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). Chemokines are a family of 70–80 aa...
The interaction of lymphocyte function–associated antigen-1 (LFA-1) integrin with its ligands intercellular adhesion molecule-1 (ICAM-1) and fibronectin, and that RANTES, IP-10, MIP-1α, and β, and MCP-1 have been described (Campanero et al., 1991, 1993; Pilduo et al., 1988, 1991; Carrera et al., 1989). The anti-ICAM-1 MAb 111 was a generous gift of Dr. V. Horejsi (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Videnka, Czech Republic). Recombinant human (rh) RANTES (specific activity 2-5 × 10^5 U/mg, purity >97%, endotoxin level <0.1 ng/μg cytokine) and rhMIP-1β (specific activity 1.6-2.5 × 10^5 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 × 10^5 U/mg, purity >95%) was produced by Genethel (San Francisco, CA), and IFN-γ (spec. act. 2 × 10^6 U/mg, purity >98%, LPS content <0.048 ng/ml) was a gift from Amgen Biologicals (Thursday Oaks, CA). Prostaglandin E2, 8-Bromo-cAMP, mutanexone monoxide, colchicine, and cytochalasin D were purchased from Sigma Chemical Co. (St. Louis, MO). Forskolin, pertussis toxin, H-89 (N-[2-(p-bromocinnamylamino)ethyl]-3-isouquinolinesulfonamide), and bisindolylmaleimide II (BIM II) were from Calbiochem (San Diego, CA).

**Protein Substrata**

Recombinant chimeric ICAM-1-Fc and pVCAM-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 cDNA cloned in pCD8/G1). After 4 d, culture supernatants were precipitated with ammonium sulphate, and then after 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. Garcia-Pardo (Centro de Investigaciones Biológicas, Madrid, Spain). Poly-L-lysine and fibroinogen 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% (Pharma-\(\)) 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.
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 mAbs, 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-ultralow 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 × 10⁴ T lymphoblasts were plated 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 mAbs were added and cells were allowed to settle in a cell incubator at 37°C and 5% CO₂ 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% NaN₃). To visualize different membrane adhesion molecules, cells were stained with specific mAbs. 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. Preparations were incubated with a biotinylated anti-avidin (Vector Labs., Inc.) for 30 min. Biotinylated avidin and uropod-bearing cells. Preparations were photographed on either ekchrome 400 (color pictures) or TMAX 400 (black and white) film (Kodak). The latter was processed to 800–1600 ASA with TMAX developer (Kallestad, Chaska, MN). For inhibition assays, cells were pre-treated 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 culuture, 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 punctuate 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., 1993a, 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
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 dis-
played a uniform distribution over the rounded surface of
the cell.

Other adhesion molecules and leukocyte cell surface an-
tigens were also tested for their ability to redistribute to-
wards 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 colo-
calized in the same cellular uropod, although ICAM-1,
CD43, and CD44 were present in a lower proportion of

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 represent-
tive experiment, out of five independent ones on each substrate and performed with T lymphoblasts obtained from different normal
donors, is shown.

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-Lys 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 inde-
pendent 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 observed 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 (PGE₂ 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-isoquenilenesulfonamide), 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 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 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 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-
Treatment: None RANTES MCP-1 MIP-1α MIP-1β IL-8 TNF-α

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 or in the absence of 2 μM PGE2, 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 three separate experiments performed with TIL from two different patients are shown.

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 PGE2, 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.

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

| Pretreatment | Prestimuli | 8-Br-cAMP | PT | PT + 8-Br-cAMP |
|--------------|------------|-----------|----|----------------|
| None         | 15 ± 2     | 44 ± 3    | 6 ± 2 | 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.

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.
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 1 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.

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-lymphot blasts 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-
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 glycosylation 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-β1 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 cytostatic 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).
VCAM-1) or ECM proteins (FN proteolytic fragments). We have found that chemokines are able to trigger uropod formation as a recruiting structure to trap more CD8+ T cells that, in their turn, 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 β2 integrin Mac-1 or its ligand iC3b have been shown to facilitate the intercellular interaction (Picker et al., 1991; Detmers et al., 1987; Hermanowski-Vosatk et al., 1988). In addition, the activated T cells bound to EC, would secrete more chemokines, resulting, all the above, in a positive feedback 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.

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., 1993a; Taub et al., 1992a,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 uropod formation, 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 β2 integrin Mac-1 or its ligand iC3b have been shown to facilitate the intercellular interaction (Picker et al., 1991; Detmers et al., 1987; Hermanowski-Vosatk et al., 1988). In addition, the activated T cells bound to EC, would secrete more chemokines, resulting, all the above, in a positive feedback 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.
Plasmodium falciparum-infected erythrocytes overlaps, but is distinct from the LFA-1 binding site. Cell 149:18.

Butcher, E. C. 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell. 57:1033-1036.

Campanero, M. R., P. Sanchez-Mateos, M. A. del Pozo, T. Heras-Campos, A. C. Carra, R. Pulido, and F. Sanchez-Madrid. 1994. 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. 1993. ICAM-3 regulates lymphocyte morphology and intracellular compartmentalization. Cell. 72:867-876.

Carré, O., P. Pallai, D. E. Staunton, and T. A. Springer. 1992. Association of integrin αvβ3 with actin-containing cytoskeletal filaments 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. 18:1056.

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. 264:15007-15013.

Chijiwa, T., A. Mishina, M. Hayashi, M. Sano, K. Hayashi, T. Inoue, K. Naito, T. Toshiko, 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. J. Biochem. 107:1549-1554.

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. Álvarez, A.umbria, 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 membrane expression on human neutrophils through a proteolytic shedding mechanism. Eur. J. Immunol. 24:2586-2594.

Pick, 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. Cebrón, A. Acevedo, M. O. de Landázuri, and F. Sánchez-Madrid. 1988. Comparative biochemical and tissue distribution study of four distinct CD45 antigen specificities. Eur. J. Immunol. 18:35-39.

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. Cebrón, A. Acevedo, M. O. de Landázuri, and F. Sánchez-Madrid. 1988. Comparative biochemical and tissue distribution study of four distinct CD45 antigen specificities. Eur. J. Immunol. 18:35-39.

Pulido, R., M. Cebrón, A. Acevedo, M. O. de Landázuri, and F. Sánchez-Madrid. 1988. Comparative biochemical and tissue distribution study of four distinct CD45 antigen specificities. Eur. J. Immunol. 18:35-39.
Tanaka, Y., D. H. Adams, and S. Shaw. 1993a. Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. *Immunol. Today.* 14:111-115.

Tanaka, Y., D. H. Adams, and S. Shaw. 1993b. 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. 1993a. 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. Ortuño, A. Harada, K. Matsushina, D. J. Kelvin, and J. J. Oppenheim. 1993b. 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. Planetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Akajane, V. Baudet, P. Boissin, E. Boursier, F. Lottiolle, et al. 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *Z. Biol. Chem.* 266:15771-15781.

Tsukita, S., K. Oishi, N. Sato, J. Sagara, A. Kawai, and S. Tsukita. 1993. 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. Dickinon, 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. Bonne, 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.

Yosumura, 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. Terazaka, 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.