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THE FUNCTIONAL SIGNIFICANCE, DISTRIBUTION, AND STRUCTURE OF LFA-1, LFA-2, AND LFA-3: CELL SURFACE ANTIGENS ASSOCIATED WITH CTL-TARGET INTERACTIONS[1]

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Three cell surface antigens associated with the cytolytic T lymphocyte(CTL)-target cell interaction were identified by generation of monoclonal antibodies (MAb) against OKT4+, HLA-DR-specific CTL and selection for inhibition of cytolysis in a ^{51}Cr-release assay. These MAb block cytolysis by both OKT4+ and OKT8+ CTL and the proliferative responses to PHA and the mixed lymphocyte response (MLR). LFA-1 is an antigen widely distributed on lymphoid tissues and is composed of two polypeptides of 177,000 and 95,000 Mr on all cell types studied. Anti-LFA-1 MAb block NK cell-mediated cytolysis in addition to T lymphocyte-mediated cytotoxicity and proliferation. LFA-2 (Mr = 55,000 to 47,000), a determinant on the sheep red blood cell receptor, is expressed by T cells but not B cells and appears specific for T cell functions. LFA-3 (Mr = 60,000) is a widely distributed antigen present on both hematopoietic and nonhematopoietic tissues and appears to only be involved in T cell functions. MAb to LFA-1 and LFA-2 inhibit function by binding to effector cell surface molecules, whereas anti-LFA-3 MAb appear to block by binding to the target cells. Together with previously described molecules, LFA-1, LFA-2, and LFA-3 demonstrate the complexity of CTL-mediated cytotoxicity at the molecular level.

Cytolytic T lymphocytes (CTL)[3] are important effectors in the cell-mediated response to viruses (1), allografts (2), and some tumors (3). The definition of cell surface molecules important in the CTL response may elucidate the general mechanisms of cellular recognition, cell interactions, and the "lethal hit" of cytotoxicity. The use of monoclonal antibody (MAb) probes to detect cell surface molecules important in the murine CTL response has repeatedly demonstrated a role for Lyt-2,3 and lymphocyte function-associated antigen #1 (LFA-1) in the CTL-target interaction (4–9). MAb to OKT3, OKT4, and OKT8 have been shown to inhibit lysis by human CTL (10–16). Recently, we generated MAb by immunization with human HLA-DR-specific CTL lines and screened for the ability to block HLA-DR-specific cytolysis. The binding of MAb to four types of molecules, LFA-1, LFA-2, LFA-3, and HLA-DR inhibited killing, suggesting that these molecules participate in the CTL-target interaction (17).

In this report, we delineate the function, cell distribution, and structure of LFA-1, LFA-2, and LFA-3. LFA-1 MAb inhibit CTL- and natural killer(NK)-mediated cytolysis, whereas LFA-2 and LFA-3 MAb block cytolysis by CTL but not NK cells. MAb recognizing all three antigens block T cell proliferative responses to alloantigens and phytohemagglutinin A (PHA). These molecules illustrate the elaborate set of requirements for the CTL-target cell interaction.

MATERIALS AND METHODS

MAb. MAb to LFA antigens were derived from subcloned lines TS1/12 (LFA-1), TS1/18 (LFA-1), TS2/18 (LFA-2), TS1/8 (LFA-2), and TS2/9 (LFA-3). Culture supernatants were routinely used, although purified immunoglobulins (Ig) from ascitic fluid gave similar results.

Commercially available MAb used included OKT3 (18), OKT8 (19), OKT4 (20), Leu-1, Leu-2a, Leu-3a, Leu-4 (15, 21), and Leu-5 (22). (Leu-1–4 antibodies were provided by Dr. Robert Evans, Sloan-Kettering Cancer Institute, NY). Nonbinding control antibodies used were M21/3.2.2, a hamster anti-mouse pan-lymphocyte MAb (23), and M22/3.2.2, a hamster anti-mouse Lyt-2,3 MAb (18).

Fluorescence-activated cell sorter (FACS) analysis. Immunofluorescence flow cytometry was performed on an FACS II after labeling cells with MAb and affinity-purified fluorescein isothiocyanate anti-mouse IgG (Zymed Laboratories, South San Francisco, CA) (5, 24).

Long-term cytolytic cell lines. OKT4+, HLA-DR-specific (10), OKT8+, HLA-A,B-specific (25), and NK (26) cell lines were generatred as described. Peripheral blood lymphocytes (PBL) from normal volunteers (M.P.: HLA-A11, Aw32, B27, Bw51, Cw2, DR7; M.C.: HLA-A3, Aw30, B7, B13, Cw6, DR4; J.F.: HLA-A2, A28, B27, Bw51, Cw1, DR5, DR7) were separated on a Ficoll/Hypaque gradient and were cocultured with irradiated lymphoblastoid cells (JY or Daudi) in interleukin 2- (IL 2) containing medium (10). All cells were propagated in complete media, i.e., RPMI 1640 (M.A. Bioproducts, Bethesda, MD) medium supplemented with 10% fetal calf serum, 2 mM L-glutamine (GIBCO, Grand Island, NY), penicillin 100 U/ml, streptomycin 100 μg/ml (GIBCO), and 25 μM 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, NY). IL 2 was the culture supernatant from PHA-stimulated Jurkatt cell line passed over an anti-PHA column and used at a final concentration of 1% (27).

^{51}Cr-release assay. CTL assays were performed in duplicate or triplicate in V-bottomed microtiter wells, and the percentage specific release was calculated (10). The human lymphoblastoid cell line JY (HLA-A2,2; B7,7; DR4,6) was used as the target for HLA-A,B- and DR-specific CTL. The human erythroid tumor cell line K562 was used to assay NK cell activity (28).

MAb were added at various concentrations directly to the microculture well at the start of the 2.5- to 5-hr incubation of the ^{51}Cr-release assay, and the percentage of blocking was calculated by the formula: Percent blocking = 100 × [(SR control) − (SR + MAb)/(SR control)] in which SR control is the specific release in the absence of MAb.

Assays were performed at effector to target ratios of 20 to 25:1 unless otherwise specified, and were harvested at 2.5 to 5 hr, whenever 50% specific release had occurred in the absence of added antibody.

Proliferation assays. Mixed lymphocyte reaction (MLR) cultures were established at 1 × 10^5 responder cells and 0.5 × 10^5 or 0.5 × 10^4 irradiated (2500 rad) stimulator cells per microtiter well (0.2 ml) and were harvested on day 5. The blastogenic response of lymphocytes to PHA was measured in a

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[3] Abbreviations used in this paper: LFA, lymphocyte function-associated antigen; CTL, cytolytic T lymphocyte; MAb, monoclonal antibody; SDS-PAGE, NaDodSO$_4$-polyacrylamide gel electrophoresis; NK, natural killer; PBL, peripheral blood lymphocytes; IL 2, interleukin 2; FACS, fluorescence-activated cell sorter; MHC, major histocompatibility complex; Mr, relative molecular mass.

microtiter system with 1×10^5 cells/well in 0.2 ml of complete media, incubated in a 5% CO_2 incubator, and harvested on day 3. Cells for MLR and PHA responses were pulsed for 18 hr with 1 μCi/well of [^{3}H]thymidine (3.3 Ci/mM; New England Nuclear, Boston, MA). The final concentrations of PHA (Sigma Chemical Co., St. Louis, MO) was 1/1000 by volume (2 μg/ml).

Iodination, immunoprecipitation, and electrophoresis. Cells were iodinated, and immunoprecipitation was carried out as described (17). Samples were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography with enhancing screens as described (17).

RESULTS

LFA-1: a broadly distributed leukocyte antigen involved in CTL- and NK-mediated cytolysis. The expression of LFA-1 by different cell types was investigated by FACS analysis and immunoprecipitation (Figs. 1 and 2). LFA-1 is expressed on 95% of PBL, including T and B cells, as well as essentially all thymocytes, PHA-activated blasts, CTL, granulocytes, monocytes, and 37% of bone marrow cells (Fig. 1). The LFA-1 MAb immunoprecipitate two polypeptide chains of Mr 177,000 and 95,000 from all cell types on which LFA-1 is expressed (Fig. 2*B*). Identical starting lysate input counts and side by side electrophoresis conditions were used for all lanes in each panel of Figure 2. Immunoprecipitation (Fig. 2) and FACS (Fig. 1) data show quantitative differences in the expression of LFA-1 on various cell types: CTL- and PHA-activated T cells > PBL > thymocytes > JY (B lymphoblastoid) cells.

The broad distribution of LFA-1 on lymphoid cells prompted us to assess the ability of LFA-1 MAb to block a variety of immune responses, including CTL- and NK-mediated cytolysis and T cell proliferation to the mitogen PHA and alloantigens in MLR. LFA-1 MAb significantly block cytolysis, not only by HLA-DR-specific CTL as previously described (17), but also by HLA-A, B-specific CTL and by cell lines displaying NK activity (Table I). A titration of LFA MAb showed that concentrations of approximately 1 μg/ml produced one-half maximal inhibition of cytolysis in a 3 to 4-hr ^{51}Cr-release assay for HLA-A2-specific CTL (Fig. 3*A*) and HLA/DR-specific CTL (Fig. 3*B*) LFA-1 MAb also block lymphocyte proliferative responses to alloantigens (MLR) and PHA (Fig. 4). Complete blocking was achieved with 1 to 5 μg/ml of antibody.

LFA-2: a molecule specifically participating in T cell functions. LFA-2 is present on approximately 55 to 80% of PBL, on all thymocytes and CTL, and on almost all PHA blasts (Fig. 1). Because LFA-2 is not expressed by granulocytes, monocytes (Fig. 1), or the B lymphoblastoid cell line JY (17), further experiments were performed to determine if LFA-2 is T cell specific. Double fluorescence staining of PBL with anti-Ig and anti-LFA-2 showed that the percentage of cells stained were additive. In addition, anti-Leu-4 (OKT3) or anti-LFA-2 alone or together stained nearly equivalent percentages of cells, but with the addition of both antibodies fluorescence intensity increased, demonstrating simultaneous staining of distinct antigenic determinants on the same cell population (data not shown). Lastly, rhodamine-labeled anti-Ig and fluorescein-labeled anti-LFA-2 stained mutually exclusive populations of PBL (data not shown). These data demonstrate that LFA-2 is present in T but not B cells.

FACS analysis (Fig. 1) and immunoprecipitation (Fig. 2*C* and *D*) demonstrate that the density of LFA-2 is greatest on PHA blasts and CTL > thymocytes > PBL, whereas Leu-4 (OKT3) expression is similar on resting and activated T cells (data not shown). PHA-activated blasts express four times as much LFA-2 antigen as normal PBL (Fig. 1, M,H).

LFA-2 has different m.w. profiles on T cells at different stages of differentiation and/or activation (Fig. 2). A polypeptide of Mr = 47,000 is immunoprecipitated from thymocytes and cultured CTL lines by anti-LFA-2 MAb (Fig. 2*C* and *D*, lanes 1, 4, and 5). In contrast, polypeptides of 55,000 and 47,000 Mr are immunoprecipitated from PHA blasts (Fig. 2*C* and *D*, lane 3).

LFA-2 was compared with other antigens associated with T cell-mediated killing. LFA-2 (Fig. 5, lanes 4 and 5, Mr = 47,000) is of different mobility in SDS-PAGE than are LFA-1 (lane 3, Mr = 177,000, 95,000), OKT3 (Leu-4, lane 6, Mr = 19,000), and OKT8 (Leu-2a, lane 7, Mr = 32,000). No precipitate was obtained for OKT4 (Leu-3a), but its reported Mr = 62,000 and its expression on a subpopulation of T cells (20) differ from LFA-2.

Because MAb to the sheep red blood cell (SRBC) receptor (Lyt-3 [9.6], Mr = 50,000 (29); OKT11A, Mr = 49,000 (30), and Leu-5, Mr = 45,000 to 50,000 (22)) and the IL 2 receptor (anti-Tac, Mr = 47,000 to 53,000 (31)) immunoprecipitate molecules of similar m.w. to LFA-2, experiments were undertaken to compare these molecules. Side by side immunoprecipitation on SDS-PAGE showed identical m.w. for LFA-2 and Leu-5 (Fig. 6*A*, lanes 3 and 4) but not Tac (data not shown). When cell lysates were precleared with anti-Leu-5, the amount of material immunoprecipitated by anti-LFA-2 MAb (Fig. 6*B*, lane 9) was greatly diminished compared with the P3X63 control preclear (Fig. 6*B*, lane 5). Conversely, preclearing with anti-LFA-2 removed the material precipitated by Leu-5 (Fig. 6*B*, compare lanes 6 and 8). Immunoprecipitation and preclearing experiments with OKT11 MAb showed that OKT11 and LFA-2 are also identical (data not shown). OKT11A antigen was immunoprecipitated more weakly, but it also shows an Mr identical to LFA-2, Leu-5, and OKT11 (data not shown).

LFA-2 has a similar cell distribution to Leu-5 (22), 9.6 (Lyt-3) (29), and OKT11 (30). They are also present on T cells but not on B cells. MAb to Leu-5 (22), 9.6 (Lyt-3) (29), and OKT11 (30), have been reported to block SRBC rosetting. Similarly, incubation of PBL with anti-LFA-2 MAb reduced the percentage of lymphocytes rosetting with SRBC from 45 to 2% whereas OKT3 did not (data not shown). Lastly, comodulation experiments showed that, when PHA blasts were incubated with anti-LFA-2 MAb for 18 hr, LFA-2 and Lyt-3 (9.6) expression were decreased by 90%, but Leu-4 (OKT3) expression was not affected (data not shown).

Anti-LFA-2 MAb blocked cytolysis by HLA-DR-specific CTL

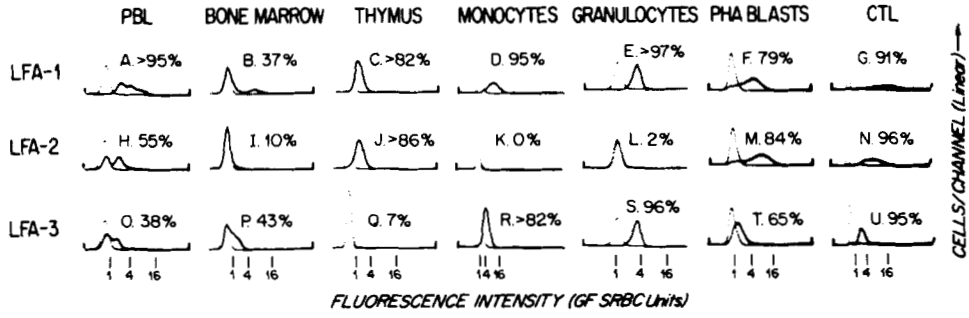

Figure 1. Immunofluorescence flow cytometry of various cell surfaces labeled with LFA MAb. Dim curves represent staining with control P3X63 supernatant. Bright curves represent staining with anti-LFA-1 (TS1/12.1.1, row 1), anti-LFA-2 (TS2/18.1.1, row 2), or anti-LFA-3 (TS2/9.1.1, row 3) MAb. Cell sources are PBL, bone marrow, thymus, adherent cells (monocytes), granulocytes, PHA-activated blasts, and a CTL clone, F1 columns from left to right). Fluorescence intensity is in glutaraldehyde-fixed SRBC units (GF SRBC) (17). Percentage of cells positive for each antigen is shown.

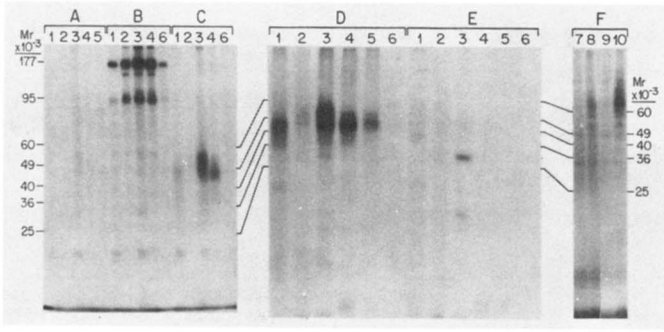

Figure 2. Biochemical characterization of LFA-1, 2, and 3 antigens expressed on different cell types. Lysates of ^{125}I-labeled human thymocytes (1), PBL (2), PHA blasts (3), CTL anti-Daudi line (4), CTL-anti-JY line (5), and JY B lymphoblastoid cell line (6) were immunoprecipitated with P3X63 supernatant as control (*A*), anti-LFA-1 MAb TS1/18.1.1 (*B*), anti-LFA-2 MAb TS2/18.1.1 (*C*), purified anti-LFA-2 MAb TS2/18.1.1 coupled to Sepharose (*D*), and purified control mouse MAb RG7/7.6 anti-rat κ-chain coupled to Sepharose (*E*). For LFA-3 antigen, lysates of ^{125}I-labeled human CTL anti-Daudi line (*F*, lanes 7 and 8) and JY (*F*, lanes 9 and 10) were immunoprecipitated with the anti-LFA-3 MAb TS2/9.1.1 (lanes 8 and 10) and P3X63 supernatant as control (lanes 7 and 9).

A–C and *F*, 100 μl (7.4 μg) of the 187-1 anti-mouse κ-chain MAb as a second antibody, and 50 μl of a 10% suspension of *Staphylococcus aureus* were used. *D* and *E*, direct immunoprecipitation by using purified MAb coupled to Sepharose was used. Reduced samples were subjected to SDS-10% (*A–C*) or 12.5% (*D–F*) PAGE and autoradiography with enhancing screens. Radioactive standard proteins of known m.w. were run in these same gels as internal controls for Mr.

TABLE I

Inhibition of cytolysis by anti-LFA-1, LFA-2, and LFA-3 MAb[a]

Antibody	OKT4⁺, HLA-DR-specific CTL		OKT8⁺, HLA-A,B-specific CTL		NK Cells	
	% Specific release	% Inhibition	% Specific release	% Inhibition	% Specific release	% Inhibition
None	51		54		48	
Control	51	0	57	0	48	0
LFA-1	20	61	25	57	24	50
LFA-2	25	51	22	62	49	0
LFA-3	21	60	21	64	49	0
OKT3	21	60	20	63	50	0

[a] Long-term cytolytic T cell lines specific for HLA-DR6, HLA-A2, and NK targets were assayed for cytotoxicity by a 4-hr ^{51}Cr-release assay effector to target ratios of 20:1. Target cells were JY for CTL and K562 for NK cells. MAb supernatants or ascites (OKT3) were added to the assay at a final concentration by volume of 1/9. The control antibody was M21/3.22, a hamster anti-mouse MAb. Other MAb used were LFA-1 (TS1/18.1.1), LFA-2 (TS2/18.1.1), LFA-3 (TS2/9.1.1), and OKT3. Data shown are percentage specific release and percentage inhibition of cytolysis by antibody.

and HLA-A, B-specific CTL but not NK cells (Table I, Fig. 3) and blocked proliferative responses to PHA and MLR (Fig. 4). The identity between LFA-2 and the SRBC receptor led us to compare the ability of anti-LFA-2 MAb and a panel of anti-SRBC receptor MAb to block CTL-mediated cytolysis. Anti-LFA-2 and anti-Leu-5 similarly blocked cytolysis by an HLA-A2-specific CTL line (Table II). OKT11A and Lyt-3 (commercially available MAb 9.6), on the other hand, did not block cytolysis. This is notable because we, and other investigators, have shown that treatment with Lyt-3 and complement (C) lyses all CTL and NK cells (26, 32), and MAb 9.6 has been reported to partially block cytolysis (32).

LFA-3: a novel, broadly distributed antigen associated with lymphocyte function. LFA-3 is expressed on 40 to 60% of PBL, including B and T lymphocytes. It is also present on essentially all monocytes, granulocytes, and CTL (Fig. 1), as well as B lymphoblastoid cell lines (JY and Daudi), K562, platelets, human vascular endothelium, vascular smooth muscle, and fibroblasts (data not shown). LFA-3 is expressed on approximately 40% of bone marrow cells (Fig. 1). LFA-3 MAb immunoprecipitates a broad band from 65,000 to 70,000 Mr from B lymphoblastoid cell lines (Fig. 2*F*, lanes 9 and 10) and a band of 60,000 Mr from

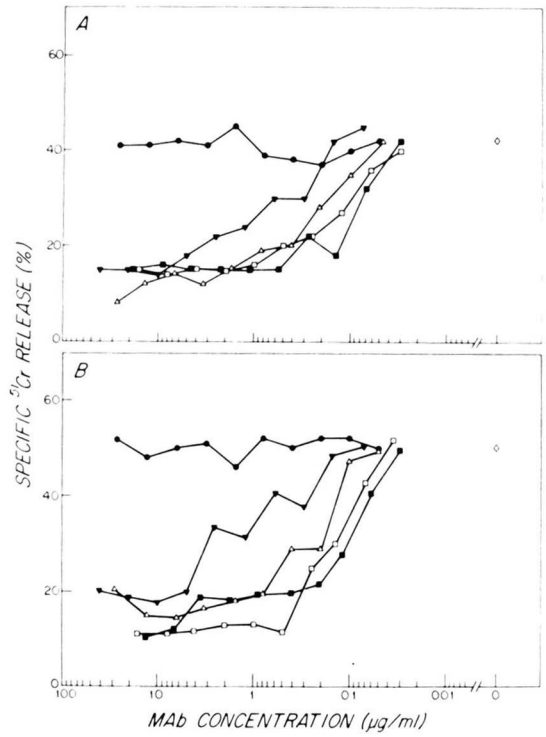

Figure 3. Inhibition of cytolytic activity by MAb to LFA-1, LFA-2, and LFA-3. Anti-HLA-DR6-specific CTL (*A*) and anti-HLA-A2-specific CTL (*B*) were assayed for cytotoxicity by a 4- to 5-hr ^{15}Cr-release assay at effector to target ratios of 20:1. MAb to LFA-1 (TS1/18.1.1; △), LFA-2 (TS1/8.1.1; ■ and TS2/18.1.2; □), and LFA-3 (TS2/9.1.2; ▼), and a hamster anti-mouse MAb (M21/3.2.2, ●) as control were added at the initiation of the assay at the indicated final dilutions. ◇, no antibody.

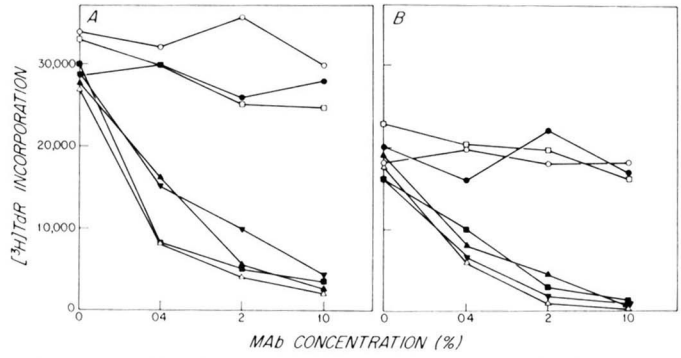

Figure 4. Inhibition of (*A*) PHA response and (*B*) MLR by anti-LFA-1, LFA-2, and LFA-3 MAb. PHA response and MLR were generated from freshly separated PBL as described in *Materials and Methods*. MAb were added at the start of the cell incubations at final antibody concentration by volume as shown. Antibodies used were to LFA-1 (TS1/18.1.1; △), LFA-2 (TS2/18.1.1; ■), LFA-3 (TS2/9.1.2; ▼), HLA-DR (TS1/16.1.1; ▲), Leu-1 (○), and hamster anti-mouse as a control (M21/3.2.2; ●). The addition of medium alone (□) is also shown. Data shown are [^{3}H]thymidine [^{3}H]TdR) incorporation at various MAb concentrations.

CTL lines (Fig. 2*F*, lanes 7 and 8). Anti-LFA-3 MAb blocked cytolysis by OKT4⁺ and OKT8⁺ CTL but not NK cells (Table I) and inhibited PHA and MLR proliferation responses (Fig. 4).

Site of blockade by anti-LFA MAb. To establish the locus of inhibition by anti-LFA MAb, effectors or targets were pretreated with LFA-1, LFA-2, or LFA-3 MAb, washed, and assessed in a standard cytotoxicity assay (Table III). Both anti-LFA-1 and LFA-2 MAb block cytolysis by binding to effector cells, whereas anti-LFA-3 MAb blocks cytolysis by binding to target cells. Because anti-major histocompatibility complex (MHC) (HLA-A, B and DR) antibodies are the only antibodies shown to inhibit allogeneic cytolysis by binding to target cells, we investigated the possibility that LFA-3 blocks lysis by binding to or near HLA-A, B or DR cell surface proteins. Competitive binding of iodinated antibody

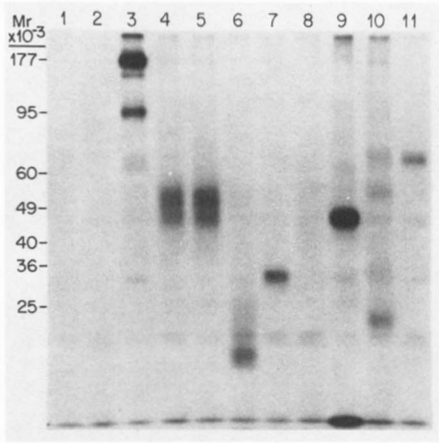

Figure 5. SDS-PAGE comparison of human antigens associated with T cell-mediated cytolysis. Lysates of ^{125}I-labeled PHA-activated human PBL were immunoprecipitated with P3X63 supernatant as control, lane 1; and the following mouse anti-human MAb: anti-LFA-3 MAb TS2/9.1.1, lane 2; anti-LFA-1 MAb TS1/18.1.1, lane 3; anti-LFA-2 MAb TS1/8.1.1 and TS2/18.1.1, lanes 4 and 5, respectively; Leu-4, lane 6; Leu-2a, lane 7; OKT4, lane 8; anti-HLA-A, B MAb W6/32, lane 9; anti-HLA-DR MAb TS1/16.1.1, lane 10; and Leu-1, lane 11. As second antibody 100 μl (7.4 μg) of the 187-1 anti-mouse κ-chain MAb together with 50 μl of a 10% suspension of *S. aureus* were used. Reduced samples were subjected to SDS 10%-PAGE and autoradiography with enhancing screens.

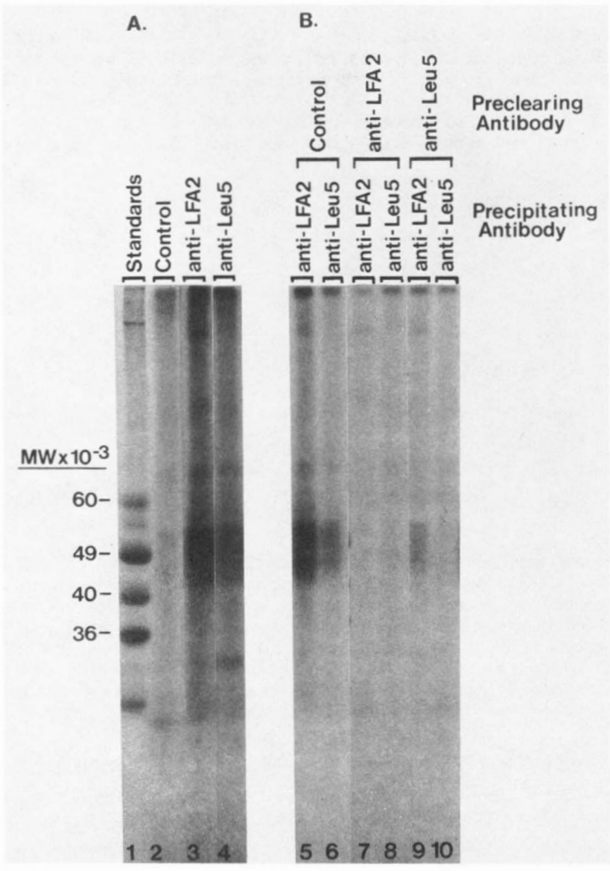

Figure 6. A. SDS-PAGE comparison of LFA-2 and Leu-5. Lysates of ^{125}I-labeled PHA-activated human PBL were immunoprecipitated with P3X63 supernatant as control, lane 2; and the following mouse anti-human MAb: anti-LFA-2 MAb TS2/18.1.1, lane 3; and anti-Leu-5 MAb ATM-1.1, lane 4, as described in the *legend* for Figure 5. Radioactive standard proteins of known m.w. were included as internal controls for Mr, lane 1.

B, Coprecipitation of LFA-2 and Leu-5. Lysates of ^{125}I-labeled PHA-activated human PBL were precleared by immunoprecipitation with P3X63 supernatant, lanes 5 and 6; anti-LFA-2 MAb TS2/18.1.1, lanes 7 and 8; and anti-Leu-5 MAb ATM-1.1, lanes 9 and 10 as described above. The resulting supernatants were then immunoprecipitated with either anti-LFA-2 MAb TS2/18.1.1, lanes 5, 7 and 9; or anti-Leu-5 AMb ATM-1.1, lanes 6, 8, and 10, and the reduced samples were subjected to SDS 10%-PAGE and autoradiography with enhancing screens.

TABLE II

Comparison of inhibition of CTL-mediated cytolysis by MAb that recognize the SRBC receptor molecule[a]

Experiment 1		Experiment 2	
Antibody	% Specific release	Antibody	% Specific release
None	56	None	58
Leu-1	55	Lyt-3	55
LFA-2	7	OKT3	17
Leu-5	12		
OKT11A	58		

[a] Anti-HLA-A2 (OKT8$^+$) CTL were assayed for cytotoxicity by a 4-hr ^{51}Cr-release assay at an effector to target ratio of 20:1. JY was the target cell used. MAb to LFA-2 (TS2/18.1.1), Leu-1, Leu-5, OKT11A, Lyt-3 (MAb 9.6), OKT3, or medium alone (None) were added to the start of the assay in final dilutions of 1/9.

TABLE III

LFA-1 and LFA-2 MAb block by binding effector cells whereas LFA-3 MAb blocks by binding target cells[a]

Treatment	Antibody					
	LFA-1		LFA-2		LFA-3	
	25:1	6:1	25:1	6:1	25:1	6:1
No antibody added	54	18	54	18	54	18
Control antibody added	55	19	52	18	54	17
LFA antibody added	22	5	10	3	20	5
Effectors pretreated	27	9	18	4	53	14
Targets pretreated	57	18	68	15	17	5

[a] A long-term cytolytic cell line specific for HLA-A2 was assayed for cytotoxicity by a 3.5-hr ^{51}Cr-release assay at effector to target ratios of 25:1 and 6:1 on JY target cells. Medium or antibody was added at the start of the assay as described (Table I). Alternatively, CTL or target cells were preincubated with LFA antibodies for 30 min at room temperature and were washed before the assay. No additional antibody was added to these assays. Control antibody was M21/3.2.2, and LFA antibodies used were TS1/18.1.1, TS2/18.1.2, and TS2/9.1.2. Data shown are percentage specific release.

TABLE IV

Effect of addition of LFA MAb[a]

Antibody Added	Experiment 1		Experiment 2	
	% Specific release	% Inhibition	% Specific release	% Inhibition
None	40		62	
LFA-1	35	12	50	20
LFA-2	38	8	21	49
LFA-3	37	5	47	25
LFA-1 and 2	22	45	7	89
LFA-1 and 3	13	68	8	88
LFA-2 and 3	33	18	38	39
LFA-1, 2, and 3	N.D.		10	84

[a] A long-term cell line specific for HLA-A2 was assayed for cytotoxicity by 4.5- (Expt. 1) and 3.5- (Expt. 2) hr ^{51}Cr-release assays at effector to target ratios of 20:1 (Expt. 1) and 25:1 (Expt. 2). JY was the target cell line. Medium or antibody was added to the assay as described (Table I). Antibodies (TS1/18.1.1, TS2/18.1.12, and TS2/9.1.12) were added at a final antibody concentration of 1/900 (Expt. 1) and 1/1000 (Expt. 2) by volume. Data shown are percentage specific release and percentage inhibition of cytolysis by addition of MAb for two separate experiments. N.D., not determined.

showed that MAb to LFA-3 does not block the binding of either HLA-A, B (w6/32) or HLA-DR (TS1/16) MAb (data not shown). Furthermore, because quantitative expression of LFA-3 by JY cells is less than 10% of HLA-A, B or HLA-DR, it is unlikely that anti-LFA-3 MAb blocks CTL lysis by steric hindrance of HLA antigens.

The effect of adding various combinations of LFA MAb to the cytotoxicity assay is shown in Table IV. The concentrations of LFA MAb were selected to give minimal inhibition of cytolysis when used alone. The combination of anti-LFA-1 and LFA-3 MAb synergistically inhibits cytolysis, whereas the combination of anti-LFA-1 and LFA-2 MAb additively inhibits cytolysis. The combination of anti-LFA-2 and LFA-3 MAb do not inhibit significantly better than either antibody alone.

DISCUSSION

Three cell surface molecules, designated LFA-1, LFA-2, and LFA-3, were previously identified by MAb selected for their ability to block cytolysis by an OKT4$^+$, HLA-DR-specific long-term CTL line (17). In this report we show that the anti-LFA MAb block all T cell cytolytic and proliferative functions studied. In addition, anti-LFA-1 MAb inhibit NK-mediated cytolysis. Anti-LFA-1 and anti-LFA-2 MAb block by binding to effector cells, whereas anti-LFA-3 MAb inhibits by binding to the target cell. The cellular distribution and functional studies of these antigens allows their classification according to paradigms.

The first class of antigens, typified by murine LFA-1 (7), are cell surface determinants that are widely distributed on both effector and target cells. Antibodies that recognize these structures appear to block lymphocyte function via interruption of cell interactions. Anti-LFA-1 MAb have been shown to block cell adhesion at the effector level. These determinants are broadly distributed, and therefore, are unlikely to be part of an antigen-specific receptor (7).

Human LFA-1 is another example of this type of antigen. It is widely distributed on lymphoid tissues and is composed of two polypeptides of 177,000 and 95,000 Mr on all cell types studied. LFA-1 MAb block antigen-specific CTL-mediated cytotoxicity, NK-mediated cytotoxicity, and T cell proliferation. CTL-mediated cytotoxicity is inhibited by anti-LFA-1 MAb binding to the effector cell rather than to the target cell. By analogy with murine LFA-1, human LFA-1 appears involved in a general process underlying cellular interaction, most likely adhesion. Thus, LFA-1 may fall into a class of molecules that are required for cell-cell interactions, and not just those specialized interactions utilized by T lymphocytes in antigen recognition. Lyt-2 typifies a second class of antigens, those associated with T cell functions. Other antigens that can be included in this class are the human antigens OKT3, OKT4, and OKT8. These antigens are expressed on T cells and may be involved in specific antigen recognition. Studies of murine lymphocyte-mediated cytolysis have shown that Lyt-2 is involved in, (33, 34) but not absolutely necessary for, the effector-target interaction (35). Lyt-2$^-$ CTL hybridomas have been isolated that are still capable of specific cell mediated cytolysis (35). Anti-OKT4 and -OKT8 antibodies may recognize monomorphic sites on the molecules involved in MHC restriction. OKT8 (Leu-2a) is the human homologue of Lyt-2 and appears to be involved in the recognition of class I MHC determinants, whereas OKT4 (Leu-3a) recognizes class II MHC determinants (10–12, 36–38). Support for this hypothesis comes from the observations that HLA-DR- (10–12, 36) and SB-(37) specific CTL and most helper/inducer cells (20) are OKT4$^+$, whereas HLA-A, B-specific CTL are OKT8$^+$ (19).

LFA-2 appears to be another example of a T cell function-specific antigen. LFA-2 MAb strongly inhibit all T cell functions studied. We have not yet established, however, whether LFA-2 participates in antigen recognition or is involved in antigen-nonspecific interactions. MAb to LFA-2 may recognize a constant region determinant on a T cell antigen receptor. We have shown that LFA-2 MAb precipitate molecules of different m.w. from T cells at different stages of activation and/or differentiation, showing that heterogeneity exists. Because LFA-2 is a glycoprotein (shown by unpublished lectin-binding studies), m.w. variation may be due to differences in either glycosylation or amino acid sequence. Experiments are currently in progress to assess LFA-2 clonal variation with the use of cloned human cell lines.

Despite the fact that LFA-2 has a wider cell distribution than OKT3, (32), LFA-2 MAb block only T cell functions. This paradox may be important in understanding the relationship between CTL and NK cells. Ortaldo *et al.* (39) have suggested that NK cells may represent an early stage of T cell differentiation. Both OKT3$^+$, Lyt-3$^+$ (40) and OKT3$^-$, Lyt-3$^+$ (41) NK cells have been described. Large granular lymphocytes are low or negative for OKT11, Lyt 3, but cultured NK cells are definitely positive (39–41). It is possible that the OKT3$^-$, Lyt-3$^+$ NK cells that we have described (26) have not yet differentiated to the stage in which LFA-2 is a functional component of the lytic process. Recently, Fast *et al.* (32) reported partial blocking of both CTL and NK activity by using the MAb 9.6 (Lyt-3). We were unable to block CTL-mediated cytolysis with OKT11A or commercially available OKT11 or Lyt-3 (9.6). These differences may result from differences in MAb affinity or recognition of different epitopes. The fact that we found no blocking of NK activity may be due to differences in NK cell subpopulations studied and/or their stage of differentiation.

Antigens present on the target cell, such as HLA-A, B and DR, represent another group of molecules involved in cell interactions (42). MAb that recognize these antigens block function by binding to target rather than effector cells. The anti-LFA-3 MAb specifically blocks function by binding to target cells, implying that LFA-3 may represent a target ligand for an effector-specific receptor. LFA-3 is broadly distributed on both lymphoid and nonlymphoid tissues (including granulocytes, platelets, vascular endothelium, smooth muscle, and fibroblasts), but LFA-3 is biochemically distinct from HLA-A, B, DR, and other MHC antigens. The possibility that anti-LFA-3 MAb can also block function at the effector level is being studied on a panel of human CTL clones that express high amounts of LFA-3. Whereas MAb to HLA-A, B block cytolysis by OKT8$^+$ CTL, and anti-HLA-DR MAb block cytolysis by OKT4$^+$ CTL, anti-LFA-3 MAb blocks cytolysis by both sets of CTL, suggesting that LFA-3 may play a role different from OKT4 and OKT8 in the CTL-target interactions.

Anti-LFA-1 and LFA-3 MAb block T cell functions synergistically. The observation that LFA-3 is also present on K562 but is not involved in NK-mediated cytolysis (i.e., it does not block), whereas LFA-1 is involved, makes it questionable that LFA-1 and LFA-3 interact as a ligand-receptor pair. It is possible, however, that due to a different type of cell interaction, LFA-1 on NK cells may interact with a different site on the LFA-3 molecule for K562 killing.

The CTL-target interaction is known to involve antigen recognition, cell adhesion, and delivery of the "lethal hit" (43). The description of at least six cell surface molecules (OKT3, OKT4/OKT8, LFA-1, LFA-2, LFA-3, and HLA-A, B/DR) involved in CTL-mediated cytotoxicity shows the complexity of this process at the molecular level.

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REFERENCES

1. Burakoff, S. J., C. S. Reiss, of Finberg, and M. F. Mescher. 1980. Cell-mediated immunity to viral glycoproteins. Rev. Infect. Dis. 2:62.

2. Cerottini, J. C., and K. T. Brunner. 1974. Cell-mediated cytotoxicity, allograft rejection, and immunity. Adv. Immunol. 18:67.
3. Herberman, R. B. 1974. Cell-mediated immunity to tumor cells. Adv. Cancer Res. 19:207.
4. Davignon, D., E. Martz, T. Reynolds, K. Kurzinger, and T. A. Springer. 1981. Monoclonal antibody to a novel lymphocyte function-associated antigen (LFA-1): mechanism of blocking of T lymphocyte-mediated killing and effects on other T and B lymphocyte functions. J. Immunol. 127:590.
5. Kurzinger, K., T. Reynolds, R. N. Germain, D. Davignon, E. Martz, and T. A. Springer. 1981. A novel lymphocyte function-associated antigen (LFA-1): cellular distribution quantitative expression and structure. J. Immunol. 127:596.
6. Sanchez-Madrid, F., D. Davignon, E. Martz, and T. A. Springer. 1982. Antigens involved in mouse cytolytic T lymphocyte (CTL)-mediated killing: Functional screening and topographic relationships. Cell. Immunol. 73:1.
7. Springer, T. A., D. Davignon, M. K. Ho, K. Kurzinger, E. Martz, and F. Sanchez-Madrid. 1982. LFA-1 and Lyt-2,3 molecules associated with T lymphocyte-mediated killing and Mac-1 and LFA-1 homologue associated with complement receptor functions. Immunol. Rev. 68:111.
8. Sarmiento, M., D. P. Dialynas, D. W. Lancki, *et al.* 1982. Cloned T lymphocytes and monoclonal antibodies as probes for cell surface molecules active in T cell-mediated cytolysis. Immunol. Rev. 68:135.
9. Golstein, P., C. Goridis, A. Schmitt-Verhulst, *et al.* 1982. Lymphoid cell surface interaction structures detected using cytolysis-inhibiting monoclonal antibodies. Immunol. Rev. 68:5.
10. Krensky, A. M., C. S. Reiss, J. W. Mier, J. L. Strominger, and S. J. Burakoff. 1982. Long-term human cytolytic T cell lines allospecific for HLA-DR6 antigen are OKT4$^+$. Proc. Natl. Acad. Sci. USA 79:2365.
11. Meuer, S. C., R. E. Hussey, J. C. Hodgdon, T. Hercend, S. F. Schlossman, and E. L. Reinherz. 1982. Surface structures involved in target recognition by human cytotoxic T lymphocytes. Science 218:471.
12. Krensky, A. M., C. Clayberger, C. S. Reiss, J. L. Strominger, and S. J. Burakoff. 1982. Specificity of OKT4$^+$ cytotoxic T lymphocyte clones. J. Immunol. 129:2001.
13. Reinherz, E. L., R. E. Hussey, K. Fitzgerald, P. Snow, C. Terhorst, and S. F. Schlossman. 1981. Antibody directed at a surface structure inhibits cytolytic but not suppressor function of human T lymphocytes. Nature 294:168.
14. Platsoucas, C. D., and R. A. Good. 1981. Inhibition of specific cell-mediated cytotoxicity by monoclonal antibodies to human T cell antigens. Proc. Natl. Acad. Sci. USA 78:4500.
15. Evans, R. L., D. W. Wall, C. D. Platsoucas, *et al.* 1981. Thymus-dependent membrane antigens in man: inhibition of cell-mediated lympholysis by monoclonal antibodies to the TH_2 antigen. Proc. Natl. Acad. Sci. USA 78:544.
16. Reinherz, E. L., R. E. Hussey, and S. F. Schlossman. 1980. A monoclonal antibody blocking human T cell function. Eur. J. Immunol. 10:758.
17. Sanchez-Madrid, F., A. M. Krensky, C. F. Ware, *et al.* 1982. Three distinct antigens associated with human T lymphocyte-mediated cytolysis: LFA-1, LFA-2, and LFA-3. Proc. Natl. Acad. Sci. USA 79:7489.
18. Kung, P. C., G. Goldstein, E. L. Reinherz, and S. F. Schlossman. 1979. Monoclonal antibodies defining distinctive human T cell surface antigens. Science 206:347.
19. Reinherz, E. L., P. C. Kung, G. Goldstein, and S. F. Schlossman. 1980. A monoclonal antibody reactive with the human cytotoxic/suppressor T cell subset previously defined by a heteroantiserum termed TH_2. J. Immunol. 124:1301.
20. Reinherz, E. L., P. C. Kung, G. Goldstein, and S. F. Schlossman. 1979. Further characterization of the human inducer T cell subset defined by monoclonal antibody. J. Immunol. 123:2894.
21. Ledbetter, J. A., R. L. Evans, M. Lipinski, C. Cunningham-Rundles, R. A. Good, and L. A Herzenberg. 1981. Evolutionary conservation of surface molecules that distinguish T lymphocyte helper/inducer and T cytotoxic/suppressor subpopulations in mouse and man. J. Exp. Med. 153:310.
22. Howard, F. D., J. A. Ledbetter, J. Wong, C. P. Bieber, E. B. Stinson, and L. A. Herzenberg. 1981. A human T lymphocyte differentiation marker defined by monoclonal antibodies that block E rosette formation. J. Immunol. 126:2117.
23. Sanchez-Madrid, F., P. Scklut, and T. A. Springer. 1983. Stable hamster-mouse hybridomas producing IgG and IgM hamster monoclonal antibodies of defined specificity. J. Immunol. 130:309.
24. Bonner, W. A., H. R. Hulett, R. G. Sweet, and L. Herzenberg. 1972. A fluorescence-activated cell sorting. Rev. Sci. Instrum. 43:404.
25. Reiss, C. S., M. E. Hemler, V. H. Engelhard, J. W. Mier, J. L. Strominger, and S. J. Burakoff. 1980. Development and characterization of allospecific long-term human cytolytic T cell lines. Proc. Natl. Acad. Sci. USA 77:5432.
26. Krensky, A. M., K. A. Ault, C. S. Reiss, J. L. Strominger, and S. J. Burakoff. 1982. Generation of long-term human cytolytic cell lines with persistent natural killer activity. J. Immunol. 129:1748.
27. Robb, R. J., A. Munck, and K. A. Smith. 1981. T cell growth factor receptors. J. Exp. Med. 154:1455.
28. West, W. H., G. B. Cannon, H. D. Kay, G. D. Bonnard, and R. B. Herbermann. 1977. Natural cytotoxicity reactivity of human lymphocytes against a myeloid cell line: characterization of effector cells. J. Immunol. 118:355.
29. Kamoun, M., P. J. Martin, J. A. Hansen, M. A. Brown, A. W. Siadak, and R. C. Nowinski. 1981. Identification of a human T lymphocyte surface protein associated with the E rosette receptor. J. Exp. Med. 153:207.
30. Palacios, R., and Martinez-Maza. 1982. Is the E receptor on human T lymphocytes a "negative signal receptor"? J. Immunol. 129:2479.
31. Leonard, W. J., J. M. Depper, T. Uchiyama, K. A. Smith, T. A. Waldman, and W. C. Greene. 1982. A monoclonal antibody that appears to recognize the receptor for human T cell growth factor; partial characterization of the receptor. Nature 300:267.
32. Fast, L. D., J. A. Hansen, and W. Newman. 1981. Human NK, ADCC, and CTL: evidence for shared T cell antigen. J. Immunol. 127:448.
33. Nakayama, E., H. Shiku, E. Stockert, H. F. Oettgen, and L. J. Old. 1979. Cytotoxic T cells: Lyt phenotype and blocking of killing activity by Lyt antisera. Proc. Natl. Acad. Sci. USA 76:1977.
34. Shinohara, N., and D. H. Sachs. 1979. Mouse alloantibodies capable of blocking cytotoxic T cell function. I. Relationship between the antigen reactive with blocking antibodies and the Lyt-2 locus. J. Exp. Med. 150:432.
35. Kaufman, Y., P. Golstein, M. Pierres, T. A. Springer, and Z. Eshhar. 1982. LFA-1 but not Lyt-2 is associated with killing activity of cytotoxic T lymphocyte hybridomas. Nature 300:357.
36. Ball, E. J., and P. Stastny. 1982. Cell-mediated cytotoxicity against HLA-D region products expressed in monocytes and B lymphocytes. IV. Characterization of effector cells using monoclonal antibodies against human T cell subsets. Immunogenetics 16:157.
37. Biddison, W. E., P. E. Rao, M. A. Talle, G. Goldstein, and S. Shaw. 1982. Possible involvement of the OKT4 molecule in T cell recognition of Class II HLA antigens. J. Exp. Med. 156:1065.
38. Engleman, E. G., C. J. Benike, C. Metzler, P. A. Gatenby, and R. L. Evans. 1983. Blocking of human T lymphocyte functions by anti-Leu-2 and anti-Leu-3 antibodies: differential inhibition of proliferation and suppression. J. Immunol. 130:2623.
39. Ortaldo, J. R., S. O. Sharrow, T. Timonen, and R. B. Herberman. 1981. Determination of surface antigens on highly purified human NK cells by flow cytometry with monoclonal antibodies. J. Immunol. 127:2401.
40. Pawelec, G. P., M. R. Hadam, E. M. Schneider, and P. Wernet. 1982. Clonally restricted natural killer cytotoxicity displayed by cloned human T cell lines. J. Immunol. 128:2271.
41. Zarling, J. M., K. A. Clouse, W. Biddison, and P. C. Kung. 1981. Phenotypes of human natural killer cell populations detected with monoclonal antibodies. J. Immunol. 127:2575.
42. Katz, D. S., and B. Benacerraf, eds. 1976. The Role of Products of the Histocompatibility Gene Complex in Immune Response. Academic Press, New York.
43. Martz, E. 1977. Mechanism of specific tumor cell lysis by alloimmune T lymphocytes: resolution and characterization of discrete steps in the cellular interaction. Contemp. Top. Immunobiol. 7:301.