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Activation by mitogens and superantigens of axolotl lymphocytes: functional characterization and ontogenic study

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SUMMARY

Urodele amphibians have weak and slow immune responses compared to mammals and anuran amphibians. Using new culture conditions, we tested the ability of lymphocytes of a well-studied salamander, the Mexican axolotl (*Ambystoma mexicanum*) to proliferate *in vitro* with diverse mitogenic agents. We demonstrated that the axolotl has a population of B lymphocytes that proliferate specifically and with a high stimulation index to the lipopolysaccharide (LPS) known as a B-cell mitogen in mammals. This proliferative capacity is observed without significant changes throughout ontogenesis. In the presence of LPS, axolotl B lymphocytes are able to synthesize and secrete both isotypes of immunoglobulin described in this species, IgM and IgY. Moreover, a distinct lymphocyte subpopulation is able to proliferate significantly in response to the mitogens usually known as T-cell specific in mammals, phytohaemagglutinin (PHA) and concanavalin A (Con A). The activated cells are T lymphocytes, as shown by depletion experiments performed *in vitro* with monoclonal antibodies, and *in vivo* by thymectomy. Splenic T lymphocytes of young axolotls (before 10 months) do not have this functional ability, which suggests maturation and/or migration phenomena during T-cell ontogenesis in this species. Axolotl lymphocytes are able to proliferate *in vitro* with a significant stimulation index to staphylococcal enterotoxins A and B (SEA and SEB). These products act on mammalian lymphocytes as superantigens: in combination with products of the major histocompatibility complex (MHC), they bind T-cell receptors with particular Vβ elements. The fact that these superantigens are able to activate lymphocytes of a primitive vertebrate suggests a striking conservation of molecular structures implied in superantigen presentation and recognition.

INTRODUCTION

In an almost identical mode as in mammals, anuran amphibians demonstrate acute cellular as well as humoral immune responses based on cellular co-operation between T and B lymphocytes.[1] The more primitive urodele amphibians, including the Mexican axolotl *Ambystoma mexicanum*, display chronic cellular and humoral immune responses that are subdued in comparison with anurans. These responses are mediated by T and B lymphocytes, characterized by monoclonal antibodies,[2–4] and are modified after thymectomy.[5] As T-cell receptor and immunoglobulin genes have been characterized in axolotl,[6–9] these distinct T and B lineages co-exist in urodeles.

However, humoral immune responses implying a T–B co-operation, with isotype switching and affinity maturation leading to an amplified secondary response, are absent.[10,11] Moreover, mixed lymphocyte reaction (MLR) of major histocompatibility complex (MHC) disparate spleen cells from outbred axolotls are very modest.[12] These defaults in urodele amphibian immune responses suggest an incompetence of helper T cells (and/or B cells), or inadequate culture conditions. In lower vertebrates, the use of fetal calf serum (FCS) strongly inhibits *in vitro* immune functions. The use of different mitogenic agents in Urodela was unsuccessful[13] until optimum culture conditions were established,[12,14] allowing the detection of T-cell growth factor (TCGF) activity.[15]

In order to analyse the functional and respective capability of the two different T and B cell lineages in adult axolotl, as wellas the ontogenesis of T- and B-lymphocyte reactivity, we performed *in vitro* polyclonal activation assays in improved culture media, using various lectins, bacterial lipopolysaccharide (LPS) and superantigens as mitogens, with or without *in vivo* and *in vitro* depletion of lymphocyte subpopulations. A specific T-cell activation was clearly demonstrated with concanavalin A (Con A) and phytohaemagglutinin (PHA); LPS had a mitogenic effect that was specific to B cells and also induced *in vitro* antibody synthesis. Staphylococcal enterotoxins A and B (SEA and SEB), which act as superantigens in mammals,[16] were also able to activate axolotl lymphocytes.

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During ontogenesis, *in vitro* stimulation assays with PHA and Con A showed a positive correlation between age and proliferative response to these agents. Assays using LPS demonstrated an absence of such a correlation for this mitogen.

MATERIALS AND METHODS

Animals

Axolotls of the Ax6 strain were bred in 16° running water in our laboratory, and fed with commercial trout pellets. Animals used in the following experiments were 4 months to 2 years old. Thymectomy was performed on 10-month axolotls as described by Charlemagne & Tournefier.[5]

Culture conditions and proliferation assays

Animals were anaesthetized in MS 222, spleens and/or thymi were removed aseptically and teased with forceps in cold amphibian phosphate-buffered saline (PBS-A). Cells were then washed three times in this buffer, and suspended at $0{\cdot}25 \times 10^6$ cells/ml in Leibovitz's-15 (L-15) diluted to amphibian osmolarity (L15-A), buffered with 10 mM HEPES (Gibco BRL Life Technologies, Grand Island, NY) and supplemented with 10 mM $NaHCO_3$, 0·1% glucose (w/v), 20 mM 2-mercaptoethanol (2-ME), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco) and 2 mM L-glutamin (Gibco). Some experiments were performed with 'complete' medium containing 0·25% bovine serum albumin (BSA/fraction V powder; Sigma, St Louis, MO) or 1% heat-inactivated FCS (Gibco). Two other media were compared with L-15 in axolotl cell culture: Dulbecco's modified Eagle's medium (DMEM), and RPMI-1640 (Gibco) diluted to amphibian osmolarity (DMEM-A and RPMI-A respectively) and supplemented with the same concentrations of HEPES, antibiotics, 2-ME and L-glutamin as in the L-15. Aliquots, 200 μl, of the cellular suspensions (5×10^4 cells) were dispensed into 96-well flat-bottomed plates (Nunc, Roskilde, Denmark).

Mitogens tested were PHA-C (IBF, Villeneuve-la-Garenne, France), Con A (Sigma), pokeweed mitogen (PWM; PolyLabo, Strasbourg, France) and LPS from *Escherichia coli* serotype 026:B6 (Sigma). SEA and SEB (Sigma) were used in several experiments. Dilutions were prepared in culture medium in appropriate concentrations and added in 20-μl aliquots to culture wells at the initiation of the culture.

Cells were cultured with mitogens, or with medium alone as a control, for 4 days at 26° with 5% CO_2. On the fourth day they were pulsed with 1 μCi [^{3}H]methyl-thymidine (Amersham, Arlington Heights, IL); 18–24 hr later they were harvested onto a glass fibre filter. β radioactivity was measured with a liquid scintillation counter.

Stimulation indices (SI) were calculated as the mean incorporation of stimulated samples divided by the mean incorporation of unstimulated samples. Three to six replicates were tested for each dilution of mitogen.

Antibodies

Monoclonal antibodies (mAb) used in this study were all as described previously.[2,3,17] Monoclonal antibodies specific for axolotl B cells were 33.101.2, 33.45.1 and 33.39.2, specific for light chains, μ heavy chain and *v* heavy chain of axolotl immunoglobulin, respectively; 19-69 is specific for axolotl immunoglobulin. The other mAb used were specific for non-B cells: 34.38.6 recognizes membrane polypeptides present on non-B lymphocytes, as well as on macrophages, and on a subpopulation of granulocytes;[2] the polyclonal rabbit antiserum L12 was directed against a 38 000 MW protein specific for the T-cell lineage.[4]

Cellular separations

Spleen cells were incubated with 20 μg/ml of purified antibodies diluted in sterile PBS-A. After 30 min incubation on ice, cells were washed twice in cold PBS-A, and incubated in 10 ml PBS-A containing 75 μl magnetic beads (approximately three beads per cell) coated with anti-mouse or anti-rabbit immunoglobulin (Dynabeads, Dynal, Oslo, Norway) for 45 min at 4° with gentle mixing. Separation was achieved by three cycles of 1 min with a magnet; adherent cells were discarded; non-adherent cells were washed and diluted in culture medium for proliferation assays as described above.

Enzyme-linked immunosorbent assay (ELISA)

A mixture of three mouse monoclonal antibodies specific for axolotl IgM and IgY (33.101.2, 33.45.1 and 33.39.2) diluted in Na carbonate buffer, pH 9·6, was bound to ELISA 96-well plates (Maxisorb; Nunc) for 8 hr at room temperature. The plates were washed with Tris-buffered saline (TBS) (Tris–HCl 10 mM, pH 7·4, NaCl 150 mM) and blocked with TBS–0·02% NaN_3 containing 3% BSA. After washing in TBS with 0·05% Tween (T–TBS), 100 μl of supernatant from cultures of axolotl spleen lymphocytes was added to the wells, and incubated 45 min at 37°. Wells were washed again three times with T–TBS. The biotinylated monoclonal antibody 19-69 (a gift from Dr V. Laurens) specific for axolotl immunoglobulin was incubated on the plates for 45 min at 37°. After washing as before, streptavidin–alkaline phosphatase was added for 45 min at 37°. Wells were washed again and developed with the substrate p-nitrophenyl phosphate, disodium (Sigma) for 45 min at room temperature. Optical density was read at 405 nm.

RESULTS

In vitro stimulation of axolotl lymphoid cells by mitogens and superantigens

As previously described by Koniski & Cohen,[14] FCS strongly inhibits PHA- or Con A-induced lymphocyte proliferation. We performed comparative assays using FCS, BSA or without any supplementary protein in the culture medium, and partly confirmed these results. In our experiments FCS was also a potent inhibitor of PHA stimulation. In addition, BSA reduced the ability of splenocytes to proliferate in response to LPS. Therefore most cultures were done in L-15 medium without serum or BSA.

A kinetic study was done for each mitogen: thymidine incorporation between the fourth and the fifth day after setting cultures was suitable for an optimal activation by all mitogens tested. Axolotl lymphoid cells were able to proliferate significantly in response to PHA, Con A, PWM and LPS under these conditions. Figure 1 shows c.p.m. results obtained in one experiment (corresponding SI were on day 4/5: 5·0 for PHA, 18·2 for LPS, 5·6 for PWM). In further experiments, results are expressed as SI, in order to compare animals and experiments. Each mitogenic agent was used at its optimal

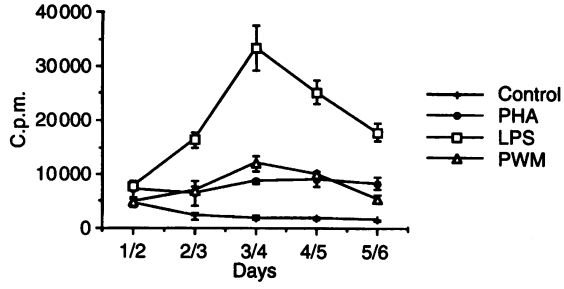

Figure 1. Splenocyte proliferation kinetics with different mitogens. Splenocytes from adult axolotls were cultured in L-15-A without any mitogen (control) or with PHA (1/100 final dilution), LPS (50 μg/ml) or PWM (5 μg/ml); [^{3}H]thymidine was added one day after, or on day 2, 3, 4 or 5, and cells were harvested 24 hr later. C.p.m. data are shown with SD values obtained from quadruplicate cultures.

concentration (previously established), which was similar in mammals (PHA, 1/100; Con A, 5 μg/ml; PWM, 5 μg/ml; LPS, 50 μg/ml).

We tested the superantigens SEA and SEB for their mitogenic properties in the axolotl. They were able to induce a significant cellular proliferation, with SI > 2 in DMEM-A or RPMI-A, whereas L-15-A medium failed to support such an activation in most assays. Individual animals were very different in their proliferative responses to enterotoxins. Although some of the axolotls seemed unable to respond to SEB with a significative index, all axolotls tested possessed a population of lymphocytes able to react with SEA (Fig. 2). Experiments showing that thymic lymphocytes significantly proliferated with SEA and SEB suggested that superantigen-reactive cells were T cells.

Characterization of proliferating cells

In order to determine whether these mitogens make it possible to characterize functional T cells, as in mammals, we used magnetic bead separations. Splenocytes were B-depleted either with a panel of monoclonal antibodies specific for light and heavy chains of axolotl immunoglobulin, or with the pan-immunoglobulin specific mAb 19-69. In every test performed, compared to total splenocytes, the population of T-enriched lymphocytes proliferated significantly better with PHA (SI raised 2–7·6 times), and also in one case with Con A. The reverse occurred with LPS (SI decreased 1·5–7·2 times) and PWM (Table 1).

We performed complementary experiments using the monoclonal antibody 34-38-6 (with or without the rabbit polyclonal serum L12) for depleting splenic population of T cells. T depletion strongly reduced PHA- and Con A-induced proliferation of splenocytes (SI means decreased threefold),

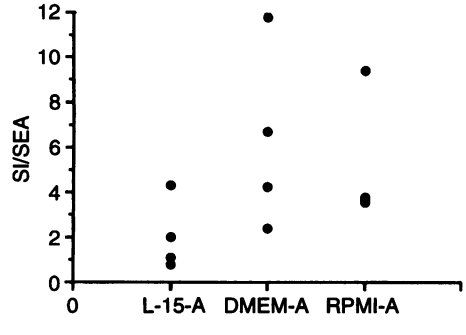

Figure 2. Proliferation to staphylococcal superantigen SEA in different culture media. Splenocytes from 12 adult axolotls were tested with SEA (5 μg/ml). Each point represents the mean SI value obtained from quadruplicate cultures.

Table 1. Effect of B depletion on splenocyte mitogenic stimulation

Mitogen	Exp.	Total cell SI	B-depleted cell SI
PHA	1*	7·2	55
	2*	2·9	12
	3†	4·5	9·2
Con A	1	4·2	24
	2	2	2
	3	1·1	1·1
PWM	1	—	—
	2	9	3·8
	3	8·5	1·4
LPS	1	21	14
	2	19	3·9
	3	18·1	2·5

Three different experiments of depletion were performed on adult splenocytes by using different antibodies specific for axolotl immunoglobulin. Total and B-depleted splenocytes were then cultured with PHA (1/100), Con A (4 mg/ml) and LPS (50 mg/ml) for 5 days in L-15-A, using standard conditions. Results are shown as SI for each mitogen and for both cell populations.

* Depletion performed with mAb 33.101.2, 33.39.2 and 33.45.1, specific respectively for the light chain and μ and ν heavy chains of axolotl immunoglobulin.

† Depletion performed with mAb 19.69.

Table 2. Effect of T depletion on splenocyte mitogenic stimulation

Mitogen	Exp.	Total cell SI	T-depleted cell SI
PHA	1*	25	6·5
	2†	21	7·5
	3†	5·1	2·2
Con A	1	25	9·1
	2	8·4	2·7
	3	3	1
PWM	1	22	16
	2	11	7·9
	3	8·4	3
LPS	1	14	28
	2	40	21
	3	12·1	4·5

Three different depletion experiments were performed with antibodies recognizing T-expressed molecules; cells were then cultured as described for Table 1.

* Depletion mediated by the purified polyclonal rabbit antiserum L12, specific for the T-cell lineage, and mAb 34.38.6, characterizing a 65 000–68 000 MW antigen present on the T-cell lineage.

† Depletion performed with mAb 34.38.6.

Table 3. Effect of thymectomy on splenocyte mitogenic stimulation

Mitogen	Exp.	Control SI	Thymectomized SI
PHA	1	11·5	5·3
	2	7.4	5
	3	3·2	2
Con A	1	2·3	0·8
	2	5·1	2
	3	2	1·1
LPS	1	82·7	63
	2	19·5	28·6
	3	23	33

Young axolotls were thymectomized at 10 months, and tested for their ability to proliferate in response to PHA, Con A and LPS between 10 and 18 months after thymectomy. Results obtained in three different experiments (1, 2 and 3), each including a control and a thymectomized animal, are shown as SI.

and had a lesser effect on PWM stimulation. B enrichment consecutive to T depletion had variable effects on LPS action (Table 2).

Thymectomy was performed on young axolotls and then the mitogenic effect of different mitogens was tested *in vitro* in these animals. This *in vivo* T depletion resulted in the same inhibitive effect on the *in vitro* PHA and Con A proliferative responses as with the *in vitro* depletion experiments. The stimulation with LPS was again variable (Table 3).

These results show that T and B lymphocytes are clearly functionally distinct in the axolotl, since they are each able to proliferate in response to different mitogens. PHA and Con A specifically stimulate T cells, whereas LPS is a potent mitogen for B lymphocytes. PWM appears to be a mitogenic agent on both lymphoid populations.

These results were also confirmed by culture experiments with axolotl thymocytes. Thymic cells were induced to proliferate with PHA and Con A, with SI comparable to those obtained with splenic cells. LPS was unable to significantly activate these cells.

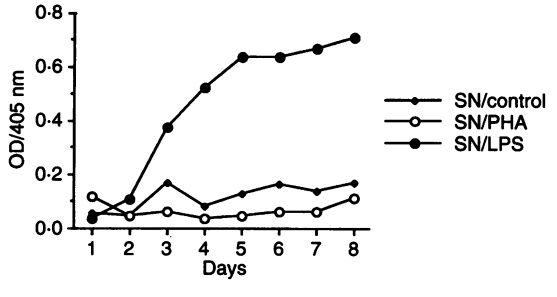

Figure 3. Presence of axolotl immunoglobulin in the supernatant of LPS-activated splenocytes. Axolotl splenocytes were cultured with 50 μg/ml LPS for 1–8 days, using usual conditions; supernatants were tested by ELISA for presence of immunoglobulin. Supernatants of cultures with PHA (SN/PHA) and without any mitogen (SN/control) were used as negative controls. Positive controls were dilutions of axolotl serum. Total secreted immunoglobulins were measured by ELISA using a panel of mAb specific for both immunoglobulin isotypes.

In vitro differentiation of B lymphocytes

Once we established the B specificity of LPS activation, we tried to demonstrate its ability to induce synthesis and secretion of immunoglobulins.

Axolotl splenocytes were cultured with LPS, PHA or without any mitogen for 8 days using standard conditions. Supernatants were harvested every day and assayed by ELISA for the presence of immunoglobulin. Data shown in Fig. 3 clearly demonstrate the ability of axolotl lymphocytes to secrete immunoglobulin after their activation by LPS. Unstimulated cells and cells cultured with PHA did not secrete a significant amount of immunoglobulin. The kinetics of immunoglobulin secretion paralleled LPS-induced proliferation: immunoglobulins were secreted from day 3, increased on day 4, and then accumulated in the supernatant. Further experiments designed to determine isotypes of secreted immunoglobulins were performed by ELISA with mAb specific for each type of heavy chain. Both types of immunoglobulin are secreted by LPS-activated lymphocytes. IgY were first present in the supernatant on day 3, IgM appeared later and their concentration was significant on day 5.

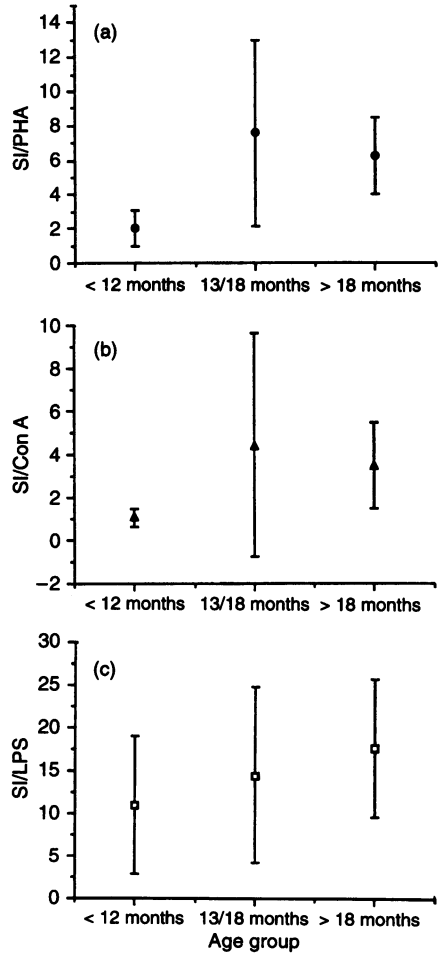

Figure 4. Mitogenic stimulation as a function of ageing. Results of proliferation tests, performed using the same experimental conditions for 60 individuals, were collected for PHA (final dilution 1/100; a) Con A (5 μg/ml; b), and LPS (50 μg/ml; c). Results are shown as means of SI values with SD values (error bars), inside each 'age group'.

Mitogen-induced proliferation during ontogenesis

Sixty axolotls between the ages of 5 and 26 months were compared for their ability to proliferate *in vitro* in the presence of PHA, Con A and/or LPS. Individual variability constituted a problem in an attempt to find a correlation between the age of individuals and their ability to respond to mitogens in culture. Therefore our study comprised a large number of individuals of different ages, all tested under the same culture conditions. Animals were separated according to age and sexual maturity, and therefore were at different stages of differentiation. Three groups were thus constituted, each of them representing a rather homogeneous proliferation ability. Juvenile axolotls (younger than 12 months) were either unable to respond or responded weakly to PHA and Con A. All sexually mature axolotls (>18 months) had a proliferative response to these lectins, with limited variation among them. A third group, comprising axolotls between 12 and 18 months, had very heterogeneous reactions, with both non-reactive and hyper-reactive individuals (Fig. 4a,b). The pattern was the same for PHA and Con A, both presenting clear T specificity, but with stronger stimulation with PHA. The LPS response did not show the same age-dependence (Fig. 4c). Even though the LPS-induced proliferation increased slightly with ageing, the three groups were not significantly different in their ability to react to LPS.

DISCUSSION

In an attempt to optimize culture conditions of axolotl lymphocytes, we looked at different critical parameters. We confirmed that FCS was a strong inhibitor of PHA- and Con A-induced proliferation; we also showed that BSA, reported by Koniski & Cohen[14] as an alternative source of protein in the medium, is convenient for PHA- and Con A-induced activation, but is a potent inhibitor of B proliferation induced by LPS. The inhibiting effect of FCS has been reported in many species, especially in fish. Pooled catfish and human sera are more convenient than FCS for mitogen-induced proliferation in channel catfish,[18] and a serum-free culture medium, including L-15, was shown to be able to support mitogen-induced proliferation and MLR in this species.[19] The serum factors preventing cell multiplication are unknown; it could be a direct toxic product for T and/or B cells, as shown in mammals with prostaglandins E1 and E2,[20] or a general toxicity due to the oxidation of the lipid fraction of the serum (this phenomenon can also occur with BSA, but is unlikely here because of the specificity of the inhibition on LPS-induced proliferation). Unknown components able to interfere with binding of the mitogen on its 'receptors' at the cell membrane, or at a later stage of signal transmission, may also explain the weak proliferation in the presence of FCS or BSA.

Individual variability is a common problem encountered in such studies with axolotl,[14] as with fish.[21] Several environmental factors could explain this high variability: immunological consequences of temperature and stress have been described.[22,23]

Endogenous features have to be considered for this high individual variability. The hypothesis of the existence of 'high responder' and 'low responder' has been described in other species such as the carp[21] and the chicken,[24] and related in this latter species to a genetic polymorphism (at least for the Con A response). The activation state of cells *in vivo* is itself rather variable, and is visible by the background proliferation (without any mitogen added *in vitro*). It is possible that these 'hyperactivated' lymphocytes (by antigens, superantigens and/or lymphokines) react in a different way *in vitro* to mitogens, or they may be induced to die by apoptosis, as sustained by the 'second signal' hypothesis.[25] The different molecules that bind mitogens at the cell surface, and act in signal transduction, could also be down- or up-regulated by previous and unknown *in vivo* activation, and interfere with measurable proliferation *in vitro*.

Specific depletion experiments mediated by monoclonal antibodies, and by thymectomy, show a clear functional dichotomy in lymphocyte subpopulations, demonstrated by their differential ability to proliferate in response to usual mitogens. B- and T-cell depletion experiments clearly identified, as in mammals and anurans, T lymphocytes able to proliferate specifically when incubated with PHA and Con A, and B lymphocytes specifically sensitive to LPS. However, some depletion experiments showed only a moderate increase or decrease of the mitogenic effect.

In vivo, with thymectomy which results in a physiological T-cell depletion, this could be the result of a partial migration of early T cells at the periphery (before 10 months). *In vitro*, more complex phenomena could explain the variable reactivity to LPS. As described in a murine model, polyclonal activation of B lymphocytes by LPS requires monocyte/macrophage-derived IL-1.[26] T depletions performed *in vitro* with mAb 34-38-6 also eliminate monocytes and could deprive B cells of a necessary signal for LPS-induced mitogenesis. In contrast, the use of very specific mAb, as in our magnetic bead depletions mediated by anti-immunoglobulin mAb, and as described by Kaufman *et al.*[12] in fluorescence-activated cell sorting (FACS) with the same mAb, allows T- and B-lymphocyte specificities to be distinguished clearly and to also demonstrate T lymphocyte functional ability in their mitogenic response.

We show here that LPS is a potent activator of axolotl B lymphocytes, stimulating growth and differentiation into IgM- or IgY-secreting cells. When incubated with LPS, murine B lymphocytes undergo proliferation and differentiation into IgM-secreting cells; class switching does not require cognate interaction between antigen-specific T and B cells, and different lymphokines regulating the subclass of secreted immunoglobulin have been identified in this experimental model.[27] The fact that both types of immunoglobulin are secreted by LPS-activated axolotl plasma cells is consistent with previous observations: expression of the two heavy chain isotypes seems mutually exclusive in this species and separate IgM and IgY cells appear at the same time in the larval spleen.[10,11] A class switching may not exist in axolotl, but cannot really be ruled out. The use of this polyclonal activation system, bypassing the requirement of T/B cellular interactions, could permit a functional study of isotype switching in the axolotl.

Staphylococcal enterotoxins (SE) have long been known for causing food poisoning; they are also mitogenic for T cells in mammals but, unlike other mitogens, they are dependent on interactions with MHC class II molecules for activation of a subpopulation of T cells expressing a peculiar T-cell receptor (TCR) Vβ. We tried to activate axolotl lymphocytes with SEA and SEB, and demonstrated that both enterotoxins were able to induce a significant proliferation of these cells. SEA seemed a

more powerful mitogen in our hands, but this could reflect non-optimal culture conditions for SEB, inasmuch as these differ for each mitogenic agent. SEA and SEB, if acting here as in mammals, bind to non-polymorphic conserved residues on MHC molecules[28] and on Vβ regions of TCR expressed by reactive T cells. This should mean that analogue structures do exist in the axolotl. Cloning of the axolotl MHC class II cDNA is in progress, and analysis of its sequence should confirm this hypothesis. This should be a good tool for a physiological characterization of these molecules. On the other hand, SE are able to induce a hyperactivation and then a deletion of reactive T cells after *in vivo* administration in mice; they have been used in a number of murine models for studying induction and modalities of negative selection in this species.[29] Nothing is known, however, in the axolotl about the thymic and/or extrathymic selection process; assays of *in vivo* and *in vitro* treatment of axolotl lymphocytes with SE are now in progress.

We undertook a large ontogenic study of axolotl lymphocyte proliferative ability, in order to look for a correlation between development and T- and B-cell functions. Individual axolotls were divided into three groups corresponding to three developmental stages. In each group, mitogen-induced proliferation had a specific pattern. In the juvenile stage group (6–12 months), we did not see any proliferation in response to T mitogenic agents; this result can be interpreted either as a lack of T cells in the spleen (a high relative proportion of immunoglobulin-positive cells has been reported at around 9 months in the spleen),[11] or as a more qualitative default in T cells present in the spleen at that moment. In the group of young adults, the clearest feature observed was the very high variability among individuals, with significant SI in the presence of PHA and Con A but high standard variation. This could be explained by the emergence around 12 months (but variable among individuals) of more or less mature cells coming from the thymus (A. Tournefier, personal communication). Finally, the adults were all able to proliferate in response to the three mitogens used, with more homogeneous results.

The B cell ability to respond to LPS was not dependent on age. This could indicate that B cells, which are present in the spleen of 7-week-old larvae and which have the same relative proportions of IgM- and IgY-synthesizing cells at 12 weeks as in adults,[11] are able to react very early in ontogenesis. Definite physiological conclusions are, however, difficult to deduce because of different activation pathways induced by LPS and immunoglobulin, the physiological B antigen receptor.

In the absence of any monoclonal antibody specifically directed against T-cell structures, the specific interactions that we were able to observe between T lymphocytes and the lectins PHA and Con A could be used in precipitation assays that would permit the characterization of urodele analogues of molecules implied in mammals in antigen binding, adhesion or signal transduction. A similar approach with the staphylococcal enterotoxins should be very useful to clarify their capacity to bind class II antigens and particular TCR Vβ in the axolotl. This would provide a new approach to the study of tolerance mechanisms in primitive vertebrates.

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