Ontogeny, thymus dependence and in vitro stimulation of lymphocyte subsets in Xenopus

Gravenor, Ian

Ontogeny, Thymus Dependence and *In Vitro* Stimulation of Lymphocyte Subsets in *Xenopus*

Ian Gravenor, BSc.

A Thesis submitted to the University of Durham
for the Degree of Doctor of Philosophy
Department of Biological Sciences,
University of Durham

August 1996

I would like to dedicate this Thesis to my parents in appreciation of all their help and support over the years

Abstract

This Thesis investigates the possibility of an extrathymic pathway of T cell development in the amphibian, *Xenopus laevis*. Initial studies examined the ontogenetic development of T cell surface antigen expression on both splenocytes and thymocytes in larvae at 7 days through to 6-8 month old adults, using the technique of dual-colour flow cytometry and employing a panel of T and B cell specific monoclonal antibodies. Flow cytometric analysis of splenocytes from thymectomized (Tx) *Xenopus* was then addressed. This revealed that early (5-7 day) larval thymectomy resulted in the ablation of T cell surface antigen expression, as defined by the monoclonal antibodies 2B1 (anti-CD5), AM22 and F17 (both anti-CD8), XT-1 (anti-XTLA-1) and D4.3 (putative anti-αβ T cell receptor). Lack of these markers was still evident in 8 month old Tx frogs, confirming the effectiveness of the operation.

In vitro studies showed that no T cell marker expression could be induced on the surface of splenocytes from Tx animals following stimulation with concanavalin A (ConA), phytohaemagglutinin (PHA) or the potent mitogenic agent, phorbol myristate acetate (PMA). Studies were also carried out to investigate whether *in vitro* stimulation induced apoptosis. Flow cytometric studies revealed that $CD5^{dull}$ expression could be induced on splenocytes from control *Xenopus* following stimulation with PMA. The nature of this induced $CD5^{dull}$ expression was investigated further in order to determine why this phenomenon was only seen in control animals. These experiments involving mixing of T and B cell populations, revealed that $CD5^{dull}$ expression was being induced upon the surface of *Xenopus* B cells, and that this PMA-induced expression required the presence of T cells, and was blocked by a protein kinase C (PKC) inhibitor.

Finally, an additional search for extrathymic T cells involved examining the intestine and liver of both control and Tx *Xenopus*, these tissues being sites of extrathymic T cell development in higher vertebrates. The intestine of control *Xenopus* was shown to contain T lymphocytes with a surface phenotype distinct to that found in spleen or liver. Studies in Tx *Xenopus* showed that although expression of some T cell markers was ablated in liver and gut, $CD5^{dull}$ and $CD8^{dull}$ (as determined by the mAb AM22) lymphocytes persisted in these organs. However, proliferative studies showed that these 'T-like' cells were unable to respond to mitogenic stimulation with ConA, suggesting that they are not functional T cells.

Declaration

No part of this Thesis has been previously submitted in support of an application for the degree of Doctor of Philosophy or equivalent qualification at the University of Durham, or any other University or Institute of Higher Education.

Some of the material presented in Chapters 2 and 4 of this Thesis has previously been reported in the following publications:

"Ontogeny and Thymus-Dependence of T Cell Surface Antigens in *Xenopus*: Flow Cytometric Studies on Monoclonal Antibody-Stained Thymus and Spleen" by Ian Gravenor, Trudy L Horton, Pamela Ritchie, Emma Flint and John D Horton. Developmental and Comparative Immunology 19(6): 507-523 (1995)

"Immune System Development in *Xenopus*" by John D Horton, Trudy L Horton, Pamela Ritchie, Ian Gravenor, Karen Horsham, pp 131-147 (1996). In: Modulators of Immune Responses ed by Stolen, J S; Fletcher, T C; Bayne, C J; Secombes, C J; Zelikoff, J T; Twerdok, L E; Anderson, D P. SOS Publications, New Jersey.

Statement of Copyright

Acknowledgements

I would firstly like to say thank you to John Horton, my supervisor, who has given me help and support throughout my time in his laboratory, and shared his knowledge and experience with me. Thanks go also to Pam (who will always be known as Lumpy to me!), Trudy and Karen who have all provided help, friendship and much needed support - both in the lab, and especially during the writing up of this Thesis.

I would also like to thank Ian Dimmick for sharing his vast knowledge on flow cytometry, especially DNA analysis, Susan Skinner for her help and use of the Epics Profile II flow cytometer at Dryburn Hospital, and to Paul Sidney for photographic work. Thanks must also go to Roche for their kind gift of the protein kinase C inhibitor used in studies found in Chapter 4, and to BBSRC for funding this project.

A very big thank you must go to my parents and to my brother Andy, not only for their financial support, but also for their encouragement and faith in me.

Lastly, I would like to thank several people who have kept me sane and and have always been there when I needed a friend: Gav, who should have had me certified a long time ago - thanks a million; Debbs, thank you for being there; and finally to all those people too numerous to mention but have given me their support and friendship - thank you!!

List of Abbreviations

Ab	Antibody
ABC	Avidin-biotin-peroxidase complex
Ag	Antigen
APBS	Amphibian phosphate buffered saline
APC	Antigen presenting cell
ASP	Apoptosis specific protein
BSA	Bovine serum albumin
CaIon	Calcium ionophore
CD	Cluster of differentiation
CMF	Calcium and magnesium free medium
ConA	Concanavalin A
DAB	3'3'-diaminobenzidine
DAG	Diacylglycerol
DPM	Disintegrations per minute
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme linked immunosorbent assay - check
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FS	Forward light scatter
GALT	Gut associated lymphoid tissue
HCG	Human chorionic gonadotrophin
HEV	High endothelial venule
HSA	Heat stable antigen
IEL	Intraepithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP_3	Inositol 1,4,5-trisphosphate
kDa	kilo Dalton
L-15	Leibovitz 15 medium
LN	Lymph node
LPS	*E coli* lipopolysaccharide

mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MS222	3-aminobenzoic acid ethyl ester
NK	Natural killer cell
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PI	Propidium iodide
PIP_2	4,5-bisphosphate
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PP	Peyer's patches
RAG-1	Recombination activating gene 1
SCID	Severely combined immunodeficient
SI	Stimulation index
SS	Side scatter
TCR	T cell receptor
^{3}H-TdR	Tritiated thymidine
TD	Thymus dependent
Th	T helper lymphocyte
TID	Thymus independent
Tx	Thymectomized

Contents

Chapter 1

1 General Introduction

This Chapter focuses attention on mammalian T cell development within the thymus and examines the controversy of extrathymic T cell maturation, presents background literature on $CD5^+$ B cell development and on apoptosis in the developing immune system. These areas are highlighted as they provide the rationale for why the experimental work in this Thesis was carried out, and why the immune system of *Xenopus* was chosen for these studies. Specific introductions to the experimental work, reviewing the appropriate literature, are given in Chapters 2-5.

1.1 Mammalian T Cell Development: intrathymic events

The mouse embryo thymus consists entirely of epithelial cells at 10 days gestation. Lymphoid stem cells then enter the thymus in waves (Jotereau et al, 1987; Coltey et al, 1987), beginning at around 11 days gestation. By 13 days there are around 1,000 cells which increase to $10x10^6$ by 18-19 days, the cortical and medullary regions being visible from 14 days. Until the thymus has developed a blood supply, thymocyte precursors enter the thymus by pushing through the thymic capsule. Once the thymus has been vascularised, thymocytes enter via the high endothelial venules (HEVs) at the cortico-medullary junction (Owen and Ritter, 1969). By 15 days the first thymocytes can be seen scattered throughout the thymus, and by 17 days the thymocytes appear to be organised with many small lymphocytes present in the cortex, and later appearing in the medulla.

1.1.1 Thymocyte Subsets

Within the thymus are 4 main lymphoid populations based on their surface expression of CD4 and CD8. These are $CD4^-CD8^-$ ('dual negative') thymocytes which account for 2-4% thymocytes, $CD4^+CD8^-$ or $CD4^-CD8^+$ ('single positive') which account for 5-10% of thymocytes, and the remainder (70-80%) are 'dual-positive', $CD4^+CD8^+$. Immature thymocytes, which include $CD4^-CD8^-$ and $CD4^+CD8^+$ populations and also some $CD4^-CD8^+$ cells - see below, can also be

identified by their high expression of peanut agglutinin (PNA) receptors, and these are usually found within the cortex. These cells also express the enzyme, nuclear terminal deoxynucleotidyl transferase (TdT), which is involved in the generation of junctional diversity during gene rearrangement of the T cell receptor (TCR) (Rothenberg, 1980). The $CD4^-CD8^-$ ('dual-negative') thymocytes can be further divided by their surface expression of the heat stable antigen (HSA), a marker found on many haemopoietic cells. Those cells that are $CD4^-CD8^-HSA^+$ tend to show precursor activity (have not fully differentiated or become committed to one lineage) and express low levels of CD5 (Rothenberg, 1980; Fowlkes et al, 1985). Those cells that are $CD4^-CD8^-HSA^-$ lack any precursor activity, tend to express high levels of CD5, and many surprisingly express the αβ TCR (Budd et al, 1987). Double-positive thymocytes ($CD4^+CD8^+$) tend to express high levels of HSA and low levels of major histocompatibility complex (MHC) I proteins and low levels of αβ TCR (Scollay et al, 1984; Roehm et al, 1984), and it appears that these double-positive thymocytes are intermediates between progenitor cells and mature single-positive T cells (Fowlkes et al, 1988; MacDonald et al, 1988a; Guidos et al, 1989), which express high levels of αβ TCR on their surface.

Although $CD4^-CD8^+$ cells are usually a sign of fully matured cytotoxic Tc cells, immature (no TCR expression) $CD4^-CD8^+$ thymocytes are also apparent early in ontogeny (Kisielow et al, 1984) at day 15-16 but very quickly acquire the $CD4^+CD8^+$ phenotype (MacDonald et al, 1988a). The transition from $CD4^-CD8^+$ to $CD4^+CD8^+$ occurs only once the cell expresses a fully rearranged αβ TCR, and the thymocyte stops dividing and is able to undergo thymic selection (Richie et al, 1988). Thymocytes that are $CD4^-CD8^-$ or $CD4^-CD8^+$ are located just beneath the thymic capsule, whilst $CD4^+CD8^+$ thymocytes are found deeper in the cortex. It is thought that dendritic cells found at the cortico-medullary junction act to screen the developing thymocytes for self-reactivity (negative selection) (Farr et al, 1985) and mature $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells can be found within the medulla.

1.1.2 Thymocyte Selection of αβ TCR^+ Cells

In order for a T cell to express a functional T cell receptor (TCR), the gene segments encoding the variable regions of the receptor (V, J (and D)) must rearrange such that one of each type is assembled. The first V region genes to be rearranged are those coding for the TCR β chain, α chain genes are rearranged subsequently. This recombination of V, (D) and J gene segments for α and β chains leads to the production of T cells with a wide range of specificities (combinatorial diversity). These regions are joined together by enzymes coded by recombinase activating

genes (eg. RAG-1) and by the enzyme, TdT. During this joining together nucleotides can be lost or gained from coding sequences, thereby further increasing the diversity of the specificity of the TCR formed.

The αβ TCR is used by mature T cells to recognise antigen, but only antigenic determinants that are presented in association with major histocompatibility complex (MHC) proteins expressed on the surface of an antigen presenting cell (APC). Mature T cells that exit the thymus have undergone selection procedures that only allow self-tolerant thymocytes to develop, whilst destroying any potentially autoreactive cells by the process of apoptosis (von Boehmer, 1992). If the TCR V region possesses high affinity binding to self antigen in association with self MHC, the thymocyte will be deleted (Kappler et al, 1987), a phenomenon called negative selection. In some mice, self-reactive T cells are not deleted in the thymus, and can be found in the periphery. However, these potentially self-reactive T cells are usually 'non-responsive' or 'anergic'. Although the molecular mechanism promoting anergy remains not fully resolved, it appears that peripheral anergy is promoted when a T cell interacts with its ligand in the absence of appropriate co-stimulatory signals (Schwartz, 1990; Roitt et al, 1995). There are several theories surrounding the induction of clonal anergy in developing thymocytes. Firstly, it could be that different epithelial cells in the thymus have different functions, for example, one type of epithelial cell may promote positive and negative selection, whilst others would induce anergy. Another possibility is that like peripheral anergy, thymocytes need a co-stimulatory signal as well as TCR-MHC II/Ag interactions - if this signal is not present, anergy is induced. This leads to the problem that if the same interactions that promote anergy are also used in positive selection, what is to stop all developing thymocytes becoming anergised. One possibility is that positive selection and anergy occur at different times in T cell ontogeny. This would mean that peripheral and thymic anergy both rely on TCR-MHC II/Ag interactions in the absence of the co-stimulatory signal - and that the thymic epithelial cells are not capable of providing this signal (Ramsdell and Fowlkes, 1990).

Positive selection of useful αβ TCR^+ T cells occurs in the thymus and involves testing thymocytes for their reactivity towards self MHC. Those cells that recognise self MHC and associated peptide with low affinity are selected and therefore survive, whilst those that do not recognise self MHC fail to survive. It appears likely that it is the affinity between the TCR and MHC that determines which T cells survive and which are deleted - those cells with a low affinity will be selected, whilst those with a strong affinity would be deleted as this could potentially lead to

autoimmunity. Another possibility is that the nature of the stromal cell interacting with the T cell is crucial. There is evidence that thymic cortical epithelial cells promote positive selection, whereas interdigitating APCs promote negative selection (Anderson et al, in press).

The time when positive selection occurs is at the double-positive stage - $CD4^+CD8^+$ (von Boehmer, 1992; Janeway and Travers, 1996). The developing T cell, with rearranged β chains of its TCR, continues to rearrange α chain genes until the cell is positively selected or dies (Janeway and Travers, 1996). Whether the TCR of a double-positive cell interacts with MHC I or MHC II will determine whether single-positive $CD8^+$ or $CD4^+$ T cells are produced respectively.

1.1.3 γδ T Cells

Selection events described above have now been quite well characterised for αβ TCR^+ T cells, whilst the development and role of T cells with a γδ TCR remains somewhat a mystery. The development of T cells in the chicken thymus consists of three waves of stem cell colonisation (Coltey et al, 1987); γδ cells are produced during the first two waves and these cells migrate to the spleen and intestine (Dunon et al, 1993b). Similarly, in mice, there appears to be several waves of γδ cell development. Moreover each wave of cells uses a different Vγ gene family (Allison, 1993; Havran and Allison, 1988). The first two waves of $\gamma\delta^+$ T cells employs Vγ3 and Vγ4 genes and additionally show very little polymorphism in their TCRs. These γδ T cells have been shown to migrate from the thymus to skin (Vγ3) and the tongue and female reproductive tract (Vγ4), and their limited TCR specificities may be important in the recognition of damaged self-cells, where there is constant shedding of the epithelium, rather than being able to recognise a wide spectrum of foreign antigens (Havran et al, 1991; Janeway et al, 1988). Later γδ T cell populations express Vγ1 and Vγ2 gene products, these cells homing to the blood and lymphoid tissues, whilst those expressing Vγ6 are found in the intestine - although there is some controversy as to whether these intestinal cells arise extrathymically (Allison, 1993).

Of all the thymocytes within the thymus, only 0.5% express the γδ TCR, indicating that this is a very small population. However, Kelly et al (1993) estimated that up to 10^4 γδ cells leave the thymus every day, indicating that these cells play an important role in the immune system. Zorbas and Scollay (1993) found that the majority of γδ cells in the thymus expressed HSA and Thy-1, and were actively dividing, and as such were likely to be a precursor population of γδ cells. The expression of HSA is

not lost by these γδ T cells until they have exited the thymus and migrated into the periphery. However, a few γδ cells in the thymus do not express HSA, and this population is thought to be a distinct lineage and not simply recirculating γδ cells that have lost their HSA expression and have returned to the thymus.

The recognition of antigen by γδ cells may differ from that of αβ cells in that there is no requirement for antigen presentation by 'classical' MHC proteins (Bluestone et al, 1991). γδ cells may recognise antigen in a similar way that αβ cells recognise super-antigens (Allison, 1993). It has been shown that γδ cells recognise host-derived proteins that are induced by stress, for example, heat shock proteins (HSPs). These are produced in response to an elevation in temperature, viral infection or mycobacterial infections, and are usually found expressed on infected or damaged host cells (Takagaki et al, 1989). Therefore, this suggests that γδ cells have a role in host defence and recognise host-derived proteins rather than foreign antigens which are recognised by αβ cells.

The numbers of γδ cells in blood and most lymphoid tissues of mice are very low, and these T cells tend to be localised at epithelial surfaces. However, in sheep and cattle γδ cells form a major population of T cells late in fetal life and early adulthood (Mackay and Hein, 1991). In sheep, up to 60% γδ cells can be found in the blood at 140 days and this level is maintained for the first three months following birth (Hein et al, 1990). This level begins to drop until by 5-8 years old, there remains a population of 5-10% $\gamma\delta^+$ T cells.

1.2 T Cell Development in the Gut - An Extrathymic Pathway?

The mammalian intestine is home to the largest population of T cells in the body. Furthermore, recent research indicates that at least some of these T cells differentiate and undergo selection procedures within the intestine, and may have developed extrathymically (Poussier and Julius, 1994).

The important lymphoid areas in the intestine consist of the lamina propria (LP) which lies just beneath the basement membrane of the intestinal villi, the intraepithelial lymphocytes (IEL) which can be found above the basement membrane between the columnar epithelial cells, and the Peyers patches (PP) which are nodules of lymphocytes that are embedded into the gut wall.

Populations of T cells can be distinguished within the mammalian intestine on the basis of their surface expression of CD4 and CD8, similar to the situation in the thymus. Dual-negative ($CD4^-CD8^-$), dual-positive ($CD4^+CD8^+$) and single-positive ($CD4^+CD8^-$ and $CD4^-CD8^+$) populations are found. Those cells expressing CD4 are located mainly within the LP and are rare within the epithelium. Also found in the LP are T cells expressing the CD8 molecule composed of an α and β chain (ie. $CD8\alpha\beta^+$ cells) and most of these cells tend to express the αβ TCR and are Thy-1^+. Those T cells found within the gut epithelium are mostly $CD8^+$, but in contrast to the $CD8\alpha\beta^+$ cells in the LP, the epithelial T cells bear two α chains, ie. are CD8 $\alpha\alpha^+$. These $CD8\alpha\alpha^+$ cells can express either the αβ or γδ TCR, but lack expression of Thy-1. Also located within the epithelium are $CD4^+CD8^+$ cells expressing the α β TCR, but these cells bear the $CD8\alpha\alpha^+$ phenotype and are thought to be mature $CD4^+$ cells that have acquired the additional CD8αα phenotype after the cell had migrated to the gut environment (Guy-Grand et al, 1993). This induction of a CD8α α phenotype within the gut occurs on both TCR^- and TCR^+ cells, eg. on TCR^- lymphocytes which are the major population of lymphocytes in nude mice (Guy-Grand et al, 1991) and the only population in SCID mice (Guy-Grand et al, 1991; Croitoru et al, 1990).

Studies on intestinal lymphocytes of nude mice have revealed that there are approximately one eighth the number of lymphocytes in the gut compared to the gut of normal mice, and $CD4^+$ and $CD8\alpha\beta^+$ cells are lacking. Of the lymphocytes present in nude animals, only about 40% express CD3 (compared with the vast majority of lymphocytes in normal mice). The majority of $CD3^+$ cells in nude mice bear the γδ TCR. As the nude mice age, their gut lymphocytes increase in number and some cells do develop that bear the αβ TCR. Rocha et al (1992) have shown that if nude mice are injected with lymphocytes from the lymph nodes (LNs) of normal mice that are $CD4^+$ and $CD8\alpha\beta^+$, these cells can later be found in the gut alongside lymphocytes of host origin ($CD4^-CD8\alpha\alpha^+$). It is suggested that $CD4^-CD8\alpha\alpha^+$ cells can develop in the absence of thymic influence. Thus mice that were thymectomized (Tx) and then lethally irradiated, were given bone marrow that had been depleted of T cells. These Tx animals developed $CD4^-CD8\alpha\alpha^+$ cells within the gut that were shown to be of host origin (Guy-Grand et al, 1991) leading to the conclusion that those cells bearing the CD8αα homodimer are generated extrathymically. Lefrançois (1993) has shown that there is a lack of αβ TCR^+ T cells in the intestines of germ-free, nude mice and also in bone marrow-

reconstituted, adult-thymectomized (ATX) mice. However, if fetal liver is used as the donor source of progenitors in ATX mice, αβ TCR^+ IELs develop.

T lymphocytes that are $CD4^+$ and $CD8\alpha\beta^+$ originate in the thymus and migrate to the Peyers patches (PP) after exiting the thymus. These lymphocytes then constantly recirculate until they become activated. Studies have shown that upon activation, lymphocytes in the PPs migrate to the thoracic duct and mesenteric LNs, and ultimately appear within the lamina propria and gut epithelium (Guy-Grand et al, 1978). Once these T cells have migrated to the lamina propria and epithelium they are terminally differentiated and remain here for the rest of their lives. This is further supported by Guy-Grand et al (1991) who have shown that germ-free mice contain only small numbers of lymphocytes in their intestines, and that this population increases once the mice are exposed to antigen. Furthermore, the greater the amount of antigen that is encountered, the greater the number of developing intestinal lymphocytes. However, these developing lymphocytes are $CD4^+$ and $CD8\alpha\beta^+$, ie are thymus-dependent (TD). Lymphocytes bearing the CD8αα homodimer are not found in the thoracic duct of normal, nude or suckling mice, and these cells do not arise from activated lymphocytes from the PPs. Instead, it appears that CD8αα cells home directly to the gut from bone marrow precursors. However, these thymus-independent (TID) lymphocytes are also found in reduced levels in germ-free mice and appear to increase as the TD population increases, suggesting that the TID lymphocytes may be influenced by factors produced by the developing TD lymphocytes.

The suggestion that $CD8\alpha\alpha^+$ lymphocytes develop within the gut is supported by the finding that RAG-1, an enzyme required for the rearrangement of the TCR genes, can be detected in these cells (Guy-Grand et al, 1992). This enzyme is found in lymphocytes in the thymus where thymocytes are rearranging their TCRs, whereas RAG-1 cannot be detected in mature T lymphocytes that are in the periphery. Levels of RAG-1 mRNA are high in thymocytes where extensive TCR gene rearrangement occurs during selection, whilst lower levels are detected in the intestine indicating that although these cells are undergoing TCR gene rearrangement, there is less arrangement occurring.

The selection processes that educate thymocytes appear not to occur in the gut. In Mls-1^aIE^+ mice, thymocytes bearing the TCR Vβ6, Vβ8.1 and Vβ11 gene families are routinely deleted within the thymus and therefore T cells employing such genetic elements are not detected in the periphery. However, although in the intestine of

these mice, the TD lymphocytes show the same deletions of these Vβ families, the TID populations express these Vβ families that should have been deleted by normal thymus selection procedures (MacDonald et al, 1988b; Bill et al, 1989). The fact that these Vβ families are not deleted in T cells developing *in situ* in the intestine may suggest that these particular TCR types are important for recognition of gut-derived antigens (eg. superantigens of bacterial origin). Therefore these intestinal-derived 'T-like' lymphocytes may play an important role in the first line of defence against certain antigens (Rocha et al, 1991).

Although it has been shown that mammalian γδ cells can develop extrathymically (as shown by their appearance in nude mice with ageing), early in life most of the γδ cells in the intestine are derived from the thymus. These cells leave the thymus before they have fully developed and undergo development and differentiation at the intestinal epithelium (Lin et al, 1994). The development of intestinal γδ cells in the chicken (Dunon et al, 1993a) and sheep (Hein et al, 1990) has been shown to be entirely thymus-dependent. Thymectomy *in utero* of lambs results in the ablation of γδ intestinal T cells, whilst age-matched siblings have normal levels of these T cells (Hein et al, 1990; Hein and Dudler, 1993).

Although some of the above mammalian experiments point to the development of thymus-independent T cell development (Guy-Grand et al, 1993; Rocha et al, 1995), the thymus-dependent vs thymus-independent development of intestinal lymphocytes has been the subject of considerable debate. For example, most IELs bear the γδ TCR, express the CD8αα homodimer and lack the T cell markers CD5 and Thy-1 (Lin et al, 1993). This is in contrast to other peripheral γδ T cells which tend to be $CD8^-$ and mostly $CD5^+$ and $Thy\text{-}1^+$. However, there are equally convincing experiments to show that the murine thymus is crucial for gut T cell development (Wang and Klein, 1994; Lin et al, 1995; Lefrançois and Puddington, 1995). Wang and Klein (1994) showed that there was a complete lack of CD4, CD8β, Thy-1, CD5 and αβ TCR expression in neonatally thymectomized (NTX) mice. If these NTX mice were treated with the neuroendocrine hormone, thyrotropin-releasing hormone (TRH) - a hormone known to have effects on T cell development - within just one week, emergence of CD4, CD8β, Thy-1, CD5 and αβ TCR expression on gut lymphocytes could be detected. By three weeks levels of these markers were approaching those found in euthymic mice. This emergence of cell surface antigen expression was also accompanied by an increase in the total number of intestinal lymphocytes in these animals. This was not simply due to a

mitogenic effect of the hormone on lymphocytes already present in the intestine as the hormone has been shown not to be mitogenic for murine lymphocytes.

1.3 Development and Significance of $CD5^+$ B Cells

In recent years there has been considerable research and controversy surrounding a small population of B cells, found in both mice and humans, that express the pan-T cell marker, CD5. These $CD5^+$ B cells develop early in ontogeny, and can be distinguished from conventional B cells in that they have a high IgM:IgD ratio, they have a tendency to produce autoreactive antibodies, and to be located in specific regions such as the peritoneal cavity, and have a self-replenishing capacity (Hardy, 1991; Kantor, 1991).

Much debate has surrounded the issue as to whether these $CD5^+$ B cells constitute a separate lineage to conventional B cells. Most workers have adopted the nomenclature that those B cells that express CD5 are referred to as B1 cells, whilst conventional B cells are termed B2 cells (Hardy, 1991; Kantor, 1991; Haughton et al, 1993). The B1 population has been further divided into those cells that express CD5, B1a cells, and those that do not express CD5, but share other properties of B1 cells, eg self-renewal, high IgM:IgD ratio, and these are termed B1b cells (Hardy, 1991; Herzenberg and Kantor, 1993; Haughton et al, 1993).

The question as to whether B1 cells represent a distinct lineage is still not resolved, with some workers providing evidence for a distinct lineage origin (Hardy, 1991; Kantor, 1991), whilst others (Wortis et al, 1991, 1992; Ying-zi et al, 1991) suggest that $CD5^+$ B cells are a result of B cell activation, and that any naive B cell has the potential to express CD5. Studies have shown that CD5 expression on B cells (both human and murine) can be induced by *in vitro* stimulation with phorbol esters (Zupo et al, 1994; Miller and Garlow, 1984; Freedman et al, 1984). However, it has been shown that not all B cells are capable of induced CD5 expression (Hardy, 1991; Haughton et al, 1993; Kawamura et al, 1994), which may suggest that CD5 is not simply an activation marker for B cells.

In mice, conventional (or B2) cells are replenished throughout life from progenitors found in the bone marrow. In contrast, B1 cells rely on self-replenishment of existing B1 cells that have developed in the first 3-6 weeks of life (Herzenberg and

Kantor, 1993). The early development of B1a cells was shown when fetal liver transferred to irradiated mice was able to give a normal distribution of $CD5^+$ B cells, whilst the transfer of adult bone marrow gave 10-20 fold less $CD5^+$ B cells (Hardy, 1991). Further studies, involving the transfer of fetal pro-B cells or adult bone marrow pro-B cells into severely combined immunodeficient (SCID) mice, showed that three weeks after reconstitution there were low levels of IgD expression, but high levels of CD5 when fetal pro-B cells were transferred. In contrast, increased levels of IgD, expression but few cells expressing CD5, occurred when bone marrow pro-B cells were used (Hardy and Hayakawa, 1991). The ability of fetal omentum to replenish the CD5 B cell population, but not the conventional B cell pool was shown by Solvasson et al (1991). This ability of fetal tissue to replenish the CD5 B cell population led to suggestions that B1a cells and γδ T cells are the most primitive type of lymphoid cells, whilst conventional B cells and αβ T cells are the 'most developed'. This was further supported by the fact that Vγ3 T cells can arise only from fetal but not from adult haemopoietic stem cells (Kantor, 1991; Havran et al, 1991). Unlike B1a cells, B1b cells are able to replenish themselves from progenitors found in both fetal liver and adult bone marrow, however, this only occurs in irradiated mice that have been given bone marrow.

B1 cells tend to show a limited range of variable heavy chain (V_H) gene usage, with V_{H11} and V_{H12} being common among these early developing cells. These B1 cells also show very little N-region insertions during rearrangements of their Ig genes, whereas later in development, B cells that have arisen from the bone marrow, virtually all show N-region insertions (Kearney, 1993). These N-region insertions lead to differences in the specificity of the CDR3 region of the encoded immunoglobulins (Ig). Those B cells produced early in life (no N-region insertions), show a very limited range of specificities, whilst those produced after birth, when N-region insertions are common, show a greater range of specificities.

Ishida et al (1992) showed that if continuous treatment of mice with anti-interleukin (IL) 10 was given from birth into adulthood, there was a dramatic reduction in the level of $CD5^+$ B cells, whilst the level and functions of conventional B cells remained unaffected. If the anti-IL-10 treatment was stopped at any time, CD5 B cells began to reappear several weeks later, indicating that the deletion is not permanent. It was suggested that the depletion of CD5 B cells may be due to increased levels of interferon (IFN) γ, which was found to increase following anti-IL-10 treatment.

The functional role of $CD5^+$ B cells has still not been resolved, but the fact that in normal mice, the levels of $CD5^+$ B cells is consistent between strains, and that these cells predominate in early life indicate that these cells play an important role in the immune system (Hayakawa et al, 1983). The ligand for CD5, CD72 is found on both B1 and B2 cells (although not expressed on plasma cells), suggesting that it may be important in T-B cell interactions (Van der Welde, 1991). The presence of CD5 on B1a cells may suggest that these cells play a role in regulating the functions of other B cell populations by interacting with CD72 found on the surface of B cells (Kawamura et al, 1994). There appears to be different signalling mechanisms between B1 and B2 cells, as shown by the need for phorbol ester alone to induce proliferation in B1 cells, but the requirement for both phorbol ester and ionomycin by B2 cells. It was also found that B1 cells do not respond to anti-Ig stimulation like B2 cells, but that their response to LPS is the same for both cell types (Kearney, 1993).

The presence of $CD5^+$ B cells has also been shown in the rabbit, but unlike mice and humans, virtually all of the peripheral B cells in the rabbit are $CD5^+$ (Raman and Knight, 1992). Most B cell development in the rabbit occurs early in ontogeny and these cells are self-replenishing therefore reducing the requirement for B cell development in the adult, a situation similar to that found in chickens (Ratcliffe, 1985). In contrast, no $CD5^+$ B cells have been detected in the rat (Vermeer et al, 1994), horse (Blanchard-Channell et al, 1994) or cat (Ackley and Cooper, 1992), although CD5 expression can be induced on the surface of B cells in the horse and cat following *in vitro* stimulation with phorbol ester.

1.4 Apoptosis in Immune System Development

Apoptosis (programmed cell death) occurs not only amongst lymphoid cells but also in all other cell types. Apoptosis occurs in primary lymphoid organs such as the thymus, liver and bone marrow to remove non-functional or aberrant cells (Krammer et al, 1994). In B cell development 95% of B cells that develop are destroyed by apoptosis if their immunoglobulin (Ig) receptors are self-reactive, or if they have non-functional Ig gene rearrangements (Cohen, 1993). Likewise, apoptosis plays a central role in T cell education in the thymus during negative selection (Von Boehmer, 1992; Korsmeyer, 1995). Apoptosis also occurs in the peripheral lymphoid organs such as the spleen and lymph nodes. In these organs the role of

apoptosis is to down-regulate the immune response or to maintain self-tolerance, thereby preventing damage or disease to the host (Krammer et al, 1994).

Apoptosis is a form of cell death that is controlled and specifically induced by a number of genes. It is distinct from necrosis, another form of cell death, whereby death is usually induced by damage to the cell itself. In necrosis, once the cell has been damaged ions are able to enter, causing the cell to take up water and swell. This continues until finally the cell ruptures, releasing the contents of the cell into the surrounding area, which will then usually initiate an inflammatory response (Farber, 1990). Apoptosis is an entirely different process, which is initiated by a whole range of stimuli, usually coming from the microenvironment, including deprivation of essential growth factors, signalling via certain surface receptors, exposure to hormones, eg. glucocorticoids, or DNA-damaging agents (Schwartz and Osborne, 1993; Núñez et al, 1994). Once the 'death signal' has been identified, apoptosis is induced and the cell begins to get smaller, there is blebbing of material from the plasma membrane releasing 'apoptotic bodies', and the chromatin condenses and finally there is cleavage of DNA into small 180 base pair fragments (Korsmeyer, 1995). The apoptotic bodies are phagocytosed by nearby macrophages thereby preventing any chance of an inflammatory response (Savil, 1993).

There are two main stages to apoptosis, the first of which is the latent phase. This is a time when cells become committed to cell death but are visually no different from other viable cells. These cells can even undergo a further round of DNA replication and cell division. The second stage, the execution phase, is when the cells become visibly detectable as apoptotic and can take as little as 15 minutes to occur. (Earnshaw, 1995). The change from the latent to the execution phase can take several hours, therefore if a sample of cells are taken from culture, there will only be a minor population of cells (20-40%) that will appear to be in the execution phase, ie. visually appear to be apoptotic. Lazebnik et al (1993) has shown that the factors that cause the induction of the execution phase are located in the cytoplasm and not in the nucleus. This is further supported by the fact that mitochondria, which are found in the cytoplasm, are important for the induction of the execution phase (Newmeyer et al, 1994).

During T and B cell development, apoptosis plays a major role in removing any lymphocytes that contain aberrant gene rearrangements for either the TCR or immunoglobulin surface receptor (von Boehmer, 1992; Deeman et al, 1990). Induction of apoptosis can be induced in mature T cells by binding of their CD3-

TCR complex, or following PHA stimulation (Kabelitz et al, 1993). Resting T cells tend to be less susceptible to Ag-induced apoptosis, but following stimulation become more susceptible (Salmon et al, 1994). It can be seen therefore that following stimulation of T cells, activated cells can go down one of two pathways - that of proliferation or apoptosis. This may be one way of controlling the immune response such that a proportion of Ag-activated T cells will undergo apoptosis and therefore limit the number of responding T cells. Wang et al (1993) have shown that upon activation of Th_1 cells, the threshold of Ag-induced apoptosis is lowered, and a fine balance between cytokine production and apoptosis is in operation. If these cells are to survive, then cytokine production must predominate, otherwise the cell will die by apoptosis. With Th_2 cells, Ag stimulation increases the threshold for Ag-induced apoptosis, therefore whether the cell dies by apoptosis or not, cytokine production by these cells is unaffected. Activated cytotoxic T cells require interleukin 2 (IL-2) in order to avoid apoptosis, and the source of IL-2 is usually from nearby helper T cells. However, repeated stimulation of helper T cells results in the loss of the helper cells ability to produce IL-2, which in turn would result in the death of activated cytotoxic T cells by apoptosis. Unless more naive helper T cells were recruited, the immune response would be terminated (Akbar and Salmon, 1995). This means that different antigen presenting cells (APCs) can stimulate different Th subsets which will result in either cytokine production or Ag-induced apoptosis, which will ultimately control the inflammatory response.

There have been several genes identified that are involved in apoptosis and its regulation which include, bcl-2, c-myc, p53 and Fas (or APO-1 in humans) amongst others. The genes c-myc and p53 have been found to promote apoptosis (Yonish-Rouach et al, 1991; Milner et al, 1993). Fas/APO-1 also induces apoptosis upon stimulation (Cohen, 1993; Krammer et al, 1994). Fas is expressed in high levels on the surface of activated T cells, whilst resting T cells display only low levels or none at all (Klas et al, 1993). In contrast, bcl-2 seems to be involved in preventing apoptosis, and is found expressed on most mature T cells (Krammer et al, 1994).

One of the most extensively studied genes is bcl-2, which produces a 26kDa integral membrane protein (Bcl-2) that is found in the outer membrane of mitochondria, the perinuclear membrane, and smooth endoplasmic reticulum (Núñez et al, 1994) The protein is anchored to the membrane whilst the majority of the protein is directed into the cytosol. It was found that increased levels of Bcl-2 in lymphocytes led to cell survival, protecting the cell from undergoing apoptosis (Akbar and Salmon, 1995; Korsmeyer, 1995). Bcl-2 is found expressed on most mature T cells

(Krammer et al, 1994) and work by Korsmeyer has revealed that there is a close interaction between Bcl-2 and another related protein, Bax (Bcl-2 associated X protein). Bcl-2 and Bax tend to form heterodimers within the cell and counter the effects of each other. If Bcl-2 predominates, homodimers of Bcl-2 form and the cell survives; if Bax predominates, homodimers of Bax form and the cell enters apoptosis. Bax shows homology to Bcl-2 in two main regions, termed BH1 and BH2, and these regions are important for dimerization. Studies have shown that if mutations are introduced into either of these regions, Bcl-2 is unable to protect the cell from apoptosis. These mutations appear to have no effect on the ability of Bcl-2 to form heterodimers with Bax, or for Bax to form homodimers, indicating that it is the formation of Bcl-2 homodimers that is critical for cell survival.

Núñez et al (1994) have shown that Bcl-2 is involved in positive selection of thymocytes in the thymus. As $CD4^+CD8^+$ thymocytes mature into single positive T cells, expression of Bcl-2 is increased. However, immature lymphocytes are still deleted by negative selection, as shown in studies using transgenic mice (Korsmeyer, 1995), which would imply that apoptosis is mediated and controlled by a range of genes and is not controlled by bcl-2 alone (Akbar and Salmon, 1995).

1.5 Use of *Xenopus* for Investigation of Lymphocyte Development

The work carried out in this Thesis is based on the animal model, *Xenopus laevis*, an anuran amphibian. At first this may seem an unusual choice of species for immunological studies, when as shown above, our understanding of components of the immune system of higher vertebrates is considerable. However, the immune system of *Xenopus* is not dissimilar to that of higher vertebrates, and includes several lymphoid organs including thymus, spleen, gut associated lymphoid tissue (GALT), the lymphomyeloid peripheral layer of the liver, and the mesonephros of the kidney (Manning and Horton, 1982; Plytycz and Bigaj, 1983; DuPasquier et al, 1989). There is a division of lymphocytes into T and B cells, and both cytotoxic and helper T cell activity has been demonstrated, as well as the existence of three classes of immunoglobulin (IgM, IgY and IgX - corresponding to the mammalian equivalents of IgM, IgG and IgA respectively) (DuPasquier et al, 1989; Horton, 1994). *Xenopus* is the most primitive vertebrate with a defined MHC (DuPasquier et al, 1989), which is referred to as *Xenopus* leukocyte antigen (XLA). The *Xenopus* MHC was discovered through skin allograft rejection studies, with both class I and

class II MHC proteins are found in *Xenopus*, but their expression pattern is different from that found in mammals (see Chapter 2).

One superior feature of *Xenopus* over higher vertebrates is the ease with which the *Xenopus* thymus can be removed at a very early point in its histogenesis, before T cell maturation has occurred. Such an effective thymectomy is possible because larvae are independent of direct maternal influences, and the thymus can be easily visualised under the transparent skin of the head. In higher vertebrates thymectomy is possible but usually at a stage in development when T lymphocytes have matured and have already escaped into the periphery. The use of strains of mice which have no thymus (nude mice and rats) abolish the need for operations to remove the thymus, but these animals have defects after their thymic loss (Lin et al, 1994) and furthermore, they have been shown to develop thymic tissue with ageing, possibly allowing the development of T cells to occur in older animals (Kennedy et al, 1992). The use of severely combined immunodeficient (SCID) mice has also been used for studies on T cell development due to the absence of both T and B cells. However, in such an immunocompromised animal model, the results must surely be questioned as to whether the findings are as a result of a lack of T cells, or due to the combined effects of the immunodeficieny overall.

1.6 Aims of the Thesis

The major aim of this Thesis is to investigate whether an extrathymic pathway of T cell development exists in the amphibian, *Xenopus laevis*. The work carried out in this Thesis aimed to study the emergence of T cell surface antigen expression in normally developing and metamorphosis-inhibited animals; to investigate the thymus-dependent nature of these cell surface antigens and their stability following *in vitro* stimulation; to probe the consequences of *in vitro* stimulation with regards to the induction of T cell surface antigens, and the occurrence of apoptosis; and finally to study the development of T lymphocytes at sites known to support the generation of extrathymic T cells in mammals.

Chapter 2

An Ontogenetic Study Exploring the Emergence of Monoclonal Antibody-Defined Cell Surface Markers in Thymus and Spleen of Control *Xenopus*

2.1 Introduction

The South African clawed toad, *Xenopus*, is an ideal animal model for immunobiological studies. It is an entirely aquatic anuran amphibian that is easy to breed and maintain in the laboratory. The immune system of *Xenopus* has been studied in considerable depth (DuPasquier et al, 1989; Horton, 1996), and bears a remarkable resemblance to that of higher vertebrates. Anatomically this includes several lymphoid organs or lymphoid cell accumulations, including thymus, spleen, gut-associated lymphoid tissue (GALT), the lymphomyeloid peripheral layer of the liver, and the mesonephros of the kidney (Plytycz and Bigaj, 1983; DuPasquier et al, 1989).

The *Xenopus* thymus is the organ responsible for the production of T cells, as is the case for mammals, and is the first lymphoid organ to develop. The paired thymus is situated on either side of the head behind the eyes. The thymic anlage buds from the pharyngeal epithelium (Horton and Manning, 1982; Turpen and Smith, 1989) at about 3 days of age and lymphoid stem cells enter the thymus at about 4 days and budding occurs. By 7 days there are approximately 1,000 lymphoid cells in the thymus, which are mostly immature as indicated by their large size (Manning and Horton, 1969). At this point there is no cortex/medulla differentiation. The overall structure of the mature larval and adult thymus is like that of mammals, consisting of an outer cortical region and an inner medulla. However, unlike mammals there is a boundary layer separating the cortex and medulla (Clothier and Balls, 1985). The cortex is full of proliferating lymphocytes in contrast to the medulla, which contains fewer lymphocytes but many diverse thymic

stromal cell types (Clothier and Balls, 1985). During metamorphosis there is involution of the thymus and the number of thymocytes is drastically reduced (DuPasquier et al, 1989). Also at this time the thymus moves position from behind the eyes backwards towards the ear. After metamorphosis a second wave of stem cells enter the thymus and this wave reaches a peak after about 2-3 months (Turpen and Smith, 1989). This new wave of cells is thought to coincide with the emergence of adult-type T cells (Rollins-Smith et al, 1992). As *Xenopus* becomes sexually mature, the thymus begins to regress once more, this time becoming embedded in fatty tissue (Manning and Horton, 1982).

Although the ontogeny of the immune system in *Xenopus* has been studied functionally (Williams and Horton, 1980; Horton, 1994), there has to date not been any in-depth analysis made of antibody-defined changes in immunocyte phenotype during development. For this reason this Thesis has begun with an ontogenetic study of *Xenopus laevis*, concentrating on the emergence of immunologically significant antigens that appear on the cell surface of tadpole splenocytes and thymocytes, and probing the changes that occur over metamorphosis and into adult life. This Chapter employs several new monoclonal antibodies directed against cell surface determinants on *Xenopus* T cells. The emergence of monoclonal antibody-defined cell populations are followed from 7 day old larvae through to 8 month old adults. This study aimed to provide a database of information regarding the development of T cells in two major lymphoid organs (thymus and spleen) of *Xenopus*, which would not only be of value for further studies in this Thesis, but also of use to other workers studying the immune system of *Xenopus*.

As an amphibian, *Xenopus* undergoes a metamorphic change from its tadpole form into an adult frog. This period of change has been extensively studied, work facilitated by the method of blocking tadpoles from metamorphosing (Rollins-Smith and Blair, 1990; DuPasquier and Flajnik, 1990). This is achieved by adding sodium perchlorate to the aquarial water which blocks the action of the thyroid gland and its hormones, preventing tadpoles from acquiring adult characteristics. This technique has been used to probe immunological changes that occur during metamorphosis, such as the emergence of class I and II MHC antigen expression, and determine whether these changes are dependent upon metamorphosis, or are simply age-dependent.
In parallel a second ontogenetic study was carried out on perchlorate-blocked larvae. By inhibiting metamorphosis, this investigated whether any major differences occurred with respect to ontogeny of lymphocyte surface antigen expression. The patterns of

expression of MHC class II surface antigens have been studied in perchlorate-blocked larvae (Rollins-Smith and Blair, 1990; DuPasquier and Flajnik, 1990). However, some of the data obtained with respect to lymphocytes was by inference due to a lack of mAbs available. Furthermore, the present study employs the extremely sensitive technique of flow cytometry, where surface antigen expression of a large number of cells can be analysed rapidly, and also probes a range of T cell surface antigens. Two-colour flow cytometric analysis was performed in these studies, thereby providing a more accurate analysis of the phenotype of developing T cell populations.

2.2 Materials and Methods

2.2.1 Animal Husbandry

Adult outbred *Xenopus laevis* were primed with 50 units of human chorionic gonadotrophin (HCG) injected into the dorsal lymph sac a day before breeding. Ovulation and mating was induced by injecting a final dose of 500 units of HCG via the dorsal lymph sac the next day. Pairs of frogs, after receiving the final dose, were placed into tanks containing cotton gauze and were left in the dark undisturbed overnight. The next day the gauze, containing the eggs, was transferred to tanks (90cm x 50cm x 25cm) filled with aerated dechlorinated water at 23±2°C.

Larvae were fed on nettle powder; the initial density was around 2-3 larvae per litre, but from 2 weeks of age they were kept at about one tadpole per litre. The stage of development was determined each week (Nieuwkoop and Faber, 1956) and the first experimental time point to be taken for flow cytometric analysis was stage 48, one week after fertilisation. From this point onwards larvae were taken at approximately weekly intervals for flow cytometric analysis. Larvae completed metamorphosis at around 8-9 weeks.

Adults were placed into smaller tanks (38cm x 25cm x 30cm), about 6 individual frogs per 3 litres of water at 23±2°C, and were fed twice weekly. Their diet consisted of either *Xenopus* Pellets (Blades Biologicals) Diet No.1 for metamorphosing or young animals until 5-6 months, or Diet No.2 for larger animals. In both cases the diet was further supplemented with bloodworms. Adults were taken for analysis at 3-4 months and again at 6-8 months of age.

2.2.2 Perchlorate Blocked Larvae

Some larvae were blocked from metamorphosing by the addition of sodium perchlorate (Sigma) at 1g per litre aquarial water beginning at 3 weeks of age (Rollins-Smith and Blair, 1990). Water was then changed and fresh perchlorate added twice a week to maintain the block. Blocked larvae only reached stage 54 (in terms of limb development) and were taken for experimental analysis at several time points from 5 weeks to 8 months of age.

2.2.3 Preparation of Thymocytes and Splenocytes

Animals were heavily anaesthetised in a solution of 3-aminobenzoic acid ethyl ester (MS222) (Sigma). Spleens were dissected out with forceps and micro-scissors and placed into a 3.5cm petri dish (Costar) containing 2ml amphibian phosphate buffered saline (APBS) containing 0.1% NaN_3 and 0.1% BSA ("FACS" medium see Appendix A). Thymuses were exposed by removing the overlying skin and were removed using a pair of tungsten needles and placed into 2ml FACS medium. Due to the small size of tadpoles and correspondingly low lymphocyte numbers, many tadpoles were used for each experimental time point, spleens and thymuses being pooled (see Results for details).

Cells were released from the organs by gentle teasing with tungsten needles in ice-cold FACS medium. The cell suspension was then transferred from the petri dish to a 5ml Falcon tube, the petri dish was then washed with a further 1ml FACS medium and the washings added to the tube. The suspension was then pipetted vigorously to further help release cells before larger pieces of tissue debris and clumps were allowed to settle before transferring the supernatant containing the cells to a fresh 5ml tube. The cells were then centrifuged for 10 minutes at 300g at 4°C and resuspended in fresh FACS medium and counted in a Neubauer haemocytometer.

2.2.4 Monoclonal Antibodies

Details concerning the monoclonal antibodies (mAbs) used are shown in Table 2.1. The origin of these anti-*Xenopus* mAbs is also shown in the table.

2.2.5 Dual Labelling of Cells for Flow Cytometric Analysis

After the cells had been adjusted to $1x10^6$ lymphocytes per millilitre, $2x10^5$ cells were added to wells of a 96 well round bottomed plate. Replicate wells were eventually stained with different antibodies. Only every other well was used, to reduce the risk of cross-contamination during the washing steps. The plate was centrifuged for 10 minutes at 300g at 4°C and the supernatant discarded. The pellets ($2x10^5$ cells) were resuspended in 50µl appropriate primary monoclonal antibody (mAb) and the plate was incubated on ice in the dark for 20 minutes. Any remaining unbound mAb was removed by two successive washing steps with 200µl FACS medium, centrifugation and discarding the supernatants. The pellets were then resuspended in 50µl 1:20 dilution of fluorescein isothiocyanate [FITC]-conjugated [$F(Ab)_2$] rabbit anti-mouse immunoglobulins (DAKO) and incubated on ice in the dark for 20 minutes. This

antibody had previously been adsorbed with 5% *Xenopus* serum to remove any non-specific anti-*Xenopus* activity. The FITC anti-mouse Ig step allows visualisation of mAb's that are unconjugated hybridoma supernatants and strengthens the signal when FITC-labelled, purified mAb's are used. The cells were then washed twice with FACS medium containing 1:100 heat-inactivated mouse serum (Sigma) to block any unbound anti-mouse Ig sites. The pellets were then resuspended in 50µl appropriate phycoerythrin [PE]-conjugated mAb and incubated on ice in the dark for 20 minutes. The cells were finally washed twice in FACS medium and resuspended in 600µl FACS medium and transferred to 5ml Falcon tubes for analysis on the flow cytometer.

2.2.5.1 Negative Controls

Due to the variable intensities of fluorescence staining on cells, it is important to distinguish what is deemed positive staining. This is achieved by staining cells with a non-*Xenopus* specific, "isotype-matched" antibody for both green and red fluorescence and setting markers to exclude 98% staining with these particular antibodies.

Throughout the surface staining studies in this Thesis, the control mAbs used were CT3 for green fluorescence, and Ig-PE for red fluorescence. CT3 is a mouse mAb of IgG_1 isotype specific for the chicken CD3 complex and therefore should not bind to *Xenopus* lymphocytes. It is not conjugated with fluorescein, and therefore must be amplified with the secondary FITC rabbit anti-mouse immunoglobulin (see section 2.2.5). Ig-PE is a mouse immunoglobulin of the IgG isotype (DAKO) that is directly conjugated with phycoerythrin. Most mAb's used were of the IgG isotype of these two "control" reagents; the exceptions being the anti-CD8 mAb's (see Table 2.1) which are IgM.

2.2.6 Flow Cytometric Analysis

Samples were analysed on either a Coulter Epics Profile II or a Coulter Epics XL-MCL flow cytometer. The ELITE software that runs the Coulter Epics XL-MCL cytometer and analyses the data produced allows manipulation of the data, both during acquisition of the data and also at a later time point if the data is saved onto computer disk. Details concerning quality control of the cytometer are shown in Appendix B.

2.2.6.1 Data Analysis

A protocol was generated to analyse surface fluorescence staining on lymphocytes from *Xenopus* using the ELITE software. This took the form of various types of histograms as shown in Figure 2.1.

1 - Forward Scatter vs Side Scatter Dot Plot and Lymphocyte Gating

Cells are shown by their physical characteristics on the screen by way of their forward scatter profile (cell size) against their side scatter profile (granularity). Therefore on the basis of cell size and granularity, the populations representing lymphocytes, granulocytes and erythrocytes can be distinguished - Figure 2.1A. During flow cytometric analysis of a cell suspension it is preferable to visualise only the information on lymphocytes. A gate can be drawn around the desired population within the forward scatter vs side scatter dot plot, and the fluorescence staining on only these cells which are known to contain mostly lymphocytes but also some macrophages (J.D. Horton, personal communication) can be analysed - Figure 2.1A(ii). During acquisition of data the cytometer was set to continue to run until 10,000 gated cells had been analysed. During subsequent data analysis, information on ungated cells (also stored on the computer) could be investigated as appropriate. The "lymphocyte gate" set excludes dead cells and debris. Dead cells can be visualised by the addition of 0.25mg/ml propidium iodide (Flukka) to tubes just before analysis using the FL3 detector (Figure 2.1C).

2 - Quadrant Plot Showing Green Fluorescence vs Red Fluorescence

Cells can be represented in the form of a quadrant plot, showing those cells that either display only FITC (green - box 4) or only PE (red - box 1) fluorescence, together with those that are dual stained with both fluorochromes (box 2), and those cells that are negative for both fluorochromes (box 3). Each cell is represented by a dot, the more concentrated the dots the greater the number of cells with that particular staining characteristic - Figure 2.1B(i).

3 - Histogram Showing Green Fluorescence

This is a histogram showing those cells that are stained with FITC fluorescence (detected by the FL1 detector) with increasing fluorescence intensity along the x-axis and relative cell number along the y-axis - Figure 2.1B(ii).

4 - Histogram Showing Red Fluoresence

This is a histogram showing those cells that are stained with PE fluorescence (detected by the FL2 detector) with increasing fluorescence intensity along the x-axis and relative cell number along the y-axis - Figure 2.1B(iii).

2.2.6.2 Colour Compensation

See Figure 2.2

Throughout the cell surface staining studies used in this thesis, antibodies labelled with fluorescein and phycoerythrin have been used. There is some natural overlap between the emission spectra of these two fluorochromes at the upper end of the FITC spectrum and the lower end of the PE spectrum. This means that when dual colour analysis of cells is performed some cells emitting a high FITC fluorescence intensity may be wrongly detected as emitting PE fluorescence by the FL2 detector, and vice versa. This overlap must be removed. In order to ascertain what amount of the signal should be subtracted, cells are singly labelled with FITC or PE, and colour compensation checked each time the machine is used, before any experimental samples are analysed. Compensation is checked by running a sample of FITC-labelled cells through the cytometer and the quadrant dot plot selected. The mean Y values for quadrants 3 and 4 should be as close as possible and these values can be changed by subtracting the FITC signal from the PE channel, ie. green colour compensation. This is then followed by a sample of cells labelled with a PE-conjugated mAb checking that the mean X values for quadrants 1 and 3 are as close as possible. These values are changed by subtracting the PE signal from the FITC channel, ie. red colour compensation.

Once these values have been set, they should not need to be changed from day to day, but it is important to run these two samples every time to make sure that the colour compensation is set correctly.

2.3 Results

2.3.1 Total Lymphocyte Numbers During Ontogeny of Control and Metamorphosis-Inhibited *Xenopus*

Normal (Control) *Xenopus*

Total thymocyte and splenocyte numbers in thymus and spleen of normally-developing *Xenopus* are shown in Figure 2.3A.

Thymocyte numbers increased rapidly from about 4000 cells at 7 days up to $2.6x10^5$ cells by 18 days. This level was then maintained, dropping slightly at metamorphosis. Thymocyte numbers then increased rapidly reaching levels of $27x10^6$ by 8 months. Splenocyte numbers were around 4000 cells at 12 days and increased to $9x10^4$ cells by 24 days. Splenocyte numbers then increased slowly until metamorphosis ($2x10^5$); thereafter a rapid expansion occurred and levels of $12x10^6$ splenocytes were achieved by 8 months.

Blocked Larvae

Total thymocyte and splenocyte numbers in blocked larvae are shown in Figure 2.3B.

In blocked larvae, thymocyte numbers remained around $3x10^5$ until 9 weeks, before expanding to $2.4x10^6$ by 8 months, ie. ten-fold less than in siblings developing normally.

There is a rapid expansion of splenocyte numbers from just under 7000 cells at 2 weeks up to $7x10^5$ by 5 weeks. During the time that metamorphosis would have occurred (9 weeks) there was a slight drop in splenocyte numbers. By 4 months splenocyte numbers were increasing, reaching levels of $4.2x10^6$ by 8 months (this compares with $12x10^6$ in normally-developing siblings).

2.3.2 Flow Cytometric Analysis of Individual Cell Surface Markers in Normal and Blocked *Xenopus*

See Figure 2.4

2.3.2.1 Normal (Control) *Xenopus*

CD5

Expression of CD5 was assessed using the mAb 2B1. The intensity of fluorescence with 2B1 on larval thymocytes was very dull (Figure 2.5) making it difficult to

determine the exact proportion of $CD5^+$ cells. By 12 days 40% thymocytes were recorded as expressing CD5; the percentage increased gradually during development, to 50-60% in the adult. 2B1 staining of larval (and adult) splenocytes was of a greater intensity (Figure 2.5) facilitating assessment of the level of $CD5^+$ cells. By 12 days CD5 surface expression was found on 30% splenocytes, this level being maintained until metamorphosis when levels increased, reaching 50-60% by 8 months.

CD8

CD8 expression was determined using the mAbs AM22 and F17, both of which stain larval and adult thymocytes and splenocytes with a bright intensity.

At 7 days thymocytes showed no surface expression of CD8, but just 5 days later the percentage of $CD8^+$ thymocytes had increased to 70-80%. This dropped to 60% by 3 weeks and this level was then maintained throughout development. Splenocytes were first assessed at 18 days when $CD8^+$ cells were less than 10%. During development the level of $CD8^+$splenocytes gradually increased, reaching 30% by 8 months.

XTLA-1

XTLA-1 expression was determined using the mAb XT-1. In the thymus at 7 days 20% thymocytes stained with XT-1. By 12 days 95-100% thymocytes are XTLA-1^+. This high level of expression was then maintained throughout development. Only 10% splenocytes expressed XTLA-1 by 18 days. The percentage of XTLA-1^+ splenocytes then increased during ontogeny, reaching around 30% by 8 months.

D4.3

Only 5% of thymocytes were recorded as $D4.3^+$ by 18 days, and this percentage was then maintained through the period of larval and adult life examined. In contrast in the spleen, by 18 days the level of $D4.3^+$ cells was 20%. The percentage of $D4.3^+$ splenocytes then increased throughout development reaching around 50% by 8 months. The intensity of staining of splenocytes with D4.3 is greater than with thymocytes (data not shown).

D12.2

There was virtually no detectable expression of D12.2 on thymocytes throughout ontogeny. The level of $D12.2^+$ splenocytes was minimal, remaining relatively constant at around 5%.

IgM

The mAb's 8E4:57 and D8 were used to identify $sIgM^+$ B cells in the spleen and thymus. No B cells were detectable in the thymus, levels of staining not being above background staining with an isotype-matched control mAb. At 12 days 40% splenocytes were IgM^+ and by 4-5 weeks this had increased to 50%. Levels of IgM^+ B cells in the spleen dropped to 30% by the end of metamorphosis and remained around this level well into adult life (8 months).

MHC Class II

The intensity of staining of both larval and adult thymocytes with AM20 (which detects MHC class II) is quite dull (Figure 2.7) and does not identify a distinct population, making accurate assessment difficult. By 2 weeks the percentage of class II^+ thymocytes was estimated to be around 20% increasing to 30-40% during larval life. After metamorphosis there is an increase in the percentage of thymocytes expressing class II, reaching 50-60% by 8 months. Splenocytes stain more distinctly with AM20, making assessment of the number of class II^+ cells more accurate. By 2 weeks 40% cells were $AM20^+$, increasing to 60% by mid larval life. There was a slight drop in class II^+ splenocytes during metamorphosis to around 50% before a rapid increase following metamorphosis, reaching levels of 80-90% class II^+ cells in the adult spleen.

2.3.2.2 Blocked Larvae

Perchlorate block was initiated at 3 weeks (stage 54).

CD5

Levels of $CD5^+$ thymocytes at 5 weeks - just 2 weeks after the block was initiated, reached 60% before dropping to around 45% at the time that metamorphosis would have occurred. From this point onwards the percentage of $CD5^+$ thymocytes increased and reached 80% by 8 months. In the spleen at 5 weeks some 35-40% splenocytes were $CD5^+$ and this increased to around 50-60% by 8 months in the blocked larvae.

CD8

The level of $CD8^+$ cells in the thymus of blocked larvae was more variable than in controls, fluctuating between 60-70% with a noticeable decline to around 50% around the time that metamorphosis would have occurred (9 weeks). In the spleen there was an increase in $CD8^+$ cells from 15-20% at 5 weeks up to 20-30% by 8 months.

XTLA-1

XTLA-1 expression in both spleen and thymus was virtually identical to that of normally developing animals. Levels of XTLA-1 in the thymus remained constant at 90-100%, and in the spleen increased from 15% at 5 weeks to around 30% by 8 months.

D4.3

Levels of D4.3 cells within the thymus were minimal remaining constant around 5-10%. In the spleen by 5 weeks D4.3 levels were around 40% and increased to around 60% by 8 months.

D12.2

There were virtually no detectable D12.2 cells in the thymus of blocked larvae. In the spleen there were around 4% D12.2 cells throughout the study.

IgM

There were no detectable $sIgM^+$ B cells in the thymus. In the spleen of blocked larvae by 5 weeks there were around 40% B cells with a general decline to around 30% by 8 months.

MHC Class II

In the thymus by 5 weeks the level of $AM20^+$ cells reached 45% and remained around this level up until 8 months. The intensity of staining with AM20 was quite dull, making accurate assessment of levels of class II^+ cells in the thymus difficult. In the spleen by 5 weeks levels of AM20 were around 60% and remained at this level till 8 months. In neither organ in blocked larvae was there any increase in the percentage of class II^+ cells, thereby contrasting the situation in adult siblings that had metamorphosed normally.

2.3.3 Dual Colour Analysis of Lymphocyte Subsets

2.3.3.1 MHC Class II Expression on T and B Lymphocytes

A) Control *Xenopus*

Dual fluorescence studies (Figure 2.6) showed that $CD5^+$ cells (the putative T cell population) in the spleen were essentially MHC class II^- until metamorphosis had occurred. From this time onwards MHC class II molecules were found to be expressed on many $CD5^+$ splenocytes. There was class II expression in the spleen before

metamorphosis but this was found on non-T cells - on IgM^+ B cells (as shown by dual staining with AM20 and D8 - see Figure 2.6) and also on Ig^- leukocytes - probably macrophages which are known to fall within the "lymphocyte" gate (J D Horton, personal communication). Class II expression is brighter on adult B cells than on adult T cells (Figure 2.6).

In the thymus there is a sizeable population of MHC II^+CD8^+ double-positive cells (up to 20%) after metamorphosis, and a low percentage of MHC II^+CD8^+ thymocytes are also seen in larvae. That this class II expression is on T cells was shown by CD8/class II double-positive staining (Figure 2.7). The CD8 antigen served to illustrate this point, since CD5 staining of thymocytes is weak.

B) Blocked Larvae

MHC class II expression on splenic T cells of perchlorate blocked larvae was prevented during and after the time that metamorphosis would normally have occurred (see Figure 2.8). By 6 months, when about 50% $CD5^+$ splenocytes were beginning to express class II in control toadlets, there was only a minimal level of $CD5^+$MHC II^+ splenocytes (5%) in blocked larvae. Only in long term blocked larvae, 8 months or more, is there a suggestion of class II molecules appearing on $CD5^+$ T cells.

2.3.3.2 D4.3 Expression on $CD5^+$ and $CD8^+$ Cells

In post-metamorphic animals dual staining with the mAb's 2B1 and D4.3 indicate that all $D4.3^+$ splenocytes are also $CD5^+$ with only a minor splenocyte population (4-6%) that are $CD5^+D4.3^-$ (Figure 2.9A). $CD8^+$ cells are virtually all also $CD5^+$ and $D4.3^+$ (see Figures 2.9B and D).

2.3.3.3 D12.2 Expression on $CD5^+$ and $CD8^+$ Cells

D12.2 stains only around 5% of adult control splenocytes. Splenocytes incubated with D12.2 and 2B1 show a small dual-stained population (3%) (Figure 2.9C). A small population (2%) of $CD5^-D12.2^+$ cells is also often evident, although not readily apparent in the traces shown. Adult splenocytes co-stained with D12.2 and AM22 show that $CD8^+$ cells tend not to express D12.2 (Figure 2.9D).

2.3.3.4 Lymphocyte Subsets Distinguished By Expression of XTLA-1, CD8 and CD5

A) Control *Xenopus*

The percentage of $CD5^+$ T cells in the spleen gradually increases during ontogeny (see previous section). The proportion of $CD5^+$ T cells co-expressing CD8 was about 33% in larvae, reached 40% in 2-4 month old adults and approached 45-50% in 6-9 month adults (see Table 2.2A). The XTLA-1 antigen in the larval spleen was found on a higher percentage of $CD8^-$ than $CD8^+$ T cells (almost 2:1). In contrast, in the adult spleen (at 6-9 months), XTLA-1 was now expressed predominantly on T cells that were $CD8^+$. In adults, the vast majority (75%) of $CD8^+$ T cells were XTLA-1^+, whereas most (77%) $CD8^-$ T cells were XTLA-1^-.

In the thymus there were only two noticeable populations staining for XTLA-1 and CD8 - those that were XTLA-1^+CD8^+ and XTLA-1^+CD8^-. These populations were found to be at a ratio of 2:1 throughout ontogeny (60%:30%). It is difficult to ascribe CD5 staining with these populations as CD5 staining in the thymus is very weak and it is therefore difficult to obtain accurate levels of CD5 (see Figure 2.5).

B) Blocked Larvae

The preference for $CD8^+$ cells to co-express XTLA-1 seen in non-blocked *Xenopus* by 6-9 months of age occurs also in blocked larvae by this age (see Table 2.2B).

2.3.4 Emergence of T Lymphocytes in the Thymus with a "Mature" T Cell Staining Pattern

In 8 month old adult control frogs, small populations in the thymus appear to have a staining intensity with the reagents to CD8, CD5 and MHC class II more reminiscent of splenocytes (see Figure 2.10). Staining intensities of thymocytes with these mAb's (especially CD5 and MHC class II) are usually quite low, whilst in the spleen the fluorescence intensity with the same reagents is much brighter, with more distinct populations of cells. In the 8 month thymus a very small, but distinct $CD5^{bright}$ population is revealed (see Figure 2.5). This is further highlighted by populations of $CD5^{bright}CD8^{bright}$ and $CD5^{bright}CD8^-$, which show splenocyte-like staining intensities compared to the major thymocyte populations that are $CD5^{dull}CD8^{dull}$, $CD5^-CD8^{dull}$ and $CD5^{dull}CD8^-$ (Figure 2.10C quadrant 2 and 3). Thymocytes from 8 month thymus dual stained for CD5 and MHC class II reveal a population of cells (18%) that are $CD5^{bright}$MHC II^{bright} indicating a mature T cell phenotype (Figure 2.10D). A small (5%), but distinct population of cells that are XTLA-1^-CD8^{bright} is

seen in the 8 month thymus (Figure 2.10B) that is absent in the 4 week thymus (Figure 2.10A).

2.3.5 XTLA-1 Variability

One phenomenon that was noted during the ontogenetic study was that pooled thymocyte preparations from several *X laevis* larvae showed two distinct levels of intensity of XTLA-1 staining from the same pool of animals. This phenomenon was not observed in the spleen, the equivalent splenocyte profiles always showing a single population of XTLA-1^+ cells. This XTLA-1^{bright} and XTLA-1^{dull} thymocyte staining was only seen in pooled populations, not within individual animals (Figure 2.11). Thus the phenomenon presumably does not relate to T cell maturational differences, but to distinct levels of expression of XTLA-1 ("high" or "low") by different individuals. Furthermore, in some *X laevis* batches XTLA-1 expression was absent in both spleen and thymus of individual animals (data not shown).

2.4 Discussion

This Chapter has provided new, in depth information on T cell antigen expression during development in the amphibian, *Xenopus laevis*. Previous work investigating T cell antigen expression was limited to the mAbs AM22 (Flajnik et al, 1990), XT-1 (Nagata, 1988) and AM20 (Flajnik et al, 1990) and included only single colour flow cytometric analysis, or fluorescence microscopy. With recent availability of additional mAbs, namely 2B1, F17, D8, D4.3 and D12.2, more extensive analysis of T cell development has become possible. In particular two colour flow cytometric analysis has been performed, which has led to more precise determination of the phenotypes of *Xenopus* T cell subsets and their development.

The mAb, 2B1, which detects the CD5 homologue in *Xenopus* (Jürgens et al, 1995), proved to be a useful T cell marker, IgM^+ B cells from the spleen failing to stain with this marker. There is a difference in CD5 fluorescence staining intensity between most lymphocytes from the thymus and spleen. The same concentrations of antibody were used for spleen and thymus, yet thymocytes consistently showed only $CD5^{dull}$ staining, compared to $CD5^{bright}$ splenocytes. This led to problems determining the exact percentage of $CD5^+$ cells within the thymus, and it is most likely that all lymphocytes in the thymus express a low level of CD5 on the cell surface (see Jürgens et al, 1995), which is upregulated once the T lymphocytes have exited the thymus. The small but distinct population of $CD5^{bright}$ cells found in the thymus of animals aged 8 months and above suggests that T cell maturation (with respect to CD5 expression) may be "completed" prior to T cell exodus from the thymus.

Dual-colour analysis of peripheral T cells confirms that there are two major populations of T cells - those that are $CD5^+CD8^+$ (ie. the cytotoxic T cells), and those that are $CD5^+CD8^-$ which are presumably the helper T cells. This latter population (representing some two thirds of T cells) cannot be identified directly as there is no mAb available that detects the *Xenopus* CD4 homologue.

Studies presented here with the mAb, D4.3 have proved interesting. The cell surface antigen recognised by D4.3 has not been fully characterised, but it was originally suggested that D4.3 is directed against the αβ T cell receptor (TCRαβ) of *Xenopus*.

Thus Ibrahim et al (1991) found that D4.3 had specificity to a cell surface determinant that was a dimer consisting of two sub-units of relative molecular weight, 40kDa and 60kDa. Immunohistological staining suggested that the tissue distribution of this determinant was similar to the distribution of both mammalian and avian TCR$\alpha\beta^+$ lymphocytes. The present ontogenetic study revealed that, like 2B1, expression of this marker in the thymus is relatively dull, whereas splenocytes showed a brighter fluorescence staining intensity, and a much higher percentage of D4.3$^+$ cells. Those splenocytes that are D4.3$^+$ all co-stain with 2B1, indicating that D4.3 is expressed on T cells. Indeed, it was found that D4.3 labels between 90-95% T cells in *Xenopus*, suggesting further evidence that D4.3 might detect the TCR$\alpha\beta$.

Another mAb that was used in the present studies was D12.2, which detects a receptor with relative molecular weight of 57kDa and which stains lymphocytes in the red pulp areas of the spleen, and the intestinal epithelium (Ibrahim et al, 1991). Levels of D12.2$^+$ cells in both the thymus and spleen are consistently low and do not appear to increase during ontogeny. It has been suggested that the mAb, D12.2 might detect the TCR$\gamma\delta$ in *Xenopus* (Ibrahim et al, 1991). In mammals and birds $\gamma\delta$ T cells are spawned early in the thymus (Zorbas and Scollay, 1993; Dunon et al, 1993; Mackay and Hein, 1991) and levels of up to 4% $\gamma\delta$ lymphocytes can usually be found in the thymus. It has also been suggested that some $\gamma\delta$ T cells develop extrathymically (Rocha et al, 1992); however experiments on sheep that were thymectomized *in utero* show a consistent depletion of $\gamma\delta$ T cells (Mackay and Hein, 1991). In *Xenopus* dual staining studies revealed that D12.2 expression is not restricted to CD5$^+$ T cells, suggesting that D12.2 may be expressed on some non-T cells. This issue is investigated in more detail in Chapter 5.

Since it is known that B lymphocytes are initially spawned in the *Xenopus* spleen (Hadji-Azimi et al, 1990) it is not surprising that sIgM$^+$ cells are the predominant lymphocyte type found in the spleen of pre-metamorphic larvae. Following metamorphosis, the percentage of sIgM$^+$ cells declines in the spleen as the percentage of T cells increases, suggesting that the role of T cells is now becoming more important in adult life.

Dual-colour analysis revealed a tendency for XTLA-1 expression to be associated with CD8$^-$ lymphocytes in young larvae, whereas following metamorphosis, XTLA-1 expression predominated on CD8$^+$ cells. This may therefore suggest that XTLA-1 is an

activation marker rather than a lineage marker for T cells. During larval life when only MHC II expression can be seen, the $CD8^-$ T cells will be the active T cell subset. However, following metamorphosis and the emergence of MHC I expression (Flajnik et al, 1987), $CD8^+$ T cells will now become an active population, thereby acquiring XTLA-1 expression. Another possible role of XTLA-1 expression is one of a maturation marker, being present only on immature T cells. This would account for the high levels of XTLA-1 expression seen in the thymus (nearly 100%), and also the small population of putative 'mature' thymocytes that are $CD5^{bright}XTLA\text{-}1^-$.

Another interesting feature concerning the expression of XTLA-1 in the thymus was the appearance of two levels of staining intensity. This was only ever seen in pooled preparations of several thymuses, and was not seen in an individual animal. These two levels of brightness were not evident in the spleen, where only a single fluorescence intensity was found. It is possible that the bright level of XTLA-1 staining could relate to expression of two dominant XT-1 coding alleles, whereas the lower intensity reflects one dominant and one recessive XT-1 allele. Some *X laevis* were found during the study that did not express any XTLA-1, and it could be that this reflects the phenotype of a double recessive XT-1 allele. Previous studies using the mAb, XT-1 (Varley, 1990), revealed that one species, *Xenopus tropicalis*, entirely lacks expression of XTLA-1 epitope on its thymic and splenic lymphocytes altogether (Varley et al, 1991). It will be interesting to further probe the role of XTLA-1 in immune functions of *Xenopus* although this is outside the scope of this Thesis. The variability in XT-1 expression illustrated in the work presented here on *X laevis* indicates that one cannot rely on the use of this T cell marker to detect a particular T cell subset.

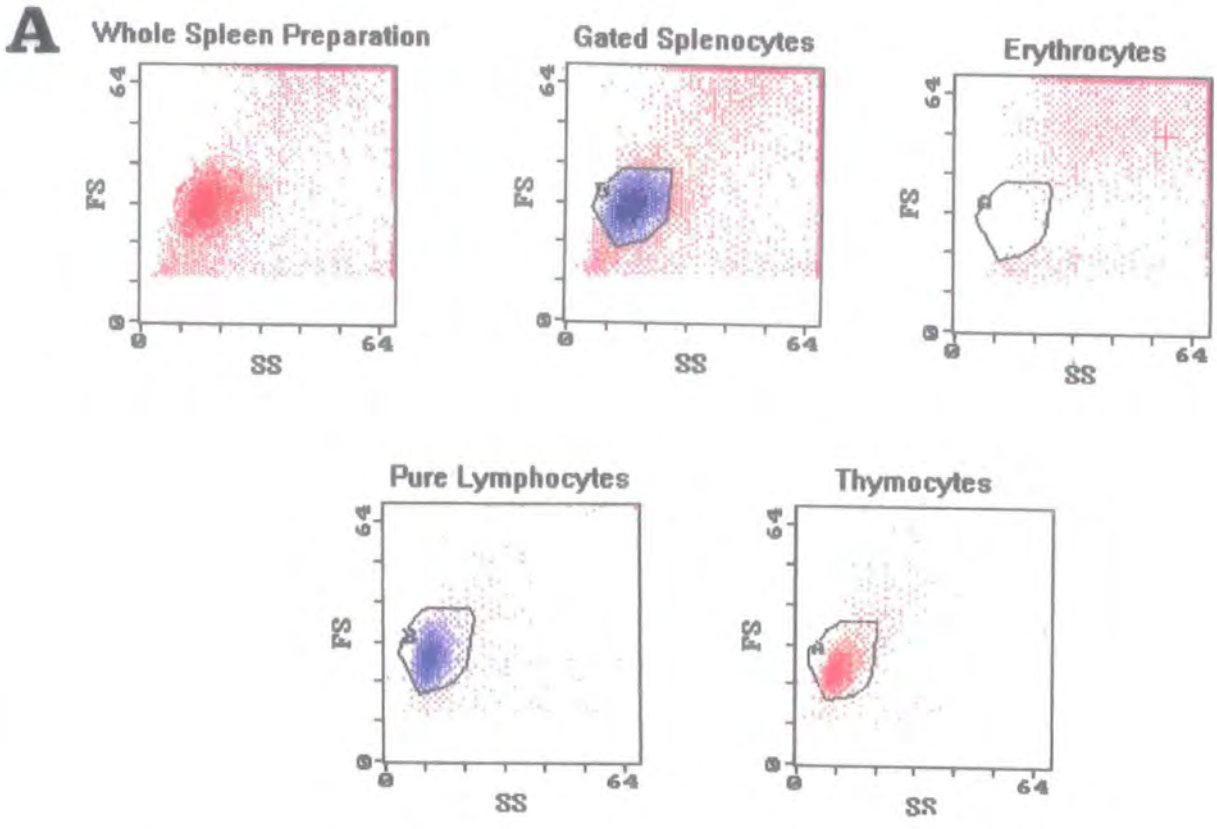
The dual-colour flow cytometric studies, especially those with perchlorate-blocked larvae, confirmed previous experiments indicating that MHC class II expression on *Xenopus* T cells was metamorphosis-dependent (Rollins-Smith and Blair, 1990). Furthermore, the present findings reveal for the first time that in metamorphic animals, B and T lymphocytes show a differential staining intensity of MHC class II expression. It was found that T cells express a low intensity of staining for MHC II, whilst B cells (and other non-T cells) show a distinctly brighter intensity of staining.

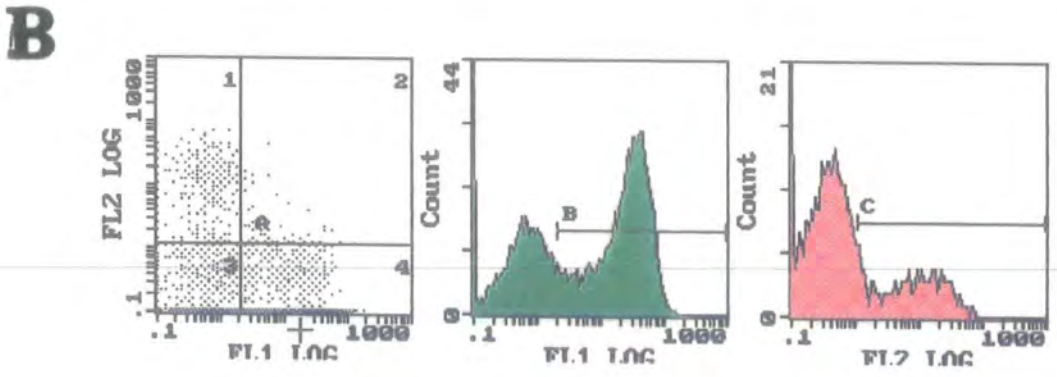
The effect of the perchlorate-block did not appear to affect the emergence of any of the cell surface determinants studied except for MHC II expression, discussed above. The perchlorate block routinely affected lymphoid cell numbers in the thymus and spleen,

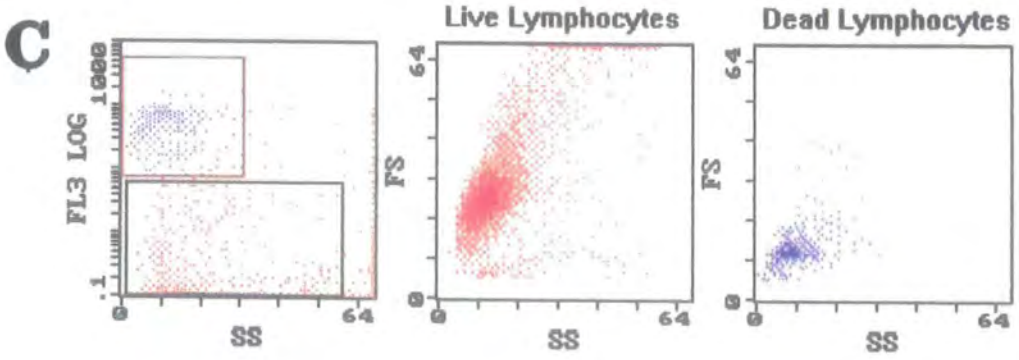
cell numbers never being as high in perchlorate blocked larvae compared to age-matched non-blocked siblings throughout ontogeny. Despite this, considerable expansion of lymphocyte numbers is seen in perchlorate-blocked larvae at a time when control froglets are also rapidly increasing thymocyte and splenocyte numbers. Overall, the present studies suggest that this increase in lymphocyte numbers and mAb-defined T cells seen in 3 month and older *Xenopus* is not dependent upon metamorphic events per se, and is independent of such phenomenon as MHC II emergence on adult T cells.

An ontogenetic study of the type presented in this Chapter has not been possible until now due to the lack of mAbs available for specific *Xenopus* T cell markers. Use of such mAbs has provided a detailed picture of the emergence of T cell subsets in both the thymus and spleen from larvae from as early as 2 weeks old, just one week after the spleen anlage has developed, throughout metamorphosis and into adult life. This provides a useful database concerning T cell ontogeny, and provided a good background for studies described in subsequent Chapters in this Thesis.

Figure 2.1 𝔸 Examples of forward scatter (cell size) vs side scatter (granularity) traces produced by the ELITE software to illustrate different cell populations: (i) whole spleen preparation containing lymphocytes, granulocytes, erythrocytes and macrophages; (ii) same preparation as in (i) but now an analysis gate has been drawn around the lymphocytes and macrophages; (iii) erythrocytes purified on a Percoll density gradient to identify where they are located; (iv) leukocytes purified on a Percoll density gradient to confirm their location within the analysis gate; (v) preparation of thymocytes to show that they have the same forward scatter and side scatter characteristics as splenocytes (note the absence of erythrocytes and granulocytes); 𝔹 Example of dual colour fluorescence measurements: (i) quadrant plot showing FL1 (FITC) vs FL2 (PE); (ii) histogram showing cells stained with FITC with increasing fluorescence intensity along the x-axis and relative cell number along the y-axis; (iii) histogram showing cells stained with PE with increasing fluorescence intensity along the x-axis and relative cell number along the y-axis; ℂ (i) Identification of dead lymphocytes by propidium iodide (PI) uptake - those cells that have incorporated PI are positive for FL3 and fall within the upper box whilst viable cells will not take up PI and remain FL3 negative and are found within the lower box; (ii) FS vs SS trace produced by back-gating on only those cells that are PI negative; (iii) trace produced by back-gating on those cells that are PI positive. The gated lymphocytes are >98% viable. These traces indicate that non-viable cells do not predominate within the lymphoid gate (<2%).


A
Whole Spleen Preparation
Gated Splenocytes
Erythrocytes
Pure Lymphocytes
Thymocytes
FS
SS
0
64


B
FL2 LOG
FL1 LOG
Count
1000
.1
1
2
3
4
44
21


C
FL3 LOG
Live Lymphocytes
Dead Lymphocytes
FS
SS
0
64
1000
.1

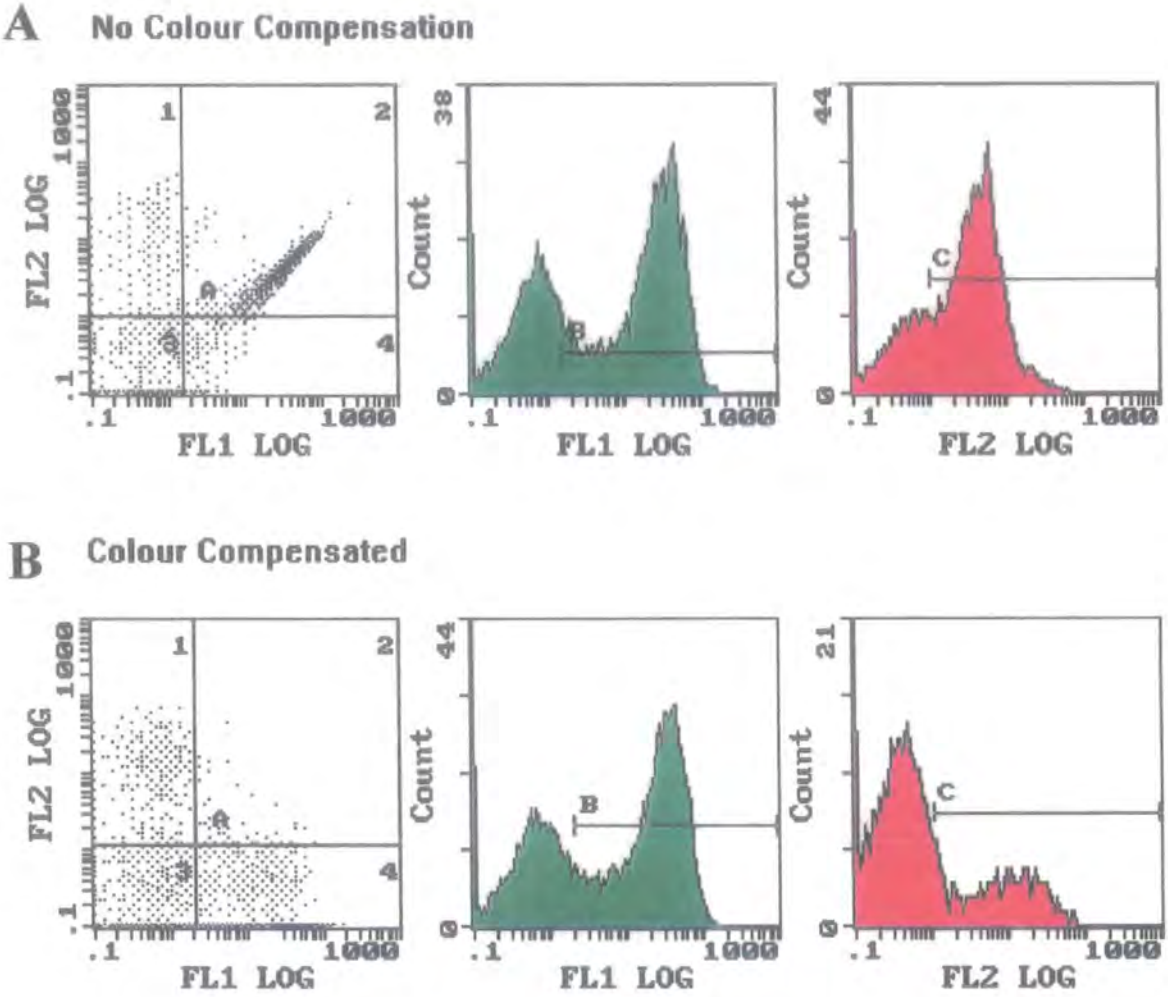
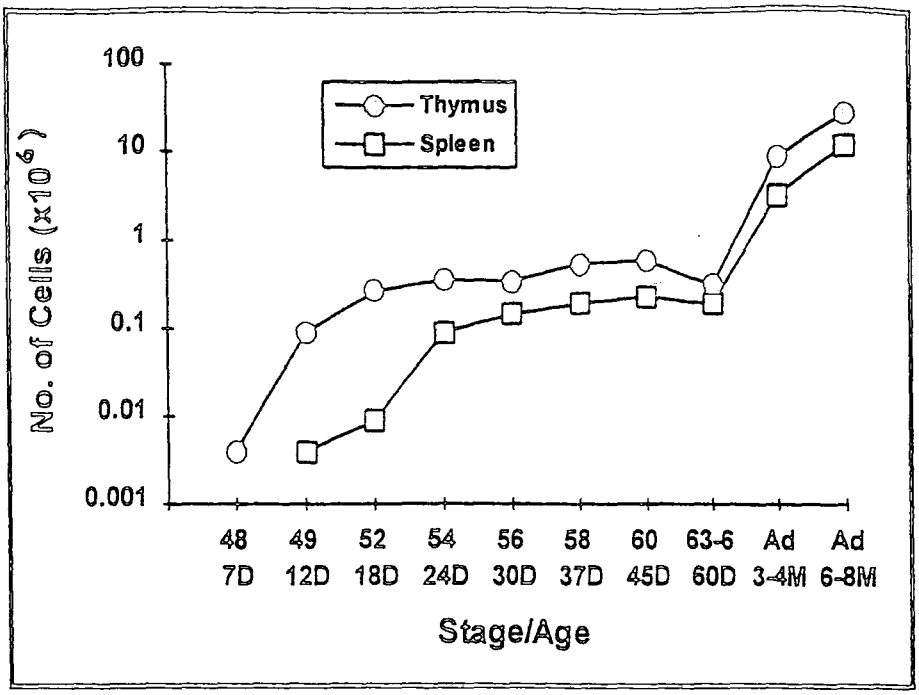


Figure 2.2 Effect of colour compensation. Sets of traces showing one sample that has been stained for two mutually exclusive populations (ie. cells known to bind to one of the mAbs used, but **not** to both mAbs). In A where no colour compensation has been used - note the population of cells in trace 1, quadrant 2 which are showing up falsely as dual-positives, further shown in trace 3 where the level of cells stained with PE is far greater than expected; **B** Traces showing the same sample, except that colour compensation has been applied - note that there are no dual positive cells and the number of positive cells in trace 3 is now the expected level.

A



B

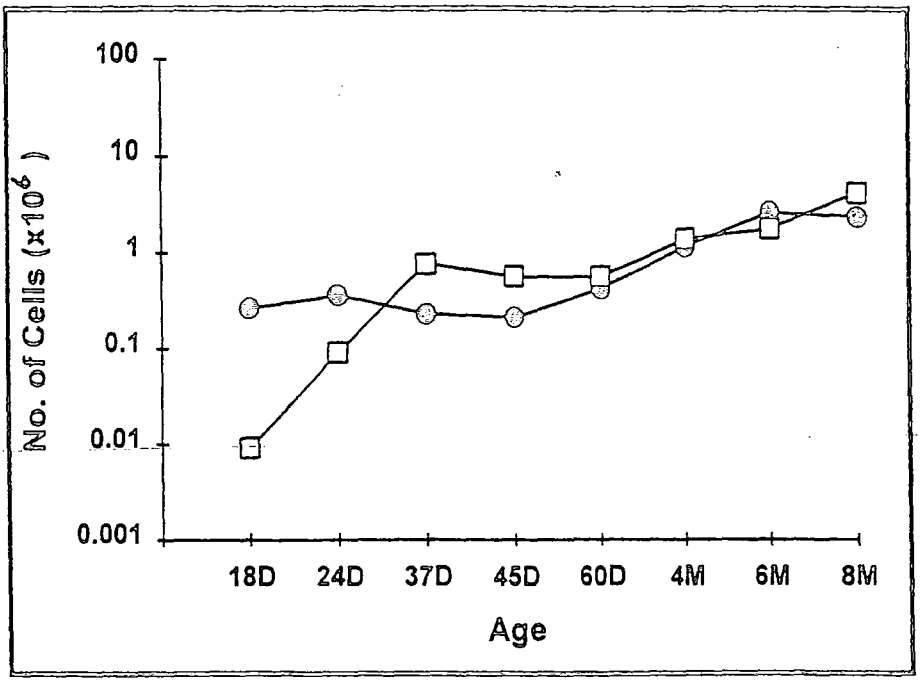
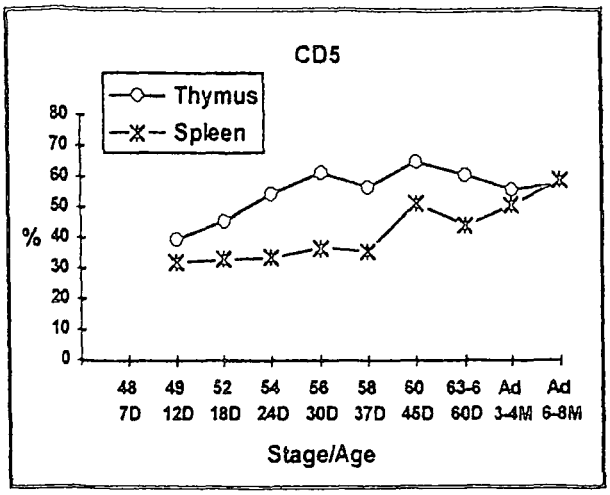
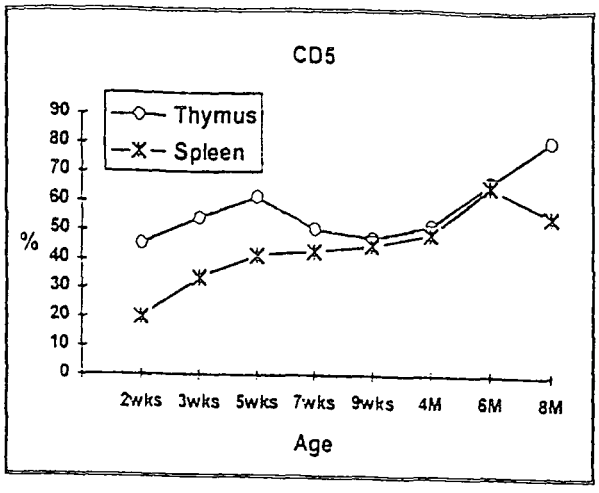


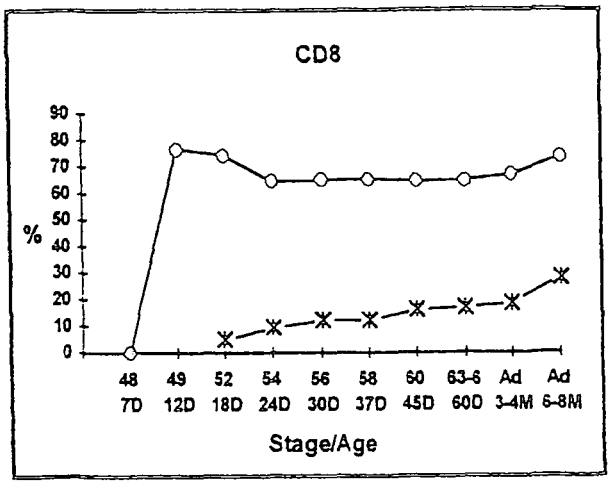
Figure 2.3 A Graph showing the number of lymphocytes ($x10^{-6}$) in the spleen and thymus during the normal development of *Xenopus laevis* from 7 day old larvae to 6-8 month old adults. Each data point represents the mean number of lymphocytes per organ from pooled animals (twenty 7-day old larvae, ten 60-day old larvae and up to 3 adults); B Graph showing the number of lymphocytes ($x10^{-6}$) in the spleen and thymus in perchlorate-blocked larvae from 18 days to 8 month old adults. Each data point represents the mean number of lymphocytes per organ from pooled larvae (fourteen 18 day-larvae, six 45-day larvae and up to 3 8-month old larvae).

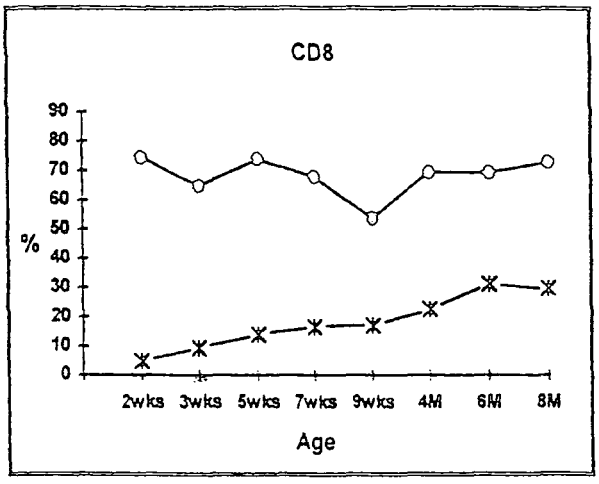
Control Animals

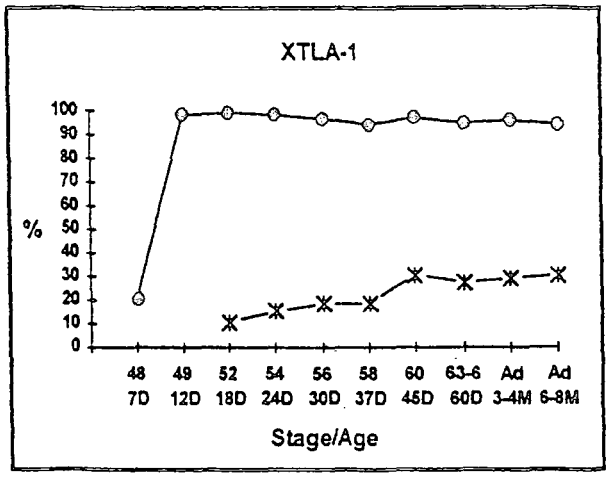
Perchlorate-Blocked Larvae











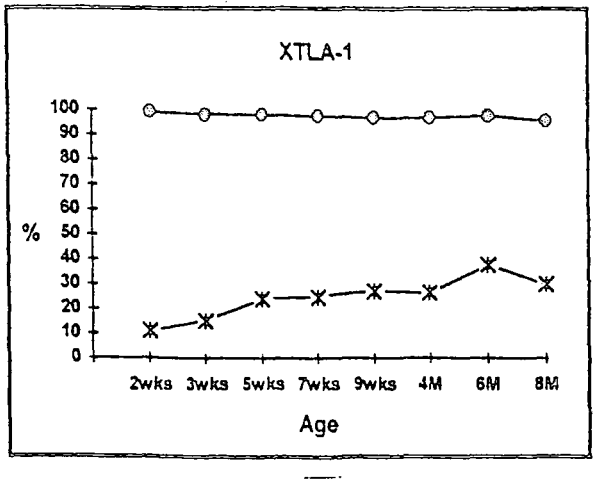
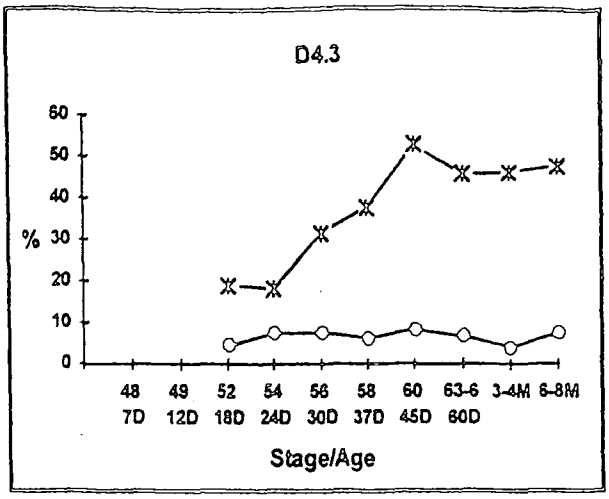
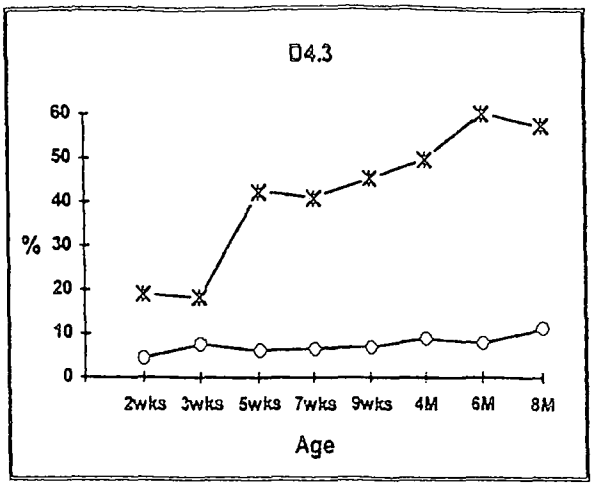
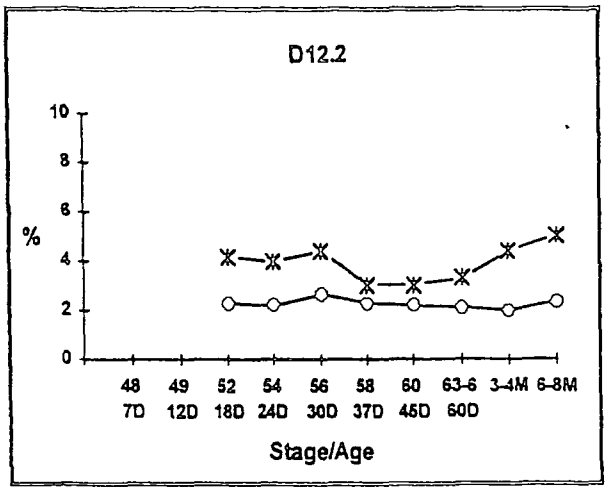
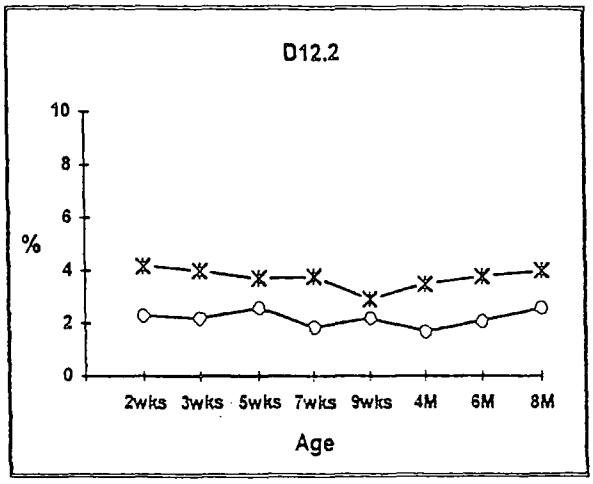


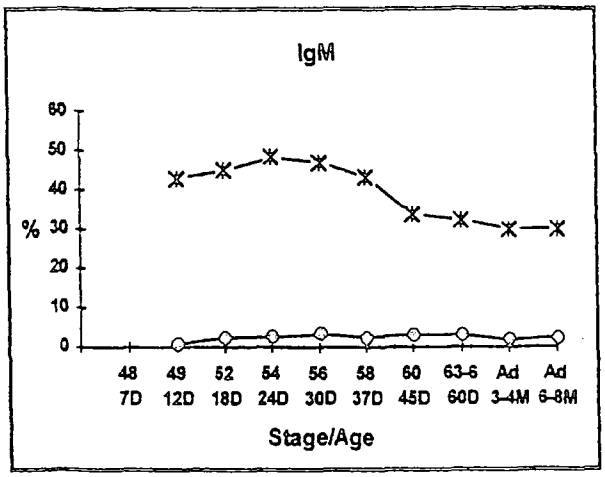
Figure 2.4 Emergence of mAb-defined T and B cell markers in the spleen and thymus during development in control *Xenopus* and perchlorate-blocked larvae. Each data point represents the mean of 2-3 separate experiments where each experiment involved a pool of 10 to 20 larvae and 2-4 adults (maximum standard deviations for thymocytes = ±10% and for splenocytes = ±7%).

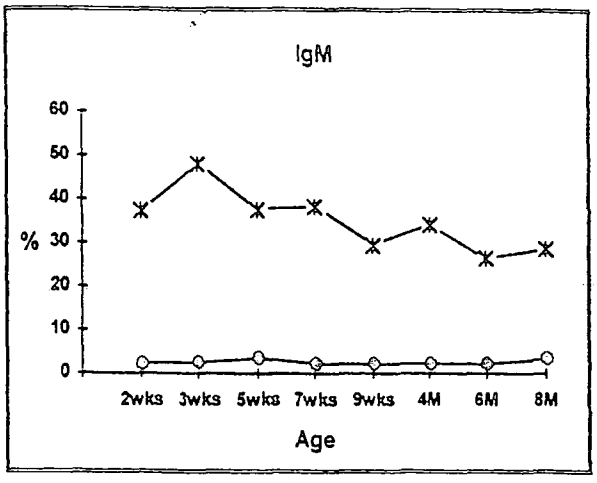

D4.3
%
0
10
20
30
40
50
60
48 49 52 54 56 58 60 63-6 3-4M 6-8M
7D 12D 18D 24D 30D 37D 45D 60D
Stage/Age

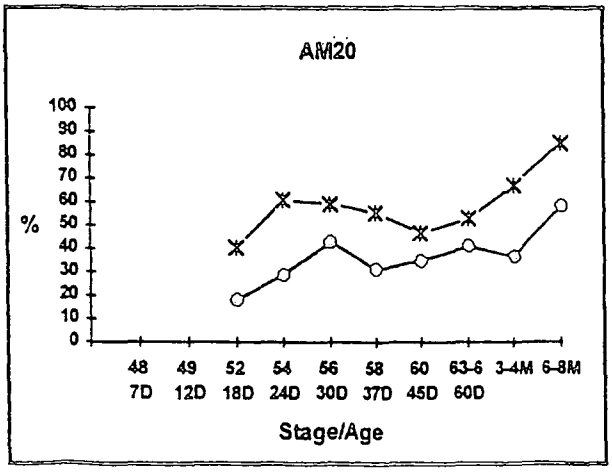

D4.3
%
0
10
20
30
40
50
60
2wks 3wks 5wks 7wks 9wks 4M 6M 8M
Age

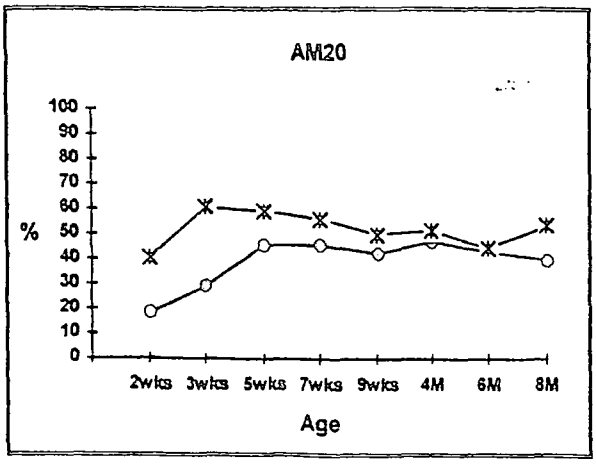

D12.2
%
0
2
4
6
8
10
48 49 52 54 56 58 60 63-6 3-4M 6-8M
7D 12D 18D 24D 30D 37D 45D 60D
Stage/Age


D12.2
%
0
2
4
6
8
10
2wks 3wks 5wks 7wks 9wks 4M 6M 8M
Age


IgM
%
0
10
20
30
40
50
60
48 49 52 54 56 58 60 63-6 Ad Ad
7D 12D 18D 24D 30D 37D 45D 60D 3-4M 6-8M
Stage/Age


IgM
%
0
10
20
30
40
50
60
2wks 3wks 5wks 7wks 9wks 4M 6M 8M
Age


AM20
%
0
10
20
30
40
50
60
70
80
90
100
48 49 52 54 56 58 60 63-6 3-4M 6-8M
7D 12D 18D 24D 30D 37D 45D 60D
Stage/Age


AM20
%
0
10
20
30
40
50
60
70
80
90
100
2wks 3wks 5wks 7wks 9wks 4M 6M 8M
Age

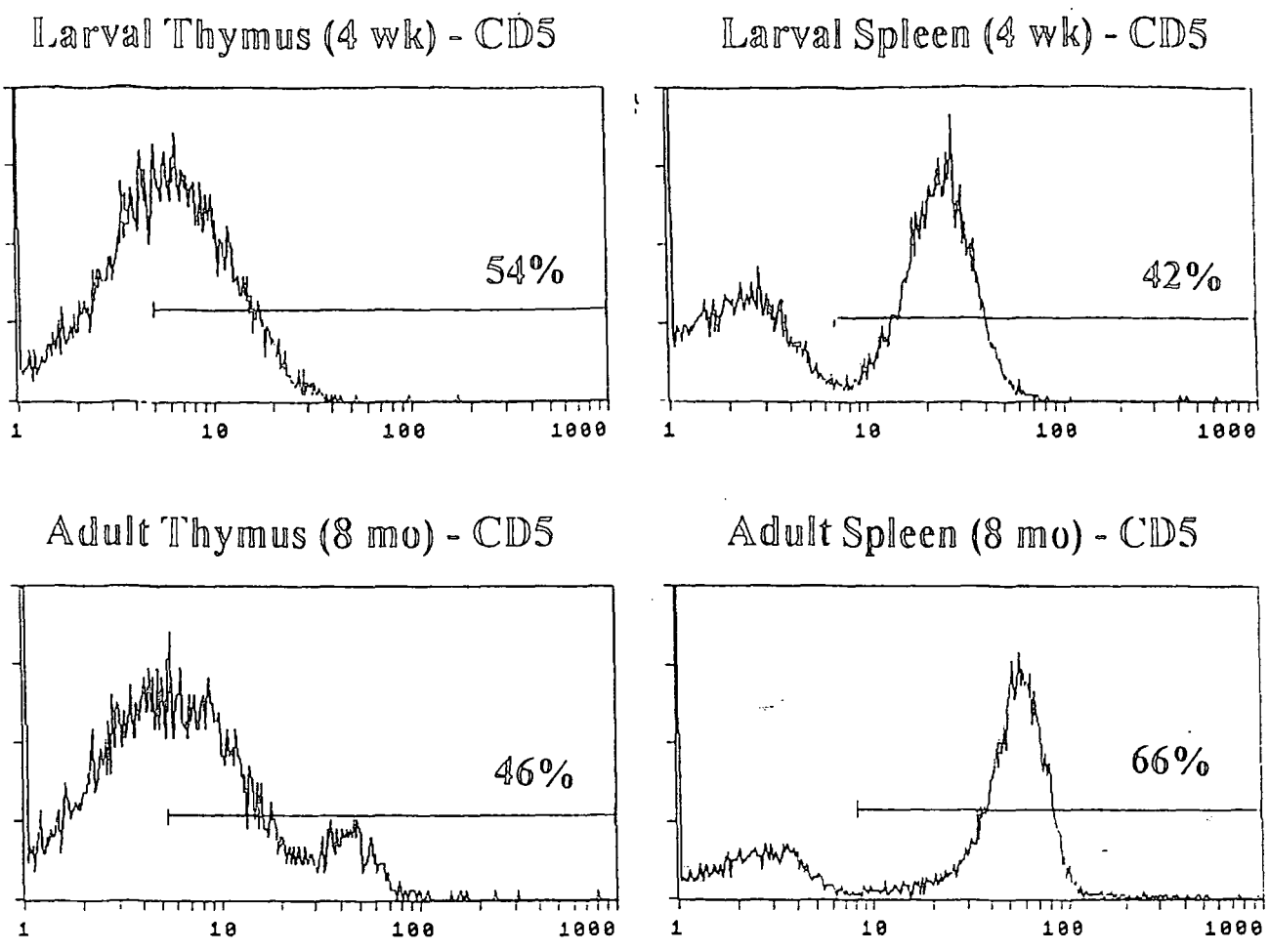


Figure 2.5 Reprentative fluorescence staining with the mAb 2B1 (CD5) in the larval (4 week) and adult (8 month) spleen and thymus. The staining of CD5 on splenocytes is distinct, with much brighter fluorescence compared to thymocytes, which show only a very dull staining intensity. In the 8 month old thymus there is a small but distinct population of cells that bear a bright, splenocyte-like staining intensity with 2B1.

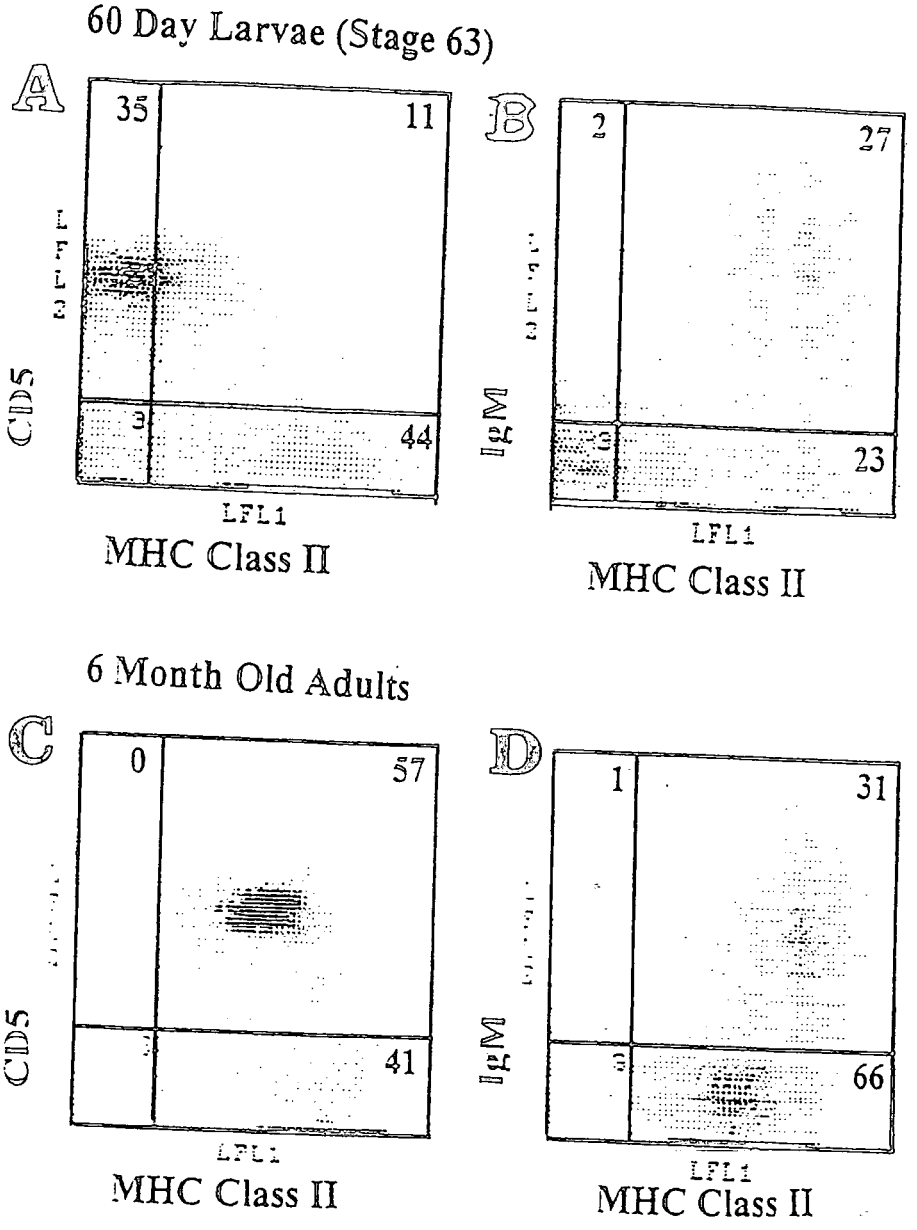


Figure 2.6 Appearance of MHC class II on T cells following metamorphosis. Larval splenocytes dual-stained for CD5 and class II and for IgM and class II show that class II molecules are not expressed on larval T cells (**A**) whilst their B cells freely express class II (**B**). After metamorphosis adult splenocytes stained for these same markers now indicate that both T and B cells express class II (**C** and **D**), but that B cells stain for class II with a greater intensity than T cells.

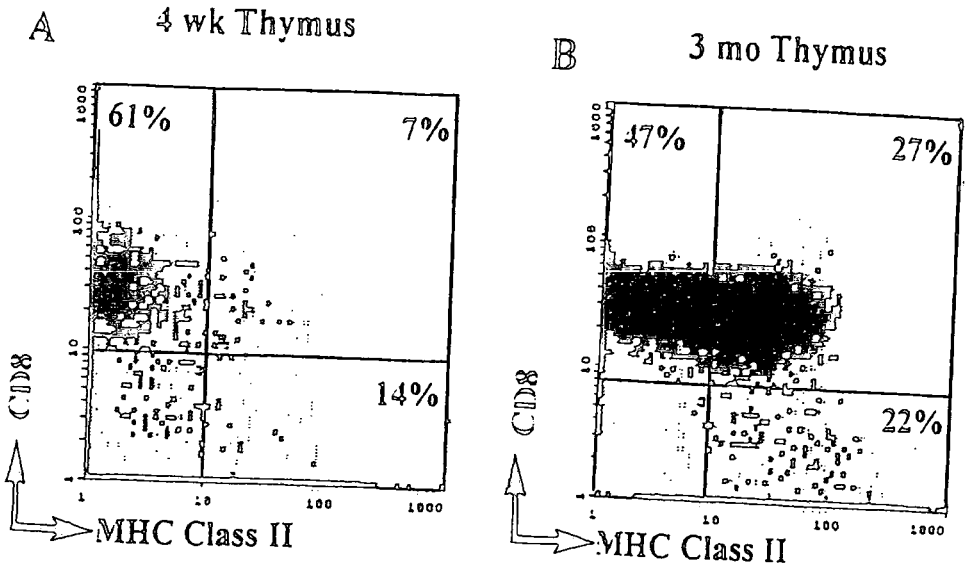


Figure 2.7 Expression of MHC class II on thymocytes from 4 week larvae and 3 month old adults. The majority of thymocytes do not express class II before metamorphosis (A). However in adult frogs (B) MHC class II is expressed in larger amounts on two major populations; $CD8^{+}$class II^{+} (27%) and $CD8^{-}$class II^{+} (22%). There is a population of $CD8^{+}$class II^{-} cells which are presumably immature thymocytes having not yet acquired class II expression.

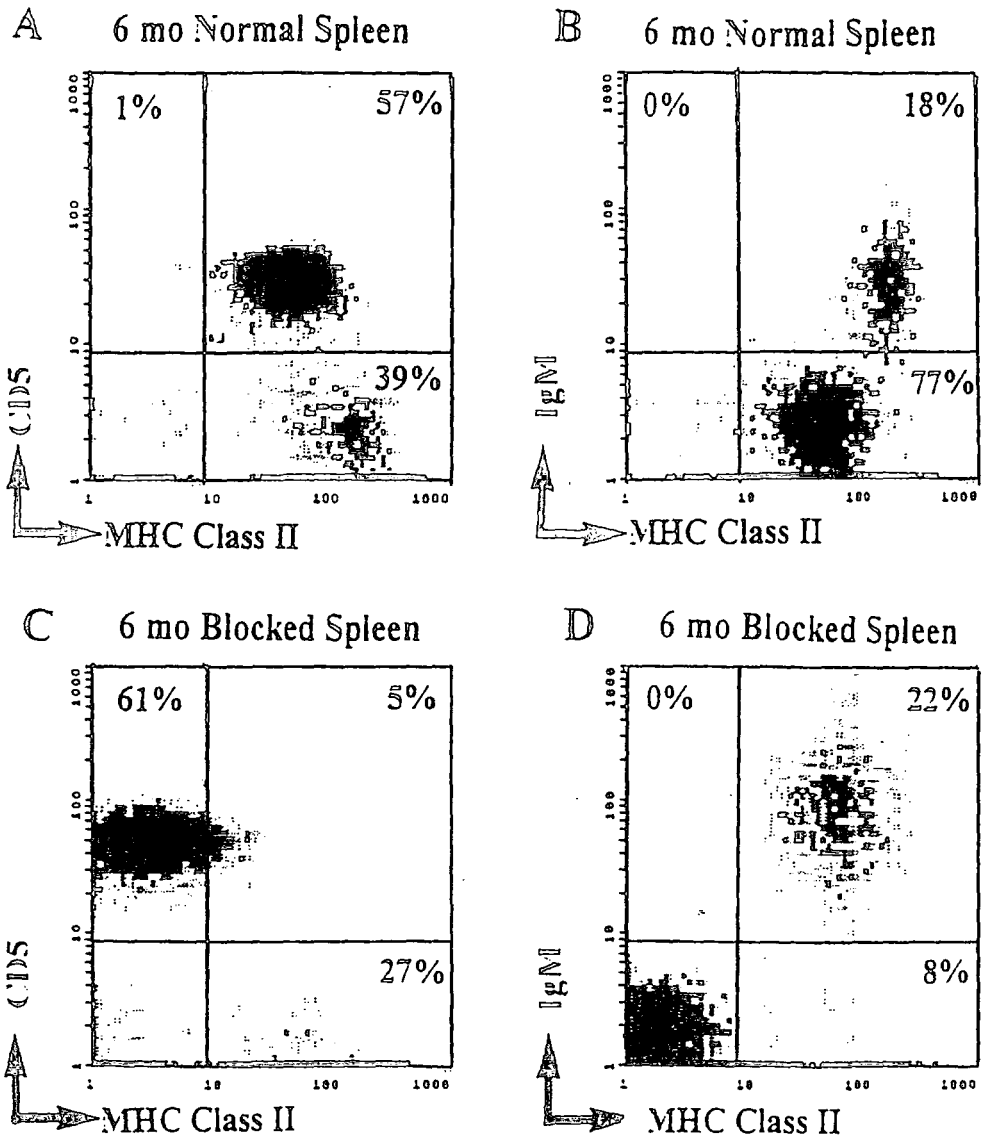


Figure 2.8 Expression of MHC class II on *Xenopus* T cells is metamorphosis-dependent. Confirmation that class II expression on adult T cells is metamorphosis-dependent is shown by dual-staining splenocytes from 6 month old control adults and 6 month old perchlorate-blocked larvae for CD5 and MHC class II and for IgM and class II. In control animals their T cells (57%) and B cells (18%) are class II^+ (**A** and **B**) whilst in perchlorate-blocked larvae of the same age their T cells (61%) are still class II^- (**C**) while their B cells (22%) are class II^+ (**D**). Note B cells of normal adult express more class II than do T cells.

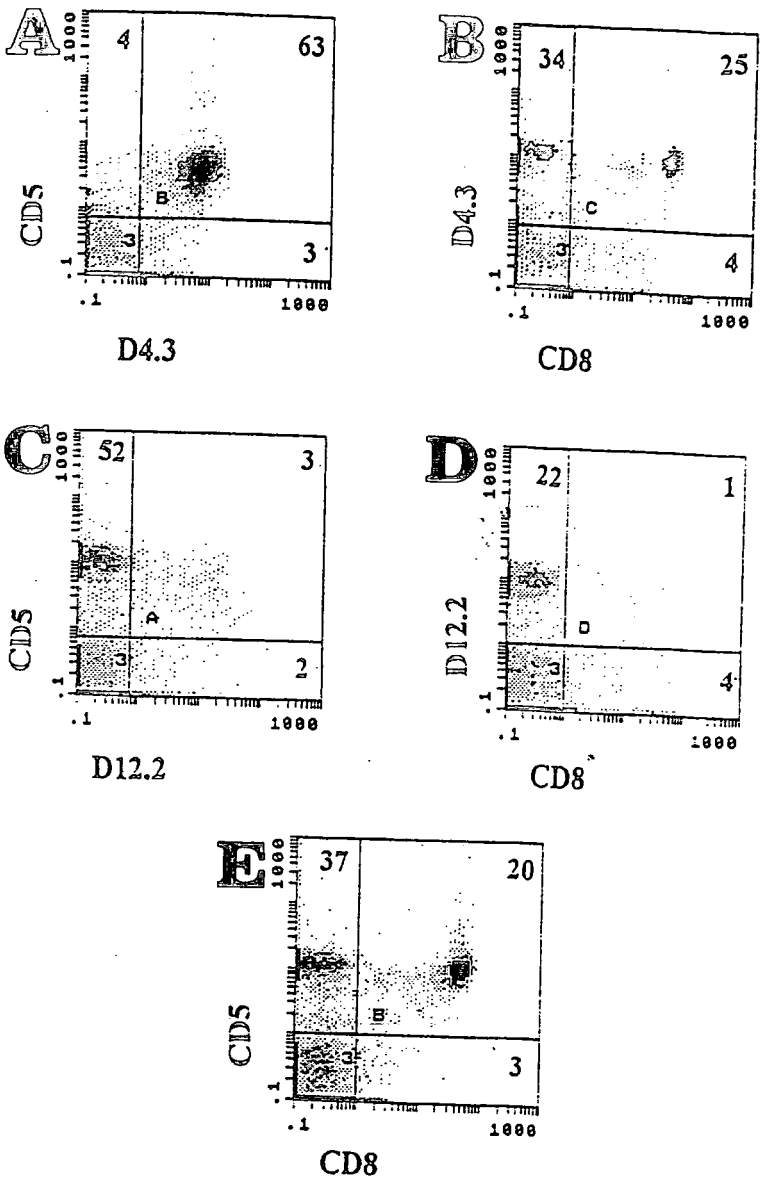


Figure 2.9 Dual staining experiments on adult splenocytes with the mAbs, D4.3, D12.2, CD5 and CD8. (**E**) Approximately a third of adult $CD5^+$ cells co-express CD8. (**A**) Co-staining for D4.3 and CD5 indicates that all $D4.3^+$ cells are $CD5^+$. (**B**) Dual staining for D4.3 and CD8 shows all $CD8^+$ cells co-express D4.3. (**C**) Splenocytes dual stained for D12.2 and CD5 (**D**) Splenocytes dual stained for D12.2 and CD8.

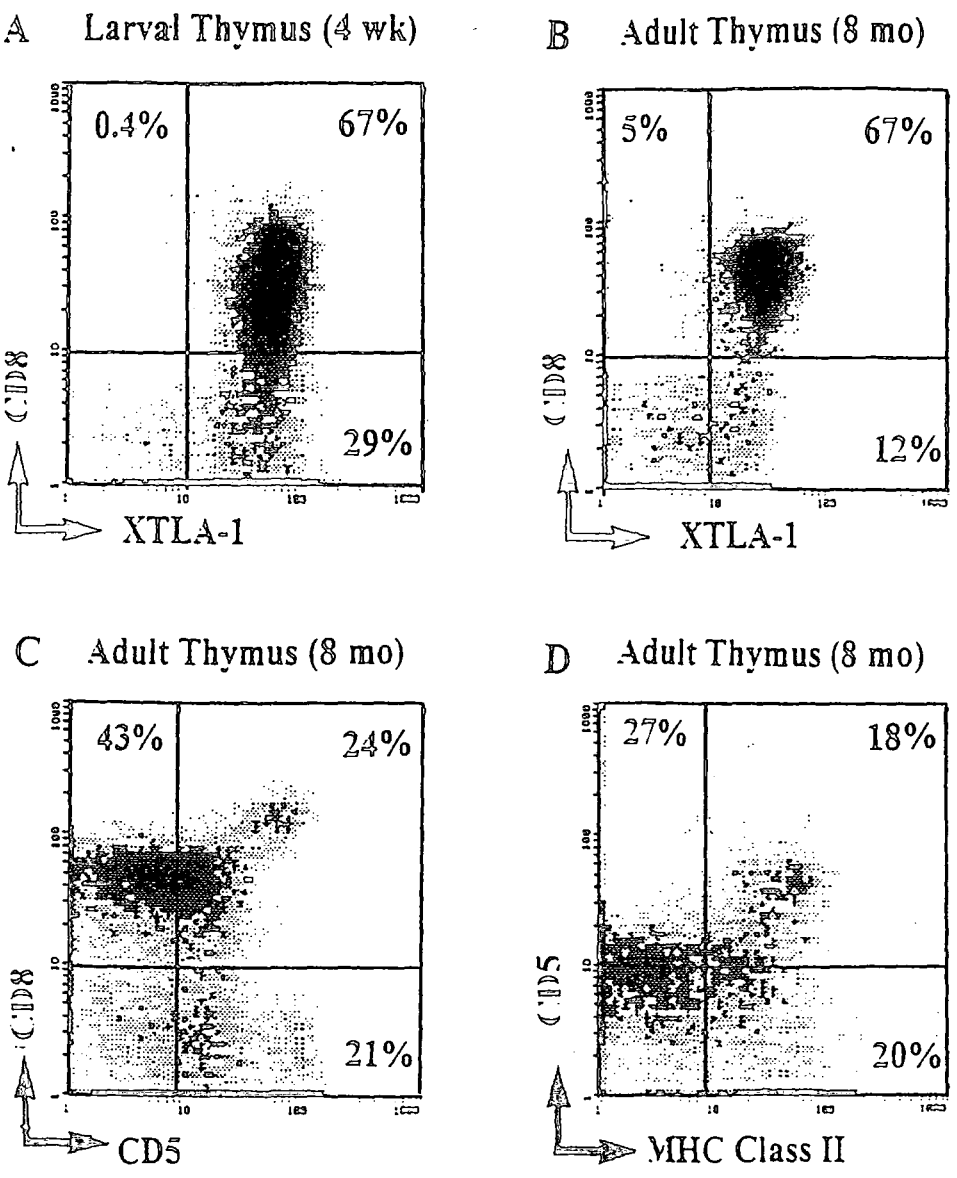
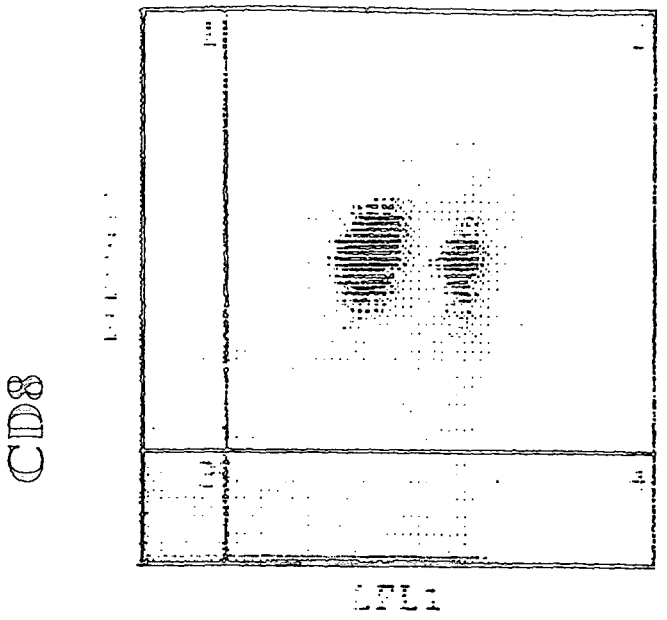


Figure 2.10 Emergence of T cells with a "mature" phenotype in the 8 month adult thymus. Dual staining studies show; a small population of $CD8^{bright}XTLA\text{-}1^{-}$ (5%) is found in the 8 month thymus (**B**) that is not present in the larval thymus (**A**); populations of $CD5^{bright}CD8^{bright}$ and $CD5^{bright}CD8^{-}$ (**C**) and a population of class $II^{+}CD5^{bright}$ cells (**D**) found at 8 months all bear the bright staining intensities more reminiscent of peripheral splenocytes.



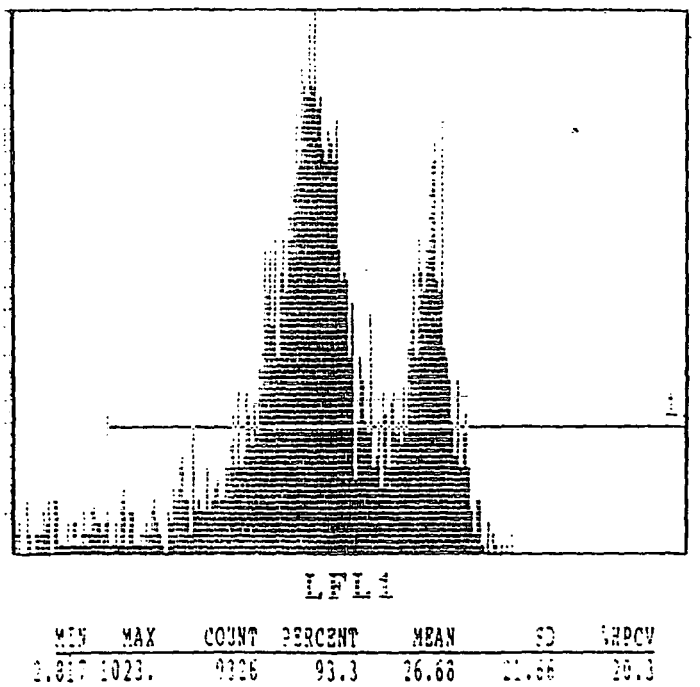


Figure 2.11 A pool of larval thymocytes dual stained for XTLA-1 and CD8 indicate two levels of XTLA-1 expression on $CD8^+$ cells, XTLA-1^{high} and XTLA-1^{low} (A). Single fluorescence trace of a pool of larval thymocytes stained for the XTLA-1 illustrating the two levels of XTLA-1 expression.

Antibody Name	Isotype	Epitope Recognised	Specificity	Reference
2B1	IgG_1	CD5 (71-82 kD)	Pan T cell marker	MD Cooper
AM22	IgM	CD8 (30kD/35kD)	T cell subset	M F Flajnik & L DuPasquier
F17	IgM	CD8 (30kD)	T cell subset	MD Cooper
XT-1	IgG_2	XTLA-1 (120 kD)	T cell subset	S Nagata
8E4:57	IgG	IgM	Pan B cell marker	R H Clothier & L N Ruben
D8	IgG_3	IgM	Pan B cell marker	MD Cooper
D4.3	IgG_1	40kD and 60kD	Not characterised	MD Cooper
D12.2	IgG_1	57kD	Not characterised	MD Cooper
AM20	IgG_{2a}	MHC class II	B cells (strong) T cells (weak)	M F Flajnik & L DuPasquier

Table 2.1 Table showing the anti-*Xenopus* monoclonal antibodies used, their isotype, the epitopes they recognise and their specificity.

A Control *Xenopus laevis* Splenocytes

Lymphocyte Subset	3-6 week old larvae	2-4 month old adults	6-9 month old adults
$CD5^+CD8^+XTLA\text{-}1^-$	4.4±2.5	6.0±4.2	6.5±5.3
$CD5^+CD8^+XTLA\text{-}1^+$	6.7±2.4	13.7±3.9	21.2±4.9
$CD5^+CD8^-XTLA\text{-}1^+$	10.5±3.5	9.5±6.4	7.4±4.5
$CD5^+CD8^-XTLA\text{-}1^-$	10.8±4.9	19.9±6.5	23.5±9.8
Total $CD5^+$	32.3±7.7	49.1±4.7	58.7±7.2

B Perchlorate Blocked *Xenopus laevis* Splenocytes

Lymphocyte Subset	3-6 week old larvae	2-4 month old adults	6-9 month old adults
$CD5^+CD8^+XTLA\text{-}1^-$	4.4±2.5	3.75±1.3	4.3±2.3
$CD5^+CD8^+XTLA\text{-}1^+$	6.7±2.4	17.3±6.8	26.3±3.8
$CD5^+CD8^-XTLA\text{-}1^+$	10.5±3.5	11.0±2.5	7.7±2.1
$CD5^+CD8^-XTLA\text{-}1^-$	1.08±4.9	12.3±7.6	24.3±5.5
Total $CD5^+$	32.3±7.7	44.3±2.2	62.7±6.8

Table 2.2 Table showing lymphocyte subsets in control and perchlorate-blocked animals stained for CD5, CD8 and XTLA-1. For control animals the number of repeat experiments for each time point were twelve 3-6 week old larvae, fifteen 2-4 month old adults and eleven 6-9 month adults. For perchlorate-blocked larvae the number of repeat experiments for each time point were twelve 3-6 week old larvae, four 2-4 month larvae and three 6-9 month larvae.

Chapter 3

In Vitro Studies Investigating the Thymus Dependence of T Cell Marker Expression and the Induction of Apoptosis

3.1 Introduction

The initial aim of this Chapter is to determine what effect removing the thymus at 5-7 days has on T cell marker development. At 7 days the thymus is still in its infancy, containing only up to 1,000 cells which have not yet reached maturity (Manning and Horton, 1969; see also Chapter 2). Many workers have examined the effect of early thymectomy on the overall functioning of the immune system of *Xenopus*. Tochinai and Katagiri (1975) showed that animals thymectomized soon after the thymus bud first appears (at around 4 days of development) are unable to reject skin allografts and revealed no signs of lymphocyte infiltration into allografts, whilst euthymic *Xenopus* rejected skin grafts from the same donors after only 16-37 days. Thymectomy at 7 days prevents splenic lymphocytes from displaying a mixed lymphocyte reaction (MLR) indicating that alloreactive T cells may not develop in thymectomized (Tx) animals (DuPasquier and Horton, 1976). On the other hand, several authors have revealed that however early thymectomy is performed, some Tx *Xenopus* can still chronically reject skin allografts (Horton and Manning, 1972; Tompkins and Kaye, 1980; Nagata and Cohen, 1983). Furthermore, following skin allograft rejection by Tx animals, splenocytes are able to mount a MLR, not only against donor stimulator cells, but against third party cells as well (Nagata and Cohen, 1983). It is doubtful that sufficient T cells could have emigrated from the thymus before thymectomy due to the earliness of

the operation. It seems more likely that the thymus can promote some degree of 'T-like' cell development in the form of thymic hormone influence on extrathymically-derived precursors, as recently suggested in the mammalian situation (Wang and Klein, 1994; Lin et al, 1995).

Early larval thymectomy abrogates antibody production against thymus-dependent antigens such as sheep red blood cells and human gamma globulin. However, the responses of Tx *Xenopus* to thymus-independent antigens such as *E coli* lipopolysaccharide and purified protein derivative from tuberculin, remain unaffected (Manning et al, 1977). In Tx *Xenopus in vitro* responses of splenocytes to the T cell mitogen, concanavalin A (ConA), were reduced to a very low, almost non-existent level (Manning et al, 1977), whilst the response to phytohaemagglutinin (PHA), another T cell mitogen, was lost completely following thymectomy (DuPasquier and Horton, 1976). The low level residual ConA response is consistent with the finding of chronic skin allograft rejection in Tx animals, supporting the possibility that an extrathymic pathway of T cell development could exist. The possible presence of such a pathway in *Xenopus* has been discussed elsewhere (Turner et al, 1991; Horton et al, 1996).

This Chapter initially examines the thymus dependency of the panel of anti-*Xenopus* T cell-specific mAbs used in Chapter 2, by comparing cell surface antigenic expression in control and Tx splenocytes. These T cell markers are also investigated after *in vitro* stimulation with a variety of mitogenic agents, to probe if changes in antigen expression occur following stimulation, and to determine whether T cell surface markers can be induced on the splenocytes from Tx animals. This will assess whether progenitor cells or immature cells exist in Tx *Xenopus* that can be pushed along the T cell development pathway by *in vitro* stimulation. To back up the flow cytometric findings, thymidine incorporation assays will also be carried out to examine whether splenocytes are induced to proliferate by these mitogenic agents. Stimulation of *in vitro* cultured cells will be attempted using agents such as calcium ionophore and phorbol esters (to activate both T and B cells), concanavalin A (to stimulate T cells) and *E coli* lipopolysaccharide (to stimulate B cells).

Since *in vivo* mitogen stimulation of *Xenopus* cells can lead to apoptosis (Ruben et al, 1994a,b), the present study will also probe whether apoptosis is being induced in mitogen-stimulated splenocytes *in vitro*. Apoptosis in mammals has been studied in great detail over recent years and is a common form of cell death that is distinct from

necrosis in that it is an active process requiring ATP and protein synthesis (Earnshaw, 1995). Necrosis is a form of cell death whereby the cell ruptures and releases its contents into the surrounding area which ensures that an immune response is initiated by nearby lymphocytes. Apoptosis on the other hand results in a decrease in cell size, a break-up of the cell's DNA and blebbing from the plasma membrane (Cohen, 1993; Korsmeyer, 1995). This ensures that the contents of the cell remains within these 'apoptotic bodies' which are then phagocytosed by macrophages, thereby avoiding an inflammatory response (Savil et al, 1993).

Two approaches are used in this Chapter to identify apoptosis in mitogen-treated lymphocytes from control *Xenopus*. These were flow cytometric analysis of DNA (to illustrate the cell cycle and identify a sub-diploid 'apoptotic' peak (Milner et al, in press)) and fluorescence microscopy of acridine orange stained cells. A novel antibody that is specific to an apoptosis specific protein (ASP) in humans (Grand et al, 1995) is also briefly assessed for its capacity to identify apoptosis in *Xenopus* thymus tumour cells.

3.2 Materials and Methods

3.2.1 Animals and Operations

3.2.1.1 Animals

Outbred *Xenopus laevis* were used throughout, and were bred and reared in the laboratory under standard conditions (Horton and Manning, 1972). Animals used in these experiments were aged between 6 and 14 months.

3.2.1.2 Thymectomy

Thymectomy operations were carried out by microcautery on tadpoles of 5-7 days of age by J D Horton as previously described (Horton and Manning, 1972). When thymectomized (Tx) animals were taken, the thymic region was routinely checked under the microscope for any signs of remaining thymic tissue or thymic regeneration - such animals were rarely found and were excluded from these experiments.

3.2.1.3 Spleen Cell Preparations

Animals were heavily anaesthetised in a solution of 3-aminobenzoic acid ethyl ester (MS222). The body was sprayed with 70% alcohol before transferring to a dissecting microscope in a laminar air flow cabinet. The spleen was then dissected out using forceps and micro-scissors and placed into a sterile 3.5cm Costar petri dish containing 2ml amphibian culture medium. Cells were released from the organ by gentle teasing with a pair of tungsten needles. The cell suspension was then transferred to a sterile 5ml Falcon tube, and the petri dish was washed with a further 1ml medium and the washings added to the tube. The suspension was then pipetted vigorously to further help release cells, before larger pieces of tissue debris and clumps were allowed to settle before transferring the supernatant suspension to a fresh tube. Splenocytes were then centrifuged at 300g, 4°C for 10 minutes and resuspended in 1ml fresh medium and counted using a Neubauer haemocytometer.

3.2.2 Amphibian Culture Medium

The culture medium used was adapted from a recipe for channel catfish medium (Luft, Clem and Bly, 1991) that was diluted to amphibian strength. Equal parts of AIM-V (Gibco), L-15 (Gibco) and autoclaved double distilled water were added to a 100ml bottle with the addition of 1.25mM L-glutamine, 0.01M Hepes buffer and heat-inactivated foetal calf serum (FCS) (either 1% for thymidine assays or 5% for culture of lymphocytes prior to flow cytometric analysis). This amphibian medium was then

filtered through a 0.2μ Gelman filter into an autoclaved bottle. Medium was stored at 4°C for up to a maximum of 2 weeks. Just prior to culture, sodium bicarbonate was added to the medium at 0.16M.

3.2.3 Tritiated Thymidine Studies on Mitogen-Treated Splenocytes from Control and Thymectomized Animals

Proliferative assays were assessed by the amount of tritiated thymidine (^{3}H-TdR) incorporated into the cells DNA after mitogenic stimulation.

3.2.3.1 Concanavalin A

Concanavalin A (ConA) (Sigma) was reconstituted in APBS to a concentration of 1mg/ml and stored at -20°C in 100μl aliquots. Splenocytes were counted and adjusted to $1x10^6$ lymphocytes per millilitre in amphibian culture medium containing 1% FCS. Aliquots of $1x10^5$ cells in 100μl were added to wells of a 96-well flat-bottomed plate (Greiner) set up in triplicate. Experimental wells were given 10μl of different concentrations of ConA (final in-well concentrations were 100μg/ml, 10μg/ml, 2.5μg/ml, 1μg/ml and 0.1μg/ml), whilst medium control wells were given an equivalent volume of medium. One hundred microlitres of distilled water was added to all wells surrounding experimental wells - this helped prevent evaporation from cultures. Plates were incubated at 27°C with 5% CO_2 in air for 18-20 hours, after which medium was changed in all wells. The plate was then incubated for a further 24 hours before each well received a one microCurie (μCi) (specific activity = 5 Ci/mmol) pulse of ^{3}H-TdR (Amersham). Cells were harvested 18-20 hours later onto fibreglass filters (Whatman) using a Skatron cell harvester. Filters were then dried at 60°C before individual filter discs containing the samples were placed into 5ml scintillation vials. Four millilitres of scintillation fluid (Betafluor) was added to each vial and the amount of incorporated thymidine was calculated on a Packard Liquid Scintillation analyser.

The level of incorporated tritiated thymidine was calculated by working out the stimulation index for each experimental sample using the disintegrations per minute (dpm):

$$\text{Stimulation Index (SI)} = \frac{\text{Mean dpm Mitogen Stimulated Samples}}{\text{Mean dpm Medium Control Samples}}$$

Once the optimal concentration of ConA had been determined, all future proliferative assays with ConA were carried out using this optimal concentration (see Figure 1).

3.2.3.2 Phorbol Myristate Acetate and Calcium Ionophore

Phorbol myristate acetate (PMA)(Sigma) was reconstituted in DMSO to a concentration of 0.5mg/ml and stored at -20°C in 50µl aliquots. Calcium ionophore (CaIon), A23187 (Sigma) was reconstituted in DMSO to a concentration of 1mg/ml and stored at -20°C in 50µl aliquots. Splenocytes were adjusted to $1x10^6$ lymphocytes per millilitre and set up in a 96-well flat bottomed plate as described above (3.2.3.1). Experimental wells were given 10µl PMA (final in-well concentrations of 100ng/ml, 50ng/ml, 10ng/ml or 5ng/ml) together with 10µl CaIon (final in-well concentrations of 0.5µg/ml, 0.25µg/ml, 0.1µg/ml and 0.01µg/ml). Plates were incubated at 27°C with 5% CO_2 in air for 18-20 hours, after which medium was changed in all wells to remove the mitogens and stop any toxic effects of these chemicals. After a further 24 hours incubation all wells received a 1µCi pulse of ^{3}H-TdR and were incubated for 18-20 hours before being harvested for liquid scintillation counting (see 3.2.3.1).

3.2.3.3 Lipopolysaccharide

E coli lipopolysaccharide (LPS) (Sigma) was reconstituted in autoclaved double distilled water to a concentration of 2mg/ml and stored at 4°C. Splenocytes were adjusted to $1x10^6$ lymphocytes per millilitre and set up in a 96-well flat-bottomed plate as described in 3.2.3.1. Experimental wells received 12.5µl LPS to give a final concentration of 250µg/ml (previously shown to be optimal, data not shown) and cultured and harvested as described in 3.2.3.1.

3.2.4 Flow Cytometric Analysis of Cell Surface Antigens Following Mitogenic Stimulation

3.2.4.1 Mitogens Used

a) Concanavalin A

Splenocytes from control and thymectomized animals were counted and adjusted to $1x10^6$ cells per mililitre in amphibian culture medium containing 5% FCS. For these flow cytometric studies, cells were cultured in 24-well plates (Greiner), each well containing $1x10^6$ lymphocytes in 1ml. One hundred microlitres of ConA was added to experimental wells at a final (optimal - see Figure 3.1) concentration of 2.5µg/ml whilst control wells received an equivalent volume of medium. The plate was incubated in a humidified

incubator at 27°C with 5% CO_2 in air. Medium was changed after 18-20 hours and fresh medium added. Cells were usually taken for flow cytometric analysis after a further 24 hours. If cells were to be cultured for longer periods of time, medium was changed on all experimental and control wells after every 3-4 days.

b) Phytohaemagglutinin

Phytohaemagglutinin (PHA-P) (Flow, ICN) was reconstituted with autoclaved distilled water to a concentration of 1mg/ml and 100µl aliquots were stored at -20°C.

Splenocytes were cultured as in 3.2.4.1a, with PHA being used at a final concentration of 1µg/ml, which had previously been shown to be optimal in terms of ^{3}H-TdR incorporation (data not shown).

c) Lipopolysaccharide

Splenocytes were cultured as in 3.2.4.1a, with 125µl LPS being added to experimental wells to give a final concentration of 250µg/ml.

d) PMA and Calcium Ionophore

Splenocytes were cultured as in 3.2.4.1a, with PMA being used at a final concentration of 10ng/ml and CaIon being used at 0.1µg/ml.

3.2.4.2 Flow Cytometry

Splenocytes were taken from the culture plate well and placed into a 5ml Falcon tube. The well was then washed out with 1ml FACS medium (see Appendix A) and the washings added to the tube. The cells were then centrifuged at 300g, 4°C for 10 minutes and adjusted to $1x10^6$/ml with fresh FACS. The cells were then labelled with fluorescent-labelled mAbs as detailed in Chapter 2 section 2.2.5.

3.2.5 Identification of Apoptosis in Mitogen-Treated Splenocytes

3.2.5.1 Flow Cytometric Analysis of Cell Cycle and DNA Changes During Apoptosis

B_3B_7 *Xenopus* thymus tumour cells (Robert et al, 1994) were initially used to show all the phases of the cell cycle as these tumour cells are constantly replicating their DNA and dividing. This is in contrast to medium cultured splenocytes which show only minimal DNA replication, with only a major peak at the G_0G_1 phase of the cell cycle.

Splenocytes were cultured with either ConA or PMA to induce apoptosis. For DNA analysis, aliquots of 200µl were dispensed into 5ml Falcon tubes. Cells were then either processed on a Coulter DNAPrep machine, or manually using reagents prepared in the laboratory - both methods gave similar results.

3.2.5.1.1 Propidium Iodide Staining of DNA

a) DNAPrep

Samples were processed automatically on the Coulter DNAPrep machine, receiving a 200µl aliquot of lysing and permeabilising reagent (LPR), followed by 2ml propidium iodide solution containing RNAse. The sample was thoroughly vortexed between each addition and was ready within about 30 seconds. Samples were then left in the dark for up to 15 minutes to allow the propidium iodide staining to saturate the nucleic acid. Samples were then analysed on a Coulter Epics XL-MCL flow cytometer.

b) Manual Preparation

Samples were first permeabilised by using 50µl of saponin solution [APBS containing 0.1% saponin (Flow Labs) and 0.1% BSA, pH 7.4]. Five hundred microlitres of propidium iodide solution (0.25mg/ml propidium iodide (Flukka) in APBS) was then added, followed by 500µl APBS and a 50µl aliquot of RNAse (stock solution made up in APBS at 1mg/ml using type I-AS RNAse (Sigma) and stored at -20°C in 200µl aliquots). Samples were thoroughly vortexed and stored in the dark for up to 15 minutes before being analysed on a Coulter Epics XL-MCL flow cytometer.

3.2.5.1.2 DNA Analysis

A protocol designed for DNA analysis was set up which consisted of 5 histograms (see Figure 3.9):-

1 - **FS vs SS** which was identical to that used for surface fluorescence studies, containing an analysis gate to include lymphoid cells.

2 - **FL3 vs FL3 Peak** - this histogram was used to ensure that only single cells were analysed. It is possible for two cells to pass through the analysis point so close together that the result would appear to be one cell with twice the DNA ie. a cell in the G_2/M phase, therefore this is a check that doublets are not analysed.

3 - **Single colour FL3 histogram (gated)** - this histogram analyses the amount of propidium iodide associated with DNA. Increasing fluorescence intensity is recorded along the x-axis (on a linear scale) with relative cell count along the y-axis. A linear scale is used for DNA analysis so that the stages in the cell cycle can be seen clearly - a logarithmic scale would condense these stages so that identification would become very difficult.

4 - **Single colour FL3 histogram (ungated)** - this histogram is identical to histogram 3 except that all cells are analysed, ie. those both inside and outside the analysis gates.

5 - **Time vs FL3** - this histogram ensures that PI binding has saturated the DNA - this line should be and remain horizontal for the analysis time, any deviation either upwards or downwards means that optimal PI binding has not been achieved.

Samples were analysed on low flow rate at a concentration of no greater than 100 events per second for a period of 5 minutes.

3.2.5.2 Apoptosis Monitored by Fluorescence Microscopy of Acridine Orange Stained Splenocytes from Control *Xenopus*

An aliquot of 200µl was taken from the cell culture and resuspended in APBS to a concentration of $2x10^6$ then placed in an 1ml Eppendorf and centrifuged at 300g at 4°C for 10 minutes. The supernatant was removed and the cells resuspended in 10µl APBS, before 10µl acridine orange (10µg/ml) was added. The cells were thoroughly resuspended before adding 10µl to an alcohol cleaned slide and a coverslip placed on top. The slides were then viewed under a Nikon Optiphot microscope fitted with episcopic fluorescent attachments using the immunofluorescence FITC filter set.

3.2.6 Use of ASP Antibody to Identify Apoptotic *Xenopus* Tumour Cells

Cytocentrifuge preparations were made of *Xenous* thymus tumour cells during log phase growth. One million cells were centrifuged at 300g at 4°C for 10 minutes before being resuspended in a 1:1 mixture of APBS and FCS at a concentration of $2x10^6$/ml. Cytocentrifuge chambers were assembled using alcohol cleaned slides and Shandon filter mats. An aliquot of 10µl FCS was added to each chamber and centrifuged at full

speed for one minute to coat the slide to give protection to the cells. Seventy five microlitres of cell suspension was then added to each chamber and centrifuged at 500rpm for 5 minutes. The slides were then carefully removed and samples allowed to air dry for 5 minutes, prior to staining with anti-ASP antibody. Slides were fixed in acetone (BDH) for 5 minutes before being incubated for 30 minutes in blocking buffer (APBS + 1% BSA). Slides were then washed 3x with wash buffer (APBS + 0.1% BSA) and an aliquot of rabbit anti-ASP antibody (1:100 made up in APBS + 10% goat serum) was added to each slide and incubated for one hour at room temperature in a humidified chamber. The slides were then washed 3x with wash buffer before the goat anti-rabbit IgG-FITC conjugated antibody (1:50) was added to the slides, and incubated for 30 minutes at room temperature as before. The slides were washed 3x with wash buffer and propidium iodide (0.25mg/ml) was added to the slides for 2 minutes before washing briefly with wash buffer. Slides were mounted in PBS/glycerol (Citifluor) and viewed under a Nikon Optiphot microscope fitted with FITC filters. Apoptosis-specific staining was seen as green fluorescence, whilst the counter-stain PI appeared red.

3.3 Results

3.3.1 Effect of Thymectomy on Splenocyte Cell Surface Markers

Following thymectomy performed on 5-7 day old larvae there are profound, reproducible changes amongst monoclonal antibody-defined cell surface markers (see Figure 3.2). Cell surface antigens CD5, CD8, D4.3 and XTLA-1 cannot be detected by flow cytometric analysis on splenocytes taken directly from the thymectomized froglets up to 8 months old. The percentage of $sIgM^+$ B cells is increased proportionately, due to the loss of T cells, but the actual number of B cells is lower in these particular spleens of thymectomized animals than in age-matched siblings. For example, the percentage of $sIgM^+$ splenocytes in a control animal (not shown in Figure 3.2) = 49.6% $\Rightarrow$ $2.70x10^6$ lymphocytes, whilst in an age-matched thymectomized sibling the $sIgM^+$ percentage = 69.3% $\Rightarrow$ $2.46x10^6$ splenic lymphocytes. The percentage of $D12.2^+$ cells increased in thymectomized animals, as shown in Figure 3.2 (11% in the thymectomized animal compared to just 4% in the control).

The percentage of macrophages in the spleen was often elevated in thymectomized animals (data not shown). Thus studies with the mAb, D4.1 - against *Xenopus* macrophages, revealed levels of to 20% positive cells in control animals, but this was doubled in several thymectomized *Xenopus*.

3.3.2 *In Vitro* Stimulation of Control and Thymectomized Splenocytes with T Cell Mitogens

3.3.2.1 Cell Size and Proliferation

Cytospins revealed that splenocytes from control *Xenopus* aged 8 months respond to PHA and ConA with increased cell size (Figure 3.3). This was confirmed by flow cytometric analysis of forward scatter vs side scatter plots (see Figure 3.4B). Splenocytes from Tx siblings cultured with T cell mitogens showed no noticeable increase in cell size (Figure 3.4D).

The level of ^{3}H-TdR incorporation was used as a measure of cell proliferation and stimulation indices were calculated in order to compare different experiments (see Table 3.1). For control splenocytes, stimulation with ConA induced cells to proliferate as judged by stimulation indices from 23 to 72 (a stimulation index of 1.0 indicates no proliferation). PHA gave stimulation indices of 18 to 51 (data not shown) indicating that both these T cell mitogens had profound effects on control splenocytes.

Splenocytes from thymectomized animals showed no indication of a proliferative response to either ConA (see Table 3.1) or PHA (data not shown). The stimulation indices achieved with these mitogens on thymectomized splenocytes were ≤ 1.

3.3.2.2 Flow Cytometric Cell Surface Analysis

Following 48 hours culture of splenocytes from 8 month old control animals in the presence of ConA or PHA, there were no apparent changes in the percentages of cells stained with the panel of monoclonal antibodies, but there were changes in the staining intensities. There was a general lowering in staining intensity for CD5, CD8 and D4.3, as well as a noticeable broadening of fluorescence intensities with CD5 and CD8 (see Figure 3.5), compared to medium controls. The staining intensity of MHC class II frequently increased following splenocyte culture in medium alone. After stimulation with the T cell mitogen, the mean intensity of MHC II staining was occasionally even more pronounced (Figure 3.6), but this was not always apparent (Figure 3.5). There appeared to be no changes to $sIgM^+$ B cells.

ConA stimulation of splenocytes from Tx *Xenopus* did not induce the expression of T cell markers - eg. CD5 and CD8 (see Figure 3.7). D12.2 and sIgM staining was usually comparable in medium cultured and ConA stimulated Tx splenocytes.

3.3.3 *In Vitro* Stimulation of Control and Thymectomized Splenocytes with a B Cell Mitogen

3.3.3.1 Cell Size and Proliferation

Following 48 hours culture in LPS, there was a slight increase in lymphoid cell size in control (Figure 3.4C) and Tx (data not shown) splenocyte suspensions.

Table 1 reveals that control splenocytes responded to LPS stimulation with stimulation indices of 3 to 5. Splenocytes from thymectomized animals responded to LPS with stimulation indices from 2 to 9.

3.3.3.2 Flow Cytometric Cell Surface Analysis

There is no appreciable change in the cell surface markers CD5, CD8 or D4.3 - there is no diminution of the staining intensity that was seen when the T cell mitogens were used. After LPS treatment, the percentage and staining intensity of $sIgM^+$ B cells was comparable to medium cultured cells. The percentage of MHC class II^+ splenocytes does not increase following stimulation, but a population of cells with increased fluorescence intensity of AM20 staining is noticeable (Figures 3.5 and 3.6C). Dual-

colour flow cytometry showed this bright MHC II population to be $sIgM^+$ B cells. Expression of D12.2 appears to be unaffected by stimulation with LPS.
For Tx splenocytes, there was no significant change in the percentage of $sIgM^+$ B cells following stimulation with LPS (Figure 3.7). The percentage of $D12.2^+$ splenocytes does not alter significantly, and there is no suggestion of the appearance of T cell markers (Figure 3.7).

3.3.4 *In Vitro* Stimulation of Splenocytes from Control and Thymectomized Animals with Phorbol Myristate Acetate and Calcium Ionophore

3.3.4.1 Cell Size and Proliferation

Phorbol myristate acetate (PMA) and calcium ionophore (CaIon) were used in conjunction with each other in initial studies on control *Xenopus*. Optimal concentrations of these agents were determined in ^{3}H-TdR dose response assays in 4 and 7 day cultures (see Figure 3.8). In a four day culture there is no one single optimal dose of PMA, as 100, 50 and 10ng/ml all result in a high level of proliferation, but it was found that 5ng/ml was sub-optimal. However, the dose of CaIon appeared critical with 0.1μg/ml proving optimal at 4 days. Doses either side of this resulted in a dramatic drop in proliferation (down from a stimulation index of 200 to just 100 for 0.25μg/ml and to just 12 for 0.01μg/ml.
Harvesting at 7 days revealed a need for more CaIon to be present in the culture - 0.25μg/ml now being optimal. This is accompanied by a decreased optimal amount of PMA - the optimal concentrations now being 10 or 5ng/ml.
Three day assays revealed that PMA could successfully achieve excellent incorporation of ^{3}H-TdR on its own (Table 3.1) without the need for CaIon co-stimulation. Moreover, cells cultured for 48 hours in PMA alone looked to be in better health than cells cultured with both PMA and CaIon or CaIon on its own. This can be seen by more events appearing below the analysis gate, indicating that these cells are damaged and therefore dead or dying (compare figures 3.4D, E and F). In the 3 day culture experiments, PMA in combination with CaIon gave SI's of only up to 38. The use of CaIon by itself resulted in a SI of maximally 15, but on average SI's were around 6.

Splenocytes from thymectomized animals also responded to these mitogenic agents - lymphocyte enlargement is noticeable 48 hours after PMA/CaIon treatment (Figure 3.4H). Stimulation with PMA alone resulted in SI's of 7 to 21 by day 3 of culture (see Table 3.1).

3.3.4.2 Flow Cytometry

In view of the forward scatter vs side scatter profiles of cells cultured for 48 hours in PMA alone (increased cell size but little cell death) (Figure 3.4D), these studies concentrated on the use of PMA alone and this data is shown in Figure 3.5. IgM is lost from the surface of B cells within 48 hours culture (or drastically reduced) of splenocytes in PMA. In contrast, both the percentage and staining intensity of CD5 is increased. The distinction between T and B cell expression of class II MHC is increased following PMA stimulation (Figure 3.6D), and was confirmed by dual-colour flow cytometry. The mean intensity of staining for CD8 is reduced following PMA stimulation and shows a broader range of intensities compared to the intensity seen in unstimulated cells. Expression of D12.2 is lost upon stimulation of control splenocytes with PMA.

Splenocytes from thymectomized animals stimulated with PMA either completely lost expression of sIgM on B cells, or in some animals $sIgM^+$ cells were drastically reduced (for example from 60% down to 10%) (see Figure 3.7). Expression of CD5, CD8 (and D4.3 - data not shown) is not seen in Tx animals either before or after stimulation with PMA (see Figure 3.7) (or PMA/CaIon). Expression of D12.2 is reduced or lost completely upon PMA stimulation.

3.3.5 Identification of Apoptosis in Splenocytes from Control *Xenopus* Stimulated with Mitogens

3.3.5.1 Background Information on Techniques

The cell cycle (see Figure 3.10) consists of three stages, G_0/G_1, where there is just one copy of the DNA (ie. n = 1), S phase where the DNA is in the process of replicating and can contain anything between one and two copies of the DNA (n >1 and <2), and G_2/M where the DNA has been replicated and the cell is about to divide (n = 2). Any cells which have less than the full complement of DNA (n<1) appear to the left of the G_0G_1 peak. This represents not only dead and dying cells, but more importantly those cells undergoing apoptosis. Typical traces of non-proliferating cells can be seen in Figure 3.9, showing non-stimulated splenocytes which are all within the G_0G_1 phase. In contrast, Figure 3.10 shows *Xenopus* thymic tumour cells to illustrate proliferating cells which demonstrate all the phases of the cell cycle.

Splenocytes stained with acridine orange and analysed by fluorescence microscopy were also used for identification of apoptosis. Activated viable cells have a single yellow/green (DNA^+) nucleus with orange/red (RNA^+) cytoplasm, whilst activated, but apoptotic cells have a fragmented nucleus with some orange/red cytoplasm.

3.3.5.2 Experimental Findings

Splenocytes were stimulated with ConA or PMA and samples taken for flow cytometry at 24, 48 and 72 hours, and for fluorescence microscopy at 48 and 96 hours.

a) Medium Cultured

Flow cytometric analysis of DNA showed few, if any cells undergoing apoptosis by 24 hours culture (Figure 3.11A). This level increased during the culture period, reaching levels of 16% by 72 hours culture. This can be clearly seen as a peak (region E) to the left of the major G_0G_1 peak of cells. Acridine orange staining of medium cultured splenocytes at 4 days culture revealed non-fragmented green nuclei (indicating no break down of the genetic material), and only a low level of RNA (red staining) in the cytoplasm (Figure 3.12A).

b) Concanavalin A Stimulated

Stimulation with the T cell mitogen ConA appeared to induce more splenocytes into the suicide pathway of apoptosis (Figure 3.11B). Initially by 24 hours there was little apoptosis detectable, but by 48 hours apoptosis had increased to 24% (compared to just 12% in medium cultured cells). By 72 hours only 19% splenocytes were apoptotic. Four day ConA treated splenocytes stained with acridine orange had transformed into blast cells (Figure 3.12B) with RNA^+ red cytoplasm. However, very few apoptotic (fragmented green nuclei) could be detected.

c) PMA + CaIon Stimulated

The effects of PMA + CaIon emerged by 48 hours, when the level of splenocyte apoptosis had reached 19%, and increased further to 25% by 72 hours (Figure 3.11C). Figure 12C shows splenocytes stained with acridine orange 4 days after stimulation with PMA + CaIon. Although there are some transformed blast cells with active red (RNA^+) cytoplasm, there is also a noticeable proportion of cells with a fragmented green nucleus, distinctive of cells undergoing apoptosis.

3.3.6 Identification of Apoptosis in Thymus Tumour Cells Using the Specific Anti-ASP Antibody

Analysis of *Xenopus* thymic tumour cells with the anti-ASP antibody showed that a low level of apoptosis was occurring in these cycling cells. Figure 3.13 shows thymus

tumour cells stained with the anti-ASP antibody. Healthy cells can be identified as having an intact red-stained nucleus and unstained cytoplasm. The cytoplasm of apoptotic cells stains green/yellow (identifying the apoptosis specific protein) and these cells have a fragmented, red-staining nucleus.

3.4 Discussion

The initial aim of this Chapter was to examine monoclonal antibody-defined lymphocyte surface antigen expression on splenocytes following early larval thymectomy. The effects of *in vitro* mitogen stimulation on cell surface antigen expression on splenocytes was then investigated and responses to the mitogens being confirmed by tritiated thymidine incorporation. Finally, the consequences of *in vitro* stimulation were examined in terms of induction of apoptosis.

Removal of the thymus from 5-7 day old larvae resulted in a dramatic loss of the T cell markers CD5, CD8, D4.3 and XTLA-1 from the spleen. At 8 months of age there is no suggestion that these markers are emerging, confirming the effectiveness of the operation in terms of T cell depletion. Splenocytes from control and Tx animals were subsequently stimulated *in vitro* with a variety of mitogenic agents to probe for changes in antigen expression and determine whether T cell markers could be induced on the surface of Tx splenocytes. Stimulation of control splenocytes with the T cell mitogens ConA and PHA, induced a reduction in the fluorescence intensity of staining for CD5 and CD8. This reduction is likely due to an increase in splenocyte cell size (as shown by an increase in their forward scatter vs side scatter profiles) but no significant increase in cell surface expression of these markers, which would result in fewer receptors per unit area. Neither ConA nor PHA resulted in any phenotypic changes in Tx splenocytes - there was no suggestion of any T cell marker expression being induced. There was no proliferative response of Tx splenocytes to the T cell mitogens, ConA or PHA, confirming the lack of T cell development following early thymic ablation. However, splenocytes from Tx animals continued to display a response to LPS and PMA.

More dramatic changes in cell surface antigen expression and tritiated thymidine incorporation were seen when splenocytes were stimulated with PMA. There was a loss of sIgM and D12.2 expression in both control and Tx frogs, confirming activation of splenocytes by this phorbol ester (Åman and Klein, 1984) and confirmed by thymidine incorporation. In control spleens stimulated with PMA there was an increase in the percentage of $CD5^{+}$ cells, with the emergence of a population of $CD5^{dull}$ splenocytes. No such emergence of $CD5^{dull}$ cells was seen in PMA stimulated Tx spleens. The increase in CD5 expression in control *Xenopus* was not altogether surprising, since stimulation of mammalian lymphocytes with phorbol esters results in an increase in

CD5 expression (Miller and Garlow, 1984; Zupo et al, 1994). However, the increase in $CD5^+$ cells in *Xenopus* was not accompanied by an increase in the expression of other T cell markers such as CD8 or D4.3. CD5 expression could well be appearing on non-T cells following PMA stimulation, since the increased expression of CD5 on PMA stimulated mammalian lymphocytes is on B cells (Clevers et al, 1985; Rothstein et al, 1986; Freedman et al, 1989a). This suggestion is probed in depth in Chapter 4, as is the finding that CD5 expression could be induced in PMA stimulated splenocytes from control *Xenopus*, but failed to appear in Tx animals.

One interesting finding highlighted during the flow cytometric analysis of mitogen stimulated splenocytes from control *Xenopus*, was the change in MHC II expression. As was shown in Chapter 2, T and B cells in adult *Xenopus* express different levels of MHC II molecules on their surface (T cells being dull and non-T cells being bright). Single colour flow cytometric analysis of MHC II does not readily distinguish these two populations. However, *in vitro* stimulation with either LPS or PMA promotes the MHC-staining distinction between these two populations, and it has been shown that phorbol ester stimulation increases MHC II expression on human B cells (Clevers et al, 1985).

Stimulating *Xenopus* splenocytes *in vitro* with mitogenic agents provides no information on whether these agents were causing deleterious effects on the cells, such as inducing cell suicide (apoptosis). Preliminary studies investigating this matter were carried out by flow cytometric analysis of DNA content. These studies were limited to splenocytes from control animals, due to the unavailability of sufficient Tx animals. Culturing splenocytes for up to 3 days in medium alone resulted in some cells undergoing apoptosis (as defined by a peak of cells at the sub-diploid position). Stimulation with ConA gave a peak of apoptotic cells by 48 hours, but this began to decline by 3 days, whilst PMA gave increased levels of apoptosis with increasing culture period. A fluorescence microscopic examination of splenocytes stained with acridine orange confirmed the flow cytometric findings, identifying increased numbers of putative apoptotic cells with fragmented nuclear material. Light microscopic observations of mammalian lymphocytes stained with acridine orange show healthy cells as having an intact green nucleus whilst apoptotic cells show the characteristic fragmented green nuclear material (A Milner & J D Horton, personal communication).

Apoptosis in thymocytes of adult *Xenopus laevis* can be induced by glucocorticoid, lectin and altered self-antigen stimulation *in vitro* (Ruben et al, 1994). This correlates with findings in mammalian thymocytes whereby apoptosis can be induced by glucocorticoid or lectin activation. However, larval thymocytes differ from those of adult frogs in that apoptosis cannot be induced by glucocorticoids, but can still be induced by lectin or altered self-antigen activation (Ruben et al, 1994). The effects of *in vivo* injection of ConA and LPS on apoptosis in *Xenopus* has recently been carried out (Grant et al, 1995). The work revealed that ConA induces a greater level of apoptosis in the spleen, and that LPS, although not as effective, is capable of inducing apoptosis in the thymic medulla and red pulp of the spleen.

Identification of apoptotic *Xenopus* lymphocytes appears to be possible with the recently described antibody that has activity against an apoptosis specific protein (ASP)(Grand et al, 1995) in cultured mammalian cells. This antibody showed cross-reactivity to *Xenopus,* and the *Xenopus* protein detected by this antibody has very recently been shown to have molecular weight characteristics similar to that identified in human and mouse cells (J D Horton & A Milner, personal communication). Due to the recent availability of this mAb, these studies were limited to *Xenopus* thymus tumour cells. Evolutionary conservation of the apoptotic specific protein is suggested from the preliminary findings presented here. Future studies on apoptosis in *Xenopus* will be aided by this straightforward histological approach.

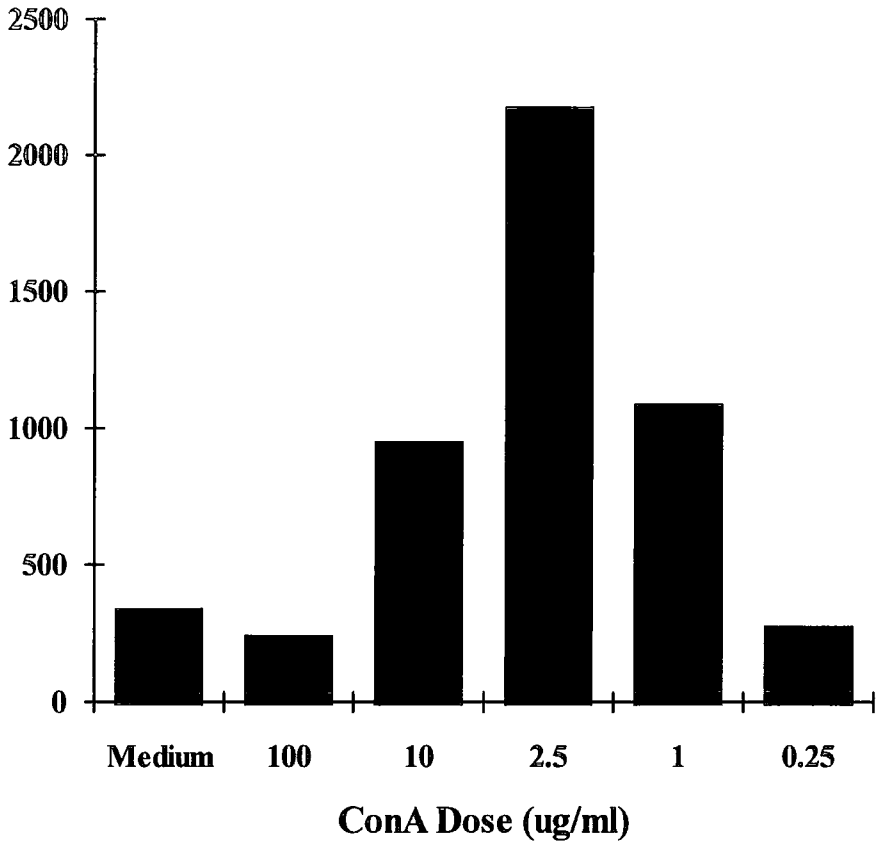


Figure 3.1 Dose response assay to ascertain the optimal dose of concanavalin A to use in cell proliferation assays. Splenocytes from outbred control *Xenopus laevis* (8 months) were cultured for 48 hours with or without various doses of ConA before receiving a pulse of ^{3}H-TdR and harvested 18-20 hours later. The level of incorporated thymidine was assessed by liquid scintillation counting by recording the number of disintegrations per minute (DPM). Representative of 4 replica experiments.

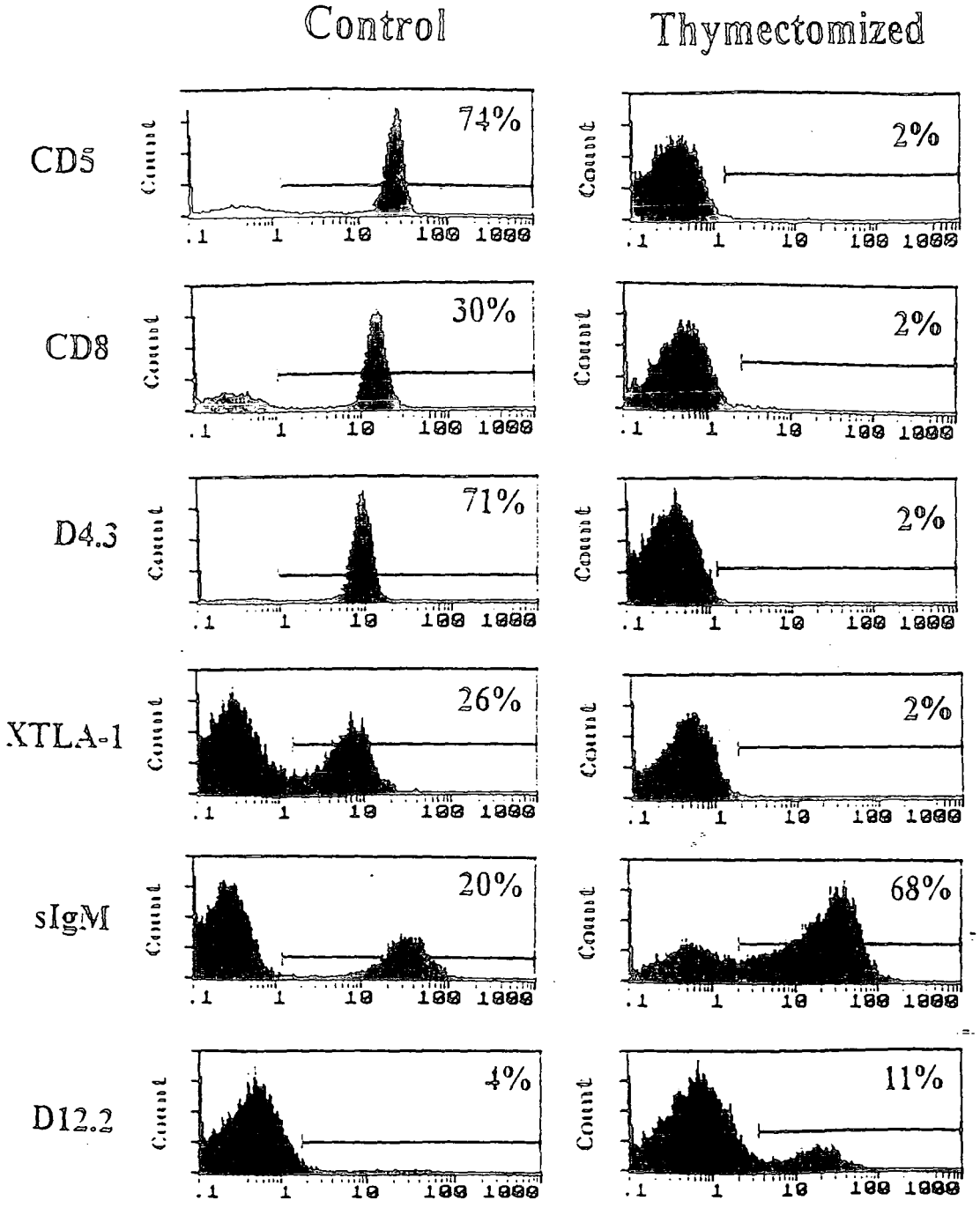
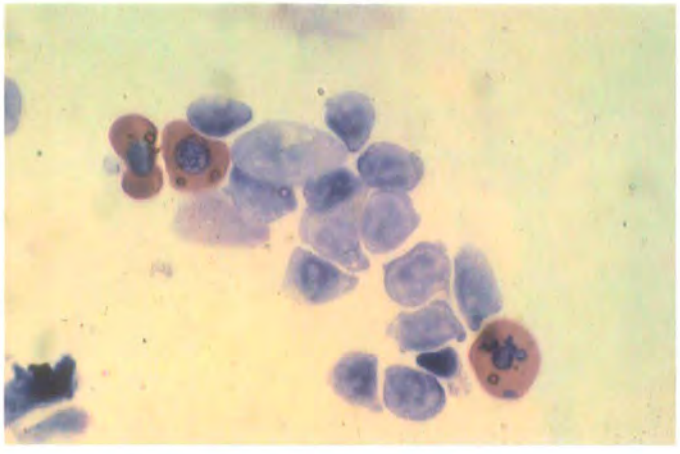


Figure 3.2 Representative flow cytometric single colour histograms comparing cell surface markers on splenocytes of control and thymectomized outbred adult *Xenopus laevis* (non-cultured cells). Analysis markers were set at 2% with isotype-matched control mAbs. Animals were aged 8 months. Representative of 4 experiments.

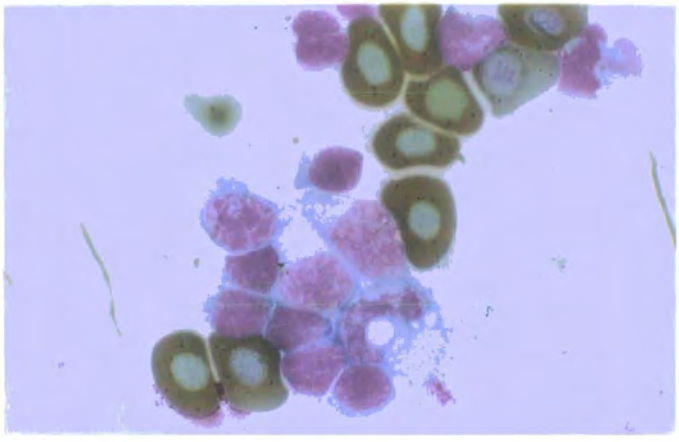
Figure 3.3 Cytocentrifuge preparations of splenocytes from 8 month old control (𝔸, ℂ and 𝔼) and Tx (𝔹, 𝔻 and 𝔽) cultured for 48 hours in amphibian culture medium alone (𝔸 and 𝔹), with the addition of 2.5μg/ml ConA (ℂ and 𝔻) or with the addition of 10ng/ml PMA + 0.1μg/ml CaIon (𝔼 and 𝔽). Wright-Giemsa stain, magnification x1250

Control Thymectomized

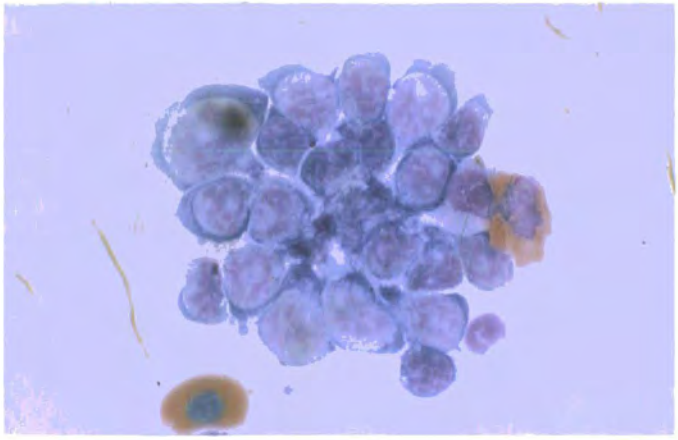
A



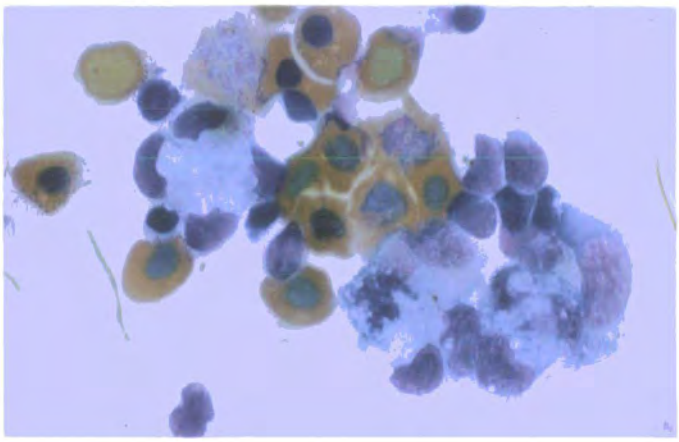
B



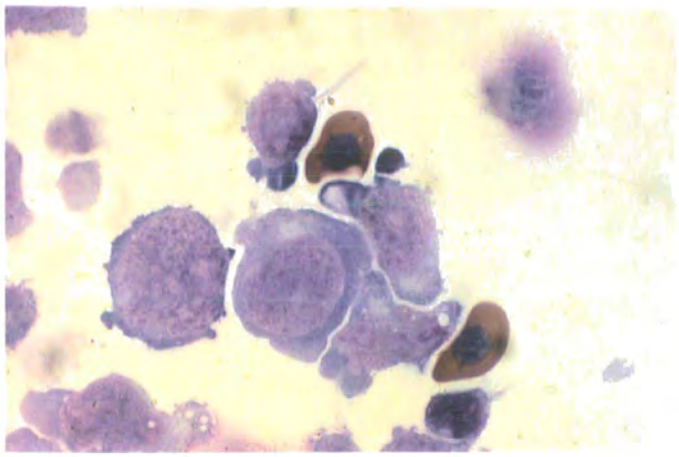
C



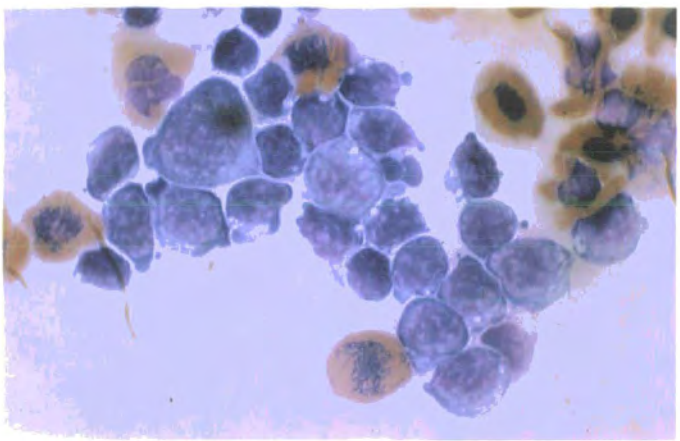
D



E



F



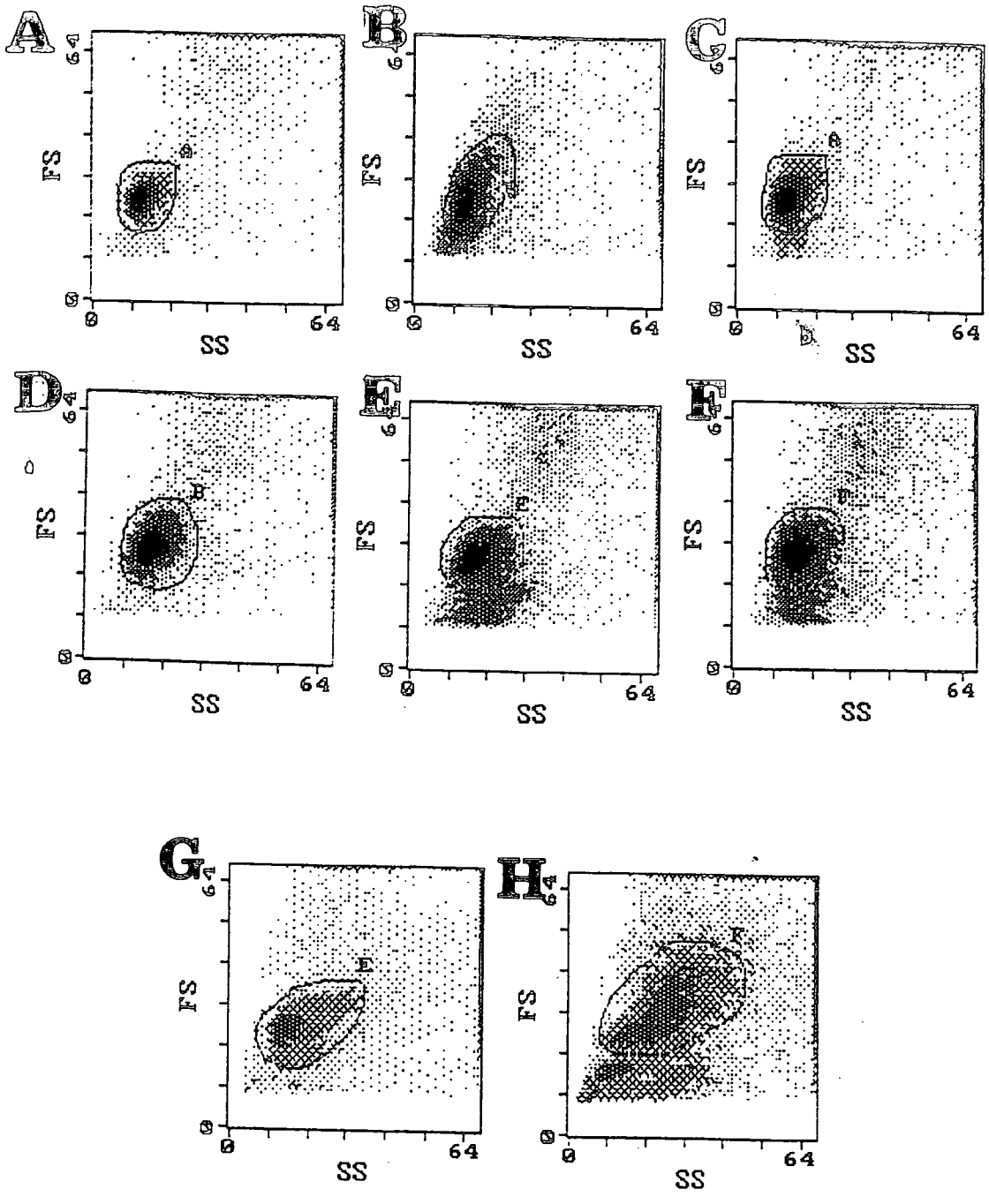
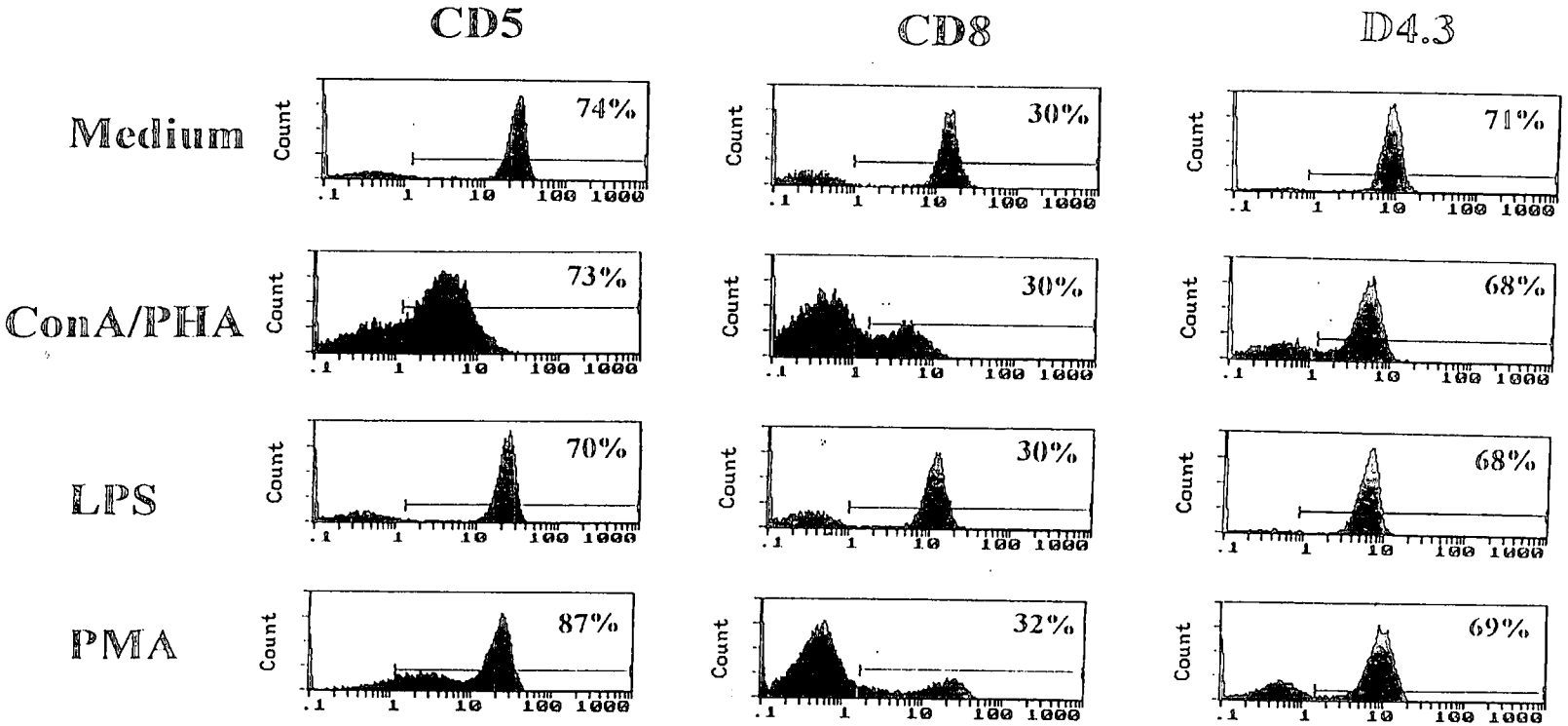
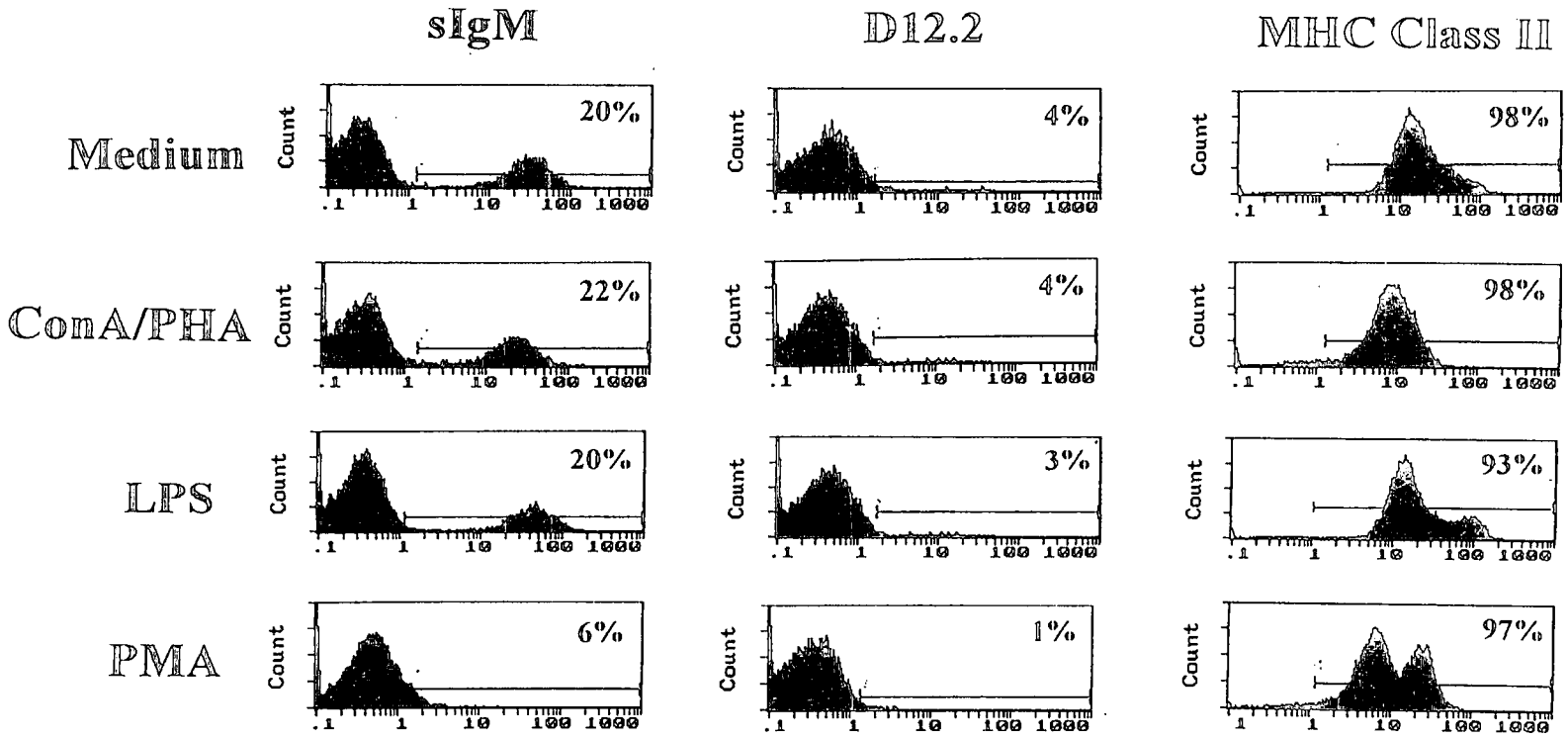


Figure 3.4 Forward scatter vs side scatter dot plot profiles of splenocyte preparations from 8 month old outbred control *Xenopus laevis* cultured for 48 hours in amphibian culture medium alone (**A**), with the addition of 2.5μg/ml ConA (**B**), 250μg/ml LPS (**C**), 10ng/ml PMA (**D**), 0.1μg/ml CaIon (**E**) or 10ng/ml PMA + 0.1μg/ml CaIon (**F**).
Splenocyte preparations from thymectomized *Xenopus laevis* cultured for 48 hours in culture medium alone (G) and PMA + CaIon (**H**).

Figure 3.5 Representative flow cytometric single-colour histograms of splenocytes from 8 month old control adult *Xenopus laevis* cultured for 48 hours. A variety of cell surface markers were compared when splenocytes were cultured in medium or stimulated with either ConA/PHA, LPS or PMA+CaIon at optimal concentrations.




sIgM
D12.2
MHC Class II
Medium
ConA/PHA
LPS
PMA
Count
20%
4%
98%
22%
4%
98%
20%
3%
93%
6%
1%
97%
.1
1
10
100
1000

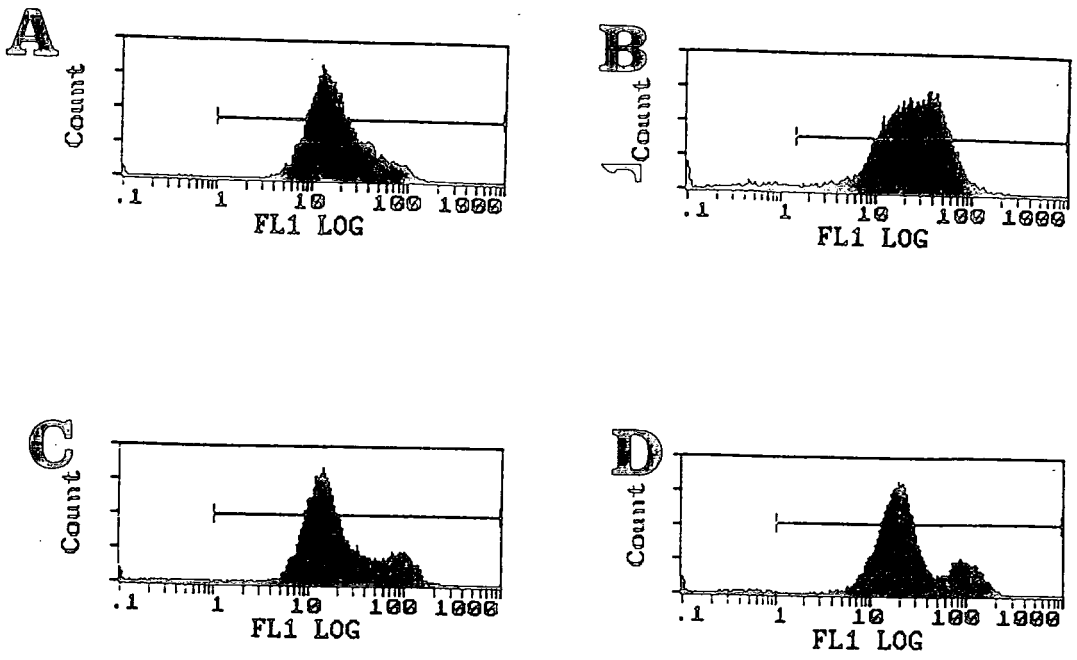


Figure 3.6 Effects of *in vitro* stimulation upon MHC class II expression on splenocytes from 8 month old control *Xenopus*. Representative flow cytometric single-colour histograms of splenocytes cultured for 48 hours in amphibian culture medium (**A**), with the addition of ConA or PHA (**B**), LPS (**C**) or PMA (**D**)

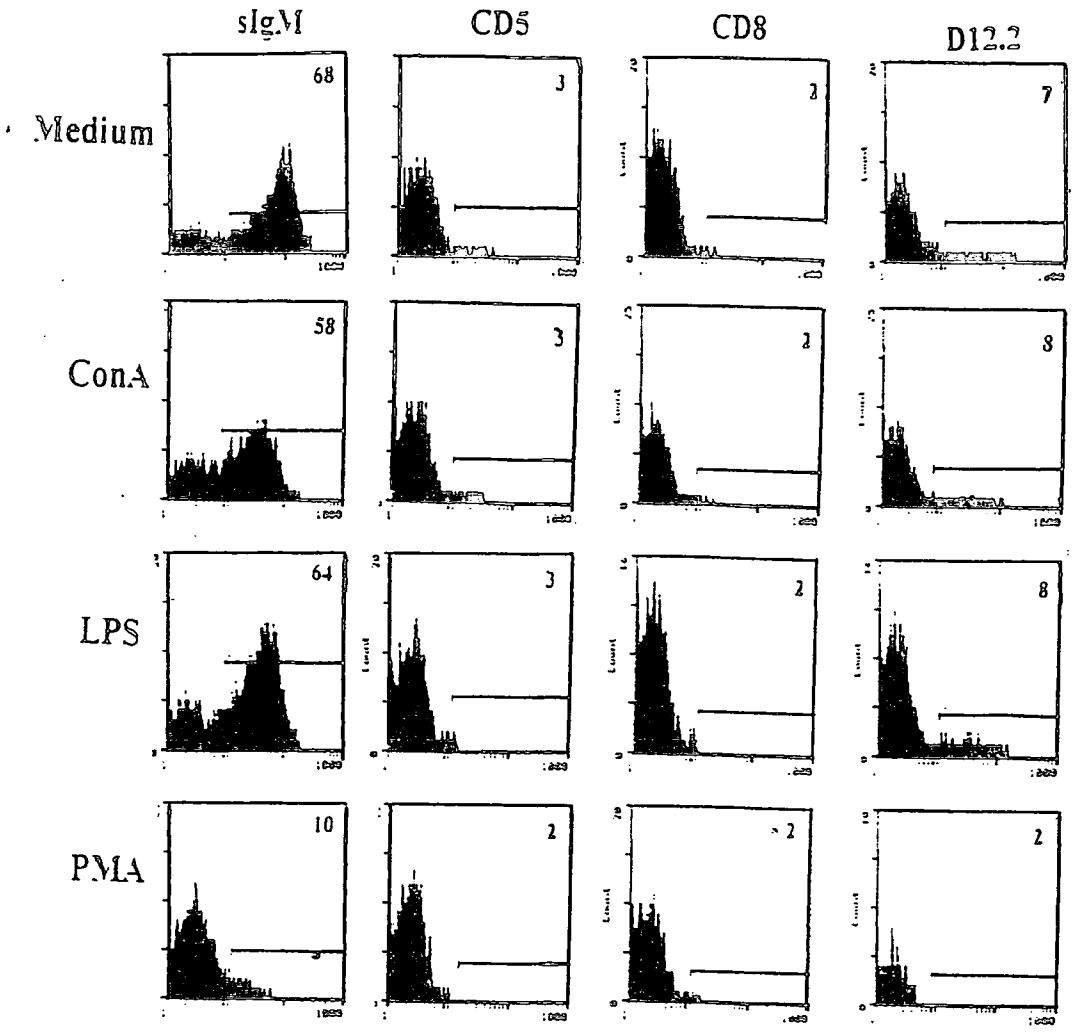
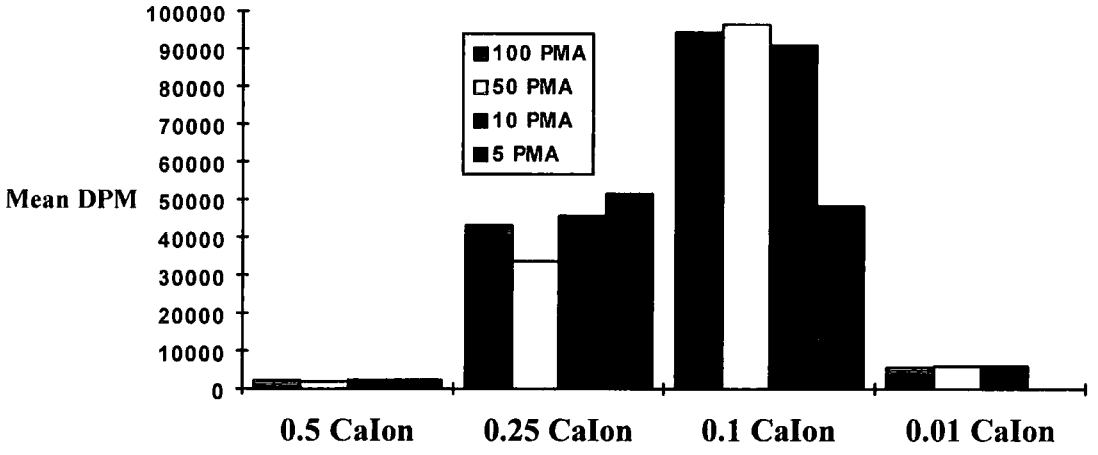


Figure 3.7 Representative flow cytometric single-colour histograms of splenocytes from 8 month old outbred thymectomized adult *Xenopus laevis*, cultured for 48 hours. Effects on cell surface markers were compared when splenocytes were stimulated with either ConA, LPS or PMA+CaIon at optimal concentrations.

A



B

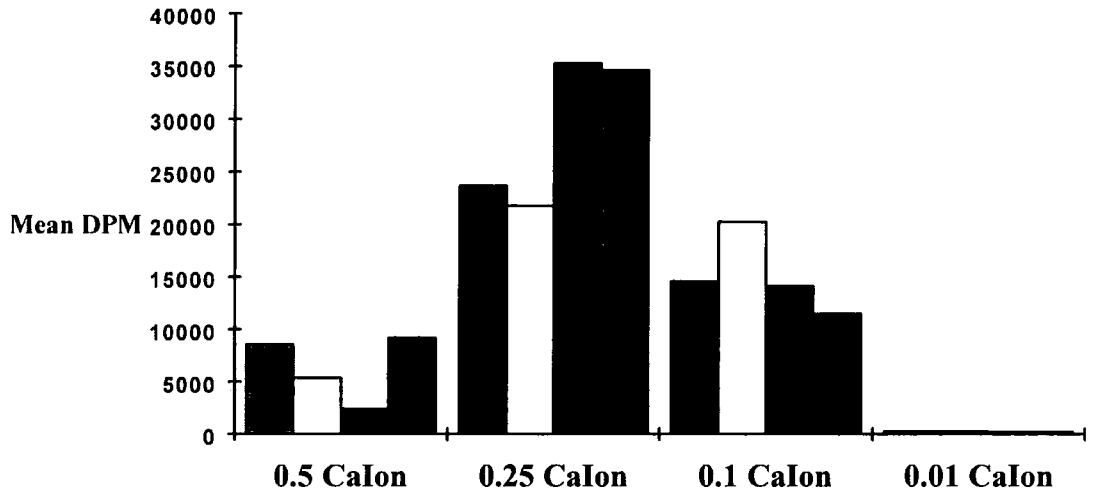


Figure 3.8 Tritiated thymidine incorporation following 4 days (**A**) or 7 days (**B**) *in vitro* stimulation with phorbol myristate acetate (PMA) and calcium ionophore (CaIon). Splenocytes from 8 month old outbred *Xenopus laevis* were cultured in 96-well plates with varying concentrations of PMA and CaIon, and received a pulse of ^{3}H-TdR 18-20 hours before harvesting the cells and carrying out liquid scintillation counting.

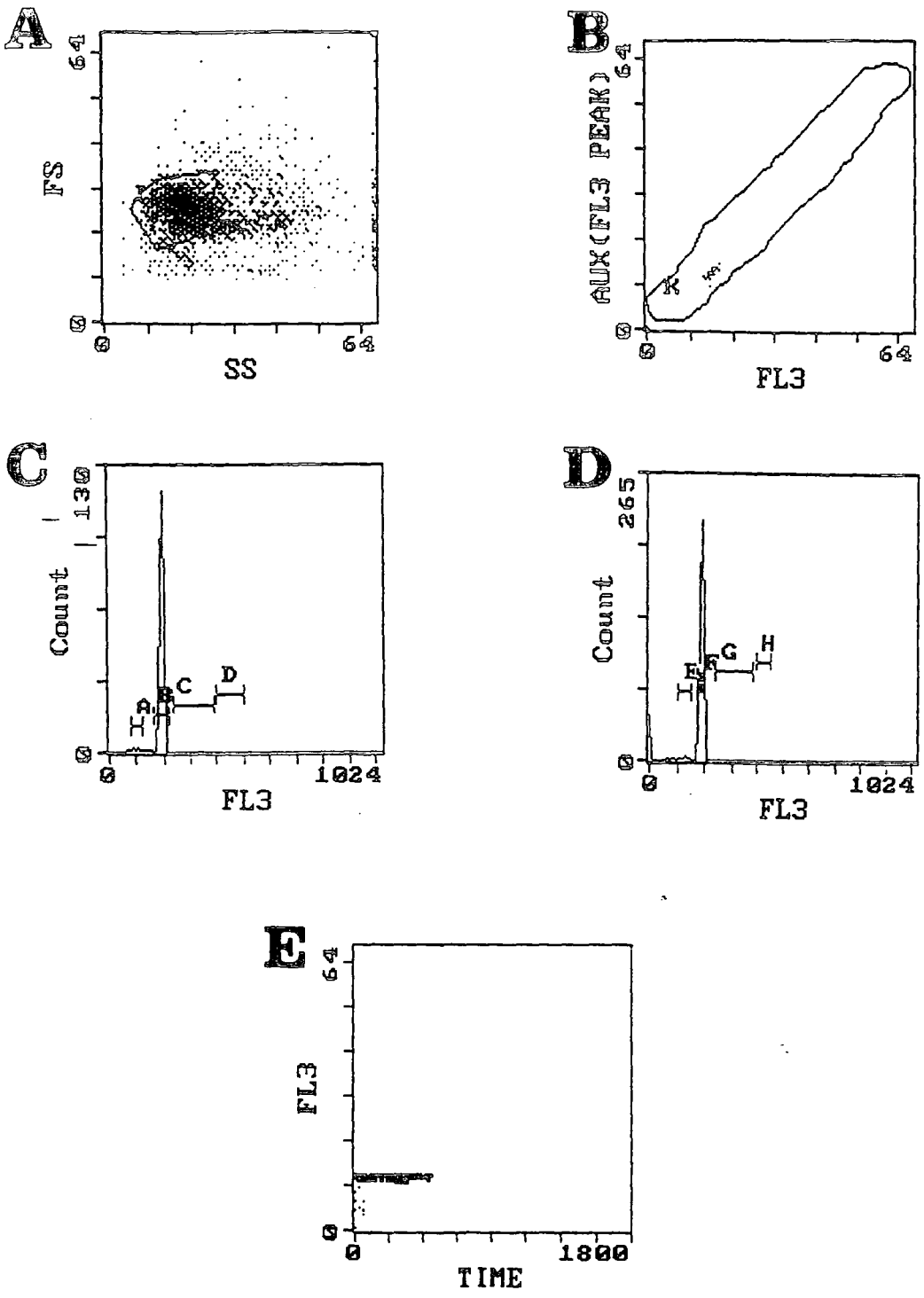


Figure 3.9 Example of traces employed during flow cytometric DNA data analysis of splenocytes. Forward scatter vs side scatter, with an analysis gate around lymphocytes (**A**), FL3 vs FL3 Peak to ensure only single cells are analysed (**B**), single colour analysis for FL3 (propidium iodide) looking at cells that fall within both the lymphocye gate (in histogram A) and the single cell gate (in histogram B) (**C**), single colour analysis for FL3 looking at all cells, ie. no gating (**D**) and time vs FL3 to ensure optimal binding of propidium iodide has been achieved, ie. should be a straight line (**E**). Only cells in G_0/G_1 are readily detectable in these medium cultured splenocytes.
The regions shown in C and D are further explained in Figure 10.

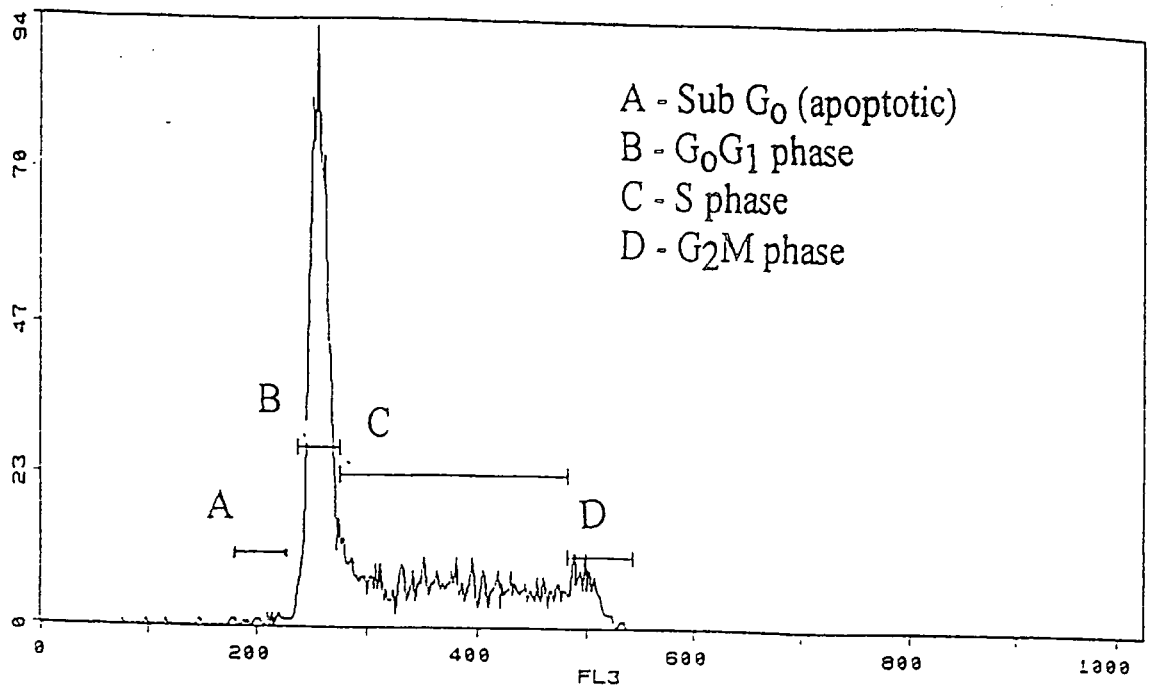


Figure 3.10 Typical trace of flow cytometric analysis of the cell cycle, visualised by propidium iodide staining of the DNA of *Xenopus* thymic tumour cells.

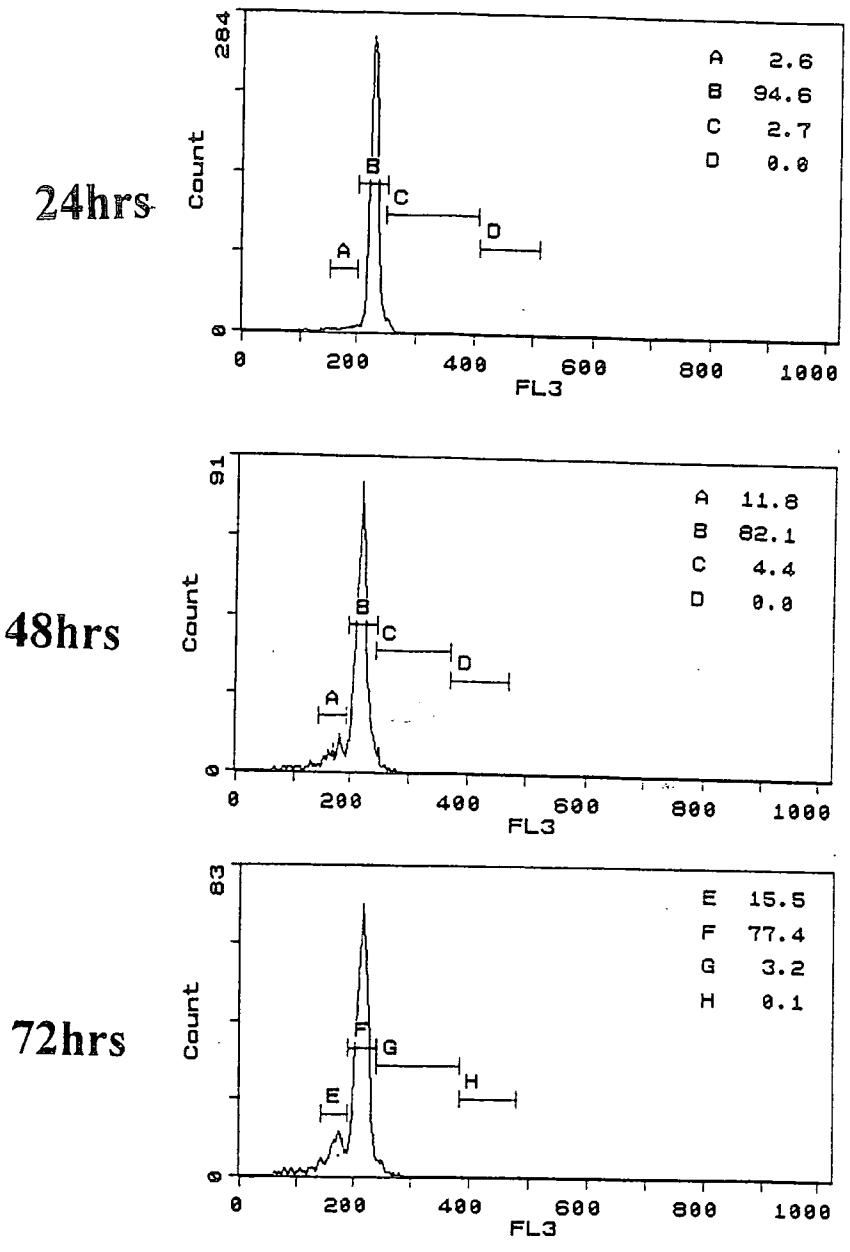


Figure 3.11A Flow cytometric profiles of DNA content of splenocytes from 8 month old control *Xenopus* cultured for 24, 48 and 72 hours in medium

ConA

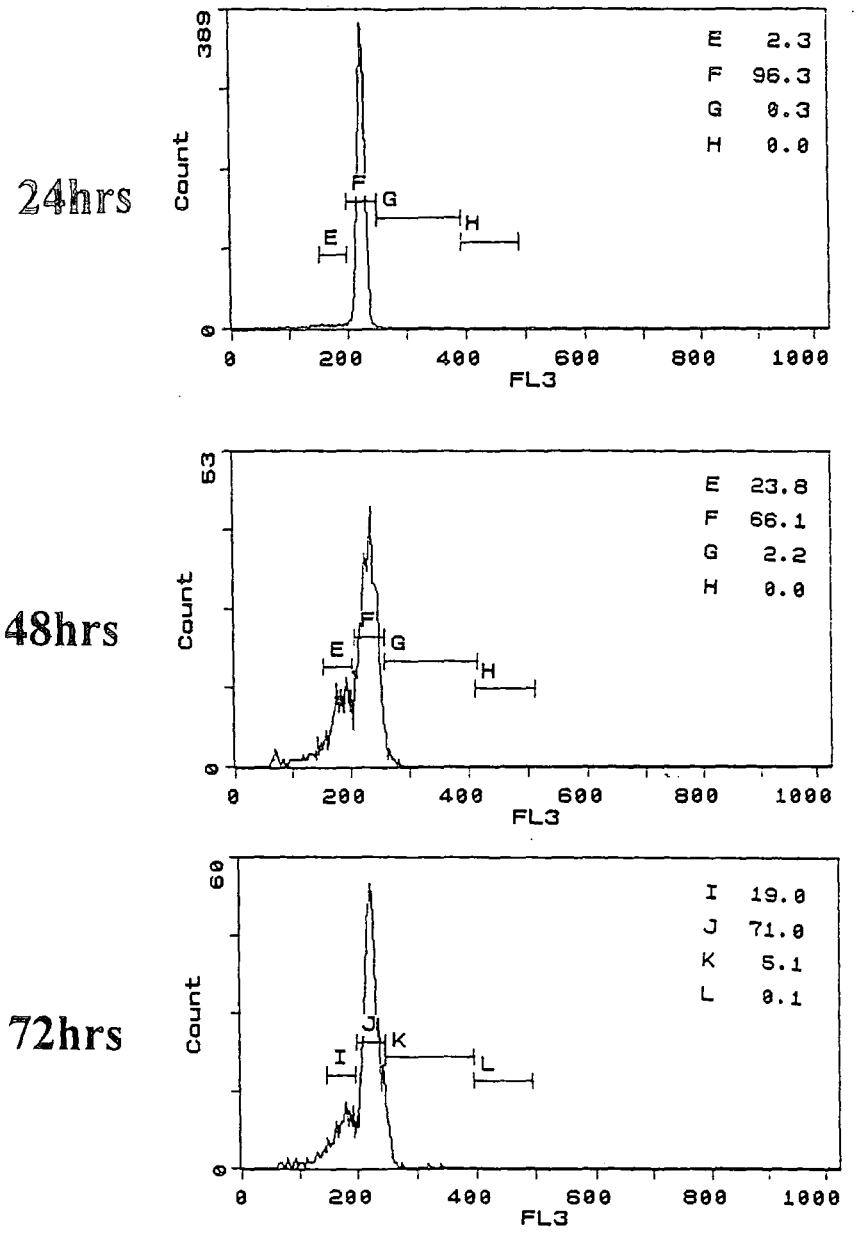


Figure 3.11B Flow cytometric profiles of DNA content of splenocytes from 8 month old control *Xenopus* cultured for 24, 48 and 72 hours in the presence of 2.5µg/ml ConA.

PMA/CaIon

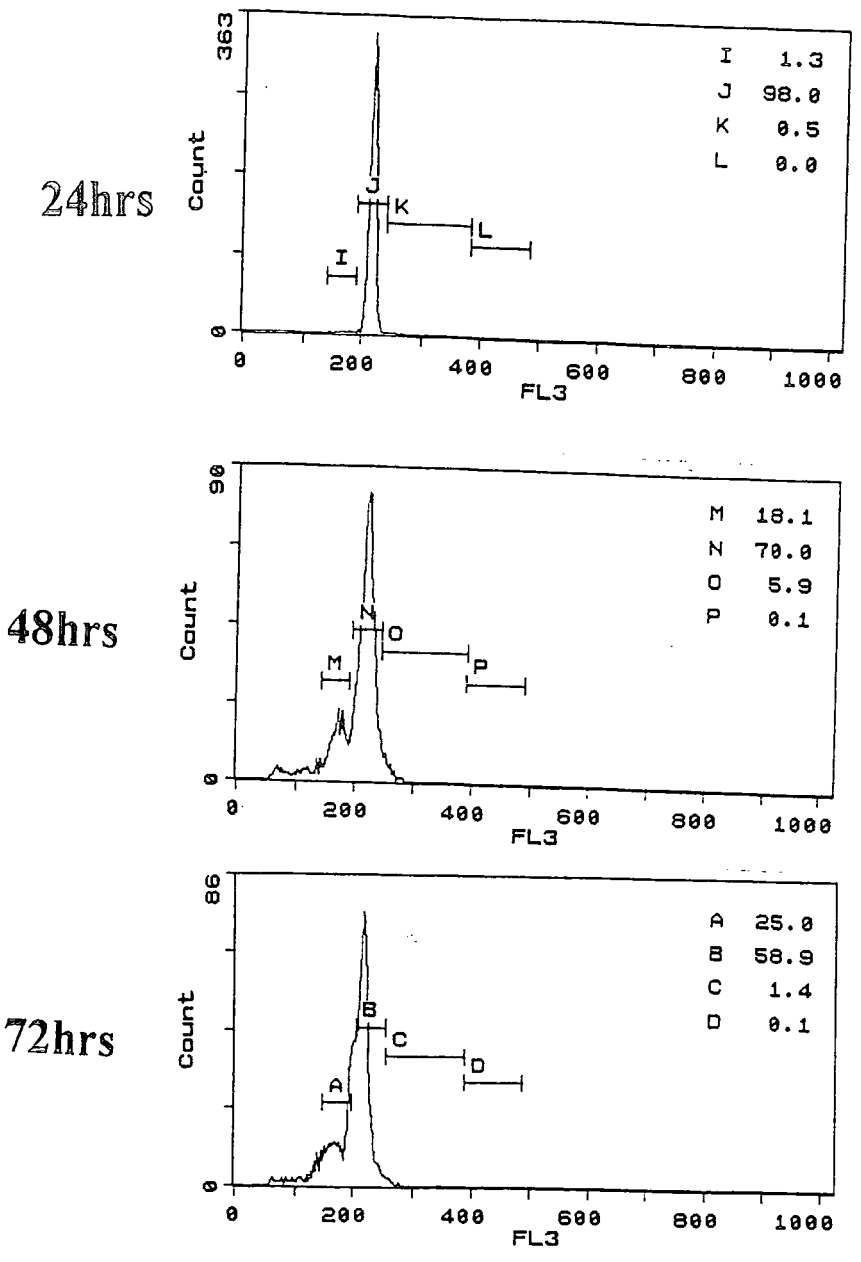
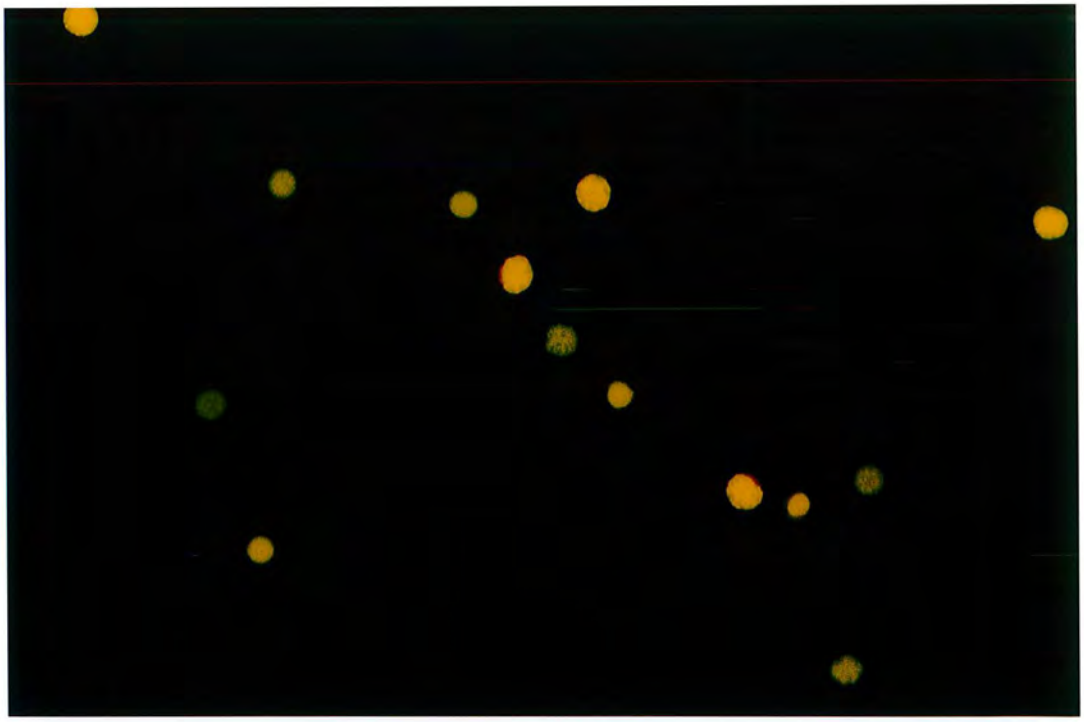


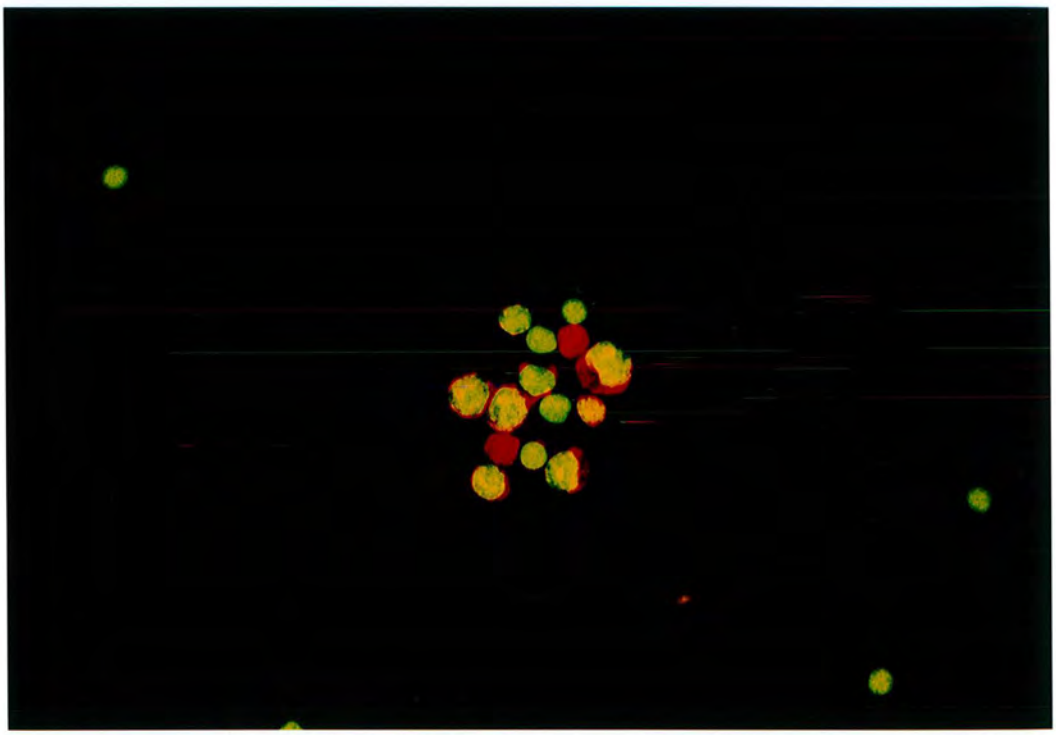
Figure 3.11C Flow cytometric profiles of DNA content of splenocytes from 8 month old control *Xenopus* cultured for 24, 48 and 72 hours in the presence of 10ng/ml PMA + 0.1μg/ml CaIon.

Figure 3.12 Cytocentrifuge preparations of splenocytes from 8 month old *Xenopus* cultured for 48 hours in amphibian culture medium alone (**A**) or in the presence of 2.5μg/ml ConA (**B**), or 10ng/ml PMA + 0.1μg/ml CaIon (**C**). Preparations were stained with acridine orange, healthy cells can be identified by their intact yellow/green nucleus, whilst apoptotic cells show a fragmented yellow/green nucleus. Activated cells can be identifed by their RNA^+ red cytoplasm.
Magnification x500

A



B



C

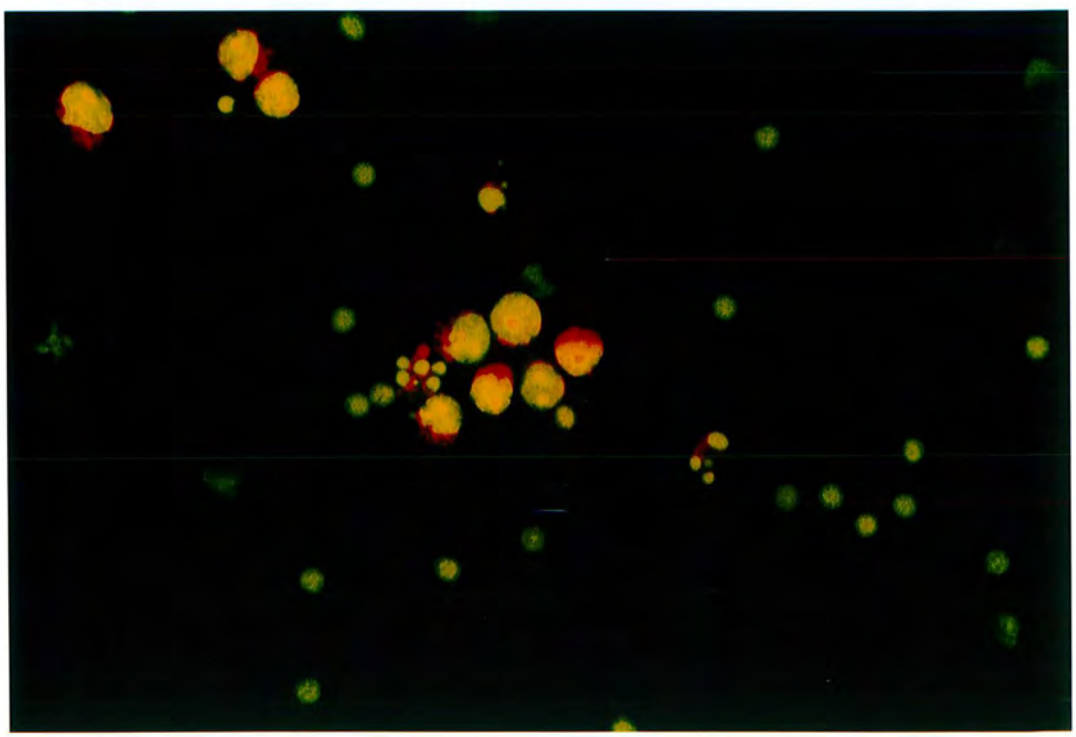
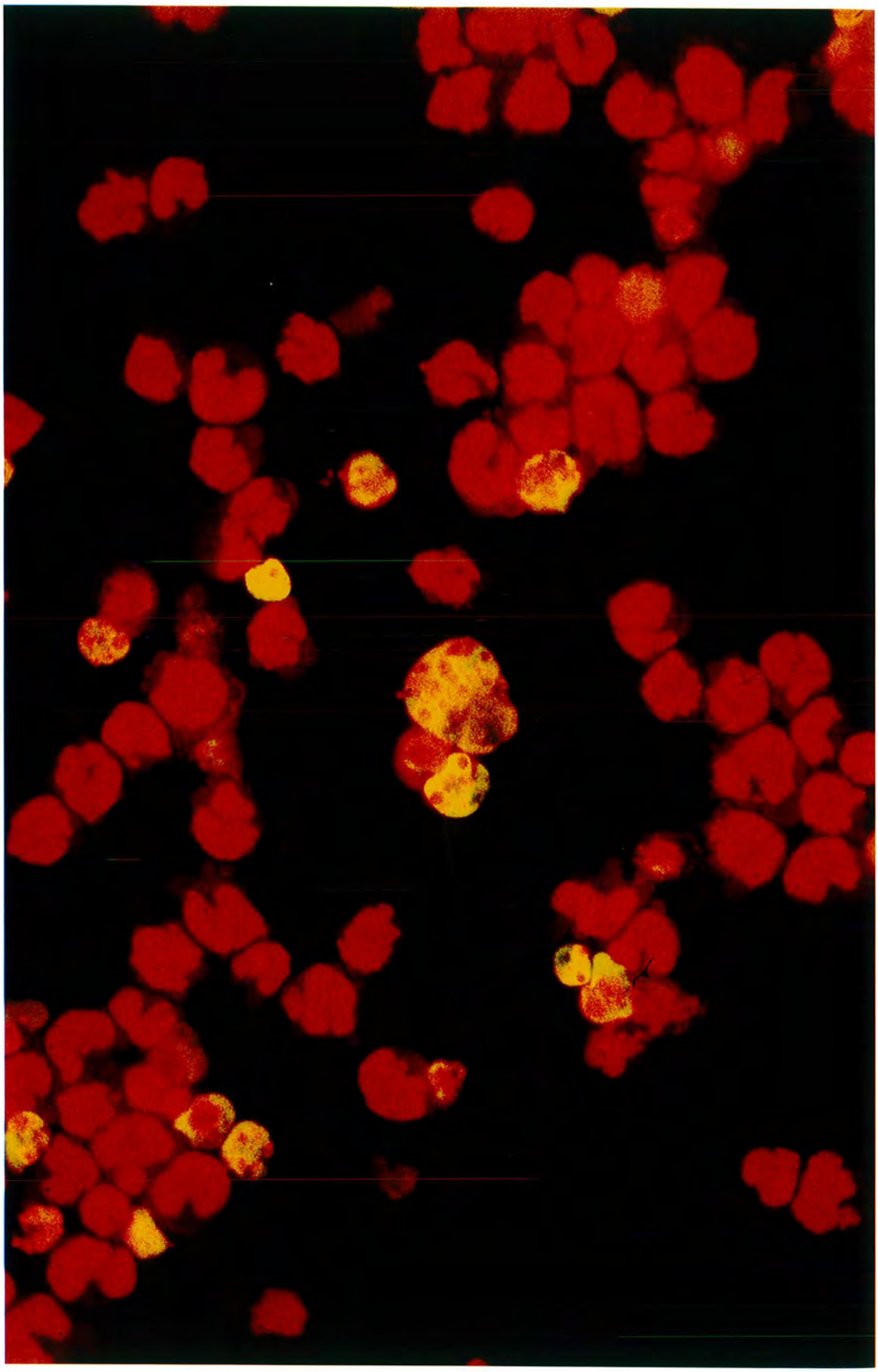


Figure 3.13 Cytocentrifuge preparation of *Xenopus* thymic tumour cells stained with the anti-ASP antibody and counter stained with propidium iodide. Healthy cells can be identified by their homogeneous red stained nucleus, whilst apoptotic cells can be identified by the presence of a fragmented red nucleus and yellow/green cytoplasm.
Magnification x 1250



Concanavalin A

Animal	Age	Mean Control DPM	Mean Experimental DPM	Stimulation Index
Control	9 months	738±58	16,455±3752	22±6
Control	12 months	1,232±277	91,552±966	73±19
Control	14 months	819±160	97,905±6410	73±6
Tx	9 months	689±48	645±213	0.94±0.3
Tx	12 months	1,449±420	1,575±460	1.04±0.2
Tx	14 months	2,208±1162	2,951±1,476	1.18±0.05

Lipopolysaccharide

Animal	Age	Mean Control DPM	Mean Experimental DPM	Stimulation Index
Control	8 months	738±25	4,947±2,765	7±4
Control	12 months	1,232±277	8,315±468	7±2
Control	14 months	819±160	4,606±708	3±0.1
Tx	8 months	689±48	1,259±250	2±0.47
Tx	12 months	1,449±420	11,296±729	8±3
Tx	14 months	2,208±1,162	9,558±277	6±2

PMA

Animal	Age	Mean Control DPM	Mean Experimental DPM	Stimulation Index
Control	8 months	738±25	49,209±58	66±2
Control	12 months	1,232±277	182,133±10,508	142±38
Control	14 months	819±160	63,132±4,517	79±21
Tx	8 months	689±48	6,822±356	10±1
Tx	12 months	1,449±420	38,310±2,771	27±11
Tx	14 months	2,208±1,162	21,715±1,336	15±7

TABLE 3.1 Representative data indicating the level of *in vitro* stimulation of splenocytes from control and Tx *Xenopus* following stimulation with either 2.5μg/ml ConA, 250μg/ml LPS, or 10ng/ml PMA.

Chapter 4

In Vitro Studies Exploring CD5 Expression Induced By Phorbol Ester Stimulation of Splenocytes from Control and Thymectomized *Xenopus*

4.1 Introduction

The role of the 67-kDa glycoprotein CD5 (also known as Leu-1 in humans and Ly-1 in mice) is still poorly understood in mammals (Kripps, 1989). CD5 is a pan T cell marker whilst its ligand, CD72 is found on B cells (van de Velde et al, 1991). The binding of CD5 with CD72 is thought to facilitate binding and therefore interactions between T and B cells. Studies have shown that the binding of anti-CD5 mAbs to both human and murine T cells causes an increase in interleukin 2 production and their proliferation (Ceuppens and Baroja, 1986). In mammals CD5 expression is not restricted to T cells alone (Kripps, 1989; Haughton et al, 1993; Kearney, 1993). In humans and in mice there is a small but distinct population of B cells (up to 10% splenic B cells) that constitutively express CD5 on the cell surface (Kipps, 1989; Kawamura et al, 1994). These $CD5^+$ B cells are thought to be derived from a distinct lineage and have been termed B1a cells as opposed to conventional B cells which have now been termed B2 cells (Herzenberg and Kantor, 1993). In the rabbit virtually all of its peripheral B cells are found to express CD5 (Raman and Knight, 1992), whilst in the rat there has been no detection of $CD5^+$ B cells despite the fact that CD5 is found on its T cells (Vermeer et al, 1994). $CD5^+$ T and B cells have also been identified in cattle (Yang et al, 1995).

With the recent production of a monoclonal antibody that identifies the amphibian (*Xenopus*) CD5 homologue (Jürgens et al, 1995), further investigations into the expression of this marker are carried out here. Studies in humans and mice have shown that CD5 expression can be induced on B cells by *in vitro* stimulation with phorbol esters (Rothstein et al, 1986; Zupo et al, 1994). In the previous chapter it was shown that CD5 expression is increased on splenocytes from control *Xenopus laevis* following stimulation with the phorbol ester PMA, whilst splenocytes from thymectomized animals failed to show any signs of CD5 expression. This chapter probes which splenocytes display this increased expression of CD5, and what conditions promote CD5 emergence on B lymphocytes. Splenocytes from control animals were separated into populations of B cells (and non-B cells) to mimic the situation found in thymectomized animals, and subsequently stimulated with phorbol ester. These experiments indicate that T cells or T cell factors are required to enable B cells to express CD5. This finding has led to cell mixing experiments, combining splenocytes from thymectomized animals with T cells and stimulating these cell mixtures with PMA to see if CD5 expression could be induced on B cells from the thymectomized animal. Finally, since it has been shown that PMA stimulates mammalian T cells via protein kinase C (PKC) activation (Nishizuka, 1984), experiments were carried out using a protein kinase C inhibitor to confirm that the increased CD5 expression induced by PMA on *Xenopus* splenocytes was acting through the protein kinase C pathway.

4.2 Materials and Methods

4.2.1 Animals and Operations

4.2.1.1 Animals

Outbred *Xenopus laevis* were used throughout, and were bred and reared in the laboratory under standard conditions (Horton and Manning, 1972). Animals were aged between 6-12 months.

4.2.1.2 Thymectomy

See 3.2.1.2

4.2.1.3 Preparation of Spleen Cell Suspension

See 3.1.2.3

4.2.2 Amphibian Culture Medium

As detailed in section 3.2.2.

4.2.3 PMA Stimulation of Control and Thymectomized Splenocytes

Splenocytes were centrifuged at 300g, 4°C for 10 minutes and resuspended in amphibian culture medium at a concentration of $1x10^6$/ml and plated into a 24-well plate with each well receiving 1ml. Experimental wells were given 100µl PMA (10ng/ml) whilst an equivalent volume of medium was added to control wells. The plate was incubated in a humidified incubator at 27°C with 5% CO_2 in air for 18-20 hours. After this time the medium was changed on all wells and the plate incubated for a further 24 hours before being analysed by flow cytometry (4.2.8).

4.2.4 Complement Mediated Lysis To Deplete T Cells

A splenocyte cell suspension was prepared (see 4.2.1.3) and the cells adjusted to $1x10^6$ cells/ml. One million cells were added to a Falcon tube and centrifuged at 300g, 4°C for 10 minutes. The pellet was resuspended in 100µl appropriate mAb (AM22 or 2B1) and incubated on ice for 20 minutes. Halfway through this incubation the cells were resuspended to ensure optimal coating of cells. The cells were then washed twice and resuspended in culture medium containing complement (guinea pig or rabbit complement was used at various concentrations - see Results) and incubated at 27°C for

45 minutes (this lower temperature was used and not 37°C as this temperature would have been harmful to amphibian cells). After this time the cells were washed twice and a viable count was made using trypan blue. Those cells that had taken up the dye were termed dead as their membranes were no longer intact presumably through the lytic action of complement-mediated lysis.

4.2.5 Panning To Enrich for B Cells

All procedures were carried out in a laminar air flow cabinet using aseptic techniques and sterile or autoclaved media.

4.2.5.1 Coating Petri Dish

Standard microbiological size (85mm) sterile petri dishes (Sterilin) were coated with anti-mouse IgG by the method of Wysicki and Sato (1978) and Bleicher and Cohen (1981). Anti-mouse Ig (20μl anti-IgG) was added to 6ml carbonate/bicarbonate buffer (pH 9.7) (see Appendix A) giving a final concentration of 10μg/ml Ab. This was then pipetted onto a sterile petri dish, making sure that the bottom of the dish was covered with the medium, and incubated at 4°C overnight. The contents of the petri dish were then discarded and 6ml blocking buffer added to the dish (carbonate/bicarbonate buffer containing 1% BSA) and incubated at room temperature for 20-30 minutes. Immediately before the cells were to be added to the dish, the blocking buffer was discarded and the dish carefully washed three times with FACS medium (see Appendix A) to remove any residual blocking buffer and reduce the pH back to 7.4 so as not to damage the cells.

4.2.5.2 Panning Procedure

A splenocyte cell suspension was prepared and the concentration adjusted to $1x10^6$/ml. An aliquot of cells was put aside that was not treated, ie. non-panned population. If the cells were not analysed the same day, they were resuspended in culture medium and put into culture until analysed. The remaining cells were split into 5ml Falcon tubes each containing $1x10^6$ cells. These were centrifuged at 300g, 4°C for 10 minutes and the pellets resuspended in 100μl anti-μ (8E4:57) mAb, and stored on ice for 30 minutes. Halfway through the incubation, the cells were agitated to ensure optimal coating with the Ab. The cells were then washed twice with FACS medium to remove any unbound mAb before the cells were pooled in a total volume of 6ml. The cells ($6x10^6$) were then added to the antibody-coated petri dish and incubated at 4°C for 40 minutes. After this

time the plate was gently swirled to maximise the number of cells binding to the plate, and the dish incubated for a further 30 minutes. The non-adherent fraction of cells was then carefully removed by pipetting and the plate washed very gently with fresh FACS medium several times to ensure that all the non-adherent cells had been recovered. At this time the petri dish was examined under an inverted microscope to ensure that all the non-adherent cells had been removed. Finally, the adherent population was removed by adding FACS medium and harshly pipetting the bottom of the dish at an angle so that the bonds between the anti-mouse IgG and the anti-μ Ab would be broken, thereby releasing the cells from the plate. This was repeated several times until there were no more cells bound to the plate as observed under the inverted microscope. Both non-adherent and adherent populations were then centrifuged and counted before being resuspended in amphibian culture medium and cultured overnight in a humidified incubator at 27°C with 5% CO_2 in air. An aliquot of cells were analysed the next day by flow cytometry (4.2.8) to check how efficient the panning procedure had been. The remaining cells were then used in cell mixing experiments as described below.

4.2.6 Cell Mixing Experiments

4.2.6.1 Mixing of Panned Populations

Splenocytes from individual control animals were panned by the method in 4.2.5 to obtain a B-cell-rich adherent population. The purity of the panning procedure was checked after overnight culture by flow cytometry. At this time the following cultures were set up in 24 well plates, with each well containing 1ml cell suspension at $1x10^6$/ml:-

i) $1x10^6$ adherent (B-cell rich) splenocytes ± 100μl PMA (10ng/ml) and 100μl CaIon (0.1μg/ml)
ii) $1x10^6$ adherent (B-cell rich) splenocytes + $1x10^6$ non-adherent (T-cell rich) splenocytes ± 100μl PMA (10ng/ml) and 100μl CaIon (0.1μg/ml)
iii) $1x10^6$ non-panned splenocytes ± 100μl PMA (10ng/ml) and 100μl CaIon (0.1μg/ml)

The plate was cultured in a humidified incubator at 27°C with 5% CO_2 in air for 18-20 hours, after which the medium was changed in all wells, and the plate incubated for a further 24 hours. After this time (approximately 3 days total culture) cells were taken for analysis by flow cytometry (section 4.2.8). CaIon was used in the culture because the cells were to be overall cultured for up to 72 hours and in the previous chapter it was

shown that the cells benefit from the presence of CaIon when cultured for this length of time.

4.2.6.2 Mixing Splenocytes from Thymectomized Animals with a $CD5^+$ T Cell Source

To determine if the presence of T cells or T cell factors were required by splenocytes from thymectomized animals for the induction of CD5 expression following PMA stimulation, two types of cell mixing experiments were used.

a) Use of Well Inserts

Splenocytes from a thymectomized (Tx) animal were plated at $1x10^6$ cells/ml in 24 well plates as detailed in section 4.2.3). A 24-well plate well insert (Falcon cell culture insert with a pore size of 3μm) was placed into the wells containing splenocytes from the Tx animal, and $1x10^6$ splenocytes from a control animal were added to the insert. To experimental wells 200μl PMA (10ng/ml) was added (2ml volume in wells therefore requires twice the amount of PMA), whilst an equivalent volume of medium was added to control wells. The plate was then incubated in a humidified incubator at 27°C with 5% CO_2 in air for 18-20 hours after which the medium was changed in all wells, and the plate incubated for a further 24 hours. After this time the well inserts containing the control splenocytes were discarded and the thymectomized splenocytes were analysed by flow cytometry (section 4.2.8).

b) Use of *Xenopus* Thymic Tumour Cells

For experiments employing the thymic tumour cells, cultures were set up using tumour culture medium (see Appendix A).

Splenocytes from a thymectomized animal were mixed with $CD5^+$ *Xenopus* tumour cells (the B_3B_7 clone of Robert, Guiet and DuPasquier (1994)) at a ratio of 1:1. The mixed cells were then plated at $1x10^6$/ml in 24 well plates, and to experimental wells 100μl PMA (10ng/ml) were added, whilst control wells received an equivalent volume of medium. The plates were then cultured in a humidified incubator at 27°C with 5% CO_2 in air for 18-20 hours, after which the medium was changed on all wells. After a further 24 hours culture the cells were taken for flow cytometric analysis (section 4.2.8).

4.2.7 Protein Kinase C Inhibition Studies

4.2.7.1 Dose Response Assay

The protein kinase C (PKC) inhibitor, RO 31-8220 was a generous gift from Roche Laboratories. It was reconstituted in DMSO at a concentration of 1mM and stored at

–20°C in 50µl aliquots.

Splenocytes from control *Xenopus* that were to receive the PKC inhibitor were adjusted to $1x10^6$/ml and aliquoted into 5ml Falcon tubes, each receiving $1x10^6$ cells. These were then centrifuged at 300g, 4°C for 10 minutes. Each pellet was resuspended in 1ml amphibian culture medium containing a different concentration of inhibitor (0.5µM, 0.25µM, 0.1µM, 0.01µM or no inhibitor for control samples), and incubated on ice for 30 minutes. Splenocytes were then centrifuged and resuspended in fresh medium containing the appropriate concentration of inhibitor. Splenocytes were then plated in triplicate in a 96 well flat bottomed plate, with two identical plates being set up. One plate received 10µl PMA (10ng/ml) to all wells whilst the second plate received an equivalent volume of medium. Both plates were then incubated for 48 hours before a pulse of tritiated thymidine was added for 18-20 hours (as detailed in section 3.2.3) - the inhibitor being present throughout the entire culture period.

4.2.7.2 Cell Surface Analysis

Splenocytes were treated as above, except that the cell pellets were resuspended in the optimal concentration of inhibitor as determined from the PKC inhibitor dose response assay. Splenocytes were plated at $1x10^6$/ml in 24 well plates ± 100µl mitogen (PMA 10ng/ml, PHA 1µg/ml or 125µl LPS 250µg/ml) and cultured for 18-20 hours in a humidified incubator at 27°C with 5% CO_2 in air. After this time medium was changed on all wells and fresh medium added - to those cells receiving the PKC inhibitor, the blocker was maintained at the appropriate concentration. After a further 24 hours culture the splenocytes were taken for flow cytometric analysis (4.2.8).

4.2.8 Flow Cytometric Analysis

Splenocytes were taken from the culture plate well and placed into a 5ml Falcon tube. The well was then washed out with 1ml FACS medium and the washings added to the tube. The cells were then centrifuged at 300g, 4°C for 10 minutes and adjusted to $1x10^6$/ml with fresh FACS medium. The cells were then labelled with fluorescently labelled mAbs as detailed in Chapter 2 section 2.2.5.

4.3 Results

4.3.1 Dual Staining Studies of PMA Stimulated Splenocytes from Control *Xenopus*

Dual staining studies with the mAbs 2B1 (anti-CD5) and 8E4:57 (anti-IgM) showed that following stimulation for 48 hours with PMA, IgM was lost from the surface of splenic B cells and a population of cells expressing $CD5^{dull}$ appeared (Figure 4.1A). Also observed was that the staining intensity of CD5 expression on T cells was also slightly increased. Dual staining studies with 2B1 and AM22/F17 (anti-CD8) or D4.3 showed that the population of $CD5^{dull}$ cells were $CD8^-$ and $D4.3^-$ (Figures 4.1B and C), indicating that these cells were not T cells. MHC class II expression was used as a marker to determine if the $CD5^{dull}$ cells were likely B cells. Dual staining for MHC II and CD5 showed that following PMA stimulation, it is a population of MHC II^{bright} cells that become $CD5^{dull}$ (Figure 4.1D). Previous studies in Chapter 2 have shown that adult *Xenopus* B cells are the major MHC II^{bright} population in the spleen, whilst adult T cells are MHC II^{dull}. This difference in MHC II expression of T and B cells was confirmed here for 48 hours cultured cells (Figure 4.2). The suggestion that it is B cells that acquire CD5 expression upon stimulation with PMA is strengthened by flow cytometric observations made 24 hours after PMA stimulation. At this time some $sIgM^{dull},CD5^{dull}$ cells are found (data not shown; Jürgens et al, 1995).

4.3.2 Flow Cytometric Analysis of PMA Stimulated Splenocytes from Thymectomized Animals

Splenocytes from thymectomized siblings of the above 6-8 month old controls were stimulated with PMA in identical conditions to control cells. It was found that in all cases B cells from these animals failed to express CD5. There was no change in phenotype of thymectomized splenocytes following PMA stimulation except for the loss of sIgM (Figure 4.3). An occasional 12 month old Tx animal was found that possessed a low percentage of $CD5^{bright}$ splenocytes (Figure 4.4). When such splenocytes were stimulated with PMA there was still no increase in CD5 expression (Figure 4.4). In such 'old' Tx frogs, residual CD5-staining, as with residual CD8 (AM22) staining, was in fact reduced within 48 hours of PMA stimulation.

4.3.3 Cell Separation Studies

Experiments were carried out to obtain pure B cell populations from control splenocytes to probe how these would respond to PMA stimulation in the absence or presence of co-cultured T cell-enriched splenocytes.

4.3.3.1 Attempt To Deplete T Cells By Complement Mediated Cell Lysis

Experiments using complement to lyse those cells labelled with anti-T cell mAbs did not prove successful. Varying concentrations of complement were used, as were different types of complement (commercial guinea-pig and rabbit complement, as well as complement obtained from fresh rabbit blood). There was some level of killing but not to the anticipated level. After numerous attempts to optimise conditions the technique was abandoned.

4.3.3.2 Enrichment of B Cells By Panning

Initial panning experiments, where the petri dish was coated with the mAb 8E4:57 and unlabelled cells were added directly to the plate ('direct' method), proved to be only partially successful. The adherent population of splenocytes was enriched from 30% to 50% $sIgM^+$ cells, but still had about 40% contaminating T cells. The 'indirect' method was later used, which involved coating the petri dish with an anti-mouse IgG and then adding to the petri dish splenocytes that had been incubated with the mAb 8E4:57. Using this method the adherent (B cell rich) population resulted in purities of >90% (Figure 4.5).

The following points were found to be beneficial to the panning technique for use with *Xenopus* splenocytes:-

1 coating the petri dish overnight at 4°C with the anti-mouse IgG - if the dish was coated at room temperature for several hours the efficiency was reduced.

2 incubating splenocytes on ice with 8E4:57 at 1:60 for 30 minutes, mixing the cells after 15 minutes to ensure optimal binding

3 washing cells twice after labelling cells and before adding cells to the petri dish - failure to do so resulted in sites on the dish being taken by unbound mAb and therefore reducing the efficiency of labelled cells binding to the plate

4 adding up to $6x10^6$ cells to any one petri dish to ensure optimal binding

5 the use of one single pan of 70 minutes with gentle swirling after 40 minutes proved to be just as effective as a double pan, ie. adding the non-adherent cells to a second petri dish after 30 minutes

4.3.4 Phorbol Ester Stimulation of Panned Populations from Control Animals

4.3.4.1 Unmixed Populations

Splenocytes from control *Xenopus* were panned to obtain a population of adherent B cells which were subsequently stimulated with PMA and CaIon. Following stimulation the population of pure B cells (87% pure) lost surface IgM expression but there was no indication of CD5 expression (Figure 4.6). Stimulation of both the non-panned and non-adherent (T cell enriched) populations resulted in an increase in the level of CD5 expression (59% $\Rightarrow$ 76% and 78% $\Rightarrow$ 85% respectively) (Figure 4.6).

4.3.4.2 Cell Mixing Experiments

Having obtained a 90% pure population of B cells by panning, an aliquot of adherent (B cell-enriched) and non-adherent (T cell enriched) were mixed back together at a ratio of 1:1 to ensure that the procedure of panning does not affect the ability of B cells to gain CD5 expression following PMA stimulation. As shown in Figure 4.7, when B cells alone were stimulated with PMA they lost their surface IgM expression but showed no signs of CD5 expression. When the adherent B cells were mixed back with the non-adherent population and then stimulated with PMA, it can be seen that now as well as losing surface IgM, there is an increase in CD5 expression (from 43% $\Rightarrow$ 63%) indicating that it is not the physical act of panning that prevents the B cells from acquiring CD5 expression.

4.3.5 Cell Mixing Experiments with Thymectomized Splenocytes and $CD5^+$ Lymphocytes

4.3.5.1 Use of Well Inserts to Separate a CD5 Cell Source from Thymectomized Splenocytes

Experiments employing the use of well inserts containing $CD5^+$ control splenocytes showed that PMA stimulation of splenocytes from a year old Tx frog was not impeded by the insert itself, as shown by the loss of surface IgM (Figure 4.8A). However, there was no significant increase in CD5 expression (16% up to 19% - Figure 4.8B) by the Tx splenocytes.

4.3.5.2 Cell Mixing Experiments Using a Thymic Tumour Cell Line as a Source of CD5

These experiments were carried out using amphibian tumour medium (see Appendix A) instead of the amphibian culture medium as used previously. To ensure that the results obtained in these experiments were not affected by the tumour medium (with its various additives), initial stimulation studies (as in 4.2.3) were carried out substituting the tumour medium in place of the amphibian culture medium. Results showed that there was no difference in the outcome of the experiments regardless of which medium was used (data not shown).

When splenocytes from a thymectomized animal were mixed with B_3B_7 thymic tumour cells, CD5 expression of gated splenic lymphocytes (based on their forward vs side scatter characteristics) increased following stimulation with PMA, from levels of 12% to 50% (Figure 4.9A). However, there was no increase in CD8 expression, and IgM was lost from the surface of these stimulated cells (Figures 4.9B and C).

One of the major problems with these particular cell mixing experiments is that flow cytometric analysis of forward scatter vs side scatter-gated cells cannot exclude all B_3B_7 tumour cells from the analysis. However, unlike splenocytes, the tumour cells do not express any MHC II molecules, even after PMA stimulation (Figure 4.10), and following PMA stimulation, the mean fluorescence intensity for CD5 is decreased on some of the cells (Figure 4.10). Therefore analyses were carried out using dual-staining of MHC II in combination with CD5 and CD8 (using the mAb F17). Those cells that are MHC II^+ are splenocytes from the thymectomized animal, by virtue of their MHC II expression. As shown in Figure 4.11, following PMA stimulation CD5 expression of Tx splenocytes (MHC II^+) increased from 3% to 18%. The percentage of CD8 expressing ($F17^+$) thymus tumour cells decreased after PMA stimulation and no CD8 appeared on the stimulated Tx splenocytes.

4.3.6 PKC Inhibitor Studies on Lymphocytes from Control *Xenopus*

A dose response assay with *Xenopus* splenocytes showed that the optimal concentration of RO 31-8220 to inhibit PMA stimulation was between 0.1μM and 0.01μM (Figure 4.12). At 0.1μM the stimulatory effect of PMA was inhibited, but it appeared that DPM values for medium-cultured cells were being impaired. Figure 4.13 shows the forward scatter vs side scatter flow cytometric profiles of cells cultured for 48 hours in medium

alone and with varying concentrations of the inhibitor to see what physical effect the inhibitor was having on the gated cells. Dead and dying cells can be seen falling below the analysis gate. At a concentration of 0.01μM the splenocytes have similar physical characteristics to medium cultured cells and appear healthy. At 0.01μM inhibitor, stimulation with PMA is not completely ablated, but is drastically reduced, mean DPM values of 65,978 without the inhibitor are down to 9,439 in the presence of the inhibitor (see Table 4.1). This dose of inhibitor did not have such dramatic effects on LPS or PHA stimulation (see Table 4.1). The response (SI) to PHA is cut by half indicating that the protein kinase C pathway is important in PHA-induced T cell activation, but is not the sole activation pathway.

The effect of the PKC inhibitor on blocking PMA induced surface antigen changes was monitored by flow cytometry (Figure 4.14). The inhibitor alone caused a drop in the percentage of splenic B cells, but an increase in the percentage of $CD5^+$ (Figure 4.14A i & ii). In contrast to cells cultured in PMA on its own, the presence of PMA + inhibitor did not increase the percentage of $CD5^+$ cells (Figure 4.14B ii). Surface IgM was lost from B cells cultured in PMA + inhibitor, indicating that B cell stimulation was still occurring (Figure 4.14B ii). However, dual-colour analysis of MHC II with CD5 showed that the inhibitor prevented PMA from inducing the B cells (MHC II^{bright}) to become $CD5^+$ (Figure 4.14B iv).

LPS stimulation (in the presence or absence of inhibitor) interestingly appeared to result in an increased percentage of splenic $CD5^+$,MHC II^{dull} T cells (compare Figures 4.14A iii and 4.14C iii & iv). This was accompanied with a decline in the percentage of $sIgM^+$ B cells (Figure 4.14C i & ii). This may be caused by B cells being stimulated and induced to die. This loss in sIgM expression would have the effect of making the level of T cells appear to increase.

The inhibitor seems to affect the stimulation with PHA. Cells dual-labelled with MHC II and CD5, show an apparent decrease in CD5 expression in the presence of the PHA + inhibitor (Figure 4.14D iv). This is likely to be due to an increase in MHC II expression which impedes the binding of the anti-CD5 mAb, thereby giving the appearance of a lack of CD5 expression (Figure 4.14D iv). This reduction of CD5 expression was not seen when the same cells were dual-labelled with sIgM and CD5 where there is no chance of steric inhibition by the two mAbs (Figure 4.14D ii). Previous dual-labelling studies with the mAb, AM20 have shown this steric inhibitory effect and was therefore not entirely surprising (data not shown).

Therefore it can be seen that in the presence of the inhibitor CD5 expression induced by PMA stimulation is inhibited, whilst in general, the inhibitor has very little effect on stimulation via LPS or PHA.

4.4 Discussion

The aims of this Chapter were to characterise the population of CD^{dull} cells that emerges following *in vitro* stimulation of splenocytes from control *Xenopus* with phorbol ester. Dual-colour flow cytometry, the use of B cell-enriched lymphocytes and cell mixing experiments with splenocytes from both control and Tx animals have indicated $CD5^{dull}$ expression occurs on PMA activated B cells, but only in the presence of neighbouring T lymphocytes. The biochemical pathway of PMA activation of amphibian lymphocytes is also probed here, by use of a PKC inhibitor.

Following *in vitro* stimulation of splenocytes from euthymic *Xenopus* with phorbol ester, two main phenotypic changes occur (see also Chapter 3). There is a general loss of IgM from the surface of B cells, and the appearance of a population of $CD5^{dull}$ cells that contrasts with the $CD5^{bright}$ T cells. Dual-colour flow cytometric analysis presented in this Chapter indicates that this dull expression of CD5 was found on B cells. In these studies, direct dual staining of sIgM and CD5 was not possible due to the loss of sIgM upon stimulation. However, the use of MHC class II expression was used in combination with CD5 to visualise B cells. Studies in Chapter 2 indicated that B cells (together with putative macrophages) are MHC II^{bright} whilst T cells are MHC II^{dull}. Here it was found that the $CD5^{dull}$ cells following PMA stimulation were MHC II^{bright} (ie. non-T cells). The likelihood that the $CD5^{dull}$ population was not a T cell population was also shown by the absence of CD8 and D4.3 on the $CD5^{dull}$ splenocytes. However, whether CD5 was simply dropping off the stimulated T cells and sticking to activated B cells could not be determined from these dual-labelling studies. *Xenopus* splenocyte culture supernatants from PMA stimulated splenocytes were first tested for the presence of CD5 by an ELISA. These preliminary results indicated that whilst this technique could detect *Xenopus* serum IgM, no CD5 could be detected in the PMA stimulated cultures. The emergence of CD5 expression on B cells by phorbol ester stimulation has been shown to occur in mice (Rothstein et al, 1986) and humans (Zupo et al, 1994). These studies have shown that mRNA coding for CD5 has been found in activated B cells, suggesting that CD5 expression on B cells is being generated *de novo* and not simply dropping off activated T cells (Freedman et al, 1989b). Miller et al (1984) also provided evidence that CD5 expression on B cells was produced *de novo* following PMA stimulation by the use of biosynthetic labelling studies.

Lymphocytes from Tx *Xenopus* were also stimulated with phorbol ester to determine whether their B cells could be induced to express CD5. The findings revealed that in the absence of T cells, IgM was lost from the surface of B cells, indicating that they had been stimulated, but these Tx B cells failed to show any sign of CD5 expression. Was this failure of CD5 expression by Tx B cells due to the lack of T cells in these splenocyte cultures following thymectomy? If this was so, were T cells required physically, ie. was direct T-B cell contact needed, or were T cell factors (cytokines) alone sufficient for the B cells? On the other hand, perhaps B cells from Tx animals were somehow different from those of euthymic *Xenopus*?

To try and begin to answer these questions studies were first carried out to find out if PMA stimulated B lymphocytes from euthymic *Xenopus* were capable of expressing CD5 in the absence of T cells. Cell enrichment studies (based on the 'panning' protocols of Bleicher and Cohen, 1981; Wysaki and Sato, 1978) provided a population of 90% pure B cells, which when stimulated *in vitro* with phorbol ester, failed to express CD5, like the situation found in Tx animals. When splenocytes enriched for T cells were added back to these purified B cells, CD5 expression could once again be induced following PMA stimulation. These experiments showed that the purification procedures did not alter the B cells' ability to respond to phorbol ester, and suggested that T cells or T cell factors were required by the B cells (from control *Xenopus*) for the induction of CD5. This contrasts findings that showed that human and murine B cells do **not** require T cells or accessory cells for their induced CD5 expression (Bertoglio, 1983; Rothstein et al, 1986; Freedman et al, 1989a,b). Indeed, Zupo et al (1994) showed that in the presence of T cells, $CD5^-$ B cells failed to become $CD5^+$ following PMA stimulation, whereas when T cells were absent, CD5 expression was induced on $CD5^-$ B cells.

The possibility that T cell derived factors were sufficient for *Xenopus* B cell expression of CD5 was investigated by the use of well inserts. When Tx splenocytes were physically separated from a T cell source by a membrane that allows the diffusion of soluble factors but prevents the T cells themselves passing through, there was a loss in surface IgM expression from the PMA stimulated Tx cells, but a minimal increase in CD5 expression. This suggests that direct T-B cell contact, rather than soluble factors alone is crucial for the induction of CD5 on *Xenopus* B cells. Restoration of the ability of Tx B cells to display PMA induced expression of CD5 was demonstrated when Tx cells were mixed directly with thymic tumour cells which could be distinguished from Tx splenocytes by virtue of their MHC II^-, $CD5^{bright}$, $CD8^{bright}$ staining features.

This contrasts the findings of Zupo et al (1994) when $CD5^-$ B cells were mixed with murine EL4 T cells (a murine thymoma cell line) in the presence of PMA and failed to express CD5.

Phorbol esters stimulate mammalian T cells via protein kinase C (PKC) activation (Clevers et al, 1985; Nishizuka, 1984). Usually PKC is activated by the breakdown product of phosphatidylinositol 4,5-bisphosphate (PIP_2), diacylglycerol (DAG) (Kikkawa et al, 1983). The breakdown of PIP_2 is usually initiated by binding of the TCR with its ligand, production of DAG occurring within a very short time. The other breakdown product of PIP_2 is inositol 1,4,5-trisphosphate (IP_3), which causes a release of intracellular calcium. The combination of DAG and Ca^{2+} causes the activation of PKC. Phorbol esters act upon PKC bringing about the activation of T cells, mimicking the effects of TCR binding. Confirmation that PMA is activating *Xenopus* T cells via PKC was shown when the PKC inhibitor, RO 31-8220 was able to block tritiated thymidine incorporation and prevent the induced expression of CD5 on B cells, although it failed to prevent loss of sIgM of PMA stimulated cells. The proliferative action of LPS was not affected by the presence of the inhibitor. Stimulation with PHA was somewhat affected by the PKC inhibitor, as noted by a drop in the level of activation in thymidine incorporation studies. This partial inhibition of PHA stimulation may not be that surprising, as stimulation via PKC is likely to be a major pathway of T cell activation. However, it is not the only pathway, as T cells can also be activated by calcium-dependent kinases which ultimately lead to transcription of T cell activation genes (Geller et al, 1987).

Although *Xenopus* is a primitive vertebrate, its B cells can be induced to express CD5 following *in vitro* stimulation with phorbol ester. The fact that only a low proportion of *Xenopus* B cells begin to express CD5 following PMA stimulation is not altogether surprising, as in mammals, not all B cells are capable of CD5 expression (Hardy, 1991; Haughton et al, 1993; Kawamura et al, 1994). Indeed, only a small percentage of the human B cell population express CD5 following *in vitro* stimulation with phorbol ester (Zupo et al, 1994). However, the work presented in this Chapter highlights the fact that *Xenopus* differs from humans and mice in that *Xenopus* B cells require T cells for CD5 expression. The immunological role of CD5 on T and B cells is still not understood in higher vertebrates. The fact that $CD5^{dull}$ B cells be induced in *Xenopus* shows that this cell type is evolutionary conserved and that CD5 is likely to be of immunologic importance to both T and B cell populations.

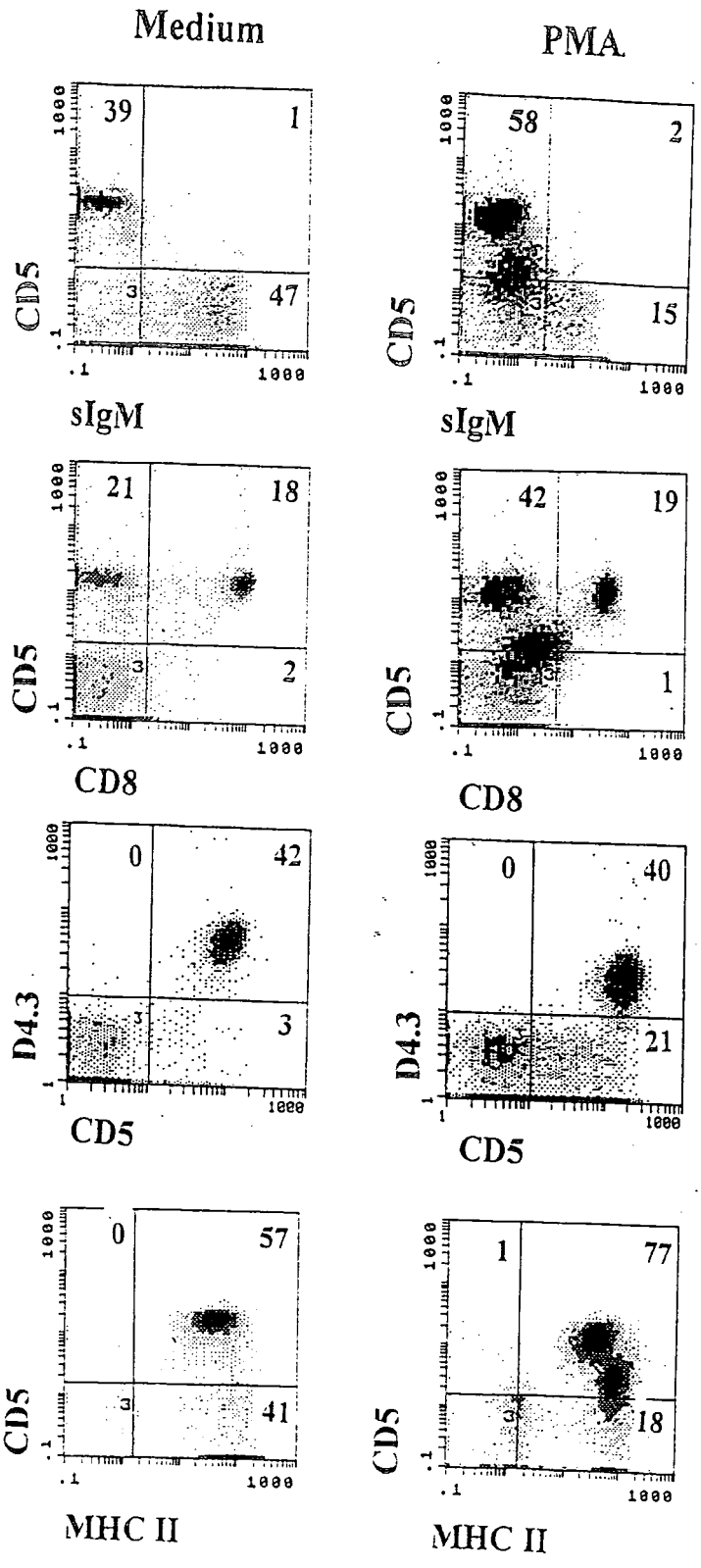


Figure 4.1 Representative traces of dual-stained splenocytes from control *Xenopus laevis* (6-8 month). Splenocytes were cultured for 48 hours in the presence and absence of PMA (10ng/ml). Traces are representative of 10 repeat experiments.

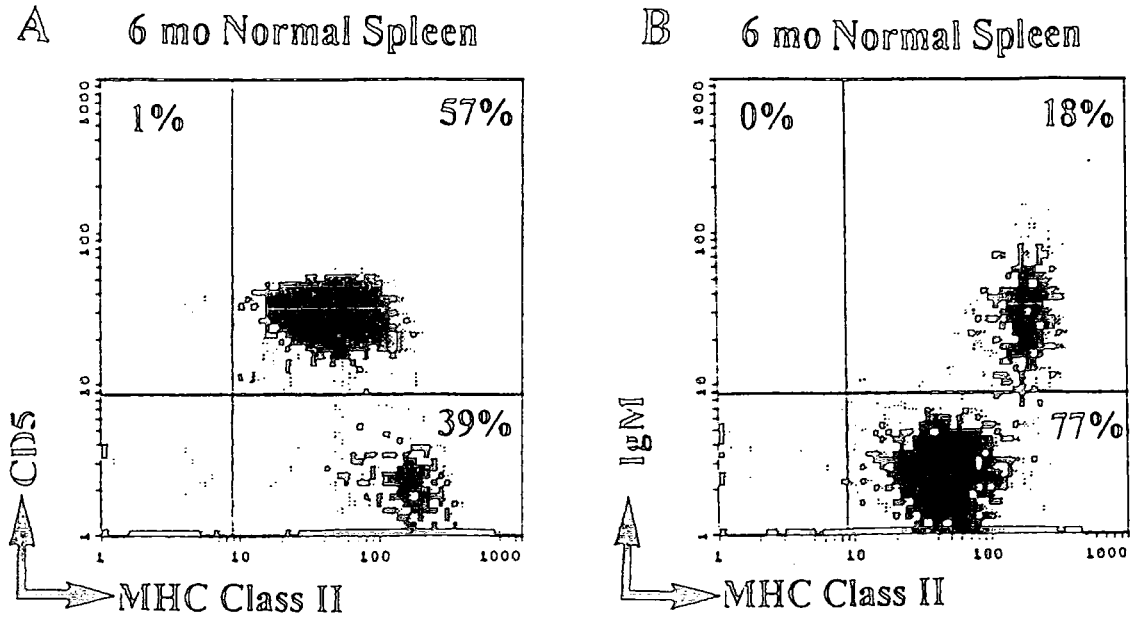


Figure 4.2 Trace showing splenocytes from control adult (6-8 month) cultured for 48 hours in medium and dual stained for either MHC II plus CD5 or MHC II plus sIgM, showing that whereas T cells (approximately 60%) stain less brightly (MHC II^{dull}), whereas B cells (approximately 20% total splenocytes) stain brightly with MHC II (ie. MHC II^{bright}). [Macrophages, approximately 16%, also stain brightly for MHC II (data not shown)]

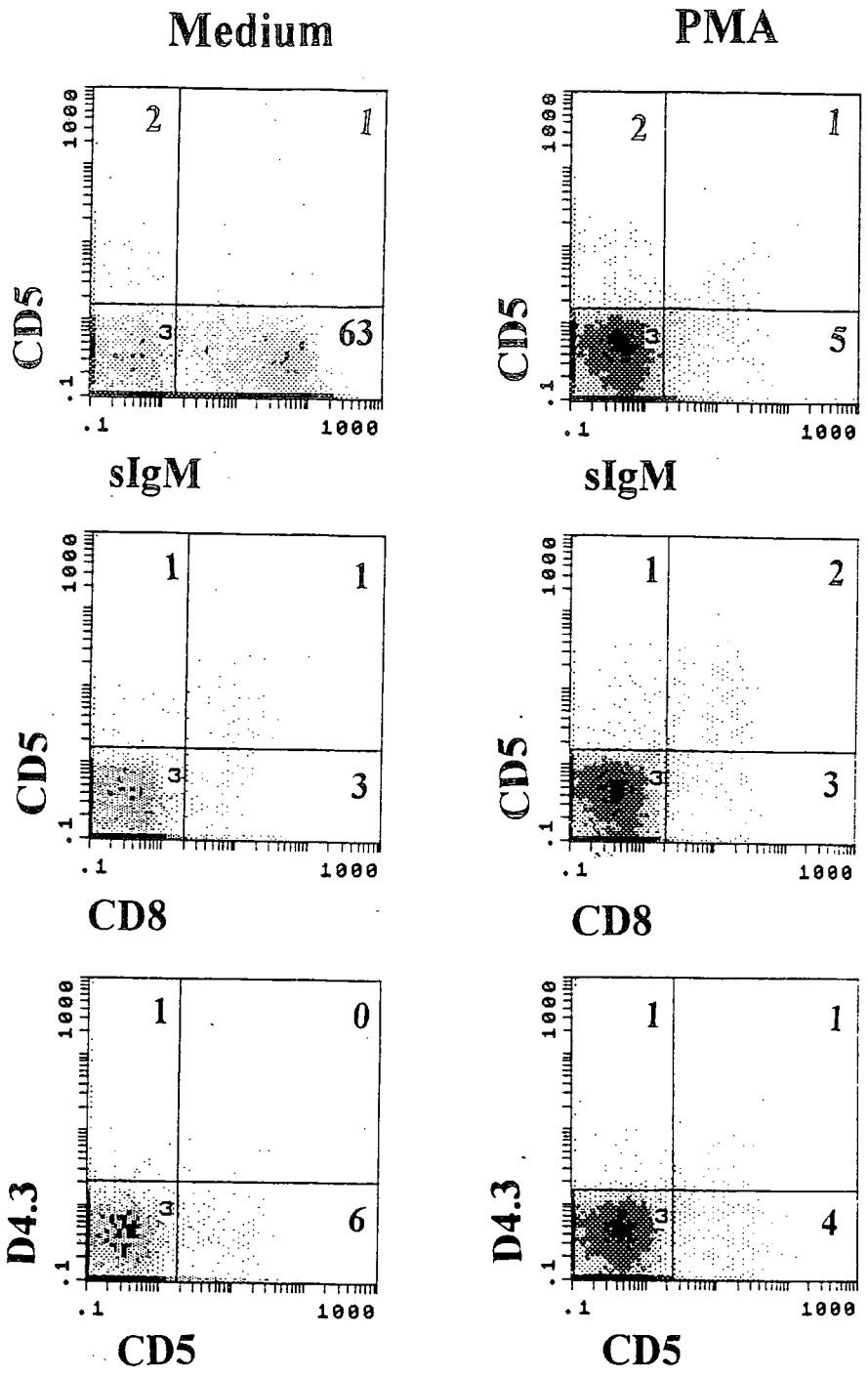


FIGURE 3 Representative traces of dual-stained splenocytes from thymectomised adult (6-8 month) Xenopus laevis. Splenocytes were cultured for 48 hours in the presence and absence of PMA (10ng/ml). Traces are representative of 5 repeat experiments.

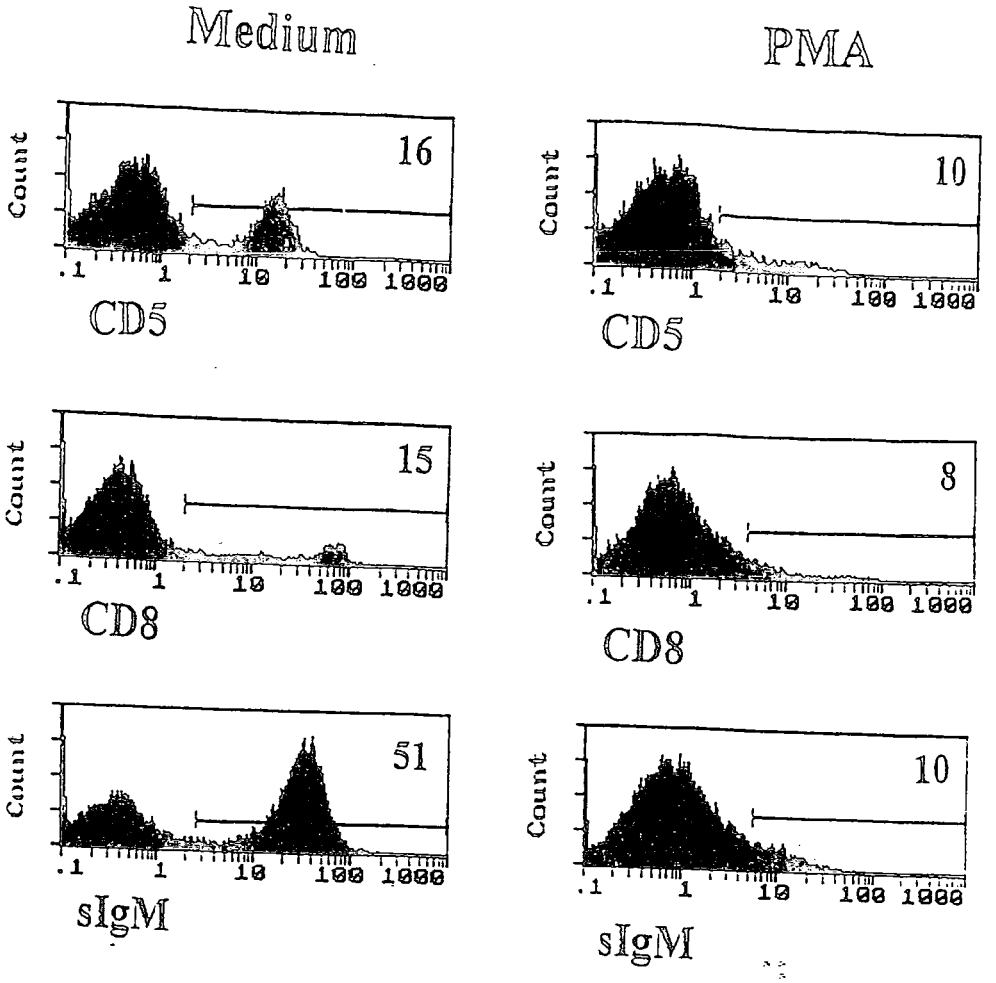
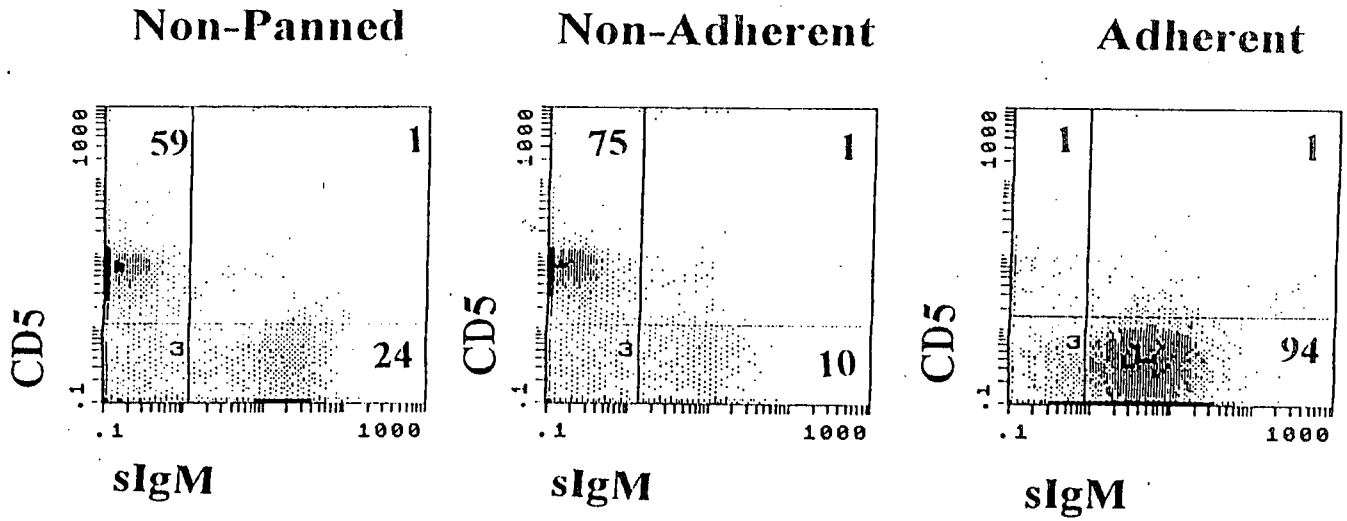


Figure 4.4 Single colour flow cytometric analysis of splenocytes from a thymectomised adult (12 month) that have been cultured for 48 hours in the presence and absence of PMA (10ng/ml). This particular animal had some CD5 expression, but failed to increase this level following PMA stimulation.

Figure 4.5 Flow cytometric analysis of splenocytes from a control adult (6-8 month) showing the efficiency of 'indirect' panning with 8E4:57 (anti-IgM) incubated cells. Splenocytes were cultured overnight following the panning procedure before being analysed by flow cytometry. Mean of 12 repeat experiments.



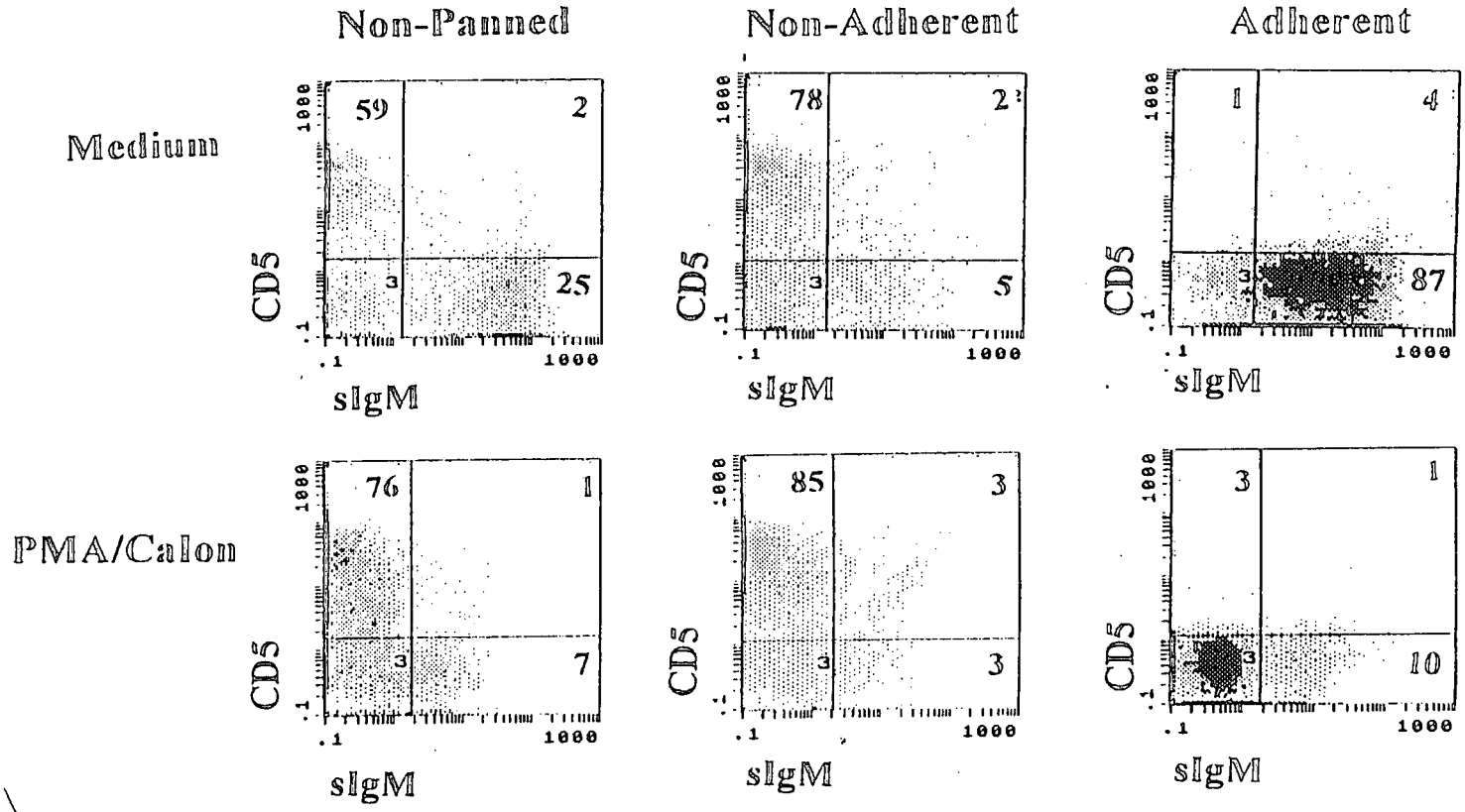


Figure 4.6 Flow cytometric analysis of splenocytes from a control adult (6-8 month) that have been panned to obtain a pure population of B cells ('adherent' population) and a 'non-adherent', T cell-enriched population. Following the panning procedure and overight culture, each population type was cultured for a further 48 hours in the presence or absence of PMA (10ng/ml) and CaIon (0.1μg/ml) before being analysed by flow cytometry. Representative of 6 repeat experiments.

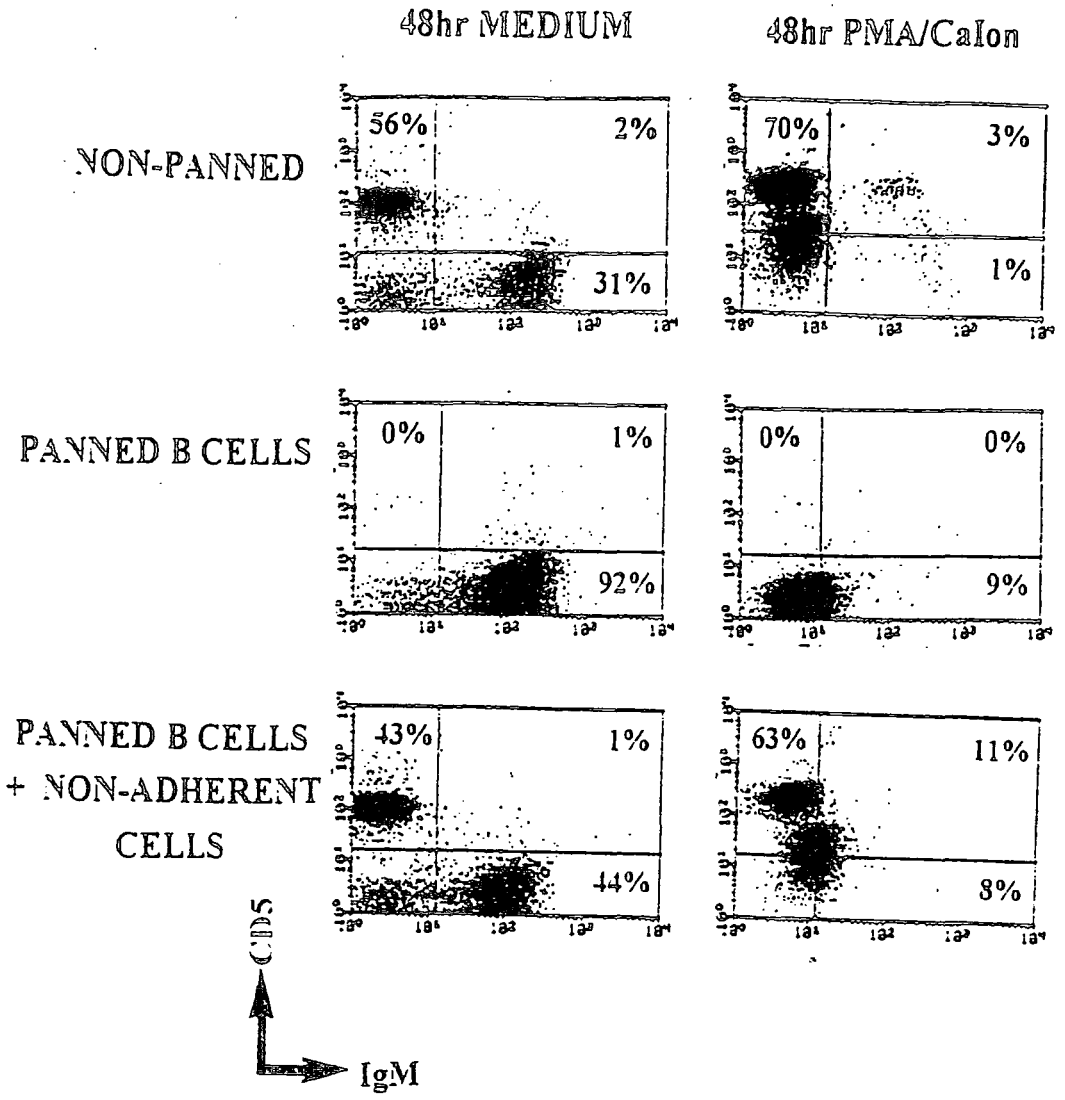


Figure 4.7 Flow cytometric analysis of splenocytes from a control adult (6-8 month) that have been panned to obtain a pure population of B cells ('adherent' population) and a T cell-enriched 'non-adherent' population. Following the panning procedure and overnight incubations, an aliquot of adherent cells were mixed 1:1 with an aliquot of the non-adherent cells, and all populations were cultured for a further 48 hours in the presence and absence of PMA and CaIon. Representative of 2 repeat experiments.

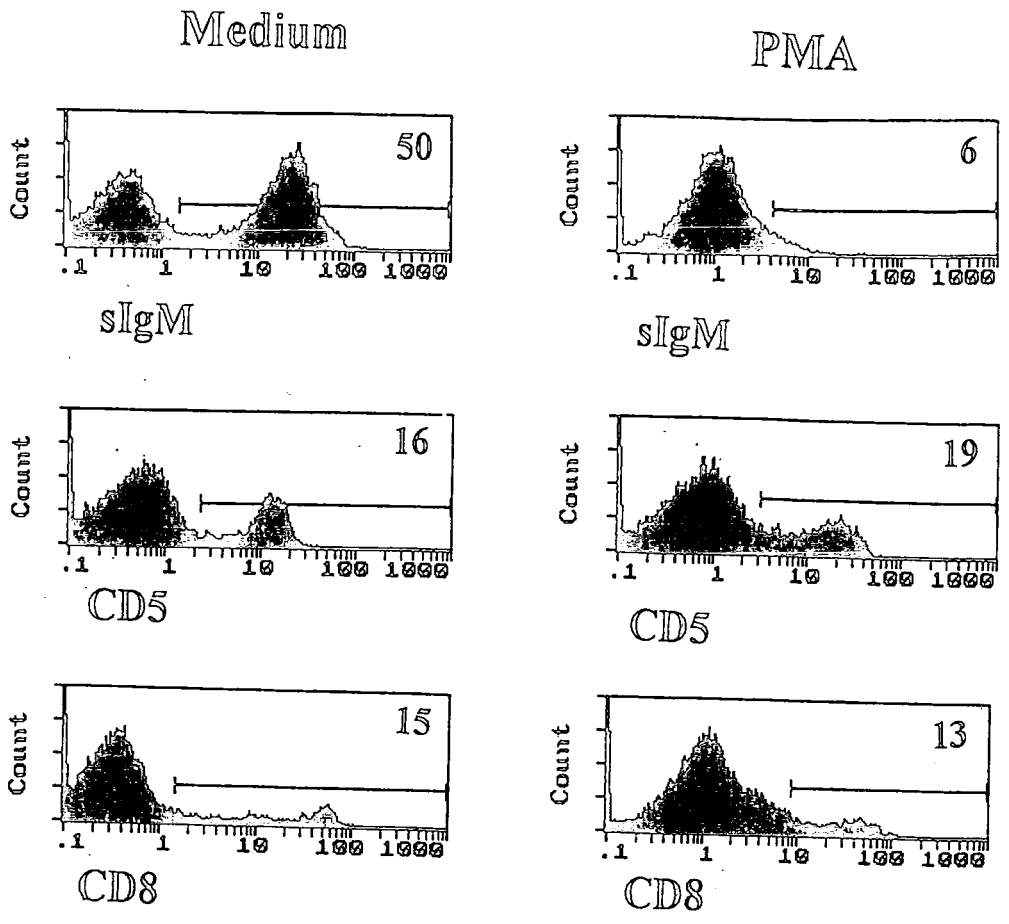


Figure 4.8 Single colour flow cytometric analysis of splenocytes from a thymectomised adult (12 month) separated from control splenocytes by a well insert, and cultured for 48 hours in the presence and absence of PMA. Representative of 2 experiments.

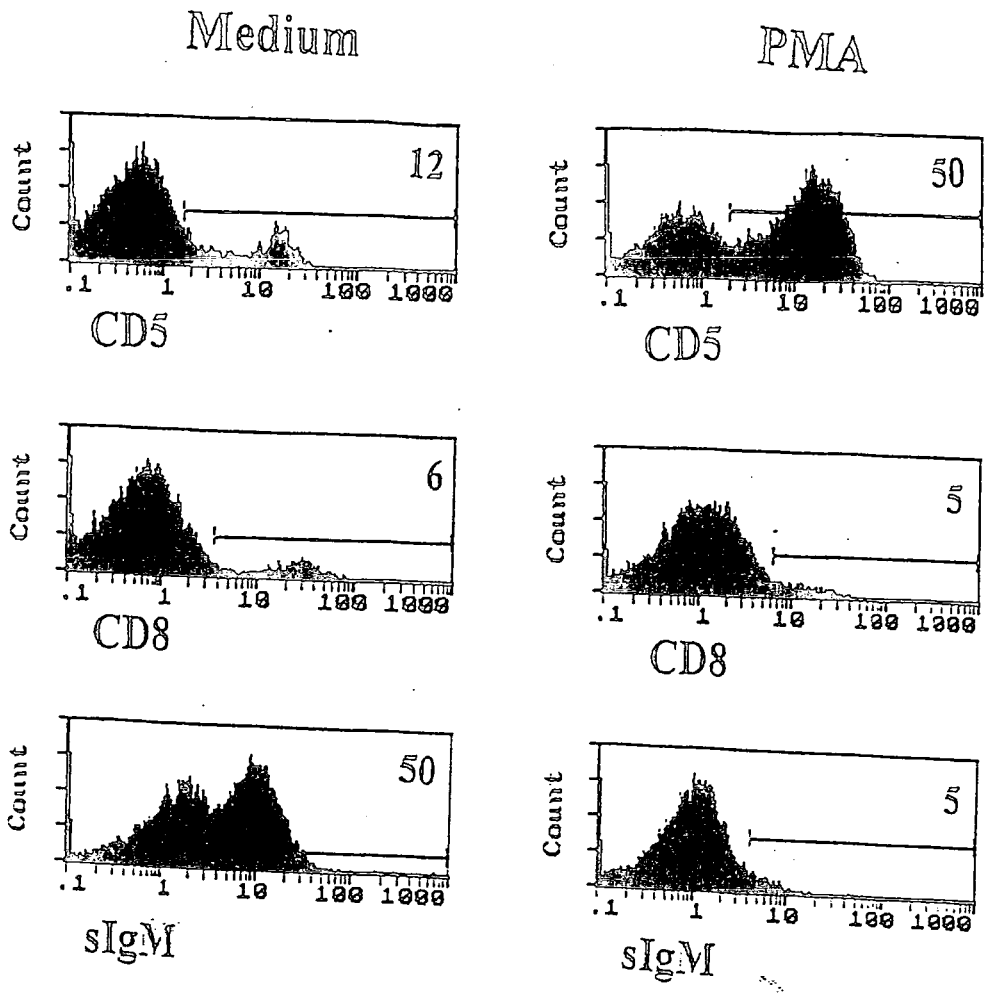


Figure 4.9 Single colour flow cytometric analysis of lymphocyte gated splenocytes from thymectomized adult (12 month) that have been cultured in the presence of B_3B_7 thymic tumour cells for 48 hours in the presence and absence of PMA. Mean of 3 repeat experiments.

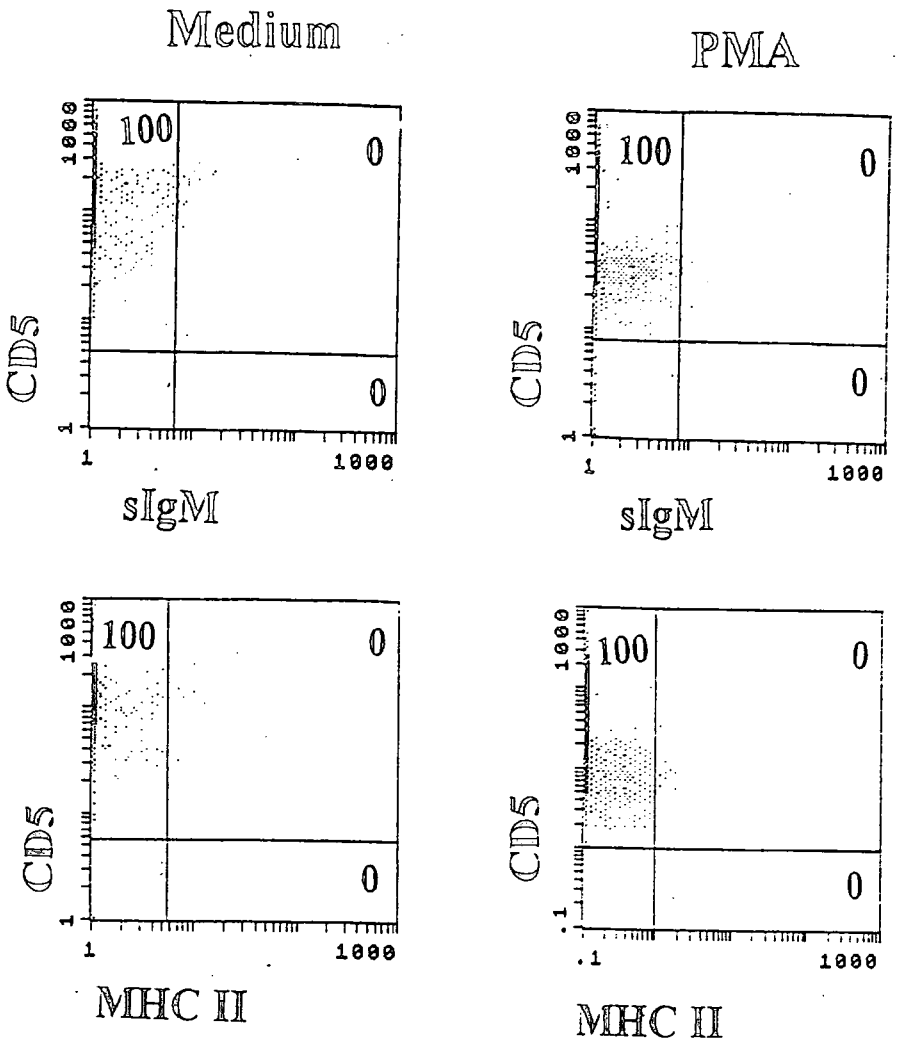


Figure 4.10 Dual-colour flow cytometric analysis of gated B_3B_7 thymic tumour cells cultured for 48 hours in the presence and absence of PMA. Note the lack of MHC II expression by the tumour cells and that expression is not induced by PMA stimulation.

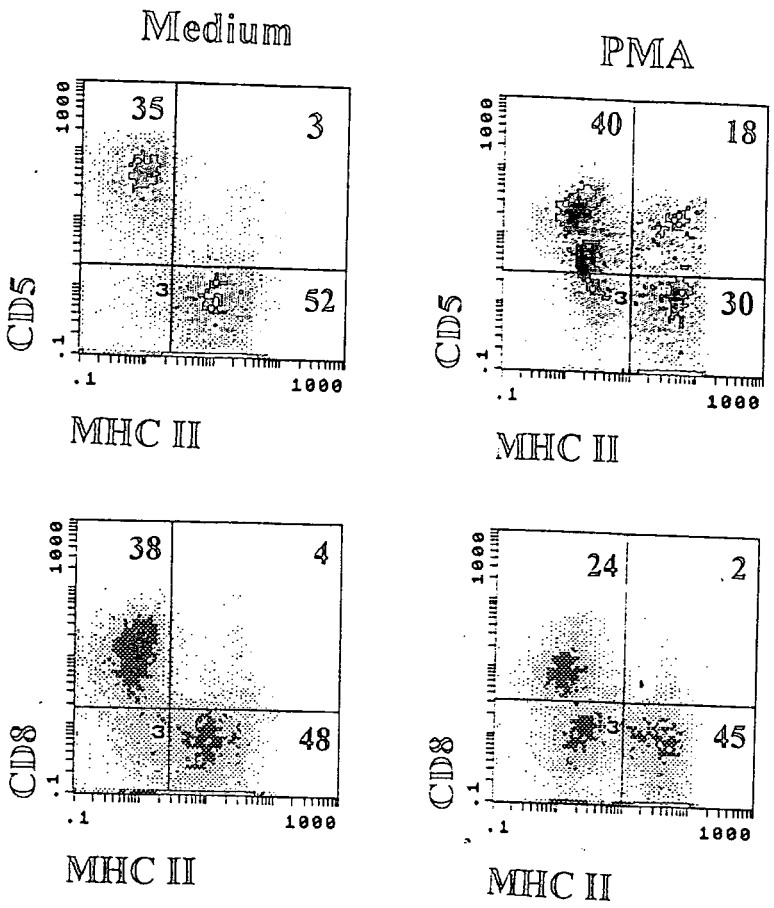


Figure 4.11 Dual-colour flow cytometric analysis of splenocytes from thymectomized adult (12 month) that have been cultured in the presence of B_3B_7 thymic tumour cells for 48 hours in the presence and absence of PMA. No FS vs SC gating has been carried out - ie. both Tx splenocytes and thymic tumour cells are being analysed. MHC II was used as a marker to discriminate the Tx splenocytes (mostly MHC II^+) from the tumour cells (all MHC II^-). Cells were co-stained with 2B1 (anti-CD5) or F17 (anti-CD8).

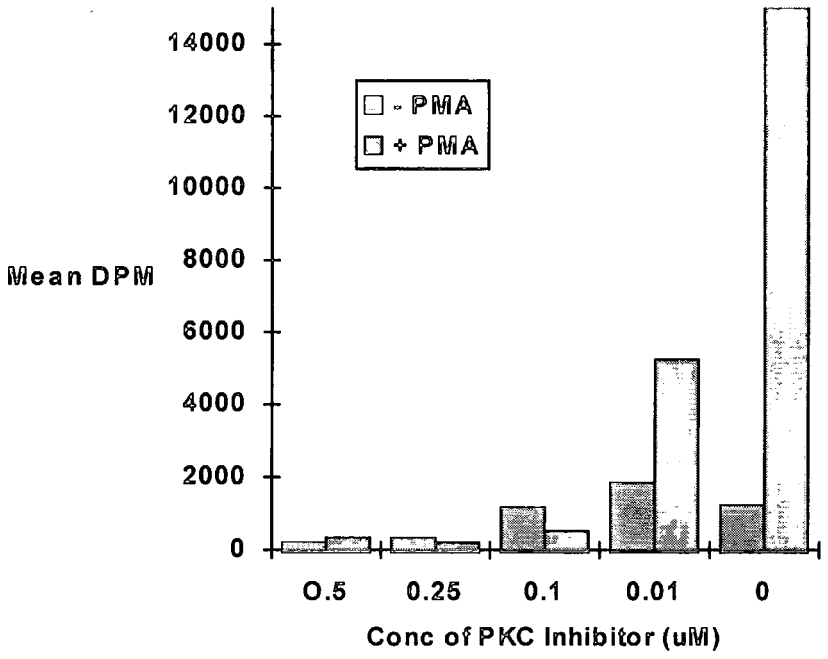


Figure 4.12 Dose response assay of the protein kinase C inhibitor to ascertain the optimal concentration for use with amphibian splenic lymphocytes. Animals were aged 8 months. Mammalian studies show an optimal concentration of 0.5μM.

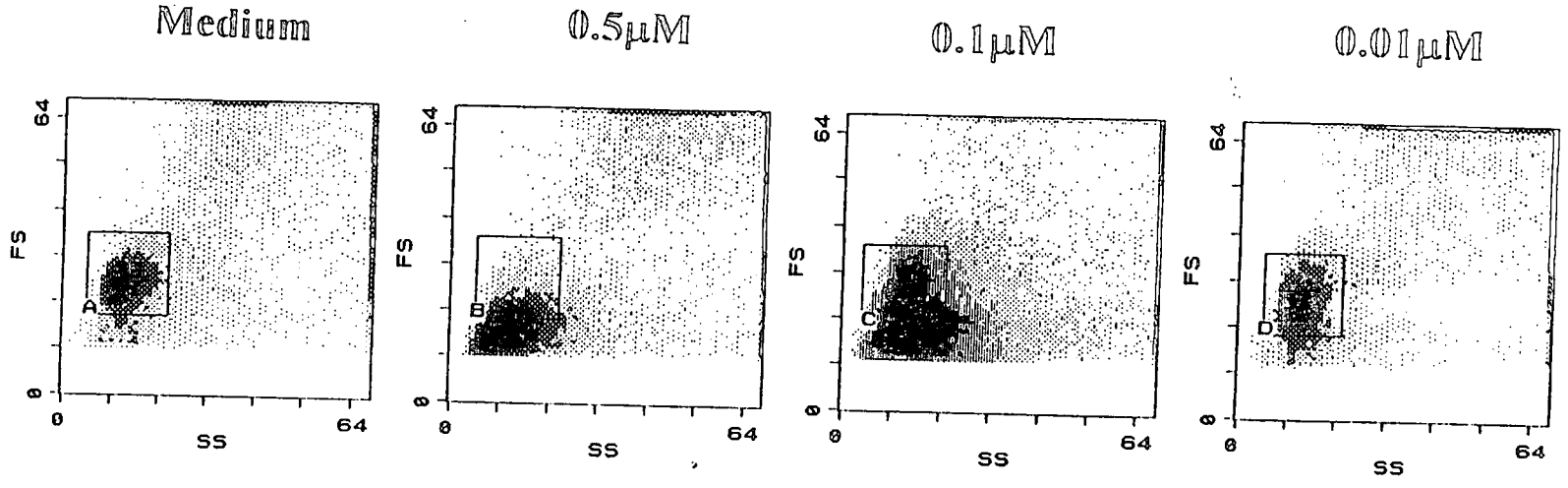


Figure 4.13 Forward scatter vs side scatter profiles of 48 hour medium-cultured splenocytes in the presence of varying concentrations of the protein kinase C (PKC) inhibitor RO 31-82230 to show the effect of the inhibitor on the health of the cells. Dead and dying cells can be seen falling below the analysis gate.

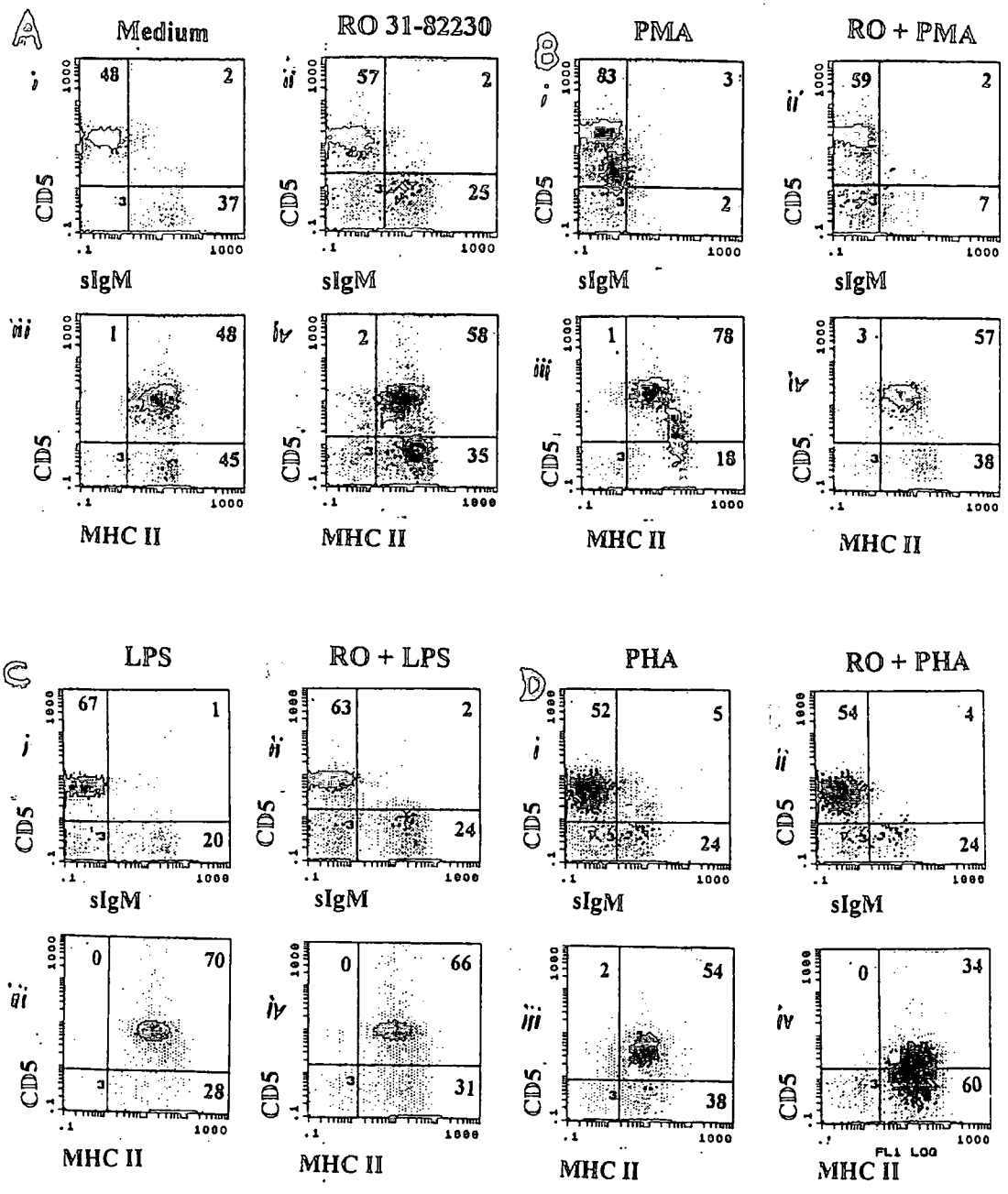


Figure 4.14 Dual-colour flow cytometric analysis of control adult (6-8 month) X laevis to show the effect of the protein kinase C inhibitor. RO 31-8220 on mitogenic stimulation. Splenocytes were cultured for 48 hours in medium (A), 10ng/ml PMA (B), 250µg/ml LPS (C) or 1µg/ml PHA (D) both in the presence and absence of the PKC inhibitor. Those cells receiving a dose of the inhibitor were pre-incubated on ice in medium containing 0.01µM inhibitor. Inhibitor was also added to the cell cultures.

Mitogen	Mean DPM No inhibitor	Stimulation Index	Mean DPM Inhibitor	Stimulation Index
PMA	65978	103	9439	15
LPS	5972	9	4394	7
PHA	32746	51	16757	26

Table 4.1 Table showing the effect of the protein kinase C inhibitor used at a concentration of 0.01μM upon stimulation of splenocytes from control adult (6-12 month) *X laevis* with the mitogens PMA (10ng/ml), LPS (250μg/ml) and PHA (0.1μg/ml). Cells were cultured for 48 hours before receiving a pulse of ^{3}H-TdR and harvested for scintillation counting 18-20 hours later.

Chapter 5

Characterisation of Lymphocytes in the Spleen, Gut and Liver of Control and Thymectomized *Xenopus*: A Search for Extrathymic T Cell Development

5.1 Introduction

Although the thymus plays a very special, perhaps unique role in T cell development the possibility of extrathymic T cell maturation is currently an issue of considerable debate. Recent work has led to the discovery that T-like cells can arise extrathymically in both congenitally athymic mammals (nude mouse and rat) (Lake et al, 1991; Vaessen et al, 1986), and in some animals that have been thymectomized at an early age (Lin et al, 1994). Extrathymic T cells have also been observed in euthymic mammals; indeed this population is a major gut lymphocyte subset (Rocha et al, 1992). Extrathymic T cells are cells that have cell surface determinants that are usually associated with T cells, but which have developed either outside of the thymus, or in the complete absence of a thymus. These T-like cells are usually found within the intestine or liver, both derived from endoderm tissue like the thymus (Rocha et al, 1995; Poussier and Julius, 1994).

Much research has been carried out on lymphocytes from the intestine, and it has been concluded that the intestine is a site of T cell development, and also houses the largest collection of T cells in the body (Poussier and Julius, 1994). Within the intestine there are many complex phenotypes of T cells that are reminiscent of those

found in the thymus (Poussier et al, 1992). There are several theories surrounding the development of these T cells and whether or not the thymus has any influence upon their development. Of the many different T cell populations found in the gut, it has been shown that some of these have been derived from the thymus and migrated to the intestine, whilst others have developed *in situ* (Guy-Grand et al, 1991; Poussier et al, 1992; Guy-Grand et al, 1993). The thymus-dependent (TD) cells tend to bear the phenotypes, TCR$\alpha\beta^{+}CD4^{+}$ and TCR$\alpha\beta^{+}CD8\alpha\beta^{+}$, originate in the thymus and migrate to the Peyers patches early in life. These cells continually recirculate until they become activated, when they leave the Peyers patches and migrate to the lamina propria (Guy-Grand et al, 1993). The thymus-independent (TID) gut lymphocytes are usually identified by their expression of the CD8$\alpha\alpha$ homodimer and are located within the intestinal epithelium, being described as intraepithelial lymphocytes (IELs). These populations can be found expressing V β families that are normally deleted in the thymus-derived lymphocytes (Guy-Grand et al, 1993; Umesaki et al, 1993) suggesting that the same selection procedures that occur in educating thymus-derived T cells do not occur here. The TID IELs may bear the TCR$\alpha\beta$ but do not have a functional TCR and do not proliferate upon stimulation with anti-TCR mAbs, unlike TD IELs (Poussier et al, 1992; Croitoru and Ernst, 1993). Early in life the major population of IELs are those bearing the $\gamma\delta$ T cell receptor (TCR), and these also tend to express CD8 in the CD8$\alpha\alpha$ homodimeric form. As the animal ages these TCR$\gamma\delta$ IELs become outnumbered by IELs expressing TCR$\alpha\beta$. However, this expansion of TCR$\alpha\beta$ IELs only occurs in those animals exposed to a conventional diet - if the animals are reared in germ-free conditions, the TCR$\gamma\delta$ IELs remain the predominant population (Poussier and Julius, 1994; Umesaki et al, 1993). However, it is important to note that recent work indicates that the IELs in euthymic animals are in fact all derived from the thymus, or through thymus-derived factors (Lin et al, 1995; Wang and Klein, 1994).

In contrast to the situation in mice, in birds it has been shown that the intestinal lymphocytes are derived exclusively from the thymus (Kasahara et al, 1993). The major population of T cells in the intestine are TCR$\gamma\delta$ cells, and it has been shown that early thymectomy in chickens completely ablates TCR$\gamma\delta$ development (Chen et al, 1989). The same situation is also found in sheep that have been thymectomized *in utero*, causing a complete ablation of TCR$\gamma\delta$ cells (Hein et al, 1990).

The liver also appears to be a possible site of extrathymic T cell development. Ilai et al (1992) found that cells bearing TCRs of intermediate intensity appear to be of extrathymic origin. These $TCR^{intermediate}$ cells were found to increase with thymic atrophy and involution, whereas TCR^{bright} (ie. thymus derived) cells decreased in numbers. These $TCR^{intermediate}$ cells were also found in the liver of nude and thymectomized mice. It was also found that these $TCR^{intermediate}$ cells could develop in the liver of euthymic mice (Okteki et al, 1992), and that these T-like cells show a high level of autoreactive clones, once again suggesting that these cells have not undergone the usual selection procedures that T cells emerging from the thymus have.

Lymphocytes in the liver that bear $TCR^{intermediate}$ have been shown to bear the CD8αα homodimer (Ohtsuka et al, 1994; Sato et al, 1995) suggesting that they are like those putative extrathymically-derived lymphocytes in the intestine. However, although putative extrathymic T lymphocytes from the liver and intestine share the expression of CD8αα, those extrathymic T lymphocytes from the intestine bear a TCR^{bright} phenotype (Ohtsuka et al, 1994). Those extrathymic T lymphocytes from the liver also differ from those in the intestine in that lymphocytes from the liver migrate into the periphery with ageing, whilst lymphocytes in the intestine of extrathymic origin remain in the intestine and do not migrate (Ohtsuka et al, 1994). The possibility of extrathymic T cells is not restricted to rodents, the appearance of extrathymic T cells has been suggested in humans. Thus Poggi et al (1993) isolated lymphocytes from human fetal liver at a time before mature thymus-derived T cells have developed in the liver.

This Chapter explores the possibility of an extrathymic pathway of T cell development in the amphibian, *Xenopus*. Having shown in Chapter 3 how effective thymectomy of 5-7 day old larvae is at preventing T cell development in the spleen, analysis of the intestine and liver of Tx *Xenopus* should show whether an extrathymic pathway exists in this primitive animal. This Chapter aims to explore the nature of T cell populations found in the intestine and liver of control animals and compare this to the picture found in Tx animals. Animals were analysed by dual-colour flow cytometry at two time points, 4-6 months of age and again when approximately 8-12 months old, in order to begin to address the ontogeny of any putative extrathymic T cells seen. Spleen, liver and gut lymphocytes from year old animals were stimulated *in vitro* with the T cell mitogen concanavalin A and also

with phorbol myristate acetate to determine whether these lymphocytes could be induced to proliferate. Immunohistochemistry on gut was also carried out to back-up the flow cytometric analysis.

5.2 Materials and Methods

5.2.1 Animals and Operations

5.2.1.1 Animals

Outbred *Xenopus laevis* were used throughout, and were bred and reared in the laboratory under standard conditions (Horton and Manning, 1972). Adult frogs aged between 4 and 12 months were used for the majority of studies carried out in this Chapter. However, some 5 week old larvae were also used in the flow cytometric analysis.

5.2.1.2 Thymectomy

Details are given in section 3.2.1.2

5.2.2 Preparation of Spleen Cell Preparation

Details are given in section 3.2.1.3

5.2.3 Extraction of Lymphocytes from *Xenopus* Intestine

The extraction process was generally in 3 stages - extraction of the cells from the gut, nylon wool purification and discontinuous Percoll gradient separation (see Figure 5.1).

5.2.3.1 Extraction of Intraepithelial Lymphocytes from the Intestine

Animals were heavily anaesthetised in MS222, the abdominal cavity was opened and the intestine dissected out, both small and large intestine being removed. The intestine was placed into Ca^{2+} and Mg^{2+} free (CMF) medium [10ml Hanks Balanced Salt Solution (Gibco) + 10ml 10x HEPES-bicarbonate buffer + 2ml FCS and made up to 100ml with distilled H_2O - see Appendix A] before being gently flushed through with 20ml CMF medium using a 10ml syringe fitted with a 21-gauge needle to remove the gut contents. The gut was then placed onto some CMF-moistened tissue, cut open longitudinally then cut into small pieces (<50mm) and placed into a 10ml conical tube containing fresh CMF. The tube was inverted 3 times and the gut pieces allowed to settle before discarding the supernatant. Fresh medium was added and the procedure repeated two more times. This step helps to remove any remaining faecal matter or mucus from the gut. The gut pieces were then transferred to a siliconised conical flask containing 20ml CMF medium

containing 0.1mM EDTA and 10% FCS (CMF/EDTA/FCS). The flask was covered with Nesco film and placed onto a magnetic stirrer, at a speed just fast enough to allow gentle agitation of the tissue, for 20 minutes. After this time the contents of the flask were transferred to a universal and vortexed on high speed for 15 seconds. Once the gut pieces had settled the supernatant was transferred to a 50ml conical centrifuge tube and stored on ice. A further 20ml CMF/EDTA/FCS was added to the universal and vortexed, and this supernatant was also added to the centrifuge tube. The gut pieces were transferred back to the conical flask and 20ml CMF/EDTA/FCS added, the flask sealed and put back on the stirrer for a further 20 minutes. Supernatants were collected into a second centrifuge tube as above. The two centrifuge tubes were then centrifuged at 300g, 4°C for 10 minutes, the supernatants were discarded and the pellets combined in 4ml sterile APBS.
The gut pieces can now be treated for the extraction of lamina propria lymphocytes (LPL).

5.2.3.2 Extraction of Lamina Propria Cells from Intestine

This extraction was carried out after the IELs had been removed. The gut pieces were placed into a universal containing 10ml APBS with 0.25% trypsin (Gibco) and 0.1mM EDTA and incubated at room temperature for 15-20 minutes with occasional vortexing. The trypsin digestion was stopped by the addition of 10ml CMF/EDTA/FCS. The universal was vortexed on high speed for 15 seconds and the supernatant transferred to a 50ml centrifuge tube. A further 20ml medium was added to the universal and vortexed and the supernatant combined with the first. The cells were then left on ice until needed.

The IELs and LPLs were either passaged separately over nylon wool and Percoll density gradients as described below, or they were pooled together as total gut lymphocytes and then passed over nylon wool and Percoll.

5.2.3.3 Nylon Wool Purification

This step removes any large pieces of tissue, clumps of cells and the larger epithelial cells. Columns were made up in advance and autoclaved. Nylon wool (Leuko-Pak Leukocyte Filter, Fenwall) was boiled in dH_2O six times and dried at 37°C. Aliquots of 0.3g nylon wool were teased apart to remove any knots or clumps and placed into 10ml nylon syringes (Vandermic) and compressed using the plunger. The plunger was removed before the column was wrapped in tin foil and autoclaved.

Just before use, the column was hydrated with sterile APBS. The cell suspension (in 4ml APBS) was added to the column, directly followed by 10ml APBS. The eluate was collected in a universal tube. Before the column could dry out, a further 10ml APBS was added to the column and the eluate collected in the same universal. Eluted cells were stored on ice ready for density gradient separation.

5.2.3.4 Discontinuous Percoll Density Gradient Separation

Percoll gradients were used to enrich for lymphocytes. A stock solution of 90% Percoll was made up using 10x APBS. Solutions of 70% and 40% Percoll were made by diluting the 90% solution with amphibian culture medium (see Chapter 3). Density gradients were made up in 10ml conical tubes by layering 2ml 90% Percoll at the bottom of the tube, overlayed with 2ml 70% followed by 4ml 40% Percoll (see Figure 5.1). Two gradients were used for each gut extraction procedure. The eluate from the nylon wool purification was centrifuged at 300g, 4°C for 10 minutes. The pellets were resuspended in 2ml APBS and 1ml layered onto each density gradient. Tubes were then centrifuged at 1800rpm at room temperature for 20 minutes. The lymphocytes were found at the 40%-70% interface and were collected into a fresh 10ml tube. Lymphocytes were washed 2-3 times with APBS to remove any contaminating Percoll and then prepared for cell culture (see 5.2.5). [Dead cells and some epithelial cells could be found in a mucoid film at the top of the gradients.]

5.2.4 Extraction of Lymphocytes from *Xenopus* Liver

Animals were heavily anaesthetised in MS222 and the two lobes of the liver dissected out taking care not to take the gall bladder. The liver was placed into a sterile 3.5cm petri dish (Costar) containing 2ml sterile APBS and teased apart using a pair of tungsten needles. The resulting cell suspension was transferred to a sterile 5ml Falcon tube, the dish washed out with a further 1ml APBS and the washings added to the tube. The cell suspension was then pipetted vigorously to help release any clumps of cells and then left for about a minute for large pieces to settle. The supernatant was then transferred to a fresh 5ml tube and centrifuged at 300g, 4°C for 10 minutes. The pellet was then washed 4 times with APBS before being resuspended in 2ml APBS. The cells were then layered onto Percoll discontinuous density gradients (as given in 5.2.3.4).

5.2.5 Culture of Lymphocytes Extracted from Gut, Spleen and Liver

Freshly prepared lymphocytes were centrifuged at 300g, 4°C for 10 minutes and resuspended in amphibian culture medium (supplemented with fungizone, penicillin and streptomycin due to the increased chances of microbial infection from the gut) at a concentration of $1x10^6$ lymphocytes per millilitre. Cells were plated out in 24-well plates (see section 5.2.7 for culture of cells for functional studies) and incubated in a humidified incubator with 5% CO_2 in air overnight. The next day cells were taken for flow cytometric analysis (see 5.2.6).

5.2.6 Flow Cytometric Analysis of Splenic, Gut and Liver Lymphocytes

Cells were transferred from the 24-well plate into a 5ml Falcon tube, and the well washed out with 1ml FACS medium. Tubes were centrifuged at 300g, 4°C for 10 minutes and the pellets resuspended in FACS medium to a concentration of $1x10^6$ lymphocytes per millilitre. Lymphocytes were incubated with the appropriate mAbs (see section 2.2.5).

5.2.7 Tritiated Thymidine Incorporation Studies

Proliferation was assessed by the amount of tritiated thymidine incorporated by DNA-replicating cells after mitogenic stimulation.

Splenocytes were cultured in triplicate at 1 x 10^6/ml in 96-well flat bottomed plates ($1x10^5$ cells/well). Ten microlitres of either ConA (2.5μg/ml) or PMA (10ng/ml) were added to experimental wells, whilst 10μl medium was added to control wells. The plate was cultured in a humidified incubator at 27°C, 5% CO_2 in air. Medium was changed on all wells after 18-20 hours to remove the mitogen and replaced with fresh medium. After a further 24 hours cells were given a 1μCi pulse of ^{3}H-TdR before being harvested 18-20 hours later. Cells were harvested onto fibreglass filters (Whatman) using a Skatron cell harvester. Filters were then dried at 60°C before individual filter discs containing the samples were placed into 5ml scintillation vials. Four millilitres of scintillation fluid was added to each vial and the amount of incorporated tritiated thymidine was calculated as degenerations per minute (DPM) on a Packard Liquid Scintillation analyser.

The level of incorporated tritiated thymidine was calculated by working out the stimulation index for each experimental sample, as follows:

Stimulation Index (SI) = Mean dpm Mitogen Stimulated Samples
Mean dpm Medium Control Samples

5.2.8 Cryostat Sectioning and Immunoperoxidase Staining of *Xenopus* Intestine

5.2.8.1 Gut Preparation

The intestine was dissected out of heavily anaesthetised animals and placed into CMF medium. The gut contents were washed out with 20ml CMF medium using a 10ml syringe and 21-gauge needle. Intestines from individual animals were placed into foil "boats", containing OCT compound (Tissue-Tek) and were slowly lowered into liquid nitrogen to freeze the OCT. The tin foil was then removed and the block was mounted onto a chuck, being left inside the cryostat for 15-20 minutes to equilibrate to cutting temperature (≈ -20°C). Six micrometre sections were captured onto poly-L-lysine coated slides and following immediate fixation in acetone for 2 minutes were placed in a slide box kept inside the cryostat. Slides were occasionally taken for haemotoxylin and eosin staining, in which case the sections were processed for H&E staining (see Appendix A). All slides were stored at -80°C prior to immunoperoxidase staining.

5.2.8.2 Immunoperoxidase Staining

All incubations were carried out in a humidified chamber at room temperature, sections never being allowed to dry out. All reagents were made up fresh and stored on ice. Sections were taken from -80°C and 2ml blocking buffer (APBS + 1% BSA) was immediately added to the slide and incubated for 30 minutes. Blocking buffer was then drained from the slide and any excess dried with tissue taking care not to touch the section. One hundred and fifty microlitres of primary mouse monoclonal antibody (2B1, F17, D12.2 all used at 1:4, or AM22 used at 1:200 - for specificities of mAbs, see Table 1 in Chapter 2) was then added to the section and incubated for 30 minutes. Slides were then washed three times by addition of wash buffer for about one minute before draining the slide and adding fresh buffer. After the third wash excess buffer was carefully removed with tissue before 150µl biotinlylated anti-mouse IgG or IgM (1:134) Ab (Vector Labs) was added to the section. Slides were then washed three times with wash buffer before 1-2ml methanol/hydrogen peroxide solution (1:100 dilution of 30% H_2O_2 in methanol) was added to each slide (to remove endogenous peroxidase) and incubated for 20

minutes. Slides were then washed three times with wash buffer before 150µl ABC solution (Vector Labs - **A**vidin-**B**iotin-peroxidase **C**onjugate) was added to each slide and incubated for 30 minutes. After this time a further 150µl ABC solution was added to each slide to help enhance the peroxidase signal, and incubated for 30 minutes. Slides were again washed three times with wash buffer. Finally 150µl DAB (3'3'-diaminobenzidine + 1:10 dilution of 30% H_2O_2) substrate was added to each slide and incubated for 5-15 minutes - sufficient time to allow a brown precipitate to form. This reaction was stopped by washing slides three times with distilled water. Slides were then counter-stained for 15 minutes with methyl green. Following dehydration through alcohol and xylene, slides were mounted in histomount and viewed under a Nikon Optiphot microscope.

5.3 Results

5.3.1 Comparison of Lymphocyte Surface Antigens on Cells from the Gut (Intraepithelial and Lamina Propria Cells) and Spleen of Euthymic *Xenopus*

Single Colour Analysis

A comparison of single colour flow cytometric traces for spleen, intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) from 6-8 month old euthymic *Xenopus laevis* was carried out (see Figure 5.2). Staining for $CD5^+$ cells revealed similar levels in all 3 cell populations, 67%, 69% and 74% respectively in the representative experiment shown. However, the major difference between lymphocytes from the spleen and those from the gut was the intensity of staining. Both the IELs and LPLs showed a broader pattern of staining, with a mean fluorescence intensity of about 4-5 units, compared to splenocytes, where mean fluorescence intensity of CD5 was about 7-8 units. The percentages of IELs and LPLs expressing the CD8 epitope recognised by AM22 were consistently higher (46% and 43%) than the percentage of splenocytes (27%) expressing this determinant. However, mean fluorescence intensities of AM22 staining were significantly lower in the IEL (11 units) and LPL (6 units) compared with the spleen (20 units). In contrast to AM22, F17 staining identified a similar level of staining of this CD8 epitope amongst splenocytes, IELs and LPLs. $F17^+$ cells in the gut also stained with low mean fluorescence intensities compared with the $F17^+$ splenocyte population. Staining for D12.2 in the spleen reached levels of only 5-10%, whilst $D12.2^+$ IELs represented 25%, and LPLs represented 14% of the lymphocyte population found at these sites. Cells from all 3 tissues showed a very broad intensity staining pattern with D12.2, with the spleen showing the greatest mean fluorescence intensity. The level of $sIgM^+$ B cells found within the gut lymphocyte population was minimal, with just 5% $sIgM^+$ cells recorded in the IELs and 4% in the LPLs, compared to levels of 26% in the spleen.

Dual-Colour Analysis

Dual-colour flow cytometric analysis of CD5 and CD8 (AM22) expression confirmed that there were 2 major lymphocyte populations in all 3 locations, namely $CD5^+CD8^-$ and $CD5^+CD8^+$ (Figure 5.3). Both IELs and LPLs contained a high proportion (50% or more) of $CD5^+$ cells that co-stained with AM22 compared with

around 40% $CD8^+$ T cells in the spleen. Also evident in the IELs and LPLs, but not in the spleen, was a small population of $CD5^-CD8^+$ cells (7% and 8% respectively). Dual staining for CD5 and D12.2 confirmed that not only was the level of $D12.2^+$ cells increased in both the IELs and LPLs, but revealed that most $D12.2^+$ cells co-expressed $CD5^+$, ie. were T cells (Figure 5.3). However, distinct $D12.2^+$ $CD5^-$ lymphocyte populations were also found in the two gut locations.

Having shown that the IEL and LPL populations were similar to each other both in levels and intensity of surface staining, both populations were subsequently pooled and analysed as a single population of gut lymphocytes. This was necessary because of low numbers of lymphocytes recovered; by pooling the populations it enabled functional studies to be carried out alongside the flow cytometric studies.

5.3.2 Comparison of Lymphocyte Surface Antigens in Spleen, Gut and Liver of Control and Thymectomized *Xenopus*

5.3.2.1 4-6 Month Old Animals

A) Control Lymphocytes

Representative traces are shown in Figure 5.4, mean percentages and mean fluorescence intensities are shown in Tables 5.1 and 5.2. The data below shows percentages from a representative experiment, but mean percentages are given in square brackets for comparsion.

Flow cytometric analysis revealed that the level of $CD5^+$ cells in all organs were virtually identical (57% [57.7%], 56% [58%] and 56% [53%] for spleen, gut and liver respectively). Although the percentages of stained cells were similar, the mean fluorescence intensities differed. For spleen and liver an almost identical single peak was found with a mean intensity of 11-14 units. In contrast, the gut lymphocytes showed a broad range of intensities with a suggestion of two CD5 peaks, with a mean fluorescence intensity of 7-9 units.

Levels of $CD8^+$ cells detected by the mAb, AM22 were found to be around 27% [28%] for spleen, 32% [34.7%] in the liver and 35% [37.3%] in the intestine. Staining intensities with the mAb, AM22 ranged from 20 to 28 units for both the spleen and liver, whilst two peaks of AM22 staining were seen in the gut, the mean fluorescence intensity being only 11 units. Staining with the mAb, F17 also directed against the CD8 homologue, generally gave similar staining patterns to AM22, for all 3 lymphocyte sites. In these particular 4-6 month old animals, there was no

significant decrease in the percentage of $F17^+$ gut cells compared with $AM22^+$ gut cells - contrast the situation with that of 6-8 month old animals (see Figure 5.2).
$D4.3^+$ cells were comparable in both spleen and liver in terms of both percentages (53% [53.7%] for spleen and 51% [52.3%] for liver), and mean fluorescence intensities (6 units for spleen and 8 units for liver). The intestine on the other hand, routinely showed a lower level of $D4.3^+$ cells (21% in the example shown) with a mean fluorescence intensity of just 2.5 units. For both spleen and liver the level of $D4.3^+$ cells was only just below that of the total level of $CD5^+$ T cells, whereas there were many $CD5^+$ T cells in the gut that did not express D4.3. Dual-colour analysis (see Figure 5.5) with the mAbs, AM22 and D4.3 showed similar staining patterns in both the spleen and liver, indicating two major populations - those that are $AM22^+D4.3^+$ and those that are $AM22^-D4.3^+$. In the gut, as well as these two populations, a further population is seen that is $AM22^+D4.3^-$ (up to 26%) - this population is only minimal in the spleen or liver.
Staining with the mAb, D12.2 showed a very minor population of $D12.2^+$ cells in the spleen (3% [5%]) which increased to 10% [9.3%] in the liver. In contrast, levels of up to 40% [36.7%] $D12.2^+$ cells were found in the gut. The mean fluorescence intensity of staining was similar for all three populations at around 3-5 units.
Figure 5.7 compares the level of $sIgM^+$ B cells found in the spleen, liver and gut - the spleen shows the highest level of B cells (41% in control animals), with the proportion of B cells in liver approximately 50% of that found in the spleen (19%), whilst levels of B cells were consistently low in the gut - 3 to 9%.

B) Thymectomized Lymphocytes

Representative traces are shown in Figure 5.6.
In thymectomized animals the levels of $CD5^+$ cells were reduced to just 5% [6.3%] in the spleen and 12% [11%] in the liver. In contrast 42% [37.7%] $CD5^+$ dull cells were still found in the intestine. The mean fluorescence intensity of CD5 staining was very low (just 3 units) in all 3 organs after thymectomy (this compares to 11-14 units in control spleen and liver and 7-9 units in control gut).
Levels of $AM22^+$ ($CD8^+$) cells in spleen, liver and gut of Tx animals were reduced to 4% [4%], 9% [10%] and 27% [26.3%] respectively. Moreover, the mean fluorescence intensities of these populations were 3, 8 and 6 units (contrast this to 20-28 units for control spleen and liver, and 11 units for control gut).

Interestingly, $F17^+$ cells (ie. $CD8^+$ cells detected by the mAb, F17) were virtually absent in spleen, liver and gut of Tx frogs. Moreover, there were no detectable levels of $D4.3^+$ cells in spleen, gut or liver following thymectomy.

Levels of $D12.2^+$ cells were only 4% [6.3%] in the Tx spleen, whereas 15% [11.3%] liver and 32% [37.3%] intestinal lymphocytes were $D12.2^+$ following thymectomy. The mean fluorescence intensity of staining in the Tx spleen was just 3 units, ie. comparable to the level seen in control animals, but this was elevated in the Tx liver and intestine from 3 to 5 and 6 units respectively.
There are high levels of B cells in the Tx spleen (86%), up to 42% in the liver, and levels from 6 to 23% in the gut (see Figure 5.7).

5.3.2.2 8-12 Month Old Animals

A) Control Lymphocytes

Mean percentages and mean fluorescence intensities are shown in Tables 5.1 and 5.2. The data below shows percentages from a representative experiment, but mean percentages are given in square brackets for comparsion.
Figure 5.8 illustrates typical patterns of staining with the mAbs, 2B1, AM22, F17, D4.3 and D12.2 for spleen, liver and gut of 8-12 month old euthymic *Xenopus*. The proportion of $CD5^+$ cells is comparable in spleen and gut, with around 75% [70% and 75.3%] lymphocytes expressing this T cell marker. This percentage has increased from that found at 4-6 months; such an increase in T cells is less evident in the liver. Fluorescence intensities of $CD5^+$ lymphocytes are comparable in the 3 tissues studied (8-12 units). The mAb's AM22 and F17, both directed against the CD8 homologue showed similar levels of $CD8^+$ cells in the spleen and liver (38% [32.7%] AM22, 36% [33%] F17 [spleen] and 33% [32.7%] AM22 and 31% [31%] F17 [liver]). Staining intensities with the anti-CD8 mAbs were also comparable in spleen and liver. In contrast, gut lymphocytes stain less intensely with both the AM22 and F17 mAbs. Furthermore, while there were 42% $AM22^+$ gut lymphocytes in the example shown, only 27% gut cells were $F17^+$. This difference in the percentage of gut lymphocytes stained by these two mAbs is consistent in different animals of this age - see also Figure 2 for 6-8 month old animals. Although the actual levels of CD8 staining vary slightly in different individuals, there is always a greater proportion of gut lymphocytes that are stained with AM22 compared with F17.

Splenocytes and liver lymphocytes stained with the mAb D4.3 reveal populations of $D4.3^+$ cells almost equal to the total level of $CD5^+$ T cells (75% [67.7%] for spleen and 57% [56%] for liver). In contrast, in the gut there is only a small population of lymphocytes that stain with D4.3, whereas the total level of $CD5^+$ cells is around 75%. Percentages of $D4.3^+$ gut cells were not consistent between animals of this age, ranging from 0 to 20%.

Levels of $D12.2^+$ cells in both the spleen and liver were minimal (both around 4% [4.3% and 5.7%]), whilst in the gut levels of between 14% and 40% [27%] are seen. Dual staining studies with CD5 and D12.2 (see Figure 5.10) show that in the spleen and liver of control *Xenopus*, there is minimal dual staining, the major population being $CD5^+D12.2^-$ (76% for spleen and 58% for liver). However, in the gut the majority of $D12.2^+$ cells co-stain with CD5 (11%) whilst there is a small population of cells (4%) that are $D12.2^+$, $CD5^-$. Dual staining studies with CD8 (AM22) and D12.2 show a similar staining pattern with virtually no $AM22^+D12.2^+$ lymphocytes found in the spleen or liver, whilst a population of dual positives (6%) can be seen in the intestine (Figure 5.11).

B) Thymectomized Lymphocytes

Figure 5.9 illustrates that in animals thymectomized between 5-7 days of age that are approximately 12 months of age lymphocytes staining for CD5 and AM22 are readily evident. In these older animals, populations of cells that stain with the mAb 2B1 have emerged that were not evident in 4-6 month old Tx animals (contrast with Figure 5.6). Some 9% [6%] $CD5^+$ cells were found in the Tx spleen, but there were 31% [21.3%] in the liver and 42% [32%] in the gut. However, the mean staining intensity of 2B1 staining on these lymphocytes in Tx *Xenopus* was not as great as that found in age-matched controls (compare 5 units in Tx frogs with 8-12 units in controls).

Distinct staining of lymphocytes from all 3 organs was also noted with the mAb, AM22, 8% [4.7%] in the Tx spleen, and 26% [20% and 26.7%] in both liver and gut. Mean AM22 staining intensity is duller in Tx animals compared to controls (compare 3-9 units in Tx frogs with 13-24 units in controls).

Staining with the other anti-CD8 mAb, F17 reveals no positive cells in Tx spleen, liver or gut (levels of 2% equalling those of isotype-matched non-specific mAbs).

Similarly, there are no D4.3$^+$ cells detectable in spleen, liver or gut of these Tx animals.

Compared with 4-6 month old Tx *Xenopus*, levels of D12.2$^+$ cells are increased in year old Tx animals, with levels of 6% [9.7%] in the spleen, 27% [20%] in the liver and 26% [28.7%] in the gut. Dual staining studies with AM22 and D12.2 indicate that in Tx animals, most of the D12.2$^+$ cells were CD8$^+$ (Figure 5.11). This was also seen when lymphocytes were dual stained for CD5 and D12.2 where the majority of D12.2$^+$ lymphocytes co-stained with CD5 (Figure 5.10).

5.3.2.3 XTLA-1 Expression in Spleen, Gut and Liver

Expression of XTLA-1 was not followed extensively in this study, but the following findings are of note. In both spleen and liver, the percentage of XTLA-1$^+$ lymphocytes were comparable (about 28%) as shown in Figure 5.12. However, in the gut, the level of XTLA-1$^+$ lymphocytes reached 80-90% (levels this high have only ever previously seen in the thymus - see Chapter 2). Dual staining studies with the anti-CD8 mAb, F17, showed that only about 30% of gut cells were dual-labelled, leaving a population of XTLA-1$^+$CD8$^-$ cells of up to 50%. In spleen and liver this population was only 7% and 5% respectively.

5.3.3 Flow Cytometric Analysis of Larval Spleen and Liver

Larval (5 week old) lymphocytes from the spleen and liver of control *Xenopus* were examined to determine if there were major differences in lymphocyte populations compared to the adult. Gut lymphocytes were not examined since the larval gut contains a dearth of lymphocytes (J D Horton, unpublished). The spleens and livers of eight 5-week old larvae were pooled and their lymphocytes extracted by the methods given in 5.2.2 and 5.2.4.

Figure 5.13 illustrates single colour analysis with the mAbs, 2B1, AM22, D4.3 and D12.2. The level of staining with the mAbs on larval splenocytes was as expected from the work carried out in Chapter 2. The staining of larval liver lymphocytes revealed significantly lower levels of CD5$^+$, CD8$^+$ and D4.3$^+$ lymphocytes compared with spleen.

5.3.4 Immunohistochemical Studies on Intestine of One Year Old Control and Thymectomized *Xenopus*

In order to provide histological support for the flow cytometric findings on T cell development in the gut, sections of intestine from year old control and Tx *Xenopus*

were examined for the presence of $CD5^+$, $CD8^+$ (using AM22) and $D12.2^+$ lymphocytes. The findings are shown as representative sections in Figure 5.14.

$CD5^+$ lymphocytes frequent the intestinal epithelium, lying just above the basement membrane. They are also found in larger numbers scattered in the lamina propria, and large nodules of $CD5^+$ lymphocytes are also found in this region of the gut (Figure 5.14A,C). $CD5^+$ lymphocytes were drastically reduced in number following thymectomy, with only a scattering of $CD5^{dull}$ cells now being seen within the gut epithelium (Figure 5.14D). There were no $CD5^+$ lymphocytes seen in the lamina propria of these Tx animals.

$AM22^+$ lymphocytes were also found in plentiful numbers in the intestine of control animals, both in the intestinal epithelium (Figure 5.14E) and in the lamina propria and nodular lymphocyte collections (data not shown). Following thymectomy, distinct $AM22^+$ cells were no longer seen, but there were some diffuse staining seen both in the epithelium and lamina propria (Figure 5.14F).

Compared with controls, there were slightly greater numbers of $D12.2^+$ lymphocytes in the intestine of Tx animals (compare Figure 5.14 G and H). These $D12.2^+$ cells appeared to be restricted to the gut epithelium.

5.3.5 Proliferative Assays Using Lymphocytes from Control and Thymectomized One Year Old *Xenopus*

See Figure 5.15

Proliferative assays were carried out on lymphocytes from control and thymectomized animals aged 8-12 months. Responses to ConA stimulation in control animals showed stimulation indices from 4 to 66 for gut, from 4 to 8 in the liver, compared to SI's from 23 to 72 in the spleen. This contrasted to ConA stimulation indices in thymectomized animals, which were less than 1 for all 3 organs. Stimulation with PMA gave indices from 22 to 120 in the gut, and up to 5 in the liver, compared to SI's from 67 to 113 in the spleen. In thymectomized animals SI's of up to 2.4 (gut), 22 (liver), and from 7 to 18 (spleen) were obtained.

It can be seen that although in these older Tx animals there are lymphocytes in all 3 organs that are $CD5^{dull}$ and CD8 $(AM22)^{dull}$, there appears to be no response to the T cell mitogen, ConA. However, these lymphocytes can respond to a non-T cell-specific mitogen such as PMA.

5.3 Discussion

This Chapter has explored the possibility of an extrathymic pathway of T cell development in *Xenopus*. In higher vertebrates there is considerable debate as to whether or not extrathymic T cells can develop in the gut and liver of both euthymic and athymic animals (see Introduction 5.1). These organs were therefore explored for signs of an extrathymic pathway of T cell development in *Xenopus*. The work presented here is the first to probe whether extrathymic T cell development is a major pathway of lymphopoeisis in an ectothermic vertebrate.

One interesting feature emerging from this study was the similarity between the staining patterns and intensities of mAb staining in the spleen and liver of euthymic *Xenopus*. This was true for both 4-6 month and 8-12 month old animals, with no significant differences in either the proportion of stained lymphocytes, or the mean fluorescence intensity of staining. However, a different pattern of mAb staining emerged in the gut, first identified in the younger animals. In particular, the intensity of staining with several mAbs was consistently lower. Indeed, the lymphocytes from the gut differed from those found in the spleen and liver in several ways. Firstly, in the spleen and liver there appeared to be a correlation between $D4.3^+$ and $CD5^+$ populations, there being only a low percentage of $CD5^+D4.3^-$ lymphocytes. In contrast, in the gut there were many $CD5^+D4.3^-$ cells noticeable at 4-6 months of age, and levels of $D4.3^+$ cells varying between animals.

Secondly, in the gut (but not in spleen and liver), a difference in the percentage of $CD8^+$ lymphocytes was revealed following staining with the anti-CD8 mAbs, AM22 and F17. This difference was only seen in older animals (8 months and above) when there was usually 10-20% more $CD8^+$ lymphocytes detected by the mAb AM22 compared with $F17^+$ cells. In younger animals, AM22 and F17 stained similar levels of gut lymphocytes. Almost two thirds of lymphocytes found in the gut, even in 4-6 month old animals, lack coexpression of D4.3, whereas a large majority of splenic and hepatic $AM22^+$ lymphocytes were also $D4.3^+$. It has been well documented that in the gut of higher vertebrates there exists a form of CD8 that is particular to the intestinal epithelium. As well as the normal CD8 molecule consisting of an α and β chain, there is also a form of CD8 consisting of 2 α chains, ie. $CD8\alpha\alpha$ (Guy-Grand et al, 1991). This $CD8\alpha\alpha$ molecule is thought by many

workers to arise extrathymically in both euthymic animals (Rocha et al, 1992) as well as in those animals lacking T cells (Lin et al, 1994). It is possible that the mAb, AM22 is able to recognise an epitope on the α chain of the *Xenopus* CD8 molecule, whilst F17 detects a different epitope not associated with the α chain. This might account for the extra level of $CD8^+$ staining seen with AM22 in the gut, which is shown here to increase with ageing - a characteristic of extrathymically derived gut lymphocytes. This point is returned to below in the section dealing with thymectomized animals.

From the studies carried out in Chapter 2, levels of $D12.2^+$ lymphocytes in the spleen of euthymic *Xenopus* were consistently low (less than 5%). It was therefore interesting to find that the liver and in particular the gut, housed larger populations of $D12.2^+$ lymphocytes. The data suggests that D12.2 marks a major subset of gut lymphocytes. In the main, $D12.2^+$ cells in the gut coexpress CD5 and CD8. In mammals, the gut houses a large collection of γδ TCR^+ lymphocytes (Viney and McDonald, 1990). Whether these gut γδ TCR^+ lymphocytes are thymus-derived remains uncertain. It appears that early in life, most γδ TCR^+ IELs in both mammals and birds do arise from the thymus (Pardoll et al, 1988; Lin et al, 1994; Kasahara et al, 1993). However, studies based on Vγ segment usage suggests that the Vγ families found in the gut are rare in thymus (Zorbas and Scollay, 1993) or may leave the thymus very early and undergo differentiation at the intestinal epithelium (Lin et al, 1994). One point that seems to be agreed upon is the fact that in later life γδ TCR^+ can and do develop extrathymically, as shown in studies using nude or thymectomized mice (Pardoll et al, 1988; Lin et al, 1994; Lefrançois, 1991; Umesaki et al, 1993). Ibrahim et al (1991) implied that the mAb, D12.2 may be directed against the *Xenopus* γδ TCR based on initial studies in characterising its molecular weight and tissue distribution. The finding here of large numbers of $D12.2^+$ lymphocytes in the *Xenopus* gut is consistent with this view. The fact that a general increase in $D12.2^+$ cells is not seen in older *Xenopus* may simply be an effect of dilution of $D12.2^+$ cells by the expansion of other $CD5^+$ T cell subsets.

One of the major goals of this Chapter was to probe for the presence of T cell markers in thymectomized animals. In young Tx animals (aged 4-6 months) there was a distinct lack of staining with the mAbs, D4.3 and F17 in the spleen, liver and gut. These two T cell markers were also absent in older animals (aged 8-12 months). In contrast, $2B1^{dull}$ and $AM22^{dull}$ lymphocytes were found in the spleen

and liver of young Tx froglets, and the levels of these increased slightly as the animals aged. By about a year of age, the percentage of $2B1^{dull}$ lymphocytes in the spleen was about 10%, whilst percentages in the liver were steadily rising, reaching about 30% at 12 months. A similar ontogenetic pattern was found for $AM22^{dull}$ lymphocytes. The gut of young Tx animals showed high levels of $2B1^{dull}$ and $AM22^{dull}$ lymphocytes (around 40% and 28% respectively), which did not increase with age. The lack of gut lymphocytes expressing F17 indicate that the CD8 molecule in Tx animals is not recognised by this mAb, fitting with earlier comments about the possible thymus-independence of $AM22^{+}F17^{-}$ gut lymphocytes.

The percentages of $D12.2^{+}$ lymphocytes in the spleen and liver of Tx animals was increased compared to age-matched euthymic 8-12 month old *Xenopus*. Levels of $D12.2^{+}$ lymphocytes in the gut of Tx animals remained at the high levels found in euthymic *Xenopus*. Also highlighted in Tx animals was the fact that most $D12.2^{+}$ lymphocytes are also $CD5^{dull}$.

Rather than being a marker for the γδ TCR, it has recently been suggested that $D12.2^{+}$ cells might represent natural killer (NK) cells. Thus $D12.2^{+}$ cells sorted by FACS and stained with Wright-Giemsa contained many lymphoid cells displaying cytoplasmic granules reminiscent of NK cells (Horton, personal communication). Natural killer cell activity in the murine intestine has been demonstrated (Tagliabue et al, 1982; Petit et al, 1985), these cells having cytoplasmic granules and showing spontaneous cytotoxic activity. These intestinal NK cells appear to share some characteristics to murine splenic NK cells, but are a distinct cell type (Tagliabue et al, 1982). Current work at Durham probing NK cell activity in *Xenopus* has indicated that NK-like activity against MHC-deficient allogeneic thymus tumour cells (Robert et al, 1994) can be shown in lymphocytes from the spleen, liver and gut of Tx animals (Horton et al, 1996 in press). Splenic NK-like activity has also been demonstrated in euthymic *Xenopus*, but only following tumour cell injection or following *in vitro* culture of splenocyte effector cells. It may not be surprising to find a higher level of NK-like activity in the Tx animals, as they lack conventional T cells and may therefore rely heavily on NK-like cells for their immune defence. To date there has been no evidence to link this NK-like activity in *Xenopus* with $D12.2^{+}$ cells, but such studies using D12.2 enriched cells are in progress.

A lack of functional T cells in Tx *Xenopus* is indicated by experiments showing the absence of *in vitro* stimulation of lymphocytes from spleen, liver and gut with ConA. This lack of response to this T cell mitogen still occurred in the older Tx animals, when expression of CD5 and CD8 (AM22) was readily evident in spleen, liver and gut. A proliferative response by lymphocytes from these organs to the mitogen, PMA still occurred in Tx frogs, attesting to the viability of cells in these animals. This rather variable response by gut lymphocytes to mitogenic stimulation demonstrated here may not be that surprising in view of the fact that studies have shown that murine gut lymphocytes respond very poorly to stimulation with ConA and also PMA (Mowat et al, 1989; Sydora et al, 1993).

This is the first time that lymphocytes from the liver and intestine of *Xenopus* have been studied in any depth immunologically. The findings indicate that the lymphocytes in the liver bear a remarkable resemblance in terms of cell surface phenotype to those from the spleen. In contrast the intestine houses a large collection of $CD5^{+}$ lymphocytes that have a complex phenotype that is specific to this tissue. Studies on thymectomized *Xenopus* revealed loss of T cell markers in 4-6 month old frogs, but with ageing, expression of $CD5^{dull}$ and $CD8^{dull}$ ($AM22^{dull}$) began to emerge in the spleen, and more specifically in liver and gut. Whether these cells can be defined and classified as extrathymic T cells cannot be confirmed. Although these Tx lymphocytes express $CD5^{dull}$ and $CD8^{dull}$, other T cell markers such as D4.3 and the CD8 epitope labelled by F17 are not seen. Furthermore, these T-like cells do not respond to conventional T cell mitogens. Whether these T-like cells (as judged by flow cytometry) are true T cells remains doubtful. Studies probing TCR molecules are required to determine whether T cells developing extrathymically can be identified in Tx *Xenopus*. Initial studies do indicate that full-length rearranged TCR β transcripts are found (at very low levels compared to euthymic *Xenopus*) amongst intestinal lymphocytes from year old Tx animals, but are absent in most Tx splenocytes (Horton and Cooper, manuscript in preparation).
If extrathymic T cell development exists in *Xenopus*, the findings presented here and above indicate that this is not a major pathway. Normal T cell lymphopoeisis in *Xenopus* undoubtedly requires the presence of an intact thymus.

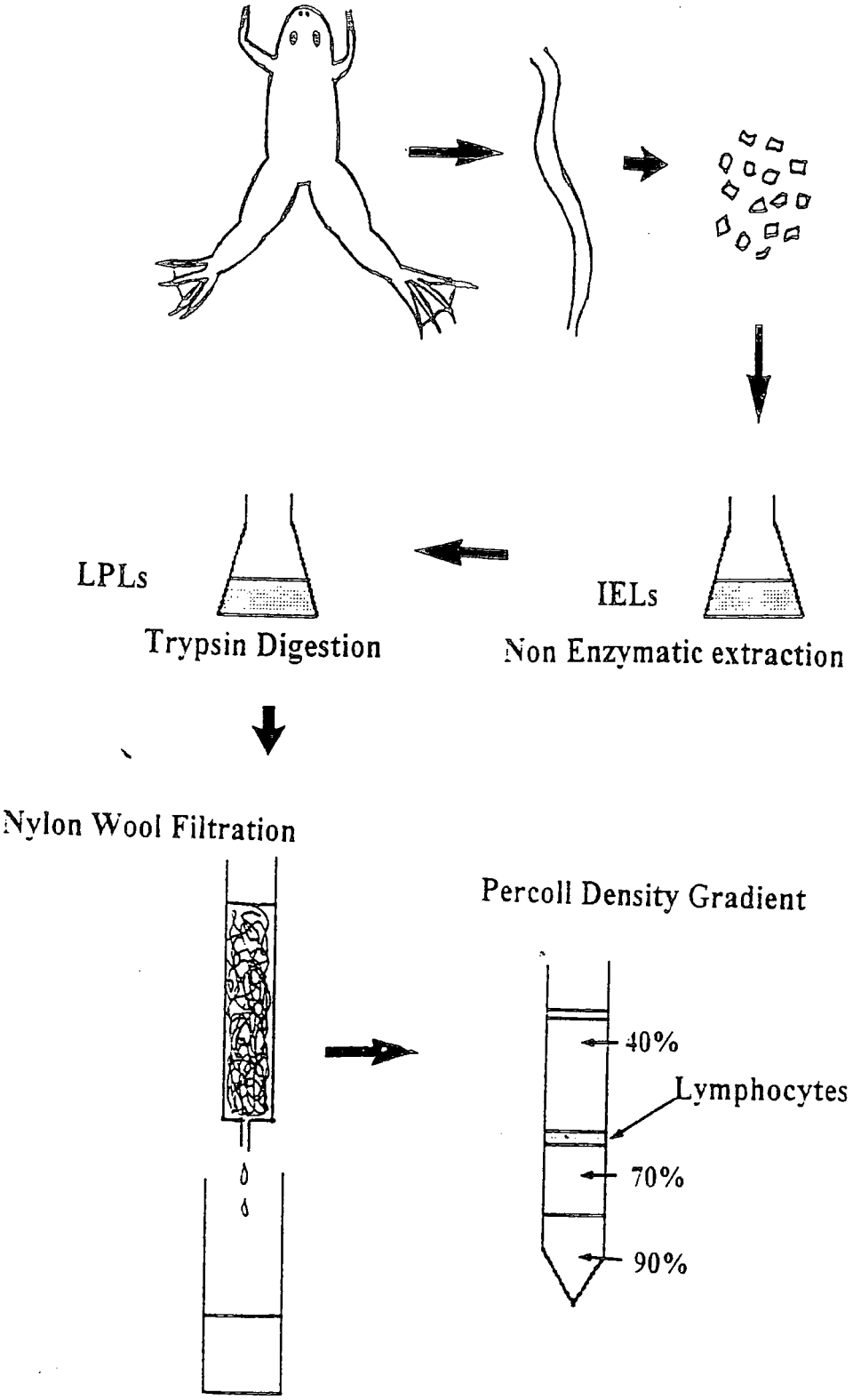


Figure 5.1 Diagramatic scheme of the intestinal lymphocyte extraction procedure. Briefly, the intestine was dissected out and flushed through with medium before being cut into small (0.5mm) pieces. Intraepithelial lymphocytes were extracted by non-enzymatic disruption on a magnetic stirrer, and once these had been extracted, the lamina propria lymphocytes were then extracted by trypsin digestion. Lymphocytes were filtered over a nylon wool column to remove large epithelial cells, and the eluate was layered onto a Percoll density gradient to enrich for lymphocytes.

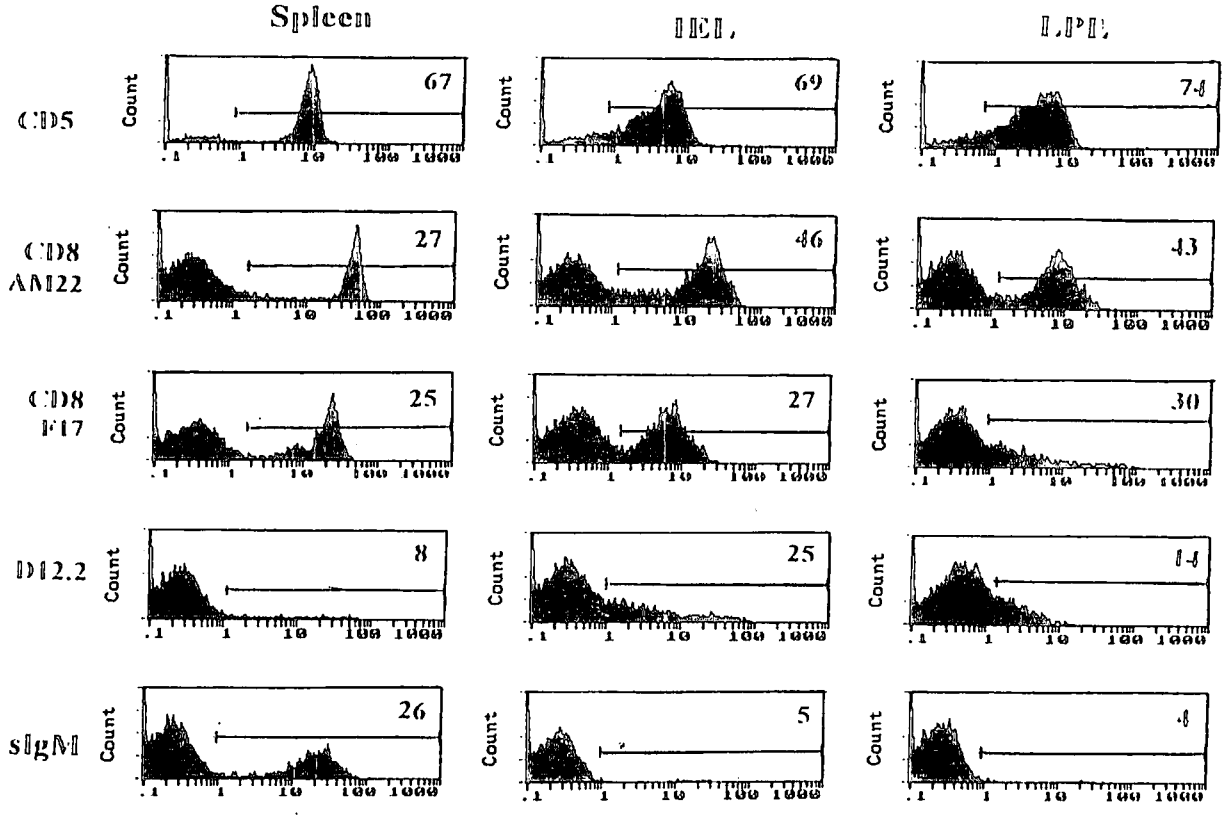


Figure 5.2 Single-colour flow cytometric analysis of splenic lymphocytes, intraepithelial lymphocytes and lamina propria lymphocytes, stained with the mAbs, 2B1 (anti-CD5), AM22 and F17 (anti-CD8), D12.2 (specificity unknown) and 8E4:57 (anti-IgM).
Traces are representative of 3 separate experiments on 6-8 month old control *Xenopus*. Animals shown in this figure were aged 8 months.

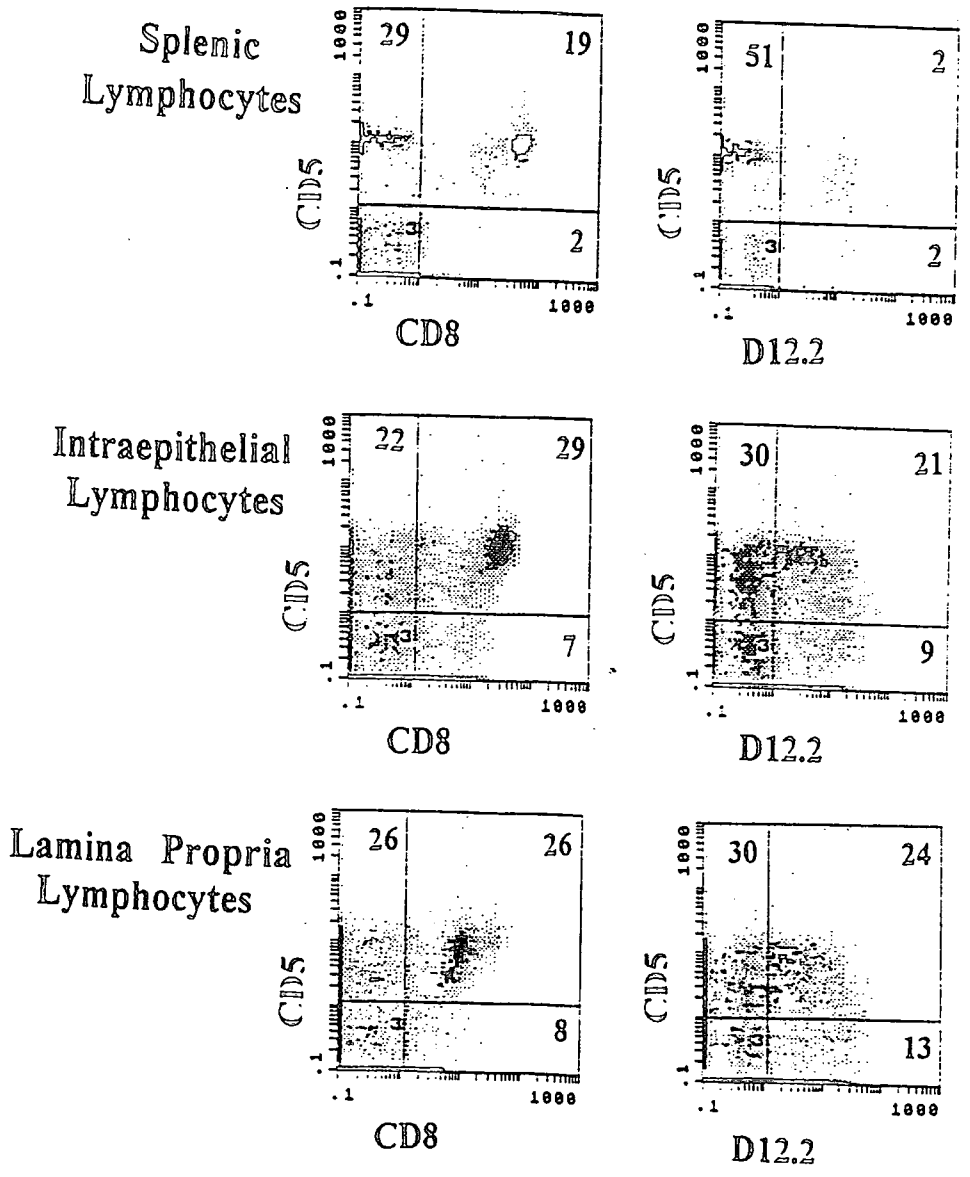


Figure 5.3 Dual-colour flow cytometric analysis of splenic lymphocytes, IELs and LPLs. Cells were dual-labelled with either AM22 and 2B1 or with D12.2 and 2B1. Representative of 3 separate experiments. Animals were control *Xenopus* aged 8 months.

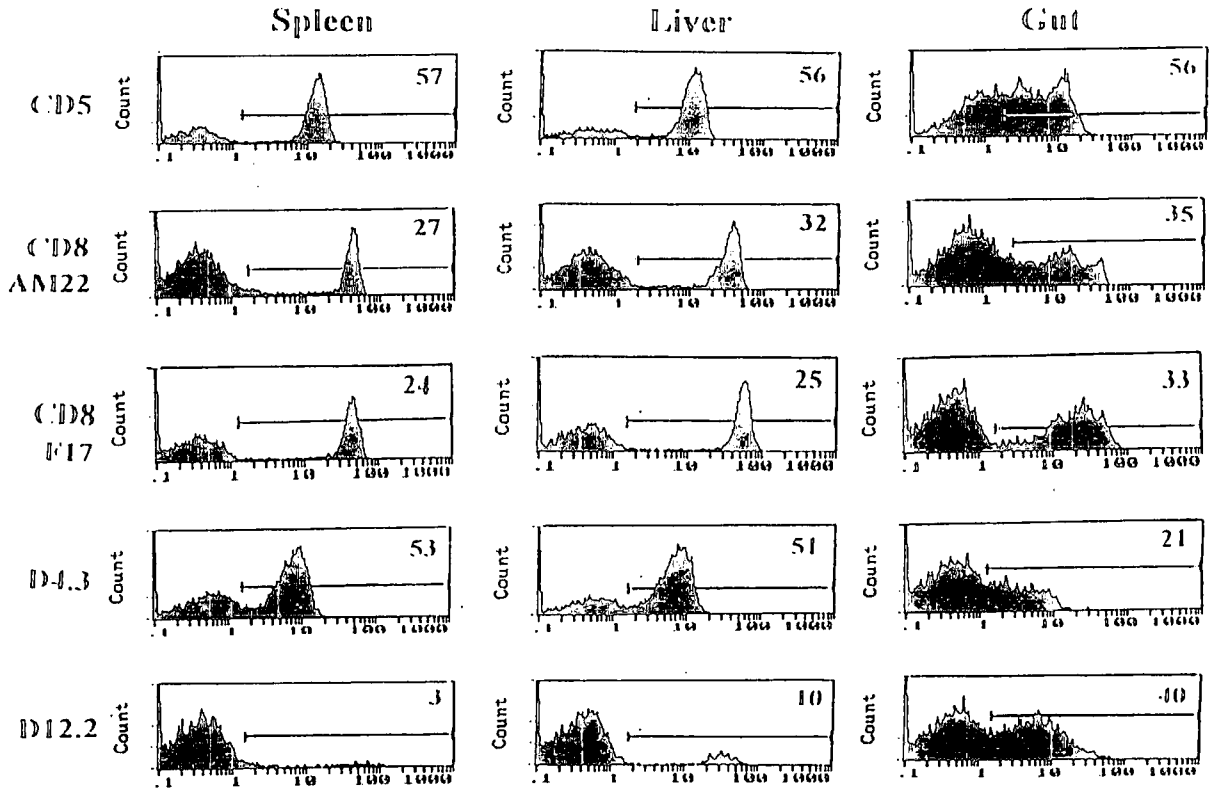


Figure 5.4 Single-colour flow cytometric analysis of lymphocytes from the spleen, gut (pooled IELs and LPLs) and liver of control *Xenopus* aged 5 months. Cells were labelled with the mAbs, 2B1, AM22, F17, D4.3 and D12.2.
Traces representative of 3 separate experiments of animals aged 4-6 months.

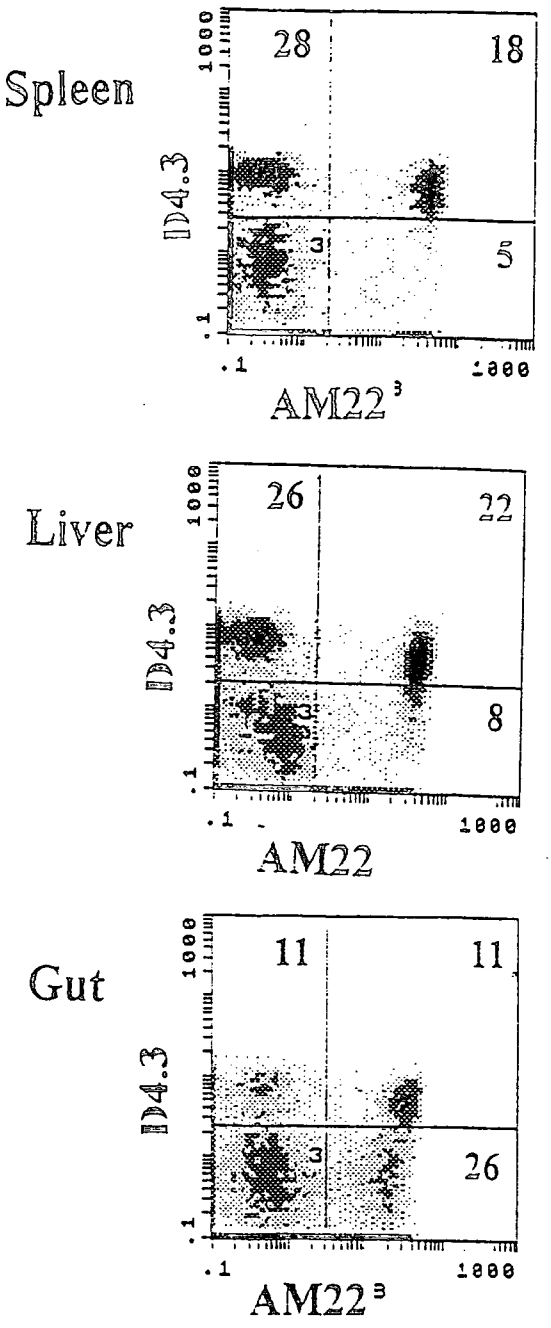


Figure 5.5 Dual-colour flow cytometric analysis of lymphocytes from the spleen, gut and liver of control *Xenopus* aged 5 months. Cells were dual-labelled with AM22 and D4.3, highlighting the presence of a population of cells that are $AM22^{+}D4.3^{-}$ in the gut which is not present in either the spleen or liver.
Traces representative of 3 separate experiments of animals aged 4-6 months.

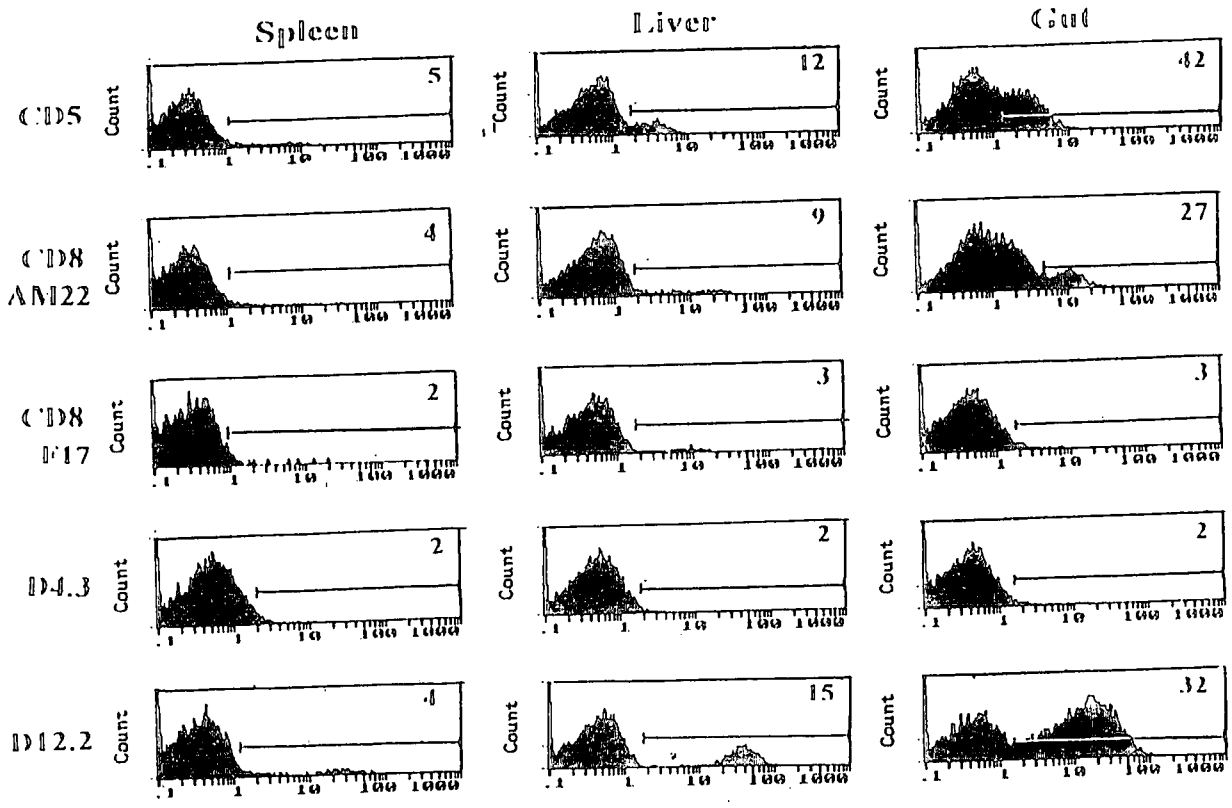


Figure 5.6 Single-colour flow cytometric analysis of lymphocytes from the spleen, gut (pooled IELs and LPLs) and liver of Tx *Xenopus* aged 5 months. Cells were labelled with the mAbs, 2B1, AM22, F17, D4.3 and D12.2.
Traces representative of 3 separate experiments of animals aged 4-6 months.

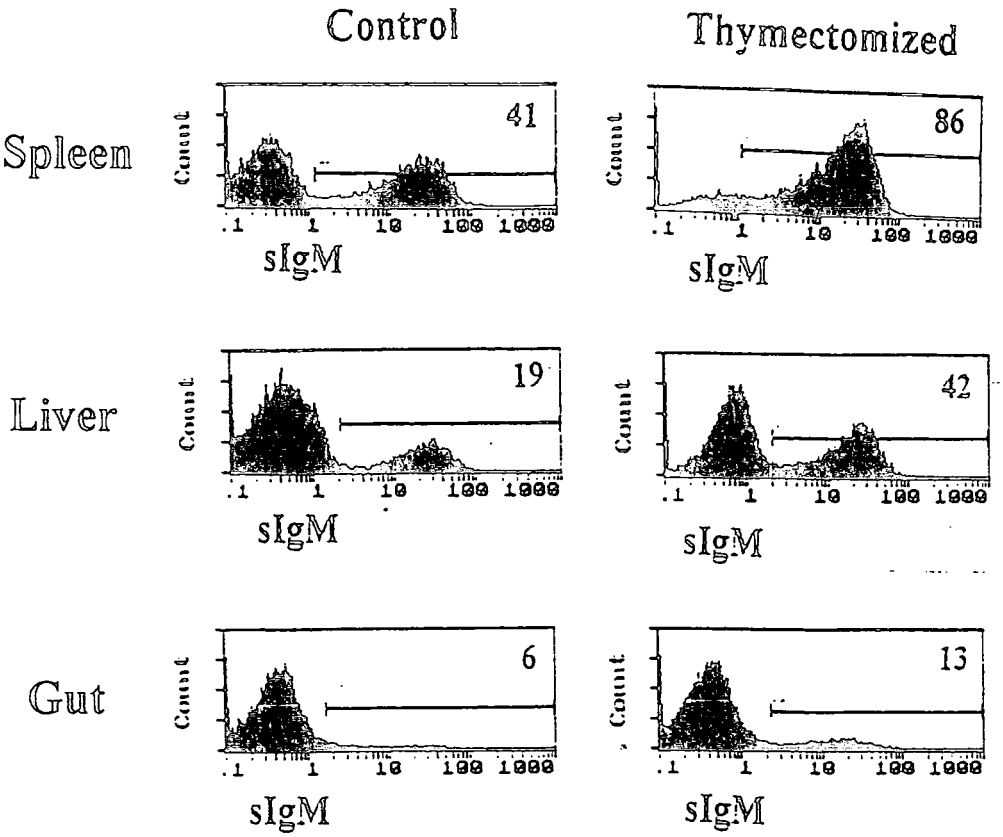


Figure 5.7 Single-colour flow cytometric analysis of lymphocytes from spleen, gut and liver of control and Tx *Xenopus* aged 5 months stained with the mAb, 8E4:57.

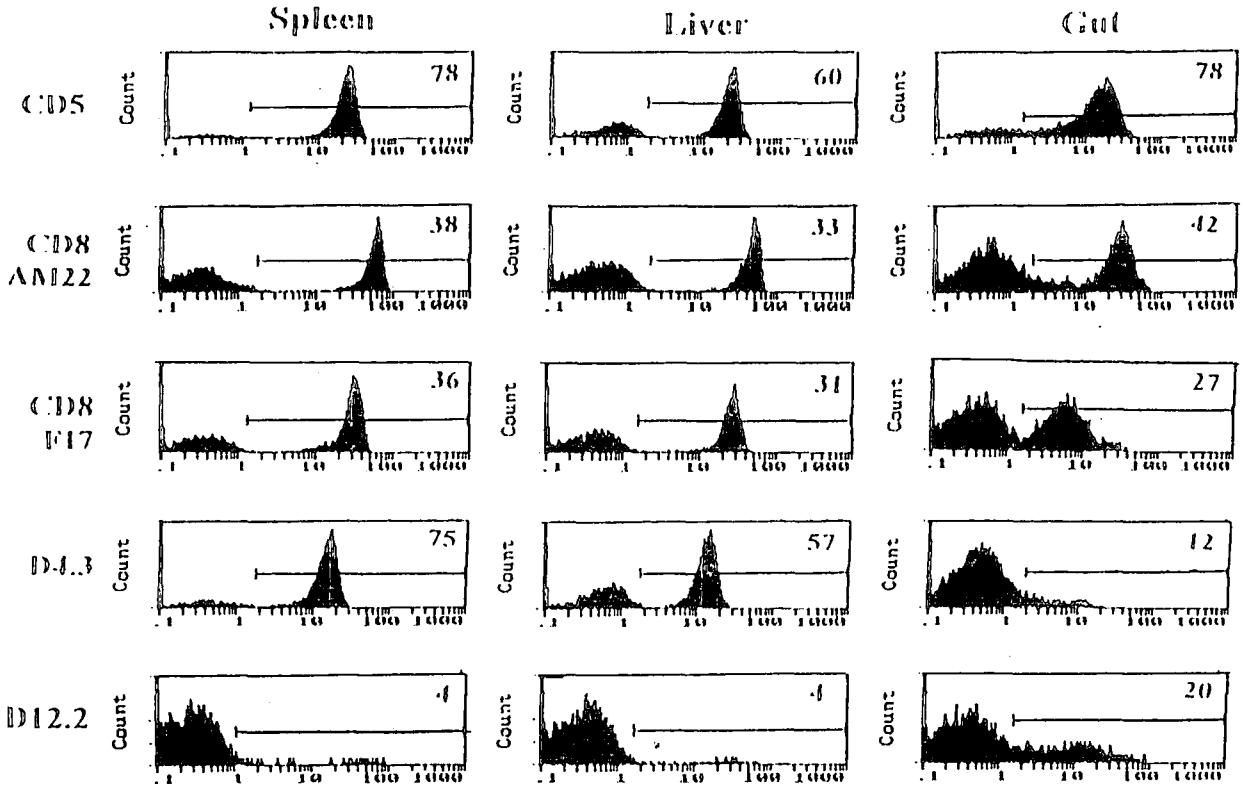


Figure 5.8 Single-colour flow cytometric analysis of lymphocytes from the spleen, gut (both IELs and LPLs) and liver of control *Xenopus* aged 11 months. Cells were labelled with the mAbs, 2B1, AM22, F17, D4.3 and D12.2.
Traces representative of 6 separate experiments of animals aged 8-12 months.

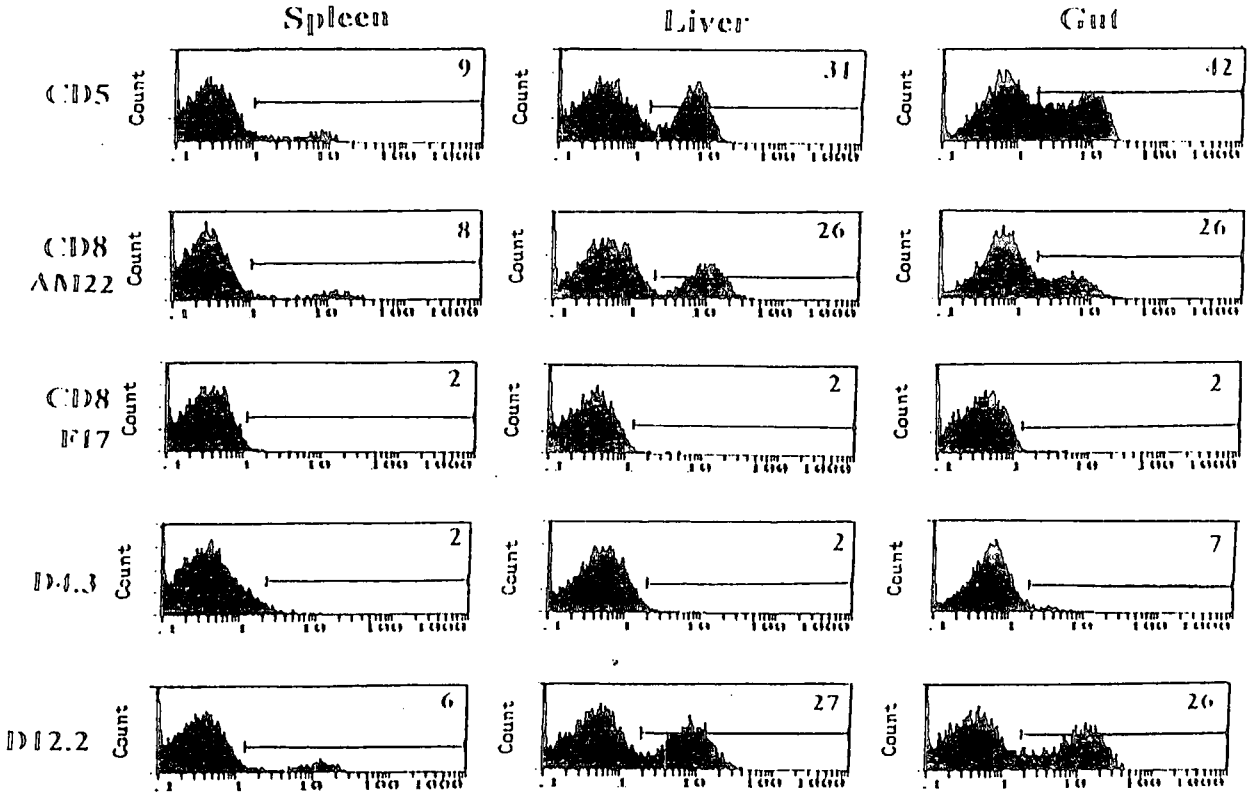


Figure 5.9 Single-colour flow cytometric analysis of lymphocytes from the spleen, gut (both IELs and LPLs) and liver of Tx *Xenopus* aged 11 months. Cells were labelled with the mAbs, 2B1, AM22, F17, D4.3 and D12.2.
Traces representative of 6 separate experiments of animals aged 8-12 months.

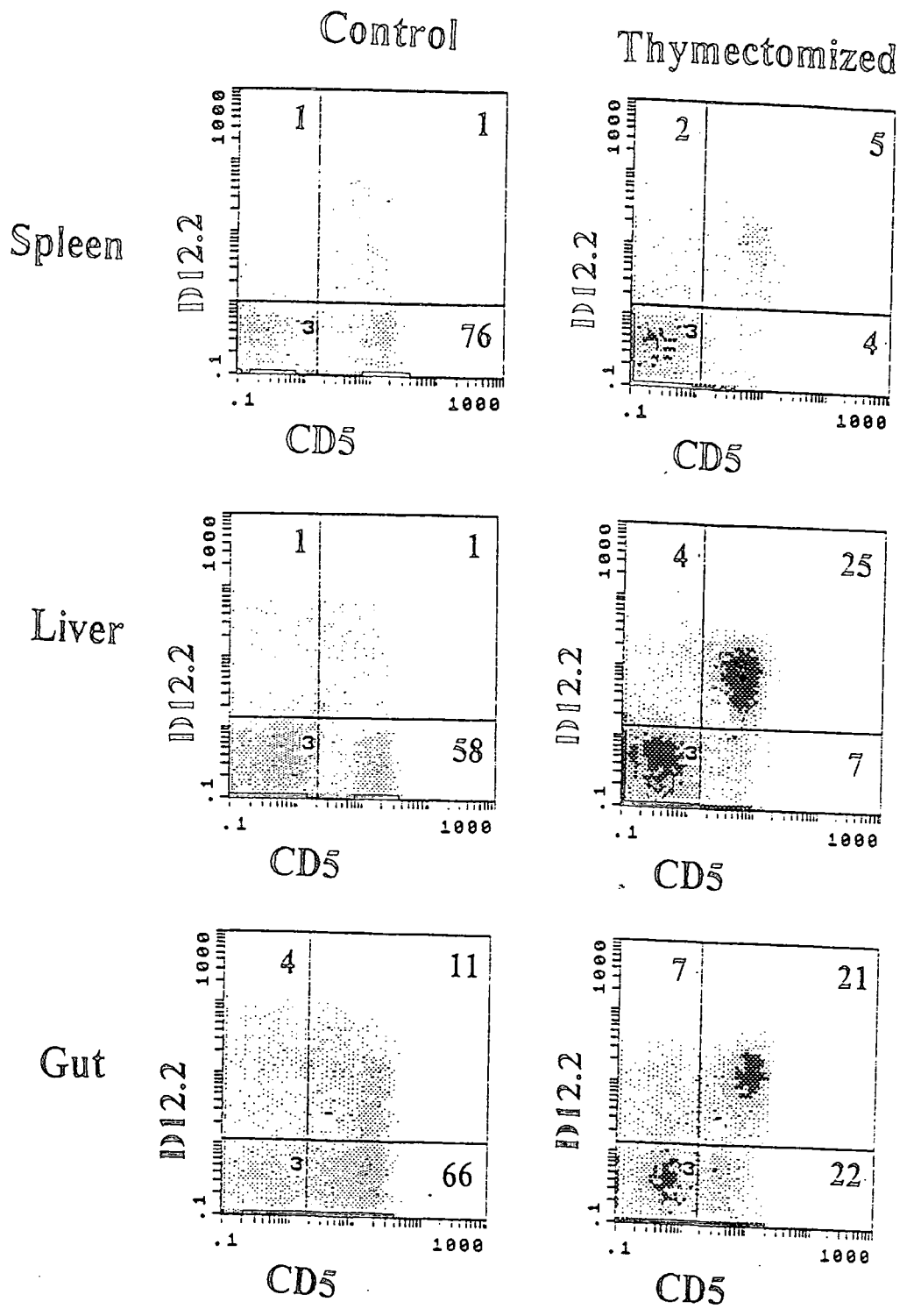


Figure 5.10 Dual-colour flow cytometric analysis of lymphocytes from the spleen, gut (IELs and LPLs) and liver of control and Tx *Xenopus* aged 11 months. Cells were dual-labelled with the mAbs 2B1 and D12.2 to highlight the minimal dual-staining population in control animals, but that this is a major population in Tx animals. Traces representative of 6 separate experiments of animals aged 8-12 months.

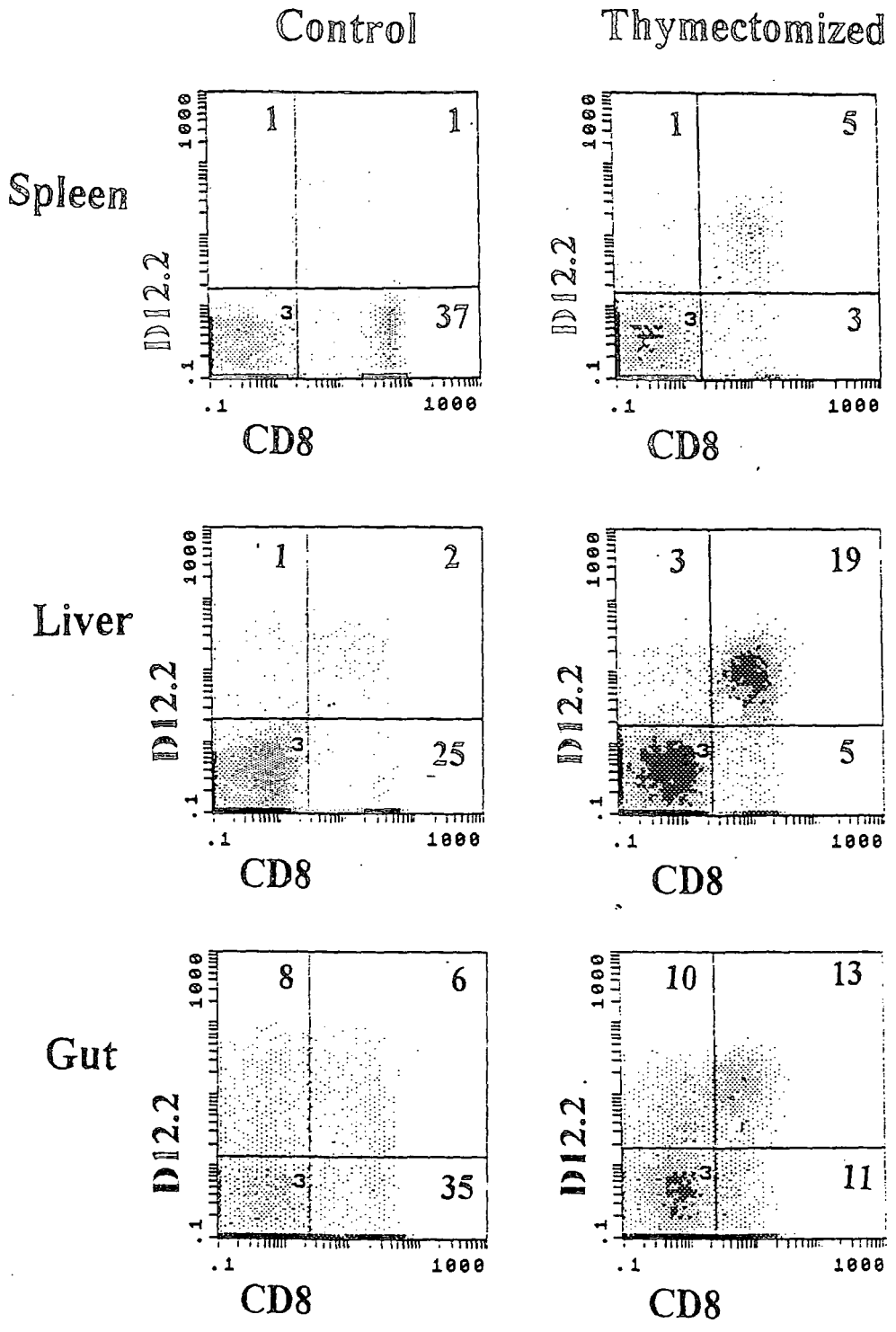


Figure 5.11 Dual-colour flow cytometric analysis of lymphocytes from the spleen, gut (IELs and LPLs) and liver of control and Tx *Xenopus* aged 11 months.
Cells were dual-labelled with the mAbs AM22 and D12.2 to highlight the minimal dual-staining population in control animals, but that this is a major population in Tx animals.
Traces representative of 6 separate experiments of animals aged 8-12 months.

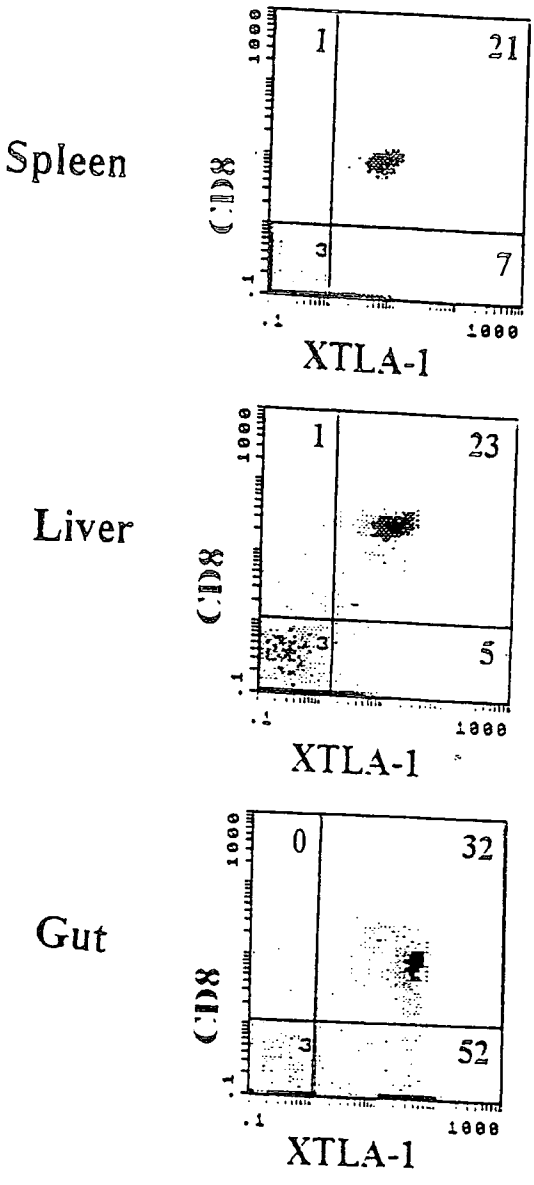


Figure 5.12 Dual-colour flow cytometric analysis of lymphocytes from spleen. gut and liver of control *Xenopus* aged 8 months. Cells were dual-stained with XT-1 and F17.
Traces representative of 3 separate experiments.

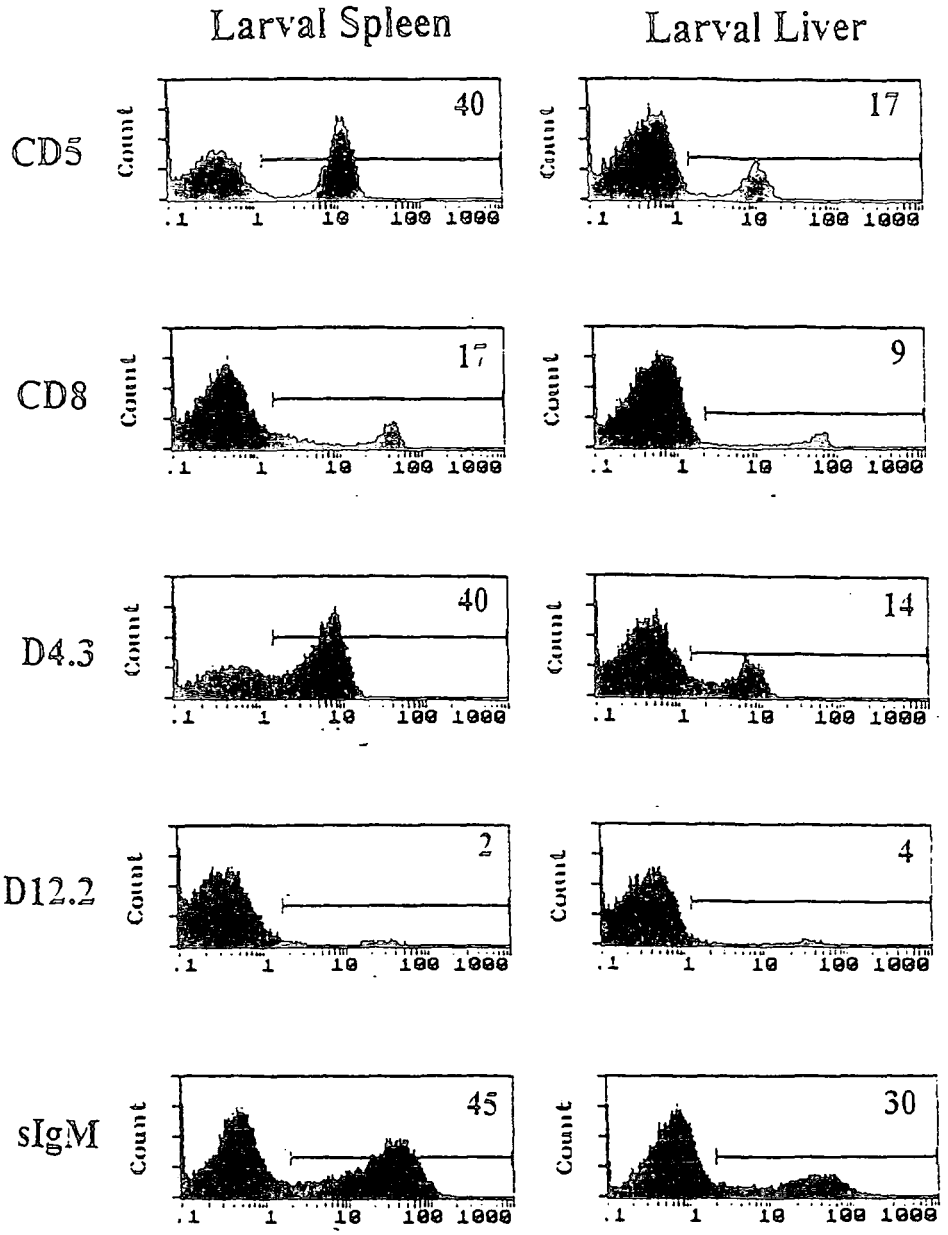
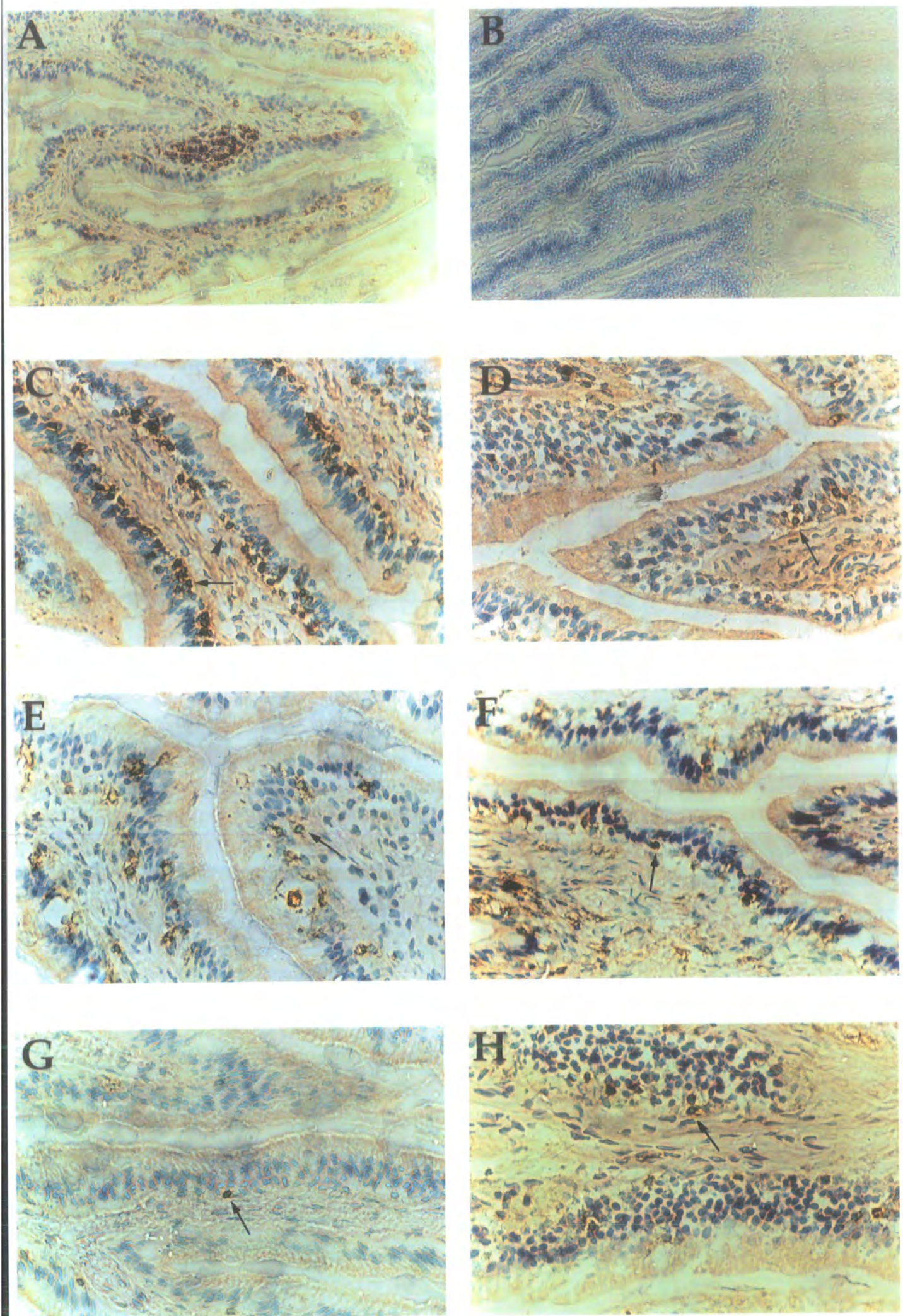


Figure 5.13 Single-colour flow cytometric analysis of lymphocytes from the spleen and liver of larvae aged 5 weeks. Cells were labelled with the mAbs, 2B1, AM22, D4.3, D12.2 and 8E4:57.
Organs from eight larvae were pooled to obtain this data.

Figure 5.14 Cryostat sections of control (A, B, C, E and G) and Tx (D, F and H) intestine (6μm) stained with 2B1 (anti-CD5) (A, C and D), with AM22 (anti-CD8) (E and F), and with D12.2 (specificity unknown) (G and H) using the immunoperoxidase technique and counter stained with methyl green. B shows a section of intestine stained with methyl green to show the structure of the gut. Magnification x1250


A
B
C
D
E
F
G
H

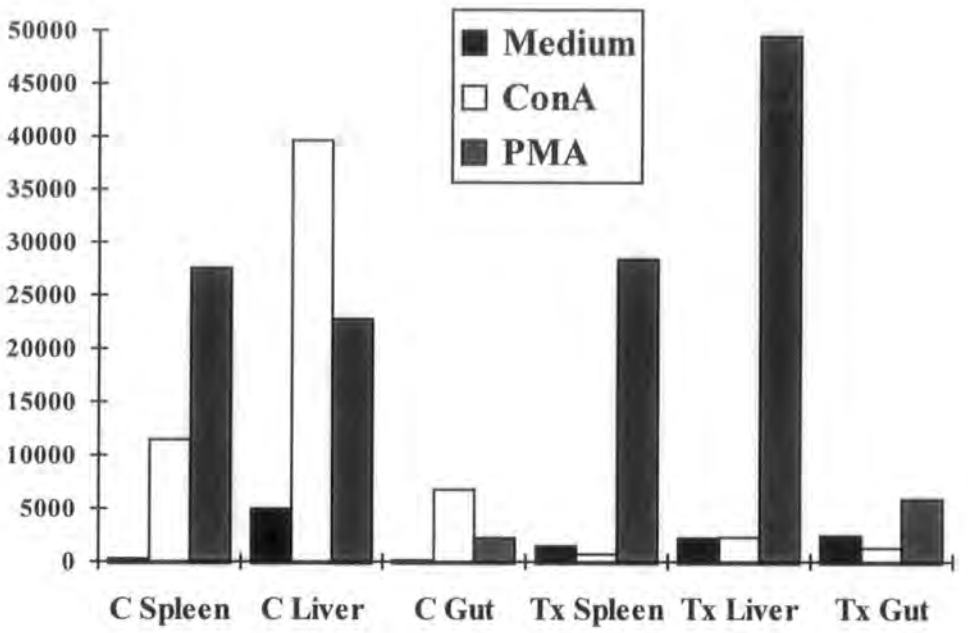


Figure 5.15 Graph showing mean DPM values for lymphocytes from spleen, liver and gut of control and Tx animals aged 11 months, stimulated *in vitro* with either ConA or PMA. Lyphocytes were cultured for 48 hours before receiving a pulse of ^{3}H-TdR 18-20 hours before harvesting and carrying out liquid scintillation counting. Data shown is from one experiment.

A 4-6 Months

Animal	Organ	Mean Percentage of Stained Cells				
		2B1	AM22	F17	D4.3	D12.2
Control	Spleen	57.7±2.1	28±1.7	23.7±0.6	53.7±4	5±1.7
	Liver	58±2.6	34.7±2.5	28±3	52.3±7.1	9.3±4
	Gut	53±12.8	37.3±8.7	32.3±4	19.7±4.2	36.7±7.6
Tx	Spleen	6.3±2.3	4±0	2.3±0.6	2.3±0.6	6.3±2.1
	Liver	11±1	10±1	3.3±1.5	2.7±1.2	11.3±3.5
	Gut	37.7±5.9	26.3±23	7.7±6.4	2.7±1.2	37.3±4.7

B 8-12 Months

Animal	Organ	Mean Percentage of Stained Cells				
		2B1	AM22	F17	D4.3	D12.2
Control	Spleen	70±13	32.7±9.2	33±7	67.7±12.7	4.3±1.5
	Liver	59±2.6	31±3.5	30.3±3.1	56±2.6	5.7±2.9
	Gut	75.3±2.5	48.7±8.3	34.7±8	17.3±11	27±12.1
Tx	Spleen	6±2.6	4.7±2.9	2±0	2±0	9.7±4.7
	Liver	21.3±10.6	20±7.9	3.7±1.5	5.3±4.2	20±8.2
	Gut	32±10	26.7±7	5.3±4.9	4±2.6	28.7±3.8

Table 5.1 Table showing mean percentage (± SD) of mAb staining with 2B1 (anti-CD5), AM22 and F17 (anti-CD8), D4.3 and D12.2 (specificity unknown). **A** - animals aged 4-6 months where n=3 for spleen, liver and gut of control and Tx; **B** - animals aged 8-12 months where n=3 for spleen, liver and gut of control and Tx.

A 4-6 Months

Animal	Organ	Mean Fluorescence Intensity of Staining				
		2B1	AM22	F17	D4.3	D12.2
Control	Spleen	13±1.4	22±4.2	24±11.3	6.5±2.1	3±0
	Liver	11±2.8	20±8.9	28±1.4	8.5±2.1	8.5±3.5
	Gut	9.3±0.6	11±2.8	12.6±0.6	2.5±0.7	3.5±0.7
Tx	Spleen	3±0	3±0	1.5±0.7	3±0	3.5±0.7
	Liver	2.5±0.7	8.5±0.7	2.5±0.7	2±1.4	4.5±0.7
	Gut	5±3.1	6±3.7	3.25±0.9	2±0	5.8±1.6

B 8-12 Months

Animal	Organ	Mean Fluorescence Intensity of Staining				
		2B1	AM22	F17	D4.3	D12.2
Control	Spleen	12.3±2.1	22.25±2.2	11±2.2	8±0	5.5±2.4
	Liver	12±2.8	23.75±2.9	12±4.4	7.5±1.3	5.25±0.9
	Gut	8±2.9	13±4.4	5.5±2.5	2±0	4.5±0.6
Tx	Spleen	3.75±0.5	2.5±0.6	3.25±0.5	1.75±0.9	14.25±2.9
	Liver	5.75±1.7	8.75±0.5	1.75±0.9	2.25±0.5	8.25±4.3
	Gut	5±0.8	4.75±1.3	2±0	1.5±0.6	7±1.2

Table 5.2 Table showing mean fluorescence intensities (± SD) of mAb staining with 2B1 (anti-CD5), AM22 and F17 (anti-CD8), D4.3 and D12.2 (specificity unknown). **A** - animals aged 4-6 months where n=3 for spleen, liver and gut of control and Tx; **B** - animals aged 8-12 months where n=4 for spleen, liver and gut of control and Tx.

Chapter 6

6.1 Conclusions and Future Work

The work presented in this Thesis attempted to investigate whether an extrathymic pathway of T cell development exists in the amphibian, *Xenopus laevis*.

An ontogenetic study was carried out in Chapter 2 on thymocytes and splenocytes from control *Xenopus* in order to probe the emergence of T cell surface antigens characterised by a panel of T cell-specific monoclonal antibodies available in the laboratory. This study provided a useful database of information on the ontogeny of T cell surface antigen expression. The findings present new insight into *Xenopus* T cell development, since such a panel of T cell-specific mAbs has not been available until recently. One important feature of the work in this Thesis has been the use of dual-colour flow cytometry. Previous studies have generally used single colour fluorescence studies, much of which has been microscopy work. The use of dual-colour flow cytometry here has revealed directly for the first time that there is differential staining of MHC II on *Xenopus* B and T cells. Thus, T cells express only a low intensity of MHC II expression, whilst B (and some other non-T cells) express a brighter intensity of staining with MHC II. Work in Chapter 2 also revealed that the inhibition of metamorphosis, by the addition of sodium perchlorate to the aquarial water, had little effect on lymphocyte surface antigen expression. Not only was the emergence of T cell surface marker expression similar, but also the percentage of cells stained with the individual mAbs was comparable in normal and metamorphosis-inhibited *Xenopus*. The present work confirmed that MHC II expression on T cells was metamorphosis-dependent (see Rollins-Smith and Blair, 1990). One important effect of inhibiting metamorphosis was that of total lymphocyte numbers.

Having characterised the emergence of T cell surface antigen expression in spleen and thymus during ontogeny, studies were carried out in Chapter 3 in order to examine the effect of early larval thymectomy (5-7 days) on these markers. Removal of the thymus at a time before any T cell markers have differentiated on thymocytes results in the ablation of T cells from the spleen at 10 months of age, as defined by the lack of T cell-specific markers recognised by the mAbs, 2B1 (anti-

CD5), AM22 (anti-CD8), F17 (anti-CD8), D4.3 (putative anti-αβ TCR) and XT-1 (XTLA-1). The loss of proliferative responses by splenocytes to the T cell mitogens, concanavalin A (ConA) and phytohaemagglutinin (PHA) also confirmed the lack of T cells functionally. These findings confirm that the thymus plays an essential role in T cell development (at least with respect to the spleen) in the amphibian, *Xenopus*.

Chapter 3 also investigated whether *in vitro* stimulation of thymectomized (Tx) splenocytes could induce the expression of T cell surface antigens. These flow cytometric studies employed the T cell mitogens ConA and PHA, the B cell mitogen lipopolysaccharide (LPS), and a mitogen that stimulates both T and B cells, phorbol myristate acetate (PMA). *In vitro* stimulation with ConA and PHA had no effect on T cell marker expression in Tx animals. However, PMA stimulation, while again achieving no induced expression of T cell markers, was able to cause the loss of surface IgM from Tx splenocytes. It was therefore concluded that *in vitro* stimulation of Tx splenocytes with either T cell mitogens or the potent mitogenic agent, PMA, was unable to induce T cell surface antigen expression. In contrast, PMA stimulation of splenocytes from control animals, as well as causing the loss of sIgM expression, also resulted in the emergence of $CD5^{dull}$ expression. This finding was further probed in Chapter 4.

As a separate focus of attention in Chapter 3, the effects of *in vitro* activation of splenocytes from control animals were analysed to determine whether cells were being pushed down the cell suicide pathway (apoptosis). Apoptosis was initially monitored by establishing flow cytometric analysis of propidium iodide stained lymphocytes and looking for sub-G_1 cells in cell cycle histograms. When lymphocytes were cultured for up to three days in medium alone, there was a low level of sub-G_1 cells induced. When lymphocytes were cultured in the presence of ConA, the level of sub-G_1 cells initially increased, suggesting that the mitogen may be exerting a toxic effect, since 24 hours after its removal, the level of apoptosis began to decline. Lymphocyte activation was being induced by ConA as shown by an increase in cell size and the detection of RNA in the cytoplasm of these lymphocytes. In contrast, stimulation of splenocytes with PMA indicated that this agent induced a higher level of apoptosis. PMA, like ConA induced an increase in cell size and achieved RNA-rich cytoplasm, but unlike stimulation with ConA, acridine orange staining revealed fragmentation of the cell's DNA, indicative of apoptosis (Korsmeyer, 1995). Future studies probing the induction of apoptosis (in both control and Tx animals) would be aided greatly by the use of an anti-apoptosis specific protein antibody that recently became available at the end of these studies

(Grand et al, 1995). Preliminary experiments revealed this antibody can detect putative apoptotic *Xenopus* thymic tumour cells. Furthermore, Western blot experiments indicate the emergence of proteins in *Xenopus* cells with similar molecular weights as emerge in mammalian apoptotic cells (A Milner & J D Horton, personal communication). It would be interesting to examine whether there is a greater basal level of apoptosis occurring *in vivo* in lymphocytes from Tx animals, and to determine whether *in vitro* mitogen stimulation results in a greater proportion of lymphocytes entering the suicide pathway. Apoptosis is a mechanism that shapes the development from larval-type tissue to adult-type tissue during metamorphosis, eg. tail reabsorption. It would therefore prove interesting to monitor the levels of apoptosis occurring in both thymocytes and splenocytes during metamorphosis in both normally developing and metamorphosis-inhibited animals.

Having established in Chapter 3 that *in vitro* stimulation of splenocytes from control *Xenopus* with phorbol ester could induce the expression of the T cell marker CD5, experiments were set up in Chapter 4 to investigate the nature of this expression. The fact that this phenomenon only occurred in control animals suggested that T cells may be an important factor in the induction of CD5 expression. 'Panning' experiments carried out to obtain a pure (>90%) population of B cells from control animals showed that B cells by themselves were not capable of induced CD5 expression. When T cells were added back to these purified B cells, CD5 expression could once again be induced and demonstrated by flow cytometric analysis. This is in contrast to similar studies in higher vertebrates where it has been shown that induced CD5 expression on B cells requires neither T cells nor any other accessory cells (Bertoglio, 1983; Rothstein et al, 1986; Freedman et al, 1989a,b). Cell mixing experiments carried out on B lymphocytes from Tx *Xenopus* suggested that CD5 expression could also be induced following phorbol ester stimulation, but again only in the presence of a T cell source (either T cells from control animals, or *Xenopus* thymic tumour cells). Future studies regarding the induction of CD5 expression should probe more precisely whether CD5 expression is being synthesised *de novo*, as is the case in mammals (Freedman et al, 1989) (by the use of RNA probes specific for CD5, or by the use of biosynthetic labelling studies), or whether the CD5 molecule is simply dropping off the T cells and sticking to the B cells. This latter possibility appears unlikely from the studies carried out in Chapter 4, but nevertheless, needs to be probed further.

Confirmation that PMA activates T cells via protein kinase C pathway was also confirmed in studies carried out in Chapter 4. The stimulatory action of PMA was

also inhibited by use of the protein kinase C inhibitor, RO 31-8220, both in terms of CD5 expression and induced lymphocyte proliferation. In contrast, the inhibitor had no effect on LPS stimulation, but reduced the proliferation with ConA or PHA by half. The fact that there was an effect of the inhibitor on stimulation with T cell mitogens is not that surprising, and indicates that the protein kinase C pathway, although being a major pathway in T cell activation, is not the sole pathway, as T cells can be stimulated via calcium-dependent kinases (Geller et al, 1987).

One important aspect for future work is to take further the information provided from these cell surface analyses. Functional studies should be undertaken to determine what the presence of these cell surface markers has on the lymphocytes functional capabilities. Will lymphocytes respond to T cell mitogens even before certain cell markers have been expressed, what effect will pre-incubating the lymphocytes with certain mAbs, to block the marker, have on the cell? Cell sorting experiments could be carried out to isolate different populations of lymphocytes, eg. D12.2^{+} cells, and to determine if this cell type behaves like a true γδ T cell, or is more natural killer cell-like.

The phenotypic analyses have only just started to reveal a more intimate picture of the lymphocyte make-up of *Xenopus*, which now needs to be followed up by functional and molecular characterisation for the complete picture to emerge.

The issue of extrathymic T cell development, which forms the major focus of this Thesis, was returned to in Chapter 5. This explored in some detail the T cell populations found in the intestine and liver of both control and Tx animals. Initial flow cytometric experiments on mAb-stained cells concentrated on the developing T cell populations in control *Xenopus*. Surface antigen markers on liver lymphocytes of these animals were very similar to those found in the spleen, in terms of mean fluorescence intensity, and percentages of mAb-defined populations. However, in the intestine, lymphocytes stained with several anti-T cell markers had a somewhat reduced mean fluorescence of intensity compared with spleen and liver. Furthermore, in older animals dual-fluorescence studies showed the emergence of populations of gut lymphocytes that were not present in the spleen or liver.

When Tx animals were examined there was no staining with the mAbs D4.3 (putative αβ TCR) or F17 (anti-CD8) in either the intestine or the liver. This was consistent both for young animals (4-6 months) and for older animals (8-12 months). In contrast, in both young and older Tx frogs, surface staining on liver and gut was

seen with the mAbs, 2B1 (anti-CD5) and AM22 (anti-CD8). However, the staining intensity with both these mAbs was duller than that found in control animals. The presence of these $CD5^{dull}$ and $CD8^{dull}$ ($AM22^{dull}$) lymphocytes in Tx animals suggests that these cells express low levels of certain T cell markers and may be 'T-like' cells developing *in situ* within the gut and liver. The discrepancy between levels of $CD8^+$ lymphocytes detected by the two anti-CD8 mAbs, namely AM22 and F17 is intriguing. It could be interpreted that the AM22 mAb detects a determinant on the α chain of the CD8 molecule and that the F17 mAb detects a determinant not found on this chain. In mammals the majority of CD8 molecules found in the intestine consist of a homodimeric α chain, and it is lymphocytes bearing this homodimer that are though to be of extrathymic origin (see Chapter 1 and 5.1). Perhaps those lymphocytes in Tx animals expressing dull AM22 may be of extrathymic origin. However, until further characterisation of the determinant detected by the mAbs, AM22 and F17 is carried out, care must be taken when interpreting this possibility.

One interesting feature to emerge from the study in Chapter 5 on both liver and intestine from control and Tx *Xenopus* was the increased level of $D12.2^+$ lymphocytes compared to that of the spleen. This mAb was previously thought to be directed against the *Xenopus* $\gamma\delta$ TCR (Ibrahim et al, 1991), however, further characterisation of this mAb was never undertaken. The findings of high levels of $D12.2^+$ lymphocytes in the intestine and liver of *Xenopus* are interesting since $\gamma\delta$ TCR^+ lymphocytes form a major intestinal population in mammals (see Chapter 1).

It will be very worthwhile to carry out further work on intestinal and hepatic lymphocyte development in *Xenopus*. Indeed only two ages were used in these studies, 4-6 months and 8-12 months. For a more detailed picture of the emergence of 'T-like' cells in Tx frogs, more time points need to be carried out between these two ages. One method to investigate the development of extrathymic T cells in the intestine could possibly make use of *Xenopus borealis*. The lymphocytes from these animals can be identified by the use of quinacrine staining, giving a characteristic speckled staining pattern (Thiébaud, 1983). The combined use of *Xenopus laevis* and *Xenopus borealis* in thymus implant studies may identify those cells that are host-derived (thymus-independent) and those that are donor-derived (thymus-dependent).

The whole issue surrounding extrathymic T cell development in *Xenopus* is still in its infancy. This was the first piece of research attempting to phenotype intestinal

lymphocytes in the amphibian, *Xenopus laevis*. It has revealed many interesting features detailed above, but has left a lot of avenues open for future work. Not only should more time points be taken between the ages used in this study, but more importantly later time points should be examined. One further site that could be examined for extrathymic T cell development is the skin, another site that has been implicated with extrathymic T cells.

Extrathymic T cells develop with ageing - animals of a year old may still be in their infancy regarding extrathymic development. Therefore it would be very interesting and indeed essential to probe whether extrathymic T cells are developing in animals aged 2 or 3 years and even up to 5 years and beyond, to be sure that T-like cells are not developing.

The possibility of extrathymic T-like cell development in *Xenopus* is currently being explored with molecular probes that can detect rearranged TCR β transcripts (Dzialo, R; Cooper, MD; Horton, JD - work in progress). These studies indicate that a low level of TCR β transcripts can sometimes be recorded in the intestine (but not spleen) of Tx animals that had been thymectomized in this laboratory. Such molecular approaches on T cell receptor expression and the nature of the CD8 co-receptor molecules are the way ahead to explore the extent to which T-like cells can develop extrathymically. At present, it is concluded that at the amphibian level of evolution, an extrathymic T cell pathway is **not** a major pathway of T cell development.

Appendix A

Solutions and Reagents

Amphibian Strength Phosphate Buffered Saline (APBS)

Make a 1:13 dilution of 10x PBS stock solution (Gibco) with dH_2O

Adjust to pH 7.4

Filter sterilise

Carbonate/Bicarbonate Buffer

Make up stock solutions of : 0.2M Na_2CO_3 (Solution A)

0.2M NaHCO3 (Solution B)

Just prior to use, mix 4.5ml solution A with 8ml solution B and make up to a final volume of 50ml with dH_2O

Adjust to pH 9.6

10x HEPES-bicarbonate Buffer

23.8g HEPES (100mM final conc)

21.0g $NaHCO_3$ (250mM final conc)

Make up volume to 1000ml with dH_2O

Adjust to pH 7.2

Autoclave and store at room temperature for up to 1 month

FACS Medium

6.6g NaCl

1.5g Na_2HPO_4

0.2g KH_2PO_4

1000ml double distilled H_2O

Add 0.1% BSA (1g per litre)

0.1% NaN_3 (1g per litre)

Adjust to pH 7.4 and filter sterilise

Staining and Blocking Buffer

50ml 10x PBS

600ml dH_2O

Add 200µl phenol indicator

Adjust pH with 1-2M NaOH until solution turns a pale peach colour
Add 0.65g NaN_3

For staining buffer:

Add 0.55g bovine serum albumin (BSA) to 550ml solution

For blocking buffer:

Add 1g BSA to remaining 100ml solution
If necessary adjust pH with NaOH

Serum Free Medium (used to make tumour medium)

500ml	Iscoves
5ml	Non essential amino acids (NEAA)
5ml	Penicilin/Streptomycin
0.5ml	Insulin
0.5ml	2 Mercaptoethanol
1.5ml	Primatone

This was filter sterilised

Tumour Medium

400ml	Serum free medium (see above)
120ml	double distilled H_2O
40ml	A6 supernatant (A6 - a fibroblast cell line)
10ml	FCS
1.3ml	Kanamycin

This was filter sterilised

Wright-Giemsa Stain

1.53g Wright's stain }
2.50g Giemsa stain } dissolved in 1 litreof methanol
100ml Glycerine (or glycerol) }

Flood slide with Wright-Giemsa stain for 5-10 minutes, then briefly rinse twice in dH_2O, dehydrate in alcohol and xylene, and mount in DPX

Appendix B

Quality Control in Flow Cytometry

Quality control in flow cytometry is a very necessary and important procedure that needs to be performed every time the machine is used. Only when the machine is performing to the highest standard can the results be accepted with any confidence.

Coulter lay down their recommendations as to how best to quality control the cytometer. This has been carried out using ImmunoCheck Beads and ImmunoBrite Beads purchased from Coulter. These beads are run through the machine each time it is to be used to make sure that the laser is correctly aligned and that all the fluorescence detectors are functional and are working properly. The quality control is carried out after the laser has warmed up (approximately 45 minutes).

ImmunoCheck Beads

These beads are of a uniform size (10μm diameter) and are coated with a fluorescent dye that emits fluorescence at all wavelengths from 525 to 700nm when excited by an argon laser at 488nm, with a peak emission of about 560nm.

An aliquot of beads is dispensed into a 5ml Falcon tube and analysed on the flow cytometer using the QC 'startup' protocol set up by the Coulter engineer when the cytometer was installed. The beads are analysed on a low flow rate and 10,000 events are accumulated. The important details to note are the half peak coefficient of variation values (HPCVs) and the mean channel of fluorescence. The HPCV gives an indication of how uniformly the beads are travelling down the centre of the stream, and Coulter state that this value should not exceed 2.5. The mean channel gives an indication of how effectively both the fluorescence and side scatter of the beads is being detected. If HPCVs are too high, repeated cleansing of the flow cell will help to correct the alignment.

ImmunoBrite Beads

These beads are used to check that the logarithmic amplifiers and photomultiplier tubes (PMTs), eg. FL1-FL4 detectors, are linear. The beads are packaged in five separate vials with each vial containing beads with a different fluorescence intensity - blank, med-low, reference-med, med-hi and brite. The beads again emit fluorescence at all wavelengths from 525-700nm.

Four drops of each level are added to a 5ml Falcon tube, mixed and analysed on the flow cytometer on the QC 'immunobrite' protocol set up by the Coulter engineer when the cytometer was installed. This protocol records the fluorescence of each level of beads for every PMT. The voltages should be set so that the level V beads (brite) are at the end of the fourth log decade. As for ImmunoCheck beads, the sample is run at a low flow rate and 10,000 events are recorded. The mean peak channel for each level is recorded and these should be plotted on log graph paper. When each of these four points (one for each PMT) are joined together they should form a straight line indicating that the machine is linear.

References

Ackley, C D; Cooper, M D; (1992), Characterization of a feline T-cell-specific monoclonal antibody reactive with a CD5-like molecule. Am J Vet Res 53: 466-471

Akbar, A N; Salmon, M; (1995), Selection and Survival of Activated Lymphocytes, Scientific Am Mar/Apr 48-57

Allison, J P; (1993), γδ T-cell development, Curr Opin Immunol 5: 241-246

Åman, P; Gordon, J; Klein, G; (1984), TPA (12-O-tetradecanoyl-phorbol-13-acetate) activation and differentiation of human peripheral B lymphocytes. Immunology 51: 27-34

Anderson, G; Anderson, K L; Conroy, L A; Hallam, T J; Moore, N C; Owen, J J T; Jenkinson, E J; (in press). Intracellular signalling events during positive and negative selection of $CD4^+CD8^+$ thymocytes *in vitro*. J Immunol in press

Bertoglio, J H; (1983), Monocyte-Independent Stimulation of Human B Lymphocytes by Phorbol Myristate Acetate. J Immunol 131: 2279-2281

Bill, J; Kanagawa, O; Woodland, D L; Palmer, E; (1989), The MHC molecule I-E is necessary but not sufficient for the clonal deletion of Vβ11-bearing T cells. J Exp Med 169: 1405-1419

Blanchard-Channell, M; Moore, P F; Stott, J L; (1994), Characterization of monoclonal antibodies specific for equine homologues of CD3 and CD5. Immunology 82: 548-554

Bleicher, P A; Cohen, N; (1981), Monoclonal Anti-IgM Can Separate T Cell from B Cell Proliferative Responses in the Frog, Xenopus laevis. J Immunol 127: 1549-1555

Bluestone, J A; Cron, R Q; Rellahan, B; Matis, L A; (1991), Ligand Specificity and Repertoire Development of Murine TCR γδ Cells, Curr Topics Microbiol Immunol 173: 133-139

von Boehmer, H; (1992), Thymic selection: a matter of life and death. Immunol Today 13: 454-458

Budd, R C; Miescher, G C; Howe, R C; Lees, R K; Bron, C; MacDonald, H R; (1987). Developmentally regulated expression of T cell receptor β chain variable domains in immature thymocytes. J Exp Med 166: 577-582

Ceuppens J L; Baroja, M L; (1986). Monoclonal antibodies to the CD5 antigen can provide the necessary second signal for activation of isolated resting T cells by solid-phase-bound OKT3. J Immunol 137: 1816-1821

Chen, C H; Sowder J T; Lahti, JM; Cihak, J; Lösch, U; Cooper, MD; (1989). A third T-cell receptor in the chicken. Proc Natl Acad Sci USA 86: 2351-2355

Clevers, H C; Versteegen, J M T; Logtenberg, T; Gmelig-Meyling, F H J; Ballieux, R E; (1985), Synergistic Action of A23187 and Phorbol Ester on Human B Cell Activation. J Immunol 135: 3827-3830

Clothier, R H; Balls, M (1985), Structural changes in the thymus glands of *Xenopus laevis* during development. In: Metamorphosis, Claredon Press, Oxford

Cohen, J J; (1993), Apoptosis, Immunol Today 14: 126-130

Coltey, M; Jotereau, F V; Le Douarin, N M; (1987), Evidence for a cyclic renewal of lymphocyte precursor cells in the embryonic chick thymus. Cell Differ 22: 71-82

Croitoru, K; Stead, R H; Bienenstock, J; Fulop, G; Harnish, D G; Shultz, L D; Jeffery, P K; Ernst, P B; (1990), Presence of intestinal inraepithelial lymphocytes in mice with severe combined immunodeficiency disease. Eur J Immunol 29: 645-651

Croitoru, K; Ernst, P B; (1993), Intraepithelial Lymphocyte Lineage and Function. In Mucosal Immunology: Intraepithelial Lymphocyes, ed Kiyono, H; McGhee, J R, Raven Press Ltd, NY

Davies, M D J; Parrott, D M V; (1981), Preparation and purification of lymphocytes from the epithelium and lamina propria of murine small intestine. Gut 22: 481-488

Deenen, G J; van Balen, I; Opstelten, D; (1990), In rat B lymphocyte genesis sixty percent is lost from the bone marrow at the transition of nondividing pre-B to $sIgM^+$ B lymphocyte, the stage of Ig light chain gene expression. Eur J Immunol 20: 557-564

Dunon, D; Cooper, M D; Imhof, B A; (1993a), Thymic Origin of Embryonic Intestinal γ/δ T Cells. J Exp Med 177: 257-263

Dunon, D; Cooper, M D; Imhof, B A; (1993b), Migration patterns of thymus-derived γδ T cells during chicken development, Eur J Immunol 23: 2545-2550

DuPasquier, L; Flajnik, M F; (1990), Expression of MHC class II antigens during *Xenopus* development. Dev Immunol 1: 85-95

DuPasquier, L; Horton, J D; (1976), The Effect of Thymectomy on the Mixed Leukocyte Reaction and Phytohemagglutinin Responsiveness in the Clawed Toad *Xenopus laevis*. Immunogenetics 3: 105-112

DuPasquier, L; Flajnik, M F; Hsu, E; Kaufman, J F; (1986), Ontogeny of the immune system in anuran amphibians. In: Prog in Immunol VI: 1079-1088

DuPasquier, L; Schwager, J; Flajnik, M F; (1989), The immune system of *Xenopus*. Annu Rev Immunol 7: 251-275

Earnshaw, W C; (1995), Apoptosis: lessons from *in vitro* systems, Trends in Cell Biol 5: 217-220

Farber, J L; (1990), The role of calcium ions in toxic cell injury. Environ Health Persp 84: 107-111

Farr, A G; Anderson, S K; Marrack, P; Kappler, J; (1985), Expression of antigen-specific, major histocompatibility complex-restricted receptors by cortical and medullary thymocytes *in situ*. Cell 43: 543-550

Flajnik, M F; Hsu, E; Kaufman, J F; DuPasquier, L; (1987), Changes in the immune system during metamorphosis of Xenopus. Immunol Today 8: 58-64

Flajnik, M F; Ferrone, S; Cohen, N; DuPasquier, L; (1990), Evolution of the MHC: Antigenicity and Unusual Tissue Distribution of *Xenopus* (Frog) Class II Molecules. Mol Immunol 27: 451-462

Fowlkes, B J; Edison, L; Mathieson, B J; Chused, T M; (1985), Early T Lymphocytes: Differentiation In Vivo of Adult Intrathymic Precursor Cells. J Exp Med 162: 802-822

Fowlkes, B J; Schwartz, R H; Pardoll, D M; (1988), Deletion of self-reactive thymocytes occurs at a $CD4^+8^+$ precursor stage. Nature 334: 620-623

Freedman, A S; Freeman, G; Whitman, J; Segil, J; Daley, J; Levine, H; Nadler, L M; (1989a), Expression and regulation of CD5 on *in vitro* activated human B cells. Eur J Immunol 19: 849-855

Freedman, A S; Freeman, G; Whitman, J; Segil, J; Daley, J; Nadler, L M; (1989b), Studies of In Vitro Activated $CD5^+$ B Cells. Blood 73: 202-208

Geller, R L; Gromo, G; Inverardi, L; Ferrero, E; Bach, F H; (1987), Stepwise Activation of T Cells: Role of the Calcium Ionophore A23187. J Immunol 139: 3930-3934

Grand, R J A; Milner, A E; Mustoe, T; Johnson, G D; Owen, D; Grant, M L; Gregory, C D; (1995), A Novel Protein Expressed in Mammalian Cells Undergoing Apoptosis, Exp Cell Res 218: 439-451

Grant, P; Clothier, R H; Johnson, R O; Schott, S; Ruben, L N; (1995), The time course, localization and quantitation of T- and B-cell mitogen-driven apoptosis *in vivo*, Immunol Lett 47: 227-231

Guidos, C J; Weissman, I L; Adkins, B; (1989), Intrathymic maturation of murine T lymphocytes from $CD8^+$ precursors. Proc Natl Acad Sci USA 86: 7542-7546

Guy-Grand,D; Griscelli, C; Vassalli, P; (1978), The mouse gut T lymphocyte, a novel type of T cell: nature, origin and traffic in mice in normal and graft-versus-host conditions. J Exp Med 148: 1661-1677

Guy-Grand, D; Cerf-Bensussan, N; Malissen, B; Malassis-Seris, M; Briottet, C; Vassalli, P; (1991), Two Gut Intraepithelial $CD8^+$ Lymphocyte Populations with Different T Cell Receptors: A Role for the Gut Epithelium in T Cell Differentiation. J Exp Med 173: 471-481

Guy-Grand, D; Vanden Broecke, C; Briottet, C; Malassis-Seris, M; Selz, F; Vassalli, P; (1992), Different expression of the recombination activity gene RAG-1 in various populations of thymocytes, peripheral T cells and gut thymus-independent intraepithelial lymphocytes suggests two pathways of T celll receptor rearrangement. Eur J Immunol 22: 505-510

Guy-Grand, D; Vassalli, P; (1993), Gut Intraepithelial T Lymphocytes. Curr Opin Immunol 5: 247-252

Guy-Grand, D; Rocha, B; Vassalli, P; (1993), Origin and Development of Gut Intraepithelial Lymphocytes. In Mucosal Immunology: Intraepithelial Lymphocyes, ed Kiyono, H; McGhee, J R, Raven Press Ltd, NY

Hadji-Azimi, I; Coosemans, V; Canicatti, C; (1990), B-Lymphocyte Populations in *Xenopus laevis* CHECK QUOTE

Hardy, R R; (1991), CD5 B cells: a separate lineage at last? Curr Biol 1: 290-292

Hardy, R R; Hayakawa, K; (1991), Generation of Ly-1 B Cells from Developmentally Distinct Precursors: Enrichment by Stromal Cell Culture or Cell Sorting. Ann N Y Acad Sci : in press

Haughton, G; Arnold, L W; Whitmore, A C; Clarke, S H; (1993), B-1 Cells are made, not born. Immunol Today 14: 84-87

Havran, W L; Allison, J P; (1988), Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. Nature 335: 443-445

Havran, W L; Carbone, A; Allison, J P; (1991), Murine T Cells with Invariant γδ Antigen Receptors: Origin, Repertoire, and Specificity, Semin Immunol 3: 89-97

Havran, W L; Chien, Y H; Allison, J P; (1991), Recognition of self antigens by skin-derived T cells with invariant γδ antigen receptors. Science 252: 1430-1432

Hayakawa, K; Hardy, R R; Parks, D R; Herzenberg, L A; (1983), The 'Ly-1 B' Cell Subpopulation in Normal, Immunodefective, and Autoimmune Mice. J Exp Med 157: 202-218

Hein, W R; Dudler, L; Morris, B; (1990), Differential peripheral expansion and in vivo antigen reactivity of αβ and γδ T cells emigrating from the early fetal lamb thymus, Eur J Immunol 20: 1805-1813

Hein, W R; Dudler, L; (1993), Divergent evolution of T cell repertoire: extensive diversity and developmentally regulated expression of the sheep γδ T cell receptor. EMBO 12: 715-724

Herzenberg, L A; Kantor, A B; (1993), B-cell lineages exist in the mouse. Immunol Today 14: 79-83

Horton, J D; (1994), Amphibians. In Immunology: A Comparative Approach, ed Turner, R J. John Wiley and Sons Ltd pp 101-136

Horton, J D; Horton, T L; Ritchie, P; (1996), Immune system of *Xenopus*: T cell biology pp 279-299 In: The Biology of Xenopus, ed Tinsley, R C; Kobel, H R

Horton, J D; Manning, M J; (1972), Response to skin allografts in *Xenopus laevis* following thymectomy at early stages of lymphoid organ maturation. Transplantation 14: 141-154

Ibrahim, B; Gartland, L A; Kishimoto, T; Dzialo, R; Kubagawa, H; Bucy, R P; Cooper, M D; (1991), Analysis of T cell development in *Xenopus*. Fed Proc 5: 7651

Iiai, T; Watanbe, H; Seki, S; Sugiura, K; Hirokawa, K; Utsuyama, M; Takahashi-Iwanga, H; Iwanga, T; Ohteki, T; Abo, T; (1992), Ontogeny and development of extrathymic T cells in mouse liver. Immunology 77: 556-563

Ishida, H; Hastings, R; Kearney, J; Howard, M; (1992), Continuous Anti-Interleukin 10 Antibody Administration Depletes Mice of Ly-1 B Cells but Not Conventional B Cells, J Exp Med 175: 1213-1220

Janeway, C A Jnr; Travers, P; (1994). Immunobiology - the immune system in health and disease. Cuurent Biology, Blackwell Scientific Publications, Garland Publishing Inc.

Janeway, C A Jnr; Jones, B; Hayday, A; (1988), Specificity and function of T cells bearing γδ receptors. Immunol Today 9: 73-76

Jotereau, F V; Hueze, F; Salomon-Vie, V; Gascan, H; (1987), Cell Kinetics in the Fetal Mouse Thymus: Precursor Cell Input, Proliferation and Emigration. J Immunol 138: 1026-1030

Jürgens, J B; Gartland, L A; DuPasquier, L; Horton, J D; Göbel, T W F; Cooper, M D; (1995), Identification of a Candidate CD5 Homologue in the Amphibian *Xenopus laevis*. J Immunol 155: 4218-4223

Kabelitz, D; Pohl, T; Pechhold, K; (1993), Activation-induced cell death (apoptosis) of mature peripheral T lymphocytes, Immunol Today 14: 338-339

Kantor, A B; (1991), The development and repertoire of B-1 cells (CD5 B cells). Immunol Today 12: 389-391

Kappler, J; Roehm, N; Marrack, P; (1987), T cell tolerance by clonal elimination in the thymus. Cell 49: 273-280

Kasahara, Y; Chen, C-L, H; Göbel, T, W, F; Bucy, R P; Cooper, M D; (1993), Intraepithelial Lymphocytes in Birds. In Mucosal Immunology: Intraepithelial Lymphocyes, ed Kiyono, H; McGhee, J R, Raven Press Ltd, NY

Kawamura, M; Wheeler, C J; Notkins, A L; Bich-Thuy, L T; (1994), Modulation of the Expression of CD5 Antigen on the Surface of Human Peripheral B Lymphocytes, Cell Immunol 159: 61-76

Kearney, J F; (1993), $CD5^+$ B-cell networks. Curr Opin Immunol 5: 223-226

Kelly, K A; Pearse, M; Lefrançois, L; Scollay, R; (1993), Emigration of selected subsets of $\gamma\delta^+$ T cells from the adult murine thymus. Int Immunol 5: 331-335

Kennedy, J D; Pierce, C W; Lake, J P; (1992), Extrathymic T Cell Maturation: Phenotypic Analysis of T Cell Subsets in Nude Mice as a Function of Age. J Immunol 148: 1620-1629

Kikkawa, U; Takai, Y; Tanaka, Y; Miyake, R; Nishizuka, Y; (1983), Protein Kinase C As a Possible Receptor Protein of Tumour-promoting Phorbol Esters. J Biol Chem 258: 11442-11445

Kisielow, P; Lieserson, W; von Boehmer, H; (1984), Differentiation of Thymocytes in Fetal Organ Culture: Analysis of Phenotypic Changes Accompanying the Appearance of Cytolytic and Interleukin 2-Producing Cells. J Immunol 133: 1117-1123

Klas, C; Debatin, K-M; Jonker, R R; Krammer, P H; (1993), Activation Interferes with the APO-1 Pathway in Mature Human T Cells, Int Immunol 5: 625-630

Korsmeyer, S J; (1995), Regulators of cell death TIG 11: 101-105

Krammer, P H; Behrmann, I; Daniel, P; Dhein, J; Debatin, K-M; (1994), Regulation of apoptosis in the immune system, Curr Opin Immunol 6: 279-289

Kripps, T J; (1989), The CD5 B Cell. Adv Immunol 47: 117-185

Lake, J P; Pierce, C W; Kennedy, J D; (1991), T Cell Receptor Expression by T Cells that Mature Extrathymically in Nude Mice. Cell Immunol 135: 259-265

Lazebnik, Y A; Cole, S; Cooke, C A; Nelson, W G; Earnshaw, W C; (1993), Nuclear events of apoptosis *in vitro* in cell-free mitotic extracts: a model system for analysis of the active phase of apoptosis. J Cell Biol 123: 7-22

Lefrançois, L; (1991), Phenotypic Complexity of Intraepithelial Lymphocytes of the Small Intestine. J Immunol 147: 1746-1751

Lefrançois, L; (1993), Differentiation and TCR Selection of Intestinal Intraepithelial Lymphocytes. In Mucosal Immunology: Intraepithelial Lymphocyes, ed Kiyono, H; McGhee, J R, Raven Press Ltd, NY

Lefrançois, L; Puddington, L; (1995), Extrathymic intestinal T-cell development: virtual reality? Immunol Today 16: 16-21

Lin, T; Matsuzaki, G; Kenai, H; Nakamura, T; Nomoto, K; (1993), Thymus influences the development of extrathymically derived intestinal intraepithelial lymphocytes. Eur J Immunol 23: 1968-1974

Lin, T; Matsuzaki, G; Kenai, H; Nomoto, K; (1994), Progenies of fetal thymocytes are the major source of $CD4^{-}CD8^{+}$ $\alpha\alpha$ intestinal intraepithelial lymphocytes early in ontogeny. Eur J Immunol 24: 1785-1791

Lin, T; Matsuzaki, G; Kenai, H; Nomoto, K; (1995), Extrathymic and thymic origin of murine IEL: Are most IEL in euthymic mice derived from the thymus? Immunol Cell Biol 73: 469-473

Luft, J C; Clem, L W; Bly, J E; (1991), A serum-free culture medium for channel catfish *in vitro* immune response. Fish & Shellfish 1:131-139

MacDonald, H R; Budd, R C; Howe, R C; (1988a), A $CD3^{-}$ subset of $CD4^{-}$ $CD8^{+}$ thymocytes: a rapidly cycling intermediate in the generation of $CD4^{+}CD8^{+}$ cells. Eur J Immunol 18: 519-523

MacDonald, H R; Hengartner, H; Pedrazzini, T; (1988b), Intrathymic deletion of self-reactive cells prevented by neonatal anti-CD4 antibody treatment. Nature 335: 174-176

Mackay, C R; Hein, W R; (1991), Marked Variations in $\gamma\delta$ T Cell Numbers and Distribution Throughout the Life of Sheep, Curr Topics in Microbiol Immunol 173: 107-111

Manning, M J; Horton, J D; (1969), Histogenesis of lymphoid organs in larvae of the South African clawed toad, *Xenopus laevis* (Daudin). J Embryol exp Morph 22: 265-277

Manning, M J; Horton, J D; (1982), Reticuloendothelial system structure and function of the amphibia. In: The Reticuloendothelial System Volume 3: Phylogeny and Ontogeny pp. 423-459

Manning, M J; Donnelly, N; Cohen, N; (1977), Thymus-Dependent and Thymus-Independent Components of the Amphibian Immune System pp 123-132 In: Phylogeny of Thymus and Bone Marrow - Bursa Cells, ed Wright, R K; Cooper, E L, Elsevier/North Holland Biomedical Press, Amsterdam

Marrack, P; McCormack, J; Kappler, J; (1989), Presentation of antigen, foreign major histocompatibility complex proteins and self by thymus cortical epithelium. Nature 338: 503-505

Miller, R A; Garlow, J; (1984), The Induction of Leu-1 Antigen Expression in Human Malignant and Normal B Cells by Phorbol Myristate Acetate (PMA). J Immunol 133: 3408-3414

Milner, A E; Grand, R J A; Waters, C M; Gregory, C D; (1993). Apoptosis in Burkitt lymphoma cells is driven by c-myc. Oncogene 8: 3385-3391

Milner, A E; Wang, H; Gregory, C D; (in press), Analysis of Apoptosis by Flow Cytometry

Mowat, A McI; McInnes, I B; Parrott, D M V; (1989), Functional properties of intra-epithelial lymphocytes from mouse small intestine IV. Investigation of the proliferative capacity of IEL using phorbol ester and calcium ionophore. Immunology 66: 398-403

Nagata, S; (1986), Development of T lymphocytes in *Xenopus laevis*: appearance of the antigen recognized by an anti-thymocyte mouse monoclonal antibody. Devel Biol 114: 389-394

Nagata, S; Cohen, N; (1983), Specific in vivo and nonspecific in vitro alloreactivites of adult frogs (*Xenopus laevis*) that were thymectomized during early larval life. Eur J Immunol 13: 541-545

Newmeyer, D D; Farschon, D M; Reed, J C; (1994), Cell-free apoptosis in *Xenopus* egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. Cell 79: 353-364

Nieuwkoop, P D; Faber, J; (1967), Normal table of *Xenopus laevis* (Daudin). Amsterdam: North Holland

Nishizuka, Y; (1984), Turnover of inositol phospholipids and signal transduction. Science 225: 1365-1370

Núñez, G; Merino, R; Grillot, D; González-García, M; (1994), Bcl-2 and Bcl-x: regulatory switches for lymphoid death and survival, Immunol Today 15: 582-588

Ohtsuka, K; Iiai, T; Watanabe, H; Tanaka, T; Miyasaka, M; Sato, K; Asakura, H; Abo, T; (1994), Similarities and Differences between Extrathymic T Cells Residing in Mouse Liver and Intestine. Cell Immunol 153: 52-66

Ohteki, T; Okuyama, R; Seki, S; Abo, T; Sugiura, K; Kusumi, A; Ohmari, T; Watanbe, H; Kumagai, K; (1992), Age-dependent increase of extrathymic T cells in the liver and their appearance in the periphery of older mice. J Immunol 149: 1562-1570

Owen, J J T; Ritter, M A; (1969), J Exp Med 129: 431

Pardoll, D M; Fowlkes, B J; Lew, A M; Maloy, W L; Weston, M A; Bluestone, J A; Schwartz, R H; Coligan, J E; Kruisbeek, A M; (1988), Thymus-Dependent and Thymus-Independent Developmental Pathways for Peripheral T Cell Receptor-γδ-Bearing Lymphocytes, J Immunol 140: 4091-4096

Petit, A; Ernst, P B; Befus, A D; Clark, D A; Rosenthal, K L; Ishizaka, T; Bienenstock, J; (1985), Murine intestinal intraepithelial lymphocytes I. Relationship of a novel Thy-1$^-$, Lyt-1$^-$, Lyt-2$^+$ granulated subpopulation to natural killer cells and mast cells. Eur J Immunol 15: 211-215

Plytycz, B; Bigaj, J; (1983), Amphibian Lymphoid Organs: A Review. Folia Biol 31: 225-236

Poggi, A; Sargiacomo, M; Biassoni, R; Pella, N; Sivori, S; Revello, V; Costa, P; Valtieri, M; Russo, G; Mingari, M C; Peschle, C; Moretta, L; (1993), Extrathymic differentiation of T lymphocytes and natural killer cells from human embryonic liver precursors. Proc Natl Acad Sci USA 90: 4465-4469

Poussier, P; Julius, M; (1994), Thymus Independent T Cell Development and Selection in the Intestinal Epithelium. Annu Rev Immunol 12: 521-553

Poussier, P; Edouard, P; Lee, C; Binnie, M; Julius, M; (1992), Thymus-independent Development and Negative Selection of T Cells Expressing T Cell Receptor α/β in the Intestinal Epithelium: Evidence for Distinct Circulation Patterns of Gut- and Thymus-Derived T Lymphocytes. J Exp Med 176: 187-199

Raman, C; Knight, K L; (1992), $CD5^+$ B Cells Predominate in Peripheral Tissues of Rabbit. J Immunol 149: 3858-3864

Ramsdell, F; Fowlkes, B J; (1990), Clonal Deletion versus Clonal Anergy: The Role of the Thymus in Inducing Self Tolerance. Science 248: 1342-1348

Ratcliffe, M J H; (1985), The ontogeny and cloning of B cells in the bursa of Fabricus. Immunol Today 6: 223

Richie, E R; McEntire, B; Crispe, N; Kimura, J; Lanier, L L; Allison, J P; (1988), Proc Natl Acad Sci USA 85: 1174

Robert, J; Guiet, C; DuPasquier, L; (1994), Lymphoid tumours of *Xenopus laevis* with different capacities for growth in larvae and adults. Dev Immunol 3: 297-307

Rocha, B; Vassalli, P; Guy-Grand, D; (1991), The $V\beta$ Repertoire of Mouse Gut Homodimeric α $CD8^+$ Intraepithelial T Cell Receptor α/β^+ Lymphocytes Reveals a Major Extrathymic Pathway of T Cell Differentiation. J Exp Med 173: 483-486

Rocha, B; Vassalli, P; Guy-Grand, D; (1992), The extrathymic T cell development pathway. Immunol Today 13: 449-454

Rocha, B; Vassalli, P; Guy-Grand, D; (1994), Thymic and Extrathymic Origin of Gut Intraepithelial Lymphocyte Populations in Mice. J Exp Med 180: 681-686

Rocha, B; Guy-Grand, D; Vassalli, P; (1995), Extrathymic T cell Differentiation. Curr Opin Immunol 7: 235-242

Roehm, N; Herron, L; Cambier, J; DiGuisto, D; Kappler, J; Marrack, P; (1984), Cell 38: 577

Roitt,I M; Brostoff, J; Male, D K; (1989). Immunology 2nd Ed Gower Medical Publishing

Rollins-Smith, L A; Blair, P J; (1990), Expression of class II major histocompatibility complex antigens on adult T cells in *Xenopus* is metamorphosis-dependent. Dev Immunol 1: 97-104

Rollins-Smith, L A; Blair, P J; Davis, A T; (1992), Thymus Ontogeny in Frogs: T-Cell Renewal at Metamorphosis. Devel Immunol 2: 207-213

Rothenberg, E; (1980), Expression of differentiation antigens in subpopulations of mouse thymocytes: regulation at the level of *de novo* synthesis. Cell 20: 1-9

Rothstein, T L; Baeker, T R; Miller, R A; Kolber, D L; (1986), Stimulation of Murine B Cells by the Combination of Calcium Ionophore plus Phorbol Ester. Cell Immunol 102: 364-373

Ruben, L N; Buchholz, D R; Ahmadi, P; Johnson, R O; Clothier, R H; Shiigi, S; (1994a), Apoptosis in thymus of adult *Xenopus laevis*. Dev Comp Immunol 18: 231-238

Ruben, L N; Ahmadi, P; Johnson, R O; Buchholz, D R; Clothier, R H; Shiigi, S; (1994b), Apoptosis in the thymus of developing *Xenopus laevis*. Dev Comp Immunol 18: 343-352

Salmon, M; Pilling, D; Borthwick, N J; Viner, N; Janossy, G; Bacon, P A; Akbar, A N; (1994), The progressive differentiation of primed T cells is associated with an increasing susceptibility to apoptosis, Eur J Immunol 24: 892-899

Sato, K; Ohtsuka, K; Hasegawa, K; Yamagiwa, S; Watanabe, H; Asakura, H; Abo, T; (1995), Evidence for Extrathymic Generation of Intermediate T Cell Receptor Cells in the Liver Revealed in Thymectomized, Irradiated Mice Subjected to Bone Marrow Transplantation. J Exp Med 182: 759-767

Savil, J; Fadok, V; Henson, P; Haslett, C; (1993), Phagocyte recognition of cells undergoing apoptosis, Immunol Today 14: 131-136

Schwartz, R H; (1990). A cell culture model for T lymphocyte clonal anergy. Science 248: 1349-1356

Schwartz, L M; Osborne, B A; (1993), Programmed cell death, apoptosis and killer genes, Immunol Today 14: 582-590

Scollay, R; Chen, W-F; Shortman, K; (1984), The Functional Capabilities of Cells Leaving the Thymus. J Immunol 132: 25-30

Solvasson, N; Lehuen, A; Kearney, J F; (1991), Int Immunol 3: 543-551

Sydora, B C; Mixter, P F; Holcombe, H R; Eghtesady, P; Williams, K; Amaral, M C; Nel, A; Kronenberg, M; (1993), Intestinal Intraepithelial Lymphocytes are Activated and Cytolytic but Do not Proliferate as well as Other T Cells in Response to Mitogenic Signals. J Immunol 150: 2179-2191

Tagakaki, Y; DeCloux, A; Bonneville, M; Tonegawa, S; (1984). Diversity of γδ T cell receptors on murine intestinal intraepithelial lymphocytes. Nature 339: 712-714

Tagliabue, A; Befus, A D; Clark, D A; Bienenstock, J; (1982), Characteristics of Natural Killer Cells in the Murine Intestinal Epithelium and Lamina Propria. J Exp Med 155: 1785-1796

Tochinai, S; Katagiri, C; (1975), Complete Abrogation of Immune Response to Skin Allografts and Rabbit Erythrocytes in the Early Thymectomized *Xenopus*. Devel Growth Diff 17: 383-394

Tompkins, R; Kaye, C; (1980). Aspects of Developmental and Comparative Immunology p403, ed. Solomon, J B, Pergamon Press, New York

Turner, S L; Horton, T L; Ritchie, P; Horton, J D; (1991), Splenocyte Response to T Cell-Derived Cytokines in Thymectomized *Xenopus*. Devel Comp Immunol 15: 319-328

Turpen, J B; Smith, P B; (1989), Precursor immigration and thymocyte succession during larval development and metamorphosis in *Xenopus*. J Immunol 142: 41-47

Umesaki, Y; Setoyama, H; Matsumoto, S; Okada, Y; (1993), Expansion of αβ T-cell receptor-bearing intestinal intraepithelial lymphocytes after microbial colonization in germ-free mice and its independence from thymus. Immunology 79: 32-37

Vaessen, L M B; Broekhuizen, R; Rozing, J; Vos, J G; Schuurman, H-J; (1986), T-Cell Development during Ageing in Congenitally Athymic (Nude) Rats. Scand J Immunol 24: 223-235

Varley, C A; (1990), T-cells and transplantation tolerance in thymectomised *Xenopus* implanted with foreign thymus. PhD Thesis

Varley, C A; Horton, J D; (1991), Lack of expression of the T-cell marker XTLA-1 in *Xenopus tropicalis*: exploration in thymus restoration studies. Dev Comp Immunol 15: 307-317

van der Velde, H; von Hoegen, I; Luo, W; Parnes, J R; Thielemans, K; (1991), The B-Cell Surface Protein CD72/Lyb-2 is the Ligand for CD5. Nature 351: 662-665

Vermeer, L A; de Boer, N K; Bucci, C; Bos, N A; Kroese, F G M; Alberti, S; (1994), MRC OX19 recognizes the rat CD5 surface glycoprotein, but does not provide evidence for a population of $CD5^{bright}$ B cells. Eur J Immunol 24: 585-592

Viney, J L; MacDonald, T T; Kilshaw, P J; (1989), T-cell receptor expression in intestinal intra-epithelial lymphocyte subpopulations of normal and athymic mice. Immunology 66: 583-587

Wang, R; Murphy, K M; Loh, D Y; Weaver, C; Russell, J H; (1993), Differential Activation of Antigen-Stimulated Suicide and Cytokine Production Pathways in $CD4^+$ T Cells is Regulated by the Antigen-Presenting Cell, J Immunol 150: 3832-3842

Wang, J; Klein, J R; (1994), Thymus-Neuroendocrine Interactions in Extrathymic T Cell Development. Science 265: 1860-1862

Williams, N H; Horton, J D; (1980), Ontogeny of Mitogen Responsiveness in Thymus and Spleen of *Xenopus laevis*. Devel Comp Immunol 1: 493-494

Wortis, H H; (1992), Surface markers, heavy chain sequences and B cell lineages. Intern Rev Immunol 8: 235-246

Wysocki, L J; Sato, V L; (1978), 'Panning' for lymphocytes: A method for cell selection. Proc Natl Acad Sci USA 75: 2844-2848

Yang, T J; Baldwin, C L; Hanby-Flarida, M; Mather, J F; Rabinovsky, E D; (1995). Monoclonal antibody BLT-1, specific for the bovine homologue of CD5, reacts with the majority of mature T cells, a subpopulation of B cells and stimulates T cell proliferation. Dev Comp Immunol 19: 337-346

Ying-zi, C; Rabin, E; Wortis, H H; (1991), Treatment of murine $CD5^-$ B cells with anti-Ig, but not LPS, induces surface CD5: two B-cell activation pathways. Int Immunol 3: 467-476

Yonish-Rouach, E; Resnitzky, D; Lotem, J; Sachs, L; Kimchi, A; Cren, M; (1991). Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature 352: 345-347

Zorbas, M; Scollay, R; (1993), Development of γδ T cells in the adult murine thymus, Eur J Immunol 23: 1655-1660

Zupo, S; Dono, M; Massara, R; Taborelli, G; Chiorazzi, N; Ferrarini, M; (1994), Expression of CD5 and CD38 by human $CD5^-$ B cells: requirement for special stimuli. Eur J Immunol 24: 1426-1433