SERUM ANTIBODY SYNTHESIS IN LARVAE OF THE BULLFROG, RANA CATESBEIANA [1]

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Much evidence now indicates that poikilothermic vertebrates are excellent forms for studies of developmental and phylogenetic aspects of immunogenetics (see reviews by Hildemann, 1962a, 1962b; Hildemann and Cooper, 1963). From a developmental viewpoint, some of these studies have been concerned with the maturation of immunologic responsiveness, with a greater emphasis devoted to the capacity to react to skin allografts, the temporal appearance of blood cell types and, more recently, the role of the thymus gland in the ontogeny of the immune system.

Although ample data are accumulating dealing with the allograft reaction in adult cold-blooded vertebrates, the immunological competence of these species as antibody producers has been at issue mainly because of insufficient study (Hildemann, 1962b; Papermaster *et al.*, 1963). Even though some reports have indicated specific precipitin production by adult amphibians (Evans and Horton, 1961; Hildemann, 1962b; Austin and Nace, 1962), no such serum antibody production has been previously demonstrated in larval amphibians. The extent and rate of maturation of the isoimmune response capacity in young poikilotherms *vis-à-vis* birds and mammals have posed interesting phylogenetic questions. Precipitins to xenogenic antigens have been induced in high titer by immunization of adult fishes (Ridgeway, 1962; Clem and Sigel, 1963) and adult amphibians (Evans, 1963; Evans and Horton, 1961; Austin and Nace, 1962), but no comparable data are at hand concerning the capacities of juvenile or larval recipients. Accordingly, this report describes new findings—the production of precipitins to xenogeneic and allogeneic serum protein antigens by larvae of the American bullfrog, *Rana catesbeiana*. This report, however, deals with the effects of thymectomy on the production of precipitins to xenogeneic antigens. Studies are now in progress which are aimed at understanding the relationship between the amphibian thymus gland, other lymphoid organs and the maturation of cellular and humoral immunity.

Materials and Methods

Thymectomy of bullfrog larvae. In anuran larvae, the thymus gland is a bilateral, white, compact organ situated in close proximity to the eye and ear.

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Although amphibians supposedly have no discrete lymph nodes comparable to those of mammals, they do have "lymph glands" which are likewise bilateral and located in the branchial region; the role of this accessory lymphoid tissue is still conjectural. The thymus gland was excised from both sides of the head after anesthetizing the tadpoles in 0.6% saline containing 42 mg. tricaine methane sulfonate/L. Sharp needles and iridectomy scissors were best for making the initial cut in the side of the head, while watchmaker forceps were used for removing the gland once it had been exposed. After the excision, as a precautionary measure, tetracycline wound powder was sprayed into the wound, which was finally closed by means of a single cat-gut suture. All operations were performed with the aid of a stereomicroscope. The tadpoles were placed in 0.6% saline for about 6–12 hours before they were returned to pond water, and were always maintained at a temperature of $25 \pm 0.5°$ C. throughout the experiments. Bullfrog larvae were collected in the late summer when they were approximately 3–4 months of age and at standard stage 25. In contrast to this condition during the first year, tadpoles rapidly grow and usually approach metamorphosis in the wild sometime during the second year.

Immunization with xenogeneic antigens. Larvae of approximately 3–4 months of age, that had been previously thymectomized bilaterally at standard stage 25, were given initially a 0.05-ml. subcutaneous injection of goldfish (*Carassius auratus* Linnaeus, 1758) serum emulsified with incomplete Freund's adjuvant. Whole serum, obtained from several adult fish by cardiac puncture, was pooled. Booster injections of the same amount of antigen alone were given intraperitoneally 10–17 days later. The interval between thymectomy and initial antigenic challenge was in all cases at least 60 days. Tadpoles were bled individually from the tail and each serum sample obtained 9–11 days after the booster was tested against four dilutions (1:4; 1:8; 1:16; 1:32) of the antigen by means of the agar gel diffusion technique of Ouchterlony. The tests, set up on microscope slides placed in humid Petri dishes, were kept at room temperature. Lines of precipitation were observed after 2–3 days in all positive tests. Since some of the tests were negative in the sham-thymectomized control group, it was concluded that naturally occurring precipitins were not present.

Immunization with allogeneic antigens. Tadpoles ranging from stages 26–29 were immunized with pooled serum obtained from several adult bullfrogs by cardiac puncture. A total of six injections, each of 0.05 ml. allogeneic serum, was given at intervals of 10–17 days. The first and fifth doses were emulsified in incomplete Freund's adjuvant and injected subcutaneously; the other injections of serum alone were given intraperitoneally. Serum samples were obtained 11 days after the last booster, and control serum from ten non-immunized larvae and adults, each tested individually, revealed no naturally occurring isoprecipitins. The remaining experimental analysis is identical to that described above when goldfish serum was used as the antigen. Methods for the successful laboratory maintenance of bullfrog larvae have been previously described (Hildemann and Haas, 1959).

Technique of bleeding. One convenient region for obtaining blood from bullfrog larvae at varying intervals was the fleshy portion of the tail just caudal to the abdomen. To prevent any excess moisture which might be mixed with the blood, the tadpoles were first held firmly and wiped dry completely with cotton

on one side. After a short quick stab into the tail with a sterile lancet, blood was collected easily and quickly with capillary pipettes and placed into culture tubes (60 × 50 mm.). When the clot had completely retracted after approximately one hour at room temperature, the tubes were centrifuged at 1500 rpm for 10 minutes. The serum was then placed in sterile culture tubes, stoppered and stored at −20° C.

Results

Three of the eight larvae immunized over a two-month period with allogeneic serum yielded serums that gave isoprecipitin bands (Fig. 1a). Thus, the existence of serum isoantigens or serum groups in this species is evident. The apparent lack of isoprecipitin production in some recipients is attributable either to insufficient immunization or to sharing of major antigens with donor serums. Inasmuch as serums obtained from these larvae prior to the last two injections gave negative results, it appears that prolonged immunization is necessary for substantial isoprecipitin production.

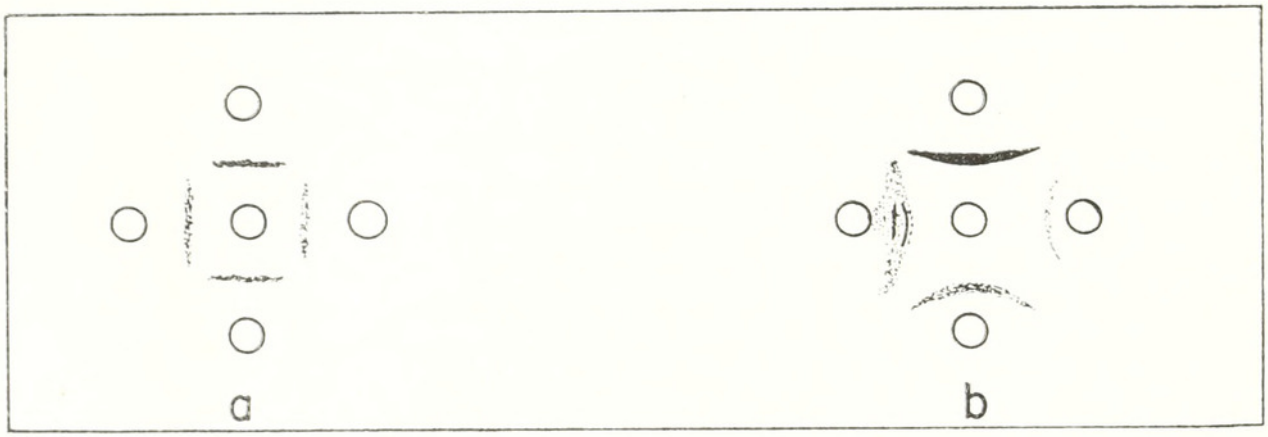

Figure 1 a, b. Characteristic precipitation patterns in double diffusion plates obtained from tadpoles immunized with adult serum (isoimmunization, Fig. 1 a), and adult goldfish serum (Fig. 1 b). Note differences in the precipitin bands between the two groups. Longer periods of immunization were necessary to produce isoprecipitins than precipitins to xenogeneic serum antigens. Six injections of antigen were required when using the allogeneic antigen. The first and fifth consisted of whole adult bullfrog serum emulsified in incomplete Freund's adjuvant. In the case of the xenogeneic group, only two injections were neecssary. The first consisted of antigen emulsified in Freund's adjuvant.

The data summarized in Table I support the thesis that bullfrog larvae are capable of synthesizing serum antibodies to xenogeneic antigens. However, the exact time at which the immune response toward diverse antigens matures has not yet been established.

In a group of 18 larvae that were sham-thymectomized and subsequently injected with goldfish serum antigen at stages 25–29, 10 showed strongly positive precipitin bands. Five serums reacted to produce weakly positive bands, while three were completely negative even after prolonged incubation. In a group of 13 larvae, however, which were thymectomized at stage 25–27, the precipitin band patterns were markedly different. In this group, only one showed a positive precipitin band, seven a weakly positive reaction, and five were negative. The

TABLE I

Precipitin reaction of bullfrog larval antiserum with goldfish serum antigen in Ouchterlony tests

No. of larvae immunized with goldfish serum	Standard stage at thymectomy	Group and standard stage at immunization and testing	Precipitin test
10		25-adult unoperated non-immunized	negative
18	25	25–29 sham-thymectomized	10 positive 5 weakly positive 3 negative
13	25	25–27 thymectomized	1 positive 7 weakly positive 5 negative

data suggest that thymectomy interfered with the humoral antibody response to serum protein antigens. However, the variation in antibody response between the two groups might to some extent depend upon differences in developmental age, *e.g.*, stage 25 versus stage 29 in relation to age at thymectomy. By comparing Figures 1a and b it is apparent that a shorter immunization schedule was sufficient to produce stronger precipitin bands to xenogeneic goldfish serum than to allogeneic serum. Because only small quantities of immune larval serum have been available, quantitative precipitin and immunoelectrophoretic analyses have yet to be performed.

DISCUSSION

Although precipitin production to soluble protein antigens by larval poikilotherms has been demonstrated in this investigation, the elucidation of the time in development when this capacity first appears remains problematical. The bullfrog has a prolonged larval period which is advantageous when dealing with developmental problems over a long period. Therefore, one important aspect of this study is to be able to show unequivocally the time of onset of the capacity to synthesize serum antibodies. It has already been demonstrated that larvae ranging in age from two months (stage 25) to two years vigorously reject skin allografts. However, newly hatched larvae at stage 24 and up to 36 days of age were sufficiently immature to become partially or completely tolerant toward allografts (Hildemann and Haas, 1959). The blood cell picture reveals that small lymphocytes begin to appear 40–45 days post-hatching during this transitional state of weak reactions to allografts (Hildemann and Haas, 1962). How the larvae resist environmental pathogens during the first six weeks post-hatching is conjectural. Abundant mucous secretions of the skin are no doubt important, while maternally-derived antibodies in the yolk may be essential until the larvae develop their own immunologically competent cells. It might be possible to correlate the disappearance of yolk, as the primary nutritive source, and the onset of feeding with the maturation of certain humoral responses after 15 days when leucocytes begin to appear.

The finding that thymectomized larvae appeared to show a weakened humoral antibody response to goldfish serum antigen is consistent with observations of mammals thymectomized early in life (Miller, 1962; Jankovic *et al.*, 1962). In avian vertebrates there exists a functional dichotomy between the thymus gland and the bursa of Fabricius. Here the thymus plays a dominant role in cellular immunity (Aspinall *et al.*, 1963) while the bursa, a cloacal lymphoid organ, is essential for humoral antibody responses (Graetzer *et al.*, 1963).

There is ample evidence now to promote vigorous debate concerning the mechanism by which the thymus mediates immunologic responsiveness (Levey *et al.*, 1963; Law *et al.*, 1964; Osoba and Miller, 1964), *i.e.*, whether it is exclusively cellular or humoral, or a combination of both mechanisms. The available evidence, supported by experiments using diffusion chambers, suggests that a humoral factor or hormone is active in juveniles and adults; however, the initial developmental acquisition of immunological competence and its subsequent maintenance may well depend upon systemic dissemination of thymocytes. The finding that bullfrog larvae thymectomized after about 50 days post-hatching show the usual acute rejection of allografts, but exhibit a weakened antibody response (Cooper *et al.*, 1963; Hildemann and Cooper, 1963) and conspicuous runting (Cooper, unpublished) supports the assumption that both cellular and humoral functions may be attributed to the thymus.

Perhaps the thymus in Amphibia continues to affect humoral antibody production and growth after the maturation of the immune response to skin allografts, but its influence is greatest on cellular immunity during the early larval period before general immunological competence is attained. In the case of skin allografts, the extent of the immune response is a function of the degree of immunogenetic unrelatedness between hosts and donors. Thus, graft survival in thymectomized mice is most prolonged in those strain combinations wherein weak histocompatibility barriers exist and least produced across strong H-2 differences (Martinez *et al.*, 1962). Although many "strong" histocompatibility alleles at various loci are evident in bullfrog populations, a high degree of immunologic specificity of allograft rejection in bullfrog larvae has also been demonstrated (Hildemann and Haas, 1961). With regard to serum antibody production, the immune response may also be substantially influenced by host genotype relative to different donor antigens, and by the schedule of immunization. Another factor which could affect the degree of immunologic impairment in thymectomized larvae is a variable regeneration of thymic rudiments and differences in maturation rate relative to age. In another study (Cooper and Hildemann, in preparation) concerned with the relationship between thymectomy and skin allograft survival, all larvae were biopsied at the end of the experimental period to determine the condition of the thymus, since it was assumed that all tissue had been removed initially. A detailed study is necessary to determine with certainty whether the thymus in the bullfrog is capable of undergoing regeneration.

In contrasting the production of precipitins to xenogeneic antigens (goldfish serum) with precipitins to allogeneic serum (*Rana catesbeiana* Shaw 1802), it seems appropriate to consider several factors. With regard to the production of antibodies against xenogeneic antigens, the class- and species-specific differences in the composition of serum proteins existing between the donor and recipients are sufficiently numerous to readily assure the induction of antibody production. By

contrast, the fewer individual differences existing between serum proteins within a species may also represent minor alterations in molecular structure that are effectively less antigenic or foreign—thus the longer period of immunization required for the production of isoantibodies. In the bullfrog subpopulation presently studied, a number of potential isoantigens (allotypic specificities) may be shared by all of the individuals tested; in other words, only a few genes determining serum protein specificity may be segregating in this population. Dray and Young (1958), in their successful demonstration of induced isoprecipitins in certain rabbits, focused attention on the importance of immunization schedules. With appropriate techniques, isoantibodies are capable of detecting not only wider antigenic differences between the several globulin fractions of serum, but also the allotypic specificities within the gamma fraction alone.

Although the present investigation has thus far been primarily concerned with the maturation of immunological competence, ancillary findings involve immunogenetic and comparative immunological concepts as well. In addition, a study of this kind devoted to an understanding of the ontogeny of the immune system in an amphibian such as the bullfrog will help to clarify concepts regarding the evolution of the immune response.

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Summary

1. Larvae of the American bullfrog, *Rana catesbeiana,* were thymectomized and sham-thymectomized at stages 25–29, representing an age range of about 60–200 days. After immunization at 25° ± 0.5° C. with goldfish serum proteins as antigens, specific precipitating antibodies were found in the majority of the sham-operated controls. In contrast, nearly all of the thymectomized group showed a markedly weakened response to goldfish serum antigens.

2. Lines of isoprecipitation were obtained in Ouchterlony tests with serums from some larvae immunized with isoantigens (whole adult serum). A longer period of immunization is apparently required to produce precipitins to serum isoantigens than to comparable xenogeneic antigens. To our knowledge, this is the first finding of serum isoantigens or allotypes and induced isoprecipitins in any poikilothermic vertebrate.

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