PURIFIED LYMPHOCYTE FUNCTION–ASSOCIATED ANTIGEN 3 BINDS TO CD2 AND MEDIATES T LYMPHOCYTE ADHESION

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T lymphocyte interactions with target and antigen presenting cells appear to require an ensemble of T lymphocyte surface proteins: the antigen receptor–CD3 (T3) complex, and the accessory molecules CD4, CD8, LFA-1, and CD2 (1). Target cells and antigen-presenting cells also express an array of accessory molecules, such as MHC antigens, LFA-3, and ICAM-1 (1). Although it is hypothesized that accessory molecules on T lymphocytes interact with molecules on other cells to mediate intercellular adhesion, there has been little direct evidence for this. However, recent studies on CD2 have suggested that it interacts with a cell surface molecule termed lymphocyte function–associated molecule 3 (LFA-3).[1]

CD2 (T11, LFA-2, E receptor) is a 50,000 M_r T lymphocyte surface glycoprotein. mAb to CD2 inhibit antigen-dependent helper T cell proliferation, IL-2 receptor expression, IL-2 secretion, cytolytic T lymphocyte–mediated killing, and adhesion to target cells (2–8). We have interpreted these results to indicate that CD2 is required for a T cell adhesion event that is critical to the initiation of T lymphocyte functional responses (1). CD2 has also recently attracted attention as a potential receptor or signal transducer for T cell activation via an alternative pathway as opposed to the classical T cell receptor–CD3 activation pathway (9–11).

LFA-3 is a widely distributed surface glycoprotein of 70,000 M_r when isolated from B lymphoblastoid cells (2). mAb to LFA-3 inhibit a spectrum of cytolytic T lymphocyte and helper T lymphocyte responses similar to that inhibited by CD2 mAb (2, 3). mAb to both molecules inhibit cytolytic T lymphocyte conjugate formation (12).

CD2 and LFA-3 have been found to be components of a single functional pathway, distinct from the LFA-1 pathway, in the adhesion of cytolytic T lymphocytes to target cells (13). CD2 mAb inhibit cytolytic T lymphocyte–

[1] *Abbreviations used in this paper:* LFA, lymphocyte function–associated antigen; OG, octyl-β-D-glucopyranoside; T11TS, T11 target structure.

mediated killing by binding to the T cell, whereas LFA-3 mAb inhibit by binding to the target cell (3, 13). Similarly, binding of thymocytes to thymic epithelial cells requires CD2 on the thymocyte and LFA-3 on the thymic epithelial cell (14, 15). Subsequently we have found that purified CD2 binds to B lymphoblastoid cells and that this binding is inhibited by LFA-3 mAb (16). Furthermore, autologous rosetting between $CD2^+$ T lymphocytes and $LFA-3^+$ human erythrocytes is mediated by CD2 and LFA-3 (17; and Makgoba, M. W., S. Shaw, E. A. Gugel, and M. E. Sanders, manuscript submitted for publication). These results suggest that LFA-3 may be the cell surface ligand for CD2.

Characterization of the biological ligand for CD2 is of great importance. In this study we have purified LFA-3 and functionally characterized its ability to mediate T cell adhesion. We show that LFA-3 binds to CD2 on the surface of T lymphocytes. Purified LFA-3 inhibits intercellular adhesion between T lymphocytes and erythrocytes, and mediates aggregation of T lymphocytes. Finally, we show that LFA-3 reconstituted into artificial membranes can efficiently mediate adhesion of human T lymphoblasts.

Materials and Methods

Cells. Human erythrocytes were stored in citrate/phosphate/dextrose/adenine of 4°C for up to 1 wk before use. Peripheral blood mononuclear cells were prepared by dextran sedimentation of whole blood and then sedimentation on Ficoll-Hypaque ($\rho = 1.077$; Sigma Chemical Co., St. Louis, MO). To obtain peripheral blood lymphocytes, monocytes were depleted from peripheral blood mononuclear cells by two incubations in complete medium (RPMI 1640, 10% FCS) in tissue culture dishes at 2×10^7 cells per 143-cm^2 plate for 60 min followed by gentle removal of nonadherent cells. T lymphoblasts were prepared by culturing peripheral blood mononuclear cells with 1 μg/ml Con A (Sigma Chemical Co.) in RPMI-1640 20% FCS at 5×10^5 cells/ml for 4 d, followed by washing out Con A and growing the cells in 1 ng/ml recombinant IL-2 for at least 3 d before use. The T lymphoma cell line SKW3 was obtained from Dr. P. Cresswell, Duke University, Durham, NC. The T lymphoma cell line, Jurkat, was obtained from Dr. M.-K. Ho, New England Nuclear, Boston, MA. The B lymphoblastoid cell line JY was obtained from Dr. J. Strominger, Dana-Farber Cancer Institute. Sheep erythrocytes were purchased from the Colorado Serum Co., Denver, CO.

Monoclonal Antibodies. The mouse anti–human mAb TS2/9 (anti-LFA-3, IgG1), TS2/18 (anti-CD2, IgG1), and TS1/22 (anti-LFA-1, IgG1) (2) were used as purified IgG or as dilutions of culture supernatants. TS2/9 mAb was purified from hybridoma culture supernatants by $(NH_4)_2SO_4$ precipitation and protein A–affinity chromatography. $F(ab')_2$ of LFA-3 mAb were prepared by papain digestion of purified IgG (18). Nonbinding control mAb was from culture supernatants of the P3X63 Ag8 IgG1-secreting myeloma.

Affinity columns. Purified IgG was coupled to Sepharose CL-4 B by a modification of the method of Cuatrecasas (19). Washed Sepharose CL-4B (Pharmacia Fine Chemicals, Upsala, Sweden) was activated with 40 mg/ml CNBr in 1 M Na_2CO_3 for 10 min on ice, and then washed with distilled water and 0.1 mM HCl. The activated Sepharose was filtered to a moist cake and added to purified antibody solution with 2–4 mg/ml IgG (LFA-3 mAb or mouse IgG) in 0.05 M NaCl and 0.1 M $NaHCO_3$, pH 8.4. The suspension was mixed end-over-end for 20 h, and any remaining reactive groups were blocked by addition of ethanolamine to 50 mM and incubation for 1 h. The supernatant was checked for uncoupled antibody by measuring absorbance at 280 nm. Coupling was usually ~90%. The Sepharose was poured into a column and washed with one cycle of pH 11 and pH 3 buffers (see below) before use for affinity chromatography.

Affinity Chromatography. LFA-3 was purified after solubilization with Triton X-100 by affinity chromatography. All operations were done at 4°C. Outdated human erythrocytes were obtained from the American Red Cross (Needham, MA). Cells from two units of

whole blood were washed three times with PBS, pH 7.2. The packed cells were pelleted to ~500 ml. Another 500 ml of PBS pH 7.2 with 2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, and 0.15 trypsin inhibitor units per milliliter aprotinin was added to the red cell suspension while stirring. After 1 h the lysate was centrifuged at 150,000 g for 2 h. The cleared lysate was passed over two columns in series at a flow rate of 20 ml/h: first a mouse IgG-Sepharose column (2 ml at 2 mg/ml) to absorb some contaminants and to filter out any particulate material, and second an LFA-3 mAb-Sepharose column (5–10 ml at 2 mg/ml). The LFA-3 mAb-Sepharose column was washed with five column volumes of 50 mM sodium phosphate pH 7.2, 0.25 M NaCl, 0.1% Triton X-100, then five column volumes of 20 mM triethylamine pH 11, 0.25 M NaCl, 0.1% Triton X-100, and again with two column volumes of the pH 7.2 buffer all at a flow rate of 1 ml/min. The remaining bound LFA-3 was then eluted with five column volumes of 50 mM glycine HCl, pH 3, 0.25 M NaCl, 0.1% Triton X-100 at a flow rate of 20 ml/h. LFA-3 generally eluted in one column volume and was neutralized by collection into 0.1 vol of 1 M Tris, pH 8.6, 0.1% Triton X-100. In some preparations, contamination with hemoglobin remained after one cycle of affinity chromatography. This could be removed by loading the pooled LFA-3-containing fractions back onto the column and eluting with pH 3 buffer after washing as above. The material obtained after two cycles of affinity chromatography contained no contaminants detectable by SDS-PAGE and silver staining. When octyl-β-D-glucopyranoside (OG) (Calbiochem-Behring, San Diego, CA) was used to elute LFA-3, all steps were the same until after the high-pH wash. The column was then washed with five volumes of phosphate buffer, pH 7.2, 0.15 M NaCl, containing 1% OG, and the LFA-3 was eluted using glycine buffer, pH 3, 0.15 M NaCl, 1% OG. The elution profile was similar to that obtained with Triton X-100. LFA-3 purification could be followed in a semiquantitative manner using a dot-blot assay (20) with ^{125}I-LFA-3 mAb. LFA-1 was purified from SKW3 cells by affinity chromatography on TS1/22 mAb–Sepharose and eluted under the same conditions as LFA-3 (R. Larson, unpublished observation). Protein concentrations were determined using the Coomassie blue dye-binding assay (21) on acetone or ethanol (1:5)-precipitated samples. Molar concentration of LFA-3 and LFA-1 were calculated using M_r of the polypeptide backbone of 25,000 (our unpublished data) and 210,000 (22), respectively.

LFA-3 Iodination and Binding Assay. LFA-3 in 0.1% Triton X-100 was dialyzed against borate-buffered saline (pH 8.2) and labelled with ^{125}I using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Pierce Chemical Co., Rockford, IL) (23) and then dialyzed against PBS. The purity of the iodinated LFA-3 was >90% by SDS-PAGE. Specific activity was 170 Ci/mmol. Binding assays were done on 2×10^6 cells in 100 μl with an input of 80,000 cpm in HBSS, 3% BSA, 10% FCS. After 60 min at 4°C the assay was terminated by centrifugation through a 0.8-ml 15% BSA cushion. The supernatant was thoroughly aspirated, and the tip of the centrifuge tube containing the pellet was cut off and counted. mAb were added 15 min before addition of the ^{125}I-LFA-3. All points were determined in triplicate.

Flow Microfluorometry. TS2/18 and TS1/22 mAb in hybridoma culture supernatants were titrated against Jurkat and peripheral blood lymphocytes to determine the lowest concentration of mAb giving optimal staining. Nonbinding control mAb was used at 5 μg/ml. All operations were carried out at 4°C. Cells (10^5) were incubated with purified membrane protein or control buffer in 20 μl of HBSS, 15% BSA for 60 min. The 15% BSA was included in this and subsequent procedures to bind detergent, and has been shown to protect cells from detergent-mediated damage (24). mAb were then added in an additional 20 μl of the same buffer, and the suspension was incubated 15 min. The cells were then washed three times and incubated with FITC–goat anti–mouse IgG (heavy and light chain–specific; Zymed Laboratories, San Francisco, CA) for 30 min, washed three times, fixed with 1% paraformaldehyde, and analyzed within 1 wk on a Coulter epics V flow cytometer.

E Rosetting. Peripheral blood lymphocytes or tumor cells (10^5) were mixed with sheep E (10^7) in 10 μl of HBSS, 15% BSA. Membrane proteins or control buffers were added in an additional 10 μl of HBSS, 15% BSA and the suspension was incubated 15–30 min

at 4°C. All samples contained the same buffer and detergent concentration, even those not receiving membrane protein. The cells were pelleted at 100 g for 10 min and held on ice for 60 min. The pellets were gently resuspended and rosettes enumerated by counting 100 nucleated cells under phase illumination.

Liposome Preparation.   Liposomes were prepared by OG dialysis (25). LFA-3 was eluted from the affinity column in the presence of 34 mM (1%) OG instead of Triton X-100. Lipids in chloroform were mixed at a ratio of egg phosphatidylcholine/cholesterol of 7:2. The lipids (0.5 mg) were dried under a nitrogen stream and then placed under reduced pressure for 1 h to remove residual chloroform. The lipid film was taken up in 1 ml of 0.15 M NaCl, 0.05 M glycine, 0.1 M Tris, pH 8, with 20–30 μg of LFA-3 and 34 mM OG and dialyzed against two changes of PBS, pH 7.2, and one of HBSS, 25 mM Hepes, pH 7.2, over 36–48 h. As a control, human glycophorin (Sigma Chemical Co.) was also reconstituted using the same procedure.

Preparation of Planar Membranes (26).   Round glass coverslips (11 mm) were boiled in 1:6 7X detergent (Linbro Chemical Co., Hamden, CT):water for 15 min. They were then washed extensively over 24 h with distilled water, soaked overnight in 70% ethanol, and allowed to dry. Drops (100 μl of liposome suspension diluted to 0.1 to 0.2 mM lipid) were placed in wells of 24-well cluster plates (Falcon Labware, Oxnard, CA). Glass coverslips were gently placed on top of the lipid suspension drops and left for 20–30 min at ambient temperature. The wells were then filled with media and the coverslips were carefully flipped over to expose the fused lipid surface. The well was washed three times with assay medium (RPMI 1640, 10% FCS, 25 mM Hepes) to remove unfused liposomes. The lipid surface was never exposed to air.

Binding Assay.   T lymphoblasts or JY B lymphoblastoid cells were labelled with ^{51}Cr by incubating 10^7 cells with 300 μCi of Na [51]CrO$_4$ (New England Nuclear) in 3 ml of RPMI-1640, 10% FCS for 90 min. The cells were then washed three times with media containing 5 mM methyl-α-D-mannopyranoside, and twice with complete medium plus 25 mM Hepes, pH 7.4. Cells and planar membranes were treated with 20 μg/ml mAb for 15 min, and in the case of pretreatment, washed before testing binding. Cells (2–5 $\times$ 10^5) were added and the plates were centrifuged at 10 g for 1 min to facilitate even and rapid cell settling. The plates were then either floated in a 37°C water bath for 15 min or placed on ice for 1 h. After the incubation the plates were washed with three changes of assay media, the wells photographed, and the contents solubilized with 0.1 N NaOH and counted in a gamma counter. Input counts were calculated to be the total input to the well multiplied by the coverslip area per well area to determine the number of cells (cpm) falling on the coverslip.

Photography.   Photomicrographs were taken using a Nikon Diaphot inverted microscope using bright-field or phase optics.

Results

We have tested human B lymphoblastoid cell lines, spleens from patients with hairy cell leukemia, and erythrocytes (E) as sources for the purification of LFA-3. Fresh human E have ~5,000 LFA-3 sites per cell (our unpublished observations). While B lymphoblastoid cell lines have ~200,000 LFA-3 molecules per cell (40-fold more than E), B lymphoblastoid cell lines contain only fourfold more LFA-3 than E on the basis of packed cell volume. Due to their ready availability in large quantity at low cost, erythrocytes were chosen for large-scale purification of LFA-3. We expected LFA-3 from erythrocytes to be fully functional, because previous studies have shown that LFA-3 on the surface of intact E binds to purified CD2 (17).

Packed E were solubilized with an equal volume of Triton X-100 lysis buffer, and LFA-3 was isolated by immunoaffinity chromatography with LFA-3 mAb. The affinity purification strategy took advantage of the unusual stability of the
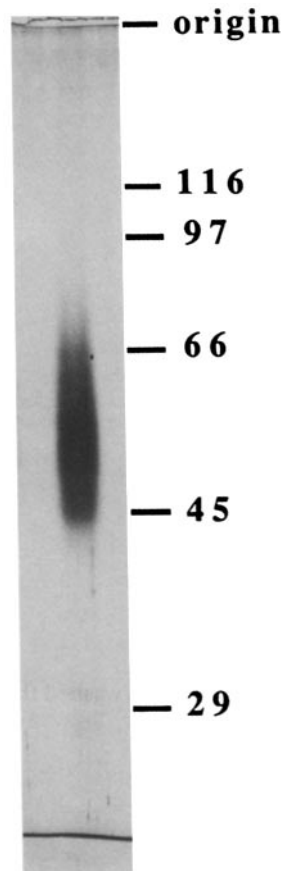
FIGURE 1.   SDS-PAGE of purified LFA-3. Purified LFA-3 (0.3 μg in 10 μl) was added to 10 μl of 2× reducing sample buffer and run on SDS-8% polyacrylamide gel (43). Protein was visualized by silver staining (44).

antigen-antibody complex in alkaline pH (pH 11), which elutes many contaminating proteins. After the high-pH wash, LFA-3 was eluted at pH 3. This procedure allowed isolation of essentially pure LFA-3 from the affinity column in a one-step isolation procedure (Fig. 1). Site number estimates suggested that erythrocytes from one unit of blood contain ~500 μg of LFA-3. Our recoveries from three isolation procedures ranged from 100 to 150 μg/unit. LFA-3 from erythrocytes migrated on SDS-PAGE as a broad band of 40,000–70,000 M_r (Fig. 1). This was somewhat lower than the 65,000–70,000 M_r observed for LFA-3 from B lymphoblastoid cells (2, 3), and was similar to LFA-3 isolated from the epidermoid carcinoma cell line A431 and from spleens from patients with hairy-cell leukemia (data not shown).

We used the purified LFA-3 to test the hypothesis that it could bind to the CD2 molecule on the surface of T lymphocytes. Purified, iodinated LFA-3 bound to the $CD2^+$ T lymphoma cell lines Jurkat and SKW3 (Table I). Binding was specific, because it was inhibited 75–78% by excess unlabelled LFA-3, and 75%–76% by LFA-3 mAb. Strikingly, CD2 mAb inhibited binding of purified

TABLE I

Binding of ^{125}I-LFA-3 to CD2$^+$ and CD2$^-$ Cells

Addition	^{125}I-LFA-3 bound		
	Jurkat	SKW3	JY
	fmol		
Control mAb	5.1	4.0	1.0
100 nM LFA-3	1.1	1.0	1.1
LFA-3 mAb	1.3	0.96	ND
CD2 mAb	1.1	0.88	0.96
CD2 mAb + LFA-3 mAb	1.1	ND	ND

After a 15-min preincubation with LFA-3 or control buffer, and with specific or control mAb at 20 μg/ml, cells (2 × 10⁶) were incubated with 190 fmol (80,000 cpm) of ^{125}I-LFA-3 in 100 μl of HBSS, 3% BSA, 10% FCS. After 1 h, free and bound radioactivity were separated as described in Materials and Methods. Each point is an average of triplicate determinations and is representative of three experiments.

^{125}I-LFA-3 to the CD2$^+$ cells by 78%. Purified LFA-3, LFA-3 mAb, CD2 mAb, and the combination of LFA-3 mAb and CD2 mAb all inhibited to the same extent. Nonspecific binding seen in the presence of these inhibitors was identical to binding of ^{125}I-LFA-3 to the CD2$^-$ B lymphoblastoid cell line JY. These results suggest that LFA-3 binds to the CD2 structure on T cells. The binding to JY and nonspecific binding to CD2$^+$ cells is probably due to some type of nonspecific association, which could involve insertion of the hydrophobic domain of the LFA-3 molecule into the cell membrane (see below).

To further test the hypothesis that LFA-3 interacts with the CD2 molecule, flow microfluorometry experiments were done to determine if preincubation of cells with LFA-3 could inhibit the subsequent binding of the TS2/18 CD2 mAb (Fig. 2). Fluorescence histograms show that Jurkat cells (Fig. 2a) and peripheral blood T lymphocytes (Fig. 2b) preincubated with 140 nM LFA-3 bound 93% and 98% less TS2/18 CD2 mAb, respectively, than cells preincubated with control buffer. Half-maximal inhibition of CD2 mAb binding to Jurkat and peripheral blood T lymphocytes was obtained at ~4 and ~1 nM LFA-3, respectively (Fig. 2e). Binding of mAb to the LFA-1 surface molecule on either cell type was not affected by preincubation with any concentration of LFA-3 (Fig. 2, c, d, and f). As an additional control, pretreatment with purified LFA-1 membrane protein (1000 nM) had no effect on CD2 mAb binding (Fig. 2e). Inhibition of ^{125}I-LFA-3 binding by CD2 mAb and reciprocal inhibition of CD2 mAb binding by purified LFA-3 demonstrates that the LFA-3 binds to the CD2 molecule, and further suggests that LFA-3 binds to CD2 at or near the TS2/18 epitope.

Purified LFA-3 was tested in functional assays to determine whether it could inhibit rosetting of human T lymphocytes with human erythrocytes or sheep erythrocytes. CD2 mAb inhibited rosetting of human T lymphocytes with both sheep and human E (Table II) as previously reported (4, 17, and Makgoba et al., manuscript submitted for publication). LFA-3 mAb reacts with human but not sheep E, and inhibited rosetting with human but not sheep E (Table II), as previously reported (17, and Makgoba et al., manuscript submitted for publica-
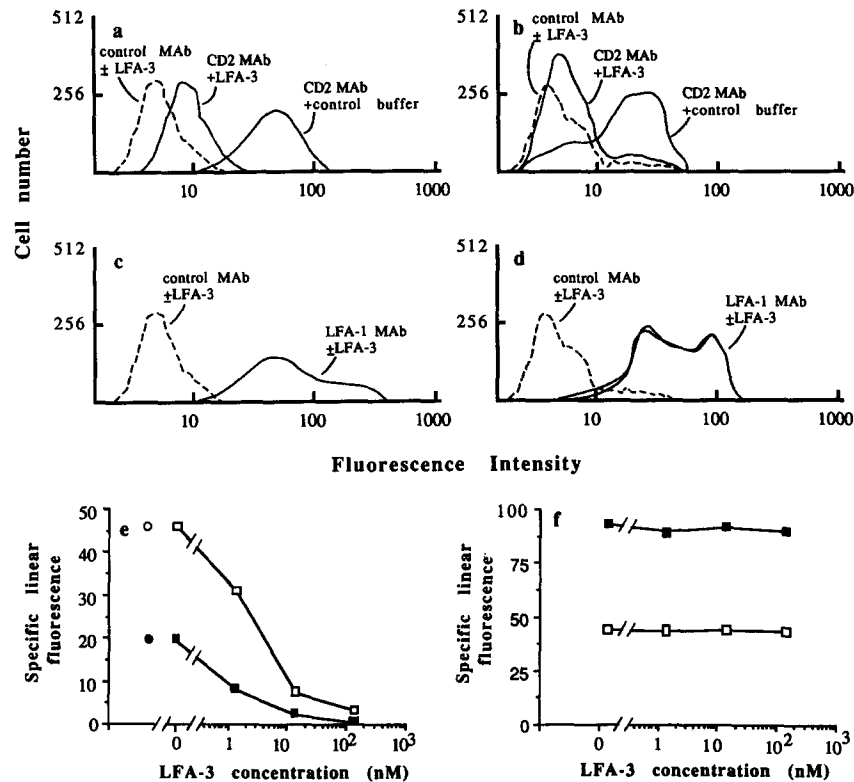
FIGURE 2. Inhibition of CD2 mAb binding by purified LFA-3. *a–d*; flow microfluorometry histograms. Jurkat (*a*, *c*) or PBL (*b*, *d*) were incubated for 1 h at 4°C with control buffer or 140 nM LFA-3, stained with a nonbinding control mAb (*dashed lines*) or specific mAb (*solid lines*) as indicated; and then stained with a FITC anti-Ig. The presence of LFA-3 in the preincubation did not influence the intensity of the nonbinding control. *e*, *f*; concentration dependence of inhibition by LFA-3 of CD2 mAb binding (*e*) and control LFA-1 mAb binding (*f*) to Jurkat (*open squares*) or PBL (*closed squares*). As an additional control Jurkat (*open circle*) and PBL (*closed circle*) were preincubated with the control membrane protein LFA-1 (1,000 nM), and then binding of CD2 mAb was determined (*e*).

tion). However, since purified LFA-3 binds to CD2 on the T lymphocyte, we predicted that it should inhibit rosetting with human T lymphocytes regardless of the erythrocyte source. Purified LFA-3 (70 nM) completely abolished autologous rosetting of Jurkat T lymphoma cells with human erythrocytes (Table II). Purified LFA-3 also completely inhibited rosetting of Jurkat cells and peripheral blood T lymphocytes with sheep erythrocytes (Table II and Fig. 3, *a–d*). Inhibition of Jurkat cell rosetting with sheep E was half-maximal at 7 nM LFA-3 (not shown). Rosetting was not affected by incubation with control buffer or up to 1,000 nM LFA-1 (Table II and data not shown). Because LFA-3 mAb did not inhibit rosetting with sheep E it was possible to test the specificity of the inhibition by purified LFA-3 by adding excess LFA-3 mAb. LFA-3 mAb completely neutralized the ability of purified LFA-3 to inhibit rosetting between human T lymphocytes and sheep E (Table II).

In addition to inhibiting rosetting, purified LFA-3 caused aggregation of the Jurkat cell line (Fig. 3 *b*). LFA-3 did not cause aggregation of erythrocytes (Fig.

TABLE II

Rosetting of Erythrocytes by T Lymphocytes

Pretreatment	Rosettes with human erythrocytes and Jurkat cells	Rosettes with sheep erythrocytes	
		Jurkat	PBL
	%		
Control mAb	54	80	30
CD2 mAb	0	0	0
LFA-3 mAb	0	76	32
Purified LFA-3	0	0	0
Purified LFA-3 + LFA-3 mAb	ND	74	36

Suspensions of 10^5 T cells and 10^7 erythrocytes were incubated for 15 min at 4°C with 20 μg/ml mAb or purified proteins. The cells were then pelleted, held on ice, and rosettes were enumerated by counting 100 nucleated cells.

3 b). We tested whether purified LFA-3 aggregated Jurkat cells by binding to CD2. Experiments were done at 4°C; at this temperature, the CD2/LFA-3 adhesion pathway but not the LFA-1 pathway is operative (13). Suspensions of Jurkat cells were treated with LFA-3 in the presence of control mAb or CD2 mAb at 4°C, pelleted, held on ice for 1 h, and then gently resuspended. In the absence of exogenous LFA-3 there was a small but significant degree of aggregation of Jurkat cells in which the majority of cells were not involved in aggregates (Fig. 3e). This aggregation was mediated by CD2 and the low quantity of endogenous LFA-3 on Jurkat (7% of expression on B lymphoblastoid cell lines, M. Plunkett and P. Selvaraj, unpublished data), because it was inhibited by CD2 mAb and LFA-3 mAb (not shown). Addition of exogenous LFA-3 greatly enhanced Jurkat cell aggregation, to the point where almost all cells were in large aggregates (Fig. 3f). This aggregation was completely inhibited by CD2 mAb and LFA-3 mAb (Fig. 3, g and h), suggesting that it is mediated by interaction between the exogenous LFA-3 and cell surface CD2. Although the physical form of LFA-3 that mediated Jurkat aggregation is not known, LFA-3 may insert into the membrane by its hydrophobic domain (see nonspecific binding in Table I) or LFA-3 may be present in a multimeric form in protein micelles.

LFA-3 normally functions as a protein anchored to the membrane by a hydrophobic domain. To demonstrate adhesion function for the purified protein in lipid bilayers, LFA-3 was reconstituted into liposomes by octyl glucoside dialysis, and the liposomes were fused onto glass coverslips (26) to allow observation of cell binding. Based on the crossectional area of lipid molecules (60 Å^2 [27]), the ratio of lipid to protein (500:1), and assuming random inside/outside orientation of LFA-3 in liposomes, the density of LFA-3 in these planar membrane would be ~7,000 molecules/μm^2. T lymphoblasts labelled with ^{51}Cr were gently centrifuged onto the planar membranes containing LFA-3, or as a control, glycophorin. >90% of cells bound to the planar membrane containing LFA-3 (Fig. 4a and Table III) but only 2% bound to those containing human glycophorin (Table III). Pretreatment of the T lymphoblasts with CD2 mAb (Fig. 4b) or of the LFA-3-bearing planar bilayer with LFA-3 mAb IgG or F(ab')$_2$ fragments
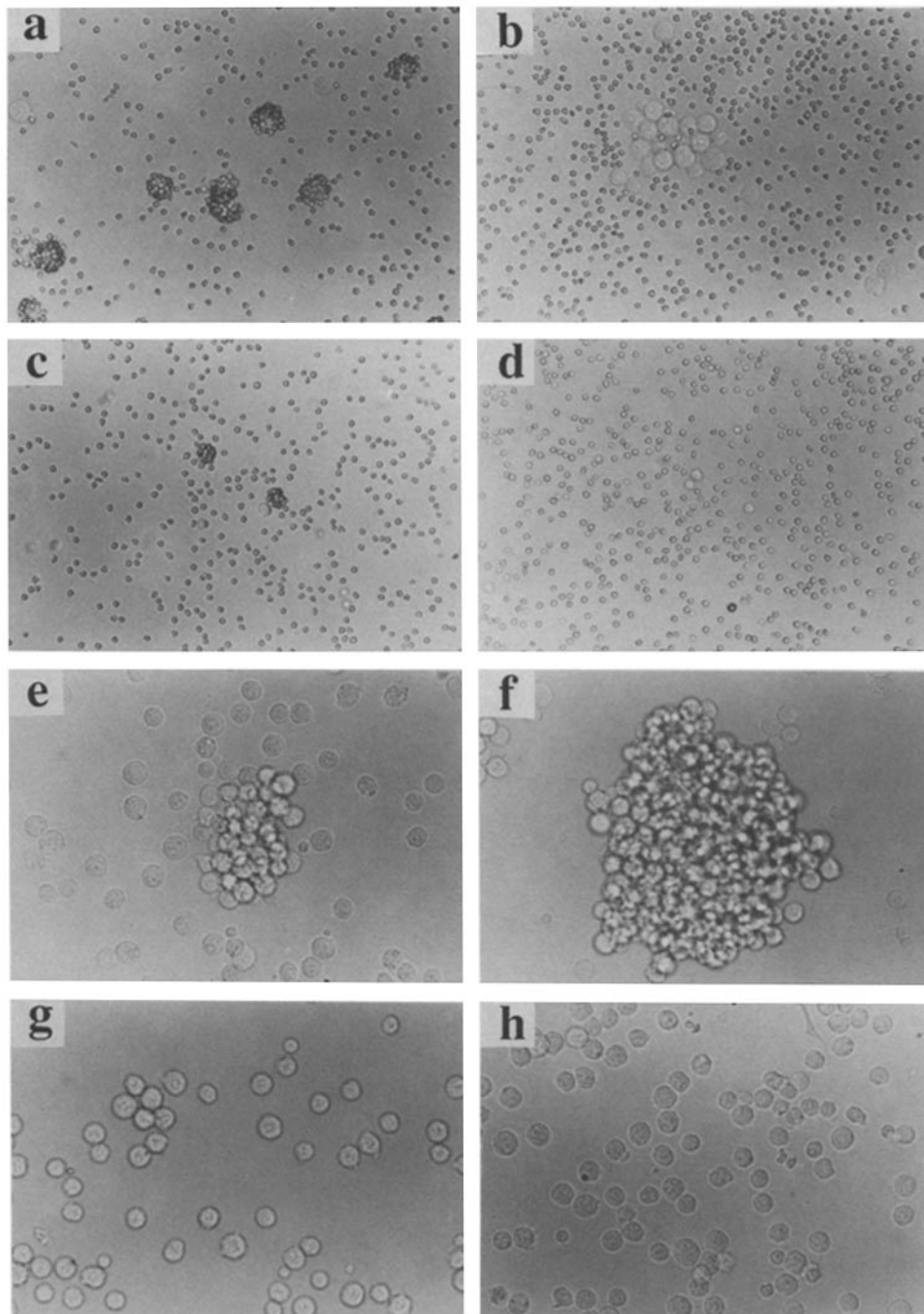
FIGURE 3. Purified LFA-3 inhibits E-rosetting and mediates aggregation of CD2+ cells. Jurkat cells (a, b) or PBL (c, d) were mixed with sheep E, incubated in suspension with control buffer (a, c), or purified LFA-3 (b, d) for 15 min and then pelleted. After 1 h on ice the cells were gently resuspended and photographed under bright-field illumination at × 500 (final). e–h; Jurkat cell aggregation. Jurkat cells (2 × 10⁵) were incubated in suspension with control buffer (e), 140 nM purified LFA-3 (f), purified LFA-3 + 20 µg/ml CD2 mAb (g) or purified LFA-3 + 20 µg/ml LFA-3 mAb (h). The cells were pelleted and left on ice for 1 h, gently resuspended and photographed under bright-field illumination at 400×. All fields are representative. Note that there are more unaggregated Jurkat cells in the absence of LFA-3 (e) than in the presence of LFA-3 (f).
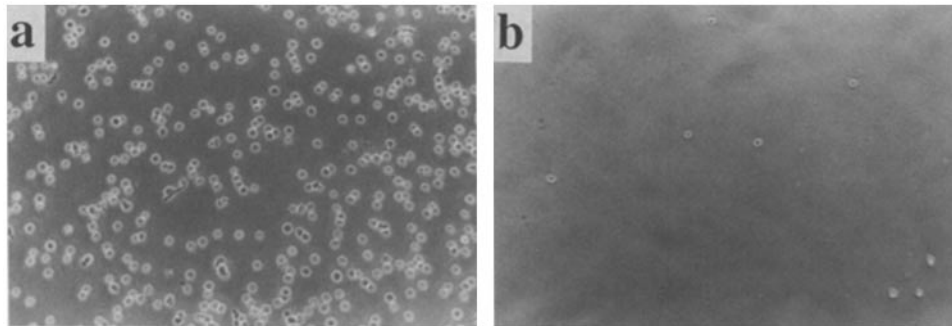
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FIGURE 4.   Adhesion to planar membranes bearing LFA-3. T lymphoblasts were added to wells containing LFA-3-bearing planar membranes. T lymphoblasts were untreated (a) or pretreated with 20 μg/ml CD2 mAb for 30 min and then washed (b). After 15 min at 37°C, the unbound cells were washed out and the wells photographed under phase illumination (× 250, final).

TABLE III

Adhesion of CD2⁺ and CD2⁻ Cells to LFA-3 and Glycophorin-bearing Planar Membranes

Condition	Cell binding (percent of input)	
	LFA-3 membrane	Glyco-phorin membrane
37°C T blasts		
Control mAb	91	1.8
CD2 mAb	2.9	2.2
CD2 mAb pretreatment of cells	2.1	2.8
LFA-3 mAb	5.1	3.6
LFA-3 mAb pretreatment of planar membranes	9.9	2.5
LFA-3 F(ab')₂ pretreatment of planar membranes	4.1	2.7
37°C JY		
Control mAb	1.2	3.9
LFA-3 mAb	1.0	2.8
4°C T blasts		
Control mAb	93	2.1
LFA-3 mAb	8.2	1.9
CD2 mAb	3.2	3.3

After mAb pretreatment or in the continued presence of specific or control mAb, T lymphoblasts or JY cells (4·× 10⁵) labeled with ⁵¹Cr (50,000 cpm) were added to wells containing liposome-fused coverslips and centrifuged at 10 g for 1 min and then incubated at 37°C for 15 min or 4°C for 1 h. Binding was determined as described in Materials and Methods. Results are either from one experiment or averaged from points duplicated between two experiments.

inhibited >95% of the binding, essentially to the level of that seen with human glycophorin-bearing planar membranes (Table III). Results were similar whether the cells were incubated on the planar membranes at 37°C for 15 min or at 4°C for 1 h (Table III). The CD2⁻ B lymphoblastoid cell line JY failed to bind to LFA-3-bearing or control planar membranes (Table III). These reconstitution experiments show that purified LFA-3 functions as a ligand for CD2-mediated T lymphocyte adhesion.

Discussion

Our results with purified LFA-3 show that it is a ligand for T cell adhesion and that adhesion is mediated by its interaction with the CD2 molecule. Iodinated LFA-3 bound to CD2⁺ T lymphocytes in a specific manner as defined by the ability of excess unlabeled LFA-3 to inhibit binding and the ability of LFA-3 mAb to inhibit binding to the same extent. This specific binding was also inhibited by CD2 mAb, suggesting that LFA-3 bound to the CD2 structure on the T lymphocyte surface. In the reciprocal experiment, purified LFA-3 was able to inhibit the binding of CD2 mAb to T cells, suggesting that LFA-3 binds to a site on CD2 at or near the TS2/18 epitope. Purified LFA-3 inhibited erythrocyte rosetting by human T lymphocytes with both human and sheep erythrocytes. This result suggests that binding of purified LFA-3 to T lymphocytes was sufficient to interfere with the interaction of T cell CD2 with LFA-3 on the human erythrocyte, and with its presumed homologue on sheep erythrocytes. While LFA-3 inhibited rosetting, it enhanced aggregation of the CD2⁺ Jurkat T lymphoma cell line. This aggregation was a specific effect of exogenous LFA-3-binding to cell surface CD2, because it was inhibited by CD2 mAb and LFA-3 mAb. This phenomenon provided evidence that LFA-3 can mediate intercellular adhesion. To study adhesion in a more defined system, LFA-3 was reconstituted into liposomes which were fused onto a glass surface to allow observation of cell binding. LFA-3 reconstituted into planar membranes could mediate very efficient CD2-dependent adhesion of T lymphoblasts at both 37°C and 4°C. These results clearly define LFA-3 as a cell surface ligand for the T lymphocyte function-associated molecule CD2.

Previously (16, 17) we have shown that purified CD2 binds to LFA-3⁺ cells and inhibits LFA-3 mAb binding. CD2 binds saturably to B lymphoblastoid cells with a dissociation constant of 50 nM. Similarly, the half-maximal concentration of CD2 required to inhibit LFA-3 mAb binding was 60 nM. Conversely, we have found here that purified LFA-3 blocks binding of CD2 mAb to both Jurkat cells and peripheral blood T lymphocytes. The half-maximal concentration of LFA-3 required to inhibit CD2 mAb binding to peripheral blood T lymphocytes and Jurkat cells was 1 and 5 nM, respectively, which is an estimate of the concentration of LFA-3 required to half saturate its binding sites or its dissociation constant. Considering the ambiguities introduced by the presence of the hydrophobic domains of each of these molecules, the estimates are in a similar range.

Purified LFA-3 was shown here to aggregate cells bearing CD2; similarly, purified CD2 has been shown to aggregate cells bearing LFA-3 (16, 17). In both cases, aggregation is mediated by interaction of CD2 with LFA-3, as shown by mAb blocking. This is evidence that both proteins can directly mediate intercel-

lular adhesion. The ability of LFA-3 to mediate adhesion was most clearly demonstrated here after reconstitution of LFA-3 into liposomes. Functional studies involving cytolytic T lymphocyte conjugation with target cell have demonstrated two distinct antigen-independent adhesion pathways, one dependent on LFA-1, and another dependent on CD2 and LFA-3 (13). The CD2 and LFA-3–dependent pathway, in contrast to the LFA-1 pathway, was not temperature-dependent and did not require Mg^{2+}. In agreement with these findings, we have found that LFA-3 reconstituted in planar membranes mediates efficient binding of T lymphoblasts both at 4°C and 37°C. Together with previous studies with soluble CD2 and functional studies on the CD2/LFA-3 pathway in T lymphocyte adhesion, the current findings provide strong evidence that the interaction between LFA-3 and CD2 mediates cell-cell adhesion.

T lymphoma cell lines, T lymphoblasts, and thymocytes form stable rosettes with human erythrocytes (28). In contrast, resting peripheral blood lymphocytes fail to form rosettes with human erythrocytes (28–31). However, we have found that purified LFA-3 binds to both Jurkat T lymphoma CD2 and peripheral blood T lymphocyte CD2 with similar affinity. Our results suggest that the difference in the ability of activated and resting T lymphocytes to form rosettes with human erythrocytes must be due to differences in parameters other than LFA-3 binding affinity. One relevant parameter is surface charge of cells, which is lower on activated cells (32) and would result in lower electrostatic repulsion between cells. Consistent with this view, peripheral blood T lymphocytes rosette with human erythrocytes after reduction of net negative surface charge by neuraminidase treatment of human erythrocytes or of peripheral blood T lymphocytes (33, 17). Along the same lines, we did not observe significant aggregation of peripheral T lymphocytes after addition of purified LFA-3, while aggregation of Jurkat cells was reproducibly augmented. This again may be due to differences in surface charge density, which would regulate the tendency of cells to come into close contact. These same factors could be responsible for regulation of CD2 dependent adhesion with other cell types.

LFA-3 is the first example of a purified ligand molecule that has been directly shown to mediate T lymphocyte adhesion. Several other T lymphocyte surface antigens are thought to be involved in receptor-ligand type interactions with structures on target cells or antigen-presenting cells. The T cell receptor is thought to interact with nominal antigen physically associated with polymorphic major histocompatability complex antigens (34). The interaction of the T cell receptor with nominal antigen alone has been reported to have a dissociation constant on the order of 10^{-5} M (35); the affinity of the same T cell receptors for nominal antigen associated with MHC could be higher. The high-affinity interaction of CD2 with LFA-3 may increase the efficiency of cell-cell interaction to a point where T cell receptor interaction with antigen can occur more efficiently. This may be particularly important for low-affinity antigen receptors. CD4 and CD8 have been suggested by functional data to interact with class II and class I MHC antigens, respectively (36). However, attempts to demonstrate an interaction between CD4 and class II molecules or CD8 and class I molecules using either purified proteins or cells overproducing these proteins have been unsuccessful thus far, using a variety of techniques (J. Strominger, personal

communication). Lymphocyte function–associated antigen-1 (LFA-1) on T lymphocytes has also been suggested to interact with ligand molecules in the human (37) and the mouse (38), but no evidence for a direct interaction has been reported.

Recently, a molecule termed the T11 target structure (T11TS) has been identified on sheep erythrocytes (39). Antibodies to T11TS inhibit rosetting of human T cells with sheep erythrocytes. While the purified T11TS has an M_r of 42,000, which is somewhat lower than LFA-3 from human erythrocytes, it is otherwise very similar in function and distribution to LFA-3 (40). Like purified LFA-3, purified T11TS blocks binding of CD2 mAb to human T lymphocytes (39). These findings suggest that sheep T11TS may be a functional homologue of human LFA-3 (1).

It has been proposed that CD2 mediates an alternative pathway of T cell activation, based on the ability of pairwise combinations of mAb to certain CD2 epitopes to trigger T cell proliferation and cytotoxic function (9). These results suggest that LFA-3, the biological ligand of CD2, may be able to trigger T cell function, at least in some contexts. LFA-3 blocks binding of the TS2/18 mAb and therefore probably interacts with CD2 at or near this site which is designated as a T11$_1$-type (9) or exterior domain III (41) epitope. Although binding to this epitope by TS2/18 does not lead to T cell activation, ligation with LFA-3 may have more profound biological consequences. The question of triggering T lymphocyte proliferation or function via CD2 is particularly interesting in the context of interactions of cells in the thymus (42). It has been shown recently that thymocytes adhere to thymic epithelial cells via a CD2/LFA-3-dependent adhesion pathway (14). This interaction may be of importance in regulating the behavior of thymocyte populations that lack the T cell receptor–CD3 complex and yet proliferate in the thymus (15, 42). In this report we have addressed adhesion-related functions of purified LFA-3. Further study is required to establish a role of LFA-3 in T lymphocyte activation or triggering via CD2.

Summary

CD2 is a T lymphocyte glycoprotein that functions in adhesion of T lymphocytes and also as a putative receptor for activation signals. Functional data suggest that LFA-3, a widely distributed cell surface glycoprotein, may be the biological ligand of CD2. We have purified LFA-3 from human erythrocytes and characterized the purified protein functionally. LFA-3 bound specifically to CD2$^+$ cells, and this binding was inhibited by CD2 mAb. Conversely, purified LFA-3 inhibited binding of CD2 mAb to cells, and the concentration required for this effect suggests that LFA-3 half-saturated CD2 at 1–5 nM LFA-3. Purified LFA-3 inhibited rosetting of human and sheep erythrocytes with CD2$^+$ T lymphoma cells and T lymphocytes, and mediated aggregation of a CD2$^+$ T lymphoma cell line. Purified LFA-3 reconstituted into planar membranes mediated efficient CD2-dependent adhesion of T lymphoblasts. These data demonstrate that LFA-3 is a ligand for CD2 and that LFA-3 can mediate T lymphocyte adhesion.

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