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Studies on the role of the thymus and  
spleen in the immune system of the  
Clawed Toad (Xenopus laevis, Daudin)

by John James Rimmer, B.Sc.

being a Thesis submitted for the Degree of

Doctor of Philosophy

in the University of Durham (Department of Zoology)

OCTOBER 1976.



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### DECLARATION

The work presented in Chapters 2 and 5 forms the basis of two publications:

(i) RIMMER, J.J. and HORTON, J.D. (1976): "Allograft rejection in larval and adult Xenopus following early thymectomy." Transplantation (in press).

(ii) HORTON, J.D., RIMMER, J.J. and HORTON, T.L. (1976): "The effect of thymectomy at different stages of larval development on the immune response of the clawed toad to sheep erythrocytes." J.exp. Zool., 196 : 243.

The work in Chapter 3 was performed in collaboration with Dr. J.D. Horton and Mrs. T.L. Horton of the Department of Zoology, Durham University.

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## ABSTRACT

- 1) A thymus-independent alloimmune system emerges early in the life of the clawed toad, independent of changes in the lymphoid system occurring at metamorphosis. Studies in Chapter 2 on first and second-set allograft rejection, both in larvae blocked from metamorphosing and in normally developing toads, show that there is no qualitative difference between the larval and adult allo-immune response.
- 2) In the intact adult, the spleen is shown to be a major site of cellular reactivity following allografting. Spleen transfer experiments between mutually tolerant partners demonstrate that this reactivity has an immunological basis. Preliminary studies in Chapter 3 implicate the spleen as a site of alloimmune memory.
- 3) Work in Chapter 4 employs the sensitive ICA technique for investigating the role of thymus and spleen in humoral immunity. RFC kinetics in the spleen following administration of different doses of sheep erythrocytes reveal a dose response curve more similar to that described in mammals than in other amphibian species. Higher immunogen doses result in more rapid and more elevated RFC production. Morphological characterisation of rosettes failed to distinguish separate functional populations (i.e. "helper" and antibody producing) of lymphocytes in Xenopus. Early thymectomy abrogates responsiveness to injected SRBC as measured by cellular proliferation and RFC levels.
- 4) Chapter 5 identifies a critical role of the thymus throughout much of larval life in the maturation of humoral immunity. Thymectomy up

to stage 5<sup>3</sup>~~4~~ abrogates RFC production to SRBC, and antibody production is impaired by thymectomy as late as stage 57. Thymectomy later <sup>at</sup> ~~than~~ stage 52 has no effect on allograft responses. The larval thymus controls development of both alloimmunity and humoral immunity but commands the maturation of the latter for a more prolonged period of development. Adult thymectomy has no effect on SRBC responsiveness up to 250 days post-surgery.

- 5) Chapter 6 provides new insight into the ultrastructure of developing thymus and emphasises the rudimentary nature of this organ at the time when thymectomy is routinely performed.

Although current concepts in immunology stem largely from studies on the immune systems of birds and mammals, immunologists are becoming increasingly aware of the importance of examining immune mechanisms in both lower vertebrates and amongst the invertebrate phyla. Such a comparative approach avoids the pitfalls inherent in generalizations based upon observations of relatively few endothermic species or "representative types" (e.g. chickens and laboratory rodents). By studying a wide range of organisms it is becoming possible to identify features which are basic to immunological reactivity, as opposed to those aspects of the immune system which represent specialized adaptations which have evolved in only a few species or animal groups. Moreover, these studies are proving to be invaluable in elucidating the phylogeny and ontogeny of mammalian immunity: see the reviews by Cooper, 1973; 1976a; DuPasquier, 1973; Good and Papermaster, 1964; Grey, 1969; Hildemann, 1972; 1974; Hildemann and Cooper, 1970; Hildemann and Reddy, 1973; Manning and Turner, 1976; Marchalonis and Cone, 1973.

Following the important discovery that annelid worms are able to destroy foreign tissue transplants, (Cooper, 1965; Duprat, 1964) the quest for prototype immune systems has, in recent years, switched to the invertebrates. Although invertebrate defence mechanisms consist primarily of non-specific phagocytosis and encapsulation, (see Cooper, 1976a; Maramarosch & Shope, 1975) recent studies suggest that various levels of immunoevolution exist amongst the diverse array of animals without backbones. (See Hildemann, 1974; Hildemann and Reddy, 1973). The lowest level, or "recognition of non-self" which is apparent in protozoans and sponges, contrasts with the more sophisticated (immune) systems displayed by more advanced invertebrate forms. Thus, the two hallmarks of an immune



response, specificity and memory, are seen in the "primordial cell-mediated immunity" which has been demonstrated from transplantation experiments in at least two invertebrate phyla. Thus amongst the Protostomia, allograft and xenograft responses, which display specificity and memory, have been demonstrated in annelids. Moreover, the cellular basis of annelid graft rejection has been examined: coelomocytes which appear at the site of graft rejection can adoptively transfer graft immunity and appear to be the 'forerunners' of the vertebrate T-lymphocyte (see review by Cooper, 1976a). Interestingly, a recent report has shown that earthworm coelomocytes respond to concanavalin A and transplantation antigens in vitro (Roche et al., 1975). The echinoderms are the second phylum in which "primordial cell-mediated immunity" has been demonstrated. These animals are deuterostomes and are considered to be more immediately ancestral to the vertebrates than the protostomes, which diverged early from the chordate-line. Specific allograft immunity with short term memory has been found in a sea cucumber (Cucumaria tricolor, Hildemann and Dix, 1972) and two species of sea-star (Protoreaster nodosus, Hildemann and Dix, 1972; and Dermasterias imbricata, Hildemann, 1974; Karp and Hildemann, 1975). Leucocytes (macrophages, small lymphocytes and granulocytes) were observed to infiltrate the allografts.

A variety of natural agglutinins and (inducible) bacteriocidal substances ("antisomes") have also been observed in the body fluids of invertebrates (Cooper, 1976a; Maramarosch and Shope, 1975). However, these bear no structural relationship to the immunoglobulins of vertebrate species (Marchalonis and Cone, 1973). Although invertebrate humoral factors lack the high degree of specificity that characterizes vertebrate antibodies, both groups of molecules share a common property of aiding the elimination of antigen (e.g. by opsonization: Cooper, 1976a). Whether or not the genes coding for immunoglobulins only arose with the evolution of the vertebrates awaits clarification. In particular, more extensive studies on invertebrate (including protochordate) cell surface receptors are required since it has

been suggested that immunoglobulin (Ig) precursors were cell-membrane associated molecules (Marchalonis and Cone, 1973).

Integrated cell mediated immunity and humoral antibody production are immune parameters unique to the vertebrates (Hildemann and Reddy, 1973). Even the primitive agnathan Hagfish possesses T- and B- lymphocyte-equivalents in the sense that it can reject allografts (Hildemann and Thoenes, 1969) and produce IgM antibody (Thoenes and Hildemann, 1970; de Ioannes and Hildemann, 1975). Although the origin and precise role of lymphocytes in ectothermic vertebrates is still controversial (see General Discussion Chapter 7), these cells have been implicated in immune responses at all levels of vertebrate phylogeny; recent studies have indicated that in agnathans (Cooper, 1971); elasmobranch and teleost fishes (Cuchens et al; 1976; Etlinger, 1976; Lopez et al., 1974; Sigel et al., 1973); and in anuran amphibians (Donnelly et al., 1976; DuPasquier et al., 1975; Goldshein and Cohen, 1972; Goldstine, Collins et al., 1975; Manning et al., 1976), lymphocytes undergo blastogenesis and proliferation when exposed to selective T- and B- cell mitogens or allogeneic cells in vitro. Basic similarities between ecto- and endothermic vertebrates in terms of humoral and cell-mediated immunity are also suggested by the demonstration of carrier-specific enhancement of anti-hapten responses (suggestive of lymphocyte co-operation?) in amphibians (Ruben, 1975) and fishes (Stolen and Mäkelä, 1975; Yocum et al., 1975), and by the existence of both strong and weak histocompatibility loci in diverse vertebrate classes (Cohen and Borysenko, 1970).

One of the better studied poikilothermic vertebrate classes with respect to their immunobiology are the Amphibia, which represent an interesting transitional stage in immunoevolution (Cooper, 1976b; DuPasquier, 1976). As a class, amphibians are remarkably heterogeneous in their degree of immunological development. Representatives of the primitive orders Urodela (Salamanders) and the little-studied Apoda (caecilians) differ markedly in

their immunobiology from the more advanced anurans. In common with the cartilagenous and primitive bony fishes (Clem, 1971; Clem and Small, 1967; Marchalonis and Edelman, 1965), the urodeles possess only a single class of immunoglobulin which corresponds to the IgM class of mammals. (Ambrosius et al., 1970; Tournefier, 1975; (newts); Ching and Wedgewood, 1967; Houdayer and Fougereau, 1972 (axolotls); Marchalonis and Cohen, 1973 (mudpuppies)). The immunoglobulins of Apoda have not been studied. Skin allografts in urodeles (Cohen, 1971) and apodans (see review by Cooper, 1976b) are rejected in chronic fashion, which, in addition to the lack of an obvious mixed lymphocyte culture (M.L.C.) reaction, has been taken as evidence for the absence of a major histocompatibility locus at this level of evolution (Cohen, 1976). Structurally, the lymphoid system of urodeles is similar to that first seen in modern Chondrichthyes (Cohen, 1976). Salamanders characteristically possess organized thymus and spleen (Cowden and Dyer, 1971; Hightower and St. Pierre, 1971) and in addition, some species possess limited lymphocytic foci in kidney (Cowden and Dyer, 1971); buccal areas (Kingsbury, 1912; Klug, 1967) and liver (Ruben et al., 1973). There is no haemopoietic bone marrow; gut associated lymphoid tissue (G.A.L.T.) is almost completely absent (Goldstine, Manickavel et al., 1975); and lymph nodes are not found (Cooper 1976a).

In sharp contrast to the situation described above, larval and adult anuran amphibians resemble mammals more closely in producing at least two antigenically distinct classes of serum immunoglobulins (Coe and Peel, 1970; Geczy et al., 1973; Marchalonis and Edelman, 1966; Steiner et al., 1975 - Rana spp., Acton et al., 1970; Diener and Marchalonis, 1970; Lin et al., 1971 - Bufo marinus; Hadji-Azimi, 1971; 1975; Lykakis, 1969; Marchalonis et al., 1970; Yamaguchi et al., 1973 - Xenopus laevis.) These two molecules (19S and 7S) have been designated IgM and 'IgG-like' on the basis of their general resemblance to the IgM and IgG of mammals. However,

it has been demonstrated (Atwell and Marchalonis, 1975; Geczy et al., 1973; Hadji-Azimi, 1971; 1975; Steiner et al., 1975) that there are certain differences between the  $\gamma$ -chains from mammalian IgG and the heavy chains of the 7S anuran Ig molecule in terms of molecular weight and electrophoretic mobility, thus indicating that mammalian IgG and anuran low molecular weight Ig molecules are not strictly homologous. Both high and low molecular weight Ig's are found prior to metamorphosis in anurans, however inducible levels of 7S antibody are difficult to detect prior to metamorphosis (DuPasquier and Haimovitch, 1976; Pross and Rowlands, 1976). Both larval and adult anurans typically reject skin allografts in acute fashion (although in some instances (e.g. Xenopus) sub-acute rejection is the rule) (Cohen, 1971). Moreover, mixtures of allogeneic lymphocytes in vitro result in significant stimulation indices (Bufo marinus, Goldshein and Cohen, 1972; Xenopus laevis, DuPasquier and Miggiano, 1973; Weiss and DuPasquier, 1973). The anuran amphibians are therefore thought to possess a major histocompatibility complex (M.H.C.) which may be homologous to that of mammals and birds (DuPasquier, et al., 1975).

The lymphoid complex of the tailless Amphibia is more advanced than that of the urodeles or primitive fishes. The primitive pipid Xenopus possesses in addition to thymus, spleen and scattered lymphocytic foci in liver and kidney; a lymphopoietic bone marrow with limited activity (Thomas and Maclean, 1974), and some G.A.L.T. (Goldstine, Manickavel et al., 1975; Horton 1969a). The lymphoid system of the ranid and bufid species is still more complex, for they also possess a number of lymph node-like structures in the neck and axillary regions (Baculi et al., 1970; Diener and Nossal, 1966; Horton, 1971; Kent et al., 1964). Although these are thought to primarily filter blood (see Cooper 1976a), it has been suggested that they are also lymph-filtering organs (Horton, 1971). These structures house both antigen-trapping (Diener and Nossal, 1966) and antibody-forming cells (Diener and Marchalonis, 1970; Cooper, 1976b). Larval forms often possess

lymph glands (see Cooper 1976b) which have been shown to be important in controlling antibody synthesis (Cooper et al., 1971). G.A.L.T. in these species is better organized and more extensive than in Xenopus (Goldstine, Manickavel et al., 1975).

Besides their obvious phylogenetic interest, the amphibians possess a number of features which render them especially suitable for immunological investigations. Thus their embryonic and larval forms are free-living, thereby facilitating studies on the ontogeny of the immune system without the attendant complications of materno-foetal interactions, whose influence may be difficult to assess in the developing amniote. In addition, experiments on mammalian embryos are rendered difficult because of their relative inaccessibility in utero. A notable exception to this rule however, are the foetal marsupials which emerge from the uterus at a fairly rudimentary stage of development and complete their maturation in the mother's pouch (Ashman and Papadimitriou, 1975; Block, 1967; Lapante et al., 1969; Stanley et al., 1972; Yadav et al., 1972). The fact that anuran amphibian larvae become immunocompetent (as judged by the ability to reject skin allografts (Horton, 1969b), give positive stimulation indices in M.L.C. (DuPasquier and Weiss, 1973), and synthesise specific antibody (DuPasquier, 1970a; Haimovitch and DuPasquier, 1973) when only small numbers of lymphocytes ( $1 - 2 \times 10^6$ ) are present (DuPasquier, 1976) provides an interesting system in which to study the problems of generation of antibody diversity (germ-line vs. somatic mutation). Progress in this sphere promises to be further aided by the recent development of isogenetic strains of Xenopus (Kobel and DuPasquier, 1975). Although "clones" of amphibians can be obtained by the process of nuclear transplantation, the technique is difficult and the yield is low.

Techniques originally devised for experimental embryology in amphibians

have also made important contributions to studies on the ontogeny of the immune system. Thus, tolerance induction may be studied by embryonic tissue grafts (Clark and Newth, 1972; Volpe, 1971). The use of triploid cell markers (Dasgupta, 1962) in normal diploid embryos allowed the detection of blood cell chimaerism following embryonic transplantation (Volpe and Gebhardt, 1966) and permitted studies on lymphocyte origins (Turpen et al., 1973; 1975, Turpen and Cohen, 1976).

Recently, Chardonnes and DuPasquier (1973) and Chardonnes (1976) have described a second period in ontogeny during which studies on the generation of tolerance to self and non-self histocompatibility antigens are possible. Thus, at metamorphosis (in Xenopus), a time when the amphibian is developing new 'adult' antigens, it is possible to induce a state of tolerance to weak histocompatibility antigens.

One final attribute of amphibian studies is that, in common with other poikilotherms, the immune response may be modified by altering the environmental temperature at which the animals are kept; this has provided a tool for dissecting various phases of the immune response (e.g. Marchalonis, 1974; Wright et al., 1975).

A major theme in immunology in recent years, and one which is pursued in this thesis, has been the study of the role of the thymus in immunity and the development of the concepts of thymus-dependency and thymus-independency of certain immune functions (see Greaves et al., 1973). A recognisable thymus is present in young specimens of all vertebrates from the elasmobranchs onwards (Cooper, 1976a). In the agnathans, the larval lamprey possesses an accumulation of lymphocytes in the gill region which has been regarded as a prototype of the thymus (Good et al., 1966). The search for an equivalent structure in the hagfish has been hampered to date by the unavailability of young specimens, however Riviere et al., (1975) have suggested that a 'protothymus' may be contained in a scattered cell population associated

with the pharyngeal velar muscles. In amphibians, the study of thymic function is facilitated by the ease with which this organ can be removed at the earliest stages of its development (see Cooper, 1973; DuPasquier, 1973; 1976; Manning, 1975; Manning and Collie, 1976). A particularly useful model in this respect, and the one employed in the present studies, is the South African Clawed Toad or Platanna, Xenopus laevis. In this species, the developing thymus may be readily located through the transparent skin and destroyed by microcautery as early as one week after fertilization. At this stage each thymus contains approximately 1000 cells with few, if any, small lymphocytes (Horton and Manning, 1972). Earlier removal (5 days) is possible, but the survival rate is much lower (Horton and Horton, 1975). Unlike other amphibian species, (e.g. Rana pipiens, Curtiss and Volpe, 1971; Rana catesbeiana, Baculi and Cooper, 1973; Alytes obstetricans, DuPasquier, 1968) thymic regeneration is a rare event in Xenopus, probably as a result of thermal damage to the pharyngeal epithelium which may otherwise give rise to a new thymic rudiment (Horton and Manning, 1972). Another remarkable feature of thymectomized Xenopus, is the absence of wasting disease (runting) (Manning, 1971; Horton and Manning, 1974a) which contrasts markedly with the high incidence of this phenomenon in other thymectomized amphibians (see DuPasquier, 1973) and thymus-deprived mammals (e.g. neonatally thymectomized and nude mice Miller, 1962; Pantelouris 1973). The lack of a wasting syndrome in Xenopus allows long-term investigations to be made on the effects of thymic ablation in otherwise healthy animals. The present study of thymic function in Xenopus is designed to give new insight into the phylogeny and ontogeny of thymic-dependence of cell-mediated and humoral immunity.

A secondary aspect to be considered in this thesis is the role of the spleen in the amphibian immune system. Previous work (Manning and Horton, 1969) has demonstrated that in Xenopus the spleen represents the most

structurally organized lymphoid tissue and, furthermore, is the organ most affected by early thymectomy in terms of cellular architecture (Manning, 1971; Horton and Manning, 1974a). In order to gain a better understanding of the precise role of lymphoid tissues in amphibian immune responses, the present work investigates the role of the spleen in cell-mediated and humoral immunity and attempts to define the extent to which splenic function is controlled by the thymus.

The effects of early thymectomy on the alloimmune response of the larva and adult is analysed in Chapter 2. Chapter 3 concentrates on the involvement of the spleen in skin allograft reactivity of control toadlets and a brief study is presented on the effect of early thymectomy on such reactivity. Chapters 4 and 5 are concerned with humoral immune responses to heterologous erythrocytes (sheep red blood cells - SRBC's). Chapter 4 characterizes the influence of immunogen dose on the rosette-forming cell response in control spleens. The effect of early thymectomy on this response is also examined. Chapter 5 investigates the effects of thymectomy at different stages of development on the maturation of the immune response to SRBC's. Chapter 6 presents a brief ultrastructural study of the amphibian thymus, particularly during the earliest stages of its differentiation. Finally, Chapter 7 reviews the major findings of this thesis and discusses these in the light of recent studies on the role of lymphoid tissues in the development of immunity.

INTRODUCTION

Participation of a thymus independent component in graft rejection in anuran amphibians has been implicated from allograft studies on Xenopus laevis toadlets. Thus, thymic removal by microcautery at 7 to 8 days of age does not result in a complete abrogation of alloimmune responsiveness but merely delays the onset of allograft rejection; many grafts are eventually infiltrated by lymphocytes and rejected in chronic fashion (Horton & Manning, 1972). Furthermore, chronic graft destruction is still apparent even when the thymus is removed at 5 days of age when it contains fewer than 500 cells (many of which are epithelial) with no small lymphocytes (Horton & Horton, 1975).

Studies to date have not assessed whether the capacity of thymectomized Xenopus to reject grafts emerges during larval life or whether it appears only in adulthood coincident with the development of new lymphomyeloid tissue at metamorphosis. Horton and Manning (1972) have reported histological observations on larval skin grafts applied to thymectomized tadpoles, but these were only carried out at 12 days post-transplantation. The eventual fate of skin allografts applied to thymectomized larvae is therefore not known. This is an important issue to resolve particularly since others (Chardonens and DuPasquier, 1973; and DuPasquier, 1973) have suggested that the larval and adult alloimmune systems are qualitatively different, the former being more thymus-dependent than the latter.

The work presented in this chapter has investigated the problem by examining first and second-set skin allograft reactivity in thymectomized larvae, some of which are held in the larval state by goitrogen treatment. New data on graft rejection in thymectomized toadlets is also presented to

allow direct comparison of larval and adult alloimmune responses.

## MATERIALS & METHODS

### Rearing and care of animals

Animals were bred and reared in the laboratory for all the studies presented in this thesis. The general conditions have been described in detail elsewhere (Horton and Manning, 1972) and only a summary is presented here. Spawning was induced by the injection of chorionic gonadotrophin into the dorsal lymph sac of male and female adult Xenopus laevis (Daudin). Larvae were reared in aerated standing water and fed nettle powder. All tadpoles were reared at  $23^{\circ} \pm 1^{\circ}\text{C}$ . After metamorphosis, stock toadlets were maintained at  $18^{\circ} - 20^{\circ}\text{C}$  and experimental toadlets were kept at  $23^{\circ}\text{C}$ , they were fed Tubifex worms twice weekly.

### Thymectomy

Thymectomy was performed on larvae at seven or eight days post-fertilization when they were at stage 47 or 48 of Nieuwkoop and Faber (1967). At this stage the thymus is a small translucent organ, approximately  $100\mu\text{m}$  in diameter. Lymphoid differentiation is only just beginning as revealed by light microscopic studies and the thymus itself contains mostly large and medium lymphoid cells with few small lymphocytes (cells  $<6\mu\text{m}$  in diam. Horton and Manning, 1972). An Electron Microscopic study of the thymus at this stage is presented later in the thesis (Chapter 6).

The thymus was removed with the aid of a Martin-Elektrotom 60 high frequency (1.75 MHz) cautery apparatus and the procedure is summarised below. Larvae are positioned ventral side down in a small petri-dish containing a layer of charcoal-agar. Under a stereomicroscope the thymus is exposed by lifting away a flap of dorsal skin using fine tungsten needles. The tip of the neutral electrode is pushed into the agar and the tip of the positive electrode (a fine tungsten needle) is placed on the

thymus. Current is applied and the localised generation of heat which ensues at the tip of the positive electrode destroys the thymus. For fuller details of the operation the reader is referred to Horton and Manning (1972). Thymic absence was always confirmed at autopsy. Both sham-thymectomized and non-operated animals served as controls.

### Experimental Design

#### A. Preliminary studies on the larva

##### (i) First-set grafts

These experiments were designed to extend previous observations which had shown an impaired lymphocytic reaction to larval skin allografts in thymectomized larvae twelve days post-grafting (Horton and Manning, 1972). Following application of a 1st set larval-skin allograft (to the head region) at 30 days of age (stage 54 of Nieuwkoop and Faber - a stage when control animals respond vigorously to allografts: see Horton, 1969a) larvae were kept for as long as it was possible to observe grafts before metamorphosis. The grafts become difficult to see at this time due to changes occurring in the shape of the head. Animals were then fixed in Bouins (from 14-20 days post-grafting) and graft viability was assessed by histological criteria on a total of 10 thymectomized and 8 sham-thymectomized animals. After fixation, individuals were embedded in paraffin wax and serial sections (8 $\mu$ m) were cut through the graft regions. Sections were stained in Ehrlich's Haematoxylin and Eosin. The methodology for larval skin grafting has been described elsewhere (Horton, 1969b).

##### (ii) Second-set grafts

Alloimmune reactivity to grafts applied in larval life was also assessed by the application of a second-set graft following metamorphosis in a further 6 thymectomized animals. Four sham-thymectomized individuals 1st-set grafted as larvae were also examined. Metamorphosis occurs at 5 to 8 weeks of age, and second-set allografts, from the original first-set

donors, were applied 100 days after first-set grafting. Seven thymectomized and two sham-thymectomized siblings, which had not received first-set grafts as larvae, were also grafted alongside the above animals with skin from the same donors.

B. Studies employing adult-skin allografts

(i) Larvae

First-set adult-skin allografts were applied to 24 thymectomized and 24 sham-thymectomized larvae at 30 days of age. 19 grafted larvae (9 thymectomized and 10 sham-thymectomized) were allowed to metamorphose in normal fashion and the fate of their grafts was followed into adult life. White, ventral-skin allografts, applied dorsolaterally in the anterior tail region (see Chardonens and DuPasquier, 1973 for technique) were employed in this group, since, in contrast to larval-skin allografts applied to the head region, the fate of these adult grafts may be followed after metamorphosis when they come to lie posterodorsally in the trunk region of the toadlet and are readily distinguishable from the surrounding host skin. (See Fig.2.1)

The remaining grafted larvae in this study (15 thymectomized and 14 sham-thymectomized) were prevented from metamorphosing by the addition of thiourea (0.05% W/V) to the water. This chemical is known to block the synthesis of thyroxin by the thyroid gland (Turner, 1960). Treatment commenced immediately post-grafting. Eight thymectomized and 8 sham-thymectomized larvae were grafted with ventral skin as described above. The remaining blocked larvae received dorsal-skin allografts to allow a more direct comparison of the rejection process in larvae and adults. The dorsal skin grafts were applied in the head region as previously described (Horton, 1969b).

To further investigate the immunological nature of graft rejection, second-set allografts from original donors were applied to 6 of the untreated animals (4 shams and 2 thymectomized) and to 11 thiourea-treated larvae (7

shams and 4 thymectomized) at various intervals following first-set rejection (see Table 2.3).

(ii) Adults

Toadlets received first-set dorsal-skin allografts at 70 to 370 days of age. This experiment employed 40 thymectomized and 34 control (12 shams and 22 non-operated) animals. The technique of skin transplantation in adults has been described previously (Simnett, 1965). Second-set grafts were applied to 10 control and 8 thymectomized toadlets at various intervals following first-set rejection. (See Table 2.3).

C. General

To ensure that alloimmune reactions observed were not peculiar to any one batch of animals, the larval-skin experiments employed 3 batches, the adult-skin experiments on larvae involved the use of 2 separate batches and the studies on adults employed the progeny of 8 different matings.

Larval-skin grafts were applied from non-sibling donors of the same age, each donor supplying skin to one host: After metamorphosis, each donor was used to provide one second-set graft to the original host and one first-set graft to a control or thymectomized host which had not received a larval first-set allograft. Donors of adult skin were non-sibling toadlets (4-8 months old) and each donor supplied skin to no more than 2 hosts.

Grafts measured from 1.5 mm<sup>2</sup> (larvae) to 2.0 mm<sup>2</sup> (toadlets).

First-set adult-skin grafts were examined three times weekly for the first 40 days post-transplantation and then at weekly intervals after this. Grafts were classified as being in either Phase 1, in which the graft appears perfectly healthy with normal pigmentation and vascularization - the latter usually occurring within 2 - 4 days. (Figs. 2.2, 2.3); Phase 2, marked by

slight pigmentary defects and vasodilation (Fig.2.3); or Phase 3, characterized by extensive haemostasis and haemorrhaging, with accompanying pigmentary destruction and general necrosis (Fig. 2.3). Complete pigment destruction marked the graft rejection end point for adult skin (Figs.2.2, 2.3). In all experiments, second-set grafts were examined daily and rejection end points only were recorded.

#### Lymphoid organ development following thiourea treatment

To assess whether or not thiourea treatment had any effect on development of lymphoid tissues, 4 thymectomized and 4 sham-thymectomized treated larvae were fixed in Bouin's following graft-rejection. Serial, 8 $\mu$ m, sections were cut through the entire animal and stained in haematoxylin and eosin.

### RESULTS

#### A) Preliminary experiments on the larva.

##### (i) Observations on first-set grafts.

All 4 larval skin grafts examined on sham-thymectomized larvae 14 days post grafting were showing signs of incompatibility. Grossly, this was manifested by vascular enlargement, and opaque patches (indicative of lymphocytic accumulations (see Horton, 1969b) below the clear graft skin. Histological examination confirmed the gross observations and revealed moderate to heavy lymphocytic infiltration of the grafted tissue. Grafts on sham-thymectomized larvae examined at later intervals post-transplantation, 2 at 18 days and 2 at 20 days, showed increasing vascular disturbances (haemostasis and haemorrhaging and melanophore destruction. All grafts were being invaded by lymphocytes (see Fig.2.4).

In contrast, grafts on thymectomized animals (4 at 14 days, 2 at 18 days and 4 at 20 days post-grafting) appeared perfect when examined grossly; there was no indication of graft destruction. Histological observations

at 14 days supported this finding. Grafts were difficult to detect histologically because they had healed in so well with the surrounding host tissue. None of these grafts showed signs of lymphocytic infiltration. One out of two grafts examined at 18 days post-grafting, however, had small numbers of lymphocytes scattered below the grafted tissue. Small numbers of lymphocytes were also seen beneath the grafts of 3 out of 4 thymectomized animals fixed at 20 days post-grafting. (See Fig.2.4) Although this type of response is markedly different from that seen under allografts on control animals, the presence of a few lymphocytes (an indication of an alloimmune response) under the grafts of thymectomized larvae confirms Horton and Manning's findings (1972). However, the minimal response, even at 20 days post-grafting, still prevented any conclusions as to the eventual fate of the grafts. The second-set experiments looked into this issue by assessing whether or not the first graft had resulted in sensitization of the host.

(ii) Second-set grafts

The results of the second part of this experiment are given in Table 2.1.

All sham-thymectomized larvae which were given first-set larval-skin grafts showed signs of incompatibility and graft-destruction prior to metamorphosis which began approximately 14 days post-grafting. These ranged from vascular enlargement of the graft in one individual through to extensive pigment destruction in another. Only one of the 4 second-set allografts applied in adult life was rejected in accelerated fashion (15 days). Grafts on the other three animals were all destroyed within the normal first-set range for controls (17 - 25 days) as were grafts on 2 siblings grafted for the first time in adult life.

None of the six thymectomized larvae in this experiment displayed any gross signs of rejecting its first set larval skin graft. All grafts

appeared perfect until the onset of metamorphosis (11 to 16 days post-grafting). It was surprising therefore to find that in adult life, 4 of these animals destroyed second-set transplants within the range of first-set rejection times normally observed for controls. (17 - 25 days). Only 2 animals displayed impaired second-set alloimmune reactivity and both bore apparently healthy grafts for 80 days before the experiment was terminated. Seven thymectomized siblings which had not received first-set grafts as larvae all maintained healthy first set adult skin allografts for >80 days.

In conclusion, these preliminary studies have confirmed previous findings (Horton and Manning, 1972) that prior to metamorphosis, the response of the thymectomized animal to first-set skin allografts is impaired, but indicate that a skin graft applied during larval life nevertheless usually (in 4 out of 6 cases in the present experiment) sensitizes the adult to a second-set graft from the same donor. The time at which this sensitization occurs is, however, not clear. To obtain more detailed information on the ontogeny of alloimmune reactivity in thymectomized animals, experiments were devised employing adult skin grafts. (see Materials and Methods). These grafts could be followed over metamorphosis and rejection monitored far more easily than with larval skin grafts (Horton, 1969a). Furthermore, to determine whether or not the thymectomized larval immune system is able to effect complete graft destruction, these studies also made use of a goitrogen (which prevented metamorphosis) to allow prolonged investigation of the larval alloimmune response.

B) Studies using adult skin grafts.

(i) First-set allografts on larvae and adults.

Rejection times for first-set adult-skin grafts applied to control and thymectomized animals are summarised in Table 2.1 and the chronology of first-set graft rejection phases in thymectomized animals is presented in Figure 2.5.

The majority of controls rejected allografts in sub-acute fashion. Of the 34 allografts applied to control toadlets, 32 were rejected in 19 to 25 days, as were 9 out of 10 grafts applied to control larvae not treated with thiourea. Chronic graft rejection, taking 40 days, was seen in 2 control toadlets. One toadlet grafted as a larva, still retained a healthy graft when observations ceased 225 days post-transplantation.

Rejection end-points for thiourea-treated control larvae were far more variable. Fifty per cent rejected grafts within 25 days and grafts on treated larvae in Phases 2 and 3 of rejection are seen in Figure 2.3. Of the remainder, 2 rejected transplants in chronic fashion (36 and 90 days) whereas 5 animals still had healthy grafts 160 days post-transplantation. The variability in alloimmune response in this group applied equally to dorsal- and ventral-skin grafts.

Table 2.2 and Figure 2.5, clearly demonstrate that first-set allograft rejection is severely impaired following early thymectomy, since in all three experimental groups grafted with adult skin, graft survival was always prolonged (>25 days). Nevertheless, 48 out of 64 thymectomized animals were able to mount a chronic alloimmune response; thus 31/40 toadlets rejected their grafts, and 4 bore grafts in phase 2 of rejection when observations were curtailed at 128 to 417 days post-grafting. Similarly, 4/9 toadlets first-set grafted as larvae and 9/15 blocked larvae rejected allografts following thymectomy. One thiourea-treated, thymectomized larva with a rejected graft is seen in Figure 2.3. Histologically, the only element of the graft remaining was the collagenous stratum compactum, which also persists in control larvae (see also Horton, 1969b). Small foci of leucocytes were seen associated with the graft collagen pad in both control and thymectomized larvae.

Alloimmune reactivity was not recorded in 16 thymectomized animals

(5 grafted as toadlets and 11 as larvae). Since observations on many of these ceased at relatively short periods post-grafting (e.g. 100 days in 7 cases) it is possible that at least some of these animals would have eventually rejected their grafts. This possibility is strengthened by the finding (see Fig.2.5) that extremely long phase ones were recorded for several thymectomized animals which subsequently destroyed allografts. Thus although phase 1 persisted for 180 and 365 days in two toadlets grafted as adults, and for 120 days in one animal grafted as a larva, these animals went on to destroy their grafts in 225, 415 and 150 days respectively. The fully viable nature of adult skin allografts on some treated larvae at prolonged intervals post-transplantation (see Fig.2.3) demonstrates that graft destruction in these animals is not simply a result of physiological incompatibility of adult skin.

Figure 2.5 emphasizes that graft rejection end points were variable in both thymectomized larvae and adults and shows that this variation was apparent in the different batches of animals used. Where graft rejection was followed through to completion, phase 1 was the most variable in duration (10-365 days), whereas the actual destructive process (phases 2 and 3) was much more uniform in nature (10 - 55 days). Extremely prolonged rejection times in thymectomized animals were generally characterised by an extended phase 1, whereas phases 2 and 3 were relatively rapid. However, degenerative phases may also occasionally become sluggish, and even impaired, in thymectomized animals as evidenced by 4 toadlets which had retained grafts in phase 2 for extended periods when observations ceased.

*(ii) Second-set allografts on larvae and adults.*

Second-set allograft rejection times are given in Table 2.3. All control Xenopus displayed accelerated rejection of second-set grafts, although in cases where first-set rejection times were relatively rapid (17 and 18 days), this acceleration was only marginal (1 or 2 days). Memory was

apparent regardless of the interval between first-set rejection and second-set application (4 to 137 days).

All 14 thymectomized animals (including 4 blocked larvae) that were second-set grafted from 3 - 112 days post-first-set rejection also displayed accelerated destruction of these transplants, in times which contrasted dramatically with the chronicity of first-set rejection. All but one of the thymectomized animals rejected second-set grafts in 21 days or less and 8 of these times fell within the time range observed for second-set grafts on controls. One thymectomized animal took 46 days to reject its second-set graft, but this time was rapid when compared to a first-set rejection of 82 days.

C) Development of Animals.

Thiourea-treated animals were successfully maintained in the larval state for the duration (22 weeks) of the study. Development proceeded no further than stage 57 (prior to forelimb emergence) and the maximum length attained was 10 cm. Treated control larvae appeared perfectly healthy and possessed the usual array of larval lymphoid tissues i.e. thymus, spleen (see Fig.2.6) ventral cavity bodies and lymphoid accumulations in the liver, mesonephros and intestine (Manning and Horton, 1969). There was no bone-marrow. Treated thymectomized larvae displayed lymphoid depletion in ventral cavity bodies and spleen comparable to that found in untreated larvae following thymectomy (Manning, 1971; Horton and Manning, 1974a). The thyroid glands of treated larvae became goitrous as shown in Fig. 2.7 (see also Colemann, et al., 1967).

There was no difference in the growth rates of thymectomized and control Xenopus (see also Horton and Manning, 1974a) whether allowed to metamorphose normally or blocked with thiourea.

## DISCUSSION

These studies have demonstrated no qualitative difference in the degree of thymic dependency of the larval and adult alloimmune response. Throughout ontogeny survival of first-set skin allografts is always prolonged following early thymectomy, but most transplants are eventually destroyed even by those animals blocked in the larval state by thiourea treatment.

A consistent feature of the present experiments was the variability of first-set graft survival times in thymectomized animals. This variation may be related to the degree of histocompatibility antigen sharing; thus in those cases where donor and host share histocompatibility antigens, extremely prolonged graft survival would be the rule. In contrast, where sharing is minimal, the rejection displayed by thymectomized animals, although still chronic, would be more rapid. It should be noted that a major histocompatibility complex, homologous to that seen in higher vertebrates, is found in Xenopus (DuPasquier, Chardonnens and Miggiano, 1975). Antigen sharing may also account for the prolonged survival times of first-set grafts on 50% of the thiourea-treated control larvae. Many of these were not effectively blocked until stage 57, i.e. within the critical metamorphic period (stage 56 - one month post-metamorphosis) when lymphocyte numbers are depleted and when tolerance to skin allografts may readily be induced if donor and host share major histocompatibility antigens (Chardonnens and DuPasquier, 1973). The use of isogenetic strains of Xenopus of known haplotype differences would allow a more thorough investigation of the role of histocompatibility antigens in graft rejection in thymectomized Xenopus (Kobel and DuPasquier, 1975).

Following chronic rejection of a first-set allograft, thymectomized larvae and adults are able to destroy second-set grafts from the same donors in rapid fashion, thereby confirming the immunological nature of

transplant rejection. Interestingly, the range of second-set allograft survival times was similar in thymectomized and control groups. This suggests that having destroyed a first-set graft, a thymectomized animal is then as immunologically competent (with respect to a particular donor) as the control animal.

The finding that control (sham-thymectomized) Xenopus grafted as larva with adult skin can reject second-set grafts applied during adult-life is in direct contrast to an earlier report by Horton (1969a) who found that application of a first-set adult skin allograft prior to metamorphosis resulted in specific tolerance of further grafts from the same donor in adult life. This difference may be explained by the fact that in Horton's experiments the second grafts were applied at approximately 70 - 90 days of age, when the hosts were quite probably in the perimetamorphic period.

The mechanism and specificity of alloimmune memory in the thymectomized toad await further investigation, but preliminary results using small numbers of thymectomized animals which had rejected first- and second-set skin allografts suggest that in some cases third-party grafts may also be rejected in rapid fashion. In the absence of genetically defined strains, interpretation of this finding is difficult, since where rapid third-party destruction was noted there may have been extensive sharing of histocompatibility antigens between donors.

The finding that alloimmune reactivity still persists following early thymectomy contrasts with a recent publication by Tochinnai and Katigiri (1975) who failed to detect any reactivity to skin allografts in 3 - 12 month old Xenopus toadlets following thymectomy during the first week of life. However, it is felt that their studies are inconclusive in demonstrating permanent allograft acceptance, since they were unable to carry out long-term graft observations due to premature death (from 71 - 146 days

post-grafting) of 12 of their 13 early-thymectomized animals. Autopsies on the latter revealed appreciable lesions and monocytic infiltrations of liver and lungs, despite rearing in antibiotics for the first 60 days post-transplantation. The prolonged viability of grafts in their experiments may, therefore, reflect the poor health of their thymectomized animals, rather than a primary consequence of thymic absence. In this respect, germ-free neonatally-thymectomized mice (which remain free from bacterial infection) show less impairment of graft rejection capacity than do conventionally-reared thymectomized animals (Miller et al. 1967). The healthy state of thymectomized Xenopus in this laboratory may be related to the absence of pathogenic microorganisms present in <sup>our</sup> their colony of toads.

It has been suggested (Cohen, 1975) that early-thymectomy fails to prevent allograft rejection in Xenopus because seeding of cells from the thymus has already begun. If early-seeded, thymus-schooled cells are involved, then one might expect such cells to subsequently expand or decline in number in the periphery with concomitant improvement or impairment of alloimmunity. However, skin graft reactivity does not vary with time interval of graft application post-thymectomy. Moreover previous reports have shown (Horton and Manning, 1972) that thymectomy as late as 14 days of age still results in an alloimmune deficiency comparable to that seen following thymectomy at 7 days (see also Chapter 5). This is remarkable when one considers that at 14 days, the thymi now contain approximately  $5 \times 10^4$  lymphocytes (DuPasquier and Weiss, 1973). If lymphocytes are already emerging from the thymus at 7 days, then a delay of 7 days in removing this organ would be expected to result in a markedly improved alloimmune response. Furthermore, chronic graft rejection displayed by toadlets thymectomized at 5 days of age (stage 46/7) (Horton & Horton, 1975) supports the view that early-seeded, thymus-dependent lymphocytes are not responsible for allograft destruction in thymectomized

Xenopus. Thus it seems unlikely that a rapid and sufficient burst of cell emigration from the thymus prior to its removal could account for chronic allograft rejection seen in toadlets thymectomized at 5 days, particularly in view of the extremely undifferentiated state of the organ at this time when it contains epithelial cells and immature lymphoid cells and comprises <500 cells (Horton & Horton, 1975.) The thymic bud only detaches from the pharyngeal epithelium at 3 days (Manning & Horton, 1969).

The present experiments indicate that a thymus independent alloimmune system emerges early in the life of the clawed toad, independent of changes in the lymphomyeloid complex occurring at metamorphosis.

TABLE 2.1

EFFECT OF APPLYING FIRST-SET LARVAL SKIN GRAFT IN  
LARVAL LIFE ON SUBSEQUENT ALLOIMMUNE RESPONSE TO  
A SECOND-SET GRAFT APPLIED AFTER METAMORPHOSIS

	CONTROLS	METAMORPHOSIS <sup>2</sup> (Days post-grafting)	SIGNS OF REJECTION <sup>3</sup> AS LARVAE	2nd SET REJECTION (Days)
<sup>1</sup> A	1	14	+	15
A	2	14	+++	22
A	3	14	++	20
A	4	14	++	22

Two shams (stock A) 1st set grafted as adults gave rejection times of 18 and 22 days.

	THYMECT- OMIZED	METAMORPHOSIS (Days post-grafting)	SIGNS OF REJECTION AS LARVAE	2nd SET REJECTION (Days)
A	1	11	-	17
A	2	11	-	22
B	3	15	-	20
B	4	15	-	>80
B	5	14	-	>80
C	6	16	-	23

Seven thymectomized (2 x A, 3 x B, 2 x C) 1st-set grafted as adults bore healthy grafts for >80 days when they were killed.

<sup>1</sup>A, B, C = Progeny of different parents. <sup>2</sup> Graft not visible after this time.  
<sup>3</sup> - = No signs of rejection.

+ = vascular enlargement;

++ = vascular enlargement and small opaque foci below graft;

+++ = extensive destruction - haemostasis, pigment destruction.

Table 2.2: Summary of first-set allograft rejection times in control and thymectomized animals grafted with adult skin.

	Number of animals grafted	Complete rejection within (days)				Grafts still intact when observations terminated
		17-25	26-50	51-100	>100	
<u>Thiourea-treated larvae</u>						
Control	14	7	1	1	0	5
Thymectomized	15	0	2	6	1	6
<u>Non-treated larvae</u>						
Control	10	9	0	0	0	1
<sup>1</sup> Thymectomized	9	0	1	2	1	5
<u>Toadlets</u>						
Control	34	32	2	0	0	0
Thymectomized	40	0	15	12	4	9 <sup>2</sup>

<sup>1</sup> These animals metamorphose before graft rejection is completed

<sup>2</sup> Four of these were in phase 2 of graft rejection



Fig. 2.1. Fig. A shows an adult ventral skin allograft applied to the anterior tail region of a thiourea-treated thymectomized larva. In non-treated larvae, grafts placed in this position come to lie postero-dorsally following metamorphosis: See Fig. B.

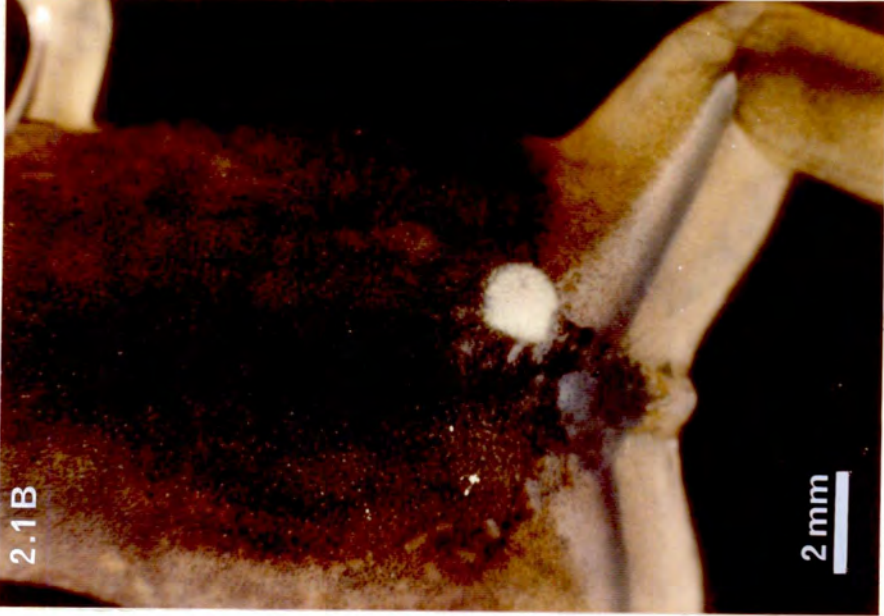


Fig. 2.2. Fig. A illustrates a healthy (phase 1) adult ventral skin allograft on a tadpole shortly (6 days) after grafting. In contrast Fig. B depicts the appearance of such a graft following rejection. The white graft-pigment is destroyed and only a dull, grey collagen pad remains. Host melanophores are visible over the rejected graft. Note the enlarged blood vessel near to the graft. Both these transplants are on thymectomized (goitrogen-treated) larvae.

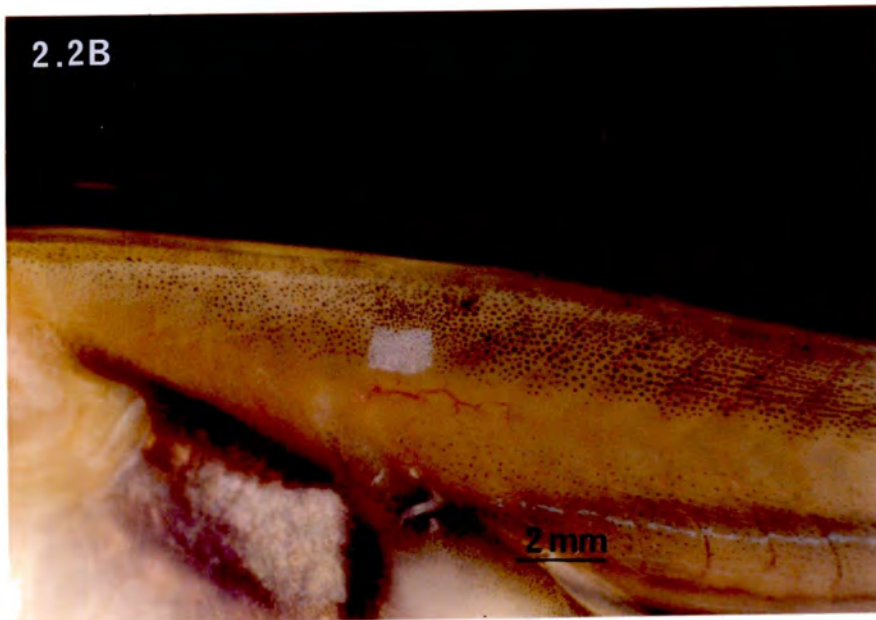
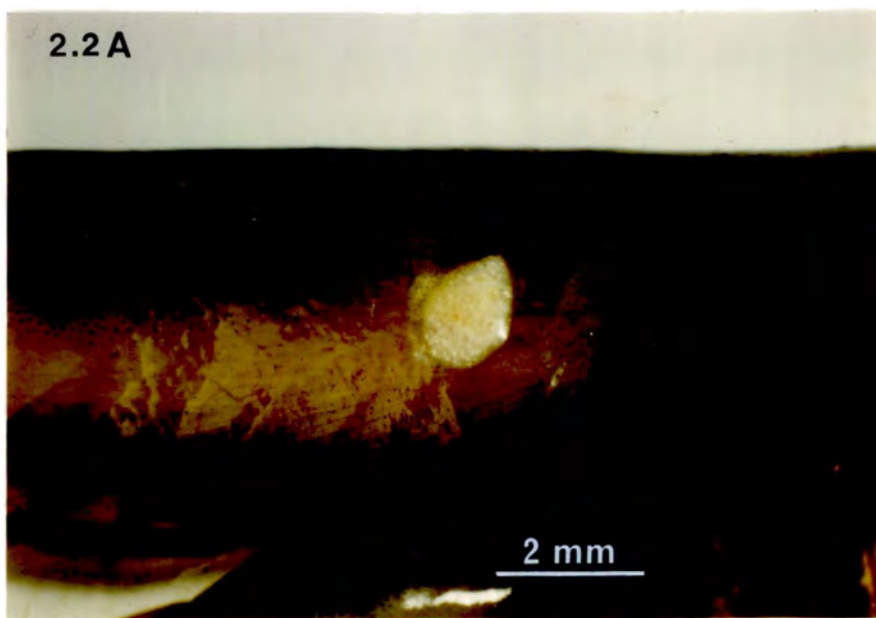


Fig. 2.3. Fig. A illustrates a graft in phase 2 (slight pigmentary defects) of rejection and Fig. B shows a transplant in phase 3 (extensive necrosis). The grafts are on two control, thiourea-treated larvae and were photographed 14 and 21 days post-transplantation respectively. A rim of host melanophores (M) surrounds the allograft of adult skin at 2 weeks. Host skin is growing in over the more degenerate graft seen in Fig. B. T = thymus  
E = eye.

Figs. C and D demonstrate the variability in graft rejection response seen in thymectomized animals. Both photographs are of stage 48 thymectomized, thiourea-treated larvae at 65 days post-transplantation. The transplant seen in Fig. C remains in perfect condition (phase 1). In contrast, the larva in Fig. D has rejected its dorsal skin allograft: host skin now overlies the collagen pad (which appears white) - all that remains of the donor tissue. Note absence of thymus in these two larvae.

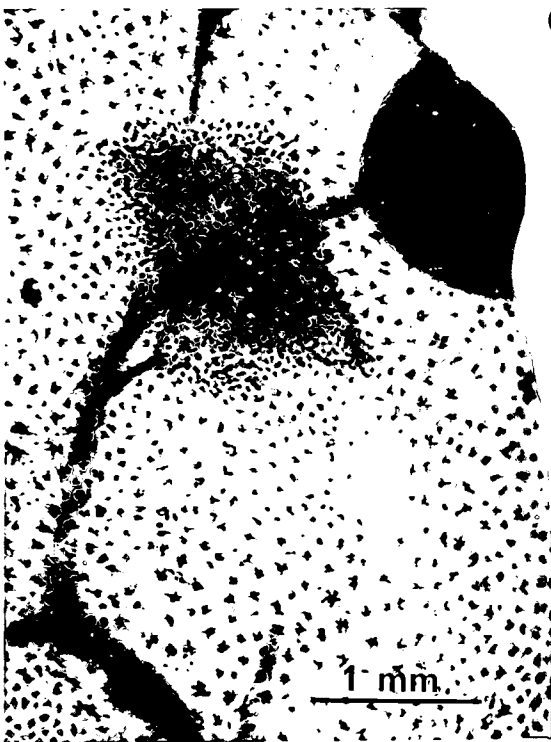


Fig. 2.4 shows the histological appearance of larval skin allografts applied to larvae at 30 days of age. Fig. A shows a graft on a control larva 18 days post-transplantation. An intense alloimmune response is evident and the graft is being invaded by a mass of cells, mostly lymphocytes. Note the breakdown of the normal larval skin histology. Fig. B shows a larval skin graft on a larval host which was thymectomized at 8 days of age. Note the healthy state of the grafted skin and the presence of small numbers of lymphocytes (arrowed) below the grafted tissue at 20 days post-grafting.

2.4

A



B



100  $\mu$ m



Fig. 2.5: Chronology of phases of first-set allograft rejection in larval and adult Xenopus thymectomized at St.48.

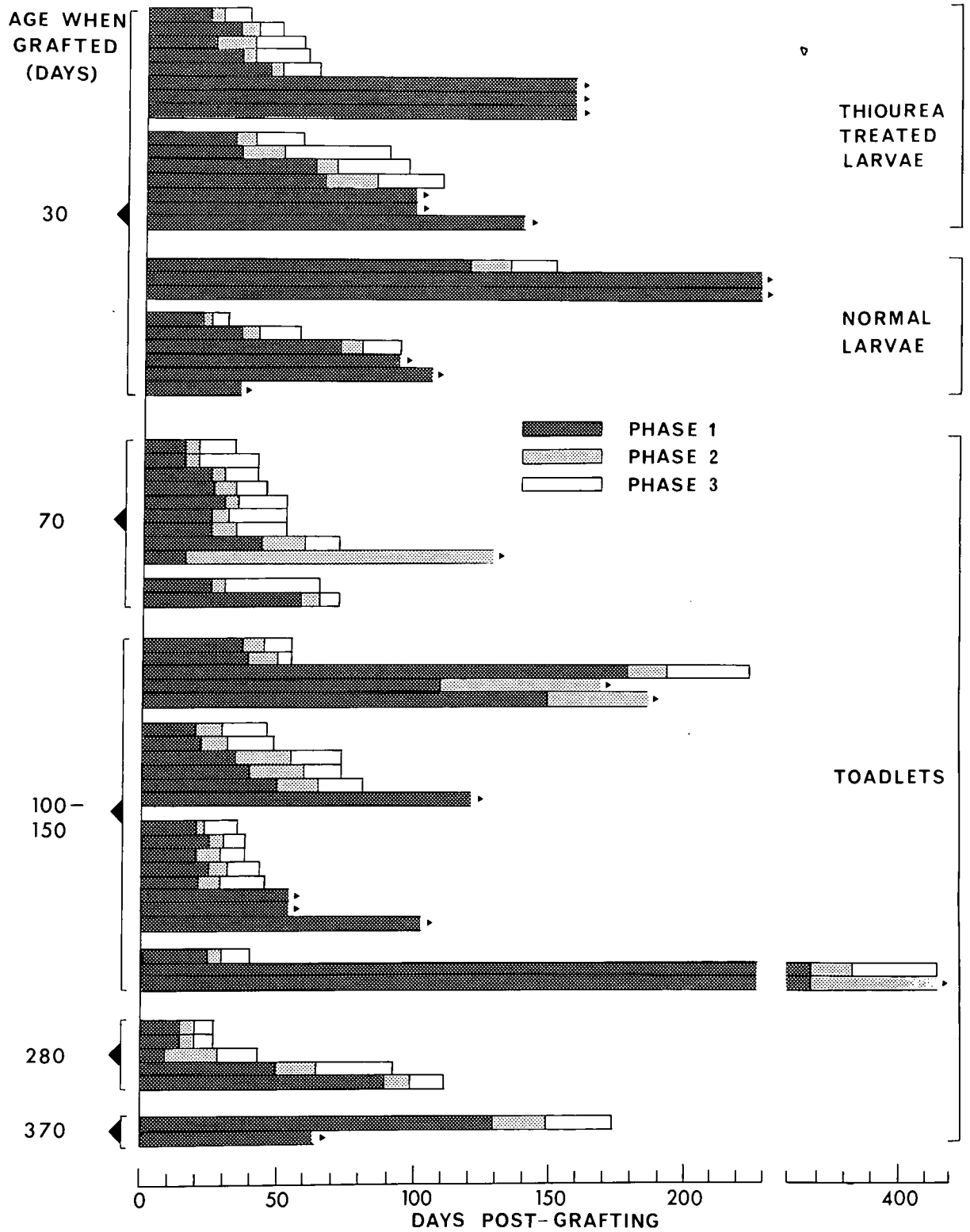
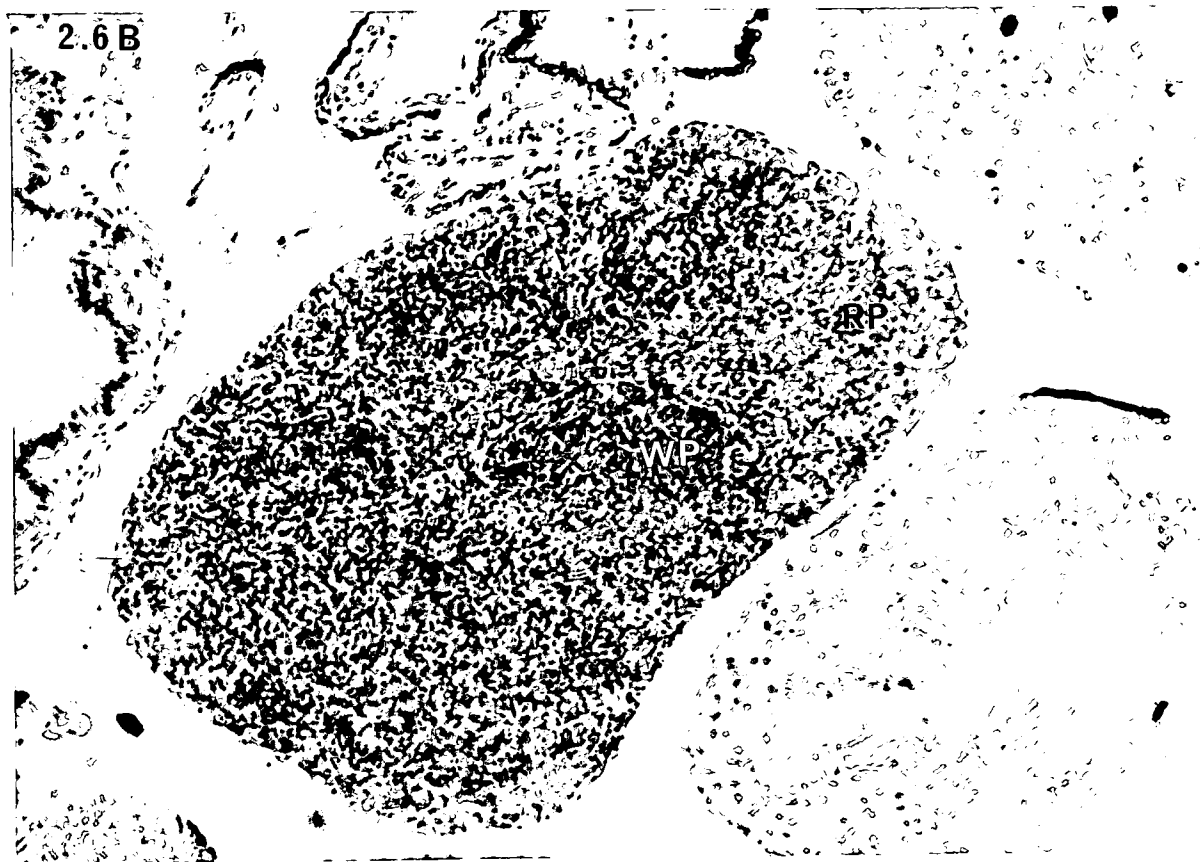
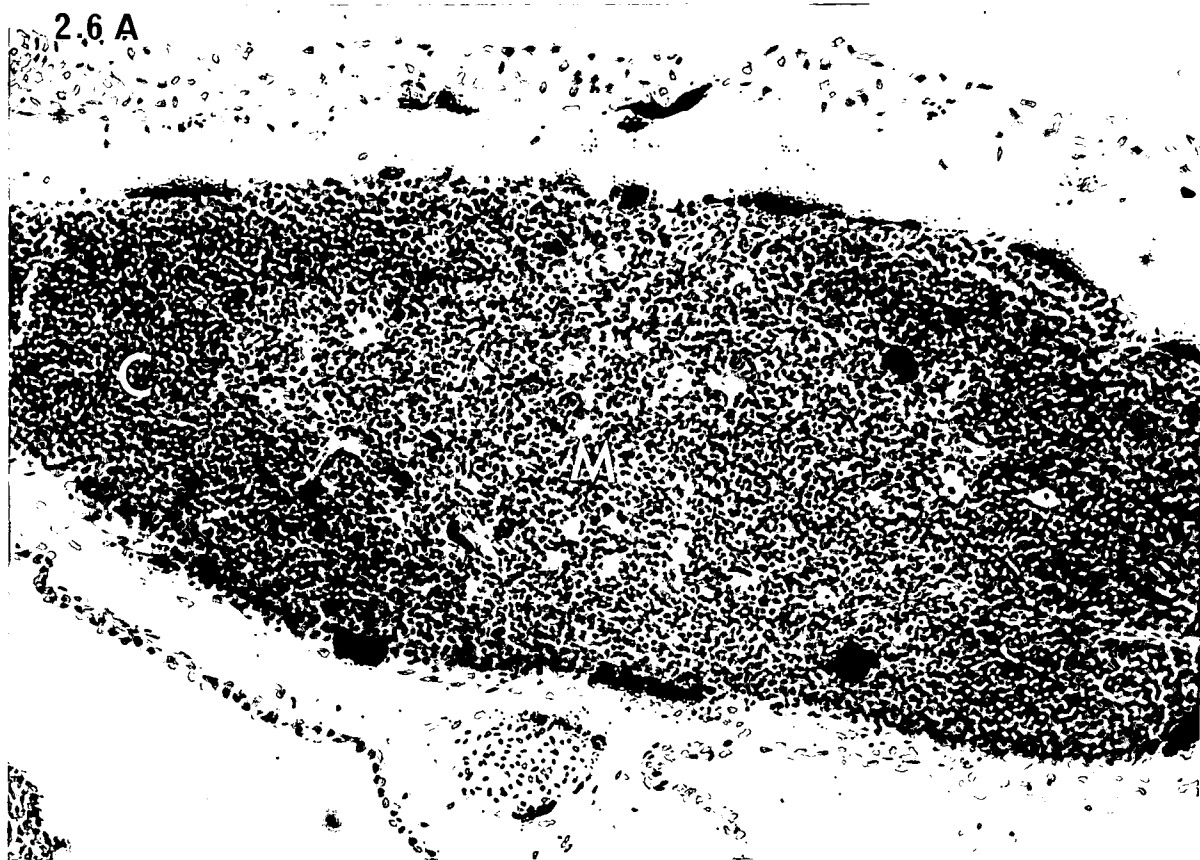


Fig. 2.6 shows the histological appearance of the thymus and spleen from a tadpole which had been kept in 0.05% W/V thiourea for 22 weeks. (The thyroid glands of this animal are depicted in Fig. 2.7). Both thymus and spleen are comparable in their histology to the organs of untreated larvae of a similar external stage. Fig. A shows the thymus (C = cortex, M = medulla) and the spleen, which is richly populated with lymphocytes, appears in Fig. B. Red pulp (RP) and white pulp (WP) regions, although visible, are not readily distinguishable in this section.



100  $\mu$ m

Fig. 2.7. Goitrous thyroid of animal described in Fig. 2.6. Despite the large size and abnormal appearance of this paired gland with hypertrophic follicular epithelial cells (FC) and reduced colloid (C), the immune response of thiourea-treated animals is apparently normal when compared with that of non-treated larvae.



0.5 mm

INTRODUCTION

Together with earlier studies (Horton and Manning, 1972; Horton and Horton, 1975) Chapter 2 has clearly established an important function of the thymus in controlling the development of the normal alloimmune response in Xenopus. It is difficult to discern the level at which this thymic governance operates without first obtaining a more detailed knowledge of how allograft rejection is effected in the intact animal and in particular, of the part played by other lymphoid organs and tissues in this process.

Following early (7 - 8 day) thymectomy, the lymphoid tissues of Xenopus generally undergo relatively normal histogenesis. After metamorphosis however, thymectomized toadlets possess spleens which are reduced in size with moderate to severe lymphoid depletion of the red pulp and in some animals a reduction in the proportion of white pulp to red pulp occurs (Horton and Manning, 1974a; Tochinai, 1975). Since thymic absence has no apparent effect on the histology of other adult lymphoid organs and tissues, it seemed important to determine therefore whether or not the spleen of the intact toadlet is involved in allograft rejection and to assess whether there is a deficiency in splenic alloreactivity in the thymectomized toad.

The role of the spleen in cell-mediated immune reactions of several amphibian species has been studied in some detail. Participation of this organ in allograft reactivity is suggested by Baculi and Cooper's study on larval bullfrogs (1970) which showed increased numbers of small lymphocytes in the spleen and other lymphoid tissues following skin grafting. Furthermore, Murikawa (1968) showed in the adult urodele amphibian, Cynops pyrrhogaster, that if the spleen is excised prior to irradiation and reautografted afterwards, the ability to reject skin allografts is restored.

In contrast, Brown & Cooper (1976), employing a similar experimental protocol, failed to restore allograft rejection in irradiated bullfrog larvae. Since the transfer of allogeneic spleens to such larvae resulted in a G.V.H. (graft versus host)-like syndrome, (Brown et al, 1975), Brown and Cooper (1976) have suggested that the larval bullfrog spleen contains 'T-cell' populations or precursors capable of evoking a G.V.H. response but does not harbour 'T-cells' capable of effecting allograft rejection. Clark and Newth (1972) were able to demonstrate G.V.H. activity in Xenopus by transplanting whole spleens to hosts rendered tolerant to the spleen donor by a massive embryonic tissue transplant. Induction of G.V.H. reactivity following allogeneic spleen implantation in irradiated Cynops pyroghaster (Japanese Newt) has been reported (Murikawa, 1968) and a GVH-like syndrome following spleen-grafting has been described for Diemictylus viridescens (Baldwin and Cohen, 1971). Spleen cells can also give rise to G.V.H. responses in turtles. (Sidky and Auerbach, 1968; Borysenko and Tulipan, 1973).

The question of splenic involvement in allograft rejection in Xenopus remains unresolved. Allogeneic spleen implants, in contrast to thymus implants, failed to restore normal alloimmune reactivity to thymectomized animals (Horton and Horton, 1975). Preliminary studies on proliferative changes in lymphoid organs of control Xenopus following skin allografting were inconclusive in implicating splenic involvement. However, of all lymphoid tissues studied (thymus, spleen, liver, kidney and intestine) there was a suggestion of increased proliferative activity in the spleen. (Horton and Horton, 1975). Some of the experiments described in this chapter were therefore designed to investigate possible proliferative changes in the spleen following allografting in more detail, and have been performed on control and thymectomized toadlets. Other experiments have investigated the capacity of spleens from sensitized (control) toadlets to transfer alloimmunity.

## MATERIALS AND METHODS

### *(i) General*

Two separate batches of animals were used as hosts in each of the three studies described below. Toadlets were 4 - 9 months old when allografted or given spleen implants. Dorsal skin grafts measuring approximately 2 mm<sup>2</sup> were applied in the dorsal trunk region. Skin allografts were from non-sibling toadlets of similar age to the hosts.

### *(ii) Autoradiography and Histology*

These experiments were primarily designed to investigate whether or not cellular changes could be detected in the spleen at a time when skin-allografts are heavily infiltrated with lymphocytes and are displaying acute rejection phenomena (Horton, 1969). Spleens from 13 allografted and 13 autografted control toadlets were therefore studied at 12 days post-transplantation. Proliferative events in the spleen prior to (5 days:- 3 allografted and 2 autografted toadlets) and following (18 days:- 4 allografted and 4 autografted toadlets) this time were also examined. Spleens from 13 non-grafted animals were also studied to assess whether the autografting procedure itself resulted in any enhanced splenic activity.

A smaller study was also made on thymectomized animals. Spleens were removed from 3 allografted and 2 autografted toadlets at 12 days post-grafting (when allografts appeared healthy) and also from 4 allografted and 2 autografted thymectomized animals at later times (55 - 100 days) when gross observations suggested that the allografts were in a similar phase of rejection to that seen at 12 days in controls. Spleens from two non-grafted thymectomized animals were also examined.

Four hours prior to spleen removal, toadlets were injected with 1  $\mu$ ci/gm.body wt. of tritiated thymidine (S.A. 22.4 Ci/mmol Radiochemical Centre Amersham) via the dorsal lymph sac. Spleens were removed from anaesthetized toadlets, fixed in Carnoy's fixative and embedded in paraffin

wax. Serial sections (8  $\mu$ m) were cut and mounted on gelatin-coated slides. In most instances individual spleens were used to provide information for both histological studies, alternate sections being stained directly with methyl green-pyronin (M.G.P.) or prepared for autoradiography. A few spleens were used for only one type of analysis. For the autoradiography, sections were dipped in Ilford K5 nuclear emulsion and exposed for 4 weeks at 4°C prior to developing and staining with M.G.P. (See Turner and Manning, 1973).

All slides were coded prior to observation. Both red and white pulp regions of the spleen were examined and estimates were made of the numbers (low, moderate or high) of large pyroninophilic or labelled lymphoid cells. The analysis of pyroninophilia was for the most part restricted to those sections stained directly with M.G.P., since the autoradiographs were relatively poorly-stained. To avoid variations in staining and labelling due to experimental technique, all the slides were processed in just two batches.

(iii) Scintillation-Counting

To confirm the histological data on elevated splenic activity in control toadlets following allografting and to determine when maximal proliferation occurs in this organ, tritiated thymidine (3HT) uptake in vitro was examined in grafted control toadlets at 3, 6, 12 and 18 days post-transplantation. At least 2 experiments were performed at each time and a total of 12 allografted and 14 autografted control animals were studied. Three thymectomized animals (2 allografted and 1 autografted) were also examined 12 days post-grafting.

Grafted animals were anaesthetized in MS222 (Sandoz) and partially exsanguinated by bleeding from the heart. This procedure reduced the numbers of erythrocytes (and, presumably other circulating blood cells) in the spleen. Spleens were removed aseptically and washed immediately

in amphibian culture medium (100 parts L-15 (Flow): 48 parts double distilled water: 16 parts foetal calf serum (Flow) decomplexed by heating for 30 minutes at 56°C: 1.6 parts Hepes buffer (Wellcome)). Spleens were then transferred to approximately 3 mls. of fresh medium in a watch glass and a cell suspension was prepared by gently teasing the spleen with fine forceps. The spleen suspension was then transferred to a sterile 10 ml centrifuge tube and cell clumps and debris allowed to settle out. The supernatant single-cell suspension was then transferred to another centrifuge tube, made up to 10 ml with fresh medium and washed twice by centrifugation at 200 x g for 10 minutes. The resulting cell pellet was resuspended in fresh culture medium and a white cell count was obtained using a Neubauer American optical haemocytometer. The cell concentration was adjusted to  $5 \times 10^6$  leucocytes/ml. The initial viability of cells prepared in this manner was >95% as assessed by trypan blue dye exclusion.

50  $\mu$ l of the spleen cell suspension ( $2.5 \times 10^5$  leucocytes) was then distributed into individual wells of sterile Cooke microtitration plates (M220 25ARTL - GIBCO) using an Oxford sampler. Cells were then pulsed by the addition of one  $\mu$ ci 3HT (S.A. 5 Ci/mmol) to each well. All cultures were set up at least in triplicate, and, following incubation for 4 hours at 28°C in a humidified atmosphere, cells were harvested with the aid of a semiautomatic cell harvester (Skatron: Flow Laboratories). Each row of wells was flushed for 1 minute with double distilled water, this transferred the cells from the culture wells to individual glass fibre filter discs and washed away any non-incorporated 3HT in the medium. Hirschberg and Thorsby (1973) have shown that harvesting of 3H - DNA in mammalian lymphocyte cultures can be effectively accomplished using only a water wash instead of TCA and methanol. The individual filters for each well were placed in glass vials and the cellular material digested for 1 hour with 0.5 ml Protosol (NEM) at 56°C. Five mls of scintillation fluid (42 mls Liquifluor

(NEM) + 1 litre of Toluene) was added to each vial and incorporated 3HT was counted in an N.E.M. liquid scintillation  $\beta$  counter.

A stimulation index (S.I.) for allografted spleens was calculated from:

$$\text{S.I.} = \frac{\text{Mean c.p.m. in spleen cells of allografted animal}}{\text{Mean c.p.m. in spleen cells of autografted animal}}$$

Autografted and allografted animals in any one experiment were siblings of similar size and weight. Where results from more than one autografted animal were available for any experiment, the highest mean c.p.m. was used to calculate the stimulation index. The coefficient of variation (S.D./Mean) for all 'autografted' cultures ranged from 3% to 35%, (mean  $17\% \pm 2.6\%$ ), stimulation was therefore considered significant if the SI was  $>1.4$ .

#### *(iv) Spleen Transfer Studies*

##### *General*

Specific immunological tolerance between 2 individual Xenopus can be induced by reciprocal exchange of large grafts of embryonic tissue (Clark and Newth, 1972). This part of the study has examined the capacity of whole spleen implants to transfer alloimmunity from sensitized control donors to mutually tolerant control and thymectomized hosts. Thymectomized animals were used to assay transference of immunity, since, if immunity was transferred by the spleen implant, it was thought that they would reject skin allografts in times which were readily distinguishable from the impaired first-set response they normally display.

##### *Induction of Tolerance*

Sibling embryos at developmental stages 21 - 23 (Nieuwkoop and Faber 1967) were selected. These were transferred to small petri dishes containing full-strength Holfreter's solution and the jelly coats and fertilization membranes removed with watchmaker's forceps. For each

experiment, 2 embryos were transferred to an operating dish, the bottom of which was covered with a layer of agar. Two oblong wells were cut side by side in the agar such that each was just deep enough to hold a single embryo. Embryos were placed on their sides, one in each well, and using fine tungsten needles a large graft of flank tissue (incorporating all 3 germ layers) was excised from each and orthotopically transplanted to its partner. The grafts were held firmly in position by a small sliver of coverslip glass, placed over each embryo. Grafts were inspected regularly to check that they had healed and had not been extruded. Coverslips were removed after 2 - 3 hours at which time the grafts were often indistinguishable from the surrounding host tissue. Operations were carried out under full strength Holfreter's solution and grafted embryos were left in this overnight. The following morning they were transferred to 50% Holfreter's solution and gradually reintroduced to water over the next few days. Mutually tolerant pairs were reared individually. The mortality within 24 hrs of the operation was extremely high (>80% in one batch of embryos) but 15 mutually tolerant pairs were successfully reared beyond metamorphosis for use in these experiments. Skin grafts were reciprocally exchanged between toadlets of each pair to test for tolerance prior to spleen transfer studies. Two sibling toadlets, which did not receive embryonic grafts, were also reciprocally skin grafted after metamorphosis to check for normal alloimmune reactivity in this family.

#### Implantation of Spleens

The spleen donor was anaesthetized and the organ removed aseptically. The intact spleen was then transplanted, through a small slit cut in the skin, to a position on the ventral surface of the mutually tolerant host's thigh where it remained visible as a red lump under the skin for the duration of the study. Revascularization was marked by the spleen flushing red some 2 - 3 days post-grafting (see also Clark and Newth, 1972). Spleen implants from 9 animals were examined histologically at the end of the

experiment from Haematoxylin and eosin stained serial sections.

### Experimental Design

This is set out in Figure 3.1 Experiments 1 to 4 employed pairs of mutually tolerant control animals. Experiment 1 examined the effect on alloimmunity of transplanting a spleen from a non-sensitized donor. Two pairs of animals were used. Skin allografts were applied to spleen recipients one week after spleen transfer.

Experiment 2 assessed the activity of spleens implanted from animals 12 days post first-set allograft application. Again, spleen recipients received 1st set skin allografts 7 days after spleen transfer.

In Experiment 3, spleens were taken from donors 1 - 3 days post first-set allograft rejection. Spleen recipients were skin allografted at 7 days (2 animals) or 21 days (2 animals) following spleen transfer.

Experiment 4 assessed the activity of spleens taken from donors within a week of rejecting a second-set allograft. Spleen recipients were skin allografted 7 days after spleen implantation.

In Experiments 5 and 6 the protocol was the same as in experiments 1 and 4 respectively, but in this case the spleen recipients had been thymectomized at 8 days of age.

Skin allografts applied to spleen donor and recipient of any one pair (in experiments 2, 3, 4 and 6) were from the same non-sibling toadlet.

## RESULTS

### A) Cellular changes in the spleen following allografting

#### i) Control toadlets.

Background levels of pyroninophilia were established from spleens of 13 non-grafted and 17 autografted animals. In all animals, pyroninophilic

cells were found scattered throughout the spleen in both the white pulp and amongst the lymphoid accumulations of the red pulp. Levels were recorded as low or moderate throughout. Autoradiographic analysis of spleens from 11 non-grafted and 15 autografted animals also showed no apparent differences between the two groups: low or moderate levels of DNA-synthetic cells were always recorded in the red pulp and in most white pulp regions (see Fig. 3.2). However, heavily-labelled white pulp was recorded in 3 spleens from both autografted and non-grafted animals. Data pooled from studies at 5, 12 and 18 days post-grafting is given in Table 3.1. Employing  $\chi^2$  with Yates' correction, no significant differences were established between non-grafted and autografted animals, i.e. autografting had no apparent effect on cellular activity within the spleen.

In contrast, allografted toadlets displayed distinct signs of elevated lymphocytic activity in the spleen. Examination of the data presented in Table 3.2 shows that at 5, 12 and 18 days post-allografting, the white pulp regions are stimulated: 13/16 show high levels of label (see Fig. 3.2) and 8/19 high levels of pyroninophilic cells (see Fig. 3.3). The data presented in Table 3.2 is summarised in Table 3.1 and a statistical analysis ( $\chi^2$  with Yates correction) confirms that both  $^3\text{H}$  labelling and pyroninophilia are significantly higher in the white pulp regions of allografted animals when compared with pooled data for autografted and non-grafted toadlets ( $P < 0.001$ ). Moreover, the pooled data suggests that pyroninophilia in the red pulp was also elevated in spleens of allografted animals ( $P < 0.05$ ) although there was no significant increase in the level of label in this region.

*ii) Thymectomized Toadlets*

Spleens taken from thymectomized toadlets were generally reduced in size. Depletion of lymphocytes from the red pulp, particularly the

marginal zone (Horton and Manning 1974a) was apparent in both non-grafted animals studied, in 3/4 autografted animals and in 6/7 allografted toadlets. The white pulp region of all spleens appeared to be richly populated with lymphocytes but, compared with controls, white pulp regions were reduced in 50% of non-grafted and autografted toadlets, in 33% of allografted animals killed 12 days post-grafting and in 75% of those killed during allograft rejection.

Pyroninophilia and labelling were similar in spleens of thymectomized non-grafted, autografted, and allografted toadlets, even when the latter were in the process of rejecting transplants. The analysis of pyroninophilia was made on spleens of 2 non-grafted, 2 autografted- and all 7 allografted animals. Low to moderate levels of pyroninophilic cells were seen in all but one of these spleens - the one exception being the white pulp of a toadlet rejecting an allograft. Autoradiographic sections were available from 2 non-grafted, 4 autografted and 6 allografted toadlets. Low or moderate levels of label were seen throughout the spleens of all animals studied, except for 1 autografted toadlet where heavy label was recorded in the red pulp.

B) Chronology of splenic proliferation following allografting

Table 3.3 shows the results of experiments on  $^3\text{HT}$  incorporation in vitro by spleen cells of control toadlets, as assessed by scintillation counting. Enhanced splenic proliferation, induced by allografting, was apparent in 2/4 animals tested at 3 days post-transplantation. (Mean S.I. 1.7). Peak stimulation indices were recorded at day 6 following allografting (Mean S.I. 4.3). By 12 days the mean index had fallen to 2.7 and by 18 days when allografts are nearing rejection end-point, the mean index was 1.8. The relatively small variation obtained in c.p.m. within triplicate cultures from the same animal is reflected in the standard errors given.

One must be wary about placing too much reliance on precise quantitative aspects of this study because of the small numbers of animals used and also because of the presence of experimental variables whose influence could not be assessed. For instance, although allografted and autografted animals within any experiment were always siblings, they were not syngeneic, therefore some variation in proliferative rates of spleen cells might be expected on genetic grounds. In addition, it was not possible to maintain animals under "germ-free" conditions so individual histories of antigenic stimulation (e.g. by microbial antigens present in the water) might also be expected to affect splenic proliferation levels.

In contrast to controls, 2 allografted thymectomized toadlets tested at 12 days post-transplantation showed no elevated proliferation in their spleens. Thus the mean ( $\pm$  S.E) c.p.m. in the one autografted thymectomized toadlet was  $4271 \pm 755$  and in the allografted animals,  $3005 \pm 263$  and  $3877 \pm 133$ .

C) Transference of immunity by spleen implants

Toads which were reciprocally grafted with flank tissue as embryos were shown to be tolerant of skin allografts from their partners. All pairs examined displayed total acceptance of their partner's skin 100 days post-grafting. In contrast, the 2 siblings which did not receive embryonic grafts, rejected skin allografts in 22 days. Histological examination of spleen implants at the end of the experiments described below were also indicative of tolerance between embryo-grafted partners. All spleens appeared healthy and there was no evidence of rejection. Implants were well vascularised and large blood vessels were apparent beneath the well-defined outer spleen capsule. The white pulp regions of implanted spleens (even in thymectomized hosts) retained apparently normal morphology and were always richly populated with lymphocytes. The red pulp regions of 6/9 spleen-implants however displayed some

lymphocytic depletion. A typical spleen implant is seen in Figure 3.4.

The results of experiments 1 - 6 (see Figure 3.1) are tabulated in Table 3.4. First-set rejection times displayed by spleen-donors ranged from 19 - 24 days and second-sets were destroyed in 13 to 16 days.

Recipients of spleens from non-grafted partners and partners which had received a skin allograft 12 days previously failed to show any enhanced reactivity to skin allografts. Indeed rather slow rejection times of 27 and 28 days were recorded in spleen recipients of experiment 1. In contrast, 3 of the animals which received spleens from partners which had already rejected 1 or 2 skin allografts, destroyed grafts from the same skin donor in accelerated fashion (13 - 15 days). Rejection times of 17 and 18 days by the other 3 toadlets in experiment 3 and 4, although accelerated with respect to the rejection times recorded in the spleen donors, fell within the normal control first-set range (17 - 25 days). The 3 animals which had rejected skin grafts rapidly had been grafted within one week of receiving their spleen implants (the other two spleen recipients had only received skin grafts 3 weeks following spleen transfer) and so in the rest of the experiments recipients generally received skin grafts within one week of spleen-transfer. The transference of a second-set response in 50% of expected cases suggests that the spleen is a site of cells or other factors involved in rapid graft rejection.

Experiments 5 and 6 employed spleen recipients which had been thymectomized at 8 days of age. Spleens from mutually tolerant, non-grafted control partners were able to restore normal first-set reactivity to skin allografts in thymectomized hosts (2 animals were tested). Furthermore, 2/3 thymectomized hosts receiving spleens from control toadlets which had already destroyed two allografts, rejected skin from the same skin

donor in less than 17 days. i.e. control second-set rejection times.

One further experiment was performed using an additional mutually tolerant pair, one partner of which was thymectomized. The control spleen donor was allowed to reject first and second-set grafts (in 23 and 12 days respectively) from one skin donor before its spleen was transferred to the thymectomized partner. After receiving the 'sensitized' spleen, the thymectomized host was challenged with skin from a 'third party donor' (that is to say a different skin donor than the one used in sensitizing the grafted spleen). This third party graft was rejected in 26 days. This finding suggests that there is some specificity in the transfer of alloimmunity accomplished by spleen implants.

#### DISCUSSION

The first two series of experiments described in this chapter were designed to investigate proliferative changes occurring in the spleen of intact animals following allografting. The high levels of label ( $^3\text{HT}$ ) in splenic lymphocytes, particularly in the white pulp regions of allografted toadlets, contrasted sharply with the relative paucity of labelled cells in spleens of non-grafted and autografted animals. Cells are known to incorporate thymidine during the DNA-synthesising (S) phase of their cycle. Following injection (in vivo) of a radioactive DNA-precursor only those cells which are in S-phase take up label (See Balls and Godsell, 1973). Since cell populations may be dividing asynchronously, a single injection of  $^3\text{H}$  Thymidine given a short time prior to sacrifice might be expected to result in the preferential labelling of rapidly proliferating populations compared with those having a larger cell cycle time. The presence of large numbers of  $^3\text{H}$  labelled spleen lymphocytes in the present experiments

is therefore likely to be an indication of intense proliferative activity. Increased synthesis of DNA has been shown following lymphocyte activation in vivo (Hanna, 1964) and in vitro (Dutton, 1967). However, a note of caution must be introduced here in view of the suggestion that DNA synthesis in mammalian lymphocyte populations following antigenic stimulation may not always be associated with mitotic activity (Pelc et al., 1972; Harris and Olsen, 1973). Nevertheless, the autoradiographic and scintillation counting studies performed here on the toad spleen indicate that this organ displays enhanced reactivity following skin allografting.

This suggestion is strengthened by the histochemical analysis using methyl green pyronin, which revealed significant increases in the number of pyroninophilic cells in spleens of allografted Xenopus. Pyronin is a histochemical agent used to demonstrate the presence of RNA, the latter being associated with the biosynthesis of protein (see Weir, 1973). Examination of well-stained autoradiographs indicated that large pyroninophilic cells were invariably  $^3\text{H}$  labelled. Their large size, pyroninophilic cytoplasm and prominent nucleoli together with  $^3\text{HT}$  incorporation in the nucleus suggests that they may be activated blast-cells. It is well known that levels of cellular RNA increase markedly following lymphocyte activation (see Ling, 1968).

The changes in cellular activity described here following stimulation by allo-antigen are similar to those reported by Turner and Manning (1973) following injection of circulating T-dependent antigens into control Xenopus. Thus they demonstrated a marked increase in cellular proliferation and formation of pyroninophilic cells in splenic lymphoid tissue, particularly in the white pulp. They suggested that the pyroninophilic cells may be involved in antibody secretion. Whether or not a similar function may be ascribed to these cells in the present study is not known, since the part played by antibody in allograft destruction in

Xenopus has not been critically assessed.

The third series of studies presented here were an attempt to investigate whether elevated splenic activity in allografted animals could be demonstrated immunologically. These studies showed that a second-set response to skin allografts could be transferred by transplanting a sensitized spleen (from an animal which had rejected 1 or 2 grafts) to a non-sensitized control host. Findings with spleens taken from donors prior to first-set rejection possibly indicate that sensitization of spleen cells only emerges after 12 days. However, interpretation of these findings is rendered difficult due to the small numbers of animals examined and the fact that spleen recipients still retained their own (fully competent) spleens, whose involvement in the response could not be assessed. In addition, the distinction between first- and second-set rejection times in controls is often marginal. These problems were resolved in part by the use of thymectomized spleen recipients, and these studies showed convincingly that the spleen of control toadlets is a major site of alloimmune reactivity.

An alternative approach to investigating splenic involvement in the alloimmune response is that of splenectomy. However it has been established that in many other amphibian species splenectomy fails to affect allograft reactivity (Rana pipiens - Vogel, 1940; Rana catesbeiana - Brown and Cooper, 1976; Pleurodeles waltlii - Deparis and Flavin, 1973; Diemictylus viridescens - Cohen, 1971). Similar findings have been made in fish, (Fundulus heteroclitus - Goss, 1961) reptiles, (Calotes versicolor - Manickavel, 1972) and mammals (Rabbit - Krohn and Zuckermann, 1954; Mice - Zunker and Agar, 1965; Haller and Jones, 1967). Moreover, splenectomy has comparatively little effect on the ability of Xenopus toadlets to respond to injected circulating antigens, either in terms of antigen clearance or antibody production. (Turner, 1973; 1974). The lack of effect of splenectomy may be due to a compensatory increase in the

activity of other organs and tissues following antigenic challenge (see Rosenquist and Wolfe, 1962; Cohen, 1971). Regeneration of splenic tissue, is also known to be a problem with Xenopus (Ruben pers comm.)

In contrast to the work on intact Xenopus, the preliminary studies on thymectomized animals demonstrated a complete lack of cellular reactivity in the spleen following allografting. Thus, histologically, levels of  $^3\text{H}$ -labelled and pyroninophilic cells in the spleen were similar in non-grafted, autografted and allografted animals - even when the latter were in the process of rejecting grafts.

In addition there was no indication of increased  $^3\text{HT}$  uptake by spleen cells of thymectomized toadlets as measured by scintillation counting 12 days post-grafting. The alloimmune deficiency in thymectomized Xenopus may therefore reflect, in part, an absence of those spleen cells capable of proliferating rapidly in response to allogeneic stimuli. This interpretation from in vivo experiments is supported by studies on the immune reactivity of spleen cells in vitro. Thus DuPasquier and Horton (1976) have demonstrated abrogation of M.L.R. reactivity in spleens from 8-day thymectomized toadlets.

Simnett (1965) and Horton and Horton (1975) have previously suggested that the mass of lymphocytes which accumulates under allografts in control animals may itself be an important centre of immunological reactivity. The relationship between the cells at the graft site, which are also a proliferating population (Horton and Horton, 1975), and those of the spleen is therefore intriguing. Whether the lymphocytes beneath the grafts of intact animals are all of splenic origin is not known. In this respect, it is of interest that despite the apparent lack of cellular activity in spleens of allografted thymectomized toadlets, these animals can chronically reject foreign skin. The proliferating lymphocytes which eventually accumulate beneath the grafts of thymectomized animals (Horton pers. comm.)

may therefore be from an extra-splenic source, possibly representing an alloimmune pathway which assumes a greater importance in the absence of the thymus.

One of the findings to emerge from the last chapter was that following chronic rejection of a first-set graft, the thymectomized toadlet was then capable of rapidly destroying subsequent grafts from the same donor. The cellular and/or humoral basis of this enhanced reactivity remains unclear, although the present studies suggest that the spleen may not be involved. It would therefore be instructive to attempt a transfer of this second-set immunity from a sensitized thymectomized animal to a non-sensitized thymectomized toadlet following introduction of various lymphoid tissues, cells or serum factors. In view of the difficulty encountered in producing large numbers of mutually tolerant pairs, a better way of investigating these issues would be to use genetically identical clones of Xenopus. (Kobel and DuPasquier, 1975). The use of such animals would also enable the experimenter to more readily assess and standardize first- and second-set graft survival times between different clones since the degree of histocompatibility antigen sharing could be strictly regulated.

Table 3.1 Tritium label and Pyroninophilia in spleens of Non-grafted, Autografted and Allografted Control Xenopus

	<u>NON-GRAFTED</u>	<u>AUTOGRAFTED<sup>1</sup></u>	<u>ALLOGRAFTED<sup>1</sup></u>
<u>LABEL IN W.P.</u>			
Low	I*	0	0
Mod.	IIIIIIII	IIIIIIIIIIII	IIII
High	III	III	IIIIIIIIIIII
	(9%)	(0%)	(0%)
	(64%)	(80%)	(24%)
	(27%)	(20%)	(76%)
<u>LABEL IN R.P.</u>			
Low	III	IIIIIIIIII	IIIIII
Mod.	IIIIIIIIII	IIIIII	IIIIIIII
High	0	0	III
	(27%)	(60%)	(38%)
	(73%)	(40%)	(44%)
	(0%)	(0%)	(18%)
<u>PYRONIN IN W.P.</u>			
Low	IIIIII	IIIIII	II
Mod.	IIIIIIII	IIIIIIIIII	IIIIIIIIII
High	0	I	IIIIIIII
	(46%)	(35%)	(11%)
	(54%)	(60%)	(47%)
	(0%)	(5%)	(42%)
<u>PYRONIN IN R.P.</u>			
Low	IIIIIIIIIIII	IIIIIIIIIIIIIIII	IIIIIIII
Mod.	I	II	IIIIII
High	0	0	0
	(92%)	(88%)	(57%)
	(8%)	(12%)	(43%)
	(0%)	(0%)	(0%)

<sup>1</sup> = pooled results for 5, 12 and 18 days post-grafting

\* I = score for one animal. Figures in parentheses = percentage of total animals in this group.

Table 3.2 Autoradiographic and M.G.P. analysis of spleens from control animals at intervals post-allografting

DAYS POST- GRAFTING		AUTORADIOGRAPHY		M.G.P.	
		w.p.	r.p.	w.p.	r.p.
<u>5 dys</u>	A24*	+++	+++	++	+
	A25	+++	++	+++	+
	A29		n.d.	+	+
<u>12 dys</u>	A31	++	+	++	+
	A32	+++	+	+++	++
	A34	+++	+	+++	+
	A35		n.d.	+++	++
	A36		n.d.	+++	+++
	A52	+++	++	+++	+
	A53	+++	+	++	+
	A54	++	++	+	+
	A55	+++	+	++	++
	A56	+++	++	++	++
	A68	+++	+++	++	++
	A69	+++	+++	+++	++
	A70	+++	+++	+++	++
	<u>18 dys</u>	A38	+++	++	
A39		++	++	++	+
A42		+++	++	++	+
A44			n.d.	++	++
Total:	+++	13/16	3/16	8/19	1/19

+ = low level of label or pyroninophilia

++ = moderate level of label or pyroninophilia

+++ = high level of label or pyroninophilia

\* = Number of individual toadlet.

TABLE 3.3 LEVELS OF  $^3\text{H}$  THYMIDINE INCORPORATION (C.P.M.) IN SPLEENS OF AUTOGRAFTED AND ALLOGRAFTED CONTROL TOADLETS AT INTERVALS POST-GRAFTING

DAYS POST-GRAFTING		AUTOGRAFTED (Mean c.p.m.)	ALLOGRAFTED (Mean c.p.m.)	S.I.
<u>3 dys</u>	Expt. 1	4809 $\pm$ 252	10994 $\pm$ 1539 1919 $\pm$ 57	2.2 (0.4)
	Expt. 2	2939 $\pm$ 589	9265 $\pm$ 590 2785 $\pm$ 89	3.2 (0.9)
<u>6 dys</u>	Expt. 1 <sup>a</sup>	1969 $\pm$ 97	8941 $\pm$ 609	4.5
	Expt. 2 <sup>b</sup>	2333 $\pm$ 171	9287 $\pm$ 932	4.0
<u>12 dys</u>	Expt. 1 <sup>c</sup>	2633 $\pm$ 223	6799 $\pm$ 1223	2.6
	Expt. 2 <sup>d</sup>	2367 $\pm$ 71	8505 $\pm$ 285	3.6
	Expt. 3	5983 $\pm$ 386	11931 $\pm$ 450	2.0
<u>18 dys</u>	Expt. 1	1950 $\pm$ 156	2447 $\pm$ 85	1.3
	Expt. 2	1909 $\pm$ 72	2958 $\pm$ 281	1.6
	Expt. 3	1611 $\pm$ 21	3873 $\pm$ 90	2.4

(a, b, c, d. Additional autograft values recorded for these times were

1670  $\pm$  153; 1691  $\pm$  153; 1691  $\pm$  134 and 2240  $\pm$  71 respectively).

TABLE 3.4. SKIN ALLOGRAFT REJECTION TIMES DISPLAYED BY CONTROL AND THYMECTOMIZED RECIPIENTS OF SPLEEN IMPLANTS FROM MUTUALLY TOLERANT

CONTROL DONORS

<u>Expt. No.</u>	<u>History</u>	<u>SPLEEN DONOR</u>		<u>SPLEEN RECIPIENT</u>		
		<u>Rej. times for skin allograft from non-sib donor</u>		<u>Time to reject skin from same donor</u>		
		<u>1st set</u>	<u>2nd set</u>			
1)	Non-grafted	-	-	28 days	Control animals	
		-	-	27 days		
2)	Grafted 12 days previously	-	-	21 days		
		-	-	21 days		
3)	Rejected 1st set allograft	19	-	17 days		
		19	-	18 days		
		19	-	15 days		
		21	-	15 days		
4)	Rejected 1st and 2nd set allografts	23	15	13 days		
		24	15	18 days		
5)	Non-grafted	-	-	22 days		Thymectomized animals
		-	-	25 days		
6)	Rejected 1st and 2nd set allografts	22	15	14 days		
		23	13	16 days		
		20	16	18 days		

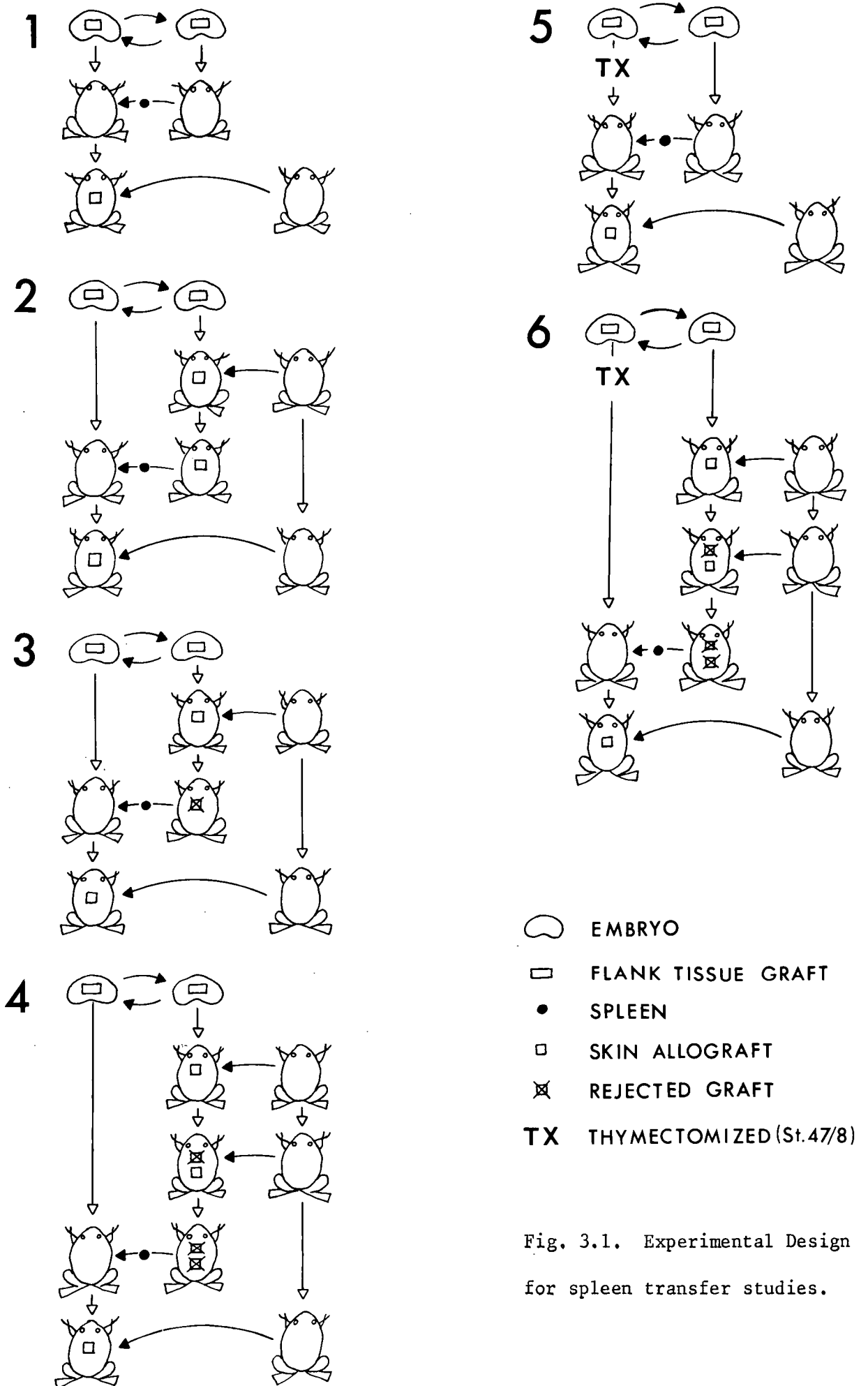
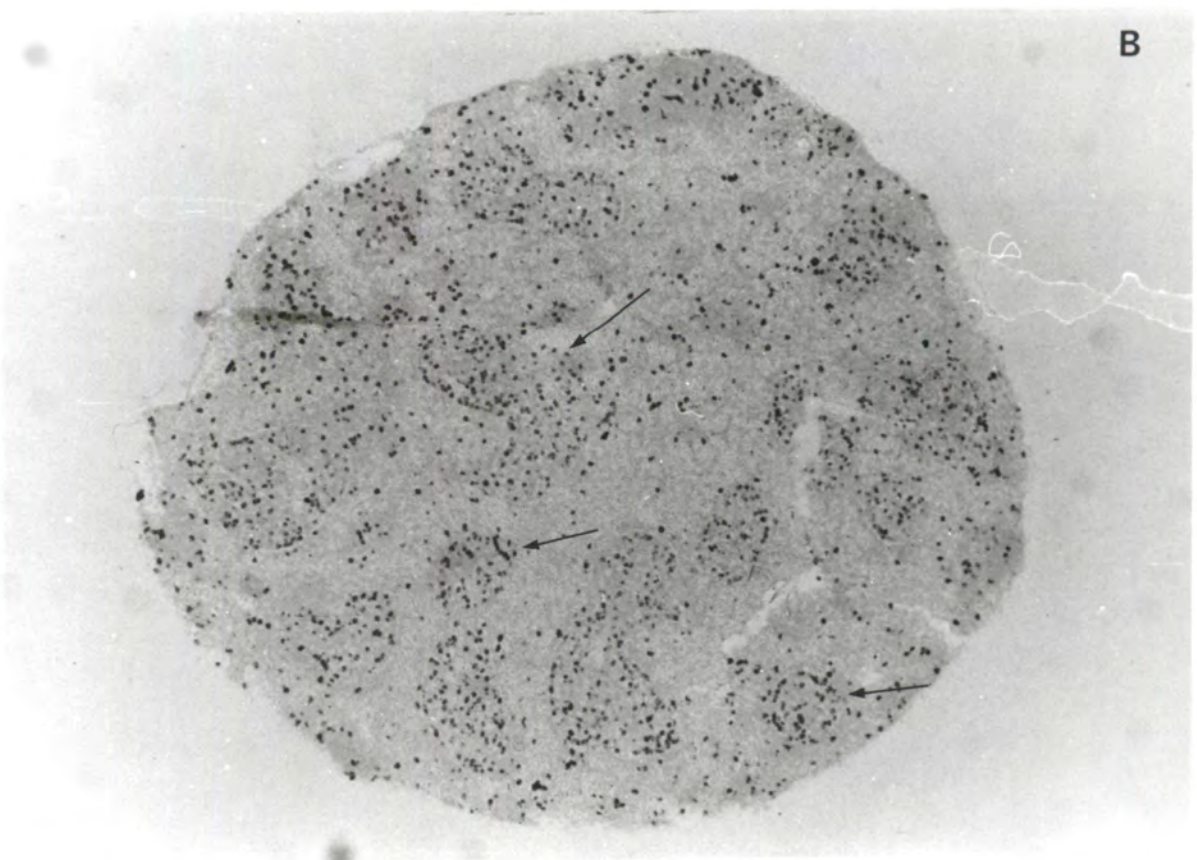
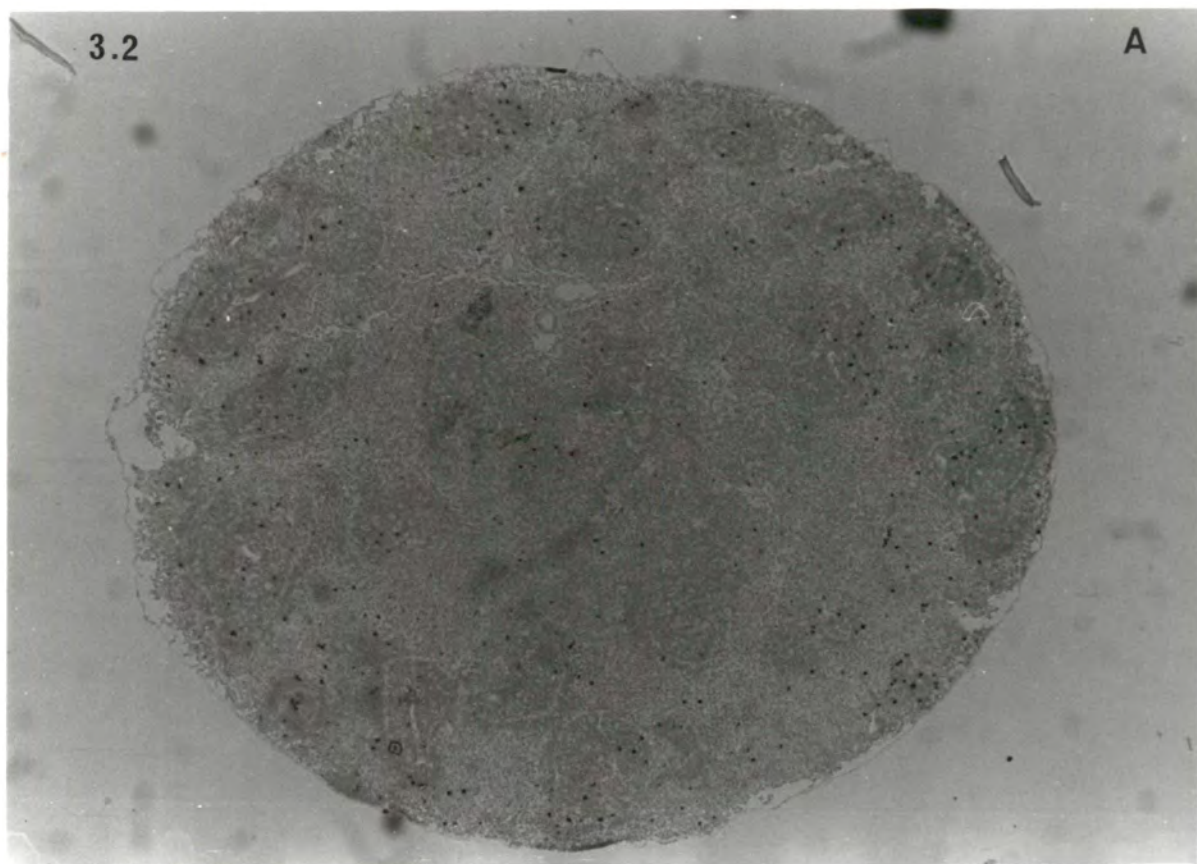


Fig. 3.1. Experimental Design for spleen transfer studies.

Fig. 3.2. Autoradiographs of adult spleen. Fig. A shows the low level of  $^3\text{HT}$  labelled cells in an autografted control Xenopus 12 days post-transplantation. In contrast, Fig. B shows the spleen of an allografted control animal at the same interval post-grafting. Here, labelled cells are abundant particularly in the white pulp regions (arrowed).



200 μm

Fig. 3.3 shows a section through part of the spleen of an allografted control animal 12 days post-transplantation. Large pyroninophilic cells (arrowed) with prominent nucleoli are present, particularly in the white pulp region (WP).

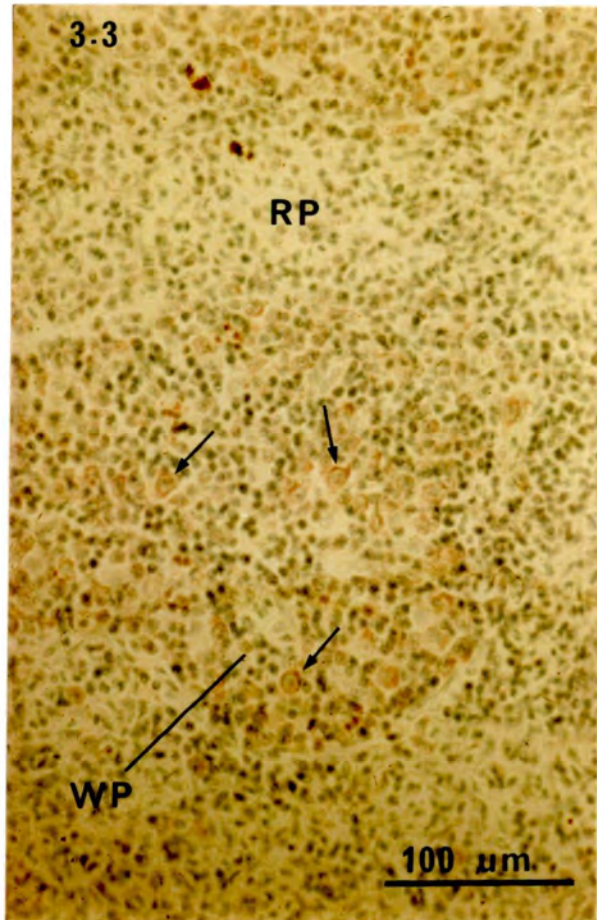
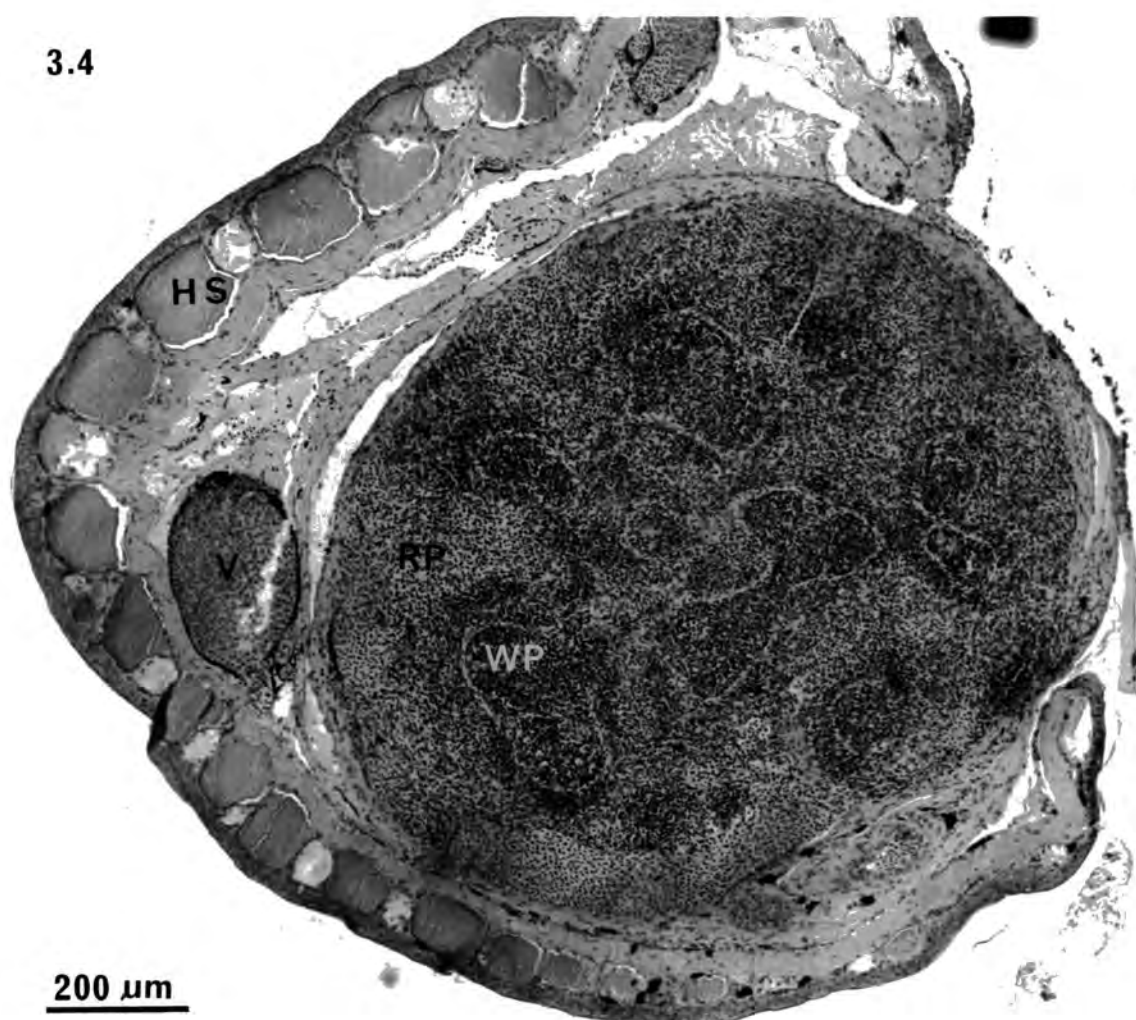


Fig. 3.4. Histological appearance of a spleen implant 20 days after transfer to a subcutaneous site in the thigh of a mutually tolerant control host. Note the normal appearance of the implant although some slight lymphocytic depletion is apparent in the red pulp (RP). The spleen recipient rejected a "first-set" skin allograft in accelerated fashion (13 days). WP = white pulp, V = blood vessel, HS = host skin.

3.4



INTRODUCTION

The work presented in the last two chapters has revealed that both thymus and spleen are intimately involved in the alloimmune response of the clawed toad. In this chapter attention is focussed upon the role of these organs in humoral immunity. Thymectomy experiments in Xenopus have established that, as in endotherms, the presence of an intact thymus during early ontogeny is essential for the development of serum antibody production to several antigens (Human gamma globulin (HGG), Sheep erythrocytes (SRBC), Rabbit erythrocytes (RRBC); Horton and Manning, 1974b; Turner and Manning, 1974; Tochinai and Katagiri, 1975). Cooper et al., (1971) and Cooper (1973) have suggested that the larval lymph gland, rather than the thymus, controls the development of antibody synthesis in Raniid frogs. Interestingly, larval Xenopus do not possess lymph glands (Manning and Horton, 1969).

Serum antibody titres in control Xenopus are relatively poor when compared with mammals (e.g. -  $\text{Log}_2$  titres of 2 - 5 following SRBC immunization, Turner and Manning, 1974). It is therefore important to investigate the apparent lack of responsiveness to T-dependent antigens in thymectomized toads at the level of those peripheral lymphoid tissues producing the antibody (e.g. spleen; Auerbach and Ruben, 1970; Collie and Turner, 1975) with the aid of sensitive techniques to document the absence of cellular reactivity. In this respect, Horton and Manning (1974b) and Collie (1974; 1976) using fluorescein-labelled antibodies have shown marked deficiencies in the trapping of T-dependent antigen (H.G.G.) in the spleen of the thymectomized toad. Moreover, the increase

in numbers of pyroninophilic cells and the increase in the size of the white pulp regions, normally seen in spleens of control toadlets following a series of injections of HGG, also failed to occur following early thymectomy (Horton and Manning 1974b).

Using the plaque assay and the sensitive immunocytoadherence assay, DuPasquier (1970a) revealed impaired cellular reactivity to SRBC and Human erythrocytes (HRBC) in spleens of thymectomized Alytes obstetricans and Tournefier (1972) has noted absence of any elevated splenic rosette forming cell (RFC) response to SRBC in the thymectomized newt (Triturus alpestris) although details of the latter experiments were not published. Ruben and his co-workers (Ruben, 1975; 1976; Edwards et al., 1976) have recently studied the binding characteristics of spleen rosette forming cells in the newt and the leopard frog in response to various doses of heterologous erythrocytes. These workers have indicated that two categories of erythrocyte-binding splenic RFC occur (c.f. mammals - Greaves et al., 1970): when the erythrocytes are bound only to the surface membrane of the lymphocyte, the latter are thought to be non-antibody-secreting, antigen-reactive cells. These antigen binding cells are called S-RFC. The other type of RFC, which binds more than one layer of foreign erythrocytes, are thought to represent antibody secreting-cells and are called S+ RFC. In amphibians, both these RFC types are readily distinguishable in non-fixed assay preparations because of the relative stability of rosettes at 4°C and the ability of amphibian spleen cells to secrete antibody at this temperature (Ruben, 1975; Edwards et al., 1976). In the 2 amphibian species studied, lower concentrations of immunogen preferentially stimulated S- RFC and also resulted in a distinctly earlier RFC peak than did high dose immunization (Ruben, 1975). Moreover, in the newt, low red-cell doses are particularly effective in achieving carrier-enhancement of anti-hapten responses (Ruben et al., 1973; Ruben, 1975). From these studies, Ruben considers that the two morpholog-

ically distinct rosette types may represent functionally distinct lymphocyte populations; and by analogy with the interacting cell populations involved in antibody production in endotherms, suggests that the S- RFC are helper cells and the S+ RFC are antibody secreting cells.

Although the kinetics of splenic RFC following SRBC administration has been examined in Xenopus (Kidder et al, 1973) the effect of immunogen dosage on the response curve and data on S- and S+ RFC in this species are lacking. It was therefore decided in this chapter to examine these issues in the control toadlet. The effect of thymectomy on rosette morphology and the extent to which the thymus governs the splenic RFC response to SRBC was then examined in the light of these findings. The relationship between RFC production and splenic proliferation following SRBC administration was also studied in control and thymectomized Xenopus, since it has been demonstrated previously that in Bufo marinus, induced splenic RFC are the product of a rapidly proliferating population of lymphoid cells (Kraft and Shortman, 1972).

## MATERIALS AND METHODS

### General

Sheep red blood cells (SRBC) in Alsevers solution (supplied by Wellcome) were washed three times in saline and resuspended in Alsevers for immunization. Toadlets were given a single injection of SRBC, (50  $\mu$ l per gram body weight via the intraperitoneal route) when they were from 20 - 30 weeks of age and weighed from 3 - 5 gms.

### A) RFC Studies

#### i) Experimental Protocol

##### Control animals

34 control toadlets received a single injection of 10% SRBC and their

spleens were assayed for RFC at 2, 4, 6, 8, 10, 14, 18 and 22 days post-injection. At least 3 injected toadlets were assayed at each time. (See Table 4.2.)

28 toadlets were injected with 0.01% SRBC and their spleens were assayed at 2, 4, 6, 8, 12, 16 and 21 days post-SRBC administration. At least 3 toadlets were examined at each time except at 6 days when only 2 animals were examined (see Table 4.3).

Background RFC counts were obtained from the spleens of 16 non-injected control animals.

#### Thymectomized animals

In the second part of this study, the RFC response to SRBC was examined in toadlets which had been thymectomized at 7 - 8 days of age. In view of the better response obtained with the higher antigen dose (see Results), thymectomized animals were tested following a single injection of 10% SRBC. ICA assays were performed on spleens of 10 thymectomized and 4 sham thymectomized toadlets at 8 days post-injection (the time of peak RFC response).

Background levels of RFC were assessed from the spleens of 16 thymectomized and 9 sham thymectomized toadlets which had not received SRBC injections.

#### *ii) The Immunocytoadherence Assay*

The protocol followed was very similar to that described by Ruben et al., (1973). Spleen cell suspensions were prepared in a culture medium, chilled on ice, consisting of 5 parts Leibowitz L-15 medium (Gibco), 4 parts of double-distilled water and one part (heat inactivated) foetal calf serum (Gibco). This medium was suggested for Xenopus cells by Balls and Ruben (1966).

Individual spleens were assayed since these yielded sufficiently

high cell concentrations ( $3-8 \times 10^6$  cells/ml excluding erythrocytes). Two assay tubes were set up for each spleen. Each tube, containing 10  $\mu$ l 1% SRBC and 50  $\mu$ l of the spleen cell suspension, was coded and incubated for 16-20 hrs at 4°C. Following gentle resuspension, by hand rolling the tubes on ice at an angle of 45°, the assay suspensions were scanned microscopically in a haemocytometer chamber (American Optical Improved Neubauer counting chamber).

Rosette forming cells were classified into one of two categories according to their SRBC binding characteristics. (Greaves et al., 1970 adopted by Ruben, 1975) Xenopus spleen cells binding more than 3 SRBC on their surface were designated S- RFC (Fig. 4.1). Cells which in addition to the membrane-bound layer, also bind one or more erythrocytes in an additional outer layer were designated S+ RFC (Fig. 4.1). In the latter case the additional erythrocyte layers are thought to be bound as a result of antibody secreted by the RFC (Ruben, 1975).

The number of RFC, expressed as RFC/ $10^6$  spleen leucocytes, was calculated from the average of at least 4 haemocytometer counts, two from each assay tube. The average number of lymphocytes scanned was approximately 15,000 per spleen assay.

## B) Proliferation Studies

### i) Experimental protocol

#### Control animals

15 control toadlets each received a single injection of 10% SRBC, and 11 toadlets received a control injection of Alsever's solution. Tritiated thymidine ( $^3\text{HT}$ ) uptake by spleen cells of each group was measured at 2, 3, 6, 8 and 10 days post-immunization.

#### Thymectomized animals

9 thymectomized toadlets were injected with 10% SRBC and 5 thymec-

tomized animals received Alsever's solution only. Proliferation in spleens of thymectomized animals was measured at 6, 8 and 10 days.

ii) Scintillation counting

The protocol for the scintillation counting technique was outlined in the last chapter and was also adopted in the present study. Spleen cells suspensions ( $5 \times 10^6$  leucocytes/ml) were pulsed with  $^3\text{HT}$  in vitro and the amount of  $^3\text{HT}$  uptake was measured by scintillation counting.  $^3\text{HT}$  uptake in spleen-cells from animals injected with Alsever's solution only were taken as 'background' and where more than one background spleen was used in any experiment, the highest recorded background level was used to calculate the stimulation index (S.I.).

$$\text{S.I.} = \frac{\text{mean c.p.m. in SRBC injected animal}}{\text{mean c.p.m. in Alsever's injected animal}}$$

Each culture was set up at least in triplicate and for "Alsevers-injected" cultures the coefficient of variation (S.D./Mean c.p.m.) ranged from 3% in some individuals to 18% in others (Mean  $\pm$  S.E.  $9.2 \pm 1.6\%$ ). Any S.I.  $>1.2$  was therefore considered positive.

## RESULTS

A) RFC levels in spleens of control toadlets

i) Non-injected animals

Background RFC were detected in the spleens of most of the animals studied (11/16) - see Table 4.1. The mean ( $\pm$  S.E.) RFC level was  $106 \pm 29$  per  $10^6$  spleen leucocytes (Range 0-411). The mean percentage of S- RFC amongst these background cells was  $73 \pm 21\%$  and the mean percentage of S+ RFC was  $27 \pm 11\%$ .

ii) Animals injected with various doses of SRBC

Tables 4.2 and 4.3, which are graphically represented in figures 4.2 and 4.3, show the kinetics of total RFC production in the spleen

following SRBC administration. Although the levels of RFC varied greatly from one animal to another, high dose (10%) SRBC gave a maximal RFC response (mean level approximately 60 x background) 8 days following injection. This is similar to the high dose peak seen in newt and leopard frog. Numbers of RFC in the spleen remained at this level until 14 days post-injection before falling to a level of approximately 30 x background at 22 days. A thousand-fold lower dose of antigen (0.01% SRBC) resulted in a slightly delayed (>8 days) and less elevated peak response (approximately 40 x background). By 21 days, levels of RFC dropped to 30 x background.

In view of the difference in RFC levels following the above two doses, an additional assay was performed with 3 control toadlets at 8 days post-injection. These animals were given 50  $\mu$ l per g.b.w. of an increased antigen dose (50% SRBC) but this resulted in a similar RFC count (Mean 5344) to that obtained after the 10% erythrocyte injection.

The upper graph in Fig. 4.3 represents the time course curve for S- RFC following 10% and 0.01% SRBC administration. Despite the great difference in antigen dose the curves are remarkably similar in outline. Thus, although the 10% curve peaks slightly earlier, the maximum numbers of S- RFC eventually produced are similar (40 - 50 x background) with both doses of antigen. S- rosettes persisted at near maximal levels for some 22 days post-injection.

The corresponding dose curves for S+ RFC indicated that the lower antigen-dose resulted in the generation of fewer secretor RFC than did the higher dose (maximally 60 x background compared with a peak of 150 x background for the 10% dose). A statistical analysis (student's 't') of the numbers of S+ RFC produced from 2 - 22 days revealed that this difference was significant ( $P < 0.001$ ). The fall in total RFC

levels occurring after 14 days following 10% SRBC administration was largely due to the fall off in numbers of S+ RFC (Fig. 4.3). Thus the proportions of S+ to S- RFC are roughly 50:50 during the first two weeks post-injection but return to a background ratio of approximately 75% S- RFC: 25% S+ RFC as the number of rosettes falls at 18 - 22 days. On the other hand, the ratio of S- : S+ RFC remains similar to the background ratio following low dose (0.01%) SRBC administration.

The lower antigen dose did not result in the generation of an earlier peak of RFC as reported by Ruben (1975) following administration of approximately 10  $\mu$ l per g.b.w. of low doses (0.25% (6 day peak) and 0.0025% (2 day peak) SRBC) of immunogen in the newt and the frog. To test the possibility that 50  $\mu$ l of 0.01% SRBC used here was too high to generate early RFC activity a further study was carried out on animals which had received a single (50  $\mu$ l per g.b.w.) injection of 0.001% SRBC. Six animals were examined at 2 days and the mean splenic RFC count for these animals was  $168 \pm 16$  RFC per  $10^6$  spleen leucocytes (Mean 65% S- RFC and 35% S+ RFC). These figures do not differ significantly from background counts. Three animals were tested at 6 days and gave a mean RFC count of  $547 \pm 113$  (70% S- RFC; 30% S+ RFC) - an indication of a weak response. Thus, there was no early RFC peak produced by the 0.001% SRBC dose.

#### Test for cytophilic antibody

To test for passive sensitization of non-immune spleen cells (in vitro) a dilution test was performed. Spleen cells of control (non-immunized) toadlets were mixed in different proportions with spleen cells from toadlets giving a known level of RFC 8 days following 10% SRBC injection. After incubation in the standard I.C.A. assay, the numbers of RFC in each mixture was counted. If no cytophilic antibody was present, then in a 50:50 mixture of immune and non-immune spleen cells for instance, the number of RFC recorded would have been half

of that counted in the 100% immune cell assay. If cytophilic antibody was present, then the number of RFC recorded in the mixture would be higher than expected. The results of this test are shown in fig. 4.4 and it may be seen that the relative number of RFC approximates to the 100% figure in all dilutions tested. Passive sensitization of non-immune cells in vitro is therefore not suspected. Whether or not cytophilic antibody binds to lymphocytes in vivo is not known.

B) RFC levels in spleen following early thymectomy

i) Non-injected animals

The mean background RFC level in spleens of 7 - 8 day thymectomized toadlets was found to be  $164 \pm 36$  per  $10^6$  spleen leucocytes (range 0-449). This figure did not differ significantly from the background levels recorded in animals sham-thymectomized at 8 days of age (Mean  $130 \pm 45$ , Range 13 - 411) or in non-operated animals (Mean  $106 \pm 29$ , Range 0 - 411).

Characterisation of S- and S+ RFC was made on 6 of the non-injected thymectomized toadlets (see Table 4.4a). The mean percentage of S- background RFC was 72% and the mean percentage of S+ background RFC 28%. These figures compare well with those obtained for non-operated controls. Early thymectomy, therefore, had no apparent effect upon the total numbers of background RFC recorded in the spleen or the relative proportions of S- and S+ RFC in this organ.

ii) RFC following injection of SRBC

Five sham-thymectomized animals all possessed high levels of RFC in their spleens 8 days after injection of 10% SRBC (Mean  $5595 \pm 902$  RFC compared with a level of  $6147 \pm 989$  RFC/ $10^6$  spleen leucocytes in spleens of non-operated controls). In contrast, none of the 10 animals thymectomized at 8 days showed any increase in RFC over background levels 8 days following SRBC injection. The mean RFC count for this group was  $121 \pm 43$  (Range 13 - 444). Interestingly the percentages of S- to S+ RFC were the

same as in non-injected thymectomized spleens (70% S-: 30% S+). (See Table 4.4b).

Since in mammals, administration of high doses of immunogen can overcome the requirement for T-dependent cells (Sinclair and Elliott 1968) a further group of 6 early-thymectomized toadlets received 50  $\mu$ l per g.b.w. 50% SRBC i.p. instead of the 10% dose used above. These animals still failed to display elevated RFC levels 8 days post-injection. Moreover, assays performed on five, 8-day thymectomized animals at 10, 12, 14, 17 and 23 days after injection of 10% SRBC also revealed background levels of RFC, evidence that a delayed response does not occur in early thymectomized toadlets.

C) Proliferative studies in control and thymectomized toadlets

Cellular proliferation in the spleens of control animals following injection of 10% SRBC was assessed by  $^3\text{HT}$  uptake, and the results are presented in Table 4.5. The variability in counts between different individual experiments is quite marked, and indeed this might be expected because of the genetic and physiological heterogeneity within the colony. However, since within each experiment all animals were siblings of the same size and weight, it seemed reasonable to compare background (Alsever's injected) c.p.m. and experimental (SRBC injected) c.p.m.

Elevated proliferation was evident 2 days post SRBC injection and maximal proliferation was seen at approximately 6 days (Mean S.I. = 4.1). By 8 days (the time of peak RFC response) proliferation in the spleen had fallen to background levels in two out of three animals tested.

Studies on the spleens of thymectomized toadlets are also shown in Table 4.5. These animals failed to show any enhanced cellular proliferation following 10% SRBC administration, indeed, in 6/9 cases, ( $^3\text{H}$ ) Thymidine uptake by spleen cells of SRBC-injected animals was lower than that recorded

in spleen cells from Alsever's-injected animals.

### DISCUSSION

The initial aim of the studies presented here was to characterize the anti-SRBC response in the spleen of control toadlets in terms of RFC produced at intervals following high (10%) and low (0.01%) dose SRBC administration. The experiments reveal overall similarities in response curves to these two doses of immunogen, although peak reactivity was achieved a little more rapidly following 10% SRBC injection and total numbers of RFC recorded were greater than with a low dose of erythrocytes.

These findings are comparable to RFC kinetics in the mammalian spleen (Greaves et al., 1970) but contrast with I.C.A. data, obtained using identical methodology to that used here, on the newt Triturus viridescens and the leopard frog Rana pipiens where an early peak of minimal binding RFC's (S-) follows low doses of immunogen (Ruben, 1975, Edwards et al., 1976). It is conceivable that in the present experiments the failure to detect a comparable early peak is due to the fragile nature of minimal binding rosettes in Xenopus early after 0.01% SRBC injection. However a further experiment suggests that an early peak of reactivity to low dose SRBC's does not occur. This experiment assessed whether any enhanced proliferative activity could be detected in the spleen within a few days of low dose SRBC administration, since, as demonstrated in this chapter, such activity is clearly associated with RFC production following 10% SRBC injection. Four animals were therefore given 50  $\mu$ l per g.b.w. 0.01% and 4 were given the same dose of 0.001% SRBC. Cell proliferation was then assessed by scintillation counting at 2 and 3 days. Positive stimulation indices were not recorded at the earlier time and indices of 1.5 and 1.3 (0.01% and 0.001% doses respectively) were seen at 3 days post-injection. These indices were no

higher than those recorded at 3 days following 10% SRBC administration. A real difference within the amphibia in the response kinetics to low dose immunogen is therefore indicated. However as noted in the introduction, it should be remembered that Ruben has suggested that the early RFC response to low immunogen doses represents helper cell reactivity. Whether or not helper cells exist in Xenopus has not been demonstrated although carrier-specific enhancement of anti-hapten responses has been reported in this species (see Edwards and Ruben, 1976). It is possible that helper activity in Xenopus cannot be readily visualized using I.C.A. and proliferative assays. It should be noted that in mice helper T-cells in the immune response to SRBC do not form rosettes (Elliott et al, 1973; Elliott and Haskill, 1975). Obviously, any further consideration of this issue awaits the necessary cell-transfer studies in the Clawed Toad. (Miller and Mitchell, 1969).

Examination of rosette morphology in the present experiments revealed that high immunogen dose preferentially stimulates the generation of S+ RFC. These cells may therefore be antibody secreting cells since, in mammals, increasing amounts of antigen are known to stimulate the production of greater numbers of plaque forming cells (Campbell and Kind, 1969). Moreover, RFC characterization in other amphibians (Ruben, 1976; Edwards et al., 1976) and also in mammals (Greaves et al., 1970, Elliott and Haskill, 1973 ) indicate that multilayered RFC's are antibody secretors. In mice, a proportion of RFC's are also known to form haemolytic plaques (McConnell 1971; Wilson, 1971). The precise relationship between RFC's and PFC's in Xenopus has not yet been examined, although PFC's can be demonstrated in some spleens 8 days after a single injection of 10% SRBC (Horton, pers. comm.) DuPasquier (pers. comm.) has studied the kinetics of PFC production in spleens of Xenopus and has found that the PFC curve lags slightly behind the RFC curve reported here. Whether or not S- and S+ RFC represent physiologically distinct sub-populations (in terms of

effector function) in Xenopus is not known. Ruben's original thesis that, in amphibians, these rosette types represent "helper" and antibody-secreting cells, may well be an oversimplification since Edwards et al., (1976) failed to demonstrate physiological differences between S- and S+ RFC in the leopard frog. Moreover, the present findings which revealed normal numbers of S- RFC in spleens of thymectomized Xenopus, fail to support Ruben's suggestion (1975) that such cells are mostly of thymic origin. It seems likely that S- RFC represent a heterogeneous population of antigen-binding cells with diverse functions. It is possible that the high levels of S- RFC, which persist until at least 3 weeks post-immunization in Xenopus, reflects a memory function for some of these spleen cells. However, the cellular basis of immunological memory, particularly with respect to humoral immunity in Xenopus, and indeed in all poikilotherms awaits elucidation (see Borysenko, 1976).

The RFC experiments on control toadlets illustrate that the I.C.A. technique is a sensitive and reliable test for anti-red cell activity and as such, is ideally suited for investigating the extent of humoral immune deficiency in the thymectomized animal. Following early thymectomy (at 7 to 8 days of age) background levels of RFC in the spleen are not affected with regard to the total numbers of RFC or the relative proportions of S- to S+ rosettes. In Xenopus it would therefore appear that peripheral cells originating from the thymus may not form background rosettes although this may not be the case in other amphibian species e.g. in the Midwife Toad (Alytes obstetricans) larval thymectomy results in approximately 50% reduction of background RFC in the spleen (DuPasquier, 1970b). However, in mice, neonatal thymectomy does not modify the numbers of spontaneous splenic RFC to SRBC (Bach and Dardenne, 1972a). Indeed, background anti-SRBC<sup>RFC</sup> appear at the same time in ontogeny in normal and neonatally thymectomized mice (Bach, 1973).

Following SRBC administration, there is no elevated RFC response in the spleen of the thymectomized toad. This lack of induced reactivity was confirmed by the proliferative studies, a finding which lends strong support to the suggestion made above, that the enhanced proliferation in the spleen following SRBC administration reflects anti-erythrocyte reactivity in control animals. The deficiency in induced reactivity may be due to the removal of helper cells by early thymectomy. Ruben and Vaughan (1974) noted substantial numbers of hydrocortisone-sensitive anti-SRBC RFC (suggestive of helper cells?) in the thymus of immunized Xenopus. On the other hand, Cooper (1973) has suggested that in amphibians the thymus also houses antibody-secreting cells during early ontogeny since PFC have been recorded in this organ in Bullfrog larvae (Moticka et al., 1973). More recently this has also been shown to be true for adult Bullfrogs (Minagawa, 1976). Cooper's proposal may be strengthened by the finding that in Xenopus, both IgG and IgM antibody production to T-dependent antigens is abrogated following 8 day thymectomy (Turner and Manning, 1974). This is in contrast to the situation in thymus-deprived mammals where normal or sub-normal titres of IgM can still be demonstrated (Taylor and Wortis, 1968; Mitchell et al., 1971; Manning et al., 1972; Pantelouris and Flisch, 1972; Torrigiani, 1972; Pritchard et al., 1973). Thus it is possible that helper and/or antibody-secreting cells are removed by larval thymectomy. However, an extra-thymic origin of a substantial population of antibody-secreting cells in amphibians seems likely in view of the finding that early thymectomy in Xenopus fails to impair antibody production to E. coli lipopolysaccharide (Collie et al., 1975) and to Polyvinylpyrrolidone (Tochinai, 1976) even when, in the latter case, the thymus is removed at 4 days of age. Thymus-independent antibody production to Salmonella typhi 'H' and 'O' antigens has been demonstrated by Tournefier and Charlemagne (1976) in urodeles (Triturus alpestris and Pleurodeles waltlii). Thymus-dependent and T-independent

lymphocyte sub-populations are known to exist in Xenopus, since in vitro studies reveal that 7 day thymectomy dramatically impairs lymphocyte reactivity to PHA and Con A, but has no suppressive effect on the proliferative response to L.P.S. and P.P.D. (DuPasquier and Horton, 1976; Manning et al., 1976; Donnelly et al., 1976).

Lack of any apparent proliferative response in the spleen of the thymectomized animal following SRBC administration (or allograft application) may implicate the absence of a mitogenic factor (or factors) following thymectomy. Studies on lymphocyte surface immunoglobulin (Ig) following early thymectomy in Xenopus have suggested that the thymus may promote mitosis, rather than differentiation, since the amount of surface Ig per cell and the percentage of Ig-bearing cells in the spleen increases following early thymectomy (see Weiss et al., 1973 for discussion). A mitogenic stimulus could be supplied by sensitized T-(helper?) cells in the intact toad and indeed there is evidence of lymphokine factors in other amphibian species (Ambrosius and Drössler, 1972; Drössler and Ambrosius, 1972; Cohen, 1975).

An alternative explanation of the immune deficiency in thymectomized toadlets does not rely directly upon the absence of thymus-derived cells, since it is possible that a thymic humoral factor may promote differentiation of helper function amongst non-thymus derived peripheral lymphocytes. In this respect, it has been shown in mice that although the numbers of background RFC may not be affected by thymectomy, the sensitivity of these cells to the drug Azothioprine is diminished. Background splenic RFC from nude mice are also less sensitive to the effects of Azothioprine in vitro than those from control mice (Bach, 1971). Interestingly, Azothioprine sensitivity is induced in RFC of mouse bone-marrow and also in RFC from spleens of nude and neonatal or adult-thymectomized mice in the presence of a thymic humoral factor, which is present in normal mouse serum but not in serum from thymectomized animals. (Bach and

Dardenne, 1972b). Miller et al. (1973) have recently demonstrated production of functional T-cells following in vitro treatment of bone marrow with thymus extract. Using the Azothioprine assay, a circulating thymic hormone has recently been detected in normal serum from three species of urodele amphibians (Pleurodeles waltlii, Ambystoma mexicanum and Triturus alpestris) but is lacking in serum from thymectomized animals (Dardenne et al., 1973).

In conclusion, the studies in this chapter confirm that the spleen is an important site of immune reactivity in humoral immune responses in amphibians and demonstrates that the larval thymus is essential for the development of induced splenic reactivity to sheep erythrocytes.

TABLE 4.1                      BACKGROUND ROSETTE FORMING CELLS AGAINST  
SRBC IN CONTROL SPLEENS

<u>INDIVIDUAL TOTALS</u>			<u>MEAN TOTAL</u>		
<u>S-</u>	<u>S+</u>	<u>Total</u>	<u>S-</u>	<u>S+</u>	<u>Total</u>
94	126	220	$77 \pm 22$	$29 \pm 12$	$106 \pm 29$
74	74	148	(73%)	(27%)	
138	0	138			
90	0	90			
0	42	42			
86	86	172			
0	0	0			
68	0	68			
0	0	0			
0	0	0			
274	137	411			
0	0	0			
75	0	75			
248	0	248			
81	0	81			
0	0	0			

All values expressed as RFC/10<sup>6</sup> spleen leucocytes.

TABLE 4.2      KINETICS OF RFC PRODUCTION IN CONTROL SPLEEN  
FOLLOWING 10% SRBC

<u>DAY OF ASSAY</u>	<u>INDIVIDUAL TOTALS OF RFC</u>			<u>MEAN TOTALS OF RFC</u>		
	<u>S-</u>	<u>S+</u>	<u>TOTAL</u>	<u>S-</u>	<u>S+</u>	<u>TOTAL</u>
2	90	0	90	152 ± 63 (63%)	91 ± 31 (37%)	243 ± 81
	90	180	270			
	278	93	371			
4	720	205	925	819 ± 55 (80%)	200 ± 35 (20%)	1019 ± 72
	905	258	1163			
	833	138	971			
6	748	125	873	1204 ± 543 (54%)	1032 ± 504 (46%)	2236 ± 1028
	590	885	1475			
	647	647	1294			
	2823	2496	5301			
8	3209	2749	5956	3464 ± 419 (56%)	2683 ± 289 (44%)	6147 ± 989
	2512	1642	4154			
	1995	2137	4132			
	4106	2576	6682			
	4320	2664	6984			
	2861	3206	6067			
	2002	1902	3904			
	5677	4617	10244			
10	4993	6991	11984	2497 ± 764 (44%)	3139 ± 1071 (56%)	5637 ± 1925
	2816	1330	4146			
	1351	2703	4054			
	602	1195	1797			
	2724	3478	6202			
14	3370	3000	6370	2994 ± 538 (52%)	2988 ± 425 (48%)	5982 ± 909
	2704	3386	6084			
	4713	3703	8416			
	1460	1379	2839			
18	1270	423	1693	2130 ± 398 (75%)	729 ± 420 (25%)	2859 ± 416
	1735	1388	3123			
	2422	531	2953			
	3092	572	3664			
22	1198	545	1743	2233 ± 1608 (79%)	589 ± 160 (21%)	2822 ± 1325
	926	337	1263			
	4575	885	5460			

All values expressed as RFC/10<sup>6</sup> spleen leucocytes.

TABLE 4.3

KINETICS OF RFC PRODUCTION IN CONTROL SPLEEN  
FOLLOWING 0.01% SRBC

<u>DAY OF ASSAY</u>	<u>INDIVIDUAL TOTALS OF RFC</u>			<u>MEAN TOTALS OF RFC</u>		
	<u>S-</u>	<u>S+</u>	<u>TOTAL</u>	<u>S-</u>	<u>S+</u>	<u>TOTAL</u>
2	75	0	75	88 ± 56 (100%)	0	88 ± 56
	188	0	188			
	0	0	0			
4	0	0	0	82 ± 23 (74%)	29 ± 29 (26%)	111 ± 79
	139	0	139			
	102	0	102			
	72	144	216			
	95	0	95			
6	184	147	311	300 ± 116 (57%)	229 ± 83 (43%)	529 ± 200
	415	311	726			
8	1893	1010	2903	1839 ± 91 (76%)	593 ± 215 (24%)	2433 ± 261
	1667	333	2000			
	1960	435	2395			
12	1213	0	1213	3072 ± 803 (71%)	1252 ± 489 (29%)	4324 ± 1288
	1620	577	2197			
	6088	3098	9186			
	2614	654	3268			
	716	0	716			
	4335	1896	6231			
	4920	2540	7460			
16	3095	893	3958	3151 ± 640 (73%)	1158 ± 218 (27%)	4309 ± 847
	4954	1801	6755			
	2680	1090	3770			
	1904	846	2750			
21	2183	397	2580	2564 ± 258 (79%)	698 ± 206 (21%)	3262 ± 712
	3107	1036	4143			
	2912	1078	3990			
	2054	280	2334			

All values expressed as RFC/10<sup>6</sup> spleen leucocytes



TABLE 4.5

## PROLIFERATION IN SPLEEN FOLLOWING INJECTION OF 10% SRBC

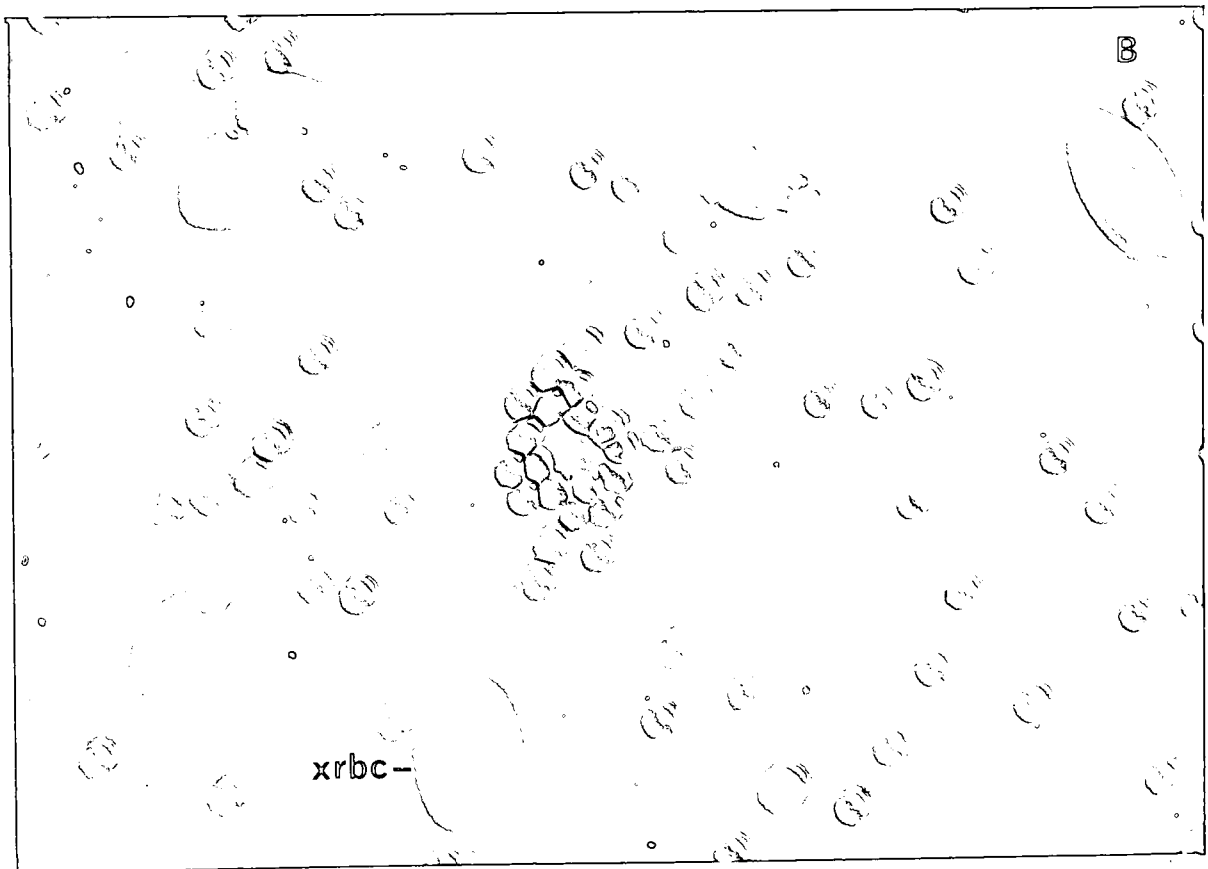
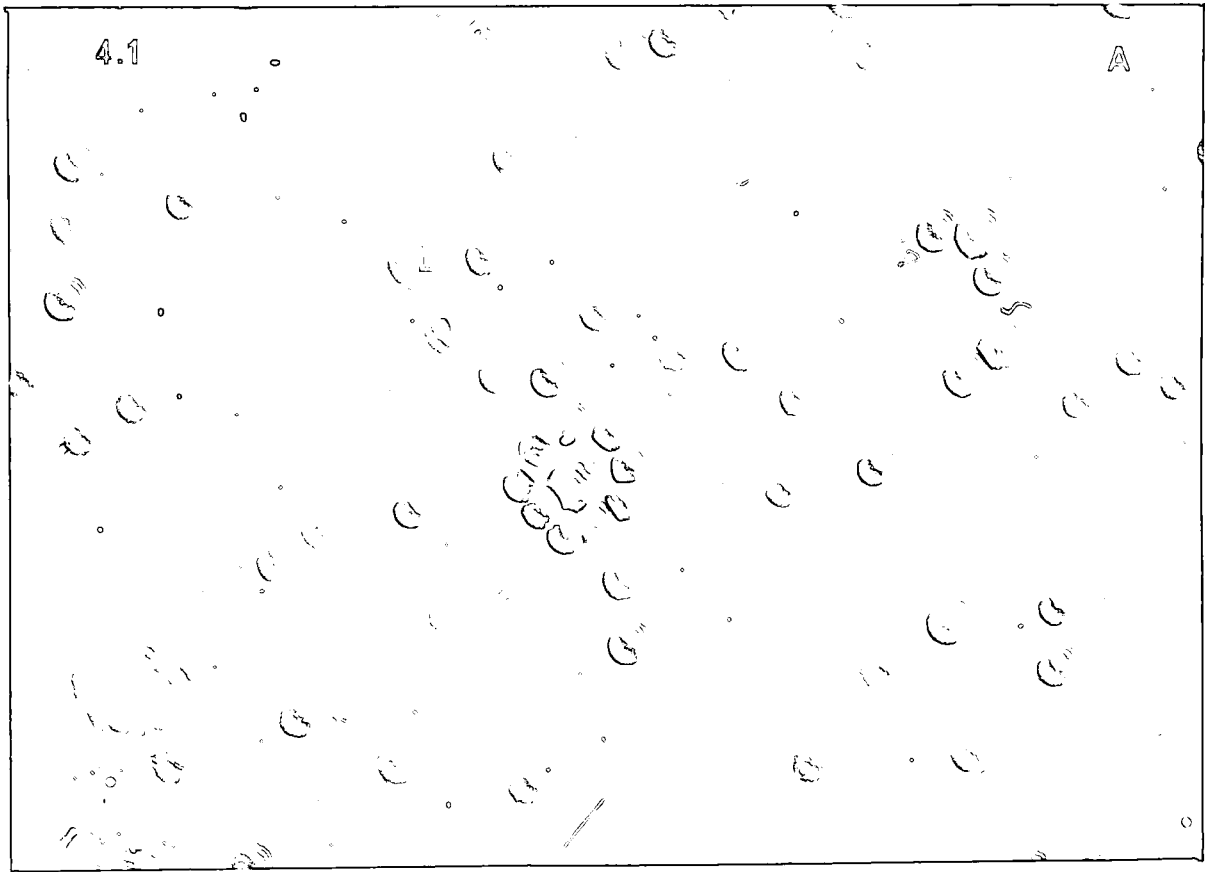
80

DAYS POST-INJECTION	CONTROL ANIMALS		S. I.	THYMECTOMIZED ANIMALS		S. I.
	ALSEVER'S INJECTED (mean c.p.m.)	SRBC INJECTED (mean c.p.m.)		ALSEVER'S INJECTED (mean c.p.m.)	SRBC INJECTED (mean c.p.m.)	
2 dys <u>Expt 1</u>	5,472 ± 290	10,685 ± 196	1.95			
3 dys <u>Expt 1</u>	5,570 ± 105	17,409 ± 525	3.1			
<u>Expt 2<sup>a</sup></u>	8,139 ± 327	7,749 ± 235	(1.0)			
		13,725 ± 197	1.7			
6 dys <u>Expt.1</u>	7,210 ± 412	48,621 ± 2090	6.7	Expt.1 3541 ± 261	712 ± 20	(0.2)
<u>Expt 2<sup>b</sup></u>	6,616 ± 211	17,358 ± 410	2.6		3221 ± 144	(0.9)
<u>Expt 3</u>	3,856 ± 92	30,645 ± 1179	4.6	Expt.2 7443 ± 350	1963 ± 150	(0.3)
<u>Expt 4</u>	10,026 ± 793	10,097 ± 120	2.6		1543 ± 116	(0.2)
		15,601 ± 269	4.0			
		45,390 ± 1005	4.5			
8 dys <u>Expt 1</u>	3,977 ± 279	2,329 ± 90	(0.6)	Expt.1 3329 ± 209	2456 ± 283	(0.7)
<u>Expt 2</u>	3,710 ± 280	6,612 ± 259	1.8		3690 ± 632	(1.1)
		2,985 ± 113	(0.8)	Expt.2 2586 ± 191	3046 ± 253	(1.1)
					2686 ± 262	(1.0)
10 dys <u>Expt 1</u>	2,197 ± 26	1,478 ± 26	(0.7)	Expt 1 7910 ± 475	2852 ± 105	(0.4)
		1,849 ± 54	(0.8)			

S. I. in parentheses are not significantly higher than background.

a,b, Additional "Alsever's injected" values recorded for these times were 7320 ± 265 & 6298 ± 96 respectively.

Fig. 4.1. Both these illustrations depict splenic RFC against sheep red blood cells (SRBC). Fig. A shows an S<sup>-</sup> RFC binding a single layer of heterologous erythrocytes. Fig. B shows an S<sup>+</sup> RFC with extra layers of adherent SRBC. XRBC = Xenopus red blood cell, L = lymphocyte (non-rosette forming).



40  $\mu$ m

Fig. 4.2: Kinetics of  $\text{Tet}^c$  RFC production in the spleen following administration of different doses of SRBC.

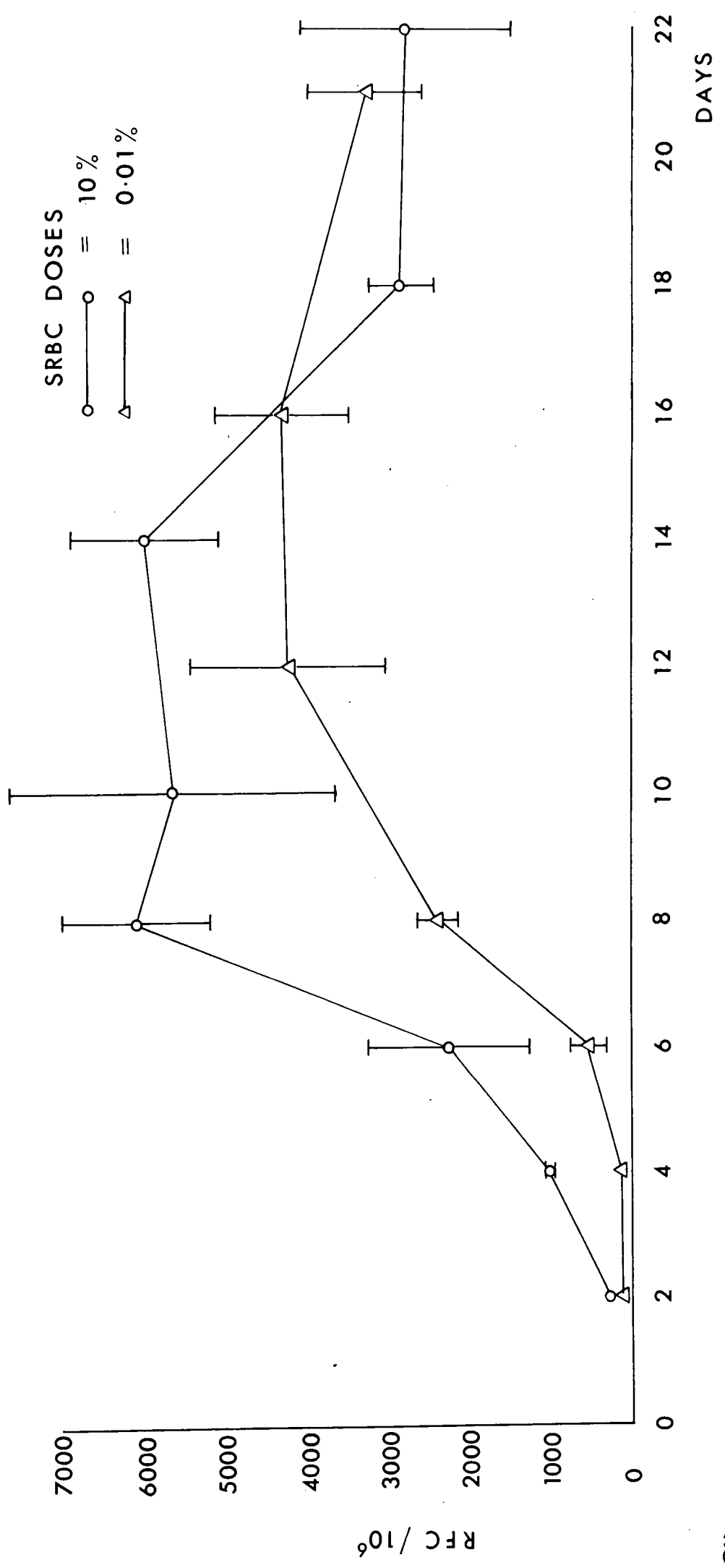


Fig. 4.3: Kinetics of S<sup>-</sup> and S+ RFC production in spleen following administration of different doses of SRBC.

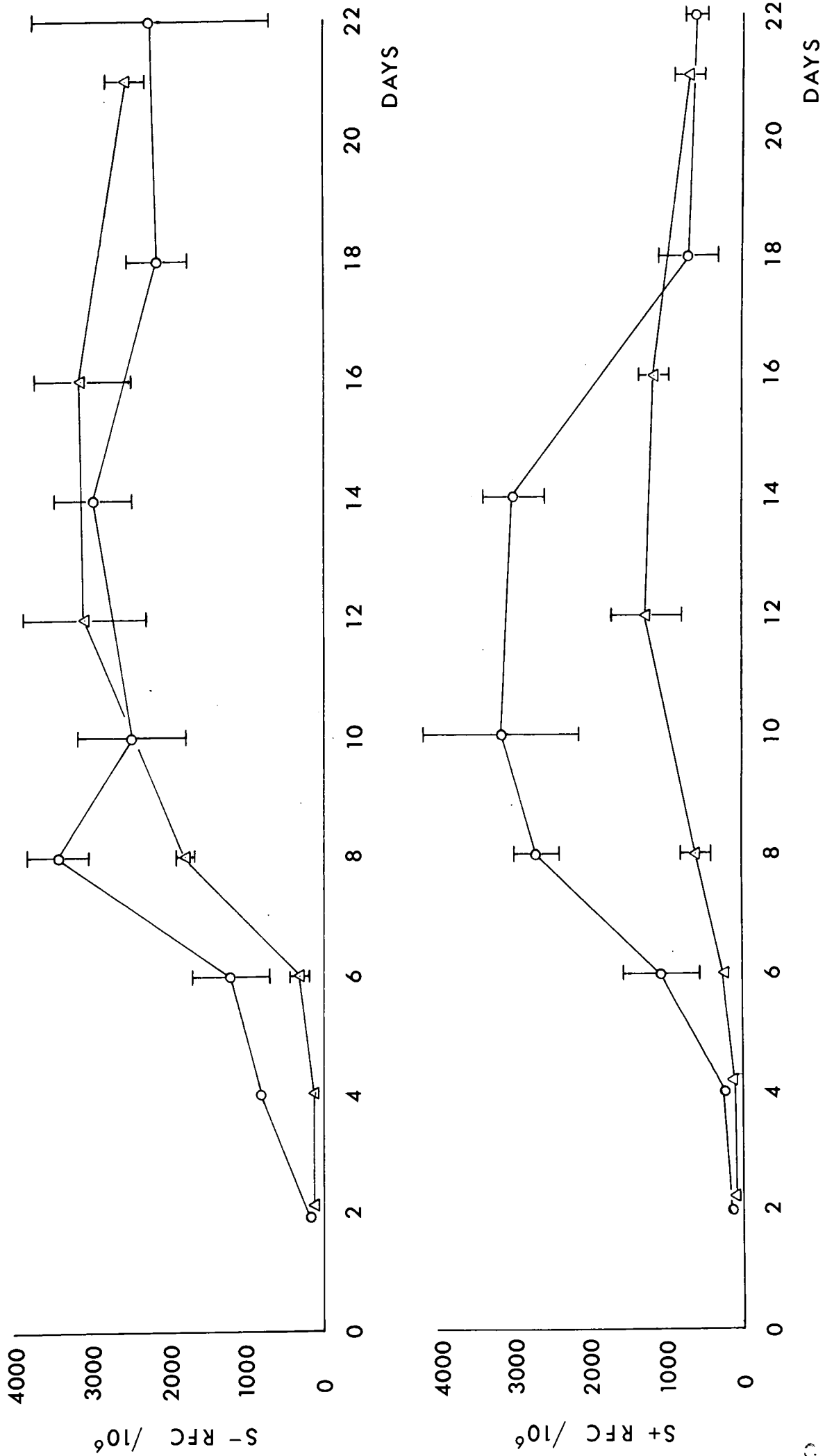
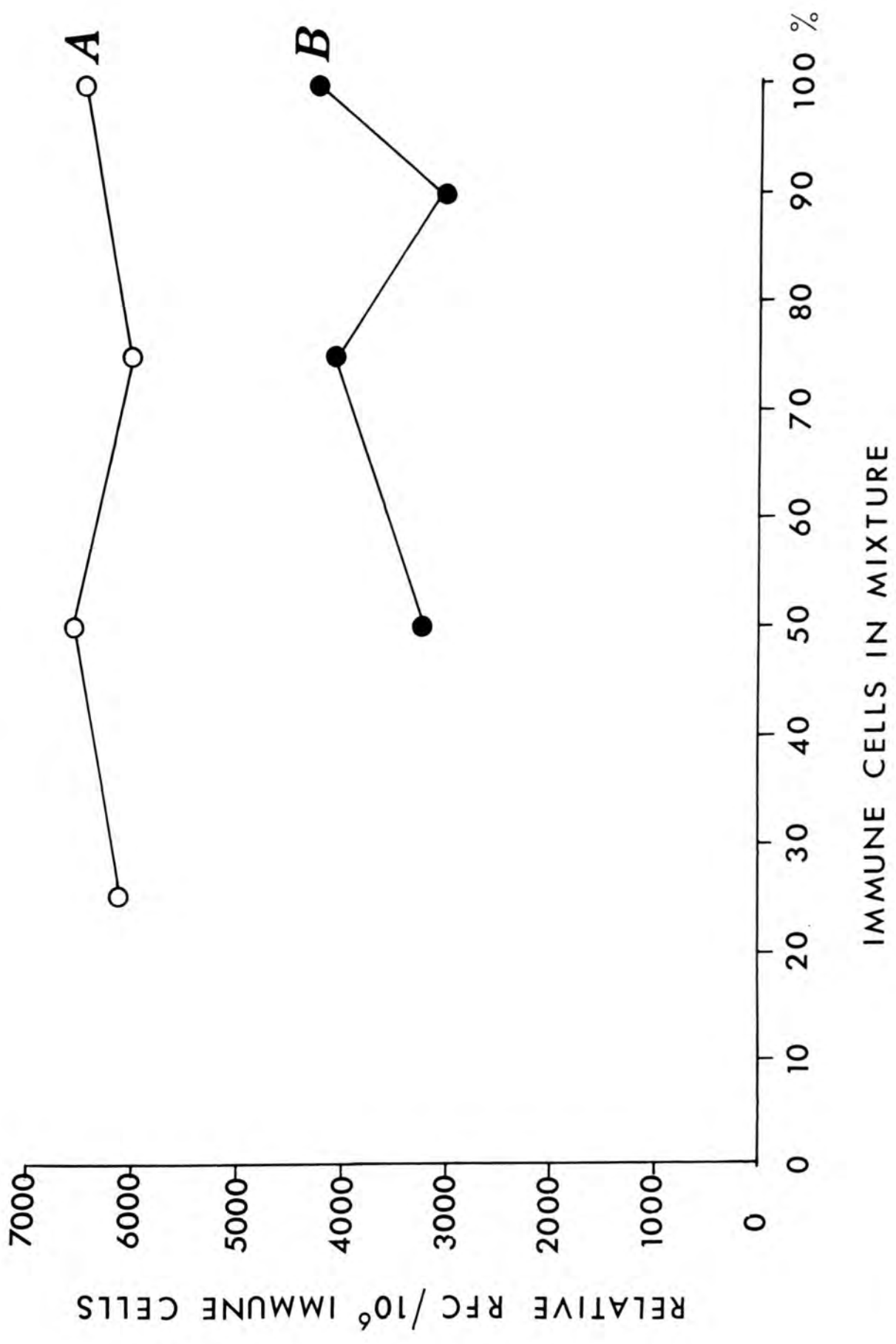


Fig. 4.4: Test for cytophilic antibody in vitro.



ERYTHROCYTESINTRODUCTION

Experiments presented so far have clearly shown that in order to establish normal cell-mediated and humoral immune responses, the thymus must remain intact for longer than the first eight days of life. But for how long? With respect to allograft immunity, this question has already been investigated by Horton and Manning (1972), who demonstrated that the thymus ceases to play an essential role during the third week of life. Thus, thymectomy at 14 days still impaired allograft reactivity, whereas thymic ablation at 17 and 19 days of age had no apparent effect on graft survival times. In contrast, the time required for the thymus to establish humoral immune responses remains an open question. The experiments presented in this chapter were therefore designed to enquire into this issue by examining the effects of thymectomy at different stages of larval development and also in adult life. Reactivity to sheep erythrocytes in the spleen was investigated at the cellular level with the aid of the I.C.A. technique. Plaque forming cells and serum antibody were also measured, following a series of injections, in control and thymectomized animals.

MATERIALS AND METHODSThymectomy

Xenopus were thymectomized or sham thymectomized at different stages of development. Table 5.1 briefly outlines the degree of lymphoid maturation and the level of immunocompetence (as suggested by earlier studies) at the time of the operations.

The effect of thymectomy at any particular stage of development was generally studied in at least two separate batches of animals. As the thymus increases in size in older larvae, more prolonged cauterization was required to effect complete destruction of the organ. The technique of thymectomy on post-metamorphic Xenopus differed from that employed for larvae. Toadlets were heavily anaesthetized and the thymus was removed surgically through a small incision made in the overlying skin. The thymus in toadlets is surrounded by fatty tissue which makes it relatively easy to excise the organ intact. Sham thymectomy involved making an incision in the skin over the thymus and manipulating the organ with sterile forceps. No sutures were required and the wound generally healed within 3 - 5 days. Thymic absence was always confirmed at autopsy.

### Experimental Design

#### A Immunization of toadlets

Animals were injected with saline-washed SRBC resuspended in Alsever's solution (50  $\mu$ l per g.b.w.) via the intraperitoneal route. Toadlets thymectomized as larvae were injected when they were from 20 to 30 weeks of age and weighed 3 to 5 grms. Adult-thymectomized toadlets were injected at various intervals from 7 to 240 days post-operation.

One group of toadlets (thymectomized and sham-thymectomized) received a single injection of 10% SRBC in Alsever's solution and were sacrificed 8 days later for the I.C.A. experiments. The numbers of toadlets assayed for each stage of thymectomy are shown in Tables 5.2 and 5.3. Total numbers of RFC in the spleen were determined as described in the last chapter.

A second group of toadlets received 3 single injections of 5% SRBC at 3 day intervals. Animals in this group were killed two weeks after the final injection when plaque and/or serum haemolysin assays were performed. Turner and Manning (1973) have previously demonstrated that good levels of

humoral antibody appear at this time. The number of toadlets assayed is shown in Table 5.2.

### B Immunization of Larvae

A brief investigation of the short term effects of thymic-removal on larvae in which the anti-SRBC response has already matured was carried out on tadpoles thymectomized at stage 53 of development. (anti SRBC responses are first detected at stage 50, Kidder et al., 1973). The results of this study are shown in table 5.4.

Larvae were given a single injection of  $5\mu$  50% SRBC intraperitoneally, since pilot experiments had shown this dose to be effective in generating elevated splenic RFC levels. I.C.A. experiments were performed 8 days post-immunization:

Injections were performed with the aid of a micropipette, drawn from a 50  $\mu$ l microcap (Shandon). The point of the micro-pipette was inserted through the ventral tail musculature into the peritoneal cavity. When the pipette was withdrawn, the muscle tissue prevented any leakage from the peritoneal cavity.

### Immunocytoadherence Assay

The technique was identical to that described in the last chapter. In the larval studies however, it was necessary to pool 10-20 larval spleens for each assay in order to give a suitable spleen cell concentration.

### Plaque Assay

The direct haemolytic plaque assay, originally described by Cunningham and Szenberg (1968) and modified by DuPasquier (1970a) for amphibian studies, was used. Spleen cell suspensions were prepared as for the I.C.A. assay and 200  $\mu$ l of spleen cells were mixed with 20  $\mu$ l of 25% SRBC and 50  $\mu$ l 1:10 guinea pig complement (Wellcome). The same batch of complement was used throughout the present studies since DuPasquier (pers.comm.) has

shown that the number of PFC recorded varies markedly depending upon the source of complement used. Control assays were also set up for each spleen using 50  $\mu$ l of medium instead of complement: these assays were uniformly negative.

The assay mixtures were prepared on ice and, after thorough resuspension, 2 x 100  $\mu$ l samples were gently pipetted into slide counting chambers (Wild and Dipper, 1974). After filling, chambers were sealed by dipping the edges in a mixture of molten paraffin wax and vaseline. The slides were then incubated for two hours at 28-30°C. After this time plaques were seen macroscopically as distinct clear areas, but were checked under the microscope. Only when a central lymphoid cell surrounded by red cell 'ghosts' (Fig. 5.1) was seen, was a PFC scored. PFC were expressed as the number per 10<sup>6</sup> spleen leucocytes.

#### Haemolysin assay

In our hands the haemolysin assay proved to be more sensitive than tests for haemagglutinating antibody and so the former test was used throughout.

Blood was collected from the ventricle and the serum de complemented by heating at 56°C for 30 minutes. The assays were set up in microtitration trays (Flow). Twenty five microlitres of serum (serially diluted) was first incubated for one hour at 28-30°C with 25  $\mu$ l of 1% SRBC. After the addition of 25  $\mu$ l 1:10 guinea pig complement and a further period of incubation for two hours, haemolysin titres were recorded and expressed as  $-\log_2$  titres.

### RESULTS

#### A Toadlet responses

##### (i) Production of RFC in the spleen

RFC levels for immunized animals following larval thymectomy are given

in Table 5.2. Data for non-injected toadlets were given in Chapter 4 and results for the 8 day thymectomized & sham-thymectomized, and non-operated injected animals also come from this chapter.

Table 5.2 shows that 8 non-operated and 11 sham-thymectomized toadlets (5 operated on at 8 days; 5 at 22 days and 1 at 30 days) all possessed high levels of RFC in the spleen. In contrast, thymectomy at 8, 15 and 22 days of age abrogated the RFC response to injected erythrocytes. There was also a partially suppressive effect of thymectomy at 30 days of age. Animals thymectomized between 37-40 and 47-60 days of age, on the other hand, displayed normal elevated levels of RFC in the spleen 8 days post-SRBC administration.

RFC levels following SRBC injection in animals thymectomized as toadlets at 6 months of age are given in Table 5.3. 'Adult'-thymectomized animals produced splenic RFC levels which were similar to those observed in sham-operated siblings, regardless of the interval between thymic removal and SRBC administration. It was noticeable that older toadlets (both sham and thymectomized) gave higher levels of immune RFC than younger animals.

In view of previous findings (Horton and Manning, 1972) that thymectomy during the third week of larval life has no subsequent effect on alloimmune reactivity, it seemed important to examine the response to allografts and SRBC in the same animal in order to confirm the differential effect of thymectomy on cell-mediated and humoral immune responses. This was also important in view of a report by Kidder et al., (1973) who found that in Xenopus tadpoles whose morphological stage was the same, the degree of lymphoid differentiation was often markedly different, the latter being more dependent upon the precise age of the animal rather than on its external appearance. Thus the present findings might have been due to

the fact that in the batches of Xenopus larvae used in the allografting studies of Horton and Manning, (1972) lymphoid development may have been more rapid than in the present studies.

Skin allografts were therefore applied to 4 toadlets thymectomized at 22 days and to 2 animals thymectomized at 30 days. All 6 animals rejected their allografts in normal first-set fashion (17-25 days). Three weeks after rejection, they were each given a single injection of 10% SRBC and assayed 8 days later for splenic RFC. The levels of RFC recorded in the 4 animals thymectomized at 22 days were 113, 0, 725 and 194 RFC/10<sup>6</sup> spleen leucocytes, i.e. they showed no enhanced anti-SRBC activity. Two control animals (sham-thymectomized at 22 days) which had also rejected allografts displayed normal RFC responses (4,700 and 6,112 RFC). The two 30-day thymectomized toadlets gave RFC levels of 3555 and 2595 both of which fall within the normal response range for control toadlets. Interestingly, these 2 grafted animals gave higher RFC counts than two animals which were not grafted prior to SRBC injection (see Table 5.2).

(ii) Cellular and humoral antibody production

This data also appears in Tables 5.2 and 5.3. Three non-injected controls were also tested for PFC and serum haemolysins: these assays yielded zero levels throughout. Negligible background levels of splenic P.F.C. and serum antibody have been recorded in previous studies on anti-SRBC responses in Xenopus (Auerbach and Ruben, 1970; Turner and Manning, 1973).

P.F.C. were detected in all non-operated and sham-thymectomized toadlets two weeks following triple SRBC injection. In contrast, no P.F.C. were recorded in spleens of 12 toadlets which had been thymectomized at 8, 15, 22 and 30 days (although only one animal was examined after 15- and 30 day-thymectomy). Moreover, 2/3 animals thymectomized between

37-40 days displayed only minimal numbers of P.F.C. in the spleen following SRBC injection. Animals thymectomized between 47-60 days of age and also during adult life, all displayed elevated levels of P.F.C. comparable with those observed in control animals following SRBC administration.

The findings on the effect of larval thymectomy on splenic P.F.C. following SRBC administration were reflected in the serum haemolysin studies. Thus, animals thymectomized at 8, 15, 22 and 30 days (14 in all) gave no detectable serum haemolysins and of 3 animals thymectomized at 37-40 days of age, serum haemolysin activity ( $-\log_2 5$ ) was detected in only one. Thymectomy at 47-60 days had no apparent effect on serum haemolysin production. The effect of adult thymectomy was examined in only two animals, which were given 3 injections of SRBC 240 days after thymic removal: both gave normal haemolytic titres.

#### B. Larval Responses

Non-operated larvae gave splenic R.F.C. levels following SRBC injection of 948-3808 R.F.C./ $10^6$  spleen leucocytes. The mean R.F.C. level from 7 separate assays was  $2131 \pm 458$  R.F.C./ $10^6$  spleen leucocytes. Background levels of 0-292 (mean  $140 \pm 35$ ) R.F.C./ $10^6$  spleen leucocytes were recorded from 7 experiments involving non-injected larvae.

Larvae thymectomized at stage 48 failed to show elevated levels of R.F.C. in the spleen when tested two weeks post operation. In this experiment the non-injected sham-thymectomized background was 292, the SRBC injected shams gave a level of 1432 and two separate pools of thymectomized spleens gave R.F.C. counts of 298 and 261 R.F.C./ $10^6$  leucocytes respectively, following SRBC injection.

R.F.C. levels in larvae thymectomized or sham-thymectomized at stage 53 are given in Table 5.4. These results indicate that the R.F.C. response to SRBC was not affected within 7 days of thymic removal and only partially affected following SRBC injection at 21 days post-thymectomy (responses

were 69% and 36% of sham response). By 33 days after thymic ablation however, the thymectomized animal no longer displayed elevated R.F.C. levels in the spleen following SRBC injection.

### DISCUSSION

The work presented in this chapter has shown that normal maturation of the immune response to sheep erythrocytes in Xenopus is dependent upon the presence of an intact thymus for much of larval life. Thus, thymectomy as late as 22 days of age completely abrogates the normal splenic R.F.C. and P.F.C. responses and also abrogates humoral antibody production to this antigen. Moreover, thymectomy at 30 days of age still impairs the anti-SRBC response, although low levels of induced R.F.C. were recorded. All animals tested following thymectomy at 37-40 days of age (stage 56/7) displayed normal levels of immune R.F.C. in the spleen, but P.F.C. and serum antibody responses were still generally impaired or abrogated. This apparent difference may simply reflect the relative sensitivity of the techniques used, but nevertheless suggests that although the proliferation of R.F.C. is unimpaired following thymectomy at 37-40 days, the capacity to synthesise and/or secrete antibody is still diminished by the operation. Recently independent studies by Collie (1976) have also shown that thymectomy up to stage 57 generally abrogates serum antibody production to SRBC. Moreover, her studies are in agreement with the work presented here which shows no defect in anti-SRBC reactivity following thymectomy after this time (i.e. during the perimetamorphic period and in adult life).

The present experiments on Xenopus indicate that those thymus-dependent cells which effect acute allograft rejection become permanently established early in ontogeny, in contrast to a more slowly established population of thymus-dependent cells involved in SRBC responses. Studies on the larva have shown that a population of SRBC-reactive cells is present in the

periphery early in larval development, (stage 50, Kidder et al, 1973) but the work in this chapter has shown that this reactivity disappears within a few weeks following later (stage 53) larval thymectomy. This finding might be due to a lack of sufficient numbers of peripheralized T-cells to form a self-replicating population in the adult (particularly since lymphocyte numbers fall dramatically over metamorphosis; see Table 5.1). Alternatively, early-seeded cells (and/or non thymus-derived cells) may require the continued presence of the larval thymus for the maintenance (possibly by a humoral factor) of their normal function. The finding that adult thymectomy has no deleterious effect on SRBC reactivity would suggest that a continued humoral influence in the periphery is not essential, at least in later life.

Adult thymectomy was shown to have no discernible effect on anti-SRBC responses even in animals injected as late as 250 days post-thymectomy - a time interval which exceeds that which normally elapses between larval thymectomy and testing after metamorphosis. Moreover, 3 toadlets thymectomized after metamorphosis were skin allografted 140 days later and displayed normal first-set allograft rejection times. A later effect of adult thymectomy in Xenopus cannot be ruled out however, since in mice defects in cell-mediated and humoral immunity may only appear in some animals 6 - 18 months post surgery (Metcalf, 1965; Miller, 1965; Taylor, 1965). Short-term effects of adult thymectomy have recently been reported by Simpson and Cantor (1975) who showed that the adult murine thymus may play an important regulatory role in both cellular and humoral responses. Thus, adult thymectomy resulted in enhanced primary killer cell responses to alloantigens in vitro and diminished helper memory responses within a month of thymic removal. Other reports have also suggested a rapid loss of regulatory function in the absence of the adult thymus e.g. mixed lymphocyte culture reactivity is increased (Mosier and Cantor, 1971), as is the primary response to certain antigens (Kerbel and Eidinger, 1972;

Andersson and Blomgren, 1971). Further evidence of a short-term effect of adult thymectomy is provided by Jacobs and Byrd (1975) who demonstrated a loss of cells responsive to T-dependent mitogens (Phytohaemagglutinin and concanavalin A) in spleens of adult mice 10 - 20 weeks after the operation. Interestingly, the response to bacterial Lipopolysaccharide (L.P.S.) was not diminished. The function of the adult thymus is therefore of a complex nature and further experimentation is required to understand its role in Xenopus.

In birds and mammals, experimental evidence has indicated that different populations of thymus dependent lymphocytes possess distinct immunological functions (e.g. helper cell activity in antibody responses, reactivity in cell-mediated responses etc.) which Droege and Zucker (1975) suggest may be generated by the development of separate populations within the thymus. The present experiments on Xenopus lend support to the concept that functionally distinct populations of lymphocytes exist in amphibians (Cohen, 1975; Ruben et al., 1973; Turpen et al., 1975) since animals thymectomized at 22 days of age display no reactivity to SRBC, but can destroy skin allografts in normal rejection times. The prolonged thymic presence required for the establishment of anti-SRBC responses when compared with the allograft response, may reflect a later differentiation and/or seeding from the thymus of those T-dependent cells involved in humoral immunity. However, recent studies have shown that the normal humoral response to HGG in adjuvant, and Rabbit erythrocytes is only abrogated by thymectomy performed earlier than stage 50 or 51 (Collie, 1976; Tochinnai and Katagiri, 1975). Regardless of the nature of the thymus-dependent cells involved in the generation of humoral antibody in Xenopus, the establishment of these cells in the periphery does not appear to be a common ontogenetic event but rather, it appears that classes of T-cells specific for different antigens may mature sequentially. In this respect, the acquisition of immunological competence to specific antigens at different stages of ontogeny is well documented in mammals

(Silverstein, 1973).

In conclusion, the experiments presented in this chapter have identified a critical role of the thymus in immune maturation after the second week of larval life, a role not apparant from previous allografting studies. In Xenopus, the thymus controls development of both cell-mediated and humoral responses but commands the maturation of the humoral response to sheep erythrocytes for a more prolonged period of development.

TABLE 5.1      THYMUS MORPHOLOGY AND IMMUNE COMPETENCE AT  
STAGES OF DEVELOPMENT WHEN THYMECTOMY WAS PERFORMED

<u>Age</u> <u>(Days)</u>	<u>Stage</u>	<u>No. of</u> <sup>1</sup> <u>thymocytes</u>	<u>Other Comments</u>
7-8	47-8	$10^3-2 \times 10^3$	Very few or no small lymphocytes present in thymus. Thymectomy at this stage impairs allograft reactivity <sup>2</sup> , abrogates M.L.R. and P.H.A. responses <sup>3</sup> , also abrogates humoral reactivity to T-dependent antigens <sup>4</sup> .
15-16	51	$5 \times 10^4$	Allograft response matures at St49 <sup>5</sup> . Anti-SRBC response detectable in spleen at St50 <sup>6</sup> . Thymus and other lymphoid organs have completed their histogenesis <sup>7</sup> . Thymectomy at 17 and 19 days has no effect on allograft response <sup>2</sup> .
22	53	$10^5$	) Increase in size of lymphoid tissues <sup>7</sup> .
30	54/5	$5 \times 10^5$	
37-40	56/7	$10^6$	Thymectomy at this stage does not affect M.L.R. response <sup>3</sup> .
47-60	Perimetamorphic	$3 \times 10^5$	Lymphocyte numbers in spleen and thymus fall during metamorphosis. <sup>1</sup> Alloimmune response atypical at this time and allograft tolerance may be induced across weak H/C differences. <sup>8</sup>
6 months	Juvenile Toadlet	$29 \times 10^6$	Numbers of thymocytes reach maximum at this time; after 1 yr thymus begins to regress. <sup>1</sup>

- Data from:
- 1 DuPasquier and Weiss, 1973.
  - 2 Horton and Manning, 1972.
  - 3 DuPasquier and Horton, 1976.
  - 4 Turner and Manning, 1974.
  - 5 Horton, 1969
  - 6 Kidder, et al., 1973
  - 7 Manning and Horton, 1969
  - 8 Chardonnens and DuPasquier, 1973

TABLE 5.2 LEVELS OF RFC, PFC AND SERUM HAEMOLYSINS IN CONTROL AND THYMECTOMIZED TOADLETS FOLLOWING SRBC ADMINISTRATION

Larval age at time of operation (days)	Type of operation	RFC Response 8 dys post single (10%) SRBC injection.		Antibody responses 2 weeks post triple (5%) SRBC injection.		Serum haemolysin titres
		No. of toadlets tested	RFC/10 <sup>6</sup> spleen cells	No. of toadlets tested	PFC/10 <sup>6</sup> spleen cells	
-	None	8	6984, 6067, 4154, 3904, 5986, 4132, 6682, 10244 (6147 ± 989)	5	41, 24, 36, 17, 51 (34 ± 6)	7 4, 4, 6, 5, 6, 4, 4 (4.7 ± 0.36)
8 (Stage 48)	Thymectomized	10	13, 17, 38, 80, 170, 26, 50, 108, 264, 444 (121 ± 44)	5	0, 0, 0, 0, 0	3 0, 0, 0.
	ShTx	5	4349, 3340, 5850, 5850, 8598 (5595 ± 902)	1	33	1 6
15 (St. 51)	Tx	2	358, 296 (327 ± 31)	1	0	5 0, 0, 0, 0, 0
	ShTx	n.d.		1	73	4 3, 4, 4, 2 (3.25 ± 0.47)
22 (St. 52/3)	Tx	5	752, 138, 342, 416, 358 (401 ± 101)	5	0, 0, 0, 0, 0	5 0, 0, 0, 0, 0
	ShTx	5	7196, 10244, 3904, 4825, 5833 (6400 ± 1108)	4	79, 45, 40, 60 (56 ± 9)	4 5, 3, 5, 7 (5 ± 0.8)
30 (St. 54/5)	Tx	2	1307, 833 (1070 ± 239)	1	0	1 0
	ShTx	1	5944	1	18	1 6
37-40 (St. 56/7)	Tx	6	2315, 2037, 8371, 2500, 7291, 10305 (5470 ± 1485)	3	2, 40, 4. (15.3 ± 14.5)	3 0, 5, 0. (1.7 ± 3.4)
	ShTx	n.d.		n.d.		n.d.
47-60 (Perimeta-morphosis)	Tx	2	3888, 6373 (5131 ± 1255)	4	31, 14, 37, 14 (24 ± 5.9)	4 4, 5, 5, 3 (4.25 ± 0.46)
	ShTx	n.d.		5	52, 28, 31, 12, 8 (26.2 ± 7.9)	5 5, 6, 4, 3, 2 (4 ± 0.7)

n.d. = not done.

TABLE 5.3

## LEVELS OF RFC, PFC AND SERUM HAEMOLYSINS IN ADULT THYMECTOMIZED AND SHAM-THYMECTOMIZED XENOPUS FOLLOWING SRBC ADMINISTRATION

RFC response 8 days post single 10% SRBC injection.

Antibody responses two weeks post triple 5% SRBC injection.

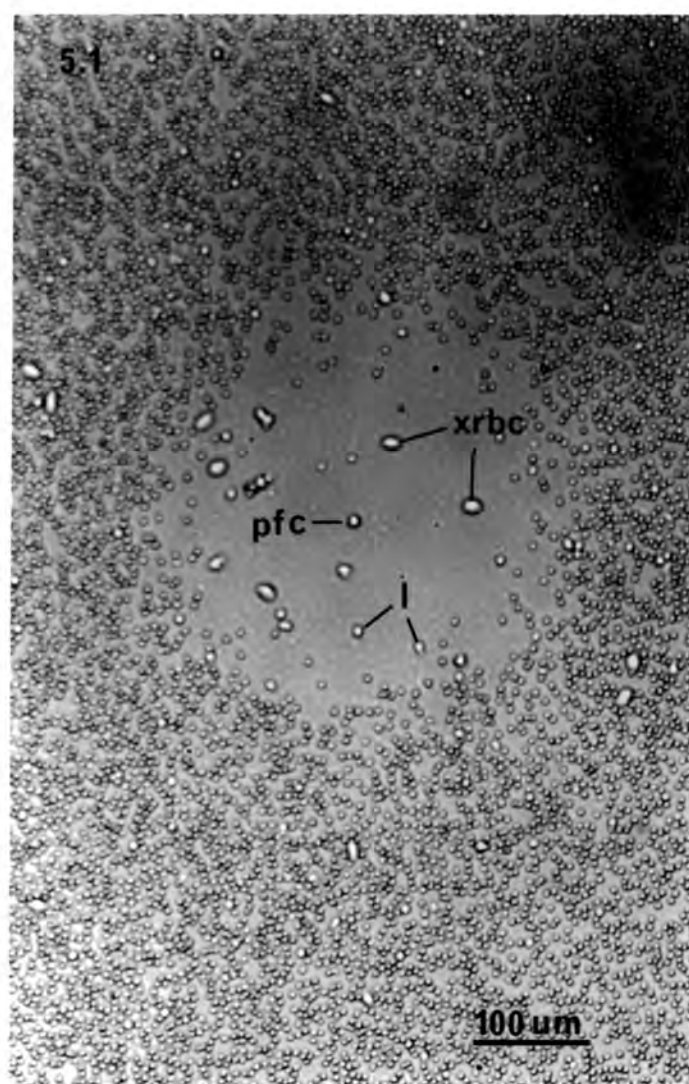
Interval between operation and SRBC injection	Type of operation	No. of toadlets tested	RFC/10 <sup>6</sup> spleen cells	No. of toadlets tested	PFC/10 <sup>6</sup> spleen cells	No. of toadlets tested	Serum haemolysin titres
7-12	Tx	6	1642, 2998, 3878, 5388, 5833, 3851 (3931 ± 628)	1	24	-	
	ShTx	2	1851, 4435 (3143 ± )	1	85	-	
20-28	Tx	5	2074, 3923, 7037, 8818, 5070 (5384 ± 1180)	5	7, 17, 85, 33, 18 (32 ± 14)	-	
	ShTx	4	3895, 6315, 5313, 3637, (4796 ± 627)	5	51, 34, 50, 46, 30 (33 ± 19)	-	
56-83	Tx	7	11607, 7541, 7705, 5128, 2276, 8822, 12036 (7859 ± 1291)	2	77, 6 (41 ± 35)	-	
	ShTx	3	6067, 1398, 2261 (3239 ± 1437)	-	-	-	
112-114	Tx	5	1250, 15406, 8888, 11666, 10573 (11806 ± 1088)	2	115, 216 (165 ± 51)		
	ShTx	5	8888, 5740, 9381, 7440, 8703 (8030 ± 657)	2	30, 10 (20 ± 10)		
200-240	Tx	3	17888, 11110, 13554, (14184 ± 2019)	-		2	4, 3 (3.5 ± 0.5)
	ShTx	2	17222, 14332, (15777 ± 1459)	-		2	3, 2 (2.5 ± 0.35)

TABLE 5.4      EFFECT OF STAGE 53 THYMECTOMY ON ANTI SRBC RESPONSE  
AT INTERVALS POST-OPERATION

Interval between operation and SRBC injection	Type of operation	RFC/10 <sup>6</sup> spleen leucocytes
7 days <sup>1</sup>	ShTx	5,300
	ShTx	5,162
	Tx	5,082
	Tx	4,450
21 days <sup>2</sup>	ShTx	3,008
	Tx	2,095
	Tx	1,100
33 days <sup>3</sup>	ShTx	1,762
	Tx	329

Background levels in ShTx larvae: <sup>1</sup>260; <sup>2</sup>150; <sup>3</sup>220.

Fig. 5.1 shows the microscopic appearance of a typical plaque. Note SRBC "ghosts" around a central plaque forming cell (pfc). Several other leucocytes (l) are present in the lysed area. XRBC = Xenopus red blood cell.



INTRODUCTION

The work in this thesis has centred largely upon the effects of early thymic removal on the development of immune responsiveness in Xenopus. To date, there exists very little detailed knowledge of the fine structure and degree of differentiation which has taken place by this stage of ontogeny. Manning and Horton (1969) have investigated thymic differentiation by light microscope studies of 8  $\mu$ m sections, but as yet there has been no detailed morphological investigation of Xenopus thymus early in development. Curtis et al. (1972) performed E.M. studies of the thymus of larval Rana pipiens (Leopard Frog), but their observations only commenced at 14 days of age, by which time thymic differentiation is quite well advanced.

The work in this chapter has examined the thymus of eight day old Xenopus larvae (the time when thymectomy was routinely performed in this thesis) and a study of 5 day thymus was also made, since this is the earliest stage at which thymic destruction has been achieved in this laboratory (Horton and Horton, 1975). The results of these studies are compared with the structure of thymus taken from 30 day old larvae; a time when lymphoid organ transformation is complete (Manning & Horton, 1969). The work provides, for the first time, insight into the ultra-structure of the amphibian thymus during the earliest stages of its ontogeny.

MATERIALS AND METHODS

Xenopus larvae were bred in the laboratory and reared at 23°C.

For the studies on 5 day and 8 day larval thymus, larvae were



heavily anaesthetized in MS-222 (Sandoz) before being fixed whole in a cold solution of glutaraldehyde (5% glutaraldehyde in 0.1M sodium cacodylate) overnight. Following fixation, larvae were washed in 0.1M cacodylate buffer overnight and post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate for 2 hrs. After washing for half an hour in 0.1M buffer the specimens were dehydrated through a graded series of methanol dilutions to 95% methanol. Dehydration was completed with two changes of absolute methanol over a period of 1 hour. Specimens were cleared in two changes of propylene oxide, each for 10 minutes, and transferred to a 1:1 mixture of Epon/Epoxypropane overnight. Infiltration was completed by immersion in pure Epon (all day) and the larvae were finally embedded in epon, the plastic being allowed to polymerize at 60°C for 48 hours. The procedure outlined above was also followed for the 30 day larval thymus except that the organ was dissected out prior to processing.

Ultrathin sections (500-600 $\text{\AA}$ ) were cut with glass knives on a Reichert OMU 3 ultramicrotome, mounted on 200 mesh copper grids and double stained with saturated uranyl acetate and lead citrate. Grids were examined with an A.E.I. EM801 electron microscope operating at 80 kV. Thick sections (1-1.5 $\mu$ ) were cut and stained in 1% Toluidine blue for the light microscopy. These sections were photographed with a Zeiss Ultraphot photomicroscope.

## RESULTS AND DISCUSSION

### A) Mature larval thymus

#### (i) Light microscopic observations

At 30 days of age, the larval thymus shows a clearly defined cortico-medullary differentiation. As reported elsewhere, (Manning & Horton, 1969) the medulla is eccentrically placed (see Fig. 6.1). The thymus is surrounded by a capsule and immediately below this is a zone of large

cells, one to two layers thick. In Toluidine blue stained sections these are pale-staining with a high nucleo-cytoplasmic ratio. Some of these cells are in mitosis (see Fig. 6.2). In appearance these sub-capsular cells resemble closely the lymphoblasts seen in the thymus of the larval urodele Pleurodeles waltlii (Charlemagne, 1974) and their position is reminiscent of the large blast cells observed in the sub-capsular zone of the adult murine thymus (see Clark, 1973). Autoradiographs of the Xenopus thymus reveal heavy  $^3\text{HT}$  uptake by sub-capsular lymphoblasts in vivo (Horton, unpublished observations). The cells of the cortical region are predominantly of lymphoid appearance i.e. round or ovoid cells with a small rim of cytoplasm and characteristic pattern of chromatin condensation in the nucleus (Fig. 6.2). There are large numbers of small lymphocytes. Large, pale-staining reticulo-epitheleal cells with evenly dispersed nuclear chromatin and prominent nucleoli are scattered throughout the inner cortical region and frequent the medulla (see Fig. 6.2). This central zone is of a highly complex nature with fewer lymphoid cells and a variety of other cell types. Cystic spaces, which resemble those described by Curtis et al (1973) in the thymus of larval Rana pipiens are occasionally seen in the thymus of mature Xenopus larvae. Medullary epitheleal cysts have been well-documented in other species including mammals, (Clark, 1963; 1966; 1968; Gad and Clark, 1968; Mandel, 1968; Mandel, 1970) birds and reptiles (Raviola and Raviola, 1967) and the urodele amphibian Ambystoma mexicanum (Klug, 1967). The role of such cells remains undefined but their products may be involved in stimulating lymphopoiesis and/or other functions.

ii) Electron Microscopic observations

A section through part of the cortex is seen in Fig. 6.3. The nuclear chromatin of the lymphocytes is organized into a series of discrete electron-dense zones and there is also a thin margin of dense

chromatin which lies just inside the nuclear membrane. The cells possess little cytoplasm with few organelles. Occasional mitochondria and small vacuoles (which resemble pinocytotic vacuoles) are apparent.

The medullary region of the thymus is shown in Fig. 6.4 and 6.5. The epithelial cells possess nuclei with evenly dispersed chromatin and prominent nucleoli. Their cytoplasm is extensive and stains less densely than the cytoplasm of lymphoid cells. Cytoplasmic projections extend in an interdigitating fashion between the lymphocytes and other cell types to form a supportive network. Myoid cells are also found in the medulla (Fig. 6.5): these have previously been described in the thymus of mammals, reptiles and other species of amphibia. (see Tör6 et al, 1969 for review; Raviola and Raviola, 1967; Curtis et al, 1972).

Typically, myoid cells possess a central nucleus, with little electron-dense chromatin but with a well developed nucleolus. The nucleus is surrounded by concentric rings of striated muscle fibres (myofibrils). The function of these cells is not understood but it has been suggested that they are involved in promoting circulation of tissue fluids within the thymus (Tör6 et al, 1969) or that they may provide a source of self-antigen (Mackay and Goldstein, 1967) which might play a part in the development of natural self-tolerance. The absence of myoid cells in the early amphibian thymus however (see below) might argue against the latter supposition. Various other unidentified medullary inclusions are also apparent (Fig. 6.4).

## B) Immature larval thymus

### i) Light microscopic observations

Toluidine blue stained sections through the middle of 5 and 8 day larval thymus are seen in Fig. 6.6. At these stages the thymus, when compared with that of the 30 day larva, is extremely small and undifferentiated. There is no apparent cortico-medullary differentiation.

*ii) Electron microscopic observations*

Fig. 6.7 shows a low power view of the thymus at 5 days. In this electronmicrograph it is possible to distinguish two types of cell; those with nuclei having evenly dispersed chromatin and well pronounced nucleoli are the epithelial cells. As in the later larval thymus their cytoplasmic processes form a network throughout the thymic rudiment. The second cell-type may be distinguished from the epithelial cells by their less well defined nucleoli and incipient chromatin condensation, often with a distinctly marginated nuclear envelope. They possess large amounts of densely-staining cytoplasm with an abundance of free ribosomes and mitochondria. These cells are thought to be blast cells and the precursors of lymphocytes seen at later stages. Both at 5 and 8 days mitoses are frequently seen, usually at the periphery.

By 8 days, the degree of chromatin condensation in some lymphoid cells is more advanced (Fig. 6.8). The vast majority of these cells still possess large amounts of cytoplasm although a slight reduction in amount is evident in some cells. Lymphocytes with a thin rim of cytoplasm as noted in the 30 day thymus are rarely seen at this stage of development.

The cytoplasmic distinction between epithelial and lymphoid cells is illustrated in Figs. 6.9 and 6.10. Epithelial cells possess some rough endoplasmic reticulum which contrasts with the dense free ribosomal arrangement observed in lymphoid cells. There are also marked differences in the mitochondria of epithelial cells and those of lymphoid cells; those in the epithelial cytoplasm are generally rounded with sparse, fingerlike cristae whereas the mitochondria of the lymphoid cells are elongate with well-defined septate cristae.

In conclusion, the thymus of the 5 and 8 day old larva is very different from that seen at 30 days of age. Ultrastructural studies of

the latter suggest that the thymic microenvironment is much more complex than that of the early thymus. At the time of its removal (7 - 8 days in this thesis) the thymus is relatively undifferentiated and composed predominantly of epithelial cells and lymphoid cells with large amounts of cytoplasm. This latter feature is not readily apparent from light microscope studies and thymic lymphocyte differentiation appears to be less advanced at 8 days than previous studies might have suggested. (Manning & Horton, 1969, Horton and Manning, 1972). At 5 days of age, these ultrastructural studies have shown that the thymus contains no cells of lymphocyte morphology, but interestingly thymectomy at this stage still fails to abrogate chronic graft rejection. As stressed earlier, the thymus first appears as a pharyngeal bud at 3 days of age and it seems unlikely that any 'T-lineage' cells could be established in the periphery at this stage; if they are, it would suggest that even at this rudimentary stage of development only the briefest thymic influence is necessary to establish at least some immunological function in later life. Further analysis of the developing amphibian thymus may provide insight into the nature of the normal factors required for T-cell maturation.

Fig. 6.1. Light microscope 1  $\mu$ m section of 30 day larval thymus.  
Note differentiation into an outer, predominantly  
lymphoid cortex (C), and an inner medulla (M)  
containing various cell types and inclusions.  
(Toluidine blue, x 430).

6.1

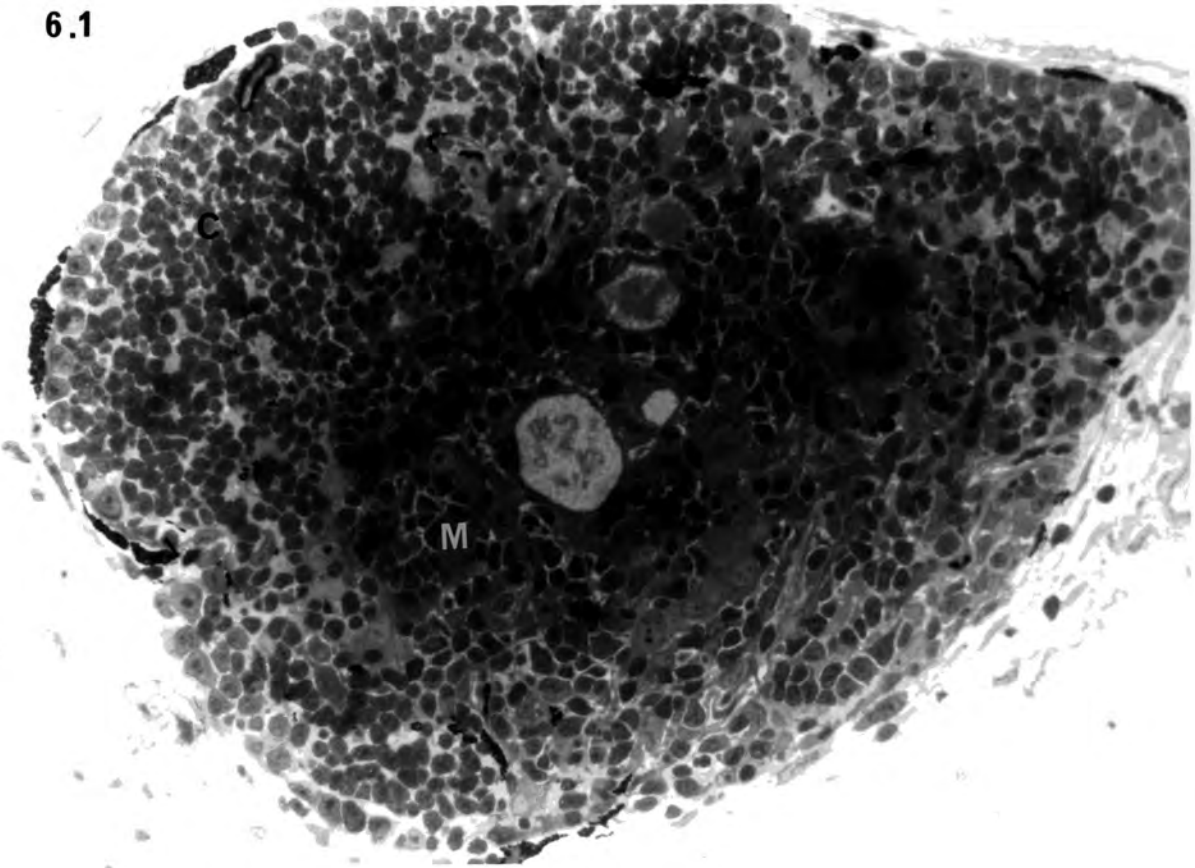
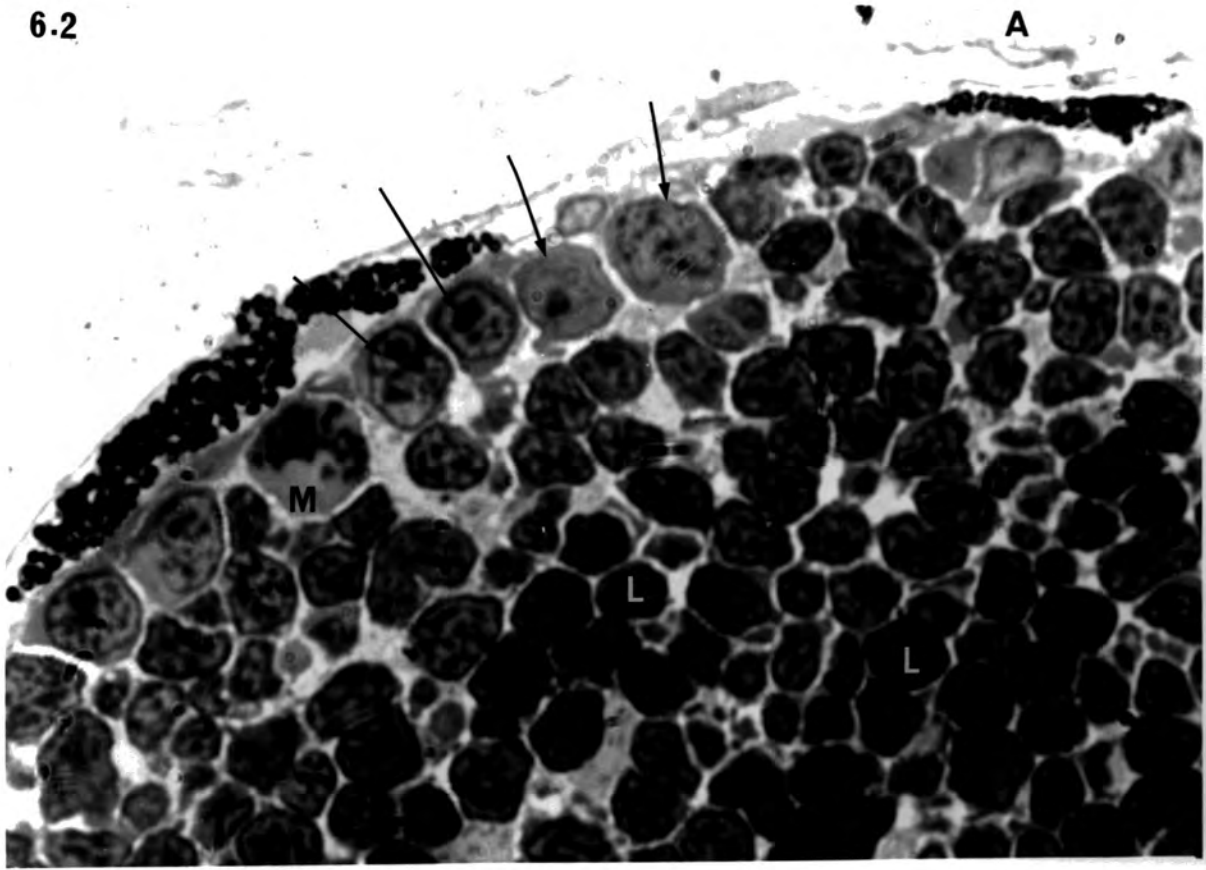


Fig. 6.2. Fig. A. shows a light microscope section through the cortical region of the 30 day larval thymus. Note the subcapsular layer of large lymphoblasts (arrowed) one of which is in mitosis (M). Numerous small lymphocytes (L) are present in the cortex.

Fig. B shows the more complex medullary region of the same thymus. Note the large, pale staining epithelial cells (E) with prominent nucleoli. Lymphocytes (L) are also present. Cy = medullary cyst.

Both 1  $\mu$ m sections are stained in Toluidine blue. (x 2,000 oil).

6.2



B

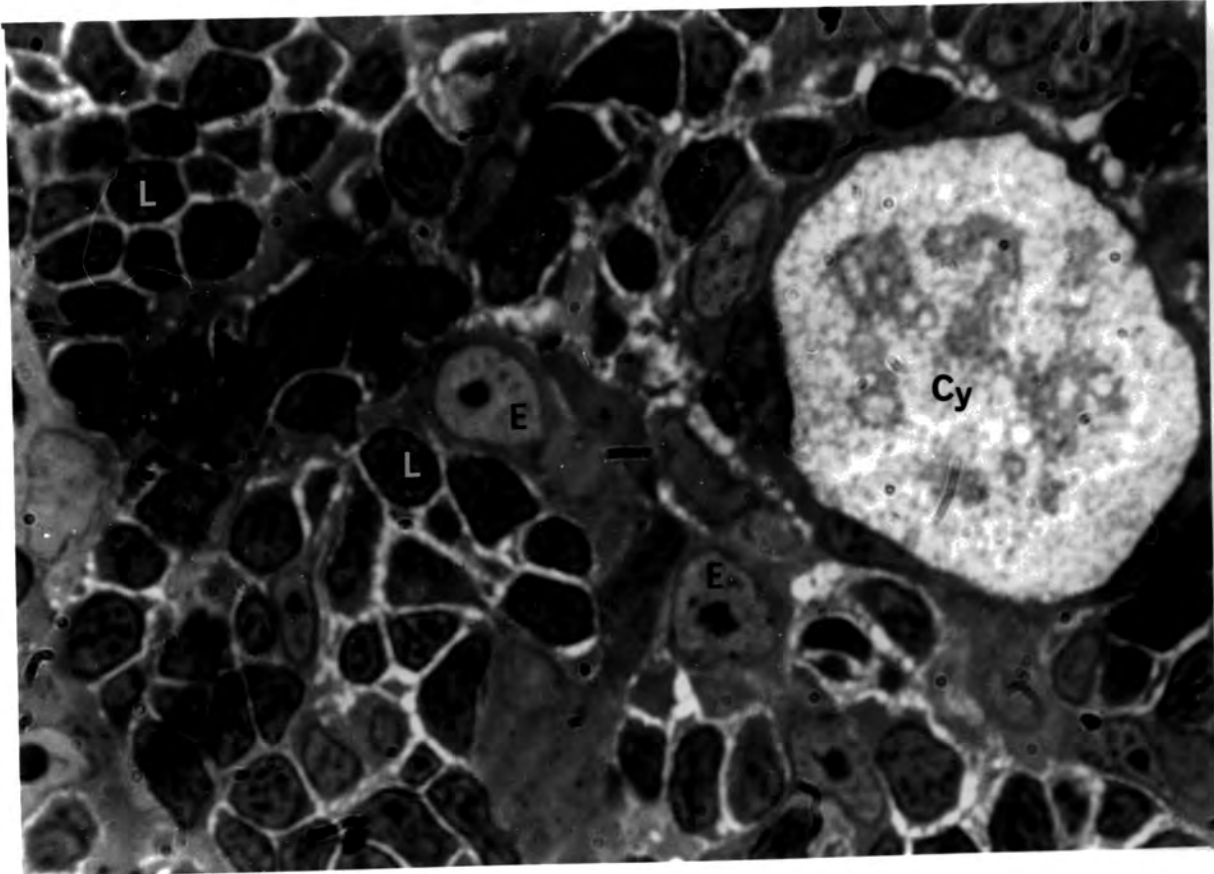


Fig. 6.3. Electron micrograph of the cortical region of a 30 day larval thymus showing small lymphocytes (L) with characteristic chromatin pattern and thin rim of cytoplasm. (x 8,000).

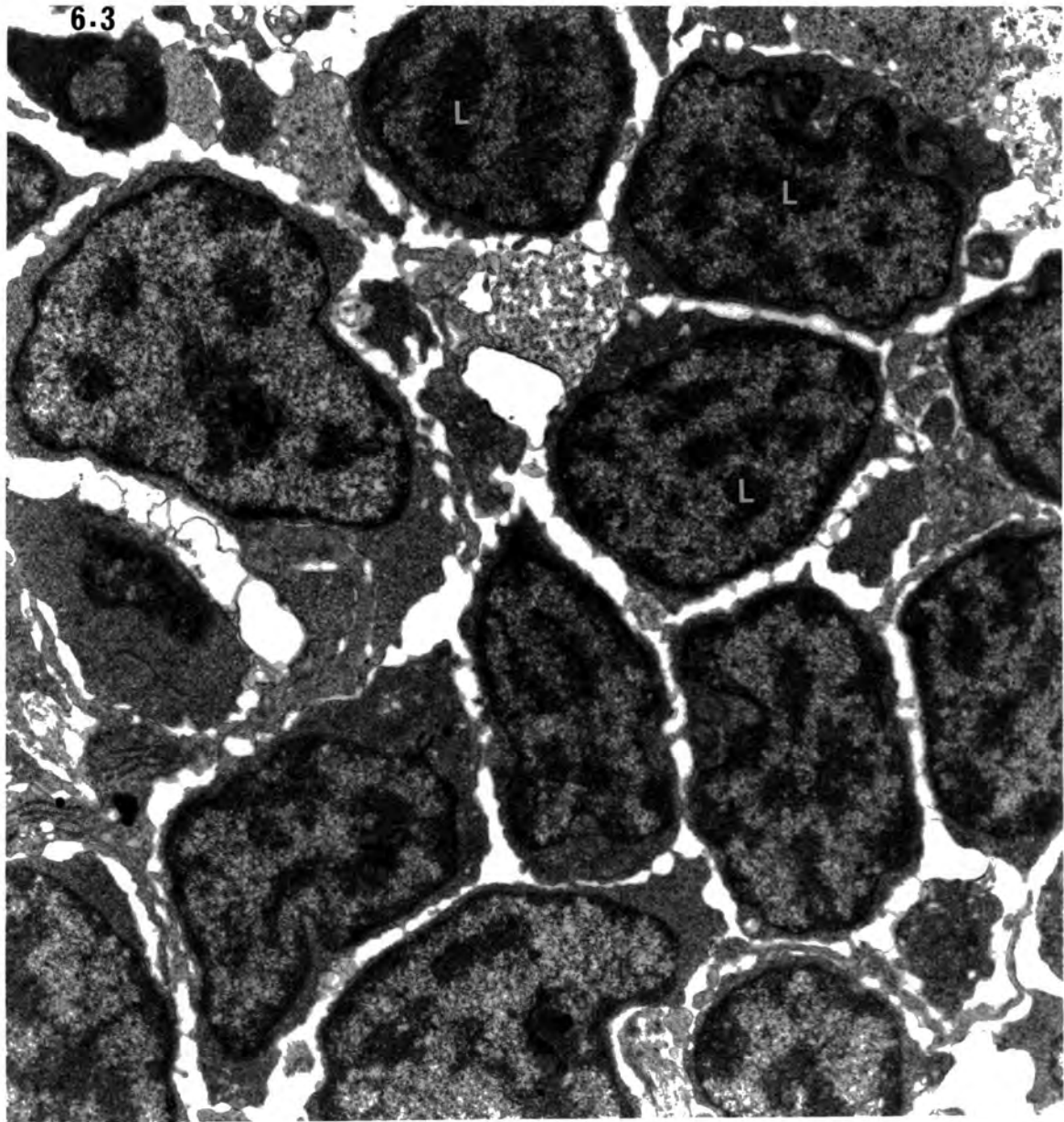


Fig. 6.4. Low power electron-micrograph depicting the medullary region of 30 day larval thymus. L=Lymphocytes, E=epithelial cells, Cy=cyst. (x 2,180).

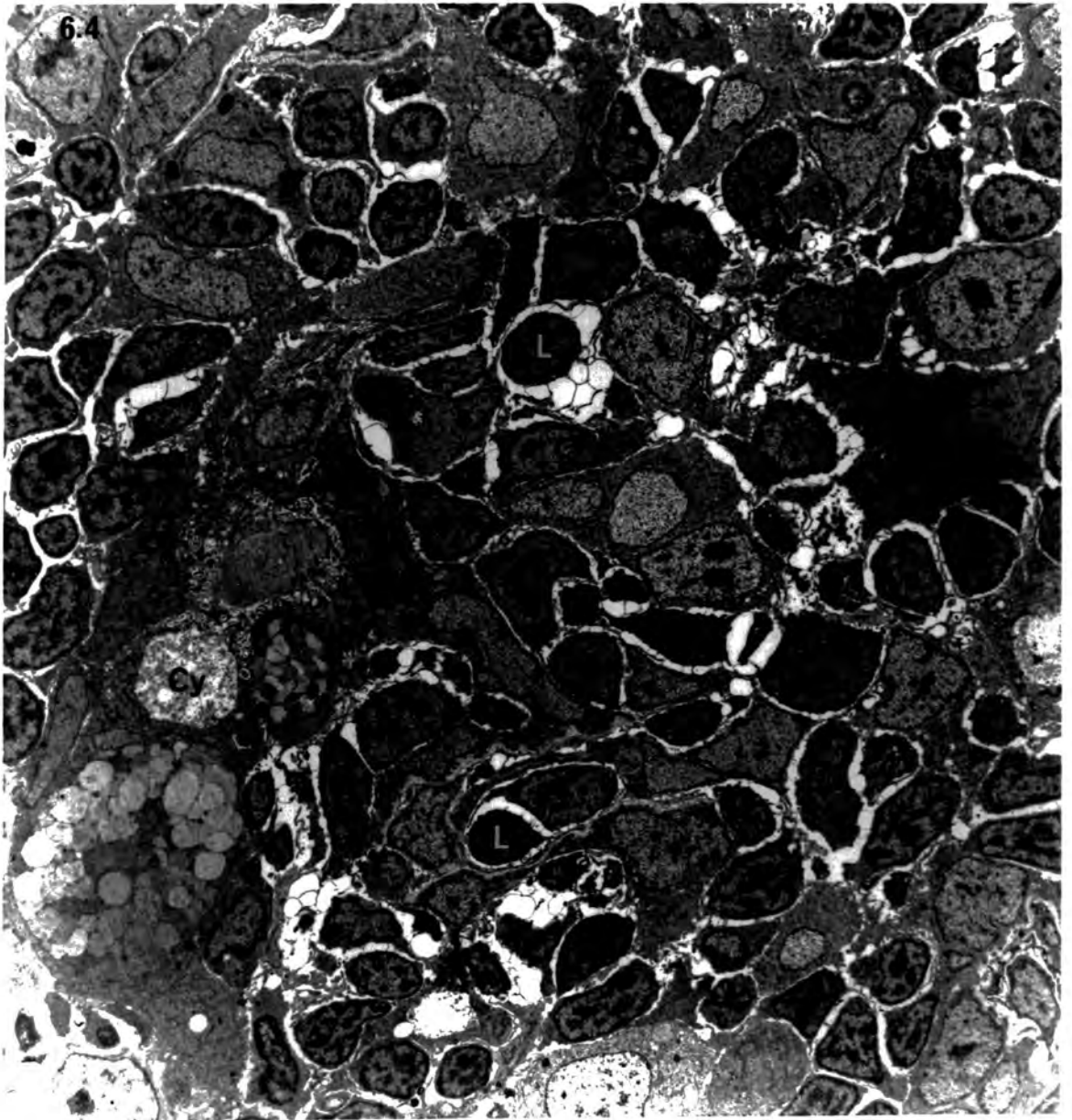


Fig. 6.5. Electron micrograph of a myoid cell in the medulla of 30 day larval thymus. Note characteristic concentrically arranged myofibrils (My) some of which are cut in cross-section (arrow).  
Nu = nucleolus; M = mitochondria; E = epithelial cell (Note cytoplasmic extension around myoid cell);  
L = lymphocyte. (x5,310).

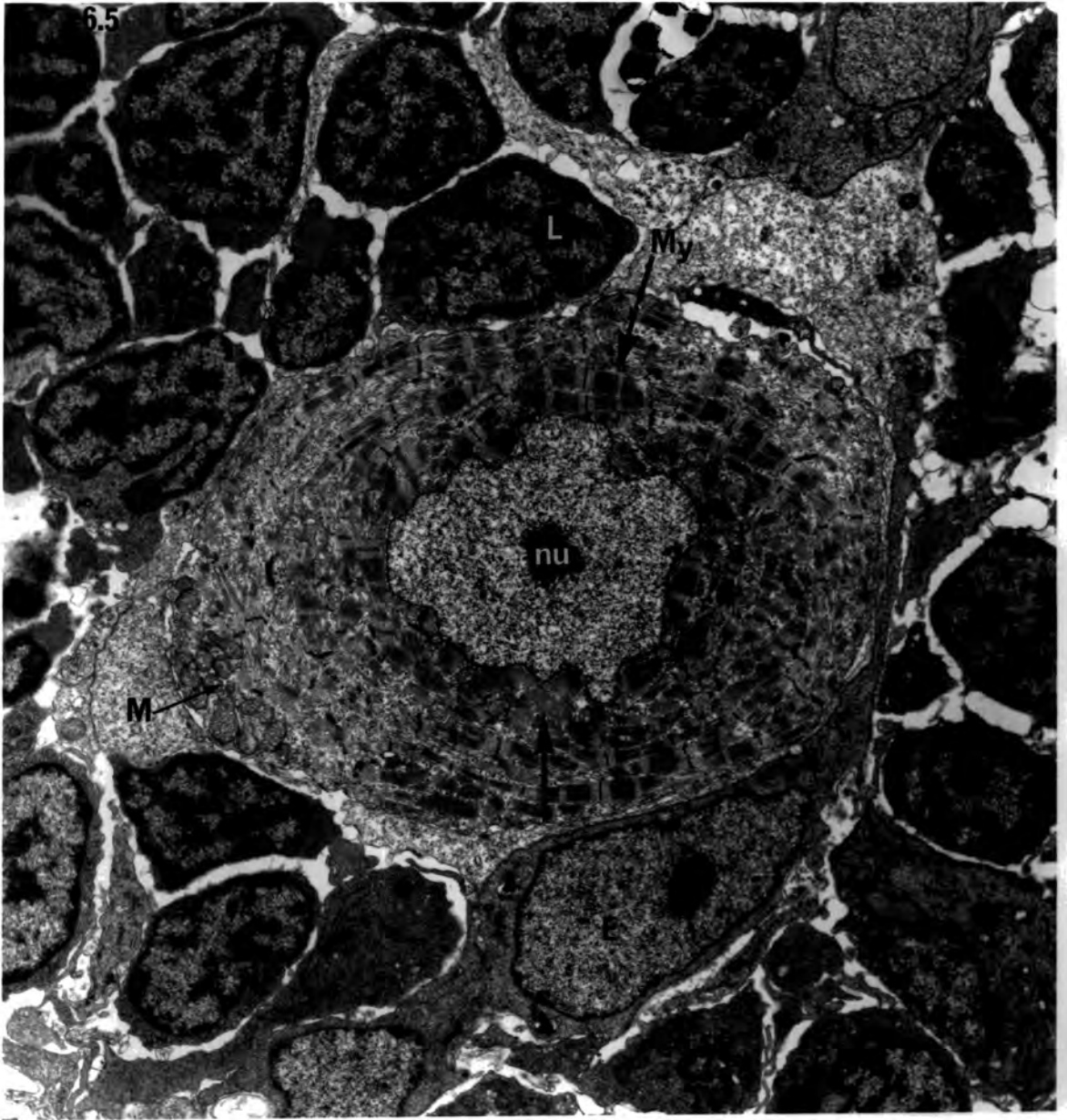


Fig. 6.6. Light microscopic sections through central regions of early larval thymi. Fig. A = 5 day thymus, Fig. B = 8 day thymus. (Toluidine blue, x600).

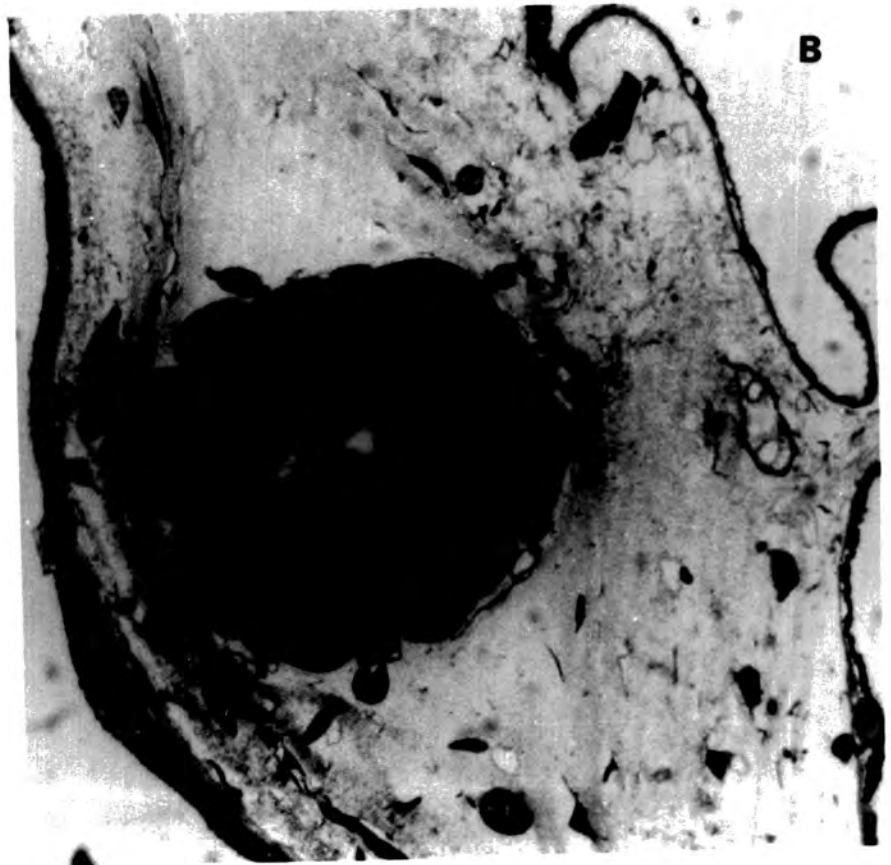
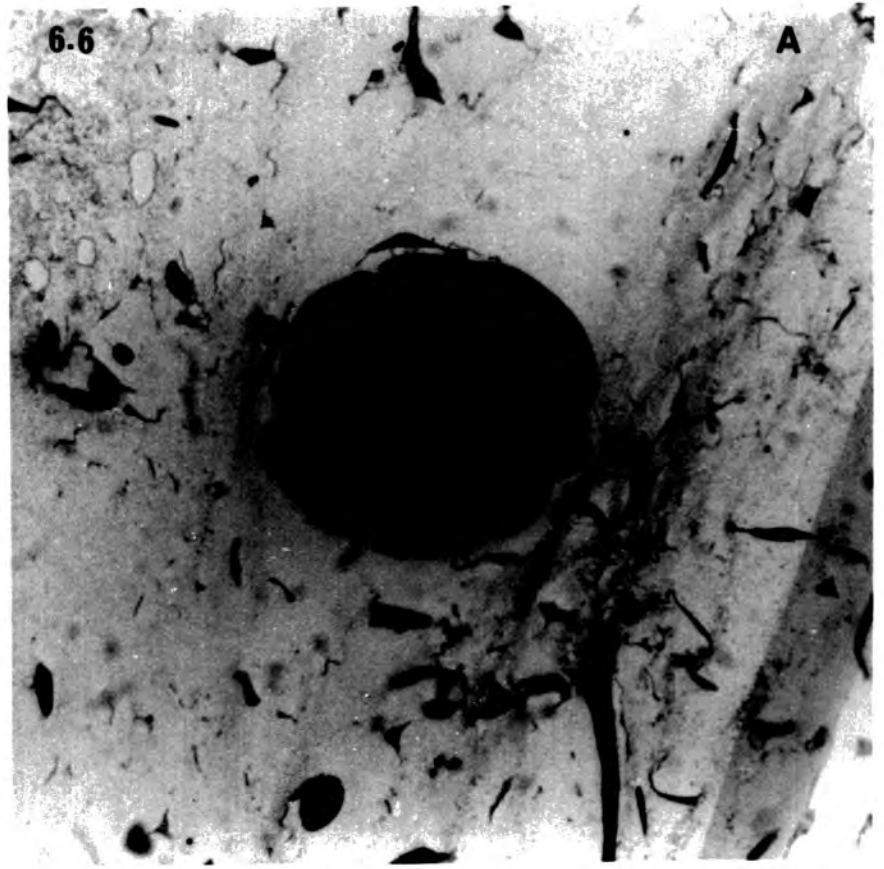


Fig. 6.7. Low magnification electron micrograph of 5 day larval thymus. Two major types of cell are distinguishable: L = lymphoid cell precursors with extensive cytoplasm, E = epithelial cell with prominent nucleolus, dispersed chromatin and paler staining cytoplasm. Note mitotic figure (M) at periphery. (x2,000).

6.7

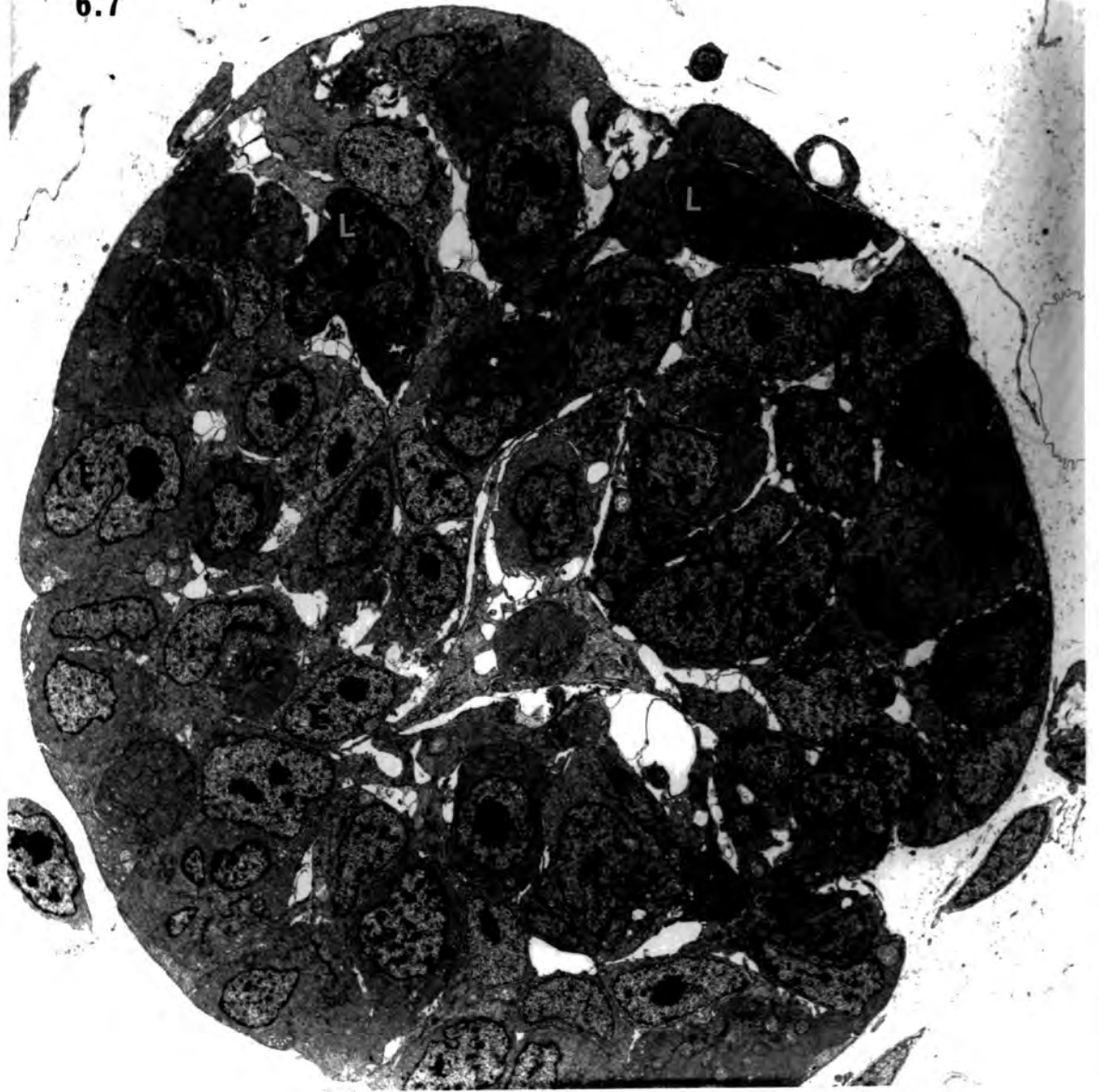


Fig. 6.8. Low magnification electron micrograph through centre of 8 day larval thymus. Despite some increase in size of the thymus, the cellular structure is essentially similar to that seen in Fig. 6.7. (x =2000, captions as in Fig. 6.7).

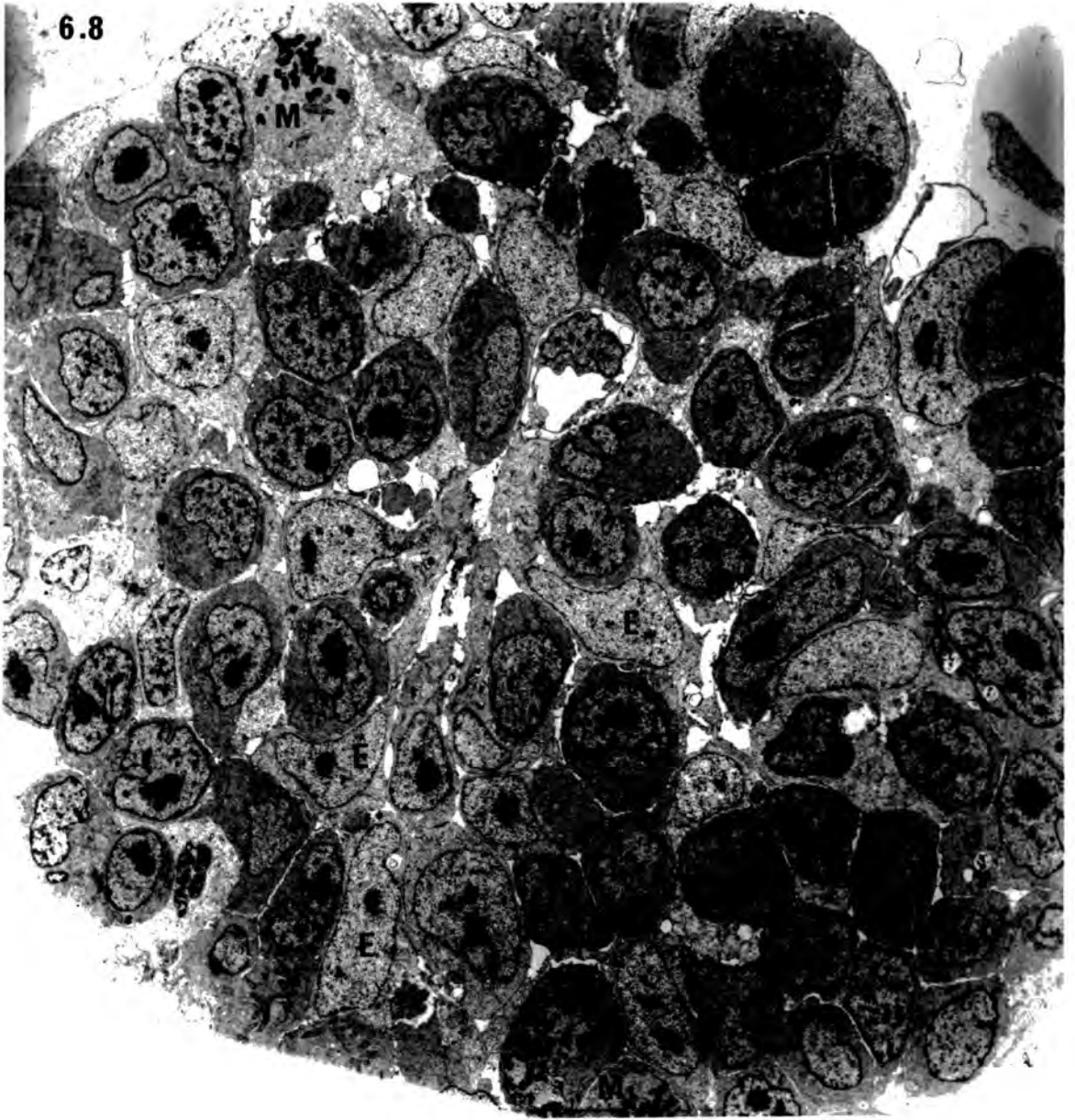


Fig. 6.9. Electron micrograph of an epithelial cell (E) in the 8 day larval thymus. Nu = nucleolus; RER = rough endoplasmic reticulum; M = mitochondria; J = junction between two adjacent epithelial cells, note desmosomes (arrowed) on either side of an intercellular canal. Large arrows indicate cytoplasmic extensions of the epithelial cell. (x8,000).

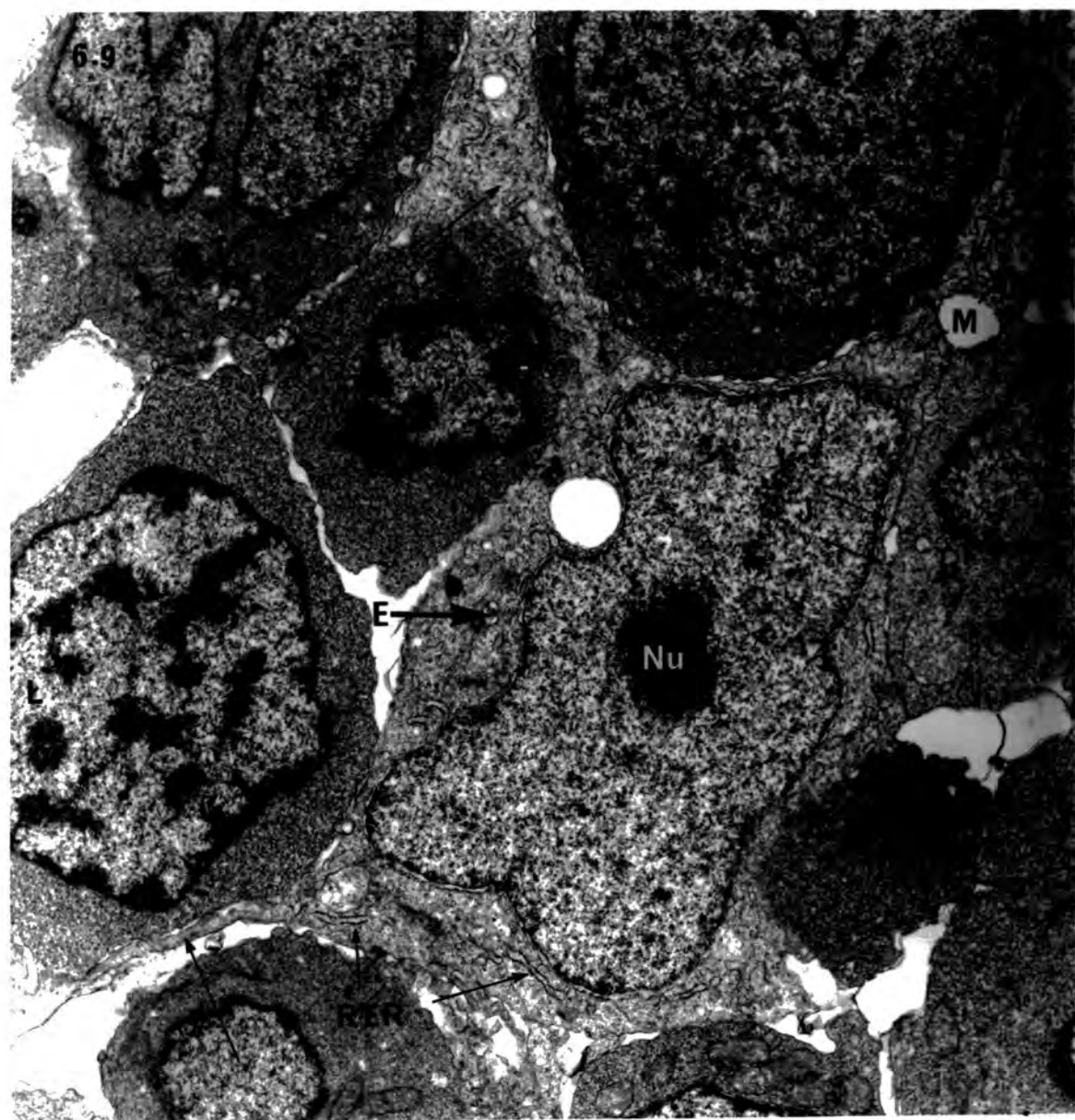
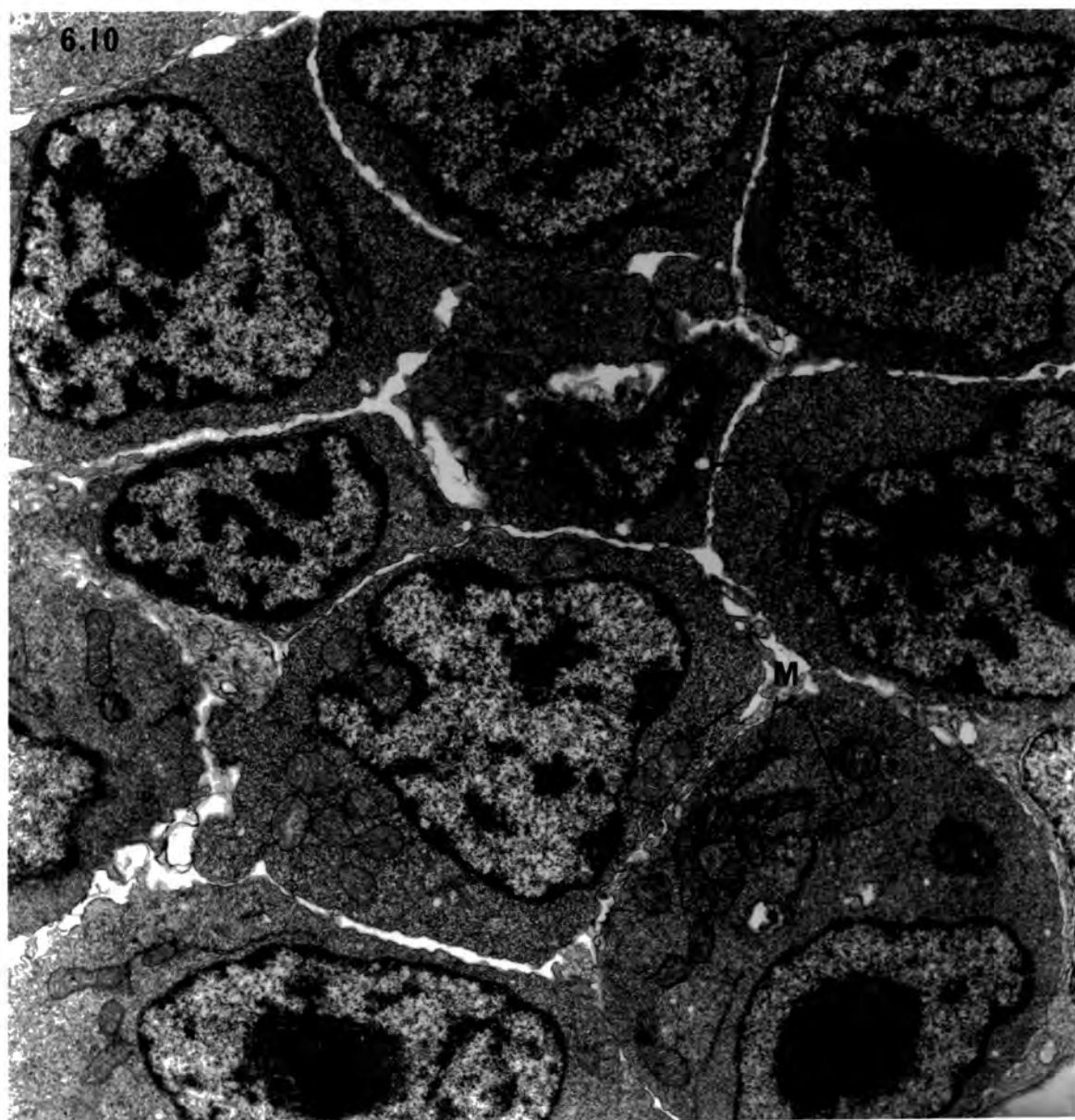


Fig. 6.10. Lymphoid cells of 8 day larval thymus. Note large amount of cytoplasm (cf Fig. 6.3) and free ribosomal arrangement. M = mitochondria; P = pinocytotic vacuole. (x 8,000).



Studies in this thesis have confirmed that early in life the thymus plays a vital role in the establishment of normal alloimmune reactivity and have shown, for the first time, that the spleen is an important centre of alloimmune reactivity in the intact amphibian. Although the detailed mechanism of transplant destruction in Xenopus still awaits clarification, the fact that early thymectomized larvae and adults can still reject first-set skin allografts in chronic fashion and display alloimmune memory offers insight into this issue. Thus the absence of other classical T-cell functions in the thymectomized toad (e.g. lack of M.L.R. or P.H.A. reactivity, DuPasquier and Horton, 1976) suggests that antibody may play a vital role in graft rejection in these animals and may also be of importance in control Xenopus. In vitro experiments employing several mammalian species (see Carpenter, 1974; MacLennan, 1973; MacLennan and Harding, 1974) have demonstrated that, in control animals, there exists a mechanism of antibody dependent lymphocyte mediated cytotoxicity. Thus a distinct sub-population of lymphoid cells (neither T- nor B- cells) has been isolated and shown to possess surface membrane receptors for the Fc region of IgG antibody. These so-called K cells are capable of killing target cells sensitized in vitro with IgG antibodies, and according to Valdimarsson (1975), the role of this system in vivo has recently been demonstrated in nude mice, where passive transfer of specific alloantibodies to skin allografts results in rejection. The development of sensitive cytotoxicity assays (eg  $^{51}\text{Cr}$  release) for use with amphibians is necessary to determine whether or not a similar mechanism can be identified in control and thymectomized Xenopus. Studies on the larva would be particularly informative since their antibody response following antigenic challenge is almost entirely IgM (DuPasquier and Haimovitch, 1976). In mammals serum antibody levels rise following rejection of first-set allografts (Roitt, 1974) and indeed low levels of alloantibody

to donor red cells (which are known to carry histocompatibility antigens DuPasquier and Chardonnens, 1975) can be demonstrated following skin-allografting in Xenopus. (Horton, pers. comm.). Such alloantibody production has not been identified in thymectomized individuals even after repeated injections of allogeneic red-cells (DuPasquier, unpublished observations). The origin of cells or factors involved in graft rejection in thymectomized Xenopus also remains unresolved, but the present studies have indicated that the spleen appears not to be involved in allograft destruction in early thymectomized animals.

The persistence of an alloimmune response in the thymectomized toad is intriguing, particularly since in endotherms it has become almost axiomatic that there is no graft rejection in the absence of the thymus. Recently however, a number of experimental studies have brought into question the rigorous thymus-dependent nature of alloimmune responses. Thus in the Quokka, a marsupial, removal of the thymus prior to the appearance of small lymphocytes does not impair the subsequent destruction of skin allografts (Ashman et al., 1975). Even more remarkable, in some respects, is the case of the sheep (reviewed by Morris, 1973) where thymectomy in utero followed by treatment with anti-lymphocyte serum (A.L.S.), which eliminates most, if not all, residual peripheral lymphocytes, fails to affect the development of a whole range of immune responses, including skin-allograft rejection. Thus, following the spontaneous re-appearance of a population of lymphoid cells after birth, these T-deprived lambs subsequently acquire full immunological competence. If, as Morris argues, these animals do completely lack T-lymphocytes, then this would appear to establish a principle of an alternative (non-thymic) pathway for the development of immunological competence. In mammalian experiments of this kind, however, it is difficult to assess the importance of maternal influences on the development of immunological competence in the offspring. Interestingly, Bryant (1974) has proposed that there may also exist an

alternative bursa-independent pathway of B-cell development in hormonally-bursectomized chickens.

In thymectomized Xenopus, the term "alternative pathway" is perhaps not strictly applicable, in view of the abrogation or severe impairment of certain T-dependent in vitro responses (Donnelly et al, 1976; DuPasquier and Horton, 1976; Manning et al., 1976). Thus in anuran amphibians, there may exist a thymus-independent component, which normally plays a role in graft destruction in the intact animal, but whose function is usually masked by the action of other, more vigorous T-dependent elements. From a phylogenetic viewpoint, it is worth mentioning that the capacity for immune recognition and chronic destruction of foreign tissue with concomitant memory function (c.f. thymectomized Xenopus) arose prior to the evolutionary appearance of the thymus, (e.g. annelids, echinoderms and hagfish - see Chapter 1). In both invertebrates and vertebrates, the thymus does not appear to provide a unique environment for the differentiation of cells (and or serum factors) capable of effecting alloimmune responses. Perhaps the primary lymphopoietic sites (i.e. thymus and bursa or its 'equivalent') of higher animals serve to exert an 'amplificatory function' on pre-existing 'T'- and 'B'- equivalent pathways, which have their phylogenetic origins in more diffuse haemopoietic sites.

The allograft reactivity displayed by thymectomized toads suggests that the amphibian thymus is not a unique source of lymphoid cells. This suggestion is strengthened further by the findings that the thymectomized animal develops relatively normal lymphoid tissues (Manning, 1971; Horton and Manning, 1974a) and a substantial population of lymphocytes bearing surface Ig (Weiss et al., 1973). Furthermore, in vivo (Collie et al., 1975; Tochinali, 1976) and in vitro (Manning et al., 1976) responses to thymus independent antigens and B-cell mitogens remain unimpaired in the thymectomized toad, even following thymectomy at 4 days of age (Tochinali, 1976).

However, the complete absence of induced reactivity to heterologous red cells in early thymectomized animals demonstrated in this thesis, along with abrogation of other in vivo and in vitro immune responses (Horton and Manning, 1974b; Manning, 1975; Turner and Manning, 1974; DuPasquier and Horton, 1976, Manning et al., 1976), indicates that the thymus is a major source of immunocompetent cells in Xenopus.

The origin of lymphocytes in amphibians is currently under debate. It has been suggested (Turpen et al., 1973; 1975; Turpen and Cohen, 1976) from experiments involving the exchange of thymic primordia between diploid and triploid embryos of the leopard frog Rana pipiens, that all lymphocytes (both T and B cells) originate from the thymus. Thymectomy experiments in Xenopus may reflect that patterns of lymphocyte origin and maturation differ in various amphibian species. Whether or not such variability exists in endotherms is unknown: however these amphibian studies stress the importance of studying diverse animal types in trying to understand the main themes of immune ontogeny. Experimental evidence from birds and mammals (see Greaves et al., 1973) has given rise to the concept that a proportion of yolk-sac derived stem cells migrate to the thymus and, following numerical expansion and 'education' in this organ, become non-Ig-secreting T lymphocytes. The latter are characterized by specific surface markers (e.g.  $\Theta$ ); a variety of recognition and effector functions (e.g. helper, 'suppressor, killer); and by other physiological properties such as their ecotaxis to T-dependent regions of peripheral lymphoid organs. Other yolk-sac derived stem cells are believed to migrate to the bursa of Fabricius (in birds) or 'bursal-equivalent' sites in mammals (e.g. foetal liver, spleen, bone marrow in mice). Here they become 'B lymphocytes' which characteristically possess readily-detectable surface Ig and upon stimulation by antigens (or polyclonal B-cell activators e.g. L.P.S.) give rise to antibody secreting cells. A variety of T-cell factors are also thought to be involved in triggering B-lymphocytes. As with T-cells there

also exists a marked degree of heterogeneity amongst the B-lymphocyte sub-population (see Transplantation Reviews, Vols 24 and 25).

In endotherms, the ability of T-lymphocytes to modulate B-lymphocyte function has been well documented: T-cells are now known to play a major role in the triggering and differentiation of B lymphocytes, and are particularly involved in promoting the IgG response to thymus-dependent antigens. T-B cell cooperation involves interaction between both populations of lymphocytes in addition to macrophages (see Greaves et al., 1973). The existence of cooperating lymphocyte populations in amphibians has been suggested on the basis of a); studies involving hapten-carrier systems (see Ruben, 1976) and b); the demonstration of T-dependent and T-independent immune responses (discussed in Chapter 4). Characterization of 'T' and 'B' lymphocyte populations in Amphibia however, and the allocation of exclusive 'helper' or antibody producing function to either is hampered by a number of experimental observations. Thus thymus cells of larval anurans, which in higher animals would be automatically classed as T-cells, possess readily demonstrable surface Ig (DuPasquier et al., 1972; DuPasquier and Weiss, 1973; Jurd and Stevenson, 1976). This finding, taken together with the demonstration of PFC in the thymus of larval and adult bullfrogs (Moticka et al., 1973; Minagawa et al., 1976), and the fact that both IgG and IgM responses to H.G.G. - a thymus-dependent antigen (Turner and Manning, 1974) are abrogated in thymectomized Xenopus; has been taken as evidence that in Amphibia, the thymus may produce, in addition to T-cells, antibody producing cells and/or their precursors (see Cooper 1973; 1976a; 1976b;). In the light of these observations, it is difficult to maintain that the abrogation of the humoral response to SRBC following larval thymectomy is not a consequence of the destruction of B-cell precursors, rather than the result of the removal of a helper T-population as might be supposed by analogy with mammalian studies.

Although there is still some controversy as to the nature of T-cell

receptors in mammals, recent reports have hinted that mammalian T-cells may possess surface Ig (see Marchalonis, 1975). Readily demonstrable surface Ig on thymocytes has been shown to be a characteristic feature in adult fishes (Ellis and Parkhouse, 1975) and urodeles (Charlemagne and Tournefier, 1976). It is conceivable therefore, that the presence of readily detectable surface Ig on thymocytes may represent a primitive condition and that surface Ig becomes vestigial later in development (e.g. anuran amphibians) or even lost in T-cells of higher animals (see Burnet, 1976). The Ig-bearing thymocytes of amphibians may therefore represent phylogenetically primitive or ontogenetically immature T-lymphocytes rather than B-cells or their precursors. Similarly, the presence of antibody forming cells in bullfrog thymus does not preclude a non-thymic origin for these cells.

The work presented in this thesis indicates that further study on the role of the amphibian thymus in antibody production is essential to determine the precise nature (i.e. helper and/or antibody production) of its involvement in humoral responses. Two major lines of research might be envisaged. In the first of these it should be possible to develop an 'adoptive transfer system' approach (Mitchison, 1957; Miller and Mitchell, 1969) to the reconstitution of the humoral immune response of animals rendered immunologically unresponsive by thymectomy and/or whole body irradiation. Such work would of course require the use of mutually tolerant or isogenetic Xenopus. The restorative efficacy of cell inocula from the thymus at different stages of its development (T-cells?) and/or (since there is only restricted haemopoietic activity in the bone marrow) spleen cells from thymectomized animals (T-independent cells?) could be tested using the haemolytic plaque assay. Techniques involving the use of anti-allotype sera (DuPasquier and Wabl, unpublished observations) and karyotypic markers could then be employed to determine the origin of the antibody-forming cells. The second approach would involve the

development of in vitro techniques for the induction of antibody synthesis. In this respect, Auerbach and Ruben (1970) have demonstrated the feasibility of such an approach using cultured spleen fragments. It should be possible therefore, to develop a 'Mishell-Dutton type' assay system to test the antibody-forming potential of putative T- and B-cells. Taken in conjunction with the experiments presented here on thymectomy at various stages of development, such studies should provide further insight into the phylogeny and ontogeny of cellular co-operation in the humoral immune response.

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