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THE FUNCTION OF LFA-1 IN CELL-MEDIATED KILLING AND ADHESION: STUDIES ON HERITABLE LFA-1, Mac-1 DEFICIENCY AND ON LYMPHOID CELL SELF-AGGREGATION

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INTRODUCTION

Lymphocyte function associated antigen-1 (LFA-1) is a cell surface glycoprotein identified in mouse and human by monoclonal antibodies which inhibit cytolytic T lymphocyte (CTL) mediated cytolysis (1-5). LFA-1 contains noncovalently associated α and β subunits of M_r = 180,000 and 95,000, respectively. Anti-LFA-1 monoclonal antibodies (MAb) inhibit cytolysis by CTL and NK cells as well as proliferative responses to mitogens, alloantigens, and soluble antigens. LFA-1 is broadly distributed on leukocytes, including lymphocytes, granulocytes, and monocytes, but is not expressed on a number of other somatic cells. Anti-LFA-1 monoclonal antibodies inhibit conjugate formation between CTL and targets (1,6,7); therefore, LFA-1 may be instrumental in cell-cell adhesion.

Recently, a number of patients with recurring, life-threatening infections were found to be deficient in LFA-1 and two other surface molecules which utilize the same β subunit, Mac-1 and p150,95 (8-11). This experiment of nature offered another means of testing the functional importance of LFA-1. We have previously suggested that LFA-1 acts as a cell adhesion molecule which can synergize with antigen-specific receptors in CTL-mediated killing, Fc receptors in antibody-dependent cellular cytotoxicity, or other receptors in natural killing to increase the avidity of cell-cell interactions (5,12,13). In addition to this, however, was raised the possibility that LFA-1 was not involved in adhesion, but that binding of anti-LFA-1 MAb provided a nonspecific "off" signal to effector cells (14).

The LFA-1-deficient patients provide a means of discriminating between these possibilities. Further experiments are also described in which it is shown that LFA-1 can mediate homotypic adhesion between cells. The results in both cases demonstrate the importance of LFA-1 in leukocyte cell-cell adhesion. Both findings also suggest that LFA-1 on target cells, as well as on effector cells, can contribute to adhesion reactions.

Mac-1, LFA-1, p150,95 IMMUNODEFICIENCY

We have investigated three patients with this disease (8-10). The αM and β subunits of Mac-1, the αL and β subunits of LFA-1, and at least the β subunit of p150,95 are deficient on the surface of patient leukocytes. Deficiency appears to be quantitative rather than qualitative, with two patients expressing about 0.5% and one patient about 5% of normal amounts on granulocytes and mononuclear cells. Quantitation by immunofluorescent flow cytometry of subunits on granulocytes from parents of these patients shows approximately half-normal surface expression. Together with absence of any evidence of mosaicism due to X chromosome inactivation in the mothers' leukocytes, the data demonstrate autosomal recessive inheritance. It appears that the primary deficiency in patient cells is of the common β subunit. Patient lymphocytes biosynthesize normal amounts of LFA-1 α chain intracellular precursor; however, it does not mature or become surface expressed (9). It appears that association with the β subunit is required for surface expression.

FUNCTIONAL ASSESSMENTS OF LFA-1 DEFICIENT LYMPHOCYTES

To examine allospecific lysis, peripheral blood lymphocytes (PBL) from LFA-1 deficient patients, family members, and unrelated individuals were stimulated with the EBV-transformed B cell line, JY. Mismatches occurred at a number of HLA loci. After six days, cells were harvested and tested for cytolysis of ^{51}Cr-labeled JY cells (Table I). All LFA-1 deficient individuals showed abnormally low levels of cytolysis of JY cells. Lytic unit calculations showed that patient 1 gave less than 10% of control lysis; patient 2 was less than 15% of control levels. In one preliminary experiment, patient 4 was approximately 50% of control levels of cytolysis (not shown). The proliferative response to JY by patient cells was 30% to 50% of normal (data not shown). Since all effector cells were adjusted to the same concentration before the CTL assay, the deficiency in cytolysis was not secondary to a failure to proliferate. Although primary human mixed lymphocyte cultures contain both specific CTL and non-specific NK cells, anti-HLA-A,B,C (w6/32) and OKT3 monoclonal antibody blocking experiments showed that greater than 50% of the JY cytolysis was allospecific and mediated by $OKT3^+$ CTL (data not shown).

Table I. Primary Cytolysis on Specific and Natural Killing Targets[a]

E:T[b]	Specific Killing JY ^{51}Cr release (%) 100:1	25:1	6:1	Natural Killing K562 ^{51}Cr release (%) 100:1	25:1	6:1
Patient 1	10	8	2	14	7	0
Mother 1	45	33	15	55	46	18
Father 1	52	49	33	67	51	27
Control 1	84	72	56	80	83	72
Patient 2	16	14	9	18	11	8
Mother 2	38	23	9	35	25	20
Father 2	53	36	29	77	55	59
Control 2	76	65	64	90	87	88

a. Cytolysis by primary cultures (6 days) of peripheral blood lymphocytes is shown as percentage specific release in a 3-4 hour assay (5). HLA types are patient 1: HLA-A2,w32; Bw35,w51; Cw4; DR3,4; mother 1: HLA-A2,2; B27,w51; Cw1; DR3,?: father 1: HLA-A3, w32; B7,w35; Cw4,7; DR2,4; patient 2: HLA-A1,2; B14,14; Cw7,w8; DR1,w6; mother 2: HLA-A1,w23; B14,w57; Cw3,w7; DRw6,w9; father 2: HLA-A2,28; B14,w62; Cw1,w8; DR1,?; JY: HLA-A2,2; B7,7; DR4,6.
b. Effector: target ratio.

To assess natural killing, PBL from LFA-1 deficient individuals, their families, and unrelated controls were cultured alone or with JY cells for six days. Natural killing was assessed on the K562 erythroleukemia cell line (HLA negative). All LFA-1 deficient individuals showed low levels of NK cell-mediated cytolysis compared to that of family members and unrelated indivuals (Table I).

PBL were also tested for proliferation after stimulation with the lectin phytohemagglutinin (PHA). PBL from LFA-1 deficient patients showed an impaired proliferative response to PHA (Table II). Responses were 20% and 10% of normal at 2.5 µg/ml and 0.25 µg/ml PHA, respectively. Interestingly, the responses by patient cells were further diminished when anti-LFA-1 MAb was added to cultures.

Patient CTL lines were maintained in long term culture by stimulation every one to two weeks with irradiated JY cells in the presence of T cell growth factor-containing medium. After three weeks in culture, the efficiency of cytolysis by LFA-1 deficient lymphocytes increased, but remained lower than that of normal lymphocytes from family members. Similar cytolytic efficiency persisted for up to twenty weeks in continuous culture. FACS analysis showed approximately 0.8% of normal expression of LFA-1 on CTL from patient 4 and < 0.1% of normal expression on CTL from patients 1 and 2 (Figure 1).

Table II. Proliferative Responses to PHA

	2.5 µg/ml	2.5 µg/ml, + anti-LFA-1	0.25 µg/ml	none
	^{3}H-thymidine incorporation ± SD (cpm x 10^{-3})			
Patient 2	28 ± 3	3.8 ± 0.2	1.5 ± 0.7	0.3 ± 0.05
Mother 2	101 ± 8	11.7 ± 2	9.1 ± 0.4	0.5 ± 0.01
Brother 2a	102 ± 2	2.9 ± 0.7	4.9 ± 2.1	0.5 ± 0.2
Brother 2b	138 ± 9	4.6 ± 0.1	10 ± 3.0	0.4 ± 0.1
Patient 4	23 ± 5	3.1 ± 0.1	0.6 ± 3.2	1.0 ± 1.0
Mother 4	75 ± 25	18.4 ± 5	13.9 ± 2.5	3.0 ± 2.4
Sister 4	108 ± 15	12.8 ± 2	9.2 ± 1.0	9.0 ± 13
Control	71 ± 14	5.5 ± 5	4.7 ± 4.9	1.1 ± 1.0

a. Proliferative responses to the indicated concentrations of PHA were measured on day 3 (5). Purified, TS1/18 F(ab')$_2$ anti-LFA-1 MAb (3 µg/ml) was added at the initiation of cultures.

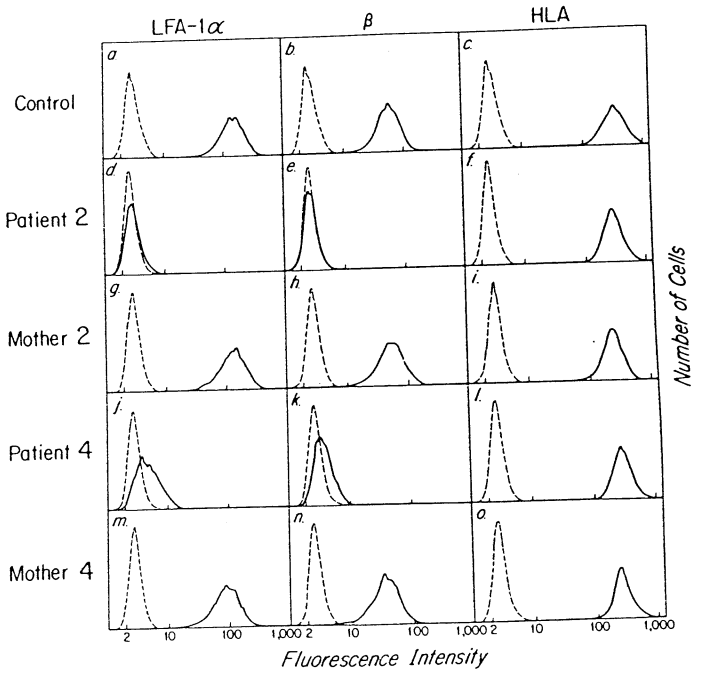

Fig. 1. Immunofluorescence Flow Cytometry of LFA-1 on Deficient and Normal CTL Lines. Long term CTL lines (four weeks) were labeled with MAb to the LFA-1 α subunit (a mixture of five MAb), to the LFA-1 β subunit (TS1/18), or to HLA (W6/32), then with fluorescin isothiocyanate-anti-mouse IgG, and analyzed on an Epics V cytometer (9).

CTL lines of patients, parents, and normal controls (Figure 1) were tested for their susceptibility to inhibition by anti-LFA-1 MAb. Effector and target cells were separately pretreated with anti-LFA-1 MAb and washed extensively prior to the ^{51}Cr release assay, and in one group anti-LFA-1 MAb was also included in the assay medium (Table III). Killing by healthy adult and parental CTL was inhibited by anti-LFA-1 MAb bound to the effector cell but not by MAb bound to the target cell as previously reported for normal CTL (5). Killing by patient 4 CTL was similarly inhibited by MAb bound to the effector. Thus, the small amount (0.8% of normal) of LFA-1 present on patient 4 is functionally important. Of note, in contrast to healthy CTL, killing by patient 4 CTL was also inhibited by MAb bound to the target cell.

Contrasting results were also found for the quantitatively more severely LFA-1 deficient CTL of patients 1 and 2. Killing by patient 2 CTL was poorly inhibited when anti-LFA-1 MAb was added to the assay, and pretreatment of killers or effectors gave equivocal or no blocking. CTL from patient 1 were inhibited when anti-LFA-1 was included in the assay. However, killing by patient 1 CTL was inhibited by MAb bound to the target cell and not by MAb bound to the effector cell. The findings with anti-JY CTL from patients 1 and 2 have been repeated many times; two representative experiments are shown in Table III.

The findings with patient 1 and 4 CTL suggest that LFA-1 on target cells can also contribute to the CTL-target cell interaction. It can be speculated that target cell LFA-1 also participates in normal CTL-mediated killing, but that its contribution to the interaction is negligible compared to that of effector cell LFA-1. The difference in anti-LFA-1 susceptibility between patient 1 and patient 2 is not understood at present. It may relate to different HLA disparities with JY as well as to differences in the LFA-1 mutation or level of expression.

Blocking by anti-LFA-1 MAb bound to the target cell might also be due to antibody bound by one combining site with target cell LFA-1 reacting through the second combining site with CTL LFA-1. Inhibition by this mechanism could be much more potent with patient CTL. Saturation of patient LFA-1 sites would occur with much less anti-LFA-1 bound per CTL, but might give equivalent inhibition of killing.

LYMPHOCYTE AGGREGATION IS INHIBITED BY ANTI-LFA-1

Another line of investigation provides independent evidence that LFA-1 on JY cells is functionally important, and supports a general role for LFA-1 in cell adhesion processes. Previous studies have demonstrated that phorbol esters induce adhesion in a wide variety of cell lines as well as in peripheral blood B and T lympho-

Table III. Inhibition of Cytolysis by Anti-LFA-1 MAb: Pre-treatment of Effectors or Targets[a]

	Experiment 1						Experiment 2	
	Control	Mom 1	Dad 1	Pat. 1	Pat. 2	Pat. 4	Pat. 1	Pat. 2
Treatment	% ^{51}Cr release from JY (at 2 different E:T ratios)							
None	61/49	64/45	64/48	49/35	66/69	52/28	52/29	48/32
Antibody in Assay	31/14	26/15	29/22	24/19	59/62	34/22	21/13	51/29
Effectors Pre-treated	46/36	37/27	43/35	47/42	54/45	25/19	55/26	42/32
Targets Pre-treated	61/48	61/39	63/47	28/19	85/68	23/22	29/21	56/41
Both Effectors and Targets Pre-treated	50/34	39/29	45/41	27/21	49/44	28/25	27/19	47/34

a. CTL-mediated killing expressed as % ^{51}Cr specific release was determined at effector:target ratios of 50:1 and 20:1 for patients and 25:1 and 10:1 for family members and the control. Effectors and target cells were separately pretreated with TS1/18 anti-LFA-1 MAb or mock pretreated with a control MAb, washed four times, then mixed together and assayed in the presence or absence of TS1/18 MAb as previously described (5). Assays were terminated after three to four hours, whenever 50% cytolysis was obtained at the higher E:T ratio.

Table IV. Effect of anti-LFA-1 MAb on PMA-Stimulated Aggregation of JY cells[a]

PMA	Additions	Exp. 1 Aggregated Cells (%)	Exp. 2 Aggregated Cells (%)
-	-	6	6.7
+	-	60	39.5
+	TS1/22 anti-LFA-1 α	0	1.3
+	TS1/18 anti-LFA-1 β	0	ND
+	TS1/18 + TS1/22	0	ND
+	TS2/18 (control)	62	ND
+	EGTA (5mM)	ND	0
+	EGTA (5mM) + Mg^{+2} (5mM)	ND	43.3

a. JY cells (3×10^6 in 300 µl of RPMI medium with 5 mM HEPES) were incubated alone, with PMA (50 ng/ml) or with PMA + MAb hybridoma supernatant (10 µl) at 37° C for 30 minutes with constant agitation. Aggregated and non-aggregated cells were counted in a hemocytometer.

cytes and monocytes (15-17). Similar divalent cation-dependent clustering occurs when autologous lymph node cells are cultured (18). LFA-1 seemed a likely candidate for mediating this adhesion process because of its wide distribution on leukocytes, its wide importance in functions requiring adhesion, its lack of antigen specificity, and its known participation in the divalent cation-dependent adhesion step in CTL-mediated killing. Therefore, the effect of LFA-1 on phorbol myristyl acetate- (PMA) mediated adhesion by JY cells was investigated (Table IV). Phorbol ester increased the percentage of aggregated JY cells from 6% to 40-60% (Table IV). Addition of MAb to the LFA-1 α or β subunit decreased aggregation to 0-1%. The decrease to below the level of spontaneous aggregation suggests anti-LFA-1 inhibits spontaneous as well as PMA-stimulated adherence, which has been confirmed in other experiments. The adhesion step is dependent on divalent cations, as shown by inhibition by EGTA, a strong Ca^{+2} and weak Mg^{+2} chelator, and the restoration of adhesion by addition of Mg^{+2} (Table IV). Previous studies had demonstrated that EDTA and EGTA inhibited (16,17). Addition of anti-LFA-1 MAb disrupted pre-formed aggregates (data not shown), in parallel with previous findings on CTL-target conjugates (13). These data suggest a strong similarity between phorbol ester-induced cell-cell aggregation and the LFA-1-dependent adhesion step in CTL-mediated killing (1,5,7,13), and suggest LFA-1 functions as an adhesion protein. Similar independent findings by Martz et. al. for spontaneous aggregation are reported elsewhere in this volume.

CONCLUSION

Patients with a genetic deficiency of LFA-1 demonstrate defects in antigen-specific killing, natural killing, and proliferative responses to lectins and alloantigens. These functional deficits conclusively demonstrate that LFA-1 is functionally important for lymphocyte cell interactions. The functional impairments of patient cells resemble those of anti-LFA-1 MAb-treated healthy control cells. This shows that MAb bound to LFA-1 inhibits LFA-1's normal physiological function, rather than activating some other mechanism such as a nonspecific "off-switch." The patients differed in the severity of their functional deficits, with the patient with the highest amount of cell surface LFA-1 showing the mildest effects on function. After repeated in vitro stimulation, functional CTL lines could be established from all patients, although their cytolytic activity was below normal. Killing by CTL effectors of the two most deficient patients was resistant to inhibition by anti-LFA-1 MAb. This may suggest that LFA-1 is an auxiliary molecule which can boost the efficiency or avidity of CTL, but is not essential for function. However, of importance in these responses could be the small amounts of cell surface LFA-1 which appear present in even the most deficient patients, as shown by immunofluorescent cytometry and the effects of anti-LFA-1 MAb on proliferation to PHA.

The results with one patient demonstrated that LFA-1 on the target cell, as well as on the effector cell, can be functionally important. A bidirectional interaction may normally occur physiologically, with LFA-1 on the effector cell of the most importance as shown by blocking experiments. A bidirectional interaction mechanism was also supported by studies on phorbol ester-induced adhesion of JY cells. Anti-LFA-1 MAb inhibited aggregation of JY cells, a process which can be regarded as homotypic as opposed to the heterotypic adhesion between effector and target cells. The importance of LFA-1 in JY cell self-aggregation is in agreement with the findings that LFA-1 on JY cells can participate in adherence to CTL. LFA-1-dependent adhesion resembled CTL-target conjugate formation in divalent cation-dependence and reversibility with MAb. It is proposed that LFA-1 acts physiologically to promote adherence between leukocytes and other cells, thereby enhancing the efficiency or avidity of interactions mediated by other leukocyte surface receptors such as the antigen-receptor of T lymphocytes.

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DISCUSSION

H. Spits: Did you look for expression of LFA-1 on PHA activated blasts of these patients?

T. Springer: Yes, absolutely. In fact, the S-35 methionine biosynthesis experiments were done partly with PHA blasts, and partly with EBV lines, and the PHA blasts are just as deficient as the patient's granulocytes. Also, I didn't show data for mononuclear cells, but they are also deficient. We don't see any increase on activated cells.

H. Spits: We have looked at similar patients. With PHA blasts LFA is coming up, but on EBV transformed B blasts they stay LFA-1 negative.

T. Springer: We would agree that the patient who has around 10% of the normal amounts on its granuloctes shows virtually nothing on its EBV lines. But our data did come from PHA blasts.

M. Sitkovsky: Can the beta chain from, let's say, Mac-1 be in association with the alpha chain from LFA-1 on monocytes? Something like a promiscuous beta chain. Can you expect that we will have always one beta chain?

T. Springer: When we've done crosslinking experiments, we can demonstrate that 100% of the molecules consist of non-covalently associated alpha and beta chains at any one moment.

M. Sitkovsky: When I do immunoprecipitation with anti-LFA-1 antibody, the alpha chain always is much darker than the beta chain. Do you think that the alpha chain has more tyrosine, or what? Or is more accessible to the iodine?

T. Springer: The sequence shows that LFA-1 has an internal tyrosine, whereas Mac-1 has an internal phenylalanine. I'm not sure that that single difference explains it but differences like that certainly might explain that phenomenon.

D. Hudig: Is there homology of the beta chain for other membrane molecules?

T. Springer: We have not been able to get any sequence information from the beta subunits. We think they're blocked. We did a computerized search of all known protein sequences, and it turns out that the LFA-1 alpha subunit shows between 28% and 42% homology to alpha interferon. There are over 8 different human interferons and 3 different rodent interferons sequenced and it shows homology to all of them. This is very statistically significant, but its a very surprising result because alpha

interferon is a much smaller molecule and its a secreted mediator rather than a surface bound one.

P. Golstein: I think you presented a very fascinating set of results, especially the results on the impairment of cell adhesion in patients. I think you said that in these patients, adherance to substrates was impaired. The question is, how do you figure in molecular terms the role that LFA-1 may have in adherance to substrates.

T. Springer: That's a good question. By the way, we don't know whether it is LFA-1 or p150,95 or Mac-1 which is important. Don Anderson is doing antibody inhibition experiments, and they suggest that actually all of these molecules may be contributing to this adherance. The adherance is affected if you look at normal glass coverslips, serum-coated coverslips, fibronectin-coated coverslips, or albumin-coated latex beads. You always see a deficit. It might be that there are specific ligand-binding sites on these molecules, as well as nonspecific sites. They're very large molecules with certainly enough room for that. There must be some specific molecules to mediate adherance to substrates. I think these molecules are very important, but the mechanism is puzzling.

P. Golstein: Is there any need for thinking that there is somewhere a receptor for LFA-1?

T. Springer: The evidence is really not in on LFA-1, but Mac-1 clearly shows specificity for C3bi. There are reports from Peter Lachman and Gordon Ross that the C3bi receptor may be a lectin-like molecule. They see inhibition by sugars, however, we do not see inhibition by the same sugars in our hands.

W. Seaman: If you do sequential immunoprecipitation with your antibodies to alpha chain, or capping, are there other structures that use the beta chain?

T. Springer: That's how we picked up p150,95, with our anti-beta chain antibody. I couldn't rule out that there are other molecules present in much smaller amount which have the same beta subunit.