0022-1767/88/1416-1904$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 141, 1904–1911, No. 6, September 15, 1988
Printed in U.S.A.

THE CD2 LIGAND LFA-3 ACTIVATES T CELLS BUT DEPENDS ON THE EXPRESSION AND FUNCTION OF THE ANTIGEN RECEPTOR[1]

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The T cell Ag receptor (CD3/Ti) and the sheep E receptor (CD2) expressed on the surface of human T cells are both capable of initiating intracellular signals necessary for T cell activation. CD3/Ti interacts with Ag to initiate cellular immune responses. Although the exact function of CD2 is unknown, lymphocyte function-associated Ag 3 (LFA-3), a 55- to 70-kDa receptor expressed on a broad spectrum of hemopoietic and nonhemopoietic cells, has recently been shown to be its natural ligand. We show here that although purified multimeric LFA-3 is not capable of initiating transmembrane signaling events on its own, the combination of LFA-3 and the anti-CD2 mAb CD2.1 induces intracellular calcium increases, phosphatidylinositol second messenger generation and lymphokine secretion in the T cell leukemic line Jurkat. In order to study the signaling requirements of CD2, we compared the ability of CD2 mAb and LFA-3 to initiate activation signals in Jurkat and in three Jurkat-derived mutants. A $CD3^-CD2^+$ mutant failed to increase calcium or exhibit phosphatidylinositol hydrolysis to either the combination of agonist CD2 mAb 9-1 and 9.6 or LFA-3 and CD2.1. Reconstitution of the Ag receptor by transfection of the Ti-β-chain restored the expression of the CD3/Ti complex and the ability to respond to either combination of CD2 ligands. However, no response to CD2 ligands was detected in a $CD3^+CD2^+$ mutant selected for signaling defects to CD3/Ti ligands. Complementation of the CD3/Ti signaling defect by cell fusion also restored competency to respond to CD2 agonists. These results demonstrate that LFA-3 under appropriate conditions can activate T cells via the CD2 complex and that this activation requires not only the cell surface expression of the CD3/Ti complex but also a functional Ag receptor pathway.

The activation of T lymphocytes results in a myriad of biologic effects, including the production of lymphokines, the appearance of new cell surface proteins, the acquisition of cytolytic function, and cellular proliferation. The use of mAb has allowed the identification of several distinct cell surface molecules that are involved in T cell activation. Some of these molecules (CD3/Ti,[3] CD2, Thy-1) are capable of inducing activation of T cells after ligand binding in the presence of accessory cells (reviewed in Ref. 1). Others (CD28, CD5, IL-1R) appear to function by augmenting the T cell activation response or by substituting for a function provided by accessory cells. Why T cells express many different receptors that, when stimulated under the appropriate conditions, exert identical biologic responses is unknown. The degree of interaction between these receptors and the commonality of the intracellular pathways through which they initiate cellular activation are also unclear.

On human T lymphocytes, two cell surface receptors have been identified to date that initiate activation signals upon stimulation: the T cell Ag receptor, CD3/Ti, and the sheep E receptor, CD2 (Thy-1 is not expressed on human peripheral T cells). CD3/Ti consists of a 90-kDa disulfide-linked heterodimer (Ti) which is noncovalently associated with five to seven invariant chains (CD3) (2–6). Ti functions to recognize antigen when presented along with the appropriate MHC molecules (7–9). The function of the CD3 complex is less clear, but it may play a role in initiating cellular activation (1).

CD2 is a 50-kDa glycoprotein found on the surface of thymocytes, mature T cells and NK cells (10, 11). Under various conditions, monoclonal antibodies to CD2 function as agonists in T cell activation (12, 13). These antibodies define several distinct epitopes on the CD2 molecule, which include $T11_1$/9.6, $T11_2$/CD2.1, $T11_3$, and 9-1 (12–14). The latter two epitopes are not expressed at high levels on normal resting T cells but are inducible either by certain anti-CD2 antibodies or by activation of T cells via CD3/Ti (12–14). Although single antibodies against these epitopes are not mitogenic, combinations of anti-CD2 antibodies are capable of inducing IL-2 production and T cell proliferation (12–15). Because this form of activation does not involve CD3/Ti ligands, this pathway has been referred to as the alternate pathway of T cell activation.

Received for publication March 31, 1988.
Accepted for publication June 9, 1988.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported, in part, by a post-doctoral fellowship to L. K. B. from the Arthritis Foundation. A. W. is an Arthritis Foundation investigator and M. A. G. is an M.D./Ph.D. candidate in the NIH Medical Scientist Training Program and is supported by the Rosalind Russell Arthritis Center.
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[3] Abbreviations used in this paper: CD3/Ti, T cell antigen receptor; LFA-3, lymphocyte function-associated Ag 3; $[Ca^{2+}]_i$, intracellular free calcium; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol.

The mechanisms by which CD3/Ti and CD2 initiate T cell activation have been only partially characterized. Antibodies to CD3 or Ti and activating combinations of antibodies to CD2 induce the hydrolysis of PIP_2 to yield the second messengers IP_3 and DAG (16–18). Indirect evidence suggests that CD3/Ti-induced PIP_2 hydrolysis is dependent upon a GTP-binding protein because cholera toxin inhibits anti-CD3 and anti-Ti-induced generation of inositol phosphates and because AlF_4^-, which is thought to activate a phospholipase C-coupled GTP-binding protein, induces hydrolysis of PIP_2 in T cells (19–21). IP_3 causes release of calcium from sequestered intracellular pools, resulting in an immediate increase in $[Ca^{2+}]_i$. IP_3 or its metabolites may be responsible for the sustained increases in $[Ca^{2+}]_i$ observed following CD3/Ti or CD2 stimulation (22–24). DAG activates protein kinase C, providing another signal necessary for cellular activation, and can be substituted for by DAG analogs or phorbol esters (25). An increase in $[Ca^{2+}]_i$ and activation of protein kinase C have been causally linked to lymphokine gene activation through as yet poorly characterized events (1).

Inasmuch as stimulation under appropriate conditions of either the antigen receptor or the alternate activation pathway results in PI second messenger generation and calcium increases, it is possible that these two distinct receptors utilize activation pathways that converge somewhere in the cascade of events leading to PIP_2 hydrolysis. Recent evidence that alternate pathway activation by using mAb requires the cell surface expression of the CD3/Ti complex (26, 27) has made this hypothesis more compelling, although the exact mechanism of this dependency is not known.

Recently, a physiologic ligand of CD2 has been identified (28–31). LFA-3 is a 55- to 70-kDa protein expressed on a wide variety of tissue types, including endothelial cells, epithelial cells, connective tissue cells, and most blood cells. Competitive binding studies and mutagenesis studies have demonstrated that LFA-3 binds to the $T11_1$ epitope of CD2 (29–32). SRBC, which express high densities of a ligand homologous to LFA-3 (31), are unable to activate T cells alone, but can induce T cell proliferation in the presence of anti-$T11_3$ mAb (33). Similarly, in preliminary experiments, combinations of purified erythrocyte LFA-3 and an anti- $T11_2$ mAb, CD2.1 (15), activated peripheral blood T cells, as measured by cellular proliferation and calcium mobilization.[4] In this report, we show that the CD2 natural ligand LFA-3 in soluble form under appropriate conditions can initiate early events characteristic of T cell activation that culminate in potent stimulation of lymphokine production, and that these events are dependent on the cell surface expression of a functional CD3/Ti molecular complex.

MATERIALS AND METHODS

Cells. The T cell leukemic line Jurkat (clone E6-1), the Ti-β-chain-deficient Jurkat mutant J.RT3-T3.5, PF-2.4 (a cell derived from J.RT3-T3.5 into which a β-chain cDNA has been transfected resulting in reexpression of CD3/Ti) and the Jurkat-derived signaling mutant J.CaM1 have been described previously (34–36). All cells were maintained in RPMI 1640 medium supplemented with 10% FCS and penicillin, streptomycin, and glutamine.

[4] **M. L. Dustin, D. Olive and T.A. Springer.** Lymphocyte function-associated 3 antigen interaction with CD2 and T lymphocyte activation. *Submitted for publication.*

mAb and reagents. Purified FITC conjugates of mouse IgG_1, the anti-CD3 mAb (anti-Leu 4) and the anti-CD2 mAb (anti-Leu 5) were obtained from Becton Dickinson Monoclonal Center, Mountain View, CA. The OKT3-producing hybridoma was obtained from the American Type Culture Collection, Rockville, MD. Purified anti-CD2 mAb 9.6 and 9-1 were generously provided by Dr. Jeffrey Ledbetter (Oncogen Corp., Seattle, WA) and Dr. Bo Dupont (Sloan Kettering Institute for Cancer Research, New York, NY), respectively. CD2.1 is an anti-CD2 antibody previously described (15). LFA-3 was isolated from solubilized human E membranes (29–32) and multimeric material was prepared by removal of octyl glucoside detergent by ultrafiltration (see footnote 4).

Immunofluorescence. Cells were stained with FITC-conjugates of the indicated mAb and flow cytometric analyses of relative immunofluorescence were performed on a FACS IV (Becton Dickinson) as previously described (34).

Indo-1 analysis. Cells were loaded with 3 μM Indo-1 (Molecular Probes, Junction City, OR) (37) as detailed previously (36), washed, and resuspended at a final concentration of 5×10^6/ml. Indo-1 fluorescence was measured in a Spex Fluorolog II spectrofluorimeter and $[Ca^{2+}]_i$ was determined in response to various stimuli as described (37).

Heterokaryon assay. This assay is described in detail elsewhere (38). Briefly, partner 1 cells were loaded with Indo-1 as for fluorimetry. Partner 2 cells were stained with a fluoresceinated mAb against HLA class I molecules. The cells were then mixed together, exposed to polyethylene glycol briefly, washed, and then incubated for 1 h. The mixed sample of cells and heterokaryons was then analyzed by FACS, with excitation beams and emission detection for both Indo-1 and FITC. Electronic gates were set to detect only partner 1 cells containing Indo-1 and heterokaryons containing Indo-1 and FITC. The "Indo-1 fluorescence ratio" is the calculated ratio of emission by intracellular Indo-1 at 404 nm (near the maximum for Ca^{2+}-bound Indo-1) vs that at 486 nm (near the maximum for Ca^{2+}-free Indo-1), with a higher ratio indicating a higher $[Ca^{2+}]_i$.

Measurement of [^{3}H]inositol phosphates. Cells were labeled with [3H]-*myo*-inositol as previously described (16), washed twice and then resuspended at 5×10^6/ml in buffer (RPMI 1640 with 10 % FCS, 10 mM HEPES) alone or buffer containing OKT3 (1:1000) or the combination of CD2.1 (1:10,000) and LFA-3 (0.3 μg/ml). [^{3}H] Inositol phosphates were then extracted and quantitated as described (16) after stimulation of the cells for 7 min, which was the optimal time for maximal IP_3 generation.

IL-2 production and biologic assay. Triplicate cultures of 4×10^5 cells in 200 μl RPMI 1640 with 10% FCS were stimulated with ionomycin (1.0 μM, Calbiochem, La Jolla, CA), OKT3 ascites (1:1000), purified E LFA-3 (0.3 μg/ml), or CD2.1 ascites (1:10,000) in the presence or absence of PMA (80 nM, Sigma Chemical Co., St. Louis, MO) for 24 h. Cell-free supernatants were harvested after 24 h of culture and were assessed for IL-2 activity (39) with the IL-2-dependent cell line CTLL-2 (clone 20) generously provided by Dr. Kendall Smith, in the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described (40).

RESULTS

CD2 ligand LFA-3 in combination with CD2.1 causes a rise in $[Ca^{2+}]_i$. In studies presented elsewhere (see footnote 4), peripheral blood T cells could be activated via the alternate pathway by the CD2 physiologic ligand LFA-3 in soluble form only in the presence of the CD2 mAb CD2.1. In order to determine the mechanism(s) by which activation of T cells stimulated with LFA-3 and CD2.1 takes place, we further examined the abilities of LFA-3 and CD2.1, alone or in combination, to cause changes in $[Ca^{2+}]_i$ in the $CD3^+CD2^+$ T cell leukemic line Jurkat. Addition of LFA-3 alone had no effect on the level of $[Ca^{2+}]_i$ in Jurkat cells as measured with the Ca^{2+}-dependent fluorescent indicator Indo-1 (Fig. 1*A*). Likewise, CD2.1 alone failed to induce an increase in $[Ca^{2+}]_i$ (data not shown). However, preincubation of Jurkat cells with LFA-3 followed by the addition of CD2.1 resulted in immediate and sustained increases in $[Ca^{2+}]_i$ (Fig. 1*B*). Similarly, preincubation with CD2.1 followed by the addition of LFA-3 as a second stimulus resulted in a comparable elevation of $[Ca^{2+}]_i$ (Fig. 1*C*). These results confirm that LFA-3 in combination with CD2.1 can initiate T cell

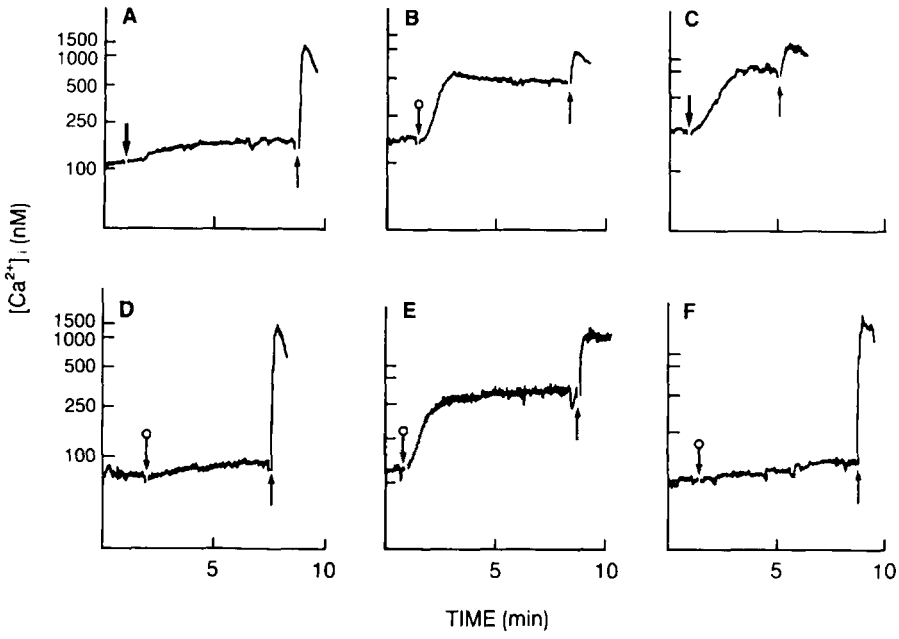

Figure 1. Calcium responses of Indo-1 loaded Jurkat and the three mutants to LFA-3 and CD2.1. *A*) Response of Jurkat to purified E LFA-3 (↓) followed by the calcium ionophore ionomycin (↑). Responses were undetectable with a dose of LFA-3 ranging from 0.01 μg/ml to 10 μg/ml (data not shown). Similarly, mAb CD2.1 in doses ranging from 1/100,000 to 1/100 dilution of ascites stimulated no significant rise in $[Ca^{2+}]_i$ (data not shown). *B*) Jurkat cells were preincubated for 20 min on ice with LFA-3, warmed to 37°C, placed in the fluorimeter, then stimulated with CD2.1 (♀) at the indicated time. *C*) Jurkat cells were pre-incubated on ice with CD2.1, warmed to 37°C, placed in the fluorimeter, then stimulated with LFA-3 (↓) at the time indicated. In *D*) J.RT3-T3.5, *E*) PF-2.4 and *F*) J.CaM1, cells were treated as in (*B*).

transmembrane signaling events via CD2 as indicated by a rise in $[Ca^{2+}]_i$.

Rise in $[Ca^{2+}]_i$ in response to LFA-3+CD2.1 is associated with PI second messenger generation. To determine the role of PI turnover in the LFA-3+CD2.1-induced $[Ca^{2+}]_i$ increases seen in Jurkat, we compared the changes in levels of individual inositol phosphates after stimulation with the anti-CD3 mAb OKT3 or the combination LFA-3+CD2.1 (Table I). Consistent with previous studies (16), OKT3 induced a five-fold increase in IP_3 in Jurkat cells. Although neither stimulation with LFA-3 nor CD2.1 alone resulted in substantial increases in individual inositol phosphates over base line (146% and 127%, respectively), the activating combination of these two ligands caused a nearly seven-fold rise in IP_3. Likewise, changes in the IP_1 + IP_2 fractions in response to OKT3 or the combination of LFA-3+CD2.1 paralleled those of IP_3. These data establish that PI second messenger generation occurs as a result of activation of CD2 by LFA-3 and CD2.1.

LFA-3 + CD2.1 can induce cellular activation. To examine whether transmembrane signaling events initiated through CD2 by LFA-3 in combination with CD2.1 could result in cellular activation, we compared the ability of Jurkat cells to produce IL-2 in response to OKT3, LFA-3, CD2.1 or the combination LFA-3+CD2.1, alone or in the presence of the phorbol ester, PMA (Table II). Jurkat cells and purified T cells produce IL-2 in response to anti-CD3 antibodies only in the presence of PMA (39, 41). As expected, none of the ligands induced the secretion of IL-2 when used as a single stimulus. However, LFA-3+CD2.1+PMA resulted in substantial IL-2 production, more than that seen with OKT3+PMA or with the combination of the calcium ionophore ionomycin + PMA. LFA-3+CD2.1 alone caused a small amount of IL-2 secretion, consistent with previous reports that synergistic combinations of CD2 mAb alone can activate T cells in the absence of signals provided by accessory cells (12, 13, 42). Collectively, these results indicate that the natural ligand of CD2, LFA-3, in combination with CD2.1 can initiate early transmembrane signaling events characteristic of T cell activation and that these events result in a potent induction of lymphokine secretion.

Functional association between CD3/Ti and CD2. Several lines of evidence suggest that there is a functional association between CD3/Ti and CD2. It has been shown that ligand-induced internalization of CD3/Ti (modulation) prevents T cell activation via CD2 even though cell surface expression of CD2 remains unchanged (26). In recent studies, $CD3^-CD2^+$ mutants of a $CD3^+CD2^+$ leukemic line lost their ability to respond to activating combinations of anti-CD2 antibodies (anti-$T11_2$ + anti-$T11_3$

TABLE I

Changes in inositol phosphates in Jurkat and its derivative mutants in response to anti-CD3 mAb or LFA-3 + CD2.1[a]

Cell	$IP_1 + IP_2$ (% of control)		IP_3 (% of control)	
	OKT3	LFA-3 + CD2.1	OKT3	LFA-3 + CD2.1
Jurkat	831 ± 23	1001 ± 19	531 ± 19	657 ± 36
J.RT3-T3.5	144 ± 5	105 ± 5	130 ± 2	110 ± 5
PF-2.4	305 ± 8	340 ± 8	264 ± 7	346 ± 21
J.CaM1	89 ± 1	148 ± 3	83 ± 10	110 ± 5

[a] Jurkat and the three mutant cells were labeled with [^{3}H]inositol as described in *Materials and Methods*, stimulated for 7 min with OKT3 or the combination of LFA-3 and CD2.1, and [^{3}H]inositol phosphate fractions were extracted. Results are the mean of triplicate cultures for each condition and are expressed as a percentage of unstimulated values ± SEM. Results are representative of at least three separate experiments.

TABLE II

LFA-3 + CD2.1-induced activation of Jurkat[a]

Stimulus	IL-2 (U/ml)	
	−PMA	+PMA
0	<2	<2
OKT3	<2	37
LFA-3	<2	<2
CD2.1	<2	<2
LFA-3 + CD2.1	10	278
Ionomycin	<2	98

[a] Jurkat cells were cultured in triplicate for 24 h with the indicated stimuli as described in *Materials and Methods*. Cell-free supernatants were assessed for IL-2 activity using the IL-2-dependent CTLL-2 cell line. The results are expressed as the mean of triplicate cultures and are representative of three independent experiments.

mAb), reportedly due to the inability of the anti-$T11_2$ mAb to induce the $T11_3$ epitope on these cells (27). However, NK cells that express CD2 but not CD3 mediate cytolytic effector function in response to activating combinations of anti-CD2 antibodies (43, 44), suggesting that CD2 can, in some instances, function independently of CD3. In addition, synergistic interactions between CD2 and CD3/Ti have also been suggested. Co-stimulation of CD2 and CD3/Ti by using single antibodies to each receptor can, under certain conditions, result in T cell activation and proliferation (14, 45). Moreover, in recent studies, transfection of human CD2 into a murine T cell hybridoma suggested that CD2 interacting with LFA-3 can synergize with Ag stimulation of CD3/Ti (46).

In order to study the interaction between the Ag receptor activation pathway (CD3/Ti) and the alternative pathway of activation (CD2), we compared the ability of LFA-3 to elicit responses characteristic of T cell activation in Jurkat and in three Jurkat-derived mutants whose phenotypes are shown in Figure 2: 1) J.RT3-T3.5, a mutant that fails to transcribe a functional Ti-β-chain, which results in the absence of cell surface expression of CD3/Ti without affecting the expression of CD2 (34, 35); 2) PF-2.4, a clone derived from J.RT3-T3.5, which has been reconstituted by transfection of a Ti-β-chain cDNA, restoring cell surface expression and function of CD3/Ti (35); and 3) J.CaM1, a signaling mutant derived from Jurkat that expresses levels of CD3 and CD2 on the cell surface comparable to the wild-type cell, yet which fails

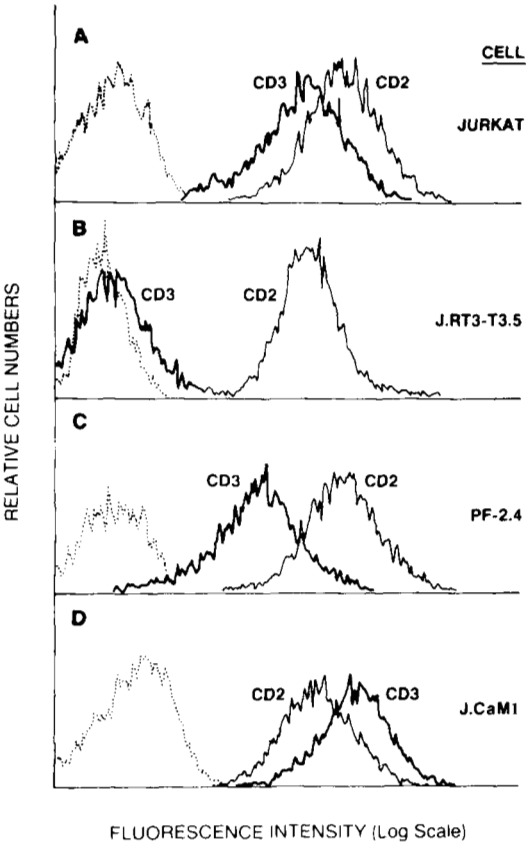

Figure 2. Cell surface expression of CD3 and CD2 on Jurkat and the three mutants as assessed by flow cytometry. Cells were stained with fluorescein-conjugated mouse control IgG (*dashed line*), the anti-CD3 mAb anti-Leu-4 (*heavy solid line*) or the anti-CD2 mAb anti-Leu-5 (*solid line*) and relative fluorescence was determined as described (34). The horizontal axis represents a scale of four log units.

to exhibit PI second messenger generation, increase $[Ca^{2+}]_i$, or produce IL-2 in response to anti-Ti or some anti-CD3 antibodies (36). Although the defect in J.CaM1 is as yet unknown, it is thought to be in the proximal portion of the signaling pathway since phospholipase C-mediated PIP_2 hydrolysis and $[Ca^{2+}]_i$ increases can be induced in this cell under certain conditions.

CD2-mediated $[Ca^{2+}]_i$ increase requires the expression of a functional CD3/Ti complex. To determine whether the Ag receptor activation pathway (CD3/Ti) influences the ability of CD2 to activate T cells via the alternate pathway, we compared the ability of activating combinations of CD2 mAb and the natural ligand LFA-3 in combination with CD2.1 to elicit calcium responses in Jurkat and in each of the three mutant cell lines. Figure 3*A* shows the responses of these four cell lines to OKT3. Only Jurkat and the reconstituted mutant PF-2.4 were capable of increasing $[Ca^{2+}]_i$ in response to OKT3, whereas J.RT3-T3.5 and J.CaM1 showed no significant change in $[Ca^{2+}]_i$. Figure 3*B* shows the responses of the same four cell lines to a combination of the activating CD2 mAb 9-1 and 9.6. As previously reported, stimulation of Jurkat with either 9-1 or 9.6 alone had no effect on $[Ca^{2+}]_i$ (47). Addition of the second antibody 9.6 to Jurkat cells prestimulated with 9-1 resulted in an immediate, substantial increase in $[Ca^{2+}]_i$. However, no change in $[Ca^{2+}]_i$ was noted in response to either antibody, alone or in combination, in J.RT3-T3.5. PF-2.4 responded in a manner similar to that of Jurkat, suggesting that reconstitution of cell surface expression of CD3/Ti concomitantly restored CD2 responsiveness to 9-1 and 9.6. However, J.CaM1, which expresses CD3/Ti, yet is unable to transmit intracellular activation signals via this receptor, was also unresponsive to the combination of mAb 9-1 and 9.6.

The absence of CD2 function in both J.CaM1 and the Ti-β-chain expression mutant J.RT3-T3.5 provides an opportunity for functional complementation between these cells. A novel assay has been developed in our laboratory to examine complementation by assessing reconstitution of the ability to mobilize Ca^{2+} in heterokaryons formed between two cells (38). In this assay one partner cell is loaded with Indo-1 and the second partner is stained with a fluoresceinated mAb against an irrelevant surface marker. The cells are then fused by polyethylene glycol, incubated for 1 h, and then evaluated in the FACS to identify fused and unfused populations of cells and to measure changes in $[Ca^{2+}]_i$ in response to stimulating mAb. Fusions between J.CaM1 and a variety of partners (including J.RT3-T3.5) allow reconstitution of CD3/Ti function (38). In the FACS, unfused Jurkat cells demonstrated an increase in the "Indo-1 fluorescence ratio" (see *Materials and Methods*) in response to the combination of 9-1 and 9.6 mAb, indicating an increase in $[Ca^{2+}]_i$ (Fig.4, *A* and *B*), and J.RT3-T3.5 cells showed no change in response to these mAb (Fig. 4, *C* and *D*). Although unfused J.CaM1 cells also demonstrated no response to these mAb (Fig. 4, *E* and *F*, *lower quadrants*), heterokaryons formed by fusion of J.CaM1 and J.RT3-T3.5 cells did respond (Fig. 4, *E* and *F*, *upper quadrants*). Thus, although neither J.CaM1 nor J.RT3-T3.5 cells alone have a complete CD2 signal transduction apparatus, the two complement each other in this reconstitution assay. These studies extend the observation that CD2

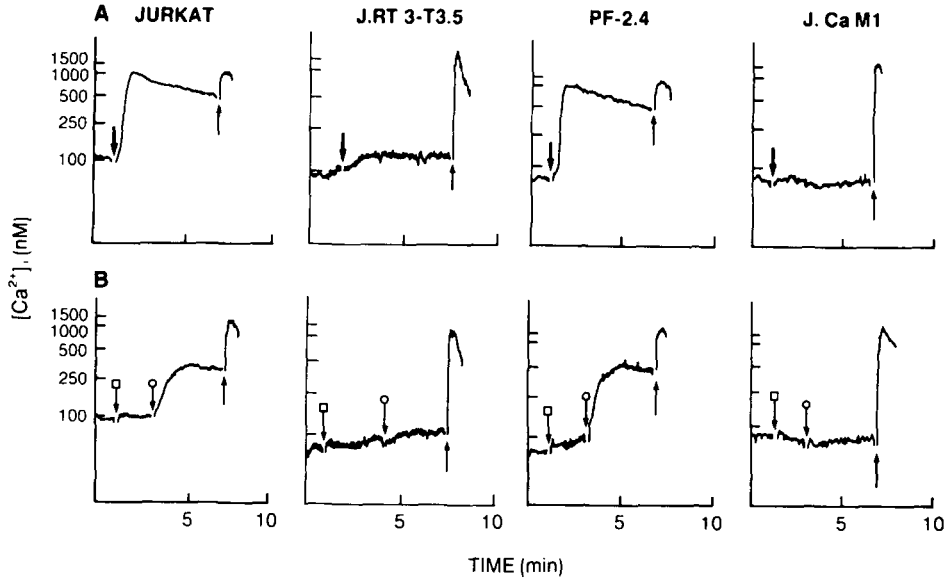

Figure 3. Calcium responses of Indo-1 loaded Jurkat and the three mutants to OKT3 or to the combination of mAbs 9-1 + 9.6. *A*) Responses of the indicated cell types to OKT3 (↓) and ionomycin (↑). *B*) Responses of Jurkat and the three mutants to purified mAb 9-1 (⇩) and 9.6 (⇩), followed by ionomycin (↑).

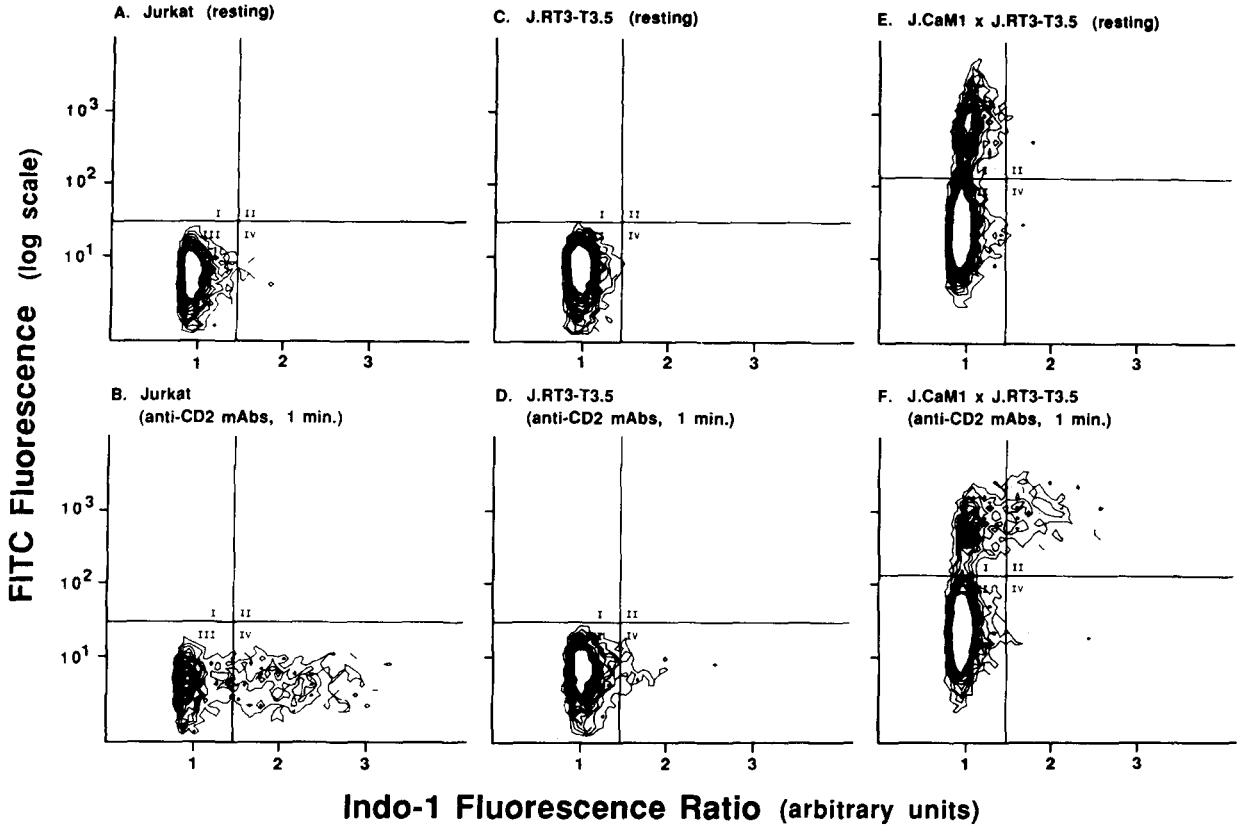

Figure 4. Heterokaryon assay of complementation. Jurkat, J.RT3-T3.5, and J.CaM1 cells were loaded with Indo-1 and J.RT3-T3.5 cells were stained with fluoresceinated anti-HLA mAb. Unfused Jurkat cells (*A* and *B*), J.RT3-T3.5 cells (*C* and *D*) and J.CaM1 cells (*E* and *F*, *lower quadrants*) and heterokaryons (*E* and *F*, *upper quadrants*) formed by fusing Indo-1-loaded J.CaM1 cells to fluorescein-stained J.RT3-T3.5 cells were analyzed for changes in $[Ca^{2+}]_i$ in response to anti-CD2 mAb. Resting cells (*A*, *C*, and *E*) were compared with cells exposed to 9-1 mAb (1 μg/ml) for 1 min followed by 9.6 mAb (1 μg/ml) for 1 min. (*B*, *D*, and *F*). Contour plots are shown with fluorescein fluorescence on the ordinate vs Indo-1 fluorescence ratio on the abscissa, with unfused cells shown in the *lower quadrants* and heterokaryons in the *upper quadrants*.

function depends on surface expression of CD3/Ti by suggesting that an intact CD3/Ti signaling apparatus is necessary for CD2 function.

Having shown that the calcium responses elicited by mAb 9-1 + 9.6 requires the cell surface expression of a functional CD3/Ti complex and proximal signaling pathway, we next sought to determine whether this dependency holds for activation of CD2 by LFA-3 and CD2.1. In contrast to the marked increase in $[Ca^{2+}]_i$ in Jurkat cells in response to these ligands (Fig. 1*B*), LFA-3+CD2.1 had no effect on calcium levels in J.RT3-T3.5 (Fig. 1*D*). As seen with 9-1 + 9.6, reconstitution of CD3/Ti cell surface expression in PF-2.4 once again restored the signaling capability of CD2 in response to LFA-3+CD2.1 (Fig. 1*E*). Similarly, J.CaM1 failed to respond to LFA-3+CD2.1 (Fig. 1*F*), suggesting that the initiation of cellular activation

by the combination of LFA-3 and mAb CD2.1 is dependent on the presence of cell surface expression of CD3/Ti and, based on the observations with J.CaM1, also on a functional CD3/Ti receptor complex.

PI second messenger generation in response to LFA-3+CD2.1 requires the expression of a functional CD3/Ti complex. A recent study has suggested that PI turnover can occur in the absence of both detectable $[Ca^{2+}]_i$ increases and cellular activation (48). To determine the role of the Ag receptor activation pathway in the LFA-3+CD2.1-induced PI turnover seen in Jurkat, we compared the changes in levels of individual inositol phosphates in the three mutant cell lines with those in Jurkat after stimulation with OKT3 or the combination LFA-3+CD2.1 (Table I). In contrast to the five- to seven-fold rise in IP_3 seen in Jurkat in response to these ligands, neither J.RT3-T3.5 nor J.CaM1 showed appreciable changes in the levels of inositol phosphates after stimulation with either OKT3 or LFA-3+CD2.1, even though both cell lines are capable of generating inositol phosphates in response to the GTP-binding protein agonist, AlF_4^- (data not shown). The reconstituted mutant PF-2.4 did show increases in IP_3 in response to both OKT3 and LFA-3+CD2.1, although the levels are somewhat less than those seen in Jurkat. This may be explained by the fact that PF-2.4 expresses only 24% of the surface CD3/Ti of Jurkat (Fig. 2). Taken together, these data confirm that the PI second messenger generation seen in response to CD2 activation by the combination LFA-3+CD2.1 has the same dependency on CD3/Ti function and expression as does the calcium response.

DISCUSSION

These results show that the CD2 natural ligand LFA-3 is capable of initiating transmembrane signaling events culminating in T cell activation when used in combination with a second CD2 stimulus delivered by the CD2 mAb CD2.1. Moreover, in accordance with other observations (see footnote 4) that T cell activation signals can be generated by LFA-3+CD2.1, this combination of ligands is a potent inducer of lymphokine secretion. Using several Jurkat-derived cell lines, we also demonstrated that in order to activate Jurkat cells via the CD2 molecule, both expression and normal function of the CD3/Ti complex is necessary.

The requirement for a second stimulus in addition to LFA-3 to initiate T cell activation is not surprising since LFA-3 is expressed on a wide variety of hematopoietic cells, including human and sheep erythrocytes (13). Under physiologic conditions, it is unlikely that LFA-3 alone would initiate T cell activation, consistent with the observations reported here and elsewhere (33) (see footnote 4). Hence, the requirement for mAb CD2.1 suggests that an additional CD2 ligand is necessary. This could be a soluble factor or a molecule on accessory cells. Alternatively, the second signal could result from CD2 interacting with another molecule on the surface of a stimulated T cell. After stimulation by antigen or CD3/Ti mAb, the CD3/Ti complex might physically interact with CD2, providing this additional ligand. This would explain the synergistic effects of CD3 and individual CD2 mAb previously reported (14, 45) as well as the findings that the CD2 molecule, when stimulated by its natural ligand LFA-3, could augment antigen-induced activation of murine T cell hybridomas (46).

With Jurkat, a CD3/Ti β-chain expression mutant of Jurkat (J.RT3-T3.5), the reconstituted variant of this expression mutant (PF-2.4), as well as the CD3/Ti signaling mutant J.CaM1, we demonstrated that transmembrane signaling via CD2 by using either mAb 9-1 + 9.6 or LFA-3+CD2.1 is dependent upon a functional CD3/Ti complex and proximal signaling pathway. Breitmeyer et al. (27) suggested that the failure of CD2 to function in the $CD3^-CD2^+$ mutants used in their studies was due to the inability of these cells to express the $T11_3$ epitope. In contrast, Jurkat and all three of the mutants used in this report express high levels of the 9-1 and 9.6 epitopes before stimulation as measured by immunofluorescence staining with the mAb 9-1 and 9.6 (data not shown). In fact, the 9-1 epitope on Jurkat is not altered by preincubation of the cells with 9.6 (data not shown). Moreover, LFA-3 and CD2.1 bind to epitopes present on resting T cells (15, 29–32) and Jurkat, eliminating the possibility that the failure to transmit activation signals via CD2 described herein is due to an inability of one of the activating ligands to bind to the cell.

Stimulation of either the CD3/Ti complex or CD2 results in activation of the PI pathway and generation of intracellular second messengers. Therefore, an alternative explanation of the apparent dependency of CD2 signaling on the CD3/Ti activation pathway is that activation via either of these molecules converges on a common intracellular pathway that includes the PI pathway. The failure of CD2 to function in a mutant selected for absence of CD3/Ti function (J.CaM1) is intriguing, and may provide some insight into the mechanism of convergence between CD2 and CD3/Ti signal transduction. Although the identity of the mutated molecule in J.CaM1 is as yet unknown, extensive complementation analyses have demonstrated that the Ti α- and β-chains are not defective in the mutant (38). Therefore, the mutation affects a molecule that is distal to the Ag-binding subunit and that is essential to the integrity of the signal transduction apparatus for the Ag receptor. It appears that CD2 function likewise depends on this molecule, implying that it converges with that of CD3/Ti either at or proximal to the mutated molecule in J.CaM1. This intermediary molecule may be a component of the CD3 complex, or it may be a molecule dependent on the expression of CD3/Ti for membrane association or for function.

Moretta et al. (49) have described $CD3^-CD2^+$ mutants of Jurkat which produce IL-2 in response to the combination of anti-CD2 mAb CD2.1 and CD2.9, yet lack CD3/Ti expression as determined by biosynthetic labeling and immunoprecipitation with anti-CD3 mAb. This is in apparent conflict with the results described above as well as with those of others (27). However, Moretta et al. did not examine transmembrane signaling events and it is possible that, under certain conditions, production of lymphokines including IL-2 as measured by the CTL-L2 line can be induced in the absence of early rises in intracellular calcium and IP_3 levels. In addition, complete lack of cell surface expression of the CD3/Ti complex was not established.

These results are not necessarily discordant with the fact that NK cells can be activated to exhibit cytotoxicity in response to activating combinations of anti-CD2 antibodies (43). Recent evidence suggests that the enhance-

ment of NK cell cytolytic activity seen with the anti-CD2 mAb 9-1 requires the low affinity FcR CD16 on these cells (44). Moreover, that one receptor should require another receptor in order to function is not without precedent. Recent studies have shown that T cell activation via Thy-1 is dependent upon the expression of CD3/Ti, although data regarding cytoplasmic free calcium changes are conflicting (50, 51). Based on our observations and those of others (26, 27) regarding CD2, as well as those made with Thy-1 (50, 51), one may speculate that the CD3/Ti complex acts as a "master" receptor on the T cell surface, through which other activation receptors mediate their signals. Whether this is a feature unique to T cells or is more generalizable to other cells is unknown.

Acknowledgments. The authors thank Drs. Bo Dupont and Jeffrey Ledbetter for generously providing the mAb 9-1 and 9.6, Dr. Kendall Smith for contributing the CTLL-2 clone, and Mr. Paul Dazin for superb technical assistance.

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