

EXPRESSION OF A FUNCTIONAL CD3-Ti ANTIGEN/MHC RECEPTOR IN THE ABSENCE OF SURFACE CD2

Analysis with Clonal Jurkat Cell Mutants

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Activation of human T lymphocytes can be initiated through two major pathways: one of these is antigen specific, whereas the other is antigen independent. Antigen-specific activation is mediated via the TCR (CD3-Ti) complex, which transduces signals after interacting with processed antigen in conjunction with MHC gene products. Similar activation can be induced experimentally by crosslinking the CD3-Ti complex with antibodies directed against individual TCR subunit proteins (1-3). An alternative antigen-independent pathway has been described based upon the in vitro observation that perturbation of the CD2 (T11) molecule with a combination of antibodies defining two different epitopes (anti-T11₂ and anti-T11₃) is able to induce cell activation and proliferation (4). The physiological counterpart of anti-T11₂₊₃ is still unknown; however, the cell adhesion structure LFA-3 appears to be one natural ligand for CD2 (5) that could provide part of the activation stimulus mediated by anti-T11 antibodies in vitro (6, 7).

Interaction of membrane receptors with appropriate mitogenic stimuli for the antigen-specific and alternative pathways results in a virtually identical series of events involving phosphatidyl inositol turnover with subsequent production of 1, 4, 5 inositol trisphosphate (IP₃)¹ and diacylglycerol (8, 9). IP₃ is directly responsible for a rise in intracellular free calcium ([Ca²⁺]_i) as a consequence of mobilization from intracellular stores (10) and/or transmembrane calcium flux (11, 12), while diacylglycerol is an endogenous activator of protein kinase C (13). Previous studies have shown that both a rise in intracellular free calcium [Ca²⁺]_i as well as phosphorylation events mediated through protein kinase C activation are necessary for IL-2 gene induction (14).

Given the similarities in early signal transduction via CD3-Ti and CD2 structures and their coexpression on the surface of mature T lymphocytes, the interdependence of the two pathways has been suggested, but remains to be fully investigated. Herein, we describe the production and characterization of CD2⁻CD3-Ti⁺ variants derived

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¹ Abbreviation used in this paper: IP₃, 1,4,5 inositol trisphosphate.

from the Jurkat CD2⁺CD3-Ti⁺ human T cell line. These mutants express normal levels of CD3-Ti- α/β molecules but lack any detectable surface CD2 molecules as judged by immunoprecipitation experiments and Scatchard analysis. Nevertheless, functional studies clearly establish that these cells can be activated via their CD3-Ti receptor, retaining the capacity to mediate all the aforementioned events associated with the transduction of activation signals. Assuming that Jurkat cells are representative of their normal cycling cellular counterparts, we conclude that CD2 is not required to activate mature T lymphocytes through their TCRs.

Materials and Methods

Cells. Variants of the Jurkat T cell line (clone J77-6.8, kindly provided by Dr. K. Smith, Dartmouth Medical School, Hanover, NH) lacking surface expression of the CD2 molecule were derived by mutagenesis, immunoselection, and all sorting, as previously described (15). Briefly, 10^7 cells were irradiated (300 rad) in a γ cell irradiator (model 1,000; Atomic Energy of Canada Ltd., Ontario, Canada) using a 137 cesium source. After 5 d in culture, cells were incubated 1 h at room temp with a mixture of anti-T11₁ (clone 3Pt2H9) and anti-T11₂ (1old241C) ascites used at a 1:100 final dilution. Rabbit sera (Pel-Freez Biologicals, Rogers, AR) at a dilution of 1:3 was added as a source of complement for 1 h at 37°C. The procedure was repeated four times and viable cells were isolated by centrifugation over a Ficoll-Hypaque density gradient (Pharmacia Fine Chemicals, Piscataway, NJ). CD3⁺Ti⁺CD2⁻ cells were sorted using an Epic V cell sorter (Coulter Electronics Inc., Hialeah, FL) and cloned at 1 cell/well. 19 CD3⁺Ti⁺CD2⁻ colonies were obtained, and three of these clones, termed T11⁻a, T11⁻b, and T11⁻c, were randomly selected and extensively characterized.

Indirect Immunofluorescence Assays. Phenotypic analyses were performed using indirect immunofluorescence assays with a fluorescein-conjugated goat anti-mouse IgG (Meloy Laboratories Inc., Springfield, VA) second antibody. 10^4 cells were analyzed in each sample and results were expressed as histograms displaying number of cells vs. fluorescence intensity on a log scale. Antibodies used in the study were anti-T3 (RW28C8, Leu-4), anti-Ti (9H5, directed against a clonotypic determinant present on Jurkat), anti-T11₁ (3Pt2H9), anti-T11₂ (1old241C), and anti-T11₃ (1mono2A6). An irrelevant ascites (1HT4-4E5) was used as a control of fluorescence background. Antibodies were used at saturating dilutions in the assay (1:200 final dilution).

Immunoprecipitations. 2×10^7 cells were surface labeled with 125 I (IMS30, Amersham Corp., Arlington Heights, IL) for 15 min at room temperature using a standard lactoperoxidase method. Labeled cells were washed and cell lysates were prepared by resuspending the cell pellet in 400 μ l of radioimmunoprecipitation assay (RIPA) buffer containing 1% Triton X-100, 0.15 M NaCl, 0.01 M NaH₂PO₄, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, and protease inhibitors, and by agitating for 40 min at 4°C. Cell lysates were then extensively precleared with formalin-fixed *Staphylococcus aureus* bacteria and protein A-Sepharose 4B beads (Pharmacia Fine Chemicals) bound to a nonreactive antibody (anti-T8). Aliquots of precleared lysates were subsequently incubated with specific antibody (either anti-T3 or anti-T11₁ antibodies) coated protein A-Sepharose beads for 6 h at 4°C. Immune precipitates were washed four times with RIPA buffer and submitted to SDS-PAGE analysis after treatment with 5% 2-ME (reducing conditions). Gels were dried and radiolabeled precipitates were visualized by autoradiography after 3 d of exposure.

Binding Assays. Purified anti-T11₁ antibody (3Pt2H9) was labeled with 10 μ l of immobilized lactoperoxidase/glucose oxidase (Enzymobeads; Bio-Rad Laboratories, Richmond, CA) in 40 mM sodium phosphate, pH 7.2, 0.4% glucose, and 1 mCi 125 I for 5 min and separated over a 1 ml Biogel P-6 column (Bio-Rad Laboratories). $0.1-6 \times 10^6$ cpm labeled antibody (sp act 10^4 cpm/pmol) was added to 10^6 cells overlaid onto 0.2 ml of a 1.5:1 mixture of dibutyl phthalate:diethyl phthalate (Aldrich Chemical Co., Milwaukee, WI). After 30 min at 4°C, tubes were centrifuged (8,500 g for 1 min), the tips of the tubes containing the cell pellets were cut, and the cell bound and free radioactivity was determined in a gamma counter. Nonspecific binding of the antibody was evaluated by performing the experiment in the pres-

ence of an excess of cold antibody (ascites used at a 1:50 dilution) and was subtracted from total binding, with specific binding representing 95% of total binding.

Northern Blot Analysis. Total RNA was extracted from 10^7 cells using the VRC method (Vanadyl Ribonucleoside Complex, New England Biolabs, Beverly, MA). 20–25 μg of RNA per lane were run over a 1.3% agarose gel and blotted onto a Gene Screen Plus nylon (New England Nuclear, Boston, MA) membrane. Prehybridized filters were hybridized with a PBI (Bam HI fragment) probe (16) labeled with ^{32}P using the random priming method to a specific activity of 10^8 cpm/ μg DNA. Filters were washed at 65°C with $2\times$ SSC 0.5% SDS, then $0.5\times$ SSC 0.5% SDS, and exposed for 18–36 h with intensifying screen (DuPont Co., Wilmington, DE). The positions of 28S and 18S RNA are given as a reference. For *HC21* gene expression (H. C. Chang and E. L. Reinherz, submitted for publication), cells were stimulated either with anti-T3, anti-T11₂ and anti-T11₃ antibodies, or calcium ionophore in the presence of PMA (5 ng/ml final concentration), before extracting the RNA. Northern blots were hybridized with the 640-bp HC21-h probe and washed as above.

Analysis of Phosphatidyl Inositol Turnover. Inositol metabolites were analyzed as previously described (17). Briefly, 10^7 cells were incubated in 1 ml of Hepes-buffered (pH 7.4) Hank's solution supplemented with 0.5% gelatin and 40 μCi of [^3H]myo inositol (New England Nuclear) for 3 h at 37°C . Cells were diluted 10-fold in RPMI 1640 medium containing 10% FCS and incubated overnight at 37°C , then washed and resuspended in 2 ml of 10% FCS, 10 mM Hepes buffer, pH 7.4, RPMI 1640 containing 10 mM LiCl (to inhibit the metabolism of inositol phosphates to inositol). Cells were then triggered either with ascites (used at a 1:200 final concentration) anti-T3 (2Ad2A2), anti-Ti, anti-T11₂ and anti-T11₃, or anti-T11₁ antibody (3Pt2H9, shown to bind to the CD2 molecule without inducing activation) (4). After a 10-min incubation at 37°C , cells were rapidly pelleted and lysed with 0.75 ml of chloroform/methanol/HCl (at a ratio of 100:200:2) before extraction of the inositol phosphates and separation over a Dowex column (Sigma Chemical Co., St. Louis, MO) (17). Inositol phosphates IP₁, IP₂, and IP₃ were eluted together with 2 M ammonium formate, 0.1 M formic acid solution. Fractions of 0.4 ml were collected and counted in the presence of aquasol (New England Nuclear).

Determination of Cytosolic Ca^{2+} . Cells were loaded with 2 $\mu\text{g}/\text{ml}$ of the acetoxymethyl ester of indo-1 (Molecular Probes, Junction City, OR) in RPMI 1640 plus 2% FCS for 45 min at 37°C , and diluted fivefold in the same medium before running on an Epics V flow cytometer using an ultraviolet laser illumination (40 mW at 351–364 nm) provided by an argon ion laser (Innova 90-5; Coherent Laser Products, Palo Alto, CA). After Ca^{2+} binding, the indo-1 dye exhibits changes in fluorescence emission wavelengths from 480 to 410 nm (18). The ratio of 410:480 nm indo-1 fluorescence was recorded vs. time. The short wavelength fluorescence was detected through a 410-nm bandpass filter after being reflected by a blue reflective dichroic, and the longer wavelength fluorescence was detected through a 480-nm bandpass filter after passing through the same dichroic. Fluorescence ratio determination allows measurements of Ca^{2+} increase independent of variability of intracellular dye concentration. Samples were analyzed at room temperature by running the indo-1-loaded cells for 1 min to determine the baseline, then adding stimulating antibodies: anti-T3 (2Ad2A2, IgM), anti-Ti (IgG2) at a 1:100 final dilution, or anti-T11₂ and anti-T11₃ (IgG2 and IgG3, respectively) at a 1:100 final dilution. In some experiments, the calcium ionophore A23187 (Sigma Chemical Co.) was added at a 1 $\mu\text{g}/\text{ml}$ final concentration as a positive control. Samples were analyzed for 6 min and results are expressed as Ca^{2+} increase in ordinate (fluorescence ratio is given in arbitrary units) vs. time in min (abscissa). One arbitrary unit represents ~ 200 nM of Ca^{2+} rise (as evaluated in quantitative parallel experiments using the quin-2 fluorescent dye).

IL-2 Production Assay. Evaluation of IL-2 production was assayed as previously described by Marrack et al. (19). 5×10^4 cells were plated in 96-well round-bottomed plates (final volume, 0.2 ml) and incubated at 37°C with anti-T3 (2Ad2A2), anti-Ti at a 1:200 final dilution or anti-T11₂ and anti-T11₃ antibodies at a 1:100 final dilution in the presence of 5 ng/ml of PMA (Sigma Chemical Co.). Supernatants were harvested after 24 h and titrated in duplicate in serial twofold dilutions (ranging from 1:4 to 1:4,096) for their ability to support the growth of the IL-2-dependent cell line CTLL20. The highest dilution of culture supernatant

able to maintain 5×10^3 CTLL20 viable cells (as evaluated in a trypan blue exclusion assay) was said to contain 1 U of IL-2. Results were expressed as arbitrary U/ml IL-2 secreted. We estimate one arbitrary unit to be equivalent to 0.25 U of rIL-2 from Biogen (Cambridge, MA).

Cell Transfection. For reconstitution of CD2 expression on T11⁻ mutants, the full-length T11 cDNA (clone PB2) (16) was inserted into the Bam HI site of the pPink-2 vector (kindly provided by Dr. P. Ohashi, Toronto, Canada), in which expression of the cDNA insert is under the control of the spleen focus-forming virus long terminal repeat (LTR), while the presence of the neomycin gene provides a selection marker for the antibiotic G418. Such generated plasmids were grown in *Escherichia coli* strain HB101 and amplified in the presence of chloramphenicol. Culture was converted to protoplasts and fused to the T11⁻ c cells in the presence of 40% polyethylene glycol 1500 as previously described (15). After 48 h, cells were plated at 30,000 cells/well in 96-well flat-bottomed microtiter plates in the presence of 2 mg/ml geneticin G418 (Gibco Laboratories, Grand Island, NY). Resistant clones were selected for CD2 expression using an indirect immunofluorescence assay, and cells were maintained in culture in the presence of 0.5 mg/ml G418.

Results

Selection of CD3-Ti⁺CD2⁻ Variants of the J77 Cell Line. Variants of the Jurkat cell line, clone J77-6.8 lacking the CD2 molecule, were obtained by mutagenesis, immunoselection, and cell sorting (see Materials and Methods). CD2⁻ colonies were screened by indirect immunofluorescence: 19 clones displaying the CD3-Ti⁺CD2⁻ phenotype were obtained and three of these clones, termed T11⁻a, T11⁻b, and T11⁻c, were randomly selected and used for further characterization. The phenotype of one of these CD2⁻ variants (T11⁻b) is shown in Fig. 1. Antibodies directed to different epitopes of the CD2 molecule failed to react with the T11⁻ variants, while all three of these epitopes were found to be expressed on the J77 parental cell line. Furthermore, CD3 and Ti expression as judged by peak mean channel fluorescence intensity was clearly comparable on J77 and T11⁻ clones, suggesting the presence of a similar number of TCR complexes with no discernable requirement for CD2 protein in CD3-Ti surface expression. An identical set of reactivities was observed for clones T11⁻a and T11⁻c (not shown).

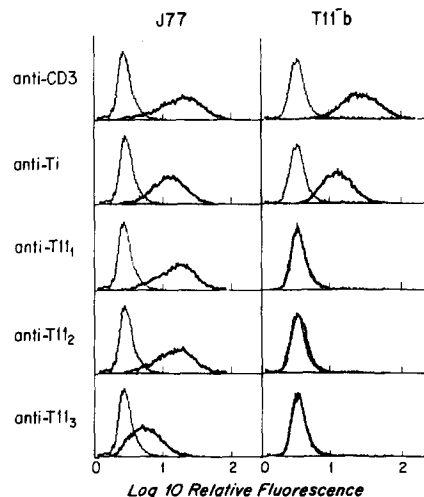


FIGURE 1. Phenotypic analysis of J77 and CD2⁻ cell lines. Reactivity of different mAbs with J77 and CD2⁻ variant cells was assayed by indirect immunofluorescence using cytofluorimetric analysis. 10^4 cells were run per sample and the diagrams represent the number of cells (ordinate) vs. intensity of fluorescence expressed in a logarithmic scale (abscissa). Reactivity of the different antibodies (thick line) is compared with background of fluorescence obtained with an irrelevant antibody (thin line).

CD2 Molecules Are Undetectable on the Surface of CD2⁻ Variants. As an alternative approach for the identification of CD2 molecules, immunoprecipitation experiments were performed on the J77 parental line, and on T11⁻a and T11⁻c clones. To this end, lysates from surface-labeled cells were immunoprecipitated either with purified anti-T11₁ or anti-T3 antibody linked to protein A-Sepharose beads and immunoprecipitates washed and analyzed by SDS-PAGE. As shown in Fig. 2 *a*, anti-T11₁ immunoprecipitates a 50-kD protein from J77 (lane C). In contrast, even overexposure of the autoradiogram failed to reveal any corresponding band from T11⁻ cells (lanes A and B). Anti-T3 antibody precipitates the CD3 subunits on the three cell lines (lanes D-F) that appear as two bands of ~27 and ~21-kD from the Jurkat cell line. Parallel immunoprecipitation conducted with the anti-Ti antibody on T11⁻ variants and J77 cells established that a Ti molecule with similar biochemical characteristics was also present on these cells (our unpublished data).

As a more quantitative means of assessing numbers of CD2 molecules on the Jurkat variants, a radioisotope binding assay was developed using ¹²⁵I-labeled anti-T11₁ antibody. As shown in Fig. 2 *b*, a high and saturable specific binding was observed on J77 cells. Scatchard analysis of the data indicated the presence of ~14,000 sites per cell (not shown). By contrast, no specific binding was detected on T11⁻c. Similar data was observed with T11⁻a and T11⁻b clones. Given the sensitivity of this binding method, we estimate that the T11⁻ mutants must express <100 T11 molecules on their cell surface. Using an identical methodology with ¹²⁵I-labeled anti-T3 and anti-Ti antibodies, we determined that J77 and T11⁻c each expressed

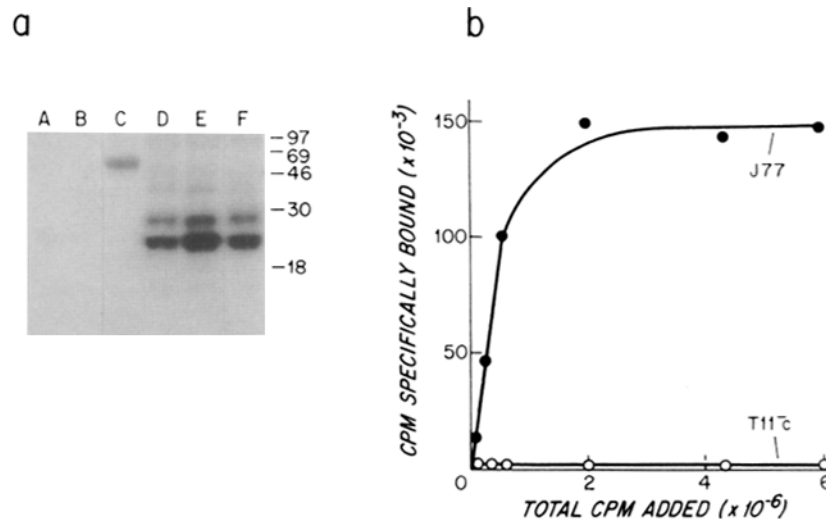


FIGURE 2. Absence of detectable CD2 protein on variant cell lines. (a) J77 and T11⁻a and T11⁻c cells were iodinated before lysis and immunoprecipitation with anti-T11₁ (lanes A-C) or anti-T3 antibodies (lanes D-F). Immunoprecipitates were analyzed in 12.5% polyacrylamide gel in the presence of 5% 2-ME. Lanes A and D, T11⁻a cells; lanes B and E, T11⁻c cells; lanes C and F, J77 cells. (b) Binding assays were conducted with ¹²⁵I anti-T11₁ as described in Materials and Methods. Specific binding was obtained by subtracting nonspecific binding from total binding. Results are expressed as mean of a triplicate determination (SD <5%).

~10,000 copies of TCRs. Thus, the level of CD3-Ti expression is not dependent upon CD2 expression.

Analysis of CD2 Gene Expression in CD2⁻ Variants. To identify whether the loss of CD2 surface expression was due to a pretranslational effect, blot analysis was performed with RNA isolated from the T11⁻a, T11⁻b, and T11⁻c cells. 25 µg of total RNA was run on a 1.3% agarose gel, transferred to Gene Screen Plus membrane, and probed with the ³²P-labeled T11/cDNA, PB1. As shown in Fig. 3, hybridization with the PB1 probe revealed two transcripts of 1.7 and 1.3 kb specifically in the T cell line J77 (lane A). In contrast, the CD2 transcripts were not detectable in the B cell line Laz 509 (lane E). Although some level of CD2 gene transcription was detected in all three T11⁻ mutants (lanes B, C, and D), this was substantially reduced in comparison with the parental cell line (up to 20-fold reduction). Note that the 1.7-kb transcript predominates in the T11⁻a and T11⁻b clones, whereas the 1.3-kb transcript predominates in the T11⁻c clone. Given the fact that either form of CD2 message can give rise to functional CD2 protein, we cannot exclude an extremely low translational level in these mutant cells. Presumably, the irradiation-induced mutagenesis has resulted in a defect in the regulatory region of the CD2 gene or another *cis*- or *trans*-acting regulatory element affecting CD2 transcription. The possibility that irradiation has introduced a point mutation in the CD2 sequence that affects message stability or translatability also cannot be ruled out.

Phosphatidyl Inositol Turnover. To characterize the status of early membrane signaling events in these T11⁻ mutants, induction of PI turnover was evaluated after stimulation via CD2 or CD3-Ti. To this end, J77 and T11⁻a and T11⁻c cells were labeled with [³H]inositol and stimulated with anti-T3, anti-Ti, or a combination of anti-T11₂ and anti-T11₃ antibodies. As a negative control, clones were stimulated with an antibody directed against a nonmitogenic epitope, anti-T11₁ (4). Results from a representative experiment are shown in Table I. As indicated, anti-T11₂ and anti-T11₃ dramatically increase PI turnover in J77; a 6.3-fold increase of inositol

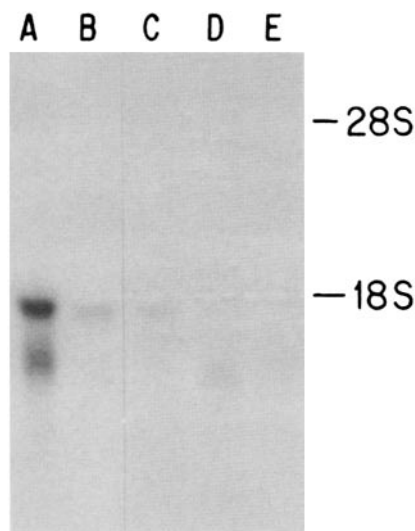


FIGURE 3. Northern blot analysis of CD2 gene expression. RNA was extracted from the cytoplasmic fraction of J77, T11⁻ variants, and the EBV-transformed B cell line (Laz 509) and 25 µg of RNA run per lane. The blot was hybridized with the ³²P-labeled PB1 cDNA probe, washed, and exposed for 22 h. Lane A, J77; lanes B-D, T11⁻a, T11⁻b, and T11⁻c, respectively; lane E, Laz 509.

TABLE I
*Determination of Phosphatidyl Inositol Turnover in
 J77 and T11⁻ Mutants upon CD2 and CD3-Ti Activation*

Cells	mAbs			
	Anti-T11 ₁	Anti-T11 ₁ + anti-T11 ₃	Anti-T3	Anti-Ti
J77	7,434	46,842 (630)	30,271 (410)	12,325 (170)
T11 ⁻ a	4,214	4,199 (99)	14,617 (350)	6,953 (160)
T11 ⁻ c	4,815	2,566 (53)	19,786 (410)	6,126 (120)

Results are expressed in cpm of [³H]myo inositol incorporated in monophosphate (IP₁) + bisphosphate (IP₂) + trisphosphate (IP₃). Numbers in parentheses represent percentage change in comparison to incubation with the nonactivating (control) anti-T11₁ antibody.

phosphates was found in comparison with stimulation with the control anti-T11₁ antibody. In contrast, these antibodies were ineffective in stimulating T11⁻a and T11⁻c cells, thereby demonstrating the functional lack of CD2 structures on these mutants. However, when anti-T3 was used to stimulate the T cell populations, a 4.1-, 3.5-, and 4.1-fold increase in PI turnover was found for J77, T11⁻a, and T11⁻c cells, respectively. Anti-Ti antibody, like anti-T3, was able to induce phosphatidyl inositol breakdown in all three cell lines, although the level of activation was found to be less than that with anti-T3 (a 1.7, 1.6, and 1.2 increase was found, respectively). Thus, PI turnover mediated via the CD3-Ti pathway occurs in the absence of detectable CD2 surface structures.

Cytosolic Ca²⁺ Measurement. To examine alteration in [Ca²⁺]_i after CD3-Ti or CD2 stimulation, cells were loaded with the fluorescent dye indo-1 and triggered with specific antibodies (Fig. 4). As previously reported (15, 20), incubation of J77

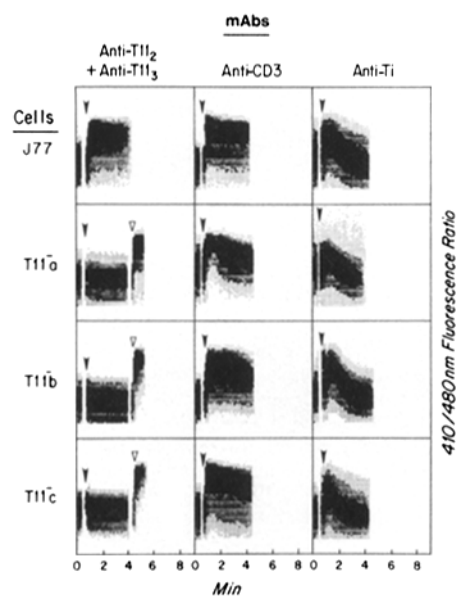


FIGURE 4. Variation of intracellular calcium after CD3-Ti and CD2 triggering. Indo-1-loaded J77 and T11⁻a, T11⁻b, and T11⁻c cell lines were incubated either with anti-T11₂₊₃, anti-T3 and anti-Ti ascites (black arrow), or calcium ionophore (open arrows), and samples were monitored continuously for 6 min on an Epics V cell sorter. Results are expressed as 410/480 nm fluorescence ratio in arbitrary units (ordinate) vs. time in minutes (abscissa).

cells with anti-T11₂ and anti-T11₃ antibodies results in a dramatic increase in cytosolic calcium as detected by a shift in the wavelength of fluorescence emission of indo-1 from blue to violet. By contrast, identical stimulation of T11⁻ cells does not induce any change in [Ca²⁺]_i. Furthermore, although not shown, this was not a consequence of a requirement for a higher antibody concentration with which to trigger, since purified anti-T11₂ and anti-T11₃ antibodies at any concentration tested also failed to stimulate a rise in [Ca²⁺]_i (not shown). This functional data adds further support for the above results in which immunoprecipitation analysis and binding assays showed absence of surface CD2. Note that treatment of these CD2⁻ cells with the calcium ionophore A23187 leads to an immediate rise in [Ca²⁺]_i, demonstrating that the CD2⁻ cells take up indo-1 without difficulty and are loaded equivalently to J77. Furthermore, incubation of cells with anti-T3 antibody induces a calcium rise in J77 as well as in the three T11⁻ mutants with similar kinetics (after ~30 s) and maintenance of an equivalent level of fluorescence during the subsequent 6 min of the study. Interestingly, stimulation with anti-Ti antibody induces a virtually identical increase in [Ca²⁺]_i in J77 and T11⁻a, T11⁻b, and T11⁻c cell lines but the rise is more transient than with anti-T3 and reverts to background level within 2 min.

HC21 Gene Induction. The above studies analyzed the early membrane signaling events known to be associated with T lymphocyte activation. To next assess other intracellular activation events, we examined the expression of early activation genes (*HC21*) and endogenous IL-2 production in CD2⁺ and CD2⁻ Jurkat cells. Induction of the *HC21* gene occurs in T lymphocytes stimulated either through the CD2 molecule or the CD3-Ti complex but not the IL-2-R (Chang, H. C. and E. L. Reinherz, submitted for publication). This gene encodes a 0.8-kb RNA of unknown function that is detectable in northern analysis within 10 min of stimulation. As shown in Fig. 5, calcium ionophore plus PMA treatment results in a high level of transcription of the *HC21* gene in both CD2⁻ (lane H) and CD2⁺ (lane D) cell lines. As expected, the 0.8-kb *HC21* mRNA is also found in J77 after stimulation with anti-T11₂ and anti-T11₃ plus PMA antibodies (lane C), while this transcript is not detected in T11⁻b cells treated under similar conditions (lane G). Importantly, comparable levels of *HC21* expression are found in J77 and T11⁻b cells after anti-T3 plus PMA stimulation (lanes B and F, respectively), strongly indicating that detectable surface CD2 expression is not required for *HC21* induction.

IL-2 Production. IL-2 production and secretion by CD2⁺ and CD2⁻ Jurkat

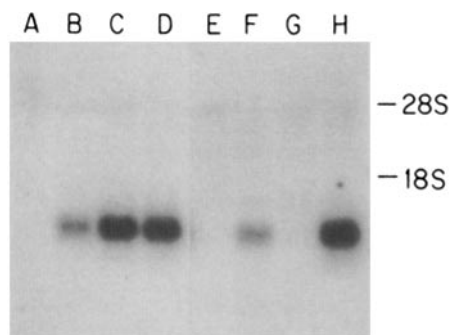


FIGURE 5. *HC21* gene induction in J77 and T11⁻b cells. Northern blot analysis was performed using 25 µg of RNA from J77 and T11⁻b cells using the ³²P-labeled *HC21*-h probe. Lanes A-D, J77 cells; lanes E-H, T11⁻b cells. Stimuli used in combination with PMA were: culture medium (lanes A and E); anti-T3 (lanes B and F); anti-T11₂₊₃ (lanes C and G); or calcium ionophore (lanes D and H).

populations was evaluated after stimulation as described in Materials and Methods. As shown in a representative experiment (Table II), supernatants of cells incubated with PMA alone contain only low levels of IL-2 (≤ 8 U/ml). Addition of anti-T11₂ and anti-T11₃ antibodies induces J77 cells to produce high levels of IL-2 (up to 2,048 U/ml), but is without any effect on CD2⁻ mutants. Anti-T3 or anti-Ti antibody stimulation leads to significant IL-2 production in all tested cell lines (128–256 U/ml). It should be noted that since serial twofold dilutions were used in the assay, we assume that results obtained by CD3-Ti stimulation and expressed in Table II do not represent any quantitative difference between J77 and T11⁻ clones. Thus, surface CD2 expression is not required for IL-2 production after CD3-Ti stimulation of Jurkat-derived cells.

Reconstitution of Surface CD2 Expression on the T11⁻ c Clone. The above studies indicated that the T11⁻ mutants could not be triggered to undergo early biochemical activation events upon stimulation with anti-T11₂ and anti-T11₃ antibodies. To prove whether this defect was a direct consequence of the absence of the CD2 gene product, we performed reconstitution analysis. To this end, a full-length cDNA clone (PB2; reference 16) was transfected into T11⁻ c cells using a previously described protoplast fusion method (15). After G418 selection and immunofluorescence screening, CD2⁺ clones were identified. Fig. 6 (*left panel*) represents the immunofluorescence analysis of one reconstituted T11⁻ c clone, termed T11⁻ cR, which clearly expresses both CD3 and CD2 molecules. Note that the level of CD2 expression in T11⁻ cR cells is less than that of the parental Jurkat cell line, but clearly detectable unlike the T11⁻ mutants (Fig. 1). Stimulation of T11⁻ cR by anti-T11₂ and anti-T11₃ antibodies induced a readily detectable rise in [Ca²⁺]_i in a substantial number of cells (Fig. 6, *right panel*). As expected, reconstitution of surface CD2 expression did not alter the capacity to stimulate cells via CD3-Ti molecules since both T11⁻ c and T11⁻ cR cell lines were found equally triggerable through their TCRs (data not shown).

Discussion

CD2⁻ mutants have been derived and characterized from the CD2⁺CD3-Ti⁺ Jurkat human T cell line. Surface expression of the CD2 molecule was undetectable using a series of mAbs directed at three different epitopes of the CD2 structure (T11₁, T11₂, T11₃) and any of three separate assays: indirect immunofluorescence,

TABLE II
IL-2 Production in J77 and T11⁻ Mutants upon CD3-Ti and CD2 Stimulation

Cells	Stimuli			
	PMA (5 ng/ml)	Anti-T11 ₂ + anti-T11 ₃ + PMA	Anti-T3 + PMA	Anti-Ti + PMA
J77	≤ 4	2,048	128	256
T11 ⁻ a	≤ 4	≤ 4	128	128
T11 ⁻ b	≤ 4	≤ 4	256	256
T11 ⁻ c	8	8	128	128

Cells were incubated for 24 h with the different stimuli and supernatants were harvested and tested in duplicate in serial dilutions for their ability to affect the growth of 5×10^3 CTLL₂₀ cells. Results are expressed in arbitrary IL-2 units as defined in Materials and Methods.

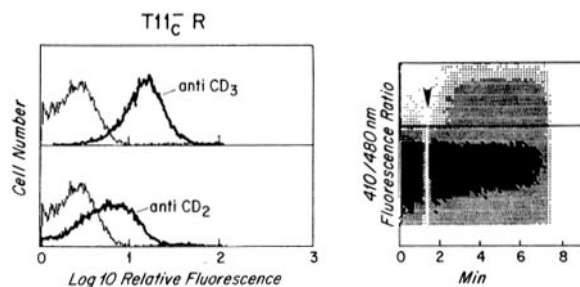


FIGURE 6. Characterization of a reconstituted T11⁻cR clone. (*Left panel*) Reactivity of anti-CD3 and anti-CD2 antibodies (*thick lines*) as assayed by indirect immunofluorescence. 10⁴ cells were analyzed per sample and the diagrams represent the number of cells (*ordinate*) vs. intensity of fluorescence expressed in a logarithmic scale (*abscissa*). Background of fluorescence (*thin line*) was obtained with an irrelevant antibody. (*Right panel*) Indo-1-loaded T11⁻cR cells were incubated with anti-T11₂ and anti-T11₃ antibodies (*black arrow*) and the sample was monitored continuously on an Epics V cell sorter. Results are expressed as 410:480 nm fluorescence ratio (*ordinate*) vs. time (*abscissa*).

immunoprecipitation-SDS-PAGE, and radiolabeled antibody binding. Furthermore, the T11⁻a, T11⁻b, and T11⁻c clones were incapable of being activated by a combination of anti-T11₂ and anti-T11₃ antibodies, which are extremely potent in stimulating the parental Jurkat clone and resting peripheral T lymphocytes (4). Thus, measurement of alterations of [Ca²⁺]_i, *HC21* gene induction and IL-2 production, all sensitive assays of T lymphocyte activation, gave no evidence for stimulation of T11⁻ mutants via the CD2 structure.

It should be noted that complete absence of CD2 structures cannot be formally excluded in the three T11⁻ variants characterized herein given that a low level of *CD2* gene transcription was detected in each mutant population. However, according to the sensitivity of the radiolabeled binding assay, these T11⁻ clones express, at most, <100 molecules/cell. This corresponds to at least a 140-fold reduction in the number of surface CD2 in comparison with the parental Jurkat line.

The basis for the defect in CD2 expression in T11⁻ variants is presently unknown. Noteworthy, at the level of RNA expression is the clear predominance of either the 1.7- or 1.3-kb message in mutant cell lines. These results raise the possibility that the mutation occurs either within a regulatory region of the *CD2* gene or *cis*- and/or *trans*-activating elements important for *CD2* expression. Moreover, since transfection of such T11⁻ mutants with human *CD2* cDNA driven by an exogenous LTR results in a high level of transcription (data not shown) with surface expression of CD2 less than the parental Jurkat cell line (compare Fig. 6, *left panel* with Fig. 1), it is possible that additional defects at the post-transcriptional level (e.g., defect in a transport system of the molecule to the membrane) could lead to lack of normal surface CD2 protein expression. However, such CD2-reconstituted cells can clearly be triggered via the CD2 molecule to augment [Ca²⁺]_i as expected.

One unequivocal finding of the present study is the observation that CD3 and Ti molecules are found on the surface of CD2⁻ mutants and that CD2 expression apparently has no effect on CD3-Ti expression since the number of molecules of CD3-Ti on Jurkat vs. CD2⁻ variants were quantitatively similar as judged by binding assay. Perhaps more importantly, these same experiments demonstrate that CD2⁻ mutants can be triggered through their CD3-Ti components to mediate phos-

phoinositol turnover, a rise in $[Ca^{2+}]_i$, nuclear activation (including *HC21* activation), and *IL-2* gene transcription, translation, and secretion. Thus, in these Jurkat variants, the CD3-Ti- α/β receptor does not require CD2 for functional activation. We observed in the present study that stimulation of the CD3-Ti structure with anti-Ti antibody was less effective than with anti-CD3 antibody at inducing early biochemical events, including phosphatidyl inositol turnover and $[Ca^{2+}]_i$ rise. In contrast, both anti-Ti and anti-CD3 antibodies induced similar levels of *IL-2* production. This result is perhaps not surprising since it is generally assumed that the CD3 subunits are involved in signal transduction or, at the least, linked more proximal to transduction elements than the antigen/MHC binding heterodimer. That *IL-2* production is equivalent after stimulation of the CD3-Ti complex via clonotypic vs. monomorphic structures is perhaps also not unexpected if only a critical threshold alteration in phosphoinositide turnover or $[Ca^{2+}]_i$ is required for *IL-2* gene induction. Nevertheless, we cannot exclude the possibility that the avidity, isotype subclass, or other intrinsic properties of the anti-CD3 and anti-Ti antibodies used in these studies (e.g., the anti-CD3 is IgM while the anti-Ti is IgG2) are themselves responsible for the differences in the early biochemical activation events.

The relative CD2 independence of the TCR complex demonstrated herein by genetic manipulation contrasts with the conclusion of certain other functional studies (21, 22). The latter have suggested that perturbation of the CD2 molecule with mAbs affects subsequent stimulation through CD3-Ti. Whether this is a direct effect of CD2 in regulating activation through CD3-Ti is controversial since other reports show that modulation of CD2 molecules has no effect on early metabolic activation events observed after CD3-Ti triggering, including inositol breakdown and alterations in $[Ca^{2+}]_i$ (23). Given that modulation experiments require preincubation of T cells for prolonged periods of time with mAbs, indirect immunoregulatory effects cannot be excluded. The present genetic approach described herein, therefore, firmly argues for the CD2-independent nature of certain TCR triggering events in Jurkat and presumably other activated T cell populations.

A CD3⁺ *IL-2*-dependent clone lacking expression of CD2 on the membrane has recently been derived from peripheral blood of a healthy subject (24). This clone was found to express components of the "second type" TCR- γ/δ complex while CD2 was undetected by indirect immunofluorescence techniques. These CD2⁻ cells were still able to be induced to proliferate and display cytotoxic activity after triggering with antibodies directed against either CD3 or Ti γ protein. Although no quantitative binding analysis was performed to rule out detectable CD2 surface protein expression by a more sensitive technique, these results establish that the physical presence of normal numbers of CD2 molecules on the membrane of this cell type is also not an absolute requirement for subsequent expression and function of its TCR (24).

The above results are in marked contrast to the unequivocal evidence indicating that CD3-Ti expression is necessary for activation of mature T lymphocytes through their CD2 structure (4, 15, 25, 26). In this regard, we recently described CD3⁻ Ti⁻ CD2⁺ mutants of the Jurkat line that failed to express a 1.3-kb Ti β gene transcript (15). These mutants were unable to be stimulated by either CD3-Ti or CD2 pathway to increase phosphoinositide turnover, mobilize calcium, or induce the *IL-2* gene. However, after reconstitution via transfection with a Ti β cDNA driven by an exogenous LTR, CD3-Ti expression was restored as well as the activation capacity through the CD2 and CD3-Ti structures (15).

Collectively, the above results suggest that while CD3-Ti is necessary for activation of T cells via CD2, the reciprocal is not true. Nevertheless, it has to be cautioned that these observations have been made on CD3-Ti- α/β Jurkat clones and one CD3-Ti- γ/δ IL-2-dependent clone, both of which represent cycling cell populations. It remains to be demonstrated whether T cell activation can proceed from the G₀ state of cells whose CD2 structure is absent or made nonfunctional. In this regard, it is a likely possibility that transduction of signaling via CD2 affects stimulation through the CD3-Ti complex. Compelling functional data has already been provided to suggest that both pathways cooperate in a synergistic fashion to induce T cell proliferation. Thus, a combination of submitogenic anti-CD3 and anti-T11 antibodies together lead to T cell proliferation (27). The ability of CD2 to augment T cell activation in the presence of low concentrations of antigenic stimuli that might be encountered during natural immune perturbations could be of substantial physiologic significance. Furthermore, the CD2 molecule appears to have an important role in T lymphocyte adhesion by facilitating the binding of regulatory and effector T cells to their cognate partners through direct interaction with the ubiquitous surface structure LFA-3 (5). Since binding of CD2 to LFA-3 leads to at least one of the signals required for T cell stimulation via CD2, a physiologic role for this pathway is suggested in both the mature peripheral T cell compartment (6, 7) and the immature thymus compartment (28, 29). In this context, the role of CD2 in regulating activation of T lineage cells at different stages of ontogeny, particularly those undergoing cell cycle entrance, remains to be fully elucidated.

Summary

To investigate the requirement for CD2 expression in activation of T lymphocytes via the CD3-Ti antigen/MHC receptor complex, we produced and characterized a series of CD2⁻ Jurkat variants. These mutants lack detectable surface CD2 as determined by indirect immunofluorescence, immunoprecipitation analysis, and specific radiolabeled antibody binding assay, but nevertheless, expressed normal numbers of CD3-Ti receptors. As expected, the combination of anti-CD2 antibodies, termed anti-T11₂ and anti-T11₃, which are mitogenic for resting T lymphocytes, failed to stimulate activation of these variants. In contrast, triggering of their CD3-Ti components resulted in the normal set of T lymphocyte-associated activation events, including phosphoinositide turnover, elevation in intracellular free calcium, early gene-induction events, and IL-2 production. Assuming that the Jurkat cell line is representative of normal cycling human T lymphocytes, we conclude that the presence of the CD2 molecule on the plasma membrane is not in itself a requirement for an operational CD3-Ti- α/β receptor.

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