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REGULATION OF LOCOMOTION AND CELL-CELL CONTACT AREA BY THE LFA-1 AND ICAM-1 ADHESION RECEPTORS[1]

MICHAEL L. DUSTIN,[2*] OLLI CARPEN,[3†] AND TIMOTHY A. SPRINGER[4†]

*From the *Center for Blood Research, 800 Huntington Avenue, Boston, MA 02115 and the †Committee on Cell and Developmental Biology and Department of Pathology, Harvard Medical School, Boston, MA 02115*

We demonstrate complementary differences in the behavior of B lymphoblastoid cells adhering to LFA-1 or its counter-receptor ICAM-1. The interaction of B lymphoblastoid cells with glass-supported planar bilayers bearing LFA-1 or ICAM-1 was observed by time-lapse video microscopy, and the distribution of adhesion receptors on cells interacting with the planar bilayers was studied by immunofluorescence microscopy. B lymphoblasts formed a large contact area and crawled rapidly (up to 25 μm/min) on planar bilayers bearing ICAM-1. In contrast, these cells attached to planar bilayers bearing LFA-1 through a fixed point about which the cells actively pivoted, using a single stalk-like projection. Phorbol ester-stimulated lymphoblasts, which adhere more strongly to ICAM-1-bearing substrates than unstimulated lymphoblasts, were still capable of locomotion on ICAM-1. Phorbol ester stimulation of B lymphoblasts on planar bilayers bearing LFA-1 promoted a rapid conversion from "stalk" attachment to symmetrical spreading of the cell on the substrate. Cellular LFA-1 remained uniformly distributed on the cell surface during interaction with bilayers bearing purified ICAM-1 as determined by immunofluorescence. In contrast, ICAM-1 was concentrated in the stalk-like structure through which the unstimulated B lymphoblasts adhered to LFA-1 in planar bilayers, but ICAM-1 immunofluorescence became more uniformly distributed over the cell surface within minutes of phorbol ester addition. Neither LFA-1 or ICAM-1 colocalized with the prominent staining of filamentous actin in the ruffling membrane regions. Interaction through cell surface LFA-1 and ICAM-1, 2, or 3 promotes different cellular morphologies and behaviors, the correlation of which with previously observed patterns of lymphocyte interaction with different cell types is discussed.

Lymphocytes partake in diverse, reversible adhesive interactions with other cells and the extracellular matrix. Adhesion of lymphocytes to other cells is essential for hematopoiesis, killing of cells by lymphocytes, communication between Th lymphocytes and APC, immune surveillance, and multiple steps of lymphocyte recirculation (1). Furthermore, lymphoid cells are thought to cluster with dendritic or stromal cell types to form critical environments in lymphoid tissues for generation of primary immune responses (2). The majority of lymphocyte adhesion mechanisms are heterophilic (between different molecules), suggesting the availability of a polarity or sidedness for the regulation of lymphocyte interactions. One of the best studied of these adhesion mechanisms is that between the integrin LFA-1 and the Ig superfamily member ICAM-1 (3, 4). This mechanism has been studied with purified components adsorbed to plastic substrates or incorporated in artificial planar phospholipid bilayers. The use of substrates containing purified LFA-1 or ICAM-1 allows examination of the activities mediated by cellular ICAMs[5] and cellular LFA-1 in a reciprocal manner. Adhesion of lymphocytes to purified ICAM-1 is dependent upon divalent cations and temperature; adhesion of lymphocytes to purified LFA-1 is also dependent on divalent cations but is only moderately temperature dependent. Purified LFA-1 supports adhesion of cells through cell-surface ICAM-1, ICAM-2, and ICAM-3 (5–7). Cellular and purified LFA-1 appear to have identical ligand binding specificity, binding to ICAMs but not other cell surface molecules, and the same relative preference for ICAMs, with a higher apparent avidity for ICAM-1, and lower and equivalent avidity for ICAM-2 and ICAM-3 (7).

Adhesion mediated by interaction of LFA-1 with ICAMs displays dramatic sidedness in the regulation of adhesiveness; avidity of LFA-1 for ICAM-1 is increased by lymphocyte activation, e.g., by TCR cross-linking or phorbol ester treatment, whereas avidity of ICAMs for LFA-1 is constitutively high (5). Such sidedness in regulation of adhesiveness may be of wide importance, because a rapid increase in avidity in response to lymphocyte activation has been established for multiple members of the integrin family and also for the Ig family member CD8 and for L-selectin (8–12). It is likely that sidedness of adhesion mechanisms may be manifested in lymphocyte activities besides the regulation of avidity. Here, a striking example of this sidedness is revealed in the motility and morphol-

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[2] Current address: Division of Hematology/Oncology, Washington University School of Medicine, Box 8125, 660 S. Euclide Ave., St Louis, MO 63110.
[3] Current address: Department of Pathology, University of Helsinki, Hartmanukatsu 3, 00290 Helsinki, Finland.
[4] Address correspondence and reprint requests to Timothy A. Springer, Ph.D., The Center for Blood Research, 800 Huntington Ave., Boston, MA 02115.

[5] Abbreviations used in this paper: ICAMs: ICAM-1, 2, or 3; f-actin; filamentous actin; HUVEC, human umbilical vein endothelial cell; OG, octyl-β-D-glucopyranoside; TS, 25 mM Tris HCl pH 8.0, 0.15 M NaCl; TSA, 25 mM Tris HCl pH 8.0, 0.15 M NaCl, 0.02% sodium azide; LGL, large granular lymphocytes.

ogy of B lymphoblasts on planar bilayers reconstituted with purified LFA-1 or ICAM-1. The results of time-lapse videomicroscopy reveal that adhesion mediated by cell surface LFA-1 or ICAMs have different sequelae. B lymphoblasts displayed locomotion on planar bilayers containing ICAM-1. In contrast, B lymphoblasts became anchored to substrates bearing LFA-1 through a stalk-like projection of the cell and did not have motion despite cell motility about the site of attachment. The distribution of LFA-1 on cells interacting with planar bilayers bearing ICAM-1 appeared uniform over the cell surface. In contrast, ICAM-1 appeared to be concentrated in the stalk of cells adhering to LFA-1 in planar bilayers. Phorbol esters triggered a redistribution of cell surface ICAM-1 over the cell surface and spreading of B lymphoblasts on planar bilayers containing LFA-1, but did not stimulate motility. These observations have implications for the mechanism of locomotion of cells over the surface of other cells and for the regulation of contact area between cells.

MATERIALS AND METHODS

Cell line. The B lymphoblastoid cell line JY was obtained from Dr. J. Strominger (Dana Farber Cancer Institute, Boston, MA) and was grown in RPMI 1640 with 10% FCS (Flow Labs, McLean, VA). The T lymphoma cell line SKW3 was obtained from Dr. P. Cresswell (Duke University, Durham, NC). The cervical carcinoma cell line HeLa was obtained from American Type Culture Collection (Rockville, MD). These lines were grown in RPMI-1640 with 10% FCS (Flow Labs, McLean, VA). HUVEC[5] were a gift of Dr. M. Gimbrone, Jr. (Brigham and Women's Hospital, Boston, MA) and were grown for 4 to 10 passages (1 : 5) with endothelial cell growth supplement (Chemicon, El Segundo, CA) and heparin (13). Monodispersed suspensions of HeLa and HUVEC were prepared identically by trypsinization with 1/2 × trypsin-EDTA (GIBCO, Grand Island, NY).

Monoclonal antibodies. Anti-LFA-1 mAb TS1/22 (IgG1)and TS2/4 (IgG1), both specific for the α subunit, TS1/18 (IgG1) specific for the β subunit (14) and anti-ICAM-1 mAb RR1/1 (IgG1) (15) and R6.5 (IgG2a) (16) were purified from ascites fluid by protein A affinity chromatography. Antibodies were coupled to CNBr-activated Sepharose CL-4B beads (17) at between 1 and 5 mg/ml. mAb were iodinated to a specific activity of 5 to 10 μCi/μg using Iodogen (18) (Pierce, Rockford, IL).

Purification of adhesion molecules. JY cells were lysed in TSA plus 1% Triton X-100, 50 millitrypsin inhibitor U/ml aprotinin, 1 mM phenylmethylsulfonylfluoride and 0.5 mM EDTA at 5 × 10^7 cells/ml. After 30 min at 4°C, the lysate was clarified by centrifugations at 1000 × g for 10 min then 100,000 × g for 1 h.

Analytical immunoaffinity isolation was performed by incubating 0.5 ml of lysate with 20 μl of mAb-Sepharose overnight at 4°C. The mAb-Sepharose was washed three times with 1% Triton X-100 in TSA and then twice in 2.5 mM Tris pH 8.0, 15 mM NaCl with no detergent in the presence or absence of 2 mM Mg^{2+} or Ca^{2+}. The mAb-Sepharose samples were subsequently processed for SDS-PAGE (19) or incubated in 0.1 M triethylamine (high pH), 0.15 M NaCl, 1% Triton X-100 at pH 11.5 for 30 min at 4°C in the presence or absence of 2 mM Mg^{2+} or Ca^{2+} as in the wash step. The supernatants from this incubation were neutralized with 50 μl of 1 M Tris pH 7.0 and then reprecipitated with 20 μl of TS1/18 or TS1/22-Sepharose in the presence of the same divalent cations as in the first wash and elution. These mAb-Sepharose samples were washed as above, and bound protein was eluted and subjected to SDS-PAGE. Prior to SDS-PAGE, proteins were eluted from mAb beads by heating to >80°C with sample buffer without a reducing agent. The eluate was separated from the beads, 2.5% 2-ME was added, the samples were heated to >80°C again and loaded on gels. Proteins in gels were detected by silver staining (20).

Preparative scale immunoaffinity isolation was performed by passing 500 ml of lysate over sequential bovine IgG, TS2/4-, and RR1/1-Sepharose CL-4B columns (5 ml at 3 mg/ml) at a flow rate of 1 ml/min. The latter two columns were washed with 10-column bed vol of 1% Triton X-100 in TSA. The TS2/4 column was then washed with 5 vol of TSA, 5 mM $MgCl_2$, 1% OG. LFA-1 was eluted with 10 vol of 1% OG in 50 mM triethylamine pH 11.5, 150 mM NaCl, 2 mM $MgCl_2$ at a flow rate of 0.3 ml/min. Fractions (2 ml) were collected into tubes with 0.2 ml 1% OG in 1 M Tris pH 7.5, 5 mM $MgCl_2$. The RR1/1 column was washed with 5 vol of 50 mM triethylamine pH 11, 150 mM NaCl, 1% OG. ICAM-1 was eluted with the same buffer adjusted to pH 12.5 with NaOH. Fractions (2 ml) were collected into 1 M Tris pH 7.0, 1% OG. Fractions were assayed for LFA-1 or ICAM-1 by immunoradiometric assay after diluting 5 μl of each fraction into 45 μl of TS in wells of polystyrene microtiter plates, incubating 1 h at 24°C to allow binding to plastic, blocking with 10 mg/ml BSA, and binding 10 μg/ml ^{125}I-mAb for 1 h at 4°C. Homogeneity of immunoaffinity purified proteins was analyzed by SDS-PAGE and silver staining (20, 21).

Reconstitution of adhesion molecules. Purified proteins in 1% OG were incorporated into liposomes of egg phosphatidylcholine and cholesterol (7 : 2) by dialysis (22). Column fractions were diluted between 1 : 1 and 1 : 63 into TS with a final concentration of 51 mM OG, 0.2 mM phospholipid and 2 mM $MgCl_2$. Mg^{2+} is not required for ICAM-1 but can be included without altering the properties of subsequently formed planar bilayers. The protein/lipid/detergent solution was dialyzed against three changes of TS plus 2 mM $MgCl_2$ over 36 h. Glass-supported planar bilayers were prepared by the procedure of Brian and McConnell (23) with the following modification. To quantitate cell adhesion and receptor densities, cleaned glass coverslips (5 mm, Bellco, Vineland, NJ) were glued to the bottom of microtiter wells with silicon adhesive (Bostik, Huntington Valley, PA, or General Electric Co., Waterford, NY). Drops (20 μl) of the 0.2 mM proteoliposome suspension were placed on the anchored coverslips and incubated for 20 to 30 min at 24°C. The wells were flooded with 200 μl of RPMI 1640, 25 mM HEPES, 5% FCS (assay media) and washed five times with assay media aspirating down to ~40 μl each time. Videomicroscopy was performed with planar bilayers formed on 12-mm coverslips in the bottom of 24-well culture plates by "flotation" of the cleaned coverslip on a 70-μl drop of 0.2 mM proteoliposome suspension exactly as described (23). Bilayers were washed with PBS/2 mM $MgCl_2$/0.025% NaN_3/1% BSA and incubated an additional 30 min in the same solution to block unbound sites on the plate. The microplate wells were then washed with the media to be used in the assay. LFA-1 or ICAM-1 density in planar bilayers was determined by ^{125}I-TS2/4 or RR1/1 binding, respectively, to bilayers in parallel to those used in adhesion and time-lapse experiments. Both mAb were used at a final concentration of 10 μg/ml, which gives >95% saturation of binding sites. Saturation conditions were used such that 10% of the labeled mAb bound. The area used for calculation of sites/μm^2 was 20 mm^2 for 5-mm coverslips, and the site number estimates were generated assuming that at saturation mAb binding occurs through only one of the combining sites (M_r = 150,000).

Binding assays. Cell lines were labeled with $Na_2{}^{51}CrO_4$. PMA was added to wells from a 1 μg/ml solution in assay media, prepared by dilution of a 1 mg/ml ethanol stock solution, to give a final concentration of 50 ng/ml. Antibodies were added to the wells 15 to 30 min prior to adding the cells. Cells (10^4-10^5) were added to microtiter wells containing coverslips with, or as a control without, LFA-1, and the plates were centrifuged at 50 × g for 5 min and incubated for 10 min at 37°C. To remove unbound cells, the wells were washed eight times with 24°C assay media using a cut-off 18-gauge hypodermic needle to aspirate all media except approximately 30 μl needed to keep the planar bilayer submerged. Bound cells were dissolved in 0.1 N NaOH and the released counts determined. Input counts were determined in parallel. The cells were examined before and after washing to assess the uniformity of settling and binding. As the coverslips cover 50% of the bottom of the wells, the percentage of cell binding was adjusted for this by dividing the input counts by 2.

Time-lapse videomicroscopy. Planar bilayers formed on one surface of 12-mm, round coverslips were washed with RPMI 1640 10% FCS and a chamber was generated in which cells could be observed with an inverted microscope after settling onto the planar bilayer. A ring of silicone grease (Dow Corning, Midland, MI) of approximately 10 mm inner diameter and 0.5 to 1 mm high was prepared on a clean glass slide. The slide was immersed in a tank of media containing the planar membrane supported on a coverslip. The coverslip was manipulated so that one edge was resting on the silicone grease ring and the other edge was raised enough to admit a micropipette tip through which 10^5 cells in 10 μl of media were slowly injected and the coverslip gently lowered so that the grease ring was uniformly compressed to give a level, sealed chamber. This procedure generates a gradient of cell densities on the planar membrane so that an appropriately low density of 10 to 20 cells/field can be obtained by scanning the membrane. Higher cell densities were not usable because of the possibility of adhesive interactions between cells. The slide was inverted and placed on the stage of a Nikon Diaphot microscope (Donsanto Co., Natick, MA) with a plexiglass incubator box warmed to 37°C. Cells were observed with phase contrast optics. The cells were allowed 15 min to warm before the beginning of the recording. The image was recorded using a Hitachi KP-130AU video camera and a Hitachi TLC 2051 video-recorder

(Donsanto) set to give 24 or 48 × normal speed on play-back. An objective lens and video projection lens were used to give a field of 366 × 288 μm on the video monitor as calibrated using the lines on a hemocytometer grid. Duration of recordings was usually 30 min, but 12- to 16-h recordings were also made using planar bilayers on the bottom of 24-well plates with 1 ml of sterile L15 media + 10% FCS. The paths of individual cells were traced from the video monitor on transparent films, and the path lengths were determined using a digitizing board and accompanying computer software (The Morphometer, Woods Hole Associates, Woods Hole, MA).

FCS was included in binding and locomotion assays to maintain viability of JY cells during experiments. Inclusion of serum, a potential source of attachment factors, did not cause interference in this system because of the inert nature of planar bilayers prepared with zwitterionic phospholipids (24). Planar bilayers without incorporated adhesion receptors did not support binding of JY cells and more impressively did not allow attachment of HeLa cells or human umbilical vein endothelial cells after 1 h at 37°C in the presence of growth media with serum (see Table I). JY cells also showed no adhesion to or locomotion over planar bilayers lacking adhesion receptors.

Immunofluorescent staining of JY cells. Permanex slides (8-chamber Lab-Tek, Nunc) were coated with purified ICAM-1 or LFA-1 in 1% OG diluted 1 : 10 with TSA overnight at 4°C, and subsequently blocked with 1% heat-treated (80°C, 20 min) BSA/2 mM $MgCl_2$ overnight at 4°C. JY cells (10^5) were added to each chamber in 200 μl and allowed to adhere for 20 min at 37°C. PMA was added to some chambers at 50 ng/ml. The cells were incubated an additional 15 min, unbound cells were aspirated from the slide, and the slides were fixed with 3.5% paraformaldehyde in PBS (pH 7.4) for 10 min at 4°C. Chambers coated with BSA were used as controls. LFA-1 was visualized with a combination of three mAb against CD11a (N217, N225, N226) obtained through the Fourth International Leukocyte Workshop and used as 1 : 500 dilution of ascites. ICAM-1 was stained with two mAb (RR1/1 and R6.5 at 10 μg/ml each). The IgG1 secreted by the myeloma P3X63 was used as a negative control for mAb staining. mAb were detected with FITC-coupled $F(ab')_2$ fragments of goat anti-mouse Ig (Tago, Burlingame, CA). After cell-surface staining, the cells were permeabilized with 0.1% Triton X-100/PBS for 5 min at 24°C. Filamentous actin was subsequently visualized with rhodamine-phallacidin (Molecular Probes, Eugene, OR). The samples were mounted in glycerol/PBS, analyzed in a Zeiss epifluorescence microscope, and photographed with Kodak T-max P3200 black and white film.

RESULTS

Immunoaffinity purification of LFA-1 heterodimers. The procedure for purifying LFA-1 with retention of its capacity to bind ICAM-1 has not been previously described. Three distinct LFA-1 mAb were compared. The α subunit mAb TS1/22 blocks LFA-1 function and binds to the denatured α subunit, whereas the α subunit mAb TS2/4 has no detectable affect on LFA-1 function and fails to bind to the α subunit when it is dissociated from the β subunit (14, 25). The β subunit specific mAb TS1/18 blocks LFA-1 function but does not bind to the dissociated β subunit (14, 25, 26). Because the LFA-1 α subunit contains putative divalent cation binding sites (27) elution from mAb-Sepharose with pH 11.5 buffer was tested in the presence or absence of Mg^{2+} or Ca^{2+}. The amount of material that remained bound to mAb-Sepharose after elution was examined directly by SDS-PAGE (Fig. 1*A*, *lanes 4–12*). The amount of eluted material that remained as intact αβ heterodimers (Fig. 1*A*, *lanes 13–21*) and the amount of eluted α subunit, whether free or complexed with the β subunit (Fig. 1*A*, *lanes 22–30*), was examined after reisolation with TS1/18 and TS1/22 mAb, respectively. Material could be eluted from the TS2/4 mAb Sepharose at pH 11.5, and, in the presence but not the absence of divalent cations, the α and β subunits remained associated in the heterodimer (Fig. 1*A*, *lanes 13–15*). Little material was eluted from TS1/22 mAb Sepharose at pH 11.5, except in the absence of divalent cations the β subunit was eluted whereas the α subunit remained bound (Fig. 1*A*, *lanes 7–9*). Most material was eluted from the TS1/18 Sepharose at pH 11.5 in the absence of divalent cations, but much less was eluted in the presence of Mg^{2+} or Ca^{2+} (Fig. 1*A*, *lanes 10–12*) and most of this material was dissociated α and β subunits. TS2/4 was superior in yield of the heterodimer (Fig. 1*A*, *lanes 13–21*). Preparative scale immunoaffinity isolation using TS2/4 with elution at pH 11.5 in the presence of 2 mM Mg^{2+} consistently yielded intact material that appeared homogeneous by SDS-PAGE and silver staining (Fig. 1*B*).

Cell binding by purified LFA-1 in planar bilayers. To validate the work described below on motility on bilayers containing LFA-1, we examined cellular adhesion to such bilayers (Table I). Binding of five human cell types to LFA-1 in glass- supported planar bilayers was efficient and was inhibited substantially by the adhesion-blocking LFA-1 mAb TS1/22 or by EDTA (Table I). ICAM-1 mAb completely inhibited binding of the epitheloid line HeLa, and inhibited binding of the B lymphoblastoid cell line JY by 57%. Binding of the Reid-Sternberg cell line L428 was inhibited 34%, and there was no inhibition of binding of HUVEC or the SKW3 T lymphoblastoid cell line. These results show that ICAM-1 is the most important of the LFA-1 ligands on JY cells and results on all five cell types corroborate recent studies with mAb to ICAM-2 and ICAM-3 (7). A combination of mAb to ICAM-1 and ICAM-2 is required to inhibit resting HUVEC adhesion to purified LFA-1, and ICAM-3 mAb inhibits SKW3 adheison to purified LFA-1 (7). The results here with LFA-1 incorporated in a lipid bilayer are very similar to results with LFA-1 adsorbed to plastic and with cell-cell homotypic adhesion (5, 7, 28) with regard to the relative contributions of different ICAMs on JY cells and the relative importance of avidity of ICAM-1, ICAM-2, and ICAM-3 on different cell types, validating the relevance of the lipid bilayer system.

Behavior of B lymphoblasts on purified LFA-1 and ICAM-1. JY cells were chosen for studies on cellular motility, morphology, and cytoskeletal organization because it was known from aggregation studies that JY and other EBV-transformed B lymphoblasts are motile cells and that their behavior in aggregation studies resembles that of lymphoblasts derived by activation of normal T and B lymphocytes (29). The density of adhesion molecules in the planar membranes was varied about the physiologic range on cell surfaces that we estimate to be on the order of 200 sites/μm^2. There was a striking difference between JY cell behavior on planar membranes bearing LFA-1 and ICAM-1. All densities of LFA-1 that allowed adhesion (50 sites/μm^2 and up) resulted in attachment of the JY cells through a stalk-like structure. The cells displayed membrane ruffling and the unattached cell body pivoted about the point of attachment (Fig. 2*A*, *arrowhead*). The site of attachment did not change over 16 h of observation with respect to the substrate, although the cell body sometimes appeared to attach to the planar membrane for periods of several minutes. In contrast, cells on bilayers bearing ICAM-1 moved several cell diameters in 10 to 20 min (up to 25 μm/min). JY cells on ICAM-1 typically resembled cells undergoing "ameboid" locomotion with a broad lamellipodium and usually a trailing uropod, although in many cases the uropod was not apparent (Fig. 2*C*). JY cells on

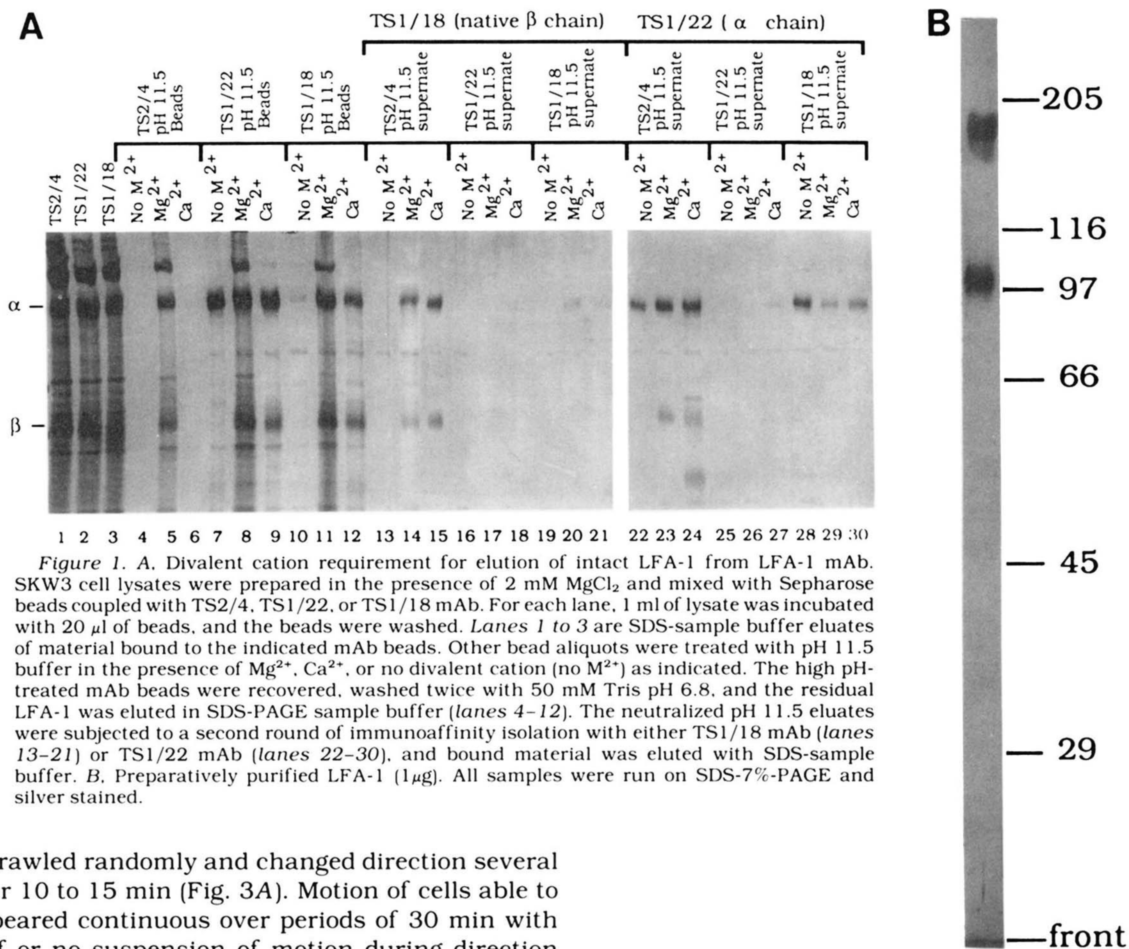

Figure 1. A. Divalent cation requirement for elution of intact LFA-1 from LFA-1 mAb. SKW3 cell lysates were prepared in the presence of 2 mM $MgCl_2$ and mixed with Sepharose beads coupled with TS2/4, TS1/22, or TS1/18 mAb. For each lane, 1 ml of lysate was incubated with 20 μl of beads, and the beads were washed. *Lanes 1 to 3* are SDS-sample buffer eluates of material bound to the indicated mAb beads. Other bead aliquots were treated with pH 11.5 buffer in the presence of Mg^{2+}, Ca^{2+}, or no divalent cation (no M^{2+}) as indicated. The high pH-treated mAb beads were recovered, washed twice with 50 mM Tris pH 6.8, and the residual LFA-1 was eluted in SDS-PAGE sample buffer (*lanes 4–12*). The neutralized pH 11.5 eluates were subjected to a second round of immunoaffinity isolation with either TS1/18 mAb (*lanes 13–21*) or TS1/22 mAb (*lanes 22–30*), and bound material was eluted with SDS-sample buffer. *B.* Preparatively purified LFA-1 (1μg). All samples were run on SDS-7%-PAGE and silver stained.

ICAM-1 crawled randomly and changed direction several times over 10 to 15 min (Fig. 3*A*). Motion of cells able to move appeared continuous over periods of 30 min with only brief or no suspension of motion during direction changes. On control bilayers lacking LFA-1 or ICAM-1, there was no adhesion and no locomotion.

Phorbol ester treatment of normal and transformed lymphocytes leads to a dramatic increase in cell surface LFA-1 avidity. Therefore, it was of interest to study whether the increase in adhesion would have any impact on cell morphology or movements on supported bilayers containing LFA-1 or ICAM-1. JY cell binding to ICAM-1 planar bilayers of 5% to 10% was increased to 60% to 80% in the presence of phorbol esters (data not shown) as was observed previously on plastic substrates (5), but a marked effect on morphology was not observed (Fig. 2*D*). In the presence of phorbol esters, the paths taken by JY cells on ICAM-1 remained apparently random with many changes of direction (Fig. 3*B*). JY cells stimulated with PMA appeared to move on ICAM-1 in a saltatory manner with stretches (5–15 min) of rapid movement punctuated by shorter (2–5 min) periods during which the cell stopped and appeared to spread. Phorbol esters had contrasting effects on JY cells on lipid bilayers containing LFA-1. Eighty percent of unstimulated JY cells adhered to LFA-1 containing planar bilayers at densities over 400 sites/μm^2 or more with half-maximal binding at 200 sites/μm^2 of LFA-1 (data not shown). Phorbol ester stimulation did not change the level of maximal binding but decreased the LFA-1 density required for half-maximal binding to 70 sites/μm^2. These results are similar to those obtained with LFA-1 adsorbed to plastic (5). Surprisingly, phorbol esters induced a dramatic change in the morphology of JY cells adhering to purified LFA-1 in planar bilayers. Populations of JY cells on LFA-1 bilayers were observed for several minutes prior to and for at least an hour after addition of phorbol esters. Initially 93.1% ± 4.3% (four experiments with 20 to 60 cells each) of JY cells were clearly attached to LFA-1 planar bilayers (200 sites/μm^2) through stalks. Within 15 min of phorbol ester addition, 73% ± 15% of the same population of JY cells spread symmetrically against the bilayer with continuation of membrane ruffling on the surface of the cells away from the LFA-1 bilayer (Fig. 2*B*). The remaining cells did not spread as dramatically but did show more restricted motion than before PMA addition.

Phorbol esters had little effect on the rate of locomotion on bilayers containing either ICAM-1 or LFA-1. The rate of movement of JY cells on ICAM-1 bilayers was broadly distributed (Fig. 4). Whereas phorbol esters tended to increase the number of immobile cells, the mean rate of movement over a wide range of ICAM-1 densities in the planar membrane was not dramatically altered (Fig. 5). However, phorbol ester treatment significantly decreased the ICAM-1 density at which random migration could be observed (Fig. 5). No cell locomotion was observed on bilayers containing LFA-1 either with or without phorbol ester addition (Fig. 4).

As JY cells moved on ICAM-1 bilayers but did not translocate on LFA-1 bilayers, it was of interest to test how the cells would behave on membranes containing both ICAM-1 and LFA-1. Bilayers containing equivalent densities of ICAM-1 and LFA-1 were prepared in parallel to the bilayers containing only ICAM-1 or LFA-1 (Fig. 4). The morphology of JY cells on mixed bilayers was more

TABLE I

Binding of cell lines expressing multiple counter-receptors to LFA-1[a]

	HeLa	JY	L428	HUVEC	SKW3
	Cells bound (%)				
Negative control (X63)	92 ± 11	70 ± 2	88 ± 6	97 ± 7	95 ± 6
LFA-1α (TS1/22)	13 ± 1	7 ± 4	5 ± 2	12 ± 2	4 ± 1
LFA-1α (TS2/4)	100 ± 5	n.d.[b]	88 ± 3	108 ± 10	95 ± 7
ICAM-1 (RR1/1)	6 ± 3	30 ± 4	58 ± 5	109 ± 3	94 ± 5
EDTA	13 ± 4	n.d.	12 ± 1	14 ± 2	4 ± 2

[a] Cells were labeled with $^{51}CrO_4$ and added to wells with planar bilayers containing LFA-1 (800 sites/μm^2) after adding mAb or EDTA. The cells were allowed to settle and bind for 1 h at 37°C. No cells were bound to LFA-1 in planar bilayers in the presence of EDTA as determined by thorough visual inspection. The counts bound in the presence of EDTA represent a systematic background from trapping of cells in the depression between the edge of the coverslip and the edge of the microtiter well. None of the cell types adhered to control membranes containing only phospholipid by visual inspection.

[b] n.d., not done.

similar to the morphology of JY cells on bilayers with ICAM-1 alone than LFA-1 alone (not shown). In all cases, a substantial proportion of cells were still able to move on these substrates, although a larger proportion of cells were immobile than on ICAM-1 alone (Fig. 4). Treatment of JY cells with phorbol esters increased the proportion of cells that moved on bilayers bearing LFA-1 and ICAM-1 (Fig. 4). Morphology of the phorbol ester-treated cells on mixed bilayers was also more similar to that of JY cells on bilayers containing ICAM-1 than LFA-1.

Localization of adhesion molecules and actin. The distribution of adhesion molecules and the force generating actin microfilament network might have a role in determining the behavior of cells on LFA-1 and ICAM-1 bearing planar bilayers. The localization of ICAM-1, LFA-1, and f-actin in cells adhering to different substrates was observed by fluorescence microscopy of fixed cells stained with fluorescein-labeled antibodies or rhodamine-phallacidin. The distribution of ICAM-1 immunofluorescence on JY cells adherent to LFA-1 bilayers was clearly nonuniform with the brightest immunofluorescence in the uropod region attached to the substrate (Fig. 6*A*). Rhodamine phallacidin fluorescence was present throughout the cytoplasm of the cells, but brighter f-actin fluorescence was present in the ruffling membrane most distant from the site of JY cell attachment to LFA-1 bilayers (Fig. 6*B*). Treatment of JY cells with phorbol esters for 15 min resulted in dispersion of ICAM-1 immunofluorescence over the surface of cells (Fig. 6*C*). Phorbol ester treatment resulted in reorganization of f-actin fluorescence into bright foci in the cytoplasm. These patches, however, did not clearly correspond to the redistributed ICAM-1 immunofluorescence, which was less patchy (Fig. 6*D*; cf. Fig. 6*C*).

The distribution of LFA-1 immunofluorescence on cells adhering to ICAM-1 in planar bilayers was uniform except for occasional brighter fluorescence in what appeared to be the trailing edge of motile cells (Fig. 6*E*). Although a leading lamellipodium was clearly identifiable in many images, no obvious increase in LFA-1 immunofluorescence in this region of the cell was observed with or without phorbol ester treatment. Cells treated with phorbol esters sometimes displayed more pronounced trailing membrane appendages attached to the substrate (Fig. 6*G*). f-Actin fluorescence was brightest in the lamellipodia of cells with polar, amoeboid morphology (Fig. 6, *F* and *H*).

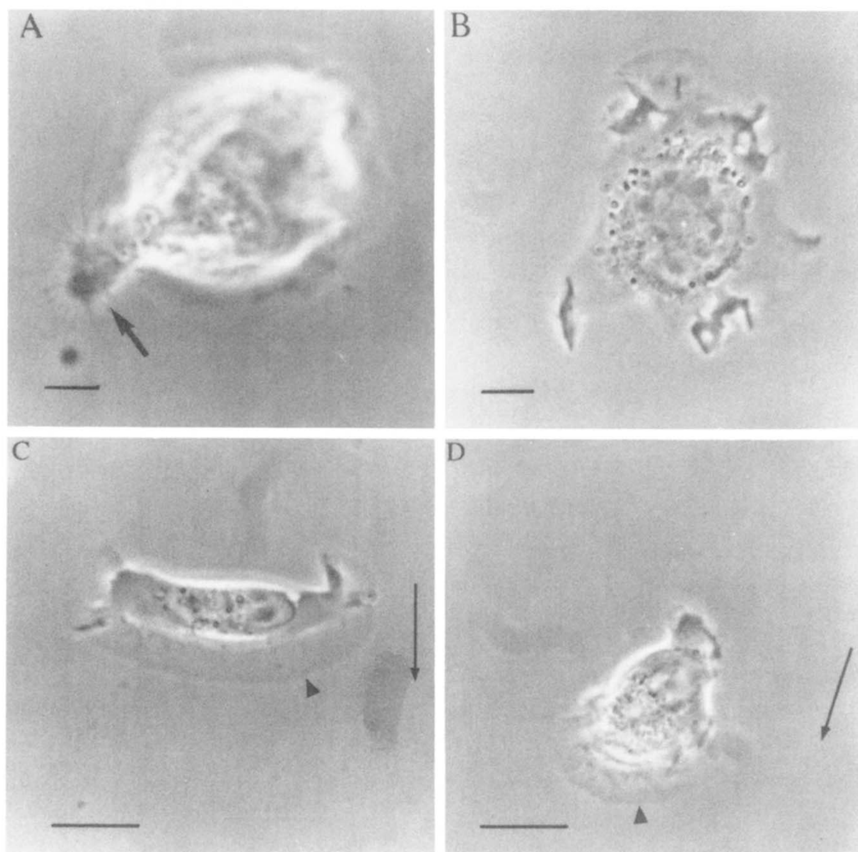

Figure 2. Morphology of JY cells adhering to LFA-1 or ICAM-1 in planar membranes. *A*, JY cells adhering to LFA-1 at 280 sites/μm^2. The *arrowheads* indicate the point of attachment to the substrate in the plane of focus. The cell body is out of focus because it is projecting out of the focal plane into the media. *B*, JY cell treated with 50 ng/ml PMA adhering to LFA-1 at 280 sites/μm^2. Note that more of the cell is in the focal plane because it is spread on the planar membrane. *C*, JY cell crawling on ICAM-1 at 350 sites/μm^2. The *arrowhead* indicates the leading edge lamellipodium. The *arrow* indicates the direction of cell motion, which was followed during photography. *D*, JY cell treated with 50 ng/ml PMA crawling on ICAM-1 present at 350 sites/μm^2. Arrows are as in *part C*. Note the more pronounced trailing extension (uropod). *Scale bar, A* and *B*, 5 μm; *C* and *D*, 12.5 μm.

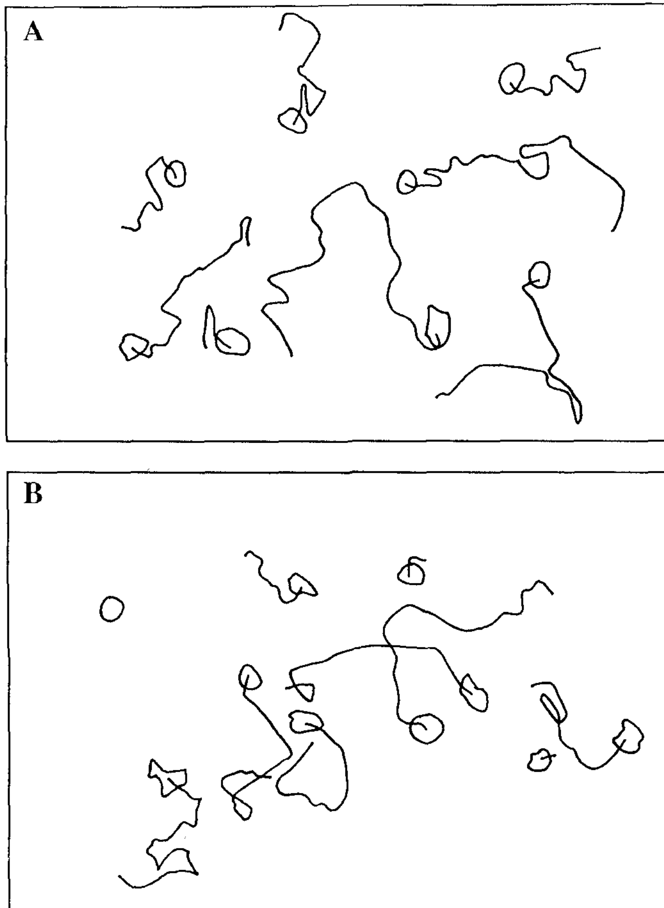

Figure 3. Random crawling of JY cells on ICAM-1 in planar bilayers. ICAM-1 was reconstituted in planar membranes at 1000 sites/μm^2. The initial position of the cell is indicated and its movement traced over a period of 21 min for untreated JY cell (*A*) or 16 min for JY cells treated with 50 ng/ml PMA (*B*). Field is 366 × 244 μm. These patterns are representative of data obtained in 11 experiments using a range of ICAM-1 densities from 300 to 1200 sites/μm^2.

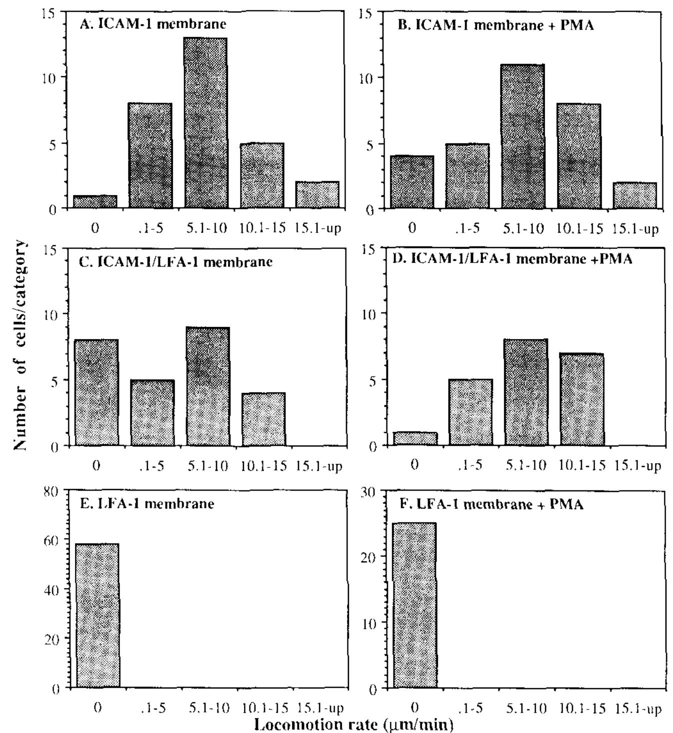

Figure 4. Locomotion rates of JY cells on adhesion receptors reconstituted in planar bilayers. Rate of locomotion of cells from one 366- × 288-μm field over a period of 20 min. ICAM-1 was at 336 sites/μm^2 and LFA-1 was at 442 sites/μm^2. PMA was at 50 ng/ml when added. Cell paths were traced from the video monitor and their length determined by digitizer and the number of cells in each range was enumerated. Similar results were obtained in a second experiment with LFA-1 and ICAM-1 combined in a single bilayer and in five different experiments comparing locomotion on ICAM-1 and LFA-1 in separate bilayers. Data for ICAM-1 and LFA-1 membranes are representative of three experiments, whereas data for mixed membranes were obtained in two of these three experiments with similar results for each trial.

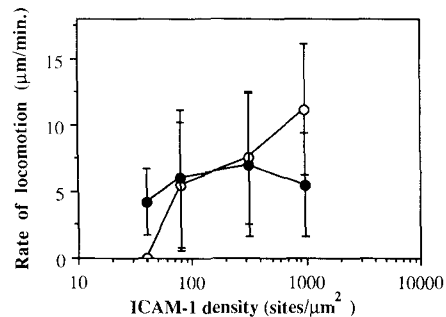

Figure 5. Effect of ICAM-1 density in the planar membrane on the average rate of JY cell locomotion. Data from a representative experiment where the rates of cell movement over 20 min were averaged for cells from one 366- × 288-μm field containing 10 to 20 cells. ○, untreated cells; ●, values (±SD) for cells treated with 50 ng/ml PMA. Two other experiments comparing at least three ICAM-1 densities yielded similar results.

DISCUSSION

We have examined the behavior of JY cells adhering to planar lipid bilayers reconstituted with purified LFA-1 or ICAM-1 cell adhesion receptors. We find that the same cells differ in ability to locomote on these substrates and show distinct differences in reorganization of contact with the substrate with regard to size and polarity. These findings demonstrate sidedness of cell behavior mediated by the distinct partners of the LFA-1/ICAM-1 adhesion mechanism.

The purification and characterization of LFA-1 were described here because of the importance to the current study, and because the purification procedure has not been described in previous reports in which such preparations have been utilized (5, 28, 30). Both the use of Mg^{2+} to stabilize LFA-1 and prevent dissociation of the α and β subunits at high pH, and the use of the TS2/4 mAb, from which LFA-1 can be efficiently eluted at pH 11.5 in the presence of Mg^{2+}, are key features of the purification procedure. The ability of Mg^{2+} to Ca^{2+} to stabilize LFA-1 demonstrates the presence of binding sites for these cations on LFA-1, and is consistent with the presence of tandem repeats predicted to bind divalent cations in the α subunit (27) and the requirement of Mg^{2+} for binding to ICAM-1 (31).

The spectrum of cell types that bind to LFA-1, and the degree of inhibition by ICAM-1 mAb, are in agreement with usage by these cell types of ICAM-1, ICAM-2, and ICAM-3 (7), confirming that purified LFA-1 reconstituted in lipid bilayers retains binding activity for all these ligands. The major ligand on JY cells that purified LFA-1 binds to is ICAM-1, as shown by substantial inhibition by ICAM-1 mAb. mAb to ICAM-2 and ICAM-3 have little effect on binding of JY cells to LFA-1, but when added together with ICAM-1 mAb give complete inhibition of

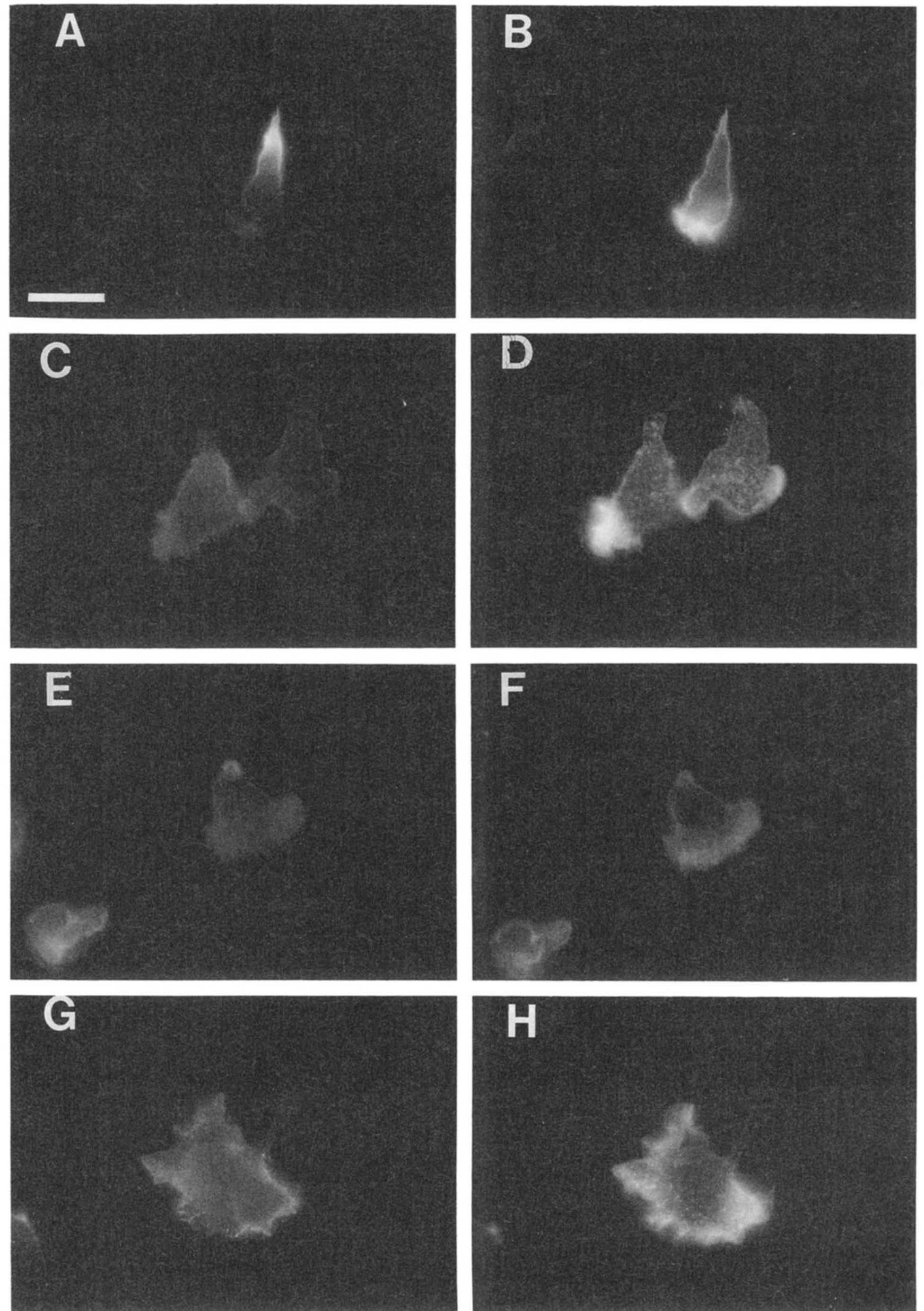

Figure 6. Immunofluorescence localization of ICAM-1, LFA-1 and filamentous actin. JY cells were allowed to adhere on LFA-1 (*A-D*) or ICAM-1 (*E-H*) substrates that were untreated (*A, B, E, F*) or treated for 15 min with 50 ng/ml PMA (*C, D, G, H*). Cells were fixed, permeabilized, and stained with ICAM-1 mAb (*A-D*) or LFA-1 mAb (*E-H*). mAb were visualized with FITC anti-mouse IgG (*A, C, E, G*) plus rhodamine phallicidin (*B, D, F, H*). *Scale bar* (for all panels), 25 μm.

binding to LFA-1 adsorbed to plastic (7). Thus, all three ligands may contribute to the behavior of JY cells on LFA-1, but we expect the primary contribution to be from ICAM-1. The binding of JY cells to purified ICAM-1 is completely inhibited by LFA-1α mAb, and JY cells do not express Mac-1 (32), the only other receptor besides LFA-1 that binds ICAM-1 (33).

JY cells attached to LFA-1 in lipid bilayers through a uropod-like stalk. This manner of attachment may be a consequence of the polarized cell surface distribution of ICAM-1. ICAM-1 was accumulated at the end of the stalk that was in contact with the bilayers containing LFA-1. JY cells in suspension show a similar polar distribution of ICAM-1 (O. Carpen, D. Staunton, P. Palai, and T. Springer, manuscript in preparation). Thus, this distribution of ICAM-1 precedes, and may therefore contribute to the formation of the stalked attachment. Similar preferential localization of ICAM-1 to the uropod of a T lymphocyte line has been described previously (34). The polar localization of ICAM-1 on JY cells is abrogated by the f-actin disrupting drug cytochalasin B (O. Carpen, P. Pallai, D. Staunton, and T. Springer, manuscript in preparation). Thus intact f-actin is required for ICAM-1 localization. Cytoskeletal elements may be required to define the stalk and an f-actin/myosin system might be involved in generating contraction and force for pivoting motility about the attachment site on the bilayer containing LFA-1.

We found that JY cells migrated on bilayers containing ICAM-1 but not LFA-1. It was surprising that the majority of nonphorbol ester-stimulated JY cells displayed locomotion at ICAM-1 densities that supported adhesion of less than 10% of JY cells in the adhesion assay. Apparently, the adhesion assay we use does not detect weak interactions that are sufficient to allow cell locomotion. Phorbol ester stimulation of JY cells increases both the proportion of cells adhering to bilayers with ICAM-1 and the proportion of immobilized cells, but did not decrease average motility. Efficient migration occurred at the highest density of ICAM-1 measured, 1000 sites/μm^2, which is near the maximal surface density of ICAM-1 found on stimulated endothelial cells (35). Others have observed that leukocyte locomotion can be inhibited on nonphysiologic substrates of high adhesiveness (36).

Parallel studies on LGL indicate that differences in the ability of cell surface LFA-1 and ICAMs to promote locomotion may be general (37). Like JY cells, LGL displayed rapid movement on planar bilayers bearing ICAM-1. Unlike JY cells, LGL translocate on bilayers containing LFA-1, but the rate of translocation is 20-fold lower than on ICAM-1. LGL have 10-fold less ICAM-1 and ICAM-2 than JY cells, but are likely to express a large amount of ICAM-3, which is the major ICAM on resting leukocytes (6, 7). The stalked attachment morphology characteristic of JY cells was not observed for LGL on bilayers containing LFA-1. Thus, ICAMs may mediate cell locomotion in some cellular contexts, and may differ in their ability to support motility and interactions with cytoskeletal components.

We found that bilayers in which ICAM-1 and LFA-1 were mixed in equal ratios promoted JY cell motile behavior similarly to bilayers containing only ICAM-1. This result rules out the possibility that the inability of JY cells to move on LFA-1 bilayers was the result of an unphysiologically strong interaction between the purified LFA-1 and cell surface ICAMs such that de-adhesion would be impossible. It also shows that motile behavior of JY cells on ICAM-1 is dominant over the sessile behavior on LFA-1.

Random locomotion of JY cells on ICAM-1 bilayers appears to require no specific stimulation. Motility is a characteristic feature of activated lymphocytes, and JY cells resemble Ag- or mitogen-stimulated T and B lymphocytes in this respect (36).

Marked lateral redistribution of LFA-1 does not appear to be required for adhesion or locomotion. Our observations suggest no difference in the amount of LFA-1 in the leading and trailing edges of cells moving on ICAM-1 bilayers. Our method of observation was less sensitive to differences between the upper (free) and lower (contact) portion of the cell membrane, but we could detect LFA-1 immunofluorescence in both focal planes suggesting no dramatic recruitment of LFA-1 to the broad area of contact with the ICAM-1 bilayer. Redistribution of LFA-1 to sites of Ag-specific cell-cell contact has been observed in some cases (38, 39) but not others (40).

LFA-1 and ICAM-1 did not colocalize with major actin concentrations in the cell. Therefore, both LFA-1 and ICAM-1 are engaged in actin-dependent functions without dictating the cellular distribution of actin filaments. This conclusion is consistent with observations of normal actin polymerization and organization in leukocytes genetically deficient in LFA-1 and related β_2 integrins (41).

The molecular mechanism of the difference in cell behavior on LFA-1 and ICAM-1 bilayers may be related to interactions of the cytoplasmic portions of the counter-receptors. It is possible that LFA-1 has the ability to associate with cytoplasmic motors that allow rearward transport of bilayer attachment sites relative to the cell, driving the cell forward (42). The cytoplasmic domain of the β subunit is required for adhesion-promoting activity of LFA-1 (43). ICAM-1 is concentrated in one region on the surface of unstimulated JY cells and becomes uniformly distributed after phorbol ester stimulation suggesting that it interacts with different cytoplasmic structures than LFA-1, leading to unique properties in regulating the nature of cell adhesion.

The use of an experimental system in which the adhesiveness of LFA-1 in the bilayer remains constant has allowed us to directly observe the consequences of ICAM-1 redistribution for the nature of adhesion. We conclude that LFA-1 regulates the strength of adhesion, but that the cellular distribution of ICAM-1 regulates the size of the contact area. The size of the contact area may have definite consequences for cell-cell communication and cell activation. Broad contact areas increase the efficacy and specificity of cell-cell communication by generating a restricted microenvironment in which exocytosed materials are maintained at high local concentrations (44). Lymphocyte activation and proliferation generally occur in clusters of lymphocytes and accessory cells in which intercellular spreading could contribute to the process of germinal center formation and lymphocyte activation.

Lymphocytes are capable of locomotion over the surface of macrophages, fibroblasts, and endothelial cells (45–47), and this may involve the LFA-1/ICAM interaction. Recently, lymphocyte transmigration through IL-1-stimulated endothelial cell monolayers was shown to be inhibited by LFA-1 mAb and ICAM-1 mAb (47, 48).

Recently, we proposed that de-adhesion of cells from other cells could be mediated by, and might physiologically require, formation of an alternative adhesion with extracellular matrix or other cells (4, 49). Because of the way we introduced JY cells onto chambers containing lipid bilayers in the experiments described above, there were variations in the cell density in the chamber. We chose area of low cell density for the observations reported above. However, we also observed areas of higher density where many of the cells form homotypic aggregates. On bilayers with from 100 to 1000 sites/μm^2 of ICAM-1, many cells crawled free of phorbol ester-stimulated aggregates by interacting with the bilayer, resulting in up to 30% of the cells remaining unaggregated. By contrast, on surfaces of lower adhesiveness, such as tissue culture-treated plastic, none of the cells escaped from phorbol ester-stimulated aggregates, despite vigorous motility that gives these spherical aggregates the appearance of a heaving mass (29). These observations lend support to the idea that formation of an alternative adhesion can be used or is required to drive de-adhesion of cells.

We have observed that complementary adhesion receptors elicit clearly distinguishable cellular behaviors. The ability of cell surface LFA-1 to promote locomotion on a bilayer containing ICAM-1 suggests that LFA-1 may have

a direct role in events such as extravasation. The surprising observation that adhesion mediated by cell surface ICAMs undergoes dramatic changes on cell activation may also be significant for our understanding of adhesion mechanisms and their use by cells. It will be interesting to see whether other integrin/Ig superfamily adhesion receptor pairs, notably VLA-4 ($\alpha 4\beta 1$) and vascular cell adhesion molecule-1 (50), also show complementarity in the locomotor and morphologic characteristics of the adhesion they mediate.

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