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Thymus independent anti-horse erythrocyte antibody response and suppressor T cells in the Mexican axolotl (*Amphibia, Urodela, Ambystoma mexicanum*)

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Summary. Anti-horse erythrocyte (anti-HRBC) antibody synthesis was studied in normal, early thymectomized and adult thymectomized axolotls. The kinetics of the responses were similar to those described in the same species for antibody synthesis against bacterial or viral antigens. Booster injections did not induce any characteristic anamnestic responses. Early and adult thymectomized axoltls gave in three experimental groups higher anti-HRBC responses than controls. It is concluded that HRBC acts in the axolotl as a thymus-independent antigen. The enhanced response in early as well as in adult thymectomized animals can be interpreted by the presence of a suppressor T-cell activity on anti-HRBC synthesis. These results do not exclude possible thymus-dependent responses for antibody synthesis in the axolotl, although such responses were not demonstrated in urodele. The questionable lacking of some functional T-cell subsets in urodele is discussed as a working hypothesis.

INTRODUCTION

The permanent abrogation of allograft rejection in early thymectomized (E-Tx) urodele amphibians is evidence for the presence of an anti-allogenic T-cell population. In *Pleurodeles waltlii*, thymectomy remains effective 9–10 weeks after fertilization, 6 weeks before metamorphosis (Charlemagne & Houillon, 1968; Fache & Charlemagne, 1975). In *Triturus alpestris*, a species whose development is faster, thymectomy is effective during the whole larval life, up to 10 weeks after fertilization, just before metamorphosis (Tournefier, 1973). This relative slowness of the alloimmune reactivity maturation in urodele is in contrast to the very early maturation of the alloimmune system in anurans; in *Xenopus laevis*, larval thymectomy is effective only during the first few days after fertilization (Horton & Manning, 1972; Tochinai & Katagiri, 1975). Urodele amphibians, when stimulated with particulate antigens can synthesize immunoglobulins restricted to the IgM class (Ching & Wedgwood, 1967; Ambrosius, Hemmerling, Richter & Schimke, 1970; Houdayer & Fougereau, 1972; Tournefier, 1974, 1975). Nevertheless, immunization with soluble antigens gives negative results. In *Pleurodeles*, E-Tx animals weakly impairs antibody synthesis to *Salmonella typhimurium*-H antigen which is a partially thymus-dependent antigen in mice (Tournefier & Charlemagne, 1975; Charlemagne & Tournefier, 1977). Sheep (SRBC) and horse (HRBC) erthrocytes are thymus-dependent antigens for

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mammals (Claman, Chaperon & Triplett, 1966; Miller & Michell, 1969), birds (McArthur, Gilmour & Thorbecke, 1973), reptiles (Kanakambika & Muthukkaruppan, 1972) and anuran amphibians (Manning & Collie, 1975). In these orders, a synergistic effect of thymus and bone marrow-like cells gives rise to specific antibody synthesis.

In the present work, the possible role of T cells on anti-HRBC antibody synthesis is studied in normal, E-Tx and adult thymectomized (A-Tx) urodele amphibian *Ambystoma mexicanum* (axolotl).

MATERIAL AND METHODS

Animals

Axolotls (black strain) were raised from natural layings obtained in our laboratory colony. Sexually mature male or female animals, from 7 to 11 months of age (body weight, 50–100 g) were kept in tap water (21 ± 2°) and fed twice a week with minced meat and living *Chironomus* larvae.

Thymectomies

Axolotls have three pairs of thymic glands arising from the 3rd, 4th and 5th visceral pouches and located dorsally, on each side of the head, near the gill roots. Thymectomies were performed 5 weeks (E-Tx) or 9–10 months (A-Tx) after fertilization. Thymuses were removed under anaesthesia (MS 222 Sandoz) with fine scissors and forceps. No mortality occurred. Search for possible thymic remnants was done by biopsy 1 month before immunization in E-Tx axolotls or at the end of the experiments in A-Tx axolotls. Incompletely thymectomized animals were discarded. E-Tx axolotls reach sexual maturity at the same time as controls; wasting (starving, cachexia) occurs 15–24 months after thymectomy in some animals, as described in larval thymectomized *Pleurodeles* (Charlemagne, 1974). A-Tx axolotls remain unaffected.

Antigens

The HRBC were always obtained from the same donor, stored in Alsever's solution and washed three times in saline before use. Intraperitoneal (i.p.) or intravenous (i.v.) injections were performed on anaesthetized animals. Preliminary studies showed that 0·2 ml i.p. doses of 25% HRBC or 0·2 ml i.v. doses of 0·25% HRBC gave the optimal responses and the most constant results. Injections were made in saline without adjuvant.

Antibody determination

Titres of serum haemagglutinating antibodies were determined on fresh sera by using a microtechnique. Two-fold serial dilutions of 25 μl of axolotl sera were made in saline. To each dilution, 25 μl of a 1% (v/v) suspension of HRBC in saline were added. The plates were incubated for 2 h at 4° and left at 12° overnight. The end-point was expressed as $-\log_2$ of the last agglutinating dilution. The effect of 2-mercaptoethanol (2-ME) treatment was assayed by incubating the sera for 2 h at 37° with 2-ME (0·1 M, final dilution) before titrating. Controls were incubated in saline.

Statistics

Student's *t*-test was used to determine levels of significance between control and thymectomized groups. Differences were considered to be significant when probability (*P*) values $<0{\cdot}05$ were obtained.

RESULTS

For each experiment, control and thymectomized axolotls were bred from the same spawning. Animals of the experimental groups I and II originate from inbred histocompatible parents selected during the past 15 years in our laboratory. Experimental group III animals originated from a more recent importation and were histoincompatible. As axolotl antibodies are inactivated within 8–12 min by heating at 56°, all the agglutination assays were performed with fresh immune serum which in most cases had no haemolytic anti-HRBC activity. About 50% of normal animals, however, present low titres ($< -\log_2 2$) of spontaneous haemagglutinating factors against HRBC.

Anti-HRBC synthesis in E-Tx axolotls

Group I. In the first experiment, 9-month-old axolotls (ten E-Tx and twelve controls) were injected i.p. with 0·2 ml of 25% HRBC in saline. A challenge of the same dose was injected i.p. 80 days later. The animals were bled 9 days before the first injection and every 20 days during the course of primary immunization and after challenge. Significant levels of anti-HRBC antibodies arose only 40 days after

priming. Titres then increased and reached a maximum at 60 days. A slight enhanced secondary response was only obtained on day 60 after challenge. E-Tx axolotls gave an higher anti-HRBC antibody response than controls. The differences were statistically significant between 40 and 60 days after priming and 40 days after challenge (Table 1).

Group II. In the second experiment, 7-month-old axolotls (fifteen E-Tx and fifteen controls) were injected i.v. with 0·2 ml of 0·25% HRBC in saline. No challenge was made. The animals were bled 8 days before the first injection every 20 days during the primary response until day 100 and a last bleeding was made 200 days after immunization. The kinetics of antibody synthesis was nearly similar to group I animals and identical anti-HRBC titres were found in control and E-Tx animals until day 40 after priming. Titres were then significantly higher in E-Tx animals on days 60 and 80 after priming (Table 1).

Anti-HRBC antibody synthesis in A-Tx axolotls

Group III. In the third experiment, 10·5-month-old axolotls (twenty A-Tx and thirty controls) were injected i.p. with 0·2 ml of 25% HRBC in saline. Adult thymectomies were done 40 days before immunization. The animals were bled 8 days before

Table 1. Anti-HRBC responses in control and thymectomized axolotls

Experimental groups*	Mean anti-HRBC titres ($-\log_2 \pm$ SD)†		Significance‡
	Control	Thymectomized	
I			
Days after priming:			
20	0·25 (±0·46)	0·70 (±0·81)	NS
40	1·25 (±1·11)	2·40 (±1·26)	$0{\cdot}05 > P > 0{\cdot}02$
60	3·60 (±2·06)	6·10 (±1·74)	$0{\cdot}01 > P > 0{\cdot}001$
80	3·20 (±1·38)	4·40 (±1·57)	NS
Days after challenge:			
20	3·0 (±1·28)	2·10 (±1·06)	NS
40	4·10 (±1·50)	6·10 (±0·98)	$0{\cdot}01 > P > 0{\cdot}001$
60	4·50 (±1·62)	5·90 (±2·22)	NS
80	4·40 (±1·70)	5·50 (±2·26)	NS
II			
Days after priming:			
20	0·70 (±0·35)	1·0 (±0·53)	NS
40	2·50 (±1·30)	2·60 (±1·70)	NS
60	3·20 (±1·52)	4·70 (±2·21)	$0{\cdot}05 > P > 0{\cdot}02$
80	2·80 (±1·60)	4·50 (±1·99)	$0{\cdot}02 > P > 0{\cdot}01$
100	2·90 (±1·97)	4·20 (±2·20)	NS
200	2·10 (±2·05)	3·50 (±2·10)	NS
III			
Days after priming:			
20	3·25 (±1·30)	4·0 (±1·16)	$0{\cdot}05 > P > 0{\cdot}02$
40	5·80 (±1·92)	7·55 (±2·87)	$0{\cdot}02 > P > 0{\cdot}01$
60	6·10 (±2·29)	8·25 (±2·40)	$0{\cdot}01 > P > 0{\cdot}001$
80	6·05 (±1·48)	7·05 (±3·60)	NS

* Group I: anti-HRBC haemagglutinin titres in twelve control and ten E-Tx 9-month-old axolotls injected i.p. on days 0 and 80 with 0·2 ml of 25% HRBC in saline. Group II: anti-HRBC haemagglutinin titres in fifteen control and fifteen E-Tx 7-month-old axolotls injected i.v. with 0·2 ml of 0·25% HRBC in saline. Group III: anti-HRBC haemagglutinin titres in thirty control and twenty A-Tx 10-month-old axolotls injected i.p. with 0·2 ml of 25% HRBC in saline.

† Mean titres expressed as $-\log_2 \pm$ standard deviation.

‡ Student's *t*-test was used to evaluate the significance of the differences observed. Difference was considered to be significant when probability (*P*) values $< 0{\cdot}05$ were obtained. NS = not significant.

the injections and every 20 days during the primary response until day 80. No challenge was made. In group III animals, the antibody synthesis occurred more quickly and strongly than those in groups I and II. Anti-HRBC synthesis could be detected on day 20 after priming. Titres rose until day 60 and then slowly decreased. A-Tx animals had significantly higher anti-HRBC titres than controls on days 20, 40 and 60 after priming (Table 1).

DISCUSSION

A single HRBC i.p. or i.v. injection elicits in the axolotl slow but specific antibody synthesis. The peaks of the responses are obtained only 2 months after priming. A booster injection does not induce any characteristic anamnestic response but only a cumulative one which is not accelerated with regard to the primary. The kinetics of anti-HRBC antibody synthesis response are similar to those obtained in the same or related species immunized with particulate antigens. Antibodies were always found to be 2-ME sensitive, so restricted to an IgM-like class, as it was clearly demonstrated in all urodele species previously studied in this respect (Ching & Wedgwood, 1967; Ambrosius *et al.*, 1970; Houdayer & Fougereau, 1972, Tournefier, 1965). Although faster, responses with the same characteristics are obtained in mammals immunized with some thymus-independent antigens (no anamnestic effect, antibodies restricted to the IgM class).

Our results clearly demonstrate that HRBC, a poor immunogenic complex for neonatally thymectomized mice, can elicit good antibody synthesis in E-Tx urodele. So, an efficient direct triggering of antibody-secreting cells by HRBC is possible in the axolotl (a single 0·2 ml i.v. dose of 0·0025% HRBC in saline remains immunogenic for thymectomized and control axolotls). At the peak of the responses, E-Tx as well as A-Tx axolotls synthesize four times or more anti-HRBC antibodies than intact animals. This enhancement can be interpreted by the presence of a suppressor T-cell activity on antibody synthesis. As the amplification of the anti-HRBC response is significant 40 days after adult thymectomy (and probably earlier), the axolotl suppressor T cells are, as in higher vertebrates, short-lived lymphocytes.

An augmentation of antibody synthesis in E-Tx (but thymus regenerated) *Rana catesbeiana* tadpoles was described in Baculi & Cooper (1973). In *Xenopus laevis*, E-Tx animals display a heightened level of serum immunoglobulins (Weiss, Horton & Du Pasquier, 1972). Nevertheless, specific anti-SRBC and anti-human gamma-globulin antibody synthesis was inhibited in E-Tx *Xenopus*, both at the IgM and IgG levels (Turner & Manning, 1974).

In mice, adult thymectomy or mild immunosuppressive treatment leads to a better immune response to some thymus-independent antigens compared to control or neonatally thymectomized animals. This phenomenon is interpreted by the presence of a short-lived suppressor T-cell population exerting a regulatory effect on the expression of stimulated B clones (Gerson & Kondo, 1970, 1971; Gerson, 1974). After adult thymectomy in mice, a remaining long-lived T-cell population (which is lacking in neonatally thymectomized animals) is supposed to amplify the immune response. The enhanced response in A-Tx *as well as in E-Tx* axolotls could be interpreted as the lack of any amplifier T cell for the anti-HRBC response. As HRBC, like most erythrocyte antigens, are thymus-dependent antigens for terrestrial vertebrates, our results can be discussed in several ways. A first hypothesis could be that, although HRBC are thymus independent for the axolotl, other putative antigens, not actually detected, may be thymus dependent. In this case, a helper T-cell subset in the axolotl remains theoretically possible. Another hypothesis could be that the axolotl definitely lacks helper T cells. It is not actually possible to justify one or other of these hypotheses in regard to our actual results. Nevertheless, several arguments argue for the lack of some T-cell subsets in urodele.

The absence of IgG-like antibodies, whatever the nature of the antigenic stimulation, and the failure to detect antibodies to the classical soluble thymus-dependent antigens might be correlated to the absence of an IgM to IgG 'switch' which is supposed to be under the control of T cells in mammals (Katz & Benacerraf, 1972).

Unlike higher vertebrates, urodele thymocytes are not stimulated to proliferate in the presence of T-cell mitogens such as PHA or Con A (Collins & Cohen, 1976). Furthermore, very low stimulation indices in mixed lymphocyte culture (MLC) have been demonstrated for lymphoid cells from axolotls of different strains (Collins, Manickavel & Cohen, 1976).

Axolotl thymus shows a more homogeneous and dense structure than higher vertebrates thymuses (Klug, 1967). The absence of a well defined cortico-

medullary differentiation in urodele thymus may be correlative with the lacking of some T-cell subset. In this respect, it is important to emphasize that in mice (reviewed in Cantor & Boyse, 1977) a cortisone-resistant T-cell population located in the thymic medulla is involved in helper function. So the prediction (see Cohen, 1976, for extensive discussion) that urodele might lack a population of T cells as well as I-like region genes themselves could be reinforced by the present data.

The appearance of suppressor T-cell function early in vertebrate evolution is not a surprising fact; this regulatory mechanism of antibody production is probably contemporary to the emergence of antibody-secreting cells themselves. Furthermore, suppressor T cells seem to be implicated in the establishment of self-non-self discrimination (Allison 1974 a, b). Such a basic phenomenon is probably related to the very early vertebrate evolution.

A T-helper function is clearly demonstrable in anuran amphibians correlatively with the emergence of a new immunoglobulin class (IgG-like). In anurans, red blood cells are thymus-dependent antigens and the early thymectomized animals, as in mammals, do not respond well to them (Manning & Collie, 1975). The emergence of thymus dependency can be considered as an efficient regulatory event; most of the antigens cannot directly trigger B cells and must in a first step be recognized by specific T-cells during a T/B collaboration mechanism. The carrier effects recently described in fishes (Stolen & Mäkelä, 1975; Yocum, Cuchens & Clem, 1975) have not yet been related to any T-cell activity and a definite T/B cellular collaboration for antibody synthesis in these systems remains to be proved.

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