

ANTIBODY-INDUCED MODULATION OF THE CD3/T CELL  
RECEPTOR COMPLEX CAUSES T CELL REFRACTORINESS  
BY INHIBITING THE EARLY METABOLIC STEPS  
INVOLVED IN T CELL ACTIVATION

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Induction of cell activation is usually the consequence of an interaction between specific surface receptors and their ligands (1). The natural ligand involved in T cell activation is the molecular complex formed by a foreign antigen and self MHC products on the surface of antigen-presenting cells. The surface TCR for the antigen/MHC complex is a heterodimeric molecule (carrying variable portions, T<sub>i</sub>) that is linked to the CD3 molecule (2). T cell activation can be artificially induced by the use of mAbs directed to CD3-TCR molecules (3) or to another surface glycoprotein, termed CD2 (4, 5), which is not physically associated with the CD3-TCR complex. Following interaction with specific mAbs, both CD3-TCR (2) and CD2 (5) molecules undergo surface capping followed by their persistent (48–72 h) disappearance (also termed modulation). As a consequence, T cells become refractory to further stimulation with mAbs of the same specificity (2, 5). Receptor modulation by specific ligands and subsequent cell refractoriness to stimuli mediated by the same ligand is a general phenomenon described in many cell types for a variety of surface receptors (6). In contrast, antibody-induced modulation of CD3-TCR receptor complex (but not of CD2 molecules) results in a general cell unresponsiveness that is not restricted to the CD3-TCR activation pathway (5). While the inhibitory effect resulting from modulation of CD3-TCR molecules has been assessed by analyzing different T cell functions, including CTL activity and lymphokine production, little is known about the actual level and mechanism of inhibition. Here we show that the early metabolic steps, such as increase in free cytoplasmic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) and inositol-3-phosphate (InsP<sub>3</sub>) formation, which usually follow receptor-ligand interactions, are inhibited in CD3-TCR-modulated cells. In addition, evidence is provided that this inhibition involves, at least in part, G proteins.

**Material and Methods**

*Cells.* The JA3 cloned cell line was derived by mutagenesis from the IL-2 producing Jurkat T-ALL by exposing these cells to gamma radiation (Cs source) (7).

This work was supported in part by grants from Italian Centro Nazionale Ricerche (P. F. Oncologia).

**Monoclonal Antibodies.** mAb with specificity for the CD3 molecule and for the antigen receptor molecule expressed on JA3 cells were derived as previously described (11). Anti-CD2 mAb (CD2.1 and CD2.9), which in combination induce in JA3 cells an increase of  $[Ca^{2+}]_i$  and  $InsP_3$  were derived as previously described (8).

**Flow Cytofluorometric Analysis.** The techniques used have been described in detail elsewhere (9). Briefly,  $2 \times 10^5$  JA3 cells were stained with the appropriate mAb followed by 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, Mountain View, CA) gated to exclude nonviable cells. Results are expressed as arbitrarily normalized histograms, i.e., number of cells vs. fluorescence intensity.

**Modulation Experiments.** Surface modulation of CD3-TCR or CD2 molecules was achieved by incubating JA3 cells for 24 h at 37°C in the presence of saturating concentrations of mAbs as described (5). To ensure the actual modulation of the various surface antigens, modulated and unmodulated JA3 cells were stained with appropriate anti-CD3, anti-TCR, or anti-CD2 mAbs and fluoresceinated second reagent, and analyzed on a flow cytometer. Only when fluorescence intensity was reduced by >90%, were JA3 cells further used in these studies.

**Determination of  $[Ca^{2+}]_i$ .** Determination of  $[Ca^{2+}]_i$  was performed as previously described (8). Briefly, JA3 cells were loaded with the acetoxymethyl ester of Fura-2 (1  $\mu$ M, final concentration) (Molecular Probes, Inc., Junction City, OR) and the fluorescence of the cellular suspension ( $5 \times 10^6$  cells per 2 ml) was monitored with a Perkin-Elmer LS-5 spectrofluorimeter using 2-ml quartz cuvette. The cell suspension was excited at 340 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.  $[Ca^{2+}]_i$  calculated by the method of Grynkiewicz et al. (10) is displayed on the vertical axis. When the stimulus was provided by  $AlF_4^-$ , a mixture of NaF (10 mM final concentration) and  $AlCl_3$  (25  $\mu$ M final concentration) was added.  $Mn^{2+}$  was used at 25  $\mu$ M, final concentration.

**Measurements of  $[^3H]$ Inositol Phosphates.** Measurements of inositol phosphates were performed as previously described (8, 11). Briefly, incorporation of  $[^3H]$ myoinositol into phospholipid was achieved by incubating JA3 cells ( $10^7$  cells/ml) in a Hepes-buffered saline solution with 30  $\mu$ Ci of  $[^3H]$ myoinositol (37 MBq/ml; Amersham Corp., Arlington Heights, IL) for 3 h at 37°C, followed by extensive washing, resuspension at  $10^7$  cells/ml in the labelling solution, and incubation for additional 60 min at 37°C. Aliquots (1 ml) of this cellular suspension were then transferred to microfuge tubes and the appropriate additions were made. Monoclonal antibodies were added to a final dilution of 1:400 of ascites fluid. When the stimulus was provided by  $AlF_4^-$ , a mixture of NaF (10 mM, final concentration) and  $AlCl_3$  (25  $\mu$ M, final concentration) was added. 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  $H_2O$  and 0.25 ml chloroform, and the upper phase was transferred to a borosilicate tube containing 5.5 ml  $H_2O$ . 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-x8 anion-exchange resin (Bio-Rad Laboratories, Richmonds, CA) in formate form (1.2 ml packed resin per 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 one to distinguish among various possible form or isomers of  $InsP_3$ .

**Treatment of JA3 Cells with Cholera Toxin.** JA3 cells ( $5 \times 10^6$  cells/ml) were incubated at 37°C for 3 h in medium alone or in the presence of 0.2  $\mu$ g/ml of cholera toxin. After incubation JA3 cells were washed twice with cold medium and used for  $Ca^{2+}$  measurements.

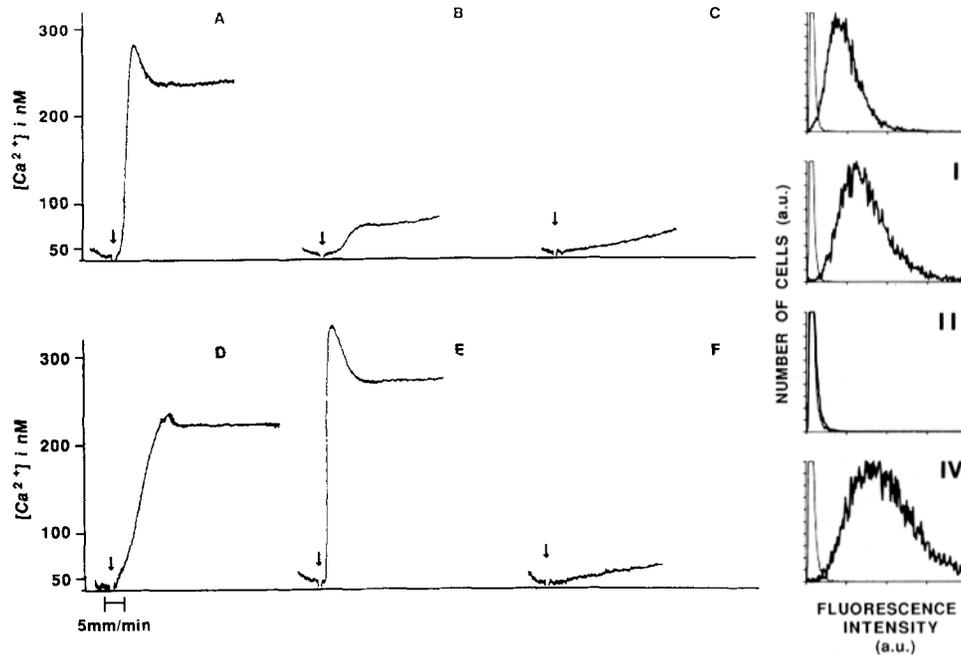


FIGURE 1. Effect of modulation of either CD3-TCR or CD2 molecules on the  $[Ca^{2+}]_i$  increments induced by stimuli acting via one or another activation pathway. Unmodulated JA3 cells stimulated with either anti-TCR (A) or anti-CD2 mAbs (D). CD3-TCR modulated JA3 cells stimulated with anti-TCR (B) and anti-CD2 (C). CD2 modulated JA3 cells stimulated with anti-TCR (E) and anti-CD2 (F). The cells were placed in the fluorimeter and stimulated with either a stimulatory combination (8, 12) of anti-CD2 mAb (1:800 of ascites fluid) or anti-TCR mAb (12) (1:800 of ascites fluid). The tracings are representative of 10 separate experiments. The different stimuli were added in the presence of 1 mM extracellular  $Ca^{2+}$ . Arrows indicate the time of addition of the stimuli. The inset photograph (right) shows that mAb-induced modulation of CD3-TCR complex did not affect surface expression of CD2 antigen. (I) TCR and (II) CD2 expression in unmodulated JA3 cells. III and IV: TCR and CD2 expression in JA3 cells modulated with anti-TCR mAb.

### Results and Discussion

In these studies we used as indicator cell system the JA3 cloned lymphocytic line, derived from the IL-2 producing Jurkat T cell leukemia. JA3 cells have been previously shown to release IL-2 following stimulation via either the CD3-TCR or the CD2 molecules (5, 7). The signal transducing mechanisms used in the CD2 pathway were found to be undistinguishable from those used in the CD3-TCR pathway (15). The experiments shown in Fig. 1 were designed to analyze the effect of either CD3-TCR or CD2 modulation on the  $[Ca^{2+}]_i$  increase induced by either mAb. After CD3-TCR modulation, a drastic reduction of  $[Ca^{2+}]_i$  rise was evident not only after stimulation with anti-TCR mAb (Fig. 1B), but also after stimulation with anti-CD2 mAbs (Fig. 1C). It should be stressed that CD3-TCR modulation does not affect surface expression of CD2 antigen (Fig. 1, inset). Modulation of CD2, on the other hand, had no effect on  $[Ca^{2+}]_i$  increase induced by anti-TCR mAb (Fig. 1E). It is noteworthy that the treatment with anti-CD3-TCR mAb did not deplete intracellular stores of  $Ca^{2+}$ , as indicated

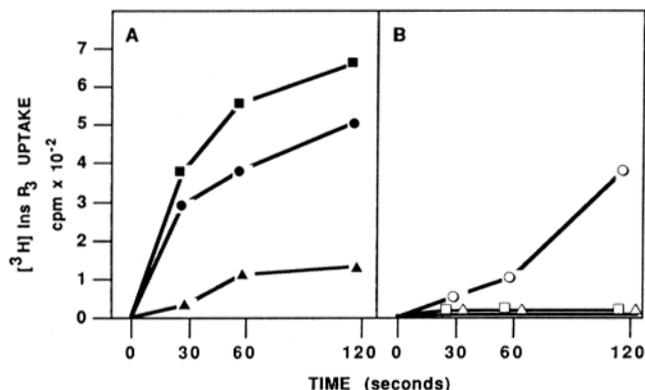


FIGURE 2. Levels of  $[^3\text{H}]\text{InsP}_3$  in unmodulated and in CD3-TCR- or CD2-modulated JA3 cells stimulated with anti-TCR or anti-CD2 mAb. In A, unmodulated (●), CD3-TCR-modulated (▲), and CD2-modulated (■) JA3 cells were stimulated with an anti-TCR mAb. In B, unmodulated (○), CD3-TCR-modulated (△), and CD2-modulated (□) cells were stimulated with anti-CD2 mAbs. The indicated times are representative of the intervals from the addition of the antibody to the lysis of the cells in chloroform/methanol (1:2). Data are expressed as cpm after the subtraction of background levels and are representative of three separate experiments.

by the fact that the response to ionomycin (a calcium ionophore that bypasses surface receptors) was preserved (data not shown).

Because it is believed that  $[\text{Ca}^{2+}]_i$  increases are mediated by  $\text{InsP}_3$  (formed by phosphatidylinositol biphosphate [ $\text{PIP}_2$ ] hydrolysis) (11, 12), we further examined whether modulation of CD3-TCR or CD2 molecules also inhibited the increase of  $\text{InsP}_3$  levels in JA3 cells stimulated with mAbs. In accordance to the data on  $[\text{Ca}^{2+}]_i$ , modulation of CD3-TCR antigens was found to strongly inhibit the  $\text{InsP}_3$  increase induced by either anti-CD3-TCR or anti-CD2 mAbs (Fig. 2). On the other hand, CD2 modulation did not block the  $\text{InsP}_3$  increases stimulated by anti-CD3-TCR mAbs.

We think it is unlikely that the refractoriness of the cells after CD3-TCR modulation is due to the persistence of one, or more, of the second messenger generated by the first stimulus. In fact (a) the levels of  $\text{InsP}_3$  are back to resting levels in  $\sim 6$  h (data not shown), (b) a persistent elevation in protein kinase C (PKC) activity is unlikely, since it has been demonstrated (13) that anti-CD3 mAb induces only a transient association with the membranes of PKC.

An alternative model may explain the general inhibitory effect observed with anti-CD3-TCR antibodies treatment. It has been suggested that the coupling between receptors and  $\text{PIP}_2$  hydrolysis by the specific membrane-bound phosphodiesterase is mediated (with different agonists and in different cell types), by a family of guanine nucleotide binding proteins (G proteins) in a fashion analogous to the coupling of receptors to adenylate cyclase (1, 14). In human T lymphocytes, the CD3-TCR complex, but not CD2 molecules may be physically associated in the membrane with the transducing machinery used by all other receptors coupled to  $\text{PIP}_2$  hydrolysis. If this is the case, modulation of the CD3-TCR complex might result in the concomitant downregulation (or inactivation) of the associated enzymatic complex, G protein, and/or phosphodiesterase. If

this hypothesis is correct, not only activation of second messenger generation by surface receptor stimulation would be inhibited by modulation of CD3-TCR complex, but also direct activation of G proteins would be prevented. As recently demonstrated  $\text{AlF}_4^-$  directly activates the  $G_s$  protein (involved in the activation of adenylate cyclase) and the G proteins coupled to  $\text{PIP}_2$  hydrolysis (15, 16). Preliminary data obtained in our laboratory indicate that modulation of surface CD3-TCR but not of CD2 molecules inhibits the  $[\text{Ca}^{2+}]_i$  and  $\text{InsP}_3$  increases mediated by  $\text{AlF}_4^-$ .

It has been demonstrated that the G proteins coupling the T cell antigen receptor to the  $\text{PIP}_2$  phosphodiesterase are inhibited by cholera toxin (17). Although not shown, cholera toxin also affected the increase of both  $[\text{Ca}^{2+}]_i$  and  $\text{InsP}_3$  induced by either anti-CD2 mAbs or  $\text{AlF}_4^-$ . It is important to note that similar regulatory mechanisms mediated by the CD3-TCR complex are involved in the downregulation of CD2-dependent activation pathway also in normal T lymphocytes (data not shown).

In conclusion, our data demonstrate that the unresponsiveness of T cell that follows modulation of CD3-TCR complex depends upon a metabolic inhibition of the very early events occurring in response to receptor stimulation. Because both  $\text{Ca}^{2+}$  mobilization and inositol metabolism were inhibited, it is likely that the signal is stopped during its transduction within the plasma membrane. The hypothesis that the block occurs at the surface receptor levels is not supported by the finding that in CD3-TCR-modulated T cells, the CD2 pathway was inhibited in spite of the unchanged expression of CD2 surface molecules. The preliminary data obtained with  $\text{AlF}_4^-$  suggest that the inhibition may occur at the level of the transducing mechanism and involve the G proteins and/or the phosphodiesterase.

### Summary

We investigated the mechanism involved in T cell unresponsiveness that follows the monoclonal antibody-induced surface modulation of the CD3-TCR complex. We determined whether modulation of CD3-TCR affected the early metabolic steps such as  $[\text{Ca}^{2+}]_i$  rise and  $\text{InsP}_3$  formation. A strong inhibition of the increase on  $[\text{Ca}^{2+}]_i$  mediated by either anti-TCR or anti-CD2 mAbs was detected. In contrast, surface modulation of CD2 molecules did not prevent the  $[\text{Ca}^{2+}]_i$  increase induced by anti-TCR mAb. Similarly,  $\text{InsP}_3$  increase was strongly reduced only after modulation of CD3-TCR complex (but not of CD2 molecules).

Therefore, it appears that surface modulation of CD3-TCR complex causes T cell refractoriness by inhibiting the very early metabolic events that follow receptor-ligand interactions.

We thank Dr. J.-C. Cerottini for helpful discussion, Alfio Bonaventura for technical assistance, and P. Brunet and J. Duc for typing the manuscript.

*Received for publication 6 April 1987 and in revised form 27 May 1987.*

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