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APOPTOSIS IN THYMUS OF ADULT *Xenopus laevis*

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□**Abstract—Thymocyte apoptosis in adult *Xenopus laevis* is demonstrated on agarose gels and is quantified by propidium iodide incorporation using flow cytometry. Basal apoptotic levels are increased after *in vitro* exposure to a glucocorticoid, dexamethasone (DEX), and to the lectin, phytohemagglutinin (PHA). To determine the role that newly introduced antigenic determinants may play in this regard, a repertoire of altered-self antigens was created by exposing thymuses *in vitro* to trinitrobenzene sulfonic acid (TNBS) thereby derivatizing self-cells and proteins via 2,4,6-trinitrophenylacetic acid conjugation. An increase in apoptosis in TNBS-treated thymuses is observed. Thus, the thymocytes of adult *Xenopus laevis* are susceptible to apoptosis when induced by a glucocorticoid, a lectin, and by altered self, antigen activation.**

□**Keywords—Amphibian; Glucocorticoid; Lectin; Antigen driven; Apoptosis.**

Introduction

Apoptosis is involved in vertebrate embryogenesis, cellular aging, and in the removal of autoreactive thymocytes of mammals. One of the major physical characteristics separating apoptosis from necrosis is nuclear DNA fragmentation (1). Because the endonuclease cleavage of DNA occurs at the internucleosomal linkers, a ladder-like pattern appears on agarose electrophoretic gels consisting of multiples of 180 base pair fragments (2).

In mammals, thymic apoptosis may be induced by glucocorticoids (3) and by antigen or lectin activation (4,5). In general, the consequences of glucocorticoid- or antigen-induced signals are thought to be dependent upon the developmental state of the target cells (6). Although the sensitivity of immature T cells to apoptosis has been demonstrated, recent evidence suggests that activated, mature lymphocytes may also be susceptible to apoptosis (7). Here, we also explore whether adult *Xenopus laevis* thymocytes, like those of mammals, are sensitive to glucocorticoid-, lectin-, and antigen-driven T-cell apoptosis.

This vertebrate species has been selected for this kind of study because it develops seriatum, with two "libraries of self," one larval, the other adult. Adult thymocytes have been exposed in vitro to dexamethasone (DEX), a synthetic glucocorticoid, phytohemagglutinin (PHA), a plant lectin, to trinitrobenzene sulfonic acid (TNBS), or amphibian phosphate-buffered saline (APBS) and tested directly for apoptosis. TNBS serves as a polyclonal stimulator by creating a great variety of novel 2,4,6-trinitrophenylacetic acid (TNP)-self cells and proteins, that is, altered self, all of which

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have the potential to stimulate the recipient's immune system. *Xenopus* will "see" TNP-conjugated cells as altered self because an enhanced capacity for a mixed lymphocyte response is observed following TNBS injections (8). Thus, we examine the capacity of new altered-self antigenicity to stimulate apoptosis, perhaps as a reflection of thymic clonal deletion in the maintenance of self-tolerance.

Materials and Methods

Animals

Laboratory bred 1–2 year-old mature *Xenopus laevis* were maintained at 23°C with a photoperiod of 12 h light and 12 h dark. They were maximally fed weekly with NASCO frog "brittle" (Fort Atkinson, WI). The animals were cared for in accordance with agreed on institutional and NIH guidelines for working with vertebrates.

Cell Culture and Stimulation of Apoptosis In Vitro

Thymocytes or splenocytes were removed from individual animals anesthesized with methyl *n*-ethyleneaminobenzoate (Fischer Scientific, Pittsburgh, PA) and teased into cell suspensions. They were cultured at a concentration of 5×10^6 cells/mL in complete Leibovitz's medium (L-15, GIBCO Laboratories, Long Island, NY), diluted to amphibian strength, with antibiotics, 2 m*M* L-glutamine, and 10% fetal bovine serum (Hyclone, Logan, UT).

The thymocytes of each individual were cultured separately in round-bottomed 96-well plates in a density of 1×10^6 cells/well at 23°C. DEX (at 10^{-3} to 10^{-12} *M*) and PHA (at 0.125 to 4.0 μg/mL), were added to some cultures and assayed for apopotsis after different intervals of time. The ethanol vehicle used to solubilize the DEX served as a control for hormone-induced apoptosis. Thymocytes from each animal were tested as separate aliquots with different concentrations of DEX or its ethanol vehicle.

TNBS-activated apoptosis was achieved by treating isolated whole thymuses with 0.03 mg/mL TNBS for 16–30 h in vitro. Controls were cultured in APBS. An experimental and a control thymus was taken for each donor, thus each experimental thymus was compared with its own control. After TNBS exposure, the thymocytes were dispersed in fresh medium for 2 h before being assayed for apoptosis.

DNA Extraction and Gel Electrophoresis

The method for DNA extraction was adapted from Sambrook et al. (9). After being cultured for different periods in round-bottomed 96-well plates, 1.5×10^6 thymocytes or splenocytes were lysed in 300 μL of lysis buffer (10 m*M* Tris, 0.01 m*M* EDTA, 0.5% sodium dodecylsulfate, and 20 μg/ml pancreatic RNAse at pH 8.0, Sigma, St. Louis, MO). After 1 h at 50–55°C, 3.3 μL of 10 mg/mL proteinase K (Sigma) was added, and the tubes were incubated for 3 h at 55°C with periodic swirling. The DNA was isolated from the digested lysate by phenol extraction followed by ethanol precipitation. The resulting DNA pellet was resuspended in 10 m*M* Tris and 1 m*M* EDTA ("TE" buffer) at pH 8.0, diluted 1/10 in loading buffer (30% glycerol, 0.1 *M* EDTA, 1% sodium dodecylsulfate, and 0.25% bromphenol blue), and run on a 1% agarose gel. The gel was stained in an ethidium bromide solution (1 μg/mL) for 20 min and counterstained in water for 5 min before DNA visualization using ultraviolet light.

Flow Cytometry

The flow cytometry used to detect and quantify apoptotic thymocytes was

adapted for amphibian cells from Nicoletti et al. (10). After various times in culture, cells were pelleted and resuspended overnight in 200 μL of ice-cold 70% ethanol in APBS. They were then repelleted and resuspended in 200–500 μL in binding buffer (APBS, 5% FCS, and 0.1% sodium azide) with 50 μg/mL propidium iodide. They were stored at 0–5°C until assayed, but not for longer than 24 h. A Coulter Epics-C flow cytometer was used to count 5000 cells for each sample.

The percent of apoptotic cells was calculated by the flow cytometer using a series of "gates." One gate, gate C, was set to the right of the genomic peak established from assays of freshly biopsied, nonapoptotic cells, in order to avoid distinctions due to higher mitotic DNA content differences or cell clumping. Gate B was set to the left of the genomic peak to detect cells with lowered DNA content. Gate A, on the far left of the histogram, was set to exclude cellular debris. The percentage of apoptotic cells was determined by dividing the number of cells with diminished fluorescence that fall between gates A and B by the total number of fluorescent cells between gates A and C × 100. On the ordinate of the histogram, the number of cells with particular fluorescence intensities were counted and plotted arithmetically. The abscissa monitors the varying levels of fluorescence intensity logarithmically. See Figure 1 for an example of the kind of visual data obtained for these experiments. The positions of the gates by channel number are shown for the L-15 control data depicted here.

Results

Establishing Assays for Apoptosis

DNA extraction and electrophoresis. DNA extracted from freshly biopsied *Xenopus laevis* lymphocytes did not produce a ladder indicative of apoptosis in the agarose gels (Fig. 2, lanes TF and SF). However, DNA fragmentation was apparent in lanes with DNA from thymocytes assayed after at least 8–10 h in vitro and from splenocytes assayed after at least 5–7 h in vitro (Fig. 2, lanes TC and SC). DNA extracted from thymocytes and splenocytes following 10 or 5 h of in vitro exposure to 2 μg/mL PHA, respectively, also demonstrated apoptosis (Fig. 2, lanes TP and SP). The negative data from gels following shorter periods in culture are not shown.

Flow cytometry. As was the case with the DNA gels described above, freshly biopsied thymocytes, unlike those that had been cultured for a minimum of 5–10 h, also showed little DNA fragmentation by this assay, that is, fewer than 10% of the propidium iodide positive cells fell to the left of Gate B (data not shown). Figure 1 is representative of data showing the basal level of thymocyte apoptosis after 20 h in vitro, using this method. Thirty six percent of the thymocytes displayed here were apoptotic.

Correlation of two assays. Samples from the same cell population were used in both the gel and flow cytometry assays. Thymocytes cultured for at least 8–10 h showed both the DNA ladder in gels *and* an apoptotic peak in the flow cytometry histograms. Freshly biopsied thymocytes and splenocytes failed to produce a DNA ladder on the gels or the appropriate apoptotic nuclear peak on the histogram.

As with mammalian studies of apoptosis, 20 h was selected for the time in culture before assay for most of the experiments described below. Preliminary tests with *Xenopus* (data not shown) demonstrated that at 20 h in vitro, the level of apoptosis of the population was at an intermediate value. Thus, after this time in vitro, there is the potential for apoptotic activity to be modulated ex-

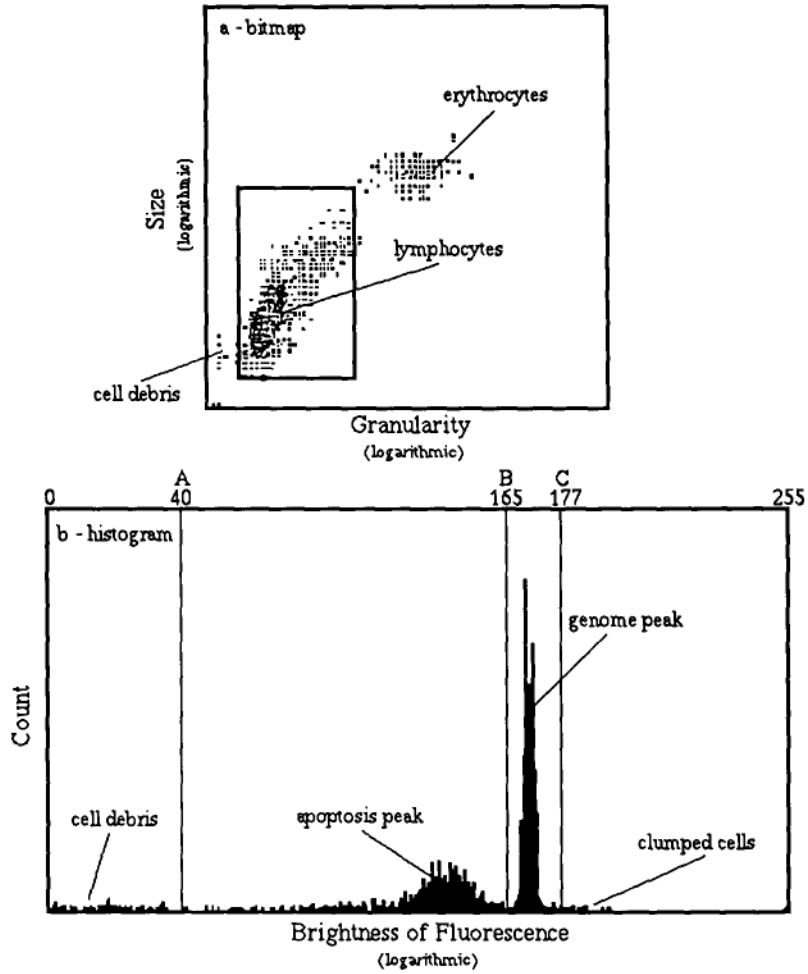

Figure 1. Two types of output are generated in flow cytometry, (a) a bitmap and (b) a histogram. The positions of the cells on the bitmap reflect differences in their size and granularity and enable different types of cells in the suspension to be distinguished so that areas of interest on the bitmap can be selected for analysis. The first 5000 cells entering the selected region are plotted on the histogram according to brightness (bound propidium iodide). The brightest cells (toward the right) have the most DNA. The population of cells with a full complement of DNA appear as a peak (genome peak) between gates B and C. The apoptotic cells between gates A and B are less bright because varying fragments of DNA have been lost. The histogram is typical of the data gathered for these studies. This histogram shows an apoptotic activity level of 36% for adult thymocytes cultured for 20 h with no additional reagents. The channel numbers used as "gates" on this run are also shown.

perimentally in *either* direction. Apoptosis is visualizable by 10 h and maximal by 30 h in vitro. Time points other than 20 h were examined when reagents, for example PHA, were tested to ensure that maximal apoptotic reactivity to these reagents would not be missed, but 20 h proved to be a suitable time for these tests as well (data not shown).

The apoptotic level in 20-h cultures of amphibian cells was not effected by the presence of fetal bovine serum (FBS) in the culture medium. Indeed, preliminary tests showed a similar level of apoptosis whether FBS was present or absent (57–62%, respectively). Thus, the apoptosis reported here after 20 h in culture is not driven by factors in the serum.

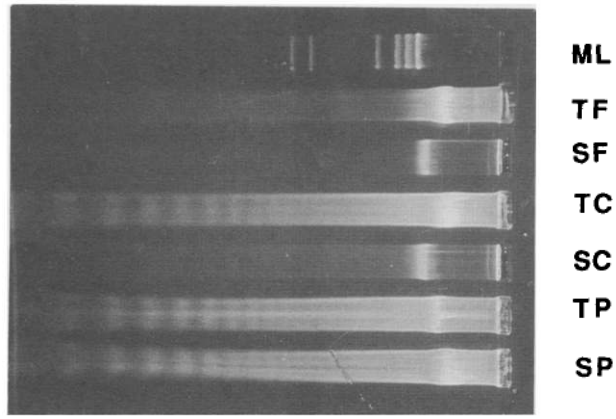

Figure 2. DNA agarose gel demonstrating the typical ladder pattern of DNA fragments in apoptotic cells. The lanes with DNA extracted from freshly biopsied adult thymocytes (TF) and splenocytes (SF) failed to show the DNA fragmentation pattern that is seen with adult thymocytes cultured for 8–10 h (TC) and splenocytes cultured for 5–7 h (SC). The typical DNA apoptotic patterns are also seen when adult thymocytes, cultured for 8–10 h (TP) and splenocytes cultured for 5–7 h (SP), are exposed to 2 μg/mL PHA in vitro. ML = the marker lane with lambda markers. The markers are from *Hin*dIII digested DNA and they are from 23.1 to 0.504 kilobase pairs in size.

Glucocorticoid-driven apoptosis. The DEX concentrations tested encompassed the natural in vivo physiological titers (11) of adults of this species. The apoptotic levels of the experimentals and controls were compared for the same population of cells within each run. Exposure to DEX significantly increased the level of thymocyte apoptosis with concentrations as low as 10^{-11} *M* in a dose-dependent fashion, as compared with the vehicle controls (Fig. 3).

PHA-driven apoptosis. The initial indication that lectin could affect apoptosis in *Xenopus* came from the DNA analyses following culture of thymocytes with 2 μg/mL PHA (Fig. 2, lanes TP and SP). The level of apoptosis produced in response to PHA depends upon the con-

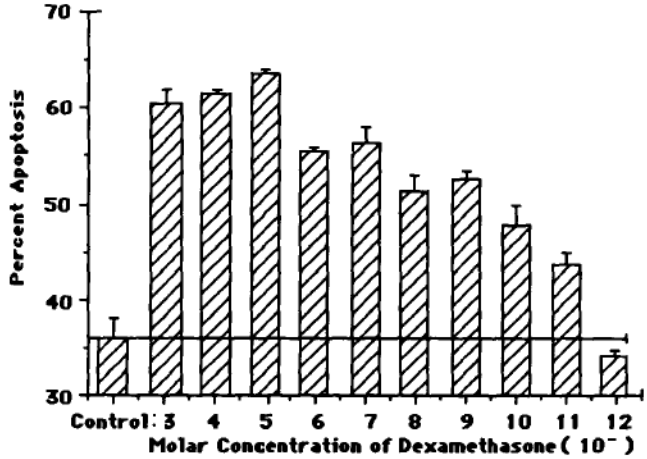

Figure 3. The effect of different molar concentrations of dexamethasone on the percentage of cells in apoptosis in 20-h replicate cultures of unpooled adult *Xenopus* thymocytes. Three experiments with four animals each were cultured separately and the results pooled. The flow cytometry assay of apoptosis involves propidium iodide incorporation.

centration being used in vitro (Fig. 4). Variation in the apoptotic levels of response to PHA or DEX stimulation in replicates of 20-h thymocyte cultures from these nonisogeneic animals is illustrated in Figures 3 and 4.

Altered-self antigen-induced apoptosis. The concentration of TNBS chosen for these studies produced 33% thymocyte cell death in preliminary tests of TNBS exposure in vitro using trypan blue uptake. Dosages of 0.003 and 3.0 mg/mL TNBS that were also examined produced 0 and 100% cell death, respectively.

Isolated adult thymuses exposed to TNBS or APBS for 16–30 h in vitro were assayed by flow cytometry 2 h after their cells had been dispersed into fresh culture medium. The whole thymuses tested showed an average (±SEM) increase in apoptosis of 68 ± 18% as compared to control levels.

Thus, all three reagents, DEX, PHA, and TNBS, stimulate increased thymocyte apoptosis after 20 h of exposure in vitro (Fig. 5). Figure 6 shows examples of the actual data observed when the three reagents were tested and compared to their controls. A separate control is shown for the TNBS test because this experiment utilized whole thymuses and an 18–32 h culture period.

Discussion

The central questions being considered in this report are whether *Xenopus laevis* thymocytes are sensitive to glucocorticoid-, lectin-, and/or antigen-driven apoptosis.

The DNA fragmentation patterns on the agarose gels and results from flow cytometry demonstrate that apoptosis does occur within these adult amphibian thymocyte populations. Because the basal percentages of the cells in apoptosis can be raised by exposure to a glucocorticoid (DEX), a lectin (PHA), and an antigenic epitope bound to self-cells and proteins (following TNBS), sensitivity of adult thymocyte apoptosis to these reagents is established. The enhanced apoptotic levels with DEX and PHA are in agreement with those reported from comparable tests that established glucocorticoid and lectin sensitivity for mammalian thymic apoptosis (4,5,12). The stimulation of increased levels of apoptosis resulting from TNBS exposure suggest that tolerance to altered-self antigens may be associated with apoptosis.

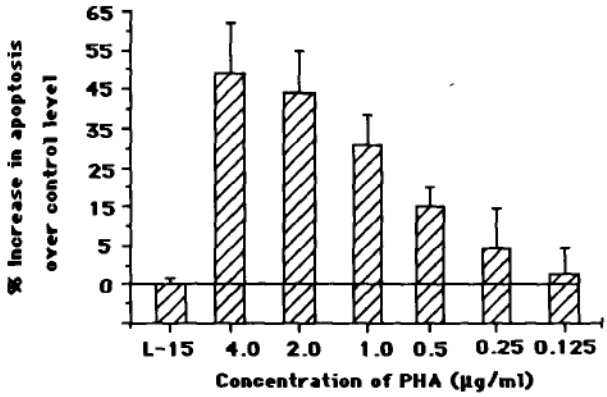

Figure 4. The dose-dependent effect of phytohemagglutinin (PHA) in vitro on the percent increase (±SD) of apoptosis over the basal level of controls (29 ± 4%) in unpooled adult thymocytes of *Xenopus laevis*. Five thousand cells have been counted for each data point and four separate tests were run. The thymocytes from individual animals were cultured for 20 h as replicates that were exposed to either culture medium alone or medium with each of the different concentrations of PHA. Apoptosis was assayed using propidium iodide binding and flow cytometry.

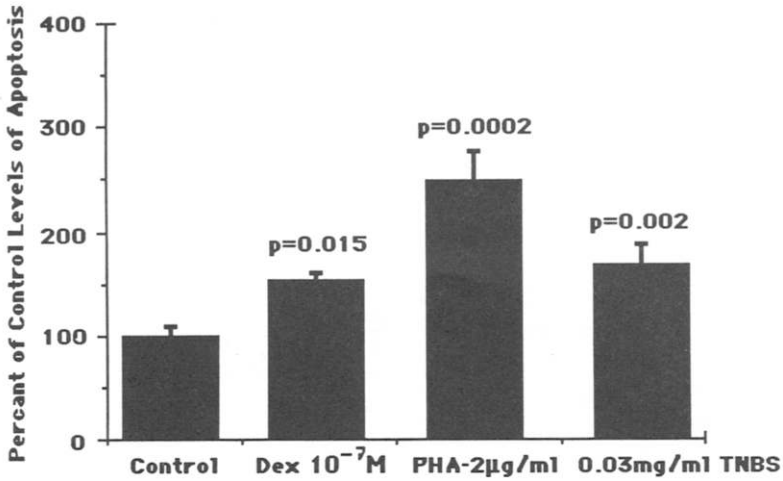

Figure 5. A summary figure showing the capacity of each of the three kinds of reagents tested to drive thymocyte apoptosis to higher levels than controls. The control and experimental DEX or PHA groups of unpooled thymocytes were cultured for 20 h. With TNBS exposures, 18–32 h was used for both experimental and control cultures. The *p* values shown indicate that when compared to their own set of controls, which were replicate cultures of the same thymic cell populations used to test the reagents, the differences are significant (paired student *t*-test, one tailed). The number of animals used to provide the thymocyte cultures to be tested with each of the reagents was 10 for the DEX, 20 for the PHA, and 14 for the TNBS.

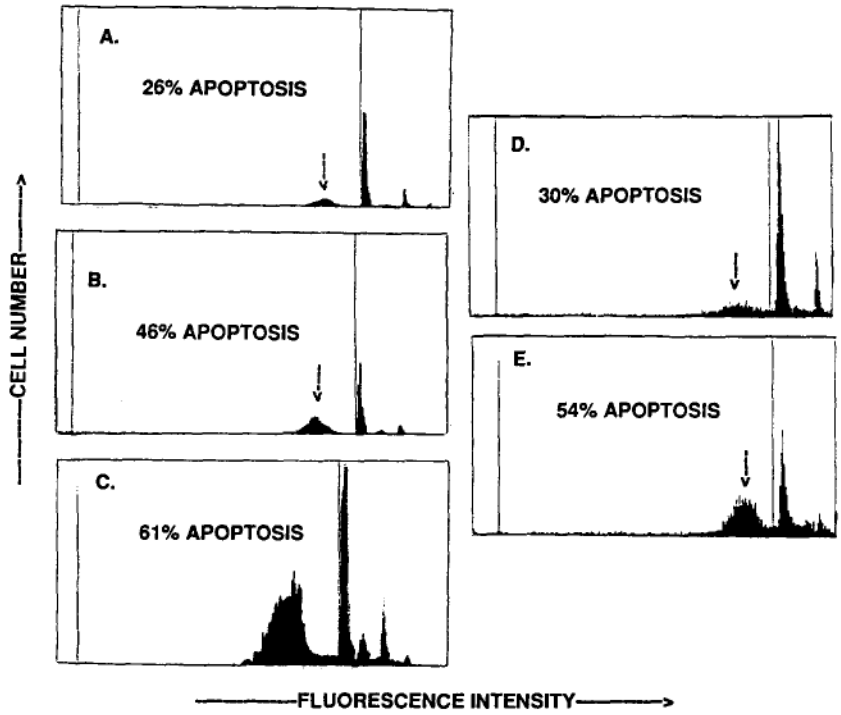

Figure 6. Histograms obtained from flow cytometry that can serve as examples of data demonstrating that exposures to (B) DEX, (C) PHA, and (E) TNBS increase basal apoptotic levels of thymocytes. (A) is from the control used for this run that tested the effects of (B) 10^{-7} *M* DEX and (C) 2 µg/mL PHA exposure in vitro on equal numbers of thymocytes from the same animal. (D) is from the control for TNBS (0.03 mg/mL) exposure in vitro (E). Separate controls are required for the TNBS tests, because whole thymuses were exposed to this reagent and different culture times were used. The percentage of apoptotic cells/5000 assayed is displayed on each histogram.

As the immune system will see the hapten as a conjugate of many different self-carriers, a broad-based increase in antigenicity can be expected and the effect on apoptosis may come close to that produced by other broad-based reagents, for example anti-CD3 antibody using mammalian T-cell hybridomas (13). Indeed, epitope-specific antigenicity following injection of TNBS does stimulate anti-TNP antibody in adult *Xenopus* (Johnson et al. unpublished results) and, as noted above, TNP conjugation of self will lead to enhanced mixed lymphocyte response capacity, suggesting that these animals do recognize the hapten conjugate as altered self (8). Thus, apoptosis in the thymus of adult *Xenopus* may mediate negative selection through the removal of self-specific cells.

We conclude that cells of the adult *Xenopus* thymus have the capacity for glucocorticoid-, lectin-, and antigen-driven apoptosis.

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