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J Clin Invest. 1986;78(5):1387-1391. <https://doi.org/10.1172/JCI112725>.

Research Article

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Abnormal Cytolytic Activity of Lymphocyte Function-associated Antigen-1-deficient Human Cytolytic T Lymphocyte Clones

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Abstract

The involvement of the lymphocyte function-associated antigen-1 (LFA-1) membrane molecule in cytolytic T lymphocyte (CTL) interactions with lymphoid target cells was investigated using CTL clones derived from two patients with a heritable deficiency of LFA-1. LFA-1 surface expression on the CTL clones was 1% of the normal level of LFA-1, unchanged with prolonged culture, and identical on 14 different CTL clones. The function of the LFA-1 molecule was addressed using the LFA-1-deficient CTL clones and LFA-1-deficient lymphoid target cells. The lytic activity of the LFA-1-deficient CTL clones was 43% of control when tested against a target cell line expressing normal levels of LFA-1 and <10% of control when tested against an LFA-1-deficient target cell line. These results demonstrate a direct involvement of LFA-1 in CTL-mediated cytolysis and suggest a more general dependence on LFA-1 in lymphoid cell-cell interactions.

Introduction

Human lymphocyte function-associated antigen-1 (LFA-1)¹ is a 177- and 95-kD glycoprotein heterodimer that is expressed on all leukocytes, most thymocytes, and on a third of bone marrow cells (1). The LFA-1 molecule was initially identified by screening monoclonal antibodies (MAb) for their ability to inhibit cytolytic T lymphocyte (CTL)-mediated cytotoxicity in the absence of complement (1, 2). MAb to LFA-1 significantly inhibited not only cell-mediated cytotoxicity but T cell proliferation as well (1). In contrast, MAb to a variety of other cell surface molecules, often present at higher cell surface density than LFA-1, did not inhibit T cell function. The ability of anti-LFA-1 MAb to inhibit a variety of T cell functions led to the suggestion that LFA-1 was an essential cell-cell interaction molecule.

A unique opportunity to assess the function of the LFA-1 molecule has been provided by patients with a genetic deficiency of the LFA-1 glycoprotein. The LFA-1 deficiency syndrome is an immunodeficiency characterized by a susceptibility to re-

current life-threatening infections (3). Biochemical analysis of the leukocytes from LFA-1-deficient patients have shown a deficiency of not only LFA-1, but of a structurally related family of molecules (4). LFA-1, complement receptor type 3 (CR3), and p150,95 are heterodimers with different heavy chains but a structurally identical 95-kD light chain. All three molecules are deficient in patients with the clinical syndrome (5). The advantage of studying CTL from these deficient patients is that significant levels of CR3 and p150,95 are not expressed on normal T cells. Thus, the CTL from these patients have a selective deficiency of the LFA-1 membrane molecule.

Previous *in vitro* studies of LFA-1-deficient CTL function have been inconclusive. Arnaout et al. (6) and Miedema et al. (7) have found no significant defect in CTL function. Krensky et al. (8), using HLA-typed related and unrelated controls, demonstrated abnormalities in both cell-mediated cytotoxicity and T cell proliferation. Similarly, Fischer et al. (9) have found severely depressed cell-mediated immune function. The inconsistencies observed *in vitro* may be explained by the use of polyclonal T cell responder populations to assess CTL function. Bulk cultures, even from the same individual, can demonstrate significant differences in cytolytic activity (10). Therefore, a comparison of clones directed at the same alloantigen should provide a more definitive evaluation of cytolytic activity.

In this report, we used the genetic deficiency of LFA-1 to investigate the role of LFA-1 in CTL-target cell interactions. The deficiency of LFA-1 on CTL clones was associated with only a 43% decrease in the lysis of a lymphoid target cell due to the ability of these CTL clones to utilize target cell LFA-1. When LFA-1 was deficient on both the CTL and the target cell, lytic activity was decreased by >90%. These results indicate a direct involvement of LFA-1 in CTL-mediated cytolysis, and suggest possible mechanisms for CTL compensation in the LFA-1 deficiency syndrome.

Methods

Peripheral blood lymphocytes. Peripheral blood lymphocytes were obtained from LFA-1-deficient patient 6 and his son (patient 8). Control lymphocytes were obtained from a healthy donor (Co1). Lymphocytes obtained from Co1 demonstrated a normal response *in vitro* based on a variety of functional and phenotypic assays (10). HLA typing was performed by the Immunology Research Laboratory at Baylor University and the Histocompatibility Laboratory at the Dana-Farber Cancer Institute: patient 6 (HLA-A1, B8, B14, DR3, DR7), patient 8 (HLA-A1, A32, B8, Bw61, DR3, DRw6), and Co1 (HLA-A11, A32, B27, Bw51, DR7).

Culture medium. Cells were grown in culture medium consisting of RPMI 1640 (M. A. Bioproducts, Walkersville, MD) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 10 mM Hepes (Sigma Chemical Co., St. Louis, MO), and 25 µM 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, NY). Long-term CTL cultures and CTL clones were maintained in 10% human conditioned medium.

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Received for publication 8 May 1986.

1. *Abbreviations used in this paper:* CR3, complement receptor type 3; CTL, cytolytic T lymphocyte; EBV, Epstein-Barr virus; F-GAM, fluorescein-conjugated F(ab')₂ goat anti-mouse antibody; IL-2, interleukin 2; LFA-1, lymphocyte function-associated antigen-1; LU, lytic units; MAb, monoclonal antibodies.

J. Clin. Invest.

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0021-9738/86/11/1387/05 \$1.00

Volume 78, November 1986, 1387-1391

Interleukin 2 (IL-2) containing human conditioned medium. The method for IL-2 production has been described elsewhere (10). Briefly, peripheral blood leukocytes obtained from five different platelet donors were mixed and washed three times. The cells were resuspended at a concentration of 3×10^6 cells/ml in RPMI 1640 (M. A. Bioproducts) containing 3% fetal calf serum and 0.15% phytohemagglutinin (Bacto PHA-P; Difco Laboratories, Detroit, MI). Special additives included 2.8 μ M indomethacin, 3 mM lithium chloride, and 50 μ M hydroxyurea, all from Sigma Chemical Co. The cells were cultured for 72 h and the supernatants were collected. The supernatants were ammonium sulfate-precipitated (50%, 75%) and dialyzed. The purified conditioned medium was filter sterilized and stored at -4°C .

Cytotoxicity assay. CTL assays were performed in triplicate in V-bottom microtiter wells (Linbro, Flow Laboratories, Hamden, CT). Effector cells were added in threefold dilutions. Target cells, preincubated in 0.1 mCi of ^{51}Cr ($\text{Na}^{51}\text{CrO}_4$, New England Nuclear, Boston, MA) for 2 h and washed three times, were added at 10^3 cells/well. Microtiter plates were centrifuged and incubated at 37°C for 4 h. After incubation, the plates were again centrifuged and the supernatants assayed for ^{51}Cr -release. Specific cytotoxicity was calculated as percent cytotoxicity = $100 \times (\text{cpm experimental release} - \text{cpm spontaneous release}) / (\text{total cpm} - \text{cpm spontaneous release})$. When blocking monoclonal antibodies were used, they were added to the wells at the start of the assay. The standard deviation of the triplicate wells rarely exceeded 2–4% of the specific lysis. Lytic units (LU) were calculated as the number of effector cells required for 50% lysis of 10^3 target cells. LU were based on effector titrations and confirmed with the following formula: $\text{LU}/10^3 = 10^3 / (\text{effector cells}/\text{target cells}) \times [(\text{plateau cpm} - \text{sample cpm}) / (\text{cpm sample})]$.

CTL clones. Peripheral blood mononuclear cells were separated on a Ficoll/Hypaque gradient (Lymphocyte Separation Medium, Bionetics, Kensington, MD). The mononuclear cells at 2×10^6 cells/ml were cocultured with irradiated JY at 1×10^5 cells/ml in 2-ml wells (Linbro) at 37°C in a 5% CO_2 incubator. The bulk culture was maintained in 9.6- cm^2 wells (Nunc, Vanguard, Neptune, NJ) by stimulation every 1–2 wk with irradiated stimulator cells. After 6 wk in culture, the cells were again isolated using a Ficoll-Hypaque gradient and cloned using limiting dilution in 96-well round-bottom microtiter plates (Linbro) with 2×10^4 irradiated JY cells as a feeder layer. Culture medium containing 10% human conditioned medium was used for cloning; fresh medium was added every 3 d. Clones were obtained at a concentration of 1 cell/10 wells. The clones chosen for further analysis were subcloned at <1 cell/well. The specificity of each clone was determined using a panel of HLA-typed target cells and monoclonal antibody blocking. Co1 clones 350, 349, and 354 were specific for HLA-A2; clones 147, 303, and 353 were specific for HLA-B7. The CTL line MMJ was specific for the cell line JY. These clones were shown to have comparable lytic activity in comparisons with clones obtained from two other normal donors.

Cell surface immunofluorescence. Cells were washed twice with phosphate-buffered saline containing 2.5% fetal calf serum and 0.02% sodium azide. Approximately 10^6 cells were incubated on ice for 30 min with an excess concentration of monoclonal antibody. The cells were washed twice and stained with fluorescein-conjugated goat F(ab')₂-anti-mouse (F-GAM) IgG antibody (Tago Inc., Burlingame, CA) diluted 1:10. The cells were incubated on ice for another 30 min. After three washes, the cells were fixed in 1% paraformaldehyde and analyzed on a FACS I (Becton-Dickinson & Co., Mountain View, CA).

Monoclonal antibodies. MAb to LFA-1 were derived from subcloned hybridomas that produced antibodies to LFA-1 as previously described (2). MAb to the LFA-1 alpha chain were TS1/22, TS2/14, TS1/11, and TS2/4. MAb to the LFA-1 beta chain was TS1/18. Titration curves were used to establish saturating concentrations of MAb. The HLA-A2-specific MAb PA2.1 was described elsewhere (11).

Tumor cell lines. The human tumor cell lines were maintained in culture media and regularly passaged. All lines were typed by the HLA tissue-typing laboratory, Dana-Farber Cancer Institute: JY (HLA-A2, B7, DR4, DR6), Priess (HLA-A2, B15, DR4), 23.1 (HLA-A2, B27, DR8), AS (HLA-A2, A24, B27, DR4), PGF (HLA-A3, B7, DR2), Thal B (HLA-A3, A10, B7, B51, DR2, DR5), Daudi (HLA-A, —, DR6), and BBN (HLA-A2, A32, B35, B51, DR3, DR4).

Results

LFA-1 surface expression. Long-term T cell lines from patients 6 and 8 of the Texas series (8) as well as parallel control lines (Co1) were maintained for >6 mo in culture. Immunofluorescence analysis of the LFA-1 alpha and beta subunits demonstrated that the T lymphocytes of moderately deficient patients' 6 and 8 (3) expressed $\sim 1\%$ of the normal amount of cell surface LFA-1. Serial immunofluorescence of the T cell lines revealed no change in the level of LFA-1 expression after 6 mo of in vitro culture. The expression of LFA-1 also did not change after treatment with IL-2 or interferon-gamma (not shown). To identify any clonal heterogeneity in the expression of LFA-1, 14 CTL clones were investigated using a similar immunofluorescence analysis. These clones were derived from six distinct bulk cultures from both patient 6 and patient 8. The 14 clones demonstrated an identical level of LFA-1 expression. Staining of a representative clone is shown in Fig. 1. Surface expression of LFA-1 on the CTL clones was also the same level as on the bulk CTL population. Thus, no heterogeneity of LFA-1 surface expression was observed on the LFA-1-deficient CTL.

Lytic activity of LFA-1-deficient CTL clones. The genetic deficiency of LFA-1 permitted the assessment of CTL interactions with lymphoid target cells when LFA-1 was (a) deficient on the CTL, (b) deficient on the target cell, or (c) deficient on both the CTL and target cell. In normal circumstances, LFA-1 is present on all lymphocytes. Although LFA-1 is expressed on both CTL and lymphoid target cells, the CTL-target cell interaction appears to depend primarily on the LFA-1 expressed on the CTL. MAb pretreatment of the effector cell maximally inhibits cytolysis, whereas MAb pretreatment of the target cell has minimal effect (1). Despite the significant deficiency of LFA-1, detailed analysis of six HLA-A2-specific CTL clones showed that the LFA-1-deficient clones were capable of effectively lysing a variety of lymphoid target cells (Table I). Based on 26 separate experiments, the lytic activity of the LFA-1-deficient CTL clones (as quantified by lytic units) was 43% of control CTL clones (Table II).

The effective lysis of lymphoid target cells suggested that LFA-1-deficient CTL could compensate for their deficiency. The molecular basis for this compensation was unclear. The LFA-1-deficient CTL clones did not express an increased number of antigen nonspecific or "accessory" molecules that have been shown to participate in the CTL-target cell interaction. The

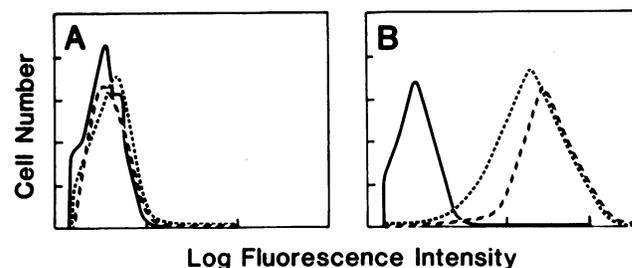


Figure 1. Surface expression of LFA-1 alpha and beta chains on patient 6 (clone F4) (A) and Co1 (clone 350) (B) CTL clones. LFA-1 alpha chain was stained with a mixture of anti-alpha chain MAb (Ts1/22, Ts1/11) and F-GAM antibody (----). The LFA-1 beta chain was stained with TS1/18 and F-GAM antibody (-----). Negative controls were stained with F-GAM antibody alone (—). The anti-LFA-1 MAb were used at a 1:200 dilution of hybridoma ascites.

Table I. Specificity of LFA-1-deficient Clones

Clone‡	% Specific lysis*						
	Priess (A2)	23.1 (A2)	AS (A2)	JY (A2, B7)	PGF (B7)	Thal B (B7)	Daudi (DR6)
F4	64	46	69	84	13	4	4
49	48	56	51	68	0	0	0
50	48	41	54	64	0	0	0
51	71	60	66	85	0	0	0
52	47	63	55	80	4	1	0
53	44	49	58	66	5	3	0

* Percent specific ⁵¹Cr release of the indicated target cell lines in a 4-h assay. Complete HLA types are listed in Methods. Relevant HLA types are noted in parentheses.

‡ Clone F4 is derived from patient 6; clones 49–53 are from patient 8. Effector/target ratios ranged from 6:1 to 9:1.

surface expression of antigen nonspecific molecules such as CD3, CD2, and CD8 was comparable on LFA-1-deficient and control CTL clones (data not shown). Another possibility suggested by MAb pretreatment experiments (8) was that LFA-1-deficient CTL clones were able to utilize LFA-1 expressed on the target cell. To investigate this possibility, B cells from patient 1 of the Texas series (8) were transformed by Epstein-Barr virus (EBV) to obtain a suitable target cell line. The B lymphoblastoid cell line derived from patient 1 (BBN) expressed ~2% of the LFA-1 and 91% of the HLA-A2 expressed on the EBV-transformed cell line JY (Table III). CTL clones specific for HLA-A2 were tested for lytic activity against JY and the LFA-1-deficient target cell BBN. Calculation of LU indicated that the lytic activity on the LFA-1-deficient target BBN by the LFA-1-deficient CTL clones was <10% of CTL clones expressing normal levels of LFA-1 (Table IV). This significant decrease in lytic activity was found with all LFA-1-deficient clones tested.

Utilization of the LFA-1 molecule. The diminished ability to lyse LFA-1-deficient target cells demonstrated that LFA-1-

Table II. Lytic Activity against the Target Cell JY*

Control		LFA-1-deficient	
Clone	LU‡	Clone	LU‡
350	363	F4	147
349	370	49	158
354	394	50	160
303	416	51	186
353	305	52	139
MMJ	<u>270</u>	53	<u>122</u>
	X = 353§		X = 152

* The target cell in all cases was the JY cell line. Control CTL were established from Co1. MMJ was a long-term CTL line. LFA-1-deficient CTL clones were obtained from patient 6 (F4) and patient 8 (49–53).

‡ LU represents the mean LU of at least three separate experiments. LU were calculated as described in Methods.

§ Lytic activity of control and LFA-1-deficient CTL clones were significantly different, *P* < 0.01, as assessed by the Wilcoxon rank sum test.

Table III. Target Cell Expression of LFA-1 and HLA-A2

Target cell	Mean relative fluorescence intensity*		
	Anti-alpha (TS1/22, TS1/11)	Anti-beta (TS1/18)	Anti-HLA-A2 (PA2.1)
BBN	2.13	2.09	158.14
JY	101.58	86.97	173.04

* The EBV-transformed target cell lines BBN and JY were stained with MAb. A pool of anti-alpha chain MAb, an anti-beta chain MAb, and an anti-HLA-A2 MAb were used at 1:200 dilution of hybridoma ascites and indirectly stained with F-GAM. Control values (F-GAM alone) were subtracted from the mean fluorescence intensity of all three samples.

deficient CTL clones, in contrast to normal CTL, utilized LFA-1 expressed on the target cell. The novel utilization of LFA-1 might also involve different functional epitopes on the LFA-1 molecule. To explore functional differences of target cell and CTL LFA-1, we used a panel of MAb to five different epitopes on the LFA-1 molecule. MAb cross-blocking experiments had previously defined different functional epitopes on the LFA-1 molecule (12). The MAb panel, composed of four anti-alpha chain MAb and one anti-beta chain MAb, was used to inhibit killing of the LFA-1⁺ target cell. Both LFA-1-deficient (F4) and control (147) CTL clones demonstrated an identical pattern of MAb inhibition (Table V).

Discussion

The genetic deficiency of the LFA-1 membrane glycoprotein has provided a unique opportunity to assess the direct involvement of this molecule in CTL function. Six HLA-A2-specific CTL clones from two patients with a deficiency of LFA-1 demonstrated abnormal cytolytic function. The cytolytic activity of these CTL clones was <50% of the lytic activity of control CTL clones. When LFA-1 expression was deficient on both the CTL and the target cell, lytic activity was <10% of control. These

Table IV. Lytic Activity against the LFA-1-deficient BBN Target Cell*

Control		LFA-1 deficient	
Clone	LU‡	Clone	LU‡
350	337	49	17
349	311	50	21
354	303	51	33
MMJ	<u>251</u>	52	<u>25</u>
	X = 301§		X = 24

* The target cell in all cases was the BBN cell line. BBN was an EBV-transformed cell line from patient 1. Control CTL clones were established from Co1. MMJ was a long-term CTL line. LFA-1-deficient CTL clones 49–52 were established from patient 8.

‡ LU represents the mean LU of at least three separate experiments. The variance of lytic activity between experiments was always <10%. § Lytic activity of control and LFA-1-deficient CTL were significantly different, *P* < 0.01, as assessed by the Wilcoxon rank sum test.

Table V. Inhibition of CTL-mediated Lysis by a Panel of Anti-LFA-1 MAb*

MAb‡	Subunit of LFA-1	% Specific lysis	
		Clone F4	Clone 147
Medium	—	47	64
TS1/18	Beta	23 (51)§	26 (59)
TS1/22	Alpha	26 (46)	25 (61)
TS2/14	Alpha	27 (43)	32 (50)
TS1/11	Alpha	40 (9)	55 (14)
TS2/4	Alpha	47 (0)	59 (8)

* Control CTL clone 147 and LFA-1-deficient CTL clone F4 were tested against the B lymphoblastoid target cell JY at an E:T ratio of 18:1 in a standard 4-h ⁵¹Cr-release assay.

‡ MAb were used at a 1:150 dilution of hybridoma ascites.

§ Numbers in parentheses indicate percent inhibition.

observations confirm the presence of abnormal CTL function in the LFA-1 deficiency syndrome. In addition, the LFA-1-deficient CTL clones and target cells studied here provide the clearest available evidence that the LFA-1 membrane molecule directly participates in CTL function, and suggest that LFA-1 may play a more general role in lymphoid cell-cell interactions.

The LFA-1 membrane molecule was initially identified as a function-associated molecule by the ability of anti-LFA-1 MAb to inhibit CTL-mediated lysis. Because LFA-1 is expressed on T cells as well as lymphoid target cells, MAb pretreatment was required to identify the relative contributions of effector and target cell LFA-1 to the killing interaction. MAb pretreatment demonstrated that anti-LFA-1 MAb inhibited cytolysis at the level of the effector cell (1). The dominant role of effector cell LFA-1 was confirmed using nonlymphoid target cells. Endothelial cells and fibroblasts do not express LFA-1. When used as target cells, endothelial cells and fibroblasts were effectively lysed by human CTL clones, and this lysis was inhibited by anti-LFA-1 MAb.

Previous experiments with LFA-1-deficient CTL and MAb blocking suggested a difference between normal and LFA-1-deficient CTL in the utilization of LFA-1 (8). To clarify this finding, we studied target cells genetically deficient in LFA-1. As expected, control CTL clones demonstrated near normal levels of lytic activity on LFA-1-deficient target cells. In contrast, LFA-1-deficient CTL clones were not able to effectively lyse the LFA-1-deficient target cell. These findings demonstrate that target cell LFA-1 can be utilized by the effector cell. The reason that target cell LFA-1 is not used in the normal CTL-target cell interaction is unclear. The relatively high density of LFA-1 on normal CTL may diminish the contribution of target cell LFA-1. The utilization of target cell LFA-1 may simply reflect the relative cell surface density of LFA-1. Alternatively, the ability of CTL to utilize target cell LFA-1 may require the enhanced expression of an as yet unidentified LFA-1 ligand. Regardless of the specific mechanism, these experiments have identified LFA-1 as an essential molecule in lymphoid cell-cell interactions.

The distinction between CTL and target cell LFA-1 suggests that the function of the LFA-1 molecule may depend on whether it is involved at the level of the CTL or the target cell. To explore the functional utilization of the LFA-1 molecule, we used a panel of anti-LFA-1 MAb (12, 13). An identical hierarchy of MAb inhibition of cytolysis was observed for both patient and control

CTL clones. These MAb blocking experiments suggest that the LFA-1 molecule retains the same functional epitopes whether it is utilized at the level of the effector or target cell. The comparable utilization of LFA-1 epitopes suggests that LFA-1 mediates a common function for both LFA-1-deficient and control CTL clones.

The ability of anti-LFA-1 MAb to inhibit CTL-target cell conjugates (14) suggested that LFA-1 may regulate cell-cell adhesion. Studies of B cell-B cell (15, 16), T cell-endothelial cell (17), T cell-T cell, myeloid cell-myeloid cell (18, 19), and T cell-fibroblast (20) adhesions have supported this interpretation. The decreased ability of LFA-1-deficient CTL to lyse an appropriate target cell may reflect a diminished adhesion between CTL and target cell. The development of sensitive biophysical measures of CTL adhesive strength will be an important contribution to the assessment of LFA-1 function.

The clinical problems in the LFA-1 deficiency syndrome primarily reflect the patients' susceptibility to bacterial infections. The syndrome is characterized by poor wound healing, severe periodontal disease, and recurrent soft tissue infections (6, 7, 9). These observations, as well as normal serum immunoglobulin levels and relatively normal delayed hypersensitivity reactions, have led to the speculation that granulocyte function is more impaired than T cell function. Only two reported LFA-1-deficient patients have developed significant infectious complications attributable to a cell-mediated immune defect. One patient died of overwhelming picornavirus infection of the tracheobronchial tree (3). The other patient developed cutaneous candidiasis (9).

The failure of most LFA-1-deficient patients to manifest a clinically significant T cell defect suggests that T cells compensate for their deficiency. There are a number of potential mechanisms of CTL compensation. The most important mechanism is that T cells, in contrast to granulocytes, respond to antigenic challenge by clonal expansion. Studies of polyclonal populations of LFA-1-deficient CTL suggest that antigenic challenge expands those T cells with the greatest capacity to proliferate and exhibit cytolytic activity. The molecular basis of this selection is unclear, but may involve an increase in the number and/or the affinity of other, as yet unidentified, cell surface molecules. An unexpected compensatory mechanism demonstrated in this study appears to be the ability of LFA-1-deficient CTL to utilize LFA-1 expressed on the target cell. Together, clonal selection and the novel utilization of cell surface molecules appear to be complementary mechanisms by which human T cells may compensate for significant genetic deficiencies. Further work will be necessary to elucidate the functional relationship of LFA-1 to other T cell surface molecules and to further define the role of LFA-1 in the CTL-target cell interaction.

Acknowledgments

The authors would like to thank M. A. V. Crimmins and M. J. Dunn for excellent technical assistance and Carolyn Gregory for the preparation of the manuscript.

This work was supported by National Institutes of Health grants CA-09280 and CA-34129.

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