CORRELATION OF CD2 BINDING AND FUNCTIONAL PROPERTIES OF MULTIMERIC AND MONOMERIC LYMPHOCYTE FUNCTION-ASSOCIATED ANTIGEN 3

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Lymphocyte function-associated antigen 3 (LFA-3)[1] is a widely distributed cell surface glycoprotein that is a ligand for the T lymphocyte CD2 glycoprotein (1–4). This receptor-ligand pair is used in many intercellular adhesion interactions, including binding of cytolytic T lymphocytes to target cells (3) and thymocytes to thymic epithelial cells (4). Both CD2 and one form of LFA-3 have membrane-spanning polypeptide segments that anchor them to the membrane (5–7), while a distinct form of LFA-3 is anchored to the membrane by a glycosylphosphatidylinositol (GPI) moiety at its COOH terminus (7–9). Despite these hydrophobic components, purified CD2 and LFA-3 can be manipulated in the absence of detergents and can be shown to bind to cells expressing LFA-3 and CD2, respectively, in radioreceptor assays (10–12). CD2 also mediates rosetting of human T lymphocytes with sheep and human erythrocytes; the sheep ligand for CD2, T11-target structure (T11TS), is a homologue of LFA-3 and can also be used in soluble form to bind to CD2 on cells (13, 14).

In addition to mediating adhesion, early studies suggested that CD2 could deliver activating signals (15, 16). Subsequently it was found that several distinct pairwise combinations of CD2 mAbs that bind to different classes of epitopes could trigger T lymphocyte proliferation and function (17–21). Hünig et al. (22) showed that sheep erythrocytes bearing T11TS were strongly mitogenic in the presence of a single CD2 mAb. Tiefenthaler et al. (23) demonstrated that purified LFA-3 or T11TS synergize with a submitogenic dose of a mitogenic CD2 mAb combination. In that study the form of LFA-3 or T11TS used was not defined and neither LFA-3 nor T11TS could stimulate T cells in combination with a single nonmitogenic CD2 mAb.

We have been interested in defining the physiochemical properties of LFA-3 important in stimulation of T cell function. There are 40,000 CD2 molecules per resting T cell (24) and 200,000 LFA-3 sites per B lymphoblast (10). Hence, signaling attendant upon interaction via these adhesion molecules might involve engagement of a higher number of surface receptors than required for signaling through many lymphokine/hormone receptors, on the order of 10^2 to 10^3 (25, 26). Furthermore, a mul-

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[1] *Abbreviations used in this paper:* GPI, glycosylphosphatidylinositol; LFA, lymphocyte function-associated; mLFA, membrane anchor intact form of LFA-3; OG, octyl-β-D-glucopyranoside; PIPLC, phosphatidylinositol-specific phospholipase C; sLFA-3, soluble form of LFA-3 released with PIPLC.

timeric form of LFA-3 might be expected to be more potent than a monomeric form, both due to its predicted ability to aggregate the CD2 receptor in the plane of the membrane and due to increased avidity. In this study we have compared the hydrodynamic, cell-binding, and cell-activating properties of multimeric LFA-3 with an intact membrane anchor (mLFA-3) and phosphatidylinositol-specific phospholipase C (PIPLC)-released monomeric LFA-3 (sLFA-3). Binding to CD2 was assessed by inhibition of adhesion and radioligand binding assays with the general finding that multimeric mLFA-3 binds much more avidly to cell surfaces than monomeric sLFA-3. Cell activation was assessed by proliferation and Ca^{2+} mobilization assays, which also indicated that mLFA-3 was much more active. Correlation of mLFA-3 binding and activation suggests that occupation of 10^4 to 10^5 CD2 sites is required for Ca^{2+} mobilization and proliferation. These results are consistent with CD2-mediated activation during adhesion where a significant proportion of CD2 sites are probably occupied.

Materials and Methods

Cells. PBMC were isolated by dextran sedimentation and Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation. Peripheral blood T lymphocytes (PBL-T) were enriched by nylon wool (Polysciences, Inc., Warrington, PA) filtration and plastic adherence. The Jurkat cell line was obtained from Dr. M. K. Ho (DuPont Co., Boston, MA). Cells were maintained in RPMI 1640, 10% FCS (Gibco Laboratories, Grand Island, NY), 5 mM L-glutamine, 50 μg/ml gentamycin (complete media). JY B-lymphoblastoid cells and Jurkat cells for LFA-3 and CD2 purification, respectively, were grown by the Massachusetts Institute of Technology Cell Culture Center (Cambridge, MA).

Monoclonal Antibodies. The TS2/9 and TS2/18 IgG1 mAbs bind to LFA-3 and CD2, respectively (1). The 9-1 IgG3 (20), D66 IgG2b (18), CD2.1 IgG1 (19), 9.6 IgG2a (24), and 35.1 IgG2a (24) bind to epitopes on CD2 that are distinct from TS2/18 by competitive binding or mutational analysis (27). 9-1 was a gift of Bo Dupont, Memorial Sloan Kettering Cancer Institute, New York, NY. D66 was a gift of Dr. Alain Bernard, Institut Gustave-Roussy, Villejuif, France. mAb 9.6 was a gift of Dr. John Hansen, Fred Hutchinson Cancer Research Center, Seattle, WA.

LFA-3 Purification. LFA-3 was purified from Triton X-100 lysates of human erythrocytes (mLFA-3) (11) or JY B-lymphoblastoid cells (6), or from supernatants of PIPLC-treated JY B-lymphoblastoid cells (sLFA-3) by immunoaffinity chromatography on TS2/9 mAb-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ). In the latter case, 50 g of viable JY cells were washed with HBSS and treated with *Bacillus thuringiensis* PIPLC (a generous gift of Dr. Martin Low, Columbia University, New York, NY) in a total volume of 100 ml for 1 h at 37°C. The concentration of enzyme used could hydrolyze 300 nmol/min/ml of [^{3}H]phosphatidylinositol at pH 7 in the presence of 0.1% deoxycholate detergent (28). The cells were pelleted at 1,000 *g* and cell debris was pelleted at 100,000 *g* for 1 h. Lysates or supernatants containing LFA-3 were passed over the affinity column and the column was washed (11). LFA-3 from erythrocytes was eluted from the immunoaffinity column at pH 3 in the presence of 1% octylglucoside (OG) detergent. LFA-3 from PIPLC supernatants was eluted at pH 3 in the absence of detergent; fractions containing LFA-3 were pooled (4–6 ml) and passed over a 1-ml phenyl-Sepharose column (Pharmacia Fine Chemicals) equilibrated with 25 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.025% NaN_3 (TSA). Some preparations were passed over a 1-ml protein A-Sepharose CL-4B column after diluting 1:1 with MAPS binding buffer (with 1% OG for mLFA-3) (Bio-Rad Laboratories, Richmond, CA). Aggregated mLFA-3 was prepared by three cycles of ultrafiltration using a Centricon 30 apparatus (Amicon Corp., Danvers, MA), adding 2 ml of PBS and reducing the volume to 50 μl with each cycle. Since the final ultrafiltrate of the last step contained the same low molecular weight components as the final retentate but not any LFA-3, this ultrafiltrate was used as a control for effects

of buffer components in subsequent experiments. SDS-PAGE and silver staining were done by the procedures of Laemmli (29) and Morrissey et al. (30), respectively.

Sucrose Gradient Sedimentation and Gel Filtration. Linear 10–34% sucrose gradients in H_2O or D_2O were prepared in 2.5 × 0.5 inch polyallomer tubes. Samples (200 μl) containing 10 mg/ml of a standard protein and 150,000 cpm ^{125}I-LFA-3 (see below) were layered on top of the gradients, the gradients were centrifuged in a SW55Ti rotor at 44,000 rpm at 4°C for 12 or 40 h, and they were decelerated without brake. Fractions were collected from the top of the gradient (31) and assayed for absorbance at 280 nm, cpm, and refractive index. The partial specific volume ($\bar{v}$) and sedimentation coefficients were calculated by the method of Clarke (32). Ovalbumin, BSA, bovine IgG, and thyroglobulin were used as standards for calculating the average viscosities (32). HPLC gel filtration was done using a DuPont GF-250 column equilibrated with PBS or PBS with 1% OG detergent. Standard proteins used were ovalbumin, BSA, bovine IgG, and thyroglobulin which have diffusion coefficients ($D \times 10^7$) of 7.76, 6.1, 4.1, and 2.65 respectively (33).

LFA-3 Iodination and Binding Assays. LFA-3 (3 or 6 μg) was iodinated with 200 μCi of Na^{125}I (New England Nuclear, Boston, MA) using 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Pierce Chemical Co., Rockford, IL) (34). The iodinated LFA-3 in the presence of 1 mg/ml BSA was dialyzed with a >95% recovery of LFA-3. For binding assays Jurkat cells or PBL-T were washed and resuspended in complete media to 2 × 10^7 cell/ml. The cells were pretreated with any reagent to be tested for blocking of LFA-3 binding for 30 min at 4°C. ^{125}I-LFA-3 (30,000 cpm/ng) was added to give a final cell concentration of 10^7 cells/ml in 400 μl and incubated an additional hour at 4°C. Three 100-μl aliquots from each binding mixture were layered on top of a 100-μl cushion of a 2:1 mixture of dibutyl and dinonylphthalates in micro sediment separation tubes (Sarstedt, Inc., Princeton, NJ) and centrifuged parallel to the centrifugal field at 10,000 *g* for 5 min. The tips containing the cells were cut off and counted. In some binding experiments the indicated number of cells were incubated with ^{125}I-mLFA-3 and mAb for 90 min at 4°C and washed three times with media over 15 min. These separation methods gave identical results with mLFA-3.

Proliferation Assays. Proliferation of PBMC or T lymphocytes was assayed by [^{3}H]thymidine incorporation. Assays were done in flat wells with 0.5 × 10^5 cells/well in 100 μl of complete media with 20% FCS (HyClone Laboratories, Logan, UT). After the indicated period of time each well was pulsed with 1 μCi of [^{3}H]thymidine (50–90 Ci/mmol, New England Nuclear) for 16 h, after which the DNA was collected using a PhD cell harvester (Cambridge Technology, Inc., Cambridge, MA).

Intracellular Ca^{2+} Measurements with Indo-1. Jurkat cells or PBL-T were loaded by incubation with 8 μM Indo-1 AM (Molecular Probes, Junction City, OR) at 10^6 cells/ml in RPMI 1640 10% FCS at 37°C for 45 min. The cells were washed three times and kept at 20–24°C until 10 min before each run, at which point the cells were raised to 37°C (35). Indo-1 was excited at 351–364 nm and emission was measured at 405–415 nm (violet) and 475–485 nm (blue) on the Epics V flow cytometer (Coulter Electronics, Hialeah, FL). The ratio between the log channel number for violet/blue was used as an indication of the relative intracellular Ca^{2+} level (indo-1 ratio in Figs. 4 and 5). The maximal ratio was determined after addition of 10 μg/ml ionomycin (Calbiochem-Behring Corp., La Jolla, CA).

Results

Purification of PIPLC-released LFA-3. LFA-3 released from the surface of B-lymphoblastoid cells by PIPLC (sLFA-3) was purified by immunoaffinity chromatography (not shown). The appearance of the LFA-3 forms by SDS-PAGE was identical to material previously purified from JY cells (6), except that peptide:N glycosidase treatment yielded a single band of 25,500 rather than two bands of 25,000 and 29,000 (not shown). The possibility of contamination by LFA-3 with an intact membrane anchor (mLFA-3) was reduced by passing the immunoaffinity purified material over a phenyl-Sepharose column. The sLFA-3 had biological activity based on its ability

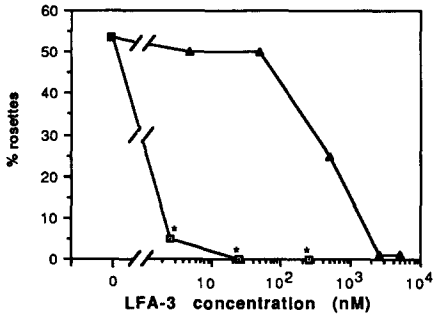

FIGURE 1. Inhibition of E rosetting by sLFA-3 and mLFA-3. Sheep erythrocytes (10^6) and Jurkat cells (10^4) were mixed in a final volume of 20 μl of RPMI 1640 with 10% FCS with sLFA-3 (▲) or mLFA-3 (⊡) and incubated 30 min at 4°C. The cells were pelleted at 50 *g* for 5 min and left at 4°C for an additional 1 h. The pellets were gently resuspended and rosettes enumerated (200 Jurkat cells counted). The asterisk near some points indicates aggregation of Jurkat cells. Averaged results of two experiments are shown.

to inhibit sheep erythrocyte binding to Jurkat cells in a 1-h assay at 4°C (Fig. 1). Maximal inhibition of rosetting by sLFA-3 was obtained at 2.6 μM and was not accompanied by the agglutination of Jurkat cells as seen when mLFA-3 was used in parallel and as previously described (11). Maximal inhibition with mLFA-3 was obtained at between 2.5 and 25 nM.

Hydrodynamic Properties of LFA-3 Forms. Gel filtration results suggested that mLFA-3 formed large, homogeneous aggregates in the absence of detergent based on comparison to sLFA-3 and protein standards. To determine how many LFA-3 monomers were present in sLFA-3 and mLFA-3 their molecular mass was determined. The partial specific volume and sedimentation coefficient of sLFA-3 were determined by sucrose gradient sedimentation in H_2O and D_2O. These values and diffusion coefficient determined by gel filtration were used to calculate the molecular mass (Table I). The molecular mass of sLFA-3 was 80,000, which is consistent with a single highly glycosylated polypeptide chain and is in good agreement with the molecular mass of 70,000 from SDS-PAGE (1, 11). The molecular mass of mLFA-3 in the presence and absence of detergent was determined from the sedimentation coefficient on H_2O sucrose gradients, the diffusion coefficient from gel filtration, and the partial specific volume of sLFA-3 adjusted for addition of diacylglycerol with or without an OG micelle associated. Molecular masses of 73 and 540 kD were determined in the presence and absence of OG, respectively, suggesting that in OG LFA-3 is a monomer, and after removal of OG the mLFA-3 micelle is an octamer. The mLFA-3

TABLE I

Hydrodynamic Properties of LFA-3 Forms

LFA-3 form	$\bar{\nu}$	$s_{20,w}$	D	M_r
	ml/g	$\times 10^{13}$	$\times 10^{7}$	
sLFA-3	0.725	3.66	4.1	80,000
mLFA-3 with OG	0.743*	3.12	4.1	73,000
mLFA-3	0.730*	12.3	2.5	540,000

Iodinated LFA-3 forms were sedimented on 10–34% sucrose gradients in H_2O or D_2O. Centrifugation was for 40 h (sLFA-3) or 12 h (mLFA-3). Diffusion coefficients were estimated from gel filtration.

* Calculated from $\bar{\nu}$ for sLFA-3 using for diacylglycerol $\bar{\nu}$ = 1.18, and for OG a micelle size of 8 kD (not subracted above) and $\bar{\nu}$ = 0.91.

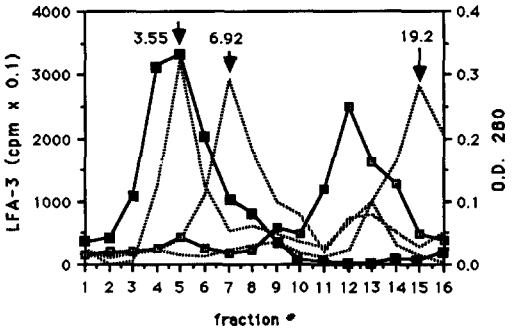

FIGURE 2. Sucrose gradient sedimentation of mLFA-3 in the presence and absence of detergent. ^{125}I-erythrocyte mLFA-3 (150,000 cpm) was sedimented for 20 h on linear 10–34% sucrose gradients in H_2O with no detergent (⊡) or 1% OG (■). Standards (-----) were ovalbumin ($s_{20,w}$ = 3.55), bovine IgG (6.92), and thyroglobulin (19.2), which were run without detergent. Fractions were collected from the top of each gradient (1 = top).

micelles were relatively homogeneous in comparison to glycoprotein standards and detergent solubilized mLFA-3 by sedimentation (Fig. 2) and gel filtration (not shown).

Binding of LFA-3 Micelles to Cell Surface CD2 at 4 and 37°C. Previously, we had shown that iodinated LFA-3 binds to CD2$^+$ cells when Triton X-100 is absorbed with 15% BSA and that this binding is inhibited by CD2 mAb and LFA-3 mAb (11); however, the aggregation state and dissociation constant were not determined. We compared the cell-binding properties of the mLFA-3 octamer with the sLFA-3 monomer. While mLFA-3 from erythrocytes or JY cells bound specifically to Jurkat T leukemic cells or resting PBL-T, sLFA-3 binding was virtually undetectable when using sLFA-3 at 4 nM (Table II). Scatchard analysis showed that erythrocyte mLFA-3 bound with a dissociation constant of 1.5 nM with 420,000 LFA-3 molecules bound per Jurkat cell and 12 nM with 65,000 LFA-3 molecules bound per resting T cell at 4°C (Fig. 3). Binding of sLFA-3 under saturating conditions was not undertaken due to the relatively large amounts of material that would be required. The amount of sLFA-3 binding in Table II suggests that the K_d for binding to cell surface CD2 is ≥400 nM. Consistent with this estimate, the K_i for inhibition of rosetting was 400 nM (Fig. 1).

Dissociation of mLFA-3 from the surface of Jurkat cells occurred very slowly with a $t_{1/2}$ >600 min. In the presence of 10 or 100 μg/ml TS2/18 mAb, the dissociation rates had $t_{1/2}$ of 60 and 2 min, respectively (not shown). Since TS2/18 mAb and mLFA-3 appear to bind CD2 in a competitive manner, the increased rate of dissoci-

TABLE II

Binding of PIPLC-released LFA-3 to Jurkat

Additions	Bound LFA-3	
	sLFA-3	mLFA-3
	cpm	
control mAb	5,553 ± 387	148,760 ± 4,003
TS2/18	4,030 ± 14	5,023 ± 23

Jurkat cells (10^6) were incubated with 200,000 cpm (4 nM final) of either sLFA-3 or mLFA-3, both prepared from JY cells, for 2 h at 4°C and aliquots were centrifuged through oil cushions. mLFA-3 was prepared from JY cells to avoid differences in glycosylation that would complicate the interpretation of the results. Similar results were obtained with erythrocyte mLFA-3.

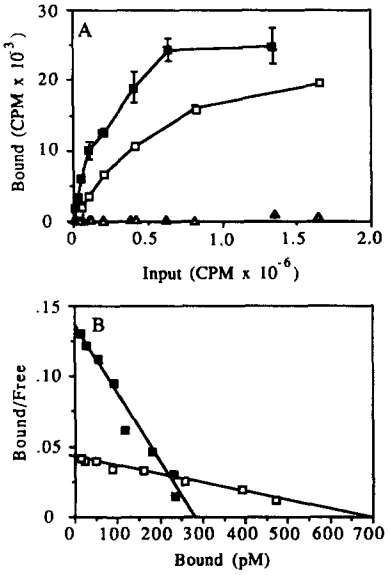

FIGURE 3. Equilibrium binding of mLFA-3 to Jurkat cells and resting T cells. (*A*) Binding of mLFA-3 was for 1 h at 4°C in the presence of control mAb (□, ■) or TS2/18 mAb (▲, △) to 5 × 10^4 Jurkat cells (*filled symbols*) or 5 × 10^5 nylon wool-enriched T cells (*open symbols*). The specific activity of mLFA-3 was not the same for Jurkat and PBL-T. Bound and free were separated by three washes with cold media. (*B*) The data were analyzed by the method of Scatchard after subtracting the binding in the presence of TS2/18 mAb.

ation in the presence of CD2 mAb suggests that mLFA-3 binds multivalently to cell surface CD2. Complete reversal of mLFA-3 binding by CD2 mAb demonstrated that LFA-3 is not endocytosed after 90 min at 4°C and that it was not inserted into membranes through its phosphatidylinositol glycan anchor during this period (9). However, mLFA-3 did appear to become internalized when Jurkat cells or PBL were elevated to 37°C, since 30–40% of cell-associated counts could not be dissociated by TS2/18 mAb after 30 min at 37°C (Table III).

We screened for CD2 mAbs that would not block binding of mLFA-3 to Jurkat cells or peripheral T cells. CD2 mAbs that inhibit rosetting were able to completely

TABLE III

Accessibility of Bound mLFA-3 to Extracellular TS2/18

Additions		Cell-associated mLFA-3	
Preincubation	Postincubation	Jurkat	PBL-T
		cpm	
4°C Control	Control	11,441 ± 500	1,543 ± 225
TS2/18	Control	86 ± 5	95 ± 14
Control	TS2/18	299 ± 26	111 ± 13
37°C Control	Control	10,990 ± 941	1,326 ± 86
TS2/18	Control	67 ± 12	89 ± 19
Control	TS2/18	4,975 ± 400	390 ± 43

Jurkat cells or PBL-T (5 × 10^5) were incubated with ^{125}I-mLFA-3 (100,000 cpm) for 60 min at 4°C or 30 min at 37°C. Jurkat cell and PBL-T CD2 was initially at ~10% occupation with LFA-3. The postincubation at 4°C without or with TS2/18 was carried out for 6 h to insure complete dissociation of accessible LFA-3. Data are means of quadruplicate determinations with standard deviations.

TABLE IV
Inhibition of mLFA-3 Binding by CD2 mAb

mAb	^{125}I-mLFA-3 bound/10^6 cells
	cpm
Control mAb	20,531 ± 596
9.6	173 ± 17
TS2/18	153 ± 57
9-2	154 ± 8
CD2.8	173 ± 29
35.1	7,171 ± 98
CD2.9	164 ± 50
CD2.1	22,817 ± 362
9-1	24,092 ± 878
D66	16,073 ± 72
OKT3	21,342 ± 487

Binding to Jurkat cells was done for 1 h at 4°C. Input counts were 100,000/10^6 cells. Antibodies were used at saturation as determined by flow cytometry. Bound and free were separated by centrifugation through a 15% BSA cushion. All the mAbs except control and OKT3 are against CD2. Results are averages of quadruplicates with standard deviation and are representative of two experiments.

block mLFA-3 binding: TS2/18, 9.6, 9-2, CD2.8, and CD2.9 (Table IV). The one exception to this was D66 mAb, which blocks all but the most stable E rosettes (36), but only blocked binding of mLFA-3 by 24%. This may be a function of low D66 epitope expression (36). The 35.1 mAb also blocked mLFA-3 binding partially (67%). The 9-1 and CD2.1 mAb, which do not inhibit rosetting, did not have any effect on mLFA-3 binding.

Effect of LFA-3 Forms on Intracellular Free Ca^{2+}. Several studies have shown that combinations of CD2 mAbs can stimulate Ca^{2+} fluxes in T cell tumor lines (37, 38) and resting PBL-T (39, 40). We have examined Ca^{2+} mobilization stimulated by LFA-3 together with CD2 mAbs that do not block LFA-3 binding. Using a flow cytometer $[Ca^{2+}]_i$ responses can be measured within minutes and the monodispersed or aggregated nature of the cells can be simultaneously evaluated, making it possible to determine whether the binding of mLFA-3 aggregates to cell surface CD2 leads to an activation signal without promoting cell-cell interaction by inserting into membranes or crosslinking CD2 molecules from different cells. When mLFA-3 (up to 240 nM) was added to Jurkat cells there was no change in the Indo-1 ratio within 15 min (not shown). Similarly, CD2.1 mAb (67 nM) alone had no effect on $[Ca^{2+}]_i$ (not shown). However, when mLFA-3 and CD2.1 mAbs were added simultaneously there was a rapid increase in $[Ca^{2+}]_i$ (Fig. 4 *A*), which was similar in magnitude and more sustained than that seen with CD3 mAb (Fig. 4 *C*). The combination of mLFA-3 and 9-1 did not stimulate a Ca^{2+} flux. The combination of sLFA-3 (40–800 nM) with CD2.1 did not result in an increase in $[Ca^{2+}]_i$ in Jurkat, while subsequent addition of mLFA-3 to the same sample resulted in a rapid increase in $[Ca^{2+}]_i$ (Fig. 4 *B*). There was no evidence of cell-cell interaction during these experiments since there was no change in light scatter (not shown).

To allow direct comparison of Ca^{2+} mobilization to equilibrium mLFA-3 binding data, Jurkat cells were equilibrated with different concentrations of mLFA-3 by in-

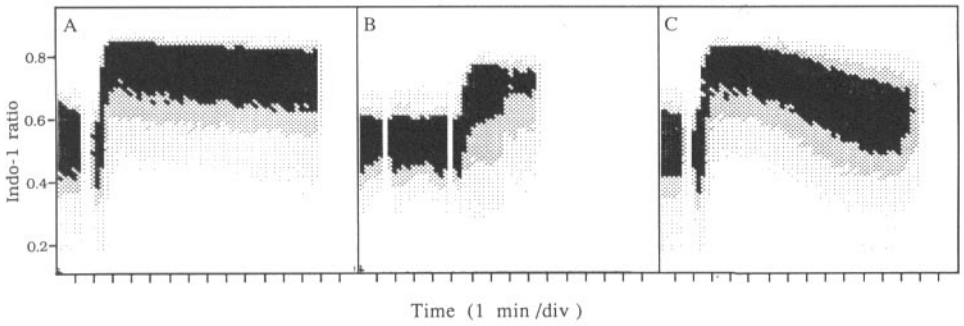

FIGURE 4. Cytoplasmic Ca^{2+} mobilization in Jurkat cells induced by CD2.1 and mLFA-3 or sLFA-3. Indo-1 loaded Jurkat cells were treated with: (*A*) mLFA-3 (40 nM) and CD2.1 (1:500 ascites); (*B*) sLFA-3 (400 nM) and CD2.1 followed by mLFA-3 (40 nM) or (*C*) OKT3 (1:10 culture supernatant). Reagents were added at 1 min (gap in data collection) and (*B*) at 1 min and 5 min.

cubation for 30 min at 37°C, then CD2.1 mAb was added to initiate Ca^{2+} mobilization (Fig. 5). Under these conditions, an approximately half-maximal increase in $[Ca^{2+}]_i$ was seen at ~1.2 nM mLFA-3, which is very close to the K_d for mLFA-3 binding to Jurkat CD2 determined above. Therefore, it appears that the signal delivered through CD2 by mLFA-3 and CD2.1 that leads to an increase in $[Ca^{2+}]_i$ is proportional to the occupation of CD2 with mLFA-3 and CD2.1 mAb up to saturation. Addition of ionomycin to Jurkat after 240 nM mLFA-3 and CD2.1 resulted

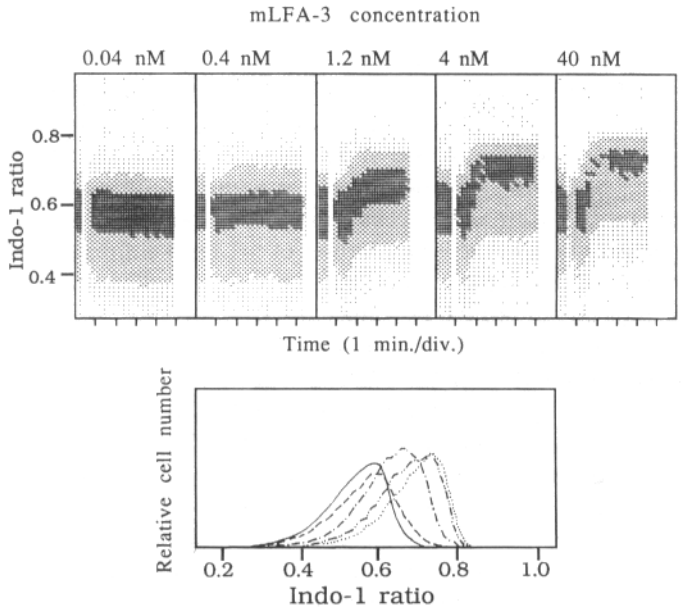

FIGURE 5. Concentration dependence of Jurkat $[Ca^{2+}]_i$ increase on mLFA-3 occupation of CD2. Jurkat cells were incubated with the indicated concentration of LFA-3 for 30 min at 37°C. CD2.1 (1:500 ascites) was added after a 30–60-s data collection on flow cytometer. The bottom of the figure shows histograms of sections through activation time plots at 3 min: 0.04 nM (—), 0.4 nM (---), 1.2 nM (·–·–), 4 nM (–··–··) and 40 nM (···).

TABLE V
Induction of T Cell Proliferation by mLFA-3 and CD2.1

Additions	^{3}H-thymidine incorporation/5 × 10^4 PBMC		
	Exp. 1	Exp. 2	Exp. 3
		cpm	
buffer	593	392	417
mLFA-3	411	645	549
CD2.1	437	839	646
IL-2	1,451	2,145	1,034
mLFA-3 + IL-2	1,345	1,399	1,284
CD2.1 + IL-2	1,609	2,039	1,545
PMA	1,713	1,382	834
mLFA-3 + PMA	1,549	978	828
CD2.1 + PMA	1,704	1,252	1,009
mLFA-3 + CD2.1	22,294	35,439	47,478
mLFA-3 + CD2.1 + IL-2	22,497	37,065	45,384
mLFA-3 + CD2.1 + PMA	77,784	114,313	153,240
sLFA-3 + CD2.1	768	–	755
sLFA + CD2.1 + PMA	926	1,156	33,535
mLFA-3 + CD2.1 + sLFA-3	23,172	–	–

Additions were as indicated on day zero: rIL-2 (0.1 ng/ml), PMA (1 nM), CD2.1 (1:500 dilution of ascites or 10 μg/ml), and mLFA-3 (40 nM). Concentrations of sLFA-3 were 800 nM in Exps. 1 and 3 and 40 nM in Exp. 2. PHA (1 μg/ml) gave ~80,000–100,000 cpm on day 3 to 4 in these experiments. Wells were pulsed with [^{3}H]thymidine on day 3 for 16 h. Results are means of triplicates and are representative of 14 experiments.

in a further increase in $[Ca^{2+}]_i$ suggesting that the Indo-1 was not at saturation for Ca^{2+} in these experiments (not shown). Similar results were obtained when mLFA-3 and CD2.1 were added simultaneously except the kinetics of the responses at lower LFA-3 concentrations were slower (not shown).

Mitogenic Effects of LFA-3 Forms on Resting T Cells. It has been shown that the aggregated form of mLFA-3 alone had no effect on PBL-T proliferation, but can induce proliferation of PBL-T in the presence of submitogenic concentrations of $T11_2$ and $T11_3$ CD2 mAb, but not with either $T11_2$ or $T11_3$ separately (23). We evaluated the ability of the CD2.1 and 9-1 mAb to induce proliferation with mLFA-3 micelles. The combination of mLFA-3 (40 nM) with the CD2.1 mAb was strongly mitogenic for PBMC from all donors tested (Table V), while mLFA-3 and 9-1 was weakly mitogenic or nonmitogenic (not shown). The mitogenic response to mLFA-3 and CD2.1 was usually (9 of 10 donors) seen in the absence of exogenous IL-2, even in donors for which CD2.1 mAb with CD2.9 mAb required exogenous IL-2 (see reference 19), although submitogenic PMA increased the response. Stimulation of DNA synthesis by mLFA-3 and CD2.1 mAb peaked on days 3-4; a pulse on day 3 was used for all experiments (not shown). In the presence of exogenously added IL-2 and 1 nM PMA, the optimal periods were days 3-4 and days 4-5, respectively. The combination of sLFA-3 between 40 and 800 nM and CD2.1 mAb with or without PMA was not mitogenic for PBMC in two of three experiments for which sufficient amount of material could be obtained (Table V). In one experiment a weak response was seen with sLFA-3, CD2.1, and PMA. Experiments with the same donors showed strong responses to mLFA-3 plus CD2.1 mAb with or without PMA.

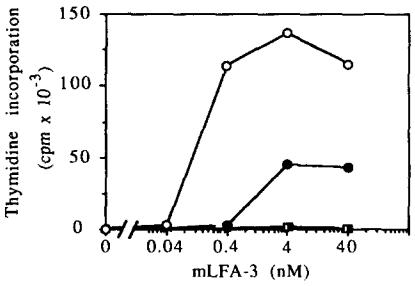

FIGURE 6. Dose-response for LFA-3 and CD2.1 induced proliferation. PBMC (5×10^4) were treated with the indicated concentration of mLFA-3 with no addition (□), PMA (1 nM) (■), CD2.1 (10 μg/ml) (●), or CD2.1 and PMA (○). Wells were pulsed on day 3 for 16 h.

The dose-responses for mLFA-3 and CD2.1 mAb were determined in the presence of saturating concentrations of the other reagent. In the presence of saturating CD2.1 mAb (67 nM) maximal PBMC or PBL-T proliferation was obtained with 4 nM mLFA-3 (Fig. 6). This is below the K_d for mLFA-3 binding to PBL-T of 12 nM. The CD2.1 mAb did not affect the K_d for ^{125}I-mLFA-3 binding to resting T cells at 4°C (not shown). The presence of exogenous IL-2 did not alter the dose-response; however, addition of 1 nM PMA decreased the mLFA-3 concentration required for maximal response 10-fold. Similarly, in the presence of excess (40 nM) mLFA-3, the CD2.1 mAb concentration giving a maximal response was 6.7 nM alone, but 0.67 nM in the presence of 1 nM PMA (not shown).

Discussion

We have compared the ability of soluble LFA-3 micelles and monomers to bind to CD2 expressing cells and stimulate T lymphocyte activation. Hydrodynamic analysis by gel filtration and sucrose gradient sedimentation suggests that PIPLC-released sLFA-3 is a single polypeptide chain in solution and thus interacts with CD2 monovalently. LFA-3 with an intact membrane anchor forms protein micelles of eight monomers. The formation of homogeneous micelles by GPI-anchored proteins such as Thy-1 has been observed previously; the LFA-3 micelles contain fewer monomers than for Thy-1, which forms micelles of 16 monomers (41). The effect of CD2 mAb on dissociation rates suggests that LFA-3 micelles bind multivalently, this accounts for the 2–3-log difference in avidity between LFA-3 micelles and monomers. The dissociation constant for mLFA-3 binding to cell surface CD2 was determined to be in the 10^{-9} to 10^{-8} M range. The 5–10-fold higher avidity of mLFA-3 for binding to Jurkat cells than to resting T cells might be due to different CD2 densities giving rise to differences in the number of CD2/LFA-3 interactions; alternatively, differences in surface charge or the glycocalyx may be important. A low affinity and rapid dissociation rate for single CD2/LFA-3 interactions is consistent with the rapidly reversible adhesion demonstrated by cytolytic T cells.

Jurkat cells and resting T cells have ~200,000 and ~40,000 CD2 antibody binding sites per cell, respectively (24, 42). Saturation binding showed that 420,000 and 65,000 mLFA-3 monomers (in octameric micelles) bind to Jurkat and resting T cells, respectively. Comparison of these numbers suggest that mLFA-3 micelles bound to Jurkat with an average of four LFA-3 sites per octamer and to resting T cells with an average of five sites per octamer. This would explain the ability of mLFA-3 to agglutinate Jurkat cells (11), since four mLFA-3 molecules per micelle would be available for binding to CD2 on another cell. The dissociation constant for monovalent interac-

tion of sLFA-3 with CD2 is suggested to be in the range of 10^{-7} to 10^{-6} M, based on the amount of binding obtained in assays with a single sLFA-3 concentration and the inhibition of sheep erythrocyte rosetting by sLFA-3.

After incubation at 37°C, some specifically bound LFA-3 micelles were not released from the cell by CD2 mAb in the media, suggesting receptor-mediated endocytosis of soluble phase mLFA-3. It is not clear whether this has any implication for CD2 function in adhesion or activation. Erythrocyte-T cell rosettes are more stable at 4°C than at 37°C (43), and it is possible that active rearrangement of ligated CD2 by a mechanism shared with the process of receptor-mediated endocytosis might be responsible for weakening adhesion.

The combination of mLFA-3 and CD2.1 mAb stimulated a rapid rise in $[Ca^{2+}]_i$ in Jurkat cells and resting T cells and induced proliferation of PBL. This system has advantages over the previously described system in which LFA-3 micelles were found to synergize with submitogenic doses of two CD2 mAbs (23). The use of a single CD2 mAb that has no mitogenic effects on its own is less complicated and allowed titration of each reagent when the other was kept at saturation.

Ca^{2+} mobilization in Jurkat was proportional to predicted occupation of CD2 by mLFA-3, with maximal stimulation occurring when ~400,000 mLFA-3 molecules (~50,000 micelles) were bound to the Jurkat cell at saturating concentrations of CD2.1 mAbs. The relative increase in $[Ca^{2+}]_i$ was less dramatic in resting T cells (data not shown), consistent with these cells having one fifth the number of CD2 molecules as Jurkat (24, 42). These results suggest that ~10^4 to 10^5 CD2 molecules must be occupied by LFA-3 and CD2.1 to stimulate an increase in $[Ca^{2+}]$.

In proliferation experiments the concentration of mLFA-3 required for a maximal response in the presence of saturating CD2.1 was just below the K_d for mLFA-3 binding. In contrast to the Ca^{2+} mobilization assay the stimulus in the proliferation assay is applied over a longer period of time and may be cumulative; furthermore, the function of accessory cells may increase the sensitivity of the T cells. Additionally, the properties of the T cells can change during culture and the actual avidity for mLFA-3 binding may have increased. The addition of submitogenic PMA decreased the amount of LFA-3 required to obtain a proliferative response 10-fold. The presence of phorbol esters has been shown to complement single CD2 mAbs and antibody pairs (19, 21) and may eliminate the requirement for a substantial increase in $[Ca^{2+}]_i$ (21).

These results suggest that CD2 is relatively inefficient in producing its effect compared with hormone receptors that are frequently effective in producing biological responses when 10^2 to 10^3 sites are occupied per cell (26, 27). The requirement for occupation of ~10^4 to 10^5 CD2 sites by LFA-3 for maximal response is consistent with signaling through CD2 during adhesion. The LFA-3 density on many cell types makes occupation of this number of CD2 molecules by LFA-3 plausible during cell-cell interactions.

In proliferation and Ca^{2+} mobilization experiments sLFA-3 and CD2.1 had no or marginal effect. It is not plausible to conclude that the sLFA-3 is intrinsically inactive in activating T cells since the degree of CD2 occupation may not have been sufficient at the sLFA-3 concentrations available. Larger (mg or greater) quantities of sLFA-3, probably generated by overexpression of cloned LFA-3, will be necessary to more accurately determine the affinity of monomeric CD2/LFA-3 interactions and to further evaluate the activating potential of monomeric LFA-3.

While mLFA-3 and CD2.1 were a potent mitogen for T cells, the combination of mLFA-3 and 9-1 was virtually inactive for stimulating proliferation in PBMC cultures and in Jurkat Ca^{2+} mobilization assays. Subsequent to our observations, it has been found that 9-1 and mLFA-3 are mitogenic for thymocytes, although not as strongly as CD2.1 and mLFA-3 (44). In contrast to the requirements of mature T cells, exogenous IL-2 or a second mAb to CD28 are requisite for proliferation in the thymocyte system (44). LFA-3 on the surface of sheep erythrocytes can stimulate human CD2 transfected murine T cell hybridomas in conjunction with 9-1 (45). However, sheep erythrocytes may present LFA-3 differently or supply other signals so these results are not directly comparable to ours. Mutational analysis indicates that CD2.1 is similar to 9-1 in that both fail to react or react weakly with a double substitution in the second Ig-like domain and a single substitution, 149Q, in the first domain (Peterson, A., and B. Seed, personal communication). However, CD2.1 binds to more sites on resting T cells than 9-1, suggesting that its interaction with CD2 differs in some way.

The natural counterpart of the CD2.1 mAb in T cell activation is not known. There may be another cell surface ligand or a soluble ligand for CD2 that binds to a different site on CD2 than LFA-3 and allows activation to occur in the absence of specific antigen. The 9-1 mAb and certain CD3 mAbs can synergize to stimulate T cell proliferation (46). Furthermore, the CD3 mAb increases expression of the 9-1 epitope on resting T cells at 4°C, suggesting a physical interaction between CD2 and CD3 in the plane of the membrane (46). Therefore, LFA-3-ligated CD2 may cooperate with the CD3-Ti complex in antigen-specific activation. In one scenario, the ligated antigen receptor–CD3 complex may contact CD2 at the CD2.1 epitope. In this case, CD2 would not offer an alternative, antigen-independent pathway of T cell activation but might synergize with the antigen-specific pathway to amplify the signal from a small number of T cell receptor–ligand interactions.

Summary

LFA-3 was purified with an intact (mLFA-3) or an enzymatically removed membrane-anchoring domain (sLFA-3). Gel filtration and sucrose gradient sedimentation showed sLFA-3 to be a single highly glycosylated polypeptide chain in solution, while mLFA-3 formed micelles of 8 LFA-3 monomers. ^{125}I-mLFA-3 bound to Jurkat T leukemic cell surface CD2 with much higher avidity than sLFA-3. mLFA-3 binding had characteristics of a multivalent interaction with cell surface CD2 and had an avidity of 1.5 nM for Jurkat cells and 12 nM for resting T cells. Two CD2 mAbs tested did not block mLFA-3 binding: 9-1 and CD2.1. These mAbs were tested in combination with LFA-3 for their ability to activate T cells. The combination of mLFA-3 and CD2.1 mAbs induced a rapid increase in cytosolic free Ca^{2+} in Jurkat cells which was proportional to mLFA-3 occupation of CD2 sites. sLFA-3 showed no activity in the Ca^{2+} flux assay. The combination of mLFA-3 and the CD2.1 mAbs significantly stimulated proliferation of PBMC. The combination of mLFA-3 and 9-1 mAbs was weakly or not mitogenic for the same cells. The combination of CD2.1 and sLFA-3 at concentrations up to 480 nM was not consistently mitogenic. Therefore, monomeric LFA-3/CD2 interaction appears to have a relatively low affinity, while multimeric LFA-3 binds with high avidity. T cell activation by binding of LFA-3

to CD2 appears to require occupation of 10^4 to 10^5 CD2 sites, which is likely to occur during adhesion, but is rare in receptor systems with soluble ligands.

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