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PURIFICATION AND CHARACTERIZATION OF THE LYMPHOCYTE FUNCTION-ASSOCIATED-2 (LFA-2) MOLECULE[1]

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The lymphocyte function-associated-2 (LFA-2) molecule, equivalent to CD2 and the E rosette receptor, was purified by MAb affinity chromatography from the Jurkat T lymphoma cell line. Jurkat was selected for its high level of expression of 1.0×10^5 sites/cell. A two-site radioimmunometric assay was developed to monitor purification. From 50 g of packed cells, 230 μg of LFA-2 was obtained with 65% yield of antigenic activity with a purification factor of 13,000. A major component of 58,000 and 54,000 was obtained that corresponded to LFA-2 antigenic activity as shown by immunoblotting and immunoprecipitation. The doublet was resolved by 2D IEF-SDS-PAGE into components of pI = 5.5 and 5.6. Smaller amounts of lower M_r components were also seen. All these components appeared related by processing or proteolytic breakdown, as shown by Cleveland peptide mapping. The LFA-2 deoxycholate complex had an apparent M_r of 68,000 by gel filtration, suggesting it was monomeric. Purified LFA-2 inhibited rosetting of T lymphocytes with sheep E, and addition to preformed rosettes caused their disruption. Inhibitory activity was absorbed by sheep E. This is the first evidence that the CD2/LFA-2 molecule can directly bind to sheep E. Purified LFA-2 should be useful for the further biochemical and functional characterization of this molecule.

The lymphocyte function-associated 2 (LFA-2)[3] molecule was initially defined by monoclonal antibodies (MAb) selected for inhibition of antigen-specific cytolytic T lymphocyte (CTL)-mediated killing (1). LFA-2 MAb block cytolysis by binding to effector CTL; preincubation of MAb with the target cell has no detectable effect (2). LFA-2 MAb block adhesion of CTL to target cells, as shown by inhibition of conjugate formation (3). The LFA-2 molecule may have physiologic importance in strengthening the adhesion of the CTL to its target, as has been suggested for the LFA-1 molecule on the effector CTL and the LFA-3 molecule on the target cell. In addition to cytolysis, a number of T helper cell functions are inhibited by anti-LFA-2, including antigen-specific proliferative responses, the mixed lymphocyte reaction, and mitogen responses (2, 4).

LFA-2 is expressed on cells of the T lineage, including thymocytes, CTL clones, peripheral blood lymphocytes (PBL), and PHA-blasts; LFA-2 is not expressed on monocytes, granulocytes, or B lymphoblastoid cell lines. Anti-LFA-2 immunoprecipitates a major diffuse band from 55,000 to 47,000 daltons. The m.w. species differ, by as much as 5000 daltons, depending on the stage of activation or differentiation of the T cell (2).

By cell distribution and coprecipitation, the LFA-2 molecule is identical to the molecules variously termed T11, Leu-5, and the E rosette receptor (4, 5, 11). Like other MAb to this molecule, LFA-2 MAb inhibit rosetting of T lymphocytes with sheep erythrocytes (E). The designation CD2 has recently been suggested for this molecule. Although CD2/LFA-2 has been termed the E rosette receptor, the only evidence for this is the ability of MAb to CD2/LFA-2 to inhibit E rosetting. It has been unclear whether the CD2 molecule participates directly in this interaction by binding to a "ligand" on E, or facilitates this interaction by some other mechanism. Rosetting with sheep E has been a convenient method for purification of human T lymphocytes; however, its relationship to physiologic responses mediated by LFA-2/CD2 is unclear. Although the T11, Leu-5, and E rosette receptor MAb were obtained before LFA-2 MAb, the significance of the CD2 molecule in T lymphocyte function was not made clear until the blocking of CTL killing studies with LFA-2 MAb were carried out. Recent blocking studies carried out in several laboratories have confirmed the physiologic importance of LFA-2/CD2 in T cell responses (6, 7). Additionally, it has been found that certain combinations of MAb that bind to different epitopes on CD2 can trigger T cell proliferation and clonal function (8).

Further understanding at the molecular level of the function of LFA-2 requires its biochemical characterization. Toward this end, we report here the purification of LFA-2 by MAb affinity chromatography and the characterization of LFA-2 by isoelectric focusing (IEF) and Cleveland peptide mapping. Furthermore, we show that the affinity-purified material can inhibit E rosette formation, and that sheep E can absorb the inhibitory activity of the LFA-2 molecule.

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[3] Abbreviations used in this paper: CD2, Cluster of differentiation 2; E, erythrocyte; LFA-1 and LFA-2, lymphocyte function-associated antigens 1 and 2; MAb, monoclonal antibody; NaDOC, sodium deoxycholate; M_r, relative molecular mass; PBL, peripheral blood lymphocyte; 2D, two-dimensional; NEPHGE, nonequilibrium pH gradient electrophoresis.

MATERIALS AND METHODS

Fluorescence-activated cell sorter (FACS) analysis. Immunofluorescence flow cytometry was performed after labeling cells with MAb and affinity-purified fluorescein isothiocyanate anti-mouse IgG (Zymed Laboratories, South San Francisco, CA) (7, 9).

Iodination of cells, protein, and MAb. Cells, MAb, and purified LFA-2 were iodinated by using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen; Pierce, Rockford, IL) according to the method of Fraker and Speck (10).

MAb to LFA-2. The MAb TS1/8 (IgG1) and TS2/18 (IgG1) were previously described (1). MAb 35.1 (IgG2a) and 9.6 (IgG2a) (11) were kindly provided by Dr. John Hansen (University of Washington, Seattle, WA).

Estimation of TS2/18 binding sites on Jurkat cells. Jurkat cells were incubated with increasing concentrations of ^{125}I-labeled TS2/18 MAb. Incubation of 2×10^5 cells and ^{125}I-MAb was in a total volume of 100 μl of cold PBS-BSA in a microtiter plate for 1 hr on ice. Cells were washed three times with 200 μl bovine serum albumin in phosphate-buffered saline (PBS-BSA) and transferred to vials to be counted. The number of TS2/18 MAb molecules bound per cell was determined from the specific cpm bound and the specific activity of the ^{125}I-TS2/18. Nonspecific binding was determined by adding a 100-fold excess of unlabeled TS2/18.

Immunoprecipitation. Cell lysates were precleared with activated, quenched Sepharose or human Ig-Sepharose before specific immunoprecipitation with a MAb to LFA-2 bound to Sepharose (1). MAb-Sepharose was incubated with cell lysates for 2 hr at 4°C. Immunoprecipitates were washed sequentially once with 10 mM Tris pH 8.0, 0.15 M NaCl, 0.1% Triton; 10 mM Tris pH 8.0, 0.15 M NaCl; and 10 mM Tris pH 6.8. Material was eluted from the Sepharose by boiling for 3 min in sample buffer containing 0.025 M Tris, pH 6.8, 3% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, and 10% glycerol. Material eluted from the Sepharose was run on Laemmli SDS-polyacrylamide gels (12). The following radiolabeled or cold proteins were used as standards: myosin, 200,000; phosphorylase B, 97,400; BSA, 68,000; ovalbumin, 43,000; lactoglobulin A, 18,367.

Sandwich radioimmunoassay (RIA) for quantitating LFA-2 in detergent extracts. The activity of LFA-2 in Jurkat cell lysates and material eluted from the affinity column was measured by a sandwich RIA. Fifty microliters per well of anti-LFA-2 MAb 35.1 (20 μg/ml in PBS, pH 6.4) were incubated in soft-well microtiter plates (Linbro S-MVC; Flow Laboratories, McLean, VA) at room temperature for 1 hr. Plates were then washed three times with 1% BSA in PBS pH 7.2. Jurkat cell lysates were obtained by incubating frozen cells with lysis buffer (0.01 M Tris pH 8.0, 0.15 M NaCl, 1% Triton X-100 [New England Nuclear, Boston, MA], 1 mM iodoacetamide [Sigma Chemical Co., St. Louis, MO], 100 kallikrein units/ml Trasylol [Sigma], and 1 mM phenylmethylsulfonyl fluoride [Sigma]) for 60 min at 4°C, and then centrifuged at 100,000 × G for 60 min. The cell extract or affinity-purified LFA-2 was diluted to various concentrations in 0.01 M Tris, 0.15 M NaCl, 0.05% Triton X-100, and 50 μl were added per well for 2 hr at room temperature. The plates were then washed three times with 1% BSA in PBS. The final incubation was with 50 μl of ^{125}I-labeled TS1/8 or TS2/18 anti-LFA-2 MAb (1 to 3×10^5 cpm) in PBS and 1% horse serum for 2 hr at room temperature. The microtiter plate was then washed three times with 1% BSA in PBS. The wells were cut and counted in a gamma counter. Nonspecific binding was determined by incubating cell lysate or affinity-purified LFA-2 in wells coated with P3-X63 myeloma supernatant, and the nonspecific binding was subtracted.

Affinity purification of LFA-2. Sixty grams of Jurkat cells were washed three times in PBS and pelleted. Cell pellets were lysed at 5×10^7 cells/ml by incubating with 1200 ml of lysis buffer for 1 hr at 4°C. The cell lysate was centrifuged at 3615 × G for 15 min. Sodium deoxycholate (NaDOC) was then added to a final concentration of 0.5% to the supernatant from this low-speed centrifugation. The cell lysate was centrifuged at 158,000 × G for 60 min at 4°C.

Human IgG (Cohn fraction II) (Miles), MAb TS2/18, or MAb TS1/8 were coupled to cyanogen bromide-activated Sepharose CL-4B (Pharmacia) to a final concentration of 0.95 mg protein/ml of packed beads. Ten-milliliter columns of each were prepared and connected in series (human IgG to TS2/18 to TS1/8). They were then prewashed, first with 50 ml of 0.5% Triton X-100, 0.1 M glycine HCl, pH 3.0, then with 50 ml of 10 mM Tris-HCl pH 8.0, 0.15 M NaCl, 1% Triton X-100, and 0.5% NaDOC, before application of the Jurkat lysate.

Approximately 1200 ml of the 100,000 × G supernatant were applied to the antibody columns at a flow rate of 1 ml/min. After the sample was loaded, the columns were separated, washed, and sequentially eluted with the following buffers: 1) 50 ml of 10 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.5% Triton X-100, and 0.5% NaDOC, 2) 50 ml of 0.1 M glycine-HCl pH 5.0 and 0.5% Triton X-100 (100 ml for TS2/18-Sepharose), 3) 50 ml of 0.1 M glycine-HCl pH 3.0 and 0.5% Triton X-100, 4) 50 ml of 0.1 M glycine-HCl pH 2.0 and 0.5% Triton X-100, and 5) 50 ml of 10 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.5% Triton X-100, and 0.5% NaDOC. Fractions of 5 ml were collected in tubes containing neutralization buffer (1 M Tris-HCl pH 11 with 0.5% Triton X-100). Fractions 23 to 28 of the glycine pH 3.0 eluted material (see Fig. 3) were pooled and concentrated to 6 ml. The pooled fractions from the TS2/18-Sepharose CL-4B column were reapplied to a second series of 1-ml columns that were prewashed and eluted as indicated above. However, 0.1% Trition X-100 instead of 0.5% Triton X-100 was used in the elution buffers.

Western blotting. Jurkat lysate and affinity-purified LFA-2 from Jurkat cells were run on a 10% polyacrylamide gel under reducing conditions, then electrophoretically transferred to nitrocellulose by the method of Towbin et al. (13). After blocking the nitrocellulose with 3% BSA, 1% hemoglobin in 0.01 M Tris, 0.15 M NaCl pH 7.8, the paper was incubated for 2 hr with ^{125}I-TS2/18, washed, dried, and autoradiographed.

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE). Radiolabeled LFA-2 and 1 μg BSA carrier were boiled for 5 min in 0.5 ml of 0.6 M Tris-HCl, 0.02 M EDTA, 9% SDS, pH 8.3 buffer and reduced under nitrogen for 90 min at 37°C with 0.025 M dithiothreitol. The proteins were then alkylated in the dark for 1 hr at room temperature by using 0.05 M iodoacetamide, and dialyzed with 0.05 M Tris-HCl, 2% 2-mercaptoethanol, 2% SDS, pH 7.5. The proteins were then precipitated with trichloroacetic acid to a final concentration of 15%, were washed successively with 15% trichloroacetic acid ethanol:ether (1:1), and ether, and were airdried. IEF and nonequilibrium pH gradient electrophoresis (NEPHGE) were performed according to the procedure of Jones (14).

Peptide mapping: Cleveland staphylococcal V8 protease digest of affinity-purified LFA-2. Iodinated affinity-purified LFA-2 was immunoprecipitated with 35.1-Sepharose and run under reducing conditions on a 10% acrylamide resolving gel, localized by autoradiography, and cut out from the wet gel. Partial peptide hydrolysis was then performed under re-electrophoresis with staphylococcal V8 protease (Miles, Naperville, IL) according to the procedure of Cleveland et al. (15) by using 1.0, 0.1, 0.01, or 0 μg of enzyme per gel.

Gel filtration of affinity-purified LFA-2. A 200-μl sample (8×10^7 cpm) of ^{125}I-LFA-2 from Jurkat cells in 10 mM Tris pH 8.0, 0.1 M NaCl, and 0.1% NaDOC with the addition of 1 mg of lysozyme as protein carrier was loaded onto a Sephadex G-200 Superfine (Pharmacia, Piscataway, NY) column (1.5 x 29 cm) that had been equilibrated with the same buffer. Fractions of 1.0 ml were collected at a flow rate of 0.1 ml/min. Aliquots (20 μl) or 35.1-Sepharose immunoprecipitates from 25-μl aliquots to which 1% Triton was added were run on 10% SDS-polyacrylamide gels run under reducing conditions.

Inhibition of rosetting by MAb to LFA-2. A modified assay of Lay et al. (16) was used to monitor sheep E rosette inhibition. Jurkat or PBL cells (2×10^6/ml) were washed three times with Hanks' balanced salt solution containing 10 mM HEPES, pH 7.2. Cells (0.1 ml) were mixed at room temperature with an equal volume of 0.5% washed sheep E and 10 μg purified monoclonal antibodies or 10 μg P3-X63 supernatant. The cells were centrifuged at room temperature for 5 min at 200 × G and then incubated on ice for 60 min. The tubes were gently rocked to resuspend the pellet and the rosettes were counted in a hemocytometer.

Inhibition of rosetting with affinity-purified LFA-2. Jurkat cells or PBL at 2.5×10^6/ml and SRBC at 2.5×10^8/ml were washed three times with Hanks' 10 mM HEPES. Each sample in this rosetting assay had a final volume of 200 μl, with a final BSA concentration of 15%. Purified LFA-2 was mixed with BSA, then 40 μl of 1×10^5 Jurkat cells were added. The mixture was incubated for 30 min at room temperature. Forty microliters of 1×10^7 sheep E were then added. Rosetting was assayed as above for each experimental sample and its control. To obtain identical detergent concentration and ionic strength as was found in the experimental sample, 20 μl of 0.01 M Tris, 0.15 M NaCl, and 0.1% Triton X-100 were added to the control sample.

Abolishing the inhibitory rosetting activity of LFA-2 by heat denaturation. In heat-denaturation experiments the proteins LFA-2 (1.8 μg), p150,95 (1.8 μg), and TS2/18 MAb (1.8 μg) were heated at 100°C for 15 min before addition of BSA.

Disruption of rosetting with affinity-purified LFA-2. The rosetting assay was also performed with Jurkat cells and PBL, with the addition of LFA-2 after rosetting had occurred. When sheep E and Jurkat cells or PBL had incubated on ice for 60 min, the control and experimental samples were placed at room temperature, and purified LFA-2 was added to the experimental sample. A buffer identical to that described above except without LFA-2 was added to the control. The samples were gently shaken and incubated for 30 min before

determining the percentage of rosettes. Sheep E rosette inhibition was calculated by the following formula:

$$\% \text{ Sheep E rosette inhibition} = 100\% \times \left(\frac{\% \text{ sheep E rosette control} - \% \text{ sheep E rosette experimental}}{\% \text{ sheep E rosette control}}\right)$$

RESULTS

Cell lines with high LFA-2 expression. T lymphoma cell lines were screened by immunofluorescent flow cytometry to find a cell source for purification of the LFA-2 molecule. Two lines, Jurkat and SKW3, consistently showed the highest staining with the MAb TS2/18 (Table I). Jurkat cells were therefore chosen as the starting material for the isolation of LFA-2.

The number of LFA-2 sites expressed on Jurkat and SKW3 cell surfaces was determined by measuring the number of Ig molecules bound to intact cells at saturating amounts of ^{125}I-labeled TS2/18 antibody (Fig. 1). From three separate experiments the estimation of the LFA-2 sites per cell was 0.8 to 1.2 × 10^5 ($\bar{x}$ = 1.0) molecules/SKW3 cells and 1.0 to 2.2 × 10^5 ($\bar{x}$ = 1.6) molecules/Jurkat cell. Scatchard plots give an estimate of the K_A for both TS1/8 and TS2/18 of 1 × 10^9 M^{-1}.

Sequential immunoprecipitation of LFA-2. Competitive inhibition studies performed with radiolabeled TS2/18 or TS1/8 with cold MAb to LFA-2 showed that the MAb 35.1 did not competitively inhibit binding of TS1/8 or TS2/18 MAb. TS1/8 and TS2/18 MAb competitively inhibited one another (data ot shown). This suggested it would be possible to use MAb 35.1 in combination with TS2/18 or TS1/8 in a sandwich RIA to monitor the purification of LFA-2 (see below). To confirm whether TS2/18 and 35.1 recognized the same molecule, sequential immunoprecipitation studies were done. Both MAb TS2/18 and 35.1 immunoprecipitated similar molecules from cell surface-iodinated Jurkat cells (Fig. 2, lanes A and B). Preclearing the detergent lysate with TS2/18-Sepharose removed molecules identified by 35.1-Sepharose (Fig. 2, compare lanes D and F). The reverse was also true; preclearing the detergent lysate with 35.1-Sepharose removed molecules identified by TS2/18-Sepharose (Fig. 2, compare lanes E and G). A molecule of 95,000 daltons was nonspecifically immunoprecipitated, as it is also immunoprecipitated with human Ig-Sepharose (lane C). Similar preclearing was found between TS1/8 and 35.1 MAb, and between TS1/8 and TS2/18 MAb (data not shown).

Affinity purification of LFA-2. For large-scale purification of LFA-2, the Jurkat cell line was chosen. Jurkat cells were solubilized with the nonionic detergent Triton X-100. After centrifugation, NaDOC was added to the supernatant, which was subjected to a second centrifugation, and the extract was applied to LFA-2 MAb affinity chromatographic columns. Preliminary experiments showed that LFA-2 could be eluted at both low and high pH, but was antigenically stable only after low pH elution. Therefore, the Triton X-100 and NaDOC detergent was exchanged for Triton X-100 while LFA-2 was bound to the LFA-2 MAb columns, and then the columns were eluted at successively lower pH. After a precolumn, two LFA-2 MAb columns, TS2/18-Sepharose and TS1/8-Sepharose, were connected in series. LFA-2 was found

TABLE I

LFA-2 expression on T cell lines[a]

Cell Type	% Positive Cells	Relative Fluorescence Intensity (GF SRBC units)
Jurkat	99	16.0
SKW3	99	14.5
HPB-MLT	90	3.6
TALL-1	16	0.1

[a] Cells were labeled with TS2/18 MAb as described in *Materials and Methods*. The scatter gates were set to exclude dead cells. Fluorescence intensity is indicated as gluteraldehyde fixed sheep E units (GF SRBC units). Fluorescence intensity was obtained with a calibration curve of GF-SRBC at different gain settings.

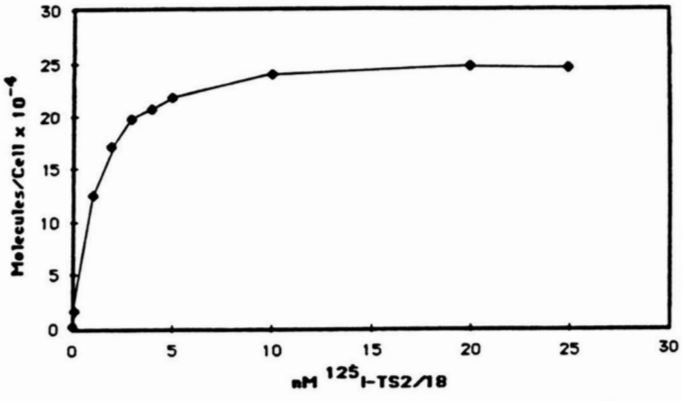

Figure 1. Saturation binding of ^{125}I-TS2/18 to Jurkat cells. ^{125}I-TS2/18 was incubated with 2 × 10^5 Jurkat cells, and binding was determined as described in *Materials and Methods*. Specific activity of ^{125}I-TS2/18 was 6.5 × 10^5 cpm/μg. The number of sites was determined by extrapolation to the *y* axis. The results represent averages of triplicate samples.

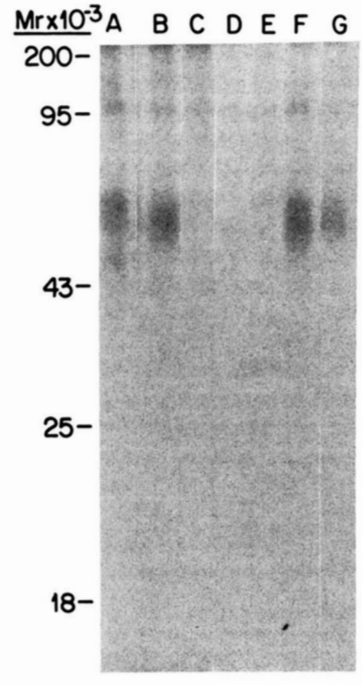

Figure 2. Immunoprecipitation of cell surface LFA-2 by MAb 35.1 and TS2/18. Lysate from ^{125}I-labeled Jurkat cells was immunoprecipitated with purified MAb coupled to Sepharose CL-4B, followed by SDS 12% PAGE under reducing conditions. *A*, TS2/18-Sepharose; *B*, 35.1-Sepharose; *C*, human Ig-Sepharose; *D*, precleared twice with TS2/18-Sepharose and subsequently immunoprecipitated with 35.1-Sepharose; *E*, precleared twice with 35.1-Sepharose and subsequently immunoprecipitated with TS2/18-Sepharose; *F*, precleared twice with human Ig-Sepharose and subsequently immunoprecipitated with 35.1-Sepharose; *G*, precleared twice with human Ig-Sepharose and subsequently immunoprecipitated with TS2/18-Sepharose. A molecule of 95,000 daltons was nonspecifically immunoprecipitated, as it is also immunoprecipitated with human Ig-Sepharose (lane C).

only in the eluate of TS2/18 column, suggesting that TS2/18 MAb-Sepharose was in excess over LFA-2 antigen, and there were no molecules which bore the TS1/8 but lacked the TS2/18 determinant.

The LFA-2 antigen eluted with pH 3 buffer as a single sharp peak in fractions 23 to 26 from the TS2/18-Sepharose column, as determined by sandwich RIA (Fig. 3*C*) and SDS-PAGE (Fig. 3*A*). The SDS-PAGE gel (Fig. 3*A*) was deliberately overloaded. It reveals a predominant band at 54,000 relative molecular mass (M_r), corresponding to LFA-2, together with smaller amounts of impurities. Western blotting confirmed that the 54,000 M_r band corresponded to LFA-2 (Fig. 3*B*, lane 1), and that there was little or no alteration in its M_r compared with the starting material (Fig. 3*B*, lane 2). A second cycle of affinity chromatography resulted in a slight increase in specific activity (Table II), but contaminants were still present (data not shown). The overall purification of LFA-2 (Table II) was 13,000-fold from the initial Jurkat extract. The yield was 230 μg of LFA-2 from 3000 mg of cell lysate. LFA-2, therefore, constitutes about 0.01% of the total protein extracted from Jurkat cells.

Biochemical characterization of affinity-purified LFA-2. Radioiodinated, affinity-purified LFA-2 was characterized by 2D IEF, 2D Cleveland mapping, and gel filtration. The 2D IEF pattern (Fig. 4*A*) showed that affinity-purified ^{125}I-LFA-2 has three major groups of iodinated bands: a) 58,000 M_r with a pI centered at 5.6, b) 54,000 M_r with a pI centered at 5.5, and c) four distinct spots at 44,000 M_r with pI of 6.0, 5.9, 5.7, and 5.6. A minor band at 50,000 M_r with a pI of 6.0 can also be seen. On longer exposure of the IEF gels, minor iodinated bands at 36,000 M_r with a pI of 6.1 to 5.5 and another minor band at 32,000 M_r with a pI of 6.1 to 5.5 can be seen. When ^{125}I-labeled purified LFA-2 was immunoprecipitated with 35.1 MAb (Fig. 4*B*) or TS2/18 MAb (Fig. 4*D*), a similar pattern was obtained.

2D Cleveland maps were used to determine the structural relationship between the LFA-2 m.w. species isolated from the affinity chromatographic column. Affinity-purified ^{125}I-LFA-2 was immunoprecipitated by 35.1-Sepharose and run on a 10% SDS-acrylamide gel under reducing conditions. Cleavage with staphylococcal V8 protease was done during re-electrophoresis on a 15% SDS-acrylamide gel. The two bands at 58,000 and 55,000 M_r, corresponding to the major LFA-2 species, gave similar Cleveland maps (Fig. 5). Bands present in lesser amounts at 44,000, 36,000, and 32,000 M_r also gave similar maps. These findings suggest these different M_r species may be related to each other by differential processing or proteolytic breakdown.

The affinity-purified ^{125}I-LFA-2 from Jurkat cells was analyzed by gel filtration on Sephadex G-200 Superfine. Aliquots of individual column fractions and aliquots of individual fractions immunoprecipitated with 35.1-Sepharose were analyzed on 10% polyacrylamide gels under reducing conditions. The LFA-2-NaDOC complex from the gel filtration profile had an apparent M_r of 68,000 (Fig. 6). Immunoprecipitated material from this peak that was run on SDS polyacrylamide gels had a major species with a M_r of 54,000. Results from the gel filtration suggest that LFA-2 is monomeric, and are compatible with some bound NaDOC.

E rosette inhibtion. We next examined whether purified LFA-2 could inhibit rosetting between sheep E and T lymphocytes. The majority of thymocytes and some T lymphocytes, but not B cells, rosette with sheep E (16, 18–22). Previous studies on rosetting have shown that

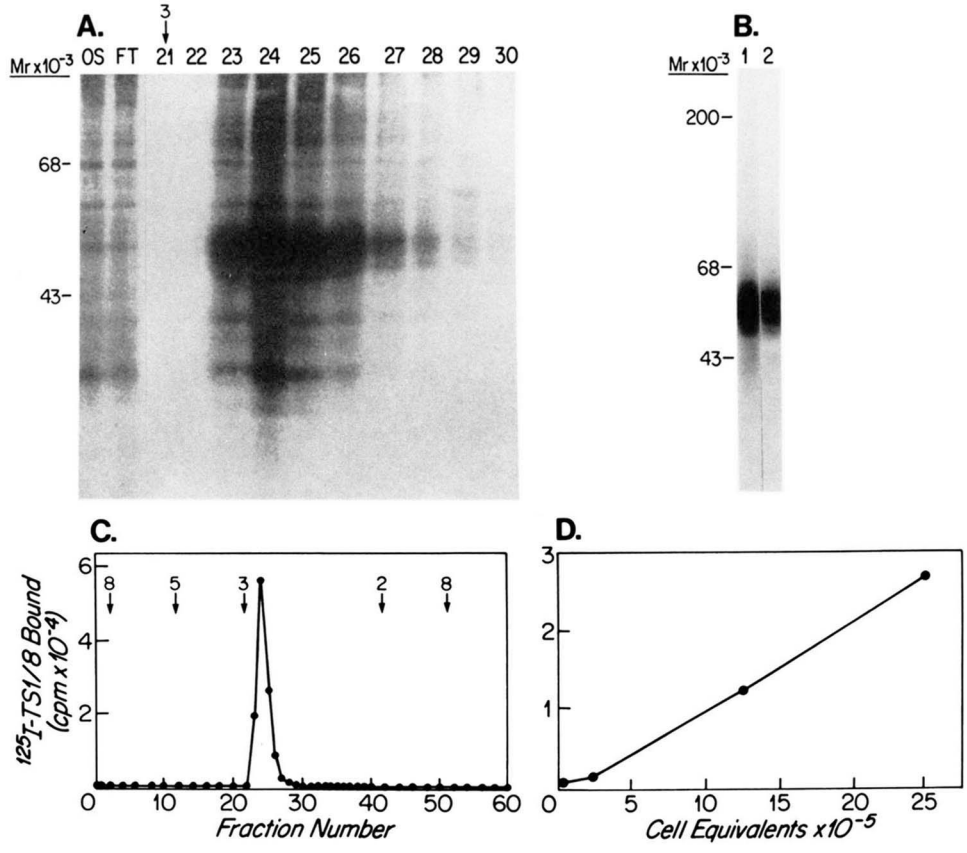

Figure 3. Purification of LFA-2. LFA-2 was purified as described in *Materials and Methods*. *A*, SDS 12% PAGE under reducing conditions, followed by silver staining of 2 μl of original lysate (OS); 2 μl of flow through (FT); and 200 μl of fractions (21 through 30) corresponding to the pH 3 elution beginning at fraction 21. *B*, Immunoblots. Samples were subjected to SDS 10% PAGE, transferred to nitrocellulose, and probed with ^{125}I-TS2/18. Lane 1, 1 μl of purified LFA-2; lane 2, 25 μl of original Jurkat lysate. *C*, Sandwich RIA of TS2/18 MAb affinity column eluates. Aliquots of 25 μl of each fraction, or for peak fractions smaller amounts in order to remain on the linear portion of the curve, were diluted to 50 μl and subjected to RIA. Amount of binding given by 25 μl or its equivalent is shown. Positions at which the pH step gradient was changed are marked. *D*, RIA standard curve. Dilutions of Jurkat cell lysate were assayed in parallel with elution fractions.

TABLE II

Purification of LFA-2 from Jurkat cells[a]

Purification Fraction	Volume (ml)	Total Protein (mg)	Specific Activity (units/mg)	Total Activity (units)	% Recovery	-Fold Purification
Cell lysate	1200	3000	2×10^5	6.4×10^8	(100)	
1st Ab affinity column	6	0.40	16×10^8	6.4×10^8	100	7,500
2nd Ab affinity column	2.5	0.23	18.2×10^8	4.2×10^8	65	13,000

[a] LFA-2 in each fraction was measured by the sandwich RIA. Protein was measured by the Bradford assay (17).

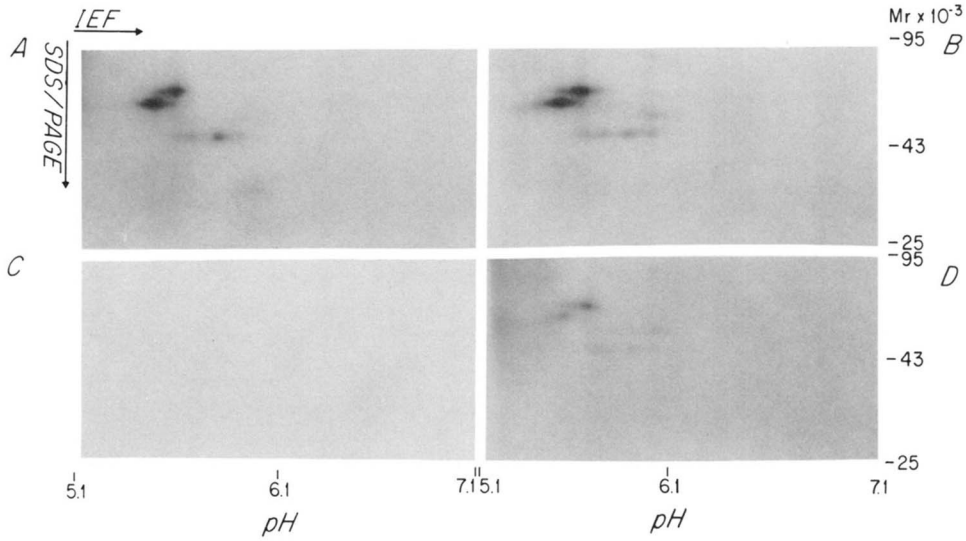

Figure 4. 2D IEF-SDS-PAGE of purified LFA-2. A sample of the eluted material was iodinated by the procedure of Fraker and Speck (10). In the first dimension, radioactive material was applied at the acidic end of the IEF gel, and the second dimension was a SDS 12% polyacrylamide gel. Equal amounts of ^{125}I-LFA-2 were subjected directly to 2D IEF-SDS-PAGE (*A*), or first subjected to immunoprecipitation with 35.1-Sepharose (*B*), human Ig-Sepharose (*C*), or TS2/18-Sepharose (*D*).

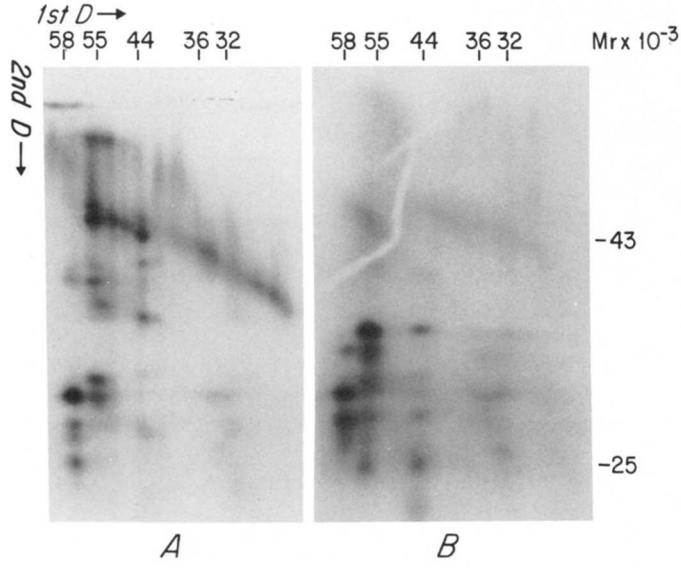

Figure 5. Cleveland 2D peptide mapping of affinity-purified ^{125}I-LFA-2. The 70,000 to 20,000 M_r region of SDS 10% PAGE lanes was placed over a second SDS 15% PAGE gel and subjected to proteolysis during reelectrophoresis by using staphylococcal V8 protease: *A*, 0.1 μg protease/gel; *B*, 1 μg protease/gel.

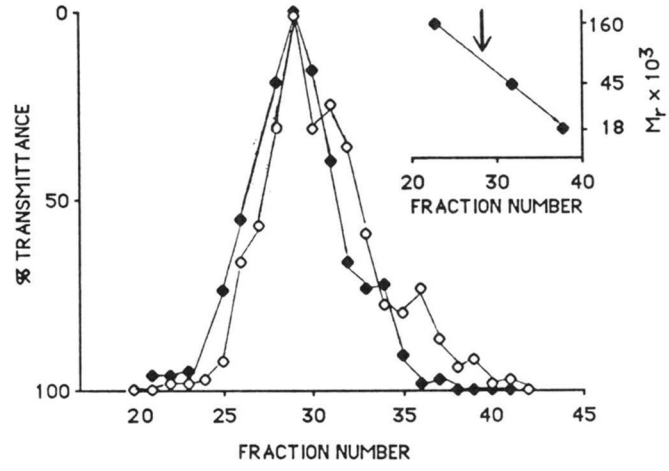

Figure 6. Gel filtration of ^{125}I-labeled LFA-2. Densitometric scan of the peak activity of the affinity purified ^{125}I-LFA-2 from the gel filtration column subjected to SDS-PAGE. LFA-2 was subjected to Sephadex G-200 gel filtration in the presence of 0.1% NaDOC, 0.01 M Tris pH 8.0, and 0.1 M NaCl. Twenty-microliter aliquots (◆—◆) or 35.1-Sepharose immunoprecipitates (◇—◇) from the gel filtration fractions were run on 10% SDS-PAGE under reducing conditions. The LFA-2 band of 54,000 M_r from SDS-PAGE was scanned with a scanning densitometer (Hoeffer Scientific Instrument GS300). *Inset*, The standard proteins for calibration of the gel filtration column were bovine γ-globulin (160,000 daltons), ovalbumin (45,000 daltons), and myoglobin (18,300 daltons). The peak from the gel filtration profile had a M_r of 68,000.

the T cells must be viable (18, 21, 22), that rosetting is optimal between pH 7.0 and pH 8.0 (20), and the maximal rosetting occurs when the initial contact of the sheep E and lymphocyte occurs at 37°C or room temperature and is followed by cooling to 4°C (16, 20).

MAb to the LFA-2 molecule were able to inhibit Jurkat and PBL from rosetting (Table III), whereas MAb TS1/18 and TS1/22 to the LFA-1 molecule had no effect on rosette formation. Affinity-purified LFA-2 also inhibited rosetting in a dose-dependent fashion, with the percentage of inhibition reaching 98.5% (Table IV). Rosetting by PBL was also inhibited by purified LFA-2 (data not shown). Interestingly, when LFA-2 was added to already formed rosettes, it could also cause disruption of rosettes (Table IV).

When LFA-2 had been boiled for 15 min before addition to the rosetting assay, it could no longer inhibit in the rosetting assay (Table V). The heat-denatured LFA-2 also showed no actvity in the sandwich RIA (data not shown). Native TS2/18 inhibits rosetting of T cells with sheep E

TABLE III
Inhibition of E rosetting by MAb[a]

MAb	Specificity	% Jurkat Cells Rosetting	% PBL Rosetting
TS1/8	LFA-2	0	0
TS2/18	LFA-2	0	0
35.1	LFA-2	0	0
TS2/18 + 35.1	LFA-2	0	0
P3x63	Conrol	25	31
TS1/18 + TS1/22	LFA-1	—	28

[a] Cells (1×10^5) in 0.1 ml Hanks' balanced salt solution 10 mM HEPES, pH 7.2, were mixed with an equal volume of 0.5% washed sheep E, and 10 μg purified MAb were added in 10 μl at room temperature. E rosettes were assayed as described in *Materials and Methods*.

TABLE IV
Inhibition by purified LFA-2 of E rosettes

LFA-2		% Jurkat Cells Rosetting	% E Rosette Inhibition
0.036 μg[a]	+	23.3	24.4
	−	30.8	
0.36 μg	+	12.5	65.0
	−	35.7	
1.8 μg	+	0.5	98.5
	−	31.7	
1.8 μg[b]	+	1.3	95.7
	−	30.5	

[a] Purified LFA-2 (+) or a buffer without LFA-2 (−) were mixed with BSA that would give a final concentration of 15%; then Jurkat cells were added, and the mixture was incubated for 30 min at room temperature. E rosettes were assayed as described in *Materials and Methods*.

[b] When rosettes had been preformed, before resuspending the pellets, the rosetted cells were incubated with or without LFA-2. The samples were gently shaken and incubated for 30 min before determining the percentage of rosettes.

TABLE V
Abolishing the inhibitory rosetting activity of LFA-2 by heat denaturation[a]

	Native Protein (% rosettes)	Denatured Protein (% rosettes)
Buffer	43	43
LFA-2	0	41
p150,95	42	43
TS2/18	0	44

[a] First, 1.8 μg of native protein or heat-denatured proteins that had been heated at 100°C for 15 min were mixed with BSA; then Jurkat cells were added, and the mixture was incubated for 30 min at room temperature. E rosettes were assayed as described in *Methods*.

TABLE VI
Absorption of LFA-2 by sheep E[a]

	First Absorption		Second Absorption	
	% Rosettes	% Inhibition	% Rosettes	% Inhibition
Sheep E-absorbed LFA-2	14	54.7	34.6	0
Chicken E-absorbed LFA-2	1.6	94.8	8.1	73.9
Jurkat-absorbed LFA-2	3.3	89.3	8.3	73.2
Control	30.9		31	

[a] Affinity purified LFA-2 (1.8 μg) was incubated either once or twice with an equal volume of packed sheep E, chicken E, or Jurkat cells for 60 min at 4°C. The control consisted of Jurkat cells and sheep E mixed with BSA and a buffer of equal ionic strength and detergent concentration without LFA-2. The supernatant from the absorption was then tested in the rosetting assay as indicated in *Materials and Methods*. Unadsorbed LFA-2 gave 100% inhibition in these experiments.

(shown in Table III and again in Table V), whereas heat-denatured TS2/18 was no longer inhibitory in the rosetting assay, and a purified membrane glycoprotein, p150,95, had no observable effect in the rosetting assay, whether native or denatured.

It seemed unlikely that inhibition of rosetting was from TS2/18 MAb leaching off of the affinity column along with the LFA-2 molecule. However, to rule out this possibility, an absorption experiment was done (Table VI). A sample of affinity-purified LFA-2 that gave complete inhibition of rosetting was absorbed with sheep E, Jurkat cells, and chicken E as specificity control (Table VI). After two rounds of absorption, sheep E were able to absorb out the inhibitory activity present in the LFA-2 purified material. In contrast, LFA-2 absorbed on chicken E and Jurkat cells still showed inhibition of rosette formation comparable to an unabsorbed sample of LFA-2. In contrast to the inability of Jurkat cells to absorb out the LFA-2 molecule, they could absorb out 1 μg of anti-LFA-2 MAb in parallel experiments (data not shown). Evidence that no detectable TS2/18 MAb was present in the affinity-purified LFA-2 also comes from NEPHGE of the radiolabeled TS2/18 and the radiolabeled affinity-purified LFA-2. There was no detectable material in the LFA-2 preparation with the m.w. and IEF pattern that corresponds to TS2/18 MAb. Thus, purified LFA-2 caused sheep E rosette inhibition.

DISCUSSION

The object of this investigation was to purify LFA-2 in an antigenically active form for biochemical characterization and functional analysis. Jurkat cells were chosen as the cell source for purification due to their high expression of LFA-2. The LFA-2 MAb bound to 1.0×10^5 sites/Jurkat cell; the actual number of LFA-2 molecules per cell may be as much as twofold higher, depending on whether the MAb is binding monovalently or divalently. LFA-2 was purified 13,000-fold from Jurkat cells by two serial affinity chromatography steps. The predominant band was 54,000 M_r and corresponded to the band identified by immunoblotting of purified material and the band previously identified by immunoprecipitation. Minor amounts of contaminants were also present; this is to be expected from the difficulty of achieving such a high degree of purification from a single type of step. We estimate that the LFA-2 is greater than 80% pure.

A two-site RIA was developed to follow the yield of LFA-2, which was found to be 65%. This can be compared with the yield predicted from the site number analysis. The protein in the detergent extract was derived from 6×10^{10} cells. Multiplying by the site number of 1.0×10^5 molecules/cell gives 6×10^{15} molecules. The amount of affinity-purified LFA-2 recovered, 230 μg, corresponds to 3×10^{15} molecules. This corresponds to a yield of 50%, which is in reasonable agreement with the RIA.

The predominant band at 54,000 M_r was resolved by IEF into a doublet focusing at pI 5.6 and 5.5. Additionally, minor components at lower M_r were seen in variable and smaller amounts. These components were compared by Cleveland peptide mapping. Inasmuch as the fragments compared by this technique are relatively large, it generally reveals identities between proteins with precursor-product relationships, but not between proteins that are products of distinct but homologous genes. The identities among the Cleveland map peptides suggest that the two components of the doublet and the lower M_r species are related by processing or proteolytic breakdown.

Biosynthetic-labeling studies showed that the major doublet at 54,000 M_r was labeled by [^{35}S]methionine and [^{3}H]leucine. No phosphorylation of LFA-2 could be detected after a 2.75-hr incubation with [^{32}P]orthophosphate. Other surface proteins were found to be phosphorylated under the same conditions (data not shown).

LFA-2 had an apparent M_r of 68,000 by gel filtration in NaDOC. This is consistent with a monomer of LFA-2 of M_r 54,000 as estimated by SDS-PAGE bound to a deoxycholate micelle, but is inconsistent with dimeric or higher m.w. forms of LFA-2.

As a first step in the functional characterization of purified LFA-2, it was tested for inhibition of E rosette formation. MAb affinity-purified, antigenically active LFA-2 was able to completely inhibit E rosetting by both Jurkat cells and PBL. This is the first evidence that the LFA-2/CD2 molecule can inhibit rosette formation. Furthermore, sheep E but not human T lymphocytes could absorb E rosette inhibitory activity. This suggests that LFA-2 is a receptor that can bind to a ligand on sheep E. Previous MAb inhibition studies had shown only that LFA-2/CD2 was required for E rosetting, and did not demonstrate that it directly interacted with a ligand on sheep E. In analogy to the large number of accessory molecules that are involved in T lymphocyte recognition of antigen (23), LFA-2/CD2 could have been an accessory molecule that did not itself interact with a ligand on sheep E. The absorption experiments presented here strongly suggest that LFA-2/CD2 is a receptor for a ligand on sheep E. Preformed rosettes could be disrupted by addition of LFA-2, showing that rosetting is a reversible interaction. The affinity-purified LFA-2 from a T lymphoma not only inhibited rosette formation with the Jurkat T lymphoma cell, but also inhibited rosette formation with PBL, suggesting that the epitope on LFA-2 that is necessary for rosette formation is probably similar or identical on PBL and on the T lymphoma cells.

The relationship between E rosetting, and the importance of LFA-2 in T cell cytolytic and helper responses, remain unclear. The availability of purified, antigenically intact LFA-2 that inhibits E rosetting will allow further functional and structural studies to be carried out. The purified LFA-2 may be useful in identifying a physiological ligand in the human system. It will be interesting to determine whether purified LFA-2 can inhibit T cell functional responses and to determine the amino acid sequence of LFA-2.

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