Pergamon

Developmental and Comparative Immunology, Vol. 18, No. 4, pp. 343–352, 1994

0145-305X/94 $6.00 + .00

0145-305X(94)00027-1

APOPTOSIS IN THE THYMUS OF DEVELOPING *Xenopus laevis*

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(Submitted April 1994; Accepted July 1994)

□Abstract—Metamorphosis in *Xenopus laevis* is a time when thyroxine and glucocorticoid levels rise, dramatic morphological and physiological changes take place, and tolerance is established to newly expressed adult antigens. In vitro exposure of thymocytes tested at different metamorphic stages, to the T-cell lectin, phytohemagglutinin (PHA), stimulates increased apoptosis, but incubation with the synthetic glucocorticoid, dexamethasone (DEX), fails in this regard. Altered-self antigenicity, following trinitrobenzene sulfonic acid (TNBS) treatment, increases apoptosis only in the late stages of metamorphosis. Developmentally blocked metamorphosing larvae demonstrate low thymic apoptotic rates that are also unaffected by in vitro exposure to DEX or by in vivo exposure to thyroxine, but are increased by PHA and in some individuals by TNBS. When released from blockade, their thymic apoptotic rates rise as progress through metamorphosis is renewed. Larval thymic apoptosis is glucocorticocoid- and thyroxine insensitive, but is lectin and altered-self antigen activated, particularly during postclimax stages.

□Keywords—Glucocorticoid-, lectin-, antigen-driven apoptosis; Amphibian metamorphosis.

Introduction

In mammals, apoptosis or programmed cell death is sensitive to glucocorticoids (1) and to antigen or lectin activation (2,3). During the metamorphic period of *Xenopus laevis*, the South African clawed toad, when thyroxine (4) and corticosterone (5) plasma levels rise, the immune system offers a unique vertebrate framework for an examination of apoptosis related to such changes. Thyroxine is responsible for driving metamorphosis, but it has little effect on the regulation of immune function during this period (6). Corticosterone, on the other hand, diminishes immune T-cell functions (7,8) by limiting T-cell clonal expansion (9,10).

Here, we examine whether metamorphic amphibian thymocytes that arise in a corticosteroid rich environment and have five times the number of adult glucocorticoid receptors (7), are sensitive to hormonally driven apoptosis. We also test whether thyroxine will affect the rate of apoptosis by using thiourea-blocked animals, which are prevented from advancing in development because of the absence of thyroxine.

In order to determine whether a functional relationship between thymic apoptosis and tolerance to adult cells that differentiate within the metamorphosing larva may exist, a T-cell lectin and a haptenated self-antigenic challenge are used prior to assessing levels of apoptosis in larval thymocytes.

Adult cells that arise within the larval body during metamorphosis are major

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histocompatibility complex (MHC) class I disparate from their larval counterparts (11). Other immunologically "visible," but non-MHC-related antigenic disparities also differentiate during metamorphosis. For example, serum proteins, especially albumin, appear and greatly increase in concentration during metamorphosis and antigenically distinct adult hemoglobin replaces the larval form (12). The potential for the interaction of larval and adult immunocytes in vivo was first demonstrated by finding that isogeneic larval and adult immunocytes would produce a mixed lymphocyte response in vitro (13). Thus, there is a clear need for tolerance by the larval immune system toward adult antigens and vice versa, as metamorphosis proceeds. How, then, is immune self-destruction avoided during this transitional period? Does clonal deletion through apoptosis play a role in providing this tolerance? In mammals, the establishment of central and peripheral self-tolerance involves, at least in part, the negative selection of anti-self specific cells through clonal deletion (14).

The use of trinitrobenzene sulfonic acid (TNBS) is particularly relevant here, because it serves as a polyclonal stimulator, creating a variety of novel trinitrophenyl (TNP)-self proteins that can stimulate the recipient's immune system to become TNP tolerant (15). Antigenicity created by TNBS exposure is seen by the recipient as altered-self because mixed lymphocyte responses are enhanced (10). Thus, recipient responses to these altered-self antigens simulate the mechanisms of central and peripheral self-tolerance used by the organism.

Materials and Methods

Animals

Nonisogeneic larval, juvenile, and adult *Xenopus laevis* were laboratory bred or purchased from NASCO (Fort Atkinson, WI) and they were fed daily with a spoonful of slurried nettle powder, pulverized frog "brittle," and/or milk powder. They were reared in large standing water tanks at 23°C. The animals were cared for in accordance with institutional and NIH guidelines for working with vertebrates.

Cell Preparation and Culture

Thymuses were removed from larvae in different stages of development (16), from juveniles and from adults after anesthesia in methyl *n*-ethyleneaminobenzoate (Fischer Scientific, Pittsburgh, PA). The thymocytes were teased into cell suspensions and cultured at 5×10^5 cells/well in a flat-bottomed 96-well plate at 23°C in complete Leibovitz's medium (L-15, GIBCO Laboratories, Long Island, NY), with antibiotics and 0.292 mg/mL L-glutamine, 10% fetal bovine serum (Hyclone, Logan, UT), and 50 μM 2-mercaptoethanol. The cells were cultured for 20 h, with phytohemagglutinin (PHA) at 2.0 μg/mL, or 10^{-1} M dexamethasone (DEX). Whereas 20 h will not be a sufficient amount of time for maximal development of apoptosis, which occurs at 30 h, an intermediate level will be produced that potentially can be modulated in either direction.

Injection of 30 μL of metyrapone, a blocker of glucocorticoid synthesis (9) was also tested with stage 60, 62, and 64 larvae. This protocol has been used previously to modulate T-cell immune functions that are inhibited by the high level of glucocorticoid during the metamorphoic period. The effect of the metyrapone is reversible with an injection of DEX. A fresh stock was prepared each time by dissolving 0.012 g of metyrapone in 200 μL of ethanol and 70 μL of Tween. This was brought up to 20 mL in amphibian phosphate buffered saline (APBS).

Tests of TNBS-activated apoptosis involved incubating whole larval thymuses in 0.03 mg/mL TNBS for 16–30 h. After

TNBS exposure, the thymocytes were dispersed in fresh medium and after 2 h, fixed in 70% ethanol overnight, stained with propidium iodide, and assayed in a flow cytometer as described below. Controls were cultured with APBS in medium alone. Adult thymocyte samples were run in parallel with the larval tests.

The results of the tests following DEX, PHA, and TNBS exposure were averaged for all larvae and adults and also separately analyzed according to developmental stage. The number of animals used in each experiment is displayed in Table 1.

Separate tests had demonstrated that the reason that freshly biopsied thymocytes do not show apoptosis is because macrophages are particularly effective at removing them in vivo. The same situation is found with rodent models. If macrophages are removed in vitro by adhesion or in vivo by injection of colliodal carbon, the percent of apoptosis in both freshly biopsied and 20-h cultured cells, increases. The apoptotic sensitivity of freshly biopsied thymocytes did not vary with developmental age. Prior tests with adult thymocytes had also shown that the amount of apoptosis seen at 20 h is independent of the presence of serum in the culture medium. Thus, apoptosis is not culture medium driven. Additional details of the protocols and illustrations of the histograms produced by flow cytometry are available elsewhere (17). These features did not vary when larval thymocytes were substituted for adult thymocytes.

The functional effect of TNBS-stimulated apoptosis on larval immune reactivity was tested by injecting 5 μL of 0.5 mg/mL TNBS on the surface of the thymus, but not into the organ itself of stage 58 larvae. The TNBS was injected in Freund's incomplete adjuvant to keep it from dispersing from around the thymus. Controls received a comparable injection of the adjuvant alone. After 1 week, the time required for the level of helper activity provided by previously peripheralized T cells to be reduced by 75% (18) the animals were all past stage 66+ and were carrier-primed (0.05% sheep red blood cells, SRBC), and 2 days thereafter were immunized with 20% TNP-SRBC. Their spleens were removed 4 days later and cultured for 7 days. The cell-free supernatant fluids of these cultured cells were tested for anti-TNP antibody by ELISA and anti-SRBC antibody by hemagglutination. In the

Table 1. Effect of Dexamethasone (DEX), Phytohemagglutinin, (PHA), and Trinitrobenzene Sulfonic Acid (TNBS) on Apoptosis in Adult, Larval, and Developmentally Blocked Metamorphosing Larval Thymuses.

Stages	% Apoptosis ± SEM			
	L-15 Controls	DEX	PHA	TNBS
Adults	36 ± 3% (n = 24)	55 ± 3 (n = 10) p = 0.03	89 ± 9 (n = 20) p = 0.004	61 ± 6 (n = 14) p = 0.003
Normal larvae*	58 ± 12% (n = 108)	58 ± 1 (n = 19) p = 0.65	63 ± 4 (n = 9) p = 0.04	52 ± 22 (n = 32) p = 0.22
Blocked larvae†	14 ± 2% (n = 10)	15 ± 1 (n = 10) p = 0.18	19 ± 1 (n = 10) p = 0.0001	20 ± 4 (n = 14) p = 0.08

Each group of experimentals is compared with its own controls made with replicate 20 h (16–30 h with TNBS) cultures. The p values measure the significance of change from control levels and n = number of animals assayed. Apoptosis is measured by flow cytometry using propidium iodide incorporation.

* The larval data from stages 52–60 in Figure 1 were pooled from the purposes of this table.

† 0.3% thiourea was used to block development at stages 57–58 in prometamorphosis. The animals were put in thiourea at stages 52–54.

ELISA, TNP-BSA at 10 μg/mL is first bound to the well surface and is used to bind *Xenopus* anti-TNP antibody in the cell-free culture supernatant fluids being examined. Rabbit anti-*Xenopus* polyclonal antisera, 1:1000 in PBSAT, and then 2 μg/mL of goat-anti-rabbit antibody-alkaline phosphatase are layered on. Three washes of PBSAT are made in between each step. The goat anti-rabbit-antibody-alkaline phosphatase antibody will react with its substrate to produce the chromagen. ELISA assays of controls, experimentals, and a reference curve are always included on the same plates.

Flow Cytometry

The protocol used to detect and quantify apoptotic cells was adapted from Nicoletti et al. (19). After 20 h in culture (16–30 h with TNBS exposure), the thymocytes were pelleted and vigorously resuspended in 200 μL of ice-cold 70% ethanol and stored for 20 h at 4°C. Then the cells were pelleted and resuspended in 200–500 μL (binding buffer) with 50 μg/mL propidium iodide (Sigma, St. Louis, MO), and stored at 4°C prior to assay. A Coulter Epics-C flow cytometer was used to count 2500 cells/sample and the percent of apoptotic cells calculated as follows. Three "gates" were set using freshly biopsied thymocytes, which had no detectable levels of apoptosis. Gate C excluded dividing cells or clumps of cells, gate B excluded background fluorescence shown with freshly biopsied thymocytes, and gate A excluded cellular debris. The stained cells that fell between gates B and A were the apoptotic population, that is, they showed a loss of DNA from the nonapoptotic (genomic) population between gates B and C. To obtain the percentage of the cells in apoptosis, the number of cells between gates B and C was divided by the total number of cells counted, that is, between gates A and C, and multiplied by 100. As noted above, illustrations of the data from flow cytometry and from DNA extractions, have been shown previously (17).

Developmentally Blocked Metamorphosing Larvae

Animals at stages 52–54 (16) were exposed to 0.03% thiourea, which blocks thyroxine synthesis (12) for a minimum of 4 weeks. Metamorphosis was stopped at stages 56–59 (16). Continuation of metamorphosis was achieved by removal from the thiourea environment with or without injection of 30 μL of 10^{-7} *M* thyroxine.

The data from all of the experiments described above were tested for statistical significance either by an analysis of variance, ANOVA, if the number of events was relatively small or by paired-Student's *t*-test.

Results

Stage-Related Apoptosis Levels

When thymocytes from various larval stages of metamorphosis were assayed after 20 h of culture, some minor stage-related differences in thymic cell apoptosis were detected (Fig. 1). The data reveal a consistantly high level of apoptosis in the premetamorphic and metamorphic stages prior to the climax stages, which rises significantly ($p = 0.04$) toward a peak at stages 63–64. These levels are much higher than those found with adult thymocytes. At least seven animals were assayed for each data point.

Modulation of Apoptosis in Normal Development

DEX, at the in vivo physiological titer for corticosterone in larvae of stage 62 (5), failed to significantly alter the level

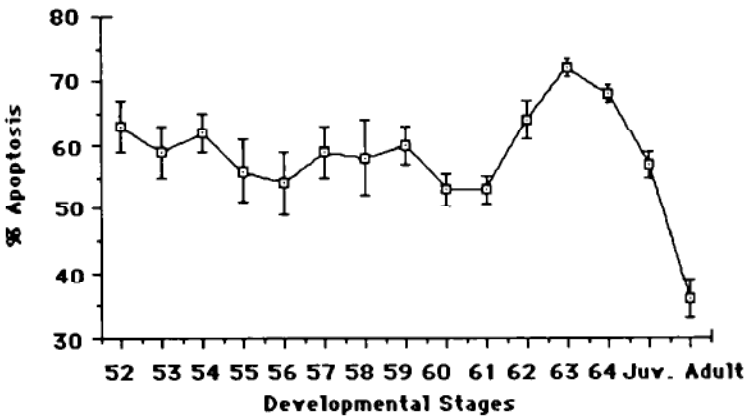

Figure 1. The levels of thymic apoptosis of 108 developing and adult *Xenopus laevis*. The data (apoptotic averages ± SDs) are displayed by developmental stage. The levels of apoptosis are measured in the thymocytes after 20 h in vitro by propidium iodide incorporation and flow cytometry. Thymocytes at Stages 62–64 have significantly more apoptosis than those at the earlier stages ($p = 0.04$, by paired Student's *t*-test). Moreover, all larval base values are clearly higher than those found with adult thymocytes. No fewer than seven animals were assayed for any data point.

of larval thymocyte apoptosis in 20-h cultures (Table 1). Dosages of DEX that were higher and lower than 10^{-7} *M* were also tested in preliminary experiments (data not shown). On the other hand, exposure of larval thymocytes to PHA stimulated small, but statistically significant, increases in apoptosis (Table 1). Analyses of the results with DEX and PHA show no variations from the average by developmental stage (data not shown). Moreover, there was no change in the level of thymic apoptotic activity when larvae at several different stages were injected with metyrapone. This was also found with stage 62 larvae, when the endogenous level of glucocorticoid has reached its peak [(5); data not shown]. Finally, although whole stage 58 thymuses treated with TNBS in vitro display no significant increase in apoptosis over controls as a group, giving an average 16 ± 9% decrease (Table 1), when the data are studied by stage (Fig. 2), it becomes clear that although larvae from the early metamorphic stages do not respond to altered-self antigenicity with increased apoptosis, those in climax and late metamorphosis do.

Adult controls, stimulated by DEX, produce a significant increase in apoptosis of 53 ± 7%. PHA had an even greater effect on average, with an increase of 148 ± 27% over controls, and TNBS exposure increased apoptosis by a significant, but lower (68 ± 18) percent above controls (Table 1).

When functional assays were run after in vivo exposure to TNBS of stage 58 thymuses, the animals were allowed to progress developmentally through the climax stages before being assayed. TNBS will affect apoptosis of thymocytes at these later stages. The data show that local thymic TNBS exposure significantly reduced the anti-TNP response. With injection of adjuvant alone, the optical density of the cell-free culture medium was 0.96 ± 0.53 SD following challenge with carrier-primed TNP-SRBC. When TNBS was included with the adjuvant, it was 0.25 ± 15 SD. This represents a loss of 74% in reactivity to the TNP epitope. There was, however, no difference between controls and experimentals in the level of anti-carrier (SRBC) reactivity as tested by hemagglutination.

Apoptotic Levels in Developmentally Blocked Larvae

The apoptotic levels found with 20-h cultures of thymocytes from thiourea-

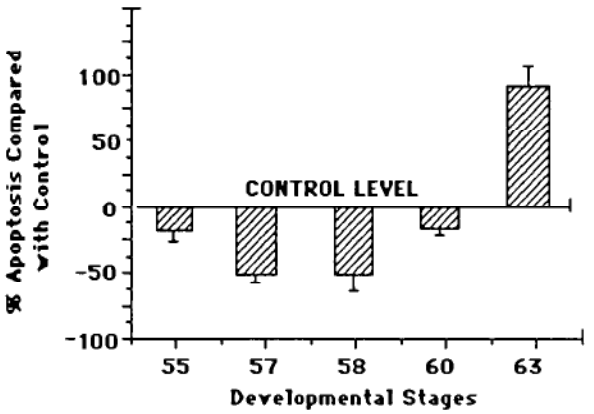

Figure 2. The effect of TNBS on apoptosis is stage-dependent in *Xenopus laevis* larvae. The data from each developmental stage are presented as the percentage of apoptosis (±SD) and are compared with the control basal apoptotic level. Whole thymuses were exposed in vitro to 0.03 mg/mL TNBS for 16–30 h and apoptosis in the cells was assayed by flow cytometry after fixation and propidium iodide incorporation. The apoptotic levels for all stages except stage 63 were below control levels. TNBS-conjugates stimulated a significant increase in apoptosis when stage 63 thymocytes were examined.

blocked larvae were found to be about 20% of those of normal developing larvae of the same developmental stage (Table 1). Even though these basal levels of apoptosis were so low in the blocked larvae, neither DEX nor TNBS were able to increase the level of apoptosis significantly. PHA increased the rate significantly by an average of 37 ± 5%, a small increase compared with the 148 ± 27% increase over controls found in adults (Table 1).

Although there was an average increase of 47 ± 25% with TNBS, this increase was not significant ($p = 0.08$). Further examination of the data after TNBS exposure showed the animals to fall into two groups, 11 having no significant increase in apoptosis (3 ± 25% above controls), and three animals responded dramatically with an average apoptotic level of 206 ± 74% above the controls.

To test whether the relative lack of thymic apoptosis in developmentally blocked larvae may have been due to the absence of circulating thyroxine (T_4) induced by the thiourea, animals were released from the developmental block after an injection of 10^{-7} *M* T_4 and removal from thiourea. Following the injection of T_4, no increase in apoptosis was evident after 2, 4, or 7 days (Table 2).

Discussion

The protocols used here measure base levels of apoptosis in larval *Xenopus* thy-

Table 2. The Effect of Thyroxine (T_4) and/or the Expression of Newly Differentiated Surface Molecules on Apoptosis.

Experimental Condition	% Apoptosis ± SD	*n*
Normal stage 58	63 ± 10	7
Blocked stage 58*	12 ± 7	10
Blocked stage 58 + T_4(2)†	10 ± 2	2
Blocked stage 58 + T_4(4)†	10 ± 5	3
Blocked stage 58 + T_4(7)†	7 ± 1	3
Normal stage 64	72 ± 2	5
Released stage 64‡	87 ± 17	4

* Blocked with 0.03% thiourea.
† Number days after a 10^{-7} *M* thyroxine injection.
‡ 18 days after removal from thiourea.

mocytes and their sensitivity to glucocorticoid-, lectin-, and antigen-driven activation in vitro and in vivo. The results show that thymocytes of normally developing larvae have levels of apoptosis that average 58% (Fig. 1), a significantly higher base level than those of adults, which average 36%. Furthermore, the lower adult apoptotic activity levels are more readily increased by the three reagents used here than are those of larval thymocytes. Thus, the regulation of thymic apoptosis in adults and larvae is different.

Exposure to DEX increases apoptosis in adult *Xenopus* thymocytes (Table 1), but fails to do so with thymocytes from normal developing larvae. Moreover, thymocytes of thiourea-blocked larvae also demonstrate unresponsiveness to DEX. These results suggest that despite the 10-fold increase in the plasma level of corticosterone and a fivefold increase in the numbers of glucocorticoid receptors in thymocytes during the relevant developmental stages (7), apoptosis in the thymus of *Xenopus* larvae is not increased by this hormone when it is added in vitro. Additionally, removal of endogenous corticosterone with metyrapone, a blocker of corticosterone synthesis in this larval organism (9), does not affect thymic apoptotic larval levels. Therefore, results from in vitro exposures to DEX, which failed to modulate apoptotic levels, are in agreement with the in vivo data. Thus, while the metamorphosing larvae bear co-existing larval and adult MHC class I disparate cells (11), the high internal corticosterone titer (5) does not appear to be solely responsible for significant amounts of thymic deletion. The apoptosis seems more likely to be related to T-cell educative requirements. An alternative explanation that would be consistent with this result, is that the unusually high endogenous titer of glucocorticoid might have removed all potential hormone sensitive cells and thus, a subsequent test using glucocorticoid exposure in vitro would find no target cells remaining.

We have previously noted (21) that glucocorticoid-induced anergy may account for functional adjustments in the immune system required to avoid immune self-destruction in the periphery during at least a portion of metamorphosis. Corticosterone, the principal endogenous glucocorticoid of this species, has been shown to be responsible for the inhibition of peripheral T-cell clonal expansion (7,10) that will impair T-cell functions during metamorphorsis (8). That anergy is involved is suggested by evidence that IL-2 will restore glucocorticoid inhibited reactivity (9,10). The data presented here suggest that while it may be an effective regulator of T-cell clonal expansion, this functional immune regulation involves neither stimulation nor reduction of apoptosis in the thymus. Removal of endogenous corticosterone by injection of metyrapone can restore T-cell function in vivo, while coinjection of metyrapone and DEX does not (9). Thus, glucocorticoids may regulate immune function by limiting *peripheral* T-cell reactivity during metamorphosis, but they do not appear to affect *central* negative selection of developing T cells through apoptosis.

Xenopus larvae exhibit a relatively stable, high level of apoptotic activity until metamorphic climax at stage 61 (Fig. 1). From this point, the rate of apoptosis increases significantly to a peak at stage 64, after which it drops rapidly to average adult levels. These results suggest that apoptosis may play a role in the redefinition of self required when new adult antigens are expressed during the late stages of metamorphosis, because it is only during these stages that new altered-self antigenicity, introduced exogenously during this time, increases the apoptotic rate (Fig. 2). Exposure to TNBS does create antigenicity in *Xenopus* that is representative of altered-self because it will enhance a mixed lympho-

cyte response using allo-splenocytes of this species (10).

Stage 64 is representative of the terminal phases of metamorphosis when growth is a minor component and differentiative events are manifest. Thus, the high level of apoptosis found at stage 64 may be related to an increased level of educative activity required by the expression of new adult antigens. TNBS-stimulated thymic apoptosis functionally reduces anti-TNP antibody production, but reactivity to the carrier, SRBC, is unaffected. In the absence of transgene technology, which has been so useful in resolving this issue in mammals, we have had to use this kind of indirect evidence to suggest a relationship between modulations of apoptosis and clonal deletion.

A relationship between increasing apoptotic rates after stage 61 and the expression of new adult antigens is also supported by the contrasting low levels of apoptosis (an average of 14%) in thiourea-blocked larvae (Table 1). Thiourea-blocked larvae are unable to proceed with differentiation and therefore, are not exposed to new adult antigens. This reduced requirement for negative selection correlated with limited anti-self reactivity may well be the reason behind their low apoptotic levels. Although one may be tempted to argue that the low level of apoptosis here is due to blocking the larvae at a stage when the thymocytes are insensitive to T_4, the level is so much lower than the comparable stage (stages 57–58) in unblocked larvae that the suggested relationship of apoptosis to exposure to new modified-self (adult) antigens seems the more compelling. That the high level of apoptosis found at stage 64 in normal development (Fig. 1) is reproduced when larvae are released from thiourea-blockade and allowed to progress through metamorphosis, is also supportive of this potential relationship. T_4, present at high levels during the metamorphic stages, is inhibited by thiourea in developmentally blocked larvae. However, our results suggest that T_4 may not be directly responsible for regulating apoptosis at any of the three time points tested (Table 2). Injection of a concentration (10^{-7} *M*), standardly used to drive larvae into premature metamorphosis, failed to increase thymic apoptotic activity in larvae released from developmental blockade. Thus, apoptotic rate increases seen in larvae allowed to progress to stage 64 after release from the thiourea block appear to have been stimulated by their renewed progression through the developmental stages rather than as a direct effect of T_4.

The inability of TNP-altered-self to stimulate an increase in apoptosis in pre-climax stages may be due to the absence of adult MHC class I expression during these stages (11). The absence of MHC class I prior to climax may remove the capacity of TNP to stimulate increased apoptosis, just as it may limit the presentation of self-epitopes within the thymus. Thus, the high rate of apoptosis observed in the thymus early in metamorphosis may, in part, reflect cells dying passively because of their inability to be positively selected through recognition and response, rather than being removed actively by the clonal deletion of anti-self reactive cells. T cells that are positively selected are those that bind non-self immunogenic peptides in association with self-MHC molecules. The role of positive selection and its MHC-peptide specificity in mammals has been reviewed recently (22).

In mammals, MHC class I molecules are important for effective presentation and thymocyte recognition of TNP in association with self (23). Larval *Xenopus* may, however, use MHC class II antigens as substitutes for class I molecules in the functioning of cytotoxic T cells (24,25). Thus, while MHC class I molecules may be lacking during early and mid-metamorphosis, MHC class II molecules may still be able to serve in thy-

mic presentation of TNP-altered self cells in larval *Xenopus*. Once MHC class I is expressed, however, and the gluoccorticortoid titer has fallen after climax, the introduction of new TNP-self antigenicity results in increased apoptosis (Fig. 2).

Acknowledgements—The authors are grateful for the partial support of this work from Grants #IM-651 from the American Cancer Society, Atlanta, GA to L.N.R.; #RR-00163 from the National Institutes of Health, Bethesda, MD to the Oregon Regional Primate Research Center; and #0735/86 from the North Atlantic Treaty Organization to R.H.C. and L.N.R. This research was also supported by a grant from the Howard Hughes Medical Institute made to Reed College under the 1991 Undergraduate Biological Sciences Initiative.

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