

HUMAN CYTOLYTIC CELL CLONES LACKING SURFACE  
EXPRESSION OF T CELL RECEPTOR  $\alpha/\beta$  OR  $\gamma/\delta$   
Evidence That Surface Structures Other Than CD3 or CD2 Molecules  
Are Required for Signal Transduction

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The surface T cell receptor (TCR) for antigen/MHC products is composed of a disulfide  $\alpha/\beta$  heterodimer noncovalently associated with CD3, a multipolypeptide cell membrane complex (1, 2). In addition, a minor subset of CD3<sup>+</sup> cells has recently been shown to express CD3-associated molecules different from  $\alpha/\beta$  molecules, which may represent the putative TCR- $\gamma$  gene product (3). T cell activation can be induced in both types of CD3<sup>+</sup> cells by the use of lectins (i.e., PHA) or mAbs directed against either the TCR, CD3, or CD2, another surface glycoprotein, which is not physically linked to the CD3/TCR complex (4-6). As previously shown (7), the interaction between surface receptors and their specific ligands elicits a series of early metabolic events, such as an increase in free cytoplasmic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) and inositol-3-phosphate (InsP<sub>3</sub>)<sup>1</sup> formation. Both events are thought to be fundamental steps in the process by which T lymphocytes are triggered to express their functional programs. We have recently shown that the signal transducing mechanisms operating in CD3<sup>+</sup> lymphocytes after stimulation via CD3 and CD2 molecules or with PHA involve activation of the classical inositol-lipid metabolism (8, 9). An important functional consequence of antibody-induced modulation of the CD3/TCR complex is represented by the T cell refractoriness to any further stimulus. This general refractoriness appears to be caused by an inhibition of the early metabolic steps involved in T cell activation (10). These results provide evidence for a regulatory role of CD3/TCR complex in the other pathways of T cell activation and suggest the existence of a physical association of the CD3/TCR complex with the transducing machinery used by all other receptors coupled to PIP<sub>2</sub> hydrolysis.

In this study we have investigated the signal-transducing mechanisms operating in human lymphocytes that lack surface expression of CD3/TCR molecules. To this end, we derived clones from CD3<sup>-</sup> peripheral blood lymphocytes. These clones expressed the CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> or the CD3<sup>-</sup>CD2<sup>-</sup>CD7<sup>+</sup> surface phenotype.

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<sup>1</sup> Abbreviation used in this paper: InsP<sub>3</sub>, inositol-3-phosphate.

We demonstrate here that a rapid increase in  $[Ca^{2+}]_i$  is detectable after anti-CD2 mAb or lectin-induced stimulation of CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> cells. This increment in  $[Ca^{2+}]_i$  is consequent to the release of  $Ca^{2+}$  from intracellular stores and to the influx from the extracellular compartment. In addition, PHA responses were not affected by the antibody-induced CD2 modulation in CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> cells. Therefore, in CD3<sup>-</sup> lymphocytes, CD2 molecules cannot exert a regulatory role comparable to that mediated by the CD3/TCR complex in mature T lymphocytes.

More importantly, we show that PHA can induce  $[Ca^{2+}]_i$  increase and inositol phosphates formation also in CD3<sup>-</sup>CD2<sup>-</sup>CD7<sup>+</sup> lymphocytes, thus indicating that in these cells surface structures other than CD3/TCR or CD2 represent the functional ligand for PHA.

### Materials and Methods

**Monoclonal Antibodies.** mAbs with specificity for the CD3, CD7, and CD28 molecules were derived as previously described (11). Anti-CD2 mAbs (CD2.1 and CD2.9), which in combination induce in lymphocyte clones an increase of  $[Ca^{2+}]_i$  and  $InsP_3$  formation (9), were derived as previously described (12).

**Flow Cytometric Analysis.** The techniques used have been described in detail elsewhere (13). Analysis of total PBMC for the distribution of CD3, CD2, and CD7 antigens was performed using two-color fluorescence cytofluorometric analysis. Cells were stained with FITC-conjugated OKT3 (IgG2a) mAb (Ortho Pharmaceuticals, Raritan, NJ) and anti-CD7 mAb (IgG1) (a generous gift of Dr. B. Haynes) followed by phycoerythrin (PE)-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Inc. Birmingham, AL). In other experiments we used PE-conjugated OKT3 (IgG2a) mAb followed by FITC-conjugated anti-Leu-5b (IgG2a) mAb (Becton Dickinson Immunocytometry Systems, Mountain View, CA), or FITC-conjugated anti-Leu-5b (IgG2a) mAb and anti-CD7 (IgG1) mAb followed by PE-conjugated goat anti-mouse IgG1. For clonal analysis,  $2 \times 10^5$  clone cells were stained with the appropriate mAb followed by fluoresceinated goat anti-mouse Ig. Control aliquots were stained with the fluorescent reagent alone. All samples were then analyzed on a flow cytometer (FACS II; Becton Dickinson Immunocytometry Systems) gated to exclude nonviable cells. Results are expressed as arbitrarily normalized histograms, i.e., number of cells vs. fluorescence intensity.

**FACS Sorting.** Before sorting cells, the sample tubing of the FACS were treated as previously described (14). Because the fluorescence profiles of PBMC stained with anti-CD3 mAb were clearly bimodal (data not shown), <5% of all cells had to be discarded because their fluorescence intensity coincided with the inflection point of the curve. Sorted cells were collected in sterile plastic tubes whose inside surfaces had been coated with sterile FCS. Once collected, cells were immediately diluted in culture medium.

**Modulation Experiments.** Surface modulation of CD2 molecules was achieved by incubating CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> clones for 24 h at 37°C in the presence of saturating concentrations of mAbs as previously described (6). To ensure the actual modulation of CD2 antigen, modulated and unmodulated cloned cells were stained with appropriate anti-CD2 mAb and fluoresceinated second reagent, and analyzed on a flow cytometer. Only when fluorescence intensity was reduced by >90% were cloned cells further used in the studies.

**Limiting Dilution Microcultures.** CD3<sup>-</sup> FACS-sorted cells were seeded in limiting numbers (from 8 to 0.5 cell/well) in round-bottomed microwells containing  $10^5$  irradiated (5,000 rad) autologous PBMC feeder cells and 1% (vol/vol) PHA in a final vol of 0.2 ml culture medium as previously described (14, 15). After a 24-h culture period, rIL-2 (100 U/ml) was added to each microwell. Microcultures were then supplemented weekly with 100  $\mu$ l of rIL-2-containing medium. After 14–21 d of culture, each microwell was assessed microscopically for growth.

**Isolation of Fresh Tumor Target Cells.** Ascitic fluid was collected by paracentesis from patients

with diffuse melanoma peritoneal metastasis into sterile 500-ml bottles containing 5,000 U heparin. The presence of neoplastic cells was confirmed by histopathological analysis. Macrophages were removed by adherence to plastic flasks at 37°C in 5% CO<sub>2</sub> for 1 h. Tumor cells were additionally purified by centrifugation over discontinuous Ficoll-Hypaque density gradients as described previously (15, 16) and were frozen in several aliquots according to standard procedures.

**Assay for Cytolytic Activities.** Cytolytic activity of cell populations or limiting dilution microcultures (14, 15) was tested against the human NK-sensitive K562 and the human NK-resistant freshly derived melanoma cells. To this end, each microculture was split into 100- $\mu$ l aliquots and was transferred into V-bottomed wells of microtiter trays with  $5 \times 10^3$  <sup>51</sup>Cr-labeled target cells in a final vol of 200  $\mu$ l. Plates were centrifuged and then incubated for 4 h at 37°C. After incubation, plates were centrifuged again and cytolysis was evaluated by counting 0.1 ml supernatant in a gamma counter. Specific lysis was evaluated as previously described (14).

**Determination of Free [Ca<sup>2+</sup>]<sub>i</sub>.** Determination of [Ca<sup>2+</sup>]<sub>i</sub> was performed as previously described (9). Briefly, CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> and CD3<sup>-</sup>CD2<sup>-</sup>CD7<sup>+</sup> clones ( $1.5 \times 10^6$ /ml) were loaded with acetoxymethyl ester of Fura-2 (1  $\mu$ M final concentration) (Molecular Probes, Inc., Junction City, OR) and the fluorescence of the cellular suspension was monitored with an LS-5 spectrofluorimeter (Perkin-Elmer Corp., Pomona, CA) using 2 ml quartz cuvette. The cell suspension was excited at 340–380 nm and fluorescence was measured at 510 nm. Five-slit widths were used for both excitation and emission. All measurements were performed at 37°C using a thermostatically controlled cuvette holder and stirring apparatus. [Ca<sup>2+</sup>]<sub>i</sub> were calculated by the method of Grynkiewicz et al. (17). In the Ca<sup>2+</sup> depletion experiments, EGTA (final concentration 5 mM) was added to the cellular suspension from a 500-mM stock (pH 7.40) 1–10 min before the addition of stimulatory mAbs (9).

**Measurements of [<sup>3</sup>H]Inositol Phosphates.** Measurements of inositol phosphates were performed as previously described (8, 9). Briefly, incorporation of [<sup>3</sup>H]myoinositol into phospholipid was achieved by incubating clone cells ( $2 \times 10^6$  cells/ml) in a Hepes-buffered saline solution, supplemented with 1% glutamine and FCS (1%), with 30  $\mu$ Ci of [<sup>3</sup>H]myoinositol (37 MBq/ml; Amersham Corp., Arlington Heights, IL) for 6 h at 37°C, followed by extensive washing, resuspension at  $2 \times 10^6$  cells/ml in the labeling solution, and incubation for additional 60 min at 37°C. 1-ml aliquots of this cellular suspension were then transferred to microfuge tubes and the appropriate additions were made. mAbs were added to a final dilution of 1:400 of ascites fluid and 1% (vol/vol) PHA. Cells were then sedimented in an Eppendorf 5414 centrifuge. The reaction was stopped by adding chloroform/methanol (1:2). The phases were separated by the addition of 0.25 ml H<sub>2</sub>O and 0.25 ml chloroform, and the upper phase was transferred to a borosilicate tube containing 5.5 ml H<sub>2</sub>O. This diluted aqueous phase (~6 ml) was mixed with 300  $\mu$ l of 100 mM disodium tetraborate (5 mM final concentration) and stored on ice until analyzed. Extracts were subjected to ion-exchange chromatography using Dowex 1- $\times$ 8 anion-exchange resin (Bio-Rad Laboratories, Richmond, CA) in formate form (1.2 ml packed resin/column). The radioactivity of the various elution fractions was quantified by scintillation counting in Aquasure (New England Nuclear, Boston, MA). This method does not allow to distinguish among various possible form or isomers of InsP<sub>3</sub>.

## Results

**Derivation and Phenotypic Analysis of CD3<sup>-</sup> Clonal Populations.** PBL were separated into CD3<sup>+</sup> and CD3<sup>-</sup> cells using the FACS and were cloned under limiting conditions in the presence of autologous feeder cells, PHA and r-IL2 as previously described (14). Most CD3<sup>+</sup> cells underwent clonal expansion, whereas ~10% CD3<sup>-</sup> cells were clonogenic under the culture conditions used (18). In view of the low numbers of CD3<sup>-</sup> cells plated per microwell and of the relatively low cloning efficiency observed in this subset, the majority of limiting dilution proliferating CD3<sup>-</sup> microcultures could be operationally considered as clones. Selection of CD3<sup>-</sup> clones was also

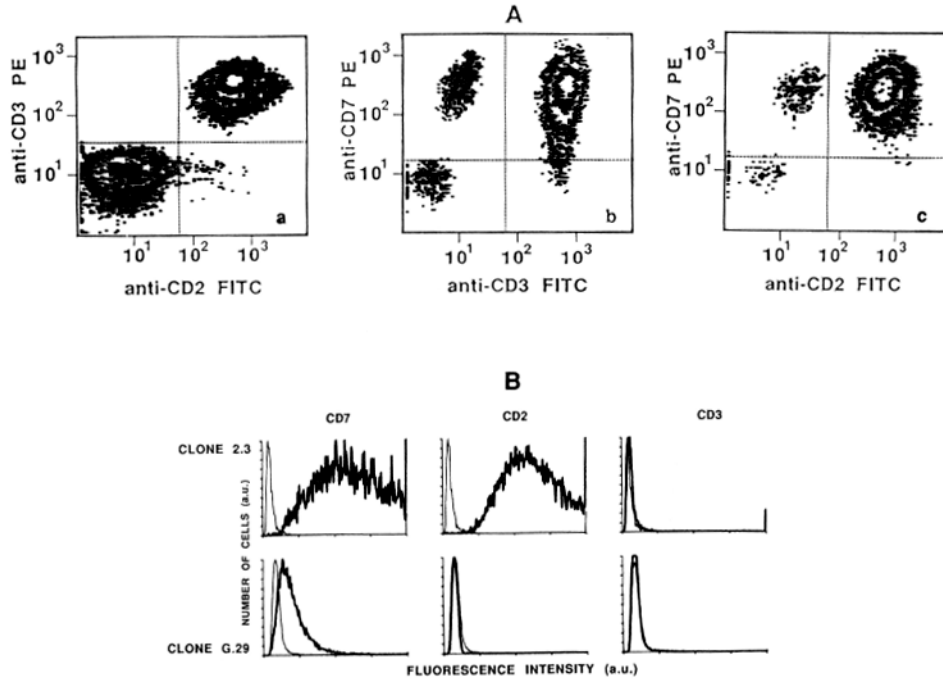


FIGURE 1. Cytofluorometric analysis of CD7, CD2, and CD3 antigen expression in peripheral blood and in cloned lymphocytes. (A) PBL were stained with FITC-anti-CD2 mAb and PE-anti-CD3 mAb (a), or with FITC-anti-CD3 and PE-anti-CD7 (b), or with FITC-anti-CD2 and PE-anti-CD7 (c) as described in Materials and Methods. Based on control samples, the contour plot was divided into quadrants representing unstained cells (*lower left*), cells with only red fluorescence (*upper left*), cells with red and green fluorescence (*upper right*) and cells with only green fluorescence (*lower right*). (B) 2.3 is a CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> clone, whereas G29 is a CD3<sup>-</sup>CD2<sup>-</sup>CD7<sup>+</sup> clone. Cells were stained with various mAbs by indirect immunofluorescence and analyzed on the FACS. Background fluorescence given by cells stained with second reagent alone is shown in each histogram.

based on their ability to proliferate sufficiently to give numbers of cells suitable for different studies, including phenotypic and functional analysis as well as analysis of the signal-transducing mechanisms.

We first analyzed the distribution of CD3 and CD2 antigens in resting PBL by two-color fluorescence cytofluorometry (Fig. 1 A, panel a). Although most CD2<sup>+</sup> lymphocytes coexpressed CD3 antigen, a subset (9.3% in the experiment shown) of CD2<sup>+</sup> cells did not. Similarly, when PBL were analyzed for expression of CD7 and CD3 (Fig. 1 A, panel b), a subset of CD7<sup>+</sup> cells (15.6%) did not express CD3. Moreover, when the same PBL population was analyzed for the expression of CD7 and CD2 antigens (Fig. 1 A, panel c) a small fraction of cells (6.3%) displayed the CD7<sup>+</sup>CD2<sup>-</sup> surface phenotype.

Clones expressing a similar CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> and CD3<sup>-</sup>CD2<sup>-</sup>CD7<sup>+</sup> surface phenotype were obtained by cloning CD3<sup>-</sup> PBL. The surface phenotype of two among the five clones selected for detailed analysis is shown in Fig. 1 B. Clone 2.3 is representative of CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> clones, whereas clone G29 is representative

TABLE I  
Cytolytic Activity of CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> and CD3<sup>-</sup>CD2<sup>-</sup>CD7<sup>+</sup> Clones

Clone <sup>†</sup>	<sup>51</sup> Cr-labeled target cells*	
	K-562	Melanoma
2.3	95	47
LG2	100	ND
2.1	87	35
G29	91	25
LA11	100	ND

\* Data are expressed as percent <sup>51</sup>Cr release. The E/T ratio used in this experiment was 2:1.

† Clones 2.3 and LG2 are CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup>. Clones 2.1, G29 and LA11 are CD3<sup>-</sup>CD2<sup>-</sup>CD7<sup>+</sup>.

of CD3<sup>-</sup>CD2<sup>-</sup>CD7<sup>+</sup> clones. Although not shown all the five clones examined were also CD4<sup>-</sup>, CD8<sup>-</sup> and CD28<sup>-</sup>.

**Functional Analysis of CD3<sup>-</sup> Clones.** Previous studies (15) performed in our laboratory have shown that CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> clones display a broad cytolytic activity against a panel of different target cells, including the NK-sensitive K562 cell line, NK-resistant cell lines, and different types of fresh tumor target cells. In contrast, the majority of CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> clones did not release significant amounts of IL-2 upon stimulation regardless of the stimulus used (including anti-CD2 mAbs, PHA, and ionomycin) (18). Table I summarizes the functional characteristics of three CD3<sup>-</sup>CD2<sup>-</sup>CD7<sup>+</sup> clones and two CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> clones. It is evident that CD3<sup>-</sup>CD2<sup>-</sup>CD7<sup>+</sup> clones, similar to CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> clones, displayed a strong cytolytic activity against the NK-sensitive K562 cell line and were also able to lyse NK-resistant freshly derived human melanoma cells.

**Analysis of Signal-transducing Mechanisms in CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> Clones.** We first investigated whether stimulation via CD2 molecules or with PHA could induce an increase in [Ca<sup>2+</sup>]<sub>i</sub> in CD3<sup>-</sup>CD2<sup>+</sup> clones. As shown in Fig. 2, the use of either a suitable combination of anti-CD2 mAbs (A and E) or of PHA (C and G) elicited a rapid increase in [Ca<sup>2+</sup>]<sub>i</sub> in both LG2 (A and C) and 2.3 (E and G) clones. When the experiments were performed in the absence of extracellular Ca<sup>2+</sup> a transient increase in [Ca<sup>2+</sup>]<sub>i</sub> was still detectable after stimulation with anti-CD2 mAbs (B and F) and PHA (D and H). These results indicate that the increase in [Ca<sup>2+</sup>]<sub>i</sub> observed in the presence of extracellular calcium was due to release of Ca<sup>2+</sup> from intracellular stores and to an influx from the extracellular compartment. It should be stressed that the small increase in [Ca<sup>2+</sup>]<sub>i</sub> detected in the absence of extracellular Ca<sup>2+</sup> after anti-CD2 mAb stimulation, has to be considered specific, since addition of Triton X-100 at the end of the stimulation period did not increase further the levels of fluorescence (Fig. 2).

The results on Ca<sup>2+</sup> mobilization suggested that in CD3<sup>-</sup> clones the metabolic pathway operating in the transduction of the signal via CD2 molecules or PHA involved the classical inositol lipid metabolism, as is the case in CD3<sup>+</sup> cells. To substantiate this hypothesis, we determined whether stimulation of CD3<sup>-</sup> cells by anti-CD2 mAbs or PHA caused an increase in the level of inositol phosphates. As shown in Fig. 3, stimulation with PHA caused a significant increase of InsP<sub>3</sub> (panel C)

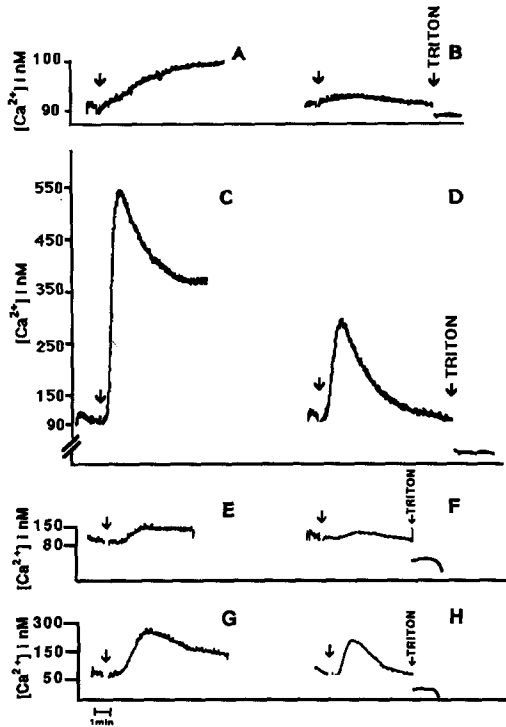


Figure 2. Increase in  $[Ca^{2+}]_i$  mediated by anti-CD2 mAbs or PHA in CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> clones. (A and E) Increase in  $[Ca^{2+}]_i$  mediated by anti-CD2 mAbs in LG2 and 2.3 clones, respectively. (C and G)  $[Ca^{2+}]_i$  increments after PHA stimulation in the same clones. The cells were placed in the fluorimeter and stimulated with either a stimulatory combination of anti-CD2 mAbs (1:800 of ascite fluid) or 1% (vol/vol) PHA. (A, C, E, and G) The different stimuli were added in the presence of 1 mM extracellular  $Ca^{2+}$ ; (B, D, F, and H)  $[Ca^{2+}]_i$  increases in response to anti-CD2 mAbs (B, F) or PHA (D, H) were examined after depletion of extracellular  $Ca^{2+}$  by EGTA (10 mM final concentration). EGTA was added 2 min before addition of the stimulus. Triton X-100 was added at the final concentration of 0.1%. Arrows indicate the time of the addition of the stimuli  $[Ca^{2+}]_i$  calculated by the method of Grynkiewicz et al. (17) is displayed on the vertical axis.

(the mobilizer of  $Ca^{2+}$  from intracellular stores) as well as of (InsP<sub>2</sub>) (panel B) and InsP<sub>1</sub> (panel A). In contrast, stimulation with anti-CD2 mAbs did not induce detectable increments in the various inositol phosphates (Fig. 3). The lack of increase in inositol phosphates, after anti-CD2 mAb stimulation, in our view, could be explained by the higher sensitivity of the  $Ca^{2+}$  assay as compared with the inositol phosphate assay.

Previous studies have suggested that the CD3/TCR complex and the CD2 molecules could represent the ligand for PHA on the surface of human T lymphocytes (19–21). The results presented above clearly demonstrate that PHA can transduce a signal in cells that lack surface expression of CD3/TCR complex. To answer the question whether the signaling mediated by PHA occurs via surface CD2 molecules, we induced surface modulation of CD2 molecules in 2.3 clone. As shown in Fig. 4 (right inset, bottom) after a 24-h incubation period in the presence of saturating doses of anti-CD2 mAbs, cells from clone 2.3 displayed a negative surface staining for anti-CD2 mAbs. The ability of anti-CD2 mAbs and PHA to induce increments in  $[Ca^{2+}]_i$  was then determined in unmodulated and CD2-modulated 2.3 cells. Fig. 4 shows that in the latter cells the increase in  $[Ca^{2+}]_i$  mediated by anti-CD2 mAbs was completely abrogated (C), whereas the  $[Ca^{2+}]_i$  increase mediated by PHA was unaffected (F).

**Transmembrane Signaling in CD3<sup>-</sup>CD2<sup>-</sup>CD7<sup>+</sup> Clones.** We next analyzed three representative clones that expressed surface CD7 antigen but did not express detectable surface CD3 and CD2 antigens. These clones represented a useful tool in order to

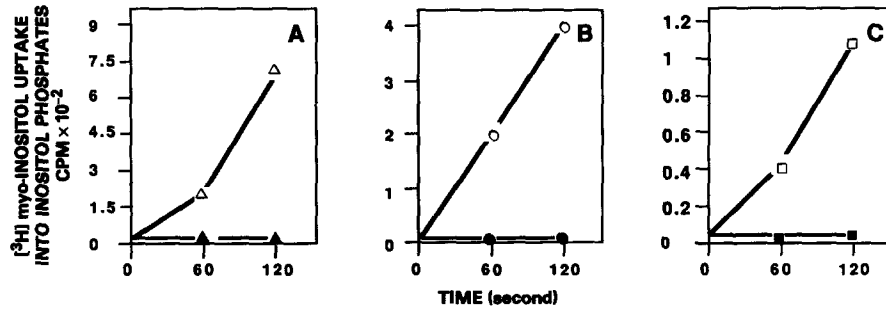


FIGURE 3. Levels of [ $^3\text{H}$ ]inositol phosphates in  $\text{CD3}^- \text{CD2}^+ \text{CD7}^+$  LG2 clone stimulated with anti-CD2 mAbs or PHA. (A) Levels of  $\text{InsP}_1$  after anti-CD2 ( $\blacktriangle$ ) or PHA ( $\triangle$ ) stimulation. (B)  $\text{InsP}_2$  levels after anti-CD2 ( $\bullet$ ) or PHA ( $\circ$ ) stimulation. (C)  $\text{InsP}_3$  levels mediated by anti-CD2 ( $\blacksquare$ ) or PHA ( $\square$ ). The indicated time points are representative of the intervals from the addition of the stimulus to the lysis in chloroform/methanol (1:2). Data are expressed as counts per minute after subtraction of background levels.

clarify whether PHA could trigger a response in the absence of CD2 molecules as previously suggested by CD2 modulation experiments in  $\text{CD3}^- \text{CD2}^+ \text{CD7}^+$  clones. To this end, clones were labeled with the fluorescent  $\text{Ca}^{2+}$  dye Fura-2 and stimulated with PHA. As shown in Fig. 5, in all instances, a rapid increase in  $[\text{Ca}^{2+}]_i$  was observed after PHA stimulation either in the presence (A, C, and E) or in the absence (B, D, and F) of extracellular  $\text{Ca}^{2+}$ . Moreover, as shown in Fig. 6, stimulation with PHA induced a significant increase in the levels of the various forms of inositol phosphates in the two clones analyzed. Since these cells express the T

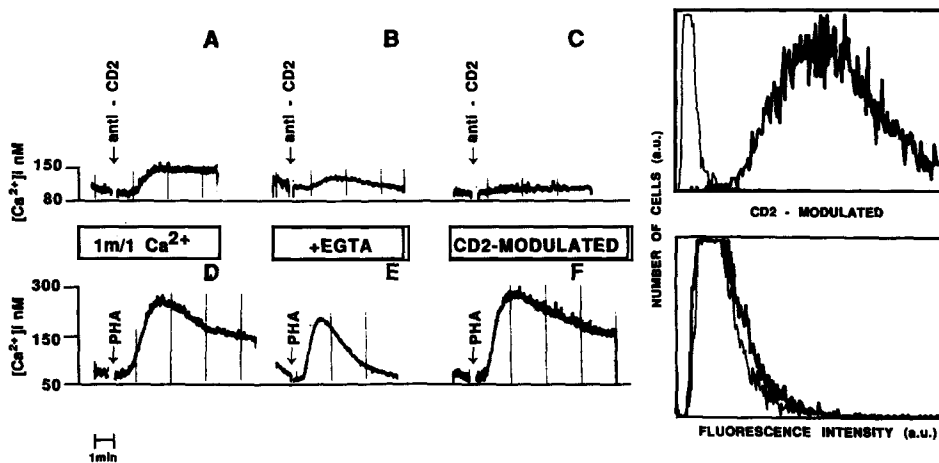


FIGURE 4. Effect of modulation of surface CD2 molecules on the  $[\text{Ca}^{2+}]_i$  increments induced by PHA in the  $\text{CD3}^- \text{CD2}^+ \text{CD7}^+$  2.3 clone. Unmodulated 2.3 cells were stimulated with anti-CD2 mAbs (A, B) or PHA (D, E) in the presence or absence of extracellular  $\text{Ca}^{2+}$ . (C) CD2-modulated 2.3 cells stimulated with anti-CD2 mAbs. (F) Increase in  $[\text{Ca}^{2+}]_i$  after PHA stimulation in CD2-modulated 2.3 cells. Arrows indicate the time of addition of the stimuli. The inset (right) shows CD2 expression in unmodulated (top) and in CD2-modulated (bottom) 2.3 cells.

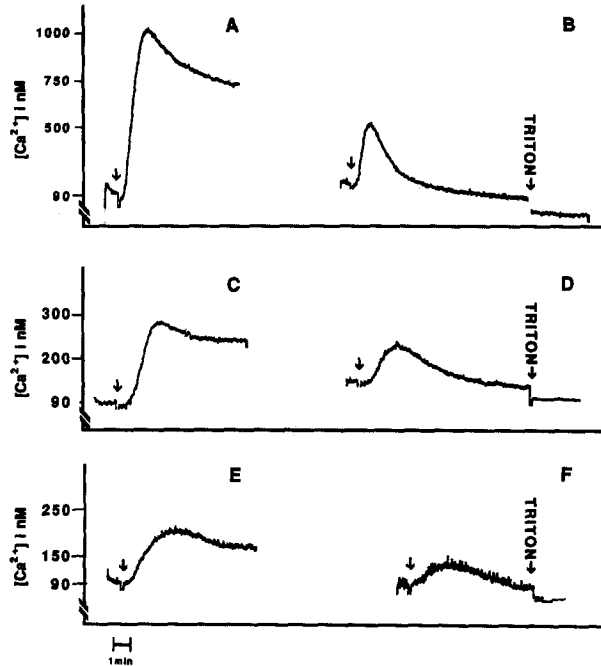


Figure 5. Analysis of  $[Ca^{2+}]_i$  increase in CD3<sup>-</sup> CD2<sup>-</sup> CD7<sup>+</sup> clones in response to PHA. (A, C, and E)  $[Ca^{2+}]_i$  increments mediated by PHA in LA11, G29, and 2.1 clones, respectively. The stimulus was added in presence of 1 mM extracellular  $Ca^{2+}$ . (B, D, and F) Increase in  $[Ca^{2+}]_i$  after depletion of extracellular  $Ca^{2+}$  by EGTA. Arrows indicate the time of the addition of the stimuli.

cell lineage-related CD7 antigen, we analyzed whether the use of anti-CD7 mAbs (either alone or in combination) would result in signal transduction similar to that observed by using PHA. In no instances could  $[Ca^{2+}]_i$  increases in the presence of anti-CD7 antibodies be observed.

### Discussion

In this study, we analyzed the signal-transducing mechanisms operating in cytolytic clones that lack surface expression of the CD3/TCR complex (CD3- $\alpha/\beta$  or CD3- $\gamma/\delta$ ). Two major phenotypically distinct clonal populations could be derived from CD3<sup>-</sup> sorted lymphocytes. The first is characterized by the surface expression of CD2 and CD7 antigens, while the second expresses surface CD7 but not CD2 antigens. Both CD3<sup>-</sup> CD2<sup>+</sup> CD7<sup>+</sup> and CD3<sup>-</sup> CD2<sup>-</sup> CD7<sup>+</sup> cells did not express surface CD4, CD8, or CD28 antigen. Functional analysis demonstrated that both types of cells had a strong cytolytic activity against NK-sensitive as well as NK-resistant tumor target cells (15). In addition, both types of cytolytic clones were usually unable to produce IL-2, whereas they released significant amounts of IFN- $\gamma$  after cell stimulation with calcium ionophore A23187 and PMA (18).

Previous studies, designed to clarify the functional interactions among the various pathways involved in T cell activation (i.e., CD3/TCR complex, CD2 molecules, activation via PHA), have clearly demonstrated that the CD3/TCR complex exerts a regulatory control on the other pathways of T cell activation (5, 6). In addition, we showed that the general T cell refractoriness that follows antibody-induced surface modulation of the CD3/TCR complex can be explained by the inhibition of



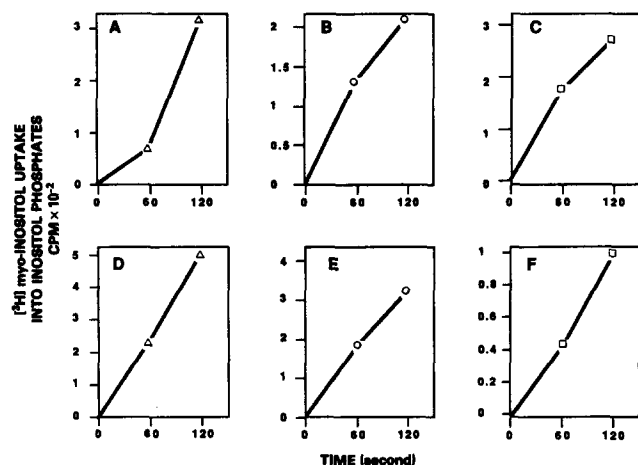


Figure 6. PHA stimulation induces increased levels of [ $^3\text{H}$ ]inositol phosphates in  $\text{CD3}^- \text{CD2}^- \text{CD7}^+$  clones. (A, B, and C) Levels of [ $^3\text{H}$ ]InsP $_1$  ( $\Delta$ ), [ $^3\text{H}$ ] InsP $_2$  (O), and [ $^3\text{H}$ ]InsP $_3$  ( $\square$ ) in G29 clone in response to PHA. (D, E, and F) [ $^3\text{H}$ ]inositol phosphate formation after PHA stimulation in the LA11 clone. The indicated time points are representative of the intervals from the addition of the stimulus to the lysis in chloroform/methanol (1:2). Data are expressed as counts per minute after subtraction of background levels.

the early metabolic steps involved in T cell activation, such as the increase in  $[\text{Ca}^{2+}]_i$  and the formation of inositol phosphates (10). An alternative approach has been to analyze  $\text{CD3}^- \text{TCR}^- \text{CD2}^+$  cell variants, derived from the IL-2-producing leukemia T cell line Jurkat. By using this approach, Weiss et al. (22) demonstrated that the loss of surface expression of the  $\text{CD3}/\text{TCR}$  complex led to functional unresponsiveness of the variant to PHA and to a metabolic block that is evidenced by the lack of  $[\text{Ca}^{2+}]_i$  increase after stimulation with PHA. However, a group of similar  $\text{CD2}^+ \text{TCR}^- \text{CD3}^-$  variants, obtained from the same cell line in our laboratory, was found to be responsive to anti- $\text{CD2}$  mAbs and at least in one case to PHA (23). Therefore, these data already suggested that the loss of the  $\text{CD3}/\text{TCR}$  complex does not necessarily lead to the loss of cell responsiveness and that at least in some  $\text{CD3}^-/\text{TCR}^-$  variants the transducing machinery may be conserved and functional. On the other hand, in these artificially obtained  $\text{CD3}^-$  cells different mutational events may be responsible for the different functional behavior of distinct T cell variants. Thus, we analyzed cells that constitutively lack surface  $\text{CD3}$  molecules.

The analysis of clones derived from normal PBL provided the opportunity to investigate whether the transmembrane signaling via  $\text{CD2}$  molecules or mediated by PHA can still be observed in cells that constitutively lack the surface  $\text{CD3}/\text{TCR}$  complex. On the basis of the proposed strict link between the  $\text{CD3}/\text{TCR}$  complex and the transducing machinery in the membrane, one may expect that the transmembrane signaling mediated by  $\text{CD2}$  molecules or PHA could be abrogated in cells lacking  $\text{CD3}/\text{TCR}$  structures. In this regard, Alcover et al. (24) showed that an increase in  $[\text{Ca}^{2+}]_i$  could be detected in  $\text{CD3}^- \text{CD2}^+$  cells. This increment was only dependent upon an influx from the extracellular compartment. However, the stimulatory combination of anti- $\text{CD2}$  mAbs used in the above study differently from our anti- $\text{CD2}$  mAbs failed to mobilize  $\text{Ca}^{2+}$  from intracellular stores also in  $\text{CD3}^+$  cells (25). Our results clearly demonstrate that in  $\text{CD3}^- \text{CD2}^+ \text{CD7}^+$  cells the signal-transducing mechanisms after receptor-ligand interaction are conserved and that an activation of the classical inositol lipid metabolism is involved. In addition, our data suggest that the regulatory function of the  $\text{CD3}/\text{TCR}$  complex on other pathways

of T cell activation occurs only in cells that bear TCR structures. Therefore, in cells that constitutively do not express the CD3/TCR complex the lack of these structures does not prevent the cell responsiveness via other pathways of activation.

The analysis of signal-transducing mechanisms in the CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> clones also allowed us to investigate whether, in the absence of surface CD3 expression, the regulation of lymphocyte responses could be assigned to CD2 molecules. Moreover, we could evaluate the involvement of CD2 molecules in the stimulation mediated by PHA. We showed that mAb-induced surface modulation of CD2 molecules completely abrogated the increase in  $[Ca^{2+}]_i$  after stimulation with anti-CD2 mAb, whereas it did not affect the  $[Ca^{2+}]_i$  increase mediated by PHA. These results indicate that CD2 molecules do not substitute for the CD3/TCR complex in regulating other pathways of activation in CD3<sup>-</sup>TCR<sup>-</sup> cells and that PHA stimulation is independent of the surface expression of CD2 molecules.

The analysis of transmembrane signaling in CD3<sup>-</sup>CD2<sup>-</sup>CD7<sup>+</sup> clones demonstrated that the PHA induced activation of this type of cells does not require surface expression of CD3/TCR or CD2 molecules. Thus, although previous studies performed in CD3<sup>+</sup> cells suggested that the CD3/TCR complex and CD2 molecules may represent the ligand for PHA (19–21), our present results indicate that the lectin-induced cell triggering may occur via other surface molecules than CD3/TCR or CD2.

Whether these other surface PHA-binding molecules present in CD3<sup>-</sup>TCR<sup>-</sup> cells are also expressed in CD3<sup>+</sup>TCR<sup>+</sup> lymphocytes has still to be determined. Thus, the downregulation of the PHA-dependent T cell activation, which follows surface modulation of the CD3/TCR complex, may not simply be due to the lack of expression of the proposed ligand (CD3/TCR complex) for PHA. On the contrary, distinct PHA-binding molecules similar to those present in CD3<sup>-</sup> cells may still be expressed after CD3/TCR modulation. If this is the case, the inhibitory effect of the antibody-induced modulation of the CD3/TCR complex on the PHA-induced cell activation is likely to be consequent to the downregulation of the transducing machinery in the membrane rather than to the loss of the cell surface ligand for PHA. One may speculate that in view of the ability to transduce activation signals provided by PHA, this molecule may represent a still undefined surface receptor involved in the MHC-nonrestricted cytolytic activity commonly observed in CD3<sup>-</sup> clones.

### Summary

We have analyzed the transmembrane signaling operating in human cytolytic lymphocytes lacking surface expression of the CD3/TCR complex. Peripheral blood lymphocytes were fractionated into CD3<sup>+</sup> and CD3<sup>-</sup> on the FACS and cloned under limiting conditions in the presence of PHA and IL-2. Approximately 90% CD3<sup>+</sup> and 10% CD3<sup>-</sup> cells underwent clonal expansion. Clones obtained from the CD3<sup>-</sup> fraction belonged to two main phenotypic groups: CD2<sup>+</sup>CD7<sup>+</sup> and CD2<sup>-</sup>CD7<sup>+</sup>. Several clones were expanded and analyzed for surface phenotype and function. All of the five clones selected for detailed analysis did not express CD4, CD8, and CD28 antigens and did not release IL-2, whereas they displayed cytolytic activity against NK-sensitive, NK-resistant, and fresh tumor target cells.

After stimulation with anti-CD2 mAbs or PHA a rapid increase in  $[Ca^{2+}]_i$  was detected in CD3<sup>-</sup>CD2<sup>+</sup>CD7<sup>+</sup> clones. This increment was caused by the release of

Ca<sup>2+</sup> from intracellular stores and by the influx from the extracellular compartment. Signaling in response to PHA did not appear to be dependent upon surface expression of CD2 molecules since antibody-induced modulation of CD2 did not prevent PHA-induced signal transduction.

Similarly, in CD3<sup>-</sup>CD2<sup>-</sup>CD7<sup>+</sup> clones [Ca<sup>2+</sup>]<sub>i</sub> increments and inositol phosphate formation occurred after stimulation with PHA.

These data indicate that the functional PHA-binding structures, expressed in both groups of CD3<sup>-</sup> clones, are distinct from CD3/TCR complex and CD2 molecules.

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