

An *in vivo* study of the ontogeny of alloreactivity in the frog, *Xenopus laevis*

SHEILA J. DIMARZO & N. COHEN *Department of Microbiology, Division of Immunology, University of Rochester School of Medicine and Dentistry, Rochester, New York, U.S.A.*

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Summary. Pre- and perimetamorphic larval and post-metamorphic juvenile frogs (*Xenopus laevis*) from a single family were grafted with skin from either of their parents (who differed from the hosts by one major histocompatibility complex (MHC) haplotype and multiple minor H loci) or from an unrelated adult (to provide a greater immunogenetic disparity). The fate of such skin grafts transplanted to hosts of various developmental stages indicated that a net destructive alloimmunity in this amphibian species develops gradually during ontogeny. In contrast to the uniform rejection of grafts on animals transplanted as post-metamorphic froglets, many recipients of grafts transplanted during larval life became tolerant. The incidence of tolerance depended on the recipients' developmental stage at the time of grafting and on the particular MHC haplotype barriers involved. In general, larvae grafted at an early developmental stage became tolerant, whereas recipients transplanted at older larval stages rejected grafts from the same donor. Even when larvae rejected their grafts, the reaction was often more chronic than that mounted by post-metamorphic froglet controls.

Present address: Dr Sheila J. DiMarzo, Department of Biology (M-001), University of California at San Diego, La Jolla, California 92093, U.S.A.

Correspondence: Dr N. Cohen, Department of Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, U.S.A.

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INTRODUCTION

Developmentally complete analyses of the ontogeny of immunity to histocompatibility alloantigens in eutherian mammals are hampered by the *in utero* location of the foetus. Although the use of marsupials can somewhat circumvent this problem, ontogenetic studies with any mammal are still complicated by maternal influences on immunological development (Silverstein, Prendergast & Kraner, 1964; Dennis, Jacoby & Griesemer, 1969; Medawar & Woodruff, 1958; Steinmuller, 1961; Ivanyi & Ivanyi, 1961; Najarian & Dixon, 1962; LaPlante, Burrell, Watne, Taylor & Zimmerman, 1969; Waring, Holmes, Cockson, Ashman & Stanley, 1978).

Amphibian larvae, unlike mammals, are free from such maternal effects. They can also be surgically manipulated at early stages and can be reared and observed throughout their relatively short developmental period. For these reasons, amphibians provide valuable tools for an inquiry into the ontogeny of cellular and humoral immunity (Du Pasquier & Wabl, 1976; Du Pasquier, 1981).

Horton (1969) demonstrated that skin transplants from adult frogs (*Xenopus laevis*) allografted to very young unrelated larvae were infiltrated by host lymphocytes shortly after such cells first appeared in the developing lymphoid organs. Chardonness & Du Pasquier (1973) not only confirmed that young pre-metamorphic *Xenopus* larvae can reject allogeneic skin grafts but demonstrated that older metamorphosing

tadpoles may be rendered tolerant of such transplants (Chardonnes & Du Pasquier, 1973; Bernardini, Chardonnes & Simon, 1969) as judged by third-party and original donor test grafting protocols (see also: Barlow, DiMarzo & Cohen, 1981).

As an initial step in a long-term systematic study of the alloreactive capabilities of pre- and perimetamorphic *Xenopus* larvae, we asked whether reactivity to foreign major histocompatibility complex (MHC) antigens appears abruptly at some predictable time in development, as it does in the foetal sheep (Silverstein *et al.*, 1964), or whether it matures gradually as described for the foetal dog (Dennis, Jacoby & Griesemer, 1969) and the neonatal rat (Medawar & Woodruff, 1958).

MATERIALS AND METHODS

Animals

A pair of field-collected adult *Xenopus* (South African Snake Farm, Fish Hoek, Capetown, South Africa) was induced to mate by injecting the male and female with 250 U. and 750 U. of human chorionic gonadotrophin, respectively (Parke-Davis, Detroit, Mich.). Larvae from this mating (Stock 40) were raised at a density of eight animals per 4 litres of dechlorinated tap water at approximately 24° and were fed nettle powder thrice weekly. Metamorphosing animals were fed white worms (*Enchytreous*, Carolina Biological Supply Co.) and ground beef heart. Larvae were developmentally staged according to the criteria established by Nieuwkoop & Faber (1967).

Beginning when larvae reached stage 51, some were continuously reared in a 0.1% aqueous solution of sodium perchlorate (Matheson, Coleman & Bell, Norwood, Ohio) until the study was completed. This concentration of goitrogen inhibits anatomical metamorphic changes, and with time, induces goiters. The precise effects of sodium perchlorate (if any) on the development and function of the immune system are unknown. Consequently, at each developmental stage, comparisons were made between treated and untreated hosts that received grafts from each of the three donors. When untreated siblings reached the particular developmental stage to be evaluated by grafting, a group of age-matched goitrogen-treated larvae were also grafted.

Grafting protocols

Separate groups of Stock 40 F₁ siblings from a single spawning received skin allografts at one of the following developmental stages (Fig. 1): premetamorphic larvae were grafted at stage 48/49, stage 51, and stage 53; perimetamorphic recipients were grafted at stage 57/58 when their forelimbs were emerging from the operculum; postmetamorphic froglets of the same sibship were transplanted at least 2 months after they had metamorphosed.

Skin graft donors were the male and female parents of this sibship, and a presumably unrelated non-parental adult. All animals were transplanted according to the procedure of Chardonnes & Du Pasquier (1973) with a single 1 mm² graft of ventral skin. Graft rejection was monitored microscopically. The rejection

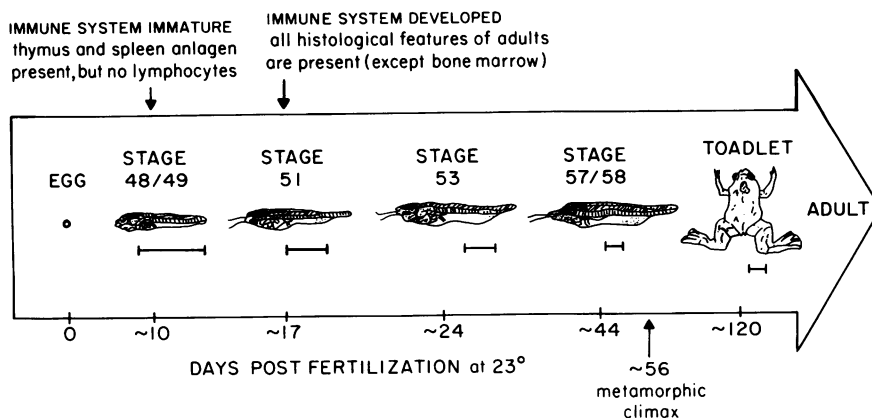


Figure 1. Developmental time scale for *X. laevis*. The developmental stage designations, drawings, and ages postfertilization are from Nieuwkoop & Faber (1967). The bar under each animal = 1 cm. Histological information about the developing immune system is from Manning & Horton (1969).

response was characterized by vascular disruptions and a decrease in the number of viable guanophores (pigment cells). Rejection was considered complete when more than 90% of the guanophores were destroyed. Animals bearing partially to fully viable skin grafts for greater than 50 days were considered tolerant (see Discussion).

Mixed leucocyte cultures

One-way mixed leucocyte cultures (MLCs) were performed according to our modifications of the method of Weiss & Du Pasquier (1973). Frogs (1–1.5 years old) were anesthetized by immersion in 0.1% MS-222 (tricane methanesulfonate; Crescent Research Chemicals, Inc., Paradise Valley, Ariz.). Spleens were removed aseptically, minced with scissors, and filtered through nylon screens. Cell suspensions were prepared in Leibovitz-15 medium diluted to amphibian osmolarity (~220 mOsm) with triple distilled water (pH 7.5) and supplemented with: 1.25×10^{-2} M HEPES buffer, 1% heat-inactivated foetal calf serum, 100 u./ml penicillin, 100 μ g/ml streptomycin (all from Gibco, Grand Island, N.Y.), 1×10^{-2} M sodium bicarbonate, and 5×10^{-5} mercaptoethanol (from Fisher Scientific Co., Rochester, N.Y.). Spleen cells were washed, counted with a haemocytometer, and resuspended at 5×10^5 viable (trypan blue-excluding) leucocytes per ml. For the MLC, 0.1 ml of responder cells were cultured with 0.1 ml of irradiated stimulator cells (6000 rad from a ^{60}Co source). This low cell density (lower than that used by Weiss & Du Pasquier, 1973), was necessary to facilitate MLC typing of many siblings in a single controlled experiment. Replicate cultures were performed in round bottom microtitre plates (Linbro, Dunkirk, N.Y.) and incubated at 26° with 5% CO_2 in Mishell-Dutton chambers (Norbo Machine and Saw Service, Huron, S.D.). After 96 hr, each well was pulsed with 2 μ Ci tritiated thymidine (SA=2 Ci/mmol; New England Nuclear, Boston, Mass.). Cultures were incubated an additional 24 hr and then harvested with a multiple automated precipitator (Otto Hiller, Madison, Wis.) onto glass fibre filters. Filters, containing trichloroacetic acid (TCA)-precipitable, ethanol insoluble material, were transferred to counting vials. Scintillation fluid (Econofluor and 0.5% protosol; New England Nuclear) was added and the samples were counted by liquid scintillation spectrometry.

MLC reactivity was determined by calculating the nett increase in stimulated counts per minute (c.p.m.,

i.e. mean c.p.m. stimulated cultures minus mean c.p.m. of control cultures).

Statistical analysis

Median survival times (MSTs) of allografts and the significance between MSTs were calculated nomographically (Litchfield, 1949). Significance of reactions in MLCs were determined using the Student's *t* test. The chi-squared test was used to compare numbers of rejected grafts. The test for goodness of fit was used to compare the observed frequency of MLC responsiveness with a theoretical frequency distribution (Dixon & Massey, 1969). For all analyses, a value of $P < 0.05$ was considered significant.

RESULTS

Immunogenetic relatedness of the parents and their F_1 siblings

MHC typing of this family by the one-way MLC was accomplished by culturing all possible combinations of stimulator and responder splenocytes from adult Stock 40 siblings. Table 1 shows that eleven siblings could be grouped into four MLC non-responsive, MHC identical, classes. Cells from members of each class did not respond significantly to irradiated stimulator splenocytes from other frogs in the same class. (There was insufficient class IV animals to test this response.) However, cells from each member of the four classes of siblings were significantly stimulated by irradiated splenocytes from individuals in the other three classes. This typing protocol was repeated four different times with other siblings; similar class assignments could be made.

Another group of siblings was tested for MLC responsiveness against irradiated female parental splenocytes. Spleen cells from all twelve siblings were significantly stimulated by irradiated allogeneic cells from their female parent (Table 2). In fact, for the ten frogs tested, the anti-parental response was as strong as the response to a non-parental, randomly selected adult female (± 2 standard errors overlap in all cases).

Allograft reactivities

Chi-squared comparisons of the percentage of goitrogen-treated and untreated animals that had rejected grafts by day 15, 20, 30 and 50 post-transplantation were performed. Neither the incidences of tolerance induction nor the kinetics of rejection of skin from any donor differed as a function of sodium perchlorate

Table 1. One-way MLC with Stock 40 F₁ splenocytes. Siblings type into four MLC identical classes

Class	Animal numbers	MLC combination	Mean c.p.m. ± SE	Δc.p.m.	
I	4,5,8	Autologous	2433 ± 332		
		MHC identical	2728 ± 208	295	<i>P</i> = 0.228*
		MHC disparate	4799 ± 158	2366	<i>P</i> < 0.001
II	2,6,7,9,11	Autologous	3150 ± 393		
		MHC identical	3471 ± 203	321	<i>P</i> = 0.242
		MHC disparate	4446 ± 254	1296	<i>P</i> < 0.03
III	1,10	Autologous	3652 ± 1144		
		MHC identical	3892 ± 213	240	<i>P</i> = 0.428
		MHC disparate	5749 ± 207	2097	<i>P</i> < 0.005
IV	3	Autologous	5857		
		MHC identical	—†	—†	
		MHC disparate	11,703 ± 558	5846	<i>P</i> < 0.01

* Probability values calculated using the Student's *t* test.

† Not applicable.

treatment. Therefore, for the following analysis (and in Tables 3 and 4), the data from goitrogen-treated and untreated animals have been pooled.

Both the donor of the skin graft and the developmental stage of the host influenced the incidence and kinetics of allograft rejection (Table 3). Regardless of the donor used, all frogs grafted during postmetamorphic life, rejected their transplants within 30 days; 90% of the grafts were completely destroyed by day 20. MSTs did not differ significantly as a function of the donor used (Table 3).

In sharp contrast to the uniformity of graft rejection by control postmetamorphic frogs, most transplants from the male parental donor to premetamorphic frogs, or to perimetamorphic recipients survived > 50 days (Table 3). Specifically, seventy-seven of the ninety grafts (86%) from this parent on larval hosts (pooled data from all larval stages) were partially to fully viable by 50 days post-transplantation. This difference in the response of larval and young adult hosts was significant for each larval stages grafted. There were no differences among the responses of individual

Table 2. Stock 40 F₁ siblings respond in one-way MLC to irradiated parental ♀ splenocytes as well as to those of a non-parental adult

Sibling number	Autologous MLC Mean c.p.m. ± SE	Anti-parental ♀ MLC		Anti-non-parental ♀ MLC	
		Mean c.p.m. ± SE	Δc.p.m.*	Mean c.p.m. ± SE	Δc.p.m.*
1	4044 ± 255	6321 ± 336	2277	6503 ± 465	2459
2	2845 ± 178	6744 ± 293	3899	6920 ± 442	4075
3	2989 ± 393	6917 ± 470	3928	10,343 ± 376	3426
4	4395 ± 350	7115 ± 487	2720	9087 ± 320	1972
5	3356 ± 252	7858 ± 649	4502	8982 ± 724	5626
6	3185 ± 237	12,853 ± 677	9668	11,746 ± 698	8561
7	12,924 ± 467	22,369 ± 1559	9445	22,482 ± 1022	9558
8	5931 ± 554	15,829 ± 468	9898	14,547 ± 562	8616
9	6857 ± 652	18,001 ± 621	11,144	ND†	ND
10	7823 ± 666	18,734 ± 720	10,911	ND	ND
11	4057 ± 259	8652 ± 447	4595	8918 ± 446	4861
12	6315 ± 342	16,694 ± 732	10,379	18,049 ± 793	11,734

* All stimulations were significant at *P* < 0.001 by the Student's *t* test.

† ND, not done.

Table 3. Allograft rejection by Stock 40 F₁ siblings according to developmental stage. Responses to parental and non-parental skin transplants.

Graft donor	Developmental stage of hosts*	Cumulative % rejected (no. rejected/no. grafted) by				MST of rejected grafts (95% confidence limits)†
		15 days	20 days	30 days	50 days	
Parental ♂	48/49	0(0/29)	0(0/29)	3(1/29)	4(1/27)	35 days‡
	51	0(0/21)	0(0/21)	5(1/21)	17(3/18)	39 days§
	53	4(1/28)	7(2/28)	21(6/28)	26(6/26)	23 days (17–33)
	57/58	0(0/19)	5(1/19)	16(3/19)	16(3/19)	23 days§
	Postmetamorphic	40(4/10)	80(8/10)	100(10/10)		17 days (17–19)
Parental ♀	48/49	0(0/25)	0(0/25)	24(6/25)	41(9/22)	32 days (27–39)
	51	86(18/21)	90(19/21)	90(19/21)	95(19/20)	12 days (11–13)
	53	18(5/28)	29(8/28)	77(20/26)	77(20/26)	19 days (17–21)
	57/58	65(13/20)	74(14/19)	74(14/19)	78(14/18)	13 days (12–14)
	Postmetamorphic	80(4/5)	100(5/5)			14 days (13–15)
Non-parental ♀	48/49	0(0/29)	4(1/26)	19(5/26)	23(5/22)	24 days (18–33)
	51	12(3/26)	28(7/25)	58(14/24)	58(14/24)	23 days (17–30)
	53	21(5/24)	42(10/24)	88(21/24)	96(22/23)	21 days (18–24)
	57/58	0(0/17)	35(6/17)	47(8/17)	67(10/15)	19 days (15–24)
	Postmetamorphic	83(5/6)	100(6/6)			14 days (12–16)

* According to Nieuwkoop & Faber (1967).

† Median survival time (MST) in days (95% confidence limits)

‡ Individual survival time.

§ MST of rejected grafts, *n* too small to compute 95% confidence limits.

Table 4. Median survival times of first- and second-set allografts on Stock 40 larvae

Graft donor	Developmental stage of hosts*	First-set grafts	
		MST of rejected grafts (95% confidence limits)†	MST of second-set‡ grafts (95% confidence limits)†
Parental ♂	48/49	35 days§	ND¶
	51	39 days**	16 days§
	53	23 days (17–33)	11 days**
Parental ♀	48/49	32 days (27–39)	17 days**
	51††	12 days (11–13)	8 days (6–10)
	53	19 days (17–21)	8 days**
Non-parental ♀	48/49	24 days (18–33)	10 days**
	51††	23 days (17–30)	9 days (6–13)
	53††	21 days (18–24)	10 days (8–14)

* According to Nieuwkoop & Faber (1967).

† Median survival time (MST) in days (95% confidence limits).

‡ Second-set grafts were transplanted at stage 57/58.

§ Individual survival time.

¶ ND, not done.

** MST of rejected grafts, *n* too small to compute confidence limits.

†† 95% confidence limits indicate that the differences in the MSTs of first- and second-set grafts are significant at $P < 0.05$; for all other groups the *n* is too small to determine the significance.

larval stages. Rejection of the few male parental grafts that were destroyed by larval hosts was delayed relative to controls with respect to both the onset of destruction and the rejection end point.

Regardless of the stage of the larva at the time of transplantation, a significantly higher proportion of female parental grafts than male parental grafts on premetamorphic and perimetamorphic recipients were rejected by day 50 (Table 3). Although almost half the larvae grafted at the earliest larval stage (stage 48/49) rejected female parental grafts, this incidence of rejection was significantly lower, and the MST of these transplants was significantly longer, than for the froglet controls. However, the incidence of rejection of skin from this female donor by larvae grafted at all the older developmental stages was the same as for postmetamorphic frogs. Although the rejection frequency was unchanged, the rapidity with which grafts of female parental skin were rejected varied among older larval groups. For example, larvae transplanted at stage 51 rejected grafts with kinetics comparable with controls, but rejection by recipients grafted as stage 53 larvae was significantly prolonged relative to stage 51 as well as to older stage hosts (Table 3). The kinetics of rejection of grafts on stage 57/58 perimetamorphic larvae were identical to those of the froglet controls.

Allografts from the non-parental donor, like transplants from the female parent, were rejected by a large percentage of hosts that had been grafted as larvae. Rejection of non-parental grafts by larvae transplanted at stage 48/49 was a significantly less frequent occurrence than was rejection by postmetamorphic

hosts (23% vs. 100%). The proportions of stage 51, stage 53, and stage 57/58 hosts that rejected these same transplants did not differ from the froglet controls. The MSTs of the rejected non-parental grafts in each of the premetamorphic groups grafted at stages 48/49, 51, 53 were significantly longer than MSTs calculated for grafts on the controls. The kinetics of graft rejection by metamorphosing stage 57/58 recipients and froglet hosts were similar.

The incidence of rejection of female parental grafts and non-parental grafts differed only for stage 51 recipients. Animals grafted at this stage rejected a significantly greater proportion of the parental grafts than non-parental grafts. The MSTs of rejected grafts from these two donors were similar for stage 48/49 and stage 53 recipients. Grafts from the female parent transplanted to stage 51 or stage 57/58 hosts, however, were rejected significantly more rapidly than grafts from the non-parental donor.

To determine whether young larvae recognize and respond to allografts immunologically, 100 of the 208 larvae grafted at stages 48/49, 51, and 53, received an original donor second-set graft as they underwent metamorphosis. The protocol was designed so that at the time of repeat grafting, each group of animals included some larvae whose first-set grafts were exhibiting each of several states of viability. That is, there was no arbitrary selection against animals that had rapidly rejected the first graft or bore fully viable transplants. As a group, hosts that had rejected first-set grafts, rejected second-set grafts with accelerated kinetics. Table 4 illustrates this point by comparing MSTs for all rejected first- and second-set grafts.

Table 5. Examples of the memory response of individual Stock 40 *Xenopus* grafted with first-set and second-set transplants as premetamorphic and perimetamorphic (stage 57/78) animals, respectively.

Skin graft donor	Host stage when first-set grafted	Individual survival times (days) of	
		First-set grafts	Second-set grafts
Male parent	53*	20	8
Male parent	53	21	14
Non-parental	48/49	36	12
Non-parental	53	26	16
Non-parental	53	24	16
Non-parental	53	22	8
Non-parental	53	24	12

* For approximate chronological ages of recipients at time of grafting see Fig. 1.

Accelerated rejection also characterized the second-set responses of individual animals as seen in the examples provided in Table 5. Thus, if any propensity for tolerance induction can be attached to the perimorphic period, it cannot 'cancel out' a nett cytotoxic response elicited during an earlier developmental stage.

When the initial transplant exhibited prolonged survival (> 50 days), the repeat graft also survived. Such viable transplants were carefully monitored for at least 50 days. In instances where animals carrying long-surviving transplants were observed for longer periods, both first- and second-set grafts appeared to survive indefinitely (> 200 days).

DISCUSSION

Genetic relationships

An MLC reaction is a marker of incompatibility at the MHC of *Xenopus laevis* (Du Pasquier, Chardonnens & Miggiano, 1975). MLC typing of Stock 40 F₁ frogs was used to identify four MHC haplotype identical (i.e., MLC non-responsive) classes of siblings. As detailed elsewhere (Barlow *et al.*, 1981), these four sibling classes indicate that either three or four different MHC haplotypes were segregating in the family. In theory, if four different MHC haplotypes were present, then the MHC haplotypes of the parents in the cross must have been AB × CD. Thus, each parent would differ from each F₁ offspring by one MHC haplotype. If the sibship contained only three different MHC haplotypes, the parental cross must have involved haplotypes AB × AC. On a statistical basis, each parent of this cross should have been MHC haplotype identical with (and MLC non-stimulatory for) 25% of the F₁ progeny. In fact, splenocytes from all twelve siblings tested were significantly stimulated by cells from the female parent (the male parent was not available for testing). According to the test of goodness of fit (Dixon & Massey, 1969), we can be statistically confident ($P \leq 0.05$) that this anti-parental response by cells from all F₁ frogs tested, proves that four different MHC haplotypes were segregating in this family. Therefore, grafts from a parent to any of its F₁ progeny involved a one MHC haplotype barrier.

Peripheral blood leucocytes from the two parents used in this investigation were mutually responsive in two-way MLCs. In addition, cells from both parents responded to irradiated stimulator cells from the non-parental donor used in this study (data not

shown). Unfortunately, without the appropriately designed breeding and genetic analysis, these MLC data are only consistent with (but do not prove) our assumption that the unrelated donor differed from *all* siblings by one or two MHC haplotypes.

Allograft reactivities

Our observations that sodium perchlorate had no overt effect on the frequency of prolonged graft survival or on the rapidity of graft rejection, are similar to the finding of Du Pasquier & Chardonnens (1975) who used another goitrogen, thiourea, to maintain grafted *Xenopus* in the perimorphic state. Nevertheless, to unequivocally evaluate whether a goitrogen-treated animal responds like its age-matched control or stage-matched control, one needs a donor–host combination in which distinctly different frequencies of tolerance induction are observed at two temporally distinct stages of development (e.g. stages 53 and 57/58).

Any discussion of the ontogeny of alloimmunity in *Xenopus* must focus on the immunogenetic disparity between donor and host. In this regard, DiMarzo (1980) noted that all grafts transplanted from adult frogs to minor H locus disparate but MHC identical (MLR unreactive) larval recipients were not rejected, regardless of when they were grafted (stage 48/49, 51, 53, or 57/58). That similar grafts on control postmetamorphic frogs were chronically destroyed provided additional evidence that the donor–host combinations she used differed only by minor H loci (LG clones 15 and 17, Kobel & Du Pasquier, 1977; JJ strain, Barlow *et al.*, 1981). It is for this reason that in the following discussion of the present study (which involved progeny of outbred parents), we will emphasize the importance of MHC antigens (or minor H antigens in association with MHC disparities) in evoking the tolerance/rejection reactions we observed.

The nett alloimmune response in this family, as visualized by the frequency and kinetics of graft rejection, depended on the donor–host combination and on the developmental stage of the recipient at the time of transplantation. The influence of the host's age was most obvious in comparisons between larval and postmetamorphic recipients. A net tolerance response was induced, in varying degrees, in all combinations with larval recipients, whereas no postmetamorphic frog accepted grafts from the same MHC incompatible donors for longer than 30 days. This difference between larval and adult responsiveness was most

clearly seen when the male parent served as the donor. In this situation, from three-fourths to virtually all of the hosts grafted at any larval stage failed to reject the transplant. Therefore, despite a one MHC haplotype difference between this donor and all recipients, a nett tolerogenic rather than a cytotoxic reaction was stimulated.

Skin from the female parent also differed from each recipient by one MHC haplotype. Although it was accepted by some larval recipients, the frequency with which it was rejected was strikingly greater than when the male parent served as the donor. Thus, at least during ontogeny, a net tolerogenic or a nett cytotoxic response can be elicited in the same set of recipients by alloantigens associated with different MHC haplotypes.

Skin from the unrelated donor was also rejected by many, but not all, larval recipients. That some grafts survived suggests that larval hosts may be predisposed to tolerization even when a putative two MHC haplotype disparity is involved. This phenomenon has been reported elsewhere for other families of *Xenopus* (Cohen, DiMarzo & Hailparn, 1980) and for more detailed immunogenetic conditions (Barlow *et al.*, 1981). No significance should be attached to the fact that in the present study the highest incidence of rejection by larval recipients was seen with female donors since we have demonstrated elsewhere that the nett immune reaction in siblings produced by a mating of outbred animals, or in MHC homozygous lines of animals (Cohen *et al.*, 1980; Barlow *et al.*, 1981), is independent of donor or host sex.

Our results suggest that during the ontogeny of *Xenopus*, there is a gradual emergence, and then a dominance of an immuno-destructive state. When very young stage 48/49 larvae (approximately 10 days postfertilization) were grafted, they were commonly rendered partially to fully tolerant of the transplant. Even when rejection occurred, it was delayed. Therefore, relative to older larvae and to postmetamorphic controls, this early stage appears to be a special time in the ontogenesis of alloreactivity that no doubt reflects (at least in part) the absence of complete differentiation of the lymphoid tissues at the time of transplantation. In general, as older and older larvae were grafted, more animals became capable of rejecting transplants. This rejection also became more and more vigorous. Generally, the ability of older larval animals to reject allografts and the rapidity with which they rejected them approached that seen in the control postmetamorphic hosts.

This maturational sequence occurred at different developmental times for each of the three donor–host combinations studied. The response to grafts from the male parent matured only after metamorphosis. Responses to the alloantigens carried by the unrelated donor and lacking in the sibling animals were essentially the same during the peri- and post-metamorphic periods. The ability to respond destructively to grafts from the female parent appeared fully developed even in very young stage 51 hosts. The extent to which these transitional periods between a nett tolerogenic and a nett destructive response reflect an increased cytotoxicity or a waning of a tolerance system has not been addressed in the present study. Nevertheless, the gradual maturation of the immune response against allografts, and the differential emergence of cytotoxic reactivities to alloantigens coded for by different MHC haplotypes at different developmental times that we observed with *Xenopus*, is identical to what has been reported for the ontogeny of alloimmunity in other species such as the dog (Dennis *et al.*, 1969), rat (Medawar & Woodruff, 1958; Steinmuller, 1961) and mouse (Boraker & Hildemann, 1965). It is, however, in direct contrast to the apparently abrupt appearance of allograft reactivity in sheep (Silverstein *et al.*, 1964).

The only significant deviation from this pattern of a gradually emerging alloreactivity was noted with the female parental donor and mid-larval stage hosts. In this combination, the MST of rejected grafts that had been transplanted at stage 51 was significantly shorter than that for grafts transplanted at stage 53. It appears that animals had the ability to acutely reject these allografts at one stage, lacked it at a later stage, and finally regained it at stage 57/58. This phenomenon, where young animals reject allografts significantly more rapidly than do animals of a slightly older developmental stage, has also been reported by Steinmuller (1961) for the neonatal rat and by Najarian & Dixon (1962) for the neonatal rabbit. This observation could reflect a differential emergence of various components of the immune system. For example, alloreactive clones of cytotoxic cells that recognize some antigens may emerge prior to some regulatory suppressor elements. Therefore, skin allografting with the appropriate antigen disparate combinations at sequential developmental stages, coupled with transfer protocols, may provide insights into the chronological development of various components of the immune system.

Chardonnes & Du Pasquier (1973) reported that premetamorphic larvae are fully immunocompetent

but that they lose some of their immune capabilities during metamorphosis. Specifically, they found that tolerance of minor transplantation antigens, and occasionally of MHC antigens, occurred only during the metamorphic transition from immunocompetent larva to immunocompetent adult. They proposed that a tolerizing mechanism emerges during metamorphosis to allow for the induction of unresponsiveness to newly appearing self-antigens. Our results in several different donor-host combinations suggest that this provocative hypothesis is perhaps too restrictive a portrayal of the maturation of alloimmunity. We have clearly shown that tolerance to MHC antigens can be induced throughout larval life and is not restricted to the one particular metamorphic period. Such tolerance clearly depends on the alloantigens that a host confronts. We have also reported that a net destructive immunity to different antigens emerges at different ages and that occasionally, the alloreactive capability seems to be compromised at subsequent developmental times. This ontogenetic pattern can be visualized in species other than *Xenopus*. Therefore, tolerizing mechanisms seem to function (and indeed, may predominate) during ontogeny of such vertebrates. Our data by no means preclude the possibility that amphibian metamorphosis may be a period when these effects become more pronounced due to the rather unique and dramatic changes in the antigenic constitution of the animal and the immune system itself (Chardonnens & Du Pasquier, 1973). Our data further speak to the value of amphibian models as powerful tools to study the ontogeny of alloimmunity and tolerance to self and nonself.

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