

The immune system is involved in *Xenopus* metamorphosis

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. *Xenopus* immune cells
4. Remodeling of immune cells during *Xenopus* metamorphosis
5. Tolerance induced in larvae but not adult *Xenopus*
6. Immune system involvement in *Xenopus* metamorphic tail regression
 - 6.1. Skin cell remodeling during metamorphosis
 - 6.2. Larval tail skin is rejected by the adult frog
 - 6.3. Larval skin cells are recognized and killed by adult T cells
 - 6.4. Isolation process and function of larval antigens recognized by the adult T cell
7. Summary
8. Acknowledgments
9. References

1. ABSTRACT

Amphibian metamorphosis provides a model to elucidate the mechanisms underlying how vertebrates reconstitute a body plan and how the immune system develops during ontogeny. In *Xenopus*, T cells are expanded from the early developmental stages just after hatching. These T cells switch from larval-type in an easily tolerizable state into an adult-type having a potent immune responsiveness comparable to that of mammals. During metamorphosis, tadpoles exhibit morphological changes in skin that completely transforms from larval-type to adult-type. Only tail tissue behaves differently; it remains a larval-type tissue until it disappears at the end of metamorphosis. Thus, at metamorphic climax, four different types of cells co-exist in a tadpole body: larval tissue cells; adult tissue cells; larval immune cells; and adult immune cells. Based on the results showing that tadpole tail skin is rejected by syngeneic adult, it is proposed that the elimination of the larval tissue cells by the adult T cells that occurs during metamorphosis is immunologically mediated. Recent results indicate that the antigenic proteins expressed in the metamorphosing skin cells participate in the process of tail regression. This chapter describes how animals adjust and survive through such crises associated with large scale replacement of entire body cells.

2. INTRODUCTION

Research on the immune system of anuran amphibians has relied heavily on *Xenopus laevis* as a model system. Studies with this species have revealed that the immune system of larvae is immature compared to that of adults. Among a multiplicity of interesting findings in the area of the ontogeny of immunity is the ease with which allotolerance can be induced during larval life. It is thought that this allotolerance prone period, which disappears after the completion of metamorphosis, reflects a normal state of larvae that is necessary for acceptance of newly appearing adult type tissues. It is less well known, but no less interesting, that the immune system of the young postmetamorphic frog appears to play a role in eliminating larval tissues, another function that disappears soon after metamorphosis. In this paper, I summarize the ontogenesis of the immune system in *Xenopus* with a focus on the transition from larvae to adults. Next, I introduce studies on remodeling of skin tissue in anuran amphibians including *Xenopus* and our finding that the immunity excludes the larval skin remaining in the tadpole at the metamorphic climax. Finally I discuss the possibility that the acquired immune system contributes not only to self-defense but also to the remodeling processes in vertebrate morphogenesis.

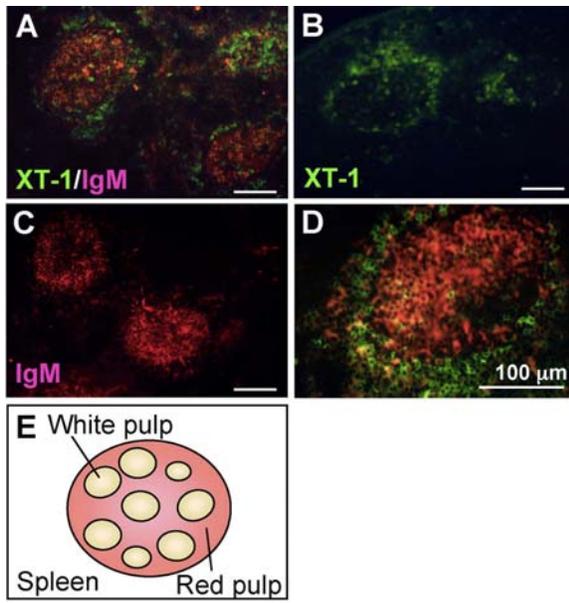


Figure 1. Distribution of *Xenopus* T cells and B cells in the adult spleen. A, Serial frozen cross sections at 4 μ m in thickness of a spleen obtained from a 2-3-Mo-old J strain frog were prepared without fixation, and then stained with anti-*Xenopus* pan- T cell mouse mAb (XT-1) and anti-*Xenopus* IgM mouse mAb (10A9) as a B cell marker. The signals were detected by using FITC or Cy3-conjugated mouse Ig antibodies shown in green color for T cells and red color for B cells, respectively. Staining represents the distribution of T cells alone (B) and B cells alone (C). D, higher magnification of a white pulp indicating that B cells are in the white pulp area and T cells are located in the red pulp area surrounding the white pulp, as illustrated in E. Scales represent 100 μ m.

3. XENOPUS IMMUNE CELLS

Electron microscopy has revealed *Xenopus* macrophage-like cells with phagocytotic activity in tadpole tails that are regressing at metamorphic climax (1). Immunocompetent *Xenopus* cells that had been noted histologically were confirmed as T cells (2, 3) and B cells (4) in the 1980's with monoclonal antibodies. These T and B cells are well distributed in the spleen (Figure 1). As shown in Figure 1, splenic B cells are found in the white pulp area (previously known as the antigen-trapping region (5)). In contrast to mammals, *Xenopus* splenic T cells are located in the red pulp area surrounding the white pulp (6). Most of the T cells are believed to be educated in the thymus since thymectomy, but not splenectomy, has a major effect on immune responsiveness (7). In *Xenopus*, the thymus anlagen arise around day 3 after fertilization at NF-stage 40 according to the Normal Table of Nieuwkoop and Faber (8). However, even after thymectomy by microcautery at the early NF developmental stage 45-47 around day 4-5 days of age, T cells still can be found (comparable to controls) in the tail tissues of tadpoles undergoing metamorphic climax (9). These T cells lack the expression of mammalian CD8 surface marker (AM22

monoclonal antibody (mAb)), which reacts with the thymus-derived cytotoxic T cell subset (10). Thus, non-thymic T cells differentiate in *Xenopus*; whether these cells differentiate in other major lymphopoietic organs, *i. e.*, the liver and the mesonephros (11) is unknown.

Before the thymus develops, leukocytes that stain with XL-1 mAb against leukocyte common antigen, appear from the early embryonic stage 35/36 and are scattered in the entire tadpole body (12). These cells seem to be macrophages although whether these cells have phagocytotic activities is still controversial.

On the other hand, Horton *et al.* reported that a *Xenopus*-specific anti-NK cell monoclonal antibody, 1F8, reacts with a subset of non-thymus derived larval cells (13). The number of the 1F8-reactive cells in the larva in the absence of thymus was almost the same as that derived from the normal larva. This 1F8 antibody begins to detect some population of cells at the metamorphic period when the MHC class Ia is also expressed. It is likely that the 1F8-reactive cells are the larval natural killer (NK) cells, which do not have cytotoxic activity against the MHC-negative cells. In contrast, the adult NK cells do (14, 15) (cf. a mAb against *Xenopus* MHC class I (TB17 mAb) as isolated by Harding *et al.*, (16) and MHC class II (AM20 mAb) identified by Flajnik *et al.*, (10)).

The immunology of *Xenopus* including these immune cells and their molecular markers as mentioned above has been well studied because, owing to its aquatic existence during larval and adult life and the ease of fertilization and husbandry in a laboratory environment, this species is ideal for immunologists to observe and manipulate from embryogenesis to organogenesis.

4. REMODELING OF IMMUNE CELLS DURING XENOPUS METAMORPHOSIS

To investigate whether T cells recognize foreign leukocytes derived from another individual or strain, the mixed leukocyte reaction (MLR) assay with radiolabeled-thymidine or BrdU-incorporation has been used as an established method for mammals (17, 18). The most remarkable finding using a MLR assay for *Xenopus* is that adult and larval immune cells are immunologically distinguishable. Specifically, when lymphocytes from cloned larval and syngeneic adult *Xenopus* are co-cultured, a significant and bi-directional prominent proliferative response has been reported (19, 20). However, in our experiments, when the lymphocytes from tadpoles were mixed with syngeneic adult tissues cells, proliferative responses were not observed, whereas adult lymphocytes showed prominent responses against the larval tissue cells (9). These proliferation responses seen in the MLR are severely impaired by early thymectomy, indicating that this response is generated by T cells (21).

Organ transplantation has been used to estimate how the immune cells are replaced during metamorphosis. Specifically, transplantation of triploid (3N) thymuses into diploid (2N) tadpoles showed a complete exchange of

Table 1. Transplantation experiments using cloned or F₁-hybrid *Xenopus*

Combination	Stage of host	Graft size	% of rejected (individual No)	Days after grafted
Exp. 1				
FF adult->JJ host	Adult	1 mm ²	100 (11/11)	20
	Larva (st57/58)	1 mm ²	90 (9/10)	
	Adult	4 mm ²	100 (13/13)	
	Larva (st57/58)	4 mm ²	53 (9/17)	
JF adult->JJ host	Adult	1 mm ²	100 (12/12)	20
	Larva (st57/58)	1 mm ²	15 (2/13)	
	Adult	4 mm ²	100 (12/12)	
	Larva (st57/58)	4 mm ²	0 (0/12)	
Exp. 2				
KK adult->JJ host	Larva (st51/52)	0.5-1 mm ²	100 (40/40)	45
		1.5-2 mm ²	0 (0/37)	
JK adult->JJ host	Larva (st51/52)	0.5-1 mm ²	41 (12/29)	45
		1.5-2 mm ²	0 (0/100-)	

Skin tissues of different sizes (surface indicated in mm²) obtained from cloned or F₁-hybrid adult donors that differs only by minor H antigens or by minor and one MHC haplotype, were grafted onto larval or adult hosts. For each combination, the percent (%) of graft rejection, and the number of individuals that have rejected versus total individuals grafted are indicated. Experiment 1 (upper panel) has been done by DiMarzo and Cohen (33) and experiment 2 (lower panel) by Nakamura and collaborators (35). After 20 or 45 days of transplantation, tadpoles at stage 57/58 or 51/52 respectively reach at the end of metamorphosis. These experiments show that adult frogs can recognize minor H antigens, whereas larvae cannot. This larval allotolerance seems to depend on the graft size from the donor. Note: these skin graft were observed under the dissection microscope, and were considered “complete rejection” when all of the iridophores in the skin grafts have been destroyed, whereas other part of grafted skins remain. In contrast, we require complete disappearance of the grafted tissue for considering as “rejected” shown in Figure 3 (50).

lymphocytes during metamorphosis (22) with large scale of apoptosis in the thymus medulla (23, 24). The number of lymphocytes both in the thymus (25, 26) and the spleen (26) is reduced to approximately 50-60 % at metamorphic climax stages (NF-stage 64-66) as compared to that of cells obtained from tadpoles at prometamorphosis (NF-stage 57-61). This renewal is not affected by thyroid hormone which induces metamorphic morphological changes (27). Taken together, immune cells seem to be totally replaced during metamorphosis from larval-type into adult-type. However, some larval immune cells do appear to remain after metamorphosis; this is based on the observation of a memory response to sheep red blood cells (SRBC) in postmetamorphic adults that had been immunized during larval life (28). From these results, it is difficult to reach a definitive conclusion due to the insufficiency of molecular probes that distinguish larval and adult immune cells. Another related but unresolved issue concerns the origin of stem cells that occur during the shift from a larval to an adult type of immunity. That is, cell lineages have not been characterized. Although the bone marrow (BM) is major lymphopoietic organs in mammals, in *Xenopus*, the BM develops after metamorphosis (29), suggesting that it is unlikely to be lymphopoietic (30, 31). Other organs *i. e.*, spleen, liver, thymus and kidney, have been proposed as candidates, but the source and indeed, the very existence of adult stem cells has yet not been confirmed.

5. TOLERANCE INDUCED IN LARVAE BUT NOT IN ADULT *XENOPUS*

Allotolerance of skin grafts is easily induced in larvae but not adults (32-35). To investigate mechanisms underlying this larval tolerance, MHC-identical clones (19, 36) or MHC-homozygous inbred strains, J (j/j) (37), K (k/k) (38) and F (f/f) (39) were established for applying skin transplantation techniques. LG clones were obtained

from endoreduplicated *Xenopus* hybrid clones between a female of *Xenopus laevis* and a male of *Xenopus gilli*, so called LG. The J strain, a completely inbred strain established in Japan (“J” for the first initial of Japan) has been maintained for almost 40 years (more than 30 generations) as a closed breeding colony. A major finding from those transplantation experiments is that adult frogs can recognize minor histocompatibility (H) antigens in essentially the same way as mammals, whereas larvae cannot reject the skin from donors that differ only by minor H antigens or in certain combinations having a one MHC haplotype disparity (33-35) (Table 1). This larval allotolerance seems to depend on the graft size from the adult donor (33, 35). Relatively large grafts were always tolerated regardless of differences in the H-antigens between donors and hosts, whereas small grafts were sometimes rejected. These results suggest that the larval tolerant state is unstable or sensitive to the balance between antigens and immunocompetent cells. These studies led us to question how amphibians confront newly appearing antigens during ontogeny and how metamorphosed animals eliminate larval antigens.

6. IMMUNE SYSTEM INVOLVEMENT IN *XENOPUS* METAMORPHIC TAIL REGRESSION

6.1. Skin cell remodeling during metamorphosis

In anuran metamorphosis, tadpoles show morphological and functional tissue remodeling from larval- to adult-type organs (40). Extreme examples of this remodeling process are seen in the epithelial organs where the epidermal cells in larvae are completely replaced by adult cells in the corresponding tissues of the frog (41-43). Skin, the largest organ in the body, is also the largest peripheral immune organ (44). Moreover, skin has the physiological function of a barrier, so that it shows most

Xenopus immune system in metamorphosis

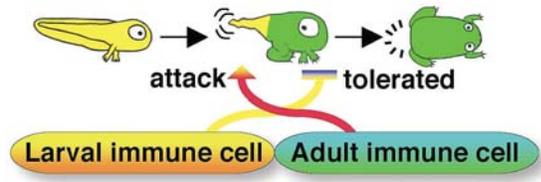


Figure 2. A new model for immune system involved in metamorphic tail regression. In metamorphosis, a tadpole shows morphological and functional changes in the skin tissue, where the epidermal cells in larvae (yellow color) completely transform into the adult cells (green color) in the frog. However, the tail skin behaves uniquely, retaining a larval-type tissue until it disappears at metamorphic climax stage. The immune system also changes during metamorphosis. Our model proposes that the adult-type immune cells, which newly appear during metamorphosis, attack and remove its own tail as non-self. On the other hand, the larval immune cells with newly appearing adult antigens are tolerated, resulting in a failure of trunk tissue to be eliminated by the immune system.

drastic changes for adaptation from an aquatic life to a terrestrial one (43). Although *Xenopus* spend their entire lives in water, they, like other amphibians, also show morphological and functional changes in the skin that are adaptive to a land environment (45). The skin of *Xenopus* larvae contains apical cells (flat and square cells that make up the uppermost single layer of the epidermis) and skein cells (located in the inner portion of the epidermis). During metamorphosis, larval basal cells appear along the basement membrane of the epidermis; these are thought to become stem cells that reconstitute the adult epidermis. By contrast, the adult epidermis does not contain the apical cells or the skein cells, and its structure is basically comparable to the mammalian epidermis (46), which includes basal germinative cells, granular cells, spinous cells and cornified cells in the outermost layer of adult skin (47). Both larval-specific apical and skein cell are eliminated during metamorphosis. However, histological observation revealed that the skein cells persist in part of the tail skin until the very end of the metamorphosis, showing mitotic activity even though they will soon be eliminated (43).

In metamorphic climax, the most remarkable change is tail regression, as the tail behaves uniquely, retaining a larval-type tissue until it disappears (43, 48). This resorption of the tail tissue, including the skin cells, has been explained solely in terms of a cell-autonomous programmed death of tissues induced by thyroid hormone (49). We, however, have focused our attention on the possible involvement of an immunological component to the elimination of larval skin during the metamorphic tissue reorganization process (Figure 2).

6.2. Larval tail skin is rejected by the adult frog

J strain of *Xenopus* is a fully homozygous inbred strain, so that frogs cannot reject skin graft from each other among adults (50) (Figure 3A). However, the grafts of larval skin, including both tail and trunk, obtained from

young tadpoles at the beginning of metamorphosis, are rejected by the syngeneic adults (Figure 3B and 3B'). Around day 2 to 9 after transplantation, the larval skin grafts appear to have been accepted by the hosts (Figure 3B', Day2). Around day 7-9, vascular infiltration is observed in the grafts (Figure 3D, Day9). Host and graft tissue are completely fused into a skin sheet, whereas accumulation of lymphocytes is observed only underneath the grafted larval skin grafts (Figure 3D, see cross section). Finally, grafted skin from early metamorphic tadpole is almost rejected around day 48 (Figure 3B'). The most important result from these transplantation experiments is that the tail skin obtained from tadpoles at metamorphic climax stage when the tadpole tail regresses, is still rejected, whereas the trunk skin from the same animals is not, suggesting that the trunk skin has already changed into adult-type skin (Figure 3C and 3C'). These rejections exhibit accelerated secondary responses, suggesting that adult T cells are involved in this disruption of larval skin grafts. During the same developmental period, adult-MHC class II positive T cells have already appeared in the metamorphosing tadpole body (Figure 4). Therefore, it seems that at the metamorphic climax stage, larval tail skin appears foreign to the "adult-type T cells" that emerge during metamorphosis. Notably, skin grafts obtained from tadpole tails are rejected irrespective of the metamorphic stage of the donors. The mean survival time of skin grafts from premetamorphic tadpoles is much longer than that from individuals at metamorphic climax stages. In other words, the rejection represents a tendency towards increasing rapidity as the metamorphic stage of the donor progresses up to stage 63 (50) (Figure 3C' compared to 3B'). Recently (as described below), this has been found to be correlated with the expression of tail antigen proteins recognized by the adult T cells that increase and reach the maximum level at metamorphic climax stages (51).

6.3. Larval skin cells are recognized and killed by adult T cells

Splenocytes including T cells from J strain metamorphic climax larvae and from adults, but not from early metamorphic larvae, show prominent proliferative responses indicated by BrdU incorporation when co-cultured with syngeneic larval tails in the presence of fetal bovine serum (9) (Figure 5A and Table 2). Furthermore, tail fragments co-cultured with adult splenocytes from the same strain in the presence of adult *Xenopus* serum, undergo apoptosis (9) (Figure 5B). Apoptosis of larval epidermal cells was confirmed by DNA ladder formation and morphological features such as nuclei fragmentation. Although these proliferation and killing assays are not able to detect both events in the same culture due to the different requisite experimental conditions, it is suggested that the larval tail cells are recognized as foreign by the T cells of metamorphic climax tadpoles. The rate of responsiveness to the syngeneic larval tail cells is comparable to that against the semi-xenogeneic adult skin obtained from the hybrid between a female of *Xenopus laevis* J strain and a male of *Xenopus borealis* B strain (52). These proliferative responses by the adult-type T cells including adult-type antigen presenting cells (APC) are completely blocked by anti-*Xenopus* MHC class II mAb (52). In *Xenopus*, MHC

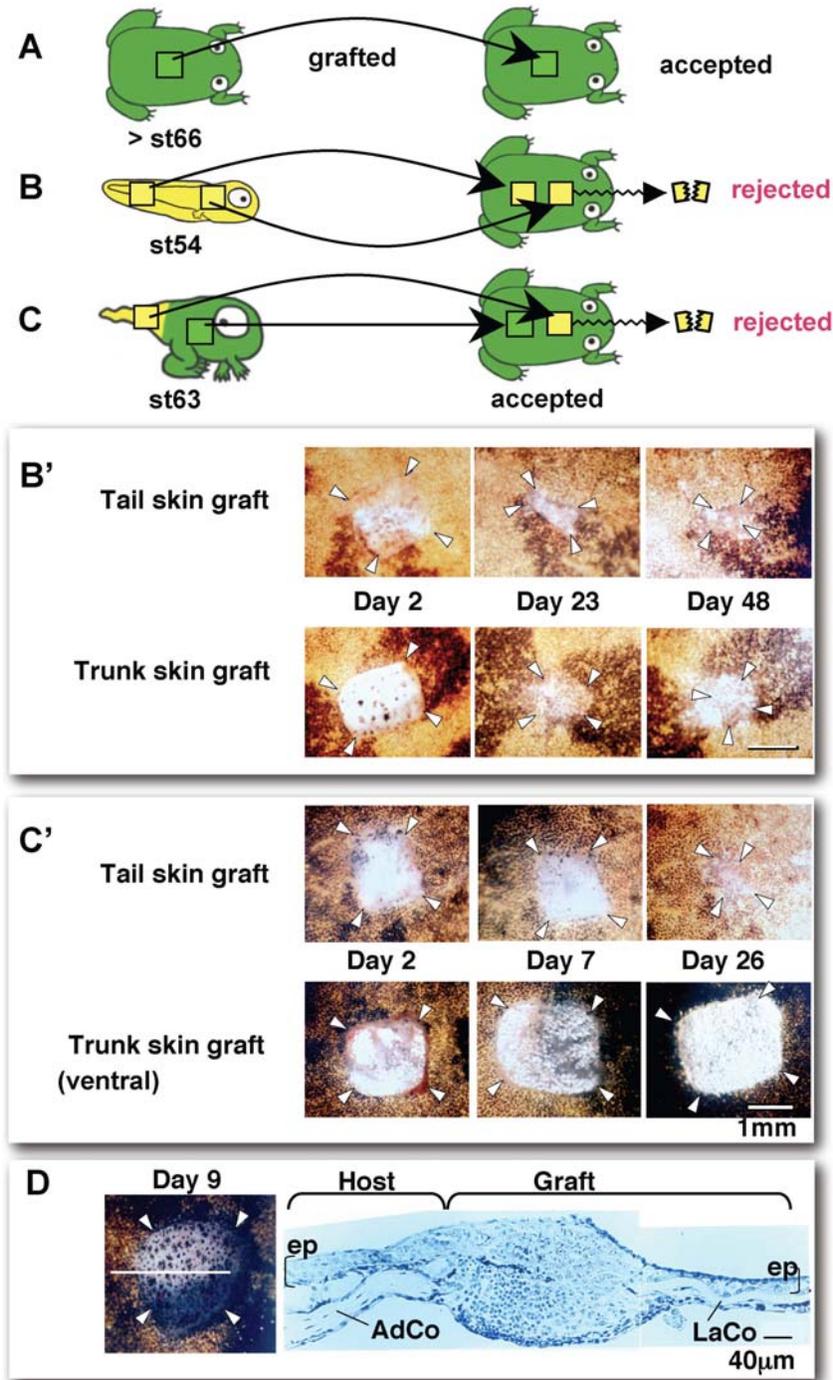


Figure 3. Larval tail skin is rejected by the adult. A, the J strain is a complete homozygous inbred strain (i.e., J frogs do not reject skin grafts from each other). B, both skin obtained from tail and trunk region of a tadpole at NF-stage 54 are rejected by the syngeneic adult frog. Larval skin is transparent as indicated in the four corners by arrowheads in B'. C, the tail skin obtained from tadpoles at the metamorphic climax NF-stage 63 is still rejected, whereas the trunk skin (ventral skin used because it appears white in C') from the same animals is accepted. This ventral trunk skin graft extends by day 26 as the growth of a host frog. D, a vertical frozen cross section at 4 µm in thickness of the skin, including a larval tail graft obtained from tadpole at NF-stage 56 and the host skin, was obtained at day 9 after transplantation, and stained with hematoxylin-eosin. Vascular reorganizations are seen in the graft. Scales in B' and C' represent 1 mm and in D 40 µm, respectively.

Table 2. T cell proliferation to the larval tail cell

Responder	Proliferative cells to each stimulator (% with SE)			
	Without	JJ larval tail tissue (st56/57)	JJ adult dorsal skin (2-3-Mo)	JB adult dorsal skin (2-3-Mo)
JJ larva (st57/58)	4.7±0.5	5.0±0.1	4.3±0.5	-
JJ larva (st63-65)	5.3±0.9	10.9±1.7	5.8±0.5	-
JJ adult (2-3-Mo)	1.9±0.4	9.9±3.5	2.1±0.1	10.5±0.9

Responder leukocytes (5×10^5 cells) obtained from spleens of larvae at stage 56/57, tadpoles at stage 63-65 and froglets at 2-3-Mo-old, were co-cultured without stimulator or with skin tissues ($2 \times 1 \text{ mm}^2$) from larvae at stage 56/57 or from dorsal area of adults at 2-3-Mo-old (9). Cells co-cultured for 5 days, were pulsed 24 hrs before to be harvested and analyzed for incorporation of BrdU by immunohistochemistry. 1000-1500 cells were counted in individual specimen per each responder leukocyte. (-); not done. Values represent means±SE obtained from three different experiments. Values of JJ adult responder T cells to the JJ larval tail tissue are comparable to those against the JB adult skin as a “semi-xenogeneic combination” (52).

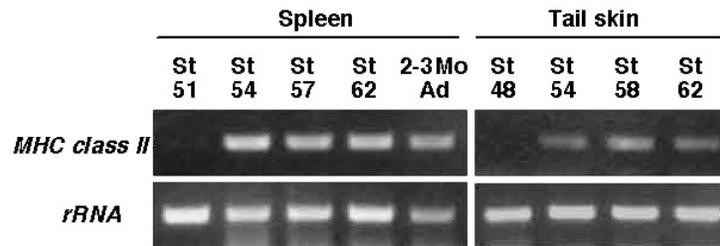


Figure 4. MHC class II expression in the spleen and the skin during *Xenopus* ontogeny. Total RNA was extracted from spleens or tail skin tissues of tadpoles of the J strain. Two μg of RNA was reverse-transcribed using random primers. Specific primers were used for MHC class II PCR reaction that was performed as described previously (52). Left panel, the immune cell exhibits transformation from the larval-type into the MHC class II positive adult-type from stage 54. Right panel, MHC class II is expressed in a coordinated fashion in the tail skin tissue cells. Expression of rRNA was used as loading control.

class II gene and its protein are expressed not only in the T cells but also in the epidermal cells in the skin during metamorphosis (52) (Figure 4). Since MHC class II molecules are expressed only in the apical cells but not in the skin cells, this implies that *in vivo* the apical cells are recognized directly by the adult T cells without the APC. *In vitro*, however, the skin cells require the APC to be recognized. Therefore, there are likely two pathways for recognition by the adult T cells in an MHC class II-restricted manner (52). Whatever the mechanism for recognition, these results suggest that a sufficient number of adult-type T cells differentiate during the course of metamorphosis, and become able to recognize larval tail cells. The effector T cells are suggested to be non-thymic (9). These results prompted us to propose that newly differentiating adult-type immune cells recognize and eliminate larval cells as “non-self” targets and led to the prediction that larva-specific antigens recognized by adult T cells may be expressed on the larval skin cells.

6.4. Isolation process and function of larval antigens recognized by the adult T cell

To investigate larval-specific antigens, an “alloantiserum” in the J strain of adults was produced by repeated grafts of syngeneic larval tail skin (53, 54). This strategy was successful because the antibody repertoire produced in the adult exclusively recognizes larval specific antigens. Using the alloantiserum, 59 kDa and 53 kDa proteins specifically expressed in the tadpole skin cells in a coordinated fashion during metamorphosis, have been isolated as candidate target antigens (51, 53). The isolated 59 kDa and 53 kDa proteins were sufficiently pure for

determining their constituent partial amino acid sequences (53). Synthetic peptides containing these amino acid sequences elicited specific rat antibodies to the same 59 kDa (51, 53) and 53 kDa larval tail molecules (unpublished data) as well as an *in vitro* T cell response of syngeneic adult frogs immunized with larval epidermis. The expression of the 59 kDa protein initially starts at the early metamorphic stages both in the trunk and the tail epidermis, then diminishes in the trunk, exhibiting a clear boundary between the tail and the trunk, and persists in the tail epidermis until the end of metamorphosis (51). This tail-specific expression of the protein can be explained in terms of a suppressive effect by thyroid hormone (51). As the hormone is present in higher concentration in the trunk than in the tail cells (49), the expression is retained only in the tail even at the metamorphic climax stage when the expression of those proteins are down regulated by the thyroid hormone. At the same period, T cell accumulation is observed only in the tail epidermis but not in the trunk regions (9). Thus far, these results are consistent with the involvement of 59 kDa and 53 kDa proteins in metamorphic tail regression as immune target antigens (50).

To further substantiate our immunological elimination hypothesis in ontogenetic tissue reorganization, the two genes encoding 59 kDa and 53 kDa proteins respectively were recently isolated in our laboratory, and farther analyses will address their functions. The possibility of innate immunity that cleans up larval cells in the tail tissue has been suggested (1). By contrast, our model propose that acquired immune response to specific

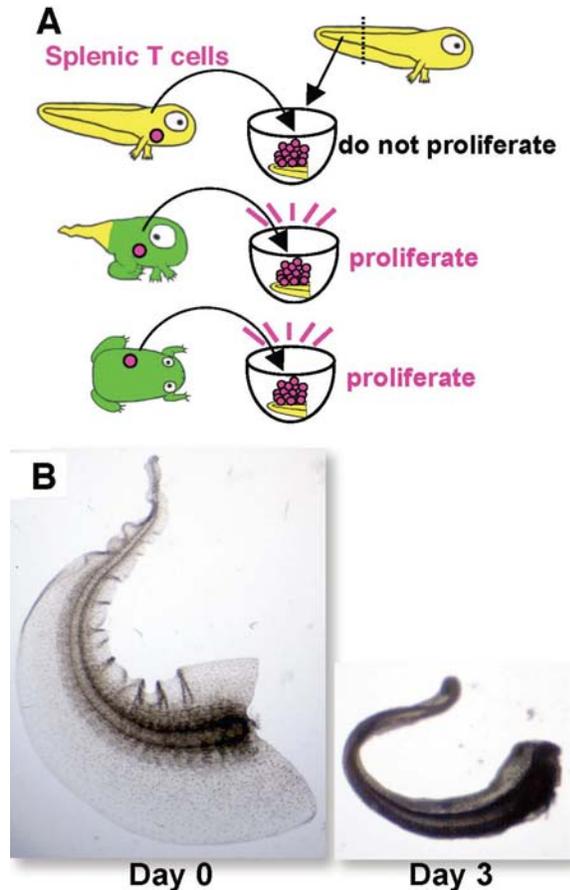


Figure 5. Larval tail skin cells are recognized and killed by the adult T cells. A, T cells from metamorphic climax tadpole (NF-stage 63-65) and adults (1-2 years-old) but not larvae (NF-stage 56/57) show *in vitro* proliferative responses against larval tails obtained from tadpoles at NF-stage 56/57. These analyses performed in 96-well U-bottom culture dishes. B, tail tip from a tadpole at NF-stage 56/57 is co-cultured with T cells obtained from adult spleens in the presence of *Xenopus* adult serum. An apoptotic destruction of the tail piece is observed after day 3 of culture.

antigen molecules selectively expressed at the metamorphic period is involved in swift elimination of larval tissues.

7. SUMMARY

The mechanism underlying amphibian metamorphosis was first considered in 1916 when it was discovered that removal of the thyroid from tadpoles inhibits their metamorphosis (55). It is currently held that amphibian metamorphosis is induced by the action of the thyroid hormone secreted from the thyroid in a cell-autonomous manner (49). Our data demonstrate that the physiological and functional replacement of the immune system is closely related to the tissue remodeling in *Xenopus*. Indeed, we propose that the immune system is an important contributor to the process of metamorphosis. These results demonstrate not only the role of the acquired

immune system for self/non-self recognition, but also how adult/larva recognition contributes to the tissue remodeling process. Whether the immune system participates more generally in tissue reorganization during vertebrate morphogenesis and to metamorphosis in urodele as well and anuran amphibians in particular, are questions worthy of future investigation.

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