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Interdependence of CD3-Ti and CD2 activation pathways in human T lymphocytes

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Human T lymphocytes can be activated through either the antigen/MHC receptor complex T3-Ti (CD3-Ti) or the T11 (CD2) molecule to proliferate via an IL-2 dependent mechanism. To investigate the relationship of these pathways to one another, we generated and characterized Jurkat mutants which selectively express either surface CD3-Ti or CD2. Here we show that CD3-Ti$^-$ mutants fail to be stimulated by either pathway to increase phosphoinositide turnover, mobilize calcium or induce the IL-2 gene. The activation capacity of these mutants via CD2 as well as CD3-Ti can be restored following reconstitution of surface CD3-Ti expression upon appropriate DNA transfer (e.g. Ti β subunit cDNA into Ti β^- Jurkat variants). Collectively, these results demonstrate that CD3-Ti and CD2 pathways are interdependent and that phosphoinositide turnover is linked to the CD3-Ti complex.

Key words: T cell receptor/IL-2/gene induction/CD2/calcium channel

Introduction

Activation of human T lymphocytes from a quiescent (G_0) state into the G_1 phase of cell cycle is initiated upon perturbation of the antigen/MHC receptor complex T3-Ti (CD3-Ti) or the T11 (CD2) structure on their surface. Subsequent induction of IL-2 receptor expression and endogenous IL-2 production lead to further cell cycle progression culminating in clonal expansion (reviewed in Weiss *et al.*, 1986; Alcover *et al.*, 1987). Although CD3-Ti and CD2 structures are not known to be physically associated despite coexpression on the same cells (Meuer *et al.*, 1984), several lines of indirect evidence suggest that they interact functionally. First, CD3-Ti crosslinking inhibits subsequent CD2-induced activation (Meuer *et al.*, 1984; Fox *et al.*, 1986; Breitmeyer *et al.*, 1987; Pantaleo *et al.*, 1987; Shipp and Reinherz, 1987) and second, submitogenic concentrations of anti-T3-Ti and anti-T11 antibodies, in combination, induce T-cell proliferation (Yang *et al.*, 1986).

Within a given T-cell population, IL-2 gene induction requires an elevation in cytosolic calcium concentration ($[Ca^{2+}]_i$) as well as phosphorylation events, one of which is mediated by protein kinase C. Consequently, combinations of agents that increase $[Ca^{2+}]_i$ and activate protein kinase C (e.g. Ca ionophores and phorbol esters) bypass the necessity of receptor-ligand binding for induction of T-cell activation including IL-2 gene transcription (Truneh *et al.*, 1985). Under physiologic conditions, transduction of membrane signalling via both CD3-Ti and CD2 occurs through the phosphatidyl inositol pathway which leads to generation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). The former is an endogenous activator of protein kinase C (Kikkawa and Nishizuka, 1978) whereas the latter induces Ca mobilization from intracellular stores as well as across the plasma membrane (perhaps in conjunction with IP_4) (Kuno and Gardner, 1987; Gardner *et al.*, in preparation).

Given the similarities in the early signal transduction events mediated by CD3-Ti and CD2 pathways, it is possible that they are one and the same. To examine the relationship of CD3-Ti and CD2 pathways to one another, we have herein created and characterized mutants of Jurkat. Because the human T-cell leukemic line Jurkat bears a mature T-cell phenotype and, like physiologic T-cell populations, can be induced to produce IL-2 in response to CD3-Ti (Weiss *et al.*, 1986) or CD2 stimulation (Moretta *et al.*, 1987), it is a suitable model system with which to study human T-lymphocyte activation.

Results and discussion

Variants of the Jurkat line clone J77-6.8 lacking surface expression of the T3-Ti (CD3-Ti) complex or the T11 (CD2) glycoprotein were derived by mutagenesis, immunoselection, fluorescence-activated cell sorting and cloning as described in Materials and methods. Analysis by immunofluorescence led to the selection of eight clones that did not express detectable levels of CD3-Ti but displayed levels of CD2 similar in magnitude to J77 (see Materials and methods for number of sites determined by Scatchard analysis). In addition, 19 clones displaying the CD3-Ti$^+$CD2$^-$ phenotype were obtained. Examples of CD3-Ti$^-$CD2$^+$ (clones 31-13 and 31-22) and CD3-Ti$^+$CD2$^-$ (clone 10) phenotypes are shown in Figure 1 and clearly demonstrate that surface expression of CD3-Ti and CD2 are independent of one another.

Further study of the CD3-Ti$^-$ mutants by Northern blot analysis showed that two of them (31-13 and 32-36) lack the 1.5-kb transcript of the Ti β gene. The latter represents the complete gene product encoding variable (V), diversity (D), joining (J) and constant (C) regions and is required for surface CD3-Ti expression (Yoshikai *et al.*, 1984; Ohashi *et al.*, 1985). In contrast, Ti α, CD3 γ, CD3 δ and CD3 ϵ transcripts and cytoplasmic CD3 proteins are detectable in these cells (data not shown). The basis for the impaired CD3-Ti expression in the other six mutants is presently unclear; each of the latter CD3-Ti$^-$CD2$^+$ mutants displays normal Ti α, Ti β, CD3 γ, CD3 δ and CD3 ϵ message levels. Their impaired CD3-Ti expression might be related to one or more defects in the subunit assembly

process, the recently described CD3 ζ chain (Weissman *et al.*, 1986), or any of a host of mutations undetected by our analysis.

Because mutants 31-13 and 32-36 express no detectable CD3 or Ti molecules by Scatchard analysis using ^{125}I-labeled monoclonal antibodies (in contrast to the parental J77 line which expresses 10 500 CD3 binding sites) and the basis of the CD3−Ti surface loss was known to reside at the level of Ti β chain expression, these mutants were studied in greater detail. As shown in Figure 2A, the parental CD3−Ti$^+$CD2$^+$ Jurkat line J77 is inducible to express IL-2 gene transcripts with a combination of Ca ionophore plus phorbol-12-myristate 13-acetate (PMA). As expected, neither ionophore or PMA alone are sufficient to lead to IL-2 gene activation. In addition, anti-T3 + PMA as well as anti-T11$_2$ + anti-T11$_3$ + PMA give rise to IL-2 gene induction. In contrast, when 31-13 (Figure 2A) or 32-36 (data not shown) are triggered with anti-T3 or anti-T11$_2$ + anti-T11$_3$ in the presence of PMA, no IL-2 gene activation occurs. This failure of IL-2 gene induction is not a consequence of general cellular unresponsiveness since the combination of Ca ionophore and PMA induces an increase in steady-state IL-2 mRNA levels comparable to the parental J77 clone. Moreover, the defect in nuclear activation was not restricted to the IL-2 gene, as an unrelated CD3−Ti and CD2 inducible gene, termed HC21, encoding a putative intracellular protein (H.C.Chang and E.L.Reinherz, unpublished) also cannot be induced in CD3−Ti$^-$CD2$^+$ mutants except with Ca ionophore and PMA (date not shown).

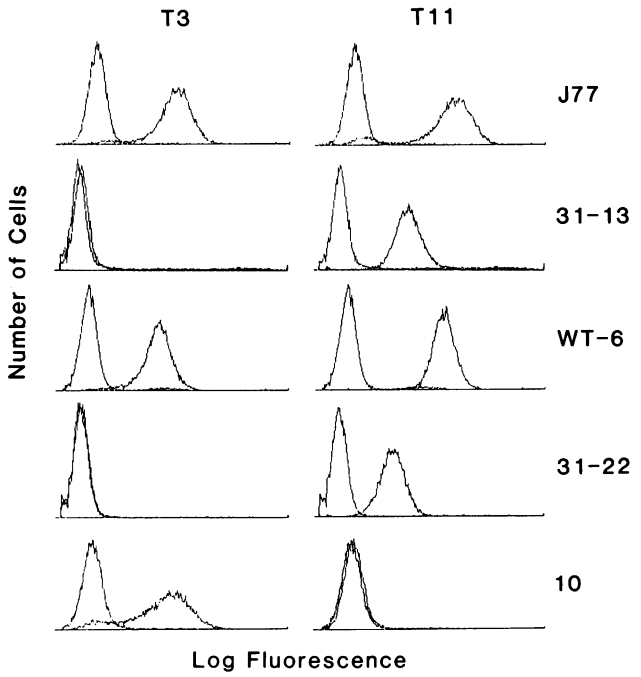

Fig. 1. Immunofluorescence analysis of CD3−Ti$^-$CD2$^+$ and CD3−Ti$^+$CD2$^-$ T-cell mutants. Fluorescence histograms of the parental CD3−Ti$^+$CD2$^+$ J77 Jurkat clone, CD3−Ti$^-$CD2$^+$ (31-13, 31-22) and CD3−Ti$^+$CD2$^-$ 10 clones. WT-6 is one example of clones obtained from the Ti β- mutant 31-13 reconstituted with a full length Ti β cDNA. Cells were reacted with mAb anti-T3, anti-T11$_1$ or an irrelevant anti-clonotypic as negative control and then stained with FITC goat anti-mouse Ig.

Note that both clone 31-13 (Figure 1) and 32-36 display readily discernible levels of CD2 (both T11$_2$ and T11$_3$ epitopes) on their surface, suggesting that impaired binding of anti-T11 antibodies is not the basis for the lack of IL-2 gene activation. Furthermore, analysis over 2−24 h shows that the lack of response is not due to a shift in IL-2 induction kinetics (data not shown). Although not shown, conventional CTLL assays also demonstrate that the negative variants, in contrast to the parental line J77, are incapable of secreting IL-2 in response to CD3−Ti or CD2 stimulation. Furthermore, consistent with these data is our finding that two other β-chain negative clones which were independently derived (clone CD3−4, G.C.Spagnoli and E.L.Reinherz, unpublished; clone JJ, kindly donated by A.Moretta, Ludwig Institute, Lausanne, Switzerland) fail to display any increase in IL-2 mRNA when triggered via CD3−Ti or CD2 even though Ca ionophore + PMA can activate their IL-2 gene. By way of contrast, CD3−Ti$^+$ clones displaying reduced (< 1000) CD2 sites per cell could be partially induced through CD3−Ti to activate IL-2 gene transcription.

Next, to determine whether the inability to activate CD3−Ti$^-$ mutants was a consequence of an early signal-

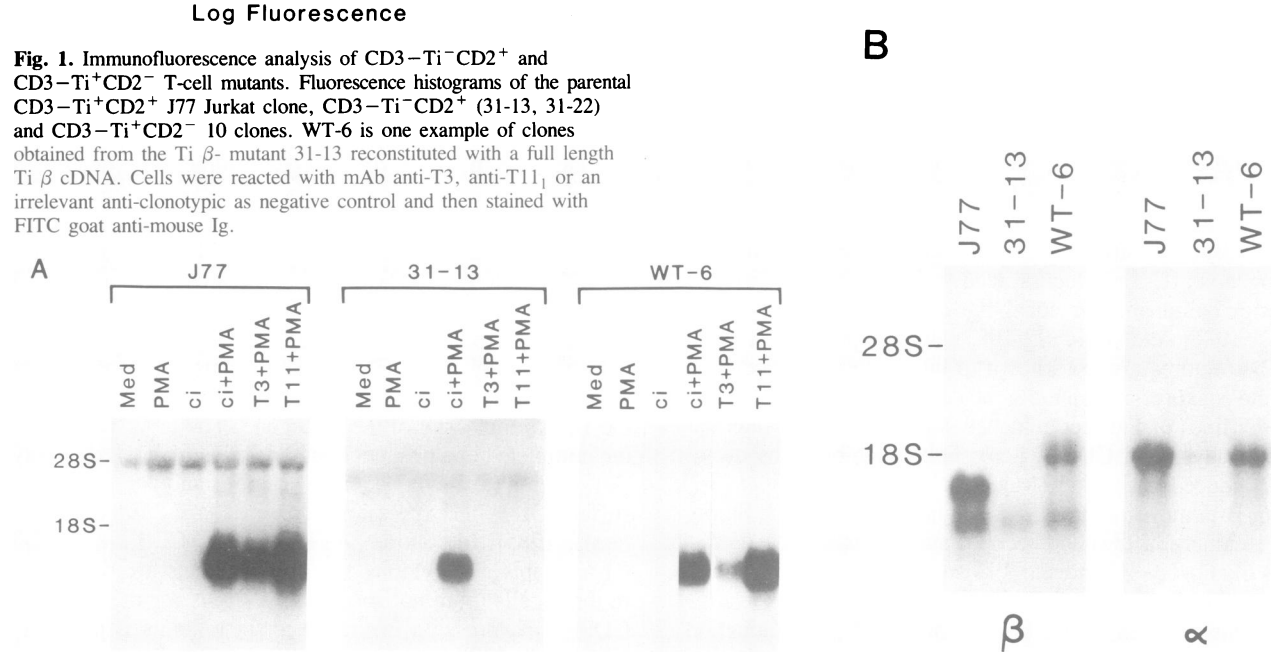

Fig. 2. IL-2 gene induction in Jurkat and variant clones. **(A)** Northern blot analysis of Jurkat cells (J77), the CD3−Ti$^-$CD2$^+$ clone 31-13 and the Ti β reconstituted clone WT-6. Cells were activated with stimuli and Northern blots hybridized with a ^{32}P-labeled cDNA probe specific for the IL-2 gene. **(B)** Northern blots of RNA obtained from J77, 31-13 and WT-6 cells and hybridized with ^{32}P-labeled cDNA probes for Ti β and Ti α.

ling defect, variations in phosphoinositide metabolites and $[Ca^{2+}]_i$ were examined after CD3−Ti and CD2 stimulation in CD3−Ti$^-$CD2$^+$ clones and compared to the J77 parent. As shown in Table I, after stimulation via CD3 or CD2, phosphoinositol levels in J77 increase to 629 and 1132% respectively. In contrast, no increment is observed when clone 31-13 is activated via either pathway. Consistent with the phosphoinositol results is the finding that, unlike with J77, there is no increment in $[Ca^{2+}]_i$ after stimulation of the

Table I. Measurement of inositol phosphate metabolites upon CD3-Ti or CD2 activation in J77, 31-13 or WT-6 cells

Clone	Phenotype	c.p.m. [^{3}H]-IP$_1$ + IP$_2$ + IP$_3$		
		Stimuli		
		Control	T3	T11
J77	T3-Ti$^+$T11$^+$	676	5036 (629)	7655 (1132)
31-13	T3-Ti$^-$T11$^+$	1022	1021 (100)	1079 (106)
WT-6	T3-Ti$^+$T11$^+$	1406	6733 (480)	17 799 (1266)

The table shows one of multiple representative experiments. The numbers given show counts per minute of [^{3}H]-inositol incorporated in inositol monophosphate (IP$_1$) + bisphosphate (IP$_2$) + trisphosphate (IP$_3$). Percentage change with respect to resting control cells is shown in parentheses.

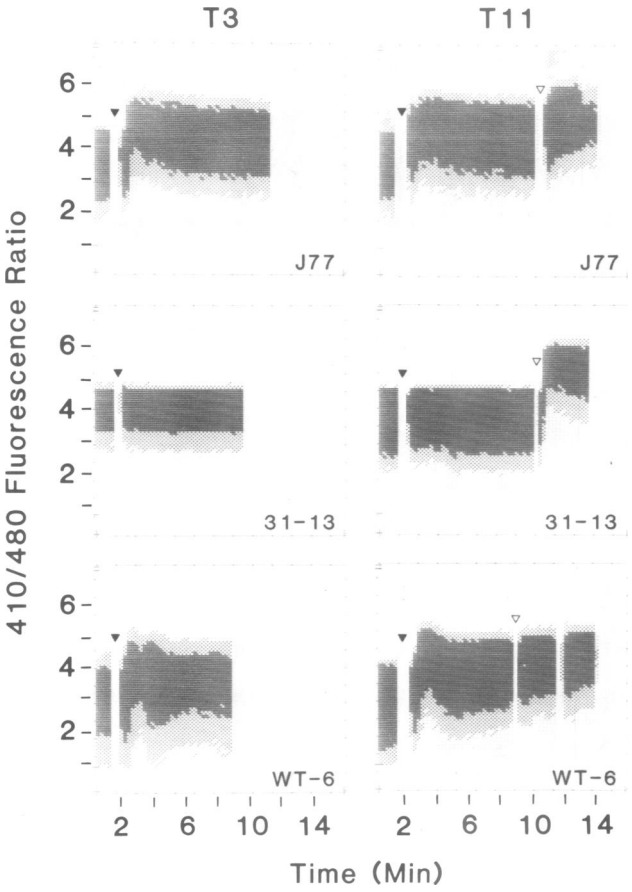

Fig. 3. Variation in $[Ca^{2+}]_i$ upon CD3−Ti and CD2 triggering. Indo-1-loaded Jurkat J77, CD3−Ti$^-$CD2$^+$ mutant 31-13 and transfectant clone WT-6 were activated with mAbs anti-T3 or anti-T11$_2$ + anti-T11$_3$ at 1:200 dilution of ascites (black arrow). Subsequent addition of calcium ionophore was used to define the maximum increase in $[Ca^{2+}]_i$ (intracellular free calcium). 410/480 nm fluorescence ratio (ordinate in arbitrary units) was recorded in real time (abscissa). The figure is representative of multiple experiments.

CD3−Ti$^-$CD2$^+$ 31-13 mutant by anti-T3 or anti-T11 (Figure 3). Virtually identical results are obtained with the other Ti β^-clone (data not shown).

To prove whether the unresponsiveness of the CD2 pathway in CD3−Ti$^-$CD2$^+$ variants is direcly related to the loss of CD3−Ti surface expression, we transfected a full length cDNA clone (Royer *et al.*, 1984) coding for the Ti β subunit of Jurkat into the Ti β- 31-13 clone (Figure 2B). Similar transfection analysis has helped to establish requirements for surface CD3−Ti expression in the Jurkat system (Saito *et al.*, 1987a,b). To this end, the Ti β cDNA was inserted into a derivative (Pink-2) of pT$_\beta$F-neo vector (Ohashi *et al.*, 1985) and transfected into 31-13 via protoplast fusion. After G418 selection and immunofluorescence screening, clones expressing levels of CD3−Ti receptors comparable to the parental line J77 were identified. Two, termed WT-1 and WT-6 (Figure 1), were chosen for further study.

Northern blot analysis demonstrates that the transfected clones, of which WT-6 is representative, display a Ti β message of substantially larger size (1.6 kb) than either full length (1.3 kb) or truncated DJC (1.0 kb) Ti β transcripts (Figure 2B; WT-6). This unique size message is probably due to differences in transcriptional initiation and/or termination sites of the DNA introduced into 31-13 via the Pink-2 vector (Ohashi *et al.*, 1985). This difference clearly indicates that the functional Ti β-chain gene in WT-6 and the other reconstituted clones is not derived from the endogenous Ti β gene. Figure 2B also shows that the level of steady-state Ti α gene transcription increases substantially in Ti β reconstituted cells, consistent with earlier findings suggesting that Ti β gene product may regulate Ti α RNA expression (Ohashi *et al.*, 1985).

When transfected surface CD3−Ti$^+$ reconstituted clones such as WT-6 are activated with anti-T3 + PMA, early signal transduction events such as phosphoinositide turnover (Table I) and elevation in $[Ca^{2+}]_i$ (Figure 3) as well as IL-2 gene (Figure 2) and HC-21 gene (not shown) induction are re-established. Furthermore, the CD2 pathway also recovers function (Figures 2 and 3, Table I; clone WT-6). The basis for the finding that IL-2 mRNA induction via CD3−Ti stimulation is quantitatively less than that followng stimulation by ionophore + PMA or anti-T11$_2$ + anti-T11$_3$ is unclear but reproducibly observed in WT-6 (Figure 2A) and a second reconstituted mutant, WT-1 (data not shown). At a minimum, these data imply that other early signals aside from inositides might be important in IL-2 gene induction. Note that restoration of function in each of the above experimental assays requires surface CD3−Ti expression since transfection of 31-13 with a truncated Ti β cDNA clone lacking the transmembrane and cytoplasmic regions fails to give rise to surface CD3−Ti expression or functional activation via CD3−Ti or CD2 (C.Alberini and O.Acuto, unpublished data).

Thus, the present findings unequivocally demonstrate that reconstitution of surface CD3−Ti expression by Ti β cDNA transfection into Ti β- Jurkat variants restores the detectable membrane and nuclear activation events mediated by CD3−Ti and CD2 stimulation. These data are consistent with earlier modulation studies (Meuer *et al.*, 1984; Pantaleo *et al.*, 1987; Breitmeyer *et al.*, 1987) demonstrating that CD2 stimulation of T-cell proliferation, elevation in $[Ca^{2+}]_i$ and phosphoinositide turnover are linked to the

CD3−Ti complex. The modulation of the CD3−Ti complex by anti-T3 antibody for a 24-h period, however, might have resulted in ineffective CD2 stimulation by a negative or regulatory signal. By constructing Jurkat mutants as shown herein, we have been able to provide direct evidence that CD2 signalling in the absence of the CD3−Ti complex cannot be achieved. Given that the CD3−Ti loss is by genetic mechanisms rather than an antibody incubation, the lack of CD2 triggering is not due to a concurrent negative signalling event.

The number of CD3−Ti receptors required to elicit signal transduction and IL-2 gene activation via CD2 appears to be quite low. In this regard, we have observed that Jurkat variants expressing <1000 CD3−Ti sites per cell (i.e. unreactive with anti-T3 monoclonal antibody by FACS) can be triggered via CD2 to give rise to inositol turnover, elevation in $[Ca^{2+}]_i$ and IL-2 gene induction. This observation probably explains the finding of Moretta *et al.* (1987) that certain Jurkat variants which are unreactive with anti-T3 antibody can be triggered via the CD2 pathway. Presumably the activation of phenotypically CD3−Ti$^-$CD2$^+$ thymocytes via CD2 may, at least in part, be a consequence of low level CD3−Ti expression (Fox *et al.*, 1985; Alcover *et al.*, 1986). We do not wish to imply, however, that CD2 stimulation cannot, by itself, give rise to activation events since it appears that the lytic program of certain Ti $\alpha^-\beta^-\gamma^-$CD3$^-$CD2$^+$ NK clones can be activated by anti-T11$_2$ + anti-T11$_3$ antibody (Siliciano *et al.*, 1985; Alcover *et al.*, 1986).

A requirement for CD3−Ti expression has also been suggested for Thy-1-mediated activation in two earlier studies employing either mouse T-cell clones or Thy-1 transfected human Jurkat cells (Gunter *et al.*, 1987; Schmitt-Verhulst *et al.*, 1987). To date, reconstitution studies have not been performed in murine cells. Nevertheless, anti-Thy-1 antibodies induce IL-2 secretion in Jurkat cells which have been transfected with the murine Thy-1 gene but not in Thy-1-transfected Jurkat variants lacking the CD3−Ti complex (Gunter *et al.*, 1987). Although there is some inconsistency in these two studies with respect to CD3−Ti requirements for Thy-1-mediated $[Ca^{2+}]_i$ elevations (Kroczek *et al.*, 1986; Gunter *et al.*, 1987; Schmitt-Verhulst *et al.*, 1987), the linkage between Thy-1 and CD3−Ti is apparent. Given that there are no structural homologies between CD2 and Thy-1 at the DNA or protein level (Sewell *et al.*, 1986; Sayre *et al.*, 1987; Seed and Aruffo, 1987), it would appear that multiple types of accessory molecules may require CD3−Ti expression for signal transduction in T-lineage cells of man and mouse.

It should also be noted that induction of IL-2 gene expression after CD3−Ti stimulation in each of five CD3−Ti$^+$CD2$^-$ mutants tested to date is reduced relative to J77. However, as we have been unsuccessful in reconstituting CD2 gene expression in these mutants, it remains to be proven whether this reduction in IL-2 gene activation is directly related to the CD2 structure. Nevertheless, it appears likely that a synergistic interaction resulting from CD3−Ti and CD2 structures is a consequence of unique signalling processes associated with each of these structures and that they collectively facilitate nuclear activation events.

Materials and methods

Mutagenesis and selection of Jurkat variants

Variants of Jurkat clone 77-6.8 (CD3−Ti$^+$CD2$^+$) (kindly provided by Dr K.Smith, Dartmouth Medical School, Hanover, NH) lacking either CD3−Ti or CD2 were derived by mutagenesis, immunoselection and fluorescence-activated cell sorting. Cells (10^7) were irradiated with 300 rad in a gamma cell 1000 irradiator (Atomic Energy of Canada, Ltd) with ^{137}Ce as a source. Cells were grown for 5 days, the CD3−Ti$^-$ fraction was enriched by complement-mediated lysis and then 10^7 cells/ml were incubated with mAb anti-T3 (2Ad2A2) (Reinherz *et al.*, 1982) and anti-Ti (5REX9H5) (Acuto *et al.*, 1983) at 1:100 dilution of ascites for 1 h at 25°C. Rabbit sera (PelFreez, Rogers, AK) was added as source of complement at 1/3 dilution and incubation proceeded at 37°C for 1 h. Dead cells were removed by centrifugation through Ficoll-Hypaque (Pharmacia) and selected cells were grown in standard conditions [RPMI 1640 media supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, penicillin and streptomycin]. The procedure was repeated four times. CD3−Ti$^+$CD2$^-$ variants were selected in a similar fashion. CD2$^+$ cells were lysed by using a combination of anti-T11$_{1A}$ (3Pt-2H9) (Meuer *et al.*, 1984) + anti-T11$_2$ (101d2-4C1) (Meuer *et al.*, 1984) mAb and complement. CD3−Ti$^-$CD2$^+$ and CD3−Ti$^+$CD2$^-$ fractions were then sorted and cloned at 1 cell/well into 96-well microtiter plates (Nunc, Denmark) using an Epics V cell sorter (Coulter, Hialeah, FL) equipped with an autoclone apparatus. Growing clones were screened by immunofluorescence using mAb anti-T3 (RW2-8C8) (Meuer *et al.*, 1983), anti-clonotypic (5REX9H5) or anti-T11$_{1A}$ (3Pt-2H9). Immunofluorescence analysis was routinely carried out with the above-described anti-T3, anti-Ti or anti-T11$_{1A}$ mAb. In addition, anti-T11$_2$ (101d2-4C1) or anti-T11$_3$ (1mono-2A6) (Meuer *et al.*, 1984) were also employed when required. Irrelevant anti-Ti mAb (2T8-2F5) was used as negative control. Cells were reacted with 1:400 dilution of ascites (30 min, 4°C) and bound antibodies detected with fluorescein-coupled goat anti-mouse Ig (Meloy, Springfield, VA) on an Epics V cell sorter. Histograms delineating qualitative differences among clones are derived from 10 000 cells. To quantitate more accurately the number of binding sites, Scatchard plot analysis was carried out as previously described (Smith and Cantrell, 1985) using ^{125}I-anti-T3 (RW2-8C8), anti-Ti (5REX-9H5) or anti-T11$_{1A}$ (3T48β5) radiolabeled with enzymobeads (Biorad) according to the manufacturer's recommendation. The binding sites per cell for anti-T3, anti-Ti and anti-T11$_{1A}$ were respectively: 10 500, 9800, 14 600 for J77; U, U, 3000 for 31-13; U, U, 10 200 for 31-22; not determined for WT-6; 3600, 4800, U for 10, where U = undetectable.

IL-2 gene induction and Northern analysis

Cells (5×10^6) were incubated for 6 h at 37°C in the presence of each of the following stimuli: medium, 10 ng/ml PMA (Sigma), 1 μg/ml Ca ionophore A23187 (Sigma), Ca ionophore + PMA, anti-T3 mAb 2Ad2A2 + PMA, anti-T11$_2$ + anti-T11$_3$ mAbs + PMA. mAbs were added at a 1:100 dilution of ascites. Northern blots were performed with 30 μg of cytoplasmic RNA/lane as previously described (Maniatis *et al.*, 1982; Royer *et al.*, 1985). Prehybridized filters were hybridized with an IL-2 cDNA probe (Taniguchi *et al.*, 1983) (kind gift of G.Crabtree, Stanford University, Stanford, CA) labeled with ^{32}P by the method of random priming to a sp. act. of $\sim 2 \times 10^8$ c.p.m./μg DNA. Filters were washed at 65°C with 2 × SSC, 0.5% SDS and exposed for 18−36 h with intensifying screen (Dupont). Position of 28 and 18S RNA is given as reference. Similar results were obtained when ionomycin was used as Ca ionophore (not shown).

Protoplast fusion of Ti α and β transcript analysis

For transfection studies, the full length β-chain cDNA clone derived from REX, a rapidly growing variant of Jurkat (Weissmann *et al.*, 1986) was inserted into the *Bam*HI site in the pPink-2 vector (kindly provided by Dr P.Ohashi, University of Toronto, Canada) by standard methodology to generate pPink Ti β. pPink-2 is a modification of the previously described pT$_\beta$F-neo (Ohashi *et al.*, 1985). In this vector, expression of the cDNA insert is under the control of the spleen focus forming virus long terminal repeat and the presence of the neomycin gene provides the selection marker for the antibiotic G418. The pPink Ti β plasmid was grown in *Escherichia coli* strain HB101 and amplified in the presence of chloramphenicol. The culture was converted to protoplasts and fused to the cells basically as previously described (Ochi *et al.*, 1983; Oi *et al.*, 1983). For the fusion 40% polyethylene glycol 1500 (Sigma, St Louis, MO), 10% dimethyl sulphoxide solution was used. Then cells were placed in 50-ml tissue culture flasks and, after 48 h, plated in the presence of 2 mg/ml Geneticin G418 (Gibco) in 96 flat-bottom microtiter plates at 30 000 cells/well. The resistant clones obtained were maintained in 0.5 mg/ml G418. Cytoplasmic RNA (20 μg) from unstimulated cells were run in formaldehyde containing gels and blotted as described above. Blots were hybridized with ^{32}P-labeled cDNA probes specific for Ti α (Royer *et al.*, 1985) and Ti β (Royer *et al.*, 1984).

Measurement of cytosolic Ca^{2+}

Cytosolic Ca^{2+} changes were determined essentially according to

Grynkiewicz *et al.* (1985). Cells were loaded with 1 μg/ml of acetoxymethyl ester of indo-1 (Molecular Probes, Junction City, OR) at 4 × 10^6 cells/ml in RPMI 1640 containing 2% FCS for 45 min at 37°C. Cells were then diluted to 10^6/ml in the same medium. Changes in fluorescence were monitored with an Epics V flow cytometer. Ultraviolet laser illumination (40 mW at 351–364 nm) was provided by an argon ion laser (Innova 90-5). Viable cells were selected for analysis on the basis of forward angle light scatter. Upon Ca^{2+} binding, indo-1 exhibits changes in fluorescence emission wavelengths from 480 to 410 nm. Short wavelength indo-1 fluorescence was detected at 90° through a 410-nm bandpass filter after being reflected by a blue reflective dichroic, with longer wavelength fluorescence being detected through a 480-nm bandpass filter after passing through the same dichroic. Both fluorescence signals were logarithmically amplified and the ratio of 410/480 nm indo-1 fluorescence was recorded versus time using a Coulter MDADS data acquisition system. Use of the ratio of intensities at these two wavelengths allows measurements of $[Ca^{2+}]_i$ independent of variabilities in intracellular dye concentration (Grynkiewicz *et al.*, 1985). All samples were analyzed at room temperature. For each determination the base line was determined by running indo-1 loaded cells for 1 min. The fluorescence ratio was set approximately in the middle of the scale. Then either anti-T3 (2Ad2A2, IgM) or anti-$T11_2$ (101d2-4C1)+ anti-$T11_3$ (1mono-2A6) or anti-$T11_{1A}$ (3Pt-2H9) (negative control) mAbs were added at a 1:200 dilution of ascites. The Ca^{2+} ionophore A23187 was added at 1 μg/ml final concentration to determine the maximum increase. Anti-$T11_{1A}$ (3Pt-2H9) did not induce any increase in $[Ca^{2+}]_i$ and was used in the experiments as negative control (data not shown). Changes in fluorescence were continuously recorded during 15 min. Ordinate axis is divided in arbitrary units.

Inositol metabolites

Inositol metabolites were isolated and quantified basically as previously described by Imboden and Stobo (1985). Cells (10^7)/ml were incubated in Hepes-buffered (pH 7.4) Hank's solution containing 0.5% gelatin and [^{3}H]-inositol (40 μCi/ml) (NEN, Boston, MA) for 3 h at 37°C. After a 10-fold dilution with RPMI 1640 medium containing 10% FCS, cells were incubated overnight at 37°C. Cells were then washed twice and resuspended at 5–10 × 10^6/ml in 10% FCS 10 mM Hepes-buffered (pH 7.4) RPMI 1640 medium containing 10 mM LiCl and incubated for 10 min at 37°C. The presence of LiCl in the medium inhibits the metabolism of inositol phosphate to inositol (Hallcher and Sherman, 1980). Cells (5–10 × 10^6/ sample were triggered either with mAbs anti-T3 (2Ad2A2) or anti-$T11_2$ + anti-$T11_3$ (101d2-4Cl + 1mono2A6) at 1:100 final dilution of ascites. A MoAb anti-$T11_{1A}$ (3Pt2H9) was used as a control since it has been shown to bind to the CD2 molecule without inducing activation (Meuer *et al.*, 1984). After 10 min at 37°C, cells are rapidly pelleted (15 s in an Eppendorf microfuge) and lysed with 0.75 ml of stop solution ($CHCl_3$:MeOH:HCl, 100:200:2). Inositol phosphates were extracted and separated in Dowex columns as previously described (Imboden and Stobo, 1985). [^{3}H]-inositol metabolites IP_1, IP_2 and IP_3 were eluted together with 20 ml of 2 M ammonium formate, 0.1 M formic acid solution. Eluted fractions were counted in the presence of aquasol (NEN). Table I shows total c.p.m. eluted from each column.

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References

Acuto, O., Hussey,R.E., Fitzgerald,K.A., Protentis, J.P., Meuer,S.C., Schlossman,S.F. and Reinherz,E.L. (1983) *Cell*, **34**, 717–726.

Alcover,A., Weiss,M.J., Daley,J.F. and Reinherz,E.L. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2614–2618.

Alcover,A., Ramarli,D., Richardson,N.E., Chang,H.C. and Reinherz,E.L. (1987) *Immunol. Rev.*, **95**, 5–36.

Breitmeyer,J.B., Daley,J.F., Levine,H.B. and Schlossman,S.F. (1987) *J. Immunol.*, **139**, 2899–2905.

Fox,D.A., Hussey,R.E., Fitzgerald,K.A., Bensussan,A., Daley,J.F., Schlossman,S.F. and Reinherz,E.L. (1985) *J. Immunol.*, **134**, 330–335.

Fox,D.A., Schlossman,S.F. and Reinherz,E.L. (1986) *J. Immunol.*, **136**, 1945–1950.

Grynkiewicz,G., Poenie,M. and Tsien,R.Y. (1985) *J. Biol. Chem.*, **260**, 3440–3450.

Gunter,K.C., Germain,R.N., Kroczek,R.A., Saito,T., Yokoyama,W.M., Chan,C., Weiss,A. and Shevach,E.M. (1987) *Nature*, **326**, 505–507.

Hallcher,L.M. and Sherman,W.R. (1980) *J. Biol. Chem.*, **255**, 10896–10901.

Imboden,J.B. and Stobo,J.D. (1985) *J. Exp. Med.*, **161**, 446–456.

Kikkawa,U. and Nishizuka,Y. (1978) *Annu. Rev. Cell Biol.*, **2**, 149–178.

Kroczek,R.A., Gunter,K.C., Germain,R.N. and Shevach,E.M. (1986) *Nature*, **322**, 181–184.

Kuno,M. and Gardner,P. (1987) *Nature*, **326**, 301–304.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 194–196.

Meuer,S.C., Hodgdon,J.C., Hussey,R.E., Protentis,J.P., Schlossman,S.F. and Reinherz,E.L. (1983) *J. Exp. Med.*, **158**, 988–993.

Meuer,S.C., Hussey,R.E., Fabbi,M., Fox,D., Acuto,O., Fitzgerald,K.A., Hodgdon,J.C., Protentis,J.P., Schlossman, S.F. and Reinherz,E.L. (1984) *Cell*, **36**, 897–906.

Moretta,A., Poggi,A., Olive,D., Bottino,C., Fortis,L., Pantaleo,G. and Moretta,L. (1987) *Proc. Natl. Acad. Sci, USA*, **84**, 1654–1658.

Ochi,A., Hawley,R.G., Shulman,M.J. and Hozumi,N. (1983) *Nature*, **302**, 340–342.

Ohashi,P.S., Mak,T.W., Van den Elsen,P., Yanagi,Y., Yoshikai,Y., Calman,A.F., Terhorst,C., Stobo,J.D. and Weiss,A. (1985) *Nature*, **316**, 606–609.

Oi,V.T., Morrison,S.L., Herzenberg,L.A. and Berg,P. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 825–829.

Pantaleo,G., Olive,D., Poggi,A., Pozzan,T., Moretta,L. and Moretta,A. (1987) *J. Exp. Med.*, **166**, 619–624.

Reinherz,E.L., Meuer,S.C., Fitzgerald,K.A., Hussey,R.E., Levine,H. and Schlossman,S.F. (1982) *Cell*, **30**, 735–743.

Royer,H.D., Acuto,O., Fabbi,M., Tizard,R., Ramachandran,K., Smart,J. and Reinherz,E.L. (1984) *Cell*, **39**, 261–266.

Royer,H.D., Ramarli,D., Acuto,O., Campen,T.J. and Reinherz,E.L. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5510–5514.

Saito,T., Weiss,A., Miller,J., Norcross,M.A. and Germain,R.N. (1987a) *Nature*, **325**, 125–130.

Saito,T., Weiss,A., Gunter,K.C., Shevach,E.M. and Germain,R.N. (1987b) *J. Immunol.*, **139**, 625–628.

Sayre,P.H., Chang,H.C.,Hussey,R.E., Brown,N.R., Richardson,N.E., Spagnoli,G., Clayton,L.K. and Reinherz,E.L. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 2941–2945.

Schmitt-Verhulst,A.M., Guimezanes,A., Boyer,C., Poenie,M., Tsien,R., Buferne,M., Hua,C. and Leserman,L. (1987) *Nature*, **325**, 628–631.

Seed,B. and Aruffo,A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 3365–3369.

Sewell,W.A., Brown,M.H., Dunne,J., Owen,M.J. and Crumpton,M.J. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 8718–8722.

Shipp,M.A. and Reinherz,E.L. (1987) *J. Immunol.*, **139**, 2143–2148.

Siliciano,R.F., Pratt,J.C., Schmidt,R.E., Ritz,J. and Reinherz,E.L. (1985) *Nature*, **317**, 428–430.

Smith,K.A. and Cantrell,D.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 864–868.

Taniguchi,T., Matsui,H., Fujita,T., Takaoka,C., Kashima,N., Yoshimoto,R. and Hamuro,J. (1983) *Nature*, **302**, 305–310.

Truneh,A., Albert,F., Goldstein,P. and Schmitt-Verhulst,A. (1985) *Nature*, **313**, 318–320.

Weiss,A., Imboden,J., Hardy,K., Manger,B., Terhorst,C. and Stubo,J. (1986) *Annu. Rev. Immunol.*, **4**, 593–619.

Weissman, P.M., Samelson,L.E. and Klausner,R.D. (1986) *Nature*, **324**, 480–482.

Yang,S.Y., Chouaib,S. and Dupont,B.O. (1986) *J. Immunol.*, **137**, 1097–1100.

Yoshikai,Y., Anatoniou,D., Clark,S.P., Yanagi,Y., Sanester, R., Van den Elsen,P., Terhorst,C. and Mak,T.W. (1984) *Nature*, **312**, 521–524.

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