

Ontogeny of immunoglobulin expression in the Mexican axolotl

JULIEN S. FELLAH¹, DANIEL VAULOT², ANNICK TOURNEFIER¹ and JACQUES CHARLEMAGNE¹

¹Laboratoire d'Immunologie Comparée, Université Pierre et Marie Curie et CNRS (UA 1135), 9 Quai Saint-Bernard, C30, 75005 PARIS, FRANCE

²Station d'Océanologie et de Biologie Marine de ROSCOFF, Université Pierre et Marie Curie et CNRS (LP 4601), 29211 ROSCOFF, FRANCE

Summary

The ontogeny of immunoglobulin (Ig) synthesis was followed at both cellular and serological levels in the Mexican axolotl (*Ambystoma mexicanum*) using polyclonal antibodies recognizing all Ig molecules and a set of monoclonal antibodies (Mabs) specific for the C μ and C ν heavy Ig chain isotypes and for the light chain constituents shared by IgM and IgY molecules. Clusters of IgM- and of IgY-synthesizing lymphocytes, often located in separate sites, are first present in spleen sections of 7-week-old 25 mm larvae, about one month after differentiation of the spleen anlage (stage 39–40). In 12-week-old 30–35 mm larvae, the relative proportion of IgM- and IgY-synthesizing cells in the spleen is the same as that in adult animals. However, a marked enhancement of the spleen B cell compartment occurs from 5 to 9 months when Ig-positive cells represent about 88 % of the lymphocyte population compared to 60 % in adults. No structures equivalent to B cell germinal centers were observed at any stage of the spleen differentiation and

cells, although often clustered in small groups, remain dispersed in the entire organ. The relative proportions of IgM and IgY B cells throughout the spleen remain constant during development (about 1 IgY⁺ cell for 5–6 IgM⁺ cells) and IgM molecules are first detected in the serum of 2.5-month-old larvae. The enhancement of the serum IgM level correlates well with the absolute number of IgM⁺ cells in the growing spleen. IgY molecules cannot be detected in the serum before the 7th month but their level quickly increases to reach about 60 % of the adult value at 10 months. Thyroxine-induced metamorphosis or hyperimmunization of 4- to 6-month-old larvae had no effect upon the temporal expression of the Ig classes in serum.

Key words: *Ambystoma mexicanum*, axolotl, B lymphocytes, flow cytometry, IgM, IgY, immunoglobulins, monoclonal antibodies, ontogeny, spleen.

Introduction

Since the development of amphibian larvae occurs in direct contact with the aquatic environment, the differentiation of an efficient immune system at an early stage seems to be essential. It is now well documented that young anuran tadpoles (*Xenopus laevis*, *Rana catesbeiana*, *Alytes obstetricans*) can produce antibodies to various antigens (Kidder *et al.* 1973; Du Pasquier and Haimowitch, 1976; Du Pasquier, 1970) in spite of the small number of lymphocytes in their spleen (10^5 – 10^6). Antibodies in tadpoles are of restricted heterogeneity, suggesting the selective usage of a limited set of V genes early in ontogeny, correlated with the rapid emergence of B lymphocytes (Du Pasquier, 1982). Anuran larvae are also able to develop vigorous alloimmune responses, correlated with the precocious differentiation of functional thymus-derived lymphocytes (Manning and Collie, 1975). Ig-synthesizing cells are first observed in the liver of stage 45 *Xenopus* tadpoles (Flajnik *et al.* 1987), and then in the spleen of stage 48 animals (respectively, 4 and 10 days after hatching). Using a

sensitive radioimmunoassay, Ig-like molecules were even detected in stage 35 *Xenopus* embryos, in the absence of differentiated lymphoid tissues (Leverone *et al.* 1979). A possible explanation of this phenomenon could be the passive transfer of maternal Ig to eggs. In urodele amphibians, larval development takes more time compared to most anurans. In the Mexican axolotl (*Ambystoma mexicanum*), hatching occurs about three weeks after fertilization, at stage 40 of the developmental table of Schreckenberg and Jacobson (1975). From the beginning of the development to the adult stage, the thymus lobes and the spleen are the unique sites of lymphocytopoiesis. Erythropoiesis and thrombocytopoiesis also occur in the spleen but granulocytes develop in a separate site, the liver cortical layer (Charlemagne, 1972). Adult axolotls reject allografts (Delanney, 1961) and synthesize IgM-like high molecular weight (HMW) molecules after specific challenge (Ching and Wedgwood, 1967; Houdayer and Fougereau, 1972; Charlemagne and Tournefier, 1977; Chardin *et al.* 1987). We recently demonstrated that axolotls are also able to synthesize 11.9 S low molecular weight

(LMW) Ig, which we called IgY with respect to their physicochemical homologies with anuran and bird IgY (Fellah and Charlemagne, 1988). The two axolotl antibody classes seem to be synthesized by independent B cell subpopulations (Tounefier *et al.* 1988) and, following antigenic stimulation, the large majority of the specifically synthesized antibodies belong to the IgM class. IgY molecules, although efficiently produced following stimulation by the hapten ARS (Warr *et al.* 1982), are only marginally synthesized against trinitrophenylated sheep red blood cells (TNP-SRBC), SRBC or HRBC even after multiple stimulation (Fellah *et al.* 1988).

The aim of the present work is to describe the ontogenic emergence of the two Ig classes of the Mexican axolotl both at the serological and cellular levels.

Materials and methods

Animals and immunization

Neotenic axolotls of the Ax 6 strain were bred in our laboratory colony. Immediately after hatching, the young larvae were fed with *Artemia* for 1 month, then with *Chironomus* larvae and finally through the adult stage with commercial trout pellets (Aqualim). The differentiation steps of embryos until hatching were determined according to the developmental table of Schreckenberg and Jacobson (1975). After hatching, age, length and body weight (b.w.) parameters were used to characterize the different steps of larval development. Some animals were immunized with HRBC or TNP-SRBC, as described previously (Charlemagne and Tounefier, 1977). 4-month-old larvae (b.w.: 10 g) and 6-month-old juvenile axolotls (b.w.: 20 g) received a single injection of 0.05 ml and 0.1 ml of 25% HRBC respectively. Blood was collected by cardiac puncture for younger animals and from a gill vessel for 6 months and older axolotls. After clotting, blood samples were centrifuged at 5000 rev min⁻¹ for 5 min at 4°C and the sera were stored at -20°C. Artificial metamorphosis was induced in some animals by intraperitoneal injection of L-thyroxine (Sigma). A single dose of 100 µg was used for sub-adult axolotls and 50 µg were injected in 10 mg larvae. Complete metamorphosis was achieved two weeks after injection.

Antibodies

The production, selection and characterization of mouse monoclonal antibodies (Mabs) to axolotl Ig have already been described (Chardin *et al.* 1987; Charlemagne, 1987; Tounefier *et al.* 1988; Fellah *et al.* 1988). Briefly, Mabs 33.45.1 and 33.39.2 are respectively specific for the μ and ν heavy (H) Ig chain isotypes. Mab 33.101.2 recognize a light (L) Ig chain determinant which is shared by the two antibody classes (IgM and IgY). These three Mabs are IgG 1 κ .

The production of a polyclonal antiserum to purified deglycosylated axolotl Ig (RAAxIg) has also been described (Fellah *et al.* 1988). This antiserum recognizes in Western blotting the two H chain isotypes and the L chains.

Immunodetection techniques

(1) Enzyme-linked-immunosorbent-assay (ELISA)

The ELISA technique was used to evaluate relative levels of Ig classes in serum of axolotls at different developmental

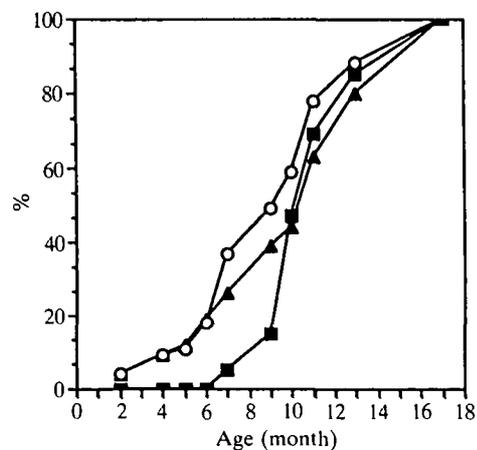


Fig. 1. ELISA analysis of axolotl serum immunoglobulins during development. Mabs 33.101.2, 33.45.1 and 33.39.2 are used for the quantitative evaluation of, respectively, total Igs (○—○), IgM (▲—▲) and IgY (■—■). Values are plotted on the ordinate as per cent of the mean corresponding values estimated for the pooled sera of 17-month-old adults (see: Materials and methods).

stages. Wells of ELISA plates (Dynatech M29) were coated overnight at 4°C with 50 µl of serum diluted 1:100 in 0.2 M-carbonate buffer, pH 9.6. Plates were then washed with A-PBS containing 0.05% Tween 20 (PBS-Tw), saturated with 3% (w/v) nonfat dry milk in A-PBS for 2 h at 37°C and incubated for 2 h at 37°C with Mabs 33.45.1, 33.39.2 or 33.101.2 (1×10^{-4} -diluted ascitic fluids). After two washes in PBS, plates were incubated with peroxidase-conjugated anti-mouse IgG antibodies (Dako) diluted 1:1000 in PBS for 2 h at 37°C and the peroxidase bound activity was revealed using *o*-phenylenediamine in the presence of H₂O₂. The reaction was stopped by 1N-H₂SO₄ and the intensity of the colored product was read at 414 nm with a multiscan photometer (Dynatech). Each assay was done in triplicate and the mean optical densities were plotted as per cent of the maximal values found for the pooled sera of 17-month-old adult axolotls.

(2) SDS-PAGE and Western blotting

Serum globulins from axolotls at different stages of development were separated by SDS-PAGE (8.7%–17.5% gradients) in reducing conditions (Laemmli, 1970). Gels were then transferred onto nitrocellulose and the separated Ig chains were revealed using RAAxIg or Mabs, as described in Fellah *et al.* (1988).

(3) Immunofluorescence of living lymphocytes

Axolotls spleens were excised from anesthetized animals (MS 222, Sandoz) and cell suspensions were prepared in amphibian-buffered saline (A-PBS) complemented with 5% fetal calf serum, as described in Tounefier *et al.* (1988). For immunofluorescence staining, 5×10^5 – 10^6 cells were incubated for 30 min in pure supernatants or in A-PBS containing 1:500-diluted ascitic fluid or RAAxIg antiserum. After three washes in A-PBS, cells were incubated with appropriate FITC-conjugated anti-Ig antibodies (Dako). Labeled cells were examined with a Leitz Orthoplan microscope equipped with a Ploem illuminator and appropriate filters. The labeled cells were also studied by flow cytometry with an EPICS 541 (Coulter). The 488 nm line (100 mW) of an argon laser (INNOVA 90-6, Coherent) was used as the excitation light

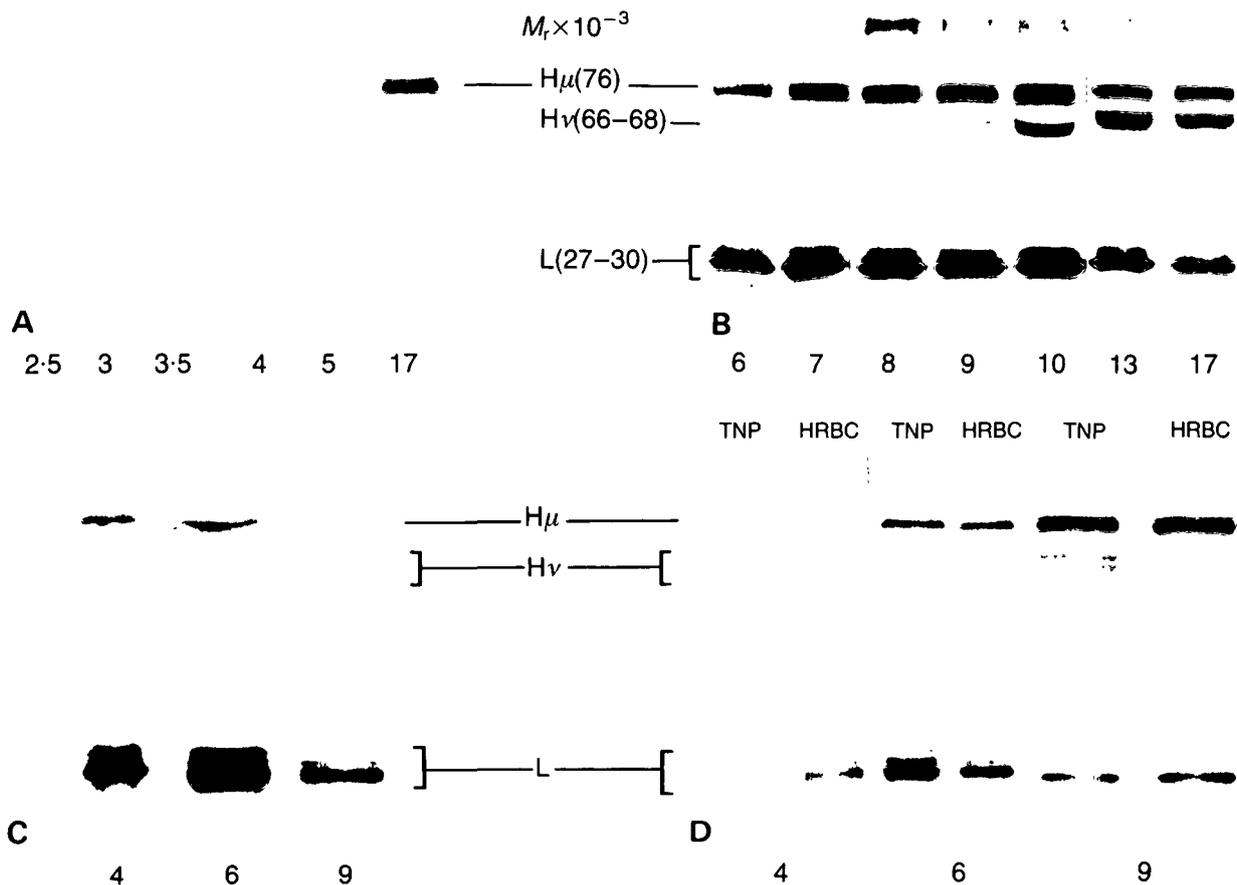


Fig. 2. A–D. Ontogenesis of serum immunoglobulin synthesis in axolotl. Samples are analyzed by SDS–PAGE in reducing conditions, electroblotted onto nitrocellulose and probed with Mab 33.45.1 (A) or RAAxIg antiserum (B–D). Ages in month are indicated above lanes, the relative molecular masses of Ig chains are shown. (A) Ontogenesis of the μ H chains expression. IgM are first detected in the serum of 2.5-month larvae. (B) vH Ig chains are first detected in the serum of 8-month larvae. (C) Expression of Ig chains in the serum of 4-, 6- and 9-month metamorphosed axolotls. Animals were treated with L-thyroxine and serum analyzed several weeks after metamorphosis. (D) Expression of Ig chains in the serum of 4-, 6- and 9-month axolotls immunized with 2,4,6-trinitrophenylated sheep red blood cells (TNP) or with horse red blood cells (HRBC). Unless significant anti-TNP or anti-HRBC antibody titres are synthesized by 4- or 6-month-old animals, no Ig of the IgY class arise.

source. Both forward and right-angle light scatters were collected as indicators of cell size. Green fluorescence was collected between 500 and 550 nm while red fluorescence was collected above 570 nm. Between 4000 and 10000 cells were analyzed per sample. The four parameters (forward and right-angle light scatters, green and red fluorescences) were recorded on 3 decade logarithmic scales mapped onto 256 channels and stored in list mode. Calibration was achieved with 10 μ m 2% (Coulter) fluorescent beads. Data collected in list mode were replayed on the MDADS unit of the EPICS 541. In a first step, lymphocytes were gated in a window based on their forward and right-angle scatter characteristics (see below). However, some red blood cells also fell within this window. Since these cells presented a marked red autofluorescence they could be eliminated by gating the lymphocytes through a second window based on both green and red fluorescence. The green FITC fluorescence histogram of the cells falling in both windows was then transferred to an IBM-AT compatible computer with the EPINET software (Coulter) and analyzed with a custom-designed software (CytoPC). We arbitrarily defined cells as positive if they were falling between channels 92 and 255, such that a control

sample (cells stained with the second FITC-antibody only) had only 5% positive cells. The percentage of positive cells and their average fluorescence relative to the reference beads were computed after conversion of the logarithmic scale (1 to 256 channels) to a linear one (1 to 1000).

(4) Histology and immunohistology

For conventional histology, whole larvae or dissected spleens, stomach and intestine were fixed in Bouin-Hollande, embedded in paraffin and 4 μ m sections were stained according to Lillie (1965). For immunohistology, larvae and lymphoid organs were fixed for 24 h in paraformaldehyde–lysine sodium periodate (PLP), according to Gendelman *et al.* (1983). Pieces were then washed for 24 h in A-PBS, dehydrated in isopropanol for 15 h and cleared for 15 min in chloroform. All the procedure was performed at 4°C. Tissues were embedded in paraffin and blocks were stored at 4°C. Sections (7 μ m) were floated onto slides previously coated with 0.01% poly-L-Lysine (Sigma) and stored for 24–48 h at room temperature before use. For immunostaining, the deparaffined sections were washed twice in A-PBS and saturated for 30 min with 3% (w/v) nonfat dry milk in A-PBS and incubated with the

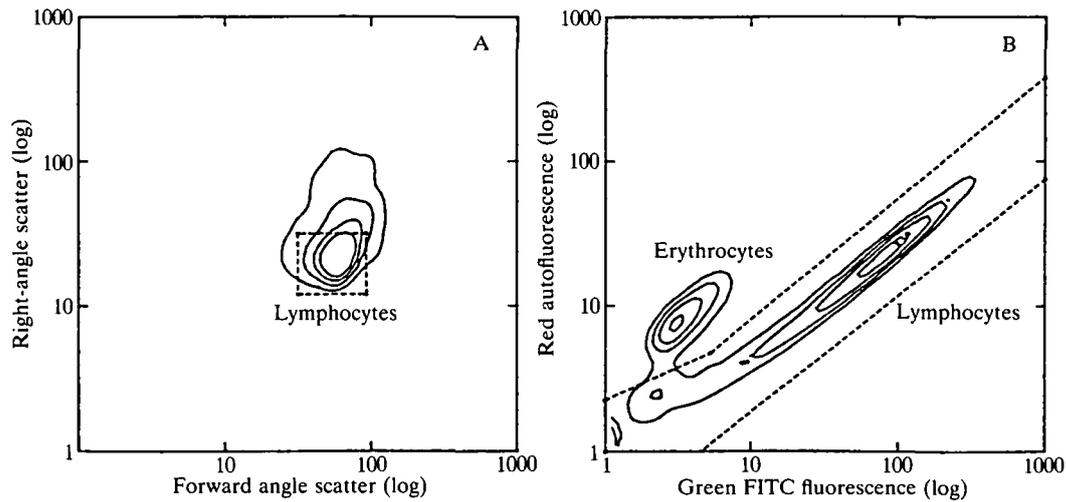


Fig. 3. Flow cytometric cytograms of axolotl spleen cells (age 9.5 months) stained with Mab 33.45.1. Axes are graduated in arbitrary logarithmic units. Contour levels correspond to 20, 50, 100 and 200 cells, respectively. (A) Forward vs right-angle scatter. Lymphocytes appear predominantly in the dashed window. (B) Green FITC fluorescence (500–550 nm) vs red autofluorescence (above 570 nm). Erythrocytes, characterized by their red autofluorescence, are eliminated by gating the lymphocytes in the dashed window.

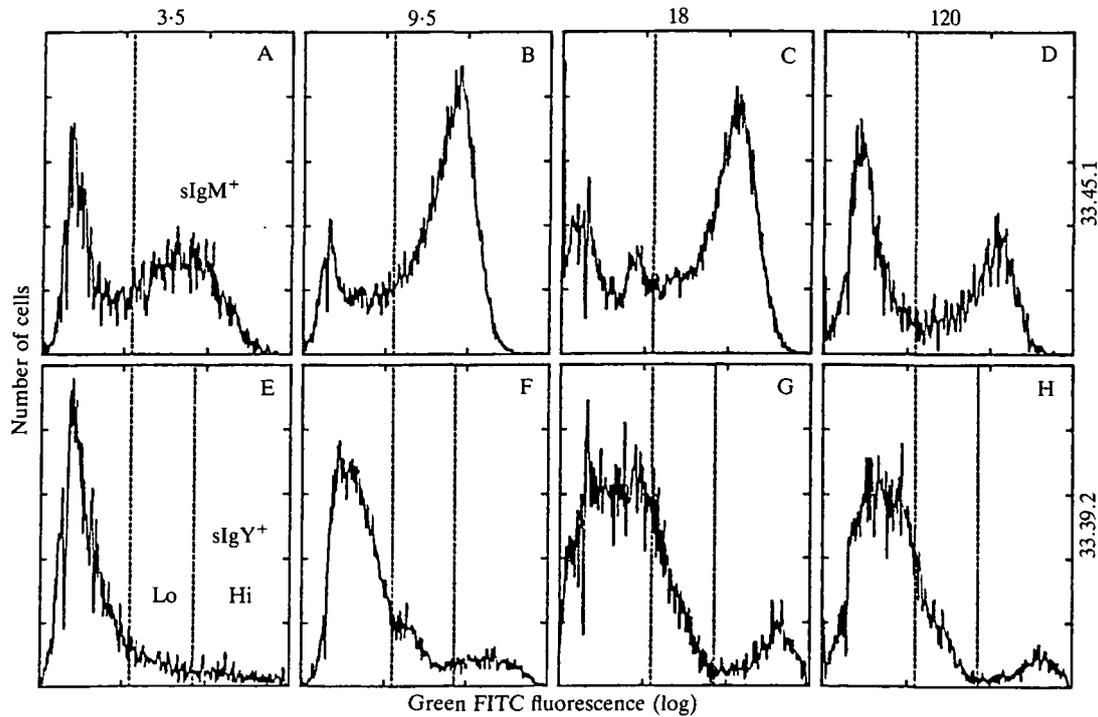


Fig. 4. Histograms of green FITC fluorescence for cells falling in the two windows defined in Fig. 3A and 3B. Upper panels (A–D): Mab 33.45.1. The dashed line corresponds to the lower limit of the sIgM⁺ cells. Lower panels (E–H): Mab 33.39.2. The left dashed line corresponds to the lower limit of the sIgY⁺ cells and the right dashed line to the limit of the sIgY⁺ Hi cells. Ages are indicated in months.

first antibody. Sections were then washed into three A-PBS changes and incubated with appropriate FITC- or peroxidase-labeled second antibodies (Dako) previously absorbed with axolotl leukocytes. FITC-labeled sections were mounted in aqueous medium (Mowiol) and peroxidase-labeled sections

were revealed using diaminobenzidine/H₂O₂, dehydrated and mounted in DPX.

(5) Controls

Complete hybridoma culture medium, ascitic fluid from mice

injected with the SP2/0 plasmocytoma and the preimmune serum from rabbit immunized with axolotl Ig (RAAxIg) were used as negative controls in all the immunodetection assays.

Results

Emergence of serum Ig classes during development

(1) ELISA

Mabs 33.45.1 and 33.39.2, respectively, recognize the μ H and the ν H chain isotypes of axolotl IgM and IgY (Fellah *et al.* 1988). These Mabs were used in an ELISA assay for comparative quantitative evaluation of the serum Ig classes in growing axolotls. Mab 33.101.2 (anti-L chains) was used in the same way to quantify total serum Igs. (Fig. 1). HMW IgM are already present in 2- to 2.5-month-old axolotl sera, about 5–7 weeks after hatching (hatching occurs 19–25 days after spawning in our laboratory conditions). At this stage, they reach only 4% of the 17-month-old adult value. A linear increase of IgM serum concentration then occurs from 5 to 13 months of development. Interestingly, LMW IgY were first detected in 7-month-old animals (5% of the adult value) and their concentration increases until 11 months to reach about 70% of the adult value, including a dramatic period of increase between 9 and 11 months. The total Ig (IgM plus IgY) values, estimated using the 33.101.2 Mab, strictly parallel the IgM synthesis during the first 6 months. A significant enhancement of total Ig comparatively to IgM values occurs after 7 months, well correlated with the newly synthesized IgY.

(2) Western blotting

Samples of axolotl serum were separated by SDS-PAGE in reducing conditions, proteins were electroblotted onto nitrocellulose and the Ig chains were detected using the RAAxIg antiserum (revelation of both μ H and ν H chains) or Mab 33.45.1 (revelation of μ chains). The $76 \times 10^3 M_r$ μ H chains were detected at all the developmental stages analyzed (Fig. 2A,B). The 66 – $68 \times 10^3 M_r$ protein doublet, which represents the ν H chains, was first detected using RAAxIg at 8–9 months of development (Fig. 2B). Overloading the

SDS gels with much larger serum samples did not improve the detection of ν H chains in young animals.

(3) Effect of immunization and metamorphosis

4-, 6- and 9-month-old axolotls were immunized with TNP-SRBC or HRBC, using our standard procedure. Animals were bled every month, starting from the first month of immunization and their sera were analyzed by Western blotting as above (Fig. 2D). Compared to age-matched controls, no change was observed in the temporal expression of the heavy chains isotypes in immunized animal. Moreover, in thyroxine-induced metamorphosed axolotls, no changes in expression of Ig isotypes was detected (Fig. 2C).

Development of Ig-synthesizing cells in the spleen

(1) Immunofluorescence and flow cytometry analysis

Axolotl spleen cells were stained for the presence of surface IgM (sIgM⁺ cells), surface IgY (sIgY⁺ cells) or surface Ig (sIg⁺ cells) using, respectively, Mabs 33.45.1, 33.39.2 and 33.101.2 and were first observed under epifluorescence microscopy. This analysis confirmed our previous results (Tournefier *et al.* 1988). Briefly, 55–65% sIgM⁺, 8–15% sIgY⁺ and 60–70% sIg⁺ cells were numbered and no important discrepancies were observed in the relative proportion of stained cells during development.

Spleen cells from 3.5-; 5-; 6-; 8-; 9.5-; 18-month-old and 10-year-old animals were analyzed by flow cytometry. In axolotls, erythropoiesis occurs mainly in the spleen and spleen cell suspensions contain a large proportion of nucleated cells. These erythrocytes cannot be simply eliminated, as can mammal red cells, by osmotic shock. Therefore, for flow cytometry analysis, lymphoid cells were first gated according to their scatter characteristics and erythrocyte contaminants were eliminated from the final counts on the basis of their red autofluorescence as described in Materials and methods (Fig. 3A,B).

The percentage of sIgM⁺ cells increases from 54% to 74% until 9.5 months and then decreases to 46% in adults. The large majority of sIgM⁺ cells fall in one peak; their average fluorescent intensity remains constant until 9.5 months and is markedly enhanced in

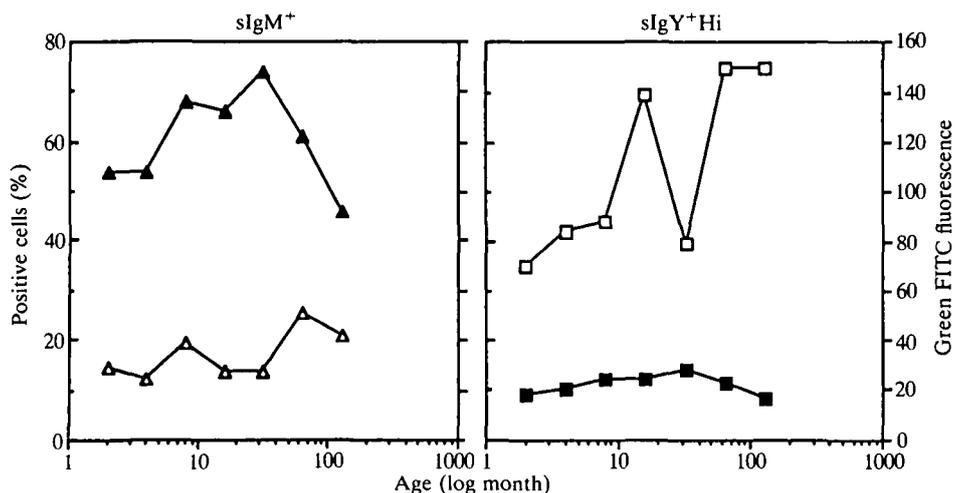


Fig. 5. Evolution of the percentage of positive cells (dark symbols, left axes) and of their average fluorescence (empty symbols, right axes) plotted to that of standard beads for Mab 33.45.1 (left panel) and Mab 33.39.2 (right panel).

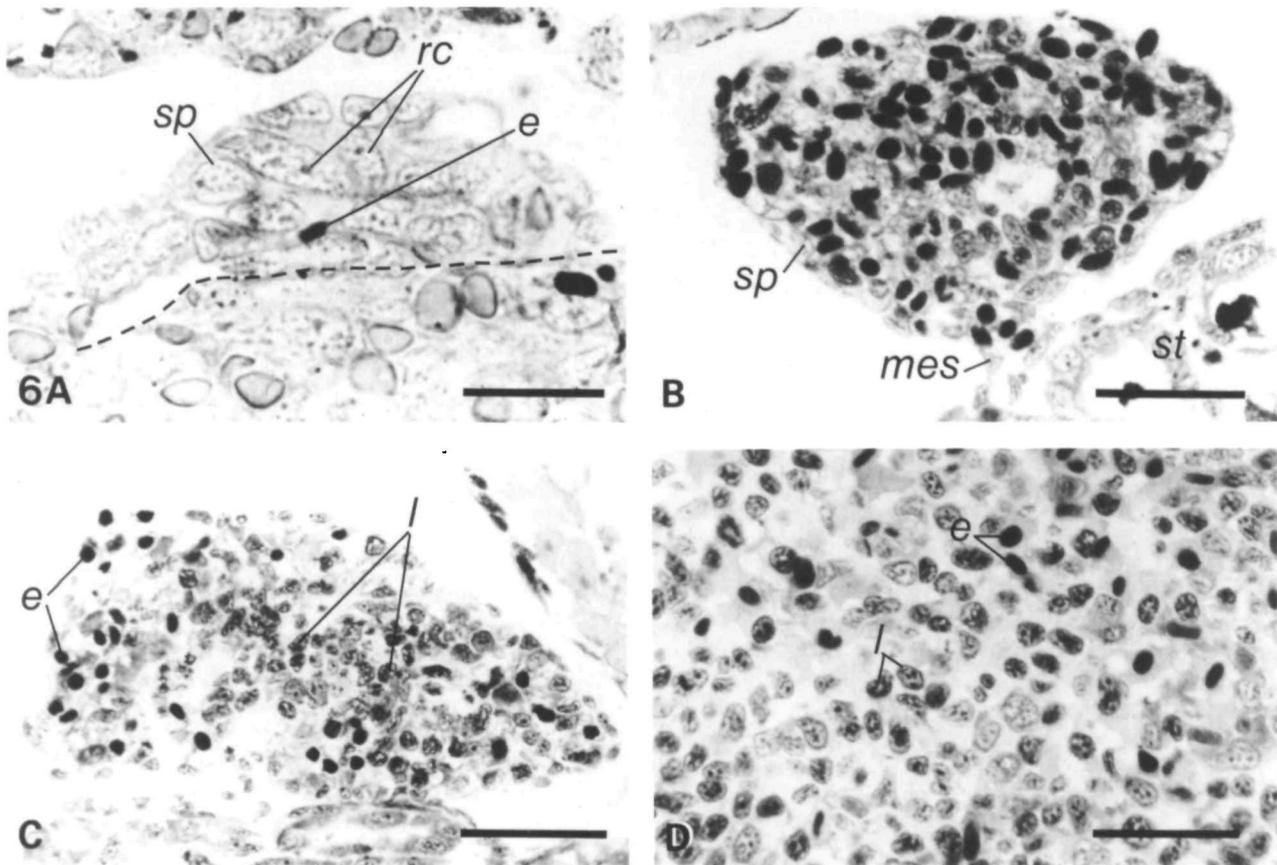


Fig. 6. Differentiation of the axolotl spleen. Bouin-Hollande fixative, 4 μ m sections, Lillie stain. (A) Stage 38. (B) Stage 40 (hatching). (C) 30–35 mm long larvae. (D) Adult. See text for explanations. e, erythrocytes; l, lymphocytes; mes, mesentery; sp, spleen; st, stomach; rc, reticular cells. Bar in A, 25 μ m; bar in B and D, 50 μ m; bar in C, 100 μ m.

adults (Fig. 4A,B,C,D). When the same analysis is applied to the sIgY⁺ cells, they divide into two populations, one discrete peak of strongly fluorescent cells (sIgY⁺Hi cells) and one ill-defined faintly stained population which seems to be more related to the background population (sIgY⁺Lo cells) (Fig. 4E,F,G,H). The sIgY⁺Hi lymphocytes represent 9–14% of the analyzed cells, a number that correlates well with the sIgY⁺ population observed under epifluorescence microscopy. At any stage, the sIgY⁺Hi cells represents 15.2 (\pm 1%) of the total (sIgM⁺ plus sIgY⁺Hi) sIg-positive cells. The average fluorescence intensity of sIgY⁺Hi cells continuously increases with age, except at 9.5 months, where a 50% drop is observed, which is not correlated to an equivalent drop in the fluorescence intensity of the sIgM⁺ cells. (Fig. 5).

(2) Immunohistology

The spleen anlage first appears at stage 39–40, 15–18 days after fertilization (a few days before hatching), as a small lenticular bud which is encapsulated within a single layer of mesenteric cells related to the dorso-anterior part of the stomach. This bud is filled with large reticular cells, erythrocytes are rare and no cells of lymphoid aspect are seen (Fig. 6A). At stage 40 (hatching, 9 mm long larvae), the spleen contains numerous

erythrocytes, reticular cells and rare cells with dense nuclei (Fig. 6B). In 6- to 8-week-old larvae (30–35 mm long), spleen erythrocytes are mainly located in large sinuses at the periphery and cells belonging to the lymphoid lineage can easily be distinguished in the center of the organ (Fig. 6C). In adult spleen, although preferential areas of erythropoiesis and lymphocytopoiesis are seen, there is no clear histological differentiation in red and white pulps (Fig. 6D). Mature erythrocytes accumulate in large sinuses at the periphery, beneath the external capsule.

Immunoglobulin-synthesizing lymphocytes are first observed in spleen of 7 weeks, 20 mm long larvae (Fig. 7A,B). Even at this early stage, two different lymphocyte populations are independently stained with Mabs 33.45.1 and 33.39.2 (data not shown). In 30 mm larvae, each population forms, in a given section, a small group of less than 10 lymphocytes, often located in the center of the spleen. When two successive sections are respectively stained with these Mabs, it is clearly observed that different clusters of lymphocytes express different heavy chain isotypes. IgM-positive clusters are more numerous and larger than IgY-positive clusters. (Fig. 7C,D,E). In 40–45 mm larvae (12 weeks old), the Ig-synthesizing cells are still located in the center of the spleen, often gathered in small

groups (Fig. 8A,B,C). In adult spleen, IgM- and IgY-positive cells are evenly scattered (Fig. 9A-E) and no germinal centers are observed even in immunized animals (data not shown).

Development of Ig-synthesizing cells in gut, liver, pronephros and mesonephros

The histological study of adult axolotl intestine and stomach sections reveals the presence of rare lymphoid cells located in both *lamina propria* and epithelium (Fig. 10A,B,C). These cells are never clustered and belong to the lymphocytic series, plasma cells are not found. Using immunofluorescence specific staining,

none of these lymphocytes are labelled by Mab 33.45.1 and only a minor subpopulation, mostly located in the *lamina propria*, faintly reacts with Mab 33.39.2. These rare IgY-positive cells are first detected in stomach and intestine sections of 4-month-old larvae and their number remain constant until the adult stage (Fig. 10D,E).

In liver sections, although active hematopoiesis occurs in the cortical layer, the majority of these cells belongs to the myeloid series (neutrophils and eosinophils polymorphonuclear cells, macrophages). The rare Ig-positive cells observed in this area are in most cases located in vascular sinuses. Lymphocytes are extremely rare in pronephros and mesonephros sections at any stage of development (data not shown).

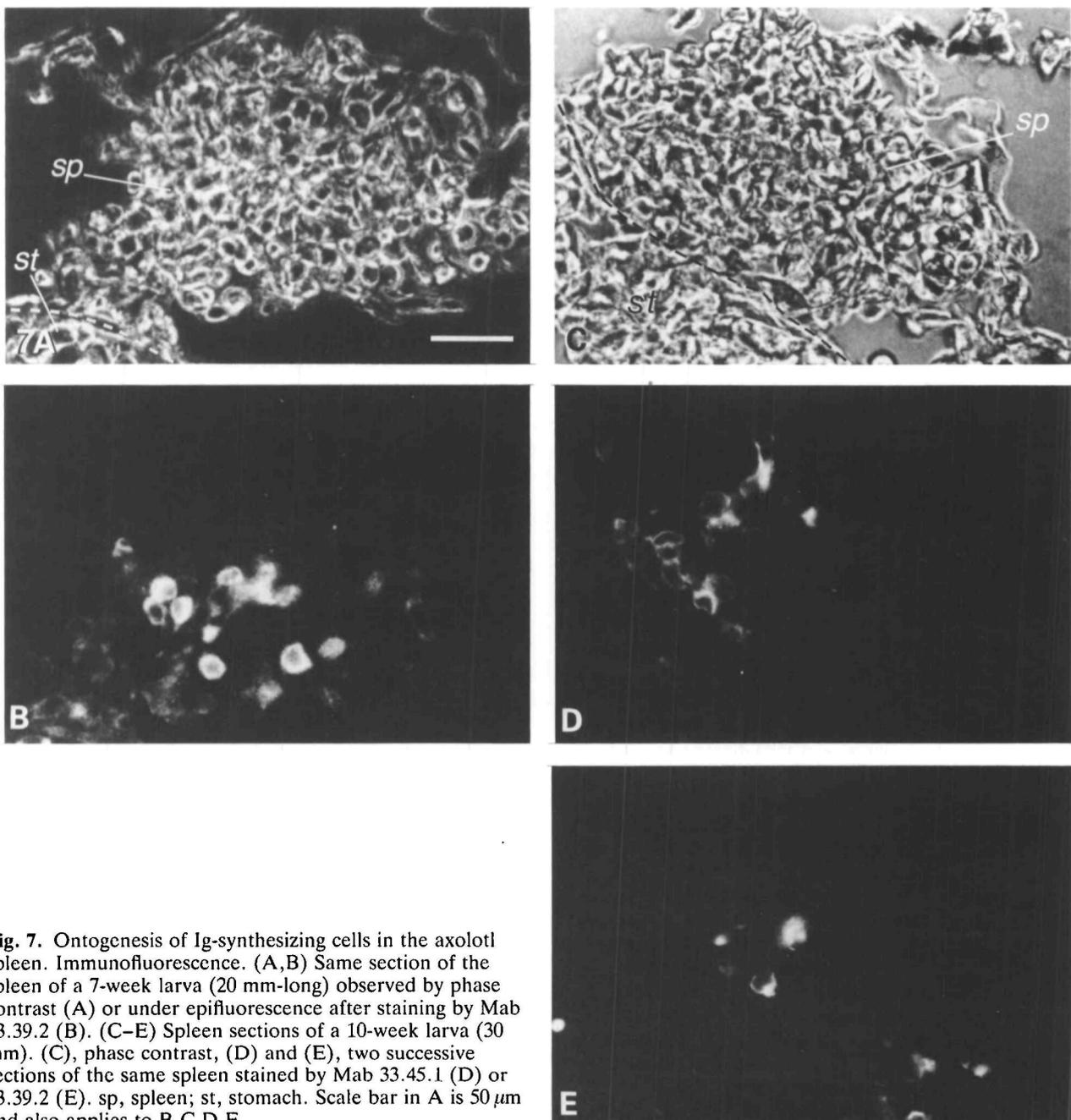


Fig. 7. Ontogenesis of Ig-synthesizing cells in the axolotl spleen. Immunofluorescence. (A,B) Same section of the spleen of a 7-week larva (20 mm-long) observed by phase contrast (A) or under epifluorescence after staining by Mab 33.39.2 (B). (C-E) Spleen sections of a 10-week larva (30 mm). (C), phase contrast, (D) and (E), two successive sections of the same spleen stained by Mab 33.45.1 (D) or 33.39.2 (E). sp, spleen; st, stomach. Scale bar in A is 50 μ m and also applies to B,C,D,E.

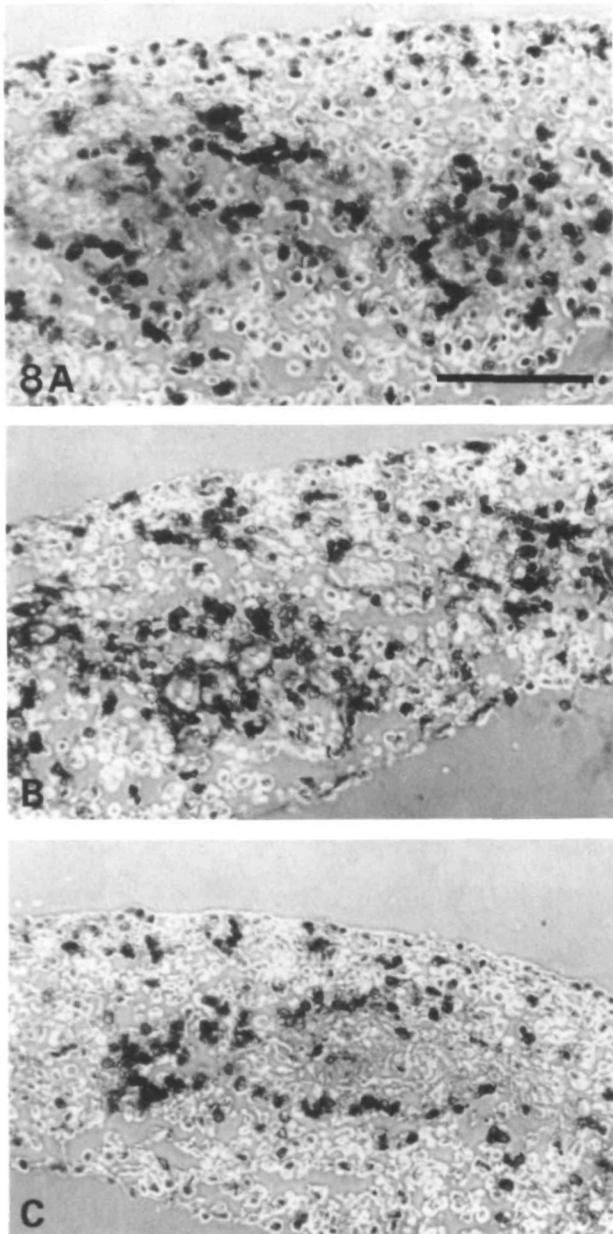


Fig. 8. Ontogenesis of Ig-synthesizing cells in the axolotl spleen. Immunoperoxidase. (A–C) Sections of a 3-month-old axolotl spleen stained with Mabs 33.101.2 (A), 33.45.1 (B) or 33.39.2 (C). Scale bar in A is 100 μ m and also applies to B and C.

Discussion

In the present paper, the ontogenic expression of serum and cellular immunoglobulins are described for the first time in an urodele amphibian. This study was made feasible by the recent availability in our laboratory of a set of monoclonal antibodies specific for different chains of the axolotl Ig molecules (Fellah *et al.* 1988). The characterization of secreted IgM and IgY in the serum, and of corresponding molecules on the surface or in the cytoplasm of immunocompetent cells, is therefore possible using these Mabs and several sensitive immunodetection techniques.

IgM-synthesizing lymphocytes are first seen in spleen sections 7 weeks after fertilization. At this stage, about 5 weeks after hatching, axolotl larvae (25 mm) are far from being fully differentiated, their posterior limbs are not yet developed and their body weight (0.2 g) is about 1/400 of the adult. Free IgM molecules are detected in the serum of 8–10 week larvae, although they could be present about one week before, considering our detection technique sensitivity limit (ELISA). For technical reasons, spleen cell suspensions analysis could be analyzed starting only with 3.5-month-old larvae. At this age, the proportion of sIgM⁺ cells is not very different from the adult value. However, from 5 until to 9 months, the proportion of sIgM⁺ spleen cells increases from 54% to 74% and then decreases to about 50% in adults. This transitory enhancement of sIgM⁺ lymphocytes will be discussed below. From the young larvae to the adult stage, a good correlation was observed between the quantitative evaluations of serum IgM and the absolute numbers of spleen lymphocytes.

In spleen sections, the IgY-synthesizing cells appear together with IgM-synthesizing cells, although located in different geographic areas. In 3.5-month larvae, the proportion of sIgY⁺ Hi cells in spleen (about 9%) is not different from the adult (8–11%). A transient enhancement of the proportion of sIgY⁺ Hi cells is observed from 5 to 9 months (9% to 14%) which strictly parallels the enhancement of sIgM⁺ cell numbers in the same period. However, the average fluorescence intensity of sIgY⁺ Hi cells continuously enhances with age, except for a transient drop at 9.5 months.

The nature of sIgY⁺ Lo lymphocyte population remains to be determined. These cells exhibit very low average fluorescence and are difficult to discriminate from the background cell population. Using Western blotting and ELISA techniques, a slight reactivity of Mab 33.39.2 for the μ H chains was observed, suggesting a possible antigenic relationship between the μ and ν heavy chains (Fellah *et al.* 1988). This cross-reactivity may explain a faint surface labeling of some sIgM⁺ cells by Mab 33.39.2 which might be taken into account by the very sensitive flow cytometry technique, according to our arbitrary background definition (see: Materials and methods).

Among the total spleen lymphocyte population, the proportion of B cells reaches maximum values at the pre-adult stage (9–10 months). The further relative diminution of B cell proportion might be correlated with the emergence, after 9 months, of new non-B lymphocyte subpopulations in the growing spleen (Kerfourn and Tournefier, in preparation).

In contrast to IgM, the secreted IgY molecules are not detected in axolotl serum before 7 months. At this time, larvae are fully differentiated, about 12 cm long and their body weight (20 g) is only one-third or one-half of adults. There is therefore a 5 months delay between the first appearance of IgY-synthesizing cells in the spleen and the presence of IgY in serum. We estimate the number of IgM-synthesizing cells in the spleen to be about 5×10^4 for 2.5-month-old animals. They have at this stage detectable serum IgM. IgM

values, as estimated using ELISA, then increase continuously with age until the adult stage. About 30×10^4 IgY-positive lymphocytes are present in the spleen of 7-month-old animals, when serum IgY molecules can first be detected. There is then a quick increase of the serum IgY quantities reaching 50% of the adult value at 9.5 months. As the relative proportions of IgM- and IgY-producing cells in the spleen remain constant at any developmental stage, the differential expression of IgY molecules in serum may reflect some peculiar metabolism characteristics of this Ig class. We recently observed that large quantities of IgY molecules are present in epithelial glandular cells of the gut during larval differentiation (Fellah *et al.* in preparation). These secretory IgY do not seem to be locally syn-

thesized by the rare gut-associated IgY-synthesizing lymphocytes and become less abundant in pre-adult and adults. A working hypothesis could therefore be that, before 7 months, most of synthesized IgY molecules are captured by the gut epithelium and that, after 7 months, the gut epithelium has less affinity for circulating IgY which rapidly accumulate in serum.

The influence of axolotl neotenic status on the development of its immune system is difficult to analyse considering that we have no information about ontogeny of Ig expression in naturally metamorphosing *Ambystoma* species. In *Ambystoma tigrinum*, natural metamorphosis arises 4–5 months after fertilization and coincides with important biochemical changes in hemoglobin components and serum proteins. In axo-

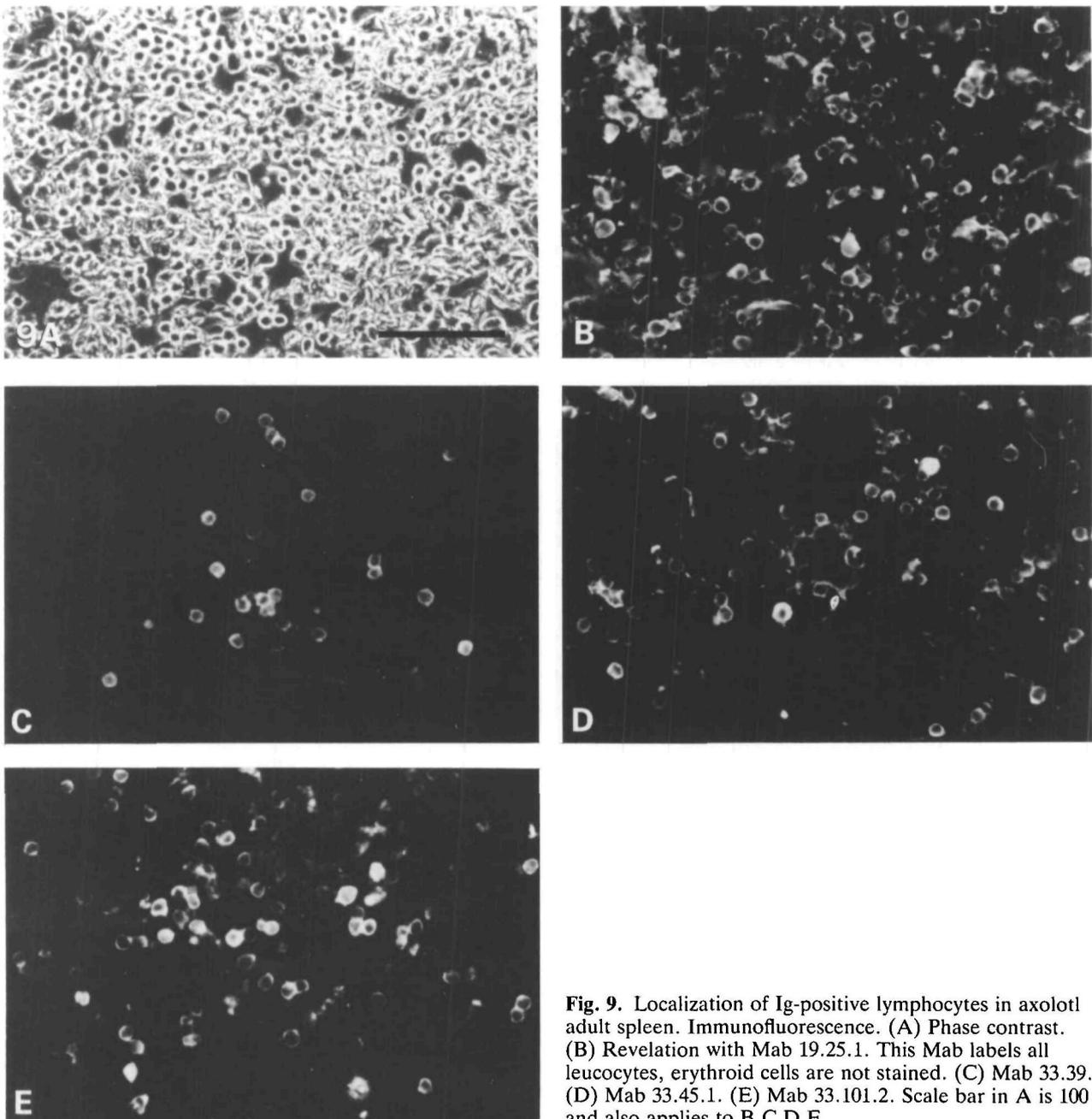


Fig. 9. Localization of Ig-positive lymphocytes in axolotl adult spleen. Immunofluorescence. (A) Phase contrast. (B) Revelation with Mab 19.25.1. This Mab labels all leucocytes, erythroid cells are not stained. (C) Mab 33.39.2. (D) Mab 33.45.1. (E) Mab 33.101.2. Scale bar in A is $100 \mu\text{m}$ and also applies to B,C,D,E.

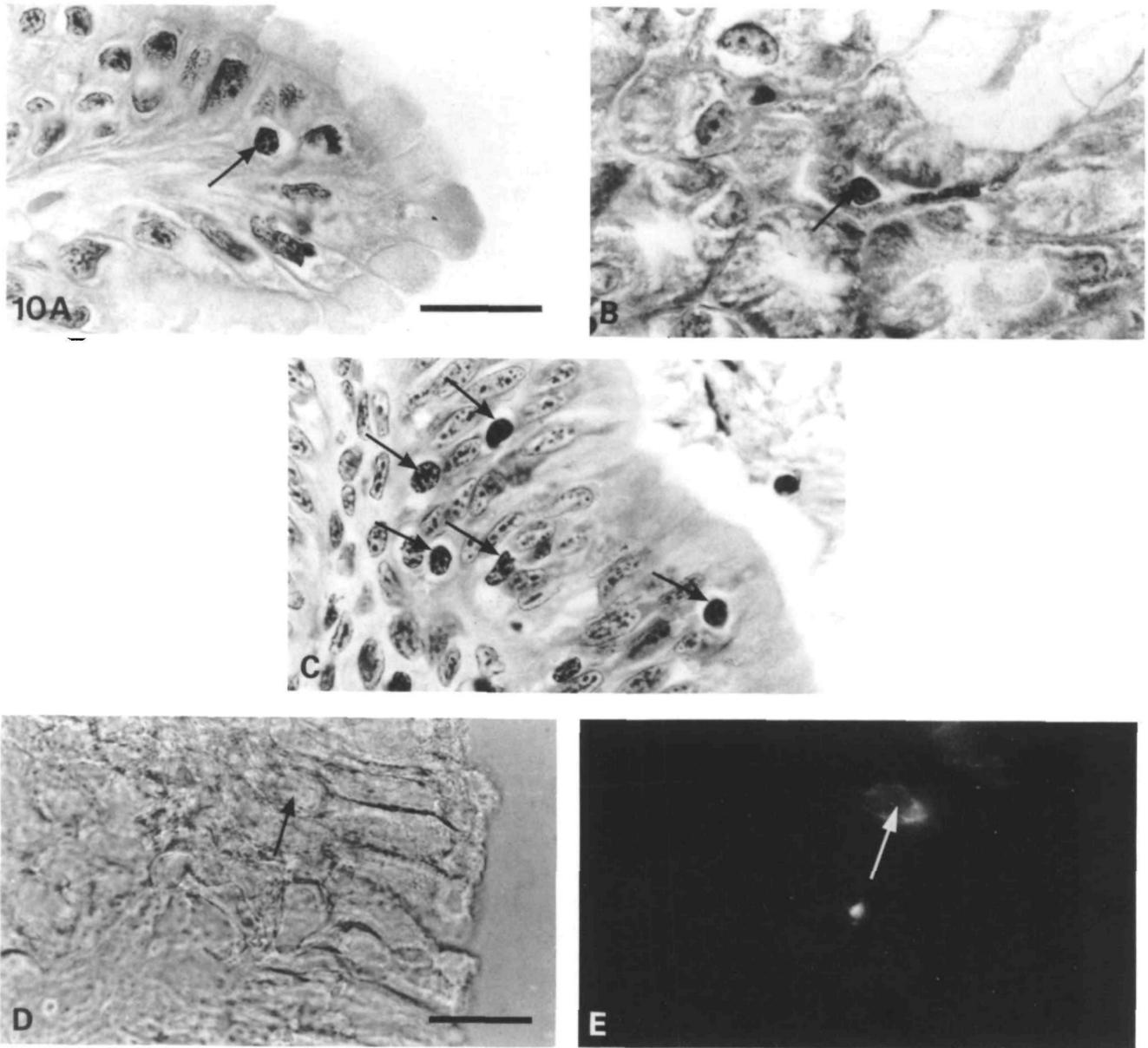


Fig. 10. Localization of lymphocytes in 8-month-old axolotl gut. (A–C) Bouin-Hollande fixative, 4 μm sections, Lillie stain. Lymphoid cells in (A) stomach epithelium, (B) stomach *lamina propria* and (C) intestine epithelium (arrows). (D,E) Same section of stomach observed by phase contrast (D) or under epifluorescence after staining by Mab 33.39.2 (E). An intraepithelial lymphocyte (arrows) is positively labelled. Scale bar in A is 40 μm and also applies to B and C. Scale bar in D and E is 25 μm .

lotl, a shift from larval to adult hemoglobin forms starts at 4 months and progressive changes in serum globulin composition can be observed from 5 to 10 months (Ducibella, 1974). The dramatic biochemical shift induced by natural metamorphosis in *A. tigrinum* should be balanced in *A. mexicanum* (a very related species) by more progressive and age-related biochemical changes. However, the induction of artificial anatomical metamorphosis by thyroxine, although producing important changes in serum globulin composition (Charlemagne, 1967; Ducibella, 1974), has no effect on serum IgY. The very late appearance of IgY molecules

in axolotl serum is related more probably to ageing than to metamorphic events.

Anuran (frogs and toads) IgY antibodies, like mammals IgG, are mostly synthesized in secondary responses against classical thymus-dependent antigens and their expression is regulated by thymus-derived cells (Manning *et al.* 1975). We recently observed that axolotl IgY antibodies are synthesized only marginally during the anti-dinitrophenyl or anti-erythrocyte responses for which IgM constitute the large majority of specific antibodies (Fellah *et al.* 1988). Moreover, these responses are thymus-independent, or even amplified

by early or adult thymectomy (Charlemagne *et al.* 1977; Charlemagne, 1979).

In mammals, B cells first synthesize and coexpress surface IgM and IgD molecules. IgM are then synthesized alone in resting B cells and, following stimulation, a recombination mechanism distinct from that involved in VH region gene assembly allows clonal B cells to replace the constant region (C μ) of the IgH locus by another CH region of the same locus while the variable region remains unaltered. This phenomenon, known as class switching, is mediated by a DNA rearrangement (S-S recombination) which results in the deletion of all C genes 5' of the expressed isotype (Davis *et al.* 1980). In *Xenopus*, the large majority of IgY-synthesizing cells in spleen also produce IgM (Hadj-Azimi and Parinello, 1978; Hsu and Du Pasquier, 1984). A physical linkage of the *Xenopus* C ν gene to the IgH locus remains to be demonstrated but is suggested by the recent characterization of Mab 10C10, which recognized a common (VH-region framework ?) determinant on μ and ν heavy chains (Hsu *et al.* 1984). In this case, the permanent cosynthesis of IgM and IgY by a significant proportion of *Xenopus* B cells could be explained by some peculiar expression mechanism (differential mRNA maturation?).

The presence of two independent and exclusive B cell subpopulations expressing either IgM or IgY surface molecules was recently demonstrated in axolotl using double immunofluorescence analysis. (Tournefier *et al.* 1988). At present, these results do not exclude the possibility that during their differentiation process, at least some B cells may successively synthesize the two Ig isotypes at the cytoplasmic level. However, the observation that separate IgM and IgY cells appear at the same time and often in different loci in the 2.5-month-old larval spleen argues for the presence of two independent B cell lineages in the axolotl.

We thank G. Aubet and J. Desrosiers for expert technical assistance and B. Cuvelier for typing the manuscript.

References

- CHARDIN, H., VILAIN, C. & CHARLEMAGNE, J. (1987). Characterization of axolotl heavy and light immunoglobulin chains by monoclonal antibodies. *Hybridoma* **6**, 627–636.
- CHARLEMAGNE, J. (1967). Etude par électrophorèse et immunoelectrophorèse des protéines sériques de l'axolotl ("*Ambystoma mexicanum*" Shaw). *Bull. Soc. Zool. France* **92**, 739–748.
- CHARLEMAGNE, J. (1972). Aspects morphologiques de la différenciation des éléments sanguins chez l'axolotl, *Ambystoma mexicanum* Shaw. *Z. Zellforsch. mikrosk. Anat.* **123**, 224–239.
- CHARLEMAGNE, J. (1979). Thymus independent anti-horse erythrocyte antibody response and suppressor T cells in the Mexican axolotl (*Amphibia*, *Urodela*, *Ambystoma mexicanum*). *Immunology* **36**, 643–648.
- CHARLEMAGNE, J. (1987). Antibody diversity in amphibians. Noninbred axolotls used the same unique heavy chain and a limited number of light chains for their anti-2,4-dinitrophenyl antibody responses. *Eur. J. Immunol.* **17**, 421–424.
- CHARLEMAGNE, J. & TOURNEFIER, A. (1977). Anti-horse red blood cells antibody synthesis in the Mexican axolotl (*Ambystoma mexicanum*). Effect of thymectomy. In *Developmental Immunobiology* (ed. J. B. Solomon & J. D. Horton), pp. 267–275. Amsterdam; North-Holland.
- CHING, Y. C. & WEDGWOOD, R. J. (1967). Immunologic response in the axolotl, *Siredon mexicanum*. *J. Immunol.* **99**, 191–200.
- DAVIS, M. M., KIM, S. K. & HOOD, L. (1980). Immunoglobulin class switching: developmentally regulated DNA rearrangements during differentiation. *Cell* **22**, 1–2.
- DELANNEY, L. E. (1961). Homografting of sexually mature Mexican axolotl. *Am. Zool.* **1**, 349.
- DUCIBELLA, T. (1974). The occurrence of biochemical metamorphic events without anatomical metamorphosis in the axolotl. *Devl Biol.* **38**, 175–186.
- DU PASQUIER, L. (1970). Ontogeny of the immune response in animals having less than one million lymphocytes: the larvae of the toad *Alytes obstetricans*. *Immunology* **19**, 353–362.
- DU PASQUIER, L. (1982). Antibody diversity in lower vertebrates. Why is it so restricted. *Nature, Lond.* **296**, 311–313.
- DU PASQUIER, L. & HAIMOWITZ, J. (1976). The antibody response during amphibian ontogeny. *Immunogenetics* **3**, 381–391.
- FELLAH, J. S. & CHARLEMAGNE, J. (1988). Characterization of an IgY-like low molecular weight immunoglobulin class in the Mexican axolotl. *Molec. Immunol.* **25**, 1377–1386.
- FLAJNIK, M. F., HSU, H., KAUFMAN, J. & DU PASQUIER, L. (1987). Changes in the immune system during metamorphosis of *Xenopus*. *Immunol. Today* **8**, 58–64.
- GENDELMAN, H. E., MOENCH, T. R., NARAYAN, O. & GRIFFIN, D. E. (1983). Selection of a fixative for identifying T cell subsets, B cells and macrophages in paraffin-embedded mouse spleen. *J. Immunol. Meth.* **65**, 137–145.
- HADJI-AZIMI, I. & PARINELLO, N. (1978). The simultaneous production of two classes of cytoplasmic immunoglobulins by single cells in *Xenopus laevis*. *Cell. Immunol.* **39**, 316–324.
- HOUDAYER, M. & FOUGEREAU, M. (1972). Phylogénie des immunoglobulines: la réaction immunitaire de l'axolotl ("*Ambystoma mexicanum*"). Cinétique de la réponse immunitaire et caractérisation des anticorps. *Annls. Inst. Pasteur* **123**, 3–28.
- HSU, H. & DU PASQUIER, L. (1984). Studies on *Xenopus* immunoglobulins using monoclonal antibodies. *Molec. Immunol.* **21**, 257–270.
- KIDDER, G. M., RUBEN, L. N. & STEVENS, J. M. (1973). Cytodynamics and ontogeny of the immune response of *Xenopus laevis* against sheep erythrocytes. *J. Embryol. exp. Morph.* **29**, 73–85.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, Lond.* **226**, 495–497.
- LEVERONE, L., SUHAR, T., BROWN, R. D. & ARMENTROUT, R. W. (1979). Characterization of immunoglobulin synthesized early in the development of *Xenopus laevis*. *Devl Biol.* **68**, 319–325.
- LILLIE, R. D. (1965). *Histopathology Technique and practical Histochemistry*, 3rd ed. New York, McGraw Hill.
- MANNING, M. J. & COLLIE, M. H. (1975). Thymic function in Amphibians. *Adv. exp. Med. Biol.* **64**, 353–362.
- SCHRECKENBERG, G. M. & JACOBSON, A. G. (1975). Normal stages of development of the axolotl *Ambystoma mexicanum*. *Devl Biol.* **42**, 391–400.
- TOURNEFIER, A., FELLAH, S. & CHARLEMAGNE, J. (1988). Monoclonal antibodies to axolotl immunoglobulins specific for different heavy chains isotypes expressed by independent lymphocyte subpopulations. *Immunol. Letts.* **18**, 145–148.
- WARR, G. W., RUBEN, L. N. & EDWARDS, B. J. (1982). Evidence for low-molecular weight antibodies in the serum of a urodele amphibian, *Ambystoma mexicanum*. *Immunol. Letts.* **4**, 99–102.