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HUMAN LYMPHOCYTE FUNCTION ASSOCIATED ANTIGEN-1 (LFA-1): IDENTIFICATION OF MULTIPLE ANTIGENIC EPITOPES AND THEIR RELATIONSHIP TO CTL-MEDIATED CYTOTOXICITY[1]

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Human lymphocyte function-associated antigen (LFA)-1, a heterodimeric lymphocyte surface glycoprotein of 177,000 and 95,000 relative molecular weight has been implicated to function in the cytotoxic T lymphocyte (CTL) effector mechanism. Seven mouse hybridoma lines producing monoclonal antibodies (MAb) reactive with this structure were studied. Three unique and 3 partially overlapping epitopes on human LFA-1 were defined by competitive cross inhibition binding assays using biosynthetically labeled anti-LFA-1 MAb. In contrast, of five rat anti-mouse LFA-1 MAb, all five recognized a common or shared epitope. An HLA-B7 specific human CTL line expressed 1.1 × 10^5 LFA-1 sites per cell with a direct saturation binding assay. Human CTL expressed two to four times more LFA-1 than peripheral blood lymphocytes or B and T lymphoblastoid cell lines. Titration of each of the anti-LFA-1 MAb in a ^{51}chromium release cytolytic assay revealed quantitative differences in the ability of the different anti-LFA-1 MAb to block cytolysis indicating distinct functional and antigenic epitopes exist on the human LFA-1 molecule. Anti-LFA-1 MAb reversibly inhibited the CTL reaction by slowing the initial rate of cytolysis. These results suggest anti-LFA-1 MAb inhibit CTL function by specific blockade of a functionally relevant molecule.

Cytotoxic T lymphocytes (CTL)[5] play an important role in the immune response to cell surface displayed antigens such as those found on virus-infected cells, tumor cells or histoincompatible tissue grafts. Extensive studies on the cellular mechanism involved in the killing reaction have indicated that lysis is a complex multi-step process resolvable into two discrete cation-requiring phases (1–4). A new approach at identifying the molecular components of the cytolytic attack mechanism has been to employ anti-CTL monoclonal antibodies (MAb) capable of inhibiting cytolysis in the absence of complement as specific probes for the identification and characterization of function-associated molecules (5–8). The validity of this approach is based largely on the fact that MAb to a variety of other CTL surface structures do not block cytolysis, thus, inferring that inhibitory MAb react with specific function-associated antigens (9).

Several monoclonal antibodies that recognize molecules on T cells or T cell subpopulations have been shown to block CTL lytic activity in the absence of complement. In man, OKT3 or Leu 4 (T3) Mab recognizes a complex glycoprotein(s) of 20,000 Mr (10–13). The antigen defined by the MAb OKT8, Leu2a, or Leu2b series (T8) is a 30–32,000 and 43,000 Mr disulfide linked glycoprotein complex(es) (11, 14–16). The OKT4 or Leu 3 antigen (T4) is 55,000 Mr (17, 18). Murine CTL are inhibited by MAb to the Lyt-2,3 complex (19–23) (the structural homologue of the T4 antigen (24)) and to the lymphocyte function-associated antigen 1 (LFA-1), a 180,000–95,000 Mr heterodimer (9, 25, 26).

In a previous report, several mouse hybridoma cell lines secreting MAb that blocked the cytolytic activity of HLA-DR allospecific human CTL were isolated (8). These MAb identified three novel human CTL surface structures and were designated lymphocyte function-associated antigens (LFA) -1, 2 and 3. Human LFA-1, a non-disulfide linked heterodimeric lymphocyte surface protein with α and β chains of 177,000 and 95,000 Mr, respectively, appears to be the structural homologue of the previously described mouse LFA-1 molecule. Functional studies on mouse LFA-1 have implicated its role in the initial recognition-adhesion phase of the CTL reaction but it is probably distinct from the CTL antigen-specific receptor (6). Anti-LFA-2 MAb precipitate a 49,000 Mr component. Anti-LFA-3 MAb recognized a diffuse 60,000 Mr protein on a human alloantigen specific CTL line.

In the present report the topographic distribution of antigenic epitopes on the human and mouse LFA-1 molecules, the quantitative expression of cell surface human LFA-1 relative to other CTL membrane antigens and the quantitative effects of the anti-LFA-1 MAb on a Class I allospecific human CTL line have been investigated. The results suggest the presence of several uniquely antigenic epitopes on the human LFA-1 molecule that reside with varying degrees of spatial proximity to the functional region(s) of the LFA-1 molecule.

MATERIALS AND METHODS

Monoclonal antibodies. Two series of mouse anti-human LFA-1 monoclonal antibodies were obtained from hybrid cell lines produced by fusion of human CTL-immune Balb/c spleen cells with the P3X63 Ag8.653 (TS1 series) or NS1 (TS2 series) myeloma cell lines as previously described (8). Both series of hybridoma cell lines have been subcloned twice and are stable. The antibodies secreted by the TS1 and TS2 series are of the IgG_1,K class subclass. Monoclonal antibody concentration in culture fluids was determined by single radial immunodiffusion (27) using a purified mouse IgG_1 MAb (PA2

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[3] Recipient of a United States Public Health Service Fogarty International Fellowship.
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[5] Abbreviations used in this paper: CTL, cytotoxic T lymphocyte(s); MAb, monoclonal antibody(ies); LFA, lymphocyte function-associated antigen; Mr, relative molecular weight; P3X63, P3X63Ag8.653 myeloma cell line; PBS, phosphate buffered saline; BSA, bovine serum albumin; SDS-PAGE, Na dodecyl sulfate polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonylfluoride; PBL, peripheral blood lymphocytes; EDTA, ethylenediaminetetraacetic acid.

(28)) as a reference standard.

Monoclonal antibodies reactive with other human CTL surface antigens, OKT3 and OKT4 were obtained from Dr. G. Goldstein (Ortho Pharmaceutical, Raritan, NJ). The B9.4 MAb (OKT8-like antigen) (7) was the kind gift of Drs. B. Malissen and C. Mawas, Centre d'Immunologie INSERM-CNRS, France. The W6/32 (IgG_{2a}) MAb, recognizing a monomorphic determinant on all HLA A,B,C antigens (29), and the anti-β_2-microglobulin MAb BBM.1 (30), were purified from ascites fluid. The rat antimouse LFA-1 MAb (M7, M15, and M17 series) were obtained as previously described (6, 31).

Monoclonal antibodies were biosynthetically labeled with [^{3}H]leucine as previously described (32). Culture supernatants containing ^{3}H-labeled MAb were dialyzed against 10 mM phosphate buffer saline (PBS), pH 7.2, and diluted into PBS containing 1% bovine serum albumin (BSA) and 0.02% NaN_3. The TS1/18.1.1 anti-LFA-1 and the W6/32 and BBM.1 MAb were purified from ascites fluid by a combination of ammonium sulfate precipitation at 45% saturation and gel filtration chromatography on a Sephacryl-S300 (Pharmacia, Newark, NJ) column (120 × 2.5 cm) at a flow rate of 10 ml/hr in 10 mM Tris, pH 8.0, 0.15 M NaCl, 1 mM EDTA, and 0.02% NaN_3. A conservative cut of the IgG fractions were pooled and dialyzed exhaustively against PBS. The concentration of mouse IgG protein was determined by absorption at 280 nm ($E_{1\%} = 14$). Analysis of IgG preparations by Na dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (7% acrylamide) indicated that they were ≥98% pure IgG.

Purified monoclonal antibodies were radioiodinated with Na ^{125}I (New England Nuclear, Boston, MA), specific activity 17 Ci/mg, using Iodogen (33) to specific activities of 0.5 to 2 × 10^6 cpm/μg and were stored in 10% PBS-0.02% BSA NaN_3 on ice. The radioiodinated MAb were used in quantitative binding studies within 1 week and retained ≥90% of their antigen binding activity. Rat MAb to mouse LFA-1 were purified and radioiodinated as previously described (31).

Competitive inhibition and quantitative MAb binding assays. Competitive cross inhibition binding assay for determination of LFA-1 epitope topography was performed with [^{3}H]L-leucine-labeled or ^{125}I MAb as previously described (32). Briefly, an optimized number of human PBL (2 × 10^5) in 25 μl of 1% BSA-PBS were dispensed into V bottom microtiter wells containing 50 μl anti-LFA-1 MAb culture supernatants serially diluted in BSA-PBS and incubated for 45–60 min on ice. Controls for nonspecific inhibition of binding included W6/32 MAb or spent P3X63 culture medium. [^{3}H]leucine-labeled anti-LFA-1 MAb (5 μl, 1.5 × 10^5 cpm) were then added and incubated for an additional 30 min on ice. This amount of labeled anti-LFA-1 MAb was 60–80% of saturating levels. Cells were then washed four times with 200 μl BSA-PBS by repeated centrifugation at 1000 × G for 2 min. Cells were transferred to vials and lysed by the addition of 100 μl 0.1% NP-40 nonionic detergent solution and 3 ml liquid scintillation cocktail (Biofluor, New England Nuclear, Boston MA) was added for counting. The percentage of inhibition of binding was calculated as a ratio of the antibody bound in the presence of inhibitor to amount bound in P3X63 culture supernatants. Generally, 1000–2000 cpm were bound for each labeled MAb in absence of any inhibitor. The nonspecific background binding was defined as the counts per minute bound in the presence of a 10-fold saturating excess (5–10 μg) of unlabeled homologous MAb (30–60 cpm). Competitive inhibition experiments for MAb to mouse LFA-1 used glutaraldehyde fixed concanavalin A stimulated mouse spleen cells as previously described (31).

A quantitative direct saturation binding assay was used to determine the number of antigenic sites on several cell types (34). The TS1/18.1.1 and W6/32 purified IgG were radioiodinated as described (33). An optimal number of cells in 25 μl (2 × 10^5 for LFA-1; 5 × 10^4 for W6/32) were incubated in increasing amounts of iodinated MAb of known specific activity in 50 μl cold BSA-PBS in microtiter plates for 60 min on ice. Cells were then washed four times with 200 μl BSA-PBS and were transferred to vials for γ counting. Nonspecific binding was defined as counts per minute bound to cells after 30 min preincubation in the presence of a 10-fold saturating excess of unlabeled homologous MAb. Nonspecific binding was subtracted before calculations.

Cell surface iodination, immunoprecipitation, and SDS-PAGE. CTL-11 (10^7 cells) were prewashed three times in warm PBS and were radiolabeled with 1.0 mCi of Na^{125}I with Iodogen (33). A detergent extract was obtained by solubilizing the labeled cells in 1 ml extraction buffer (10 mM TRIS (pH 8.0), 0.15 M NaCl, 1% v/v NP-40 nonionic detergent, 40 mM iodoacetamide, 1 mM phenylmethylsulfonylfluoride (PMSF) and 10 mg/ml bovine hemoglobin) on ice for 1 hr. Cell nuclei and insoluble material were removed by centrifugation at 25,000 × G for 20 min and the extract was dialyzed against 500 ml PBS at 4°C for 18 hr.

Immunoprecipitation was carried out by the sequential addition of: 1)100 μl of the indicated MAb as culture supernatant or purified IgG (3 μg) diluted with extraction buffer; 2) 10 μl of the ^{125}I extract (2.5 × 10^5 cpm, 1 × 10^5 cell equivalents) for 1 hr on ice; 3) 100 μl 187.1 MAb (4 μg) (35–37); and 4) 50 μl of a 10% suspension of fixed *Staphylococcus aureus* Cowan I strain protein A bacteria (38). The incubation was continued for 30 min with shaking. The immune complex adsorbed bacteria were washed three times in 10 mM TRIS-saline, pH 8.0, containing 0.5% NP-40, 0.5% Na deoxycholate and 0.05% Na dodecyl sulfate buffer. Purified TS1/18.1.1 and BBM.1 IgG covalently coupled to Sepharose CL4B at 2 mg/ml beads by the method of March (39) modified by Kolb (40), were used to directly immunoprecipitate LFA-1 and HLA. The MAb-CL4B beads were incubated and washed as described above.

The washed immunoadsorbed antigens were eluted with SDS-PAGE sample buffer: 50 mM TRIS, pH 6.8, 2 mM EDTA, 4% SDS, 10% glycerol, 0.02% bromphenol blue and 5% (v/v) β-mercaptoethanol. Samples were subjected to electrophoresis on 10% polyacrylamide slab gels (41). Autoradiography was performed on dried gels using intensifying screens (Kronex, DuPont, Wilmington, DE) for 48 hr at −85°C (42).

Fluorescence activated flow cytometry. CTL-11 cells were allowed to culture without any target feeder cells for 5 days before analysis. Cells were 85–95% viable as judged by the trypan blue dye exclusion. CTL-11 (2 × 10^6 cells) were divided into four aliquots and were incubated on ice with 1 μg TS1/18.1.1, OKT4, B9.4, OKT3 or, as control, the 2A2 anti-mycoplasma MAb (1:50 dilution of ascites) or normal mouse serum (1:10 dilution) for 30 min. Cells were washed two times in cold BSA-PBS and were incubated with 25 μl of a 1:20 dilution of fluorescein labeled F(ab')$_2$ fragments of caprine anti-mouse Ig (Cappel Laboratories, Cochranville, PA) and were washed four times in cold BSA-PBS. Analysis was performed on a flow cytometer system as previously described in detail (43, 44).

Human alloimmune CTL lines. Tissue culture medium used to grow CTL lines and target cell lines was RPMI-1640 supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μg/ml gentamycin. T cell growth factor (TCGF) was produced by the JURKAT (J6.2 subclone) cell line by stimulation with phytohemagglutinin-P lectin and phorbol myristic acetate according to Robb *et al.* (45). Residual lectin was removed by immunoadsorption with rabbit anti-PHA globulin covalently linked to Sepharose CL4B[6]. The human CTL-11 line is a T cell growth factor dependent line with cytolytic activity directed primarily at the HLA-B7 alloantigen and was grown and tested for alloantigen specificity as previously described[6] (18). Briefly, the CTL-11 line was produced from normal PBL in a one-way mixed lymphocyte culture with irradiated (2500 rad) allogeneic B lymphoblastoid cell line JY (HLA-A2, B7, DR4,6) as a stimulating allogeneic target cell. After a secondary stimulation with the JY cell line, alloreactive CTL were selected for HLA-B7 reactive clones by subculture in medium containing 2 units/ml of TCGF and irradiated MST B-lymphoblastoid cell line (HLA-A3, B7, DR2); MST shares only the HLA-B7 alloantigen with the JY cell line. This method has been used successfully to select for HLA-A2 and DR4,6 reactive human CTL lines[6] (18).

Cytolytic assay. Anti-HLA-B7 CTL effector cells (2 to 10 × 10^3 cells in 50 μl) were mixed with 100 μl of MAb-containing supernatants diluted in medium or medium alone in U bottom microtiter wells in triplicate for 15 to 30 min at 37°C. MST target cells (10^3 cells in 50 μl) labeled with Na ^{51}Chromate (^{51}Cr) (305 mCi/mg, New England Nuclear) were added, the plates were centrifuged at 500 × G for 2 min and subsequently incubated 2 to 4 hr at 37°C. ^{51}Cr released into the supernatant was determined with the aid of the Titer-Tek supernatant collection system (Flow Laboratories, Inglewood, CA). The release of label from target cells without CTL was defined as: spontaneous release (sr); experimental (exp), the ^{51}Cr label released from targets in the presence of CTL; and the total label released (t), determined by addition of 150 μl of a 3% SDS solution. The percentage of cytolysis was calculated according to the formula: [(EXP-SR)/(T-SR)] × 100. The SD of triplicate wells rarely exceeded 2–4% lysis. Spontaneous release of label from the MST line was 3–4%/hr.

RESULTS

Human LFA-1 expresses multiple antigenic epitopes. Isolation and initial characterization of seven stable mouse hydridoma cell lines secreting MAb (all of the $\gamma_1\kappa$ subclass) that precipitated a noncovalently associated heterodimer of 177,000 and 95,000 Mr from human T and B lymphocytes have been reported (8). The topographical relationship of the antigenic epitope(s) recognized by this series of anti-human LFA-1 MAb was investigated with a competitive cross-inhibition binding assay using [^{3}H]leucine biosynthetically labeled MAb (Fig. 1). Two clearly distinct and unique epitopes were defined by MAb TS1/18 and TS2/14 since only the homologous antibody acted as a competitive inhibitor. Another distinct epitope was defined by the TS1/12 and TS1/11 MAb which acted as reciprocal competitive inhibitors. Monoclonal antibodies TS2/4, TS2/6, and TS1/22 defined complex partially overlapping antigenic epitopes on the human LFA-1 molecule. TS2/14 was a nonreciprocal inhibitor of the TS1/22 MAb. W6/32 anti-HLA did not inhibit the binding of any of the anti-LFA-1 MAb.

To confirm that the unique epitopes defined by these MAb

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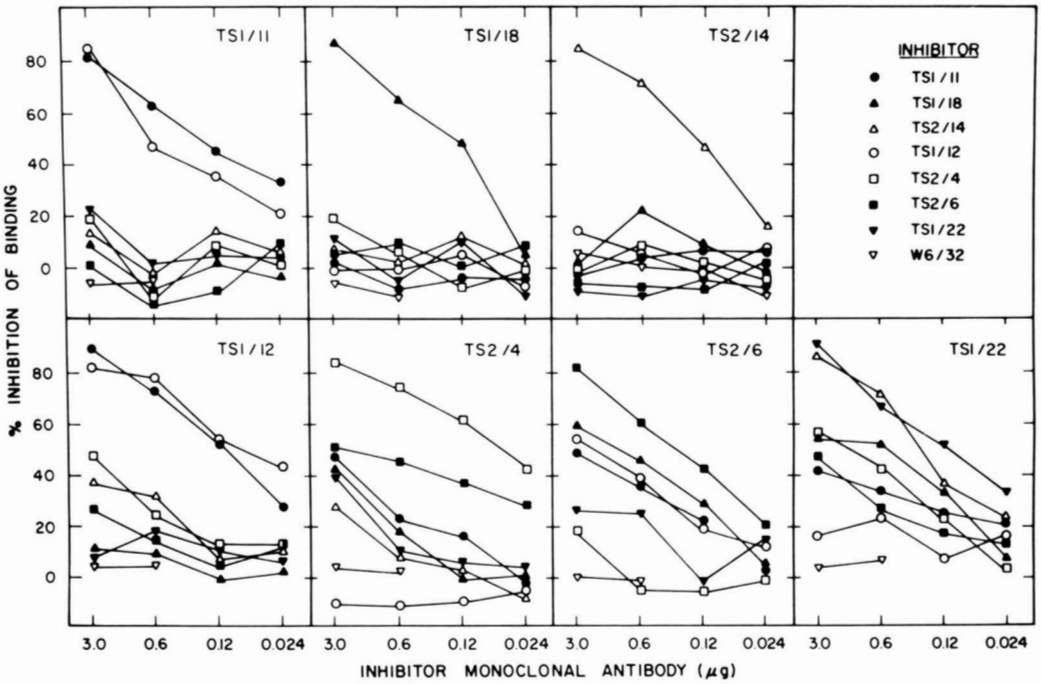

Figure 1. Competitive cross inhibition binding assay of mouse anti-human LFA-1 MAb. Biosynthetically labeled ([^{3}H]leucine) monoclonal antibody culture supernatants from the anti-human LFA-1 series (*upper right corner of each panel*) were tested for binding to 2×10^5 human PBL pretreated with the medium alone, W6/32 MAb or the other anti-LFA-1 MAb (as culture supernatants). The percentage of inhibition of binding is relative to the MAb bound in the presence of medium alone. For each MAb, 1000–2000 cpm were bound. Data represent mean of duplicate wells; SD was less than 4–5%.

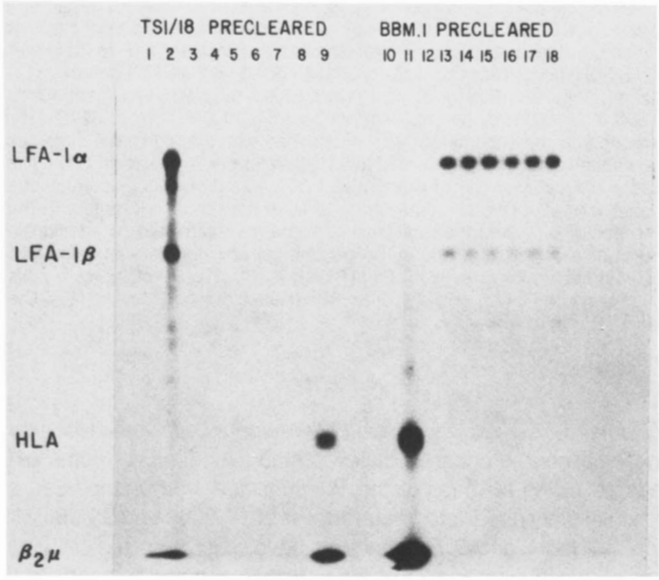

Figure 2. The epitopes defined by the anti-LFA-1 MAb reside on the same molecule. The TS1/18-Sepharose CL-4B anti-LFA-1 and BBM.1-Sepharose CL-4B anti-β_2 microglobulin beads were used to preclear an ^{125}I-labeled NP-40 extract of CTL-11 as described in the *Results* section. The precleared extracts were immunoprecipitated and analyzed by SDS-PAGE (10% acrylamide) and autoradiography. *Lane 1*, second cycle preclear with TS1/18-4B; *lane 2*, first cycle preclear with TS1/18-4B; *lanes 3–9*, immunoprecipitates of TS1/18-4B precleared extract with TS1/18, TS1/11, TS1/22, TS2/14, TS2/4, TS2/6, BBM.1, respectively; *lanes 10* and *11*, second and first cycle preclears with BBM.1-4B, respectively; *lanes 12–18*, immunoprecipitates of BBM.1 precleared extract with 2A2 MAb (for control), TS1/18, TS1/11, TS1/22, TS2/14, TS2/4, TS2/6.

resided on the same LFA-1 molecule, the TS1/18 MAb was isolated in purified form and linked to Sepharose CL4B (see *Materials and Methods*). Radioiodinated ^{125}I detergent extract of the human CTL-11 line was immunoprecipitated with the TS1/18-CL4B to remove all reactive antigens (preclearing). As a control, a parallel extract was precleared with BBM.1 MAb (anti-β_2-microglobulin) coupled to Sepharose-CL4B. This procedure was repeated to ensure complete removal of the antigens from the extracts. The other anti-LFA-1 and BBM.1 MAb were then used to immunoprecipitate their antigens from the precleared extracts before analysis of the immunoprecipitates by SDS-PAGE and autoradiography (Fig. 2). The TS1/18-4BCl quantitatively removed all of the LFA-1 from the extract (Fig. 2, *lanes 1* and *2*) as well as antigens that were reactive with all the other anti-LFA-1 MAb (*lanes 3–8*) without removing BBM.1 antigen (*lane 9*). Similarly the BBM.1-4BCL quantitatively removed all β_2-microglobulin (as HLA) without affecting the ability of anti-LFA-1 MAb to immunoprecipitate LFA-1. Thus, the unique epitopes defined by this collection of anti-LFA-1 MAb reside on the same molecule as that bound by the TS1/18 MAb.

Several rat MAb reactive with mouse LFA-1 (M17/4, M7/14, M17/7, and M17/5) were investigated for the topographic distribution of their epitopes on the mouse LFA-1 molecule (Fig. 3). The results indicated that M17/4, M17/5, M17/4 and M17/7 were efficient reciprocal competitive inhibitors of each other and thus define a common epitope. The M15/15 MAb was a partial competitive inhibitor of M17/4 and failed to compete with the others indicating M15/5 defined a partially shared but distinct epitope. Thus, of the rat MAb currently available, two partially related epitopes were recognized on the LFA-1 molecule.

Quantitation of cell surface LFA-1. The TS1/18 anti-human LFA-1 MAb was purified, iodinated, and used as a probe to determine the number of antigen binding sites per cell on a functional CTL line using a direct saturation binding assay. ^{125}I-TS1/18 and ^{125}I-W6/32 binding to CTL-11 was saturable and highly specific (Fig. 4). In six separate experiments human CTL-11 expressed 0.7 to 1.6×10^5 LFA/sites/cell (mean $1.03 \times 10^5 \pm 3 \times 10^4$). Long term cultures (≥6 weeks) of CTL-11 expressed higher levels of LFA-1 than CTL-11 cultured for only 1–2 weeks. HLA-A, B, C antigenic sites were expressed at a 10-fold higher level than LFA-1 on the same cells ranging from 5.2 to 17×10^5 sites/cell (mean $9.8 \times 10^5 \pm 3.8 \times 10^5$).

Quantitative expression of LFA-1 and HLA-A, B, C on several

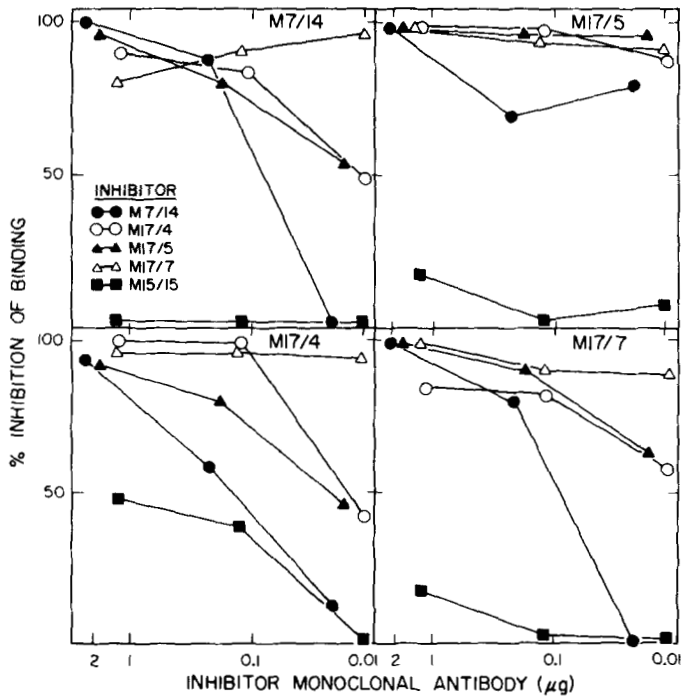

Figure 3. Competitive cross inhibition binding assay of rat anti-mouse LFA-1 MAb. Experiments were carried out on mouse Con A stimulated splenic lymphocytes as described in *Materials and Methods*. No inhibition and 100% inhibition were determined with nonactive M1/69HK culture supernatant and the homologous unlabeled MAb, respectively.

human cell lines and peripheral blood lymphocytes (PBL) (Table I) showed a relatively constant ratio of HLA to LFA-1 of almost 10, with the exception of the Epstein-Barr virus transformed JY B lymphoblastoid line which displayed 10-fold enhanced expression of HLA-A, B, C antigens (46). Analysis of CTL-11 by fluorescence activated flow cytometry (43, 44) (*Materials and Methods*) indicated LFA-1 was expressed on ≥98% of the cells. The T3, T8, and T4 antigens were expressed on 93%, 33%, and 70% of the cells, respectively.

Functional topography of the LFA-1 epitopes. The ability of an antibody to sterically inhibit the biological activity of a protein is thought to be related to the proximity or spatial organization of the antigenic epitope to the functional site(s) of the protein. This series of anti-LFA-1 MAb, although selected on the basis of their ability to inhibit human CTL-activity, showed considerable variation in blocking cytolysis. Titration of each anti-LFA-1 MAb on the HLA-B7 specific CTL-11 line (Fig. 5) revealed significant and reproducible differences in the inhibitory activity of these anti-LFA-1 MAb. The TS1/18 MAb showed the greatest level of inhibition with 50% maximum inhibition of lysis occurring at 0.5–0.7 μg/ml; TS1/22 and TS2/6 were moderate inhibitors requiring 1–2 μg/ml to achieve 50% maximum inhibition; TS1/11 and TS2/14 inhibited lysis at high concentrations (≥10 μg/ml) of MAb. In contrast TS2/4 showed no inhibitory activity (less than 10%) in this particular assay. An identical pattern of reactivity with these same MAb has been observed with $T4^+$ HLA-DR-specific human CTL (8). This indicated the variability in the degree of inhibition was not due to the antigen specificity of the CTL effectors. From the quantitative binding analyses presented earlier (Fig. 4) all antibodies were in considerable excess (10- to 50-fold) required for saturation of LFA-1 sites. This result suggested that the antigenic epitopes defined by these anti-LFA-1 MAb may be associated with unique functional regions on the LFA-1 molecule.

A comparison of the CTL-inhibiting activities of the anti-LFA-1 MAb with their epitope reactivities indicated that a complex relationship between the antigenic epitopes and the functional region(s) on the LFA-1 molecule (Table II). The TS1/18 MAb, which defined a unique epitope, showed the strongest inhibition of CTL activity. A moderate CTL-inhibiting MAb, TS2/6, defined an antigenic epitope partially related to a strongly (TS1/18) and a weakly (TS1/11) CTL-inhibiting MAb. In contrast, TS2/4, a non-CTL-inhibitory MAb, shares partial reactivity with antigenic epitopes exhibiting strong (TS1/18), moderate (TS1/22 and TS2/6), and weak (TS1/11) associations with CTL activity. The TS2/14 MAb, which defines a unique epitope, but also non-reciprocally cross inhibited binding of TS1/22, inhibited cytolysis to essentially the same degree as TS2/6 and TS1/22.

The rat anti-mouse LFA-1 MAb (M7 and M17 series) which recognize a common epitope, have previously been shown to inhibit mouse CTL function by 80–100%. In contrast, the anti-mouse LFA-1 MAb, M15/15, which recognized a partially related or different epitope, inhibited CTL killing by 50% (6, 31).

Anti-LFA-1 reversibly inhibits cytolysis. A moderate fluctuation in the inhibitory activity of TS1/18 was observed in several experiments. Since the variation may have been linked to the length of the CTL assay the effects of TS1/18 on the kinetics of CTL-11 mediated lysis of MST target cells was studied. CTL-11 was mixed in 100 μl medium containing 3 μg TS1/18.1.1 or 2A2 control MAb. ^{51}Cr-labeled MST target cells were added to the mixture and the plates were centrifuged to start the reaction and were incubated at 37°C for various lengths of time. The results indicated TS1/18 MAb-treated cells showed a significant delay (1.5–2 hr) in the initial onset of target cytolysis with a subsequent increase in the rate of cytolysis to 10% lysis/hr (through the first 4 hr), approaching the rate observed in the untreated control

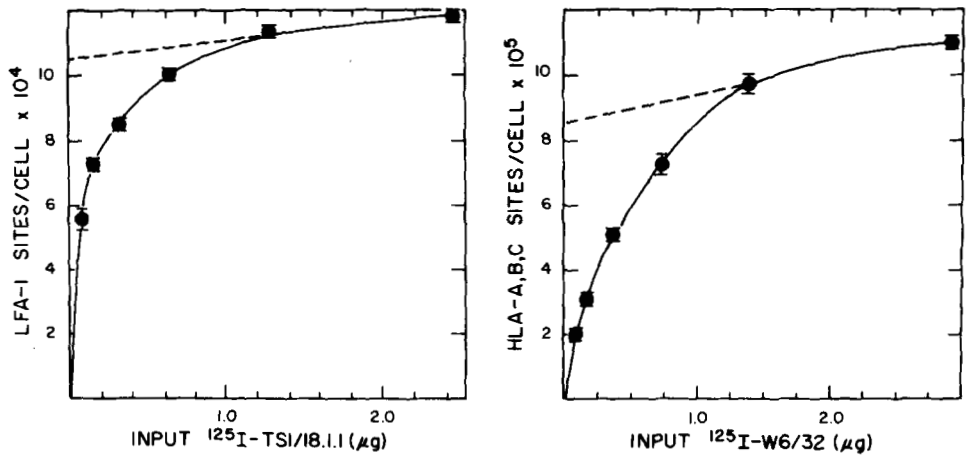

Figure 4. Quantitative saturation binding assay of anti-human LFA-1 and Anti-HLA MAb on the human CTL-11 line. Saturation binding assay was performed as described in *Materials and Methods* section. The specific activity of [^{125}I]TS1/18.1.1 and [^{125}I]W6/32 was 1.01 × 10^6 cpm/μg and 1.65 × 10^6 cpm/μg, respectively. The CTL-11 line was 95% viable and in week 4 of growth. For TS1/18, 2.0 × 10^5 cells were used and 5 × 10^4 for W6/32. Data represent mean ±SD of triplicate samples.

TABLE I

Quantitation of LFA-1 and HLA-A,B,C sites on various cell types[a]

Cell[b]	LFA-1 cpm Bound at saturation × 10^{-3}[c]	LFA-1 Sites/cell × 10^{-5}	HLA-A,B,C cpm Bound at saturation × 10^{-3}[c]	HLA-A,B,C Sites/cell × 10^{-5}	Ratio HLA/LFA-1
CTL-11	9.35 ± 0.05	0.88	33.0 ± 01.9	9.5	10.6
PBL	3.64 ± 0.28	0.34	7.2 ± 02.0	2.1	6.2
JY	4.45 ± 0.08	0.42	164.0 ± 12.0	47.0	112
CEM	2.88 ± 0.28	0.27	12.8 ± 00.4	3.6	13

[a] Direct saturation binding was determined as described in *Materials and Methods* by titration of ^{125}I-TS1/18.1.1 (anti-LFA-1, specific activity 2.1 × 10^6 cpm/μg) or ^{125}I-W6/32 (anti-HLA, specific activity 2.8 × 10^6 cpm/μg) on 2 × 10^5 or 5 × 10^4 cells, respectively.

[b] Cell types: CTL-11 (Week 3 of growth), Anti-HLA-B7 TCGF-dependent human CTL line. PBL, peripheral blood lymphocytes, are the original source of CTL-11 and were stored in 90% calf serum/10% dimethylsulfoxide in liquid N_2. Nonviable cells were removed before the binding study by Ficoll-hypaque density centrifugation. JY, Human Epstein-Barr virus transformed B lymphoblastoid cell line. CEM, human T lymphoblastoid cell line.

[c] Data represent mean ± SD of triplicate samples.

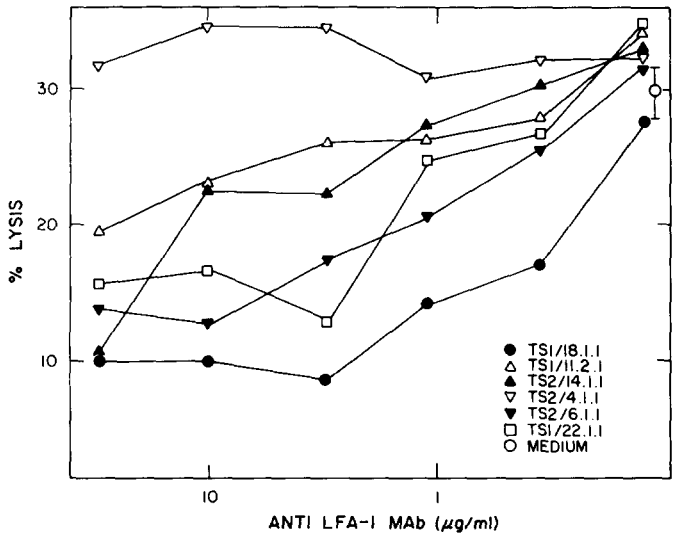

Figure 5. Anti-human LFA-1 monoclonal antibody inhibition of a HLA-B7 specific human CTL line. CTL-11 cells were preincubated in various concentrations of MAb for 20 min at 37°C before addition of ^{51}Cr-labeled MST target cells (E:T 10:1). The mixtures were incubated at 37°C for 4 hr. The percentage of cytolysis was determined as described in *Materials and Methods* section. Spontaneous release was 11%.

TABLE II

Comparison of CTL-inhibiting activity and antigenic epitopes of anti-human LFA-1 monoclonal antibodies

Anti-LFA-1 Monoclonal Antibody	% Inhibition[a] of Cytolysis	Epitope[b] Reactivity
TS1/18	51 ± 14	unique
TS1/22	40 ± 11	shared (14,4,18,6,11)
TS2/6	35 ± 10	shared (18,11)
TS2/14	29 ± 3	unique
TS1/11	12 ± 5	unique
TS2/4	6 ± 6	shared (6,11,18,22)

[a] The percentage of inhibition of cytolysis was calculated as a ratio of the % lysis in the presence of 3 μg/ml of the indicaed MAb to the % lysis in medium alone during a 4-hr ^{51}Cr-release assay. The data represent the mean ± SD of several different experiments using CTL-11 line on JY or MST target cells at 10:1 to 20:1 effector: target ratio. n = 14 for TS1/18; n = 2 to 4 experiments for the other LFA-1 MAb.

[b] The epitope reactivity as defined from data in Figure 1. A unique epitope showed inhibition only by homologous MAb. A shared epitope showed competitive inhibition by other anti-LFA-1 MAb. Numbers in parenthesis indicate the abbreviation for the anti-LFA-1 MAb showing complete (underlined) or partial reactivity with the indicated MAb.

reaction (12% lysis/hr) (Fig. 6). As a consequence, the degree of inhibition mediated by the TS1/18 MAb decreased from 73% inhibition measured at 1 hr to 21% measured at 8 hr incubation. The asymptote in the lytic curve for both populations at 8 hr probably represents two factors: 1) Incomplete release of the

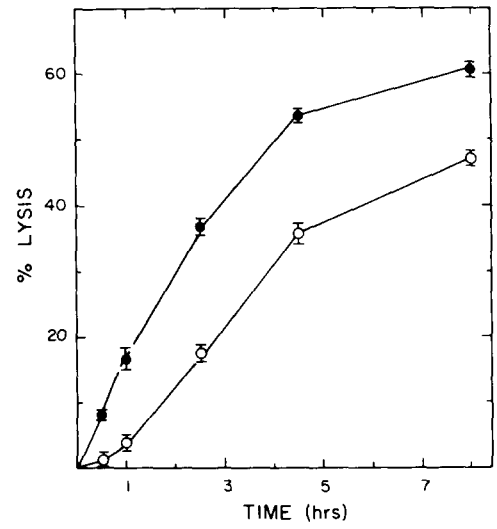

Figure 6. The effect of TS1/18 MAb on the kinetics of lysis of MST by CTL-11. The CTL-11 (10^4 cells) were incubated in 100 μl medium (●) or medium containing 3 μg of TS1/18 (○) MAb in microtiter wells for 20 min at 37°C. ^{51}Cr-labeled MST target cells (10^3) were then added; the plate was centrifuged at 500 × G for 2 min to initiate contact and incubated at 37°C. At the indicated time points the plate was centrifuged at 500 × G for 1 min and a row of wells was harvested as described in *Materials and Methods*. The plate was then returned to the incubator; total time out of the incubator was 3 min. The spontaneous release at the indicated time points was 2.5%, 3%, 4%, 11%, and 29%. The data represent mean of triplicate wells ± SD and is representative of four experiments.

^{51}Cr label from lysed target cells; and 2) increased spontaneous release of label from target cells. These factors may have underestimated the actual degree of lysed target cells.

DISCUSSION

The studies presented here have investigated the immunochemical properties of the hyman LFA-1 molecule, quantitated LFA-1 expression, and described the topographic relationship of the antigenic epitopes and the functional site(s) of human LFA-1 in comparison with the epitope topography of mouse LFA-1. Three unique and three complex epitopes on the human LFA-1 molecule (summarized in Table II) were identified with Balb/c-derived IgG_1 monoclonal antibodies by using a competitive cross inhibition binding assay (Fig. 1). Immunoprecipitation preclearing studies indicated the epitopes resided on the same LFA-1 molecule (Fig. 2). Several rat anti-mouse LFA-1 MAb similarly tested showed significant competitive cross-inhibition, and thus by this criteria, identify a common determinant and one partially shared epitope on mouse LFA-1 (Fig. 3). These rat anti-mouse LFA-1 MAb were derived from several fusions representing an extensive search for CTL inhibiting MAb (6). In addition, several other rat anti-LFA-1 MAb derived in other laboratories (25, 26) were also competitive inhibitors of the M7/14 MAb [T. A. Springer unpublished observations, (47)]. These results indicate that cytolysis-inhibiting rat MAb recognize a very limited number of epitopes on mouse LFA-1. The antigenic diversity observed with human LFA-1 as defined by mouse MAb most likely reflects the species diversity between the immunized host and the eliciting antigen. This suggests that xenogenic immunization with human cells will be useful in obtaining MAb which recognize highly conserved molecules involved in CTL function. This suggestion finds additional support by the discoveries of the T3, LFA-2 and LFA-3 antigens in man; their equivalents, as of yet, have not been found in the mouse even though extensive studies have been conducted (5, 6).

Quantitation of LFA-1 sites on various cell types revealed that a cytolytically active human CTL line contained 2 to 4 times more

LFA-1 than normal PBL or T and B lymphoblastoid cell lines (Table I). HLA-A, B, C antigen expression was 20-fold higher on a B lymphoblastoid cell than on PBL, while these cells expressed similar levels of LFA-1. In the mouse, T cells were found to express higher levels than B cells, and activated mouse T lymphocytes (primary MLC-sensitized CTL and Concanavalin A blasts) were found to express significantly higher levels of LFA-1 than normal splenocytes (48). This may be analogous to the higher level of LFA-1 expression observed on human CTL-11 line compared to PBL. Collectively, these data indicate that LFA-1 expression is quantitatively increased on functionally active lymphocyte populations and suggest that higher LFA-1 expression may be related to a gain in T cell effector function.

The studies presented here provide several important results germane to the mechanism of anti-LFA-1 MAb inhibition of human CTL-mediated cytotoxicity. The identification of several unique epitopes on human LFA-1 provides, in part, an explanation for the variability in the inhibitory activity of the TS1 and TS2 anti-human LFA-1 MAb. All of these MAb are of the $\gamma 1,\kappa$ chain subclass and thus antibody subclass differences would not account for the differential blocking activities. Differences in individual MAb affinity constants may have contributed to this variability although this seems unlikely since the concentrations of MAb used in the inhibition studies were in considerable excess of saturation (100 ng TS1/18 is 50% saturating for 2.5×10^5 cells; 1–3 μg/ml was required to maximally inhibit 10^4 CTL cells) and the anti-LFA-1 MAb are of sufficiently high avidity to immunoprecipitate LFA-1.

The ability of anti-LFA-1 MAb to inhibit CTL function cannot be attributed to trivial means but appears to be through a specific interaction with the effector cell surface LFA-1 molecules. Anti-LFA-1 MAb did not agglutinate nor was it directly cytotoxic to CTL as evidenced after staining of treated cells with the vital dye trypan blue (C. Ware, unpublished) and the effects of anti-LFA-1 were largely reversible within a short time (Fig. 6). Although the B lymphoblastoid target cells express LFA-1, studies to be presented elsewhere (Krensky *et al.*, manuscript in preparation) have shown that pretreatment of the CTL population with anti-LFA-1 MAb will block CTL function, i.e., precoating of the target cell was not sufficient to block lysis. The relative inability of the TS2/4 MAb to inhibit CTL function provides an excellent specificity control for blocking effects of the other anti-LFA-1 MAb. Of interest to note, the TS2/4 MAb should prove to be an excellent reagent for cytochemical localization of LFA-1 during CTL-target cell interactions without perturbation of LFA-1 biological activity.

The high density of HLA relative to LFA-1 (10:1) on the CTL surface coupled with the fact that anti-HLA antibodies such as W6/32 when bound to the effector CTL do not block the cytolytic reaction, even though they do when bound to the target cell surface, strongly suggests that the mechanism of anti-LFA-1 MAb blockade was not through a nonspecific "blanketing" effect of antibody on the CTL surface. A summation of these results in conjunction with similar studies on the effect of MAb to LFA-1 on mouse CTL function lead us to conclude that anti-LFA-1 MAb blockade of human CTL function was by the inactivation of a functionally relevant molecule; it is not yet clear whether anti-LFA-1 MAb modulate (by capping or shedding) LFA-1, or inhibit by direct steric hindrance of a functional site(s). Studies with antibody Fab fragments should resolve this issue.

The functional role of LFA-1 in human CTL killing reaction has not yet been determined, however, the similarities in cellular distribution and structural properties of mouse and human LFA-1 would suggest they may have similar functions. Studies on mouse LFA-1 have implicated its role in the initial Mg^{++} dependent recognition-adhesion step (5, 6, 25). LFA-1 may function as a general cell-cell interaction molecule contributing to the overall avidity of the T cell antigen receptor promoting firm contact (adhesion) between the CTL and target cell membranes (6). In light of this model LFA-1 may be less essential to establish efficient contact in CTL populations with high avidity antigen receptors. By increasing the reaction time after treatment of CTL with anti-LFA-1 MAb (as in Fig. 6) sufficient low avidity contacts could have occurred to promote target cell lysis, thus reversing the effect of anti-LFA-1 MAb on CTL activity.

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