Durham E-Theses

Studies on the thymus and ontogeny of lymphocyte heterogeneity in the clawed toad Xenopus laevis (Daudin)

Williams, Nigel Howard

How to cite:

Williams, Nigel Howard (1981) *Studies on the thymus and ontogeny of lymphocyte heterogeneity in the clawed toad Xenopus laevis (Daudin)*, Durham theses, Durham University. Available at Durham E-Theses Online: http://etheses.dur.ac.uk/7834/

Use policy

The full-text may be used and/or reproduced, and given to third parties in any format or medium, without prior permission or charge, for personal research or study, educational, or not-for-profit purposes provided that:

- a full bibliographic reference is made to the original source
- a link is made to the metadata record in Durham E-Theses
- the full-text is not changed in any way

The full-text must not be sold in any format or medium without the formal permission of the copyright holders.

Please consult the full Durham E-Theses policy for further details.

Academic Support Office, The Palatine Centre, Durham University, Stockton Road, Durham, DH1 3LE
e-mail: e-theses.admin@durham.ac.uk Tel: +44 0191 334 6107
http://etheses.dur.ac.uk

Studies on the thymus and ontogeny of lymphocyte heterogeneity in the clawed toad *Xenopus laevis* (Daudin)

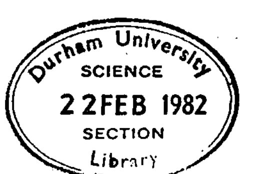
by

Nigel Howard Williams
B.Sc. (Dunelm)

The copyright of this thesis rests with the author. No quotation from it should be published without his prior written consent and information derived from it should be acknowledged.

.... being a thesis presented in candidature for the degree of Doctor of Philosophy in the University of Durham.

November
1981



Thesis
1981/WIL

CONTENTS

Page

Abstract (iii)

CHAPTER 1: General Introduction 1

CHAPTER 2: Lymphocyte reactivity to 'T' and 'B' cell mitogens: Studies on thymus and spleen of the adult. 12

Introduction 12
Materials and Methods 14
Results 17
(a) ^{3}HTdR incorporation and viability of lymphocytes in mitogen-free cultures
(b) Proliferative responses of thymocytes and splenocytes to T and B cell mitogens
Discussion 20
Tables 26
Figures 31

CHAPTER 3: Ontogeny of mitogen responsiveness in thymus and spleen 32

Introduction 32
Materials and Methods 33
Results 37
(a) Preliminary studies comparing miniaturised and standard culture techniques 37
(b) Ontogeny of mitogen responsiveness 38
(c) Characterisation of thymocyte mitogen responses 40
(i) Autoradiographic evidence of T and B mitogen stimulation 40
(ii) The effect of nylon wool treatment on T and B mitogen stimulation 41
Discussion 42
Tables 50
Figures 61

CHAPTER 4: Mitogen and mixed lymphocyte culture responses following thymectomy: studies investigating putative T lymphocyte heterogeneity 71

Introduction 71
Materials and Methods 73
Results 76
(a) Preliminary studies on mitogen stimulation of PBL 76
(b) Mitogen studies on lymphocytes from allo-immune toadlets 77
(c) MLC and mitogen studies on lymphocytes from toadlets thymectomixed at 3-4 weeks of age 78
Discussion 78
Tables 83
Figures 87

Page

CHAPTER 5: Ontogeny of *in vivo* lymphocyte reactivity to the hapten trinitrophenyl conjugated to a thymus-dependent and thymus-independent carrier 89

Introduction 89
Materials and Methods 91
Results 95
(a) Ontogeny of induced splenocyte PFC reactivity 95
(b) Ontogeny of induced antigen-binding cells in the spleen 96
(c) Antibody forming cells within the thymus 98
Discussion 98
Tables 104
Figures 110

CHAPTER 6: Thymus histogenesis over metamorphosis and in organ culture 113

Introduction 113
Materials and Methods 114
Results 117
(a) Thymus morphology 117
(b) Organ culture experiments 119
Discussion 120
Tables 123
Figures 124

CHAPTER 7: Concluding remarks 132

Acknowledgements 138

Bibliography 139

DECLARATION

Some of the work presented in Chapters 2,3 and 4 form the basis of four publications*. Assistance of collaborators in Chapters 2 and 4 is gratefully acknowledged.

*(i) Horton, J.D., Smith, A.R., Williams, N.H., Smith, A., and Sherif, N.E.H.S. (1980). "Lymphocyte reactivity to 'T' and 'B' cell mitogens in _Xenopus laevis_: studies on thymus and spleen". Devel. Comp. Immunol. _4_: 75-86.

(ii) Horton, J.D., Smith, A.R., Williams, N.H., Edwards, B.F. and Ruben, L.N. (1980). "B-equivalent lymphocyte development in the amphibian thymus?". In: Development and Differentiation of Vertebrate Lymphocytes, Ed. J.D. Horton. Elsevier/Amsterdam. pp. 173-182.

(iii) Smith, A.R., Williams, N.H. and Horton, J,D. (1980). "_In vitro_ reactivity of _Xenopus_ lymphocytes to T cell mitogens following allograft rejection and to sheep erythrocytes after _in vivo_ priming". In: Phylogeny of Immunological Memory, Ed. M.J. Manning. Elsevier/Amsterdam. pp. 197-205.

(iv) Williams, N.H. and Horton, J.D. (1981). "Ontogeny of mitogen responsiveness in thymus and spleen of _Xenopus laevis_". In: "Aspects of Developmental and Comparative Immunology I" Ed. J.B. Solomon. Pergamon: Oxford. pp.493-494.

STATEMENT OF COPYRIGHT

"The copyright of this thesis rests with the author. No quotation from it should be published without the prior written consent and information derived from it should be acknowledged".

ABSTRACT

(1) Functionally mature lymphocytes can be demonstrated *in vitro* by their proliferation (measured by scintillation counting) in response to mitogens. Studies in Chapter 2 examine the responsiveness of adult *Xenopus* lymphocytes from both the thymus and the spleen to B and T cell mitogens. Optimum mitogen doses and levels of foetal calf serum supplementation are established. Good stimulation indices are found when splenocytes are stimulated with both PHA and Con A. Thymocytes also respond well to these T cell mitogens, but a small response to B cell mitogens (*E. coli* LPS and PPD) is also found.

(2) The ontogeny of lymphocyte responsiveness to T and B cell mitogens is examined in Chapter 3. Very little stimulation is found in the larval thymus, but shortly after the end of metamorphosis a thymocyte response begins to emerge and by 6 months of age good stimulation is found with both T *and* B cell mitogens. While good levels of thymocyte stimulation with the T cell mitogens are maintained in later life, the response to the B cell mitogen LPS declines to only a low level by 12 months of age. Mitogenic responses emerge in the spleen after the end of metamorphosis in a similar manner to thymocytes and good responses were found in all the subsequent ages tested. The transient response of thymic cells to LPS, which is maximal at 6 months of age, was confirmed by auto-radiography and was shown to be dependent on a population of nylon-wool adherent lymphocytes.

(3) The larval thymus plays a critical role in the establishment of T cell functions, and Chapter 4 looks at the effect of thymectomy at different stages of larval development on the subsequent alloimmune response, mixed lymphocyte reactivity and T cell mitogen responsiveness. The results reveal that (a) skin allograft rejection displayed by 7-day thymectomized animals is effected in the absence of spleen and

blood lymphocytes reactive to T cell mitogens, (b) T-mitogen responsive cells and MLR T cells cannot easily be separated into distinct populations in terms of their degree of thymus dependency.

(4) Chapter 5 looks at the ontogeny of both T helper cell and B cell function by examining the in vivo antigen-binding reactivity of splenocytes to a T-dependent (TNP-SRBC) and a T-independent (TNP-LPS) antigen. Good levels of TNP binding cells can be induced in the spleen when larvae are only 3 weeks old following injection of TNP-SRBC (after low dose priming with SRBC) or TNP-LPS. The level of antigen binding in both cases appears to peak at metamorphosis. However, when cellular antibody production was measured, no plaque forming cells could be induced in the spleen until after the end of metamorphosis.

(5) Chapter 6 presents (a) a brief histological study of the thymus in late larval life and at metamorphosis. This work characterises the dramatic depletion of thymocyte numbers that occurs at the end of metamorphosis and the renewed lymphopoiesis immediately afterwards, and (b) preliminary results on the effect of 7-day organ culture on thymus histogenesis.

CHAPTER 1

General Introduction

Thymus function and lymphocyte heterogeneity in birds and mammals

It is now beyond question that the thymus is of central immunological importance (Miller, 1979). The first major breakthrough that clearly linked the thymus with the development of the immune system came in 1961, when Miller published his findings on the effects of thymectomy in the newborn mouse. He revealed that neonatal thymectomy (but not adult thymectomy) was associated with reduced lymphocyte numbers in blood and lymphoid tissues and abrogated the ability of the mouse to reject skin allografts. In the next few years it became apparent that neonatal thymectomy in mammals not only affected cellular immunity, but also impaired antibody responses to some antigens (Miller, 1962, 1963). It was realised later that it was only the performance, not the level of antibody-forming cells which was altered by neonatal thymectomy (see discussion in Miller, 1979).

In birds it became clear early on that two separate 'central' lymphoid organs were responsible for establishing humoral and cellular immunity. Thus neonatal removal of the bursa of Fabricius (a cloacal lymphoid organ found only in birds) impaired antibody production but not allograft rejection in chickens, whereas early thymectomy impaired alloimmune reactivity but had only variable effects on antibody production (Glick, 1956; Szenberg and Warner, 1962; Cooper, Peterson, South and Good, 1966). The search for a mammalian equivalent of the avian bursa involved protracted investigations (see Greaves, Owen and Raff, 1973, for discussion). It now appears that the foetal liver and spleen are centrally involved in production of antibody-forming cell precursors in the

Durham University SCIENCE 22 FEB 1982 SECTION Library

mammalian embryo (Owen, Raff and Cooper, 1975), the bone marrow assuming this role in later life.

These extirpation experiments, backed up with observations on mammals with congenital immunodeficient states (such as those on the nude mouse, with its hypoplastic, non-lymphoid thymus - see Pantelouris, 1971, 1973; Rygaard, 1973) gave rise to the concept of thymus-dependent (T) and bursa-(or mammalian bursal-equivalent)-dependent (B) lymphocytes for the 2 ontogenically-distinct cellular populations found in the 'peripheral' lymphoid tissues (e.g. lymph nodes). T and B lymphocytes are clearly also functionally distinct populations; thus T cells, were shown to be centrally involved in cellular immunity, while B lymphocytes gave rise to antibody-producing cell precursors.

Why the thymus should play a role in antibody production to certain antigens (called 'thymus-dependent') remained a mystery until the late '60s. Then Claman, Chaperon and Triplett (1966) revealed that cells from mouse bone marrow and thymus (each independently capable of very little antibody production) could, when injected together into irradiated recipients, act synergistically to give a good antibody response when challenged with foreign erythrocytes. Moreover, all the antibody in this type of experiment was shown to be produced by injected bone marrow cells (Davies, Leuchars, Walls, Marchant and Elliott, 1967; Mitchell and Miller, 1968). It appeared that the T lymphocytes somehow 'helped' bone marrow derived cells to secrete antibody. The other line of evidence showing T/B cell cooperation came from hapten-carrier experiments. A hapten is a small, chemically defined substance (e.g. trinitrophenyl (TNP)) which can combine

specifically with antibody, but cannot by itself induce an immune response; that is it is antigenic but not immunogenic. To induce an immune response a hapten must be coupled to a macromolecular carrier to form an immunogenic complex. To obtain a good restoration of secondary anti-hapten IgG antibody response in irradiated mice, it was necessary to have present two separate lymphocyte populations, one responsive to the carrier and one to the hapten (Mitchison, 1971). Carrier-sensitive cells were subsequently shown to be thymus-derived, whereas hapten-sensitive lymphocytes were bone marrow-derived (Raff, 1970). The precise mechanism of T cell help is still under debate, but it undoubtedly involves one or more signals from the T lymphocyte that triggers the B cell directly or indirectly via macrophages (see Miller, 1979).

In more recent years, extensive research has shown that mammalian effector T cells (unlike B cells, which seem functionally fairly homogeneous) are a functionally heterogeneous family of lymphocytes. Cytotoxic (Miller, Brunner, Sprent, Russell and Mitchell, 1971), helper and suppressor (Gershon and Kondo, 1972) T cell sub-populations, as well as those involved in delayed-type hypersensitivity reactions, have been identified and, furthermore, characterised by both their surface differentiation antigens (e.g. the Ly antigens described by Cantor and Boyse, 1977) and by their expression of antigens encoded by the major histocompatibility gene complex (MHC). It is now beginning to be appreciated that MHC antigens play an important role in T lymphocyte reactivity. Thus in inbred rodents, and possibly also in humans and chickens, reactivity of helper and cytotoxic T cells are believed to be restricted by the MHC. It has been known for some time that MHC

antigens are molecular markers of individuality: thus when transplants are performed between MHC-disparate individuals, graft rejection will occur rapidly, in contrast to the much better transplant survival if MHC identity exists between donor and host. The more recent finding is that the MHC is also of vital importance in T cell recognition of foreign antigens where the MHC is not serving as the immunising difference (Miller, 1979). Thus T helper cell collaboration with resting B lymphocytes will, in general, occur only if these lymphocytes express the same, lymphocyte-defined MHC antigens (Katz and Benacerraf, 1975; Andersson, Schreier and Melchers, 1980), and T cell-mediated cytotoxicity specific for virus-altered (Zinkernagel and Doherty, 1974), or hapten-modified cells (Shearer, Rehn and Garbarino, 1975) requires serologically-defined MHC identity of T lymphocytes with altered target cells. Although it has seemed likely from mouse chimera studies that it is within the thymus that T cells learn to become MHC restricted during development (Zinkernagel, 1978), recent experiments suggest that this 'learning' within the thymus may not alone be responsible for determining the range of MHC restriction (see Howard, 1980).

Concomitant with the discoveries of the functional complexity of the thymus-dependent lymphocyte population in mammals, and the possible role of the thymus in MHC restriction, have been investigations to determine the precise role that the thymus plays in T lymphocyte development and differentiation (see review by Jordan and Robinson, 1981 and by Owen et al. 1977). It has become apparent that the thymus does not play a 'static' role in this context but, rather,

is involved in a precise ontogenetic sequence. Early work by Moore and Owen (1967) on parabiosed chick embryos showed that there was an input of haemopoietic stem cells (T and B lymphocytes are believed to derive from a common stem cell source) into the embryonic thymus. Owen and Ritter (1969) showed (using an organ culture technique) that stem cell inflow to the thymus begins at a precise time. Thus explants of 6-day chicken thymuses failed to become lymphoid _in vitro_, whereas 7-day old thymuses displayed lymphocyte development when placed in culture. The quail-chick chimera system has subsequently proved valuable in analysing the brief periods of development when the thymus becomes attractive for stem cells (see Le Douarin, 1977). After entering the thymus, stem cells proliferate in the thymic cortex and then lymphocytes migrate towards the medulla (Borum, 1968). Lymphocytes are thought to leave the thymus through the medullary venules (Saint-Marie, 1973). It has been suggested that intrathymic MHC restriction of mammalian T cells is dependent on contact of thymus lymphocyte with the thymic epithelium during ontogeny, and is determined by the haplotype of the thymic epithelial cells rather than the thymus-lymphocytes themselves (Zinkernagel, 1978). This restricting property of thymus epithelial cells is believed to extend through ontogeny at least until juvenile stages (see Triesman, 1981). Organ culture studies on the thymus (see reviews by Robinson, 1980; Robinson and Owen, 1976, 1977; Mandel and Kennedy, 1978) and sequential thymectomy experiments (Shimamoto, 1980) reveal that there is a sequential emergence of T lymphocyte populations in mammals.

Phylogeny of T and B equivalent lymphocytes

The central importance of ontogenetic events in the establishment of the immune system in mammals and birds makes the study of the

phylogeny of the immune system particularly important. The relationship between the development of the individual and the evolution of species and lineages has been an enduring question in evolutionary biology. Ontogeny recapitulates phylogeny was Haekel's answer in the nineteenth century (see Gould, 1978). Although a belief in recapitulation is no longer tenable, further understanding of the relationship between these two phenomena is of continuing interest. Heterochronic differences in the ontogeny of diverse immune systems highlight the importance of phylogenetic study, of not only vertebrates but invertebrates too. Developmental and comparative immunology is now a well established area of research, and several symposia (see Hildemann and Benedict, 1975; Wright and Cooper, 1976; Solomon and Horton, 1977; Horton, 1980; Manning, 1980; Solomon, 1981), review articles (Du Pasquier, 1973, 1976; Borysenko, 1976; Cohen, 1977) and books (Manning and Turner, 1976; Cooper, 1976; Gershwin and Cooper, 1978; Marchalonis, 1977), have defined the central issues of this research field. Primordial cell-mediated immunity, characterised by cytotoxic reactivity following allograft sensitisation and by specifically inducible memory, has been demonstrated in parazoans, coelenterates, annelids and echinoderms. Furthermore, mixed lymphocyte culture (MLC) reactivity has been demonstrated in tunicates. These findings suggest that immunocytes functionally-equivalent to certain mammalian T lymphocytes can exist in the absence of a thymus. Thus, although invertebrate immunocytes often develop within special cellular aggregations (such as the axial organ of echinoderms and the gill-associated nodules of tunicates), no true thymus exists in any backboneless animal. Although humoral factors are known to augment phagocytosis in many

invertebrates, no immunoglobulin (Ig) molecules have yet been found.

Functionally-equivalent T and B cells, specific immunological memory and Ig's exist in all vertebrates, including agnathan fishes which are the only vertebrates where there is no convincing evidence for the presence of a thymus. (Good, Finstad, Pollara and Gabrielsen, (1966) have described an aggregation of lymphocytes in the larval lamprey which they think may be a protothymus, and Riviera, Cooper, Reddy and Hildemann (1975) suggest the presence of a protothymus within the velar muscle complex of the Pacific hagfish). T cell-equivalent functions, such as acute graft rejection and strong MLC reactions, are two of the functional assays associated with disparity at the MHC. The presence of these reactions in 'advanced' amphibians (anurans), 'advanced' fishes (teleosts), birds ('advanced' reptiles?) and mammals, but their absence in 'primitive' fishes (e.g. agnathans, chondrichthyans and chondrosteans), 'primitive' amphibians (urodeles and apodans) and reptiles, has led Cohen and Collins (1977) to suggest that the MHC may have evolved independently on four separate occasions during vertebrate phylogeny, and may reflect convergent gene evolution. It seems likely, however, that components of the MHC also exist in some of the 'primitive' forms, but allelic polymorphism may be minimal in these.

The functional dissociation of the ectothermic immune system into heterogeneous populations of lymphocytes has been a major consideration of comparative immunology, and a variety of techniques have been used for this purpose. Differential mitogenesis has been of importance in this respect. In mammals different mitogens specifically stimulate either T or B lymphocytes and studies using

these mitogens have now been performed in all vertebrate groups (see Chapter 2 for review). A second potent method for study of lymphocyte heterogeneity in poikilotherms has involved the use of hapten-carrier experiments (see Chapter 5 for review). Lymphocyte cooperation (suggesting at least two different lymphoid populations) in humoral immune responses has been suggested in both teleost fish and amphibians, but seems to be absent in agnathans (Fujii, Nakagawa and Murakawa, 1979).

The extent to which immune maturation is dependent upon the thymus throughout the vertberates has been examined in thymus ablation experiments. Thymectomy in the teleost fish, the Mozambique mouth brooder (Sailendri, 1973) reveals a slow ontogeny of T-dependent immune functions in this species. Thymectomy of 4 month old fish still caused extended survival of scale allografts, while at 2 months it suppressed humoral immune reactivity to subsequent challenge with sheep erythrocytes. The effect of thymectomy on T-independent antigens was not examined by Sailendri, so we cannot conclude that impaired humoral immunity is due to deletion of T cells. It is possible that both T and B cells are spawned in the thymus of some fish (see Chapter 2). Adult thymectomy has no effect on the allograft response of rainbow trout (Botham, Grace and Manning, 1980), as in the lizard, _Calotes versicolor_ (Manickavel, 1972), although a vital role of the reptilian thymus in lymphocyte maturation is suggested by the use of anti-[illegible]-thymocyte serum in this species, which results in a profound lymphopoenia in thymus (and spleen) and suppresses allograft rejection.

Amongst the ectothermic vertebrates, it is the amphibian class, in particular the anuran order, which has received most attention with respect to the effect of thymectomy on immune maturation. The thymus is clearly the major source of graft rejection cells in both urodeles (Charlemagne and Houillon, 1968; Cohen, 1969, 1970; Tournefier, 1972, 1973; Charlemagne, 1974; Fache and Charlemagne, 1975) and anurans (Cooper and Hildemann, 1965; Du Pasquier, 1965; Curtis and Volpe, 1971; Horton and Manning, 1972; Tochinai and Katagiri, 1975; Rimmer and Horton, 1977). Although the role of the anuran thymus in antibody production seems broadly comparable to that occurring in mammals (see below), the situation in urodeles seems more uncertain in this respect. Thus responses to mammalian T-dependent antigens are either minimally impaired (Tournefier and Charlemagne, 1975) or increased (Charlemagne and Tournefier, 1977) following thymectomy. Possibly humoral immune regulation in urodeles is not effected by helper T cells. Indeed it may be that urodeles recognise all antigens as T-independent (see Ruben and Edwards, 1980, for discussion).

The clawed toad (actually a pipid frog - see Deuchar, 1972), Xenopus laevis, has proved particularly useful in the study of functional dichotomy of the anuran immune system into T-dependent and T-independent components (see Cohen and Turpen, 1980, for review). Thymectomy early in life can easily be achieved in this species, which does not runt after the operation (Horton and Manning, 1974). It has been shown that early thymectomy impairs allograft rejection (Horton and Manning, 1972), abrogates humoral immune reactivity to

classical T-dependent antigens (Tochinai and Katagiri, 1975; Turner and Manning, 1974; Horton, Rimmer and Horton, 1976) and abolishes *in vitro* mixed leucocyte culture (MLC) reactivity (Du Pasquier and Horton, 1976) and responsiveness to the T cell mitogens phytohaemagglutinin (PHA) and Concanavalin A (Con A) (Du Pasquier and Horton, 1976; Manning, Donnelly and Cohen, 1976; Donnelly, Manning and Cohen, 1976), whereas it has no apparent effect on antibody production to classical T-independent antigens (Collie, Turner and Manning, 1975; Tochinai, 1976) or on *in vitro* lymphocyte stimulation by the putative B cell mitogens *E.coli* lipopolysaccharide (LPS) and purified protein derivative of tuberculin (PPD) (Manning et al., 1976). Horton and Sherif's (1977) sequential thymectomy studies have suggested the possibility of sequential establishment of different T cell subsets in *Xenopus* (see Chapter 4).

The work in this Thesis attempts to gain further insight into the development of thymus function and lymphocyte heterogeneity in *Xenopus*. Chapter 2 examines the culture conditions necessary for obtaining good mitogen responses in thymus and spleen to putative T and B cell mitogens, and forms the basis for work in the next Chapter. Chapter 3 investigates the ontogeny of mitogen reactive cells in thymus and spleen, to gain direct insight into the time when lymphocytes responsive to putative T and B cell mitogens first appear, and to follow this responsiveness over the immunologically-interesting period of metamorphosis. The possibility of T lymphocyte heterogeneity in *Xenopus* is assessed in thymectomy experiments performed in Chapter 4. This work centres on the *in vitro*

proliferative capabilities (mitogen and MLC reactivity) of lymphocytes taken from toadlets thymectomised in early or mid-larval life. Chapter 5 looks at the *in vivo* ontogeny of antigen-reactive T-and B-equivalent cells in the spleen with the aid of T-dependent and T-independent hapten-carrier complexes. As in Chapter 3, it is the larval and perimetamorphic periods which are the main focus of study. The ontogeny of splenic antibody-secreting cells, and the possibility of antigen production within the thymus are also briefly considered in Chapter 5. The penultimate Chapter is concerned with the histological changes occurring in the thymus over metamorphosis. It also examines the histological outcome of organ culturing the larval thymus. The major findings from the work in this Thesis are outlined in Chapter 7.

CHAPTER 2

Lymphocyte reactivity to 'T' and 'B' cell mitogens : Studies on Thymus and Spleen of the adult

Introduction

The differential responsiveness of lymphocytes to mitogens has been a major means of distinguishing T and B cells in mammals and birds Greaves and Janossy, 1972). The phytomitogens PHA, an extract of the red kidney bean *Phaseolus vulgaris* (Nowell, 1960), and Con A, an extract of the Jack bean *Canavalia ensiformis* (Powell and Leon, 1970), exclusively stimulate T cells. In contrast LPS, an extract of the bacterium *Escherichia coli*, and PPD induce proliferation exclusively in B cells. Pokeweed mitogen (PWM), an extract of the stem of *Phytolacca americana* (Borejeson *et al*., 1966), seems to stimulate both T and B lymphocytes.

The use of these mitogens in a variety of endothermic vertebrates has demonstrated the presence of both T- and B-mitogen responsive cells in the peripheral lymphoid system. In contrast, the thymus was shown to contain lymphocytes responsive only to T cell mitogens (Robinson and Owen, 1976) suggesting that this central lymphoid organ is exclusively a T cell domain. Furthermore, the importance of the thymus early in ontogeny for the subsequent establishment of functional T cells was confirmed by the absence of T mitogen responses in the periphery following early thymectomy.

The phylogeny of lymphocyte heterogeneity and, in particular, the role of the thymus in the establishment of functionally-distinct lymphocyte populations, is an important question in immunology.

Differential mitogenesis is a useful means of detecting lymphocytes functionally-equivalent to mammalian T and B cells throughout the vertebrates. Of the agnathan fishes, both the hagfish (Riviere et al., 1975) and lamprey (Cooper, 1971) possess peripheral lymphocytes that are stimulated by PHA. Amongst the elasmobranch fishes, nurse shark peripheral blood leucocytes (PBL) respond rather poorly to T cell mitogens. They undergo blastogenesis to Con A, but only when large amounts of mitogen (1mg/10^6 cells) are used, and the response to PHA is found only in a population of gradient-separated PBL (Lopez, Sigel and Lee, 1974; Sigel, Lee, McKinney and Lopez, 1978). It has recently been shown, however, that nurse shark PBL are stimulated by PHA, Con A and LPS to become cytotoxic to xenogeneic target cells (Pettey and McKinney, 1981). In the teleosts (Osteichthyes) responsiveness to both T and B mitogens can readily be demonstrated in the periphery. Thus lymphocytes from spleen and pronephros of the bream respond to PHA, Con A and LPS (Cuchens, McLean and Clem, 1976). These latter workers also found that the bream thymus contains lymphocytes reactive to both T and B mitogens (see also Cuchens and Clem, 1977). In contrast, the rainbow trout behaves more like the mammalian thymus in that it responds to Con A but not to LPS (Etlinger, Hodgins and Chiller, 1976; Chilmonczyk, 1978).

Recent studies on mitogen responsiveness in anuran amphibians have revealed similarities between poikilotherm and endotherm lymphoid systems, to the extent that normal mitogen reactivity of Xenopus lymphocytes to PHA and Con A in the periphery is dependent on the presence of the thymus early in development, in contrast to the thymic independence of LPS and PPD responsiveness (Du Pasquier and Horton, 1976; Manning, Donnelly and Cohen, 1976). The work in this

Chapter was designed to complement these studies by investigating the *in vitro* proliferative behaviour of adult *Xenopus* lymphocytes from the thymus to mammalian T and B cell mitogens. Additionally it compares thymocyte responses with those of lymphocytes from the spleen, the latter being the major peripheral lymphoid organ in this species. The experiments investigate optimal culture conditions, in particular mitogen concentration and level of serum supplementation - for mitogen reactivity of thymocytes and splenocytes.

Materials and Methods

(a) Rearing and care of animals

All animals used in this Thesis were bred and reared in the laboratory. Spawning was induced by injection of chorionic gonadotrophin (Xenopus Ltd.) into the dorsal lymph sac of male and female *Xenopus laevis* (Daudin). Animals mated overnight and the eggs laid were transferred to aerated, standing water and maintained at $23^{o}C \pm 2^{o}C$. After hatching, the tadpoles were distributed at a density of about 10 per tank (one per litre) and were fed on nettle powder twice a week up until metamorphosis, after which toadlets were kept 4 per tank and were fed on *Tubifex* worms and later on minced meat (liver or heart) twice a week. All animals used in this Chapter were aged 10-15 months.

(b) Lymphocyte culture

Thymus and spleen were removed from the animal aseptically in a laminar flow hood, following anaesthesia with MS 222 (Sandoz). Organs were transferred to watch glasses containing modified Leibovitz L-15 culture medium (Flow Laboratories). For each 58 cm^3 of L-15 medium the following additions were made:- 24 cm^3 double-distilled water, 1.6 cm^3 2mM $^{2-}$mercaptoethanol, 0.8 cm^3 1M Hepes buffer,

0.8 cm^3 penicillin/streptomycin (Flow), 0.5 cm^3 fungizone (Flow) and 0.5 cm^3 L-glutamine (Flow). This amphibian medium was sterilised by filtration through a 0.22 µm 'Millex' filter (Millipore). Tissues were teased apart under a steremicroscope using watchmaker's forceps. The teased tissues were then transferred to glass centrifuge tubes and the contents allowed to settle for a minute. The supernatant cell suspension, devoid of all large clumps, was washed twice (using a refrigerated centrifuge at 350 x g for 10 mins) and resuspended in medium. Lymphocytes were counted in a Neubauer American Optical counting chamber and their concentration adjusted to 5×10^6 cm^{-3}.

Triplicate lymphocyte cultures prepared from individual adults were set up in microtest plates (M25 ARTL, Sterilin), which have v-shaped wells. Forty µl cell suspension (2×10^5 lymphocytes) were distributed to each well. Ten µl mitogen (or medium in control cultures) and finally 10 µl heat-inactivated foetal calf serum (FCS, batch 427006, Flow) were then added (both mitogen and serum being freshly diluted with amphibian medium). The extent to which the level of serum supplementation affected lymphocyte viability and reactivity to various mitogens was examined:- FCS was added to give a final concentration of either 10% or 1%; serum-free cultures were also set up, the ten µl being replaced by 10 µl medium.

Plated cultures were placed in a humidified incubator at 28°C for 2 days. At this time the cultures were pulsed with 10 µl (1µCi) tritiated thymidine (^{3}HTdR, specific activity 5 Ci $mmol^{-1}$, Radiochemical Centre, Amersham). The cells were then incubated for a further 18-24 hours at 28°C before they were harvested with a Skatron cell harvester (water wash model, Flow). The glass fibre

discs containing the recovered cells from individual wells were placed into separate glass scintillation vials, air dried for 30 minutes, 0.5 cm^3 tissue solubilizer (Protosol, New England Nuclear) was then added to each vial and incubated at 55-60°C for 1 hour. Finally 5 cm^3 scintillation fluid (42 cm^3Liquifluor (NEN) per litre of toluene) was added. ^{3}HTdR incorporation was measured with a Nuclear Enterprise liquid scintillation counter.

Stimulation indices (S.I.'s) for each individual experiment were measured by dividing the mean counts per minute (cpm) for the triplicate cultures with mitogen added by the mean cpm obtained with control (no mitogen) cultures. Stimulation indices from, on average, 8 separate experiments provided each mean S.I. given in Figures 2.1-2.4. S.I.'s were considered positive only when they reached 1.5, since the mean ($\pm$ SE) coefficient of variation (SD/mean) in cpm for triplicate cultures was 23% $\pm$ 3.5%, the highest variation being 47%.

(c) Lymphocyte viability

The effect of FCS supplementation on lymphocyte viability was examined in 72 hour mitogen-free cultures. Cells from both thymus and spleen from 5 separate experiments were stained in 0.2% nigrosine for 10 minutes and the percentage of dye-excluding lymphocytes calculated from the mean of triplicate cultures.

(d) Mitogens

The following mitogens were used:- PHA M, Con A, *E.coli* LPS 055 :B5 (all from Difco), PPD 298 (Ministry of Agriculture, Fisheries and Food, Weybridge) and PWM (Flow Laboratories).

Results

(a) ^{3}HTdR incorporation and viability of lymphocytes in mitogen-free cultures.

The level of ^{3}HTdR uptake after 3 days in culture by thymocytes and splenocytes in the absence of mitogen is given in Table 2.1. Addition of 1% FCS to spleen and thymus cultures increased mean background cpm 2-3 fold when compared with serum-free cultures. A further 3-4 fold increase in cpm occurred when splenocytes were cultured with 10% FCS. In contrast, thymocytes in 10% FCS displayed only marginally elevated cpm when compared with 1% FCS cultures. Thymocyte background cpm were noticeably lower than splenocyte cpm for each FCS concentration. With respect to 0% and 1% serum supplementation, this could well be related to the poorer viability of thymus lymphocytes (35% and 55% viable respectively) when compared with spleen lymphocytes (65% and 79%) at 72 hours of culture. Ten per cent FCS supplementation resulted in improved viability of thymus lymphocytes (84%), directly comparable to spleen lymphocyte viability (86%). This equally good viability of lymphocytes from both thymus and spleen in 10% FCS does not explain the very high levels of ^{3}HTdR incorporation seen in the splenocyte cultures at this level of serum supplementation. This feature might be related to antigenic stimulation of spleen lymphocytes by FCS, a stimulation not readily apparent in thymus cultures. Or it could be that 10% FCS culture conditions are still not optimum for thymocyte proliferation.

(b) Proliferative responses of thymocytes and splenocytes to T and B cell mitogens

The dose-response curves (based on mean S.I.'s) of thymocytes and splenocytes to Con A, PHA and LPS are shown in Figs. 2.1-2.3

respectively. Table 2.1 shows details of cpm recorded in these experiments, but only for optimally-stimulated and mitogen-free cultures.

Con A: With 1% FCS supplementation the response of thymocytes and splenocytes to this mitogen was almost identical. Maximal S.I.'s of just over 12 were recorded with cells from both organs when 0.5μg cm^{-3} Con A was used. With 10% FCS, more mitogen (2.5μg cm^{-3}) was required to optimally stimulate splenocytes and thymocytes, and maximal S.I.'s were lower. The particularly low mean S.I. (4.3) found with splenocytes cultured in 10% FCS appeared to be primarily related to the high background counts under these serum conditions. Con A responses in serum-free media were not examined.

PHA: In contrast to Con A, where substantial stimulation occurred over only a narrow dose range of mitogen, a wider range of PHA concentrations effected good levels of proliferation. With 10% FCS supplementation, thymocytes and splenocytes displayed identical dose-response curves. For both cell types 40μg cm^{-3} PHA proved optimal, with mean S.I.'s of around 7. The maximal stimulation of thymic lymphocytes was unchanged when cultured in 1% FCS or in the absence of serum, although under these conditions 8μg cm^{-3} PHA also proved to be 'optimal'. Maximal stimulation of splenocytes cultured in 1% FCS or with no serum was almost doubled when compared with the 10% FCS spleen cultures. Forty μg cm^{-3} and 8μg cm^{-3} proved optimal for the 1% FCS cultures, whereas 8μg cm^{-3} achieved 'maximal' stimulation of serum-free cultures.

LPS: Splenocytes responded vigorously, especially in serum-free conditions to this B cell mitogen, displaying a mean maximal S.I. of 22 with the highest concentration of LPS used (2mg cm^{-3}). Addition of FCS resulted in a significantly lower maximal S.I. (7.4). In both serum-free and 1% FCS spleen cell cultures, the more LPS added, the greater the uptake of $^{3}HTdR$. Thymus lymphocytes of these 10-15 month old animals responded relatively poorly to LPS when compared with spleen cultures. In serum-free or 1% FCS conditions, mean maximal S.I.'s were only just greater than 2, with only 4/8 and 8/10 thymus cultures (respectively) displaying significant S.I.'s. However, with 10% FCS all 13 thymocyte cultures set up consistently displayed significant S.I.'s with optimal LPS concentration (1 mg cm^{-3}). The mean S.I. of these maximally-responding cultures was 4 (range 2.2 - 8.8).

The potential of thymus lymphocytes to respond to 2 other mitogens - PPD and PWM - was also briefly examined (see Fig. 2.4).

PPD: With 10% serum supplementation, the maximal mean S.I. (3.3) was attained with 1.0 mg cm^{-3} PPD, with only one culture failing to respond. The background mean cpm in these experiments was 811 $\pm$ 145, whereas cpm following PPD stimulation rose to 2,365 $\pm$ 270.

PWM: With 1% FCS supplementation the maximal mean S.I. of thymocytes was 4.6 when 25 µg cm^{-3} PWM was used. Background mean cpm was 790 $\pm$ 206 in contrast to the mean of 2631 $\pm$ 292

cpm recorded in the PWM stimulated cultures.

Combined PHA and LPS: In order to gather some circumstantial evidence that the T and B mitogens used here were, in fact, stimulating distinct populations of *Xenopus* lymphocytes, an additional experiment was carried out with splenocytes. Thymocytes were not used because of their relatively poor response to LPS. In this experiment, cultures from the same spleen were set up with (a) PHA (20 μg cm^{-3}) alone, (b) LPS (2 mg cm^{-3}) alone and (c) PHA and LPS combined in the same well. One % FCS supplementation was used. The results are shown in Table 2.2. The stimulations achieved when the two mitogens were combined in culture proved to be approximately that expected from adding the S.I.'s achieved by use of PHA or LPS in separate wells. This suggests that these two mitogens may well be stimulating separate splenocyte populations, i.e. T and B-equivalent cells respectively.

Discussion

The experiments have shown that both thymocytes and splenocytes from 10-15 month old *Xenopus* respond well to the T cell mitogens PHA and Con A, PHA having a broader dose-response curve than that of Con A. The proliferative response of cells from these 2 organs to Con A was virtually identical in terms of optimal mitogen dose, level of stimulation and effect of serum supplementation. PHA reactivity was also comparable in the two organs, although maximal stimulation of thymocytes was lower than that recorded in splenocyte cultures when serum-free or 1% FCS supplementation

conditions were used, i.e. under conditions that allow only poor thymocyte viability. In terms of mitogen reactivity, therefore, it appears that functionally-mature T-equivalent cells exist in good numbers in both organs in Xenopus.

Reactivity of anuran lymphocytes to these T cell mitogens has been examined in some detail (Bufo marinus, Goldshein and Cohen, 1972; Rana pipiens, Goldstine, Collins and Cohen, 1976; Xenopus laevis, Du Pasquier and Horton, 1976; Manning, Donnelly and Cohen, 1976; Donnelly, Manning and Cohen, 1976; Horton and Sherif, 1977; Green and Cohen, 1979). In terms of optimal mitogen dose, these studies are comparable with mammalian findings (see for example, Coutinho, Möller, Andersson and Bullock, 1973; Janossy and Greaves, 1971). The present experiments on Xenopus highlight the requirement for higher doses of mitogen to optimally stimulate lymphocytes when these are cultured with increasing levels of FCS. This finding is well characterised in mammals and is thought to be related to binding of mitogen to FCS proteins (see Coutinho et al., 1973). The finding of good responsiveness to T cell mitogens in the anuran thymus contrasts with the results from the urodele, Ambystoma mexicanum, where no response to T cell mitogens was found in the thymus (Collins and Cohen, 1976). This is a good example of the distinct differences that exist between anuran and urodele immune systems (Jurd, 1978).

The demonstration of a substantial response of Xenopus thymocytes to Con A and PHA contrasts to some extent with the work of Donnelly, Manning and Cohen (1976), Green and Cohen (1979),

who reported only minimal stimulation of adult *Xenopus* thymocytes with these mitogens. The use of a higher concentration of lymphocytes per culture in the experiments reported here may, to some extent, explain this disparity. Thus experiments reported in the third Chapter of this Thesis, attempting to reduce cell numbers in microtest plate mitogen cultures, were not successful in procuring good S.I.'s. Donnelly *et al* (1976) also noted that detection of residual Con A reactivity in spleens of early-thymectomized *Xenopus* was dependent upon maintaining a sufficiently high concentration of cells *in vitro*. In contrast to unfractionated thymocytes, their experiments did demonstrate an excellent Con A and PHA response in the low density fraction of bovine serum albumin separated thymus lymphocytes, revealing heterogeneity of T-equivalent cells in the anuran thymus, which has been previously demonstrated in mammals and birds (Stobo and Paul, 1973).

Work in this Chapter reveals that *Xenopus* thymocytes are stimulated by the mitogen PWM, although this response is relatively poor compared with that to PHA and Con A. Work on mammals suggests that PWM stimulates a subset of T lymphocytes that have helper activity, and that this response can be influenced by suppressor T lymphocytes (Greaves, 1972; Hirano, 1977; Keigthley *et al*., 1976). In humans PWM stimulation of helper T cells can lead to polyclonal B cell activation (Hammerström *et al*., 1979; Lanzavecchia *et al*., 1979).

Under optimal conditions, the splenic response of Xenopus to LPS was better than that seen with PHA or Con A. Manning et al. (1976) revealed quite the opposite in their studies. However they added 1% FCS in their experiments, which we have shown are suboptimal for LPS reactivity. The fact that very high concentrations (1 mg cm^{-3}) of LPS are required to 'optimally' stimulate Xenopus splenocytes was, however, also demonstrated in their experiments. Less LPS (100-400 µg cm^{-3} 055 : B5 preparation) is required to optimally stimulate splenocytes in other amphibians (Goldstine, Collins and Cohen 1976; Collins and Cohen, 1976), and fishes (Etlinger, Hodgins and Chiller, 1976), higher doses of LPS proving to be suboptimal. The reasons for these differences are unclear. A parallel between the findings in Xenopus and experiments performed in mammals exists, however. Thus the more LPS (055 : B5) added to mouse spleen cells (maximum concentration used = 100 µg cm^{-3}), the greater the degree of induced proliferation (Coutinho et al. 1973).

In contrast to spleen cells, Xenopus thymocytes respond relatively poorly to LPS. Nevertheless, the positive stimulation of many thymocyte preparations with this putative B cell mitogen and also with PPD suggests the possibility of a population of B-equivalent cells within the anuran thymus. However, one must be cautious in drawing this conclusion since the highest levels of stimulation with LPS were seen in those thymocyte cultures supplemented with 10% FCS. It could therefore be that the induced reactivity was achieved by synergistic interaction of foreign serum components with mitogen, possibly effecting alteration

of the specificity of this mitogen for B-equivalent cells (see Etlinger et al, 1976, for discussion). However, the significant response to this mitogen in half of the serum-free thymocyte cultures implies that the better response of thymocytes in 10% FCS might simply be related to increased viability of LPS-reactive cells under these culture conditions. It should also be pointed out that LPS may be indirectly stimulating *Xenopus* T lymphocytes. Thus LPS can, via interaction with macrophages, cause triggering of some T cells in mice (Roelants, 1978). LPS-reactive *Xenopus* thymocytes do, however, appear to be a different population to PHA and Con A responsive thymocytes (see nylon wool separation experiments performed in Chapter 3). Although the double mitogen stimulation experiments with *Xenopus* also suggest that PHA and LPS stimulate separate spleen lymphocyte populations, similar studies with thymocytes would ascertain that this holds for the thymus.

Experiments are currently in progress (Cribbin, Horton and Zettergren) in the laboratory to determine whether B-equivalent cells can be identified directly in LPS stimulated thymus cell cultures. These experiments involve the use of anti-*Xenopus* Ig reagents to detect the appearance of cytoplasmic-Ig-labelled lymphocytes. LPS is used in mammals to stimulate B cells to develop into antibody secreting plasma cells (see Melchers, 1979). In the teleost, the trout, LPS stimulation *in vitro* does induce B-equivalent cell production in the spleen (assayed by induction of PFC's and by the appearance of cells with the ultrastructure of plasma cells) but *not* in the thymus (Etlinger, Hodgins and Chiller, 1978). The finding of a B cell activity within the anuran thymus would not be novel since functioning antibody forming cells have been found in the thymus of diverse vertebrate

species, e.g. mice (Herzenberg and Herzenberg, 1974; Bosma and Bosma, 1974; Micklem et al., 1976); rabbits (Chou, 1966; Jentz, 1979), chickens (Seto, 1978), snakes (Kawaguchi, Kina and Muramatsu, 1978), frogs (Moticka, Brown and Cooper, 1973; Minagawa, Ohnishi and Murakawa, 1976) and fishes (Sailendri and Muthukkaruppan, 1975; Ortiz-Muniz and Sigel, 1971).

In endotherms intrathymic antibody forming cells may well be spawned in the periphery and then have migrated to the thymus, since a clear separation of T and B cell systems is believed to exist early in their development, the thymus effecting T cell maturation, the bursa of Fabricius or bursal-equivalent being the primary site of B lymphocyte differentiation. There is no direct bursal equivalent in mammals since B cell differentiation is multifocal, beginning in the placenta and then later emerging in other sites, e.g. the foetal liver and spleen and eventually in the bone marrow (Melchers, 1979; Owen, Raff and Cooper, 1975).

In lower vertebrates there is substantial evidence that the thymus is the primary site of T-equivalent lymphocyte differentiation (see the Introduction to this Thesis) but uncertainties remain concerning the origins of B-equivalent lymphocytes. This is discussed in the next Chapter in the light of experiments investigating the ontogeny of mitogen reactivity to putative T and B mitogens in thymus and spleen of larval and young adult Xenopus, since these studies provide additional characterisation of the thymocyte response to LPS.

TABLE 2.1 Comparison of cpm obtained in mitogen-free and optimally-stimulated thymus and spleen cell cultures under different conditions of FCS supplementation.

Mitogen	Organ	FCS conc. %	Optimal mitogen conc. µg cm^{-3}	Background mean cpm ± S.E.	Stimulated mean cpm ± S.E.
Con A	Spleen	1	0.5	2,555 ± 737	21,500 ± 5,296
		10	2.5	10,016 ± 1,251	41,000 ± 5,489
	Thymus	1	0.5	823 ± 127	8,336 ± 3,735
		10	2.5	1,063 ± 390	12,365 ± 9,831
PHA	Spleen	0	8	1,651 ± 452	17,302 ± 667
		1	40	3,522 ± 887	32,426 ± 6,031
		10	40	9,131 ± 1,294	56,115 ± 6,268
	Thymus	0	8	496 ± 72	4,815 ± 3,513
		1	8	1,677 ± 494	7,578 ± 1,793
		10	40	1,936 ± 633	12,726 ± 4,030
LPS	Spleen	0	2000	822 ± 166	12,568 ± 4,085
		1	2000	2,341 ± 489	19,206 ± 3,473
	Thymus	0	2000	302 ± 33	540 ± 130
		1	1000	957 ± 238	2,217 ± 611
		10	1000	1,073 ± 304	4,065 ± 1,084

Although maximal S.I.'s can be calculated from the Table, such indices will not agree precisely with those shown in Figs. 2.1-2.3, since the latter are based on differences between background and stimulated cpm recorded in individual experiments, rather than on pooled cpm data (see Methods).

TABLE 2.2 Effect on spleen lymphocyte proliferation of combining PHA and LPS in the same culture

Mitogen	Spleen 1 cpm (individual wells)	Spleen 1 Mean cpm	Spleen 1 S.I.	Spleen 2 cpm (individual wells)	Spleen 2 Mean cpm	Spleen 2 S.I.
-	1,825 2,298 3,497	2,540		1,831 1,129	1,480	
LPS (2mg cm^{-3})	29,077 28,389 31,222	29,563	11.6	19,263 23,188 16,221	19,557	13.2
PHA (20 µg cm^{-3})	17,358 36,643 12,547	22,182	8.7	14,100 9,831 10,669	11,533	7.8
2mg cm^{-3} LPS + PHA 20µg cm^{-3}	53,979 72,045 68,316	64,780	25.5	41,328 28,500 30,579	33,469	22.6

Figs. 2.1 - 2.4

o	no FCS supplementation
●	1% " "
Δ	10% " "

Each point represents the mean S.I. ($\pm$ S.E.) calculated from (an average) 8 separate experiments.

Fig. 2.1

Dose response curves to concanavalin A

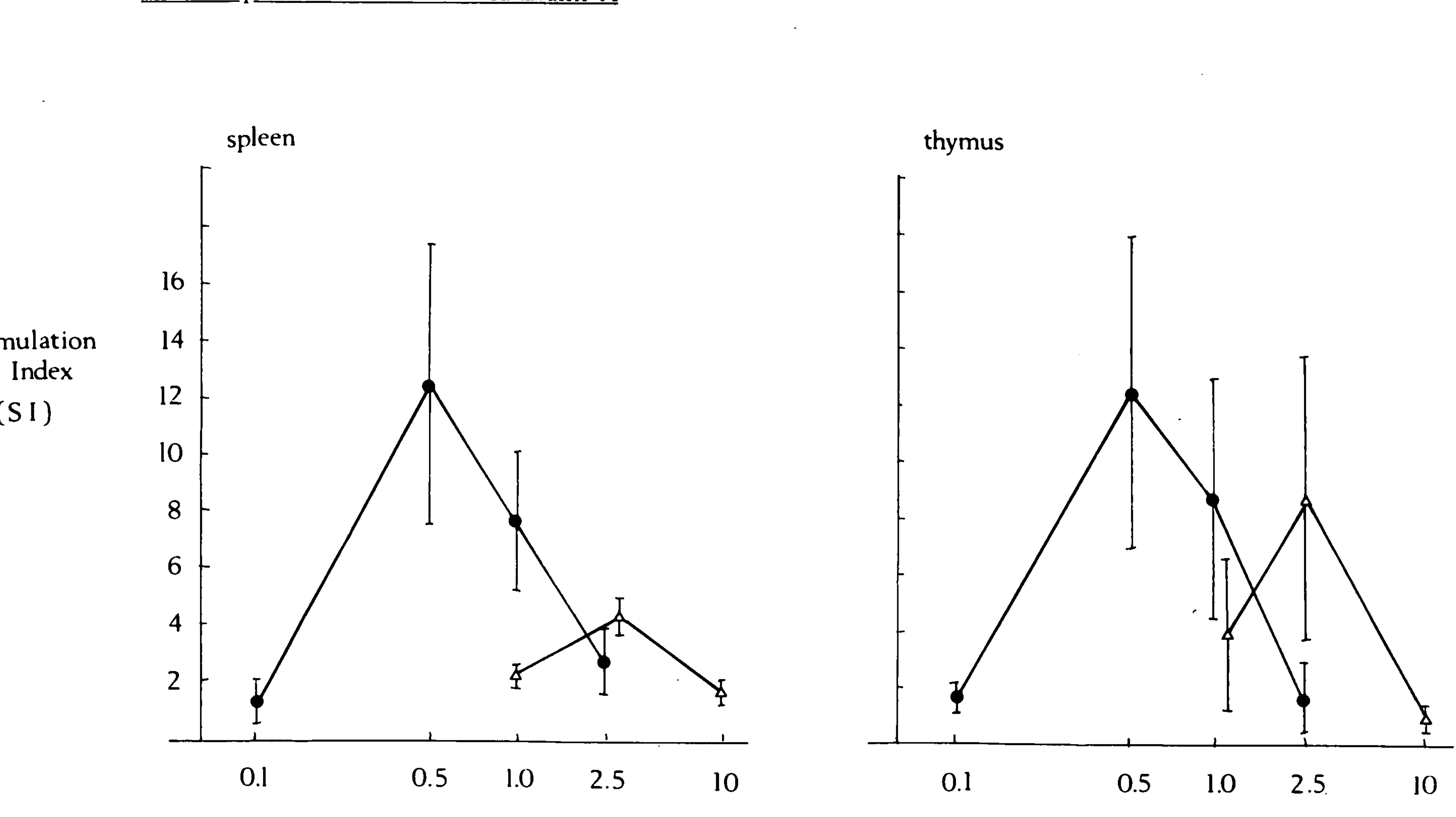


Fig. 2.2

Dose response curves to Phytohæmagglutinin

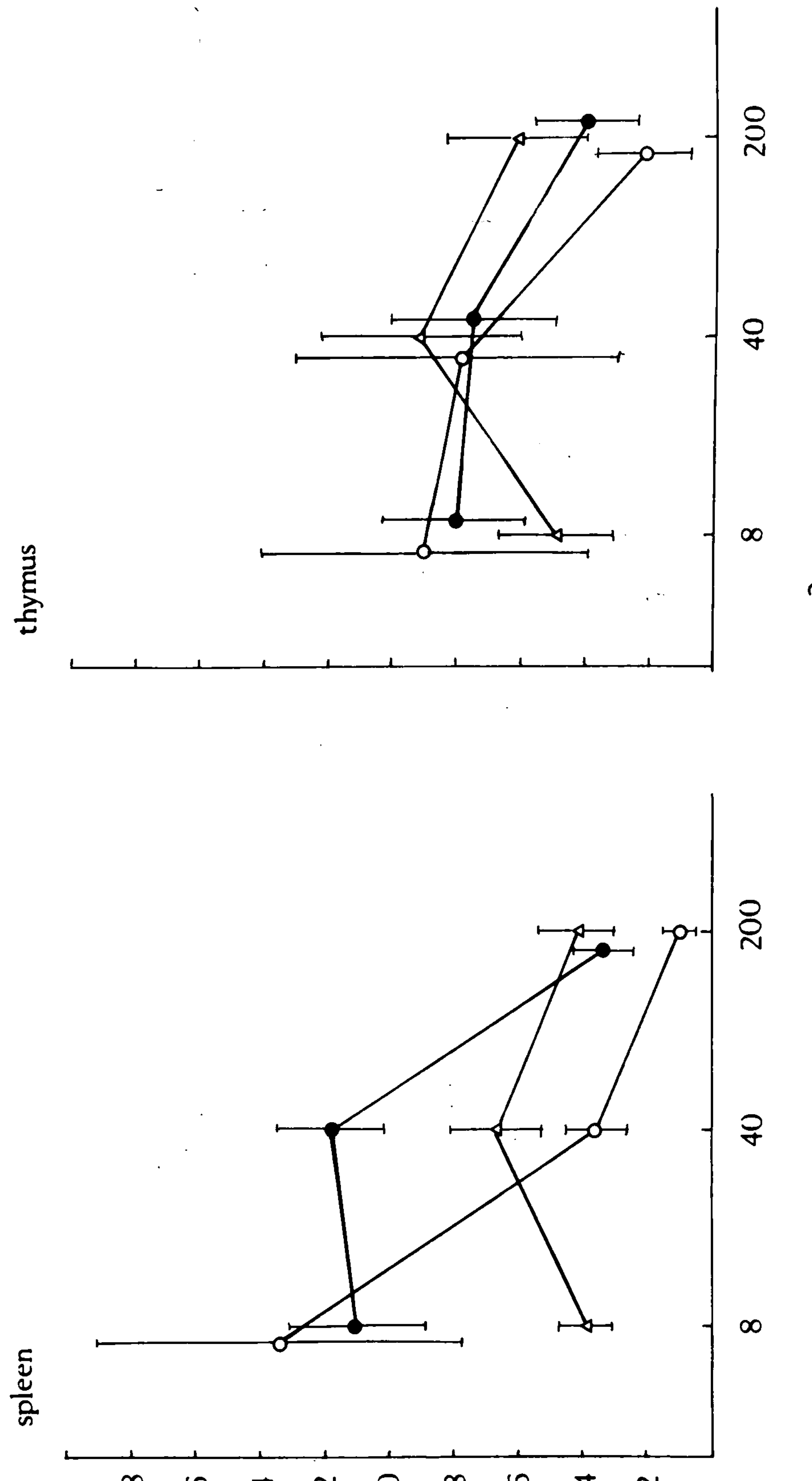


Fig. 2.3

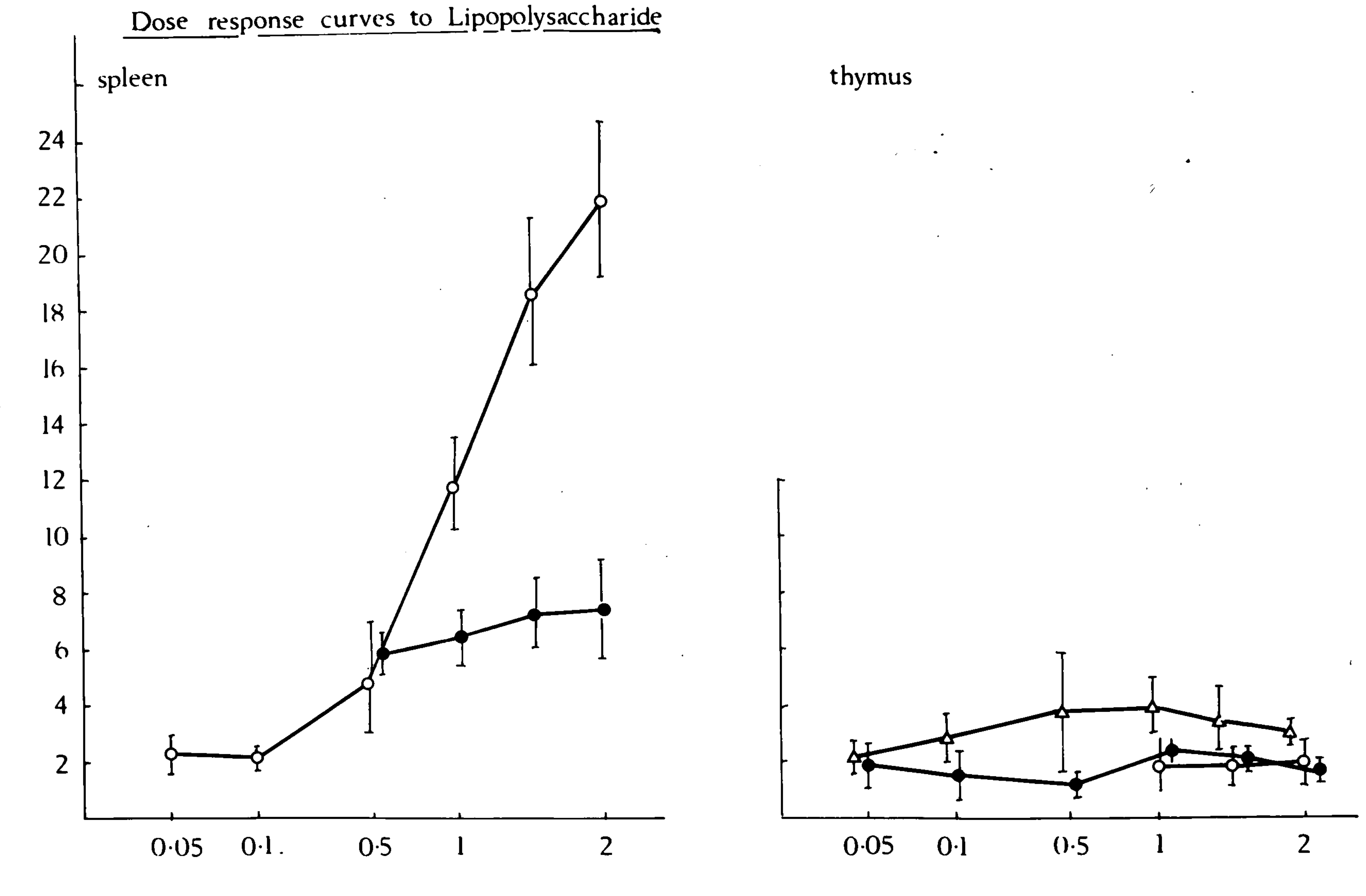


Fig. 2.4

Thymus dose response curves

Purified Protein Derivative of tuberculin

Pokeweed Mitogen

S I

8 6 4 2

0·05 0·1 0·5 1

mg cm^{-3}

10 25 50 300

µg cm^{-3}

Concentration

CHAPTER 3

Ontogeny of mitogen responsiveness in thymus and spleen

Introduction

Proliferative reactivity to both T and B cell mitogens is not an inherent property of embryonic lymphocytes. Indeed the emergence of mitogen reactivity in mammals is considered to be a marker for a certain level of functional maturity (Janossy & Greaves, 1972; Stobo and Paul, 1973; Mosier, 1974; Kruisbeek, 1979). The mitogen studies in this Chapter were designed to investigate the ontogeny of T- and B-equivalent lymphocytes within the anuran thymus during larval and post-metamorphic life. The competence of intra-thymic larval and young toadlet lymphocytes to respond *in vitro* has been examined by others with respect to MLC reactivity (Du Pasquier & Weiss, 1973), but there have been no reports concerning the development of mitogen-reactive cells within this organ. The emergence of thymocyte reactivity during the first two years of life to Con A, PHA and LPS are examined here and proliferative responses are compared, wherever possible, with those occurring in the spleen. The experiments on ontogeny of mitogen reactivity pay particular attention to the thymocyte LPS response, which proves to be maximal in the young toadlet. The cells involved in the proliferative response to this mammalian B cell mitogen are compared with thymocytes stimulated with PHA following autoradiography and nylon wool separation. The effect of metamorphosis on emergence of thymocyte mitogen reactivity is also discussed in some detail.

Materials and Methods

(a) Miniaturised cell culture technique

Larval lymphoid tissues possess relatively few lymphocytes compared with the adult, so the standard technique of lymphocyte culture described in Chapter 2 had to be miniaturised. Initial experiments with toadlet lymphocytes showed that reducing cell numbers in the standard microtest plate reduced the subsequent scintillation counts to an unacceptably low and variable level. However, it was possible to reduce cell numbers and volume by 25% (5×10^4 lymphocytes in 10µl) in flat-bottomed Terasaki microculture plates (Sterilin): this yielded S.I.'s comparable to the standard technique (2×10^5 lymphocytes in 40µl). These experiments are described below. Success with miniaturisation made it possible to work with individual larval thymuses, but larval spleens still proved to contain too few lymphocytes for individual analysis. The standard cell culture technique described in Chapter 2 was used with toadlet tissues.

The miniaturised technique is illustrated in Fig. 3.1. The larval thymus was dissected out and placed in a microtest plate containing medium (see Chapter 2) where it was broken up mechanically under a stereomicroscope using tungsten needles. Thymocytes were then washed by centrifugation for 10 minutes at 350 x g. The resuspended cells were counted and adjusted to 5×10^6 lymphocytes cm^{-3}. Ten µl of the cells and 2.5 µl of mitogen (medium in control cultures) were added to each well of a Terasaki plate. Finally 2.5 µl of FCS was added to give a final concentration of 1%.

One per cent FCS supplementation was used throughout all the ontogeny experiments, since this helped keep background counts down (although larval thymocyte background cpm were still very high in the Terasaki plates - see Results). The mitogen concentrations used are given in the Tables. Triplicate lymphocyte cultures were set up.

After 48 hours at 28°C the cells were pulsed with 0.25 μCi ^{3}HTdR (2.5 μl of a 1 : 10 dilution 1mCi cm^{-3} ^{3}HTdR, specific activity 5 Ci $mmol^{-1}$). 18 - 24 hours later the contents from individual wells were transferred to separate positions on harvesting mats of the Skatron cell harvester by manual pipetting. The mats were then inserted into the head of the harvester and the cells washed thoroughly and prepared for scintillation counting as in the previous Chapter. The coefficient of variation using the miniaturised technique was comparable with that in the standard technique. Therefore S.I.'s of 1.5 and above were considered positive.

(b) Autoradiography

At the end of the 3 day culture, instead of harvesting the radioactive wells the latter were washed with amphibian phosphate-buffered saline (PB) and then lymphocytes counted. Approximately 1 x 10^{6} thymus lymphocytes were resuspended in 50 μl FCS and a smear of cells prepared using a cytocentrifuge (Shandon) as follows. The cells were inserted into cuvettes and centrifuged for 4 minutes at 600 rpm, which forces the cells onto a circular area of a microscope slide. The slides were then fixed in methanol and air-dried. In the darkroom they were dipped in Ilford K5 nuclear

emulsion, following the technique described by Rogers (1973), and incubated for 1 week at 4°C to expose the silver grains. Following photographic development they were stained with Leishman's stain, and a permanent mount prepared.

The method for counting labelled and blast lymphoid cells was as follows:- Fifty fields, using the microscope at x1000 magnification, from the centre of the smear were examined and all lymphoid cells counted and identified. Coded slides were used and gave consistently-repeatable results. Cells were intentionally heavily-labelled to facilitate counting.

(c) Nylon wool separation

Thymocytes were separated on a nylon wool column using the method of Blomberg, Bernard & Du Pasquier (1980). Briefly 1 gm nylon wool ("Leuko-Pak", Fenwal Laboratories - obtained from Travenol) was washed by boiling 6 times in distilled water. It was then dried and teased to remove knots, loosely packed into a 10 cm^3 syringe and autoclaved. Lymphocyte separation then proceeded as follows. The column was saturated with sterile amphibian strength PBS containing 10% FCS for at least 1 hour prior to use. The thymocyte suspension was then added in 1-2 cm^3 medium. Two to three cm^3 of the PBS/FCS solution was added and the column incubated at 30°C for 1 hour. After this time the non-adherent cells were flushed through with about 20 cm^3 of PBS/FCS, centrifuged and resuspended in medium and the lymphocyte concentration readjusted to 5×10^6 cm^{-3} for culture. The nylon wool-passaged cells were compared for mitogen reactivity with unseparated cells from the same thymocyte suspension. In these mitogen experiments the miniaturised technique was used throughout.

(d) Experimental design

(i) Preliminary studies on miniaturisation

Mitogen responses and background cpm of individual thymus and spleen cell suspensions (taken from 4-8 month old toadlets) were examined using the microtest and Terasaki plates, with standard and miniaturised techniques respectively.

(ii) Ontogeny of mitogen responsiveness

This major series of experiments was designed to investigate the ontogeny of responsiveness of thymocytes, from thymuses taken from individual larvae of stage 52 (3 weeks old) and continuing until about 2 years of age, to the T cell mitogens PHA and Con A and the B cell mitogen LPS. Figure 3.2 shows the relationship between age and developmental stage, that has been used throughout the thesis, and follows the normal table of *Xenopus laevis* development of Nieuwkoop & Faber, 1956. As already mentioned, the very low lymphocyte numbers found in larval spleens precluded mitogen studies on this organ until after metamorphosis.

(iii) Characterisation of the thymocyte mitogen response

This involved autoradiography and nylon wool separation experiments. The autoradiography provided morphologic evidence of stimulation with T and B cell mitogens. Autoradiographs were prepared from thymocyte cultures (taken from 6 month old animals) treated with the mitogens PHA and LPS. The nylon wool experiments looked at the effect of removing nylon wool-adherent cells on mitogen responses. Thymocyte suspensions from 4 animals (6 or 12 months old - see Results) were separated on nylon wool columns. After separation, the passaged cells were stimulated with either PHA, Con A or LPS.

Results

(a) Preliminary studies comparing miniaturised and standard culture techniques.

Figure 3.3 examines mitogen stimulation of thymus and spleen cells from individual toadlets with standard and miniaturised techniques. Although levels of mitogen stimulation of thymocytes proved to be rather poor at 4 months of age (see subsequent findings below), the microtest (standard technique) and Terasaki (microtechnique) systems nevertheless proved comparable. With the spleens (taken from 8 month old toadlets) good and comparable stimulations were seen with both techniques. Findings with thymocytes suggested that the miniaturised technique could be employed for the ontogenetic studies on the larvae.

Analysis of background counts is given in Table 3.1. It had been expected that the level of tritium counts in the Terasaki plates would be 25% of those with the microtest system. This proved to be the case for splenocytes, whereas thymocyte counts in the Terasaki plates were, on average, more than sixfold higher than expected from the counts recorded in the microtest plates. This difference also occurred when 10% FCS supplementation was used (this concentration of FCS promotes greater viability of thymocytes in microtest plates compared with 1% supplementation - see Chapter 2). It is not known why the miniaturised technique promotes elevated ^{3}HTdR uptake only in the thymocyte cultures. Perhaps thymic cultures are more sensitive than spleen cultures to *in vitro* conditions (e.g. plate shape and cell number per well) because the thymus contains (at the outset of culture) a much higher

proportion of rapidly proliferating lymphocytes than does the spleen.

(b) Ontogeny of mitogen responsiveness

(i) Studies with Con A

Thymus. The results are given in Tables 3.2 and 3.3 and Fig. 3.4. At the first stage examined (stage 52/3, ≃ 3 weeks old) no response was found; in fact the wells containing mitogen all gave counts lower than that found in control wells. The next stage examined was stage 54/5 (≃ 4 weeks old) and again the cultures containing Con A generally gave a depressed cpm compared with unstimulated cells. Thymocytes from 2 animals in this group did display a S.I. of just >1.5, possibly indicating the emergence of Con A reactive thymocytes at this time. By stage 56/7 (6 weeks old) a small response to Con A was found in all cultures tested, with a mean S.I. of 1.71. This response disappears at the onset of metamorphosis (stage 58/9, ≃ 7 weeks old) and recovers only slowly after metamorphosis. At 4 months of age stimulation was positive in only one animal of 6 tested, but after this age all toadlet thymuses responded to Con A. The maximum mean level of stimulation seen (mean S.I. = 10) occurred in 1 year old toadlets.

Spleen. The results are given in Table 3.4 and Fig. 3.4. At 4 months of age, which was the earliest tested, 3 out of 4 toadlets responded with mean S.I. = 2.3. Splenocyte responsiveness to Con A remained constant (mean S.I. ≃ 6) in 6-9 month old animals, higher levels being recorded only in older animals. The highest S.I. (17.6) was seen in a 2 year old animal.

(ii) Studies with PHA

Thymus. The results are given in Tables 3.5 and 3.6 and Fig. 3.5. With this mitogen, no consistent proliferative response was found in the 4 larval stages tested (3-8 weeks of age). Just one stage 56/7 larva gave a significant (S.I. = 1.6) response. At 3½ months of age (i.e. 6 weeks after the end of metamorphosis) 2 out of 4 animals showed a positive PHA thymocyte response. As with Con A, the PHA response then increased slowly with age, giving a maximum mean level (S.I. ≈ 18) by about 9 months of age. This level of response appears to decline in later life, since the mean S.I. at 2 years of age was just under 6.

Spleen. The results are given in Table 3.7 and Fig. 3.5. The youngest toadlets tested (3.5 months) gave negative responses, but a constant positive PHA response appeared-soon afterwards, by 4 months of age. A high level of PHA stimulation (S.I.≅ 18) was seen in spleens taken from 6-7 month old toadlets. In 9 and 12 month old animals the mean S.I. of splenocytes to PHA was reduced, but some individual animals still displayed S.I.'s of > 10.

(iii) Studies with LPS

Thymus. The results are given in Tables 3.8 and 3.9 and Fig. 3.6. There was no mitogenic response to LPS at the earliest stages tested (stages 52-55). The mitogen-treated thymocytes from these young larvae all showed a depressed cpm level compared with unstimulated cells. At stage 56/7 a small response was detected in all 3 larvae tested (mean S.I. = 2.1). LPS reactivity of thymocytes could no longer be detected at the onset of and just after the completion of metamorphosis, but re-emerged in 2 out of 3 toadlets tested at 4 months

of age. After this time consistent LPS responsiveness of thymocytes was seen in young toadlets, rising to a peak mean S.I. of ≃ 6 at about six months of age. The thymocyte LPS response gradually diminished after 6 months and by 12 months of age it had dwindled to a low level (mean S.I. = 1.6) and this was also found in later life. These ontogenetic studies not only confirm that LPS responsiveness occurs in post-metamorphic *Xenopus* thymus, but revealed that the response is found consistently in young toadlets, reaching levels that compare favourably with reactivity to T cell mitogens.

Spleen. The results are given in Table 3.10 and Fig. 3.6. The youngest animals tested for splenocyte responsiveness to LPS were toadlets of 3½ and 4 months old. Only 1 of the 4 animals tested responded. After 4 months a response rapidly emerged, with a mean S.I. at 6 months being 7.8. Unlike the thymus, this level of LPS responsiveness is maintained in older animals.

(c) Characterisation of thymocyte mitogen responses

(i) Autoradiographic evidence of T and B mitogen stimulation

These results confirm those obtained by scintillation counting in as much as stimulation with both PHA and LPS results in increased levels of ^{3}HTdR uptake and blast cell transformation in thymocytes. Similar studies on a few spleen cell preparations also provided morphologic evidence for enhanced ^{3}HTdR uptake in mitogen-treated cultures. The percentage of tritium-labelled cells in the unstimulated cultures was about 1% (see Table 3.11 and Fig. 3.7). In the LPS-stimulated cultures from the 6 month old animals, the percentage of

labelled cells increased to 5.5%. The majority of these labelled cells were larger than 10μm in diameter. In the PHA stimulated cultures the percentage of labelled thymocytes increased to 11.2%. Again the majority of labelled cells were of lymphoblast morphology (>10 m). This value for PHA fits quite well with mammalian work where 15% of murine thymocytes respond to Con A (Gerhart et al., 1976). Figure 3.8 (a-c) shows cytocentrifuge preparations of thymocytes (cultured in medium, LPS or PHA) that have been prepared for autoradiography. It can be seen that thymocytes are agglutinated in the mitogen treated cultures, particularly with PHA. Proliferating lymphoblasts were generally heavily labelled (see Fig. 3.8,d) although some blasts, with fewer exposed silver grains, were also found.

(ii) The effect of nylon wool treatment on T and B mitogen stimulation

These experiments were designed to look at the effect of removing nylon wool adherent cells on the proliferative response of thymocytes to putative T and B mitogens. Of the 4 animals used in these experiments, 2 were six months old (i.e. at an age when the maximum response of thymocytes to LPS occurs) and 2 were twelve months old (when thymocytes respond well only to T cell mitogens). The results are shown in Fig. 3.9. In the younger animals a good response to LPS was found in control, non-nylon wool treated cells (S.I.'s = 6.5 and 3.5). Mitogen responses to Con A and PHA were also as expected. Passage through nylon wool eliminated the response to LPS (S.I.'s now 0.9 and 0.3). Interestingly responses to both T cell mitogens were also appreciably reduced, but reactivity to these generally remained at around 50% the level found in unseparated thymocytes.

In the thymocyte cultures prepared from the 2 older animals the level of LPS response in unseparated cells was fairly low (S.I.'s = 2.5 and 2.1) but the response to the T cell mitogens was still consistently good. Nylon wool passaged thymocytes from these older animals showed no response to LPS. Thymocyte reactivity to PHA was now either unaffected or increased by nylon wool passage. Con A reactivity in these 1 year old animals was only slightly lowered by nylon wool passage.

Discussion

By about mid-larval development, anuran larvae are competent to reject skin allografts (Horton, 1969) and respond to circulating thymus-dependent antigens (Kidder, Ruben & Stevens, 1973). Since both of these immune mechanisms necessitate thepresence of T lymphocytes (see Horton & Manning, 1972; Horton, Rimmer & Horton, 1977) it might be expected that functional T cells would be found within the thymus itself during larval life. In the mouse the ability of thymic lymphocytes to respond to T cell mitogens occurs by parturition and is used as the first hallmark for the differentiation of functional T cells (Robinson & Owen, 1976), However, the results of thymocyte mitogen reactivity in developing *Xenopus* suggest, at best, only a minimal response of larval thymocytes to Con A, and no response at all to PHA.

The results presented here show that an appreciable and constant T mitogen response in the *Xenopus* thymus emerges only after metamorphosis and maximum levels are not seen in thymus or spleen until about 6 months or later. This seems to be rather slow when

compared to mammals, since sustained adult levels of mitogen reponsiveness in the thymus to Con A and PWM are found by 3.5 days after birth in the mouse (Robinson, 1976; Mosier, 1977). Adult levels of responsiveness in the thymus to T cell mitogens are found in the guinea pig by mid-gestation (Leiper and Solomon, 1977) as is also the case with sheep (Leino, 1978) and humans (Stites et al.,1974). In *Xenopus* PHA responsiveness (but not Con A reactivity) declines in older animals. This decline in PHA responsiveness with age has been reported for mammals (Onódy *et al.*, 1980).

The low and transient response of larval *Xenopus* thymocytes to Con A and the apparent lack of their reactivity to PHA could be due to several factors. First, sub-optimal microculture conditions used with the larval thymus may be responsible. As mentioned in the Results, the Terasaki plates did encourage high thymocyte background cpm, which may have tended to mask any proliferation induced by the T cell mitogens. However these plates were used successfully in several experiments on thymocytes. The use of an inappropriate dose of mitogen to stimulate larval thymocytes seems unlikely, since at the outset of these ontogenetic experiments other doses of Con A and PHA were tested (without any greater success). Secondly, the final stages of functional maturation of T cells may, in the larva, occur predominantly in the periphery, perhaps achieved by thymus humoral factors that are known to exist in amphibians (Dardenne *et al.*, 1973). Although this may be true for mitogen-reactive T cells, Du Pasquier and Weiss (1973) studies on *Xenopus* showing that MLC-reactive thymocytes are present at least by stage 54, indicate that this is not the case for all lymphocyte populations.

In mammals, PHA responsiveness of spleen cells occurs prior to PHA reactivity of thymocytes, suggesting post-thymic maturation of this population (Kruisbeek, 1979). Horton and Sherif (1977) found that an intact thymus was necessary in Xenopus until stage 54 to establish PHA responsiveness in the adult spleen. Similar findings have also been shown for Con A (Manning and Collie, 1977). Peripheral T cells capable of reacting to T cell mitogens could therefore be expected to be found in the spleen during late larval life. (Experiments were in fact carried out on pooled larval spleens, but the results were too inconsistent for conclusions to be drawn, probably due to mixed lymphocyte reactivity between allogeneic cells). However, the results with the spleen after metamorphosis suggest that if this is so, then their numbers probably remain small until well into adult life. Studies on several larval and post-metamorphic lymphoid organs are obviously required to investigate this issue, although these experiments will require the pooling of lymphocytes from histocompatible animals to yield sufficient cells for analysis. Thirdly, Con A and PHA might, in anurans, selectively activate only helper T cells, rather than cytotoxic and MLC reactive subsets. This is dealt with in more detail in the next Chapter, but alloimmune lymphocyte populations are known to be established in the periphery early in life, whereas those helper subsets involved in humoral immunity are dependent upon an intact thymus for a much longer period of development - even until after metamorphosis when low doses of antigen are used (Gruenewald and Ruben, 1979). Indeed significant levels of Ig "G" antibody production (which depends heavily on T cell help) occur only in the adult anuran (Du Pasquier & Haimovich, 1976).

Differences in the emergence of Con A and PHA reactive thymocytes in Xenopus are possibly indicated from the data presented here. It seems that the PHA responsive population is slower to mature, first appearing only after metamorphosis. This could be because the two T cell mitogens are stimulating different T cell subsets. This has been shown to occur in mammals (Hayry et al., 1976). Stobo and Paul (1973) have shown in mice that only mature thymic lymphocytes respond to T cell mitogens, with one subset responding equally well to Con A and PHA, whereas another subset responds almost exclusively to Con A. Additional work (Paetkau et al., 1976; Larsson *et al.*, 1980) has shown that in some strains of mice thymocytes respond well to Con A, but only poorly to PHA. However it was found that the addition of Con A to thymocyte cultures causes an increase in the rate of synthesis of a costimultor factor (Interleukin), which is required for effective stimulation of Con A responsive lymphocytes. Moreover, addition of this cofactor (taken from the supernatant of Con A stimulated cells) to thymocyte cultures enabled these to become good responders to PHA. Whether or not cofactors are involved in anuran lymphocyte mitogen responses is not yet known.

The emergence of LPS reactive cells in the larval and young adult thymus (and their disappearance over metamorphosis) parallels the ontogenetic pattern of thymocyte responsiveness to Con A. This suggests, perhaps, that the LPS response is manifested by cells actually spawned within the thymus rather than through cells casually migrating through this organ from the periphery.

LPS responsiveness in the thymus reaches a maximum level at about six months and then declines, in contrast to the gradual elevation of LPS reactivity displayed by splenocytes taken from older toadlets.

Morphologic confirmation that LPS results in thymocyte stimulation in six month old toadlets was provided in the autoradiographic experiments presented. Moreover, the likelihood that LPS reactive thymocytes are a subset distinct from PHA and Con A reactive thymocytes was suggested by the nylon wool separation studies. Thus, under conditions which are known to substantially reduce surface Ig + ve lymphocytes (i.e. B-equivalent cells) from the spleen (Blomberg et al., 1980), thymocyte reactivity remains only to T cell mitogens. Interestingly at six months of age, but not at 12 months, T mitogen reactivity of thymocytes is also affected by nylon wool passage, implying that some T-equivalent cells are also removed by this procedure. This could be explained by the finding that thymocytes of young adult Xenopus are still rich in surface Ig (Du Pasquier & Weiss, 1973).

Organ culture studies in mammals have clearly shown that B cell differentiation is multifocal (Owen, Raff and Cooper, 1975). Thus B cells develop first in the placenta (Melchers, 1979), then in the foetal liver and spleen, and later in the bone marrow in mice. In Xenopus, 19Sand 7S immunoglobulins are first found at stage 35 (2 days old) when the larva is just hatching (Leverone et al., 1979), before a thymic rudiment has even appeared (Manning & Horton, 1969). Furthermore, cytoplasmic Ig^{+} cells are found in larval Rana kidney very early in ontogeny (Zettergren et al., 1980). These and other findings (showing normal LPS reactivity

in early-thymectomized *Xenopus* - see Turner and Manning, 1974) indicate an important extra-thymic origin of certain B-equivalent populations. B-equivalent lymphocyte development in anurans may well be multifocal as in mammals; it appears that the bone marrow is a site of B cell development in adult frogs (Zettergren *et al*., 1980).

It is possible however, that some B-equivalent cell subsets differentiate within the anuran thymus. A number of experimental observations support this hypothesis. Following early thymectomy in *Xenopus*, both IgG and IgM antibody production to thymus-dependent antigens is abrogated and also reactivity to TNP-Ficoll is dramatically impaired (Horton, Edwards, Ruben & Mette, 1979). This hapten-carrier complex was, until recently, thought to activate a subset of mammalian B lymphocytes and was considered to be a thymic independent antigen(Mosier, Mond & Goldings, 1977). However, recent studies suggest a degree of thymic-dependence of TNP-Ficoll reactivity in mammals (Mond et al., 1980). Certain embryological experiments, in which gill bud regions (containing thymic rudiments) were transferred to ploidy-distinct hosts, suggested the possibility that virtually all peripheral lymphocytes in leopard frogs normally originate in the thymus (Turpen, Volpe & Cohen, 1975; Turpen & Cohen, 1976). However, more recent findings on the potentiality of the gill bud region suggest that in addition to housing the thymus anlage, it also contains the pronephric rudiment, which may be an important source of lymphoid stem cells, particularly of B lymphocytes (Volpe *et al*., 1981; Zettergren *et al*., 1980).

The possibility that the mammalian thymus spawns some B cells has also been considered. Micklem *et al.* (1976) discuss the possibility that multipotential stem cells exist in the murine thymus which are capable of differentiating *in situ* into B cells. Jentz (1979) has found that, by cell transfer experiments, B cells in the thymus have different rates of function to B cells isolated from other organs (34 times more productive of antibody than spleen B cells), in rabbits.

Other experimental observations suggest that the amphibian thymus is not a source of antibody-producing cells. Thus thymocyte reconstitution experiments on thymectomized *Xenopus*, both *in vivo* (Katagiri *et al.*, 1980) and *in vitro* (Ruben *et al.*, 1977 indicate that the thymus provides only helper activity rather than antibody producing cells.

One major feature of these experiments has been to demonstrate that metamorphosis effects a temporary disruption of the emergence of thymocyte mitogen reactivity. This is not surprising since this period of development is associated with severe depletion of thymocyte numbers (Du Pasquier and Weiss, 1973; see also Chapter 6). During metamorphosis adult-specific antigens are appearing and there could be a danger that these antigens will be destroyed by an immunologically mature immune system. However it has been shown that there is an increased susceptibility of the metamorphosing anuran to tolerance induction of MHC antigens, probably achieved through the activity of (T) suppressor cells, which have been demonstrated at this time in *Xenopus* (Du Pasquier and Bernard, 1980). Active suppression of lymphocyte reactivity appears to develop in the larvae at the very time that mitogen responsiveness is

beginning to emerge. The gradual diminution of suppressor cell activity within the thymus after adult antigens are established may effect, in part, the gradual increase in thymus (and spleen) mitogen responsiveness seen in early adult life. The co-existence of mitogen-responsive and suppressor subsets in the same lymphoid tissue has been demonstrated in rats by density gradient separation (Rocha et al., 1979). There is circumstantial evidence for suppressor activity within the thymus of adult *Xenopus*, since Donnelly, Manning & Cohen (1976) were able to detect T mitogen reactive cells only in a certain fraction of separated thymocytes, whereas unseparated cells failed to respond to Con A and PHA. Active suppression within the thymus during larval life is also suspected (Du Pasquier, personal communication) and this could account for the poor mitogen responses displayed by thymocytes prior to metamorphosis.

TABLE 3.1 Comparison of ^{3}HTdR cpm obtained in unstimulated cultures in Terasaki and microtest plates

Splenocyte cultures from 8 month old toadlets

40μl in microtest plate	10μl in Terasaki plate		Terasaki / microtest %
480	260		54.2
3355	847		25.2
2963	347		11.7
3367	647		19.2
1292	634		49.1
1994	1665		83.5
		Observed	40.4 ± 24.6%
		Expected	25.0%

Thymocyte cultures for 4 month old toadlets

Microtest plate	Terasaki plate		Terasaki / microtest %
177	207		116.9
202	209		103.5
501	1029		205.4
705	1789		253.8
485	754		152.5
249	344		142.1
724	1103		152.3
		Observed	161.9 ± 51.0%
		Expected	25.0 %

Each horizontal row of data reflects cpm obtained for the same cell suspension.

TABLE 3.2 Ontogeny of thymocyte response to Concanavalin A 1.0μg cm^{-3}

Larval responses up to metamorphosis

Stage/age		Mean cpm unstimulated	Mean cpm stimulated	Stimulation index
52/3 3 weeks	1)	908	688	0.76
	2)	809	693	0.77
	3)	4815	813	0.17
				0.57 $\pm$ 0.1
54/5 4 weeks	1)	1330	1129	0.85
	2)	972	1482	1.52
	3)	1886	1301	0.69
	4)	2064	1727	0.84
	5)	2421	1151	0.48
	6)	1554	2460	1.58
	7)	1800	1648	0.92
	8)	1580	720	0.46
				0.92 $\pm$ 0.25
56/7 6 weeks	1)	862	1390	1.61
	2)	1520	2474	1.63
	3)	1477	2618	1.77
	4)	522	943	1.81
				1.71 $\pm$ 0.05
58/9 7-8 weeks	1)	904	585	0.65
	2)	1220	357	0.29
	3)	291	177	0.61
				0.52 $\pm$ 0.11

TABLE 3.3 Ontogeny of thymocyte response to Concanavalin A 1.0μg cm^{-3}

Responses after metamorphosis

Age (months)		Mean cpm unstimulated	Mean cpm stimulated	Stimulation index
3.5	1)	1673	2296	1.4
4	1)	623	1544	2.48
	2)	757	1036	1.37
	3)	501	469	0.94
	4)	705	391	0.60
	5)	3808	2591	0.70
	6)	2991	1808	0.60
				1.10 $\pm$ 0.30
5	1)	535	1431	2.70
	2)	1328	2959	2.20
	3)	485	1845	3.80
				2.90 $\pm$ 0.47
7	1)	452	1492	3.30
	2)	1078	9593	9.00
				6.15 $\pm$ 1.69
8	1)	249	1927	7.70
	2)	389	4693	12.10
	3)	1163	3690	3.20
	4)	446	2826	6.30
				7.33 $\pm$ 1.84
10	1)	353	1853	5.30
	2)	873	7733	8.90
				7.10 $\pm$ 1.34
12	1)	353	2712	7.70
	2)	531	5629	10.60
	3)	1211	14412	11.90
				10.00 $\pm$ 1.1

TABLE 3.4 Ontogeny of the splenocyte response to Concanavalin A 1.0μg cm^{-3}

Age (months)		Mean cpm unstimulated	Mean cpm stimulated	Stimulation index
4	1)	3402	1059	0.31
	2)	6637	11611	1.74
	3)	5024	25297	5.03
	4)	4621	9427	2.04
				2.28 ± 0.99
5	1)	652	834	1.28
	2)	1269	6465	5.10
				3.19 ± 1.9
6	1)	1861	6457	3.47
	2)	1233	8138	6.60
	3)	952	6940	7.29
	4)	3367	12655	3.80
				5.95 ± 1.37
7	1)	4034	30176	7.50
	2)	3193	13308	4.20
				5.85 ± 1.28
9	1)	1292	7036	5.50
	2)	1239	7775	6.30
	3)	1057	4948	4.68
				5.49 ± 1.14
12	1)	669	5486	8.20
24	1)	2147	37720	17.60
	2)	5125	26650	5.20
				11.4 ± 3.1

TABLE 3.5 Ontogeny of thymocyte response to Phytohaemagglutinin 20μg cm^{-3}

Larval responses up to metamorphosis

Stage/age		Mean cpm unstimulated	Mean cpm stimulated	Stimulation index
52/3 3 weeks	1)	4532	515	0.11
	2)	1323	1660	1.25
				0.68 ± 0.57
54/5 4 weeks	1)	1800	1602	0.90
	2)	2102	846	0.40
	3)	1580	1580	1.00
				0.76 ± 0.59
56/7 6 weeks	1)	1269	1482	1.20
	2)	1892	2046	1.10
	3)	2290	2106	0.92
	4)	1851	1703	0.92
	5)	681	728	1.10
	6)	340	540	1.60
				1.16 ± 0.15
58/9 7-8 weeks	1)	1549	862	0.56
	2)	1460	1076	0.74
	3)	1643	1448	0.88
	4)	1102	1185	1.10
				0.82 ± 0.11

TABLE 3.6 Ontogeny of thymocyte response to Phytohaemagglutinin 20μg cm^{-3}

Responses after metamorphosis

Age (months)		Mean cpm unstimulated	Mean cpm stimulated	Stimulation index
3.5	1)	3808	1474	0.40
	2)	2991	9789	3.30
	3)	1029	1525	1.50
	4)	1789	1610	0.90
				1.50 ± 0.75
4	1)	1119	2221	1.97
	2)	669	1893	2.82
		885	4124	4.66
				3.15 ± 0.79
7	1)	1078	9177	8.50
	2)	356	1361	3.80
	3)	4034	42765	10.60
	4)	3193	16818	5.30
	5)	1274	4634	3.60
	6)	1428	13621	9.30
	7)	841	20665	24.50
				9.37 ± 2.73
8	1)	1163	14002	12.00
	2)	446	5810	13.00
	3)	389	8064	20.70
				15.23 ± 2.76
9.5	1)	970	19497	20.10
	2)	533	6703	12.60
	3)	247	5987	24.20
	4)	353	3783	10.70
	5)	873	18905	21.70
				17.86 ± 2.6
24	1)	2271	12600	5.50
	2)	1123	7394	6.60
	3)	1988	11381	5.70
				5.93 ± 0.37

TABLE 3.7 Ontogeny of splenocyte response to Phytohaemagglutinin 20μg cm^{-3}

Age/stage		Mean cpm unstimulated	Mean cpm stimulated	Stimulation index
3.5	1)	1832	1020	0.56
	2)	2076	908	0.43
				0.50 ± 0.5
4	1)	3402	19086	5.60
	2)	6638	16149	2.41
	3)	221	505	2.28
	4)	183	853	4.66
	5)	11440	58403	5.10
	6)	5024	43103	8.57
				4.39 ± 1.26
4.5	1)	1632	4290	2.62
	2)	3242	37765	11.60
	3)	4195	18256	4.30
	4)	1269	3122	2.50
				5.25 ± 2.31
5	1)	3860	38170	9.90
	2)	3975	53116	13.10
				11.50 ± 1.26
6	1)	4896	35755	7.30
	2)	1137	9813	8.63
	3)	1913	13285	6.94
	4)	978	9193	9.71
	5)	846	34444	40.70
	6)	308	13570	44.09
	7)	3174	25315	8.00
				17.91 ± 6.29
7	1)	1148	42668	37.20
	2)	1693	14782	8.70
	3)	2875	24779	8.60
	4)	2114	35711	7.00
	5)	438	11960	27.30
				17.28 ± 5.03
9	1)	2066	8537	4.10
	2)	6660	76265	11.50
				7.80 ± 1.9
12	1)	2540	22182	8.70
		539	3368	6.30
		408	2620	6.40
		379	6685	17.60
		517	1254	2.40
		1620	1940	1.20
		338	1780	5.30
				6.53 ± 1.78

TABLE 3.8 Ontogeny of thymocyte response to *E.coli* Lipopolysaccharide 2.0mg cm^{-3}

Larval responses up to metamorphosis

Stage/age		Mean cpm unstimulated	Mean cpm stimulated	Stimulation index
52/3 3 weeks	1)	2111	750	0.36
	2)	599	293	0.49
	3)	1292	247	0.19
	4)	1606	797	0.49
				0.38 ± 0.1
54/5 4 weeks	1)	1159	357	0.31
	2)	2597	691	0.27
	3)	2306	928	0.40
				0.33 ± 0.03
56/7 6 weeks	1)	862	1332	1.55
	2)	1520	4802	3.56
	3)	1477	2301	1.56
				2.09 ± 0.5
58/9 7-8 weeks	1)	904	555	0.61
	2)	1220	566	0.46
	3)	291	200	0.69
				0.59 ± 0.1

TABLE 3.9 Ontogeny of thymocytes reponse to *E. coli* Lipopolysaccharide 2.0mg cm^{-3}

Responses after metamorphosis

Age (months)		Mean cpm unstimulated	Mean cpm stimulated	Stimulation index
3.5	1)	1673	927	0.60
	2)	2991	2351	0.80
				0.70 ± 0.1
4	1)	534	1582	3.00
	2)	1029	829	0.80
	3)	1789	2753	1.50
				1.80 ± 0.6
5	1)	1328	3133	2.40
6	1)	1746	9722	5.60
	2)	933	5549	5.90
	3)	650	4199	6.50
	4)	556	3000	5.40
				5.90 ± 0.2
7	1)	301	1353	4.40
8	1)	689	3171	4.50
	2)	389	1304	3.40
	3)	1163	7313	6.30
	4)	446	977	2.20
				3.20 ± 0.9
10	1)	1970	6695	3.60
	2)	1289	3773	2.90
	3)	373	1062	2.80
	4)	353	1366	3.90
	5)	873	2465	2.80 ± 0.2
12	1)	646	1194	1.80
	2)	254	329	1.30
	3)	587	528	0.90
	4)	792	1902	2.40
	5)	524	577	1.10
	6)	426	640	1.50
	7)	569	1139	2.00
				1.60 ± 0.01
24	1)	831	1664	2.00
	2)	1107	1550	1.40
	3)	582	873	1.50
	4)	778	1481	1.90
				1.70 ± 0.2

TABLE 3.10 Ontogeny of splenocyte response to *E.coli* Lipopolysaccharide

2.0mg cm^{-3}

Age (months)		Mean cpm unstimulated	Mean cpm stimulated	Stimulation index
3.5	1)	2277	3228	1.41
4	1)	2076	4346	2.09
	2)	1832	2199	1.10
	3)	652	698	1.07
				1.42 ± 0.33
6	1)	1055	3336	3.20
	2)	1222	8800	7.20
	3)	2006	4768	2.30
	4)	2238	41159	18.30
				7.80 ± 3.64
9	1)	1072	22826	21.20
	2)	2066	7277	3.50
	3)	6660	29813	4.50
	4)	909	5324	5.90
				8.78 ± 4.17
24	1)	1218	11329	9.30
	2)	921	12157	13.20
	3)	3217	33779	10.50
	4)	1260	12348	9.80
				10.80 ± 0.8

TABLE 3.11 Autoradiograph Data

% of labelled and unlabelled thymocytes in relation to cell size following mitogen stimulation

	Proportion (%) of unlabelled cells		Proportion (%) of labelled cells		
	0-10μm	>10μm	0-10μm	>10μm	Total
Control (unstimulated)	98.9	1.1	0	0	0
	99.1	0.9	0	0	0
	99.9	0.1	0	0	0
	98.4	0.4	0.4	0.8	1.2
	78.0	17.8	0.5	3.7	4.2
	94.3	4.8	0.9	0	0.9
					1.05 ± 0.55
LPS	96.9	0.6	0	2.5	2.5
	95.8	0.8	1.1	2.3	3.4
	95.5	0.9	0.4	3.2	3.6
	92.8	0.8	2.7	3.7	6.4
	77.7	11.4	0	10.9	10.9
	93.8	0.3	0.5	5.4	5.9
					5.45 ± 1.25
PHA	84.3	0.6	5.3	9.7	15.0
	82.4	2.7	5.4	9.5	14.9
	86.4	0	6.1	7.5	13.6
	95.5	1.2	2.2	1.2	3.4
	74.6	14.7	1.1	9.6	10.7
	87.7	2.8	2.6	6.8	9.4
					11.17 ± 1.81

Fig. 3.1

Organs were transferred to a Microtest plate where a lymphocyte suspension was prepared and washed by centrifugation. Following counting and adjusting to a concentration of 5×10^6 lymphocytes cm^{-3}, the cells were distributed into a Terasaki plate for culture. After 2 days, the cells were pulsed for 24 hours and then hand harvested onto a glass fibre mat and prepared for scintillation counting.

Fig. 3.1

Technique of cell culture in Terasaki plates

10 µl cells
2·5µl medium/
mitogen

Day 2

2·5ul ^{3}H thymidine

Day 3

cells harvested

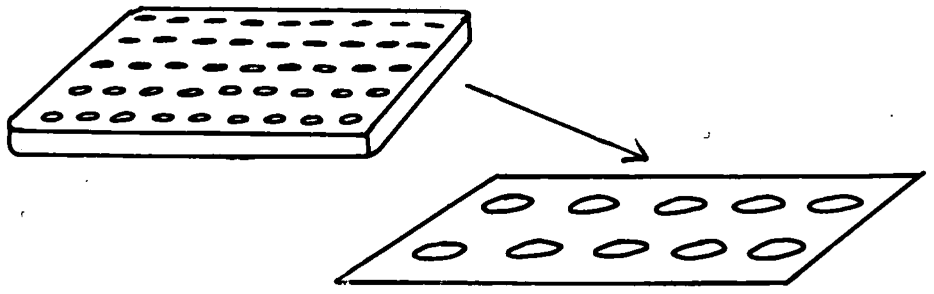


Fig. 3.2

Ontogeny of *Xenopus laevis* larvae

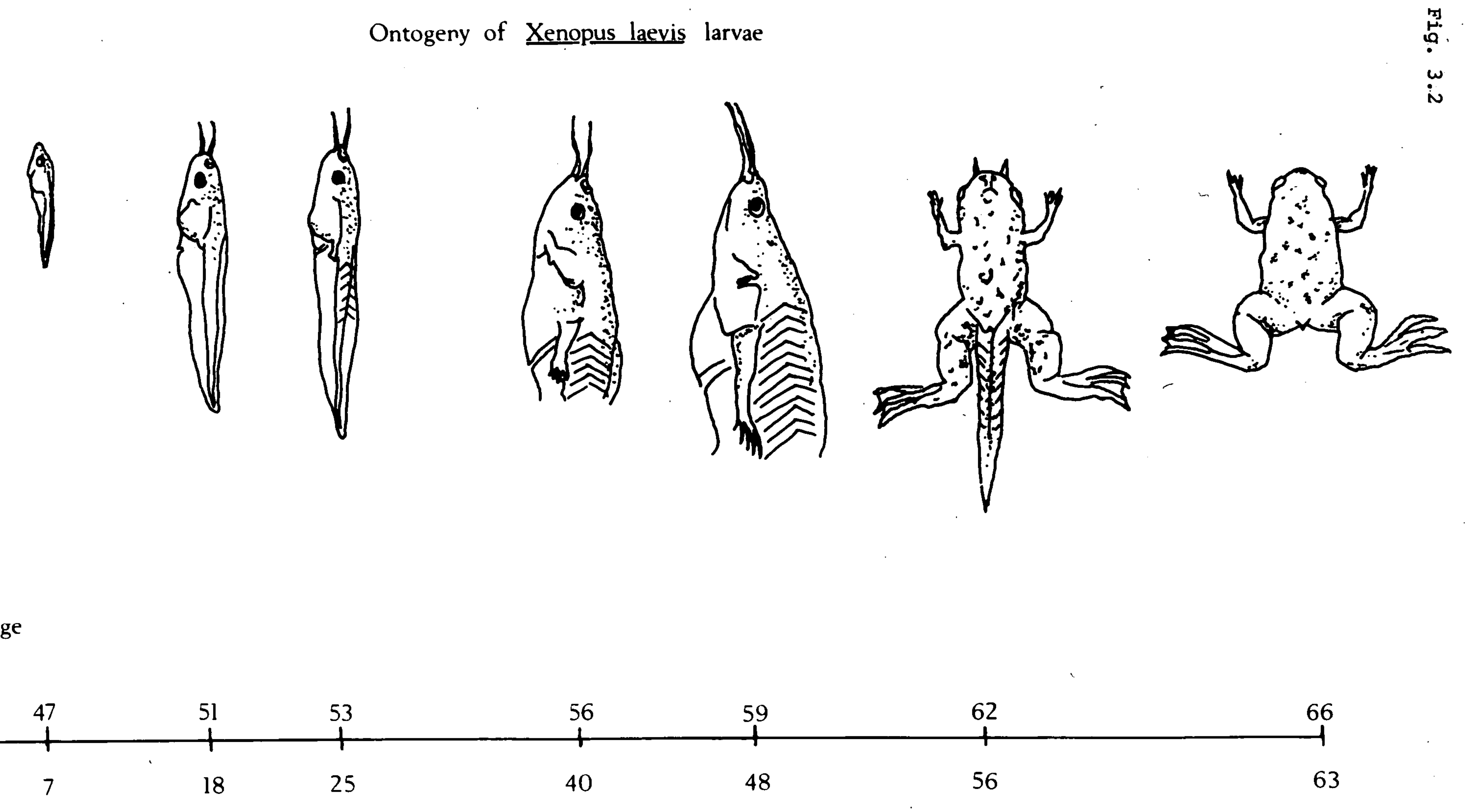
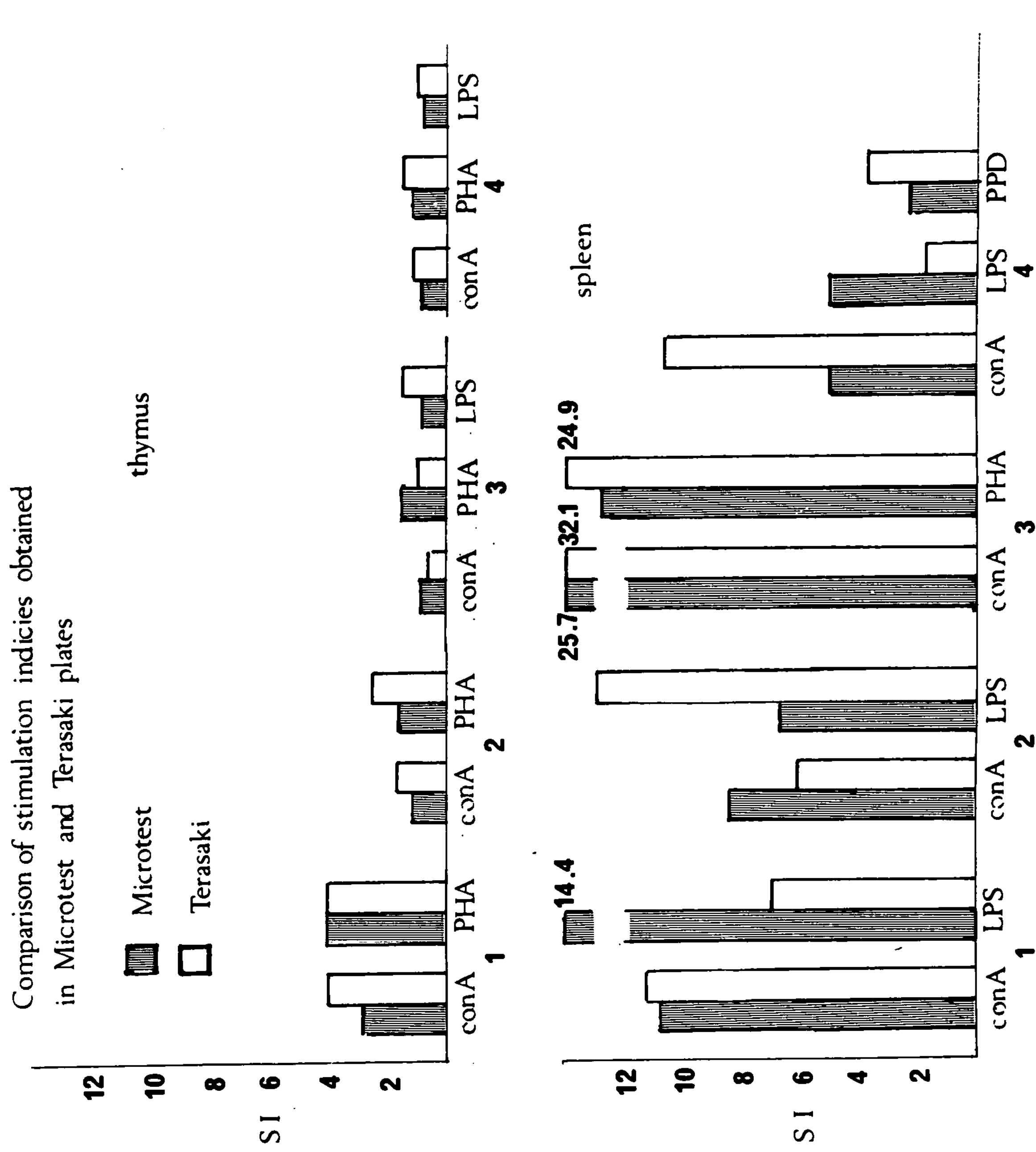


Fig. 3.3



Figs. 3.4 - 3.6

Each point represents the mean S.I. ($\pm$ S.E.). For number of animals used, see Tables 3.2-3.10.

Fig. 3.4

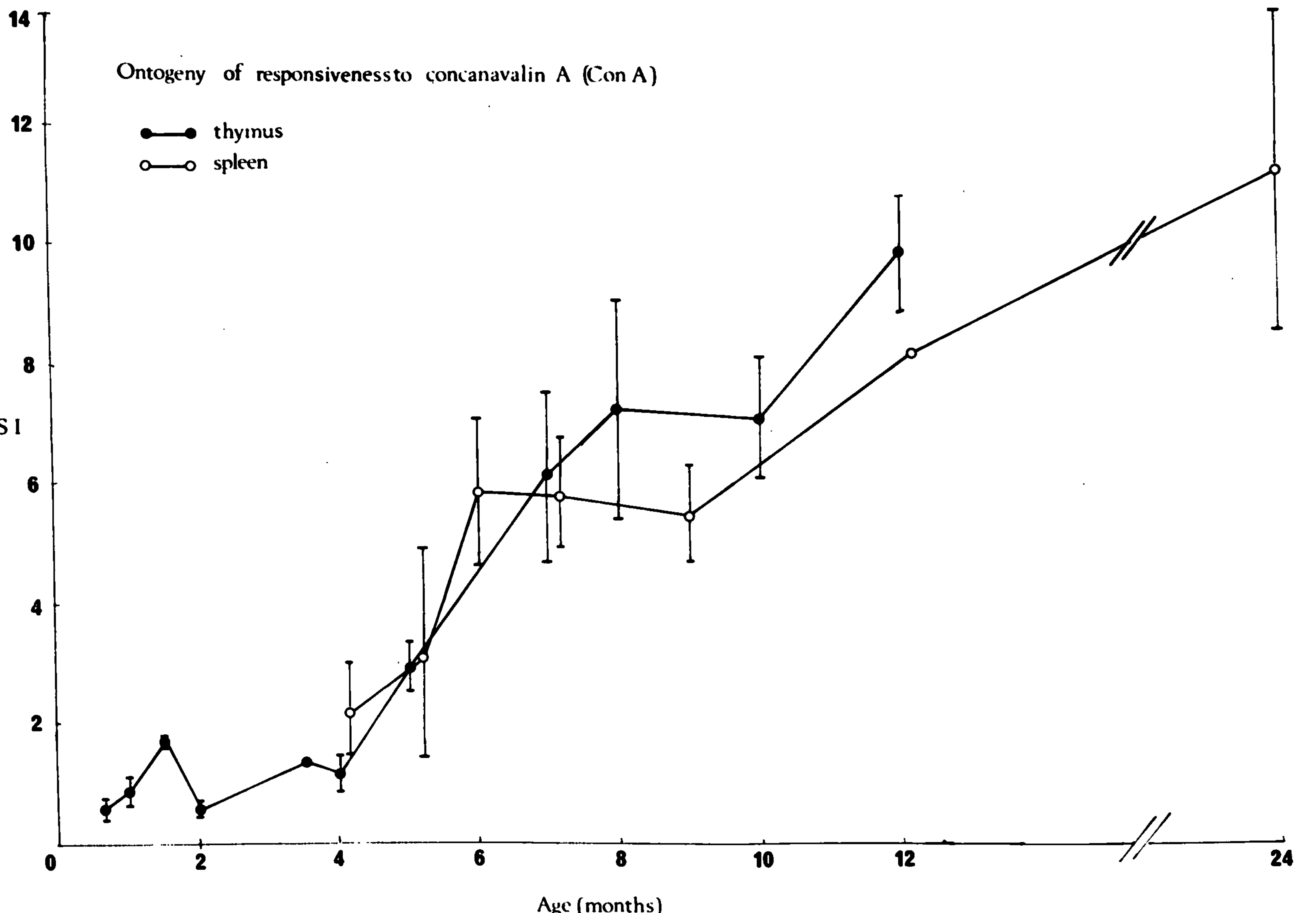


Fig. 3.5

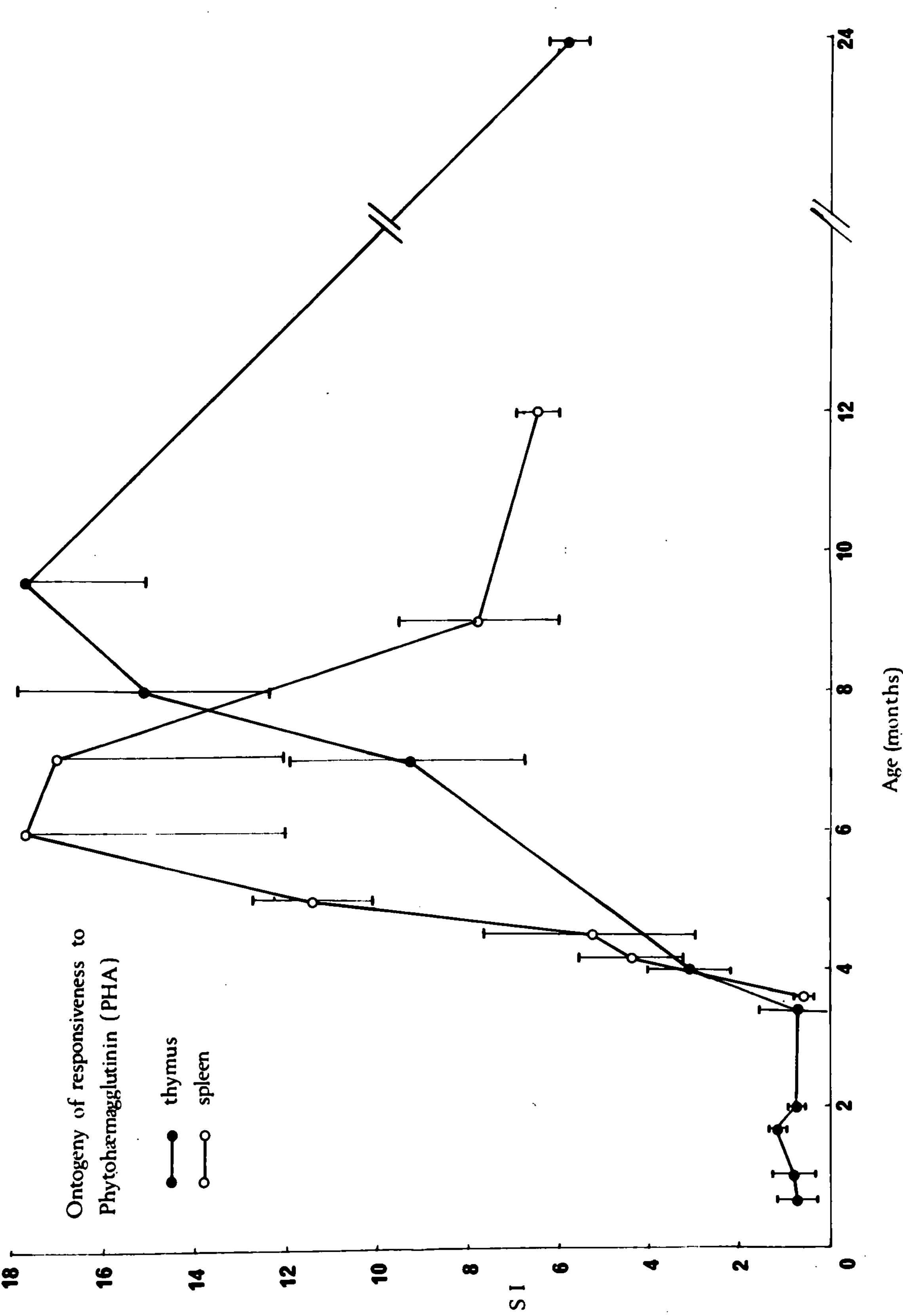


Fig. 3.6

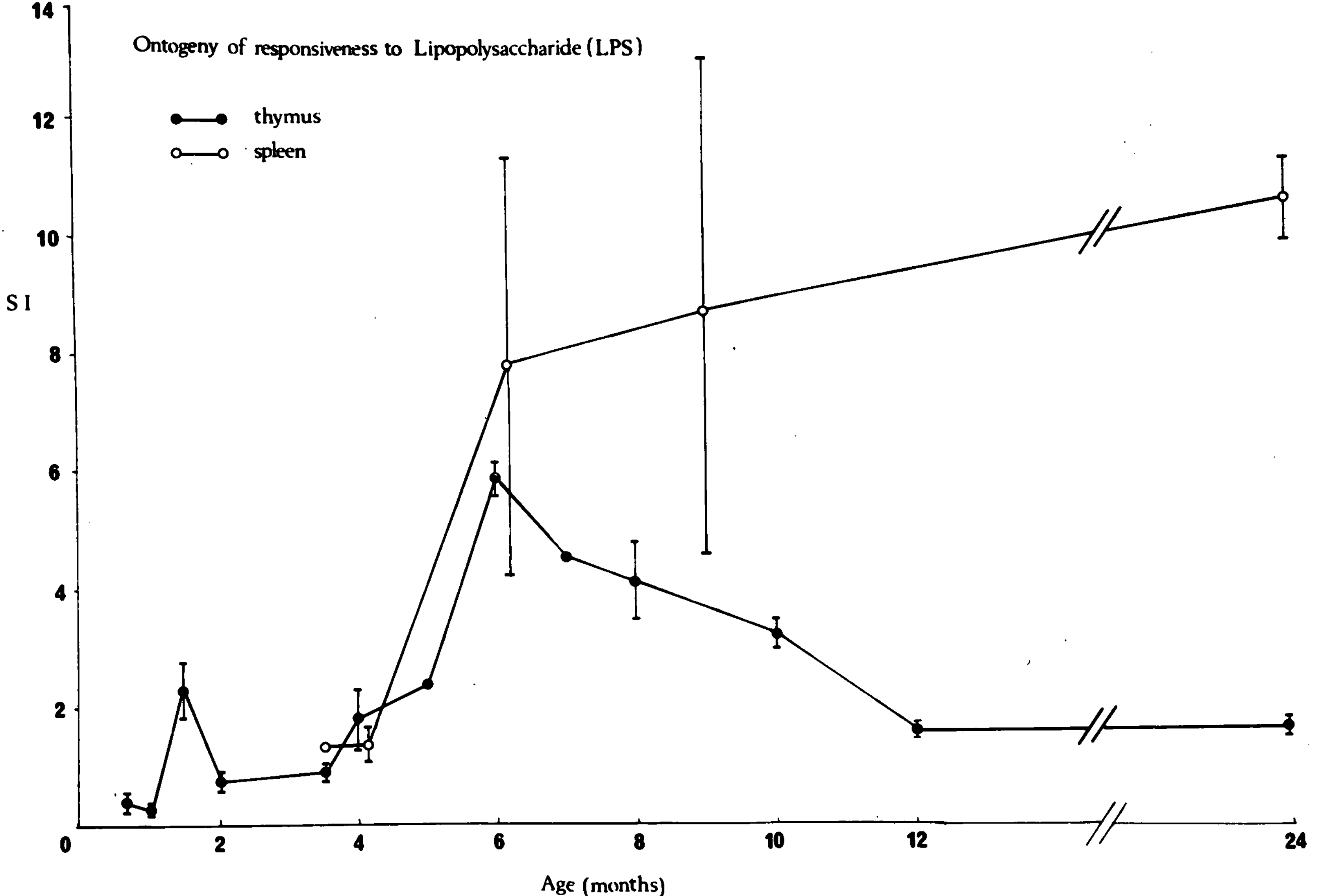


Fig. 3.7

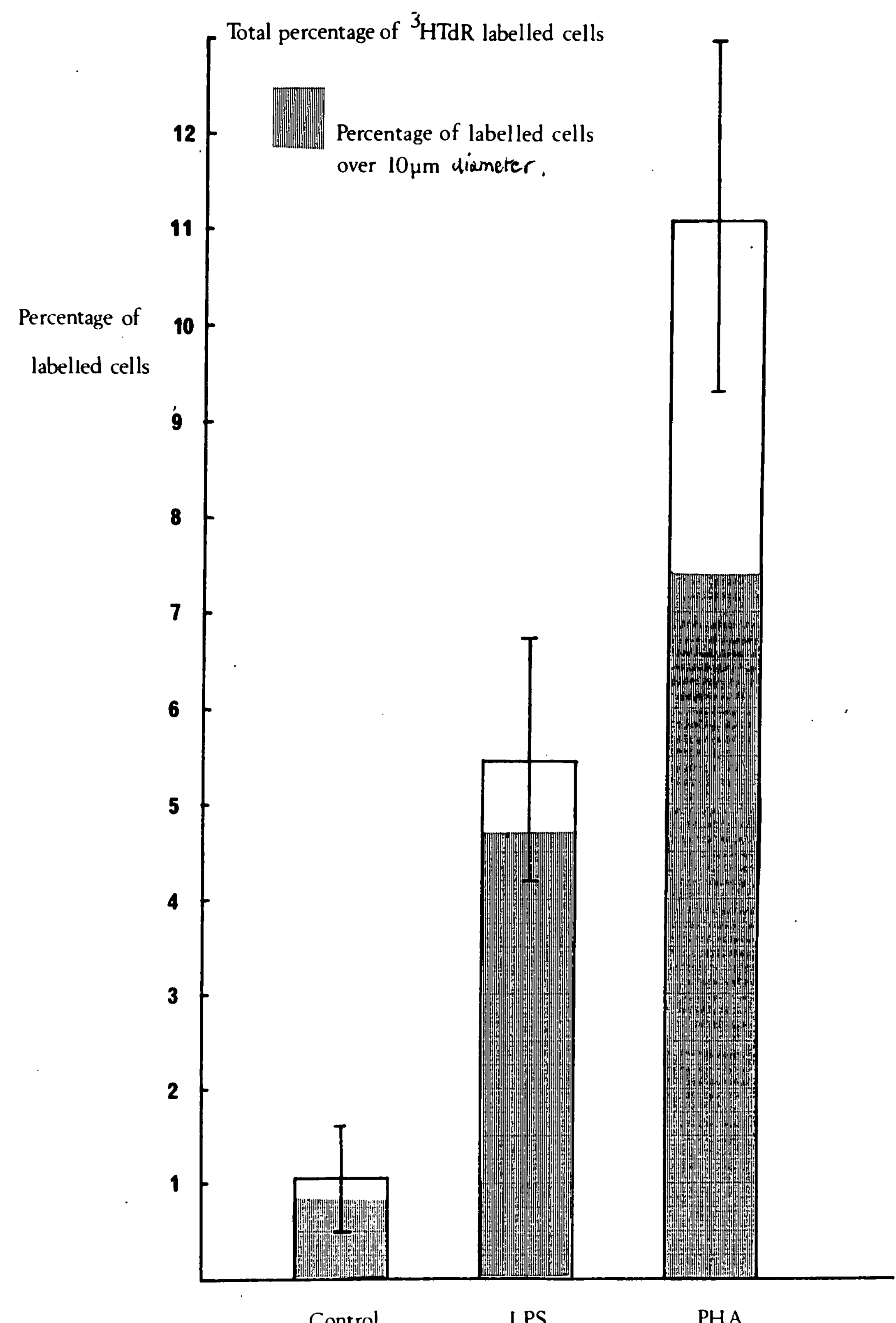


Fig. 3.8

Autoradiographs of control and mitogen stimulated thymus cells from 6 month old animals.

(a) Control cells showing background levels of labelling in the absence of mitogen. (L-labelled cell).

(b) Thymus cells stimulated with LPS. Some clumping of the cells has occurred. Several thymocytes with exposed silver grains can be clearly seen.

(c) Thymus cells stimulated with PHA. Considerable agglutination has occurred. ^{3}HTdR incorporation is extensive, with many thymocytes silver-labelled.

(d) Higher magnification micrograph of PHA-stimulated thymocytes.

Note that the majority of labelled lymphocytes are blast cells. Most blasts are heavily-labelled, while others possess fewer exposed silver grains.

a

L

b

c

1cm = 50μm

d

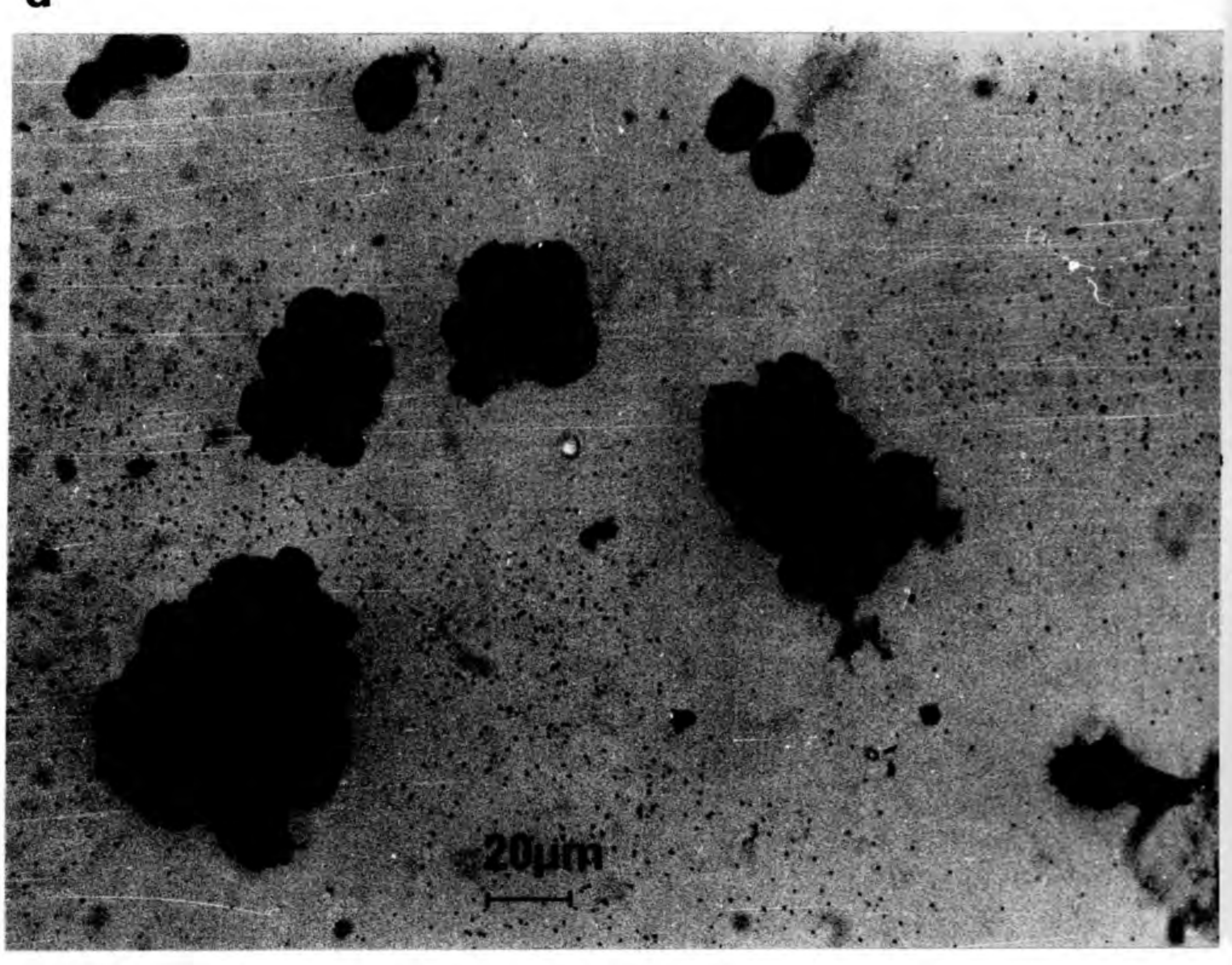


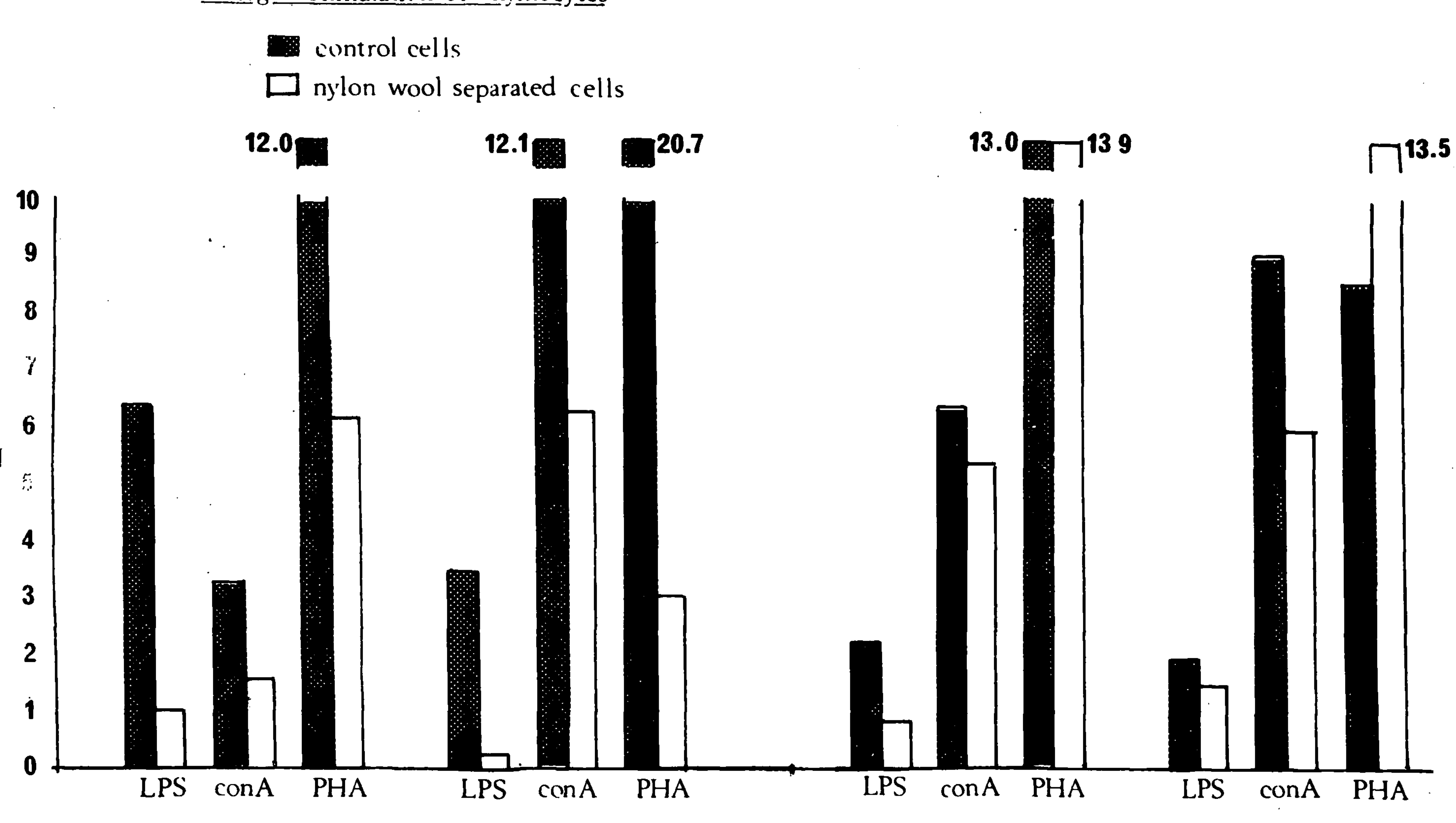
Fig. 3.9

All mitogens were used at the optimum doses used for the ontogenetic studies. Cultures were supplemented with 1% FCS.

Animals 1 and 2 were 6 months old.

Animals 3 and 4 were 12 months old.

Viability of separated cells was comparable to control cells.


Mitogen stimulation of thymocytes
control cells
nylon wool separated cells
12.0
12.1
20.7
13.0
13 9
13.5
10
9
8
7
6
S I
4
3
2
1
0
LPS
conA
PHA
LPS
conA
PHA
LPS
conA
PHA
LPS
conA
PHA
1
2
3
4

CHAPTER 4

Mitogen and mixed lymphocyte culture responses following thymectomy : Studies investigating putative T lymphocyte heterogeneity

Introduction

In this Chapter a different approach to the study of the ontogeny of thymus function in the *Xenopus* larvae is used and two issues, emanating from previous studies on thymectomised *Xenopus*, are investigated. A few years ago it was demonstrated that following thymic ablation by microcautery at 7 or 8 days (Horton & Manning, 1972; Rimmer & Horton, 1977), and even as early as 5 days of age (Horton and Horton, 1975)(when the thymus contains, in total, <500 cells), a chronic first-set allograft rejection eventually occurs, usually several months post-grafting at 23°C. Moreover, second-set grafts, applied at various intervals (3-112 days) after first-set rejection, were rejected rapidly within 2-3 weeks by these early-thymectomized (tx) *Xenopus*. It was suggested that this rejection capacity is probably achieved by a thymus-independent component, possibly one that is not normally active in the non-tx animal, or only of secondary importance. However, thymectomy experiments performed at even earlier stages by others (Tochinai and Katagiri, 1975; Tompkins and Kaye, 1981) point to the distinct possibility that very early seeding from the thymus of a few lymphocytes might have occurred prior to thymectomy at 5-8 days of age. If this were true, the memory response displayed by early-tx toads could well be achieved by expansion of this small T cell population, possibly induced by histocompatibility antigens of the grafted skin. In an attempt

to investigate whether such restoration occurs, experiments were set up to look for the reappearance of T cell markers, such as reactivity to the T cell mitogens (PHA and Con A), in allografted, early-tx toads, since these markers are virtually undetectable in non-grafted, early-tx animals (Horton and Sherif, 1977; Manning and Collie, 1977; Du Pasquier and Horton, 1976; Manning, Donnelly and Cohen, 1976; Green and Cohen, 1979). The investigation concentrates on splenic lymphocytes, but peripheral blood lymphocytes (PBL) are also used, since it has previously been shown (Horton, Horton and Rimmer, 1977) that the spleen is possibly not centrally involved in skin graft destruction in tx animals (i.e. may not be a site that houses any restored T cell population), in contrast to its central involvement in alloimmunity in control toads.

The second part of this investigation concerns a re-examination of the effects of later larval thymectomy on *in vitro* proliferative responses. In a study of the effect of sequential thymectomy on the ability of *Xenopus* splenocytes to display mixed lymphocyte culture (MLC) reactivity and to respond to T cell mitogens, Horton and Sherif (1977) indicated that the thymus may be required for a longer period of larval life to establish mitogen responsiveness than is necessary for the establishment of MLC reactivity. For example they showed that thymectomy at 3-4 weeks of age (stages 52-54) no longer abrogates splenic MLC reactivity(compared to the effect of 7 day thymectomy), whereas PHA reactivity of spleen cells taken from other 3-4 week-tx toadlets was abolished. They suggested the possibility

that MLC and PHA reactive lymphocytes in _Xenopus_ may therefore be distinct T cell populations. In order to determine more rigorously whether MLC positive lymphocytes, that are PHA negative can be detected within the spleen, splenocytes from _individual_ toadlets thymectomized at 3-4 weeks of age were here tested in both MLC and mitogen assays. Mitogen studies on PBL in these animals were also carried out to examine whether other peripheral lymphocyte populations also require a prolonged thymus presence _in vivo_ to permanently establish their PHA reactivity.

Materials and Methods

(a) Thymectomy

Thymectomy was performed by microcautery, following the technique described by Horton and Manning (1972), either at stage 47 (1 week) or at stages 52-54 (3-4 weeks). At one week of age the thymus is a small translucent organ, approximately 100μm in diameter, thymus lymphocyte differentiation has begun, as revealed by light and electron microscope studies (Horton and Manning, 1972; Nagata, 1977; Rimmer, 1977), but few small lymphocytes are found. By 3-4 weeks of age the thymus has enlarged to a diameter of about 1mm, displays cortico-medullary differentiation, and contains large numbers of small lymphocytes (particularly in the cortex). Thymic ablation was confirmed when the animals were killed. Non-operated animals served as controls.

(b) Grafting

The grafting technique of Horton and Manning (1972) was followed. Dorsal skin ($2mm^2$) from a non-sibling donor was transplanted.

After grafting the toadlets were kept at 26°C to encourage more rapid allograft rejection in the tx animals than occurs at 23°C.

(c) Separation of PBL

A mixture of 34% Triosil ("Isopaque," Vestric) and 9% Ficoll 400 (Pharmacia) was prepared which gave a final density of 1.094. This mixture was then distributed into aliquots, autoclaved, stored in the dark at 4°C until use. Blood was collected under sterile conditions from anaesthetised animals by cardiac puncture, and was transferred by pipette into amphibian L 15 medium containing 20 units heparin (Roche) cm^{-3}. The blood/medium mixture was made up to 2 cm^3 by adding more medium and was then carefully layered onto 2 cm^3 of the Ficoll/Triosil mixture in a 10 cm^3 centrifuge tube. This was centrifuged for 2 minutes at 160x g. The top 1 cm^3 of medium was then discarded and the bottom 1 cm^3 containing lymphocytes at the interface was carefully pipetted off. The cells were finally washed 3 times in medium, prior to their use in the mitogen assays.

(d) Mixed lymphocyte culture

Preparation of cells for MLC is similar to that described for the standard mitogen culture in Chapter 2. Control cultures contained 40μl spleen cells from one animal at a concentration of 5×10^6 lymphocytes cm^{-3}. The mixed lymphocyte cultures used 20μl of cells from each animal and consisted of splenocytes from either 2 control or 2 tx animals. Control and thymectomized

toadlets belonged to the same family. In addition to 1% FCS supplementation, 10µl *Xenopus* serum (final concentration 0.5%) was added. *Xenopus* serum has been shown to lower background counts, so resulting in better S.I.'s (Du Pasquier and Horton, 1976). Pilot experiments tested the effect of 3 different pools of *Xenopus* serum on MLC reactivity and the best pool was used throughout all experiments described here. The cultures were pulsed at 3 days with 1 µCi ^{3}HTdR and cells harvested 18-24 hours later, prior to processing for scintillation counting. Stimulation indices were calculated by dividing mean cpm of mixed cultures by the average of the mean cpm of the corresponding control culture values. S.I.'s were considered positive when they reached 1.5.

(e) Experimental design

(i) Preliminary studies on mitogen stimulation of PBL

The ability of PBL to respond to PHA and LPS with our culture technique had to be assessed prior to the use of these cells in the 2 major investigations. The standard culture technique was used and involved separated PBL at 5×10^{6} cm^{-3} with 1% FCS supplementation. Both 7-day-tx and control toadlets (8-10 months old) were used. PHA was added at a final concentration of 20 µg cm^{-3} and LPS at 2 mg cm^{-3}. LPS was used primarily to check for functional viability of lymphocytes in tx animals.

(ii) Mitogen studies on lymphocytes from control and thymectomized alloimmune toadlets.

Control and 7-day-tx toadlets were grafted when 8-10 months old. (Grafts for tx hosts came from control or tx donors but this did not alter their fate!) Grafts were inspected every 2-3 days.

Eighteen weeks post-grafting splenocytes and PBL were examined for mitogen reactivity by standard culture technique. The following concentrations of mitogens were used:- PHA - 20μg cm^{-3}; Con A - 1 μg cm^{-3}; LPS - 2 mg cm^{-3}. The 18 week interval between grafting and the mitogen experiments was selected because second-set grafts applied to early-tx animals at this post-transplantation interval should be rejected rapidly - i.e. any T cell restoration occurring should be detectable by this time.

(iii) Mitogen and MLC studies on lymphocytes from toadlets thymectomized at 3-4 weeks of age.

Animals used in these studies were 8 months old. PBL were used only for mitogen experiments and were cultured with the standard technique. Splenocytes from individual animals were usually tested for mitogen reactivity (miniaturised technique) and in MLC (standard technique). LPS was again used to check functional lymphocyte viability. Doses of mitogen used were as in (i) above.

Results

(a) Preliminary studies on mitogen stimulation of PBL

Results are illustrated in Table 4.1 and Fig. 4.1. They show that although the response of PBL from control toadlets to PHA was generally low (mean S.I. $\pm$ S.E. = 5.5 $\pm$ 2.8), all the cultures gave positive S.I.'s. In contrast PHA stimulated PBL cultures on thymectomized animals gave negative S.I.'s throughout (mean S.I. = 0.9 $\pm$ 0.2. With LPS both control and tx PBL gave positive S.I.'s (means of 6.0 $\pm$ 3.4 and 12.0 $\pm$ 3.0 respectively). These results confirm that responsiveness to T cell mitogens is abrogated by early tx, whereas reactivity to B

mitogens is left intact. The data is in agreement with recent findings (Green and Cohen, 1979) on mitogen reactivity of tx *Xenopus* PBL.

(b) Mitogen studies on lymphocytes from alloimmune toadlets

Counts per minute are given in Table 4.2. S.I.'s and graft rejection times are illustrated in Fig. 4.2. At 26°C the 7 control toadlets on average rejected allografts in 16 $\pm$ 0.8 days. The 7 tx animals took, on average, nearly three times as long (43 $\pm$ 8.3 days).

Following graft rejection, splenocytes from controls displayed good levels of reactivity to both PHA and Con A (S.I.'s were 15.4 ($\pm$4.4) and 7.4 ($\pm$3.2) respectively. PBL from these animals responded well to both PHA (mean S.I. = 6) and LPS (mean S.I. = 12.2). All the spleens from early-tx allografted toadlets failed to respond to Con A (mean S.I. = 0.5 ($\pm$0.2)). Reactivity to PHA was also abolished in spleen cell cultures from 4 out of 5 animals (mean S.I. = 0.9 $\pm$ 0.3), the other showing a dramatically impaired response (S.I. = 1.8). The possibility that sites other than the spleen could contain restored populations of T-mitogen-reactive cells seems unlikely in view of the results with PBL from 2 tx allografted animals. Thus PBL from these failed to respond to PHA, although they showed good mitogen reactivity to LPS. It appears, then, that allograft rejection occurs in tx animals in the absence of lymphocytes able to respond to T cell mitogens.

(c) MLC and mitogen studies on lymphocytes from toadlets thymectomized at 3-4 weeks of age

MLC data for splenocytes of control and tx animals is given in Table 4.3. All 9 allogeneic combinations between controls resulted in enhanced ^{3}HTdR uptake compared with background (autologous) cultures (mean S.I. $\pm$ S.E. = 3.4 $\pm$ 0.5; range 1.5-5.9). In contrast, only half (6/12) MLC combinations between the 11 tx animals used displayed positive S.I.'s (overall the mean S.I. was 1.6 $\pm$ 0.3, ranging from 0.6-3.4). It appears that in these experiments thymectomy during mid-larval life has impaired the normal maturation of MLC-reactive lymphocytes in some, but not all, toadlets.

Mitogen responses of splenocytes and PHA of 8 of these same tx animals are given in Table 4.4. Only 3/8 splenocyte cultures responded to PHA (mean S.I. = 2.2 $\pm$ 0.6), the best S.I. being 6.0. Likewise only 2/7 PBL cultures displayed PHA reactivity (mean S.I. = 1.1 $\pm$ 0.3). Reactivity of splenocytes and PBL to LPS was, as expected, unaffected by mid-larval thymectomy (mean S.I.'s were 7.6 $\pm$ 1.9 and 9.4 $\pm$ 1.9 respectively).

Since only one individual case of positive MLC reactivity (animals 10 x 11) but negative PHA reactivity of both partners occurred in the tx group, it seems that tx at 3-4 weeks of age has here impaired both PHA and MLC reactivities to such an extent that the experiments provide no clear evidence that these functions are afforded by distinct T lymphocyte populations.

Discussion

One major objective of this Chapter was to determine whether lymphocyte reactivity to T cell mitogens occurs in early-

tx animals that have rejected skin allografts. Responsiveness to T cell mitogens is often considered to be a hallmark of all T cell populations, one which transcends their specific immunologic function. Indeed T cell mitogens are widely used as an indicator that competent T lymphocytes are present in an individual (see Chapter 3). In the present experiments on tx *Xenopus*, these mitogens have been used to investigate the possibility of a general restoration of T-equivalent cells.

Considering first the speed of graft rejection in the 7-day-tx toadlets, it is interesting to note that keeping the animals at 26°C rather than 23°C after transplantation considerably reduces the time taken to effect graft rejection (cf. Rimmer and Horton, 1977). Elevating the temperature by 3°C results in a more rapid and uniform alloimmune rejection in early-tx toads. Perhaps acute graft rejection at 23°C is critically dependent on amplifier T cells (MLC-positive lymphocytes are absent from tx, non-grafted animals - see Du Pasquier and Horton, 1976), whereas at higher temperatures these cells assume less importance.

Early-tx animals that have rejected grafts were shown to be unresponsive to 2 T cell mitogens, at least with respect to their splenocytes and PBL. As these peripheral populations of lymphocytes responded well to LPS, the non-reactivity seems specific for T, rather than B cell mitogens. The mitogen assays were performed 18 weeks post-first-set grafting - i.e. at a time when second-set grafts should be rejected rapidly -

therefore it seems likely that even acute allograft destruction in tx animals is effected by lymphocytes that are not able to respond to PHA or Con A. This interpretation fits well with previous sequential tx data, where thymic ablation as late as 30 days still abrogates splenic PHA reactivity, but leaves allograft rejection capacity to develop quite normally (Horton and Manning, 1972; Horton and Sherif, 1977; Manning and Collie, 1977).

It will now be important to determine whether MLC reactivity (particularly towards donor MHC antigens) is restored following allograft destruction in tx animals. If it is not, then in order to argue that allograft immunity in early-tx toadlets is mediated by a thymus-dependent lymphocyte subset (rather than a thymus-independent component), one would have to suggest that this is achieved by an early-seeded T-equivalent cell population that is PHA and MLC negative. This line of research should be a useful approach in determining whether cytotoxic T lymphocytes (recently demonstrated in *Xenopus* by Bernard, Bordmann, Blomberg and Du Pasquier, 1979) represent a separate T-equivalent subset from MLC- and PHA-responsive lymphocytes in amphibians. Interestingly it has been shown in mammals that MLC reactive populations can respond to PHA and Con A, but that certain alloreactive T cells are non-responsive to these T cell mitogens (Watenabe *et al.*, 1977). Indeed Häyry *et al.* (1976) have found, by long term selective culture, that there is only a small overlap between these functions in mice.

The second major investigation performed in this Chapter involved a fresh look at the possibility that thymectomy in mid-larval life could reveal that T-mitogen-reactive cells are, at least

to some extent, a population of lymphocytes distinct from MLC-reactive cells. However the studies on *in vitro* proliferative behaviour of lymphocytes from toadlets tx at 3-4 weeks of age have provided no clear distinction between these two populations in terms of their degrees of thymus-dependency. The establishment of both PHA- and MLC-reactive lymphocyte populations in the periphery would appear to require a prolonged thymus presence *in vivo* compared with the more rapid, but equally gradual, establishment by the thymus of *in vivo* alloimmune reactivity (with respect to the latter, see Tompkins and Kaye, 1981). Although a distinction between the duration of thymus-dependence of MLC and PHA reactive populations was suggested by Horton and Sherif (1977) (as noted in the Introduction), these workers did indicate some impairment of MLC responses regularly occurs following thymectomy at the stages of development studied here.

Further investigations on sequential thymectomy and MLC reactivity should make use of defined strains of *Xenopus*, using MLC combinations where the mixed lymphocytes come from toadlets with strong MHC differences. Under such conditions MLC reactivity of control cells would be far more uniform than seen here in experiments which, unfortunately, had to make use of sibling combinations. In *Xenopus*, antigens stimulating in the MLC reaction segregate as if controlled by a single genetic locus, and acute graft rejection seems to be governed by this same region. This polymorphic genetic locus has been called the XLA MHC system (Du Pasquier and Kobel, 1977) and bears certain homology with mammalian MHC's. In *Xenopus*, some erythrocyte antigens segregate with MLC determinants, while others belong to different linkage groups (Du Pasquier and Kobel, 1977). As in endotherms, there

must also be minor HC antigens in *Xenopus*, since MLC identical toadlets are still capable of rejecting grafts exchanged between each other. Du Pasquier and Kobel (1977) have shown that graft rejection times can be predicted by MHC haplotype differences, and that the level of MLC reactivity is also related to this MHC disparity (2 haplotype differences giving greater MLC S.I.'s than 1 haplotype difference). This linkage between MLC reactivity and speed of allograft rejection is discussed by Cohen and Collins (1977) who conclude that there is some correlation between these two functions throughout all vertebrates so far investigated, although Cohen and Horan (1977) found no such correlation in the newt.

TABLE 4.1 **Mitogen stimulation of peripheral blood lymphocytes in non-grafted toadlets : the effect of 7-day thymectomy**

Controls

PHA 20μgcm^{-3}

Background counts per min.	Stimulated counts per min.	SI
124	284	2.3
377	1604	4.3
78	1370	22.0
630	1037	1.7
593	1147	1.9
626	2067	3.3
146	464	3.2
	Mean ± S.E.	5.5 ± 2.8

LPS 2 mg cm^{-3}

Background counts per min.	Stimulated counts per min.	SI
680	2220	3.2
146	1885	12.8
593	1213	2.1
	Mean ± S.E.	6.0 ± 3.4

7-day thymectomized

PHA 20μgcm^{-3}

Background counts per min.	Stimulated counts per min.	SI
599	675	1.1
1241	195	0.2
1315	1196	0.9
1180	1159	1.0
457	726	1.5
657	558	0.9
	Mean ± S.E.	0.9 ± 0.2

LPS 2 mg cm^{-3}

Background counts per min.	Stimulated counts per min.	SI
599	16852	28.1
1241	8570	6.9
183	1928	10.3
1315	17108	13.0
1180	6350	5.4
457	2399	5.2
1967	2945	15.3
	Mean ± S.E.	12.0 ± 3.0

TABLE 4.2 **Mitogen stimulation of splenocytes from control and 7-day thymectomized toadlets following graft rejection**

Controls

	Unstimulated cpm	PHA stimulated cpm	Con A stimulated cpm
1	4034	42765	30176
2	3193	16818	13308
3	438	11960	7120
4	841	20665	nd
5	1428	13261	2122

7 day thymectomized

	Unstimulated cpm	PHA stimulated cpm	Con A stimulated cpm
1	188	185	181
2	2807	1427	593
3	2790	802	623
4	1426	1639	287
5	327	597	253

TABLE 4.3 The effect of thymectomy at stage 52-54 on MLC reactivity of splenocytes

CONTROLS

	1	2	3
1	3275	20272 (4.7)	18612 (3.7)
2		5328	9260 (1.5)
3			6919

	4	5	6
4	897	3027 (2.5)	2793 (2.8)
5		1560	4903 (3.7)
6			1101

	7	8	9
7	2938	56921 (5.9)	8361 (4.5)
8		16295	13428 (1.6)
9			802

THYMECTOMIZED

	1	2	3
1	271	709 (3.1)	794 (0.9)
2		182	1466 (1.9)
3			1356

	4	5	6
4	1053	5035 (3.4)	6512 (1.1)
5		1930	8283 (1.3)
6			10677

	7	8	9	10	11
7	133	259 (0.7)	nd	nd	1780 (0.9)
8		446	nd	nd	1159 (0.6)
9			5257	8074 (2.0)	7321 (1.5)
10				2428	5702 (1.8)
11					3864

S.I.'s given in parentheses

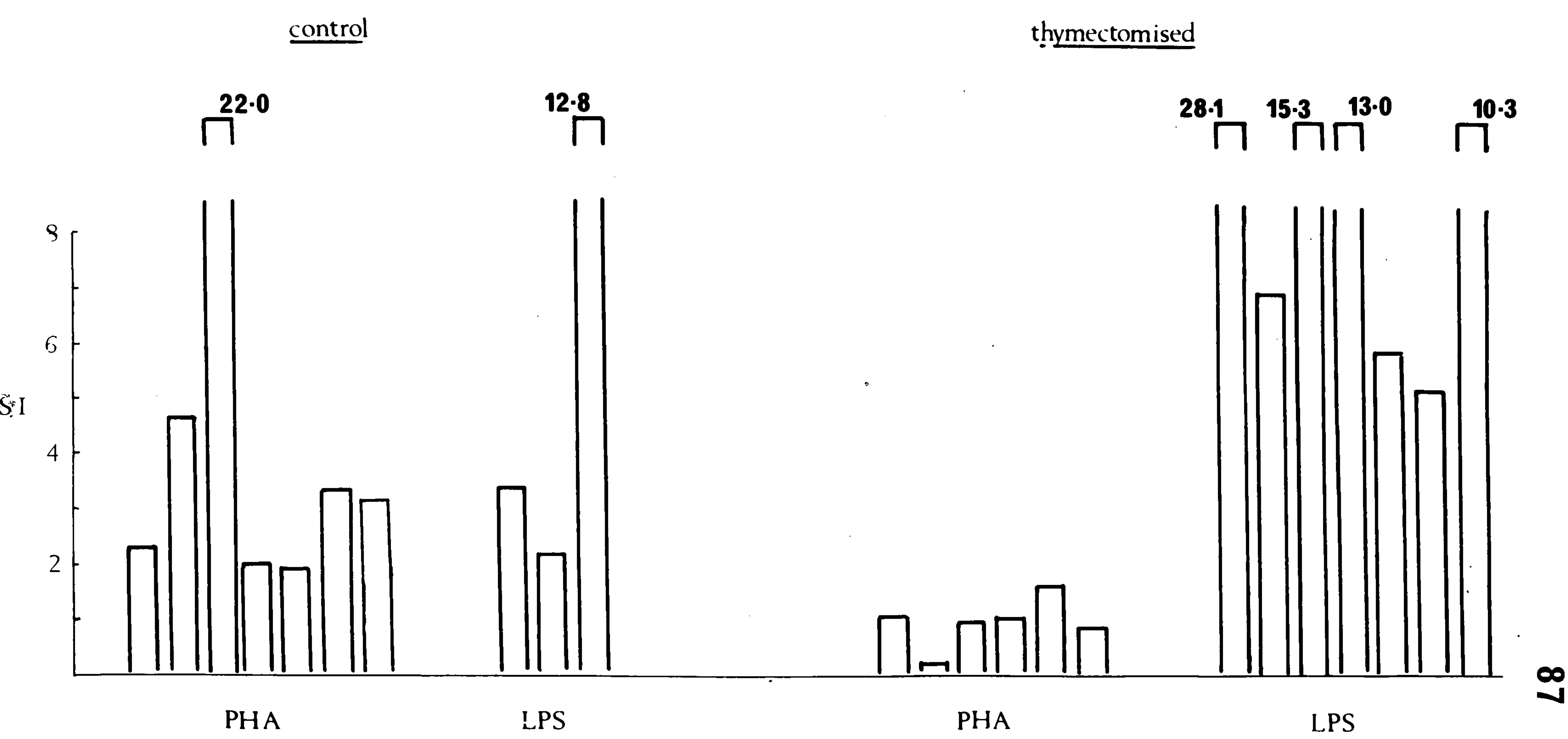
TABLE 4.4 The effect of thymectomy at stage 52-54 on reactivity of splenocytes and PBL to PHA and LPS

Animal No.	Spleen		Blood	
	PHA	LPS	PHA	LPS
4	1.9	11.9	0.9	3.3
5	1.2	8.4	2.6	17.7
6	1.1	5.9	0.8	10.6
7	3.9	0.5		
8	1.1	1.3	1.6	5.2
9	6.0	17.6	0.2	10.3
10	1.3	7.8	0.9	13.0
11	1.3	7.6	1.0	5.4

Animals 4-11 are the same tx toadlets as in the MLC experiments in Table 4.3.

Fig. 4.1

Mitogen responsiveness of peripheral blood leucocytes (PBL) from control and thymectomized animals. PHA was at 20μgcm^{-3} and LPS 2.0mg cm^{3}. One per cent FCS supplementation was used throughout.



control
thymectomised
22·0
12·8
28·1
15·3
13·0
10·3
SI
8
6
4
2
PHA
LPS
PHA
LPS

Fig. 4.2

Mitogen responsiveness of control and thymectomized animals that have rejected allografts.

spleen cells

PHA at 20µgcm^{-3}

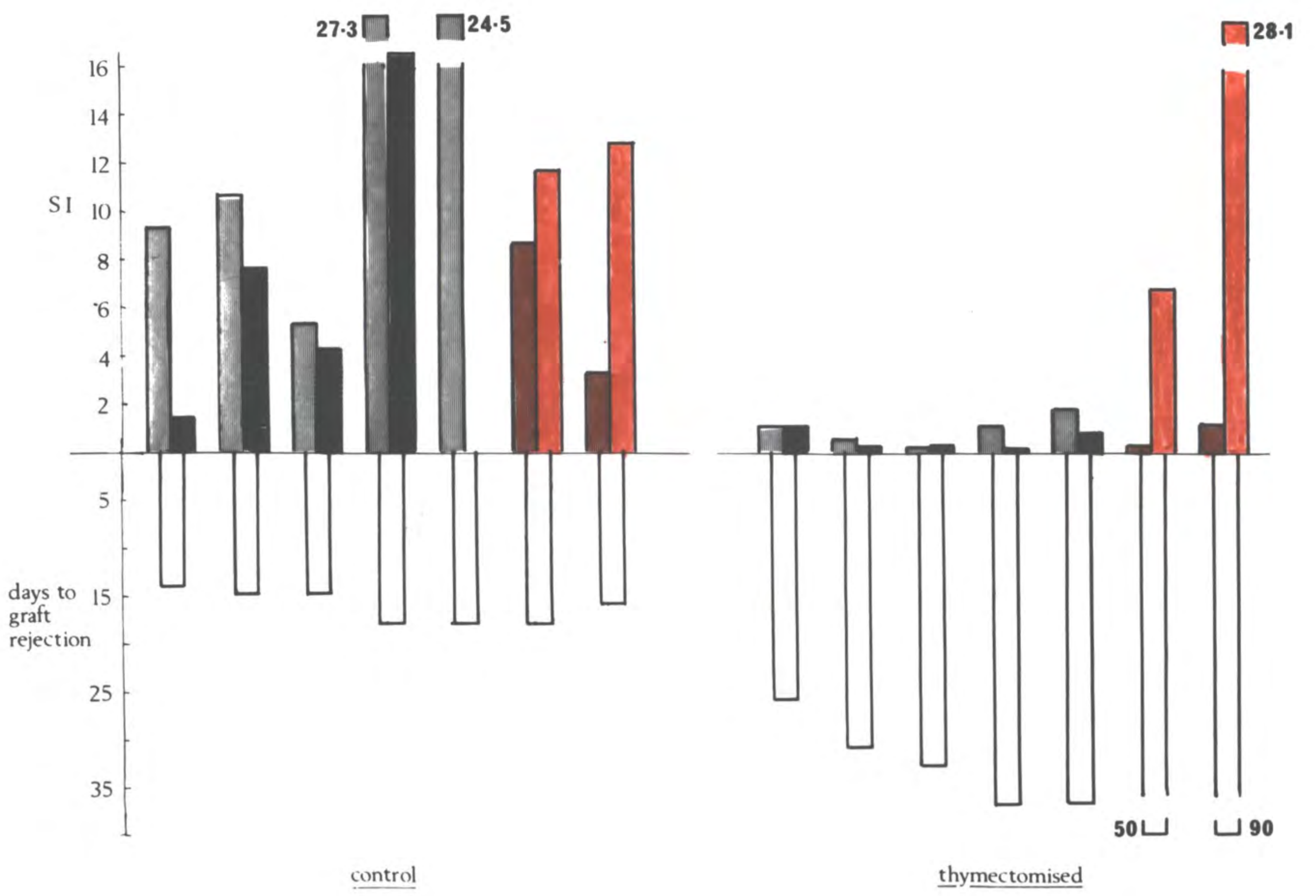
Con A at 1.0µgcm^{-3}

LPS at 2 mg cm^{-3}

peripheral blood leucocytes

LPS at 2mg cm^{-3}

PHA at 20µgcm^{-3}


27·3
24·5
28·1
SI
16
14
12
10
8
6
4
2
5
days to graft rejection
15
25
35
50
90
control
thymectomised

CHAPTER 5

Ontogeny of in vivo lymphocyte reactivity to the hapten trinitrophenyl conjugated to a thymus-dependent and thymus independent carrier.

Introduction

Central to the functioning of all immune systems is the ability to recognise antigenic determinants, and crucial to this initial recognition phase is the binding of antigen to specific receptors on the surfaces of cells of the immune system. An immune response to circulating antigen can be characterised by an increase in both number of lymphocytes capable of binding antigen and the affinity of this binding capacity. Although some antigens stimulate B cells directly to produce antibody, most ('thymus-dependent') antigens require the involvement of helper T cells before a response can be elicited. The importance of this collaboration between T and B cell was first investigated in thymectomized and restored mice, using xenogeneic erythrocytes as a thymus-dependent antigen (Claman _et_. _al_, 1966; Davies, _et al_, 1967; Mitchell and Miller, 1968). Hapten-carrier experiments have also been centrally involved in this respect (see Mitchison, 1971). Lymphocyte collaboration has since been demonstrated (through hapten-carrier studies) on diverse vertebrates that include teleost fish (Yocum _et al_., 1975; Stolen & Makela, 1975, 1976; Ruben, Warr, Decker & Marchalonis, 1977), urodeles (Ruben, van der Hoven & Dutton, 1973) and anuran amphibians (Ruben, 1975; Ruben & Edwards, 1980).

This chapter exploits the hapten-carrier system to investigate the _in vivo_ ontogeny of T-helper cell activity and B-equivalent

lymphocytes in the *Xenopus* spleen. In the present studies sheep red blood cells (SRBC) have been used as a thymus-dependent carrier, and LPS as a thymus-independent carrier for the hapten trinitrophenyl (TNP). Recent studies on *Xenopus* (Horton, Edwards, Ruben and Mette, 1979) have confirmed the thymus-dependent/independent status respectively of these two carriers. Carrier pre-immunisation is required to achieve good levels of anti-TNP responses following TNP-RBC injections. In contrast TNP-LPS requires no such carrier pre-immunisation and so one can examine functional B cell ontogeny directly.

While at metamorphosis many anuran immune responses seem to decline or disappear (e.g. thymocyte mitogen reactivity) (Chapter 3) it has recently been reported that *in vivo* induced antigen-binding splenocyte reactivity to a thymus-dependent hapten-carrier complex actually increases at this time (Ruben *et al.*, 1980). These authors suggest that this elevation in 'humoral' immune reactivity may be caused by an enhancement of helper T cell activity at metamorphosis, necessary perhaps as a counterbalance to the suppressed cellular immunity found at this time (Du Pasquier and Bernard, 1980). The present experiments further investigate the influence of metamorphosis on the ontogeny of humoral immune reactivity, and examine the possibility that both T- *and* B-equivalent lymphocyte reactivity is elevated at this time.

The work begins with a comparison of plaque-forming cell (PFC) responses in spleens taken from different aged animals to three antigens - SRBC, TNP-SRBC and TNP-LPS. The ontogeny of splenocyte reactivity to the 2hapten-carrier conjugates (using the more sensitive immuno-

cytoadherence (ICA) assay) is then considered in detail. The chapter ends with a consideration of the thymus as a site containing antibody-forming cells, since mitogen studies earlier in the Thesis suggest this organ contains some B-equivalent lymphocytes.

Materials and Methods

(a) Antigens and injections

Sheep red blood cells (SRBC) and horse red blood cells (HRBC) in Alsever's solution (Flow Laboratories) were washed three times in saline prior to use in injection and/or assays).

Toadlets received a single injection of 10% SRBC, 50µl per gram body weight, via the intraperitoneal (i.p.) route. Larvae were immunized with a single injection of 5µl 50% SRBC i.p. Larval injections were performed with the aid of a micropipette, drawn from a 50µl microcap (Shandon). The tip of the micropipette was inserted through the ventral tail musculature into the peritoneal cavity. When the pipette was withdrawn, the muscle prevented leakage of injected material from the peritoneal cavity.

Haptenation of erythrocytes with TNP followed the procedure of Rittenberg and Pratt (1969). TNP-SRBC for injection were prepared by adding 2.5 cm^3 of packed SRBC to 7 cm^3 cacodylate buffer (pH 6.9), containing 0.065g TNBS (BDH). The mixture was stirred constantly in a foil-covered flask for 30 minutes at room temperature, and then 15 cm^3 saline were added to stop the reaction. These 'heavily'-conjugated cells were then washed in glycyl-glycine (4.5×10^{-3}M) to remove any unbound TNP, and following this were washed three times in saline. These haptenated cells were injected at the same

concentration and dose as the unhaptenated SRBC. Carrier pre-immunisation was necessary to obtain a good RFC response to TNP-SRBC (see Results). Here 0.0025% SRBC were injected 2 days prior to the TNP-SRBC injection, 50µl (g.b.w.) of this low carrier dose for toadlets, 5µl for larvae.

TNP-LPS was prepared as described elsewhere (Jacobs and Morrison, 1975) and was injected at a concentration of 50µg cm^{-3} for toadlets (50µl g.b.w.) and 0.5mg cm^{-3} for larvae (5µl).

For the PFC and ICA assays, HRBC were used as TNP-carrier, but with a lighter haptenation protocol than used for TNP-SRBC injection; only 0.0025g TNBS was used and the reaction was stopped after 10 minutes.

(b) PFC assay

The slide haemolytic plaque assay, originally described by Cunningham and Szenberg (1968) and modified by Du Pasquier (1970) for amphibian studies, was used. Cell suspensions were prepared in culture medium as described in Chapter 2, but here chilled on ice, and adjusted to a maximum of 5 x 10^6 lymphocytes cm^{-3}. Each PFC assay mixture consisted of 200µl spleen cells mixed with 20µl of 25% assay RBC and 50µl 1:10 guinea pig serum (Wellcome) as a source of complement. Tests of several different complement batches were made and the best was selected for use throughout all the experiments. Control assays were also set up for each experiment, using 50µl medium instead of complement; these assays were uniformly negative.

The assay mixtures were prepared on ice, but then allowed to reach room temperature to prevent formation of air bubbles when inserted into the PFC chambers. After thorough resuspension, duplicate 100µl assay samples were gently pipetted into PFC slide chambers

(Wild and Dipper, 1974). Chambers were sealed with molten paraffin wax and vasoline and were then incubated for 2-3 hours at 28°C. After this time plaques could be seen macroscopically as distinct clear areas, but they were checked under the microscope. Only when a central *Xenopus* lymphoid cell could be seen surrounded by mammalian red cell ghosts was a PFC scored. PFC were expressed as the number 10^{-6} lymphocytes. Anti-TNP PFC were estimated from the difference in numbers of PFC v. TNP-HRBC minus HRBC (see Results).

(c) ICA assay

Cell suspensions were prepared exactly as described for the PFC assay. Two tubes were set up for each assay, each tube containing 10µl test RBC (1%), and 50µl of the lymphocyte suspension. Tubes were coded (to avoid bias when counting RFC's) and incubated overnight at 4°C. Following gentle resuspension, the assay suspensions were scanned microscopically using the whole .9 mm^3 of an American Optical Neubauer counting chamber. RFC were scored when more than 3 test RBC adhered to the lymphocyte membrane (see Fig. 5.3). The number of RFC, expressed as RFC/10^6 lymphocytes, was calculated from at least 4 haemacytometer counts (2 from each duplicate tube set up).

However, anti-TNP RFC were usually estimated from the difference in numbers v. TNP-HRBC-HRBC. In a few ICA experiments (see Results), the specificity of anti-TNP reactivity was checked by incubating the assay suspensions in either TNP-glycine (10^{-3}M) or glycine (10^{-3}M) alone.

(d) Experimental design

(i) Ontogeny of induced splenocyte PFC reactivity

The kinetics of the PFC response of toadlets to a single injection of SRBC were examined first to ascertain optimum times to assay. Injected animals were kept at the elevated temperature of 26°C to try and increase the uniformity of the antibody response (which had been realised with the allograft rejection studies performed in Chapter 5). Sixteen animals, aged 9-15 months, were given a single injection of SRBC and spleen cell suspensions from at least 2 separate animals were assayed on days 4,6,7,8,9,12 and 14.

These initial kinetic experiments were preliminary to a second series of experiments (again carried out at 26°C), which compared ontogeny of PFC responsiveness to the T-dependent antigens SRBC and TNP-SRBC with that occurring to the T-independent antigen TNP-LPS. For each larval assay it was necessary to pool 10-20 individual spleens (depending on age) due to the very low lymphocyte numbers. In these experiments some of the assays on toadlet spleens were performed on pooled spleen suspensions, to ensure that the larval results were not an artifact of the pooling of allogeneic cells in the PFC assay. These pooling experiments are marked in the Tables.

(ii) Ontogeny of induced antigen-binding cells in the spleen

Larval assays consisted of a pool of 10-20 spleens. Initial experiments on larvae examined TNP-SRBC immunisation schedules required for good anti TNP-HRBC binding activity in the spleen. These studies included an experiment to check whether or not

low-dose priming with the erythrocyte carrier was necessary to effect elevated levels of RFC's over background (assays from saline injected animals). Ontogenetic ICA experiments (all carried out at 23°C) with TNP-SRBC and also with TNP-LPS were then examined, with particular emphasis on events at metamorphosis. Again some of the toadlet assays were performed on pooled splenocyte suspensions, to ensure that larval antigen binding results were not an artifact of pooling allogeneic lymphocytes.

(iii) Antibody forming cells within the thymus

In the third study, the thymus itself is briefly examined as a site of PFC activity following immunisation with TNP-SRBC. Procedures were exactly as for the spleen, but each assay was prepared from an individual thymus. The thymuses came from 6 month old toadlets, which had been kept at 26°C after injection.

Results

(a) Ontogeny of induced splenocyte PFC reactivity

The results showing the kinetics of the toadlet response to a single injection of SRBC show (see Table 5.1) that moderate levels of PFC were induced in the spleen. In contrast to the absence of splenic PFC recorded in non-injected toadlets kept at 26°C, by 4 days post-SRBC injection PFC had appeared. Maximum PFC levels were found by 6-8 days post-injection, after which time the level of response declined. The kinetics and level of PFC responsiveness correspond quite well with PFC results from other poikilotherms (e.g. cyprinid fish, Rijkers and Muiswinkel, 1977).

Table 5.2 shows the results of experiments comparing the PFC responses of different aged animals to the three antigens SRBC,

TNP-SRBC and TNP-LPS. No splenic PFC to any of the antigens were seen here before 3 months of age - i.e. until post-metamorphic life. By 4 months of age (two months after the end of metamorphosis) small numbers of PFC were found to SRBC, and by 8 months, adult anti-SRBC PFC levels were seen. With regard to TNP-SRBC, anti-TNP PFC were found in 7 month old animals, although the numbers were rather low. The assay on the 12 month animals was performed on a pool of spleen cells and these again showed a (low level) anti-TNP PFC response, suggesting that the lack of PFC responses to TNP-SRBC before and over metamorphosis is not an artefact of pooling cells. The results with TNP-LPS are similar to those with the T-dependent antigens, in that up to 3 months of age no splenic PFC response was found. At six months, however, reasonable levels of anti-TNP PFC were found after TNP-LPS injection, as they were at 12 months.

It appears from these results that a significant change in some immune component(s) takes place after the end of metamorphosis which allows the subsequent detection of splenic PFC. However, a second series of ontogenetic experiments was undertaken using the more sensitive ICA assay.

(b) Ontogeny of induced antigen-binding cells in the spleen

Antigen-binding reactivity to TNP-SRBC and TNP-LPS during larval, metamorphic and adult life is shown in Tables 5.4 and 5.5, also in Figures 5.1 and 5.2. Table 5.3 shows the results of initial experiments investigating the TNP-SRBC response with pooled larval spleen cell suspensions. These experiments confirmed the need for low dose carrier priming (as demonstrated by Ruben and Edwards, 1977) for adult *Xenopus*, given 2 days before the main injection of TNP-erythrocyte complex to produce good levels of TNP-HRBC splenic RFC.

Lack of such carrier pre-immunisation - i.e. giving TNP-SRBC alone did, however, appear to give a low level anti-TNP-HRBC response (c.f. saline injected controls). Furthermore, injection of 50% SRBC alone failed to induce TNP-HRBC binding above background levels. Background levels of anti TNP-HRBC RFC's following NaCl injection were found to be low throughout ontogeny with a mean level of 782 $\pm$ 204.

Table 5.4 and Fig. 5.1 show the results of the complete ontogenetic study on anti-TNP-SRBC responses, using the standard immunisation (i.e. low dose priming) schedule. Anti-TNP binding lymphocytes are found in the earliest-injected animals (injected at stage 52/3). In these larvae modest RFC numbers are also seen with HRBC: high background activity to erythrocyte antigens early in ontogeny is expected in view of Du Pasquier's findings on Alytes obstetricans (1970). The mean level of anti-TNP RFC's following TNP-SRBC injection increased through ontogeny, reaching maximum levels of 6418 $\pm$ 3641 and 7684 $\pm$ 3776 RFC/10^6 lymphocytes at stages 56/7 and 58/9 respectively. Animals injected at stage 65/6 (which is at the end of metamorphosis, when lymphocyte numbers are at their lowest) showed a lower mean level of anti-TNP response than did those larvae injected at stages 56-59 (although S.E.'s are very high at these stages of maximal response, which precludes any statistical significance of this apparent difference). The anti-TNP RFC level (3402 $\pm$ 260) at stage 65/6 TNP-SRBC injected animals is similar to the mean anti-TNP levels found in the juvenile and adult animals assayed.

Table 5.5 and Fig. 5.2 show the ontogeny of splenocyte RFC activity to TNP when the hapten is coupled to the T-independent carrier LPS. Again a reasonable anti-TNP response is seen in the earliest stage larvae injected (52/3) 4622 $\pm$ 755 RFC's). A rather high background RFC count against HRBC was again seen in one spleen pool at this stage. By the onset of metamorphosis (stage 56/7) the level of anti-TNP RFC following TNP-LPS injection was significantly elevated (11,937 $\pm$ 3579) and this high level was sustained right through metamorphosis. At stage 65/6 mean anti-TNP RFC numbers were 13,203 $\pm$ 357. Anti-TNP RFC levels in juvenile and adult stages tested were 7276 $\pm$ 554 and 8396 $\pm$ 462 respectively, which were significantly lower than the levels found over metamorphosis. Some assays were incubated with TNP-glycine (see Table 5.4) to demonstrate the specificity of the anti-TNP RFC response.

(c) <u>Antibody forming cells within the thymus</u>

Table 5.6 shows the results of a brief investigation of the thymus as a centre of PFC activity following TNP-SRBC injection. A low level of response was consistently found in thymocyte suspensions assayed 10 days after low dose carrier priming, although the PFC response was afforded almost exclusively to the carrier (SRBC). Thus there were no PFC's in 5/6 thymic suspensions to TNP-HRBC and no thymic PFC at all to HRBC. The thymic, anti-carrier PFC response appeared to have disappeared by 14 days. Other preliminary findings (not reported) with the ICA assay support these PFC results. Thus low levels of anti-carrier RFC's are seen on day 10, but little, if any, response to the hapten occurs.

Discussion

The initial experiments reported in this Chapter demonstrated that splenic PFC can consistently be induced in 9-15 month old toadlets

following injection with the thymus-dependent antigen SRBC. However, when PFC responses to SRBC, TNP-SRBC and TNP-LPS were examined in younger toadlets and larvae, no antibody forming cells were found in the spleen until 1-2 months after metamorphosis. This seems rather surprising, since serum immunoglobulins in Xenopus have been reported to be present from a very early stage of development (stage 35 - see Leverone et al., 1979) and because PFC have been found in other anuran larvae (Alytes obstetricans, Du Pasquier, 1970); Rana catesbeiana, Moticka et al., 1973).

There are a number of possible reasons why PFC could not be detected in spleens of young Xenopus. First, it is possible that the spleen is not established as a site of antibody production until after metamorphosis, but this seems unlikely as RFC's are found in this organ from an early age, and antigen binding is a prerequisite for PFC activity. Perhaps the kinetics of PFC production differ before metamorphosis and hence the larval assays were performed at the 'wrong' times after injection. Secondly, it seems that metamorphosis in Xenopus signals major changes with respect to immunoglobulin production; these changes include alterations in both surface Ig expression on thymocytes and the capacity for Ig 'G' antibody production. With respect to the latter, it has been found that although larval serum contains Ig'G'-like antibodies, such antibody is not detected as readily as in the adult (Du Pasquier and Haimovich, 1976). Although deficient Ig'G' antibody production would not appear to be a causative factor for the lack of PFC's encountered here (the assays measured direct plaques, presumably IgM-secreting cells) it is possible that larval Xenopus IgM antibody is, for some reason, unable to fix mammalian complement efficiently.

Possibly amphibian complement could be used successfully here, although it may be necessary to use adult serum. It has been shown that some genes of the mammalian MHC code for, and control expression of, complement components (see Schreffler, 1978 for review). If the _Xenopus_ MHC also controls some aspects of complement activation, it could be that the complement genes involved are activated only in the adult. In this respect Du Pasquier _et al_ (1979) suggest that serologically-detectable MHC antigens in _Xenopus_ are detected at or only after metamorphosis.

Although no PFC were found until after metamorphosis, antigen binding cells can readily be induced in the _Xenopus_ spleen from early in ontogeny. Thus Kidder _et al_. (1973) demonstrated anti-SRBC binding cells in larval spleens from animals injected at stage 50 onwards. The experiments reported here employed the hapten-carrier system to examine the ontogeny of the RFC response of spleen cells to TNP-SRBC and to TNP-LPS. Good levels of induced antigen-binding cells in the larva to TNP after TNP-SRBC injection required low dose carrier priming. In the adult amphibian such carrier-reactive lymphocytes are believed to represent T-helper cells in view of their antigen-binding characteristics (they are minimal-binding cells of the non-secretory type - see Ruben, 1975) and their thymus-dependency (Horton _et al_., 1979). Therefore it would appear from the present experiments with TNP-SRBC that some T-helper cell populations are functioning at the earliest stages injected (stage 52/3). This finding is in agreement with the work of Ruben, Welch and Jones, 1980. On the other hand in order to explain the deficient Ig'G' antibody responses displayed by anuran larvae (see above), full establishment of T helper cell activity may develop only after metamorphosis.

The experiments here reveal that stage 52/3 Xenopus larvae also contain antigen-reactive, B-equivalent, splenic lymphocytes, since antigen binding reactivity to TNP occurred following TNP-LPS administration to such animals.

Maximum anti-TNP RFC levels following low dose SRBC priming and TNP-SRBC injection 2 days later were seen in the spleen at the onset of metamorphosis. This finding is in agreement with similar experiments on Xenopus reported by Ruben, Welch and Jones (1980). These workers suggest that the elevated TNP response found in the spleens of metamorphosing animals may be due to the effect of metamorphosis on a population of cells that normally suppresses helper function in larval and adult Xenopus: if this suppressor population was selectively lost or blocked early in metamorphosis, then higher levels of anti-TNP binding cells in the spleen following TNP-RBC injection might be expected, due to unrestrained T cell help. However, the elevated response to TNP-LPS at metamorphosis demonstrated here does not altogether fit with their suggestion. Thus the TNP-LPS results suggest that elevated B cell reactivity may be occurring at metamorphosis. Alternatively, their could simply be a temporary increase in proportion of (antigen-binding) B cells over T cells in the small spleen found at metamorphosis (Du Pasquier and Weiss, 1973), possibly related to reduced T cell migration from the thymus that may be occurring at this time (see Chapter 6). Whether the RFC's recorded in the spleen against TNP-HRBC represent B- rather than T-equivalent antigen-binding lymphocytes is uncertain, although it is pertinent to point out that the majority of such RFC's were of the secretory type which, it has been suggested elsewhere (Ruben and Edwards, 1980), are antibody-secreting lymphocytes.

Durham University SCIENCE 22 FEB 1982 SECTION Library

Very recently Jones and Ruben (1981) have re-examined the effect of metamorphosis on the splenic antigen-binding response to TNP when conjugated to an erythrocyte carrier. They reveal that stage 57/8 is a unique period in which a (low level) anti-TNP RFC response (3.5 x 10^3 RFC's/10^6 lymphocytes) can occur in the absence of carrier (erythrocyte) pre-immunisation. (Interestingly a low level anti-TNP response was also seen here in stage 57/8 animals injected with TNP-SRBC without low dose SRBC priming (see Table 5.3). Jones and Ruben suggest that an internal histoincompatibility between larval and adult lymphoid cells, which is likely to be occurring at this time (see Du Pasquier, Blomberg and Bernard, 1979), provides a substitute for carrier priming)i.e. overrides the absolute necessity for T cell help). Interestingly their experiments provide no evidence for elevated B cell reactivity at metamorphosis, since although anti-TNP splenic RFC numbers following TNP-LPS injection at stage 57/8 were higher ≃10,500 RFC/10^6 lymphocytes) than in the adult ≃4,000 RFC/10^6 lymphocytes), they were no different from RFC numbers in the group of animals injected with TNP-LPS at stages 51-54 (10,000 RFC). This contrasts with the findings presented here, where larvae injected with TNP-LPS at stage 52/3 yielded significantly fewer TNP-binding splenocytes than those animals injected at stage 57/8. The stage 52/3 larvae used in this Chapter were all 3 weeks old, whereas the ages of the stage 51-54 larvae used by Jones and Ruben were not given. If their stage 51-54 animals were in fact older than the stage 52/3 larvae used here- i.e. had a more developed lymphoid system (closer to metamorphosis?) than their external stage suggested (see Ruben, Stevens and Kidder, 1972 for discussion of this age/stage effect) - this could conceivably provide an explanation for the discrepancies encountered.

The final experiment in this Chapter revealed that (*in vivo*) the young adult thymus contains anti-SRBC PFC (and RFC) after TNP-SRBC immunisation, whereas anti-TNP plaques in this organ were not detected. Although further experiments are obviously required to investigate the significance of positive anti-carrier, but negative anti-hapten antibody production, the findings do suggest that antigen administration can achieve PFC appearance in the thymus. This has previously been demonstrated in thymuses of several vertebrate species (see Discussion in Chapter 2). The experiments here do not define whether the thymus is itself a site for reaction to the hapten-carrier complex, but the results do suggest that cellular antibody production in this organ is not achieved simply by a random recirculation of PFC, as only PFC against the carrier were found in the thymus. Recent findings (Hsu and Du Pasquier, 1981) demonstrating the high levels of PFC production *in vitro* by *Xenopus* thymocytes (following in vivo priming with DNP-KLH and reimmunisation *in vitro*) point again to the thymus as a major site of B-equivalent cells in this species.

TABLE 5.1 Splenic PFC response of toadlets, 9-15 months of age after single injection of 50µl gram body weight^{-1} 10% SRBC

Assay Day	4	6	7	8	9	12	14
Mean PFC counts/ chamber (in each animal)	7.5,2	178,153	128,78	113,55	40,55	34,27,19	3,14
PFC/10^6 leucocytes (in each animal)	101,16	1049,784	155,636	832,291	326,325	239,157,34	19,134
Mean PFC/10^6 leucocytes	59	917	696	562	326	143	77

Non-injected animals showed an absence of anti-SRBC PFC in these experiments.

All animals were kept at 26°C after injection.

TABLE 5.2

(i) Ontogeny of PFC response to SRBC

Immunisation protocol		Stages when injected					
		56/7	58/9	3 months	4 months	8 months	12 months
Day 0: Inj SRBC 10%		(pool)			pool		pool
Day 7: Assay	PFCvSRBC	0	-	0,0,0,0	42,3	489,670	243,122,755,636

(ii) Ontogeny of PFC response to TNP-SRBC

Immunisation protocol		56/7	58/9	65/6	7 months	12 months
Day 0: Inj 0.0025% SRBC						
Day 2: Inj 10% TNP-SRBC		pools	pool	pool		pool
Day 10: Assay	PFCvSRBC	0,0	0	0	93,77	
	PFCvHRBC	0,0	0	0	0 0	15
	PFCvTNP-HRBC	0,0	0	0	31,67	128

(iii) Ontogeny of PFC response to TNP-LPS

Immunisation protocol							
Day 0: Inj TNP-LPS							
Day 8: Assay		56/7	58/9	65/6	3 months	6 months	12 months
		pool	pool	pool		pool	
	PFCvHRBC	0	0	0	0,0	0	20,0
	PFCvTNP-HRBC	0	0	0	0,0	286	448,327

All animals kept at 26°C post-injection

PFC=PFC/10^6 lymphocytes

Spleen lymphocytes used in all the experiments

TABLE 5.3 RFC studies on TNP-SRBC responses following various immunisation schedules

		Experiment 1	Experiment 2	Experiment 3	Experiment 4
Injected at stage 56/7	Immunisation protocol	A*	B	S	S
	Assay v SRBC	2430	-	-	1728
	HRBC	-	-	556	725
	TNP-HRBC	1388	635	3333	10,785
		Experiment 1	Experiment 2	Experiment 3	
Injected at stage 58/9	Immunisation protocol	S	S	S	
	Assay v SRBC	-	3232	236	
	HRBC	163	1382	236	
	TNP-HRBC	4970	15,353	3310	
		Experiment 1	Experiment 2	Experiment 3	
Injected at stage 65/6	Immunisation protocol	C	S	S	
	Assay v SRBC	O	O	-	
	HRBC	453	1122	164	
	TNP-HRBC	226	3142	3826	

*Key A 10µl 50% TNP-SRBC Day O - Assay Day 8
B 10µl 50% SRBC Day O - Assay Day 8
C 10µl 0.65% NaCl Day O - Assay Day 10
S 5µl 0.0025% SRBC Day O - 10µl 50% TNP-SRBC Day 2 - Assay Day 10. (standard protocol)

For each experiment a pool of spleen cells was assayed v SRBC, HRBC and TNP-HRBC. Each experiment consisted of a pool of 10-20 larval spleens and the data represent the number of RFC/10^6 lymphocytes.

All animals kept at 23°C after injection.
Spleen lymphocytes used in all the experiments

TABLE 5.4 Ontogeny of the ICA responses to TNP-SRBC

Stage injected	RFC/10^6 lymphocytes v. HRBC	v.TNP-HRBC	anti-TNP	v.TNP-HRBC + glycine	v. TNP-HRBC + TNP-glycine
52/3	1481	3704	2223		
	982	3782	2800		
			2512 ± 289		
54/5	423	3597	3174		
	516	5278	4762		
			3978 ± 804		
56/7	556	3333	2777		
	725	10,785	10,060		
			6418 ± 3641		
58/9	163	4970	4807		
	182	15,353	15,171		
	236	3310	3074		
			7684 ± 3776		
65/6	0	3142	3142		
	164	3826	3662		
			3402 ± 260		
5 months			3279	3279	0
			3404	3612	208
			3342 ± 63		
7 months	910	3642	2732		
	0	5151	5151		
	0	4533	4533		
			4145 ± 727		
12 months	389	5456	5275		
	322	7407	7274		
			1526	2114	588
			2899	3033	194
			4637 ± 1293		

Immunisation schedule: Day 0: 5µl 0.0025% SRBC; Day 2: 10µl 50% TNP-SRBC; Day 10: Assay

Animals kept at 23°C post-immunisation. Spleen lymphocytes tested in all the experiments

TABLE 5.5 Ontogeny of the ICA response to TNP-LPS

RFC/10^6 lymphocytes

Stage injected	v.HRBC	v.TNP-HRBC	anti-TNP	v.TNP-HRBC +glycine	v.TNP-HRBC +TNP-glycine
52/3	0	4753	4753		
	385	3703	3318		
	2222	8148	5926		
			4622 ± 755		
56/7	-	8358	8358		
	837	16,352	15,515		
			11,937 ± 3579		
58/9	666	13,111	12,445		
	363	16,575	16,212		
	0	11,252	11,252		
			13,303 ± 1495		
65/6	174	13,020	12,846		
	582	14,142	13,560		
			13,203 ± 357		
6 months	285	8114	7826		
	333	7055	6722		
			7276 ± 554		
12 months			8859	10,285	1425
			7934	8815	881
			8396 ± 462		

Immunisation schedule was:

Day 0 : 5μl 0.5mg TNP-LPS

Day 8 : Assay

Animals kept at 23°C post-immunisation

Spleen lymphocytes tested in all the experiments

TABLE 5.6 PFC response by thymus cells following TNP-SRBC immunisation

Immunisation protocol

Day 0 : Inj 0.0025% SRBC

Day 2 : Inj 10% TNP-SRBC

	PFC/10^6 lymphocytes		
	v.HRBC	v. TNP-HRBC	v. SRBC
Assay Day 10	0,0,0,0,0,0	0,0,0,9,0,0	25,17,25,43,121,4
Assay Day 14	0,0,0	0,0,0	0,0,0,0

Animals kept at 26°C post-injection

Fig. 5.1

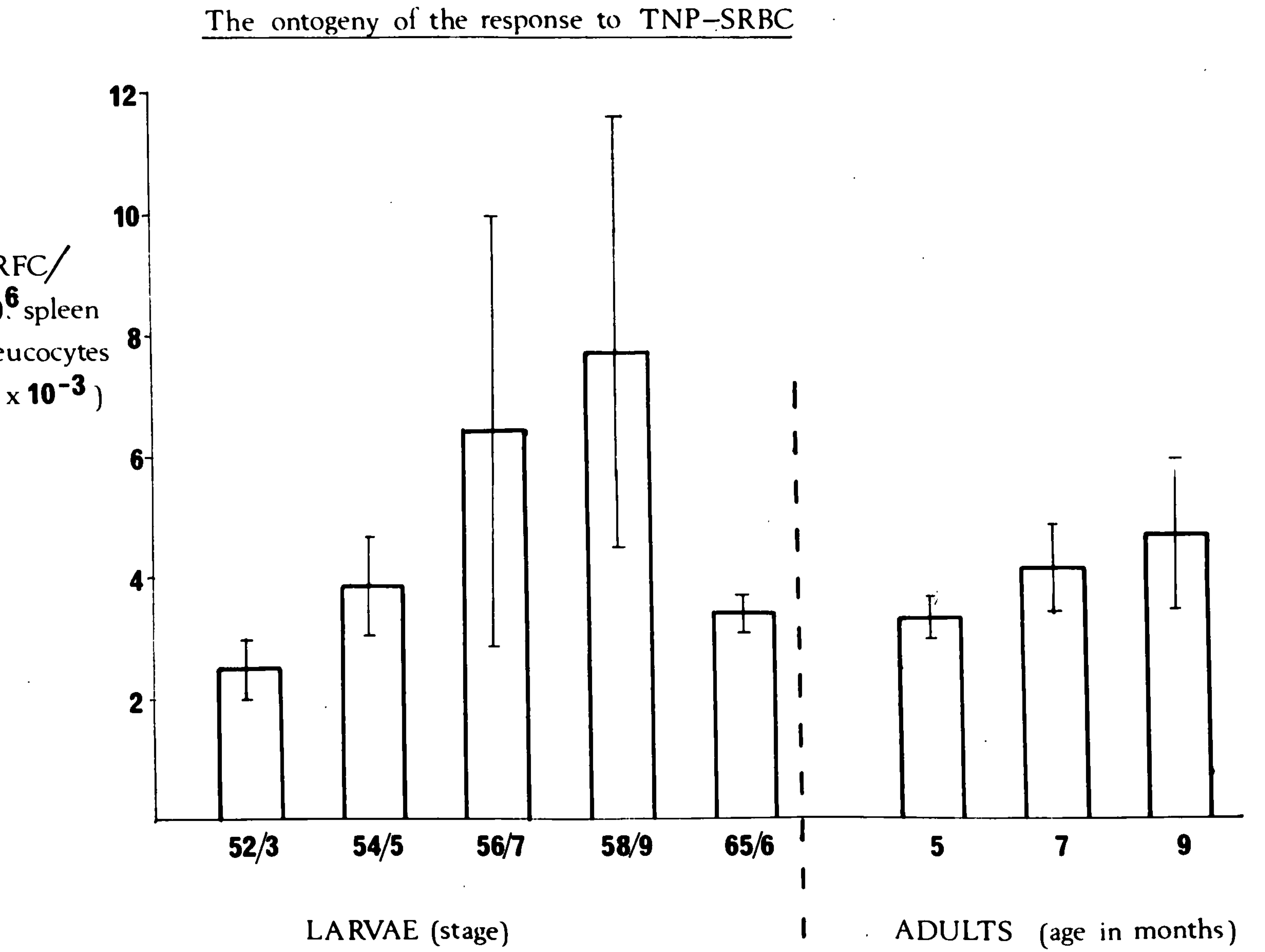
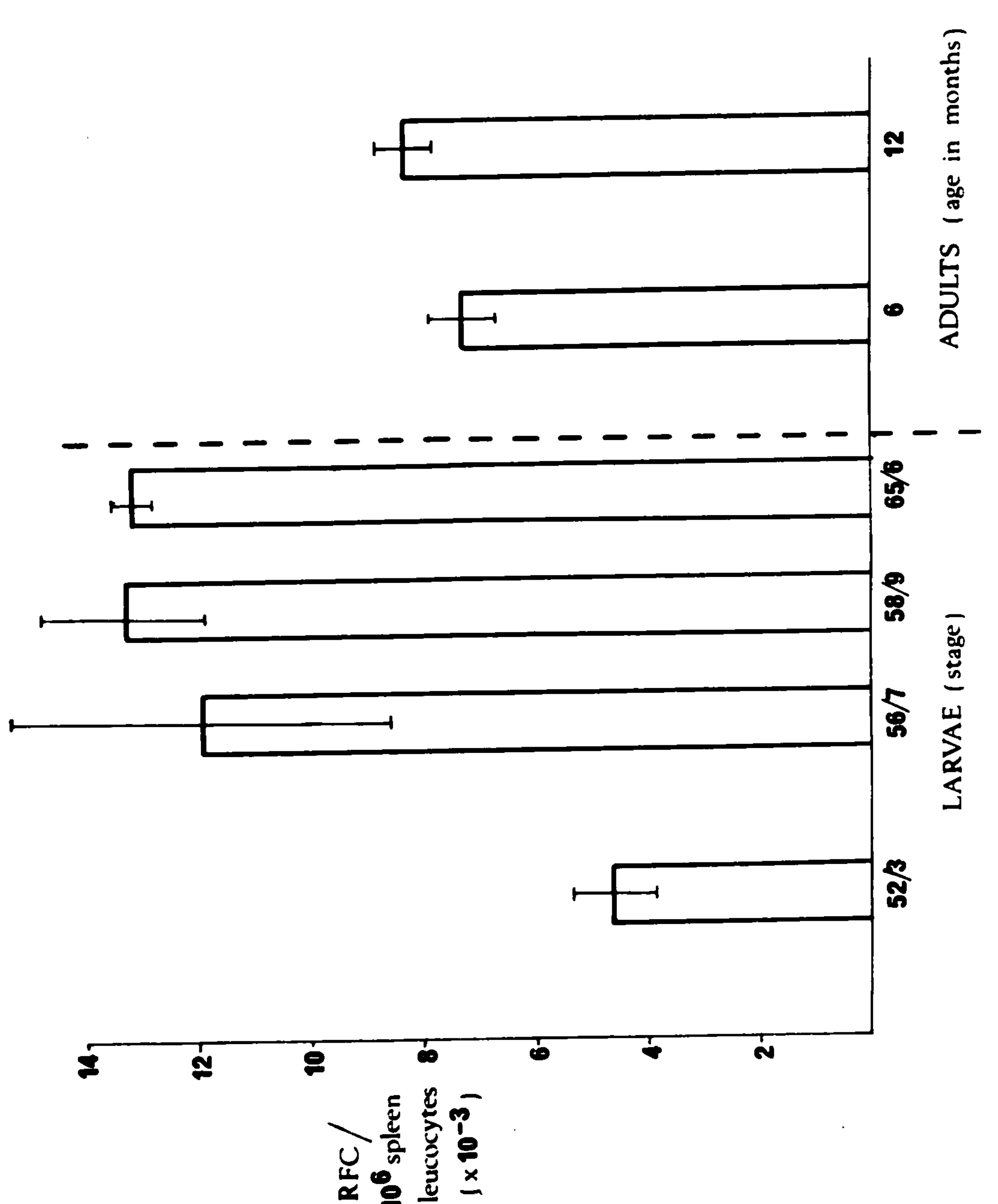


Fig. 5.2



The ontogeny of the response to TNP–LPS

Fig. 5.3

Cytocentrifuge preparations of cells from an ICA assay using splenocytes from toadlets immunised against sheep erythrocytes (SRBC). Rosette forming cells (RFC) can be seen in each micrograph and comprise a central lymphoid cell (small or large lymphocyte) binding one or additional layers of SRBC.

XRBC = Xenopus erythrocyte.

XL = Xenopus lymphocyte

Scale 1cm = 25μm

RFC(XL)

RFC

SRBC

RFC

XRBC

CHAPTER 6

Thymus histogenesis over metamorphosis and in organ culture

Introduction

Until quite recently, studies on the ontogeny of the immune system in anurans have concentrated on events during embryonic and larval life. Both cellular and humoral immunity develop prior to metamorphosis, the acquisition of immunocompetence being concommitant with the differentiation of lymphoid tissues, particularly the thymus (see Horton, 1969; Kidder et al., 1973). In *Xenopus* early thymus histogenesis has been studied in considerable detail. Thus ultrastructural investigations have shown the appearance of large basophilic cells within (between the thymic epithelial cells) and immediately adjacent to the thymus at 3 days (stage 40) (Nagata, 1977). Recent experimental embryological evidence on ploidy-labelled *Xenopus* reveal that the extra-thymic origin of these stem cells may well be the nephric region of the embryo (Volpe et al. 1981). The basophilic stem cells gradually increase in number within the thymus and are thought to be the precursors of the first small lymphocytes seen at around 7 days of age (Manning and Horton, 1969; Rimmer, 1978). An extrinsic origin of thymic lymphocytes in other vertebrates has also received considerable experimental support (see Le Douarin, 1977).

In *Xenopus* the immune system as a whole is thought to undergo major changes at metamorphosis (which occurs about 7-8 weeks after the first appearance of small lymphocytes in the thymus and the initiation of immunocompetence). Thus the number of recoverable thymocytes is known to drop dramatically at this time (Du Pasquier and Weiss,

1973), alloimmune reactivity is 'suppressed' (Chardonnens, 1976) and regulatory T cell activities (e.g. help and suppression) are altered (see Discussion in Chapters 3 and 5 and also Du Pasquier and Bernard, 1980). Details of histologic changes in the thymus over metamorphosis are, however, not available (see Manning and Horton, 1969). The first part of this Chapter therefore looks at the effect of metamorphosis on thymus histogenesis and attempts to correlate the structural changes observed during larval, metamorphic and early post-metamorphic life with the numbers of thymic lymphocytes that can be recovered when the organ is teased apart *in vitro*.

The second part of this Chapter is a preliminary investigation on the effect of organ culture on larval thymus lymphopoiesis. Organ culturing of the amphibian thymus should, if technically feasible, prove to be a powerful tool to examine development and differentiation of thymic lymphocytes (e.g. in early larval life and at metamorphosis) in the absence of *in vivo* cellular migrations or humoral effects. It has been shown in mice (Robinson, 1980; Mandel and Kennedy, 1978) and chickens (Sallstrom and Alm, 1973) that transfer of the thymus early in ontogeny into organ culture does not prevent proliferation and differentiation of lymphocytes. Precursor cells, present in the embryonic thymus before removal, continue to differentiate normally with respect to their surface antigenic markers and T cell functions, this differentiation paralleling the developmental sequence observed *in vivo*.

Materials and Methods

(a) Thymus morphology

The first experiment considered the number of thymic lymphocytes recoverable from animals of various stages of development, including

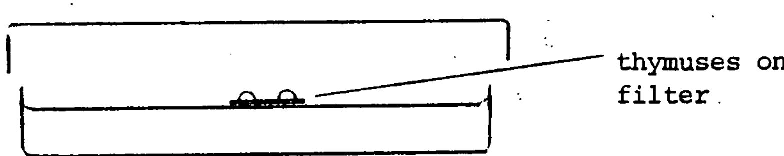
the period over metamorphosis. The cells were prepared as described in Chapter 3. For each stage examined, an average of 10 animals was used.

For the histological studies, 3 animals were selected at the following stages; stage 54/5 (4 weeks), stage 58 (7 weeks), stage 60 (8 weeks), stage 66 (9 weeks, the end of metamorphosis), one week post-metamorphosis, 2 weeks post-metamorphosis and 3 weeks post-metamorphosis. The selected animals were heavily anaesthetised in MS 222 then fixed in Bouins fixative. After dehydration, the specimens were wax embedded and 8μm sections cut and stained with haematoxylin and eosin. Thymuses from two additional stage 66 animals were prepared for 1μm sectioning. These thymuses were fixed in cold gluteraldehyde (5% gluteraldehyde in 0.1M sodium cacodylate) overnight, then washed in 0.1M cacodylate buffer for 24 hours and post-fixed in osmium tetroxide for half an hour. After washing for half an hour in 0.1M cacodylate buffer, the specimens were dehydrated in methanol, cleared in propylene oxide and then transferred to a 1:1 mixture of epon/epoxypropane overnight. They were finally embedded in epon, the plastic being allowed to polymerise at 60°C for 48 hours. 1μm sections were cut using a Reichert ultramicrotome and were stained in toluidine blue. To assess the location of proliferating lymphocytes within the young adult thymus, two 3-week post-metamorphic toadlets were injected with tritiated thymidine (S.A. 22.4 Ci/mmol) 4 hours before killing. Thymuses were then prepared for autoradiography as described in Chapter 3.

(b) Thymus organ culture

The technique used followed that developed by Robinson and Owen (1976) for mammalian thymus organ culture. Thymuses were

dissected out of anaesthetised larvae aseptically, care being taken to remove as much connective tissue as possible from around the thymic capsule. Thymuses were placed in a watchglass containing amphibian culture medium (see Chapter 2) but with the addition of sodium bicarbonate (0.085 g cm^{-3}). Ten cm^3 culture medium was added to a Falcon sterile plastic Petri dish, which was supplemented with 1.5 cm^3 decomplemented FCS. Several batches of FCS were tried in preliminary organ culture experiments, but the batch (purchased from Gibco) showing maximum lymphocyte recovery was selected and used in all experiments presented here. On the surface of the medium were floated 13mm diameter Nucleopore polycarbonate filters (Sterilin) with 0.4μm pore size. These had been boiled in distilled water, with 3 changes, and then autoclaved before use. Each pair of thymuses was placed on the prepared filter, as in the diagram below.



Such thymus organ cultures were incubated in a humidified incubator at 28°C with 5% CO_2 in air. Two stages of development were selected for experimentation; stage 52, as a young thymus where lymphocyte numbers are rapidly increasing, and stage 58, when metamorphosis has just begun. At both stages 9 animals were used. Thymocyte suspensions were prepared directly from 3, as described in Chapter 3, except that after the mechanical disruption the thymus was broken up by incubation in trypsin/EDTA for half an hour. (Trypsinisation

proved to be necessary for releasing lymphocytes from organ-cultured thymuses). Three animals were kept as controls and allowed to develop normally to determine the extent of in vivo changes in thymus morphology over the experimental period. The final 3 animals provided thymuses for organ culture. On day 7 the organ cultured thymuses were taken off the filters and cell suspensions prepared by teasing and trypsinisation. Thymocytes were then counted in a haemacytometer and a cytospin preparation made (see Chapter 3). The cytospins were used to count the numbers of lymphocytes and epithelial cells present. Thymocyte cytospin preparations from the 3 control animals were also made at 7 days. One pair of 7-day organ-cultured thymuses from a stage 52 larvae were plastic-embedded, sectioned at 1μm and stained with toluidine blue (as described in (a) above).

Results

(a) Thymus morphology

Figure 6.1 shows the number of thymic lymphocytes that can be recovered at various stages of development from stage 50/51 (2 weeks old) up to 3 weeks after the end of metamorphosis (i.e. 12 weeks total age). The data show that in the larva a maximum level of about 10^6 thymic lymphocytes occurs at the very beginning of metamorphosis (stage56/7(6 weeks)). This level is similar to that found by Du Pasquier and Weiss (1973) at this stage. Du Pasquier and Weiss recorded that thymocyte numbers decline to 3×10^5 over the peri-metamorphic period, whereas the results here show a sharper fall to only 3.5×10^4 at 9 weeks (the end of metamorphosis). Thymocyte numbers increase dramatically within 3 weeks post-metamorphosis, when 1.2×10^6 lymphocytes are recoverable.

The histology of the thymus in larval, metamorphosing and post-metamorphic *Xenopus* is shown in Figs. 6.2 - 6.5. At stage 54 (Fig. 6.2a) and stage 58 (Fig. 6.2b) the thymus is highly lymphoid. At stage 60 the thymus (Fig. 6.2c) is still large and continues to be an extremely lymphoid structure. Cortex and medulla are clearly defined at all the above 3 stages, with a greater density of lymphocytes in the cortex. Lymphoblasts can be seen in the subcapsular region of the cortex (Fig. 6.3a) but these become less evident by stage 60 (Fig. 6.3b). By the end of metamorphosis (stage 66) the thymus is not reduced in size but histologically now looks quite different (Fig. 6.4a). There is a large, essentially epithelial, medullary region, in which many basophilic granulocytes are found. The cortex appears narrower than at early stages. Overall the number of small lymphocytes appears greatly reduced. Furthermore, there is now no indication of a subcapsular layer of lymphoblasts (Fig. 6.4a). By one week post-metamorphosis, the thymus appears to be slightly reduced in size and is again sparsely populated with lymphocytes (Fig. 6.4b). However, there now appears a broad and discreet band of lymphoblasts beneath the thymic capsule (Fig. 6.5a). Towards the medulla there is a small cortical band of lymphocytes, and lymphocytes are now seen again in the medulla. Two weeks after the end of metamorphosis, the discreet peripheral band of lymphoblasts is particularly noticeable (Fig. 6.4c and 6.5b). By this time the area occupied by cortical small lymphocytes has enlarged and the thymus as a whole has grown. Increasing numbers of lymphocytes are visible in the medulla. At 3 weeks post-metamorphosis the thymus has enlarged considerably (Fig. 6.4d) and takes on its 'adult' form; it is highly lymphoid in both cortex and medulla,

with a narrow band of lymphoblasts around the periphery of the cortex. Autoradiographs of such thymuses (Fig. 6.6) show that it is the lymphoblasts of the peripheral cortex which are actively synthesising DNA. This zone therefore represents the region where thymic lymphocyte proliferation is occurring.

(b) Organ culture experiments

Development of the stage 52 thymus in organ culture does not mirror development occurring *in vivo* (see Table 6.1). After culture, the proportion of lymphocytes to epithelial cells decreases when compared with the thymuses prepared directly from the control larvae. Many large epithelial cell types are noticeable in the 7-day cultured thymus (see Fig.6.7-6.8). The total number of thymocytes also declined after 7 days in culture, in relation both to the number at the start of the culture period (i.e. at stage 52) and the number in the control thymuses, which remained *in vivo* for the duration of the experiment. However, the cells remaining after 7 days in culture showed good viability (70-80%, as assessed by dye exclusion).

The results were essentially the same when stage 58 thymus was cultured. Although thymocyte numbers in the *in vivo* developing controls declined (as expected) at this time from 0.94×10^6 to 0.40×10^6, in organ culture the decline was greater (0.17×10^6 cell recovered at 7 days of culture). The proportion of lymphocytes to epithelial cells was only marginally decreased in organ-cultured thymuses compared with *in vivo* development.

Figure 6.7 shows a 1μm section of a 7-day organ cultured thymus taken from a stage 52 animal. It can be seen that the cortico-medullary structure becomes rather indistinct. Furthermore, compared with the *in vivo* picture expected at this time (see Fig. 6.1a), there are large vesicles in the medulla, an overall reduction in number of lymphocytes and a flattening of the thymus onto the filter. Melanin deposits are noticeable following organ culture.

Discussion

The work in the first part of this Chapter investigated morphologic events occurring in the thymus at metamorphosis. The data on cell recovery confirms previous findings (Du Pasquier and Weiss, 1973) that this period of development is associated with a considerable diminution of thymocyte numbers but highlights the end of metamorphosis as being the most dramatic period in this respect. The histological study of thymus ontogeny also reveals that the end of metamorphosis is associated with significant structural alterations in this organ. Thus thymic medullary lymphocytes are virtually absent at this time and the subcapsular lymphoblast zone, normally found in the cortex, has disappeared. Interestingly these morphologic events occur at about the stage when serologically-detectable MHC antigens can first be detected on leucocytes in *Xenopus* (Du Pasquier, Blomberg and Bernard, 1979). If the thymus is centrally involved in MHC restriction of certain T lymphocyte subsets in *Xenopus* (see Bernard, Bordmann, Blomberg and Du Pasquier, 1981) then perhaps substantial reduction in thymocyte numbers, and discontinuity of normal lymphopoiesis,is an essential prerequisite to reprogramming a new population of T

cells to interact correctly with, and become tolerant to, the new adult MHC antigens emerging at, or soon after, metamorphosis. Certainly the data presented here suggest a new wave of thymus lymphocyte proliferation occurs rapidly just after the completion of metamorphosis, since the peripheral cortical lymphoblast zone reappears and lymphocyte numbers throughout the thymus increase at this time. Thymus lymphopoiesis in *Xenopus* may well be similar to that seen in mice, to the extent that lymphocytes at the periphery of the cortex divide rapidly and produce a maturing population which moves toward the centre of the organ (Weissman, 1967). Whether or not the surge in thymus lymphopoiesis seen immediately after metamorphosis is dependent upon a new wave of stem cell input into the organ at this time (from newly emerging bone marrow?) is unknown (see below).

The organ culture experiments on *Xenopus* thymus, revealing relatively low lymphocyte/epithelial cell ratios and low overall thymocyte numbers compared to thymuses developing *in vivo*, appear to conflict with findings in birds and mammals, which show that removal and organ culture of the embryonic thymus does not prevent proliferation and differentiation of functional T lymphocytes (see Introduction). Before considering the possibility of real physiologic differences, technical factors must be considered. Although many amphibian organs have been cultured (Monnickendam and Balls, 1973), this is the first time that intact *Xenopus* thymus has been kept *in vitro* and examined histologically after several days in organ culture. Although the culture technique used was adapted from a successful mammalian system (Robinson and Owen, 1976), and one used successfully for culturing the chicken thymus (Sällström and Alm, 1973), it may be unsuitable for the amphibian thymus.

It must be remembered that initial attempts at thymus organ culture in the mouse led to largely epithelial cultures. Improved culture techniques led to the first demonstration of thymic lymphopoiesis _in vitro_ (see Robinson, 1980, for review). In the present experiments on the _Xenopus_ thymus, lymphocytes were found at the end of the culture period and their viability was quite good. However, the decrease in thymus lymphocyte numbers revealed after culture might suggest that a constant input of precursor cells into the amphibian thymus is required. Proliferation of a small number of stem cells that enter the thymus early in ontogeny may be sufficient for lymphopoiesis in mammals but not in amphibians. Although this hypothesis could help to explain the rather gradual establishment of T-dependent functions in _Xenopus_, further work on the organ culture system is obviously required.

TABLE 6.1 Results of organ culture experiments - analysis of cytospins

Experiment 1

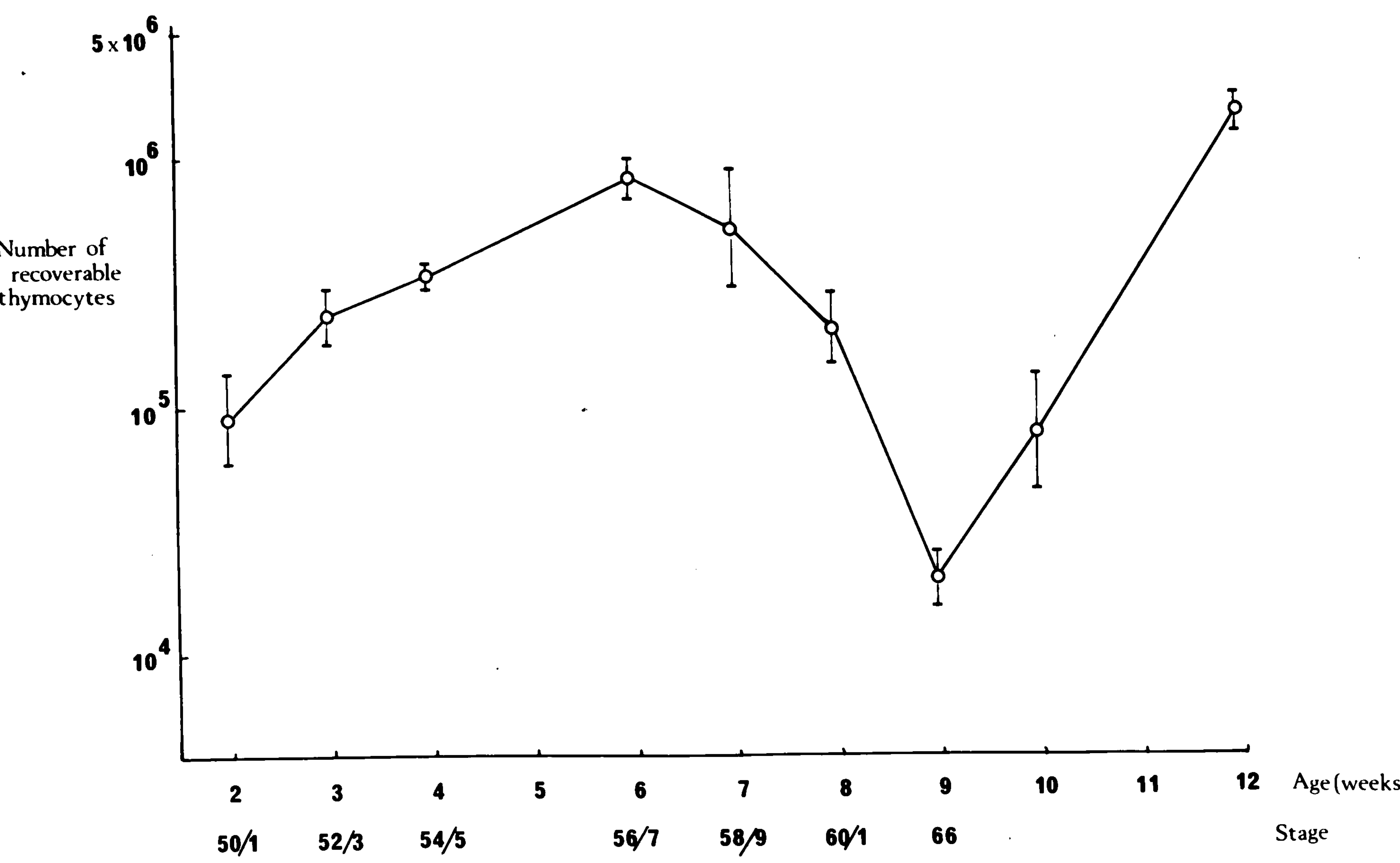
	Stage 52 thymuses		Development in organ culture	Development *in vivo*
Mean number of thymus cells ± S.E. per thymus	$0.055 \times 10^6 \pm 0.02$	7 days	$0.032 \times 10^6 \pm 0.21$	$0.12 \times 10^6 \pm 0.03$
% of lymphoid cells in each animal	96.1,77.3,86.8		69.3, 75.6	96.9, 82.8, 95.8

Experiment 2

	Stage 58 thymuses		Development in organ culture	Development *in vivo*
Mean number of thymus cells ± S.E.	$0.94 \times 10^6 \pm 0.24$	7 days	$0.17 \times 10^6 \pm 0.06$	$0.40 \times 10^6 \pm 0.08$
% of lymphoid cells in each animal	80,9,82.3,76.9		58.3, 51.1, 62.0	69.2, 79.8, 67.8

Fig. 6.1

Number of recoverable thymocytes at various stages of development from 2-12 weeks of age. The end of metamorphosis is at 9 weeks.



5×10^6
10^6
Number of recoverable thymocytes
10^5
10^4
2 3 4 5 6 7 8 9 10 11 12
Age (weeks)
50/1 52/3 54/5 56/7 58/9 60/1 66
Stage

Fig. 6.2

Thymus histology during larval and early metamorphic life showing the increase in size and the highly lymphoid structure of cortex (c) and medulla (m)

(a) Stage 54

(b) Stage 58

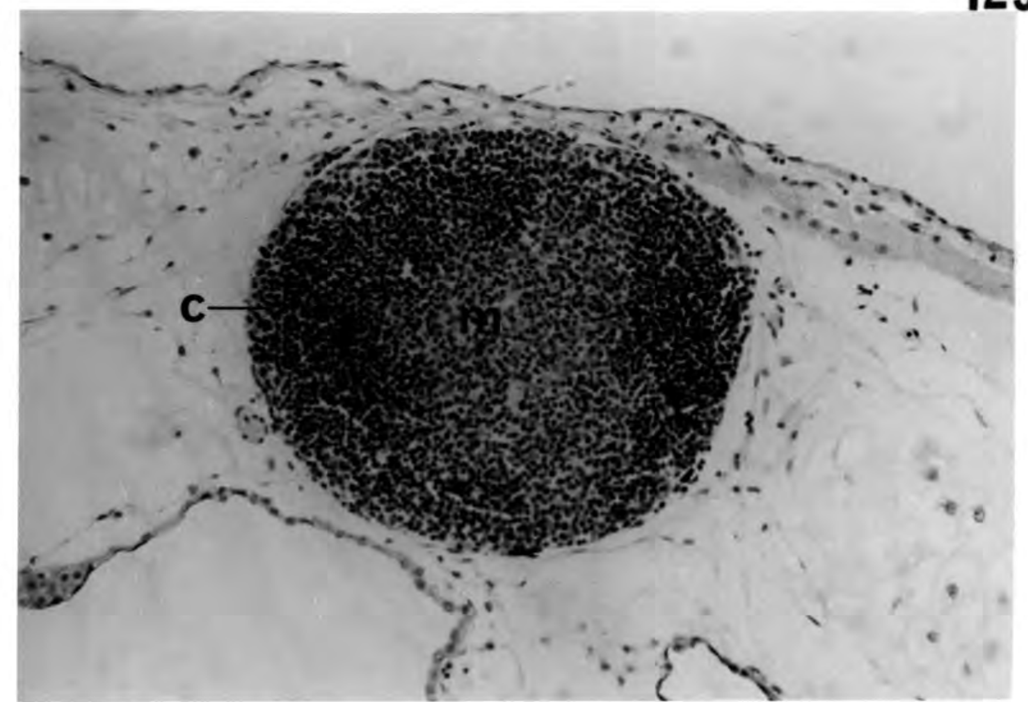
(c) Stage 60

8μm sections

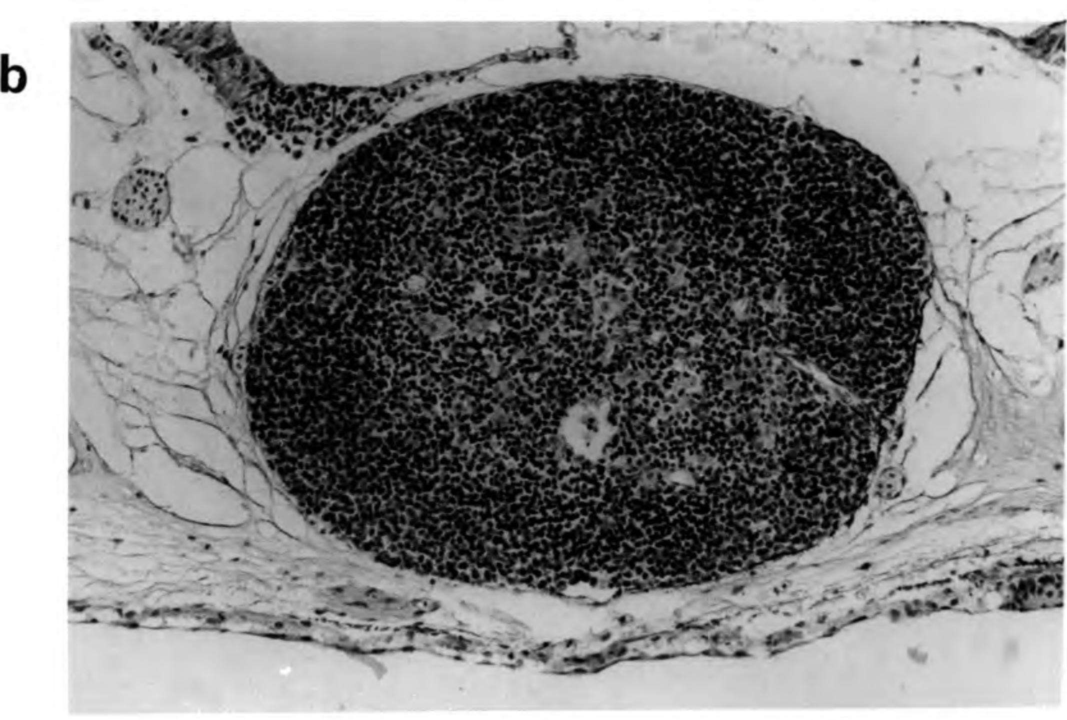
Stain H and E

Magnification x100

a



b



c

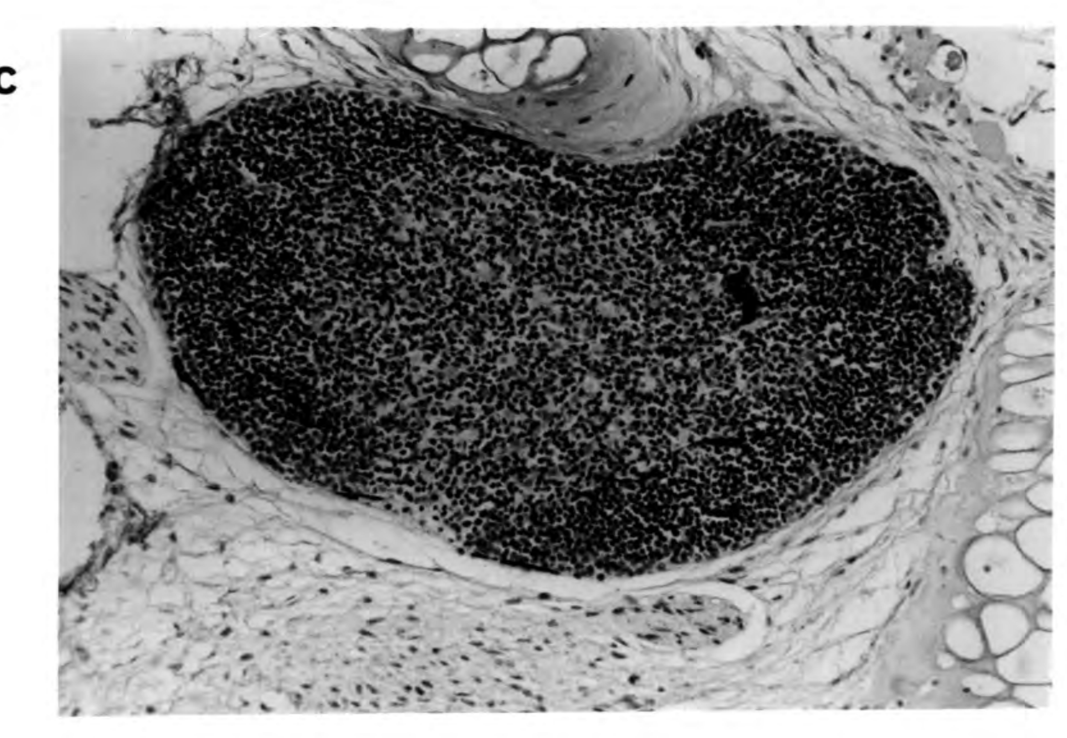


Fig. 6.3

(a) This micrograph shows the band of lymphoblasts (L) found at the periphery of the cortex at stage 54.

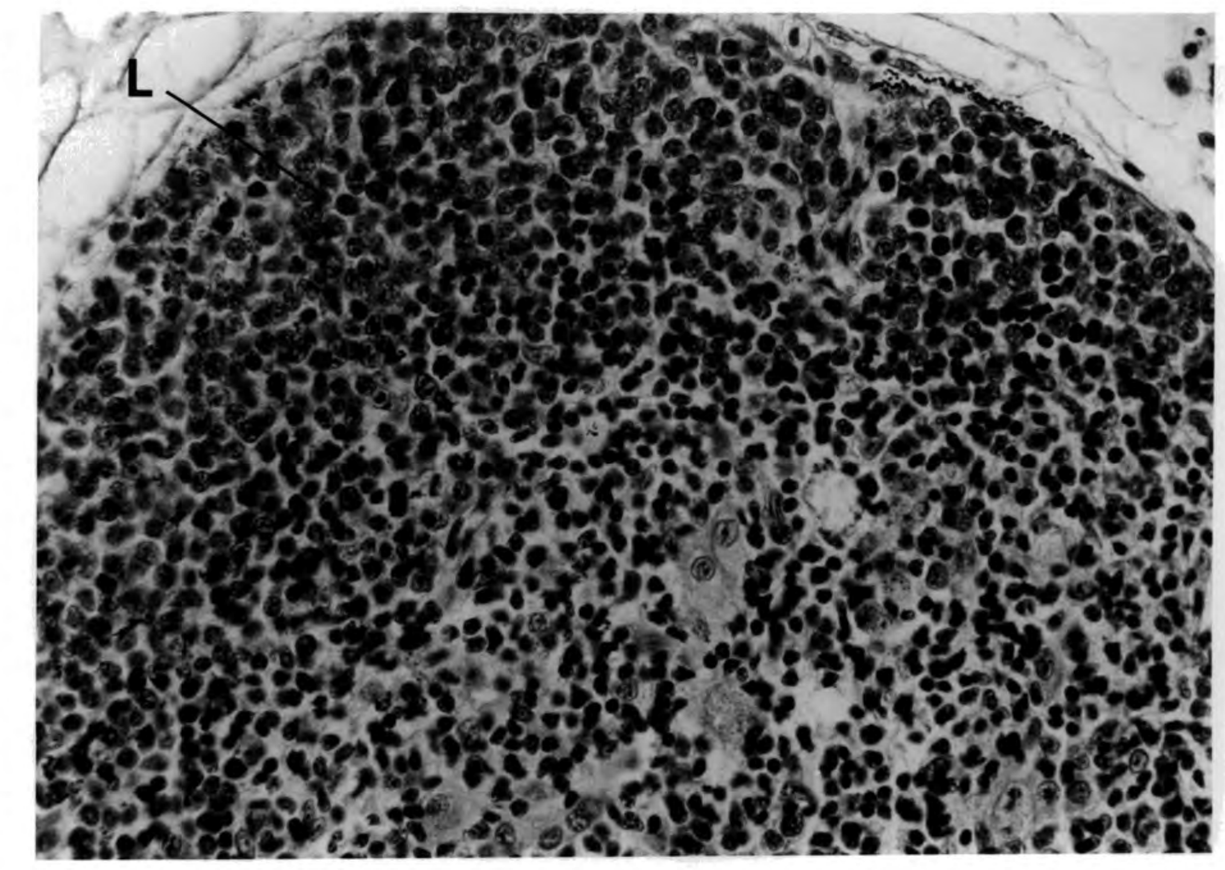
(b) By stage 60, although the thymus is still lymphoid, this layer of large, rapidly dividing lymphocytes, is no longer so readily apparent.

8μm

Stain H and E

Magnification x 250

a



b

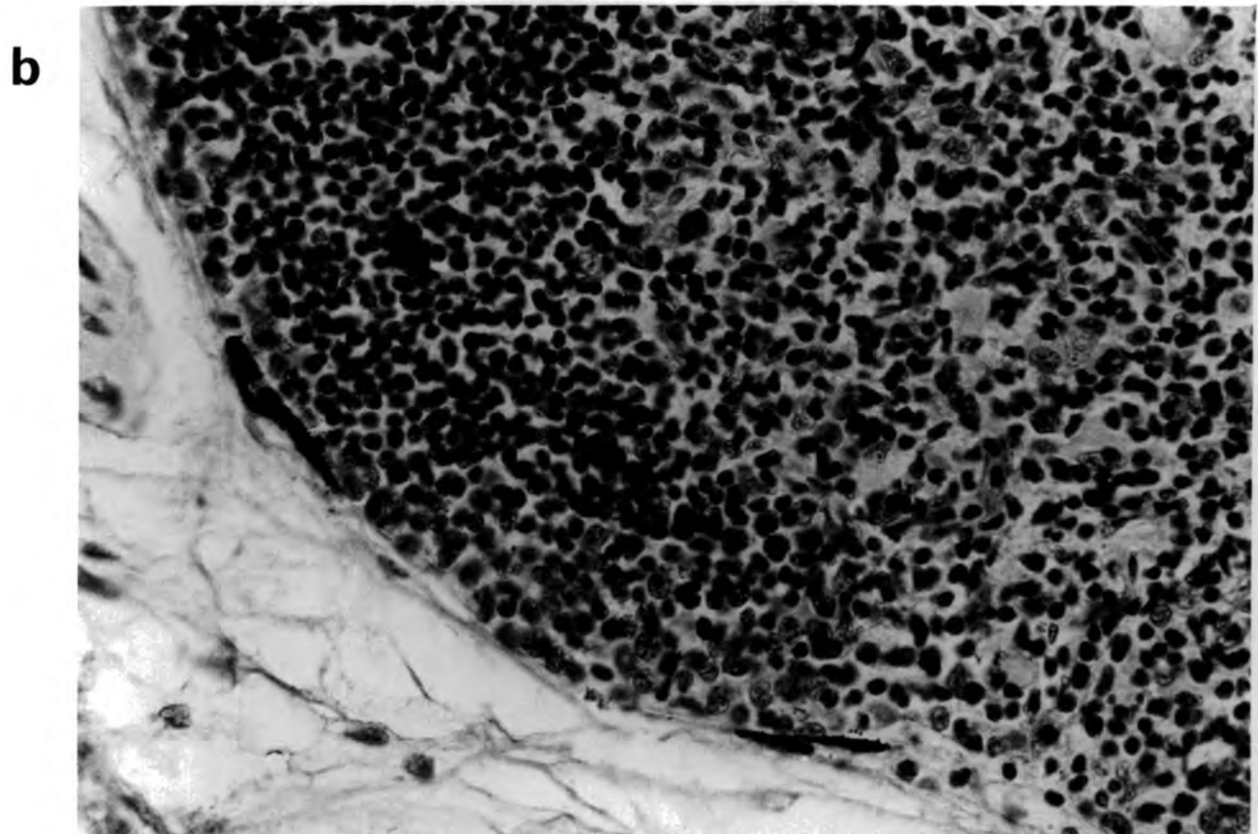


Fig. 6.4

Thymus histology during late metamorphosis and early post-metamorphic life.

(a) Stage 66 - the end of metamorphosis, 1μm

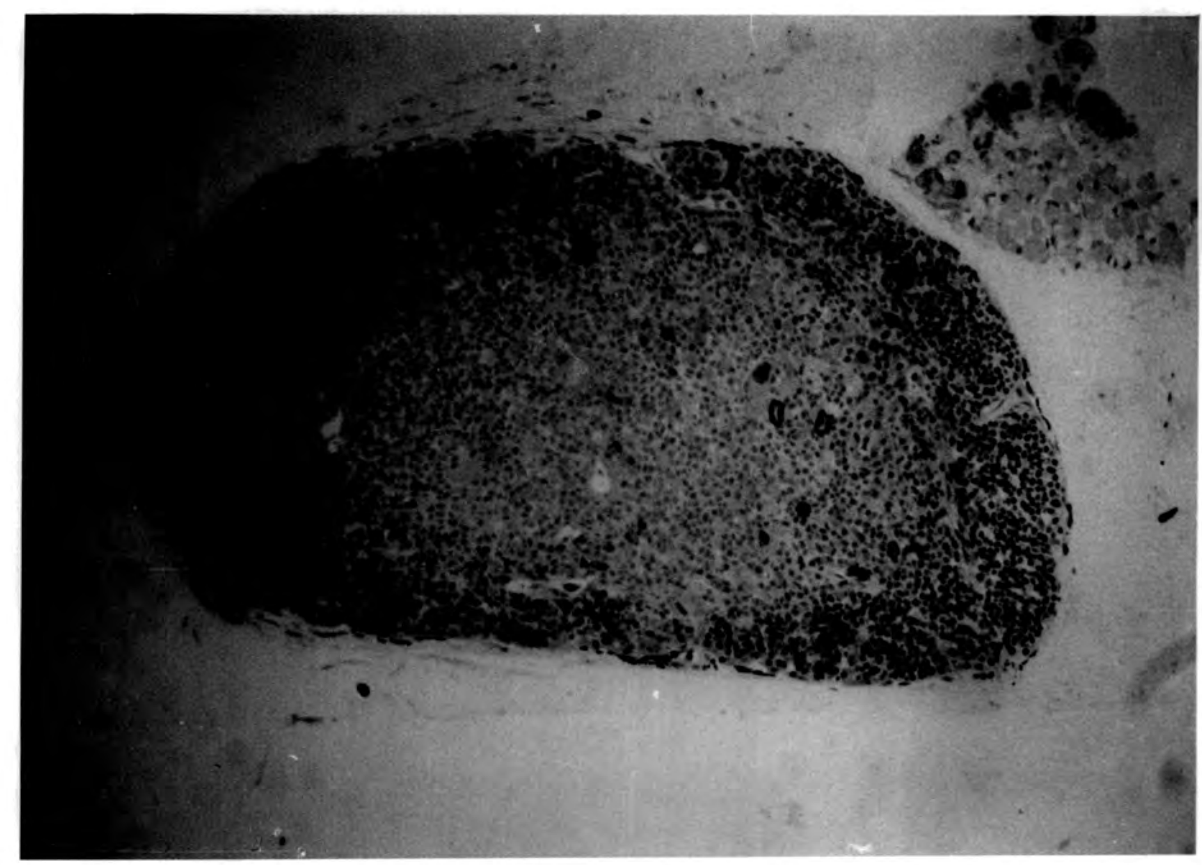
(b) 1 week after the end of metamorphosis) 8 μm

(c) 2 weeks " " " " ") H and E

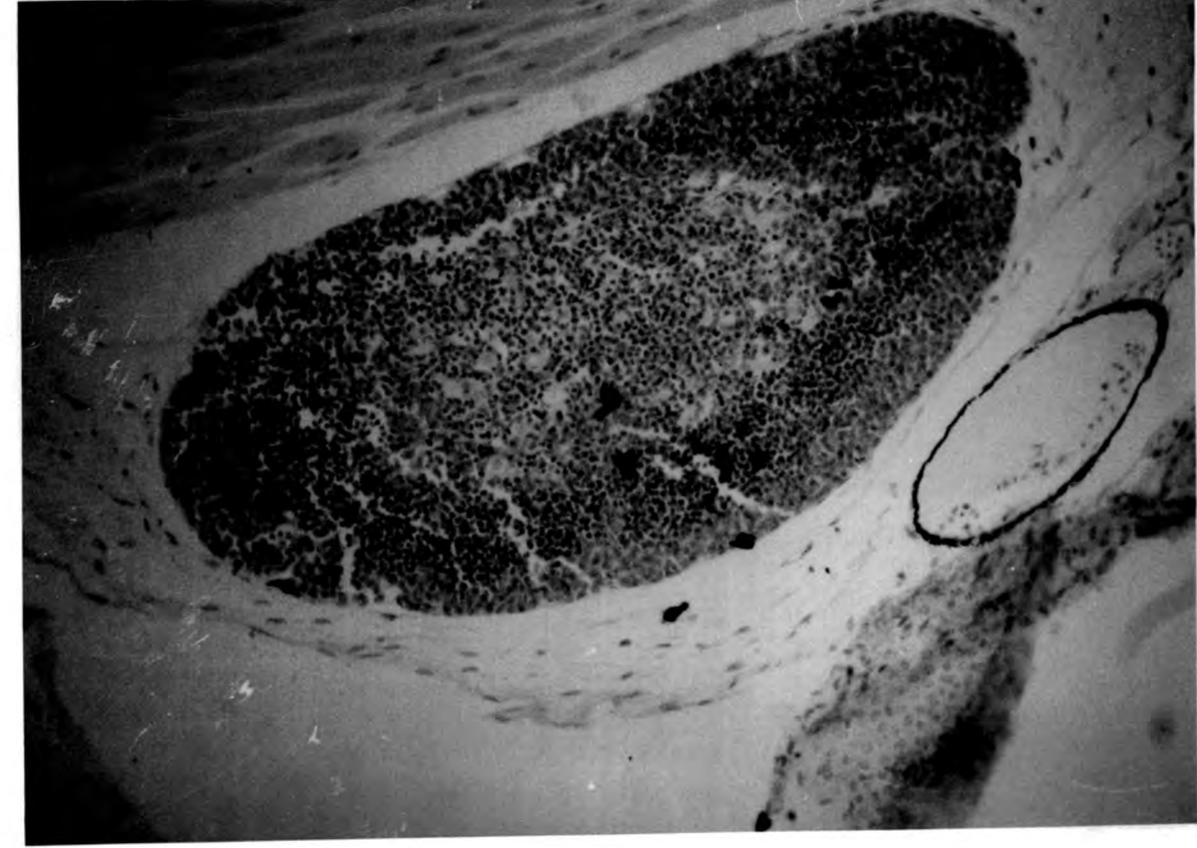
(d) 3 weeks " " " " "

Magnification x 100

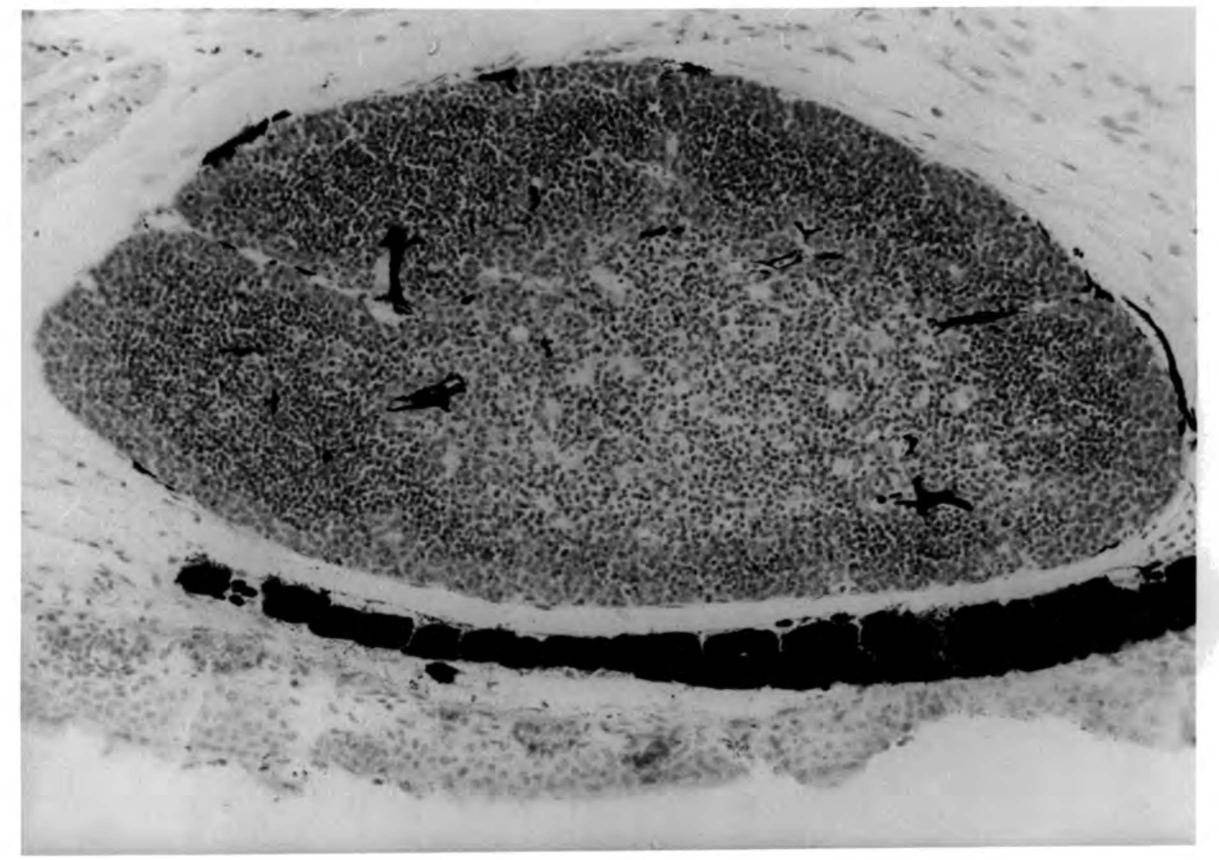
a



b



c



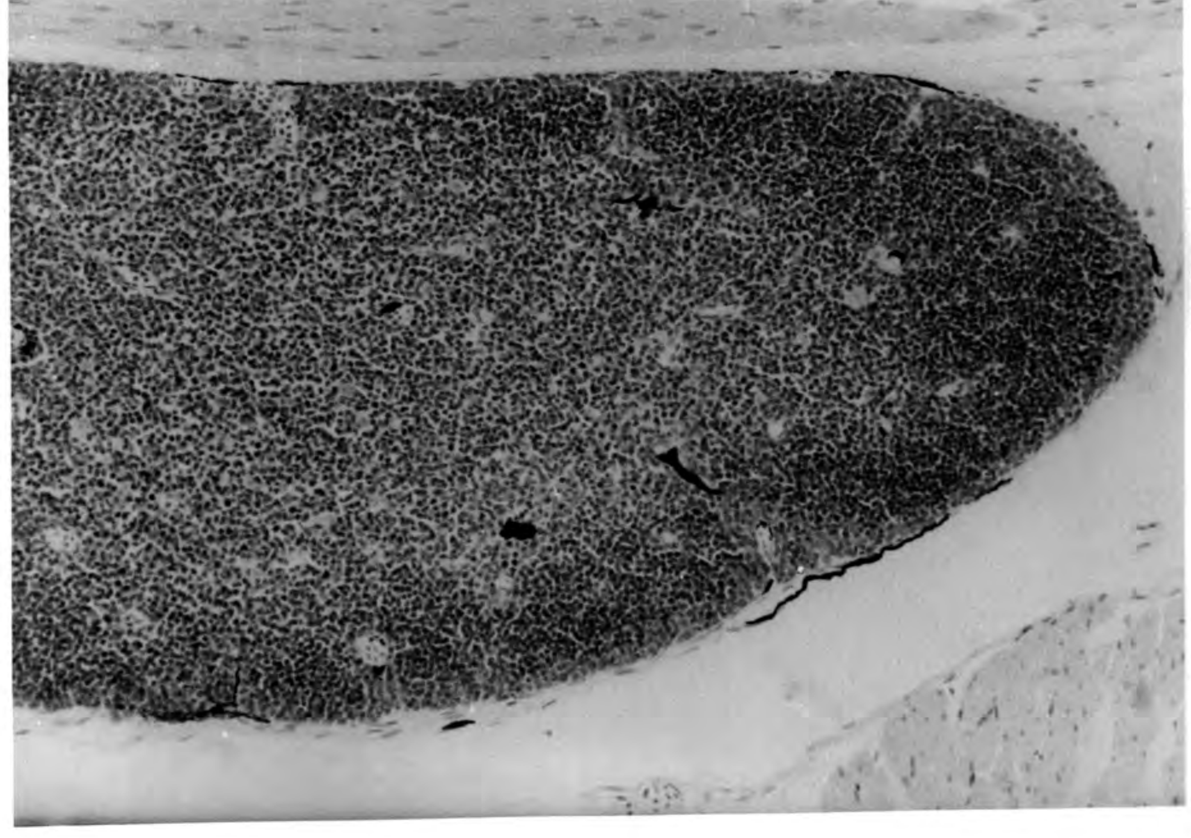


Fig. 6.5

Micrographs of the thymus

(a) 1 week and

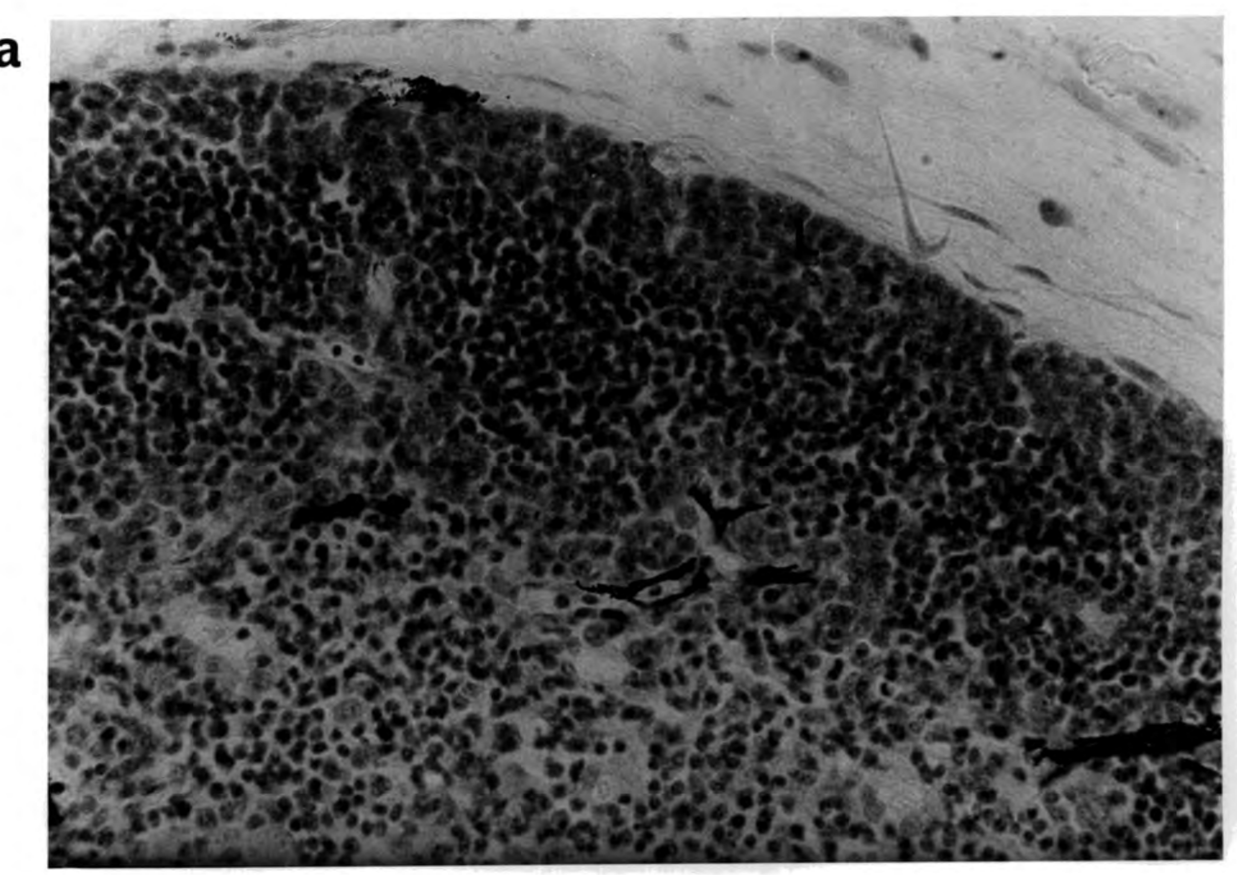
(b) 2 weeks after the end of metamorphosis.

A discrete band of lymphoblasts (L) can be clearly seen in the peripheral cortical region. Lymphocyte numbers are increasing in both cortex and medulla.

8µm

magnification x 250

a



b

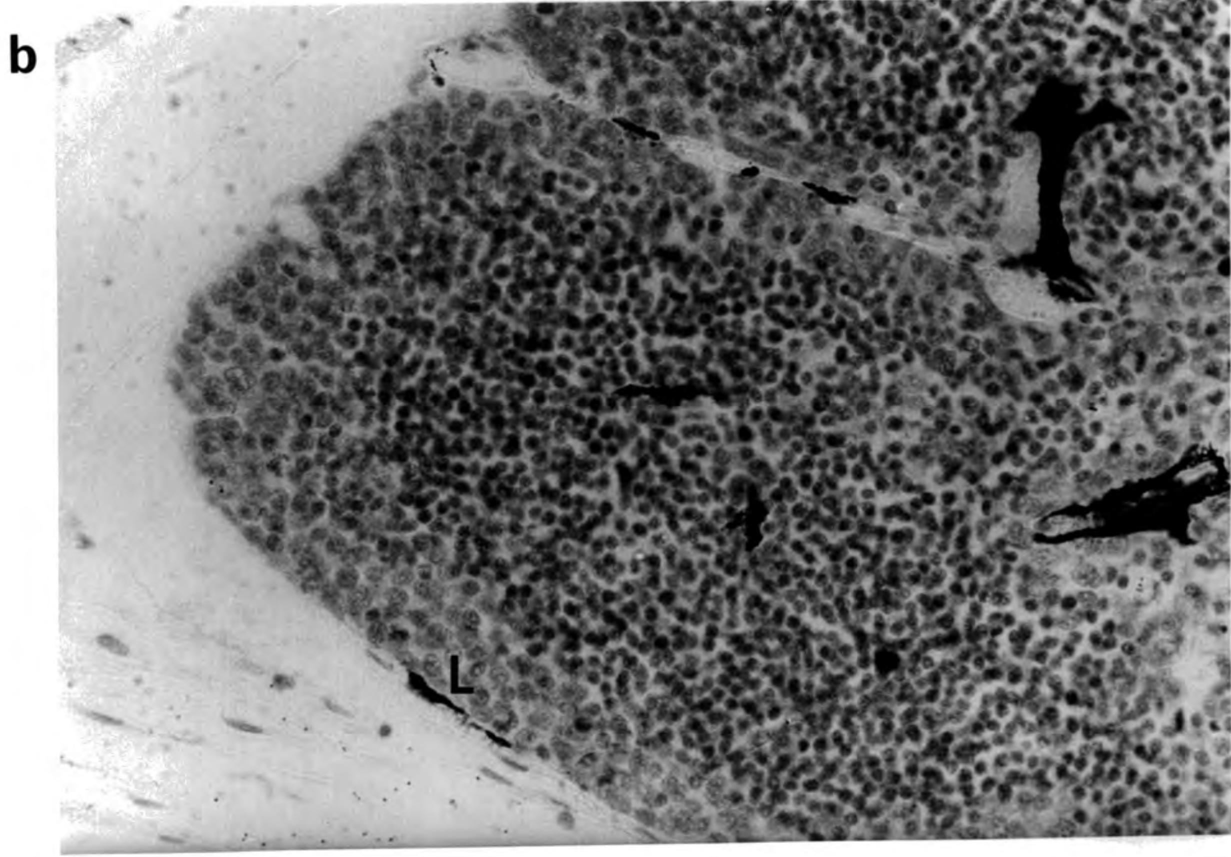


Fig. 6.6

Autoradiograph of thymus from 3-week toadlet following *in vivo* injection of tritiated thymidine.

LL= Labelled lymphocytes in peripheral cortex.

Magnification x 100

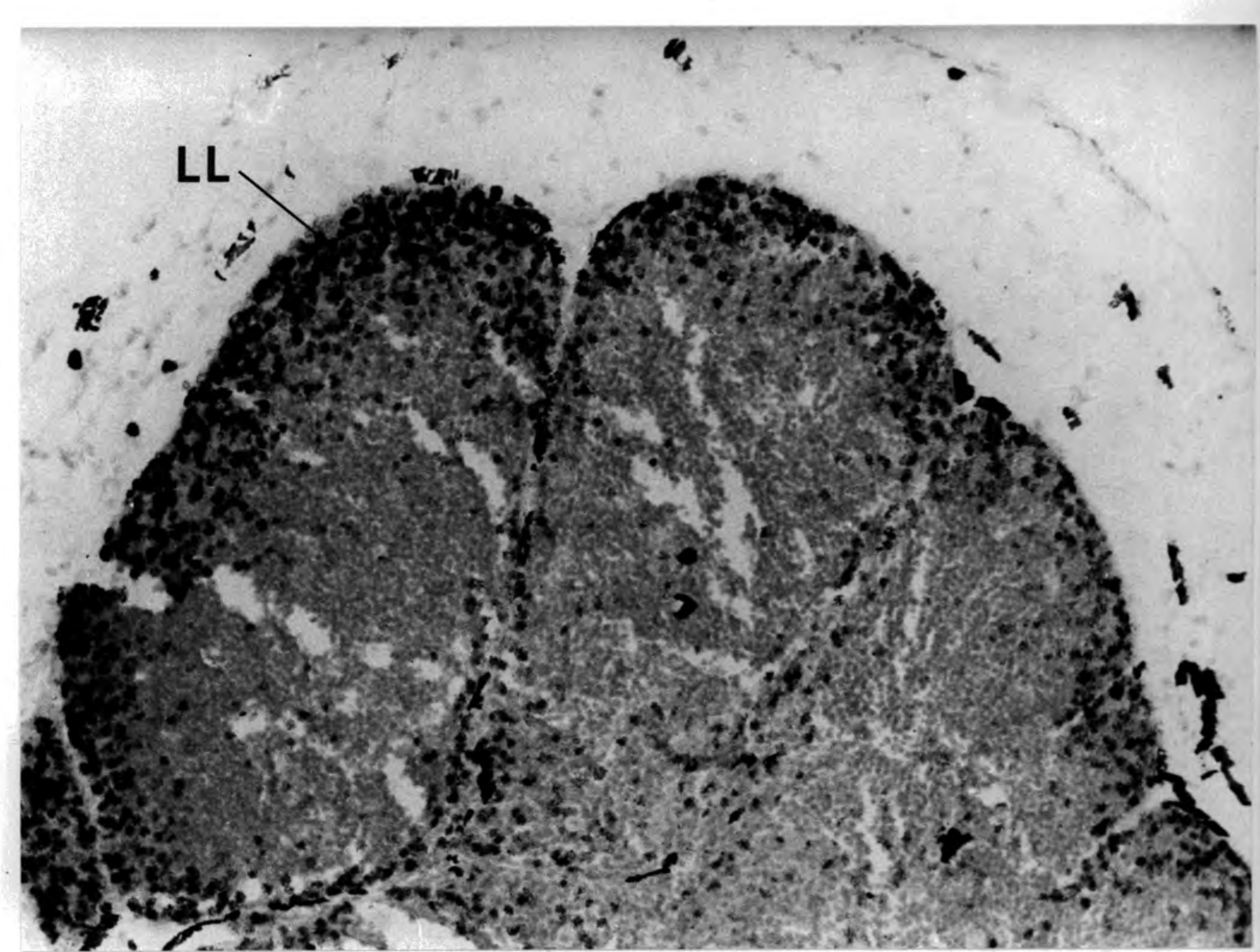

LL

Fig. 6.7

1μm plastic section of a thymus (stain toluidine blue) from a stage 52 animal following organ culture for 7 days. The organ has reduced lymphocyte numbers with many large non-lymphoid elements in the medulla (M) membrane to which thymus is attached.
Mag. x 100

Fig. 6.8

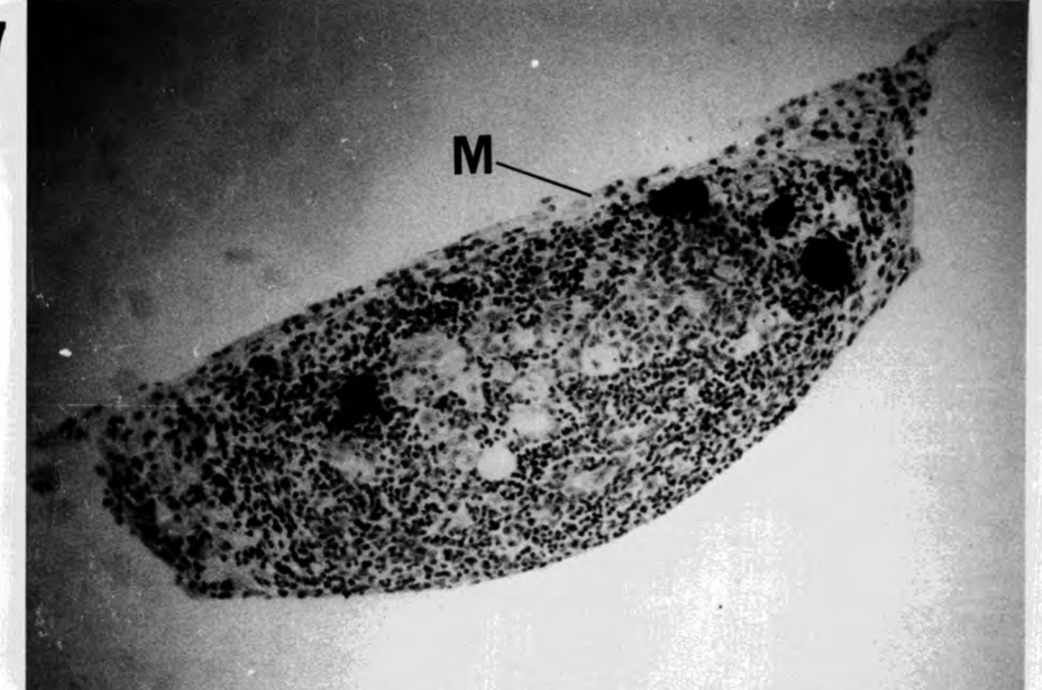
Cytospin preparations of thymocytes taken from larvae that were at stage 52 at the start of the experiment.

(a) control cells prepared directly from the larva after 7 days *in vivo*.

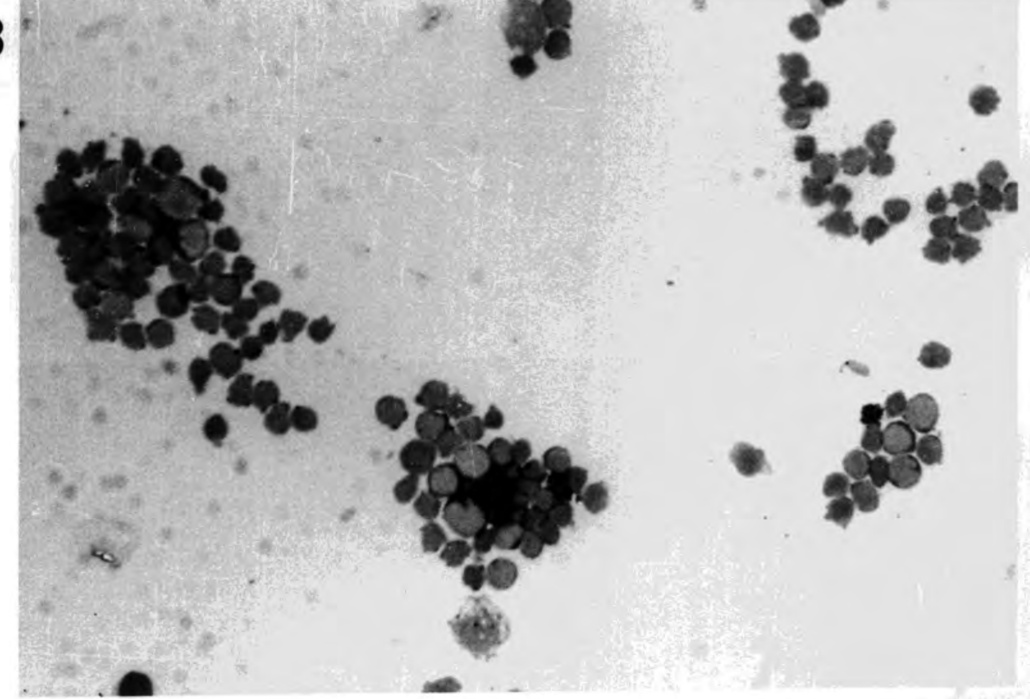
(b) cells following organ culture *in vitro* for 7 days. Although lymphocytes (L) can be seen, there are many large epithelial cells (E) present.

Mag. x 400

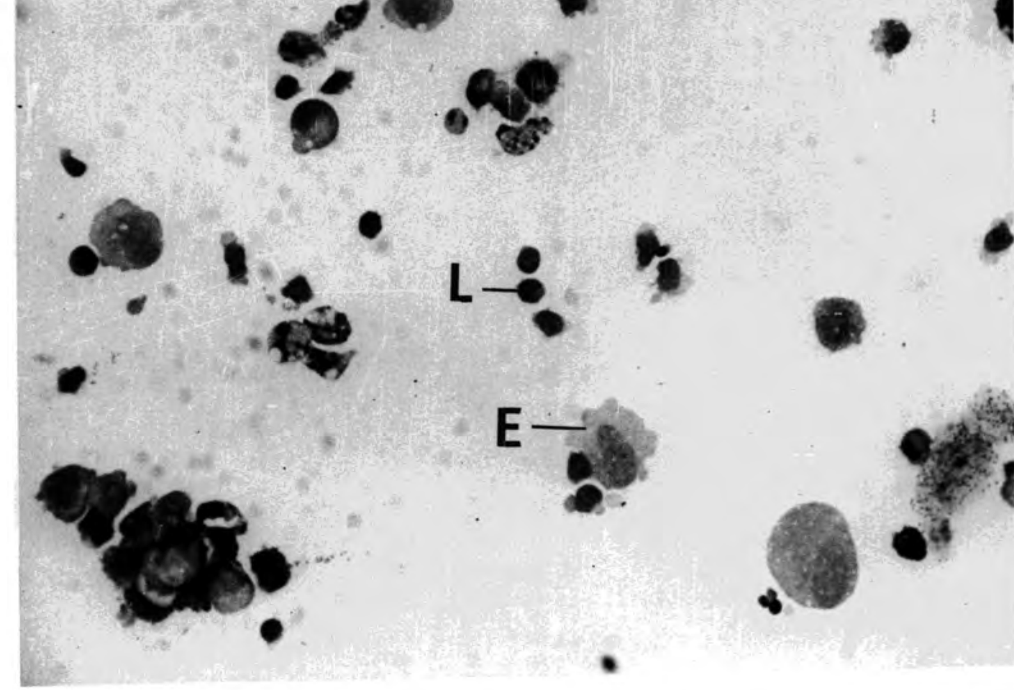
6.7



6.8
a



b



CHAPTER 7

Concluding remarks

The major findings of this Thesis are outlined in Figure 7.1 and can be considered in four sections, representing the main technical approaches used.

(1) Mitogen reactivity of lymphocytes in vitro

Initial culture experiments investigated the capacity of adult *Xenopus* thymus and spleen cells to proliferate following treatment with an array of mitogens which, in mammals, selectively activate either T or B lymphocytes. Good proliferative responses to putative T cell mitogens were recorded (using scintillation counting) for both thymocytes and splenocytes. These cells required comparable optimal PHA and Con A doses and displayed similar levels of stimulation. Splenocytes responded well to high doses of the B cell mitogen LPS. Interestingly thymocytes also displayed a consistent, albeit low level, proliferative response to LPS and PPD, suggesting the possibility that a population of B-equivalent lymphocytes resides within the adult anuran thymus.

The use of a miniaturised culture technique (5×10^4 lymphocytes per well) allowed, for the first time, mitogen experiments to be performed on thymocytes taken from individual larvae and metamorphosing *Xenopus*. During these stages of development thymocytes appear to display little sign of enhanced ^{3}HTdR uptake following Con A, PHA or LPS stimulation. A low level response to Con A and LPS was, however, recorded just prior to metamorphosis, when the thymus reaches its maximal larval size. Metamorphosis

effected a temporary disruption of the emergence of thymocyte mitogen reactivity. Appreciable and constant T and B mitogen responses in the Xenopus thymus and spleen gradually emerge after metamorphosis. Maximum levels of LPS responsiveness of thymocytes were seen at 6 months of age (when the mean S.I. reached 6). LPS reactivity of thymocytes at this time was confirmed by autoradiography; moreover, nylon wool separation experiments showed that this responsiveness lies within, or is dependent upon, a nylon wool adherent population of thymocytes. The distinct ontogenetic pattern of thymocyte responsiveness to LPS suggests that these B-equivalent cells may actually be spawned within the thymus rather than be casually migrating through this organ from the periphery.

The capacity of larval lymphoid cells other than those from the thymus to react to mitogens was not examined in any detail here. Such experiments are needed to determine the sites where functionally-equivalent T and B lymphocytes first emerge in the anuran. These experiments will require the use of histocompatible animals where cell pooling will allow study of very early stages (and during metamorphosis), when lymphocyte numbers are small. In Xenopus some hybrid laevis/gilli females lay a proportion of diploid eggs, which can be activated by irradiated sperm into gynogenetic development (Kobel and Du Pasquier, 1975). LG hybrids from a particular clone produced in this way have been shown to be identical to one another in a variety of immunological assays (e.g. grafting, MLC reactivity and antibody patterns (see Kobel and Du Pasquier, 1977). Gynogenetic development of anuran eggs through pressure (Tompkins, 1978) or cold shock treatment (Kawahara, 1978) following activation with irradiated sperm are other procedures that are being successfully used to procure (by suppressing elimination

of the second polar body) MHC compatible animals for immunological study (see discussion in Kobel and Du Pasquier, 1977).

Histocompatible colonies of *Xenopus* occurring naturally have also been described in some laboratories (Katagiri, 1978). The increasing availability of histocompatible strains of *Xenopus* increases the value of this model system for future immunologic studies.

(2) Thymectomy

Thymectomy was used in conjunction with lymphocyte culture and grafting studies to investigate the extent to which different T cell functions are dependent upon an intact thymus. *Xenopus* thymectomized at 7 days of age are often still capable of rejecting skin allografts, albeit in very chronic fashion. Spleen and blood lymphocytes taken from such thymectomized and alloimmune animals were, however, shown to be unresponsive to the T cell mitogens Con A and PHA, although they responded normally by proliferation following LPS treatment. Lymphocytes involved in graft rejection and T-mitogen-reactive cells might therefore represent distinct T cell populations in *Xenopus*, a suggestion which fits with previous sequential thymectomy studies (Horton and Sherif, 1977). Experiments in this Thesis involving thymectomy during mid-larval life failed to provide support for Horton and Sherif's suggestion that T mitogen reactive cells and MLC reactive lymphocytes represent separate T cell lineages.

Early thymectomy in *Xenopus* will continue to be a valuable technique with which to probe the developing immune system. With isogeneic animals, reconstitution experiments are readily feasible to further dissect possible T lymphocyte heterogeneity. The role

of the thymus in MHC restriction can also be profitably examined in this anuran: thus thymuses of known MHC haplotype can be implanted to early-thymectomized animals, whose subsequent cytotoxic T cell reactivity and T-B cell interactions can be assessed. The lack of runting or wasting disease in early-thymectomized Xenopus suggests, perhaps, that natural killer (NK)-like cells assume considerable importance in this anuran. This is certainly an interesting area for future work.

(3) Hapten-carrier immunisation

Three main findings came from these experiments, which employed the hapten TNP conjugated eo either a T-dependent (SRBC) or T-independent carrier (LPS). Firstly, it was shown that responsiveness to these injected antigens can be detected (by antigen binding) in the spleen from an early larval age, revealing that T-helper cells and B-equivalent lymphocytes are functioning from this time. The level of induced RFC's against TNP reached adult levels by the onset of metamorphosis, irrespective of whether the hapten was presented on the T-dependent or T-independent carrier. In fact the RFC response to TNP-LPS during metamorphosis was elevated when compared with the adult. Secondly, antibody-secreting PFC to injected SRBC, TNP-SRBC and TNP-LPS were detected in the spleen only after the end of metamorphosis. Thirdly, small numbers of anti SRBC PFC were found in the adult thymus following TNP-SRBC administration.

This work suggests that metamorphosis is associated with altered humoral immune regulation. Further experiments are required to characterise the TNP response at metamorphosis to determine the basis for the enhanced splenic reactivity recorded at this time. The apparent lack of PFC response until after metamorphosis is

intriguing and also warrants further investigation.

(4) Histologic studies on thymus development at metamorphosis

The histological studies highlighted the dramatic drop in lymphocyte numbers that occurs at the end of metamorphosis. A dearth of lymphocytes throughout the thymus occurs at this time, just prior to a rapid reappearance of lymphocytes in the peripheral cortex. Three weeks after the end of metamorphosis the thymus is considerably enlarged and is again highly lymphoid. These findings correlate with altered immune reactivity demonstrated both in this Thesis and elsewhere during metamorphosis.

The concept of specific suppressor cells as a discrete T cell subpopulation, which faced some resistance from immunologists when first proposed, has now come of age (Golub, 1981), and advances in understanding immunoregulation in the next few years will, in part, focus on the central involvement of these cells in modulating immune responses. Amphibian metamorphosis (a time when an internal histoincompatibility in the form of the emergence of new adult-specific antigens is presented to a competent immune system) presents a unique opportunity for examining immunoregulatory T cell populations, which may have significance outside of purely phylogenetic consideration.

Figure 7.1 Summary of major findings in Thesis concerning the development of the immune system in Xenopus laevis.

Age (weeks)

0

1 — Chronic allograft rejection capacity but not reactivity to T cell mitogens can develop if the thymus is removed at this stage.

3 — Antigen binding responsiveness to T-dependent and T-independent antigens is found in the spleen. Thymectomy at this stage causes impairment of both T mitogen reactivity and MLC responsiveness

6 — Small response to both T and B cell mitogens first found: the thymus. Larval thymic lymphocyte numbers reach maximum level.

7 — Thymic lymphocyte numbers are declining Mitogen responsiveness no longer observed in the thymus. Elevated levels of RFC reactivity to TNP-SRBC and TNP-LPS are found in the spleen.

9 — Thymic lymphocyte numbers are at a minimum. No mitogen responsiveness observed in the thymus. Elevated levels of antigen binding to TNP-LPS found in the spleen.

24 — Thymic lymphocyte numbers reach maximum adult levels. Good mitogen responsiveness to both T and B cell mitogens now found in both thymus and spleen. Maximum levels of thymus response to LPS found. PFC responsiveness to T-dependent and T-independent antigens is established. PFC's detectable in thymus.

ACKNOWLEDGEMENTS

I should like to express my thanks to my supervisor Dr.J.D. Horton for his unstinting guidance and help during the course of this study and in particular my appreciation of his enthusiasm and commitment to this work and developmental and comparative immunology in general.

Thanks are due to Professor D. Barker and the Department of Zoology for providing the facilities to make this study possible.

I would also like to thank those who have provided technical assistance at various times throughout this study, in particular Miss Judith Chambers, Miss Margaret I'Anson and Mrs. Christine Richardson.

I am especially indebted to Mrs. Pauline Bransden and Mr, David Hutchinson for the expert help received in the preparation of this thesis.

Finally I should like to thank Durham University for providing my Research Studentship for the duration of these studies.

REFERENCES

Andersson, J., Schreier, M.H. & Melchers, F. (1980). T-cell-dependent B-cell stimulation in H-2 restricted and antigen dependent only at the resting B-cell level. Proc. Natl. Acad. Sci. 77, 1612-.

Bernard, C.C.A., Bordmann, G., Blomberg, B. & Du Pasquier, L. (1979). Immunogenetic studies on the cell-mediated cytotoxicity in the clawed toad Xenopus laevis. Immunogenetics. 9: 443-.

Bernard, C.C.A., Bordmann, G., Blomberg, B. & Du Pasquier, L. (1981). Genetic control of T helper cell function in the clawed toad Xenopus laevis. Eur. J. Immunol. 11: 151-.

Blomberg, B., Bernard, C.C.A. & Du Pasquier, L. (1980). In vitro evidence for T-B lymphocyte collaboration in the clawed toad, Xenopus. Eur. J. Immunol. 10: 869-.

Borejeson, J., Reisfield, R., Chessin, L.N., Welsh, P. & Douglas, S.D. (1966). Studies on human peripheral blood lymphocytes in vitro. I. Biological and physiochemical properties of Pokeweed mitogen. J. exp. Med. 124: 859-.

Borum, K. (1968). Studies on the rat thymus. Scand. J. Haematol. 5: 339-.

Bosma, M. & Bosma, G. (1974). Production of donor allotype and suppression of host allotype in a thymocyte transfer system between Balb/c congenic mice. In: Progress in Immunology II Vol. L (Ed. by L. Brent & E.J.Holborow). North-Holland: Amsterdam, p. 119.

Botham, J.W., Grace, M.F. & Manning, M.J. (1980). Ontogeny of first-set and second-set alloimmune reactivity in fishes. In: Phylogeny of Immunological Memory. (Ed.M.J.Manning) Elsevier, Amsterdam. 83-.

Borysenko, M. (1976) Phylogeny of Immunity: an overview. Immunogenetics 3: 305-.

Cantor, H. & Boyse, E.A. (1977). Lymphocytes as models for the study of mammalian cellular differentiation. Immunol. Rev. 33: 105-.

Charlemagne, J. (1974). Larval thymectomy and transplantation immunity in the urodele Pleurodeles waltlii Michah (Salamandridae). Eur. J. Immunol. 4: 390-.

Charlemagne, J. & Houillon, C. (1968). Effets de la thymectomie larvaire chez l'amphibian urodèle, Pleurodeles waltlii Michah. Production a l'état adulte d'une tolérance aux homograffes cutanées. C.r. Lebd. Séanc. Acad. Sci., Paris. 267: 253-.

Charlemagne, J. & Tournefier, A. (1977). Anti-horse red blood cells antibody synthesis in the Mexican axolotol, Ambystoma mexicanum: Effects of thymectomy. In: Developmental Immunobiology (Eds. Solomon, J.B. & Horton, J.D.) Elsevier, Amsterdam 265-.

Chardonnens, X. (1976) La tolérance aux antigènes d'histocompatibilité pendant la métamorphose de l'Amphibien Anoure, Xenopus laevis: Un modèle pour l'étude de la tolerance au self. Thesis. University of Geneva (No. 1748).

Chilmoncyk, C. (1978). In vitro stimulation by mitogens of peripheral blood lymphocytes from rainbow trout (Salmo gairdneri) Annales d'Immunol. 129:3

Chou, C.T., Dubiski, S. & Cinader, B. (1966). Antibody formation and experimentally induced chimerism in very young rabbits. Nature (Lond.) 211: 34-.

Claman, H.N., Chaperon, E.A. & Triplett, R.F. (1966). Thymus-marrow cell combinations - synergism in antibody production. Proc. Soc. Exp. Biol. Med. 122: 1167-.

Cohen, N. (1969). Immunogenetic and developmental aspects of tissue transplantation immunity in urodele amphibians. In: Biology of Amphibian Tumours. Ed. M.M. Migell. Springer-Verlag, New York. 153-.

Cohen, N. (1970). Tissue transplantation immunity and immunological memory in Urodela and Apoda. Transpl. Proc. 2: 275-.

Cohen, N. (1977). Phylogenetic emergence of lymphoid tissues and cells. In: The Lymphocyte: Structure and Function. Ed. J.J.Marchalonis. Marcel Dekker. 149-.

Cohen, N. & Collins, N.H. (1977). Major and minor histocompatibility systems in ectothermic vertebrates. In: The Major Histocompatibility System in Man and Animals. Ed. D. Gotze. Springer-Verlag, Berlin. 313-.

Cohen, N. & Horan, M. (1977). Lack of correlation between the rapidity of newt allograft rejection and the frequency and magnitude of stimulation in the mixed lymphocyte culture reaction. In: Developmental Immunobiology. Eds. J.B.Solomon and J.D. Horton. Elsevier/Amsterdam. p. 259.

Cohen, N. and Turpen, J.B. (1980). Experimental analysis of lymphocyte ontogeny and differentiation in an amphibian model system. In: Biological Basis of Immunodeficiency. Eds. E.W. Gelfand & H.M. Dosch. Raven Press, New York. p.25-.

Collie, M.H., Turner, R.J. & Manning, M.J. (1975). Antibody production to lipopolysaccharide in thymectomized Xenopus. Eur. J. Immunol. 5: 426-.

Collins, N.H. & Cohen, N. (1976). Phylogeny of immunocompetent cells. II. In vitro behaviour of lymphocytes from the spleen, blood and thymus of the urodele Ambystoma mexicanum. In: Phylogeny of Thymus and Bone-Marrow-Bursa Cells. Eds. R.K. Wright & E.L.Cooper, p. 143.

Cooper, A.J. (1971). Ammocoete lymphoid cell population in vitro. In: Fourth Leukocyte Culture Conference., p.137, Ed. O.R. McIntyre Appleton Century Crofts, New York.

Cooper, E.L. & Hildemann, W.H. (1965). Allograft reactions in bullfrog larvae in relation to thymectomy. Transplantation 3: 446-.

Cooper, E.L. (1976). Comparative Immunology. Prentice-Hall, New Jersey, 338pp.

Cooper, M.D., Peterson, R.D.A., South, M.A. & Good, R.A. (1966). The functions of the thymus and bursa system in the chicken. J. Exp. Med. 123: 75-.

Coutinho, A., Möller, G., Andersson, J. & Bullock, W.W. (1973). In vitro activation of mouse lymphocytes in serum-free medium: effect of T and B cell mitogens on proliferation and antibody synthesis. Eur. J. Immunol. 3: 299.

Cuchens, M.A. & Clem, W.L. (1977). Phylogeny of lymphocyte heterogeneity. II. Differential effects of temperature on fish T-like and B-like cells. Cell. Immunol. 34, 2:219-.

Cuchens, M., McLean, E. & Clem, W.L. (1976). Lymphocyte heterogeneity in fish and reptiles. In: Phylogeny of Thymus and Bone-Marrow Bursa Cells. (Ed. R.K. Wright & E.L. Cooper) p.205-213. Elsevier/North Holland Biomedical Press, Amsterdam.

Cunningham, A.J. & Szenberg, A. (1968). Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology 14: 599.

Curtis, S.K. & Volpe, E.P. (1971). Modification of responsiveness to allografts in larvae of the leopard frog by thymectomy. Devl. Biol. 25: 177-.

Dardenne, M., Tournefier, A., Charlemagne, J. & Bach, J.F. (1973). Studies on thymic products VII. Presence of thymic hormone in urodele serum. Ann. Immunol.(Inst,Pasteur), 124c: 465-.

Davis, A.J.S., Leuchars, E., Wallis, V., Marchant, R. & Elliott, E.V. (1967). The failure of thymus-derived cells to make antibody. Transplantation. 5: 222

Deuchar, E.M. (1972). Xenopus laevis and developmental biology. Biol. Rev. 47: 37-112.

Donnelly, N., Manning, M.J. & Cohen, N. (1976). Thymus dependence of lymphocyte subpopulations in Xenopus laevis. In: Phylogeny of Thymus and Bone-Marrow-Bursa Cells. (Eds. R.K. Wright & E.L. Cooper) Elsevier, Amsterdam, 133-.

Du Pasquier, L. (1965). Aspects cellulaires et numeraux de l'intolérance aux homogreffes de tissu musculaire chêz le têtard d'Alytes obstetricans; rôle du thymus. C.r. Lebd. Séanc. Acad. Sci, Paris. 261: 1144-

Du Pasquier, L. (1970). L'aquisition de la compétence immunologique chêz les vértébrés. Etude chez la larve du Crapaud accoucheur Alytes obstetricans. Thèse doctorat ès Sciences. Université de Bordeaux. No. 290.

Du Pasquier, L. (1973). Ontogeny of the immune response in cold-blooded vertebrates. Current Topics in Microbiology and Immunology, 61: 37.

Du Pasquier, L. (1976). Phylogenesis of the vertebrate immune system. In: Mosbacher Colloqium (Eds. F.Melchers & K. Rajowsky). Springer-Verlag, Berlin. 101-.

Du Pasquier, L. & Bernard, C.C.A. (1980). Active suppression of the allogeneic histocompatibility reactions during the metamorphosis of the clawed toad Xenopus. Differentiation. 16: 1-.

Du Pasquier, L., Blomberg, B. & Bernard, C.C.A. (1979). Ontogeny of immunity in amphibians: changes in antibody repertoire and appearance of adult major histocompatibility antigens in Xenopus. Eur. J. Immunol. 9: 900-.

Du Pasquier, L. & Haimovitch, J. (1976). The antibody response during amphibian ontogeny. Immunogenetics. 3: 1-.

Du Pasquier, L. & Horton, J.D. (1976). The effect of thymectomy on the mixed leukocyte reaction and phytohaemagglutination responsiveness in the clawed toad, Xenopus laevis. Immunogenetics. 3: 105-.

Du Pasquier, L. & Kobel, H.R. (1979). Histocompatibility antigens and immunoglobulin genes in the clawed toad: expression and linkage studies in recombinant and hyperdiploid Xenopus hybrids. Immunogenetics 8: 299-.

Du Pasquier, L. & Weiss, N. (1973). The thymus during the ontogeny of the toad Xenopus laevis: growth, membrane-bound immunoglobulins and mixed lymphocyte reaction. Eur. J. Immunol., 3: 773.

Etlinger, H.M., Hodgins, H.V. & Chiller, J.M. (1976). Evolution of the lymphoid system. I. Evidence for lymphocyte heterogeneity in rainbow trout revealed by organ distribution of mitogenic responses. J. Immunol. 116: 1547-.

Etlinger, H.M. Hodgins, H.V. & Chiller, J.M. (1978). Evolution of the lymphoid system. III. Morphological and functional consequences of mitogen stimulation of rainbow trout lymphocytes. Devl. Comp. Immunol. 2: 263-.

Fache, B. & Charlemagne, J. (1975). Influence on allograft rejection at different stages of larval development in urodele amphibian Pleurodeles waltlii Michah (Salamadridae). Eur. J. Immunol. 5: 155-.

Fujii, T., Nakagawa, H. & Mirakawa, S. (1979). Immunity in lamprey II. Antigen-binding response to sheep erythrocytes and hapten in the ammocoete. Devl. Comp. Immunol. 3: 609-.

Gershon, R.K. & Kondo, K. (1972). Infectious immunological tolerance. Immunology 21: 903.

Gershwin, E. & Cooper, E.L. (1978). Animal Models of Comparative and Developmental Aspects of Immunity and Disease. Pergamon, New York. 396pp.

Glick, B., Chang, T.S. and Jaap, R.G. (1956). The bursa of Fabricius and antibody production. Poultry Sci. 35: 224-.

Goldshein, S.J. & Cohen, N. (1972). Phylogeny of immune competent cells I. In vitro blastogenesis and mitosis of toad Bufo marinus) splenic lymphocytes to phytohaemagglutinin and in mixed lymphocyte cultures. J. Immunol. 108: 1025-.

Goldstine, S.N., Collins, N.H. & Cohen, N. (1975). Mitogens as probes of lymphocyte heterogeneity in anuran amphibians. In: Immunologic Phylogeny (Eds. W.H.Hildemann & A.A.Benedict), Adv. exp. Med. Biol., 64: 343-. Plenum, New York.

Golub, E.S. (1981). Suppressor T cells and their possible role in the regulation of autoreactivity. Cell 24: 595-.

Good, R.A., Finstad, J., Pollara, B. & Gabrielson, A.E. (1966). Morphological studies on the evolution of the lymphoid tissues among the lower vertebrates. In: Phylogeny of Immunity. (Eds. R.T.Smith., P.A. Miescher & R.A.Good). University of Florida Press, Gainesville, p. 149.

Gould, S.J. (1977). Ontogeny and Phylogeny. The Berknap Press of Harvard Univ. Press. Cambridge, Massachusetts.

Greaves, M.F. & Janossy, G. (1972). Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. Transplant. Rev. 11, 87.

Greaves, M.F., Owen, J.J.T. & Raff, M.C. (1973). T and B lymphocytes. Origins, properties and role in immure responses. Excerpta Medica, North Holland, Amsterdam.

Green, N.& Cohen, N. (1979). Phylogeny of immunocompetent cells. III. Mitogen response characteristics of lymphocyte subpopulations from normal and thymectomized frogs (Xenopus laevis). Cell. Immunol. 48: 59.

Gruenwald, D.A. & Ruben, L.N. (1979). The effect of adult thymectomy upon helper function in Xenopus laevis: the South African clawed toad. Immunology 38: 191-.

Hammerström, L., Bird, A.G., Britton, S. & Smith, C.I.E. (1979). Pokeweed mitogen induced differentiation of human B cells: evaluation by a protein A haemolytic plaque assay. Immunology 38: 181-.

Häyry, P., Roberts, P.J., Ranki, A. & Weber, T. (1976). Selective responses of mouse T cells to different T mitogens and in mixed lymphocyte culture induced cytolysis reaction. Cell. Imm. 25: 121-.

Herzenberg, L.A. & Herzenberg, L.A. (1974). Allotype suppression and production by thymocytes and thymus derived cells. In: Progress in Immunology II Vol. 2 (Eds. L.Brent & E.J. Holborow) p. 111. North Holland, Amsterdam.

Hildemann, W.H. & Benedict, A.A. (1975). (Eds.) Immunolic Phylogeny. Plenum, New York. 485pp.

Hirano, T., Kuritani, T., Kishimoto, T. & Yamamura, Y. (1977). In vitro immune response of human peripheral lymphocytes. I The mechanism(s) involved in T helper cell functions in the pokeweed-induced differentiation and proliferation of B cells. J. Immunol. 119: 1235-.

Horton, J.D. (Ed.) (1980). Development and Differentiation of Vertebrate Lymphocytes. Elsevier, Amsterdam 280pp.

Horton, J.D., Edwards, B.F., Ruben, L.N. & Mette, S. (1979). Use of different carriers to demonstrate thymic dependent and thymic independent anti-trinitrophenyl reactivity in the amphibian Xenopus laevis. DCI 3: 621-.

Horton, J.D. & Manning, M.J. (1972). Response to skin allografts in Xenopus laevis following thymectomy at early stages of lymphoid organ maturation. Transplantation. 14: 141-.

Horton, J.D. & Manning, M.J. (1974). Lymphoid organ developmen- in Xenopus thymectomized at eight days of age. J. Morph. 143: 385-.

Horton, J.D., Rimmer, J.J. & Horton, T.L. (1976). Critical role of the thymus in establishing humoral immunity in amphibians: studies on Xenopus thymectomized in larval and adult life. Devl. Comp. Immunol. 1: 119-.

Horton, J.D. & Sherif, N.E.H.S. (1977). Sequential thymectomy in the clawed toad: effect on mixed leucocyte reactivity and phytohaemagglutinin responsiveness. In: Developmental Immunobiology. J.B. Solomon & J.D. Horton (Eds.) Elsevier, Amsterdam, p. 283-.

Howard, J. (1980). MHC restriction, self-tolerance and the thymus. Nature 286: 15.

Hsu, E. & Du Pasquier, L. (1981). B and T cell differentiation in Xenopus. In: Basel Institute for Immunology Annual Report 1980, p. 44.

Jacobs, D.M. & Morrison, D.C. (1975). Stimulation of a T-independent primary anti-hapten response in vitro by TNP-lipopolysaccharide (TNP-LPS). J. Immunol. 114, 360.

Janossy, G. & Greaves, M.F. (1972). Lymphocyte activation II Discriminating stimulation of lymphocyte subpopulations by phytomitogens and heterologous antilymphocyte sera. Clin. exp. Immunol. 10: 525-.

Jentz, P., Chou, C.T., Szymanska, I. Rivas Alcata R, Dubiski S. & Cinader, B. (1979). Immunoglobulin synthesis by thymus B cells. Immunology 38: 275-.

Jones, S.E., & Ruben, L.N. (1981). Internal histoincompatibility during amphibian metamorphosis? Immunology (In press).

Jordan, R.K. & Robinson, J.H. (1981). T lymphocyte differentiation In: Anatomy Society Symposium "The Thymus Gland". Ed. M. Kendall, Academic Press, London. (In press).

Jurd, R. (1978). Crisis of Identity. Devl. Comp. Immunol. 2: 229-.

Katagiri, C., Kawahara, H., Nagata, S. & Tochinai, S. (1980). The mode of participation of T-cells in immune reactions as studied by transfer of triploid lymphocytes into early-thymectomized diploid Xenopus. In: Development and Differentiation of Vertebrate Lymphocytes. (Ed.) J.D. Horton p. 163.

Katz, D.H. & Bonacerraf, B. (1975). The function and inter-relationship of T cell receptors, Ir genes and other histocompatibility gene products. Transpl. Rev. 22: 175-.

Kawaguchi, S., Kina, T. & Muramatsu, S. (1978). Natural haemolytic activity of snake serum: III. Plaque-forming cells in adult and newly-hatched snakes (Elaphe quadrivirgata) Devl. Comp. Immunol. 2, 657.

Kawahara, H. (1978). Production of triploid and gynogenetic diploid Xenopus by cold treatment. Devl. Growth and Differentiation 20: 227.

Keigthley, R.G., Cooper, M.D. & Lawton, A.R. (1976). The T cell dependence of B cell differentiation induced by pokeweed mitogen. J. Immunol. 117: 1538-.

Kidder, G.M., Ruben, L.N, & Stevens, J.M. (1973). Cytodynamics and ontogeny of the immune response of Xenopus laevis against sheep erythrocytes. J. Embryol. exp. Morph. 29: 73-85.

Kobel, H.R. & Du Pasquier, L. (1975). Production of large clones of histocompatible, fully identical clawed toads (Xenopus). Immunogenetics 2: 87-.

Kruisbeek, A.M. (1979). Thymic factors and T cell maturation in vitro: a comparison of the effects of thymic epithelial cultures with thymic extracts and thymus dependent serum factors. Thymus 1: 163-.

Lanzavecchia, A., Sacchi, R., Maccario, R., Vitiello, A., Nespoli, L. & Ugazio, A.G. (1979). Plaque assay with protein A-coated erythrocytes for the evaluation of human immunoglobulin-secreting cells induced by pokeweed mitogen. Scand. J. Immunol. 10: 297-.

Larsson, E-L, Coutinho, A. & Martinez - A,C. (1980). A suggested mechanism for T lymphocyte activation: Implications on the acquisition of functional reactivities. Immunolical Rev. 51: 61-.

Leino, A. (1978). Ontogeny of PHA, Con A responses in the fetal lamb. Clin.Immun. & Immunopath 11: 6-.

Leiper, J.B. & Solomon, J.B. (1977). Ontogeny of mitogenic responsiveness to PHA and sheep erythrocytes and lymphokine production in foetal guinea-pigs. Immunology 32: 215-.

Le Douarin, N.M. (1977). Ontogeny of primary lymphoid organs. In: B and T cells in immune recognition. Eds. F. Low & G.E. Roelants. Wiley, London. 1-.

Leverone, L., Suhar, T., Brown, R.D. & Armentrout, R.W. (1979). Characteristics of immunoglobulin synthesised early in the development of Xenopus laevis. Devel. Biol. 68: 319-.

Lopez, D.M., Sigel, M.M. & Lee, J.C. (1974). Phylogenetic studies on T cells. I. Lymphocytes of the shark with differential response to phytohaemagglutinin and concanavalin A. Cell. Immunol. 10: 287-.

Mandel, T.E., & Kennedy, M.M. (1978). The differentiation of murine thymocytes in vitro and in vivo. Immunol. 35: 317-.

Manning, M.J. (1980). (Ed.) Phylogeny of Immunological Memory. Elsevier, Amsterdam. 318pp.

Manning, M.J. & Collie, M.H. (1977). The ontogeny of thymic dependence in the amphibian Xenopus laevis. In: Developmental Immunobiology (Eds.) J.B. Solomon & J.D. Horton, Elsevier/North Holland Biomedical Press, Amsterdam. p.291.

Manning, M.J., Donnelly, N. & Cohen, N. (1976). Thymus-dependent and thymus-independent components of the amphibian immune system. In: Phylogeny of Thymus and Bone-Marrow-Bursa Cells. (Eds.) R.K. Wright & E.L. Cooper, Elsevier/Amsterdam. p.123-.

Manning, M.J. & Turner, R.J. (1976). Comparative Immunobiology. Blackie, Glasgow & Halsted Press. 184pp.

Manickavel, V. (1972). Studies on skin transplantation immunity in the lizard, Calotes versicolor. Ph.D. thesis, Annamalai University, India.

Marchalonis, J.J. (1977). Immunity in Evolution. Edward Arnold, London. 316pp.

Melchers, F. (1979). Murine embryonic B-lymphocyte development in the placenta. Nature 277: 219-.

Micklem, H.S., Anderson, N., Ure, J. & Parry-Jones, H. (1976). Long term immunoglobulin G production by transplanted thymus cells. Eur. J. Immunol. 6: 425-.

Miller, J.F.A.P. (1961). Immunological function of the thymus. Lancet 2: 748-.

Miller, J.F.A.P. (1962). Immunological significance of the thymus of the adult mouse. Nature (London) 195: 1318.

Miller, J.F.A.P. (1963). Tolerance in the thymectomized animal. In: La Tolérance Acquise et la Tolérance Naturelle à l'égard de Substances Antigéniques Définies. Colloque du Centre National de la Recherche Scientifique. p. 47.

Miller, J.F.A.P. (1979). Experimental thymology has come of age. Thymus 1: 3-25.

Miller, J.F.A.P., Brunner, K.T., Sprent, J.; Russell, P.J. & Mitchell, G.F. (1971). Thymus-derived cells as killer cells in cell-mediated immunity. In: Proc. of the Third International Congress of the Transplantation Society (The Hague, 1970). Transpl. Proc. 3: 915-.

Minagawa, Y., Ohnishi, K. & Murakawa, S. (1976). Structure and immunological function of lymphomyeloid organs in the bullfrog, Rana catesbeiana. In: Immunologic Phylogeny (Eds.) W.H. Hildemann & A.A. Benedict, Plenum, New York. p. 257.

Mitchell, G.F. & Miller, J.F.A.P. (1968). Cell to cell interaction in the immune response. II. The source of haemolysis-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. J. Exp. Med. 128: 821.

Mitchison, N.A. (1971). The carrier effect in the secondary response to hapten-protein conjugates. II. Cellular cooperation. Eur. J. Immunol. 1, 18-.

Mond, J.J., Mongini, P.K.A., Sieckmann, D. & Paul, W.E. (1980). Role of T lymphocytes in the response to TNP-AECM-Ficoll. J. Immunol. 125: 1066-.

Monickendam, M.A., & Balls, M. (1973). Amphibian organ culture. Experientia 29: 1-.

Moore, M.A.S. & Owen, J.J.T. (1967). Experimental studies on the development of the thymus. J. exp. Med. 126: 715.

Mosier, D.E. (1974). Ontogeny of mouse lymphocyte function: I. Paradoxical elevation of reactivity to allogeneic cells and phytohaemagglutinin in BALB/C foetal thymocytes. J. Immunol. 112: 305-.

Mosier, D.E. (1977). Ontogeny of T cell function in the neonatal mouse. In: Development of host defenses. (Eds.) M.D. Cooper & D.H. Dayton. Raven Press, New York.

Mosier, D.E., Mond, J.J. & Goldings, E.A. (1977). The ontogeny of thymic independent antibody responses in vitro in normal mice and mice with an X-linked B cell defect. J. Immunol. 119; 1874.

Moticka, E.J., Brown, B.A. & Cooper, E.L. (1973). Immunoglobulin synthesis in bullfrog larvae. J. Immunol. 110: 855-.

Nagata, S. (1977). An electron microscopic study on the thymus of larvae and metamorphosed toads Xenopus laevis. Daudin. Journal of the Faculty of Science, Hokkaido Univ. Series VI Zoology 20: 263-.

Nieuwkoop, P.D. & Faber, J. (1956). Normal Table of Xenopus laevis (Daudin) North Holland, Amsterdam.

Nowell, P.C. (1960). Phytohaemagglutinin: an initiator of mitosis in cultures of human leukocytes. Cancer Res. 20: 462-.

Ónody, C., Bátory, G. & Petranyi, G.G.Y. (1980). Age-dependent responsiveness of T lymphocytes to allogeneic and PHA stimulation. Thymus 1: 205-.

Ortiz-Muniz, G. & Sigel, M.M. (1971). Antibody synthesis in lymphoid organs of two marine teleosts. J. Reticuoendothelial Soc. 9: 42-.

Owen, J.J.T., Raff, M.D. & Cooper, M.D. (1975). Studies on the generation of B lymphocytes in the mouse embryo. Eur. J. Immunol. 5: 468-.

Owen, J.J.T. & Ritter, M.A. (1969). Tissue interaction in the development of thymus lymphocytes. J.exp. Med. 129: 431-.

Owen, J.J.T., Andrew, T., Jordan, R.K., McElroy, P., Robinson, J.H., Singh, U. & Willcox, H.N.A. (1977). Factors influencing the primary differentiation of lymphocyte populations. In: Developmental Immunobiology. (Eds.) J.B.Solomon & J.D. Horton.
pp. 131-.

Paetkau, V., Mills, G., Gerhardt, S. & Monicorrie, V. (1976) Proliferation of murine thymic lymphocytes in vitro in mediated by the Concanavalin A induced release of a lymphokine (costimulator). J. Immunol. 117: 1320-.

Pantelouris, E.M. (1971). Observations on the immunobiology of 'nude' mice. Immunology, 20: 247.

Pantelouris, E.M. (1973). Athymic development in the mouse. Differentiation 1: 437-.

Pettey, C.L., & McKinney, E.C. (1981). Mitogen induced cytotoxicity in the nurse shark. Devel. Comp. Immunol. 5: 53-.

Powell, A.E. & Leon, M.A. (1970). Reversible interaction of human lymphocytes with the mitogen Concanavalin A. Exp. Cell. Res. 62: 315-.

Raff, M.C. (1970). Role of thymus derived lymphocytes in the secondary humoral immune response in mice. Nature (Lond). 226: 1257-.

Rijkers, G.T. & van Muiswinkel, W.B. (1977). The immune system of cyprinoid fish. The development of cellular and humoral responsiveness in the cosybarb (Barbus conchonius). In: Developmental Immunobiology. (Eds.) J.B. Solomon & J.D. Horton, Elsevier,North Holland Amsterdam, p. 233.

Rimmer, J.J. (1977). Electron microscopic studies of the developing amphibian thymus. Devel. Comp. Immunol. 1: 321-.

Rimmer, J.J. & Horton, J.D. (1977). Allograft rejection in larval and adult Xenopus following early thymectomy. Transplantation 23: 142-148.

Rittenberg, M.B. & Pratt, K.L. (1969). Anti-trinitrophenyl (TNP) plaque assay. Primary response of Balb/c mice to soluble and particulate immunogen. Proc. Soc. exp. Biol. Med. 132: 575-.

Riviere, H.B., Cooper, E.L., Reddy, A.L. & Hildemann, W.H. (1975). In search of the hagfish thymus. Amer. Zool. 15: 39-49.

Robinson, J.H. (1980). A review of thymus ontogeny in organ culture. In: Development and Differentiation of Vertebrate Lymphocytes. (Ed.)J.D. Horton, Elsevier, Amsterdam. p. 111-.

Robinson, J.H. & Owen, J.T.T. (1976). Generation of T-cell function in organ culture of foetal mouse thymus. I. Mitogen responsiveness. Clin. exp. Immunol. 23: 347-.

Robinson, J.H. & Owen, J.J.T. (1977). Generation of T-cell function in organ culture of foetal mouse thymus. II. Mixed lymphocyte culture reactivity. Clin. exp. Immunol. 27: 322-.

Rocha, B., Freilas, A. & De Sousa, M. (1979). Characterisation of rat spleen cell populations. I Cell interactions in the regulation of in vitro response to Concanavalin A. Immunology 36: 619-.

Roelants, G.E. (1978). The regulatory role of macrophages in immune recognition. In: B and T cells in immune recognition. (Eds.) F. Loor & G.E. Roelants, John Wiley & Sons, London, p.103.

Ruben, L.N. (1975). Ontogeny, phylogeny and cellular cooperation. Amer. Zool. 15: 93.

Ruben, L.N., Clothier, R., Hodgson, R., & Balls, M. (1977). The in vitro reconstitution of a thymus cell dependent humoral immune response in spleens of thymectomized Xenopus laevis with allogeneic thymocytes. In: Developmental Immunobiology (Eds.) J.B. Solomon & J.D. Horton, Elsevier/North Holland Biomedical Press, Amsterdam, p. 277.

Ruben, L.N. & Edwards, B.F. (1977). Phenotypic restriction of antigen binding specificity on immunised amphibian spleen cells. Cell Immunol. 33: 437-.

Ruben, L.N. & Edwards, B.F. (1980). The phylogeny of the emergence of "T-B" collaboration in humoral immunity. In: Contemporary Topics in Immunobiology. (Eds.) N.Cohen & J.J. Marchalonis, Vol. 9, Plenum Press, New York, pp.

Ruben, L.N., Stevens, J.M. & Kidder, G.M. (1972). Suppression of the allograft response by implants of mature lymphoid tissues in larval Xenopus. J. Morphol. 138: 457-.

Ruben, L.N., van der Hoven, A. & Dutton, R.W. (1973). Cellular cooperation in hapten carrier responses in the newt Triturus viridescens. Cell Immunol. 6:300-.

Ruben, L.N., Warr, G.W., Decker, J.M. & Marchalonis, J.J. (1977). Phylogenetic origins of immune recognition: lymphoid heterogeneity and the hapten/carrier effect in the goldfish, Carassius auratus. Cell. Immunol. 31: 266.

Ruben, L.N., Welch, J.M. & Jones, J.E. (1980). Carrier primed anti-hapten responses in larval and metamorphosing Xenopus laevis, the South African clawed toad. In: Development & Differentiation of Vertebrate Lymphocytes (Ed. J.D. Horton), Elsevier/Amsterdam p. 227-.

Rygaard, J. (1973). Thymus and self; immunobiology of the mouse mutant nude. Wiley, London.

Sailendri, K. (1973). Studies on the development of lymphoid organs and immune responses in the teleost, Tilapia mossambica (Peters). Ph.D. thesis, Madurai University, 106 pp.

Sailendri, K. & Muthukkaruppan, V. (1975). The immune response to the teleost, Tilapia mossambica to soluble and cellular antigens. J. exp. Zool. 191: 371-.

Saint-Marie, G. (1973). Cell migration and the thymus. In: Contemporary Topics in Immunobiology, Vol. 2. "Thymus Dependency". (Eds.) A.J.J.Davies & R.L.Carter, p. 111-.

Sällstrom, J.F. & Alm, G.C. (1973). The in vitro maturation of the embryonic chicken thymus. II. Development in organ culture of lymphocytes responsive to PHA and Con A in vitro. Acta. path. microbiol. Scand. Section A 81: 75-.

Schreffler, D.C., Atkinson, J.P., Brown, L.J., Parker, K.L. & Ross, M.H. (1981). In: Immunology of the major histocompatibility complex (Eds. M.B.Zaleski, C.J.Abeyonnis, & K.J.Kano). Pub. S. Karger A.G. Basel, 1981.

Seto, F. (1978). Apparent antibody producing capacity of chicken thymocytes. Devel. Comp. Immunol. 2: 667-.

Shearer, G.M., Rehn, T.G. & Garbarino, C.A. (1975). Cell mediated lympholysis of trinitrophenyl-modified autologous lymphocytes. Effector cell specificity to modified cell surface components controlled by the H-2K and H-2D serological regions of the murine major histocompatibility complex. J. exp. Med. 141: 1348-.

Shimamoto, Y., Taniguchi, C., Kubo, C. & Nomoto, K. (1980). Differences in thymus-dependency among various T-cell functions. Immunol. 41: 167-.

Sigel, M.M., Lee, J.C., McKinney, E.C. & Lopez, D.M. (1978). Cellular immunity in fish as measured by lymphocyte stimulation. Mar. Fish. Rev. 40, 6-11.

Solomon, J.B. (Ed.) (1981). Aspects of Developmental and Comparative Immunology I. Pergamon, Oxford, 572pp.

Solomon, J.B. & Horton, J.D. (Eds.) (1977). Developmental Immunobiology. Elsevier, Amsterdam. 456pp.

Stites, D.P., Carr, M.C. & Fudenberg, H.H. (1974). Ontogeny of cellular immunity in the human fetus. Development of responses to phytohaemagglutinin and to allogeneic cells. Cell. Immunol. 11, 257-.

Stobo, J.D. & Paul, W.E. (1973). Functional heterogeneity of murine lymphoid cells. III. J. Immunol. 110: 362-.

Stolen, J.S. & Mäkelä, O. (1975). Carrier preimmunization in the anti-hapten response of a marine fish. Nature, 254: 718-.

Stolen, J.S. & Mäkelä, O. (1976). Cell collaboration in marine fish. The effect of carrier preimmunization on the anti-hapten response to NIP and NNP. In: Phylogeny of Thymus and Bone Marrow-Bursa Cells. (Eds).R.K.Wright & E.L. Cooper. Elsevier/Amsterdam, p. 93.

Szenberg, A. & Warner, N.L. (1962). Dissociation of immunological responsiveness in fowls with a hormonally arrested development of lymphoid tissue. Nature (London) 194: 146-.

Tochinai, S. (1976). Demonstration of thymus-independent immune system in Xenopus laevis. Response to polyvinylpyrrolidone. Immunology 31: 125-128.

Tochinai, S. & Katagiri, C. (1975). Complete abrogation of immune response to skin allografts and rabbit erythrocytes in the early thymectomized Xenopus. Development, Growth & Differentiation. 17: 383-394.

Tompkins, R. (1978). Triploid and gyrogenetic diploid Xenopus laevis. J. exp. Zool. 203: 251.

Tompkins, R. & Kaye, C. (1981). Effects of thymectomy during early development of Xenopus laevis. In: Aspects of Developmental and Comparative Immunology. I. Ed. J.B. Solomon. Pergamon Press, Oxford, p. 403.

Tournefier, A. (1972). Les reactions immunitaires chez les Amphibiens Urodèles. III. Rôle du thymus dans l'immunité de transplantation. Capacité d'immunisation aux antigènes particulaires chez le Pleurodèle et le Triton alpestre adultes. In: Phylogenic and Ontogenic Study of the Immune Response and its contribution to the Immunological Theory. Colloque Inserm. pp.105-. Ministry of Public Health, Paris.

Tournefier, A. (1973). Developpement des organes lymphoïdes chez l'Amphibien Urodèle Triturus alpestris Laur; tolérance des allogreffes après la thymectomie larvaire. J.Embryol. Exp. Morph. 29: 383-.

Tournefier, A. & Charlemagne, J. (1975). Antibodies against salmonella and SRBC in Urodele amphibians: synthesis and characterization. In: Immunologic Phylogeny. (Eds.) W.H.Hildemann &A.A.Benedict, Plenum, New York pp.161-.

Triesman, M. (1981). The significance of immunity restriction by the major histocompatibility complex; and of the occurrence of high polymorphism at MHC Loci. Two hypotheses. J. Theoret. Biol. 89: 409-.

Turner, R.J. & Manning, M.J. (1974). Thymic dependence of amphibian antibody responses. Eur. J. Immunol. 4: 343-.

Turpen, J.B. & Cohen, N. (1976). Alternative sites of lymphopoeisis in the amphibian embryo. Ann. Immunol. (Inst.Pasteur) 127C: 841-.

Turpen, J.B., Volpe, E.P. & Cohen, N. (1975). On the origin of thymic lymphocytes. Amer. Zool. 15: 51-.

Volpe, E.P., Tompkins, R., & Reinschmidt, D.C. (1981). Evolutionary modifications of nephrogenic mesoderm to establish the embryonic centers of hemopoiesis. In: Aspects of Developmental and Comparative Immunology. I. (Ed.) J.B. Solomon, Pergamon Press, Oxford, p. 193.

Watanabe, T., Fathman, C.G. & Coutinho, A. (1977). T cells which proliferate in response to concanavalin A include cells which proliferate in mixed leucocyte reactions. Eur. J. Immunol. 7: 603-.

Weissman, I.L. (1967). Thymus cell migration. J. exp. Med. 126: 291-.

Wild, A.E. & Dipper, F.A. (1974). A simple method for demonstrating specificity of antibody production by single cells. J. Immunol. Methods 4: 275-.

Wright, R.K. & Cooper, E.L. (Eds.) (1976). Phylogeny of thymus and bone marrow-bursa cells. North Holland, Amsterdam, 325pp.

Yocum, D., Cuchens, M. & Clem, L.W. (1975). The hapten-carrier effect in teleost fish. J. Immunol. 114: 925.

Zinkernagel, R.M. (1978). The thymus: its influence on recognition of "self major histocompatibility antigens" by T cells and consequences for reconstitution of immunodeficiency. Springer Seminars in Immunopathology 1: 405-.

Zinkernagel, R.M. & Doherty, D.E. (1974). Immunological surveillance against self components by sensitised T lymphocytes in lymphocyte chorio-meningitis. Nature (Lond). 251: 457-.

Zettergren, L.D., Kubagawa, H. & Cooper, M.D. (1980). Development of B cells in Rana pipiens. In: Phylogeny of Immunological Memory (Ed.) M.J. Manning, Elsevier/ North Holland Biomedical Press, Amsterdam, p. 177.

Durham University
SCIENCE
22 FEB 1982
SECTION
Library