## *T-cells and transplantation tolerance in Thymectomised Xenopus implanted with foreign thymus*

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# T-cells and Transplantation Tolerance in Thymectomised Xenopus implanted with foreign thymus

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## Abstract

This thesis investigates the expression of a T-cell differentiation antigen, (XTLA-1), in various strains and species of *Xenopus*, and demonstrates the effect of early-thymectomy, (by microcautery), on XTLA-1 expression. It further examines restoration of the T-cell dependent immune system, (particularly with respect to transplantation responses), and the extent to which tolerance to donor antigens is achieved by implantation of xenogeneic, as well as allogeneic, thymi into early-thymectomised *Xenopus* larvae. The means by which transplantation tolerance is maintained in intact, control *Xenopus*, following perimetamorphic skin grafting, is also addressed.

Initial work, reported in Chapter 2, showed that XTLA-1 is expressed by the majority of thymocytes and by a proportion of splenocytes from all *X.laevis*, *X.borealis*, and hybrid clonal *Xenopus*,(*X.laevis* x *X.gilli* and hybrid *X.laevis* x *X.muelleri*),examined. *X.tropicalis* lymphocytes, however, do not express XTLA-1. Early-thymectomy by microcautery effectively removes T-cells, as detected by XTLA-1 expression. In Chapter 3, normal adult and larval tissue distribution of XTLA-1 positive cells is described, and the XTLA-1 and *X.borealis*,(quinacrine fluorescence), markers are employed to demonstrate the differentiation of T-cells derived from early- thymectomised hosts within xenogeneic, (*X.tropicalis*), thymus implants. The effects of implantation of allogeneic and xenogeneic larval thymi into early-thymectomised hosts, in terms of T-cell responses and of induction of tolerance to thymus donor antigens, is explored in Chapter 4; *X.borealis* xenogeneic thymus implants are apparently as effective in these regards as are allogeneic implants, but *X.tropicalis* xenogeneic thymus implants do not fully restore thymus-dependent immune responses. Preliminary investigations of skin graft rejection, mixed leukocyte culture and T-cell mitogen responses of *X.tropicalis*, in comparison to those of other *Xenopus* species, are reported in Chapter 5; the results of these experiments raise the possibility that *X.tropicalis* splenocytes are less responsive, in mixed leukocyte culture, to xenogeneic stimulators than are splenocytes of other *Xenopus* species. In Chapter 6, histological examination of skin grafts, accepted by virtue of the tolerance induced by prior implantation of a thymus gland from the skin graft donor into the early-thymectomised hosts, reveals some rapid alteration in the composition of these skin grafts; infiltration of the tolerated skin grafts by host-derived lymphocytes suggests that tolerance induced by thymus implantation does not abrogate recognition of thymus donor antigens. Finally, also in Chapter 6, tolerance induced in control, intact *Xenopus* by perimetamorphic skin grafting is shown to be susceptible to cyclophosphamide injection, suggesting that the maintenance of this tolerance is mediated by suppressor cells.

## Declaration

Some of the material presented in Chapters 4 and 6 of this Thesis has previously been reported in the following publications:

"Attempts to break perimetamorphically induced skin graft tolerance by treatment of *Xenopus* with cyclophosphamide and interleukin-2", by John D. Horton, Trudy L. Horton, Claire A. Varley and Laurens N. Ruben. Transplantation, (1989), vol. 47, No. 5, pp. 883-887.

"Use of the *X.borealis* fluorescence marker to determine the fate of 'tolerated' skin xenografts", Claire A. Varley, Trudy L. Horton, Pamela Aitchison and John D. Horton (1989), Devel. Comp. Immunol., vol. 13, No. 4, 391-392.

"Demonstration of anti-donor alloreactivity in skin graft- tolerant *Xenopus*," John D. Horton, Trudy L. Horton, Claire A. Varley and Laurens N. Ruben (1989), Devel. Comp. Immunol., vol. 13, No. 4, 420.

"Characterisation of 'split tolerance' in thymectomised *Xenopus* implanted with quinacrine-marked, histo incompatible thymus", by John D. Horton, Claire A. Varley, Trudy L. Horton and Pamela Aitchison, submitted to J. Exp. Zool.

Assays of T-cell responses in thymectomised LG15 hosts implanted with *X.tropicalis* thymi, (described in Chapter 4),were performed in collaboration with Trudy L. Horton.

Some assays of *X.tropicalis* lymphocyte responses to T-cell mitogens, reported in Chapter 5,were performed in collaboration with Sarah L. Turner.

## Statement of Copyright

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# Chapter 1

# Introduction

Thymus-dependent lymphocytes, (T-cells), are known to recognise antigen in the context of cell-membrane associated molecules encoded by the major histocompatibility complex, (MHC), a phenomenon called MHC-restriction. It has been demonstrated that MHC- restriction is not an intrinsic property of T-cells, but that it must be 'learned' during T-cell differentiation; Bevan, (1977), and Zinkernagel, (1978 ), showed, using bone marrow and thymus chimaeric mice, (thymectomised, irradiated mice, reconstituted with bone marrow and thymus from different sources), that mature T-cells preferentially recognise antigen in association with MHC molecules of the same genotype as those expressed on the radioresistant cells of the thymus within which the T-cells develop. Thus, the thymus clearly plays a major role in T-cell education. Although recognition of 'self' MHC plus (non-MHC encoded) antigen is necessary for an effective T-cell response, recognition of 'self' leading to destructive autoimmune responses must be avoided. The process by which potentially autoreactive T-cell clones are destroyed, suppressed or otherwise made inactive is known as self-tolerance induction, and the thymus is also thought to play a central part in this.

Recent developments in the study of mammalian immunology, (the description of the T-cell receptor, (TCR), and of genes coding for parts of the constituent chains of the TCR; the discovery of the specificity conferred upon TCRs by their inclusion of particular gene products; and the production of monoclonal antibodies specific for some TCR constituents), have permitted the direct observation of tolerance induction by clonal deletion of self-reactive T-lineage cells within the murine thymus. The TCR of mouse and man is composed of two polypeptide chains; the bulk of mature T-cells express TCRs composed of $\alpha$ and $\beta$ chains, (although some express, instead, TCRs composed of $\gamma$ and $\delta$ chains). The $\alpha$ chains are the product of genes functionally rearranged to bring together one each of many possible $V_\alpha$ and $J_\alpha$ gene segments, (about 50 of each exist in mice); functional $\beta$ genes are rearranged from about 21 $V_\beta$, 12 $J_\beta$ and 2 $D_\beta$ gene segments, in mice, (see recent reviews by Marrack & Kappler, 1987; Davis & Bjorkman, 1988). One particular murine $V_\beta$ gene segment, $V_\beta$17a, appears to confer specificity for I-E, (MHC class II), molecules of various murine MHC haplotypes on T-cells bearing TCRs encoded in part by this gene element, (Kappler, Wade et al, 1987). Analysis of mouse T-cells immunofluorescently labelled with KJ-23 monoclonal antibody,

(anti- $V_\beta 17a$ gene product), revealed that, in strains expressing I-E molecules, $V_\beta 17a$ positive, ($V_\beta 17a^+$), mature T-cells were very few, (0.6% of the T-cell population), whereas, in non-I-E-expressing strains, $V_\beta 17a^+$ T-cells comprised the expected 10% of the T-cell population, (Kappler et al, 1987). In the same study, Kappler and coworkers showed that $V_\beta 17a^+$ positive T-cells were deleted within the thymus of I-E expressing mice, before these cells became functionally mature. Intrathymic deletion of T-lymphocytes bearing $V_\beta 8.1^+$ and $V_\beta 6^+$ TCRs, (specific for particular minor lymphocyte stimulating, (Mls), antigens, (non-MHC encoded antigens which stimulate strong primary proliferative T-cell responses in mixed lymphocyte cultures involving MHC- identical cells, as recently reviewed by Abe & Hodes, 1988)), has also been observed in mice expressing the allele of the Mls locus for which these TCRs are specific, (Kappler et al, 1988; MacDonald et al, 1988). Furthermore, in transgenic mice, (which contain 'foreign' genes, artificially introduced into the germ-line, often together with a tissue- enhancer and a particular promoter to determine the tissue in which the products of the introduced genes are expressed, see Jaenisch, 1988), expressing the rearranged $\alpha$ and $\beta$ chain genes from a class I (H-2 Db) - restricted, H-Y (male antigen) -specific cytotoxic T-lymphocyte clone, tolerance induction in males has been shown to result from intrathymic deletion of functional T-cells expressing the transgenic TCR, (Kisielow et al, 1988).

The stage of T-cell differentiation at which potentially autoreactive clones are deleted has also recently come under scrutiny. Briefly, T-cell precursors entering the thymus have not yet rearranged their TCR genes and so do not express TCRs. They are also negative with respect to the surface expression of CD4 and CD8 antigens; these differentiation antigens, when expressed on mature peripheral T-cells, appear to stimulate the interactions of T-cells with their target cells, to contribute to MHC- restriction, (CD4 expression correlates with MHC class II recognition and CD8 expression with class I recognition), and possibly to influence intrathymic T-cell repertoire selection, (see Emmrich, 1988). During intrathymic differentiation T-cells develop through a series of intermediate steps, including a double positive, ($CD4^+CD8^+$), $TCR^+$ stage, the majority finally emerging as $CD4^+CD8^-$ or $CD4^-CD8^+$ cells, expressing a selected TCR repertoire. Clonal deletion to prevent the emergence of functional autoreactive T-cells may operate at any of a number of junctures between TCR expression and maturity. In mice expressing the relevant antigens, $V_\beta 8.1^+$ and $V_\beta 17a^+$ T-cells are eliminated from the mature $CD4^+$ helper and $CD8^+$ cytotoxic lymphocyte pool, while immature $CD4^+CD8^+$ thymocytes expressing TCRs containing these gene products are unaffected, (Kappler et al, 1987; Kappler et al, 1988). On the other hand, in the transgenic, H-Y-specific TCR model of Kisielow and coworkers, (1988), deletion of immature, double-positive thymocytes is observed. To add a further twist to this story, investigation of an unusual transgenic mouse model in which the transgenic receptor is specific for both LCM virus in the context of H-2b and for $Mls^a$, (since the $\beta$ chain of this TCR uses $V_\beta 8.1$), suggests that the stage at which developing T-cells are deleted may depend upon the nature of the tolerising antigen, (Pircher et al, 1989). Thus, in these TCR transgenic mice, induction of tolerance to $Mls^a$ does not affect double-positive thymocytes, while tolerance induced to LCM virus

does. The mechanics of clonal deletion are not, as yet, understood.

As differentiating T-cells learn MHC-restriction within the thymus, and as tolerance can be imposed here on at least some developing T-cells, the most plausible hypothesis is that both of these phenomena are mediated through interaction of the TCR with class I and class II MHC-expressing cells of the thymus, (discussed by Marrack & Kappler, 1987, 1988; Fink, 1988). Both thymic epithelium and bone-marrow derived thymic macrophages and dendritic cells express MHC molecules; much work has been carried out in an attempt to decide which of these cell types is responsible for tolerance induction. Putative epithelial thymi, produced by low temperature organ cuture of foetal thymus, do not induce tolerance when transplanted to histoincompatible intact recipients, but do induce tolerance to donor MHC antigens when allografted into athymic recipients; the presence of donor-type dendritic cells in these cultured thymi could not be ruled out, (Jordan et al, 1985). 2-deoxyguanosine treated, epithelial foetal thymus glands, implanted into intact recipients, (Ready et al, 1984), or into athymic, nude mice, (von Boehmer & Schubiger, 1984; von Boehmer & Hafen, 1986), do not appear to induce tolerance to class I or class II MHC molecules. However, von Boehmer & Hafen, (1986), found that nude mouse recipients of allogeneic 2-deoxyguanosine-treated thymus implants were tolerised to minor histocompatibility antigens expressed on the thymic epithelium. Cultured fragments of mouse thymus, containing reduced but demonstrable numbers of thymic macrophages, were found to both restore immune functions and induce tolerance to thymus-donor H-2 antigens when implanted into nude mouse recipients, (Hong & Klopp, 1982). Furthermore, whole neonatal allogeneic thymi were found to induce MLC tolerance to donor antigens in nude mice otherwise functionally restored by this procedure, (Kindred, 1976); skin grafts of thymus donor type were, however, sometimes rejected by implanted animals. Recently, using irradiated, bone- marrow grafted mice, which carried I-E molecules only on the graft cells, Marrack and coworkers found that these I-$E^+$ bone-marrow derived cells were sufficient to induce tolerance by clonal deletion of $V_\beta 17a^+$ cells, (Marrack & Kappler, 1987). Kyewski, (reported in Crispe, 1988), has concluded that interaction between I-$A^+$ dendritic cells and $CD4^+8^+$ thymocytes could trigger clonal deletion of self-reactive cells when these arrive at the cortico-medullary junction of the thymus.

Despite the above evidence, from experiments using mice, implicating bone-marrow derived cells in tolerance induction, transplantation of quail epithelial thymi, (removed from the donor quails prior to colonisation with haemopoietic precursor cells), into early chick embryos lead to tolerance towards tissues of the thymus donor, (Ohki et al, 1987, 1988; Belo et al, 1989). Belo and coworkers also established that if only one third of the total thymus epithelium was of quail type this was sufficient to induce tolerance.

Although tolerance induction by clonal deletion has been shown to occur, and is thought by many to be the main mechanism by which neonatal tolerance of T- cells to self antigens is established, the work of Ohki, Belo and coworkers on quail/chick chimaeras, (quoted above), and of Morrissey et al, (1983), and of Zamoyska et al, (1989), on mice raises the possibility that post-thymic mechanisms may also induce effective T-cell tolerance. In the models used by all these authors, T-cells

were allowed to differentiate in one of two thymus glands, expressing different intrathymic antigens, within the same individual. Despite evidence that both thymi generated mature functional T-cells tolerant to the particular intrathymic antigens encountered during differentiation, all peripheral T-cells appeared to be tolerant to both sets of thymic antigens. As two rounds of thymic education, one within each thymus, are difficult to envisage, and since thymic antigen presenting cells do not, apparently, migrate between thymi within an individual, (Morrissey et al, 1983), Zamoyska et al, (1989), feel that some form of suppression is at work in this situation. They further suggest that this mechanism may operate normally to quell autoreactive responses to tissue-specific antigens encountered in the periphery, which are not expressed in the thymus.

Coutinho and Bandeira, (1989), hypothesise, on the basis of their own work and of that quoted in the last paragraph above, that T-cell tolerance is actually maintained through self-assertion by activated, autoreactive T-cell clones with appropriate specificities. Thus, they suggest, normal immune systems include a set of internally activated lymphocytes which are essentially self-reactive; these serve to ensure recursive selection of B- and T-cell repertoires. These authors are also of the opinion that although clonal deletion is observed, this phenomenon may not be causally related to tolerance induction.

Recently, tolerance induced in adult animals, (Mls-$1^{b+}$ mice made specifically unresponsive to Mls-$1^{a}$ antigens), has been shown to result from clonal anergy, rather than deletion, (Rammensee et al, 1989). Anergic T-cells in these animals expressed interleukin-2, (IL-2), receptors and underwent limited blastogenesis upon stimulation, but failed to produce IL- 2 themselves. Furthermore, Burkly et al, (1989), have recently noted that T-cell tolerance by clonal anergy occurs naturally in transgenic INS-I-E mice, which express I-E on pancreatic $\beta$ cells, but not in the thymus or peripheral lymphoid organs. In these animals, T-cells bearing TCRs comprising $V\beta17^{a}$ gene products, (which confer specificity for I-E molecules), are not deleted, but are not responsive either. Depending upon the circumstances of its induction, then, tolerance may be achieved in a number of ways.

The amphibian, *Xenopus*, is a useful model system in which to explore developmental aspects of T-cell tolerance induction, in that it is known to possess an immune system similar in many ways to, although perhaps less complex than, those of mammals, (see reviews by Manning & Horton, 1982; Flajnik et al, 1987; Du Pasquier et al, 1989).

*Xenopus* possesses a major histocompatibility complex which, although not as polymorphic as its mammalian equivalent, governs acute allograft rejection, mixed leukocyte culture responses, cell mediated cytotoxicity responses and the genetic restriction of T-B lymphocyte collaboration. Furthermore, *Xenopus* is easily bred and reared in the laboratory, has a free-living larval stage, (which can be surgically manipulated at very early developmental stages), and passes through metamorphosis, (a process involving extensive rearrangement of existing tissues and the generation of entirely new self antigens), when already immunocompetent. Inbred strains of *Xenopus* and isogeneic clonal animals are now available, (see Kobel & Du Pasquier, 1977), and monoclonal antibodies directed against *Xenopus* immunoglobulins, a surface membrane expressed T-cell differentiation

antigen, (XTLA-1), and various MHC-molecules have been produced, (see Flajnik et al, 1988). Cell marker systems, such as the quinacrine marker of *X.borealis*, (Thiébaud, 1983), are additional useful tools available to comparative immunologists. Although no congenitally athymic *Xenopus* strains, (equivalent to nu/nu mice and rats), exist, thymectomy can be performed very early in life without causing runting of the thymectomised frog. Both surgical, (Tochinai & Katagiri, 1975), and microcautery, (Horton & Manning, 1972), thymectomy techniques appear to be fully effective in the abrogation of thymus-dependent responses.

Reconstitution of T-dependent responses in thymectomised *Xenopus* can be achieved by implantation of adult or larval, normal or lymphocyte-depleted thymi which are syngeneic, allogeneic or, as shown in this thesis, xenogeneic to the recipient, (Horton & Horton, 1975; Tochinai et al, 1976; Nagata & Kawahara, 1982; Nagata & Cohen, 1984; Arnall & Horton, 1986). Tolerance to thymus-donor antigens, of varying degrees of profundity, can be induced by this thymectomy/thymus implantation technique; tolerance can also be achieved by grafting of skin to perimetamorphic *Xenopus*, (Bernadini et al, 1969, 1970; Chardonnens & Du Pasquier, 1973; Cohen et al, 1980; Barlow et al, 1981; Barlow & Cohen, 1983), although the unresponsiveness achieved by these two techniques may be maintained by different mechanisms, (Nakamura et al, 1987; Maeno et al, 1987).

The major aims of the work reported in this thesis were: to investigate the expression of XTLA-1 in various strains and species of *Xenopus* and to determine the effect of early- thymectomy by microcautery on XTLA-1 expression; to explore restoration of the T-cell dependent immune system, (particularly with respect to transplantation immunity), and the extent to which tolerance to donor antigens is achieved following foreign thymus implantation to early- thymectomised larvae, (making central use of xenogeneic donors which can provide useful discriminatory markers); and finally to study the means by which transplantation tolerance is maintained in control *Xenopus* following perimetamorphic skin grafting.

# Chapter 2

# Immunofluorescence experiments with the T-cell specific monoclonal antibody, XT-1:Species distribution of XTLA-1 positive lymphocytes and the effect of early thymectomy.

## 2.1 Introduction

Antibodies to *Xenopus* immunoglobulins, which facilitate the identification and separation of cells of the B-lineage, have been available for some time, (DuPasquier, Weiss and Loor, 1972; DuPasquier and Weiss, 1973; Jurd and Stephenson, 1976; Hadji-Azimi, 1977; Hadji-Azimi and Schwager, 1980; Bleicher and Cohen, 1981; Hsu, Julius and DuPasquier, 1983). Until recently, however, no antibodies were available with specificity for other *Xenopus* leukocyte surface markers. Now two such mouse monoclonal antibodies are available. One of these, XL-1, is directed against a determinant expressed on all lymphocytes, granulocytes, thrombocytes and macrophages of both adult and larval *Xenopus*, (Ohinata, Tochinai and Katagiri, 1990). The second, XT-1, permits the labelling and isolation of *Xenopus* thymocytes and of a proportion of peripheral T-lineage cells, (Nagata, 1985).

XT-1 secreting hybridoma cells were originally generated following the immunisation of BALB/C mice with viable thymocytes from inbred J strain *X.laevis*. Splenocytes from these immunised mice were then fused to P3-X63-Ag8.653 mouse myeloma cells. The XT-1 clone represents the one hybridoma line of 1013 cultures, generated from 5 fusions, which was shown, on the basis of a number of criteria, to be specific for a cell- surface antigen expressed by *Xenopus* T-lineage cells. Initial screening, (Nagata, 1985), of the XT-1 monoclonal antibody established that it

reacts with > 95% thymocytes and 27–30% spleen cells from intact J strain frogs, but with less than 2% splenocytes from early thymectomised animals, and not at all with red blood cells, in indirect immunofluorescent staining of cell suspensions. Staining of frozen sections of various J strain tissues with XT-1, in this same study, confirmed that expression of the XT-1 determinant was confined to cells of the *Xenopus* immune system. Interestingly, sections of brain were unstained, indicating that the XT-1 determinant is not a *Xenopus* equivalent of the murine Thy-1 antigen. The XT-1 monoclonal antibody was shown to belong to the IgG $2_b(\lambda)$ subclass, (Nagata, 1985); it may be affinity purified on Protein A Sepharose CL4B columns, (Nagata, 1985), and has been found not to fix guinea pig, rabbit or mouse complement, (Nagata, 1986a).

The cell surface antigen recognised by the XT-1 monoclonal antibody, named XTLA-1, has been partially characterised, (Nagata, 1988). SDS-PAGE and two-dimensional electrophoresis analyses of immunoprecipitated lysates of radioiodinated J strain splenocytes and thymocytes revealed that the XTLA-1 antigen is a glycoprotein consisting of a single polypeptide. In its reduced form, this glycoprotein has an apparent molecular weight of 120 kD, and an isoelectric point between pH 5.3–6.7. Under non-reducing conditions, XTLA-1 ran on SDS-PAGE as two bands of 120 kD and 110 kD apparent molecular weights; both these peptides have an identical mobility on reduced SDS-PAGE. Nagata suggests that these two bands "may reflect different forms of the 120 kD membrane peptide that has some 2-mercaptoethanol sensitive structures, such as intra-peptide chain disulphide bonds". He also offers the alternative suggestion that these two forms may result from damage to the molecular conformation of the antigen during the extraction and immunoprecipitation processes. In addition, the XTLA-1 antigen was found, by Endo-F treatment, to have N-linked glycans; the presence of extensive charge heterogeneity in 2-D electrophoresis following this treatment suggested the presence of additional, O-linked glycans.

Direct double immunofluorescence staining studies have been performed, (Nagata, 1985), utilising the XT-1 monoclonal antibody and an anti-*Xenopus* IgM monoclonal antibody, (6.16, raised by Bleicher and Cohen, 1981), bound to red or green fluorescent microbeads, respectively. No viable J strain splenocytes were found to be labelled with both red and green microbeads, indicating that XTLA-1 and surface IgM are mutually exclusive, i.e. that XT-1 is specific for a non-B cell antigen. It is worth noting that 43% of the adult J splenic lymphocytes enumerated by fluorescence microscopy in this study were found to be both XT-1 negative and surface IgM negative, suggesting that XT-1 is not a pan-*Xenopus* T-cell marker. Further data, (Nagata, 1986a), confirmed that XTLA-1 was expressed only on functionally-defined T-lineage cells and supported the suggestion that not all T-lineage cells carried this surface marker. J strain spleen cells, enriched by cell affinity chromatography to between 69% and 81% XT-1 positive cells, showed a significantly augmented proliferative response, in culture, to an optimal dose of phytohaemaggl utinin A, (PHA). The response of this cell population to an optimal dose of concanavalin A, (Con A), was slightly reduced, compared to that of control, unseparated cultures. Cultures depleted of XT-1 positive lymphocytes, (to 2-6% positives), by this method showed significantly reduced, but by

no means abrogated, responses to optimal doses of PHA, and Con A, compared to those of control, unseparated cultures. Furthermore, 3 of 4 similarly depleted J strain spleen cell cultures continued to show significant, if slightly decreased, mixed lymphocyte culture, (MLC), responses to irradiated, allogeneic, (inbred K strain *X.laevis*), stimulator cells.

While it is possible that the 2–6% XT-1 positive lymphocytes left in these depleted cultures might be sufficient to generate significant PHA, Con A and MLC responses, Nagata considers that it is more likely that there is a population of surface IgM-negative, XT-1-negative thymus-dependent lymphocytes in the spleen, which are responsive to PHA and ConA and in MLC. Since virtually all thymocytes express XTLA-1, then this determinant must be lost or otherwise become undetectable on some T-lineage cells after their emigration to the periphery. The XT-1 positive and negative T-cell subpopulations may, then, represent different maturation stages, or different functional subsets of T-lineage cells. Equally, the possibility remains that XTLA-1 negative peripheral T-cells may never have passed through the thymus, but may have matured *in situ* in response to factors, perhaps hormones, liberated from the thymus, or by other T-cells.

Nagata's initial studies of the tissue distribution of XTLA-1 positive lymphocytes, (Nagata, 1985), were carried out on cells from adult and larval J strain *Xenopus* and also on cells from adult K strain and A8 strain *X.laevis*, (A8xJ)$F_1$, (KxJ)$F_1$, and outbred *X.laevis*. In these studies, indirect immunofluorescent staining of cell suspensions was performed using XT-1 hybridoma culture supernatants and FITC-labelled anti-mouse immunoglobulin antibodies. The proportion of labelled cells was assessed by fluorescence-activated cell sorting, (FACS), analysis. Thymocyte suspensions from all of these animals were found to contain similar proportions, (92–95%), of XTLA-1 positive cells, with approximately equal intensity of fluorescent staining, implying that the density of XTLA-1 antigen per cell was similar. However, while 30± 3% of J strain splenocytes were XT-1 positive, A8 and K strain splenocytes showed no peak of labelled cells, indicating that peripheral T-cells of these animals did not express XTLA-1. Despite exhibiting significantly lower fluorescence intensity than J strain cells, splenocytes from (A8 x J)$F_1$ and (K x J)$F_1$ frogs comprised approximately 30% XTLA-1 positive cells.

In a more recent publication, (Nagata, 1988), reported on the use of high titre XT-1 ascites fluids and affinity purified IgG fractions of XT-1 in indirect immunofluorescent staining for FACS analysis. While the results previously obtained for adult J strain lymphocytes were confirmed, in terms of the proportion of XTLA-1 positive cells, splenocytes from K and A8 strain *X.laevis*, as well as those from hybrid, clonal(*X.laevis x X.gilli*)LG15, and *X.borealis* frogs labelled in this way were now found to contain 20-35% XTLA-1 positive lymphocytes. Splenocytes from early-thymectomised representatives of these animals were depleted of XT-1-labelled lymphocytes. Similar analyses of cells from the newt, (*Cynops pyrrhogaster*), the frog, (*Rana nigromaculata*), the toad, (*Bufo japonicus*), human, mouse and fish, (Japanese medaka), revealed that the XTLA-1 determinant was not expressed. Furthermore, immunoprecipitation of labelled cell surface moieties from radioiodinated thymocytes of J and K strain *X.laevis*, LG15 and *X.borealis* animals resulted in peptide bands with an identical molecular weight, (120 kD).

Nagata, (1988), therefore comments that "the use of culture supernatants could give misleading results". Although the initial fluorescence microscopy reported in this Chapter was performed on live cell suspensions indirectly labelled with XT-1 hybridoma supernatants, observations on identical cell suspensions labelled with XT-1 monoclonal antibody affinity purified from ascites fluid, (a gift from S.Nagata), were periodically made, to assess the effects of possible variations in antibody titre of supernatant batches. Furthermore, in subsequent experiments involving the use of a FACS machine, one stock preparation of XT-1 monoclonal antibody, affinity purified in our laboratory from pooled hybridoma supernatants, was used throughout to standardise the procedures.

Initial studies reported in this Chapter examine the expression, as assessed by fluorescence microscopy and FACS analyses, of XTLA-1 by thymocytes and splenocytes of a wide range of *Xenopus* strains and species held in our laboratory. This work was initiated prior to Nagata's recent studies, (1988), which suggested that XTLA-1 expression was common to the *Xenopus* species he tested, and which indicated that this surface marker was expressed by these species with a similar antigen density. The goal of the initial experiments reported here was to determine if a strain or species with a significantly and consistently different pattern of XTLA-1 expression could be identified. Animals of this type would be useful as donors or as thymectomised hosts in thymus implantation/reconstitution experiments. For example, one would then be able to trace the origins of T-cells appearing in such animals following implantation.

The second objective of the studies reported in this Chapter was to confirm that XTLA-1 positive lymphocytes were lost after early thymectomy achieved by microcautery. Standard procedures of early thymectomy operations differ slightly in both timing and technique between laboratories. For example, in our hands, early thymectomy is routinely performed at 7 days post-fertilisation, (Nieuwkoop and Faber stage 47/48), whereas in Nagata's studies, (1985, 1988), this operation was carried out at stage 46, (5 days post-fertilisation), after the method of Tochinai and Katagiri, (1975). Also, techniques of early thymectomy vary between establishments, since both direct surgical intervention, (Tochinai and Katagiri, 1975; Nagata, 1985; Nagata, 1988), and microcautery techniques, (Horton and Manning, 1972), are effective at removing thymic rudiments. Nagata and Cohen, (1983), thymectomised *Xenopus* larvae at both time points and by both techniques, and reported no difference in the immune response capacity of the resulting animals; we wished to confirm their functional results in terms of XT-1 determinant expression.

## 2.2 Materials and Methods

### 2.2.1 Animals and operations

#### 2.2.1.1 Animals

Outbred *X.laevis*, inbred MHC-compatible J strain *X.laevis*, (MHC = jj), interspecies hybrid (*X.laevis* x *X.gilli*) clones LG5, (MHC = bc), LG15 and LG17, (MHC = ac), interspecies hybrid (*X.laevis* x *X.muelleri*) LM3, (MHC = wy), outbred *X.borealis*, interspecies (*X.borealis* x *X.laevis* J strain) XbJ $F_1$ crosses and outbred *X.tropicalis* animals were used in these studies. All animals were bred and reared in this laboratory, at 22-24°C, as previously described in Horton and Manning, (1972), and Kobel and DuPasquier, (1975).

Animals were 3-20 months of age when examined, with the majority being 4-6 months of age.

#### 2.2.1.2 Early-thymectomy Operations

Early thymectomy operations were performed, by microcautery, on J, LG15, XbJ and LM3 tadpoles at 7 days of age, (stage 47-48 of Nieuwkoop and Faber), after the method of Horton & Manning, (1972). Additional outbred *X.laevis* tadpoles were thymectomised at 5.5 and 7.5 days of age for FACS analysis studies. Age-matched, control groups of outbred *X.laevis* tadpoles underwent sham thymectomy operations at either 5.5 or 7.5 days post-fertilisation. Thymic regions of early thymectomised animals were examined when these animals were sacrificed. If there was any suggestion of thymic regrowth, these animals were not included in experiments.

### 2.2.2 Antibodies

#### 2.2.2.1 XT-1 Hybridoma Supernatant

XT-1 hybridoma cells, a generous gift from S. Nagata, were cultured in RPMI medium, (Flow Labs.), containing 2mM L-glutamine, (Flow Labs.), 50$\mu$M 2-mercaptoethanol, (BDH Chemicals), 100 IU/ml Penicillin, (Flow Labs.), 100 $\mu$g/ml Streptomycin, (Flow Labs.), 2.5$\mu$g/ml Fungizone, (Flow Labs.), and 7% foetal calf serum, (Flow Labs.), in 50 ml plastic tissue culture flasks, (Falcon T.C. Plastics, Becton Dickinson). The flasks were maintained at 37°C, in an atmosphere of 5% $CO_2$ in air, in a humidified incubator. Every 3 days, when the hybridoma cells were dense, the cells were passaged and the culture supernatant collected, thus: following pipetting to remove any adherent cells from the sides of the flask, 1 ml of cell suspension was passaged into 9 mls of supplemented RPMI, in a fresh culture flask. The remaining 9 mls of cell suspension was aseptically transferred to a 10 ml sterile polystyrene centrifuge tube, (Sterilin Ltd.), and spun down at 300g for 10 minutes at 4°C. The XT-1 containing supernatant was decanted into a fresh tube,

and recentrifuged at 1,400g for 10 minutes to remove any debris. The supernatant was then either transferred to sterile polythene freezing vials, (NUNC, Gibco Ltd), and frozen at -20°C, or pooled together with several batches and stored in sterile glass bottles at 4°C with the addition of 0.1% sodium azide to inhibit microbial growth.

#### 2.2.2.2 Ammonium sulphate precipitation and Protein A affinity purification of XT-1 from hybridoma supernatant

In light of comments, (Nagata, 1988), that the use of XT-1 hybridoma supernatants in indirect immunofluorescence analyses could be misleading, it was decided to affinity purify a stock of XT-1 *monoclonal* antibody, (mAb), for future labelling studies. Rather than purify the $IgG2_b$ component of XT-1 ascites fluid, which may well contain irrelevant and possibly cross-reactive antibodies of the same mouse immunoglobulin subclass, it was decided to use hybridoma supernatant as the mAb source. Although our hybridoma supernatants contained foetal calf serum, (FCS), the concentration of protein-A binding IgG in FCS is usually negligible, (Goding 1986), and this is the only immunoglobulin contaminant in supernatants. Also, even in the unlikely case that low concentrations of cross-reactive calf antibodies were eluted from the Protein A- Sepharose column together with XT-1, the use in labelling studies of a secondary antibody specific for mouse immunoglobulin should obviate the detection of any spurious antibody specificities.

The method of choice for affinity purification of mouse $IgG2_b$ from hybridoma supernatants is affinity chromatography on Protein A-Sepharose, (Goding, 1986; Harlow and Lane, 1988). Protein A is a 42kD polypeptide, which is a normal constituent of the cell wall of *Staphylococcus aureus*, although *S.aureus* isolates are available which secrete rather than incorporate Protein A. Protein A binds IgG of a number of species with high affinity and specificity, (reviewed by Goding, 1978), although the strength of binding of different IgG subclasses varies in each species. When immobilised on a matrix such as agarose gel beads, Protein A may be used to isolate IgG by: (1) introducing the IgG-containing medium into a column of Protein A-agarose beads and allowing the immobilised Protein A to bind the IgG molecules by their Fc regions. (2) washing the column well to remove unbound contaminants. (3) eluting the bound IgG from the column in a small volume of buffer, thereby purifying, and often concentrating, the IgG antibody fraction.

Commercially available Protein A-agarose beads, (Protein A-Sepharose CL4B, Pharmacia LKB), have a binding capacity of approximately 20 mg IgG/ml swollen gel, (quoted for human IgG, in Affinity Chromatography: Principles and Methods, Pharmacia LKB Biotech). As hybridoma supernatants typically contain relatively low concentrations of antibody, (50-100 $\mu$g/ml, (Goding, 1986); 20-50 $\mu$g/ml, (Harlow and Lane, 1988)), in order to obtain a maximally concentrated purified antibody stock, it would be necessary to pass quite large volumes, (roughly between 200 and 1,000 ml), of supernatant through our 2 ml Protein A-Sepharose column. To circumvent the disadvantages inherent in loading large volumes of tissue culture supernatant, (long loading times with the possibilities of antibody

deterioration, and microbial contamination of supernatant and column), it was decided to preconcentrate the supernatant antibody fraction by ammonium sulphate precipitation.

The technique of ammonium sulphate precipitation of immunoglobulins is based on the observation that individual proteins are precipitated by different concentrations of this salt. Proteins in aqueous solution form hydrogen bonds with water molecules, through their exposed polar and ionic groups. Adding into the solution high concentrations of small, highly charged groups, such as ammonium or sulphate ions, interrupts the hydrogen bonding between protein groups and water, and results in much decreased protein solubility. The ammonium sulphate concentration required to precipitate a particular protein depends upon various parameters: (A) the number and position of polar groups within the protein, (B) the molecular weight of the protein, (C) the pH of the solution, and (D) the temperature at which the precipitation is performed, (Harlow and Lane, 1988).

Immunoglobulins are precipitated by lower ammonium sulphate concentrations than are most serum proteins, although the precise concentration required varies between species. For most applications it is sufficient to increase the ammonium sulphate concentration to 50% to precipitate out the immunoglobulin fraction, but it must be remembered that other proteins may co-precipitate, or be trapped within the large, flocculate immunoglobulin precipitate. Consequently, subsequent purifications steps are required to obtain a very clean immunoglobulin preparation.

### Ammonium sulphate precipitation

The precise method used was adapted from Weir, (1978):

1. A saturated solution of ammonium sulphate, (Sigma Chemicals), was prepared by addition of 1,000 g solid ammonium sulphate to 1 l double distilled water at room temperature. (Addition of measured amounts of solid ammonium sulphate to immunoglobulin-rich fluids, although eventually resulting in a homogeneous 50% solution, may give rise to transient, localised high salt concentrations, with resultant precipitation of lower molecular weight proteins). The solution was stirred for 2-3 hours, and then heated with stirring until all the solid dissolved. The hot solution was filtered into a clean glass vessel, (all glassware used in this preparation was thoroughly rinsed in double distilled water before use, as Goding, (1986), reports that traces of detergent on vessels used can interfere with immunoglobulin precipitation), and left to stand overnight at room temperature. Following overnight cooling, during which time excess ammonium sulphate precipitated out, the pH of the solution was adjusted to 7.0.

2. Saturated ammonium sulphate solution was added slowly, with constant stirring, over the course of $1\frac{1}{2}$ to 2 hours, to 1l pooled XT-1 hybridoma supernatant, (the product of several cell passages), containing 10 mM sodium azide, to give a 50% solution. This step was performed at room temperature. As the ammonium sulphate concentration reached approximately 40% a fine, 'milky' precipitate began to form, and this became more pronounced as the ammonium sulphate concentration increased.

3. The 1:1 saturated ammonium sulphate solution: supernatant mixture was then incubated overnight on ice, since immunoglobulins present in low concentration may take some time to precipitate, (Mishel & Shiigi, 1980), and finally was centrifuged for 20 minutes at 9,000 g.

4. The pellet of precipitate was washed twice in fresh 50% saturated ammonium sulphate solution to remove soluble contaminating proteins, then redissolved in a small volume, (approx. 5ml), sterile phosphate, buffered saline (PBS).

5. The redissolved fraction was dialyzed over 24 hours at 4°C, against 3 changes of 1l each sterile PBS, to remove excess salt. The dialysis tubing was boiled to remove plasticisers, (glycerin), treated with 0.01% EDTA-potassium salt for 10 minutes before use, (to leach out metallic ions), and then washed extensively in hot and then cold sterile distilled water, (Mishel & Shiigi, 1980). The redissolved immunoglobulin fraction was handled aseptically throughout this procedure to prevent microbial contamination, and potential proteolytic degradation. During the dialysis step the volume of the immunoglobulin fraction increased slightly.

6. 10 mM sodium azide was added to the dialysed preparation, and this was stored in sterile plastic freezing vials, (Sterilin Ltd.), at 4°C.

### Affinity purification on Protein A-Sepharose CL4B

For this step in the purification of an XT-1 monoclonal antibody stock preparation, a modification of the methods suggested by Ey et al., (1978), Goding, (1978), and MacKenzie et al., (1978), was used. These methods were originally devised to allow separation of IgG of different subclasses bound to the same Protein A-Sepharose column, by successive elution with buffers of decreasing pH. $IgG_1$, $IgG_{2a}$ and $IgG_{2b}$ were found by these authors to elute separately at pH 6.0, 4.5 and 3.5, respectively.

Although, given the relative crudity of the precipitation procedure, our redissolved ammonium sulphate precipitate of XT-1 hybridoma supernatant was potentially contaminated with serum proteins, mouse $IgG_{2b}$, (the XT-1 monoclonal antibody subclass), was the only Protein A- binding immunoglobulin expected to be present in appreciable quantities. Thus very nearly all immunoglobulin bound to the Protein A-Sepharose column was expected to elute from the column at pH 4.5. To check that this was, indeed, the case, a trial run was performed in which a small volume of redissolved ammonium sulphate precipitate was loaded onto the column and, after careful washing, pH 6.0, 4.0 and 3.0 buffers were successively applied. Only when the pH 4.0 buffer was applied was any measurable quantity of protein eluted from the column, as assessed by the absorption at 280 nm of the fractions collected. Since all unbound, non-immunoglobulin proteins were washed from the column prior to application of elution buffers, we were confident that a pure immunoglobulin preparation could be eluted from the column. Furthermore, since no immunoglobulins were found to be eluted at any pH other than that appropriate to $IgG_{2b}$, we could also be sure that any non-XT-1 immunoglobulin contamination was minimal. Thereafter, only immunoglobulins eluted by pH 4.0 buffer were collected.

**Preparation of buffers** Binding buffers, (1.5m glycine (Sigma Chemicals)/3M NaCl (BDH Chemicals), pH8.9); elution buffer, (0.1M citric acid (Sigma Chemicals), pH 4.0); and regeneration buffer, (0.1M citric acid (Sigma Chemicals) pH 3.0), were prepared in double distilled water. Very great care was taken to adjust the pH of these solutions accurately.

**Column preparation** 0.3g Protein A-Sepharose CL4B, (Sigma Chemicals), was weighed out, and gently suspended in 20ml binding buffer, including 0.02% sodium azide to deter microbial growth. This was left overnight at 4°C to allow the agarose beads to swell. The following day, the swollen Protein A-Sepharose beads were loaded under gravity, into a C10 glass column, (Pharmacia), and then slowly washed through with binding buffer until the beads were packed. A flow rate of 0.8ml/minute was provided by a peristaltic pump, (Pharmacia).

**Immunoglobulin separation** Following careful washing of the column with 10 bed volumes, (approximately 10 ml), of binding buffer, the redissolved ammonium sulphate precipitated XT-1 was loaded slowly onto the column. A flow rate of 0.8 ml/minute, provided by the peristaltic pump,

was used throughout the procedure. Once the sample had run down to the top of the Protein A-Sepharose bed, the column was refilled with fresh binding buffer, taking great care not to disturb the agarose matrix. 10 bed volumes of binding buffer were, next, run through the column to wash out all unbound, non-immunoglobulin protein. Purified XT-1 was washed from the column by addition of approximately 10 bed volumes of pH 4.0 elution buffer and small fractions, (0.8 ml/fraction, corresponding to one minute running time per fraction), were collected as this buffer was applied. All fractions were immediately adjusted to neutral pH by the addition of a few drops of 1M Tris base, (Sigma Chemicals), pH 8.4, and stored at 4°C.

**Column regeneration** Following elution of the $IgG_{2b}$ fraction, the Protein A - Sepharose column was washed with large volumes, (approximately 15 ml), of regeneration buffer to remove any traces of contaminating immunoglobulins. The column was then re-equilibrated with binding buffer, including 0.02% sodium azide, and stored at 4°C.

**Protein concentration estimation and pooling of fractions** The protein, (immunoglobulin), concentration of each fraction was estimated by the method of Warburg and Christian, (1942): fractions were placed into separate quartz glass cuvettes, and the optical densities, (O.D.s), of each fraction were measured at both 280 nm and 260 nm, against a blank of elution buffer. O.D. measurements were made using a Gilford U.V. spectrophotometer.

The ratio O.D. (280nm)/O.D.(260nm) was calculated for each fraction, this ratio was compared with a published standard curve to give an approximate correction factor, and the protein concentration of each factor was then calculated

from the formula:

protein concentration = O.D.(280nm) x Correction factor x 1/light path length

All protein was found to elute within the first five fractions collected. These fractions were pooled to give three preparations with the following estimated protein concentrations: Prep I : 0.09mg/ml, Prep II : 0.05 mg/ml, Prep III : 0.47 mg/ml. Affinity purified XT-1 Prep III was used for indirect immunofluorescent labelling for FACS analysis. 10mM sodium azide was added to these preparations, and they were stored at 4°C.

#### 2.2.2.3 Secondary antibodies

Several different commercially available fluorescein isothiocyanate (FITC) - labelled anti-mouse immunoglobulin preparations were used as secondary antibodies in the indirect immunofluorescence analyses reported later in this chapter. Each secondary antibody was initially tested at a variety of dilutions on samples from the same suspension of known XTLA-1 positive cells, previously incubated with XT-1 monoclonal antibody, to determine that which gave the most intense fluorescent staining. Secondary antibodies were used at concentrations slightly above those giving the brightest staining, as advised in Goding, (1986).

Furthermore, each secondary antibody was incubated with samples of similar cells which had not been preincubated with XT-1 monoclonal antibody, to assay for its non-specific binding to *Xenopus* cells. In initial tests, sheep anti-mouse immunoglobulins - FITC, (Sigma Chemicals), was found to show some degree of staining, (> 2%), in control preparations, (data not shown). As it was suspected that this might be due to Fc binding of labelled secondary antibody molecules by macrophages, etc., subsequent work reported in this Chapter was performed with FITC-labelled $F(ab')_2$ fragment anti-mouse immunoglobulin antibodies which, by fluorescence microscopy, failed to label > 2% of cells. Both primary and secondary antibodies were centrifuged for 10 minutes at 5,500 rpm before use to remove aggregates, ('deaggregation').

Despite these precautions, initial trials of FACS analysis still revealed some unacceptable background staining levels with sheep anti-mouse IgG $F(ab')_2$ - FITC, (Sigma, Cat. No. F2883). This problem was overcome in FACS experiments by preadsorbtion of the stock antibody preparation before use with 50$\mu$l/ml normal *Xenopus* serum, followed by careful deaggregation.

### 2.2.3 Indirect immunofluorescence studies

#### 2.2.3.1 Staining media

Two different media were used for the preparation of single cell suspensions, washing steps and dilution of antibodies.

For fluorescence microscopy studies the medium used was : Leibovitz L-15 medium,(Flow Labs), diluted to amphibian isotonicity, (60%), with double distilled water. To this was added 1% foetal calf serum to act as a source of irrelevant protein, 0.01M HEPES, (Flow Labs), to help maintain physiological pH throughout the staining procedure, and 0.1% sodium azide to discourage microbial growth and to inhibit cell metabolism, thereby helping to prevent 'capping' and subsequent endocytosis or shedding of labelled cell surface antigens.

For FACS analyses, the medium used, ('staining buffer'), was : amphibian-isotonicity phosphate buffered saline, (APBS), (6.6g NaCl, 1.5g $Na_2H\ PO_4$, 0.2g $KH_2PO_4$ per litre double distilled water, pH 7.4; Flajnik, 1983), with 0.1% bovine serum albumen, (Sigma Chemicals, Cat. No. A7906), as a source of irrelevant protein, and 0.1% sodium azide, as above.

#### 2.2.3.2 Preparation of thymocyte/splenocyte single cell suspensions

Spleens and/or thymi were removed from anaesthetised donor animals into individual 30 mm plastic petri dishes, (Sterilin Ltd.), each containing 2 ml staining medium. The organs were mechanically fragmented using fine watchmakers forceps and tungsten needles, and the fragments were then transferred, in suspension, to 4 ml plastic test tubes, (Sarstedt Ltd.). Each petri dish was washed with a further 1 ml staining medium by repeated pipetting to ensure that as many cells as possible were collected, and this additional 1 ml was added to the contents of the plastic test tube. The contents of the tube were vigorously pipetted to free as many cells as possible from the organ fragments, and then left to settle briefly to allow debris and cell clumps to fall out of suspension. The supernatant single cell suspension was then removed to a fresh plastic test tube, and the cells were washed by two cycles of centrifugation, (1,400 rpm 10 minutes), followed by resuspension in 2 ml fresh staining medium. Cell suspensions were counted in a haemocytometer, and the volumes were adjusted to give a concentration of $1 \times 10^6$ leukocytes/ml. Throughout this preparation process, cell suspensions were kept on ice.

#### 2.2.3.3 Indirect immunofluorescence staining for fluorescence microscopy

Each single cell suspension, prepared as above was divided into 'control' and 'experimental' aliquots, in 4 ml non-sterile plastic test tubes, to give between $0.5 \times 10^6$ and $2 \times 10^6$ cells per tube, (aliquots of less than $0.5 \times 10^6$ were found difficult to handle during the staining protocol). All tubes were centrifuged for 10 mins at 1,400 g to pellet the cells, and the supernatant medium was removed and discarded. 'Experimental' aliquots were resuspended in 0.3ml/tube XT-1 mAb, diluted in staining medium. Most labelling procedures were carried out using a 1:3 dilution of XT-1 hybridoma supernatant 'cocktail', (a mixture of several different supernatant batches, prepared to give a large amount of a standard supernatant preparation), but, for reasons outlined previously, later stainings involved the use of an affinity purified XT-1 mAb preparation. The proportions of XTLA-1 positive leukocytes in aliquots of cells from a single suspension, labelled with 1:3 supernatant cocktail or a 1:50 dilution of our own affinity purified XT-1 stock,

were compared with those labelled with a 1:100 dilution of affinity purified XT-1, (a gift from S. Nagata), and were found to be statistically comparable, (data not shown). Thus, the nature, (hybridoma cocktail or affinity purified preparation), of the XT-1 mAb used in each staining has not been shown in Tables 1–3.

'Control' aliquots were resuspended in either 0.3 ml/tube 1:3 GD5 hybridoma supernatant, (an anti-fungal monoclonal antibody, used as an irrelevant control antibody), or in staining medium alone. Control aliquots were always found to display a background, (< 2% positive cells), level of staining.

Following incubation for one hour on ice, all aliquots were washed by two cycles of centrifugation, (1,400rpm,10 mins), and resuspension in 2 ml fresh staining medium.

After the second wash, the supernatant medium was discarded, and the cell pellet was resuspended in 0.3 ml/tube secondary FITC-labelled antibody. Two different commercially available secondary antibodies were used, and each was assessed for lack of, (< 2%), non-specific binding to control preparations, and titred to find the appropriate dilution prior to experimental use. Secondary antibodies used were:

Sheep anti-mouse IgG $F(ab')_2$ - FITC, (Sigma Chemicals Cat. No. F2883).

Rabbit anti-mouse immunoglobulins $F(ab')_2$-FITC, (Dako Ltd. Cat. No. F313).

All tubes were protected as much as possible from light during, and subsequent to, secondary antibody incubation, to avoid 'bleaching' of the fluorochrome label.

Following incubation in secondary antibody for 1 hour on ice, aliquots were washed as after primary incubation, and then resuspended in 25–50 $\mu$l staining medium. Approximately 10$\mu$l cell suspension was placed on a clean glass microscope slide, covered with a clean, grade O coverslip and observed in the fluorescence microscope. The microscope used in these studies was a Nikon Optiphot fitted with an epifluorescence illumination system and a FITC filter set, (Nikon DM 510).

Cells to be counted were first observed by phase contrast microscopy under a x 40 objective, (Nikon x 40 Ph3 DL N.A. 0.85), to assess the condition of the cells, and then fluorescent cells in successive fields were counted under epifluorescent illumination at 495nm, and the proportions of positive and negative leukocytes was calculated. Between 100 and 650 cells/preparation were counted.

#### 2.2.3.4 Photography of labelled cell suspensions

Representative populations of labelled and control cells were photographed using phase-contrast and epifluorescence illumination systems. The camera system used, (Nikon Microflex UFX-II) was fitted to our Nikon Optiphot microscope. Phase-contrast photographs were automatically light metered by this system, with no exposure adjustment. For black and white photography, a green filter was introduced into the transmitted light path to enhance contrast. Fluorescence photographs were also automatically exposure metered, using the '–2 exposure ad-

justment' setting. For black and white photography, KODAK Technical Pan or Ilford TMAX film was used, rated at 400 ASA. Colour photographs were taken using FUJI 400 ASA transparency film.

#### 2.2.3.5 Indirect immunofluorescence staining for FACS analyses

Single cell suspensions were prepared as above. Each cell suspension was divided into aliquots to give, wherever cell numbers allowed, 3 aliquots of between $5 \times 10^5$ and $2 \times 10^6$ cells/tube, thus:

- Unstained Control was left completely unstained, to act as a control for cellular autofluorescence.
- Secondary Antibody Control was incubated with FITC-labelled antibody only, to act as a control for non-specific binding of the secondary antibody.
- Experimental - the experimental sample was incubated with XT-1 mAb, followed by incubation with the secondary antibody.

Samples from the same stock preparation of affinity purified XT-1, (prepared in our laboratory), were used throughout this series of experiments, diluted 1:50 in staining buffer.

The secondary antibody, rabbit anti-mouse immunoglobulins $F(ab')_2$ - FITC, (Dako Ltd. Cat. No. F313), was diluted 1:30 in staining buffer prior to incubation.

Cell suspensions were centrifuged, (1,400 rpm 10 mins), the supernatant staining buffer was removed, and the cell pellet was resuspended in 0.3 ml/tube primary antibody dilution. The cells were incubated in primary antibody for 45 minutes, on ice to prevent capping and subsequent endocytosis or shedding of labelled cell surface antigens. Washing between incubation steps was by two cycles of centrifugation, followed by resuspension in 2 ml/tube fresh staining buffer. Following this washing step, the cells were pelleted, (1,400rpm, 10 mins), the supernatant buffer was discarded, and the pellet was resuspended in 0.3 ml/tube secondary antibody dilution. During, and after, secondary antibody incubation, all preparations were protected from light to avoid 'bleaching' of the fluorochrome label. After a 45 minute incubation, on ice, the cell suspensions were washed as after primary antibody incubation, and finally resuspended in a volume of staining buffer adjusted to return the cell concentration to $1 \times 10^6$ cells/ml.

#### 2.2.3.6 FACS analysis of XT-1 labelled cells

Following indirect immunofluorescent labelling, cell suspensions were transported, on ice, to the Department of Surgery, Newcastle Medical School, for FACS analysis. Here, they were analysed, using an Argon ion laser with 400mW output at 488nm, on a FACS 420 fluorescence activated cell sorter, (Becton Dickinson), which has been upgraded to measure four parameters. The fluorescent channels

of this machine are fitted with either linear or log amplifiers, the latter being calibrated such that full scale, (256 channels), is equivalent to a fluorescent intensity range of 3 log decades.

As cells pass through the sorter, its integral computer collects and stores values of each of four different parameters for every 'event' or cell counted. The parameters measured are: low angle forward light scatter, (an indirect measurement of cell size), granularity, (correlated with the degree of right angle light scatter caused by the cell), and fluorescence intensity at each of two different wavelengths, (530 and 575 nm).

Cells were gated, using low-angle forward light scatter and granularity measurements, such that the majority of live leukocytes were analysed, whilst most red blood cells and thrombocytes were excluded. This was accomplished initially by separating *Xenopus* blood over a density gradient, to give one fraction comprising red cells and thrombocytes, (assessed from their morphology when viewed by phase contrast microscopy), and another containing other leukocyte types. Gating parameters were set to exclude the former and include the latter fractions. As non-viable cells appear 'smaller' than viable ones, in FACS terms, the viability of leukocytes falling within the established gating parameters was assessed by use of the fluorescent vital dye propidium iodide, (Fluka Chemie AG). Non-viable cells take up this dye very readily, while viable cells do not. When a suspension of cells incubated with propidium iodide was run through the sorter, very few brightly fluorescent cells were recorded, indicating that our gating parameters also tended to exclude non-viable cells from those counted, on the basis of low-angle forward light scatter, (see Appendix 1).

This gating was particularly important for analysis of splenocyte suspensions, which were not density-gradient separated prior to staining, and which, consequently, contained relatively high proportions of non-lymphoid cells.

Attempts were also made to correlate low-angle forward light scatter measurements with particular leukocyte populations, identified by both direct and indirect immunofluorescent labelling techniques. *Xenopus* B-cells were indirectly labelled with a monoclonal anti-*Xenopus* IgM immunoglobulin antibody, (8E4:57. A gift from R. Clothier, originally raised by Langeberg and Ruben), and the size and granularity of the resulting fluorescent population were examined. These parameters overlapped with those of the XTLA-1 positive population, and so morphological separation of lymphoid populations was not possible. Directly FITC-labelled anti-human leukocyte antibodies M1, M3 and $\alpha$-Leu, (anti-CD15, anti-CD14 and anti-CD45, respectively; Becton Dickinson), were tested for their cross-reactivity with *Xenopus laevis* splenocytes, in a further effort to differentiate particular leukocyte populations, with a view to gating out non-lymphoid cells on the basis of granularity and size. Unfortunately, the determinants recognised by these antibodies were not detectable on *Xenopus* cells, and so no positive discrimination of leukocyte populations could be made in this way.

For each sample analysed, 10,000 events were recorded. Statistical analysis of these data was performed, using the BDIS Consort 30-version F2/88 program, (Becton Dickinson), and colour graphic representations were generated using the

Lysis, (Becton Dickinson), program.

#### 2.2.3.7 Presentation of results of FACS analysis of FITC-labelled cells

These take the form of computer-generated histograms graphically portraying the relationship between cell numbers and log FITC fluorescence intensity. From the raw data collected, the BDIS Consort program assigns each cell counted to one of 256 channels, representing increasing log fluorescence intensity. (Log values are used to greatly extend the scale of fluorescence intensity measurable). Histograms are then generated, showing the number of cells assigned to each channel.

For each cell suspension, three samples are run through the sorter: firstly, a completely unstained preparation, to check for autofluorescence at the wavelength measured. Next, a secondary antibody control, to assay for non-specific binding, and to allow one to note the background staining level. Finally, the experimental preparation is counted. By inserting markers into successive histograms, it is possible to measure the proportion of fluorescent cells, (refer to Fig. 1): providing that no autofluorescence is found, and that there is little or no non-specific binding, the histograms generated from these two sets of data should very nearly coincide. Markers may be inserted into these graphs on either side of the peak of control histograms, conventionally within 2% of the total cell count, on either tail of the curve. Next, the original markers are inserted into the histogram of the data generated during analysis of the experimental sample. The BDIS consort program then calculates the percentage of the total number of cells which are assigned to channels between the second and third markers, i.e. the percentage of cells counted in the experimental sample which are more intensely fluorescent than those in the control samples. The mean fluorescence intensity value for the cells in each interval between two markers is also calculated. These individual means were further averaged to give a group mean. No further statistical analyses have been made of these data.

In some figures, (Figs. 7,8,9,12b,13b), in order more clearly to show the variation in fluorescence intensity observed in experimental, stained preparations, control histograms have been omitted from the graphs.

## 2.3 Results

### 2.3.1 Fluorescence microscopy analyses of XTLA-1 expression in control and thymectomised *Xenopus*

#### 2.3.1.1 Proportions of XTLA-1 positive cells

As shown in Tables 1 and 2, in the majority of *Xenopus* strains/species examined a large proportion, (76–88%), of thymocytes and a subpopulation, (14–34%), of splenocytes were positively stained by indirect immunofluorescence with XT-1 monoclonal antibody. LG17 control animals showed lower proportions of stained

cells, with just 61% thymocytes and only 4% splenocytes, (mean percentages), labelled. By contrast, none of the *X.tropicalis* animals examined, ($n$ = 8, age range 3–20 months post-fertilisation), possessed XTLA-1 bearing thymic or splenic lymphocytes which were detectable by this technique.

Single cell suspensions of splenocytes from early, (7 day), thymectomised J, XbJ, LG15 and LM3 *Xenopus* were also examined for the presence of XTLA-1 positive cells; these stainings were performed concurrently with those on age-matched non-thymectomised animals from the same strain/species group. Table 3 shows that splenocyte suspensions from these early thymectomised animals contained only 4–10% XT-1 positive cells. The proportion of stained cells was always much lower than that found in the non-thymectomised age and strain/ species matched group of animals.

#### 2.3.1.2 Appearance of XT-1 labelled cell suspensions

Positively-labelled thymocytes and splenocytes had a 'capped' or 'spotted' appearance under epifluorescent illumination, as described by Nagata, (1986b), (see Figure 2.A-C). It should be noted that this 'capping' effect was seen despite the fact that staining procedures took place in the presence of the metabolic inhibitor, sodium azide, and were performed on ice.

The appearance of positively-stained cells from different strain/species groups was similar, although 'caps' on LG17 clone cells tended to appear smaller and more delicate than those on cells from animals of other groups.

Labelled thymocytes were uniformly much more intensely fluorescent than labelled splenocytes. Furthermore, the relatively greater homogeneity of thymocyte suspensions made observation and enumeration of these cells quite straight forward; splenocyte suspensions contained varying proportions of cell types other than lymphocytes, (red blood cells and other non-lymphoid leukocytes), which, while not specifically stained by this protocol, did not facilitate observations. Splenocyte suspensions from thymectomised animals often contained large proportions of these irrelevant cell types.

#### 2.3.1.3 Statistical comparison of proportions of XT-1 labelled cells within and between control and thymectomised groups

Within groups, percentages of XT-1-labelled thymocytes and splenocytes varied quite widely, as indicated by the standard deviations of group means, (see Tables 1–3).

Statistical comparison of the group means of XT-1 labelled thymocytes and of XT-1 labelled splenocytes from different strain/species groups was made using the Kruskall-Wallis one-way analysis of variance test. This is a non-parametric statistical test, which does not rely upon the normal distribution of the groups of data compared. Given the small numbers of samples within each group, it is quite possible that these data are not normally distributed, and, in this case, parametric statistical analysis may give anomalous results. By the Kruskall-Wallis test,

comparison of the group means, including that of *X.tropicalis*, of XT-1 labelled thymocytes indicated a statistically significant difference between the groups. A similar analysis performed between the groups, with the exception of *X.tropicalis*, indicated that there was no statistically significant, ($p > 0.05$), difference between these data. That is, proportions of XT-1 labelled thymocytes do not vary significantly between the XTLA-1 expressing strains and species of *Xenopus* examined in this study.

Using the Kruskall-Wallis analysis, comparison of the group means, including that of *X.tropicalis*, of XT-1 labelled splenocytes again indicated a statistically significant difference between the control groups. A similar test performed between the groups, with the exception of *X.tropicalis*, showed that, by contrast with the thymocyte data, there were still significant, ($p < 0.01$), differences between these group means. In other words, differences in the proportions of XT-1 labelled splenocytes between the XTLA-1 expressing strains and species of *Xenopus* examined here <u>are</u> significant. The one-way analysis of variance does not allow one to identify which particular data vary significantly, hence a Duncan range analysis test was performed. This test can identify which data vary significantly from others analysed, but has the drawback, in this instance, that it is a parametric test. By this analysis it was suggested that the proportion of XTLA-1 positive splenocytes in LG17 controls was significantly, ($p < 0.05$), lower than that in all other groups examined, with the exception of LG15 and outbred *X.laevis*, while the group mean of *X.borealis* labelled splenocytes was significantly higher than that of all other groups, except XbJ and LG5 controls.

To compare the group means of XT-1 labelled splenocytes from control animals with those from thymectomised animals of the same strain or species, another non-parametric test, the Mann-Whitney test, was employed. Proportions of XT-1 labelled splenocytes from control and thymectomised J strain, and XbJ animals were significantly different, ($p < 0.01$), as were those from LG15 animals, ($p < 0.05$), whereas group means of control and thymectomised LM3 frogs were not statistically different. The latter finding may reflect the fact that control and thymectomised groups of LM3 frogs were particularly small, ($n = 5$ and $n = 3$, respectively), and the within-group variation was particularly wide in this strain.

By fluorescence microscopical analysis, the number of cells counted per animal was necessarily quite low, as each count took some time, and because the condition of the cell suspensions deteriorated with time, despite being kept on ice and in the dark. Also, although the proportions of XT-1-positive and -negative cells could be assessed by this method, no measure could realistically, repeatedly be made of the relative intensity of fluorescence of each cell. For these reasons, further analyses were performed using a fluorescence activated cell sorter, (FACS).

### 2.3.2 FACS analysis of XTLA-1 expression in control and thymectomised *Xenopus*

#### 2.3.2.1 Studies on control animals

As in the previous section, single cell suspensions of thymocytes and splenocytes from a number of different strains and species of *Xenopus* were studied. Due to the lack of availability of animals, not all groups examined in the previous section were re-studied by FACS analysis. Control, non-thymectomised animals investigated were from the following groups: outbred *X.laevis*, J strain inbred *X.laevis*, *X.borealis*, LG15, LG5 and *X. tropicalis*.

With the exception of *X.tropicalis* animals, all control individuals examined possessed significant numbers of XTLA-1 positive thymocytes and splenocytes, (see Figures 2–6). In these intact, control groups there was marked variation, both within and between groups, in terms of the proportion of positively stained thymocytes and splenocytes, (see Table 4). The group mean percentage of XT-1 labelled thymocytes varied between groups from 79%, (6.5 day sham thymectomised *X.laevis*, $n = 3$), to 91%, (*X.borealis*, $n = 3$). The group mean percentage of positively labelled splenocytes varied between groups from 8.5%,, (*X.borealis*, $n = 1$), to 24%, (7.5 day sham thymectomised *X.laevis*, $n = 5$).

Typical examples of FACS profiles obtained for thymocytes and splenocytes of these different groups are shown in Figures 2–6. Note also that the fluorescence intensity of labelled thymocytes is consistently greater than that of splenocytes from the same animal.

In terms of the relative fluorescence intensity of staining, (equivalent to the XTLA-1 antigen density), there were, again, marked variations within and between groups, in both thymocyte and splenocyte preparations, (see Figures 7 and 8). The significance of these variations in intensity is made more difficult to assess by the following considerations:

1. Although the antibodies used and the protocol followed, were identical in each staining experiment, the staining procedures were not all carried out simultaneously, therefore some day to day variation might be involved.

2. The FACS machine was not independently calibrated, (on fluorescent microbeads, for example), before each analysis was performed. However, proportions of positively stained cells, (and their relative fluorescence intensities), from similar animals analysed one after another on the same day were often markedly different, (see Figure 2.9, for example). When the first experimental, (fluorescent), sample analysed was reassessed at the end of a run of samples, to check for 'wobble' on the machine, this was not found to be appreciable, (see Figure 2.9A).

In contrast to the other intact *Xenopus* studied, in the five thymocyte and four splenocyte *X.tropicalis* preparations examined, only background levels of stained cells, (group means 1.4% and 0.6%, respectively) were found, (see Figures 10 and 11).

#### 2.3.2.2 Studies on the effect of thymectomy

In view of the equivocal results from fluorescence microscopical analyses of early, (7 day), thymectomised animals presented in the previous section, extensive FACS studies were carried out on early-thymectomised *X.laevis*.

The expression of XTLA-1 by splenocytes of early-thymectomised outbred *Xenopus laevis* was analysed, in comparison with that of similar cells from sham thymectomised, age-matched control sibling animals. Early thymectomy, both at 5.5 days and 7.5 days post-fertilisation, was found to reduce the proportions of detectable XTLA-1 expressing splenocytes to background levels, (2.3% and 2.1% for 5.5 day, ($n = 7$), and 7.5 day, ($n = 3$), early thymectomy, respectively). (See Figures 12 and 13). Sham thymectomised sibling control splenocytes were 14.3%, (5.5 day, $n = 4$), and 24.6%, (7.5 day, $n = 5$), positive.

Thus, in terms of the XTLA-1 T-cell surface marker, FACS analysis reveals that our thymectomy protocol is fully effective at depleting XT-1 positive T-cells.

## 2.4 Discussion

The data reported in this Chapter extend work previously reported, (Nagata, 1985; Nagata, 1988), on the species distribution of the XTLA-1 T-cell surface antigen, on the relative frequency of XTLA-1 expressing populations in the thymus and spleen and on the density of expression of XTLA-1 on cells of these populations. Furthermore, they allow comparison of two different early thymectomy techniques, in terms of the efficiency of depletion of detectable T-cell populations.

In his original publication on the subject, Nagata, (1985), reported that thymocytes of J strain, K strain and A8 strain inbred *X.laevis* all expressed the XTLA-1 antigen. However, at that time splenocytes of only J strain and hybrid $F_1$s, (where one parent was J strain), were said to be XTLA-1 positive. In later work, employing purified XT-1 monoclonal antibody rather than hybridoma culture supernatant, (Nagata, 1988), it emerged that, in fact, not only did a proportion of K and A8 strain *X.laevis* splenocytes express XTLA-1, but also that thymocytes and some splenocytes of LG15 and *X. borealis* animals were positive for this marker. We can confirm the results of Nagata's recent experiments, in terms of the presence of XTLA-1 antigen on the surface of lymphocytes of these *Xenopus*. Furthermore, it is revealed here that thymic and some splenic lymphocytes of outbred *X.laevis*, LM3, XbJ, LG17 and LG5 animals also express XTLA-1.

We are unable to confirm, from either our microscopy or our FACS analysis studies, Nagata's report, (1988), that the fluorescence intensity of positively-labelled cells, (and, hence the approximate number of determinants expressed per cell), is similar either within or between species/strain groups. In his publication, Nagata shows FACS-generated histograms of XT-1 labelled thymocytes and splenocytes from *X.laevis*, *X.borealis* and LG15 frogs. The peaks of labelled cells in all these histograms apparently coincide, although as the axes are unlabelled, it is impossible to confirm this impression. In our hands, microscopical observation

of labelled splenocytes was always found to be more difficult than similar observation of stained thymocytes, largely due to the generally much duller fluorescence observed on individual spleen cells. Furthermore, FACS analysis of thymocyte and splenocyte suspensions underlined the fact that, for suspensions of these two cell types, even from the same donor animal, the majority of labelled splenocytes were less intensely fluorescent than the majority of labelled thymocytes, i.e. the histogram peaks did not coincide. A lack of coincidence in intensity peaks is seen not only in the comparison between stained splenocytes and thymocytes, but also when comparing stained splenocyte preparations, (or stained thymocyte preparations), from different animals, both within and between strain/species groups.

The individual variation recorded in proportions of XTLA-1 positive thymocytes and splenocytes, and in density of antigen expression, even within groups of isogeneic, clonal animals may be due to technical variation. Studies of larger groups, particularly of isogeneic clonal animals, would help to resolve this issue. Also, calibration of the FACS machine would make comparison between different staining experiments easier. This phenomenon is also worthy of further investigation in terms of the animals' general state of immune responsiveness, comparing this with their XTLA-1 expression. As XTLA-1 is apparently not a pan T-cell marker, it may be restricted to particular functional T-cell subsets. Nagata's studies, (Nagata, 1986a), showed that enrichment of XT-1 positive lymphocytes lead to augmentation of responses to PHA and to allogeneic cells in MLR, but not to increased response to ConA, suggesting that XT-1 positive T-cells are functionally different from XT-1 negative T-cells. Although all animals used in our studies were apparently healthy, variations in XT-1 determinant expression may reflect their immune responses to unknown environmental factors. Investigation of XTLA-1 expression by peripheral lymphocytes of animals responding to known immunological stimuli, (such as skin allografts or xenografts, T-dependent antigens, etc.), may cast some light on this matter.

FACS analysis of splenocyte populations from *Xenopus* thymectomised by microcautery at 5.5 and 7.5 days post-fertilisation underlines the fact that this procedure is as effective at ablating detectable XT-1 positive T-cells as the surgical technique devised by Tochinai and Katagiri, (1975). Nagata and Cohen, (1983), investigated the immune response capacity, (skin allograft rejection), of J strain animals which had been thymectomised at 4–7 days post fertilisation by either surgical or microcautery techniques. They found no difference in the rejection capacities of these animals, and also concluded that such technical differences were not influential.

In the present study it is not clear why splenocytes from thymectomised animals were seen, by fluorescence microscopy, to contain XTLA-1 positive cells, albeit only a low proportion of labelled cells. Although the fluorescence microscopy studies were generally carried out using hybridoma supernatants rather than purified XT-1, as stated in the introduction to this Chapter, periodical checks were made with an affinity purified XT-1 mAb to confirm the reliability of supernatant staining. What is more, Nagata's, (1988), comments suggested that, if anything, the use of hybridoma supernatants might lead to an underestimate of XTLA-1 expression, rather than to spuriously high counts. A possible explanation for

the unexpectedly high proportion of labelled cells observed in the splenocytes of thymectomised animals by fluorescence microscopy, but not by FACS analysis, relates to the FACS gating procedure described earlier, (Section 2.3.1.6). If the labelled cells observed by fluorescence microscopy in thymectomised splenocyte populations were not lymphocytes, and were somehow specifically or non-specifically bound by XT-1, then these cells, having different low-angle forward light scatter and granularity properties from those of lymphocytes, may be gated out of FACS analyses. Certainly for the analysis of splenocyte populations with this antibody, the use of FACS techniques and high titre antibodies has proved to be more reliable.

One of the main aims of the work in this Chapter, was to identify a strain or species of *Xenopus* with a significantly and consistently different pattern of XTLA-1 expression from other *Xenopus*. This aim was achieved with the finding that *X.tropicalis* animals have no XTLA-1 positive lymphocytes, as detectable by indirect immunofluorescence analysis. It will be important to clarify whether lack of detectable XTLA-1 expression in *X.tropicalis* is due to total non-expression of this antigen, or to masking of its expression. Immunoprecipitation experiments using the XT-1 monoclonal antibody should finally resolve this question.

The putative absence of the XTLA-1 antigen by *Xenopus tropicalis* thymocytes and splenocytes is interesting from a number of aspects. *X.tropicalis* is the most primitive of the various extant *Xenopus* species, dating back to the Palaeocene, (Estes, 1975 cited in Kobel and Du Pasquier, 1986). This species has only 20 chromosomes, compared with the 36 chromosomes of the other strains and species examined in this study. As these other species are thought to have come about by allopolyploidisation from original, diploid species, (Kobel and Du Pasquier, 1986), then presumably *X.tropicalis* is the present day descendent of a non-XTLA-1-expressing ancestor species.

Since the functional relevance of XTLA-1 is not yet known, it would perhaps be enlightening to compare T-dependent immune responses of *X.tropicalis* with those of species which do express this surface antigen. Some preliminary studies investigating several immune responses of *X.tropicalis*, shown to be T-dependent in other *Xenopus* species have now been made, and are presented in Chapter 5.

With regard to experiments designed to investigate the effects of thymus implantation into early-thymectomised animals, (in terms of differentiation and expansion of host T-cell populations demonstrable with XT-1 labelling within a xenogeneic environment, and the appearance of these cells in the peripheral lymphoid organs), *X.tropicalis* are useful thymus donor animals. Experiments using this model system are reported in the next two Chapters.

In Chapter 3 the histological distribution of XTLA-1 positive lymphocytes within the lymphoid tissues of normal adult and larval *Xenopus* is examined. Furthermore, the differentiation of XTLA-1 positive T-cells in early-thymectomised *X.borealis*, following their implantation with *X.tropicalis* thymi, is described.

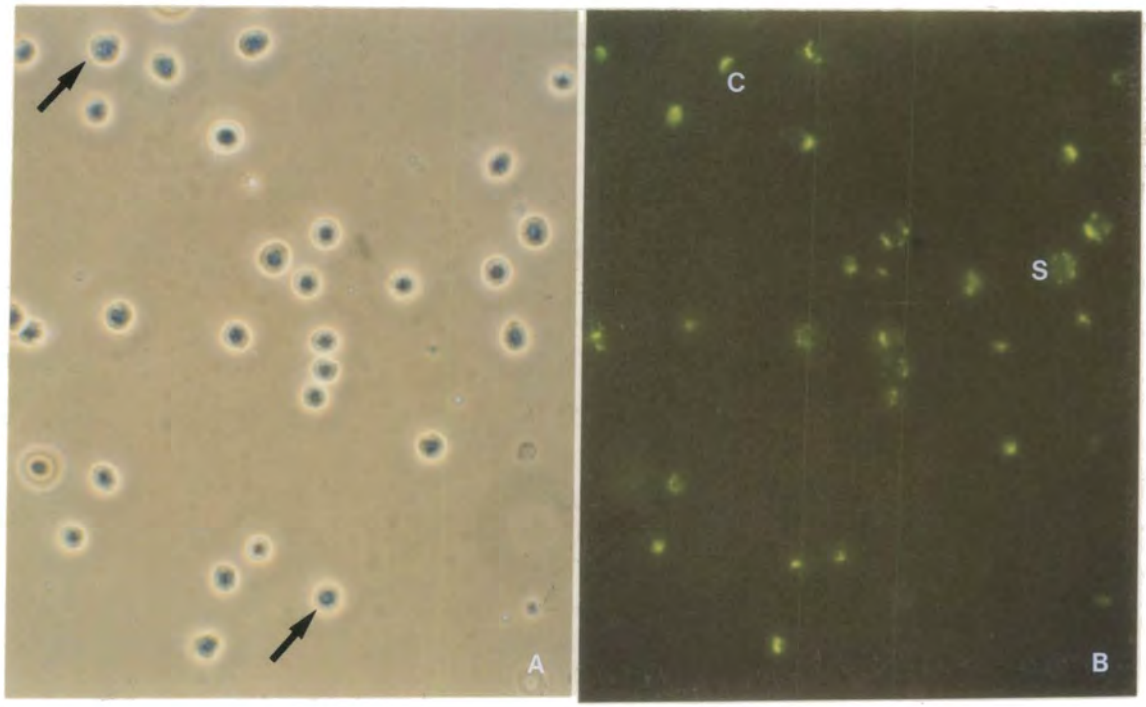
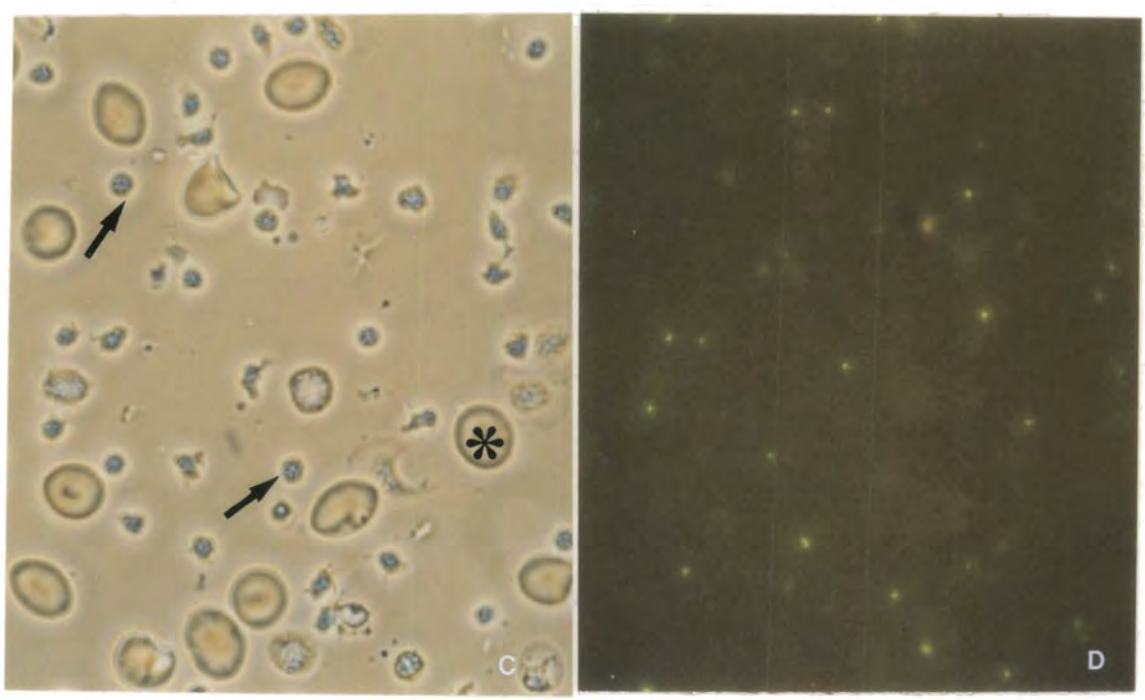



**Figure 2A** XT-1 labelled X.borealis lymphocytes. Phase contrast and fluorescence views of thymocytes, (a and b), and splenocytes, (c and d), indirectly immunofluorescently labelled with XT-1 mAb and secondary antibody, showing 'capped', (c), or 'spotted', (s), appearance. Note brighter fluorescence of thymocytes. (arrows = unlabelled lymphocytes, * = red blood cells). x360.



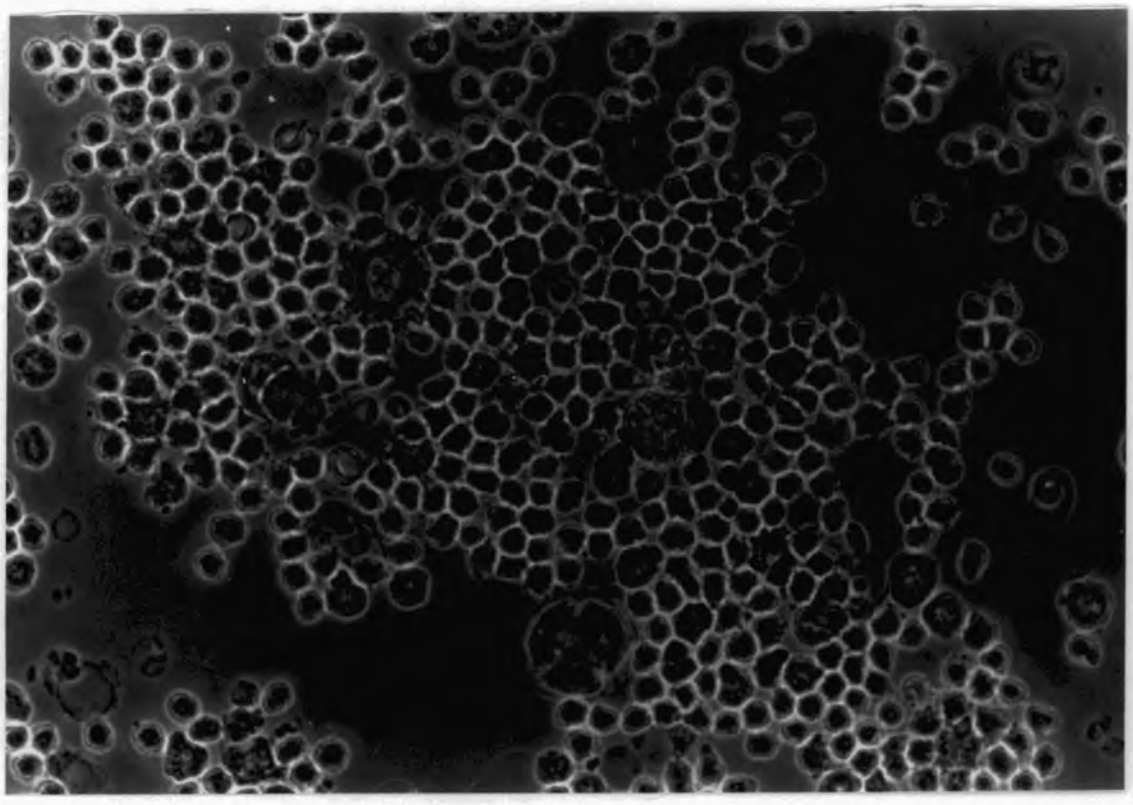
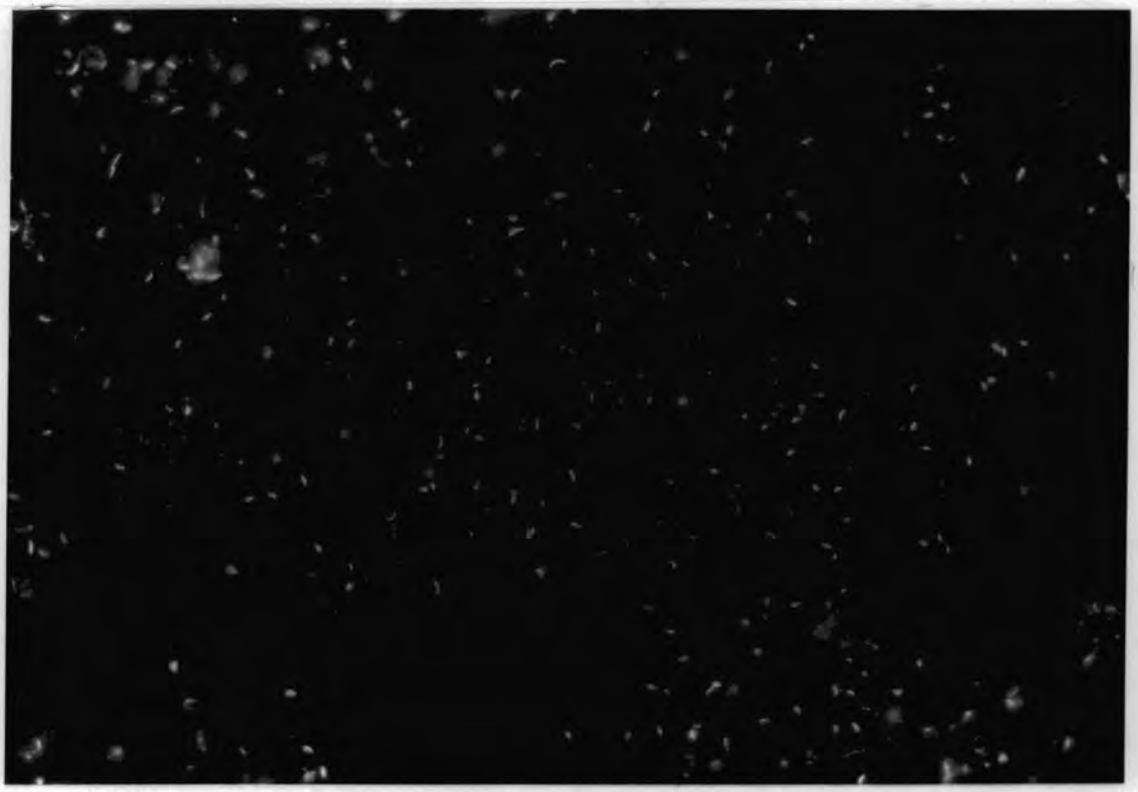


**Figure 2.B** XT-1 labelled LG5 thymocytes, Phase contrast and fluorescence views. Note large, XTLA-1 -ve non-lymphoid cells. x430



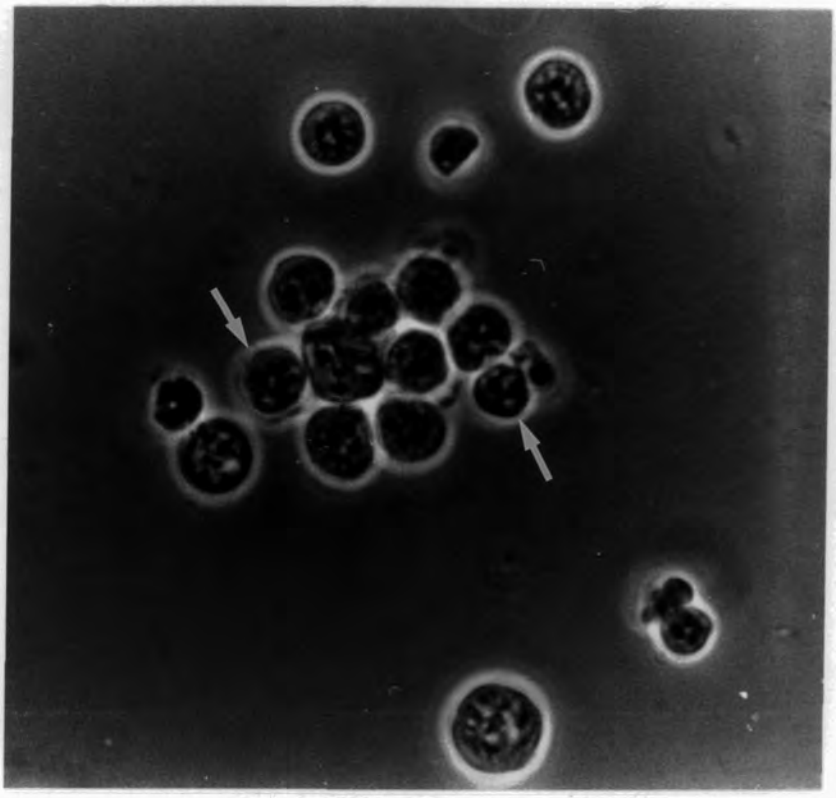


**Figure 2.C** XT-1 labelled LM3 splenocytes. Phase contrast and fluorescence views. (arrows = unlabelled lymphocytes). x1,100

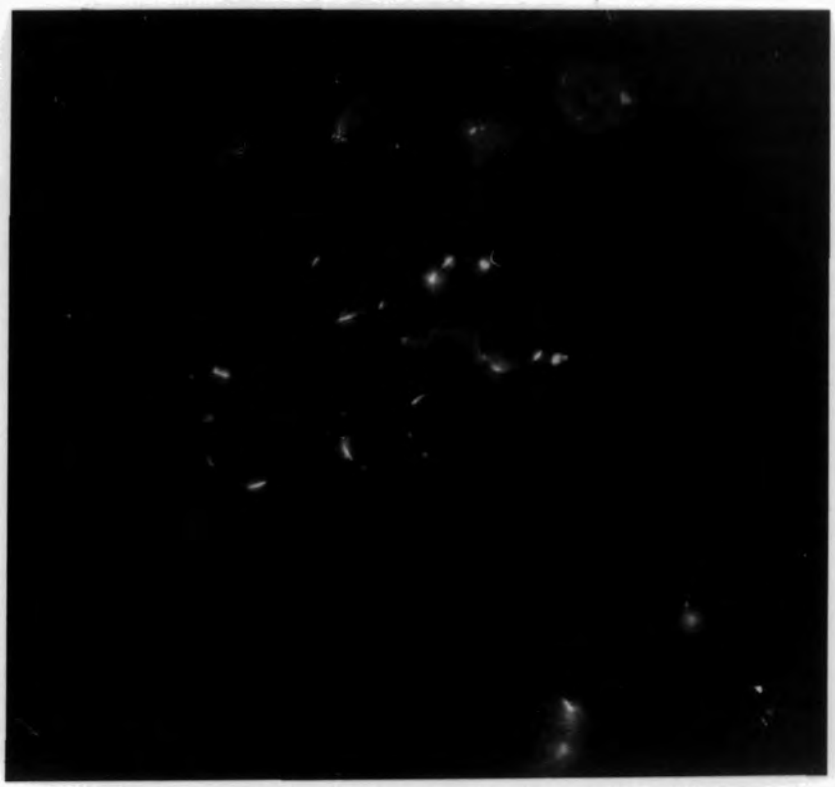
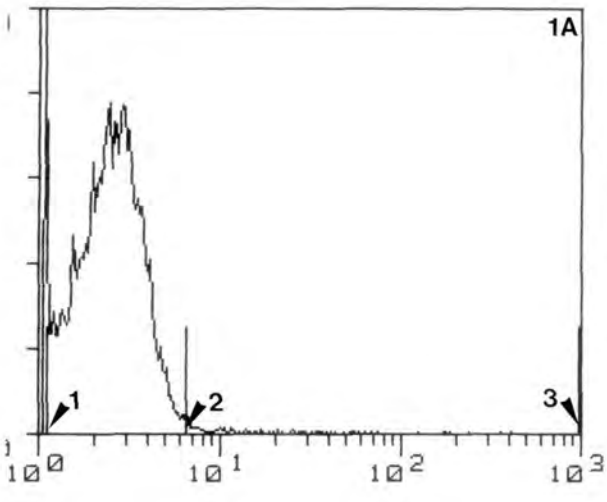


**Figure 2.1, A to D** Statistical analysis of FACS-generated data by the BDIS Consort program and graphic representations generated by the LYSIS program. Figure 2.1A shows fluorescence intensity histogram of unstained, control preparation, showing markers, (arrowed and numbered), inserted. Fig. 2.1B shows fluorescence intensity histograms of secondary antibody control preparation, following insertion of identical markers. Note coincidence of two control peaks. Figure 2.1C shows fluorescence intensity histogram of 'experimental' preparation, including the markers; in this case, 94.3% of cells counted fell between markers 2 and 3, ie were fluorescent compared to control preparations. The mean fluorescence intensity value for this 'experimental' peak was 43.2. Figure 2.1D shows fluorescence intensity histograms from 2.1A, 2.1B and 2.1C superimposed, one on another, (green = unstained control, blue = secondary antibody control, red = 'experimental preparation), in a diagram generated by the LYSIS program. (X-axis = log fluorescence intensity, (530 nm), Y-axis = relative cell number, (10,000 events / sample)).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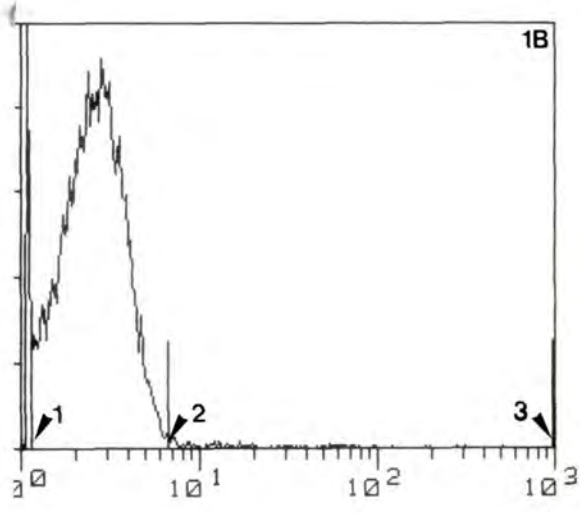
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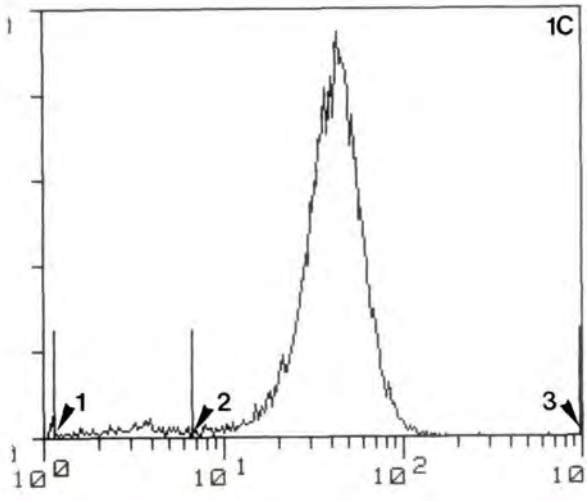
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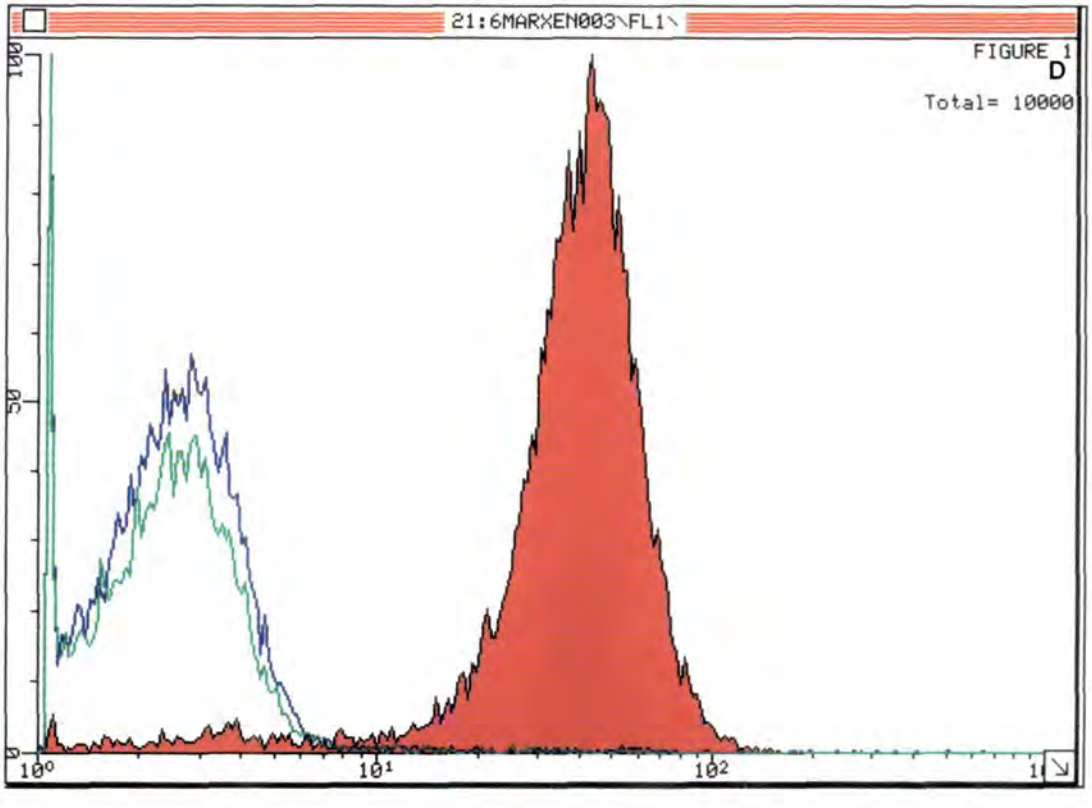
10 3


21:6MARXEN003\FL1\
FIGURE 1
D
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0
10⁰
10¹
10²

**Figures 2.2-2.6** are representative FACS-generated fluorescence intensity histograms of thymocytes and splenocytes from individuals of different strains / species of *Xenopus*.Green = unstained control preparation; blue = secondary antibody control preparation; red = 'experimental', (XT-1 + secondary antibody), preparation. X-axis = log fluorescence intensity, (530 nm), Y- axis = relative cell number, (10,000 events / sample).

Note that the majority of thymocytes, and a proportion of splenocytes, express XTLA-1, although the density of this antigen, related to the fluorescence intensity, is variable, (note position of peak of fluorescent cells along log scale of X-axis). Note, also, that the fluorescence intensity of labelled thymocytes is consistently greater than that of splenocytes from the same animal.

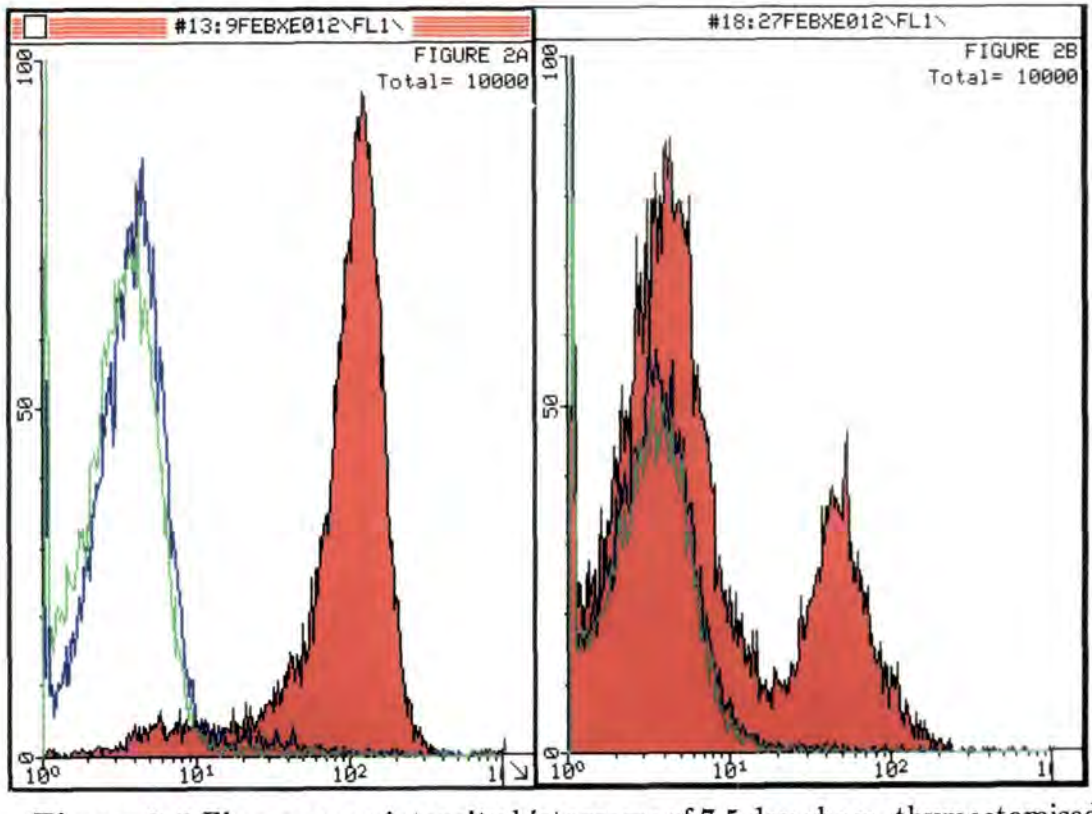



**Figure 2.2** Fluorescence intensity histograms of 7.5 day sham- thymectomised, outbred *X.laevis* thymocytes, (2A), and splenocytes, (2B).

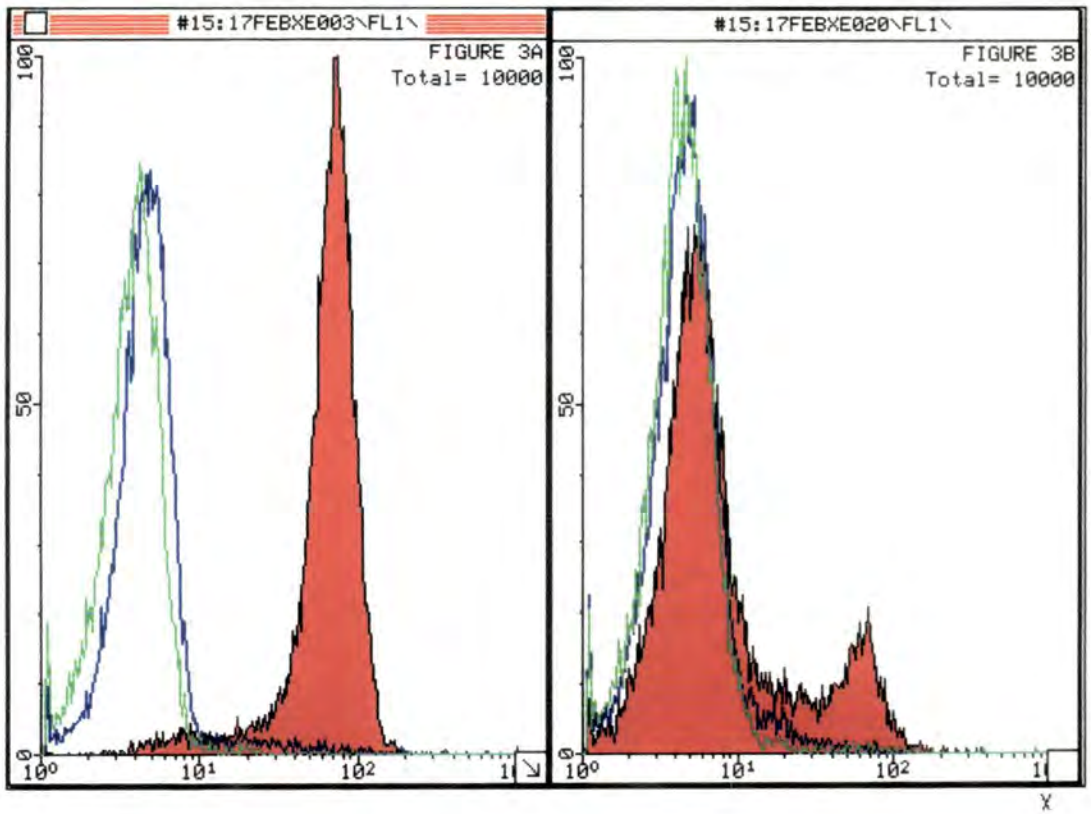



**Figure 2.3** Fluorescence intensity histograms of J strain *X.laevis* thymocytes, (2.3A), and splenocytes, (2.3B).

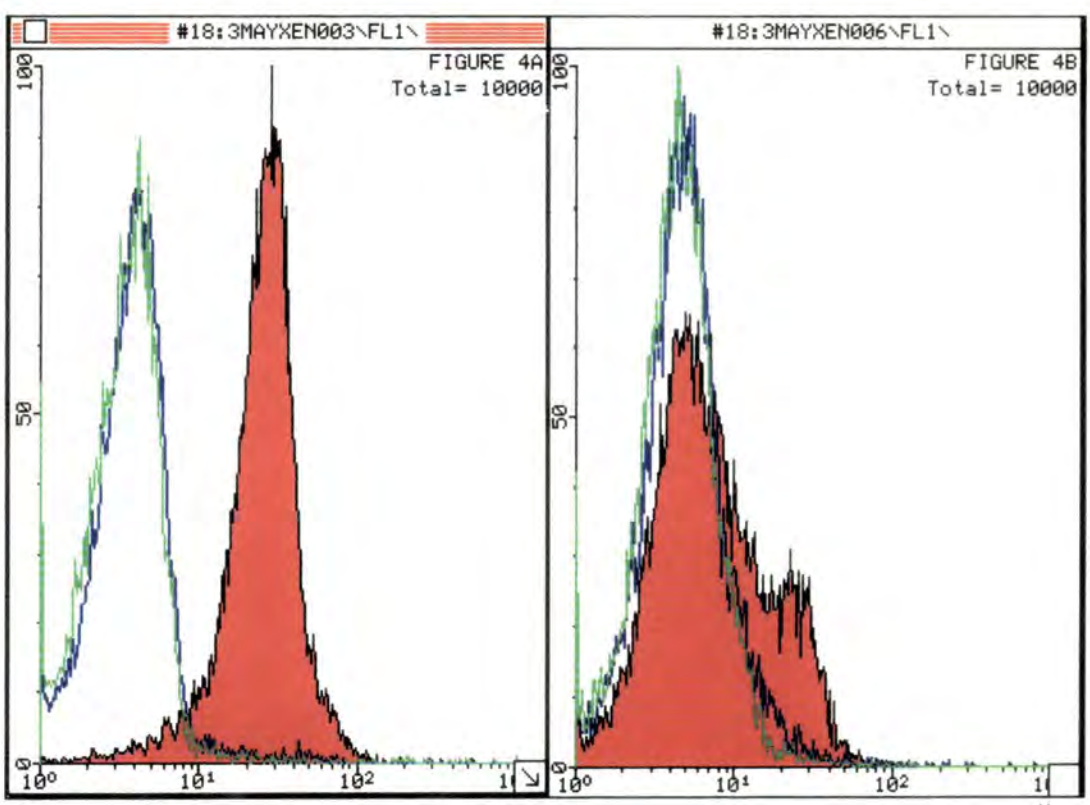



**Figure 2.4** Fluorescence intensity histograms of X.borealis thymocytes, (2.4A), and splenocytes, (2.4B).

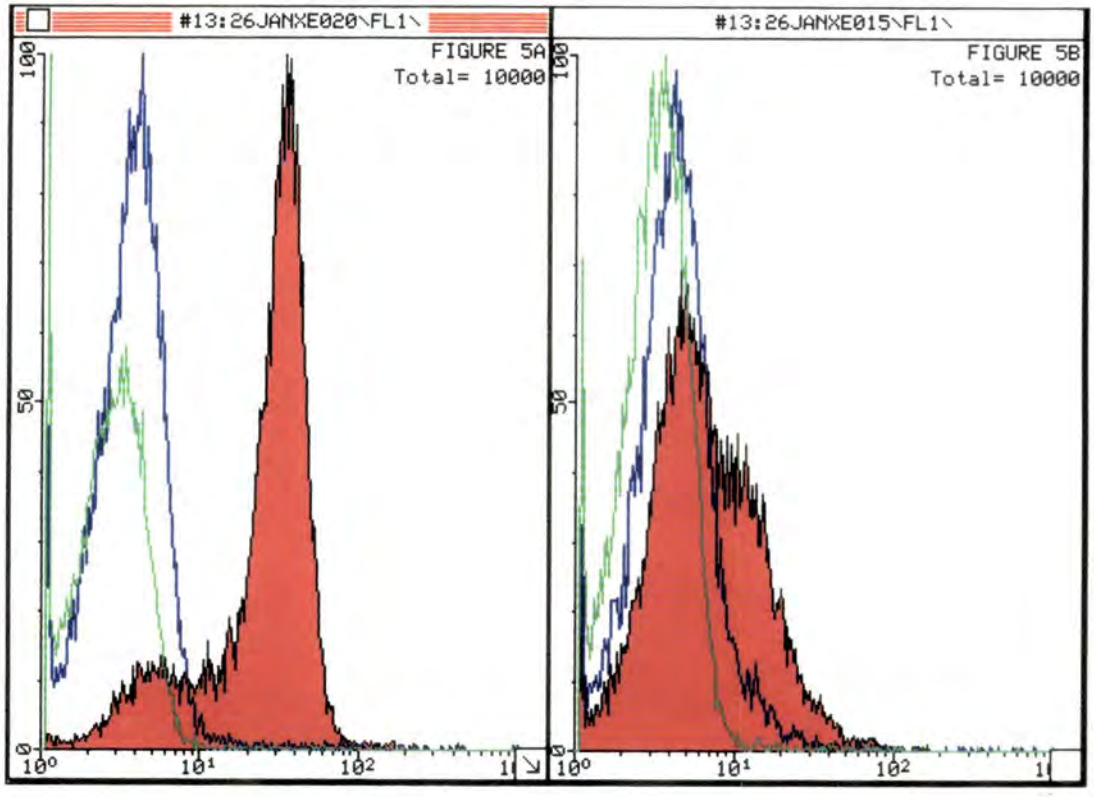



**Figure 2.5** Fluorescence intensity histograms of LG15 thymocytes, (2.5A), and splenocytes, (2.5B).

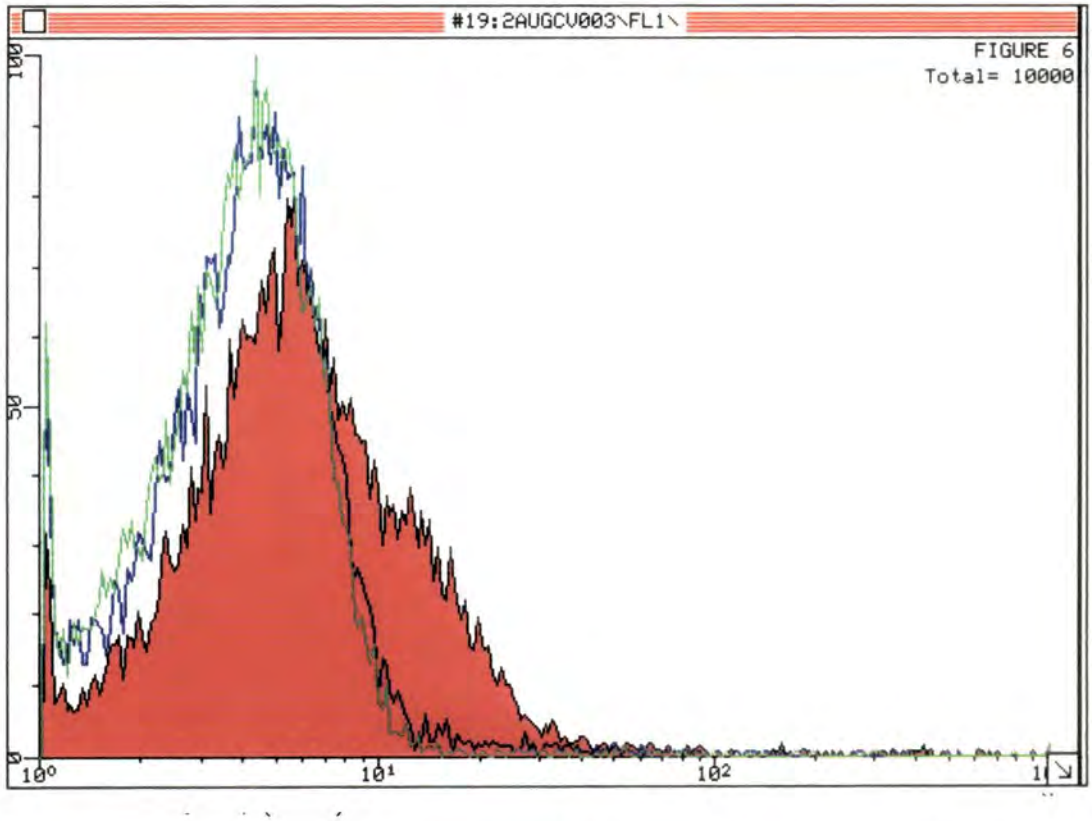
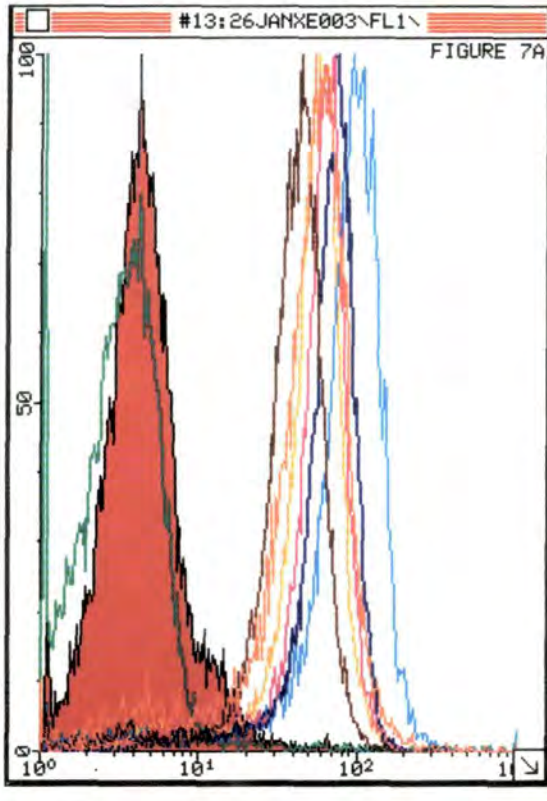
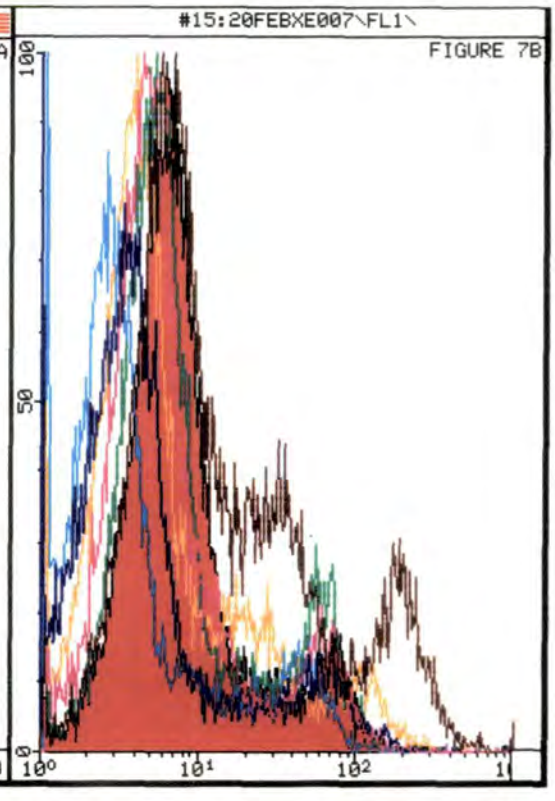
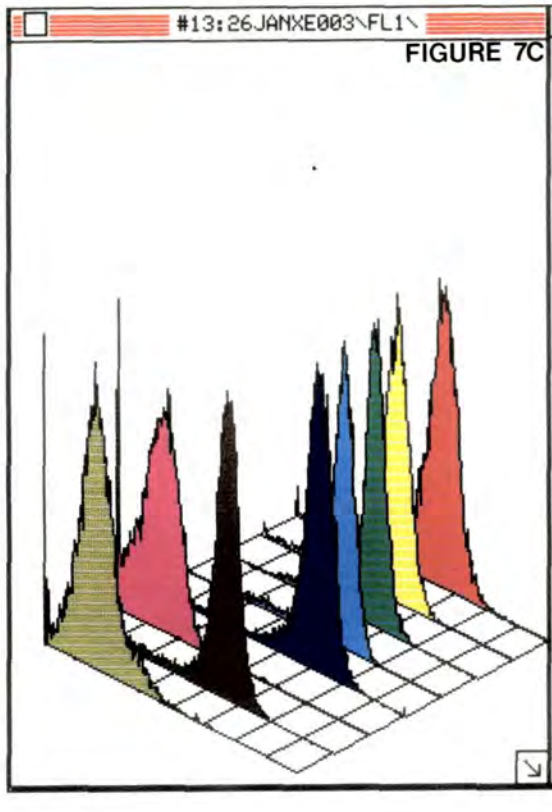



**Figure 2.6** Fluorescence intensity histogram of LG5 splenocytes.

**Figure 2.7** Variation in intensity of fluorescence is observed within strain / species groups. Histograms of 'experimental'; (X T-1 mAb + secondary antibody), preparations of J strain *X.laevis* thymocytes, (2.7A), and splenocytes, (2.7B). Note that peaks of fluorescent cells do not coincide. Figures 2.7C and 2.7D are 3-dimensional representations of above thymocyte, (2.7C), and splenocyte, (2.7D), histograms, X-axis = log fluorescence intensity, (530nm), Y-axis = relative cell number, (10,000 events / sample). NB. Colours used for individual histograms in Figures 7A&7B are not the same as those used in Figures 7C&7D.


#13:26JANXE003\FL1\
FIGURE 7A
100
50
0
10⁰
10¹
10²


#15:20FEBXE007\FL1\
FIGURE 7B
100
50
0
10⁰
10¹
10²


#13:26JANXE003\FL1\
FIGURE 7C

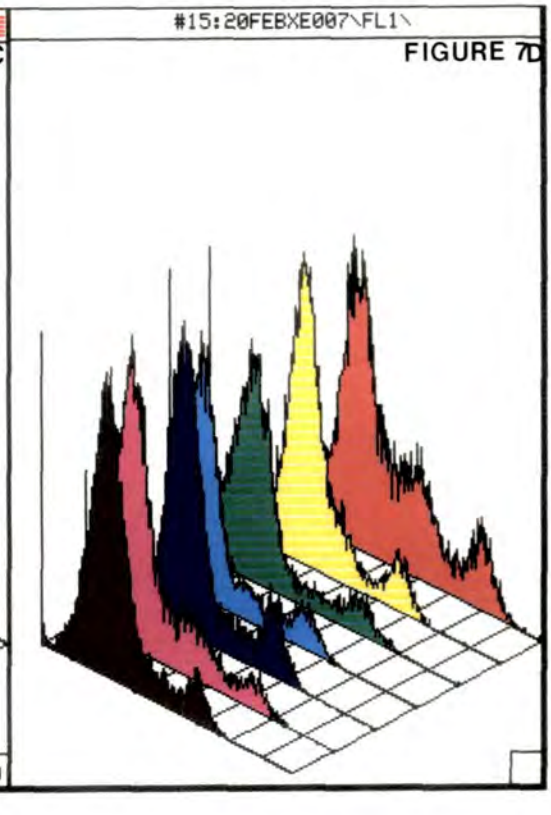
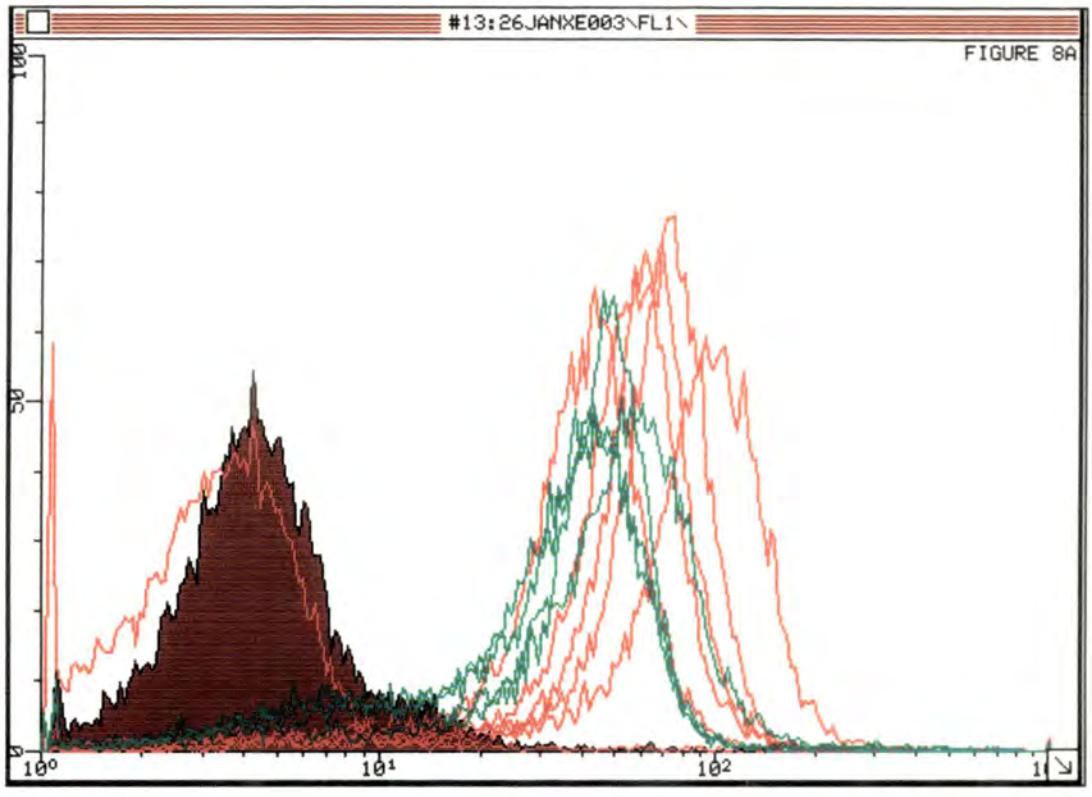

#15:20FEBXE007\FL1\
FIGURE 7D

**Figure 2.8A** Variation in intensity of fluorescence is observed both within and between strain / species groups. Histograms of 'experimental', (XT-1 mAb + secondary antibody), preparations of J strain *X.laevis*, (dark red / red), and outbred *X.laevis*, (green), thymocytes to show lack of coincidence of peaks of fluorescent cells both within and between strain / species groups. X-axis = log fluorescence intensity, (530 nm), Y-axis = relative cell number, (10,000 events / sample).

**Figure 2.8B** 3-dimensional representation of histograms of 'experimental', (XT-1 mAb + secondary antibody), preparations of J strain *X.laevis*, (red) and outbred *X.laevis*, (green), splenocytes. Note lack of coincidence of peaks of fluorescent cells both within and between strain / species groups. X-axis = log fluorescence intensity, (530 nm), Y-axis = relative cell number, (10,000 events / sample).


#13:26JANXE003\FL1\
FIGURE 8A
100
50
0
10⁰
10¹
10²

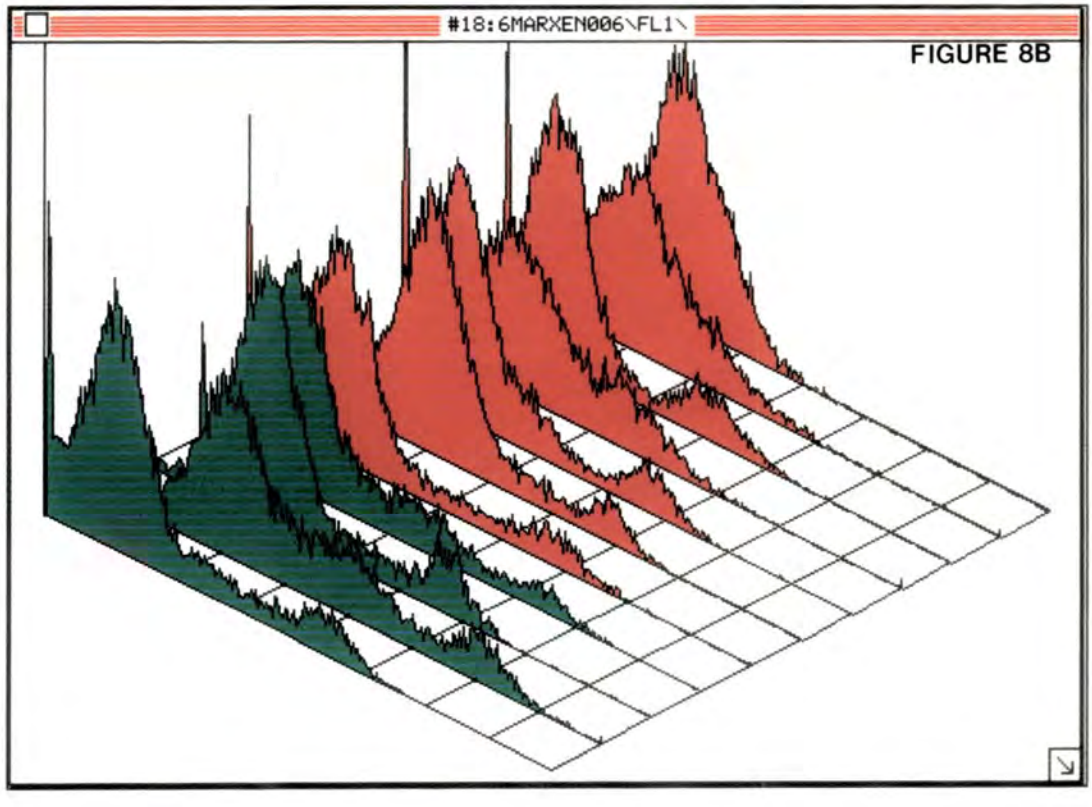
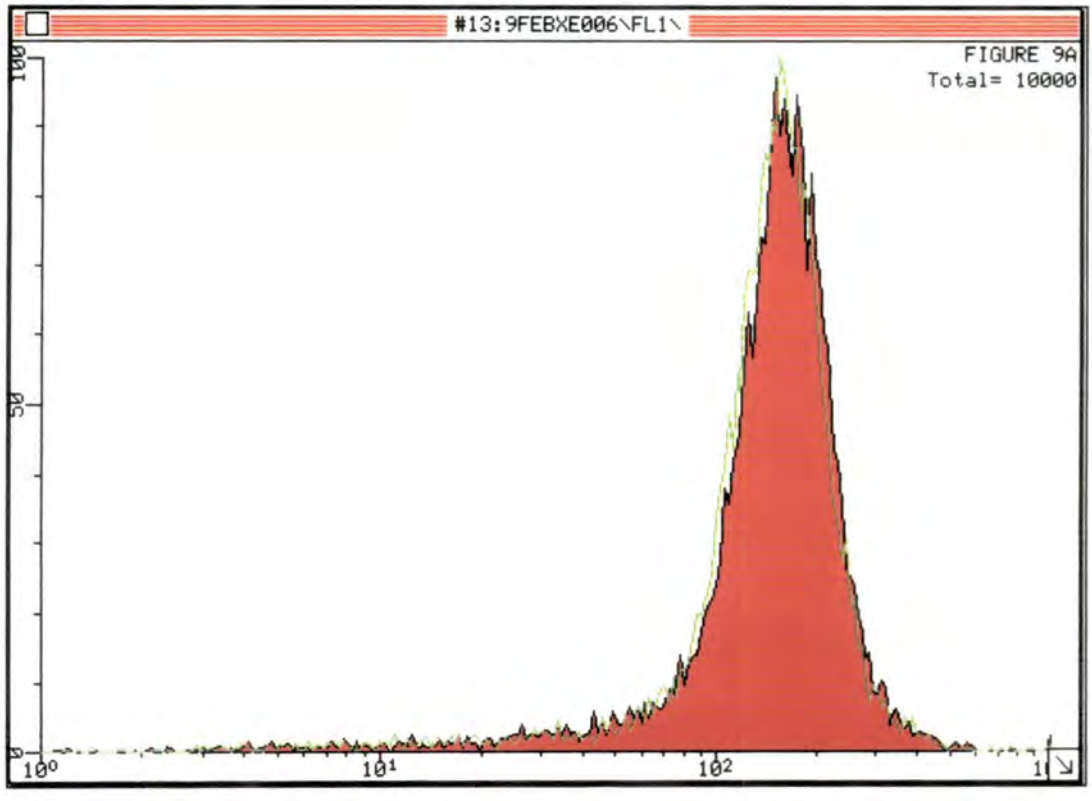

#18:6MARXEN006\FL1\
FIGURE 8B

**Figure 2.9A** Variations in intensity of fluorescent labelling are unlikely to be due to drift in the settings of the FACS machine. Fluorescence intensity histograms of the same 'experimental'(XT-1 mAb + secondary antibody), preparation of J strain thymocytes analysed at the beginning, (red), and end, (green), of a run of samples. Note the coincidence of the histograms. X-axis = log fluorescence intensity, (530 nm), Y-axis = relative cell number, (10,000 events / sample).

**Figures 2.9B and 2.9C** Variations in fluorescence intensity seem unlikely to be due to day to day inconsistencies in the staining protocol. Histograms of thymocytes, (2.9B), and splenocytes, (2.9C), from 3 sham- thymectomised outbred *X.laevis*. These 'experimental', (XT-1 mAb + secondary antibody), preparations were antibody-labelled and analysed on the same day, but show differences in fluorescence intensity. X-axis = log fluorescence intensity, (530 nm), Y-axis = relative cell number, (10,000 events / sample).


#13:9FEBXE006\FL1\
FIGURE 9A
Total= 10000
100
50
0
10⁰
10¹
10²
1

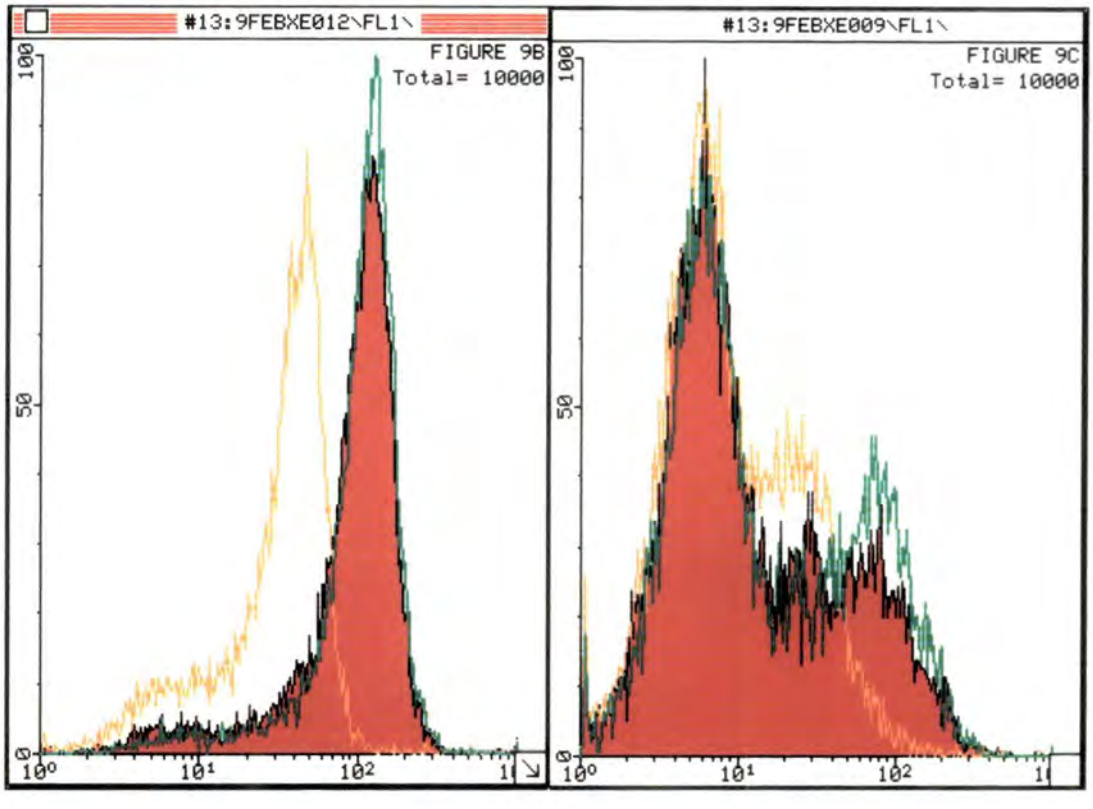
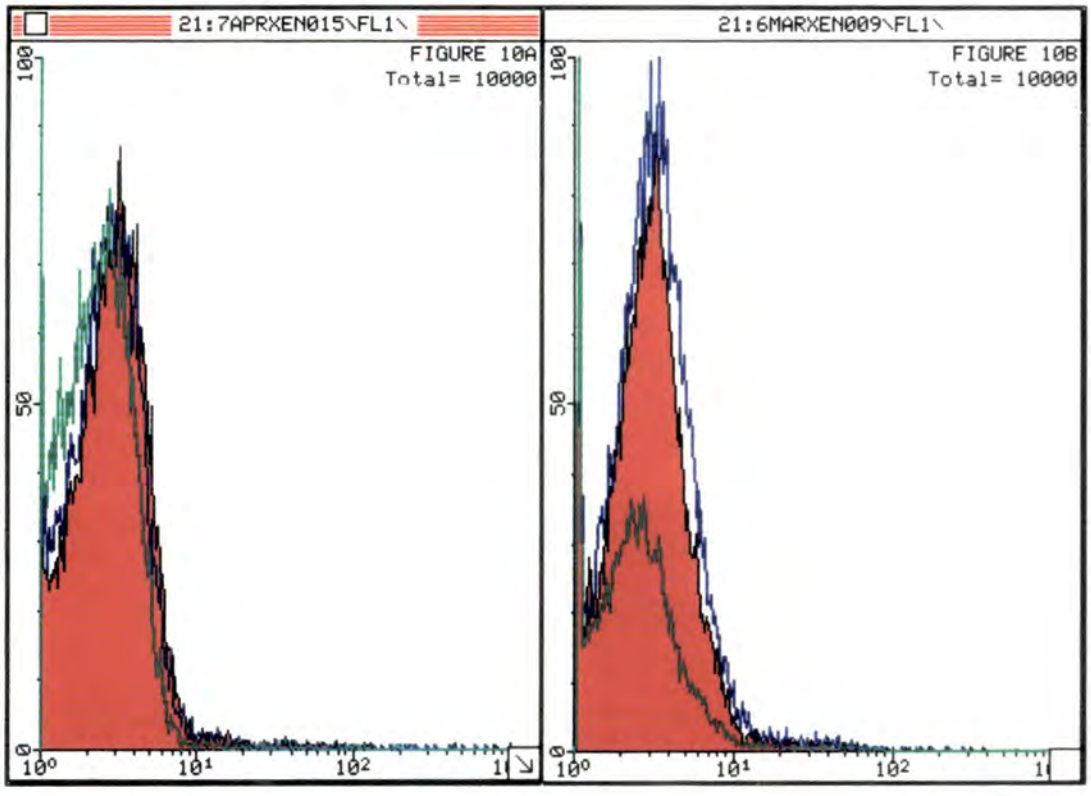

#13:9FEBXE012\FL1\
FIGURE 9B
Total= 10000
#13:9FEBXE009\FL1\
FIGURE 9C
Total= 10000
100
50
0
10⁰
10¹
10²
1

**Figure 2.10** *X.tropicalis* lymphocytes do not express detectable levels of XTLA-1 antigen. Fluorescence intensity histograms of thymocytes, (2.10A), and splenocytes, (2.10B). No peak of positively-labelled cells is seen. Unstained control preparation = green; secondary antibody control preparation = blue; 'experimental', (XT-1 mAb + secondary antibody), preparation = red. X-axis = log fluorescence intensity, (530 nm), Y- axis = relative cell number, (10,000 events / sample).

**Figure 2.11** No significant levels of XTLA-1 positive *X.tropicalis* lymphocytes were found in any 'experimental', (XT-1 mAb + secondary antibody), preparations examined. Fluorescence intensity histograms of several 'experimental' preparations of thymocytes, (2.11A), and splenocytes, (2.11B). X-axis = log fluorescence intensity, (530 nm), Y-axis = relative cell number, (10,000 events / sample).


21:7APRXEN015\FL1\
FIGURE 10A
Total= 10000
21:6MARXEN009\FL1\
FIGURE 10B
Total= 10000

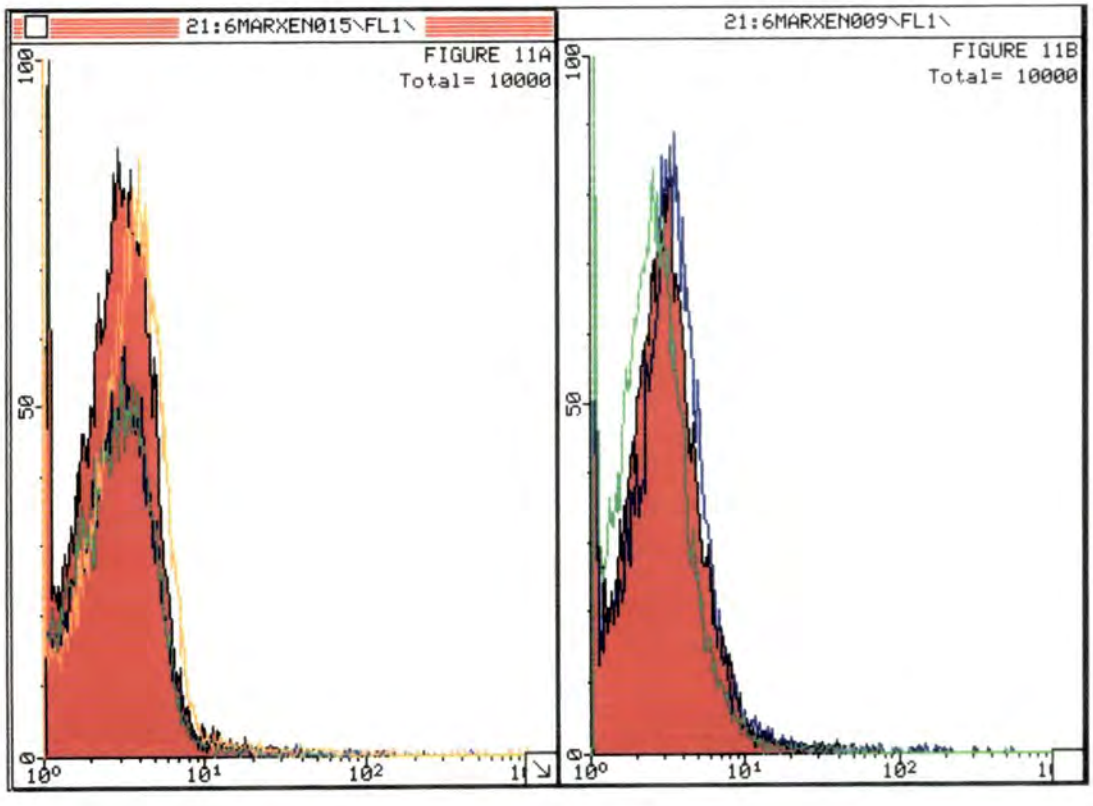
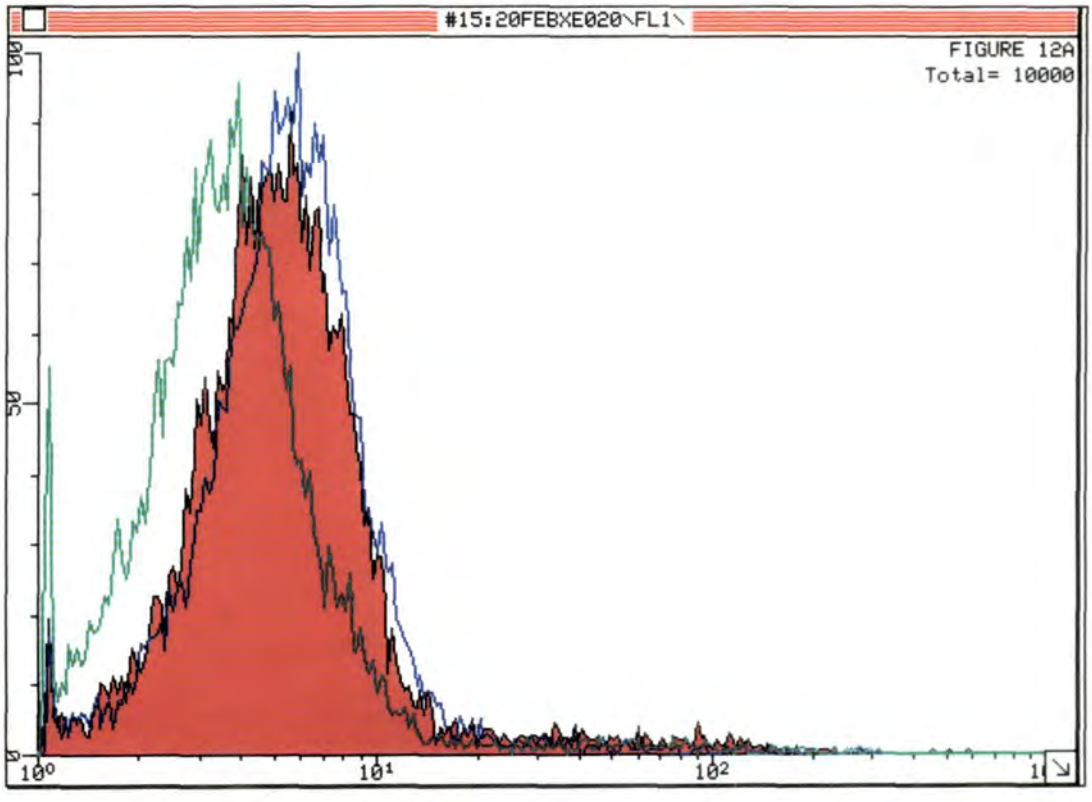
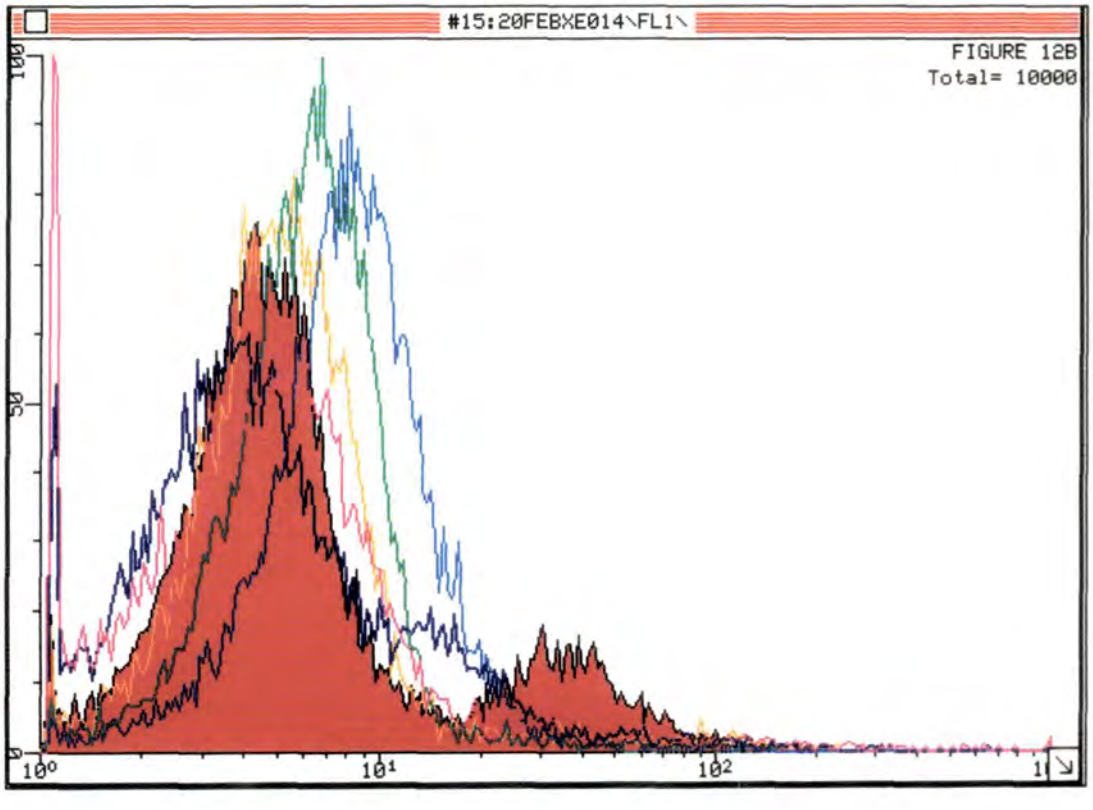

21:6MARXEN015\FL1\
FIGURE 11A
Total= 10000
21:6MARXEN009\FL1\
FIGURE 11B
Total= 10000

**Figure 2.12A** 5.5 day thymectomy by microcautery reduces levels of XTLA-1 positive cells to background. Fluorescence intensity histograms of splenocytes from a 5.5 day thymectomised outbred *X.laevis*. Green = unstained control preparation; blue = secondary antibody preparation; red = 'experimental', (XT-1 + secondary antibody), preparation. X-axis = log fluorescence intensity, (530 nm), Y- axis = relative cell number, (10,000 events / sample).

**Figure 2.12B** Comparison of XTLA-1 expression by splenocytes from sham-thymectomised and 5.5 day thymectomised outbred *X.laevis*. Fluorescence intensity histograms of 'experimental', (XT-1 mAb + secondary antibody), preparations of sham-thymectomised, (red), and 5.5 day thymectomised splenocytes showing disappearance of peak of XTLA-1 positive cells after thymectomy. X-axis = log fluorescence intensity, (530 nm), Y-axis = relative cell number, (10,000 events / sample).


#15:20FEBXE020\FL1\
FIGURE 12A
Total= 10000
100
50
0
10⁰
10¹
10²


#15:20FEBXE014\FL1\
FIGURE 12B
Total= 10000
100
50
0
10⁰
10¹
10²

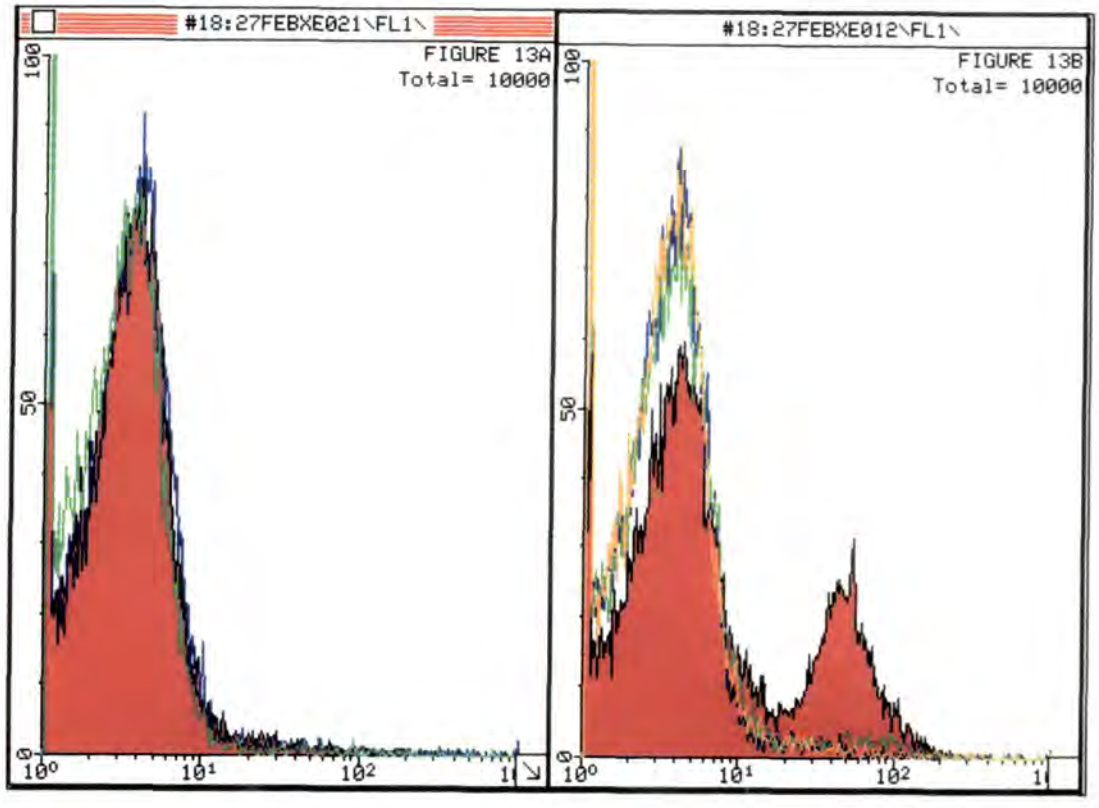



**Figure 2.13** No peak of XT-1 labelled cells is seen in 7.5 day thymectomised splenocytes. Fluorescence intensity histograms of unstained control, (green), secondary antibody control, (blue) and 'experimental', (XT-1 mAb + secondary antibody), (red), preparations of splenocytes from 7.5 day thymectomised outbred *X.laevis*, (2.13A). Figure 2.13B shows fluorescence intensity histograms of 'experimental' preparations of splenocytes from a single sham-thymectomised, (red), and several 7.5 day thymectomised outbred *X.laevis*, showing the effectiveness of the thymectomy. X-axis = log fluorescence intensity, (530 nm), Y-axis = relative cell number, (10,000 events / sample).

**Table 2.1** Fluorescence microscopical analysis of XTLA-1 +ve thymocytes from different strains / species of *Xenopus*.

| Strain/ species | Cells counted | % positive cells | Group mean positive cells (+/-S.D.) | Number of animals |
|---|---|---|---|---|
| J strain | 205 | 92.7 | 87.7 (+/-7) | 2 |
| *X.laevis* | 110 | 82.7 | | |
| Outbred | 110 | 46.4 | | |
| *X.laevis* | 165 | 97.6 | 75.6 (+/-26) | 3 |
| | 110 | 82.7 | | |
| | 274 | 88.3 | | |
| Outbred | 144 | 82.6 | | |
| *X.borealis* | 309 | 94.8 | 80.6 (+/-12) | 5 |
| | 324 | 64.8 | | |
| | 155 | 72.3 | | |
| | 115 | 76.5 | | |
| XbJ | 185 | 73 | 79.3 (+/-6) | 4 |
| | 108 | 81.5 | | |
| | 86 | 86 | | |
| LG5 | 198 | 77.3 | 79.5 (+/-3) | 2 |
| | 246 | 81.7 | | |
| | 305 | 86.9 | | |
| | 267 | 90.6 | | |
| | 450 | 84 | | |
| LG15 | 423 | 87.5 | 83.8 (+/-5) | 8 |
| | 537 | 78 | | |
| | 357 | 87.9 | | |
| | 200 | 80 | | |
| | 413 | 75.3 | | |
| | 342 | 64.3 | | |
| LG17 | 267 | 71.2 | 60.8 (+/-12) | 4 |
| | 214 | 64.5 | | |
| | 250 | 43.2 | | |
| | 300 | 85 | | |
| | 439 | 88 | | |
| | 94 | 70.2 | | |
| | 194 | 70.1 | | |
| LM3 | 255 | 61.2 | 76.9 (+/-13) | 9 |
| | 275 | 86.9 | | |
| | 227 | 55.9 | | |
| | 368 | 84.8 | | |
| | 252 | 90.5 | | |
| | 617 | 0 | | |
| | 357 | 0 | | |
| | 429 | 0 | | |
| *X.tropicalis* | 232 | 0 | 0 | 8 |
| | 165 | 0 | | |
| | 384 | 0 | | |
| | 503 | 0 | | |
| | 311 | 0 | | |

**Table 2.2** Fluorescence microscopical analysis of XTLA-1 +ve splenocytes from different strains / species of *Xenopus*.

| Strain/ species | Cells counted | % positive cells | Group mean positive cells (+/- S.D.) | Number of animals |
|---|---|---|---|---|
| | 347 | 17 | | |
| | 357 | 11.2 | | |
| J strain | 217 | 18 | | |
| *X.laevis* | 101 | 29.7 | 18.1 (+/-6) | 7 |
| | 495 | 16.2 | | |
| | 473 | 17.5 | | |
| | 384 | 16.9 | | |
| Outbred | 109 | 4.6 | | |
| *X.laevis* | 63 | 20.6 | 16.3 (+/-10) | 3 |
| | 168 | 23.8 | | |
| | 193 | 14 | | |
| | 230 | 18.7 | | |
| Outbred | 279 | 46.6 | | |
| *X.borealis* | 179 | 63.1 | 32.3 (+/-18) | 7 |
| | 261 | 26 | | |
| | 126 | 20.6 | | |
| | 234 | 37.1 | | |
| | 203 | 23.6 | | |
| | 178 | 27 | | |
| XbJ | 110 | 29 | 25.5 (+/-4) | 5 |
| | 136 | 27.9 | | |
| | 209 | 20 | | |
| LG5 | 197 | 34.5 | | 1 |
| | 244 | 13.9 | | |
| | 387 | 16.8 | | |
| | 367 | 17.4 | | |
| LG15 | 392 | 17.3 | 14.1 (+/-4) | 7 |
| | 554 | 8.5 | | |
| | 260 | 14.6 | | |
| | 486 | 9.9 | | |
| | 327 | 3 | | |
| LG17 | 311 | 3.5 | 4.7 (+/-3) | 4 |
| | 357 | 2.5 | | |
| | 247 | 9.7 | | |
| | 532 | 11.5 | | |
| | 202 | 22.8 | | |
| LM3 | 243 | 23.9 | 19.3 (+/-8) | 5 |
| | 307 | 11.1 | | |
| | 33 | 27.3 | | |
| | 323 | 0 | | |
| | 510 | 0 | | |
| | 407 | 0 | | |
| *X.tropicalis* | 262 | 0 | 0.00 | 8 |
| | 113 | 0 | | |
| | 298 | 0 | | |
| | 150 | 0 | | |
| | 78 | 0 | | |

**Table 2.3** Fluorescence microscopical analysis of XTLA-1 +ve splenocytes from early-thymectomised *Xenopus*.

| Strain/ species | Cells counted | % positive cells | Group mean positive cells (+/-S.D.) | Number of animals |
|---|---|---|---|---|
| J strain *X.laevis* | 97 | 6.2 | 4.3 (+/-1) | 5 |
| | 114 | 2.6 | | |
| | 468 | 4.9 | | |
| | 501 | 3.5 | | |
| | 446 | 4.3 | | |
| XbJ | 239 | 10.9 | 10.9 (+/-4) | 6 |
| | 64 | 17.2 | | |
| | 187 | 10.2 | | |
| | 136 | 10.3 | | |
| | 48 | 6.3 | | |
| | 95 | 10.6 | | |
| LG15 | 633 | 7.1 | 8.7 (+/-2) | 4 |
| | 324 | 6.5 | | |
| | 294 | 9.5 | | |
| | 220 | 11.8 | | |
| LM3 | 213 | 2.3 | 7.5 (+/-6) | 3 |
| | 203 | 5.9 | | |
| | 218 | 14.2 | | |

Table 2.4 FACS analysis of thymocytes and splenocytes from different strains / species of *Xenopus*.

| Strain/ species | % positive thymocytes | Group mean positive cells | % positive splenocytes | Group mean positive cells |
|---|---|---|---|---|
| **CONTROL** | | | | |
| | 86.2 | | 29.4 | |
| | 88 | | 24 | |
| | 92 | | 21 | |
| **J strain** | 91.2 | 90.5 | 15.7 | 18.7 |
| ***X.laevis*** | 91.9 | | 19.9 | |
| | 93.4 | | 7 | |
| | 93.3 | | 16.4 | |
| | 87.8 | | 16.5 | |
| | 77.9 | | 21.4 | |
| **5.5 day sham TX** | 77.8 | 79.3 | 2.5 | 14.3 |
| ***X.laevis*** | 82.3 | | 18.9 | |
| | | | 14.4 | |
| | 89.4 | | 25.9 | |
| **7.5 day sham TX** | 80 | | 21.7 | |
| ***X.laevis*** | 91.2 | 86.5 | 31.7 | 24.6 |
| | 80.9 | | 16.8 | |
| | 91.1 | | 27 | |
| | 92.1 | | | |
| ***X.borealis*** | 91.3 | 91.8 | 8.5 | |
| | 92 | | | |
| **LG5** | | | 18.8 | |
| **LG15** | 86 | | 30 | 19.3 |
| | | | 8.6 | |
| | 0 | | 1 | |
| | 1.5 | | 0 | |
| ***X.tropicalis*** | 2.5 | 1.4 | 1.4 | 0.6 |
| | 1.5 | | 0 | |
| | 1.5 | | | |
| **THYMECTOMISED** | | | | |
| | | | 1 | |
| | | | 3.1 | |
| **5.5 day TX** | | | 0.6 | 2.3 |
| ***X.laevis*** | | | 1.6 | |
| | | | 3 | |
| | | | 4.4 | |
| | | | 2.1 | |
| **7.5 day TX** | | | 4 | 2.1 |
| ***X.laevis*** | | | 0.2 | |

# Chapter 3

# Use of the XT-1 monoclonal antibody to explore the distribution of T-cells within larval and adult lymphoid organs and the development of host T-lineage cells in thymus-implanted, thymectomised Xenopus

## 3.1 Introduction

During the ontogeny of *Xenopus*, the first organ to become lymphoid is the thymus. This organ first arises as an epithelial invagination at about 3 days post-fertilisation, (stage 40 of Nieuwkoop and Faber), (Manning and Horton, 1982). Stem cells, (thymocyte precursors), initially migrate into the larval thymus at 3–4 days of age, (stage 42–43), (Tochinai, 1978; Kurihara and Kato, 1986). This first period of precursor immigration is completed by 20 days of age, (stage 52), (Turpen and Smith, 1989). The immigrant large lymphoblasts undergo many mitoses within the larval thymus, and, by day 6–8 post- fertilisation, the thymus has a normal cortex- medulla architecture, (Manning and Horton, 1969). The expression of class II MHC molecules on thymic epithelium is not a pre-requisite for stem cell immigration, since Du Pasquier and Flajnik, (1987), revealed that class II molecules are first expressed within the thymus medulla at day 8. Larval thymocytes apparently do not express class II antigens, (Du Pasquier and Flajnik, 1986). Nagata's, (1986b), immunofluorescence studies on XT-1 labelled cell suspensions showed that XTLA-1 positive thymocytes, (comprising 8% of the total lymphoid population), are found at 7 days of age, (stage 48), in J strain larvae.

The proportions of XT-1 labelled cells rose sharply by stage 49, (10 days post-fertilisation), reaching adult levels, (approximately 95%), by stage 52, (20 days). The density of XTLA-1 antigen was observed to increase from stage 50 up until stage 56, when fluorescence intensity profiles, as measured by flow cytometry, were similar to those seen on adult thymocytes, (Nagata, 1986b).

There is an involution of the thymus during metamorphosis, in *Xenopus*, involving a large reduction in thymic lymphocyte numbers by the end of metamorphosis, (stages 65–66), (Du Pasquier and Weiss, 1973; Flajnik et al., 1987). It has recently been revealed that another period of thymic colonisation by stem cells occurs during metamorphosis, between 38 and 57 days post-fertilisation, (Turpen and Smith, 1989). Following metamorphosis, thymocyte numbers increase again, reaching their maximum level at 15–16 months post-fertilisation, (Du Pasquier and Weiss, 1973). The cortical thymocytes which appear in the second wave of histogenesis express class II MHC antigens, (Du Pasquier and Flajnik, 1987).

The adult *Xenopus* thymus has a structure similar to that of other vertebrates; it is enclosed in a thin connective tissue capsule, from which trabeculae penetrate into the organ, (Manning and Horton, 1982). Lymphocytes are tightly packed into the cortex of the thymus, between epithelial cells. The thymic medulla, which is separated from the cortex by a distinct cellular barrier, (Nagata, 1976a, 1977), is far more heterogeneous, containing, for example, epithelial and epithelial reticular cells, myoid cells, granulocytes and mucous cells, (Clothier and Balls, 1985; Russ, 1986).

The larval spleen first appears in *Xenopus* shortly after the thymus, at about 12–14 days post-fertilisation, (Manning and Horton, 1982). XTLA-1 positive cells were not found at stage 50, (15 days), but reached significant levels at stage 52, (20 days), and increased to adult levels, (30%), by stage 56, (40 days), (Nagata, 1986b). The spleen continues to differentiate throughout larval life, until by metamorphosis it consists of three to five white pulp areas delineated from the red pulp by boundary layers, (Manning and Horton, 1982). Adult *Xenopus* spleen is located in the mesentery of the gut. It is organised into red and white pulp areas, these latter areas consisting of a central arteriole surrounded by small lymphocytes. The majority of white pulp lymphocytes are B-lymphocytes, as assessed by cytoplasmic immunoglobulin staining, (Baldwin and Cohen, 1981a). XL cells, thought to resemble mammalian follicular dendritic cells, (Baldwin and Cohen, 1981a&b; Baldwin and Sminia, 1982), are also found in both larval and adult spleen; these are peripherally located in the adult white pulp. Obara, (1982), suggested that there was a preferential localisation of thymus- dependent cells in the red pulp, and of thymus-independent cells in the white pulp areas. Manning, (1971), also reported a decrease in the ratio of lymphocytes to reticular cells in the red pulp marginal zone, (which lies just outside the white pulp region), in early-thymectomised animals.

In addition to the thymus and spleen, lymphoid tissues in larval *Xenopus* include 4 pairs of ventral cavity bodies, (VCBs), and 2 dorsal cavity bodies in the branchial region, (Manning and Horton, 1982; Tochinai, 1975). These are described as densely packed lymphoid accumulations, consisting of predominantly

small to medium-sized lymphocytes, with occasional macrophages and other leukocyte types. The percentage of XTLA-1 positive cells in larval liver and kidney increased from background levels at stage 50 to adult levels by stage 56, (Nagata, 1986b). In adult *Xenopus*, nodules of gut-associated lymphoid tissue are found, (Manning and Horton, 1982). Lymphomyeloid tissue is also found within the kidney, liver and bone marrow in *Xenopus*, (Manning and Horton, 1982).

Thymectomy at early stages of thymus histogenesis, (4–8 days), abrogates or severely impairs classical T-dependent responses in *Xenopus*, (Horton and Manning, 1972; Tochinai and Katagiri, 1975). Injection of suspensions of adult histocompatible or incompatible thymocytes were found to restore skin allograft rejection capacity in early-thymectomised, (TX), frogs, (Nagata and Tochinai, 1978). A later study, (Kawahara, Nagata and Katagiri, 1980), involving the injection of TX *Xenopus* with histocompatible ploidy-marked lymphocytes, suggested that this restoration of graft rejection was largely due to the activity of the injected cells, rather than to any resultant maturation of host alloreactive cells. In contrast, the injected cells contributed only indirectly to antibody production against rabbit erythrocytes, by helping host B-cells, (Katagiri et al., 1980). Immune responses, (e.g. skin allograft rejection and thymus-dependent antibody production), of TX individuals were also shown to be restored by implantation of normal adult MHC-compatible, (Tochinai et al., 1976), or incompatible, (Horton and Horton, 1975), thymus, or by MHC-incompatible larval thymus grafts, either normal or gamma-irradiated, (Du Pasquier and Horton, 1982; Gearing et al., 1984).

Nagata and Kawahara, (1982), implanted either histocompatible or incompatible adult triploid thymi into diploid TX recipients in adult life. These authors then tested the skin allograft response of thymus-implanted *Xenopus* and, in contrast to the work mentioned above, found complete restoration of this response only in those animals given MHC-compatible implants. Implantation of MHC-incompatible adult thymus restored graft rejection capacity, but graft survival times were extended, compared to those on control animals. Investigation of the ploidy of lymphocytes developing within thymus implants showed that more than 90% of those in compatible implants were of donor origin, (116 days post-implantation). However, in allogeneic implants, (153 days post-implantation), the majority of implant lymphocytes had been replaced by host-type cells.

Further work on the differentiation of host lymphocytes in early-thymectomised, thymus-implanted animals was carried out by Nagata and Cohen, (1984). They implanted normal adult MHC-matched, partially matched or totally mismatched thymi into adult *Xenopus* thymectomised on day 4 or 5 post-fertilisation. Triploid implants were inserted into diploid hosts, and *vice versa*, and the differentiation of host cells within the implants and in the periphery was examined by flow cytometry. Regardless of the donor-host combination, thymus grafts restored T-cell dependent immune responses, both **in vivo** and **in vitro**. Most donor thymocytes in implanted thymus grafts from MHC- matched or mismatched donors were replaced by lymphocytes of host origin within 6–9 months after thymus grafting. Depending upon the donor-host combination, cells from partially or totally MHC-mismatched donor implants could persist within the spleen for at least one year after implantation. In certain donor-host combinations, however, no allogeneic

donor cells could be found in the periphery 7 months post-implantation. Overall, Nagata and Cohen's experiments confirmed that thymus implants, even from MHC-incompatible donors, could promote the differentiation of host precursor T-cells. The time course of host cell input into these implants, (performed in adult life), and their location within the implants, was not, however, addressed in these studies.

Using the *X.borealis* quinacrine marker to discriminate between host and donor cells, Russ, (1986), and Horton et al., (1987), investigated the sequence of input of host cells into normal and irradiated, (lymphocyte depleted, Russ and Horton, 1987), adult thymi implanted into larval TX *Xenopus*. The quinacrine marker system, (first described by Thiébaud, 1983), is based on the differential staining of nuclei of different *Xenopus* species with the fluorescent dye, quinacrine. Cells of *X.borealis* origin stained with this dye show bright nuclear spots of fluorescence, thought possibly to correspond to areas of A-T rich DNA on the chromosomes, (Weisblum and de Haseth, 1972; Brown et al., 1977),although this has been challenged, (Comings, 1978). Other *Xenopus* species examined in Thiébaud's study did not, (with the exception of *X.fraseri*), show fluorescent spots. Thus, quinacrine staining can be used to demonstrate the species origin of cells in smears and tissue sections. Horton et al.'s, (1987), time-course study found that normal, lymphoid allogeneic and xenogeneic thymus implants began to be infiltrated by host thymocytes by 2 weeks post-implantation. The xenogeneic implants were predominantly populated by donor cells at 8–9 weeks post-implantation, whereas the allogeneic implants still contained mostly host thymocytes. Stromal cells within both types of implant remained of donor origin. Irradiated adult implants were already well repopulated by host cells at 2 weeks after implantation, but these implants did not persist, presumably due to irradiation damage.

The studies reported in this Chapter had two aims. The first of these was to describe the histological distribution of XTLA-1 positive lymphocytes within the lymphoid tissues of normal adult and larval *Xenopus*, since Nagata's, (1986b), ontogenetic studies had examined XTLA-1 expression only in lymphocyte suspensions. The second aim was to examine the origin of thymocytes and, in particular, to investigate the differentiation of the XTLA-1 T-cell marker within larval thymus implants and in the periphery of thymus-restored, TX *Xenopus*. For this study, TX recipients were *X.borealis*, carrying the quinacrine marker, while thymus donors were *X.tropicalis*, which were shown in Chapter 2 not to express the XTLA-1 T-cell marker.

## 3.2 Materials and Methods

### 3.2.1 Animals and Operations

#### 3.2.1.1 Animals

All animals were bred in this laboratory at 22–24°C, as described by Horton and Manning, (1972), and Kobel and Du Pasquier, (1975).

Thymi and spleens for frozen sectioning were removed from 6 month old outbred *X.tropicalis* froglets. Whole 35 day old, (stage 54), outbred *X.laevis* and 44 day old, (stage 56), *X.borealis* tadpoles were also sectioned.

#### 3.2.1.2 Early-thymectomy Operations

Thymectomies were carried out on *X.borealis* stage 47–48, (7 day old), larvae by microcautery, after the method of Horton and Manning, (1972).

#### 3.2.1.3 Thymus implantation operations

Thymectomised *X. borealis* larvae were implanted when 4 weeks old, (stage 53-54), with pairs of normal lymphoid thymi from age- and stage- matched *X.tropicalis* larvae. Thymi were aseptically dissected out of anaesthetised donor tadpoles into sterile APBS. These thymi were implanted into sub-cutaneous pockets made on each side of the head, (medial to the eye), as described by Horton and Horton, (1975). Host animals had previously been found, by microscopical observation, not to possess any thymic tissue. Any suggestion of thymic regeneration in potential host animals lead to their exclusion from the experiment; such regeneration occurred only rarely. Implanted hosts were placed into amphibian Ringers' solution, diluted 1:2 with distilled water, for 24 hours, following which the Ringers' solution was further diluted by the repeated addition of aerated standing water. Implanted hosts were finally, (after a total of 48-72 hours), returned to fresh aerated standing water and reared like control, unoperated siblings.

Implanted thymi were translocated during metamorphosis, coming to rest at the posterior margin of the eye, where they remained visible as bumps under the hosts' skin. The persistence of these thymus implants was monitored by microscopical observation at intervals throughout the course of the experiment. FACS analysis, using XT-1 and propidium iodide staining techniques, was carried out on thymus/thymus implant and spleen cell suspensions from implanted host animals and on control *X.borealis* and *X.tropicalis* animals, (siblings of host and donor animals, respectively).

### 3.2.2 XT-1 Staining of frozen sections

#### 3.2.2.1 Preparation of frozen sections

Adult lymphoid organs, (thymus/thymus-implants and spleen), were dissected out of heavily-anaesthetised donor animals, and immediately placed into a pool of Cryomatrix embedding medium, (Shandon), on a metal chuck. Composite blocks of adult organs were generally made, containing both thymi/thymus-implants and spleen from an individual animal. Fragments of overlying skin often remained attached to dissected thymi. Care was taken to fully surround all organs to support the frozen tissues and to also avoid the introduction of air bubbles into the medium. The organs were then frozen on the metal chucks, using $CO_2$ gas. The freezing

process took less than one minute to complete. The blocks were then placed in the chamber of a Bright cryostat, (Bright Instruments Ltd.), which was maintained at -20°C and were left to thermally equilibrate for 10–15 minutes prior to cutting.

Dissected tadpole lymphoid organs were found to be too small to handle conveniently, and so whole, anaesthetised tadpoles were divided transversely, to give segments containing the organs under study. These fragments were placed into Cryomatrix on metal chucks, orientated such that transverse sections could be taken, and frozen in the same manner as adult organs.

Serial transverse 6$\mu$ sections of frozen blocks were cut, and were collected onto clean microscope slides. The sections on slides were air dried for 1–2 hours at room temperature, then placed in slide trays and sealed in polythene bags. They were stored frozen at -20°C.

#### 3.2.2.2 Fixation of sections

Slides were taken from storage in the -20°C freezer, and air dried, at room temperature, for 30 minutes.

Individual sections on slides, (usually 2–4 consecutive sections per slide), were scored round with a diamond pencil, to facilitate standing drop formation for blocking buffer/antibody incubations, and to make it easier to locate sections under the microscope.

Attempts were made to carry out indirect immunofluorescence staining on unfixed frozen sections, but generally the tissue preservation was so poor that analysis was impossible. Therefore, fixation in cold, (-20°C), absolute methanol was adopted as routine procedure, as this had previously been shown, (Nagata, 1985), not to interfere with the binding properties of the XTLA-1 determinant. Absolute methanol, rather than 70% methanol, was used as, in initial tests, fixation in the latter lead to duller staining. Sections were fixed for 20 minutes, as longer fixation times were also found, in initial studies, to diminish the fluorescence intensity.

Following fixation, the slides were washed to remove the methanol, in 2 changes of 50 ml staining medium, (APBS with 0.1% BSA and 0.1% sodium azide), with 5 minutes incubation per wash, at room temperature.

#### 3.2.2.3 Indirect immunofluorescent staining of frozen sections with XT-1

This procedure was carried out at room temperature throughout. All incubations took place in a moist chamber, (a sealed plastic box lined with dampened filter paper, above which the slides were supported on glass rods), to ensure that the sections did not dry out during staining, an event which tends to produce high levels of non-specific sticking of antibodies. All reagents were applied in 20–50$\mu$l per section standing drops. Washing steps consisted of 3 incubations for 5 minutes each, in 50ml per change staining medium.

Prior to the first incubation step, all slides were incubated for one hour in

blocking buffer. Blocking buffer consists of APBS with 0.1% sodium azide, but with a higher, (1%), BSA content than the staining buffer. This blocking step was found, in trial staining procedures, to be necessary to prevent non-specific sticking of antibodies to the sections. Following the blocking step, this buffer was tipped off the slides, which were carefully dried around the ringed sections.

Experimental sections were next incubated in deaggregated 1:50 dilution of XT-1 ascites fluid, for 1 hour, or, in experiments to confirm the specificity of binding of the ascites fluid used, in 1:20 deaggregated dilution of affinity purified XT-1 mAb.

Control sections were incubated either in staining medium alone, or in 1:40 mouse IgG, (Sigma Chemicals Cat. No. I-5381), to assay for non-specific IgG binding.

Following the primary incubation, all reagents were tipped off the slides, which were then washed as previously described, and again dried around the ringed sections before application of the secondary antibody to all sections.

The secondary antibody used was sheep anti-mouse IgG $F(ab')_2$ - FITC, (Sigma Chemicals, Cat. No. F2883). This was diluted 1:20 in staining medium, and deaggregated before use. Secondary antibody incubation was for one hour.

Following the secondary antibody incubation, the slides were again washed as previously described and dried, after which they were mounted for observation.

#### 3.2.2.4 Mounting of stained sections for fluorescence microscopy

Several different mounting media were tested in initial stainings, (staining medium, UV free aqueous mounting medium, (Difco Ltd.), and Citifluor PBS/ glycerol mounting medium, (Citifluor Ltd.)). Of those tested, the Citifluor PBS/glycerol medium performed best in terms of the ease of handling and of the intensity and persistence of staining observed. This mounting medium contains an anti-fading agent, (p-phenylene diamine), which helps to prevent bleaching of fluorescence during observation and photography, probably by acting as a scavenger of free radicals liberated by excitation of fluorochromes, thereby preventing destructive reactions taking place between these free radicals and other fluorochrome molecules, (Harlow and Lane, 1988).

A drop of Citifluor PBS/glycerol mountant was placed over each section, and these were covered with grade O coverslips, care being taken to exclude air bubbles.

Slides were then observed and photographed following the procedures as described in the previous Chapter.

### 3.2.3 Quinacrine hydrochloride staining protocol for frozen sections

#### 3.2.3.1 Fixation of sections

Frozen sections were prepared as described in 3.2.2.1.

Slides were taken from storage in the -20°C freezer and air dried, at room temperature, for 30 minutes.

Individual sections on slides, (usually 2–4 consecutive sections per slide), were scored round with a diamond pencil to facilitate their location under the microscope.

Slides were immersed in Carnoy's fixative, (10% glacial acetic acid, 60% absolute alcohol, 30% chloroform), for 10 minutes at room temperature.

Following fixation, the slides were washed in 2 changes of 50 ml quinacrine staining buffer, (18.15% 0.1M citric acid, 81.85% 0.2M $Na_2HPO_4$pH 7.0), for approximately 2 minutes per change to wash off the fixative solution, and washed for a further 10 minutes in fresh quinacrine staining buffer, (50 ml).

#### 3.2.3.2 Quinacrine hydrochloride staining of frozen sections

Washed slides were incubated for 15 minutes in 0.5% quinacrine hydrochloride, (Sigma Chemicals Ltd.), in staining buffer. At the end of this time, the slides were rinsed briefly in staining buffer, and then washed in 2 changes of 50 ml quinacrine staining buffer, for 10 minutes per change, to remove excess dye. Throughout this staining procedure, slides and solutions were protected from light.

#### 3.2.3.3 Mounting and observation of stained sections

Washed slides were carefully dried around the ringed sections and a drop of 50% sucrose solution was placed over each section, then these were covered with grade O coverslips, care being taken to avoid air bubbles.

Coverlips were sealed by painting a thin layer of 50:50 vaseline: paraffin wax mixture around the edges, and leaving this to cool before observation.

#### 3.2.3.4 Observation and photography of sections

The sections were examined under a Nikon Optiphot microscope fitted with epi-fluorescence illumination and blue-violet UV filters (Nikon Cat. No. DM455), and photographed with an attached Microflex UFX-II camera system.

### 3.2.4 FACS analysis of XT-1 labelled and propidium iodide stained thymus/thymus implant and spleen single cell suspensions

In addition to the histological, time-course study of T-cell differentiation within implanted *X.tropicalis* thymi and in host *X.borealis* spleens, FACS analyses of lymphoid cell suspensions from two implanted animals were made 78 days post-implantation. Adult *X.borealis* and *X.tropicalis* control thymocyte and splenocyte suspensions were also examined.

#### 3.2.4.1 Single cell suspensions

Thymus/thymus-implants and spleens were removed from implanted and control animals into staining buffer, (ABBS/0.1% BSA/0.1% sodium azide). Two thymus implants were recovered from each host animal; the implants appeared to be in good condition and were cellular, although small in comparison to both *X.borealis* and *X.tropicalis* control thymi.

Single cell suspensions were made from implants, control thymi and spleens, as described in the previous chapter, (2.2.3). Thymus/ thymus-implant cell suspensions from each individual animal were pooled.

#### 3.2.4.2 Indirect immunofluorescence staining with XT-1 for FACS analysis

Cell suspensions, prepared as above, were divided where possible, into control and experimental aliquots and were stained with XT-1 monoclonal antibody as described in Chapter 2 (2.2.3).

#### 3.2.4.3 Propidium iodide staining

Following FACS analysis of XT-1 labelled cell suspensions, these suspensions were further processed, by the method of Taylor, (1980), to permit assessment of the species origin of individual cells. By this method, cell membranes are removed by the action of the non-ionic detergent, Triton-X-100, and the DNA of the remaining cell nuclei is labelled with the fluorescent dye, propidium iodide, (P.I.). The fluorescence intensity of cell nuclei labelled with P.I. is directly related to their nucleic acid content. To avoid any contribution to the staining intensity other than from DNA, Ribonuclease A is included in the staining solution to break down any nuclear RNA present. *X.tropicalis* cells have fewer chromosomes than those of *X.borealis*, and hence much less DNA, so they are less intensely stained with P.I. The species origin of cells in thymus/thymus implant and spleen cell suspensions could thus be assessed.

**Propidium iodide staining solutions.** Propidium iodide, (Fluka, Cat. No. 81845), was dissolved at a concentration of 250 $\mu$g/ml in 1% Triton-X-100, (Sigma Chemicals), solution, and stored at 4°C, protected from light. Ribonuclease A,

Type IAS, (Sigma Chemicals, Cat. No. R5503), was dissolved in distilled water at a concentration of 25 mg/ml. This solution was freshly made before each staining.

**Propidium iodide staining protocol.** To each experimental, (XT-1 stained), aliquot propidium iodide solution was added in the ratio of 0.25 ml P.I. solution to 1 ml cell suspension. 50$\mu$l Ribonuclease A solution per ml cell suspension was also added. Aliquots were then incubated briefly, (5 minutes), at room temperature, and then run through the cell sorter.

The intensity of fluorescence at 575nm was recorded for each 'event' or cell nucleus counted, and histograms of number of events vs fluorescence intensity, (575nm), were generated for each sample. 10,000 events were recorded for each sample counted.

## 3.3 Results

### 3.3.1 Observations on XT-1 labelled adult thymus and spleen sections

In LM3 and LG5 thymus 'experimental' sections, (incubated with XT-1 mAb and sheep anti- mouse IgG F(ab')$_2$ - FITC), bright fluorescence was observed, outlining all the lymphoid cells of the cortex, in a 'honeycomb' pattern of staining. The subcapsular cortical layer, (4–5 cells deep), was often more intensely stained than the deeper layers of the cortex, (Figure 3.1, A and B). The thymus medulla was overall less brightly fluorescent than the cortex and cells in this area were less uniformly stained around their circumference, (Figure 3.2, A and B). The thymus capsule, trabeculae and blood vessels, identified by phase contrast microscopy, were not stained by this protocol.

Control sections, (incubated with staining medium or non-immune mouse IgG followed by secondary antibody), were uniformly unstained, although there were occasional deposits of fluorochrome randomly distributed on some of these sections, (see Figure 3.1, C and D; Figure 3.2, C and D).

Experimental sections of adult *X.tropicalis* thymus were uniformly unstained, as were control sections, confirming results obtained for thymocyte single cell suspensions described in Chapter 2, (see Figure 3.3, A and B).

Sections of LM3 and *X.tropicalis* adult spleens were examined. In LM3 experimental sections, cells with fluorescent outlines were observed, distributed, often in clumps, in the red pulp; there was a suggestion that these accumulations were particularly prevalent in the marginal zone near the boundary layer of white pulp follicles, (as discerned by phase contrast microscopy), (Figure 3.4, A and B). A few positively stained cells were seen dispersed within the white pulp follicles, (see Figure 3.4, C and D). Control LM3 sections did not contain fluorescently-outlined cells, (Figure 3.4, E and F). Experimental and control sections of *X.tropicalis* adult spleen were also unstained, although observation of spleen sections was complicated by a generalised yellowish autofluorescence in the red pulp, (Figure 3.5, A

to D).

### 3.3.2 Observations of XT-1 labelled larval tissues

In preparation for the study of larvally implanted thymi reported later in this chapter, sections of outbred *X.laevis*, (stage 54), and *X.borealis*, (stage 56), larvae were examined. The sections observed were cut through the thymic region, and also anterior to the thymi.

Staining patterns in experimental sections of both *X.laevis* and *X.borealis* larvae were similar. The thymi were brightly stained, with individual cells outlined in a regular, 'honeycomb' pattern in the cortical region, and less regularly so in the medullary area, (Figure 3.6, A to D). Again, the staining intensity of the medulla was overall less than that of the cortex. Interestingly, there was again a difference between the intensity of staining observed in the immediate subcapsular region of the cortex and that of the bulk of the cortex. However, in contrast to the situation seen in adult thymus, the larval subcapsular cortex appeared less intensely stained than the rest of the cortex.

XTLA-1 positive, (XTLA1 +ve), cells were also observed scattered in the pharyngeal tissue outside the thymus, (Figure 3.7, A and B), in both *X.laevis* and *X.borealis*. Distinct accumulations of positive cells were seen in the *X.borealis* pharyngeal region. From their location, as assessed by phase contrast microscopy, these accumulations correspond to ventral cavity bodies, (see Figure 3.8, A to C).

Control sections of *X.laevis* and *X.borealis* tadpoles were unstained, (see Figure 3.8, C and D).

### 3.3.3 Observations on quinacrine hydrochloride stained adult *X.tropicalis* thymus and larval *X.borealis* thymic region sections

These observations were undertaken to confirm that *X.tropicalis* cells do not carry the quinacrine marker possessed by *X.borealis* cells.

In quinacrine stained sections of X.borealis larval thymic regions, all nuclei showed characteristic bright spots of fluorescence, (Figure 3.9A), while concurrently stained sections of *X.tropicalis* adult thymus, although displaying heavy generalised uptake of this dye, showed no bright nuclear spots of fluorescence, (see Figure 3.9B).

### 3.3.4 Immigration of *X.borealis* cells into implanted *X.tropicalis* thymi and their differentiation into XTLA-1 positive T-cells

At intervals from 9 to 55 days after implantation of larval *X.tropicalis* thymi, recipient *X.borealis* TX animals were sacrificed. Serial frozen sections were cut through

the thymic region, (prior to metamorphosis), or through the dissected implants and the spleen of post-metamorphic hosts. Sections of non-thymectomised, unimplanted sibling *X.borealis* were examined alongside the experimental preparations.

Sections were either stained with quinacrine hydrochloride, to demonstrate the presence within the thymus implants of quinacrine positive (Q +ve) cells of host origin or by indirect immunofluorescence with XT-1 mAb, to show the presence and distribution of XTLA-1 expressing, (host), T-lineage cells.

Attempts were made to restain sections, (previously labelled by indirect immunofluorescence using XT-1 mAb), with quinacrine, to provide direct comparison between XTLA-1 expression and cell origin. Unfortunately, this did not prove to be possible for the following reasons: the fragile sectioned material did not withstand this extended procedure in good condition, and the nuclear staining with quinacrine was very dull, possibly due to the incompatibility of fixation solutions required for the two techniques.

#### 3.3.4.1 Observations on sectioned thymus implants and spleens from host animals

Observations on implanted animals were made as follows:

- 9d. post-implantation, (n = 1)
- 27–28d. post-implantation, (n = 2)
- 33d. post-implantation, (n = 1)
- 48d. post-implantation, (n = 2)
- 55d. post-implantation, (n = 2)

At all but the first time point, host animals were newly metamorphosed froglets.

**9d Post-implantation:** (1 larva, 2 implants).

Morphology: Both implants were large and cellular with good cortico-medullary distinction.

Quinacrine staining: The majority of cells in these implants were small *X.tropicalis*, (Q negative), lymphocytes. However, larger, Q +ve, (*X.borealis*), cells were frequently seen, scattered both singly and in small foci throughout these implants. Most cells of *X.borealis* origin were found in the cortex, (Figure 3.10, A and B).

XT-1 mAb staining: Very few XTLA-1 +ve cells were found in these implants, (Figure 3.10, C and D). 1–2 positive cells/section were observed in implant 1, and 2–3 positive cells/section were seen in implant 2. These were exclusively in the cortical region of the implants.

**27d and 28d post implantation** (2 froglets, 2 implants and spleen recovered from each).

Morphology: Although still large and cellular, these implants showed some disruption of normal cortico-medullary organisation, in that lymphocytes arranged in the usual tightly packed cortical arrangement were found in patches, rather than in a continuous band, below the capsule.

Quinacrine staining: Three of the four implants contained large numbers of Q +ve *X.borealis* cells, (Figure 3.11, B), estimated to comprise 30–50% of the total cell number. One of the four implants, (recovered 28 days after implantation), still contained few Q+ve cells. The 'spotted' cells had the appearance of lymphocytes, and were distributed in both the cortical and medullary regions, (as far as these could be determined), but predominantly in the former. Q+ve cells were often found in groups within the implant.

XT-1 mAb staining: XTLA-1 +ve lymphocytes were present in moderate numbers in 3 of 4 implants, (those which were 30–50% Q +ve), and in small numbers in the remaining implant. The XTLA-1 +ve cells, often in small clusters, were found both in putative cortex and medulla but, as was the case with the quinacrine stained material, the outer, cortical areas were more heavily stained with XT-1 mAb.

Spleen sections from these two thymus-implanted *X.borealis* contained no detectable XTLA-1 +ve cells.

**33d post-implantation:** (1 froglet, 2 implants, (one was too small to examine), and spleen).

Morphology: As at 27–28d.

Quinacrine staining: This implant was well infiltrated throughout with *X.borealis* lymphocytes, although these cells were most prevalent in the outer, cortical area.

XT-1 staining: Again, as at 27 and 28 days post-implantation, XTLA-1 +ve cells were present throughout the implant, often grouped together, and frequently towards the thymus capsule, (Figure 3.11, B and C).

The spleen of this animal did contain small numbers of XTLA-1 +ve cells, in both red and white pulps, as discriminated by phase contrast microscopy, (Figure 3.11, D and E).

**48d post-implantation.** (2 froglets, 2 implants and spleen recovered from each).

Morphology: Three of the four implants examined at this time point were quite large and cellular, although the cortico-medullary distinction was not clear. One of the four was small and very disorganised, with loosely associated cells and some evidence of necrosis.

Quinacrine staining: In the 3 largest implants, there were many Q +ve cells, estimated to comprise 60% in the subcapsular region, (areas described refer to the organisation of a normal thymus. In view of the morphology described above, designation of tissue as cortical or medullary can only be approximate), 30–50% in the deeper cortex, (with localised areas which were completely *X.borealis* type) , and 10–20% of medullary cells. Smaller, non-spotted *X.tropicalis* nuclei were still visible throughout the implants, (Figure 3.12, A and B).

The one small implant was largely composed of quinacrine-positive cells.

XT-1 staining: In three of the four implants, the staining pattern with XT-1 mAb approached that seen in a normal, adult control XTLA-1 +ve thymus; that is, there was a 'subcapsular' band of brightly staining 'honeycomb' pattern, duller staining in the rest of the cortex and scattered positive staining in the medullary region, (Figure 3.12, C to F).

The smallest implant showed one cluster of 'honeycomb' outlined XTLA-1 +ve lymphocytes towards the capsule.

Again, spleens contained few XTLA-1+ve lymphocytes detectable by fluorescence microscopy. The majority of those seen were in the splenic red pulp.

**55d post-implantation:** (2 froglets, 2 implants and spleen from each animal).

Morphology: Although each animal had two recognisable thymic implants, one was very small and flattened, and was not processed for analysis. The remaining 3 implants were of medium size and were cellular; cortico-medullary organisation was a little better in these implants than in many of those taken after shorter implantation times.

Quinacrine staining: Quinacrine staining was not carried out on these sections.

XT-1 staining: These implants even more closely approached control thymi, in terms of their XTLA-1 +ve cell number and distribution. There was often a brighter subcapsular layer, characteristic of adult control thymus sections, although this was not necessarily continuous. Beneath this layer, the bright 'honeycomb' cortical staining was apparent, in quite a wide band, and the medulla also contained many positively outlined cells, (Figure 3.13, A to C).

Spleen sections, however, were not heavily labelled with, again, only scattered single XTLA-1 +ve cells, unlike the pattern seen in control *X.borealis* spleens.

## 3.3.5 FACS analysis of thymocytes from normal and implanted thymus and of spleen cells

### 3.3.5.1 XT-1 staining

Analysis of thymocytes and splenocytes of the control *X.borealis* and *X.tropicalis* animals examined here was included in the FACS data presented in Chapter 2, (see Table 4). To reiterate these results: the sibling control *X.borealis* animal possessed 92% XTLA-1 +ve thymocytes, (secondary antibody background staining = 1.1%), and 22.5% XTLA-1 +ve splenocytes, (2° antibody background staining = 2%).The *X.tropicalis* donor sibling control possessed no XTLA-1 +ve thymocytes. *X.tropicalis* splenocytes were not analysed during this experiment.

Cell numbers from *X.tropicalis* thymus implants, removed from *X.borealis* hosts 3 months post-implantation, were too low to allow for unlabelled and secondary antibody control aliquots to be prepared. Only 'experimental', (XT −1 and then secondary antibody labelled), samples were prepared from implant cells. In order to assess the proportion of XT-1 +ve cells in these samples, the fluores-

cence intensity histograms generated by their FACS analyses were compared with background fluorescence levels recorded for unlabelled and secondary antibody control preparations of thymocytes from unimplanted *X.borealis* animals. Using this comparison, implanted animal I possessed 72% XTLA-1 +ve implant cells while implanted animal II possessed 89% XTLA-1 +ve implant cells, (see Figure 3.14).

Spleen cell suspensions from thymus-implanted animals were also analysed. These organs were a little smaller than those from control *X.borealis* and *X.tropicalis* animals, and contained fewer cells. Enough cells were obtained from the spleen of implanted animal I to prepare unlabelled control, secondary antibody control and experimental samples. However, only enough splenocytes to prepare an experimental sample were obtained from implanted animal II. In order to estimate the proportion of XTLA-1 +ve cells in implanted animal II's spleen, this experimental sample was compared with background fluorescence levels, (from unlabelled and secondary antibody control samples), from animal I's splenocytes. Implanted animal I was found to have 11.3% XTLA-1 +ve splenocytes, while implanted animal II was estimated to have 19% XTLA-1 +ve cells within its spleen, (see Figure 3.15).

#### 3.3.5.2 Propidium iodide staining

Histograms of intensity of P.I. fluorescence against 'event', (cell nucleus), number were generated first for adult *X.borealis* control thymocytes and then for adult *X.tropicalis* thymocyte suspensions. Markers were then inserted to exclude non-fluorescent events, and to bracket fluorescent *X.borealis* nuclei.

When the *X.tropicalis* fluorescence intensity histogram was overlaid onto that of *X.borealis* cell nuclei, a small overlap, of 8% of the total nuclei counted was observed, (Figure 3.16). This overlap probably corresponds to *X.tropicalis* thymocyte nuclei in 'S' phase, or in mitosis, which contain twice the usual amount of DNA. (Darzynkiewicz et al., (1981), reported that Taylor's single step method for P.I. staining resulted in the loss of mitotic cells, due to their total disintegration in the presence of 1% Triton X-100, but Taylor, (1981), has refuted this report).

When P.I. staining intensity histograms were generated for nuclei of cells of thymus implants, implants from both animals were seen to contain small peaks of *X.tropicalis* nuclei and much larger peaks of *X.borealis* nuclei, (Figure 3.16). The *X.tropicalis* nuclei positively identified comprised 8%, (implanted animal I), and 10%, (implanted animal II), of the total nuclei counted. It is important to bear in mind that up to 8% of *X.tropicalis* thymocyte nuclei examined overlapped with those of *X.borealis*, thus estimates made by this method of the proportions of *X.tropicalis* cells in these implants may be up to 8% inaccurate. From examination of the *X.tropicalis* peaks in these implant nuclei, however, it appears unlikely that very many *X.tropicalis* cells are in mitosis at the time sampled; the small *X.tropicalis* peaks tail off to the baseline some distance short of the marker dividing diploid *X.tropicalis* cells from diploid *X.borealis* cells.

P.I. fluorescence intensity profiles for splenocyte suspensions from implanted animals, (Figure 3.17), show no peak of *X.tropicalis* nuclei; this finding suggests that there are no, or only very few, thymus donor cells in the periphery of host, *X.borealis*, animals at this time after implantation. It is important to remember, however, that spleen cell suspensions contain many red blood cells; these are nucleated, in *Xenopus* and, although whole red cells are gated out of XT-1 FACS analysis, red cell nuclei will be included in P.I. staining analyses. Thus, if the proportion of donor cells remaining in the spleen is low, then very few of these may be represented in a sample of 10,000 spleen cell nuclei.

## 3.4 Discussion

Initial studies reported in this Chapter described the organisation of XTLA-1 expressing T-lymphocytes in thymus and spleen of normal adult LG5 and LM3 animals. Given that fluorescence microscopy and FACS analysis studies, (reported in Chapter 2), had shown that the vast majority of their thymocytes do express this antigen, the bright 'honeycomb' staining pattern, in which apparently all cortical thymocytes were outlined, was not surprising. Also, Nagata, (1985), had previously reported that, in frozen sections of adult J strain thymus, all cortical thymocytes were stained. Equally, the observation that cells of the medulla were by no means all labelled was expected, considering the diversity of cell types commonly found in this area, (epithelial and epithelial reticular cells, myoid cells, granulocytes, etc. as well as lymphocytes), (Clothier & Balls, 1985; Russ, 1986). Some of these non-lymphoid cells may have been present in the single-cell suspensions studied in the previous Chapter, and may have contributed to the very small negatively stained population found. However, many non-lymphoid cell types are lost in the preparation of suspensions. Nagata, (1985), reported that all thymocytes in medullary areas were XTLA-1 positive. Particularly intense staining was seen in the immediate subcapsular layer of these two adult thymus sections, a zone which is frequented by lymphoblasts, which are found in the intestices between epithelial and epithelial reticular cells, (Russ, 1986). Interestingly in the sections of stage 54 and stage 56 larval thymi examined, the subcapsular layer, which also contains many large lymphocytes, often undergoing mitosis, (Nagata, 1976), appeared duller than the bulk of the cortex.

The ventral cavity bodies of *Xenopus*, which disappear at metamorphosis, appear to be thymus-dependent regions, since they are depleted of lymphocytes following early thymectomy, (Manning, 1971). The present study confirms that these pharyngeal lymphoid nodules of controls contain a great many T-lineage cells. XT-1 staining patterns in adult spleen to some extent corresponded to the distribution of T-dependent lymphocytes in this organ as previously described, (Horton and Manning, 1974; Obara, 1982). That is, the majority of the fluorescent cells were observed in the splenic red pulp, with particular accumulations in the perifollicular, (marginal zone), areas of the spleen. However, some XTLA-1 +ve cells were also found within the B-cell rich white pulp zones.

XT-1 staining of adult *X.tropicalis* thymus and spleen sections has confirmed

the observations made in the previous chapter, that this species does not express the XTLA-1 antigen. Furthermore, quinacrine-stained sections of *X.tropicalis* material did not show bright nuclear spots exhibited by *X.borealis* cells. Thiébaud, (1983), commented that of all the species held in her laboratory, only *X.borealis* and *X.fraseri* showed this 'spotting' pattern. No mention was made of *X.tropicalis* in her report, but we can now state that cells of this species are negative for this marker.

With regard to the study of the kinetics of infiltration of, and differentiation within, *X.tropicalis* larval thymus by *X.borealis* TX host cells, a number of points arise. From comparisons of the number of positively labelled cells observed in quinacrine and XT-1 stainings of 9 day post-implantation, (p.-i.), sections, it is strongly suggested that newly immigrating *X.borealis* T-lymphocyte precursors are XTLA-1 negative, (XTLA-1-ve). Thus, although Q +ve cells with the morphological characteristics of lymphocytes are seen in moderate numbers in implants examined at this time point, very few of these are yet XTLA-1 +ve. Nagata, (1986b), in his ontogenetic study of XTLA-1 expression, found that only 8% of thymocytes from stage 48 larvae carry this marker, implying that T-lymphocyte precursors of *Xenopus* are XTLA-1-ve.

The majority of Q+ve cells, and all XTLA-1 +ve cells observed at 9 days p.-i were found in the cortex. In Horton, Russ et al.'s, (1987), studies, Q+ve *X.borealis* TX larval hosts were implanted with thymi from donors which were apparently *X.borealis*, but which did not carry the quinacrine marker. These latter animals are referred to as Q-ve "*X.borealis*". In these author's observations of Q-ve "*X.borealis*" implants into Q+ve *X.borealis* TX larval hosts, host lymphocytes were observed mainly in the medulla. Although comparisons will be drawn during this discussion, with Russ's, (1986), and with Horton, Russ et al's, (1987), observations, it is important to note that there are two major differences between their protocols and those used in the current studies: thus, all implants described here were from age- and stage-matched donors, whilst their studies employed adult normal or gamma-irradiated, (lymphocyte-depleted), implants. Also, the current experiments involved transplantation of thymi across species barriers, while earlier work, at least with Q-ve into Q+ve *X.borealis* transfers, probably involved less genetic disparity between donor and host.

By 4 weeks p.-i, implants had lost their normal cortico-medullary organisation. This would appear to be a usual development during thymus implantation; Horton, Russ et al., (1987), noted a similar feature in Q-ve to Q+ve host implants examined at 3 weeks p.-i. Presumably this reflects the death or migration of large numbers of donor-type cortical lymphocytes and the, as yet, limited immigration and/or multiplication of host-type lymphocyte precursors. Of the four implants observed at this time, three now contained 30–50% Q+ve cells, predominantly located in the cortex. However, XTLA-1+ve cell numbers were still appreciably lower, underlining the point made earlier that XTLA-1 expression is delayed following arrival or proliferation in the thymus. The host animals had, by 4 weeks p.-i, passed through metamorphosis. This point may well be significant, considering Turpen and Smith's, (1989), findings of a second, (or possibly third), wave of thymic colonisation during larval life, which occurred between 38

antigens and engendering a response to other, 'foreign' tissue implants.

Differentiation of XTLA-1 +ve cells proceeded steadily until 55 days p.-i, when XTLA-1 +ve cell distribution approached that of control froglet thymi. Cortico-medullary definition was beginning to be re-established in these implants and, although perhaps smaller than equivalent control thymi, they gave every indication of persisting in the host froglets.

FACS analysis of thymus implant cells made 78 days after operation, revealed that in the two animals examined at this time point, approximately 72% and 89% of the cells within the implant were XTLA-1 +ve. Considering the persistence of *X.tropicalis* cells within the implants, (approximately 8–10%, as assessed by propidium iodide staining), this slightly lower proportion of XT-1+ve cells than might be expected for thymocytes may be explained simply by persistance of non-XTLA-1 expressing donor cells. Complete replacement by host-derived thymocytes may take a considerable time to complete, or, as suggested by Nagata and Cohen, (1984), lymphocyte chimaerism within implants may be persistent in some MHC-mismatched donor-host combinations. Persistence of allogeneic donor-type lymphocytes has also been noted in thymus-grafted nude mice, (Loor, Kindred and Hogg, 1976).

Sections of host spleen analysed at each post-metamorphic sampling time up to 55 days post-implantation, showed very few XTLA-1 +ve cells. These histological findings were unexpected, for several reasons. Nagata's study of the ontogeny of XTLA-1 expression, (1986b), showed that, although the percentage of XTLA-1 +ve cells in the spleen was at background levels at stage 50, (15 days post-fertilisation), significant staining levels, (6%), were reached by stage 52, (20 days post- fertilisation). At stage 56, (40 days post-fertilisation), adult levels, (30%), of XTLA-1+ve splenocytes were observed, and these levels persisted, with no detectable drop until the end of the study, when animals were one year old. Thus detectable proportions of T-lineage splenocytes were found only 12 days after XTLA-1+ve cells were found in the thymus. Even allowing for the disruption of normal development caused by the absence of thymic influence during 3 weeks of larval life, (from thymectomy at day 7 until implantation at day 28 post-fertilisation), and by the possible emigration of XTLA-1-ve *X.tropicalis* T-cells into the host periphery, the apparent lack of significant detectable XTLA-1 expression in the host spleen at 55 days post-implantation is unusual.

Previous thymus-implantation studies, (Nagata and Kawahara, 1982; Nagata and Cohen, 1984; Horton and Horton, 1975, Horton et al., 1990 in press), have shown that restoration of T-dependent responses in TX animals can be achieved even by implantation of xenogeneic thymi. Where this question has been addressed, (e.g. by Nagata and Cohen, 1984), host-derived T-cells have been found in the peripheral lymphoid organs of these thymus- implanted animals, implying that restoration is not solely due to the action of donor-derived T-lymphocytes.

Thus, although no functional studies were undertaken in these *X.borealis* animals implanted with thymus from *X.tropicalis*, the differentiation of large numbers of host thymocytes and the experience of previous experimenters with thymus-implanted models lead us to expect that these host animals should eventually

contain host-derived peripheral T-lineage cells. In contrast to the observations on XT-1 labelled spleen sections examined up to 55 days post- implantation, FACS analysis of XT-1 labelled splenocyte suspensions from two thymus-implanted TX animals, made 78 days after implantation, showed that 11.3% and 19% of their splenocytes were XTLA-1 +ve.

The number of XT-1 labelled splenocytes seen in the two animals examined by FACS at 78 days post thymus implantation was considerably greater than that seen in any sections of spleens from thymus-implanted animals at earlier time points. Thus, either the technique of XT-1 staining of frozen spleen sections underestimated the number of positively labelled cells present, or there had been a substantial emigration of XTLA-1 +ve cells into the periphery between 55 and 78 days post-implantation. Since frozen sections of control spleens, XT-1 labelled by the same protocol, show moderately high numbers of labelled cells, distributed in areas previously described as being thymus dependent, the former possibility seems unlikely. Therefore, the establishment of a complement of XTLA-1 +ve splenocytes approaching that found in control *X.borealis* animals is apparently delayed for several months after thymus-implantation, and then takes place within a few weeks.

It is important to note that, since XT-1 is not a pan-T-cell marker, functional thymus-dependent cells which did not express XTLA-1 may have been present in host spleens much sooner after thymus- implantation than those XTLA-1 +ve cells detected in this study. Further experimentation with this model system, including functional analyses such as kinetic tests of splenocyte T-cell mitogen responsivity, and MLC responsivity are necessary to clarify this point.

The relatively late arrival in the host spleen of appreciable numbers of XTLA-1 +ve lymphocytes, or their delayed differentiation in situ, under the influence of the implanted thymus, may relate to the very foreign nature of the implanted thymus. One can hypothesise that, in this donor-host combination, thymic stromal elements required to interact with developing XTLA-1 +ve thymic cells, to induce their maturation into XTLA-1 +ve peripheral T-cells, are missing from newly implanted thymi. With time, either host stromal elements may colonise the implanted thymi, permitting normal development of XTLA-1 +ve splenocytes or possibly donor thymic elements which interfere with this process are lost. A further possibility, if peripheral T-cells can normally develop in situ under the influence of humoral factors from the thymus, is that *X.tropicalis* thymic stroma do not secrete factors that promote XTLA-1 +ve splenocyte differentiation.

Experiments to investigate the functional development of TX animals given donor thymi of various incompatibilities are examined in the next Chapter. These studies, in part, explore whether xenogeneic *X.tropicalis* thymus implants effect restoration of a variety of T-cell functions.

**Figure 3.1, A and B** Phase contrast and fluorescence views of 6$\mu$ 'experimental', (XT-1 mAb + secondary antibody), frozen section of adult LM3 thymus cortical region. Note typical 'honeycomb' staining pattern, with bright fluorescence in the immediate subcapsular layer. The thymus capsule, (c), and trabeculae, (t), are unstained. m = melanin. x500

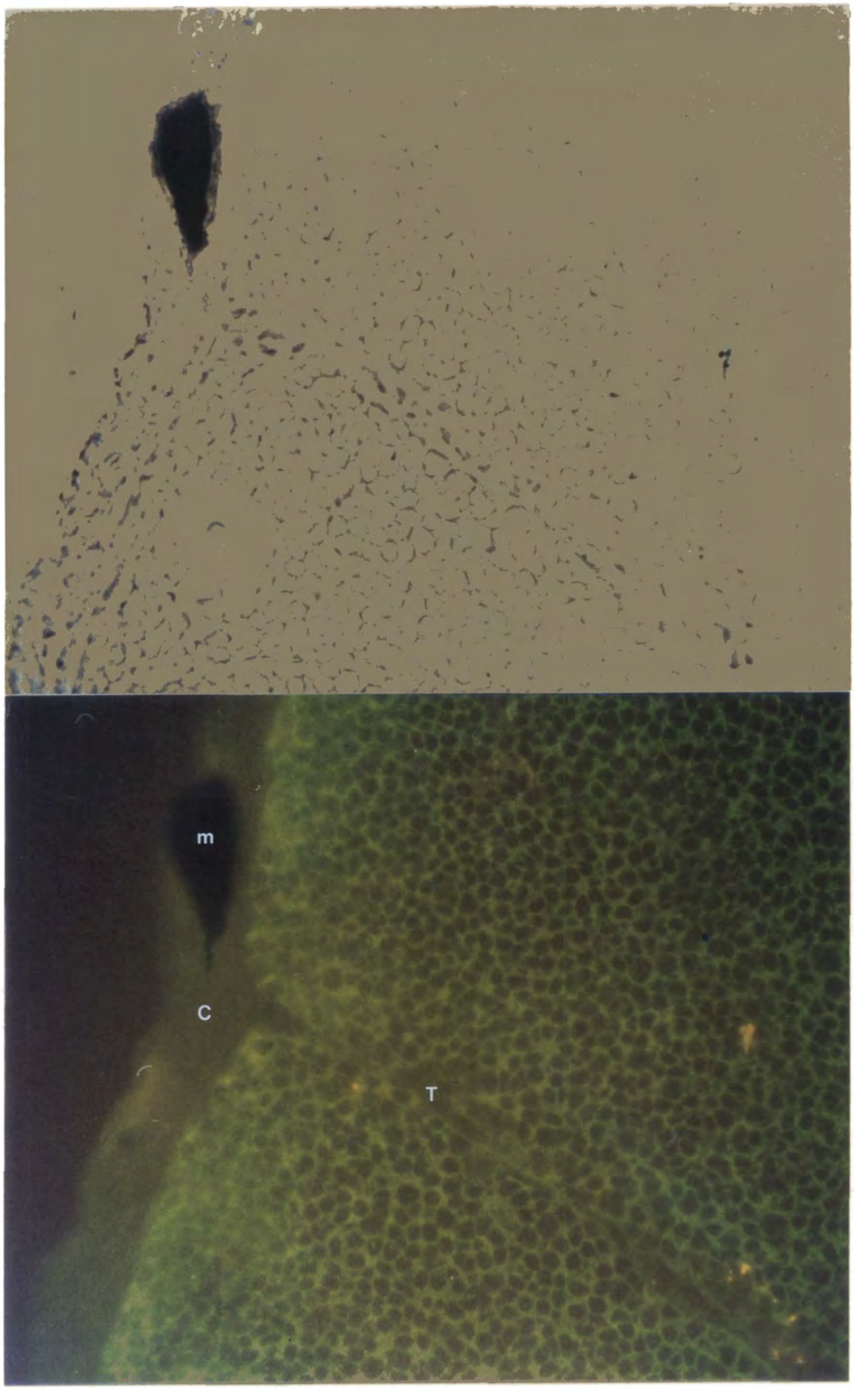

m
C
T

**Figure 3.1, C and D** Phase contrast and fluorescence views of 6$\mu$ 'control' frozen section of adult LM3 thymus cortical region. Thymus capsule = c; trabeculae = t; blood vessel = bv. x500

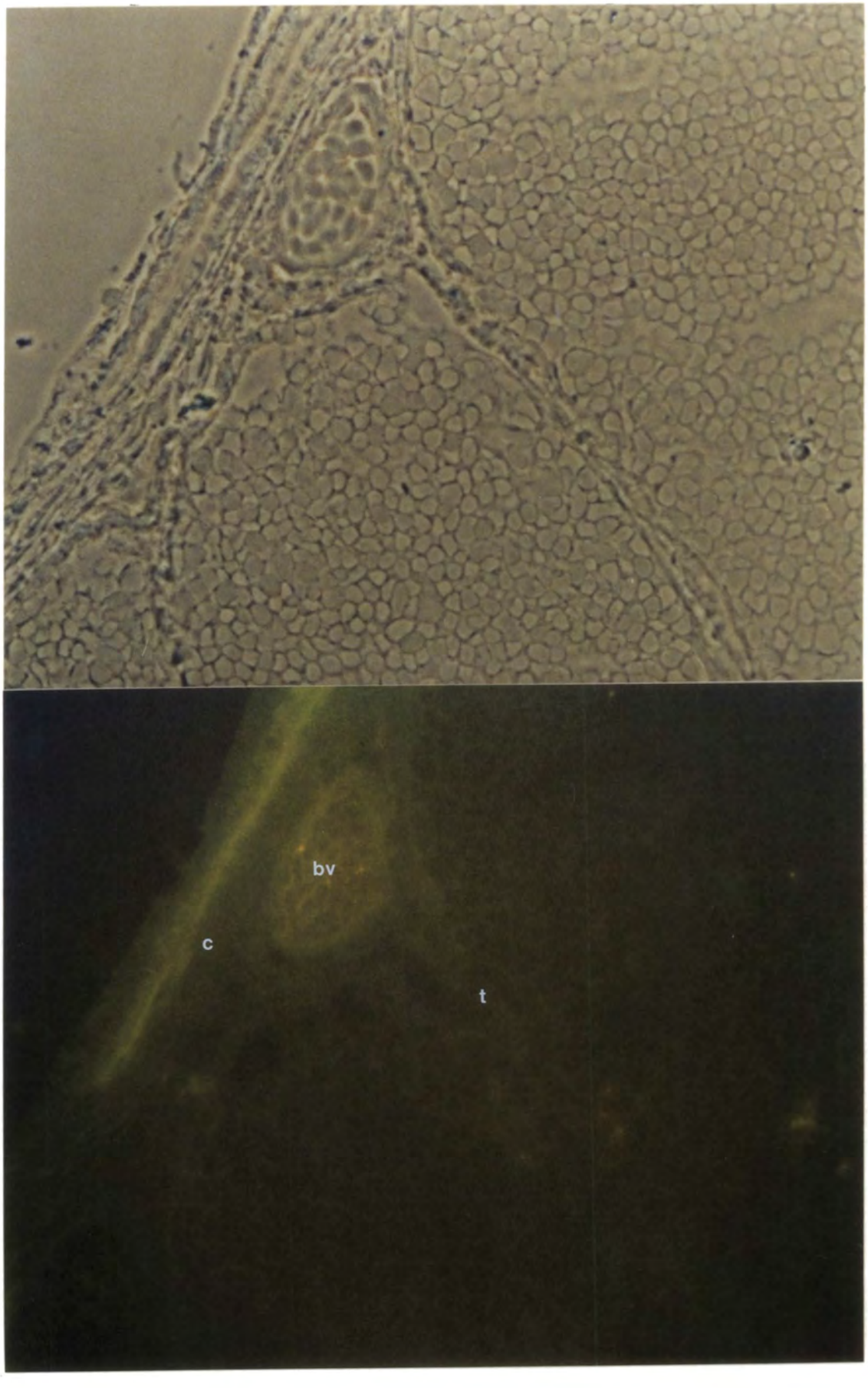

bv
c
t

**Figure 3.2, A and B** Phase contrast and fluorescence views of 6$\mu$ 'experimental', (XT-1 mAb + secondary antibody), section of adult LM3 thymus medulla. Observe labelled medullary lymphocytes, and unstained, non-lymphoid cells, (arrowed). x500

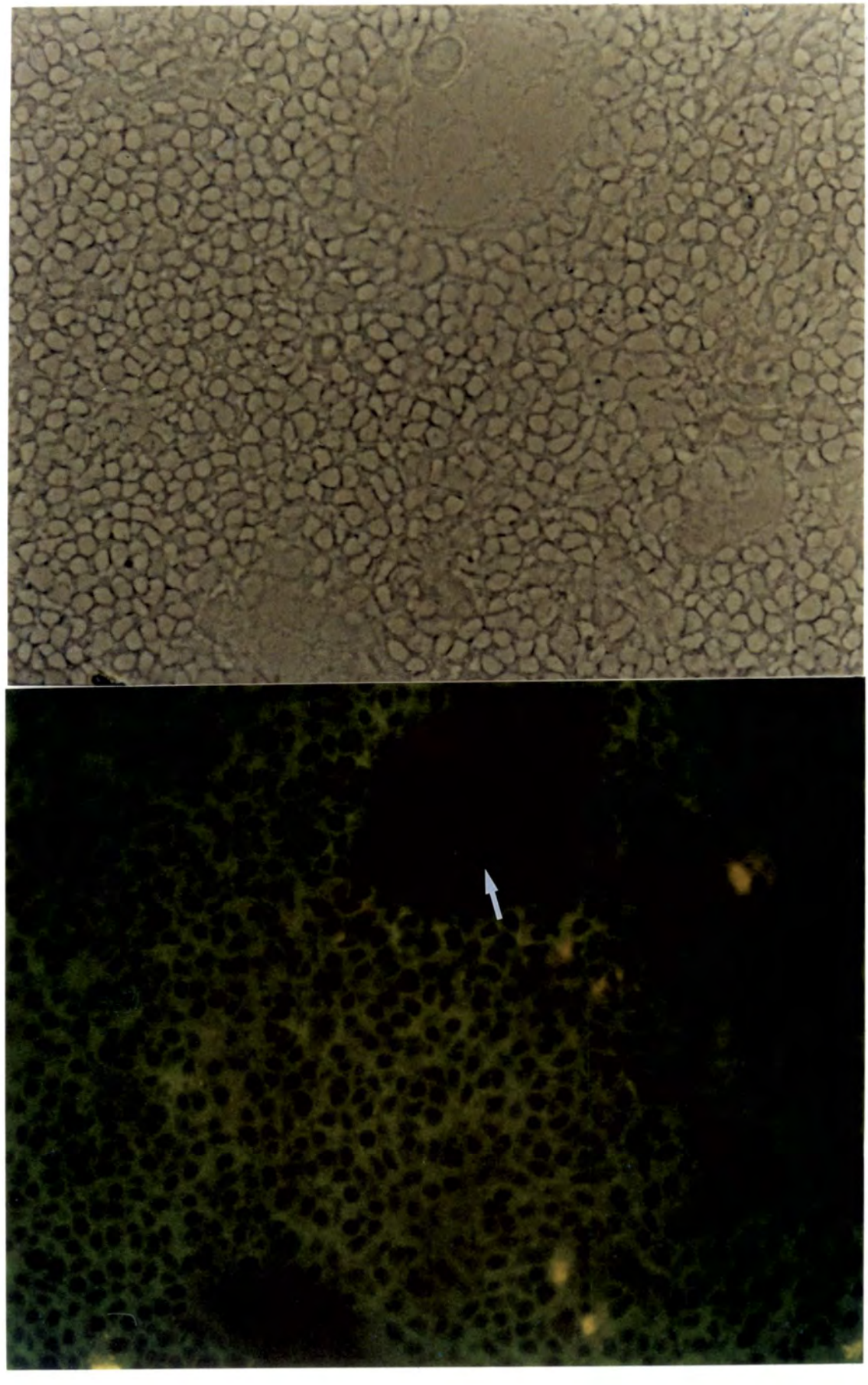
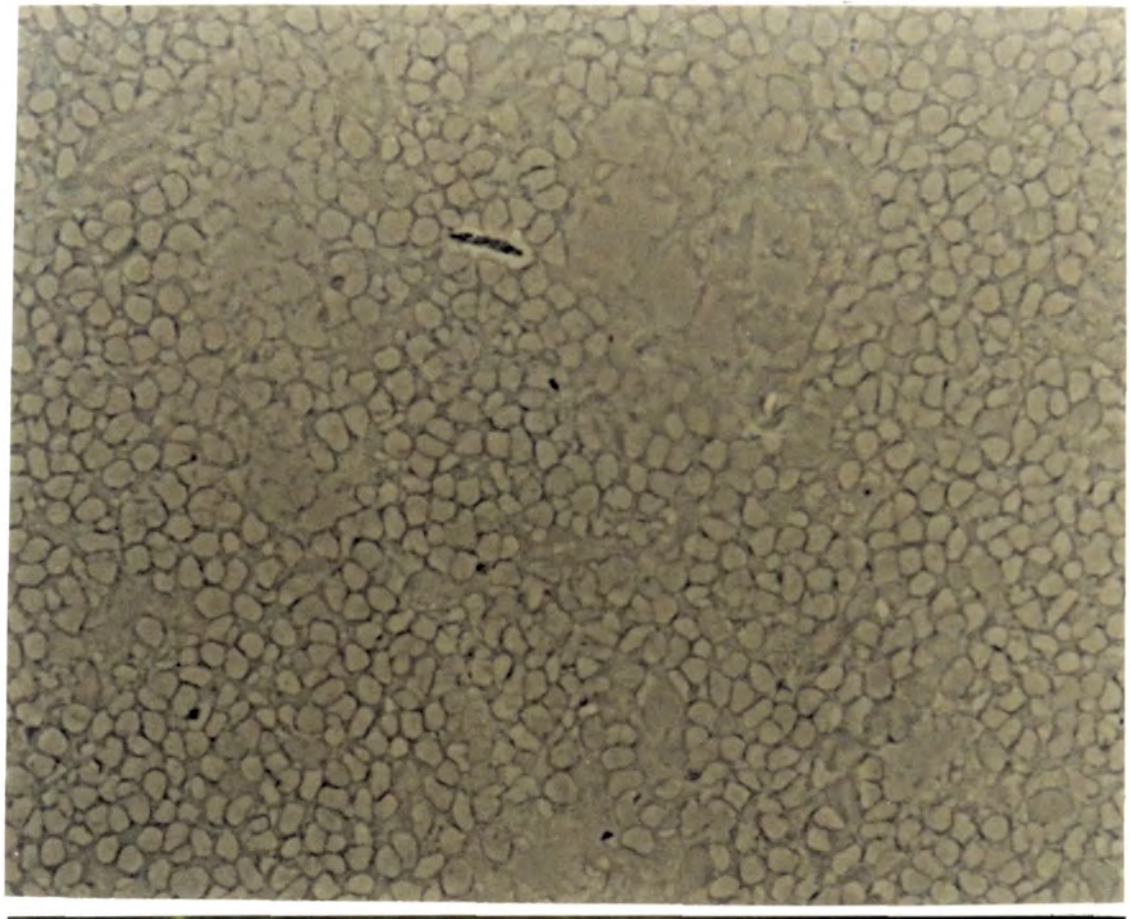


**Figure 3.2, C and D** Phase contrast and fluorescence views of 6$\mu$ 'control' frozen section of adult LM3 thymus medulla. x500



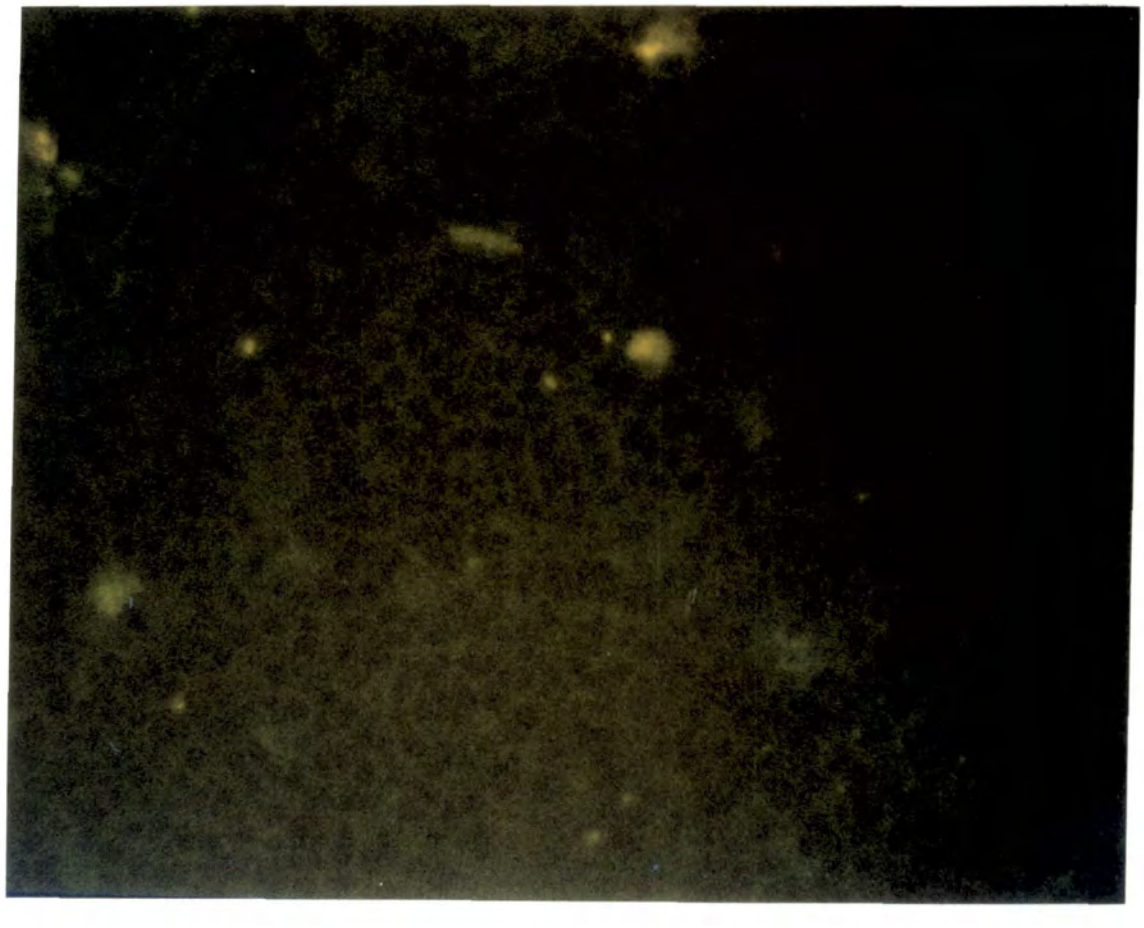


**Figure 3.3, A and B** Phase contrast and fluorescence views of 6$\mu$ 'experimental' section of adult *X.tropicalis* thymus cortex. Note lack of labelling with XT-1 mAb. Thymus capsule = c; melanin = m. x500

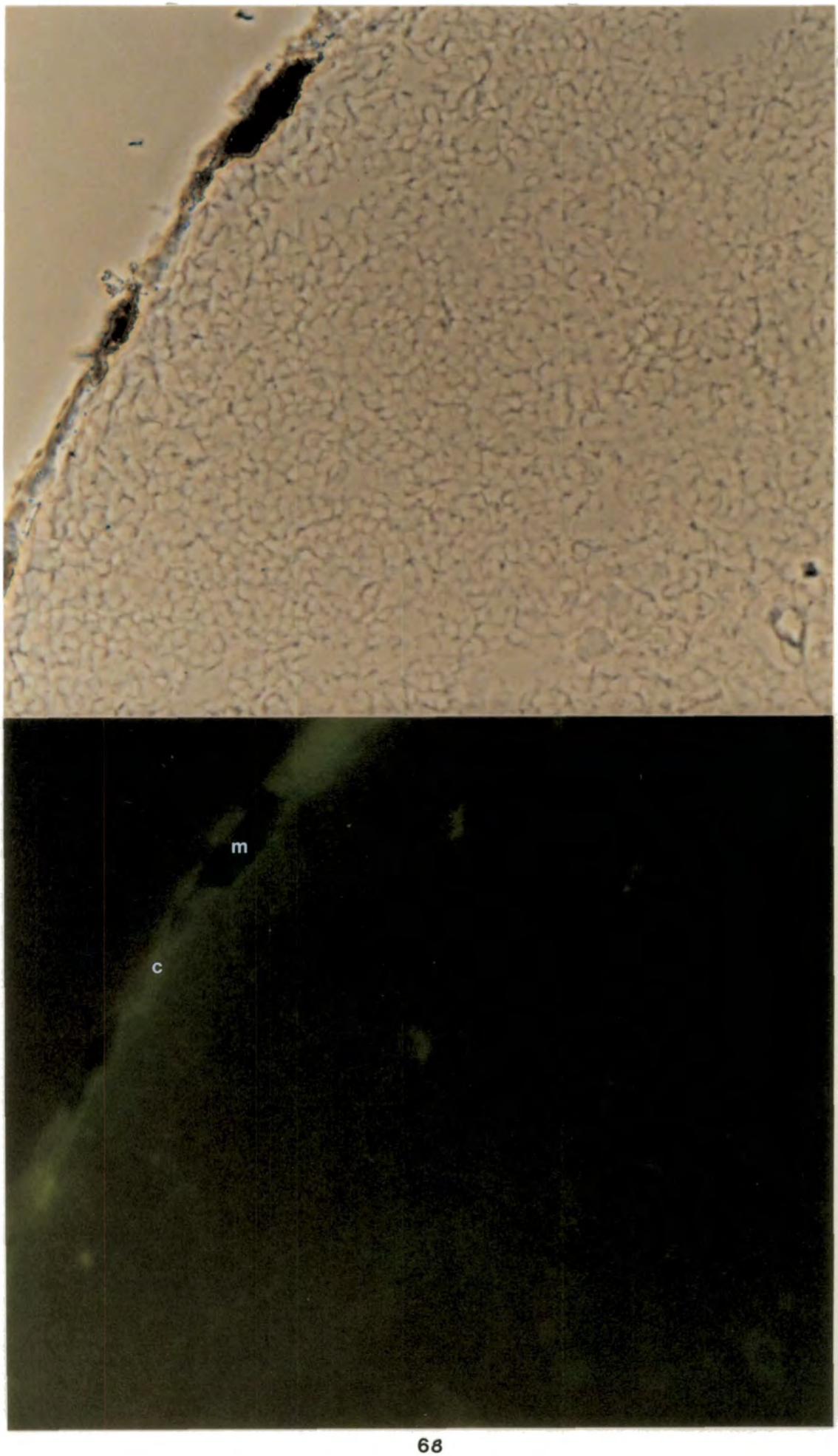

m
c

**Figure 3.4, A and B** Phase contrast and fluorescence views of 6$\mu$ 'experimental', (XT-1 mAb + secondary antibody), section of adult LM3 spleen. XTLA-1 positive cells are seen in the red pulp, (rp), particularly in the marginal zone,(mz), around the white pulp follicles, (wp). Labelled cells are also seen within the white pulp follicles. x500

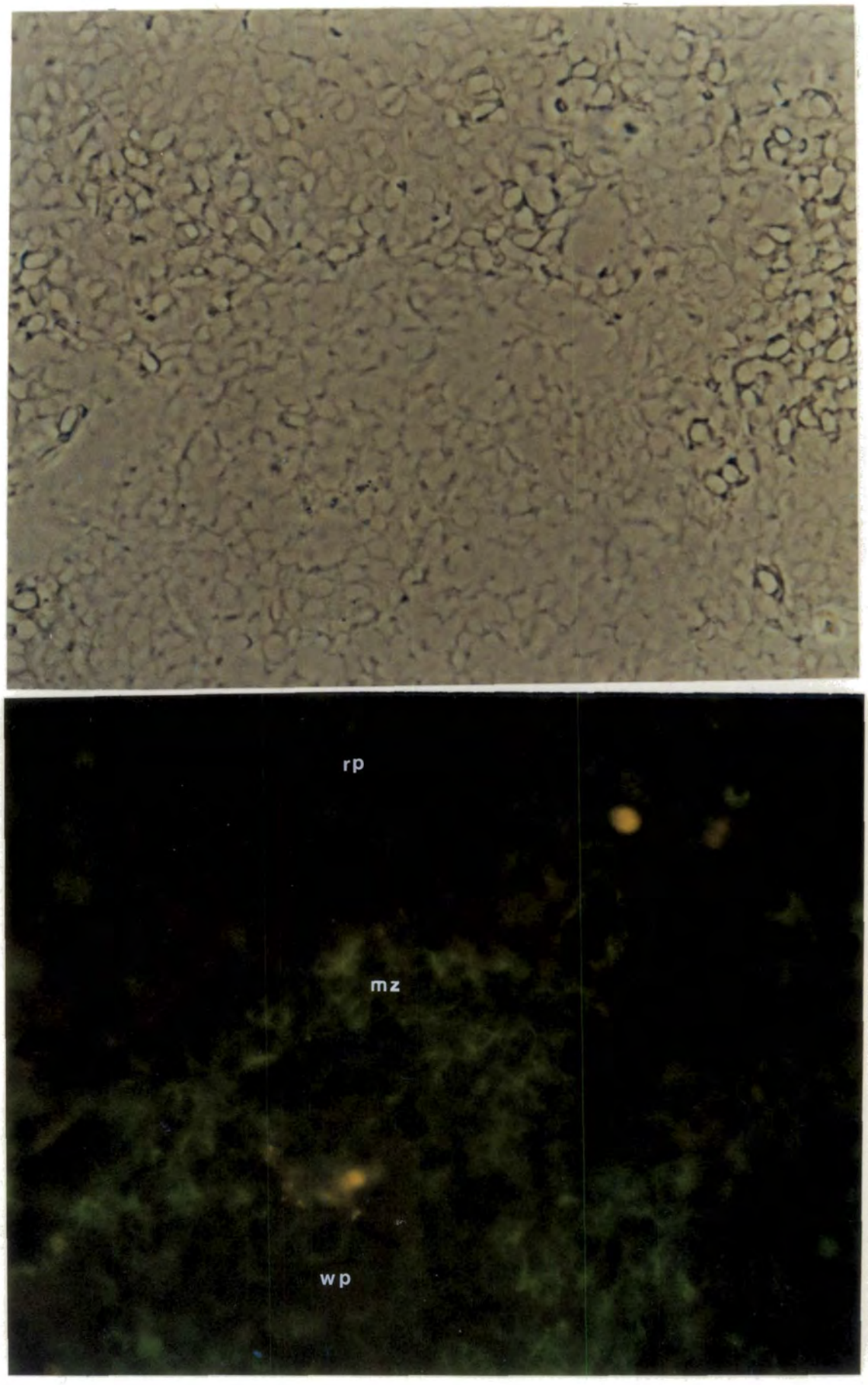

rp
mz
wp

**Figure 3.4, C and D** Phase contrast and fluorescence views of 6$\mu$ 'experimental', (XT-1 mAb + secondary antibody), section of adult LM3 spleen. XTLA-1 +ve cells are seen in the marginal zone, outside the boundary layer, (arrowed), and scattered in a white pulp follicle, (wp). x500

**Figure 3.4, E and F** Phase contrast and fluorescence views of 6$\mu$ 'control' frozen section of adult LM3 spleen. White pulp follicle = wp; arrows point to boundary layer. x500

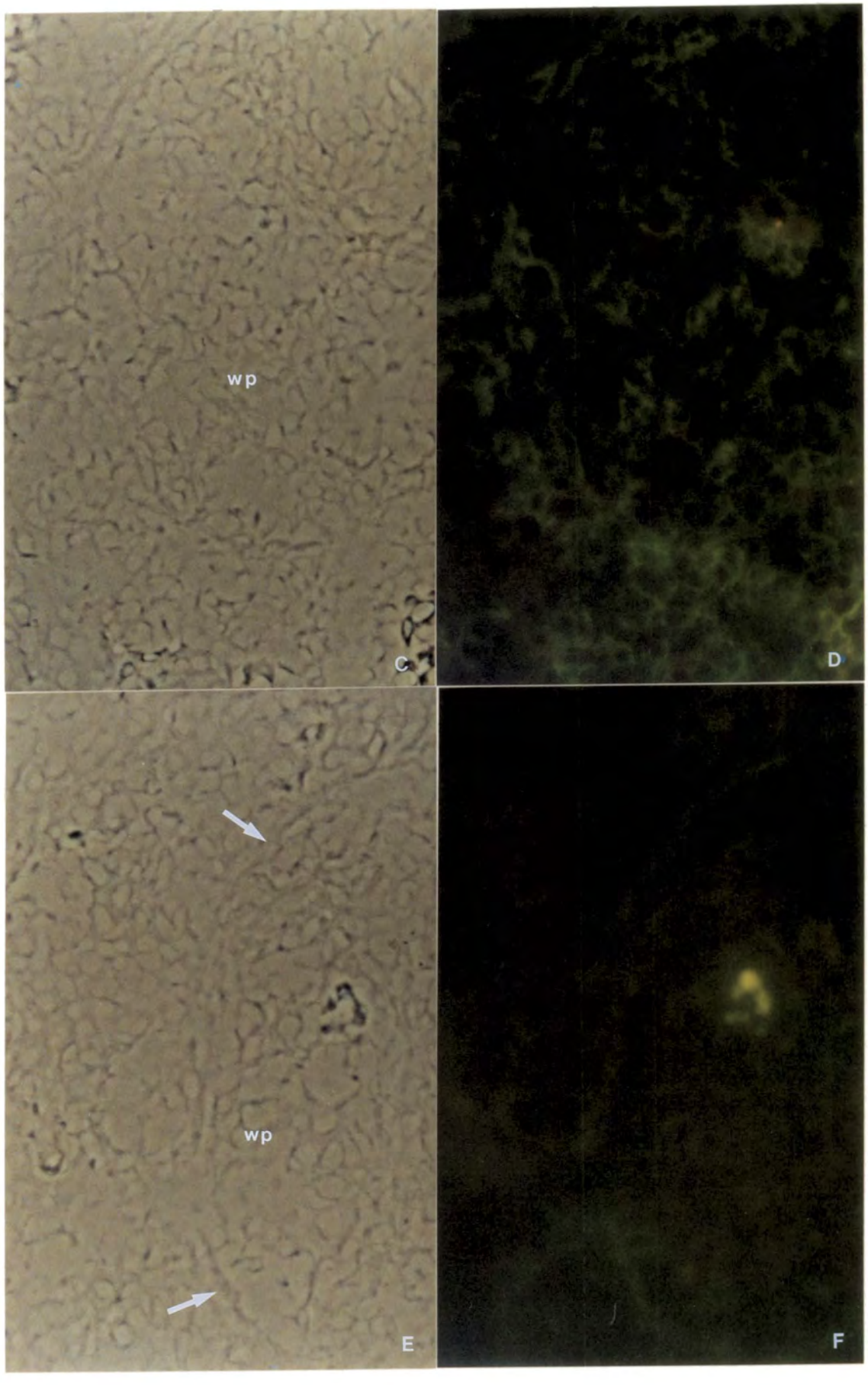

wp
C
D
wp
E
F

**Figure 3.4, A and B** Phase contrast and fluorescence views of 6$\mu$ 'experimental', (XT-1 mAb + secondary antibody), section of adult LM3 spleen. XTLA-1 positive cells are seen in the red pulp, (rp), particularly in the marginal zone,(mz), around the white pulp follicles, (wp). Labelled cells are also seen within the white pulp follicles. x500

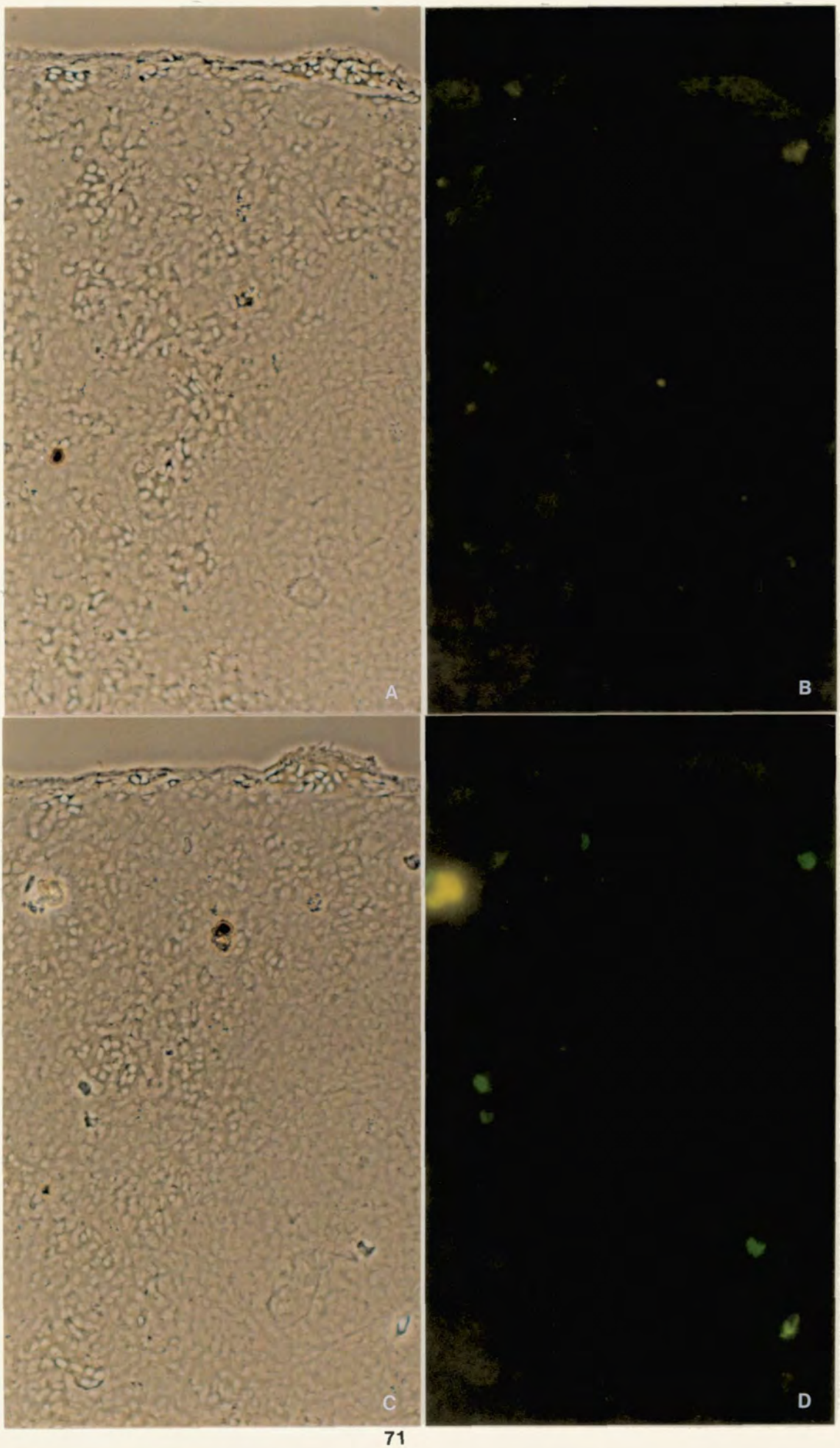

A
B
C
D

**Figure 3.6, A and B** Phase contrast and fluorescence views of 6$\mu$ 'experimental', (XT-1 mAb + secondary antibody), section of outbred *X.laevis* larval, (stage 54), thymus cortex. Bright 'honeycomb' staining is observed in most of the cortex, with duller staining in the immediate subcapsular layer. Melanin = m. x500

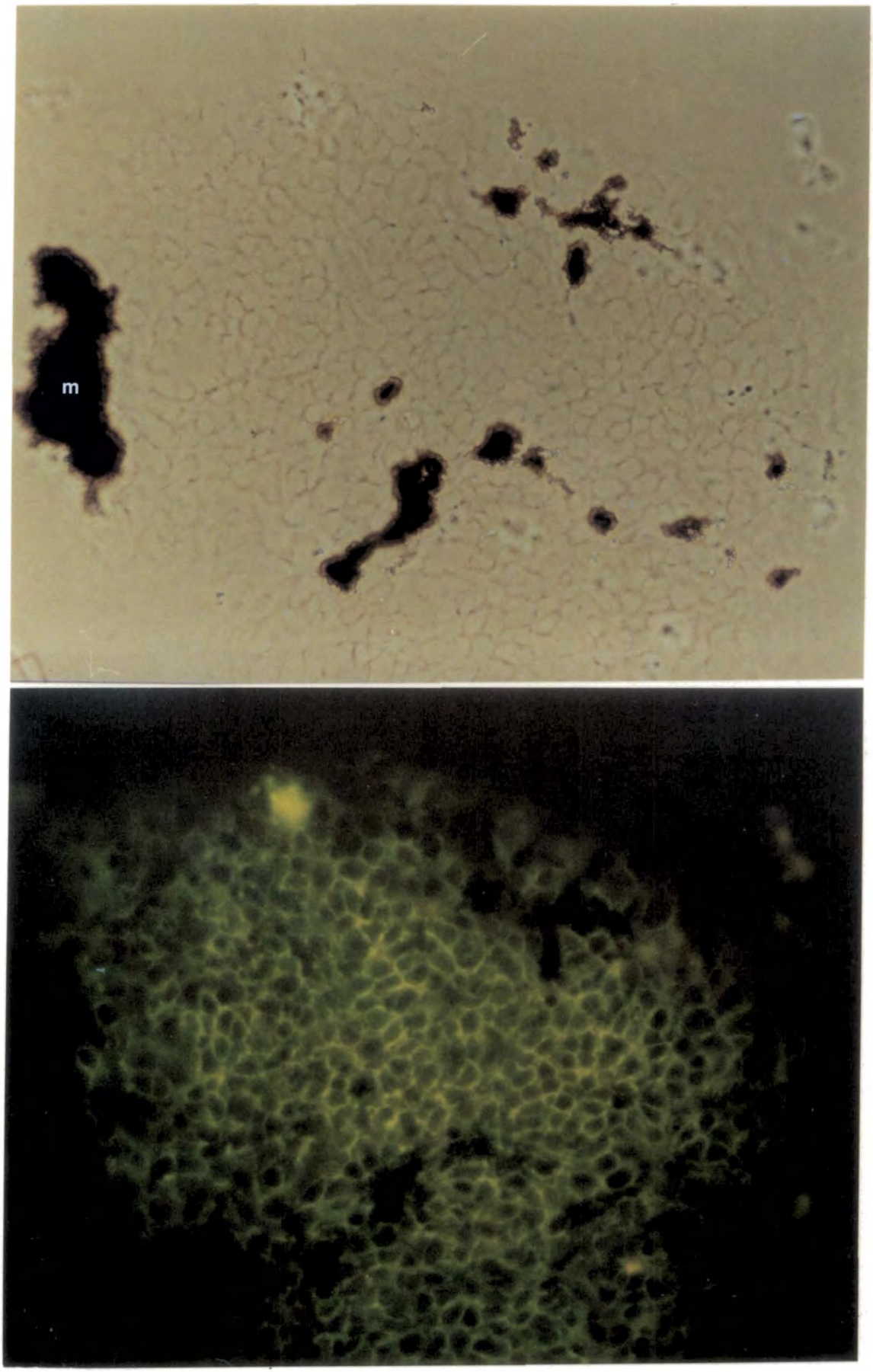
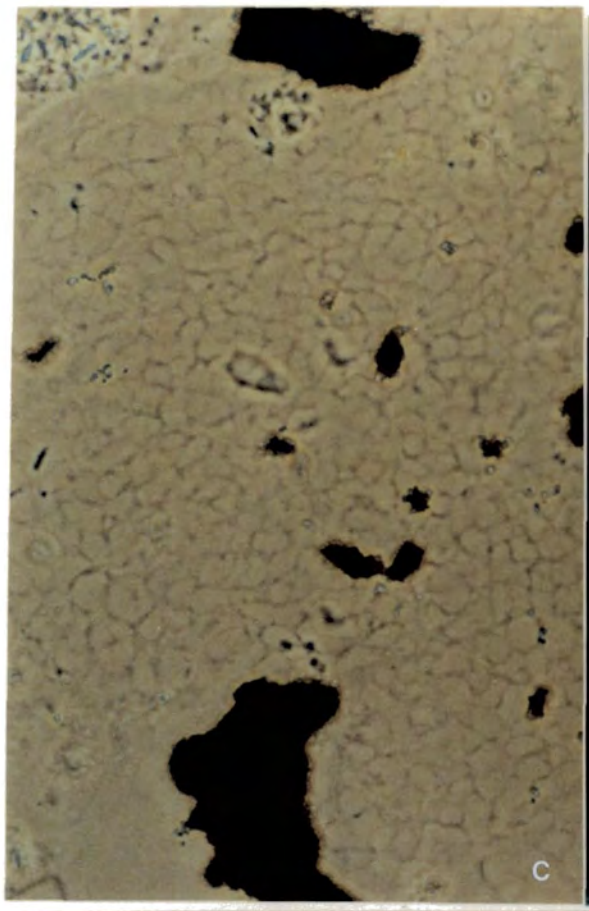
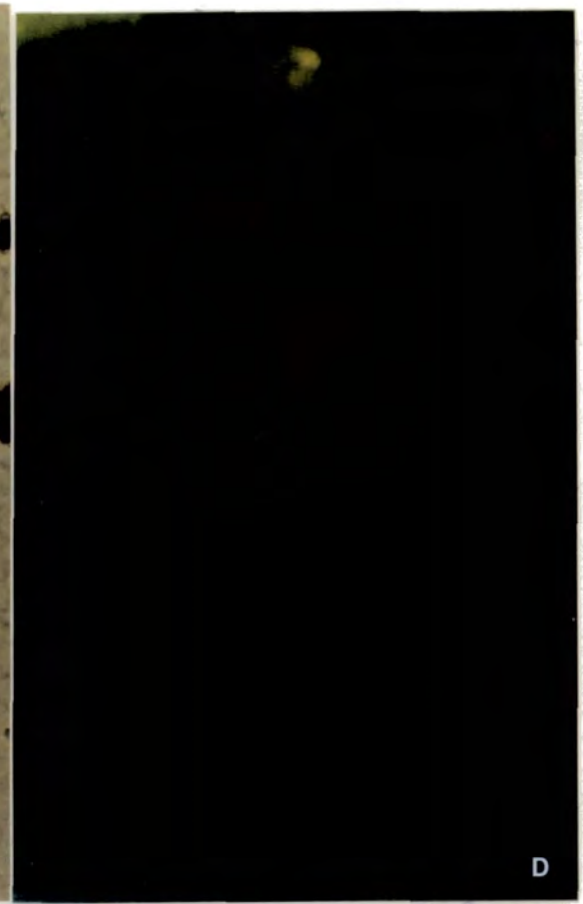

m

**Figure 3.6, C and D** Phase contrast and fluorescence views of 6$\mu$ 'control' frozen section of outbred *X.laevis* larval, (stage 54), thymus cortex. x500

**Figure 3.7A** Phase contrast view of 6$\mu$ 'experimental' section of *X.borealis* larval, (stage 56), pharyngeal region. x250


C


D

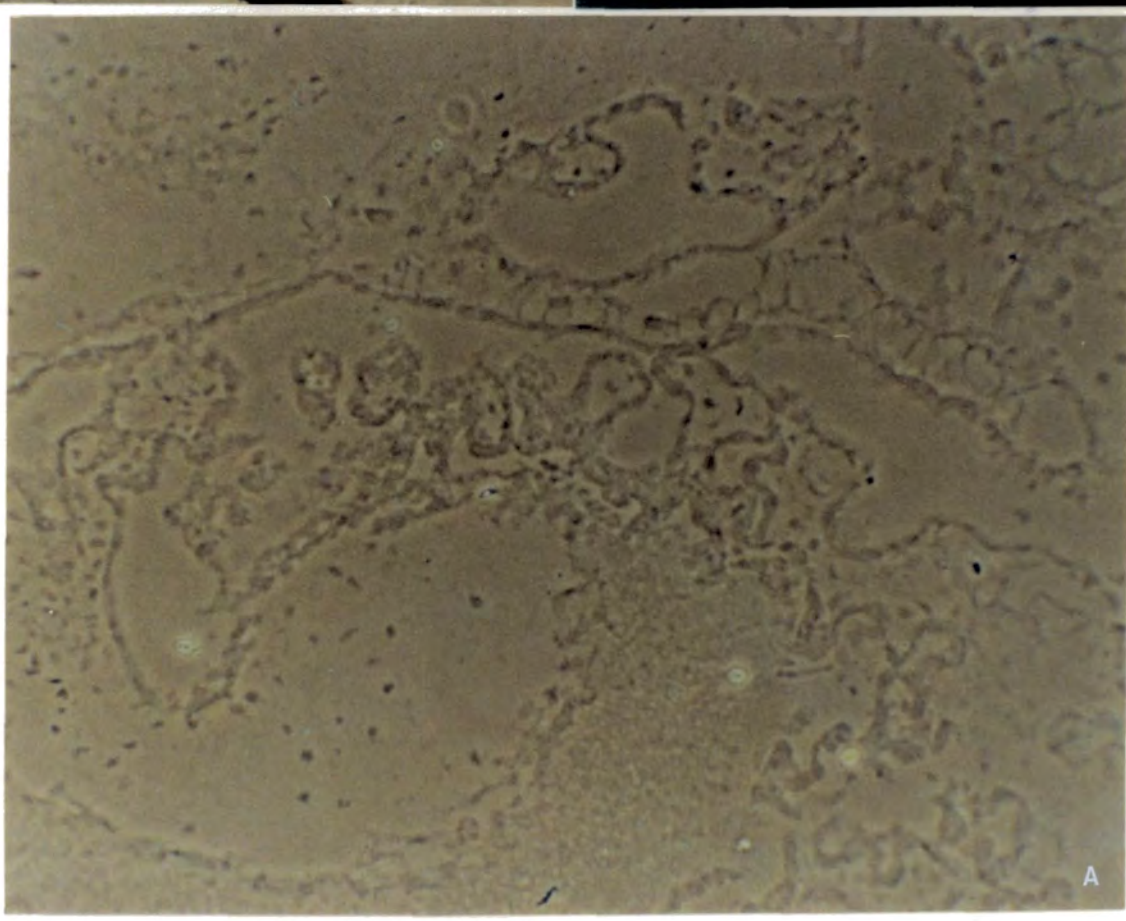

A

**Figure 3.7B** Fluorescence view of 6$\mu$ 'experimental', (XT-1 mAb + secondary antibody), section of *X.borealis* larval, (stage 56), pharyngeal region. (see Figure 3.7A). Small accumulations of XTLA-1 positive cells are seen in the gill region. x500

**Figure 3.8A** Phase contrast view of 6$\mu$ 'experimental', (XT-1 mAb + secondary antibody), section of *X.borealis*, (stage 56), pharyngeal region, showing position of ventral cavity body, (arrowed). x250

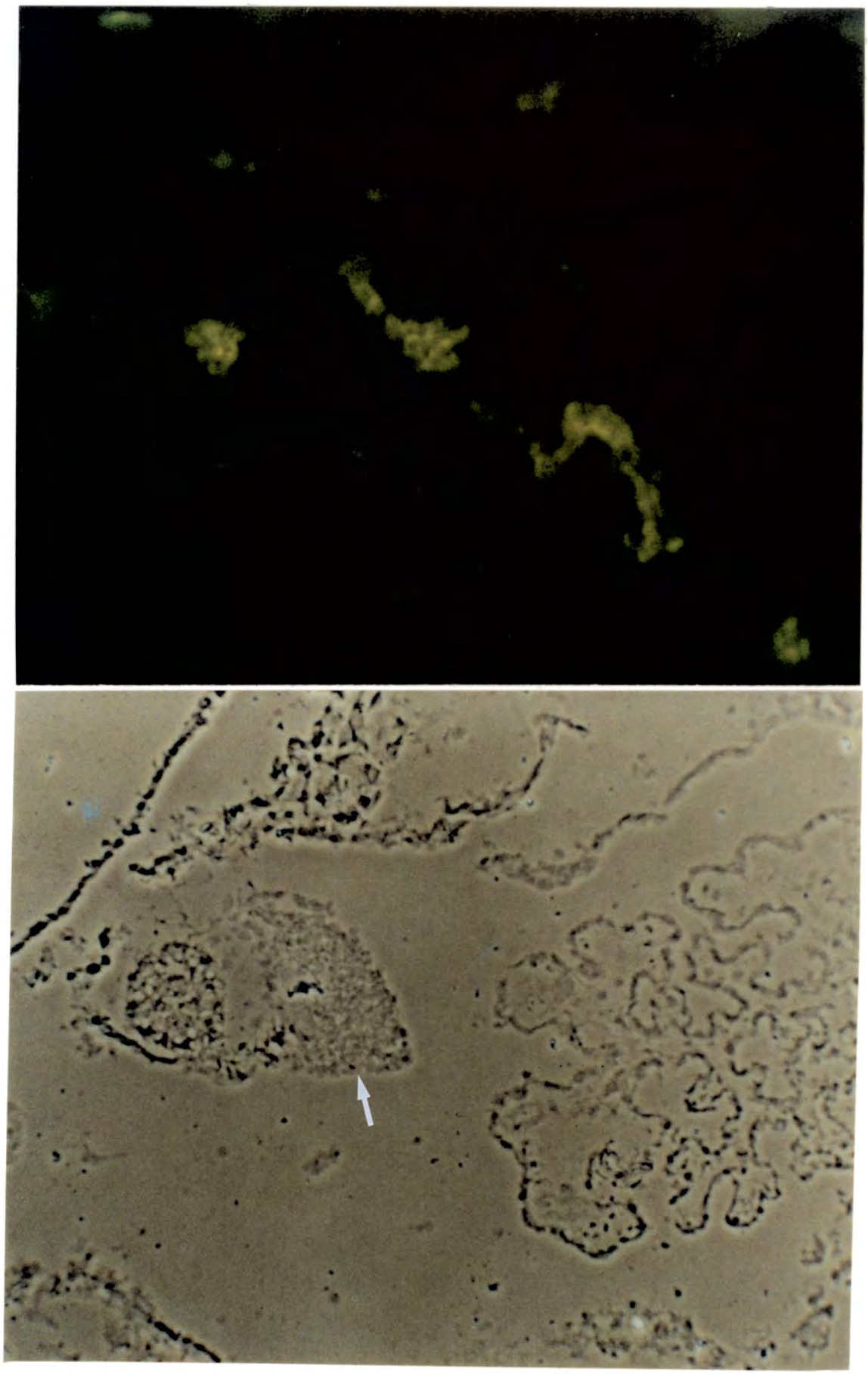
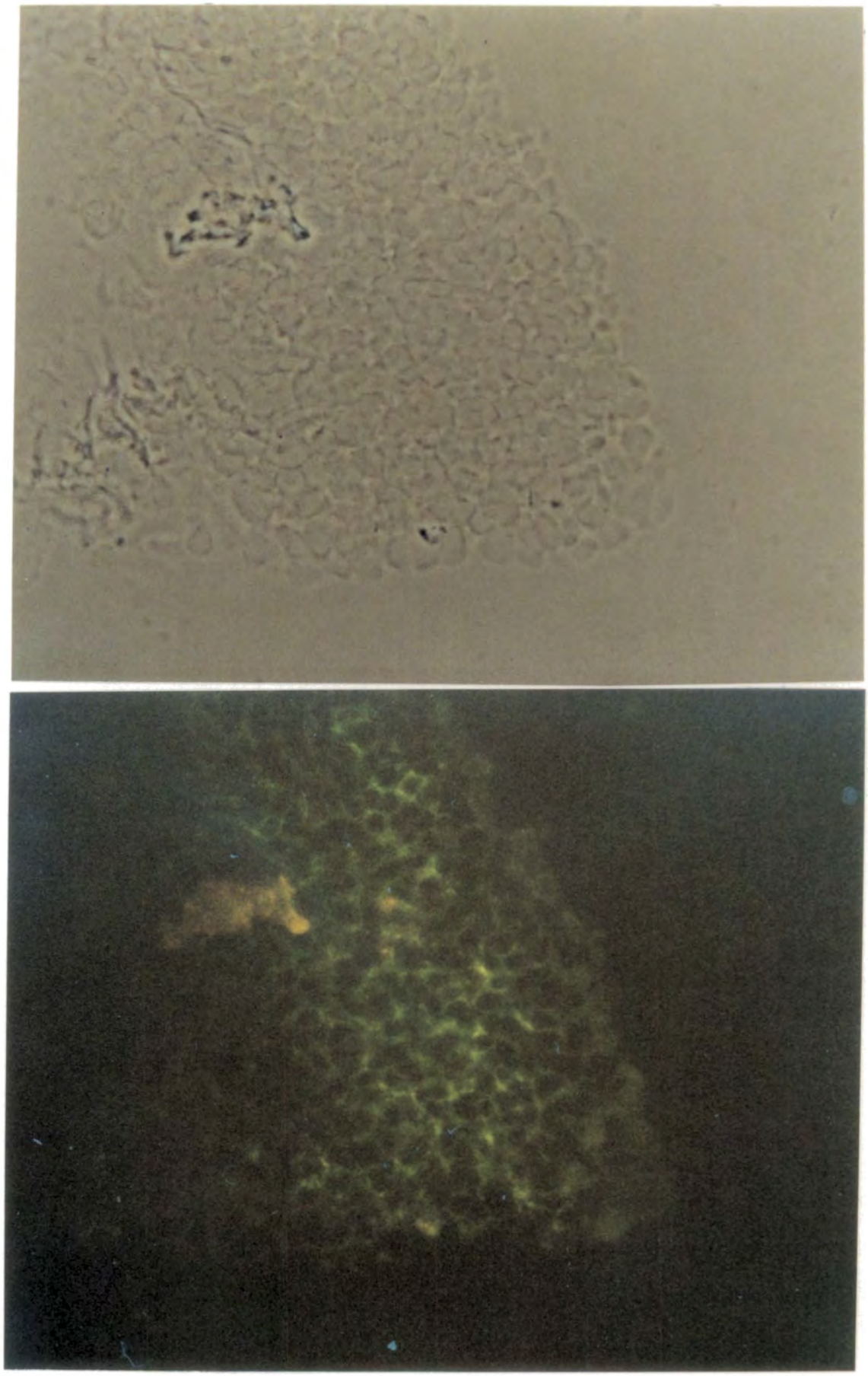


**Figure 3.8, B and C** Phase contrast and fluorescence views of 6$\mu$ 'experimental', (XT-1 mAb + secondary antibody), section of ventral cavity body from stage 56 larval *X.borealis*. (See Figure 3.8A). This organ contains XTLA-1 positive lymphocytes. x500



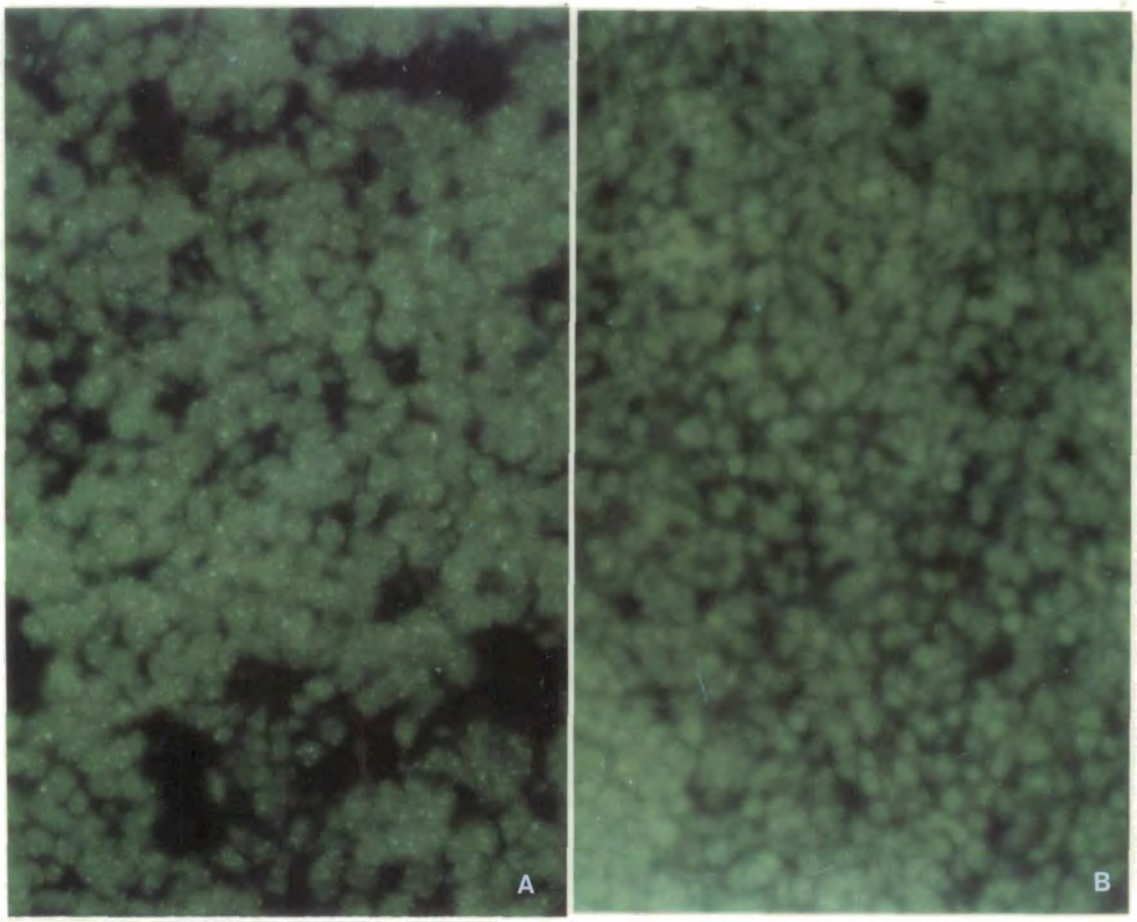
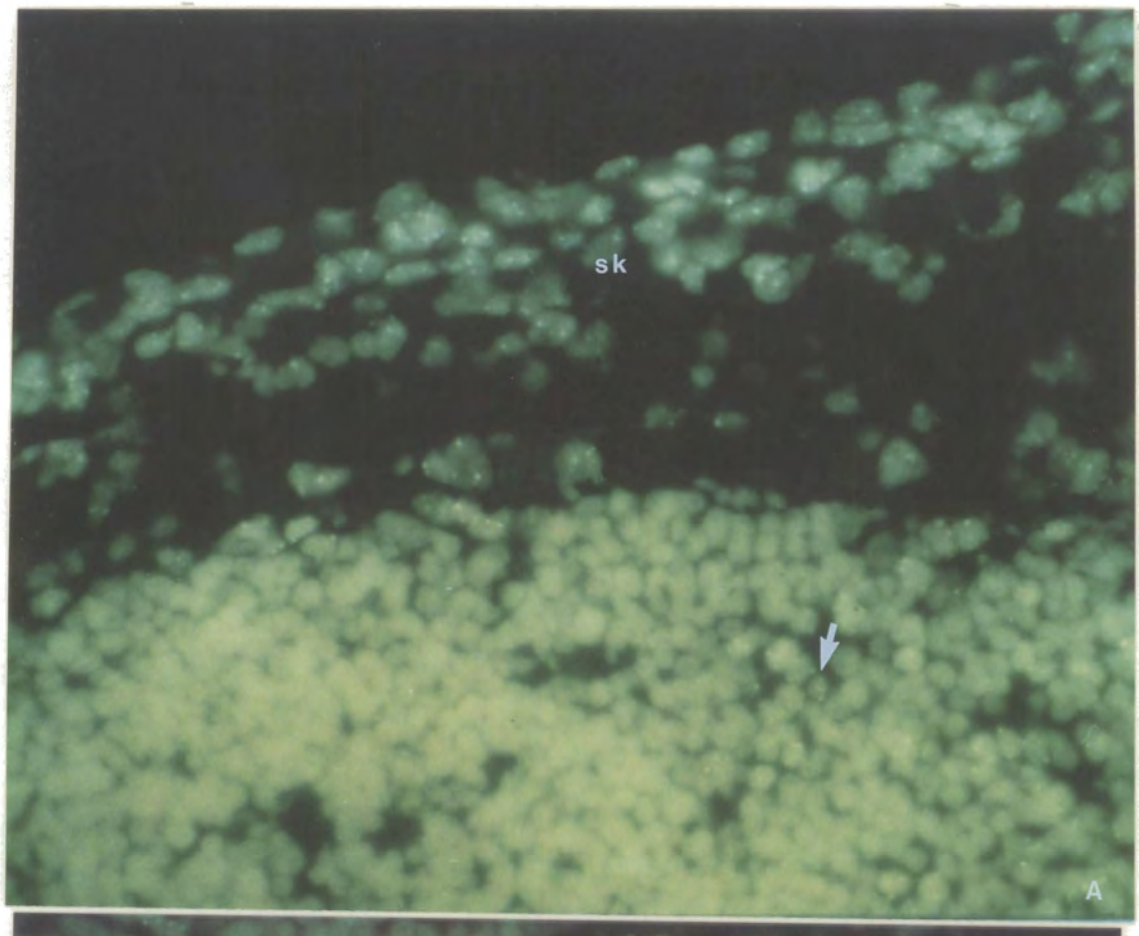



**Figure 3.9, A and B** Quinacrine-stained 6$\mu$ frozen sections of *X.borealis*, (3.9A), and *X.tropicalis*, (3.9B), thymi, showing 'spotted' appearance of *X.borealis* nuclei and 'non-spotted' appearance of *X.tropicalis* nuclei. x500

**Figures 3.10 - 3.13** show 6$\mu$ frozen sections of *X.tropicalis* thymi which were implanted into TX *X.borealis* hosts during larval life, and 6$\mu$ frozen sections of host spleens.

**Figure 3.10, A and B** *X.tropicalis* thymus implanted into TX *X.borealis* host, 9 days post-implantation. Quinacrine-stained 6$\mu$ frozen sections show small numbers of Q+ve host lymphocytes, (arrowed), in the cortex of predominantly Q-ve thymus implants. Note Q+ve host skin, (sk), above thymus implant in Figure 3.10A. x500


sk
A

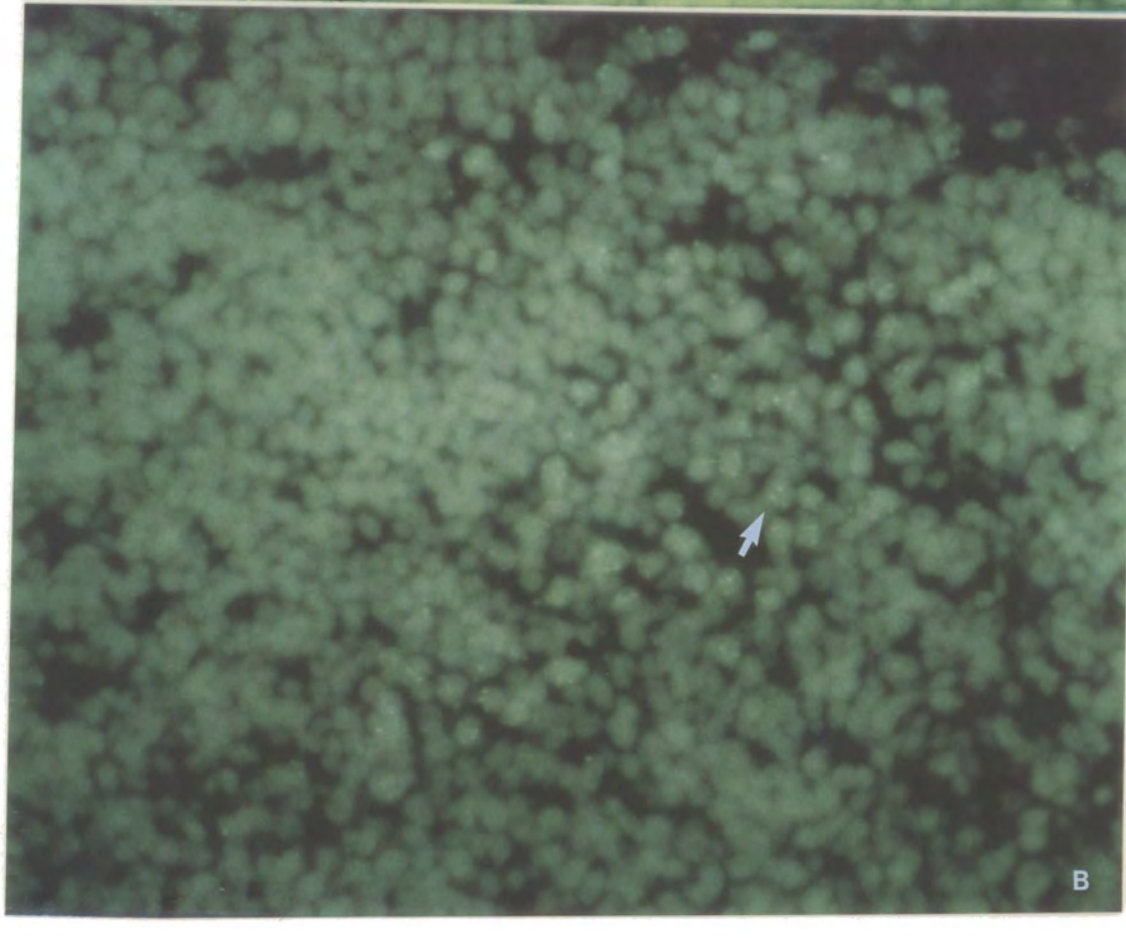
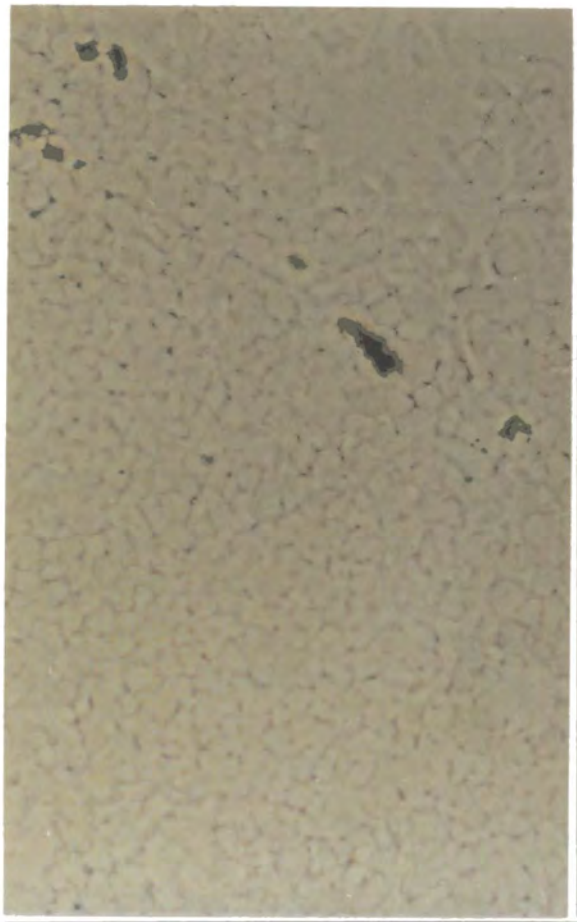

B

**Figure 3.10, C and D** *X.tropicalis* thymus implanted into *X.borealis* host, 9 days post-implantation. Phase contrast and fluorescence views of XT-1 labelled implant cortex, showing a single XTLA-1 +ve lymphocyte. x500

**Figure 3.11A** *X.tropicalis* thymus implanted into *X.borealis* host,28 days post-implantation. Quinacrine-stained 6$\mu$ frozen section of thymus implant cortex, showing large numbers of Q+ve lymphocytes, (arrowed). x500



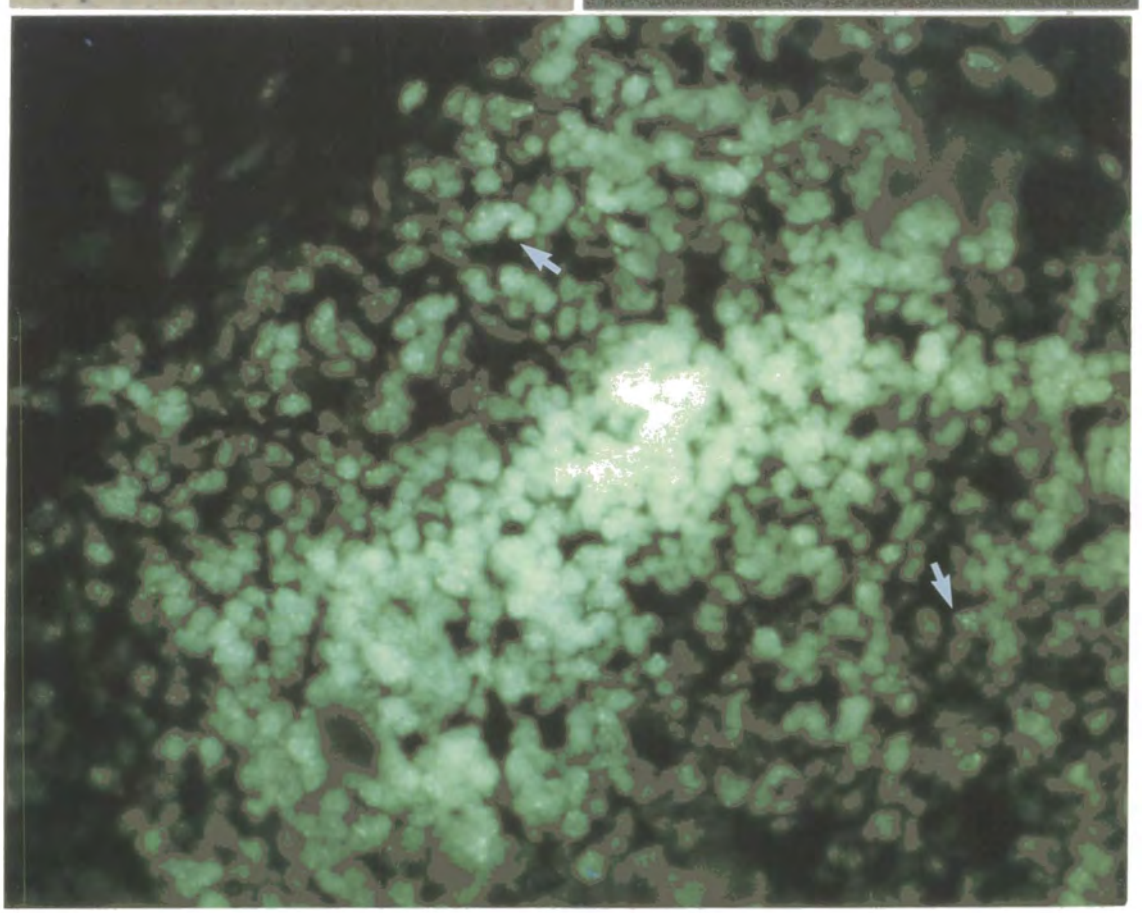
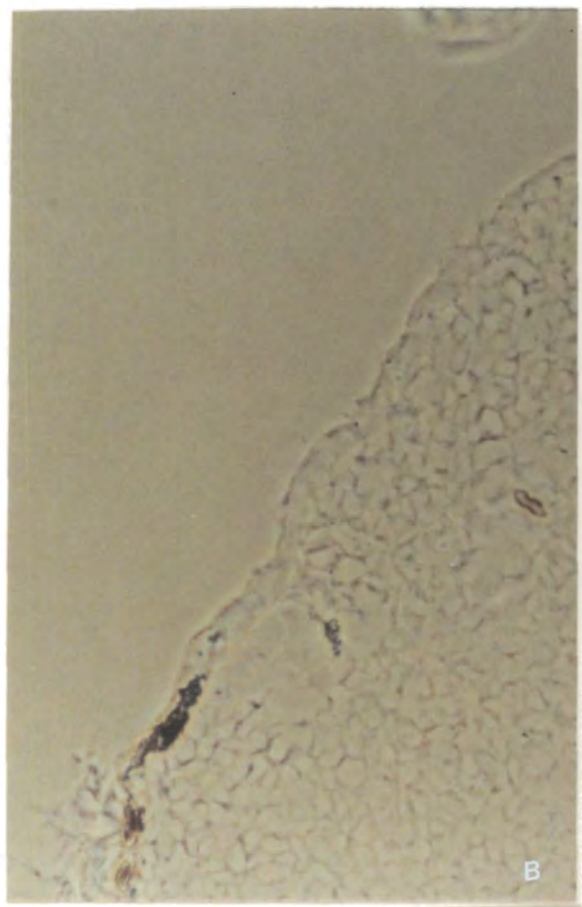
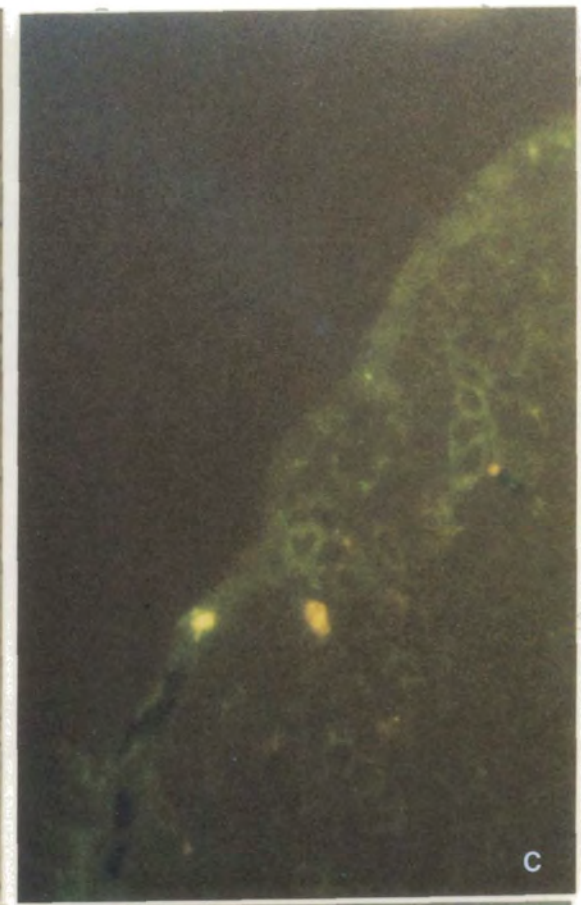
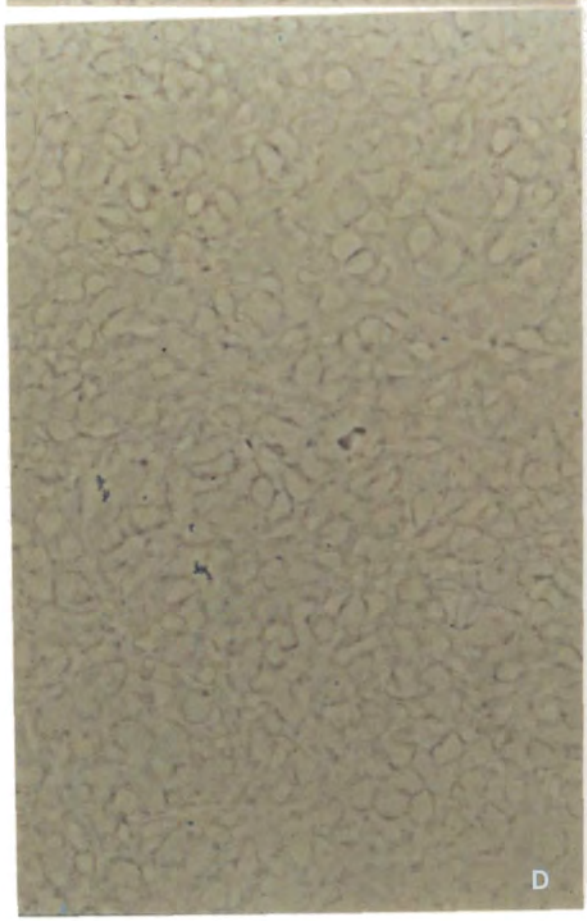


**Figure 3.11, B and C** *X.tropicalis* thymus implanted into *X.borealis* host, 33 days post-implantation. Phase contrast and fluorescence views of XT-1 labelled $6\mu$ frozen section of thymus implant, showing several small groups of XTLA-1 positive cells in cortical region. x500

**Figure 3.11, D and E** *X.tropicalis* thymus implanted into *X.borealis* host, 33 days post-implantation. Phase contrast and fluorescence views of XT-1 labelled $6\mu$ frozen section of host spleen, showing a single XTLA-1 positive lymphocyte in the red pulp. x500


B


C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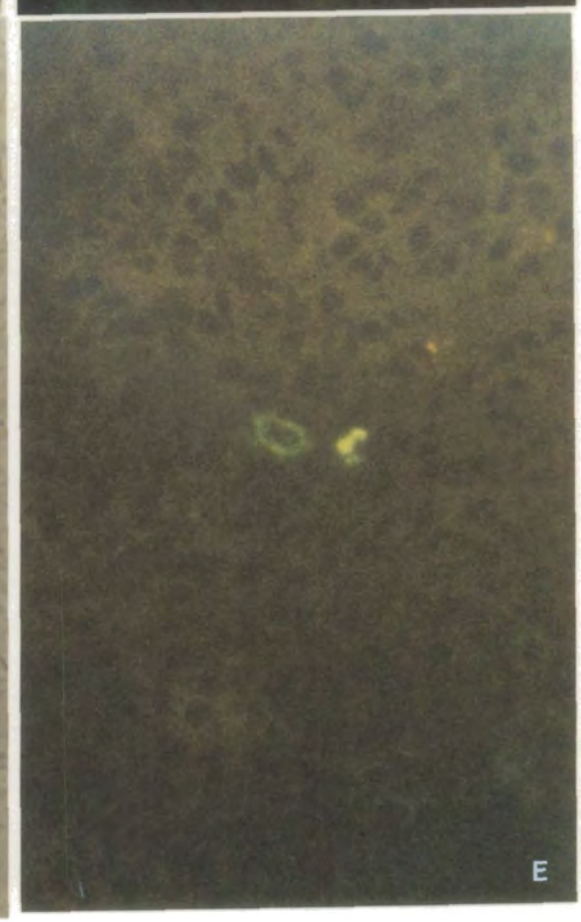

E

**Figure 3.12, A and B** *X.tropicalis* thymus implanted into *X.borealis* host, 48 days post-implantation. Quinacrine-stained 6$\mu$ frozen section of thymus implant cortex, (3.12A), and medulla, (3.12B), showing large numbers of Q+ve cells, particularly in the cortex. Smaller, 'non-spotted' *X.tropicalis* nuclei are still detectable, (arrowed). x500

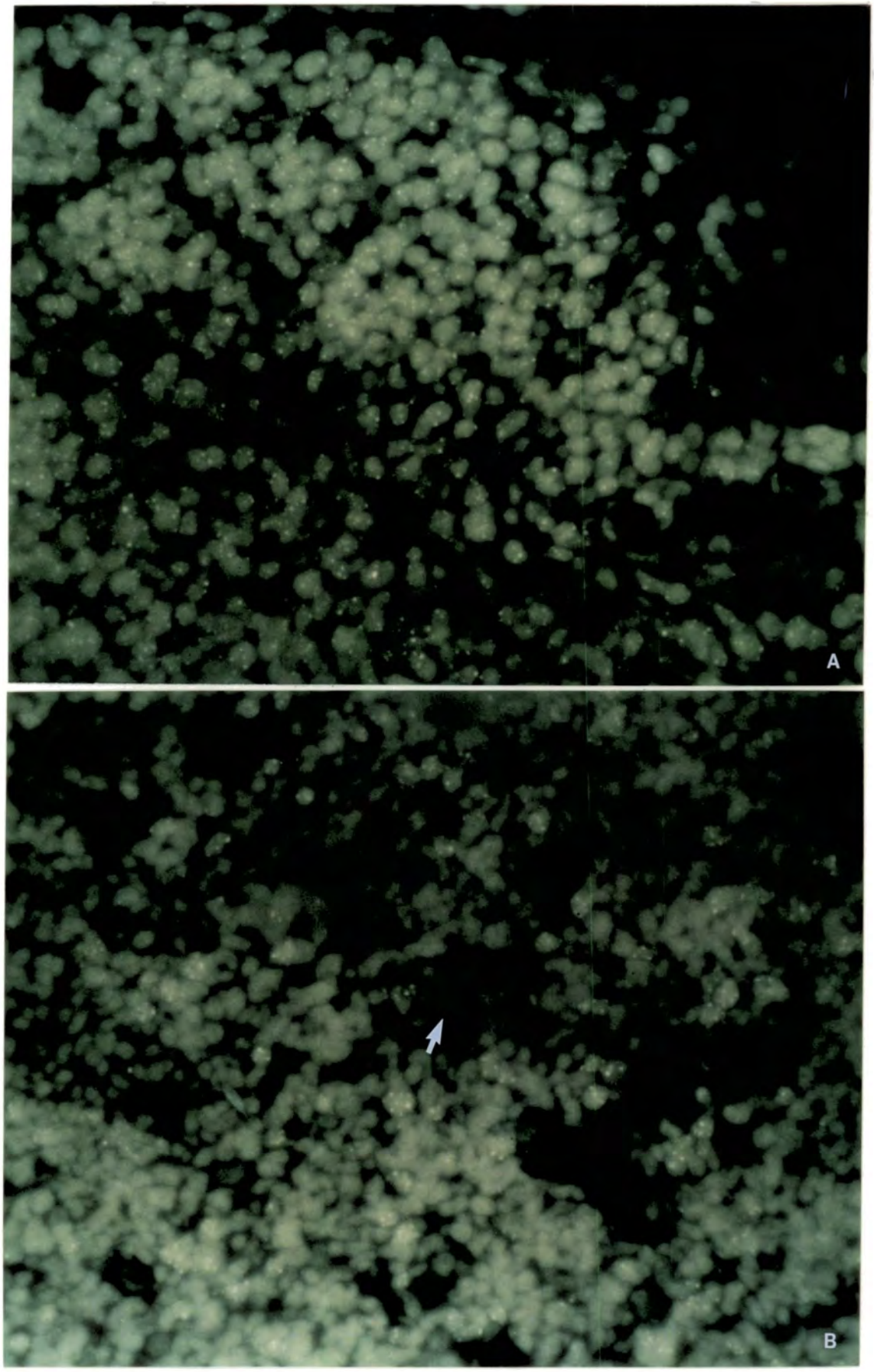
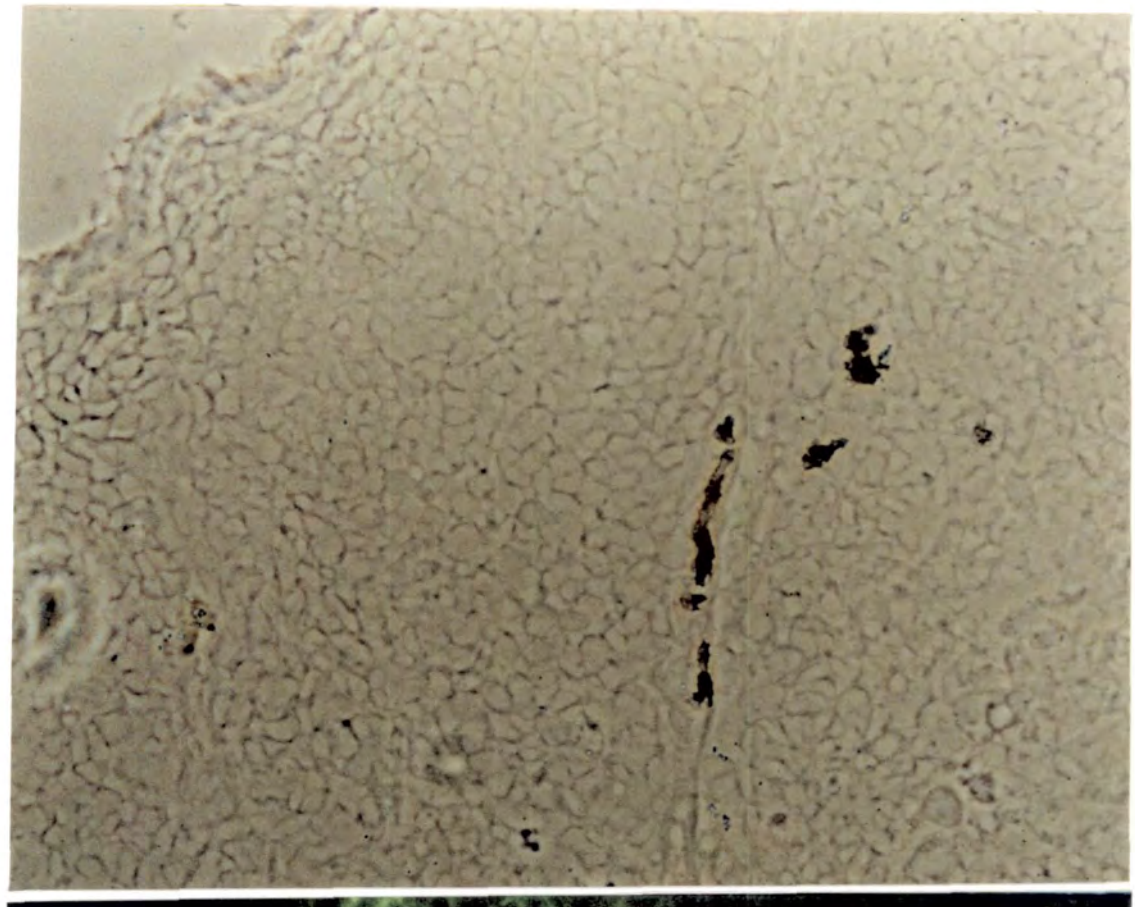

A
B

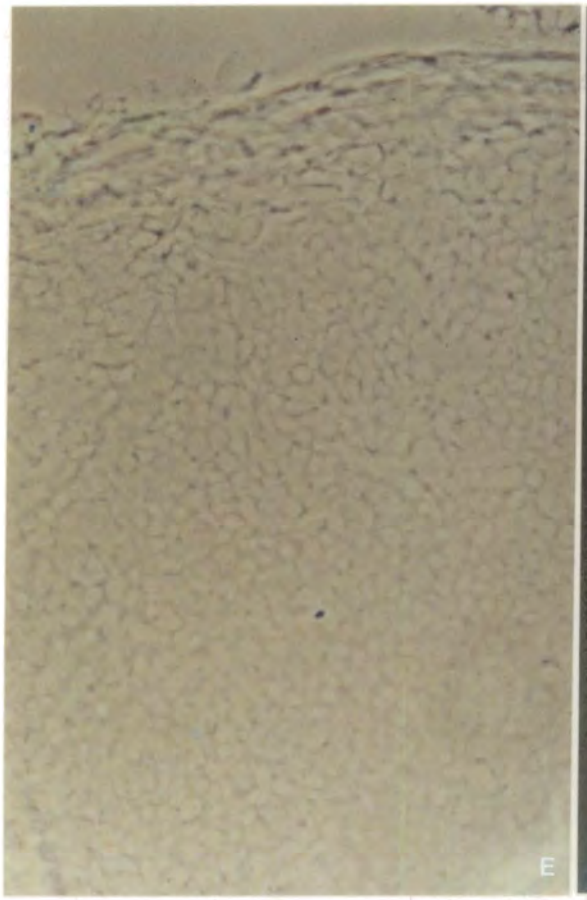
**Figure 3.12, C and D** *X.tropicalis* thymus implanted into *X.borealis* host, 48 days post-implantation. Phase contrast and fluorescence views of XT-1 labelled 6$\mu$ frozen section of thymus implant, showing bright 'honeycomb' pattern of staining in cortex, particularly in the immediate subcapsular layer, but little staining in the medullary region, (m). x500




m

**Figure 3.12, E and F** *X.tropicalis* thymus implanted into *X.borealis* host, 48 days post-implantation. Phase contrast and fluorescence views of 'control', (no XT-1 mAb), 6$\mu$ frozen section of implanted thymus cortex. x500

**Figure 3.13A** *X.tropicalis* thymus implanted into *X.borealis* host, 55 days post-implantation. Fluorescence view of XT-1 labelled 6$\mu$ frozen section of thymus implant cortex, showing bright 'honeycomb' staining of XTLA-1 positive cells. x500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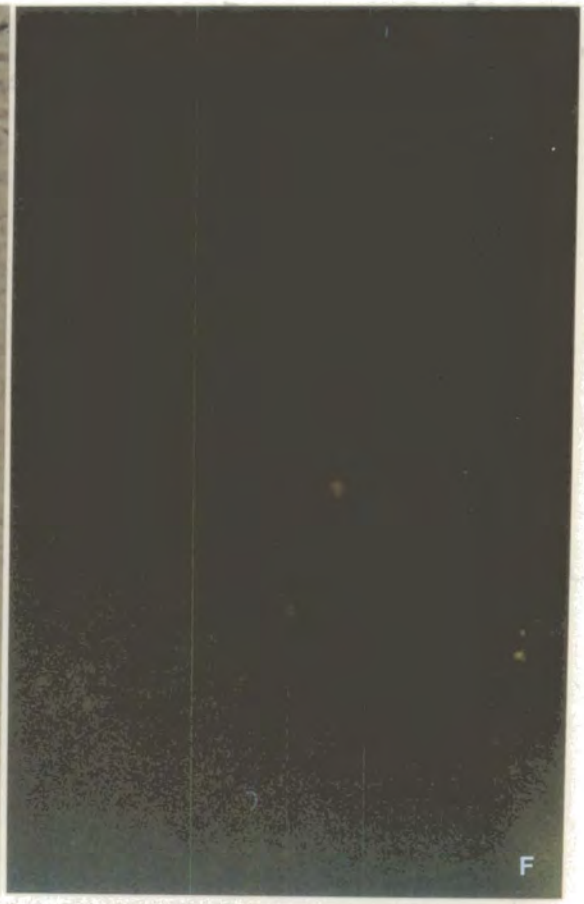
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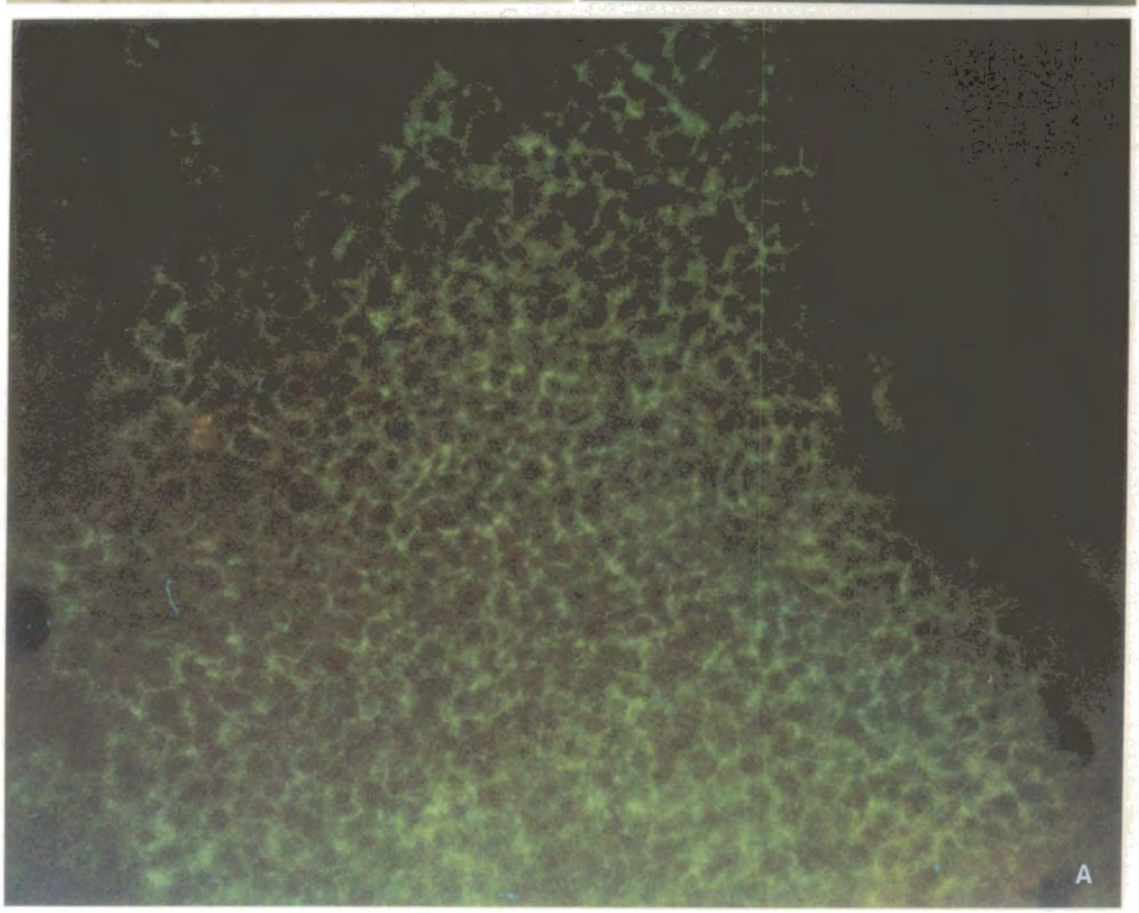
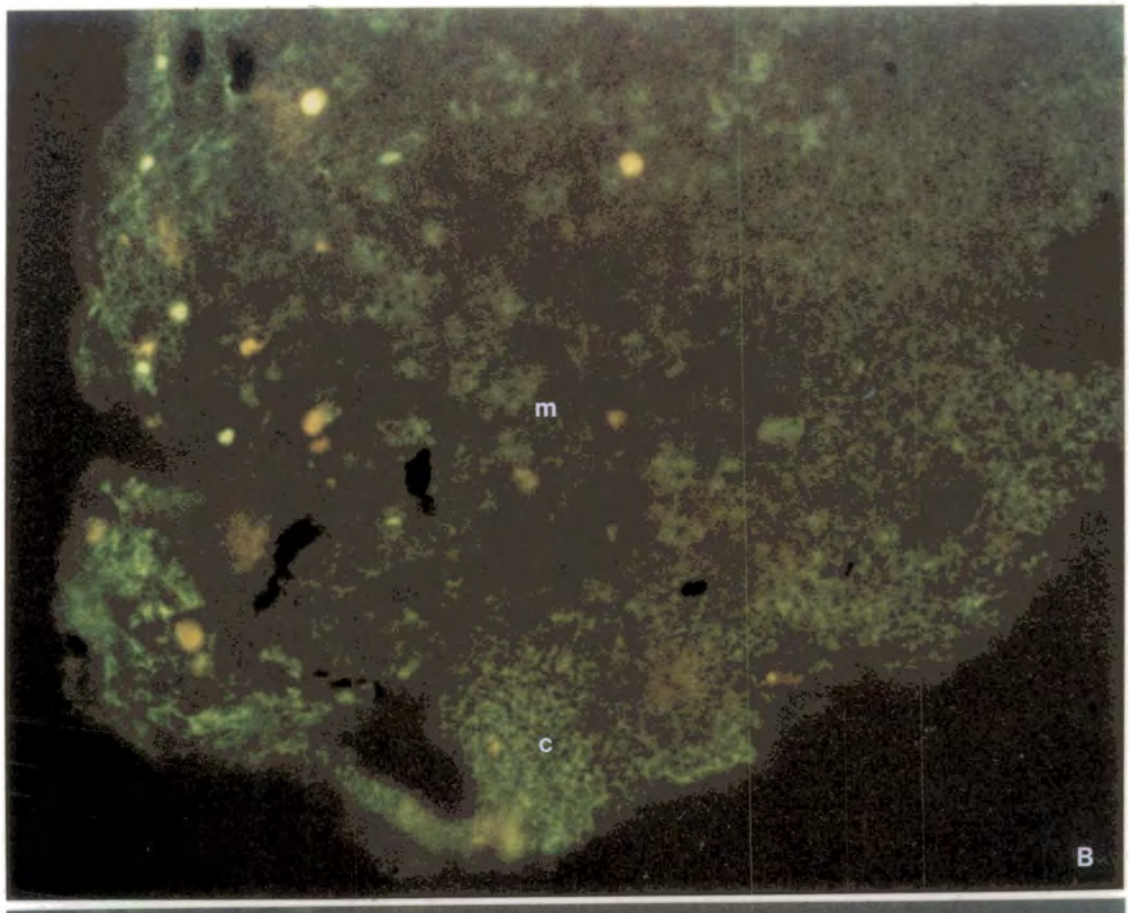

A

**Figure 3.13, B and C** *X.tropicalis* thymus implanted into *X.borealis* host, 55 days post-implantation. Fluorescence view of XT-1 labelled and 'control' 6$\mu$ sections of thymus implant, showing large numbers of XTLA-1 positive lymphocytes in cortex, (c), and medulla, (m), in 3.13B. Control section, (3.13C), shows scattered deposits of fluorochrome, (green), and some yellow autofluorescence, but is not specifically stained. x250


m
c
B

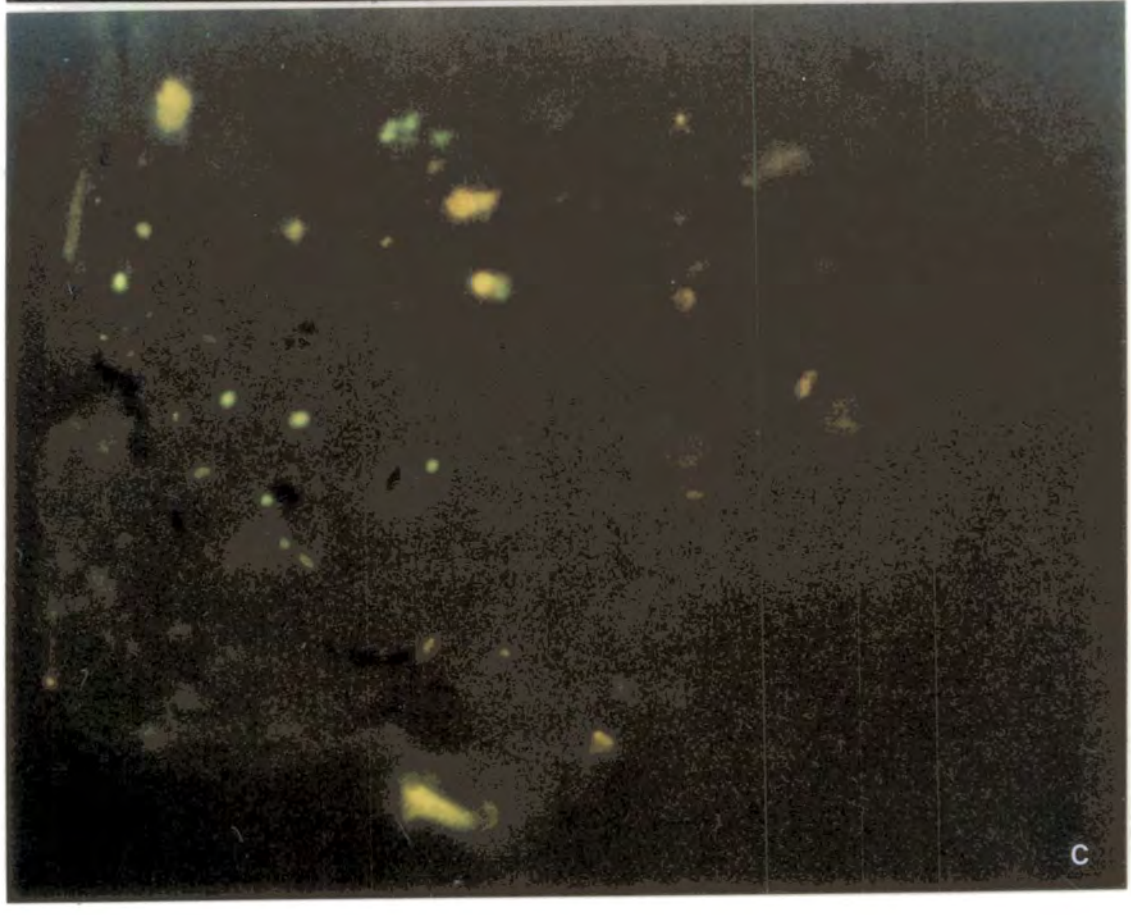
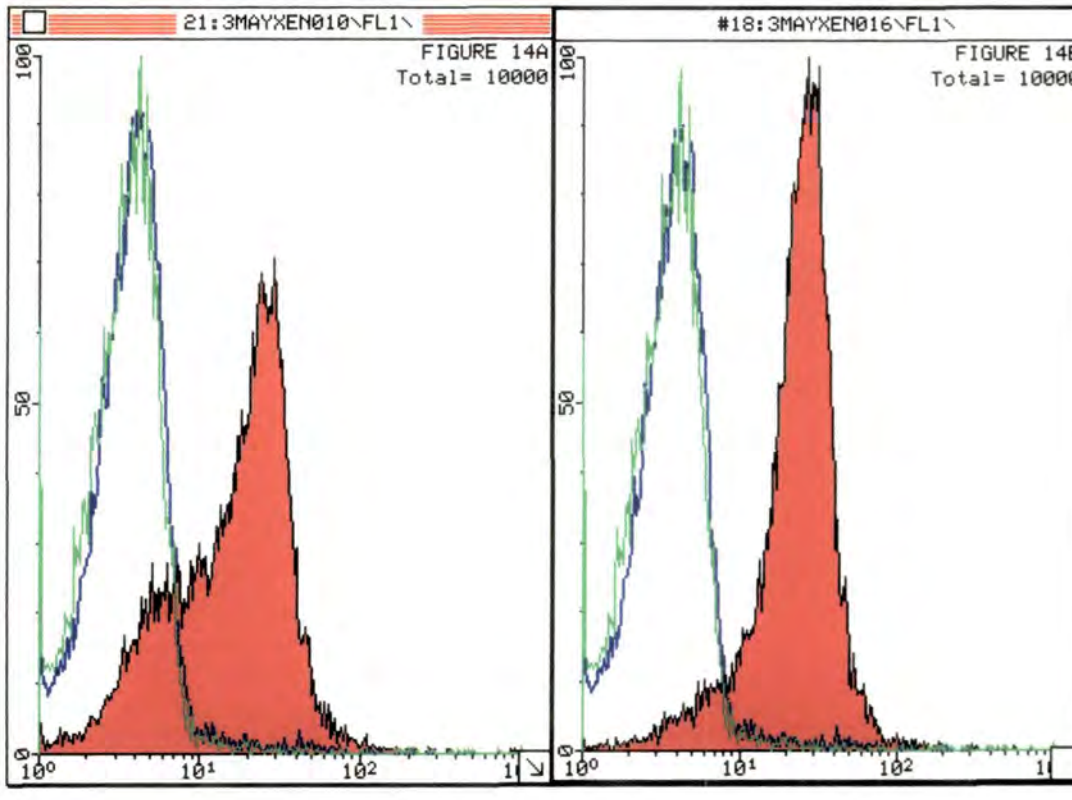

C

**Figure 3.14, A and B** *X.tropicalis* thymus implanted into *X.borealis* host, 78 days post-implantation. FACS-generated fluorescence intensity histograms of 'experimental', (XT-1 mAb + secondary antibody), preparations, (red), and of unstained control, (green), and secondary antibody, (blue), preparations of *X.borealis* thymocytes. Note large peaks of XTLA-1 positive thymocytes in implant cell preparations. X-axis = log fluorescence intensity,(530 nm), Y-axis = relative cell number, (10,000 events / sample).

**Figure 3.15, A and B** *X.tropicalis* thymus-implanted *X.borealis* host, implanted in the *X.tropicalis* thymus, 78 days post-implantation. FACS generated fluorescence intensity histograms of 'experimental', and control preparations of host splenocytes. Green = unstained control preparation; blue = secondary antibody preparation; red = 'experimental', (XT-1 mAb + secondary antibody), preparation. Spleen of both implanted animal I, (3.15A), and implanted animal II, (3.15B), contained XTLA-1 positive lymphocytes. Note that the same control histograms are shown in both A and B, (see text). X-axis = log fluorescence intensity, (530 nm), Y-axis = relative cell number, (10,000 events / sample).


21:3MAYXEN010\FL1\
FIGURE 14A
Total= 10000
#18:3MAYXEN016\FL1\
FIGURE 14B
Total= 10000

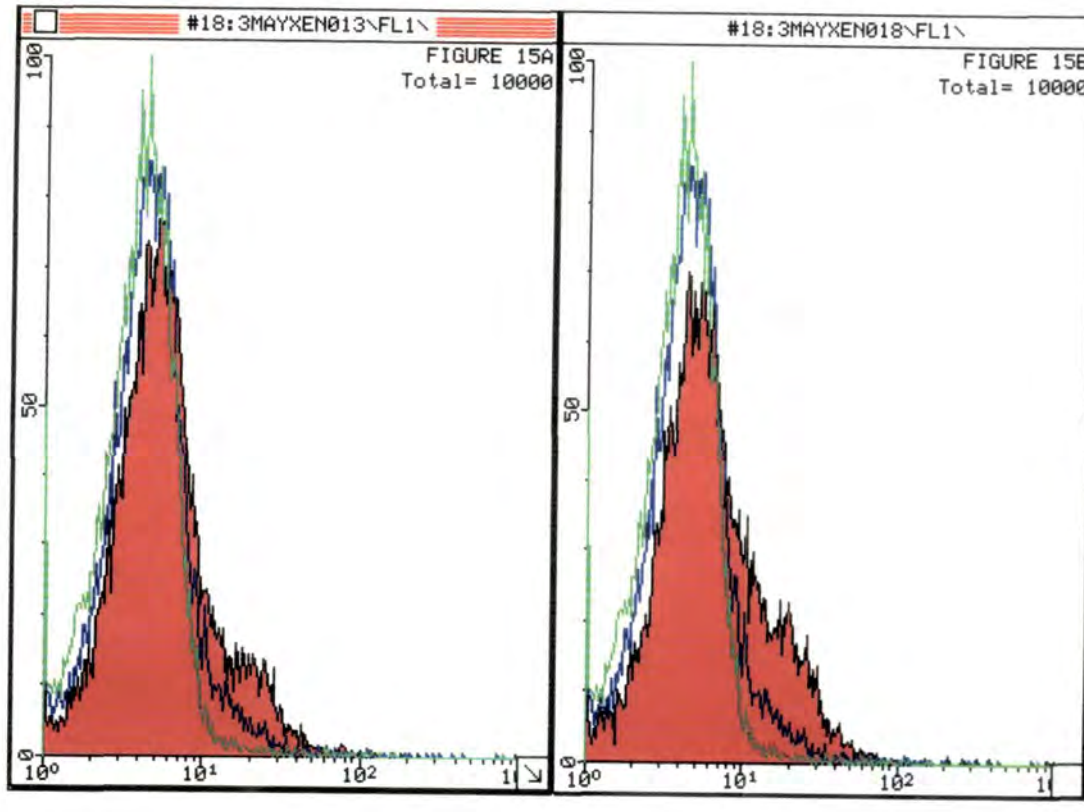
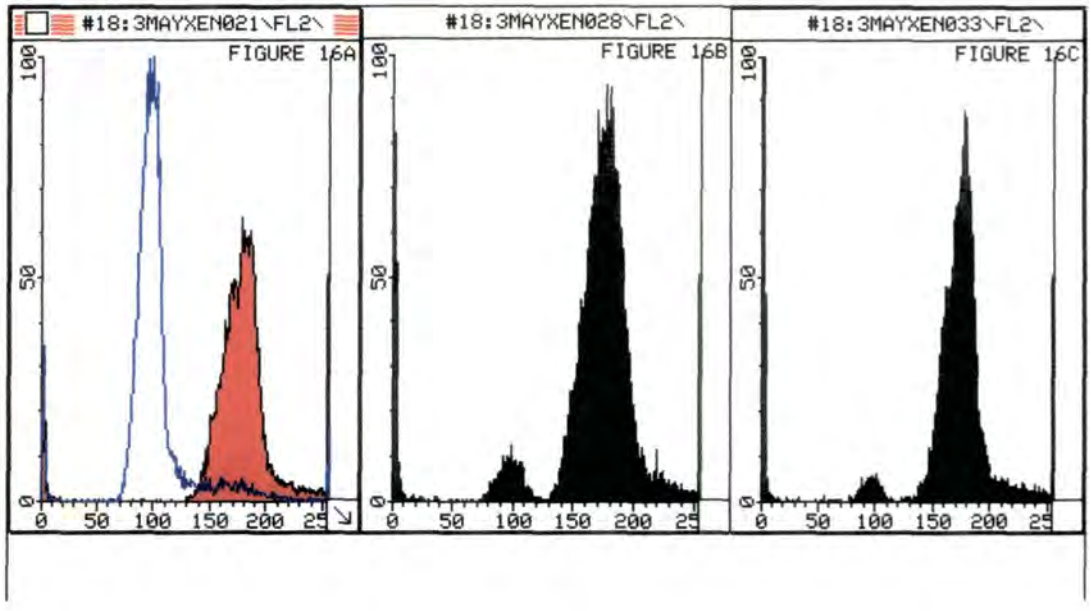
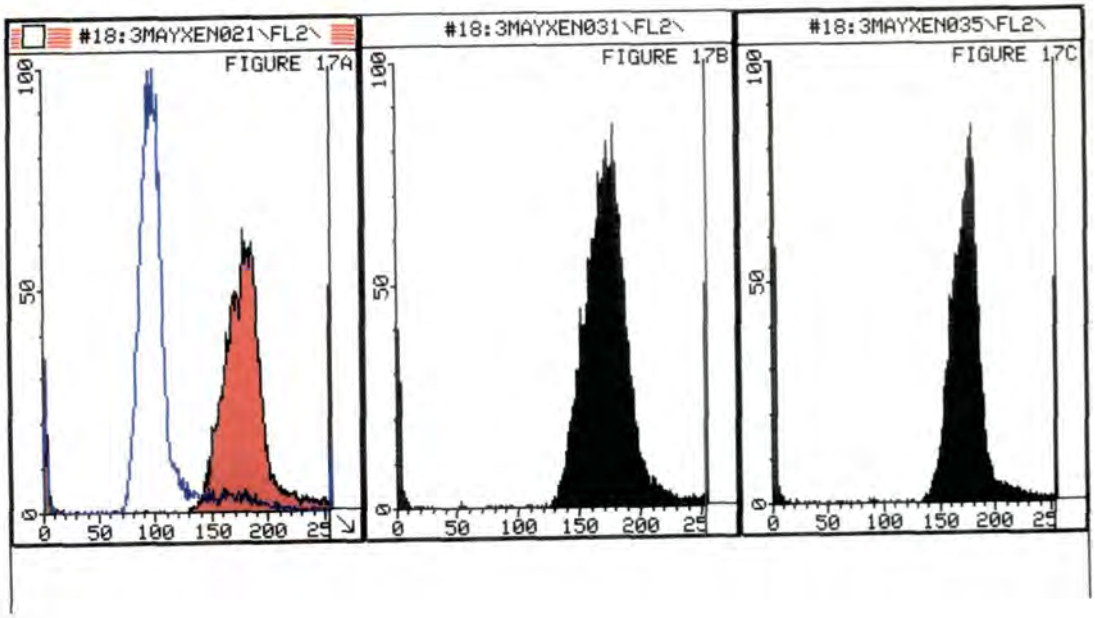

#18:3MAYXEN013\FL1\
FIGURE 15A
Total= 10000
#18:3MAYXEN018\FL1\
FIGURE 15B
Total= 10000

**Figure 3.16, A to C** *X.tropicalis* thymus implanted into *X.borealis* host, 78 days post-implantation. FACS-generated fluorescence intensity histograms of propidium iodide stained cell nuclei. Figure 3.16A shows propidium iodide stained *X.tropicalis* nuclei, (blue), and *X.borealis* nuclei, (red). Note overlap of histograms, probably corresponding to *X.tropicalis* nuclei in 'S' phase or mitosis, (8%). Figures 3.16, B and C show p.-i. stained thymus-implant cell nuclei from two animals; each comprises a large peak of host-derived,(*X.borealis*), nuclei and a smaller peak of persisting donor- derived, (*X.tropicalis*), nuclei. X-axis = fluorescence intensity, (575 nm). NB This is a linear scale. Y-axis = relative number of cell nuclei, (10,000 events/sample).

**Figure 3.17, A to C** *X.tropicalis* thymus implanted *X.borealis* host, 78 days post implantation. FACS-generated fluorescence intensity histograms of propidium iodide stained cell nuclei. Figure 3.17A shows *X.tropicalis* nuclei, (blue), and *X.borealis* nuclei, (red). Figures 3.17, B and C show host splenocyte nuclei from two thymus-implanted animals. No thymus-donor derived nuclei are detectable in these preparations. X-axis = fluorescence intensity, (575 nm). NB this is a linear scale. Y-axis = relative number of cell nuclei, (10,000 events/sample).


#18:3MAYXEN021\FL2\
FIGURE 16A
#18:3MAYXEN028\FL2\
FIGURE 16B
#18:3MAYXEN033\FL2\
FIGURE 16C


#18:3MAYXEN021\FL2\
FIGURE 17A
#18:3MAYXEN031\FL2\
FIGURE 17B
#18:3MAYXEN035\FL2\
FIGURE 17C

# Chapter 4

# Thymus dependent immune responses in thymectomised Xenopus implanted with semi- or fully- xenogeneic thymus

## 4.1 Introduction

A number of authors have described the effect of early larval thymectomy on the immune responses of *Xenopus*. Thymectomy during the first week of life, either by microcautery, Horton & Manning, (1972), or by surgical intervention, (Tochinai & Katagiri, 1975), was found to severely delay or to abrogate skin allograft rejection. Tompkins & Kaye, (1981), reported that, while progressively earlier thymectomy, (from stages 51 to 42), resulted in increasingly poor allograft responses, even to multiple grafts, some animals thymectomised at stage 42 still retained the ability to chronically reject grafts. Furthermore, Nagata & Cohen, (1983), demonstrated that, following chronic first-set graft rejection, J strain *Xenopus* thymectomised at 4–7 days, rejected second-set skin grafts in an accelerated fashion, (although still more slowly than second set rejection displayed by control animals), which was specific to grafts from the original skin donor. In contrast to allograft rejection, it has been reported that the rejection of skin xenografts is unaffected by early-thymectomy, (James et al, 1982b; Clothier et al, 1990). Furthermore, these authors revealed that prior treatment of host animals with NMU, (which abrogates skin allograft responses), failed to halt xenograft destruction, (see also Balls et al, 1981).

Du Pasquier & Horton, (1976), and Horton & Sherif, (1977), reported that 7-8 day thymectomy abrogated mixed leukocyte culture, (MLC), responses of *Xenopus* splenocytes to allogeneic cells. Following chronic first-set skin allograft rejection, however, early-thymectomised *Xenopus* can display non-specific primary MLC responses, i.e., responses which are not limited to stimulator cells from the skin donor, (Nagata & Cohen, 1983). Proliferative responses to T-cell mitogens, such as phytohaemagglutinin-A, (PHA), and concanavalin A, (Con A), have been

shown to be abrogated, (Du Pasquier & Horton, 1976; Horton & Sherif, 1977), or severely reduced, (Manning et al, 1976; Manning & Collie, 1977); Ruben & Edwards, 1979), following early thymectomy. Antibody responses to human gamma globulin, (Turner & Manning, 1974), rabbit red blood cells, (Tochinai & Katagiri, 1975), and sheep red blood cells, (Turner & Manning, 1974; Manning & Collie, 1975; Horton, Rimmer & Horton, 1976, 1977; Ruben & Edwards, 1979), have all been shown to be abrogated following early thymectomy. Rosette-forming cell activity of *Xenopus* towards TNP-SRBC and TNP-Ficoll is abrogated by early-thymectomy, (Horton et al, 1979). Clonal *Xenopus* thymectomised at 7 days, have been shown to be capable of mounting a low- affinity IgM antibody response to DNP-KLH, but, unlike the humoral response of intact controls to this antigen, thymectomised *Xenopus* produced no "IgG- like" anti- DNP-KLH antibodies, (Du Pasquier & Horton, 1982).

Other authors have described the functional effects of implantation of one or more thymi into early-thymectomised *Xenopus.* Acute rejection of third-party skin grafts was restored in early-thymectomised outbred *X.laevis* implanted in larval life with allogeneic larval thymi, (Horton & Horton, 1975), whereas skin grafts from thymus donors were tolerated. Implantation of MHC-disparate larval thymi into early-thymectomised tadpole hosts, (clonal hybrid animals), was found to induce tolerance to thymus-donor type skin, while restoring the ability to reject major and even minor histocompatibility disparate grafts, (Du Pasquier & Horton, 1982).

Implantation of minor-H-disparate J strain larval or adult, normal or gamma-irradiated, (lymphocyte-depleted), thymi into early-thymectomised J strain tadpole hosts has been found to restore acute graft rejection of MHC-disparate skin in the majority of implanted animals, (Arnall & Horton, 1986). MHC-disparate adult, (normal or irradiated), implants into thymectomised clonal tadpole hosts were shown, by these authors, to fully restore responses to third-party MHC-disparate skin grafts. In contrast, reactivity to thymus donor-derived grafts was abolished; responses to skin grafts where only minor histocompatibility differences were on view were impaired. Early-thymectomised *Xenopus*, implanted post-metamorphically with MHC-compatible or -incompatible adult thymi have also been shown to be capable of at least partially restored skin graft rejection responses. For example, Tochinai et al, (1976), reported that J strain adults, following thymectomy at 4-5 days of age and subsequent implantation, in adult life, with histocompatible froglet thymi, rejected first-set skin allografts more slowly than intact controls, but acutely rejected second- set grafts. Nagata & Kawahara, (1982), studied skin allograft responses of J strain hosts thymectomised at stage 45 and implanted in adult life with age-matched MHC-compatible triploid or MHC-incompatible thymi. In their experiments, animals implanted with histocompatible thymi rejected third-party skin grafts as acutely as did controls, while histoincompatible thymus-implanted hosts showed chronic rejection of third-party skin. In an extensive study, involving the implantation in adult life of MHC-matched or partially or totally MHC-mismatched froglet thymi into early- thymectomised J strain or LG clonal individuals, Nagata & Cohen, (1984), noted third-party skin graft rejection times which were not significantly different from those of con-

trols, regardless of the genetic relatedness of thymus donor and host. Nakamura et al, (1987), and Maeno et al, (1987), have reported that early-thymectomised *Xenopus*, implanted post-metamorphically with irradiated or non-irradiated adult semi-allogeneic thymi show restored rejection of third-party skin grafts, but specifically tolerate grafts of donor-type skin.

*In vitro* MLC responses of early-thymectomised *Xenopus* implanted in larval or adult life with normal or irradiated, larval or adult thymi have also been studied. It has been generally found, (Arnall & Horton, 1986; Maeno et al, 1987; Nakamura et al, 1987), that thymus implantation results in restoration of MLC responses to third-party cells. Some researchers have also noted the phenomenon of 'split tolerance', in which thymus-implanted animals do not reject thymus-donor type skin, but are responsive in MLC to donor-type stimulator cells, (Nagata & Cohen, 1984; Arnall & Horton, 1986). Arnall & Horton, (1987), showed that thymus-implanted thymectomised host animals were susceptible to GvH disease induced by injection of splenocytes primed to the thymectomised host, but only when these came from the thymus donor strain. Overall the above experiments suggested that it is the cytotoxic, rather than the proliferative, response to thymus donor that is missing in these thymus-implanted animals.

Proliferative responses to the T-cell mitogens PHA and Con A were found by Nagata & Cohen, (1984), to be significantly lower in MHC-matched or partially, or totally, allogeneic thymus-implanted animals than in controls, but to be increased over those seen in thymectomised siblings. Russ, (1986), however, noted complete restoration of splenocyte response to PHA in outbred *X.laevis* implanted with allogeneic thymi.

Antibody responses requiring T-cell help have generally been shown to be restored in early-thymectomised *Xenopus* by the implantation of MHC- matched or -mismatched thymi. Plaque forming cells to sheep red blood cells were found, although in reduced numbers compared to those in controls, in all implanted hosts, (Nagata & Cohen, 1984). Gearing et al, (1984), also found some restoration of plaque forming cell and serum haemolysin responses to sheep red blood cells in early-thymectomised *X.laevis* implanted with larval irradiated or non-irradiated MHC- incompatible thymi, or with MHC-compatible or -incompatible adult thymi. Isogeneic and fully allogeneic thymus implants were shown to restore host IgM responses to DNP-KLH, in terms of quantity, affinity and specificity, (Du Pasquier & Horton, 1982). These authors also reported that low molecular weight immunoglobulin responses were restored in each thymus donor/thymectomised host combination examined, and that, by isoelectric focusing, antibody spectrotypes were of the host type.

In the studies reported in this Chapter, the effect of implantation of partially- or totally-xenogeneic thymi into early-thymectomised tadpole hosts is investigated, since previous functional studies have concentrated on restoration with allogeneic implants, and since morphological studies have already suggested that *X.borealis* and *X.tropicalis* carry markers useful in the examination of the immune system of thymus-implanted animals. A variety of functional tests for T-cell activity were performed, in vitro and in vivo, to assess the degree of restoration of thymus-

implanted animals. The extent to which restoration of third-party graft rejection and tolerance to thymus donor-type cells and tissues is induced by semi- and fully-xenogeneic thymus implants forms the main thrust of this Chapter.

# 4.2 Materials and Methods

## 4.2.1 Animals, Operations and Injections

### 4.2.1.1 Animals

Larval J strain *X.laevis*, (MHC = jj), and interspecies hybrids LG17 and LG15, (MHC = ac), were used as thymectomised hosts in these experiments.

Larval thymus donors were interspecies (*X.borealis* x J strain *X.laevis*) XbJ or outbred *X.borealis* or *X.tropicalis* tadpoles. Adult donor thymi were taken from 12 month old *X.tropicalis*, (a gift from R.Clothier). All larval donor and host animals were bred and reared in this laboratory, at 22-24°C, as previously described in Horton & Manning, (1972), and Kobel & Du Pasquier, (1975). Adult *X.tropicalis* animals were maintained under similar conditions.

Third-party skin donor animals were adult *X.borealis*, LM3, (MHC = wy), LG5, (MHC = bc), and outbred *X.laevis*. All of these animals were bred and reared in this laboratory as described above.

Adult, female, outbred *X.laevis* were bought from Xenopus Ltd. for immune and non-immune sera production.

### 4.2.1.2 Early-thymectomy Operations

Early-thymectomy operations were performed, by microcautery, on 7 day old, (stage 47-48 of Nieuwkoop & Faber), hosts, after the method of Horton & Manning, (1972)

### 4.2.1.3 Donor Thymus Preparation

#### Irradiated thymi

Adult *X.tropicalis* thymus donors were $\gamma$-irradiated with a dose of 3,000 Rads, using a $^{60}$Cobalt-source, (see Appendix II for details of calculation of irradiation doses). Five days after irradiation, both thymi from each anaesthetised donor were aseptically dissected out, into sterile amphibian-strength phosphate buffered saline, (APBS: NaCl 6.6 g/l, $Na_2HPO_4$ 1.5 g/l, $KH_2PO_4$ 0.2 g/l, pH 7.4; Flajnik, 1983). At this time, irradiated donor thymi had the classical shrunken, darkened appearance described by Russ & Horton, (1987).

**Non-irradiated thymi**

Larval thymus donors were age-matched with host animals. Thymuses were aseptically dissected out of anaesthetised donor tadpoles into sterile APBS. *X.borealis* and XbJ donors, each having contributed one thymus, were kept individually to allow later skin grafting to their thymus-implant hosts.

#### 4.2.1.4 Thymus Implantation Operations

Donor thymi were implanted into anaesthetised, thymectomised stage 56-59 hosts as described in Chapter 3, (3.2.1.3).

#### 4.2.1.5 Skin Grafting Operations

Control, thymectomised, (TX), or TX,thymus-implanted animals were 3-7½months of age when skin grafted. The interval between thymus implantation and skin grafting was 2-5$\frac{1}{2}$ months.

Skin graft beds were prepared on anaesthetised hosts by removing a small piece of skin from the centre of the back, at the level of the front limbs; care was taken to avoid damaging the surrounding host skin during this procedure. A 2mm$^2$ piece of, usually, ventral skin, (to facilitate monitering of graft survival), was transferred from the anaesthetised donor into the prepared graft bed. As this was made slightly smaller than the graft, the edges of the graft could be tucked under those of the host skin, to hold the graft in place until it was completely healed in.

Newly grafted hosts were placed in shallow, dilute (1:3 with distilled water), amphibian Ringers' solution, (insufficiently deep to cover their dorsal surface), and kept undisturbed in a darkened room to recover. Beginning approximately 12 hours after operation, the Ringers' solution was further diluted with aerated standing water, until the animals could be returned to fresh standing tap water.

Two to three days after operation, when the skin grafts had healed in place,the edges of the surrounding host skin were trimmed back to the level of the graft, such that host and donor skin edges butted up together.

Skin graft survival was assessed by regular microscopical observation, the rejection end point being taken as the time at which all donor pigment cells had been destroyed.

#### 4.2.1.6 Sheep blood cell immunisations and serum collection

Sheep blood cells in Alsevers' solution were purchased from Tissue Culture Services. Aliquots of SBC solution were washed twice in large volumes of saline, (0.85% sodium chloride solution), and resuspended in fresh saline at a concentration of 25% by volume.

TX, control and thymus-implanted froglets, (7 months of age),were immunised on days 0, 7, 14 and 42 with a total of 0.1 ml per immunisation 25% SBC solution, (0.05 ml intraperitoneally, 0.05 ml into the dorsal lymph sac).

Additionally, two large, unoperated outbred *X.laevis* females were immunised on days 0, 7, 14 and 42 with a total of 0.4 ml per immunisation 25% SBC solution, (0.2 ml intraperitoneally, 0.2 ml into the dorsal lymph sac), in an attempt to provide a source of high titre *Xenopus* anti-sheep blood cell serum.

Seven days after the final immunisation, anaesthetised frogs were bled out by cardiac puncture, using finely drawn glass needles. Blood samples, (approximately 0.5-1.0 ml whole *Xenopus* blood), were left overnight at 4°C to clot, and then centrifuged, (5,500rpm 10 minutes). The supernatant serum was aseptically collected into sterile tubes, and sodium azide was added to a final concentration of 0.02%. Serum samples were stored at 4°C. Unimmunised large *X.laevis* females were also bled, by the same technique, to provide non-immune *Xenopus* serum. TX, control and thymus-implanted animals were pre-bled, prior to immunisation, from a vein in the foot, to assay for any naturally occurring haemagglutinins / haemolysins. Small volumes, (100-200 $\mu$l/sample), of whole *Xenopus* blood were collected using this protocol, and serum from each sample was prepared and stored as described above.

## 4.2.2 In Vitro Assays for *Xenopus* T-Cell Activity

### 4.2.2.1 Tissue Culture Medium

Leibovitz L-15 culture medium, (Flow Labs.), was diluted to amphibian tonicity, (60%), with sterile double distilled water, and was supplemented with 1.25 mM L-glutamine, 50 I.U./ml penicillin, 50 $\mu$g/ml streptomycin, 0.01 M HEPES buffer, 2.5$\mu$g/ml fungizone, (all from Flow Labs.), and 0.083 mM 2-mercaptoethanol, (BDH). This medium was further supplemented with heat- inactivated foetal calf serum, (FCS, Flow Labs.), to 1% for splenocyte cultures or to 10% for thymocyte cultures. Mixed thymocyte/splenocyte cultures were carried out in 5.5% FCS-supplemented medium. All media were aseptically prepared in a laminar air flow cabinet, and were filtered through 0.2 micron pore-size membrane filters, (Millipore), before use. Washing, counting, etc. of cell suspensions was carried out in the above media, but cells were transferred into fresh medium containing 0.01 M sodium bicarbonate buffer, (Flow Labs.), just prior to plating out and incubation.

### 4.2.2.2 Preparation of Thymus/Thymus Implant and Spleen Single Cell Suspensions

Organs were removed from anaesthetised 4-8$\frac{1}{2}$ month old animals, which had been previously washed in 70% ethanol to reduce the risk of microbial contamination, into tissue culture medium. Organ removal was performed as aseptically as possible, using sterile instruments, in a laminar air flow cabinet.

The dissected organs were then transferred to a separate tissue culture facility for preparation of single cell suspensions. Separation of organ collection and further procedures was intended to reduce the risk of microbial contamination of cultures.

Preparation of single cell suspensions was carried out as described in Chapter 2, except that all procedures were performed under aseptic conditions, and that tissue culture medium, rather than staining medium, was used.

#### 4.2.2.3 Irradiated Splenocyte Suspensions

Splenocyte suspensions destined to act as stimulator cells in mixed leukocyte cultures were $\gamma$-irradiated, (6,000 Rads), by exposure to a $^{60}$Cobalt source. This dose of irradiation was found, in preliminary studies, (see Appendix II), to abrogate proliferative responses to phytohaemagglutinin A, and lead to low background levels of proliferation in mixed leukocyte culture experiments, (for example: mean dpms, ($\pm$ S.D.), for 4 day culture of 2 x $10^5$ irradiated cells: J strain *X.laevis* = 100 ($\pm$7), *X.borealis* = 87 ($\pm$35), LG15 = 84 ($\pm$21)).

#### 4.2.2.4 Culture Conditions.

All cultures were maintained, at 26°C, in a humidified incubator, (Flow Labs.), in an atmosphere of 5% $CO_2$ in air.

#### 4.2.2.5 T-Cell Mitogen Response Assays

Concentrations of purified phytohaemagglutinin A, (PHA-P), and Concanavalin A, (Con A), required to give optimal stimulation, and the kinetics of optimal T-cell mitogen responses, were examined in studies carried out in this laboratory by T.L.Horton and P.Ritchie. Following the results of their kinetic studies, all mitogen-stimulated cultures described in this Chapter were pulsed with tritiated thymidine solution, ($^3$HTdr, Amersham; specific activity 5$\mu$Ci/mole), after 48 hours culture, and were harvested 24 hours later.

**PHA-P Response Assays**

These assays were carried out in hanging drop cultures made in Terasaki plates, (Sterilin Ltd.)., (see Knight's, (1982), description of this technique for original mammalian lymphocyte cultures). Miniaturised assays were used to allow collection of the maximum amount of data from the relatively small number of cells available from thymus-implanted hosts. Splenocyte suspensions were adjusted to give a concentration of 0.6-1.5 x $10^6$ cells/ml and were plated out at a volume of 18$\mu$l per well. 2$\mu$l of PHA-P, (Flow Labs.), dilution was added to each experimental well, to give a final concentration of either 0.2$\mu$g/ml or 0.02$\mu$g/ml. Control wells received 2$\mu$l tissue culture medium.

20$\mu$l per well sterile double distilled water was placed in outer wells of the plate to reduce evaporation and subsequent 'edge effects'. The Terasaki plates were inverted before culture, and were maintained in this inverted position throughout the $^3$HTdr pulsing procedure, (2 $\mu$Ci per well), and subsequent culture. Five replicate cultures were set up for each cell suspension/PHA-P combination.

**Con A Response Assays**

These assays were carried out in 96-well U-bottomed microtitre plates, (Sterilin Ltd.), on 100$\mu$l cultures of 1 x $10^6$ splenocytes per ml. Con A, (Flow Labs.), was added to experimental cultures to give a final concentration of 1 $\mu$g/ml. Control cultures received tissue culture medium. Outer wells of plates contained 100$\mu$l per well sterile double distilled water to avoid drying 'edge effects'. Cultures were pulsed with 1$\mu$Ci per well $^3$HTdr. Three replicate cultures were set up for each cell suspension/Con A concentration combination.

### 4.2.2.6 One-way Mixed Leukocyte Culture, (MLC), Responses

The kinetics of one-way MLCs had previously been studied in this laboratory, (T.L.Horton, personal communication). Following the results of these studies, MLCs described in this Chapter were pulsed with $^3$HTdr after 72 hours culture and were harvested 24 hours later.

These assays were carried out in 96-well microtitre plates, (Sterilin Ltd.). Splenocyte suspensions were adjusted to give a concentration of 1 x $10^6$ cells per ml, while thymocytes were adjusted to 2 x $10^6$ cells per ml. 'Experimental' splenocyte cultures consisted of either 100$\mu$l or 50$\mu$l responder splenocyte suspension, plus an equal volume of irradiated stimulator splenocytes. Thymocyte/splenocyte 'experimental' cultures consisted of either 100$\mu$l or 50$\mu$l responder cells plus an equal volume of irradiated stimulator splenocytes. 'Irradiated control' cultures, consisting of only irradiated stimulator or irradiated responder cells, in appropriate volumes, were set up, to allow measurement of the $^3$HTdr uptake of these cells. 'Background control' cultures, consisting of equal volumes of responder thymocytes/splenocytes and irradiated, MHC-identical splenocytes were set up, to allow measurement of the $^3$HTdr uptake of cells cocultured with isogeneic stimulators. Cultures were pulsed with either 2$\mu$Ci per well, (200 $\mu$l cultures), or 1 $\mu$Ci per well, (100 $\mu$l cultures), $^3$HTdr. Three replicate cultures were set up for each combination.

### 4.2.2.7 Cell Harvesting and Scintillation Counting

Harvesting of the inverted cultures in Terasaki plates was carried out using a Flow Labs. Titertek cell harvester, onto glass fibre filters, (Flow Labs.). 96-well microtitre plates were harvested onto glass fibre filter mats, using a Skatron semi-automatic cell harvester.

Following collection onto individual filter discs, all cultures were extensively washed with distilled water to remove unincorporated tritiated thymidine. The

filter discs were dried at 60°C, then placed into individual scintillation vials, ('Pico hang-in vials',Packard), containing 2 ml per vial Betafluor, (National Diagnostics), scintillation fluid.

Counting was performed in an automatic liquid scintillation counter, (Tricarb 300C, Packard). The level of proliferation of each culture was measured as disintegrations, (quenched counts), per minute, (dpm).

#### 4.2.2.8 Calculation of Stimulation Indices and Assessment of Statistical Significance

Mean dpm for each set of replicate cultures was calculated.

Stimulation indices, (S.I.s), for mitogen-stimulated cultures were assessed by the formula:

$$\text{S.I.} = \frac{\text{mean dpm PHA-P mitogen-stimulated cultures}}{\text{mean dpm unstimulated cultures}}$$

Stimulation indices for one-way MLCs were assessed by the formula:

$$\text{S.I.} = \frac{\text{mean dpm 'experimental' - mean dpm irradiated stimulator cultures}}{\text{mean dpm 'background control' - mean dpm irradiated responder cultures}}$$

A S.I. of 1.0, therefore, indicates that no increase in proliferation was seen in 'experimental' or mitogen-stimulated cultures, compared with control cultures. For S.I.s >1.0, mean dpms of 'experimental' and control cultures were statistically compared, using the Students' t-test. When the probability, p, of any difference in dpm was <0.05, the S.I. was considered significant.

### 4.2.3 Haemolysis and Haemagglutination Assays

Initial tests were performed in 96–well microtitre plates, (Sterilin Ltd.), but, since this technique required greater volumes of serum than were often available in prebleed samples, the technique was later modified for use in small volumes, in Terasaki plates, (Sterilin Ltd.).

SBC in Alsevers' solution were washed three times in large volumes of saline and then diluted to give a 1% by volume suspension of SBC in saline. Guinea pig complement, (Tissue Culture Services), was diluted to 1/10 stock concentration with saline.

**Microtitre Plate Assay**

To each of 96 wells, 25 $\mu$l 1% SBC solution was added. For haemolysis assays, 25 $\mu$l diluted guinea pig complement was added to each well, while, for haemagglutination assays, 25$\mu$l saline was added. Finally, each sample of *Xenopus* serum was serially doubly diluted along a row of 12 wells, 25 $\mu$l serum dilution being added to each well. The plates were sealed and incubated for one hour, at room

temperature, and then visually assessed for lysis or agglutination of SBC. The highest dilution at which these effects were seen was noted for each serum tested and this dilution, expressed as - $\log_2$, represented the effective antibody titre for that serum.

**Terasaki Plate Assay**

In this assay, the same protocol as that described above was adopted, but lower volumes, (6$\mu$l), of each solution were added per well.

In initial tests on positive and negative control sera, results from the two assays were comparable, (see Table 5), and, thus, results were pooled.

## 4.3 Results

### 4.3.1 Skin Grafting Experiments

Several different groups of TX, control and TX,thymus-implanted *Xenopus* were assayed for their responses to donor and to third-party skin grafts. Where this was possible, all third-party grafts of outbred donor skin made within a single experiment came from the same donor animal.

**J Strain *X.laevis* hosts.** (See Figure 4.1).

Unoperated Js, (n=7), rejected *X.borealis* skin grafts in 18-22 days; XbJ grafts, (n=4), were rejected in 18-28 days. Three of six TX animals grafted with *X.borealis* skin rejected these grafts chronically, (30-96 days), while the remaining 3 of 6 grafts were in perfect condition 100 days after application. All 4 XbJ grafts applied to TX animals were retained in perfect condition for 100 days. TX animals, each implanted in larval life with a single larval XbJ thymus, (n=3), rejected third-party *X.borealis* skin within 3 weeks, but tolerated XbJ skin from their thymus donor in perfect condition for 100 days.

**LG17 Hosts.** (See Figure 4.2).

These animals formed part of a larger group, set up to histologically examine the condition of grafts tolerated by TX clonal animals, following implantation with *X.borealis* thymi, (see Chapter 6 for details of this study). The results reported here are from a preliminary grafting experiment, performed to establish that xenogeneic thymus-implanted animals were restored to reject third-party grafts, but did not reject thymus- donor skin grafts.

Controls, (n=4) rejected *X.borealis* skin very quickly, (11-15 days). One of three TX animals grafted with *X.borealis* skin also rejected its' graft within 15 days, even though no regenerate thymus was found when it was autopsied; the remaining 2 of these TX LG17s carried intact *X.borealis* grafts when they were killed, 26 days after grafting, although one of the two showed pigmentation disturbances indicative of chronic rejection. A further 2 TX LG17s were grafted with LM3 skin, which was still in perfect condition 50 days after application. Four of 5 TX LG17s, each implanted in larval life with a single larval *X.borealis* thymus, rejected LM3 skin

grafts acutely, (15-19 days), but tolerated grafts from their individual thymus-donors. Two LG17 TX, thymus implanted animals acutely rejected third-party *X.borealis* skin grafts, (13 days), (data not shown in Figure 4.2).

**LG15 Hosts.** (See Figure 4.3).

LG15 controls, (n=6), rejected LG5 skin grafts in 18-28 days. Outbred *X.laevis* grafts, (n=4), subsequently applied to these animals were also rejected rapidly, (12-17 days). LG15 TX animals, (n=2), carried intact LG5 skin grafts until they were sacrificed, (76 days and >100 days after grafting); these same animals, regrafted with outbred *X.laevis* skin, were able to reject these grafts in 19 and 28 days. In contrast to the responses of the majority of thymus-implanted animals reported so far, LG15 TX animals (n=3), each implanted in larval life with 2 *X.tropicalis* larval thymi, carried intact third-party LG5 grafts when sacrificed, [>100 days, (n=1); 76 days, (n=2)]; two LG15 TX larvally implanted with $\gamma$-irradiated adult *X.tropicalis* thymi also failed to show signs of rejection of LG5 skin within 35 days after grafting. The three LG15 TX given larval *X.tropicalis* implants, previously grafted and shown to be tolerant of LG5 skin, also failed to reject outbred *X.laevis* skin grafts within 76-100 days, although slight pigment disturbances were noted in these grafts. ( To confirm that the outbred *X.laevis* skin donor was MHC-disparate to the LG5 skin donor, and, therefore, that the thymus-implanted LG15 TX showed a failure to respond to 2 antigenically different skin grafts, 2 LG5 control animals were grafted with skin from the outbred *X.laevis* donor. These grafts were rejected in 14 days).

## 4.3.2 In Vitro Assays for *Xenopus* T-Cell Activity

### 4.3.2.1 MLC Responses

#### J Strain Hosts

The one-way MLC reactivity of splenocytes from J TX animals, each implanted in larval life with a single XbJ larval thymus, was tested against stimulator splenocytes from their thymus donors and from third-party XbJ animals. One-way MLC responses of thymic implant cells from these hosts to donor XbJ splenocytes were also examined. Results from these tests were compared with those from MLCs of splenocytes of control and TX Js to XbJ and to *X.borealis* irradiated splenocytes. The mean dpm, ($\pm$ S.D.), from these cultures, and the S.I.s and p values of the differences between responses to XbJ, (donor or third-party), or to *X.borealis* cells, compared with those to J cells, are shown in Tables 1 and 2.

Splenocytes from control J strain froglets routinely showed significantly increased $^3$HTdr incorporation when stimulated with XbJ or *X.borealis* irradiated cells, as compared with J strain stimulators. In contrast, splenocytes from TX J strain froglets uniformly failed to respond to XbJ irradiated stimulators. However, TX splenocytes did show significant stimulation when cocultured with irradiated *X.borealis* splenocytes.

Splenocytes from all J strain TX implanted with XbJ larval thymi showed

elevated levels of $^{3}$HTdr incorporation when cultured with third- party XbJ stimulators, compared with those cocultured with J strain stimulators, but in only 2 of 4 cases, (animals 1 and 4), were these differences statistically significant. When the $^{3}$HTdr uptake of splenocytes from thymus-implanted animals cocultured with J strain stimulators was compared to that following coculture with thymus donor irradiated splenocytes, only animals 3 and 4 were seen to respond significantly to donor cells.

Cells taken from the implanted thymi of all four of these animals were significantly stimulated by coculture with thymus donor irradiated splenocytes. Unfortunately, insufficient implant cells were recovered to examine their responses to third-party stimulators, but thymocytes taken from a sibling, control J thymus showed a mean, (±S.D.), dpm of 1490, (± 416), when stimulated with irradiated XbJ cells and of 490, (± 83), following coculture with J strain splenocyte stimulators.

**LG15 Hosts**

The MLC reactivity of splenocytes and thymus-implant cells from a single LG15 TX animal, implanted in larval life with a pair of *X.tropicalis* larval thymi, was tested against irradiated splenocytes from host-type and from two third-party types, (LM3 and LG5). This animal was carrying, at the time of sacrifice, intact skin grafts from LG5 and outbred *X.laevis* donors which had been applied >100 days previously.

The responses of implanted host splenocytes were compared with those of splenocytes from two similarly skin grafted controls, (which had acutely rejected LG5 and *X.laevis* grafts), and one TX, (still carrying a perfect LG5 graft), LG15 animal. The results of this experiment, in terms of mean dpms, (± S.D.), S.I.s and p values, are shown in Table 3. MLC cultures were set up with two different volumes of responder and stimulator suspensions, either 100 $\mu$l, or 50 $\mu$l, of each suspension. Generally, mean dpms and S.I.s were higher when larger volumes of cells, (two-fold cell number), were cocultured.

Splenocytes from control LG15 froglets generally showed elevated levels of $^{3}$HTdr incorporation when cocultured with third-party irradiated stimulators, compared to those recorded following coculture with LG15 irradiated splenocytes, and S.I.s were particularly high when 1 x $10^{5}$ control splenocytes were cocultured with equal numbers of irradiated LG5 splenocytes, (S.I.s=4.8, 27.1). By Students' t-test, however, control animal 1 showed significant MLC reactivity only when 1 x $10^{5}$ responder cells were cocultured with LG5 irradiated splenocytes. Control animal 2 responded significantly to both LM3 and LG5 stimulator cells in all cultures.

In contrast, splenocytes from the LG15 TX froglet showed no significant increase in proliferation, (over that seen following coculture with irradiated isogeneic cells), when cocultured with either LM3 or LG5 stimulators. Splenocytes from the LG15 TX animal, implanted in larval life with *X.tropicalis* larval thymi, were not able either to respond significantly to either LM3 or LG5 stimulators, when 50 $\mu$l or 100 $\mu$l aliquots of responders and stimulators were cocultured, although the S.I.

for coculture of 100 $\mu$l of splenocytes from this animal with an equal volume of LM3 stimulators, (S.I. = 3.5), suggested that some proliferation had been induced.

Control LG15 thymocytes showed significantly elevated levels of proliferation when cocultured with 100 $\mu$l volumes of LM3 and LG5 irradiated splenocytes, compared to the proliferation seen when cocultured with LG15 splenocytes. Similarly, thymus implant cells from the LG15 TX host implanted with *X.tropicalis* thymus were found to respond significantly to both LM3 and LG5 third-party stimulators.

#### 4.3.2.2 T-Cell Mitogen Responses

The proliferative responses of splenocytes from control LG15 froglets following stimulation with PHA-P, (n=1), at two different concentrations, and with Con A, (n=2), were examined. Significantly elevated levels of $^3$HTdr incorporation were seen in all mitogen-stimulated cultures, compared with those recorded for unstimulated cultures, (see Table 4). Splenocytes from LG15 TX animals stimulated with PHA-P, (n=1) or with Con A, (n=1), showed a significant residual response to both 0.2 $\mu$g/ml PHA-P and to 1.0 $\mu$g/ml Con A, compared to unstimulated cultures, but failed to respond to 0.02 $\mu$g/ml PHA-P, (see Table 4).

Splenocytes from 2 LG15 TX,each implanted in larval life with a pair of $\gamma$-irradiated adult *X.tropicalis* thymi, were cultured with or without PHA-P, while those from a single LG15 TX host implanted with larval *X.tropicalis* thymi were cultured with or without Con A, (see Table 4). T-cell mitogen responses of all three of these host animals were significant, and, in fact, the responses of thymus-implanted animals to 0.02 $\mu$g/ml and to 0.2 $\mu$g/ml PHA-P were found, by Students' t-test, to significantly surpass those of control LG15s, ($p<0.001$).

### 4.3.3 Antibody Production to Sheep Blood Cells

The serum antibody titres of control and TX LG17 and of LG17 TX hosts, each implanted during larval life with a single *X.borealis* larval thymus, were compared by haemagglutination and haemolysis assays, (see Table 5). Sera from control, TX and thymus-implanted LG17s bled prior to immunisation were routinely found to be inactive , as was serum from unimmunised outbred *X.laevis*. Following multiple immunisation with SBC, control LG17, (n=3), serum haemagglutination titres, (-log2), ranged from 1.6-2.6, (mean=1.9), while serum haemolysis titres were 1.6-4.6, (mean=2.9). Of sera from two TX LG17s immunised with SBC, antibody activity was found in only one, (mean haemolysis/haemagglutination titres = 0.8). In sera from LG17 TX, each implanted with a single *X.borealis* larval thymus, (n=4), both haemagglutinating, (titre range:3.6-3.9, mean=3.8), and haemolytic, (titre range:2.6-4.6, mean = 3.8), anti-SBC antibodies were found. Multiply immunised *X.laevis* positive control animals showed serum haemagglutinin titres of 6.6 and 5.6, (mean = 6.1), and serum haemolysis titres of 9.6 and 8.6, (mean = 9.1).

## 4.4 Discussion

In this Chapter, 'preliminary' data on T-cell mitogen reactivity and on antibody production to a T-dependent antigen by TX hosts implanted with xenogeneic thymus glands, are reported. These responses are compared with responses made by control and TX, unimplanted animals.

Responses of TX LG15 animals to the T-cell mitogens, PHA-P and Con A were found in these studies to be lower than those of either control or thymus-implanted, TX siblings, but were still statistically significant. Other authors have also reported an ability of TX *Xenopus* to display low level responses to T-cell mitogens, (see Introduction to this Chapter). For example Donnelly et al, (1976), and Green-Donnelly & Cohen, (1979), investigating mitogen responses of density-fractionated splenocytes from intact and TX *Xenopus*, noted residual PHA and Con A responsivity in some TX fractions. These authors suggested the presence of a suppressor cell population, of a different density to that of PHA responsive cells, in TX animals, since, in their hands, unfractionated TX splenocytes did not respond to PHA.

TX LG15 implanted with *X.tropicalis* thymi responded well to PHA-P, and, in fact, produced higher stimulation indices at both concentrations tested than did control animals. Con A reactivity of the single LG15 TX, implanted with *X.tropicalis* thymus, examined was also good. These markers of T-cell maturity are, therefore, well developed in TX animals given xenogeneic thymus-implants.

Anti-sheep blood cell antibody responses were also found to be restored in TX animals, (LG17), implanted with xenogeneic, (*X.borealis*),larval thymi. Furthermore, contrary to the findings of Nagata & Cohen, (1984), these responses surpassed those seen in control siblings. Du Pasquier & Horton, (1982), found that, in LG15, LG3 and LM3 TX hosts implanted with isogeneic or fully MHC-disparate larval thymi, IgM responses to DNP-KLH were often higher in allogeneic than in isogeneic donor/host combinations.

In view of Bernard et als', (1981), analysis of the genetic control of T-B-cell cooperation in LG clonal animals, which demonstrated that totally MHC-mismatched T- and B- cells could not collaborate to generate splenic plaque-forming cell responses to the T-dependent antigen, KLH, our findings suggest that host- type T-helper cells, despite education in xenogeneic *X.borealis* thymus-implants, can still interact efficiently with host-type B-cells. An alternative possibility is that anti-SBC antibody responses in these implanted animals were made by thymus-derived *X.borealis* B-cells in collaboration with persistant *X. borealis* T-helper cells. B-cells appear from mitogen studies to be present in stage 56/57 larval thymus, (Horton et al., 1980; Williams et al, 1983), although, from Protein A plaque screening assays, (Hsu et al, 1983), immunoglobulin-secreting thymic B-cells are few in number. This alternative suggestion is unlikely, for two reasons. *X.borealis* lymphocytes within the thymic implants were found to largely replaced by LG17 lymphocytes and cells of *X.borealis* origin were not found in the spleens of implanted host animals at two months post-thymus-implantation, (see data reported in Chapter 6). Furthermore, ploidy-labelling experiments on thymus-

implanted TX *Xenopus* have shown that, in these animals, antibody-forming cells are host-derived, (Kawahara et al., 1980). No indication of the existance of the natural heterohaemagglutinins to sheep red cells reported by Jurd, (1978), was found in either unimmunised control *X.laevis* or unimmunised LG17 'prebleed' sera.

The main goal of the work described in this Chapter, was to assess the extent to which transplantation immunity was affected following early larval thymectomy and the subsequent larval implantation of semi- or fully- xenogeneic thymi.

Third-party, (allogeneic, semi- or fully-xenogeneic), skin grafts placed onto intact control J strain, LG17 or LG15 animals were all acutely rejected. In contrast, thymectomised animals grafted with allogeneic or semi-xenogeneic skin generally retained their grafts intact for long periods. Of the nine thymectomised animals grafted with fully xenogeneic *X.borealis* skin, five tolerated these grafts for the duration of the experiments; the remaining four were able to reject *X.borealis* skin, although in all but one case the rejection responses seen were chronic. These findings suggest that rejection of xenogeneic skin does not procede simply by a thymus-independent mechanism, as suggested by James et al, (1982a & b) and Clothier et al, (1990). In view of the suggested thymus-independency of responses to xenogeneic tissues,it is worthy of note here that XbJ, *X.borealis* and *X.tropicalis* thymus grafts themselves persisted in TX host animals for several months in our experiments.

The subacute rejection in 19 and 28 days, by two TX LG15 frogs, (carrying intact LG5 skin grafts), of subsequently-applied grafts of outbred *X.laevis* skin is somewhat more rapid than one might expect from a TX animal, (J strain TX animals were found to reject first-set outbred *X.laevis* grafts in 50-149 days, A8 grafts in 90 $\pm$ 7 days and LG15 grafts, (applied following accelerated rejection of second-set A8 skin), in 94 $\pm$ 16 days, (Nagata & Cohen, 1983)). Nagata & Cohen, (1983), described accelerated rejection of second-set grafts applied to TX *Xenopus*, following chronic first-set rejections, but found that these memory responses were specific for the original skin donor type. In the experiments reported in this Chapter, it is conceivable that the *X.laevis* skin donor used shared some histocompatibility antigens with the original LG5 donor and that the LG5 skin graft had immunised the TX recipient to these antigens, despite, itself, being tolerated.

Of the TX animals implanted with larval *X.borealis* or XbJ thymi, (n=8), all tolerated skin grafts of thymus donor origin for extended periods, whereas 7 of 8 rejected third-party skin acutely. One of these host animals, an LG17 TX implanted with a single *X.borealis* larval thymus, failed to reject third-party skin grafts. In two 8 month old TX J strain animals, implanted with larval XbJ thymi in late larval life, levels of XT-1 labelled splenocytes, (16.3%), assessed by indirect immunofluorescent staining and fluorescence microscopy, approached that seen in control animals, (16.9%), (data not shown); this correlated with restored third-party skin graft and MLC reactivity. It is not known whether these XTLA-1 positive cells were donor - or host-derived. In this regard, Nagata & Cohen, (1984), found that, depending on the thymus donor/TX host combination employed, persistant splenocyte chimaerism could result, (donor cells were found in

host spleens for at least one year after adult thymus implantation, in some cases). Furthermore, Horton et al, (1987), found a low level of persisting donor cells in the periphery of TX J strain animals implanted with normal adult *X.borealis* thymi 9 weeks after implantation. In contrast examination of serial sections of spleens of TX, LG17 hosts, implanted with larval *X.borealis* thymus 3-4 months previously showed that no donor-type cells were present, (see Chapter 6).

TX LG15 hosts, (n=5), implanted with either normal,larval or adult, irradiated *X.tropicalis* thymi, were found, in contrast to the majority of allo- and xeno-thymus implanted animals, to tolerate third-party skin grafts from LG5 and outbred *X.laevis* donors. Responses of these animals to thymus donor type skin were not explored, since donor animals were sacrificed at the time of thymus implantation, and since inbred lines of *X. tropicalis* are not available. It would, however, be interesting in future experiments to test the responses of implanted animals to donor *X.tropicalis* skin, and also to determine if similarly implanted host animals could reject third- party *X.tropicalis* skin grafts. This lack of response to foreign skin grafts occurred despite the fact that several of these same animals were subsequently shown to respond to the T-cell mitogens PHA and Con A. Furthermore, two LG15 TX animals implanted with larval *X.tropicalis* thymi were shown, by FACS analysis, to have large numbers (> 50%) of XTLA-1 positive cells within their thymus implants, and moderate numbers, (approximately 8%), of XTLA-1 positive peripheral lymphocytes, (data not shown). Data reported in the previous Chapter also underlines the fact that host, XTLA-1 positive, T-cells can differentiate in *X.tropicalis* thymus implants, and that these cells eventually colonise the periphery. Although there is apparently a protracted lag time between infiltration of, and differentiation within, these implants of host XT-1 staining cells and their appearance in the spleen, third-party skin grafts, applied two months after thymus-implantation, were tolerated in some cases for over 100 days without rejection. Thus, it appears that host XTLA-1 positive peripheral T-cells, which have differentiated within the xenogeneic *X.tropicalis* thymic environment, are either unable to recognise, or unable to reject, MHC antigens of a third party donor.

The limited data available on MLC reactivity of hosts implanted with larval *X.tropicalis* thymi, (data from only one animal), is equivocal, since splenocytes from this animal responded to third-party stimulators with stimulation indices greater than 1.0, but mean dpms of these cultures were not found to be significantly different to those from cultures of host splenocytes with irradiated self stimulators. Thymus implant cells from this host, on the other hand, responded significantly to both third-party stimulator types. Currently, we do not know whether the lack of graft rejection in TX animals implanted with *X.tropicalis* thymi is the result of a paucity of a particular (XTLA-1 negative?) T-cell subset, for example cytotoxic T-cells, or if the relevant cells exist, but are suppressed or are unable to function in this context due to inappropriate thymic education. Further MLC, CML and adoptive transfer experiments are required to investigate this issue. However,the effect of implantation of *X.tropicalis* thymi is certainly worthy of further investigation and such studies may be able to throw light on fundamental questions relating to thymic T-cell education.



MLC responses of splenocytes from TX J strain frogs stimulated with irradiated XbJ and *X.borealis* splenocytes were examined in some detail in this Chapter. While thymectomy was found to abrogate response to XbJ stimulator cells, **in vitro** splenocyte responses to *X.borealis* cells were found to be thymus-independent. It is quite likely that this latter response to fully xenogeneic stimulators is mediated by back stimulation. Thus, irradiated *X.borealis* cells could recognise J responders as foreign, and could release factors which stimulate TX J strain cells, (perhaps B- cells), to proliferate. In the coculture of J strain cells with XbJ stimulators, back stimulation in MLC was not possible, since XbJ cells would not recognise any MHC-antigens expressed on J strain cells. However, the nature of the proliferating cells in these xenogeneic cocultures remains to be resolved. From previous studies, (reported in Chapter 2), involving FACS analysis of TX J strain splenocytes, indirectly immunofluorescently labelled with the XT-1 monoclonal antibody, TX animals appear to lack XTLA-1 positive cells. Although XT-1 is not a pan-T-cell marker, studies which showed an absence of PHA response in TX J strain animals examined here, (unpublished data, discussed in Horton et al, 1990), indicates that T-cells were not present.

Splenocytes from TX J strain animals implanted with larval XbJ thymi showed significant MLC responses to third-party XbJ stimulators in two of four cases, although levels of $^{3}$HTdr incorporation were elevated when splenocytes from all implanted animals were cultured with third-party XbJ stimulators, compared with coculture with J strain cells. Furthermore, splenocytes from two implanted animals responded significantly to coculture with thymus-donor splenocytes. Such ' split tolerance' confirms previous findings from TX, thymus-implanted *Xenopus*, (see Introduction to this Chapter). A new finding reported here is that lymphocytes taken from xenogeneic thymus-implants responded to donor-type stimulators. Previous reports on MLC responses of thymus-restored TX *Xenopus* have concerned only splenocyte reactivity. Host splenocytes may have only limited aquaintance with thymus donor MHC antigens, since some T-cell populations may mature in the periphery under humoral influence from the implanted thymus, (Dardenne et al, 1973). It is, therefore, interesting to note that cells, (probably host-derived, see Horton & Russ, (1987), and Chapter 3), developing alongside the persisting implanted thymic stroma, (see Horton & Russ, 1987, for a description of this persistance), still display MLC reactivity. Evidence of the nature of the responding cells, i.e. that they are T- lineage cells, and of the continued expression of donor-type MHC antigens on the persisting stroma, remains to be uncovered. Panning experiments, using the XT-1 monoclonal antibody, should help to confirm that this split tolerance is a T-cell mediated phenomenon, and, thus, that donor-educated host T-cells can proliferate **in vitro** but cannot implement graft destruction.

## Figures

**Figure 4.1** Fate of *X.borealis* and XbJ skin grafts applied to control, TX and TX, thymus-implanted J strain *X.laevis* hosts. TX, thymus-implanted J hosts received XbJ skin grafts from their original thymus donors. Y-axis = end point of graft destruction, in days, as assessed by destruction of skin graft pigment cells.

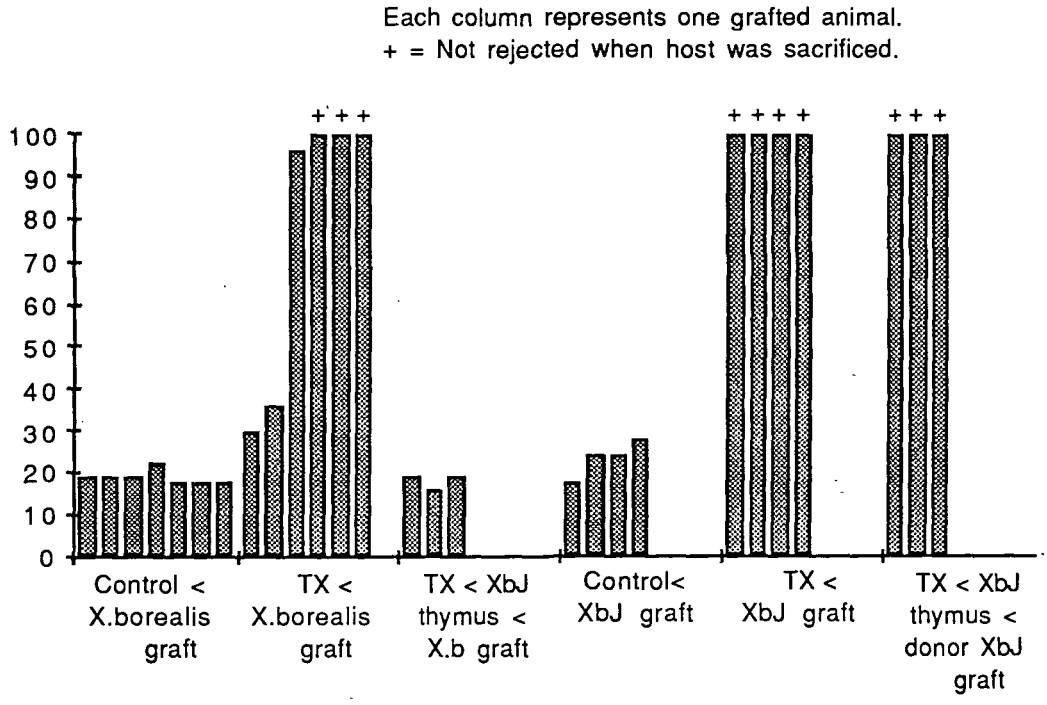


**Figure 4.2** Fate of *X.borealis* and LM3 skin grafts applied to control, and TX, thymus-implanted LG17 hosts. TX, thymus-implanted LG17 hosts received *X.borealis* skin grafts from their original thymus donor. Y-axis = end point of graft destruction, in days.

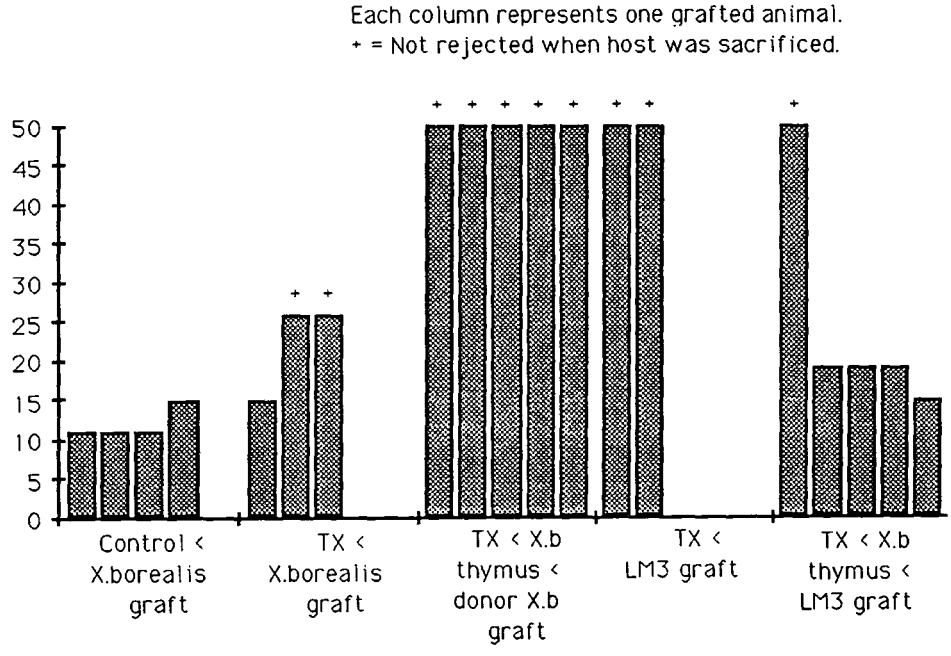


**Figure 4.3** Fate of LG5 and outbred *X.laevis* skin grafts applied to control, TX and TX LG15, implanted during larval life with *X.tropicalis* thymi. Y-axis = end point of graft destruction, in days.

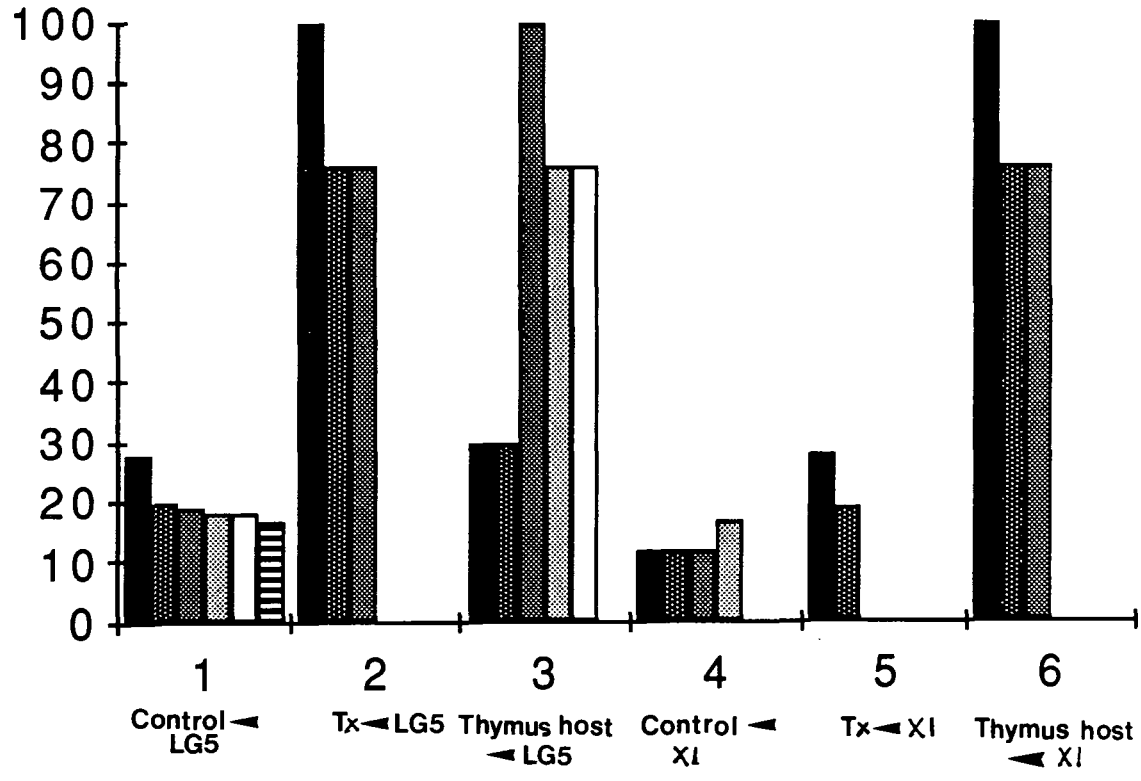


Table 4.1 MLC reactivity of splenocytes from control and T x J strain *X.laevis* to *X.borealis* and XbJ stimulators.

| Animal number | Vs. irradiated J strain Mean dpm (+/-S.D.) | Vs. irradiated XbJ Mean dpm (+/-S.D.) | S.I. | p | Vs. irradiated X.borealis Mean dpm (+/-S.D.) | S.I. | p |
|---|---|---|---|---|---|---|---|
| **CONTROL** | | | | | | | |
| 1 | 353 (+/-251) | 800 (+/-62) | 2.3 | .05 | 3204 (+/-909) | 9.1 | .01 |
| 2 | 131 (+/-9) | 448 (+/-57) | 3.4 | .001 | 921 (+/-438) | 7 | .05 |
| 3 | 1109 (+/-523) | 3860 (+/-504) | 3.5 | .01 | 6773 (+/-443) | 6.1 | .001 |
| 4 | 772 (+/-275) | 3296 (+/-239) | 4.3 | .001 | 4888 (+/-152) | 6.3 | .001 |
| 5 | 456 (+/-326) | N.D. | - | - | 6243 (+/-908) | 13.7 | .001 |
| 6 | 1131 (+/-902) | N.D. | - | - | 7928 (+/-332) | 7 | .001 |
| 7 | 736 (+/-119) | 7113 (+/-1095) | 9.7 | .001 | 8152 (+/-1571) | 11.1 | .001 |
| 8 | 2564 (+/-584) | 5223 (+/-417) | 2 | .01 | 8996 (+/-365) | 3.5 | .001 |
| **THYMECTOMISED** | | | | | | | |
| 1 | 225 (+/-48) | N.D. | - | - | 1431 (+/-531) | 6.4 | .02 |
| 2 | 285 (+/-71) | N.D. | - | - | 1554 (+/-278) | 5.5 | .002 |
| 3 | 163 (+/-72) | 251 (+/-117) | 1.5 | N.S. | 721 (+/-265) | 4.4 | .05 |
| 4 | 396 (+/-150) | 447 (+/-84) | 1.1 | N.S. | 2522 (+/-1099) | 6.4 | .05 |
| 5 | 323 (+/-20) | 438 (+/-131) | 1.4 | N.S. | 6496 (+/-3240) | 20 | .05 |
| 6 | 430 (+/-30) | 447 (+/-46) | 1 | N.S. | 3496 (+/-1473) | 8.1 | .05 |

N.D. = Not done.
N.S. = Not significant, p> 0.05

Table 4.2 MLC reactivity of splenocytes and thymus-implant cells from 7 day-TX J strain *X.laevis*, implanted with larval XbJ thymus in late larval life.

| Animal number | Vs. irradiated self Mean dpm (+/-S.D.) | Vs. irradiated XbJ thymus donor Mean dpm (+/-S.D.) | S.I. | p | Vs. irradiated XbJ third party Mean dpm (+/-S.D.) | S.I. | p |
|---|---|---|---|---|---|---|---|
| **SPLENOCYTES** | | | | | | | |
| 1 | 1750 (+/-533) | 2888 (+/-1538) | 1.7 | N.S. | 3805 (+/-448) | 2.2 | .01 |
| 2 | 598 (+/-379) | 1413 (+/-595) | 2.4 | N.S. | 1703 (+/-1302) | 2.8 | N.S. |
| 3 | 324 (+/-235) | 1280 (+/-325) | 4 | .02 | 670 (+/-147) | 2.1 | N.S. |
| 4 | 184 (+/-34) | 588 (+/-190) | 3.2 | .05 | 926 (+/-58) | 5 | .001 |
| **THYMUS GRAFT CELLS** | | | | | | | |
| 1 | 1115 (+/-157) | 2810 (+/-426) | 2.5 | .01 | N.D. | - | - |
| 2 | 474 (+/-230) | 1991 (+/-509) | 4.2 | .01 | N.D. | - | - |
| 3 | 573 (+/-174) | 3135 (+/-530) | 5.5 | .002 | N.D. | - | - |
| 4 | 814 (+/-407) | 2683 (+/-639) | 3.3 | .02 | N.D. | - | - |

N.D. = Not done.
N.S. = Not significant, p>0.05.

**Table 4.3** MLC reactivity of splenocytes from TX LG15, of splenocytes and thymocytes from control LG15 and of splenocytes and thymus-implant cells from TX LG15, implanted in late larval life with *X.tropicalis* thymi.

**SPLENOCYTE RESPONDERS**

| Splenocyte source | Volume (ul)* | vs. self (LG15) dpm (+/- S.D.) | | vs. LM3 dpm (+/- S.D.) | | S.I. | p | vs. LG5 dpm (+/- S.D.) | | S.I. | p |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control 1 | 50 | 146 | (+/-44) | 147 | (+/-22) | 1 | - | 311 | (+/-105) | 2.1 | NS |
| | 100 | 166 | (+/-48) | 310 | (+/-97) | 1.9 | NS | 801 | (+/-265) | 4.8 | .025 |
| Control 2 | 50 | 108 | (+/-28) | 195 | (+/-43) | 1.8 | .05 | 479 | (+/-60) | 4.4 | .001 |
| | 100 | 69 | (+/-19) | 584 | (+/-13) | 8.5 | .001 | 1872 | (+/-245) | 27.1 | .001 |
| LG15 TX | 50 | 186 | (+/-90) | 126 | (+/-19) | 0.7 | - | 115 | (+/-13) | 0.6 | - |
| | 100 | 274 | (+/-330) | 331 | (+/-232) | 1.2 | NS | 194 | (+/-119) | 0.7 | - |
| LG15 TX given X.tropicalis thymus | 50 | 124 | (+/-57) | 114 | (+/-25) | 0.9 | - | 143 | (+/-37) | 1 | - |
| | 100 | 125 | (+/-39) | 439 | (+/-215) | 3.5 | NS | 177 | (+/-39) | 1.4 | NS |

**THYMOCYTE RESPONDERS**

| Thymocyte source | Volume (ul)* | vs. self (LG15) dpm (+/- S.D.) | | vs. LM3 dpm (+/- S.D.) | | S.I. | p | vs. LG5 dpm (+/- S.D.) | | S.I. | p |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control 1 | 50 | 526 | (+/-127) | 795 | (+/-150) | 1.5 | NS | 1355 | (+/-49) | 2.6 | .005 |
| | 100 | 590 | (+/-49) | 2039 | (+/-550) | 3.5 | .025 | 1808 | (+/-195) | 3.1 | .005 |
| LG15 TX given X.tropicalis thymus | 50 | 644 | (+/-86) | 2100 | (+/-879) | 3.3 | NS | 937 | (+/-148) | 1.5 | NS |
| | 100 | 697 | (+/-84) | 2217 | (+/-411) | 3.2 | .01 | 1430 | (+/-290) | 2.1 | .025 |

NS = Not Significant, p>0.05

* = Volume of responder and stimulator suspensions cocultured.

**Table 4.4** T-cell mitogen responses of splenocytes from control and TX LG15 and from TX LG15, implanted in late larval life with *X.tropicalis* thymi.

| Splenocyte source | Unstimulated mean dpm (+/-S.D.) | + 1 ug/ml Con A mean dpm (+/-S.D.) | S.I. | + 0.02 ug/ml PHA-P mean dpm (+/- S.D.) | S.I. | p | + 0.2 ug/ml PHA-P mean dpm (+/- S.D.) | S.I. | p |
|---|---|---|---|---|---|---|---|---|---|
| Control 1 | 387 (+/-144) | 3192 (+/-57) | 8.2 | Not Done | | | Not Done | | |
| Control 2 | 142 (+/-24 | 6493 (+/-467) | 45.7 | Not Done | | | Not Done | | |
| Control 3 | 73 (+/-13) | Not Done | | 152 (+/-42) | 2 | .025 | 804 (+/-191) | 11 | .001 |
| TX 1 | 241 (+/-92) | 1183 (+/-265) | 4.9 | Not Done | | | Not Done | | |
| TX 2 | 98 (+/-86) | Not Done | | 93 (+/-12) | 0.9 | - | 503 (+/-171) | 5.1 | .005 |
| TX + X.tropicalis thymus 1 | 218 (+/-74) | 3795 (+/-314) | 17.4 | Not Done | | | Not Done | | |
| TX + X.tropicalis thymus 2 | 62 (+/-1) | Not Done | | 655 (+/-119) | 11 | .005 | 2099 (+/-214) | 33.8 | .001 |
| TX + X.tropicalis thymus 3 | 188 (+/-102) | Not Done | | 613 (+/-268) | 3.3 | .025 | 4235 (+/-980) | 22.5 | .001 |

**Table 4.5** Serum haemagglutination and haemolysis titres from SBC- immunised and unimmunised *X.laevis* controls and from SBC-immunised and unimmunised LG17s,(control, TX and TX, *X. borealis* thymus implanted).

| Animal number. | *X.laevis* | | | |
|---|---|---|---|---|
| | Negative control | | Positive control | |
| | + C' | - C' | + C' | - C' |
| | 0 | 0 | 9.6 | 6.6 |
| | | | 8.6 | 5.6 |

**LG17 Control**

| | Prebleed | | Immune | |
|---|---|---|---|---|
| | + C' | - C' | + C' | - C' |
| 1 | 0 | Not Done | 4.6 | 2.6 |
| 2 | 0 | Not Done | 1.6 | 1.6 |
| 3 | Not Done | Not Done | 2.6 | 1.6 |

**TX**

| | Prebleed | | Immune | |
|---|---|---|---|---|
| | + C' | - C' | + C' | - C' |
| 1 | 0 | Not Done | 0 | 0 |
| 2 | Not Done | Not Done | 1.6 | 1.6 |

**TX, thymus implanted**

| | Prebleed | | Immune | |
|---|---|---|---|---|
| | + C' | - C' | + C' | - C' |
| 1 | 0 | Not Done | 4.6 | 3.6 |
| 2 | 0 | Not Done | 3.9 | 3.9 |
| 3 | 0 | Not Done | 2.6 | 3.6 |
| 4 | Not Done | Not Done | 3.9 | 3.9 |

+C' = Included guinea pig complement, ie heamolysin titre.
-C' = No guinea pig complement, ie haemagglutinin titre.
Titres expressed as -log2

# Chapter 5

# In vivo and In vitro immunological responses of Xenopus tropicalis

## 5.1 Introduction

Both larval and adult *X.laevis* are able to reject skin allografts acutely, (Horton, 1969; Tochinai & Katagiri, 1975; Manning & Turner, 1976). Mixed leukocyte responses, (MLCs), are effected by *Xenopus* laevis thymocytes from larval stage 54 onwards, (Du Pasquier & Weiss, 1973), although there is a depression of this MLC at metamorphosis,and fully effective responses do not recover until two months post-metamorphosis, (Du Pasquier & Chardonnens, 1975). Du Pasquier et al., (1975), found, from family studies using outbred *X.laevis* adults, that MLC responses of peripheral blood leukocytes and acute graft rejection were apparently controlled by the same genetic region; these studies revealed four groups of MLC identical siblings within one family, implying heterozygosity of the parent animals and the expression by their progeny of 2 MLC haplotypes each. Furthermore, skin graft rejection was seen between MLC-identical siblings, implying the existence of further, independent, histocompatibility loci. Studies on the genetic control of histocompatibility reactions, (MLC responses and acute skin graft rejections), in other *Xenopus* species, (*X.vestitus*, and *X.ruwenzoriensis*), and in laboratory-created hybrids, (hybrids between *X.ruwenzoriensis* and *X.laevis* and trispecies *X.laevis/X.gilli/X.clivii* hybrids), were also made, (Du Pasquier et al, 1977). Results consistent with expression of a single MHC complex were found for *X.vestitus*, (2n = 72 chromosomes), but not for *X.ruwenzoriensis* animals, (2n = 108 chromosomes). Laboratory made hybrid polyploid individuals also showed MLC and graft rejection responses which indicated codominant expression of each constituent MHC complex. The two or more loci found in *X.ruwenzoriensis* did not appear to be equivalent in strength, one eliciting stronger MLC and more acute graft rejection than the others. Kobel & Du Pasquier, (1986), comment that *X.ruwenzoriensis* is considered for other reasons to be a recent, naturally-occurring polyploid. These authors also quote data from MLC experiments per-

formed with *X.tropicalis* cells, which indicates that this species expresses one MLC locus per gamete.

Recent work, (Flajnik et al., 1984; Kaufman et al., 1985a & b), involving the immunoprecipitation with *Xenopus* alloantisera, or with rabbit anti- human class II molecule antibodies, of *X.laevis* and LG clonal cell surface antigens revealed the existance of class I and class II MHC antigens in these animals. The class I antigens immunoprecipitated from leukocytes and erythrocytes were found to comprise a polymorphic intrinsic glycoprotein heavy chain of 40 - 45 kD, in noncovalent association with a minimally polymorphic light chain, equivalent to $\beta_2$-microglobulin. Unfortunately, the alloantisera used in these studies contained various unwanted specificities, and so were not useful for immunofluorescence work, making it impossible to tell whether the class I antigens recognised were classical transplantation antigens, (present on all cells), or much less polymorphic class I antigens with restricted tissue distributions, like the Tla/Qa antigens. In fact, in a recent review Flajnik et al, (1988), comment that, as yet, no evidence has been found of Qa or Tl-like class I molecules either on haemopoietic cells, or secreted in the serum. *Xenopus* class II MHC antigens, immunoprecipitated from lymphocytes, were composed of two 30 - 35 kD transmembrane glycoproteins. Mouse monoclonal antibodies to *Xenopus* class I and class II antigens have now been produced, (Du Pasquier & Flajnik, 1987; Flajnik et al., 1988). It is known, (see Du Pasquier et al., 1989, for review), that class I heavy chains and class II chains are encoded by the *Xenopus* MHC complex, (named the XLA complex, Kobel & Du Pasquier, 1977), and that, for each XLA haplotype, there are 2$\alpha$- and up to 5 $\beta$-class II loci. Patterns of class II antigens run on 2D gels were found to match precisely with MLC data from family studies, implying that recognition of these antigens is the normal cue for T-cell proliferative responses to foreign lymphocytes. It was also found in these studies, however, that MLC tests may underestimate class II antigen polymorphism, (reviewed by Flajnik et al., 1985).

Larval *Xenopus* lymphocytes express $\alpha$- and $\beta$- class II chains identical to those of adults, but the tissue distribution of these antigens is different in tadpoles, (only 50% - 70% of tadpole splenic lymphocytes, including B-cells, express class II, whereas all adult lymphocytes are class II positive), (Du Pasquier & Flajnik, 1987). From immunoprecipitation studies, class I MHC antigens are apparently not expressed before metamorphosis, in *Xenopus*, (Du Pasquier et al., 1979; Flajnik et al., 1986). Moreover, it has been reported that tadpole lymphocytes proliferate when cultured with isogeneic adult cells **in vitro**, (Du Pasquier et al, 1979), possibly in response to the class I antigens expressed by adult cells.

Cell-mediated lympholysis, (CML), responses can be generated in *Xenopus*, although prior **in vivo** and **in vitro** priming of responders is required. CML-reactive cells specifically recognise MHC-linked target molecules on lymphoblasts, (reviewed by Du Pasquier et al., 1989). Lallone & Horton, (1985), assessed both MLC and CML as **in vitro** correlates of **in vivo** transplantation reactions. These workers found that CML responsivity was a poor correlate of **in vivo** skin graft rejection, since this response was difficult to demonstrate against minor histocompatibility antigen-disparate targets, but that MLC responses correlated well with skin graft reactivity.

The thymus-dependency of acute skin graft rejection and MLC responses has been shown by a number of authors, in studies on early-thymectomised *Xenopus*, (see reviews by Flajnik et al., 1987 and Du Pasquier et al, 1989; see, also, introduction to Chapter 4). Proliferative responses to mammalian T-cell mitogens, such as phytohaemagglutinin-A, (PHA), and Concanavalin A, (Con A), have also been demonstrated in *Xenopus*, and have been shown to be abrogated or significantly reduced, following early-thymectomy, indicating the thymus-dependent nature of these reactions, ( see review by Du Pasquier et al.,1989; see also introduction to Chapter 4). Restoration of thymus- dependent immune responses to early-thymectomised *Xenopus* with implanted irradiated or non-irradiated, larval or adult MHC-matched or partially or totally mismatched thymi has also been demonstrated, (see Chapter 4).

*X.tropicalis* differ from other strains and species of *Xenopus* so far examined in non-expression of the XTLA-1 T-cell surface antigen, (see Chapters 2 and 3). Moreover, in preliminary investigations, xeno-implanted larval or adult *X.tropicalis* thymi failed to restore, (third-party), skin graft rejection responses to early-thymectomised individuals. Implantation of adult irradiated or larval *X.tropicalis* thymi into early-thymectomised LG15 hosts was shown, however, to restore responsivity to PHA and to Con A and to facilitate the differentiation of XTLA-1 positive thymocytes and splenocytes, (see Chapters 3 and 4).

Reports of the use of *X.tropicalis* as skin graft donors, (Clothier et al, 1990), and of irradiated *X.tropicalis* splenocytes as xenogeneic stimulators in studies of *X.laevis* immune responses, have shown that they provoke strong reactions, comparable to those seen in response to allogeneic tissues and cells, (Lallone & Horton, 1985). Furthermore, Kobel & Du Pasquier, (1986), quote data from unpublished experiments designed to establish the number of MLC haplotypes expressed by *X.tropicalis* lymphocytes, implying that MLC reactivity to allogeneic *X.tropicalis* stimulators is seen in this species. In general, however, the immune system of *X.tropicalis* appears not to have been examined in any depth.

Since, as discussed in the previous Chapter, TX LG15 larvally implanted with *X.tropicalis* may prove to represent an interesting model for the study of thymic control over transplantation reactivity in *Xenopus*, we felt that it was important to investigate a range of classical thymus-dependent immune responses in this species. The experiments reported in these Chapters explore skin graft, MLC and T-cell mitogen responses in intact *Xenopus* tropicalis.

## 5.2 Materials and Methods

### 5.2.1 Animals and Skin Grafting

#### 5.2.1.1 Animals

Adult *X.tropicalis* animals were a gift from Dr. R.H. Clothier, University of Nottingham. Members of this group of animals were the parents of three further sibships of *X.tropicalis* bred in our laboratory. Additionally, outbred and inbred

J strain *X.laevis*, outbred *X.borealis*, isogeneic clonal LG15 and (LG15 x outbred *X.laevis*) $F_1$ animals were bred and reared in our laboratory as described in Horton & Manning, (1972), and in Kobel & Du Pasquier, (1975).

#### 5.2.1.2 Skin grafting operations

Skin grafting operations were performed on 12-15 month old frogs, as described in Chapter 4, except that dorsal, rather than ventral, *X.tropicalis* skin was applied to recipients, since ventral skin of these animals lacks the irridiophores and chromatophores usually used to determine the end point of graft destruction.

### 5.2.2 In Vitro Assays for T-cell activity

#### 5.2.2.1 Tissue Culture Medium

Amphibian-strength Leibovitz L-15, as described in Chapter 4, was used for in vitro assays, except that, in initial PHA response assays, additional cultures were set up in L-15 medium supplemented with 0.25% bovine serum albumen, (BSA), (Sigma Chemicals).

#### 5.2.2.2 MLC Responses

Splenocyte single cell suspensions were prepared from 5-17 month old *Xenopus*, as described in Chapter 4, and were adjusted to a concentration of 1 x $10^6$/ml Irradiated stimulator cells were gamma-irradiated, (6,000 Rads), as previously described. 100 $\mu$l each of responder and stimulator splenocytes were plated out into 96-well V-bottomed microtitre plates, (Sterilin Ltd.), and were cultured as previously described. Since the kinetics of the *X.tropicalis* MLC response were unknown, pulsing of cultures with 1 $\mu$Ci $^3$HTdr and subsequent harvesting were performed after two different intervals, as shown in the Results section of this Chapter.

#### 5.2.2.3 T-cell Mitogen Responses

**PHA-P Responses**

PHA-P response assays described in this Chapter were carried out in 96- well V-bottomed microtitre plates, (Sterilin Ltd.), using 1 x $10^5$ splenocytes or thymocytes, from 5-17 month old *Xenopus*, in 100 $\mu$l tissue culture medium. PHA-P was added to experimental wells to give a range of final concentrations, designed to determine the optimal dose for stimulation of *X.tropicalis* cells. Control cultures received tissue culture medium rather than mitogen. Cultures were pulsed with 1 $\mu$Ci per well $^3$HTdr 24 hours prior to harvesting. In order to investigate the kinetics of responses of *X.tropicalis* cells to this mitogen, cultures were harvested

at a range of times after initiation, as shown in Tables 5, 6 and 7. Three replicates were set up for each cell suspension/PHA-P concentration combination.

**Con A Responses**

Con A response assays reported in this Chapter were set up and pulsed as for PHA-P assays, described above, except that experimental wells contained various Con A concentrations, with or without the addition of $\alpha$- methyl mannoside. Splenocyte cultures were harvested at a range of times after initiation, as shown in Tables 8 and 9. Thymocyte cultures were harvested on day 3. Again, three replicate cultures were set up for each cell suspension/Con A concentration, ($\pm$ $\alpha$-methyl mannoside), combination.

Cell harvesting, scintillation counting and calculation of S.I.s and statistical significance for all **in vitro** assays reported in this Chapter were performed as described in Chapter 4.

## 5.3 Results

### 5.3.1 Skin Grafting Experiments

The responses of adult, (12 - 15 months of age), *X.tropicalis* to a skin graft from an outbred *X.laevis* or an inbred, (J strain), *X.laevis* adult, (6 - 7 months old), or from a non-sibling *X.tropicalis* adult, (12-14 months old), were examined. Further outbred *X.laevis* animals were grafted with either *X.tropicalis* dorsal skin grafts, or with skin from non-sibling outbred *X.laevis* animals. Additionally, *X.tropicalis* animals were given autografts, to ascertain the effect on both grafts and hosts of this manipulation, (see Table 1).

Outbred *X.laevis* frogs rejected inbred J strain grafts, (n=3), and outbred *X.laevis* grafts, (n=1), acutely, (19 - 24 days). *X.tropicalis* grafts, (n=5), were also rejected acutely, (16 -23 days).

*X.tropicalis* adults, (n=5), grafted with *X.laevis* skin, (from either inbred J strain or outbred donors), rejected these grafts in 19-33 days. Allogeneic *X.tropicalis* grafts, (n=4), were rejected in 19-29 days. *X.tropicalis* animals which were autografted showed no sign of immunological activity towards these grafts within 60 days.

### 5.3.2 In Vitro Assays

#### 5.3.2.1 MLC Responses

The proliferative responses shown by splenocytes of *X.laevis*, (J strain or outbred), and of *X.borealis* to irradiated xenogeneic *X.tropicalis* stimulator splenocytes were examined at two time points, ($^3$HTdr pulse at 3 days, harvest at 4 days; $^3$HTdr

pulse at 4 days, harvest at 5 days), (see Table 2). All *X.laevis*, (n=7 combinations), and *X.borealis*, (n=1), responder cells harvested at 4 days, (found to be optimal for these cells), showed significantly elevated mean dpms, following coculture with *X.tropicalis* stimulators, compared to those recorded following cocultures with irradiated responder type cells. J strain responder splenocytes from 2/3 cocultures with different *X.tropicalis* stimulators also showed significantly elevated proliferation levels, when harvested at 5 days. The stimulation indices from some of these J vs. *X.tropicalis* cocultures were rather high, due in part to the low mean dpms from J vs. irradiated self background control cultures. Mean dpms of *X.tropicalis* irradiated cells, alone, (shown in Table 2), ranged from 39-470 and, although these are generally higher than counts recorded for J irradiated splenocytes, (mean dpms shown in Table 3), preliminary experiments had shown that irradiated *X.tropicalis* splenocytes did not proliferate when stimulated with T-cell mitogens, thus the high S.I.s from J vs. *X.tropicalis* irradiated stimulators are not artefactual. Due to low numbers of *X.laevis* splenocytes, 5 day *X.laevis* background cocultures, (responder vs. irradiated responder cells), for 5 day cultures were not set up. In calculating 5 day S.I.s, therefore, background control dpms used were those obtained from 4 day cultures.

In contrast, *X.tropicalis* responder splenocytes were not significantly stimulated by either *X.laevis* or *X.borealis* irradiated splenocytes at either 4 or 5 days, (see Table 3). In fact, in a number of cultures, the mean dpm of *X.tropicalis* responder cells cocultured with *X.laevis* or *X.borealis* stimulators was significantly reduced, compared with those of *X.tropicalis* irradiated/non-irradiated splenocyte cultures. Again, this appears to be a genuine phenomenon, as differences between mean dpms from cultures of irradiated *X.tropicalis* and of irradiated *X.laevis* or *X.borealis* splenocytes do not account for the significantly reduced levels of proliferation observed in cocultures of *X.tropicalis* responders with *X.laevis* or *X.borealis* stimulators.

MLC responses, at 4 and 5 days, of *X.tropicalis* splenocytes cocultured with irradiated allogeneic *X.tropicalis* stimulator cells were also tested, (see Table 4). At 4 days, 6 of 8 combinations of *X.tropicalis* responder splenocytes with irradiated allogeneic stimulators showed elevated $^{3}$HTdr incorporation, compared to levels of incorporation seen when responders were cultured with irradiated self stimulators. At 5 days, however, only 1 of 6 allogeneic combinations tested showed significant stimulation.

#### 5.3.2.2 Responses to T-Cell Mitogens

#### PHA-P Responses

*X.tropicalis* thymocytes, from two animals, were cultured in either 1% FCS-supplemented or 0.25% BSA-supplemented culture medium, with PHA-P at a range of concentrations, (from 0.01 $\mu$g/ml to 5.0 $\mu$g/ml), or without PHA- P. Since neither the optimal PHA-P concentration required to stimulate *X.tropicalis* lymphocytes, nor the kinetics of their responses to this mitogen were known, dif-

ferent cultures were harvested at several intervals after initiation, (see Table 5). Significantly elevated proliferation levels, compared to those of unstimulated cultures, were seen when *X.tropicalis* thymocytes were cultured with 0.01 $\mu$g/ml, 0.05 $\mu$g/ml, 0.1 $\mu$g/ml or 0.5 $\mu$g/ml PHA-P. The highest stimulation indices were found in 3- 4 day cultures including 0.1 $\mu$g/ml PHA-P. Stimulation indices were generally higher in BSA-supplemented medium than in FCS-supplemented medium, but mean dpms of both control and PHA-P stimulated cultures set up in BSA-medium were lower.

Splenocyte suspensions from four *X.tropicalis* were also cultured in either 1% FCS-supplemented or 0.25% BSA- supplemented medium and stimulated with PHA-P, (see Table 6). Cultures set up in FCS-supplemented medium responded with significantly elevated dpms to all PHA-P concentrations tested at 2, 3 and 4 days, and to all but the two highest concentrations at 5 days. Cultures set up in BSA-supplemented medium responded significantly to 0.05 $\mu$g/ml and to 0.1 $\mu$g/ml at 2, 3 and 4 days, and also to 0.5 $\mu$g/ml and to 1.0 $\mu$g/ml at 2 days. No significant stimulation was seen at 5 days by any of the PHA-P concentrations used, in cultures set up in BSA- supplemented medium. Optimal responses from cultures in BSA-medium, in terms of stimulation indices, were obtained at 4 days, with 0.05 $\mu$g/ml and 0.1 $\mu$g/ml PHA-P.

Table 7 shows mean dpm, ($\pm$ S.D.), and PHA-stimulation indices recorded for splenocytes from two (LG15 x outbred *X.laevis*)$F_1$ animals and for one outbred *X.laevis* animal. Mitogen-stimulated cultures, (containing 0.1 $\mu$g/ml PHA-P), harvested at 2, 3, 4 and 5 days showed significant stimulation, compared with control cultures.

It can be seen in these experiments that proliferation levels of unstimulated *X.tropicalis* splenocytes were high, (dpms between 2,200- 8,500), compared with those of *X.laevis* and (LG15 x wild)$F_1$ unstimulated splenocyte cultures, (dpms between 235-1,685), hence *X.tropicalis* splenocyte S.I.s tended to be lower.

### Concanavalin A Responses

Cultures of thymocytes and splenocytes from *X.laevis*, LG15, *X.borealis* and *X.tropicalis* were set up in FCS-supplemented culture medium, including Con A at a range of concentrations. Again, since the kinetics of the response of *X.tropicalis* cells to this mitogen were unknown, 3, 4 and 5 day harvesting times were used. Furthermore, to assay for the specificity of response to Con A, a number of cultures included 0.1 M $\alpha$-methyl mannoside, the specific ligand for Con A, which, in this concentration, has proved in this laboratory to successfully prevent mitogenesis of *X.laevis* T-cells. Mean dpms, ($\pm$ S.D.), and stimulation indices recorded from this series of experiments are shown in Tables 8 and 9. The highest stimulation indices recorded, from both splenocyte and thymocyte cultures, were noted with 0.5 $\mu$g/ml and 1.0 $\mu$g/ml Con A, for all strains and species of *Xenopus* examined. Significantly elevated mean dpms were found for Con A stimulated splenocyte cultures harvested at 3, 4 and 5 days. Thymocyte cultures were all harvested on day 3. Addition of $\alpha$-methyl mannoside to the culture medium abrogated the mitogenic effect of Con A in the majority of cases, although small residual

responses were occasionally seen.

Again, mean dpms from unstimulated *X.tropicalis* cultures were generally much higher than those from unstimulated *X.laevis*, *X.borealis* or LG15 cultures.

## 5.4 Discussion

Thymocytes and splenocytes of *X.tropicalis* were compared to those of other *Xenopus* species, in terms of their responsivity to T-cell mitogens. These experiments revealed that *X.tropicalis* cells were sensitive to these mitogens, responding with significantly elevated levels of proliferation to both PHA-P and to Con A, although stimulation indices were often lower in *X.tropicalis* cultures than in those of other *Xenopus* species examined, due to high background proliferation levels of unstimulated *X.tropicalis* cells. The optimal dosage and kinetics of Con A polyclonal stimulation were found to be similar for all *Xenopus* species examined, and addition of $\alpha$-methyl mannoside generally abrogated this effect, confirming the specificity of the stimulation. Although the kinetics of *X.tropicalis* lymphocyte stimulation by PHA-P resembled those of other *Xenopus* species, *X.tropicalis* cells responded optimally to a 10-fold lower PHA-P concentration. Further studies, employing early-thymectomised *X.tropicalis* individuals are required to confirm the thymus-dependency of PHA-P and Con A responses in this species.

Rejection of both skin allografts and xenografts by *X.laevis* hosts is acute, as described by Lallone & Horton, (1985), and by Clothier et al, (1990), although the latter authors consider that xenograft response is thymus-independent and that it procedes by a different mechanism to allograft rejection, (discussed further in the next Chapter). We are now able to report that *X.tropicalis* adults also acutely reject both allografts and xenografts, in times not significantly different from those taken by *X.laevis*, ( comparison, by Students' T-test, of mean rejection times for allografts and xenografts by each species revealed no significant differences, $p>0.05$). The histological appearance of grafts being rejected by *X.tropicalis* was not examined in these studies. Furthermore, the thymus- dependency, or otherwise, of these responses was not addressed, since thymectomised *X.tropicalis* animals were not available. However, graft rejections seen in this species were not due to the effects of experimental manipulation on either grafts or hosts, since autografts survived for long periods in perfect condition.

MLC responses of *X.laevis* and of *X.borealis* splenocytes to *X.tropicalis* stimulators, found to be optimal after 4 days culture, were significant and showed stimulation indices in some cases higher than those recorded for allogeneic stimulation, (c.f. control LG15 responses to LG5 stimulators, quoted in Chapter 4, for example). In cocultures of *X.tropicalis* splenocytes with irradiated allogeneic cells the majority of combinations harvested after 4 days culture, (but only one of those harvested after 5 days culture), were found to be stimulatory, although stimulation indices were still quite low. Kobel and Du Pasquier, (1986), quote data from other sources which indicate that *X.tropicalis* lymphocytes express two MLC haplotypes per cell. To obtain this data, MLC tests must have been performed using allogeneic *X.tropicalis* stimulator cells, and significant stimulations

recorded. Unfortunately, the original source of these results could not be found, so it was impossible to compare our protocols with those used in previous studies

Responder splenocytes from *X.tropicalis* animals, (n=8), showed, on the other hand, no increased proliferation, following coculture with *X.laevis* or *X.borealis* stimulators, after either 4 or 5 days of culture.This apparent lack of MLC stimulation of *X.tropicalis* splenocytes by xenogeneic irradiated splenocytes is puzzling. Previous studies, (Lallone & Horton, 1985), have shown that splenic MLC is a good in vitro correlate of acute skin allograft rejection, and such rejection occurred following xenografting of *X.tropicalis*. The lack of correlation between acute skin xenograft rejection and splenic MLC response to xenogeneic stimulators seen here requires further investigation.

Taken together, stimulation indices recorded for MLC responses of *X.tropicalis* splenocytes to allo- and, particularly, to xeno-stimulators might be seen to reflect inappropriate culture conditions for the demonstration of strong reactivity to histocompatibility antigens. However, T-cell mitogen-stimulated *X.tropicalis* splenocytes, cultured in identical medium, showed significant stimulation at both 4 and 5 days of culture, suggesting that at least some T-cells survive in culture for these periods. One noticeable feature of these experiments with *X.tropicalis* was that background control, (coculture with irradiated self cells), dpms were relatively high; high background proliferation levels were also seen in mitogen experiments. Interestingly, mean dpms of irradiated *X.tropicalis* stimulators were generally higher, (2-15 fold), than those of irradiated *X.laevis* or *X.borealis*, but preliminary tests had shown that 6,000 Rad irradiation reduced polyclonal mitogenic stimulation of *X.tropicalis* splenocytes to insignificant levels, suggesting that this irradiation dose was fully effective in abrogating the capacity for proliferation. High background counts may have precluded, or reduced, the visualisation of induced mitogenesis, particularly in MLC experiments, where S.I.s were much lower than in mitogen experiments. It is possible that the kinetics of *X.tropicalis* splenocyte MLC responses are very different from those of other *Xenopus* species, and, consequently, that protocols used in the studies reported here did not permit demonstration of reactivity of these cells to xenogeneic stimulators.

In summary, then, *X.tropicalis* show many classical T-cell responses, despite their lack of expression of the XTLA-1 T-cell marker. Although it has not been shown here that these responses are, indeed, dependent upon the thymus, this species may be regarded as a useful and appropriate thymus-donor with which to explore T-cell development in xenogeneic thymus chimaeras.

**Table 5.1** Skin allo- and xenograft rejection by *X.laevis* and *X.tropicalis* recipients.

| Host animal | Outbred X.laevis donor | | | J strain X.laevis donor | | |
|---|---|---|---|---|---|---|
| | Rejection times (days) | n | Mean graft survival time (+/- S.D.) | Rejection times (days) | n | Mean graft survival time (+/- S.D.) |
| Outbred X.laevis | 19 | 1 | - | 19,19,24 | 3 | 20.7 (+/-3) |
| X.tropicalis | 19,19 | 2 | 19 | 19,29,33 | 3 | 27 (+/-7) |

| | X.tropicalis (allograft) donor | | | X.tropicalis (autograft) donor | |
|---|---|---|---|---|---|
| | Rejection times (days) | n | Mean graft survival time (+/- S.D.) | Rejection times (days) | n |
| Outbred X.laevis | 19,23,23,19,16 | 5 | 20 (+/-3) | Not done | - |
| X.tropicalis | 24,24,29,19 | 4 | 24 (+/-4) | No rejection >60 days | 2 |

**Table 5.2** MLC reactivity of *X.laevis* and *X.borealis* splenocytes to *X.tropicalis* stimulators.

| | 4 day harvest | | | | | | 5 day harvest | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Responder type.** | **Vs. irradiated self Mean dpm (+/-S.D.)** | | **Vs. irradiated X.tropicalis Mean dpm (+/-S.D.)** | | **S.I.** | **p** | **Vs. irradiated self Mean dpm (+/-S.D.)** | | **Vs. irradiated X.tropicalis Mean dpm (+/-S.D.)** | | **S.I.** | **p** |
| **Outbred X.laevis** | 371 | (+/-346) | 8879 | (+/-960) [322]* | 25.5 | <0.001 | | | | | | |
| **Outbred X.laevis** | 4247 | (+/-173) | 10181 | (+/-668) [247]* | 2.4 | <0.001 | 4247 | (+/-173)a | 13514 | (+/-636) | 3.2 b | <0.001 |
| **J strain X.laevis** | 544 | (+/-192) | 7254 | (+/-483) [937]* | 13.1 | <0.001 | | | | | | |
| | | | 6718 | (+/-98) [78]* | 12.9 | <0.001 | | | | | | |
| **J strain X.laevis** | 88 | (+/-34) | 8502 | (+/-1288) [445]* | 140.3 | <0.001 | | | 3230 | (+/-3102) [239]* | 52.7b | N.S. |
| | | | 7697 | (+/-280) [551]* | 125.8 | <0.001 | 88 | (+/-34)a | 7571 | (+/-4024) [236]* | 126.3b | 0.05 |
| | | | 7434 | (+/-1016) [184]* | 124.4 | <0.001 | | | 6539 | (+/-1197) [90]* | 110.1b | <0.001 |
| **X.borealis** | 3079 | (+/-769) | 15613 | (+/-1180) [922]* | 5 | <0.001 | 3660 | (+/-421) | 15329 | (+/-1579) [424]* | 4.2 | <0.001 |

* = Irradiated vs. irradiated background dpm.
a = Data from 4 day harvest.
b = Calculated using 4 day background levels.
N.S. = Not significant, p >0.05.

**Table 5.3** MLC reactivity of *X.tropicalis* splenocytes to *X.laevis* or *X.borealis* stimulators.

| 4 day harvest | | | | | | 5 day harvest | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Vs. irradiated self | | Vs. irradiated stimulators | | | | Vs. irradiated self | | Vs. irradiated stimulators | | | |
| Mean dpm | (+/-S.D.) | Mean dpm | (+/-S.D.) | S.I. | p | Mean dpm | (+/-S.D.) | Mean dpm | (+/-S.D.) | S.I. | p |
| 1331 | (+/-173) | 606 | (+/-77) [57]*x | 0.5 | <0.001 | | | | | | |
| 1750 | (+/-189) | 1417 | (+/-174) [42]*x | 0.8 | N.S. | 2838 | (+/-571) | 2030 | (+/-571) | 0.7 | N.S. |
| 5611 | (+/-316) | 3265 | (+/-198) [56] *j | 0.6 | 0.005 | | | | | | |
| 3313 | (+/-500) | 528 | (+/-41) [56]* j | 0.2 | <0.001 | | | | | | |
| 3351 | (+/-630) | 2251 | (+/-214) [58]* j | 0.7 | 0.05 | 2621 | (+/-561) | 1436 | (+/-548) j | 0.6 | N.S. |
| 3210 | (+/-247) | 3792 | (+/-277) [58]* j | 1.3 | N.S. | 3809 | (+/-1308) | 2208 | (+/-242) j | 0.6 | N.S. |
| 3481 | (+/-111) | 1255 | (+/-89) [58]* j | 0.4 | <0.001 | 5433 | (+/-4556) | 3045 | (n = 1) j | 0.6 | - |
| 9491 | (+/-487) | 6875 | (+/-633) [46]* b | 0.8 | 0.025 | 7310 | (+/-776) | 6064 | (+/-456) b | 0.9 | N.S. |

* = Irradiated vs. irradiated background dpm.
x = Outbred X.laevis stimulators.
j = J strain X.laevis stimulators.
b = X.borealis stimulators.
N.S. = Not significant, p>0.05.

**Table 5.4** MLC reactivity of *X.tropicalis* splenocytes to allogeneic stimulators.

| 4 day harvest | | | | | 5 day harvest | | | |
|---|---|---|---|---|---|---|---|---|
| Vs. irradiated self | Vs. irradiated allogeneic stimulators | | | | Vs. irradiated self | Vs. irradiated allogeneic stimulators | | |
| Mean dpm (+/-S.D.) | Mean dpm (+/-S.D.) | S.I. | p | | Mean dpm (+/-S.D.) | Mean dpm (+/-S.D.) | S.I. | p |
| 5611 (+/-316) | 10246 (+/-728) | 2 | .005 | | | | | |
| 3313 (+/-500) | 2940 (+/-165) | 0.8 | N.S. | | | | | |
| 3351 (+/-630) | 3811 (+/-423) | 1.1 | N.S. | | 2621 (+/-561) | 2262 (+/-822) | 0.9 | N.S. |
| | 7859 (+/-314) | 2.5 | <0.001 | | | 4821 (+/-1562) | 1.4 | N.S. |
| 3210 (+/-247) | 7069 (+/-489) | 2.3 | <0.001 | | 3809 (+/-1308) | 3654 (+/-302) | 1 | N.S. |
| | 16971 (+/-1175) | 5.8 | <0.001 | | | 15942 (+/-5939) | 4.3 | 0.05 |
| 3480 (+/-111) | 4509 (+/-203) | 1.3 | 0.005 | | 5433 (+/-4556) | 3845 (+/-1042) | 0.7 | N.S. |
| | 5931 (+/-670) | 1.7 | 0.005 | | | 4201 (+/-1268) | 0.8 | N.S. |

**Table 5.5** Proliferative responses of (LG15 x wild)F1 and of *X.tropicalis* thymocytes to PHA.

| PHA-P concentration (ug/ml) | 2 day harvest FCS mean dpm (+/-S.D.) | 2 day harvest BSA mean dpm (+/-S.D.) | 3 day harvest FCS mean dpm (+/-S.D.) | 3 day harvest BSA mean dpm (+/-S.D.) | 4 day harvest FCS mean dpm (+/-S.D.) | 4 day harvest BSA mean dpm (+/-S.D.) | 5 day harvest FCS mean dpm (+/-S.D.) | 5 day harvest BSA mean dpm (+/-S.D.) | 7 day harvest FCS mean dpm (+/-S.D.) | 7 day harvest BSA mean dpm (+/-S.D.) |
|---|---|---|---|---|---|---|---|---|---|---|
| **X.TROPICALIS** | | | | | | | | | | |
| No PHA-P | 5562 (+/-769) | 3195 (+/-314) | 3135 (+/-477) | 2014 (+/-241) | 1680 (+/-303) | 964 (+/-30) | | | | |
| | | | 1721 (+/-157) | 1308 (+/-240) | | | 650 (+/-126) | 540 (+/-83) | 472 (+/-37) | 307 (+/-4) |
| 0.01 ug/ml | 6206 (+/-785) | 3585 (+/-311) | 3774 (+/-534) | 2340 (+/-70) | 1929 (+/-164) | 1081 (+/-98) | | | | |
| | S.I.=1.1 | S.I.=1.1 | S.I.=1.2 | S.I.=1.2 | S.I.=1.1 | S.I.=1.1 | | | | |
| | | | 2072 (+/-87) | 1904 (+/-74) | | | 798 (+/128) | 648 (+/-67) | 341 (+/-51) | |
| | | | S.I.=1.2* | S.I.=1.4* | | | S.I.=1.2 | S.I.=1.2 | S.I.=0.7 | |
| 0.05 ug/ml | 7989 (+/-483) | 4899 (+/-609) | 6313 (+/-296) | 6810 (=/-25) | 3214 (+/-318) | 4288 (+/-531) | | | | |
| | S.I.=1.4* | S.I.=1.5* | S.I.=2* | S.I.=3.4* | S.I.=1.9* | S.I.=4.4* | | | | |
| 0.1 ug/ml | 11388 (+/-835) | 4568 (+/-547) | 1115 (+/-1959) | 11572 (+/-615) | 5148 (+/-292) | 6281 (+/-292) | | | | |
| | S.I.=2.05* | S.I.=1.2* | S.I.=3.5* | S.I.=5.7* | S.I.=3.1* | S.I.=6.5* | | | | |
| | | | 2974 (+/-38) | 6137 (+/-1652) | | | 960 (+/-121) | 1502 (+/-99) | | |
| | | | S.I.=1.7* | S.I.=4.7* | | | S.I.=1.5* | S.I.=2.8* | | |
| 0.5 ug/ml | 10048 (+/-857) | 2732 (+/-389) | 12006 (+/-281) | 4235 (+/-312) | 5024 (+/-677) | 1095 (+/-116) | | | | |
| | S.I.=1.8* | S.I.=0.85 | S.I.=3.8* | S.I.=2.1* | S.I.=2.9* | S.I.=1.1 | | | | |
| 1 ug/ml | 3269 (+/-390) | 1197 (+/-403) | 2831 (+/-705) | 982 (+/-320) | 615 (+/-128) | 379 (+/-92) | | | | |
| | S.I.=0.58 | S.I.=0.4 | S.I.=0.9 | S.I.=0.5 | S.I.=0.36 | S.I.=0.39 | | | | |
| | | | 718 (+/-80) | 953 (+/-207) | | | 218 (+/-36) | 284 (+/-74) | | |
| | | | S.I.=0.4 | S.I.=0.7 | | | S.I.=0.33 | S.I.=0.5 | | |
| 5 ug/ml | | | 172 (+/-49) | 239 (+/-80) | | | 86 (+/-39) | 62 (+/-16) | | |
| | | | S.I.=0.1 | S.I.=0.2 | | | S.I.=0.13 | S.I.=0.1 | | |
| **(LG15 x WILD) F1** | | | | | | | | | | |
| NO PHA-P | | | 1555 (+/-100) | | | | 558 (+/-50) | | 140 (+/-12) | |
| 1 ug/ml PHA-P | | | 2264 (+/-209) | | | | 246 (+/-16) | | 177 (+/-37) | |
| | | | S.I.=1.45* | | | | S.I.=0.4 | | S.I.=1.26 | |

* = Significant ,p<0.05.

**Table 5.6** Proliferative responses of *X.tropicalis* splenocytes to PHA.

| PHA-P concentration (ug/ml) | Animal number | 2 day harvest FCS mean dpm (+/-S.D.) | 2 day harvest BSA mean dpm (+/-S.D.) | 3 day harvest FCS mean dpm (+/-S.D.) | 3 day harvest BSA mean dpm (+/-S.D.) | 4 day harvest FCS mean dpm (+/-S.D.) | 4 day harvest BSA mean dpm (+/-S.D.) | 5 day harvest FCS mean dpm (+/-S.D.) | 5 day harvest BSA mean dpm (+/-S.D.) |
|---|---|---|---|---|---|---|---|---|---|
| No PHA-P | 1 | 6029 (+/-351) | 6064 (+/-291) | 8525 (+/-1163) | 5644 (+/-801) | 7973 (+/-481) | 4850 (+/-399) | | |
| | 2 | | | | | 11817 (+/-2854) | | | |
| | 3 | | | | | 2233 (+/-364) | | 3305 (+/-135) | |
| | 4 | | | | | | | 7253 (+/-2356) | 7712 (+/-6010) |
| 0.01 ug/ml | 1 | 12583 (+/-1173) S.I.=2.1* | 8145 (+/-1567) S.I.=1.3 | 21074 (+/-2611) S.I.=2.5* | 10527 (+/-5580) S.I.=1.9 | 21650 (+/-2353) S.I.=2.7* | 13228 (+/-6552) S.I.=2.7 | | |
| | 4 | | | | | | | 34694 (+/-2765) S.I.=4.8* | 15980 (+/-138) S.I.=2.1 |
| 0.05 ug/ml | 1 | 58131 (+/-6331) S.I.=9.6* | 18489 (+/-2275) S.I.=3.0* | 84035 (+/-2286) S.I.=9.8* | 33020 (+/-2035) S.I.=5.8* | 61799 (+/-4657) S.I.=7.7* | 37811 (+/-2072) S.I.=7.8* | | |
| | 3 | | | | | 16129 (+/-1329) S.I.=7.2* | | 19223 (+/-1027) S.I.=5.8* | |
| 0.1 ug/ml | 1 | 64734 (+/-3149) S.I.=10.7 | 27737 (+/-2004) S.I.=4.6* | 92231 (+/-1953) S.I.=10.8* | 37591 (+/-1465) S.I.=6.7* | 51542 (+/-1359) S.I.=6.5* | 36134 (+/-5796) S.I.=7.4* | | |
| | 2 | | | | | 173515 (+/-13671) S.I.=14.7* | | | |
| | 3 | | | | | 22104 (+/-577) S.I.=9.9* | | 21327 (+/-4942) S.I.=6.5* | |
| | 4 | | | | | | | 50048 (+/-1260) S.I.=6.9* | 13496 (+/-617) S.I.=1.8 |
| 0.5 ug/ml | 1 | 33446 (+/-2824) S.I.=5.5* | 9661 (+/-1871) S.I.=1.6* | 34553 (+/-189) S.I.=4.0* | 9702 (+/-3567) S.I.=1.7 | 25789 (+/-2135) S.I.=3.2 | 7871 (+/-1991) S.I.=1.6 | | |
| 1 ug/ml | 1 | 18546 (+/-1586) S.I.=3.1* | 6738 (+/-242) S.I.-1.1* | 15162 (+/-578) S.I.=1.8* | 6714 (+/-393) S.I.=1.2 | 11112 (+/-1160) S.I.=1.4* | 5343 (+/-233) S.I.=1.1 | | |
| | 4 | | | | | | | 9761 (+/-1258) S.I.=1.3 | 3925 (+/-158) S.I.=0.5 |
| 5 ug/ml | 4 | | | | | | | 1628 (+/-338) S.I.=0.2 | 1998 (+/-218) S.I.=0.3 |

**Table 5.7** Proliferative responses of *X.laevis* and (LG15 x wild)F1 splenocytes to PHA.

| PHA-P concentration (ug/ml) | Animal number | 2 day harvest mean dpm (+/-S.D.) | 3 day harvest mean dpm (+/-S.D.) | 4 day harvest mean dpm (+/-S.D.) | 5 day harvest mean dpm (+/-S.D.) |
|---|---|---|---|---|---|
| No PHA-P | (LG15 x wild)F1 1 | 518 (+/-102) | 1586 (+/-52) | 235 (+/-88) | |
| | (LG15 x wild)F1 2 | | | | 637 (+/-139) |
| | Outbred X.laevis 1 | | | 1683 (+/-167) | |
| 0.1 ug/ml | (LG15 x wild)F1 1 | 9700 (+/-5509) S.I. = 18.7* | 23390 (+/-9756) S.I. = 88.6* | 24655 (+/-5900) S.I. = 105* | |
| | (LG15 x wild)F1 2 | | | | 83636 (+/-706) S.I. = 131* |
| | Outbred X.laevis 1 | | | 25519 (+/-3282) S.I. = 15.2* | |

Table 5.8 Proliferative responses of *X.tropicalis* thymocytes and splenocytes to Con A.

| Animal number | CON A concentration (ug/ml) | 3 day harvest mean dpm (+/-S.D.) | S.I. | p | 4 day harvest mean dpm (+/-S.D.) | S.I. | p | 5 day harvest mean dpm (+/-S.D.) | S.I. | p |
|---|---|---|---|---|---|---|---|---|---|---|
| **THYMOCYTES** | | | | | | | | | | |
| | No CONA | 1035 (+/-22) [1095 (+/-26)]* | | | | | | | | |
| | 0.1 ug/ml | 1222 (+/-69) [972 (+/-102),N.S.]* | 1.1 | 0.025 | | | | | | |
| 1 | 0.5 ug/ml | 7190 (+/-665) [1154 (+/-184),N.S.]* | 6.9 | 0.001 | | | | | | |
| | 1 ug/ml | 7288 (+/-1297) [1067 (+/-180),N.S.]* | 7 | 0.005 | | | | | | |
| | 5 ug/ml | N.D. | - | - | | | | | | |
| **SPLENOCYTES** | | | | | | | | | | |
| 1 | No CONA | 3621 (+/-228) | | | | | | | | |
| | 1 ug/ml | 36343 (+/-1748) | 10 | 0.001 | | | | | | |
| | No CONA | 6334 (+/-878) [6480 (+/-1882)]* | | | | | | 7295 (+/-893) | | |
| | 0.1 ug/ml | 7196 (+/-925) [6477 (+/-568),N.S.]* | 1.1 | N.S. | | | | | | |
| 2 | 0.5 ug/ml | 34609 (+/-4415) [4304 (+/-789),N.S.]* | 5.5 | 0.001 | | | | | | |
| | 1 ug/ml | 24934 (+/-1562) [7250 (+/-1392),N.S.]* | 3.9 | 0.001 | | | | 23516 (+/-1690) | 3.2 | 0.001 |
| | No CONA | | | | 6880 (+/-327) | | | | | |
| | 0.1 ug/ml | | | | 15410 (+/-8177) | 2.2 | N.S. | | | |
| 3 | 0.5 ug/ml | | | | 22680 (+/-1832) | 3.3 | 0.001 | | | |
| | 1 ug/ml | | | | 59121 (+/-3122) | 8.6 | 0.001 | | | |
| | 5 ug/ml | | | | 27357 (+/-6392) | 4 | 0.01 | | | |

[ ] + 0.1M $\alpha$-methyl mannoside.

**Table 5.9** Proliferative responses of *X.laevis* splenocytes and of LG15 and X.borealis thymocytes and splenocytes to Con A.

| Animal number | CON A concentration (ug/ml) | 3 day harvest mean dpm (+/-S.D.) | S.I. | p | 4 day harvest mean dpm (+/-S.D.) | S.I. | p | 5 day harvest mean dpm (+/-S.D.) | S.I. | p |
|---|---|---|---|---|---|---|---|---|---|---|
| **THYMOCYTES** | | | | | | | | | | |
| | NoCONA | 698 (+/-39) [593 (+/-47)]* | | | | | | | | |
| | 0.1 ug/ml | 671 (+/-66) [697 (+/-40)]* | 1 [1.2] | - [.05] | | | | | | |
| LG15 1 | 0.5 ug/ml | 1246 (+/-48) [675 (+/-78),N.S.]* | 1.8 | 0.001 | | | | | | |
| | 1 ug/ml | 1717 (+/-390) [727 (+/-73),N.S.]* | 2.5 | 0.025 | | | | | | |
| X.borealis 1 | NoCONA | 595 (+/-86) | | | | | | | | |
| | 1.0 ug/ml | 4908 (+/-551) | 8.3 | 0.001 | | | | | | |
| **SPLENOCYTES** | | | | | | | | | | |
| | NoCONA | 301 (+/-245) [319 (+/-203)]* | | | | | | 107 (+/-26) | | |
| LG15 1 | 0.1 ug/ml | 621 (+/-74) [275 (+/-74),N.S.]* | 1.9 | N.S. | | | | | | |
| | 0.5 ug/ml | 2899 (+/-865) [1057 (+/-286)]* | 9.6 [3.3] | 0.01 [.025] | | | | | | |
| | 1 ug/ml | 9999 (+/-2602) [212 (+/-31),N.S.]* | 33 | 0.005 | | | | 10555 (+/-518) | 99 | 0.00 |
| | NoCONA | | | | 246 (+/-174) | | | | | |
| | 0.1 ug/ml | | | | 9613 (+/-1405) | 39 | 0.001 | | | |
| J strain X.laevis 1 | 0.5 ug/ml | | | | 5188 (+/-5361) | 21 | N.S. | | | |
| | 1 ug/ml | | | | 23543 (+/-226) | 96 | 0.001 | | | |
| | 5 ug/ml | | | | 1541 (+/-761) | 6.3 | 0.05 | | | |
| | NoCONA | 274 (+/-70) [280 (+/-26)]* | | | | | | 228 (+/-25) | | |
| | 0.1 ug/ml | 316 (+/-51) [234 (+/-58),N.S.]* | 1.1 | N.S. | | | | | | |
| X.borealis 1 | 0.5 ug/ml | 335 (+/-43) [360 (+/-28)]* | 1.2 [1.3] | N.S. [.01] | | | | | | |
| | 1 ug/ml | 1679 (+/-347) [376 (+/-76),N.S.]* | 6.1 | 0.005 | | | | 1700 (+/-467) | 7.4 | 0.0 |
| X.borealis 2 | NoCONA | 709 (+/-193) | | | | | | 615 (+/-228) | | |
| | 1.0 ug/ml | 5862 (+/-397) | 8.3 | 0.001 | | | | 6935 (+/-2624) | 11 | 0.02 |

[ ]= Cultured + 0.1M α-methyl mannoside.
N.S. = Not significant, p>0.05.

# Chapter 6

# Histological observations on donor skin grafts tolerated by thymus- implanted hosts and attempts to break perimetamorphically-induced skin graft tolerance

## 6.1 Introduction

Skin allografts can be rejected by *Xenopus* from very early stages of development, in fact from the time that small lymphocytes first appear in the larval thymus, (stage 49). Furthermore, larvae given skin grafts from non-identical donors before they reach stage 49 can respond to these grafts once they reach stage 49, (Horton, 1969). Acute allograft rejection takes approximately three weeks to complete in *Xenopus*, (although the process is temperature dependent), and procedes through the following sequence of events: capillary dilation with haemostasis and breakdown, lymphocyte infiltration, disintegration of pigment cells and death of the epithelial and glandular components of the graft, (Horton 1969). Clothier et al, (1990) observed that, after 21 days post-grafting, host epidermis had replaced donor epidermis and only a thin, fibrous layer remained of the graft, between epidermis and collagen layer. This graft collagen persists for prolonged periods. Second-set responses are accelarated, graft rejection being complete in 6-9 days, (Tochinai & Katagiri, 1975), and second-set grafts may not be vascularised before rejection, (Manning & Turner, 1976).

The histological features of skin xenograft rejection have recently been described by Clothier et al, (1990), who examined the rejection of *X.borealis* skin by intact *X.laevis* hosts. These authors found that, although morphologically similar, the processes of allo- and xenograft rejection appeared histologically distinct, in that xenografts were not infiltrated by host lymphocytes. Instead, a heavy lym-

phocytic accumulation was observed around the xenograft, while macrophages and neutrophils were seen to invade the xenograft, with active phagocytosis occurring in the graft dermis by day 12 after application. Clothier et al feel that xenograft rejection does have an immunological basis, however, as rejection of second-set xenografts is accelerated and since antibodies specific for *X.borealis* were found in the blood of grafted *X.laevis* hosts. This latter phenomenon is not restricted to xenografted hosts; in fact, skin graft rejection is an essential step in the production of 'strong' alloantisera, in *Xenopus*, (reviewed by Flajnik et al, 1988).

Although early-thymectomised *Xenopus* may still reject first-set allografts chronically, and second-set grafts, from the original donor, in an accelerated fashion, (Horton & Horton, 1975; Tompkins & Kaye, 1981; Nagata & Cohen, 1983), acute skin allograft rejection has been demonstrated, by a number of authors, to be thymus-dependent, (Horton & Manning, 1972; Tochinai & Katagiri, 1975; Horton & Horton, 1975; Tompkins & Kaye, 1981; Clothier et al, 1990). Furthermore, acute skin allograft rejection in *Xenopus* has been shown to be under the genetic control of the XLA complex, (Du Pasquier et al, 1975; Du Pasquier et al, 1977), which encodes polymorphic class I, II, III and IV molecules having strong structural homologies to those of mammals, (see reviews by Du Pasquier et al, 1989 and Flajnik et al, 1988). Studies with anti-*Xenopus* class I MHC antisera have shown that these transplantation antigens are expressed in the highest amounts on haemopoietic tissue and skin, (Flajnik et al, 1986). Also, class II MHC positive dendritic cells, (possibly Langerhans equivalents), have been described within *Xenopus* epidermis, (Du Pasquier & Flajnik, 1987), and these 'passenger leukocytes' appear to be the critical stimulators of skin graft rejection in larval, but not in adult, *Xenopus*, (Flajnik & Du Pasquier, 1984).

Skin xenograft rejection has been reported, (Balls et al, 1981; James et al, 1982a; Clothier et al, 1990), to be unaffected by either early- thymectomy or by treatment with NMU, (a chemical carcinogen which leads to permanent loss of the thymic cortex and an inability to mount rejection responses to skin allografts, (Balls et al, 1980)). Clothier et al, (1990), suggest that xenograft rejection is not MHC-restricted and, therefore, is not based on the recognition of the xenograft as altered self.

Induction of tolerance to histoincompatible skin can be induced in *Xenopus* in a number of ways. Clark & Newth, (1972), noted that exchange of massive grafts, (including presumptive skin, body wall and gut and probably blood-forming cells), between pairs of stage 22 *Xenopus* embryos resulted in mutual tolerance in 93% of cases. The basis of this tolerance induction was investigated by Manning & Botham, (1980), and by Botham & Manning, (1980). These authors found that splenocytes from post-metamorphic, mutually- tolerant animals showed no, or only a very low, reactivity to those of their partners in MLC, but showed a noticeably heightened response to third-party cells. Furthermore, the third-party skin allograft response was particularly strong in tolerised individuals. Manning & Botham suggested from these findings that mutually-tolerant animals were chimaeric, with two sets of alloreactive lymphocytes. Adult thymectomy did not disrupt embryonically-induced tolerance, suggesting that this state was not mediated by short-lived thymus-derived suppressor cells.

Grafting of major or minor H antigen disparate skin onto intact larval and perimetamorphic *Xenopus* has been shown to induce long-lasting tolerance to subsequent grafts from the same donor or donor-type, (Bernadini et al, 1969, 1970; Chardonnens and Du Pasquier, 1973; Cohen et al, 1980; Barlow et al, 1981; Barlow & Cohen, 1983; Nakamura et al, 1987). The success of tolerance induction by this method appears to depend on the size of the grafts applied, the antigen disparity between donor and host and the developmental stage of the recipient and graft at the time of grafting, (see reviews by Du Pasquier, 1982; Flajnik et al, 1987; Katagiri & Tochinai, 1987; Du Pasquier et al, 1989).

It has been reported that maintenance of this allotolerance depends upon the continuous presence of the tolerating graft, (Chardonnens, 1976; Kaye et al, 1983), although Horton et al, (1989), found that removal of perimetamorphically applied first-set grafts after four weeks did not break allotolerance in every case, when second-set grafts were applied up to 14 weeks after first-set graft removal. The mechanism whereby perimetamorphically-induced tolerance is maintained has been examined in several studies. Du Pasquier & Bernard, (1980), obtained evidence that thymus-dependent suppressor cells play a part in this process; using LG15 and LG17 clonal animals, they found that rejection of minor H-disparate grafts by adult recipients could be delayed by repeated injections of thymus/spleen or thymus cells from metamorphosing frogs. Rejection of these minor antigen disparate grafts commenced as soon as injections of perimetamorphic cells stopped. The specificity of suppression by cells from metamorphosing animals was not explored. Perimetamorphically-induced tolerance to minor disparate skin could not be broken, six months after grafting, by injection of peripheral blood cells from adults primed to the skin graft donor type. However, if LG17 animals, tolerating one perimetamorphically-applied LG15 graft, were regrafted with LG15 skin and simultaneously injected with primed, adult anti-LG15 cells, the original graft remained unaffected, but the new graft began to be rejected. The rejection process stopped part-way through, presum ably as host suppressor cells began to inactivate the injected, primed cells.

Nakamura et al, (1987), analysed the cellular basis of transplantation tolerance to semi-allogeneic skin induced by grafting during larval life. These authors found that, whereas 1-2 $mm^2$ semi-allogeneic or fully allogeneic grafts routinely engendered tolerance, grafting of small, (0.5-1 $mm^2$), pieces of (J x K)$F_1$ skin to larval, J strain recipients did not always successfully induce tolerance. Furthermore, in those J strain animals tolerised by small (J x K)$F_1$ grafts, tolerance of secondary and tertiary grafts was not always complete; MLC responses to semi-allogeneic skin graft donor-type splenocytes were strong, (comparable to that seen to third-party stimulators), in 7/8 perimetamorphically tolerised J strain hosts. Injection of up to 5 x $10^6$ splenocytes from non-tolerised adult frogs into syngeneic, larvally skin grafted hosts failed to break tolerance of their perimetamorphically-applied grafts. In fact, even when the injected cells had been previously stimulated with (J x K)$F_1$, (skin donor type), cells, tolerance of larvally-applied skin grafts was maintained, despite seeding, to levels of between 28 and 51%, of the ploidy-marked, primed, injected cells into the spleen of the skin-grafted host. Taking these results as evidence of maintenance of tolerance by suppression, rather than by clonal

deletion of alloreactive cells, Nakamura et al went on to test the transferance of this suppression, with splenocytes of J strain hosts, larvally grafted with (J x K)$F_1$ skin; these splenocytes were injected into intact, syngeneic J strain frogs, or into TX syngeneic frogs. (J x K)$F_1$ grafts, applied to intact J strain hosts the day after injection of 'tolerant' splenocytes, were rejected, but more slowly than might have been expected for this donor/host combination, (in 22-30 days, whereas non- injected Js rejected (J x K)$F_1$ skin in 14 days). Injection of 2 x $10^6$ 'tolerant' splenocytes into TX, syngeneic frogs restored their graft rejection responses to third-party skin, but not to (J x K)F1 skin. Injection of TX Js with increasing proportions of splenocytes from normal, unmanipulated Js, together with splenocytes from (J x K)F1 tolerant splenocytes, again restored third-party rejection, but rejection of (J x K)F1 skin grafts, varying from chronic to acute, was observed. The intensity of rejection increased as the proportion of injected 'tolerant' splenocytes decreased. Grafting of irradiated or non-irradiated thymi from J strain animals, larvally tolerised to (J x K)$F_1$ skin, into TX Js restored graft rejection responses in the thymus-implanted hosts, including rejection of (J x K)$F_1$ skin. Nakamura et al interpret their results to mean that, in skin grafted animals, tolerance is maintained by a dynamic balance between suppressor cells, (probably peripheral, since grafting of thymus from tolerised frogs into TX recipients did not transfer tolerance), and cytotoxic peripheral lymphocytes. They speculate that tolerance of larvally-applied skin grafts, with concurrent MLC reactivity to graft donor cells, ('split tolerance'), occurs because relatively immature class I MHC reactive cells, found in perimetamorphic hosts at the time of grafting, can be suppressed, whereas class II MHC reactive cells are more differentiated in these animals, and so resist suppression.

Induction of tolerance to donor MHC type histoincompatible skin grafts is also observed following grafting of adult or larval, normal or irradiated thymi into TX hosts, in both larval and adult life, or by the construction of head/body chimaeras, (in which the thymic epithelium is of one MHC type, while lymphoid cells are of another). TX J strain recipients, implanted in adult life with adult, normal HD strain thymi, tolerated subsequent skin grafts from thymus donor animals, with no lymphoid accumulations or infiltration, when these grafts were examined 100 days after application, (Nagata & Kawahara, 1982). MHC-defined TX hosts, implanted in adult life with 1 or 2 MHC-type disparate thymi, all accepted subsequent skin grafts which shared MHC-antigens with the thymus donor, (Nagata & Cohen, 1984). Irradiated adult (J x K)F1 thymi, implanted into TX J strain hosts during adult life, similarly induced tolerance to donor skin grafts, (Nakamura et al, 1987; Maeno et al, 1987). Implantation of histoincompatible larval thymi into TX larval recipients, (Horton & Horton, 1975; Du Pasquier & Horton, 1982; Arnall & Horton, 1986), was equally effective. Tolerance of thymus donor type skin grafts was also observed in LG15(head)/LG3(body) chimaeras, (Flajnik et al, 1985).

Furthermore, implantation of semi- or fully xenogeneic XbJ or *X.borealis* larval thymi into TX J strain or LG17 hosts, during larval life, was found, in studies reported in Chapter 4, to induce specific unresponsiveness to subsequent grafts of donor skin.

The basis of allotolerance induced in early-thymectomised J strain *X.laevis*, implanted in larval life with normal or irradiated (J x K)F1 thymi, was investigated by Maeno et al, (1987). Implantation of either type of thymus restored third-party graft rejection responses within two months, but induced specific tolerance to skin grafts from the thymus donor type. Furthermore, MLC activity towards splenocyte stimulators of thymus donor type was found to be specifically suppressed in these implanted animals. Since splenocytes of normal thymus-implanted animals were found to comprise 4-32% donor-derived lymphocytes, whereas those of TX animals given irradiated implants were entirely host-derived, further analyses were made using the latter animals. Splenocytes from triploid J strain frogs, tolerised by implantation of (J x K)F1 irradiated thymi, were transferred to further TX J frogs by injection, 90 days after thymus grafting. Splenocyte-recipient TX animals were found to be restored to third-party skin grafts, but were tolerant to (J x K)$F_1$, in terms of skin grafting and MLC responses. This transferred skin graft tolerance could be broken by injection of 2 x $10^6$ splenocytes from normal, syngeneic animals. On the other hand, transfer of 2 x $10^6$ splenocytes from thymus-grafted, tolerant frogs to normal, syngeneic animals failed to suppress acute rejection of a subsequent skin graft of thymus-donor type. These results were suggested to indicate the induction of tolerance by clonal deletion, in thymus-induced tolerant animals, since they appeared to possess no suppressor cells specific for (J x K)$F_1$ MHC antigens.

The lack of 'split tolerance' seen by Maeno et al in thymus-implanted tolerised *Xenopus* is in contrast to that seen by Nagata & Cohen, (1984), after implantation of adult MHC-mismatched thymi into larvally- thymectomised adult *Xenopus*, by Arnall & Horton, (1986), following grafting of larval LG3 thymus into TX LG17 larval hosts and to that seen when J strain TX, implanted with semi-xenogeneic XbJ larval thymi during larval life, were examined in our own studies, (see Chapter 4).

In the first studies reported in this Chapter, LG17 TX, each implanted in larval life with a single larval *X.borealis* thymus, were grafted with skin from their individual thymus donor. These tolerated grafts, which carried the distinctive *X.borealis* quinacrine marker, (described by Thiébaud, 1983, and in Chapter 3), were histologically examined, at several time points after grafting, to assay for any signs of immunological activity towards them, which, if Maeno et al's theories of thymus-induced tolerance by clonal deletion in this model system are correct, should not be present. Moreover, the *X.borealis* quinacrine marker allowed investigation of the persistance of donor elements in tolerated grafts.

In mice, injection of interleukin-2, (IL-2), has been shown to prevent the induction of tolerance to semi-allogeneic cells, (Malkovsky & Medawar, 1984), and the maintenance of tolerance to later skin grafts, (Malkovsky & Medawar, 1984; Asherson et al, 1985; Loveland et al, 1986). In contrast, in recent studies on chickens, IL-2 injection failed to break neonatally- induced transplantation tolerance, (Tempelis et al, 1988). Cyclophosphamide, an alkylating agent, has been shown to accelerate mammalian allograft rejection, (Shin et al, 1984), probably by directly interacting with suppressor T-cell subsets to inhibit suppression mechanisms. Ruben et al, (1987), have shown that both human recombinant IL- 2 and

Cyclophosphamide are effective, in *Xenopus*, in breaking hapten- specific tolerance in humoral responses. In light of these findings and Nakamura's suggestion that perimetamorphic tolerance induced by skin grafting in control *Xenopus* is maintained by suppression, the second goal of the experiments reported in this Chapter was an attempt to break this skin-induced tolerance using recombinant human interleukin-2 or Cyclophosphamide.

## 6.2 Materials and Methods

### 6.2.1 Animals, Operations and Injections

#### 6.2.1.1 Animals

Clonal LG17, LG15, LG5 and LM3 *Xenopus* and outbred *X.borealis* animals were bred and raised in this laboratory, after the methods of Kobel & Du Pasquier, (1975), and Manning & Horton, (1972).

#### 6.2.1.2 Early thymectomy and thymus implantation operations

LG17 larvae destined to be hosts for implanted xenogeneic thymi were thymectomised, by microcautery, after the method of Horton & Manning, (1972), when they were 7 days old, (stage 47-48). These LG17 TX host larvae were each implanted, when 5 weeks old, (stage 56-57), with a single *X.borealis* larval thymus, taken from an age-matched donor tadpole, as described in Chapter 3. Other LG17 TXs were left unimplanted, to act as controls. Outbred *X.borealis* thymus donors were kept separately, to allow later grafting of thymus-implanted hosts with skin from their individual thymus donors.

#### 6.2.1.3 Skin grafting operations

Perimetamorphic, (stages 57-60), LG5, LG15 or LM3 control tadpoles were given approximately 2 $mm^2$ ventral skin grafts, (see Chapter 4), from 4-6 month old froglet donors; these grafts were placed dorsolaterally above the hindlimb of the recipients. LG5 and LM3 hosts received LG17 skin, while LG15 hosts received LG5 skin.

At the time that some animals were sacrificed for histological examination of skin grafts, they were also bled, by cardiac puncture, using finely drawn, heparinised glass needles.

#### 6.2.1.4 Injections

Human recombinant interleukin-2, (rIL-2), was purchased from Amersham International, (Cat.No. ARN.7010). This was dissolved in Leibovitz L-15 medium, (Flow Labs.), (diluted to amphibian isotonicity with sterile double distilled water,

and supplemented with 0.1% human serum albumen, (Sigma Chemicals)). 100 $\mu$l volumes, containing either 50 or 250 Units rIL-2, were injected intraperitoneally into 3-4 g body-weight froglets, as described in section 6.3.2.2. (HSA was included as a carrier protein; controls received HSA alone).

Cyclophosphamide, (Sigma Chemicals), was made up in human serum albumen-supplemented, amphibian-isotonicity L-15 medium, at a concentration of 3 mg/ml. 100 $\mu$l volumes were injected intraperitoneally into 3-4 g body- weight froglets, as described in section 6.3.2.2.

## 6.2.2 Histology and Cytology

### 6.2.2.1 Fixation of tissues

Skin grafts, spleens and thymus-implants were dissected from heavily- anaesthetised thymus-implanted host animals and were immediately fixed for 30-45 minutes in Carnoy's fixative, (10% glacial acetic acid, 60% absolute alcohol, 30% chloroform).

### 6.2.2.2 Dehydration procedure

Following fixation, tissues were dehydrated through 2 changes each of 70% and 95% alcohol, 30 minutes per change. Complete dehydration is not a prerequisite for embedding in Historesin, as the final embedding solution contains 6–8% water.

### 6.2.2.3 Historesin embedding solutions

Intermediate infiltration solution, infiltration solution and embedding medium were made using a Historesin embedding kit, (L.K.B.), as follows:Infiltration solution contains 50 ml basic resin, mixed with one packet activator. Intermediate infiltration solution is prepared by mixing equal quantities of 95% alcohol and infiltration solution. Embedding medium, which is prepared immediately before use, consists of 15 ml infiltration solution with 1 ml hardener solution.

### 6.2.2.4 Embedding procedure

The partially dehydrated specimens were immersed for 1-2 hours in intermediate infiltration solution, at room temperature, with constant agitation on a rotary mixer. Next, the tissues were transferred to infiltration solution for 1-2 hours, or until the tissues appeared translucent and sank to the bottom of infiltration vessels. Rotary mixing was continued throughout this step. Finally, specimens were placed into embedding medium, in individual plastic capsules, (Agar Aids Ltd.), orientated to allow transverse sections to be cut. The plastic capsules were sealed and the blocks were left for 40-120 minutes, at room temperature, to polymerise.

#### 6.2.2.5 Section preparation

4$\mu$m transverse serial sections of skin grafts, thymus implants and spleens were cut, using glass knives, on a Reichert Ultramicrotome. These were spread, on pools of distilled water, on glass microscope slides and then dried on a hotplate.

#### 6.2.2.6 Quinacrine staining of sections

Sections were rehydrated in quinacrine staining buffer, (18.15% 0.1M citric acid, 81.85% 0.2M $Na_2HPO_4$, pH 7.0), for 10 minutes. They were then stained in 0.5% quinacrine hydrochloride solution, washed and mounted, as described in Chapter 3.

#### 6.2.2.7 Preparation of blood smears

Small drops of whole *Xenopus* blood, collected in heparinised glass needles, as described above, were placed on clean glass microscope slides. Thin blood films were produced, by smearing each droplet of blood across a slide, using the edge of another microscope slide. Blood smears were air dried for 30 minutes, at room temperature, and then fixed in Carnoys' fixative, washed in quinacrine staining buffer and quinacrine stained, as described above.

## 6.3 Results

### 6.3.1 Skin Graft Tolerance Induced by Implantation of Larval *X.borealis* Thymus to Thymectomised LG17 Hosts

In an initial experiment, designed to confirm that *X.borealis*-thymus- implanted LG17 TX hosts were restored to reject third-party skin grafts, but tolerated grafts from their individual donor, 5 host animals were grafted, when 3 months old, with *X.borealis* donor and with LM3 skin. Two of these host animals were later regrafted with third-party *X.borealis* skin. Control LG17s, (n=4), were grafted with *X.borealis* skin from animals which had acted as thymus donors, while TX LG17s were grafted with *X.borealis*, (n=3), or LM3, (n=2), skin. The results of this experiment were reported in Chapter 4, but, to reiterate: control animals acutely rejected all grafts, TX animals did not respond to LM3 grafts, but 1/3 acutely rejected *X.borealis* skin and a further TX showed signs of chronic rejection, while 4/5 thymus-implanted hosts were able to acutely reject third-party skin, but tolerated donor grafts.

Following this experiment, 3-4 month old LG17 TX, each implanted with a single *X.borealis* thymus in larval life, (n=8), were grafted with skin from their individual donors. One control LG17 and 1 TX LG17 were also grafted with skin from each *X.borealis* thymus donor

#### 6.3.1.1 Histological Appearance of Quinacrine-Stained *X.borealis*, (thymus donor), Skin Grafts, on Thymus-Implanted, TX LG17 Hosts

*X.borealis* donor skin grafts, from TX LG17 hosts implanted with *X.borealis* thymi, taken for Historesin embedding and histological examination at several times after application, appeared fully viable when the host animals were sacrificed.

**10-14 Days Post-Grafting, (n=6)**

These grafts appeared healthy, and had the general structure of normal skin (see Figure 6.1). Several grafts, however, contained small foci of host-derived lymphocytes which were found particularly within the glandular layer of the grafts (see Figure 6.2, A to C). Examination of the quinacrine staining pattern of these grafts showed that, in most cases, (5/6), the graft epidermis had been replaced by host-type, non-spotted cells. Neck cells of both granular and mucous glands, which project through the epidermis, were found to be of skin donor origin. The glandular layer, *stratum spongiosum*, and the collagen layer, *stratum compactum*, of these grafts were still of donor origin (see Figure 6.3, A and B).

**26 Days Post-Grafting, (n=2), and 12-20 Weeks Post-Grafting, (n=2)**

Again, these skin grafts appeared normal, although containing small accumulations of host-derived lymphocytes within the *stratum spongiosum*. In three out of four grafts, however, the epidermis had been completely replaced by host-derived cells. Glandular structures, including gland neck cells, remained of donor, quinacrine-positive, type, as did cells of the *stratum compactum*.

#### 6.3.1.2 Histological Appearance of Quinacrine-Stained *X.borealis* Grafts Applied to Unimplanted Thymectomised LG17 Hosts

Sections of *X.borealis* grafts applied 26 days previously to 2 non- thymus-implanted LG17 TX, were examined following quinacrine staining. The epidermal layers of both these grafts had been replaced by host-derived cells. One of the two TX hosts appeared to be in the process of chronically rejecting its xenograft, which was heavily infitrated with host lymphocytes, and showed some loss of normal glandular structure. The second of the two was normal in structure, but contained small foci of host lymphocytes within the glandular layer.

#### 6.3.1.3 Observations on Quinacrine-Stained Spleen and Thymus Implant Sections and Blood Smears

Quinacrine-stained serial sections of spleens from LG17 control, (n=1), and from LG17 TX, *X.borealis* thymus-implanted animals, (n=2), which had been subsequently grafted with *X.borealis* donor skin were examined 26 days post-grafting. No quinacrine-positive cells were found in the control spleen, although a few were found in spleens of thymus-implanted, (three months previously), skin grafted hosts. Quinacrine-positive cells found in *X.borealis* thymus-implanted, donor skin grafted, host LG17 spleens were located in the red pulp and marginal zone areas.

Spleens of LG17 TX animals, each implanted 3-4 months previously with a single *X.borealis* thymus, (n=2), which had not received *X.borealis* skin grafts were also serial sectioned, quinacrine stained and examined. No quinacrine-positive cells were found in these sections.

Quinacrine-stained sections of thymus-implants, examined 3-4 months after implantation, were found to contain almost exclusively host-derived lymphocytes. Stromal elements were of donor origin and occasional Q+ve myoid cells were seen in the medulla, although the cortico-medullary organisation in these implants was often poor, and thymus-implants were small (see Figure 6.5).

Quinacrine-stained blood smears from LG17 controls, (n=2), and from LG17 TX, implanted with *X.borealis* thymi, (n=2), were made 26 days after these animals had been grafted with *X.borealis* skin. Control LG17 blood smears contained no Q+ve cells. Blood smears from LG17 TX, thymus-implanted animals contained a very low number, (approximately 0.1%), Q+ve cells.

A single non-skin grafted LG17 TX, thymus-implanted animal was also bled at this time, (4 months post-implantation); quinacrine stained blood smears from this animal were completely Q-ve.

## 6.3.2 Attempts to Break Maintenance of Perimetamorphically Induced Skin Graft Tolerance

### 6.3.2.1 Perimetamorphic tolerance induction to semi- or fully- allogeneic skin

Three groups of 5-6 week old, (stage 57–60), perimetamorphic control *Xenopus* were grafted with skin from donors which were one or two MHC antigen disparate. Thus: group A comprised LG15s, (MHC=ac), grafted with LG5, (MHC=bc), skin; group B comprised LG5s grafted with LG17, (MHC=ac), skin; group C comprised LM3s, (MHC=wy), grafted with LG17 skin. By 100 days post-grafting, all first-set semi-allogeneic grafts applied to group A and to group B animals were still being tolerated in perfect condition. Of the group C animals, only 50% still carried tolerated grafts, the rest having rejected their grafts in 3–5 weeks.

### 6.3.2.2 Attempts to break skin graft tolerance

Animals from groups A, B and C which were still tolerating their perimetamorphically applied grafts in perfect condition after 100 days were divided into treatment groups. All of these animals were regrafted with skin of the same type as their original donor, while undergoing one of three treatments: HSA controls were injected three times, on days 1, 4 and 7 after second-set grafting with 0.1% human serum albumen, (HSA), supplemented L-15 medium; rIL-2 injected animals were given 50 Units rIL-2 on day 1 after grafting and 250 Units on days 4 and 7 after grafting; Cyclophosphamide, (CyP), injected animals were given 300 $\mu$g CyP two days prior to second-set grafting. All injections were given intraperitoneally.

(See Figure 6.6). All HSA control animals, from groups A, B and C, retained their first- and second-set grafts throughout the experiment, (i.e. for 50 days after second-set grafting). Injection of animals of these three groups with rIL-2 failed to break tolerance of either first or second-set grafts; 3/6 group C animals, (LM3 grafted with LG17), however, showed some signs of chronic rejection of their first-set grafts, (dilated blood vessels and some pigmentary disturbances), at 30 days post-second-set grafting, but these grafts appeared healthy 20 days later.

In contrast, injection of CyP 2 days prior to second-set grafting was effective in breaking perimetamorphically-induced tolerance, in certain donor/host combinations. In group C animals, where a two MHC-haplotype disparity existed between donor and host, 6/7 second-set grafts showed extensive vasodilation within 10 days and were completely rejected, as assessed by total destruction of graft iridiophores, within 25-28 days. First-set grafts on these animals, previously retained in perfect condition for over 100 days, rapidly broke down after CyP injection, being rejected 20–25 days later. Furthermore, 2/5 group A froglets, (LG15 given LG5), rejected their second-set grafts 21 and 28 days after CyP injection, whereas their first-set grafts showed only mild rejection phenomena by 50 days after second-set grafting. The remaining 3/5 CyP-injected group A animals retained both first- and second-set grafts intact. No CyP-injected group B animals showed any signs of alloimmune destruction of either graft.

## 6.4 Discussion

Quinacrine-stained sections of *X.borealis* larval thymus implants, examined 3–4$\frac{1}{2}$ months after implantation into LG17 TX larval hosts, were found to contain almost exclusively host-derived lymphocytes, within donor- type stroma. These findings, therefore, support Horton , Russ et al's (1987), observations, (made 8–9 weeks post-implantation), of adult, (6–8 month old), *X.borealis* thymus glands implanted into TX J strain *X.laevis* or TX LG clonal larval hosts. Replacement of thymic-implant lymphocytes, within 8 weeks, by those of host type was also seen in studies reported in Chapter 3, in which xenogeneic *X.tropicalis* larval thymi were implanted into larval *X.borealis* TX recipients. Although no time-course study of the turnover from donor- to host-derived thymocytes was made in the experiments reported in this Chapter, it is likely that the metamorphosis of the recipient animals was a critical period in this process. Thus, Turpen & Smith, (1989), showed that there is extensive colonisation of the *Xenopus* thymus by lymphoid precursors between 38 and 57 days of age, (i.e. just before and during metamorphosis). Also, in experiments reported in Chapter 3, XTLA-1 negative *X.tropicalis* thymus implants showed noteable XTLA-1 positive, host-derived thymocyte content following the metamorphosis of the thymus-graft recipient.

Sections of the spleens of two LG17 TX, each implanted 3-4 months previously with a single *X.borealis* larval thymus, which had subsequently been grafted with *X.borealis* skin from the thymus donor, were examined, following quinacrine staining. The low number of Q+ve cells found, in the red pulp and marginal zone areas of these spleens are likely to have emanated from the *X.borealis* skin grafts, rather

than from the earlier thymus implants, since spleens of thymus-implanted, non-skin-grafted animals contained no demonstrable Q+ve cells. Quinacrine-stained blood smears from thymus-implanted hosts, either skin grafted or non-skin grafted, were also examined, and a similar situation to that seen in spleen sections prevailed in these specimens, i.e. skin grafted animals possessed Q+ve cells while ungrafted animals did not. Other workers in this laboratory have also found skin graft derived Q+ve cells within the spleens of Q-ve TX animals grafted with *X.borealis* skin, (Horton et al, 1990 in press). The nature of these donor-derived cells is uncertain, but they warrant further investigation, since they might either promote or deter anti-donor reactivity, depending on the immune status of the recipient. Interestingly, no such cells were found in quinacrine stained sections of the spleens of control LG17 animals, following rejection of *X.borealis* skin grafts.

The apparent lack of donor-derived cells in spleens of LG17 TX animals, implanted 3-4 months previously with larval *X.borealis* thymus, but left without subsequent skin grafts contrasts with the observations of Horton et al, (1987), who saw *X.borealis* thymus-graft derived cells scattered in the spleen, and occasionally in the blood, of TX J strain hosts. Since their observations were made on hosts implanted with adult thymi, it is possible that emigrants from adult thymi are more persistant in the host periphery. Equally, the extra 1-2 months which elapsed between thymus implantation and observation of the host spleens in the present study may have allowed the death or replacement of large numbers of donor-derived cells. In Nagata & Kawahara's, (1982), studies, ploidy-marked allogeneic thymus-implant cells were few in number, (less than 8% of the total), when TX host splenocytes were examined 5 months after thymus-implantation. Equally, Nagata & Cohen, (1984), noted variable persistance within TX host spleens of donor cells from adult thymus implants; in their experiments, the degree of persistance appeared to depend on the donor/host combination.

Following implantation of *X.borealis* larval thymi into larval LG17 TX hosts, thymus-donor skin grafts were tolerated for over 50 days. Histological examination of similar, tolerated grafts up to 20 weeks post- application, showed that these grafts remained in good condition, despite occasional accumulations of host-derived lymphocytes within the *stratum spongiosum*. Du Pasquier and Flajnik, (1987), also observed invasion of tolerated skin grafts by host derived, MHC class II positive cells. Interestingly, though, fluorescence microscopical examination of quinacrine-stained sections of tolerated *X.borealis* grafts revealed that the epidermis of these grafts is often replaced, within two weeks, by host type epidermis. This replacement did not include the neck cells of donor glands, which open onto the skin surface, a dichotomy which may reflect the differing embryonic origin of glandular and epidermal layers, or may be a consequence of a particular sensitivity of the germinative epidermal layer of amphibian skin to interruption of vascular and neural supply occasioned by the process of skin grafting. Graft epidermis may, therefore, be unable to achieve the necessary rate of mitosis required, and so may be overgrown by adjacent, intact host epidermis. Rapid epidermal replacement was also observed in quinacrine-stained sections of *X.borealis* skin grafts applied to unimplanted LG17 TX recipients. Replacement of graft epidermis is probably not unique to xenografts, in that such replacement has been postulated in studies

of allografts applied to *Xenopus*, (Horton, 1969), and in studies of skin allografted newts, (Cohen, 1966). The process of epidermal replacement of grafts by host cells may have immunological consequences, in that class II MHC positive dendritic cells, (suggested to be Langerhans cells-equivalents), have been described within the *Xenopus* epidermis, (Du Pasquier & Flajnik, 1987) and these 'passenger leukocytes' seem to be critical stimulators of skin allograft rejection in larval *Xenopus*, although not in adult animals, (Flajnik & Du Pasquier, 1984). It has, moreover, been suggested that inactivation of, or removal of, Langerhans cells from the skin might be of importance in promoting skin allograft acceptance in mammals, (reviewed by Silberberg-Sinakin et al, 1980). Thus, loss of many donor-derived dendritic cells by epidermal replacement may help to establish tolerance in thymus-implanted animals. It should be noted, however, that epidermal replacement, assuming that this is a general feature of skin grafting, does not render tolerated grafts less antigenic to intact recipients. Thus, Nakamura et al, (1987), transferred (J x K)$F_1$ skin grafts, which had been tolerated by J strain hosts for at least 150 days following perimetamorphic application, to intact J strain adult hosts. These grafts were acutely rejected. Horton et al, (1989), demonstrated that perimetamorphically-applied LG5 skin grafts, tolerated for 24 weeks by LG17 recipients, were fully accepted when grafted back onto naive LG5 animals. Transfer of similar grafts onto LG5 animals previously primed to LG17 skin, however, resulted in alloimmune reactivity towards the putative LG5 grafts, demonstrating some alteration in the antigen expression of these grafts.

Our skin xenografting experiments, reported here and in Chapter 4, suggest that this response in *Xenopus*, like the response to skin allografts in this species, is thymus dependent. Thus, TX J strain and TX LG17 hosts generally failed to reject, or only chronically rejected, XbJ or *X.borealis* skin. When TX animals were implanted with xenogeneic thymi, third-party graft rejection was usually restored, while skin xenografts from the thymus donor and, it should be noted, the xenogeneic thymus tissue itself, were tolerated. In contrast to the findings from this laboratory, Balls, Clothier and colleagues have suggested that skin xenograft rejection in *Xenopus* is thymus-independent. They base their theory, in part, on the use of N-methyl-N-nitrosourea, (NMU), which selectively destroys the thymic cortex and T-cell areas of peripheral lymphoid organs and results in permanent loss of T-helper function in *Xenopus*, (Clothier et al, 1990). NMU-treated outbred *X.laevis* in their studies failed to reject skin allografts while showing relatively normal rejection of various types of xenograft, (from *Rana pipiens, X.tropicalis* and *X.borealis*). In earlier studies, though, (Balls et al, 1981), rejection of *X.borealis* skin by NMU- treated hosts was markedly delayed, from 15+/-1 to 34+/-2 days. Furthermore, work on TX *X.laevis* by this group, (James et al, 1982a; Clothier et al, 1990), indicated that this operation had a minimal effect on skin xenograft destruction, but that it delayed allograft rejction by some 5 weeks. In their recent study, these workers suggest that xenograft rejection does not rely on lymphocytic infiltration. In contrast, unpublished histological studies conducted in this laboratory have shown that *X.borealis* skin grafts, in the process of rejection by intact control animals are extensively infiltrated by host lymphocytes. Furthermore, our histological examination of *X.borealis* skin grafts in the process of chronic graft re-

jection by TX hosts also revealed extensive infiltration of host lymphocytes. Thus, we feel that in vivo cytotoxic mechanisms involved in allo- and xenograft rejection are fundamentally similar.

In light of Maeno et al's suggestion that implantation of histoincompatible thymus to TX *Xenopus* results in a tolerance to donor type mediated by clonal deletion, it is perhaps strange that small foci of host- derived lymphocytes were regularly observed in the glandular layer of tolerated grafts. These lymphocyte accumulations are not a normal feature of *Xenopus* skin, but did not appear to be correlated with any effective graft rejection response. Furthermore, we have found, in MLC experiments reported in Chapter 4, evidence of positive allorecognition of donor-type stimulator cells in thymus-implanted, donor-skin graft tolerant hosts.

With regard to allotolerance induced perimetamorphically, (by skin grafting), in control *Xenopus*, attempts to break the maintenance of tolerance by rIL-2 injection were not succesful, here. The physiological relevance of using human rIL-2 in *Xenopus* may be questioned, since Watkins & Cohen, (1987), reported that human rIL-2 did not have growth-promoting activity for *Xenopus* PHA-induced lymphoblasts. However, Ruben et al have shown that anti- human Tac antibodies, (which bind the $\beta$-chain of human lymphocyte IL-2 receptors), bind to fixed and unfixed *Xenopus* lymphocytes, (Langeberg et al, 1987); the level of binding of these antibodies to *Xenopus* lymphocytes was found to increase after stimulation of these cells with PHA. Effective competition for binding to *Xenopus* splenocytes was found by these workers between rIL-2 and anti-Tac antibodies. Furthermore, functional activity of rIL-2 in *Xenopus* has been reported by Ruben, (1986), who found that it could promote helper cell activity required to generate anti-hapten antibody responses. Preliminary studies, (Ruben & Horton, personal communication), had suggested that rIL-2 injection could interfere with perimetamorphic induction of tolerance in J strain hosts grafted with J/LG5 skin. High corticosteroid levels found in *Xenopus* during metamorphosis, (Marx et al, 1987), might well interfere with IL-1 and IL-2 production, and by so doing compromise various regulatory and effector mechanisms.

The findings reported here, that perimetamorphic tolerance induced by skin grafting control *Xenopus* can be broken down by treatment with CyP, particularly when a 2 MHC-haplotype disparity exists between donor and host, add weight to Du Pasquier & Bernard's, (1980), and to Nakamura et al's, (1987), evidence that this state is maintained by a suppressive mechanism. Furthermore, since Du Pasquier & Bernard, (1980), found that transfer of perimetamorphic thymocytes to adult, newly-skin grafted hosts could delay graft rejection, suppressive mechanisms may well promote acquisition of self-tolerance during metamorphosis. Midlarval thymectomy experiments, (Barlow & Cohen, 1983), demonstrated that this operation could increase the frequency of rejection of perimetamorphically-applied semiallogeneic grafts, perhaps by removal of suppressor subsets.

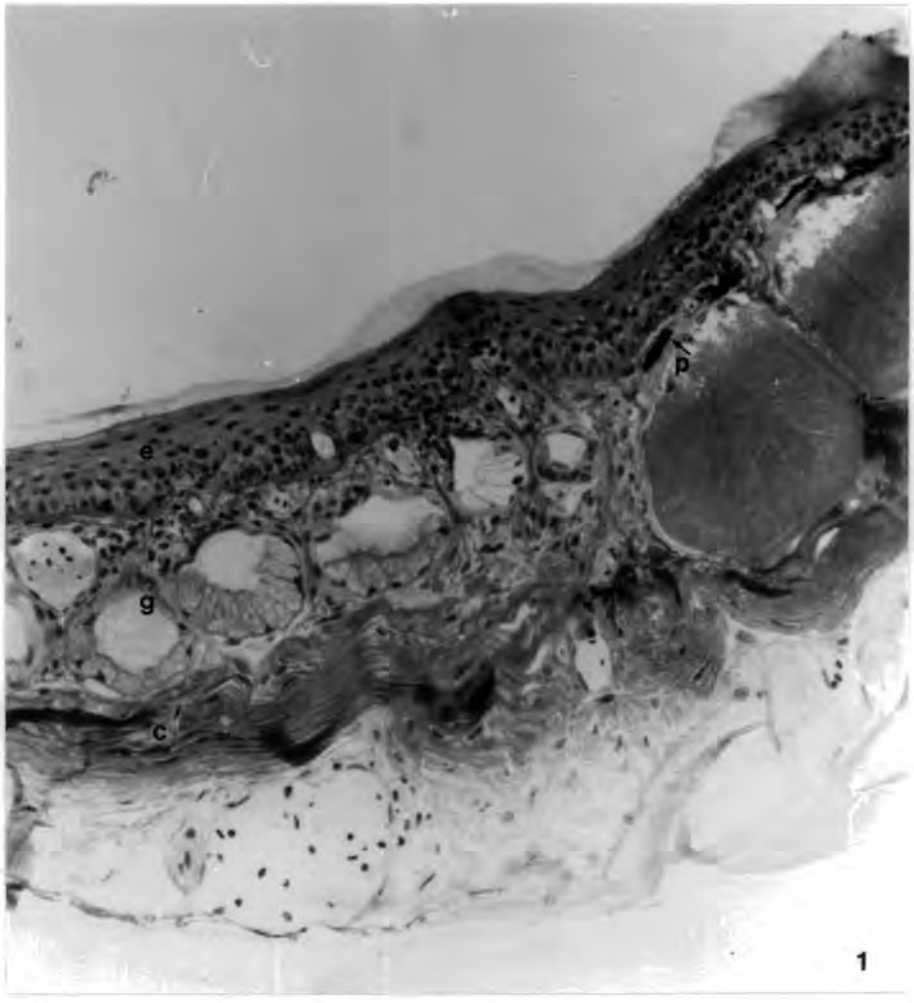
In preliminary experiments, in which thymus-induced tolerant animals were treated with CyP, this treatment did not break tolerance towards grafts of donor-type skin, (data not shown).

Further work, using the protocols described in this Chapter, is required to

determine if thymus-induced tolerance in TX hosts is really mediated by a mechanism different to that which operates in skin-induced tolerance in control animals.

**Figure 6.1** H & E stained 4$\mu$ sections of Historesin-embedded, tolerated skin graft, (12 days after application), at the junction with surrounding host skin. Pigment cells ,(p), are seen in host dorsal skin, but are absent from ventral skin graft. The TX LG17 skin graft host had been implanted, in larval life, with a single thymus from the same *X.borealis* donor which supplied the skin graft. The skin graft shows normal structure; e = epithelium; g = glandular layer; c = collagen layer. x160

**Figure 6.2A** Phase contrast view of 4$\mu$ section of Historesin-embedded *X.borealis* skin graft tolerated by TX, thymus-implanted LG17 host, 12 days after application, showing normal structure despite small focus of lymphocytes. e =epidermis; g = glandular layer; c = collagen layer; f = lymphocyte focus. x250

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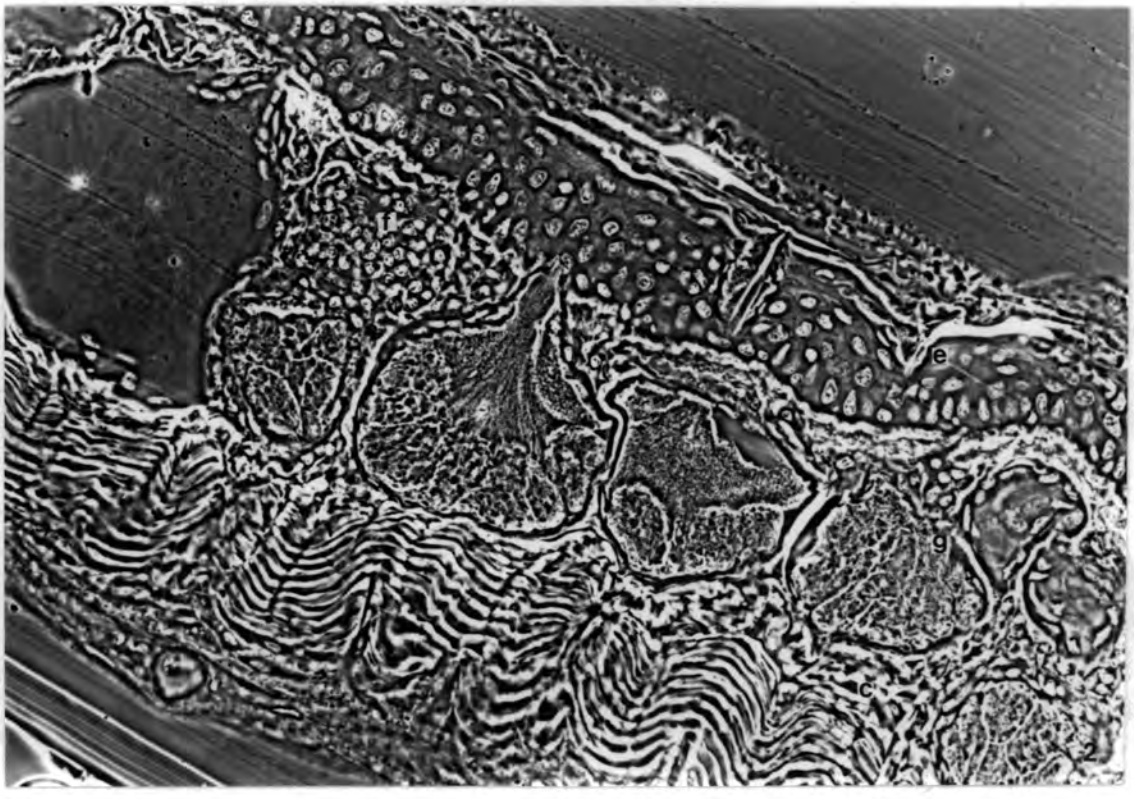
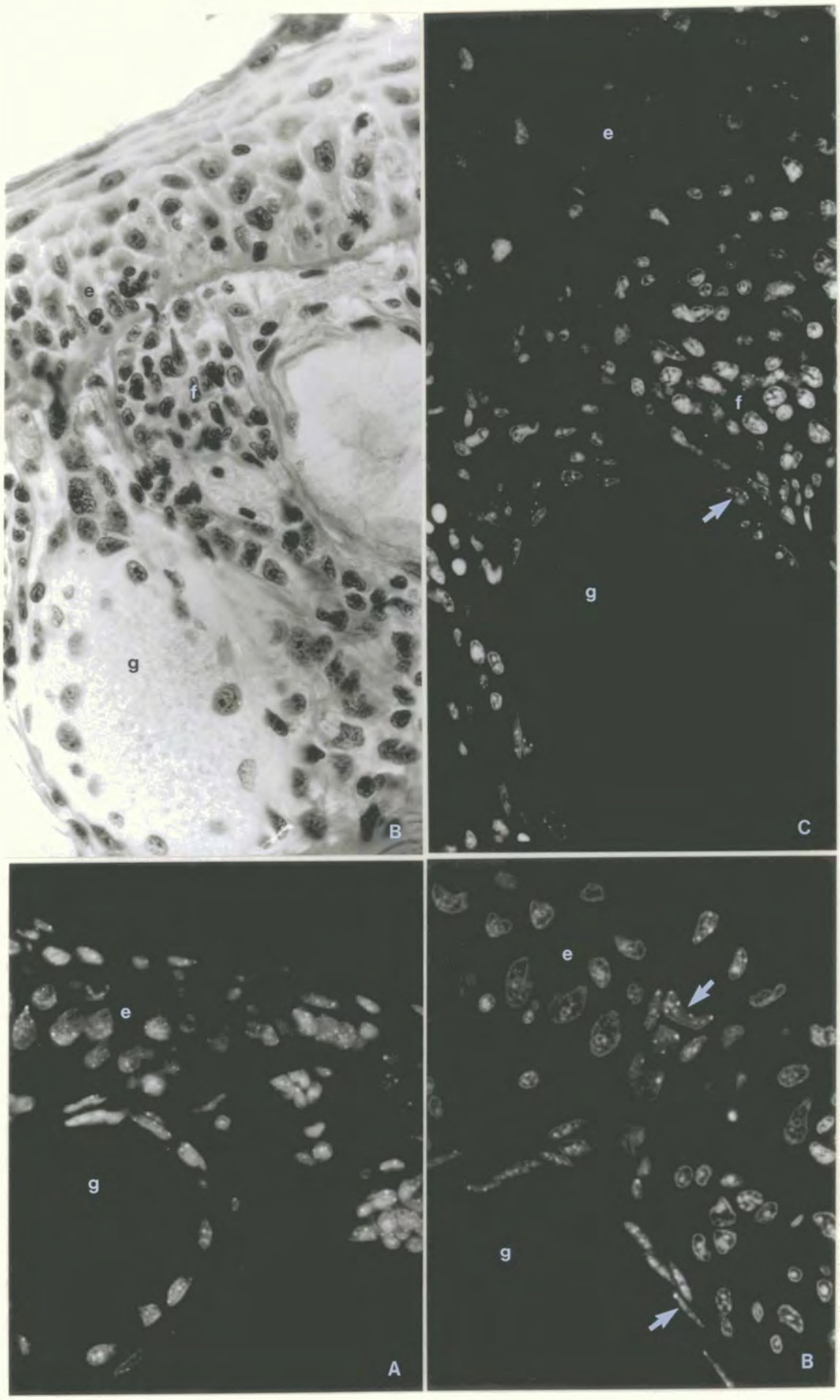


**Figure 6.2, B and C** H & E stained,(6.2 B), and quinacrine stained,(6.2 C), sections of tolerated *X.borealis* skin graft,(12 days after application), showing focus of lymphocytes in glandular layer. Quinacrine staining reveals host-derived, ('non-spotted'), origin of lymphocytes. Note epidermal replacement by host cells and persistance of donor, ('spotted'), glandular elements, (arrowed). e = epidermis; g = glandular layer; f = lymphocyte focus. 6.2B: x450; 6.2C: x520

**Figure 6.3, A and B** Quinacrine stained 4$\mu$ sections of Historesin-embedded normal *X.borealis* skin and tolerated *X.borealis* skin graft. Figure 6.3A shows normal *X.borealis* skin epidermis, (e), and glandular layer, (g). Note 'spotted' appearance of cell nuclei. Figure 6.3B shows epidermis and part of the glandular layer of a tolerated *X.borealis* skin graft, 12 days after application. Note that the graft epidermis has been replaced by host cells, while the glands, including gland neck cells, remain of donor origin, (arrows point to donor cell nuclei). 6.3A: x640; 6.3B: x520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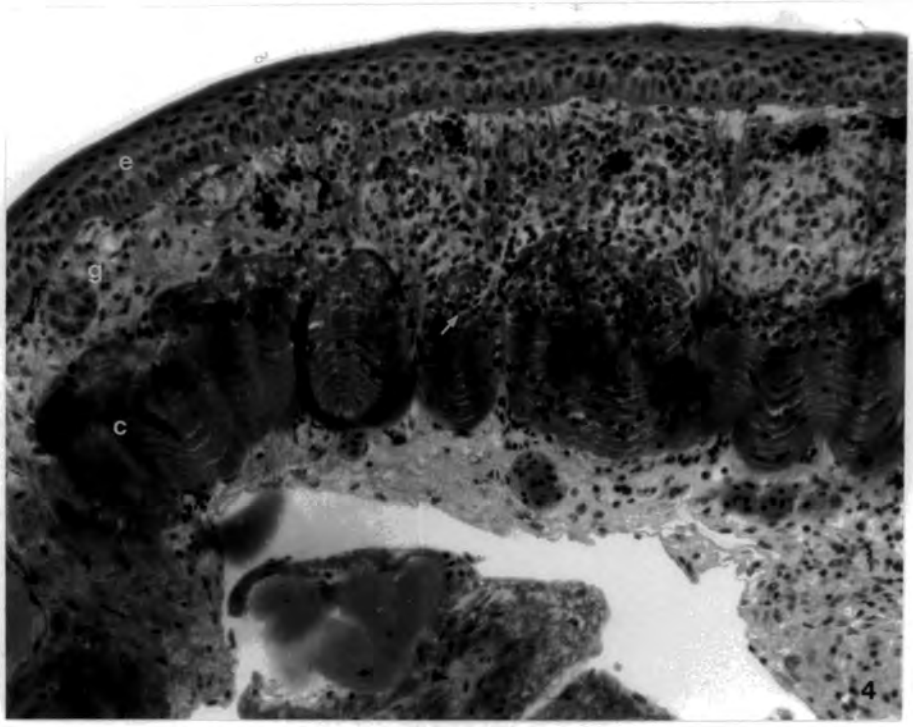
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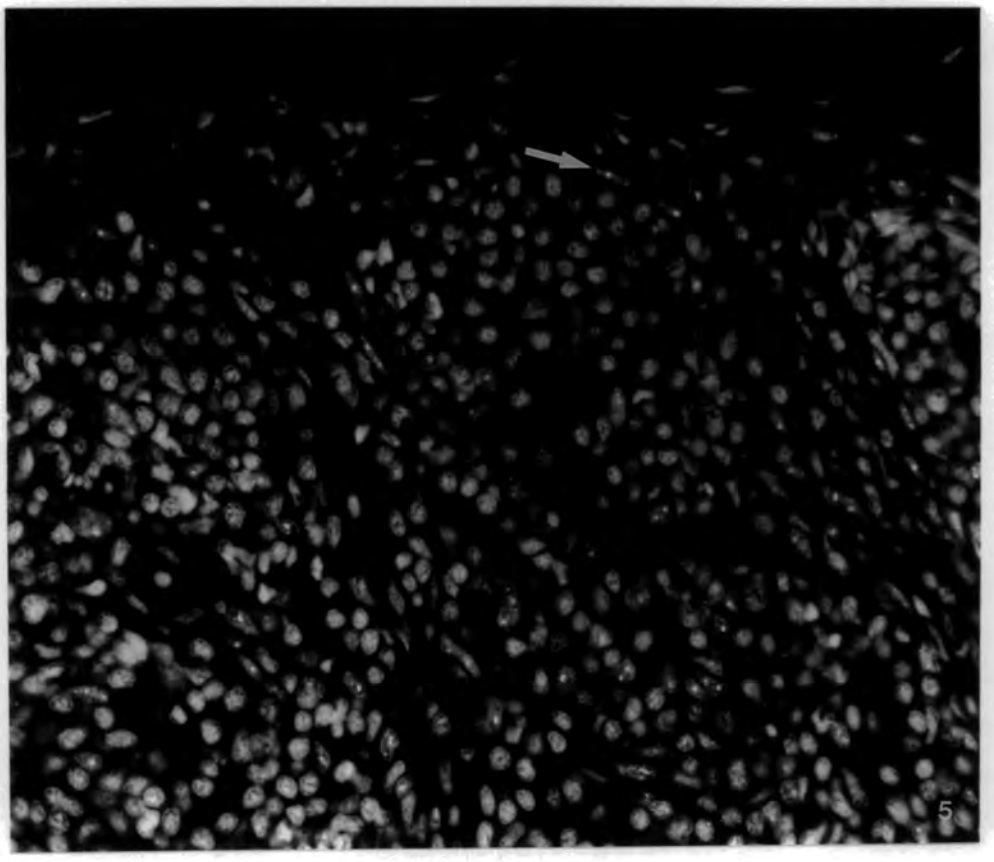
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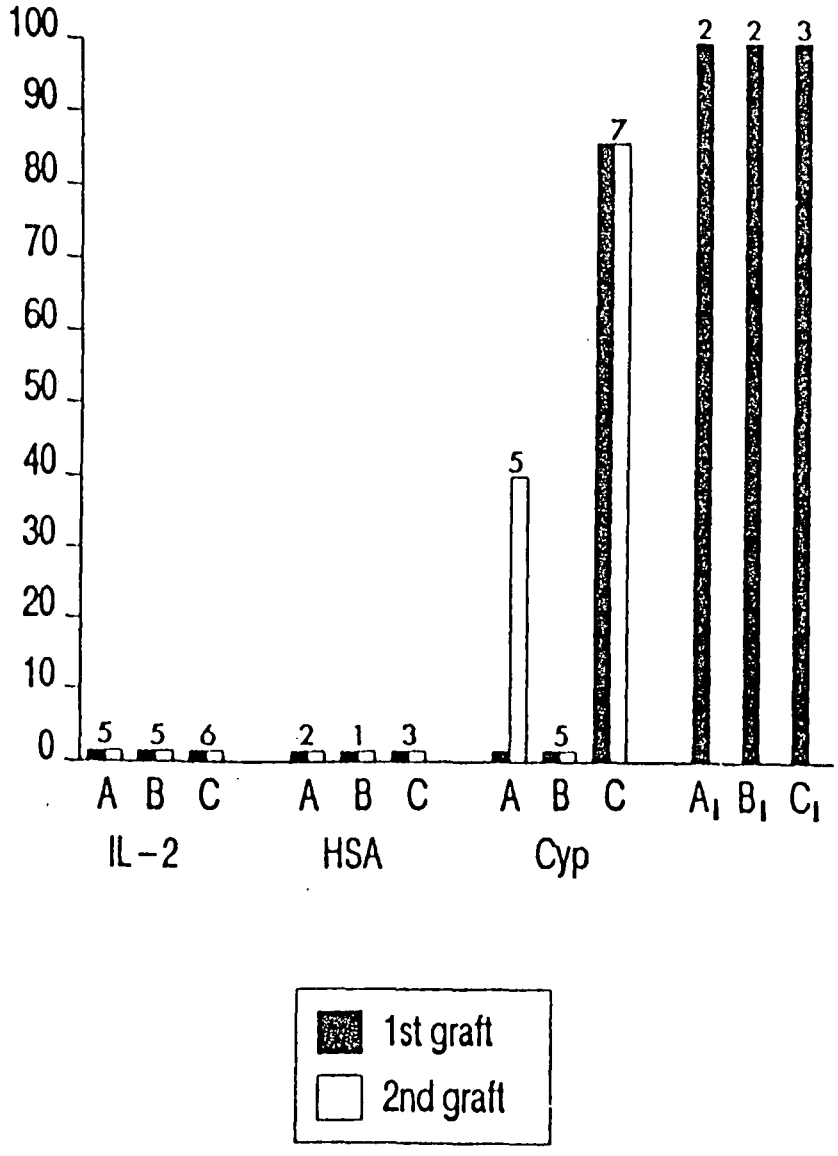
**Figure 6.6** Injection of froglets with cyclophosphamide, but not with IL- 2, can break skin allotolerance. Bar graphs show percentage of grafts rejected after grafting at 100 days postmetamorphosis. The 3 columns $A_1$, $B_1$, $C_1$ on the right of the figure refer to the outcome of applying first- set skin grafts to noninjected froglets when 100 days postmetamorphosis. Injected froglets received their first-set grafts during metamorphosis, (stages 54–56), and their second set grafts when 100 post- metamorphosis. Group A = LG15 hosts given LG5 skin; Group B = LG5 hosts given LG17 skin; Group C = LM3 hosts given LG17 skin. Numbers at the top of columns refer to animals used. X-axis captions refer to treatment groups, IL-2 = interleukin 2 injections, HSA = control injections, CyP = cyclophosphamide injections, (see text for details).



# Chapter 7

# Summary

This thesis concentrates on aspects of T-cell differentiation in foreign, (allo- or xeno-), thymus implanted, early-thymectomised *Xenopus*. T-cells were discriminated by the demonstration of a *Xenopus* T-cell marker, XTLA-1, and the species distribution of this antigen was examined. A variety of functional assays were employed to assess the development of thymus- dependent immune responses and induction of tolerance. Immunoregulatory mechanisms involved in the maintenance of transplant tolerance in control animals were also assessed.

Initial work, reported in Chapter 2, surveyed the expression of the XTLA-1 surface antigen by *Xenopus* T-cells in the thymus and periphery of various strains and species of *Xenopus*, extending Nagata's, (1985, 1988), studies. All *X.laevis* strains, and all hybrid clonal animals, derived in part from *X.laevis* parentage, thus far examined have proved to express XTLA-1 on virtually all thymic and on a proportion of peripheral T-cells, as have the *X.borealis* individuals examined. Our flow cytofluorometric data stand in contrast to those of Nagata, however, with respect to the antigen density of XTLA-1 on thymic and peripheral T-lymphocytes of these strains and species. In our hands, splenic T-cells were generally shown to be less intensely fluorescent than thymocytes, (when labelled with XT-1 and FITC- conjugated secondary antibodies), regardless of the strain or species from which thay were obtained. FACS analysis of XT-1 labelled splenocytes from *Xenopus* thymectomised by microcautery confirmed the effective depletion of T-cell numbers by this thymectomy technique, reinforcing the conclusions drawn from previous functional studies carried out in this laboratory, and elsewhere, (e.g.by Nagata & Cohen, (1983)).

Further immunofluorescent staining with XT-1 on frozen sections, (Chapter 3), lead to a description of the distribution of XTLA-1 positive lymphocytes in normal adult thymus and spleen and in the thymus and pharyngeal region of late-stage larvae. It was shown that adult cortical lymphocytes of the immediate subcapsular zone apparently express high densities of this antigen. Curiously, the same does not necessarily hold for cells from the same region of larval thymus, although previous histological and E.M. studies, (Nagata, 1976; Clothier and Balls, 1985; Russ, 1986), suggest that this zone is occupied by large, actively dividing lymphocytes in both adult and larval thymi. Splenic XTLA-1 positive lymphocytes were generally,

although not exclusively, found in areas previously described as thymus-dependent. Furthermore, the VCBs of late stage larvae, suggested by Manning, (1971), to be thymus-dependent areas, were shown in the current work to contain large numbers of XTLA-1 positive lymphocytes. Larval spleen was not examined.

The finding of a species, *X.tropicalis*, in which XTLA-1 expression is not detectable at all, either by fluorescence microscopy or by FACS analysis, (Chapters 2 and 3), is novel and of interest. Immunoprecipitation, using the XT-1 monoclonal antibody, should now be carried out to determine if XTLA-1 is not expressed at all by thymus-dependent cells of this species, or, alternatively, if expression of this antigen is masked in some way, or is, perhaps, below the threshold of detection by immunofluorescent labelling techniques.

In view of the potential use, (described below), of *X.tropicalis* in further studies, preliminary investigations were made of the skin graft rejection, MLC and T-cell mitogen responses of *X.tropicalis*, (Chapter 5). Skin allo- and xenograft rejection were found to be as acute in this species as in *X.laevis*, and appeared morphologically similar to graft rejections performed by other *Xenopus*. PHA-P and Con A responses by *X.tropicalis* splenocytes and thymocytes were significant, although S.I.s were generally lower than those of either *X.laevis* or *X.borealis*, probably due to the high background dpm recorded for unstimulated *X.tropicalis* cells. MLC responses to allogeneic *X.tropicalis* splenocytes were positive, but again low, while irradiated xenogeneic splenocytes provoked no detectable proliferation by *X.tropicalis* responder splenocytes. Once again, background proliferation levels of *X.tropicalis* responder splenocytes and of irradiated *X.tropicalis* stimulator cells were relatively high, compared with those observed for other *Xenopus* species. Due to lack of thymectomised *X.tropicalis* animals, the thymus-dependency of these reactions awaits confirmation.

Markers which allow the distinction of the origin of individual cells are invaluable in any system which involves the transfer of cells or tissues from one animal to another, and the assessment of the effect of this transfer. In *Xenopus*, the quinacrine marker, (described by Thiébaud, 1983), is carried by cells of *X.borealis* and *X.fraseri*, and has been used in previous studies in this laboratory, (Russ, 1986; Horton & Russ et al, 1987), to examine the persistance of elements of transplanted thymi. Additionally, the finding that *X.tropicalis* lymphocytes do not express detectable levels of XTLA-1 makes this species a useful thymus donor source, for examination of the differentiation of XTLA-1 positive host-derived lymphocytes following xeno-thymus implantation. Studies were, therefore carried out to assess the effects of implantation of these xenogeneic thymi, in terms of functional restoration, tolerance induction and XTLA-1 positive lymphocyte generation, (Chapter 4).

Implantation of semi- or fully-xenogeneic larval XbJ or *X.borealis* thymus- implants into TX larval recipients generally appears to be as effective at restoring proliferative and cytotoxic responses to third-party cells and tissues as implantation of allogeneic thymi.

Implantation of *X.borealis* larval thymus into TX LG17 hosts was found to restore the capacity for antibody production against the thymus-dependent anti-

gen, sheep blood cells, and, in fact, anti-SBC antibody titres in thymus-implanted animals surpassed those found in similarly immunised intact controls.

In terms of tolerance induction by XbJ or *X.borealis* thymus implantation into TX hosts, this, again, appeared to follow the 'rules' of allo-thymus implantation-induced tolerance. That is, skin grafts from thymus donor animals were uniformly tolerated. Histological examination of *X.borealis* thymus-donor grafts onto thymus-implanted LG17 TX hosts revealed the rapid replacement of donor epidermis with that of host type. This phenomenon is probably common to allo- and xenografts, from previous histological observations of allografting in *Xenopus*, (Horton, 1969), but apparently does not affect the antigenicity of grafts applied to intact recipients. MLC tests, using thymus-implant cells and splenocytes of semi-xenogeneic thymus-implanted hosts, showed that 'split tolerance', previously noted for splenocytes of allo-thymus-implanted recipients by some authors, (Nagata & Cohen, 1984: Arnall & Horton, 1986), although not by others, (Maeno et al, 1987), prevailed in these animals. ('Split tolerance' describes the state of unresponsivity to foreign skin graft antigens, with concurrent proliferative reactivity to lymphocytes of the skin graft donor.) MLC responses shown to the thymus-donor are T-dependent, but the proliferation observed may not be due to T-cell division. This situation could be clarified by depletion of either the B-cell or the T-cell populations from splenocytes, prior to MLC. We have found that splenocytes from TX J strain animals showed significant proliferative responses to *X.borealis* stimulators; this phenomenon is probably the result of back-stimulation by irradiated stimulators of J strain B-cells. Nakamura et al, (1987), commenting on the 'split tolerance' observed in perimetamorphically- skin grafted *Xenopus*, suggested that emerging, relatively immature larval anti- class I MHC reactive lymphocytes were amenable to suppression, whereas more mature anti-class II MHC reactive lymphocytes were not. Maeno et al, (1987), however, consider that tolerance induced by thymus-implantation is effected by clonal deletion. In light of both the 'split tolerance' observed following allo- or xeno-thymus implantation, and our observation that donor type skin grafts applied to thymus implanted animals, despite being tolerated for long periods, were infiltrated by small foci of host- derived lymphocytes, it is likely that the cytotoxic effector cells responsive to donor MHC antigens are deleted, while recognition of donor antigens remains.

Implantation of adult, irradiated or larval *X.tropicalis* thymi into TX LG15 hosts, however, did not appear to follow the allo-thymus implantation 'rules'. Thus, restoration of third-party skin graft rejection was not observed in these animals, and significant MLC response to third-party splenocyte stimulators was only seen in thymus-implant cells and not in host splenocytes,. Previous studies, on *X.borealis* Tx hosts implanted with *X.tropicalis* thymi, (reported in Chapter 3), had suggested that the majority of thymus-implant cells would be of host origin, by the time that these functional studies were undertaken and the limited FACS analyses carried out on *X.tropicalis* thymus implanted LG15 animals indicated that over 50% thymus-implant cells and approximately 8% splenocytes were XTLA-1 positive host-derived T-cells. Despite poor MLC and skin graft responses, host splenocyte responses to PHA-P and Con A were good, suggesting the restoration of at least some peripheral T-cells. Tolerance induction was not examined in this

study; further experiments should now be carried out to probe both this question, and to examine the response of *X.tropicalis* thymus implanted animals to skin grafts from donors allogeneic to the thymus donor.

Using both XT-1 labelling and the quinacrine marker,the infiltration of and differentiation within these implants of host-derived T-cells was followed, in frozen sections of larval *X.tropicalis* thymi, implanted into TX *X.borealis* host. It was strongly suggested in this study that immigrating T-lymphocyte precursors are XTLA-1 negative, reinforcing Nagata's, (1986), ontogenetic study of XTLA-1 expression. The substantial increase in host-derived thymocytes within implants removed after the recipient had metamorphosed was significant, in light of Turpen & Smiths', (1989), report of a wave of thymic colonisation by stem cells, occurring between 38 and 57 days post-fertilisation. It would appear that, given an appropriate donor thymus, T-stem cell migration procedes with normal chronology in TX animals. The dual XTLA-1/quinacrine marker model used here might prove to be a useful tool for developmental immunology studies, especially if XT-1 labelling and quinacrine staining could be carried out on the same section.

Use of *X.tropicalis* thymus implants in these studies has the further advantage that single-step propidium iodide staining, (Taylor, 1980), of splenocyte nuclei can reveal the presence of donor-type cells in the host periphery. It is important to be able to gauge donor-cell persistance, when conducting functional tests of reconstitution by normal, non-irradiated implants, since, obviously, any positive response measured may be due in part to the activity of donor-derived cells, if these are present. In the single FACS experiment reported in Chapter 3, no *X.tropicalis* thymus-donor cells were found in the host periphery 2 months after implantation, although this finding should be checked, using density-gradient separated splenocyte suspensions, to avoid the contribution of red cell nuclei to this P.I. staining analysis.

In the final Chapter, the mechanism of perimetamorphically-induced allo- tolerance was examined, using injection of rIL-2 and CyP. The findings reported in this Chapter reinforce Nakamura et als', (1987), suggestion that this tolerant state is mediated by suppression of cytotoxic response. Further work should be carried out, particularly using CyP injection, to see if the mechanisms involved in thymus-induced tolerance are different to those at work in perimetamorphic skin graft tolerance. It has been suggested that the mechanisms of xenograft and allograft response are different, and that the former is thymus-independent, (James et al, 1982; Clothier et al, 1990). If this is so, then the mechanism of xenograft rejection is certainly amenable to some form of control exerted by the thymus, since XbJ and *X.borealis* thymus implantation induced long-lasting tolerance to donor type xenogeneic skin, and the thymus implants themselves persisted for several months, (as did those of *X.tropicalis* donors). If xenothymus induced tolerance is maintained by clonal deletion , as Maeno et al have suggested for allothymus-induced tolerance, it is difficult to see how this process could affect a thymus-independent mechanism. Cell transfer experiments, involving the injection of xenothymus-induced tolerant splenocytes into intact syngeneic recipients, concurrently with thymus donor skin grafting of the intact, injected recipients might indicate whether the tolerance observed in this model is mediated by suppressor

cells.

# Appendix A

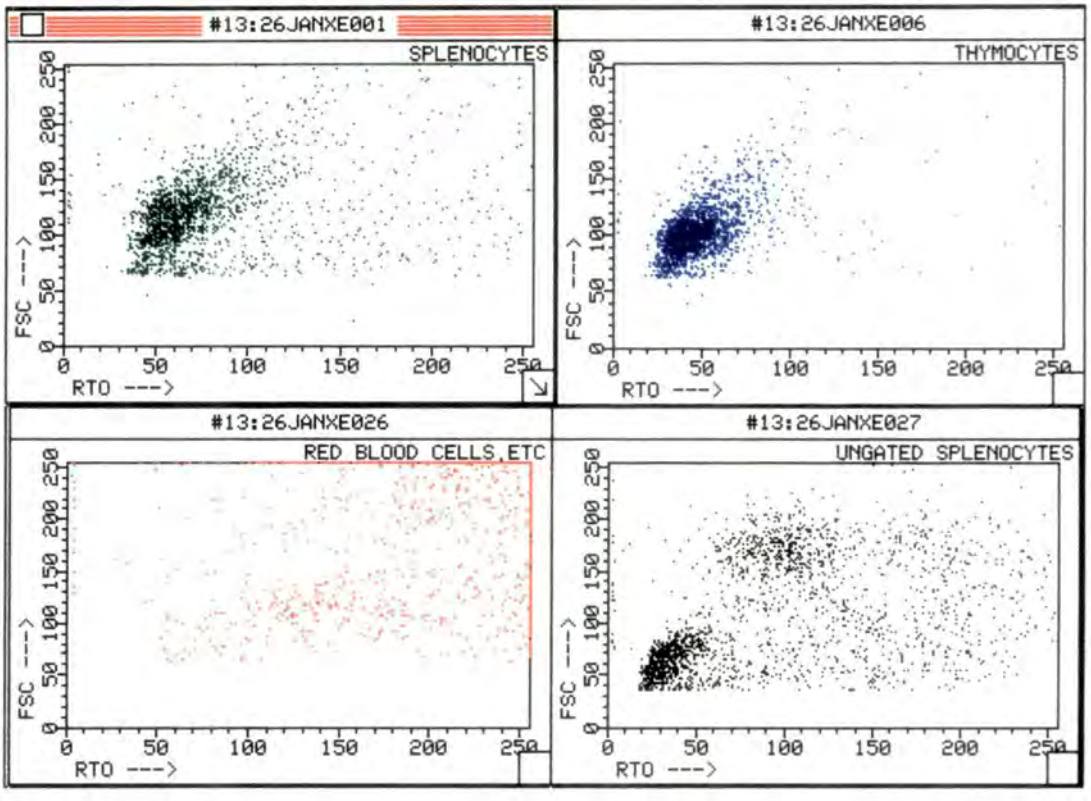
**Gating of samples for FACS analysis, and the viability of cells falling within these gating parameters.**

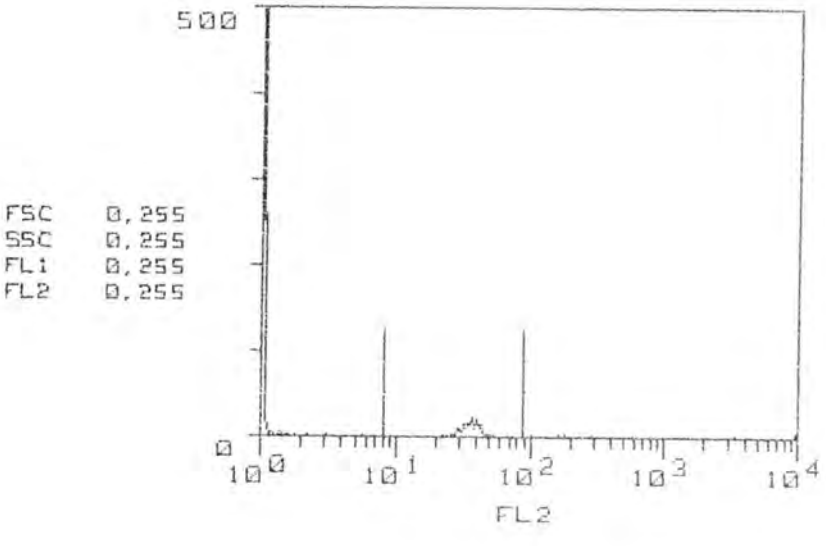
**Figure A.1**

Dot-plots showing the distribution of unseparated *X.laevis* splenocyte, (green), and thymocyte, (blue), suspensions, (x axis = granularity, Y-axis = frontal light scatter, (related to cell size)). Gates for all analyses of whole cells were set to include the cell populations shown in these two windows. The red dot-plot shows the small proportion of non-lymphoid cells which fall within the gating parameters, (this preparation was separated from lymphoid cells by density gradient centrifugation over Ficoll, and comprised mostly red cells and thombocytes as assessed by microscopical observation). The black dot-plot represents ungated data collection, from a suspension of unseparated *X.laevis* splenocytes.

**Figure A.2**

FACS-generated fluorescence intensity histogram, showing the very small proportion of non-viable cells which fall within the standard gating parameters used for studies reported in this thesis; when incubated with propidium iodide, (without detergent), non-viable cells take up this fluorescent dye, while viable cells do not. Non-viable cells in this sample comprised 1.5% of the total number analysed. X-axis = fluorescence intensity, (575 nm), Y-axis = relative cell number, (10,000 events analysed).


#13:26JANXE001
SPLENOCYTES
#13:26JANXE006
THYMOCYTES
#13:26JANXE026
RED BLOOD CELLS,ETC
#13:26JANXE027
UNGATED SPLENOCYTES
FSC --->
RTO --->
0
50
100
150
200
250


FSC 0,255
SSC 0,255
FL1 0,255
FL2 0,255
500
0
10^0
10^1
10^2
10^3
10^4
FL2

# Appendix B

**Calculation of $\gamma$-irradiation doses, and the effect of various dosages on the proliferative response of LG15 splenocytes to the T-cell mitogen, PHA.**

The $\gamma$-irradiation dosage received by cells and tissues left at measured distances from a $^{60}$Cobalt source, for known durations, was assessed using the Fricke chemical dosimeter, (Spinks and Woods, 1976).

LG15 splenocytes, irradiated with 3,000, 4,000 or 6,000 rads, or unirradiated were placed in culture, in 96 well v-bottomed microtitre plates. Half of these cultures were stimulated with 0.1 $\mu$g/ml PHA, while the other half were left unstimulated. Cultures, ($1 \times 10^5$ splenocytes/culture in 100$\mu$l tissue culture medium), were pulsed with 1$\mu$Ci/well $^3$HTdr after 3 days, and harvested 24 hours later, as described in Chapter 4. Stimulation indices were calculated as described in Chapter 4, and were as follows: Non-irradiated splenocytes S.I. = 16.0, 3,000 rad irradiated splenocytes S.I = 10.0. 4,000 rad irradiated splenocytes S.I. = 4.9 6,000 rad irradiated splenocytes S.I. = 1.4. Although the S.I. for 6,000 rad irradiated splenocytes was slightly above 1.0, the mean dpm, (irradiated + PHA), was not significantly different from the mean dpm, (irradiated - PHA), by students t-test, (data not shown).

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