

LEUKOCYTE FUNCTION-ASSOCIATED ANTIGEN 1 IS AN ACTIVATION MOLECULE FOR HUMAN T CELLS

BY MARY C. WACHOLTZ, S. S. PATEL, AND P. E. LIPSKY

*From the Harold C. Simmons Arthritis Research Center, Medical Scientist Training Program,
and Immunology Graduate Program, Department of Internal Medicine,
University of Texas Southwestern Medical Center, Dallas, Texas 75235*

The leukocyte function-associated antigen 1 (LFA-1)¹ molecule belongs to the integrin family of adhesion proteins (1, 2). This group contains LFA-1, Mac-1, and p150,95, which share a common β chain, but have different α chains (3). The common β subunit is now designated CD18, whereas the α subunits are designated CD11a for LFA-1, CD11b for Mac-1, and CD11c for p150,95 (4). Each of these molecules has a somewhat different cellular distribution among hematopoietic cells (5). mAbs to these determinants have been useful in determining that these proteins are involved in interactions of the cell with its environment as in cell-cell adhesion, cell-ligand interactions, or cellular migration and homing (5-7). LFA-1 was first detected by the recognition of mAbs that could block CTL-mediated killing (8). The LFA-1 molecule has since been shown to be involved in the adhesion of target and effector cells in NK-mediated killing, the induction of T cell proliferation by accessory cell-dependent stimuli, and in some T cell-dependent B cell responses (5-8). Children with a genetic deficiency in the surface expression of LFA-1 have diminished cell contact-dependent functional responses (5).

The LFA-1 molecule, therefore, is important in mediating events external to the cell membrane, but there is little information about whether perturbation of LFA-1 may also transmit information to the lymphocyte. Such a possibility cannot be examined with mixed cell populations, as the anti-LFA-1 mAb may inhibit the final functional outcome by preventing cell contact. There are circumstances, however, where the anti-LFA-1 mAb can augment T cell activation in the absence of accessory cells. Thus, for example, the response of highly purified T cells to immobilized mAbs to CD3 was enhanced by coimmobilized mAbs to LFA-1 (9), suggesting the possibility that LFA-1 molecules could transmit signals to the cell. However, the generation of a positive signal by the anti-LFA-1 mAb could not be distinguished from increased adherence or stabilization of the T cell to the surface, thereby permitting more effective interaction with the anti-CD3 mAb. More recently, purified T cells were shown to proliferate modestly in response to the combination of immobilized anti-LFA-1 mAb and PMA (10). This suggested the possibility that the anti-LFA-1

This work was supported by grants AR-09989 and AR-36169 from the National Institutes of Health. S. S. Patel was supported by Medical Scientist Training Grant GM-08014.

¹ *Abbreviations used in this paper:* [Ca²⁺]_i, intracellular free calcium concentration; LAD, leukocyte adhesion deficiency; LFA-1, leukocyte function-associated antigen 1.

mAb could provide a costimulatory signal similar to that provided by calcium ionophores, which raise intracellular calcium ($[Ca^{2+}]_i$) (11). In other studies, however, soluble anti-LFA-1 mAbs have been reported to modify T cell proliferative responses to immobilized anti-CD3 mAbs in either a positive or negative manner (12). Thus, anti-CD11a mAbs were costimulatory whereas anti-CD18 mAbs were inhibitory. These findings raised the possibility that binding to the α or β chains might differentially affect T cell activation.

We have recently shown enhanced activation of human T cell clones when cells were pretreated with mAbs that recognized two different surface antigens, including the combinations of mAbs to CD3 + HLA-A,B,C, CD3 + CD4/CD8, or HLA-A,B,C + CD4/CD8 followed by crosslinking (13). As this experimental system provided a homogeneous population of cells free of accessory cells, it was possible to use T cell clones to examine the ability of mAb to LFA-1 to costimulate T cells in the absence of either accessory cells or an immobilized stimulus. Both the ability to increase $[Ca^{2+}]_i$ as a marker of transmembrane signaling and the ability to activate the cells functionally were examined. The results show that crosslinking anti-LFA-1 mAb alone does not increase $[Ca^{2+}]_i$ nor does it stimulate T cells. However, crosslinking LFA-1 and CD3 simultaneously, but independently, results in a prolongation of the increase in $[Ca^{2+}]_i$ and in enhancement of both IL-2 production and proliferation. The LFA-1 molecule, therefore, can transmit a costimulatory signal to T lymphocytes that results in enhanced activation when the CD3 complex is also engaged.

Materials and Methods

mAbs and Reagents. The mAbs included OKT3 (Ortho Pharmaceutical, Raritan, NJ), an IgG2a mAb directed to the CD3 complex on mature T cells (14); Leu-4 (Becton Dickinson Immunocytometry Systems, Mountain View, CA), an IgG1 mAb against CD3; 158.86 (gift from Dr. Jefferson Paslay; Upjohn, Kalamazoo, MI), an IgG2b mAb against CD3; W6/32, (American Type Culture Collection [ATCC], Rockville, MD), an IgG2a mAb directed to nonpolymorphic class I MHC molecules; MB40.5 (ATCC), an IgG1 mAb directed to nonpolymorphic class I MHC determinants; 60.3 (gift from Dr. Patrick Beatty, Fred Hutchinson Cancer Center, Seattle, WA), an IgG2a mAb directed to an epitope common to the β chain (CD18) of LFA-1, Mac-1, and 150,95; TS1/18 (gift of Dr. T. A. Springer, Harvard Medical School, Boston, MA), an IgG1 mAb directed to CD18; TS1/22 (gift of Dr. T. A. Springer, Harvard Medical School, Boston, MA), an IgG1 mAb directed against the α chain of the LFA-1 molecule (CD11a); and anti-Tac (gift of Dr. Thomas Waldmann, National Cancer Institute, Bethesda, MD), an mAb against the p55 component of the IL-2-R (CD25). The Fab fragments of the anti-CD3 mAb 64.1 were the kind gift of Dr. Ellen Vitetta (University of Texas Southwestern Medical Center, Dallas, TX). Control mAbs included MOPC (ATCC, IgG1) and P1.17 (ATCC, IgG2a).

Affinity-purified goat anti-mouse Ig used in cultures (GaMIg; Cappel Laboratories, Malvern, PA) was diluted in culture medium to a final concentration of 10 μ g/ml. GaMIg antiserum obtained from Antibodies Inc. (Davis, CA) was also used in some experiments. No difference was observed in the response elicited by either preparation. Affinity-purified isotype-specific goat anti-mouse Ig (anti-IgG2a, anti-IgG1, and anti-IgG2b; Fisher Scientific Co., Pittsburgh, PA) was used at a final concentration of 10 μ g/ml in cultures or at 5 μ g/ml in flow cytometric determinations of $[Ca^{2+}]_i$. According to the manufacturer, crossreactivity of the isotype-specific antibodies was never >3%. rIL-2 was the gift of Cetus Corp., (Emeryville, CA). PHA was obtained from Burroughs Wellcome Co., Research Triangle Park, NC.

T Cell Clones. T cell clones were prepared as previously described (15). Before use, clones were passed every 7 d for three to four passages by culturing equal numbers of the clone

(3×10^5 cells/well) and irradiated syngeneic or allogeneic PBMC with PHA and IL-2 to generate sufficient numbers of cells. The cells were then cultured in fresh medium containing IL-2, but no PHA or feeder cells for 12–19 d. Cells were used during the 12–19-d period because the cellular responses were stable and reproducible. If maintained longer without restimulation, responsiveness declined. Cells were washed on at least three occasions during this period and recultured with IL-2 (20 U/ml). No feeder cell contamination could be detected. Data concerning the phenotype and function for some of these clones (NP1, NP7, NP19, NP18, NP13, LM2, and LM5) have been presented previously (13, 16). All these clones were >97% CD18 or CD11a positive. Clonality for these cells was demonstrated by analysis of the rearrangement patterns of the genes encoding the β chain of the TCR (16). These clones have also been shown to mediate MHC-unrestricted cytotoxicity and to provide help for B cell differentiation (15, 16). Clones BR2, BR4, and BR10 were isolated for this study from a child with leukocyte adhesion deficiency (LAD) and expressed <1% surface CD18 or CD11a. Clones NP18 and NP13 were CD8⁺; all other clones were CD4⁺.

Reacting T Cells with mAb. To stimulate clones for both analysis of $[Ca^{2+}]_i$ and functional responses, the cells were suspended at 1.5×10^7 cells/ml in cold PBS containing 2% FCS. The cells were incubated with saturating concentrations of the mAb for 30 min at 4°C and then washed twice. After washing, the cells were resuspended in appropriate medium for analysis of functional responses.

Phenotypic Analysis. Phenotypic analysis of the clones was carried out by staining cells on ice with saturating concentrations of the mAb listed above. Control cells were stained with an isotype-matched irrelevant mAb. Cells were washed, and then treated with a secondary fluorescein-conjugated GaMIg (CooperBiomedical, Inc., Malvern, PA). The stained clones were analyzed by flow cytometry using a 50HH flow cytometer (Ortho Diagnostic Systems Inc., Westwood, MA) as previously described (17, 18). Dead cells were gated out after incubation with propidium iodide. Surface expression of these antigens remained constant during the study period.

Measurement of $[^3H]$ Thymidine Incorporation. Cell culture was carried out in medium RPMI 1640 supplemented with 10% FCS, penicillin G (200 U/ml), and L-glutamine (0.3 mg/ml) in 96-well flat-bottomed microtiter plates (Costar; Cambridge, MA) in a total volume of 200 μ l. T cell clones previously treated with various mAbs were cultured at 10^5 cells/well with or without GaMIg or isotype-specific secondary antibodies (10 μ g/ml) for a total of 36 h, as this was previously determined to give the optimal response. 18 h before harvesting on glass fiber filter paper, 1 μ Ci of $[^3H]$ thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) was added to the culture. $[^3H]$ Thymidine incorporation was measured by liquid scintillation counting. All data are expressed as mean cpm \pm SEM of triplicate determinations.

Assay for IL-2 Production. T cells pretreated as described above were cultured for 18 h with or without GaMIg (10 μ g/ml) in the presence of anti-Tac mAb (2 μ g/ml) to prevent absorption of IL-2. To assess IL-2 content in the supernatants, IL-2-dependent CTLL-2 cells (3.5×10^3 cells/well) were cultured in RPMI 1640 medium containing 10% FCS with diluted culture supernatants or different concentrations of IL-2 to define the standard curve. The cells were incubated for 14 h at 37°C, then pulsed with 1 μ Ci of $[^3H]$ thymidine (6.7 Ci/mmol; New England Nuclear) and harvested 14 h later. $[^3H]$ Thymidine incorporation was measured by liquid scintillation counting. The amount of IL-2 in the culture supernatants was computed by comparison with the standard curve.

Analysis of Cytosolic Free Calcium ($[Ca^{2+}]_i$). Flow cytometry using cells loaded with the fluorescent calcium-sensitive dye indo-1 was used to analyze changes in $[Ca^{2+}]_i$, as previously described (19). Cells suspended in RPMI 1640 medium containing 5% FCS and 10 mM Hepes (pH 7.4) at a density of $4\text{--}10 \times 10^6$ cells/ml were incubated with 5 μ M indo-1/AM (Molecular Probes; Eugene, OR) for 30 min at 37°C. The suspension was diluted 1:10 with the same medium and incubated an additional 30 min at 37°C. The cells were then stained at 4°C with the appropriate mAb as described above. Before use, an aliquot of cells was resuspended in warm HBSS containing 1% human serum and 10 mM Hepes (pH 7.4) to give a final concentration of 10^6 cells/ml.

Flow cytometric analysis was done with the 50HH flow cytometer and associated 2150 computer (Ortho Diagnostics Systems Inc.). An argon laser (Coherent Innova 90-5, Coherent

Inc., Palo Alto, CA) focused in the UV (351–368 nm) was used to excite indo-1. The violet fluorescent emission, which increases as the dye binds calcium, was selected using a DF 405/20 bandpass filter (Omega Optical, Inc., Brattleboro, VT) after separation with a 430-nm dichroic mirror (Ortho Diagnostics Systems Inc.). Blue emission, which decreases as calcium is bound, was selected using a DF 485/22 bandpass filter (Omega Optical, Inc.). Cells were maintained at 37°C, and baseline data were collected for 1.5–2 min before addition of crosslinking antibody. A modified version of the “Ratiorg” protocol was used to calculate the violet to blue ratio, which is proportional to intracellular calcium (20), and to display it as a function of time. Data were smoothed using the program “Normcon,” and the program “Cyto2D” was used to determine the mean ratio and percentage of responding cells (both provided by Dr. Peter Rabinovitch through the Naval Medical Research Institute, Bethesda, MD).

To determine the approximate $[Ca^{2+}]_i$ that corresponds to a measured violet to blue ratio, indo-1-loaded cells were simultaneously studied in the flow cytometer and in suspension using a fluorimeter (MPF-44B; Perkin-Elmer Corp., Pomona, CA). Ionomycin (Calbiochem-Behring Corp., San Diego, CA), a calcium ionophore, was used to vary $[Ca^{2+}]_i$, which was calculated for cells in suspension as described by Grynkiwicz et al. (20). This value was related to the simultaneously obtained violet-to-blue ratio determined with the flow cytometer to derive a standard curve.

Results

Crosslinking CD3 with CD18 or CD11a Prolongs the Calcium Signal. When human T cell clones are pretreated with an anti-CD3 or an anti-HLA-A,B,C mAb followed by crosslinking with a secondary GaMIg, two different, but characteristic changes in $[Ca^{2+}]_i$ are observed (13, 21). As shown in Fig. 1, crosslinking CD3 resulted in a rapid increase in $[Ca^{2+}]_i$ in ~90% of cells followed by a rapid decline. Crosslinking HLA-A,B,C caused a delayed, but more sustained increase in a smaller percentage

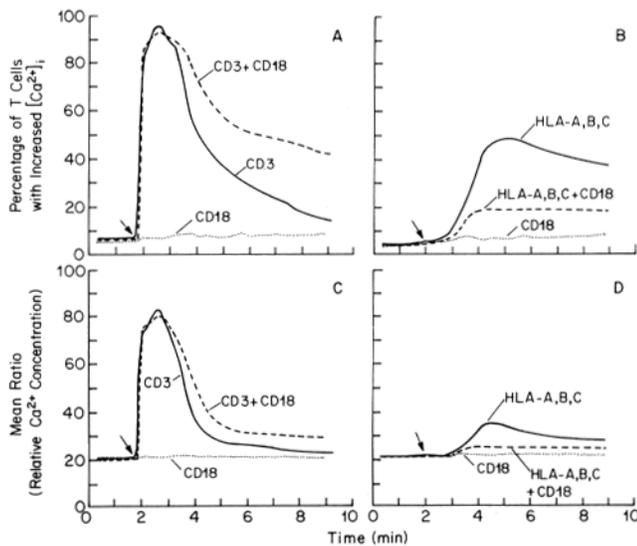


FIGURE 1. Crosslinking CD3 and CD18 results in a prolonged increase in $[Ca^{2+}]_i$. Clone NP18 ($CD8^+$) cells were pretreated with the indicated mAb (anti-CD3-OKT3; anti-HLA-A,B,C-W6/32; anti-CD18-60.3) at 4°C. Cells were warmed to 37°C and were analyzed by flow cytometry for 1.5–2 min to establish the baseline $[Ca^{2+}]_i$. At the arrow, the crosslinking secondary antibody, GaMIg, was added and the measurement continued for up to 8 min. The data were analyzed in terms of the percentage of T cells that increased $[Ca^{2+}]_i$ (A and B), and in terms of the mean fluorescence ratio (violet to blue), which reflects the mean $[Ca^{2+}]_i$ of the population (C and D). In the absence of GaMIg, the mAb did not induce an increase in $[Ca^{2+}]_i$. GaMIg induced no increase in $[Ca^{2+}]_i$ in cells stained with an irrelevant control mAb (data not shown).

of cells at any given time point. Crosslinking LFA-1 using the mAb 60.3 (anti- β chain, CD18) did not increase $[Ca^{2+}]_i$. However, when cells were pretreated with saturating concentrations of both an anti-CD3 and an anti-CD18 mAb, the initial increase in $[Ca^{2+}]_i$ was as rapid as with crosslinking CD3 alone, but the increase in $[Ca^{2+}]_i$ was prolonged (Fig. 1, *A* and *C*). The average $[Ca^{2+}]_i$ 7 min after adding GaMIg was increased by 200 nM, compared with that observed after crosslinking CD3 alone. This enhancement by crosslinking CD18 was observed only with concomitant crosslinking of CD3, as crosslinking HLA-A,B,C with LFA-1 (Fig. 1, *B* and *D*) or crosslinking CD4/CD8 with LFA-1 in appropriate clones (data not shown) did not enhance the response and often decreased it.

The enhancement of the CD3 response could also be demonstrated using a mAb directed to CD11a (LFA-1 α). Crosslinking CD11a alone with GaMIg did not change the baseline $[Ca^{2+}]_i$ (data not shown). However, as shown in Fig. 2, crosslinking CD3 and CD11a prolonged the increase in $[Ca^{2+}]_i$, whereas crosslinking HLA-A,B,C and CD11a diminished the observed calcium response. 7 min after adding GaMIg, the $[Ca^{2+}]_i$ of cells pretreated with both the anti-CD3 and anti-LFA-1 mAb was ~ 100 nM greater than that observed after crosslinking CD3 alone.

Fig. 3 shows that cloned T cells isolated from an individual who cannot make a productive β chain and thus cannot express LFA-1 (5) do not generate a prolonged calcium signal after costimulation with anti-CD3 and anti-LFA-1 mAbs followed by GaMIg. In addition, the response to crosslinking class I MHC molecules was not modified by treatment with an anti-LFA-1 mAb. Thus, although the prolonged increase in $[Ca^{2+}]_i$ elicited by crosslinking CD3 and LFA-1 was small in magnitude, it occurred reproducibly, was detected when either the α or β chain was crosslinked, and required expression of LFA-1.

Crosslinking CD3 with LFA-1 Enhances the Proliferative Response of Human T Cell Clones.
As crosslinking surface LFA-1 could prolong the calcium signal produced by cross-

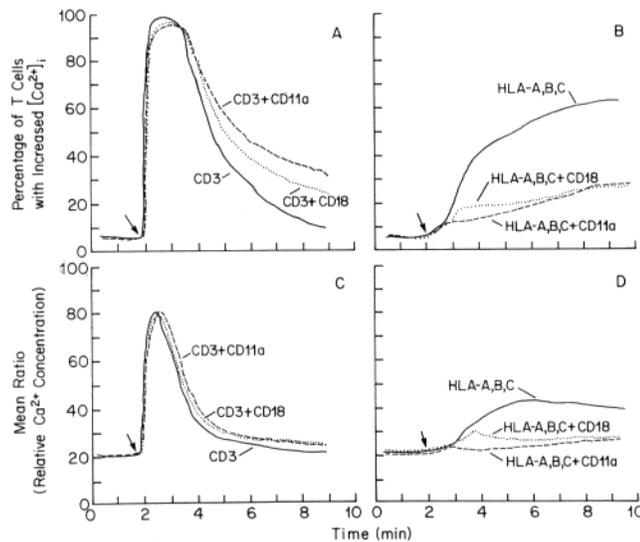


FIGURE 2. Crosslinking either the α or the β chain of the LFA-1 molecule prolongs the $[Ca^{2+}]_i$ response induced by anti-CD3 mAb. Clone NP1 ($CD4^+$) cells were pretreated at $4^\circ C$ with the indicated mAb (anti-CD3-OKT3; anti-CD18-TS1/18; anti-CD11a-TS1/22) and washed. Subsequent analysis was done as described in Fig. 1.

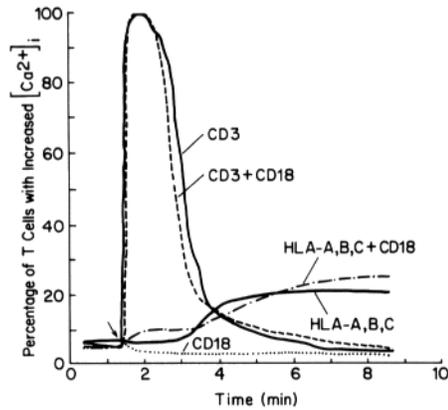


FIGURE 3. Anti-LFA-1-induced prolongation of the anti-CD3 calcium signal is not seen in T cell clones from a child with LAD. Clone BR10 ($CD4^+$; $<1\%$ LFA-1 $^+$) cells isolated from a child with LAD were pretreated at 4°C with the indicated mAb (anti-CD3-OKT3; anti-HLA-A,B,C-W6/32; anti-CD18-60.3), washed, and analyzed as described in Fig. 1.

linking CD3, the effect of crosslinking LFA-1 on the functional responses of T cell clones was examined. Table I shows the proliferative response of both a $CD4^+$ and a $CD8^+$ clone. Treatment with an anti-CD18 mAb with or without crosslinking secondary antibody did not affect DNA synthesis. The $CD4^+$ clone, but not the $CD8^+$ clone responded somewhat to pretreatment with the anti-CD3 mAb alone, and this response was increased by crosslinking. Crosslinking both CD3 and CD18 markedly enhanced [^3H]thymidine incorporation in both the $CD4^+$ and the $CD8^+$ clone. As previously observed (19, 21), crosslinking class I MHC determinants increased [^3H]thymidine incorporation. Crosslinking HLA-A,B,C and CD18, however, did not change this response. As shown in Table II, crosslinking CD3 and CD11a also increased [^3H]thymidine incorporation compared with the response to either mAb alone. The increase in [^3H]thymidine incorporation was smaller than that seen in the experiment in Table I, demonstrating the variation in the magnitude of responses of different clones. In addition, crosslinking a different anti-CD18 mAb (TS1/18)

TABLE I
Crosslinking CD3 and CD18 Enhances Proliferation of Human T Cell Clones

Specificity of mAb	[^3H]Thymidine incorporation			
	$CD4^+$ clone		$CD8^+$ clone	
	Control	+ GaMIg	Control	+ GaMIg
	<i>cpm</i> $\times 10^{-3} \pm SEM$			
Control	0.6 \pm 0.0	0.5 \pm 0.0	1.4 \pm 0.1	1.3 \pm 0.2
CD18	0.5 \pm 0.0	0.6 \pm 0.0	1.0 \pm 0.0	1.6 \pm 0.2
CD3	2.4 \pm 0.1	8.8 \pm 0.0	1.5 \pm 0.2	0.9 \pm 0.2
CD3 + CD18	1.4 \pm 0.1	44.2 \pm 0.4	3.0 \pm 0.3	22.4 \pm 0.4
HLA-A,B,C	0.8 \pm 0.0	7.9 \pm 0.4	1.2 \pm 0.0	16.8 \pm 0.5
HLA-A,B,C + CD18	0.7 \pm 0.0	8.4 \pm 0.1	0.9 \pm 0.0	11.8 \pm 0.4

Clone LM2 ($CD4^+$) or NP18 ($CD8^+$) was pretreated with saturating concentrations of the indicated mAb (control-P1.17; anti-CD18-60.3; anti-CD3-OKT3; anti-HLA-A,B,C-W6/32), washed, and incubated for 36 h. 18 h before harvesting, $1 \mu\text{Ci}$ of [^3H]thymidine was added. All data are expressed as mean $cpm \pm SEM$.

TABLE II
*Crosslinking Either the α or the β Chain of the LFA-1 Molecule
 Enhances Proliferation of Anti-CD3-stimulated T Cell Clones*

Specificity of mAb	$[^3\text{H}]$ Thymidine incorporation	
	Control	+ GaMIg
	<i>cpm $\times 10^{-3} \pm \text{SEM}$</i>	
Control	1.6 \pm 0.1	1.4 \pm 0.1
CD3	1.6 \pm 0.1	1.2 \pm 0.1
CD18	1.3 \pm 0.2	1.3 \pm 0.2
CD11a	1.6 \pm 0.1	1.7 \pm 0.0
CD3 + CD18	2.1 \pm 0.0	5.2 \pm 0.6
CD3 + CD11a	2.3 \pm 0.1	8.1 \pm 0.3

Clone NP7 (CD4⁺) was pretreated with saturating concentrations of the indicated mAb (control-P1.17; anti-CD3-OKT3; anti-CD18-TS1/18; anti-CD11a-TS1/22), washed, and incubated (10^5 cells/well) either with or without GaMIg. Cells were cultured for 36 h. 18 h before harvesting, 1 μCi of $[^3\text{H}]$ thymidine was added. All data are given as mean cpm \pm SEM.

from that used in Table I (60.3) also enhanced proliferation of anti-CD3-stimulated clones. Thus, three different anti-LFA-1 mAbs enhanced the proliferative response to anti-CD3 mAb.

Crosslinking CD3 and Either CD18 or CD11a Enhances IL-2 Production. The enhancement of anti-CD3 mAb induced $[^3\text{H}]$ thymidine incorporation by the anti-LFA-1 mAb was apparent even when no additional exogenous IL-2 was added, suggesting that IL-2 was being produced in these cultures. When IL-2 production was directly examined, as shown in Table III, crosslinking the surface LFA-1 molecule via either the α or β chain or crosslinking CD3 alone resulted in little or no IL-2 production. However, pretreating cells with both an anti-CD3 mAb and an anti-LFA-1 mAb

TABLE III
*Crosslinking CD3 and CD18 or CD11a Increases IL-2 Production
 of Anti-CD3-stimulated T Cell Clones*

Specificity of mAb	IL-2 production			
	Clone 1		Clone 2	
	Control	+ GaMIg	Control	+ GaMIg
	<i>U/10⁶ cells</i>			
Control	0.0	0.0	0.0	0.0
CD18 ¹	0.0	0.0	0.0	0.0
CD18 ²	0.0	0.0	0.0	0.0
CD11a	0.0	0.0	0.0	0.0
CD3	1.0	0.0	0.0	0.6
CD3 + CD18 ¹	0.3	4.2	0.0	9.6
CD3 + CD18 ²	0.6	5.8	0.0	9.6
CD3 + CD11a	0.6	4.8	0.3	8.3

Clone (NP1; CD4⁺) or clone 2 (LM5; CD4⁺) was pretreated with the indicated mAb (control-P1.17; anti-CD18¹-60.3; anti-CD18²-TS1/18; anti-CD11a-TS1/22; anti-CD3-OKT3), washed, and incubated (10^5 cells/well) with or without GaMIg. Supernatants were harvested at 18 h and assayed for IL-2 production.

markedly increased IL-2 production. The enhancement could be demonstrated using mAbs directed at either the β chain (anti-CD18) or the α chain (CD11a), and the magnitude of increase was comparable. Table IV demonstrates that the enhancement of IL-2 production by crosslinking CD3 and LFA-1 does not occur in cells isolated from an individual with LAD, although crosslinking class I MHC molecules did stimulate IL-2 release. Thus, the LFA-1-mediated enhancement of IL-2 production requires costimulation of CD3, can be induced by crosslinking either the α or β chain, and requires LFA-1 surface expression.

Crosslinking LFA-1 to LFA-1 Enhances the Functional Response to CD3, but not to HLA-A,B,C. To determine whether crosslinking LFA-1 to LFA-1 was sufficient to increase the response to CD3 or whether crosslinking CD3 to LFA-1 was necessary, experiments using isotype-specific crosslinking secondary antibodies were carried out. Fig. 4 demonstrates the typical responses observed using three different clones. In the first clone, NP1 (CD8⁺), the IgG1 anti-CD3 mAb elicited some IL-2 production whether crosslinking antibody was added or not. IL-2 production by the IgG2a anti-CD3 mAb was seen when anti-IgG2a crosslinking antibody was added. No IL-2 production resulted from pretreatment and crosslinking with either of two anti-CD18 mAbs. When cells were pretreated with both an anti-CD3 and either of the two anti-CD18 mAbs, the usual enhancement of IL-2 production was observed after crosslinking by GaMIg. When surface LFA-1 was specifically crosslinked on cells also carrying an anti-CD3 mAb of a different isotype, IL-2 production was significantly greater than that produced when either CD3 or LFA-1 was crosslinked on cells pretreated with a single mAb. This was observed regardless of whether the anti-LFA-1 mAb was of the IgG1 or IgG2a isotype. Crosslinking the anti-CD3 mAb of the IgG2a isotype on cells stained with both an IgG2a anti-CD3 mAb and the IgG1 anti-CD18 mAb also increased IL-2 production above the level induced by crosslinking CD3 alone, suggesting that the presence of an anti-LFA-1 mAb, although

TABLE IV
*Anti-LFA-1 Does not Increase IL-2 Produced by Anti-CD3-stimulated
T Cell Clones Deficient in the Expression of LFA-1*

Specificity of mAb	IL-2 production					
	LAD 1		LAD 2		NP1	
	NIL	+ GaMIg	NIL	+ GaMIg	NIL	+ GaMIg
	<i>U/10⁶ cells</i>					
Control	0.0	0.0	0.0	0.0	0.0	0.0
CD3	0.0	1.0	0.0	1.0	0.0	0.0
CD18	0.0	0.0	0.0	0.0	0.0	0.0
CD3 + CD18	0.0	1.0	0.0	1.0	0.0	4.0
HLA-A,B,C	0.0	7.2	0.0	6.0	0.0	6.4
HLA-A,B,C + CD18	0.0	6.8	0.0	6.4	0.0	3.6

Clone LAD 1 (BR2; CD4⁺) and clone LAD 2 (BR4; CD4⁺), which do not express detectable surface LFA-1, and clone NP1 (CD4⁺, LFA-1⁺) were pretreated with the indicated mAb (control-P1.17; anti-CD3-OKT3; anti-CD18-60.3; anti-HLA-A,B,C-W6/32), washed, and incubated (10⁵ cells/well) with or without GaMIg. Supernatants were harvested at 18 h and assayed for IL-2 production.

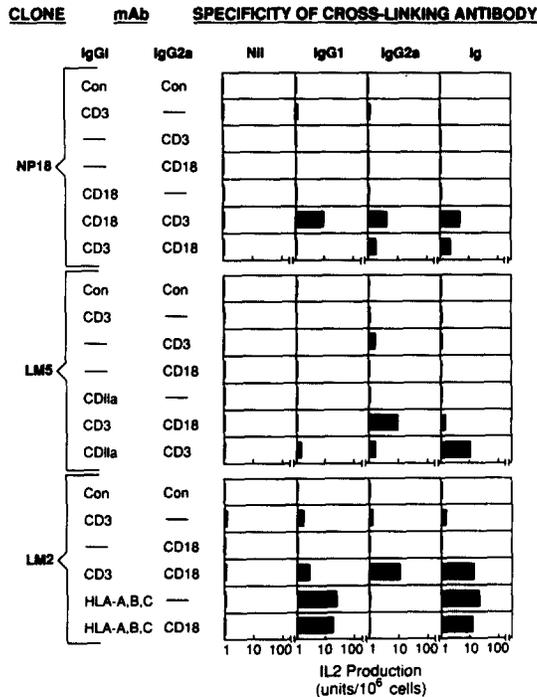


FIGURE 4. Specifically crosslinking LFA-1 to LFA-1 augments the functional response of anti-CD3-stimulated clones. Clones NP18 (CD8⁺), LM5 (CD4⁺), or LM2 (CD4⁺) were pretreated with saturating concentrations of the indicated mAb (control-P1.17 [IgG2a], MOPC [IgG1]; anti-CD3-Leu-4 [IgG1], OKT3 [IgG2a]; anti-CD18-TS1/18 [IgG1], 60.3 [IgG2a]; anti-CD11a-TS1/22 [IgG1]; anti-HLA-A,B,C-MB40.5 [IgG1]), washed and incubated (10⁵ cells/well) with isotype-specific anti-IgG1 or anti-IgG2a secondary antibodies or GaMIg. When anti-IgG1 or anti-IgG2a isotype-specific antibodies were used to crosslink, P1.17 (IgG2a) or MOPC (IgG1), respectively, were added to the cultures to prevent nonspecific crosslinking. Supernatants were harvested at 18 h and assayed for IL-2 production as described in Materials and Methods.

not crosslinked with a secondary antibody, might reciprocally enhance the anti-CD3 response.

The second experiment using clone LM5 showed that crosslinking the α chain (CD11a) when cells were pretreated with an anti-CD3 mAb and an anti-CD11a mAb increased IL-2 production, although the amount produced was small compared with crosslinking via CD18 in clone NP18. In the final experiment with clone LM2, the response to crosslinking CD18 by cells that were also pretreated with an anti-CD3 mAb was again increased over the response to crosslinking either CD3 or CD18 on cells pretreated with either mAb alone. In addition, when cells were pretreated with an anti-HLA-A,B,C mAb, crosslinking with either the isotype-specific antibodies or GaMIg stimulated IL-2 production. However, pretreatment of cells with an anti-CD18 mAb in addition to an anti-HLA-A,B,C mAb not only did not stimulate IL-2 production when only CD18 was crosslinked, but also somewhat decreased IL-2 production when HLA-A,B,C was crosslinked either using the isotype-specific antibody or GaMIg. In all of these experiments, the amount of IL-2 generated by crosslinking LFA-1 on cells pretreated with both an anti-CD3 and an anti-LFA-1 mAb was at least as great that generated by random crosslinking using GaMIg. It should be noted that in these experiments, excess control mAb of the alternate isotype was added to the cultures to eliminate any isotype-nonspecific crosslinking. Moreover, a high degree of specificity was observed in that IL-2 production was elicited only by the crosslinking antibody of the correct isotype. Thus, although the clones varied in their ability to respond to the stimuli provided, crosslinking LFA-1 alone on cells

carrying both an anti-CD3 mAb and an anti-LFA-1 mAb consistently enhanced IL-2 production above that observed by crosslinking either surface protein on cells treated with a single mAb.

Specifically Crosslinking LFA-1 Increases $[Ca^{2+}]_i$ if Cells Are Pretreated with Both an Anti-CD3 and an Anti-LFA-1 mAb. The previous experiments with isotype-specific crosslinking suggested that crosslinking LFA-1 could transmit an independent signal to the T cell. This was confirmed by measuring the change in $[Ca^{2+}]_i$ generated by isotype-specific crosslinking of individual surface proteins as shown in Fig. 5. As seen previously, crosslinking LFA-1 via either the α or the β chain with either the isotype-specific antibody or GaMIg on cells pretreated only with the anti-LFA-1 mAb did not increase $[Ca^{2+}]_i$. However, when cells were pretreated with both an anti-CD3 and an anti-CD18 mAb, followed by specific crosslinking of only the anti-LFA-1 mAb, a delayed, but sustained increase in $[Ca^{2+}]_i$ by 30–70% of T cells was observed. In Fig. 5 A, the delayed response that occurred after crosslinking only CD18 when cells were also pretreated with an anti-CD3 mAb was compared with the rapid response elicited by crosslinking only the anti-CD3 mAb with the appropriate isotype-specific antibody. The lack of an immediate increase in $[Ca^{2+}]_i$ when CD18 was crosslinked on cells also reacted with an anti-CD3 mAb demonstrates the specificity of the isotype-specific secondary antibody. Fig. 5 B shows that increasing $[Ca^{2+}]_i$ by crosslinking LFA-1 using an anti-CD18 mAb of a different isotype also required pretreating the cells with an anti-CD3 mAb. In addition, GaMIg-induced aggregation was compared with isotype-specific crosslinking. GaMIg-induced crosslinking of CD3 and CD18 resulted in an increased and more sustained signal. In Fig. 5 C, crosslinking either the α chain (CD11a) or the β chain (CD18) of LFA-1 when cells were also reacted with an anti-CD3 mAb was shown to be equally effective in increasing $[Ca^{2+}]_i$. Fig. 5 C also shows that specifically crosslinking CD3 on cells pretreated with both mAbs resulted in a more sustained increase in $[Ca^{2+}]_i$ than crosslinking CD3 on cells reacted with an anti-CD3 mAb alone. These results indicate that enhancement of the increase in $[Ca^{2+}]_i$ resulted from independently cross-

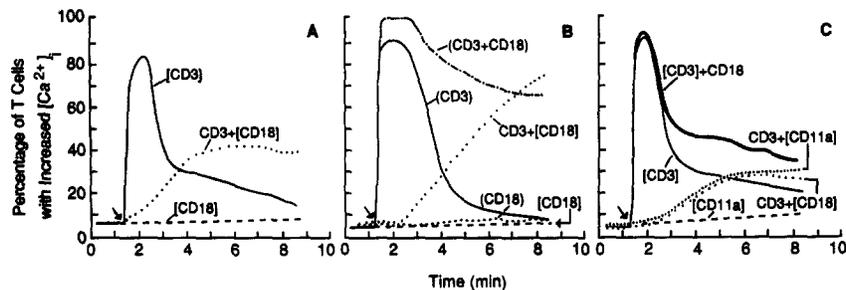


FIGURE 5. Crosslinking LFA-1 to LFA-1 when cells are also pretreated with an anti-CD3 mAb results in increased $[Ca^{2+}]_i$. (A) Clone NP1 ($CD4^+$) was pretreated with the following mAb: anti-CD3-OKT3 (IgG2a); anti-CD18-TS1/18 (IgG1). (B) Clone NP18 ($CD8^+$) was pretreated with the following mAb: anti-CD3-158.86 (IgG2b); anti-CD18-60.3 (IgG2a). (C) Clone NP13 ($CD8^+$) was pretreated with the following mAb: anti-CD3-OKT3 (IgG2a); anti-CD18-TS1/18 (IgG1); anti-CD11a-TS1/22 (IgG1). After washing, cells were analyzed as described in Fig. 1. Crosslinking by the appropriate isotype-specific secondary antibodies is indicated by brackets. Crosslinking by GaMIg (B) is indicated by parentheses.

linking either CD3 or CD11a/CD18 on cells reacted with mAbs to both determinants and did not require purposeful crosslinking of these structures. Thus, it was not necessary to aggregate CD3 to LFA-1 directly to generate and transmit an activation signal to the T cell clones.

The Enhancement of Signaling by the Combination of mAbs to LFA-1 and CD3 Requires Intact, Bivalent Anti-CD3 mAb. As shown above, crosslinking the LFA-1 molecule transmitted an activation signal to the cell if an anti-CD3 mAb was also bound to the cell. To determine whether bivalent anti-CD3 mAb was required or whether binding by a Fab fragment of an anti-CD3 mAb was sufficient, both the change in $[Ca^{2+}]_i$ and IL-2 production were examined. Fig. 6 shows that when cells were pretreated with an Fab fragment of an anti-CD3 mAb, a typical rapid increase in $[Ca^{2+}]_i$ occurred after crosslinking with GaMIg. Moreover, when cells were pretreated with both an anti-CD3 Fab fragment and either an anti-CD18 or an anti-CD11a mAb followed by crosslinking with GaMIg, a more sustained increase in $[Ca^{2+}]_i$ was seen similar to the pattern observed when CD3 and LFA-1 were crosslinked using a bivalent anti-CD3 mAb. However, when cells were pretreated with both the Fab fragment of an anti-CD3 mAb and an anti-CD18 mAb (Fig. 6 A) or an anti-CD11a mAb (Fig. 6 B) followed by crosslinking with an isotype-specific secondary antibody to crosslink only the LFA-1 molecule, no enhanced signal was observed.

When IL-2 production was examined (Table V), similar results were observed. Thus, neither the Fab fragment of the anti-CD3 mAb, nor crosslinking LFA-1 alone, resulted in IL-2 production, whereas crosslinking the Fab fragment of the anti-CD3 mAb with either an isotype-specific secondary antibody or GaMIg induced IL-2 production. However, specifically crosslinking the LFA-1 molecule when cells were

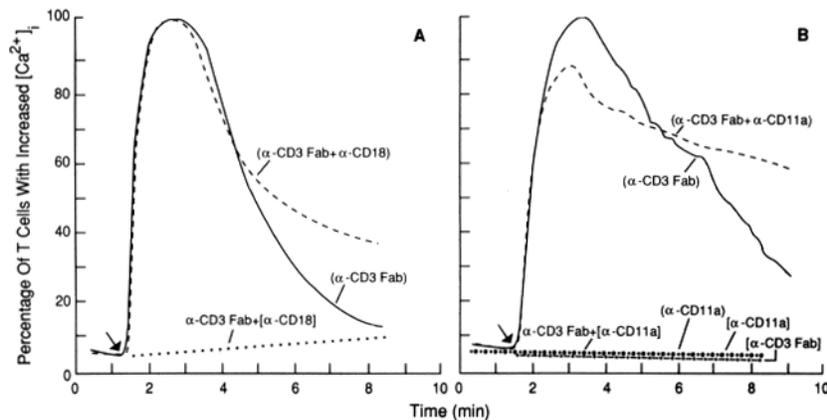


FIGURE 6. Crosslinking LFA-1 specifically when cells are also pretreated with a univalent anti-CD3 mAb Fab does not increase $[Ca^{2+}]_i$. (A) Clone NP19 ($CD4^+$) was pretreated with the following mAb: (α -CD3 Fab-64.1, IgG2a; α -CD18-TS1/18, IgG1). (B) Clone NP13 ($CD8^+$) was pretreated with the following mAb: (α -CD3 Fab-64.1, IgG2a; α -CD11a-TS1/22, IgG1). The brackets or parentheses indicate the surface antigen that was being crosslinked. Crosslinking of the α -CD3 Fab alone and the α -CD3 Fab + α -CD18/ α -CD11a was accomplished with GaMIg. Specific crosslinking of α -CD18/CD11a when cells also were reacted with the α -CD3 Fab was done with the anti-IgG1 isotype-specific antibody.

TABLE V
*Crosslinking LFA-1 Increases IL-2 Production When CD3 is
 Bound by Bivalent Anti-CD3 mAb*

Specificity of mAb	IL-2 production			
	Control*	IgG1*	IgG2a*	Ig*
	<i>U/10⁶ cells</i>			
Control	0.0	0.0	0.0	0.0
CD3(Fab)	0.0	0.0	14.5	2.0
CD11a	0.0	0.0	0.0	0.0
CD3(Fab) + CD11a	0.1	0.0	26.8	44.7

Clone NP18 (CD8⁺) was pretreated with the indicated mAb (control-P1.17 or MOPC; anti-CD3(Fab)-64.1, IgG2a; anti-CD11a-TS1/22, IgG1), washed, and incubated (10⁵ cells/well) with or without the indicated crosslinking antibody. When cells were incubated with an anti-IgG anti-IgG1 or an anti-IgG2a antibody, control mAb of the alternate isotype (P1.17-IgG2a or MOPC-IgG1, respectively) were added to block potential crossreactivity. Supernatants were harvested at 18 h and assayed for IL-2 production.

* Specificity of crosslinking antibody.

also reacted with the Fab fragment of the anti-CD3 mAb failed to increase IL-2 production, whereas crosslinking the Fab fragment of the anti-CD3 mAb on cells also reacted with anti-LFA-1 mAb resulted in marked enhancement of IL-2 production. These experiments suggest that intact bivalent anti-CD3 mAb must be bound on the cell surface in order for crosslinking of LFA-1 to transmit an activation signal to the cell.

Discussion

The LFA-1 molecule has a well-established role in mediating the adhesion or interaction of T cells and a variety of other cell types (1, 5, 6). By using human T cell clones that can be activated by crosslinking particular surface antigens in the absence of T cell-accessory cell or T cell-solid phase interactions, these studies have shown that LFA-1 can act as a signaling molecule as well. The costimulation of CD3-induced responses required expression of the LFA-1 molecule, and, therefore, could not be explained by unanticipated crossreactivity of the mAb. Thus, T cells cloned from a child with LAD and no detectable surface α or β chain did not exhibit the prolongation of the anti-CD3-induced calcium signal or the enhancement of anti-CD3-stimulated IL-2 production. The lack of a requirement for cellular interactions in the generation of activation signals by crosslinking LFA-1 was confirmed during the measurement of [Ca²⁺]_i by flow cytometry. Not only are the cells in dilute suspension and constantly being stirred, but only single cells, not doublets or aggregates, were analyzed in the flow cytometer.

Study of the change in [Ca²⁺]_i first suggested that crosslinking LFA-1 molecules delivers direct signals to the T cells. The change in [Ca²⁺]_i is important because an increase in [Ca²⁺]_i is one of the critical early biochemical changes in T cell activation (22). Moreover, a change in [Ca²⁺]_i demonstrates that transmembrane sig-

naling has occurred. Crosslinking LFA-1 via either the α or the β chain using GaMIg or an isotype-specific secondary antibody induced little or no change in $[Ca^{2+}]_i$ in cells reacted with only a single mAb. This is consistent with our previous results (19) and those of others (23) using freshly prepared human peripheral T lymphocytes, but different from another study in which a small increase in $[Ca^{2+}]_i$ was observed after crosslinking (24). The cells used in that report were not purified, however, and the minimal increase in $[Ca^{2+}]_i$ was limited to a small percentage of cells. Therefore, it was possible that anti-LFA-1 mAb directly induced an increase in $[Ca^{2+}]_i$ in cells other than T cells.

Although mAbs to LFA-1 did not induce a change in $[Ca^{2+}]_i$ alone, they did alter the calcium response induced by anti-CD3 mAb. Thus, when clones were pretreated with an anti-CD3 mAb and either an anti-CD18 or CD11a mAb followed by crosslinking, a prolongation of the anti-CD3-induced elevation in $[Ca^{2+}]_i$ was observed. This increase, though small, was consistently observed in the different clones and was detected whether LFA-1 was crosslinked via the α or β chain. This augmentation was limited to CD3 as crosslinking LFA-1 and HLA-A,B,C or CD4/CD8 (data not shown) diminished the typical delayed, sustained calcium response seen after crosslinking these surface antigens. The costimulation via LFA-1 is different from that observed with mAbs to other surface molecules that are co-mitogenic for anti-CD3-stimulated cells. Thus, mAb to HLA-A,B,C, CD2, CD4/CD8, CD5, or CD28 can induce readily detectable increases in $[Ca^{2+}]_i$ when crosslinked alone on cells not reacted with an anti-CD3 mAb (13, 19, 24, 25), whereas mAbs to LFA-1 do not. These results indicate that the ability of a mAb that recognizes a surface antigen to costimulate with anti-CD3 is not predicted by its ability to induce a change in $[Ca^{2+}]_i$ alone.

Crosslinking CD3 with LFA-1 not only prolonged the increase in $[Ca^{2+}]_i$ but also augmented T cell activation as measured by $[^3H]$ thymidine incorporation or IL-2 production. The enhancement of responses induced by crosslinking LFA-1 did not occur with all modes of stimulation as concomitant crosslinking of HLA-A,B,C with LFA-1 did not affect or somewhat diminished the response to crosslinking HLA-A,B,C alone. There is no clear explanation for the difference between the response induced by the combination of anti-CD3 + LFA-1 and that from anti-HLA-A,B,C + LFA-1, although the results suggest that stimulation by the CD3/TCR molecular complex might be uniquely costimulated by engagement of LFA-1 molecules. Thus, costimulation via LFA-1 may be of special importance during the accessory cell-T cell interaction involved in antigen presentation.

Because activation by LFA-1 could only be demonstrated when CD3 was engaged, the relationship between crosslinking CD3 and LFA-1 was further examined by specifically crosslinking one or the other surface antigen with isotype-specific secondary antibodies when cells were pretreated with two mAbs of different isotypes. It was important in these experiments that the isotype-specific crosslinking antibodies did not nonspecifically bind mAbs of another isotype. In our experiments, a high degree of isotype specificity was present in that IL-2 production in cells reacted with a single mAb was elicited only by crosslinking antibody of the correct isotype, even when control mAb of the alternate isotype normally added in excess was not added

to the cultures. In addition, the isotype-specific antibodies increased $[Ca^{2+}]_i$ when the surface mAb was of the correct isotype, but not when cells were stained with mAbs of a different isotype in control experiments (data not shown). Thus, no cross-reactivity was detected at the level of sensitivity of the techniques used. The isotype-specific crosslinking experiments demonstrated that it was not necessary to directly crosslink CD3 to LFA-1 to generate an activation signal. Although crosslinking cells reacted with the anti-LFA-1 mAb alone did not stimulate the cells or increase $[Ca^{2+}]_i$, crosslinking LFA-1 when the cells carried an anti-CD3 mAb that was not further crosslinked was a strong stimulus for IL-2 production and increasing $[Ca^{2+}]_i$. Of note, the stimulation of IL-2 production by independently crosslinking LFA-1 on cells pretreated with both an anti-CD3 and an anti-LFA-1 mAb was at least as effective as aggregating CD3 + LFA-1 by crosslinking with GaMIg. However, univalent binding of the CD3 complex by an anti-CD3 Fab fragment was not sufficient to augment the LFA-1 signal. The requirement for bivalent anti-CD3 mAb indicates that some aggregation of CD3 molecules is necessary for costimulation.

Although our data are the most direct demonstration of signaling via the LFA-1 molecule, other work has been consistent with such a role for this surface antigen. A mAb that recognized the α chain of mouse LFA-1 stimulated proliferation of CTL clones without additional crosslinking of this IgM mAb (26). However, this mAb coprecipitated proteins in addition to the LFA-1 α subunit so that the stimulatory effect could not be definitely ascribed to its binding of the LFA-1 molecule. The observation that immobilized anti-LFA-1 mAb could stimulate peripheral blood T cells if PMA was present suggested that immobilized anti-LFA-1 mAb triggered costimulatory increases in $[Ca^{2+}]_i$ (10). However, the current findings that mAbs to LFA-1 failed to induce measurable increases in $[Ca^{2+}]_i$, even if crosslinked, question that conclusion. Our results also differ somewhat from those of van Noesel et al. (12), who found that soluble anti-CD18 mAbs were inhibitory of immobilized anti-CD3-induced proliferation, whereas soluble anti-CD11a mAbs increased responses. We have previously been unable to show inhibition of immobilized anti-CD3-induced proliferation by soluble anti-CD18 mAbs (9). Moreover, the findings of van Noesel et al. (12) are difficult to reconcile with the current results that clearly demonstrate that augmentation of anti-CD3-induced T cell activation can occur when either anti-CD18 or anti-CD11a mAb is used as a costimulant. No major differences in the signaling potential of the α or β chains of the LFA-1 molecule could be documented. Thus, although previous work has suggested that LFA-1 might have a signaling role, consistent results to support this possibility have not emerged. The current experiments using T cell clones avoided some of the pitfalls of previous studies and clearly demonstrated a costimulatory role for LFA-1 in anti-CD3-activated T cells.

Perturbation of other molecules of the integrin family may also result in signal transduction. Thus, for example, epinephrine-induced stimulation of the Na^+/H^+ antiporter in platelets was found to be dependent upon fibrinogen binding of the integrin glycoprotein IIb-IIIa (27), and binding of gp IIb-IIIa resulted in cytoskeletal association of the protein (28, 29). In 3T3 cells, fibronectin binding to a fibronectin receptor resulted in differential expression of a number of genes (30). In addition, a mAb that recognized CD11b (Mac-1) has been noted to provide a costimulatory signal for macrophage activation (31). Although these experiments are more indirect

than those of the present study, they support the possibility that signal transduction is a more general property of the integrin family of molecules.

Association with the cytoskeleton may be an important mechanism by which LFA-1 or other integrin molecules can participate in cellular activation. Cytoskeletal components localize in areas of cell contact when integrin family molecules are engaged (1, 32, 33). In addition, a plasma membrane fibronectin receptor, the cell-substrate attachment antigen, physically binds a cytoskeletal component, talin (34). Cross-linking of LFA-1 on murine T cells is claimed to cause redistribution of talin, but not other cytoskeletal proteins if the cells are also treated with phorbol esters (35). These results, although preliminary, suggest that the receptor-ligand binding of integrin family molecules may result in establishment of a bridge to the cytoskeleton that may be important in cellular activation.

Additional evidence that the integrin molecules may be involved in signal transduction derives from studies of protein phosphorylation. Thus, the α chain of the LFA-1 molecule is constitutively phosphorylated and β chain phosphorylation can be induced by PMA (36). Moreover, the cell-substrate attachment antigen sequence has been determined and contains a tyrosine residue in a region homologous with the phosphorylation site of epidermal growth factor, suggesting that tyrosine phosphorylation of this molecule may be important in regulating its function (37). The data thus suggest the possibility that LFA-1 and other integrin molecules can be phosphorylated and can associate with the cytoskeleton. Either or both of these mechanisms may be important in their capacity to function as signaling molecules.

These studies demonstrate cooperativity between the signal-transducing capabilities of CD3 and LFA-1. Independently crosslinking LFA-1 generates an activation signal for the cell, but only when CD3 is also engaged by a bivalent mAb. There are a number of potential explanations for this finding, including the possibility that there is no functional or physical interaction between the two molecules, but that the signals generated independently synergize in activating the cell. Since it is difficult to show any signaling capability of LFA-1 alone, this does not appear to be likely. Alternatively, engagement of one of the molecules might lead to enhanced signaling capability of the other and thereby lead to an enhanced functional response. One potential mechanism for the latter phenomenon could be through cytoskeletal reorganization, as binding of either the CD3/TCR or LFA-1 is associated with directed movement of a number of components of the cytoskeleton (35, 38). Although the details of these rearrangements are not known, it is possible that movement of cytoskeletal proteins triggered by crosslinking of one of these surface determinants could result in an organization of the other molecule that is more conducive to signaling. Both CD3 and LFA-1 also undergo phosphorylation of cytoplasmic sequences (36, 39). Thus, activation of protein kinases, including protein kinase C or A, by engagement of these surface antigens could be used to communicate a positive influence to other signaling molecules in the cell membrane. Either or both of these events could result in cooperativity between CD3- and LFA-1-induced transmembrane signaling. In this regard, it is tempting to speculate that binding of T cells and APC via LFA-1-ICAM interactions might lead to enhanced signaling through the CD3/TCR complex when antigen is recognized. Thus, interaction with LFA-1 under physiological circumstances may not only facilitate cellular adhesion, but in doing so, may deliver important costimulatory signals to amplify T cell activation.

Summary

The leukocyte function-associated antigen 1 (LFA-1) molecule is well established as a surface protein involved in cellular adhesion and interaction, but there has been little information about whether engagement of this molecule can also directly modify cellular activation. These studies demonstrate that crosslinking the LFA-1 molecule on human T cell clones transmits a unique signal to the cell. Crosslinking LFA-1 alone did not increase intracellular calcium ($[Ca^{2+}]_i$), nor did crosslinking LFA-1 activate the cells as measured by IL-2 production or $[^3H]$ thymidine incorporation. However, when CD3 and LFA-1 were crosslinked, a more prolonged calcium signal was observed than when CD3 alone was crosslinked. Moreover, IL-2 production and DNA synthesis were greatly augmented. These responses could be demonstrated when LFA-1 was crosslinked via either the α or the β chain, and required surface expression of the LFA-1 molecule as no enhancement was observed in T cell clones from a child with leukocyte adhesion deficiency. The enhancement of cellular activation by LFA-1 did not require that it be directly crosslinked to the CD3 complex. Thus, crosslinking LFA-1 alone with isotype-specific secondary antibodies on cells also pretreated with an anti-CD3 mAb of a different Ig isotype stimulated the cells as effectively as crosslinking both surface antigens with GaMIg. Similarly, a delayed, but sustained increase in $[Ca^{2+}]_i$ was elicited. This increase in $[Ca^{2+}]_i$ and the enhanced functional responses required engagement of CD3 with an intact bivalent anti-CD3 mAb, as crosslinking LFA-1 on cells also reacted with Fab fragments of an anti-CD3 mAb did not increase $[Ca^{2+}]_i$, nor activate the cells. These data indicate that LFA-1 can convey activation signals to T cells. Synergism in signaling can be observed upon crosslinking of LFA-1 and independently crosslinking CD3. In the physiologic interaction between T cells and accessory cells, the interaction of LFA-1 with its ligand, intercellular adhesion molecule 1, may therefore not only facilitate cellular adhesion, but also may amplify T cell activation by delivering costimulatory signals.

We wish to thank Dr. T. A. Springer, Dr. E. Vitetta, and Dr. P. Beatty for their generous gifts of many of the mAb used in these studies, Dr. D. Anderson for his help in obtaining peripheral blood from a child with leukocyte adhesion deficiency, Gary Goff for his excellent technical assistance, and Lisa Wicktor for her help in the flow cytometric studies.

Received for publication 10 April 1989.

References

1. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell*. 46:549.
2. Kishimoto, T. K., K. O'Connor, A. Lee, T. M. Roberts, and T. A. Springer. 1987. Cloning of the β subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. *Cell*. 48:681.
3. Sanchez-Madrid, F., J. A. Nagy, E. Robbins, P. Simon, and T. A. Springer. 1983. A human leukocyte differentiation family with distinct α subunits and a common β subunit: the lymphocyte function-associated antigen (LFA-1), the C3bi complement receptor (OKM1/Mac-1), and the p150,95 molecule. *J. Exp. Med.* 158:1785.
4. McMichael, A. J. 1987. Leukocyte Typing III: White Cell Differentiation Antigens. Oxford University Press, New York. 949 pp.

5. Anderson, D. C., and T. A. Springer. 1987. Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. *Annu. Rev. Med.* 38:175.
6. Springer, T. A., M. L. Dustin, T. K. Kishimoto, and S. D. Marlin. 1987. The lymphocyte function associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. *Annu. Rev. Immunol.* 5:223.
7. Martz, E. 1987. LFA-1 and other accessory molecules functioning in adhesions of T and B lymphocytes. *Hum. Immunol.* 18:3.
8. Davignon, D., E. Martz, T. Reynolds, K. Kurzinger, and T. A. Springer. 1981. Lymphocyte function-associated antigen one (LFA-1): a surface antigen distinct from Lyt-2/3 that participates in T lymphocyte-mediated killing. *Proc. Natl. Acad. Sci. USA.* 78:4535.
9. Geppert, T. D., and P. E. Lipsky. 1988. Activation of T lymphocytes by immobilized monoclonal antibodies to CD3. Regulatory influences of monoclonal antibodies to additional T cell determinants. *J. Clin. Invest.* 81:1497.
10. Carrera, A. C., M. Rincon, F. Sanchez-Madrid, M. Lopez-Botet, and M. O. de Landazurei. 1988. Triggering of co-mitogenic signals in T cell proliferation by anti-LFA-1 (CD18, CD11a), LFA-3, and CD7 monoclonal antibodies. *J. Immunol.* 141:1919.
11. Truneh, A., F. Alber, P. Golstein, and A.-M. Schmitt-Verhulst. 1985. Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. *Nature (Lond.)* 313:318.
12. van Noesel, C., F. Miedemann, M. Brouwer, M. A. de Rie, L. A. Aarden, and R. A. W. van Lier. 1988. Regulatory properties of LFA-1 α and β chains in human T lymphocyte activation. *Nature (Lond.)* 333:850.
13. Wacholtz, M. C., S. S. Patel, and P. E. Lipsky. 1989. Patterns of co-stimulation of T cell clones by cross-linking CD3, CD4/CD8, and class I MHC molecules. *J. Immunol.* In press.
14. Van-Wauwe, J. P., J. R. De-Mey, and J. G. Goossens. 1980. OKT3: a monoclonal anti-human T lymphocyte antibody with potent mitogenic properties. *J. Immunol.* 124:2708.
15. Patel, S. S., D. L. Thiele, and P. E. Lipsky. 1987. Major histocompatibility complex-unrestricted cytolytic activity of human T cells. Analysis of precursor frequency and effector phenotype. *J. Immunol.* 139:3886.
16. Patel, S. S., A. D. Duby, D. L. Thiele, and P. E. Lipsky. 1988. Phenotypic and functional characterization of human T cell clones. *J. Immunol.* 141:3726.
17. Davis, L., R. Vida, and P. E. Lipsky. 1986. Regulation of human T lymphocyte mitogenesis by antibodies to CD3. *J. Immunol.* 137:3758.
18. Thiele, D. L., S. S. Patel, and P. E. Lipsky. 1988. Anti-CD3 and phorbol myristate acetate regulation of MHC unrestricted T cell cytotoxicity. Lack of a requirement for CD3/T cell receptor complex expression during tumor cell lysis. *J. Immunol.* 140:3252.
19. Geppert, T. D., M. C. Wacholtz, L. S. Davis, and P. E. Lipsky. 1988. Activation of T4 cells by cross-linking class I MHC molecules. *J. Immunol.* 140:2155.
20. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440.
21. Geppert, T. D., M. C. Wacholtz, S. S. Patel, E. Lightfoot, and P. E. Lipsky. 1989. Activation of human T cell clones and Jurkat cells by cross-linking class I major histocompatibility complex molecules. *J. Immunol.* 142:3763.
22. Weiss, A., J. Imboden, K. Hardy, B. Manger, C. Terhorst, and J. Stobo. 1986. The role of the T3/antigen receptor complex in T cell activation. *Annu. Rev. Immunol.* 4:593.
23. O'Flynn, K., A. M. Krensky, P. C. L. Beverley, S. J. Burakoff, and D. C. Linch. 1985. Phytohaemagglutinin activation of T cells through the sheep red blood cell receptor. *Nature (Lond.)* 313:686.
24. Ledbetter, J. A., C. H. June, L. S. Grosmaire, and P. S. Rabinovitch. 1987. Crosslinking

- of surface antigens causes mobilization of intracellular ionized calcium in T lymphocytes. *Proc. Natl. Acad. Sci. USA*. 84:1384.
25. June, C. H., P. S. Rabinovitch, and J. A. Ledbetter. 1987. CD5 antibodies increase intracellular ionized calcium concentration in T cells. *J. Immunol.* 138:2782.
 26. Pucher, H., P. Groscurth, S. Baumhutter, M. Aguet, R. M. Zinkernagel, and H. Hengartner. 1986. A monoclonal antibody against altered LFA-1 induces proliferation and lymphokine release of cloned T cells. *Eur. J. Immunol.* 16:172.
 27. Banga, H. S., E. R. Simons, L. F. Bass, and S. E. Rittenhouse. 1986. Activation of phospholipases A and C in human platelets exposed to epinephrine: role of glycoproteins IIb/IIIa and dual role of epinephrine. *Proc. Natl. Acad. Sci. USA*. 83:9197.
 28. Phillips, D. R., L. H. Jennings, and H. H. Edwards. 1980. Identification of membrane proteins mediating the interaction of human platelets. *J. Cell. Biol.* 86:77.
 29. Painter, R. G., and M. Ginsburg. 1982. Concanavalin A induces interactions between surface glycoproteins and the platelet cytoskeleton. *J. Cell. Biol.* 92:565.
 30. Spiegelman, B. M., and C. A. Ginty. 1983. Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes. *Cell*. 35:657.
 31. Ding, A., S. D. Wright, and C. Nathan. 1987. Activation of mouse peritoneal macrophages by monoclonal antibodies to Mac-1 (complement receptor type 3). *J. Exp. Med.* 165:733.
 32. Chen, W.-T., E. Hasegawa, T. Hasegawa, C. Weinstock, and K. M. Yamada. 1985. Development of cell surface linkage complexes in cultured fibroblasts. *J. Cell Biol.* 100:1103.
 33. Damsky, C. H., K. A. Knudsen, D. Bradley, C. A. Buck, and A. F. Horwitz. 1985. Distribution of the cell substratum attachment (CSAT) antigen on myogenic and fibroblastic cells in culture. *J. Cell Biol.* 100:1528.
 34. Horwitz, A., K. Kuggan, C. Buck, M. C. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin: a transmembrane linkage. *Nature (Lond.)*. 320:531.
 35. Burn, P., A. Kupfer, and S. J. Singer. 1988. Dynamic membrane-cytoskeletal interactions: Specific association of integrin and talin arises in vivo after phorbol ester treatment of peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. USA*. 85:497.
 36. Hara, T., and S. M. Fu. 1986. Phosphorylation of α , β subunits of 180/100-Kd polypeptides (LFA-1) and related antigens. In *Leukocyte Typing II. Volume III. Human Myeloid and Hematopoietic Cells*. E. L. Reinherz, B. F. Haynes, L. M. Nadler, and I. D. Bernstein, editors. Springer-Verlag, New York, Inc., New York. 77-84.
 37. Tamkin, J. W., D. W. DeSimone, D. Fonda, R. S. Patel, C. Burk, A. F. Horwitz, and R. O. Hynes. 1986. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell*. 46:271.
 38. Kupfer, A., S. J. Singer, C. A. Janeway, and S. L. Swain. 1987. Coclustering of CD4 (L3T4) molecule with the T-cell receptor is induced by specific direct interaction of helper T cells and antigen-presenting cells. *Proc. Natl. Acad. Sci. USA*. 84:5888.
 39. Patel, M. D., L. E. Samuelson, and R. D. Klausner. 1987. Multiple kinases and signal transduction. Phosphorylation of the T cell receptor complex. *J. Biol. Chem.* 262:5831.