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# Thymic Lymphomas Are Resistant to Nur77-Mediated Apoptosis

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**We reported previously that thymic lymphomas from mice expressing transgenic TCR autoreactive against male (HY) antigen were resistant to anti-CD3 antibody-mediated induction of apoptosis although they were responding to TCR triggering. To test whether thymic lymphomas were specifically resistant to TCR-dependent $Ca^{++}$-mediated induction of apoptosis, we have measured apoptosis of cells treated with $Ca^{++}$-dependent (ionomycin, A23187) and $Ca^{++}$-independent (etoposide, dexamethasone) inducers of apoptosis. Here we show that, unlike thymocytes, all thymic lymphomas were resistant to $Ca^{++}$-dependent but not to $Ca^{++}$-independent induction of apoptosis. These results excluded a general defect of apoptosis in lymphoma cells and suggested a specific inhibition of the calcium-mediated (TCR-dependent) pathway of apoptosis in lymphomas. Interestingly however, nuclear expression of a specific mediator of TCR-dependent apoptosis Nur77 was induced in ionomycin-resistant lymphomas indicating that, unlike normal thymocytes, thymic lymphomas are resistant to Nur77-mediated apoptosis.** 

***Key Words:*** **apoptosis; Nur77; TCR; transgenic mice; thymic lymphoma.**

Apoptosis, a genetically programmed cell death (PCD), regulates development of T cells by preventing survival of potentially dangerous thymocytes expressing autoreactive T cell receptor (TCR) and useless thymocytes expressing receptors unable to recognize any antigens (negative selection). Only cells able to recognize foreign antigen in the context of self MHC molecules are rescued from PCD by positive selection and become mature T cells [2-4]. The abrogation of PCD during development of thymocytes could contribute to the lymphomagenesis of immature thymocytes which otherwise would be deleted. In agreement with this possibility we have found that cells of thymic lymphomas from TCR transgenic (Tg) mice were largely resistant to TCR-mediated apoptosis [1]. All molecular participants of signal transduction pathways leading to negative selection of thymocytes are not yet known, but one of the consequences of TCR triggering associated with the induction of PCD is an increase of cytoplasmic $Ca^{++}$ concentration [5, 6] which leads to induction of nuclear expression of the transcription factor Nur77 [7]. Specific engagement of Nur77 in TCR-dependent but not dexamethasone-induced apoptosis and defective clonal deletion of autoreactive thymocytes with impairment in Nur77 function has been reported [8].

Here, to learn more about molecular mechanism responsible for resistance to TCR-mediated induction of apoptosis in these lymphomas, we examine whether they display a specific blockade in calcium-dependent signalling pathway or, alternatively, a general defect not associated with TCR signalling in the executional phase of PCD.

## MATERIALS AND METHODS

*Thymocytes.* Thymocytes were isolated from C57BL/6/BoyIiw mice obtained from the colony at the Institute of Immunology and Experimental Therapy, Wroclaw or from C57BL/6 mice expressing transgenic TCR specific for the male antigen (HY) in the context of H-$2D^b$ molecule [9]. Suspensions of thymocytes were prepared in RPMI medium by pressing the thymus through a fine nylon mesh.

*Lymphoma cell lines.* Established cell lines from primary thymic tumors of mice with transgenic TCR were cultured (37°C, 5% $CO_2$) in 24-well Costar tissue culture plates in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20 $\mu$M $\beta$-mercaptoethanol, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 10% FCS. Phenotypic characterization of lymphoma cell lines was previously described [1]. In some experiments cells freshly isolated from tumor and characterized with anti-CD4, CD8 (Becton Dickinson) antibodies were used directly in experiments or after short-term culture.

*Induction of apoptosis.* Thymocytes and lymphoma cells were cultured for 20 hours (37°C, 5% $CO_2$) in 96-well Costar flat bottom tissue culture plates in IMDM with 10% FCS ($2x10^5$ cells per well) and

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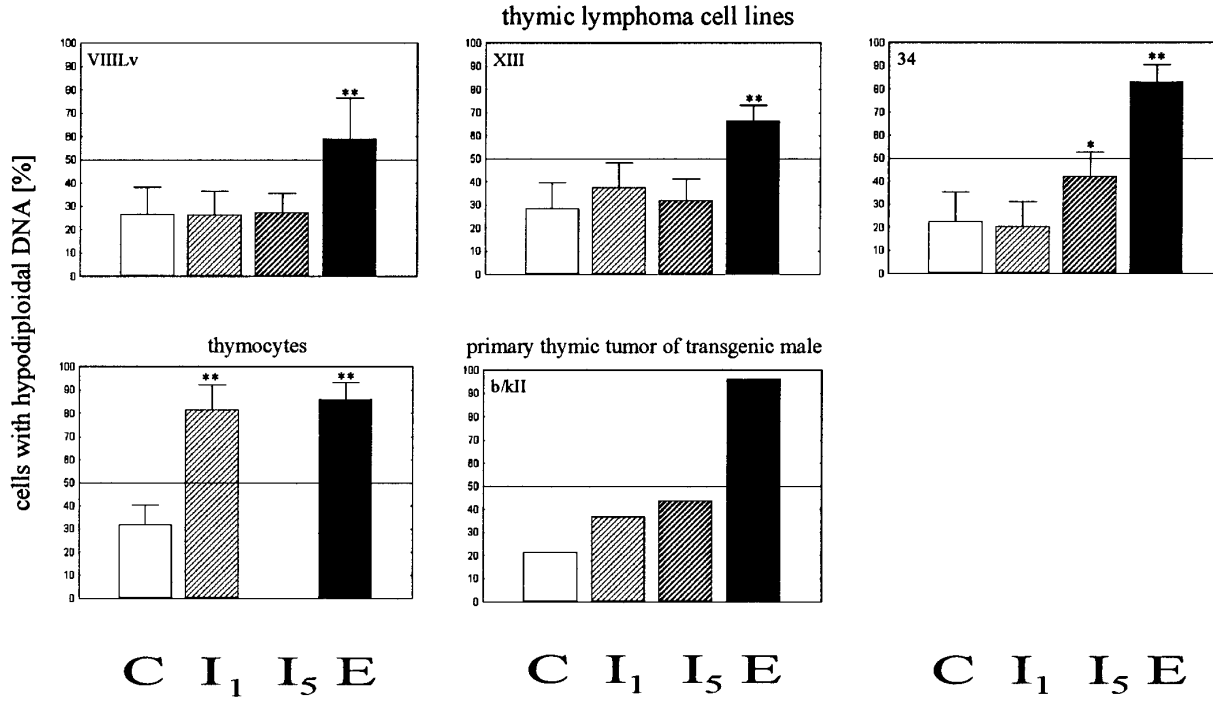


**FIG. 1.** Sensitivity of thymic lymphoma cell lines to calcium- or etoposide-mediated induction of apoptosis. Lymphoma cell lines, thymocytes from non-transgenic C57BL/6 mice, and cells isolated from the primary male thymic tumor were treated with ionomycin in concentrations of 1 $\mu$g/ml ($I_1$) or 5 $\mu$g/ml ($I_5$), or with 50 $\mu$M of etoposide (E), or with 0.05% of DMSO in the case of controls (C). Bars with whiskers express mean percentages of apoptotic cells from 7-12 experiments and standard deviations. Statistically significant differences between controls and tested samples are marked with asterisks, *$p < 0.05$, and **$p < 0.001$.

with a continuous presence of one of the applied inducers of apoptosis (Sigma) at concentrations in culture medium in the range of: 0.5-5.0 $\mu$g/ml of ionomycin, 0.25-1.0 $\mu$M of A23187, 1 $\mu$M of dexamethasone, 50 $\mu$M of etoposide. Cells treated with the solvent only (0.05% DMSO in IMDM with 10% FCS) were taken as controls in all experiments.

*Analysis of cell DNA content by flow cytometry.* DNA content was evaluated by the method using propidium iodide and flow cytometry [10]. Briefly, cells after treatment with inducer of apoptosis were washed in phosphate buffered saline (PBS), fixed with 70% ethanol for an hour at 4°C, then digested for an hour at 37°C with RNase A (Sigma) and following washings were stained with propidium iodide (Sigma, 50 $\mu$g/ml in 0.1% Triton X-100, 0.1 mM EDTA). Cell suspensions were then analysed with a FACSCalibur flow cytometer (Becton Dickinson). Cell debris were excluded from analysis by electronic gating. Histograms of DNA content were drawn using PC Lysis program for Windows. Apoptosis was quantified as percentages of cells with hypodiploidal DNA content.

*Western blot analysis of nuclear Nur77 induced expression.* The lymphoma cells or freshly isolated thymocytes were cultured for 2 hours in the presence of ionomycin (0.5 $\mu$M) and PMA (10 ng/ml, Sigma). The nuclear extracts were prepared from cultured cells as described before [11] with modifications. Briefly, nuclei were isolated from cells treated for 10 min with buffer containing 5 mM $KH_2PO_4$, pH 6.4, 150 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.3% Triton X-100, 5 mM $MgCl_2$, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 15 $\mu$g/ml aprotynin, 15 $\mu$g/ml leupeptin, and then centrifuged 1000 x g for 10 min at 4°C. The pellets were resuspended and nuclear proteins were extracted with the same buffer containing 350 mM NaCl for 30 min on ice and sonicated for 2 min at the end of incubation period. Extracts were then centrifuged 12,000 x g for 15 min at 4°C. Nuclear extracts were electrophoresed (35 $\mu$g of protein in each lane) on 8% polyacrylamide gel with SDS and electroblotted (Bio-Rad) onto a nitrocellulose membrane which was then stained with Ponceau S to verify equal amounts of transferred proteins in each lanes. The membrane was probed with anti-Nur77 rabbit antiserum (Santa Cruz, sc 990) followed by a donkey anti-rabbit immunoglobulin-horseradish peroxidase linked whole antibody (Amersham, NA 934). The antibody reactivity was detected with an Amersham ECL kit.

*Statistical analysis.* The results were evaluated by t-test for independent samples (Statistica for Windows).

## RESULTS

*Resistance of thymic lymphomas to ionomycin.* All lymphoma cell lines tested were completely resistant to ionomycin at concentration ranging from 1 to 5 $\mu$g/ml which induced apoptosis in normal thymocytes (Fig. 1) as well as in thymocytes from healthy transgenic females (data not shown). Lymphomas also survived treatment with A23187 (another calcium ionophore) at concentration of 0.25 $\mu$M which induced apoptosis in thymocytes (data not shown). These results indicate a complete unresponsiveness of leukemic cells to calcium-mediated apoptotic signals.

To check whether demonstrated resistance of leukemic cells to ionomycin was not an artefact generated during in vitro culture, sensitivity of cells directly isolated from the primary tumor to inducers of apoptosis was examined. Result demonstrated in Fig. 1. shows that freshly

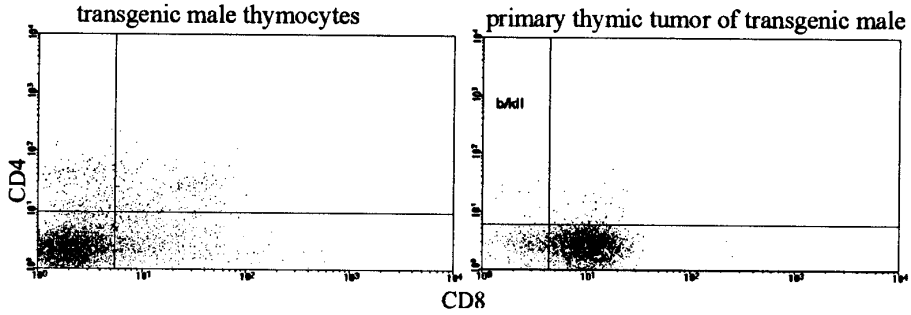


**FIG. 2.** CD4/8 phenotypes of thymocytes from transgenic male and primary thymic tumor of transgenic male.

isolated cells from the spontaneous thymic tumor of transgenic male were also resistant to ionomycin. Expression of CD8 on these lymphoma cells (Fig. 2) suggests that the transformed thymocytes escaped clonal deletion process since in normal transgenic males the majority of $CD8^+$ thymocytes become deleted [9].

*Sensitivity of lymphomas to etoposide and dexamethasone.* All lymphoma cell lines tested appeared to be sensitive to 50 $\mu$M etoposide, a topoisomerase inhibitor, which induced apoptosis of above 60% of cells (Fig. 1). This agent induces apoptosis by the mechanism not related to TCR-dependent signal transduction pathway but rather as a response to damaged DNA engaging p53 protein in signal transduction [12, 13]. In addition, we found that lymphoma cells were sensitive to dexamethasone (1 $\mu$M), an inducer of apoptosis in immature thymocytes (data not shown). Also ionomycin-resistant cells isolated directly from the primary tumor were sensitive to etoposide (Fig. 1) and dexamethasone (not shown).

*Nuclear expression of Nur77 induced by ionomycin.* Presented results show resistance of thymic lymphomas to calcium-mediated induction of apoptosis and sensitivity to PCD triggered by calcium-independent signalling pathways. To find out at which point in calcium mediated signalling pathway the blockade exists, we studied induction of Nur77 with ionomycin. We assumed a defect of calcium-induced Nur77 nuclear expression in the lymphomas but unexpectedly stimulation with ionomycin and PMA induced Nur77 nuclear expression in ionomycin-resistant lymphomas as well as in ionomycin-sensitive thymocytes from healthy mice (Fig. 3).

## DISCUSSION

Results of our previous paper [1] demonstrated sensitivity of transgenic thymocytes undergoing oncogenic transformation to TCR-mediated negative selection and yet we observed male lymphomas with phenotype corresponding to those subpopulations of thymocytes which are deleted due to negative selection in healthy transgenic males, i.e., $CD8^+TgTCR^+$ [9]. We proposed a hypothesis that one of the necessary leukemogenic events which enabled transformed thymocytes to escape normal regulatory mechanisms, could be a defect of TCR-dependent apoptosis.

A strong indication of a block of apoptosis at the level of execution would be resistance of lymphoma cells to induction of apoptosis by different agents inducing cell death. The results of the present paper demonstrated however, that thymic lymphomas, unlike normal thymocytes, specifically resisted calcium-mediated induction of apoptosis by ionomycin but were sensitive to calcium-independent induction of apoptosis by etoposide or dexamethasone. These observations excluded defects of apoptosis at the level of execution common to different signalling pathways and suggested a specific inhibition of TCR-triggered apoptosis, mimicked by treatment of cells with calcium ionophores.

Defective thymic clonal deletion was reported for thymocytes of doubly transgenic mice expressing transgenic TCR anti-HY and transgenic Nur77 with dominant negative mutation [8]. Those self-reacting thymocytes were demonstrated to contain $CD8^+$ cells and $CD8^+$ phenotype was also observed in the case of thymic lymphomas with self-reacting TCR anti-HY suggesting failure of their clonal deletion [1]. In view of above observations [8] one could expect the lack of Nur77 induction in response to

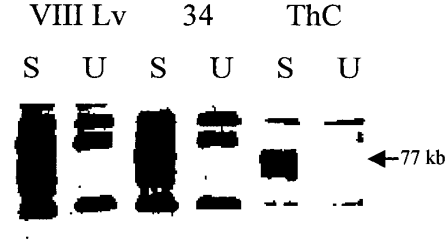


**FIG. 3.** Western blot analysis of the Nur77 protein induced in nucleus by ionomycin and PMA. Shown are results for thymocytes (Thc) and two lymphoma cell lines (#34) and (#VIII Lv) stimulated for 2 h with ionomycin (0.5 $\mu$M) and PMA (10 ng/ml). Nuclear extracts were prepared from stimulated (S) or unstimulated cells (U).

increased calcium concentration in thymic lymphomas but we were able to induce nuclear expression of Nur77 by ionomycin in ionomycin-resistant cells. This indicates that, unlike in thymocytes [14], Nur77 does not lead to apoptosis in thymocyte-derived lymphomas.

Thus, our results suggest that a defect of apoptosis in these cells is located downstream of the Nur77 induction and upstream from the execution of PCD. Alternatively, in the lymphomas originated from immature thymocytes Nur77 mediates signals to other cellular programs than apoptosis, possibly to proliferation as suggested in other report [15].

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