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# The *c-myc* gene regulates the polyamine pathway in DMSO-induced apoptosis.

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**Abstract.** It is accepted that apoptosis is a gene-controlled process of cellular self-destruction. It occurs during physiological regulation and in pathological situations in the life of a cell. In the immune system, several different intracellular and extracellular factors have been associated with the induction of apoptosis, and the final responses depend on the cell system and the acquired signals. In lymphoid cells, dexamethasone-induced apoptosis is associated with *c-myc* downregulation in cells that remain in G0–G1 until the point of death. Ornithine decarboxylase (ODC), a key enzyme involved in polyamine biosynthesis, is regulated by c-myc, which is a transcriptional activator implicated not only in the control of cell proliferation and differentiation but also in programmed cell death. As dimethylsulphoxide (DMSO) induces apoptosis in the RPMI-8402 human pre-T cell line, the present study analysed the involvement of the *c-myc* proto-oncogene and polyamine pathway as mediators of apoptosis. Cell growth, programmed cell death, c-myc expression, ODC activity and intracellular polyamine content were detected after DMSO and difluoromethylornithine (DFMO) treatment. DMSO-treated cells exhibit a decrease in ODC activity and polyamine levels associated with cell growth arrest and programmed cell death induction. The expression of *c-myc* proto-oncogene, as its mRNA or protein, is specifically down-regulated. DFMO, a well defined polyamine biosynthesis inhibitor, completely blocks ODC activity, resulting in growth inhibition but not apoptosis. Moreover, in these samples no evidence of changes of *c-myc* expression were found. The results obtained suggest that, in RPMI-8402 cells, DMSO provokes a c-myc-dependent decrease of ODC activity followed by a depletion of intracellular polyamine levels, associated with programmed cell death and cell growth arrest.

## INTRODUCTION

Polyamines such as putrescine, spermidine, and spermine are aliphatic cations present in all mammalian cells (Tabor & Tabor 1984, Heby & Persson 1990). Polyamines are involved in a

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large variety of biochemical processes and their apparent interaction with DNA is a very interesting characteristic (Hougard *et al.* 1987, Pegg 1988). The depletion of intracellular polyamine levels inhibits cell growth (Heby 1981, Seiler 1990) and the treatment of mammalian cells with inhibitors of polyamine biosynthesis leads to a cessation of cell division suggesting that they play a strategic role in cell proliferation. Difluoromethylornithine (DFMO) is one of the most important polyamine biosynthesis inhibitors, acting at the first rate-limiting step in polyamine synthesis as an irreversible ornithine decarboxylase (ODC) inhibitor (Sugiura, Shafman & Kufe 1984, Seiler 1990).

ODC, which catalyses the decarboxylation of ornithine to form putrescine, is a highly regulated enzyme with control points at the level of transcription, translation, and protein degradation (Heby & Persson 1990, Seiler 1990). Recently, it has been observed that the *ODC* gene is a myc transcriptional target (Bello-Fernandez, Packman & Cleveland 1993) and that its ability to promote cell cycle progression is a result of, in part, transcriptional activation of the *ODC* gene. The expression of *c-myc* proto-oncogene is then closely associated with cell growth and is down-regulated by growth-inhibitory agents, while an enforced expression promotes cell cycle progression. These results suggest a direct correlation between *c-myc*, ODC activity and polyamine levels during cell proliferation and differentiation. Cell growth and differentiation are events often regulated by programmed cell death.

Programmed cell death or apoptosis is now widely recognized as a common form of cell death and represents a mechanism of cell clearance in many physiological situations where the deletion of cells is required (Ashwell *et al.* 1994, D'Andrea 1994, Linette & Korsmeyer 1994). Previously, it has been hypothesised that apoptosis might be considered as an abortive mitosis owing to deregulated cell cycle progression associated with polyamine deregulation. (Desiderio *et al.* 1995, Grassilli *et al.* 1995). A biochemical marker of apoptosis is the activation of $Ca^{2+}$ and $Mg^{2+}$-dependant endonucleases that cleave host chromatin into oligonucleosome-length fragments. In thymocytes, the prevention of endonuclease activation by spermine shows that DNA fragmentation can be modulated by polyamines, indicating its dependence on the structural arrangement of the chromatin (Brüne *et al.* 1991).

Dimethylsulphoxide (DMSO) is a polyvalent agent in cell biology. In fact, its multiple roles encompass a cryoprotectant of cells in freezing-thawing (Glofcheski *et al.* 1993), a carrier of many compounds (Mintz *et al.* 1993), a differentiating reagent of myeloid cells (Yung *et al.* 1994, Brackman, Lund-Johansen & Aarskog 1995), an inducer for arresting cell growth in the G1/S phase (Sawai *et al.* 1990, Teraoka *et al.* 1991) and a protective agent to differentiated lymphoid cells from apoptosis (Lin *et al.* 1995). Since DMSO (Trubiani *et al.* 1996) can specifically induce RPMI-8402 human pre-T lymphoblastoid cells into apoptosis, this experimental model was used to investigate the involvement of the polyamine pathway in the regulation of DMSO-dependent apoptosis. Using DMSO and DFMO treatment, the relationship of intracellular polyamines metabolism to cell proliferation and programmed cell death was examined.

## MATERIALS AND METHODS

### Cell cultures

The human RPMI-8402 cell line, described by Huang *et al.* (1974), is a thymic lymphoma showing immunological features of pre-T cells. Cells were maintained in continuous suspension culture in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 mM Na–pyruvate, and 25 mM Hepes. Cells were grown at $2.5 \times 10^5$/ml, with

more than 98% viability as determined by the trypan blue exclusion test Cell number was established by averaging at least five counts using a haemocytometer During the log growth phase, the cells were treated with 1 5% (v/v) DMSO (gas chromatography grade) for a time ranging between zero to 72 h (Trubiani *et al* 1996) In an attempt to characterize the role of ODC activity and polyamines in the development of programmed cell death, the ODC suicide substrate inhibitor DFMO was used RPMI-8402 cells were incubated, up to 72 h, with a 5-mM final concentration of DFMO as previously suggested (Sugiura *et al* 1984) The concentrations of DMSO and DFMO used were obtained from values in the literature (Pegg 1988, Seiler 1990, Basu *et al* 1993, Brackman *et al* 1995, Lin *et al* 1995, Trubiani *et al* 1996) and from personal experience These concentrations exerted suitable pharmacological effects without aspecific toxicity (unpublished data)

**Apoptosis estimation**

Apoptosis was detected by morphological examinations, at the light and the electron microscope level, DNA laddering assessment and by flow cytometry as previously described (Sawai *et al* 1990, Trubiani *et al* 1994a, Trubiani *et al* 1996)

**Cell cycle distribution**

The measurement of nuclear DNA content was carried out using a CycleTEST PLUS DNA Reagent Kit as described by the manufacturer (Becton Dickinson, Mountain View, CA, USA) with analysis on a FACStar$^{plus}$ flow cytometer (Becton Dickinson) More than 12 000 events were acquired and the resulting histogram profiles were analysed using CellFit software for ploidy analysis The apoptotic peak was defined as the peak that occurs in a channel number less than in G0, as described by others (Darzynkiewicz *et al* 1992)

**Polyamine determination**

Cellular polyamines were extracted and derivatized with dansyl-chloride as previously described (Trubiani *et al* 1994b) The HPLC equipment was composed of two 420 pumps, a mixer M491 and a SFM25 fluorescence detector (excitation $\lambda$ 338 nm, emission $\lambda$ 425 nm), a Data System 450 (Kontron Instruments, Amersham, UK) and a 7000 injectors (Rheodyne, Cotati, CA, USA) Separation was achieved with a Spherisorb S50DS2 5-m column (250 × 4 6 mm i d ) (Kontron Instruments) The DNS-derivatives of polyamines were separated with a solvent comprising water–acetonitrile–methanol (5 3 2) as solvent A and acetonitrile–methanol (3 2) as solvent B The sample was eluted with a linear gradient from 28 to 90% solvent B in solvent A over 15 min at a flow rate of 1 0 ml/min

**Ornithine decarboxylase determination**

The ODC enzyme assay was carried out in total volume of 50 $\mu$l, of which 40 $\mu$l was cell extract The assay mixture contained (final concentration) 50 mM Tris–HCl, pH 7 5, 0 1 mM EDTA, 5 mM dithiothreitol, 0 05 mM pyridoxal 5′-phosphate, 0 5% Triton X-100, 0 4 mM L-ornithine and 0 1 $\mu$l [$^{14}$C]ornithine (specific activity 58 mCi/mmol) ODC activity was determined according to the method of Seely & Pegg (1983)

**c-myc Determination**

Untreated and DMSO or DFMO-treated cells were collected in cold hypotonic lysis buffer (1 mM $NaHCO_3$, 5 mM $MgCl_2$, 100 $\mu$M PMSF, 10 $\mu$M leupeptin and 10 $\mu$g/ml soybean trypsin inhibitor) Protein concentration was assayed by a dye binding technique using BIO-RAD (Milano, Italy) protein assay reagent and measured spectrophotometrically Subsequently, 30 $\mu$g

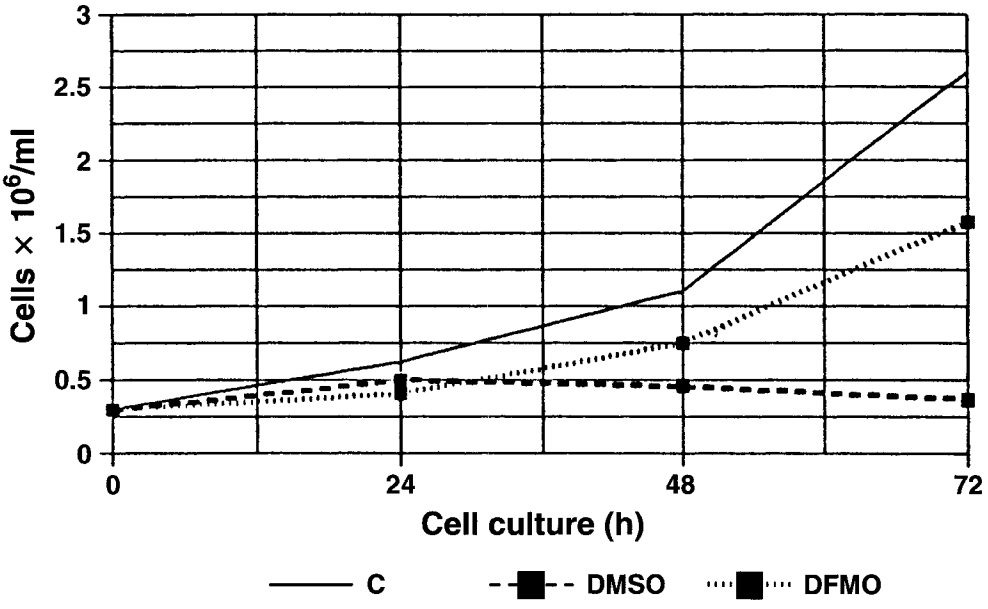


**Figure 1.** RPMI-8402 cell growth evaluation during DMSO and DFMO treatment. It is possible to observe, by comparing with the untreated samples (C), a light decrease of cell growth induced by DFMO while DMSO inhibits completely the cell expansion. Means of five different experiments. (SD ± 3%).

of each cell sample was separated on a 0.1% SDS, 8% polyacrylamide gel. Proteins were then transferred to nitrocellulose using a buffer containing 0.192 M glycine, 0.025 M Tris–HCl pH 8.3, 20% methanol at 40 V (constant) for 24 h at 4 °C. Nitrocellulose strips were saturated for 1 h at 37°C in phosphate-buffered saline (PBS), pH 7.4 containing 4% bovine serum albumin (BSA). They were made to react for 6 h at room temperature in PBS, 10% NGS, 4% BSA containing a 1 : 100 dilution of rabbit anti-c-myc polyclonal antibody recognizing the epitope corresponding to amino acids 408–421 found within the carboxy terminal domain of c-myc of human origin. After washing, samples were incubated for 2 h at room temperature in PBS, containing a 1 : 500 dilution of a peroxidase-conjugated anti-rabbit IgG. Strips were reacted with 0.05 mg/ml DAB, 50 mM Tris–HCl, pH 7.6, and 0.015% $H_2O_2$ as substrate.

**Table 1.** Cell cycle analysis of untreated, DFMO and DMSO-treated cells. The cell-cycle distribution was determined by propidium iodide staining and flow cytometry. DMSO treatment at 24 h provokes an arrest in the G1 phase. DFMO treatment did not induce substantial modification of cell cycle distribution. The data enclosed within brackets, in the DMSO-treated samples, indicate the apoptotic sub-G1 peak. Values are the average of five separate experiments (SD ± 2%) and are expressed as percentages of cell cycle distribution

| Hours | 24 h | | | 48 h | | | 72 h | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | DMSO | DMFO | Control | DMSO | DMFO | Control | DMSO | DMFO |
| G0 + G1 | 41 | 64(6) | 50 | 45 | 84 (18) | 58 | 58 | 87(34) | 65 |
| S | 42 | 22 | 30 | 39 | 12 | 29 | 27 | 7 | 21 |
| G2 + M | 17 | 14 | 20 | 16 | 4 | 13 | 15 | 6 | 14 |

Immunochemical control was performed using pre-immune serum as the primary antibody or by omitting either the primary or secondary antibody NGS 10% and BSA 4% were added to the washing buffer

**mRNA quantification by PCR**

mRNA was extracted from $1 \times 10^6$ untreated and DMSO-treated cells using the Oligotex Direct mRNA Kits (Qiagen, Chatsworth, CA, USA) Serial dilutions (10-fold) of this RNA were used as a template for a cDNA synthesis reaction *c-myc* mRNA levels were evaluated by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using the Promega Access RT-PCR System (Promega Corporation, Madison, WI, USA) RT-PCR was performed according to the manufacturer's procedure Primers used for amplification were oligonucleotides recognizing sequences in *c-myc* exon 1 (5′-GCACTGGAACTTACAACACC-3 and 5′-GGTGCTTACCTGGTTTTCCA-3) They were used to amplify a 155-bp fragment spanning the sequence 27162871 (Neri *et al* 1989) After amplification, 10 $\mu$l of the PCR products were run on a 2% agarose gel

**Source of materials**

DMSO, all cell lines culture material, and all reagent grade materials were from Sigma (St Louis, MO, USA) DFMO was from Merrell Dow Research Institute (Cincinnati, OH, USA) Antibodies were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA) Reagents for electron microscopy were from Polyscience (Warrington, PA, USA) Radionucleotides were from Amersham (Milan, Italy)

## RESULTS

In human pre-T RPMI-8402 cells DMSO and DFMO treatment generated several biological modifications Figure 1 and Table 1 show, in treated samples, cell growth arrest with a concomitant modification of cell cycle phases After 72 h of DMSO treatment, more than 80% of the cells were arrested in the G1 phase while only 7% of the cells were in the S or G2 + M phase Cell growth appears nearly completely inhibited DFMO provoked a less

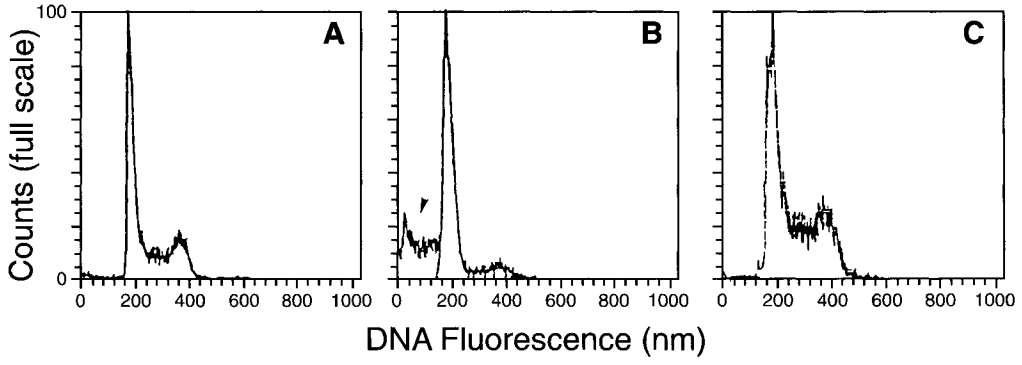


**Figure 2.** Histogram, at 72 h of cell culture, of treated and untreated RPMI-8402 cells The DNA fluorescence has been obtained as described under *Materials and methods* (a) Control cells (b) DMSO-treated cells showing the modification of DNA content The peak indicative of apoptosis (arrow) is evident (c) DFMO-treated sample showing the absence of apoptosis

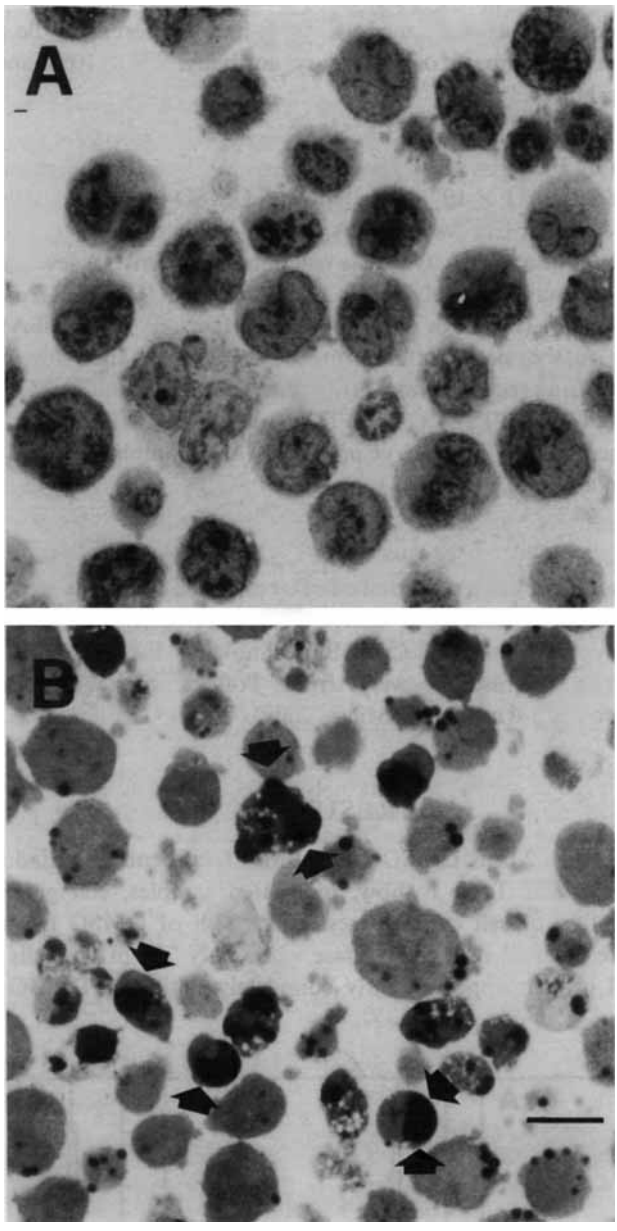


**Figure 3.** Morphology of untreated (a) and DMSO-treated (b) RPMI-8402 cells at 72 h of culture DMSO-treated sample exhibits the presence of several apoptotic cells with the typical nuclear features of chromatin condensation, and apoptotic bodies (arrows) Bar 15 $\mu$m

evident change of cell cycle distribution, 21% of the cells were in the S phase, associated with a reduction of cell growth DMSO induced more than 86% of cell growth inhibition, while DFMO provoked a lesser reduction of 36% (Figure 1) This is in accordance with the data in the literature (Heby 1981, Pegg 1988) The distribution of cellular DNA content, as analysed by FACS, demonstrated a hypodiploid peak in DMSO-treated samples indicative of

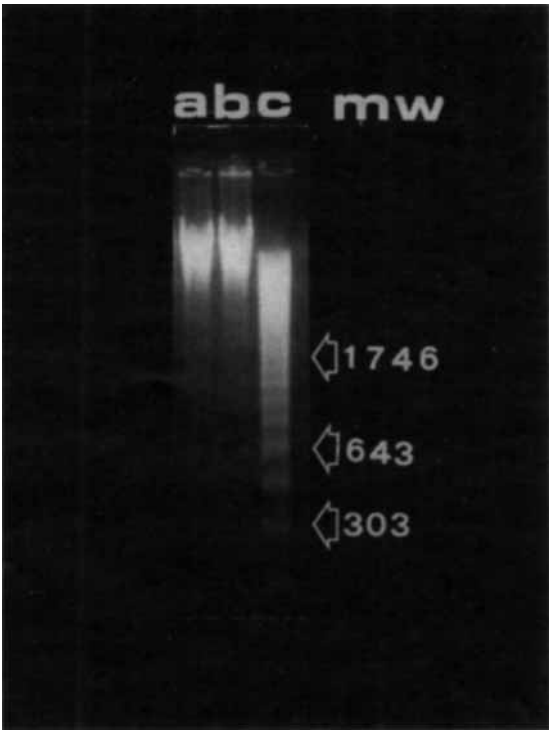


**Figure 4.** Electrophoretic analysis of DNA, recovered at 72 h of a cell culture, showing the ladder-like pattern of DNA banding, indicative of apoptosis, only in the DMSO-treated sample. (a) Untreated, (b) DFMO-treated, and (c) DMSO-treated cells. Lane mw, molecular weight.

apoptosis (Figure 2), recognizable after 24 h of treatment. Untreated and DFMO-treated cells never displayed the presence of an apoptotic peak, and maintained a normal DNA distribution. Apoptosis was also examined by morphological analysis and DNA electrophoresis. In DMSO-treated samples, apoptotic cells can be recognized by their nuclear modifications, i.e. chromatin condensation, micronuclei and apoptotic bodies. At 72 h of treatment, more than 40% of the cells displayed these morphological features (Figure 3). Moreover, DNA electrophoresis showed the presence of ladder-type fragmentation, indicative of apoptotic cells, exclusively in the DMSO-treated sample (Figure 4). DFMO did not provoke morphological changes (data not shown) or DNA modifications (Fig. 4). The ODC activity and polyamine levels are reported in Figure 5. ODC decreased both in DMSO and DFMO-treated cells even if DFMO induced a rapid and sustained arrest of ODC activity. ODC activity decreased by more than 70% after 24 h of DFMO exposure, against 20% for DMSO-treated cells. The level of polyamines was decreased, in a different fashion, in both treated samples. DFMO caused an immediate and intense depletion of putrescine and spermidine content, while DMSO induced a constant decrease of putrescine with a consequential down-regulation of spermidine and spermine. From these results it is possible to conclude that DMSO provoked a decrease of ODC activity followed by a down-regulation of polyamine levels associated with cell growth arrest and progressive apoptosis induction. DFMO, however, provoked a decrease of ODC activity, polyamine amounts and cell growth inhibition was unable to induce apoptosis. As Ryan & Birnie (1996) proposed a close association between ODC activity and the *c-myc* proto-oncogene and Thulasi *et al.* (1993)

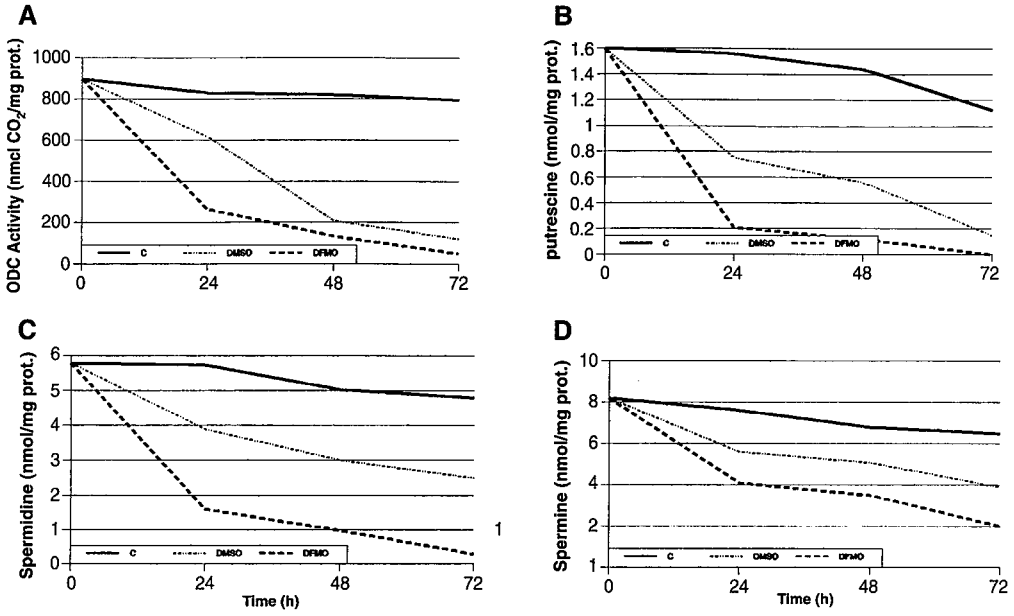


**Figure 5.** ODC activity and polyamines levels in untreated and DMSO or DFMO-treated cells (a) ODC activity; and levels of (b) putrescine, (c) spermidine, and (d) spermine. Results are the average of six separate experiments (SD ± 4%).

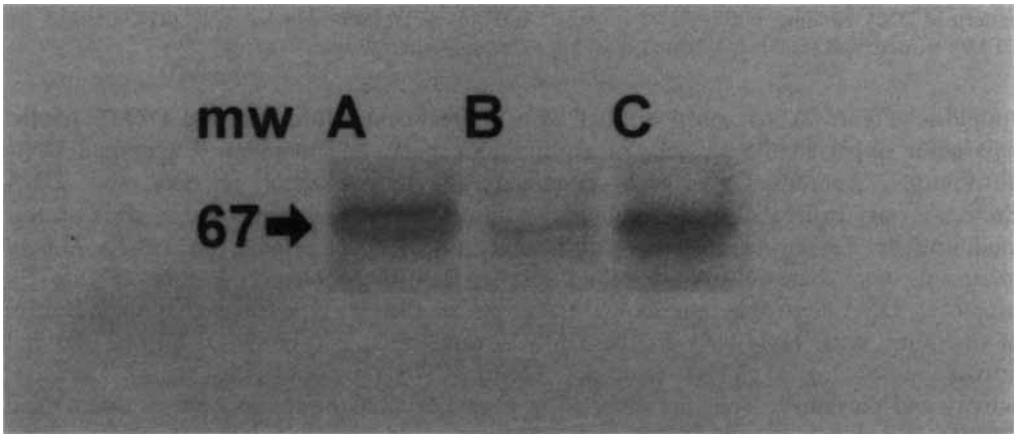


**Figure 6.** Detection of c-myc protein in RPMI-8402 cells by immunoblot/enzyme immunoassay. SDS–8% polyacrylamide gel electrophoresis and immunoblot were carried out as described under *Material and methods*. (a) Untreated cells. (b) DMSO-treated cells showing a decrease of c-myc protein. (c) DFMO-treated cells. mw, molecular weight.

showed a suppression of *c-myc* in dexametasone-treated lymphoid cells, the expression of this proto-oncogene was analysed at the level of mRNA and protein synthesis. Immunocytochemical (data not shown) and immunoblot analysis (Figure 6) shows that c-myc levels in DMSO-treated RPMI-8402 cells were decreased. This down-regulation is a result of a reduction in its mRNA. The RT-PCR showed that the level of the *c-myc* transcript was

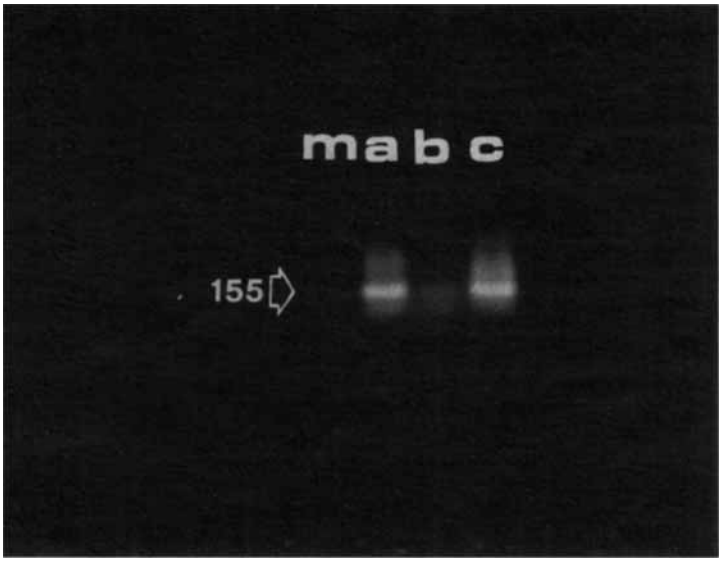


**Figure 7.** RT-PCR expression analysis of c-myc. Cellular mRNA was collected at 72 h of culture. (a) Log-phase cells; (b) DMSO-treated cells; and (c) DFMO-treated cells. It is possible to note the down-regulation of c-myc RNA in the DMSO-treated sample. Lane m, fragment size.

down-regulated by DMSO (Fig. 7). In DFMO-treated cells there were no detectable changes in c-myc expression, as shown by mRNA and protein (Figure 6 and Figure 7).

## DISCUSSION

The present data show that in the human pre-T cell line DMSO inhibits, via *c-myc*, the activity of ODC, which in turn provokes a decrease in the intracellular level of polyamines and cell growth arrest. These data agree with other results establishing that ODC catalyses the conversion of ornithine to putrescine, and that ODC is required for entry into the S phase of the cell cycle (Heby 1981, Pegg 1986). The DMSO-dependent decrease in ODC activity, and the secondary depletion of intracellular polyamine levels, is associated with the induction of programmed cell death. DFMO treatment inhibits ODC activity not via *c-myc*, but presumably by blocking its own catalytic activity. (Sugiura *et al.* 1984, Seiler 1990). The down-regulation of polyamine levels through this mechanism did not significantly alter the rate of cell growth and did not provoke apoptosis. At this point, it is suggested that in the present experimental model a complex situation exists in which polyamines are essential but insufficient to induce programmed cell death, suggesting that they intervene as regulating agents of apoptosis. In fact, although apoptosis can be induced by a *c-myc* independent pathway (Packham & Cleveland 1995, Ryan & Birnie 1996), the DMSO-induced apoptosis in RPMI-8402 cells is regulated by a down-expression of *c-myc* that generates a decrease in ODC and polyamines. The decline in intracellular polyamine content increases the sensitivity of the cell to DNA fragmentation. Other studies have shown the key role of polyamines in the regulation of nucleic acid structure (Feurstein & Marton 1989, Snyder 1989, Basu *et al.* 1993), and that the activity of particular nucleases and DNA polymerases is modulated by polyamine biosynthesis (Snyder 1989, Brüne *et al.* 1991, Basu *et al.* 1993). Moreover, since it has been described that programmed cell death consists of an ordered sequence of

biochemical events (Ashwell *et al.* 1994, Kroemer *et al.* 1995), the hypothesis suggested by the present data is that DMSO drives a cell type gene-dependent induction phase of programmed cell death. The specific response of RPMI-8402 to DMSO treatment could demonstrate this. The positive response to DMSO induces the second step or effector phase of programmed cell death. The biochemical pathway activated during this phase provokes, via *c-myc* inhibition, the specific down-regulation of ODC activity and polyamine levels as well as the arrest of cell growth and the modification of the cell cycle. The last phase, which in the present experimental model occurs after 24 h of DMSO treatment, corresponds to the degradation phase of apoptosis and provides an explanation for the DNA degradation and morphological changes. Finally, this study may present an original probe that gives the opportunity to understand, by a new experimental approach, the specific role of *c-myc* and the polyamine pathway in the regulation of nucleic acid secondary structure during the cell suicide process.

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