

# Susceptibility of different subsets of immature thymocytes to apoptosis

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**Abstract** In the present study the susceptibility of different subsets of immature rat thymocytes to undergo apoptosis was examined. Unfractionated rat thymocytes were negatively enriched into immature double positive (CD4<sup>+</sup>CD8<sup>+</sup>), immature single positive (CD4<sup>-</sup>CD8<sup>+</sup>CD3<sup>-</sup>) and triple negative (CD4<sup>-</sup>CD8<sup>-</sup>CD3<sup>-</sup>) thymocytes. These enriched subsets of immature thymocytes were then exposed to various apoptotic stimuli such as dexamethasone, etoposide and thapsigargin which readily induced apoptosis in unfractionated rat thymocytes. We found that the double positive thymocytes and their precursor cells, i.e. the single positive immature thymocytes, were equally sensitive to apoptosis after treatment with the apoptotic stimuli. In sharp contrast, the early migrants or precursor-containing thymocytes which are triple negative have a lower spontaneous apoptosis rate and were relatively resistant to all the apoptotic stimuli. These findings showed a breakpoint in thymocyte sensitivity to apoptosis which occurs after the onset of CD8 expression, suggesting that susceptibility of thymocytes to apoptosis is developmentally regulated.

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**Key words:** Thymocyte; Etoposide; Thapsigargin; Dexamethasone; Apoptosis

## 1. Introduction

Apoptosis is a form of controlled cell death that is distinct from necrosis. It plays an important role in diverse biological processes and occurs in most tissues at some stage during development [1,2]. Distinct morphological and biochemical changes take place during apoptosis which lead to the controlled destruction of the cell. These changes include cell shrinkage, plasma and nuclear membrane blebbing, nuclear condensation, and cleavage of chromatin into both high molecular weight (HMW) and oligonucleosomal-length DNA fragments presumably as a result of endonuclease activity [3–5]. The cell eventually disintegrates into small apoptotic bodies, which are recognised and engulfed by neighbouring cells or macrophages [6].

A model system that has come to the forefront of these studies in the last decade is the use of isolated thymocytes *in vitro*. It is well established that a variety of agents including glucocorticoids, compounds reactive with DNA topoisomerase II, and intracellular Ca<sup>2+</sup> elevating agents, induce exten-

sive apoptosis in isolated thymocytes [7–9]. However, isolated thymocytes constitute a very heterogeneous population, consisting of at least five different subsets of thymocytes at different stages of development, which can be distinguished by the expression of certain cell surface antigens [10,11]. The early migrants from the bone marrow or progenitor T cells are essentially CD4<sup>-</sup>CD8<sup>-</sup>CD3<sup>-</sup> or triple negative (TN) cells. This population expands into the CD8<sup>+</sup>CD3<sup>-</sup> single positive (SP) immature thymocytes, which are also precursor cells for the CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP) thymocytes [12,13]. More than 80% of the cells in unseparated thymocytes express the DP phenotype [14]. The other two subsets are the mature T cells which have survived the clonal deletion, i.e. the CD4<sup>+</sup>CD8<sup>-</sup>CD3<sup>+</sup> SP and CD8<sup>+</sup>CD4<sup>-</sup>CD3<sup>+</sup> SP mature thymocytes [10,11,14].

Various reports have suggested that most of the cells dying of apoptosis in unseparated thymocytes after glucocorticoid treatment or an elevation of cytosolic free calcium concentration were of the CD4<sup>+</sup>CD8<sup>+</sup> DP phenotype [15,16]. The supporting evidence for this came from studies using flow cytometry and phenotyping the surviving cells after induction of apoptosis. However, phenotyping thymocytes after induction of apoptosis may not provide a clear indication of the population of cells surviving or undergoing apoptosis as some cell surface antigens, for instance CD4 and CD8, are down-regulated in thymocytes undergoing apoptosis [16]. Furthermore, one of the earliest characteristic changes in apoptotic cells is the profound effect on plasma membrane lipid packing, which could affect the presentation of various surface antigens [17].

In the present study, the susceptibility of different subsets of immature thymocytes to undergo apoptosis was examined in greater detail. The different subsets of immature thymocytes, i.e. DP, CD8<sup>+</sup> SP and TN were negatively enriched from unfractionated thymocytes and their propensity to undergo apoptosis examined using various apoptosis-inducing agents. Our present findings indicate that TN cells are relatively resistant to the induction of apoptosis by various stimuli, whereas both CD8<sup>+</sup> SP and DP cells are equally sensitive to apoptosis induced by these agents.

## 2. Materials and methods

### 2.1. Animals

Male Fischer 344 rats 4–5 weeks old, bred at the Medical Research Council Toxicology Unit (Leicester University, UK) under pathogen-free conditions were used.

### 2.2. Reagents

The hybridomas producing the rat monoclonal antibodies (mAb) OX-8, anti-CD8 [14], OX-44, anti-CD53 [18], W3-25, anti-CD4 [14], OX-19, anti-CD5 [19] were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK), and used as culture supernatants. The hybridoma producing anti-T cell receptor (TCR), R73 (pan  $\alpha\beta$ ) was kindly provided by Dr. T. Hunig (Würzburg, Germany) [20].

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**Abbreviations:** DP, double positive; SP, single positive; TN, triple negative; HMW, high molecular weight; TCR, T cell receptor; Dex, dexamethasone; FCS, fetal calf serum; PE, R-phycoerythrin; FITC, fluorescein isothiocyanate

Purified anti-CD4 coupled to FITC, biotin-labelled anti-CD8 and phycoerythrin (PE)-coupled streptavidin were obtained from Serotec (UK). Dexamethasone (Dex) and etoposide (VP-16) (a DNA topoisomerase II inhibitor) were obtained from Sigma Chemical Co. (UK), and thapsigargin was from Gibco BRL (UK). Goat anti-mouse IgG (GAMIgG)-coated BioMag beads were from Advance Magnetics (UK) and GAMIgG-coated Dynal beads were from Dynal (Norway).

### 2.3. Thymocyte isolation and subset enrichment

Thymocytes were isolated as previously described [8] and resuspended in RPMI 1640 supplemented with 10% FCS. All the subsets of immature thymocytes, CD4<sup>-</sup>CD8<sup>-</sup>CD3<sup>-</sup> TN, CD4<sup>-</sup>CD8<sup>+</sup>CD3<sup>-</sup> SP and CD4<sup>+</sup>CD8<sup>+</sup> DP cells, were enriched by negative selection. Total thymocytes were incubated with different cocktails of saturating mAb-containing hybridoma culture supernatants, OK-8, W3/25 and R73 (TN cells), W3/25, OX-44 and R73 (CD8<sup>+</sup> SP cells) or OX-44 (DP cells) for 30 min on ice. The cells were washed twice with ice-cold PBS and then incubated with GAMIgG-coated Biomag beads at 10:1 beads to cell ratio. The cell suspensions were incubated on ice for 30 min with gentle mixing every 5 min. Magnetic beads and adhering cells were removed using a magnetic particle separator (Dynal MPC-6). The procedure was repeated twice, with Biomag beads at 5:1 bead to cell ratio followed by GAMIgG-coated Dynal beads at 2:1 beads to cell ratio. The cells recovered were used as TN, CD8<sup>+</sup> SP and DP cells in experiments.

### 2.4. Cell culture

For in vitro culture and treatment of thymocytes,  $1.25 \times 10^6$  cells/ml were cultured in 2 ml RPMI 1640 medium supplemented with 10% FCS, in 24-well plates with the agents of interest in a humidified incubator with 5% CO<sub>2</sub> in air. After 4 h, aliquots of  $1 \times 10^6$  cells from each treatment were taken for HMW as well as nucleosomal DNA analysis, and  $0.5 \times 10^6$  cells taken for quantification of apoptosis using flow cytometry.

### 2.5. Flow cytometric analysis

Flow cytometric analysis was used to (a) phenotype the cell surface markers on the enriched thymocyte subsets and (b) quantitate the percentage of cells undergoing apoptosis in thymocytes after treatment with various apoptosis-inducing agents. In phenotyping analysis, all the antibodies used were at saturating concentrations. For two-colour immunofluorescence labelling,  $1 \times 10^6$  cells in 50  $\mu$ l PBS containing 0.2% FCS and 0.2% sodium azide were sequentially exposed to (a) biotin-conjugated mAb to the first marker and FITC-conjugated mAb to the second marker; (b) streptavidin-conjugated PE. Analysis was performed on a FACScan flow cytometer (Becton Dickinson). Light scatter gates were set to include all viable nucleated cells. Samples were analysed using the LYSIS II software and are displayed as dotplots. Populations of cells expressing markers were calculated from quadrants set using the appropriate negative controls.

To detect apoptotic thymocytes, the cells were prepared as previously described [21] before analysis using a Vantage flow cytometer (Becton Dickinson) equipped with an argon laser tuned to the blue line (488 nm) and a krypton laser tuned to 352 nm. In brief, after incubation the cells were centrifuged to remove the medium. The cell pellets were dispersed gently, resuspended in 1 ml RPMI medium and incubated with the fluorescent dye, Hoechst 33342, at 1.5  $\mu$ g/ml for 10 min at 37°C. The cells were centrifuged to remove the excess dye in the PBS and the pellet was resuspended in PBS with 0.005  $\mu$ g/ml propidium iodide. The stained cells were stored on ice until analysis.

### 2.6. Analysis of nucleosomal DNA fragments and the formation of HMW DNA fragments

The analysis of nucleosomal DNA fragments was essentially that described by Sorenson et al. [22]. In brief, after incubation, an aliquot of thymocyte suspension from each treatment containing  $1 \times 10^6$  cells was dispensed in Eppendorf tubes. The cells were centrifuged at  $200 \times g$  for 5 min and the medium removed. The cell pellet was resuspended in 15  $\mu$ l water followed by 5  $\mu$ l RNase (stock, 50 mg/ml)

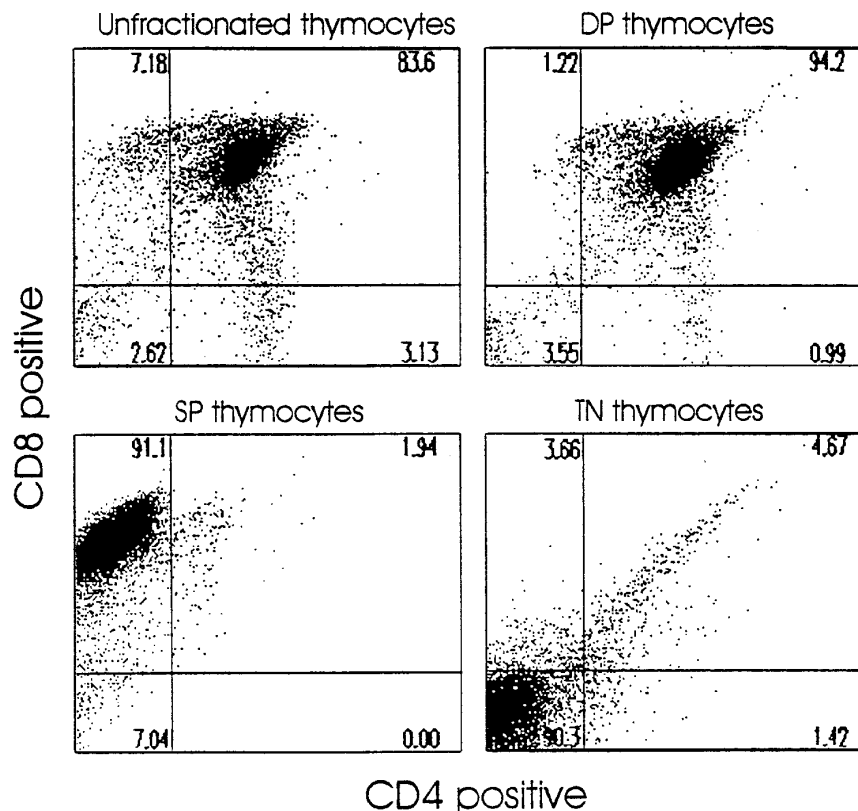


Fig. 1. Phenotypes of the enriched subsets of immature thymocytes. Unseparated thymocytes were incubated with various cocktails of mAbs and then positively depleted using GAM magnetic beads. The enriched negative selected DP, SP, TN and unfractionated thymocytes were phenotyped for CD4 and CD8 antigens using a FACScan flow cytometer as outlined in Section 2. The numbers represent the percentage of cells in each quadrant.

and 5  $\mu$ l loading buffer. The cell mixture was mixed thoroughly before 10  $\mu$ l from each sample was loaded into each well of a 1.8% agarose gel with a precast digestion gel. The gel was electrophoresed for 1 h at 20 V followed by 3 h at 90 V before being visualised under UV illumination. The formation of HMW DNA fragments was analysed using field inversion gel electrophoresis as previously reported [5]. In brief, after incubation  $1 \times 10^6$  cells from each treatment were centrifuged and the medium was removed. The cell pellets were resuspended in soft melting agarose and pipetted into the wells of a plug mould. The agarose plugs were digested for 48 h at 50°C with pronase (1 mg/ml final concentration) before being inserted into the wells of a 1% agarose gel. The HMW DNA fragments were electrophoresed using conditions previously described [5], and visualised using UV illumination.

### 3. Results and discussion

In the present investigation, the susceptibility of different subsets of immature thymocytes to undergo apoptosis after treatment with various apoptotic stimuli was examined. To obtain relatively pure populations of different subsets of immature thymocytes, unfractionated thymocytes were negatively selected by the positive depletion of unwanted cells on the basis of their cell surface markers which are expressed differentially during thymocyte development. This procedure avoided the possible activation of cells through various cell surface antigens, which may affect their sensitivity to apoptosis if the cells had been purified by positive selection. Immature DP thymocytes, which represent  $\sim 85\%$  of unseparated thymocytes, do not express the cell surface antigen, OX-44 (rat homologue of CD53 [18]) which is present in mature SP and immature DN thymocytes. Accordingly, total thymocytes were depleted of CD53<sup>+</sup> cells by magnetic cell sorting with

anti-CD53 mAbs. This procedure routinely yielded DP cells of greater than 90% purity (Fig. 1). Immature CD8<sup>+</sup> SP thymocytes, which are the precursors for DP cells, represent only  $\sim 2\%$  of the total unseparated thymocytes [14]. Similar to DP cells, CD8<sup>+</sup> SP cells do not express the CD53 cell surface antigen [13], and were enriched by depleting cells expressing CD53, CD4 and TCR cell surface antigens using magnetic cell sorting with anti-CD53, anti-CD4 and anti-TCR mAbs. Routinely 85–90% CD8<sup>+</sup> SP cells were obtained in this fashion, which represented a 40-fold enrichment (Fig. 1). Enrichment of TN rat T cell progenitor thymocytes was achieved by depleting thymocytes expressing CD8, CD4 and TCR cell surface antigens using magnetic cell sorting with anti-CD8, anti-CD4 and anti-TCR mAbs. This procedure routinely yielded a cell population of  $\sim 95\%$  purity, which did not express CD4 and CD8 antigens (Fig. 1).

To determine the susceptibility of these subsets of immature thymocytes to apoptosis, negatively enriched TN, CD8<sup>+</sup> SP and DP immature thymocytes were incubated with Dex (0.1  $\mu$ M), etoposide (10  $\mu$ M) and thapsigargin (50 nM). These compounds have all been shown previously to induce extensive apoptosis in unfractionated thymocyte populations [4,7–9]. Apoptosis was determined using three different assays, i.e. a flow cytometry to quantitate apoptotic as well as viable cells [21], field inversion gel electrophoresis to detect the formation of HMW DNA fragments  $> 50$  kb due to chromatin cleavage [5], and the standard agarose gel assay for separating nucleosomal-length DNA fragments  $< 50$  kb. After 4 h incubation, cell viability in all the untreated control thymocyte subsets as well as unseparated thymocytes was 90–95% as determined by PI staining (Table 1). Cell viability in treated thymocytes var-

Table 1  
Apoptosis and viability of immature thymocytes in response to various apoptotic stimuli

Subset	Treatment	Apoptosis (%)	Viability (%)
Unfractionated thymocytes	–	14.9 $\pm$ 2.5	90.6 $\pm$ 2.0
	Dex	39.8 $\pm$ 4.3	89.2 $\pm$ 1.5
	etoposide	60.9 $\pm$ 2.4	89.2 $\pm$ 3.5
	thapsigargin	43.2 $\pm$ 12	85.6 $\pm$ 3.5
CD4 <sup>+</sup> CD8 <sup>+</sup> DP thymocytes	–	11.4 $\pm$ 0.6	95.4 $\pm$ 1.0
	Dex	43.7 $\pm$ 3.9	89.0 $\pm$ 2.3
	etoposide	61.2 $\pm$ 1.1	89.4 $\pm$ 1.4
	thapsigargin	52.8 $\pm$ 14	84.8 $\pm$ 3.9
CD4 <sup>–</sup> CD8 <sup>+</sup> CD3 <sup>–</sup> SP thymocytes	–	20.8 $\pm$ 2.7	91.4 $\pm$ 0.6
	Dex	48.7 $\pm$ 3.0	89.2 $\pm$ 1.2
	etoposide	70.3 $\pm$ 5.0	88.6 $\pm$ 1.9
	thapsigargin	56.9 $\pm$ 7.7	87.4 $\pm$ 2.8
CD4 <sup>–</sup> CD8 <sup>–</sup> CD3 <sup>–</sup> TN thymocytes	–	NA <sup>a</sup>	93.3 $\pm$ 0.2
	Dex	NA	91.0 $\pm$ 0.3
	etoposide	NA	87.8 $\pm$ 1.4
	thapsigargin	NA	90.4 $\pm$ 1.6

Unfractionated and negatively enriched DP, SP and TN subsets of thymocytes ( $5 \times 10^6$  cells/ml) were treated with various apoptotic stimuli for 3 h and the percent of cells undergoing apoptosis and cell viability were assayed using flow cytometry as outlined in Section 2. Data are the means  $\pm$  S.E.M. of 3–5 experiments. NA: not available.

<sup>a</sup>More than 50% of this subset of cells do not take up Hoechst 33342.

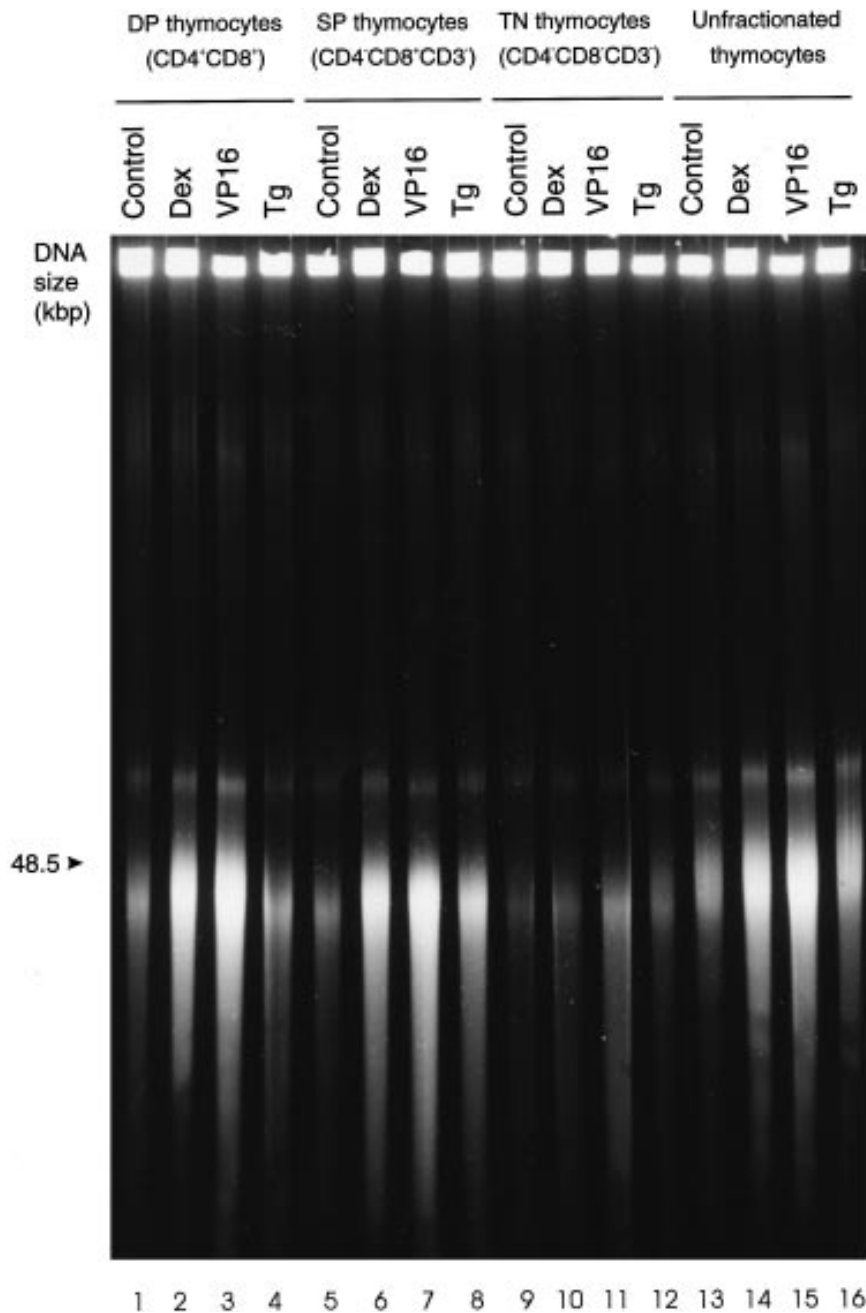


Fig. 2. Effect of various apoptotic stimuli on the formation of HMW DNA fragments in enriched subsets of immature thymocytes. The enriched subsets of immature DP, SP, TN and unfractionated thymocytes were treated with 0.1  $\mu$ M Dex, 10  $\mu$ M VP16 (etoposide) and 50 nM thapsigargin (Tg) for 4 h. Thereafter agarose plugs were prepared with these cells and the formation of HMW DNA fragments was analysed using FIGE as described in Section 2.

ied between 85 and 91% (Table 1) and was not significantly different from control values. Treatment of unfractionated thymocytes with Dex, etoposide and thapsigargin resulted in 39.8, 60.9 and 43.2% apoptotic cells, respectively, as compared to  $\sim$ 14.8% untreated cells (Table 1). As expected, a similar extent of apoptotic cells was observed with the enriched DP cell population after treatment with Dex, etoposide and thapsigargin (Table 1) confirming previous observations that the DP cells in unseparated thymocytes are highly sensitive to apoptosis [15,16]. Although the enriched CD8<sup>+</sup> SP cells appear to have a higher percentage of cells undergoing apoptosis after exposure to Dex, etoposide and thapsigargin compared

to the DP thymocytes (Table 1), its spontaneous apoptosis rate was also higher. With the enriched TN cells about 50% of this population of cells did not take up the Hoechst 33342 dye, and therefore, the flow cytometric assay could not be used to quantify apoptosis in this subpopulation of cells. This finding indicated that under these conditions the Hoechst 33342 DNA dye did not permeate all cells and therefore, caution must be exercised to carefully evaluate the permeability of each cell type to the dye before using this method.

The susceptibility of the enriched immature subsets of thymocytes to undergo apoptosis after treatment with various apoptotic stimuli was further examined by the HMW and

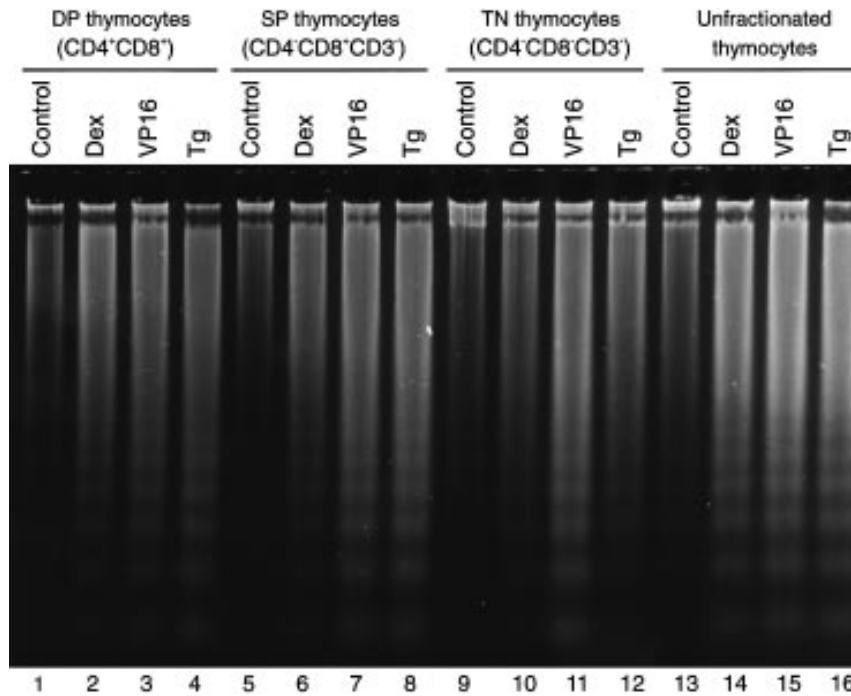


Fig. 3. Formation of oligonucleosomal-length DNA fragments in different subsets of immature thymocytes after exposure to different apoptotic stimuli. The enriched subsets of immature DP, SP, TN and unfractionated thymocytes were treated with 0.1  $\mu$ M Dex, 10  $\mu$ M VP16 (etoposide) and 50 nM thapsigargin (Tg) for 4 h and the formation of oligonucleosomal-length DNA fragments was analysed using conventional gel electrophoresis as described in Section 2.

oligonucleosomal-length DNA fragments formed. As illustrated in Fig. 2, HMW DNA fragments of 50 kb were formed in control unfractionated thymocytes (lane 13) due to spontaneous apoptosis, which corresponded with a low level of DNA degraded to oligonucleosomal-length fragments (Fig. 3, lane 13). Upon treatment with Dex, etoposide and thapsigargin, an extensive level of HMW DNA fragments was formed and accumulated at around 50 kb (Fig. 2, lanes 14, 15 and 16, respectively), which correlated with a similar increase in the formation of oligonucleosomal-length fragments (Fig. 3, lanes 14, 15 and 16). Similar to unfractionated thymocytes, the formation of 50 kb HMW DNA fragments in both DP as well as CD8<sup>+</sup> SP thymocytes was extensive after apoptotic stimuli treatment (Fig. 2, lanes 1–4 for DP cells, and lanes 5–8 for CD8<sup>+</sup> SP cells). As previously observed with unfractionated thymocytes, the degree of DNA degradation into oligonucleosomal-length DNA fragments in these cells (Fig. 3, lanes 1–4 for DP cells, and lanes 5–8 for CD8<sup>+</sup> SP cells) correlated well with the formation of HMW DNA. With the TN cells only a small amount of HMW DNA fragments (Fig. 2, lane 9) was observed in the control cells, which was much lower than that seen with the DP, SP or unfractionated thymocytes. This indicated that the TN cells had a lower spontaneous apoptotic rate compared to the other immature subsets of thymocytes. Upon treatment with apoptotic stimuli only a small increase in the formation of 50 kb HMW DNA fragments (Fig. 2, lanes 10–12) was seen with the TN cells, suggesting that these cells were less sensitive to the apoptotic stimuli compared to the other immature thymocyte subsets. Etoposide, which caused extensive accumulation of 50 kb HMW DNA fragments in both the DP and CD8<sup>+</sup> SP subsets, was only moderately effective in TN cells (Fig. 2, lane 11), whereas both Dex and thapsigargin had little effect. Similarly,

a corresponding decrease in oligonucleosomal-length DNA was also observed in the TN cells after exposure to the apoptotic stimuli (Fig. 3, lanes 10–12). Thus, TN cells were less susceptible to apoptosis after Dex, etoposide and thapsigargin treatment compared to DP, CD8<sup>+</sup> SP immature thymocytes.

These findings are in part compatible with the observations of Andjelic et al. [23] that in mice the TN cells are insensitive to calcium-mediated apoptosis. This resistance of TN cells was proposed to be related to a high level of expressed bcl-2 mRNA [23]. Since over-expression of bcl-2 also blocks steroid-induced apoptosis [24,25], it is possible that bcl-2 is responsible for the resistance to Dex- or etoposide-induced apoptosis in TN cells. Thymic regeneration studies in mice after *in vivo* steroid treatment suggest that a population of cells other than SP mature thymocytes is capable of regenerating thymocytes after thymic involution [26,27]. Because TN cells consist of early progenitor T cells or pro-thymocytes, it is likely that the steroid-insensitive population of cells in steroid-treated mice are the TN cells. Our present results also indicate that beside the DP cells, the CD8<sup>+</sup> SP thymocyte subset are also susceptible to apoptosis. It has been shown previously that the CD8<sup>+</sup> SP subset is a transition population of cells from CD4<sup>-</sup>CD8<sup>-</sup> to CD4<sup>+</sup>CD8<sup>+</sup>, having no IL-2 receptors and expressing low or undetectable TCR [13,28]. This suggests that the CD8<sup>+</sup> SP cells may be involved in positive selection *in vivo*, since the loss of IL-2 receptors and the beginning of TCR expression in TN cells marks the start of negative selection during thymocyte development [10,11]. Taken together, these findings suggest that the susceptibility of immature thymocytes to apoptosis may be developmentally regulated and that the onset of antigen expression involved in thymic selection marks the breakpoint of this sensitivity.

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