Changes in antibody and complement production in *Xenopus laevis* during postmetamorphic development revealed in a primary *in vivo* or in *vitro* antibody response

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SUMMARY

Xenopus laevis (G-line) mounts a primary plaque forming cell (PFC) response either *in vivo* or *in vitro* following challenge with foreign erythrocytes. Methods are described for generating and assaying the response, which specify criteria such as antigen dose, antigen choice, response kinetics, and complement source. The results suggest that at the peak of the primary response (approximately day 6), animals of different ages produce predominantly different 'classes' of antibody which display markedly different complement-fixing characteristics. Antibodies produced by larvae and 4-month-old postmetamorphic animals appear here to be unable to fix either guinea pig complement (GPC') or adult *Xenopus* complement, but can readily fix complement from 6-month-old *Xenopus*. The proportion of spleen PFC's producing antibody capable of fixing GPC' progressively increases from about six months to 18 months of age. Possible explanations for such ontogenetic changes are discussed.

INTRODUCTION

Adult frog and toad immunoglobulins are able to fix guinea pig complement (Ohnishi, 1980; Romano, Geczy & Steiner, 1973) and this combination is commonly used in direct plaque-forming-cell (PFC) assays to study haemolytic antibody responses to foreign erythrocytes. Antibodies of *Xenopus* tadpoles (and those of young postmetamorphic *Xenopus*) appear less able (Du Pasquier & Cunningham, unpublished data) or altogether unable (Williams, 1981) to fix guinea pig complement, and rosette-forming-cell assays often must be used as an alternative to PFC assays (Kidder, Ruben & Stevens, 1973). In this report primary *in vivo* and *in vitro* responses of *Xenopus* have been examined in animals ranging in age from 2 months (tadpole) to several years (full-grown adults). The results describe a modified Mishell–Dutton (1966) culture system for generating primary PFC responses in *Xenopus* and outline possible ontogenetic changes in the complement-fixing ability of *Xenopus* antibodies. Three sources of complement have been compared for activity in the PFC assay,

including 6-month-old and adult *Xenopus* serum and commercial guinea pig serum.

<div align="center">MATERIALS AND METHODS</div>

Animals

Inbred *Xenopus laevis* have been used throughout these experiments. Inbred *Xenopus* (G-line) (Katagiri, 1978), which appear to be MHC homozygous (JJ) (Di Marzo & Cohen, 1982), were donated by C. Katagiri (Hokkaido University, Sapporo, Japan) or purchased commercially (Nippon Life Sciences, Sapporo, Japan). Animals used for breeding or experimentation were healthy, feeding, and free from obvious infection. Offspring were obtained through artifical breedings induced by repeated injection of human chorionic gonadotrophin (Griffin and George, Sussex, England). Animals were reared at constant temperature (23 °C) and were immunized at elevated temperature (26 °C). Prior to metamorphosis animals were fed nettle powder, for 3–4 months following metamorphosis they were fed live *Tubifex* worms, and thereafter minced ox liver.

Antigens

Sheep red blood cells (SRBC) from a single sheep were purchased commercially (Tissue Culture Services, Slough, England) and rabbit red blood cells (RRBC) from a single rabbit were collected via an ear vein. Prior to storage, erythrocytes were centrifuged (350 *g*) and the buffy coats were aspirated and discarded. Erythrocytes were stored in Alsever's solution (Flow Labs, Irvine, Scotland) for at least one week and up to four weeks prior to use.

In vivo *immunization*

Immunization of *Xenopus* was performed by injection of washed and intact sheep or rabbit red blood cells suspended in amphibian strength saline. Postmetamorphic *Xenopus* received standard 0·05ml per gram body weight injections of a 0·0025% suspension or 10% suspension, via the dorsal lymph sac. *Xenopus* tadpoles received 0·005ml injections of a 50% suspension, intraperitoneally.

In vitro *immunization*

Immunization of *Xenopus* spleen cells was performed by culturing 3×10^6 spleen leukocytes (at 10×10^6 per ml) in flat-bottom 24-well plates (Linbro No. 76-033-05, lot No. 76091102, Flow Labs, Irvine, Scotland) with varying numbers or equal numbers of intact sheep or rabbit red blood cells. The complete culture medium consisted of 60% Leibovitz-15 (L-15) supplemented with 10% heat-inactivated foetal calf serum (Lot number 29072126), 10mM-HEPES buffer, 20mM-sodium bicarbonate, 2mM-1-glutamine, 50 units/ml penicillin,

$50\mu g$/ml streptomycin, $2\cdot5\mu g$/ml fungizone (all from Flow Labs, Irvine, Scotland) and $0\cdot05$mM-2-mercaptoethanol (British Drug Houses, Poole, England). Cultures were fed on days 1, 3 and 5 with one drop ($30\mu l$) of a nutritive mixture consisting of 60% L-15 supplemented with 20% FCS, 20mM-HEPES buffer, 40mM-sodium bicarbonate, 10mM-1-glutamine, 5 × Eagles non-essential amino acids, 5 × Eagles essential amino acids (all from Flow Labs, Irvine, Scotland) and 1% (w/v) D-glucose (Sigma Chemical Co., Poole, England). Cultures were incubated (at 26–27 °C), in a water-saturated atmosphere of 5% CO_2 in air prior to harvest. Cultures were harvested by pipetting and gentle scraping and the contents were transferred to 12 × 75mm Falcon tubes (A. J. Beveridge Co., Newcastle, England).

PFC assay

In vivo and *in vitro* PFC responses were measured by the thin-layer direct-slide technique (Cunningham & Szenberg, 1968). Cells to be assayed for PFC activity were suspended at varying dilutions in 60% L-15 supplemented only with 10% FCS. A $150\mu l$ sample of each spleen cell suspension was combined with $15\mu l$ of a 25% suspension of indicator erythrocytes and $40\mu l$ of antigen-absorbed, 1/10 diluted guinea pig or 1/10 diluted *Xenopus* serum.

Guinea pig serum used as a source of complement was purchased commercially in lyophilized form (Wellcome Reagents Ltd., Beckenham, England) and reconstituted immediately prior to use. *Xenopus* serum used as a source of complement was collected from non-immune animals by cardiac puncture, maintained on ice, and used fresh (not frozen).

The mixtures were pipetted into double microscope slide chambers which were sealed with a 2:1 mixture of paraffin wax and petroleum jelly. After 1–2 h at 30 °C, PFC were counted on two separate slides at dilutions which gave up to 100 plaques per slide, under low power of a dissecting microscope and only plaques with a clear central lymphocyte were scored as PFC. Data is expressed as *in vivo* PFC per 10^6 originally recovered spleen leukocytes, or as *in vitro* PFC per 10^6 originally cultured leukocytes.

RESULTS
In vivo *and* in vitro *PFC responses of 6–9-month-old* Xenopus

In vivo challenge with sheep or rabbit erythrocytes results in a specific primary anti-SRBC or anti-RRBC PFC response which peaks on approximately day 7 (Tables 1 and 2). Responses are elicited by low doses and by high doses of RBC. Using *Xenopus* (homologous) serum rather than guinea pig (heterologous) serum as a source of complement increases the sensitivity of the PFC assay (no further increase occurs when both *Xenopus* and guinea pig sera are used together). Both types of complement must be absorbed and *Xenopus* serum must be used fresh (not frozen).

Table 1. *Antigen and complement dependence of the* in vivo Xenopus *anti-SRBC and anti-RRBC PFC response*

		PFC/10^6 originally recovered spleen leukocytes			
	RBC	ANTI-SRBC-PFC		ANTI-RRBC-PFC	
In vivo Immunization	dose %	GPC'	6 mo XLC'	GPC'	6 mo XLC'
SRBC injected	0	0	0	0	0
	0·0025	1	110	0	0
	10	30	380	0	0
RRBC injected	0	0	0	0	0
	0·0025	0	0	0	180
	10	0	0	45	431

PFC were assayed using 1/50 diluted *Xenopus* serum from 6-month-old G-line animals or guinea pig serum as a source of complement.
PFC were measured 6 days following immunization
Responder animals are 6-month-old G-line.
Individual measurements from pools of three animals.

In vitro challenge with sheep or rabbit erythrocytes also results in a specific primary anti-RRBC PFC response which peaks on approximately day 7, but not an anti-SRBC PFC response (Tables 3 and 4). In absence of RRBC or SRBC low levels of PFC arises spontaneously against both types of red cell. The presence of spontaneous PFC's following *in vitro* culture contrasts with our failure to record background anti-SRBC or anti-RRBC PFC's in splenocytes assayed directly after removal from the animal. It is possible that *in vitro* conditions (where FCS is present) achieve polyclonal differentiation of B cells, which include anti-red-cell reactive clones. Thus FCS added to L15 medium enhances tritiated thymidine uptake of spleen lymphocytes (Williams, 1981) and anti-RRBC PFC numbers increase during culture of splenocytes (in the absence of RRBC's) taken from early-thymectomized *Xenopus* (Lallone, 1984). In the presence of SRBC, and at all RBC-to-leukocyte ratios tested, the formation of anti-SRBC PFC is specifically decreased, while RRBC specifically increase the formation of anti-RRBC PFC. The reasons for this differential effect of SRBC and RRBC remain unclear, but may be a unique feature of the *in vitro* PFC response of inbred (G-line) animals reared in our laboratory. Detecting an *in vitro* PFC response is at least as dependent on the use of *Xenopus* serum rather than guinea pig serum as a source of complement as is the *in vivo* response.

Choice of complement source in the 6-day PFC response of different-aged animals

Parallel changes occur in antibody and complement systems during

Table 2. *Time courses of the* in vivo *Xenopus anti-SRBC and anti-RRBC PFC response*

In vivo Immunization	RBC Dose %	Target RBC	C' Source	PFC/10^6 originally recovered spleen leukocytes			
				Day 3	Day 7	Day 11	Day 15
Non injected	0	R	6 mo XLC'	0			0
			GPC'	0			0
	0	S	6 mo XLC'	0			0
			GPC'	0			0
SRBC injected	10	R	6 mo XLC'	0	0	0	0
			GPC'	0	0	0	0
	10	S	6 mo XLC'	0	588	39	5
			GPC'	0	117	173	89
RRBC injected	10	R	6 mo XLC'	0	874	45	18
			GPC'	0	189	157	15
	10	S	6 mo XLC'	0	184	0	0
			GPC'	0	0	0	0

PFC were assayed using 1/50 diluted *Xenopus* serum from 6-month-old G-line animals or guinea pig serum as a source of complement.
Responder animals are 9-month-old G-line.
Individual measurements made from pools of three animals.

Table 3. *Antigen and complement dependence of the* in vitro *Xenopus*
anti-SRBC and anti-RRBC PFC response

| | | PFC/10^6 originally cultured spleen leukocytes | | | |
| | | ANTI-SRBC-PFC | | ANTI-RRBC-PFC | |
In vitro Immunization	RBC/leuko ratio	GPC′	6 mo XLC′	GPC′	6 mo XLC′
SRBC stimulated	0	0	35	0	60
	0·001	0	15	0	61
	0·01	0	1	0	55
	0·1	0	0	0	63
	1·0	0	0	0	58
	10	0	0	0	49
RRBC stimulated	0	0	25	0	57
	0·001	0	20	0	61
	0·01	0	18	0	155
	0·1	0	20	0	270
	1.0	0	21	0	510
	10	0	19	0	103

PFC were assayed using 1/50 diluted *Xenopus* serum from 6-month-old G-line animals or guinea pig serum as a source of complement.
PFC were measured on day 6 of culture
Responder cells are from 6-month-old G-line animals
Individual cultures from pools of six animals.

development and aging in *Xenopus* which can be detected in either an *in vivo* or an *in vitro* PFC response. Tadpoles and 4-month-old postmetamorphic animals generate PFC in their spleens 6 days following *in vivo* SRBC challenge and their antibodies appear able to fix 6-month-old *Xenopus* complement (XLC′) but neither adult XLC′ nor guinea pig complement (GPC′) (Table 5). Adult animals also generate PFC in their spleens following SRBC challenge and their antibodies appear able to fix complement from any of these three sources, since responses can be detected equally well using complement from guinea pig, young *Xenopus*, or adult *Xenopus* donors. The development of *in vivo* generated PFC able to fix GPC′ occurs gradually and the ratio of PFC detected using young (6-month-old) XLC′ to PFC detected using GPC′ appears to be inversely proportional to the age of the spleen cell donor. PFC from the spleens of adult mice can be easily detected using guinea pig complement and yet their antibodies appear unable to fix complement from *Xenopus* of any age.

Spleen cells from animals 4 months old generate PFC in culture 6 days following RRBC challenge and their antibodies appear able to fix 6-month-old XLC′ but neither adult XLC′ nor GPC′ (Table 6). As in the *in vivo* response, 12-month-old *Xenopus* possess *in vitro*-generated antibodies that can fix GPC′.

Table 4. *Time course of the* in vitro *Xenopus anti-SRBC and anti-RRBC PFC response*

In vitro Immunization	RBC/ leuko ratio	Target RBC	C' source	PFC/10^6 originally cultured spleen leukocytes			
				Day 3	Day 7	Day 11	Day 15
Non-stimulated	0	R	6 mo XLC'	28	63	12	2
			GPC'	0	0	0	0
	0	S	6 mo XLC'	10	2	0	0
			GPC'	0	0	0	0
SRBC stimulated	1·0	R	6 mo XLC'	18	43	20	3
			GPC'	0	0	0	0
	1·0	S	6 mo XLC'	1	0	0	0
			GPC'	0	0	0	0
RRBC stimulated	1·0	R	6 mo XLC'	241	594	268	55
			GPC'	3	3	0	0
	1·0	S	6 mo XLC'	15	0	0	0
			GPC'	0	0	0	0

PFC were assayed using 1/50 diluted *Xenopus* serum from 6-month-old G-line animals or guinea pig serum as a source of complement.
Responder animals are 6-month-old G-line animals.
Individual cultures from pools of ten animals.

Table 5. *Parallel changes in the antibody and complement systems during aging in Xenopus, demonstrated in a primary in vivo anti-SRBC PFC response*

Responder age	SRBC dose %	PFC/10^6 originally recovered spleen leukocytes and PFC/spleen					
		GPC'		6 mo XLC'		Adult XLC'	
		10^{-6}	spleen^{-1}	10^{-6}	spleen^{-1}	10^{-6}	spleen^{-1}
2 mo Xenopus (tadpole – stage 56/7)	0	0	0	0	0	ND	ND
	50	0	0	322	16	ND	ND
4 mo Xenopus	0	0	0	4	9	0	0
	10	0	0	894	2056	0	0
12 mo Xenopus	0	0	0	0	0	0	0
	10	51	331	431	2801	0	0
18 mo Xenopus	0	0	0	0	0	0	0
	10	282	3384	535	6420	0	0
Adult Xenopus (Full grown, unknown age)	0	0	0	0	0	0	0
	10	59	2832	72	3456	109	5232
Adult Mouse	0	0	0	0	0	0	0
	10	556	10564	0	0	0	0

PFC were assayed using 1/50 diluted *Xenopus* serum from 6-month-old or adult G-line animals or guinea pig serum as a source of complement.
PFC were measured 6 days following immunization.
Responder animals are G-line *Xenopus* or Swiss Albino Mouse.
Individual measurements from pools of 20 tadpoles or one to four frogs.

Table 6. *Parallel changes in the antibody and complement systems during aging in* Xenopus, *demonstrated in a primary* in vitro *anti-RRBC PFC response.*

Donor age	RRBC/ leuko ratio	PFC/10^6 originally cultured spleen leukocytes		
		GPC'	6 mo XLC'	Ad XLC'
4 mo *Xenopus*	0	0	53	0
	1.0	0	525	0
12 mo *Xenopus*	0	17	33	0
	1·0	120	412	0
18 mo *Xenopus*	0	58	56	ND
	1·0	593	605	ND
Adult *Xenopus* (Full grown, unknown age)	0	77	ND	ND
	1.0	573	ND	ND

PFC were assayed using 1/50 diluted *Xenopus* serum from 6-month-old or adult G-line animals or guinea pig serum as a source of complement.
PFC were measured on day 6 of culture.
Responder cells are from G-line animals.
Individual cultures from pools of one to four animals.

However, the antigen-induced *in vitro* response recorded with GPC' becomes comparable with assays using young XLC' only when splenocytes are taken from 18-month-old animals.

DISCUSSION

These experiments provide two useful pieces of technical information concerning the generation and assay of primary spleen PFC responses in *Xenopus*. First, they suggest that *Xenopus* of any age may be able to mount a primary PFC response to foreign erythrocytes (including an *in vitro* response, which can be abolished by removal of nylon wool or glass bead adherent cells, by 3000 R irradiation, or by early larval thymectomy (Lallone, 1984)). Second, they suggest that in animals less than 6 months old, such a PFC response is detectable only using homologous serum (i.e. fresh serum from non-immune 6-month-old *Xenopus*) rather than heterologous serum (i.e. lyophilized guinea pig serum) as a source of haemolytic complement. The findings serve to illustrate the point made by the late W. Hildemann (1978) that complement from different vertebrates is not always naturally interchangeable and that the most efficient haemolytic activity is often obtained only with homologous serum. The sources of antibody, complement and target cell need careful choice in order to maximise lysis.

Standard amphibian culture conditions (with minor modifications) have been used before to generate *in vitro* antibody responses in *Xenopus* (and antigen-binding responses in *Bufo marinus*; see Azzolina, 1975). A good PFC

response to soluble protein antigens has been generated in cultures of dissociated *Xenopus* spleen cells; however, no response could be detected without prior *in vivo* hapten and carrier priming (Blomberg, Bernard & Du Pasquier, 1980). A primary PFC response to foreign erythrocytes has been generated in cultures of *Xenopus* spleen fragments; however, this response required at least 14 days to become readily detectable by a direct PFC assay (Auerbach & Ruben, 1970).

Anuran amphibians (including *Xenopus*) are known to produce high and low relative molecular mass immunoglobulins (Hadji-Azimi, 1979) and a complement system which is superficially similar to that of mammals (Weinheimer, Evans & Acton, 1971; Legler *et al.*, 1979; Ruben, Edwards & Rising, 1977; Moticka, Brown & Cooper, 1973; Donnelly & Cohen, 1977). Following metamorphosis there is an overall switch from larval to adult life and a significant increase in physical size. To accommodate these changes a variety of new adult proteins appear and replace larval proteins which subsequently disappear (Wald, 1958; Wise, 1970; Manwell, 1966). Not unexpectedly, changes occur in the nature and relative concentration of various serum proteins and this may include certain immunoglobulin classes and certain complement components (Richmond, 1968; Du Pasquier, Blomberg & Bernard, 1979; Geczy, Green & Steiner, 1973). A postmetamorphic shift in the predominant immunoglobulin class produced in a primary PFC response could stem directly from the differential activation of certain immunoglobulin genes (resulting either in the increased production of a low relative molecular mass Ig'G' class, see Du Pasquier & Haimovich, 1976, or the decreased production of a less-well-defined, possibly larval, Ig class; see Hadji-Azimi, 1979). That *Xenopus* may possess more than two Ig isotypes is suggested by very recent, preliminary, findings of Du Pasquier (personal communication). The shift could also arise indirectly from the progressive turnover and replacement of certain B cell subsets (caused possibly by a decrease in the number of intrathymic- or thymus-derived B cells, see Du Pasquier, Weiss & Loor, 1972; Du Pasquier & Weiss, 1973; Hsu, Julius & Du Pasquier, 1984; Williams, Cribbin, Zettergren & Horton, 1983).

In most cases an obvious advantage can be associated with making a larval to adult protein switch. For example, tadpole and adult haemoglobins have markedly different oxygen-binding affinities (Hamada, Sakai, Tsushima & Shukuya, 1966). However, in the case of immunoglobulins such an advantage is not so obvious. Larval antibodies appear able to bind antigen specifically, to bind complement specifically, and appear perfectly adequate with respect to the size of their antigen repertoire, despite having far fewer lymphocytes (B cells included) than do their adult counterparts (Haimovich & Du Pasquier, 1973; Du Pasquier & Wabl, 1976). Perhaps young *Xenopus* (less than 6 months old) retain the ability to produce a predominantly or uniquely larval class of immunoglobulin (i.e. one which is capable of fixing only young *Xenopus*

complement but not the (modified?) complement components from adult *Xenopus*) when antigen challenge places severe stress on their immune system. There are examples of other protein changes that can take place after metamorphosis which set a precedent. For example, following metamorphosis, individual tadpole erythrocytes begin production of adult haemoglobin; eventually these cells are replaced by adult erythrocytes, and yet under severe anaemic stress (induced by phenyl-hydrazine) adult cells can be induced to reactivate production of tadpole haemoglobin (Moss & Ingram, 1965; Maniatis & Ingram, 1972; Maniatis, Steiner & Ingram, 1969; Hamada & Shukuya, 1966).

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