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Original Article

Conversion of red blood cells (RBCs) from the larval to the adult type during metamorphosis in *Xenopus*: specific removal of mature larval-type RBCs by apoptosis

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ABSTRACT The conversion of hemoglobins (Hbs) and red blood cells (RBCs) from the larval to the adult type was monitored during normal metamorphosis in *Xenopus laevis,* and in artificially induced metamorphosis-arrested and precociously metamorphosed animals by means of SDS-PAGE, Hb immunohistochemistry, and double-staining with *in situ* DNA nick-end labeling (TUNEL) for detection of apoptosis and Hb immunostain. During normal metamorphosis, larval RBCs gradually decreased and, conversely, adult RBCs increased in number. However, in metamorphosis-arrested tadpoles, the larval-adult conversion of RBCs did not occur within 4 weeks, but did rather within 6 months after the controls metamorphosed. In order to identify possible mechanisms for the specific removal of larval RBCs from circulation in metamorphosing and metamorphosed animals, double-staining experiments with TUNEL and Hb immunostain were carried out. During metamorphic climax, many larval RBCs expressed TUNEL-positive reactions in the spleen, suggesting that the larval RBCs were specifically removed from the spleen during metamorphosis. When the larval RBCs were transferred to the circulatory system of histocompatible control adults, they survived for a long time, and no transferred RBCs showed TUNEL-positive reactions. In contrast, larval RBCs transferred to histocompatible adults that had been treated with T_3 were reduced in number in the circulatory system of the recipients. Double-staining experiments demonstrated that the transferred larval RBCs underwent apoptosis in the spleen and liver of the adult recipients treated with T_3, indicating that the mature larval-type RBCs were specifically removed from metamorphosing animals by apoptotic cell death under the influence of THs.

KEY WORDS: *Xenopus laevis, RBCs, apoptosis, metamorphosis, TUNEL.*

Introduction

Hemoglobin (Hb) switching during vertebrate development has long been one of the leading models for investigating the regulation of gene expression during animal development. The Hb transition may be physiologically important for inducing the change in oxygen affinity required for the adaptation from an embryonic or fetal environment to an atmospheric environment in mammals and birds, or from an aquatic environment to terrestrial life in amphibians. In amphibians, Hb switching from the larval to the adult type has been investigated with special interest in metamorphosis (Cardellini and Sala, 1979; Ducibella, 1974a,b; Hosbach *et al.*, 1982; MacLean and Jurd, 1972; Satoh and Wakahara, 1997, 1999; Yamaguchi and Wakahara, 1997; Yamaguchi *et al.*, 2000), which is a complete reconstruction of the body at the biochemical as well as the morphological level triggered by thyroid hormones (THs) (see Weber, 1967; Tata, 1996; Yoshizato, 1989, 1992).

Although the Hb transition has been demonstrated to occur within a single erythroid cell population in a salamander, *Hynobius retardatus* (Yamaguchi and Wakahara, 1997; Yamaguchi *et al.*, 2000), it involves replacement of the larval RBCs by adult RBCs in many other amphibians, such as *Rana pipiens* (Hollyfield,

Abbreviations used in this paper: DIG, digoxigenin; Hb, hemoglobin; HC, hydrocortisone; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; RBC, red blood cell; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; T_3, 3, 3', 5'-triiodo-L-thyronine; T_4, thyroxine; TH, thyroid hormone; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling; TU-SPC, thiourea-sodium perchlorate.

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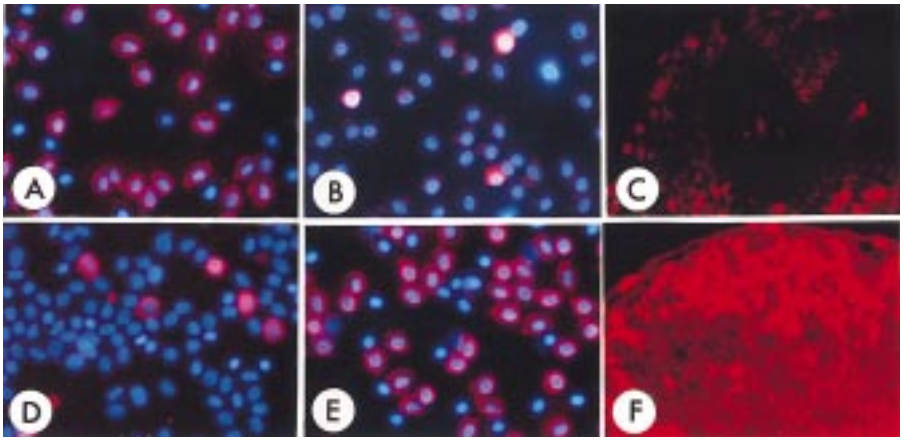

Fig. 1. Immunostaining of RBCs during metamorphosis in *Xenopus laevis*. *(A, B, C) RBCs stained with antibody to larval Hb. (D, E, F) RBCs stained with antibody to adult Hb. The secondary antibody was conjugated with Cy3, and thus each RBC emitted red fluorescence. Nuclei of the RBCs were stained with Hoechst (blue fluorescence).* **(A, D)** *RBCs of control larvae at stage 56 on a smear preparation. Almost all RBCs express the larval Hb.* **(B, E)** *RBCs of a control juvenile 2 weeks after metamorphosis on smear preparation. Almost all RBCs are stained with the adult antibody.* **(C, F)** *Histological sections of spleens from TU-SPC treated, metamorphosis-arrested larvae 4 weeks (C) and 6 months (F) after the controls metamorphosed. Almost all RBCs express the adult Hb, even those from TU-SPC treated larvae 6 months after the controls metamorphosed.*

1966), *Rana catesbeiana* (Dorn and Broyles, 1982; Just and Klaus-Just, 1996; Moss and Ingram, 1968), and *Xenopus laevis* (Weber *et al.*, 1989). In *Xenopus*, however, Jurd and MacLean (1970) reported that approximately 25% of the RBCs contained both adult and larval globins using larval- and adult-specific antibodies, suggesting that Hb switching occurs within a single RBC population. Whatever the modes of RBC transition, either replacement of old-(larval) type RBCs by new (adult) ones or Hb switching in a single RBC population, Hb switching itself is believed to occur during metamorphosis triggered by THs in many amphibian species (Hourdry, 1993). It is thus important to clarify how larval RBCs are specifically removed from circulation and how adult RBCs or erythroblasts proliferate during amphibian metamorphosis.

Recently, Hasebe *et al.* (1999) demonstrated by means of *in vitro* fluorescence labeling of RBCs and injection of the labeled cells into animals at various metamorphic stages that the larval RBCs were selectively removed from systemic circulation at the time of metamorphic climax in *Rana catesbeiana*. The labeled larval RBCs were ingested by hepatic and splenic macrophages, indicating that macrophages are involved in the splenic elimination of larval cells. In this respect, Nishikawa and Hayashi (1999) showed by means of double-staining experiments with *in situ* DNA nick-end labeling (TUNEL) and Hb immunostaining that larval erythroblasts decreased through the apoptotic process in *Xenopus laevis*. Their results indicate that the erythropoietic system is converted during metamorphosis effectively by two distinct hormonal mechanisms, T_3-hydrocortisone (HC) synergism on adult erythroblast proliferation and T_3-mediated programmed death of larval erythroblasts.

In this study, we investigated mechanisms of erythropoietic conversion from the larval to the adult type during normal metamorphosis of *Xenopus laevis* and in goitrogen-treated metamorphosis-arrested and TH-treated precociously metamorphosed animals by means of immunohistochemistry using larval- and adult-specific Hb antibodies and TUNEL. Furthermore, larval RBCs were transferred to the adult circulatory system of the *Xenopus* histocompatible strain J (Nakamura *et al.*, 1985, 1987), and their fate in adult recipients was monitored with or without T_3 treatment in order to identify possible mechanisms of RBC conversion during metamorphosis. From these observations, we propose an important role for apoptosis in the removal of mature larval-type RBCs from circulation during and after metamorphosis in *Xenopus laevis*.

Results

RBC changes during metamorphosis

Larval and adult RBCs were readily distinguished on smear preparations of RBCs by means of immunohistochemistry using larval- and adult-specific antibodies to Hbs (Fig. 1). During metamorphosis, some RBCs were stained with larval antibody, but others with adult antibody. Figure 2 shows the conversion over time of RBCs from the larval to the adult type during normal metamorphosis and in metamorphosis-arrested tadpoles of *Xenopus laevis*. In normal controls, only larval RBCs were observed in circulation before stage 52, a premetamorphic stage.

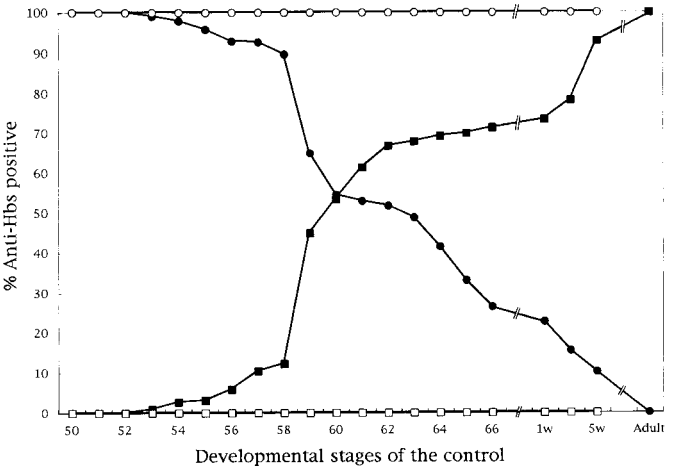

Fig. 2. Chronological changes in the proportion of larval and adult RBCs to total RBCs. *RBCs were collected from normal, control animals (closed symbols) before, during, and after metamorphosis and from TU-SPC treated metamorphosis-arrested larvae (open symbols) at the same intervals as the controls. RBCs were stained with antibodies to larval (circle) and adult (square) Hbs, and counterstained with Hoechst on smear preparations. Numbers of RBCs expressing either larval or adult antigens were counted and their ratios (%) to total RBCs were calculated. Larval RBCs gradually decrease in number during normal metamorphosis, and adult RBCs, conversely, increase. At stage 60, the onset of metamorphic climax, RBC conversion is at the halfway point. In contrast, no conversion of RBCs from the larval to the adult type occurred in the metamorphosis-arrested tadpoles within our experimental period (to 4 weeks after the controls metamorphosed).*

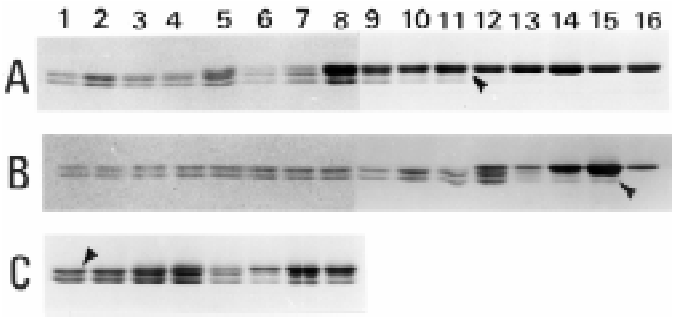

Fig. 3. Transition of Hbs from larval to adult types in *Xenopus laevis*. **(A)** *Hbs from normal controls,* **(B)** *TU-SPC treated metamorphosis-arrested tadpoles and* **(C)** *T_3-treated precociously metamorphosing animals were analyzed on SDS-PAGE at different stages. Lane numbers from 1 to 16 correspond to the time or stage of sampling for controls: 1, 14 days or st. 50; 2, 24 days or st. 55; 3, 32 days or st. 57; 4, 36 days or st. 59; 5, 40 days or st. 61; 6, 42 days or st. 63; 7, 44 days or st. 65; 8, 44 days or st. 66; 9, 1 week; 10, 2 weeks; 11, 3 weeks; 12, 5 weeks; 13, 2 months; 14, 3 months; 15, 5 months and 16, 6 months after metamorphosis. Typical larval Hb is separated into two relatively fast-migrating bands, and adult Hb shows a single, more slowly migrating band. Even at stage 66, the completion of metamorphosis, larval bands are still observed in the controls (lane 8 of* **A***). Two or three weeks after stage 66, the larval bands begin to disappear (arrowhead on lane 11 of* **A***). In TU-SPC-treated metamorphosis-arrested tadpoles, larval bands are still observed 5 months after the controls metamorphosed (arrowhead on lane 15 of* **B***). The Hb transition in the metamorphosis-arrested tadpoles was completed 6 months after the controls metamorphosed. In T_3-treated, precociously metamorphosing tadpoles, the adult-specific band appears much earlier than in the controls (arrowhead on lane 1 of* **C***).*

No adult RBCs at all were observed at this stage. The proportion of larval RBCs to total RBCs gradually decreased thereafter, while, conversely, the proportion of adult RBCs to total RBCs increased. At stage 60, the beginning of metamorphic climax, the proportion of both larval and adult RBCs to total RBCs became approximately 50%, showing that the conversion of RBCs from the larval to the adult type was at the half way point. Although adult RBCs became predominant during and after metamorphosis, the conversion was not complete even after the animals had completed morphological metamorphosis (stage 66). Three weeks after the completion of metamorphosis, approximately 10% of the RBCs still expressed larval Hbs. The conversion was completed 6 months after metamorphosis (data not shown). Because the sum of the percentages of larval and adult RBCs relative to total RBCs did not exceed 100% at any time during the period of RBC conversion, it was concluded that no RBCs expressed both larval and adult Hbs concurrently.

In contrast, larval to adult conversion of RBCs did not occur in metamorphosis-arrested tadpoles at all within 4 weeks after the controls had metamorphosed. All RBCs examined so far showed larval Hbs exclusively. In other words, no adult Hbs were expressed in RBCs in metamorphosis-arrested tadpoles, even after almost all RBCs in the control animals expressed adult Hbs. Adult RBCs, however, were detected in goitrogen-treated metamorphosis-arrested tadpoles 6 months after the controls metamorphosed (Fig. 1F), showing that the Hb/RBC conversion occurred even in metamorphosis-arrested tadpoles, although it took an extraordinarily long time.

Hb changes in metamorphosis-arrested and precociously metamorphosed animals

In order to obtain further information on RBC and Hb transitions from the larval to the adult type during the later developmental stages after metamorphosis and on the possible involvement of THs in the conversion, Hb changes were examined in normally metamorphosed controls, artificially induced metamorphosis-arrested and precociously metamorphosed *Xenopus* using SDS-PAGE, which conveniently, but with less sensitivity, allowed us to identify the Hb transition (Fig. 3). Larval and adult Hbs were readily distinguished by their different mobilities on the gel, which showed two bands of larval Hb migrating rather faster and a more slowly migrating adult band.

In normal controls, the Hb transition was almost completed 2 or 3 weeks after morphological metamorphosis (Fig. 3A). In contrast, larval bands were still observed in the metamorphosis-arrested tadpoles 5 months after the controls had metamorphosed. Six months after the controls had metamorphosed, the Hb transition from the larval to the adult type was completed in the metamorphosis-arrested tadpoles, suggesting that the conversion of the Hbs, and thus of the RBCs, occurred even in the metamorphosis-arrested tadpoles (Fig. 3B). In precociously metamorphosing and metamorphosed animals treated with T_3, the adult-specific band had already appeared only 14 days after fertilization, when the controls were at stage 50, much earlier than metamorphosis (Fig. 3C).

Apoptosis in larval RBCs

Apoptotic cell death in larval or adult RBCs was detected by means of double-staining with TUNEL and Hb immunostain (Fig. 4) in the spleen during normal metamorphosis in *Xenopus laevis*. Using different fluorescent dyes conjugated with secondary antibodies, Cy3 (red) for Hbs (Fig. 4A) and fluorescein (green) for DIG (Fig. 4B) antibodies, it was possible to identify the origins of the RBCs that underwent apoptosis. Double-stained RBCs, i.e., larval RBCs undergoing apoptotic cell death, emitted a bright orange fluorescence mixed with red and green (Fig. 4C). Figure 5 shows chronological changes in the proportions of RBCs of the larval and adult types and in the proportion of TUNEL-positive RBCs to total RBCs. As in the case of the conversion of RBCs in circulation, which were examined using smear preparations (Figs. 1, 2), the conversion was confirmed in RBCs in the spleen. The conversion of RBCs was at the half-way point at stage 59 in the spleen, a little earlier than in circulation. Even at the end of the metamorphosis (stage 66), 30% of the RBCs showed larval Hbs, basically identical to the case of RBCs in circulation. Although larval RBCs that also stained with TUNEL were not observed before stage 57, a prometamorphic stage, they began to be observed at and after stage 58. During stages 60 to 62, the climax stage of metamorphosis, 5% to 7% of larval RBCs showed TUNEL-positive staining, suggesting that a certain population of the larval RBCs underwent apoptosis. The proportion of double-stained larval RBCs gradually decreased thereafter until the end of metamorphosis. In contrast, no adult RBCs showing the TUNEL reaction were observed during any developmental stages examined so far, suggesting that adult RBCs were not subjected to apoptotic cell death during the conversion of RBCs.

Fate of transferred larval RBCs in adult recipients

The transfer of mature larval RBCs to the circulation of adults, using larvae and adults of the histocompatible J strain of *Xenopus*

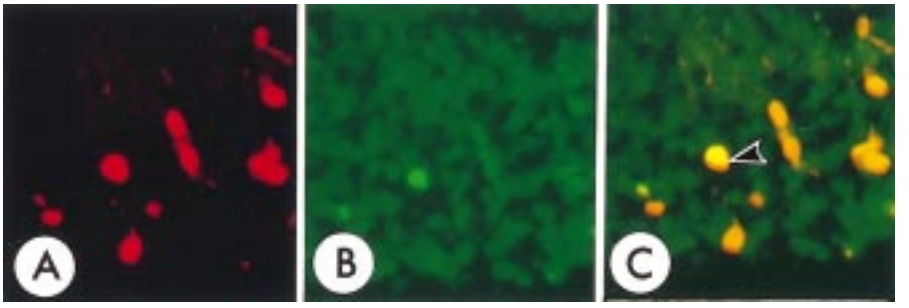

Fig. 4. RBCs double-stained with TUNEL and Hb immunohistochemistry. *A section of the spleen of a larva at metamorphic climax (st. 62) was stained with antibody to larval Hb conjugated with Cy3 (red) and with TUNEL (fluorescein, green). The section was observed with different excitation filters for* **(A)** *Cy3 (larval Hb),* **(B)** *for fluorescein (TUNEL), and* **(C)** *for Cy3 and fluorescein (double-stained with larval Hb and TUNEL). A double-positive RBC (i.e., larval RBC undergoing apoptosis) is clearly shown, emitting bright yellow fluorescence mixed with red and green (arrowhead).*

laevis (Nakamura *et al*., 1985), was performed to identify possible mechanisms of specific removal of larval RBCs during and after metamorphosis. The fate of transferred larval RBCs in the circulatory system of the adult recipients was monitored immunohistochemically using a larval-specific antibody. It was confirmed preliminarily that the transferred larval RBCs expressed larval Hbs exclusively, but that the adult RBCs did not, and that the original RBCs circulating in the adult recipients expressed adult Hbs exclusively (Fig. 6A). Thus the transferred RBCs were readily distinguished immunohistochemically from the RBCs of the recipients (Fig. 6B). RBCs expressing larval antigens were definitively detected in the recipients' circulation, even though the proportion was relatively low (approximately 0.5%).

Figure 7 shows chronological changes in the proportion of transferred larval RBCs that stained with larval-specific antibody to total RBCs on smear preparations. Although the proportion of the larval RBCs in circulation in the recipients was monitored every other day, the proportion did not change substantially during our experimental period (31 days after the transfer), suggesting that the transferred larval RBCs survived for a long time in recipients. In contrast, the transferred larval RBCs showed a different behavior in T_3-treated recipients from that exhibited in control recipients. When adult recipients were treated with a high concentration of T_3 ($1x10^{-7}$ M), the transferred larval RBCs drastically decreased in number as early as 3 days after the transfer and continued to decrease thereafter. In recipients treated with a lower concentration of T_3 ($1x10^{-9}$ M), relatively many larval RBCs were found 1 and 3 days after the transfer. They, however, gradually began to decrease 5 days after the transfer and continued to decrease as days passed. Furthermore, when adult recipients were treated with T_3 ($1x10^{-7}$ M) 21 days after the transfer (arrow in Fig. 7), the proportion of the transferred larval RBCs to total RBCs drastically decreased as early as 2 days after the T_3 treatment, suggesting again selective removal of the transferred larval RBCs from recipients under the influence of T_3.

In order to obtain further information with respect to mechanisms for the specific removal of larval RBCs in T_3-treated recipients, the spleen, liver, kidney, and heart of the recipients were examined by means of double-staining with TUNEL (fluorescein, green) and Hb immunostain (Cy3, red). Figure 8 shows double-positive RBCs with TUNEL and Hb antibody in the spleen and liver of T_3-treated recipients. A few RBCs emitting orange fluorescence (mixed with red and green) were encountered in the spleen (Fig. 8A) and liver (Fig. 8B) when stained with larval Hb antibody, whereas double-positive RBCs were not observed at all with adult Hb antibody (Fig. 8C). One day after the transfer, double-stained larval RBCs were not observed in either control or T_3-treated recipients. In all organs examined from the control recipients, double-stained larval RBCs were not observed during our experimental period (31 days after the injection). In recipients treated with $1x10^{-9}$ M T_3, double-stained larval RBCs were frequently observed in the spleen and liver 7 and 14 days after the injection. Even 21 days after injection, they were detected in the spleen and liver, although the proportion was relatively lower than previously. In the kidney and heart, however, double-stained, larval RBCs were not observed at all throughout our experimental period (data not shown). These results suggest that the spleen and liver are the major sites of the specific removal of larval RBCs during and after metamorphosis.

Discussion

Hormonal regulation in Hb transition

Adult Hb in *Xenopus laevis* was readily distinguished from larval Hb by their globin subunits (Fig. 3), as in many other amphibians (*Rana catesbeiana*, Moss and Ingram, 1968; *Bombina variegata*,

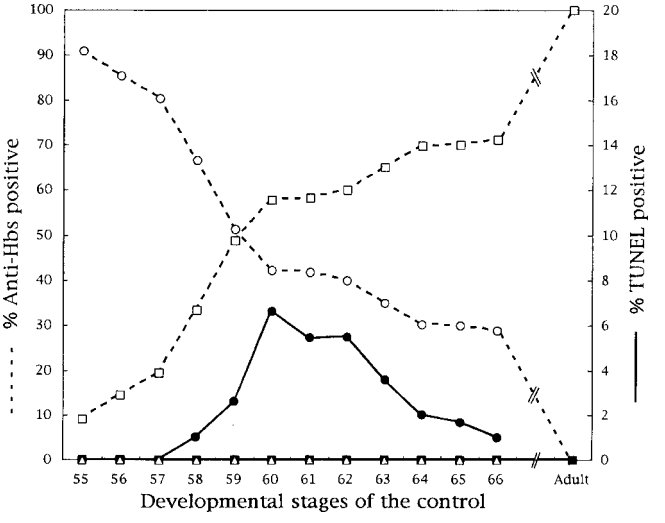

Fig. 5. Chronological changes in the proportion of larval and adult RBCs to total RBCs in the spleen and in TUNEL-positive RBCs. *The spleens were sectioned and double-stained with TUNEL and Hb for immunohistochemistry. Numbers of RBCs stained with antibodies (open symbols) to larval (circle) and adult (square) Hbs, and with TUNEL (closed symbols) were counted, and their ratios (%) to total RBCs were calculated. Double-positive RBCs (larval Hb-positive and TUNEL-positive, closed circle) were detected during and after the metamorphic climax, although adult Hb-positive and TUNEL-positive RBCs (closed square) were not detected at all throughout metamorphosis. Open triangles indicate the percentage of adult RBCs in TU-SPC treated metamorphosis-arrested larvae.*

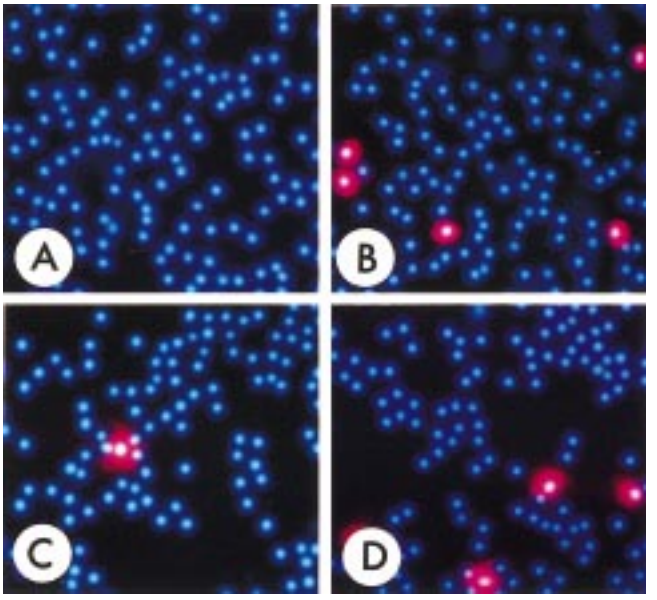

Fig. 6. Immunostains of transferred RBCs with larval Hb antibody. *Larval RBCs were transferred to adult recipients of the histocompatible J strain of Xenopus laevis. RBCs were collected from recipients and stained with larval Hb antibody (Cy3, red). Nuclei were counterstained with Hoechst (blue).* **(A)** *RBCs from a recipient that was not injected with larval RBCs. No RBCs are stained with the larval antibody.* **(B)** *RBCs from a recipient that was injected with larval RBCs and treated with T_3 for 1 day. A certain number of RBCs are stained with the larval antibody.* **(C)** *RBCs from a recipient that was injected with larval RBCs and treated with T_3 for 21 days, showing few cells stained with the larval antibody.* **(D)** *RBCs from a T_3-free recipient for 21 days, showing many RBCs stained with the larval antibody.*

Cardellini and Sala, 1979; *Ambystoma mexicanum*, Ducibella, 1974a; Maclean and Jurd, 1971; *Hynobius retardatus*, Arai and Wakahara, 1993; Yamaguchi and Wakahara, 1997). In almost all amphibian species examined so far, the Hb transition is triggered by THs, T_4 and/or T_3 (Hourdry, 1993). Even in the axolotl, the transition has been reported to occur, depending on a very low concentration of THs (Ducibella, 1974b; Jurd, 1985). Because the Hb transition was accelerated by administration of T_3 (Fig. 3), it must be true that the Hb transition is more or less dependent on THs in *Xenopus*. At present, however, this is a problem to be solved in the future. Adult Hb as well as adult RBCs appeared in TU-SPC-treated tadpoles in which morphological metamorphosis was completely arrested at stage 54, even though it took a long time (Figs. 1F and 3B). Similar observations have been reported in metamorphosis-arrested *Xenopus* treated with a different potent goitrogen, propylthiouracil (MacLean and Turner, 1976). In this respect, it is worthy to note that the Hb transition in *H. retardatus* is independent of thyroid activity (Satoh and Wakahara, 1997; Wakahara and Yamaguchi, 1996) and thus of THs (Arai and Wakahara, 1993; Wakahara *et al.*, 1994). It must be induced during metamorphosis by pituitary gland activity (Satoh and Wakahara, 1997, 1999), or by an unknown temporal regulation of genes encoding globin subunits, as is known to occur in chicken and mammalian globin genes (Choi and Engel, 1988; Engel, 1993). Precise examination of the down regulation of larval globin genes and the up regulation of adult globin genes by THs through TH receptors and other factors, such as pituitary-gland factors, using proper molecular probes in *Xenopus* is necessary for the elucidation of the role of hormones in the Hb transition in metamorphosis-arrested animals.

RBC transition during metamorphosis

There are two concepts that can explain the Hb/RBC transition from the larval to the adult type in amphibians: either the transition involves the replacement of the larval RBCs by adult RBCs (the "RBC replacement" model, postulated in *Rana*, Hollyfield, 1966; Dorn and Broyles, 1982; Just and Klaus-Just, 1996; in *Xenopus*, Weber *et al.*, 1989; 1991), or the Hb transition occurs within a single erythroid population, and thus no RBCs are replaced (the "Hb switching" model, postulated in *Hynobius*, Yamaguchi and Wakahara, 1997; Yamaguchi *et al.*, 2000). The present study shows convincingly that there are no RBCs expressing both larval and adult Hbs concurrently (Fig. 2), and that larval RBCs but not adult RBCs are specifically removed from circulation during metamorphosis (Fig. 5), which is triggered by THs, thus favoring the "RBC replacement" model in *Xenopus*.

In *Xenopus,* transplantation experiments demonstrate that hematopoiesis occurs in two waves: primitive RBCs that produce larval Hb exclusively are derived from the ventral blood island (VBI)(Ohinata and Enami, 1991), while definitive ones (expressing adult Hb) originate mainly from the dorsolateral plate (DLP)(Turpen *et al.*, 1997), although cells of the VBI contribute to adult erythro-

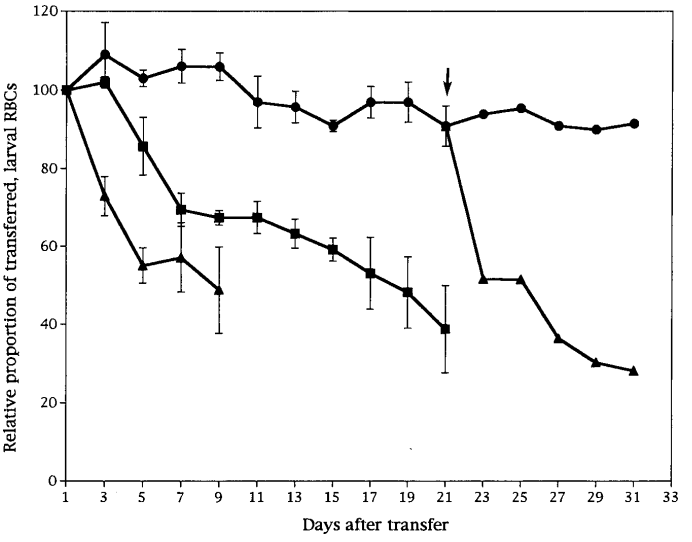

Fig. 7. Fates of transferred, larval RBCs in adult recipients of the histocompatible J strain of *Xenopus laevis*. *Larval RBCs were injected directly into the heart of either control or T_3-treated adults, and then RBCs were collected from recipients by cutting the toes every other day. The transferred larval RBCs were distinguished from adult RBCs of recipients immunohistochemically using larval Hb antibody on smear preparations. Numbers of larval RBCs among approximately 3000 RBCs from the recipients' circulation were counted every other day, and the proportion of larval to total RBCs was calculated. Because the recovery of larval RBCs differed in each recipient even at 1 day after injection (larval RBCs/total RBCs = 0.33% - 0.58%), the proportion was recalculated as the percentage of larval RBCs in recipients 1 day after the transfer became 100. Although the transferred larval RBCs survived for a long time in control adults, and thus did not decrease in number (closed circle), they gradually decreased in number in T_3-treated adults (closed triangle, 10^{-7} M T_3; closed square, 10^{-9} M T_3).*

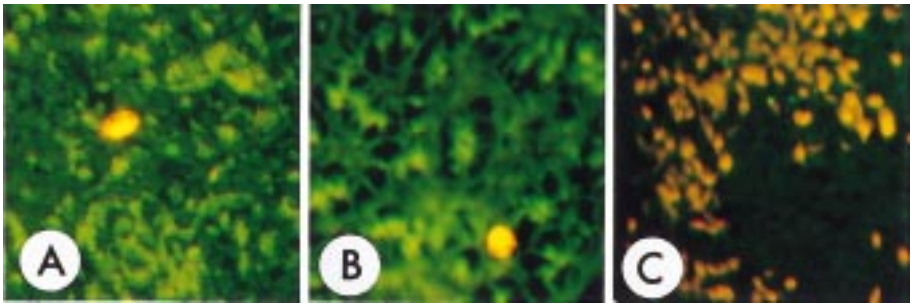

Fig. 8. Immunohistochemistry of transferred larval RBCs showing apoptosis in the spleen of recipients. *Larval RBCs were transferred to the hearts of adults. Sections of the spleen and liver of the recipients were double-stained with TUNEL (fluorescein, green) and Hb antibody (Cy3, orange). Double-positive RBCs emit bright, yellow fluorescence mixed with green and orange.* **(A)** *Section of the spleen of a recipient treated with T_3 for 14 days, showing an RBC double-stained with TUNEL and larval antibody.* **(B)** *Section of the liver of the same recipient as (A), showing a double-stained RBC.* **(C)** *Similar section of the spleen of (A) double-stained with TUNEL and adult antibody. No adult RBCs are stained with TUNEL.*

poiesis to some extent (Maeno *et al*., 1985a,b; Rollins-Smith and Blair, 1990). A switch from larval to adult globin gene expression in *Xenopus* has been reported to be mediated by erythroid cells from two distinct compartments (Weber *et al*., 1991). The different responses of larval and adult RBCs to THs, apoptotic cell death in larval but not in adult RBCs, might reflect the different origins of these two RBC populations. Selective removal of larval RBCs and the proliferation of adult RBCs might be regulated under certain influences of THs as described below.

RBC transfer in Xenopus *J-strain*

In order to examine the fate of transferred cells in the recipients, reliable markers or signals to identify or distinguish them from the recipients' cells are absolutely necessary. When cells are chemically labeled there is always a risk that the labeled cells will be damaged by the coupling reaction, resulting in a reduced life span, or that the recognition of labeled larval cells by recipients will be altered by possible modification of the surface configuration. In this respect, the use of biological markers such as larval-specific antigens is promising. From this point of view, transfer experiments have been done using donor larval RBCs and adults as recipients in *Rana catesbeiana* (Hasebe *et al*., 1999). Unfortunately, because the transfer experiments in *R. catesbeiana* were done between wild animals purchased from dealers, the transferred, larval RBCs were non-self to the recipients, and thus were eliminated immunologically from recipients. Therefore, the splenic and hepatic elimination of larval RBCs by macrophages reported in *Rana* might be attributed, at least in part, to an immunological rejection. In this respect, the transfer of RBCs between tadpoles and adults of the histocompatible J strain of *Xenopus laevis*, which has been produced at Hokkaido University (Nakamura *et al*., 1985, 1987), can eliminate all these problems. The larval RBCs transferred to adults in this study can survive for a long time without immunological rejection, although Izutsu and Yoshizato (1993) reported that larval skins from the histocompatible J strain were immunologically rejected when transplanted to adults of the same strain. This might imply that larval RBCs do not express stage-specific surface antigens recognized by the adult immune system, which are known to be present in larval skin cells (Izutsu and Yoshizato, 1993; Izutsu *et al*., 1996). Selective removal of larval RBCs from circulation in metamorphosing and metamorphosed animals can thus be examined more simply by using the histocompatible J strain of *Xenopus* than by using wild animals, without the necessity of considering factors such as immunological rejection of transferred cells.

Selective removal of larval RBCs

This study is the first to demonstrate that transferred larval RBCs cannot survive in T_3-treated adults, while they can survive in control adults (Fig. 7). This result suggests that the mature larval-type RBCs are selectively removed from circulation under the influences of THs. The TUNEL method has been employed to detect apoptosis in larval cells that must be removed during amphibian metamorphosis (Nishikawa and Hayashi, 1995, 1999; Ohmura and Wakahara, 1998; Hasebe *et al*., 1999). Double-staining experiments with TUNEL and Hb immunostain in this study convincingly demonstrate that mature larval-type RBCs are subjected to apoptosis in the spleen and liver of the recipients under the influence of THs (Figs. 7 and 8). It is thus assumed that the larval RBCs are specifically removed from circulation by apoptotic cell death during the metamorphic climax. Nishikawa and Hayashi (1999) have shown that larval-type erythroblasts are subjected to apoptosis in the liver, suggesting that the larval-adult conversion of RBCs is conducted by T_3-mediated programmed death of larval precursor cells. In this model, however, it is difficult to explain how the mature larval-type RBCs circulating in metamorphosing tadpoles are selectively removed from circulation. Because selective apoptosis of mature larval-type RBCs in the spleen was demonstrated *in vivo* at the metamorphic climax (Fig. 5) and in adult recipients treated with T_3 in RBC-transfer experiments (Figs. 7 and 8), the larval-adult conversion of RBCs in *Xenopus* is conducted by a mechanism similar to that known to be used in the general transformation in anuran metamorphosis: the selective removal of many kinds of mature larval-specific cells (Yoshizato, 1992).

In the goitrogen-treated metamorphosis-arrested larvae of *Xenopus*, selective removal of larval RBCs is not expected to occur, because there is not enough TH to elicit morphological metamorphosis as well as apoptosis in RBCs. In spite of this, larval Hbs as well as RBCs finally disappeared even in the metamorphosis-arrested tadpoles, although it took as long as 6 months (Figs. 1F and 3B). It is thus assumed that larval RBCs in the metamorphosis-arrested tadpoles disappeared through mechanisms other than their active removal. Probably, larval RBCs in the metamorphosis-arrested tadpoles passively disappeared by the exhaustion of their source. This assumption is consistent with previous observations that the primitive erythropoiesis that is the source of larval RBCs has a limited potentiality to differentiate and proliferate (Flores and Frieden, 1972; Widmer *et al*., 1983). The appearance of adult RBCs in metamorphosis-arrested larvae, even though it takes a long time, suggests at least that there is a certain process inde-

pendent of THs in RBC conversion in *Xenopus laevis* (Jurd and MacLean, 1970). Although it must be true that during normal metamorphosis in *Xenopus* that specific removal of mature larval-type RBCs takes place by apoptotic cell death under the influence of T_3 (this study), and that specific proliferation of adult erythroblasts is elicited by T_3-hydrocortisone (HC) synergism (Nishikawa and Hayashi, 1999), the possible contribution of TH-independent events to RBC conversion, e.g., chronological age or size, or some other independent factor such as a pituitary factor (Satoh and Wakahara, 1999), should be kept in mind for a comprehensive clarification of the mechanisms regulating conversion.

Materials and Methods

Animals

The MHC-homozygous inbred strain J of *Xenopus laevis* (MHC haplotype, *jlj*), which was produced and characterized by Nakamura *et al.* (1985), was used. Fertilized eggs were obtained after ovulation, and mating was induced by gonadotropic hormones (Gonatropin, Teikokuzoki, Tokyo, Japan). Tadpoles were fed creamed beans and froglets were fed commercial fish meal (Oriental Kobo, Tokyo, Japan). Tadpoles were staged as described by Nieuwkoop and Faber (1956).

Artificial manipulation of metamorphosis

Tadpoles were reared in a goitrogenic solution (0.2% thiourea and 0.4% sodium perchlorate, TU-SPC) to arrest their metamorphosis. The goitrogen-treated tadpoles grew without any sign of morphological metamorphosis, but arrested at stage 54, even after the controls metamorphosed. Some tadpoles were treated with 1×10^{-9} M 3, 3', 5'-triiodo-L-thyronine (T_3), to induce precocious metamorphosis.

Electrophoresis

Four to six animals from each collection were anesthetized in an aqueous solution of MS222 (Sandoz), and then they were bled in ice-chilled 50% PBS with 10 mM EDTA, either by puncturing the heart or by cutting the tail. Procedures for preparation of hemolysates were previously described (Arai and Wakahara, 1993; Wakahara *et al.*, 1994). After the amount of protein in each hemolysate was determined using BCA Protein Assay reagent (Pierce Chem Co.), each sample from individual animals was electrophoresed or frozen at -80°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970), using 15% separating gels. All electrophoresed gels were stained with Coomassie Brilliant Blue and then photographed.

Transfer of larval RBCs to adult circulation

Larval RBCs were collected from premetamorphic tadpoles (stage 54). They were washed with amphibian phosphate-buffered saline (APBS, 70% strength of mammalian PBS, pH 7.2) twice, and then lightly centrifuged (300 g) for 5 min. RBCs from 15 tadpoles were pelleted and suspended in 200 µl of APBS, and then transferred into the circulation of the recipient adults (6 months old, approximately 5 g in body weight) via a direct injection into the heart. The injected adults were treated with or without T_3 (1×10^{-7} M and 1×10^{-9} M). Small portions of RBCs were collected from the injected adults every day by cutting the tips of the toes to monitor the fate of the transferred, larval RBCs in the recipients. Smear preparations of the RBCs were double-stained with TUNEL and Hb immunostain as described below. The spleen, liver, kidney, and heart from the recipients were fixed with 4% paraformaldehyde in APBS and processed for histological sections. They were also double-stained with TUNEL and Hb immunostain.

Immunohistochemistry for Hb staining

RBCs on smear preparations were fixed immediately with 100% methanol for 3 min and then washed in APBS 3 times. After blocking for 2 h with 10% FSC-PBS at 37°C, these smears were treated with larval Hb antibody (L4.27) or adult Hb antibody (A7.2) for 2 h at 37°C. After washing in APBS 3 times, Cy3-conjugated goat anti-mouse IgG (1:1000, in dilution)(Chemicon International Inc.) was applied to the preparations as a secondary antibody, and then incubated for 2 h at 37°C. These were finally washed with APBS 3 times and stained with Hoechst 33342 for nuclear localization, mounted in glycerol, and observed under a fluorescence microscope. Organs from each animal were fixed with 4% paraformaldehyde in APBS, dehydrated, and embedded in Tissue-Prep (Fischer, Fair Lawn). Histological sections 5-µm thick were immunostained for smear preparations as described above.

Double-staining with TUNEL method and Hb immunostain

Apoptotic cell death in RBCs was detected by means of the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end-labeling (TUNEL) method, which distinguishes apoptosis from necrosis by specifically detecting DNA cleavage and chromatin condensation associated with apoptosis (Gavrieli *et al.*, 1992), using an "*In Situ* Apoptosis Detection Kit" (Apop Tag Plus Fluorescein, S7111-KIT, Oncor) according to the manufacturer's instructions. Briefly, smear preparations or deparaffinized sections were pretreated with 5 µg/ml Proteinase K (Boehringer-Mannheim) for 20 min at room temperature, washed in APBS 2 times, and treated with an "equilibration buffer" for 30 min and a "working strength TdT enzyme" (70% reaction buffer, 30% TdT enzyme) for 1 h at 37°C. After incubation, the preparations were treated with a "working strength stop/wash buffer" (1 ml stop/wash buffer, 34 ml distilled H_2O) for 10 min at room temperature and washed in PBS 3 times. For indirect fluorescent staining, the preparations were treated with "anti-digoxigenin conjugate" (53% blocking solution, 47% anti-digoxigenin conjugate) for 30 min at 37°C, washed in PBS 4 times, and then immunostained with Hb antibodies in the same way as described above. For positive controls, the sections were treated with DNase I (1 mg/ml, Boehringer-Mannheim) for 10 min at room temperature.

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