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Russ, Jane Hannah

USE OF ORGAN-CULTURE, IRRADIATION AND ADOPTIVE-TRANSFER TO INVESTIGATE THE ROLE OF THE XENOPUS THYMUS IN T LYMPHOCYTE DEVELOPMENT

by

JANE HANNAH RUSS

B.Sc. (Dunelm)

A Thesis submitted for the degree of Doctor of Philosophy

Department of Zoology, University of Durham.

August 1986

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Abstract

This Thesis attempts to develop an amphibian model system for exploring the role of the thymus, particularly its stromal cells, in the acquisition of allotolerance. Inbred, clonal and cytogenetically-marked Xenopus are used in this work.

The initial experiments (Chapter 2) describe in vitro attempts to deplete the larval and post-metamorphic thymus of its lymphocyte component, while leaving its stromal elements intact. The histologic effects of deoxyguanosine-treatment and δ-irradiation on the organ-cultured thymus are examined in wax sections. In vivo whole-body irradiation (3000R) and subsequent in situ residence for ~ 10 days proves the most successful technique for depleting thymic lymphocyte numbers from the froglet thymus (Chapter 3). This technique provides a lymphocyte-depleted thymus with a fairly normal 3-d stromal network. Furthermore, these thymuses show no sign of lymphocyte regeneration when organ-cultured for ~ 2 weeks. In Chapter 3, 1 µm sections and electron microscopy of plastic-embedded sections provide a detailed picture of the froglet thymus following irradiation.

Chapters 4 and 5 employ ploidy labelling and the X. borealis (quinacrine-fluorescence) cell marker system, to show that larval and adult (normal or in vivo irradiated) thymuses, implanted to early-thymectomized Xenopus (MHC-compatible or -incompatible), become infiltrated by host lymphoid cells, the thymic epithelium remaining donor-derived. A time-course study shows that for normal thymus implants, host cells begin to immigrate in good number only after metamorphosis is complete; with these thymus implants, donor-derived lymphocytes can still be found in the blood and spleen of thymectomized

hosts several months post-implantation. Irradiated thymus implants attracted host cells more rapidly, their lymphoid complement becoming almost exclusively host-type within 2 weeks post-implantation when animals were at a late larval stage of development. Despite rapid colonization of irradiated implants by host lymphoid cells, these thymuses degenerate soon after metamorphosis, presumably due to irradiation damage of stromal elements.

The experiments in Chapter 6 compare the proliferative responses of thymocytes from normal and organ-cultured thymuses. The technical conditions (e.g. cell numbers per well) for obtaining good stimulation indices with T cell mitogens, and in mixed leucocyte culture (MLC), are examined. Thymocytes organ-cultured for 12 days generally display elevated 3HTdR uptake compared with cells tested straight from the animal. Both control and experimental (mitogen- or alloantigen-treated) cultures of organ-cultured thymocytes show these elevated DPM. Surprisingly, in vivo-irradiated, organ-cultured thymocytes, are still stimulated by phytohaemagglutinin (PHA)-treatment.

Chapter 7 investigates in vitro T-cell proliferative responses of splenocytes and thymocytes in allothymus-implanted, early-thymectomized Xenopus. Splenocyte reactivity to PHA and to third-party alloantigens (in MLC) is restored when normal allogeneic thymus is implanted ("adoptively-transferred"). However, MLC reactivity of thymocytes and splenocytes to thymus-donor strain cells is generally lacking; allothymus-implanted animals are also tolerant to thymus-donor strain skin grafts. Unfortunately, thymectomized animals implanted with in vivo irradiated, allothymuses died prior to in vitro assaying.

Preliminary attempts to generate supernatants (by treating cultured splenocytes with PHA and/or phorbol myristate acetate) that would routinely enhance T cell proliferative responses of thymocytes, are outlined in Chapter 8.

General conclusions to be drawn from this Thesis and suggestions for future work with this amphibian model are to be found in Chapter 9.

ACKNOWLEDGEMENTS

Foremost, I would like to thank my supervisor, Dr. John Horton for his help, advice and enthusiasm throughout the course of this study.

I would also like to thank Miss Pamela Aitchison, Mrs. Trudy Horton, Miss Margaret I'Anson, Mrs. Christine Richardson and Mr. Jack Warner for their excellent technical assistance.

I am also very grateful to Dr. A. Hepher, Dr. B. Banks and Dr. M. Stacey for their help with the fluorescence, light and electron microscopy.

Thanks go to Dr. A. Gearing and Dr. R. Lallone for their valuable guidance during the early part of this research.

I am especially indebted to Mr. David Hutchinson and Mr. David Young for preparation of the photographic material, and to Mrs. C. Dowson for typing this thesis.

Finally, I should like to thank Professor D. Barker, Professor K. Bowler and the Department of Zoology for providing the facilities to make this study possible.

This work was supported by a grant from the Science and Engineering Research Council.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 MAMMALIAN THYMOLOGY

Thymus Structure and Function

The thymus is a primary lymphoid organ present in all vertebrates, with the possible exception of jawless fish and some cartilagenous fish (McCumber et al., 1982). The mammalian thymus is active early in life, since it is essential for the development of a healthy immune system. In the mouse, for example, both cell-mediated immunity (e.g., to intracellular phases of bacterial and viral infections and to fungal and protozoal organisms) and humoral antibody responses (to many antigens living extracellularly) are usually severely impaired by neonatal thymectomy (Miller, 1979). An intact thymus is also necessary for normal reactivity against tumours and foreign transplants.

The thymus provides a microenvironment in which immature thymocyte precursor cells can proliferate and differentiate into more mature T-cells, free from the influence of exogenous antigens. Intrathymic T-cell maturation involves the complex interaction of T-lymphocyte progenitors with the stromal cellular components of the thymus, as well as with thymic humoral factors (Crouse et al., 1985). The end result of this maturation is a functionally diverse population of T-lymphocytes, which will interact specifically with self cells presenting foreign antigens that are in association with MHC (major histocompatibility complex) glycoproteins, and which will also respond to foreign MHC antigens. These "educated" T-cells leave the thymus and circulate within the blood and lymph, or settle in thymus-dependent regions of the secondary lymphoid organs (e.g. spleen, lymph nodes). In the periphery, thymus-schooled lymphocytes may

need to be influenced by thymic hormonal factors to complete their T-cell differentiation (Dardenne and Bach, 1981; Low and Goldstein, 1985). However, fluorescence cell sorter experiments (Scollay, 1984), on the functional properties of T-cells that have recently emigrated from the thymus, indicate that this extra-thymic maturation step is not a pre-requisite for T-cell development. The importance of extra-thymic maturation pathways for T-lineage cells have, in contrast, recently been stressed by Dosch et al. (1985).

Thymus glands of all animals contain morphologically similar cells, although cell size and numbers vary greatly between species (Kendall, 1981a). The mammalian thymus consists of three parts: an outer (subcapsular) cortex, an inner (deep) cortex and a medulla. The outer cortex contains many lymphoblasts, the inner cortex mainly lymphocytes. The medulla also houses lymphocytes, but is rich in epithelial cells and dendritic antigen-presenting cells (for example see Roitt, Brostoff and Male, 1985). It is at the cortico-medullary junction, that T-cells, selected for seeding the periphery, leave the thymus.

The thymus is surrounded by a connective tissue capsule from which septae project into the organ, dividing it into lobules. The capsule and septae are composed of collagen and contain fibroblasts, adipocytes, macrophages, mast cells, eosinophils, neutrophils, plasma cells, lymphocytes as well as nervous and vascular components (Crouse et al., 1985). The blood-thymic barrier is formed from endothelial cells (lining blood vessels) with basal laminae, peri-vascular cells (smooth muscles or pericytes), a connective tissue space and basal laminae of associated epithelial cells. The barrier (first demonstrated morphologically by Clarke, 1963) effectively separates blood-borne antigens from the majority of the cortical lymphoepithelial components.

The supporting meshwork of the thymus consists of epithelial cells; these contain tonofilaments and are connected to one another via their irregularly-branched cytoplasmic processes (Hwang et al., 1974). The epithelial component forms a three-dimensional network of cells, with lymphocytes in the interstices. Epithelial cells in the deep cortex are spider-shaped, whereas the medulla contains more squamous and closely packed epithelial cells (van Ewijk, 1984). Crouse et al. (1984) propose that the majority of medullary epithelial cells, together with the epithelial cells of the subcapsular cortex, are of ectodermal origin, whereas the epithelial cells of the deep cortex (and probably those of the outer medulla) are of endodermal origin (Haynes, 1984). Ultrastructural analysis of thymic epithelial cells has shown them to be heterogeneous in morphology (van de Wijngaert et al., 1984; van Vliet, 1984). For example, "pale" and "dark" epithelial cells have been described (Singh, 1981) on the basis of electron density. These epithelial types may represent stages in a differentiation process (van de Wijngaert et al., 1984), or reflect their separate embryological origins (Crouse et al., 1984). A special type of epithelium-lymphocyte interaction (so-called "thymic nurse cells"), visible as epithelial cells with internalized lymphocytes, has been demonstrated in the thymus cortex (Wekerle, 1980; Ritter et al., 1981; van de Wijngaert et al, 1983). Interactions between developing T-lineage cells and self MHC antigens expressed on thymic epithelial cells may well be of importance in promoting some aspects of T-cell maturation (see Ready et al., 1984). One of the functions of thymus epithelial cells is to synthesize humoral factors, also thought by some to be intimately involved in T-lymphocyte maturation (Gelfand et al., 1980; Janossy et al., 1983). The presence of pronounced rough endoplasmic reticulum, a well-developed Golgi complex and an electron-lucent nucleus are indications of factor-synthesizing cells.

Derivatives of the epithelial component include myoid cells, Hassal's corpuscles, extra- and intra-cellular cysts and granulocytes (see Chapter 3 for further discussion of these cell types). Hassal's (thymic) corpuscles are unique to the medulla, and consist of epithelial cells wound about each other in a concentric pattern. Blau (1967a) suggests that the corpuscles are "graveyards" for dead cellular material; alternatively they may be regions where unwanted antigens are accumulated and disposed of (Blau, 1967b). More recently, humoral factors have been detected in thymic corpuscles, leading to speculation of a thymic endocrine function (Bach, 1983).

Other non-lymphoid cells providing the thymic stroma include macrophages (MHC - Class II positive and negative - see below) and interdigitating dendritic cells (IDC - Steinman and Nussenzweig, 1980). These immigrant, haemopoietically-derived, stromal cells are discussed further in Chapter 3. Briefly, macrophages (phagocytic) and IDC (non-phagocytic) are both capable of antigen presentation (Poulter, 1983). The exact role of these antigen-presenting cells in T-cell differentiation has not been elucidated, although it has been suggested that interactions of these cells with developing lymphocytes promotes the differentiation of T helper lymphocytes (Robinson, 1984; Zepp et al., 1984).

The involution of the thymus is among the first noticed indications of aging. In man, thymic involution begins between the ages of 11 and 15 years; the reduction in size is accompanied by an increase in adipose tissue surrounding the thymus (Kendall, 1981b).

T-cell Differentiation

The thymic rudiment develops as an outpushing of the pharyngeal pouch epithelium, and becomes seeded with blood-borne lymphoid stem cells of mesodermal origin (Lassilla, 1981). Whether or not the stem cells which colonize the thymus are precommitted to becoming

T-cells is controversial (Jordan and Robinson, 1981; Owen and Jenkinson, 1984). Immigration of thymic stem cells may be directed by a thymus-derived chemotactic factor (Le Douarin et al., 1984; Potworowski and Pyke, 1985). The stem cells then begin to differentiate into T-cell subpopulations (T helper, T cytotoxic and T suppressor cells) directed by cell-cell contact with the thymic stroma and also by humoral factors secreted by the stroma (Kingston et al., 1984b).

T-cell surface markers, including receptors (e.g. the T-cell antigen receptor - see Tonegawa, 1985), other glycoproteins and enzymes, have proved very useful in defining the pathways of T-cell differentiation. For example, the Lyt 1,2,3 (lymphocyte-specific) series of differentiation antigens, expressed on mouse T lymphocytes, can be identified with polyclonal antibodies. Mouse T cells are either Lyt 1+ (T helper (Th) cells) or Lyt 23+ (cytotoxic/suppressor T cells (Tc/s)); the acquisition or loss of these markers corresponds to specific maturational events (Jordan and Robinson, 1981). Monoclonal reagents recognizing glycoproteins found only on Th cells (T4) or unique to mature Tc/s lymphocytes (T8) are also proving invaluable in elucidating T-cell differentiation pathways in humans (see Roitt et al., 1985).

It has been elucidated that the subcapsular cortex contains blastoid cells which are rapidly proliferating; these lymphoblasts (about 3% of total thymocyte population) are believed to represent "stem" cells newly-arrived in the thymus. The deep cortex comprises lymphocytes that have begun to differentiate new T-cell surface antigens, but are still functionally immature, i.e. they do not produce interleukin-2 or become cytolytic T cells in response to alloantigen or lectin. The deep cortical cells include both actively-dividing and more quiescent lymphocytes: together these make up 80-85% of

thymic lymphocytes; many of these cells die in the thymus, rather than being exported. The medulla (comprising the remaining 10-15% thymocytes) houses functionally-mature thymocytes, which are phenotypically indistinguishable from peripheral cells. Medullary T cells can be separated into subpopulations (helper, cytotoxic and suppressor T cells) by phenotypic markers (reviewed in Scollay, 1983; Smith, 1984).

T-cell Education

During their differentiation within the thymus, T-lineage cells learn to distinguish between "self" and "non-self" and are rendered tolerant to self antigens (particularly self-MHC antigens - Klein, 1982). This process of self-tolerance is vital, since it prevents destructive autoimmune reactions occurring. Thymic lymphocytes also acquire MHC-restriction, meaning that they recognize and react to foreign antigen only when the latter is presented on the cell surface in association with self-MHC gene products (Zinkernagel, 1978; Bevan and Fink, 1978). Cells bearing foreign MHC antigens also appear to provide the necessary stimulus to activate T cells. It is not yet clear whether T cells possess separate receptors, one for each component of this dual recognition system, or whether foreign antigens can become so closely associated with self-MHC glycoproteins that a single T cell receptor can embrace both at the same time (reviewed in Lallone, 1984).

In vivo studies have shown that MHC antigens expressed on the thymic non-lymphoid cells, are essential for the development of self-tolerance and MHC-restriction. However, the precise mechanism(s) involved in the T cell differentiation events and the nature of the thymic stromal components (epithelial cells, macrophages, IDC) involved

remain controversial, despite, for example, many experiments on thymus/bone marrow reconstituted, irradiated mice and with thymus-implanted, nude mice (Longo and Schwartz, 1980; Kindred, 1981; Zinkernagel, 1982; Kruisbeek et al., 1983; Lo and Sprent, 1986).

1.2 AMPHIBIAN (XENOPUS) THYMOLOGY

The thymic cytoarchitecture of anuran amphibians will be discussed in Chapter 3. There appear to be many morphologic similarities between the amphibian and mammalian thymus (e.g. Duijvestijn and Hoefsmit, 1981; Manning and Horton, 1982). One possible difference is the absence of true Hassal's corpuscles from the thymus of some amphibians. However, it has been suggested that a similar role to that of thymic corpuscles is played in amphibians by degenerated epithelial cells filled with dense bundles of cytoplasmic tonofilaments (Kapa et al., 1968; Bigaj and Plytycz, 1984).

Evidence for the presence of T cells in the clawed frog, Xenopus, stems from the ability of larval thymectomy to abrogate graft rejection (Horton and Manning, 1972; Nagata and Cohen, 1984), mixed lymphocyte culture (MLC) reactivity (Du Pasquier and Horton, 1976) and phytohaemagglutinin (PHA) and concanavalin A (Con A) responsiveness (Du Pasquier and Horton, 1976; Green-Donelly and Cohen, 1979; Nagata and Cohen, 1983). There is also evidence that the Xenopus thymus is the site to which "pre-T cells" migrate (Volpe et al., 1979) to be matured into both helper and cytotoxic T cells (Tochinai et al., 1976; Kawahara et al., 1980) and to be conferred with MHC-restriction (Flajnik et al., 1984b).

Other features of the mammalian immune system appear to be present in Xenopus, for example, thymus (T)-dependent and T-independent humoral immune responses (see Manning and Horton, 1982), B cell populations (against which monoclonal antibodies are now beginning to be prepared -

Bleicher and Cohen, 1981), an MHC (Flajnik et al., 1984c and 1985a; Kaufman et al., 1985a,b), genetically restricted T-B collaboration *in vitro* (Bernard et al., 1981), IgM and low molecular weight IgY (IgG-like) antibody production. For detailed reviews of the amphibian immune system see recent reviews by Flajnik (1983), Cribbin (1984), Lallone (1984), Watkins (1985) and Varley (1986).

Xenopus offers a unique model to study the involvement of the thymus in self-tolerance induction and MHC-restriction of developing T cells, since the anuran can be surgically manipulated, with relative ease, throughout larval development. Thus, chimeric *Xenopus* can be created by joining the anterior portion of one 24-hour embryo (containing the thymic anlagen = epithelium) to the posterior portion of an MHC-incompatible embryo (from which the haemopoietic stem cells, including lymphocytes, arise - Flajnik et al., 1984a). Alternatively, *Xenopus* that have been early-thymectomized when only 4-7 days old, can be implanted during late larval or adult life with thymuses (irradiated or not) from either isogeneic or allogeneic donors with a defined MHC (Du Pasquier and Horton, 1982; Nagata and Cohen, 1984; Gearing et al., 1984; Horton et al., 1986). These types of "chimera" offer a far more physiologically normal experimental model to study thymic influence on T-cell education, than lethally irradiated, bone-marrow/thymus reconstituted chimeric mice, used to initially describe thymic education.

1.3 PURPOSE OF THESIS

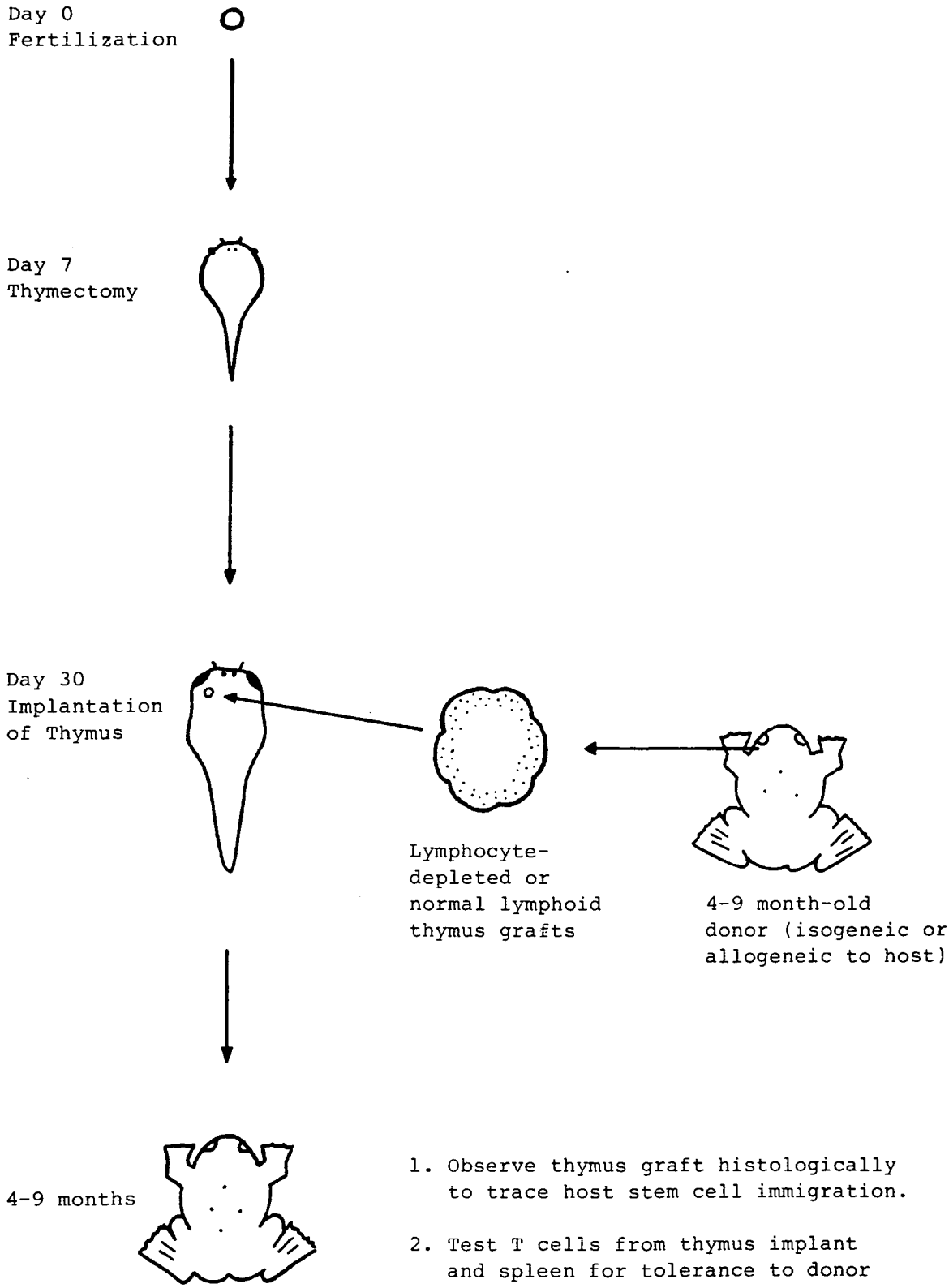
The experiments reported in this Thesis were carried out in an attempt to develop the *Xenopus* model system for exploring the role of the thymic microenvironment in T-lymphocyte differentiation. The basic experimental model I wished to develop is illustrated in Figure 1.1, and involves implanting lymphocyte-depleted (virtually

epithelial) thymus glands (or normal lymphoid thymus implants) into allogeneic or isogeneic, thymectomized tadpole hosts. The purpose of using lymphocyte-depleted implants was so that the role of the thymic stromal compartment in development of host cells could be assessed, without the complication of donor-derived lymphocyte effects. At various time intervals after transplantation, the implants would be examined histologically to observe the extent of repopulation with host-type lymphoid cells. The experimental plan was then to examine *in vitro* alloimmune responses of host lymphocytes towards thymus donor and third-party MHC antigens using the mixed lymphocyte culture (MLC) assay. It was intended to concentrate the work on thymus implants from young adult (froglet) donors, because it is only during or after metamorphosis that both Class I and II MHC antigens are expressed on *Xenopus* cells (Du Pasquier et al., 1979; Flajnik et al., 1985a).

The Thesis is subdivided into Chapters as follows. Chapter 2 describes initial *in vitro* attempts made to deplete the *Xenopus* thymus of lymphocytes. Chapter 3 illustrates in some detail the effect of *in vivo* γ-irradiation on thymic structure by light- and electron-microscopic analysis of plastic-embedded material. Chapter 4 begins to investigate the extent to which normal and irradiated (lymphocyte-depleted) thymus grafts are infiltrated by host lymphoid cells, using a ploidy cell-marker system. In Chapter 5 an alternative (improved) cell-marker system is illustrated (the *Xenopus borealis*/quinacrine fluorescence marker) and is used to histologially examine the timing of immigration of host-derived cells into thymus implants. Chapter 6 describes T cell proliferative assays (MLC and mitogen experiments) on normal and organ-cultured thymocytes. The reconstitution of these T cell proliferative responses and acquisition of tolerance to thymus donor strain lymphocytes in 7 day-thymectomized *Xenopus*, implanted

with histoincompatible thymuses, is reported in Chapter 7. Chapter 8 outlines preliminary attempts to produce supernatants, from *in vitro*-stimulated splenocytes, which would have immunoenhancing properties for thymocyte reactivity. In the final Chapter (9) the major findings of this Thesis are outlined and some suggestions for future experiments discussed.

Figure 1.1 **Basic plan for investigating the influence of the thymic microenvironment on T cell tolerance acquisition**



CHAPTER 2

ORGAN CULTURE OF THE XENOPUS THYMUS: ATTEMPTS TO DEPLETE THYMIC LYMPHOCYTES

2.1 INTRODUCTION

A variety of techniques have been employed to deplete the thymus of its lymphocyte complement but leave its stromal elements intact (Crouse et al., 1985); these techniques have included *in vivo* maintenance of thymuses in diffusion chambers (Owen and Ritter, 1969), monolayer culture techniques (cited in Jordan and Crouse, 1979), sub-optimal culture (Loor and Hägg, 1977; Hong et al., 1979), and irradiation (Pritchard and Micklem, 1973; Loor and Hägg, 1977) of post-natal thymus explants. More recently, low temperature organ culture alone (Jordan and Crouse, 1979), or low temperature culture combined with high oxygen environment (Pyke et al., 1983) have proved effective in producing lymphocyte-depleted murine embryonic thymuses. *In vitro* exposure of developing murine thymus rudiments to deoxyguanosine is also successful in procuring an organ depleted of lymphoid cells but allows stromal elements to remain viable (Jenkinson et al., 1982).

The culture of thymic rudiments from the chick and mouse embryo in diffusion chambers on the avian chorioallantoic membrane was designed to study stem cell/organ rudiment interaction. Consequently, when thymic primordia from early embryos (10-11 days gestation for the mouse, 6-7 days for the chick) were cultured in this manner, anlage remained epithelial due to the absence of a stem cell supply (Owen and Ritter, 1969).

In contrast, monolayer techniques utilize an *in vitro* approach in selecting for thymic epithelium, where cells are encouraged to grow in two-dimensional array submerged in medium. However, thymus monolayers have been found to yield heterogeneous cultures in which cells other

than the epithelium predominate, and to lack the 3-d microenvironmental milieu of the intact organ (Robinson and Jordan, 1983).

The use of sub-optimal culture of thymic explants to achieve lymphocyte depletion involves culturing thymuses in vitro in medium which does not support the growth of rapidly-dividing cells. Lymphocytes die quickly under poor culture conditions, but the viability of the slowly-dividing epithelial cells is less affected. Usually, after 3-4 days culture in medium unable to support lymphopoiesis, thymuses are transferred to fresh growth-supporting medium for the remaining culture period (total time 1 to 2 weeks) - Loor and Hägg (1977).

Similarly, the preparation of viable epithelial thymus by irradiation treatment makes use of the different sensitivities of thymus epithelial cells and lymphocytes to potentially harmful X- or δ -rays. In the mouse, virtually all thymocytes are destroyed by an X-irradiation dose of 500 rads, while the thymus epithelium resists doses up to 5000 rads (Trowell, 1961). X-ray doses of from 500 to 2000 rads have commonly been used to produce epithelial murine thymuses (cited in Loor and Hägg, 1977). Lymphocyte regeneration can occur in organ culture when less than 1000 rads is given to the 14-day foetal thymus (Pyke et al, 1983).

Low-temperature organ culture of the foetal (14 day) mouse thymus (the stage of initial haemopoietic colonization) has proved particularly successful in deleting the lymphohaemopoietic component, leaving the epithelial matrix intact. Thymuses are placed on ultra-thin poly-carbonate filters and cultured at a gas-liquid interface for 7 days at 24°C in an atmosphere of 5% carbon dioxide in air. Cultures are subsequently allowed to recover under conventional conditions (37°C; 5% CO_2 in air) for a further 7 days (Jordan and Crouse, 1979).

A modification of the above technique is used by Pyke et al. (1983). They have found that 7 day incubation of 14 day foetal murine

thymus at room temperature (22 - 24°C) in 10% CO_2 in O_2 will deplete most of the lymphoid cells and all the Ia-positive (MHC Class II) populations, but these conditions spare the epithelial component. Use of 10% CO_2 in air did not remove all Ia-positive cells from the thymus. Of importance to transplantation biology are experiments which show that culturing organs (e.g. murine thyroid) in high O_2 concentration (Lafferty et al., 1975) or hyperbaric oxygen and hydrocortisone (Talmage and Dart, 1978) prior to allotransplantation, extends the survival of grafts in allogeneic hosts. A popular explanation proposed is that O_2 selectively kills "passenger leukocytes" (e.g. Class II MHC-antigen presenting cells), which are efficient stimulators of allograft immunity.

Alternatively, thymic lobes from 14-day mouse embryos can be depleted of lymphocytes by organ culture under conventional conditions (mentioned above) for 5 days in medium containing 1.35 mM deoxyguanosine and then for a further 7 days post-treatment in fresh medium only (Jenkinson et al., 1982). Deoxyguanosine is selectively toxic to the immature, spontaneously dividing thymic lymphocytes (Cohen, 1980) and also depletes other haemopoietic cells (e.g. Ia-positive thymic dendritic cells and their precursors) that are found within the thymus (Ready et al., 1984). Deoxyguanosine does not affect the epithelial framework of the thymus, which survives the treatment and continues to express both Class I and II MHC antigens (Ready et al., 1984).

This Chapter investigates the potential of organ culture in association with temperature effects, deoxyguanosine treatment and γ-irradiation, to procure normal or lymphocyte-depleted thymuses taken from various stages of development of Xenopus.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Several strains of histocompatible Xenopus are available in our laboratory. This chapter makes particular use of thymuses taken from a partially inbred strain of X. laevis; these animals, originally called G-line by Katigiri (1978), are now referred to as J strain, to reflect their Japanese "origin" (Di Marzo and Cohen, 1982). These Xenopus are MHC homozygous (haplotype jj), but display minor histocompatibility (H) antigenic differences (Di Marzo and Cohen, 1982).

Clonal X. laevis/X. gilli (LG) hybrids, family LG17 (haplotype ac) were also used in some experiments. LG animals were obtained by in vitro activation of the endoreduplicated, diploid eggs (that these hybrid Xenopus lay) with UV-irradiated sperm (Kobel and Du Pasquier, 1975). Animals within a particular clone are isogeneic.

Diploid eggs of another LG clone (LG5) were fertilized with J sperm to produce J x LG5 (jad) Xenopus, that were also used here. The triploidy of these JLG5 animals was confirmed by silver-staining of nucleoli (see Chapter 4).

Eggs from mating outbred (wild) X. laevis (purchased from Xenopus Ltd.) were the final group used in these organ culture studies.

Male and female Xenopus were given injections of human chorionic gonadotrophin (HCG, Xenopus Ltd.) to induce gamete maturation, ovulation and mating (where appropriate). Fertilized eggs were transferred to aerated, standing water. Animals were reared at 23 $\pm$ 2°C, under conditions described elsewhere (Horton and Manning, 1972). Larvae were fed hydrated nettle powder and froglets (for the first few post-matamorphic months) were fed Tubifex worms. Older animals were given both Tubifex and minced ox liver.

Developmental stages were determined according to the Normal Table of Nieuwkoop and Faber (1956) - see Figure 2.1.

2.2.2 Organ Culture

a) Normal conditions

Thymus glands were removed from MS222 (Sandoz) - anaesthetized animals (either larvae or froglets) using sterile instruments and working in a laminar airflow hood. Thymuses were washed briefly in amphibian strength Leibovitz (L15) culture medium and placed on a 13 mm diameter polycarbonate filter with 0.4 µm pore-size (Nuclepore Corporation - obtained from Sterilin Ltd.). The filters had been previously boiled three times in double distilled water and autoclaved. An individual filter was positioned on a piece of gelatin foam sponge (Sterispon No.1, Allen & Hanbury) that had been placed in a 35 x 10 mm plastic petri dish (Costar) containing approximately 1.5 ml amphibian L15 culture medium. The technique of culturing thymuses in this way was developed from the paper on foetal mouse thymus organ culture by Jenkinson et al. (1982). A diagram of the culture arrangement is seen in Figure 2.2.

The amphibian organ culture medium consisted of an L15 base (Flow labs) diluted to 60% with double-distilled water, and supplemented with 10% heat-inactivated (56°C for 1 hour) foetal calf serum (FCS, Flow labs), and also with 10 mM Hepes buffer, 20 mM sodium bicarbonate, 2 mM L-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, 2.5 µg/ml Fungizone (all from Flow labs) and 0.05 mM 2-mercaptoethanol (British Drug Houses, Poole, England). The culture medium was sterilized by filtration through a 0.22 µm Millipore filter (Millipore, U.K.). Thymuses were cultured in a water-saturated atmosphere in a 5% CO_2 incubator at 26-27°C, and culture medium was changed every 3 or 4 days. Table 2.1 gives the list of experiments using "normal" organ culture.

b) Organ cultures at varying temperatures

In these experiments (see Table 2.2) thymuses from larvae and froglets were organ cultured at various temperatures, either above or

below the usual organ culture temperature (26°C). Temperatures of 4°, 10°, 15° and 34°C were used. These thymuses were cultured in $NaHCO_3$-free medium, since an atmosphere of 5% CO_2 in air was not available at the temperatures used. Preliminary work suggested that thymuses cultured in the absence of bicarbonate at 26°C were morphologically comparable to thymuses cultured at this temperature in the presence of $NaHCO_3$.

c) Deoxyguanosine - treatment of organ cultures

DG (Sigma, 2' - Deoxyguanosine Grade II) was made up to a 9mM stock solution in amphibian-strength L15 medium, aliquotted and stored frozen. Since DG is only moderately soluble and optimal solubility is achieved at pH2 (using 0.01 M HCl), it was important to check the molarity of the stock solution. This was done by obtaining a spectrum of DG absorbance over a range of wavelengths using a spectrophotometer. The concentration of DG can then be calculated since:

$$\text{molarity} = \frac{OD \times 100}{\varepsilon^{255}}$$

where OD = optimum absorbance of DG at 255 nm

100 = dilution of DG stock solution so that the absorbance at 255 nm can be read on the spec.

ε^{255} = 12,300 (DG molar extinction coefficient).

Several experiments were carried out (see Table 2.3) in which the molarity of DG was varied in order to find a concentration that would effectively deplete thymuses of their lymphocyte population. The optimal concentration of DG used with foetal mouse thymus is 1.35 mM for 5 days culture (Jenkinson et al., 1982). In preliminary experiments with larval Xenopus thymus, concentrations of DG < 1.5 mM had no apparent short-term effect on the histological appearance of thymuses. Treated thymuses were placed in DG (1.5 - 2.8 mM) for several days followed by culture in DG-free medium. Control thymuses were cultured

for the same total culture period, but in DG-free medium exclusively.

d) The effect of γ -irradiation in vitro

The radioactive source used was Cobalt-60 (Co^{60}), which emits gamma rays. The dose of radiation administered was varied according to the distance of the thymus from the source and the length of time of irradiation. The doses of 1000, 2000 and 3000 rads were achieved by positioning thymuses (removed from froglets and directly placed in medium in Falcon 12 x 75 mm tubes) at 17 cm, 13 cm and 10½ cm away from the source respectively for 5 mins. irradiation time. The total irradiation dose received after 5 minutes exposure at various distances from the source was measured using a ferrous ion chemical dosimeter by R.L. Lalone (personal communication; Lallone 1984). Table 2.4 shows the number of experiments with in vitro-irradiated and organ-cultured thymuses.

2.2.3 Histology

Organ-cultured thymuses were fixed in Bouin's for approximately 24 hrs, dehydrated and then embedded in paraffin wax. 7 µm sections were stained in haematoxylin and eosin.

2.3 RESULTS

2.3.1 Histology of thymuses organ-cultured under normal conditions

Table 2.1 shows the outcome of representative experiments on larval and froglet thymuses organ cultured under normal conditions. Larval thymuses (taken from 3-5 week old animals) spread out onto the Nuclepore filters and became rather flattened (Figure 2.3a) and somewhat reduced in overall size. However, during the first week of culture, thymuses retain their cortex/medulla differentiation and are still very lymphoid. During the second week of culture, cortico-medullary differentiation was no longer visible and lymphocyte numbers are now significantly reduced (Figure 2.3b). Unlike the in vivo situation,

3-week larval thymuses failed to grow in organ culture; whether this is related to poor organ-culture conditions or absence of progenitor cell input in the in vitro situation, is unknown.

Thymuses taken from peri-metamorphic stages (st. 60/61 - 8 weeks old) faired similarly to the larval organ cultures - see Figure 2.4. Figure 2.4a shows a normal stage 60/61 thymus, whereas Figure 2.4b reveals a 7 day organ cultured peri-metamorphic thymus that is in condition > C <, (see key to Table 2.1 - 2.4) i.e. shows significantly reduced lymphocyte numbers and loss of cortico-medullary differentiation. This extensive lymphocyte depletion is not typical of a 9 week old in vivo thymus (see Williams, Ph.D. thesis 1981), although near the end of metamorphosis the Xenopus thymus does naturally display reduced lymphocyte numbers.

Froglet thymuses retained a far more normal structure after 2 weeks of organ culture. This is shown in Figure 2.5b. Lymphocyte numbers are only minimally depleted and cortex/medulla differentiation remains. The cortex was observed to develop a "follicular-like" arrangement, with distinct lymphoid-filled follicles, separated by connective tissue septae and inter-follicular "channels" (Figure 2.5c).

2.3.2 Histology of thymuses following organ-culture at various temperatures

Table 2.2 summarizes the results of these experiments.

Cold temperature treatment (4°, 10° and 15°C) had a profound influence on short-term (2-4 day) cultured larval thymus and longer-term (7-12 day) cultured froglet thymus. The larval thymuses kept at 10° or 15°C appeared healthier than normal organ cultured controls (see Figure 2.6a, contrast with Figure 2.3a); they were far more lymphoid, with obvious cortex and medulla differentiation. Both larval and froglet thymuses cultured at low temperatures maintained their size and shape well (Figures 2.6a and b). Sections through one 5 month froglet thymus, that had been cultured for one week at

4°C, were of particular interest (see Figure 2.7). Large "cells", inside of which there appeared to be several lymphocytes, were occasionally seen in this thymus. These complexes bear superficial resemblance to thymic nurse cells.

In contrast to organ-cultured thymuses kept at low temperatures, those kept at an elevated temperature (34°C) for only 7 days resulted in degenerating, necrotic thymuses, full of pyknotic cells (see Figure 2.8).

2.3.3 Histology of organ-cultured thymuses after DG-treatment

Table 2.3 shows the results of these experiments. Thymuses from larvae and adults were cultured in DG (1.5 - 2.8 mM final concentration) for culture periods ranging from 2-6 days, followed by a period of culture in DG-free medium. Representative sections of such DG-treated thymuses are provided in Figure 2.9. Three-week larval thymus seems to become virtually lymphocyte-free, but very much reduced in size after treatment with DG (Figure 2.9a). In contrast, DG has far less dramatic effect on lymphocyte numbers in the froglet thymus. The length of time the 3½ month organ cultured thymus has to be in 2.2 mM DG to cause lymphocyte depletion must approach 4 days, since 4-day DG treatment (Figure 2.9b) results in reduction of lymphocyte numbers, whereas 2-day treatment has little effect apart from causing an overall mild reduction in size. Another experiment with thymuses from 6 month old froglets involved two separate 3-day treatments with 2.0 mM DG treatment, in an attempt to poison the majority of proliferating lymphocytes. However, this experiment had no more dramatic effect on depleting lymphocyte numbers (Figure 2.9c). With the oldest thymuses (8 months) even two 3-day treatments of 2.8 mM DG failed to remove all thymic lymphocytes, although the thymus becomes very small.

2.3.4 The effect of in vitro irradiation on histology of organ-cultured thymus

The results of representative experiments are shown in Table 2.4. Irradiation proved more effective than deoxyguanosine in destroying lymphocytes in froglet thymuses. Following just 2 days organ culture, many pyknotic cells were visible in the irradiated thymuses (Figure 2.10a), particularly in the cortex. By 14 days, 1000 rad-irradiated thymuses become reduced in size and display significantly reduced lymphocyte numbers. The effect of 2-3000 rads was similar, but more dramatic. Now very few lymphocytes were seen at 14 days of culture, and thymuses were extremely small.

Thymuses given 1000 rads on day 1 and again on day 6 were also virtually devoid of lymphocytes by 2 weeks, but reduction in their size was less marked than those given a single 2000 rad dose (see Figure 2.10b). Irradiated thymus cultured for 4 weeks became degenerate (Figure 2.10c), and surviving epithelial cells had become arranged into convoluted epithelial layers, with cystic spaces being prominent. The observation of epithelial outgrowths at this time supports the concept that epithelial cells are the major radiation-resistant cell type in the thymus.

2.4 DISCUSSION

As noted in the General Introduction, organ culture of the embryonic mouse thymus is currently proving to be of considerable value in probing the role of the thymic microenvironment (e.g. the stromal cells - see Weiss and Sakai, 1984) in procuring self- or allo-tolerance acquisition by developing T lymphocytes. In this Chapter, larval and post-metamorphic Xenopus thymuses have been organ cultured under a variety of experimental conditions to determine the most appropriate treatment to achieve survival of stromal elements, but depletion of thymic lymphocytes.

A variety of tissues taken from X. laevis have been successfully organ cultured by other workers (see review by Monnickendam and Balls, 1973); the list includes lymphoid tissues such as kidney, liver and spleen, but excludes the thymus gland. Amphibian organs can be successfully cultured submerged in tissue culture medium, but success has also been achieved by placing organs, such as the spleen, at a gas-liquid interface, comparable to the technique used in this Chapter (Balls, Simnett and Arthur, 1969). Spleen fragments from both immature and mature, post-metamorphic Xenopus can be cultured in serum-supplemented L15 medium for 18 days at temperatures ranging from 10°C to 25°C, with good histological preservation. (Balls et al. (1969) used a culture medium without added buffers - Hepes and sodium bicarbonate). Such Xenopus spleen organ cultures have been used to study in vitro aspects of antibody production (see, for example, the early work of Auerbach and Ruben, 1970).

Williams (1981) was the first to attempt organ culture of the Xenopus thymus. He used a similar technique to the culture system described here, and organ cultured thymuses from stage 52 and 58 larvae for 7 days at 28°C in 5% CO_2 in air. He found a considerable decrease in thymic lymphocyte numbers after culture, a finding that is in agreement with the present experiments on the larval and peri-metamorphic thymus. That this lymphocyte depletion may not directly relate to poor organ-culture conditions is suggested by the present work on post-metamorphic thymuses. These retained a far more normal structure, even after 2 weeks of organ culture. Perhaps a "regular" input of lymphoid stem cells is required into the larval thymus, whereas following metamorphosis, the organ becomes relatively independent of such stem cell immigration, and therefore organ cultures retain their lymphoid complement more readily. Tochinai (1978) has shown

that there is an early (3-4 days) stem cell immigration to the Xenopus thymus, but whether or not later "windows" of input occur is still unknown. A propensity of larval thymocytes for a short life span (compared with the adult) could, of course, also be a contributory factor in the differences observed between larval and adult thymus organ cultures.

The tendency for larval and peri-metamorphic thymuses to flatten onto the Nuclepore filters more readily than froglet thymuses may be due to the strength and thickness of the connective tissue capsule enclosing the thymus. The thinner, more flexible larval capsule may collapse more easily in culture than the stronger adult thymus capsule. Also, the arrangement of trabeculae (continuous with the capsule) penetrating the cortex and dividing it into "follicles" may enable the cultured froglet thymus to better retain its morphology.

In contrast to the poor growth of larval Xenopus thymus organ cultures, thymus lobes from 14 day foetal mouse (i.e. 2 days after initial haematopoietic colonization - see Robinson and Jordan, 1983) increase in size approximately five-fold over a 7 day culture period (Robinson, 1980). Such growth is due to proliferation of both epithelial and lymphoid cells and results in a ten-fold increase in lymphocyte numbers. However, following longer-term organ culture, the mouse embryonic thymus falls far short of its in vivo growth potential (Robinson, 1980).

Initial attempts to produce lymphocyte-depleted thymuses involving the use of low (and high) organ-culture temperatures were unsuccessful. Interestingly, froglet thymuses cultured for up to 12 days at 10°C or 15°C were extremely lymphoid and appeared very healthy. (Unfortunately experiments with the larval thymus were really too short-term to make any sound comment on low temperature effects). These results contrast with the mammalian situation, i.e. when 14 day mouse embryo thymus is cultured for 7 days at 24°C

(rather than 37°C), the result is an "epithelial" organ (Robinson and Jordan, 1983). The disparity in these findings may relate to the fact that Xenopus is a poikilotherm, and hence its lymphocytes are able to adapt to survive at the low environmental temperatures used here in organ culture. In contrast the mammalian lymphocyte is unable to tolerate temperatures outside the normal physiological range.

Organ culture of the Xenopus spleen at 18°C rather than 25°C is known to lower the mitotic rate (Balls et al., 1969). Presumably in the present study, where thymuses were kept at 10°C and 15°C, the rate of lymphocyte turnover is lowered. In this event, the numbers of thymic lymphocytes dying might be dramatically decreased, leading (in the short-term) to an increase in thymocyte numbers within the cultured organ.

Of some relevance to the present findings that short-term organ culture of the Xenopus thymus at low environmental temperatures causes an increase in lymphocyte cellularity, are the in vivo findings of Plytycz and Bigaj (1983a/b) on seasonal changes in the cytoarchitecture of the thymus of Rana temporaria. These authors found that the thymus is lymphocyte-rich in the warm summer months, but during hibernation in the cold winter months the thymus involutes and becomes lymphocyte-depleted, consisting mostly of secretory cells and cysts. The marked winter involution (in October) was followed by gradual recovery of the organ terminating in May. Plytycz and Bigaj suggested that the lymphocyte depletion in winter is related to migration of cells out from the thymus rather than to increased intrathymic death of thymocytes.

The presence of complexes, bearing superficial resemblance to thymuc nurse cells (TNCs), within a 4°C organ cultured Xenopus thymus deserves comment. In mammals, it has been suggested that TNCs are reticulo-epithelial cells which have internalized lymphocytes and that

play a role in intrathymic T cell maturation (Wekerle et al., 1980). The observation of an immature immunologic phenotype of these lymphocytes (Kyewski and Kaplan, 1982) is compatible with this hypothesis. TNC-like cells have been visualized in electron micrographs in the cortex of human pediatric thymuses (Wijngaert et al., 1983), the rat thymus (Hwang et al., 1974; Duijvestijn and Hoefsmit, 1981) and the mouse thymus (Wekerle et al., 1980). However, Farr and Nakane (1983) report that TNC were not observed in either the cortex or medulla, in a light and electron-microscopic examination of murine thymus (6-10 weeks of age). However, they admit that this may be due to the low number of TNCs recovered from murine thymic tissue - about 10^4 per thymus.

Holtfreter and Cohen (1986) working with the Rana tadpole thymus have observed TNC-like complexes, which possess "a smooth contour suggestive of it being a membrane-limited unit body and containing many actively mobile thymocytes". The diameter of Rana TNC-like complexes ranged from 20 μm to 60 μm. The putative TNC illustrated in Figure 2.7b/c is within this range (taking the diameter of cortical thymocytes as varying between 5 μm and 10 μm). Holtfreter and Cohen question the assumption that the TNC is a single giant cell that has engulfed lymphocytes. From observations of mechanically- and enzymatically-dissociated thymuses cultured in hanging-drops for 6-8 days, they conclude that the Rana "TNC" is, in fact, a complex of epithelial cells, lymphocytes and other stromal cells. They also propose that TNCs described in other animals might be in vitro artifacts, caused by the extensive trypsin treatment necessary to obtain them; they suggest that TNCs are actually small fragments of the thymus architecture. An alternative theory, proposed by Wood (1985), is that TNCs are macrophages filled up with viable lymphocytes, where the rate of internalization has

exceeded the store of lysosomal enzymes, thereby preventing phagocytosis of the lymphocytes.

Deoxyguanosine (DG) - treatment (1.5 mM) of organ-cultured, larval (3 week) Xenopus thymus was successful in depleting lymphocytes, but the epithelial organ remaining was very much reduced in size. Whether it will be technically feasible to work with these tiny epithelial thymuses in thymus restoration experiments planned in this laboratory, remains to be answered. DG-treatment of post-metamorphic thymus organ cultures was far less successful in selectively depleting lymphocytes. Even high DG-doses (2.8 mM) failed to deplete all thymic lymphocytes and such doses caused substantial reduction in overall thymus size.

DG has an inhibitory effect on proliferating T lymphocytes (Cohen et al., 1980). There is extensive evidence that DG interferes with DNA synthesis in cultured cells via its phosphorylated product deoxy GTP (deoxyguanosine triphosphate), which inhibits ribonucleotide reductase and hence interferes with purine and pyrimidine metabolism and consequently DNA synthesis. DG-sensitivity appears to be related to an enhanced ability of immature cells for uptake and phosphorylation of DG to deoxy GTP and by their reduced ability to degrade accumulated deoxy GTP. These findings may explain why DG-treatment of larval thymuses is effective in lymphocyte depletion, since premetamorphic thymocytes (certainly at stage 53) are functionally immature - for example there are no PHA-reactive thymocytes at this stage of development (Williams et al., 1983). The selective elimination by DG of cortical (rather than medullary) lymphocytes, evident in some cultured froglet thymuses, may occur because the mitotically active population of T cells residing in the cortex (Williams, 1981) are sensitive to DG toxicity.

The experiments with in vitro irradiation and subsequent thymus

organ culture were the most promising in terms of producing a thymus depleted of lymphocytes, but with intact stromal elements. This work concentrated on the post-metamorphic thymus, since it is only after metamorphosis that true Class I MHC antigens become expressed on cells within the thymus and elsewhere in the body (Flajnik, Kaufman and Du Pasquier, 1985a). Class II MHC antigens appear to be expressed on both adult and larval cells (Flajnik et al., 1985a). γ -irradiation initially depletes the more highly mitotic cortical lymphocytes. Doses of 2-3000 rads also achieved significant depletion of medullary lymphocytes by 14 days of culture, but overall thymus size was much reduced. Irradiating on day 1 and 6 of culture with 1000 rads procured a thymus with very few lymphocytes, but with relatively little effect on overall size. The preliminary irradiation studies in this Chapter suggest that the different sensitivities of thymus stromal cells and lymphocytes to γ -irradiation can be exploited to produce viable, epithelial-looking, froglet thymuses. A much closer look at the effect of irradiation on thymus architecture is undertaken in the next Chapter.

Key to Tables 2.1 - 2.4

(Histology of thymus at end of experiment)

A	=	Completely normal sturcture e.g. thymuses taken straight from the animal.
B	=	Still very lymphoid, but with fewer lymphocytes than in vivo thymuses. Cortex and medulla nearly always present.
C	=	Lymphocyte numbers significantly reduced. Cortex and medulla differentiation obscure. However, lymphoid areas still seen scattered within the thymus.
D	=	Thymuses appear to contain mostly stromal cells, but with scattered lymphocytes still remaining. Epithelial cells predominate.
E	=	Thymus becoming degenerate and structurally destroyed. Some epithelial cells, but with many pyknotic cells.
> <	=	Thymus significantly reduced in size.

Table 2.1

Outcome of experiments with thymuses organ cultured under normal conditions

Age (Stage)	Strains used	Thymus organ culture		
		(No. of thymuses)	Days in vitro	Histological outcome (see Key)
3-5 weeks (st.53-56) Larva	J/JLG5	(3)	4	> B <
	J	(3)	12	> C <
8 weeks (st.60/61) Peri-metamorphic	J	(4)	0	A
		(3)	7	> C <
3 months Froglet	J	(4)	14	B
5-8 mths Froglet	J/WILD	(6)	0	A
	J/JLG5	(4)	6-7	B
	J/JLG5/LG17	(18)	11-15	B

Table 2.2

The effect of different temperatures on organ-cultured thymuses

Age (Stage)	Strains used	Organ culture of thymus			
		Treatment	(No. thymuses)	Days in vitro	Histological outcome (see Key)
5 week (st. 56) Larva	JLG5	10°C	(4)	2-4	A
		15°C	(3)	2-4	A
5 months Froglet	JLG5	4°C	(4)	7	A
				12	A
		15°C	(4)	7	A
				12	A
		34°C	(4)	7	E
				12	E

Table 2.3

Effect of Deoxyguanosine on organ-cultured thymuses

Age (Stage)	Strains used	(No. thymuses)	Organ Culture				
			DG mM	Time of culture in: DG	Time of culture in: DG-free medium	Total culture time	Histological Outcome (see Key)
3 weeks (st. 52/3)	J	(6)	1.5	4-5	7	11-12	> D <
Larva	J	(6)	1.8	4-5	7	11-12	> D <
3½ months froglet	J	(4)	2.2	2	9	11	> B <
	J	(4)	2.2	4	7	11	> C <
6-8 months	J	(5)	2.5	5	10	15	> C <
	J	(16)	Range 2.0-2.8	2 x 3*	2 x 3	12	> B/C <

* Thymuses were treated alternately with DG for 3 days, then DG-free medium for 3 days and then this cycle repeated.

Table 2.4

Effect of in vitro ɣ -irradiation on organ-cultured thymuses

Age	Strain	(Nos. Thymuses)	Dose of Irradiation	Length of Culture (Days)	Histological Outcome (see Key)
3-6 months Froglets	J	(4)	1000R	2-6	> C <+
	J/LG17	(4)		14	> C <
	J	(3)		28	> E <
	J	(6)	2-3000R	2-6	> C <+
	J/LG17	(4)		14	> D <
	J	(4)		28	> E <
	J/LG17	(9)	1000R x 2*	14	> C < or > D <

* Irradiate twice, the second irradiation being half way through the culture period.

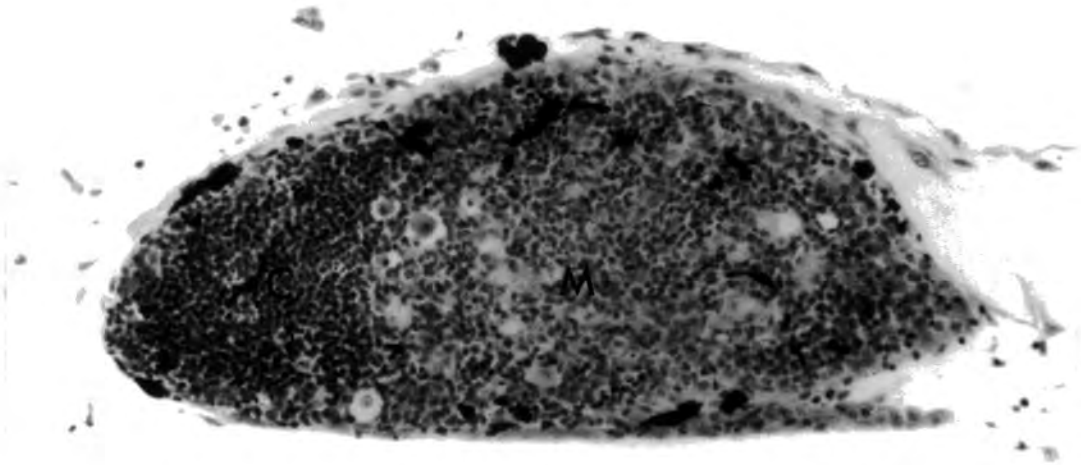
+ These thymuses had many pyknotic cells.

Figure 2.3. Larval thymus following organ culture

a) 5 week stage 55/6 larval thymus cultured for 4 days under normal conditions. Thymus is slightly flattened onto the Nuclepore filter, but is still very lymphoid, with clear cut cortex (C) and medulla (M) differentiation. Thymus of category > B <. (x 200)

b) 3 week stage 52/3 larval thymus cultured for 12 days under normal conditions. Although lymphocytes are found scattered in foci (f), the epithelial (e) component of the thymus is particularly apparent. Category > C <. (x 125)

2.3a



2.3b

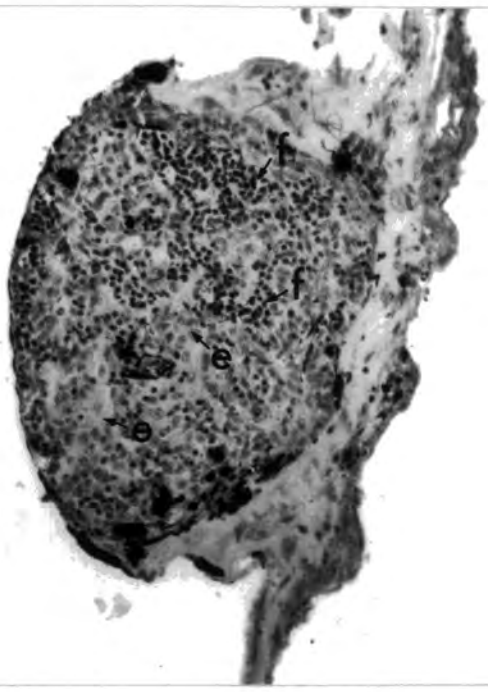
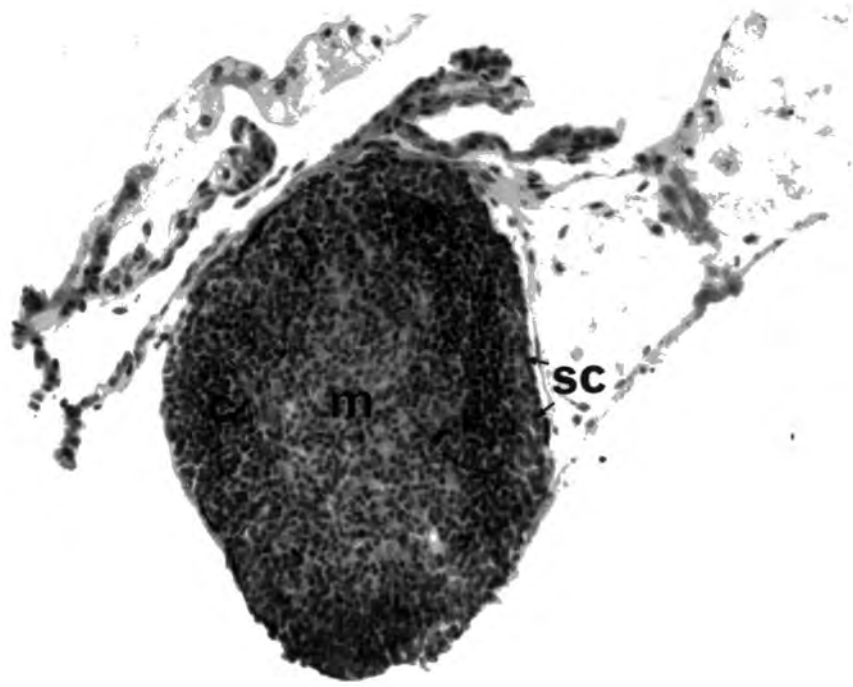


Figure 2.4. Peri-metamorphic thymus following organ culture

a) Thymus removed from stage 60/1 metamorphosing Xenopus and directly prepared for histological examination. The cortex (c) is densely-populated with lymphocytes and a subcapsular zone (sc) of less densely-stained lymphoid cells is noticeable. Paler-staining epithelial cells are prominent where lymphocyte numbers are more diffuse in the medulla (m). Category A. (x 125)

b) Thymus removed from 8 week, stage 60/1 Xenopus and cultured for 7 days under normal organ culture conditions. The 3-d arrangement of the thymus has been affected by organ culture. The thymus has spread onto the Nuclepore (n) filter and differentiation into cortex and medulla is no longer apparent. The lymphocytes, found in foci, are far fewer in number than in the in vivo situation. The non-lymphoid component of the thymus is particularly noticeable. At a higher magnification, elongated reticular cells can be seen lined up alongside the capsule of the thymus, next to and parallel to the filter. Category > C <. (x 125)

2.4a



2.4b

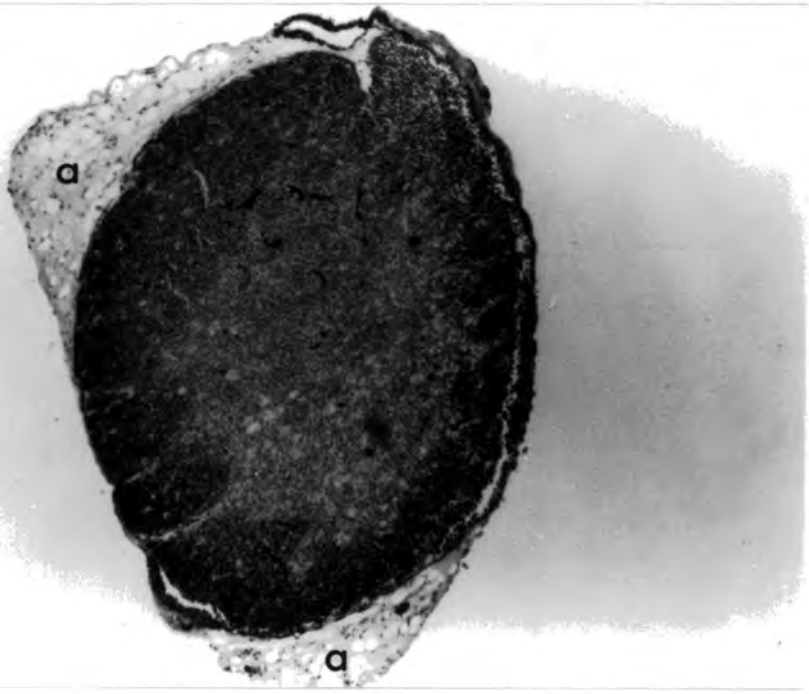
Figure 2.5. Froglet thymus histology following normal organ culture

a) 8 month froglet *in vivo* thymus. Thymus shows typical cortex/medulla differentiation and is very lymphoid. Connective tissue trabeculae penetrate in from the capsule through the cortex. Some adipose tissue (a) is seen adjacent to the thymus. Category A. (x 80)

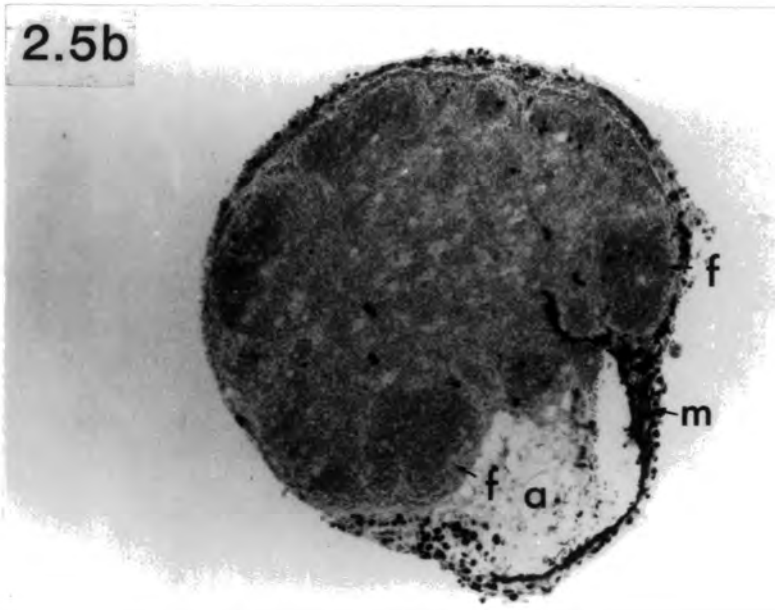
b) 8 month froglet thymus organ cultured for 15 days under normal organ culture conditions. Lymphocyte numbers have minimally declined during culture, and cortex and medulla are still apparent. Note follicle-like (f) arrangement of cortical lymphocytes. a = adipose tissue, m = melanin. Category B. (x 80)

c) "Follicular arrangement" or cortical lymphocytes is seen in this (12 day) organ cultured 5 month old froglet thymus. "Follicles" are separated by connective tissue (ct) septae. Lymphoid-filled "channels" penetrate between the "follicles". c = capsule, ep = epithelial cells, m = melanin. Category B. (x 400)

2.5a



2.5b



2.5c

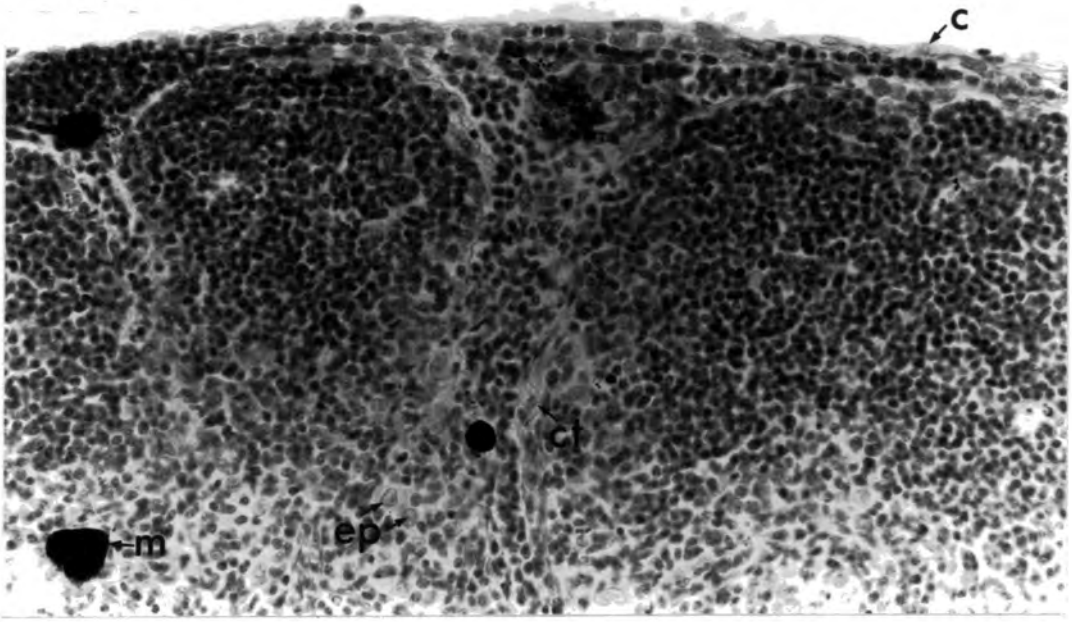


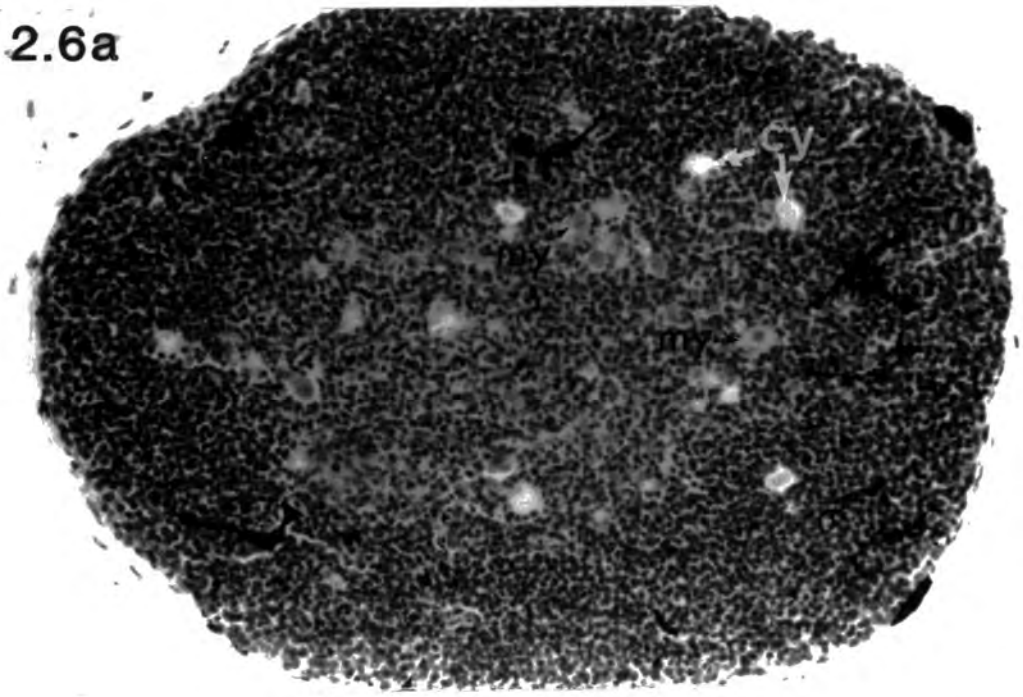
Figure 2.6. Cold temperature treatment of larval and froglet thymus

a) 5 week stage 55/6 larval thymus cultured for 4 days at 10°C. Thymuses look healthy and have maintained their size and shape well. Lymphocyte numbers directly compare with the in vivo situation. cy = cysts, my = myoid cells. Category A. (x 200)

b) 5 month froglet thymus organ cultured at 4°C for 12 days. The thymus is large, extremely lymphoid, and resembles the in vivo thymus. Category A. (x 100)

Thymuses organ cultured at 15°C resembled those cultured at 4°C.

2.6a



2.6b

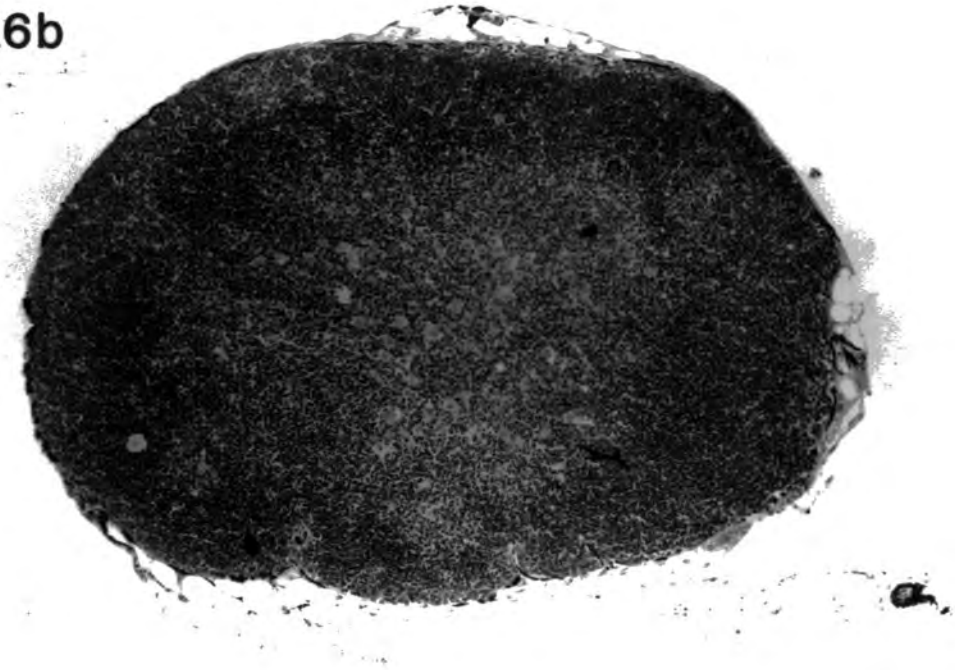
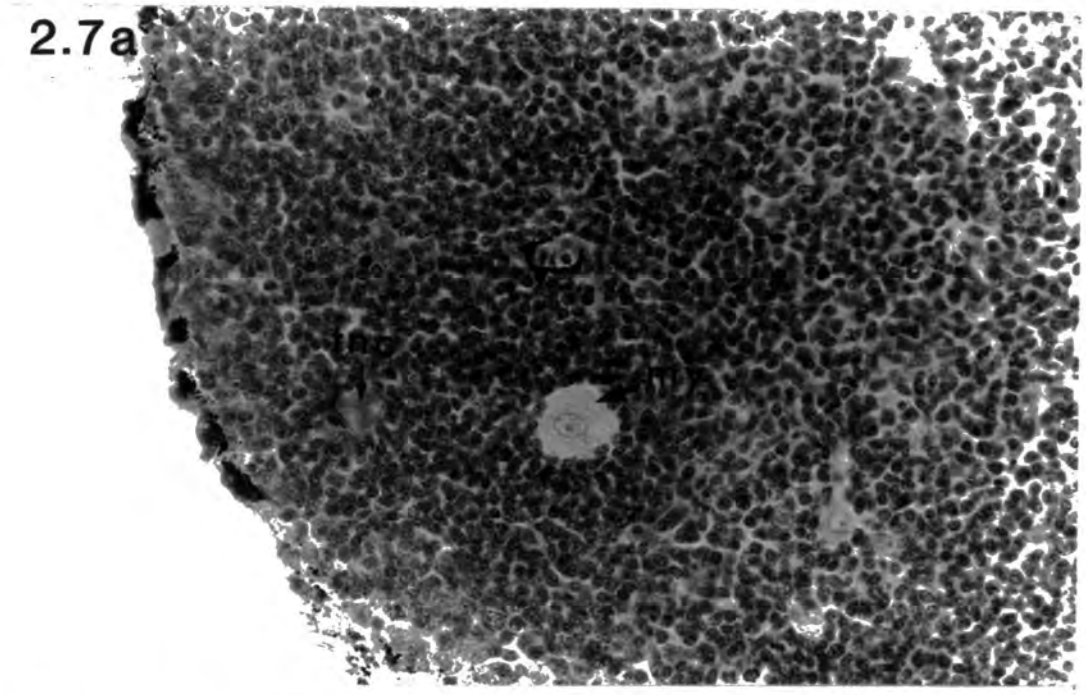


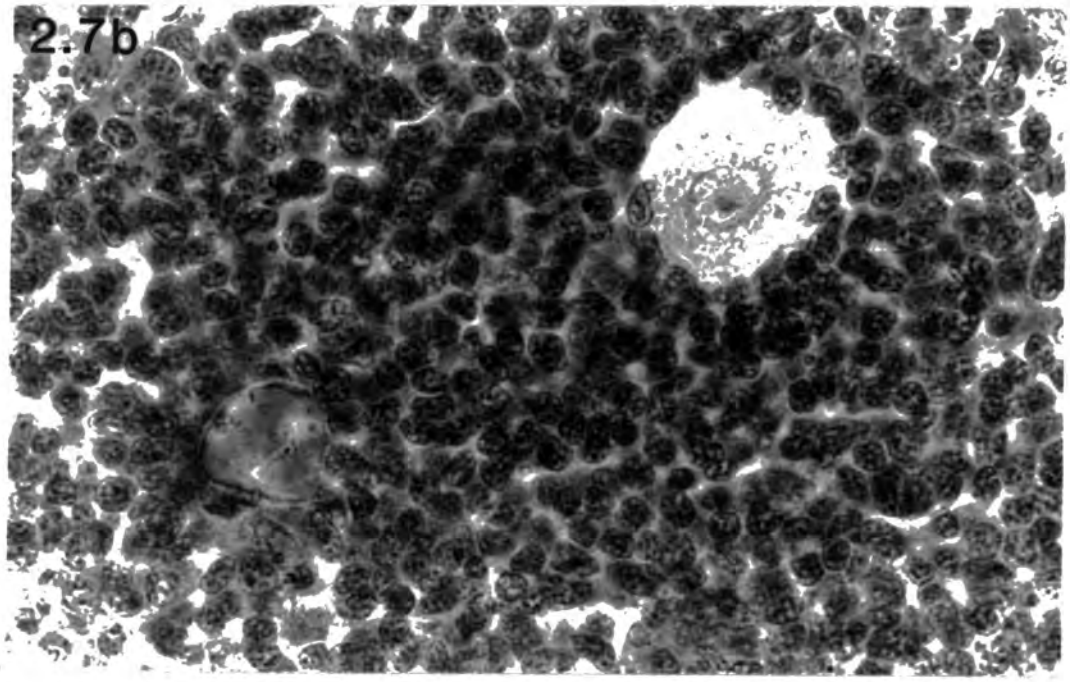
Figure 2.7. 5 month froglet thymus following 7 days organ culture at 4°C.

Category A.

a) Shows a portion of the cortex with a myoid cell (my) putative "thymic nurse cell" (tnc) - x 400.

b and c) were taken at different optical planes of focus to show inclusions within the "nurse cell". In 7b the focal plane is on the surface of the complex, whereas in 7c, several cells appear within the structure. (x 850)


2.7a


2.7b

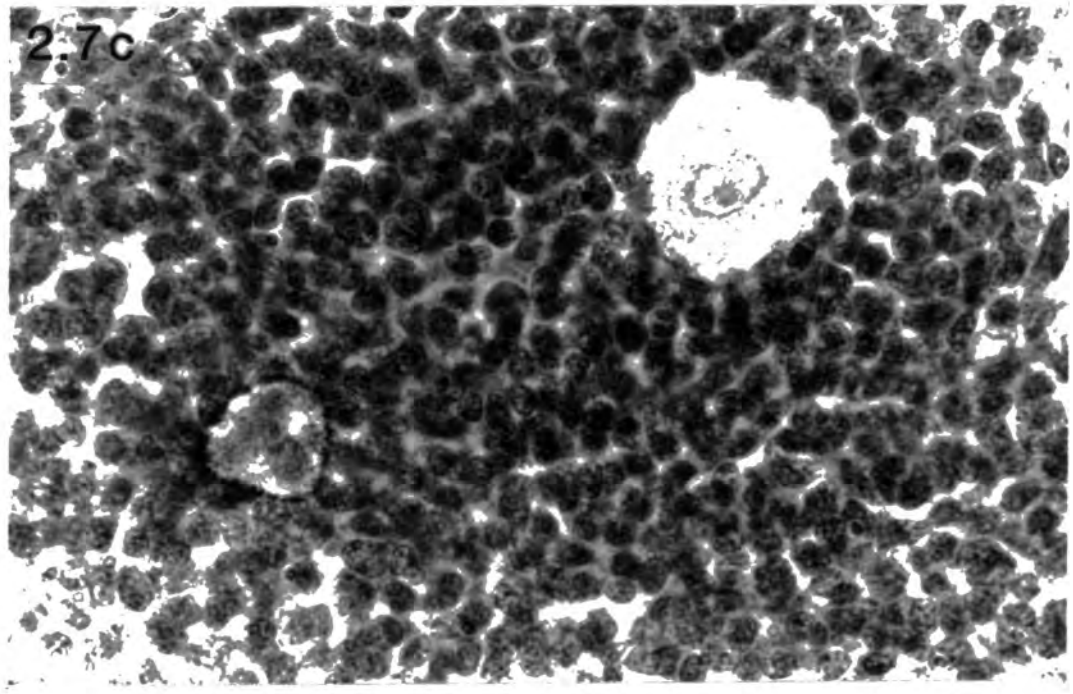
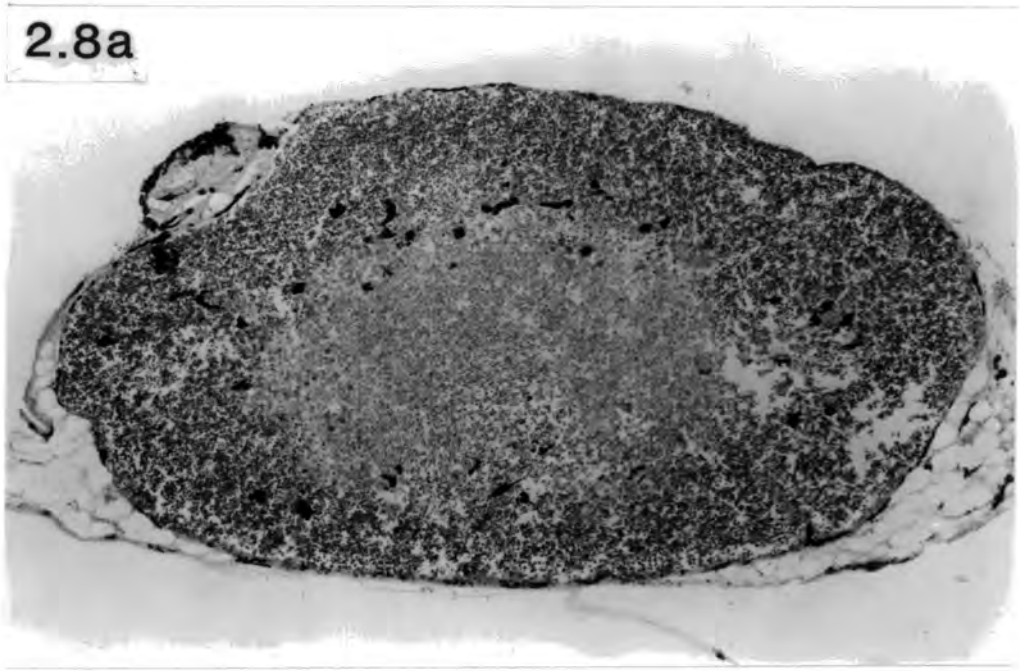

2.7c

Figure 2.8. 5 month froglet thymus cultured for 12 days at 34°C

Category E.

a) Thymus shows cortex/medulla demarcation, but has become necrotic and full of pyknotic cells. (x 100)

b) High power view of the same thymus to show the degenerating thymus in more detail. A few medullary epithelial cells (e) still remain intact. (x 400)


2.8a

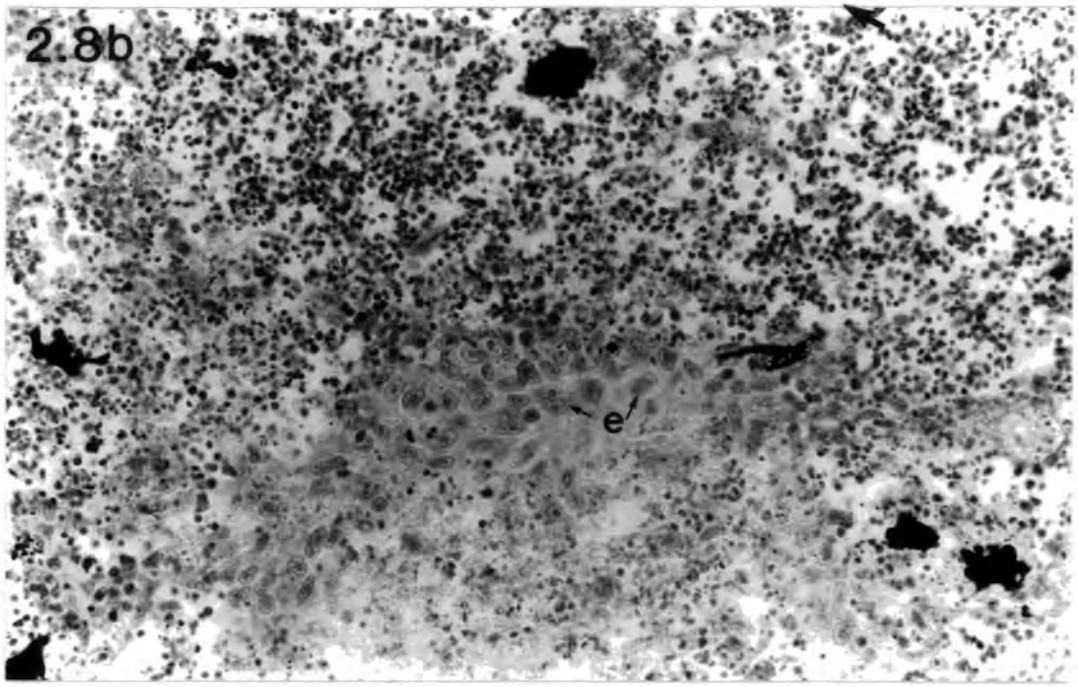
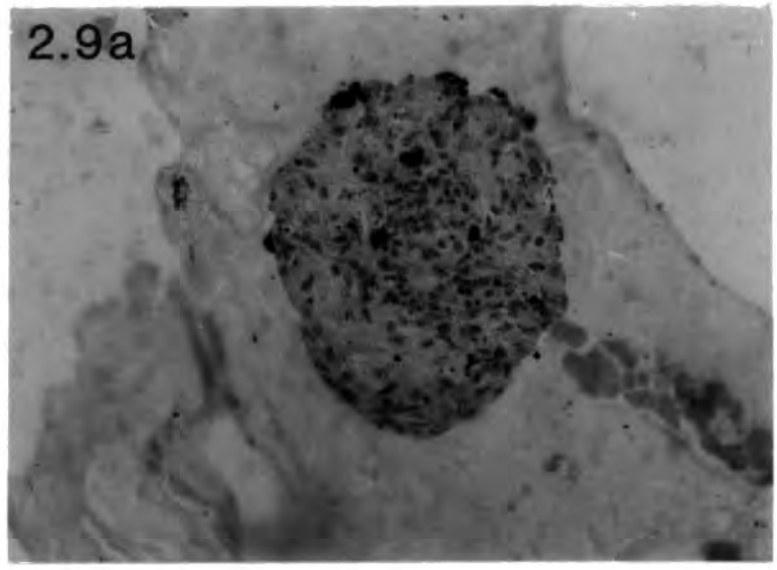

2.8b
e

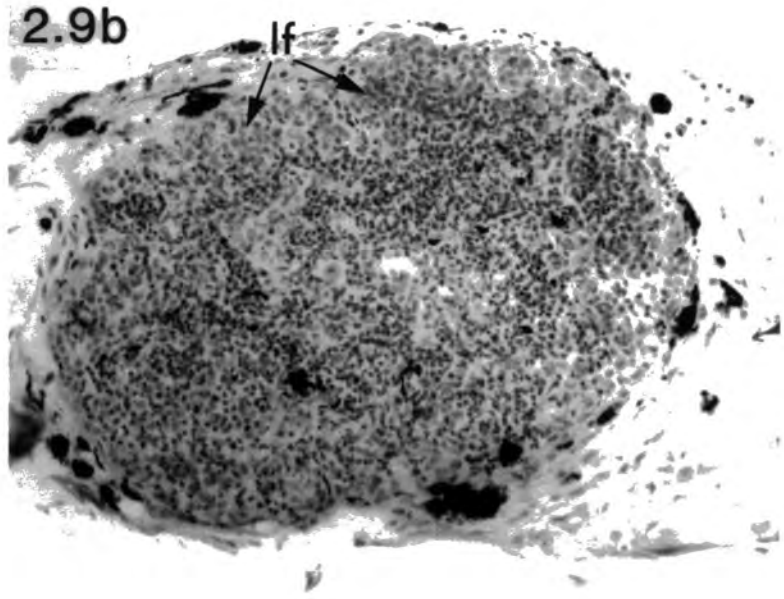
Figure 2.9. Deoxyguanosine treatment of larval and froglet thymus

a) 3 week stage 52/3 larval thymus treated for 5 days in 1.5 mM DG and a further 7 days in DG-free medium. The thymus is significantly reduced in size and is virtually epithelial. Category > D <. (x 125)

b) 3½ month froglet thymus cultured for 4 days in 2.2 mM DG and then in DG-free medium for 7 days (total culture time 11 days). Thymus possesses significantly fewer thymocytes than normal. The structural difference between the cortex and medulla is much less defined. The DG appears to have been particularly effective in destroying cortical, rather than medullary, lymphocytes. Category > C <. (x 110). lf = lymphocyte-free region of cortex.

c) 6 month froglet thymus cultured in 2.0 mM DG for 3 days then DG-free medium for 3 days, then 2.0 mM DG again for 3 days and finally DG-free medium for 3 days (total culture time 12 days). Thymus is still very lymphoid, although small areas of cortex appear lymphocyte depleted. Category > B/C <. (x 110). m = melanin clumps.





2.9c

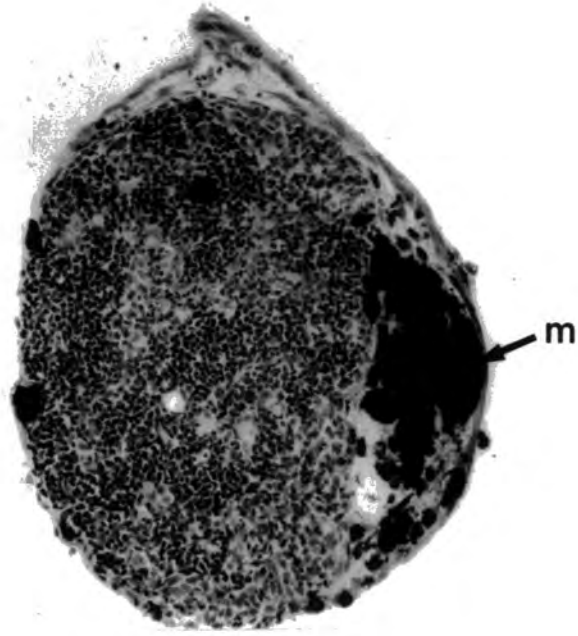
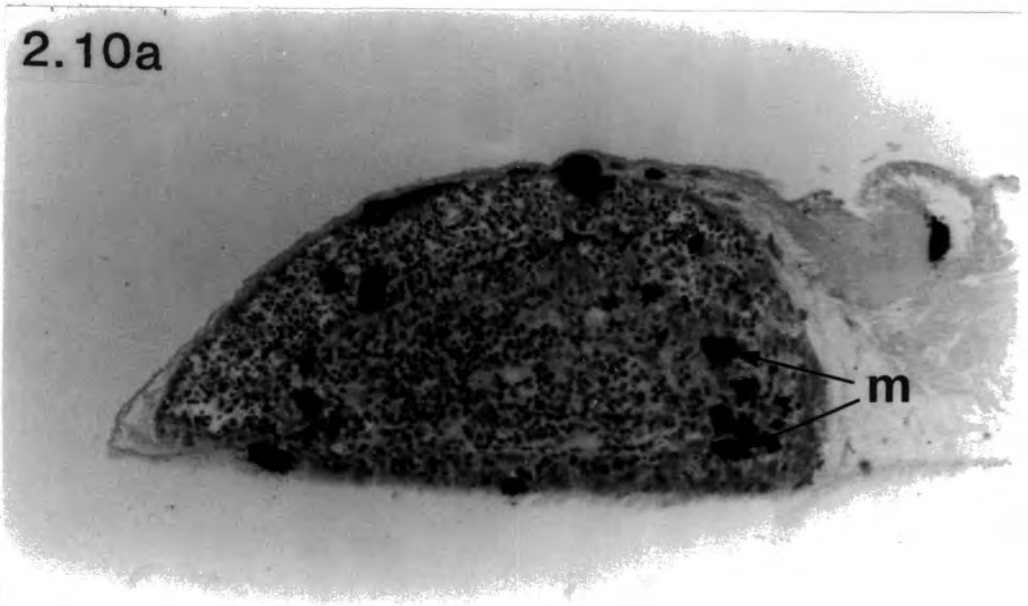


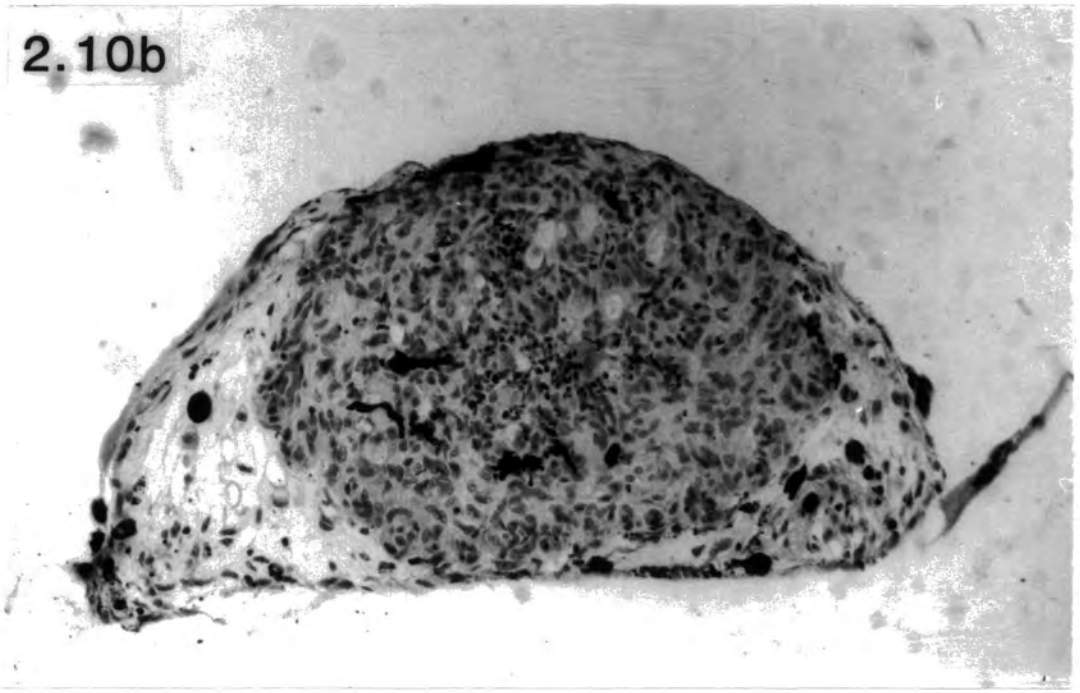
Figure 2.10. In vitro δ -irradiation and organ culture of froglet thymuses

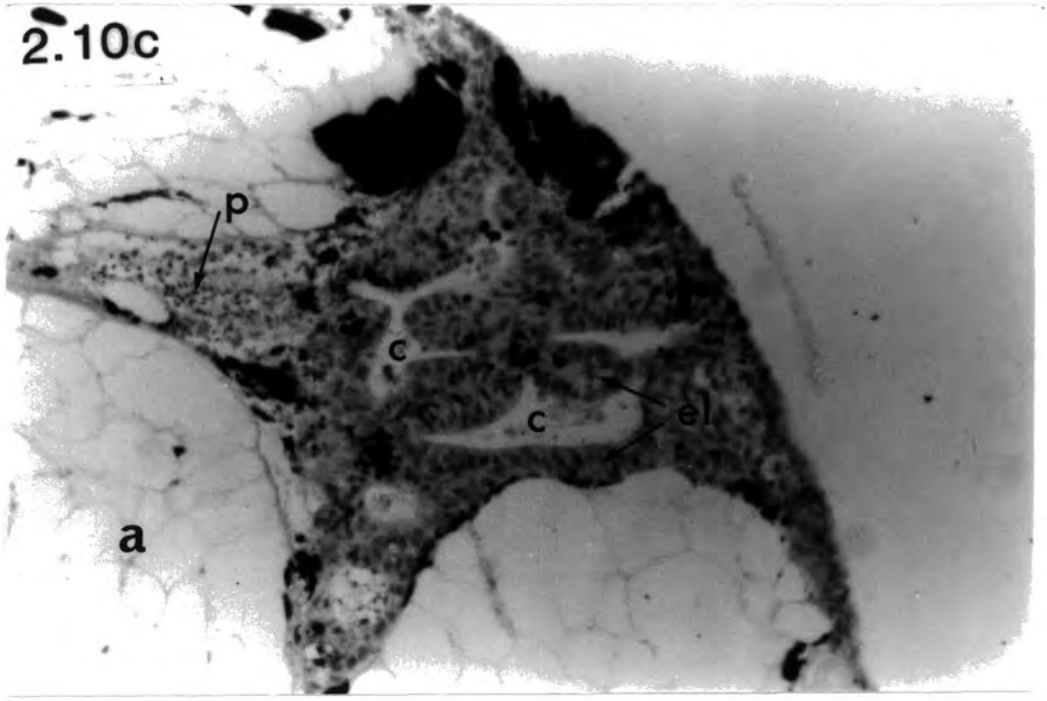
a) 5 month froglet thymus cultured for 2 days after receiving a dose of 1000 rads. Many pyknotic cells are present and are especially visible in the cortical zone although lymphocytes are still apparent in the medulla. Category > C <. (x 125). m = clumps of melanin.

b) 6 month old froglet thymus cultured in medium for 14 days having been treated with a dose of 1000 rads at the beginning of culture and on day 6 of culture. Only very few lymphocytes remain (these are found in the centre of the thymus). The thymus is now mainly composed of epithelial/epithelial reticular cells. Category > D <. (x 125)

c) 4 months froglet thymus irradiated with 2000 rads and cultured for 4 weeks. Thymus is very degenerate and pyknotic cells (p) are evident. The epithelial cells have become arranged into convoluted epithelial layers (el). Large cystic (c) spaces are seen. Melanin and adipose tissue (a) surround the thymic area. Category > E <. (x 200).


2.10a
m


2.10b


2.10c
p
c
c
el
a

CHAPTER 3

EFFECT OF IN VIVO IRRADIATION ON THYMUS STRUCTURE: LIGHT AND ELECTRON MICROSCOPIC STUDIES

3.1 INTRODUCTION

The last Chapter showed that irradiation can successfully be used to destroy lymphocytes in the froglet thymus. After a period in organ culture, irradiated thymuses appeared mainly epithelial (when examined in 8 μm wax sections) and there was no evidence of lymphocyte regeneration. However, lymphocyte-depleted thymuses produced in this way were generally very reduced in size and, therefore, were considered unsuitable for use in the planned *in vivo* transfer experiments, since immigration of host cells into a small thymus might be compromised. In this Chapter I show that *in vivo* whole body irradiation and subsequent residence in the irradiated froglet is a more successful way of producing a lymphocyte-depleted thymus, that retains a more normal 3-dimensional stromal network, with intact cortical and medullary areas and relatively few necrotic cells. This work also presents a detailed light and electron microscopic investigation of the froglet thymus before and after *in vivo* irradiation.

3.2 MATERIALS AND METHODS

3.2.1 Animals

J strain *X. laevis* were used in this Chapter. All animals were post-metamorphic and ranged from 5-9 months old.

3.2.2 In vivo irradiation to deplete thymic lymphocytes

Froglets were placed in 50 mm diameter plastic beakers and whole-body irradiated with either 1,000 or 3,000 rads, using the Cobalt-60 source as described in Chapter 2. Animals generally remained healthy during the first 7-10 days after irradiation, but after this

time the froglets often became sluggish and deaths were frequent from 10 days onwards, particularly in the 3,000 rad irradiated group.

3.2.3 Experimental plan

(i) Time-course study on effects of in vivo irradiation

Thymuses were dissected from control froglets. The interval between irradiation and thymus removal varied from 5-14 days (see Table 3.1). Thymuses were fixed in Bouin's and embedded in paraffin wax. 7 µm sections were stained in haematoxylin and eosin (H and E). The effect of irradiation on a major peripheral lymphoid organ - the spleen - was also assessed 8 days post 3,000 rads. These 7 µm sections were stained with a triple stain (periodic acid Schiff's (PAS), orange G and haemalum), so as to highlight irradiation-induced changes. The stain is a modification of the techniques described by Pearse (1953). Routine fixatives are suitable, e.g. Bouin's 7 µm wax sections were placed in xylene for approximately 10 mins to dewax, then taken down a series of alcohols (3 changes in absolute, one change in 95% and one in 70% alcohol) to tap water and rinsed in distilled water. Sections were then oxidized in 1% periodic acid for 10 mins and rinsed in distilled water. Slides were placed in Schiff's reagent (PAS) at room temperature for 15 mins, rinsed in distilled water, and left in running tap water for 20 mins to turn pink. Nuclei were stained in Mayers haemalum for 1 min. and sections placed in running tap water for 10 mins to blue. Slides were dipped in orange G for 1 min, and then placed immediately into 70% alcohol and dehydrated in a series of alcohols and xylene. Slides were mounted in DPX for microscopic examination.

(ii) Organ-culture of in vivo-irradiated thymuses

Thymuses were removed from 3,000 rad-irradiated froglets at 8 days post-irradiation, and were placed in organ culture for either 7 or 12 days, to determine if lymphoid "regeneration" of the thymuses would

take place in the absence of any extrinsic haemopoietic source. At the end of organ culture these (5 in total) thymuses were fixed in Bouin's, embedded in paraffin wax and 7 μm sections stained in H and E.

(iii) Light and electron microscopic observations on plastic-embedded thymuses 9 days after 3,000 rads total body irradiation

Three control and three irradiated thymuses were initially fixed in Karnovsky's fixative for 1½ hrs at 4°C [Karnovsky, 1965 - this fixative was made from two solutions A and B, kept separate and refridgerated before use. Solution A comprised 2 g paraformaldehyde and 40 mls distilled water shaken together and stirred. To this 2-6 drops of IN NaOH was slowly added until the precipitate dissolved. Solution B was comprised of 10 mls 25% gluteraldehyde mixed with 50 mls 0.2 M sodium cacodylate buffer pH 7.3. A and B were stirred together just before use and cooled.] Thymuses were then post-fixed in 1% buffered osmium tetroxide for ½ - 1 hour at 4°C [1% osmium tetroxide in 0.1 M buffer was made by mixing 2% OsO_4 in equal amounts with 0.2 M sodium cacodylate.] The organs were continually rotated during fixation to ensure maximum infiltration by each fixative. Tissues were dehydrated at room temperature by leaving in 70% alcohol for 15 mins, 95% alcohol for 15 mins and in absolute alcohol for 30 mins (three changes for each alcohol strength). Thymuses were passed through three changes of propylene oxide (or acetone)/araldite for 30 mins at 45°C, and finally embedded in araldite for 12 hours at 45°C and for a further 2 days at 60°C. The araldite mixture was made from 10 mls araldite (CY212), 10 mls DDSA (dodecenyl succinic anhydride), 1 ml dibutyl phthalate and 0.5 ml DMP 30 [2, 4, 6 tri(dimethyl aminomethyl) phenol], stirred together for 5-10 mins (Glauert and Glauert, 1958).

One micron sections were cut through the thymus at its maximal diameter using a Reichert Ultramicrotome, stained in Toluidine blue for light microscopy, and photographed with a Zeiss Ultraphot photomicroscope.

The toluidine blue stain comprised 0.1 gm toluidine blue, 0.1 gm sodium tetraborate and 0.05 gm pyronin, dissolved in 60 mls distilled water. The solution was pipetted onto the slides (on a hotplate) and left for 2-3 mins, then washed off with distilled water and sections mounted in DPX.

Ultra thin sections were mounted on copper grids and double-stained with saturated uranyl acetate and lead citrate. Grids were observed with a Phillips 400T electron microscope operating at 100 kv.

3.3 RESULTS

3.3.1 Time-course study on effects of in vivo irradiation

The results are summarized in Table 3.1. 5-8 days after 1,000 rad irradiation, thymuses appeared healthy, with only a small decrease in size; they contained very reduced lymphocyte numbers, the latter mainly being found in the medulla. Pyknotic cells were scarce. By 12 days post-irradiation, lymphocytes were now also very evident in the cortex of 2/3 thymuses examined (see Figure 3.1). Presumably, in these thymuses, 1,000 rad-resistant lymphocytes have proliferated and have partially repopulated the organ.

Thymuses removed 5 days after 3,000 rad total body irradiation again showed clearly the initial destruction of cortical lymphocytes; in contrast, the medulla remained fairly richly populated with lymphoid cells (Figure 3.2). Within 7 days of 3,000 rad irradiation, thymuses were more depleted of lymphocytes, the medulla now containing only scattered lymphocyte-rich foci (Figure 3.3). By 8-14 days post 3,000 rads, thymuses consisted mainly of stromal cells, although a few scattered lymphoid cells appeared to persist in the medulla (Figure 3.4). The cortex appears to have become reduced in size and is virtually lymphocyte-free. Cystic spaces and myoid cells are frequently noticed in these irradiated thymuses.

Overall then, 3,000 rad irradiation in vivo appeared to be successful in procuring a lymphocyte-depleted thymus, which retained a fairly normal overall 3-D arrangement - i.e. in terms of persistent cortex/medulla differentiation. Although reduced in size and lymphocyte content, in vivo irradiated thymuses have a more normal architecture than the in vitro irradiated organs described in Chapter 2.

Spleens removed from 3,000 rad-irradiated froglets 8 days after irradiation were severely depleted of lymphocytes - such that white pulp, marginal zone and red pulp lymphoid accumulations had virtually disappeared (compare Figure 3.5 with 3.6). The white pulp regions have shrunk and the vascular network (stained pink with PAS) appears prominent in the irradiated spleens.

3.3.2 Organ culture of in vivo irradiated thymus

Five thymuses were taken from froglets 8 days after 3,000 R whole body irradiation. The 2 thymuses organ-cultured for 8 days displayed loss of cortex/medulla differentiation. However, some lymphocytes were still seen scattered in the organs. After 12 days of organ culture, the irradiated thymuses had become small and looked rather abnormal in structure. They were still comprised mainly of stromal cells, with very few lymphocytes (Figure 3.7).

3.3.3 Light microscopic observations on 1 μm plastic-embedded thymuses, 9 days post 3,000 rad total body irradiation

Figures 3.8 to 3.13 highlight the principle effects of irradiation on thymus architecture.

Figures 3.8 to 3.9 provide low magnification views of normal and irradiated thymuses. The latter are about one third to one half the size of the normal thymus. Irradiated thymuses are, as expected, extremely depleted of lymphocytes, although cortex and medulla can still easily be recognized. Large fat cells can be seen around both

normal and irradiated thymuses, but are particularly obvious around the irradiated organ. Clumps of melanin are very noticeable in irradiated thymuses.

Figures 3.10 and 3.11 are higher magnification views of cortex and medulla of control thymuses. Blood capillaries are noticeable at the edge of the thymus and extend down through the cortex; they are also seen in the medulla. Penetrating blood capillaries are associated with connective tissue trabeculae, that penetrate down from the capsule, through the cortex. These trabeculae are continuous with the thymic capsule.

In the subcapsular cortex (see Figure 3.10) are found relatively paler-staining, large lymphoid cells. These cells are the lymphoblasts; mitotic figures are frequently seen in this region (Figures 3.10, 3.11a). Epithelial cells and epithelial reticular cells can be seen in the subcapsular cortex.

The inner (or deep) cortex is very rich in small lymphocytes, that contain densely-stained nuclear chromatin and have minimal cytoplasm. Within this deep cortex (and medulla) are seen some noticeable, very pale-staining cells (Figure 3.10); these have large pale-staining nuclei with prominent nucleoli and voluminous, pale-staining cytoplasm, that appears to interdigitate with surrounding lymphoid cells. These cells could represent the amphibian equivalent of interdigitating antigen-presenting cells (see Bigaj and Plytycz, 1984).

The medulla is rich in epithelial and epithelial reticular cells (Figure 3.10, 3.11b and c), although it still has large numbers of lymphocytes. The latter are less-densely stained than the small lymphocytes of the deep cortex (compare Figure 3.11a and b). Lymphocytes can be distinguished from adjacent epithelial cells by their

distinctive chromatin pattern. Thus, hetero- and euchromatin patches with distinct nuclear membrane staining are lymphocyte characteristics, whereas epithelial cell types have paler-staining nuclei, often prominent nucleoli and a far more extensive cytoplasm (Figures 3.11b and c). Myoid cells (Figure 3.10) and cystic spaces are occasionally seen in the medulla. Granulocytes are seen frequently (Figure 3.10, 3.11 b and c). Macrophages are also found (Figure 3.11a), but are not particularly common in the control thymuses.

Figures 3.12 and 3.13 concentrate on the irradiated thymus. Figure 3.12 shows in some detail the "epithelial" nature of the thymus. In the absence of lymphocytes, the epithelial cells in the subcapsular cortex are very noticeable and contain prominent nucleoli; some are rounded and others (the epithelial reticular cells) are elongated. Both pale and darker-staining (Figures 3.13a and c) epithelial reticular cells are found; one part of the cortical region of the thymus shown in Figure 3.9 contained large numbers of these epithelial reticular cells (Figure 3.13c).

Just beneath the subcapsular cortex, the deeper cortex is now very reduced in size and is comprised of epithelial cells and noticeable intercellular spaces (Figures 3.12 and 3.13a), which presumably were previously (in the normal thymus) occupied by the deep-cortical lymphocytes.

The medulla contains a scattering of lymphoid cells (Figures 3.12 and 3.13b), but is mainly comprised of epithelial cells and a variety of other cell types. These include myoid cells, cystic structures (3.13b) and various types of granulocyte (3.13b). Deposits of melanin are also noticeable in the medulla (Figure 3.12). Lipid inclusions are commonly found (Figure 3.13b). Macrophages (3.13 b and c) containing phagocytosed material are frequently seen in irradiated thymuses,

although these cells are not always easy to identify without the use of macrophage-specific stains. The granulocyte population includes mast cells (containing relatively-round granules) (Figure 3.13b) and large granulocytic cells that are comparable to the granular glands found in frog skin - these contain ellipsoid granules, typical of eosinophils (Figure 3.13b).

3.3.4 Electron microscopic observations on normal and 3,000 rad-irradiated thymuses

Figures 3.14 - 3.17 are representative views of cortex and medulla of a control thymus. The subcapsular cortical layer is seen in Figure 3.14. This contains many lymphoblasts, 2 of which are shown in stages of mitosis. Figure 3.15 shows an electron micrograph of the deep cortex, packed with small lymphocytes. A large cell, with pale-staining nucleus and cytoplasm is also seen - whether this is an epithelial cell or an "interdigitating APC" is unclear. Its cytoplasm appears relatively smooth contoured and lacks the finger-like extensions expected of an interdigitating cell. Figure 3.16 shows the cellular nature of the medulla. Lymphocytes are seen interspersed between the cytoplasmic extensions of much paler-staining cells. It is uncertain whether these cytoplasmic processes belong to interdigitating cells (of extrinsic origin - see Discussion) or epithelial cells. The putative interdigitating cells referred to here were not seen in the irradiated thymuses, i.e. they are radiosensitive. A medullary myoid cell, surrounded by small lymphocytes and epithelial reticular cells, is seen in Figure 3.17.

Figures 3.18 - 3.22 are representative views of irradiated thymuses. Figure 3.18 shows epithelial cells that predominate in the cotical region. Their nuclei are generally pale-staining, except for the nucleolus and nuclear membrane. Their cytoplasm is rich in ribosomes, mitochondria and some rough endoplasmic reticulum is also seen.

Figure 3.19 shows a macrophage in the outer cortical region - it contains a rich supply of densely-staining lysosomes, for eliminating phagocytosed material.

Figure 3.20a and b show a portion of the medulla and reveal the nature of cystic structures that are frequently seen following irradiation. Numerous microvilli, and classical cilia (9 + 2 arrangement of fibrils), project into the lumen of the cyst, which contains much debris. The cytoplasm of the cells associated with the cysts is sometimes rich in granular material. Adjacent epithelial cells have an abundance of rough endoplasmic reticulum, indicating active protein synthesis. Myoid cells of normal appearance (Figure 3.21) and mucous cells (3.22) are also seen in irradiated thymuses. Some degenerate-looking myoid cells were also seen.

This brief ultrastructural analysis of normal and irradiated thymus reveals that although the irradiated organ is dramatically depleted of lymphocytes, it does still contain many normal-looking stromal elements.

3.4 DISCUSSION

This Chapter has examined, in some detail, the outcome of whole-body irradiation on the cytoarchitecture of the 5-9 month old froglet thymus. To my knowledge, this is the first time that the effects of irradiation on amphibian thymus structure have been reported. Early research by Cooper and Schaffer (1970) examined the effects of 5000R γ -irradiation (from a cobalt-60 source) on aspects of the immune system of the leopard frog. The numbers of peripheral blood lymphocytes declined sharply following 5000R irradiation, although other blood cell types, e.g. granulocytes and monocytes, seemed unaffected. My studies reveal that following a dose of 3,000 rads, thymuses taken in the second week post-irradiation show a predominance of stromal cells, with

loss of cortical lymphocytes and depletion of medullary lymphocytes, although some lymphocytes still persist in this latter zone. Many workers have previously noticed the relatively radio-resistant population of lymphocytes in the (mammalian) thymic medulla compared with those in the cortex (e.g. Trowell, 1961; Bartel, 1984). Regaud and Crémieu (1912) first described the phenomenon of thymus inversion following irradiation, i.e. the atrophy of cortical lymphocytes and the persistence of epithelial cells, making the cortex resemble the medulla. Today, it is generally thought (Sharp and Watkins, 1981; Roitt et al., 1985) that the difference in radiobiological properties of cortical and medullary lymphocytes represents the existence of two different subpopulations of thymocytes - immature and proliferating lymphocytes (about 85% of the total) in the cortex, while the remaining 15% of thymic lymphocytes found in the medulla are a functionally mature population and may resemble fully-differentiated helper, cytotixic and suppressor T cells.

The nature of the thymic lymphocyte subsets that can survive 3,000 rads irradiation in the frog is not known. Certainly the radiation sensitive B cells found in the Xenopus thymus (Williams et al., 1983; Hsu et al., 1983) should be effectively removed (see discussion by Gearing et al., 1984); T suppressor cells are also known to be irradiation sensitive in both mammals (see Sharp and Watkins, 1981) and in Xenopus (Ruben et al., 1985 - where 1000 rads eliminates suppressor inducer function). That Xenopus T helper cells from the spleen may be able to withstand 3,000 rads is suggested by Cribbin (1984) from her in vivo reconstitution studies of primary antibody responses and by Lallone (see Lallone, 1984 and Horton et al., 1986) in his experiments on in vitro reconstitution. However, Ruben et al., (1985) show that 2,000 rads prevents thymus cells from helping cocultured spleen fragments to produce antibody.

Thymuses removed from total-body irradiated (3000R) Xenopus after 8 days and placed in organ culture showed no signs of lymphocyte regeneration. In contrast, 1,000 rad irradiation resulted in lymphoid regeneration, as seen when thymuses were removed 12 days post irradiation. Similar thymus recovery after adult whole-body irradiation has been reported in mammals (summarized in Sharp and Watkins, 1981). Mammalian studies suggest that the thymic progenitor cell is relatively radio-resistant. However, the ability of the thymic microenvironment to support thymocyte differentiation in vivo declines significantly when the thymus experiences radiation doses greater than 1,000 rads (Sharp and Watkins, 1981).

Spleens from 3,000 rad irradiated Xenopus also showed extensive lymphocyte depletion. The lymphocyte-rich areas of the spleen, i.e. white pulp and marginal zone (extrafollicular region encircling the white pulp) were especially devoid of lymphocytes. From studies of spleen from early-TX Xenopus (Horton and Manning, 1974a; Tochinai, 1976a,b) and NMU-carcinogen-treated animals (Balls et al, 1980; Clothier et al., 1980) the marginal zone and red pulp are believed to concentrate thymus-derived lymphocytes (T cells) whereas white pulp follicles are thought to be rich in cytoplasmic Ig-positive B lymphocytes (Baldwin and Cohen, 1981). Both marginal zone T cell and white pulp B cell lymphocyte populations seem to be radiosensitive to 3000R.

The studies reported here have revealed several interesting features with respect to the structure of the amphibian thymus. The first point worthy of comment is that the cortex has distinct sub-capsular and deep cortical zones. This situation is also found in the mammalian thymus, where the subcapsular region houses a population of blasts ($\sim$10% of the total thymic lymphocyte population), whereas the deep

cortex contains lymphocytes of smaller size (some 75% of the total), many of which are destined to die within the thymus (Scollay, 1983). The precise inter-relationship between the cortex/medulla and cell traffic through the mammalian thymus is still uncertain, although several theories have been proposed (reviewed by Scollay, 1983). Thus the differentiation of stem cells to "mature" T cells in the thymus may occur by various routes, involving the cortex and medulla compartments either independently or jointly.

Another feature concerning froglet thymic structure suggested here is the presence of interdigitating cells (IDC) in the normal thymus, but their possible absence following irradiation. These large, pale-staining cells appear morphologically similar to the mammalian IDCs (Kaiserling et al., 1974) and have been noticed by other amphibian workers (Bigaj and Plytycz, 1984; Clothier and Balls, 1985; Turpen and Smith, 1986). IDCs in both mammals and amphibians (see Turpen and Smith, 1986) are thought to be migratory haematopoietic stromal cells and resemble both the Langerhan's cells in the skin and the IDCs in the spleen and lymph nodes. In the peripheral tissues their purpose is to trap antigen and present it to immunocompetent lymphocytes. Mammalian IDCs are rich in surface Class II MHC antigens and in the periphery they are found closely associated, through junctional complexes, with the surrounding lymphocytes (Weiss and Sakai, 1984). In the thymus, the many projections on the IDCs that interdigitate with surrounding thymocytes give them an extremely suitable structure for an antigen presenting task. As well as thymic epithelial cells, IDCs and macrophages (these latter two cell types classified together as accessory cells, and are both haemopoietically-derived) are believed to play a critical role in T cell selection, education and maturation. It has been proposed that interactions occurring between MHC rich

accessory cells and lymphoid elements may be involved in the acquisition of MHC-restriction, whereas the epithelial component may be involved in the acquisition of self-tolerance (reviewed in Turpen and Smith, 1986). It has also been suggested that thymic antigen presenting cells (IDCs/macrophages) may have a more significant role in educating T cells that will eventually recognize Class II MHC products (T helper cells), whereas the thymic epithelium itself imparts restriction specificity on T cytotoxic cells which recognize Class I molecules plus antigen (reviewed by Watkins, 1985).

Mammalian IDCs are most likely derived from monocytes and therefore are members of the mononuclear phagocyte system (Duijvestijn and Hoefsmit, 1981); however some results have suggested that IDCs have a reticular origin in birds (reviewed in Bigaj and Plytycz, 1984). Turpen and Smith (1986) have studied the ontogeny of both phagocytic and non-phagocytic (dendritic) accessory cells in the amphibian thymus, by grafting haemopoietic stem cells into cytogenetically distinct Xenopus embryos (2N or 3N) prior to thymic colonization. Their experiments provide circumstantial evidence suggesting thymocytes and thymic accessory cells could arise from a bipotential precursor, that diverges into these separate lineages after colonization of the epithelial rudiment, during early development. An alternative theory concerning the identity of thymic IDCs is proposed by Wood (1985), who believes that thymic interdigitating/dendritic cells do not exist as more than morphologic variants of macrophages, and that the terms interdigitating or dendritic cell are misleading and useful for descriptive purposes only. He also suggests that while thymic epithelial cells may provide signals which attract lymphocytes to the thymus and which stimulate precursor cell multiplication, it is the macrophages which provide the stimuli for thymocyte maturation. In the present

study on irradiated Xenopus thymus, macrophages were frequently seen, illustrating that these cells must be considered as part of the radiation-resistant stromal population.

The light microscopic observations reported here are in agreement with the detailed ultrastructural observations on the developing Xenopus thymus made by Clothier and Balls (1985), who described the presence of pale- and dark-staining epithelial reticular cells. These 2 populations were especially clearly seen here in the 1 μm sections of irradiated thymuses. The morphologic difference between these epithelial cells may indicate varying states of metabolic activity of a single epithelial cell-type (Singh, 1981), or may reflect their different embryologic origins (Farr and Nakane, 1983; Crouse et al., 1984). It has been reported that ectoderm gives rise to cortical epithelium, while the epithelium of the thymic medulla is endodermal in origin (cited in Farr and Nakane, 1983). Crouse et al. (1984) have also found two apparently distinct epithelial types based on differences in staining (light and dark) and other morphologic criteria, when studying lymphoid-free, low temperature-cultured, embryonic mouse thymic epithelium. Transplantation of this thymic epithelium (that was rich in light-staining epithelial cells) to syngeneic and allogeneic immunodificient mice, and subsequent studies on splenocytes from these hosts, revealed that spleens were not reconstituted either in T-cell function or relevant T-cell surface phenotypes. Crouse et al. (1984) propose that the lightly-stained epithelial cells are ectodermal in origin and are responsible for inducing stem cell immigration and their early proliferation, whereas the endodermally-derived epithelium (dark epithelial cells) is responsible for the emigration and/or maturation of the T-cell population found in the periphery.

As noted in some detail by Clothier and Balls (1985), a number of secretory cell types containing distinctive granules are found

within the Xenopus thymus. The present emperiments reveal that 3,000 rads whole-body irradiation did not remove these cells from the thymus; thus mucous cells, mast cells/basophils and large granular cells with ellipsoid cytoplasmic granules (the latter resembling skin granular glands) were readily seen in irradiated thymuses. Clothier and Balls (1985) have recorded TRH (thyrotrophin-releasing hormone) and a variety of neuroactive amines in Xenopus dermal granular glands. Furthermore, they reveal significant amounts of TRH in the stage 56/7 X. laevis thymus, prior to its appearance in the dermal granular glands (Balls et al., 1985). Plytycz and Bigaj (1983b) reported that, during hibernation and mating of the adult frog R. temporaria, the thymus becomes depleted of lymphocytes and consists of numerous secretory cells filled with secretory granules and degeneration cysts. This accompanied an increase in thymic endocrine activity, which they suggest may promote the final steps of peripheral T cell maturation ready for the start of the frog's active life in the summer.

A particularly noticeable effect of irradiation on the thymus gland, apart from the obvious reduction in lymphocytes, was the increase in number of cysts; myoid cells also became very obvious. Myoid cells are epithelial derivatives (Törö et al., 1969) and contain actin and myosin myofilaments arranged as in striated muscle. It is thought that contraction of these cells may facilitate thymic fluid circulation and/or the cells may act as a source of muscle-specific self-antigens (Törö et al., 1969; Rimmer, 1980). Many degenerating myoid cells, as well as normal-looking myoid cells, were observed in irradiated thymus. It seemed possible that the degenerating myoid cells might contribute,in some way, to the formation of cystic structures. These cysts often contain cells with microvilli and cilia, which presumably aid in movement of fluid in the lumen. The cysts may

well act as sites for the deposition fo dead cellular material (Kendall, 1981a). The cystic cells, which contain secretory-looking granules, may be involved in the release of secretory material into the cyst lumen, or may reabsorb this material for transport to the vicinity of thymocytes or into the bloodstream (Curtis et al., 1979). It is possible that material resulting from lymphocyte breakdown is salvaged (Clothier and Balls, 1985), this could partly explain the increase in "cystic activity" within the irradiated thymus.

In conclusion, the 1 μm light and electron microscopic observations presented here reveal that, in the short-term, a radiation dose of 3,000 rads given in vivo does not cause appreciable damage to the overall thymic stromal architecture, but does dramatically reduce lymphocyte numbers. Whether 3000R irradiation affects MHC antigen expression of thymic stromal cells is not known. It was felt that such an irradiated thymus should prove useful for the implantation studies planned in this Thesis, where I wished to observe the influence of the donor thymic stroma in attracting host lymphoid cells and inducing them to become tolerant to donor MHC antigens. It should be noted that longer-term defects caused by irradiation of the thymus do become apparent, as seen in the cell traffic studies (see Chapters 4 and 5).

Key to Table 3.1

A	=	Completely normal structure: (comparable with thymuses taken straight from the animal).
B	=	Still very lymphoid, but with fewer lymphocytes than the *in vivo* thymus. Cortex and medulla nearly always present.
C	=	Lymphocyte numbers reduced. Cortex depleted of lymphocytes, but some lymphocyte-rich areas are visible in the medulla.
D	=	Thymuses contain mostly stromal cells (e.g. epithelial cells). However, some scattered lymphocytes are still seen in the medulla. Cortex is still a distinct zone.
> <	=	Thymuses reduced in size, although this reduction is never as dramatic as with *in vitro*-irradiated thymuses (see Chapter 2).

Table 3.1

Thymus histology (7 μm sections) 5-12 days post 1,000 or 3,000 rads in vivo irradiation

Dose of *in vivo* irradiation	Days post-irradiation when thymuses removed	(No. of Thymuses)	Thymic appearance (see Key)
Controls (no irradiation)	-	(4)	A
1000R	5	(3)	> C <
	8	(3)	> C <
	12	(3)	> B/C <
3000R	5	(3)	> C <
	7/8	(6)	> C <
	10	(3)	> D <
	12	(3)	> D <
	14	(3)	> D <

Animals = J

Age = 5-9 months

Figure Legends (Figures 3.1 - 3.6)

Figures 3.1 to 3.6 are representative 7 μm wax sections of thymus or spleen taken from 4-9 month old J strain X. laevis, at various times following total body γ -irradiation. Sections were stained with haematoxylin and eosin (Figures 3.1 - 3.4) or triple stain (Figures 3.5 and 3.6).

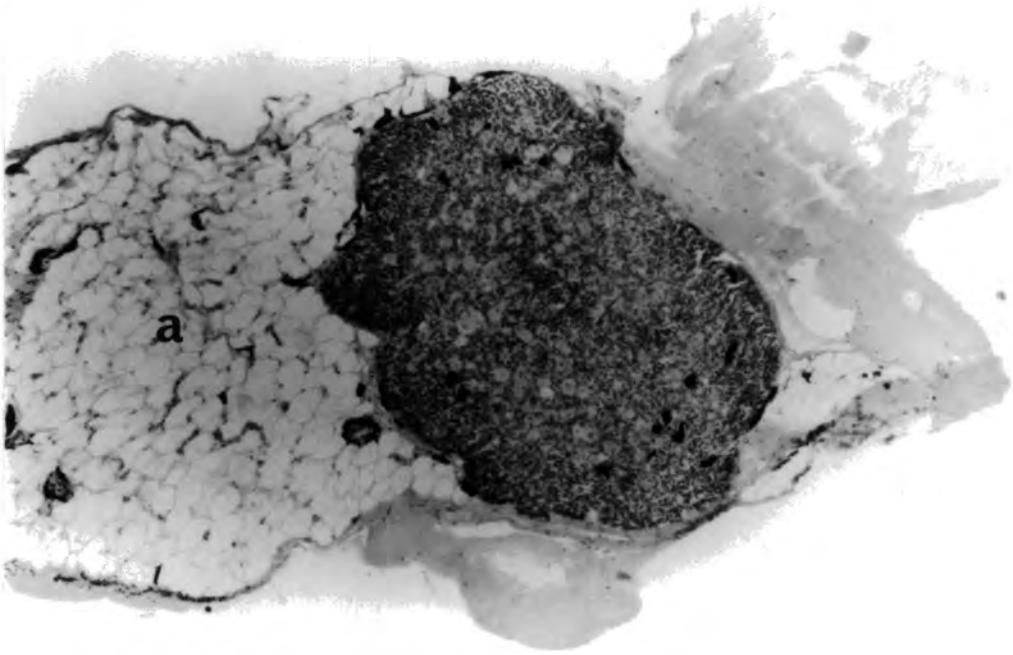
Figure 3.1

1,000 rad-irradiated thymus removed 12 days post-irradiation. Notice that lymphocytes are found scattered in the medulla and are also particularly evident in the cortex. a = adipose tissue. Thymus category > B <. (x 80)

Figure 3.2

3,000 rad-treated thymus, removed 5 days post-irradiation. Shows the cortex (c) severely depleted or lymphocytes. Contrast the lymphoid rich medulla (m). Thymus category > C <. (x 200)

3.1



3.2

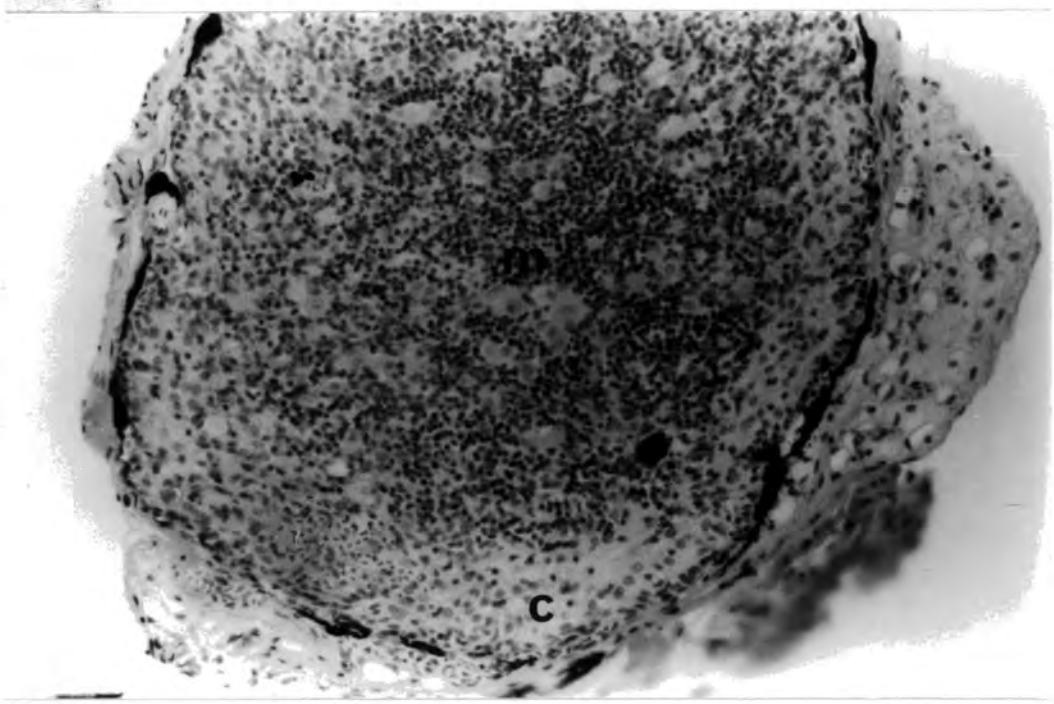


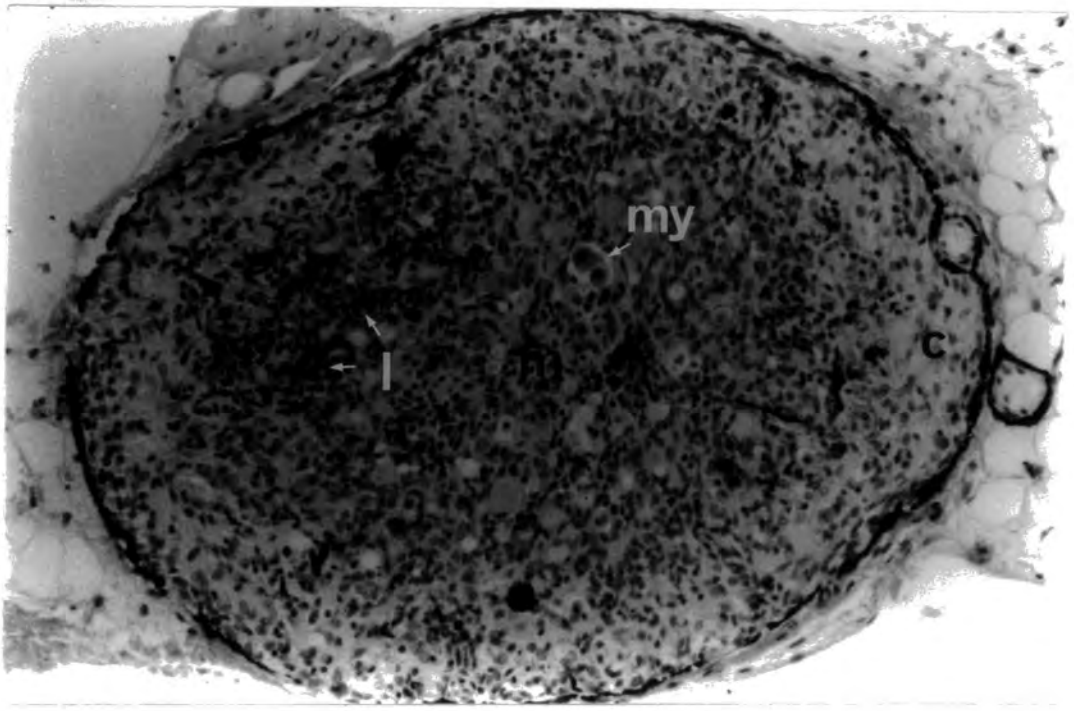
Figure 3.3

3,000 rad-treated thymus, removed 7 days post-irradiation. Cortex (c) contains very few lymphocytes and is mainly epithelial. Medulla (m) still retains a reduced lymphocyte (l) population. (my) myoid cells. Thymus category > C <. (x 200)

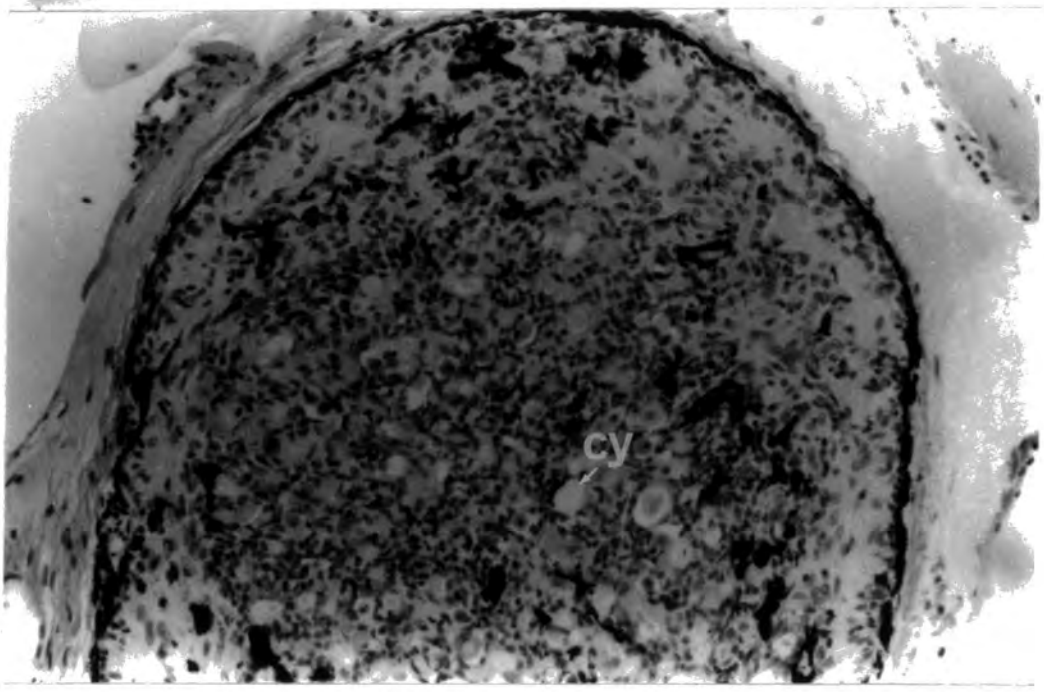
Figure 3.4

3,000 rad-treated thymus, removed 10 days post-irradiation. Thymus now consists mostly of stromal elements. The cortex is rather reduced and virtually devoid of lymphocytes. (cy) cyst. Thymus category > D <. (x 200).

3.3



3.4

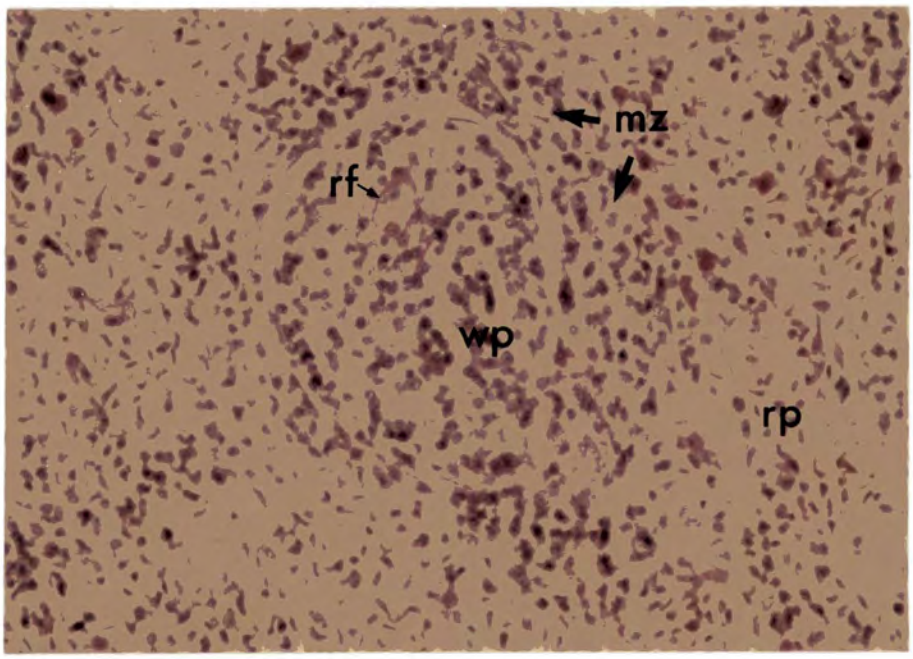


Figures 3.5 and 3.6. 7 μm spleen sections stained with "triple stain"

Figure 3.5 is a control spleen showing typical white pulp (wp) region, rich in lymphocytes, that surrounds a central artery (the latter associated with PAS-stained reticulin fibres - rf). Note also, the marginal zone (mz) with many (purple-stained) lymphocytes and red pulp (rp), which is rich in erythrocytes and lymphocytes. Some PAS positive cells are seen in the red pulp. (x 350)

Figure 3.6 is a spleen removed from a 3,000 rad-treated animal, 8 days after irradiation, showing extensive lymphocyte-depletion. White pulp areas (wp) have shrivelled, leaving the reticulin fibres (rf) of the vascular component evident. Erythrocytes (e), mesenchymal cells (m) and some PAS positive cells are seen in the red pulp. (x 350)

3.5



3.6

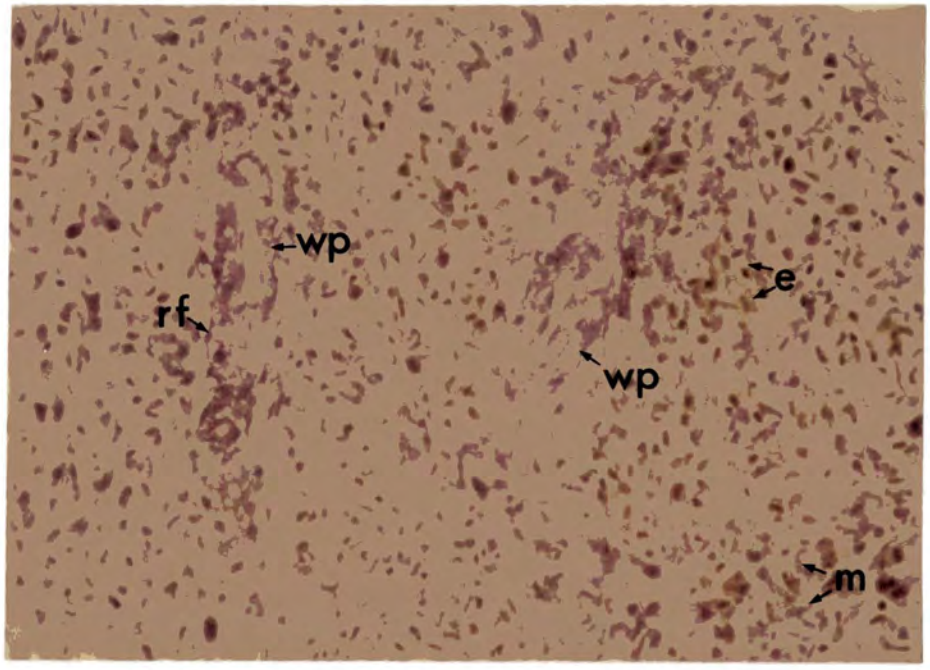


Figure 3.7

3,000 rad-irradiated thymus removed 8 days post-irradiation and subsequently cultured for 12 days *in vitro*. Thymus has an abnormal structure and there is no evidence of thymocyte regeneration. It is comprised mostly of stromal cells although some scattered lymphocytes (l) are seen. (ep) epithelial cells. (m) melanin clumps. Arrow points to artifact. (x 200)

3.7

Figure Legends (Figures 3.8 - 3.13)

Toluidine blue stained, 1 μm plastic embedded sections of control and *in vivo* (3,000 rad) irradiated thymuses (9 days post-irradiation) from J strain *X. laevis* (9 months old).

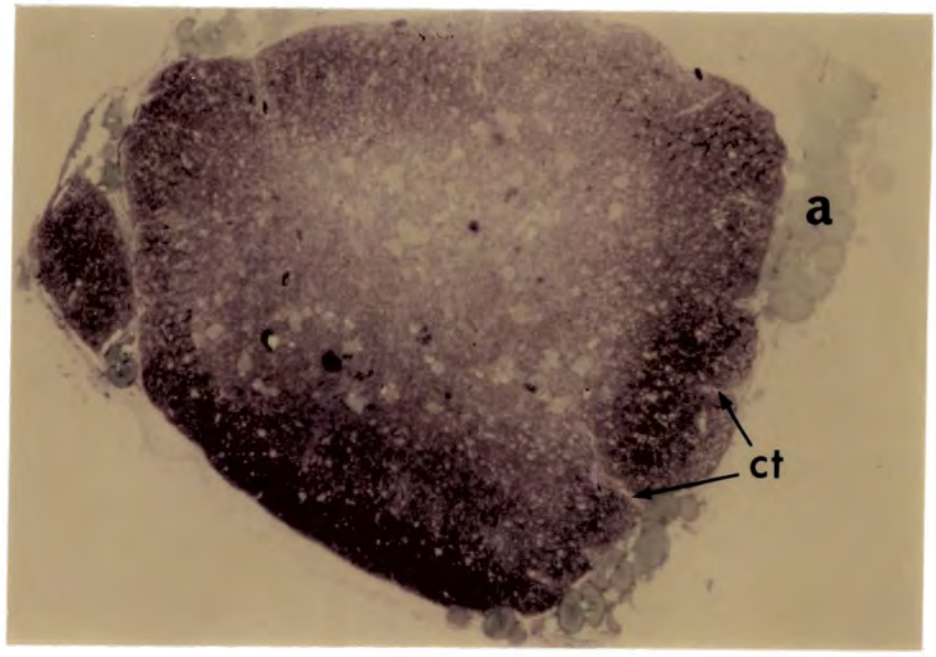
Figure 3.8

Control thymus. Extensive, and extremely lymphoid, cortex. Paler-staining, less lymphoid, medulla. Connective tissue trabeculae (ct) penetrate the cortex. (a) adipose tissue. (x 70)

Figure 3.9

Irradiated thymus. Note dramatic loss of lymphocytes from cortex (c). Some lymphocytes (l) retained in medulla (m). Thymus reduced in size, but cortex/medulla differentiation is still evident. (a) adipose tissue. (x 175)

3.8



3.9

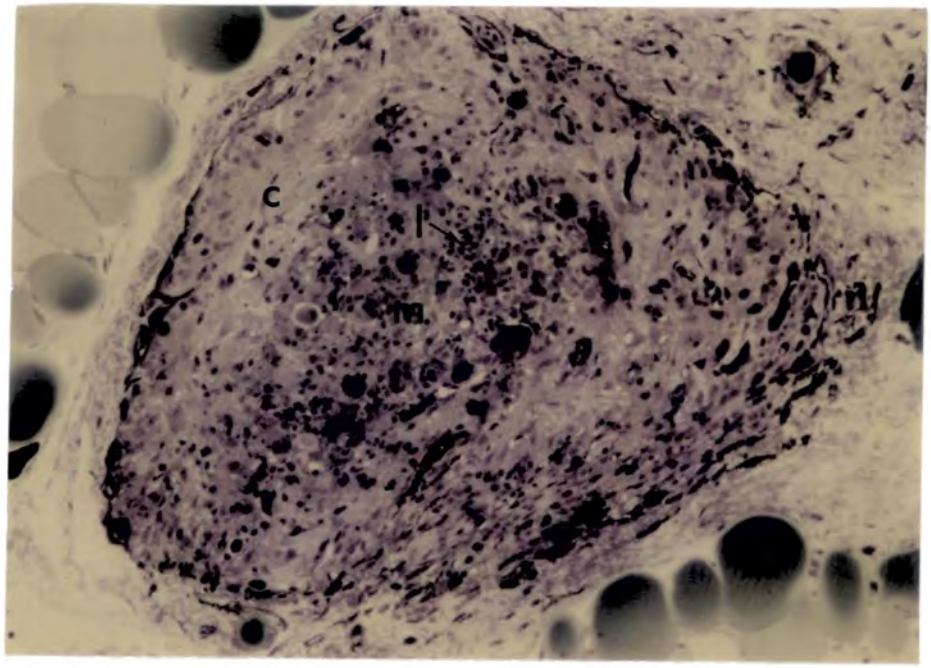


Figure 3.10

A montage through a segment of cortex and part of medulla of a control thymus. (x 700)

(bc) blood capillaries

(c) capsule

(e) erythrocytes

(ep) epithelial cells

(gr) granulocyte

(i) "interdigitating cells"

(l) lymphocytes

(lb) lymphoblasts

(my) myoid cell

3.10

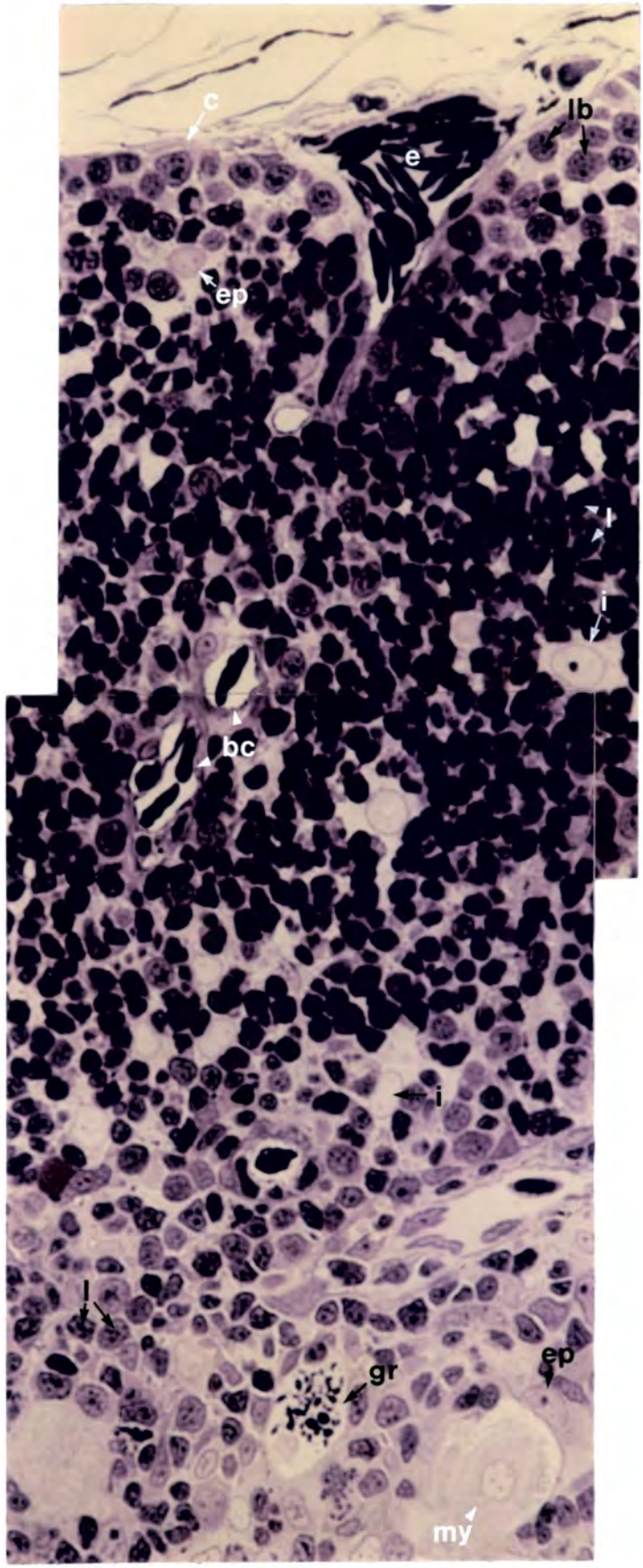

c
e
lb
ep
l
i
bc
i
l
gr
ep
my
sub-capsular cortex
deep cortex
cortico-medullary junction
medulla

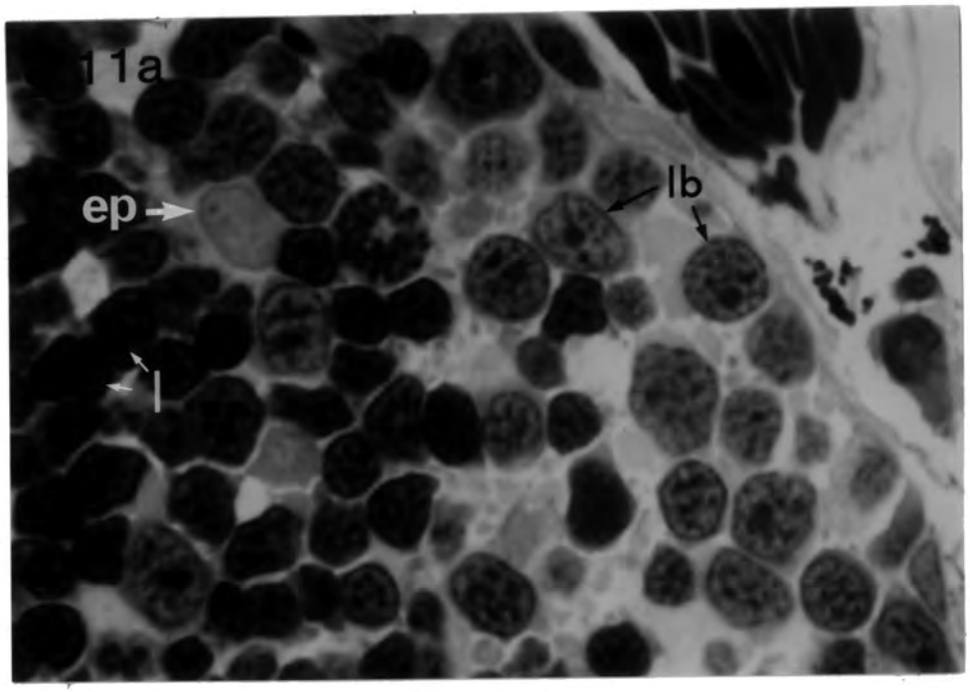
Figure 3.11. High magnification views through regions of a control thymus

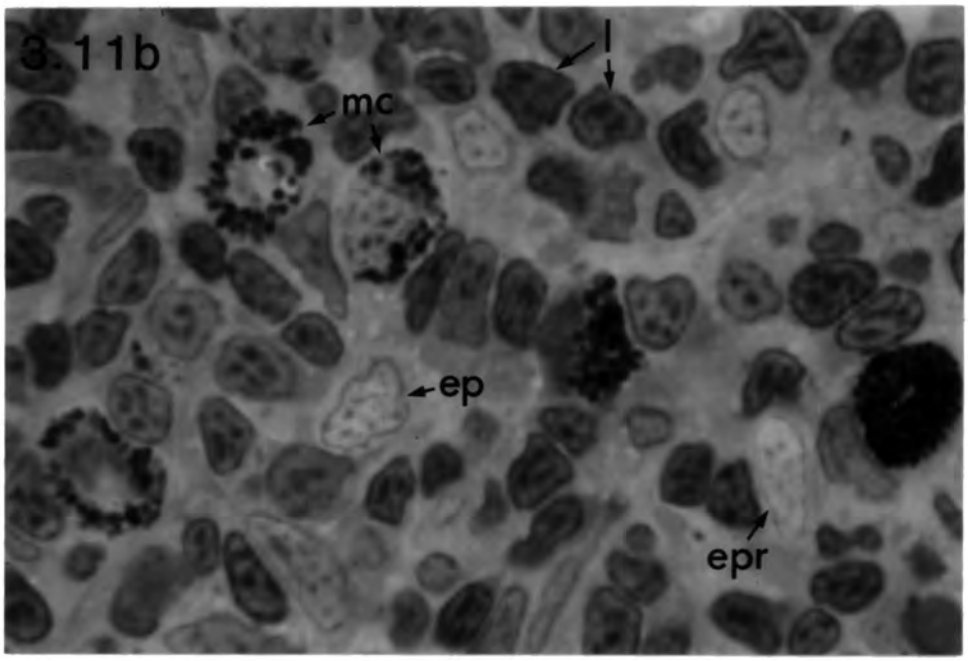
(x 1750)

a) Lymphoblasts (lb), some in mitosis (arrowed) in sub-capsular cortex. (ep) epithelial cell, (l) lymphocytes.

b) Mast cells (mc) - tissue basophils - in medulla. (l) lymphocytes, (ep) epithelial cell, (epr) epithelial reticular cell.

c) A large granular cell (lgc), containing ellipsoid granules is seen in the medulla. A mast cell (mc) and other unidentified granular cells are also seen.


11a
ep
lb
l


3.11b
l
mc
ep
epr

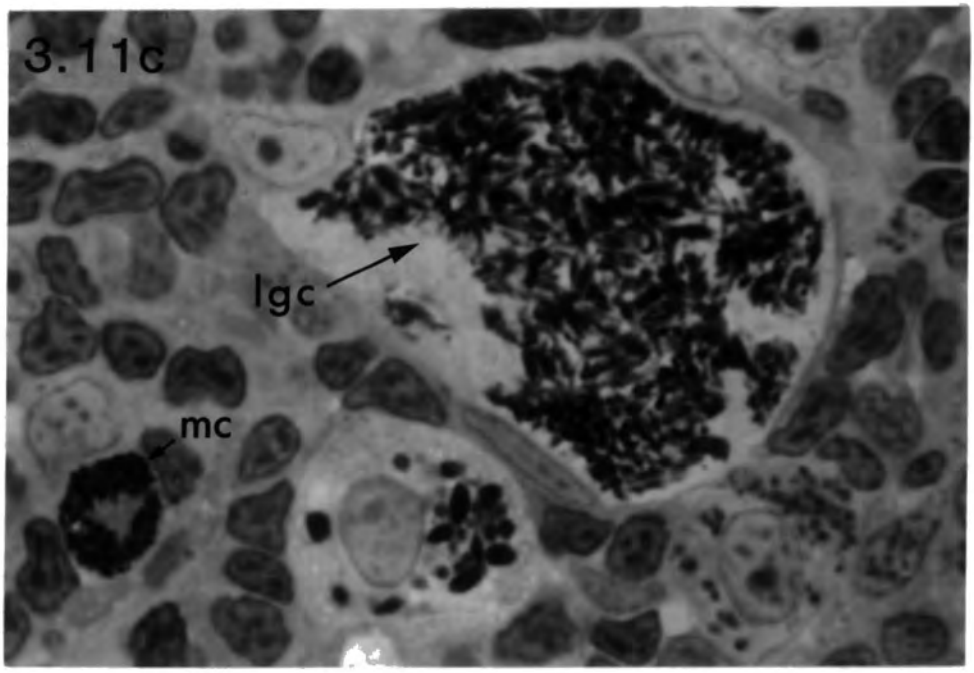

3.11c
lgc
mc

Figure 3.12

A montage through a segment of the cortex and medulla of an irradiated thymus (x 700). Enlarged views of this thymus are shown in Figures 3.13a and b.

(ep) epithelial cells

(epr) epithelial reticular cells

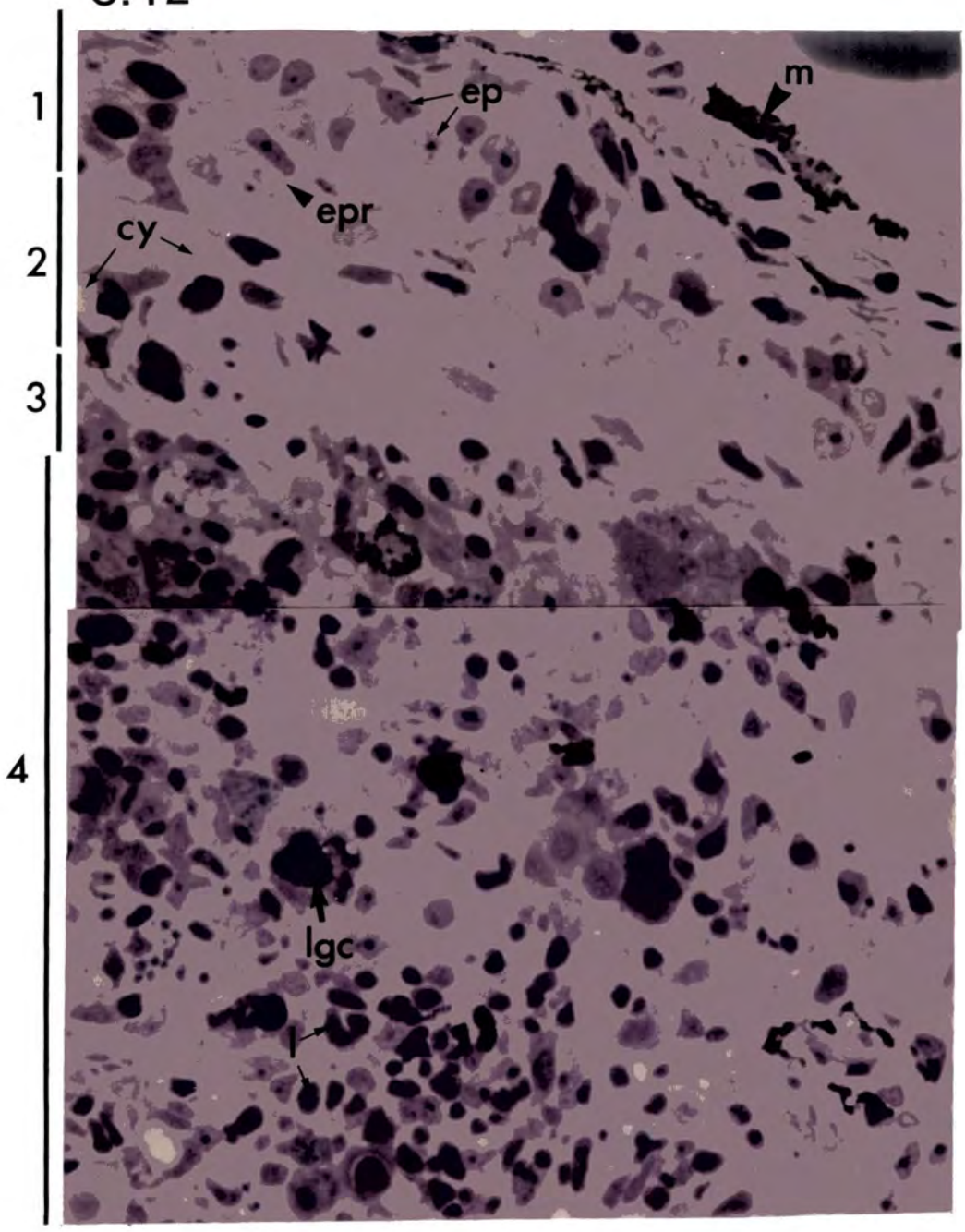
(l) lymphocytes

(m) melanin

(cy) cystic spaces in deep cortex region

(lgc) large granular cell

3.12



1 sub-capsular cortex

2 deep cortex

3 cortico-medullary junction

4 medulla

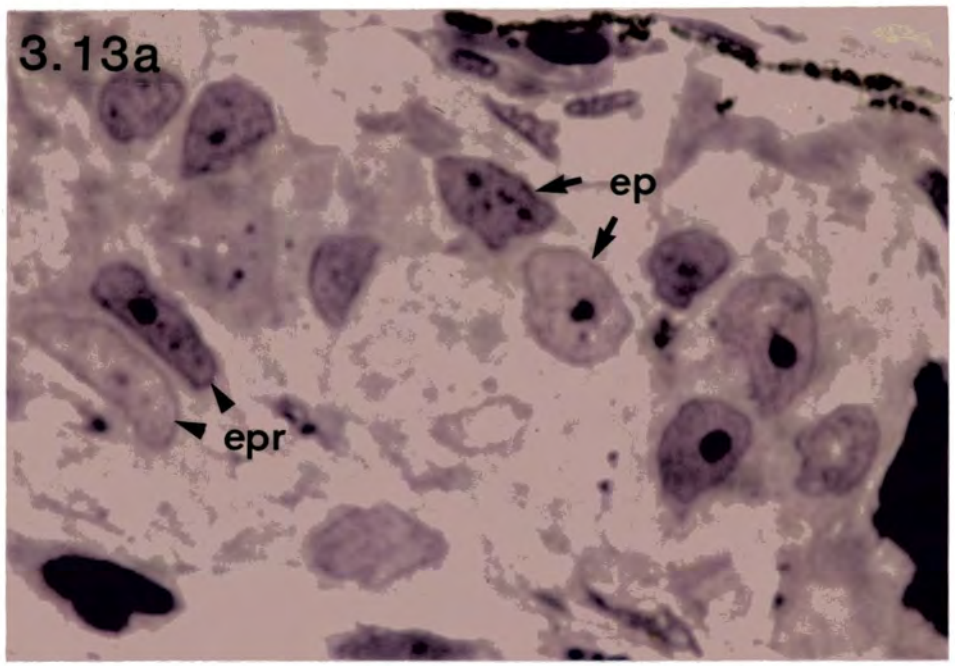
Figure 3.13

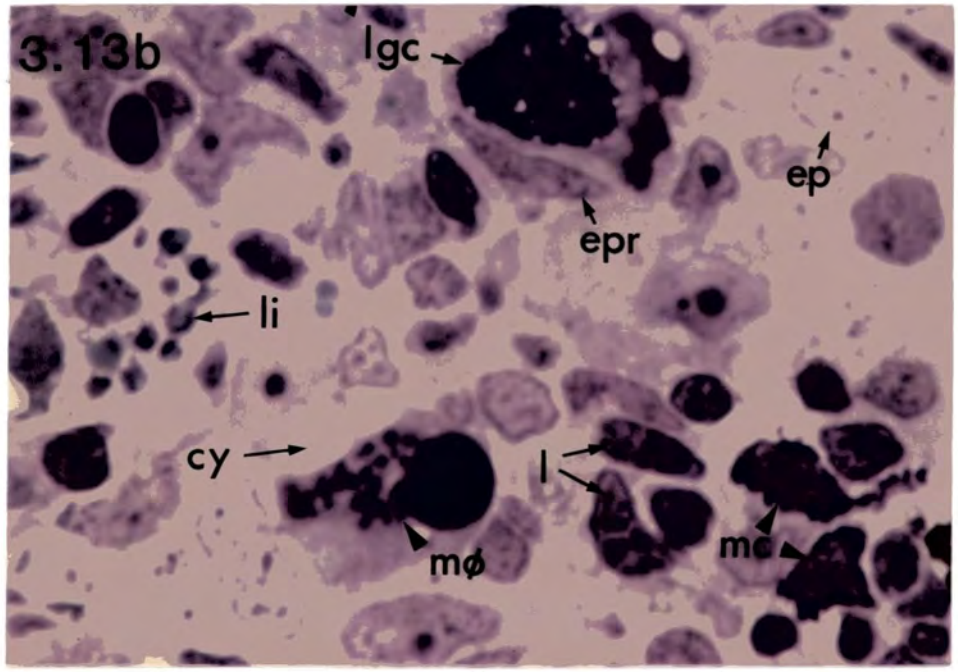
High magnification views through 3,000 rad irradiated thymus. (x 1750).

a) Subcapsular cortex, showing epithelial cells (ep) and epithelial reticular cells (epr). The deep cortex begins at the bottom of the photograph.

b) Medullary region, showing epithelial cells (ep), epithelial reticular cells (epr), lymphocytes (l), a large granulocyte (lgc), mast cells (mc), a macrophage (mø), and lipid inclusions (li). Cystic spaces (cy) are frequent.

c) Cortical region of part of the thymus shown in Figure 3.9 shows many epithelial reticular cells (epr) and macrophages (mø), with ingested material.


3.13a
ep
epr


3.13b
lgc
ep
epr
li
cy
l
mø
mø

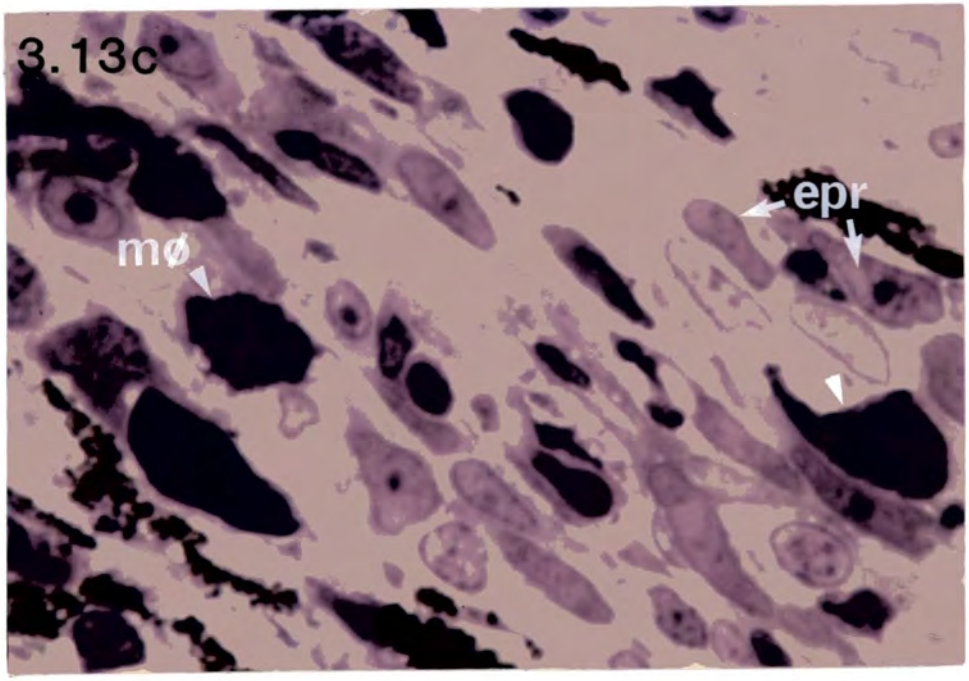

3.13c
epr
mø

Figure Legends (Figures 3.14 - 3.17)

Figures 3.14 - 3.17 are electron micrographs of thymuses from normal, 9 month, J strain.

Figure 3.14

Subcapsular cortex, revealing many lymphoblasts, two of which are in mitosis (M). (C) capsule, (P) pigment. (x 7200)

3.14

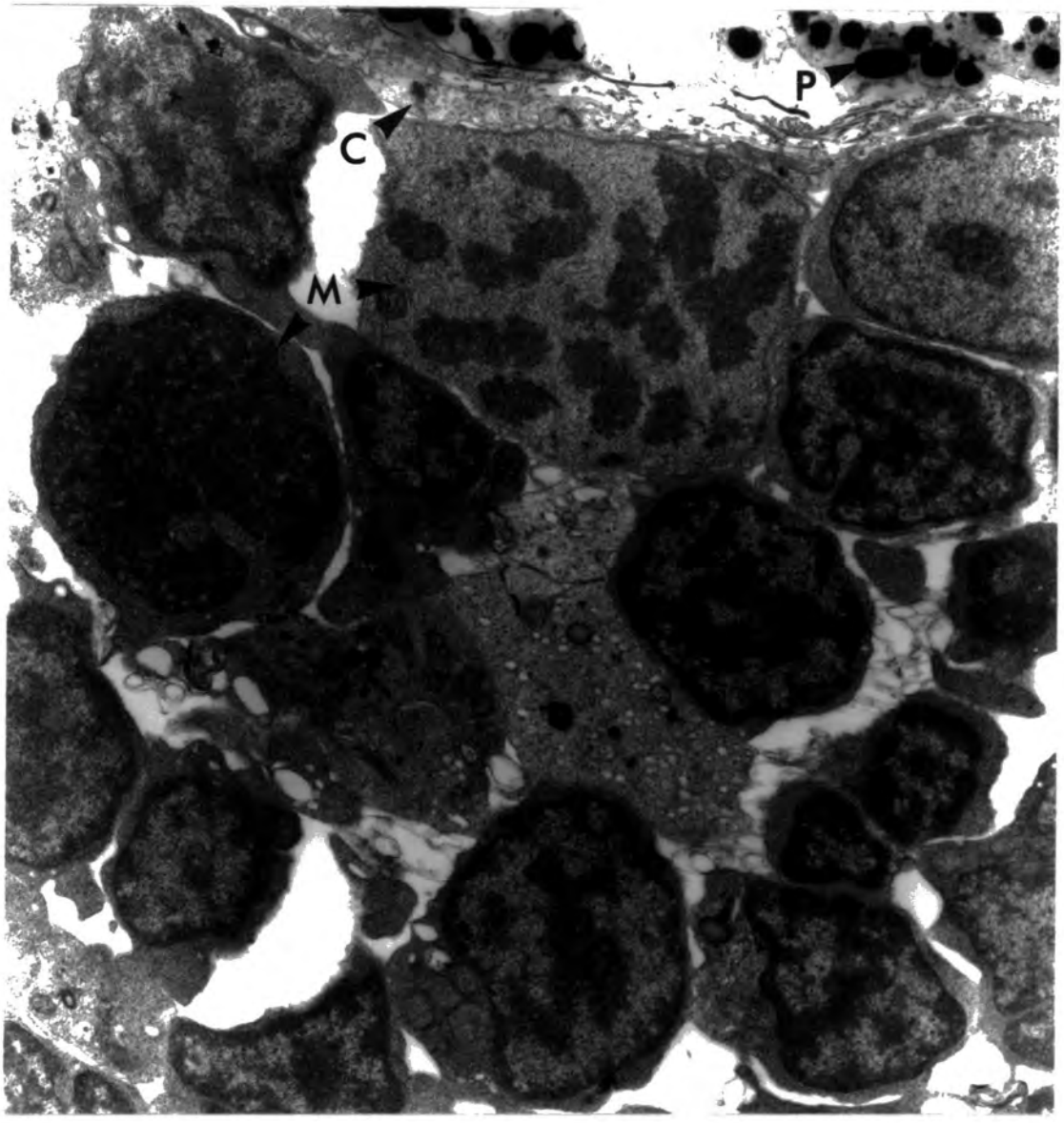


Figure 3.15

Deep cortex - showing small lymphocytes, and pale-staining cell - probably an epithelial cell. (x 5600)

3.15

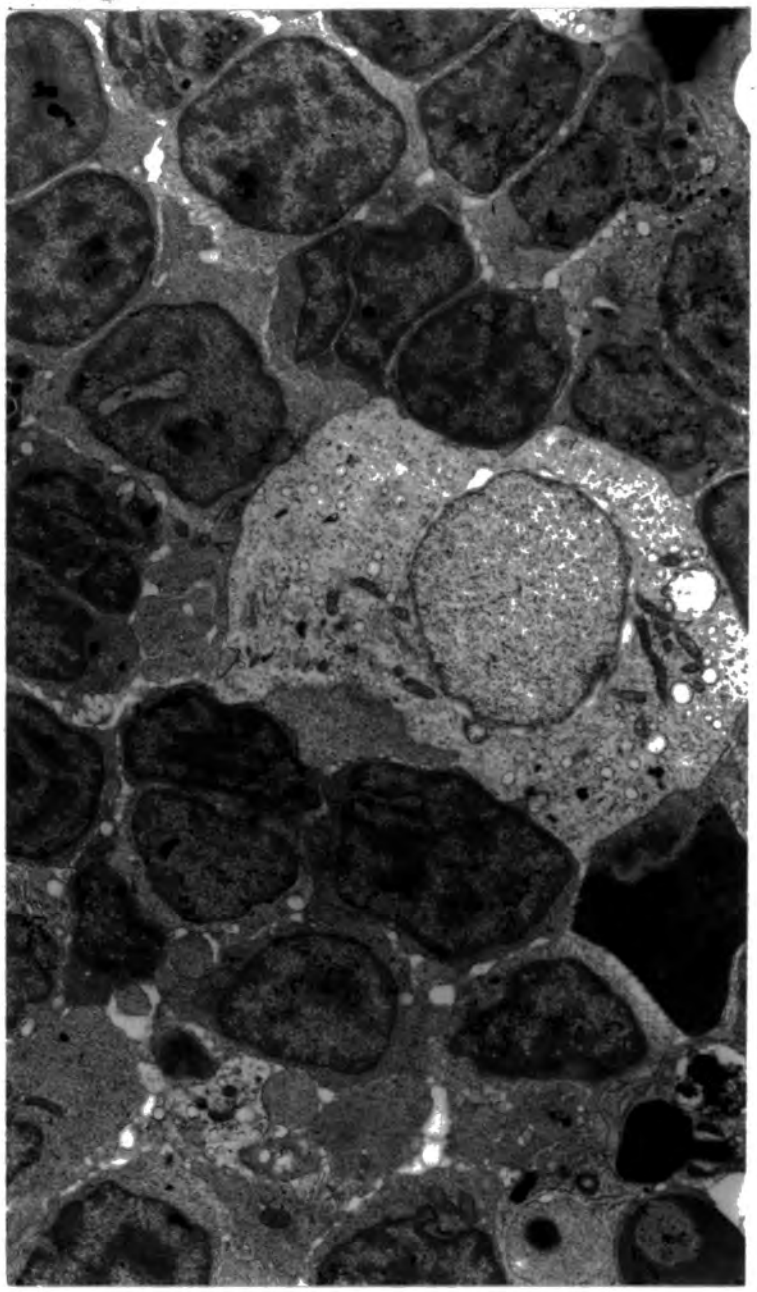


Figure 3.16

Medullary region showing lymphocytes (L) interspersed between cytoplasmic extensions of paler-staining cells - the latter being either epithelial cells or interdigitating cells. (I) putative interdigitating cell with extensive cytoplasmic processes. (x 5600)

3.16

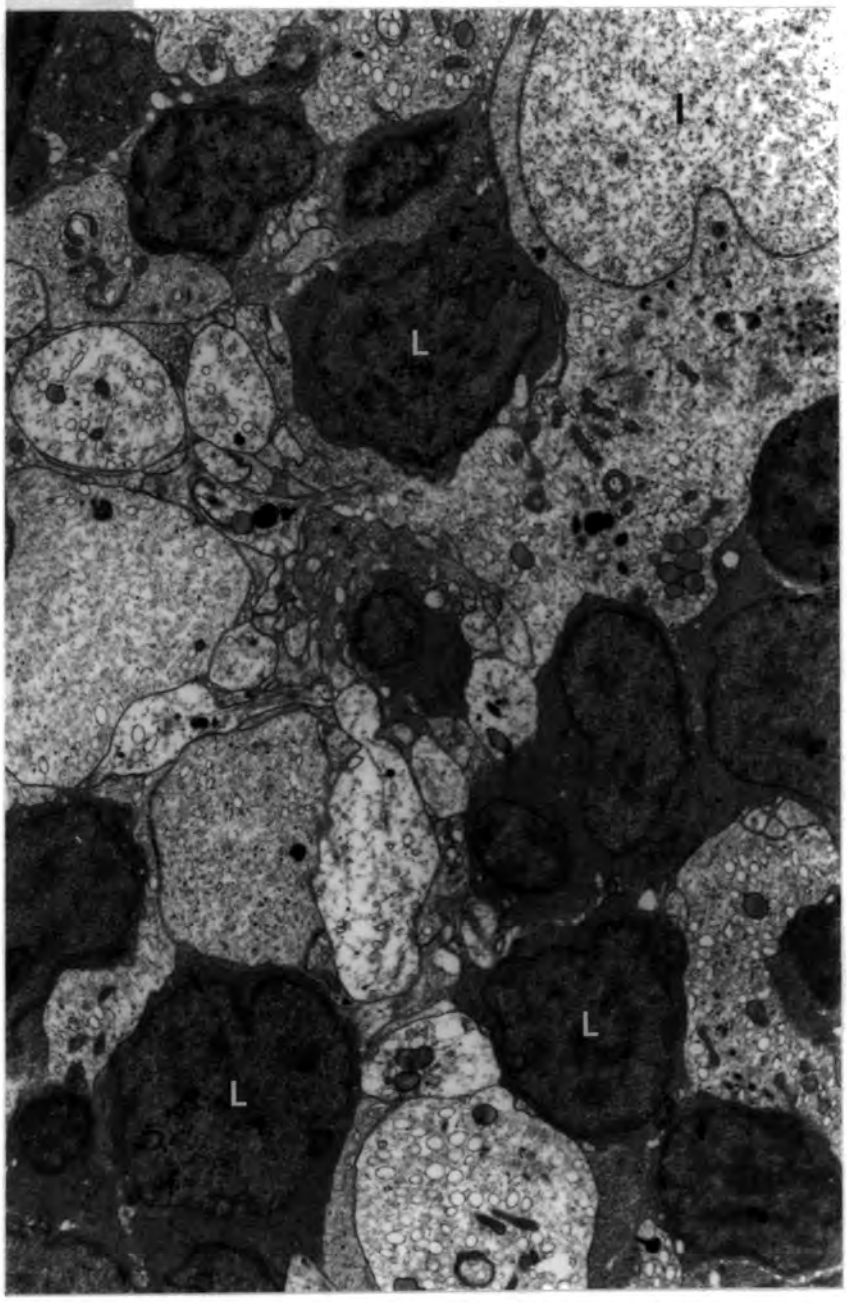


Figure 3.17

Medullary region, showing large myoid cell (MY), with striated myofibrils. Surrounding the myoid cell are epithelial reticular cells (EPR) and lymphocytes (L). (x 5600)

3.17

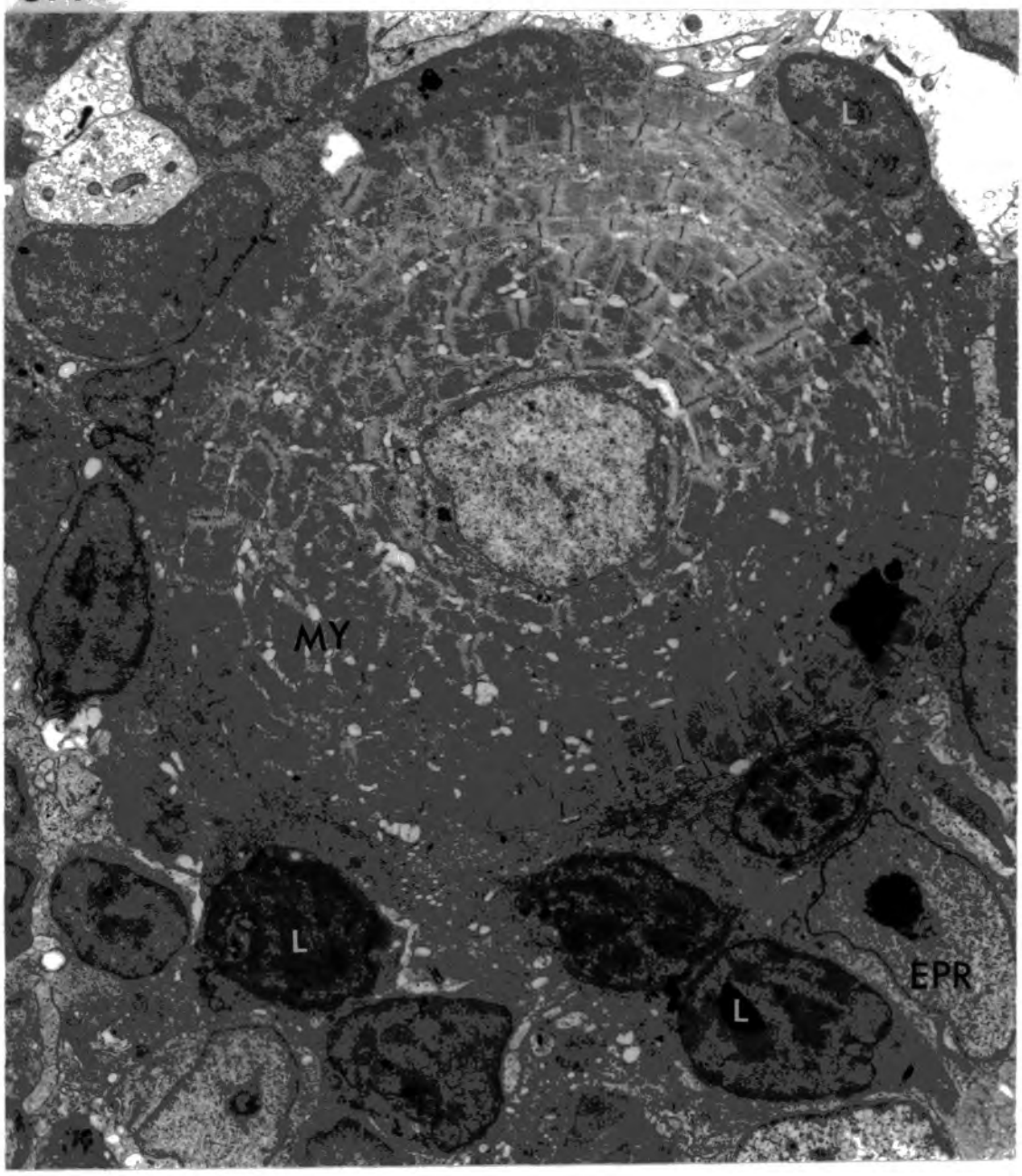


Figure Legends (Figures 3.18 - 3.22)

Figures 3.18 - 3.22 are electron micrographs of thymus taken from toadlets 9 days post 3,000 rads irradiation.

Figure 3.18

The cortical region shows epithelial cells (E) and an epithelial reticular cell (EPR) with extensive cytoplasmic processes.
N.B. Intercellular spaces are evident. (x 7200)

3.18

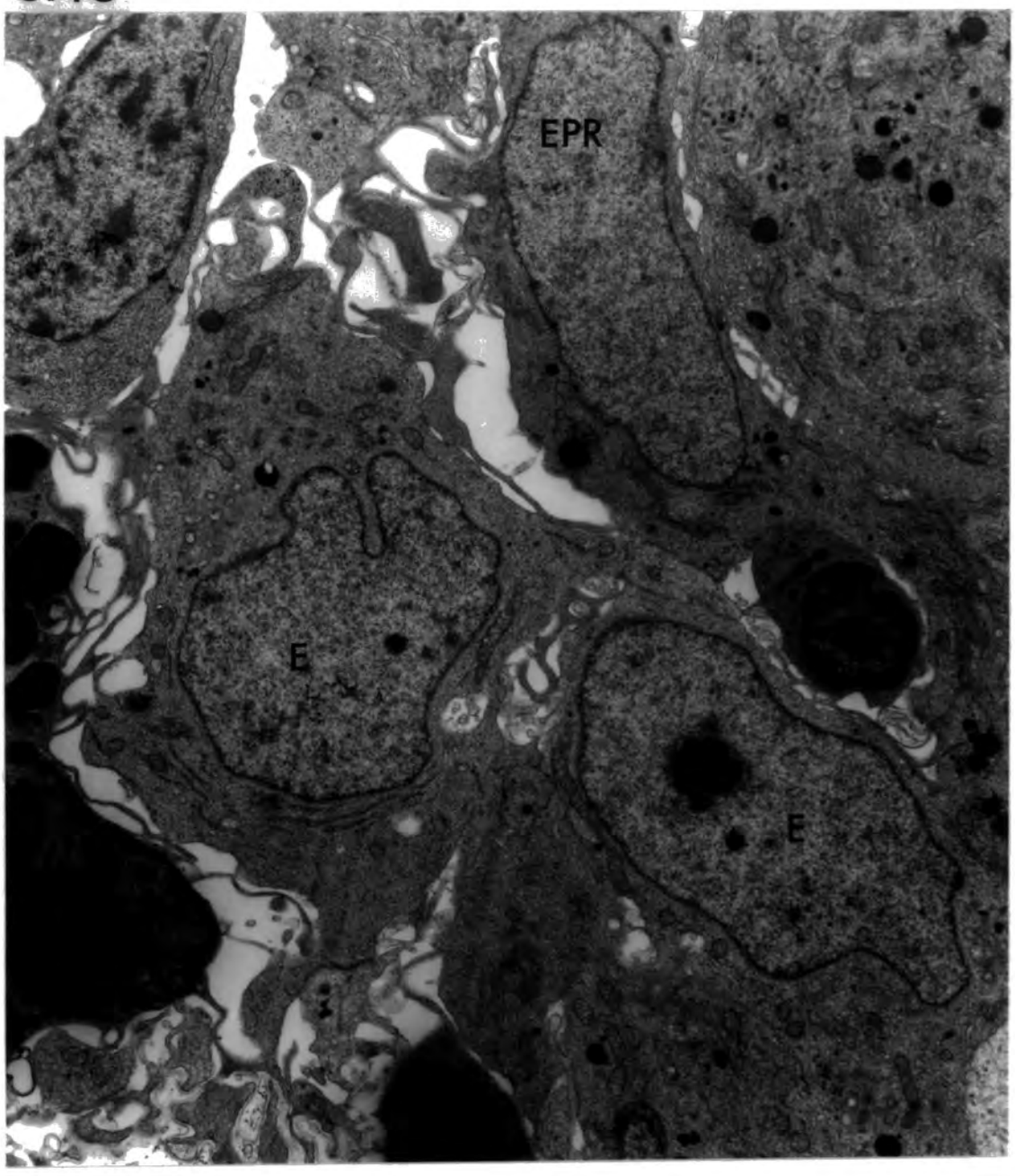


Figure 3.19

A macrophage in the subcapsular cortex. The cell contains many lysosomes and has long pseudopodial extensions. (x 9200)

3.19

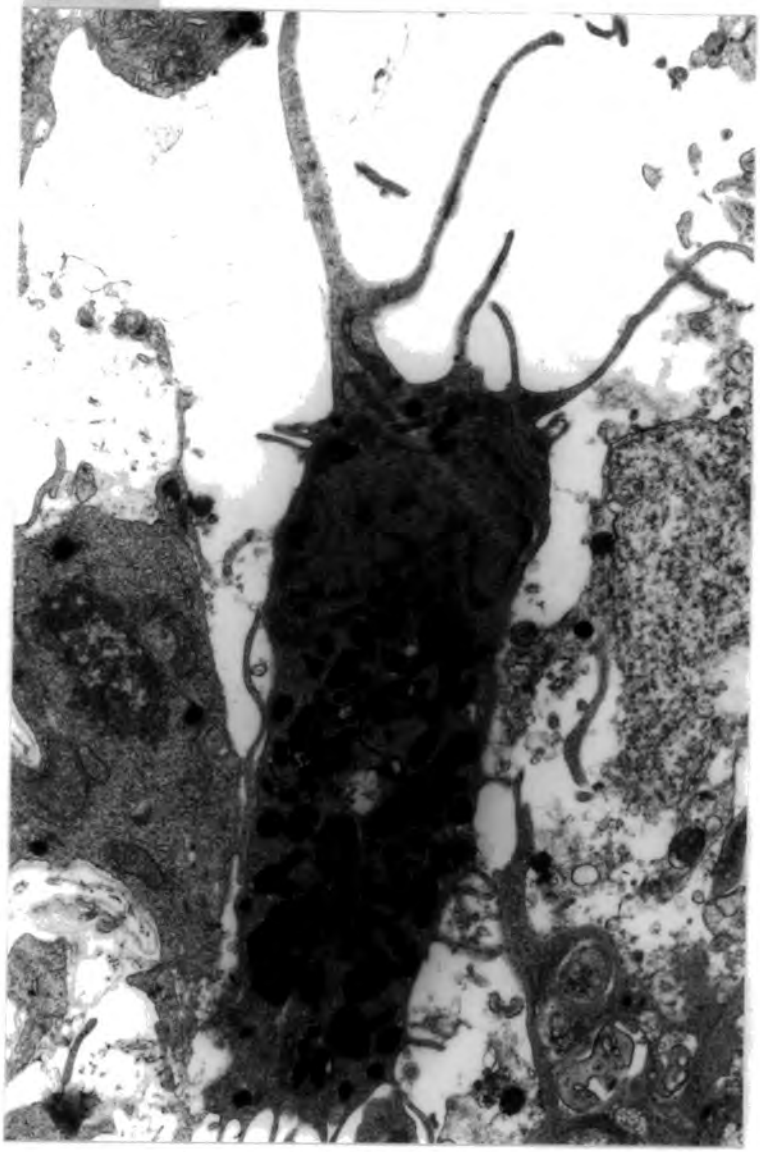


Figure 3.20a

Two cysts (Cy) in the medulla. EP = epithelial cell with an abundance of rough endoplasmic reticulum (Er). Cellular debris is seen within the cyst. (x 7200)

3.20a

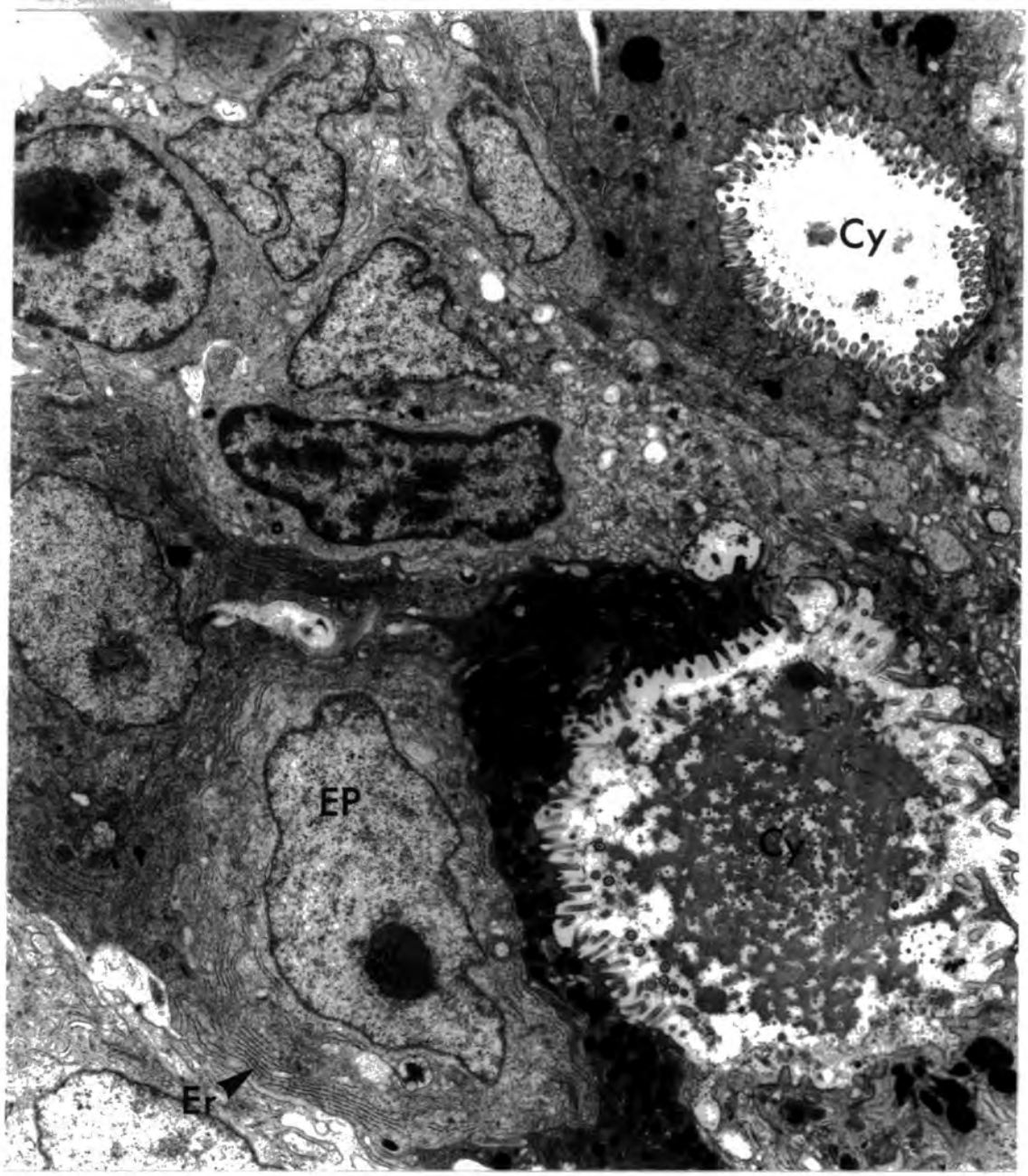


Figure 3.20b

Shows more clearly the cilia projecting into the cyst interior. The cell is rich in granular material. A distinct cell boundary between the ciliated cell and adjacent epithelial cell can be seen (arrow) in places. (x 34,000)

3.20b

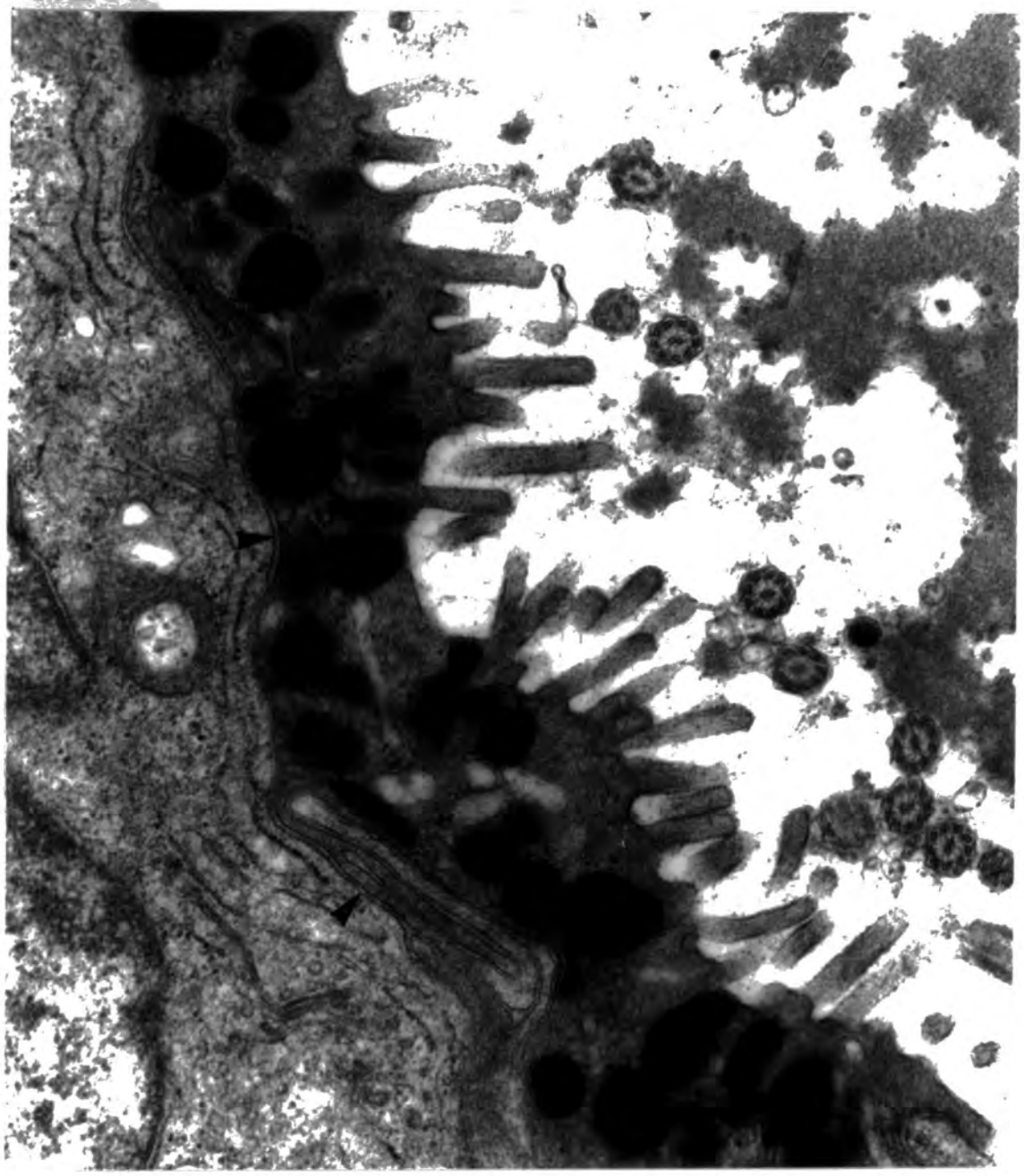


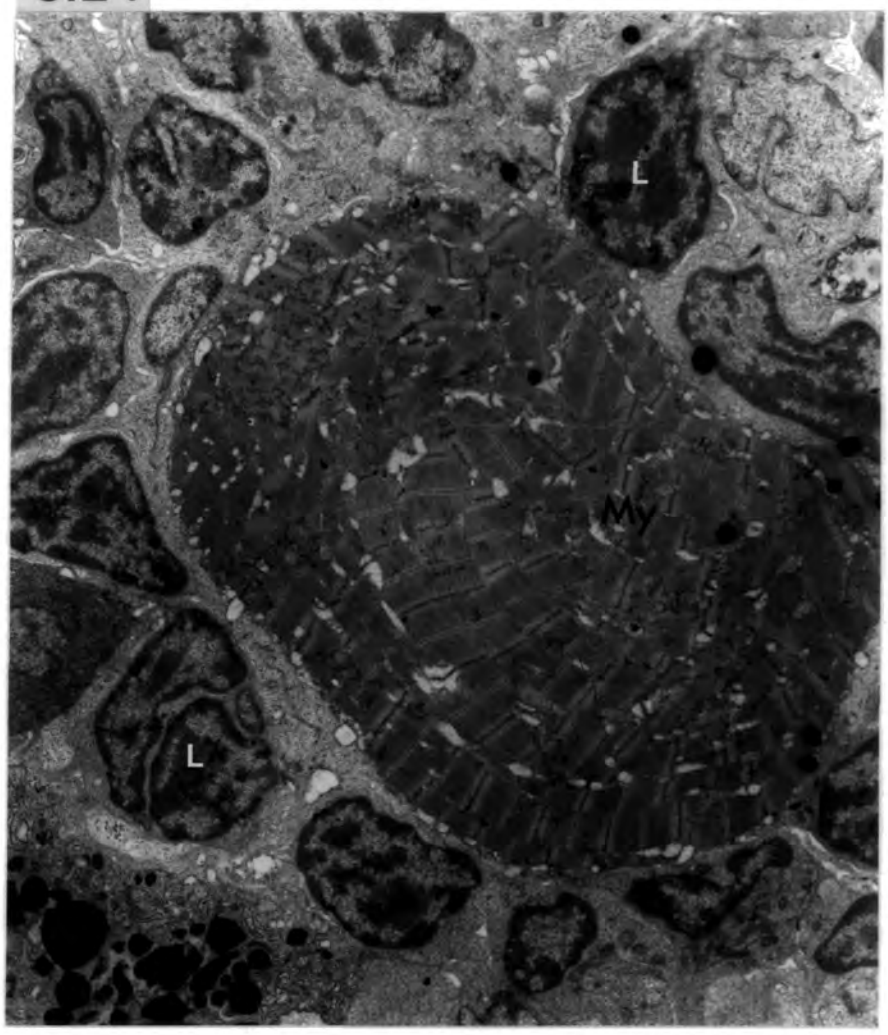
Figure 3.21

A myoid cell (My) in the medulla; some lymphocytes (L) still remain. (x 5600)

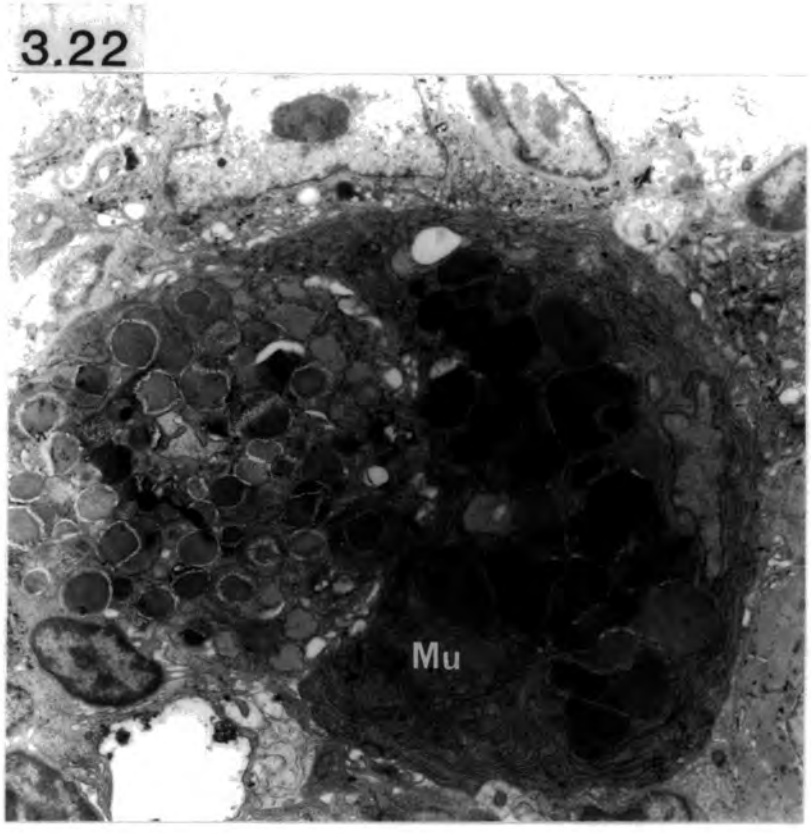
Figure 3.22

Medulla, showing a mucous cell (Mu). (x 5600)

3.21



3.22



CHAPTER 4

REPOPULATION OF NORMAL AND LYMPHOCYTE-DEPLETED THYMUS IMPLANTS WITH HOST-DERIVED CELLS: INITIAL EXPERIMENTS USING PLOIDY-LABELLING

4.1 INTRODUCTION

Previous work in this laboratory (Gearing et al, 1984) had examined the extent to which incompatible larvel thymus (irradiated or non-irradiated) could restore a T-cell dependent antibody response of TX Xenopus laevis. In those experiments a few larval implants (both normal and in vitro irradiated) were examined histologically at 4 and 30 days after transplantation to 4-6 week old MHC-incompatible larvae. Control implants were found to develop normally, and use of a triploid/diploid marker system revealed repopulation, by lymphocytes of the TX host, had occurred extensively by 6-8 months of age. 1000 rad and 5000 rad in vitro irradiated thymus implants were initially predominantly "epithelial" but became lymphoid within 30 days. However, irradiated implants remained much smaller than controls and usually disappeared after the hosts metamorphosed. The origin of lymphocytes developing within the irradiated thymus implants was not assessed. This is an important issue to resolve, since one major reason for the work being carried out in this Thesis is to provide a system for exploring the alloimmune capabilities of host lymphocytes that have developed within a foreign ("epithelial") environment. In mammals, lymphocyte-depleted thymuses are infiltrated by host cells following transfer across MHC barriers (Robinson and Jordan, 1983; Crouse et al., 1984; Jordan et al., 1985), although the precise role of the thymic epithelium in inducing allotolerance of developing T cells is still under discussion (see recent papers by Ready et al., 1984; Jordan et al., 1985b; also discussion in Chapter 7).

In this Chapter, the extent to which larval and adult (normal or irradiated) Xenopus thymuses are infiltrated by host cells 7 weeks following implantation to MHC-matched larval hosts (control or TX) is briefly explored. (The morphology of thymus implants at 7 and 16 weeks is also noted). Thymuses were irradiated by the in vivo technique described in the last Chapter, which provides a thymus extremely depleted of lymphocytes, but with its stroma left relatively intact, with few necrotic cells.

In order to begin to explore whether in vitro recolonization of the lymphocyte-depleted amphibian thymus can be achieved, as it can in mammals (Jenkinson et al., 1982), a pilot study employing transfilter or coculture of irradiated froglet thymus with other, MHC-compatible, lymphoid tissues, is also carried out here. Previous studies in the chick (Jotereau et al., 1980) and mouse (Fontaine-Perus et al., 1981) have shown that thymus anlage (prior to colonization by stem cells) explants can become lymphoid by in vitro transfilter culture with a suitable source of precursors, thus implying that the "empty" thymus is attractive to these cells (see Le Douarin, 1978). Furthermore, Jenkinson et al. (1982) have achieved in vitro transfilter recolonization of lymphoid-depleted (by deoxyguanosine treatment) 14 day foetal mouse thymus, with allogeneic lymphoid precursor cells from foetal liver or 13-day thymus. Similarly, Pyke et al. (1983) have demonstrated in vitro repopulation of 14-day murine foetal thymus previously depleted of lymphocytes by organ culture at low temperature with high oxygen. Their system involved in vitro coculture of thymuses with allogeneic foetal liver. Coculture of the 14-day foetal mouse thymus with allogeneic spleen/thymus has been used to explore the development of allotolerance by developing thymocytes (Robinson and Owen, 1978; De Luca et al., 1980). The above experiments are particularly exciting, since they should provide in vitro approaches to understanding the nature of the thymic

cells involved in tolerance acquisition and MHC-restriction by developing T cells.

4.2 MATERIALS AND METHODS

4.2.1 Animals

The strains of Xenopus used in this Chapter were diploid and triploid J strain Xenopus laevis and triploid JLG5 Xenopus. Triploid J Xenopus were obtained by placing in vitro-fertilized eggs at low temperature (3°C) for 15 mins, 12-13 mins post-fertilization. This suppresses extrusion of the second polar body (Kawahara, 1978) and hence a double complement of maternal genes are incorporated in the zygote. Triploid JLG5 eggs were procured by in vitro fertilization of diploid LG5 eggs with J sperm. To check the ploidy of individuals, smears of small pieces of tail tip epithelium, or blood smears, were stained with silver nitrate (see below).

4.2.2 In vivo repopulation of normal and lymphocyte-depleted thymus

a) Surgery

Thymus glands removed from larvae (4 weeks) or adult (6 mths) donors (either normal froglets or froglets kept for 10 days post-3000 rads) were cleared of excess connective tissue and fat. A single thymus was then implanted into lightly-anaesthetized larval (stage 56/7) hosts by insertion under the skin just beside the eye. Hosts were either thymectomized larvae, whose thymuses has been removed by microcautery at 7 days of age, or were control animals. Implanted larvae were placed for 24 hours in diluted (and aerated) Ringer's solution (1 part Amphibian strength Ringers:2 parts distilled water). Aerated standing water was then gradually added over the next 24-48 hours until the animals could be returned to water, and reared in the normal way (described in Chapter 2). Host and donor combinations used are considered in the Results section.

b) Histology of implants

After 7 weeks or 4 months, some of the thymus grafts were examined histologically by fixing in Bouin's, embedding in paraffin wax and staining 7 µm sections with H and E. Other thymus implants taken at 7 weeks were checked for their ploidy by silver-staining of thymocyte smears (cytospin preparations).

c) Silver staining

Thymuses or thymus implants were first teased apart in amphibian L15 medium and single-cell suspensions made by repeated pipetting to dissociate clumps. Cells, mainly lymphocytes, were then washed, counted and adjusted to 2.0×10^6 thymocytes/ml. 50 µl aliquots (1×10^5 cells) were then mixed with an equal quantity of FCS in order to protect the cells during the subsequent centrifugation step. 100 µl samples were transferred to a cytocentrifuge (Shandon) and centrifuged for 5 min at 600 rpm. Thymocyte smears were then air dried for 30 mins and fixed in methanol for 10 mins, prior to silver staining for estimating ploidy by nulceolar number. The stain was 50% $AgNO_3$ (e.g. 0.125 g $AgNO_3$ plus 250 µl double-distilled water) mixed with $\frac{1}{7}$ volume (36 µl) formic acid (pH 2.9) (see Olert, 1979). A few drops of silver stain were pipetted onto the slides, which were left to stain for 15-20 mins, covered with a coverslip. Stained slides were then washed with a weak NaOH solution (0.001 M), left to air dry then cleared in xylene and mounted in DPX. The number of nucleoli per nucleus was counted under oil emersion (x 100) using coded slides. Cells in at least 10 different fields of vision (approximately 200 cells) were counted on each slide. Thymocytes undergoing cell division and erythrocytes were not included. Only when nucleolar counts had been made were the identities of the slides revealed.

Host blood smears were also analysed for ploidy. Fine glass pipettes (drawn from Pasteur pipettes) were used to obtain blood from the heart. Blood smears were air dried, fixed in methanol and silver-stained

as above 10-12 minutes staining was usually sufficient. Ploidies of blood cells were counted as above.

4.2.3 Preliminary in vitro attempts to repopulate lymphocyte-depleted thymuses

Figure 4.1 illustrates two types of culture system set up to attempt lymphoid repopulation of organ-cultured thymuses that had been irradiated in vitro with 2-3000 rads as described in Chapter 2. Immediately following irradiation, or up to 7 days in organ culture after irradiation, thymuses were deliberately ruptured, before placing individually in transfilter or co-culture with a similar sized liver, kidney or spleen fragment (i.e. putative "sources" of stem cells). Both thymuses and lymphoid fragments were J strain, 3-6 months old. The cultures were incubated (see conditions in Chapter 2) for various periods up to 3 weeks and then thymuses were examined histologically for lymphoid repopulation.

4.3 RESULTS

4.3.1 In vivo repopulation of normal and lymphocyte-depleted thymus

It was desirable in these initial implantation experiments to implant thymuses from donors MHC-compatible with the larval hosts, in order to prevent the latter (particularly the controls) effecting allo-destruction of the implanted tissue. Since J and JLG5 animals alone were available for the experiments, it was decided to implant J strain thymuses into either JLG5 or J recipients; this combination meant that hosts could visualize only minor H antigen differences on the cells of the implanted thymus (see Chapter 6 for studies on J versus JLG5 combination). Larvae fail to mount an alloimmune response against minor H disparate skin (Dimarzo and Cohen, 1982).

a) Histological observations

Table 4.1 summarizes the histological outcome of observations on thymus implants at 7 and 16 weeks post-implantation.

Thymuses implanted from J 2N larvae into JLG5 3N hosts (either control or TX) looked healthy when examined at 16 weeks. All 4 thymus implants examined were lymphoid and had relatively normal cortex/medulla differentiation. Lymphocyte numbers seemed rather reduced when compared with normal froglet thymuses.

Thymuses from two 6 month old J 2N froglets implanted into JLG5 3N hosts appeared to be in perfectly normal condition when examined 7 weeks post-implantation, as did thymus implants from 4 J 3N froglets that had resided in J 2N TX hosts for either 7 (Figure 4.2a) or 16 weeks.

Thymus implants taken from 3,000R-irradiated J donors (either 2N or 3N donors) and transplanted to JLG5 3N controls or J2N TX respectively appeared to have become repopulated with lymphocytes at 7 weeks. At this time these thymuses displayed cortex/medulla differentiation, although were slightly reduced in size compared with non-irradiated implants (Figure 4.2b). These implanted irradiated thymuses contrasted with the appearance of 2 thymuses, taken directly for histology, from the same 3,000R-irradiated donors as used to supply the implants. These non-implanted thymuses were extremely depleted of lymphocytes, as described in Chapter 3, and resembled Figure 3.4.

By 16 weeks post-implantation, however, irradiated thymuses were very reduced in size and lymphocyte numbers. Cortex and medulla differentiation was by now lost and melanin deposits were noticeable.

b) Evidence for host-cell immigration into thymus implants, 7 weeks post-implantation

Nucleolar counts on silver-stained thymocyte cytospins (and blood smears) are summarized in Table 4.2. Nucleolar counts can be used to give an overall assessment of movement of cells between 3N and 2N

individuals, although it should be noted that the technique is imprecise on a per cell basis, in view of variable nucleolar expression (Kobel and Du Pasquier, 1975) - i.e. not all 3N cells will express all 3 nucleoli, only a proportion. However, a population containing triploid cells can be distinguished from a diploid cell population, since the latter cells should generally stain with only 2 or 1 nucleoli. The silver staining of control diploid (J) and triploid (JLG5) blood smears is shown in Figure 4.3a and b. These animals were of the same age as the implanted froglets used here.

Thymus implants from 2N J larval donors implanted into control JLG5 3N hosts appeared to have been heavily infiltrated with host cells 7 weeks post-implantation, since more than 50% of thymocytes were recorded with 3 nucleoli, which compared well with nucleolar counts on the host's own thymuses. Infiltration of donor J 2N larval thymus implants by 3N cells also occurs when implanted to 3N JLG5 TX hosts, although nucleolar counts on one thymocyte preparation revealed only 30% of cells with 3 nucleoli.

Host derived (JLG5 3N control) thymocytes were also extensive within thymus implants taken from adult J 2N donors (43 and 48%) - Figure 4.4a. Irradiated thymuses had 60% of their thymocytes recorded as containing 3 nucleoli (Figure 4.4b).

Nucleolar counts on thymocytes of the originally diploid implants and in the triploid host's own thymuses were always within 15% of each other.

Host blood cell (erythrocyte) nucleolar counts are shown to confirm that hosts were all triploid.

4.3.2 Preliminary in vitro attempts to monitor cell migration into lymphocyte-depleted, organ-cultured thymus

Transfilter and coculture experiments set up using some 40 thymuses and associated lymphoid organ fragments were unsuccessful in revealing

any lymphoid repopulation of the irradiated thymuses. The latter always remained greatly reduced in size and usually structurally destroyed. Some visible stromal cells were found, but many pyknotic areas were usually seen.

4.4 DISCUSSION

These preliminary studies, showing the presence of significant numbers of host cells within implanted normal thymuses, confirm the restoration work of others on Xenopus (Gearing et al., 1984; Nagata and Cohen, 1984). Additionally, my experiments show that the 3,000 rad in vivo irradiation treatment of thymuses does not preclude these from being colonized by host lymphoid cells, at least when examined 7 weeks post-implantation. At this time, in vivo irradiated thymuses displayed a normal histology, and were very lymphoid, although slightly reduced in size compared with unirradiated thymus grafts. However at 16 weeks, the irradiated thymuses appeared much less lymphoid than controls and no longer showed cortex/medulla demarcation, due to reduction in lymphocyte numbers. This may represent the onset of degeneration of the irradiated thymus noticed previously by Gearing et al. (1984). However, in vivo irradiated (3,000 rad) thymus grafts certainly survive well beyond metamorphosis, hopefully time sufficient to restore a TX animal's impaired immune system. Long term failure of irradiated thymus implants to survive might suggest that functional viability of stromal cells is not permanently retained.

In this Chapter, it was found that the most reliable estimate of host cell input into the thymus could be judged by using triploid (3N) hosts and diploid (2N) thymus implants, since it was found easier to observe (by silver staining) migration of 3N cells into a 2N thymus than vice versa. Thus diploid animals contain very few cells recorded with 3 nucleoli, whereas triploid animals possess a substantial proportion

of cells in which only 2 nucleoli can be seen (see Table 4.2, Figures 4.3a and b). There is, however, data suggesting that polyploid (tetraploid or triploid) amphibian lymphoid cells may be less active in migrating through diploid tissues compared with diploid lymphocytes (Deparis and Jaylet, 1976; Turpen et al., 1977; Nagata and Cohen, 1984).

The silver-staining method of measuring repopulation of thymus implants by recording nucleolar number of host lymphocytes does not give a precise, quantitative analysis of the extent of recolonization. Other workers, for example Flajnik et al. (1984a), have used flow cytometry to determine the ploidy of 3N/2N lymphocytes. In that technique cells are initially stained with the fluorochrome-mithramycin and then passed through a fluorescence activated cell sorter (FACS) to determine the fluorescence intensity of lymphocytes per single cell. This gives a much more accurate measurement of cell composition in 2N/3N thymus "chimeras" than silver-staining. However, neither of these methods gives an accurate picture of the position of 3N/2N cells within the thymus. Such a picture would give important information on the site of host cell entry and cell movement in the donor thymus and about the nature of any persisting donor cell types. This issue is examined in the next Chapter.

The mechanism of stem cell homing to thymic primordia or lymphoid-depleted thymus <u>in vivo</u> and <u>in vitro</u> has not been elucidated. Le Douarin and Jotereau (1975) have shown in quail-chick transplantation experiments, that thymic stem cells are available in the embryo for some days prior to colonization. Therefore, the onset of stem cell immigration is more likely to be regulated by the thymic primordium itself, as stem cell availability is not a limiting factor. It has been suggested that thymic receptivity might involve a chemotactic mechanism, resulting from the production by the thymic epithelium of a factor (or factors) attractive

for the circulating haemopoietic cells (Le Douarin, 1978; Le Douarin et al., 1984). It is thought that the lymphoid cell content within the thymus might also be involved in regulating the entry of stem cells. For example, if their numbers drop below a certain critical level, the thymus may release a homing signal for more lymphoid precursor cells to rejuvenate the lymphoid population.

Transfilter - and co-culture of "epithelial" froglet thymuses with putative stem cell sources was attempted in this Chapter, but these experiments showed no sign of thymocyte regeneration. Unfortunately, these in vitro experiments were carried out before it was realized that in vivo 3,000R irradiation (and in situ residence for 10 days) procured a more structurally intact, lymphocyte-depleted froglet thymus, than did in vitro irradiation followed by organ culture. Thus, future organ culture experiments should benefit from the use not only of "more structurally normal" lymphocyte-depleted thymuses, but also by employment of thymus (and stem cell source) from earlier developmental stages, when stem cell traffic to the thymus may be more likely to occur, due to increased chemotactic thymic influence (see below). For example, a likely stage may be around the completion of metamorphosis, since thymocyte numbers are known to fall sharply at the end of metamorphosis (Du Pasquier and Weiss, 1973; Williams, 1981) and then rapidly increase in number within a few weeks. The "dip" in thymocyte numbers could possibly reflect a cell changeover during metamorphosis, so that "new" thymocytes can acquire MHC-restriction and tolerance to "adult-type". MHC antigens (Du Pasquier and Weiss, 1973; Du Pasquier et al., 1979; Williams, 1981). Thymus explants, taken from animals when thymocyte numbers are minimal, may be particularly receptive to stem cells in transfilter - or co-culture. Additionally, the use of larval (e.g. liver, kidney) and embryonic (e.g. lateral-plate mesoderm) tissues may be better sources of stem cells (Turpen et al., 1982; Turpen and Smith,

1985) in future *in vitro* attempts to recolonize a thymus.

Owen and Jenkinson (1984) have reported successful recolonization of lymphocyte-depleted, 14-day foetal mouse thymus (DG-treated), in hanging-drop cultures made by inverting full wells of Terasaki microwell plates. The wells are seeded with stem cells from a 13-day mouse embryo thymus. After 2 days in hanging drops, the recolonized thymic lobes are transferred to organ culture to allow maturation of stem cells. They have found that as few as five stem cells, or even a single cell (Kingston, Jenkinson and Owen, 1984a) derived from 13-day thymus can achieve lymphoid repopulation of an alymphoid thymus. These workers prefer to use DG-treated thymus, as it provides a much greater bulk of thymic epithelium for colonization, rather than has been obtained by Pyke et al. (1981) using murine epithelial primordia produced by low temperature treatment. As an alternative to transfilter or coculture the hanging-drop system would be useful to try with the lymphocyte-depleted *Xenopus* thymus.

In conclusion, this Chapter indicates that the *in vivo* irradiated "epithelial" froglet thymus retains its functional ability to attract host lymphoid precursor cells. However, the experiments do not indicate the exact timing of host cell input. Similarly, normal froglet and larval thymus implants show a replacement of donor-type lymphocytes with host-type cells by 7 weeks post-implantation, the timing of cell changeover can only be speculated. For example, the process may be gradual, beginning at the time of transplantation, or it may occur at a specific time during ontogeny; e.g. during metamorphosis (see above). Thymus glands from mice, transplanted to F_1 progeny show (by chromosome analysis) an initial proliferation of donor cells, which continues for about 2 weeks, after which dividing host cells begin to appear and completely replace the donor cells by 21 days (cited in Yoffey, 1981).

A study of the timing of host cell immigration into normal and irradiated thymus implants in thymectomized Xenopus is carried out in the next Chapter.

Table 4.1

Light microscopic examination of thymus implants at 7 and 16 weeks post-implantation

Host Type	Thymus Donor Type	Appearance at:	
		7 weeks	16 weeks
JLG5 3N	J 2N Larval	ND	A/B
JLG5 TX 3N	J 2N Larval	ND	A/B
JLG5 3N	J 2N Adult	A	ND
JLG5 3N	J 2N Adult irradiated (3000R 10 days in vivo)	> B <	> C <
J 2N TX	J 3N Adult	A	A
J 2N TX	J 3N Adult irradiated (3000R 10 days in vivo)	> A <	> C <

2 thymus implants were examined to give each data point.
Hosts were larvae (stage 56/7) at implantation.
All normal and irradiated adult thymus donors = 6 mths old.
Larval donors = 4 weeks.

(N.B. 2 thymuses removed 10 days post 3000 rads and examined histologically directly, consisted almost entirely of stromal elements, with very few lymphycytes.)

Code A = completely normal structure.

B = Still very lymphoid, but with fewer lymphocytes than normal thymus. Cortex and medulla nearly always distinct zones.

C = Lymphocyte numbers greatly reduced. Cortex and medulla differentiation lost. However, lymphoid areas still seen within the thymus.

> < = Thymus reduced in size.

Table 4.2

Ploidy analysis of blood and thymocytes from control and thymus-implanted animals, 7 weeks post thymus implantation

Host	Donor Thymus	Percentage Cells with 3 Nucleoli		
		Host Blood (Erythrocytes)	Host Thymocytes	Thymocytes of Thymus Implant
J Triploid (3N) Control		51%	61 ± 7*%	
J Diploid (2N) Control		2%	4 ± 4*%	
JLG5 3N	J 2N Larval	38% 33%	57% 46%	55% 55%
JLG5 3N TX	J 2N Larval	39% 37%	- -	30% 54%
JLG5 3N	J 2N Adult	58% 40%	55% 63%	43% 48%
JLG5 3N	J 2N Adult irradiated (3000R 10 days *in vivo*)	47% 48%	59% 69%	61% 59%

Each line of data represents counts made on an individual host animal, 7 weeks post-thymus implantation.
At least 200 thymocytes/erythrocytes counted in each animal.
Adult thymus donors = 6 mths old. Larval thymus donors = 4 weeks old.
Larval hosts (stage 56/7) at implantation.

* Thymocyte data from Gearing et al. (1984).
No. animals = 15 (3N controls), 6 (2N controls).

Figure 4.1. Diagrammatic representations of culture systems

Figure 4.1a. Transfilter-culture

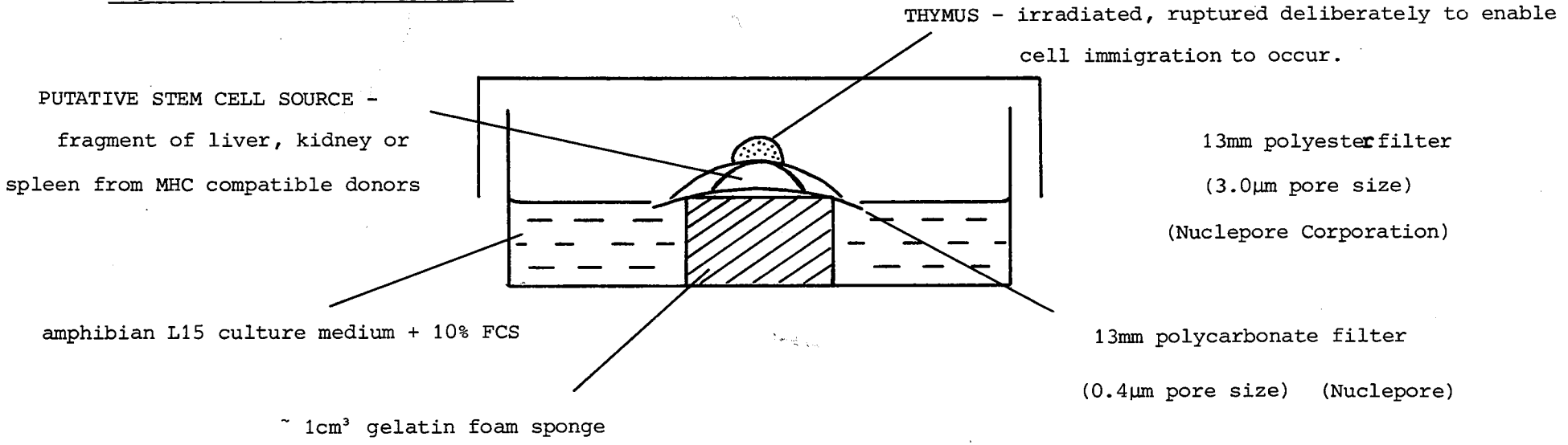
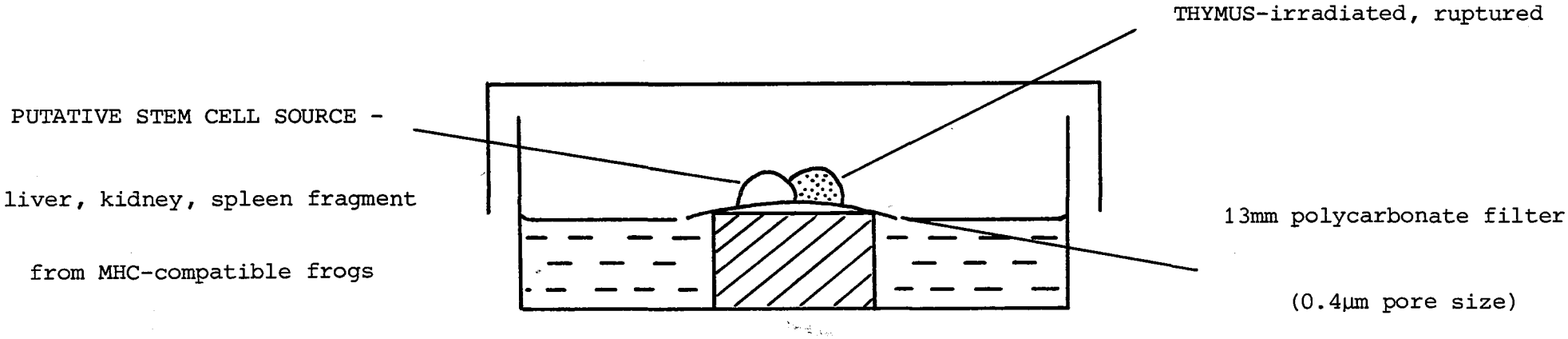


Figure 4.1b. Co-culture



Figures 4.2a and b:

Histology of thymuses examined 7 weeks post-implantation.

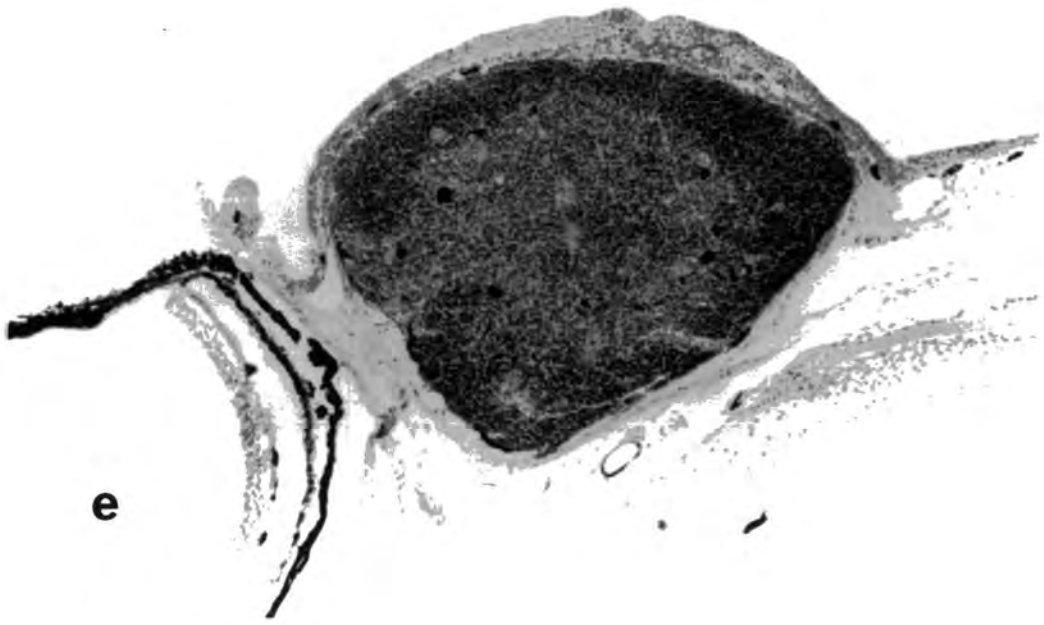
(a) J 3N adult thymus grafted to J 2N larval TX host; note the normal appearance.

(b) J 3N adult, irradiated (3000R 10 days *in vivo*), thymus grafted to J 2N larval TX host; the cortex/medulla differentiation is maintained, although the thymus is slightly reduced in size.

N.B. Thymus implants come to lie close to the eye (e) after metamorphosis. (Magnification x 80).

4.2a

4.2b



Figures 4.3a and b:

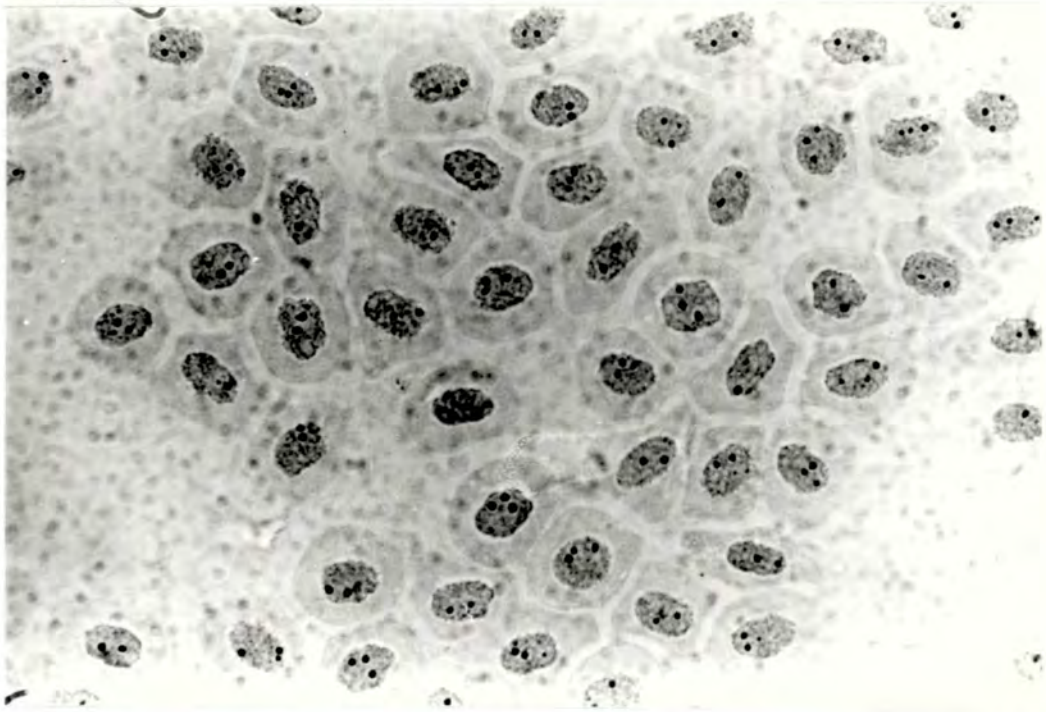
Silver-stained blood smears.

(a) JLG5 triploid control (11 weeks old); many cells are seen with 3 nucleoli.

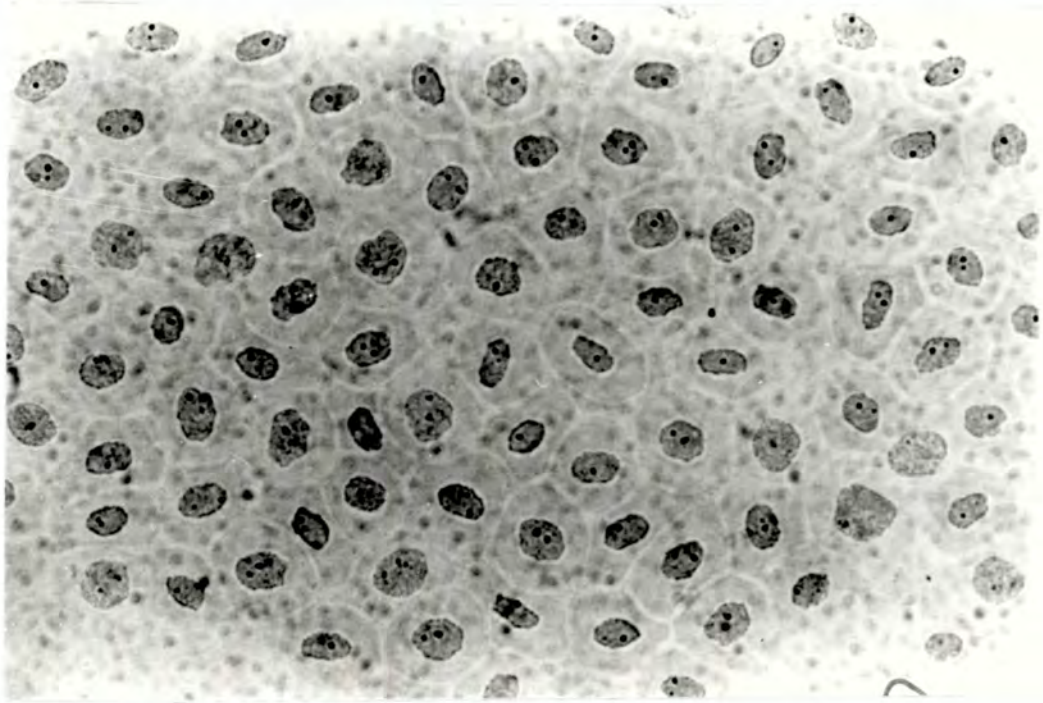
(b) J diploid control (11 weeks old); cells with either 2 or 1 nucleoli are seen.

(Magnification x 800).

4.3a



4.3b



Figures 4.4a and b:

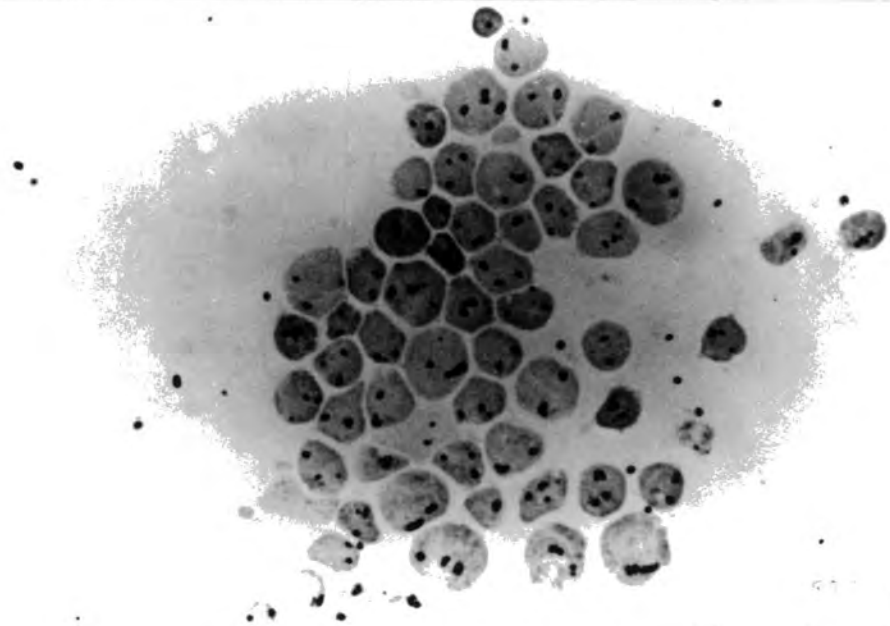
Silver-stained cytospin smears taken from thymus grafts 7 weeks post-implantation.

(a) J 2N normal adult thymus implanted to JLG5 3N host.

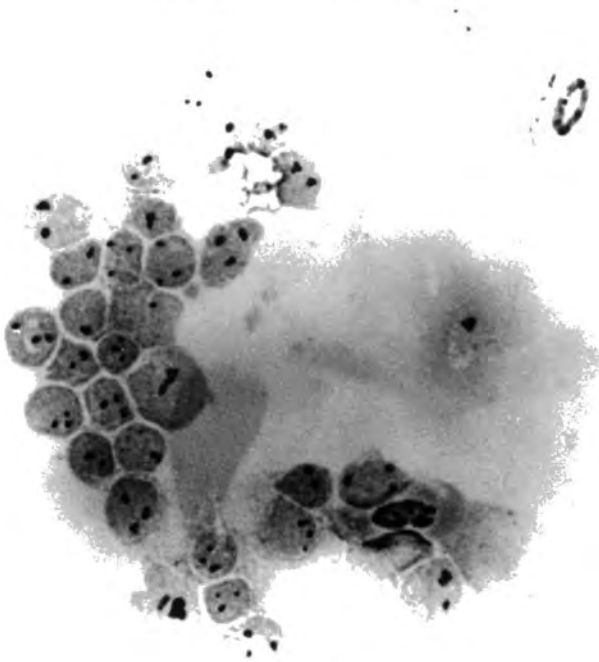
(b) J 2N irradiated (3000R 10 days *in vivo*) adult thymus implanted to JLG5 3N recipient.

Note that many thymocytes in both smears contain 3 nucleoli - i.e. many lymphoid cells are of host origin. (Magnification x 800).

4.4a



4.4b



CHAPTER 5

USE OF THE X. BOREALIS FLUORESCENCE CELL MARKER TO STUDY IMMIGRATION OF HOST-DERIVED CELLS INTO NORMAL AND LYMPHOCYTE-DEPLETED THYMUS IMPLANTS

5.1 INTRODUCTION

Cell marker systems, that can distinguish cells of different genetic origin, have proved particularly useful in developmental immunology. For example, a variety of cell markers [e.g. sex and other chromosome markers (Moore and Owen, 1967; Owen and Ritter, 1969), species-specific characteristics (Le Douarin and Jotereau, 1973), allotypic markers (Dieterlen-Lièvre et al., 1980) and ploidy (Turpen et al., 1982)] have been used in diverse vertebrate species to verify the extrinsic origin of thymic lymphocyte precursors, and the timing of stem cell colonization of the thymus. Furthermore, cell markers have been critical in investigations on the embryonic origin of haemopoietic cells, including T-lineage stem cells (Turpen and Smith, 1985).

In birds, the marker system most extensively used is based on structural differences found between the interphase nuclei of two closely related species, the japanese quail and the chicken (Le Douarin and Jotereau, 1973). In the quail, the chromatin in each cell is condensed into either a single central mass or several heterochromatic masses. In the chicken the chromatin is dispersed in a fine network. These nuclear characteristics can easily be distinguished after Feulgen-Rossenbeck staining.

In mice, the allogeneic surface differentiation antigens Thy 1.1 and Thy 1.2 have recently been used to investigate thymocyte repopulation in chimeras, by examining frozen sections stained with monoclonal anti-Thy 1.1 and anti-Thy 1.2 antibodies (Fontaine-Perus et al., 1981; Jenkinson et al., 1982; Ezine et al., 1984).

In amphibians, the ease with which diploid and triploid animals can be produced experimentally has provided an important and widely used cell marker system (see Chapter 4). Until recently, the ploidy of chimeric tissues was determined by laborious or rather imprecise techniques - e.g. evaluating cell size, estimating nucleolar number (using the silver stain technique described in Chapter 4), counting the number of chromosomes in metaphase plates, or microdensitometry of Feulgen-stained cells (reviewed in Flajnik et al., 1984a). However, a far more accurate, fairly rapid method of detecting diploid and triploid cells has now been developed; this employs flow cytometry of mithramycin-stained cells (Nagata and Cohen, 1984; Flajnik et al., 1984a).

A recently-described marker technique, that holds considerable promise for those working with the Xenopus immune system, is based on the differential fluorochrome staining of the nuclei of different Xenopus species, e.g. X. laevis and X. borealis (Thiébaud, 1983). When stained with the fluorescent dye, quinacrine, the nuclei of X. borealis cells display (under U.V. illumination) a number of bright fluorescent spots on a homogeneous background, whereas X. laevis quinacrine-stained nuclei have no bright spots. Some workers believe that the bright fluorescent spots may be associated with AT-rich sequences of DNA along the chromosomes (Weisblum and de Haseth, 1972; Brown et al., 1977). However, there is also evidence that AT-rich DNA and quinacrine-stained fluorescent spots may not be so strictly related in situ (Comings, 1978). With the quinacrine-staining technique, the origin of a variety of cell types in X. borealis/laevis chimeras are clearly visible (Thiébaud, 1983). As an added advantage (compared with ploidy-marking), the quinacrine fluorescence is applicable to sectioned material as well as smears.

Although quinacrine-staining properties of lymphocytes was not specifically examined in Thiébaud's paper, it seemed likely that the use of the X. borealis marker system would prove invaluable for examining lymphoid cell migration into and out of thymus implants, in more detail than was possible in Chapter 4. In this Chapter, thymuses were therefore transferred between X. borealis and "non-quinacrine-staining" species. Recipients were early-thymectomized larvae and thymus donors were either normal froglets, or froglets that had been irradiated with 3,000 rads to deplete thymic lymphocyte numbers. Specifically, the quinacrine marker system (along with toluidine-blue stained sections) is here used to probe the timing of host lymphoid cell input to normal and lymphocyte-depleted foreign thymus implants, to identify the origins of stromal and lymphoid cells within the implants, and to briefly assess the extent to which donor thymocytes persist in the host blood and spleen.

5.2 MATERIALS AND METHODS

5.2. Animals

X. borealis adults carrying the quinacrine fluorescent cell marker were a generous gift from Dr. R. H. Clothier, University of Nottingham, U.K. The parents of that borealis colony had originally been purchased several years ago from Xenopus Ltd., who had field collected them from Lake Nukuru in Southern Kenya. Putative X. borealis adults were also purchased by us directly from Xenopus Ltd. Apparently this stock had originally been collected from the N.W. Province of Kenya (by Mr. M.P. Simmonds, School of Biological Sciences, Queen Mary College, London) and we have found them not to possess the quinacrine-spotting nuclear pattern. We have not formally identified these quinacrine-negative Xenopus, although the males do possess the

same distinctive mating call as the quinacrine-positive X. borealis. The quinacrine-positive and -negative "borealis" are referred to as X. borealis +ve and X. borealis -ve respectively in this Chapter. Offspring were obtained from X. borealis by the usual injections of human chorionic gonadotrophin hormone into mature males and females to induce ovulation and mating. Animals were reared as described in Chapter 2.

J strain X. laevis and LG hybrid Xenopus were also used in this Chapter.

5.2.2 Thymus implantation

Either normal or lymphycyte-depleted thymuses (i.e. thymus taken from froglets 7-10 days post 3,000 rads in vivo irradiation) were implanted to 7-day TX larvae when 4-6 weeks old (as described in Chapter 4). Donor/host combinations used are shown in the Tables. MHC-disparate combinations were used in this Chapter.

5.2.3 Blood and thymocyte smears

These were prepared as in Chapter 4. However, smears were stained here with quinacrine.

5.2.4 Histologic preparation of thymus implants and host spleens

Thymus implants and spleens were embedded in historesin, which proved excellent material (superior to paraffin wax) for preparing 4 μm sections, found to be the most suitable thickness for subsequent toluidine blue or quinacrine staining.

For historesin embedding, tissues were first fixed in Carnoy's (60% absolute alcohol, 30% chloroform and 10% acetic acid) for 30 mins, then dehydrated in a series of dilute ethanol (70-95% ethanol). Total dehydration to 100% ethanol is not necessary as the final historesin embedding medium contains 6-8% water. The infiltration solution was prepared by dissolving 50 ml basic resin in 1 packet of activator,

both provided in the L.K.B. Historesin Kit (No. 2218/500). The intermediate infiltration solution was made by mixing 1:1 (95% ethanol/infiltration solution). Dehydrated specimens were immersed in intermediate solution for 1½ hours, then in pure infiltration solution until specimens appeared slightly translucent and sank to the bottom of vials. Tissues were then placed in embedding medium in plastic capsules and left to polymerize for 40-120 mins at room temperature. The embedding medium consisted of 15 mls infiltration solution and 1 ml hardener. 4 μm sections were cut on a Reichert Ultramicrotome and either stained in toluidine blue (as described in Chapter 3) or quinacrine (see below). Toluidine blue sections were photographed using a Kodak Ektachrome film (200 ASA).

5.2.5 Fluorescent staining with quinacrine dye

Slides were first placed in buffer (18% citric acid 0.1 M, 82% pH 7.0 Na_2HPO_4 0.2 M) for 10 mins. They were then stained with fluorescent dye (0.5% solution of quinacrine dihydrochloride - Sigma - in buffer) for 40 mins using a staining dish covered in foil. Dye was washed off by dipping slides in 3 changes of buffer, sections or smears were left for 20 mins in the last buffer wash, then mounted in 50% sucrose. The slides were sealed with wax (50:50 wax/vaseline) and stored in the dark until observation with a Nikon fluorescence microscope using a BV (blue/violet) filter. Black and white photographs of sections were taken using a Technical Pan film [rated at 50 ASA]; colour photos were taken using either Kodak Ektachrome or Fuji films [400 ASA].

5.2.6 Experimental design

a) Pilot studies on developing quinacrine staining for thymocyte cytospins and thymus/spleen sections were carried out first.

b) A preliminary thymus implantation experiment was carried out to assess both the usefulness of the boreàlis spotting marker

to observe cell immigration to the thymus and to determine when a more detailed time-course study on lymphoid repopulation should take place. Hosts in this preliminary experiment were either X. borealis +ve TX larvae or LG TX larvae. Donor thymuses were from 4-6 month old froglets (either normal animals or 3,000 rad-irradiated). LG thymuses were given to X.borealis +ve hosts, whereas X. borealis +ve thymuses were given to LG hosts. Thymuses were taken for sectioning and quinacrine staining at 12-15 days or at 16 weeks post thymus implantation; spleens were additionally removed from LG TX animals given X. borealis +ve normal thymuses at 16 weeks.

c) A detailed time-course study at 1, 2, 3 and 9 weeks post thymus implantation was carried out on i) X. borealis +ve TX larval hosts given 11 month old X. borealis -ve thymuses (normal or 3,000 rad irradiated), and ii) J TX larval hosts given 6 month old X. borealis +ve thymuses. Thymuses were examined at each time point, in both toluidine-blue stained sections, and following quinacrine staining for fluorescence microscopy. Spleen sections and blood smears were also examined in those animals given control borealis +ve thymuses.

5.3 RESULTS

5.3.1 Pilot studies developing quinacrine staining for lymphoid tissues

(i) Blood smears from X. borealis were stained with quinacrine to confirm the nuclear spotting pattern of fluorescence (Figure 5.1). Animals with such spotted nuclei were called X. borealis +ve individuals:- all the F1s from the X. borealis adults given by Dr. Clothier had such "spotted nuclei".

(ii) Thymus cytospins were prepared from X. borealis +ve (Figure 5.2a) froglets (4 months old) and also from similar aged J strain Xenopus laevis (Figure 5.2b) to confirm the quinacrine-staining properties of thymocytes. The usefulness of the quinacrine fluorescence to detect the origin of individual cells was indicated by preparing 50:50 in vitro mixes of X. borealix +ve and J strain Xenopus thymocytes (Figure 5.3). Unfortunately, in the preparation of thymocyte smears, the majority of epithelial and other stromal cells, e.g. the adherent cells such as dendritic antigen-presenting cells and macrophages (Turpen and Smith, 1986) are lost, and the majority of cells examined are thymic lymphocytes. Therefore it was necessary in the planned lymphocyte repopulation studies to examine quinacrine-stained sectioned material, which would be able to reveal far more about the origin of various cell types in the implanted thymuses.

(iii) Quinacrine-stained historesin sections of 4 μm thickness proved optimal for observing the spotted appearance of X. borealis nuclei and the lack of spotting in X. laevis nuclei (compare Figure 5.4 with 5.5). Thinner sections revealed too few spots for fluorescence analysis, and thicker sections gave rather high background fluorochrome staining.

5.3.2 Trial fluorescence experiments with normal and irradiated thymus implants

The outcome of this quinacrine-staining experiment is shown in Table 5.1. Non-irradiated thymus implants were mainly comprised of donor cells (category X - see legend to Table) at 12-15 days post-implantation, although a few host cells were seen mainly in the medulla. By 16 weeks, the vast majority of lymphocytes in the thymus cortex are now host-derived (category Z - see Figures 5.6 and 5.7a), whereas stromal cells throughout the thymus appear to be predominantly donor origin (Figure 5.6). In the medulla some donor lymphocytes are found alongside host lymphocytes (Figure 5.7b).

Spleens removed from LGTX animals given a X. borealis +ve normal thymus 16 weeks previously revealed an occasional thymus implant-drived cell both in the perifollicular regions (Figure 5.8) and in the red pulp. Within 15 days implantation of a 3,000 rad-irradiated thymus, the majority of thymocytes found in the implants were already of host origin (see Table 5.1). By 16 weeks, irradiated thymus implants could no longer be located.

5.3.3. Time-course study comparing cell migration into normal and irradiated thymuses: toluidine-blue and quinacrine-stained preparations

A summary of the data is given in Table 5.2. The more detailed findings are illustrated in Figures 5.9 to 5.24.

(i) One week post thymus implantation (Hosts at stages 56/57)

a) Normal adult X.borealis -ve thymus implants in X. borealis +ve, TX hosts

Thymuses were healthy (Figure 5.9a) with relatively normal cortex and medulla. Quinacrine-stained skin, removed along with the thymus, provided a clear indication that host cells were spotted (Figure 5.9b). Between skin and thymus implants were found cellular accumulations comprising both donor- and host-derived cells. Host polymorphs (particularly neutrophils) were frequently seen here (Figure 5.9b). Within the thymus, the subcapsular zone was rich in lymphoblasts, often seen in division (Figures 5.10a and b). The deep cortex was packed with small lymphocytes. Enlarged blood vessels traversing the cortex contain both host- and, occasionally, donor-derived blood cells (Figure 5.10b). Spotted (host) cells, often of irregular shape, were seen in the cortex, although the majority of cortical cells, including mitotic cells, were still donor in origin (Figure 5.10b).

Enlarged blood vessels were also found in the medulla (Figure 5.11a). These vessels often contained large cells, with basophilic cytoplasm, resembling haemocytoblasts; neutrophils

were also evident in these vessels. Quinacrine-staining revealed that, overall, the medulla was far more of a chimeric cellular population than the cortex - i.e. both host and donor cells were found interspersed. Host cells included lymphocytes, polymorphs and cells with pale, irregular-shaped nuclei. The identity of the latter was unclear (Figure 5.11b).

b) Irradiated X. borealis -ve thymus implants in X. borealis +ve, TX hosts

Irradiated thymuses were greatly reduced in size (contrast with Figure 5.9a) and contained only a scattered array of lymphocytes, with no very obvious cortex/medulla differentiation, under low magnification (Figure 5.12a). Much adipose tissue surrounds the irradiated thymus. The "cortical" region is shown in Figure 5.12b and reveals a dearth of lymphocytes, but noticeable epithelial and epithelial reticular cells. Neutrophils were very frequent around and within the irradiated thymus (Figure 5.12b). These, presumably phagocytic, cells were of host origin (Figure 5.12c). Epithelial cells of the "cortical" region were clearly of donor (non-spotted) origin (Figure 5.12c), whereas the few lymphocytes found emanated from both donor and host. Haemocytoblasts were seen in the "cortical" region (Figure 5.12b). In the "medulla" are seen many multinucleate cystic cells (Figure 5.13a) filled with lightly-stained material; myoid cells are also frequent. The "medulla" is heavily infiltrated with a variety of host cell types, including haemocytoblasts, lymphocytes and cells with irregularly-shaped nuclei (Figure 5.13b).

c) Normal X. borealis +ve thymus implants in J, TX recipients

In contrast to a) above, here the host is "non-spotted" and the donor "spotted", in quinacrine fluorescence terms! Thymuses appeared healthy and normal in appearance. Quinacrine staining

revealed mostly host cells perithymically, although the occasional donor cell was observed outside the thymus itself (Figure 5.14a). It appeared difficult to find host-derived cells amongst the "spotted" cells of the thymus cortex and medulla, i.e. there seemed to have been little host cell immigration at one week post implantation in this donor/host combination. An occasional host cell was however recorded in the medulla (Figure 5.14b).

This donor/host combination was useful for tracing donor-derived cells in the periphery. Quinacrine-stained blood smears revealed a significant number of donor lymphocytes (Figure 5.15a) and occasionally other cell-types. The spleens from J recipients also contain a high level of thymus donor-derived cells (Figure 5.15b). These are mostly noticeable in the red pulp and perifollicular zones, but some donor lymphocytes were also seen within the white pulp. Donor cells comprise both lymphoid (small and circular) and possibly non-lymphoid (with more irregularily-shaped nuclei) cells.

(ii) Two-weeks post thymus implantation
(Hosts at stages 59/60)

a) Normal X. borealis -ve thymus implants
in X. borealis +ve, TX hosts

Thymus implants were less lymphoid than at one week. The cortex was particularly reduced in lymphoid content and some necrosis was visible in the medulla (Figure 5.16a). Blood vessels associated with thymuses appeared enlarged and haemocytoblasts were frequently seen within these capillaries. The vast majority of cells in both cortex and medulla were shown by quinacrine staining to be of donor origin (non-spotted), but some host cells of varied morphology were found scattered throughout the thymus (see Figures 5.16b and c).

b) Irradiated X. borealis -ve thymus implants in X. borealis +ve, TX hosts

Compared with week 1, irradiated thymus implants contained noticeably more lymphocytes, seen in discrete clusters in the cortical region. Thymuses were still very small (see Figure 5.17a). Many cysts and myoid cells frequented the medulla (Figure 5.17a). In quinacrine-stained preparations, clusters of lymphocytes in the cortex were seen to be of host origin (Figure 5.17b); the medulla also contained some host-derived lymphocytes.

c) Normal X. borealis +ve thymus implants in J, TX recipients

Thymus implants were closely comparable to those removed at one week post-implantation - i.e. there were very few host-derived cells in the lymphoid thymuses. Thymus donor-derived lymphocytes were again seen in the blood smears prepared. Spleens appeared normal in structure (as at week one) with distinct white pulp areas bordered by a double-layer of boundary cells. Lobular cells, thought to be antigen presenting cells (Baldwin and Cohen, 1981) were evident in the white pulp (see Figure 5.18a). Spleens were well-populated with lymphocytes, many of which are thymus donor-derived (Figure 5.18b). The red pulp and perifollicular zones were especially rich in spotted, thymus-derived lymphocytes. Many donor cells were also found now within the white pulp.

(iii) Three weeks post thymus implantation (Hosts at stages 60-65 i.e. in the last stages of metamorphosis)

a) Normal X. Borealis -ve thymus implants in X. borealis +ve, TX hosts

Nodules of small lymphocytes were found in the thymus cortex. Cysts, mucous cells and myoid cells were now frequently seen throughout much of the thymus. The centre of the organ appeared necrotic (Figure 5.19a). Very few host cells were found in these thymuses at 3 weeks post implantation (see Figure 5.19b).

b) Irradiated X. borealis -ve thymus implants in X. borealis +ve, TX hosts

These thymus implants have now a greatly restored lymphoid population. The subcapsular cortex contains many lymphoblasts with frequent mitotic figures visible (Figures 5.20a, b). Host polymorphs were often seen just outside the thymus. Quinacrine staining revealed that the cortex has a highpercentage of host-derived lymphoid cells (Figure 5.20c). Mitotic figures in both cortex and medulla were of host origin. Stromal elements were of donor origin, including cystic cells and myoid cells.

c) X. borealis +ve thymus implants in J, TX hosts

These were similar to the 2 week picture. Implants had been minimally infiltrated by host cells. Thymus-derived cells were frequent in the spleen, particularly in red pulp and marginal zone, but also some donor cells were again found in the white pulp follicles. The blood was also chimeric with donor-derived lymphocytes representing 5-10% of the PBL.

(iv) 9 weeks post-implantation (Recipients were now froglets)

a) Normal X. borealis -ve thymus implants in X. borealis +ve, TX hosts

These thymuses (11 months old when transplanted) had by now decreased in size and, although still containing many lymphocytes, had little suggestion of cortex and medulla. Cystic spaces and myoid cells were frequently seen (Figure 5.21a). There were still very few host-derived cells within these thymuses; those that had immigrated were scattered throughout the organ.

b) Irradiated X. borealis -ve thymus implants in X. borealis +ve, TX hosts

In contrast to 3 weeks post-implantation, these thymuses now appeared very reduced in lymphoid content and seemed to be degenerating (Figure 5.22a). Epithelial and reticular elements

were of donor origin, whereas lymphoid cells remaining were host-derived (Figure 5.22b).

c) X. borealis +ve thymuses in J, TX hosts

In contrast to a) above, these thymuses - from younger (6 month old) donors - were very healthy, with masses of lymphocytes and cortex/medulla differentiation (Figure 5.23a). Quinacrine staining revealed that the vast majority of lymphocytes were, at 9 weeks post-implantation, host-derived (i.e. non-spotted, see Figure 5.23b). With these thymuses, then, we see a dramatic development of host-derived lymphocytes in the thymus soon after metamorphosis. Donor-derived lymphocytes are still very frequent in the spleen (mainly in the red pulp), and also occasionally seen in the blood.

5.4 DISCUSSION

In this Chapter, the X. borealis marker system has proved very useful for studying cell traffic to and from thymuses implanted to early-TX Xenopus. The identity of quinacrine positive/negative cells is especially clear in smears and cytospin preparations, since the whole of each cell is seen and hence the fluorescence is bright. However, this Chapter has also shown that quinacrine-staining can be successfully used with resin-embedded material that is sectioned at 4 μm thickness, even though the fluorescence intensity is naturally lower, as only part of each cell is visible. This success with tracing cells in sectioned material means that the origin of thymic lymphoid and non-lymphoid cells can readily be examined in situ, a distinct advantage over previous ploidy-labelling studies.

The results presented here with 4-6 month old MHC-incompatible thymus implants confirm the preliminary data of Chapter 4 (where MHC-

compatible thymus implants were examined). Thus, they reveal that the lymphoid population developing within a non-irradiated thymus has become predominantly of host origin when the implant is examined several months post-transplantation. Furthermore, they extend similar observations of others (Gearing et al., 1984; Nagata and Cohen, 1984) by revealing that the epithelial component of the thymus remains essentially donor-derived. This finding lends credence to the suggestion that cells of a TX host can enter the donor thymus and therein come under the influence of a foreign stromal environment (see Nagata and Cohen, 1984). In contrast to the excellent growth and lymphoid nature of the thymus implants taken from 6 month old or younger froglets, those from 11 month old animals displayed relatively few lymphocytes, which were mainly of donor origin, at 9 weeks post-implantation. Perhaps such thymuses no longer "attract" lymphoid stem cells, or are particularly susceptible to the trauma of the operation. Interestingly, when Xenopus approaches one year old, their thymuses are known to begin to involute in situ (Du Pasquier and Weiss, 1973). The age of the thymic donor must therefore be taken into careful consideration in future experiments employing Xenopus froglets.

The time-course investigations, looking at colonization of a lymphoid thymus implant by cells of the TX host, imply that metamorphosis may be a critical stage. Thus when hosts are still in the final stages of transformation (i.e. 3 weeks post thymus implantation), their thymus implants are still predominantly of donor origin. However, by nine weeks the picture has dramatically changed to a mainly host-derived thymocyte population. It is possible that the post-metamorphic immigration of host cells is directly related to a decline in the number of donor lymphocytes within the thymus implant just after metamorphosis is completed (thymic lymphocyte numbers decrease naturally at this time -

see Williams, 1981). Thus, depletion of thymic lymphocyte numbers effected naturally, or by γ-irradiation, could well trigger the release of chemoattractants from the thymus and thereby elevate host precursor cell immigration. There is evidence (from avian and mammalian experiments - see Le Douarin et al., 1982) to suggest that when thymuses are lymphocyte-depleted, they become more attractive to stem cells.

Lack of available lymphoid stem cells in metamorphosing Xenopus would seem not to be a factor that delays repopulation of lymphoid thymus implants, since 3,000 rad-irradiated implants appear to become rapidly recolonized by host cells, their lymphoid complement being almost exclusively host-type within 2 weeks of implantation. The rapid immigration of host cells into irradiated thymus implants might well be associated with the release of excessive amounts of chemoattractant from irradiation-damaged cells. The particularly heavy infiltration of irradiated thymuses (noticed soon after implantation) with host neutrophils presumably represents the need for phagocytosis of dead or dying donor material. The nature of other host cells, with irregularly-shaped nuclei, that were frequently seen within thymus implants soon after transplantation is uncertain. These could well be thymic accessory cells, that include both macrophages and inter-digitating cells (see Chapter 3).

It seems that 3,000 rad-irradiation causes irreparable, long-term damage to thymic stromal cells, since although such thymuses initially become repopulated with host lymphocytes, etc., they fail to grow in the froglet and eventually disappear. Irradiation is known to affect the ability of cells to divide, and so the relatively slow cell turnover rate of thymic stromal cells (compared with lymphocytes) means that the effect of irradiation on the stromal cells takes many weeks/months

to manifest itself. These findings indicate a critical role of donor stromal cells in the long-term survival of implanted thymuses. When donor stromal cells die, the thymus degenerates, presumably because the essential (epithelial?) cells cannot be supplied by the host.

TX J strain animals given X. borealis +ve normal thymuses displayed extensive numbers of donor cells in both blood and spleen soon after implantation. Chimerism of peripheral lymphocytes was still apparent several months post-implantation - as has also been revealed by mithramycin-staining following transfer of thymuses to post-metamorphic, early-TX Xenopus (Nagata and Cohen, 1984). Thymus donor lymphoid cells were mainly located in the marginal zone and red pulp lymphoid tissue of the spleen - known to be T-dependent lymphoid regions (see Horton and Manning, 1974a and b). However, some cells of thymic origin were also found in the splenic white pulp - a B-cell rich zone (Baldwin and Cohen, 1981). Perhaps the thymic-derived cells in the white pulp seen here represent the B-cell population known to exist in the Xenopus thymus (Williams et al., 1983; Hsu et al., 1983). Lack of thymus implant-derived lymphocytes in the periphery of TX animals given irradiated thymus implants was not investigated here. These experiments will require an in depth study using X. borealis +ve irradiated thymic implants. The question of whether cells from the donor thymus are found in the periphery is important, since peripheral chimerism may well contribute to the level of host tolerance towards donor antigens.

The so-called "X. borealis negative" animals used in some of the experiments here were raised from adults which we believed to have been originally collected from N.W. Kenya. Those Xenopus morphologically appeared to be true X. borealis (M.P. Simmonds, personal communication). However, it is known that considerable hybridization between Xenopus species occurs - this is certainly so in the N.W. Province of Kenya, where hybridization of X. borealis with X. laevis victorianus is suspected

(M.P. Simmonds, pers. comm.). Perhaps such hybridization has "diluted out" the quinacrine-specific marker. F_1 hybrids (produced in our laboratory) between X. borealis +ve and X. laevis still carry the fluorescent marker, but this is less intense than in X. borealis. It seems to us unlikely that our "X. borealis negative" animals are actually X. muelleri, although these two species of Xenopus have apparently often been mistaken for one another in the past (Brown et al., 1977; Thiébaud, 1983). The origin of our quinacrine-negative animals (the highlands of N.W. Kenya near to the town of Eldoret, between Kisumu and Tambach), is believed to be outside of the distribution of X. muelleri (M. Simmonds, pers. comm.) Thiébaud (1983) reveals that X. borealis show extremely clear quinacrine fluorescence, while X. muelleri have been found to be entirely negative. The present study suggests that some X. borealis may also not carry this fluorescent cell marker. One problem with the use of the X. borealis marker system is that chimeras established are naturally allo- or xenogeneic combinations. Control experiments, using MHC-compatible thymus donors and host animals, were not attempted in this Chapter. Cell immigration to MHC-compatible thymus implants was, however, preliminarily assessed by ploidy analysis in Chapter 4. In the future, it will be possible to implant thymuses from "non-spotted" J strain X. laevis donors to "quinacrine-spotted" hosts, where the thymus implant is only minor-antigen disparate to the recipient. Thus X. laevis/X. borealis hybrids, produced by crossing an X. borealis male with a J female, have now been raised in this laboratory (J. D. Horton, personal communication). These hybrids have cells that retain the quinacrine-spotting pattern (although, as mentioned above, less intense than true X. borealis).

In conclusion, this Chapter has revealed that an irradiated thymus implant can provide only a temporary site for TX host lymphoid

cells to develop. In contrast, a lymphocyte-rich implant, although more slowly infiltrated by host cells, continues to grow well and provides a long-term environment for host thymocyte development. Attention is now turned to examining the alloimmune *in vitro* capabilities of lymphocytes developing in TX animals implanted with normal or lymphocyte-depleted thymuses.

Table 5.1

Trial fluorescence experiments to detect the origin of cells in thymus implants transplanted to 7-day thymectomized Xenopus

Hosts (Stage 55/6 when implanted)	Donor Thymus	Origin* of cells in quinacrine-stained thymus implants	
		12-15 days (no. animals)	16 weeks (no. animals)
LGTX	X. borealis +ve (4-6 months)	X (2)	Z (2)
LGTX	Irradiated X. borealis +ve (4-6 months) (8 days post 3000 rads in vivo irradiation)	> Z < (2)	N.D.
X. borealis +ve TX	LG (4-6 months)	X (2)	Z (2)
X. borealis +ve TX	Irradiated LG (4-6 months) (8 days post 3000 rads in vivo irradiation)	N.D.	Not Found (3)

* Code:- X = Mostly donor cells, although a few host cells mainly in the medulla. (Low level of host cell input).

Y = Host cells more noticeable throughout the thymus. (Medium to high level of host cell input.)

Z = Host cells the major lymphoid population. But stromal cells and some lymphocytes remain donor-type. (Very high level of host cell input.)

> < = Thymuses reduced in size.

N.B. Some irradiated X. borealis and LG thymuses were taken directly for histological analysis: these appeared mostly "epithelial" except for a few lymphocytes remaining in the medulla.

Table 5.2

Simplified summary of time course study to observe migration of cells into normal and lymphoid-depleted thymuses

Host	Donor Thymus	Appearance* of thymus implants stained with toluidine blue (TB) or quinacrine (Q)							
		Weeks post-implantation							
		One		Two		Three		Nine	
		TB	Q	TB	Q	TB	Q	TB	Q
X. borealis +ve TX	*X. borealis* -ve 11 months	A/B	X	B/C	X	B/C	X	> C <	X
X. borealis +ve TX	Irradiated *X. borealis* -ve 11 months (*in vivo* irradiation 7 days post 3000 rads)	> C <	> Y <	> C <	> Z <	> B <	> Z <	> C < Degenerating	> Z <
JTX	*X. borealis* +ve 6 months	A	X	A	X	B	X	A	Z

Hosts = larvae (stage 55) at time of implantation. 2 thymus implants tested each week per group of animals.

* Code:- A = Completely normal structure, e.g. compare with thymuses taken straight from the animal.

B = Still very lymphoid, but with fewer lymphocytes than *in vivo* thymuses. Cortex and medulla nearly always present

C = Lymphocyte numbers significantly reduced. Cortex and medulla differentiation lost. However, lymphoid areas still seen scattered within the thymus.

X, Y, Z See Table 5.1.

Figure 5.1

Quinacrine-stained blood smear showing erythrocyte nuclei with fluorescent spots, thereby confirming that this was an X. borealis +ve individual. (x 400)

5.1

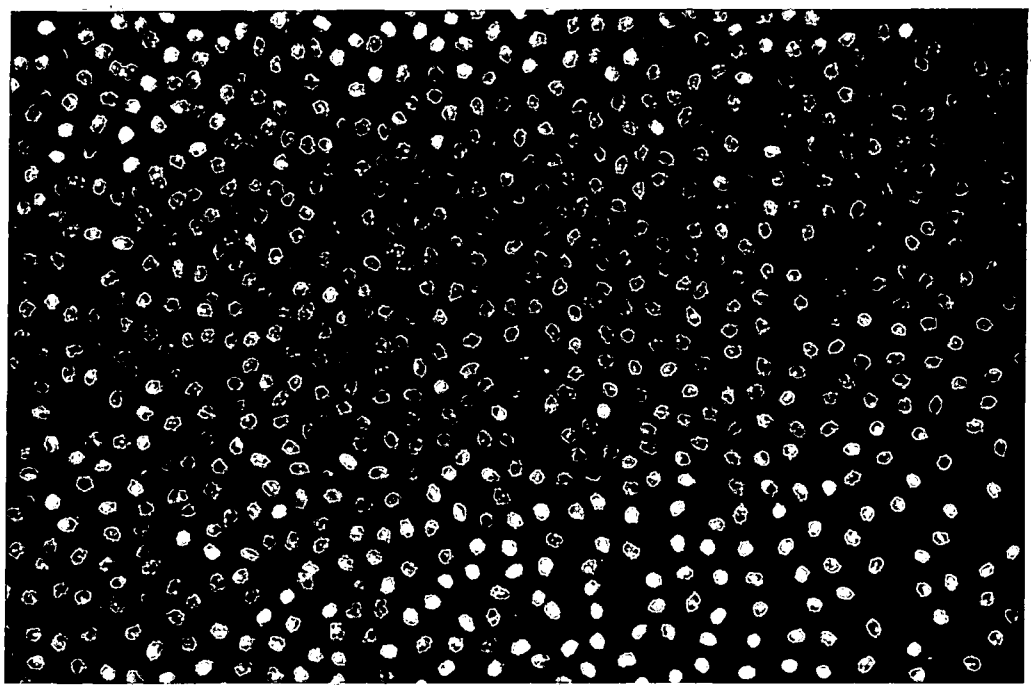


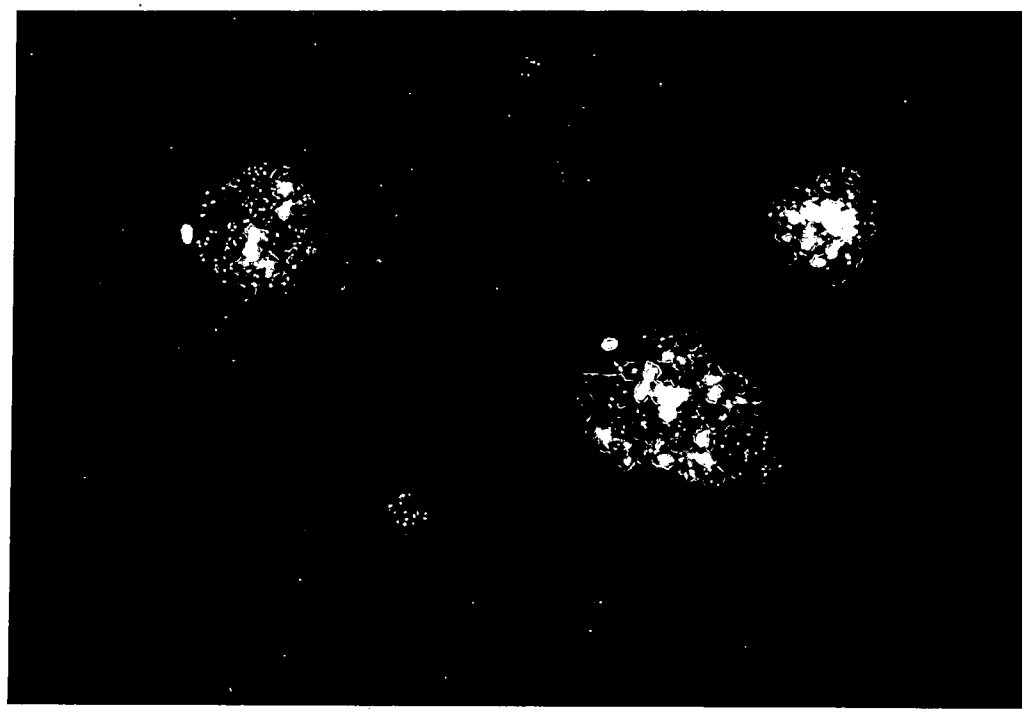
Figure 5.2

Quinacrine-stained thymocyte cytospins from 4 month old froglets.

a) *X. borealis*, with spotted nuclear fluorescence.

b) J strain *X. laevis* with unstained nuclei. (x 400)

5.2a



5.2b

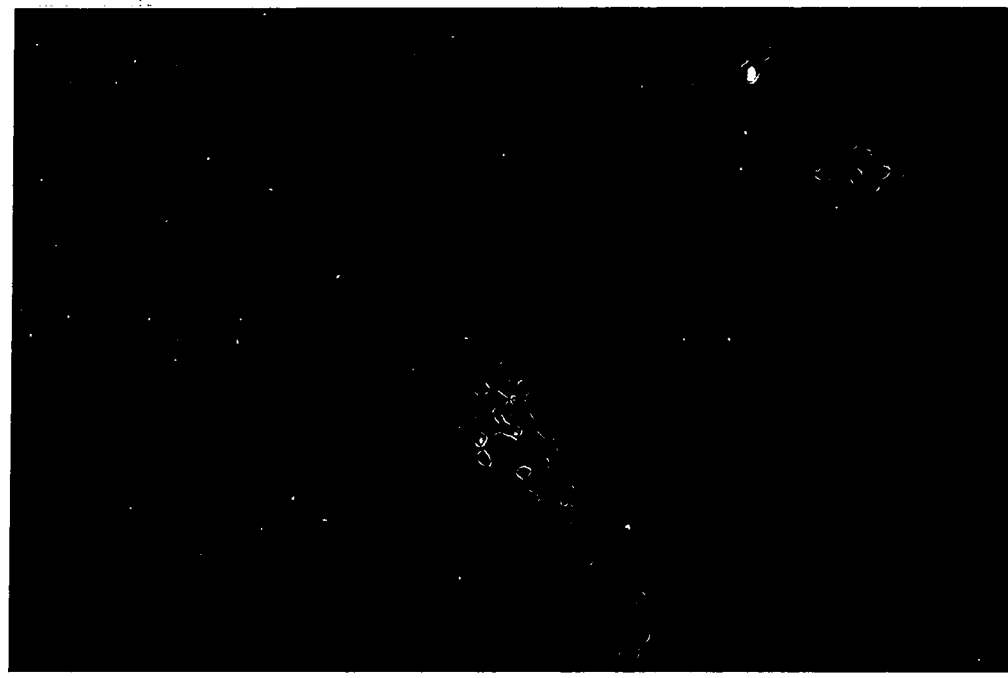
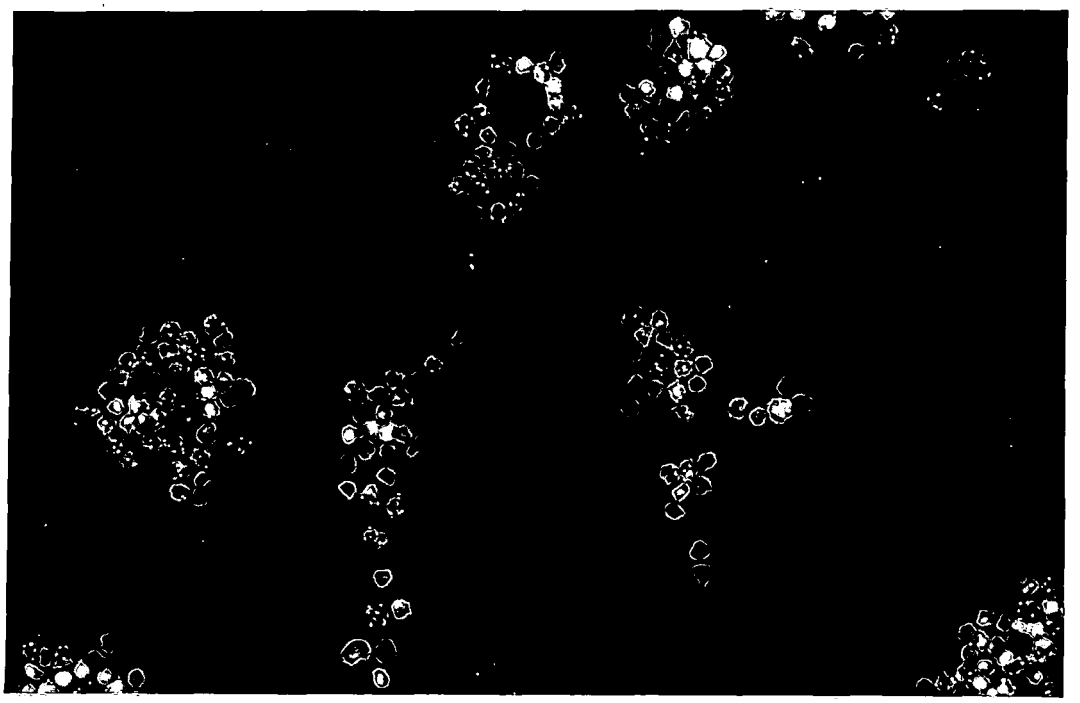


Figure 5.3

Thymocyte cytospin prepared from a 50:50 mixture of X. borealis +ve and J X. laevis thymus cells. The origin of each cell can be easily identified following quinacrine staining and fluorescence microscopy. (x 400)

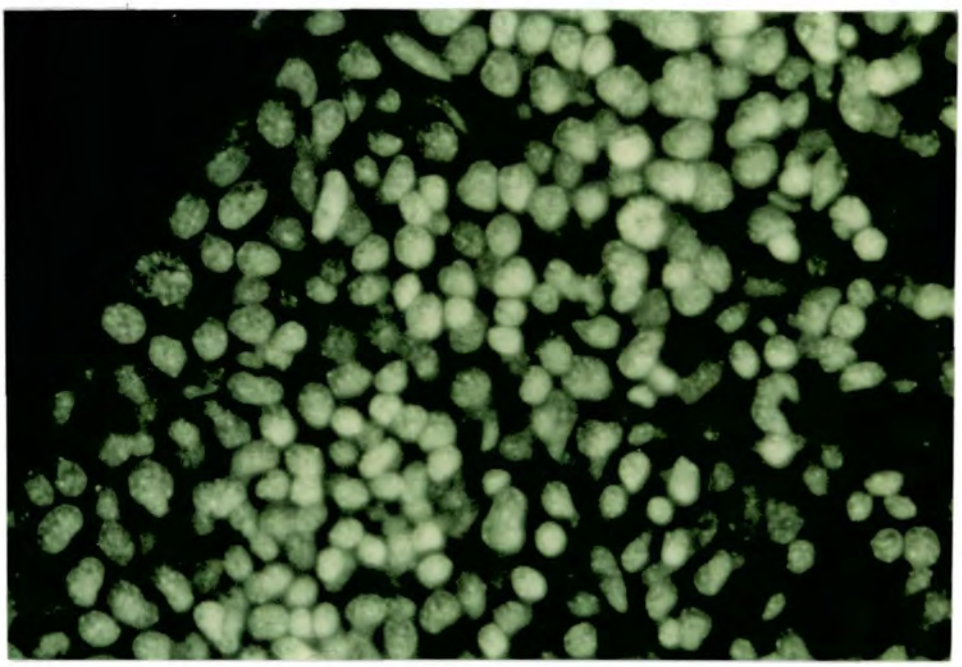
5.3



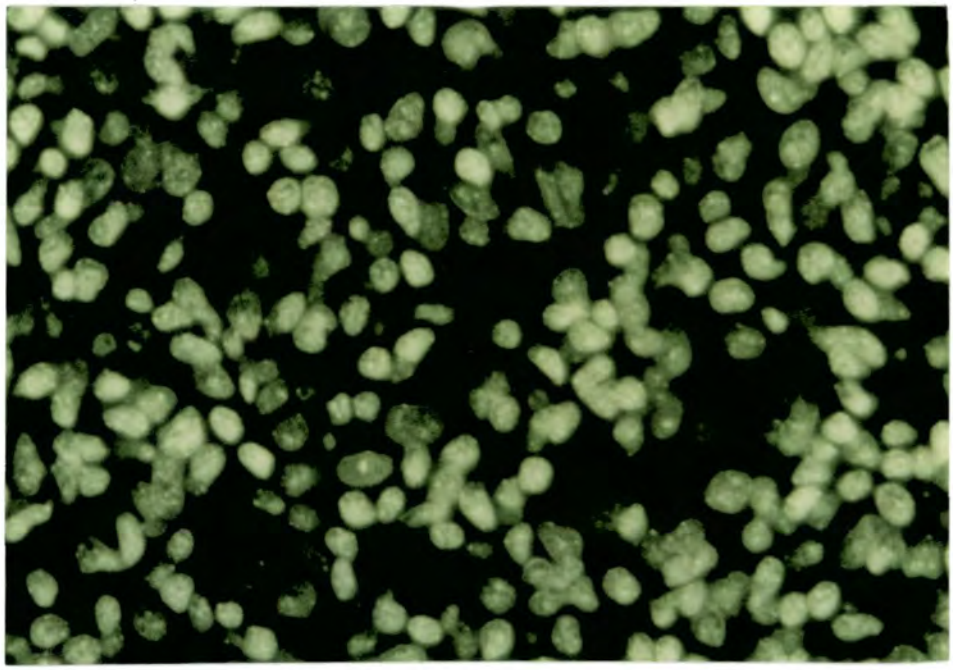
Figures 5.4a and b

Quinacrine-stained 4 μm historesin sections from X borealis +ve stage 56/7 larva. Immunofluorescence reveals spotted appearance of cells in both cortex (a) and medulla (b). (x 850)

5.4a



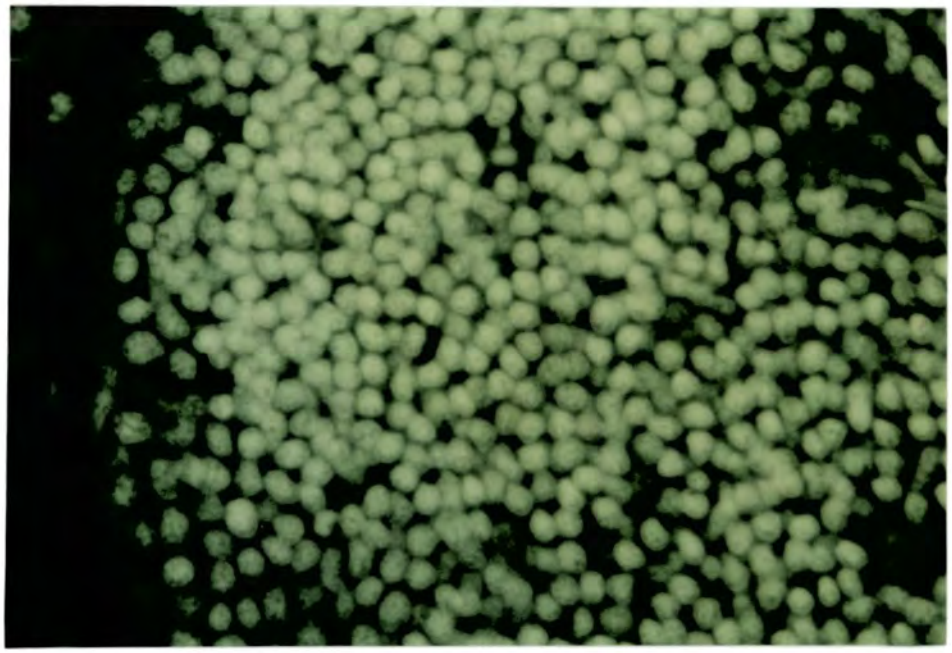
5.4b



Figures 5.5a and b

Quinacrine-stained 4 μm historesin sections from J strain X. laevis, stage 56/7 larva showing absence of spotted nuclei in cortex (a) and medulla (b). (x 850)

5.5a



5.5b

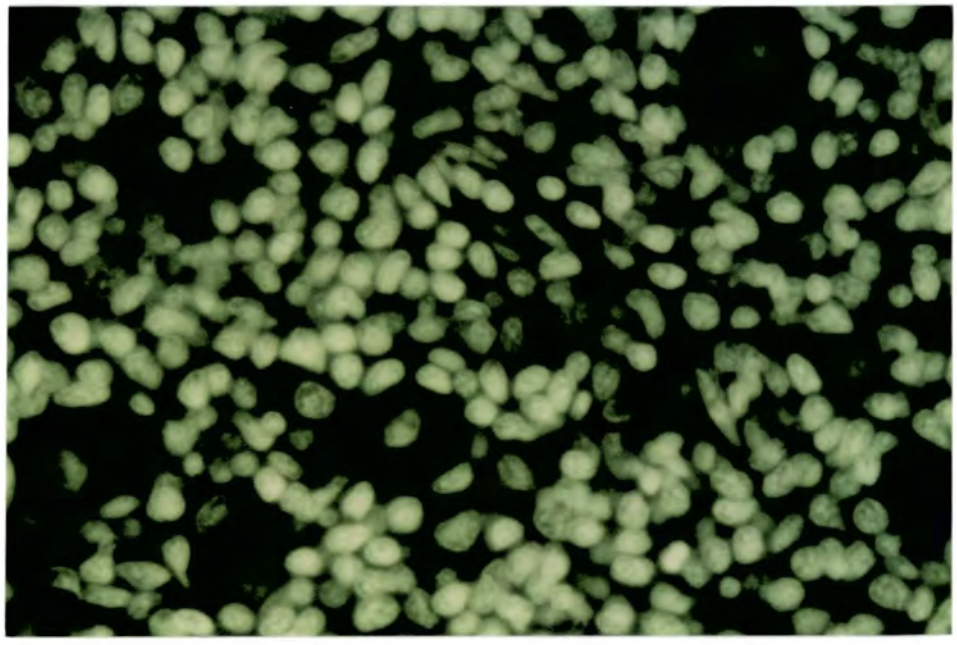
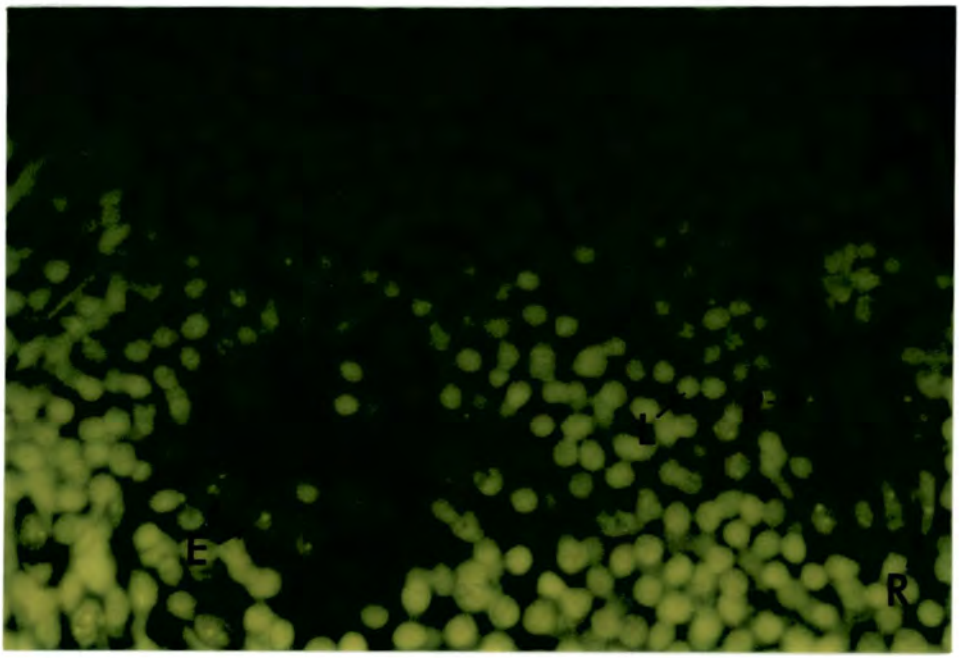


Figure 5.6

Quinacrine-stained 4 μm section through the cortex of a thymus implant. Thymus donor was X. borealis +ve froglet (4-6 months). Thymus implant removed for fluorescence studies 16 weeks post implantation. Recipient of thymus implant was an LG3 TX larva. The lymphocytes (L) are mainly host-type although the epithelial (E)/reticular (R) cells remain of donor (spotted) origin. (x 850)

5.6



Figures 5.7a and b

This figure shows the reciprocal combination to Figure 5.6; *X. borealis* +ve TX animal is the recipient of an LG thymus graft. Implant sectioned 16 weeks post-grafting; (a) shows host lymphocytes (spotted) have seeded the cortex and stromal cells (arrowed) remain of donor-type, (b) the medulla contains a mixture of host lymphocytes and thymus donor-derived epithelial cells (e). A few donor-derived lymphocytes (l) are also found. (x 800)

Figure 5.12

X. borealis -ve, irradiated (removed from donor 7 days post 3000 rads) thymus, grafted to X. borealis +ve, TX host. One week post-implantation.

a) Toluidine blue-stained section showing reduction in size of thymus, loss of cortex and medulla and few lymphocytes. Category > C <. (x 70). Note extensive adipose (a) tissue.

b) Toluidine blue-stained section of "cortical" region. Many neutrophils (n) are seen within and around the thymus. (ep) epithelial cells, (l) lymphocytes, (h) haemocytoblasts. (x 700)

c) Quinacrine-stained "cortical" region, to show host origin of neutrophils (n) and donor origin of epithelial cells (ep). Some spotted lymphocytes (sl) and non-spotted lymphocytes (l) are found. (x 800)

Figure 5.13

X. borealis -ve, irradiated thymus, grafted to X. borealis +ve, TX host. One week post implantation.

a) Toluidine blue-stained "medulla".
(cy) cysts, (my) myoid cell. (x 350)

b) Quinacrine fluorescence of "medulla".
Host cells have heavily infiltrated the medulla. These include: lymphoid cells (l), some haemocytoblasts (h) and some cells with irregularly-shaped nuclei (arrows). (x 800)

Figure 5.14

X. borealis +ve thymus implanted to TX, J recipient and removed one week later for quinacrine staining.

a) Extra-thymic (et) tissue contains mostly host, but occasional donor cells. Cortex is still packed with donor lymphocytes (dl). (x 800)

b) The medulla is also comprised mainly of donor cells, although the occasional host cell (arrowed) is evident, usually associated with blood capillaries. (x 800)

Figure 5.15

X. borealis +ve thymus implanted to TX, J recipient one week post-implantation: observations on quainacrine-stained blood and spleen.

a) Blood smear, revealing donor thymus-derived lymphocytes (l) and a much larger cell type. The majority of nuclei (erythrocytes and an occasional lymphocyte) are non-spotted i.e. host-derived.

b) Spleen section, revealing high level of thymus-derived lymphocytes in perifollicular zone (pf), red pulp (rp) and also an occasional donor-derived cell in the white pulp (wp). (bl) boundary layer cells. (x 800)

Figure 5.16

X. borealis -ve thymus implanted to X. borealis +ve, TX host. Two weeks post-implantation.

a) Toluidine blue section, showing some loss of cortical lymphocytes and some necrosis in medulla (m). Thymus category B. (x 70)

b) and c) Quinacrine-stained cortex and medulla respectively. Most cells in thymus are of donor origin. However host cells of varied morphology are seen throughout the thymus. (bv) blood vessel, (p) perithymic, host cells.

Figure 5.17

In vivo irradiated *borealis* -ve thymus graft resident in *borealis* +ve, TX host for 2 weeks.

a) Toluidine blue stained section. Thymus is more lobular than at one week and more lymphoid. Many cystic and myoid cells are obvious in the medulla. Thymus category > C <. (x 70)

b) Quinacrine-stained section. Cortex contains clusters of host-derived (spotted) lymphocytes (hl), although thymic stromal elements (e) are still predominantly donor-type. (x 800)

Figure 5.18

Spleen from J TX hosts 2 weeks after implantation with an *X. borealis* +ve graft. Spleen appears normal in structure.

a) Toluidine blue staining:- white pulp area enclosed by a double-layer of boundary cells (bl) and containing lymphocytes and lobular "antigen-presenting" cells (apc). (x 1750)

b) Quinacrine fluorescence:- the perifollicular zone (pf) and red pulp (rp) are rich in thymus donor-derived cells. (wp) white pulp. Donor-derived cells are also obvious in the white pulp. (x 800)

Figure 5.19

X. borealis -ve thymus graft 3 weeks post-implantation to X. borealis +ve, TX host.

a) Toluidine blue. Thymus looks rather unhealthy and there is a necrotic centre (to the right of the figure); cystic cells are a prominent feature. Category B/C. (x 175)

b) Quinacrine staining reveals that the vast majority of cells in the cortex and throughout the thymus are still negatively-staining, donor-type. (x 800)

Figure 5.20

In vivo irradiated X. borealis -ve thymus implant 3 weeks post-grafting to X. borealis +ve, TX host.

a) and b) Toluidine blue-stained sections. Thymus has become repopulated with lymphocytes (Figure a) (x 175) Thymus category > B <. In b) the subcapsular cortex is seen with many prominent lymphoblasts (with basophilic cytoplasm); a mitotic figure is clearly visible. (x 1750)

c) Quinacrine-stained section through cortex, showing many host-derived lymphoid cells. Stromal elements are of donor origin. (x 800)

Figure 5.21

X. borealis -ve control thymus implanted into X. borealis +ve, TX host. 9 weeks post-implantation.

Toluidine blue-stained section showing:- No cortex/medulla. Many cysts and myoid cells. Lymphocytes still in good numbers throughout the organ. Note absence of subcapsular lymphoblast zone. Thymus category > C <. (x 175)

Figure 5.22

Irradiated X. borealis -ve thymus in X. borealis +ve, Tx host. 9 weeks post-implantation.

a) Toluidine blue. Thymus very small and full of cysts. Some lymphocytes remaining, however. Thymus category > C <. (x 175)

b) Quinacrine fluorescence. Shows host-derived lymphocytes and donor-derived (non-spotted) epithelial and reticular cells. (x 800)

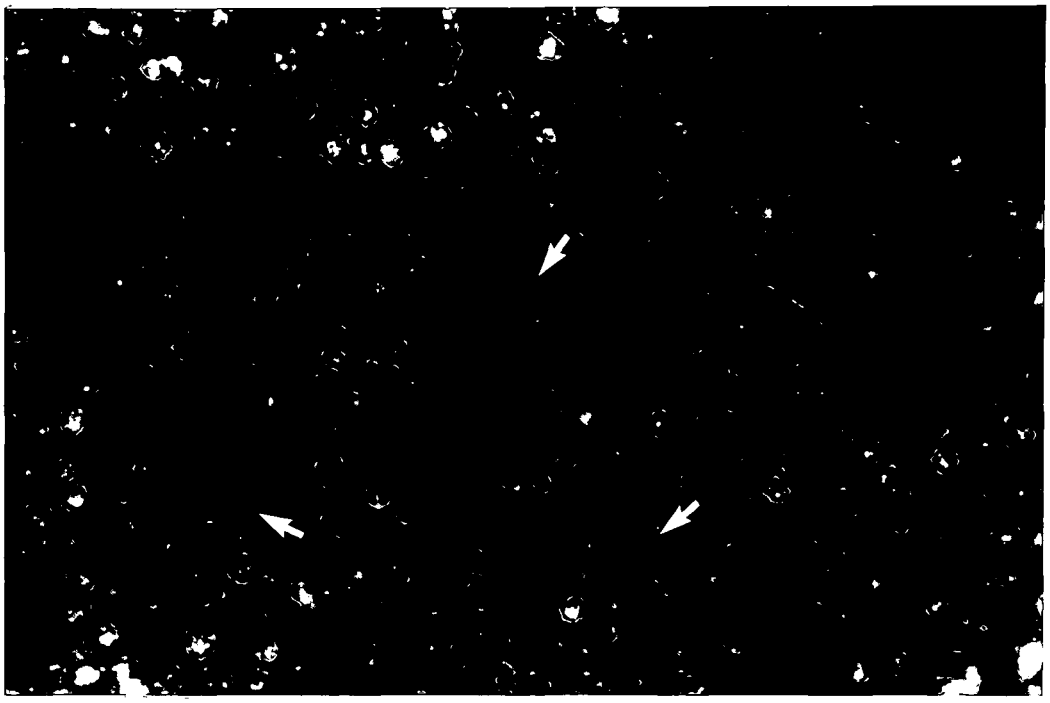
Figure 5.23

Normal X. borealis +ve thymuses in J, TX hosts.
9 weeks post-implantation.

a) Toluidine blue-stained section shows very lymphoid nature of thymus. Note the nodules of lymphocytes separated by trabeculae in the cortex. Thymus category A. (x 175)

b) Quinacrine fluorescence, reveals that lymphocytes are predominantly host-derived, whereas stromal elements, including cysts, etc. are of spotted, donor origin. (x 850)

5.7a



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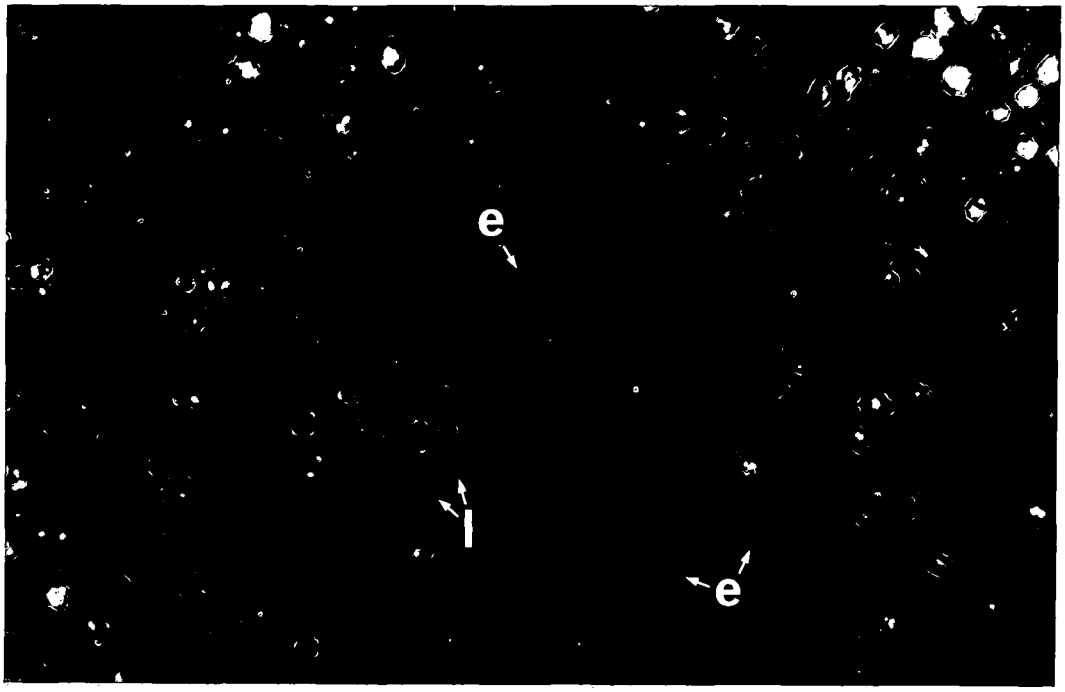


Figure 5.8

Quinacrine-stained 4 µm section of spleen from LG TX that had 16 weeks previously received an X. borealis +ve thymus. Occasional thymus donor cells (arrowed) occur in the perifollicular region. (x 850).
(bl) boundary layer, (wp) white pulp.

5.8

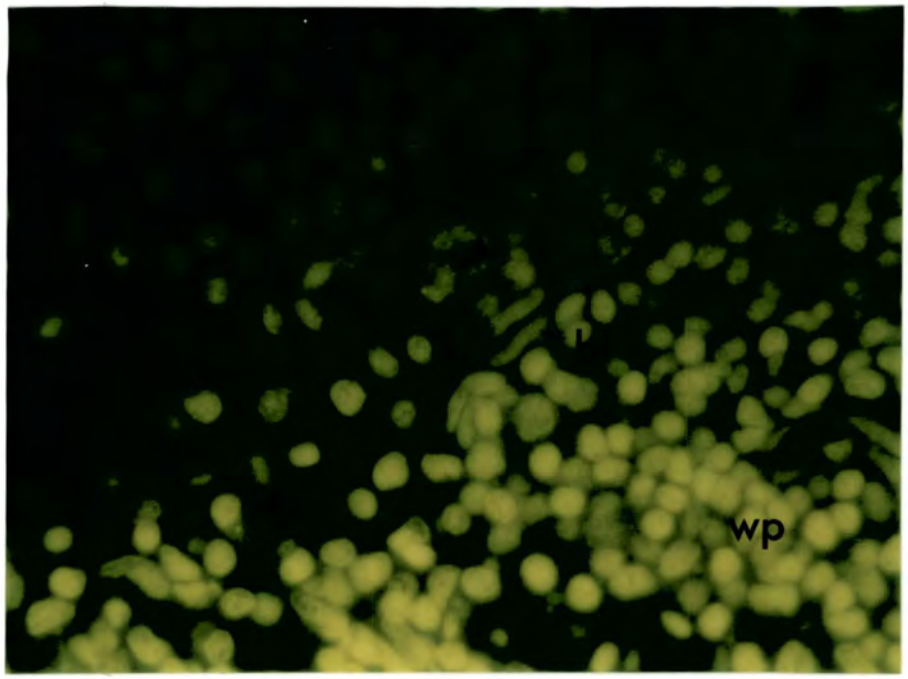


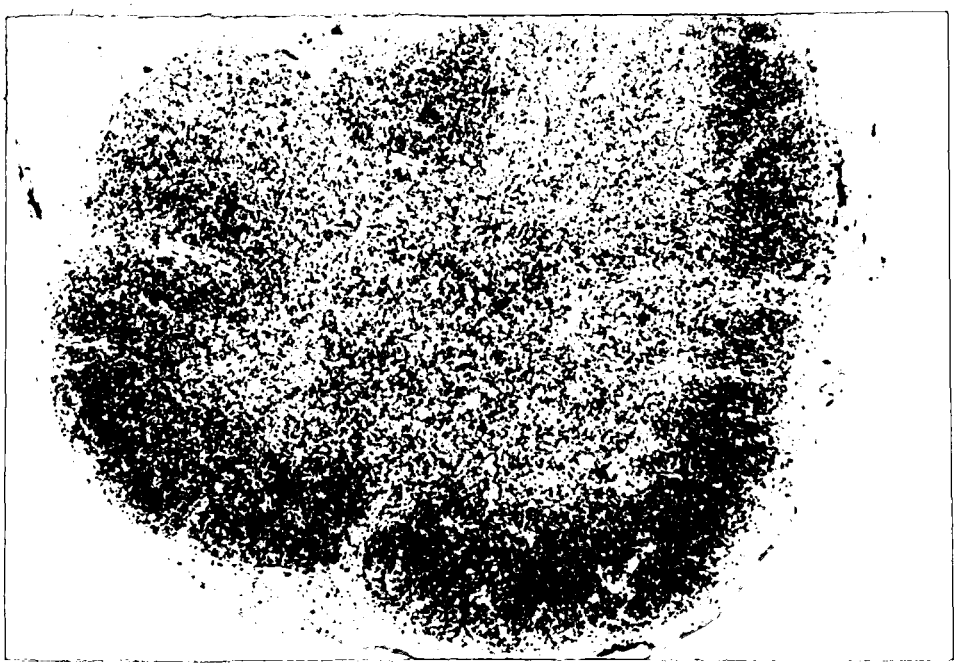
Figure 5.9

X. borealis -ve thymus grafted to *X. borealis* +ve, TX host. Examined one week post-implantation.

a) Toluidine blue stained section, showing normal structure of thymus implant. Category A. (x 70)

b) Quinacrine fluorescence of region just outside of implant. Host skin (sk) comprised of cells with spotted nuclei; extra-thymic tissue (et) contains a variety of cell types, including neutrophils (n). (x 800)

5.9a



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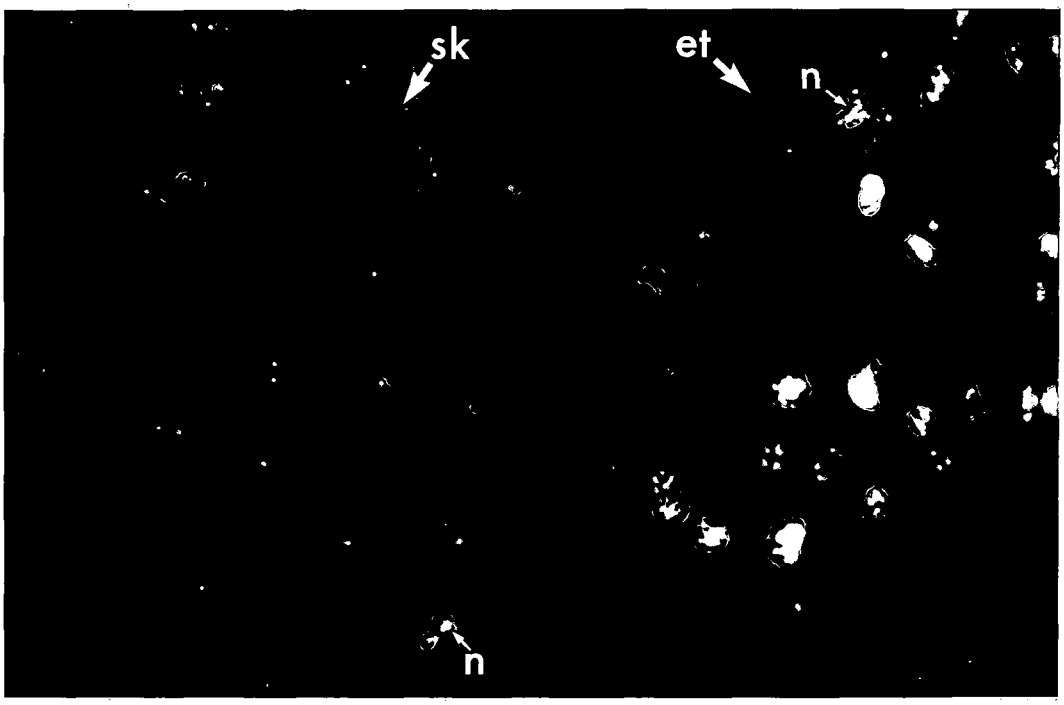


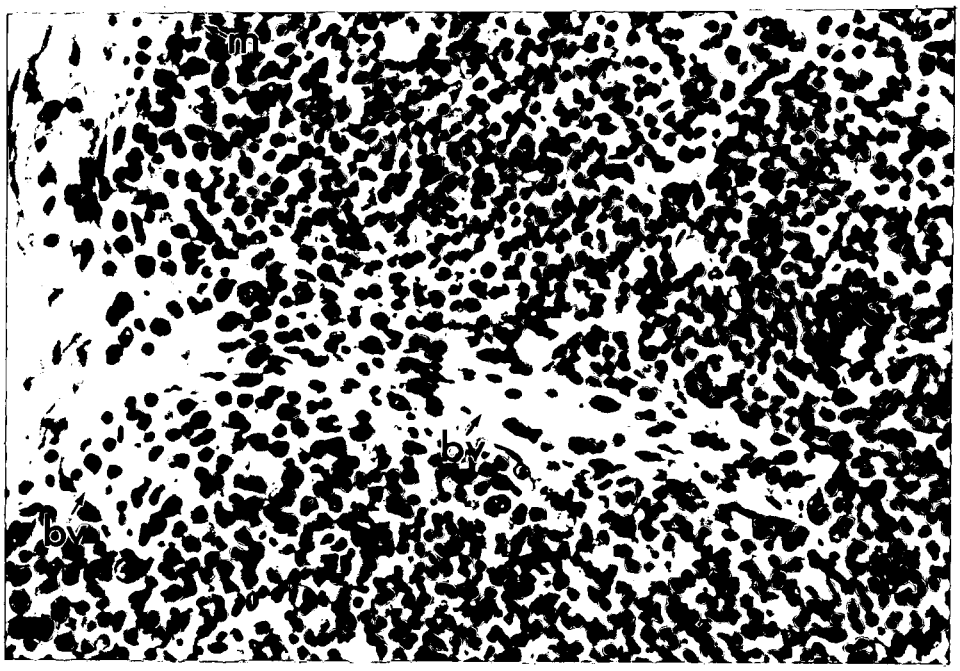
Figure 5.10

X. borealis -ve thymus grafted to X. borealis +ve, TX host one week post-implantation.

a) Toluidine blue-stained section of cortex shows lymphoblasts of subcapsular cortex, some in mitosis (m), and small lymphocytes in deep cortex. Enlarged blood vessels (bv) are seen just outside and passing through the cortex. (x 350)

b) Quinacrine fluorescence through cortical region. Lymphoblasts and lymphocytes of cortex still mostly of donor origin. A few host (spotted) cells (arrowed) are seen within a blood vessel (bv) and also in the thymic parenchyma. (x 800)
(m) mitotic cell.

5.10a



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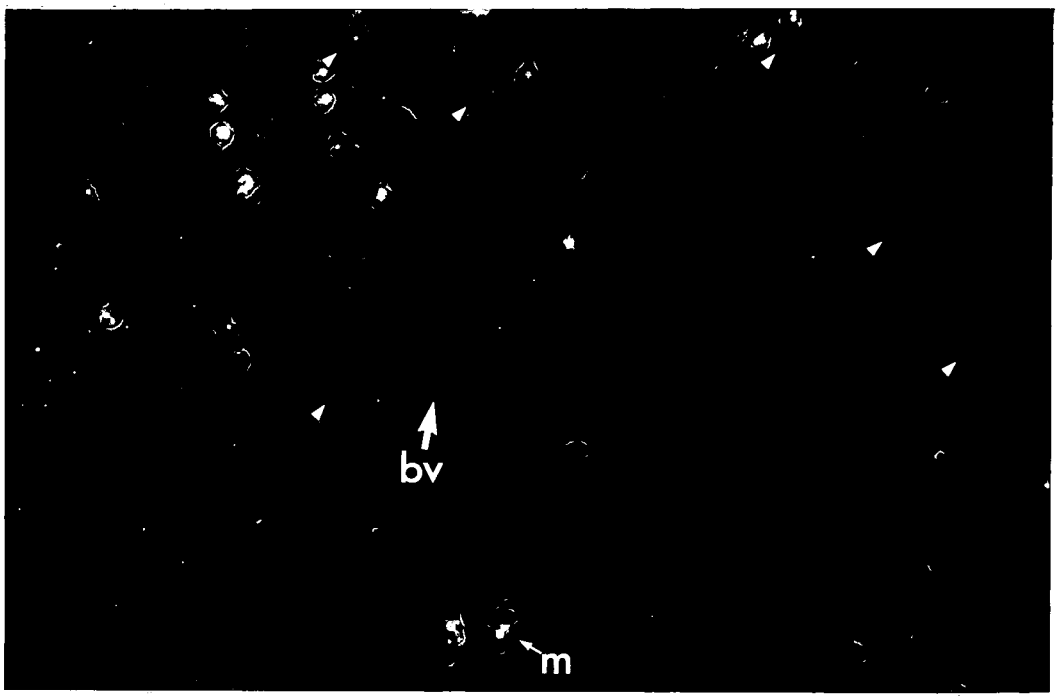


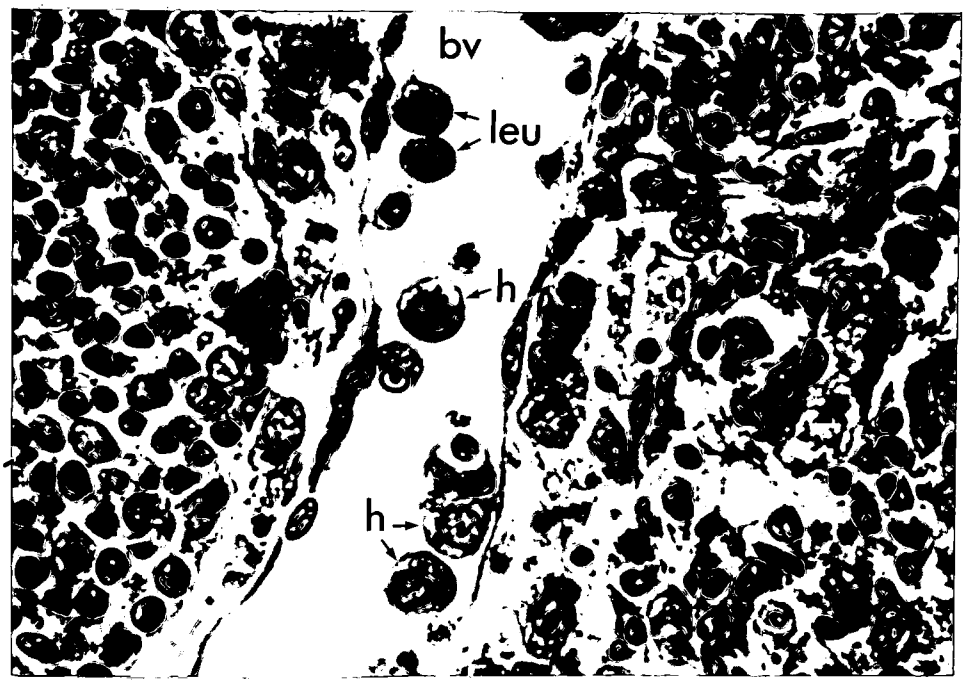
Figure 5.11

X. borealis -ve thymus grafted to X. borealis +ve, TX host. One week post-implantation.

a) Toluidine-blue section showing enlarged blood vessel (bv) at medulla (right)/cortex (left) interface.
(h) haemocytoblasts, (leu) leukocytes (probably neutrophils).
(x 700)

b) Quinacrine-stained medulla, to show chimerism of cells present. Host cells include neutrophils (n), lymphocytes (l) and cells with irregularly-shaped nuclei (arrowed). (x 800)

5.11a



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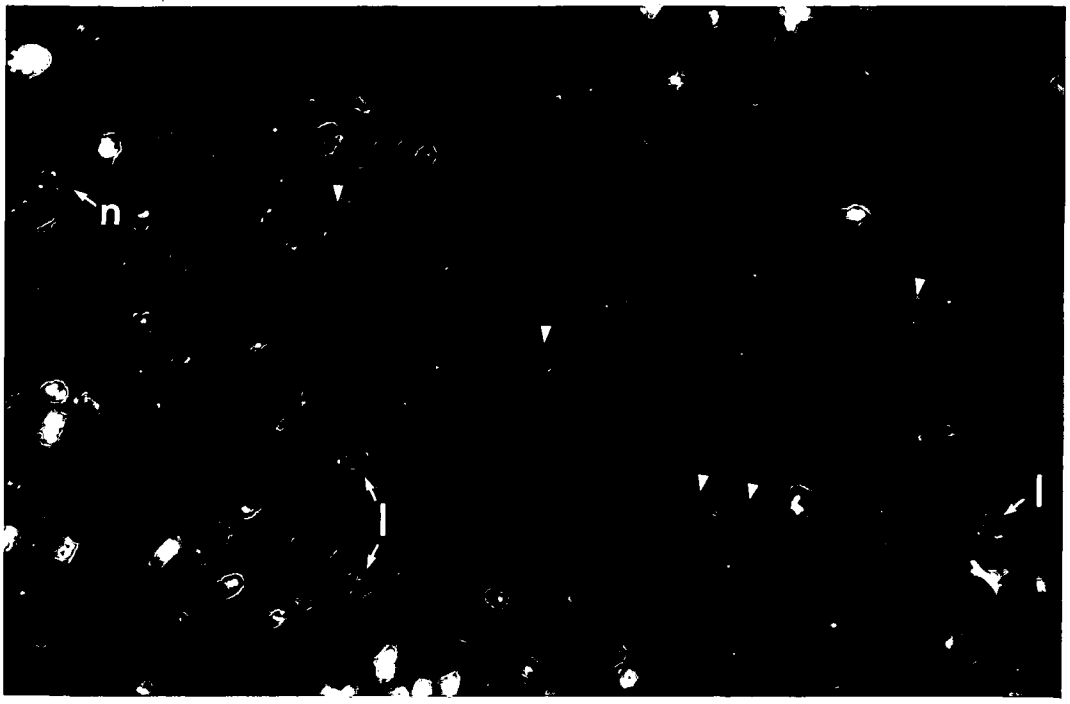

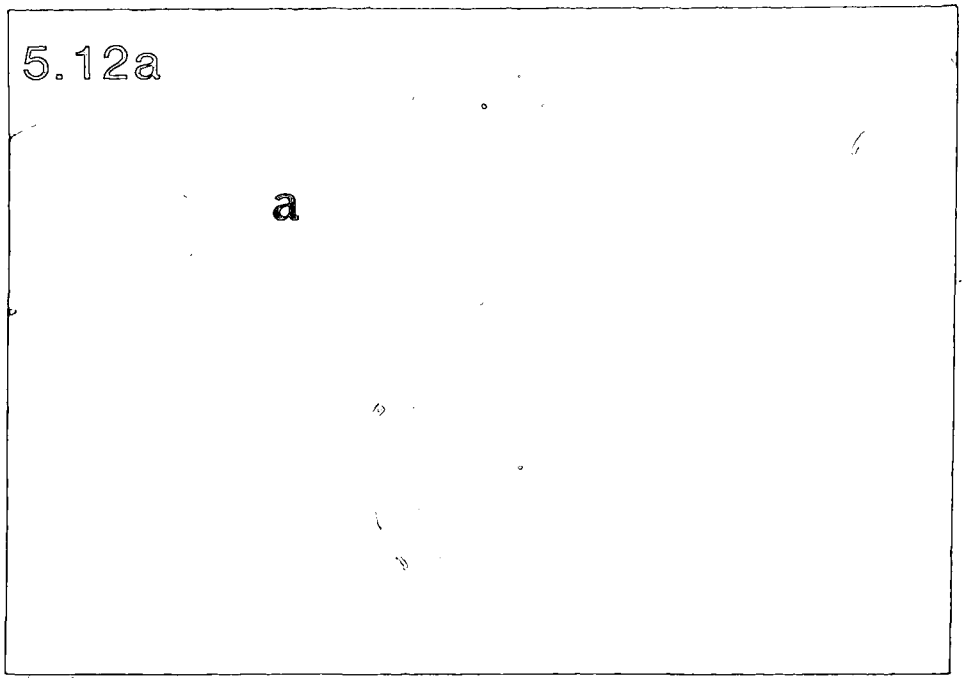
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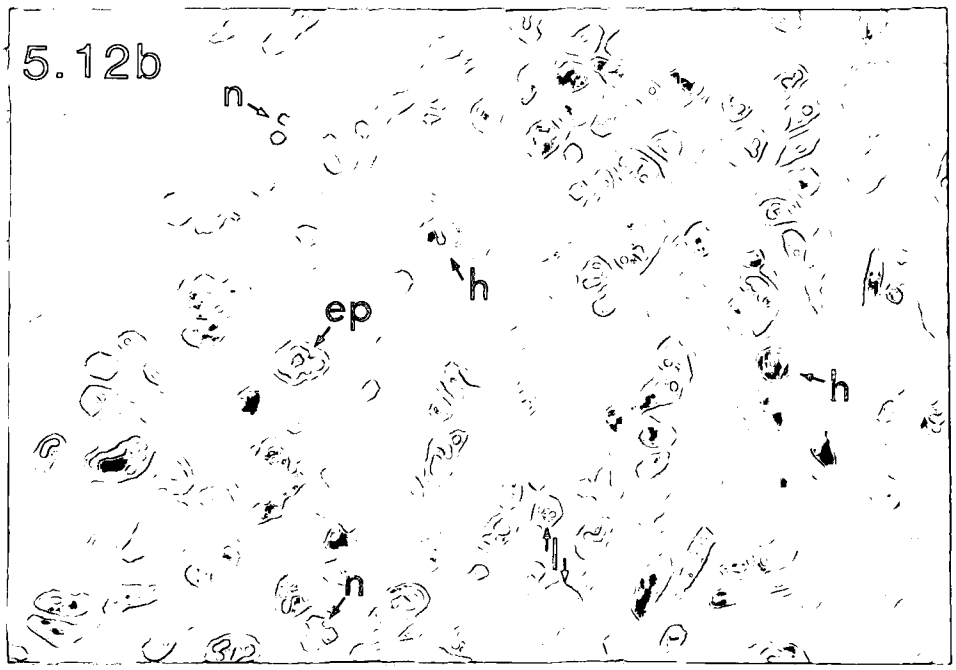
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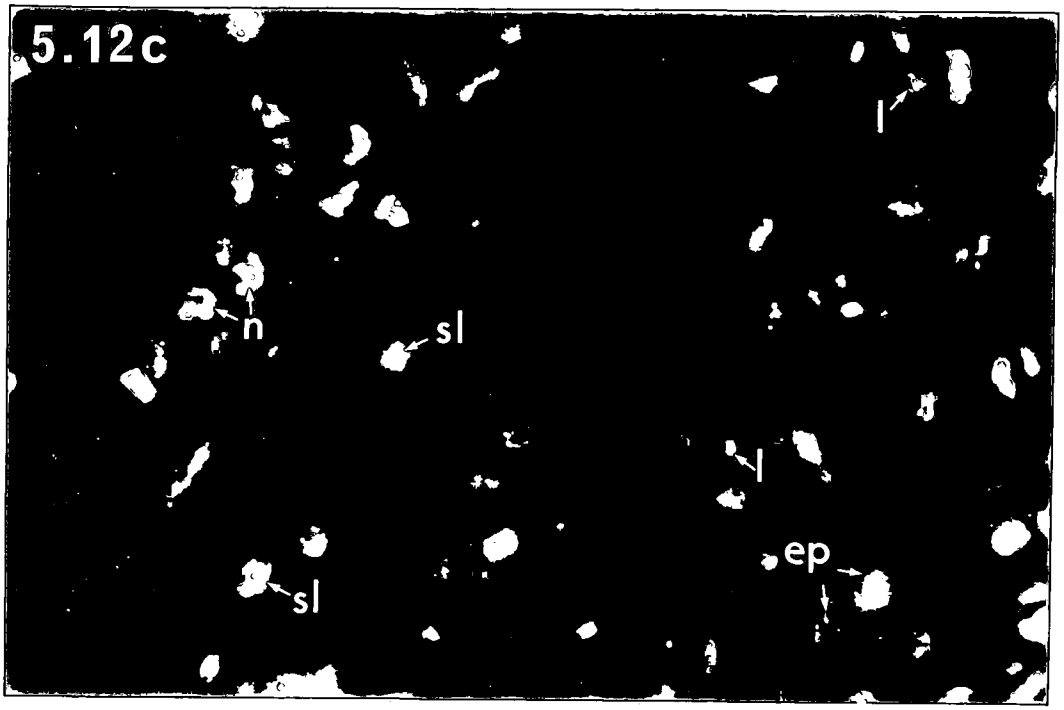
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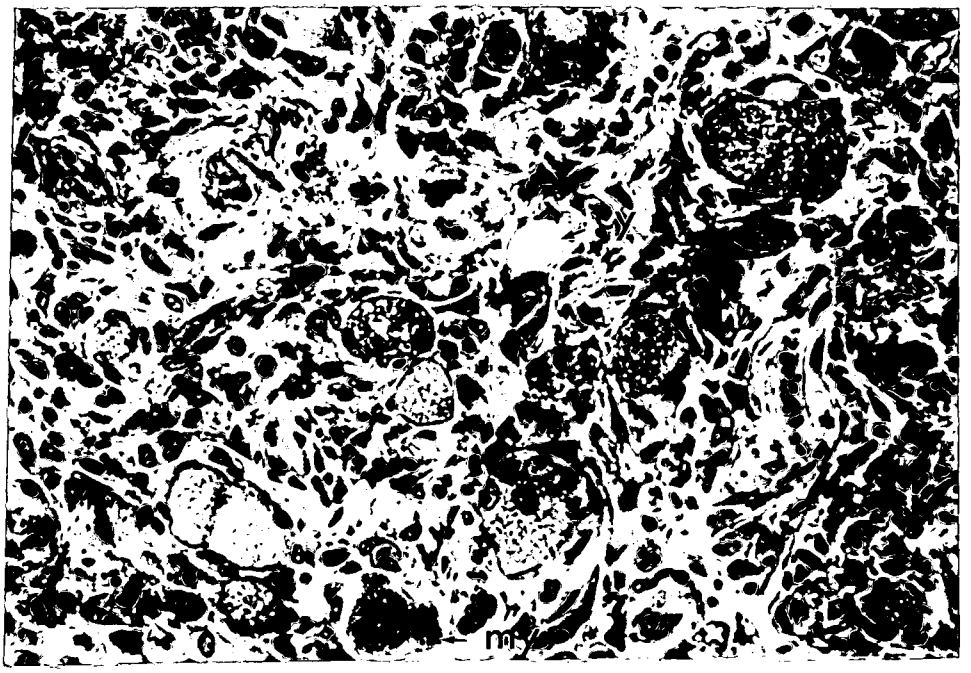

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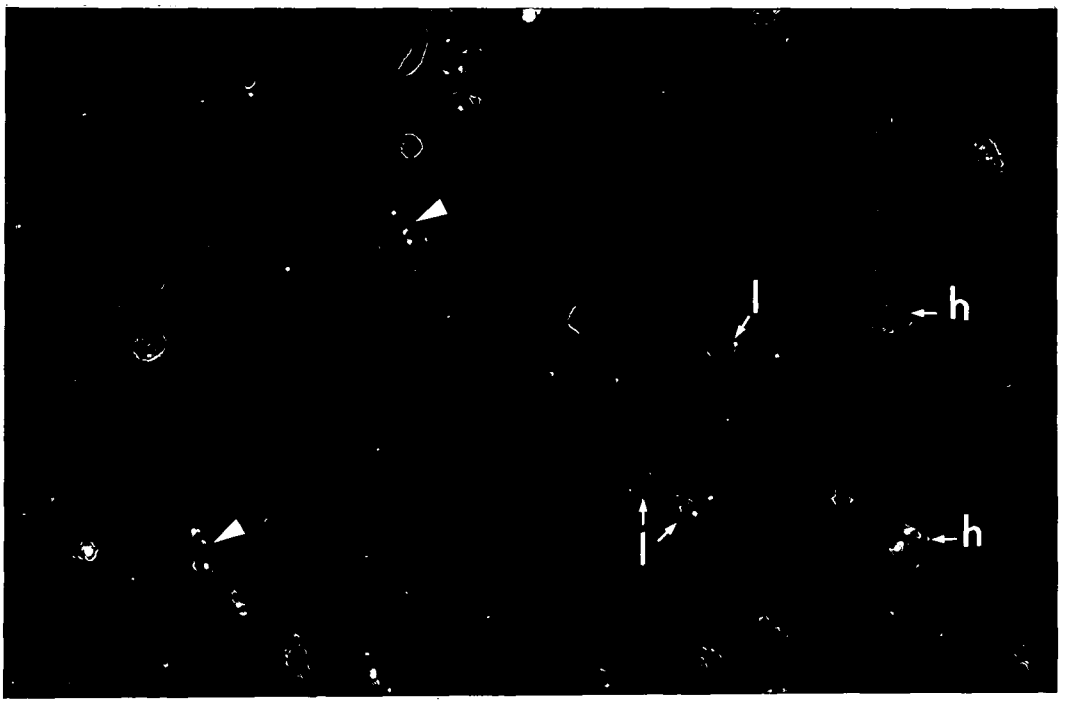

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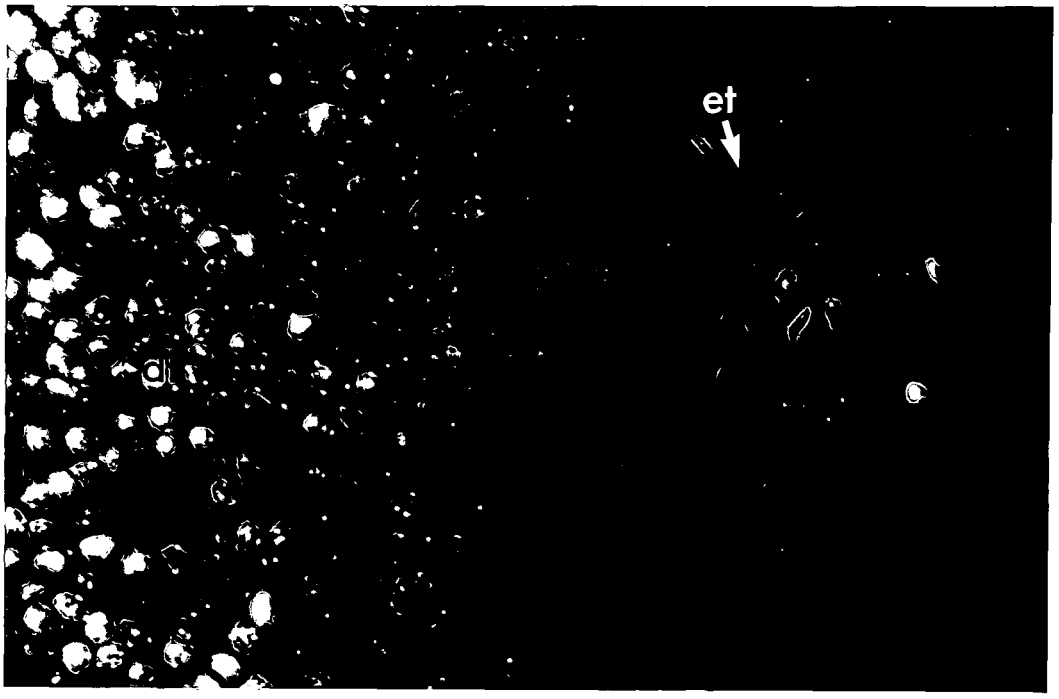

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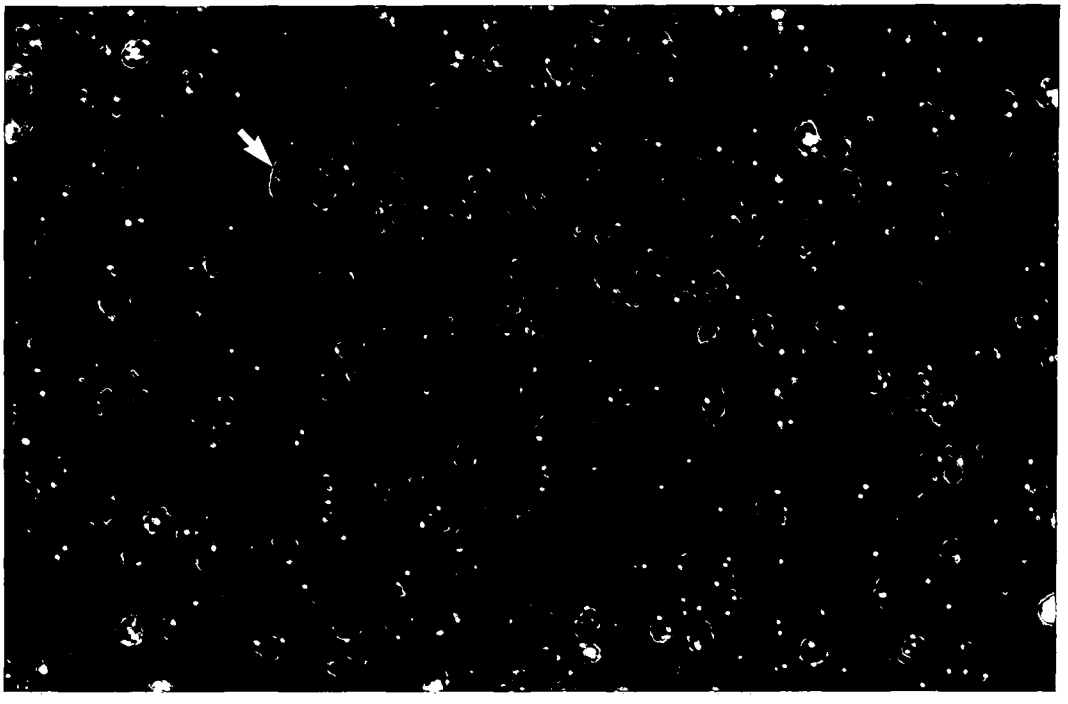

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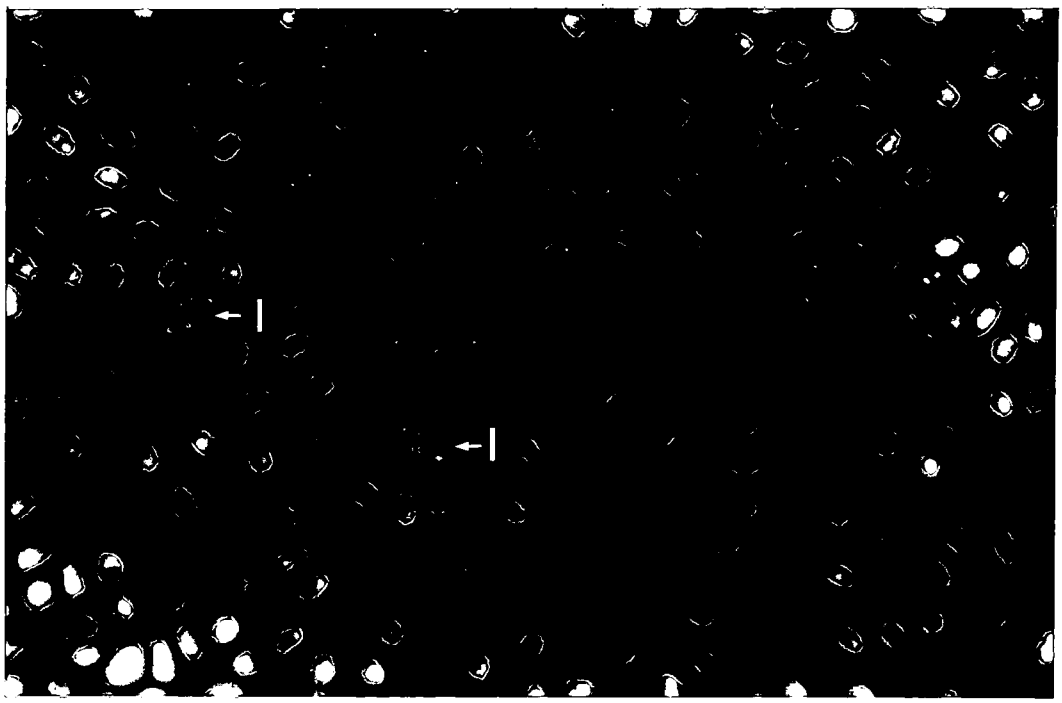

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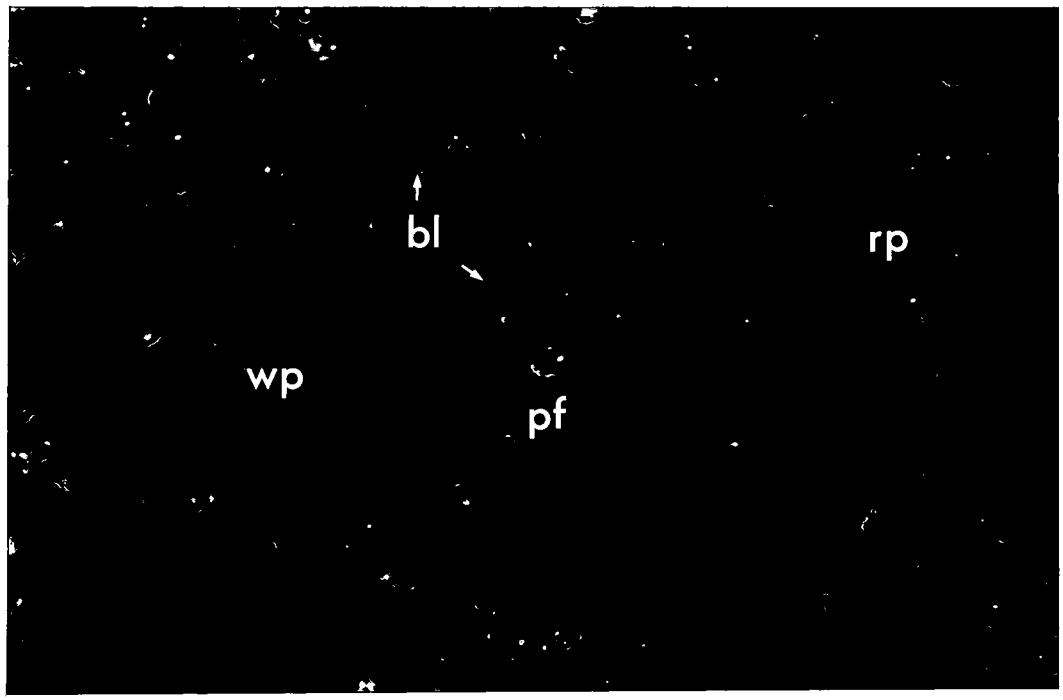

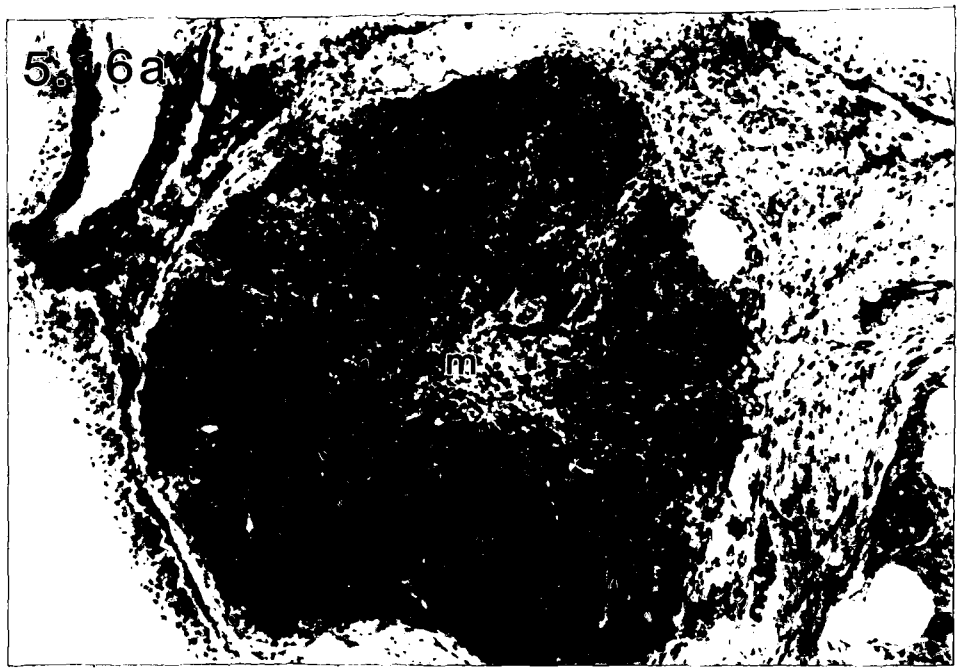

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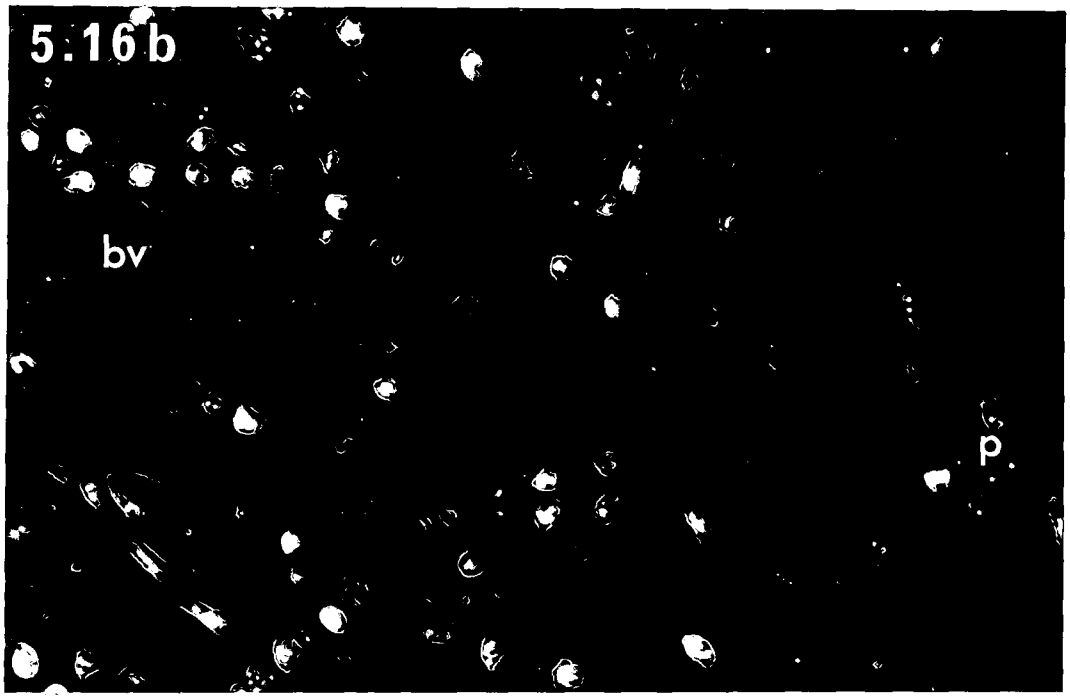

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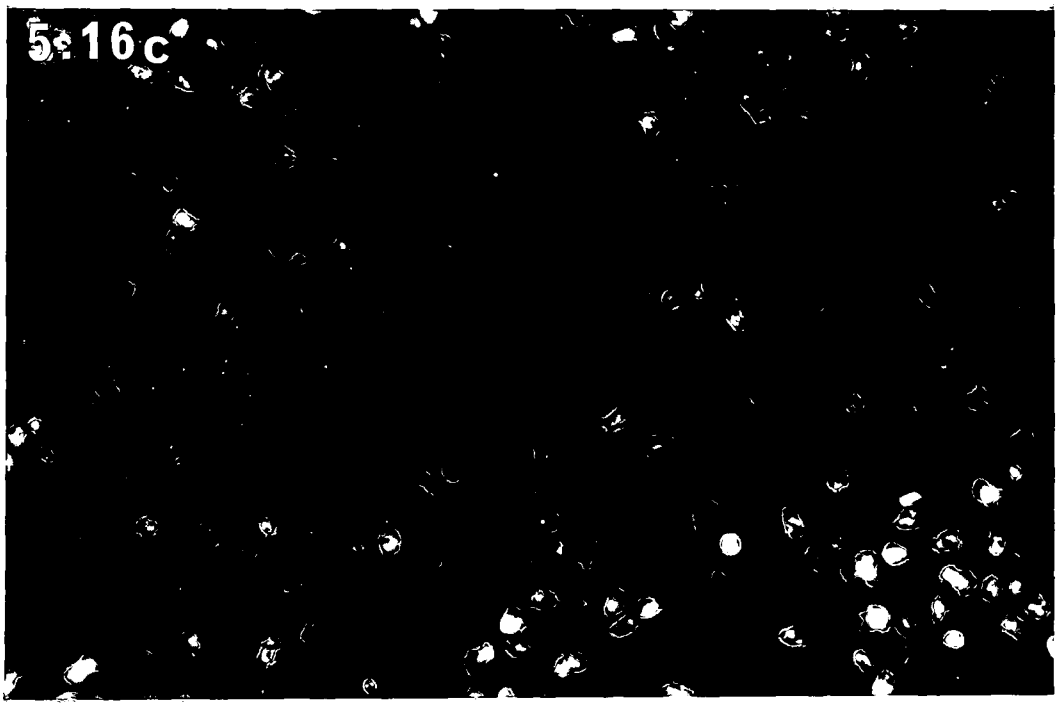

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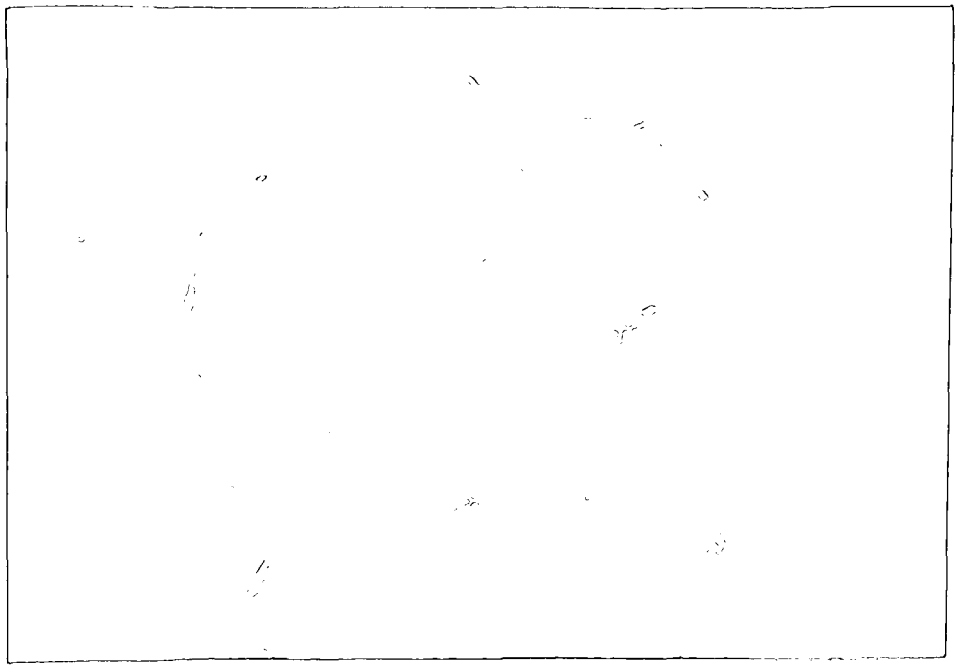

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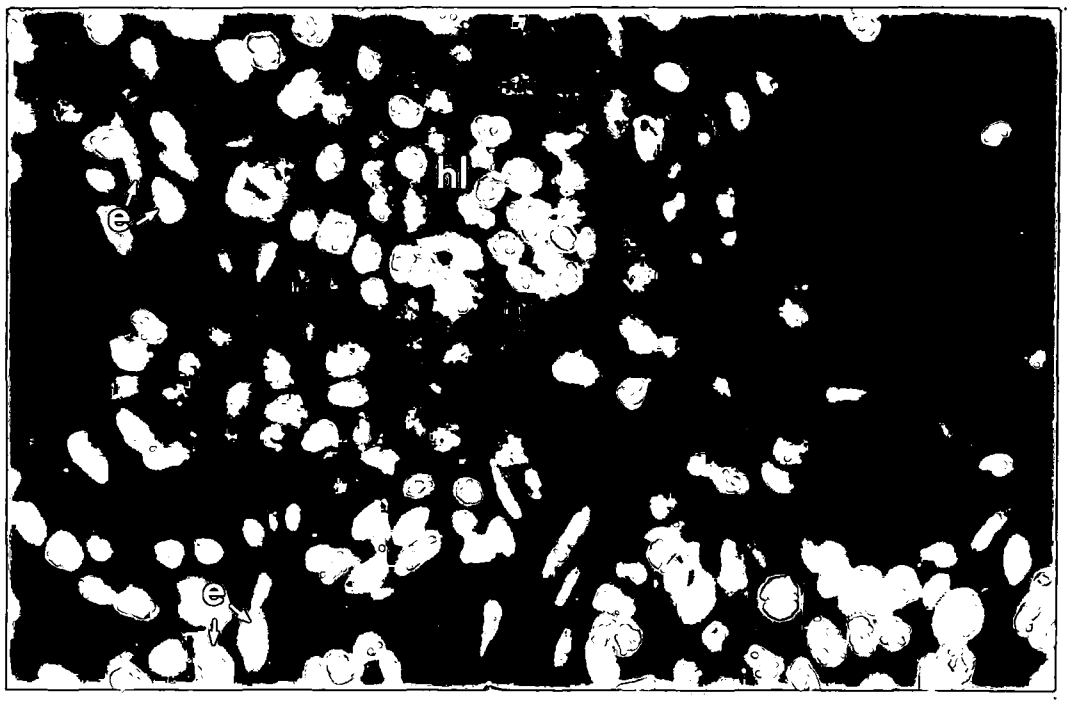

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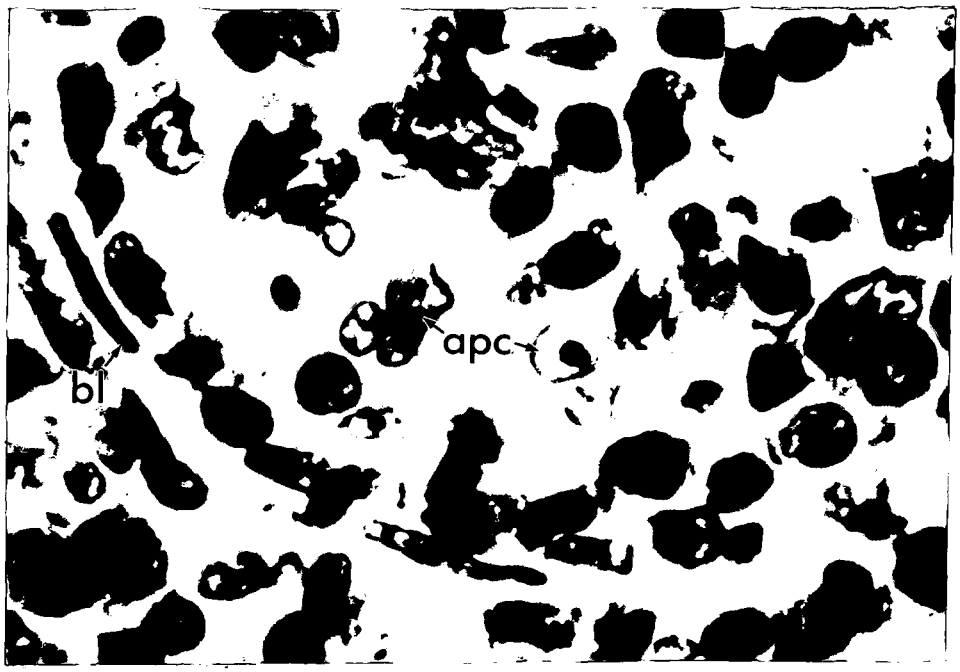

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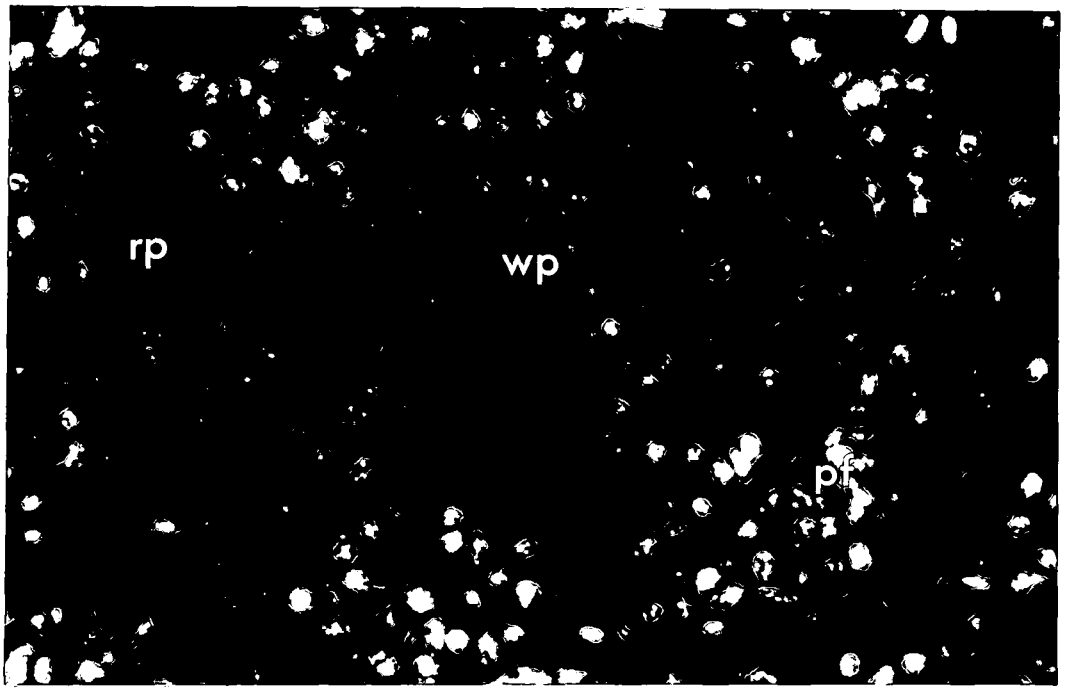

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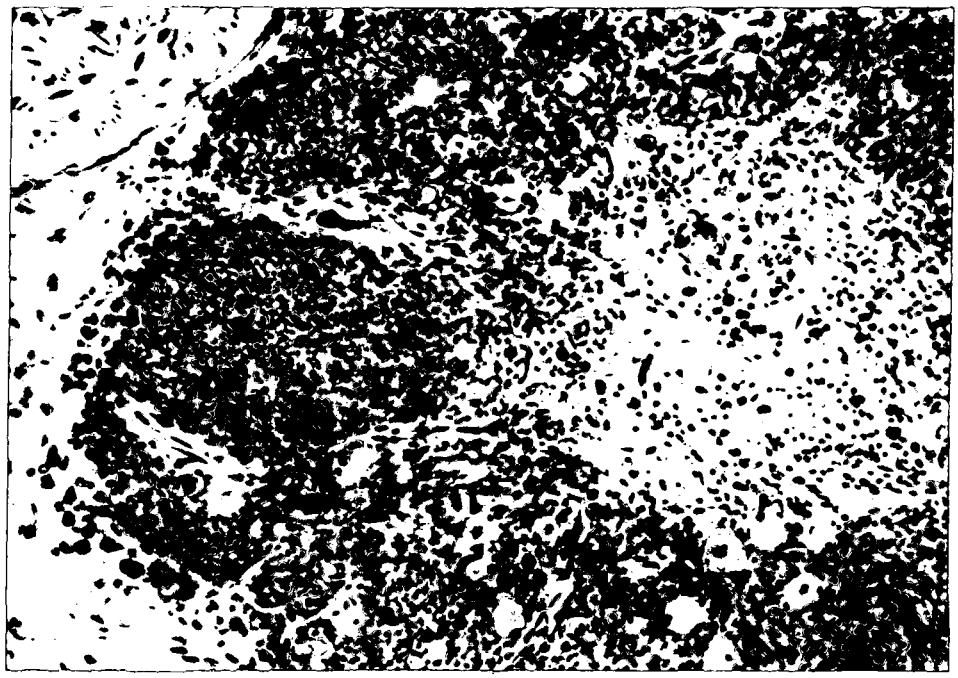

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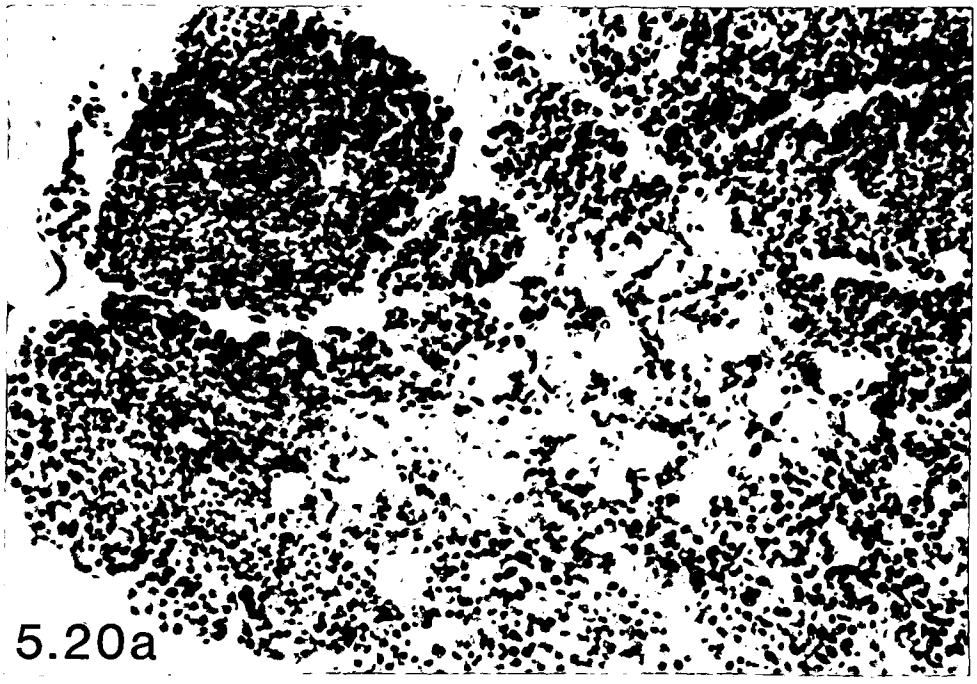

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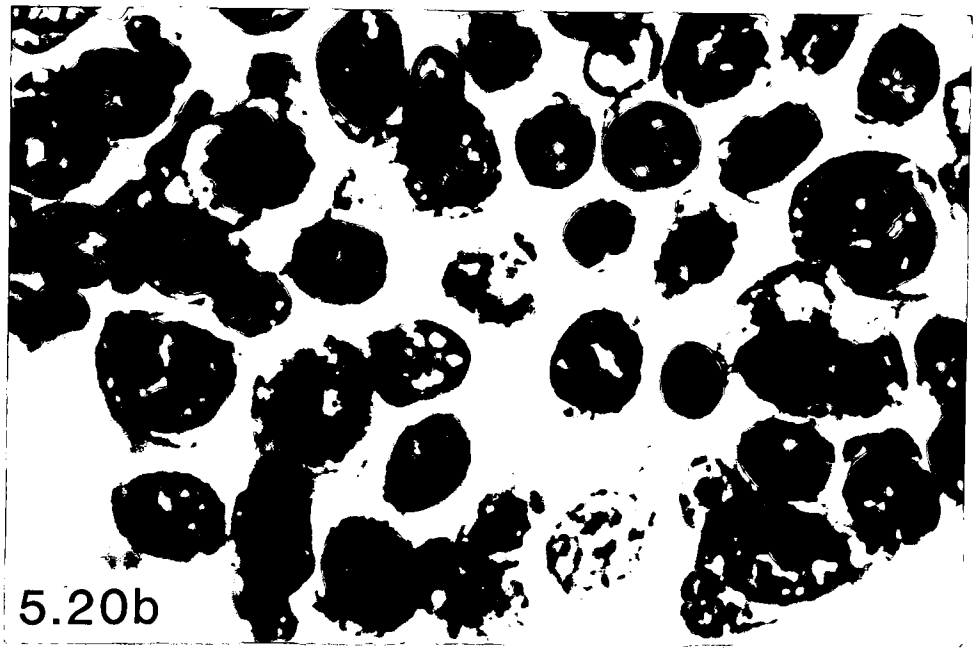

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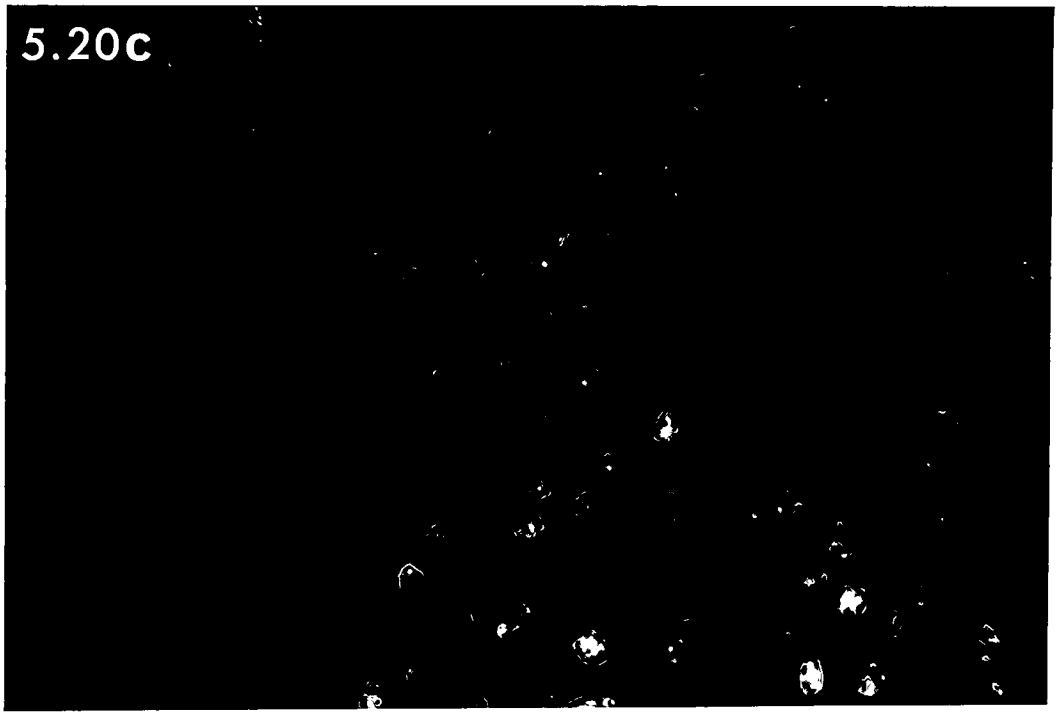

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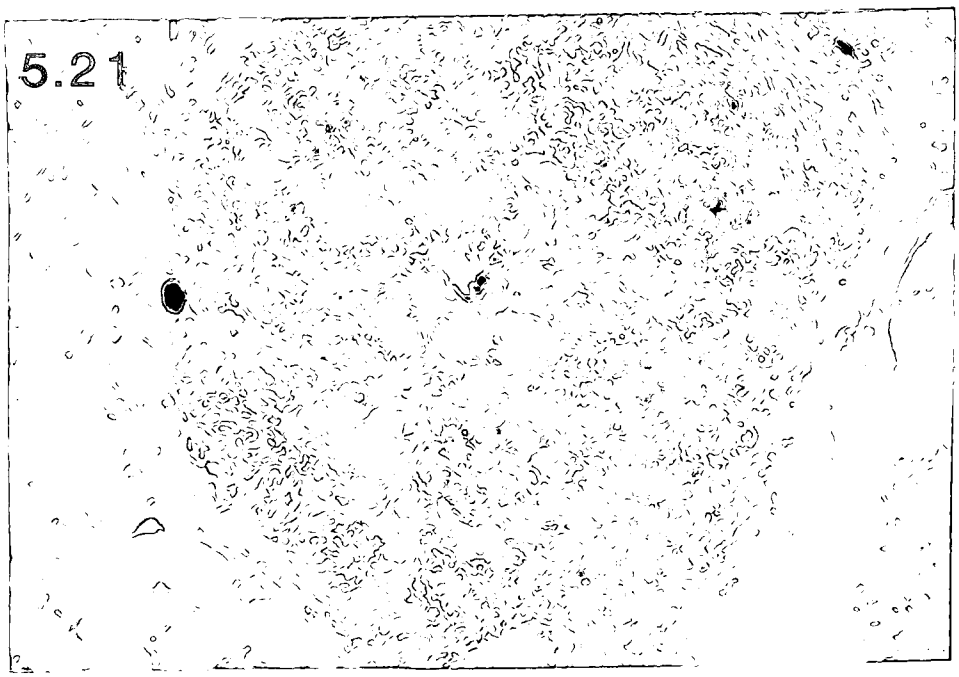
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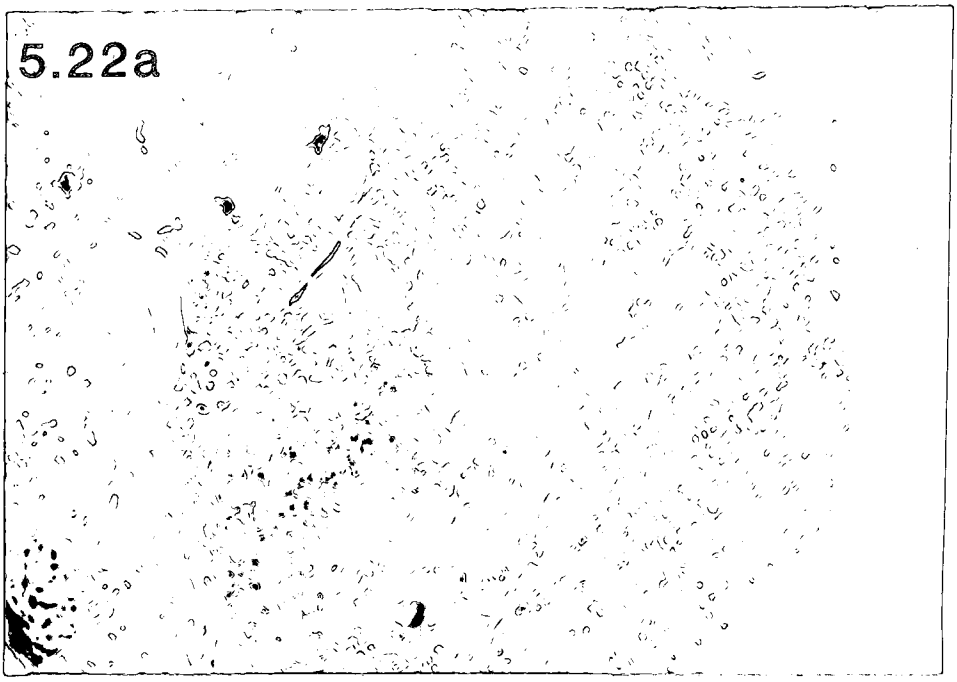
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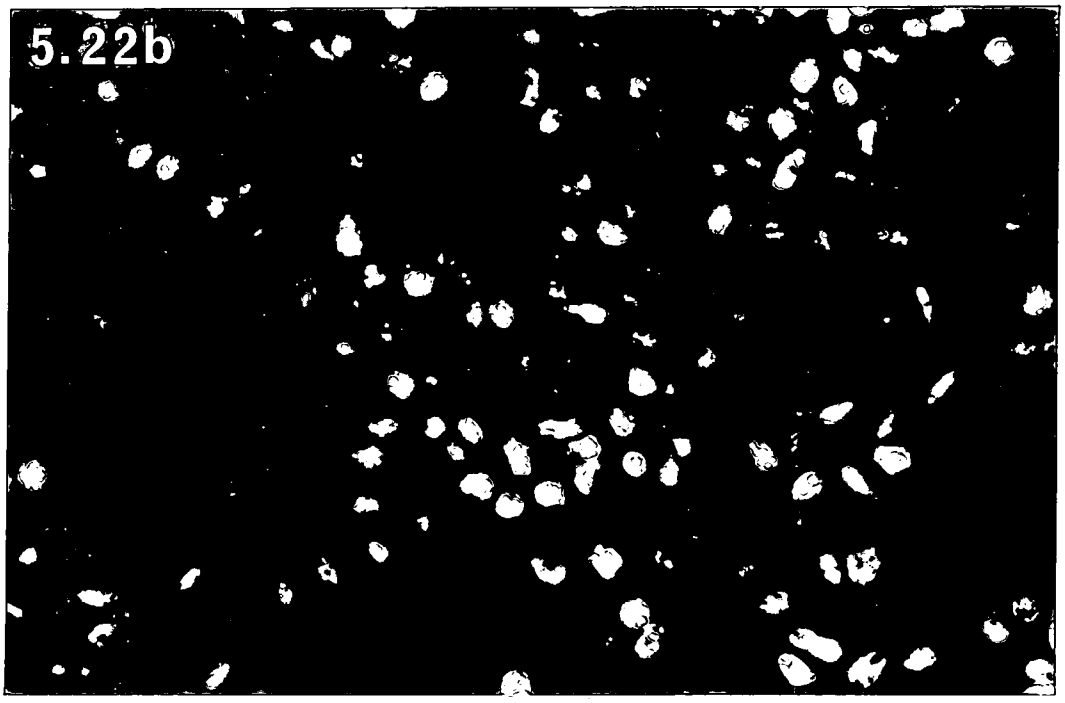
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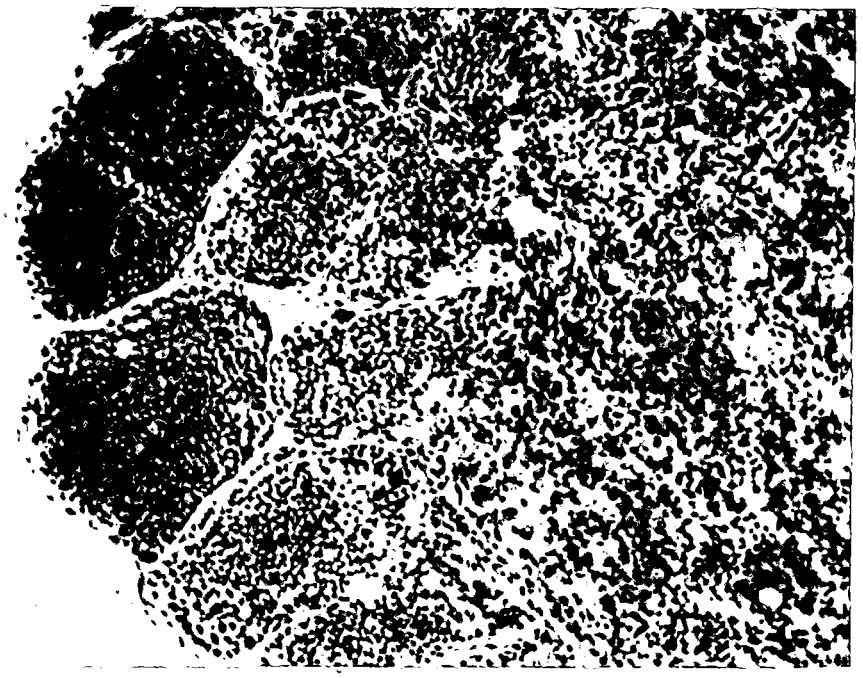

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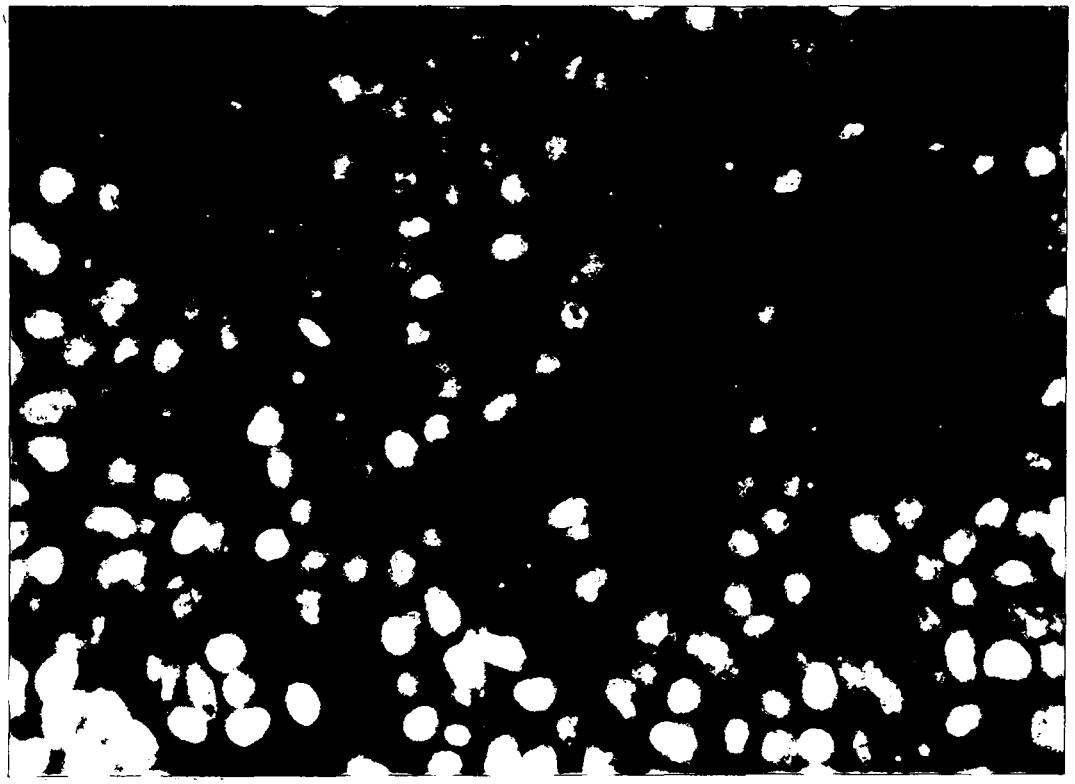

CHAPTER 6

PROLIFERATIVE RESPONSES OF THYMOCYTES TO T CELL MITOGENS AND TO ALLOANTIGENS : IN VITRO STUDIES ON NORMAL AND ORGAN-CULTURED THYMUSES

6.1 INTRODUCTION

This Chapter focusses attention on functional aspects of Xenopus thymocytes. Specifically, the experiments examine T cell proliferative responses of thymocytes from normal and organ-cultured thymuses to the mitogens PHA (phytohaemagglutinin) and Con A (concanavalin A), and in MLC (mixed lymphocyte culture).

Conditions required for obtaining optimal in vitro stimulation of thymocytes taken from in vivo-maintained froglet thymuses are examined first, as a forerunner to the studies in the next Chapter, which examine MLC reactivity of thymus implants. PHA and Con A are known to act as T-cell mitogens in anurans (Du Pasquier and Horton, 1976; Manning et al., 1976; Donnelly et al., 1976; Green-Donelly and Cohen, 1979). These mitogens induce polyclonal activation of lymphocytes (taken from a variety of lymphoid tissues - Green-Donelly and Cohen, 1979), causing them to proliferate and differentiate. In mammals, mitogens have been widely used as probes for functional sub-populations of thymocytes in vivo and in vitro (see Robinson, 1980 for review). Furthermore, the emergence of lymphocyte reactivity to mitogens is considered a sign of immunological maturation and has been used by many workers to examine the ontogeny of immunocompetence in various vertebrate species, including Xenopus (see Williams et al., 1983 for review; Rollins-Smith et al., 1984). MLC reactivity is also commonly used as a marker of immunocompetence. Thus when two populations of allogeneic lymphoid cells are mixed in vitro T cells are induced to proliferate in response to cell surface alloantigens.

Alloantigens encoded by genes of the major histocompatibility complex are the major stimuli of this response (see, for example, Roitt et al., 1985). MLC reactivity of peripheral lymphocytes, like T cell mitogen responsiveness, has been shown to be a T cell-dependent proliferative response in Xenopus (Du Pasquier and Horton, 1976). The ontogeny of MLC reactivity of thymocytes has been studied in detail by Du Pasquier and Weiss (1973).

Several workers have shown that the ontogeny of T cell function in organ-cultured foetal mouse thymus closely parallels the in vivo situation (Robinson and Owen, 1976 and 1977; Tufveson et al., 1976; Juhlin et al., 1977 and 1978). In these studies, immunocompetence was tested by measuring the ontogeny of MLC and cell mediated lympholysis reactivity, and by the development of mitogen responsiveness. Additionally, it was shown that the ontogenetic pattern of appearance of T-lineage-specific alloantigens (e.g. Thy-1, TL, Lyt) on in vitro-cultured thymocytes was characteristic of thymocytes developing in vivo. These findings demonstrate the validity of the in vitro model in mice, and show that T-lineage development can be supported by the thymic microenvironment alone, independent of extrinsic influences in vivo. The experiments on lymphocyte proliferation carried out here on the organ-cultured Xenopus thymus were designed to determine whether T cell reactivity remains comparable to the normal in vivo situation. This part of the study is seen as preliminary to any future work examining T cell responses of chimeric thymuses obtained by in vitro recolonization (e.g. transfilter experiments).

6.2 MATERIALS AND METHODS

6.2.1 Animals

The strains and ages of Xenopus used in these experiments are given in the Tables.

6.2.2 Cell culture of normal thymus

A. Standard Culture Conditions

Thymuses were removed aseptically from MS222-anaesthetized animals and placed in amphibian L15 culture medium (for formula see Chapter 2). Organs were teased apart in 30 mm plastic petri dishes with tungsten needles and clumps were dispersed by gently pipetting with a 1 ml pipetter (Anachem). Cells were transferred to Falcon 12 x 75 mm tubes and washed twice by 300 g centrifugation for 10 mins and removal of supernatant medium. Cells were resuspended in medium supplemented with 10% FCS.

For the mitogen experiments, the cell concentrations were adjusted to 2.0×10^6 lymphocytes/ml. 50 µl cells (i.e. 1×10^5 lymphocytes) were then plated out in tissue-culture treated, 96 well (V-shaped) places (Sterilin). Background and mitogen-treated cultures were set up in 3 or more replicate wells. Mitogens (freshly diluted in medium) were added in 10 µl aliquots. Background cultures received 10 µl medium instead. Cells were pulsed after 48 hrs of culture at 26 ± 1°C in a water saturated 5% CO_2 atmosphere, with 1 µCi 3HTdR (S.A. 5Ci/Mmol) (Amersham International). They were then harvested with a Skatron cell harvester at 72 hours; filters were dried at 60°C for 12 hours and then filter discs (with attached cellular material) were placed in Packard scintillation vials and 4 mls scintillation fluid (Betafluor, National Diagnostics) added. Samples were counted using a Packard β counter. S.I.s were calculated by dividing mean DPM mitogen-treated cultures by mean DPM background cultures. Mitogens used were

phytohaemagglutinin M (PHA, Flow) and Concanavalin A (Con A, Sigma). Final dilutions/concentrations of mitogens in culture were:-

PHA; 1:2000, 1:1000 and 1:500 (dilutions of the reconstituted sample),

Con A; 1 µg/ml, 5 µg/ml and 10 µg/ml.

For the MLC experiments, 50 µl (2×10^5) responder lymphocytes were cultured in 3 or more replicate V wells at 26± 1°C in a 5% CO_2 incubator, with 50 µl (2×10^5) stimulator cells. For the one-way experiments, the latter were irradiated with 6000 rads from the Co^{60} source. Cells were pulsed at 48-72 hrs with 1 µCi 3HTdR and harvested 24 hrs later. Scintillation counting was performed as above. For the two-way MLCs, stimulation indices were calculated by dividing the mean DPM of the mixed triplicate cultures by the mean counts of the corresponding controls.

$$\text{S.I.} = \frac{\text{mean DPM of mixed culture (A x B)}}{\text{mean DPM A alone + mean DPM B alone}}$$

$$\text{i.e. S.I.} = \frac{(A + B)}{(A + A \div 2) + (B + B \div 2)}$$

where (A + B) = total of 4×10^5 thymocytes from 2 different strains of Xenopus (experimental cultures)

(A + A) = 4×10^5 thymocytes from strain 'A'

(B + B) = 4×10^5 thymocytes from strain 'B'

) control triplicates

For one-way MLCs, stimulation indices are calculated by dividing the mean DPM of experimental cultures by the mean DPM of control cultures.

$$\text{S.I.} = \frac{(A + RB) - (RB + RB \div 2)}{(A + RA) - (RA + RA \div 2)}$$

where (A + RB) = Mean DPMs of 2×10^5A + 2×10^5 irradiated B (experimental cultures).

(A + RA) = Mean DPMs of 2×10^5A + 2×10^5 irradiated A (control cultures)

(RB + RB) and (RA x RA) = mean DPMs of triplicates comprising 4 x 10^5 irradiated cells of either strain 'A' or 'B'.

Statistical evaluation

The significance of stimulation indices in both PHA and MLC experiments were evaluated using the Student's 't' test , where P must be less than 0.05 for S.I.s to be considered positive.

B. Modifications of Culture Conditions

Attempts were made to optimize induced proliferative (particularly MLC) reactivity of thymocytes from normal thymuses by altering the standard culture conditions used above. Various parameters were examined such as the effect of varying cell concentration, using different shaped wells and culturing in serum-free medium supplemented instead with 0.5% glucose.

6.2.3 Mitogen and MLC studies on organ-cultured thymuses

Thymuses from normal or 3000 R-irradiated Xenopus (7 days post-in vivo irradiation) were organ-cultured as described in Chapters 2 and 3. In vivo maintained and organ-cultured thymuses were then dissociated as above for mitogen or MLC reactivity. Details of culture conditions are given in the Table legends. Some thymocyte cultures were fed at 24 hrs with 10 µl of a nutritive cocktail, as mentioned in the legends. The nutritive mixture consisted of 60% L15 base supplemented with 20% FCS, 20 mM Hepes buffer, 40 mM sodium bicarbonate, 10 mM l-glutamine, 5 x Eagles essential amino acids (from 50 x stock, Flow) and 5 x non-essential amino acids (from 100 x stock, Flow) (see Lallone, 1984).

6.3 RESULTS

6.3.1 Cell culture of normal thymus

A. Mitogen experiments

Thymocytes from all froglets (3.5 - 8.0 months of age) display proliferative responses to both the T cell mitogens PHA and Con A (Table 6.1). The best response to PHA in this particular experiment occurred at PHA concentrations of 1:1000 - 1:1250. Stimulation of thymocyte proliferation with Con A was highest when 5 µg Con A/ml was used.

A brief study on the effect of varying the cell concentration on PHA stimulation was carried out. Here 100 µl cells were added to each well (+ 20 µl PHA or medium). This experiment showed that as the number of thymocytes per well is increased so too were the mean DPMs of stimulated and control cultures (Table 6.2). Stimulation indices increased until 4.0×10^5 cells/well were used; with 8.0×10^5 lymphocytes/well, S.I.s at each PHA dilution declined. At all cell concentrations, PHA dilutions ranging from 1:500 - 1:2000 were stimulating, with PHA 1:500 and 1:1000 proving optimal. A concentration of 4.0×10^6 thymocytes/ml (4.0×10^5 cells/well produces the highest stimulations at 1:1000 and 1:500 dilutions.

B. MLC experiments

Two-way MLC between thymocytes taken from Xenopus of the same isogeneic (LG15) clone results in no induced proliferation (Table 6.3). In contrast, 2-way MLC between MHC-disparate strains of Xenopus generally effected good stimulation indices, when cultures were harvested at day 3. The outcome of harvesting cells on day 4 of culture varied. Thus in experiment number 3 (J x LG3 coculture - see Table 6.3) a higher S.I. occurred on day 4 than on day 3, when 4.0×10^5 thymocytes were cocultured, whereas in two other experiments (numbers 1 and 2) (J x LG3

and J x LM3) comparing S.I.s at day 3 and 4 when numbers of cocultured cells were only 2.0 x 10^5 cells, the 3 day harvest time proved optimal. The mean DPMs of both background and mixed cultures increase as cell numbers increase (see Experiments 1 and 2), but this does not necessarily result in higher S.I.s. The experiments suggest that if sufficient thymocyte numbers are available for study, then 4.0 x 10^5 cells/well and harvesting on day 4 may be the most suitable method for obtaining good S.I.s in 2-way MLC. If fewer thymocytes are available, 2.0 x 10^5 cells plated per well and harvesting the MLC on day 3 appears adequate. The data illustrates the necessity of performing kinetic studies, wherever possible.

One-way MLC stimulation, where a total of 4 x 10^5cells/well are cultured (2 x 10^5responders + 2 x 10^5irradiated stimulators) are shown in Table 6.4 Excellent MLC indices are given in all allogeneic thymocyte combinations used, when the cultures were harvested on day 3. The few cultures harvested on day 4 proved to display poorer, or insignificant stimulation.

The effect of varying the cell concentration (from 1-4 x 10^5thymocytes per well) was examined in Table 6.5 Wells with 1 and 2 x 10^5 responders displayed consistently good S.I.s, whereas 5 x 10^4responders appeared, generally, too few to consistently respond to cocultured (5 x 10^4) irradiated stimulators, since in $^3/_5$ cases S.I.s were not significant.

C. Attempts to improve MLC stimulation indices

(i) Well-shape

Neither U- nor flat-bottomed wells proved satisfactory for thymocytes in one-way MLC (Table 6.6). The V-shaped wells may provide closer cell contact, essential for inducing thymocyte MLCs.

(ii) Glucose supplementation

Thymocytes respond better in one-way MLC when 10% FCS is used to supplement the culture medium than when 0.5% glucose was added to serum-free amphibian medium (Table 6.7). Although mean DPMs of control cultures remain about the same in both types of medium, counts in the mixed (experimental) cultures were much lower in the glucose-supplemented cells. FCS must therefore improve conditions for allo-antigen driven proliferation of T cells. 0.5% glucose has been used successfully by other workers culturing Xenopus lymphocytes (L. Du Pasquier, personal communication).

6.3.2 Mitogen and MLC studies on organ-cultured thymuses

An initial experiment showed that thymocytes from 12-day organ-cultured thymuses display excellent T cell proliferative ability (comparable to in vivo-maintained organs) when stimulated by the mitogens PHA and Con A (Table 6.8). Organ-cultured thymuses had to be pooled prior to use, approximately 3 were required for each experiment to get sufficient numbers of lymphocytes to plate out 1×10^5 cells per well. Further experiments (see Table 6.9) showed that mean DPMs of both background and PHA-stimulated cultures were generally elevated following organ-culture. Higher S.I.s of PHA-treated organ-cultured thymocytes were achieved than when thymocytes were taken straight from the animal. In the MLC experiments (Table 6.9) mean DPMs of control and experimental (mixed) cultures (when 1×10^5 responders were used) were again higher for organ-cultured thymocytes, compared with cells taken directly from the animal. MLC S.I.s were similar between experimental groups, although when 5×10^4 organ-cultured thymocytes were placed in MLC, S.I.s were particularly good.

One final experiment examined the proliferative potential of thymocytes remaining 7 days post 3000R - *in vivo* irradiation, and following organ-culture for 10 days (Table 6.9). These cells could, surprisingly, be induced to proliferate well with PHA. This one experiment necessitated the use of a pool of 12 organ-cultured thymuses!

6.4 DISCUSSION

The initial experiments, examining proliferative responses of cells taken from normal froglet thymuses, confirmed the work of others that Xenopus thymocytes can proliferate well to T cell mitogens (see Williams et al., 1983) and to alloantigens (Du Pasquier and Weiss, 1973). Thymocytes from animals ranging from 3½ to 8 months and thymocyte numbers ranging from 1-8 x 10^5 per well were effectively stimulated to proliferate when treated with either PHA or Con A. Thymocytes from Xenopus froglets respond in one-way MLC when responder cell numbers were 2 x 10^5/well or as low as 1 x 10^5/well, whereas 5 x 10^4 responders gave inconsistent MLC stimulation indices. Technical requirements for obtaining MLC reactivity of Xenopus thymocytes (and splenocytes) have been examined previously. Parameters such as culture medium, serum concentration, antibiotics, cell density, duration of culture, area of culture surface, time of pulsing and specific activity of tritiated thymidine, were all found to affect DNA synthesis in vitro of both control and stimulated cultures (see Weiss and Du Pasquier, 1973 for review). Here, modifying the well-shape, by testing U- and flat-bottomed wells instead of V-wells, and altering the culture medium by using 0.5% glucose instead of 10% FCS, did not increase 3HTdr uptake by thymocytes, nor improve thymocyte alloreactivity in MLC.

Functional studies on thymocytes from organ-cultured embryonic mouse thymuses by Robinson and Owen (1976) have demonstrated a five-fold increase in the magnitude of mitogen responses, compared with reactivity of in vivo-maintained thymus. Mitogen responses were maximal in organ cultures of around 12 days duration, when TL negative, cortisone-resistant cells were reaching a peak. Tufveson et al. (1976) and Juhlin et al. (1977 and 1978) also found that lymphocytes which

had developed in 14-day organ-cultured embryonic mouse thymus (14 days old at the beginning of culture) using serum-free medium, showed strong mitogen- and allo-reactivity. My results also suggest an increased mitogen response of thymocytes taken from froglet thymuses organ cultured for 12 days. This enhanced mitogen reactivity might be due to the accumulation of PHA-reactive cells that would otherwise have emigrated from the thymus in vivo. Also, intrathymic stem cells may not proliferate indefinitely and the enrichment of mature (PHA-reactive) thymocytes could be due to the maturation of stem cells and the inability of new stem cells to enter the thymus in vitro and give rise to further populations of immature cells (Robinson and Owen, 1976). The higher levels of background 3HTdR incorporation seen in organ-cultured thymocytes may relate to this increased number of mitogen-reactive cells, some of which are also able to proliferate in response to FCS antigens - as can splenic lymphocytes cultured in FCS-supplemented media (see Horton et al., 1980).

This brief functional study on organ-cultured froglet thymus suggests that the culture system used can support normal T cell function. This finding proves promising for future in vitro thymus recolonization experiments with allogeneic/syngeneic stem cells, using, for example, transfilter, coculture or hanging-drop culture techniques (see Chapter 4, Discussion).

Surprisingly, in vivo-irradiated, organ-cultured thymocytes, although very few in number, still reacted well to PHA stimulation. It has already been shown in this Thesis that 3000-rad treated thymus, removed 7 days post-irradiation, still retains a reduced lymphocyte population within the thymic medulla (see Figure 3.3, Chapter 3). These radioresistant, culture-resistant, immunoreactive (medullary) lymphocytes must therefore not be excluded from playing a role in

the restoration of T-cell reactivity seen when irradiated thymuses are transferred to T x *Xenopus*.

MLC reactivity of thymocytes removed from thymus implants, which are often reduced in size compared with the normal thymus, should therefore present no major technical problems. This issue is the subject of the next Chapter, where MLC reactivity of cells within thymus implants is compared with the *in vitro* allorecognition capabilities of the host's splenic lymphocyte population.

Table 6.1

The proliferative response of thymocytes from froglets of various ages to PHA and Con A using standard culture conditions

Animal Age (months)	Background DPM ± S.E.	PHA Final Dilution DPM ± S.E. (S.I. + P value)			Con A Final Concentrations DPM ± .S.E. (S.I. + P value)		
		1:1250	1:1000	1:500	1 µg/ml	5 µg/ml	10 µg/ml
3.5	623 ± 56	2877 ± 208 (4.6 < 0.001)	2269 ± 208 (3.6 < 0.002)	1136 ± 105 (1.8 < 0.02)	1969 ± 176 (3.2 < 0.002)	3378 ± 382 (5.4 < 0.002)	734 ± 19 (1.2 N.S.)
3.5	727 ± 85	3304 ± 146 (4.6 < 0.001)	2918 ± 368 (4.0 < 0.01)	2009 ± 103 (2.8 < 0.001)	2477 ± 614 (3.4 < 0.005)	2900 ± 330 (4.0 < 0.01)	808 ± 100 (1.1 N.S.)
5.0	362 ± 87	1908 ± 92 (5.3 < 0.001)	2577 ± 286 (7.1 < 0.002)	N.D.	N.D.	N.D.	N.D.
7.0	645 ± 67	N.D.	7703 ± 259 (11.9 < 0.001)	N.D.	N.D.	6192 ± 320 (9.6 < 0.001)	4294 ± 374 (6.7 < 0.001)
8.0	575 ± 42	N.D.	2169 ± 400 (3.8 < 0.02)	N.D.	N.D.	N.D.	N.D.

Pulse: 48 hrs. Harvest: 72 hrs.

10% FCS. Means from triplicate (or more) cultures.

Cell no. = 1 x 10^5 lymphocytes/well (2 x 10^6 lymphocytes/ml)

J strain X.laevis. 50 µl cells + 10 µl mitogen (or medium)

Table 6.2

PHA stimulation of thymocytes from normal thymus: effect of cell concentration

Cell Nos. per well (x 10^5)	Background DPM (± SE)	PHA 1:2000		PHA 1:1000		PHA 1:500	
		DPM	S.I.	DPM	S.I.	DPM	S.I.
1.0	456 (±251)	2273 (±294)	5.0	4275 (±434)	9.4	4253 (±321)	9.3
2.0	834 (±166)	5369 (±228)	6.4	9867 (±980)	11.8	8128 (±672)	9.8
4.0	1066 (±223)	11987 (±1325)	11.3	17530 (±1325)	16.5	17757 (±2135)	16.7
8.0	4412 (±764)	22628 (±602)	5.1	47332 (±2281)	10.7	47721 (±3803)	10.8

Pulse: 48 hrs. Harvest: 72 hrs.

10% FCS. Triplicate Cultures.

6 month wild Xenopus laevis.

All S.I.s = significant.

100 µl cells + 20 µl PHA or medium.

Table 6.3

Two-way stimulation of thymocytes from normal thymus: effect of cell concentration

Expt.	Combination (Ages of thymocyte donors)	Total lymphocytes nos. per well (x 10^5)	Harvest day 3 DPM ± SE Background cultures	Harvest day 3 Mixed culture	Harvest day 3 S.I.	Harvest day 3 P value	Harvest day 4 DPM ± SE Background cultures	Harvest day 4 Mixed culture	Harvest day 4 S.I.	Harvest day 4 P value
1.	J x LM3 (5 mths) (7 mths)	4	(LM) 2007 ± 389 (J) 447 ± 36	3707 ±539	3.0	<0.001				
		2	(LM) 820 ± 105 (J) 335 ± 162	2010 ±375	3.5	<0.02	(LM) 1121 ± 227 (J) 368 ± 147	996 ±150	1.1	N.S.
		1	(LM) 741 ± 99 (J) 179 ± 47	1792 ± 203	3.9	<0.001				
2.	J x LG3 (6 mths) (6 mths)	4	(LG) 5980 ± 1046 (J) 1093 ± 158	8077 ±296	2.3	<0.001				
		2	(LG) 2355 ± 411 (J) 436 ± 50	4149 ±585	3.5	<0.01	(LG) 1575 ± 375 (J) 528 ± 51	2723 ±234	2.6	<0.001
3.	J x LG5 (6 mths) (4 mths)	2	(J) 600 ± 47 (LG) 2238 ± 250	2621 ±485	1.9	<0.05				
	J x LG3 (6 mths) (5 mths)	4	(J) 2844 ± 647 (LG) 3174 ± 1092	7576 ±202	2.5	<0.001	(J) 1038 ± 64 (LG) 2169 ± 200	8917 ±173	5.6	<0.001
	LG15 x LG15 (1) (2) (9 mths) (9 mths)	4	(1) 1036 ± 329 (2) 1187 ± 187	1078 ±122	1.0	N.S.				

Pulse 24 hrs prior to harvest.
10% FCS. Triplicate (or more) cultures.
100 µl lymphocytes per well (i.e. 50 µl from each partner in MLC).

Table 6.4

One-way MLC stimulation of thymocytes from normal thymus: standard culture conditions

Expt. No.	Cell combination (Ages of thymocyte donors)	Total thymocyte no. per well ($\times 10^5$)	Harvest day 3				Harvest day 4			
			DPM ± SE				DPM ± SE			
			Resp. x R.Autol.	Resp. x R.Allo.+	S.I.	P value	Resp. x R.Autol.	Resp. x R. Allo.	S.I.	P value
1.	10 mths J x RLG15	4.0*	455 ± 9	4354 ± 436	9.9	< .001	812 ± 217	4130 ± 446	5.2	< .001
	10 mths J x RLG3	4.0	455 ± 9	3561 ± 182	8.1	< .001				
	9 mths LG15 x RJ	4.0	340 ± 24	1199 ± 262	5.3	< .01	410 ± 113	587 ± 42	1.4	N.S.
	9 mths LG15 x RLG3	4.0	340 ± 24	1754 ± 424	7.5	< .01				
	14 mths LG3 x RJ	4.0	175 ± 22	671 ± 233	6.8	< .05	306 ± 70	401 ± 73	1.2	N.S.
	14 mths LG3 x RLG15	4.0	175 ± 22	645 ± 109	5.6	< .001				
2.	10 mths J x RLG3	4.0	815 ± 411	5495 ± 181	6.7	< .001	N.D.			
	12 mths LG17 x RJ	4.0	418 ± 33	1854 ± 505	4.4	< .05				
	14 mths LG3 x RJ	4.0	193 ± 62	1190 ± 242	6.2	< .02				
	14 mths LG3 x RLG17	4.0	193 ± 62	2762 ± 117	14.3	< .001				

Pulse: 24 hrs prior to harvest.
10% FCS. Triplicate (or more) cultures.
* Responder cell nos. = 2×10^5/well. Total volume per well = 100 µl.
\+ Stimulators = 6,000R irradiated cells from one of the animals that also supplied responder cells in that experiment.

Table 6.5

One-way MLC stimulation of thymocytes from normal thymus: effect of cell concentration

Expt. No.	Age responders	Cell combination	Total cell no. per well ($\times 10^5$)*	Harvest day 3 DPM ± SE Resp. x R.Autol.	Harvest day 3 DPM ± SE Resp. x R. Allo.	Harvest day 3 S.I.	Harvest day 3 P value	Harvest day 4 DPM ± SE Resp. x R.Autol.	Harvest day 4 DPM ± SE Resp. x R. Allo.	Harvest day 4 S.I.	Harvest day 4 P value
1.	7 mth	LM3 x RJ	4.0	893±148	2869±326	3.5	<.001	ND			
	5 mth	J x RLM3	4.0	346±53	901±86	2.5	<.001				
		LM3 x RJ	2.0	488±80	1283±178	3.1	<.001	555±72	849±92	1.7	N.S.
		LM3 x RJ	1.0	525±98	823±242	1.7	N.S.	ND			
		J x RLM3	1.0	338±127	372±55	1.1	N.S.				
2.	5 mth	LG3 x RJ	4.0	1638±233	4025±386	2.6	<.001	ND			
	8 mth	J x RLG3	4.0	591±141	2358±367	3.9	<.001				
		LG3 x RJ	2.0	664±154	1516±201	2.3	<.01	536±99	1372±198	2.5	<.02
		J x RLG3	2.0	490±113	1132±262	2.3	<.05	164±62	698±154	4.7	<.05
		LG3 x RJ	1.0	381±42	450±53	1.2	N.S.	ND			
		J x RLG3	1.0	216±48	741±49	3.7	<.002				
3.	6 mth	J x RLG5	2.0	403±73	1076±92	3.8	<.001	ND			
		J x RLG5	1.0+	477±102	1012±180	2.9	<.01				

Pulse: 24 hrs prior to harvest.
10% FCS. Triplicate (or more) cultures.
* Total volume = 100 µl (i.e. 50 µl cells from both responder and stimulator with responder cell nos. varying from 0.5 - 2 x 10^5).
+ In this experiment, only 25 µl (5 x 10^4) were used from each partners.

Table 6.6

One-way MLC stimulation of thymocytes from normal thymus: Effect of well shape

Cell combination	Total no. cells/well (x 10^5)	Well Shape	DPM ± SE Responder x R.Autol.	Responder x R. Allo.	S.I.	P value
J x RLG5 6 mths 4 mths	2.0	V	403 ± 73	1076 ± 92	3.8	<0.001
	2.0	U*	482 ± 150	566 ± 32	1.1	N.S.
	2.0	⌊⌋+	281 ± 27	375 ± 77	1.3	N.S.

10% FCS.

Pulsed on day 2.

Harvest day 3.

Triplicate (or more) cultures.

100µl cells total (1 x 10^5 from responder, 1 x 10^5 stimulator)

* = U-shaped wells (Costar)

+ = ⌊⌋-shaped wells - ½ size diameter (Costar)

Table 6.7

One-way MLC stimulation of thymocytes from normal thymus: Effect of supplementation with 0.5% glucose rather than 10% FCS.

Cell combination	Total no. cells/well (x 10^5)	Type of Supplementation	DPM ± SE Responder x R.Autol.	Responder x R. Allo.	S.I.	P value
J x RLG5 6 mths	4.0	10% FCS	626 ± 147	3116 ± 258	5.1	<0.001
LG5 x RJ 5 mths	4.0	10% FCS	853 ± 46	2327 ± 170	3.0	<0.001
J x RLG5	4.0	0.5% glucose	935 ± 85	1713 ± 220	1.9	<0.01
LG5 x RJ	4.0	0.5% glucose	773 ± 64	1668 ± 99	1.8	<0.001

Pulsed day 2.

Harvest day 3

Triplicate (or more) cultures.

100 µl cells total (i.e. 2 x 10^5 responder, 2 x 10^5 stimulator)

Cultures were supplemented with either FCS or glucose.

Table 6.8

PHA and Con A stimulation of thymocytes from organ-cultured and normal thymus

Origin thymocytes	Total cell nos. per well ($\times 10^5$)	Background DPM ± SE	PHA 1:1000		Con A 5 µg/ml		
			DPM ± SE	S.I.	Background DPM ± SE	DPM ± SE	S.I.
In vivo							
maintained	1.0	1257 ± 92*	7205 ± 552	5.7	1257 ± 92	5838	4.6
	1.0	438 ± 116+	2785 ± 136	6.4			
	1.0	322 ± 140+	1516 ± 169	4.7			
In vitro							
maintained	1.0	1167 ± 62*	11760 ± 207	10.1	1167 ± 62	7800	6.7
(organ cultured for 12 days)	1.0	1315 ± 256+	6828 ± 746	5.2			

Pulse: 48 hr. Harvest: 72 hrs.

10% FCS, triplicate cultures.

All stimulation indices are significant.

Animals+ Wild X.laevis - 7 month

* J Xenopus laevis - 4 month.

Table 6.9

PHA and MLC experiments with normal and in vitro maintained J strain froglet thymuses

J strain thymocytes	Mitogen Experiments				MLC Experiments			
	Day Harvest	Background DPM ± SE	PHA 1:500 DPM ± SE	S.I.	Day Harvest	Responder x R. Autol.	Responder x R. Allo. (LG17 or LG15)	S.I.
In vivo maintained	3	750 ± 41	2118 ± 173	2.8	3	719 ± 159	1537 ± 294	2.1
	3	639 ± 30	2491 ± 272	3.9	3	571 ± 34	1235 ± 98	2.2
	3	912 ± 307	2068 ± 226	2.3	3	571 ± 51	1059 ± 66	1.9
	3	468 ± 47	1546 ± 127	3.3	4	724 ± 79	1246 ± 217	1.7
In Vitro maintained	3	4925 ± 322	28592 ± 1421	5.8	3	2326 ± 137	3609 ± 464	1.6
	3	2987 ± 323	15273 ± 2239	5.1	3	5838 ± 394	9732 ± 695	1.7
	3	1610 ± 149	11316 ± 802	7.1	4	2242 ± 205	5626 ± 439	2.5
	3	435 ± 77[a]	3833 ± 392	8.8	4	671 ± 128[a]	4570 ± 613	6.8
					4	479 ± 60[a]	3117 ± 333	6.5
					4	367 ± 73[a]	1584 ± 43	4.3
In vivo irradiated (3000R 7 days) In vitro 10 days	3	1861 ± 111[a]*	17786 ± 3327	9.6	N.D.			

No. of responders = 1 x 10^5 per well, except for those marked with an 'a', which were plated at 0.5 x 10^5 per well.
Either 2 or 3 thymuses were pooled for experiments on normal organ-cultured cells.
* Numbers of pooled lymphocytes were very limited from irradiated, organ-cultured thymuses - 12 thymuses were needed for these experiments.
10% FCS, triplicate (or more) cultures.
Cultures fed nutrient cocktail at 24 hrs. Animals 4 - 9 months.
All stimulation indices significant (student's t test).

CHAPTER 7

T CELL PROLIFERATIVE RESPONSES OF THYMOCYTES AND SPLENOCYTES IN ALLOTHYMUS-IMPLANTED, EARLY-THYMECTOMIZED XENOPUS

7.1 INTRODUCTION

Although the thymus is thought to play a central role in inducing T-lineage lymphocytes to become tolerant to self, the nature of the stromal cell types (epithelial cells, dendritic cells, etc.) which are responsible remains controversial. For example, Ready et al. (1984) transplanted epithelial (deoxyguanosine-treated) embryonic mouse thymus (expressing MHC antigens) to histoincompatible, normal mice. These implants failed to be rejected, suggesting to Ready et al. that MHC antigens expressed on the epithelial cells may not play a critical role in transplantation tolerance, this function being attributable to haemopoietically-derived accessory cells. Earlier work by Zinkernagel et al. (1980), involving normal and irradiated thymus implants to nude mice, suggested that alloantigens presented on epithelial cells and other radioresistant thymic cells were probably not tolerogenic. In contrast, Jordan et al. (1985b) revealed that embryonic, histoincompatible thymus, depleted of lymphocytes by low temperature treatment, results in recipient nude mice being restored in MLC to third-party stimulators, whereas their thymic lymphocytes fail to respond to MHC antigens of the thymic donor - i.e. they suggest that the thymic epithelium may be of importance in the generation of allo- (and self-) tolerance.

This Chapter outlines experiments that begin to probe the role of the amphibian thymus in deleting auto-reactive T cells. The experiments involve the transplantation of normal and δ-irradiated thymuses to early-thymectomized, MHC-compatible or -incompatible larval

Xenopus. It was the initial intention of the study to subsequently test the ability of lymphocytes taken from all these implanted animals to respond in MLC to thymic donor and third-party MHC antigens, checking cells both in the periphery (e.g. splenocytes) and also those within the thymus implant. It seemed important to check whether thymocytes (as well as splenocytes) were tolerant to thymic donor antigens, since host-derived thymic lymphocytes would undoubtedly have experienced the MHC antigens (Class II and Class I - in the froglet - see Flajnik et al., 1985a) on the thymic stromal cells. In contrast, it is conceivable that splenic T lymphocytes could develop in the periphery under a hormonal thymic influence and therefore would never come into direct contact with donor (stromal) MHC antigens, unless these stromal cells had emigrated from the thymus implant (see Discussion by von Boehmer and Schubiger, 1984, on this point).

Unfortunately the MLC experiments reported here became limited to those Tx animals receiving normal, lymphoid, thymus implants, since in the experiments set up with Xenopus given irradiated thymuses, all animals died prematurely (as did their sibling controls), before functional assays were possible. Also included in this Chapter were experiments investigating restoration of splenocyte reactivity to the T cell mitogen PHA in allothymus-implanted, Tx animals.

7.2 MATERIALS AND METHODS

7.2.1 Animals

This Chapter makes use of J strain X. laevis, outbred (Wild) X. laevis and isogeneic LG Xenopus. Additionally hybrid Xenopus, namely JLG5 (3N) and JLM3 (3N) were also used; these hybrids were produced by the in vitro fertilization of LG5 or LM3 diploid eggs with J sperm. JLG5 and JLM3 were useful as stimulators in one-way MLC reactions,

since (as shown in the Results section) they were incapable of effecting "back stimulation" following irradiation and coculture with J responder lymphocytes. Back stimulation can occur in one-way MLC response in mammals, when irradiated stimulators are able to recognize determinants on the allogeneic responding cells, causing the former to secrete factors that can induce proliferation of the responding population (see Flajnik, 1983). Assays (skin grafting or *in vitro* tests) were performed on animals ranging from 4½ - 10 months of age.

7.2.2 Thymectomy and thymus implantation

J strain larvae at 7-8 days of age (Stage 48) were thymectomized (Tx) by electrocautery (Horton and Manning, 1972). At 4-5 weeks of age (Stage 56) some Tx animals were implanted sub-cutaneously with a normal thymus from an MHC-disparate JLG5 larval donor (4 weeks) or LG5 adult donor (5 months). Other JTx larvae were given a thymus from an adult J (minor H disparate donor). Animals were reared through metamorphosis. An additional experiment was set up with MHC-disparate or -compatible thymuses coming from donors that had been irradiated *in vivo* with 3000 rads 10 days prior to thymus removal. Unfortunately, the animals in this experiment (and their sublings) died prematurely, excluding planned MLC experiments.

7.2.3 MLC (and PHA) reactivity

Splenocytes and thymocytes from control animals, splenocytes from Tx froglets, and splenocytes and thymocytes from thymus-implanted Tx *Xenopus* were compared in one-way MLC, following coculture with various 6000 rad-irradiated stimulator splenocytes and/or thymocytes, following the technique described in Chapter 6. For thymocytes, 1 or 2 x 10^5 responders were cocultured with 1 or 2 x 10^5 irradiated thymocyte stimulators respectively in a total volume of 100 µl. For splenocytes, 1 x 10^5 responders were cocultured with 1 x 10^5 irradiated splenocyte

stimulators in a total volume of 200 µl. Splenocytes were cultured in 1% FCS-supplemented L15 amphibian medium, whereas thymocytes were kept in 10% FCS-supplemented medium. Thymocytes were pulsed at 48 hrs, splenocytes at 72 hrs, with 1 µ Ci 3HTdR; both cell types were harvested 24 hrs later. Other experimental details are given in the Tables.

The ability of splenocytes from control, Tx and thymus-implanted animals to respond to PHA was also compared. Spleen cells were here plated out at 1 x 10^5 cells/well in a total of 50 µl, and 10 µl mitogen (or medium) was then added to each well. PHA cultures were supplemented with 1% FCS and extra nutrients (where indicated), details of the nutritive cocktail are given in Chapter 6, Materials and Methods. PHA at a final concentration of 1:1500 was used.

7.2.4 Skin grafting

Skin grafts were exchanged between J and JLG5 control froglets to confirm the inability of JLG5 to respond to J, in contrast to the potential alloreactivity that should occur in the reciprocal combination. Some JTx animals implanted with JLG5 or LG5 thymuses were tested for in vivo tolerance to LG5 skin prior to MLC assays. The technique of skin transplantation was as described previously - (Horton, 1969). Dorsal skin grafts approximately 2 mm^2 were used.

7.3 RESULTS

7.3.1 Reciprocal skin grafting between J and JLG5 froglets

Six J froglets (5 months old) rejected JLG5 skin in a mean time of 26 ($\pm$1) days. In contrast, six JLG5 froglets of the same age failed to reject J skin, grafts surviving in perfect health throughout the observation period (>100 days).

7.3.2 MLC experiments with thymocytes and splenocytes taken from control J, JLG5 and JLM3 froglets

These experiments revealed that alloreactivity can be induced in vitro when J splenocytes or thymocytes are cocultured with JLG5 or JLM3 stimulators, but that no induced proliferation occurs in the reciprocal combination. Table 7.1 summarizes stimulation indices obtained from all MLCs performed, whereas Table 7.2 provides information on 3HTdR incorporation in selected experiments.

In addition, Table 7.1 shows that J splenocytes and thymocytes tend to be more reactive in MLC against irradiated LG5 stimulators than JLG5 stimulators. Others have also observed the lower response to irradiated hybrid stimulators (see Flajnik, 1983). The present findings may occur because LG5 lymphocytes provide a stronger alloantigenic stimulus than JLG5 cells to J strain lymphocytes. Alternatively, irradiated LG5 splenocytes and thymocytes may be stimulated by the J strain responders (back stimulation) into producing factors that then help to cause proliferation of the latter. This cannot occur with JLG5 stimulators since they express the J haplotype and are therefore tolerant to J strain MHC antigens. Table 7.2 shows that mean DPMs for (1 or 2 x 10^5) thymocyte background cultures are generally lower than background splenocyte (1 x 10^5) cultures, despite the use of 10% FCS-supplementation used with thymocytes, compared with only 1% FCS added to splenocyte cultures. Similar findings have been noted by others previously (see Horton et al., 1980).

7.3.3 MLC experiments on thymocytes and splenocytes from control, Tx and thymus-implanted, Tx J strain animals

Table 7.3 summarizes the results of these experiments by giving stimulation indices only. Table 7.4 shows DPM values for the experiments involving MHC-incompatible thymus implantation. In all cases, J control

splenocytes and thymocytes responded successfully to alloantigens expressed on JLG5 and JLM3 lymphocytes. Thymectomized J froglets generally failed to respond to the same allogeneic stimulators. The one Tx froglet that did respond probably indicates that the animal was not properly thymectomized. In contrast, JTx froglets grafted with MHC-compatible (J) thymuses react well against JLG5 and JLM3 alloantigens, showing that alloreactivity has been restored by thymus implantation.

In 5 out of 6 experiments where JTx froglets were implanted with MHC incompatible thymus (either larval or adult thymus), splenocytes failed to respond to irradiated splenocytes of the thymus donor MHC-type, although induced proliferation to third party JLM3 was positive in 4 out of 5 cases. Lymphocytes from thymus implants of the same animals uniformly failed to proliferate when cocultured with thymocytes of the thymus donor MHC-type, although the same thymocytes did respond (in 3 out of 3 cases) to third party JLM3 cells. Mean DPM values for cultures involving responder cells from TxJs given MHC-incompatible thymuses are given in Table 7.4.

Some JTx froglets that received either JLG5 or LG5 implants were grafted (prior to MLC assay) with LG5 skin to check for in vivo tolerance induction. Fifty days later, all LG5 grafts on these animals were in perfect condition.

Additional JTx larvae to those reviewed in Tables 7.3 and 7.4 were implanted with MHC-disparate thymus implants (6 were given in vivo irradiated thymuses, another 6 received normal thymus grants). These were to have been used for MLC assays, to assess potential differences in alloreactivity/tolerance induction to donor thymus MHC-type between Tx animals given normal and irradiated ("epithelial") implants. Unfortunately, all these animals died prematurely and so the MLC tests

could not be performed. Therefore, the influence of a lymphocyte-depleted thymic microenvironment, compared with a normal thymus environment, on T cell tolerance induction to thymic donor antigens still awaits clarification.

7.3.4 PHA responses of splenocytes in control, Tx and allo-thymus implanted animals

Induced proliferative reactivity of splenocytes from Tx animals to the T cell mitogen PHA was non-existent or very poor compared with controls (see Table 7.5). Cultures from control Xenopus supplemented with a nutritive mixture (see Methods in Chapter 6) proliferated particularly well with PHA. Proliferative ability of T cells to PHA is restored when allogeneic thymus is grafted to Tx, outbred X. laevis (Table 7.6). In these latter experiments, thymus donor and Tx host were presumed to be of at least one MHC-haplotype disparity, since skin grafts exchanged between family Y and Z are rejected in less than 3 weeks (data not shown).

7.4 DISCUSSION

The MLC experiments in this Chapter have made use of hybrid stimulator cells (e.g. JLG5) in an attempt to prevent any possible "back stimulation" of responder (J) lymphocytes. The tissue-typing experiments confirmed that J animals reject JLG5 and JLM3 skin within 3 weeks, whereas the hybrids fail to reject J strain skin grafts and fail to display MLC reactivity to J stimulators. These findings verify that hybrids (used as stimulators in one-way MLC) were tolerant to J strain MHC antigens. Any positive stimulation indices in MLC were therefore due to the reactivity of the responder population alone.

The results of MLC experiments on TxJ strain froglets implanted with normal allogeneic (MHC-disparate) thymus show that splenocytes and implant thymocytes both respond to third-party stimulator cells,

but generally fail to proliferate when cocultured with thymus donor strain cells. The only positive MLC response to thymus donor-type cells was shown by splenocytes from one Tx animal given an LG5 adult thymus. Unfortunately, MLC experiments with Tx animals implanted with in vivo-irradiated allogeneic thymuses were not attempted because all these experimental animals died prematurely. Obviously this experiment will be crucial to repeat in order to investigate the immunological outcome when relatively few donor lymphocytes, but intact thymic stroma, is implanted.

In the present experimental situation, i.e. when fully lymphoid thymuses are implanted, it is quite possible that lack of MLC reactivity to donor antigens by host lymphocytes and splenocytes is achieved by the extensive complement of donor lymphocytes within the thymus and in the periphery (see Chapter 5), rather than being caused by thymic stromal elements. Other workers in this laboratory (Arnall and Horton, 1986) have recently revealed that splenocytes from the majority of Tx Xenopus given adult MHC-disparate thymus implants fail to respond in MLC against thymus strain stimulators (but could respond to third-party stimulators). In contrast, those Tx animals implanted with a larval allogeneic thymus (either normal or 3,000R in vitro irradiated) possessed splenocytes that could proliferate to thymus-type MLC antigens. Arnall and Horton have recently (1986) shown that $^{2}/3$ Tx J strain Xenopus given in vitro irradiated (3000R) adult LG5 implants could, unlike $^{4}/4$ animals given normal LG5 thymuses, display splenic MLC reactivity to thymus donor alloantigens. Arnall and Horton (1986) suggest that the immunologic outcome following allothymus implantation to Tx Xenopus might be related to the number of lymphocytes remaining within thymus implants.

The phenomenon of "split tolerance" following allothymus implantation to adult Tx Xenopus has been described by Nagata and Cohen (1984). "Split tolerance" also occurs in embryonic (histo-incompatible) anterior/posterior chimeras (Flajnik et al., 1985b) - where lymphoid stem cells of one MHC type develop under the influence of a thymus epithelium of different MHC type. "Split tolerance" is referred to here as the induced tolerance to thymus donor-strain skin grafts, but positive in vitro reactivity of host splenocytes to thymus donor-strain lymphocytes in one-way MLC. Lymphoid cells from mice or rats bearing tolerated skin grafts, after neonatal tolerance induction, display MLC tolerance to donor cells in some cases, but not in others (Hăsek and Chutnă, 1979). In contrast, lymphocytes from nude mice implanted with allogeneic thymus, routinely display MLC tolerance to thymus donor strain cells, whereas in vivo reactivity to thymus donor skin grafts may (Kindred, 1976) or may not (Manning and Hong, 1983) occur. "Split tolerance" seen in the amphibian experiments may represent tolerance to Class I, but not to Class II MHC antigens (Arnall and Horton, 1986; Flajnik, 1983), since MLC reactivity in Xenopus (as in mammals) is known to be a Class II related function (Kaufman et al., 1985).

Finally, the experiments revealing restoration of PHA reactivity by splenocytes in Tx animals given allogeneic thymic implants fits with experiments performed on mammals. For example, nude mice given thymic fragments depleted of lymphocytes by organ culture, display restoration of PHA reactivity (by lymph node cells), providing sufficient amount of thymic tissue is implanted (Manning and Hong, 1983). In contrast, thymic epithelium (produced by low temperature treatment) failed to reconstitute PHA reactivity of splenocytes from adult thymectomized, lethally irradiated, foetal liver reconstituted host

mice (Crouse et al., 1984). These latter authors consider that endodermally-derived epithelial cells are lacking in their low-temperature cultured thymuses, and suggest that it is these cells that are responsible for normal maturation and/or peripheralization of T cells.

Table 7.1

One-way MLC reactivity of splenocytes and thymocytes taken from J, JLG5 and JLM3 froglets

Responder Cells	S.I. v. 6000R-irradiated stimulators				
	JLG5	LG5	JLM3	LM3	J
Splenocytes					
J	1.7 2.6 1.7 1.4 1.6	2.9 5.0	1.7	ND	ND
JLG5	ND	1.0* 1.1*	3.9 2.6	4.3	0.7* 1.0* 1.1* 1.2* 1.0*
JLM3	4.2 1.4	ND	ND	ND	1.2*
Thymocytes					
J	2.1 2.3 1.8 2.7 1.5 2.2	3.0 3.8 2.1 2.9	2.8 2.8	2.5 3.5	ND
JLG5	ND	1.1* 1.2*	1.2* 1.5	ND	0.9* 0.9* 1.0* 1.0* 1.1* 1.4*

* = stimulation indices not significant. Rest of S.I.s significant by t test.
No. of responders/well = 1.0×10^5 splenocytes, 1 or 2.0×10^5 thymocytes. Equal nos. of stimulators were used.
Splenocytes harvested at 96 hrs, thymocytes at 72 hrs.
Each data point against a particular stimulator represents the outcome of an MLC response effected by different responder animals.
The same responder population was often tested against several different stimulators.
Animals 6 - 7 months old.
Other conditions, see methods.

Table 7.2

Levels of 3HTdR incorporation in reciprocal 1-way MLCs between J and JLG5

Cell Combination	DPM ± SE			
	Responder x R Autol.	Responder x R Allo.	S.I.	P value
Splenocytes				
J x RJLG5	1739 ± 233	2898 ± 110	1.7	<.02
	5569 ± 274	7882 ± 218	1.4	<.05
	3494 ± 327	5730 ± 156	1.6	<.01
	235 ± 45	609 ± 68	2.6	<.02
	1627 ± 98	2766 ± 202	1.7	<.01
JLG5 x RJ	7976 ± 1735	5782 ± 1067	0.7	NS
	6230 ± 949	6399 ± 415	1.0	NS
	972 ± 147	960 ± 192	1.0	NS
	4332 ± 297	5186 ± 716	1.2	NS
Thymocytes				
J x RJLG5	1062 ± 93	2270 ± 140	2.1	<.001
	1021 ± 88	2342 ± 157	2.3	<.001
	483 ± 53	1290 ± 58	2.7	<.001
	1213 ± 131	2615 ± 221	2.2	<.001
	991 ± 146	1773 ± 98	1.8	<.01
	1185 ± 64	1825 ± 106	1.5	<.01
JLG5 x RJ	662 ± 85	578 ± 63	0.9	NS
	1077 ± 146	988 ± 95	0.9	NS
	1114 ± 140	1083 ± 62	1.0	NS
	1740 ± 209	1646 ± 129	1.0	NS
	978 ± 64	1104 ± 78	1.1	NS
	799 ± 85	1108 ± 140	1.4	NS

Legend as in Table 7.1

Table 7.3

MLC reactivity (stimulation indices) of splenocytes and thymocytes from TX J strain Xenopus froglets following implantation of allogeneic thymus in larval life

Responder Cells	S.I. v. 6000R Irradiated Stimulator Cells	
	JLG5	JLM3
Splenocytes		
J control	2.6 1.7 1.7 1.4 1.6	1.7
J TX	1.0* 1.0* 2.6 0.9*	1.0* 0.9* 0.8*
J TX ← J adult thymus	5.4 2.5	3.6 5.8
J TX ← JLG5 larval thymus	1.1* 0.6* 0.5*	1.9 1.5
J TX ← LG5 adult thymus	2.3 1.3* 0.8*	5.0 2.9 1.5*
Thymocytes		
J control	2.3 2.7 2.1 1.8 1.5 2.2	2.8 2.8
J TX ← JLG5 larval thymus	1.0* 0.9*	1.9 1.9
J TX ← LG5 adult thymus	1.0* 0.9*	1.5

All stimulation indices significant by students t test, except for those marked with *.
Indices underlined represent animals which carried perfect LG5 skin grafts for 50 days before MLC tested.
No. of responders/well = 1 x 10^5 splenocytes, 1 or 2 x 10^5 thymocytes.
Spleen cells harvested at 96 hours, thymocytes at 72 hours.
Each data point represents the outcome of an MLC response effected by different responder animals.
The same responder population was often tested against both JLG5 and JLM3 stimulators.
Animals 5 - 6 months except J TX ← J thymus (= 10 mths).
For other details see Methods.

Table 7.4

Levels of 3HTdR incorporation for one-way MLCs of spleen and thymus lymphocytes from TX J strain Xenopus following larval implantation of MHC-incompatible thymus

Responder Cells	DPM ± SE						
	Responder x R.Autol.	Responder x R.JLG5	S.I.	P value	Responder x R JLM3	S.I.	P value
Splenocytes							
JTX ← JLG5 larval thymus	425 ± 97	463 ± 103	1.1	NS			
	526 ± 44	316 ± 33	0.6	NS	978 ± 169	1.9	<.02
	858 ± 102	467 ± 76	0.5	NS	1320 ± 125	1.5	<.01
JTX ← LG5 adult thymus	463 ± 104	1075 ± 184	2.3	<.01	2307 ± 298	5.0	<.001
	878 ± 151	1114 ± 255	1.3	NS	2538 ± 163	2.9	<.001
	1460 ± 198	1200 ± 280	0.8	NS	2119 ± 377	1.5	NS
Thymocytes							
JTX ← JLG5 larval thymus	465 ± 71	447 ± 35	1.0	NS	876 ± 78	1.9	<.01
	1305 ± 89	1161 ± 87	0.9	NS	2448 ± 170	1.9	<.001
JTX ← LG5 adult thymus	440 ± 38	454 ± 32	1.0	NS			
	785 ± 102	720 ± 42	0.9	NS	1205 ± 52	1.5	<.01

Legend same as Table 7.3.
Individual J recipients were usually tested against both RJLG5 and RJLM3 stimulators.

Table 7.5

Effect of 7-day TX on PHA responses of splenocytes

Strain	DPM ± SE			
	Background	PHA 1:1500	S.I.	P value
Controls				
JLG5	1,285 ± 158	27,077 ± 217	21.1	<.001
	649 ± 71	91,752 ± 2,757	141.4	<.001
LG5	4,353 ± 905	108,153 ± 3,505	24.9	<.001
LG3	1,595 ± 428	69,502 ± 1,021	43.6	<.001
	725 ± 296	11,009 ± 2,145	15.2	<.001
	1,291 ± 144	65,997 ± 3,232	51.1	<.001
TX				
J	4,460 ± 597	2,662 ± 309	0.6	NS
JLG5	2,853 ± 1,065	2,939 ± 179	1.0	NS
	757 ± 98	3,231 ± 625	4.3	<.01
LG3	1,397 ± 230	3,739 ± 111	2.3	<.001

Indices underlined represent cultures fed extra nutrients.
1% FCS supplementation. No. of responders/well = 1.0×10^5.
Pulse 48 hrs, harvest 72 hrs.
Each row of data indicate spleen cells from different responder animals.

Table 7.6

Restoration of in vitro PHA reactivity of splenocytes from outbred, thymectomized Xenopus laevis following larval implantation of allogeneic larval thymus

Stimulation Indices		
Control (Family Y)	TX (Family Y)	TX + thymus (Family Y) (Family Z)
6,0, 10,5, 3.3, 6.3, 7,0, 8.1.	0.5*, 0.5*	2.8, 11.5, 21.7, 19.2, 12.7, 10.5, 10.8, 13.0.
$\bar{x} \pm$ SD = 6.9 $\pm$ 2.4	$\bar{x}$ = 0.5*	$\bar{x}$ = 12.8 $\pm$ 5.7

All stimulation indices significant, except those asterixed.
1 x 10^5 spleen leucocytes per well.
Pulsed 48 hrs, harvested 72 hrs.
PHA concn. = 1:500.
Each data point represents splenocyte cultures from separate individuals.
Animals aged 6-8 mths.
Families Y and Z are from outbred *X. laevis* ♀ x ♂ no. 14, ♀ x ♂ no.16, respectively.

CHAPTER 8

ATTEMPTS TO GENERATE SUPERNATANTS FROM PHA- AND PHORBOL MYRISTATE ACETATE-STIMULATED SPLENOCYTES WHICH CAN IMPROVE THYMOCYTE PROLIFERATIVE RESPONSES

8.1 INTRODUCTION

In mammals, splenocytes stimulated with T cell mitogens (e.g. PHA and Con A) or allogeneic cells, produce a variety of lymphokines, including a soluble, antigen non-specific, immunoenhancing factor with growth activity for T cells (Farrar et al., 1982). This factor is now known as interleukin-2 (IL-2), and is known to bind to IL-2 receptors on activated T cells, inducing their proliferation. Only those T lymphocytes that have interacted with antigen (or mitogen) are capable of IL-2 receptor synthesis and expression (Kay et al., 1984). Interleukin (IL-1), secreted by macrophages, induces the release of IL-2 from T helper cells (Schreier, 1984). IL-2 has been reported to induce thymocyte proliferation in the presence of a sub-optimal dose of T cell mitogen; IL-2 can also augment the proliferation and generation of cytotoxic T cells, following alloantigen-stimulation of T-cells (reviewed in Farrar et al., 1982).

In amphibians, supernatants (SNs) produced from MLC- and PHA-stimulated Xenopus splenocytes will co-stimulate thymocytes (enabling them to proliferate when cultured with sub-optimal doses of T cell mitogen) and induce enhanced proliferation of splenic lymphoblasts, suggesting that these SNs contain IL-2 like molecules (Gearing, 1985; Watkins, 1985; Watkins and Cohen, 1986).

In this Chapter, attempts are made to generate SNs (from PHA-stimulated Xenopus spleen cells) which have immuno-enhancing properties. It was thought that active SNs might be helpful in improving thymocyte viability and reactivity in MLC, which would be useful when only limited numbers of thymocytes are available for testing, e.g. when thymus implants

are being studied. The ability of the SNs to co-stimulate T cell mitogen reactivity of thymocytes is also examined here.

Phorbol myristate acetate (PMA) has been used to optimize the production of immuno-enhancing lymphokines by murine spleen cells (Fuller-Farrar et al., 1981). It is thought that PMA acts as a pharmacological analoque of macrophage-derived IL-1 (Farrar et al., 1982). PMA is believed to enhance IL-2 production, either by providing an inducing signal to the precursors of IL-2 producing cells, or by selectively inactivating/ inhibiting cells which suppress IL-2 production. PMA has been used by mammalian workers to produce SNs containing a more concentrated form of growth factors, e.g. 10^{-8} M PMA is used in combination with Con A to induce IL-2 production from murine splenocytes (Fuller-Farrar et al., 1981). These same authors found that PMA stimulates the production of heterogeneous factors, i.e. not purely IL-2, therefore a purification step was required to obtain IL-2. PMA was itself found not to be mitogenic for thymocytes, nor did it synergize with PHA to stimulate thymocyte proliferation. However, Watkins (1985) reports that PMA induces dramatic proliferation of Xenopus thymocytes, splenocytes and blasts, suggesting that the mechanism of Xenopus and mammalian T cell activation may differ. This Chapter also reports on preliminary experiments that use PMA as either a stimulant or co-stimulant (with PHA) of splenocytes, in order to procure from these cells factors that can increase MLC reactivity of Xenopus thymocytes.

8.2 MATERIALS AND METHODS

8.2.1 Animals

The strains of Xenopus used are given in the Tables.

8.2.2 Preparation of supernatants

a) PHA-activated supernatants (PHA.SN)

Spleens were teased apart in petri dishes, using amphibian L15

medium, with 1% FCS supplementation. Cells were washed twice, counted with a haemocytometer, and resuspended in 500 µl aliquots in 12 x 75 mm test tubes (Falcon) at 5-10 x 10^6 leucocytes/ml. PHA was then added so that the final concentration in the tubes was 1:1000 or 1:500. Control supernatants were set up in a similar fashion, medium rather than mitogen being added to the spleen cell cultures. Control supernatants are referred to as CSN in the Tables. After 4-5 hrs incubation at 26-27°C*, control and PHA-stimulated cultures were centrifuged and washed three times in excess (3-4 mls) medium to "remove" the PHA. Cells were then resuspended at a final concentration of either 5.0 or 10.0 x 10^6 leucocytes/ml in amphibian medium (containing 1% FCS) and plated out in 24 well plates (Flow Labs - well dimensions 1.7 x 1.6 cm), 0.5 mls cell suspension/well. The cells were then incubated for a further 20 hrs at 26-27°C. The supernatant (500 µl) from each well was then transferred to separate test tubes, centrifuged at 300 g for 10 mins and the cell-free supernatants collected and stored frozen for later use.

b) Phorbol myristate acetate-activated supernatants (PMA.SN)

162 µl culture medium was added aseptically to 1 mg PMA (Sigma, No. 8139) in an ampule to obtain .01 M PMA stock solution. 20 µl aliquots of .01 M. PMA were placed into small capped vials and stored frozen. Different concentrations of PMA were tested (see Table 8.4), the stock solution (.01 M) being freshly diluted with amphibian L15 medium just before use. PMA dilutions were added to splenocytes (4-5 x 10^6/ml) in 12 x 75 mm tubes. Cells were incubated with PMA for 20 hours at 26-27°C. After this incubation, the cells were centrifuged (300 g for 10 mins) and washed three times in excess medium to "remove"

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* Splenocytes pulsed for just 4 hours with PHA, washed three times and then cultured for 72 hours in medium alone, have been shown to display elevated 3HTdR DPM (S.I. $\hat{=}$ 4) when compared with non-mitogen treated cells (data not shown).

the PMA, and resuspended at 4-5 x 10^6 leucocytes/ml. Some cells were then placed directly into 24 well plates (0.5 mls cells per well) and cultured in normal medium for a further 20 hours. Other PMA-treated cells were placed (4-5 x 10^6 leucocytes/ml) in 12 x 75 mm tubes and pulsed with 1:500 PHA for 4 hours. These PMA plus PHA-treated cells were then washed three times (see preparation of PHA.SN above) and then cultured (at 4-5 x 10^6 leucocytes/ml) in 24 well plates (0.5 mls per well) for a further 20 hours.

After incubation (with or without PHA) the PMA supernatants were harvested as in a) above and stored frozen for later use. The supernatants from cells treated with both PMA and PHA are designated "PMA/PHA.SN", whereas the supernatants from cells treated only with PMA are referred to as "PMA.SN".

At the time of harvesting supernatants, a sample of the cells/supernatant remaining in the plates was examined to confirm absence of contamination by micro-organisms.

8.2.3 Assays for measuring supernatant activity

Various dilutions (in amphibian medium) of supernatants (see Tables) were tested for their ability either a) to co-stimulate thymocytes that were treated with a sub-optimal PHA dose (see Table 8.1), or b) to potentiate bi-directional or one-way thymocyte MLC reactivity. PHA and MLC techniques were the standard ones described in Chapter 6. Supernatants were added at the beginning of culture. Supernatant titrations of 1:3, 1:5, 1:10 and 1:30 were tested, these ratios indicate the final dilution of SN in each well. For further details - see Table legends.

8.3 RESULTS

8.3.1 The effect of supernatants on thymocyte responsiveness to PHA

i) Calculation of a sub-optimal PHA dose for use in thymocyte co-stimulation assay

The co-stimulation assay requires that a sub-optimal dose of PHA be used, so as to reveal any potentiating effects supernatants might have on the proliferation of thymocytes. PHA at 1:1000 was shown to result in good S.I.s in all experiments (see Table 8.1). Moreover, PHA concentrations of 1:2000 and 1:4000 still achieved S.I.s of 4 or greater. PHA diluted 1:8000 was chosen as the sub-optimal dose of mitogen to be used in conjunction with the supernatants.

(ii) The effect of control and PHA supernatants on thymocyte reactivity to PHA

Table 8.2 illustrates the outcome of two typical experiments set up to examine the co-stimulating capacity of PHA supernatants on thymocyte reactivity to sub-optimal PHA doses. In experiment 1, both a 1:3 and 1:10 dilution of PHA-supernatant (raised from J splenocytes) appeared to enable J thymocytes to respond to 1:8000 PHA. The 1:30 dilution of the supernatant had no apparent effect. In contrast, no co-stimulating capacity of supernatants was shown in experiment 2 - where LG15 thymocytes were examined. Here the response of thymocytes to 1:8000 PHA, without added SN, was quite reasonable (SI = 4.6). Both PHA.SN and CSN (raised from LG15 splenocytes) failed to augment this PHA response. In both experiments, supernatants appeared to elevate background 3HTdR uptake by thymocytes (i.e. cells not cultured with PHA). Indeed the (1:5) PHA.SN achieved an eleven-fold increase in DPM of LG15 thymocytes.

8.3.2 Effect of supernatants on thymocyte MLC reactivity

i) Use of PHA supernatants to potentiate thymocyte one-way MLC reactivity

Table 8.3 reflects the typical outcome of this type of experiment.

Both control and PHA.SNs failed to significantly elevant MLC responsiveness of J thymocytes to LG thymocyte stimulators. In MLC experiments other than shown here, PHA.SNs (1:10) often appeared to elevate background DPMs two- to three-fold.

ii) Use of PHA- and/or PMA-induced supernatants to potentiate 2-way MLC reactivity of thymocytes

Table 8.4 illustrates an initial experiment with J and LG3 co-cultured thymocytes, where PMA and PHA were used to generate the supernatants tested. For supernatant production from splenocytes, PHA was used at 1:500 and PMA at 3×10^{-4} M. Control and PHA supernatants raised from J splenocytes failed to significantly elevate background and experimental (MLC) DPM. In contrast, the combined use of PHA and PMA to generate SNs caused a distinct increase in background DPM, but lowered the stimulation index achieved in the MLC. Supernatants raised from LG splenocytes also failed to elevate MLC reactivity. Again the PMA/PHA supernatants caused elevated background and experimental DPM.

Table 8.5 shows the outcome of a subsequent experiment with J and LG5 thymocytes, in which the concentrations of PMA (used in conjunction with PHA) to activate spleen cells for supernatant production were varied. The combined use of PMA (2×10^{-6} M and 2×10^{-9} M) and PHA (1:500) failed to elevate background DPM, but did potentiate 2-way MLR (SIs of 3.5 and 3.0 respectively). The PHA.SN on its own here also resulted in an increase in stimulation index from 1.9 (no SN) to 3.0. 2×10^{-3} M PMA, used in conjunction with 1:500 PHA, to induce splenocyte supernatants, proved to be stimulatory for background thymocyte cultures - e.g. a 9-fold increase in DPM with the J thymocytes. However, this PMA/PHA.SN failed to elevate the 2-way MLC response of thymocytes.

8.4 DISCUSSION

Overall, these preliminary attempts to generate supernatants that would consistently enhance mitogen and MLC reactivity when added to cultured Xenopus thymocytes were not particularly successful. PHA SNs did appear to be able to co-stimulate thymocytes to respond to sub-optimal doses of PHA in some experiments, but not in others. Such SNs only occasionally enhanced thymocyte MLC reactivity. Supernatants generated from PMA-stimulated splenocytes also failed to routinely enhance thymocyte MLC reactivity. SNs generated from the spleen had a tendency to elevate 3HTdR uptake of not only allo-antigen/mitogen stimulated thymocytes but also background cultures.

In this preliminary study no attempt was made to remove any residual PHA (by absorption with chicken red blood cells - see Watkins, 1985) or PMA (by absorption with activated charcoal) from supernatants. Therefore, any potentiating effects supernatants had on the proliferation of thymocytes in the absence or presence of allo-antigen/sub-optimal mitogen, may have been caused partly or wholly by contaminating lectin or phorbol esters. However, it might well be that the present experiments are revealing lymphokine factor release from splenocytes and the ability of these factors to (occasionally) promote thymocyte proliferation in vitro.

Further studies with PMA- and PHA-treated lymphocytes should be carried out to determine if growth factors can be generated that will routinely promote alloreactivity of thymocytes. Already it is known that amphibian T cell growth factors do exist (Gearing, 1985; Watkins and Cohen, 1986). However, the potential for using such (purified) factors for the long-term maintenance and reactivity of frog lymphocyte subsets in culture is only now beginning to be realized.

Table 8.1

Thymocyte responses to low PHA concentrations

Animal	Background DPM ± SE	PHA treated DPM ± SE			
		1:1000	1:2000	1:4000	1:8000
J 5 mths	362 ± 87	2577 ± 286 S.I. = 7.1	1908 ± 92 S.I. = 5.3	1392 ± 178 S.I. = 3.9	1031 ± 51 S.I. = 2.9
LG15 9 mths	306 ± 12	3394 ± 185 S.I. = 11.1	N.D.	N.D.	1401 ± 367 S.I. = 4.6
J 6 mths	1312 ± 93	7210 ± 413 S.I. = 5.5	N.D.	N.D.	3751 ± 550 S.I. = 2.9
Wild 10 mths	531 ± 79	4333 ± 95 S.I. = 8.2	N.D.	N.D.	1059 ± 66 S.I. = 2.0

Pulse 48 hrs, harvest 72 hrs.
10% FCS. 1×10^5 cells/well.
All S.I.s significant.

Table 8.2

The effect of control- and PHA-supernatants on thymocyte response to a sub-optimal PHA dose

Experiment	Origin of Thymocytes	Origin of SN	Type of SN (concentration)	Background DPM ± SE	PHA 1:8000 DPM ± SE	S.I.	p value
1	J 6 mths	J 6 mths	None	459 ± 44	477 ± 18	1.0	NS
			PHA.SN 1:3	727 ± 72	2878 ± 387	4.0	<.05
			PHA.SN 1:10	715 ± 186	1322 ± 112	1.9	<.05
			PHA.SN 1:30	583 ± 66	714 ± 46	1.2	NS
2	LG15 9 mths	LG15 9 mths	None	306 ± 12	1401 ± 367	4.6	<.05
			CSN 1:5	674 ± 61	1360 ± 92	2.0	<.01
			PHA.ASN 1:5	3394 ± 185	5597 ± 320	1.7	<.01

Total cell nos./well = 2×10^5 (J) or 1×10^5 (LG15)
Pulse 48 hrs, harvest 72 hrs.
10% FCS,
NS = S.I.s not significant.

Table 8.3

The effect of control and PHA supernatants on thymocyte MLC reactivity

MLC Combination		Origin of SN	Type of SN (concentration)	J x RJ DPM ± SE	J x RLG17 DPM ± SE	S.I.	p value
Responders	Stimulators						
J 8 mths	RLG17 10 mths	J 8 mths	None	571 ± 34	1235 ± 98	2.2	<.001
			CSN 1:5	540 ± 39	1398 ± 134	2.6	<.001
			CSN 1:10	632 ± 70	1655 ± 240	2.6	<.01
			PHA.SN 1:5	570 ± 41	1065 ± 106	1.9	<.01
			PHA.SN 1:10	524 ± 55	1658 ± 282	3.2	<.01

2×10^5 Responders/well. R = 2×10^5 irradiated (1000R) Stimulators.

Pulse 48 hrs. Harvest 72 hrs.

10% FCS.

Table 8.4

Effect of PHA.SNs and/or PMA.SNs on thymocyte 2-way MLC reactivity: Experiment 1

MLC Cell Combination	Origin or SN	Type of SN needed	Background J	DPM ± SE LG3	Experimental DPM ± SE J x LG3	S.I.	P value
J x LG3 6 mths 5 mths		None	1038 ± 64	2169 ± 200	8917 ± 173	5.6	<.001
	J 6 mths	CSN	1049 ± 95	1443 ± 73	8992 ± 525	7.2	<.001
		PHA.SN	1481 ± 65	2361 ± 58	9653 ± 954	5.0	<.001
		PMA/PHA.SN	4484 ± 401	4667 ± 439	9544 ± 1304	2.1	<.01
	LG 5 mths	CSN	1330 ± 213	1160 ± 124	7277 ± 839	5.9	<.001
		PHA.SN	2349 ± 140	3620 ± 292	9271 ± 367	3.1	<.001
		PMA.SN	2824 ± 397	2530 ± 150	9526 ± 729	3.6	<.001
		PMA/PHA.SN	4960 ± 620	4748 ± 388	11390 ± 773	2.4	<.001

Total cell no. per well = 4.0×10^5

Pulse 72 hrs. Harvest 96 hrs.

SNs generated by use of 3×10^{-4} M PMA; 1:500 PHA.

SN concn. = 1:10.

Table 8.5

Effect of PHA.SNs and PHA/PMA.SNs on thymocyte 2-way MLC reactivity: Experiment 2

MLC Cell Combination	Type of SN added	Background DPM ± SE		Experimental DPM ± SE J x LG	S.I.	p value
		J	LG			
J x LG5 6 mths 4 mths	None	600 ± 47	2238 ± 250	2621 ± 485	1.9	<.05
	PHA.SN	824 ± 99	2174 ± 264	4505 ± 616	3.0	<.01
	PMA/PHA.SN (PMA = 2×10^{-3} M)	5256 ± 193	6063 ± 301	8162 ± 750	1.4	<.02
	PMA/PHA.SN (PMA = 2×10^{-6} M)	693 ± 102	1865 ± 266	4499 ± 1004	3.5	<.02
	PMA/PHA.SN (PMA = 2×10^{-9} M)	660 ± 72	2229 ± 252	4336 ± 544	3.0	<.002

Total cell nos./well = 2×10^5

Pulse 48 hrs. Harvest 72 hrs.

PHA.SNs generated by 1:500 PHA.

Supernatant concn. = 1:10

SNs generated from J (5 mths) splenocytes.

CHAPTER 9

CONCLUDING REMARKS

In mammals both thymic epithelial cells and thymic antigen-presenting cells (APCs, e.g. thymic macrophages) are known to express MHC antigens and are present early in ontogeny (Robinson and Jordan, 1983). However, which of these cell types effect tolerance induction of T-lineage lymphocytes to self- and allo-MHC antigens remains uncertain (see also Chapter 3, Discussion). The main purpose of this Thesis was to develop an amphibian model system for future, in depth, examination of the role of the Xenopus thymic micro-environment, particularly its stromal components, in T cell tolerance induction. The basic plan (see Figure 1.1) was to implant irradiated thymus grafts (with a much reduced lymphocyte complement) and normal lymphoid grafts into MHC-incompatible or -compatible, thymectomized tadpole hosts. At various time intervals after transplantation the implants would be examined histologically to observe the extent of repopulation with host-type lymphoid cells. After 4-9 months the animal's thymus and spleen were to be tested in vitro for immune reactivity to thymus donor third-party MHC antigens, using the mixed leucocyte culture (MLC) assay. The major achievements of this Thesis are outlined below:-

1. The initial goal had to be to find a method of depleting the thymus of its lymphoid component, whilst maintaining a healthy thymic stroma. Deoxyguanosine treatment and subsequent organ culture proved successful in procuring "epithelial" larval thymus (Chapter 2), whereas in vivo irradiation (3000 rads and ~10 days residence in situ) was most effective for young adult frog thymus (Chapter 3).

2. Originally, a triploid/diploid cell marker system was used to identify the origin of various cell types developing within thymus

implants (Chapter 4). However, in Chapter 5 an alternative method was described, which proved more precise for visualizing the timing of host cell immigration to thymus implants; this involved staining cells with the fluorescent dye quinacrine. X. laevis cells show no nuclear fluorescent spotting, whereas X. borealis (positive) cells show fluorescent spots, due to AT rich nuclear chromatic.

3. Functional studies on thymocytes began (Chapter 6) by assessing T cell mitogen and MLC responses of normal, organ-cultured and in vivo irradiated (then organ-cultured) thymus lymphocytes. Leucocytes from 12-day organ-cultured thymus displayed excellent proliferative reactivity to T cell mitogens and in one-way MLC. The in vitro assays in Chapter 7 revealed that splenocyte reactivity to PHA and third-party alloantigens was restored when normal allogeneic thymus is implanted to early-thymectomized Xenopus. In contrast, MLC reactivity of thymus and spleen lymphocytes to thymus-donor strain cells was generally absent; allothymus-implanted animals were also tolerant to thymus-donor type skin grafts. Unfortunately, thymectomized animals implanted with irradiated allothymuses died prior to in vitro assaying.

The research in this Thesis has provided a basis for several areas of work in the future. Firstly, experiments should attempt to define the role(s) that allogeneic froglet/larval thymus stromal elements (procured by in vivo irradiation/deoxyguanosine treatment, respectively play in in vivo allotolerance induction. The use of fluorescent-labelled antisera to identify MHC Class I and II antigens (see papers by Flajnik et al., 1984c and 1985a; Kaufman et al., 1985a and b) could be profitably used to reveal the nature of MHC antigen expression on various Xenopus thymic stromal cells. The X. borealis marker system may also be of considerable use to identify origins of cells in these experiments, particularly if inbred strains of these animals can be developed.

Secondly, the *in vivo* irradiation technique developed for procuring "epithelial" thymus glands, and success with thymus organ culture, could be employed to "build" chimeric thymuses *in vitro*. This would be useful for probing which thymic stromal cell types are required to impart (self-) and allo-tolerance to developing immigrant thymocytes (see also Chapter 4, Discussion). Similarly, deoxyguanosine treatment of organ-cultured larval thymus may also be useful in this respect. Deoxyguanosine (DG) in mammals is known to be selectively toxic to immature thymic lymphocytes, dendritic cells (which are one population of thymic APCs) and the precursors of these cell types (Ready et al., 1984).

Thirdly, a long-term goal should be to create thymuses from reaggregated, MHC-disparate thymus epithelial cells and antigen-presenting thymic macrophages, and then to repopulate these, either *in vitro* (as above) or by implanting *in vivo*, with lymphoid precursors of a third MHC-type, before MLC typing the developing T lymphocytes. This approach is currently being considered in the mouse (Robinson and Jordan, 1983) for examining the role of thymic APCs in allotolerance acquisition. Thymic macrophages may be identified by their phagocytosis of opsonized yeast or latex beads, and can be separated from other cells by their adherent and/or buoyant density characteristics (Robinson, 1983; Turpen and Smith, 1986).

Finally, the *Xenopus borealis* cell marker could be used to examine cell traffic from normal and lymphocyte-depleted allothymus implants to the (thymectomized) host's periphery. This should tell us more about the extent to which peripheralised donor cells contribute to the acquisition and maintenance of tolerance to thymus donor-type MHC antigens (see Chapter 5 and 6, Discussions). The *X. borealis* marker system could well be exploited for other purposes; for example,

for histologically examining cell inflow to, and emigration from, skin allografts that are being rejected or tolerated by their hosts. Such a study might provide important clues as to the cellular basis of transplantation tolerance often seen at metamorphosis, and throw some new light as to why alloimmune impairment is seen following early larval thymectomy.

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