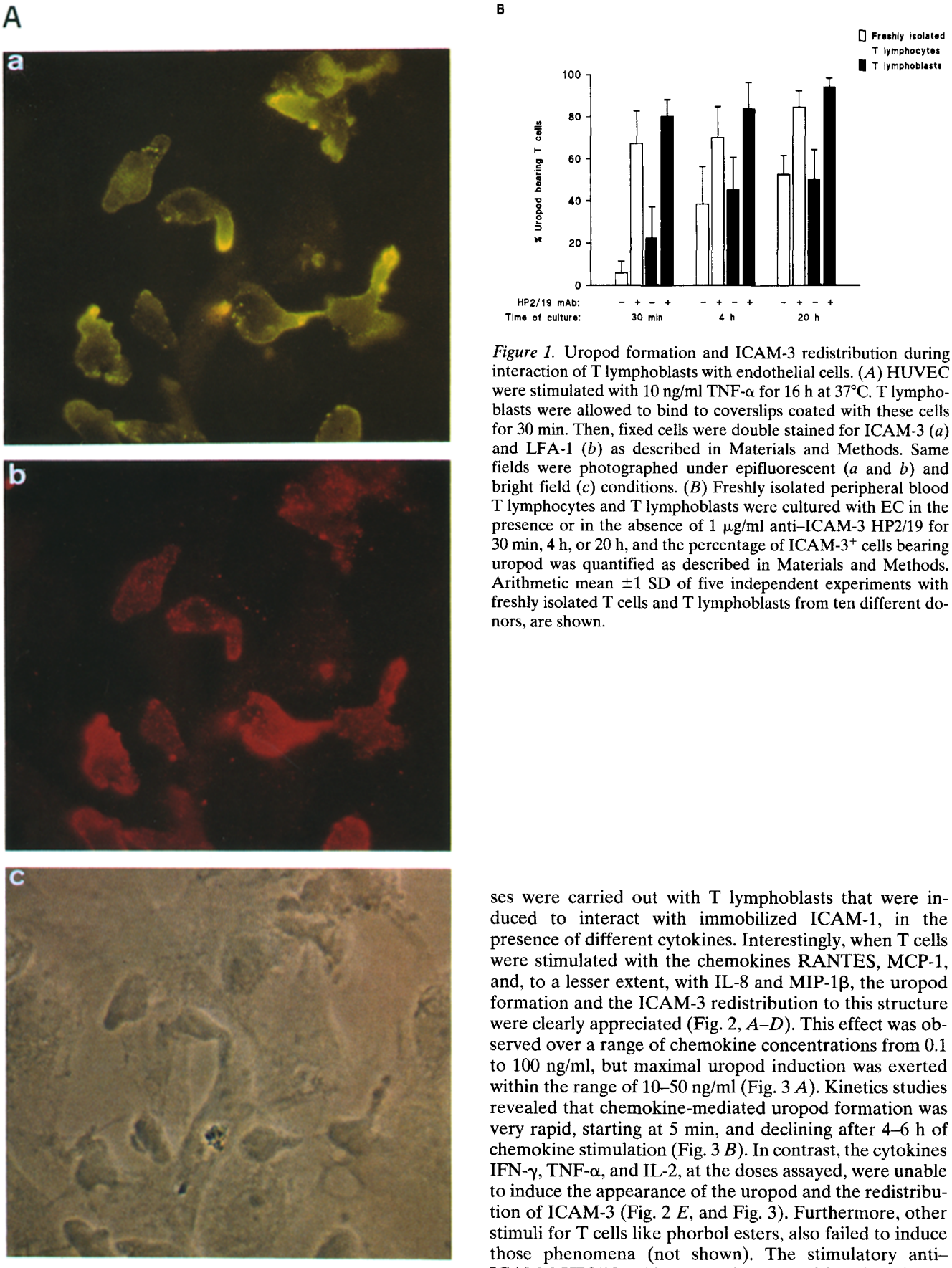


Figure 1. Uropod formation and ICAM-3 redistribution during interaction of T lymphoblasts with endothelial cells. (*A*) HUVEC were stimulated with 10 ng/ml TNF-α for 16 h at 37°C. T lymphoblasts were allowed to bind to coverslips coated with these cells for 30 min. Then, fixed cells were double stained for ICAM-3 (*a*) and LFA-1 (*b*) as described in Materials and Methods. Same fields were photographed under epifluorescent (*a* and *b*) and bright field (*c*) conditions. (*B*) Freshly isolated peripheral blood T lymphocytes and T lymphoblasts were cultured with EC in the presence or in the absence of 1 μg/ml anti–ICAM-3 HP2/19 for 30 min, 4 h, or 20 h, and the percentage of ICAM-3$^+$ cells bearing uropod was quantified as described in Materials and Methods. Arithmetic mean ±1 SD of five independent experiments with freshly isolated T cells and T lymphoblasts from ten different donors, are shown.

ses were carried out with T lymphoblasts that were induced to interact with immobilized ICAM-1, in the presence of different cytokines. Interestingly, when T cells were stimulated with the chemokines RANTES, MCP-1, and, to a lesser extent, with IL-8 and MIP-1β, the uropod formation and the ICAM-3 redistribution to this structure were clearly appreciated (Fig. 2, *A–D*). This effect was observed over a range of chemokine concentrations from 0.1 to 100 ng/ml, but maximal uropod induction was exerted within the range of 10–50 ng/ml (Fig. 3 *A*). Kinetics studies revealed that chemokine-mediated uropod formation was very rapid, starting at 5 min, and declining after 4–6 h of chemokine stimulation (Fig. 3 *B*). In contrast, the cytokines IFN-γ, TNF-α, and IL-2, at the doses assayed, were unable to induce the appearance of the uropod and the redistribution of ICAM-3 (Fig. 2 *E*, and Fig. 3). Furthermore, other stimuli for T cells like phorbol esters, also failed to induce those phenomena (not shown). The stimulatory anti–ICAM-3 HP2/19 mAb was used as control for triggering of uropod formation (Fig. 2 *F*, and Fig. 3 *B*).

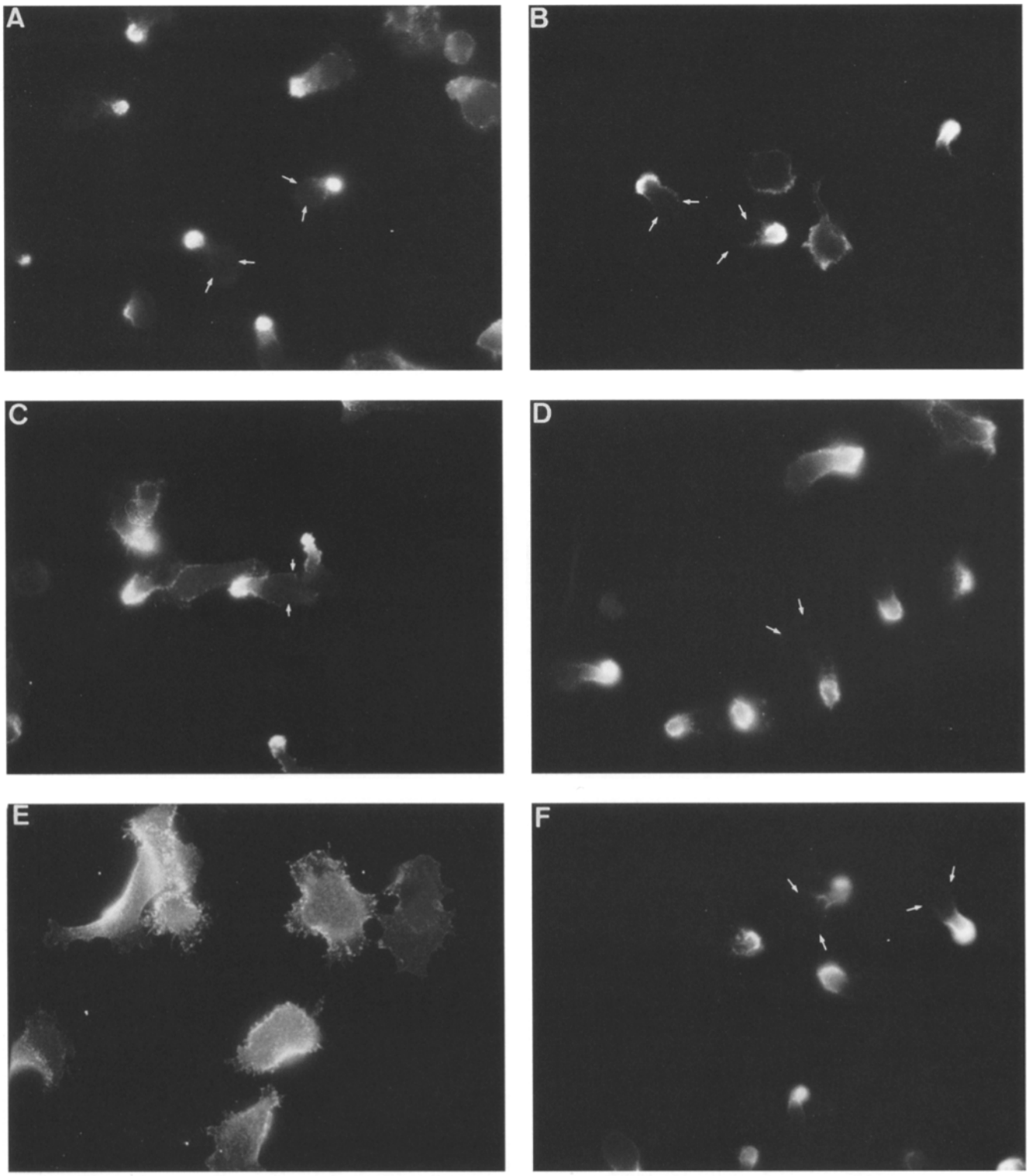


Figure 2. Chemokines induce uropod formation and ICAM-3 redistribution during T lymphoblast attachment to ICAM-1. T lymphoblasts were allowed to bind to coverslips coated with 20 μg/ml ICAM-1-Fc for 30 min at 37°C in the presence of 10 ng/ml RANTES (*A*), MCP-1 (*B*), MIP-1β (*C*), IL-8 (*D*), IFN-γ (*E*), or 5 μg/ml anti–ICAM-3 HP2/19 mAb (*F*). Then, fixed cells were stained for ICAM-3 with TP1/24 mAb as described in Materials and Methods. The cellular uropod and the cell contact area with the substratum were indeed in a distinct plane of focus. Arrows have been added to indicate the cell bodies.

We next examined the ability of the chemokines RANTES and MCP-1 to induce cell polarization on other substrates such as VCAM-1, or the fibronectin fragments FN40 and FN80, that contain binding sites for very late activation antigen (VLA)-4 and VLA-5. Interestingly, these two chemokines were able to trigger uropod formation and ICAM-3 redistribution in lymphoblasts incubated on ICAM-1, VCAM-1, and FN80, and in a lower extent on

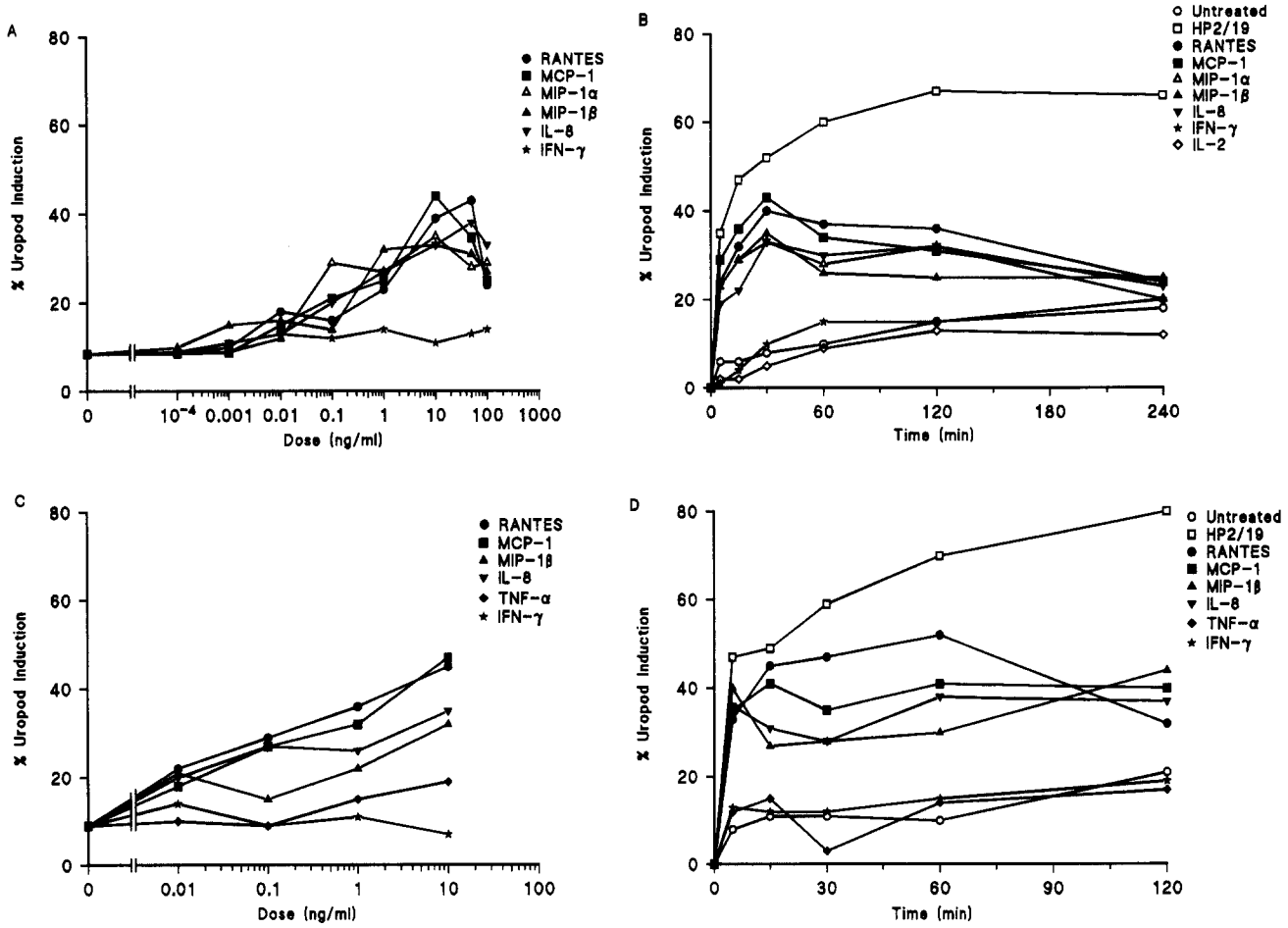


Figure 3. Dose dependence and kinetics of the uropod formation effect induced by chemokines. T lymphoblasts were allowed to bind to coverslips coated with 20 μg/ml ICAM-1 (*A* and *B*) or FN80 (*C* and *D*) in the presence of several doses of the different chemokines and cytokines for 30 min (*A* and *C*) and for different times of incubation with cytokines at 10 ng/ml or 50 U/ml IL-2 (*B* and *D*). As control, cells treated with 5 μg/ml anti–ICAM-3 HP2/19 mAb and untreated were also included in time course experiments. Then, fixed cells were stained for ICAM-3 and the percentage of uropod-bearing cells was quantified as described in Materials and Methods. A representative experiment, out of five independent ones on each substrate and performed with T lymphoblasts obtained from different normal donors, is shown.

the 38-kD FN fragment containing CS-1 (Fig. 4). Dose-response and time-course studies performed on FN80 yielded similar results to those obtained with ICAM-1 (Fig. 3, *C* and *D*). In contrast, these chemokines did not trigger uropod formation when T blasts were settled on E-selectin, poly-L-lysine, BSA, or fibrinogen (Fig. 4, and data not shown). Under these conditions, ICAM-3 displayed a uniform distribution over the rounded surface of the cell.

Other adhesion molecules and leukocyte cell surface antigens were also tested for their ability to redistribute towards the uropod, on T cells induced to adhere to FN80 in the presence of RANTES. Very interestingly, ICAM-1, was also found to localize in the uropod upon RANTES stimulation, although to a lower extent than ICAM-3 (over 15% of the cells) (Fig. 5 *A*). Two other adhesion molecules, CD43 and CD44, were also localized in that structure in a small proportion (14–18%) of T cells upon treatment with this chemokine (Fig. 5, *B* and *C*). Double fluorescence studies showed that all these molecules colocalized in the same cellular uropod, although ICAM-1, CD43, and CD44 were present in a lower proportion of

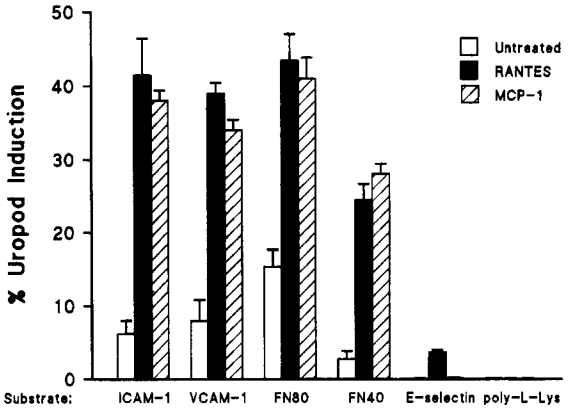


Figure 4. Chemokine-mediated cellular polarization during T lymphoblasts adhesion to different substrates. T lymphoblasts were allowed to bind to coverslips coated with either 20 μg/ml ICAM-1-Fc, 10 μg/ml VCAM-1-Fc, 20 μg/ml FN80, 20 μg/ml FN40, 20 μg/ml rsE-selectin, or 100 μg/ml poly-L-Lysine for 30 min at 37°C either in the presence or in the absence of 10 ng/ml of RANTES or MCP-1. Coverslips were processed as described in Materials and Methods. Arithmetic mean ±1 SD of three independent experiments are shown.

cells than ICAM-3 molecule (not shown). In contrast, the majority of molecules tested, including LFA-1, β1 integrins, CD45, CD3, CD2, CD7, and CD5, did not redistribute to the cellular uropod, (Fig. 5, *D–F*, and data not shown).

Selective Uropod Induction by Chemokines in Different T Cell Subsets

It has previously been described that different chemokines act as chemotactic factors for different T cell subsets and/

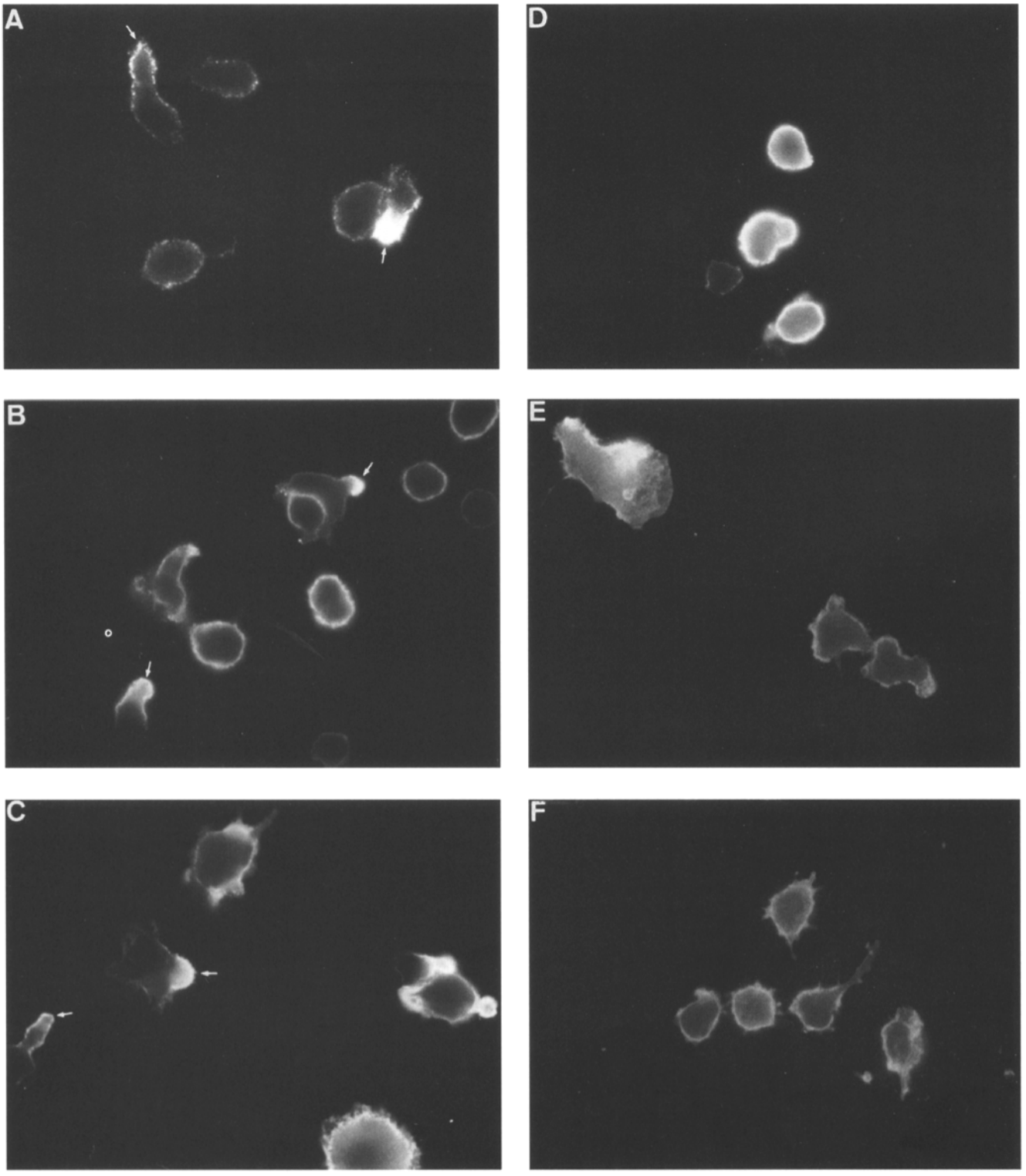


Figure 5. Redistribution of adhesion molecules to the cellular uropod upon chemokine treatment. T lymphoblasts were allowed to adhere to FN80-coated coverslips for 30 min at 37°C in the presence of 10 ng/ml RANTES. Then, fixed cells were stained for ICAM-1 (*A*), CD43 (*B*), CD44 (*C*), CD7 (*D*), LFA-1α (*E*), or CD45 (*F*) as described in Materials and Methods. Arrows have been added to indicate the cell uropods.

or cells at different stages of differentiation. Therefore, it was of interest to explore whether different chemokines were able to trigger uropod formation selectively in the major mature T cell subsets. Whereas in $CD4^+$ cells the effect was exerted mainly by RANTES, MIP-1α, and MCP-1, in $CD8^+$ cells MIP-1β displayed the highest activity (Fig. 6). Furthermore, the effect of chemokines was also studied on $CD8^+$ tumor infiltrating lymphocytes (TIL) isolated from two different patients with melanoma. We found that over 60% of the CD8 TIL showed cellular uropodia where ICAM-3 was redistributed after treatment with MIP-1β, whereas little effect was obtained with RANTES or MIP-1α, that selectively targeted $CD4^+$ T cells (Fig. 7 *A*). Interestingly, the long cytoplasmic projections, resembling neuronal axons, that TIL displayed in normal culture, tended to disappear after chemokine treatment (Fig. 7 *B*).

Involvement of cAMP Signaling Pathway in Uropod Formation

Chemokines bind to receptor molecules from the seven–transmembrane-domain rhodopsin-like family expressed on various immune cells, that couple through G proteins to adenylate cyclase (Murphy, 1994). To examine the involvement of cAMP signaling pathway in uropod formation, T cells were treated with pharmacological agents that increase intracellular levels of cAMP (PGE_2 and forskolin) and with 8-Br-cAMP, a membrane permeable cAMP analogue. All these reagents were able to induce uropod formation and ICAM-3 redistribution on T lymphoblasts adhering to ICAM-1 (Fig. 8) or FN80 (not shown). Moreover, they exerted an additive effect in combination with either RANTES and MCP-1, but not with the anti–ICAM-3 HP2/19 mAb (Fig. 8). However, an additive effect was indeed observed between suboptimal doses of the anti–ICAM-3 mAb and cAMP agonists (data not shown). Next, we examined whether the interference with cAMP signaling could affect the development of cell uropodia induced by chemokines and anti–ICAM-3 mAb. To this end, we treated T lymphoblasts with H-89 (N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquenilesulfonamide), a selective inhibitor of cAMP-dependent protein kinase (Chijiwa et al., 1990). This agent prevented uropod formation and ICAM-3 redistribution mediated by RANTES and MCP-1, and to a lower extent by the anti–ICAM-3 activating mAb (Fig. 9 *A*). No inhibitory effects of H-89 was exerted on T lymphoblasts cell adhesion to FN80 (see below Fig. 10 *B*) or ICAM-1-Fc (not shown). In contrast, the selective inhibitor of protein kinase C bisindolylmaleimide II (Toullec et al., 1991) did not inhibit uropod formation. Furthermore, this agent was able to induce by itself a certain level of uropod formation and ICAM-3 redistribution (Fig. 9 *A*), thus suggesting a "cross-talk" between cAMP- and PKC-dependent signaling pathways (Kozawa et al., 1992; Zong et al., 1994).

Most of the biological effects of chemokines are inhibited by pertussis toxin (PT), a specific inhibitor of certain G proteins, although a small PT-resistant component can usually be detected (Wu et al., 1993; Murphy, 1994; Maghazachi et al., 1994). To examine the involvement of G proteins-dependent signaling in chemokine-mediated uropod formation, T cells were pretreated with PT, and then

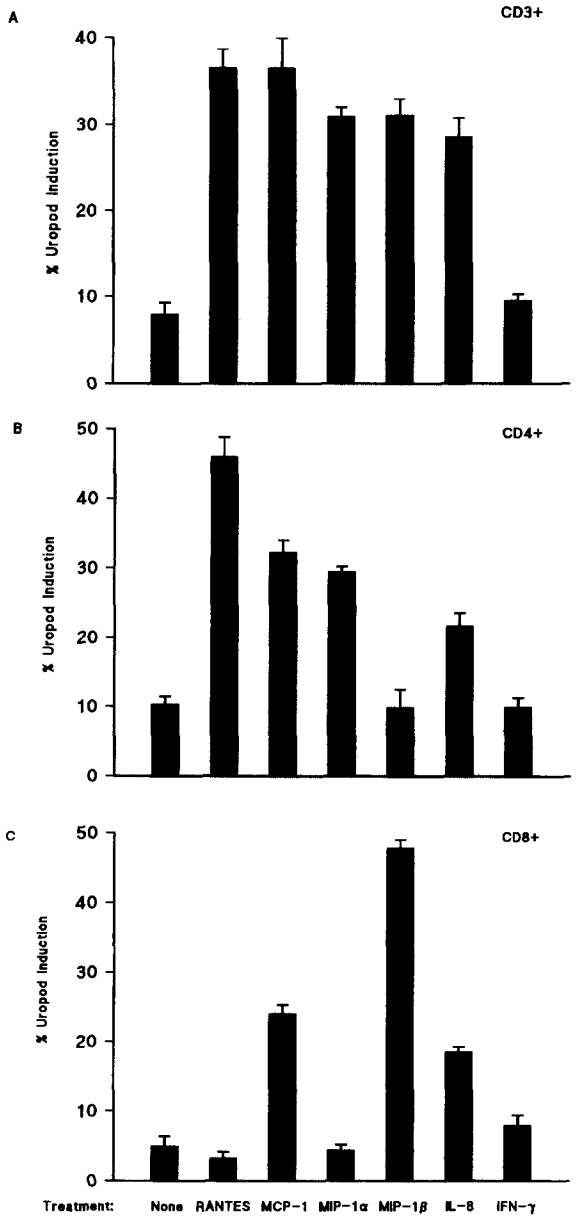
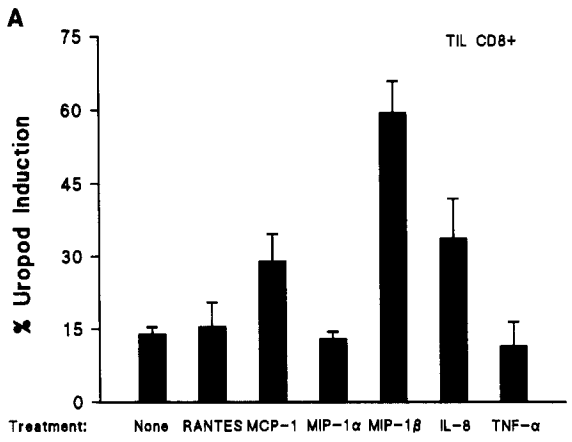


Figure 6. Selective uropod induction by chemokines in different T cell subsets. $CD3^+$ (*A*), $CD4^+$ (*B*), or $CD8^+$ (*C*) T lymphoblasts obtained as described in Materials and Methods were allowed to interact with ICAM-1-Fc–coated coverslips for 30 min at 37°C either untreated or in the presence of 10 ng/ml RANTES, MCP-1, MIP-1α, MIP-1β, IL-8, and IFN-γ; then fixed cells were stained for ICAM-3 and the proportion of cells bearing uropod was calculated as described above. Arithmetic mean ±1 SD of three independent experiments are shown.

allowed to interact with immobilized ICAM-1 in the presence of either RANTES, MCP-1, or the anti–ICAM-3 mAb HP2/19. The raising of the uropod and the ICAM-3 relocation induced by RANTES and MCP-1 was completely prevented by PT (Fig. 9 *A*). A similar inhibitory effect of PT was observed over uropodia formation induced by MIP-1α, MIP-1β, and IL-8 (data not shown). However, the dose of PT employed (1 μg/ml) induced an incomplete inhibition of the uropod formation by the anti–ICAM-3 mAb (Fig. 9 *A*); a high dose of PT (2 μg/ml) completely abrogated uropod formation induced by this antibody. In-



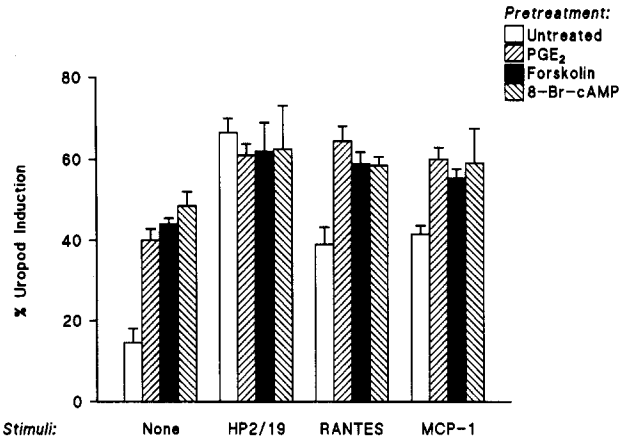


Figure 8. cAMP agonists induce uropod formation and ICAM-3 redistribution during T lymphoblasts attachment to ICAM-1. T lymphoblasts were incubated for 30 min at 37°C in the presence or in the absence of 2 μM PGE_2, 50 μM forskolin, or 1 mM 8-Br-cAMP, and then allowed to bind to coverslips coated with ICAM-1-Fc for 30 min at 37°C, in the presence or in the absence of 10 ng/ml RANTES, MCP-1, or 5 μg/ml anti–ICAM-3 HP2/19 mAb. Then, fixed cells were stained for ICAM-3 and the percentage of cells bearing uropodia was measured as described in Materials and Methods. Data represent mean values (±SD) of five independent experiments performed.

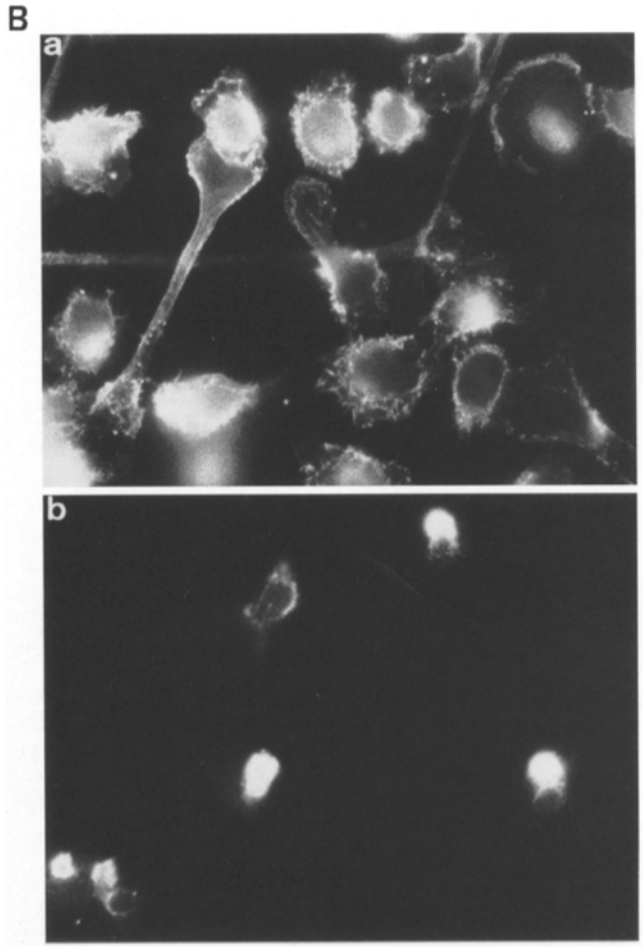


Figure 7. Uropod induction by chemokines in $CD8^+$ TIL. $CD8^+$ TIL were allowed to bind to coverslips coated with ICAM-1-Fc for 30 min at 37°C in the presence of RPMI 1640 or 10 ng/ml RANTES, MCP-1, MIP-1α, MIP-1β, IL-8, or TNF-α. Then, ICAM-3–stained coverslips were processed as described in Materials and Methods (*A*). In parallel, some cells treated with MIP-1β (*b*), or untreated (*a*) were photographed (*B*). Note the morphological changes of TIL after chemokine treatment. Arithmetic mean ±1 SD of three separate experiments performed with TIL from two different patients are shown.

terestingly, the addition of 8-Br-cAMP after PT treatment of T lymphoblasts partially reverted the inhibitory effect of PT (Table I), indicating that PT has no irreversible effect on T cells. No inhibitory effect of PT was exerted on T lymphoblasts cell adhesion to FN80 (see below Fig. 10) or ICAM-1-Fc (not shown).

Similar inhibition experiments were performed in the T lymphoblast-like cell line HSB-2 (Dougherty et al., 1988), which also bears the cytoplasmic uropod when the cells are allowed to adhere to FN80 (not shown). As it occurs with T lymphoblasts, both PT and H-89 cAMP-dependent kinase inhibitor, but not bisindolylmaleimide II were able to abrogate uropod appearance in HSB-2 cells (Fig. 9 *B*).

Role of Myosin in Uropod Formation and Cell Aggregation

We have previously described the presence of linear arrays of myosin in cell uropods induced through ICAM-3, and the abrogation of this structure by the myosin-disrupting drug butanedione monoxime (Campanero et al., 1994). To explore the involvement of the myosin motor in the uropod formation induced by chemokines, we used this myosin-disrupting agent. As shown in Fig. 10, butanedione monoxime completely prevented the uropod formation

Table I. Reversion by 8-Br-cAMP of Pertussis Toxin Effect on Uropod Formation

	Pretreatment			
	None	8-Br-cAMP	PT	PT +
Simuli				8-Br-cAMP
None	15 ± 2	44 ± 3	6 ± 1	33 ± 3
HP2/19	54 ± 5	79 ± 4	25 ± 2	51 ± 2

T lymphoblasts were incubated for 30 min in the presence or in the absence of 1 μg/ml pertussis toxin, and then 1 mM 8-Br-cAMP was either added or not. After 30 min, T cells were allowed to bind to coverslips coated with ICAM-1-Fc in the presence or in the absence of anti–ICAM-3 HP2/19 mAb. Then, fixed cells were stained for ICAM-3 and the percentage of cells bearing uropodia was calculated as described in Materials and Methods. Arithmetic mean ±1 SD of two separate experiments are shown.

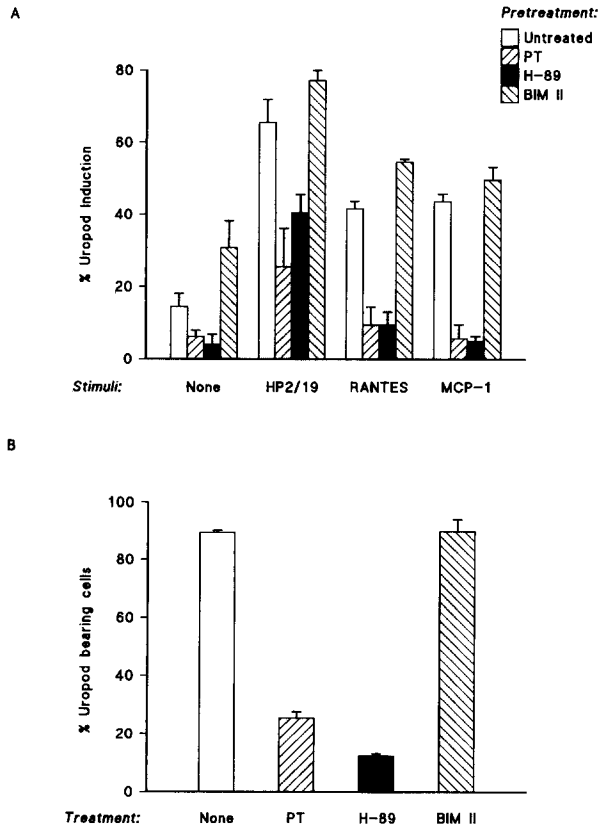


Figure 9. Pertussis toxin and the cAMP-dependent kinase selective inhibitor H-89 prevent chemokines-mediated uropod formation and ICAM-3 redistribution. T lymphoblasts (*A*) and HSB-2 T cells (*B*) were incubated for 30 min at 37°C in the presence or absence of 1 μg/ml pertussis toxin, 30 μM H-89, or 1 μM bisindolylmaleimide II. Then, T lymphoblasts (*A*) were allowed to bind to coverslips coated with ICAM-1-Fc for 30 min at 37°C, in the presence or in the absence of 10 ng/ml RANTES, MCP-1, or 5 μg/ml anti–ICAM-3 HP2/19 mAb. HSB-2 cells were allowed to bind to coverslips coated with FN80 for 30 min at 37°C. Then, fixed cells were stained for ICAM-3 and the percentage of cells bearing uropodia was measured as described in Materials and Methods. Data represent mean values (±SD) of four independent experiments performed.

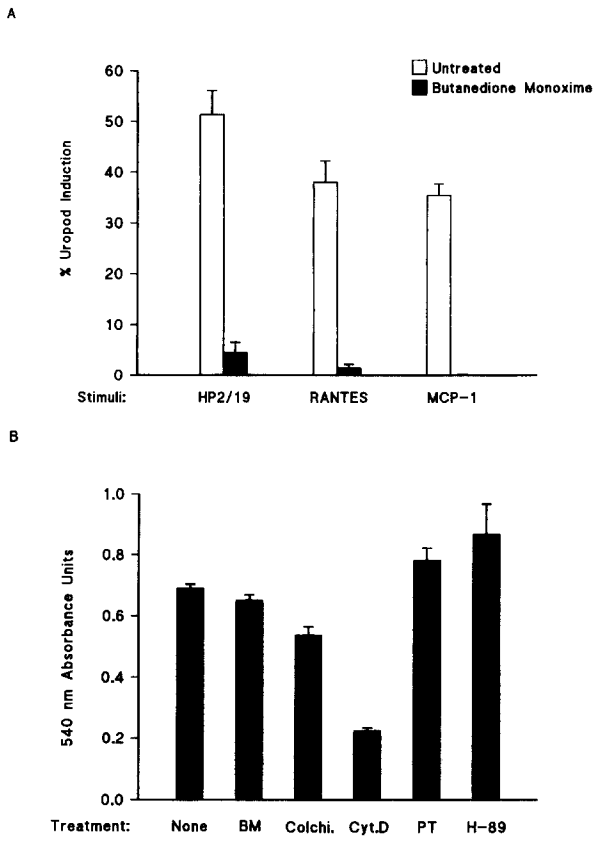


Figure 10. Myosin-disrupting drug butanedione monoxime prevents uropod formation but does not affect cell adhesion. (*A*) T lymphoblasts were incubated for 30 min at 37°C in the presence or absence of 10 mM butanedione monoxime, and then allowed to bind to coverslips coated with ICAM-1-Fc for 30 min at 37°C. Then, fixed cells were stained for ICAM-3 and the percentage of cells bearing uropodia was measured as described in Materials and Methods. Arithmetic mean ±1 SD of four separate experiments are shown. (*B*) T lymphoblasts were incubated for 30 min at 37°C in the presence or in the absence of 10 mM butanedione monoxime, 20 μM colchicine, 20 μM cytochalasin D, 1 μg/ml PT, or 30 μM H-89. Then, anti–ICAM-3 HP2/19 mAb-induced adhesion to microtiter wells coated with 20 μg/ml FN80 was assayed as previously described (Campanero et al., 1994). Arithmetic mean ±1 SD of five separate experiments run by duplicate are shown. Adhesion to albumin-coated wells was always lower than 0.12 optical density units.

and ICAM-3 reorganization induced by RANTES and MCP-1, whereas disruption of microtubules with colchicine did not inhibit the uropod formation (not shown). We have also tested the role of butanedione monoxime on cell adhesion and cell aggregation induced by the anti–ICAM-3 mAb (Campanero et al., 1993, 1994). Interestingly, butanedione monoxime did not inhibit the induction of anti–ICAM-3–mediated cell adhesion to FN80 or ICAM-1 (Fig. 10 *B*, and data not shown). The role of uropod formation and cell polarization in the acquisition of locomotor capacity was investigated through a cell aggregation assay. In this assay, cells locomote on a dish to contact each other forming cell aggregates. Cell aggregation was assayed in the presence of butanedione monoxime, and although this drug did not affect cell adhesion (Fig. 10 *B*), it completely blocked anti–ICAM-3–mediated aggregation of T cells settled on culture dishes (Fig. 11). Colchicine did neither inhibit uropod formation (data not shown) nor cell aggregation induced by the activating anti–ICAM-3 mAb (Fig. 11). These results suggest that myosin motor is required for cell polarization and aggregation but not for cell adhesion.

Discussion

It has been described that upon lymphocyte activation, cells become polarized, displaying a uropod (Wilkinson and Higgins, 1987). Lymphocyte polarization with the generation of cell uropod and ICAM-3 redistribution to this structure takes place during the interaction of T-lymphoblasts with endothelial cells, and under stimulation with an anti–ICAM-3 mAb (Campanero et al., 1994). Interestingly enough, we report herein that several chemokines are efficient physiologic triggers of uropod formation and ICAM-3, ICAM-1, CD43 and CD44 redistribution when T lym-

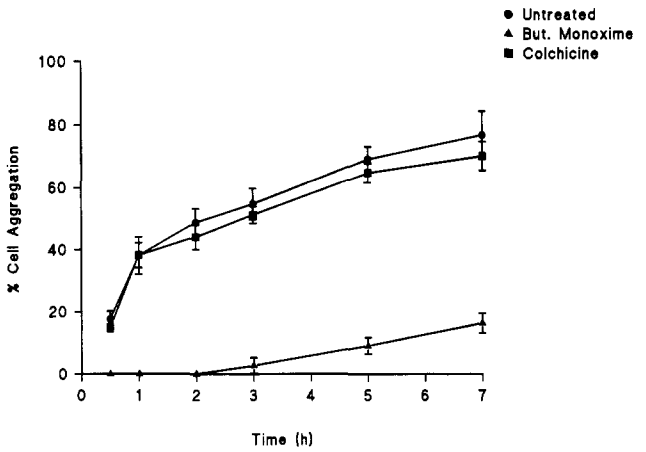


Figure 11. Myosin disruption inhibits ICAM-3–mediated cell aggregation. T lymphoblasts were incubated for 30 min at 37°C in the presence or in the absence of 10 mM butanedione monoxime, or 20 μM colchicine, before the addition of anti–ICAM-3 HP2/19 mAb. Percent of aggregation was calculated as described in Materials and Methods, at different times from the beginning of the assay. Data are representative of six experiments.

phocytes adhered to either endothelial cell adhesion molecules (VCAM-1, ICAM-1) or ECM proteins (fibronectin).

We have confirmed that in absence of stimulatory antibody, only a small and variable proportion of T lymphoblasts spontaneously display cellular uropods and ICAM-3 redistribution, and that the frequency of these phenomena is dramatically increased after the prolonged interaction of T cells with EC. This is in accordance with a previous report describing the formation of a T cell pseudopod where other adhesion molecules (namely CD2, CD44, and L-selectin) were polarized during coculture of lymphocytes and EC (Rosenman et al., 1993). Both endothelial and T cells are able to secrete a large array of chemokines during their interaction or upon specific activation with proinflammatory stimuli (Oppenheim et al., 1991). Thus, the local production of chemokines may account for the uropod formation and adhesion receptor redistribution observed during lymphocyte-EC adhesion. This phenomenon could also explain the presence of uropodia and ICAM-3 redistribution in some isolated T lymphoblasts induced by PHA.

ICAM-1, another ligand for LFA-1, was also driven to the cellular uropod upon T lymphocyte stimulation with chemokines, although in a lower proportion of cells than ICAM-3. This is in agreement with a previous report where ICAM-1 redistribution to an uropod-like structure was detected on B lymphoblastoid cells that were interacting with LFA-1–coated surfaces (Dustin et al., 1992), as well as on cytospin preparations of T and B cell lines (Dougherty et al., 1988; Carpén et al., 1992). Two other lymphocyte adhesion molecules, CD43 and CD44 were also found to be redistributed to the cellular uropod upon chemokine treatment. These two glycoproteins share some features with ICAM-3, such as their abundant expression on leukocytes, high glycosilation and involvement in leukocyte activation and aggregation. The location of all these adhesion receptors within the uropod supports a possible proadhesive role of this cellular structure. An alternative possibility is that the induced surface redistribution of these highly charged molecules to a cellular uropod may be part of a regulatory mechanism controlling the distribution of surface charges. Therefore, the recruitment of highly glycosylated molecules to the uropod, could dramatically modify the negative charge distribution of lymphocyte membrane, which might be accompanied by reduced repulsion in the area of cell adhesion. In agreement with this hypothesis, it has been reported that CD43 may have an anti-adhesive function (Ardman et al., 1992; Manjunath et al., 1993). However, other highly charged molecules as CD45 did not redistribute to the cellular uropod. On the other hand, the redistribution of certain molecules within the uropod could be part of a multi-step regulatory system of lymphocyte adhesiveness: first, the physiological stimuli, that could be the interaction with a natural ligand plus other factors as chemokines, would elicit a rapid adhesion receptor redistribution towards the uropod; second, sustained stimulation might lead to proteolytic cleavage of those molecules. In this regard, the different molecules found herein to redistribute to the cellular uropod have been described that, under certain conditions, are proteolytically cleaved and shed to the external milieu (Campanero et al., 1991; Del Pozo et al., 1994; Bazil et al., 1995).

Cell adhesion to specific integrin ligands, namely ICAM-1, VCAM-1, or FN, appears to be required for chemokine-mediated uropod induction, since mere physical adhesion of lymphocytes to poly-L-lysine is not sufficient for triggering uropod formation in the presence of chemokines. On the other hand, stimuli capable to induce cell spreading such as PMA or anti-β_1 integrin TS2/16 mAb, fail to trigger cellular uropod (Campanero et al., 1994). Therefore, cell adhesion to integrin ligands seems to be an obligatory but not sufficient requirement in the induction of lymphocyte uropod by chemokines. Interestingly, uropod induction and cell adhesion appear to be independently regulated, since they display different cytoplasmic requirements. Our findings with a myosin-disrupting drug suggest that the force generated by the contraction of myosin can drive both cell polarization and ICAM-3–induced cell aggregation, whereas the ICAM-3 induction of cell adhesion is independently regulated.

G proteins couple seven-transmembrane domain receptors to enzymatic systems like adenylate cyclase and phospholipase C (Wu et al., 1993; Murphy, 1994; Mantel et al., 1995). cAMP-dependent pathway appears to be involved in the mobility of human T lymphocyte surface molecules (Kammer et al., 1988). On the other hand, it has recently been described that MIP-1α induces an increase in intracellular cAMP level in the megakaryocytic leukemia cell line M07e (Mantel et al., 1995). Therefore, we assessed the possible implication of cAMP signaling pathways in the regulation of uropod formation and cell adhesion receptor redistribution. Our results that a membrane-permeable cAMP synthetic analogue as well as other different upregulators of cAMP induce these cellular events, underscore the role of cAMP metabolism in the regulation of these phenomena. This is further supported by the prevention of either chemokine- or ICAM-3–induced uropod formation by H-89, an inhibitor of cAMP-dependent protein kinase. The cytoskeletal association of all of the adhesion molecules redistributed (Carpén et al., 1992; Campanero et al.,

1994; Yonemura et al., 1993; Tsukita et al., 1994; Lokeshwar et al., 1994) along with the ability of cAMP-dependent kinase to trigger cytoskeletal protein phosphorylation (Selden and Pollard, 1983; Kammer et al., 1988) suggest a possible linkage. Nevertheless, this issue deserves a further understanding of the signal transduction mechanisms associated with chemokine-mediated uropod formation.

Chemokines-directed ICAM-3 redistribution requires the coupling of the G-protein signaling pathway, as suggested by their inhibition with PT. In this context, other functions induced by chemokines such as leukocyte chemotaxis (Spangrude et al., 1985), or firm adhesion (arrest) and migration of lymphocytes across EC (Bargatze and Butcher, 1993) have also been found to be dependent on this pathway, as they are blocked by PT. Interestingly, we have found that PT also blocks uropod formation induced by the activating anti–ICAM-3 mAb. This is in accordance with results recently described showing that production of cAMP is sensitive to PT (Pian and Dobbs, 1995). These data along with the inhibition of uropod formation induced by anti–ICAM-3 mAb with H-89, strongly suggest the involvement of G-protein and cAMP-dependent pathway in ICAM-3 signaling. This is not an astonishing finding since it has been described that other adhesion receptors such as CD2 are also linked to cAMP-dependent signaling (Carrera et al., 1988; Kammer et al., 1988; Hahn et al., 1991). This interesting point deserves further investigation.

The precise cell subset that is targeted by each chemokine is still in dispute, specially in the case of MIP-1α and MIP-1β (Tanaka et al., 1993*a*; Taub et al., 1993*a*,*b*; Schall et al., 1993). Moreover, it has been described that a chemokine can attract at different concentrations different lymphocyte subsets (e.g., MIP-1α for $CD8^+$ and $CD4^+$ T cells). On the other hand, RANTES and MCP-1 β-chemokines attract selectively memory T cells, T helper lymphocytes in the case of RANTES (Schall et al., 1990), and both helper and cytotoxic T cells in that of MCP-1 (Woldemar Carr et al., 1994). Interestingly, lymphocytes that are recruited into inflamed tissues bear preferentially the memory/activated $CD45RA^-$ $CD45RO^+$ phenotype (Woldemar Carr et al., 1994). Since we found that the chemokines RANTES and MCP-1 displayed the stronger capability in inducing uropods, it is feasible that this structure is involved in the recruitment and migration of memory T cells in vivo. MIP-1β showed little or no uropod inducing-effect over the $CD4^+$ T cell population; however, its effect was very strong over the $CD8^+$ T cells, isolated both from normal donors and from melanoma tumor infiltrating lymphocytes. The cellular uropod induced in TIL by chemokines may play an important role in vivo, acting as a recruiting structure to trap more $CD8^+$ T cells that, in turn, will be cytotoxic for tumor cells.

It has been proposed that chemokines exert their effects either as soluble/diffusible mediators (chemotactic mechanism) or immobilized on a cell surface (haptotactic mechanism) (Rot, 1993; Springer, 1994). Interestingly enough, we have found that chemokines are able to trigger uropod formation and ICAM-3 redistribution when T cells are bound to either EC adhesion molecules (ICAM-1 and VCAM-1) or ECM proteins (FN proteolytic fragments). Thus, it is very feasible that uropod formation occurs in vivo, during the firm adhesion of lymphocytes to endothelium.

The chemokine-mediated regulation of lymphocyte morphology and redistribution of leukocyte receptors like ICAMs, CD43, and CD44, may represent a novel mechanism contributing to the recruitment of leukocytes to inflammatory sites. In this model, chemokines would attract different leukocyte subsets which would be tethered by endothelial selectins. Chemokines would then trigger functional activation of leukocyte integrins, which mediate strong adhesion to endothelium through their interaction with their counter-receptors ICAM-1, -2, and VCAM-1. Then, leukocytes would spread on the endothelial cell surface, and at the same time the cellular uropod induced by the chemokines would emerge towards the lumen of the vessel. In this structure, the high amount of ICAM-1, -3, and likely other molecules with similar features, would facilitate the adhesion, recruitment, and activation of new passing leukocytes. Accordingly, a good exposure of the L-selectin on neutrophils or the local clustering of the β_2 integrin Mac-1 or its ligand iC3b have been shown to facilitate the intercellular interaction (Picker et al., 1991; Detmers et al., 1987; Hermanowski-Vosatka et al., 1988). In addition, the activated T cells bound to EC, would secrete more chemokines, resulting, all the above, in a positive feed back loop that could significantly reinforce the inflammatory response. Therefore, the redistribution of ICAM-3 and other adhesion receptors on the uropod may represent an amplifier mechanism of a chemotactic cascade initiated by chemokines.

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References

Acevedo, A., M. A. del Pozo, A. G. Arroyo, P. Sánchez-Mateos, R. González-Amaro, and F. Sánchez-Madrid. 1993. Distribution of ICAM-3-bearing cells in normal human tissues. Expression of a novel counter-receptor for LFA-1 in epidermal Langerhans cells. *Am. J. Pathol.* 143:774–783.

Ardman, B., M. A. Sikorski, and D. E. Staunton. 1992. CD43 interferes with T lymphocyte adhesion. *Proc. Natl. Acad. Sci. USA.* 89:5001–5005.

Arroyo, A. G., P. Sánchez-Mateos, M. R. Campanero, I. Martín-Padura, E. Dejana, and F. Sánchez-Madrid. 1992. Regulations of the VLA integrin-ligand interactions through the β1 subunit. *J. Cell Biol.* 117:659–670.

Arroyo, A. G., M. R. Campanero, P. Sánchez-Mateos, J. M. Zapata, M. A. Ursa, M. A. del Pozo, and F. Sánchez-Madrid. 1994. Induction of tyrosine phosphorylation during ICAM-3 and LFA-1-mediated intercellular adhesion, and its regulation by the CD45 tyrosine phosphatase. *J. Cell Biol.* 126: 1277–1286.

Aruffo, A., I. Stamenkovic, M. Melnick, C. B. Underhill, and B. Seed. 1990. CD44 is the principal cell surface receptor for hyaluronate. *Cell.* 61:1303–1313.

Bargatze, R. F., and E. C. Butcher. 1993. Rapid G protein-regulated activation event involved in lymphocyte binding to high endothelial venules. *J. Exp. Med.* 178:367–372.

Bazil, V. 1995. Physiological enzymatic cleavage of leukocyte membrane molecules. *Immunol. Today.* 16:135–140.

Berendt, A. R., A. McDowall, A. G. Craig, P. A. Bates, M. J. E. Sternberg, K. Marsh, C. I. Newbold, and N. Hogg. 1992. The binding site on ICAM-1 for

Plasmodium falciparum-infected erythrocytes overlaps, but is distinct from the LFA-1 binding site. *Cell.* 14:9–18.

Butcher, E. C. 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell.* 67:1033–1036.

Campanero, M. R., R. Pulido, J. L. Alonso, J. P. Pivel, F. X. Pimentel-Muiños, M. Fresno, and F. Sánchez-Madrid. 1991. Down-regulation by tumor necrosis factor alpha of neutrophil cell surface expression of the sialophorin CD43 and the hyaluronate receptor CD44 through a proteolytic mechanism. *Eur. J. Immunol.* 21:3045–3048.

Campanero, M. R., M. A. del Pozo, A. G. Arroyo, P. Sánchez-Mateos, T. Hernández-Caselles, A. Craig, R. Pulido, and F. Sánchez-Madrid. 1993. ICAM-3 interacts with LFA-1 and regulates the LFA-1/ICAM-1 cell adhesion pathway. *J. Cell Biol.* 123:1007–1016.

Campanero, M. R., P. Sánchez-Mateos, M. A. del Pozo, and F. Sánchez-Madrid. 1994. ICAM-3 regulates lymphocyte morphology and integrin-mediated T cell interaction with endothelial cell and extracellular matrix ligands. *J. Cell Biol.* 127:867–878.

Carpén, O., P. Pallai, D. E. Staunton, and T. A. Springer. 1992. Association of intercellular adhesion molecule-1 (ICAM-1) with actin-containing cytoskeleton and α-actinin. *J. Cell Biol.* 118:1223–1234.

Carrera, A. C., M. Rincón, M. O. de Landázuri, and M. López-Botet. 1988. CD2 is involved in regulating cyclic AMP levels in T cells. *Eur. J. Immunol.* 18:961–964.

Carrera, A. C., L. Cárdenas, A. Tugores, M. A. Alonso, F. Sánchez-Madrid, and M. O. de Landázuri. 1989. Activators of protein kinase up-regulate the cell surface expression of CD2 and CD5 T cell glycoproteins. *J. Biol. Chem.* 264:15650–15655.

Chijiwa, T., A. Mishima, M. Hagiwara, M. Sano, K. Hayashi, T. Inoue, K. Naito, T. Toshioka, and H. Hidaka. 1990. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquenilesulfonamide (H-89), of PC12D pheocromocitoma cells. *J. Biol. Chem.* 265:5267–5272.

de Fougerolles, A. R., and T. A. Springer. 1992. Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. *J. Exp. Med.* 175:185–190.

de Fougerolles, A. R., L. B. Klickstein, and T. A. Springer. 1993. Cloning and expression of intercellular adhesion molecule-3 reveals strong homology to other immunoglobulin family counter-receptors for lymphocyte function-associated antigen-1. *J. Exp. Med.* 177:1187–1192.

de Fougerolles, A. R., X. Qin, and T. A. Springer. 1994. Characterization of the function of intercellular adhesion molecule (ICAM)-3 and comparison with ICAM-1 and ICAM-2 in immune responses. *J. Exp. Med.* 179:619–629.

Dejana, E., S. Colella, L. R. Languino, G. Balconi, G. C. Corbascio, and P. C. Marchisio. 1987. Fibrinogen induces adhesion, spreading and microfilament organization of human endothelial cells in vitro. *J. Cell Biol.* 104:1403–1411.

del Pozo, M. A., R. Pulido, C. Muñoz, V. Alvarez, A. Humbría, M. R. Campanero, and F. Sánchez-Madrid. 1994. Regulation of ICAM-3 (CD50) membrane expression on human neutrophils through a proteolytic shedding mechanism. *Eur. J. Immunol.* 24:2586–2594.

Detmers, P. A., S. D. Wright, E. Olsen, B. Kimball, and Z. A. Cohn. 1987. Aggregation of complement receptors on human neutrophils in the absence of ligand. *J. Cell Biol.* 105:1137–1145.

Dougherty, G. J., S. Murdoch, and N. Hogg. 1988. The function of human intercellular adhesion molecule-1 (ICAM-1) in the generation of an immune response. *Eur. J. Immunol.* 18:35–39.

Dustin, M. L., O. Carpén, and T. A. Springer. 1992. Regulation of locomotion and cell-cell contact area by the LFA-1 and ICAM-1 adhesion receptors. *J. Immunol.* 148:2654–2663.

Fawcett, J., C. L. L. Holness, L. A. Needham, H. Turley, K. C. Gatter, D. Y. Mason, and D. L. Simmons. 1992. Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes. *Nature (Lond.).* 360:481–484.

Gilat, D., R. Hershoviz, Y. A. Mekori, I. Vlodavsky, and O. Lider. 1994. Regulation of adhesion of $CD4^+$ T lymphocytes to intact or heparinase-treated subendothelial extracellular matrix by diffusible or anchored RANTES and MIP-1β. *J. Immunol.* 153:4899–4905.

Hahn, W. C., Y. Rosenstein, S. J. Burakoff, and B. E. Bierer. 1991. Interaction of CD2 with its ligand lymphocyte function-associated antigen-3 induces adenosine 3′,5′-cyclic monophosphate production in T lymphocytes. *J. Immunol.* 147:14–21.

Hermanowski-Vosatka, A., P. A. Detmers, O. Gotze, S. C. Silverstein, and S. D. Wright. 1988. Clustering of ligand on the surface of a particle enhances adhesion to receptor-bearing cells. *J. Biol. Chem.* 263:17822–17827.

Hernández-Caselles, T., G. Rubio, M. R. Campanero, M. A. del Pozo, M. Muro, F. Sánchez-Madrid, and P. Aparicio. 1993. ICAM-3, the third LFA-1 counterreceptor, is a co-stimulatory molecule for both resting and activated T lymphocytes. *Eur. J. Immunol.* 23:2799–2806.

Huber, A. R., S. L. Kunkel, R. F. Todd, and S. J. Weiss. 1991. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Science (Wash. DC).* 254:99–102.

Juan, M., O. Viñas, M. R. Pino-Otín, L. Places, E. Martínez-Cáceres, J. J. Barceló, A. Miralles, R. Vilella, M. A. de la Fuente, J. Vives, et al. 1994. CD50 (intercellular adhesion molecule 3) stimulation induces calcium mobilization and tyrosine phosphorylation through $p59^{fyn}$ and $p56^{lck}$ in Jurkatt T cell line. *J. Exp. Med.* 179:1747–1756.

Kammer, G. M., C. A. Boehm, S. A. Rudolph, and L. A. Schultz. 1988. Mobility of human T lymphocyte surface molecules CD3, CD4, and CD8: regulation by cAMP-dependent pathway. *Proc. Natl. Acad. Sci. USA.* 85:792–796.

Kelner, G. S., J. Kennedy, K. B. Bacon, S. Kleyensteuber, D. A. Largaespada, N. A. Jenkins, N. G. Copeland, J. Fernando Bazan, K. W. Moore, T. J. Schall, et al. 1994. Lymphotactin: a cytokine that represents a new class of chemokine. *Science (Wash. DC).* 266:1395–1399.

Koopman, G., Y. van Kooyk, M. de Graaff, C. J. L. M. Meyer, C. G. Figdor, and S. T. Pals. 1990. Triggering of the CD44 antigen on T lymphocytes promotes T cell adhesion through the LFA-1 pathway. *J. Immunol.* 145:3589–3593.

Kozawa, O., H. Tokuda, M. Miwa, J. Kotoyori, and Y. Oiso. 1992. Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E_2 in osteoblast-like cells. *Exp. Cell Res.* 198:130–134.

Lokeshwar, V. B., N. Freigen, and L. Y. W. Bourguignon. 1994. Ankyrin-binding domain of CD44 (GP85) is required for the expression of hyaluronic acid–mediated adhesion function. *J. Cell Biol.* 126:1099–1109.

Maghazachi, A. A., A. Al-Aoukaty, and T. J. Schall. 1994. C-C Chemokines induce the chemotaxis of NK and IL-2-activated NK cells. Role for G proteins. *J. Immunol.* 153:4969–4977.

Manjunath, N., R. S. Johnson, D. E. Staunton, R. Pasqualini, and B. Ardman. 1993. Targeted disruption of CD43 gene enhances T lymphocyte adhesion. *J. Immunol.* 151:1528–1534.

Mantel, C., S. Aronica, Z. Luo, M. S. Marshall, Y. J. Kim, S. Cooper, N. Hague, and H. E. Broxmeyer. 1995. Macrophage inflammatory protein-1α enhances growth factor-stimulated phosphatidylcholine metabolism and increases cAMP levels in the human growth factor-dependent cell line M07e, events associated with growth suppression. *J. Immunol.* 154:2342–2350.

Miller, D., and M. Krangel. 1992. Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. *Crit. Rev. Immunol.* 12: 17–46.

Murphy, P. M. 1994. The molecular biology of chemoattractant receptors. *Annu. Rev. Immunol.* 12:593–633.

Nong, Y. H., E. Remold-O'Donnel, T. W. Le Bien, and H. G. Remold. 1989. A monoclonal antibody to sialophorin (CD43) induces homotypic adhesion and activation of human monocytes. *J. Exp. Med.* 170:259–267.

Oppenheim, J. J., C. O. Zacharie, N. Mukaida, and K. Matsushima. 1991. Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu. Rev. Immunol.* 9:617–648.

Pian, M. S., and L. G. Dobbs. 1995. Evidence for Gβγ-mediated cross-talk in primary culture of lung alveolar cells. Pertussis toxin-sensitive production of cAMP. *J. Biol. Chem.* 270:7427–7430.

Picker, L. J., R. A. Warnock, A. R. Burns, C. M. Doerschuk, E. L. Berg, and E. C. Butcher. 1991. The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. *Cell.* 66:921–933.

Pulido, R., M. Cebrián, A. Acevedo, M. O. de Landázuri, and F. Sánchez-Madrid. 1988. Comparative biochemical and tissue distribution study of four distinct CD45 antigen specificities. *J. Immunol.* 140:3851–3857.

Pulido, R., M. J. Elices, M. R. Campanero, L. Osborn, S. Schiffer, A. García-Pardo, R. Lobb, M. E. Hemler, and F. Sánchez-Madrid. 1991. Functional evidence for three distinct and independent inhibitable adhesion activities mediated by the human integrin VLA-4. *J. Biol. Chem.* 266:10241–10245.

Rosenman, S. J., A. A. Ganji, T. F. Tedder, and W. M. Gallatin. 1993. *Syn*-capping of human T lymphocyte adhesion/activation molecules and their redistribution during interaction with endothelial cells. *J. Leuk. Biol.* 53:1–10.

Rot, A. 1993. Neutrophil attractant/activation protein-1 (interleukin-8) induces *in vitro* neutrophil migration by haptotactic mechanism. *Eur. J. Immunol.* 23: 303–306.

Sánchez-Mateos, P., A. G. Arroyo, M. A. Balboa, and F. Sánchez-Madrid. 1993. Post-receptor occupancy events in leukocytes during β1 integrin-ligand interactions. *Eur. J. Immunol.* 23:2642–2648.

Schall, T. J. 1991. Biology of the RANTES/SIS cytokine family. *Cytokine.* 3: 165–183.

Schall, T. J., K. Bacon, K. J. Toy, and D. V. Goeddel. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature (Lond.).* 347:669–671.

Schall, T. J., and K. B. Bacon. 1994. Chemokines, leukocyte trafficking, and inflammation. *Curr. Opin. Immunol.* 6:865–873.

Schall, T. J., K. Bacon, R. D. R. Camp, J. W. Kaspari, and D. V. Goeddel. 1993. Human macrophage inflammatory protein α (MIP-1α) and MIP-1β chemokines attract distinct populations of lymphocytes. *J. Exp. Med.* 177:1821–1825.

Selden, S. C., and T. D. Pollard. 1983. Phosphorylation of microtubule-associated proteins regulates their interactions with actin filaments. *J. Biol. Chem.* 258:7064–7071.

Spangrude, G. J., F. Sacchi, H. R. Hill, D. E. van Epps, and R. A. Daynes. 1985. Inhibition of lymphocyte and neutrophil chemotaxis by pertussis toxin. *J. Immunol.* 135:4135–4143.

Springer, T. A. 1990. Adhesion receptors of the immune system. *Nature (Lond.).* 346:425–434.

Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell.* 76:301–314.

Tanaka, Y., D. H. Adams, S. Hubscher, H. Hirano, U. Siebenlist, and S. Shaw. 1993*a*. T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-

1β. *Nature (Lond.)*. 361:79–82.

Tanaka, Y., D. H. Adams, and S. Shaw. 1993*b*. Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. *Immunol. Today*. 14:111–115.

Tanaka, Y., D. H. Adams, and S. Shaw. 1993*c*. Regulation of leukocyte recruitment by proadhesive cytokines immobilized on endothelial proteoglycan. *Curr. Top. Microbiol. Immunol.* 184:99-106.

Taub, D. D., K. Conlon, A. R. Lloyd, J. J. Oppenheim, and D. J. Kelvin. 1993*a*. Preferential migration of activated $CD4^+$ and $CD8^+$ T cells in response to MIP-1α and MIP-1β. *Science (Wash. DC)*. 260:355–358.

Taub, D. D., A. R. LLoyd, K. Conlon, J. M. Wang, J. R. Ortaldo, A. Harada, K. Matsushima, D. J. Kelvin, and J. J. Oppenheim. 1993*b*. Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. *J. Exp. Med.* 177:1809–1814.

Toullec, D., P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Akajane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle, et al. 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* 266:15771–15781.

Tsukita, S., K. Oishi, N. Sato, J. Sagara, A. Kawai, and S. Tsukita. 1994. ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. *J. Cell Biol.* 126:391–401.

Vazeux, R., P. A. Hoffman, J. K. Tomita, E. S. Dickinson, R. L. Jasman, T. St. John, and W. M. Gallatin. 1992. Cloning and characterization of a new intercellular adhesion molecule ICAM-R. *Nature (Lond.)*. 360:485–488.

Vartdal, F., G. Kvalheim, T. E. Lea, V. Bosnes, G. Gaudernack, J. Ugelstad, and D. Albrechtsen. 1987. Depletion of T lymphocytes from human bone marrow. Use of magnetic monosized polymer microspheres coated with T lymphocyte-specific monoclonal antibodies. *Transplantation*. 43:366–371.

Wilkinson, P. C., and A. Higgins. 1987. OKT3-activated locomotion of human blood lymphocytes: a phenomenon requiring contact of T cell with Fc receptor-bearing cells. *Immunology*. 60:445–451.

Woldemar Carr, M., S. J. Roth, E. Luther, S. S. Rose, and T. A. Springer. 1994. Monocyte chemoattractant protein 1 acts as a T lymphocyte chemoattractant. *Proc. Natl. Acad. Sci. USA*. 91:3652–3656.

Wu, D., G. J. LaRosa, and M. I. Simon. 1993. G-protein coupled signal transduction pathways for interleukin-8. *Science (Wash. DC)*. 261:101–103.

Yonemura, S., A. Nagafuchi, N. Sato, and S. Tsukita. 1993. Concentration of an integral membrane protein, CD43 (leukosialin, sialophorin), in the cleavage furrow through the interactions of its cytoplasmic domain with actin-based cytoskeletons. *J. Cell Biol.* 120:437–449.

Zong, Z. P., K. Fujikawa-Yamamoto, K. Teraoka, H. Yamagishi, M. Tanino, and S. Odashima. 1994. Potentiation of K252a, a protein kinase inhibitor-induced polyploidization by cAMP in cultured fibrosarcoma cell line. *Biochem. Biophys. Res. Commun.* 205:746–750.