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CHARACTERIZATION OF ALLOIMMUNE RESPONSES AND
THE GENERATION OF TRANSPLANTATION TOLERANCE IN
THYMECTOMIZED, THYMUS-IMPLANTED XENOPUS.

by

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B.Sc. (Dunelm)

A Thesis submitted for the degree of Doctor of Philosophy
Department of Zoology, University of Durham

February 1987

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21. MAY 1987

Characterization of alloimmune responses and the generation of transplantation tolerance in thymectomized, thymus-implanted *Xenopus* by Jane C. Arnall.

Abstract - This Thesis investigates the role of the *Xenopus* thymus in educating T cells to destroy minor histocompatibility (H) antigen-disparate skin grafts and probes the extent to which allotolerance is established to major histocompatibility complex (MHC) antigens first encountered at metamorphosis. The work began with intrastrain skin grafting and mixed leucocyte culture (MLC) (Chapter 2) which confirmed that only minor H antigen differences exist between J strain *Xenopus laevis* individuals, thus making them suitable as an additional animal model to the *X.laevis/X.gilli* (LG) isogeneic hybrids available. Chapter 3 revealed that rejection of minor H disparate grafts was completely thymus-dependent, while 7-day thymectomy (Tx) severely impaired rejection of MHC disparate grafts. Implantation (at 4-6 weeks of age) of larval or adult thymus from MHC-compatible or MHC-disparate donors restored the ability of Tx *Xenopus* to reject 3rd-party MHC antigen-disparate grafts, while tolerance to skin of the thymus donor type always ensued. Restoration of minor H graft rejection was impaired when thymus donor and Tx host were MHC- or minor H antigen-mismatched. The use of LG hybrids revealed that minor H graft rejection was restored in Tx hosts only by implantation of a fully identical thymus. In Chapter 4, skin graft tolerance to donor MHC antigens was demonstrated following perimetamorphic allografting of skin to control J and LG recipients; the skin graft tolerance induced in certain donor/host combinations was shown to be not entirely specific for minor H antigens. In Chapter 5, *in vitro*, 1-way MLC reactivity of splenocytes from thymus-implanted Tx animals to thymus donor-type cells was shown to be variable, but occasionally positive; control *Xenopus*, made allotolerant of skin grafts by prior skin implantation retained a splenic MLC towards skin donor strain splenocytes. *In vivo* MLR assays in Chapter 6 also detected proliferation towards alloantigens of the skin or thymus donor, but the nature of these alloreactive (T-dependent) cells remains uncertain. Preliminary graft-versus-host experiments indicated that the tolerance induced by allothymus restoration of Tx hosts was more complete than following skin alloimplantation to metamorphosing controls; these studies also suggested that it is the cytotoxic effector component of tolerant animals that is defective.

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DECLARATION

Some of the material presented in Chapters 2, 3, 4 and 5 of this Thesis formed the basis for the following publications;

"Impaired rejection of minor-histocompatibility-antigen-disparate skin grafts and acquisition of tolerance to thymus donor antigens in allothymus-implanted, thymectomized Xenopus" by Jane C. Arnall and John D. Horton, *Transplantation*, (1986) Vol.41, No.6, pp.766-776.

"Restoration of alloimmunity and acquisition of allotolerance following thymus implantation in thymectomized Xenopus" by Jane C. Arnall and John D. Horton; an abstract from the 3rd International Congress of Developmental and Comparative Immunology, Reims, France, July 7-13 1985. *DCI* Vol.10, No.1, p.120.

The preliminary studies in Chapter 6 have been communicated to the International Symposium on the Immunology of Ectothermic Vertebrates, Rochester, New York, July 12-14 1986 and will appear as the following abstract;

"In vivo and in vitro studies on perimetamorphically induced allotolerance in Xenopus" by Jane Arnall, John Horton and Trudy Horton, In Press.

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ACKNOWLEDGEMENTS

Foremost, I am most grateful to my supervisor Dr. John Horton for his all his help, guidance and friendship during the course of my study. His patience during the writing of this thesis was very much appreciated.

Thanks also go to Miss Pamela Aitchison and Mrs Trudy Horton for tea and technical assistance. In addition, I wish to thank other members of the Department of Zoology who have been of assistance during the production of this Thesis, in particular Dr. Nigel Dunstone for giving his time to help me with the word processor, Dr. M. Stacey and Dr. R. Banks for the use of their microscope and David Hutchinson and David Young for their help in the production of the photographs. I am also grateful to Professor D. Barker and Professor K. Bowler of the Department of Zoology for providing the facilities to allow this study to take place.

Finally, I would like to thank my parents and husband Peter for their moral support during this time.

This work was supported by a Research Studentship provided by the University of Durham.

CHAPTER ONE

GENERAL INTRODUCTION

Major histocompatibility complex (MHC) restriction and self tolerance.

It is now widely accepted that thymus-dependent lymphocytes (T cells) recognize foreign (non-MHC) antigens only when the latter are in association with self MHC molecules. This concept was first put forward in 1974 by Zinkernagel and Doherty, who found that the mammalian cytotoxic T cell (Tc) response required associative recognition of viral antigen + self MHC and that effective killing of targets by Tc cells required compatibility of Tc and target cells at the K or D region of the mouse MHC (which code for class I MHC glycoproteins), (Zinkernagel and Doherty, 1974a,b; Doherty and Zinkernagel, 1975). Cytotoxic T cells generated against minor histocompatibility (H) antigens (Bevan, 1975) or chemically-modified cells (Shearer, Rehn and Garbarino, 1975) were also found to be self MHC restricted in their ability to kill. Studies of T cell interactions with macrophages in delayed-type hypersensitivity (DTH) responses (Miller et al, 1976), and of helper T cells (Th) with macrophages (Erb and Feldmann, 1975; Kappler and Marrack, 1976) revealed that these T cell subpopulations are also restricted to self MHC, but that the restriction involved gene products (class II antigens) of the I region of the mouse MHC in contrast to the class I



restriction of cytotoxic T cells. However, since non-self MHC antigens are able to supply the necessary antigenic display to stimulate T cells in the absence of associative recognition of self MHC markers, the requirement for such associative recognition for T cell activation is not absolute.

The MHC-restricted ability of T cells to recognize foreign antigen is not germ-line encoded. T cells must "learn" (become "educated") to recognize antigen in association with self MHC (class I or class II) molecules during their development (Bevan, 1977). Although a central role for the thymus in the differentiation of stem cells along the T lymphocyte pathway has been recognized for many years (Miller, 1961; Cantor and Weissman, 1976), it was not until the experiments by Bevan (1977) and Zinkernagel et al, (1978) that it appeared likely that (at least some) learning of MHC restriction specificity by T cells also occurs within the thymus. The principal mammalian model systems employed in such "thymic restriction" studies have been radiation-induced chimeras (ie. mice, which as adults are thymectomized, lethally irradiated, injected with bone marrow cells, implanted with irradiated thymus and then immunized) and thymus-reconstituted nudes (mice which congenitally lack a functional thymus (Wortis, Nehlsen and Owen, 1971)).

Using the first model, Zinkernagel et al, (1978) suggested that some radioresistant element(s) in the thymus was responsible for the process of "educating" cytotoxic T

cells. However, this assumption has been challenged by Howard (1980), who suggests that the thymic preference seen in such radiation chimeras was not due to positive selection of T cells restricted to the thymus MHC type (A), but rather to a suppression of clones restricted to the bone marrow donor MHC type (B). Thymic education was also reported for DTH (Miller et al, 1979) and helper T cells (Kappler and Marrack, 1978; Sprent, 1978). Other experiments on radiation chimeras apparently revealed that the self MHC-restriction imposed by the thymus was not absolute (Katz et al, 1979; Matzinger and Mirkwood, 1978), although Longo, Matis and Schwartz (1981), suggested that these latter findings might be due to contamination of the donor bone marrow inoculum by donor T cells.

Interpretations based on restriction studies with thymus-reconstituted nude mice (Kindred, 1978; Zinkernagel et al, 1980) conflicted with the thymic education hypothesis. Nude mice of MHC type (A) grafted with a B type thymus were shown to have T cells (Th and Tc) restricted to recognizing non-MHC antigen in the context of (A). Bevan (1981) suggests that nudes may have "pre-T cells", already self-restricted, which mature extrathymically under the influence of thymic factors, following thymus grafting, and subsequently display a host- rather than thymus- restricted response. Nude mice have indeed been shown to possess Thy-1 positive cells which, after stimulation with a T cell growth factor (IL-2), differentiate into cytotoxic T cells

restricted to self-MHC in the absence of a thymus (Gillis et al, 1979; Hunig and Bevan, 1980).

From the evidence of mammalian studies to date, the consensus of opinion appears to be that, although the influence of the thymus gland may not be absolute, it plays a critical role during the initial development of the immune system, and is centrally involved, not only in the acquisition of T cell MHC restriction specificities, but also in the induction of (self) tolerance (Kindred and Sordat, 1977; Robinson and Owen, 1978; Zinkernagel et al, 1980).

In mammals, the nature of the thymic elements responsible for MHC restriction and self-tolerance acquisition has recently come under scrutiny, but the specific mechanisms involved in the intrathymic differentiation of T-lineage lymphocytes are still poorly understood. Both thymic epithelium and thymic antigen presenting cells (APC's) could theoretically impart MHC restriction specificity on developing T cells. Both mouse thymic APC's and epithelial cells have been shown to express class I and class II antigens (Rouse et al, 1979; Jenkinson, van Ewijk and Owen, 1981) during early development. Since thymic accessory cells (eg. thymic macrophages and dendritic cells) have been shown to be critically involved in either selecting or instructing the MHC recognition specificity of class II-restricted T cells (Longo and Schwartz, 1980; Longo and Davis, 1983; Kruisbeek et al, 1983), a primary role of the thymus might be the generation of T cells of class II-restricted specificity (Th cells). Although thymic APC's

may impart MHC restriction specificities on T cells restricted to class II antigens, these APC's do not, however, appear to be involved in the MHC class I restriction of cytotoxic T cells (Zinkernagel, 1982). In addition, thymocytes appear to ignore MHC class I antigens expressed on the thymic epithelium itself (Von Boehmer and Schubiger, 1984). The thymic epithelium, on the other hand, may have a role in the induction of tolerance in T lymphocytes (Good, Pike and Nossal, 1983; Jordan et al, 1985), although this issue remains controversial (see Ready et al, 1984).

The amphibian model and the scope of this Thesis.

Since the precise role of the mammalian thymus during early development in T cell education and self tolerance acquisition is still unresolved, the initial aim of this Thesis was to explore this issue, using the anuran amphibian Xenopus. Unlike mammalian radiation chimeras and nude mice, the amphibian model has the advantage that it has a free-living embryo and can be surgically manipulated at very early stages of development, prior to the maturation of the immune system. Amongst amphibians, the immunobiology of the clawed frog, Xenopus, has been particularly well studied; this primitive anuran is known to possess an immune system similar in many respects to that of mammals (see Cohen and Turpen, 1980; Manning and Horton, 1982 for recent reviews). Xenopus is easily reared and maintained in the laboratory

and inbred "strains" and isogeneic clones are now becoming more readily available.

Xenopus has been shown to possess functionally distinct T and B lymphocytes through the use of selective T and B cell mitogens (Donnelly, Manning and Cohen 1976; Green and Cohen, 1979; Horton et al, 1980; Williams et al, 1983), thymus-dependent and thymus-independent antigens (Turner and Manning, 1974; Collie, Turner and Manning, 1975; Tochinai and Katagiri, 1975; Tochinai, 1976; Horton, Rimmer and Horton, 1976; Du Pasquier and Wabl, 1976), and by the use of monoclonal reagents against Xenopus immunoglobulins (Bleicher and Cohen, 1981) and thymocyte determinants (Nagata, 1985).

This amphibian species also displays the functional characteristics typical of a mammalian MHC. Acute rejection of allografts, the mixed leucocyte response (MLR), cell-mediated lysis and the genetic restriction of T-B lymphocyte collaboration in the generation of an in vitro secondary antibody response have been shown to be genetically linked, segregating together within Xenopus families (Du Pasquier, Chardonnens and Miggiano, 1975; Bernard et al, 1979; Du Pasquier, Blomberg and Bernard, 1979; Bernard et al, 1981). Very recently, biochemical studies have demonstrated the presence of molecules homologous to their class I and class II mammalian counterparts on the surface of Xenopus haemopoietic cells (Flajnik et al, 1984; Kaufman et al, 1985; Kaufman, Flajnik and Du Pasquier, 1985).

In Xenopus, thymic ablation can be performed as early as 4 days after fertilization (Tochinai and Katagiri, 1975); stage 45 of Nieuwkoop and Faber (1967). This is prior to the differentiation of small lymphocytes, which does not occur in Xenopus until stage 49 (12 days) (Nagata, 1977). In contrast to neonatally thymectomized (Tx) mammals, such 4-8 day thymectomy in Xenopus does not lead to runting, but results in either severe impairment or abrogation of allograft rejection (Horton and Manning, 1972; Tochinai and Katagiri, 1975; Rimmer and Horton, 1977; Kaye and Tompkins, 1983; Nagata and Cohen, 1983), depending on the precise relatedness of donor and host. The MLC response in Xenopus to foreign MHC antigens has also been reported to be thymus dependent (Du Pasquier and Horton, 1976; Horton and Sherif, 1977). Early thymectomy also abolishes, or severely impairs, the response to the T cell mitogens phytohaemagglutinin (PHA) and concanavalin A (Con A) (Du Pasquier and Horton, 1976; Manning, Donnelly and Cohen, 1976; Horton and Sherif, 1977; Green (Donnelly) and Cohen, 1979; Nagata and Cohen, 1983), the plaque-forming cell antibody response to heterologous red blood cells (Horton and Manning, 1974; Tochinai and Katagiri, 1975) and the low molecular weight immunoglobulin (IgY) response to dinitrophenyl-keyhole limpet haemocyanin (DNP-KLH) (Du Pasquier and Horton, 1982).

Experimental reconstitution of T cell-dependent immune responses has already been attempted with thymectomized Xenopus. In 1975, Horton and Horton demonstrated that larval implantation of early (7-8 day) thymectomized (Tx)

animals with genetically undefined thymuses restored both the ability to acutely reject third-party skin allografts and the response to sheep red blood cells (SRBC's). Injection of histocompatible or incompatible thymocytes was also found to be effective in restoring the antibody response of Tx Xenopus to heterologous erythrocytes (Kawahara, Nagata and Katagiri, 1980) and human gamma globulin (Nagata, 1980); the use of ploidy-marked cells in the former work demonstrating that, although the injected cells participated directly in graft rejection, they only provided a helper function in the antibody response. Experiments performed using genetically-defined, Tx animals have since demonstrated that the antibody response to both DNP-KLH and SRBC can be restored using either isogeneic or allogeneic thymus implants (Du Pasquier and Horton, 1982; Gearing, Cribbin and Horton, 1984). In addition, thymic reconstitution studies have revealed that, although the ability to reject skin grafts from third-party donors is restored, tolerance to thymus donor strain antigens ensues (Horton and Horton, 1975; Nagata and Cohen, 1984).

The thymus reconstitution experiments reported in this Thesis were initially performed to further probe the immune capabilities of Tx/thymus implanted animals and, in particular, to examine their ability to display reactivity to minor H antigen-disparate skin grafts. These experiments were designed to determine whether the thymus, during late larval and early adult life, plays a central role in the education of minor H antigen-reactive T cells. It was first

necessary to characterize histocompatibility reactions displayed within the J strain Xenopus, which were to be used extensively in part of this study; the responses of these inbred frogs to intra-strain skin grafts and in mixed leucocyte culture are presented in Chapter Two. The ability of perimetamorphically thymus-implanted, Tx inbred and clonal animals to reject skin grafts displaying novel major or minor histocompatibility antigens, when tested as adults, is examined in Chapter Three. The extent to which non-specific perimetamorphic tolerance is the cause of the poor reactivity towards minor H antigens observed in Tx Xenopus restored with allogeneic thymus is examined in Chapter Four, where allotolerance is induced in control Xenopus following perimetamorphic skin grafting. To investigate the extent of the allotolerance induced to donor MHC antigens by thymus or skin grafting in late larval life, the in vitro MLC reactivity of T cells from animals made tolerant by both protocols is studied in Chapter Five. Following the discovery that splenocytes from some animals tolerant of donor skin retain the ability to proliferate to donor (irradiated) splenocytes in vitro, Chapter Six explores whether host spleen cells (from tolerant animals) can proliferate in vivo to injected, irradiated, splenocytes (of the tolerance-inducing MHC type). This Chapter also probes (through a graft-versus-host assay) the ability of tolerant animals to defend against potentially hostile, viable splenocytes, and assesses whether their cells can mount a GVH reaction on adoptive transfer. Future

experiments to explore the cellular basis of allotolerance in Xenopus are discussed.

CHAPTER TWO

CHARACTERIZATION OF HISTOCOMPATIBILITY REACTIONS WITHIN J STRAIN XENOPUS LAEVIS.

INTRODUCTION

The G strain of Xenopus laevis was produced through over fifteen years of inbreeding within C. Katagiri's colony of X.laevis at Hokkaido University, Sapporo, Japan. They were later rechristened J (DiMarzo and Cohen, 1982a) to reflect their Japanese origin. J strain Xenopus are MHC-identical (haplotype jj) and were initially thought not to reject skin and other tissues from one another (Katagiri, 1978; Tochinai and Katagiri, 1975). However, DiMarzo and Cohen (1982a) subsequently reported that these J strain clawed frogs display minor histocompatibility (H) antigen disparities and presented evidence for chronic intra-J strain skin graft destruction.

Central use of J strain Xenopus in the immunological restoration and tolerance experiments in this Thesis was envisaged, since they are easily bred and produce large numbers of offspring. But first, in view of these conflicting reports about J strain histocompatibility, it seemed essential to examine the alloimmune capabilities and genetic relatedness of our own J colony. Therefore a detailed study of intra-J strain responses to first-set,

second-set and third-party J skin grafts is presented in this Chapter.

The mixed leucocyte response (MLR) is known to be an in vitro marker of MHC disparity (Du Pasquier and Miggiano, 1973; Du Pasquier, Chardonnens and Miggiano, 1975 and Du Pasquier et al, 1977). In contrast, MLC reactivity is non-existent or, at best, poor when splenic leucocytes (from ungrafted animals) expressing only minor H antigen differences are mixed and co-cultured in vitro (Du Pasquier and Miggiano, 1973).

In their 1982a paper, DiMarzo and Cohen mentioned that lymphocytes from their J colony were mutually unreactive in MLC. Therefore, in addition to the in vivo graft rejection experiments, the response of splenic leucocytes from ungrafted J animals to J alloantigens in one- and two- way MLC is also examined, to confirm whether only minor H antigen differences exist between individuals of our colony. The effect of in vivo immunization with J skin on subsequent intra-J MLC reactivity is also examined, since others have shown that spleen lymphocytes from J frogs that had rejected MHC-disparate skin, exhibit a specific increased incorporation of tritiated thymidine when cocultured with skin donor lymphocytes, relative to MLC indices effected by non-immune responder cells (Barlow and Cohen, 1981).

Skin graft rejection and MLC responses of our J strain animals to MHC-disparate donors are studied alongside the intra-J experiments.

MATERIALS AND METHODS

Animals.

The J strain X.laevis used in this Thesis were descended from adults donated by C. Katagiri from his original Japanese stock. They were bred and reared in the laboratory at $23 \pm 1^{\circ}\text{C}$, as described fully elsewhere (Horton and Manning, 1972). Briefly, male and female adults were induced to spawn by injection of chorionic gonadotrophin (Griffin and George) into the dorsal lymph sac. Naturally-fertilized embryos were collected on nylon netting and transferred to aerated, dechlorinated water. Hatched larvae were fed with nettle powder. Post-metamorphic animals were given Tubifex worms; older frogs received ground ox liver twice-weekly.

Some isogenic X.laevis/X.gilli hybrids (LG clone numbers 5, 3 and 17) were used in this Chapter as donors of MHC-disparate skin, or as sources of stimulator spleen leucocytes in MLC. These clonal frogs were produced from LG females (or their F_1 offspring) donated to our laboratory by L. Du Pasquier (Basel Institute for Immunology, Basel, Switzerland), by the gynogenetic development of their endoreduplicated eggs, after activation with UV-irradiated sperm (Kobel and Du Pasquier, 1975).

The outbred, "wild" Xenopus laevis used to provide an additional source of allogeneic lymphocytes in the culture work were bred in the laboratory from adults purchased from Xenopus Ltd.

Skin grafting.

Froglets were lightly-anaesthetised in MS222 (Sandoz) and grafted dorsally with one or more 2-3mm² dorsal, froglet skin grafts. Hosts were kept in shallow amphibian Ringer's solution (to keep the grafted areas dry) for 12 hours to allow the grafts to heal in; they were then returned to tanks of fresh water and maintained at an elevated temperature of 25-26°C for the observation period to mimic the higher temperatures used in MLC. Grafts were observed under a binocular microscope every 2 or 3 days for 100 days. Irregular observations were also made at later post-grafting periods. Rejection was considered complete at the time of total pigment cell destruction; histologically, only collagen pad of the donor skin remains at the rejection end point chosen here (Horton, 1969 and see Fig. 2.1h).

Preparation of splenic leucocyte suspensions.

All surgical instruments and plastics used in cell preparation and culture were sterile. Culture medium was sterilized by filtration through a 0.22 µm filter (Millipore). Animals were anaesthetised with MS222 (Sandoz) and rinsed with 70% ethanol, before removal of the spleen in a laminar air flow hood. Using watchmaker's forceps, spleens were gently teased apart in small petri dishes (Falcon, 3001F) containing Leibovitz L-15 culture medium (Flow), supplemented with 50 IU penicillin/ml, 50 µg streptomycin/ml, 2.5 µg fungizone/ml, 1.25mM l-glutamine, 0.01M sodium bicarbonate buffer, 0.01M HEPES buffer (all

from Flow) and 0.083mM 2-mercaptoethanol (BDH), diluted with 2x distilled water to amphibian tonicity. One percent decomplemented foetal calf serum (Flow) was added to protect the cells during preparation and to increase cell viability in culture. Spleen cell suspensions were transferred from petri dishes to 5ml plastic tubes (Falcon, 2058 Tube). Tissue fragments were gently pipetted to release leucocytes and the debris allowed to settle before removal of the suspended spleen cell supernatant to fresh test tubes. Cells were washed 3 times in the above culture medium by centrifugation at 350xg for 10 minutes at 4°C. After the final wash, the cells were resuspended in a known volume of medium appropriate for counting (usually 1ml) and the leucocyte concentration for each suspension determined using a Neubauer haemocytometer. Leucocyte numbers were adjusted to 1×10^6 viable cells/ml in preparation for culture.

Splenic leucocytes to be used as irradiated stimulator or autologous cells in 1 way MLC were inactivated in suspension by exposure to a total irradiation dose of 6000 rads at a rate of 1200 rads/minute from a 60 Co source (see Lallone, 1984). After irradiation, the cells were washed once more and resuspended in fresh medium at culture concentration.

All spleen cell suspensions used in this Thesis were prepared by this method.

Mixed Leucocyte Culture.

The MLC assay methods used in this Thesis are modifications of those devised by Weiss and Du Pasquier (1973).

Splenic leucocytes suspended at 1×10^6 viable cells/ml were placed in the appropriate assay combination (see later), in triplicate (unless stated otherwise in Table Legends), in 96 well, conical-based tissue culture plates (Sterilin, M25 ARTL). Each well contained a total of 2×10^5 splenic leucocytes in 200 μ ls of modified L-15 culture medium. Cells were incubated at 26°C in a water-saturated atmosphere of 5% CO_2 in air. Each well was pulsed after 72 hours with $1 \mu\text{Ci}$ tritiated thymidine (specific activity 5 Ci/mmol, Amersham) in 10 μ ls of medium, and transferred after a further 24 hours incubation (using a semi-automatic cell harvester (Skatron, Flow)) onto individual glass fibre filter discs. The filters were dried at 60°C for 1 hour and punched out into individual scintillation vials (Packard, Pico hang-in vials) and 2.5mls toluene scintillator fluid (Packard) was then added. Tritiated thymidine uptake and incorporation by dividing cells was measured in each culture sample by an automatic liquid scintillation counter (Packard, Tricarb 300C). The proliferation level for each triplicate culture was expressed as mean disintegrations (quenched counts) per minute (dpm) \pm standard error of the mean (sem). The index of stimulated proliferation was calculated as follows:

$$\text{Stimulation index} = \frac{\text{mean dpm experimental cultures}}{\text{mean dpm control cultures}}$$

Therefore a stimulation index (SI) of 1.00 indicates that no increase in proliferation is seen in the experimental cultures when compared with control cultures. For indices >1.00, mean dpms for experimental and control cultures were statistically compared using the Student's "t" test. When the probability level, *p*, of any difference in dpm was <0.05, the SI was considered significant.

In one- and two-way MLC assays on ungrafted J animals in this Chapter, experimental mean dpm \pm sem were provided by co-culturing 1×10^5 cells from each partner (irradiated where applicable). The background, non-stimulated (control) level of cell proliferation in these experiments was obtained by culturing 2×10^5 cells from each partner (normal or irradiated) used in the MLC separately and then halving the mean dpm \pm sem before use as denominators in calculation of the SI. For one-way MLCs with grafted animals and all other MLC experiments described later in this Thesis, mean control dpms were obtained by co-culturing triplicates of 1×10^5 responder cells with 1×10^5 irradiated autologous cells: experimental dpms were provided as above. In these latter experiments, dpm from 1×10^5 irradiated cells were also measured and subtracted from the control and experimental mean values, before calculating SIs; (see MLC Table Legends). The irradiation dose of 6000 rads employed here is routinely found to reduce the dpm of irradiated

cultures to <20% of the proliferation shown by non-irradiated cells (data not shown).

EXPERIMENTAL DESIGN

(a) Skin grafting.

To determine the extent of minor H antigen disparities within J strain X.laevis, 1st-set J skin grafts were applied to 42, six month-old J hosts. Animals were given 1 or 2 allografts each: 21 froglets received 2 allografts and 21 were grafted with a single J allograft and an autograft, the latter served as a control for damage or infection due to the operative technique.

The chronology of intra-J strain rejection phases was examined in detail in 18 recipients. These hosts received 1 or 2 1st-set allografts; 100-120 days after 1st-set grafting, these 18 animals were given a single (2nd-set) graft from one of the original donors and also a 3rd-party graft from a new J donor. These secondary grafts were applied to see if rejection was accelerated by immunization with a 2nd-set graft, and whether alloimmune memory was specific to the original J donor. This experiment, therefore, was (in part) an attempt to examine the degree of H antigen sharing amongst our J colony.

In the above experiments, skin donors for any particular host were non-siblings both to that host and to each other to maximize histocompatibility differences. Donors were of a similar age to the hosts.

To check that J strain Xenopus were capable of normal MHC-disparate allograft rejection, 12, six month-old froglets were given a single foreign graft from an LG (either LG3, LG5 or LG17) donor. These hosts did not receive any skin grafts from J donors.

(b) MLC reactivity of J strain splenocytes.

First, to show that ungrafted Js were in fact capable of proliferating to MHC-disparate cells in vitro, splenic leucocytes from 5 frogs were cultured in 1-way MLC with irradiated splenocyte stimulators from outbred X.laevis. The outbred animals were assumed to differ by one or two MHC alleles from the inbred J strain Xenopus, since skin grafts exchanged between them were rejected "acutely"; ie. in approximately 3 weeks at 26°C (J.D. Horton, personal communication).

The responses of splenic leucocytes from 9, ungrafted, nine month-old J animals to splenocytes from other, ungrafted, non-sibling Js were examined in both 1- and 2-way mixed leucocyte cultures. The 2-way cultures were performed in an attempt to visualize responses that might be too low to be detected in assays which only measured the proliferation levels of one population of cells.

Finally, to determine whether alloreactivity to first-set J skin grafts results in an increase in the number of splenocytes reactive to J to a level where induced proliferation in MLC could be detected, three animals (Fig. 2.2, host nos. 29, 31 and 32) were tested in 1-way MLC

against their respective 1st- and 2nd-set skin graft donors, 8 months after application of the 2nd-set grafts. Specificity was checked with irradiated, 3rd-party J cells from a different donor to that which supplied the 3rd-party skin grafts. MLC reactivity of these grafted Js to MHC-disparate antigens was confirmed with irradiated cells from outbred X.laevis, LG3 or LG17 isogeneic clones.

RESULTS

(a) Skin grafting studies on J strain X.laevis.

In this Thesis, allografts are referred to as being "perfect" only while they resemble autografted skin transplants (Fig. 2.1a). Since destruction of minor H antigen-disparate allografts can be of a prolonged, chronic nature, rejection phenomena have been described in two phases. A graft in "Phase I" of rejection displays vascular disturbances such as vasodilation and haemostasis (Fig. 2.1b). The graft and graft bed may therefore appear reddened and contain pools of extravascular blood. Slight pigmentary defects become noticeable as melanophores contract and take on a "punctate" appearance (Fig. 2.1c). During "Phase II", dermal skin glands are often revealed and become noticeable under the more necrotic epidermis (Fig. 2.1d). Some intra-J skin allografts remain for prolonged periods in this condition (see Fig. 2.2 and Fig. 2.1e), rather than continuing to become more necrotic; "late" phase II witnesses further destruction of pigment cells, which now

release their melanin content (Fig. 2.1f), giving the graft a blackish appearance. The edges of the dorsal skin graft eventually begin to whiten (Fig. 2.1g). "Rejection End Point" is reached when pigment and skin glands have been completely destroyed. The rejected dorsal skin graft appears predominantly white, with occasional clumps of necrotic, blackened tissue (Fig. 2.1h).

Table 2.1 reveals the fate of first-, second-set and third-party J strain allografts, and also the response of Xenopus to MHC-disparate LG grafts. The 12, MHC-disparate skin grafts from either LG3, 5 or 17 donors were all rejected by their J hosts within 30 days ("acute" fashion). In contrast, none of the 1st-set J strain grafts were fully rejected by this time, but 63% were fully rejected by 100 days. Indeed, by then only 2/63 grafts remained in "perfect" condition; the other 21 non-rejected grafts displayed chronic rejection phenomena. Autografts placed alongside allografts always survived in perfect condition. Of the 18 J froglets that were subsequently grafted with 2nd-set and 3rd-party J skin, to probe the immunological basis of intra-J graft rejection, 77% now rejected their 2nd-set grafts "acutely" (here defined as within 30 days). Considerable minor H antigen sharing between animals in the colony was shown by the fact that this accelerated graft rejection was not always specific to the original J donor; indeed 44% of the 3rd-party grafts were completely destroyed acutely. However, a certain degree of minor H antigen polymorphism is indicated, since the proportion of secondary

grafts not fully rejected by 100 days was higher when the grafts were 3rd-party (50%-9/18) than when from the original donor (17%-3/18).

Figure 2.2 illustrates the chronology of graft rejection phases in the 18 regrafted J animals. First-set J grafts remained in perfect condition for prolonged post-transplantation periods when compared with MHC-disparate grafts on other J hosts; the latter reach phase I within one week of grafting. Phase I and II of graft rejection in some J hosts given J grafts were of quite short duration, eg. host nos. 1 and 3. In many cases, however, phases I and II were prolonged and 5 1st-set grafts on the 18 recipients shown in this Figure did not complete rejection within the 100 day observation period. There were found to be no obvious differences in the outcome of graft rejection by hosts given a single 1st-set graft and those given two. With respect to secondary grafts, six hosts (nos. 1,4,6,7,28 and 33) revealed accelerated (acute) rejection specific to the 2nd-set transplant only. In contrast, 8 J hosts (nos. 2,3,5,19,21,27,31 and 32) displayed acute rejection of both 2nd-set and 3rd-party grafts. A final group of 4 hosts (nos. 8,22,29 and 30) failed to show acute destruction of either 2nd-set or 3rd-party skin grafts. Only 3 hosts (22,29 and 30) failed to complete rejection of any of their J grafts within 100 days. It should be noted that in all 18 regrafted hosts, onset of rejection phenomena in both 2nd-set and 3rd-party grafts was more rapid than with

the 1st-set J grafts; the elevated response of grafted Js is further examined in the MLC studies described below.

(b) MLC reactivity of J strain splenic leucocytes.

The response of ungrafted J strain X.laevis to MHC-disparate cells in 1-way MLC is shown in Table 2.2.

Splenocyte populations from 3 froglets, (B,D and E), were each tested with one different, outbred, X.laevis stimulator population. Splenocytes from two additional outbred X.laevis provided stimulators for both G and H. In all the cultures, the responder J strain leucocytes displayed significantly elevated levels of proliferation induced by the cocultured, irradiated stimulator cells, although a large variation (1.45-6.87) was seen in the stimulation indices. Variation was also observed in the level of dpm obtained in different cultures. For example, in cultures of cells relating to animals B and E, relatively high proliferation was recorded from both irradiated (stimulator) and non-irradiated (responder) control cultures. The mean dpm of irradiated cells was, however, always reduced to <20% of non-irradiated cells (data not shown).

One- and 2-way MLC reactivity of ungrafted Js to J leucocytes is examined in Table 2.3. Splenocytes from 4 J animals (A,B,C and D) were tested in 1-way cultures against a panel of irradiated J stimulators. Cells from 6 Js were used to establish three, bi-directional MLC experiments. No significant induced proliferation was detected in either the

1- or 2-way cultures. In contrast, as shown in Table 2.4, one J animal, host 31 of Fig 2.2, that had rejected 1st- and 2nd-set minor H antigen-disparate J skin grafts from donor 32, showed a significant proliferative response in 1-way MLC when cocultured with irradiated splenocytes from this graft donor. Host 32, that had also rapidly rejected its 2nd-set J graft (from animal 31), failed to show a significant MLC response to splenocytes from its donor, although the SI suggests otherwise. Host 29, which had failed to completely reject any J graft also showed no evidence of MLC reactivity to graft donor cells. In agreement with the skin grafting experiments, the MLC response of host 31 to J stimulators appears not to be specific for cells of the skin donor, as shown by the SI achieved when splenocytes from this froglet were cocultured with 3rd-party J cells. All 3 allografted J hosts examined in this MLC experiment showed significant SIs when their splenocytes were cocultured with MHC-disparate stimulators.

DISCUSSION

The in vivo and in vitro experiments on alloreactivity between J strain X.laevis presented here confirm and extend the observations of others (DiMarzo and Cohen, 1982a; Nagata and Cohen, 1983) that these partially-inbred frogs are MHC-compatible, but display minor H antigen differences.

Experiments on more than 60 intra-J skin allografts reveal that these were never rejected acutely, but that the

majority of grafts were eventually completely destroyed in chronic fashion. DiMarzo and Cohen (1982a) have previously revealed that at 24°C, 72% (13/18) of J froglets had rejected 1mm² J skin by 75 days post-grafting; larger grafts (4mm²) fared slightly better, with only 50% being rejected in the same time. The median survival time of small and large rejected grafts in their experiments was approximately 50 days. In the experiments reported here, 63% of 1st-set J grafts (2mm²) had been rejected by 75 days ie. a proportion directly comparable to that found in DiMarzo and Cohen's study; the median survival time of 44 days obtained for rejected J grafts in the present experiment is also similar. The fate of J grafts not rejected by 75 days was not discussed by DiMarzo and Cohen, whereas the present work reveals that nearly all J grafts display chronic rejection phenomena by 100 days post-transplantation.

The present experiments extend our knowledge about intra-J graft rejection to the 2nd-set situation. The finding that both 2nd-set and 3rd-party J grafts are often both rejected in acute fashion, indicates a lack of extensive H antigen polymorphism within the J strain. On the other hand, the very high proportion of 1st-set J grafts that undergo allorecognition and the finding that 2nd-set skin graft destruction can be specific, reveal a certain degree of diversity of minor H antigens. The interpretation of limited polymorphism of minor H loci in J strain Xenopus is consistent with the views of Obara, Kawahara and Katagiri (1983), who studied skin graft reactivity among siblings of

gynogenetic, genetically-undefined X.laevis. These authors confirmed the findings of DiMarzo and Cohen (1982a), that Xenopus larvae respond poorly, if at all to disparate minor H antigens; this was not studied in the present experiments.

The in vitro MLC experiments recorded here are in agreement with a recent report, (Nagata and Cohen, 1983), that cocultures of splenocytes from ungrafted J froglets fail to display enhanced proliferative reactivity compared to unstimulated control cultures, thereby confirming the absence of MHC differences within the J strain. Lack of, or extremely poor, MLC reactivity between splenocytes taken from minor H antigen-disparate, non-grafted LG clones (Kobel and Du Pasquier, 1975; Lallone and Horton, 1985) also confirms that this proliferative response is a correlate of MHC disparity in Xenopus, class II MHC antigens being thought to effect MLC reactivity when MHC-disparate lymphocytes are cocultured (Kaufman et al, 1985). However, MLC reactivity between minor disparate lymphocytes can occur if allografted animals are used. Thus, in the present study J strain Xenopus that have rejected (J) skin allografts can sometimes then display MLC reactivity which, like in vivo skin grafting, is not entirely (J) donor-specific. Lallone and Horton (1985) have recently shown, in this laboratory, that splenocytes taken from minor H graft-sensitized LG17 Xenopus can display quite good MLC reactivity to leucocytes of the skin donor (LG15). Positive MLC reactivity between minor H-disparate leucocytes in these situations could relate to both increased numbers and activity of

alloreactive cells and elevated non-specific factors (eg. production of interleukin(s), macrophage activity), changes promoted by in vivo exposure to minor H antigens. Enhanced proliferative ability of alloreactive cells could account for the finding that rejection of secondary J grafts (both 2nd-set and 3rd-party) is always initiated more rapidly than primary J graft rejection (see Fig. 2.2). Interestingly, the rapidity of onset of secondary graft rejection appeared not to be related to speed of eventual graft destruction. Although MHC-disparate graft rejection in Xenopus may well involve the activity of cytotoxic T cells (see Bernard et al, 1979), the extent to which these cells contribute to minor H graft destruction remains controversial. Thus Lallone and Horton (1985) failed to demonstrate the generation of cytotoxic cells in minor H-grafted LG Xenopus, whereas Watkins (1985) has recently provided some evidence to the contrary. Lack of correlation between MLC reactivity and skin graft rejection capacity is discussed again later in this Thesis.

Confirmation that individuals of our J strain colony are compatible at the MHC, but minor H antigen-disparate, made them suitable candidates for use in the projected thymus-implantation studies in the following Chapter where I wished to examine development of alloimmune responses and allotolerance to major and minor H antigens in thymectomized animals, following restoration with allothymus implants.

Table 2.1 Legend

MHC-disparate grafts:	A single MHC-disparate graft from an isogenic LG hybrid, clone number 3, 5 or 17 was applied to an ungrafted J individual when 6 months old. These hosts did not receive any J skin grafts.
1st-set J grafts:	Animals received 1 or 2 grafts from separate J individuals when 6 months old. (Those toadlets (N=21) given only 1 allograft were also given a control autograft; the latter were always in perfect condition >100 days).
2nd-set J grafts:	Applied 100-120 days following 1st-set grafting. Animals received a single 2nd-set graft (same donor as 1st-set).
3rd-party grafts:	A single 3rd-party graft (from a new J donor) was applied alongside the 2nd-set graft.

† Phase I: vascular disturbances and slight pigmentary defects.

Phase II: pigment destruction modest to severe, glandular appearance to the graft.

Rejected: complete pigment and skin gland destruction.

Table 2.1 Fate of MHC-disparate (LG) and first- and second-set J skin grafts applied to J strain Xenopus laevis.

Donor skin type	No. of grafts	Graft rejection times in days						Condition of grafts† not fully rejected 100+ days			% grafts fully rejected within	
		0-15	16-30	31-45	46-60	61-75	76-100	<u>Chronic rejection</u>			100 days	30 days
								Phase II	Phase I	Perfect		
LG 3, 5 or 17 MHC-disparate grafts	12	-	12	-	-	-	-	-	-	-	100	100
J strain <u>X. laevis</u> 1st-set grafts	63	-	-	24	11	5	-	19	2	2	63	0
J strain <u>X. laevis</u> 2nd-set grafts	18	-	14	1	-	-	-	3	-	-	83	77
J strain <u>X. laevis</u> 3rd-party grafts	18	-	8	1	-	-	-	9	-	-	50	44

Table 2.2 Legend

† Letters here and in Table 2.3 refer to individual J froglets.

$$\text{Stimulation Index} = \frac{\text{Mean dpm experimental culture (J x r. X1)}}{\text{Mean dpm control cultures (J responder + r.X1) cultured separately}}$$

SI's with $p < 0.05$ by Student's t-test were considered significant.

6000 rad-irradiated splenocytes from outbred X. laevis (r.X1) were used as MHC-disparate stimulators in mixed leucocyte cultures.

Experimental cultures (J x r.X1): mean dpm \pm sem were for 2×10^5 splenic leucocytes (1×10^5 responder cells cocultured with 1×10^5 stimulators).

J responder control cultures: mean dpm \pm sem were for 1×10^5 splenic leucocytes (half the actual counts recorded from 2×10^5 cells plated per well.

r. X1 stimulator control cultures: mean dpm \pm sem were for 1×10^5 irradiated, splenic leucocytes ($\frac{1}{2}$ the actual counts recorded from 2×10^5 cells).

All cells cultured at 1×10^6 cells/ml in 200 μ l modified L-15 medium/well in 96, well, conical-based plates for 72 hours, then pulsed with 1 μ Ci tritiated thymidine/well for 24 hours before harvesting.

Cultures were set up in triplicate except where marked *, these were duplicate cultures only.

Animals G and H were each tested against two different outbred X. laevis spleen stimulators.

Animals were aged approximately 9 months when assayed in culture.

Table 2.2 Response of ungrafted J strain X. laevis splenocytes in one-way mixed leucocyte culture to irradiated, outbred X. laevis (MHC-disparate) stimulators.

J strain animal	Stimulation Index	Statistical p value	Experimental Cultures		Control Cultures	
			(J x r.X1) mean dpm ± sem	(J responder) mean dpm ± sem	(r.X1 stimulator) mean dpm ± sem	
† B	1.45	< 0.01	6658 ± 362	2923 ± 77*	1662 ± 155	
D	4.54	< 0.001	3908 ± 86*	784 ± 468	77 ± 41	
E	2.87	< 0.002	15680 ± 1647	4304 ± 410	1163 ± 647*	
G	4.53	< 0.01	8033 ± 1041	1731 ± 840	43 ± 11	
G	6.87	< 0.001	12094 ± 266	1731 ± 840	29 ± 10	
H	4.25	< 0.01	3735 ± 450	836 ± 483	43 ± 11	
H	6.77	< 0.001	5859 ± 659	836 ± 483	29 ± 10	

Table 2.3 Legend

For one-way MLC, stimulation index =
$$\frac{\text{mean dpm experimental culture } (J_1 \times r.J_2)}{\text{mean dpm control cultures } (J_1 + r.J_2)\text{cultured separately}}$$

For other details see Table 2.2 Legend.

For two-way MLC, stimulation index =
$$\frac{\text{mean dpm experimental culture } (J_1 \times J_2)}{\text{mean dpm control cultures } (J_1 + J_2)}$$

Experimental cultures ($J_1 \times J_2$), mean dpm \pm sem given for 2×10^5 splenic leucocytes (1×10^5 cells J_1 cocultured with 1×10^5 cells J_2).

Control J_1 and J_2 cultures, mean dpm \pm sem given for 1×10^5 splenic leucocytes.

All cells cultured at 1×10^6 cells/ml in 200 μ l modified L-15 medium/well in 96 well, conical-based plates for 72 hours, then pulsed with 1 μ Ci tritiated thymidine/well for 24 hours before harvesting.

Cultures set up in triplicate except where marked * on the table; these were duplicate cultures only.

Stimulation indices with a p value > 0.05 were not significant (NS) by Student's t-test.

Animals tested in culture were aged approximately 9 months.

Table 2.3 Response of ungrafted J strain X. laevis splenic leucocytes to J stimulators in one- and two-way mixed leucocyte cultures.

(a) One-way MLC

J strain animal combination (J ₁) x (r.J ₂)	Stimulation Index	p value	Experimental Cultures		Control Cultures	
			(J ₁ x r. J ₂) mean dpm ± sem		J ₁ mean dpm ± sem	r.J ₂ mean dpm ± sem
A x r. E	1.37	NS	4057 ± 840	2142 ± 328	810 ± 418	
B x r. C	0.95	NS	3133 ± 786	2923 ± 77*	368 ± 19*	
C x r. B	0.74	NS	2336 ± 504	2160 ± 379*	991 ± 220*	
D x r. I	1.08	NS	1006 ± 132	784 ± 468	150 ± 23	
D x r. K	0.91	NS	909 ± 144	784 ± 468	211 ± 98	

(b) Two-way MLC

(J ₁) x (J ₂)	Experimental Cultures		Control Cultures	
	(J ₁ x J ₂) mean dpm ± sem		J ₁ mean dpm ± sem	J ₂ mean dpm ± sem
A x E	0.77	NS	4947 ± 938	4304 ± 410
F x L	1.33	NS	8241 ± 548	3005 ± 656
G x M	2.25	NS	5782 ± 1564*	836 ± 483

Table 2.4 Legend

Stimulation Index = $\frac{\text{experimental mean dpm} \pm \text{sem (responder} \times \text{r. stimulator)}}{\text{control mean dpm} \pm \text{sem (responder} \times \text{r. autologous)}}$

control mean dpm \pm sem (responder \times r. autologous)

Stimulation indices with $p < 0.05$ by Student's t-test were considered significant.

The J animals used as 1st- and 2nd-set skin donors, 3rd-party J (unrelated to 3rd-party skin donors) and MHC-disparate Xenopus (outbred X. laevis, LG clones 3 and 17) were used as sources of 6000 rad-irradiated leucocytes in the mixed leucocyte cultures.

Experimental and control mean dpm \pm sem were from 2×10^5 splenic leucocytes.

All cells were cultured at 1×10^6 cells/ml in 200 μ l modified medium/well, in 96 well, conical-based plates for 72 hours, then pulsed with 1 μ Ci tritiated thymidine/well for 24 hours before harvesting.

All cultures were set up in triplicate.

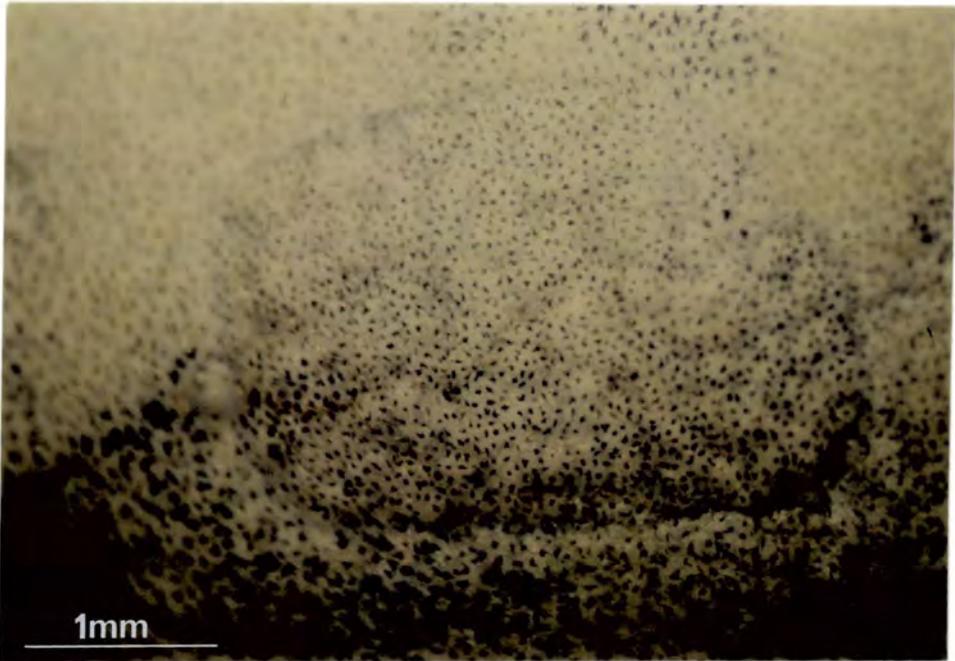
Animals were aged approximately 18 months and had previously received J skin grafts as shown in Fig. 2.2.

Table 2.4 One-way MLC reactivity of grafted J strain X. laevis.

MLC Combination	Animal No. (see Fig.2.2)	Stimulation Index	Statistical p value	Experimental Cultures (responder x r.stimulator) mean dpm ± sem	Control Cultures (responder x r.autologous) mean dpm ± sem
Grafted J versus J graft donor	29 x r. 30 31 x r. 32 32 x r. 31	0.85 5.31 1.79	NS <0.01 NS	1374 ± 595 4259 ± 909 2477 ± 498	1624 ± 400 802 ± 58 1382 ± 488
Grafted J v. 3rd party J	31 x r. J 32 x r. J	3.35 2.03	<0.002 NS	2687 ± 226 2806 ± 657	802 ± 58 1382 ± 488
Grafted J v. MHC-disparate <u>X.laevis</u> or <u>X.laevis</u> / <u>gilli</u> clones 3 or 17.	29 x r. LG17 29 x r. X.1. 31 x r. LG17 31 x r. LG 3 32 x r. LG17 32 x r. LG 3	5.11 4.59 7.57 6.63 3.62 2.70	<0.001 <0.001 <0.01 <0.001 <0.01 <0.02	8301 ± 490 7455 ± 440 6068 ± 798 5317 ± 256 5005 ± 499 3726 ± 365	1624 ± 400 1624 ± 400 802 ± 58 802 ± 58 1382 ± 488 1382 ± 488

Figure 2.1 Phases of skin allograft rejection in Xenopus laevis

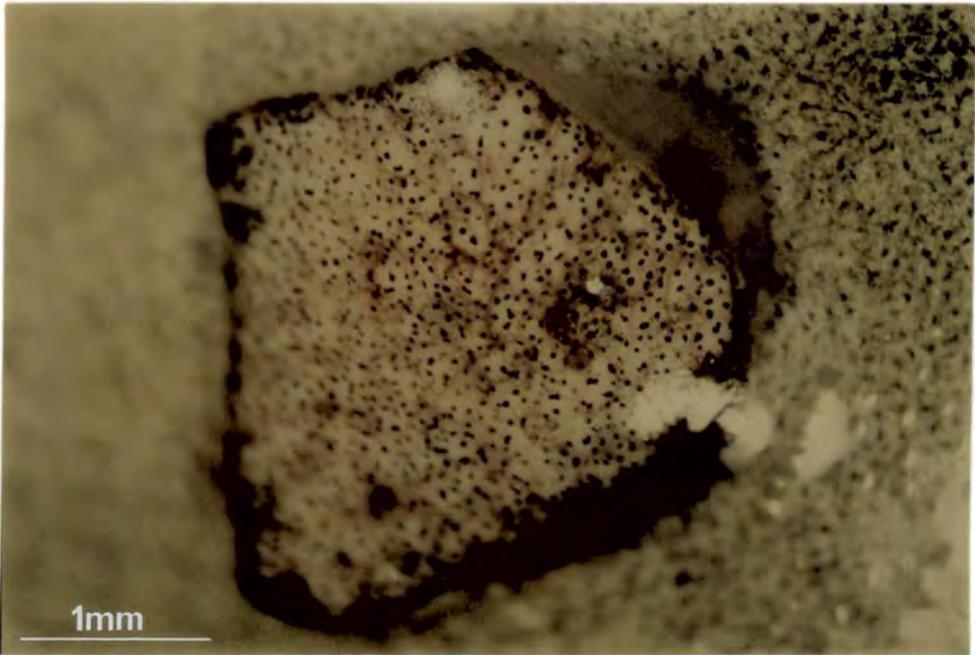
2.1 a Perfect condition



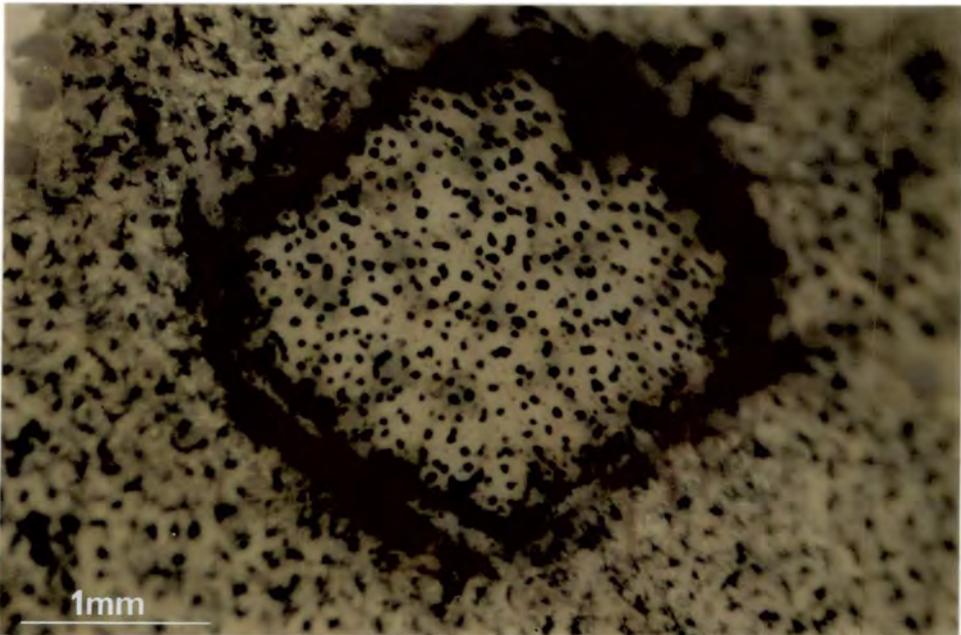
No signs of rejection. Graft has healed in and has become revascularized.
N.B. This allograft was photographed several months after transplantation.

2.1 b and c Phase I allograft rejection

(b)



(c)

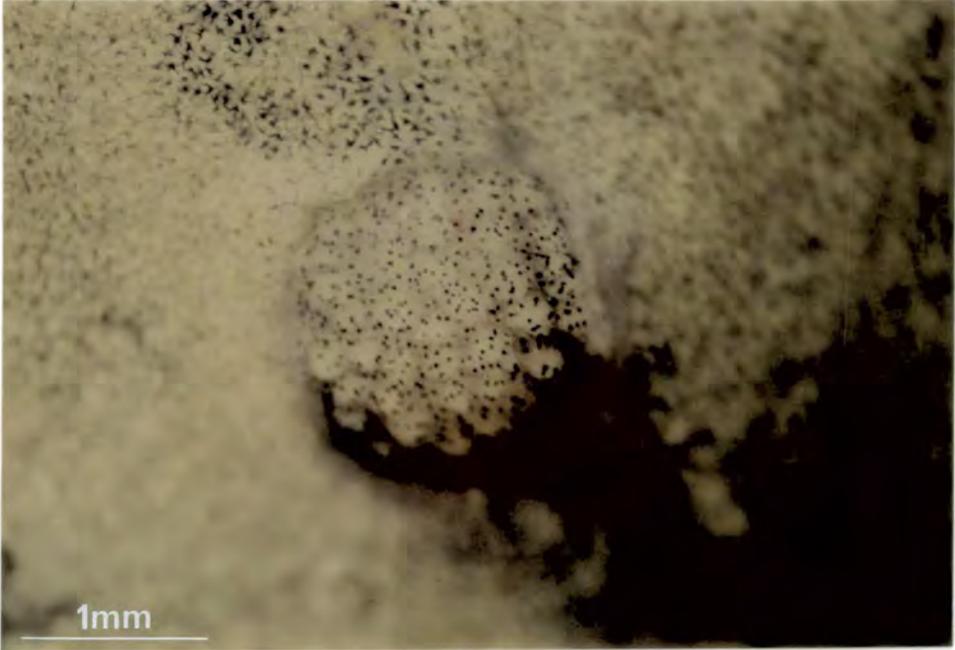


Noticeable features are:- (i) Vasodilation of blood capillaries (Fig 2.1b), followed by haemostasis; (ii) pigmentary defects becoming noticeable as the melanophores in the skin contract, giving a 'punctate' and rather pale appearance to the graft, (2.1c). N.b. Figs 2.1 b and c were of MHC-disparate graft rejection, during the first 2 post-operative weeks. The graft bed is still particularly evident in Fig 2.1b.

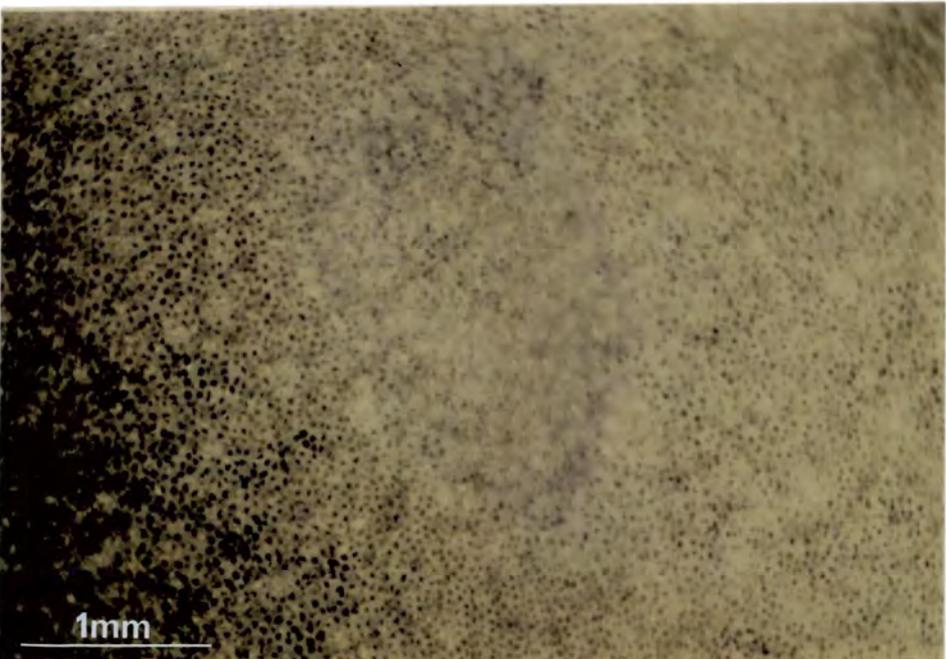
2.1 d - g Phase II allograft rejection

Pigment destruction is now modest to severe, with underlying glandular layers of the skin becoming prominent, (2.1d). Minor-disparate grafts may stay like this for a prolonged period, (2.1e), rather than enter 'late' Phase II. In those grafts reaching the rejection end point, black patches of melanin are now seen, as melanophores are destroyed, (2.1f). The edges of the graft begin to whiten, (2,1g).

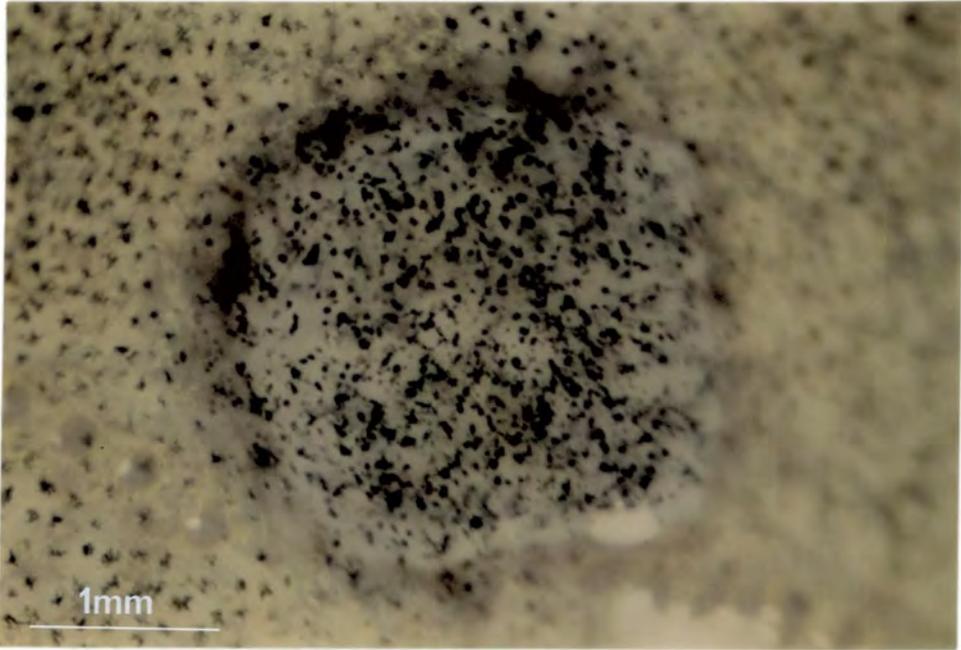
(d)



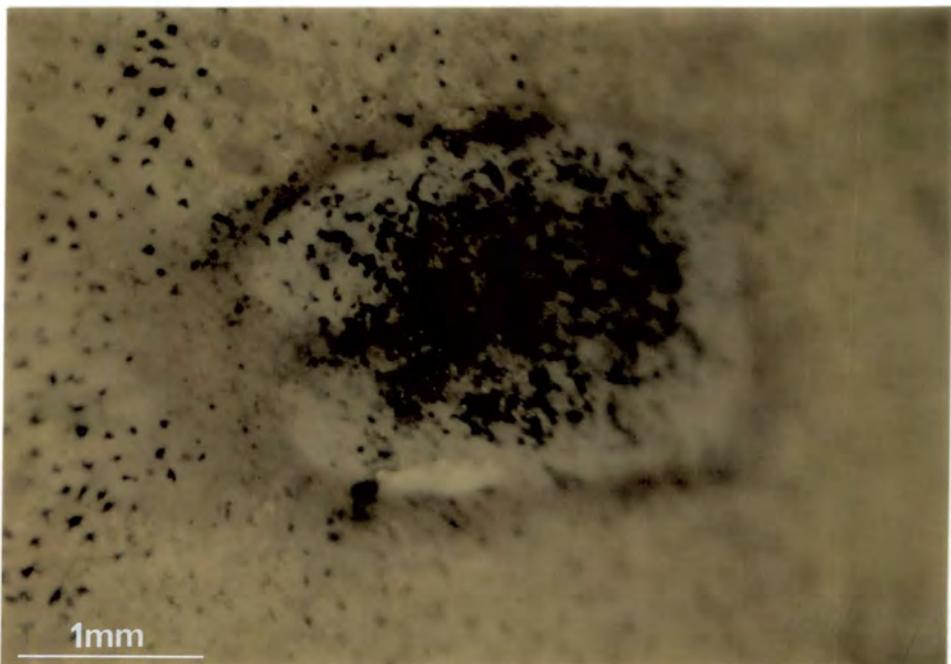
(e)

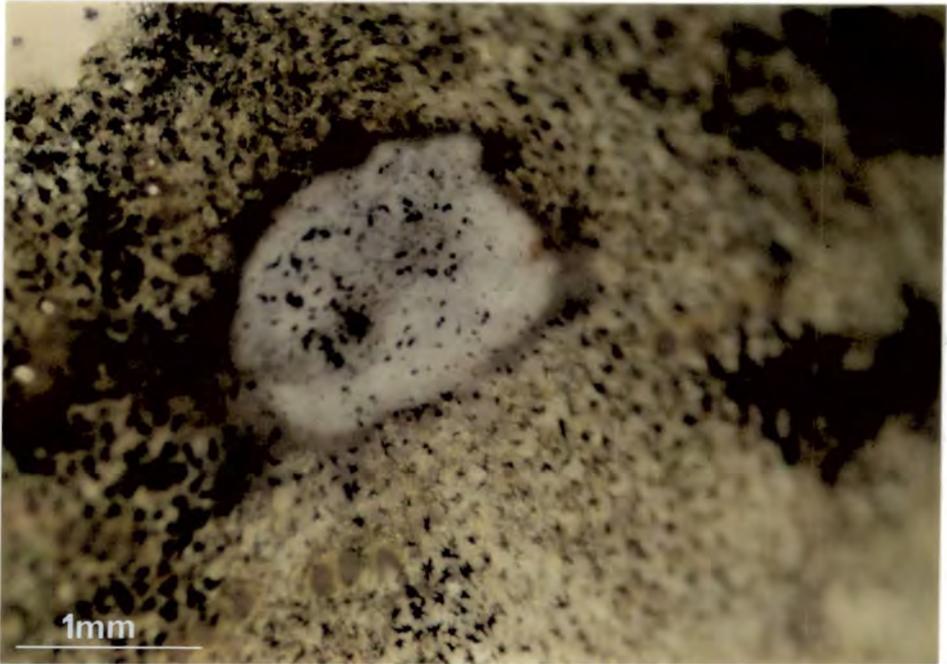


(f)



(g)



2.1 h Rejection end point

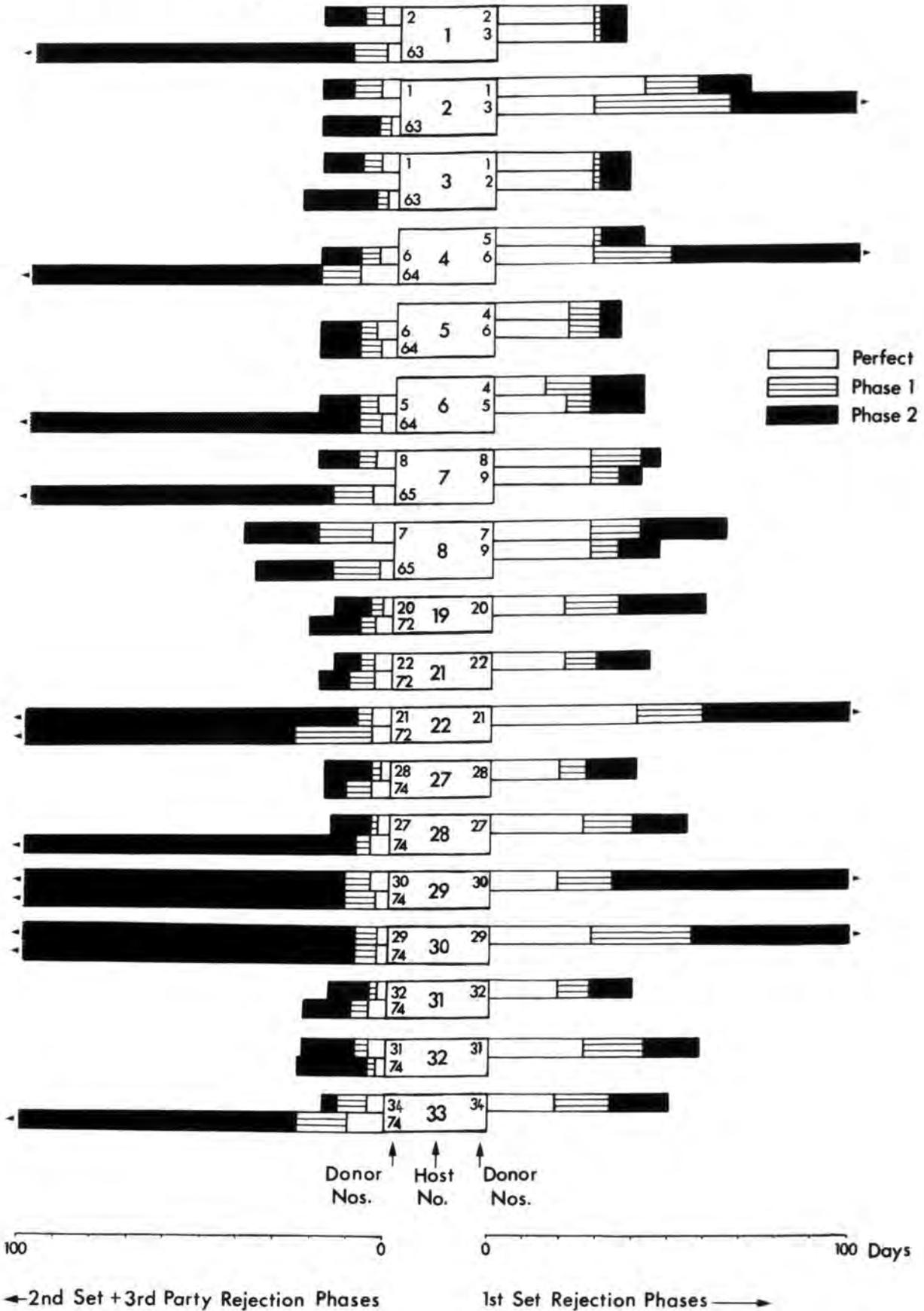
Pigment cells and skin glands are now completely destroyed. The graft bed of collagen has a whitish appearance and the host epidermis gradually grows inwards to cover the scar.

Figure 2.2 Legend.

Eighteen J strain Xenopus hosts were given second-set and third-party J skin allografts 100-120 days following first-set grafting.

For other experimental details see Legend to Table 2.1 and 'Experimental Design' section of text.

Fig.2.2 Chronology of graft rejection phases in J strain *Xenopus laevis*.



CHAPTER THREEIMPAIRED REJECTION OF MINOR H-DISPARATE SKIN
GRAFTS AND ACQUISITION OF ALLOTOLERANCE IN
THYMECTOMIZED XENOPUS IMPLANTED WITH
HISTOINCOMPATIBLE THYMUS.

INTRODUCTION

Thymectomy of 4-7 day-old Xenopus larvae is known to severely impair in vivo T cell functions such as skin allograft rejection (Horton and Manning, 1972; Tochinai and Katagiri, 1975; Rimmer and Horton, 1977) and helper activity in antibody responses (Turner and Manning, 1974; Tochinai and Katagiri, 1975; Horton, Rimmer and Horton, 1977; Horton et al, 1979).

Previous studies have shown that the response to skin allografts can be restored in such early-Tx Xenopus by subcutaneous implantation of either allogeneic larval thymus into larval hosts (Horton and Horton, 1975), or adult thymus into an histocompatible adult host (Tochinai, Nagata and Katagiri, 1976), provided that the test graft is MHC-disparate to both thymus donor and host.

The results of recent experiments involving implantation of adult (MHC- compatible or incompatible), ploidy-marked thymuses and larval allothymuses into early-Tx Xenopus (Nagata and Kawahara, 1982; Nagata and Cohen, 1984;

Gearing, Cribbin and Horton, 1984) suggest that, like the nude mouse (Kindred, 1978), the Tx amphibian possesses precursor cells capable of differentiating into T cells under the influence of a thymic microenvironment. This Chapter examines whether T cells, that have developed as a consequence of thymus implantation into Tx hosts, can restore skin graft alloimmunity, in particular to skin bearing only minor H antigen differences to the thymus donor and/or the Tx host.

The experiments make use of 7-day-thymectomized J strain Xenopus and X.laevis/X.gilli (LG) hybrids, which were implanted with either an isogeneic or allogeneic (minor H or MHC antigen-disparate) thymus in late larval life. The primary intention of these experiments was to study the role of the thymus during late larval and early adult life in the education of minor H antigen reactive T cells, since such alloreactive T cell populations are thought to be MHC-restricted in mammals (Bevan, 1975). It was necessary to extend this work to include LG animals, which, although not so readily available as J strain, possess better defined histocompatibility antigens, in the sense that some isogeneic strains exist that are of the same MHC, but differ from other clones only in terms of minor H antigens (Kobel and Du Pasquier, 1977).

In this Chapter, then, thymus-implanted, Tx Xenopus were examined in postmetamorphic life for their ability to reject skin grafts that should be displaying histocompatibility antigens (either MHC or minor H antigens)

not found on host or on thymus donor cells. They were also tested with skin from donors that were isogenic to the thymus donor to examine whether specific in vivo tolerance to thymus strain antigens is achieved.

Also included in this Chapter is a brief investigation confirming the host origin of lymphocytes within thymus implants and examining whether donor thymocytes survive for a prolonged period in the periphery; this study makes use of the X.borealis fluorescence cell marker (see Thiébaud, 1983).

MATERIALS AND METHODS

Animals.

The MHC haplotypes of the X.laevis/X.gilli (LG) hybrid clones used in this Thesis were designated by Kobel and Du Pasquier (1977), on the basis of relative speeds of skin graft rejection and magnitudes of mixed leucocyte reactions between members of different clones. Clones 15 and 17 were assigned the haplotypes (ac) and differ by minor H antigens only. Both clones are disparate to clone LG5 (originally designated with MHC haplotypes (bc) (Kobel and Du Pasquier, 1977), although more recently referred to as being (ad) (Bernard et al, 1981) and in this Thesis), by a single MHC haplotype and differ from clone LG3 (haplotypes bd) by 2 MHC haplotypes. Animals within a clone are identical with respect to both their minor and major H antigens. LG clonal

animals were produced, as mentioned in detail in Chapter 2, by in vitro fertilization of the endoreduplicated (2N) eggs with UV-irradiated sperm (see Kobel and Du Pasquier, 1975).

Xenopus borealis were also used in this Chapter. The adult animals were a generous gift from Dr R.H. Clothier, University of Nottingham, U.K., from which offspring were obtained by the usual breeding procedure.

Thymectomy.

Larval thymectomy was carried out using the microcautery method of Horton and Manning (1972). Thymuses were bilaterally removed when larvae were 7 days old (Stage 47 of Nieuwkoop and Faber, 1967). The larvae were checked for absence of thymic regeneration before metamorphosis and also at post mortem.

Thymus implantation.

Thymuses for subsequent implantation were dissected from donors under anaesthesia in MS222 (Sandoz) using tungsten needles, fine forceps and spring scissors; they were then transferred to petri dishes containing Leibovitz L-15 culture medium (Flow) diluted 5:3 with 2x distilled water. Donor thymuses were either from larvae of similar age and stage to the host, or from 4-6 month old adults. Sometimes these adult thymuses were too large for implantation and required halving before use. To deplete thymic lymphocyte numbers, irradiation of some donor thymuses was performed just prior to implantation; thymuses

were placed in 12x75 mm Falcon tubes in 5:3 medium and irradiated with a ⁶⁰Cobalt source at a dosage of either 1000 rads (200 rads/min) for larval thymus, or 3000 rads (600 rads/min) for adult thymus.

Implantation was performed under light anaesthesia in MS222, when the thymectomized (Tx) larval hosts were 4-6 weeks old, stages 56/58 (hind-limbs are well developed, fore-limbs erupt during stage 57; ie. metamorphosis is beginning). A single, whole or halved thymus was implanted subcutaneously, just posterior and medial to an eye (see Fig. 3.1a). The thymus was pushed into a groove prepared in the host using glass or tungsten needles). Operated larvae were allowed to recover in aerated 1:2 amphibian Ringer's solution diluted with 2x distilled water and then gradually reintroduced to standing water over the next 24 hours. Larvae were checked for the presence of an implant before, and again after metamorphosis, when the non-irradiated thymus can usually be seen as a distinct lump near the eye (Fig. 3.1b).

Skin grafting.

Dorsal skin grafts from donors of similar age to the hosts were applied to 5-9 month old, Tx froglets, to thymus-implanted Tx animals and also to control Xenopus, and monitored as previously described in Chapter 2.

EXPERIMENTAL DESIGN**(a) Skin grafting studies on thymus-implanted animals.**

Thymectomized J strain Xenopus were implanted as larvae with an MHC-compatible (but minor H-disparate, J strain) or MHC-incompatible (LG3, 5 or 17) thymus. Tx LG clones were given either an isogeneic thymus or an allogeneic thymus implant (one or two MHC haplotype-disparate). The possible influence of the maturity of the implant (n.b. expression of class I MHC antigens is only seen on "adult" cells; see Flajnik, 1984 and Discussion) on repopulation by T cell precursors and subsequent restoration of alloimmunity, and/or induction of tolerance to foreign thymus donor antigens, was examined by the use of both larval and adult thymus grafts. Some thymuses were also irradiated in vitro prior to implantation to reduce the contribution probably made by donor lymphocytes (see Nagata and Cohen, 1984) to the establishment of the peripheral T cell population.

As young (5-9 month old), postmetamorphic froglets, the thymus-implanted Tx hosts were tested for restoration of acute allograft rejection by the application of a 3rd party skin graft (MHC-disparate to both thymus donor and host strain by at least one MHC haplotype).

Restoration of minor H antigen allograft rejection was assessed by grafting Tx J strain hosts, given J larval (normal or irradiated) or adult, MHC-compatible or incompatible thymus implants, with (putative) 3rd party J skin. Since minor H antigen polymorphism within the J strain

appears to be limited, some hosts received grafts from more than one J donor to maximize the likelihood of minor H antigen disparity between host, thymus donor and test graft. The effect of implantation of an MHC-disparate thymus on the hosts ability to reject minor H antigen-disparate skin was also examined with LG frogs, where comparisons could be made directly with other animals given isogeneic thymus implants.

In addition to these experiments on restoration of skin alloimmunity, some allothymus-implanted Tx hosts were tested for specific in vivo skin graft tolerance of thymus donor MHC antigens.

Typical rejection times for minor H and MHC antigen-disparate skin grafts were obtained by grafting unoperated J and LG control froglets of similar age to the implanted animals. The thymus dependency of both minor H and MHC antigen-disparate graft rejection by J strain Xenopus and LG clones 15 and 17 was demonstrated by skin grafting studies with non-implanted, Tx animals.

(b) Origin of lymphocytes within thymus implants and in the periphery.

J strain Tx larvae were implanted with either a normal or 3000 rad-irradiated X.borealis froglet thymus. At 5 months of age, they were grafted with LG15 skin to check that this implantation of allothymus had restored in vivo alloreactivity. Following rejection of the skin grafts, non-irradiated thymuses were removed, fixed in Carnoy's, embedded in historesin (see Russ, 1986) and 4 μ m sections

were stained in either toluidine blue or in quinacrine: a fluorochrome that stains AT-rich X.borealis cells (but not X.laevis cells) in a "spotted" fashion, when viewed by fluorescence microscopy (Thiébaud, 1983). Gamma-irradiated implants had, by now, disappeared and therefore were not available for histologic examination. Cytospin preparations (see Williams, 1981) of splenocytes and blood smears from the above Tx animals (given either normal or irradiated thymus implants) were also stained with quinacrine and observed for the presence of X.borealis cells in the host periphery.

RESULTS

(a) Gross and histologic observations on thymus implants.

Thymus implants could readily be seen during larval life, through the transparent overlying skin (Fig. 3.1a), but gamma-irradiated thymuses became very much reduced in size.

Histologic observations on normal and irradiated larval allogeneic thymus implants during the first month post-implantation have recently been made by others in this laboratory (Gearing, Cribbin and Horton, 1984). Briefly, their studies showed that irradiated (1000 or 5000 rad) alloimplants initially appear to be predominantly epithelial, in contrast to the normal appearance of non-irradiated thymuses (see Fig. 3.2a and b). By early

postmetamorphic life, Gearing et al (1984), revealed that the tiny, irradiated implants have become lymphoid; however, as noted in the present work, irradiated thymus implants (both larval and adult) soon become undetectable. In contrast, non-irradiated compatible and incompatible implants continue to grow (Fig. 3.1b) in postmetamorphic life and retain a perfectly normal histological structure (Fig. 3.2c).

(b) Origins of lymphocytes in J strain X.laevis given X.borealis adult thymus implants.

Non-irradiated X.borealis thymus implants removed from 6-month old, Tx J strain X.laevis, sectioned and stained with toluidine blue were useful for illustrating the histologic differences between lymphocytes and stromal cells (eg. epithelial cells); see Fig. 3.3a. Quinacrine staining and fluorescence microscopy of thymus implant sections revealed that the vast majority of thymus lymphocytes were by now of host origin; in contrast, epithelial cells (and cells of the thymic capsule) appeared to have remained as X.borealis (donor) type (Fig. 3.3b and c).

X.borealis lymphocytes were never seen in the blood or spleen of those 5 Tx animals given irradiated X.borealis thymuses. The situation appeared to be the same in 4 of the 5 animals given non-irradiated implants. However, in the other animal, significant numbers of donor cells were seen in the spleen cytopins (Fig. 3.4a/b) and in the blood.

(c) Skin graft experiments with control, Tx and thymus-implanted J strain recipients.

The results are illustrated in Figure 3.5. The data from control J strain animals is taken from experiments performed during the skin grafting study in Chapter 2. In contrast to the sub-acute (30-50 days) or chronic rejection (>50 days) seen with minor H-disparate skin, MHC-disparate grafts are rejected acutely (within 30 days). Early thymectomy generally abrogated the ability to destroy MHC-disparate grafts, although 3 LG17 transplants were rejected in sub-acute or chronic fashion. Implantation of minor H-disparate J thymus, be it larval or adult, normal or irradiated, restored the allograft response of Tx J animals to MHC antigens; most grafts from LG or wild *X.laevis* donors were now rejected in acute fashion, although a few transplants were rejected only sub-acutely. In contrast, 24/26 J grafts (placed alongside the recognized MHC-disparate grafts) remained perfect for >100 days, only 2 being rejected in sub-acute fashion.

Implantation of MHC-disparate (LG) adult thymus (normal or irradiated) also restored the ability of Tx J animals to reject skin either acutely or sub-acutely from donors MHC-disparate to host or thymus donor. Skin graft tolerance, specific to thymus donor histocompatibility antigens, is induced by this protocol, since skin grafts from the thymus donor LG strain in 20/21 cases remained in perfect condition for >100 days, whilst neighbouring, unrelated transplants

were being rejected. J transplants also remained in excellent health in this group of animals.

(d) Skin graft experiments with LG recipients.

The results are illustrated in Figure 3.6. MHC-disparate skin grafts applied to LG17 and LG15 controls are rejected acutely, whereas minor graft destruction is generally sub-acute (30-50 days). Early thymectomy impairs (to varying degrees dependent on the particular donor-host combination) rejection of MHC-incompatible grafts, but abrogates minor H-incompatible graft rejection. As with the experiments on J strain Xenopus, MHC-incompatible, larval and adult thymuses restore the ability of Tx animals to reject third-party, MHC-incompatible skin, but achieve tolerance to thymus donor strain skin grafts.

Implantation of an isogeneic larval thymus to Tx LG17 froglets fully restores both MHC- and minor H- disparate graft rejection, revealing that absence of a thymus for a few weeks of larval life is not, in itself, the causative factor for the disturbed recognition of minor antigens that often occurs here following allogeneic thymus implantation. LG17 and LG15 Tx animals implanted with thymus incompatible by one or two MHC haplotypes displayed (in all cases but one) impaired rejection of skin from donors which bore only minor H differences that can be recognized as foreign. It appears that impaired rejection of skin on which minor antigens were expected to be "on view" occurs both when the thymus is of completely different MHC to the test skin graft

donor (eg. LG17 (ac) Tx implanted with LG3 (bd) thymus and subsequently tested with LG15 (ac) skin) and even when the thymus shares one haplotype with the test skin graft (eg. LG17 (ac) Tx implanted with LG3 (bd) thymus and grafted with LG5 (ad) skin). Furthermore, when the thymus implant was of identical MHC to the test skin graft (eg. LG3 (bd) Tx implanted with LG15 (ac) thymus and assayed with LG17 (ac) skin) nearly all froglets failed to complete rejection prior to 100 days.

DISCUSSION

In this Thesis, implantation was deliberately performed before metamorphosis was complete, to allow sufficient time for the recipient to recover from the thymectomy operation, but not sufficient to allow the Tx animals to develop, perhaps abnormally, for long in the absence of a thymic environment. Operating at this early stage also enables the organ to be transplanted before the period of rapid growth of the in situ thymus, which occurs just after the end of metamorphosis (Du Pasquier and Weiss, 1973; Williams, 1981), following a temporary decrease in thymic size a few days earlier. Both adult and larval thymuses were implanted to Tx animals in the present work, since it is only after metamorphosis that class I MHC antigens become detectable on Xenopus cells; class II MHC antigens have been recorded in larvae (Flajnik, Kaufman and Du Pasquier, 1983).

The experiments reported in this Chapter reveal, using a variety of donor/host strain combinations, that larval or adult thymus from histocompatible or allogeneic donors (normal or irradiated), implanted a few weeks after early larval thymectomy, will fully restore the ability of Xenopus to reject third-party, MHC-disparate skin grafts, while tolerance to skin of the MHC-disparate donor type is induced. These findings confirm and extend previous observations from this laboratory (Horton and Horton, 1975). They are also in agreement with the recent findings of Nagata and Cohen (1984), who implanted adult MHC- matched or mismatched thymus to adult, early Tx Xenopus and noted normal third-party allodestruction.

Tochinai, Nagata and Katagiri reported in 1976 that although implantation of histocompatible adult thymus, performed as late as 1.5 years after early thymectomy did restore skin allograft rejection capacity in 4-5-day-Tx Xenopus, rejection was somewhat delayed relative to controls. Nagata and Kawahara (1982) found that third-party allograft rejection was also delayed following allothyms implantation into young, early-Tx adult hosts when compared to hosts given histocompatible implants. In the present study, however, delayed rejection of third-party allografts following implantation of larval and adult allogeneic thymus before metamorphosis was not observed. A possible explanation for the differing results might be that the establishment of sufficient numbers of T lymphocytes in the periphery is retarded when the thymic environment and host

stem cells are histoincompatible, and this time lag in allorestoration is reflected in the results of the relatively "short term" (8 weeks between thymus implantation and skin grafting) experiments of Nagata and Kawahara. It should be noted that they found that the adult allothymus implants were fairly rapidly recolonized by lymphoid cells of the Tx host (>50% host-derived cells within 12 weeks) whereas MHC-compatible thymuses were, surprisingly, minimally (<10%) recolonized by host cells within 116 days post-implantation. Such a cell changeover in the allogeneic implants may cause a delayed establishment of a new peripheral T cell pool. Perhaps the longer interval between implantation and test-grafting used here may allow time for both host precursor T cells to differentiate within the allogeneic thymus and for the T lymphocytes to migrate to the periphery in sufficient numbers to fully restore alloreactivity.

An obvious question arises as to whether the cells involved in the restoration of allograft rejection in these thymus implantation experiment are of donor origin (emanating from the thymus implant) or represent host-derived T-lineage cells, that have migrated to the thymus implant, or been influenced hormonally (Dardenne et al, 1973) by the implant.

There can be little doubt from the cell marker studies presented here and elsewhere (Nagata and Kawahara, 1982; Gearing, Cribbin and Horton, 1984; Nagata and Cohen, 1984) that host cells do actively colonize allothymus implants

(both larval and adult) and form the major lymphoid population of the thymus implant after several months. The present, and the more detailed studies of Russ (1986), which make use of the X.borealis marker to observe cell migration into thymus implants, reveal clearly that the epithelial cells of the foreign thymus implant remain predominantly donor in origin. Overall, the skin grafting and cell traffic experiments give credence to the view (Nagata and Cohen, 1984) that, as in the nude mouse (Wortis, Nehlsen and Owen, 1971; Kindred, 1978), donor thymic elements promote the differentiation of host precursor cells in Tx Xenopus along a T cell pathway. This situation also seems to hold true for 3000 rad-irradiated, adult allothymus implants. Thus Russ (1986) has shown that such implants are heavily infiltrated by host cells within the first few weeks post-implantation, this infiltration being initially more extensive than in non-irradiated thymus implants, but that stromal elements remain of donor type. Just why these irradiated, repopulated thymuses disappear early in postmetamorphic life remains uncertain; presumably the dose of irradiation used is sufficient to damage not only donor thymic lymphocytes, but also leads to the eventual loss of the donor stromal cells that earlier attracted host lymphoid precursors.

Although it appears that the implanted thymus houses host-derived T-lineage cells, the possibility that thymic donor-derived lymphocytes persist in the peripheral circulation (eg. spleen, blood etc.) and play a key role in allorestoration must also be considered. Lack of involvement

of donor-derived T-lineage cells in allorestoration is suggested by previous observations in this laboratory (Horton and Horton, 1975) that non-irradiated, larval spleen alloimplants (which contain T lymphocytes able to display MLC reactivity) fail to reconstitute the response of Tx Xenopus to MHC-disparate skin grafts. If donor cells residing in the periphery were mainly responsible for allorestoration, then injection of viable, isolated thymocytes might be expected to restore allograft rejection as effectively as whole thymus implantation. Nagata and Tochinnai (1978) report that such an injection of isolated, histocompatible lymphocytes can restore the allograft rejection capability of early Tx Xenopus, but that injection of a similar number of allogeneic thymocytes only results in poor third-party allogeneic skin rejection. Similarly, injection of congenic but not allogeneic thymocytes restores T cell function in nude mice (Kindred, 1978). It would appear that injected allogeneic donor lymphocytes either fail to survive in adequate numbers, are inactivated, or else cannot co-operate effectively with other components of the host's immune system (such as antigen presenting cells) to produce normal graft rejection. In this Chapter, thymus-derived cells were observed in the spleen and blood of only one J Tx animal, several months after receiving an X.borealis non-irradiated, adult thymus. However, large numbers of donor-derived cells appear to enter the circulation within the first few weeks following implantation of non-irradiated, allothymuses (Russ, 1986) to

Tx Xenopus. Early seeding of thymus implant-derived lymphocytes following use of irradiated thymus transplants awaits examination, since the possible involvement of radiation-resistant T helper cells must not be overlooked (see discussion by Gearing, Cribbin and Horton, 1984). Nagata and Cohen (1984) have shown (by mithramycin staining of ploidy-labelled cells) that from 0-32% donor-derived lymphoid cells persist in the spleen and up to 46% in the implant, 6-7 months after allothymus implantation to adult, Tx Xenopus.

The nature of the donor-derived cells involved with acquisition and maintenance of allotolerance to skin grafts of the thymus strain also awaits clarification. Presumably the donor (stromal) cells within the thymus are centrally involved, although the critical involvement of donor cells in the periphery must also be considered, as has been suggested from recent experiments on thymus-implanted, nude mice (Von Boehmer and Schubiger, 1984). The mechanism underlying allotolerance following implantation of irradiated, "epithelial", thymus may be interesting to explore, particularly since these thymic implants often disappear after metamorphosis. Furthermore the role of thymic epithelium in creating transplantation tolerance is currently under considerable debate (see Jenkinson, van Ewijk and Owen, 1981; Jordan et al, 1985).

In this Chapter, it has been shown that rejection of minor antigen-disparate grafts is completely thymus dependent, while thymectomy severely impairs MHC-disparate

skin graft rejection. When Tx J strain individuals were implanted with MHC-disparate thymus, it was not surprising to find that J skin grafts subsequently failed to be rejected, if it is postulated that the T lymphocytes (developing from J host stem cells under the influence of the thymus implant) which effect rejection were attempting to visualize minor H antigens following education within an MHC-disparate thymic environment. On the other hand, even when the thymus was of identical MHC to the Tx recipient (ie. J thymus to Tx J), subsequent J skin grafts (in 24/26 cases) were also tolerated. The limited minor H antigen polymorphism suspected within the J group might account for a proportion of the "tolerant" situations with thymus-restored, Tx animals, since many of the skin graft donors would not present any novel minor antigens to those already expressed by either the host or thymus donor. It is possible that the only two J skin grafts to be rejected were on Tx animals that had received thymuses from donors that happened to be identical to the recipient in terms of minor antigens (note only 2/63 skin grafts exchanged between J strain controls remained perfect at >100 days).

To gain a better understanding of this poor restoration of minor H antigen-disparate graft rejection, it was decided to use clonal Xenopus (with their more clearly defined H differences) as recipients and donors, in addition to the J strain X.laevis, in this study. The outcome of allothymus restoration with the LG Tx recipients essentially confirmed the findings from the J strain study. Thus rejection of skin

expressing only minor H antigen differences is impaired when thymus and host are MHC-mismatched. When the experimental combination was LG17 Tx (ac) implanted with LG3 (bd) thymus and then tested with LG15 (ac) skin (Fig. 3.6), it seemed possible that the deficiency in rejecting the latter could be related to the recipients bearing the "wrong" MHC type of thymus; ie. the T cells in these Tx LG17 animals learn to recognize minor antigens in the context of the MHC expressed on cells within the implanted LG3 thymus, and are therefore inefficient at dealing with LG15 minor antigens expressed on an (ac) cell background. However, such a straightforward interpretation is not possible in view of the experiments on Tx LG3 recipients given an LG15 thymus. Even with the "right" thymus MHC type, these animals still dealt with subsequently grafted LG17 skin extremely poorly. Only when thymuses isogeneic to the host were implanted, was the Tx animals restored in its ability to visualize minor antigens on skin grafts.

Very recently, Flajnik, Du Pasquier and Cohen (1984; 1985), using a different microsurgical approach, have presented data which suggests that, during early development, the Xenopus thymus is involved, to a limited extent, in establishing the MHC type with which T helper cells preferentially interact. They studied chimeric Xenopus; made by joining the anterior portion of one 24-hour embryo (containing the thymic anlagen; ie. thymus epithelium) to the posterior portion of an MHC-incompatible embryo (from which the haemopoietic stem cells, including

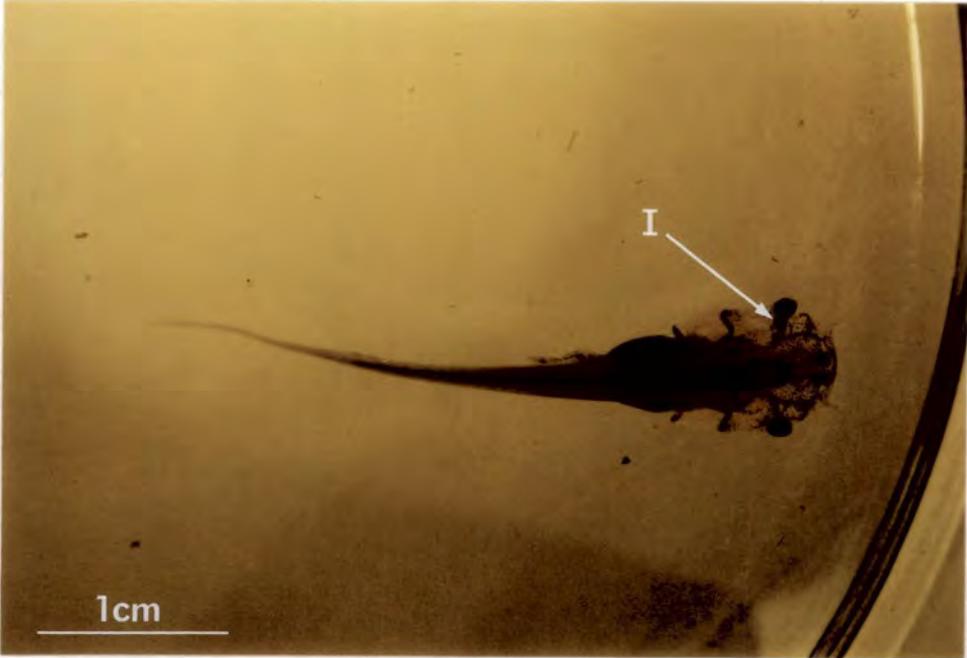
lymphocytes arise). When assayed for antibody production against DNP-KLH, such chimeric animals generally displayed normal IgM reactivity but kinetically-delayed, or low, IgY antibody titres. This is in contrast to the results from experiments using the thymectomy/thymus implant model, which has not demonstrated a central role for the amphibian thymus in the establishment of MHC restriction of helper T cells during late larval and early postmetamorphic life (Du Pasquier and Horton, 1982; Gearing et al, 1984; Horton et al, 1986).

Of particular relevance to the present study are the recent experiments of Flajnik, Du Pasquier and Cohen (1985) on embryonically-established chimeras, which revealed minor H antigen-disparate skin graft rejection of normal kinetics, only when the chimera had been constructed with its anterior region (ie. thymus epithelium) MHC-compatible to the skin donor. Embryos created with their lymphocytes MHC-compatible to the skin donor, but with their thymic anlagen MHC-incompatible, were able to respond to the minor antigens on display, only in a delayed fashion. Evidence that the MHC haplotype of the thymus epithelium may govern the MHC antigen interaction preference of minor H antigen-reactive cells comes from studies on mammalian cytotoxic T cells (Bevan, 1975). A major consideration in this Chapter was whether the thymectomy/thymus implantation model could be used to demonstrate a role of the amphibian thymus in the education of minor H antigen-reactive T cells. The finding that defective minor H-disparate graft destruction occurs,

even when the allothymus implant and skin donor are both of the same MHC type as the Tx host, may indicate that "non-specific" tolerance at the level of minor H antigens is being induced in some experiments and is obscuring the educative role of the thymic stroma in the MHC restriction of minor antigen rejection. This is quite possibly the case, since the thymus implants were transferred at the beginning of a temporary period (peri-metamorphosis) when it is readily possible to achieve allotolerance to (skin) grafts (Bernardini, Chardonnens and Simon, 1970; Chardonnens and DuPasquier, 1973). Whether MHC restriction of the T cells responsible for minor H antigen rejection takes place, and the part that the thymus plays in this education remains unresolved in these Tx/thymus implantation experiments. In the next Chapter, the ability of perimetamorphic implantation of skin to non-thymectomized (control) Xenopus to induce tolerance to minor and major histocompatibility antigens is examined. These experiments address the issue of whether tolerance to minor H antigens around the time of metamorphosis is donor specific, and further examine aspects of allotolerance induction in Xenopus.

Figure 3.1 Photographs of adult thymus implants in situ.

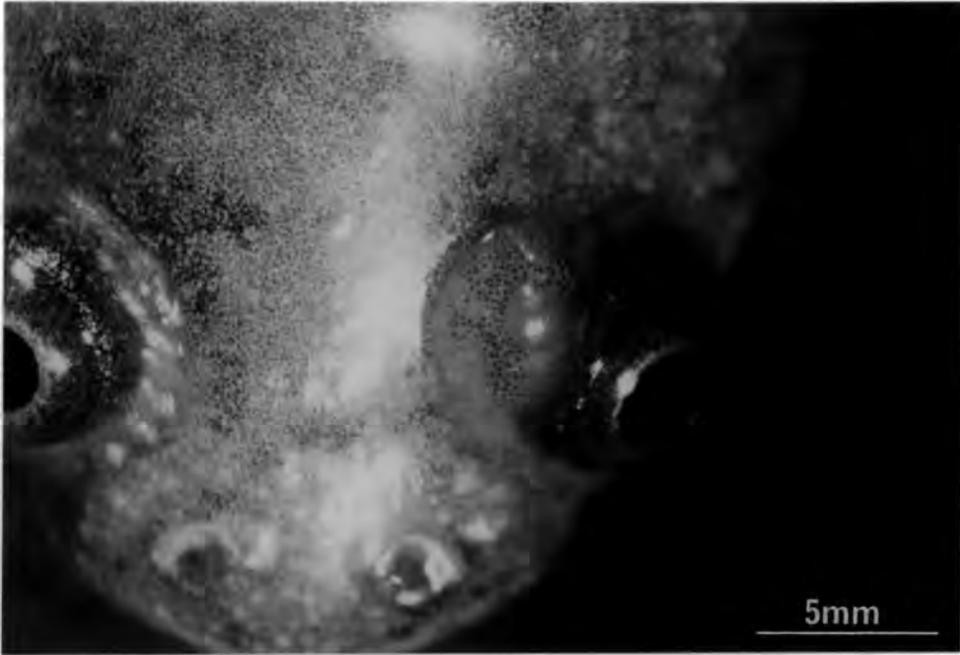
(a) Position of thymus implant in the thymectomized larva.



The non-irradiated, froglet thymus implant (I) is visible subcutaneously, adjacent to the eye. This photograph was taken 1 week post-implantation.

Figure 3.1 Continued

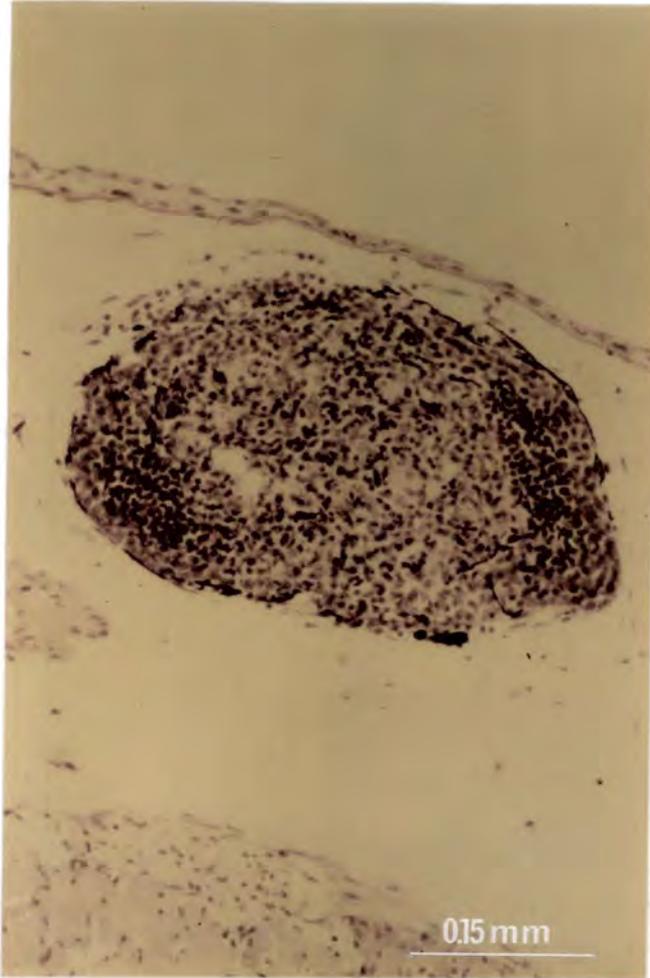
(b) Position of thymus implant in the young froglet.



The non-irradiated thymus implant is noticeable in the froglet medial to the eye. It has become vascularized and has grown in size. This photograph was taken 3 months post-implantation, by which time irradiated thymus implants have usually disappeared from view.

Figure 3.2 Histology of thymus implants.

- (a) Section through the centre of a non-irradiated larval thymus implant.



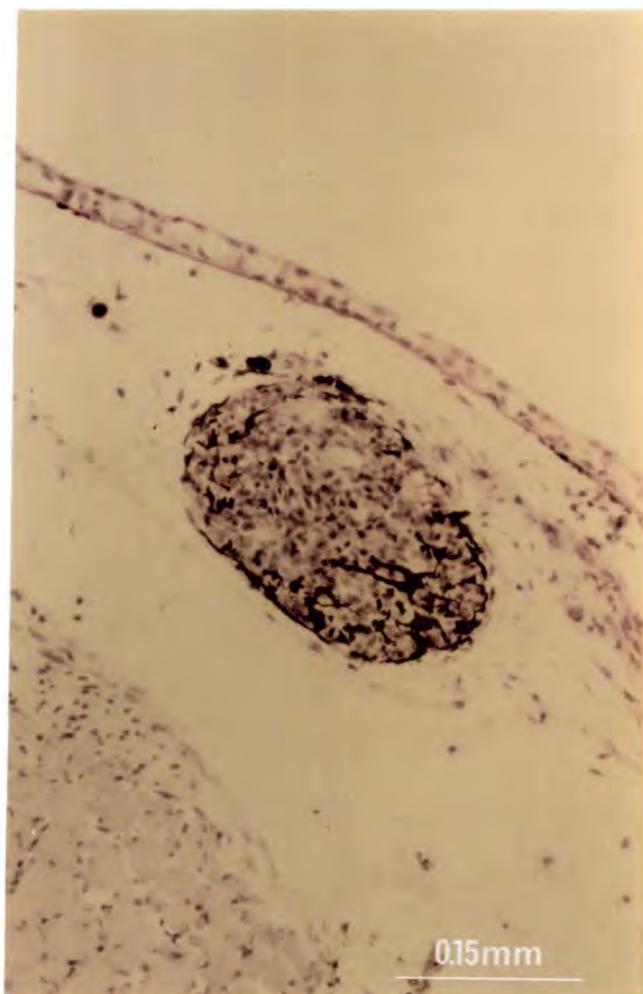
Non-irradiated thymus has normal cortex/medulla differentiation and is very lymphoid.

Photograph taken 4 days post-implantation and is courtesy of Dr. J.D. Horton.

Stain = haematoxylin and eosin.

Figure 3.2 continued

- (b) Section through the centre of a 5000 rad-irradiated larval thymus implant.



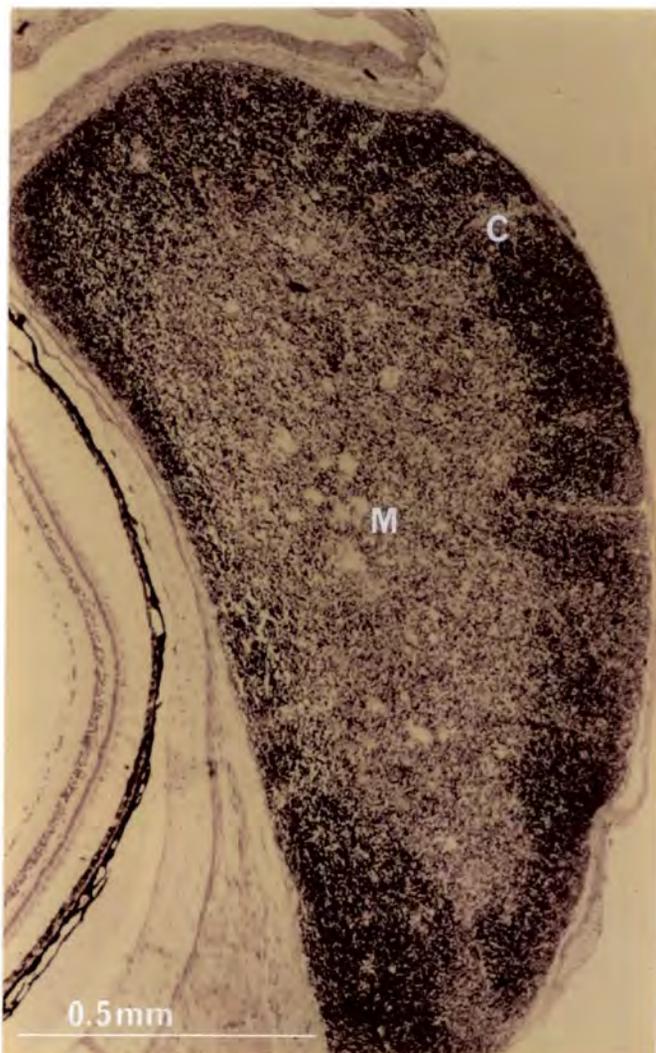
In contrast to the non-irradiated thymus implant in (a), the irradiated thymus is smaller and predominantly comprised of stromal cells.

Photograph taken 4 days post-implantation and is courtesy of Dr. J.D. Horton.

Stain = haematoxylin and eosin.

Figure 3.2 continued

- (c) Section through the centre of a non-irradiated, larval allogeneic thymus implant, 30 days post-implantation.

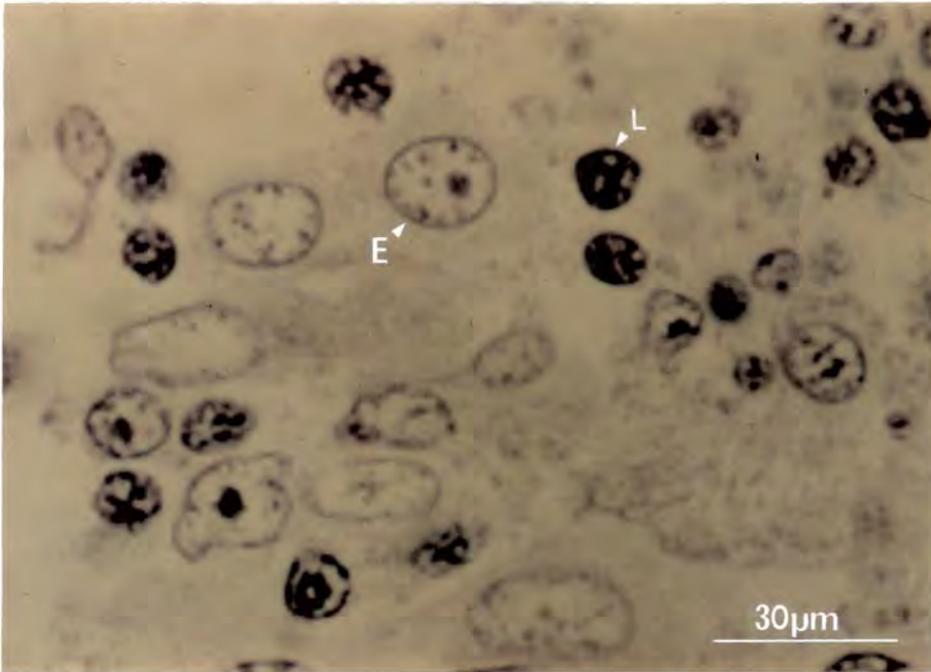


Implant contains lymphocytes and displays normal cortex/medulla differentiation. It has also increased in size. Photograph is courtesy of Dr. J.D. Horton.

Stain = haematoxylin and eosin.

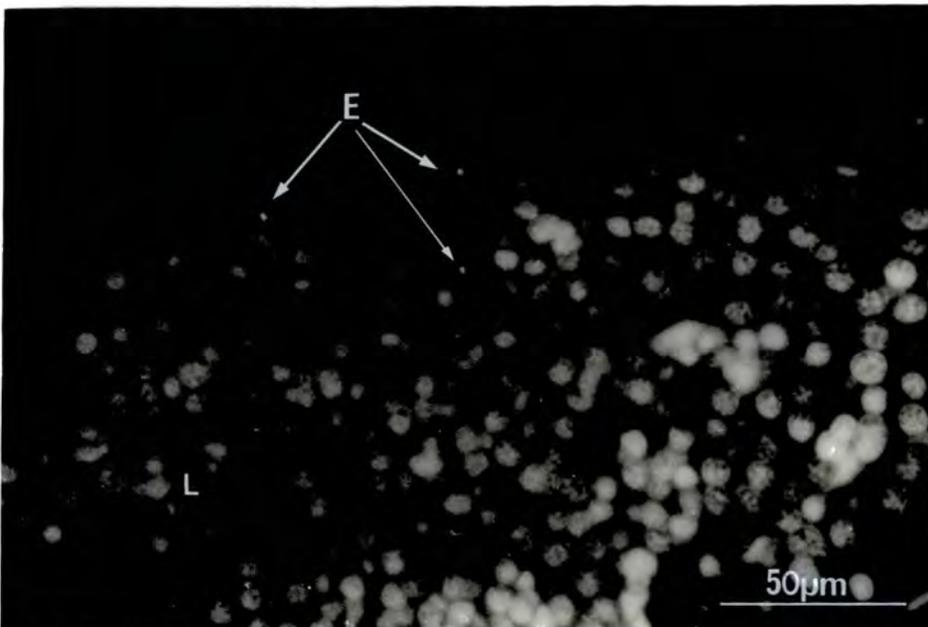
Figure 3.3 Use of the X.borealis fluorescent cell marker: sections through adult, non-irradiated X.borealis thymus, 5 months post-implantation into Tx J strain X.laevis hosts.

- (a) Histological differences between lymphocytes and stromal (eg. epithelial) cells.



Stain = toluidine blue

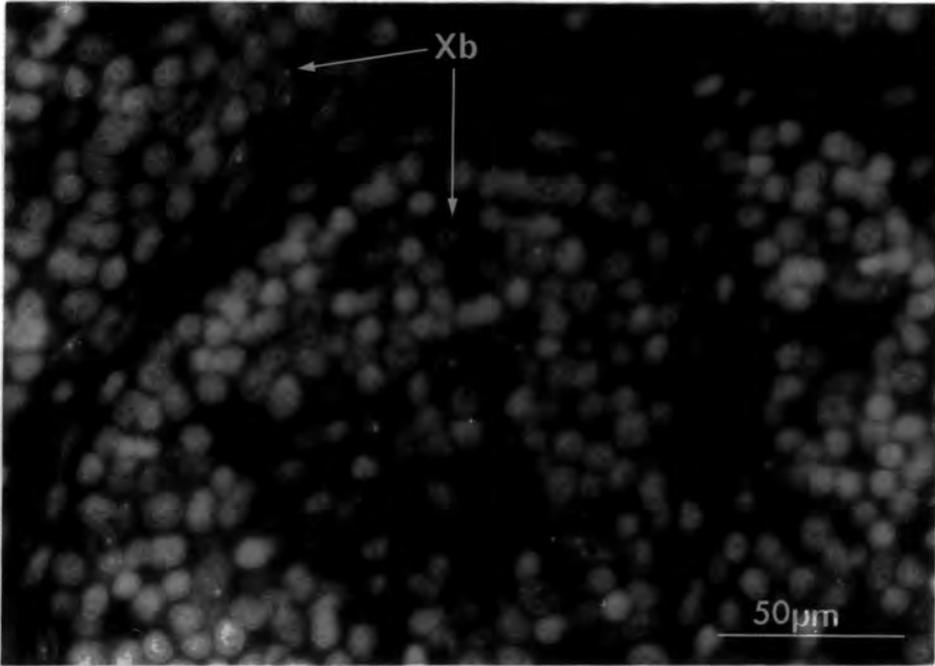
- (b) Epithelial cells and cells of the thymic capsule remain as X.borealis type (ie. donor type) following thymic implantation. Note fluorescent "spotting" of X.borealis cells. Lymphocytes are now of host origin.



Stain = quinacrine

Figure 3.3 continued

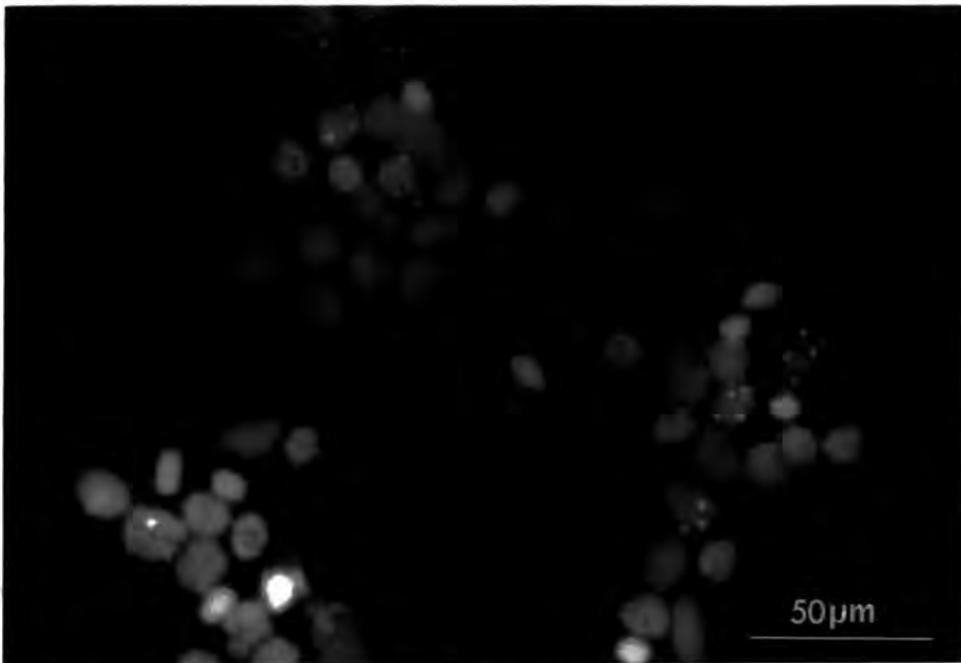
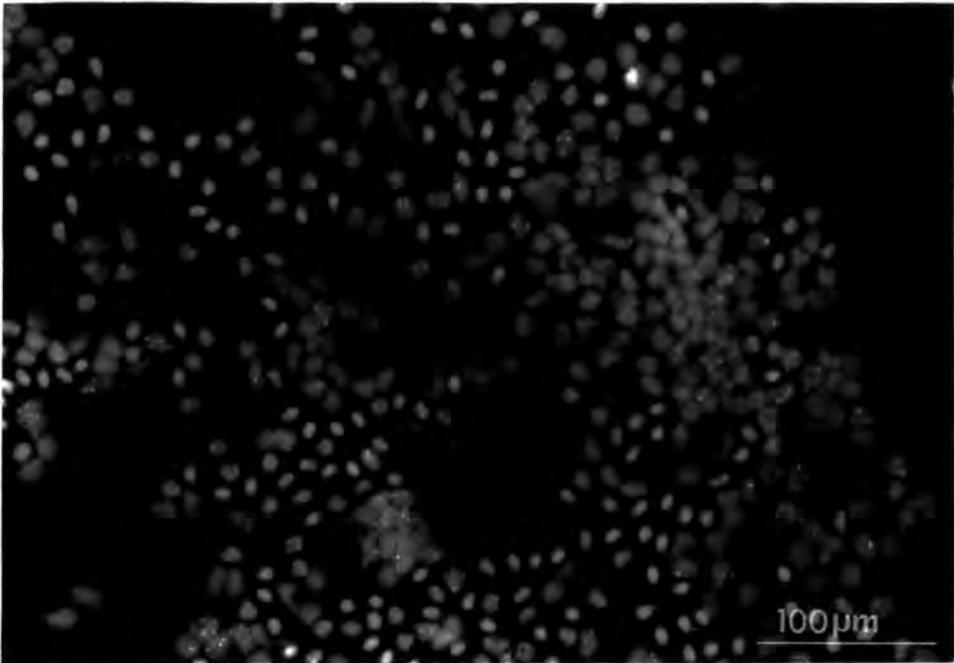
(c) X. borealis, "spotted" cells remain in the thymus implant.



Stain = quinacrine

Figure 3.4 Cytospin preparations of spleen from Tx J host implanted 5 months previously with non-irradiated adult X.borealis thymus.

(a) and (b) Donor, X.borealis "spotted" cells present in the spleen of a single J Tx host.



Stain = quinacrine

Figure 3.5 Legend

Effect of implantation of MHC-disparate or minor H antigen-disparate thymus on skin allograft responses of Tx J strain *Xenopus laevis*.

Thymectomy at 7 days; thymus implantation at 4-6 weeks (stage 56-58). Skin grafts were applied at 5-9 months. Each recipient usually received a total of 2 skin grafts that were from donors that were MHC-disparate to one another; exceptions to this included some controls, which were grafted with skin from 2 different J individuals only (see Table 2.1), and some J-thymus-implanted animals that received a total of 3 grafts - 2 from different J individuals as well as one MHC-disparate graft. For the thymus-implanted animals, individual J donors supplied maximally 2 skin grafts. J skin donor individuals were always different from J thymus donors.

Figure 3.6 Legend

Effect of implantation of isogeneic or MHC-disparate thymus on skin allograft responses of Tx LG Xenopus.

Experimental details mostly as in Figure 3.5. Here, each recipient usually received a total of 2 skin grafts from donors that were MHC-disparate to one another. A few animals received only a single skin allograft.

CHAPTER FOUR

PERIMETAMORPHIC ALLOTOLERANCE INDUCTION IN CONTROL LG AND J STRAIN XENOPUS

INTRODUCTION

In the preceding Chapter it was revealed that Tx, allothymus-implanted Xenopus displayed uniformly poor allograft responses to skin displaying only minor H antigen difference to the thymus donor or Tx host. It was suggested that either allotolerance induced by perimetamorphic grafting is "non-specific" at the level of minor antigens, or that reactivity to such antigens is only fully established when the thymus is isogeneic to the Tx host. In order to determine the extent to which the implantation of allogeneic tissue at metamorphosis can, by itself, affect subsequent rejection of minor H-disparate skin grafts, this Chapter investigates the alloimmune outcome of implanting skin and, occasionally, thymus from minor H antigen-incompatible and MHC antigen-disparate donors to non-thymectomized Xenopus in late larval life.

Anuran amphibians acquire immunocompetence early in development. During much of larval life they can reject allografts and synthesize antibodies (see review by Du Pasquier, 1982). During metamorphosis, the developing frog

encounters novel adult-specific self-antigens, which can be shown to be immunogenic to the larva, if it is challenged appropriately (Triplett, 1962; Maniatis, Steiner and Ingram, 1968; Du Pasquier, Blomberg and Bernard, 1979). Since normally the animal does not die of an autoimmune disease at metamorphosis, it has been supposed that there must be periods during development which are conducive to the induction of self-tolerance (Du Pasquier and Bernard, 1980). Responses to allogeneic material, which may well reflect reactivity towards self antigens, confirm that MHC antigen-specific allograft tolerance can be experimentally induced in Xenopus laevis during perimetamorphosis (Bernardini, Chardonens and Simon, 1969; Chardonens and Du Pasquier, 1973; Cohen, DiMarzo and Hailparn-Barlow, 1980; Barlow, DiMarzo and Cohen, 1981), a period characterized by several changes in the immune system (Du Pasquier and Weiss, 1973; Du Pasquier and Chardonens, 1975). The ease with which perimetamorphic (and, indeed, larval) allografting of skin induces tolerance to subsequent skin allografts is known to be dependent on a variety of factors, that include donor-host combination (ie. the particular MHC haplotype barriers involved), graft size and precise stage of development of the host (Bernardini, Chardonens and Simon, 1970; Chardonens and Du Pasquier, 1973; DiMarzo and Cohen, 1979; Barlow, DiMarzo and Cohen, 1981). Du Pasquier (1982), has some evidence to suggest that in isogeneic Xenopus such perimetamorphically-induced tolerance to MHC haplotype(s) may be somewhat "non-specific"; extending to all minor H

antigens associated with the skin donor LG family. This issue is investigated in this Chapter using LG and J strain Xenopus hosts.

MATERIALS AND METHODS

Animals.

The diploid J strain Xenopus laevis and X.laevis/X.gilli isogeneic hybrids used as implant donors and hosts were bred and raised as described in Chapter 2. Some triploid J strain Xenopus, were used here to provide skin for implantation to 2N J hosts. The reason why 3N donors were used in the experiment was that originally the grafted hosts were to have acted as controls in a ploidy-marker experiment. However, with the advent of the X.borealis marker they became redundant for that purpose. They were reutilized here, since 3N skin may provide a wider display of minor antigens and would therefore be more likely to present novel minor H antigens to J recipients. (The question of minor H antigen sharing in J strain Xenopus was discussed in Chapter 2). Triploids were produced by preventing extrusion of the 2nd polar body by cold (3°C) shock treatment 12 minutes after in vitro fertilisation of J eggs with J sperm (Kawahara, 1978). A proportion of the viable J embryos produced in this way contain a double complement of maternal genes, in addition to the paternal chromosomes. The ploidy of the offspring can be determined

Postmetamorphic grafting.

Assay grafts of dorsal skin, measuring $2-3\text{mm}^2$, were applied (as previously described in Chapter 2) several months after metamorphosis to froglets previously implanted at stage 56/8 with either skin or thymus.

EXPERIMENTAL DESIGN**(a) Implantation of skin or thymus to stage 56/8 larvae.**

The extent to which minor H and MHC antigen-disparate skin, implanted at metamorphosis, is tolerated was examined first (see Table 4.1). Larvae of LG clones 15, 3 and 5 were implanted with allogeneic skin (disparate by either one or two MHC haplotypes) from appropriate adult donor LG animals. In total, 18 LG hosts received skin disparate by one MHC haplotype, while 24 animals were implanted with a two haplotype-disparate graft. Four LG17 hosts were implanted with minor H-disparate skin from an LG15 donor. Five LG15 and 5 LG3 larvae were implanted with an identical graft to act as operative controls. The fate of minor H- and MHC-disparate skin implants was also examined with J strain Xenopus. Here, 5 diploid J hosts were given grafts from a 3N J donor, and 8 were implanted with LG5 skin. The fate of these implants was followed by regular (weekly) observations that continued for 2-3 months after metamorphosis. Implants were considered tolerated when in perfect condition >100 days after implantation.

The use of thymus implants at perimetamorphosis to generate allotolerance in control Xenopus was also examined. Thus 7 non-thymectomized J Xenopus were implanted with minor H-disparate J thymus, while 11 J stage 56/58, larvae were given MHC-disparate LG5 thymus. These thymuses were not carefully monitored for their survival, since this particular experiment concentrated on the outcome of a subsequently-applied skin graft (see below and Fig. 4.3).

(b) Outcome of assay skin grafts applied to skin and thymus -implanted animals.

Three to 4 months after metamorphosis, 2 or 3 dorsal skin assay grafts from various donor strains were applied to most of those skin-implanted LG animals which were bearing tolerated implants (see Fig. 4.2). Animals that had rejected the perimetamorphically-applied skin implants were not given an assay graft. LG 15, 3 and 5 hybrids, bearing fully viable allogeneic skin implants, were grafted with dorsal skin isogenic to the implant, to check that the failure to reject the original MHC-disparate implant was due to tolerance induction. Inability to reject allogeneic skin at metamorphosis might reflect a "failure" to recognize alloantigens at this stage; furthermore, the immunogenicity of the implanted skin may become reduced after a long period of residence in the host. In addition, the MHC antigen-specificity of the tolerance induced by perimetamorphic grafting was tested in LG15 and LG5 hosts by the application

of skin from a donor disparate to both host and implant donor by at least one MHC haplotype.

To investigate possible poor reactivity towards minor H antigens, LG5 (ad) and LG3 (bd) animals that were carrying perfect perimetamorphically-applied LG15 (ac) skin, were now tested for alloreactivity to LG17 (ac) skin, ie. of the same MHC (ac), but with different minor H antigens to the implant donor. Additionally, 12 LG15 animals, implanted at metamorphosis with one MHC haplotype (LG5)- or two haplotype (LG3)- disparate skin, were tested for their ability to visualize assay skin grafts from donors (LG17) of minor H antigen-disparity to the hosts (see Fig. 4.2).

Reactivity towards minor H-disparate skin effected by J animals previously implanted with (and tolerant to) either 3N J skin or LG5 skin were compared by applying assay skin grafts from new J individuals (see Fig.4.2). Each J host was here given 2 dorsal skin grafts from 2 separate diploid J donors. All J skin graft donors were of different parentage to the hosts, to maximize minor H antigen disparity.

Thymus-implanted J hosts (6 months of age) were grafted only with skin of the thymus donor MHC type (see Fig. 4.3). J assay skin donors were of different parentage to either host or J thymus implant donor. Single dorsal skin grafts were applied in these experiments.

RESULTS

(a) Fate of perimetamorphic skin implants.

Figure 4.1a shows a healthy graft of allogeneic skin 2 weeks post-implantation, lying in the anterior tail region of the perimetamorphic larval host. Alloimplants that were destined to be permanently tolerated generally remained either in perfect condition over metamorphosis and into early adult life; others initially displayed mild signs of an alloimmune reaction, ie. very slight vasodilation and pigmentary disturbances similar to those found during early Phase I of a normal alloimmune response (see Chapter 2), then seemed to recover and, along with those grafts that remained in good health throughout, subsequently appeared perfectly healthy for >100 days - indeed permanently. The white skin implants contrast dramatically with the pigmented dorsal skin of the adult host (see Fig. 4.1b).

When alloimplants were not tolerated by their hosts, rejection phenomena were similar to those described in previous Chapters, although graft rejection end points were a little delayed compared with MHC-disparate skin grafts applied to froglets. Initial vascular disturbances and pigment destruction begins during metamorphosis. These are followed by necrosis of the glandular elements of the skin, culminating in the eventual destruction of the entire graft.

Table 4.1 illustrates how the fate of adult skin grafts implanted to stage 56/58 larvae is dependent on the genetic relatedness of donor and host. One hundred percent of the

isografts and minor H antigen-disparate grafts (in both LG and J strain Xenopus) remain in perfect condition for >100 days. In contrast, 89% of skin grafts bearing one MHC haplotype disparity (eg. LG5/LG15 combinations) are tolerated, whereas only 57% (LG3 to LG15), 40% (LG15 to LG3), and 38% (LG5 to J) of fully allogeneic grafts survive.

(b) Effect of perimetamorphic skin implants on subsequent alloimmune reactivity.

Permanently surviving allografts, applied perimetamorphically, induce in vivo tolerance to donor MHC antigens expressed on skin. This is indicated by the permanent survival of subsequent skin grafts from the same MHC-disparate donor strain (Fig. 4.2). Those LG15 recipients tolerant of a perimetamorphic skin graft from LG3 or LG5 are still able to reject 3rd-party (J) skin (skin expressing new MHC antigens) in acute fashion. Tolerance to minor H antigens can also be induced at metamorphosis by skin implantation. This was demonstrated by an additional experiment, not shown in Fig. 4.2. Here, four LG17 (ac) hosts were implanted with LG15 (ac) skin at perimetamorphosis. These grafts remained healthy and subsequent LG15 grafts also stayed in perfect condition for >100 days. However, these LG17 hosts rejected MHC-disparate (LG3) skin assay grafts in normal times (25, 28 and 29 days).

The ability of LG15 hosts, bearing viable MHC-disparate skin implants, to deal with minor antigens (eg. LG17)

displayed in the context of host type MHC appears relatively normal; thus, 4/6 animals implanted with, and tolerant to, LG3 skin rejected LG17 skin sub-acute and 6/6 LG15s, bearing intact LG5 skin implants, rejected LG17 skin in under 50 days (cf. Fig. 3.6). Minor H-disparate graft rejection is also unimpaired in J hosts following implantation with LG5 skin, since all 3 J hosts bearing tolerated LG5 skin rejected both the J assay grafts within the normal time range for the J-J combination (cf. Fig. 3.5).

In contrast, the ability of LG recipients perimetamorphically implanted with MHC-disparate skin to visualize minor H antigens displayed in the context of the skin implant donor MHC (rather than host MHC type) is deficient (see Fig. 4.2). Here, 1/2 LG3 Xenopus and 6/6 LG5 recipients (all given, and tolerant to, LG15 skin) failed to reject LG17 grafts within 100 days. On the other hand, when J controls were given jjj skin grafts (ie. only minor H antigen disparate) at stage 56/58 and then asked to reject a subsequent graft from a new jj donor, rejection of these new, minor-disparate, grafts was effected in 9/10 cases within 100 days (7 in sub-acute manner, 2 in chronic fashion).

(c) Effect of perimetamorphic thymus implantation on subsequent alloreactivity.

Implantation of 11 perimetamorphic control J strain hosts with foreign LG5 thymus promoted "tolerance" to

subsequently-applied skin grafts from LG5 in 36% of cases (see Figure 4.3). Interestingly, this proportion reflects the percentage of J recipients that become tolerant of LG5 skin grafts implanted at perimetamorphosis (see Table 4.1). The four "tolerated" assay LG5 grafts on LG5 thymus-implanted Js all underwent a visible rejection "crisis" soon after transplantation, reaching late Phase I, ie. displayed vasodilation and punctate melanophores, revealing the underlying glandular tissue, before recovering at later post-grafting periods. Seven Js given LG5 thymus implants rejected assay LG5 skin grafts in typical (20-29 days) 1st-set fashion. No very rapid, 2nd-set, graft rejection reactions were seen.

All seven J controls given J thymus at perimetamorphosis were later able to reject skin grafts from 3rd-party J donors within normal 1st-set time range. They therefore behaved like J controls given J skin implants at metamorphosis, which were able to reject skin from a new J skin donor.

DISCUSSION

The results of this study confirm the findings of others (Bernardini, Chardonnens and Simon, 1969; Chardonnens and Du Pasquier, 1973), that MHC-specific allograft tolerance in adult Xenopus can be induced following allogeneic skin grafting of larvae during metamorphosis. As

in the studies by Du Pasquier and Chardonnens (1975), DiMarzo and Cohen (1979) and Barlow, DiMarzo and Cohen (1981), the proportion of tolerant individuals produced, was found to be dependent on the genetic relatedness of the donor and perimetamorphic host, with increased MHC haplotype disparity between them leading to fewer hosts becoming tolerant. Also in agreement with previous studies was the finding that all MHC-identical, but minor H-disparate, implants remained in perfect condition and generated tolerance towards secondary assay grafts of the same genotype. As with allothymus-induced tolerance in Tx animals, tolerance induced to MHC alloantigens by perimetamorphic skin implantation is MHC-specific, ie. it does not extend to subsequent grafts displaying new MHC antigens.

It has been suggested that the alloimmune system matures only gradually during larval life, with cytotoxic reactivity towards skin alloantigens coded for by the various MHC haplotypes emerging at different developmental times (DiMarzo and Cohen, 1982a). These considerations may, in part, account for the variation in extent of perimetamorphic tolerance achieved with different strain combinations noted here (see Table 4.1). However, why (within a particular donor/host strain combination) some hosts are rendered tolerant to the MHC-disparate skin, yet other genetically identical recipients reject skin implants of the same donor type, is uncertain. The precise state of maturation of the larva and the graft:host size ratio may

both affect the alloimmune outcome, since whether or not tolerance is induced probably depends on a fine balance between the nett tolerogenic and nett destructive responses of the immune system during development. Although allograft rejection can occur as soon as the thymus becomes lymphoid (Horton, 1969), tolerance to MHC antigens can also be generated (particularly when large skin allografts are applied) throughout larval life (DiMarzo and Cohen, 1982b), this tolerance depending on the presence of an intact thymus gland. However, metamorphosis appears to be a special period during which tolerance to alloantigens is readily induced, since this critical time in development normally allows for the induction of unresponsiveness to newly-appearing self-antigens (Chardonens and Du Pasquier, 1973). There is some evidence (Du Pasquier and Bernard, 1980) that perimetamorphic allotolerance is effected, in part, by suppressor T cells.

The outcome of the skin implantation experiments with LG recipients indicates the possibility that perimetamorphic application of MHC-disparate tissue (eg. thymus or skin) can induce in vivo tolerance to additional minor antigens associated with the implant donor MHC. Thus both LG3 and LG5 hosts, implanted during larval life with LG15 skin, showed impaired reactivity to subsequent LG17 grafts (see Fig. 4.2). The basis of such "non-specific" (minor H antigen) perimetamorphic tolerance is uncertain. It could be related to minor H antigen-sharing between different families of the same MHC, or may represent a "non-specific" blockade of the

immune system, preventing T cells from reacting against any donor cells that carry the same MHC antigens as the skin implant. The absence of alloreactivity to LG17 skin shown by the above LG3 and LG5 recipients, could be related to the fact that these animals have a population of T cells preferentially restricted to recognise minor antigens presented by cells possessing their own thymus MHC antigen type (bd and ad respectively), rather than by cells of the skin assay donor MHC type (ac). This is the explanation given by Flajnik, Du Pasquier and Cohen (1985), to explain the failure of LG3 (bd) Xenopus (ie. with an LG3 thymus) that had been made tolerant to ac antigens by embryonic implantation of an LG15 eye, to reject LG6 (ac, but with minor H differences to LG15) skin in normal fashion.

The present experiments with skin-implanted, control LG3 and LG5 animals, demonstrate lack of reactivity towards assay skin (eg. LG17) bearing disparate minor H antigens, when these antigens are associated with the implant donor MHC (eg. LG15); relatively normal reactivity towards minor H antigen-disparate skin of host MHC type (ie. LG15 hosts, implanted with LG5 skin and tested with LG17 skin) is also revealed. This latter situation is not the same as when MHC-disparate thymuses had been implanted at metamorphosis to Tx animals. In the thymus implantation study (see Chapter 3), rejection of minor H antigen-disparate skin assay grafts was always inefficient, whether the minor-disparate skin graft was related to thymus or host MHC type. These skin implantation experiments infer that the uniformly defective

ability of Tx animals given MHC-disparate thymus to reject minor-disparate assay skin grafts is not always due to non-specific perimetamorphic tolerance induction, but related in some donor/host combinations to a thymic defect.

The ability of J controls, made tolerant to J skin by perimetamorphic grafting, to reject skin from 3rd-party J donors (Fig. 4.2), suggests that a specific allotolerance to minor H antigens of the original implant donor can ensue in Xenopus, if recipient and implant donor are only minor H antigen disparate. However, the donor/host strain combination may well affect the outcome, since Du Pasquier (1982), has noted that LG17 made tolerant at metamorphosis to LG15 skin, although able to reject subsequent MHC-disparate (LG3) skin grafts, were unable to reject LG7 skin of only minor H disparity to both LG17 and LG15. Since induction of minor H antigen-specific allotolerance is here shown to occur following J to J implantation at metamorphosis, then the reason why J Tx animals given a J thymus subsequently display such poor rejection of J skin grafts (see Chapter 3, Fig. 3.2) is unlikely to be due to non-specific tolerance. The differences in outcome between experiments in Chapters 3 and 4 appear not to be related to the fact that the skin implant donors (Chapter 4) were 3N, whereas the thymus donors (Chapter 3) were 2N, since 5/6 J Tx recipients given a 3N J thymus have recently been shown to carry intact 3rd-party J grafts for >100 days following transplantation. This Chapter has also shown that the thymus appears to be no more effective than skin in the induction

of "non-specific" perimetamorphic tolerance, since all seven control Js implanted with J thymus displayed typical chronic rejection of subsequent 3rd-party J skin grafts. It is uncertain in the experiments with Js that these hosts were tolerant to skin of the original implant donor, as the latter were unavailable to provide skin for later testing. However, it seems probable that these J hosts were tolerant of the original J donor, since LG17 controls, implanted at metamorphosis with minor H-disparate LG15 skin, displayed 100% tolerance towards subsequent LG15 skin grafts.

For the moment, it seems likely from the work presented here that the defective rejection of minor H antigen-disparate skin seen in Chapter 3 in the Tx animals given even minor H antigen-incompatible thymuses may relate primarily to poor restoration, rather than being due to allothymus-induced, "non-specific" tolerance. Minor H-disparate thymuses implanted in late larval life may have a compromised ability to receive and/or educate sufficient numbers of host stem cells, because such histoincompatibility is reflected on cell membranes and affects cell-cell interactions. Interestingly, the migratory activity of triploid lymphoid cells through diploid tissues (eg. thymus) seems to be impaired when compared with the migration of diploid cells (Turpen, Volpe and Cohen, 1977; Nagata and Cohen, 1984). Such compromised interactions between stem cells and thymic stromal cells might result in failure of those T lymphocytes involved in minor graft rejection to become established in sufficient numbers.

Interestingly, Tx J Xenopus implanted with J thymus have been reported to display additional defects in their immune system; thus their splenic "T" lymphocytes fail to help cocultured J strain B cells produce antibody to rabbit erythrocytes in vitro, whereas T cells from normal J froglets do cooperate (Lallone, 1984). The defective development of T cells in Tx animals given even minor H antigen-disparate thymus implants makes it extremely difficult to interpret the reasons for the poor minor H antigen-disparate graft rejection in Tx Xenopus following restoration with MHC-incompatible thymus glands.

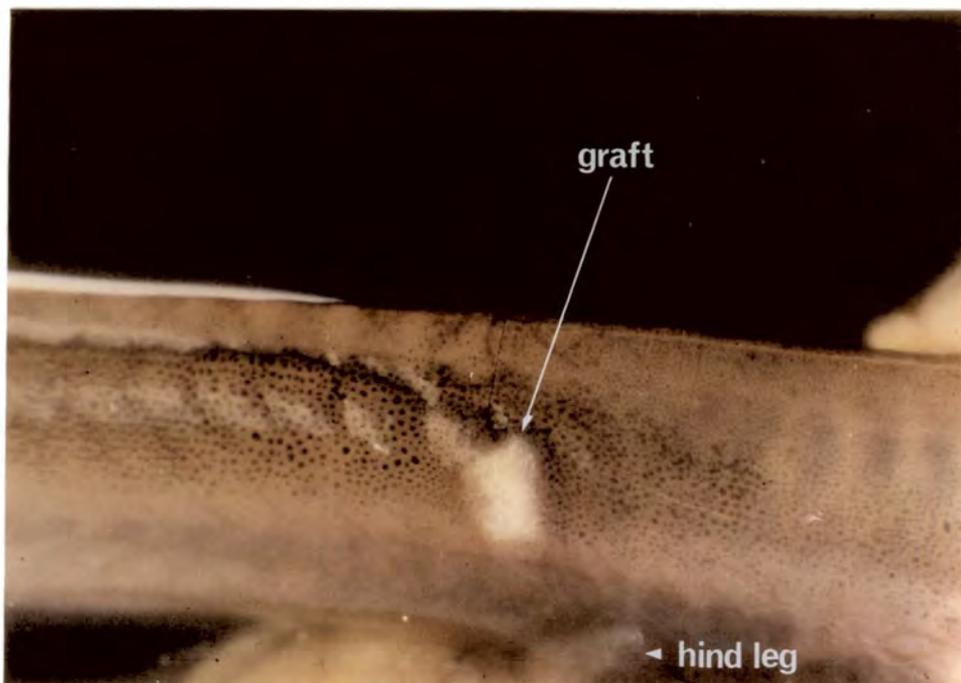
Table 4.1 Fate of adult skin grafts applied to larvae at stage 56-58 (ie. during perimetamorphosis).

Recipient (MHC haplotypes)	Skin Donor (MHC haplotypes)	No. of Animals	Number Tolerant*	Percentage Tolerant*
LG15 (ac)	LG15 (ac)	5	5	100%
LG 3 (bd)	LG 3 (bd)	5	5	100%
LG17 (ac)	LG15 (ac)	4	4	100%
LG15 (ac)	LG 5 (ad)	9	8	89%
LG 5 (ad)	LG15 (ac)	9	8	89%
LG15 (ac)	LG 3 (bd)	14	8	57%
LG 3 (bd)	LG15 (ac)	10	4	40%
J 2N (jj)	J 3N (jjj)	5	5	100%
J 2N (jj)	LG 5 (ad)	8	3	38%

Tolerant* = grafts in perfect condition 100 days + after implantation.

Figure 4.1 Photographs of 2mm^2 ventral skin grafts implanted into control Xenopus at perimetamorphosis.

(a)



Stage 58 (perimetamorphic) larva, 2 weeks after implantation of allogeneic ventral skin.

(b)



Postmetamorphic froglet bearing a fully viable ventral skin allograft, implanted at the stage shown above in (a).

Figure 4.2 Legend

Effect of implantation of MHC-disparate or minor H antigen-disparate skin on subsequent allograft responses of control LG and J strain Xenopus.

The initial skin implant was applied 4-6 weeks (stage 56-58) after fertilization and then the subsequent skin grafts were applied at 5-9 months.

Implanted LG hosts were given 2 or 3 assay skin grafts from disparate donor strains.

J recipients received 2 J assay grafts from separate J donors only. Only those recipients bearing "tolerated", larvally-applied skin implants are listed in this Figure.

Figure 4.3 Legend

Effect of implantation of adult MHC-disparate or minor H antigen-disparate thymus on subsequent allograft responses to skin of the thymus donor MHC type.

J recipients, J thymus donors and J assay skin donors were all of different parentage to maximize H antigen disparity. Thymuses were implanted when hosts were 4-6 weeks old (stage 56-58). Assay skin grafts were applied at 6 months of age. Implanted animals were each given 1 assay skin graft. Only grafts which were in perfect condition more than 100 days post-grafting were scored as "tolerated".

CHAPTER FIVE

IN VITRO STUDIES WITH THYMUS-IMPLANTED, THYMECTOMIZED XENOPUS AND SKIN-IMPLANTED, CONTROL FROGS.

INTRODUCTION

In previous Chapters, in vivo studies have revealed that third-party skin allograft (MHC-incompatible) rejection is restored following implantation of Tx Xenopus with foreign thymus, while MHC-specific tolerance is induced to assay skin grafts of thymus donor type; additionally it has been shown that MHC-specific tolerance can also be achieved by perimetamorphic allografting of skin (and thymus) to control larvae. Attention is turned in this Chapter to in vitro proliferative capabilities of T lymphocytes removed from these experimental animals.

Thymectomy at very early stages of larval development has been reported to eliminate the in vitro proliferative responses of Xenopus lymphocytes to T cell mitogens (such as phytohaemagglutinin (PHA) and Concanavalin A) and to allogeneic cells in mixed leucocyte culture (Du Pasquier and Horton, 1976; Manning, Donnelly and Cohen, 1976; Donnelly, Manning and Cohen, 1976; Green (Donnelly) and Cohen, 1979). PHA and MLC reactivity of splenocytes from thymus-implanted, Tx animals is assessed here.

Previous studies on early Tx Xenopus have demonstrated restoration of antibody production (both IgM and IgG) to thymus-dependent antigens following implantation of histocompatible or incompatible adult thymus (Tochinai, Nagata and Katagiri, 1976; Nagata and Cohen, 1984), implantation of MHC-mismatched larval thymus (Du Pasquier and Horton, 1982; Gearing, Cribbin and Horton, 1984) and by injection of isolated histocompatible and incompatible thymocytes (Nagata, 1980). Although restoration of alloimmunity in Tx Xenopus has been studied in some detail (see Chapter 3), no information concerning in vitro T cell functions had been performed when this study was undertaken.

In the mammalian equivalent of the early-Tx Xenopus, the nude mouse, grafting with neonatal (isogenic or allogeneic) thymus results in erratic, or partial, reconstitution of the immune response to T cell mitogens and T-dependent antibody production: reactivity to skin grafts is also variable (Kindred and Loor, 1974).

The major purpose of this Chapter was to investigate MLC reactivity of splenocytes from allotolerant animals (effected either by thymus implantation to Tx individuals or skin implantation to control, perimetamorphic Xenopus) to MHC antigens of the implant donor strain. Using the technique of embryonic exchange of flank tissue developed by Clark and Newth (1972), Manning and Botham (1980) were able to demonstrate considerable reduction, or absence, of specific MLC reactivity between mutually tolerant animals in Xenopus. Brooks (1975) found that neonatal mice, fully

tolerized by injection of allogeneic cells, showed no MLR or cytotoxicity in in vitro tests, whereas partially tolerant animals often responded in MLC; this response being related to the cell dose administered.

MATERIALS AND METHODS

Animals and operations.

J and LG strains of Xenopus were used in this study. In some of the one-way MLC experiments using J responder cells, hybrid JxLG5 and JxLM3 (laevis/muelleri) Xenopus were required to prevent the possibility of back stimulation effected by irradiated stimulator cells (Von Boehmer, 1974). These animals were produced in the laboratory by fertilizing the endoreduplicated (2N) eggs laid by LG and LM Xenopus with J strain sperm. The hybrid offspring were checked for triploidy by silver staining of blood cell smears (Olert, 1979). We have shown that J animals reject JLG5 (jad) and JLM3 (jwy) skin grafts within 3 weeks, whereas the hybrids fail to respond acutely to J strain skin grafts and do not display MLC reactivity to J stimulators (unpublished data).

Seven-day thymectomies and implantation of thymus or skin grafts in late larval life, were performed as described in Chapters 3 and 4.

Animals were 6-12 months old when in vitro assays were performed.



Mitogen Culture.

Splenic leucocyte suspensions were prepared in fully supplemented Leibovitz L-15 medium (Flow) as described in Chapter 2. Leucocyte cultures were plated in triplicate from individual toadlets. One hundred microlitre aliquots of splenocyte suspension (1×10^5 leucocytes/well) were distributed to individual wells of Cooke V-well microtest plates (M25-ARTL, Sterilin). Ten microlitres of the mitogen PHA (M) (Difco) was added to each test well (10 μ l medium was added to control cultures). The final concentration of PHA in the culture was 10 μ g/ml. Cultures were incubated at 26°C for 48 hours and were then pulsed with 1 μ Ci/well tritiated thymidine (specific activity 5Ci/mmol, Amersham). Tritiated thymidine incorporation was measured after a further 18-24 hours culture as described previously for the MLCs in Chapter 2.

Stimulation indices for each individual experiment were calculated by dividing the mean quenched counts per minute (dpm) for triplicate mitogen cultures by the mean dpm obtained with control cultures.

Mixed leucocyte culture.

Splenic leucocytes were examined in one-way MLC, as described in Chapter 2. The significance of the difference between experimental and control dpm values was compared using the Student's t-test.

EXPERIMENTAL DESIGN

(a) In vitro studies following thymectomy and thymus implantation.

Splenocyte proliferation to the T cell mitogen PHA.

To briefly assess whether the proliferative response of splenic leucocytes to PHA is restored to normal levels by thymus implantation, J strain and LG Tx animals were implanted at 4 weeks with MHC-identical or MHC-incompatible thymus (either adult or larval, normal or irradiated - see Table 5.1). Splenocytes from restored, control and Tx Xenopus were then tested after metamorphosis.

Thymus dependency of the mixed leucocyte culture response.

The effect of 7-day thymectomy on the mixed leucocyte response was initially examined here using J strain Xenopus. Controls and Tx animals were stimulated in one-way MLC with irradiated cells from various MHC-disparate Xenopus. To remove any possible proliferation of responder cells induced by release of factors by the irradiated stimulator splenocytes following recognition of responder cells, irradiated hybrid stimulator cells (JLG5 and JLM3) were used in some cultures (see Table 5.2).

MLC reactivity of splenocytes from thymus-implanted, Tx animals.

In the first experiment, splenocytes were taken from Tx LG animals, implanted with either larval or adult, compatible or disparate, normal or irradiated thymus (see Table 5.3). All the thymus-implanted hosts used in this experiment had previously been skin allografted as froglets, and had been shown to tolerate skin of the thymus MHC type, whilst rejecting a 3rd-party foreign graft (see Chapter 3). Splenocytes were cultured in one-way MLC with irradiated stimulator cells of thymus donor MHC and also with cells of 3rd-party origin.

The second experiment examined MLC responses of Tx J strain animals following implantation of either minor H antigen (J) or MHC (LG5) antigen -disparate thymuses (Table 5.4). JLG5 and JLM3 irradiated stimulator cells were used to avoid possible back stimulation effects. The responder animals in this experiment were not skin grafted prior to the in vitro MLC assay, since alloimmunization by application of a skin graft can influence proliferation in the MLC (Barlow and Cohen, 1981).

(b) Splenocyte MLC reactivity following perimetamorphic grafting with MHC disparate skin.

Splenocytes from LG clones 17, 15 and 3 bearing fully viable larvally-applied MHC-disparate skin implants, and which had also received (as froglets) and tolerated (>100

days) further test skin grafts of donor MHC type, were tested for their ability to proliferate in vitro to cocultured splenocytes from implant donor strain and from a 3rd-party Xenopus.

RESULTS

(a) In vitro studies following thymectomy and subsequent thymus implantation.

PHA reactivity.

In Table 5.1 it can be seen that splenocytes from all 4 control Xenopus tested proliferated in vitro to the T cell mitogen PHA, with a mean stimulation index \pm sem of 10.37 ± 1.9 . The thymus dependency of this response was confirmed; thus splenocytes from an early-tx froglet failed to show any increased proliferation when PHA was added to the culture.

Implantation of an MHC-compatible thymus, either larval or (irradiated) adult, to a Tx host before metamorphosis restored PHA reactivity to within the control range. The mean SI of 9.06 ± 2.6 was not found to be significantly different ($p > 0.05$) to that of the control group.

In all 7 Tx given MHC-disparate thymus as larvae, splenocyte proliferation to PHA was again found to be restored. As with control animals, levels of stimulation were variable (range 4.9-23.3). Both larval and adult thymus

implants were found to be effective and 3000 rad-irradiation of adult implants did not preclude restoration of this T cell response. The mean SI of 11.66 ± 2.4 for this group of animals did not differ ($p > 0.05$) from those of either the controls or the Tx animals given MHC-compatible thymus implants.

MLC reactivity.

Results given in Table 5.2 show the initial experiment with J strain recipients. Early Tx routinely impairs the MLR of J strain Xenopus. Splenocytes from the control J froglets, all displayed significant proliferation to the panel of MHC-disparate stimulators in one-way culture. Significant proliferation in MLC was found in only 3/11 cultures of splenocytes from Tx animals. Two of the positive stimulations were very low (1.21 versus LM3 and 1.32 versus JLG5), indeed no SI with Tx cells was as high as an SI from any control animal.

The results from the MLC experiments on LG Tx hosts that had been implanted with thymuses are given in Table 5.3. Responder cells from LG15 and 17 control froglets all proliferated against MHC disparate cells from various donors in one-way MLC. No increased proliferation was observed when cells from LG15 Tx were cultured with MHC disparate irradiated stimulators. The MLC response by 5 Tx LG (2 LG5 and 3 LG17) animals was consistently found to be restored following implantation before metamorphosis of MHC identical, larval thymus; each host proved MLC positive when

its splenocytes were tested against two different allogeneic stimulators. Both LG5 Tx animals given an LG17 larval thymus with one MHC haplotype disparity, and all 6 LG17 Tx implanted with a MHC incompatible LG3 larval thymus (either normal or 1000 rad-irradiated) were found to contain spleen lymphocytes capable of proliferating against 3rd-party and thymus type, irradiated stimulator cells. In contrast, the 4 LG15 Tx implanted with one MHC haplotype disparate (LG5) adult thymus, uniformly failed to display MLC reactivity against LG5 stimulators, while responding well to 3rd-party cells. The very high SIs recorded with some LG15 splenocytes seem to be due to the particularly low background dpm seen with these cells (see Legend, Table 5.3).

Subsequent experiments (Table 5.4) involved responder cells from non skin-grafted J strain animals and the use of hybrid stimulator cells. It has been shown previously in Chapter 2 that ungrafted J strain Xenopus fail to respond to minor H-disparate J stimulators in MLC, but recognize MHC-disparate cells in normal fashion. The 3 control Js tested here displayed positive responses to the allogeneic hybrid stimulators, as did the 2 Tx Js implanted with adult, minor H disparate J (3N) thymus. Cells from the 3 Tx Js generally failed to proliferate significantly to the stimulators in MLC; only 1/6 cultures proving positive (SI only 1.32) statistically. All 5 J Tx implanted with LG5 adult thymus (normal or irradiated) responded to JLM3 stimulators. In contrast, the 2 Tx animals given a normal LG5 thymus failed to show significant stimulation when their

splenocytes were cocultured with JLG5 stimulators. Of the 3 given an irradiated LG5 implant, 2 were stimulated by thymus-type stimulators.

(b) MLC reactivity of splenocytes from control *Xenopus* following perimetamorphic skin implantation.

One-way MLC responses of LG control *Xenopus* (bearing viable, larvally-applied skin alloimplants and assay skin grafts) to splenocyte stimulators of the skin donor MHC type and 3rd-party are given in Table 5.5 (stimulation indices for certain culture combinations involving non-implanted control LG17 and 15 are taken from Table 5.3). Non-implanted controls displayed positive proliferation in MLC against MHC-disparate cells except in one culture where J stimulators were used. LG15 implanted with isogeneic LG15 skin as late larvae also responded to 1 and 2 haplotype-disparate cells. Splenocytes from LG15 grafted with skin of a single MHC haplotype disparity (LG5) were stimulated in 1-way MLC with both 3rd-party and also with donor type cells (in 7/8 cases). Although fewer animals grafted at metamorphosis with totally disparate skin were available for testing in MLC (since a smaller proportion initially become tolerant to the skin graft *in vivo*), splenocytes from the 3 LGs tested (1 LG15, 1 LG17 and 1 LG3) also showed induced proliferation when cocultured with either 3rd-party or skin donor strain stimulators.

DISCUSSION

The proliferative response of splenocytes to the T cell mitogen PHA in early-tx Xenopus has been shown in this Chapter to be restored by thymus implantation, regardless of the maturity of the thymus, or donor-host combination. Recent experiments by Nagata and Cohen (1984), involving grafting of adult MHC-matched and mismatched thymuses to early Tx, but adult Xenopus, are in agreement with the findings presented here. Nagata and Cohen also suggest that these thymus grafts can promote the differentiation of host precursor T cells along a T cell pathway, after their discovery that PHA-responsive cells in the spleens of implanted animals are of host type.

The PHA response in Xenopus is known to be T cell mediated (Du Pasquier and Horton, 1976; Bleicher and Cohen, 1981) and to be dependent on the maturation stage of the lymphocytes and/or the proportion of PHA-reactive subsets in the culture (Green (Donnelly) and Cohen, 1979). PHA- and MLC-reactive lymphocytes may represent distinct T cell populations, since sequential thymectomy studies during larval development indicate that the thymus is required for a longer period during development to establish splenocyte reactivity to T cell mitogens, than for responsiveness in MLC (Horton and Sherif, 1977).

The MLC experiments reported here reveal that cells from early-tx frogs can still occasionally respond, albeit at low levels, to allogeneic stimulators in MLC, even when

the possibility of "back stimulation" has been excluded. Interestingly, Tompkins and Kaye (1980) observed chronic allograft rejection in some frogs thymectomized as early as 3 days post fertilization, when the thymus contains less than a few hundred cells. Similar observations have also been made by Nagata and Cohen (1983) on J Xenopus thymectomized at 4-7 days of age. Seven-day Tx Xenopus, that have rejected (chronically) 1st-set allografts, reject 2nd-set grafts in accelerated fashion (Rimmer and Horton, 1977) and also can then display a splenic MLC response, which is not specific to cells of graft donor origin (Nagata and Cohen, 1983). In this Chapter, a low level MLC response has been demonstrated in a few Tx Xenopus, even before skin grafting. These features of early-Tx Xenopus have led to the suggestions, first proposed by Horton and Manning in 1972, that cells derived neither from, nor influenced by, the thymus are involved in chronic allograft rejection and can be driven to proliferate in in vitro MLR. However, it is possible that the thymus rudiment may have already influenced alloimmunity before thymectomy by very early seeding of T-lineage cells to the periphery or by secreting humoral factors (Dardenne et al, 1974). The possibility that the thymus is not solely responsible for T cell development in the mammal has also recently come under discussion (Dosch, White and Grant, 1985), thus development of host T cells can be shown to proceed in the absence of thymus in nude mice if interleukin-2 is provided, or following injection of irradiated, foreign T cells.

Splenocytes taken from Tx animals rendered in vivo tolerant to skin allografts of the thymus implant strain, display variable in vitro reactivity to thymus donor strain lymphocytes when tested in one-way MLC. Others have previously reported similar "split tolerance" (in vivo to skin grafts, but not in vitro, in MLC) following allothymus implantation to Tx Xenopus in adult life (Nagata and Cohen, 1984) and in embryonic (histoincompatible) anterior/posterior chimeras (Flajnik, Du Pasquier and Cohen, 1984 and 1985). Lymphoid cells from mice or rats bearing tolerated skin grafts, following neonatal tolerance induction, also display MLC tolerance to donor cells in some cases, but not in others (Hásek and Chutná, 1979). In contrast to the split tolerance seen in some Tx/restored Xenopus, lymphocytes from nude mice implanted with allogeneic thymus, routinely display MLC tolerance to thymus donor strain cells, whereas in vivo reactivity to thymus strain skin grafts can occur (Kindred, 1978).

Cohen et al (1985) have recently reported that following perimetamorphic skin allografting and the acquisition of in vivo non-reactivity to subsequent grafts from the same donor, the extent of in vitro MLR tolerance of splenocytes to cells of the skin donor is variable. However, of the 11 MLC experiments on LG Xenopus implanted as larvae with (and rendered tolerant to) allogeneic skin reported in this Chapter, only one culture failed to display significant proliferation when stimulated with skin donor strain splenocytes.

Variability in MLC reactivity to donor-type cells has been found here following thymus implantation to Tx Xenopus. Moreover, these experiments suggest that the nature of the thymus implanted is a critical factor in the in vitro immunological outcome. Thus splenocytes from the majority of Tx Xenopus given adult thymus implants failed to respond in MLC against thymus strain stimulators, whereas those implanted with a larval thymus could proliferate to thymus-type MHC antigens. Whether or not the immunologic outcome relates to the lymphocyte numbers within the thymus implant, or is associated with qualitative changes (MHC antigen expression?) on thymic cells during ontogeny, remains an open question. Interestingly, 2/3 J animals given irradiated LG5 adult implants were able to display MLC reactivity to JLG5 stimulators. Possibly the induction and/or maintenance of tolerance in the cellular population normally responsible for proliferation in MLR requires a certain degree of lymphocyte chimerism; a criterion which may not be fulfilled in Tx animals implanted with larval or, in some cases, irradiated adult thymus; thus irradiated thymuses in particular, contain low numbers of lymphocytes (Russ, 1986). If allotolerance in vivo (generated in Tx animals with allothymus and in control Xenopus following perimetamorphic skin grafting) was due, for the most part, to peripheral suppression mechanisms, then proliferation of splenocytes in MLC to donor antigens could arguably be caused either by the failure of suppression to operate in vitro, or else to proliferation of suppressor cells

themselves. In either case, it would seem more likely that splenic MLC reactivity to donor antigens would be detected in skin-implanted rather than Tx/thymus implanted frogs. Thus, in vivo tolerance induced by allogeneic skin implantation is likely to be maintained by a suppressive mechanism operating in the periphery, while it is easier to envisage clonal deletion operating following implantation of Tx hosts with foreign thymus, since host precursor T cells are known to pass the implant following implantation (Russ, 1986). The phenomenon of in vivo tolerance to skin grafts of a particular MHC type, but variable in vitro MLC reactivity to this same MHC, may truly represent split tolerance. Thus tolerance may be achieved to MHC determinants centrally involved in skin allograft rejection, but not to those molecules (class II antigens) known to effect MLC reactivity in Xenopus.

The next Chapter begins to probe the mechanism of thymus- and skin-induced transplantation allotolerance, induced in vivo, but not always in vitro, by examining the ability of host splenocytes to display in vivo MLR proliferation, and by investigating the extent to which tolerant hosts can defend themselves against injected donor strain splenocytes and promote graft-versus-host reactivity.

Table 5.1 Restoration of in vitro PHA reactivity of splenocytes from Tx Xenopus following thymus implantation.

Control	Tx.	Tx + MHC-compatible thymus.	Tx + MHC-disparate thymus.
Stimulation Indices			
9.46, 12.21 13.70, 6.11	0.32	6.50 ^ℓ , 11.62 [†]	10.50 ^ℓ , 5.39 14.90, 23.29 [†] 4.92 [†] , 9.53 [†] 13.10 [†]
x= 10.37 ± 1.9	x= 0.32	x= 9.06 ± 2.6	x= 11.66 ± 2.4

Legend

Animals used were either J strain X. laevis, or LG Xenopus.
 ℓ - implanted with larval thymus; others were given adult.
 † - thymus 3000 rad-irradiated prior to implantation.

Culture conditions: 1×10^5 splenic leucocytes/100 μ l/well.
 Pulsed with 1 μ Ci tritiated thymidine/well after 48 hours
 for 24 hours. PHA added at a concentration of 10 μ g/ml medium.

No significant difference between mean SI's for control PHA cultures and PHA cultures with Tx + thymus, restored with either MHC-compatible or MHC-disparate thymus ($p > 0.05$ by t-test)
 There was no significant difference in the mean SI's for PHA reactivity for cells from Tx implanted with MHC-compatible thymus and Tx animals implanted with MHC-disparate thymus.

Table 5.2 The effect of early thymectomy on the MLC reactivity of splenic leucocytes.

Responder	6000 R Stimulators					
	LG3	LG17	LM3	JLG5	JLM3	
J control	2.49	N.D.	4.63	4.15	4.80	
	4.22			3.28	3.28	
				6.93	8.73	
J Tx	2.00	0.88*	1.21	0.53*	0.63*	
	0.94*			0.51*	1.32	1.19*
	0.95*				1.50*	1.80*

Legend:

All animals tested were ungrafted. Thymectomized animals were checked post mortem for absence of thymus glands.

Experimental dpm were found to be significantly elevated by the Student's t-test ($p < 0.05$), unless asterixed.

Table 5.3 MLC reactivity of splenocytes from skin grafted Tx LG clonal Xenopus following implantation of larval or adult thymus in late larval life.

Recipient	Thymus donor	6000 R stimulators				
		LG17 (ac)	LG 5 (ad)	LG 3 (bd)	J (jj)	JLM3 (jwy)
LG 5 Tx (ad)	LG 5 larval (ad)	3.49	N.D.	8.07	N.D.	2.35
		5.02				
LG 5 Tx (ad)	LG17 larval (ac)	2.97	N.D.	6.06	N.D.	2.51
		2.52				
LG17 control (ac)	---	N.D.	2.75	1.55	3.43	N.D.
			3.54	2.07		
				2.50		
				3.00		
LG17 Tx (ac)	LG17 larval (ac)	N.D.	2.53	2.44	2.08	N.D.
				1.92	2.04	
				2.03		
LG17 Tx (ac)	LG 3 larval (bd)	N.D.	N.D.	2.36	2.18	N.D.
				3.51	2.49	
				2.03	2.01	
				2.88	3.34	
				2.82†	3.04†	
				2.57†	2.70†	
LG15 control (ac)	---	N.D.	9.92	N.D.	N.D.	12.96
			11.40			15.86
			8.49			9.17
LG15 Tx (ac)	---	N.D.	0.84*	N.D.	N.D.	1.42*
LG15 Tx (ac)	LG 5 adult (ad)	N.D.	0.94*	N.D.	N.D.	17.98
			0.76*			9.76
			0.83*			3.30
			1.19*			3.17

Tx at 7 days, thymus implants at 4-6 weeks. Assayed at 6-8 months. Splenocytes from each implanted animal were tested against 6000 R stimulator cells of thymus donor strain and 3rd-party origin. MLC results expressed as stimulation indices (see method).

† given 3000 rad-irradiated thymus

* Experimental dpms not significantly elevated; $p > 0.05$ by t-test.

Mean dpm levels ($\bar{x} \pm \text{sem}$) in autologous cultures (responder x irradiated autologous stimulator):-

LG5Tx+LG5 larval thymus $\bar{x} = 6470 \pm 623$,
 LG5Tx+LG17 larval thymus $\bar{x} = 5147 \pm 762$,
 LG17 control $\bar{x} = 5505 \pm 3538$,
 LG17Tx+LG17 larval thymus $\bar{x} = 4000 \pm 2112$,
 LG17Tx+LG3 larval thymus $\bar{x} = 2928 \pm 1249$,
 LG15 control $\bar{x} = 381 \pm 118$,
 LG15 Tx $\bar{x} = 549$,
 LG15Tx+LG5 adult thymus $\bar{x} = 1110 \pm 347$

Table 5.4 MLC reactivity of splenocytes from Tx J strain Xenopus following implantation of adult thymus during late larval life.

Recipient (MHC haplotypes)	Thymus donor (MHC haplotypes)	6000 R Stimulators	
		JLG5 (jad)	JLM3 (jwy)
J control (jj)	---	6.93	8.73
		3.28	3.26
		3.01	2.34
J Tx (jj)	---	1.50*	1.80*
		0.53*	0.68*
		1.32	1.19*
J Tx (jj)	J 3N (jjj)	4.15	4.80
		4.59	6.62
J Tx (jj)	LG5 (ad)	1.88*	2.82
		0.94*	2.36
J Tx (jj)	LG5 3000 R (ad)	3.82	8.97
		3.99	2.27
		0.97*	2.11

Legend

All experimental dpm's were significantly elevated by Student's t-test ($p < 0.05$) except those SI's marked *. Three J control and Tx animals were the same as those depicted in Table 5.2.

Mean tritiated thymidine incorporation ($x \pm \text{sem}$) in autologous cultures (responder \times irradiated autologous stimulator):-

J control $x = 766 \pm 243$,

J Tx $x = 1213 \pm 159$,

J Tx \leftarrow J thymus $x = 904 \pm 99$,

J Tx \leftarrow LG5 thymus $x = 2155 \pm 664$,

J Tx \leftarrow 3000 rad-irradiated LG5 thymus $x = 1405 \pm 1141$

Animals were assayed in MLC when 6-8 months old.

Table 5.5 MLC reactivity of splenic leucocytes from control LG Xenopus following perimetamorphic implantation of MHC-disparate skin.

Recipient	Skin donor	6000 R stimulators				
		LG15 (ac)	LG 3 (bd)	LG 5 (ad)	J (jj)	JLM3 (jwy)
LG15 control (ac)	---	N.D.	6.83 10.99	9.92 8.42 11.40	1.65* 6.06	12.96 9.17 15.86
LG15 control (ac)	LG15 (ac)	N.D.	2.77 2.14	3.86 2.93	N.D.	N.D.
LG15 control (ac)	LG 5 (ad)	N.D.	3.70 5.77 4.14	4.31 9.92 5.55	N.D.	11.70 10.97 4.21 4.14 5.65
LG15 control (ac)	LG 3 (bd)	N.D.	3.44	N.D.	4.97	N.D.
LG17 control (ac)	---	N.D.	3.00	N.D.	3.43	N.D.
LG17 control (ac)	LG 3 (bd)	N.D.	3.00	N.D.	6.32	N.D.
LG 3 control (bd)	---	6.92	N.D.	N.D.	4.95	N.D.
LG 3 control (bd)	LG15 (ac)	3.88	N.D.	N.D.	3.53	N.D.

Only animals 'tolerating' larvally-applied skin grafts appear in this Table. All hosts 'tolerating' a MHC-disparate, larvally-applied graft also tolerated test grafts of skin donor MHC type applied after metamorphosis for >100 days. All animals showed significant proliferation in MLC as judged by Student's t-test ($p < 0.05$), except those results marked * in the Table.

Mean dpm levels ($\bar{x} \pm \text{sem}$) in autologous cultures:-

LG15 control $\bar{x} = 523 \pm 204$, LG15/15 skin $\bar{x} = 2036 \pm 462$,
 LG15/5 skin $\bar{x} = 1087 \pm 235$, LG15/3 skin $\bar{x} = 1205$,
 LG17 control $\bar{x} = 1793$, LG17/3 skin $\bar{x} = 1117$,
 LG3 control $\bar{x} = 320$, LG3/15 skin $\bar{x} = 423$.

Animals were aged 7-12 months when assayed.

CHAPTER SIXIN VIVO STUDIES ON
XENOPUS FOLLOWING PERIMETAMORPHIC
TOLERANCE INDUCTION.INTRODUCTION

The extent of allotolerance induced by implantation of MHC-disparate thymus or skin into Tx and control Xenopus respectively during late larval life has so far been examined in this Thesis by skin grafting and in vitro MLC assays. It has been found that, despite the indefinite survival of subsequent donor strain skin grafts, in vitro proliferative activity in MLC remains to donor strain MHC antigens in skin-implanted hosts and in many Tx frogs reconstituted with allogeneic thymus grafts. In this Chapter, "tolerant" animals are tested in an in vivo version of the mixed leucocyte response, to discover whether the observed host T cell proliferation in response to cocultured (irradiated) donor strain splenocytes is blocked in vivo. Thus it is possible that suppressive factors may normally limit in vivo proliferative responses to donor antigen, thereby preventing rejection of assay skin grafts.

The nature of the alloreactive cells persisting in skin graft tolerant Xenopus is examined further in graft-versus-host response (GVHR) assays. In mammals, the

proliferative phase of injected T cells in the GVHR, resulting in enlargement of the host spleen, is thought to correspond to the proliferative response in MLR and to be directed against Class II products of the MHC. In contrast, the cytotoxic, effector phase of injected T cells, which results in host cell death, is believed to be a response to Class I MHC antigens and to correspond to the CML reaction (Simonsen, 1962; Elkins, 1976). Recently, lethal GVHR has been demonstrated in the absence of any killer cell differentiation (Jadus and Peck, 1983).

There have been a few reports concerning GVHR in Xenopus. In 1972, Clark and Newth observed some occurrence of GVHR following transplantation of allogeneic spleens to recipient frogs, in which tolerance to the spleen donor had been induced by transfer of embryonic flank tissues. More recently, Hsu, Julius and Du Pasquier (1983) reported that mortality (due to GVHR) could be induced in thymectomized larvae by injection of allogeneic splenic T lymphocytes. Very recently, Nakamura (1985) has shown that Xenopus (J x K) F₁ hybrid offspring succumb to a lethal GVH disease when given primed Xenopus spleen or splenocytes from the J parental strain.

The susceptibility of Tx Xenopus, reconstituted with allogeneic thymus in late larval life, to injected splenocytes (of thymus donor strain type) is examined here. These experiments were designed to determine whether such hosts have lost the cytotoxic potential to eliminate the injected cells, before the latter kill the host, through a

GVHR. The second GVHR assay, employed to probe thymus-induced allotolerance, assesses the ability of splenocytes removed from thymus-implanted Tx hosts to mount the proliferative phase of a GVHR against donor MHC antigens, following adoptive transfer to a suitable host.

MATERIALS AND METHODS

Animals.

The animals used for experiments in this Chapter were 12-18 months old. Those frogs implanted perimetamorphically with MHC-disparate thymus or skin had subsequently received assay skin grafts as young adults both from original donor and 3rd-party strains; all had specifically tolerated the original donor strain graft for more than 100 days, but had rejected the unrelated skin in normal fashion. Thymectomy, thymus implantation and skin implantation were carried out as mentioned previously.

Preparation of cells for injection.

All splenic leucocyte suspensions used in the in vivo MLR and GVHR assays were prepared for injection in serum-free amphibian L-15 culture medium and adjusted to 12.5×10^6 viable leucocytes/ml. Stimulator leucocytes for the in vivo MLR assay were inactivated by exposure for 5 minutes to a ^{60}Co source at a dose rate of 1000 rad/min and then washed in medium. In all experiments, each host received

2.5×10^6 pooled donor leucocytes (in 200 μ ls), injected via the dorsal lymph sac with a sterile 1ml disposable syringe.

In vivo MLR assay.

Animals in five experimental groups, a-e (see Figure 6.1), were injected with 5000 rad-irradiated MHC-disparate splenic leucocytes. Other animals in each group received irradiated isogeneic (or MHC-compatible) splenocytes to provide base-line (control) tritiated thymidine uptake for calculating stimulation indices. Injected animals were maintained for 72 hours at 26°C. Spleens were then removed aseptically and cell suspensions prepared in fully-supplemented L-15 culture medium. Cells from each spleen were plated in V-well culture plates (minimum of 5 replicates) at 2×10^5 leucocytes/200 μ ls. Each well was then pulsed with 1 μ Ci tritiated thymidine (5Ci/mmol) for only 7-12 hours (sufficient time to allow incorporation of thymidine, but keep any in vitro effects on the cells to a minimum). Tritiated thymidine uptake by splenocytes taken from each individual animal injected with MHC-disparate cells was compared with 2 or 3 individuals (of the same experimental group) given MHC-compatible or isogeneic cells; the difference was represented as a stimulation index (mean "allogeneic" dpm/"autologous" dpm). The proportion of individual SIs that were significant ($p < 0.05$) was determined by comparing individual experimental and control dpm with Student's t-test. All experiments on a particular group (a-e) of animals were performed together to avoid

variability due to physiological effects in vivo and in vitro.

GVHR Assays.

Two types of experiment were carried out. In the first, viable splenic leucocytes were taken from donors that had been primed (by skin grafting) against one or both MHC haplotypes of the (allotolerant) recipient into which they were now injected. Injected animals were maintained at 26°C and were examined daily for external signs of GVHR; eg. vascular disturbances and overall sickness of the recipient (described by Nakamura, 1985). The spleen and liver were autopsied and histologically compared with animals given control injections. In the second experiment, splenic leucocytes were removed from allotolerant animals and their ability to cause the splenic enlargement phase of a GVHR was assessed 10 days after adoptive transfer. Body weight (g) and spleen weight (mg) of recipients were determined at this time. Spleen enlargement in a GVHR is believed to be attributable to migration of injected leucocytes to host spleen, where they proliferate in response to host MHC antigens. A spleen enlargement index, SEI, can therefore be used as a measure of the severity of the GVHR and was calculated as follows:

SEI = Relative spleen weight for experimental animal

Mean rel. spleen wt. of minimum control group

where the relative spleen weight (rsw) = spleen weight (mg)/100g body weight. An index of 1.00 is thus, by definition, indicative of no change in spleen weight relative to the control group. The minimum index considered significantly increased is 1.30 (see Simonsen, 1962).

EXPERIMENTAL DESIGN

(a) In vivo MLR experiments.

To see whether increased splenic leucocyte proliferation could be detected by the in vivo MLR protocol described above, JLG5 hybrid hosts were injected with irradiated, X.borealis splenocytes. Control levels of thymidine uptake were provided by injecting other JLG5 hybrids from the same breeding with irradiated MHC-compatible splenocytes from J donors. These animals formed experimental group (a). The thymus dependency of the in vivo proliferation was tested; J strain, 7-day Tx frogs were injected with irradiated X.borealis splenic leucocytes and proliferation levels in each animal compared with Tx animals given J cells (group b). The in vivo MLR of control LG15 Xenopus given LG3 irradiated cells was examined in group (c). Tx LG15 implanted before metamorphosis with adult LG3 thymus (group d) were tested for evidence of enhanced splenocyte proliferation following injection of irradiated thymus donor strain LG3 leucocytes. Finally, in experimental group (e), LG15 control hosts, tolerating skin implants of

LG3 or LG5 (due to perimetamorphic skin grafting) were injected with irradiated splenocytes from the implant donor strain, or with isogeneic cells. The experimental animals tested in groups (d) and (e) had all been grafted with test skin grafts of thymus or skin implant donor type as young adults. These grafts were still in perfect condition at the time of assay, 5 months postgrafting.

(b) GVHR assays.

Initially, 7-day Tx Xenopus and Tx hosts reconstituted with isogeneic thymus during late larval life were tested for their susceptibility to GVH disease by injection with a suspension of viable splenic leucocytes, pooled from MHC-disparate donors. These LG3 hosts received either LG15 anti-LG3 cells (ie. from an LG15 (ac) donor that had previously rejected a LG3 (bd) skin graft), or J anti-LG5 (ad) cells. LG3 Tx Xenopus implanted with LG15 thymuses (either larval or adult thymus) were then tested for their ability to deal with potentially hazardous LG15 anti-LG3 splenocytes or J anti-LG5 cells. Two control LG3 hosts bearing tolerated LG15 skin implants were also tested in this "mortality assay", by injection of LG15 anti-LG3 splenocytes.

In the second assay, splenic leucocytes were prepared from J Tx frogs implanted with LG5 thymus. Pooled cells were then injected into JLG5 triploid hosts. Hybrids were used to prevent allorecognition of MHC antigens carried by the injected cells and to allow any donor anti-LG5 cells to

proliferate. Other JLG5 hosts were injected with either JLG5 or J splenocytes to provide minimum and maximum GVHR controls respectively.

RESULTS

In vivo MLR assay.

The stimulation indices calculated from comparison of mean dpm from each experimental animal with that for each control are shown in Tables 6.1-6.5. The overall mean stimulation index for each experimental group was also determined and the data summarized in Figure 6.1.

Injection of irradiated X.borealis splenic leucocytes into 3, MHC-disparate JLG5 hybrids caused elevated ($SI > 1.0$) tritiated thymidine uptake by host spleen cells, as determined by comparison with those 3 recipients given J cells (Table 6.1), although mean stimulation indices for each of 3 test animals proved to be quite variable (2.18-7.02). The overall mean SI for group (a) was found to be 4.85.

The results from group (b) are shown in Table 6.2. These indicate that enhanced splenic proliferation following injection of MHC-disparate cells in this in vivo assay is thymus dependent. Moreover, they confirm that the foreign X.borealis cells do not themselves proliferate after 5000 rad-irradiation and injection into Tx J hosts, since all 4 Tx animals injected with X.borealis cells failed to display

tritiated thymidine incorporation levels significantly above controls given J splenocytes.

Data for LG15 controls injected with irradiated LG3 splenocytes (Table 6.3) revealed a mean SI of 5.61 ± 1.86 . All individual SIs were rather variable (2.98-11.10). Three of the 4 LG15 Tx implanted with LG3 adult thymus (see Table 6.4) similarly displayed elevated tritiated thymidine uptake following injection of irradiated splenocytes from the thymus donor strain (LG3), when compared to splenic proliferation levels in animals given irradiated LG15 splenocytes. It would therefore seem that these animals contain splenic T cells capable of proliferation in in vivo MLR against LG3, despite bearing tolerated LG3 skin grafts.

Finally, splenocytes from all allogeneic skin-implanted, control LG15 hosts, that were tolerating test grafts of the original LG3 or LG5 implant strain, were shown (Table 6.5) to be capable of enhanced proliferation in response to injected implant strain cells, when tested in this assay. In experimental groups (d) and (e), the mean stimulation indices for individual tested animals varied widely, but since this was also observed in group (c) (as noted above), this high variability most probably reflects the very nature of this type of in vivo proliferative assay. In the in vivo MLR, proliferative responses of splenocytes from separate individuals are compared, whereas in the in vitro MLR, SIs are calculated by comparing thymidine uptake by cells from a single animal, cocultured with allogeneic or autologous, irradiated stimulators.

GVHR Experiments.**(i) Susceptability of thymectomized and allothymus-restored Tx animals to GVHR.****Symptoms of graft-versus-host disease:**

Onset of GVH disease in Xenopus in these experiments was found to be characterized by the appearance of dilated blood vessels and contraction of melanophores in the skin of the injected host, similar to that seen in an allograft during rejection (see Figure 6.2a). These effects were especially pronounced around the eyes and on the ventral surface of the body about 1-2 weeks after injection. Affected animals also suffered from oedema and usually died within a couple of weeks of the onset of visible symptoms. At autopsy, spleens from animals undergoing a GVH attack were generally found to be considerably enlarged compared to normal (see for example Figs. 6.2c and b respectively) and white pulp regions seemed much reduced when closely examined histologically (Fig. 6.2e). The enlarged spleens were found to contain many lymphoblasts concentrated in the white pulp areas, and contrasted the small lymphocyte population seen in the white pulp follicles and perifollicular regions of normal spleens (Fig. 6.2d). Eosinophilia and active phagocytosis (eg. of melanin) were also found to be common phenomena in the enlarged spleens. Striking effects were also observed in sections of liver from a host undergoing GVH attack (see Fig. 6.2g). Compared with liver from a

healthy frog (Fig. 6.2f) the lobules were packed with large lymphocytes, obliterating the normal architecture of the organ. Whether the lymphoblasts recorded in large number in the spleens and livers of animals undergoing GVH attack represent donor or host cells remains uncertain.

Mortality assay:

The outcome of these GVH experiments on thymus reconstituted Tx hosts is shown in Table 6.6. When non-irradiated, MHC-disparate (LG15 anti-LG3) splenocytes were injected into 3 Tx LG3 (bd) frogs, the host animals rapidly developed the gross symptoms of GVH attack described above and died approximately 2 weeks after injection. In contrast, all 6 LG3 Tx hosts reconstituted with isogeneic LG3 thymus remained perfectly healthy following injection with either LG15 anti-LG3 or J anti-LG5 (ad) allogeneic splenocytes during the observation period (>100 days). The ability to effectively counter a GVH attack would therefore appear to be thymus dependent. However, LG3 Tx hosts reconstituted with allogeneic LG15 larval or adult thymus were found to be specifically susceptible to killing by (bd-primed) LG15 splenic leucocytes (ie. of the thymus donor strain) since cells primed against the (d) haplotype of the host, but coming from a donor strain (J anti-LG5) failed to induce a GVHR. Onset of the disease was slower in the thymus reconstituted Tx hosts than in the LG3 Tx hosts, the animals starting to display symptoms 3-4 weeks after injection. The

illness was also more protracted in some of these frogs, death not occurring until some weeks later.

Two LG3 control frogs implanted with LG15 skin at metamorphosis were also available for testing in this GVH assay. Unlike the thymus-implanted Tx hosts, injection with splenocytes of donor strain type (LG15 anti-LG3) here failed to induce GVH disease in either host.

(ii) Spleen enlargement (splenomegaly) assay.

The results from this assay are shown in Table 6.7. The mean relative spleen weight from 3 JLG5 hosts injected with minor H antigen-disparate JLG5 splenic leucocytes provided a control, base line, of 70.26 by which to estimate the degree of splenomegaly in the maximum controls and test animals. These minimum control animals were displaying no signs of ill health when they were killed at 10 days. The 3 JLG5 hosts injected with J splenic leucocytes, however, all had acute vasodilation in the skin and appeared close to death by 10 days from GVH disease resulting from attack by the injected J cells following their allorecognition of the MHC-disparate JLG5 host. The host spleens were visibly enlarged and they proved to have SEIs of 1.50 and greater.

Splenic leucocytes from J Tx frogs reconstituted with LG5 adult thymus, and shown to be tolerant to subsequent LG5 skin grafts, did not induce noticeable symptoms of GVHR in the JLG5 hosts and when the SEIs were calculated for these

three hosts, the spleens were found to be not significantly enlarged compared to those of the minimum controls.

DISCUSSION

Overall, the in vivo MLR experiments would seem consistent with the in vitro MLR studies performed in Chapter 5, at least as far as the skin-implanted animals are concerned. Thus spleens from all the skin-implanted froglets tested appear to contain leucocytes able to proliferate in response to in vivo exposure to implant donor strain antigens expressed on injected, irradiated splenocytes. In the in vitro MLC study, it was found that although Tx animals reconstituted with larval or irradiated adult thymus generally responded in MLC, the majority of Tx hosts with adult allothymus implants were unable to respond significantly in vitro. However, following in vivo exposure to donor strain splenocytes, 3/4 Tx LG15 hosts reconstituted with normal, adult, LG3 thymus here appear capable of responding, by proliferation, to the injected cells. The in vivo findings reported here suggest that animals made tolerant of MHC-disparate skin by perimetamorphic implantation of thymus or skin still retain a population of lymphocytes (presumably T-dependent) reactive towards alloantigens of the original donor, but concomitantly fail to reject skin from the same donor; positive (in vitro) MLRs recorded are not simply due to culture conditions

artificially relieving suppression or allowing interaction of cell types normally separated in vivo. Whether the host splenocyte (T cell) proliferative response also occurs following application of a skin graft destined to be tolerated is currently being investigated in this laboratory; initial studies suggest that the host spleen does respond (J.D. Horton, personal communication) presumably indicating that sufficient class II-bearing cells are present in the skin, or within its vasculature, to stimulate a MLR.

The nature of the T cells in tolerant animals capable of alloreactivity towards cells of the donor MHC type inducing the tolerance remains uncertain. One possibility (discussed briefly in Chapter 5) is that these dividing cells represent suppressor T lymphocytes. In mammals, it is thought by some workers that in transplantation tolerance situations, suppressor cells may proliferate in response to idiotypic determinants carried on syngeneic T cells stimulated by the allograft, thereby suppressing the alloimmune response (Lancaster, Chui and Batchelor, 1985). Preliminary attempts to adoptively transfer suppression of allograft rejection from LG15 Xenopus tolerant to either LG3 or LG5 skin (following perimetamorphic skin implantation) by injecting five normal LG15 individuals with 6×10^6 splenic and peripheral blood leucocytes each via the dorsal lymph sac, just after application of an LG3 or LG5 skin graft, were also performed here. These attempts failed to significantly prolong allograft survival (rejection times

18, 21, 22, 23 and 25 days) in comparison with control times (18, 18 and 18 days). However, in view of the fact that adoptive transfer of suppression of alloreactivity in metamorphosing Xenopus is known to be difficult to achieve, requiring the continual supply of large numbers of injected cells (Du Pasquier and Bernard, 1980), these preliminary findings are not given too much weight.

It seems unlikely that the cells proliferating in MLR are cytotoxic T cells, since the GVHR experiments suggest that the cytotoxic effector component in some tolerant animals appears to be defective. Thus Tx hosts implanted with foreign thymus, either larval or adult, all failed to survive in the GVHR mortality assay; symptoms were specifically induced by thymus-type splenocytes rather than 3rd-party leucocytes. However, these restored animals appear to possess some advantage over non-restored Tx hosts, afforded, perhaps, by thymus-dependent helper cells, eg. through T-dependent antibody production? Interestingly, two animals implanted with allogeneic skin at metamorphosis failed to succumb to potentially hazardous, viable splenocytes. This might indicate that allotolerance effected by perimetamorphic application of skin is less "complete" than that effected in Tx Xenopus following implantation of a foreign thymus. Direct measurement of cytotoxic T cell reactivity in animals rendered tolerant to allogeneic skin by perimetamorphic thymus or skin implantation is now required to formally reveal whether a defect in the

generation of this effector T cell population is the reason why skin grafts survive indefinitely.

A third possibility is that MLR-reactive cells in allotolerant Xenopus are T helper cells. Their presence in this situation would, perhaps, be surprising in view of the widely held belief that, in mammals, cells with surface antigen markers of the T helper cell lineage, appear to be centrally involved in allograft rejection (see review by Mason, 1983). Moreover, the preliminary GVHR data presented here suggest the possibility that T helpers in allotolerant Xenopus are functionally impaired. Thus splenocytes taken from allothymus-implanted, Tx animals fail to produce spleen enlargement when adoptively transferred to appropriate hosts. In mammals, GVH disease can be induced by cloned T helper cells (Jadus and Peck, 1983). Further, more comprehensive studies with the GVHR system will be useful in probing the extent to which helper and cytotoxic T cell reactivity towards the donor (inducing the tolerance) are impaired in allotolerant Xenopus.

Nakamura (1985) has recently stressed the importance of using primed donor leucocytes sensitized with host antigens, either by skin grafting or in MLR, to produce a satisfactory GVH response. Prestimulation in vivo and in vitro is also known to be required with Xenopus to produce cytotoxicity in CML (Bernard et al, 1979; Lallone and Horton, 1985). It would therefore be of interest in future GVH experiments to presensitize splenocytes of allotolerant (allothymus-restored, Tx or skin-implanted, control) Xenopus

in MLR, rather than by skin grafting, since donor skin is tolerated. One can then observe whether these stimulated cells are capable of responding to and killing a host in GVHR following adoptive transfer.

Future experiments to study the defect in allotolerant animals will be aided by the increasing availability of monoclonal reagents to detect T cells in J strain Xenopus (Nagata, 1985), and alloantisera against class I and II antigens of the Xenopus MHC; the XLA locus (Flajnik et al, 1984; Kaufman et al, 1985). Now that an amphibian T cell growth factor, apparently functionally and structurally homologous to mammalian IL-2, has been partially characterized (Watkins and Cohen, 1986), the possibility of long-term culturing and marking of Xenopus T cell subsets may not be far away.

Table 6.1 Stimulation indices for in vivo MLR.

Group (a); JLG5 controls injected with irradiated X.borealis splenic leucocytes.

Mean dpm ± sem Experimental splenocytes	Mean dpm ± sem Control splenocytes			Mean SI (for each experimental animal)
	JLG5 + r.J x = 2222 ± 70	JLG5 + r.J x = 1512 ± 55	JLG5 + r.J x = 1952 ± 72	
	Stimulation Indices (individual comparisons)			
+ JLG5 + r.Xb x = 12976 ± 519	5.84	8.58	6.65	7.02
JLG5 + r.Xb x = 9984 ± 385	4.45	6.54	5.06	5.35
JLG5 + r.Xb x = 4019 ± 166	1.81	2.66	2.06	2.18
	Mean SI for Group (a) = 4.85 ± 1.42			

+ JLG5 injected with 2.5×10^6 irradiated X.borealis splenocytes.

Hosts spleens removed 72 hours post-injection of irradiated (r.) splenocytes, plated at 2×10^5 leucocytes/200µl/well and pulsed for 7-12 hours with 1µCi tritiated thymidine/well.

All SI's significant by Student's t-test, the dpm of each experimental splenocyte population is compared with the dpm of each control population to give a stimulation index.

Table 6.2 Stimulation indices for in vivo MLR.
Group (b); J Tx injected with irradiated X.borealis splenic leucocytes.

Mean dpm \pm sem Experimental splenocytes	Mean dpm \pm sem control splenocytes		Mean SI (for each experimental animal)
	J Tx \leftarrow r.J x = 2273 \pm 168	J Tx \leftarrow r.J x = 2992 \pm 149	
	Stimulation Indices (individual comparisons)		
J Tx \leftarrow r.Xb x = 2774 \pm 102	1.22	0.93	1.08
J Tx \leftarrow r.Xb x = 1965 \pm 211	0.86	0.66	0.76
	Mean SI for Group (b) = 0.92 \pm 0.16		

See Table 6.1, Legend for experimental details.
0/4 Stimulation indices were found to be significant with Student's t-test.

Table 6.3 Stimulation indices for in vivo MLR.
Group (c); LG15 controls injected with irradiated LG3 splenic leucocytes.

Mean dpm \pm sem Experimental splenocytes	Mean dpm \pm sem Control splenocytes				Stimulation Indices (individual comparisons)	Mean SI (for each experimental animal)
	LG15 + r.LG15 x = 2863 \pm 32	LG15 + r.LG15 x = 4317 \pm 101	LG15 + r.LG15 x = 513 \pm 27	LG15 + r.LG15 x = 463 \pm 57		
LG15 + r.LG3 x = 10239 \pm 424	3.58	2.37	---	---	2.98	
LG15 + r.LG3 x = 15544 \pm 228	5.43	3.60	---	---	4.52	
LG15 + r.LG3 x = 5402 \pm 456	---	---	10.53	11.67	11.10	
LG15 + r.LG3 x = 1806 \pm 66	---	---	3.63	4.02	3.83	
Mean SI for Group (c), = 5.61 \pm 1.86						

See Table 6.1, Legend for experimental details.

8/8 Stimulation indices were found to be significant with Student's t-test.

Table 6.4 Stimulation indices for in vivo MLR.

Group (d); LG15 Tx implanted with adult LG3 thymus (LG15/3) injected with irradiated LG3 splenic leucocytes.

Mean dpm ± sem Experimental splenocytes	Mean dpm ± sem Control splenocytes				Mean SI (for each experimental animal)
	LG15/3+r.LG15 x = 3890 ±140	LG15/3+r.LG15 x = 3779 ± 77	LG15/3+r.LG15 x = 622 ± 50	LG15/3+r.LG15 x = 786 ± 39	
	Stimulation Indices (individual comparisons)				
† LG15/3 + r.LG3 x = 24475 ± 1999	6.29	6.48	---	---	6.39
LG15/3 + r.LG3 x = 28687 ± 4755	7.37	7.59	---	---	7.48
LG15/3 + r.LG3 x = 1253 ± 92	---	---	2.01	1.59	1.80
LG15/3 + r.LG3 x = 657 ± 31	---	---	1.06*	0.84*	0.95
	Mean SI for Group (d) = 4.10 ± 1.66				

See Table 6.1, Legend for experimental details.

† LG15/3 = LG15 Tx implanted with LG3 thymus.

* These Stimulation indices not significant with Student's t-test.

6/8 SIs were significant with Student's t-test.

Table 6.5 Stimulation indices for in vivo MLR.

Group (e); LG15, implanted with LG3 (LG15-3) or LG5 (LG15-5) skin, injected with irradiated implant MHC-type splenic leucocytes.

Mean dpm ± sem Experimental splenocytes	Mean dpm ± sem control splenocytes					Mean SI (for each experimental animal)
	LG15-3+r.LG15	LG15-3+r.LG15	LG15-5+r.LG15	LG15-5+r.LG15	LG15-5+r.LG15	
	x = 513 ± 27	x = 463 ± 57	x = 5194 ± 580	x = 4869 ± 347	x = 4948 ± 467	
	Stimulation indices (individual comparisons)					
† LG15-3 + r.LG3 x = 1768 ± 87	3.45	3.82	---	---	---	3.64
LG15-3 + r.LG3 x = 976 ± 38	1.90	2.11	---	---	---	2.01
† LG15-5 + r.LG5 x = 9419 ± 379	---	---	1.81	1.93	1.90	1.88
LG15-5 + r.LG5 x = 25860 ± 1184	---	---	4.98	5.31	5.23	5.17
	Mean SI for Group (e) = 3.18 ± 0.78					

See Table 6.1, Legend for experimental details.

† LG15-3 = LG15 implanted with LG3 skin.

† LG15-5 = LG15 implanted with LG5 skin.

10/10 Stimulation indices were significant with Student's t-test.

Table 6.6 GVH disease mortality following injection of immunized splenic leucocytes to thymectomized and thymus-implanted animals.

Host Animal	Cell type Injected	Number of animals injected	Time of death (Days post-injection)	Number surviving >100 days post-injection
LG 3 Tx	LG15 anti-LG 3	3	13, 13, 13	0
LG 3 Tx with LG 3 thymus	LG15 anti-LG 3	4	--	4
LG 3 Tx with LG 3 thymus	J anti-LG 5	2	--	2
LG 3 Tx with LG15 thymus	LG15 anti-LG 3	5	26 ^ℓ , 38 ^ℓ , 25, 42, 62	0
LG 3 Tx with LG15 thymus	J anti-LG 5	3	--	3

Legend: MHC haplotypes for X.laevis/gilli hybrid strains; LG 3 = bd, LG 5 = ad, LG15 = ac
X.laevis J strain haplotype = jj
 Animals were injected with 2.5×10^6 viable splenic leucocytes via the dorsal lymph sac.
 ℓ = larval thymus implant.

Table 6.7 Splenomegaly assay for GVH proliferation.

Experimental Group	Relative spleen weight spleen wt. (mg)/100g body weight.	Mean Control Relative spleen weight.	Spleen Enlargement Index = $\frac{\text{experimental rel. spleen wt.}}{\text{mean Control rel. spleen wt.}}$
Control minimum:	81.48		
JLG5 injected with	78.88	70.26	1.00
JLG5 splenic leucocytes.	50.41		
Control maximum:	105.63		1.50
JLG5 injected with	139.93	--	1.99
J splenic leucocytes.	196.72		2.80
Test:			
JLG5 injected with splenic leucocytes	74.66		1.06
from J Tx implanted	80.43	--	1.14
with LG5 adult thymus	90.61		1.29

Legend: Splenomegaly assays performed 10 days post -injection of splenocytes. J splenic leucocytes used in the maximum control were from J animals that were previously grafted with, and had rejected LG5 skin. Host animals were JLG5 3N hybrids and were each injected with pooled 2.5×10^6 viable splenic leucocytes from the appropriate donors. Only J Tx implanted with LG5 adult thymus that were carrying a perfect LG5 skin graft >100 days post-grafting were used as cell donors for the test assay.

Figure 6.1 Legend

Key to experimental groups:

- a = JLG5 controls injected with irradiated X. borealis splenic leucocytes.
- b = J Tx. injected with irradiated X. borealis splenic leucocytes.
- c = LG15 controls injected with irradiated LG3 splenic leucocytes.
- d = LG15 Tx. implanted with adult LG3 thymus, injected with irradiated LG3 splenic leucocytes.
- e = LG15 controls implanted with LG3 or LG5 skin, injected with irradiated implant donor (LG3 or 5) splenic leucocytes.

Fig.6.1 Summary of in vivo MLR data

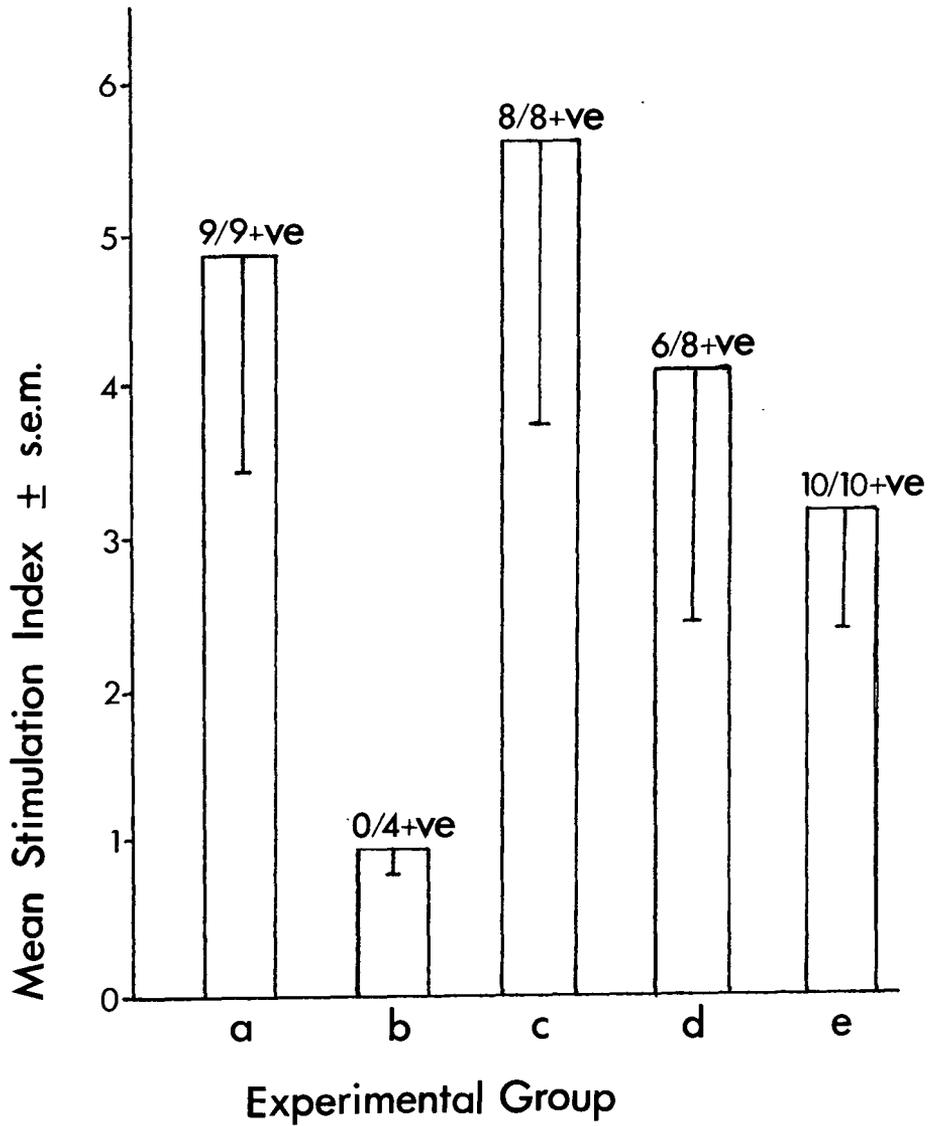


Figure 6.2 Symptoms of a Graft-versus-host reaction.

(a) External signs of a GVHR.



LG3 Tx implanted with LG15 thymus, injected with LG15 anti-LG3 splenic leucocytes. Appearance of the skin after 60 days. Note vasodilation of skin capillaries and pools of extravascular blood. Melanophores are contracted, giving the skin a "punctate" and pale coloured appearance. This animal was close to death on day 62 post-injection when it's spleen was removed for histologic study (see Figure 6.2 c).

Figure 6.2b Legend

GVHR Control: LG3 Tx. implanted with LG3 thymus, injected with 2×10^6 LG15 anti-LG3 splenic leucocytes 62 days previously.

Whole spleen section 6-7 μ m taken through centre.

Note distinct white and red pulp regions.

Stain = haematoxylin and eosin.

Magnification x 6.25

Figure 6.2c Legend

GVHR Test: LG3 Tx. implanted with LG15 thymus, injected with 2×10^6 LG15 anti-LG3 splenic leucocytes 62 days previously.

Whole spleen section 6-7 μ m taken through centre.

Note enlargement of the organ in comparison with control (b), with the diameter approx. twice that of the control. Also loss of clear white/red pulp definition.

Stain = haematoxylin and eosin.

Magnification x 6,25

Figure 6.2 continued

(b)



(c)

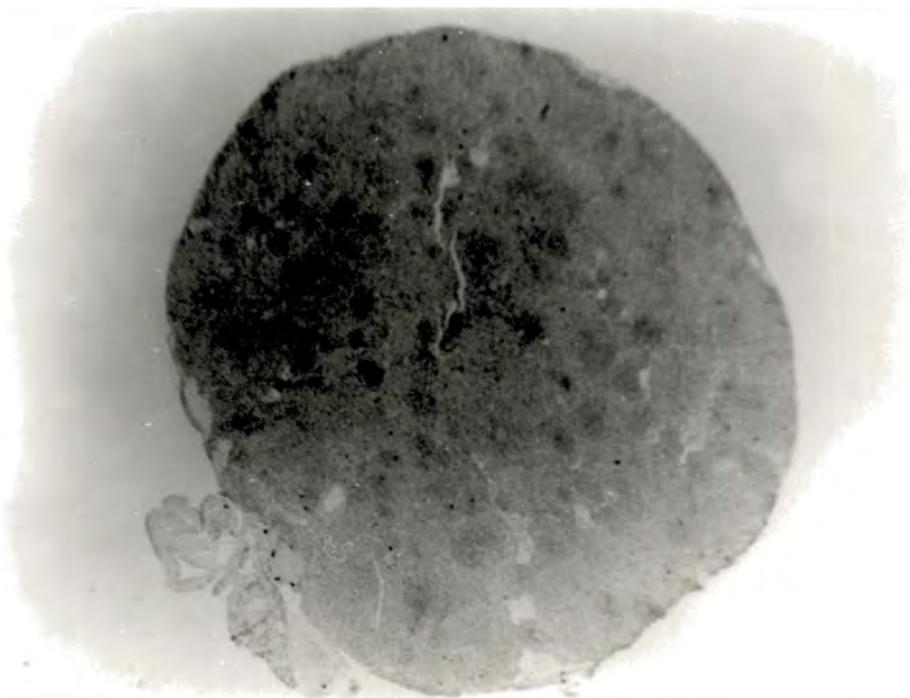


Figure 6.2d Legend

GVHR Control: LG3 Tx. implanted with LG3 whole adult thymus, injected with 2×10^6 LG15 anti-LG3 splenic leucocytes 62 days previously.

Higher magnification to show rich small lymphocyte population in white pulp and marginal zone.

Stain = haematoxylin and eosin.

Magnification x 100

Figure 6.2e Legend

GVHR Test: LG3 Tx. implanted with LG15 whole adult thymus, injected with 2×10^6 LG15 anti-LG3 splenic leucocytes 62 days previously.

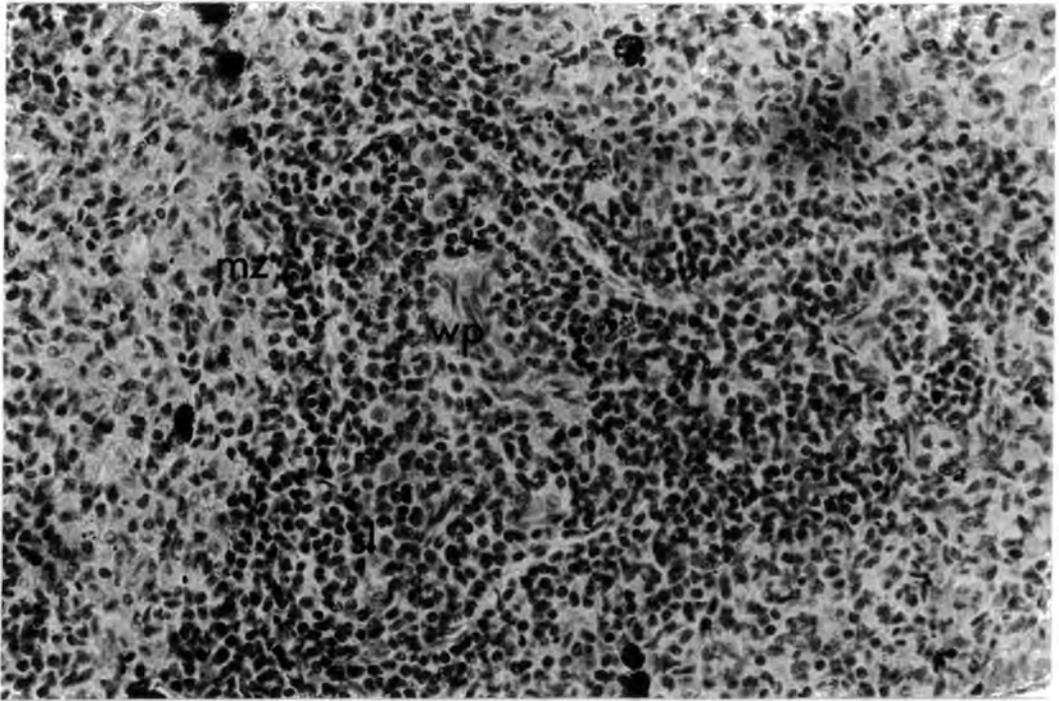
Note many lymphoblasts in the white pulp, but lack of small lymphocytes from white pulp and marginal zone.

Stain = haematoxylin and eosin.

Magnification x 100

Figure 6.2 continued

(d)



(e)

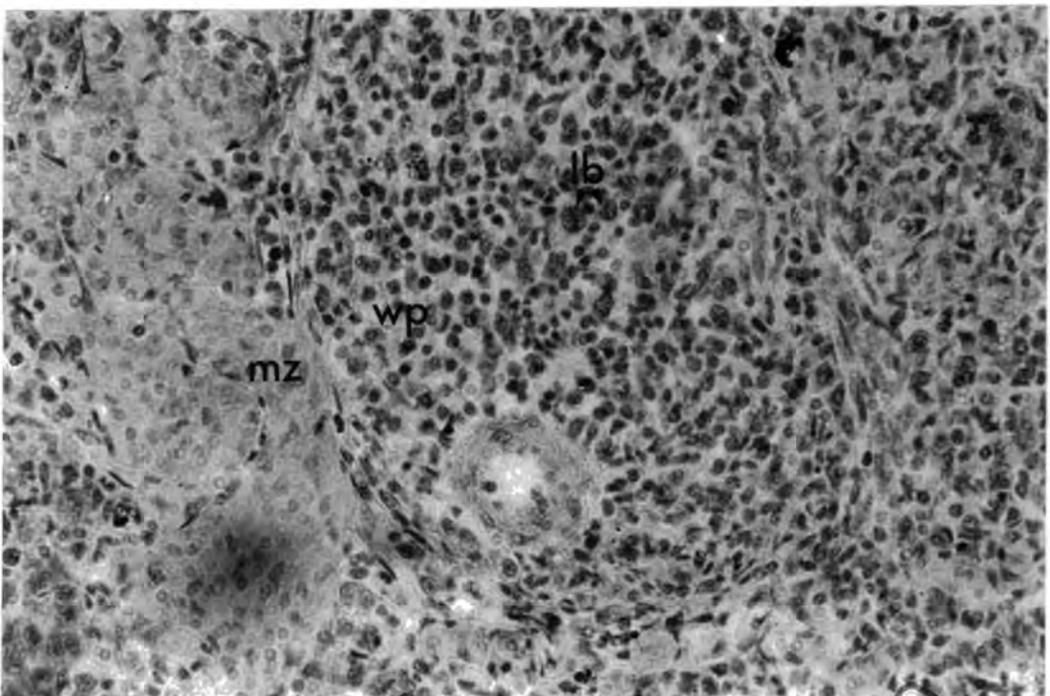


Figure 6.2f Legend

GVHR Control: LG3 Tx. implanted with whole adult LG3 thymus, injected with 2×10^6 LG15 anti-LG3 splenic leucocytes 62 days previously.

Section through liver (6-7 μ m) to show normal liver structure.

Stain = haematoxylin and eosin

Magnification x 50

Figure 6.2g Legend

GVHR Test: LG3 Tx. implanted with whole adult LG15 thymus, injected with 2×10^6 LG15 anti-LG3 splenic leucocytes 62 days previously.

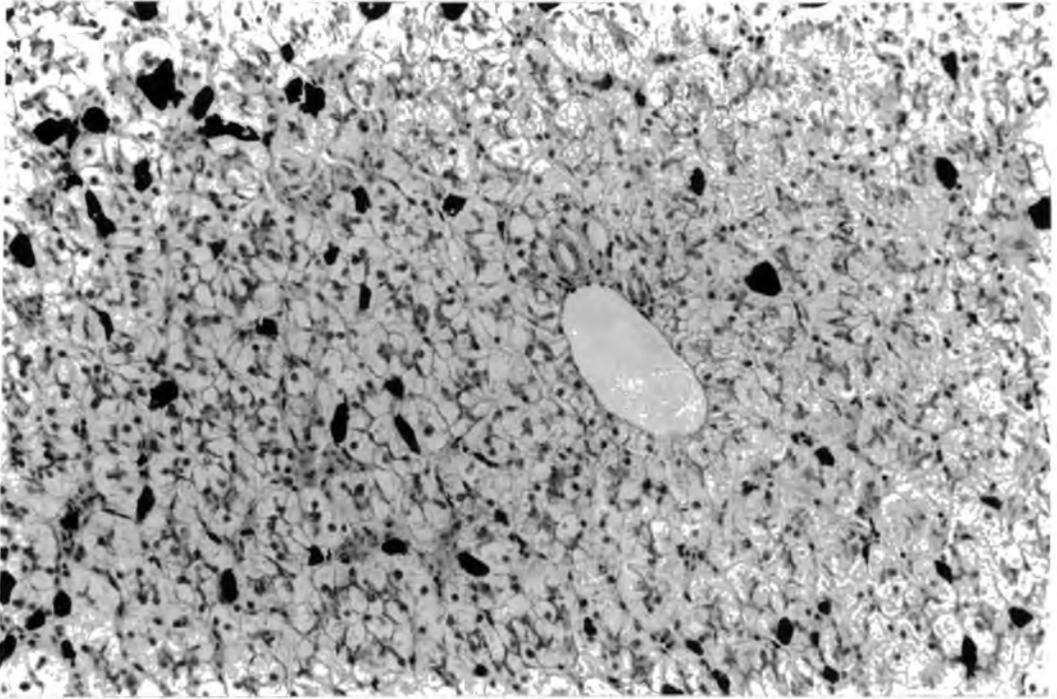
6-7 μ m section through liver. Note many lymphoblasts and lymphocytes are present, totally obscuring the normal structure.

Stain = haematoxylin and eosin

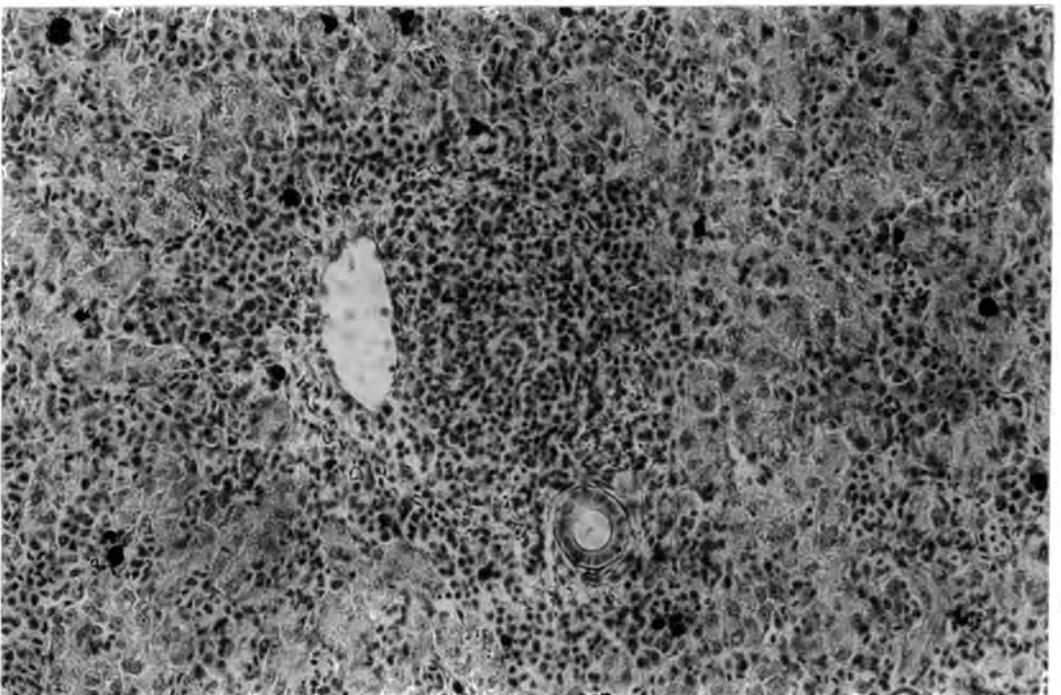
Magnification x 50

Figure 6.2 continued

(f)



(g)



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