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TCR and immunophenotype changes in dimethyl sulfoxide-dependent programmed cell death

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ABSTRACT: In the thymus most deleted cells are immature thymocytes and the high rate of cell death within the thymus is involved in the development of the initial T-cell receptor repertoire. Functional T-cell receptor recognition units are created by somatic rearrangements of gene segments, and the expression of successfully assembled TCR complex is the key to molecular events that culminate in T-cell activation, growth and differentiation. Previously, we reported that DMSO induces apoptosis in RPMI-8402 human pre-T cells. Here we examine the fate of pre-T cells undergoing negative selection analysing the responsiveness to DMSO-enforced TCR expression and immunophenotype modulation. Our results demonstrate that DMSO induces cell growth inhibition, cell phenotype changes, with down-regulation of CD2 and CD7, and increases in α/β or γ/δ TCR chains led by TdT, RAG-1 and RAG-2 activity. These modifications are associated with an apoptotic program. Taken together, these data suggest the existence of an early checkpoint that ensures *in vivo* the effective intrathymic differentiation supported from another point of view, the linkage between immunophenotypes and TCR regulation in T-cell differentiation and programmed cell death. (J Biol Regul Homeost Agents 2000; 14: 269-74)

KEY WORDS: *Programmed cell death, Differentiation, TCR, Immunophenotype, DMSO, Thymocytes*

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Introduction

Programmed cell death (PCD) is a stereotyped form of cell death occurring in a variety of biological Systems (1-4). It is the principal form of cell death for lymphocytes in processes such as autoreactive T-cell elimination in the thymus, and antigen-driven B-cell selection in germinal centres (5-9). Early T cell progenitors, generated in the fetal liver or in the bone marrow, migrate to the thymus where they mature and differentiate through positive or negative selection processes (10-12). Particularly, positive selection occurs in thymocytes that successfully assemble functional receptor genes expressing the heterodimeric (α/β or γ/δ) T cell receptor (TCR) on the cell surface in association with the CD3 protein complex.

TdT is a creative DNA polymerase acting in early lymphoid cells as a somatic mutagen. TdT can generate diversity in the DNA sequence by adding nucleotides to the gene producing N-regions in the immunoglobulin heavy chain or to the T cell receptor (13, 14). If the TdT is implicated in nucleotide addition, RAG-1 and RAG-2 (recombination activating genes) are two genes critically involved in the TCR V(D)J rearrangement processes (15). Only T cells that express appropriate TCR and that recognize self MHC molecules in the thymus are selected for potential export to the periphery and if not die by programmed cell death.

Apoptosis in lymphoid cells can be induced in different ways depending on the cell system and acquired signals. Activated human peripheral blood T-lymphocytes, and human thymocytes undergo apoptosis when exposed to dexamethasone or to monoclonal antibodies directed at CD2 or CD3 (16). Previously we found that dimethyl sulfoxide (DMSO) provokes cell death by PCD in human pre-T cell line RPMI-8402 (17).

DMSO, which is a well known inducer of cell differentiation, acts as second messenger in several cell lines and this effect is associated with its ability to growth-arrest cells at the G0/G1 phase of the cell cycle (18-20). In this study we analysed the effect of DMSO on the expression of TCR and immunophenotype and how the modulation of these molecules can be related to programmed cell death.

Materials and Methods

Cell cultures

Human RPMI-8402 cell line is a thymic lymphoma showing immunological features of pre-T cells as previously analysed (17, 21). Cells were maintained in continuous suspension culture in RPMI-1640 medium supplemented with 10% FBS, 4 mM L-glutamine, 100 mM Na-pyruvate and 25 mM hepes. Cells were grown

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at $2.5x10^5$/ml, with more than 98% viability as determined by trypan blue exclusion test. During the log growth phase, the cells were treated with 1.5% (v/v) of DMSO (gas chromatography grade) for a time ranging between 0 and 72 hours.

Apoptosis estimation

Apoptosis was detected by morphological examination, DNA laddering assessment and by flow cytometry as previously described (17). Light morphological evaluation was performed by mixing 10µl of $1x10^6$ cells/ml with 10µl of ethidium bromide and acridine orange dye solution and examination by fluorescence microscopy. Cells were scored as either viable or non-viable and either normal or apoptotic. Cells with bright green chromatin were scored as viable; those with bright orange chromatin were scored as non-viable. Normal nuclei are those with chromatin in organised structures while apoptotic nuclei are those with highly condensed or fragmented nuclei.

Fluorescence-activated cell sorter (FACS) analysis

Untreated and DMSO-treated cells were analysed according to their physical parameters or immunophenotype (22-24). The identification of CD3 and TdT-intracellular antigens was done for permeabilised cells. PBS-washed cells were immediately treated for 5 min by FixPerm (Caltag, Milan, Italy) a particular agent containing paraformaldehyde and triton. FixPerm is able to produce a light cell permeabilization while conserving the specific antigenic properties. Permeabilised and whole cells were washed with PBS and suspended in RPMI-1640 medium containing 10% FBS to a density of $2x10^6$ cells/ml. A 0.1 ml aliquot of the cell suspension was placed in a plastic tube and treated with human Ig at a final concentration of 15 mg/ml at 4° C for 15 min to reduce the non specific antibody binding. Samples were then incubated at 4° C for 30 min with an appropriate amount of FITC-conjugated mAb anti-CD2, CD3, CD4, CD7, CD8, CD10, CD16, CD19, CD34, CD45, CD56, CD117, TdT and α/β or γ/δ TCR chains. Fluorescence-stained cells were analysed with FACS-440 (Becton-Dickinson, Mountain View, CA, USA). Human peripheral mononuclear cells were used as negative or positive controls for surface and intracytoplasmic staining.

mRNA quantitation by PCR

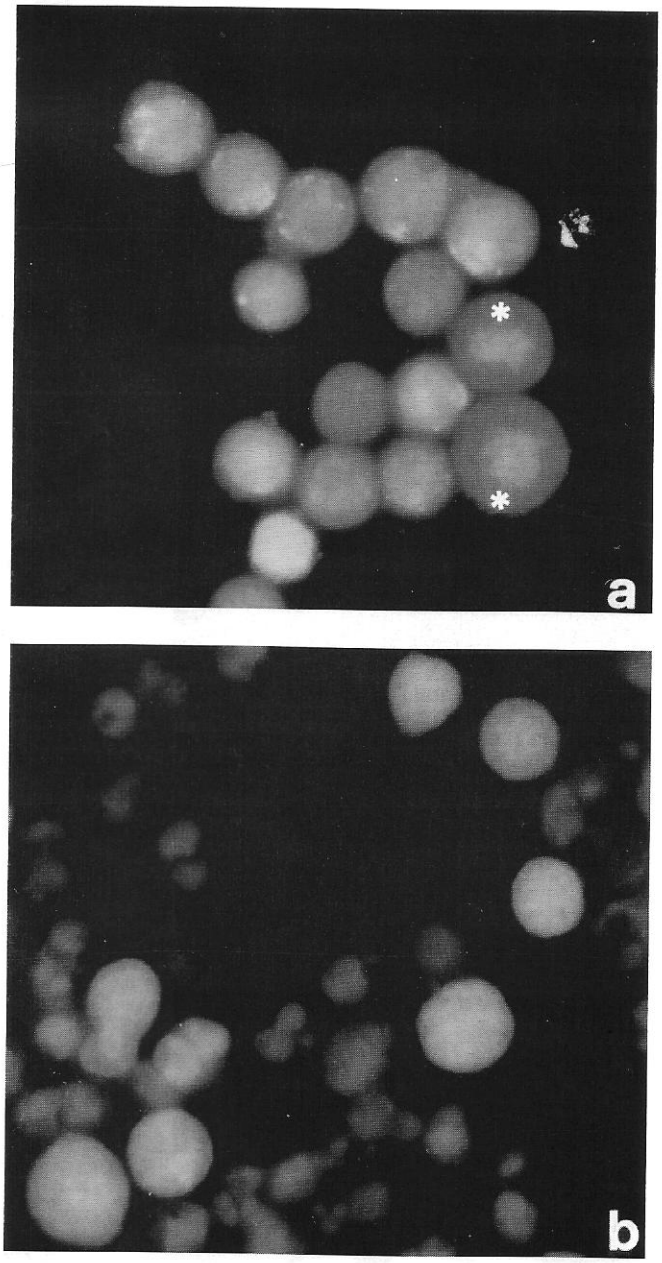
TCR α/β or γ/δ chains, RAG-1, RAG-2 and TdT mRNA levels were evaluated by RT-PCR. In brief, mRNA was extracted from $1x10^6$ untreated and DMSO-treated cells using the Oligotex Direct mRNA Kits (Quiagen Inc. Chatsworth, CA, USA). Serial dilutions (10-fold) of this RNA were used as template for a cDNA synthesis reaction. mRNA levels were then evaluated by quantitative reverse transcriptase PCR using the Promega Access RT-PCR System (Promega Corporation. Madison, WI, USA). Using the same amounts of samples, RT-PCR was performed including positive and negative control (data not shown) according to the manufacturer's procedure. Primers used for TdT, RAG-1 and RAG-2 amplification were the same as those described by Chan et al (6), while oligonucleotides recognising sequences of TCR chains are those described by Sensi et al (25). After amplification, 10µl of PCR products were run on 2% agarose gel. Specificity of the amplified fragments was validated by their predicted size.

Source of Materials

Mouse monoclonal antibodies anti-CD2, CD3, CD4, CD7, CD8, CD10, CD16, CD19, CD34, CD45, CD56, CD 117, α/β or γ/δ-TCR were from Becton Dickinson (Mountain View, CA, USA). Anti-TdT monoclonal antibody was from Supertech (Rockville, MD, USA). Primer sequences were from Genenco (M-Medical, Florence, Italy). All cell line culture material and reagent grade materials were from Sigma (St. Louis, MO, USA).

Results

We previously described that in human pre-T cell line RPMI-8402 DMSO induces a significant modification of the cell cycle associated with PCD (17). Figure 1 shows the presence of apoptotic cells recognizable by typical nuclear modification and staining. These cells after 72 hours of DMSO treatment add up to more than 50% of the total cell population. Because the differentiation of normal and leukemic T-cells can be evaluated by antigenic profile modification, we analysed DMSO-induced differentiation by testing cell-surface markers and expression of TCR-gene products, which commonly lead to the generation of a complete CD3/TCR complex on differentiated cells. A detailed and quantitative antigenic profile of RPMI-8402 cells was obtained using a panel of mAbs in combination with flow cytometric measurements. As shown in Table I untreated cells display the presence of cell surface antigens CD2, CD7, CD10, CD34 and CD45, but lack the CD117, CD4, CD8, CD19, CD16 and CD57 antigens. The presence of intracellular levels of CD3 and TdT have been detected using permeabilised cells. Untreated cells show the phenotype $CD2^+C3^+CD7^+CD10^+CD34^+CD45^+$ TdT^+. The treatment by DMSO induces (Tab. I) changes of immunophenotype profile with a decrease in CD2, CD7 which are completely down regulated respectively after 24 and 48 hours of treatment. TdT turns-down-regulated with the presence of less than 30% positivity after 72 hours while CD3, CD10, CD34 and CD45 antigens appear unaffected by treatment. CD3 remains ever expressed at the cytoplasmic level. It



has been reported that in the thymus, the modification of the immunophenotype is combined with TCR gene transcription and rearrangement (14, 26), suggesting the existence of different control points that regulate the progression of thymocytes from the earliest subset to mature cells (27). Then, we thought it very intriguing to analyse the presence and the modulation of TCR associated to programmed cell death. Figure 2 reports during the DMSO-treatment, cell death progression evaluated by physical parameters (top panels, X and Y area) and TCR chain expression detected by immunofluorescence intensity (corresponding to the lower graphs, X and Y).

After 24 hours of treatment, it is possible to individuate two different cell populations. The first, shown in upper Y areas of sectors 1-4, corresponds to non-apoptotic cells. During the treatment these cells are arrested in G0/G1 phase of the cell cycle and synthesize TCR molecules as reported in peaks b (α/β-chain) and c (γ/δ-chain) of bottom graphs Y. This increase results reliable at 48 and 72 hours of treatment. The second subpopulation (upper X areas, sectors 1-4) is made up of apoptotic cells. The steady increase in this cell population is associated with a concomitant decrease in α/β and γ/δ-TCR positivity (peaks b and c of apoptotic subpopulation), suggesting the hypothesis that apoptosis may occur after the up-regulation of TCR when the molecular machinery of protein synthesis is not yet inhibited (1, 2). To support this data we analysed the level of TCR-mRNAs. RT-PCR analysis (Fig. 3) displays the increase in TCR-mRNAs levels explaining the DMSO-dependent increase in TCR proved by flow cytometric evaluation. At this

◀ **Fig. 1 - Light morphology analysis performed as described in Materials and Methods section. a) Untreated cells. It is possible to observe two mitotic cells (asterisk). b) 72 hours DMSO-treated cells. Note the presence of many cells showing typical apoptotic features as chromatin fragmentation or apoptotic bodies. Magnification x500.**

TABLE I - IMMUNOLOGICAL PROFILE

Time ⇒ ⇓ Phenotype	0 hours	24 hours	48 hours	72 hours
TdT	97	85	48	30
CD34	97	93	95	96
CD45	94	90	90	91
CD10	99	95	96	96
CD2	46	12	a	a
CD3	98	95	93	90
CD4/CD8	a/a	a/a	a/a	a/a
CD7	95	81	38	a/a
CD16/CD57	a/a	a/a	a/a	a/a
CD117/CD19	a/a	a/a	a/a	a/a

Effect of DMSO treatment on CD antigens and TdT. The results are reported as percentage of cell positivity at different times of treatment and are the means of eight different experiments (SD ± 3%). As reported in Materials and Methods intracellular CD3 and TdT antigens are detected in permeabilised cells. The letter a indicates absence of positivity

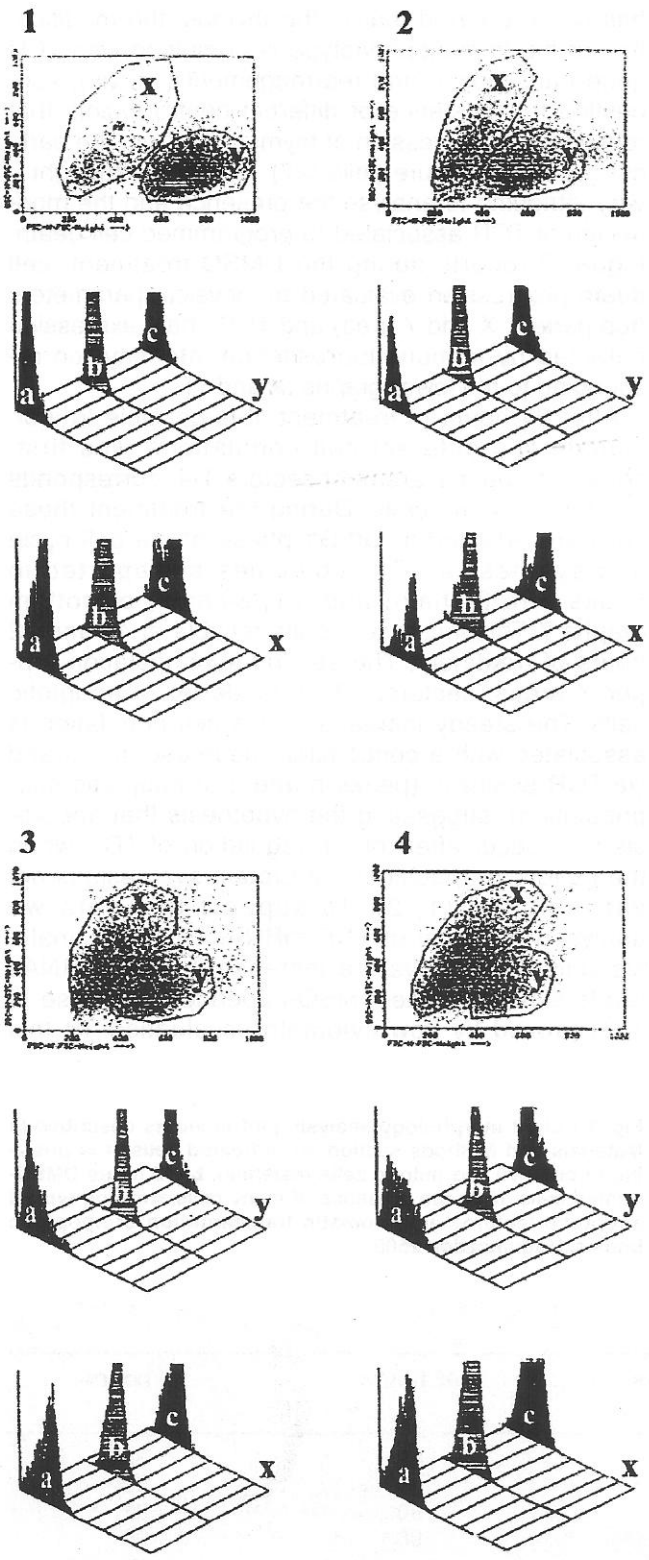


Fig. 2 - Flow cytometric analyses of the forward/sideward scatter of untreated compared with DMSO treated RPMI-8402 cells. X area shows apoptotic subpopulation while Y area shows growing cells. The sectors 1, 2, 3 and 4 report the data obtained respectively at zero, 24, 48 and 72 hours of DMSO treatment. Quantitative comparison of α/β (peak b) and γ/δ (peak c) TCR chains expressed in non apoptotic (Y) and in apoptotic (X) sub-populations. The peak a represents the background of fluorescence. The data represent one out of six independent experiments.

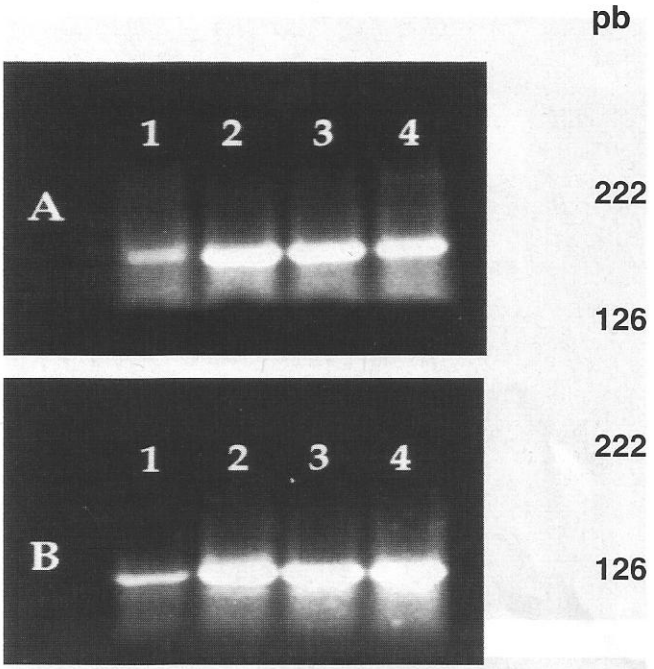


Fig. 3 - TCR α (a) and TCR β (b) gene expression of RPMI-8402 pre-T cell line. mRNA from untreated (lane 1) and 24 (lane 2), 48 (lane 3) and 72 (lane 4) hours of DMSO-treated samples. Amplified products were run on a 2% agarose gel and visualized by EtBr staining. An increase of mRNA levels of α and β TCR chains can be observed after 24 hours of treatment. The data represent one out of four independent experiments.

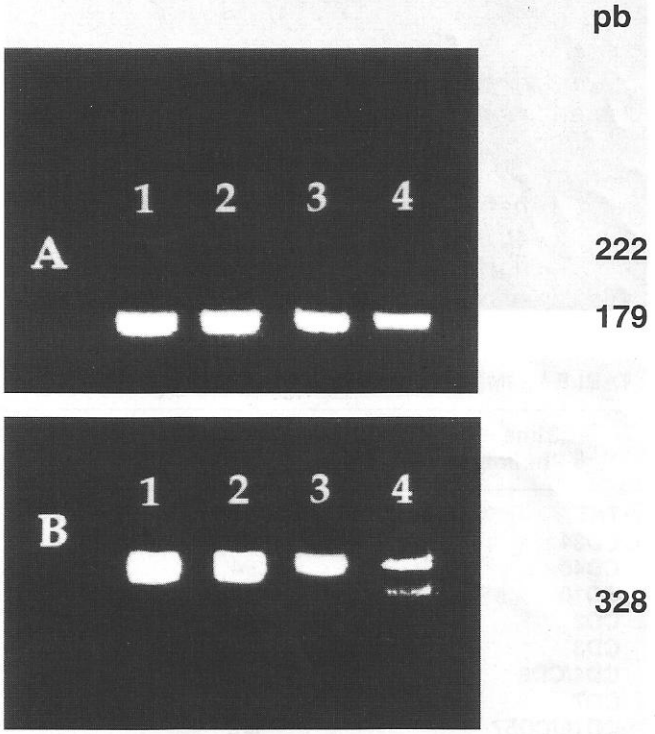


Fig. 4 - RT-PCR detection of RAG-1 (a) and TdT (b) mRNA during DMSO treatment. Lane 1: Untreated cells. Lanes: 2,3 and 4 indicate respectively 24, 48 and 72 hours of treatment. It is possible to note the down-expression of TdT and RAG-1 mRNA levels during the treatment. Similar results were obtained in four other experiments.

point we considered the possible involvement of TdT, RAG-1 and RAG-2, as key regulating agents of TCR expression. RT-PCR analysis confirm the progressive down-regulation of TdT as obtained by FACS (Tab. I) and this is due to the decrease in its mRNA (Fig. 4). Analogous results have been obtained analysing the presence of RAG-1 and RAG-2. In fact, DMSO provokes the down-regulation of RAG-1 and RAG-2 in terms of protein and mRNA synthesis (Fig. 4). All data obtained converge to indicate that DMSO other than cell cycle arrest affects the intracellular pathway of TCR and immunophenotype expression and these changes are related to cell death progression.

Discussion

Cell differentiation is a complex biological process which can be experimentally modulated. In many cell types it has been established that the expression of differentiated phenotype is preceded by growth arrest and that, in some cell lines, growth arrest at a definite cell cycle state must occur before differentiation (28). During the differentiation process, unnecessary or deleterious cells are eliminated by an irreversible process named programmed cell death or apoptosis. PCD consists in ordered sequences of biochemical events occurring in different physiological stages (29). Following a signal, which may be either intrinsic or extrinsic to the cell, the cell reaches a committed phase of an apoptotic program that ends in a final degradation phase. The latter, which includes all of the known morphological stages of apoptosis, is brief but decisive. In the thymus it has been observed that a clonal negative selection by PCD can be activated in cases of inappropriate cell differentiation. In part, this event originates from developing lymphocytes assembling antigen receptor genes by a recombination mechanism with a high probability of generating out-of-frame joins (30, 31). Consequently, most newly generated T cells lack a functional antigen receptor and must be purged. Moreover, those that have generated a receptor that happens to recognise a self antigen must be eliminated to prevent the development of autoimmunity (32, 33). The results reported here help to explain that DMSO-induced PCD may be related to defective cell differentiation with TCR synthesis playing a primary role. Moreover, as previously reported (34), the intensity of TRC signalling and the ability to undergo apoptosis is influenced by the level of CD2 expression.

This is in accord with the results of Mixter et al (35) who demonstrated that in normal mice TCR-α/β^+ $CD4^-$ $CD8^-$ thymocytes contain a high proportion of cells undergoing apoptosis. This apoptotic subpopulation is identified by the absence of surface CD2. Thus, the down-regulation in CD2 level observed in RPMI-8402 treated cells confirms the association of this cell surface glycoprotein with T-CR and apoptosis regulation. A possible scenario suggested could be that DMSO-enforced synthesis of TCR, the deregulation of cell growth and immunophenotype provoke an imbalance in normal cell differentiation. Newly generated ineffective cells activate an endogenous apoptotic program able to provoke a negative clonal selection. Dale Godfrey and Albert Zlotinik (27) proposed an updated model for early T-cell differentiation with five different control-points decisive to the fate of developing thymocytes. As suggested, we prove the existence of a selection point at $CD10^+C2^-CD7^-CD3^-CD4^-CD8$ stage subsequent to TCR expression and TdT and RAGs down-regulation.

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