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Original Article

Hemoglobin transition from larval to adult types occurs within a single erythroid cell population during metamorphosis of the salamander *Hynobius retardatus*

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ABSTRACT In amphibians transitions of hemoglobins (Hbs) and red blood cells (RBCs) from larval to adult types have been reported to occur at metamorphosis. The transition of Hbs in the salamander *Hynobius retardatus* also occurs during metamorphosis, but almost independently of thyroid activity. Changes in several properties of RBCs, including their morphology, buoyant density and Hb phenotypes, were analyzed during the normal development of *Hynobius retardatus*. Typical larval RBCs were distinguished from typical adult ones by their different morphology and different buoyant density, while RBCs from metamorphosing animals had a single buoyant density and thus could not be separated into two populations on a Percoll density gradient. When RBCs from metamorphosing animals were examined immunohistochemically using larval or adult globin-specific antibodies, all the RBCs from any developmental stages from early larvae (36 days after hatching) to metamorphosed juveniles contained varying quantities of both antigens recognized by these antibodies. Immunohistochemical observation also demonstrated that the erythropoietic organs were the liver and spleen at early larval stages, but limited to the spleen in metamorphosing larvae and metamorphosed adults. These findings support the idea that the Hb switching in *Hynobius retardatus* occurs in a single RBC population, rather than the concept that larval RBCs are replaced by new, adult RBCs, as is known to occur in many amphibians.

KEY WORDS: *RBCs, hemoglobins, hematopoiesis, metamorphosis, Hynobius retardatus*

Introduction

Hemoglobin (Hb) switching has long been one of the leading models for investigating the regulation of gene expression during animal development. In most species of vertebrates Hb genes are organized in clusters in which different globin sequences are closely spaced. The expression of these genes is typically regulated both at a tissue-specific and at a stage-specific level (see Gilbert, 1994). In amphibians, Hb switching from larval to adult types has been investigated with special interest in metamorphosis (MacLean and Jurd, 1972; Ducibella, 1974a,b; Cardellini and Sala, 1979; Hosbach *et al.*, 1982), which is a complete reconstruction of the body at the biochemical as well as the morphological level triggered by thyroid hormones (Weber, 1967; Yoshizato, 1989, 1992).

In *Xenopus laevis*, a switch in Hb synthesis occurs at metamorphosis resulting from the replacement of the larval globin subunits by a set of distinct adult ones (Hosbach *et al.*, 1982; Sadmeyer *et al.*, 1988). The transition of Hbs during metamorphosis in *Xenopus* has been reported to involve replacement of the larval RBCs by adult RBCs (Weber *et al.*, 1989). Similar replacement of the larval RBCs by adult ones has been reported in *Rana pipiens* (Hollyfield, 1966) and *Rana catesbeiana* (Moss and Ingram, 1968; Dorn and Broyles, 1982; Just and Klaus-Just, 1996). 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 the 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 the metamorphosis triggered by thyroid hormones in many amphibian species (Hourdry, 1993).

The transition of globin subunits from larval to adult types in *Hynobius retardatus*, which has been reported to show neotenic reproduction in a specific environment of Lake Kuttara in Hokkaido, Japan (Sasaki, 1924; Sasaki and Nakamura, 1937) but whose neotenic population in Lake Kuttara is believed to be extinct at present, is somewhat different from the other amphibians: the Hb transition occurs in almost the same time schedule in normally

Abbreviations used in this paper: Hb, hemoglobin; RBC, red blood cell.

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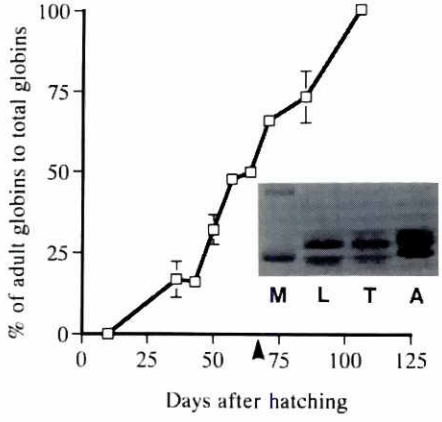

Fig. 1. Transitions of globin subunits from larval to adult types during normal development of *Hynobius retardatus*. *At 10 days after hatching, only larval globins are detected, and thus the ratio of adult globins to total globins is zero. Adult globins appear as early as 36 days after hatching, and their proportion to total globins rapidly increases thereafter. The transition is completed 100 days after hatching. An arrowhead indicates the time of the completion of metamorphosis according to external morphology. Inset: electrophoretic patterns of globin subunits on SDS-PAGE.* **(M)** *Molecular markers (upper, 20 kDa; lower, 14 kDa);* **(L)** *typical larval globins;* **(T)** *globins in transition;* **(A)** *typical adult globins.*

metamorphosing animals, in metamorphosis-arrested (goitrogen-treated) larvae (Arai and Wakahara, 1993; Wakahara and Yamaguchi, 1996), and in precociously metamorphosed animals induced by exogenously applied thyroxine (Wakahara *et al.*, 1994). Furthermore, the Hb transition is extraordinarily retarded in metamorphosis-arrested larvae whose pituitary glands have been surgically removed, whereas the transition in thyroidectomized larvae occurs in the same time schedule as normally metamorphosed controls (Satoh and Wakahara, 1997). These observations suggest that Hb switching depends on the activity of pituitary gland, but not on that of the thyroid gland in this species. This is a unique phenomenon among amphibian species in which the Hb transition has been analyzed so far. In this respect, it would be of great interest to know whether the changeover from larval to adult Hb occurs within individual RBCs, or whether separate populations of RBCs exist, each exclusively containing one or the other of the two types of Hb. To investigate this problem we prepared antibodies against *Hynobius* larval and adult Hbs and used them to determine which type of Hb was present within each RBC in normally metamorphosing and metamorphosed animals of *Hynobius retardatus*.

Results

Hb changes during the metamorphosis

Larval and adult globin subunits were easily distinguished on SDS-PAGE by their different mobilities (Fig. 1, inset). Figure 1 shows chronological changes in globin subunits from larval to adult types during normal development of *Hynobius retardatus*. At 10 days after hatching, only larval globins were detected, and thus the proportion of adult to total globins was zero. Adult globins appeared as early as 36 days after hatching. Their proportion to total globins increased rapidly thereafter, and the transition was completed approximately 100 days after hatching, a little later than morphological metamorphosis (see Fig. 5 in Arai and Wakahara, 1993).

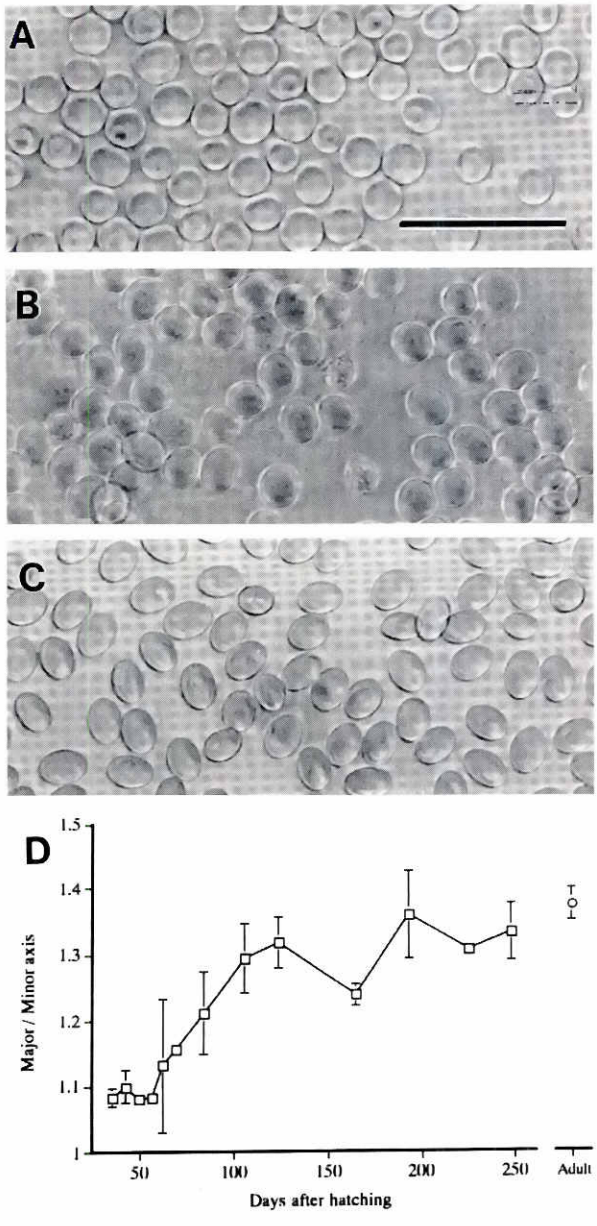

Fig. 2. Changes in morphology of RBCs in 30% PBS during normal development of *Hynobius retardatus*. **(A)** *Typical larval RBCs showing a round or spherical shape.* **(B)** *RBCs from a metamorphosed juvenile (89 days after hatching), showing an "intermediate" morphology between larval and adult RBCs.* **(C)** *Typical adult RBCs showing an elliptical or oval shape.* **(D)** *Chronological changes in the morphology of RBCs from larval to the adult types. Bar, 100 μm.*

Morphological changes in RBCs

Larval and adult RBCs showed different morphology in 30% PBS; the former was characterized by a round or spherical shape (Fig. 2A), while the latter by an oval or elliptical shape (Fig. 2C). Approximate major and minor axes of the round, larval RBCs were

24.4 μm and 21.1 μm, whereas those of the elliptical, adult RBCs were 28.3 μm and 21.0 μm, respectively. During the transition, RBCs showed an "intermediate" form between typical larval and adult RBCs (Fig. 2B), indicating that they could not be separated into two distinctive (larval and adult) RBC populations during their transition. The ratio of major to minor axes of typical larval RBCs was approximately 1.1, showing the almost spherical shape. That of adult RBCs was 1.35, showing their elliptical shape. Figure 2D shows chronological changes in the morphology of RBCs during normal development. After metamorphosis (approximately 70 days after hatching), the ratio of the major to minor axes of RBCs gradually increased to the level of adult RBCs.

In ACG (0.08% citric acid, 2.2% trisodium citrate dihydrate, and 2.4% glucose) solution, a hypertonic solution for larval cells in urodeles (Ducibella, 1974b), typical larval RBCs were strikingly shrunk by dehydration (Fig. 3A). In contrast, adult RBCs exhibited a rather smooth, elliptical shape (Fig. 3C), suggesting that RBCs could be classified into two groups according to their osmotic properties. During the transition, RBCs of "intermediate" form, which partially shrank, were also found (Fig. 3B). In typical adults, however, no "intermediate" forms were found. Figure 3D shows chronological changes in the osmotic properties of RBCs during normal development. Between 70 and 150 days after hatching, the proportion of smooth, elliptical RBCs in ACG solution increased markedly from 0% to approximately 75%. At the end of this experiment (250 days after hatching), about 25% of RBCs were still of larval form. Because more than 95% of RBCs showed the smooth, elliptical shape in typical adults, the transition of osmotic properties in RBCs was not completed even at the end of this experiment, and thus did not occur simultaneously with the Hb switching, which was completed at 100 days after hatching (Fig. 1). This is in contrast with observations reported in *Ambystoma mexicanum* (Ducibella, 1974b).

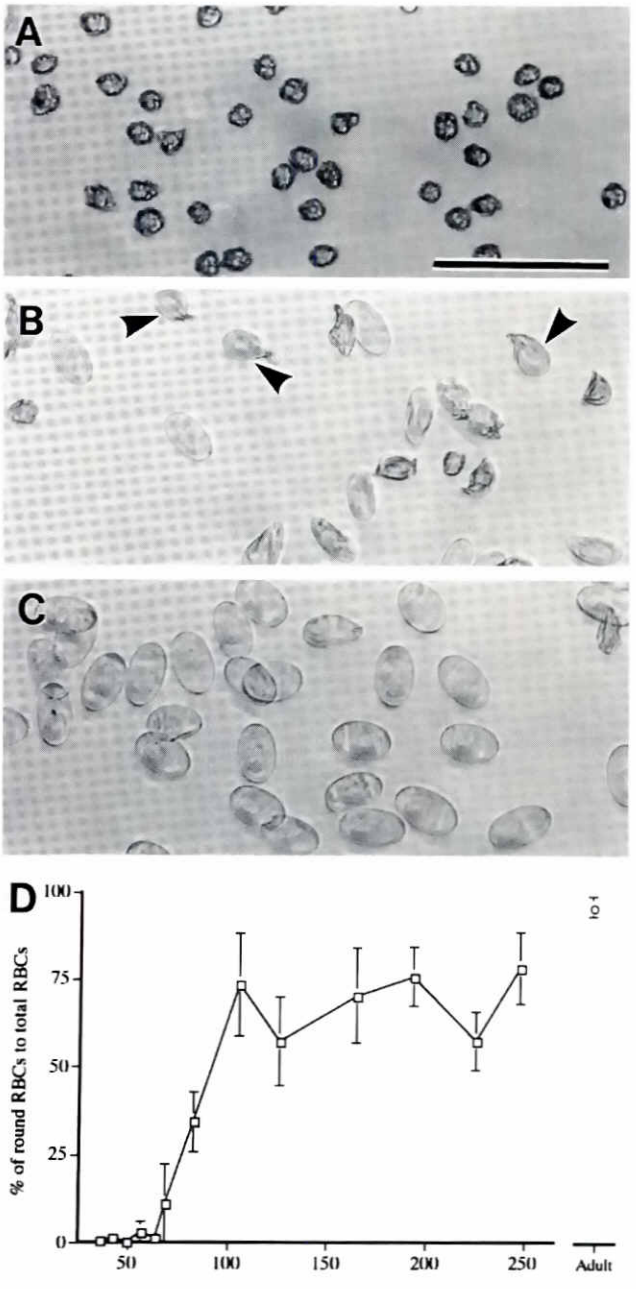

Fig. 3. Changes in osmotic properties of RBCs in ACG solution (a hypertonic solution for larval cells). (A) *Completely shrunken by dehydration, typical larval RBCs.* **(B)** *RBCs of "intermediate" form partially shrunken (arrowheads), encountered during the transition.* **(C)** *Typical adult RBCs which show an oval or elliptical shape with smooth cell surface.* **(D)** *Chronological changes in the osmotic properties of RBCs from larval to adult types. Bar, 100 μm.*

Changes in buoyant density of RBCs

Typical larval RBCs had a lower density (approximately 1.051 g/ml) (Fig. 4A) than typical adult RBCs (approximately 1.068 g/ml) (Fig. 4C). When RBCs from normally metamorphosing larvae (60 days after hatching, and thus containing both the larval and adult globin subunits, see Fig. 1) were analyzed on Percoll density gradients, they were not separated into two distinctive populations, but showed a single band of an intermediate density (1.060 mg/ml) between the larval and adult densities (Fig. 4B). Chronological changes in the buoyant density of RBCs during normal development are shown in Figure 4D. Fifty days after hatching, the density of RBCs began to increase and reached nearly 1.066 g/ml at 70 days. It remained at this level until 230 days after hatching, when it reached an adult level, 1.068 g/ml. During the transition, each RBC fraction showed a uniform density and could not be separated into two populations, a result completely different from results reported in *Rana catesbeiana* (Dorn and Broyles, 1982).

Specificity of antibodies

In order to test the specificity of the antibodies obtained in this study, three types of hemolysates were employed for immunoblots; typical larval (from 3 to 10 days after hatching), typical adult (from sexually mature male), and larval-adult transition (from metamorphosing larvae). Figure 5 shows typical Western blots stained with the polyclonal antibody to larval-specific Hb (Pab-HbL) and the monoclonal antibody to adult-specific Hb (Mab-HbA). The larval Hb was recognized only by the Pab-HbL, but not by the Mab-HbA (Fig. 5, lanes 1 and 4). On the other hand, the adult Hb was recognized only by the Mab-HbA, but not by the Pab-HbL (Fig. 5, lanes 2 and 5). Hbs from metamorphosing animals (larval-adult transition), whose RBCs contained both larval and adult types of

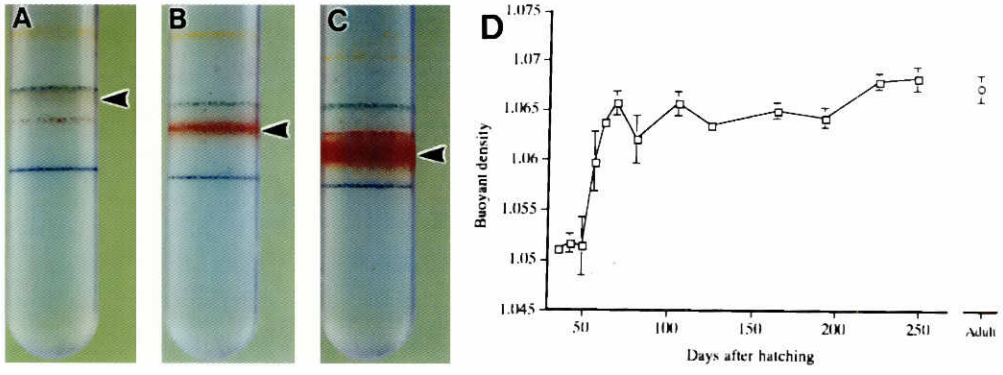

Fig. 4. Changes in a buoyant density of RBCs on Percoll gradient density with color density markers. **(A)** *Typical larval RBCs showing a density of 1.051 gm/ml.* **(B)** *RBCs from metamorphosing larvae (60 days after hatching) showing a density of 1.060 gm/ml.* **(C)** *Typical adult RBCs showing a density of 1.068 gm/ml.* **(D)** *Chronological changes in the buoyant density of RBCs during normal development of Hynobius retardatus. During the transition in density of RBCs, each RBC fraction cannot be separated into two distinctive populations, but shows a single density. Arrowheads indicate the position of RBCs. Color density marker: yellow, 1.035; green, 1.051; red, 1.060; blue, 1.074 gm/ml.*

Hbs, were recognized by both the Pab-HbL and Mab-HbA (Fig. 5, lanes 3 and 6). From the estimated molecular weights, the components recognized by these antibodies were assumed to be larval and adult globin subunits (Arai and Wakahara, 1993).

The specificity of these antibodies was also confirmed on smear preparations of larval and adult RBCs. Figure 6 shows immunohistochemistry of typical larval and typical adult RBCs, using the Pab-HbL and Mab-HbA, respectively, and the corresponding phase-contrast micrography. The Pab-HbL stained only the larval RBCs, but not the adult RBCs (Fig. 6A,C). In contrast, the Mab-HbA stained only the adult RBCs, but not the larval RBCs (Fig. 6B,D).

Coexistence of larval and adult Hbs in individual RBCs

Figure 7 shows immunohistochemistry of RBCs during normal development of *H. retardatus*, using the Pab-HbL and Mab-HbA. All RBCs from any developmental stages from early larval (36 days after hatching, Fig. 7A,B) to metamorphosing (50 days, Fig. 7C,D; 64 days, Fig. 7E,F) stages contained both antigens recognized by the Pab-HbL and Mab-HbA. At 89 days after hatching (post-metamorphic juvenile), RBCs stained strongly with the Mab-HbA, but more weakly with the Pab-HbL compared to RBCs from younger larvae (Fig. 7G,H). At 105 days after hatching, the RBCs were stained only with the Mab-HbA, but not with the Pab-HbL (Fig. 7I,J).

Erythropoietic organs

In order to determine possible erythropoietic organs in larvae and adults, several organs were examined histologically and immunohistochemically. Erythroid cells whose cytoplasm was stained weakly with eosin were frequently observed in the spleen (Fig. 8B). Mitotic figures of these cells were also often encountered. In accordance with morphological criteria, they were assumed to be RBC-precursor cells (Grasso, 1973a). When two successive sections were immunostained with the Pab-HbL and the Mab-HbA at 10 days after hatching, many of the erythroid cells in the spleen were stained with the larval Hb-specific (Pab-HbL) antibody (Fig. 8A), but not with the adult Hb-specific (Mab-HbA) antibody (data not shown). At 36 days after hatching all RBCs and/or RBC-precursor cells in the spleen were stained with both antibodies (Fig. 8C,D). RBCs and RBC-precursor cells from the same region on two consecutive sections were stained with both antibodies. RBCs from adult spleens were stained only with the Mab-HbA (Fig. 8E), but not with the Pab-HbL (data not shown).

In contrast, the liver behaved differently from the spleen as an erythropoietic organ. At 10 days after hatching, the liver had many RBCs and/or RBC-precursor cells which were recognized with the Pab-HbL (Fig. 8F). The immunoreactive RBCs and/or RBC-precursor cells were nested among larval hepatocytes. At 36 days after hatching, however, the RBC-precursor cells were hardly observed in the liver (Fig. 8G). A few RBCs in capillaries were stained with the Mab-HbA, but no nested RBC-precursor cells were observed at all. Similar results were obtained from the livers of post-metamorphosed juveniles and adults (data not shown). In kidney from early developmental phases to post-metamorphic stages, RBC-precursor cells were observed much less than in liver, suggesting that this organ did not function as an erythropoietic organ at any stage.

Discussion

Hb switching in Hynobius retardatus

The results described in this study convincingly demonstrated that RBCs from early larvae to metamorphosed juveniles in *Hynobius retardatus* expressed, more or less, concurrently both larval and adult phenotypes of Hb (Figs. 7, 8). Furthermore, the RBCs from metamorphosing animals could not be separated into two distinctive populations, but showed a single density intermediate between typical larval and adult densities on a Percoll density

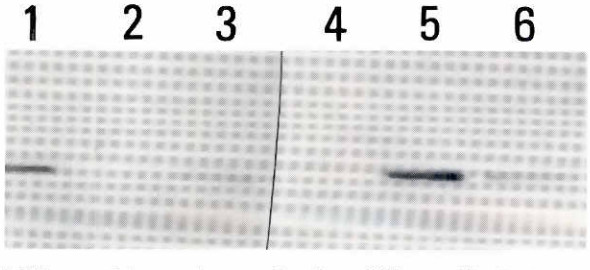

Fig. 5. Western blot analyses using larval Hb-specific (Pab-HbL) and adult Hb-specific (Mab-HbA) antibodies. *Hemolysates from typical larval-* **(lanes 1,4)** *and typical adult-RBCs* **(lanes 2,5)**, *and RBCs from metamorphosing larvae (larval-adult transition)* **(lanes 3,6)** *were electrophoresed and blotted. The Pab-HbL recognizes specifically larval globins (lane 1), but not adult ones (lane 2). The Mab-HbA recognizes specifically adult globins (lane 5), but not larval ones (lane 4). Larval and adult globins from metamorphosing larvae were recognized by both the Pab-HbL and Mab-HbA (lanes 3 and 6).*

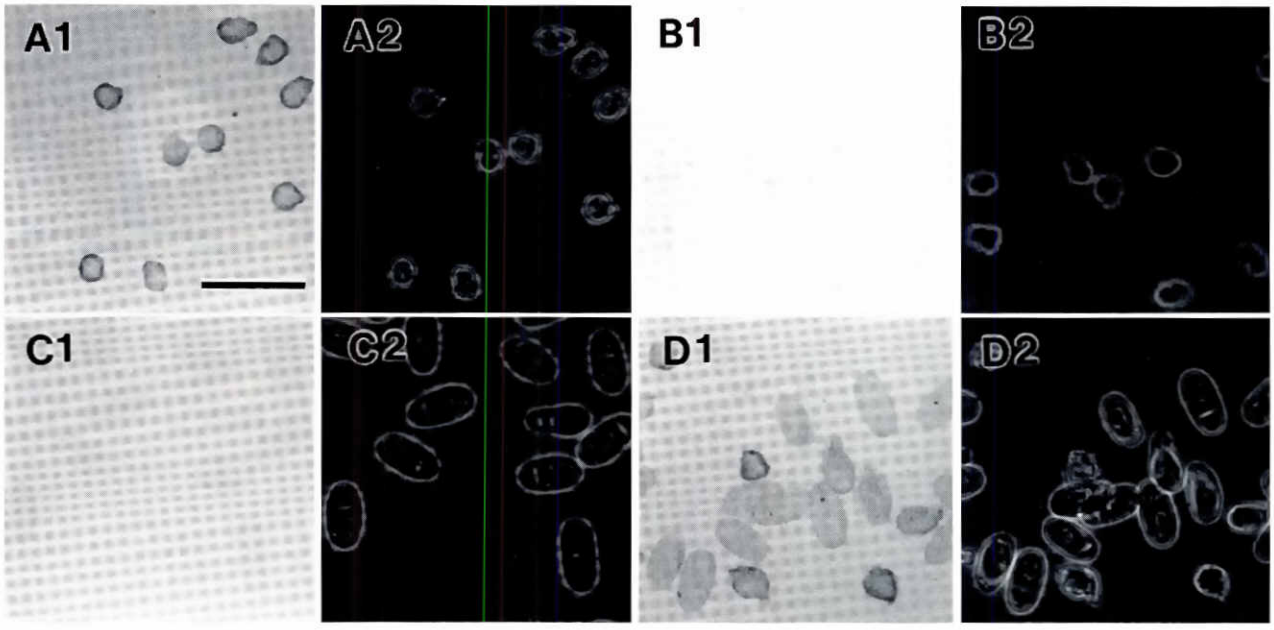

Fig. 6. Enzyme-immunohistochemistry of RBCs stained with Pab-HbL and Mab-HbA. (A1,B1) *RBCs from typical larvae (3 to 10 days after hatching).* **(C1,D1)** *RBCs from a typical adult (a sexually mature male). The corresponding phase-contrast micrographs of the immunohistochemistry are shown in* **(A2-D2)**. *RBCs were fixed with 100% methanol, and stained with larval Hb-specific polyclonal antibody (Pab-HbL)* **(A1,C1)**, *or with adult Hb-specific monoclonal antibody (Mab-HbA)* **(B1,D1)**. *The Pab-HbL stains only larval RBCs* **(A1)**, *but not adult ones* **(C1)**. *The Mab-HbA stains only adult RBCs* **(D1)**, *but not larval ones* **(B1)**. *Bar, 50 μm.*

gradient (Fig. 4), in contrast to what has been reported in *Rana catesbeiana* (Dorn and Broyles, 1982). These observations favor the idea that the Hb switching occurs in a single RBC population ("Hb switching" model), rather than the concept that larval RBCs are replaced by new, adult RBCs ("RBC replacement" model) (see Broyles, 1981). Continuous changes in the RBC morphology during their transition from larval to adult types in 30% PBS (Fig. 2) as well as in ACG solution (Fig. 3) are also consistent with the "Hb switching" model in this species. Although studies of cell morphology, Hb phenotypes, as well as DNA and protein synthesis in the circulating RBCs of various amphibian species have suggested that an Hb transition reflects the replacement of larval RBCs by a new population of erythroid cells committed to adult Hb synthesis (Broyles, 1981), Hb switching in *Hynobius retardatus* may occur in a single RBC population.

The specificity of the antibodies used was demonstrated by Western blot (Fig. 5) as well as by immunohistochemistry (Fig. 6). Weber *et al.* (1989) have clearly demonstrated, using a similar procedure, that a certain fraction of RBCs contains only larval globins and the remaining fraction contains adult globins during the Hb transition in *Xenopus*. The concurrent presence of the larval and adult Hbs in an individual RBC in *Hynobius* was undoubtedly demonstrated by all the RBCs being recognized by both antibodies in smear preparations of RBCs (Fig. 7) as well as in histological sections of the spleen (Fig. 8).

Erythropoietic organs in H. retardatus

In amphibians, erythropoietic organs are known to change dramatically during ontogeny, from embryonic (ventral blood island) to larval, and from larval to adult. The exact erythropoietic organ(s) in larvae, however, have been controversial: kidney in *Xenopus* (Turner, 1988), liver and kidney in *Rana* (Broyles *et al.*, 1981), peripheral to liver (Weber *et al.*, 1991) or liver (Ohinata and Enami, 1991) in *Xenopus*, or spleen in *Triturus* (Tournefier, 1973). In *Hynobius retardatus*, the erythropoietic organs were demonstrated to be liver and spleen in early larval stages, and limited to the spleen in adults, in *Triturus* (Grasso, 1973b). According to the "RBC replacement" model, different classes of Hbs are expected to be expressed in different erythroid precursor cells which differentiate in the specific erythropoietic organs (Weber *et al.*, 1991). In contrast, in the "Hb switching" model, as in *Hynobius retardatus*, the Hb switching may occur in the same erythroid precursor cells, which differentiate in the spleen. Figure 9 shows a tentative model for RBC transition in *Hynobius retardatus*. During early developmental stages, the spleen and liver must be major erythropoietic organs. In the liver, however, erythropoietic activity ceases and thus RBC-precursor cells are hardly observed in metamorphosing larvae and metamorphosed juveniles. This suggests that the liver functions as an erythropoietic organ only at early larval stages, from which RBCs containing only larval globins differentiate, as in the kidney of bullfrog (Broyles *et al.*, 1981), the blood island of rainbow trout (Iuchi and Yamamoto, 1983), and the yolk sac or liver of human (Wetherall and Clegg, 1979). The spleen becomes the major erythropoietic organ just before the Hb transition in *H. retardatus*. It is thus suggested that the spleen functions as a major erythropoietic organ throughout life in this species, in contrast to *Rana* and *Xenopus* (Broyles, 1981; Weber *et al.*, 1991).

Hormonal regulation in RBC transition

Adult RBCs in *Hynobius retardatus* were readily distinguished from larval RBCs not only by their globin subunits (Fig. 6) and density on a Percoll gradient (Fig. 4), but also by their cell shape

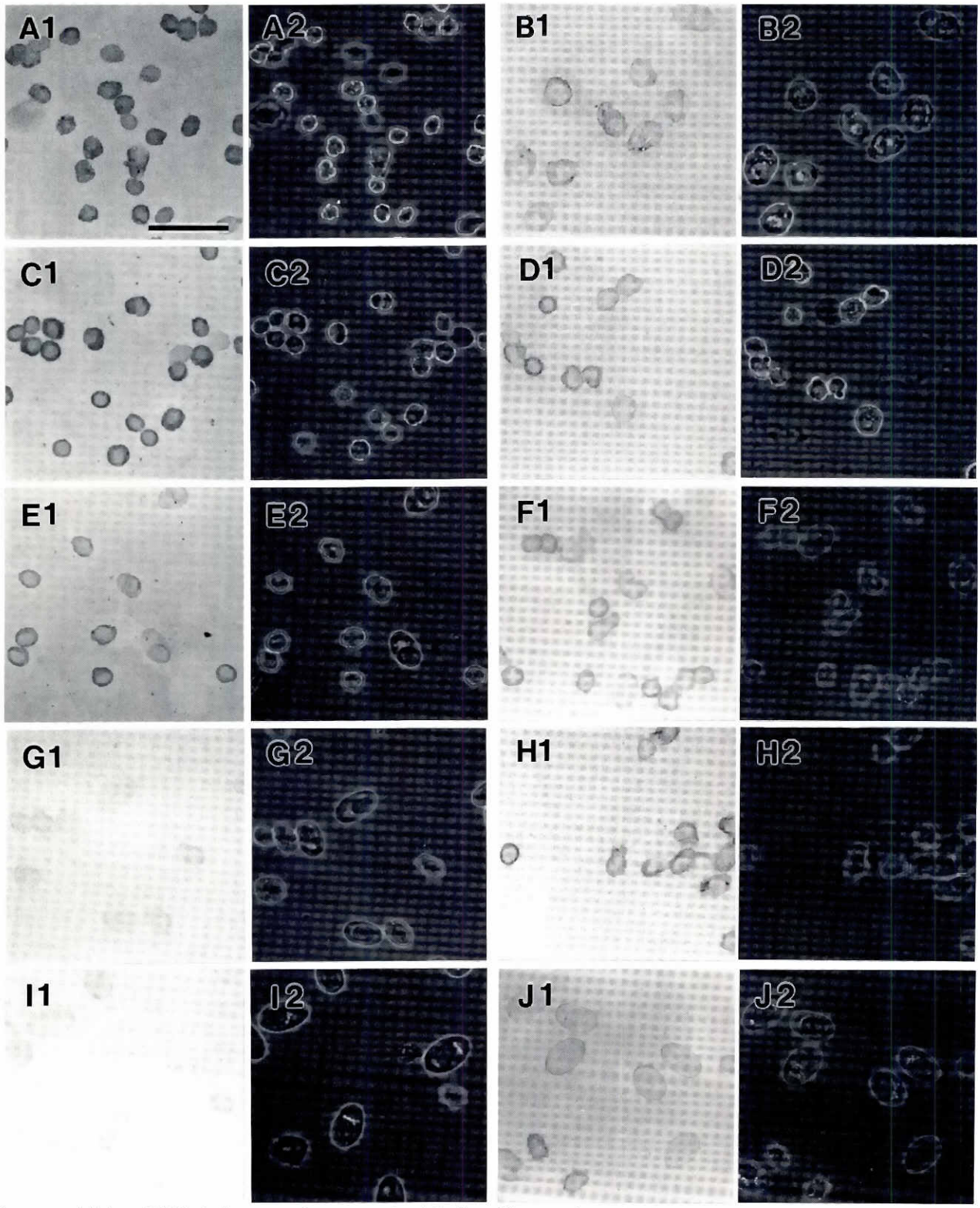

Fig. 7. Immunostaining of RBCs during normal metamorphosis in *Hynobius retardatus*. **(A1,C1,E1,G1 and I1)** *RBCs stained with larval Hb-specific polyclonal antibody (Pab-HbL).* **(B1,D1,F1,H1 and J1)** *RBCs stained with adult Hb-specific antibody (Mab-HbA). The corresponding phase-contrast micrographs are shown in* **(A2-J2)**, *respectively.* **(A,B)** *36 days after hatching;* **(C,D)** *50 days after hatching;* **(E,F)** *64 days after hatching;* **(G,H)** *89 days after hatching;* **(I,J)** *105 days after hatching. All RBCs from any developmental stages before (36 days) and during (50 days, 64 days) metamorphosis were recognized by both the Pab-HbL and Mab-HbA, suggesting that the RBCs contained both larval and adult Hbs, concurrently. After metamorphosis, the immunostaining with the larval antibody diminished (89 days), and then was completely lost (105 days). Bar, 50 μm.*

(Fig. 2) and behavior in a hypertonic solution (Fig. 3), in the axolotl *Ambystoma mexicanum* (Ducibella, 1974b). In the axolotl the transition has been reported to occur depending on a very low concentration of thyroid hormones (Ducibella, 1974b; Jurd, 1985). In contrast, the Hb transition in *Hynobius retardatus* is independent of a thyroid activity (Satoh and Wakahara, 1997), and thus of thyroid hormones (Arai and Wakahara, 1993; Wakahara *et al.*, 1994). It must be induced during metamorphosis by pituitary gland activity (Satoh and Wakahara, 1997), and/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). Thyroid hormone-dependent globin gene expression during amphibian metamorphosis is a useful model to investigate hormone-dependent gene expression (Widmer *et al.*, 1981; Hosbach *et al.*, 1982; Banville and Williams, 1985; Weber *et al.*, 1991). In contrast, Hb switching in *Hynobius retardatus* may provide a new model for investigating the temporal regulation of gene expression or control of developmental timing during ontogeny (see Wakahara, 1996).

The concurrent presence of larval and adult Hbs in a single RBC population demonstrated in this study does not mean simply that both genes encoding larval and adult globins are simultaneously expressed in RBCs and/or RBC-precursor cells. In fact, it is unclear whether changes in the proportion of adult Hbs to larval ones occur in individual RBCs, or whether the proportion remains the same from the time an individual RBC differentiates until its death. The next step of the study will be to obtain molecular probes to elucidate further mechanisms controlling the temporal regulation of Hb genes in *Hynobius retardatus*.

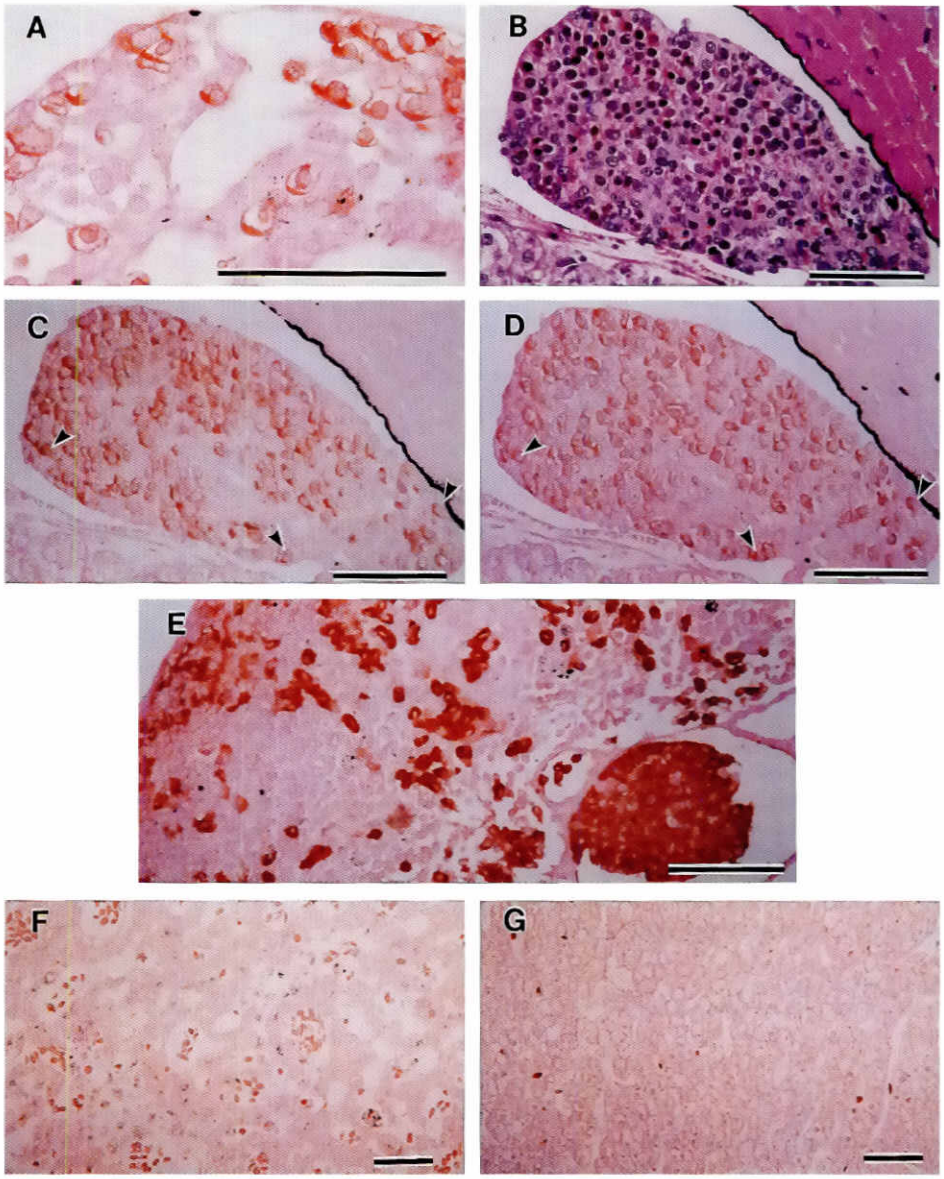

Fig. 8. Immunohistochemistry showing the erythropoietic organs. *Tissue sections were treated with a larval Hb-specific antibody (Pab-HbL) or adult Hb-specific antibody (Mab-HbA) as a primary antibody, and peroxidase-labeled anti-mouse IgG (secondary antibody). Peroxidase activity was detected by DAB.* **(A)** *The spleen at 10 days after hatching immunostained with Pab-HbL.* **(B,C,D)** *Consecutive sections of the spleen at 36 days after hatching stained with hematoxylin and eosin, Pab-HbL, and Mab-HbA, respectively. The same erythroid cells are stained with both antibodies on consecutive sections (C,D, arrowheads).* **(E)** *Adult spleen stained with Mab-HbA.* **(F)** *Section through the liver at 10 days after hatching stained with Pab-HbA, showing a lot of nesting erythroid cells.* **(G)** *Section through the liver at 36 days after hatching stained with Mab-HbA, showing no erythroid cells except for RBCs in capillary. Bars, 100 μm.*

Materials and Methods

Animals

Sexually mature adult males and fertilized eggs of *Hynobius retardatus* were collected from several ponds or small streams in the vicinity of Sapporo during the breeding season. The embryos hatched from their egg sacs were reared in dechlorinated tap water at room temperature. Mature males were stored at 4°C until use. Blood samples were collected from very early larvae at 3 to 10 days after hatching (typical larval RBCs), from developing larvae (RBCs in transition), and from sexually mature males (typical adult RBCs), respectively, after anesthesia by immersing in MS222 (Sandoz, 1:2000 in Steinberg's solution for larvae and 1:500 in De Boer's solution for adult).

Electrophoresis

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. Electrophoretic patterns were photographed and the transition of globin subunits from larval to adult types was analyzed by measuring the ratio of adult to total globins using a computerized image analyzer (NIH-image).

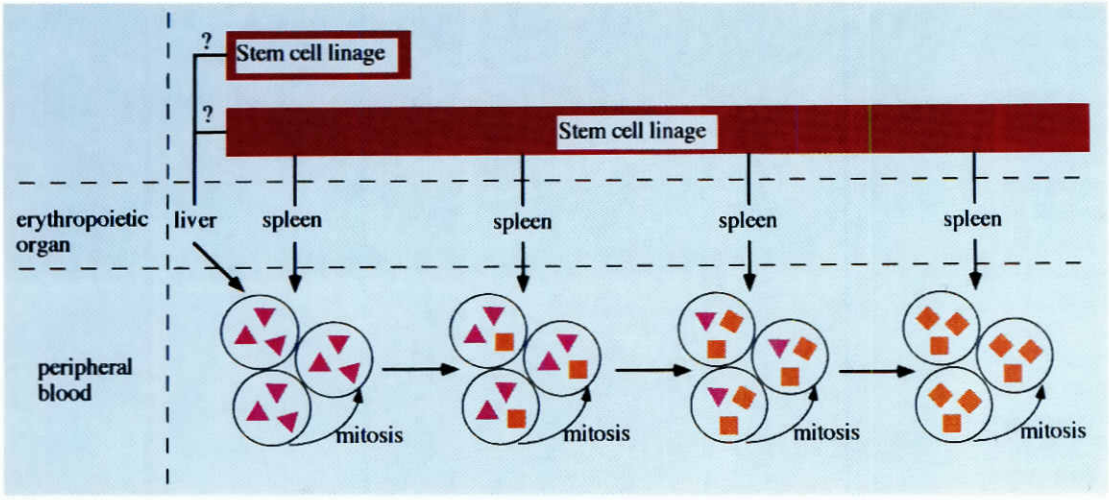

Fig. 9. A tentative model for Hb and RBC transitions in *Hynobius retardatus*. *At early developmental stages, the spleen and liver must be erythropoietic organs, and differentiating erythroblasts in both organs synthesize larval-specific Hbs only. In the liver, however, erythropoietic activity ceases and thus erythroblasts are hardly observed in metamorphosing larvae. The spleen becomes the major erythropoietic organ as development proceeds. In the spleen, erythroblasts initially synthesize larval-specific Hbs exclusively, then larval- and adult-specific Hbs concurrently, and finally synthesize adult-specific Hbs after metamorphosis. The origin and kinds of erythropoietic stem cell lineage(s) are unknown. Pink triangles indicate larval Hbs, and orange squares indicate adult Hbs.*

RBC morphology

Freshly prepared RBCs were observed directly by inverted phase contrast microscopy in 30% PBS (73 mM NaCl, 18 mM KH_2PO_4, 57 mM Na_2HPO_4; pH 7.2) and photographed. Major and minor axes of the RBCs were measured and analyzed using a computerized image analyzer (NIH-image). The morphology of RBCs was also analyzed in ACG solution (0.08% citric acid, 2.2% trisodium citrate dihydrate, and 2.4% glucose; Ducibella, 1974b), which must be hypertonic to larval RBCs. Larval and adult RBCs were readily distinguished by their different morphology in the ACG solution. Oval or elliptical RBCs with smooth cell surfaces were counted as adult RBCs, and extremely shrunken RBCs as larval RBCs. The percentage of adult to total RBCs was calculated.

Buoyant density

A continuous density gradient of Percoll (Pharmacia) from 0% to 83% in 50% PBS was prepared in a centrifugation tube. Freshly collected RBCs were layered onto the Percoll gradient with colored density markers. After centrifugation (700g, 30 min), the position of the RBCs was measured and then the density of the RBCs was estimated according to their position relative to the density markers.

Preparation of antibodies

To prepare mouse monoclonal antibodies against either larval or adult Hbs, hemolysates from typical larval and typical adult RBCs were obtained according to the method described previously (Arai and Wakahara, 1993). Mice received four subcapsular injections of a total of 4 mg of hemolysate proteins. Three days after the final injection, the spleen cells were removed and fused with myeloma cells, according to the method devised by Köhler and Milstein (1975). Diluted hybridoma supernatants were screened in order to select specific antibodies that reacted with either typical larval or adult hemolysates, but not with both. Several specific clones to the adult Hbs were isolated by the limiting dilution technique. One of them, named Mab-HbA was used in this study. Because monoclonal antibodies specific enough to recognize only the larval Hbs were not cloned, serum from mice immunized with larval hemolysate was diluted 1:500 and extensively absorbed by the adult hemolysate, and used as a polyclonal antibody (Pab-HbL) which reacted specifically to the larval Hbs.

Immunoblot

Typical larval and typical adult hemolysates were applied to SDS-PAGE, using 15% separating gels, and blotted to Immobilon PVDF transfer membrane (Nihon Millipore, Tokyo). After blocking in 5% dry milk with 0.1% sodium azide, the membrane was incubated for 30 min with primary antibody (Pab-HbL, 1: 3000; Mab-HbA, 1:500, in dilution) at 4°C, and then for 90 min with alkaline phosphatase-labeled secondary antibody (rabbit anti-mouse IgG, diluted 1: 500). Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as the substrate for detecting the reaction.

Immunohistochemistry

Freshly prepared blood samples were smeared on clean glass slides, air-dried and fixed with 100% methanol. The smear preparations were incubated for 30 min in 0.3% H_2O_2 in methanol to decolor. After blocking for 2 h with 10% FCS-PBS at 37°C, the smear preparations were overlaid with the Pab-HbL (1:3000) or Mab-HbA (1:100) for 30 min at 4°C, and then with alkaline phosphatase-labeled anti-mouse IgG. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as the substrate for detecting the reaction.

To determine the erythropoietic organ(s) in larvae and adults, several organs were fixed with methanol-acetic acid (3:1) mixture and embedded in Tissue-Prep (Fischer, Fair Lawn). They were cut at 5 μm thickness and stained immunohistochemically with methods basically identical to those described above. Horseradish peroxidase-conjugated rabbit anti-mouse IgG (diluted 1: 500) was used as a secondary antibody. DAB was used as the substrate for detecting the reaction with H_2O_2.

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