Use of monoclonal antibodies to study alloreactivity and T cell development in Xenopus

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ABSTRACT

This study investigated T-cell surface antigenic changes in the spleen of *Xenopus* following *in vivo* and *in vitro* allogeneic challenge, using novel mouse monoclonal antibodies (mAb's) against all T cells (CD5), the putative αβ-T cell receptor (TCR), the putative γδ-TCR, the CD8 receptor and against major histocompatibility complex (MHC) class II proteins.

Flow cytometric analyses of splenocytes from skin allografted intact, 5-day thymectomised (Tx) and skin allotolerant animals were examined. There was an increase in the number of cells expressing γδ TCR's in those froges which rejected test allografts. The Tx *Xenopus laevis* and allotolerant LG3 animals that tolerated grafts showed no increase in the γδ TCR. Twenty-four days post-grafting *X. laevis*, most of the γδ TCR positive cells surprisingly co-expressed the αβ TCR. Following mixed lymphocyte reactions (MLR) for 9-14 days, of previously grafted *X. laevis* and LG15 splenocytes, there was an increase in the number of T cells with a significant increase of γδ TCR positive cells.

Stimulation indices of MLR's were increased by pre-culturing the stimulator cells with concanavalin A (ConA). This increase was apparently not due to changes in cytokine production or cell surface antigen expression, of the stimulated cells. Little increased MHC class II expression was seen, in flow cytometric analyses. The reasons for elevated MLR with ConA-activated stimulators therefore remain to be assessed.

The *in vitro* response of *Xenopus* splenocytes to the superantigen, staphylococcal enterotoxin B (SEB), was tested. The response was weakly significant but was significantly less than that seen in mice.

USE OF MONOCLONAL ANTIBODIES TO STUDY ALLOREACTIVITY AND T CELL DEVELOPMENT IN *XENOPUS*

by

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Submitted in accordance with the requirements for the degree of Master of Science

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October 1993

To my Mum and Dad with affection and gratitude

ACKNOWLEDGEMENTS

I would first like to express my thanks to my supervisor Dr John Horton for supervising this project and for his encouragement and guidance in the writing of this thesis.

I would also like to thank Trudy Horton and Pam Ritchie for all their friendly advice and guidance. Also, for making my time in Durham interesting and enjoyable.

Thanks are also due to Mr Ian Dimmick, at Dryburn Hospital, Durham and Dr John Robinson, at Newcastle University Medical School, for the use of the Coulter Epics flow cytometer and the Becton Dickinson FACS Scan, respectively. Also to Mr David Hutchinson, at Durham University, for the photographs.

Thank you also to Cathy Higgins and Jane Oakes, who have had to suffer me pestering them for time on their computers whilst writing.

I would like to express my heartfelt appreciation to all my friends, especially Steve and Kate who have always been at hand to encourage (and bully!) me to continue with this thesis, without them I feel that it would have never reached this present form.

Last but by no means least, I would like to say a very big thank you to all my family. They have always believed in me (even when I didn't!) and given me support (including financial!) and encouragement, throughout my life.

DECLARATION

No part of this thesis has been previously submitted in support of an application for a MSc. degree or equivalent qualification at the University of Durham, or any other university or institute of higher education.

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ABBREVIATIONS

APC	Antigen presenting cell
APBS	Amphibian phosphate buffered saline
BSA	bovine serum albumin
Ci	Curie
CML	cell mediated lympholysis
CO_2	carbon dioxide
ConA	concanavalin A
dpm	disintegrations per minute
FCS	foetal calf serum
FITC	flourescein isothiocyanate
[^{3}H]TdR	tritiated thymidine
IEL	intra-epithelial lymphocytes
Ig	immunoglobulin
IL-1 or -2	interleukin-1 or -2
IU	international units
kDa	kilo Daltons
L-15	Leibowitz 15 culture medium
LG + number	*X. laevis* x *X. gilli* hybrid gynogenetic clones
LM + number	*X. laevis* x *X. mülleri* hybrid gynogenetic clones
M	molar
mAb	monoclonal antibody
MHC	major histocompatibility complex
ml	millilitres
MLC	mixed leukocyte culture
MLR	mixed lymphocyte/leukocyte reaction
mM	millimolar
mmol	millimole
PE	phycoerythrin
PHA	phytohaemmaglutinin
R	irradiated at 6000 rads
SEB	*Staphylococcal* enterotoxin B
TCGF	T cell growth factor
TCR	T cell receptor
µCi	micro Curie
µg	micrograms
µl	microlitres

μm	micrometers
UV	ultra violet
v	incubated with
XLA	*Xenopus* lymphocyte antigen
1°	primary
2°	secondary

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CHAPTER 1

INTRODUCTION

1.1 Comparative Immunology

The basic requirements of an immune system, specificity (molecules and cells which react with foreign molecules and not self), diversity (an antibody can be produced against any foreign antigen), adaptivity (memory cells combating a second infection more quickly than the first) and the ability to respond to unexpected stimuli, have all been met by vertebrates. The defence systems of invertebrates, on the other hand, are more primitive, often relying solely on non-specific phagocytic cells.

Consistent signs of adaptive immunity, together with histological evidence of organised lymphoid tissue first appear in the lowest vertebrates, the cyclostomes (Roitt, Brostoff and Male, 1987). However it is only once the true fishes are reached that a well developed immune system, essentially of the mammalian kind, is seen (Cunningham, 1978).

If, therefore, cold blooded vertebrates mount a fundamentally identical immune response to that of mammals they act as extremely useful models to study the ontogenic development of immunologic capacities of the more evolved vertebrates. Use of comparative immunology in the study of non-mammalian vertebrates enables better appreciation and understanding of the more complex biological systems.

1.1.1 *Xenopus* as a model for comparative immunology

The immune systems of many amphibians have been well studied. There are two classes of Amphibia: the Urodela (for example, newts) and the Anura (for example, frogs and toads). The most studied class in terms of the ontogenic aspects of immunity is the Anura, with the African clawed toad *Xenopus*, providing the most information. The genus *Xenopus* comprises six species which cover much of the

African continent. *Xenopus laevis*, the major species studied, is a non-tropical species and is the only South African amphibian to occupy a completely aquatic habitat, feeding and breeding underwater.

There are several properties of *Xenopus* which have led to its major use as an experimental animal model. It is easy to maintain in the laboratory, since it will readily consume food and shows low mortality rate, also it is relatively easy to breed, females can be used several times a year for breeding. Frog embryos, tadpoles and adults are all amenable to experimental manipulation, permitting *in vivo* experiments, such as grafting studies to be performed at all stages of ontogeny (Obara, Kawahara and Katagiri, 1983). Partially inbred major histocompatibility complex (MHC) homozygous strains and isogeneic clones (Kobel and Du Pasquier, 1975) exist, which allow unambiguous attribution of polymorphic molecules or functions to the MHC (Flajnik and Du Pasquier, 1990). Metamorphosis separates the life of *Xenopus* to two distinct periods and provides a useful method to study self tolerance to new self antigens (Ags) in an immunocompetent tadpole.

The immune system of *Xenopus* has been well characterised. It is less complex than that of mammals but exhibits many of the hallmarks of the mammalian immune system (Du Pasquier, Schwager and Flajnik, 1989; Flajnik and Du Pasquier, 1990). The ability to mount an immune response develops early in *Xenopus* (Hsu and Du Pasquier, 1984) by twelve days after fertilisation tadpoles are able to mount specific humoral and cellular immune responses which become maximal approximately two weeks later. Investigations into the immune system of *Xenopus* have revealed that *Xenopus* lymphocytes can be separated into T and B cells (Goldstine, Collins and Cohen, 1976) by functional (Bleicher and Cohen, 1981; Green (Donnelly) and Cohen, 1979) and biochemical (Nagata, 1985; Schwager and Hadji-Azimi, 1985; Schwager and Hadji-Azimi, 1984) analyses. The production of cytokines analogous to interleukins (IL) -1 and -2 has been demonstrated in *Xenopus* (Watkins and Cohen, 1987; Watkins, Parsons and Cohen, 1987) and polymorphic MHC molecules homologous to mammalian class I and II have been described (Flajnik and Du

Pasquier, 1990). Three isotypes of immunoglobulin heavy chain are present, which are arranged in the genome in the classical mammalian type organisation (Du Pasquier, Schwager and Flajnik, 1989).

1.2 The Thymus

In all vertebrates the first organ to become lymphoid is the thymus. This occurs in *Xenopus* at stage 48 (Manning and Horton, 1969), when the animal is about 8 days of age. By this time the larvae are already feeding and free-swimming. The cellular content (Clothier and Balls, 1985) and role of the amphibian thymus (Horton, Horton and Ritchie, In preparation) are homologous to that of mammals, that is it is a primary lymphoid organ producing and exporting T lymphocytes to the periphery. A small population of B lymphocytes is also routinely present in the *Xenopus* thymus (Williams and Horton, 1980); this contrasts with the mammalian thymus, in which B cells are scarce. It is unlikely, however, that the *Xenopus* thymus spawns these B cells. The size of the organ increases rapidly at 2-3 months after metamorphosis, when about 1-3 x 10^7 lymphocytes are present; it then undergoes regression at sexual maturity.

The function of the thymus was first determined from studies on mice in the 1950's (Miller, 1961). Miller showed that certain immune responses, such as an antibody response, are to some extent thymus-independent, while others, such as foreign graft rejection, are wholly thymus-dependent, therefore identifying the thymus as a vital organ supplying T lymphocytes as defence against infection (Miller, 1961). In 1968 investigation of the spontaneous mouse mutant 'nude' (Pantelouris, 1968), in which the thymus is hypoplastic, confirmed Miller's observations and irrevocably established the concept of separate T and B lymphocyte compartments.

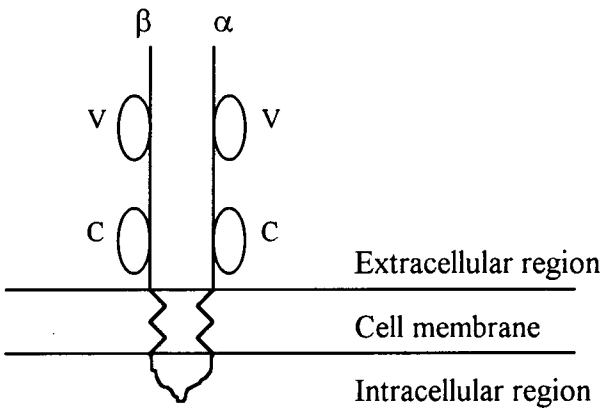
1.2.1 T lymphocyte development and T cell receptors in birds and mammals

The lymphocytes of amphibians, birds and mammals have been shown to be the executive cells of the specific immune system, and evidence suggests that the same is true of fishes (Ellis, 1977). In birds and mammals there are 2 major populations of lymphocytes, which are functionally distinct. The T lymphocytes which mature in the thymus are involved in cell mediated immunity and exert a regulatory role on the second class, the B lymphocytes. The latter are derived from the bursa of Fabricus in birds, whereas in mammals they arise from the liver and spleen during foetal life and bone marrow post-natally. The B lymphocytes are responsible for antibody production.

The primary role of T lymphocytes in the immune system is to distinguish between self and non-self determinants. Recognition of non-self antigens normally leads to the activation of distinct subclasses of functional T lymphocytes which , in turn, will induce antibody production by B cells and cytolysis of pathogen-infected host cells. To distinguish between self and non-self antigens, T lymphocytes utilise their antigen receptors. In birds and mammals it is known that these antigen receptors are two-chain heterodimers non-covalently associated with the CD3 complex. There are two distinct T cell receptor (TCR) complexes, which have been identified in both mammals and birds, known as the αβ- and γδ-TCR's, both of which are similar in structure and genomic organisation (Brenner *et al.*, 1986; Lahti *et al.*, 1988). The structural features of the αβ heterodimer are shown in figure 1.1.

The genes encoding the α, β, γ and δ chains are composed of variable, V, diversity, D (except the γ chain), joining, J and constant, C, regions. These undergo somatic rearrangement during development to create diversity and are located on chromosomes 14, 6, 13 and 14 respectively, in the mouse; in fact the δ chain gene is located within that of the α chain locus.

Figure 1.1 Structure of the human TCR heterodimer (Roitt, Brostoff and Male, 1993). The TCR α and β chains each comprise two external immunoglobulin-like domains (V and C), a transmembrane segment and a short cytoplasmic tail. The α and β chains are disulphide linked. The form of the γδ TCR is similar to its αβ counterpart. In humans the γ and δ chains are either disulphide or non-disulphide linked; non-disulphide linked forms have not been reported in mice.



Although T cells expressing αβ and γδ receptors share some common characteristics, they appear to be separate lineages. In the mouse they differ significantly in their ontogeny, anatomical distribution and physiological functions:

1.2.1.1 Ontogeny The αβ-TCR develops within $CD4^+8^+$ 'double positive' thymocytes destined to generate $CD4^+8^-$ (helper T cells) and $CD4^-8^+$ (cytolytic T cells) 'single positive' T cells, which exit to the periphery (Scollay *et al.*, 1988). Earlier in development $CD4^-8^-$ 'double negative' thymocytes express the γδ-receptor (Pardoll *et al.*, 1987), while at later times γδ-expression on $CD4^-8^-$ thymocytes is greatly reduced or absent.

In the mouse the expression of γδ precedes that of αβ-TCR by 1-2 days. The sequential appearance of molecules during ontogeny is in general a reflection of

their phylogenetic history. It is likely therefore that the γδ-receptor arose in evolution before its αβ counterpart.

The lineage relationship between αβ and γδ T cells has been analysed by the use of various T cell receptor (TCR) transgenic mice. Commitment to the γδ cell lineage seems to be determined not by productive γ and δ gene rearrangements, but by nuclear proteins which control γ and perhaps δ chain expression (Haas, Kaufman and Martinez-A, 1990); otherwise the αβ TCR is formed.

1.2.1.2 Distribution T cells expressing the αβ receptor are the major lymphocyte population in the peripheral lymphoid organs, while the γδ T cells are the major lymphocyte subset in the epithelia of various non-lymphoid tissues. γδ T cells represent about 1-5% of the total cells in the adult thymus.

1.2.1.3 Physiological functions The repertoire of peripheral αβ TCR positive T lymphocytes is largely determined by selective events within the thymus, so that T cells in the periphery respond to exogenous or endogenous peptides presented in the context of self major histocompatibility complex (MHC) molecules. During development in the thymus T lymphocytes that express αβ T-cell receptors with sufficient affinity to MHC molecules expressed on thymic epithelial cells are initially positively selected. These cells then undergo a negative selection process, where T cells which can react strongly with self-MHC/self-antigens (expressed on dendritic cells of the thymus) are inactivated. T cells remaining after these two selection processes are allowed to mature to functional T cells (Sha *et al.*, 1988).

At present the selective mechanisms operative for the γδ T cells are poorly understood. However a preliminary investigation into the influence of MHC antigens on TCR γδ usage in murine $CD8^+$ intraepithelial lymphocytes (IELs) demonstrated an extrathymic selection mechanism for these cells, perhaps in the intestinal epithelium and suggested that these cells may exhibit MHC class II-restricted antigen recognition (Lefrancois *et al.*, 1990).

1.2.2 Extra-thymic T cell development

There is increasing evidence that T cell development can to some extent occur outside the thymus in mammals (Kennedy, Pierce and Lake, 1992; Ferrick *et al.*, 1989; Hunig, 1983). Indeed it has been known for some time that there is not a complete absence of T cells in the nude ('thymusless') mouse, as shown by identification of cells bearing the θ-antigen (Thy-1) (Raff, 1973).

The concept of extrathymic T cell development in mammals has been reinforced by reports of cells in nude mice showing rearrangements of the genes coding for γδ (Yoshikai, Reis and Mak, 1986) and αβ T cell receptors (Kishihara *et al.*, 1987) and the numbers of cells bearing the T cell markers Thy1, CD4 and CD8 increase substantially with age in both nude mice and nude rats (Chen *et al.*, 1984; Vaessen *et al.*, 1986; Kennedy, Pierce and Lake, 1992). However the number of Thy-1 positive cells that also express HSA (heat stable antigen - a marker of immature thymocytes that usually decreases with age of the mouse) remains at 50%, leading to the concept that these cells may resemble immature thymocytes. Also 40% of Thy-1^+ cells in nude mice express the $CD4^-CD8^-$ phenotype in contrast to 1-5% in euthymic mice. Moreover, the $CD4^+$ cells of athymic animals do not reject allografts, do not induce T-dependent antibody responses, do not evoke delayed-type hypersensitivity reactions and do not proliferate to T cell mitogens (Kung and Thomas, 1988; Bell, 1989), which contrasts with cells which have undergone thymic processing. $CD8^+$ cells in athymic animals have a low degree of specific lysis and do not proliferate in response to the T cell mitogen, concanavalin A.

Two populations of $CD8^+$ cells have been demonstrated using expression of a specific epitope of the differentiation antigen Ly6. Both subsets show cytotoxic activity against class I and class II MHC antigens in allogeneic mixed lymphocyte culture. Euthymic mice have both $CD8^+Ly6C.2^-$ and $CD8^+Ly6C.2^+$ subsets in roughly equal numbers (Leo *et al.*, 1988), however only the $CD8^+Ly6C.2^-$ are thymus-derived. The Ly6C.2 antigen has been found on 85% of $CD8^+$ cells in athymic animals (Leo *et al.*, 1988). Perhaps the most persuasive evidence suggesting

that T cells can develop extrathymically comes from the fact that nude spleen cells stimulated *in vitro* or *in vivo* with allogeneic cells and exogenous IL-2 develop into specific cytotoxic effector T cells (Hunig and Bevan, 1980; Hale, 1980).

Kennedy *et al.* have presented evidence that suggests that the spleen and lymph nodes in nude mice may serve as sites of this T cell maturation, although $CD4^+$ T cell maturation is driven inefficiently (Kennedy, Pierce, and Lake, 1992). These organs do contain T cell subsets characterised as immature, transitional and mature by analogy to thymocyte subsets and with age, these T cell subsets progress from immature to mainly mature phenotypes.

The hypothesis that extrathymic T cell development occurs in nude animals is not, however, supported by the compromised immunocompetence displayed by these animals (Kung and Thomas, 1988). Thus, although $CD8^+$ T cells in nude mice can serve as CTL, the CTL repertoire appears restricted (Hunig and Bevan, 1980). There exists a non-thymus derived, natural killer (NK)-like mechanism in nude and euthymic mice and rats known as allogeneic lymphocyte cytotoxicity (ALC), which can be blocked by anti-CD8 antibody (Bell, 1989). The existence of this phenomenon indicates that there is an efficient effector mechanism outside the conventional CTL mechanisms, able to recognise alloantigens and kill specific targets. This may explain why athymic animals can survive and why they chronically reject allografts.

1.2.3 γδ T-cell receptor positive lymphocytes

It has been proposed that γδ receptor bearing cells and other T-like cells in nude mice arise from a pre- or non-T cell lineage and belong to a primitive cytotoxic defence system. The γδ receptor is, in evolutionary terms, considered to be pre-thymic and arose before the αβ-TCR. The αβ-TCR is considered a phylogenetic advance linked with the evolution of the thymus (Bell, 1989). The level of expression of γδ-receptor-bearing cells in nudes is equivalent to that of euthymics, suggesting that these cells can arise by a thymus independent pathway (Yoshikai *et al.*, 1988). Other types of lymphoid cells and non-lymphoid cells also bear the γδ receptor, for

example NK-like cells including IL-2 dependent large granular lymphocytes, intra-epithelial lymphocytes of the intestine and cell lines of dendritic epidermal cells. However, recent work on chickens (Dunon, Cooper and Imhof, 1993) does not support the concept of thymus-independent γδ T cell development. The γδ positive T cells in the intestine of chicken appear to be thymus-derived.

The function of cells bearing the γδ receptor is not yet known. The fact that they can be induced to become cytotoxic effector cells suggests that they are part of a defence system against environmental insult, or are designed to survey the organism for mutant clones. Janeway *et al.* (Janeway, Jones and Hayday, 1988) speculate that the γδ receptors may be largely specific for a group of class I MHC antigens different from those expressed on most somatic cells. The intraepithelial distribution of γδ cells and their cytotoxic activity has led to the hypothesis that they are involved in immune surveillance of epithelial surfaces (Janeway, Jones and Hayday, 1988) acting as the first line of defence against invading pathogens, for example *Mycobacterium tuberculosis*, which can stimulate γδ bearing cells.

On activation of γδ splenocytes and thymocytes with anti-CD3 (plus exogenous IL-1 or 2) or ConA, there is increased expression of CD8 molecules and IL-2 receptors on the cell surface, respectively. γδ cells employ CD3-mediated activation pathways very similar to those of αβ T cells (Marüsïc-Galësïc *et al.*, 1988) and also mediate spontaneous cytolysis against a variety of tumour targets as do CD8 positive αβ TCR bearing cells (Roitt, Brostoff and Male, 1993).

Several lines of γδ receptor expressing cells have been established in mice which have cytotoxic activity. Therefore γδ positive T cells may account for some of the 'non-MHC restricted' cytotoxic activity seen in human and murine peripheral T cells. Murine and human γδ T cells have been reported to lyse various targets, such as chicken erythrocytes, cells treated with mycobacteria or staphylococcal enterotoxin A (SEA) and various leukaemic cells, for example Molt 4. They also produce lymphokines, such as IL-4 and suppress B cell responses (Haas, Kaufman and Martinez-A, 1990).

Attempts to identify the ligands for γδ TCR have focused on MHC class I-like proteins, mycobacteria and heat shock proteins (hsp) (Ferrick, 1989, Allison and Havran, 1991). It appears that γδ T cells do not predominantly recognise antigens presented by 'classical' class I and class II MHC proteins, the repertoire specificity of the γδ-receptor appears to be more limited than that of the αβ receptor and includes class I or class I-like MHC molecules (Matis, Cron and Bluestone, 1987). Recognition is reported to be NK-like and MHC unrestricted.

1.3 The Immune System of *Xenopus*

1.3.1 Lymphoid tissues

The role of the *Xenopus* thymus in T lymphocyte dependent events has been examined in some depth by early thymectomy (Tx) experiments (Manning, 1971). The larval thymus is clearly visible through the transparent skin between the eyes and tympanum, which facilitates early Tx. Following Tx *Xenopus* grow at a normal rate and, although lymphoid tissues develop relatively normally, the spleen is reduced in weight (Horton and Manning, 1974).

Tx has a profound effect on immunologic development: it abrogates acute allograft rejection and mixed leukocyte reaction (MLR) (Du Pasquier and Horton, 1976; Horton and Manning, 1972); impairs responsiveness to ConA and PHA (Du Pasquier and Wabl, 1977; Du Pasquier and Horton, 1976); and it abolishes cellular and humoral responses to T lymphocyte dependent antigens and the low molecular weight, IgY, response to DNP-KLH (Tochinai and Katagiri, 1975). However *in vitro* proliferative responses to T-independent mitogens, for example *Escherichia coli*, LPS are not affected by early Tx (Donnelly, Manning and Cohen, 1976; Collie, Turner and Manning, 1975) and reactivity towards xenografts are relatively thymic independent (Horton *et al.*, 1992; Clothier *et al.*, 1989). Tx thus showed the existence of thymus (T) -dependent and -independent components of the immune system of *Xenopus*

(Manning, 1971). Similar effects of Tx have also been found in other amphibian species (Rollins-Smith and Cohen, 1982).

Immune responses abrogated by early Tx can be restored by grafting of allogeneic thymus or injection of dissociated thymocytes into Tx *Xenopus* (Katagiri and Tochinai, 1987; Du Pasquier and Horton, 1982; Rimmer and Horton, 1977).

A second major lymphoid tissue in all amphibians is the spleen. In the adult, this organ is mainly involved in trapping antigen (Ag) and housing proliferating lymphoid cells after stimulation by Ag. In the larva it is a major source of B lymphocytes (Hadji-Azimi *et al.*, 1990; Du Pasquier and Weiss, 1973). The mature spleen has regions of red and white pulp and is a convenient source of both T and B lymphocytes (maximum of about 4 x 10^7 lymphocytes in 300g adults) for experimental use. The splenocytes of *Xenopus* can be selectively stimulated to proliferate by classic T- and B-cell mitogens, induced to react specifically in a mixed leukocyte reaction (MLR) and are capable of differentiation into cytotoxic lymphocytes. Perhaps surprisingly splenectomy has no major effect on immune responses (Turner, 1973).

Other sources of lymphoid cells in *Xenopus* include liver, kidney, mesentery, gills and blood, also the ventral and dorsal cavity bodies in larvae and the adult intestinal lymphoid tissue.

1.3.2 Mitogen reactivity and cytokine production

As already mentioned *Xenopus* T and B lymphocytes have been shown to proliferate in response to classic T- and B-cell mitogens (Nagata, S., 1986) respectively. A mitogen is a substance which causes cells, particularly lymphocytes to undergo cell division, stimulating them to polyclonal proliferation and subsequent maturation. This contrasts with conventional antigens which stimulate specific clones of T or B cells, limited by antigen/MHC expression or antigen specificity, respectively, of their cell surface receptors. This means that usually only a fraction of 1% of all the available T or B cells are activated.

Soluble concanavalin A (ConA) and phytohaemmaglutinin (PHA) are plant polyvalent carbohydrate binding proteins (lectins). These specifically stimulate T lymphocytes to synthesise deoxyribonucleic acid (DNA), divide and differentiate into effector cells capable of either helping or suppressing a B or T lymphocyte response, or acting as killer cells in a cytotoxic reaction. A product of the cell wall of *E. coli*, lipopolysaccharide (LPS) and purified protein derivative of tuberculin (PPD) both stimulate B lymphocytes to synthesise DNA, proliferate and differentiate into plasma cells, capable of secreting large amounts of immunoglobulins (Igs). This polyclonal responsiveness is commonly measured by the incorporation of tritiated thymidine ([^{3}H]TdR) into newly synthesised DNA which is considered to represent an increase in the numbers of dividing cells, a hallmark of the immune response to specific antigens. Continued presence of polyclonal mitogens achieves growth and differentiation (from precursors to effectors) of the activated lymphocytes.

The proliferative response of *Xenopus* to PHA and ConA is apparent as soon as the spleen becomes lymphoid, stage 51 (Rollins-Smith, Parsons and Cohen, 1984). There is a slight drop in responsiveness at stages 57-59 and then an increase to maximal proliferation at about 3 months after metamorphosis. Larval thymocytes respond weakly to PHA and ConA, the thymic response to these mitogens largely emerging in post-metamorphic life (Williams *et al.*, 1983). This contrasts with mammalian development in which thymocytes become responsive to mitogens in foetal stages and mitogen responsiveness appears in the spleen around the time of birth (Robinson and Owen, 1976).

The existence of T and B cells in *Xenopus* was demonstrated by the fact that the traditional mammalian T and B cell mitogens also stimulate thymus dependent lymphocytes in *Xenopus* (Nagata, 1986). The cells stimulated by ConA and PHA are surface immunoglobulin (sIg) negative (Bleicher and Cohen, 1981). Mitogen reactivities of lymphocyte suspensions from the spleen, blood, liver, bone marrow and thymus reveal organ compartmentalisation for reactivities to thymus-dependent and independent antigens. The splenocytes and thymocytes have a greater response to the

thymus dependent mitogens whereas cells from the liver and blood respond equally well to both groups of mitogens (Green (Donnelly) and Cohen, 1979).

Splenocytes, but not thymocytes, from *Xenopus* stimulated *in vitro* with alloantigens or mitogens (PHA, ConA) produce supernatants with T cell growth factor like activity (Turner *et al.*, 1991; Watkins and Cohen, 1987; Gearing, 1985). Maximal activity is obtained after 24 hours of culture while at 72 hours supernatants are no longer stimulatory. Splenocytes from seven day thymectomised animals do not produce this T-cell growth factor (TCGF) (Turner *et al.*, 1991). More detailed studies of PHA splenic lymphocyte culture supernatants showed that they can effect growth of thymic or splenic T lymphoblasts; promote growth of alloreactive T cell lines; and costimulate, with a submitogenic dose of PHA, thymocytes (Watkins and Cohen, 1987, Watkins, Harding and Cohen, 1988). However, these supernatants could not effect growth of resting splenocytes. This frog TCGF has a molecular mass of 14-21kDa.

Additional studies demonstrated that PHA-generated supernatants promote proliferation of surface immunoglobulin negative cells from Tx *Xenopus* (Turner *et al.*, 1991). Thus it appears that T lymphocytes are involved in producing a number of cytokines with mitogenic activity. These cytokines are thought to include interleukin-1 (IL-1), -2, -4 and/or -5 like molecules.

1.3.3 Alloreactivity and the major histocompatibility complex

Allogeneic stimulation (stimulation due to intraspecies genetic variation) of T lymphocytes is most effective when foreign MHC antigens are found on the surface of the stimulator cells. Although the MHC was discovered in transplantation immunology it is now well established that in several species of mammals and birds products of the MHC play key roles in antigen presentation to T lymphocytes via the TCR, which recognises an antigen/MHC complex (see section 1.2.1). MHC molecules are receptors for small polypeptides. They are partly responsible, along with the TCR, for the properties we recognise as being characteristic of vertebrate

adaptive immunity, that is memory, specificity and self recognition. The role of MHC proteins is to present processed antigenic peptides to T lymphocytes. Binding of intracellular antigenic peptides by MHC molecules occurs within the endoplasmic reticulum prior to transport of the nascent MHC class I molecules to the cell surface. While, binding of extracellularly derived antigens by MHC class II proteins occurs within endosomes of antigen presenting cells (APC's), that house internalised antigens.

T cells are MHC restricted. This describes the necessity of an antigen-specific T cell receptor (TCR) to interact with processed immunogenetic polypeptide in association with an MHC encoded molecule of the self type. This interaction can lead to activation of the T cell. The generation of cytotoxic T lymphocytes and collaboration between T and B cells are all self MHC restricted. However many T cells can also recognise foreign MHC proteins, for example in graft rejection and in mixed leukocyte reactions. This is believed to be due to cross reactivity of the T cells concerned, that is foreign MHC appears to be able to mimic the antigen/self MHC complex for which the TCR is specific.

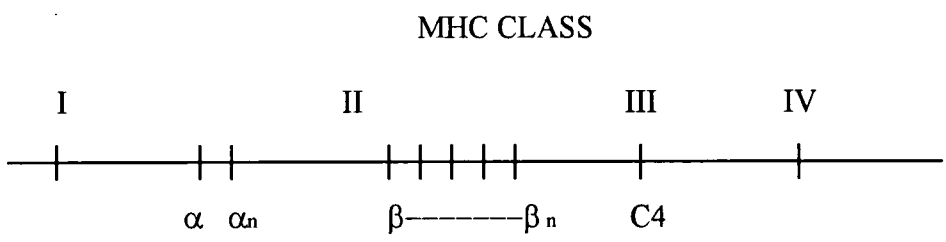
Lower down the phylogenetic scale, the South African toad, *Xenopus*, possesses the equivalent of an MHC. The existence of an MHC in *Xenopus* was first suggested in the 1970's (Du Pasquier, Chardonnens and Miggiano, 1975; Du Pasquier and Miggiano, 1973), from *in vitro* experiments involving the mixed lymphocyte reaction (MLR) and the generation of cytotoxic T lymphocytes (CTL), by *in vivo* skin grafting and by the use of antisera that recognise red cell antigens segregating with MLR determinants. The generation of CTL's (Bernard *et al.*, 1979), T-B collaboration (Bernard *et al.*, 1981) and the graft versus host (GVH) reaction (Nakamura, 1985) were all found to be genetically controlled by the MHC. In addition, the role of the MHC in the positive and negative selection of T cells by the thymus of *Xenopus* was also suggested from experiments with thymus reconstitution of thymectomised animals (Du Pasquier, and Horton, 1982) and thymus/haematopoietic cell embryonic chimeras (Flajnik, Du Pasquier and Cohen,

1985). In *Xenopus* the thymus, transplanted embryonically or into thymectomised larval hosts, can confer *in vivo* tolerance to MHC antigens of the thymus and to subsequent skin grafts. However this tolerance is not complete since T cells reactive to the MHC of the thymus can be detected by MLR in almost all situations (Arnall and Horton, 1987; Flajnik, Du Pasquier and Cohen, 1985; Nagata and Cohen, 1984).

Experiments using *Xenopus* chimeras indicate that thymus epithelium can effect suppression, rather than deletion, of developing T cells. Thus such chimeras will accept skin grafts of the head type, but their lymphocytes can still respond in MLC to head type stimulators. Transplanting of thymi, which had been depleted of their lymphocytes by γ-irradiation, into Tx hosts has enabled studies into the role of thymic stromal cells in T cell education (Russ and Horton, 1987). These have shown that the irradiated thymus can restore T cell dependent antibody production and alloreactivity to early Tx *Xenopus* and induce *in vivo* allotolerance towards thymic donor strain skin grafts (Arnall and Horton, 1986; Gearing, Cribbin and Horton, 1984).

The MHC of *Xenopus*, *Xenopus* lymphocyte antigen (XLA), has now been documented both biochemically and functionally (Flajnik and Du Pasquier, 1990). The MHC of *Xenopus* is expressed as a single genetic region (figure 1.2)

Figure 1.2 Diagrammatic representation of the *Xenopus* major histocompatibility complex (Roitt, Brostoff and Male, 1993).



Class I, class II and class III molecules have all been described in *Xenopus*. *Xenopus* is the only ectothermic species where both polymorphic class I and class II MHC gene products have been formally documented (Flajnik and Du Pasquier, 1990; Flajnik *et al.*, 1986; Kaufman *et al.*, 1985; Kaufman, Flajnik and Du Pasquier, 1985; Flajnik *et al.*, 1984). Evidence suggests that there are also minor histocompatible (H) loci in *Xenopus*, but that these exhibit limited polymorphism (Obara, Kawahara and Katagiri, 1983).

The *Xenopus* class II molecules look much like their mammalian counterparts and the α and β chains can be cross-linked. Class II molecules are composed of two 30-35 kDa integral membrane glycoproteins. Each XLA haplotype seems to carry 2α and up to 5β genes (figure 1.2). Tadpoles and adults express identical class II molecules but with different tissue distribution. Studies with anti-*Xenopus* class II mAbs (Du Pasquier and Flajnik, 1990; Flajnik, 1990) have shown that a subpopulation of splenic leukocytes in larvae (including B cells) are positive for class II, but larval T cells do not express class II. In the adult both B and T cells express class II constitutively. This contrasts with the mammalian distribution were only B cells express class II. Class II positive Langerhans-like cells are found in the epidermis of *Xenopus* skin.

Xenopus class I MHC also appears to be very similar to mammalian class I. There appears to be only one class I locus, there being no evidence for the K, D or L antigens found in the mouse. The class I α chain has a molecular weight ranging from 40-44 kDa (depending on the allele examined), while the light chain (the homologue of β_2-microglobulin), has a molecular weight of 13 kDa. Family studies confirmed that the heavy chains are encoded by XLA genes. Class I molecules are found on the cell surface of all tissues of the adult, with the highest expression on haematopoietic cells including erythrocytes. Tadpoles do not express class I molecules at the cell surface - they only appear at metamorphosis, which suggests that class I is not essential for function of the larval immune system. There does, however, appear to be cytoplasmic expression of α chains in several organs of the tadpole tissues, with

highest expression in the thymus (Flajnik and Du Pasquier, 1990). The reason for this lack of expression of class I during embryonic life may be that tadpoles commit their adaptive immune system to humoral immunity at the expense of the class I-restricted cell mediated immunity. That is to say, because tadpoles possess few lymphocytes, selection of a class I-restricted population is precluded and the T cells are then dedicated to antigen recognition only in the context of class II.

1.3.3.1 *In vitro* assays to study alloreactivity

The immune response to MHC incompatibility in *Xenopus* has been studied *in vitro* by the MLR and cell mediated lympholysis (CML) and *in vivo* by graft rejection (Horton, Horton and Varley, 1989; Lallone and Horton, 1985; Bernard *et al.*, 1979). As in mammals these assays indicate that allogeneic MHC molecules stimulate a large proportion of T cells (Bernard *et al.*, 1979; Du Pasquier and Horton, 1976; Tochinai and Katagiri, 1975).

MLR is a model which represents the recognition phase of a response to an allogeneic stimulus. While a one-way MLR (in which the stimulating cells are inactivated, for example, by irradiation), provides a model for the study of the behaviour of cells which recognise and respond to the antigenic stimulation only.

The standard conditions for MLC with *Xenopus* cells were initially developed in 1973 (Weiss and Du Pasquier, 1973), which led onto much wider use of the MLC in genetic and ontogenic studies. It was early studies using this technique that indicated that the characteristics described for the genetic control of MLR for mammals are also true for *Xenopus* (Du Pasquier and Miggiano, 1973). That is, one genetic region, a high degree of polymorphism and haplotype effect (a difference of 2 haplotypes resulting in a higher stimulation than a difference of only 1 haplotype). Therefore these studies were first to suggest that an MHC exists in *Xenopus*.

Generation of T cell lines specific for certain MHC haplotypes (Watkins, Harding and Cohen, 1988) and use of mAbs against class I and class II molecules, have shown that the MLR is class II specific (Harding, Flajnik and Cohen, In press).

Cell mediated lympholysis (CML) in adult *Xenopus* has been demonstrated against both adult (Lallone and Horton, 1985; Bernard *et al.* 1979) and larval (Horton, Horton and Varley, 1989) MHC disparate targets and also against minor H antigens (Cohen, 1971), by application of several minor histoincompatible skin grafts to adult *Xenopus* over an 18 month period (Horton, Horton and Varley, 1989).

The cytotoxic activity of MLR-restimulated lymphocytes is mediated by T cells (Bernard *et al.*, 1979), as shown by the fact that removal of B lymphocytes, by passage of effector spleen cells through a nylon wool column, improves killing on a per cell basis. It therefore appears that the genes responsible for the highly specialised function of T killer cells emerged early in evolution at least at the time of the emergence of the amphibians ($\cong$ 300 million years ago) and that they were already linked to the MHC of this species. The responding cytotoxic T-cells specifically recognise MHC-linked target molecules on PHA induced lymphoblasts (Bernard *et al.*, 1979) as no lysis is observed when the target cells differ from the specific stimulators by 2 MHC haplotypes.

Tadpole cells can be used as target lymphoblasts to be lysed in CML, this suggests that class II restriction of killer cells exists in *Xenopus*, as larval cells are believed only to express class II (see above). The possibility that higher specific chromium-51 (^{51}Cr) release occurs when adult rather than larval targets are used (Horton, Horton and Varley, 1989) may indicate that anti-class I cytotoxicity is also important in the adult.

A study carried out to compare the *in vivo* and *in vitro* assays to determine which most accurately reflects MHC incompatibility and T lymphocyte function (Lallone and Horton, 1985) showed MLR but not CML to be a good *in vitro* correlate of the *in vivo* skin graft rejection. Cytotoxic cells could not be convincingly demonstrated against minor histocompatibility antigen disparate targets.

1.3.4 Transplantation immunology

A large volume of research has been carried out to study the response to self and foreign grafts in *Xenopus*. This work, as well as numerous other *Xenopus* studies, has been greatly helped by the development of gynogenetic clones of *Xenopus* (Kobel and Du Pasquier, 1975).

Production of gynogenetic clones is a very useful method for obtaining large numbers of identical individuals of *Xenopus*. When *X. gilli* or *X. mülleri* are crossed with *X. laevis* individuals some of the hybrid females lay two types of eggs, one almost twice as large as the other. The larger eggs are diploid which arises due to endoreduplication during meiosis (see figure 1.3). After fertilisation, with normal sperm, the larger eggs give rise to triploid individuals, whereas most of the embryos of the smaller eggs die during development. The diploid eggs have one complete chromosome set from each parental genome therefore such eggs have a genotype identical to that of the hybrid mother, as they carry both the Z and W chromosomes. In *Xenopus* the female is heterogametic, ZW and the male homogametic ZZ (Chang and Witschi, 1956). Gynogenetic (development of the ovum without fertilisation) development of the diploid eggs thus results in isogeneic offspring. The use of ultra-violet (UV) irradiated sperm ensures gynogenetic development of the eggs, without the participation of the spermatozoan nucleus.

Gynogenetic development can be confirmed by use of four tests. First by ploidy: all tadpoles developing from diploid eggs activated by irradiated sperm will be diploid. Counts of nucleoli in epidermal cells of a tadpole show if the egg was diploid (1.6-1.9 nucleoli/cell) or triploid (2.5-2.9 nucleoli/cell) (Kobel and Du Pasquier, 1975). Secondly the sex of the offspring: all large eggs develop into females. Thirdly by the MLR: cells from gynogenetic siblings give a negative MLR, whereas MLR between triploid individuals would give positive activation. Fourthly skin graft reactions: skin grafts exchanged between gynogenetic siblings survive for greater than 100 days with no signs of rejection (Obara, Kawahara and Katagiri, 1983) whereas grafts exchanged between triploid siblings will acutely reject the graft.

Different families of *X. laevis* x *X. gilli* cross (LG) clones exists whose MHC haplotypes have been characterised (Bernard *et al.*, 1979). These clones are extremely useful in studies of the amphibian immune system. Antibody diversity and heterogeneity can be studied in identical individuals. Experiments involving cell transfers can be performed. Also investigations into the nature of the generation of cell tolerance at metamorphosis, can be performed by studying the interactions of populations of larval and adult cells exhibiting no other differences than those due to age.

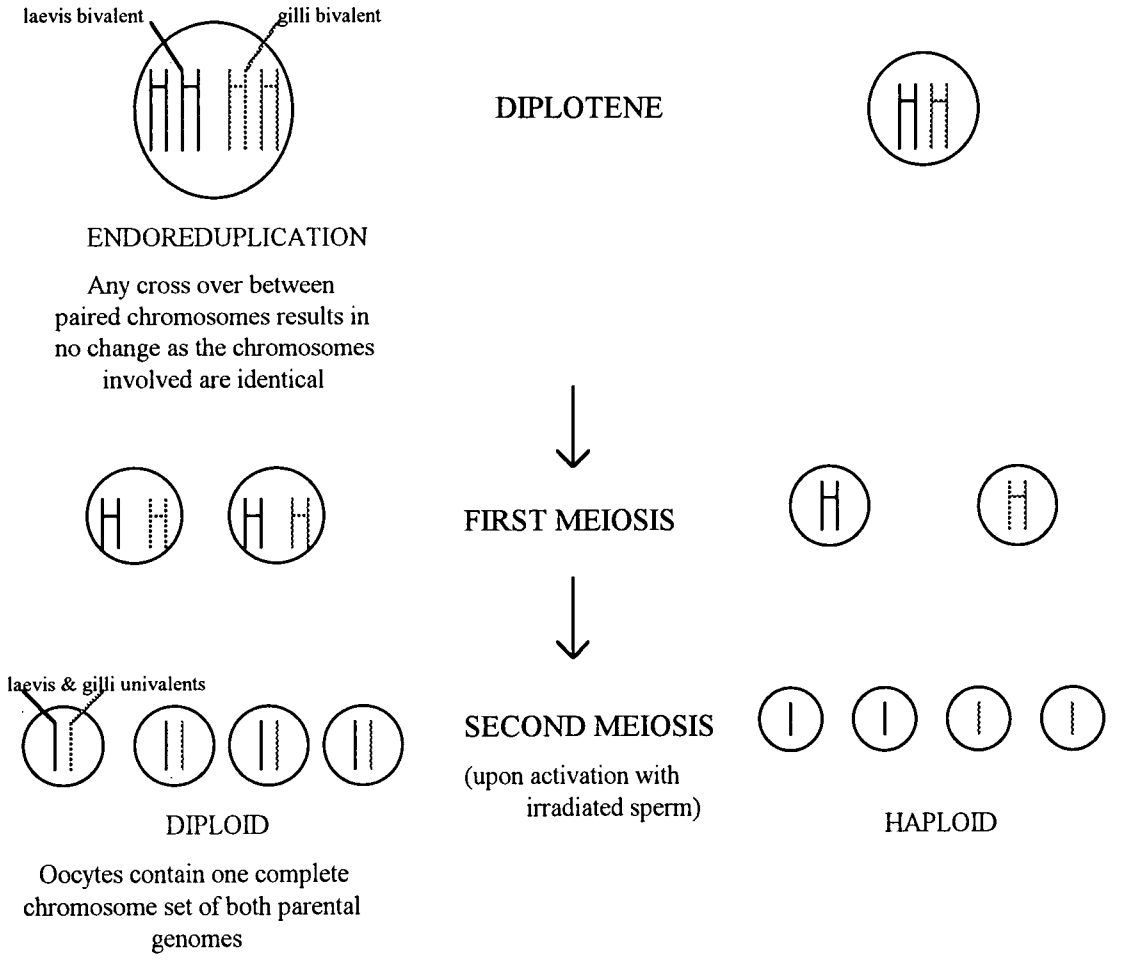
The response to grafts has been studied in depth in adult *Xenopus* (Horton *et al.*, 1992; Horton, Horton and Varley, 1989; Lallone and Horton, 1985; Obara, Kawahara and Katagiri, 1983). *Xenopus* will tolerate isografts and autografts, but reject allografts and xenografts within 3·5 weeks at 23°C (Nakamura *et al.*, 1987). Grafts with just minor histocompatibility differences are rejected in 5-8 weeks in adults (Obara, Kawahara and Katagiri, 1983; DiMarzo and Cohen, 1982a; DiMarzo and Cohen, 1982b).

Following application of a foreign skin graft the main sequence of events during rejection are: vascularisation of the graft; haemostasis (the graft becomes red in appearance, due to breakdown of blood vessels); loss of graft pigment cells; finally complete pigment cell breakdown - this is usually taken as the endpoint of rejection. Incompatible grafts are invaded by lymphocytes within one week, which forms the predominant part of the host cellular invasion. Two to three weeks postgrafting necrosis of the epidermis occurs, the graft being heavily invaded by lymphocytes, eosinophils and fibroblasts.

T cells are intimately involved in allograft rejection, as has been shown by early Tx, which results in failure of or, at best, chronic graft rejection (Kaye and Tompkins, 1983; Du Pasquier and Horton, 1976; Horton and Manning, 1972). The rejection of allografts in *Xenopus* is, at the cellular level, different to that of xenografts. T cells predominate within allografts whereas B lineage cells, macrophages and polymorphonuclear leukocytes are responsible for the destruction of

xenografts (Horton *et al.*, 1992; Clothier *et al.*, 1989). This agrees with observations that show that early Tx does not impair xenograft rejection in *Xenopus*. This may be due to the xenogeneic MHC antigens being 'too different' from the self MHC antigens to be recognised as altered self by the cytotoxic T cells. Xenoantigens would be presented by host antigen presenting cells, thereby leading preferentially to T-helper cell activation and eventually to an antibody response.

Figure 1.3 Formation of haploid (small) and diploid (large) eggs during meiosis in hybrid *Xenopus*.



1.3.4.1 Larval transplantation immunology and tolerance

An interesting time to study the response to allografts in *Xenopus* is at metamorphosis. This is a time when *Xenopus* must become tolerant to new self antigens, for example class I antigens and adult forms of keratin and haemoglobin. Profound immunological changes occur as tadpoles metamorphose into adult amphibians. These include the expression of a different antibody repertoire, a lessening of skin graft tolerance and the appearance of class I MHC antigens on leukocytes. Larvae of amphibians are immunologically mature, being capable of acutely rejecting MHC-disparate allografts in 15-30 days (Horton, 1969). However many cases of tolerance have been observed, in fact minor H antigen disparate grafts are routinely tolerated by larvae (DiMarzo and Cohen, 1982a). It is thought that the tolerance to allografts at metamorphosis reveals a dampening of the immune response when tolerance to newly arising adult-specific self antigens must occur. Tadpole anti-adult MLR between lymphocytes of an isogeneic clone has been observed (Du Pasquier, Schwager and Flajnik, 1989; Kaye, Schermer and Tompkins, 1983) which may relate to the tadpole recognising adult determinants to which it is not yet tolerant.

In the larva the ultimate fate of MHC disparate grafts, tolerance or rejection, has been shown to depend on a number of factors. In 1969 it was noted that a significant number of grafts transplanted among siblings survived longer when they were transplanted during metamorphosis than when they were grafted after metamorphosis. This was shown to be a property of the host and not the graft. Chardonnens and Du Pasquier grafted larval and post-metamorphic siblings with skin from post-metamorphic members of the same sibship. Their analyses showed that for a period around metamorphosis *Xenopus* is predisposed to becoming tolerant (Chardonnens and Du Pasquier, 1973). They also proposed that perimetamorphic tolerance was preferentially induced to minor histocompatibility antigens (Chardonnens and Du Pasquier, 1975). In 1985 Cohen and colleagues carried out further experiments to study allo- and self-tolerance in larval *Xenopus* (Cohen *et al.*, 1985). Family studies showed that a significant number of perimetamorphic recipients

(10-84% depending on the particular MHC haplotype combination), of relatively large skin grafts, $4mm^2$, from a parent enjoyed prolonged survival, compared to grafts from unrelated individuals. These studies revealed that perimetamorphic tolerance can be induced to MHC antigens and that the frequency of tolerance to MHC antigens depends on the particular MHC haplotype by which the donor and host differ. These experiments also showed that survival times of MHC disparate grafts on metamorphosing frogs are similar to those associated with MHC identical but minor H locus disparate skin grafts on adult *Xenopus*. Although tolerance to grafts from unrelated individuals was shown to be significantly less easy to induce than when parental skin was used, the mean survival times of unrelated grafts were still longer than those seen in postmetamorphic recipients. Therefore partial tolerance can be induced to alloantigens encoded for by two as well as by one MHC haplotype disparate skin grafts.

Cohen *et al.* extended the above findings to studies with MHC-defined strains, looking at three combinations, MHC identical but minor H locus disparate grafts, one MHC- and two MHC-haplotype disparate grafts. The first combination showed tolerance to the minor H antigens in every instance (DiMarzo and Cohen, 1982a). Tolerance was also seen in the second combination, this tolerance being more frequent when the donor graft was larger, 89% compared to 10% for smaller grafts. As expected from the family studies, tolerance to a two MHC haplotype disparity was significantly less frequent, however it was still present.

Although all the above work was carried out on perimetamorphic individuals, this period is not unique with respect to tolerance induction (Cohen *et al.*, 1985; DiMarzo and Cohen, 1982a; DiMarzo and Cohen, 1982b). The ultimate outcome of a graft, tolerance or rejection, placed on a larva at any developmental stage depends on all the above parameters that affect allotolerance expression at the perimetamorphic period.

Frogs which have tolerated a skin graft during premetamorphic or perimetamorphic life, have been shown to accept repeat skin grafts from the original

donor (but not from third party donors), when transplanted two and a half years afterwards whereas such grafts applied to control siblings, not 'tolerised' as tadpoles, are rejected (DiMarzo and Cohen, 1982a; DiMarzo and Cohen, 1982b; Barlow and Cohen, 1981). Moreover frogs that have received and rejected a graft during larval life have been found to reject a second-set graft with accelerated kinetics, showing that memory can develop in frogs. Third party grafts are always rejected (Flajnik *et al.*, 1987).

Skin grafts not rejected by tadpoles are not simply undetected by the immune system. Allografts are invaded by lymphocytes whether they are tolerated or rejected whilst lymphocytes are absent from autografts. This implies that despite tolerance allografts are always recognised as foreign (Barlow and Cohen, 1983).

There are several lines of evidence from *in vivo* and *in vitro* experiments to suggest that allotolerance, effected by larval grafting, is not due to the absence of alloreactive T cell clones, in the tolerant animal. When lymphocytes from tolerant animals are placed in mixed leukocyte cultures, with irradiated donor cells, an MLR is often seen (Cohen *et al.*, 1985; Barlow and Cohen, 1981). This split tolerance has also been observed in mammals.

Cell transfer (multiple injections of thymocytes and splenocytes into an isogeneic adult from a metamorphosing animal) studies have shown that the higher frequency of chronic responses or tolerance to skin allografts in larval and metamorphosing animals may relate to suppression of the immune responses to histocompatibility antigens (Nakamura *et al.*, 1987; Barlow and Cohen, 1983; DiMarzo and Cohen, 1982a; DiMarzo and Cohen, 1982b; Du Pasquier and Bernard, 1980). Suppressor function in *Xenopus* is sensitive to cyclophosphamide (Kamali, Ruben and Gregg, 1986).

Late larval thymectomy has also revealed that tolerance to MHC antigens induced during the perimetamorphic period is a thymus dependent event (Barlow and Cohen, 1983). When larval siblings were thymectomised one day before being grafted the frequency of hosts that rejected grafts increased and mean survival times

decreased. However some degree of tolerance was still seen indicating that there are also thymus independent suppressor systems.

It has been noted that there is a high corticosteroid plasma level present during *Xenopus* metamorphosis. Corticosteroids are known to inhibit suppressor activity therefore this suggests that suppressor function is not responsible for the allotolerance seen at metamorphosis (Ruben *et al.*, 1989). Corticosteriods are also known to prevent proliferation of T cells by PHA. It is possible therefore that the compromised T cell function seen at metamorphosis may be due to a limited capacity for T cell clonal expansion in immune responses.

1.4 Purpose of Study

The main aim of this thesis was to probe aspects of transplantation immunity in *Xenopus*.

The availability of monoclonal antibodies directed against *Xenopus* T cells and MHC antigens, which have recently been produced allow one to probe the *Xenopus* immune system in some considerable depth. Monoclonal antibodies (mAb's) directed against all *Xenopus* T cells, putative αβ and γδ TCR's, the CD8 receptor, MHC antigens and putative IL-2 receptors were utilised here to study the effect allogeneic stimuli have on the splenocyte T cell populations of *Xenopus*. To my knowledge little work has been carried out regarding this issue. It is known that responding cells within the allograft consist largely of T-lymphocytes (Horton *et al.*, 1992) and that the spleen is a centre of alloimmune reactivity in *Xenopus* (Horton, Horton and Rimmer, 1977). In particular mAb's were used here to give insight into the possible role in graft rejection of γδ TCR positive T cells within *Xenopus*. Little is known about this subpopulation of T cells in *Xenopus* - where they originate and their function. Evidence from nude mice (Bell, 1989) shows that cells expressing γδ chains can develop independently of the thymus; moreover γδ TCR bearing T cells may be involved in cytotoxic responses against alloantigens (Haas, Kaufman and Martinez-A,

1990). It was therefore of interest to probe whether putative γδ positive T cells are involved in allograft rejection in *Xenopus*, especially as following early Tx, this species can often chronically reject allogeneic transplants (Horton and Manning, 1972).

In initial experiments in Chapter 2 flow cytometry was used with the anti-*Xenopus* monoclonal antibodies, to identify possible cell surface antigen changes on splenocytes during *in vivo* allograft rejection, with an emphasis on the level of expression of *Xenopus* αβ and γδ TCR bearing cells. Flow cytometric studies were also carried out on spleens from *Xenopus* rendered tolerant to MHC-disparate skin, following skin transplantation in larval life. These animals allowed me to probe whether cellular changes occurring during rejection also occurred in animals where test grafts were being tolerated. Early-thymectomised *Xenopus* were also grafted to observe whether alloimmunisation affects the splenic profile as detected by the mAb's.

In Chapter 3 various parameters of the mixed lymphocyte reaction (MLR) were explored. Initial experiments addressed the issue of whether *in vitro* reactivity would be significantly elevated by the use of T cell mitogen-activated stimulator cells. The eventual goal of this work (had time permitted!) was to avoid the necessity (Du Pasquier, Schwager and Flajnik, 1989; Bernard *et al.*, 1979) of having to immunise animals *in vivo* (by skin allograft rejection), prior to restimulation of lymphocytes in MLR, in order to generate cytotoxic T lymphocyte effectors *in vitro*. The avoidance of having to carry out an *in vivo* grafting step would perhaps aid studies probing whether cytotoxic T cell responses can be generated in larvae, which are class I MHC deficient. It is known that mitogenic stimulation of human lymphocytes results in an increased expression of class II MHC molecules (Roitt, Brostoff and Male, 1993), therefore as *Xenopus* T cells also express class II molecules, it was predicted that the ConA stimulated *Xenopus* splenocytes would also have increased MHC class II expression. Furthermore a recent report by Harding *et al.* (Harding, Flajnik and Cohen, In press) has suggested that stimulating *Xenopus* lymphocytes with an alloantigen or a mitogen does result in increased expression of class II MHC antigen.

The use of ConA activated stimulators could result in improved alloantigen presentation by these cells, since Harding *et al.* revealed that class II is the effective stimulus of *in vitro* MLR.

Work in chapter 3 also characterised, by flow cytometry, the phenotypes of T cells from the spleen of *Xenopus* after alloantigenic and T cell mitogen stimulation *in vitro*.

In chapter 4 a preliminary investigation into the effects of the superantigen staphylococcal enterotoxin B (SEB), on stimulation of *Xenopus* lymphocytes, was also carried out. Since there are no published reports on whether *Xenopus* lymphocytes respond to such superantigens. In mammals superantigens are known to stimulate all T cells with certain Vβ epitopes (Marrack and Kappler, 1990; White *et al.*, J., 1989; Pullen, Marrack and Kappler, 1988). Although not directly related to the main theme of the work presented in this thesis, these novel studies are reported here.

Conclusions are outlined in chapter 5, which also considers future directions this work may take.

CHAPTER 2

FLOW CYTOMETRIC STUDIES FOLLOWING SKIN ALLOGRAFTING: COMPARISON OF T CELL MARKER ANTIGEN EXPRESSION IN CONTROL, EARLY-THYMECTOMISED AND SKIN ALLOTOLERANT *XENOPUS*

2.1 INTRODUCTION

The nature of T lymphocyte changes in the spleen of *Xenopus*, when presented with an allogeneic stimulus, has been investigated.

The major histocompatibility complex (MHC) of *Xenopus* is responsible for acute skin graft rejection and generation of cytotoxic responses to allogeneic cells (Bernard *et al.*, 1979). Adult *Xenopus* will accept grafts from MHC identical clones but reject MHC-disparate and xenogeneic grafts in 24 days. Minor histocompatibility antigen disparate grafts are rejected much more slowly. Thymectomy (Tx) results in prolonged survival of allografts (Horton and Manning, 1972) which shows that allograft rejection is a thymus dependent event. However, Tx animals can still reject xenografts. Allografts and xenografts are rejected by two distinct mechanisms. Allograft rejection involves extensive lymphocytic infiltration of the graft tissues, while macrophages and polymorphonuclear leukocytes appear to be responsible for the destruction of xenografts (Horton *et al.*, 1992; Clothier *et al.*, 1989).

The main way in which the immune response to MHC incompatibility, *in vivo*, has been studied in *Xenopus*, is by grafting specific organs, primarily skin. Following application of a foreign skin graft the main events in rejection are: vascularisation of the graft; normal blood flow in the graft; haemostasis; the graft becomes red in appearance, due to breakdown of blood vessels; and loss of graft pigment cells, with complete pigment cell breakdown usually being taken as the endpoint of rejection. During rejection of an allograft, many elements of the graft are

taken up by macrophages and transported via the blood to the liver and spleen (Du Pasquier, 1973), which may result in modification of the proportions of various leukocytes within these organs. Therefore the effect that skin allografting has on the splenocyte populations, within grafted animals, has been explored here with the use of several newly created monoclonal antibodies (mAb's). The availability of mouse mAb's raised against *Xenopus* T cells and MHC antigens provide a very useful method to probe aspects of T cell development and use within *Xenopus*. Particular emphasis is placed here on the expression of the receptors, in grafted animals, to which the mouse mAb's D12.2 and D4.3 react. These mAb's react with discrete subpopulations of immunoglobulin-negative lymphocytes. In this laboratory D12.2 usually stains between 5 and 10% of splenocytes, while D4.3 stains ≥ 20% thymocytes and approximately 55% splenocytes (which represents most of the 60% T cells found in the adult). D4.3 and D12.2 mAb's are thought to recognise the *Xenopus* TCR αβ and γδ homologues, respectively (Ibrahim *et al.*, 1991). F17, a putative *Xenopus* anti-CD8 receptor mAb, is also used in these studies, as is the pan-T cell marker 2B1. This chapter also probes whether T cell phenotype changes occur in spleens of skin allografted Tx animals.

Events following application of skin to specific allotolerant animals, have also been followed. Although larvae and metamorphosing *Xenopus* are capable of acutely rejecting allogeneic skin grafts (Obara, Kawahara and Katagiri, 1983) they do not always reject grafts, in fact very often there is tolerance of MHC-incompatible skin (Cohen *et al.*, 1985; Chardonnens, and Du Pasquier, 1973). The ultimate fate of MHC disparate grafts (tolerance or rejection) in larvae depends on size of the graft, degree of alloantigen disparity between donor and host and developmental stage of the recipient at the time of grafting (Cohen *et al.*, 1985); tolerance particularly occurs to minor H antigens. Tolerance is immunologically specific to the host and is thought to arise as a consequence of the fact that larvae have to become tolerant to new self antigens at metamorphosis. It appears that tolerance is due mostly to anergy or suppression of reactive T lymphocytes (Cohen *et al.*, 1985).

2.2 MATERIALS AND METHODS

2.2.1 Animals

Animals were bred and reared in the laboratory under standard conditions (Nieuwkoop and Faber, 1967). They were either outbred *Xenopus laevis* or gynogenetic clones (LG or LM hybrids (Kobel and Du Pasquier, 1975)), from 3-4 week old larvae up to 12 month old adults. The animals, and their ages, used in each experiment can be found in the Results section.

The animals were reared at 23 ± 3°C, in dechlorinated tap water. Larvae were fed nettle powder twice weekly and after metamorphosis the froglets were fed *Tubifex* worms and ground pig's heart.

2.2.2 Early Thymectomy

Animals were thymectomised (Tx) by microcautery at 5-6 days as previously described (Horton and Manning, 1972). The absence of a thymus was routinely checked in larval life by stereomicroscopic observations and by dissection of adults on killing.

2.2.3 Skin Grafting

The donor animal was heavily anaesthetised in a solution of 3-aminobenzoic acid ethyl ester and the appropriate number of squares, $3mm^2$, of dorsal or ventral skin removed for grafting. Care was taken not to bruise the skin with the forceps as some areas of the graft could then show signs of non-specific damage.

Allo- or isogeneic skin was then transplanted to the graft bed, prepared by removing an appropriate piece of dorsal skin from an anaesthetised adult. Autografts were also performed, here a $3mm^2$ square was cut in the dorsal skin of an anaesthetised adult; one side of the graft was not cut through so as to help retention of the autograft (see Plate 2.1). After application of the graft the adult hosts were initially kept in a sufficient volume of water to keep just their ventral side wet for 8-10

hours and then placed in a normal volume of water (approximately 5 litres (l) in a 9l tank) by which time the grafts had usually adhered to the host skin.

Larvae (stage 54) received ventral skin allografts from adult donors, to enable long term monitoring. A 1.5mm^2 graft was placed in a prepared graft bed just posterior to the eye. Grafted larvae were kept with their ventral surfaces moist for 1-2 hours, prior to returning to aquaria (8l dechlorinated water).

All animals were kept at 23°C and the grafts observed stereo-microscopically 3 days a week for up to 6 weeks post-grafting, the state of the grafts being noted and photographs taken. Rejection was considered complete when all signs of guanophores (pigment cells) had been lost.

2.2.4 Indirect-Immunofluorescent Staining and Flow Cytometry

Immunofluorescent staining of the froglets splenocytes was carried out at various times post-grafting, to observe change in cell surface markers of the splenocyte population.

The animals were heavily anaesthetised in 3-aminobenzoic acid ethyl ester, the spleen removed and placed in amphibian phosphate buffered saline (APBS) (see Appendix A) containing 0.1% sodium azide and 0.1% bovine serum albumin (BSA), as a source of irrelevant protein. A splenocyte suspension was then prepared by teasing the spleen with fine needles. The cells were washed in APBS, counted and adjusted to 1x10^6 cells/ml. The cell suspension was then divided into 200µl aliquots (2x10^5 cells for staining) in Falcon 5ml tubes, centrifuged at 300g, at 4°C for 10 minutes, the supernatant removed and the cells incubated on ice for 20 minutes with 50µl of the appropriate monoclonal antibody (mAb - see Table 2.1). These stained cells were pelleted and washed twice in 2ml APBS. The cells were then incubated, on ice for 30 minutes in the dark, with 50µl of a 1:20 suspension of a flourescein isothiocyanate (FITC)-conjugated secondary rabbit anti-mouse immunoglobulin (Ig) (Fab-fragment). The FITC-conjugated secondary antibody had been preabsorbed with a 1:20 dilution of *Xenopus* serum, to reduce non-specific staining. For single

staining, that is FITC only, the cells were then pelleted, washed twice in 2ml APBS and finally resuspended in 400µl APBS. For dual staining, after incubation with the FITC-conjugated secondary antibody, the cells were washed twice in 2ml APBS containing mouse serum (1:100 dilution). The mouse serum was required to absorb any of the FITC-labelled anti-mouse antibody not bound to the primary mAb's. Cells were then stained with 50µl of a phycoerythrin (PE)-conjugated mAb (see Table 2.1) for 20 minutes, on ice. Cells were finally washed twice in APBS and resuspended in 400µl APBS.

Cells (10,000 per sample) were analysed on either a Coulter Epics flow cytometer or a Becton Dickinson FACS Scan. During analysis the cells were gated to exclude erythrocytes and dead leukocytes (see Appendix B), from the statistical analysis. Any counts that could be due to non-specific binding of the mAb's were eliminated by staining 1 aliquot of the cells with either the FITC control - a mouse anti-chicken thymocyte mAb (CT3), or the PE control - a non-specific mouse IgG1 PE-conjugated mAb. These control samples were run through the flow cytometer and a gate set so that 97-98% of the CT3 or Ig-PE binding was not included when counting.

2.2.5 Activation of Splenic T Cells with Concanavalin A

Spleens were removed from intact and Tx *X. laevis* at 8, 21 and 27 days post-grafting to monitor the response of their T lymphocytes to concanavalin A (ConA). The spleen was dissociated by crushing between the frosted ends of 2 sterile glass slides and washed in amphibian-strength Leibowitz-15 (L-15 - Flow Labs) amphibian culture media, supplemented with 1% foetal calf serum (FCS - Gibco), 0.01 M HEPES buffer, 50 IU/ml penicillin, 50µg/ml streptomycin, 2.5µg/ml amphotericin B, 1.25 mM L-glutamine, 18µl/ml sodium bicarbonate (all from Flow Labs) and 0.083mM mercaptoethanol (BDH). The number of viable lymphocytes was counted using a haemacytometer, by trypan blue exclusion: cells were diluted 1:1 in trypan blue, viable cells excluded trypan blue whereas dead cells took up the dye.

The lymphocytes were then adjusted to $1x10^6$ cells/ml and plated out in V-bottomed 96 well tissue culture plates at $1x10^5$ cells/well in L-15 amphibian medium either with or without 2.5μg/ml ConA (see Appendix C). The cells were cultured at 26°C in a humidified atmosphere of 5% carbon dioxide (CO_2) in air, for 48 hours, then 1 microCurie (μCi) tritiated thymidine ([^{3}H]TdR), specific activity 5 Ci/mmol, was added per well and the cells incubated for a further 24 hours. The cells were then harvested using a Skatron cell harvester onto 0.2μm Whatman filters, the filters dried and placed into 5ml scintillation vials. Four ml of scintillation cocktail (Betaflour, National Diagnostics) was added and the disintegrations per minute (dpm) calculated on a Packard scintillation counter.

2.3 RESULTS

2.3.1 Comparison of the Response to Skin Allografts in Three Experimental Groups

2.3.1.1 Control animals

The external appearance following the typical stages of rejection of primary LG15 grafts on an adult *X. laevis* (thymus intact) are shown in Plate 2.1. The response to allografts seen on all the intact hosts was typical of allogeneic rejection seen by others, with vascularisation of the graft, followed by haemostasis and then pigment cell breakdown.

Autografts were always permanently tolerated. As can be seen from Table 2.2, intact *X. laevis*, LG15 and LG3 of 3 months and older rejected allogeneic primary grafts from 16-22 days. Secondary grafts, carried out 1-2 months following primary skin graft rejection, were generally rejected in an accelerated manner. LG3 and LG5 *Xenopus* clones have the *b* MHC haplotype in common. This may explain the accelerated rejection of an LG5 skin graft applied to the LG15 animal which had previously received an LG3 skin graft (Table 2.2).

2.3.1.2 Thymectomised animals

Plate 2.2 shows the appearance of a healthy tolerated LG15 skin graft 21 days post-grafting onto a 5 month old 5-day-Tx animal.

Table 2.3 shows typical results of the outcome of allografting 5 and 6 day Tx *X. laevis* and LG3 animals. On animals of up to 5 months of age allografts showed no signs of rejection and were tolerated for at least 100 days. However the results reveal that four 6 month old Tx *X. laevis* acutely rejected LG15 skin grafts in 21 ± 4 days. This was an unexpected result as Tx animals of this age usually chronically reject allografts in greater than 60 days, if at all (data from this laboratory).

2.3.1.3 Tolerant animals

The LG3 animals which initially received semi- and fully-MHC disparate, primary skin grafts when 3 week old larvae (stage 54) achieved vascularisation of the grafts and permanently tolerated them (Table 2.4). Only one transplant, from an LM3 donor, was chronically rejected in 33 days. These animals had all passed through metamorphosis by 28 days post grafting. They were specifically tolerant only to the haplotype of the primary graft, as shown by regrafting at 7 and 9 months old (Table 2.4). Thus LG3 animals which had tolerated LG5 or LM3 adult skin grafts tolerated subsequent skin grafts of the same origin whereas LG3, tolerised to LG5, subsequently acutely rejected LM3 skin grafts.

2.3.2 Flow Cytometry of mAb-Stained Splenocytes from Grafted Animals

Flow cytometry was carried out on intact and Tx *X. laevis* animals grafted with LG15 skin and also on LG3 animals, including those rendered tolerant by larval grafting.

2.3.2.1 Control *Xenopus laevis*

On harvesting the spleen for staining the number of lymphocytes was counted. This often revealed an increase in the number of cells from intact animals following allografting compared to control and autografted animals.

Flow cytometry revealed no obvious increase in the size of the *X. laevis* lymphocytes following application of primary LG15 skin grafts. This can be seen from the dot plots, which show the size of the cells plotted against the granularity (see Appendix B). However there was a slight increase in the mean fluorescence of the 2B1 and D12.2 positive cells between the control and allografted animals, for example at 14 days post-grafting, see Figure 2.1. The mean fluorescence reflects the number of markers per cell, so this suggests that following allogeneic challenge there is an increase in expression of these 2 cell surface receptors.

Slight variations were seen in the percentages of T cell markers in control and grafted intact *X. laevis*. There was a notable increase in the number of D12.2 positive cells (see Figure 2.1 and Tables 2.5-2.7). However, no definite differences in the level of staining of any of the other mAb's could be concluded. Table 2.5 compares T cell surface antigen expression 14 days post-autografting/allografting to 5 month old *Xenopus*, when no dramatic differences in markers was evident, except for the 14% D12.2, noted in one of the allografted animals. Table 2.6 probes T cell markers, using dual colour fluorescence, at 24 days post-grafting. An increased level of D12.2 positive cells is again suggested, also these mostly co-express 2B1, F17 and, surprisingly, D4.3 (see Figure 2.2). Table 2.7A probes the splenic phenotypes in 5 and 6 month old intact animals following allografting. Increased numbers of D12.2 positive cells, which also bore the 2B1 T cell marker (indicating that they are indeed T cells) were found. The 2B1 marker is thought, in *Xenopus*, to stain the equivalent of the CD5 cell surface receptor (Cooper, M. D., Unpublished), which is found on all mammalian T cells and a sub-population of B cells.

2.3.2.2 Thymectomised *Xenopus laevis*

Flow cytometry was also carried out on splenocytes taken from siblings of the above *X. laevis*, which had been Tx at 5 days and had received LG15 grafts (Table 2.7B). Tx animals had a greatly reduced number of 'T-like' cells compared to intact animals. By 5-6 months there was approximately 8-9% 2B1 positive cells, compared to no T cells in younger Tx animals (Horton *et al.*, In preparation). The mean fluorescence of the 2B1 positive cells from the Tx animals was always lower than that seen in intact animals (Figure 2.3), indicating less markers per cell. There was no obvious increase in the percentage or expression of any particular T-cell marker in the Tx animals, following application and tolerance of allografts. However 4 of the Tx animals, studied here, had acutely rejected LG15 skin allografts (see Table 2.3). Flow cytometry suggested a slightly increased percentage of D12.2 positive cells (11%) in one of these latter animals, when examined at 20 days post-grafting.

This same Tx animal had 14% F17 positive cells compared to 3-7% seen in the other Tx animals (Figure 2.3). *In vitro* experiments showed that the low level of T cells, found in the Tx animals, did not allow a response to ConA (see section 3.2.2.1 and Table 2.8). However, the presence of the D12.2/F17 positive lymphocytes could explain the fairly acute allograft rejection.

2.3.2.2.1 Response to ConA of splenocytes from 6 month old intact and Tx allografted *X. laevis*

Response to ConA was carried out on splenocytes from 6 month old intact *X. laevis* and 5 day Tx *X. laevis* which had received LG15 grafts (Tables 2.2 and 2.3). The intact animals had rejected their allografts within 17 days and the Tx animals were from the group which had rejected allografts within 28 days.

Controls responded well to the ConA (see Table 2.8). The Tx animals did not respond to the mitogen (SI $\approx$ 1, Table 2.8), at 8-27 days following allografting. This showed an absence of reactive T cell clones in the Tx animals, although members of this group had rejected allografts in an equivalent time to that seen in intact *Xenopus* (Table 2.3).

2.3.2.3 Studies on LG3, including skin allotolerant animals

These animals were used to probe whether the increase seen in D12.2 positive cells, following allografting of normal controls, would appear when animals tolerant of a donor received a second graft from that donor. Since LG3 were used here, the studies began by observing cellular events in the spleens of adult LG3 animals (which had not been rendered tolerant) following primary skin grafting. Table 2.9 shows the splenocyte phenotypes, of intact LG3 animals grafted with LG15 skin. The intact LG3 animals did not show the increase in D12.2 and F17 positive cells seen in *X. laevis* following allograft rejection. However these animals had rejected the LG15 skin graft in 20 days (Table 2.2) and their splenocytes were stained 32 days

post-grafting. It is possible that the observed splenocyte phenotypes had returned to that of an unstimulated population.

Studies on animals rendered tolerant in larval life are shown in Table 2.10 (A and B). An increase in D12.2 positive T cells was not observed following grafting (at 9 months of age) with a transplant destined to be tolerated. There was however, an increase in D12.2 staining in two animals tolerant to LG5, which at 9 months had received LM3 grafts, 3 and 4 weeks prior to testing.

2.4 DISCUSSION

Flow cytometry was used with anti-*Xenopus* monoclonal antibodies, to identify possible cell surface antigen changes on splenocytes during *in vivo* allograft rejection, with an emphasis on the level of expression of *Xenopus* T cell markers. The spleen has previously been identified as a centre of alloimmune reactivity in *Xenopus* (Horton, Horton and Rimmer, 1977).

2.4.1 The Effect of Allografting on Splenocyte Phenotype in Intact Animals

The acute rejection of skin allografts observed by the intact adults was as expected (Obara, Kawahara and Katagiri, 1983). T lymphocytes are intimately involved in this allograft rejection (Horton *et al.*, 1992; Clothier *et al.* 1989). There is extensive lymphocytic infiltration of the graft tissues which could be related to T-helper or T-cytotoxic activity, which is involved in allograft rejection in mammals.

In *Xenopus* the spleen contains specific alloreactive cells capable of differentiation into cytotoxic lymphocytes which specifically recognise MHC-linked target molecules on lymphoblasts (Bernard *et al.*, 1979). In mammals cytotoxic T cells express the CD8 cell surface receptor and so during allograft rejection an increase in the number or expression of these cell surface molecules would be expected, probably in the spleen (Horton, Horton and Rimmer, 1977). The putative *Xenopus* anti-CD8 mAb, F17, was therefore utilised to see whether there was an increase in these cells in the spleen during the graft rejection response, to determine if these cells could possibly be the cytotoxic effector cells. Such a projected increase was not obvious, there being only a slight increase in CD8 positive cells during rejection, approximately 27% to 31%. However there was a difference in the number of CD8 positive cells which also express the γδ TCR: from 4% in autografted control spleens up to 11% of F17 positive splenocytes from animals which have received LG15 skin grafts. The majority of the γδ positive cells in allografted *Xenopus* also express the putative CD5 marker, as labelled by the mAb 2B1. After stimulation of

murine $CD3^+CD4^-CD8^-$ γδ splenocytes, with anti-CD3 mAbs, or ConA, there is an increase of the γδ cells which co-express CD8 on their cell surface (Han Cheng *et al.*, 1991). The findings in *Xenopus* may reflect an increase in the number of γδ TCR positive cells with cytolytic properties, following allogeneic challenge. It is thought that the D12.2 antibody recognises the *Xenopus* γδ TCR homologue (Ibrahim *et al.*, 1991). Little is known about this subpopulation of T cells in *Xenopus*, regarding their origin and their function. Conversely, in mice and humans much is known of their origin and location. In humans there are approximately 12% CD3-γδ positive T cells in the spleen and a variable subset, 30-80% of γδ positive lymphocytes also express CD5 and/or CD8 cell surface markers (Inghirami *et al.*, 1990).

The function of γδ TCR positive cells in mammals is under debate. Two potential roles of γδ TCR positive cells have been suggested: surveillance of epithelia, monitoring the integrity of the cell layers that separate the internal from the external milieu; and differentiation of T cells bearing αβ receptors in the thymus (Janeway, Jones and Hayday, 1988). All human CD3-γδ cell lines express a broad range of cytolytic activities (Marüsïc-Galësïc *et al.* 1988) and in mice epithelial γδ positive cells can be induced to become cytotoxic effector cells. The ligands of γδ TCR positive cells in mammals remain to be fully elucidated, but they have been found to recognise evolutionary highly conserved antigens such as mycobacterium and/or heat shock proteins (Haas, Kaufman and Martinez-A, 1990; Lefrancois *et al.*, 1990). In the majority of cases this recognition is not MHC restricted, however, they can also recognise class I or class I-like molecules in both humans and mice (Ferrick *et al.*, 1989).

It has been suggested in mice that γδ TCR-bearing T cells may be involved in cytotoxic responses against alloantigens (Haas, Kaufman and Martinez-A, 1990). This is in agreement with the results shown here which show an increase of γδ TCR positive/CD5 positive cells following allogeneic challenge in *Xenopus*. The expression of CD5 by these cells would seem to suggest that they are not NK-cells, which are also γδ receptor positive in mice.

Stress proteins have also been suggested as ligands for γδ cells. These stress proteins are highly conserved through phylogeny from prokaryotes to eukaryotes (Lindquist and Craig, 1988). There is increased expression of these in response to environmental stress or following infections. An increased expression of stress proteins following application of allografts could therefore occur, resulting in the increased expression of γδ cells seen in *Xenopus*. Stress proteins are also major immunogens for αβ receptor positive T cells and B cells.

The increase of γδ T cells in the splenocyte population seen in animals rejecting allografts, indicates that γδ T cells in *Xenopus* are involved in the rejection process. They may act as accessory cells, producing lymphokines which enhance cytotoxicity, or could be directly involved, making use of their cytolytic properties to destroy allogeneic cells.

2.4.2 The Effect of Allografting on Splenocyte Phenotype in Thymectomised Animals

As expected the Tx animals generally tolerated allografts (Flajnik *et al.*, 1987; Du Pasquier, Schwager and Flajnik, 1989). However, unexpectedly, four Tx *X. laevis* acutely rejected LG15 skin grafts, although their splenocytes were unable to respond to the T cell mitogen, ConA.

Early thymectomy resulted in a great reduction in the number and level of expression of cells staining with the T cell mAbs, as has been seen elsewhere (Horton *et al.* 1992). However, by 6 months there were approximately 8-9% 2B1 positive 'T-like' cells. In nude mice an increase of CD8 and 'T-like' cells is seen with age (Bell, 1989). Normal levels of γδ TCR positive cells can mature in nude mice, while there are greatly reduced levels of αβ TCR positive cells. This was also seen in the Tx *Xenopus* where similar levels of γδ TCR positive cells to intact animals were seen, but there was a profound reduction of αβ TCR positive cells. In Tx *Xenopus* there was a 1:1 ratio of αβ:γδ TCR positive cells, whereas in intact animals this ratio was nearer

6-8:1. This may explain the inefficient allograft rejection, which involves αβ TCR positive cells in mammals.

The Tx animals which had rejected LG15 skin grafts displayed a slight rise in the number of γδ TCR positive cells. Such cells could account for the alloreactivity seen in these animals.

The level of 2B1 positive cells in the Tx animals at 6 months of age deserves comment. At this time there was approximately $1x10^6$ cells present in the spleen of these Tx animals, of which 9% (that is, $0.9x10^5$ cells) would be 2B1 positive. It has been shown that injection of just $1x10^5$ T lymphocytes is required to restore skin allograft rejection in nude mice (Bell, 1989). This suggests that these Tx *Xenopus* should be able to reject their allografts. However, the fact that the majority of these Tx animals tolerated allografts suggests that there may be a fundamental flaw in their TCR's, resulting in an inability to provide help, cytotoxicity or release cytokines. It has also been suggested with nude mice that there is an intrinsic defect in their T cell surface receptors which results in an inability to carry out normal functions such as response to T cell mitogens. This was also seen in the Tx *Xenopus* since the 'T-like' cells from these animals did not proliferate in the presence of ConA. This showed an absence of reactive T cell clones in these animals, although they had acutely rejected the skin grafts.

Evidence from nude mice (Bell, 1989) shows that cells expressing γδ chains can develop independently of the thymus, maybe in the spleen or lymph nodes (Lefrancois *et al.*, 1990), and therefore these could be the origin of the 'T-like' cells seen in the Tx *Xenopus* of above 5-6 months of age. XT-1 (an anti-T cell mAb) positive cells have been found in the basal epidermal layer of intact and Tx *Xenopus* skin (Horton *et al.*, 1992). These cells may represent one population of non-thymus derived 'T-like' cells found in mammalian epithelia. It would be interesting to see if these cells also express the γδ TCR.

2.4.3 Flow Cytometric Studies on Allotolerant *Xenopus*

In *Xenopus* the allodestructive mode of immunity matures gradually during the entire larval period. Larvae are capable of acutely rejecting allogeneic skin grafts (Obara, Kawahara and Katagiri, 1983), however larvae and metamorphosing *Xenopus* do not always reject grafts, in fact very often there is tolerance of MHC-incompatible skin (Cohen *et al.*, 1985; Chardonnens, and Du Pasquier, 1973).

The artificial induction of tolerance to non-self has served as a model for understanding the events responsible for induction and maintenance of self-tolerance. Specific immunological tolerance to alloantigens encoded for by major and minor H loci can be induced by simply skin grafting *X. laevis* at an appropriate stage of development and in an appropriate donor-host combination. This induction of tolerance was shown here by grafting LG3, stage 54, larvae with semi- and fully-disparate MHC skin grafts. This tolerance was also shown to be specific for the MHC type of the larval donor, which confirms previous reports (Cohen *et al.*, 1985). The fact that this tolerance inducibility is dependent on the host and not lack of antigenicity of the graft (Chardonnens and Du Pasquier, 1973) was confirmed here by the fact that LG3 animals not 'tolerised' as larvae rejected equivalent skin grafts.

There is a higher incidence of tolerance when the donor and host share H antigens (Cohen *et al.*, 1985; Chardonnens and Du Pasquier, 1973), which may explain why one of the LM3 grafts was rejected by an LG3 larva. LM3 differs from LG3 by two MHC loci, MHC haplotypes *wy* and *bd* respectively, whereas LG5 and LG3 share the *b* MHC locus. Tolerance to two MHC haplotype disparate grafts is usually significantly less than that seen with one MHC haplotype difference (Cohen *et al.*, 1985). The rejection of the LM3 (*wy*) graft by LG3 (*bd*) larvae was probably due to the double haplotype difference. Also the LM3 clone is a result of a cross between *X. laevis* and *X. mülleri*, and so contains MHC haplotypes from a different *Xenopus* species to that of the LG3 animals. This may therefore have been recognised as xenogeneic by the LG3 and have been rejected by an humoral rather

than a cellular response (Horton *et al.*, 1992). However, this would not explain why six of the LM3 grafts were permanently tolerated.

The splenocyte phenotypes of tolerant animals were observed to see if there was any decrease in the number of cells thought to be involved in allograft rejection. There was no obvious decrease in the cell numbers or level of expressions by any of the observed cell surface markers.

It has been suggested that the cytotoxic effector part of the alloresponse is inhibited in tolerant *Xenopus*. The flow cytometric observations presented here revealed no alteration in the splenic T cell antigens following application of grafts from the tolerance-inducer donor, whereas increased γδ cell percentages were seen following third party graft application. Suppressor effector cells have been demonstrated in the spleen, but not the thymus of *Xenopus*. However, the thymus is thought to induce suppressor effector cells in splenic tissue by production of a humoral factor (Ruben *et al.*, 1985). Therefore, although here there was no obvious increase or decrease of any of the T cell phenotypes in the tolerant *Xenopus*, there could conceivably be a rise of suppressor cells following test graft application, to which a marker has not been attributed.

It appears from the above work that skin tolerant animals may not display alterations in splenocyte phenotype following application of skin from the tolerance-inducing donor.

Overall, the findings indicate that events such as elevated γδ T cells, in the spleen during normal graft rejection are associated with the rejection process. The increase not being caused by a property of the foreign skin, for example release of cytokines, which are unrelated to the rejection events.

Table 2.1 Summary of the Properties of the Monoclonal Antibodies which were used to probe changes in Splenocyte Phenotype

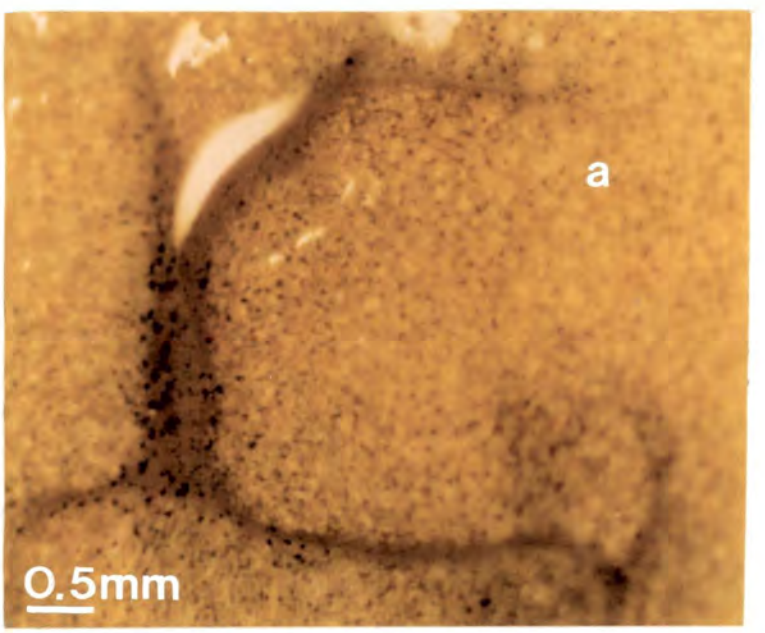
All the mAb's used were raised in mice. The following table describes the properties and nature of the antibodies used:

mAb	Cell surface receptor stained	Concentration used for staining	Nature of mAb used
CT3[a]	chicken thymocytes	1:10	SN
Ig-PE[b]	mouse IgG1 (specificity unknown)	1:40	P
2B1	All *Xenopus* T cells	1:200[c]	P
D4-3	*Xenopus* αβ TCR?	1:200	P
D12-2	*Xenopus* γδ TCR?	1:200	P
F17	*Xenopus* CD8 receptor	1:200[d]	P
AM22	*Xenopus* CD8 receptor	1:4	SN
8E4:57	*Xenopus* surface IgM[e]	1:20	SN
XT1	*Xenopus* XTLA receptor	1:4	SN
AM20	*Xenopus* MHC class II	1:4	SN
FJ17 & 20G2	*Xenopus* IL-2 receptor	1:4	SN

a = FITC negative control; b = PE negative control; c = used at 1:100 for PE staining; d = PE-labelled only; e = shows the total number of B cells; TCR = T cell receptor; P = purified mAb solution; SN = crude hybridoma supernatant.

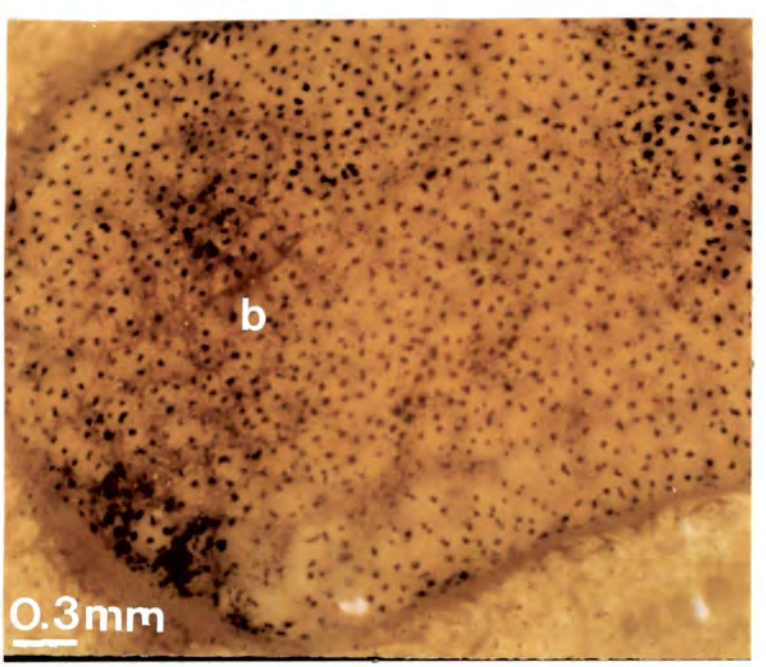
Plate 2.1 External appearance of (A) a healthy autograft on intact *X. laevis* 6 days post grafting, showing where the skin has been left uncut to aid autograft retention (a); and (B) primary LG15 skin grafts on control *X. laevis* (i) 6, (ii) 13, (iii) 16 and (iv) 22 days post-grafting, showing enlarged blood vessels (b) and pigment cell destruction (c and d), leading to completion of rejection at 22 days.

(A) Autograft

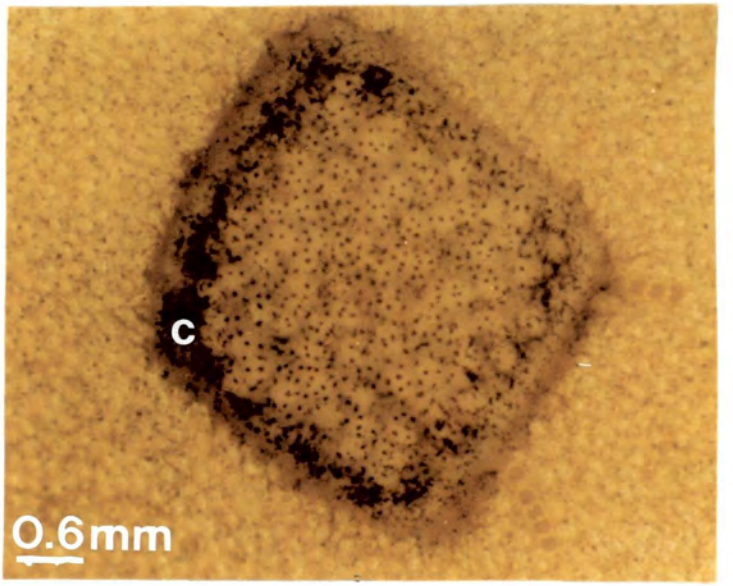


(B) Primary LG15 skin graft

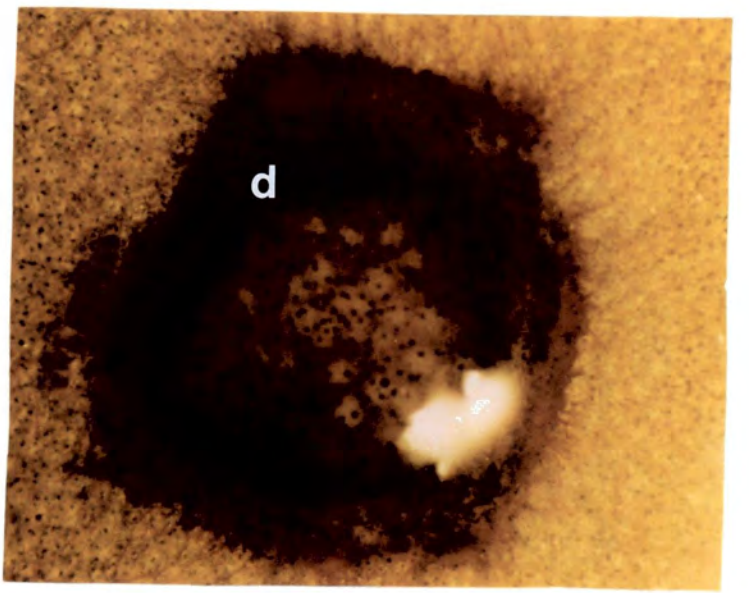
(1)



(ii)



(iii)



(iv)

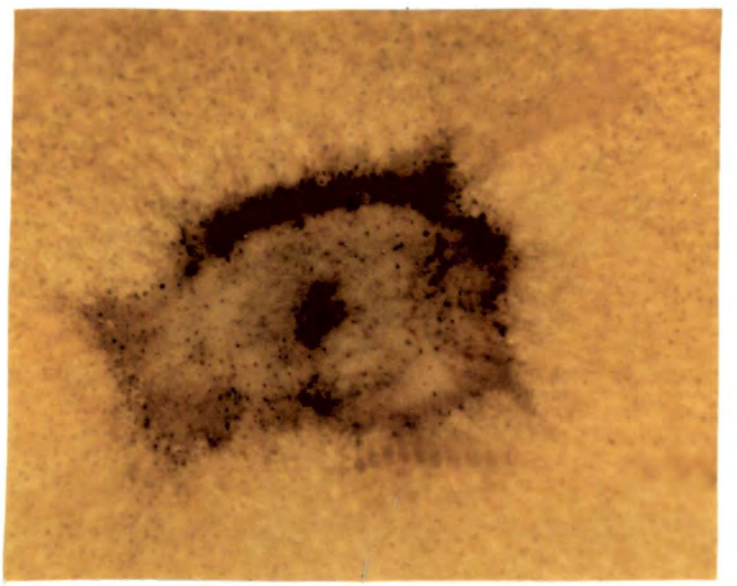
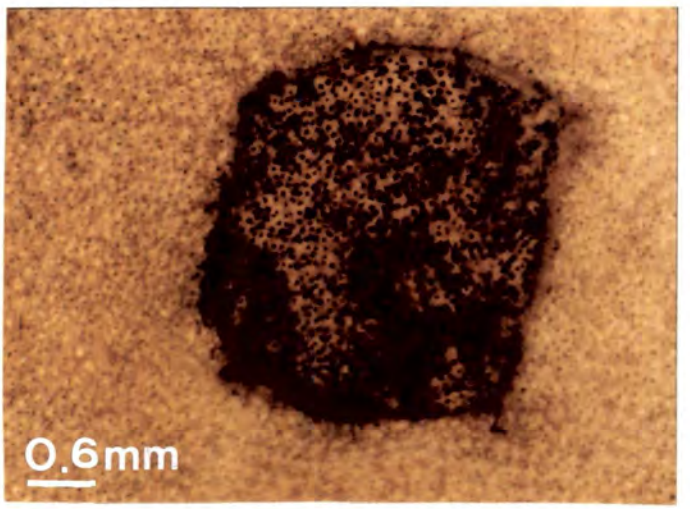


Plate 2.2 External appearance of (A) tolerised and (B) rejected LG15 skin grafts on 5 day Tx and intact *X. laevis*, respectively, 21 days post grafting.

(A)



(B)

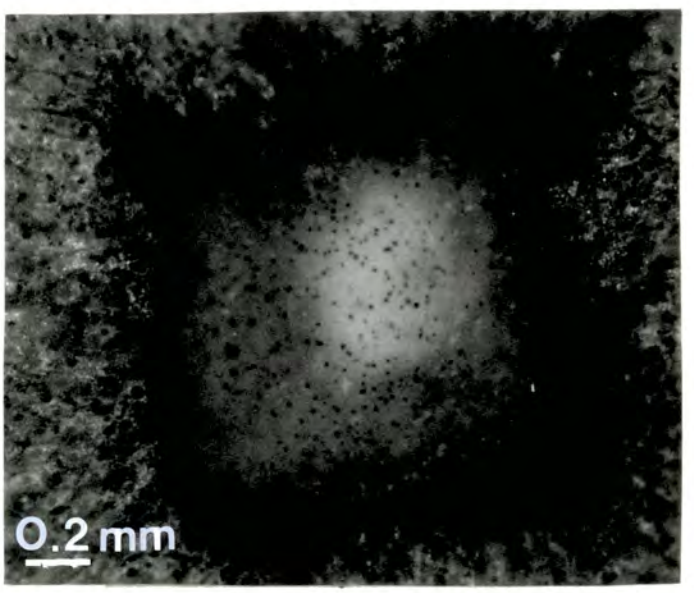


Table 2.2 Characterisation of the outcome of primary and secondary skin allografts in intact adults.

Host (age in months)	Donor		Survival of primary graft[a]	Survival of secondary graft[a]
	Primary	Secondary		
X. laevis (3)	LG15[b]	LG15	19 ± 3 (n=5)	13 ± 2
X. laevis (5)	LG15	nd	19 ± 1	
X. laevis (7)	LG15	nd	16 ± 1	
LG15 (10)	LG3[c]	LG3	20	14 ± 1
LG15 (10)	LG3	LG5	22 ± 2	16 (n=1)
LG3 (3.5)	LG15	nd	20	
LG3 (7)	LG5[d]	LG5	18 ± 2	20 (n=1)

a = Survival times are shown in days ± standard deviation, n = 3 or 2 animals for primary and secondary grafts respectively, unless otherwise stated; b = MHC haplotype, *ac*; c = MHC haplotype, *bd*; d = MHC haplotype, *bc*; nd = not done

Table 2.3 Outcome of primary and secondary skin allografts in 5 day Tx adults.

Host (age in months)	Donor		Survival of primary graft[a]	Survival of secondary graft[a]
	Primary	Secondary		
X. laevis (3 & 5)	LG15	LG15	> 100	> 100
X. laevis (6)	LG15	nd	21 ± 4 (n=4)	
LG3 (3)	LG15	nd	> 100 (n=5)	

a = Survival times are shown in days ± standard deviation, n = 3 unless otherwise stated.

Table 2.4 Shows tolerance induction in LG3 individuals as a result of allografting larvae at 3 weeks.

Host	Primary donor (at 3 weeks)	Survival of primary graft[a]	Secondary donor (at 7 months)	Survival of secondary graft[a]	Tertiary donor (at 9 months)	Survival of tertiary graft[a]
LG3	LG5	> 100 (n=8)	LG5	> 80 (n=2)	LG5 LM3	> 30 (n=2) 20 (n=1)
LG3	LM3	33, >100 (n=7)	LM3	> 80 (n=2)	LM3	> 30 (n=2)
LG3	LG5	> 100 (n=8)	LM3	18, 20	nd	
LG3	LG15	> 100 (n=7)	nd		nd	

The number of animals used for each grafting protocol is shown in brackets; a = survival times of the grafts are shown in days; nd = not done.

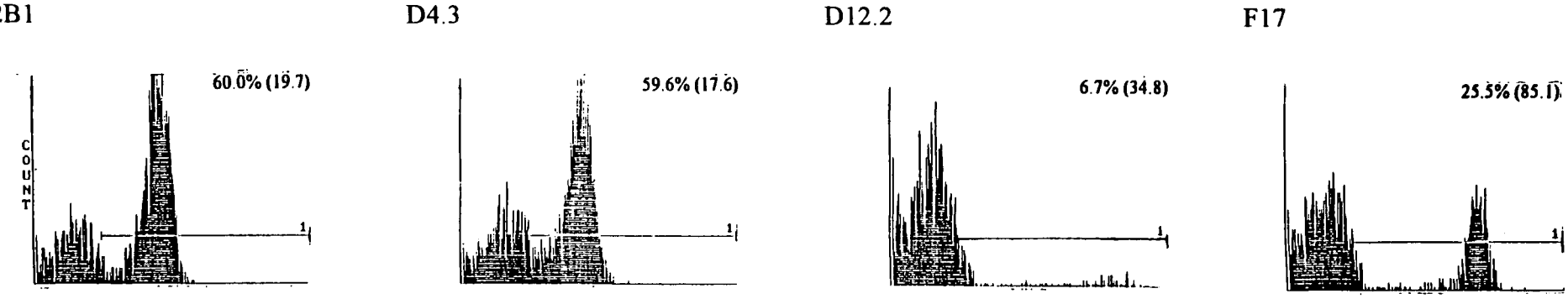
Table 2.5 Distribution of T cell subgroups in the spleen of 5 month old intact *X. laevis* 14 days post grafting with either an autograft or a primary LG15 skin graft.

Treatment of *X. laevis* prior to staining[a]	Percentage of positive stained cells[b]			
	mAb's used			
	2B1	D4-3	D12-2	F17
Autograft	50	48	7	21
Autograft	60	60	5	26
LG15	60	54	14	24
LG15	53	56	8	19

a = graft survival times are shown in Table 2.2; b = the lymphocytes were stained with FITC-labelled antibody only.

Figure 2.1 Showing the typical histograms obtained from the Coulter Epics flow cytometer, following staining of 5 month old *X. laevis* splenocytes, with FITC-labelled monoclonal antibodies only, at 14 days post grafting with either an (a) autograft or (b) LG15 skin. The percentage staining and mean fluorescence (in parenthesis) are indicated.

(A) T cell subgroups following autografting.



(B) T cell subgroups following application of an LG15 skin allograft

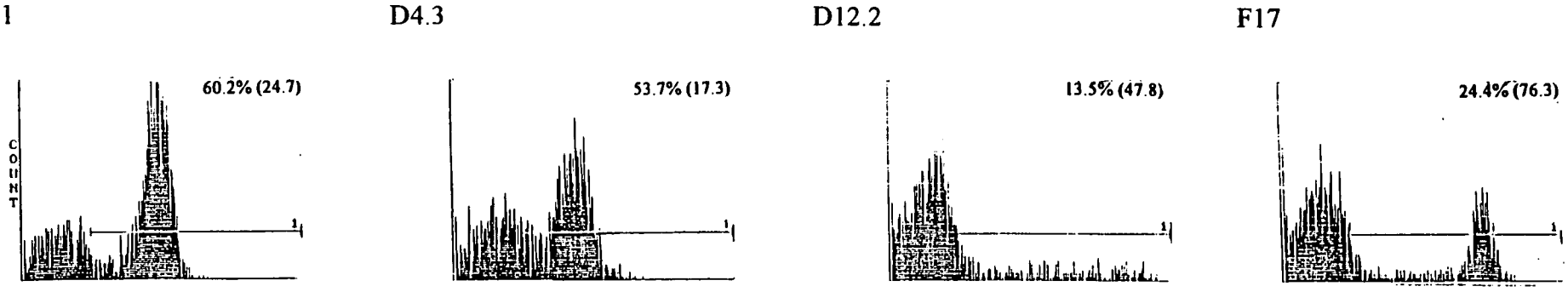


Table 2.6 Distribution of T cell subgroups in the spleen of 5 month intact *X. laevis* 24 days post grafting with either an autograft or a primary LG15 skin graft.

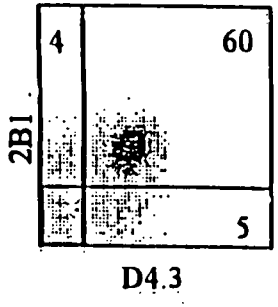
Primary graft[b]	Percentage of positive stained cells[a]								
	mAb's used								
	2B1	D4.3$^+$		D12.2$^+$		D4.3$^+$		F17$^+$	
		2B1$^+$	2B1$^-$	2B1$^+$	2B1$^-$	D12.2$^+$	D12.2$^-$	D12.2$^+$	D12.2$^-$
Autograft	55	46	3	6	2	2	29	4	16
LG15	57	52	2	13	2	15	40	12	14
LG15	62	56	2	14	2	12	48	11	20

a = lymphocytes were double stained with FITC- and PE-labelled antibodies; b = graft survival times are shown in Table 2.2.

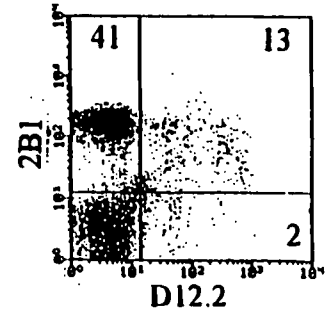
Figure 2.2 Dot plots taken from the Becton Dickinson FACS Scan showing the typical increase of γδ TCR and CD8 positive cells in the spleen of 5 month old intact *X. laevis* following application of primary LG15 skin grafts. These dot plots also show the increase of expression of 2B1/D12.2, D4.3/D12.2 and D12.2/F17 double positive cells following allografting. Splenocytes were stained 3 weeks post grafting. The data shown in Tables 2.5-2.7 was derived using the percentages, shown in each quadrant of the dot plots.

(A) Dual staining of control *X. laevis* splenocytes.

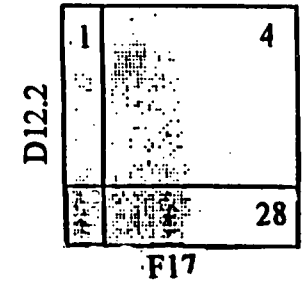
D4.3/2B1



D12.2/2B1

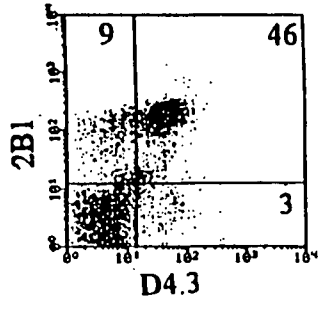


F17/D12.2

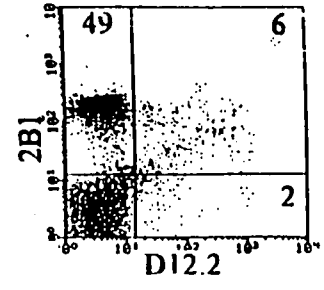


(B) Dual staining of *X. laevis* splenocytes following autografting.

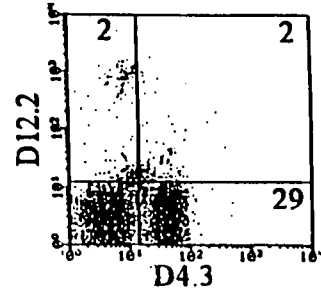
D4.3/2B1



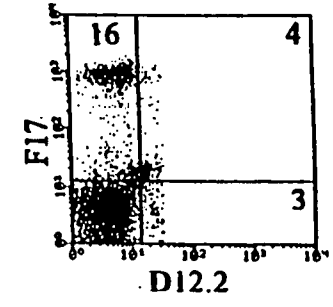
D12.2/2B1



D4.3/D12.2

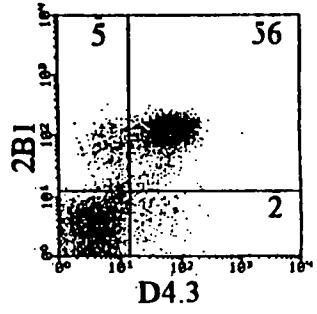


D12.2/F17

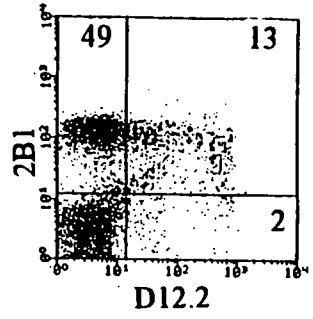


(C) Dual staining of *X. laevis* splenocytes following allografting

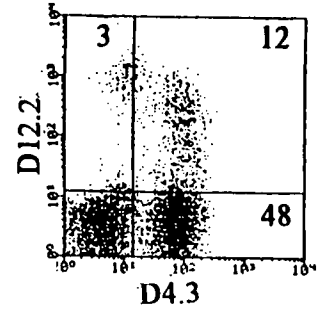
D4.3/2B1



D12.2/2B1



D4.3/D12.2



D12.2/F17

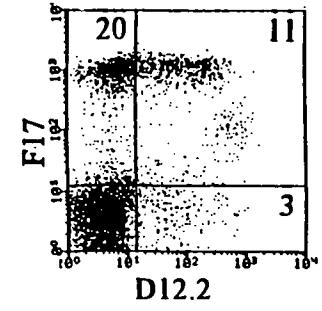


Table 2.7 Distribution of T cell subgroups in the spleen of 5-6 month old intact and 5 day Tx *X. laevis* following grafting with a primary LG15 skin graft.

A: Intact *X. laevis*

Treatment of *X. laevis* prior to staining[a]	Percentage of positive stained cells[b]					
	mAb's used					
	2B1	D4.3+		D12.2+		F17
		2B1+	2B1−	2B1+	2B1−	
20 days post grafting:						
No graft	64	60	5	5	5	32
Autograft	76	65	2	6	3	38
LG15	75	70	3	22	4	47
27 days post grafting:						
No graft	67	57	2	5	2	37
LG15	59	51	3	17	2	36

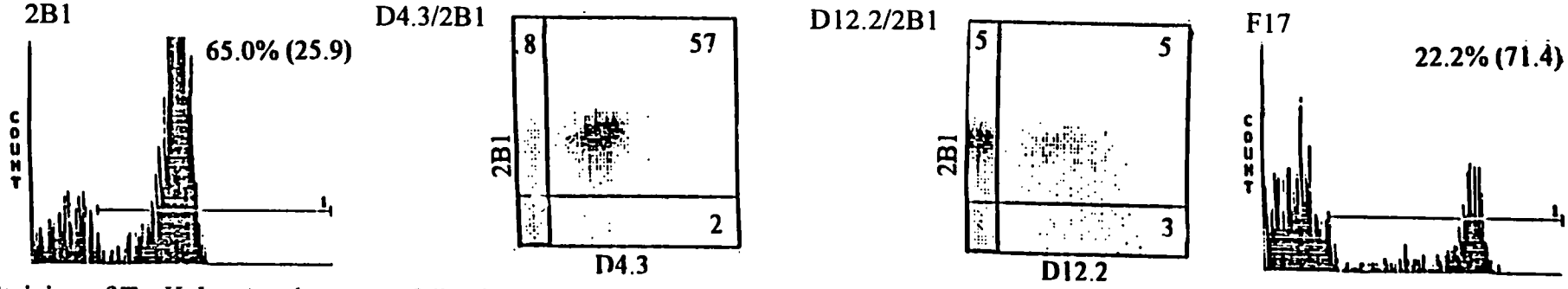
B: 5 day Tx *X. laevis*

Treatment of *X. laevis* prior to staining[a]	Percentage of positive stained cells[b]					
	mAb's used					
	2B1	D4.3+		D12.2+		F17
		2B1+	2B1−	2B1+	2B1−	
20 days post grafting:						
LG15[c]	9	5	7	3	8	14
LG15[c]	9	5	5	3	6	7
27 days post grafting:						
No graft[c]	8	4	5	4	4	7
LG15[c]	8	4	4	4	3	7
LG15[c]	8	2	2	5	3	3
131 days post 1st, and 72 days post 2nd set grafting:						
2nd set LG15[d]	6	0.6	6	1	4	nd
2nd set LG15[d]	9	0.6	2	2	4	nd

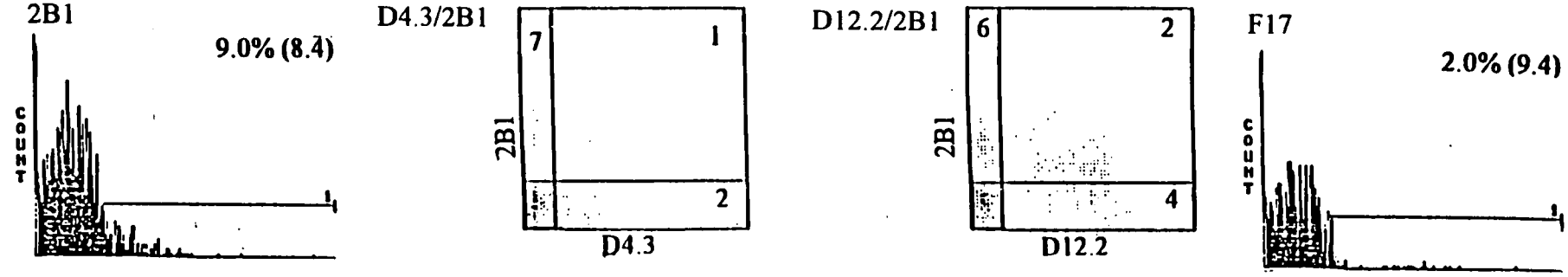
a = graft survival times are shown in Tables 2.2 and 2.3; b = lymphocytes were stained with both FITC- and PE-labelled antibody; c = where grafted rejection was in 18-28 days; d = tolerated both primary and secondary grafts; nd = not done

Figure 2.3 Dot plots and histograms taken from the Coulter Epics flow cytometer, showing the typical distribution of 2B1, D4.3, D12.2 and F17 positive cells in the spleen of 5-6 month old intact and 5 day Tx *X. laevis* following application of primary LG15 skin grafts, as shown in the lower parts of Table 2.7. The dot plots show the reduced staining and mean fluorescence of 2B1 positive cells from the Tx animals compared to the intact animals of the same age. The percentage staining and mean fluorescence (in parenthesis) are indicated.

(A) Staining of control intact *X. laevis* splenocytes.



(B) Staining of Tx *X. laevis* splenocytes following application of an LG15 skin graft, which was tolerated.



(C) Staining of Tx *X. laevis* splenocytes following application of an LG15 skin graft, which was rejected in 18 days.

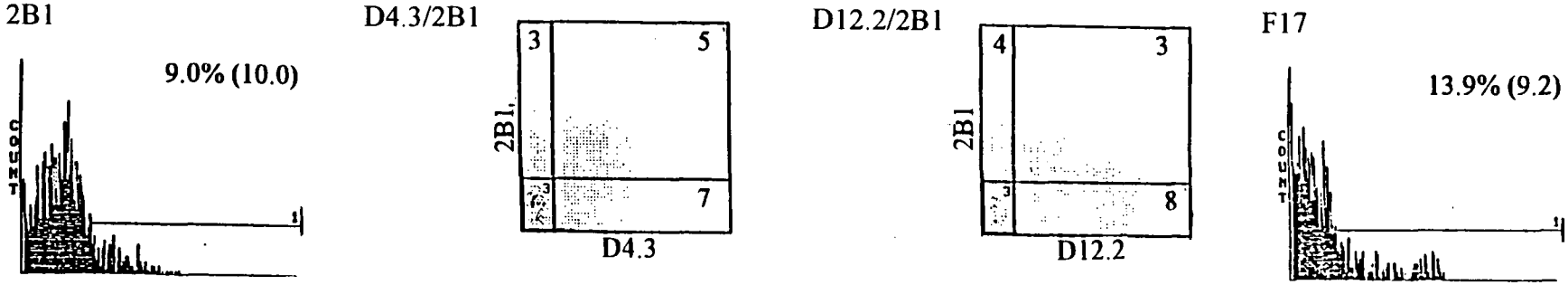


Table 2.8 Summary of the disintegrations per minute (dpm) and stimulation indices (SI's) of 6 month control and Tx *X. laevis* splenocytes to 2.5µg/ml ConA harvested at 8, 21 and 27 days post-grafting.

Treatment of *X. laevis* prior to stimulation	8 days post-grafting		21 days post-grafting		27 days post-grafting	
	dpm	SI	dpm	SI	dpm	SI
Controls:						
No graft	26,281	75	28,206	30	61,281	20
Autograft			68,132	30		
LG15 skin graft	45,354	45	44,888	62	71,493	24
5 day Tx:						
No graft					6,513	1
Autograft	785	0.8				
LG15 skin graft	225	0.7	793	1.3	8,778	0.7

Table 2.9 Distribution of T cell subgroups in the spleen of 4 and 7 month old LG3 32 days post-first set grafting with LG15 skin.

Treatment of LG3 prior to staining[a]	Percentage of positive stained cells[b]					
	mAb used					
	2B1	D4.3$^+$		D12.2$^+$		F17
		2B1$^+$	2B1$^-$	2B1$^+$	2B1$^-$	
no graft	53	45	3	3	3	30
LG15	54	44	3	5	4	33
LG15	53	44	3	4	4	30

a = graft survival times for are shown in Tables 2.2 and 2.3; b = lymphocytes were stained with both FITC- and PE-labelled antibodies.

Table 2.10 Distribution of T cell subgroups in the spleen of 9 month old LG3 animals, rendered tolerant by larval grafting, comparing animals which had tolerated or rejected skin allografts

A: 3 Weeks Post-Tertiary Grafting

Prior treatment of LG3[a]		Graft applied at 9 months[a]	Percentage of positive stained cells[b]						
			mAb's used						
3 weeks (larval)	6 months		2B1	D4.3$^+$		D12.2$^+$		D12.2$^+$		F17
				2B1$^+$	2B1$^-$	2B1$^+$	2B1$^-$	D4.3$^+$	D4.3$^-$	
LG3	no graft	Autograft	49	39	2	3	2	1	4	28
LG5	LG5	LM3	64	49	2	13	4	7	9	34
LM3	LM3	LM3	60	51	3	5	4	2	5	35

B : 4 Weeks Post-Tertiary Grafting

Prior treatment of LG3[a]		Graft applied at 9 months[a]	Percentage of positive stained cells[b]						
			mAb's used						
3 weeks (larval)	6 months		2B1	D4.3+		D12.2+		D12.2+	F17
				2B1+	2B1−	2B1+	2B1−	D4.3+	D4.3−	
LG3	no graft	Autograft	51	41	4	4	3	1	4	27
LG5	LG5	LM3	52	40	3	3	7	4	6	30
LM3	LM3	LM3	59	48	2	2	4	2	5	26
no graft	LG5	LG5	48	38	4	4	4	2	4	28
LG5	LG5	LG5	45	38	2	2	3	1	5	23

The LG3 animals used here had been grafted when 3 week old larvae with either LG5 or LM3 adult skin, these were then shown at 6 and 9 months to be specifically tolerant to the primary donor skin grafts; a = survival times of the grafts are shown in Table 2.4; b = lymphocytes were stained with both FITC- and PE-labelled antibodies.

CHAPTER 3

USE OF MITOGEN ACTIVATED STIMULATORS TO ENHANCE THE MIXED LEUKOCYTE REACTION AND CHANGES IN RESPONDER CELL PHENOTYPE FOLLOWING *IN VITRO* ALLOSTIMULATION

3.1 INTRODUCTION

T cells recognise antigens in a MHC restricted fashion through a T-cell receptor (TCR) consisting of two disulphide linked glycoprotein chains (termed αβ or γδ). These are noncovalently associated with an invariant complex of proteins, termed CD3, which appear to be responsible for signal transduction (Marüsïc-Galësïc *et al.*, 1988). The αβ TCR positive cells recognise antigens as peptide fragments in the context of class I or class II MHC. They are helped in this by expressing the co-receptors either CD8 or CD4 respectively. CD8 positive cells mediate cytotoxic reactions, while CD4 positive cells are referred to as helper cells (Allison and Lanier, 1987). The functional TCR repertoire of the αβ T-cell population is shaped by positive and negative selection events that occur during intrathymic maturation. Positive selection skews the T-cell repertoire to recognise foreign antigen in the context of 'self'-MHC molecules, while negative selection results in clonal deletion of T cells which react strongly against self MHC. αβ and γδ TCR positive cells have very similar activation properties and functional capacities (Marüsïc-Galësïc *et al.*, 1988). Induction of function of γδ TCR positive cells can be achieved by a variety of stimuli known to elicit activation signals on αβ positive cells, for example concanavalin A (ConA).

T cells are also able to respond to, and recognise, allogeneic MHC. This response is considered to be due to cross reactivity of TCR's, whose normal ligands would be self-MHC bound to peptide.

The MHC class I and II molecules of *Xenopus* have fundamentally the same roles and structure as those of mammals (Flajnik *et al.*, 1984; Kaufman *et al.*, 1985). *Xenopus* is the only ectothermic species were polymorphic class I and class II MHC gene products have been formally documented (Flajnik and Du Pasquier, 1990; Kaufman *et al.*, 1985; Kaufman, Flajnik and Du Pasquier, 1985; Flajnik *et al.*, 1984) and clones and inbred lines are available. As in mammals, graft rejection, MLR's and cell mediated cytotoxicity indicate that allogeneic MHC molecules stimulate a large proportion of T cells. Therefore *Xenopus* represents an evolutionary significant and practically useful model for studying MHC function in lower vertebrates.

The *in vitro* correlate of allograft rejection, the mixed lymphocyte reaction (MLR), is investigated in this chapter. MLR is an assay system for T cell recognition of allogeneic cells in which the response is measured by the extent of T cell proliferation. The MLR has been shown to be an accurate *in vitro* correlate of *in vivo* allograft rejection in *Xenopus* (Lallone and Horton, 1985). Thymectomy (Tx) was used to show that, like acute graft rejection, T lymphocytes are the responder cells in MLR in *Xenopus* (Flajnik *et al.*, 1987; Du Pasquier and Horton, 1976). In adult *Xenopus* the spleen is a good source of helper T cells and MLR effector cells (Du Pasquier *et al.*, 1985).

It was first suggested in the early 1970's that MLR in *Xenopus* is under genetic control (Du Pasquier and Miggiano, 1973). It has later been confirmed to be controlled by class II MHC molecules (Harding, Flajnik and Cohen, In press). That is, MLR alloreactivity in *Xenopus* is generated by T cells in response to class II MHC molecules (Flajnik, Du Pasquier and Cohen, 1985; Du Pasquier and Horton, 1976).

In *Xenopus* all adult T cells are positive for class II and the latter can be up-regulated after stimulation with alloantigens or T-cell mitogens (Harding, Flajnik and Cohen, In press; Ho, 1992). In this Chapter attempts are made to elevate *in vitro* MLR by culturing MLR stimulator cells with the T-cell mitogen ConA prior to setting up the assay. The eventual goal of this work was to avoid the necessity of having to immunise animals *in vivo* (by skin allograft rejection), prior to restimulation of

lymphocytes in MLR, in order to generate cytotoxic T lymphocyte effectors *in vitro* (Du Pasquier, Schwager and Flajnik, 1989; Bernard *et al*, 1979). If this goal could be achieved, then studies on cytotoxic T lymphocytes in larvae would be more feasible. The causative factors of any elevated MLR seen following use of T cell mitogen-activated stimulator cells, was investigated by carrying out flow cytometry of the stimulating cells. Particular emphasis was placed on the level of expression of class II MHC molecules and T cell antigen specific markers.

As a result of the findings in Chapter 2 that *in vivo* skin allograft rejection can sometimes result in changes in the splenocyte phenotypes of the recipient animal, phenotypes of the responding cell population during *in vitro* MLR were examined here. Flow cytometry, making use of the anti-T cell surface receptor monoclonal antibodies (mAb's), 2B1, D4.3, D12.2 and F17, was utilised.

Some splenocytes were set up in long term MLR's in order to represent, more accurately, the time scale seen in *in vivo* allograft rejection. Viability of the responding cell populations was maintained by adding T-cell growth factor- (TCGF) rich supernatants to the culture medium. TCGF is produced by ConA-and PHA-stimulated *Xenopus* splenocytes. It is capable of stimulating proliferation of splenic and thymic lymphoblasts and supporting growth of alloreactive T-cell lines (Watkins and Cohen, 1987; Harding, Flajnik and Cohen, In press). Continued presence of both irradiated allogeneic cells and TCGF was required to maintain a high rate of proliferation of the responding cells.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of a Lymphocyte Cell Suspension

Spleens from animals of 6-12 months of age were used for preparation of lymphocyte cell suspensions.

The animal was heavily anaesthetised in a solution of 3-aminobenzoic acid ethyl ester. The spleen was then asceptically removed and placed in 2ml Leibowitz-15 (L-15 - Flow Labs) amphibian strength culture medium, supplemented with 1% foetal calf serum (FCS - Gibco), 0.01 M HEPES buffer, 50 IU/ml penicillin, 50μg/ml streptomycin, 2.5μg/ml amphotericin B, 1.25 mM L-glutamine, 18μl/ml sodium bicarbonate (all from Flow Labs) and 0.083mM mercaptoethanol (BDH) in a Costar 35 x 10mm petri dish. Single cell suspensions were then prepared by gently crushing the spleen between the frosted ends of two sterile glass slides. The splenocyte suspension was washed back into the culture dish with cold L-15 amphibian medium and the cell suspension transferred to a Falcon 5ml plastic tube and allowed to stand for a few minutes for the splenic capsule pieces to settle to the bottom of the tube. The supernatant cell suspension was transferred to a fresh tube and the cells washed twice by centrifugation at 300g, at 4°C for 10 minutes and then resuspension in 1ml L-15 amphibian medium. The supernatant was removed and the cells resuspended in 1ml L-15 amphibian medium. The cells were then diluted 1:1 in trypan blue and viable lymphocytes counted in a haemacytometer. Viable cells exclude trypan blue whereas dead cells take up the dye.

3.2.2 Preparation of Stimulator Cells for Mixed Leukocyte Reactions

These were either splenocytes freshly-harvested from the froglet or were cells that had been pre-cultured in the presence or absence of ConA (2.5μg/ml) for 2-3 days.

Splenocytes to be used in pre-culture were prepared as in section 2.1 and washed in fully supplemented (as above) L-15 amphibian medium (complete medium).

Viable lymphocytes were counted and then plated out in 2ml L-15 complete medium at $3x10^6$ cells/well in 24 well tissue culture plates. These were cultured for 2 or 3 days at 26°C in a water saturated atmosphere of 5% carbon dioxide (CO_2) in air, with or without ConA.

Freshly taken or *in vitro* pre-cultured cells were placed in 5ml plastic tubes on ice. The cells were then subjected to γ radiation from a Cobalt-60 source for 20 minutes, 16cm from the source. This gave a total of 6,668 rads which was adequate to inactivate the cells (Appendices D and E). Fully irradiated cells were then washed twice prior to use as stimulators in one-way MLR. The pre-cultured cells were washed in complete L-15 medium containing 1mM α-methyl mannoside, to bind the ConA present in the medium surrounding the cells.

3.2.3 Tritiated Thymidine Incorporation Following One-way MLR

3.2.3.1 Technical details of the MLR

The responder spleen cell suspension was prepared directly from the animal and the cells washed twice in amphibian strength complete L-15 medium. Two hundred microlitres of L-15 containing $1x10^5$ responder splenocytes and $1x10^5$ stimulators were plated out into individual wells of a 96 well V-bottomed tissue culture plate. Responder splenocytes were either from a non-grafted animal or an allo- or autografted animal. Control cultures were also set up which contained $1x10^5$ responders with $1x10^5$ irradiated responders. These provided background counts emanating from 'self-stimulated' cells. Cultures were also established with $2x10^5$ irradiated stimulator or responder cells only, to calculate counts coming from such stimulators. Each culture was carried out in triplicate, the cells being incubated at 26°C, in a water saturated atmosphere of 5% CO_2 in air, for 3 days. One microCurie (μCi) of tritiated thymidine ([^{3}H]TdR) was then added per well (pulsed) and the cells cultured for a further 24 hours. The cells were then harvested using a Skatron cell harvester onto 0.2μm Whatman filters.

The filters were dried and then placed into 5ml scintillation vials. Four ml of scintillation cocktail (Betaflour, National Diagnostics) was then added and the disintegrations per minute (dpm) calculated on a Packard scintillation counter.

The stimulation index (SI) for each culture was then calculated by use of the following formula:

$$SI = \frac{\text{dpm (Responders v. R Stimulators)} - \frac{\text{dpm (R Stimulators v. R Stimulators)}}{2}}{\text{dpm (Responders v. R Responders)} - \frac{\text{dpm (R Responders v. R Responders)}}{2}}$$

where dpm = disintegrations per minute; v = incubated with; R = irradiated.

3.2.3.2 Probing the use of concanavalin A activated stimulator cells to enhance MLR

Several assays investigating the use of ConA-activated cells as stimulators in MLR were carried out. These were used to determine why pre-culturing the stimulator cells resulted in increased thymidine incorporation of mixed cultures, compared to those seen when the stimulator cells were not pre-cultured.

3.2.3.2.1 Dynamics of thymidine incorporation of ConA-activated cells following irradiation

A lymphocyte cell suspension was prepared as in section 2.1. The cells were then plated out in a 24 well tissue culture plate, at $3x10^6$ lymphocytes/well with no, 2.5μg/ml or 5μg/ml ConA. They were then incubated at 26°C, for 48 hours, in a water saturated atmosphere of 5% CO_2 in air. Each sample was then treated with 1mM α-methyl mannoside to bind any free ConA and split into 2 aliquots. One aliquot of each sample was irradiated and then plated out in triplicate, at

$1x10^5$cells/well in a flat bottomed 96 well plate. Four replica plates were set up. The non-irradiated cells were plated out similarly. Plates were pulsed 1-4 days following irradiation, incubated for a further 24 hours, then thymidine incorporation was assessed as above.

In a second experiment splenocytes from *X. laevis* animals were either pre-cultured, for 48 hours, with or without 2.5μg/ml ConA or freshly prepared and then split into 2 aliquots. One aliquot of each sample was then irradiated. All the samples were then plated out in triplicate, at $1x10^5$ cells/well in a flat bottomed 96 well plate and incubated as above for 4 hours and then pulsed. The cells were incubated for a further 24 hours after which thymidine incorporation assessed, as above.

3.2.3.2.2 To investigate if ConA pre-stimulated cells still respond to alloantigens or ConA following irradiation

These experiments were carried out to determine if the ConA pre-stimulated cells could still proliferate in response to alloantigens or ConA following irradiation. If so, this could contribute to the high SI's seen when ConA-pre-cultured stimulators are used to establish MLR's.

Spleens were removed from 12 month old *X. laevis* or LG15 animals and a spleen cell suspension prepared. The lymphocytes were then cultured at $3x10^6$cells/well, in a 24 well plate, with or without 2.5μg/ml ConA for 48 hours at 2°C, 5% CO_2 in air. After 48 hours incubation a fresh splenocyte population was also prepared. Each sample was then washed in L-15 complete containing 1mM α-methyl mannoside and split into 2 aliquots, one of which was irradiated.

The *X. laevis* splenocytes were then plated out in triplicate with or without 2.5μg/ml ConA (see Tables 3.4 and 3.5) at $1x10^5$cells/well in 2 V-bottomed 96 well plates. One plate was pulsed with [^{3}H]TdR on the day of irradiation and the other after a further 48 hours incubation. Plates were harvested and thymidine incorporation assessed after overnight incubation with [^{3}H]TdR.

Pre-cultured and freshly taken LG15 splenocytes were also set up in MLR with *X. laevis* splenocytes in various combinations (see Table 3.6). This was in order to determine whether irradiated freshly harvested or ConA-pre-cultured LG15 cells could respond to the *X. laevis* cells, thereby giving a 2-way MLR.

3.2.3.2.3 To investigate whether the high stimulation indices are due to cytokine release

The high SI's seen in MLR's employing ConA-activated stimulators could be partly due to cytokines released by the ConA-pre-cultured irradiated cells or to ConA remaining on the surface of the stimulator cells, even after washing in L-15 complete plus 1mM α-methyl mannoside.

LG15 lymphocytes were incubated at $3x10^6$ cells/well, in a 24 well plate, with 2.5μg/ml ConA for 48 hours, as above. These cells were then irradiated and established in MLR's with freshly-harvested LG15 responders. This determined whether the ConA-pre-cultured LG15 stimulator cells could, after irradiation, stimulate elevated thymidine incorporation of self responders.

3.2.4 Flow Cytometry of *In Vitro* Cultured Cells

3.2.4.1 Cells cultured with concanavalin A

Spleens from 6-12 month old LG15 animals were used. A splenocyte cell suspension was prepared. $3x10^6$ splenocytes were then cultured, in 2ml complete L-15 amphibian medium (1% FCS), for 3 or 7 days, with or without 2.5μg/ml ConA, in 24 well tissue culture plates at 26°C, 5% CO_2 in air. These cells were then harvested, stained with various mAb's and analysed on a Coulter Epics flow cytometer (see Chapter 2, section 2.4).

3.2.4.2 Cells established in MLR following *in vivo* grafting

3.2.4.2.1 Experiment 1

Ten month old LG15 animals were immunised *in vivo* by grafting skin from LG5 donors. After graft rejection (18 days) spleens were removed, dissociated and cultured in 24 well tissue culture plates. Each well contained equal numbers of responders (either 2 or $5x10^6$) and irradiated stimulators, either allogeneic (LG5) or isogeneic, in 2ml L-15 amphibian complete medium supplemented with 5% FCS. The cells were cultured for 1 week at 26°C, in 5% CO_2 in air. To act as controls spleens were also taken from non-grafted LG15's and placed in culture with irradiated isogeneic cells, as above. After 1 week incubation the cells were washed once and fractionated over Histopaque, δ=1.077 (Sigma). Cells at the interface (containing mostly viable responder lymphocytes) were collected, washed and plated out into 24 well tissue culture plates in the ratio 10:1 responders ($1x10^6$ or $2x10^5$) to irradiated stimulators, in 2ml medium containing 25% T-cell growth factor (TCGF). [TCGF = supernatant collected from the culture of cells stimulated with 2.5μg/ml ConA for 24 hours and then treated with 1mM α-methyl mannoside to remove the ConA]. After a total of 2 weeks *in vitro* the cells were harvested, stained with various mAb's and processed for flow cytometry as in Chapter 2, section 2.4. Flow cytometry was also carried out on freshly-harvested LG15 splenocytes from animals which had or had not rejected LG15 skin grafts.

The degree of proliferation was also measured after 2 weeks in culture. $1x10^5$ of the viable cultured cells were incubated with 1μCi [^{3}H]TdR for 24 hours, harvested and counted by liquid scintillation.

3.2.4.2.2 Experiment 2

A second experiment was carried out using 5 month old *X. laevis* animals. Spleens were removed from both non-grafted animals and animals which had rejected an LG15 skin graft (20 days). These spleens were dissociated, the splenocytes

washed and set up as responders at $3.5x10^6$ splenocytes with or without $3.5x10^6$ irradiated LG15 stimulators, in 2 ml L-15 amphibian medium, in 24 well tissue culture plates. They were then cultured for 3 or 9 days at 26°C in 5% CO_2 in air. The cells cultured for 9 days were separated over Histopaque after 6 days *in vitro* and the splenic lymphocytes restimulated for a further 3 days in 2ml L-15 containing 25% TCGF with or without $1x10^5$ irradiated LG15 stimulators, in a 1:1 ratio. All the populations were then processed for flow cytometry, see Chapter 2, section 2.4.

3.3 RESULTS

3.3.1 Tritiated Thymidine Incorporation Following One-way MLR

3.3.1.1 Effect of prior allografting

Splenocytes from LG15 *Xenopus* (MHC haplotype, *ac*) that had rejected LG5 (MHC haplotype, *bc*) skin, designated LG15 anti-LG5, gave SI's of 5 and 7 (calculated by use of the dpm shown in Table 3.1) when established in 4 day MLR's with irradiated (R) LG5 splenocytes. The control non-grafted LG15 gave an SI of 3 in both experiments. This suggests that the LG15 anti-LG5 animals had been 'sensitised' to the LG5 MHC haplotype, thus giving a secondary MLR.

After 2 weeks of culture, thymidine incorporation was assessed for the long-term MLR cultures used in flow cytometry, experiment 1 (section 2.4.2.1). This revealed that the cells incubated with irradiated allogeneic cells, at the higher concentration, were proliferating at a significantly higher rate (19,028dpm), compared to those incubated with irradiated isogeneic cells (465dpm). Continued presence of the allogeneic cells was required to achieve this high rate of proliferation.

3.3.1.2 Use of ConA-activated cells as stimulators in MLR

The MLR's using stimulators which had been pre-cultured in medium alone gave higher SI's compared to MLR's in which the stimulator cells were prepared fresh (16 and 8, respectively). However, this elevated SI was even more marked when ConA pre-cultured T lymphoblasts were used as stimulators, SI = 56 (Table 3.2).

3.3.1.2.1 Dynamics of thymidine incorporation of ConA-activated cells following irradiation

Tables 3.3 and 3.4 show that the LG15 and *X. laevis* lymphocytes respectively had been stimulated to proliferate by ConA (SI's of approximately 200 and 39, respectively). Irradiation 24 hours prior to harvest inhibited this proliferation

by 4-6 fold (Table 3.4). The proliferation rate of the LG15 splenocytes continued to fall over the 5 days following irradiation (Table 3.3), whereas the non-irradiated cells (which had been ConA pre-stimulated) maintained a high rate of proliferation throughout the 7 days of this assay (Table 3.3).

An MLR assay is over a 4 day period. Therefore by pulsing on the third day of the assay, that is 3 days after the ConA pre-cultured cells have been irradiated, these stimulators would not incorporate sufficient tritiated thymidine to contribute significantly to the disintegrations per minute seen. Moreover, any counts due to incomplete inactivation of the responders are removed, during calculation of the SI (see section 2.3), by use of control cultures which contain either irradiated stimulators or irradiated responders, only and responders with irradiated self.

3.3.1.2.2 To investigate if ConA pre-stimulated cells still respond to ConA or alloantigens following irradiation

The results shown in Table 3.5 indicate the degree of proliferation of *X. laevis* splenocytes following 48 hours pre-culture with or without ConA, followed by their irradiation and a further 72 hours incubation with or without ConA. The non-irradiated, freshly harvested *X. laevis* cells had responded well to the ConA (SI = 66). However these cells showed no ConA-induced proliferation following irradiation.

The ConA- and medium pre-cultured, non-irradiated cells could still respond to the mitogen, however ConA-pre-cultured cells only responded poorly. After irradiation the medium pre-cultured cells showed minimal response to ConA, whereas the ConA pre-cultured cells showed a significant response to the second ConA pulse (SI = 69, in experiment 1). Interestingly, the irradiated cells which had initially been pre-cultured for 2 days with ConA prior to irradiation did not show a high thymidine incorporation after a further 3 days in culture without additional ConA.

Table 3.6 shows the results of MLR's to investigate whether irradiated ConA stimulated LG15 splenocytes could respond to alloantigens. *X. laevis* cells responded

well to irradiated ConA-treated LG15 stimulators (dpm = 4,765). However, this was not due to counts from the stimulators, but to counts from the responders. This can be concluded from the observation that when the same ConA-treated LG15 stimulators were mixed with irradiated *X. laevis* cells, the dpm decreased to 432.

3.3.1.2.3 To investigate whether the high SI's are due to cytokine release

The results, shown in Table 3.7, indicate that the fresh LG15 responders have not been induced to proliferate in the presence of the ConA-pre-cultured irradiated LG15 stimulators as the SI were 1.4 and 3.5 in 2 experiments. However, the *X. laevis* responders gave SI's of 42 and 10, to the same ConA-pre-cultured LG15 stimulators.

3.3.2 Flow Cytometric Studies of *In Vitro* Cultured Cells

3.3.2.1 Cells cultured with concanavalin A

Flow cytometric studies were carried out to investigate if the increase in stimulation seen in the MLR's using ConA pre-cultured irradiated stimulators was reflected by a change in cell surface marker expression of the ConA-stimulated cells. An increased class II expression of these cells, as has been seen elsewhere following ConA stimulation (Harding, Flajnik and Cohen, In press), could lead to increased stimulation of allogeneic responders in MLR and explain the high SI's seen above.

Flow cytometry of the cells incubated with ConA showed that by 3 days of culture they were larger than those incubated in medium alone or taken fresh (see Figure 3.1), as shown by the dot plots of forward scatter (cell size) against the logarithm of side scatter (cell granularity), see Appendix B. This indicated that the lymphocytes were responding to ConA and forming lymphoblasts.

The percentages of splenocytes expressing various antigens after 3 or 7 days in culture, with or without ConA, are shown in Table 3.8. There was a marginal increase in the number of 2B1 positive cells (but a distinct drop in the percentage of

D4.3 positive cells), following incubation with ConA. There were very few B cells remaining after 7 days *in vitro* with ConA. Figure 3.2 shows that the AM22 positive cells appeared to become less brightly stained as the cultures proceeded, which indicates a decreased expression of this cell surface marker. After 3 and 7 days in culture, mean fluorescence intensities with AM20 (anti-MHC class II mAb) of ConA-stimulated cells were compared with those found in fresh or medium pre-cultured cells. A small peak of very brightly stained AM20 positive cells could be seen in the 3 day ConA treated cells, but this had disappeared by 7 days culture. Interestingly, the mean fluorescence intensity with AM20 was greater (mean = 124) with medium cultured cells, possibly explaining why such cells are better stimulators in MLR's than fresh cells.

3.3.2.2 Cells established in MLR after *in vivo* grafting

Tables 3.9 and 3.10 summarise the results of 2 separate experiments. There were variations in the number of 2B1, D4.3, XT-1, AM22/F17 and 8E4:57 positive cells following grafting and *in vitro* culture but no definite trends could be seen (see Table 3.9). However as shown in Table 3.10, the percentage of *X. laevis* cells expressing 2B1 following *in vivo* grafting with LG15 skin and restimulation for 9 days *in vitro* with LG15 stimulators, reached 77%. The LG15 anti-LG5 (cells from an LG15 animal which had rejected an LG5 skin graft) and *X. laevis* anti-LG15 cells did, however, show a significant increase in cells staining with the mAb D12.2, following *in vitro* allogeneic challenge, with the same stimulators as used *in vivo*. Thus, D12.2 positive percentages were 15% and 19% respectively, from 3-8%, in control cultures (see Tables 3.9 and 3.10). Typical staining profiles of several mAb's on *X. laevis* cells are shown in Figure 3.3.

The cells taken from *X. laevis* which had been grafted with LG15 skin and then cultured for 9 days with irradiated LG15 cells (Table 3.10) had 2 distinct populations of lymphocytes (Figure 3.4), based on forward and side scatter. The

larger lymphocyte population was relatively rich in D12.2 positive cells, containing 22% γδ TCR positive cells.

3.4 DISCUSSION

The *in vitro* correlate of skin graft rejection, that is the mixed lymphocyte reaction (MLR), was examined here. Firstly, experiments were carried out to try to enhance stimulation of the responding cells by pre-incubation of the stimulator splenocytes with the T cell mitogen concanavalin A (ConA). This is known to increase the expression of class II MHC molecules per cell (Harding, Flajnik and Cohen, In press). Furthermore, class II MHC is the molecule which achieves stimulation in MLR, as shown by blocking experiments with anti-class II mAbs (Harding, Flajnik and Cohen, In press). Second, long term MLR's were established to examine the responder cell phenotypes. Flow cytometry was used with anti-*Xenopus* monoclonal antibodies to identify possible cell surface antigen changes on splenocytes following mitogenic or allogeneic challenge *in vitro*, with an emphasis on the level of expression of *Xenopus* T-cell surface antigens and class II MHC expression.

3.4.1 Use of Concanavalin A Activated Cells as Stimulators in MLR

The proliferative response of *Xenopus* splenocytes to ConA is evident as soon as the spleen becomes lymphoid (stage 51). As expected (Rollins-Smith, Parsons and Cohen, 1984; Williams *et al.*, 1983) the splenic lymphocytes were highly stimulated by incubation with ConA, as shown by the disintegrations per minute (dpm) following 72 hours *in vitro* . Disintegrations per minute show the amount of tritiated thymidine incorporated into newly synthesised DNA and thus give an estimate of the degree of proliferation of the lymphocytes in culture.

Use of ConA to enhance stimulation capacity of allogeneic stimulators in MLR was first carried out following a report by Harding *et al.* (Harding Flajnik and Cohen, In press). This suggested that stimulating *Xenopus* lymphocytes with an alloantigen or a mitogen results in increased expression of the class II MHC antigens. MLR's involving such mitogen-activated stimulators should, therefore, be enhanced as MLR in *Xenopus* is known to be under control of the class II MHC molecule

(Harding Flajnik and Cohen, In press). All T and B lymphocytes of adult *Xenopus* constitutively express class II molecules (Du Pasquier and Flajnik, 1990; Flajnik *et al.*, 1990). This indicates that T cells, as well as B cells, in frogs may act as antigen presenting cells. T cells could therefore act as stimulating cells in an MLR in *Xenopus* but this has not been proven, as yet.

The results shown in this Chapter indicate that incubation of the stimulators *in vitro* prior to use as irradiated stimulators in MLR does indeed increase their stimulatory properties. However, this was especially marked if the stimulator cells were pre-incubated with ConA. Pre-culturing of responders and stimulators for use in MLR's has been shown elsewhere to increase stimulation indices (Lallone and Horton, 1985). This could be as a result of increased class II MHC expression per cell due to stimulation by foetal calf serum present in the culture medium (Flajnik *et al.*, 1990); this phenomenon is also indicated in the present study.

The increased MLR stimulation, achieved by use of irradiated ConA-pre-cultured stimulators, could be due to one or more of 5 factors. Firstly, ConA still present in the medium or on the surface of the stimulators. This should be removed by washing the cells in medium containing 1mM α–methyl mannoside. Secondly, incomplete inactivation of the pre-cultured LG15 cells by irradiation. However, any counts due to incomplete inactivation of the responders are removed, during calculation of the SI (see section 2.3), by setting up control cultures which contain irradiated stimulators only, irradiated responders only and responders with irradiated self. Thirdly, 'enhanced' recognition of responders by irradiated ConA pre-treated, stimulators, causing back stimulation. Fourthly, production of cytokines, for example, IL-2, by the pre-cultured LG15 cells. Fifthly, change of cell surface markers producing a greater alloimmunity or change in antigen presentation abilities of stimulators. These possibilities were investigated (see section 2.3.1) and are discussed below.

3.4.1.1 Incomplete inactivation of the stimulators by irradiation

As the T lymphocytes stimulated with ConA would have been proliferating rapidly (SI approximately 100), it was thought that the irradiation may not have inactivated all the cells. If so, it would be possible for them to continue to proliferate sufficiently to take up a significant amount of tritiated-thymidine, to respond to the alloantigen of the responding cells (giving a two-way MLR), or give T cell help by producing cytokines. These factors would have contributed to the high dpm's seen. *Xenopus* helper T cells are known to more resistant to inactivation by irradiation than other T cells (Ruben *et al.*, 1985).

The experiments reported here indicate that the high proliferation rate of the ConA stimulated cells was immediately inhibited by irradiation. By three days following irradiation the ConA stimulated cells were proliferating only minimally. It was therefore concluded that the dpm seen from the MLR's, when irradiated ConA-stimulators were used was mainly due to proliferation of the non-irradiated responders, following activation in response to the stimulators and not due to tritiated thymidine uptake by the irradiated stimulators.

Incomplete inactivation of the responders was, however, suggested by the investigation as to whether the irradiated ConA-activated cells could be further stimulated by ConA. Further incubation of these irradiated cells with ConA did indeed result in enhanced proliferation, with SI's of 69 and 19 in two consecutive experiments. Cells which had been incubated in medium alone for 48 hours were also slightly resistant to the irradiation; following irradiation and incubation with ConA they gave stimulation indices of approximately 18 and 3 in the two experiments. In contrast, the cells which had been harvested from the animals immediately before irradiation were unable to respond to the ConA. This suggests that preincubation with medium or ConA does make cells more resistant to the irradiation. However, when in an MLR situation the cells would not be subjected to further stimulation by ConA as it is removed by the use of α-methyl mannoside. It was shown in this experiment that the irradiated cells from all three populations when subsequently

incubated in medium alone gave very low dpm (see Table 3.5). This therefore suggests that the high dpm seen in the MLR's, when ConA stimulators are used, is due to thymidine uptake by the responding cells.

The irradiated pre-cultured cells would, however, be in the presence of alloantigens and so could possibly respond to these, resulting in a 2-way MLR. However, data presented in Table 3.6 indicated there was no 2-way MLR occurring. Here, high stimulation indices seen in the MLR's using ConA pre-cultured stimulators are due to the *X. laevis* splenocytes responding to the irradiated pre-cultured LG15 lymphocytes and not *vice versa*.

3.4.1.2 Investigation to determine if the increased activation in the MLR's is due to cytokines released by the mitogen stimulated cells

It is possible that the increased MLR, achieved by use of ConA-activated stimulators, could be due to cytokines released by the ConA stimulated cells. A variety of cytokines, collectively known as T-cell growth factor (TCGF), have been identified in culture supernatants harvested from *Xenopus* splenocytes after stimulation with T-cell mitogens or alloantigens (Watkins and Cohen, 1987). Such culture supernatants can achieve growth and proliferation of *Xenopus* splenic T lymphoblasts (Watkins and Cohen, 1987) and also of unstimulated *Xenopus* splenocytes (Turner and Horton, 1991). In mammals T lymphoblasts, but not unstimulated T cells, express high affinity interleukin-2 (IL-2) receptors comprised of both α and β chains (Smith, 1988), which promote their responsiveness to IL-2, formerly called TCGF. IL-2 is a lymphotropic hormone-like polypeptide that plays a critical role in regulation of the immune response. It binds to receptors on activated T cells and triggers their proliferation (Watkins and Cohen, 1987). IL-2 is known to be present in *Xenopus* (Ruben *et al.*, 1985). TCGF supernatants are thought to contain a variety of cytokines, including IL-2 (Turner *et al.*, 1991).

In an MLR the cells are being stimulated by the presence of allogeneic MHC molecules, which would presumably result in expression of receptors for different

cytokines, for example IL-2. The target of TCGF, T lymphocytes, are the responding cells in MLR (Du Pasquier and Horton, 1976; Horton and Manning, 1972); any cytokines produced by the ConA stimulated cell, still present in the medium could therefore lead to increased stimulation of the responding cells.

It appears that any TCGF produced by the ConA pre-stimulated cells is no longer present in the above assays, as freshly taken LG15 cells have not been stimulated by the presence of the irradiated ConA pre-cultured LG15 lymphocytes (SI $\approx$ 2.5; dpm $\approx$ 77). The TCGF was probably removed when washing the cells prior to setting up in MLR and no more produced following irradiation.

This experiment also shows that the LG15 cells have not been markedly altered by the ConA stimulation, making them appear 'foreign' to isogeneic cells.

3.4.1.3 Flow cytometric studies of the concanavalin A stimulated cells

The increased stimulation properties shown by the ConA pre-incubated cells in MLR studies presented here, together with previous published data (Harding, Flajnik and Cohen, In press), suggested a possible change of cell surface antigens on these cells. Flow cytometric analysis was therefore carried out on these cells, concentrating on the level of expression of class II MHC molecules and T-cell surface receptors.

This revealed that the ConA stimulated cells were larger than non-stimulated or freshly-harvested cells. An increase in the number of T cells following incubation with ConA is also indicated. Surprisingly, following incubation of the LG15 splenocytes with ConA for 3 and 7 days, there was an almost complete loss of T cells bearing the putative $\alpha\beta$ T-cell receptor. This could possibly be due to endocytosis of the $\alpha\beta$ TCR by activated T cells, without re-expression.

As expected (Flajnik and Du Pasquier, 1990), the anti-class II MHC mAb AM20 stained approximately 100% of the LG15 splenocytes. However, there was little sign of any increased expression of class II in the short term ConA studies reported here. Indeed, the mean fluorescence was highest for the LG15 cells

incubated without ConA than those with, possibly explaining why such cells are better stimulators in MLR's than fresh cells. If the high SI's in MLR's are solely due to class II MHC expression on the stimulators, then enhanced SI's would have been expected to have been seen with medium pre-cultured stimulators rather than with ConA-activated stimulators. As this was not the case, it appears that increased class II MHC expression alone does not explain the very high SI's given by the ConA pre-incubated stimulators, compared to the slightly raised SI's given by the stimulators pre-incubated in medium alone.

After three days in culture with ConA there was a small peak of very brightly stained class II MHC positive cells. This may be significant as the MLR's were carried out over a three day period. This peak of brightly stained class II positive splenocytes had disappeared by 7 days *in vitro* with ConA. An MLR set up using stimulators that had been cultured for 10 days with ConA failed to show the increased stimulation seen when using 3 day ConA pre-cultured stimulators (data not shown). This could relate to the loss of the bright peak of AM20 positive cells.

3.4.2 Flow Cytometric Studies on *In Vitro* Allostimulated Cells

In Chapter 2, changes in the expression of certain cell surface markers (for example, elevation of D12.2 positive cells) were noted in the spleen as a result of allografting. MLR was utilised here to further examine these cell surface receptor changes. The main advantages of this *in vitro* approach are that cell maturation or change of cell surface markers of T cells can be observed without the complexities arising due to cell migration to, or from, the spleen.

Allografting LG15 animals with LG5 skin prior to setting up in MLR with LG5 stimulators, resulted in an increased proliferation of the responding cells, compared with MLR's where the responders came from non-grafted *Xenopus*. This is in agreement with previous studies (Barlow and Cohen, 1981). The results show that the LG15 animals had been sensitised to the LG5 haplotype, *in vivo*, giving a secondary MLR.

In vivo grafted animals re-stimulated with the same donor cell type *in vitro* showed a significant increase in cells staining with the putative anti-γδ TCR mAb, D12.2 (Ibrahim *et al.*, 1991), following *in vitro* allogeneic challenge, for 9-14 days. This consequence of allostimulation has been observed elsewhere in Chapter 2, section 3.2. The increase in the number of γδ TCR positive cells was not seen after only 3 days in culture or after incubation with isogeneic cells.

The assay protocol used in the above assays (*in vivo* allogeneic stimulation by skin grafting followed by *in vitro* allostimulation) is similar to that used to generate allospecific cytotoxic effector cells for use in cell mediated lympholysis (CML) assays (Horton, Horton and Varley, 1989; Lallone and Horton, 1985; Bernard *et al.*, 1979). Following graft rejection and subsequent restimulation in MLC, splenic T cells become competent to kill donor target cells *in vitro* (DuPasquier, Schwager and Flajnik, 1989), therefore cytolytic cells would be present in these cultures. In mammals and chickens γδ TCR positive cells are known to possess a number of cytolytic properties (Han Cheng *et al.*, 1991; Haas, Kaufman and Martinez-A, 1990; Lefrancois *et al.*, 1990; Marüsïc-Galësïc *et al.*, 1988), thus the rise of γδ TCR positive cells, here, could reflect a rise of alloreactive cytotoxic effector cells, which are active in CML's.

Although the functions of αβ TCR positive cells in mammals are well characterised, relatively little is known of the functional properties of γδ TCR positive cells in mammals. Accumulating evidence has shown that γδ cells exhibit typical effector functions of the T lymphocytes (cytolysis of target cells and release of lymphokines), as well as recognising a wide range of ligands, including classical and non-classical MHC antigens, bacterial stress proteins, and some self-antigens (Han Cheng *et al*, 1991; Haas, Kaufman and Martinez-A, 1990; Ferrick *et al.*, 1989).

There is at least one human γδ cytolytic T-lymphocyte line which is specific for MHC class I-like molecules (Han Cheng *et al.*, 1991) and murine γδ cell lines reactive with allogeneic MHC molecules have been found (Matis, Cron and Bluestone, 1987). This alloreactivity includes recognition of MHC class II I-E and

class I H-2D-encoded molecules. Moreover, γδ cell lines which recognise non-polymorphic MHC class I antigens encoded within the TL and Q regions, commonly known as class Ib molecules have been described (Han Cheng *et al.*, 1991; Matis, Cron and Bluestone, 1987). MHC unrestricted, NK-like recognition by γδ-receptor positive cells has also been reported (Moingeon *et al.*, 1987; Moingeon *et al.*, 1986). MHC class II-restricted antigen recognition has been suggested for γδ TCR/CD8 positive intraepithelial lymphocytes (IEL's) (Lefrancois *et al.*, 1990).

Many of the putative γδ TCR positive spleen cells found in *X. laevis* which had been grafted with LG15 skin followed by 9 days culture with irradiated LG15 cells, appeared to represent T lymphoblasts. Although it seems likely that *Xenopus* putative γδ TCR positive cells carry out a role in the response to the allogeneic stimulators, a study of over 2000 murine T cell hybridomas revealed many alloreactive αβ T-cell hybridomas, but there were no alloreactive γδ T-cell hybridomas. Also, no alloreactivity has been found amongst chicken γδ T-cells (Haas, Kaufman and Martinez-A, 1990). The increase of putative γδ TCR positive cells shown here following allogeneic stimulation may reflect a different role for these cells in the evolutionary more primitive vertebrate *Xenopus*. *Xenopus* γδ TCR positive cells may exhibit properties more commonly associated with the αβ TCR in higher vertebrates.

Since it is known that γδ receptor positive cells are very responsive to IL-2 in mice (Bell, 1989) and that these express IL-2 receptors (Han Cheng *et al.*, 1991), the addition of the TCGF to *Xenopus* MLR cultures may have contributed to the increase seen in D12.2 positive cells. The TCGF was added after one week *in vitro* and the increase of γδ receptor positive cells was only seen in allogeneic stimulated splenocyte populations taken for staining after this time.

The findings in this Chapter add further weight to the results shown in Chapter 2, namely that the rise in the putative γδ-receptor positive T cell population in the spleen following allostimulation, is a property of the graft rejection process.

This may indicate that γδ TCR positive cells play a more important role in alloreactivity in *Xenopus* than they do in more evolutionary advanced mammals and birds. These cells now require further investigation to probe their precise role in the *Xenopus* immune system.

Table 3.1 Disintegrations per minute (dpm) ± standard error (SE) following MLR of splenocytes from *in vivo* grafted and non-grafted LG15 animals stimulated by irradiated LG5 splenocytes.

MLR (4 day)	dpm ± SE	
	Experiment 1	Experiment 2
LG15[a] v R LG5	3108 ± 141	6448 ± 749
LG15[a] v R LG15	970 ± 222	2155 ± 966
LG15[b] v R LG5	4440 ± 326	3489 ± 1952
LG15[b] v R LG15	934 ± 207	627 ± 326
R LG15 v R LG15	108 ± 38	166 ± 82
R LG5 v R LG5	77 ± 10	112 ± 26

LG15[a] = splenocytes taken from non-grafted LG15 animals; LG15[b] = splenocytes taken from LG15 animals which had rejected an LG5 skin graft; R = irradiated cells; v = incubated with.

Table 3.2 Summary of SI's and increase over background[a] disintegrations per minute (δdpm) of MLR's where the irradiated LG15 stimulators had been freshly taken or pre-cultured, with or without ConA.

MLR (4 day)	Experiment 1		Experiment 2		Experiment 3	
	SI	δdpm	SI	δdpm	SI	δdpm
X. laevis v R LG15	8	509	1.7	61		
X. laevis v R LG15[b]	16	1159				
X. laevis v R LG15[c]	56	4116	58	4591	42	3311

a = see section 2.3.1 for explanation of control cultures; LG15 = cells taken on the day the MLR was established; LG15[b] = cells incubated for 48 hours in medium before the MLR was established; LG15[c] = cells incubated for 48 hours with ConA before the MLR was established; R = irradiated; v = incubated with.

Table 3.3 Summary of the thymidine incorporation following *in vitro* incubation of ConA-activated LG15 splenocytes; effect of irradiation.

Cell culture[a]	dpm at indicated days of incubation			
	4	5	6	7
LG15[b]	177	318	278	155
R LG15[b]	101	74	79	115
LG15[c]	33,321	56,318	45,495	26,911
R LG15[c]	8092	3028	1265	1011
LG15[d]	37,197	68,403	50,215	24,205
R LG15[d]	6998	2869	683	1178

a= On day 2 the cells were treated with α-methyl mannoside and were either irradiated or not, the cells were pulsed with [^{3}H]TdR 24 hours prior to harvesting; b = cells incubated in L-15 medium alone prior to irradiation; c = cells incubated in complete L-15 medium and 2.5μg/ml ConA prior to irradiation; d = cells incubated in L-15 medium and 5μg/ml ConA prior to irradiation.

Table 3.4 Summary of thymidine incorporation of *X. laevis* following ConA stimulation and irradiation.

Cell culture[a]	dpm ± SE	
	Experiment 1	Experiment 2
X. laevis[b]	1400 ± 152	3342 ± 162
R *X. laevis*[b]	183 ± 12	326 ± 51
X. laevis[c]	1251 ± 22	2564 ± 1026
R *X. laevis*[c]	201 ± 21	120 ± 45
X. laevis[d]	72,314 ± 2193	87,736 ± 6387
R *X. laevis*[d]	14,488 ± 602	7044 ± 3291

a = cells pulsed 4 hours after irradiation and harvested 24 hours later; b = cells freshly-harvested on the day of irradiation; c = cells incubated for 48 hours prior to irradiation; d = cells incubated for 48 hours with ConA prior to irradiation; R= irradiated.

Table 3.5 Summary of thymidine incorporation of *X. laevis* ConA-activated cultures at 120 hours total incubation, which were irradiated after 48 hours pre-culture and re-stimulated with ConA.

Cell culture	dpm ± SE	
	Experiment 1	Experiment 2
X. laevis[a]	364 ± 86	398 ± 79
X. laevis[a] + ConA	24,808 ± 1130	25,421 ± 1157
R *X. laevis*[a]	54 ± 12	66 ± 4
R *X. laevis*[a] + ConA	64 ± 15	86 ± 25
X. laevis[b]	850 ± 207	1157 ± 193
X. laevis[b] + ConA	42,143 ± 3033	44,992 ± 5013
R *X. laevis*[b]	66 ± 4	93 ± 42
R *X. laevis*[b] + ConA	1190 ± 166	307 ± 115
X. laevis[c]	2017 ± 229	10,714 ± 2897
X. laevis[c] + ConA	19,622 ± 3471	66,628 ± 4295
R *X. laevis*[c]	168 ± 100	137 ± 22
R *X. laevis*[c] + ConA	11,623 ± 1323	2665 ± 147

a = cells freshly-harvested on the day of irradiation; b = cells pre-cultured for 48 hours prior to irradiation; c = cells pre-cultured for 48 hours with ConA prior to irradiation; R = irradiated.

Table 3.6 Summary of the SI's and δdpm[a] of MLR's to investigate whether the increased SI's, seen after using ConA pre-cultured irradiated stimulators, is due to a 2-way MLR occurring.

MLR	dpm ± SE	SI	δdpm
X. laevis v R LG15[b]	4765 ± 443	58	4591
X. laevis v R LG15	116 ± 39	1.7	61
R LG15[b] v R *X. laevis*	432 ± 197	4	308
R LG15 v R *X. laevis*	106 ± 32	2	42.5

a = δdpm is calculated by dpm (responders v R stimulators) - dpm (responders v R responders) - dpm (R responders v R responders); b = cells incubated with ConA prior to the MLR; R = irradiated; v = incubated with.

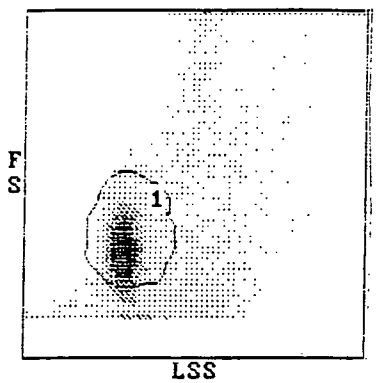
Table 3.7 Summary of the SI's and δdpm of MLR's to investigate whether the increased SI's, seen after using ConA pre-cultured stimulators, is due to release of cytokines.

MLR	Experiment 1		Experiment 2	
	SI	δdpm	SI	δdpm
X. laevis v R LG15[a]	42.0	3311	10.0	874
LG15 v R LG15[a]	1.4	32	3.5	122

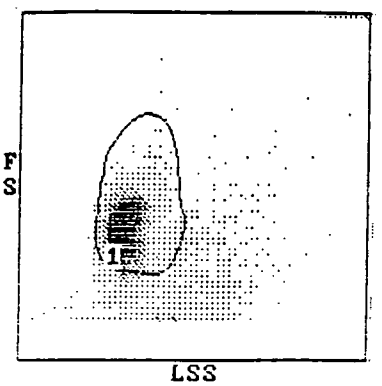
a = cells incubated with ConA prior to MLR; R = irradiated; v = incubated with.

Figure 3.1 Dot plots of LG15 spleen cells stained when freshly-harvested and after 3 days in culture with or without ConA - showing increased cell size after incubation with the mitogen.

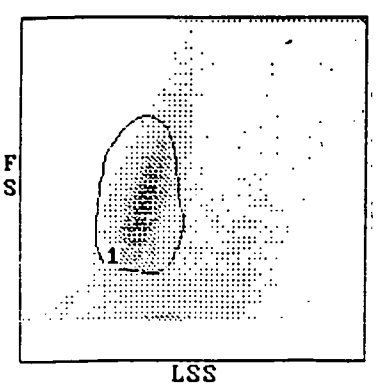
(A) LG15 splenocytes freshly-harvested.



(B) LG15 splenocytes cultured in L-15 medium for 3 days prior to staining.



(C) LG15 splenocytes cultured in L-15 medium and 2.5µg/ml ConA for 3 days prior to staining.



FS = forward scatter ('cell size'); LSS = log side scatter ('cell granularity').

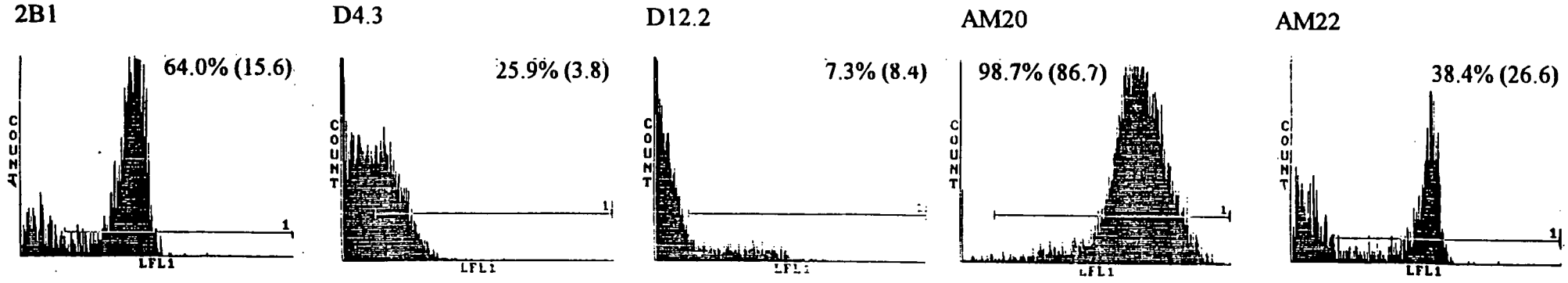
Table 3.8 Distribution of splenocyte phenotypes from LG15 animals, freshly harvested or following 3-7 days culture with or without ConA.

Treatment of cells prior to staining	Percentage of positive stained cells[a]						
	mAb's used						
	2B1	D4.3	D12.2	AM22	AM20	8E4:57	FJ17
Freshly harvested cells	64			38	99	15	25
Freshly harvested cells	50	27	8	26	98	12	18
3 days *in vitro*	60			34	94	16	23
3 days *in vitro* + ConA	72	4	6	28	99		29
7 days *in vitro* + ConA	75	5	5	37	99	5	

a = cells stained with FITC-labelled antibodies only

Figure 3.2 Histograms of LG15 splenocytes, freshly-harvested and after 3 and 7 days incubation with or without ConA. The percentage positive staining and mean fluorescence (in parenthesis) are indicated.

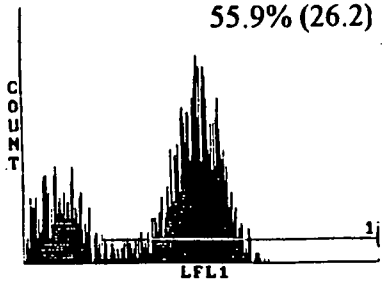
(A) LG15 splenocytes freshly-harvested.



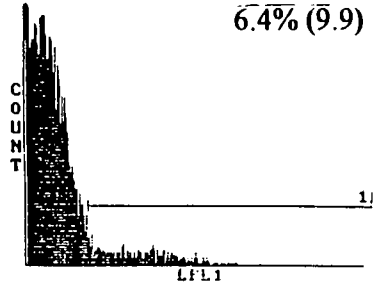
(B) LG15 splenocytes cultured in L-15 medium for 3 days prior to staining.

(C) LG15 splenocytes cultured in L-15 medium and 2.5μg/ml ConA for 3 days prior to staining.

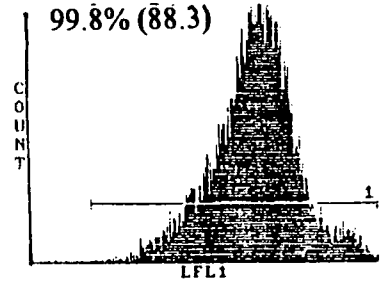
2B1



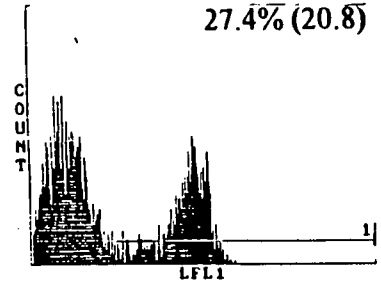
D12.2



AM20

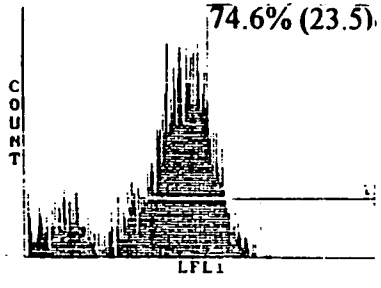


AM22

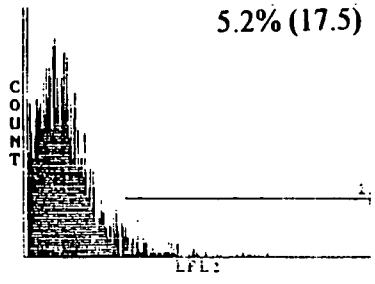


(D) LG15 splenocytes cultured in L-15 medium and 2.5μg/ml ConA for 7 days prior to staining.

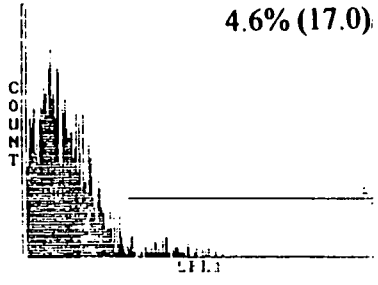
2B1



D4.3



D12.2



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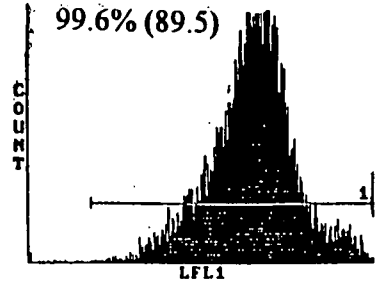


AM22

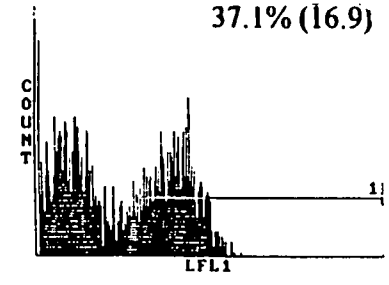


Table 3.9 Distribution of splenocyte phenotypes from skin allografted LG15 animals, either freshly harvested or after 14 days culture with irradiated stimulators.

Treatment of LG15 cells prior to staining		Percentage of positive stained cells					
		mAb's used					
in vivo	*in vitro*[a]	2B1	D4.3	D12.2	XT-1	AM22	8E4:57
	fhc	38 **59**	18 **35**	3 **8**	20	24 **35**	19
LG5 skin graft	fhc	40	12	3	17	28	14
	R LG15	52	20	4	24	27	13
LG5 skin graft	R LG5	59 **42**	28	16 **14**	30	25 **26**	16
LG5 skin graft	R LG15			 **10**		 **11**	

fhc = freshly-harvested cells; **Bold** = cell proportions from a repeat experiment; a = splenocytes cultured for 14 days with shown irradiated stimulators, first week in a 1:1 ratio and the second week in a 10:1 ratio (Experiment 1 described in section 2.4.2.1); R = irradiated.

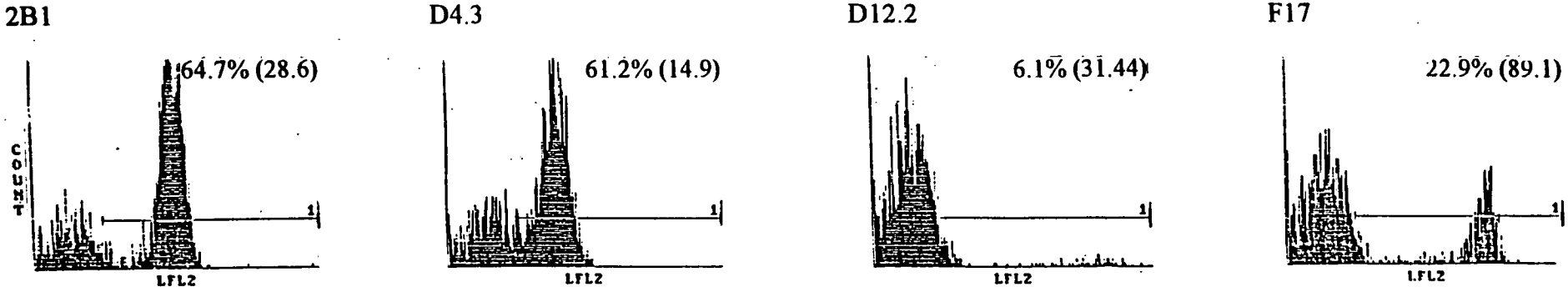
Table 3.10 Distribution of T cell splenocyte phenotypes of skin allografted *X. laevis*, taken fresh or after 3 or 9 days culture with irradiated stimulators.

Treatment of *X. laevis* cells prior to staining		Percentage of positive stained cells			
		mAb's used			
in vivo	*in vitro*	2B1	D4.3	D12.2	F17
Autograft	fhc	65	61	6	23
LG15 skin graft	R LG15[a]	38		7	
Autograft	R LG15[a]	55	43	4	22
LG15 skin graft	R LG15[b]	77	54	19	
	[b]	57		6	

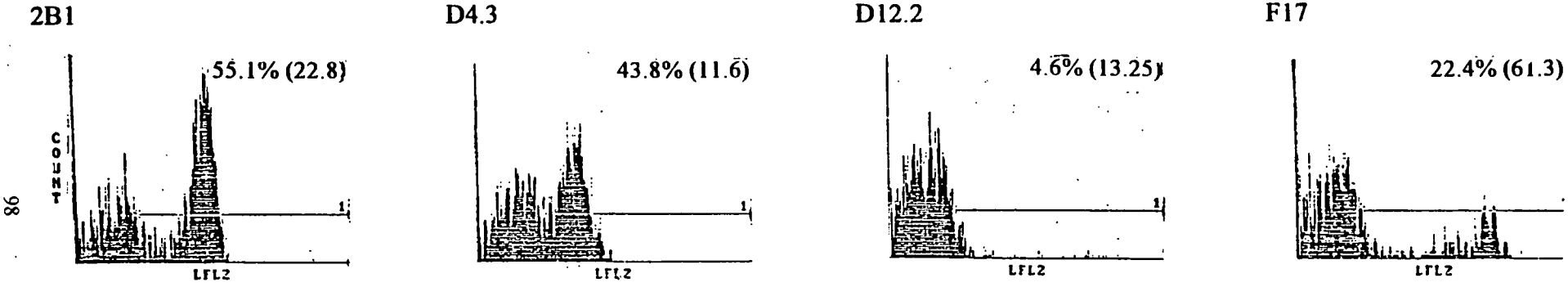
fhc = splenocytes taken on the day of flow cytometry; Experiment 2 described in section 2.4.2.2 - a = splenocytes cultured for 3 days with shown stimulators; b = splenocytes cultured for 9 days alone or with shown irradiated stimulators; R = irradiated.

Figure 3.3 Histograms of *X. laevis* splenocytes freshly-harvested or following *in vivo* and *in vitro* allostimulation. The percentage positive staining and mean fluorescence (in parenthesis) are indicated.

(A) *X. laevis* splenocytes freshly-harvested

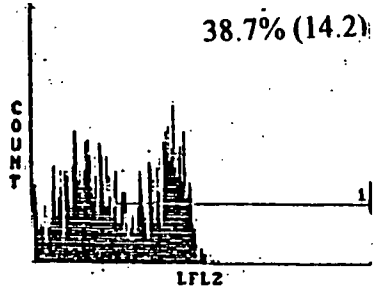


(B) *X. laevis* splenocytes cultured with irradiated LG15 stimulators for 3 days prior to staining.

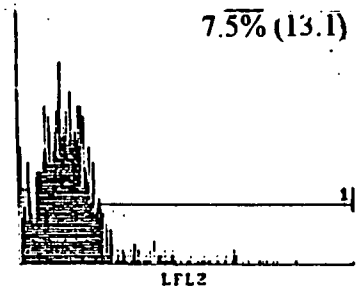


(C) *X. laevis* splenocytes, following *in vivo* LG15 skin graft rejection (20 days) and culture with irradiated LG15 stimulators for 3 days prior to staining.

2B1

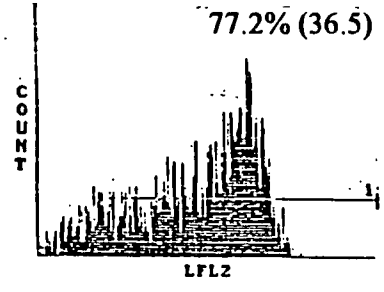


D12.2



(D) *X. laevis* splenocytes, following *in vivo* LG15 skin graft rejection (20 days) and culture with irradiated LG15 stimulators for 9 days prior to staining.

2B1



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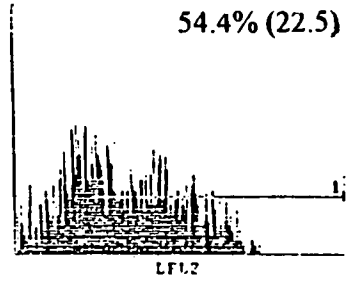


D12.2

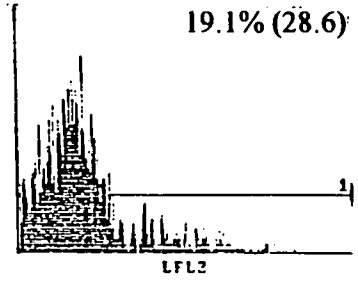
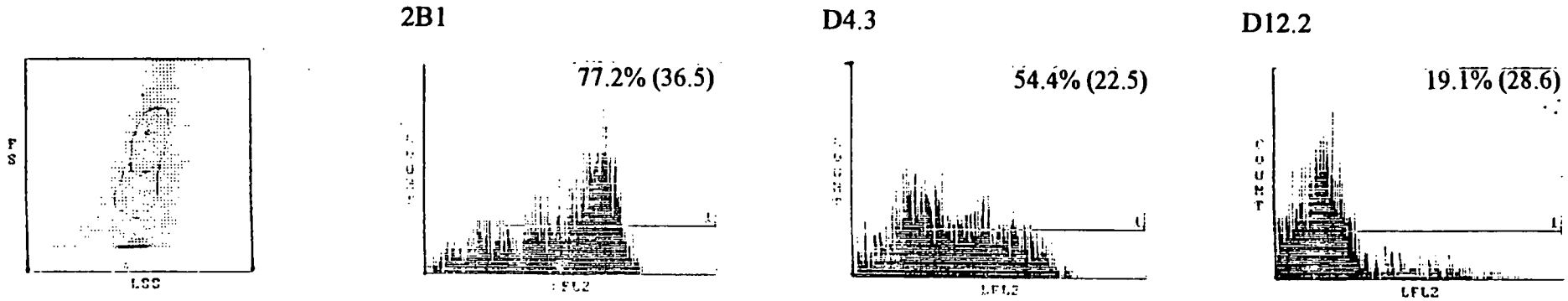
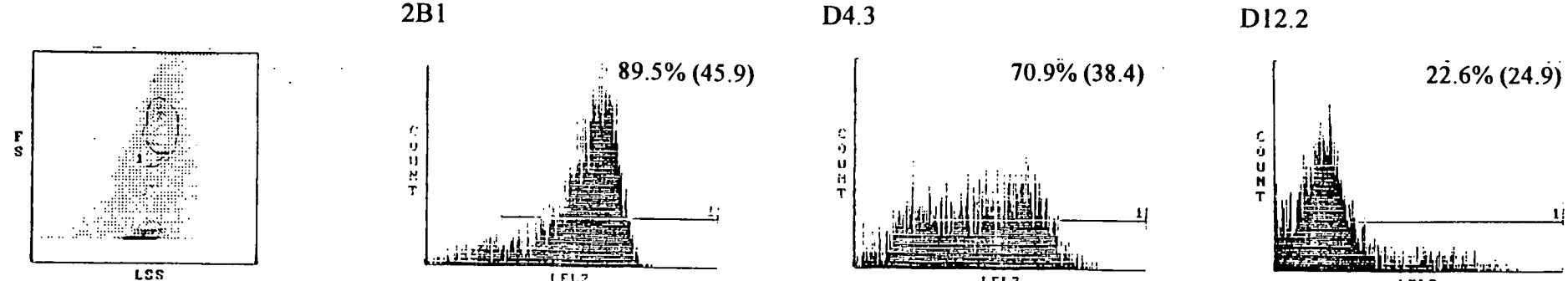


Figure 3.4 Dot plots and histograms of *X. laevis* splenocytes, taken from animals which had rejected an LG15 skin graft in 20 days and then were cultured with irradiated LG15 splenocytes for 9 days - showing 2 populations of lymphocytes. The percentage positive staining and mean fluorescence (in parenthesis) are indicated.

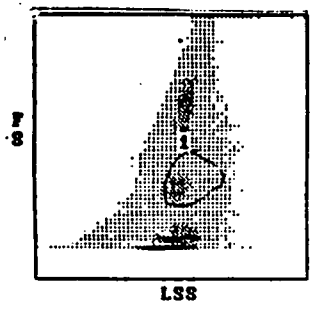
(A) Entire lymphocyte population



(B) Larger lymphocyte population



(C) Smaller lymphocyte population



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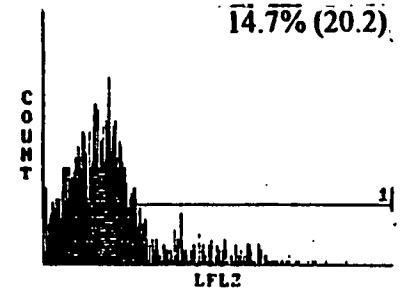


CHAPTER 4

DOES STAPHYLOCOCCAL ENTEROTOXIN B INDUCE STIMULATION OF *XENOPUS* SPLENIC LYMPHOCYTES ?

4.1 INTRODUCTION

A group of mitogens in which there has been considerable interest in recent years, as they stimulate proliferation of specific clones of T cells in mice and humans, are the superantigens. These were first detected in the early 1970's, through their ability to stimulate a strong primary mixed lymphocyte reaction (MLR) between cells from mice of the same MHC haplotype (Festenstein, 1973). It has also been known for many years that certain bacterial enterotoxins are potent T cell mitogens (Peavy, Adler and Smith, 1970).

There are 2 main groups of superantigens, those produced endogenously by mice (minor lymphocyte stimulating (Mls) determinants), and toxins produced by certain bacteria of the gut. Among the best studied enterotoxins are those produced by *Staphylococcus aureus*; these include a number of structurally related, small basic proteins of 20-30 kDa, termed staphylococcal enterotoxin (SE) A, B, C1, C2, C3, D and E (Drake and Kotzin, 1992; Herman *et al.*, 1991).

The bacterial enterotoxins have been implicated as the causative agents in a number of diseases, for example the staphylococcal enterotoxins cause almost a quarter of food poisoning cases in the United States. Also the organisms that produce these toxins have been implemented in certain autoimmune disorders, for example, *Mycoplasma arthriditis* which causes arthritis in rats.

There are 3 characteristics common to all superantigens. Firstly, they are Vβ TCR family specific (Marrack and Kappler, 1990; White *et al.*, J., 1989). Secondly, they are MHC class II dependent, that is they need to be bound to class II in order to activate T cells (Tomai *et al.*, 1992; Qasim, Kehoe and Robinson, 1991). All MHC

class II haplotypes will bind superantigens although some are more efficient than others (Robinson, Pyle and Kehoe, 1991). Thirdly, T cells recognise the intact superantigen on the MHC, that is superantigens are not processed and presented in the antigen presenting groove of the MHC like conventional antigens (Herman *et al.*, 1991). Also there is no requirement for accessory molecules, such as CD4 and CD8, on the surface of T cells to effect stimulation (Quaratino *et al.*, 1991).

Superantigens are so named as they stimulate virtually all T lymphocytes bearing specific T cell receptors (TCR's). TCR's are coded for by 5 variable gene segments, Vα, Jα, Vβ, Dβ and Jβ, all of which contribute to the specific interaction of T lymphocytes with conventional peptide antigens presented in the context of MHC molecules. There are potentially millions of possible combinations of these genetic elements and so the frequency of responding T cells to a given antigen is usually very low, only a fraction of 1% of all T cells. Superantigens stimulate such large numbers of T cells, as many as 5-25% of a mouse's T cell repertoire, because they stimulate virtually all T cells expressing particular Vβ genes (Kappler *et al.*, 1988; Pullen, Marrack and Kappler, 1988). These superantigens are not acting as mitogens as in the case of concanavalin A, as not all clones of T cells are activated. In mice SEB stimulates virtually all T cells bearing Vβ3 and Vβ8.1, 8.2 and 8.3 epitopes, plus a few others. Administration of SEB to neonatal mice results in elimination of all mature and some immature T cells bearing these Vβ elements, resulting in tolerance, by clonal deletion, to SEB (White *et al.*, 1989). Similarly there are 'gaps' in the Vβ repertoire of mice which reflect the Vβ regions which Mls elements stimulate. It is thought that endogenous superantigens may have evolved to eliminate T cells which would be reactive to exogenous superantigens (Janeway, 1990).

Superantigens combine with MHC class II to form ligands that stimulate T cells (Herman *et al.*, 1991; Marrack and Kappler, 1990). They stimulate via sites that are distinct from those involved in presentation of conventional antigens: they do not bind to the groove on the surface of the MHC (Dellabona *et al.*, 1990). Instead, it is thought that they act like a vice clamping the antigen presenting cells to T cells (see

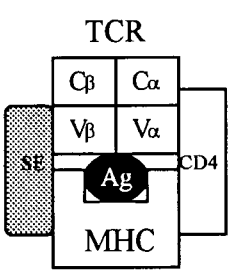
Figure 4.1), resulting in T cell activation (Janeway *et al.*, 1989; Marrack and Kappler, 1990).

Although the MHC allele is not important for superantigen presentation, several of the SE's show differential binding to class II isotypes. For example, most of the SE's appear to bind with higher affinity to murine I-E than to I-A (Yagi *et al.*, 1990).

Metabolically active antigen presenting cells (APC's) are required for stimulation of optimal T cell proliferation, by the superantigen streptococcal M protein, since active APC's are capable of producing co-stimulatory signals, for example cytokines. However, metabolically inactive APC's are capable of presenting SEB to T cells, therefore the requirements for presentation of different superantigens clearly vary (Tomai *et al.*, 1992).

There are no published reports on whether *Xenopus* lymphocytes respond to these antigens. Therefore it was decided to carry out some preliminary experiments to determine if *Xenopus* lymphocytes are stimulated by superantigens.

Figure 4.1 The current consensus model of responses to staphylococcal enterotoxins (SE) showing binding of the superantigen to the outer faces of class II MHC and the Vβ region of the TCR (Janeway, 1990).



4.2 MATERIALS AND METHODS

Splenic lymphocytes from 6 month old outbred *X. laevis* were plated out in V-bottomed 96 well tissue culture plates and the appropriate quantity of SEB (Sigma) added to give a range of concentrations. All cultures were carried out in triplicate. The cells were cultured in amphibian strength L-15 complete medium (see Chapter 2, section 2.5) at 26°C, in a humidified atmosphere of 5% CO_2 in air, for 2-6 days. One μCi [^{3}H]TdR was then added per well and the cells incubated for a further 24 hours. The cells were then harvested and thymidine incorporation assessed, as in Chapter 2, section 2.5.

Various parameters of the assay were investigated, including concentration of SEB, splenocyte concentration, the period of culture before pulsing and percentage of foetal calf serum (FCS) added to cultures.

4.3 RESULTS

There has been some indication that *X. laevis* lymphocytes respond weakly to SEB at doses above 10^{-6}M and the response decreases as the dose becomes more dilute (Table 4.1). The response to SEB appears to be strongest at 3 days (Figure 4.2) when the cells are cultured at $1x10^5$cells/well (Table 4.1), with 10^{-5}M SEB (Table 4.2), in L-15 complete medium. Supplementing the media with higher than 1% FCS made little difference to the stimulation index. Higher doses of FCS resulted in a general increase in disintegrations per minute as a result of the mitogenicity of this medium supplement.

Control cultures, stimulating the cells with ConA, were set up in conjunction with all the assays and these all gave high stimulation indices (SI ≈ 102, Table 4.2), indicating that the T lymphocytes in the cultures were viable.

The increase in disintegrations per minute of the activated cultures from the control culture was analysed by Students' t test to determine if there was a significant

increase in lymphocyte proliferation following incubation with SEB (Appendix F). This gave a p value of 0.1 with 4 degrees of freedom. This indicates that the maximum proliferation seen is significantly different from the negative control and that the superantigen SEB exerts only minimal mitogenic influence on *Xenopus* lymphocytes.

4.4 DISCUSSION

Initial studies have shown that *Xenopus* T cells respond only weakly to SEB. It appears thymidine incorporation is greatest at 3 days incubation with 10^{-5}M SEB (Figure 4.2). Under similar assay conditions to those used with murine T cell lines, *Xenopus* splenocytes showed a 400-fold lower proliferation rate. This may indicate that *Xenopus* class II molecules or TCR's, or both, may have a lower affinity for SEB, than mice. The affinities for the enterotoxins are usually within the nanomolar range, so only very few SE molecules need to be bound to a class II positive cell for it to trigger a T cell response (Herman *et al.*, 1991). The lower proliferation may also be due to inappropriate culture conditions. The assays with *Xenopus* have used a mixed population of cells from the spleen, compared to the T cell lines used in most murine and human studies. Enrichment of T lymphocytes (by panning or cell sorting) would seem advisable prior to subsequent experiments with *Xenopus* cells.

In mice and humans the superantigen SEB has been found to activate T cells polyclonally in an MHC class II dependent, but haplotype unrestricted manner (Drake and Kotzin, 1992; Herman *et al.*, 1991; Marrack and Kappler, 1990). The biochemical and functional attributes of *Xenopus* MHC and TCR molecules are quite similar to those of mammals (see Chapter 1, sections 1.2.1 and 1.3.3). Additionally there are clues that indicate the basic structure of the *Xenopus* MHC is also fundamentally the same as those seen in mammals. For example, rabbit anti-human class II β chain antibody can precipitate *Xenopus* class II MHC molecules (Kaufman *et al.*, 1985), indicating sequence similarity. Also very recently, cDNA clones for *Xenopus* class II β chain have been identified which show structures fundamentally similar to their mammalian counterparts and nearly 50% amino acid homology with mammalian class II β chains (Sato *et al.*, 1993). Although the *Xenopus* MHC α chain has some N-terminal sequence homology with mammalian α chains, the genes encoding the α chains have yet to be cloned (Flajnik and Du Pasquier, 1990; Du Pasquier, Schwager and Flajnik, 1989).

Although activation of T cells by SEB is haplotype unrestricted there is a preference for certain MHC isotypes, for example SEB is preferentially presented by DR and I-E antigens expressed on humans and mice APC's respectively. Although antibodies to mammalian class II molecules cross-react with *Xenopus* class II there is no evidence for *Xenopus* isotypes homologous to the mammalian families, DQ/I-A or DP. However an antibody directed against human DR antigens has been shown to be capable of immunoprecipitating almost all of the same class II molecules on *Xenopus* cells as various alloantisera reactive with the class II molecules of several different haplotypes (Kaufman, Flajnik and Du Pasquier, 1985) suggesting that *Xenopus* has DR-like molecules. There is also evidence for the presence of I-E-like molecules in frogs, since I-E negative but not I-E positive mice produce class II specific antibodies after immunisation with frog lymphocytes (Flajnik *et al.*., 1990; Shinohara *et al.*, 1981).

In mammals SEB activates T cells via the Vβ elements of their TCR. It does not bind the TCR where conventional peptides bind, but engages Vβ on an exposed face, a region predicted to be a β pleated sheet (Marrack and Kappler, 1990). *Xenopus* may not possess Vβ elements which recognise SEB. As the regions to which SEB binds are outside the conventional antigen binding site for both the MHC and TCR molecules, the sequences of these regions may not have been strictly conserved between mammals and amphibians. This may indicate why culture with SEB resulted in low proliferation.

Staphylococcal strains are natural pathogens of mice and humans. It is possible, therefore, that these organisms have evolved so their toxins specifically bind sequences found on mammalian class II molecules, sequences not present in *Xenopus* class II molecules. It appears that their ability to cause disease comes from their property to activate a large number of T cells. *S. aureus* toxins do bind less well to mouse class II than to human class II proteins, since *S. aureus* is indigenous to humans. Indeed a different species, *S. xylosis*, is found in mice, so the differential

binding affinities may be the consequence of evolutionary divergence of the bacteria with their hosts.

Further work will clearly have to be carried out to investigate whether *Xenopus* T lymphocytes can be made to proliferate more readily in response to SEB. Confirmation that it is T cells which are responding could be achieved by use of Tx animals and by the use of mAbs which block T cell markers or class II antigens. These techniques have been used very successfully to reveal that the *in vitro* mixed lymphocyte response in *Xenopus* is mounted against class II MHC proteins (Harding, Flajnik and Cohen, 1993).

Table 4.1 Stimulation indices of splenic lymphocytes following incubation, at different cell concentrations, with SEB for 3 days.

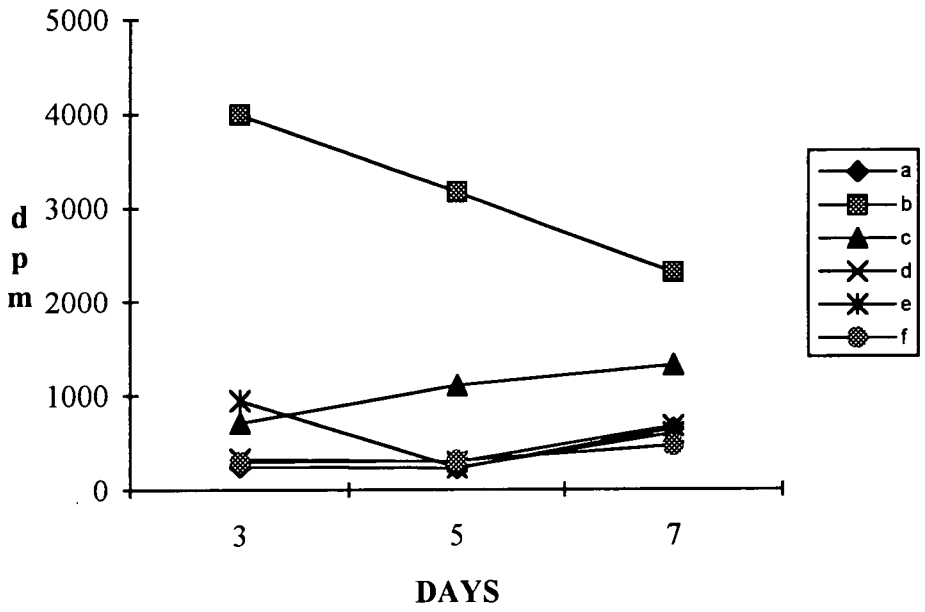
Dose SEB (Molar)	SI at various cell concentration (cells/ml)		
	2×10^4	1×10^5	3×10^5
10^{-5}	3	16	8
10^{-6}	1	2	2
10^{-7}	1	1	1
10^{-8}	1	1	1
10^{-9}	1	1	1

Table 4.2 Typical dose response of splenic lymphocytes[a], when cultured at $1x10^5$cells/well, with SEB for 3 days.

Dose SEB (Molar)	dpm ± SE[b]	SI
0	402 ± 138	
10^{-4}	2193	5.5
10^{-5}	3900 ± 2819	10.0
10^{-6}	981 ± 423	2.4
10^{-7}	487 ± 110	1.2
10^{-8}	545 ± 348	1.3
10^{-9}	40 ± 99	1.0

a = these cells were also cultured with 2.5μg/ml ConA in L-15 complete medium, which gave dpm = 35,859 ± 4,029 and SI = 89; b = n = 3, except where the cells were cultured with 10^{-4}M SEB. Here only one well was tested due to lack of cells.

Figure 4.2 Kinetics of the response of *X. laevis* splenocytes (1 x 10^5 cells/well) to various concentrations of SEB following incubation for 3, 5 and 7 days.



where a = the negative control, b-f = incubation with 10^{-5}M, 10^{-6}M, 10^{-7}M, 10^{-8}M and 10^{-9}M SEB.

CHAPTER 5

CONCLUDING REMARKS AND FUTURE WORK

The aim of this thesis was to investigate developmental aspects of the *Xenopus* immune system, especially T cell development and transplantation immunity. Work in Chapter 2 concentrated on the effects of *in vivo* allostimulation on the distribution of T cell surface antigens within the adult splenic population. Intact, thymectomised and skin tolerant *Xenopus* were used in these flow cytometric experiments, which employed a range of new monoclonal antibodies. The proliferative response of cultured *Xenopus* splenocytes to irradiated allogeneic stimulus was examined in Chapter 3 by carrying out thymidine incorporation studies. Such MLR's employed responder splenocytes from non-grafted or grafted animals and also compared the use of normal or T cell mitogen activated, irradiated stimulator cells. Surface antigenic profiles of cultured cells were again monitored by flow cytometry. The effect of thymectomy on alloreactivity, T cell mitogen responsiveness and on T cell phenotypes was also briefly assessed. In Chapter 4 preliminary experiments probing the response of *Xenopus* splenocytes to the superantigen, staphylococcal enterotoxin B (SEB), were carried out.

The major findings of this work and suggestions for follow-up experiments are discussed below.

5.1 Skin Graft Rejection

As expected intact *X. laevis*, LG3 and LG15 animals acutely rejected allografts, while 5-day-thymectomised *X. laevis* and LG3 tolerated or chronically rejected allografts. Control LG3 animals given foreign skin in larval life subsequently tolerated secondary grafts from the same donor, but could reject third party grafts. Skin allografting LG15 animals with LG5 skin prior to restimulation in MLR resulted

in an increased thymidine incorporation of the responding cells, compared with MLR's where the responders came from non-grafted animals. This showed that the LG15 animals had been sensitised to the LG5 haplotype, giving a secondary MLR.

5.2 Mixed Leukocyte Reactions

Pre-incubation of stimulator cells with ConA enabled these to affect very high levels of responder cell proliferation. Reasons for such enhanced stimulatory capacity were studied by carrying out flow cytometry of the stimulating cells, with particular emphasis on the level of expression of class II MHC molecules and T cell antigen specific markers. The possibility of elevated cytokine production by the T-cell-mitogen activated stimulators was also investigated. It was shown that the increase in stimulation did not appear to be solely due to cytokines in the medium or to correspond to any difference of cell surface antigen expression, as detected by flow cytometry, of the cells incubated with ConA. Also, there was little indication of any increased expression of class II in the short term ConA studies reported here. The reasons for the elevated MLR achieved by the use of ConA-pre-incubated stimulators therefore remain to be assessed, although it would appear from the work of Harding *et al.* that increased class II MHC expression will be a likely causative factor (Harding, Flajnik and Cohen, 1993). Blocking experiments with the anti-class II MHC monoclonal antibodies could be carried out to see if this reduces the high stimulation indices seen.

The ultimate goal of these assays was to probe the possibility of developing a CML assay using larval cells, where it is not readily possible to go through graft rejection (which takes 3 weeks) prior to *in vitro* MLR/CML. A CML assay in larvae would show if cytotoxic cells exist in the class I deficient larva.

5.2.1 Expression of the putative αβ T-cell receptor following incubation with concanavalin A

The complete loss of the putative αβ T cell receptor positive (TCR) cells, following incubation with ConA, was interesting. This could be further investigated to see if this affects stimulatory properties of these cells. To see if there is a correlation between loss of αβ TCR positive cells and increase in allo-stimulation. The loss of αβ TCR positive cells could possibly make the stimulating cells' MHC class II molecules more accessible, thus increasing the allostimulation.

5.3 Expression of the Putative γδ T-Cell Receptor following *In Vivo* and *In Vitro* Allostimulation

Flow cytometry of the *in vivo* and *in vitro* allostimulated splenocytes from normal *Xenopus* revealed no significant changes in the level of expression of any of the cell surface antigens examined, except for an increase in the number of cells staining with the monoclonal antibody D12.2, the putative γδ TCR positive cells. It also appears that these γδ TCR positive cells are active in the alloimmune response, as many of the γδ TCR positive splenocytes from *X. laevis* (which had been cultured for 9 days with irradiated LG15 cells) appeared to represent T lymphoblasts.

Flow cytometry of LG3 animals which had been induced to become specifically tolerant to certain MHC haplotypes, following application of skin allografts during larval life, revealed no obvious difference in the splenocyte phenotypes of these populations, compared with those from animals not rendered tolerant. That is to say, there was no obvious decrease in the spleen cell numbers or level of expressions of any of the observed cell surface markers, thought to be involved in allograft rejection. Furthermore, no increase in γδ expression was seen in the spleen when tolerant animals were given a new graft from the donor that induced tolerance. This suggests that where such an increase occurs in normal animals this is not simply due to properties of foreign skin unrelated to rejection events, rather it is associated with the rejection process. The increase in expression of the γδ-receptor

when third party allografts were given to these allo-tolerant animals further confirms this suggestion.

It is thought that the γδ receptor evolved before the αβ complex and that the latter represents an advance, linked with the evolution of the thymus. It would be interesting to further explore the appearance and function of γδ TCR positive cells in *Xenopus*, following allostimulation, in intact and Tx animals, by flow cytometry and transplantation reactions. By use of a fluorescence activated cell sorter (FACS) it would be possible to purify these γδ TCR positive cells and therefore determine whether they respond to the conventional T cell mitogens PHA and ConA. It may also be possible to inject the sorted γδ TCR positive cells into isogeneic animals to determine whether this enhances their response to allogeneic skin grafts.

The increase of putative γδ TCR positive cells, shown here following allogeneic stimulation, may reflect a different role for these cells in the evolutionary more primitive vertebrate *Xenopus*. It may indicate that γδ TCR positive cells play a more important role in alloreactivity in *Xenopus* than they do in more evolutionary advanced mammals and birds. *Xenopus* γδ TCR positive cells may exhibit properties more commonly associated with the αβ TCR in higher vertebrates.

5.4 Response of *Xenopus* Splenocytes to the Superantigen, SEB

Chapter 4 showed that *Xenopus* T cells could respond weakly to the superantigen SEB. The induced proliferation rate was 400-fold less than that seen in mice under similar assay conditions, suggesting a lower affinity of either *Xenopus* class II MHC molecules or TCR's, or both, for the superantigen than those of mice.

Further work is required to investigate whether *Xenopus* T lymphocytes can be made to proliferate more readily in response to SEB, by changing the assay conditions, for example. Also the response of *Xenopus* T cells to other superantigens, for example SEA or SEE could be investigated. Confirmation that it is T cells which are responding can be achieved by use of Tx animals and by the use of monoclonal antibodies which block T cell markers or class II antigens. These techniques have

been used very successfully to reveal that the *in vitro* mixed lymphocyte response in *Xenopus* is mounted against class II MHC proteins (Harding, Flajnik and Cohen, 1993).

Appendix A - Amphibian Phosphate Buffered Saline

Double distilled water	1000ml
Sodium chloride (NaCl)	6.6g
Di-sodium hydrogen phosphate (Na_2HPO_4)	1.5g
Potassium di-hydrogen orthophosphate (KH_2PO_4)	0.2g

For immunostaining wash buffer add 1g bovine serum albumin
1g sodium azide (NaNs)

Adjust to pH 7.4

Appendix B - Gating of Samples for Fluorescence Activated Cell Sorter Analysis

Splenocyte suspensions were stained with the FITC control, CT3 and/or the PE control, Ig-PE and run through the flow cytometer. From this a dot plot was obtained (Figure B1) of forward scatter (cell size) against the logarithm of side scatter (cell granularity). A gate could be manually placed around the concentrated lymphocyte population (labelled as 1 in Figure B1), to exclude the larger and more granular cell populations, for example erythrocytes.

A histogram (Figure B2 - Coulter Epics flow cytometer) or a dot plot (Figure B3 - Becton Dickinson FACS Scan) of fluorescence intensities, was derived from this gated lymphocyte population containing 10,000 events. Markers were set using the FITC- or PE-controls (1 in Figure B2 and quadrants in Figure B3), so that the equivalent of 97-98% of the CT3 and/or Ig-PE labelled cells would not be included as positively stained cells during analyses. This eliminated events, due to non-specific binding of the mAb's, being included in the counts.

Figure B1 Typical dot plot obtained when splenocytes stained with CT3 or Ig-PE controls are run through the flow cytometer, showing the gate around the concentrated lymphocyte population (1).

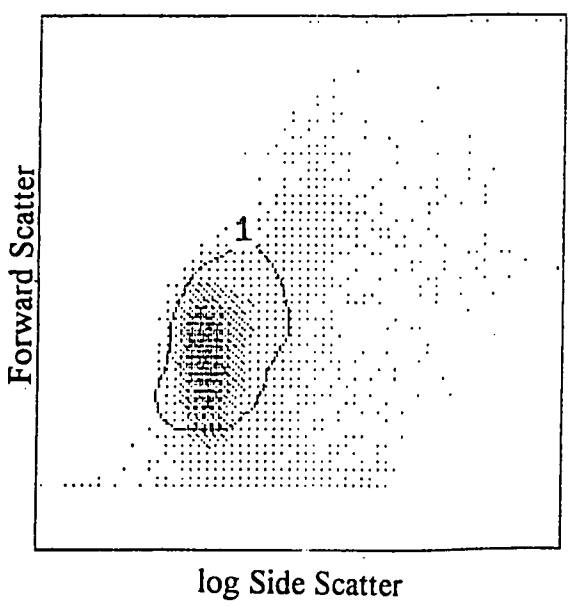
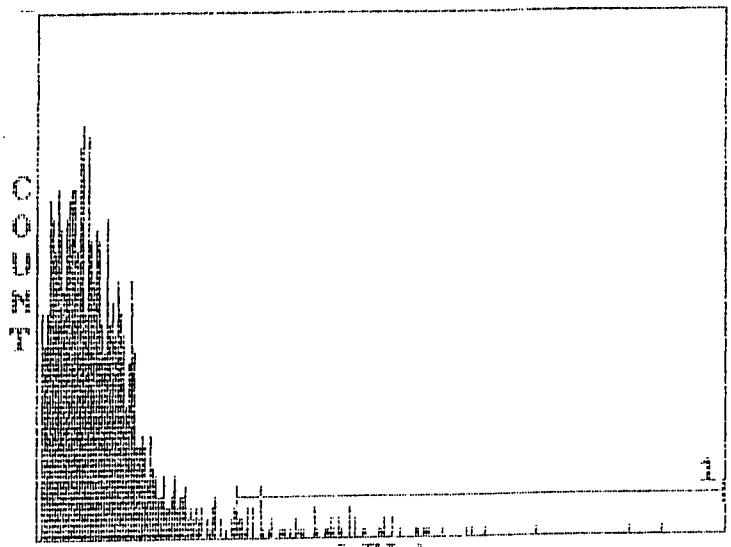
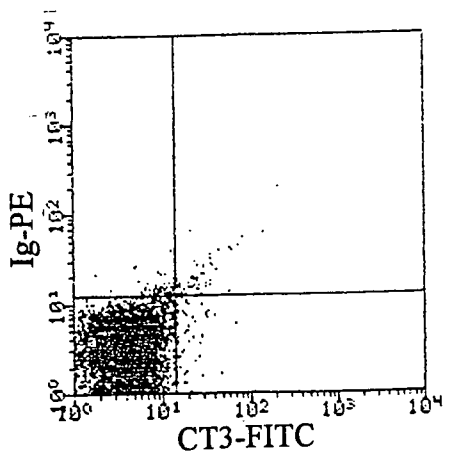


Figure B2 Typical histogram obtained, on the Coulter Epics flow cytometer, following gating as in Figure B1, showing the marker (1) set to remove 98% of the non-specifically labelled cells from being included in the analyses.



MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
7.391	1023.	265	2.0	21.43	2.99	0.17

Figure B3 Typical dot plot obtained, on the Becton Dickinson FACS Scan, following gating as in Figure B1, showing the automatic placing of quadrants to remove 98% of the non-specifically labelled cells from being included in the analyses.



----- Quad Stats -----
File: U3:T1N28-8013 Sample: 013
Date: 8/28/92 Gate G1= R1
Parameters: FL1-H(LOG),FL2-H(LOG) Quad Location: 14.33,11.97
Total= 10000 Gated= 7070

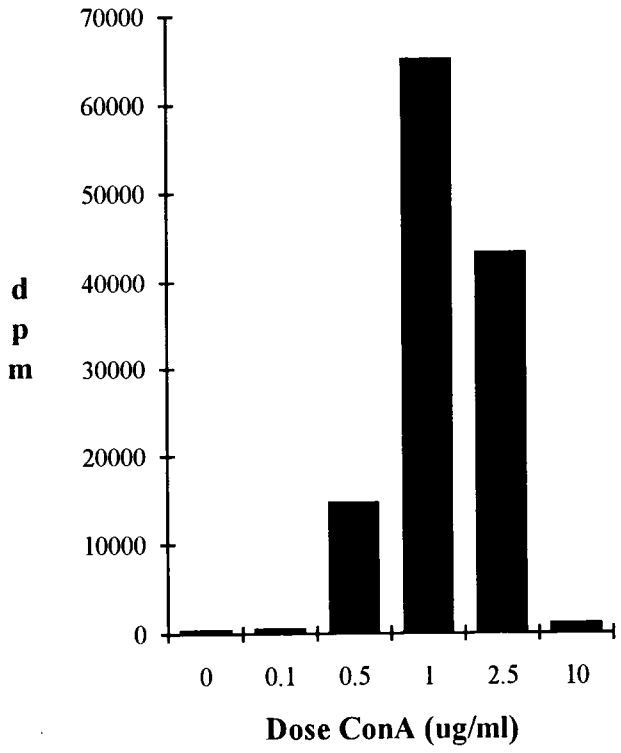
Quad	Events	% Gated	% Total	Xmean	Ymean
1 UL	70	0.99	0.70	9.18	14.83
2 UR	56	0.79	0.56	27.93	21.01
3 LL	6833	97.36	68.33	3.97	2.99
4 LR	61	0.86	0.61	20.58	4.09

Appendix C - Determination of the Optimal Dose of ConA to Induce T Cell Proliferation

A suspension of splenic lymphocytes was prepared and these were plated out in V-bottomed 96 well tissue culture plates at $1x10^5$ cells/well or in 24 well tissue culture plates at $3x10^6$ cells/well and the appropriate quantity of mitogen added, to give a range of concentrations. The cells were cultured at 26°C for 2 days, then 1μCi [^{3}H]TdR was added per well and the cells incubated for a further 24 hours. The cells were then harvested and thymidine incorporation assessed as in Chapter 2, section 2.5.

The optimal dose at $1x10^5$ cells/well, in a 96 well V-bottomed plate was either 1 or 2.5μg/ml ConA (Figure C1) and in a 24 well plate, at $3x10^6$ cells/well the optimal dose was 2.5μg/ml ConA.

Figure C1 Dose response of *X. laevis* splenic T cells to ConA at $1x10^6$ cells/well in V-bottomed 96 well plates



Appendix D - Calculation of the Radiation Dose, from the Cobalt-60 Source, used to Irradiate MLR Stimulator Cells

In order to calculate the dose rate at various distances from the source the Inverse Square Law is used:

Dose rate at an unknown distance from the Co-60 source (Krads/hr)

$$= \left(\frac{\text{Known distance (cm)}}{\text{Unknown distance (cm)}}\right)^2 \text{ x dose rate at known distance from the Co-60 source (Krads/hr)}$$

By use of a curve of the change in dose rate of the Cobalt-60 source over 5.25 years (Figure D1), the dose rate at 8cm from the source in January 1992 was 80.025 Krads/hr.

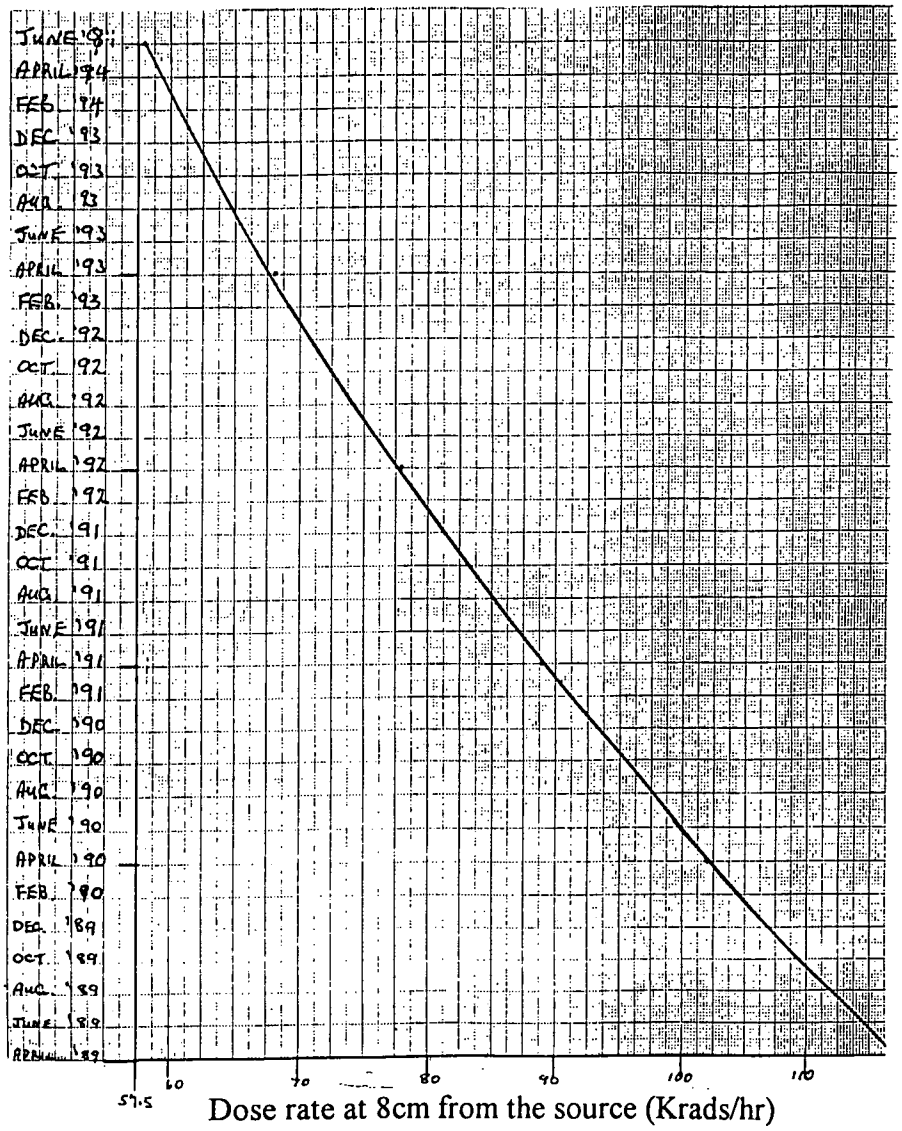
Therefore the dose rate at 16cm from the source

$$= \left(\frac{8}{16}\right)^2 \text{ x } 80.025$$

$$= 20.006 \text{ Krads/hr}$$

$$= 6.668 \text{ Krads/20 minutes}$$

Figure D1 Change in dose rate of the cobalt-60 source over 5.25 years



Appendix E - Dose of Irradiation Required to Inactivate ConA Stimulated Cells

This was carried out in order to confirm that following irradiation the T cell mitogen stimulated cells are unable to proliferate and therefore are suitable for use as MLR stimulators.

Splenocytes from 1 year old *X. laevis* and LG46 were cultured in L-15 complete medium with or without 2.5μg/ml ConA, for 48 hours. Each culture was then split into 6 aliquots and irradiated at 5 different doses, ranging from 5300 rads to 8700 rads, with one aliquot left unirradiated. The cells were subsequently plated out in triplicates at $1x10^5$ cells/well, in a 96 well V-bottomed plate and pulsed with 1μCi [^{3}H]TdR after 4 hours incubation. The cells were incubated for a further 24 hours and thymidine incorporation assessed as in Chapter 2, section 2.5.

Table E1 shows that the T cells had been stimulated by the ConA after 48 hours incubation. Thymidine incorporation was greatly reduced after irradiation at all doses. It was calculated using the half life of the cobalt-60 source that 16cm from the source gave 6,668rads in 20 minutes. This was chosen as the dose to be given as 16cm was a convenient distance from the source.

Table E1 Tritiated thymidine incorporation of splenic lymphocytes following ConA stimulation and irradiation.

Dose radiation (rads)	dpm of the splenocytes 24 hours post irradiation			
	LG46 + ConA	LG46	*X. laevis* + ConA	*X. laevis*
0	5558	85	874	71
5300	318	94	107	76
5900	269	70	162	69
6668	279	145	107	57
7590	525	74	71	71
8700	260	91	162	58

Appendix F - Students t test to Determine if there was a Significant Increase in Lymphocyte Proliferation following Incubation with SEB

For the dpm of the cells cultured with 10^{-5}M SEB, the calculation was as follows:

$$\text{variance } (s_c^2) = \frac{[\Sigma X_1^2 - (\Sigma X_1)^2/ N_1 + \Sigma X_2^2 - (\Sigma X_2)^2/ N_2]}{N_1 + N_2 - 2}$$

$$s_c^2 = \frac{[61.47 \times 10^6 - 1.36 \times 10^8/3 + 5.24 \times 10^5 - 1.45 \times 10^6/3]}{3 + 3 - 2}$$

$$s_c^2 = \frac{1.588 \times 10^7}{4}$$

$$s_c^2 = 3.969 \times 10^6$$

The value for t was then calculated using the value for s_c^2 by use of the following formula:

$$t = \frac{X_1 - X_2}{s_c\sqrt{1/N_1 + 1/N_2}}$$

$$t = \frac{3900 - 402}{1992.2\sqrt{1/3 + 1/3}}$$

$$t = 2.15$$

With 4 degrees of freedom, $p = 0.1$

Therefore the maximum proliferation seen is weakly significantly different from the negative control, indicating that the superantigen SEB exerts only a small mitogenic influence on *Xenopus* lymphocytes.

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