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Immune Response against Skin Allograft and Rabbit Red Blood Cells in Metamorphosing and Metamorphosis-inhibited *Xenopus laevis*

By

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(With 8 Text-figures and 1 Table)

During the last decade, several lines of immunobiological studies have established that larval amphibians, thanks to their free-living state, provide an excellent material for studying the ontogeny of immune reactivities (for a review, see Du Pasquier, 1973). Although the anuran amphibians evidently acquire immune responsiveness in the early larval stages, there are some controversies as to whether or not their acquired capacity retains its level through metamorphosis, a phenomenon unique in this class of vertebrates. In *Xenopus*, a high proportion of skin allograft tolerance has been reported to occur in the metamorphic stages, provided the grafting is made between siblings (Bernardini *et al.*, 1969, 1970; Chardonnens and Du Pasquier, 1973; Chardonnens, 1975). Depression of serum antibody production was also observed during this period in *Rana catesbeiana* (Moticka *et al.*, 1973). Such a depression of immune reactivities may be possible in this particular stage, in view of the reorganization of the lymphoid system accompanying this period (Cooper, 1967; Manning and Horton, 1969). On the other hand, Hildemann and Haas (1959) and Bovbjerg (1966) have presented evidence for the occurrence of normal skin allograft rejection in *Ranidae*.

The present experiment was undertaken as a part of a series of studies which are intended to explore the ontogeny of immune response in the anuran *Xenopus laevis*. To compare the animals' immune reactivities *during* metamorphosis with those of larvae and adults, persistent larvae were obtained by larval thyroidectomy or treatment with thiourea (TU). The results will be described and discussed below.

Materials and Methods

Materials: The material used was the South African clawed toad, *Xenopus laevis*. Immune responses were studied using the siblings which have been

maintained in our laboratory for several years. This group of animals, tentatively referred to as "G group", share histocompatibility antigens as defined by the lack of allograft rejection among individuals (*cf.*, Tochinai and Katagiri, 1975).

Fertilized eggs were obtained by induced mating, after an injection of chorionic gonadotropin (Gonotropin, Teikoku Zoki Co.) into mature males and females. Embryos and larvae were maintained in dechlorinated tap water, with a constant aeration at 23°C. Larvae older than 4 days post fertilization were fed boiled alfalfa leaf powder every other day. Developmental stages were determined by the Normal Table of Nieuwkoop and Faber (1956).

Inhibition of metamorphosis: Thyroidectomy was performed on stage 51 (17-day-old) larvae. The organs in this stage of the larvae are a pair of oval glands approximately 300 μm in size, situated immediately anterior to the branches of the ventral carotid arteries which occur ventral to the hyoid. The larvae were anesthetized in 1/5,000 MS222 (Sandoz) in Steinberg's solution, and placed dorsal side downward on the operation plate set on ice-chilled water. This plate was composed of a glass slide to which the glass rods were affixed about 2 mm apart, so that a larva to be operated on was fixed firmly. The operation was performed under the dissecting microscope through transmission light. A cut was first made on the ventral skin along the midline. Then, using a hooked, sharp steel needle, both thyroid glands were removed. Tissues at the operated area were then pipetted off to minimize the residual thyroid tissue. After the operation, the larvae were placed overnight in Steinberg's solution, followed by transfer to water for rearing under the usual conditions. The operated larvae developed normally to stage 56 (20 days after operation). Following that stage, the individuals which were successfully thyroidectomized ceased metamorphosis. About 30 days after the operation, when non-operated siblings were at stage 58, those operated larvae which were undergoing metamorphosis were omitted. Of 99 operated animals, 86 survived, and 15 individuals did not metamorphose. Twelve metamorphosis inhibited larvae were reared for approximately 100 days to test their immune responsiveness.

From stage 48 (10 days old) on, the larvae were reared in 0.01% thiourea (TU). Fifty larvae thus treated developed normally to stage 57, and ceased metamorphosis at this stage over 70 days, when non TU-treated siblings metamorphosed to toadlets.

Allograft rejection: Normally developing and metamorphosis-inhibited G-group larvae received skin allografts from "HD-group" toadlets, histocompatibly unrelated donors. The skin transplantation was made according to the procedures described by Chardonnens and Du Pasquier (1973). Skin grafts were given to thyroidectomized individuals 40 days after the arrest of metamorphosis, and to TU-treated ones 20 days after the arrest. Normally-metamorphosing stage 56 and 58 larvae also received a graft. To minimize the difference in the relationship between hosts and donors, each 4-5 host animals received grafts from a single HD-group donor. In addition, some individuals received autografts. Following the

transplantation, the fate of the skin grafts was observed and recorded every day under the dissecting microscope.

Serum antibody response: Animals 45 days after TU-treatment and non-treated stage 57 larvae received an intraperitoneal injection of 5 μ l rabbit red blood cells (RRBC), suspended at 50% in phosphate buffered saline (PBS). Since a direct intraperitoneal injection caused a considerable leakage of antigen, injection was made so that a microsyringe was inserted through the tail muscle. Each larva received 3 injections at 2 day intervals. Four weeks after the last injection of antigen, the animals were bled, their serum collected, and hemagglutinating activities were determined by a microtiter technique, using 1% RRBC suspension as a test antigen. Decomplementation of serum was made by the addition of 0.04M EDTA.

Histological: The animals were fixed in Bouin's fluid. The lymphoid organs to be examined were dissected, embedded in paraffin, and serially sectioned at 7 μ m. The sections were stained with Delafield's hematoxylin and eosin.

Results

During the period of the experiments to be described below, thyroidectomized and TU-treated individuals retained the appearance of stages 56 and 57 larvae, respectively (Fig. 1). Their size measured about 12 cm in length, as contrasted with 8cm in normally developing stage 56-57 larvae. About 30 days after the arrest of metamorphosis, a bending of the tail and a contraction of the epidermal melanophores became apparent. In particular, thyroidectomized individuals ceased to take food 60 days after the operation.

Response to skin allograft

Fig. 2 summarizes the fate of skin allografts in metamorphosing and metamorphosis-inhibited individuals. Before the end of the graft rejection, all 18 individuals which had been neither thyroidectomized nor TU-treated underwent metamorphosis quite normally. Of the total of 37 animals which received skin grafts, 1 TU-treated larva was dead during the post-grafting days. All 19 autografts applied to normal stage 58 and TU-treated larvae were accepted. It is clear from the figure that, in spite of the apparent physiological difference, all 35 animals rejected allografts in a similar rate of time, with the median survival times (MSTs): 19.1 \pm 0.5 days (stage 56), 19.7 \pm 0.5 days (stage 58), 20.0 \pm 1.1 days (thyroidectomized) and 19.7 \pm 0.4 days (TU-treated). All second-set grafts were rejected in an apparently accelerated fashion, without the restoration of blood circulation in 14 cases. The MSTs were 10.0 \pm 0.8 days, 9.7 \pm 0.6 days and 10.2 \pm 0.4 days for the three experimental groups, respectively. These results show that both metamorphosing and metamorphosis-inhibited animals possess an allotransplantation immunity equal to that of adults (*cf.*, Tochinal and Katagiri, 1975).

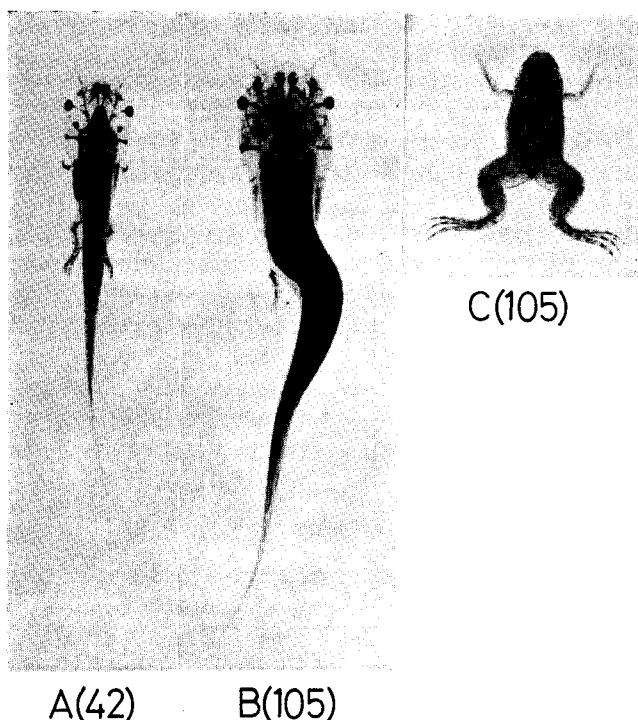


Fig. 1. Photographs of *Xenopus*, showing stage 57 larva (A), thiourea-treated (B) and non-treated (C) animals. Numbers in parentheses indicate the age in days after fertilization.

Response to RRBC

Table 1 summarizes the antibody response against RRBC in normally metamorphosing and TU-treated animals. Both groups of animals possessed no naturally occurring antibody to RRBC. After receiving RRBC at stage 57, the animals which were not treated with TU underwent metamorphosis, so that 4 weeks later they were bled as toadlets. It is evident in the table that, as found in the case of allograft rejection, the serum antibody response is again unaffected by the physiological changes accompanying metamorphosis.

Histology of lymphoid organs

Examination of thymus from TU-treated larvae sacrificed 80 days after the treatment showed a well-developed cortex similar to that found in the thymus of stage 57 larvae (Figs. 3 and 4). It is contrasted with the relative increase of medulla in the thymus of normally metamorphosed toadlets of the same age (Fig. 5). On the other hand, in the thyroidectomized larvae fixed 100 days after the

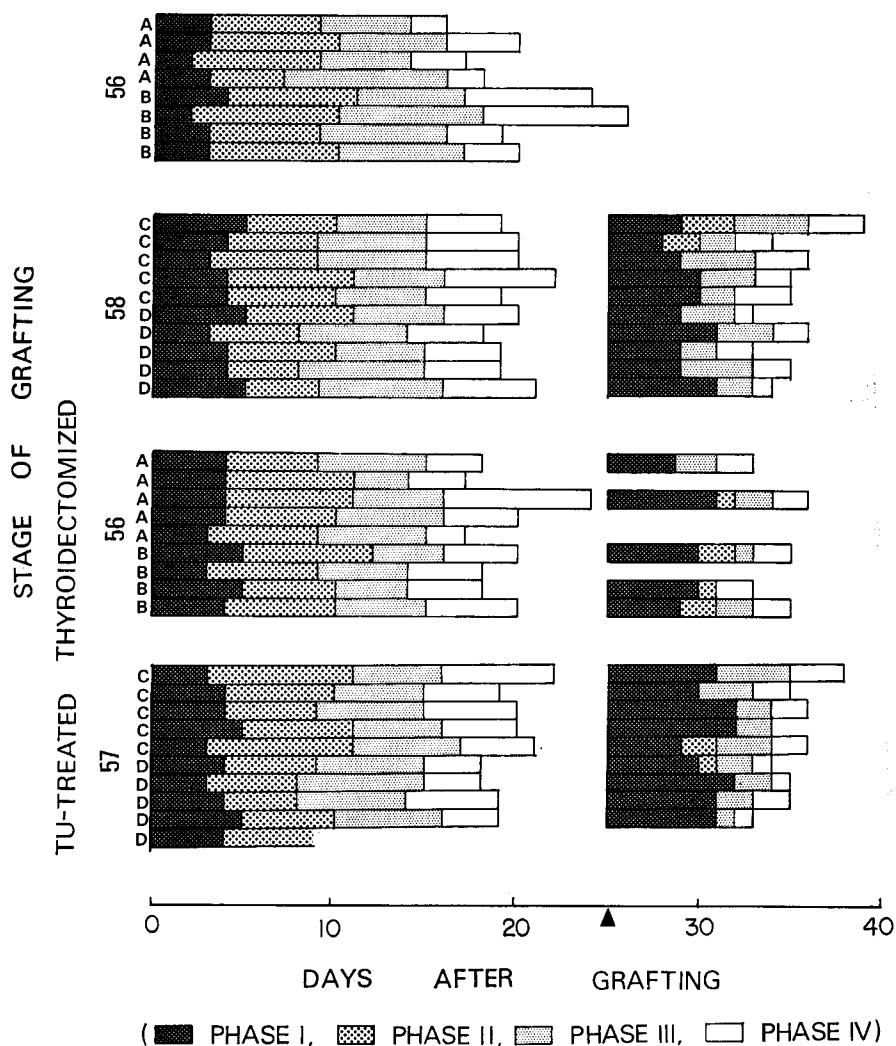
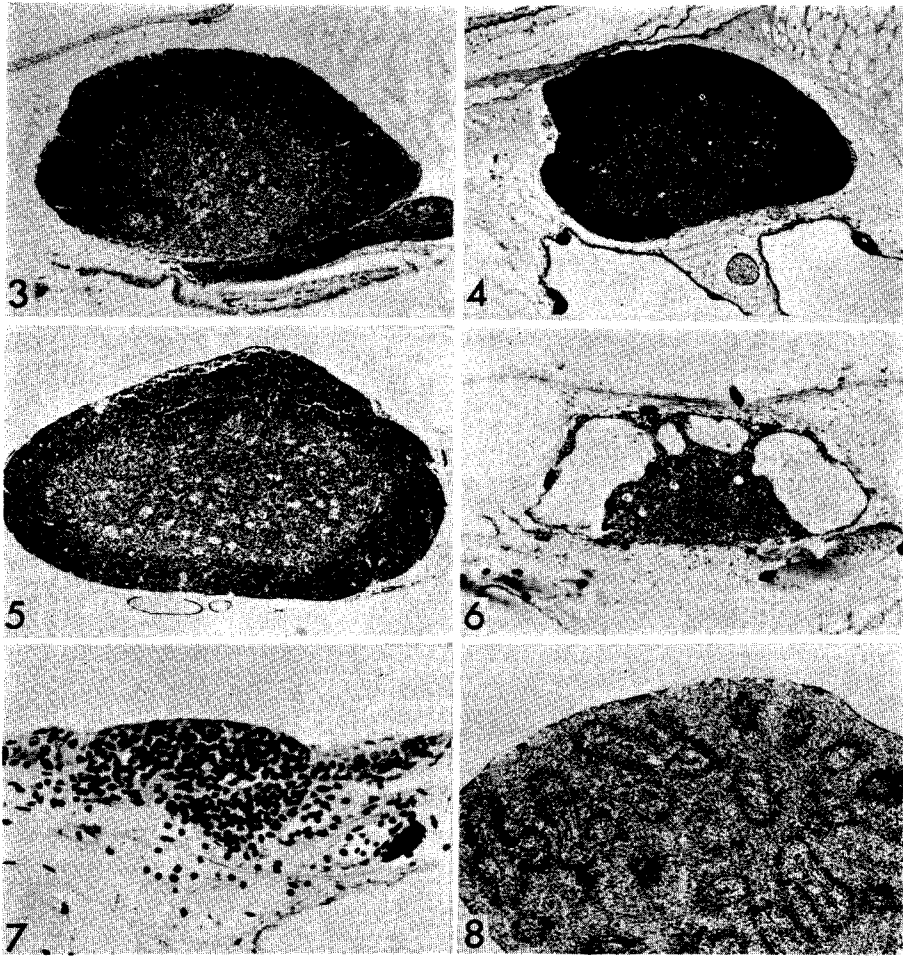


Fig. 2. Fate of skin allografts in metamorphosing and metamorphosis-inhibited "G-group" hosts. A-D on the ordinate show "HD-group" donors who provided grafts. For criteria of graft rejection phases (I-IV), see Tochinal and Katagiri (1975).

operation, a reduction of the total volume of thymus was apparent (Fig. 6). Remarkable was the occurrence of several large vacuoles occupying the outer part of the organ. Lymphocytes were seen to be packed only in particular parts, but the differentiation of cortex and medulla was not appreciable. Coincident with the arrest of metamorphosis at stage 56-57, both thyroidectomized and TU-treated



Figs. 3-6. Sections through thymus from thiourea-treated animal (100 days old; Fig. 3), stage 57 larva (40 days old; Fig. 4), 120-day-old toadlet (Fig. 5) and thyroidectomized animal (120 days old; Fig. 6). Note the normal appearance in Fig. 3, and the diminution in size and occurrence of vacuoles in Fig. 6. $\times 40$

Figs. 7 and 8. Sections through first ventral cavity body (Fig. 7; $\times 200$) and spleen (Fig. 8; $\times 40$) of thiourea treated animal 100 days after fertilization (60 days after the arrest of metamorphosis), showing heavy accumulation of lymphocytes in both organs.

animals possessed larval branchial lymphoid tissues; *i e.*, 4 pairs of ventral cavity bodies (VCBs; Fig. 7) and 2 pairs of dorsal cavity bodies (DCBs; *cf.*, also Tochinai, 1975). Likewise, the degree of lymphocytic accumulation in the spleen and gut-

Table 1. Formation of antibody against RRBC in metamorphosing and metamorphosis-inhibited *Xenopus laevis*

Type of Animals	Number of Animals								
	Ab Titer (-log ₂)								
	0	1	2	3	4	5	6	7	8
Thiourea-treated Immunized					2	3			
Non-immunized	5								
Metamorphosing Immunized						2	2	1	
Non-immunized	5								

associated lymphoid tissues was quite similar in both metamorphosis-inhibited and normal stage 57 larvae (Fig. 8).

Discussion

Both metamorphosing and metamorphosis-inhibited individuals used in the present study showed the same order of response, against allografts and RRBC, as that observed in adults (*cf.*, Tochinai and Katagiri, 1975). It is evident, therefore, that neither the physiological changes accompanying metamorphosis nor its artificial inhibition have appreciable effects on the immune responsiveness.

The normal allograft rejection observed in the present study is consistent with the results obtained in hypophysectomized or thyroxine-treated larvae of *Rana pipiens* (Bovbjerg, 1966) and metamorphosing *Rana catesbeiana* (Hildemann and Haas, 1959). However, these results differ from those of Bernardini *et al.* (1969, 1970) and Chardonens and Du Pasquier (1973), who recorded that *Xenopus* during the metamorphic and post-metamorphic stages displays a unique depression of allograft rejection. It should be noticed that, as suggested by a recent analysis using TU-treated *Xenopus* (Chardonens, 1975), this impaired immunity is appreciable only in a particular combination of sibling pairs which possess a minimal difference of histocompatibility genes. Although their genetic background is not well defined, the heterogeneity between G- and HD-group toads used in the present study is clear. Thus, the difference between the present and above-cited results could quite conceivably be dependent on the difference in the degree of heterogeneity of genomes between hosts and donors. In view of their phylogenetic level, together with their availability as inbred laboratory animals, it would be possible to obtain a *Xenopus* colony with a high degree of shared histocompatibility genes. The occurrence of histocompatible "G-group" *Xenopus* may well be explained on the same ground (*cf.*, Tochinai and Katagiri, 1975).

The ability of larval amphibians to produce specific antibodies has been well established, both by immuno-cytoadherence and hemagglutination tests (for *Alytes*, Du Pasquier, 1970; for *Rana*, Moticka *et al.*, 1973; for *Xenopus*, Kidder *et*

al., 1973). The present result that antibody production is independent of metamorphosis is not consistent with the result obtained in *Rana catesbeiana* (Moticka *et al.*, 1973), where the antibody reaction to SRBC was markedly depressed during metamorphosis. The exact reason for this contradiction is not known. Besides the materials used, the difference in the condition between these studies is very wide as to the antigen employed, its dose, and the schedule of its injection as well as the period between the antigenic stimulation and bleeding.

In spite of an apparent aberrant feature of the thymus caused by thyroidectomy, the immune responsiveness of metamorphosis-inhibited animals was entirely normal. This is not surprising in view of the thymectomy experiments (Turner and Manning, 1974; Tochinai and Katagiri, 1975), where the immune responsiveness in *Xenopus* was shown to be established as early as stage 47-48, and maintained to the adult stage. During metamorphosis, remarkable changes or reorganization occur in the lymphoid system (Cooper, 1967; Manning and Horton, 1969) and, possibly, in the composition of serum proteins as well (Marchalonis, 1971; Geczy *et al.*, 1973; Jurd *et al.*, 1975). Because of a profound reorganization of the organism as a whole, it is plausible that a weakening of immune reactivity, but not of the immune recognition mechanism, may take place during the metamorphic stage of amphibians. Thus, even in a case where the "tolerance" of skin allografts was recorded (Chardonens and Du Pasquier, 1973), a lymphoid invasion to grafts has been observed. Hence, the impairment during metamorphosis of immune reactivities as reported by the previous researchers may be attributable to a certain physiological state in general inherent in this stage.

Summary

In an attempt to examine the immune responsiveness during metamorphosis of *Xenopus laevis*, the allograft rejection and circulating antibody response were studied of the metamorphosing and metamorphosis-inhibited animals. Persistent larvae of stage 56-57 were obtained by thyroidectomy or treatment with thiourea (TU). Both metamorphosing and metamorphosis-inhibited "G-group" animals rejected skin allografts from incompatible "HD-group" toads in the same range of days as that of adults (MSTs, 19.1-20.0 days). A definite, accelerated second-set response was observed (MSTs, 9.7-10.2 days). In addition, all animals tested produced a specific antibody against rabbit red blood cells (RRBC) in a titer of 2^4 - 2^7 . These results indicate that the immune responsiveness established in the early stages of development functions and is maintained beyond metamorphosis. A temporary impairment of immunity during metamorphosis, if present, could be ascribed to the profound reorganization of the organism as a whole at this stage.

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