Amphibian Immunology with a Special Emphasis on Axolotl Haematology

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Phylogeny of Blood Cells

In all vertebrates, erythrocytes transport oxygen, thrombocytes or platelets initiate haemostasis, and leucocytes defend against foreign invasion. Even the lowest vertebrates, the agnathans, synthesize antibodies, exhibit allogeneic recognition, and possess lymphocyte heterogeneity. Cooperation of subsets of cells and establishment of immunological memory occur for the first time in vertebrates. This specialization in the mechanisms of discriminating "self" from "non-self" develops in association with increased body size, (generally) longer life span, and reduced reproductive potential, as compared to invertebrates.

Within vertebrates, amphibians comprise a diverse group of animals. Amphibians constitute a link between the aquatic fishes and the terrestrial amniotes, in that the larval form is free living and aquatic, and the adult form (in most cases) emerges onto land. In amphibians, bone marrow occurs phylogenetically for the first time, connected with terrestrial life stages.

In amniotes, with the loss of the free-living larva, the needs of the developing embryo (or fetus) for circulating cells are mainly respiratory (that is: erythrocytes), whereas leucocytes for protection against infection are of minor importance until hatching or birth. The protected environment of the embryo will thus allow long periods for the production and maturation of the cells of the immune system.

Amphibian ontogeny may give clues to phylogenetic development in vertebrates; the free-living larva is, in most cases, devoid of maternal care and protection. It has to provide immune defences for itself. During metamorphosis, the animal rapidly changes into the adult form, changing organs and body shape to cope with new habits and habitats. Also blood cells meet new demands during this transformation: erythrocytes with new types of haemoglobin (suitable for terrestrial existence) appear, and the immune system adapts to new surroundings. New pathogens are encountered, and adult tissues with new antigens influence the establishment of recognition patterns of *self* and *non-self*.

The amphibian spleen is the site for the development of erythrocytes and thrombocytes. Eosinophilic and neutrophilic granulocytes develop in the liver, whereas basophilic cells are formed in the thymuses. Some anurans have lymphomyeloid nodes, which resemble the lymph nodes of higher vertebrates, though they filter the blood, not the lymph. Bone marrow is present in all anurans, though poorly developed in aquatic species. Lymphoid accumulations are found in the kidneys (intertubular regions), and in the liver (subcapsular zone and small, deeper foci). *Gut associated lymphoid tissue* (GALT) has also been demonstrated in amphibians.

The sizes of erythrocytes vary considerably within the class Amphibia. Urodeles have larger cells, whereas anurans—especially high altitude forms—have much smaller cells, present in large numbers. Amitotic divisions occur, resulting in nucleate as well as anucleate red cells of various, irregular shapes. The cells become round later, when the rearrangement of the cytoskeleton has been completed (Turner 1988, Ginsburg et al. 1989). The DNA in mature erythrocytes is inactive and situated along the nuclear envelope. The nuclear lumen, like the rest of the cell, contains haemoglobin. Amphibian haemoglobins are tetrameric. The Bohr effect is enhanced by the aggregation of deoxygenated haemoglobin molecules (Turner 1988). The aggregates dissociate as oxygenation proceeds. In the bullfrog, *Rana catesbeiana*, four larval-type and four adult-type haemoglobins have been described (Turner 1988).

Four different types of granulocytes have been described in amphibians. The descriptions are mainly morphological, and it should not be taken for granted that they are homologous, or even analogous, to mammalian cells of similar appearance.

T and B subsets of lymphocytes have been demonstrated in anurans. In urodeles, there is evidence of cooperation among different subsets of cells following antigen challenge. A tetrameric IgM is produced early in the response; later an IgG-like monomer is synthesized. The presence of antibody produc-

ing cells of non-lymphocytic origin has been reported in an anuran (Turner 1988). The origin of this blast-like cell type is still to be discovered.

Amphibian Immunology

The clawed toad (*Xenopus laevis*). Descriptions of amphibian immunology are mainly based upon studies of the clawed toad, or South African frog, *Xenopus laevis* (Figure 1), since this species is the most thoroughly examined. However, the clawed toad, belonging to the family Discoglossidae, is by no means representative of the "average" amphibian (or even anuran), nor is it among the most highly developed amphibians. In *Xenopus*, the immunological organs (DuPasquier et al. 1989) are: the thymuses, the spleen, and the perihepatic zone (in larval and adult life). Lymphoid tissue is found in the pharynx (before metamorphosis) and in the kidneys. Bone marrow develops at metamorphosis (DuPasquier et al. 1989) and shows seasonal variation (Hadji-Azimi et al. 1987). Lymphomyeloid cells are found in association with gills and gut (DuPasquier et al. 1989, Jurd 1987). *Xenopus* does not possess lymphomyeloid nodes. Blood cells have been described in detail (Hadji-Azimi et al. 1987) in *Xenopus*, which has erythrocytes, thrombocytes (responsible for clotting), lymphocytes, monocytes/macrophages, and four distinct types of granulocytes. B- and T-lymphocytes have been identified, and active plasma cells are found in circulation in immunized toads (DuPasquier et al. 1989). Two classes of immunoglobulins have been demonstrated (Jurd 1987): a high molecular weight IgM, and a low molecular weight IgRAA.

Immune function during metamorphosis. Immune development is closely linked with other developmental changes during metamorphosis. The free-living amphibian larvae are immunocompetent (Flajnik et al. 1986), and thus able to defend themselves against microorganisms. The larval antibody repertoire is less heterogenous, and consists of other idiotypes (DuPasquier et al, 1979) than that of the adult, metamorphosed amphibian.

Metamorphosis includes formation of a high number of new antigeneic compounds and disappearance of a number of larval antigens. During metamorphic development, these antigens will be present simultaneously. The antigen change is correlated with changes in the immune system. Lymphocytes of larval type are tolerant of larval antigens, but both disappear in the course of metamorphosis. During maturation of the new "adult type" immune system, the new lymphocytes become tolerant of the new antigens, but not of the larval antigens (Flajnik et al. 1987, Horton et al. 1989), probably because they do not coexist with them long enough for induction of immunological tolerance (DiMarzo and Cohen 1982). Thus, there may be a transient state of internal histoincompatibility (Horton et al.

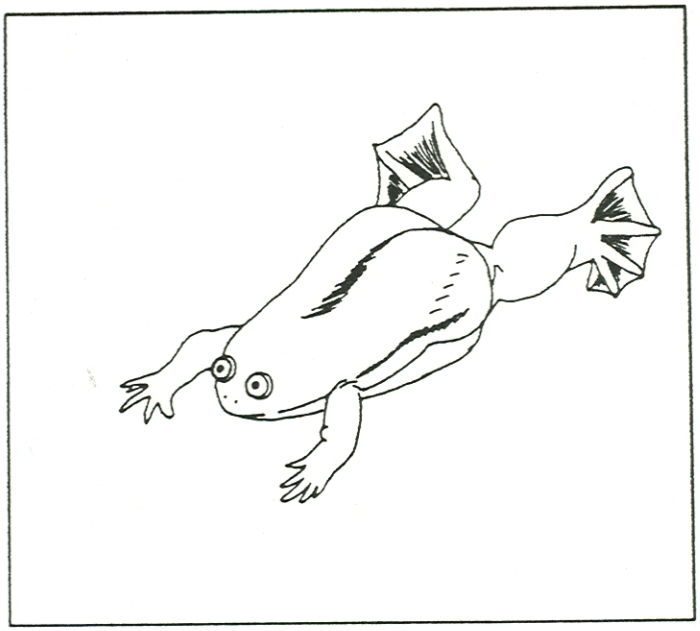


Figure 1. The clawed toad, *Xenopus laevis*. The adult *Xenopus* is aquatic, but goes to the surface to breathe.

1989, Jones and Ruben 1981) in the metamorphosing amphibian. Studies of the clawed toad, *Xenopus laevis* (DuPasquier et al. 1979, Kalami et al. 1986), have revealed that the immune system undergoes major alterations during metamorphosis. MHC class II antigens, involved in mutual recognition of immunocompetent cells, are present at larval stages, whereas MHC class I antigens ("transplantation antigens"), are not found until anatomical metamorphosis has taken place (Jurd 1977, Flajnik et al. 1986). Allogeneic skin transplants last longer (Bernardini et al 1969) in metamorphosing animals than in postmetamorphic adults, and immunological tolerance may be induced more readily during metamorphic development (Chardonnens and DuPasquier 1973, DiMarzo and Cohen 1982) than in other periods of life. This indicates the possibility of immunosuppression during metamorphosis (DuPasquier and Bernard 1980).

The Mexican Axolotl (*Ambystoma mexicanum*)

Immunological organs. In the axolotl, the most important immunological organs are the spleen, the thymuses (at least during development), and the liver. The axolotl has no lymphomyeloid nodes, and neither bone marrow nor GALT has been demonstrated. The six distinct thymuses are situated on the dorsal side of the head, proximal to the gills. In the axolotl spleen, there is no distinction between red and white pulp, as in higher vertebrates. In the liver, the perihepatic zone has haemopoietic function.

Immune function. Axolotls appear to have a well-functioning immune system, since animals maintained in captivity are not prone to infections. However, few studies have been undertaken regarding immune functions. Skin graft rejection is slow, lasting several months (Ching and Wedgwood 1967, Ussing 1991). *In vitro* mixed lymphocyte reaction (MLR) is weak (Jones and Ruben 1981). Two classes of antibodies have been described, a high molecular weight IgM (Charlemagne 1987), and a low molecular weight IgY (Warr et al. 1982, Fellah and Charlemagne 1988). Antibody response is slow; maximum serum titer is reached 60-80 days after immunization (Charlemagne and Tournefier 1977). Secondary response (after repeated immunizations) is not markedly accelerated (Charlemagne and Tournefier 1977). Thymocytes appear to be mainly of suppressor function (TS cells), since early thymectomy, or irradiation of the thymus, leads to increased antibody response (Charlemagne 1979 and 1981).

Available reports on the axolotl immune system mainly concern adult, neotenic animals. We therefore decided to study blood cells in young larval axolotls before, during, and after induced metamorphosis. Metamorphosis was induced with T3, as previously described (Ussing and Rosenkilde 1993a). Blood smears (Figure 2) and organ imprints were prepared, following routine procedures for haematological staining and identification (Ussing 1991, Ussing and Rosenkilde 1993b).

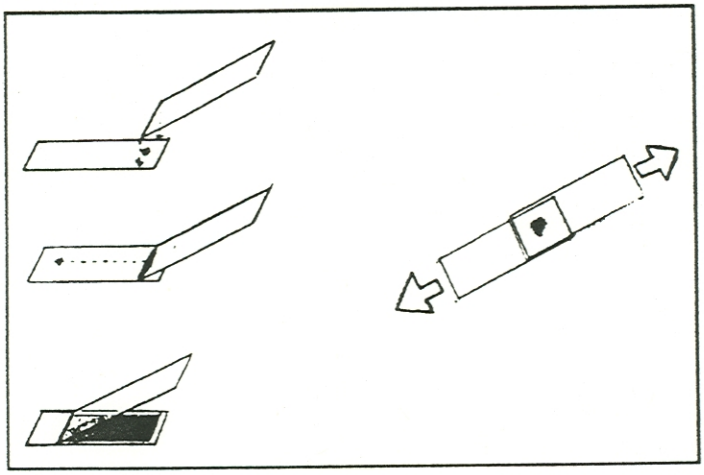


Figure 2. Methods for making blood smears. Blood from axolotls is "sticky" and will not spread to an even film with the technique used for mammalian blood (left). Instead, cytosmears were prepared by placing a 20-40 µl drop between two specimen slides. Blood was allowed to spread to the edges of the glass slides (held horizontally), which were then drawn from each other with a smooth movement (right).

Axolotl haematology. All blood cell types described for anurans were also found in axolotls at all the examined stages: erythrocytes, small and large thrombocytes, lymphocytes, monocytes, macrophages, and granulocytes. In addition to the well-known granulocyte types (eosinophils, basophils, and polymorphnucleated neutrophils), we found neutrophilic granulocytes with bilobed nuclei. This peculiar cell type, designated the *Pelger-Hüet-anomaly-like cell*, or "PH-cell," was first described in an amphibian by Hadji-Azimi et al. (1987). These cells resemble leucocytes found in the circulation of leukaemic patients with *Pelger-Hüet anomaly*.

The effect of metamorphosis on blood cell pattern. Prior to metamorphosis, eosinophilic granulocytes were the most abundant WBCs. Eosinophils showed a decrease in number during metamorphosis. Lymphocyte numbers decreased temporarily during metamorphosis, but grew more abundant after metamorphosis than before. Large, weakly staining thrombocytes were most abundant in neotenic axolotls, whereas the small, densely staining thrombocytes predominated in postmetamorphic animals. Erythrocytes undergoing amitotic division were found in animals at all stages, whereas mitotic divisions (with visible chromosomal figures), occurred only during metamorphosis (Ussing 1991). The results are summarized in Table 1.

In two independant studies, we found ciliates in the blood from axolotls in midmetamorphosis or metamorphic climax. The parasite (Figure 3) was identified as *Chilodonella sp.*, using the key by Hoffman et al. (1975). Protozoans of this genus are usually reported as external parasites of fishes, feeding on the gills (Ali et al. 1988a and b). The ciliate was not encountered in blood samples from neotenes, nor in animals that had completed metamorphosis. Premetamorphic blood samples even from the same axolotls that were infected during metamorphosis (Figure 4) were free from parasites. In some cases, the parasites were found in numbers as high as 10% of the WBC count.

Quantitative determinations of cells were obtained using an electronic differential blood

Table 1. Circulating cells found in axolotls before, during, and after experimentally induced metamorphosis.

	METAMORPHIC STAGE		
	pre-	mid-	post-
ERYTHROCYTES			
nucleate:	+	+	+
anucleate:	(+)	(+)	(+)
mitotic division:	–	+	–
amitotic division:	+	+	+
THROMBOCYTES			
large (immature):	+	+	+
small (mature):	+	+	+
LYMPHOCYTES:	+	+	+
GRANULOCYTES			
eosinophils:	+	+	+
basophils:	(+)	(+)	(+)
Neutrophils			
polymorphnucleated:	+	+	+
PH-cells (bilobed n.):	(+)	(+)	(+)
MONOCYTES/MACROPHAGES:	(+)	(+)	(+)
PARASITES			
Chilodonella:	–	+/–	–

( ): minor contribution; <: increase in relative numbers during development; >: decrease in relative numbers during development.

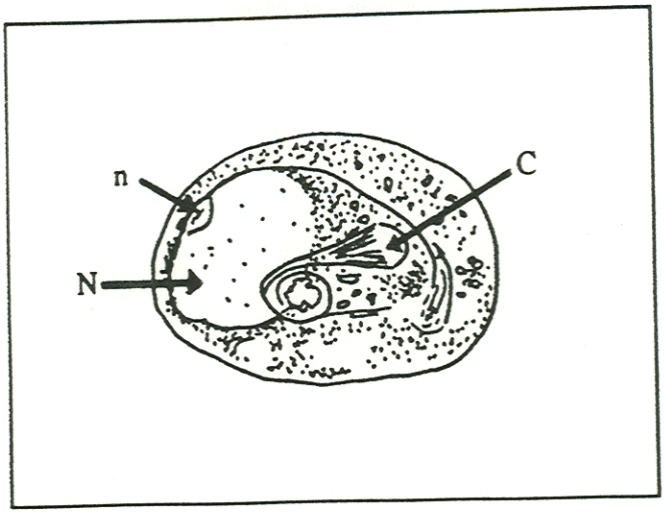


Figure 3. The invasive ciliate *Chilodonella*. N: macronucleus, n: micronucleus, C: cytostoma.

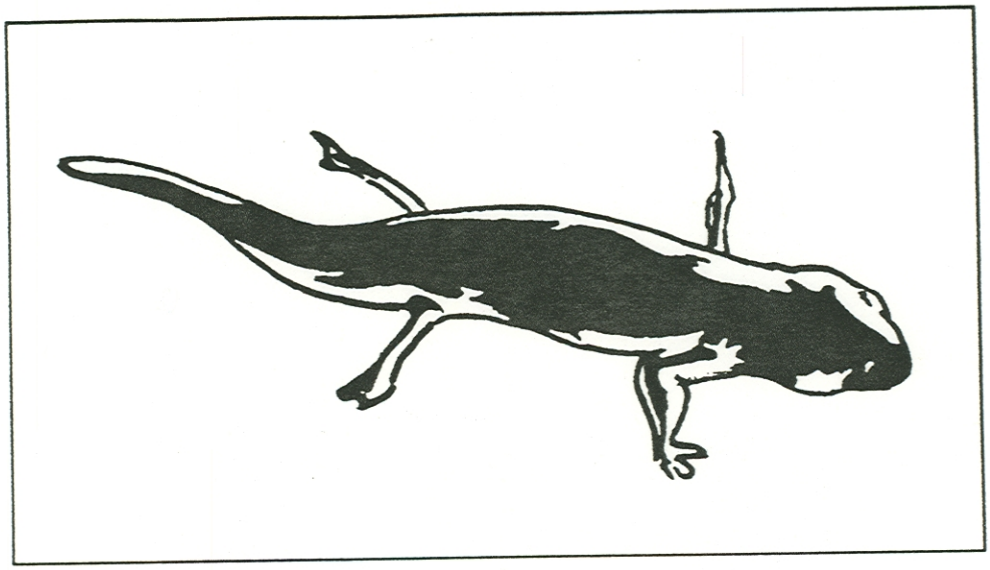


Figure 4. Infected axolotl: a young animal at metamorphic climax (gill remnants overgrown but not completely resorbed).

cell counter (Ussing 1991). To obtain a functional quantitation of leucocyte types (and not only relative values), the ratio of each leucocyte type to RBC numbers was calculated. The WBC/RBC ratio decreased during metamorphosis (Table 2). Lymphocytes, eosinophils, and polymorphnucleated neutrophils showed marked alterations in relative as well as in corrected numbers during metamorphic development. The values for these three cell types are shown in Figures 5 and 6.

Haemocytoblasts (RBC stem cells) were found in circulation at all stages, though more frequently in young animals. Mitotic divisions of mature erythrocytes were seen in midmetamorphic animals only. Spleens underwent

Table 2. Number of WBC/1,000 RBC (mean ± s.d.):

Neotenes:	36.4 ± 27.2	(n = 7)
Midmetamorphic:	26.0 ± 12.1	(n = 8)
Newly metamorphosed:	17.2 ± 3.1	(n = 4)
One month postmetamorphic:	4.5 ± 3.8	(n = 4)

drastic changes during metamorphosis: the contents of mature red cells disappeared during midmetamorphosis and metamorphic climax, and were only gradually replaced in postmetamorphic animals. There were no signs of anaemia, or of increased haematopoiesis (i.e., haemocytoblasts or proerythroblasts) in circulation during metamorphic climax or early postmetamorphosis. The switch from larval to adult haemoglobin takes place early in neotenic development (corresponding to metamorphosis in transforming species), whereas functional changes in oxygen affinity, presumably regulated by organic phosphates (Turner 1988), occurs in relation to anatomical metamorphosis. The macro- and microscopic changes in spleens occurred during induced metamorphosis in both young (< 6 months) and adult (≥1 year) axolotls.

Discussion. The observations on circulating cells show that the axolotl blood cell system is as diverse as that described for anuran amphibians. Despite the lower phylogenetic level and the neotenic condition, all morphological cell types described in *Xenopus* were also found in our axolotls.

The observed decrease in lymphocyte numbers in metamorphosing axolotls suggests a decrease in humoral immune response during TH-induced metamorphosis in the axolotl. The transient increase in eosinophil percentage during metamorphosis, combined with the decrease in relative numbers of all other leucocytes points to a major role of the eosinophilic granulocyte in immune defence during metamorphic climax. Comparison of relative and corrected ("absolute") numbers indicate that the transient increase in eosinophilic percentage is caused by a decrease in number of all other leucocyte types during metamorphic climax. During late metamorphic and early postmetamorphic stages, eosinophil numbers continue to decrease, whereas lymphocytes and polymorphnucleated neutrophils increase in number. In adult axolotls, polymorphnucleated neutrophils become the most abundant leucocyte type after completion of metamorphosis. Also lymphocytes become more important in postmetamorphic axolotls, since immune reactivity is enhanced, as seen through activation/release of plasma cells in(to) circulation after antigen challenge.

The Pelger-Hüet cells known from *Xenopus* (Hadji-Azimi et al. 1987), were found in both neotenic, metamorphosing, and postmetamorphic axolotls. The fact that PH-cells have been demonstrated in adult *Rana temporaria*, but not in newly metamorphosed froglets (Ussing 1991), suggests that this cell type does not exert important functions until later in life.

The infection with the ciliate *Chilodonella* could take place if the protozoans were internalized with parts of the shrinking gills, or they might have penetrated the surface actively. The parasites were most probably the cause of the poor survival of metamorphosing axolotls during the spring when the infections were discovered. Invasive *Chilodonella* ciliates in fish are reported to be cytophagous, and infections are lethal within 2 or 3 weeks, depending upon the condition and gender of the fish (Hoffman et al. 1975). In recent studies (unpublished) we have found other protozoan species in axolotl blood smears, always from animals in midmetamorphosis. We have also observed protozoans in blood drawn from *Rana dalmatina* during metamorphic climax. The fact that the parasites occur in the organism during metamorphosis, and only then, suggests that the immune defence is weakened in some as yet undefined ways during metamorphosis.

The question of active immunosuppression during amphibian metamorphosis has been raised (DuPasquier and Bernard 1980). In the axolotl, the developmental changes may be more gradual than in anurans and transforming urodeles. The transient decrease during metamorphosis of all WBC types except eosinophils support other indications that at

Figure 5. Relative numbers (% of WBC).

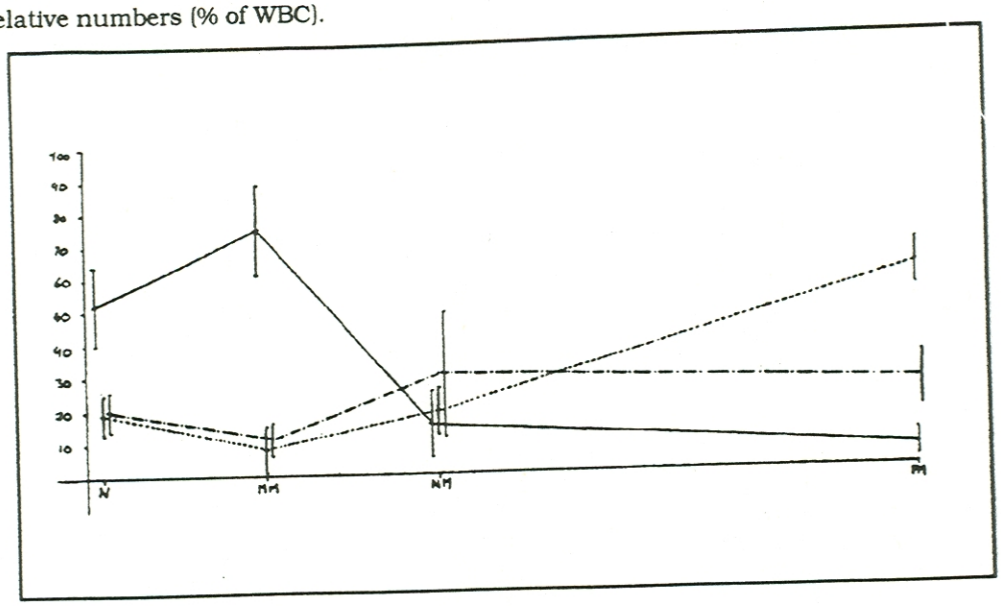
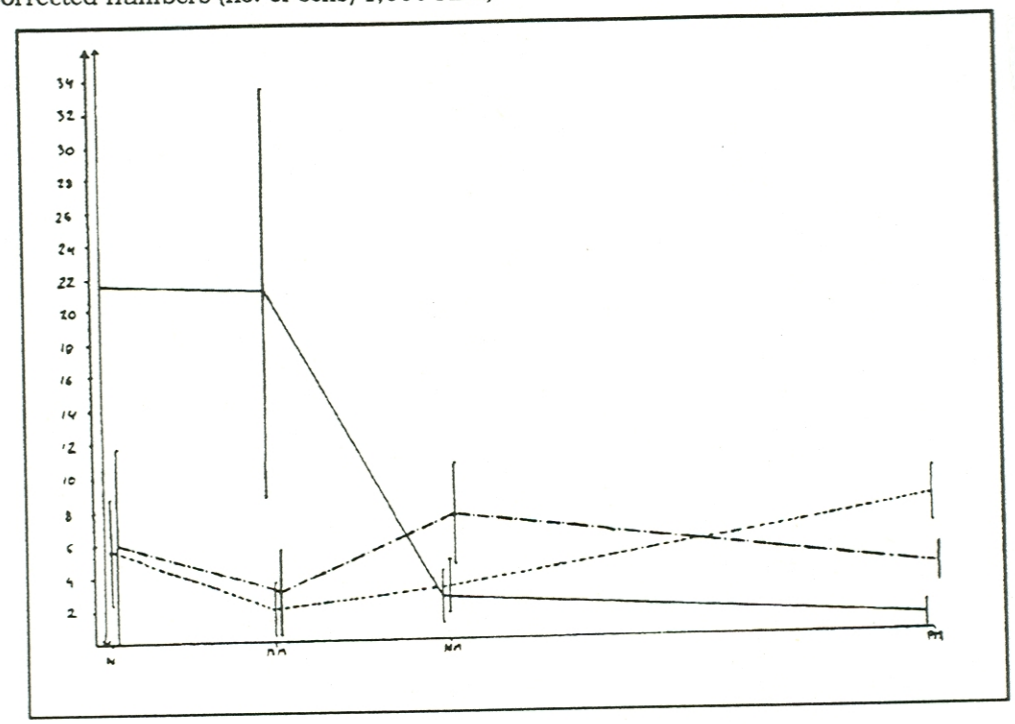


Figure 6. Corrected numbers (no. of cells/1,000 RBC).



Figures 5 and 6. Frequency of leucocyte types during metamorphosis. _____ eosinophils; —.—.— lymphocytes; ..... polymorphnucleated neutrophils. N: neotenic; MM: midmetamorphic; NM: newly metamorphosed, PM postmetamorphic axolotls. Y = mean ± s.d.

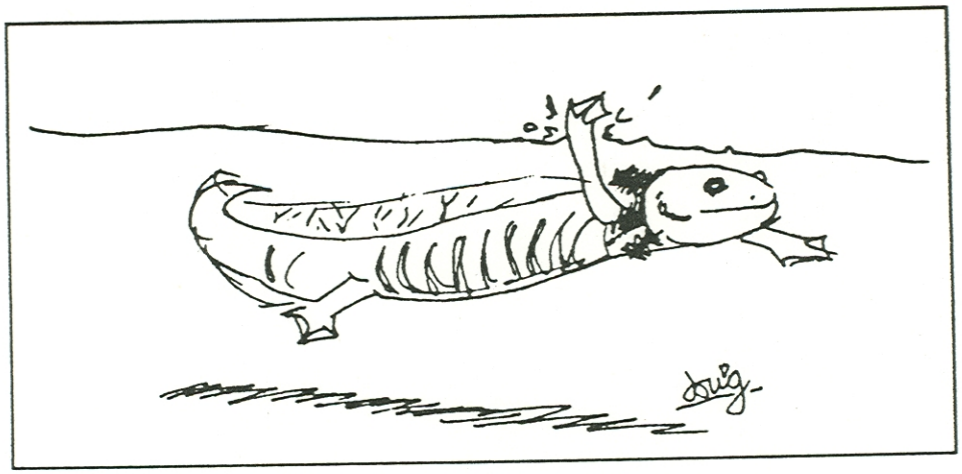
least some immune functions are suppressed. The *Chilodonella* infections could be facilitated during a period of immunosuppression. The mechanisms leading to decreased immune activity are yet to be described. *In vitro* investigations (Ruben et al. 1990), indicate that low levels of *interleukin* (IL-2) may be of importance.

Immunization results (Ussing 1991) show that neotenic and metamorphosed axolotls differ in the mechanisms evoked by immunization, using a soluble (protein) antigen, and a reaction period of three weeks. There were no changes in the blood cell pattern of neotenic axolotls after 1, 2, or 3 antigen challenges. In newly metamorphosed axolotls, a 3- to 4-fold increase in active plasma cells was observed in animals challenged two or three times, whereas animals challenged only once showed no difference from controls injected with saline. The observed reaction after repeated injections may be caused either by the repetition (second and third challenges) or by the summation of the antigen load (increased total dose).

Axolotl immunology is still a new field to be investigated. Available reports are not always comparable, since animal colonies may show considerable variation. Also environmental factors such as temperature (Tahan and Jurd 1978), season of the year (Garrido et al. 1989, Saad et al. 1990), or laboratory conditions (Dulak and Plytycz 1989) may exert influence on immune functions of lower vertebrates.

Conclusion. The axolotl is diverse and well-functioning with respect to the immunological and haematological features studied so far. The immune system comprises a variety of leucocyte types. Serum complement (Avila and Lambris 1990) and two classes of immunoglobulins have been demonstrated. Induced metamorphosis led to alterations in ratios of different leucocyte types, suggesting a qualitative difference in mechanism of immune response in neotenic versus metamorphosed axolotls. Fellah et al. (1989) have demonstrated that neither thyroxine (T4)-induced metamorphosis nor repeated antigen challenge was capable of promoting formation of low molecular weight antibodies (IgY) in very young axolotl larvae.

While thyroid hormone induced metamorphosis exerts direct influence on immunological cells, developmental factors such as growth are also important for maturation of the "adult type" immune functions of metamorphosed amphibians. The latter is especially the case for lymphocytes and neutrophilic granulocytes. The observations suggest that development of the adult amphibian immune system requires maturation, as reached through increased age and size, and underlines the fact that metamorphosis and growth are integrative and not separate events of amphibian development.



Drawings by Jonas Ussing.

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