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Functional Studies of Cytokines Produced by *Xenopus* Lymphocytes following T Cell Mitogen Stimulation

by

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(B.Sc., Dunelm)

A Thesis Submitted to the University of Durham
in Candidature for the Degree of Master of Science
Department of Biological Sciences
University of Durham
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21 JUL 1992

Dedication

To God my Lord, Jesus Christ,

for Your joyous love in my happy days;

for Your unfailing mercy in my darkest hours;

and for Your truth and just that shape my life

for a better future

Declaration

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

Mitogen studies reported in Table 3.3 were performed in collaboration with Mrs. Trudy L Horton and Mrs. Pamela Ritchie.

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I am forever indebted to my supervisor, Dr. John D Horton, for his invaluable advice, guidance and patience throughout the course of my studies and in particular his encouragement and help during the writing-up of this thesis; and especially his initial courage and faith in taking me onto this research.

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Thanks are due to Dr. Brian Shenton for the use of the FACS and its associated computing facility at the Department of Surgery, University of Newcastle-upon-Tyne, and also to Dr. Alice Givan for operating the FACS and her helpfulness.

I also would like to express my sincere appreciation to all my friends at Durham, in particular, Po-Lin, William and Winston, whose support, encouragement, advice and other ' moral-boosting ' gestures are much treasured.

Last, but by no mean least, all my love and thank you to my family, without whom nothing would have been possible; to Mom and Dad, for being so marvellous parents and all your trust, understanding, moral and financial support, and everything all these years; to Kathleen, for being such a wonderful sister always on my side. We are forever together at hearts.

Abstract

In vitro culture conditions for the production and assay of T cell mitogen-induced culture supernatants have been investigated in an amphibian model system, the clawed toad, *Xenopus*. The thesis has paid particular attention to probing the ability of such supernatants (and T cell mitogen) to activate lymphocytes from early (7 day) - thymectomized (Tx) animals, in order to investigate possible thymus-independent ' T - like ' cell emergence.

Supernatants from 24-hour-cultures of concanavalin A (ConA)-treated *Xenopus* splenocytes were first generated. Following neutralization of ConA by methyl-mannopyranoside, active supernatants (ASNs) were shown to induce proliferation of freshly-prepared adult splenocytes (supposedly unstimulated) from control *Xenopus* equally as well as *in vitro* pre-stimulated cells. ASNs were also able to enhance tritiated thymidine (^{3}HTdR) incorporation by larval thymocytes which had been ' co-stimulated ' with a sub-optimal concentration of phytohaemagglutinin. Elevated ^{3}HTdR incorporation was also observed when such supernatants were tested on splenocytes from Tx toadlets. Purification of *Xenopus* ASN was not attempted here, but such material has been shown elsewhere to contain cytokines such as interleukin-2 (IL-2). Human recombinant IL-2 proved unable to co-stimulate larval thymocytes.

The use of a panel of monoclonal antibodies investigated the possibility that T cell markers might be up-regulated on control and Tx splenocytes following *in vitro* incubation with ASN, or after *in vivo* injection with such ' cytokine ' - rich material. These flow cytometric studies on animals of various developmental stages failed to reveal experimentally-inducible expression of the T cell specific markers —— XTLA-1 and the putative CD8 marker. [Additional studies illustrated that cultured control splenocytes display blastogenesis and increased expression of major histocompatibility complex class II molecules, following ConA stimulation.] Flow cytometry interestingly

revealed the natural, *in vivo*, emergence of a low percentage of XTLA-1 and CD8 - expressing splenocytes removed from thymectomized *Xenopus* aged 6 months and older. Such presence of a few ' T - like ' cells in Tx animals of this age (but not in younger toads) was confirmed functionally through ^{3}HTdR studies on these splenocytes following ConA stimulation.

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List of Abbreviations

Ab	Antibody
Ag	Antigen
APBS	Amphibian strength phosphate buffered saline
APC	Antigen Presenting Cell
ASN	Active supernatant
αmm	α-methyl-d-mannoside (methyl-mannopyranoside)
BSA	Bovine serum albumin
ConA	Concanavalin A
ConA SN	crude supernatant produced from ConA stimulated lymphocytes
CSN	Control supernatant
DLS	Dorsal lymph sac
DNP-KLH	Dinitrophenyl - keyhole limpet haemocyanin
D.P.M.	Disintegrations per minute
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein iso-thio-cyanate
HCG	Human chorionic gonadotrophin
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
i.p.	intraperitoneal
kD	kiloDalton
L-15	Leibovitz-15
LN	Lymph node
LPS	Lipopolysaccharides
McAb	Monoclonal Antibody
MHC	Major histocompatibility complex
MLC	Mixed Leucocyte Culture
MLR	Mixed Lymphocyte Reaction
MW	Molecular weight
NK	Natural Killer
PBS	Phosphate Buffered Saline

PHA	Phytohaemagglutinin
P.I.	Propidium Iodide
s.d.	Standard Deviation
S.I.	Stimulation Index
SN	Supernatant
Tc	cytotoxic T cell
TCGF	T cell growth factor
Th	helper T cell
Ts	suppressor T cell
Tx	Thymectomized
UV	Ultra Violet

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Chapter 1

General Introduction

1.1 Phylogeny of Immunity

Comparative immunology is concerned with the study of the immune system of all classes of living creatures in the animal kingdom. The goal of such research is to obtain a clearer picture and better understanding of how the immune system evolved. Additionally, studies of the immune system of the vertebrates provide research models for immunologists to gain further insight into the human immune system. The evolution of vertebrate immunity is difficult to trace since the ancestors of vertebrates are now extinct and therefore interpretations have to be made from living animals.

Immunological studies of the invertebrates have mainly concentrated on the Protostomes rather than the Deuterostomes from which the vertebrates are believed to have evolved. This is because the arthropods and molluscs frequently have pest status, transmit disease and are of considerable economic importance.

A summary of the immuno-potentialities of invertebrates is shown in **Table 1.1** (table courtesy of Horton and Ratcliffe, 1992, in press).

In contrast to the diversity of forms seen within invertebrate phyla, all vertebrates possess a fairly basic plan of organization, belonging to one phylum —— the Chordata. The basic cellular and molecular components of immunity are common to all vertebrates, whose immuno-potentialities are also shown in **Table 1.1**.

Table 1.1

Summary of the Immune System Found in Invertebrate and Vertebrate Phyla

Invertebrates

phagocytosis / encapsulation important in eliminating non-self material

cell-mediated immunity evident early in evolution

different classes and subclasses of immunocyte plus haemopoietic tissue present in coelomates

inducible, broad spectrum, humoral immunity in some coelomates, but immunoglobulins absent from all phyla

lectins and prophenoloxidase involved in recognition of self / non-self in at least some phyla

Vertebrates

all display cell-mediated and humoral immunity

all possess a range of lymphoid tissues, which become more complex in ' higher ' forms

all possess B lymphocytes; T - equivalent cells identified in bony fishes; all ' higher ' forms have IgM, with jawed vertebrates possessing additional, non-μ, heavy chains

molecular and genetic characterization of immunoglobulins, MHC glycoproteins, T cell receptors, complement and cytokines of lower vertebrates is in progress

1.2 Use of *Xenopus* for Immunological Studies

Amphibians are ectothermic tetrapods and are represented by 3 orders; namely, Apoda, Urodela and Anura, the latter to which frogs and toads belong.

Extensive research has been made on the immune system of anurans, which provide excellent models for studying immunity as far as ontogenetic aspects are concerned. The most in-depth-studied anuran animal model is the South African clawed toad, *Xenopus* (Horton, 1988; Du Pasquier, Schwager & Flajnik, 1989). The immune system of *Xenopus* has revealed a range of immunological features comparable to that found in mammals; namely, T and B cells, a major histocompatibility complex, diverse immunoglobulin classes and several cytokines (Du Pasquier, 1982; Horton, 1992). The availability of either MHC-compatible [entirely histocompatible (Kobel & Du Pasquier, 1975) or displaying minor histocompatibility differences (Katagiri, 1978)] or MHC-incompatible *Xenopus*, together with the fact that larvae are free-living, with no maternal influence during larval development, offer immunologists an interesting model to understand fundamental immunobiological processes. The ease of breeding and husbandry of these entirely aquatic creatures in the laboratory has made *Xenopus* an attractive and convenient model for the study of immunity. Additionally, *Xenopus* can be experimentally manipulated from an early developmental age, allowing procedures such as transplantation (Horton, 1969) and early thymectomy (Horton and Manning, 1972) to be readily performed during larval life. Such a thymectomy, followed by thymus grafting experiments, has allowed studies on T cell education (Horton *et al*, 1989*a*; Horton, Horton & Varley, 1989) and T cell tolerance induction (Nagata & Cohen, 1984; Cohen *et al*, 1985; Arnall & Horton, 1986; Arnall & Horton, 1987; Horton *et al*, 1989*b*) to be performed at the amphibian level of evolution. An ever-increasing availability of monoclonal antibodies to a variety of *Xenopus* cell surface molecules (Du Pasquier, Schwager & Flajnik, 1989; Flajnik *et al*, 1988) has further potentiated studies on this

species. Additionally, species-specific markers, such as the quinacrine fluorescence spotting pattern of *Xenopus borealis* DNA (Thiébaud, 1983) and ploidy-labelling techniques (making use of triploid and diploid *Xenopus*) have been useful for tracing cell movements within transplanted tissue (Turpen *et al*, 1982; Horton *et al*, 1987).

1.2.1 Cellular Aspects of Immunity in *Xenopus*

Xenopus has several organs of immunological significance, including the thymus, spleen, gut-associated lymphoid tissue (GALT), kidney and liver.

The thymus anlagen of *Xenopus* first become distinguishable when larvae are about 3 days old / st. 40 (Manning & Horton, 1969; Katagiri & Tochinai, 1987). Each of the paired thymic buds appears as a small epithelial proliferation of the pharyngeal epithelium (Manning & Horton, 1982) but detachment soon occurs (on day 5) and the thymus becomes a separate entity that lies posterior to the eyes of the transparent larva. At 6 - 7 days, the thymus (which comprises less than 1000 cells) is visible to the naked eye through the transparent skin, and the organ can be removed relatively easily at this time (Horton & Manning, 1972). The thymus in adult *Xenopus* is found lying just under the skin, posterior to the middle ear. The thymus comprises an outer cortex and a central medulla. Cortical lymphocytes, which are rapidly-proliferating, are particularly sensitive to γ - irradiation (Russ, 1986; Russ & Horton, 1987). This organ is known to be primarily concerned with the production of T-lineage cells and there is evidence (Manning & Horton; 1982) that, like as in mammals, such T cells see antigens in the context of self MHC peptides (Du Pasquier, Schwager & Flajnik, 1989; Kaufman, Flajnik & Du Pasquier, 1991 and see below).

The existence of T and B lymphocytes in *Xenopus* has been confirmed by numerous studies using, for example, mitogens (Green-Donnelly & Cohen, 1979),

negative selection processes (Bleicher & Cohen, 1981; Nagata, 1985) and thymectomy. *Xenopus* T cells proliferate in the presence of classical mammalian T cell mitogens such as phytohaemagglutinin (PHA) and concanavalin A (ConA) (Watkins, 1985). Due to the ease of thymectomy on 6 / 7 day-old larvae, the crucial role played by T cells in the immune system of *Xenopus* has been confirmed. Early thymectomy not only impairs the ability of cultured T cells to respond to either PHA or ConA (Du Pasquier & Horton, 1976; Manning, Donnelly & Cohen, 1976; Green-Donnelly & Cohen, 1979; Nagata & Cohen, 1983), but also results in failure of acute graft rejection and impairs mixed lymphocyte reactions (MLRs: Du Pasquier & Horton, 1976; Horton & Manning, 1972; Tochinai & Katagiri, 1975; Kaye & Tompkins, 1983; Nagata & Cohen, 1983). Abolition of the plaque-forming-cell response to heterologous red blood cells (Tochinai & Katagiri, 1975; Du Pasquier & Wabl, 1977) and the low molecular weight Ig (IgY) response to dinitrophenyl - keyhole limpet haemocyanin (DNP-KLH: Du Pasquier & Horton, 1982) have been observed. However, immunological responses to T-independent antigens, such as lipopolysaccharide (LPS, a B cell mitogen: Collie, Turner & Manning, 1975), polyvinylpyrrolidone (Tochinai, 1976; Clothier *et al*, 1988; Clothier *et al*, 1989) and PPD (a protein derivative of tuberculin: Manning, Donnelly & Cohen, 1976) are not affected by early thymectomy. Interestingly, if thymus transplants (either MHC-compatible or MHC-incompatible) are given to thymectomized *Xenopus*, their antibody response to sheep erythrocytes could be restored (Gearing, Cribbin & Horton, 1984).

The T and B lymphocytes in *Xenopus* are functionally different. Anuran helper T (Th) cells are relatively γ - irradiation resistant (up to 3000 RADS), nylon wool non-adherent and surface immunoglobulin (Ig)- negative; whereas the antibody producing B cells are nylon wool adherent, surface Ig positive and γ - irradiation sensitive (500 RADS) (Blomberg, Bernard & Du Pasquier, 1980).

The spleen is a major peripheral lymphoid organ in *Xenopus*, as in all jawed vertebrates. It not only traps antigens, but is also a site for the release of lymphocytes and their proteinaceous products following antigen stimulation (Manning & Horton, 1982). There is a similarity between the histology of the mammalian and amphibian spleens. The amphibian spleen possesses thymus-dependent (perifollocular red-pulp, T cell-rich) and thymus-independent (white pulp, B cell-rich) compartments at maturity (Volpe, 1971; Manning & Turner, 1976). The spleen is also a major site of B cell development in *Xenopus* (Hadji-Azimi, Coosemans & Canicatti, 1990). This becomes evident when the white pulp areas in the spleens of thymectomized *Xenopus* remain lymphoid and stain well with anti-Ig antibodies (Bleicher & Cohen, 1981).

The gut-associated lymphoid tissue forms the front-line immune defence in *Xenopus*. This organ is enriched with the presence of IgM and IgX - secreting plasma cells. IgX, which is the amphibian analogue to the mammalian IgA, is exclusively associated with this tissue (Du Pasquier, 1989; Du Pasquier, Schwager & Flajnik, 1989).

The kidney of *Xenopus* is shown to house accumulations of B cells and is involved in the retention of experimentally-introduced antigens (Du Pasquier, 1982*a*). The liver, on the other hand, is an important site for the initial development of B lymphocytes in *Xenopus* (Manning & Horton, 1982). Bone marrow in *Xenopus* appears to be rudimentary and not actively involved in lymphopoiesis; yet this tissue is involved in the differentiation of neutrophilic granulocytes in the adult (Hadji-Azimi, Coosemans & Canicatti, 1990).

1.2.2 Molecular Aspects of Immunity in *Xenopus*

The immune system molecules of *Xenopus* show many similarities to those of endotherms. A family of glycoproteins, known as the major histocompatibility complex molecules, play a vital role in the regulation of the mammalian immune system (Klein, 1986). These highly polymorphic molecules are encoded by a large, but closely linked, set of genes called the major histocompatibility complex (MHC). An MHC has been shown to exist in diverse mammals, birds (the B locus) and in *Xenopus* (the XLA locus). The two best-studied MHC are both mammalian, the murine MHC, H-2 (located on chromosome 17) and the human MHC, HLA (located on chromosome 6). Each complex controls the synthesis of different classes of MHC proteins (for example, MHC class I and II proteins) which differ in structure, function and cellular distribution.

In mammals, T lymphocytes will only recognise antigen or antigenic peptide when it is presented in association with an appropriate class of MHC-encoded molecules. This phenomenon is known as MHC restriction and is believed to be a learned process. This has been demonstrated by Zinkernagel and Doherty (1974*a*&*b*) in the mid-1970s. They observed that MHC restriction of T cells is established not only by their own genotype but also is effected by the MHC molecules that T cell precursors see and encounter in the thymus during their differentiation and maturation. Such MHC molecules are usually of ' self ' origin and so T cell responses are self-MHC restricted, but , if foreign thymi are implanted to thymusless recipients, such response can become allo-restricted. In mammals, MHC class I molecules are expressed on almost all nucleated somatic cells and present fragments of viral proteins produced within cells to cytotoxic T cells. In contrast, MHC class II molecules are expressed constitutively on relatively few cells, including B cells and specialised antigen-presenting cells (APCs); these class II molecules present fragments of extrinsically-derived foreign antigens to helper T cells. Helper T (Th) and cytotoxic T (Tc) lymphocytes bear surface

molecules (known as the ' markers ') CD4 and CD8 respectively. These markers help to ' enhance ' the recognition and close interaction between the specific T cell concerned and MHC class II and I (respectively) on target cells. Helper T cells collaborate with B cells (and APCs) to initiate antibody production; they are also involved in the generation of cytotoxic T cells, whose function *in vivo* is the destruction of virally-infected cells. Th cells preferentially interact with antigen delivered by class II^{+ve} cells, whereas Tc recognize antigen presented class I molecules. However, a minority of mammalian Tc cells that are class II restricted have been identified. Considerable polymorphism is shown by the MHC within a particular species. Such MHC polyporphism is an important factor in promoting rapid destruction of tissues grafted between unrelated individuals.

The suggestion of an MHC existing in *Xenopus* initially surfaced in the 1970s. Now the *Xenopus* MHC [known as the XLA (for *Xenopus*-leucocyte-associated antigens)] has been extensively studied. Functional (Du Pasquier & Miggiano, 1973), genetic (Bernard *et al*, 1981) and molecular (Du Pasquier, 1989; Du Pasquier, Schwager & Flajnik, 1989; Flajnik & Du Pasquier, 1990*b*; Kaufman, Flajnik & Du Pasquier, 1991) studies have been involved in the identification of the XLA complex. With the use of alloantisera and xenoantisera (Flajnik *et al*, 1984; Kaufman *et al*, 1990*a*&*b*; Nakamura *et al*, 1986), class I , class II and class III - like molecules have been detected on the surface of *Xenopus* cells; the subregions encoding these molecules have subsequently been identified within the *Xenopus* XLA. The compatibility or incompatibility of different *Xenopus* strain tissue antigens [as determined by mixed leucocyte culture (MLC) and speed of graft rejection] together with biochemical and serological studies, have established the different MHC haplotypes expressed by these animals (Kaufman, Flajnik & Du Pasquier, 1991). Tc and Th cell populations in *Xenopus* display MHC-restricted killing and helper roles respectively. Tc targets may include both MHC class I and II molecules (Watkins, Hardings & Cohen, 1988; Horton,

Horton & Varley, 1989). Th lymphocytes in *Xenopus*, however, appear to recognize class II proteins only (Du Pasquier & Flajnik, 1990; Flajnik *et al*, 1990).

There are three classes of immunoglobulin produced by *Xenopus* B cells; IgM, IgY, IgX (Du Pasquier, 1989; Du Pasquier, Schwager & Flajnik, 1989). They are the analogues to the mammalian IgM, IgG and IgA respectively (Brandt *et al*, 1980). The repertoire of antibody found in *Xenopus* is relatively restricted in size compared to mammals (Du Pasquier, 1982*b*; Du Pasquier, Schwager & Flajnik, 1989). This restriction may relate to the need for efficient utilization of body resources, especially during larval life when lymphocyte numbers are low (only a few millions). The wastage of lymphocytes (created by random somatic mutations) is affordable only in an immune system where cells proliferate quickly and there is frequent generation of lymphocytes when compared with the life of the individual. However, it has been shown that cell cycle can be prolonged at low temperatures; as such, in the situation of cold-blooded vertebrates like amphibians, the turnover of lymphocyte generations is likely to be low compared with mammals. Furthermore, since individuals not borne *in utero* are under great selection pressure to develop an efficient immune system as soon as possible to fight against external pathogenic infections, no time can be lost on a process involving selection of randomly-appearing somatic mutations of lymphocyte clones. Absence of somatic mutational mechanisms in generating immunoglobulin diversity in *Xenopus* may explain the limited antibody diversity found in this species (Du Pasquier, 1982*b*; Schwager *et al*, 1989).

Xenopus B cells are able to mount a good proliferative response to mammalian B cell mitogens such as pokeweed mitogen (PWM: Schwager & Hadji-Azimi, 1984), anti-IgM antibodies (Schwager & Hadji-Azimi, 1985), but respond minimally *in vitro* to *E.coli*-produced LPS (Bleicher *et al*, 1983). Nevertheless, LPS can induce synthesis of immunoglobulins (Williams *et al*, 1983).

A wide range of monoclonal antibodies (McAbs) are now available for the identification of specific T and B cell surface molecules, and initial studies have been carried out using either fluorescence microscopy or flow cytometry. This panorama of McAb includes those that recognize putative αβ and γδ chains of the *Xenopus* T cell receptor (courtesy of Dr. M.D. Cooper, University of Alabama, unpublished data).

1.3 Cytokines and Interleukin-2 (IL-2)

Cytokines are soluble polypeptides involved in the regulation of both natural and acquired immunity. They serve as ' signal ' molecules for cell activation and as mediators in intercellular communication, through specific ligand-receptor interaction (Hamblin, 1988; Meager, 1990) in an autocrine or paracrine fashion. Cytokines are potent regulators, being required only in minute quantities to mediate immune responses. The actions of different cytokines are redundant and influence the action of one another. Cytokines are primarily synthesized in response to immunological stimulations and act locally; if produced in sufficient amount they may circulate and exert endocrine effects. The important roles played by cytokines are T cell activation, B cell activation, haematopoiesis, lymphotoxicity and inflammation (Watson, 1981; Schreier, 1984; Balkwill, 1988; Meager, 1990).

To-date, five groups of cytokines have been categorized, according to their principal actions in the mammalian immune system. They are: interleukins, colony-stimulating factors, interferons, tumour necrosis factors and 'others' (Gearing, 1989*a&b*; Arai *et al*, 1990; Balkwell & Burke, 1989). Nowadays, ' cytokine ' is a generic term that includes the whole spectrum of soluble mediators released by cells of any type (even non-lymphoid). ' Interleukins ' were designated as cytokines secreted by lymphoid cells

and which act as communication signals between lymphocytes; however, this is probably too narrow a definition.

Ten interleukins have so far been identified [including new interleukins like ILs -7 (Chazen *et al*, 1989), -8 (Baggiolin, Walz & Kunkel, 1989), -9 (Donahue, Yang & Clark, 1990) & -10 (Zlotnik & Chen, 1991)] in mammals, many of which have also been characterized. IL-1 and IL-2 - like molecules appear to be found in diverse vertebrate groups (see review by Secombes, 1991). In *Xenopus*, IL-1 - like material has been described in culture supernatants from LPS-stimulated peritoneal macrophages (Watkins, Parsons & Cohen, 1987), whereas a factor with IL-2 - like function is secreted into culture medium following T cell mitogen activation of splenic lymphocytes (Watkins & Cohen, 1987*b*; Turner, 1990). This thesis explores the production and utilization of active supernatants derived from *Xenopus* splenocytes stimulated with T cell mitogen and details of the relevant literature on this topic are given in Chapter 2. Since such supernatants are known to be rich in ' IL-2 - like ' cytokine, some introductory remarks concerning mammalian IL-2 are given here.

Mammalian interleukin-2 (IL-2) was the first T cell derived cytokine to be extensively characterized, since its discovery in the 1970s. It was originally called ' T cell growth factor (TCGF) ', a name describing its biological activity (Morgan, Rucetti & Gallo, 1976). It is principally responsible for progression of T lymphocytes from G_1 to S phase of the cell cycle, and in turn regulates their proliferation and subsequent clonal expansion. The mammalian secreted IL-2 is a 14 - 17kD glycoprotein encoded by a single gene on chromosome 4 (Hamblin, 1988). IL-2 is mainly produced by antigen - or mitogen -stimulated $CD4^{+ve}$ Th cells (Smith, 1990*a*&*b*) but $CD8^{+ve}$ T cells can also produce this cytokine (Grannelli-Piperno, Vassalli & Reich, 1981). IL-2 is both a paracrine and autocrine growth factor acting as a signal molecule to neighbouring lymphocytes or to the T cell that produced it (Smith, 1990*a*&*b*).

The ability of IL-2 to activate cells is shown to be dependent upon the expression of interleukin-receptors on the responding cells; there are two forms of IL-2 receptors, each with differing affinity for IL-2; these being found on both activated T cells (Smith, 1988*a*) and B cells (Muraguchi *et al*, 1985) in mammals. The high affinity IL-2 receptor is composed of two non-covalently linked polypeptides, each of which independently has some affinity for IL-2 (Tsudo *et al*, 1986; Smith, 1987; Teshigawara *et al*, 1987). The smaller polypeptide, called the β chain, is of MW 55kD and is recognized by the anti-Tac (T cell activation) monoclonal antibodies. This glycoprotein 55 (p55) has been found in soluble form (i.e., in serum —— see, e.g., Lambert, Stura & Wilson, 1989) being released by activated T lymphocytes (Motoi *et al*, 1988; Zambello *et al*, 1989) but the significance of this soluble form is not yet certain (Smith, 1989; Young *et al*, 1991). It has been suggested that the shedding of this IL-2r is due to chronic T cell stimulation (Reddy & Grieco, 1988; Campen *et al*, 1988). Shedded receptor proteins may bind free IL-2, thus intercepting its interactions with cell-based receptors. There is now accumulated evidence (see Famularo *et al*, 1990; Rubin & Nelson, 1990) that , clinically, an increased level of soluble p55 in the serum is a sign of strong antigenic stimulation , as seen in chronic infections [such as in the cases of atopic-dermatitis (Kapp, Schopf & Piskorski, 1988), autoimmune diseases (Keystone *et al*, 1988; Hussain *et al*, 1990) and active multiple-sclerosis (Adachi, Kumamoto & Araki, 1990)] and acute rejection of organ transplants (Perkins *et al*, 1989; Stockenhuber *et al*, 1990; Ippoliti, Vigano & Pizzolo, 1991). Recent studies have shown that p55 β chains are expressed on non-T cells such as NK cells and also activated B cells (Nakanishi *et al*, 1984; Zubler *et al*, 1984; Zola *et al*, 1991). The other IL-2 receptor peptide is known as the α chain, which is of 70kD and is expressed naturally on resting / unstimulated T cells. Upon antigenic / mitogenic stimulation of the T cell, the α chain, which has a longer intracytoplasmic section, acts as a ' signal ' for the induction, synthesis and expression of the β chain. p55 and p70 together become a high affinity IL-2 binding

site for exogenous IL-2 to interact with. Endogenous IL-2 is then produced by the activated T cell, which also increases p55 expression, which in turn leads to more IL-2 synthesis via a positive amplification system. Once the stimuli imposed on T cells are removed, transcription of IL-2 and its receptor genes eventually decreases, proliferation then stops and the cells return to the G0 phase of the cell cycle.

Other than inducing T cell proliferation and upregulation of IL-2 receptor expression, the biological activities of IL-2 include: (i) growth factor for immature T cells and thymocytes; (ii) stimulant to the growth of NK cells, enhancing their cytolytic function, eventually leading to the proliferation of lymphokine activated killer (LAK) cells; (iii) upregulation of macrophage phagocytic activity; (iv) stimulation of B cells both as a growth factor and as a stimulus for antibody synthesis (Smith, 1990*a*&*b*).

Mammalian IL-2 was initially generated and purified from T cell mitogen - induced leucocyte culture supernatants from sources such as human (Mier & Gallo, 1982), cow (Brown & Grab, 1985), rat (Di Sabato, 1982), mouse (Granelli-Piperno, Vassali & Reich, 1981; Riendeau *et al*, 1983) and gibbon (Henderson *et al*, 1983). However, with the advancement in molecular cloning techniques, the protein purification procedure was superseded, as recombinant IL-2 became available (see, for example, Kato, 1988).

1.4 Purpose of Thesis

This thesis was initiated to probe the nature of ' cytokine - like ' substances released into culture medium supernatant by T cell mitogen - activated *Xenopus* lymphocytes *in vitro* and to investigate the responsiveness of lymphocytes taken from early-thymectomized animals to such active supernatants as recently described by Turner (1990) and Turner *et al* (1991)

The initial objective of this research, as presented in Chapter 2, was to investigate *in vitro* culture conditions for the production and assay of such ' cytokines '. In this Chapter, particular attention is paid to probing the ability of such ConA-derived active supernatants (and T cell mitogens) to induce proliferation in lymphocytes from early-thymectomized *Xenopus*.

The second goal of this thesis (Chapter 3) was to determine if *in vitro* stimulation of lymphocytes with ' cytokines ' or *in vivo* injection of active culture supernatant would result in changes in expression of certain lymphocyte surface antigens, such as the appearance of T cell markers on lymphocytes from early-thymectomized toadlets. These experiments involved flow cytometry of monoclonal antibody-stained lymphocytes. The studies were especially designed to explore the possibility of ' T-like ' cell development in thymectomized *Xenopus*.

Chapter 2

Studies on Lymphocyte Proliferation Induced by Cytokines Secreted from T Cell Mitogen - Stimulated *Xenopus* Splenocytes

2.1 Introduction

Lectins have long been widely used in many fields of biology for preparative and analytical purposes. The binding mechanism between cells and lectins initiates a variety of biological reactions. One of the most dramatic effects of the interaction of lectins with lymphocytes is mitogen stimulation, i.e., the triggering of quiescent, non-dividing lymphocytes into a state of growth and proliferation. In contrast to antigenic stimulation, in which specific clones of lymphocytes with appropriate antigen receptors are induced to proliferate, lectins activate multiple lymphocyte clones irrespective of their antigenic specificity. Following polyclonal mitogenic stimulation, changes such as increased permeability for various metabolites and an accelerated turnover of phospholipids take place at the cell membrane. Intracellularly, genetic derepression (RNA, protein synthesis) leads to morphological changes and ultimately the cells synthesize DNA and enter mitosis. Continued presence of polyclonal mitogens achieves growth and differentiation (from precursors to effectors) of the activated lymphocytes.

In addition to these events, which are common to most cells undergoing active growth, stimulated lymphocytes exhibit certain unique features. Soon after activation, stimulated lymphocytes release a variety of biologically-active polypeptides, known as cytokines, such as IL-2, γ-interferon and so on.

Mitogens have been extensively used to investigate the immune system of a wide spectrum of animals, such as mammals, birds, reptiles, amphibians and fishes. In amphibians, as in ' higher ' vertebrates, certain mitogenic lectins stimulate only the thymus-dependent population of lymphocytes (T cells) and fail to achieve mitosis of thymus-independent (B) cells. Plant lectins such as concananvalin A (ConA) and phytohaemagglutin (PHA) are T cell mitogens, whereas lipopolysaccharide prepared from *E.coli* is mitogenic for B cells. In endotherms, following T cell mitogen challenge, IL-2 (and other cytokines) are released by the activated T lymphocytes into the culture medium. Such culture medium was initially described as ' conditioned ', and was noted to be able to sustain culture of normal T cells for prolonged periods (Morgan, Rucetti & Gallo, 1976). Mitogen-induced culture supernatants, with interleukin-2 - like properties have been identified in echinoderms (starfish, *Asterias rubens* : Leclerc *et al*, 1988), fish (carp, *Cyprinus carpio* : Caspi & Avtalion, 1984), reptiles (snake, *Spalerosophis diadema* : El Ridi, Wahby & Saad, 1986), birds (chicken : Schauenstein *et al*, 1982; Schnetzler *et al*, 1983) and amphibians (*Xenopus* : Gearing, 1985; Watkins & Cohen, 1987; Turner, 1990). Studies attempting to use IL-2 - like factors across phylogenetic barriers (between and within these classes) have also been carried out. Most of the results show uni-directional stimulation (**Table 2.1**).

An amphibian ' T cell growth factor ' - like substance has been identified from culture supernatants of control, but not thymectomized, *Xenopus* splenocytes following T cell mitogen stimulation or MLC - reactivity (Cohen, Watkins & Parsons, 1987; Watkins & Cohen, 1987*b*; Turner & Horton, 1989). Biochemical characterization of this putative ' IL-2 ' - like activity present in such active supernatants has been carried out (Watkins & Cohen, 1987*a*). Following purification with SDS-PAGE, it was revealed that the amphibian IL-2 is ascribed to a protein of 14kD (Haynes & Cohen, 1991). These authors also suggested that there is no cross-reactivity between the human and amphibian IL-2.

Table 2.1

Summary of IL-2 Reactivity on Lymphoblasts

		Types of Lymphoblasts						
		Hu	Ra	Mo	Gi	Ca	Ch	*X.l.*
Source of IL-2	Human	√[1]		√[2]		√[3]		X[4]
from TCGF-rich	Rat	X[5]	√[5]	√[2]		√[3]		
Supernatants	Mouse	X[2]		√[2]		X[3]	X[7]	X[8]
	Gibbon	X[6]		√[2]	√[6]	√[3]		
	Carp					√[3]		
	Chicken			X[7]			√[7]	
	Amphibian: *X.laevis*	X[4]		X[4]				√[8]

√ indicates positive cross-reactivity whereas X indicates negative cross-reactivity. Empty spaces represent unavailable information. Numbers by each sign are references.

[1]Mier & Gallo (1982)

[2]Granelli-Piperno, Vassali & Reich (1981)

[3]Caspi & Atvalion (1984)

[4]Watkins (1985)

[5]Di Sabato (1982)

[6]Henderson, Hewetson, Hopkins, Sowder, Neubauer & Rabin (1983)

[7]Schnetzler, Oommen, Nowak & Franklin (1983)

[8]Watkins & Cohen (1987*b*)

This Chapter further investigates aspects of lymphocyte activation - assayed by tritiated thymidine incorporation - induced by cytokines secreted from T cell mitogen-stimulated *Xenopus* splenocytes. The ' optimal ' dose of ConA for directly stimulating *Xenopus* splenocytes and thymocytes and for the generation of cytokine-rich supernatants is initially assessed. The ability of ConA supernatants to stimulate freshly-prepared splenocytes and pre-cultured lymphocytes was then investigated. Thus Watkins and Cohen (1987*b*) reported that only T cell mitogen pre-cultured lymphocytes (i.e., lymphoblasts) are able to proliferate when treated with TCGF - rich culture supernatant, in contrast to freshly-prepared or medium pre-cultured lymphocytes, which did not respond.

One major consideration of this Chapter has been to use, as assay cells, lymphocytes removed from early-thymectomized animals. It has been well documented that following early-thymectomy, Tx *Xenopus* are unable to acutely reject skin allografts (Horton & Manning, 1972; Horton & Horton, 1975; Nagata & Cohen, 1983), and also display impairment of *in vitro* T cell proliferative activities, as mentioned in the General Introduction (Chapter 1). However, it has been shown that both in a mammalian (Gillis *et al*, 1979) and amphibian (Watkins, Parsons & Cohen, 1987) situation, the proliferative response of splenocytes from thymus-impaired animals to T cell mitogen can be restored if exogenous T cell growth factor is supplied. In this Chapter, the ability of active supernatants (presumably rich in an array of cytokines) to directly achieve mitogenesis of freshly-prepared splenocytes from Tx animals is examined. Additionally, the capacity of ASNs to promote (co-stimulate) the proliferation of larval thymocytes to T cell mitogen stimulation is examined. The last part of this Chapter re-assesses (see Watkins, Parsons & Cohen, 1987) whether human recombinant interleukin-2 is able to stimulate *Xenopus* lymphocytes *in vitro*, since this human molecule has been shown to be able to modulate *Xenopus* immune response *in vivo* (Ruben, Clothier & Balls, 1985; Ruben, 1986; Ruben *et al*, 1987; Horton *et al*, 1989*a*).

2.2 Materials and Methods

2.2.1 Animals: Species, Breeding and Husbandry

Xenopus used were all bred and reared in the laboratory. Outbred *Xenopus laevis* used for breeding were commercially purchased from Xenopus Ltd. The parents of J strain *X.laevis* (formally known as G strain) were themselves the offspring from animals donated by Dr. C. Katagiri of Hokkaido University in Japan. The *X.laevis / X.gilli* (LG15) and *X.laevis / X.muelleri* (LM3) clonal adult females were generous gifts from Dr. L. Du Pasquier at the Basel Institute of Immunology, Switzerland.

Xenopus were stimulated to spawn by injection of human chorionic gonadotrophin (HCG; Griffin & George Ltd.). Outbred *Xenopus laevis* are MHC undefined (as there is no record on their MHCs) and can be bred naturally. An initial 250IU of HCG was injected into the dorsal lymph sacs of males and females so that they could be ' primed ' a few days before breeding. On the actual day for breeding another 500IU of HCG was given to each animal. One male and one female were put together in an aquarium, in which a piece of nylon gauze was placed. They were kept in the dark and left undisturbed overnight. The next morning fertilized embryos would have attached to the gauze, which could then be placed in aquaria filled with aerated and dechlorinated water.

An identical breeding protocol was used for the J strain *X.laevis*. This strain was initially inbred and developed in the laboratory of Dr. Katagiri (Tochinai & Katagiri, 1975). J strain animals are homozygous for their MHC (jj MHC haplotype). However, each animal differs from one another by minor histocompatibility antigens. The latter became apparent when these animals were shown to reject grafts from each other chronically (Di Marzo & Cohen, 1982).

Clonal *Xenopus* were originally generated by crossing *Xenopus laevis* with *Xenopus gilli* or *Xenopus laevis* with *Xenopus muelleri* for producing *Xenopus* LG and LM clones respectively (Kobel & Du Pasquier, 1975). Female toads from these interspecies crosses produce both large, diploid and small, haploid eggs. The large eggs have become diploid since they underwent a process of endoreduplication during oogenesis, whereas the small eggs did not. During meiosis, identical (endoreduplicated) chromosomes pair in large eggs only, whereas in small eggs, synapsing between *gilli* and *laevis* homologues is often unsuccessful. When fertilized with ultra-violet irradiated sperm, the large diploid eggs will produce clonal offspring that are identical to their mother and each other. The small haploid eggs fertilized with the same sperm, which have their DNA destroyed during irradiation, will start development, yet eventually die due to lack of sufficient genetic material.

Clonal *Xenopus* were produced by *in vitro* fertilization. The females were given 50 - 100IU of HCG into their dorsal lymph sacs the day before they were due to spawn. 500IU was then given to each animal early in the morning when eggs were required. Approximately 5 hours later these female toads were ready to spawn, judging from the redness of their cloacae. Sperm suspension was prepared from a pair of testes, which were taken from an anaesthetised male *X.laevis*; testes were teased apart using sterile scissors and forceps in 2 - 3ml amphibian strength L-15 culture medium. In order not to introduce paternal genetic material to the LG eggs, a thin layer of sperm suspension was spread on a number of sterile slides, and the latter placed under an ultra-violet lamp. The wavelength of this lamp is 254nm and sperms were irradiated for a total of 6 and a half minutes (a few drops of water were added to each slide after 3 minutes). This UV-irradiated sperm suspension was then collected and kept on ice. A ready-to-spawn female was then taken out from water and induced to lay eggs by squeezing her abdomen firmly. Eggs were added to UV-irradiated sperm in a watch glass, and after 2 minutes were transferred to a dish containing 10% amphibian ringer. All these

procedures were carried out at room temperature. Gradually embryos were transferred into aquaria of aerated and dechlorinated water.

All animals were reared in aerated and dechlorinated water at 23°C 1°C. As soon as embryos developed into tadpoles, they were fed with nettle powder until the end of their metamorphosis. Toadlets were fed with *Tubifex* worms initially and then gradually introduced to minced bovine / pig heart. Animals were fed twice a week on the day before their aquaria were cleaned.

Ages of animals used for various experiments are shown in the Results Section.

2.2.2 Thymectomy

Thymectomy was carried out on *Xenopus* tadpoles at 6 - 7 days of age / at stage 47 - 48 (Nieuwkoop & Faber, 1967). Thymectomy was by electromicrocautery, following the method of Horton & Manning (1972). The pair of thymi was destroyed with a fine tungsten needle, to which a high frequency voltage was applied; the operation being performed under a stereo-microscope. Some animals also were sham-thymectomized (cautery applied, but thymus left intact). Operated tadpoles were initially kept in aquaria part-filled with 30% amphibian ringer, and were left undisturbed in the dark overnight to recover. The thymic regions of these animals were frequently examined during larval life under a stereo-microscope. If there were signs of the appearance of a thymus, such animals would be discarded; however, thymic regeneration was extremely rare.

Thymectomy operations were performed by Dr. J.D. Horton or Mrs. T.L. Horton, who hold animal licences granted by HM Home Office. There was insufficient

time for the author to learn this operation procedure within the time-limit of this research. Close observation of the thymectomy procedure was, however, carried out.

2.2.3 Media

The principle culture medium used for all *in vitro* experiments was Leibovitz-15 (L-15) medium (Flow Labs), adjusted to amphibian osmolarity at 220mosm with autoclaved double distilled water. 0.01M H.E.P.E.S. buffer, 1.25mM L-glutamine, 2.5μg/ml Fungizone, 50μg/ml Streptomycin, 50IU/ml Penicillin (all from Flow Labs) and 0.083mM β-mercapto-ethenol (BDH) were added to modify the medium. It was further supplemented either with heat-inactivated (decomplemented) foetal calf serum (FCS; Gibco batch 1060) or bovine serum albumin (BSA; Fraction V, Sigma). The final concentration of FCS was always at 1% of the total volume of medium when used in proliferative assays. Five percent or 10% FCS was used for long term *in vitro* culture work. BSA was added at a final concentration of 0.25% w/v and this medium was used for amphibian cytokine / supernatant generation. All media were prepared aseptically in a laminar air flow hood and were sterilized by passing through 0.22μm filters (Flowpore). Finally, 0.01M sodium bicarbonate (Flow Labs) was added to the media, in which cell cultures were incubated, as a buffer and for nutritional purposes.

The medium used to prepare sperm suspensions for *in vitro* fertilization constituted 4 parts of double distilled water to 6 parts of L-15 medium.

Amphibian ringer comprised 6.6g NaCl, 0.15g KCl, 1.35ml CaCl and 0.3g $NaHCO_3$ (all from Sigma), dissolved in one litre of double distilled water. It was adjusted to pH 7.6 - 7.8.

2.2.4 Preparation of Leucocyte Suspensions

Removal of lymphoid organs was carried out aseptically in a laminar air flow hood, its working surface having been swabbed down with 70% alcohol. All instruments were treated and kept in 95% alcohol. Donor animals were given an overdose of anaesthetic in a solution of 3-amino-benzoic acid ethyl ester (Sigma) in double distilled water. When the animal was heavily anaesthetized, its ventral skin and the head area were spread liberally with 70% alcohol for removal of spleen and / or thymus.

For the removal of spleen, the skin and the body wall on the left hand side of the abdomen were removed using scissors and watch-makers forceps. The spleen was carefully excised and placed in a small, sterile Petri-dish (Costar), containing 2ml sterile culture medium. Similar procedures were used for the removal of the thymus, except that the region of skin between the eye and the forearm was removed. The pigmented thymus, on either side of the head, was carefully excised, with the surrounding fatty tissue trimmed away. This pair of cleaned thymi was then placed in a small, sterile Petri-dish (Costar) containing 2ml sterile culture medium.

After the removal of spleen from a thymectomized animal, its thymic region was carefully examined under a microscope to confirm that thymus regeneration had not occurred. If there were any signs or doubt, this donor would have to be discarded.

Spleens, or pairs of thymi, were gently crushed between the frosted ends of two microscopic slides (previously sterilized with 100% alcohol and flamed). When tadpoles or young post-metamorphic toadlets were used, thymus and / or spleen were teased apart using tungsten needles to yield sufficient cells. The cells were then transferred to a 5ml plastic sterile test tube (Falcon). An additional 1ml sterile culture medium was used to wash the Petri-dish and collect any remaining cells. The tube was

left on ice while clumps settled to the bottom. The supernatant cell suspension was then carefully taken off, leaving clumps behind, and transferred to a fresh sterile test tube. Cells were spun at 300g for 10 minutes at 4°C twice, by taking off supernatant and resuspending the pellet with fresh and cold sterile medium. Cells were counted in a Neubauer haemocytometer (American Optical) before the last washing procedure. Viable leucocytes alone were counted; these were distinguished by their ability to exclude the uptake of the vital dye, trypan blue (NBL). The appropriate cell concentration was then adjusted in the bicarbonated culture medium.

2.2.5 Enrichment of Lymphocytes using Density Gradient Centrifugation

This protocol was used frequently with the larger spleens (from 1 - 2 years old *Xenopus*), which are often rather erythroid. When the initial spleen cell suspension was prepared, it was spun at 300g for 10 mins at 4°C. The cell pellet was subsequently resuspended in 1 - 2ml fresh sterile medium and layered gently over 3 - 4ml Histopaque 1077 (density = 1.077; Sigma). This was then spun at 250g for 10 mins at 4°C. A white and opaque band, which contained mainly lymphocytes, was formed between the medium and Histopaque, whereas erythrocytes were found at the bottom of the tube. Lymphocytes were carefully recovered from the Histopaque with the use of an autoclaved Pasteur glass pipette and transferred to a fresh test tube, where they were resuspended in about 1ml fresh medium, washed and trypan blue - excluding (viable) cells counted. Histopaque-treated lymphocytes were treated as usual from this step onwards.

2.2.6 Tritiated Thymidine Incorporation following Mitogen Stimulation

The concentration of cells for proliferative assays was adjusted to 1X 10^6 splenocytes or 2X 10^6 thymocytes per ml of sterile culture medium, which was either BSA- or FCS- supplemented. Cultures were set up in (at least) triplicate, with an aliquot of 100µl of cell suspension into each well, using 96-well V-shape-bottomed tissue culture plates (Sterilin; Cell Cult). Various concentrations of ConA (Flow Labs) were prepared freshly in the bicarbonated medium, and for each well, a 10µl aliquot was added. 100µl of autoclaved double distilled water was put into each well surrounding the culture triplicates; this was to prevent dehydration of cultured cells and so as to minimize the " edge effect " (which may affect the experiment). Cells were then cultured for 3 - 5 days (see Results) in a humidified incubator, set at 26°C ± 1°C with 5% CO_2 in the atmosphere. Twenty four hours before harvesting and scintillation counting, 1µCi tritiated thymidine (^{3}HTdR, Amersham; Sp. Act. = 5Ci / mmol) was added to each well, using a multi-dispensing pipetter (BCL).

In order to confirm that the dose of ConA used to generate active supernatants from 5X 10^6 splenocytes per ml in 24-well flat-bottemed plates (Sterilin; Cell Cult) was in fact inducing good levels of proliferation, an additional experiment was set up as follows. Splenocytes were here cultured at 5X 10^6 / ml and 100µl of cells were stimulated with 10µl of various doses of ConA, in flat-bottomed 96-well plates.

A semi-automatic harvester (Skatron) is used to harvest radioactively-labelled cells from microtitre plates onto fibre-glass filter mats. Mats were kept in a 60°C oven until dry. Individual discs containing harvested cells were then punched out into scintillation vials (Pico ' Hang-In ' vials; Packard). Each vial was dispensed with 4ml of scintillation fluid (β-fluor; National Diagnostics). Vials were then placed in racks and

analysed for radioactivity emissions from assay cells (that had incorporated tritiated thymidine in their DNA) in a scintillation β-counter (Tricarb; Packard).

2.2.7 Generation of Supernatants using ConA and Neutralization of this Mitogen using Methyl-mannopyranoside

Splenocytes from 1 - 2 years old outbred or J strain *Xenopus laevis* were cultured in 0.25% BSA- supplemented medium. Cell suspensions were initially prepared as in **2.2.4&5** and then adjusted to 5X 10^6 leucocytes per ml. 24-well flat-bottomed plates (Sterilin; Cell Cult) were used and 1ml aliquot of cell suspension was dispensed into each well. ConA supernatants (SNs) were initially generated with 1μg of ConA to 5X 10^6 cells / ml, but were later generated by adding 2.5μg/ml ConA to the same cell concentration. 100μl of the appropriate dilution of mitogen was added into every well to give the desirable ' in-well ' concentration (as stated above). Control SNs were generated using 1ml of splenocytes cultured with 100μl medium instead of mitogen. These cell cultures were incubated for 24 hours at 26°C ± 1°C in a 5% CO_2 incubator. Contents of each well were then gently resuspended. Plates were subsequently centrifuged at 300g for 10 mins at 4°C. Culture SNs were then removed by gentle pipetting and were transferred to clean tubes. Both experimental and control SNs were treated with methyl-mannopyranoside [α-methyl-d-mannoside (αmm); Sigma], which neutralizes ConA (see below). These supernatants were finally filtered through 0.2μm filters (Flowpore) and stored in freezing cryovials (Nunc) at -20°C.

Methyl-mannopyranoside was dissolved in autoclaved double distilled water and filtered to make a 1M solution. Various ' in-tube ' concentrations of αmm, namely 0.01M, 0.02M, 0.05M and 0.1M were initially tested on a ConA solution at 10μg per ml (which became approximately 1μg per ml when 10μl of this ConA solution was

added to a well containing 100µl cell suspension). The above concentrations of αmm were prepared by taking 1 part of 1M αmm into 99, 49, 19 and 9 parts of the ConA solution respectively. The effectiveness of αmm in neutralizing ConA was assessed by the failure of these αmm ' treated ' mitogen solutions to stimulate freshly-prepared splenocytes. The most effective dose of αmm was 0.1M and so this concentration was selected for standard absorption of ConA from supernatant, i.e., 1 part of 1M αmm to 9 parts supernatant.

2.2.8 Proliferative Assay for Supernatant Activity: Use of Freshly-prepared and Pre-cultured Lymphocytes

The assay cells used for testing the proliferative activity of supernatants were either splenocytes or thymocytes. These were used either ' fresh ' (i.e., directly from the animal) or following pre-culture, for various periods of time, with or without ConA. ConA was used in an attempt to drive the expression of T cell growth factor receptors (Watkins & Cohen, 1987*b*).

Pre-cultured lymphocytes were prepared by setting up splenocytes at 4 - 5X 10^6 leucocytes / ml and by establishing 2X 10^6 thymocytes / ml, both with 1µg/ml ConA (Flow Labs); pre-culture was set up in 24-well flat-bottomed plates (Sterilin; Cell Cult) in 10% FCS L-15 medium. Lymphocytes pre-cultured in medium alone acted as controls. Cells were fed with fresh 10% FCS medium every 3 - 4 days; here plates were spun at 300g for 10 mins at 4°C, supernatant of 1ml taken up from one side of the well without disturbing the cells, and finally the pellet of cells gently resuspended in fresh medium. When cells were ready to be assayed, they were harvested and viable cells counted.

1X 10^6 splenocytes or 2X 10^6 thymocytes either ' fresh ' or ' pre-cultured ' were used for the supernatant assay; cells were now kept in 1% FCS L-15 medium. 100µl aliquots of cell suspensions were pipetted into individual wells of a 96-well flat-bottomed tissue culture plates (Sterilin; Cell Cult). 50µl of medium was dispensed into each well before adding 50µl of stimulants, i.e., ASN in the experimental culture, CSN in the control culture or medium alone. Thus the final volume per well was 200µl , with the amount of SN in each well 1 in 4. Cultures were set up in triplicates and incubated at 26°C ± 1°C in a humidified 5% CO_2 incubator for 48 hours before each well was pulsed with 1µCi ^{3}HTdR (Amersham; Sp. Act. = 5Ci / mmol). They were incubated for a further 24 hours prior to harvesting and scintillation counting, as described above.

2.2.9 Co-stimulation Assay for Supernatant Activity

Splenocytes and thymocytes from late larval stage (st. 56 - 59) and young post-metamorphic animals were used to determine whether ASN could interact with a sub-optimal dose of T cell mitogen, to drive lymphocyte proliferation. Due to the low number of cells obtainable from each tadpole, workable populations of splenocytes and thymocytes were routinely prepared by pooling lymphocytes from several animals together. Cell suspensions were prepared with tungsten needles, as described in **2.2.4**, and were adjusted to 5X 10^5 leucocytes / ml for splenocytes and 2X 10^6 leucocytes / ml for thymocytes in 1% FCS L-15 medium. Then into each well of a 96-well flat-bottomed plate (Sterilin; Cell Cult) a 100µl of cell suspension was dispensed. 50µl of sub-optimal doses of PHA (Flow Labs), which either became 0.5µg / ml and 0.05µg / ml ' in-well ', was added to every well. PHA, rather than ConA, was used because the ConA inhibitor αmm was present in the culture supernatants. 50µl supernatant under test was finally added to each well. Controls included cells cultured in medium alone, PHA alone or SN

alone. In all cases the final volume per well was 200µl. Proliferation was assayed as in **2.2.8.**

2.2.10 Experiments Employing Mammalian (Human) Recombinant Interleukin-2 (rIL-2)

2.2.10.1 Stimulation of Murine Lymphocytes with rIL-2

Animals used for this study were BALB/c mice. They were between 6 - 10 weeks old at the time of experimentation. The medium used was RPMI 1640, supplemented with 2mM L-glutamine, 100IU/ml Penicillin, 100µg/ml Streptomycin, 20mM H.E.P.E.S. , 2.5µg/ml Fungizone (all from Flow Labs), and 50µM β-mercaptoethenol (BDH), and 10% heat-inactivated foetal calf serum (Flow Labs). Prior to setting up culture, 23.8mM sodium bicarbonate (Flow Labs) was added into the medium, which was prepared aseptically and filter-sterilized.

A mouse was killed by breaking its neck; under aseptic conditions, both lobes of the thymus and the spleen were removed, and then placed in separate Petri-dishes (Costar), each containing 2ml RPMI 1640 cell culture medium. Organs were chopped gently under the medium and clumps were dissociated between two sterile ground glass slides to obtain single cell suspensions, which were transferred to clean test tubes (Falcon) to allow any tissue debris to settle. The supernatant cell suspensions were then transferred to other tubes and centrifuged at 150g for 10 mins at 4°C. The pellets were resuspended in 3ml medium and layered over 4ml Histopaque 1077 (Sigma; density = 1.077) for lymphocyte enrichment (see **2.2.5**). Lymphocytes were collected and resuspended in fresh medium. They were spun twice before cultures were set up,

with the cell numbers counted and viability determined with trypan blue (NBL) exclusion prior to the last wash.

A dose response assay was initially set up to find the optimal ConA dosage for both murine splenocytes and thymocytes, with similar procedures to the amphibian model as described in **2.2.6**, but with cell concentrations at 2X 10^6 / ml and 1X 10^7 / ml respectively, and incubation temperature set at 37°C.

For the IL-2 assay, murine splenocytes (2X 10^6 leucocytes / ml) and murine thymocytes (1X 10^7 leucocytes / ml) were cultured in 24-well plates (Sterilin; Cell Cult), with 1ml aliquots of cell suspensions being dispensed into individual wells. To each well was added either 100µl of 25µg/ml ConA (2.5µg/ml ConA ' in-well '), or medium. Cells were cultured for 24 hours at 37°C in a 5% CO_2 humidified incubator. ConA-treated cells (now ' blasts ') and medium pre-cultured cells were then washed three times with RPMI 1640 containing 25mM methyl-mannopyranoside (Sigma). 50µl of splenocytes (1X 10^6 / ml) or thymocytes (2X 10^6 / ml) were then distributed into the wells of 96-well flat-bottomed plates (Sterilin; Cell Cult). The rIL-2 added at this stage was human recombinant interleukin-2 from Hazleton Laboratories, U.S.A.. Serial dilutions (1 in 2) were prepared from the initial concentration of 500IU/ml ' in-well '. 50µl of each dilution of rIL-2 was dispensed into each triplicate set of cells. The negative control was culture medium alone. Plates were incubated for 72 hours at 37°C. At 18 - 24 hours before harvesting, each well was pulsed with 1µCi ^{3}HTdR (Amersham; Sp. Act. = 5Ci / mmol).

2.2.10.2 Stimulation of Amphibian Lymphocytes with rIL-2

The assay cells used here were splenocytes and thymocytes from late larval stages (st. 56 - 59) and young post-metamorphic animals. The assay was the same as the lymphocyte co-stimulation assay described in **2.2.9**, except that rIL-2 (60IU

/ ml) was used instead of supernatant. The final volume per well was 200µl and rIL-2 represented 25% of the well volume. That is, ' in-well ' concentration for IL-2 was 15IU/ml, which was a saturating level for murine lymphocytes (see Results, and personal communication with Dr. E. Ingham, Leeds University). This experiment was a 3-day assay, which was cultured, pulsed and harvested as described before.

2.2.11 Statistical Representation of Data

Data is shown in mean D.P.M. (disintegrations per minute) per replicate culture, with the respective standard error, i.e. $X \pm \sigma_{n-1}$.

Stimulation Indices (S.I.) are useful indicators of how well the assay cells respond to a particular stimulant when compared with the control, and are calculated as follows:

i> For mitogen and rIL-2 studies,

$$S.I. = \frac{\text{Mean D.P.M. of Culture Treated with the Mitogen or rIL-2}}{\text{Mean D.P.M. of Culture Treated with Medium}}$$

ii> For supernatant assays,

$$S.I. = \frac{\text{Mean D.P.M. of Culture in } \alpha\text{mm-treated ASN}}{\text{Mean D.P.M. of Culture in } \alpha\text{mm-treated CSN}}$$

In the latter case, S.I.s were not calculated using the D.P.M. from assay lymphocytes cultured in medium alone as the denominator, since medium itself does not act as a ' real ' control to ASN.

The Student's *t* test was used to test the significance of results. This test was chosen because the sample size (per culture replicate) was less than 30 and the population where the sample was drawn from was assumed to be normally or near-normally distributed. Any pair of data is compared in such a way that the significant level (the *p* value) is set at 5%.

2.3 Results

Initial experiments assessed the optimal dose of ConA to give maximum stimulation of *Xenopus* splenocytes and thymocytes from animals aged between 6 to 18 months (sections **2.3.1&2**).

2.3.1 Proliferative Responses of *Xenopus* Splenocytes and Thymocytes to ConA in FCS Medium

Splenocytes from outbred *Xenopus laevis* showed maximum incorporation of tritiated thymidine at the concentration of 1μg/ml ('in-well') in a standard 3-day proliferative assay (**Fig.2.1**). This was also the dose at which thymocytes responded best.

To ensure the reproducibility of results between experiments, splenocytes of an individual outbred *X.laevis* were used to set up replicate dose response assays in 2 microtitre plates. 1μg/ml was found to be optimal and counts of both plates at a particular ConA dilution were very similar (**Fig.2.2**).

Lymphocytes from other *Xenopus*, namely LG and LM clonal animals, *X.tropicalis* and *X.borealis* also respond optimally to 1μg/ml ConA (data not shown) at the cell concentrations used above.

In another experiment, the effect of density gradient centrifugation (using Histopaque 1077) was examined. Results on two separate splenocyte populations, one untreated and the other passed over Histopaque, are shown in **Fig.2.3**. In the 3-day assay, Histopaque-enrichment of lymphocytes appeared not to interfere significantly with their ability to respond to ConA at 0.5 and 1μg/ml. Depression of mitogen-reactivity was noticeable, however, when 0.1μg/ml ConA was used. In the 5-day assay, Histopaque-enriched splenocytes showed marginally elevated D.P.M.s compared with untreated cells at most ConA concentrations (and even in medium alone). Such separated lymphocytes responded especially well to ConA at the concentration of 1μg/ml after 5 days of culture. Additional assays (not shown) indicated that higher ^{3}HTdR incorporation was routinely seen after Histopaque separation in medium-cultured splenocytes. It was also observed that such cells sustained higher viability in culture than unseparated splenocytes. Histopaque separation was therefore routinely used in the preparation of erythrocyte-rich splenocytes suspension from ' old ' *Xenopus* . It was considered that death of erythrocytes (which have a relatively short life-span *in vitro*) might well tend to ' spoil ' the culture medium and that removal of erythrocytes would therefore promote leucocyte viability.

2.3.2 Proliferative Studies Defining Conditions for Generating and Assaying ConA-stimulated Culture Supernatants

2.3.2.1 Dose-response of Splenocytes to ConA in BSA Medium

Since BSA is used instead of FCS (to minimize foreign serum components) in the process of amphibian cytokine (' supernatant ') generation, a dose response assay had to be set up in this BSA medium, while maintaining all other culturing conditions the same (e.g. cell concentration, number of cells per well, amount of stimulant per well), to find out the optimal stimulatory dosage of ConA. 0.5μg/ml ConA for 1X 10^6 cells / ml was found to give the best stimulation (**Fig.2.4**).

2.3.2.2 Defining Optimal ConA Concentration for Stimulating 5X 10^6 Splenocytes in BSA Medium

In the production of amphibian cytokine, 1μg/ml ConA for 5X 10^6 splenocytes (in one ml of culture medium) was initially tested, following the method of Turner (1990). However, the active supernatant produced in this manner was found to have only low stimulating capacity in preliminary cytokine assays (data not shown). Therefore a dose response proliferative assay was set up in BSA medium using 5X 10^6 splenocytes per ml. To further mimic the conditions of supernatant generation, a 96-well flat-bottomed plate was used in this experiment. 2.5μg/ml ConA proved to be the optimal dose (**Fig.2.5**). Although, 2.5μg/ml ConA and 1μg/ml ConA were not significantly different in terms of inducing lymphocyte proliferation (**Appendix A1**), it was decided to use the higher dose of ConA (2.5μg/ml) in supernatant generation experiments. This dose proved successful in this respect (see later sections).

2.3.2.3 Inhibition of ConA's Mitogenic Ability using Methyl-mannopyranoside (αmm)

A] Ascertaining Concentration of αmm Most Effective in Absorbing ConA in FCS-supplemented Medium

The capacity of 1μg/ml ConA to stimulate splenocytes was completely abrogated by use of 0.1M αmm (**Fig.2.6**). When lower concentrations of αmm was used, mitogenic activity was still present, as seen by the tritiated thymidine incorporation of responding cells. In fact, the lower the concentration of αmm used, the higher the D.P.M. counts from responding cells and the closer the counts to the original unabsorbed ConA dosage. Even though αmm is a sugar-complex, and therefore potentially nutritive, 0.1M of this compound added to medium slightly depressed, rather than elevated, thymidine incorporation (data not shown).

B] Specificity of Methyl-mannopyranoside Absorption: Comparison of ConA and PHA Stimulation

Fig.2.7 illustrates that 1μg/ml ConA (for 1X 10^6 cells / ml) was again shown to be the optimal dose. 2.5μg/ml ConA was, however, minimally stimulatory —— contrast the situation when 5X 10^6 splenocytes / ml are cultured, when the higher dose is optimal (see **Fig.2.5**). 0.1M αmm added to these ConA solutions resulted in disappearance of induced proliferation, as expected. PHA achieved good proliferation of splenocytes, even after treatment with 0.1M αmm. This observation confirmed the specificity of αmm binding to ConA only.

2.3.3 Mitogenesis of Freshly-harvested Splenocytes Induced by Active Supernatant from ConA-treated Cells

Experiments were carried out to test the stimulatory ability of various batches of supernatants, produced by stimulating 5X 10^6 splenocytes (from 1 - 2 year old adult *Xenopus*) with either 1μg/ml or 2.5μg/ml ConA (see **Figs.2.8&9**) for 24 hours respectively. Assay cells used here were freshly-harvested splenocytes. Use of CSN, which was absorbed with 0.1M αmm resulted in cell proliferation levels comparable to medium-cultured splenocytes. ASNs, in contrast, produced with the lower dose of ConA (**Fig.2.8**) induced enhanced tritiated thymidine incorporation, but this dose was not as effective at producing active supernatant as 2.5μg/ml ConA (**Fig.2.9**). As both types of ASN had been absorbed with 0.1M αmm, which is found to be effective in abrogating the stimulating effects of these 2 ConA dosages, the induced proliferation of cells effected by ASNs is likely to be due to factors secreted from the ConA-activated splenocytes (probably cytokines). Yet their effect still fell short of the proliferation level induced by ConA.

2.3.4 Comparison of Medium- or ConA- Pre-cultured Lymphocytes to Respond to ASNs

Since ASN generated from ConA-stimulated splenocytes resulted in elevated ^{3}HTdR incorporation of freshly-prepared cells, experiments were set up to investigate if pre-cultured lymphocytes (especially those cultured with ConA) could be driven into even more intense proliferation, when treated with ASN [as Watkins & Cohen (1987*b*) had previously shown that mitogen-induced lymphoblasts, but not

lymphocytes taken directly from *Xenopus* toadlets, responded well to ASN; max. S.I. = 30].

In the first experiment, thymocytes (1x 10^6 leucocytes / ml) from a *X.borealis* (9 months old) were pre-cultured with either 1μg/ml ConA or medium for 3 days. Then both kinds of cells were assayed for their reactivity to ConA, supernatants and medium (**Fig.2.10**). Not only did both cell populations respond to ConA, as expected, but also to ASN. There were no significant differences between counts of ConA- pre-cultured and medium- pre-cultured cells when these were assayed with the same stimulant. When data was expressed as a stimulation index, ASN seemed to be slightly more stimulatory for medium- pre-cultured cells (**Table 2.2**), partly due to their lower background D.P.M. in medium.

Since 3-day pre-culture of thymocytes in ConA had no apparent effect on sensitivity of cells to ASNs, splenocytes from *X. laevis* were therefore pre-cultured, and for a longer period, prior to assay (**Fig.2.11**). Interestingly enough, the cells pre-cultured in ConA for 7 days responded better (in terms of S.I.) to one batch of supernatant (batch 7) than the unstimulated cells, but the reverse happened when they were cultured with ASN of batch 6 (**Table 2.3**). Cells pre-cultured for 8 days in medium consistently responded better (in terms of S.I.s) to ASNs than did the splenocytes pre-cultured in ConA for 8 days. It therefore appears that ConA pre-culture did not enhance responsiveness of splenocytes to ASNs.

2.3.5 Ability of ASN to Stimulate Splenocytes from Thymectomized *Xenopus*

This study compared proliferation of splenocytes from Tx and control *Xenopus* (4 months old) cultured with ConA-induced ASN. Animals with their thymi

destroyed at the age of 6 / 7 days should possess no thymus-dependent lymphocytes in their immune systems. Despite this, as shown in **Fig.2.12**, when cultured with αmm-treated ASN, splenocytes from Tx toadlets not only responded quite well (S.I. = 10.3), but even achieved higher thymidine incorporation than control splenocytes. The possibility that residual T cells present in the Tx animals were responding to the ASN appears unlikely, since their splenocytes were shown unable to respond to ConA or PHA, contrasting the excellent response of their control siblings to these T cell mitogens.

2.3.6 Induction of Murine Lymphocyte Proliferation by Human Recombinant Interleukin-2

Initially, a dose response assay was set up to determine the optimal dose of ConA for stimulating murine thymocytes and splenocytes, in terms of tritiated thymidine incorporation (**Fig.2.13**). Splenocytes (1X 10^7 leucocytes / ml) achieved maximal proliferation when stimulated by 5 or 6μg/ml ConA (two doses statistically indifferent, see **Appendix A2**) whereas thymocytes (2X 10^6 leucocytes / ml) were most reactive to 6μg/ml ConA.

Since the same cell concentrations were used in the IL-2 assay, 2.5μg/ml ConA (a sub-optimal dose) was chosen to pre-stimulate both murine splenocytes and thymocytes 24 hours before various dilutions of human recombinant IL-2 (HrIL-2) were tested on them. The use of this particular dose of ConA was to drive the expression of IL-2 receptors on both cell types. However, it was hoped that this dose would not be sufficiently stimulatory for the responding cells to secrete enough IL-2 to drive extensive proliferation, or else the potency of the exogenous IL-2 could not be ascertained. Both ConA- pre-cultured splenocytes and thymocytes responded extremely well to the

exogenous IL-2 (**Fig.2.14**), showing the best S.I.s in the range of 15IU (1 in 32) to 500IU/ml. Lymphocytes which were incubated without ConA- pre-stimulation, in distinct contrast, responded minimally to rIL-2, with their D.P.M.s very close to the background (medium) counts (**Table 2.4**). This experiment not only proved the potency of the rIL-2, but also confirmed that IL-2 receptor expression needed to occur for the cytokine to induce proliferation. For the latter reason, when this rIL-2 was tested on amphibian lymphocytes, a co-stimulation assay (involving sub-optimal T cell mitogen plus rIL-2) was used.

2.3.7 Attempt to Induce Tritiated Thymidine Incorporation in *Xenopus* Lymphocytes Co-cultured with PHA and ASN / rIL-2

2.3.7.1 Response of Larval Lymphocytes to Co-stimulation using *Xenopus* - derived ' Cytokine '

These experiments (**Fig.2.15**) were conducted on larval cells, since it has been suggested elsewhere (Watkins & Cohen, 1987) that larval thymocytes are sensitive to co-stimulation when using T cell mitogen and mitogen-induced ASN. Pooled lymphocytes from several *X.laevis* tadpoles (at larval st. 56 - 58) were treated with ASN, CSN or medium, in the presence of various dilutions of PHA. PHA was used here as a ' co-stimulator ' instead of ConA, since the presence of any un-bound αmm in the supernatants would ' interfere ' with the activity of ConA and reduce the proliferative ability of such ConA concentrations. When medium alone or CSN was added, larval thymocytes responded poorly to various PHA doses. In contrast, in the presence of ASN, a healthy stimulation was observed (best S.I. = 4.4, when comparing ASN with CSN, at

PHA = 0.25μg/ml), i.e., ' co-stimulation ' for larval thymocytes was observed. Larval thymocytes showed a low level response to ASN, in the absence of PHA (S.I. = 2.1).

Larval splenocytes responded only minimally to ASN, in the absence of PHA (S.I. = 1.5). The unresponsiveness of larval splenocytes to ASN was in contrast to adult splenocytes, which showed enhanced tritiated thymidine incorporation (see previous section and **Fig.2.12**). These splenocytes were, however, able to respond massively to PHA doses, with or without ASN added. There was no sign of co-stimulation for larval splenocytes.

2.3.7.2 Comparison of Amphibian ASN and Human rIL-2 to Stimulate Larval Lymphocytes

Lymphocytes from tadpoles (at larval st. 57 - 59) were treated with *Xenopus* culture supernatant, medium or HrIL-2, and co-stimulated with various doses of PHA. As noted above, co-stimulation induced by ASN / PHA was observed with larval thymocytes, but not with larval splenocytes (**Fig.2.17**). Larval thymocytes responded minimally to PHA in the absence of ASN, whereas splenocytes did not require ASN to display a good response to this mitogen. Recombinant human interleukin-2 had no apparent effect on either type of larval cells. Thus rIL-2 failed to directly stimulate thymocytes or splenocytes cultured in medium and also showed no co-stimulatory capacity when used in conjunction with PHA on thymocytes.

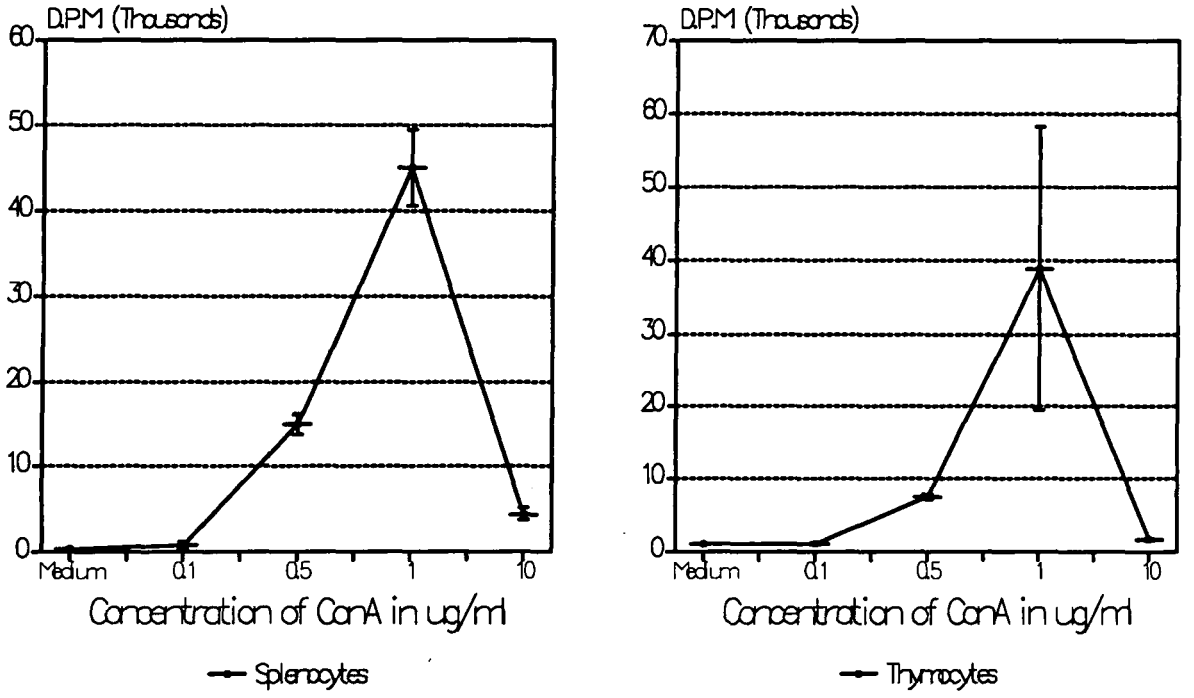

Fig.2.1 Dose Response to ConA of *Xenopus* Lymphocytes Cultured in FCS Medium

Freshly-prepared outbred *X.laevis* splenocytes (1x 10^6 leucocytes / ml) and thymocytes (2x 10^6 leucocytes / ml) were dispensed into 96-well V-bottomed plates in 100µl volume per well in 1% FCS L-15.

ConA was added to produce ' in-well ' dilutions of 0.1, 0.5, 1 and 10µg/ml. Cultures were set up in triplicate. Each well was pulsed with 1µCi ^{3}HTdR 24 hours before harvesting. Lymphocytes were cultured for 3 days.

The above graphs were plotted with various dilutions of ConA on the X-axis against ^{3}HTdR incorporation in D.P.M. on the Y-axis. Error bar of each co-ordinate represents its corresponding standard deviation.

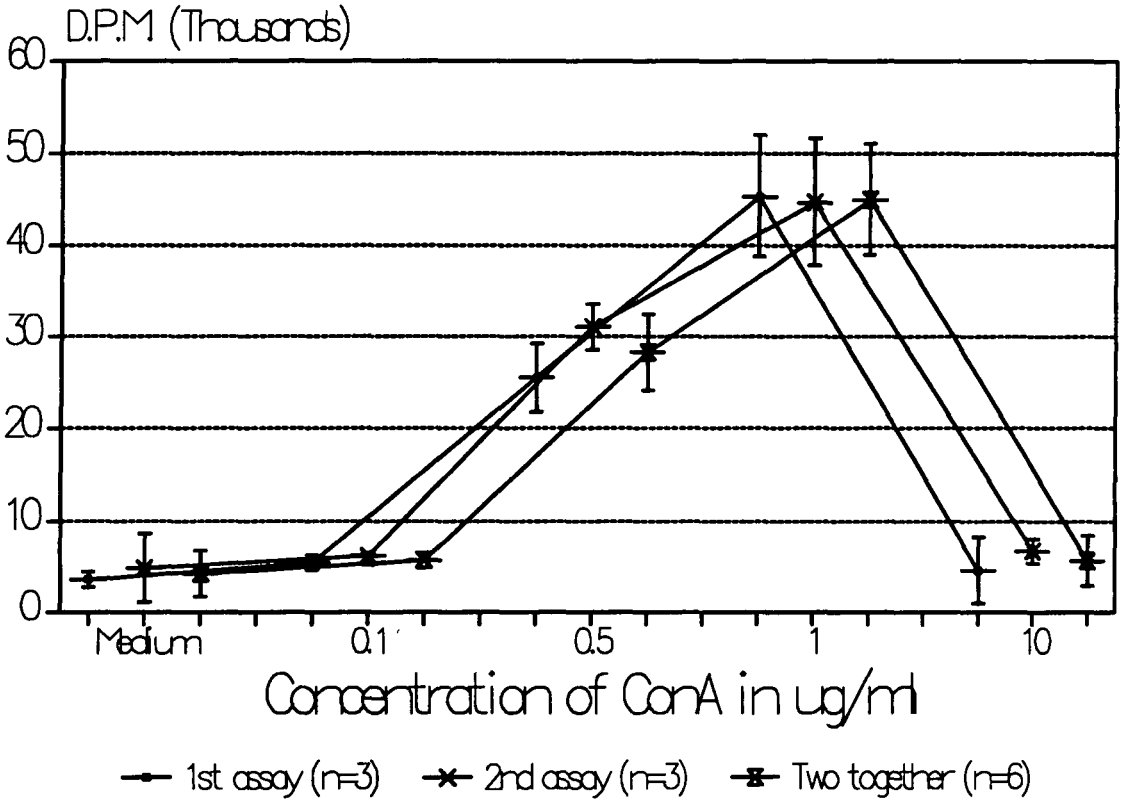

Fig.2.2 Comparison of ^{3}HTdR Incorporation of Splenocytes between Replicate Mitogen Assays

Freshly-prepared splenocytes from an individual outbred *X.laevis* were dispensed (1x 10^{5} leucocytes / well) into two 96-well V-bottomed plates in 1% FCS L-15 medium.

ConA was added to produce ' in-well ' dilutions of 0.1, 0.5, 1 and 10μg/ml. Cultures were set up in triplicate. After 48 hours (2 days) in culture each well was pulsed with 1μCi ^{3}HTdR and harvested 24 hours later.

These dose response graphs were plotted with various ConA dilutions on the X-axis against radioactivity incorporations on the Y-axis. Error bars of the co-ordinates represent their respective standard deviation.

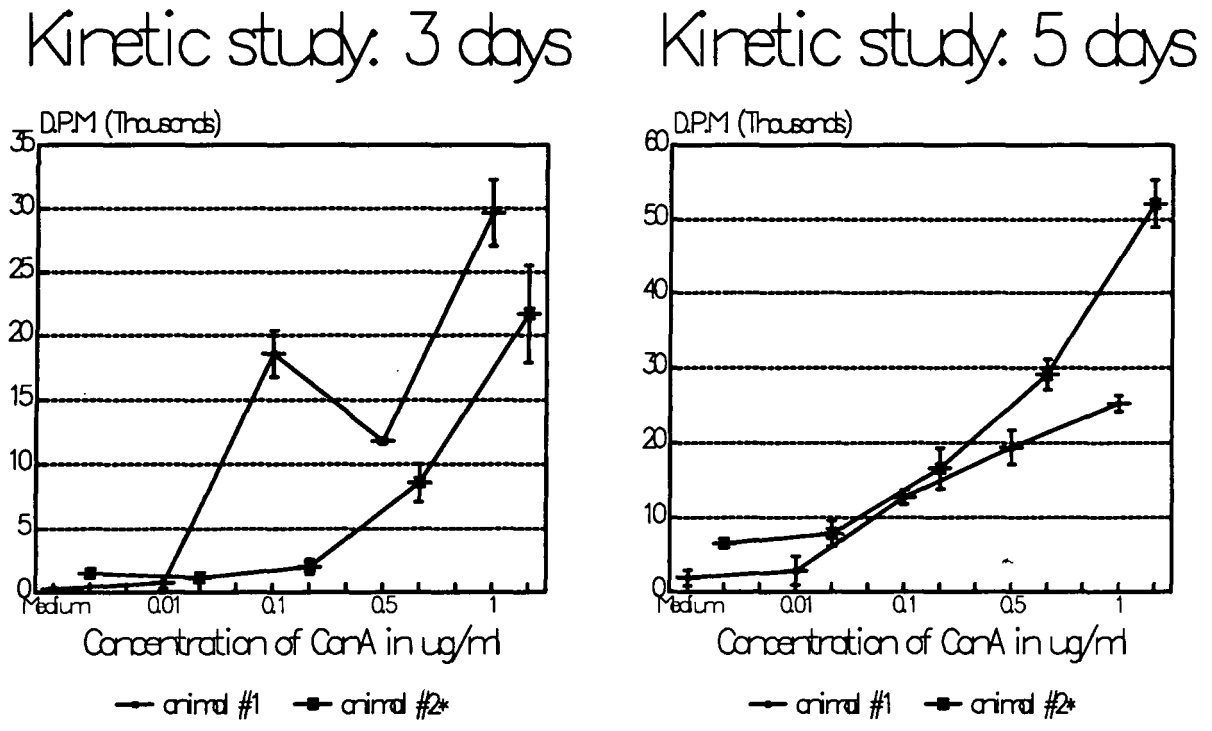

Fig.2.3 Effect of Density Gradient Centrifugation on ^{3}HTdR Incorporation

Freshly-prepared splenocytes of two individual outbred *X.laevis*, with one population passed over Histopaque and the other population left untreated, were dispensed (1x 10^{6} leucocytes / ml) in 100µl per well into 96-well V-bottomed plates. Splenocytes were cultured in 1% FCS L-15 medium.

ConA was added to produce ' in-well ' dilutions of 0.01, 0.1, 0.5 and 1µg/ml. Cultures were set up in triplicate. Harvesting of cultures took place after 72 hours (3 days) or 120 hours (5 days), with each well pulsed with 1µCi ^{3}HTdR 24 hours earlier.

#1 and #2 designate individual animals, and * is the splenocyte population that underwent Histopaque treatment. Curves were plotted with ConA dilutions against D.P.M. and error bars are standard deviations.

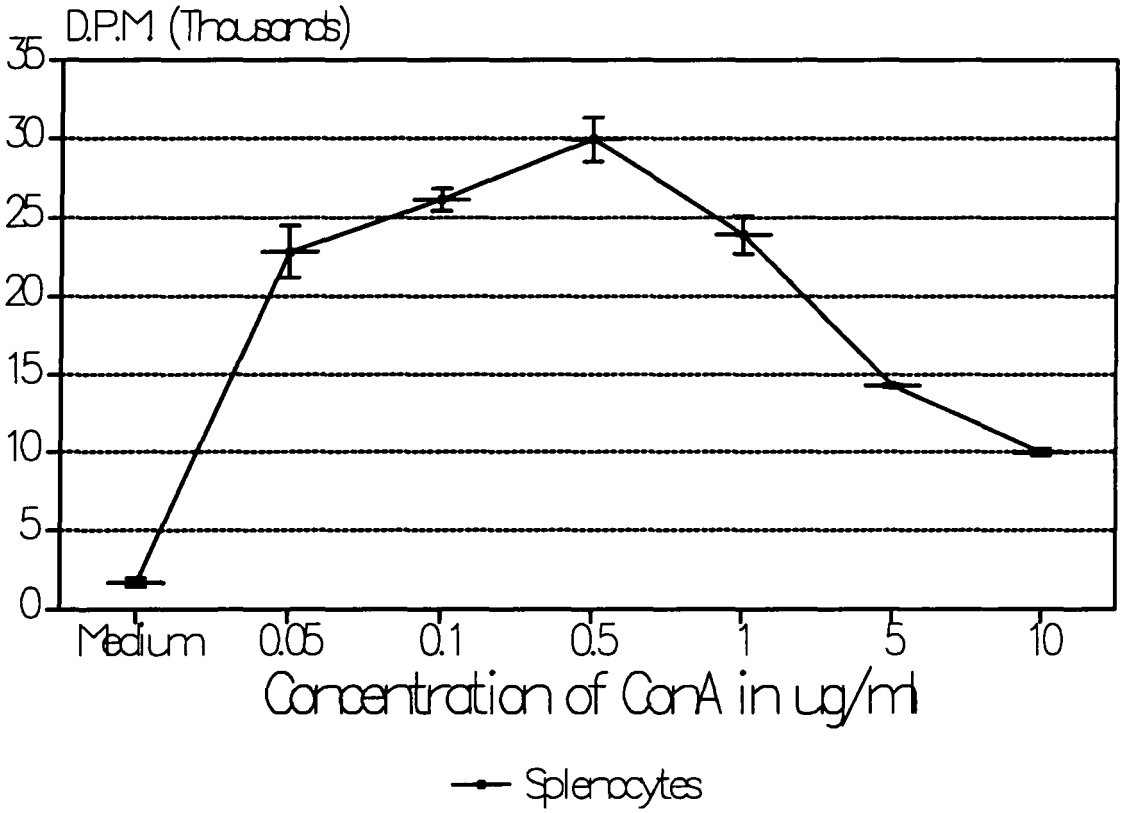

Fig.2.4 Assessment of Optimal ConA Dose for *Xenopus* Splenocytes in BSA Medium

Freshly-prepared splenocytes from an outbred *X.laevis* were dispensed into each well of a 96-well V-bottomed plate at 100µl aliquots (1x 10^6 leucocytes / ml) in 0.25% BSA L-15 medium.

ConA dilutions refer to ' in-well ' concentrations. Cultures were set up in triplicate, with each well pulsed with 1µCi ^{3}HTdR at 48 hours, and harvested at 72 hours.

The graph was plotted with various ConA dilutions against D.P.M.. The standard deviation of each data point is represented with an error bar.

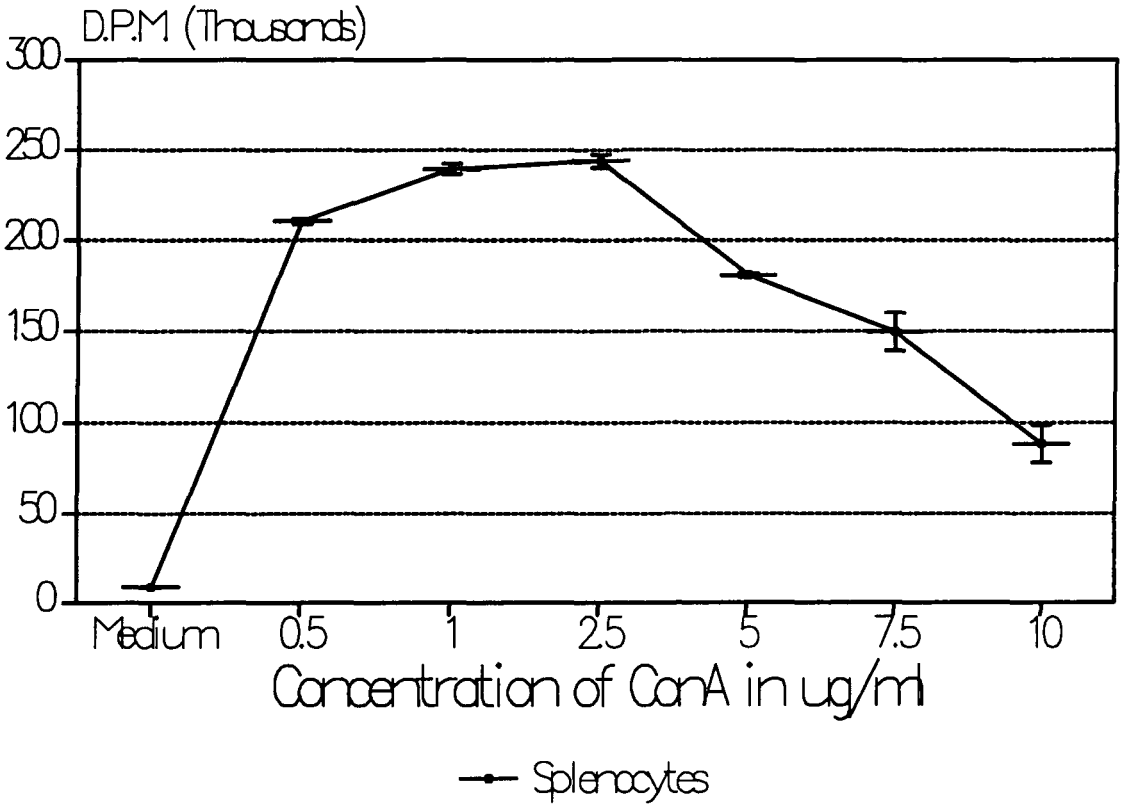

Fig.2.5 Assessment of Optimal ConA Dose for Amphibian ' Cytokine ' Production

Freshly-prepared splenocytes from a J strain *X.laevis* were dispensed (5x 10^5 leucocytes / well) into a 96-well flat-bottomed plate in 0.25% BSA L-15 medium after passage over Histopaque. Cultures were set up in triplicate for 3 days. Each well was pulsed with 1μCi ^{3}HTdR 24 hours before harvesting.

The graph was plotted with various ConA dilutions against D.P.M., with the error bars representing the standard deviations.

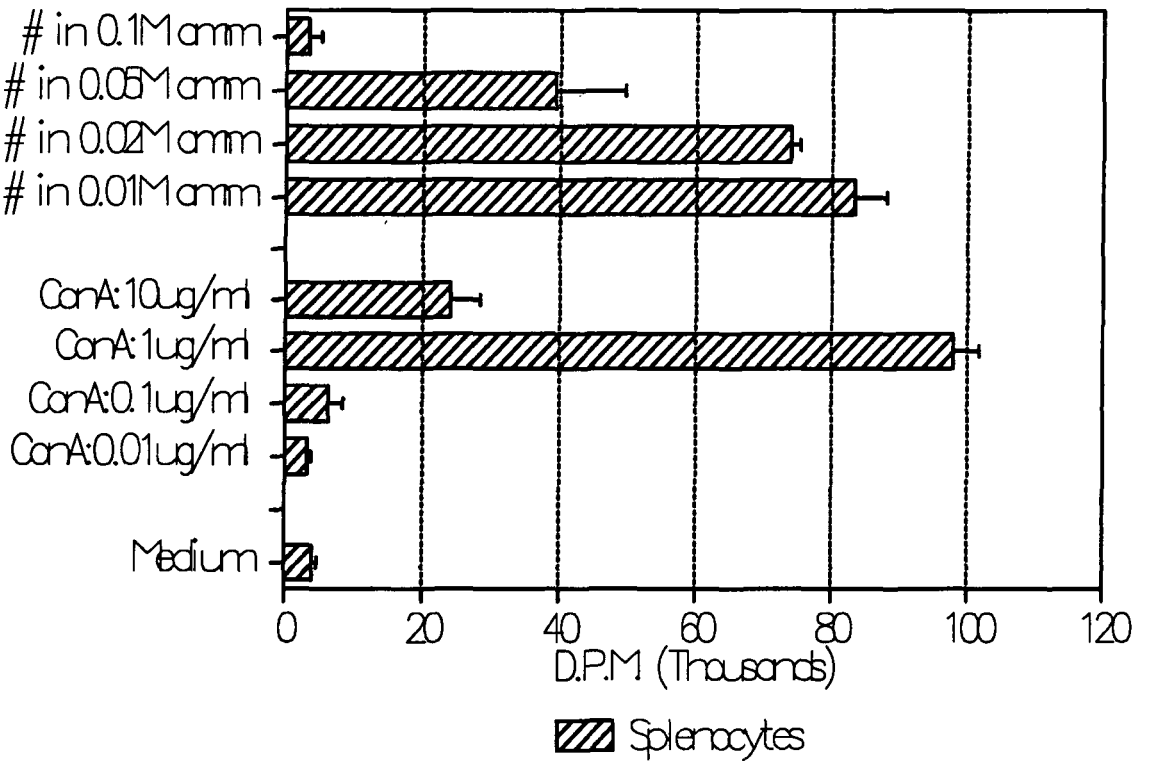

Fig.2.6 Abrogation of Proliferative Effect of ConA with Methyl-Mannopyranoside

Freshly-prepared splenocytes (1x 10^6 leucocytes / ml) of a J strain *X.laevis* were dispensed in 100µl aliquot per well into a 96-well V-bottomed plate in 1% FCS L-15 medium. Cells were treated with various concentrations of ' unabsorbed ' ConA, and also with 1µg/ml ConA absorbed with various concentrations of αmm. # denotes the ConA that was ' absorbed '. Cells were cultured for 48 hours, then pulsed with ^{3}HTdR and harvested at 72 hours.

Each bar represents the mean D.P.M. counts of a culture triplicate treated with a particular ' stimulant '. The corresponding standard deviation is shown with an error bar.

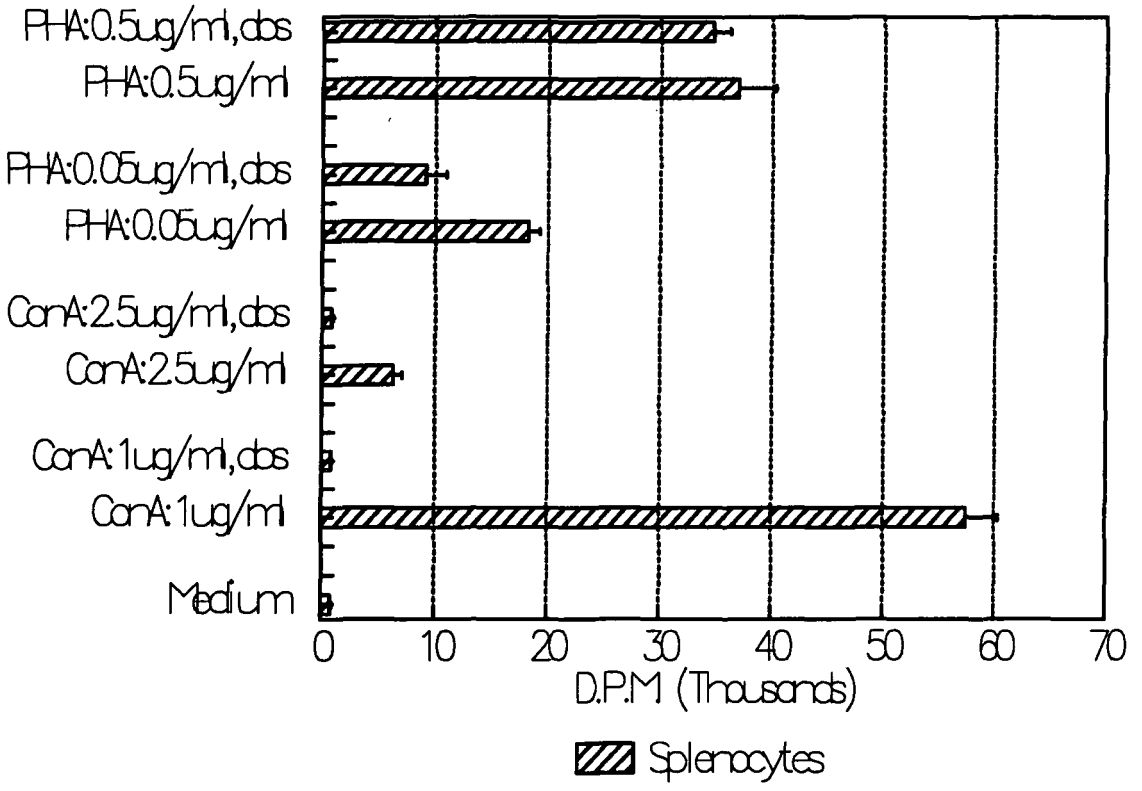

Fig.2.7 Absorption of ConA, but not PHA, Effected by Methyl-Mannopyranoside

Splenocytes of a JLG5 (hybrid of J strain and LG5 *Xenopus laevis*) were dispensed in 100µl (1x 10^5 leucocytes) / well into a 96-well flat-bottomed plate in 1% FCS L-15 medium.

ConA was added in to give ' in-well ' concentrations of 1 and 2.5µg/ml; whereas PHA was at 0.05 and 0.5µg/ml. These T-cell mitogens were also absorbed with 0.1M αmm. This assay was for 3 days, culture being pulsed with ^{3}HTdR 24 hours before harvesting.

Shaded bars represent the mean intensity of radioactivity incorporation of each experimental triplicate. Error bars indicate standard deviations.

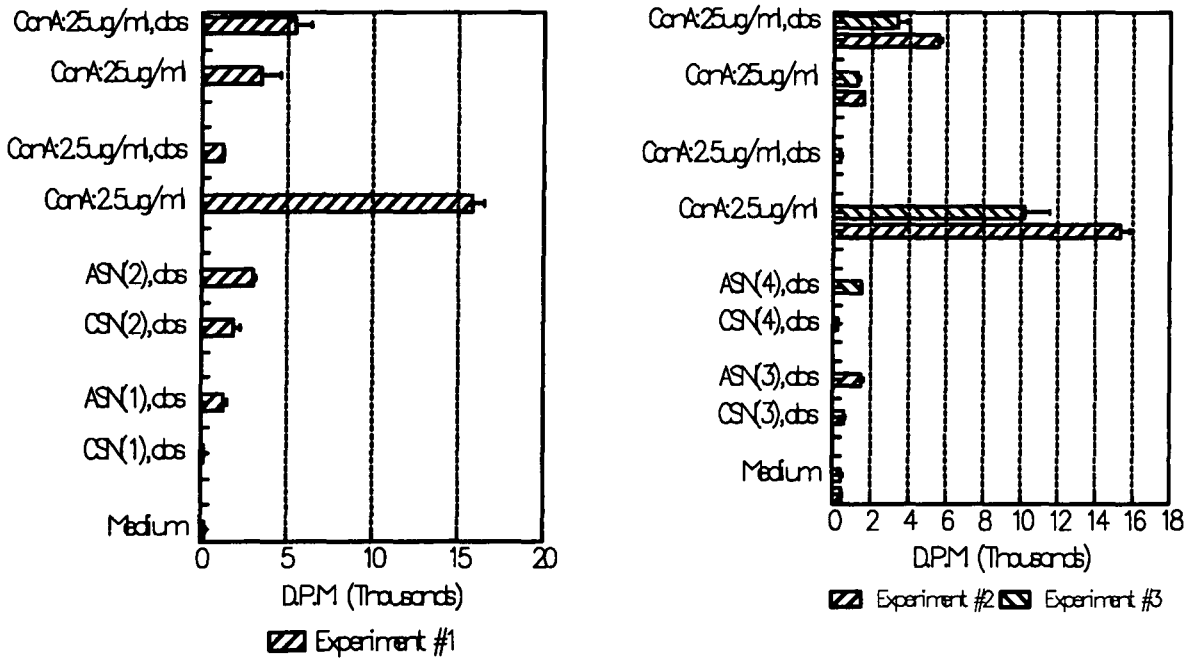

Fig.2.8 Testing Mitogenic Ability of Supernatants on Freshly-Prepared Splenocytes

Splenocytes of outbred *X.laevis* were dispensed (1x 10^5 leucocytes / well) into 96-well flat-bottomed plates in 1% FCS L-15 medium. Individual supernatant were added in at 50µl / well. ConA was added in to produce ' in-well ' concentrations of 2.5 and 25µg/ml. Their ɑmm-absorbed dilutions were also prepared . Final volume per well was 200µl. Data shown above were 3-day assays. Each well was pulsed with 1µCi ^{3}HTdR after being in culture for 48 hours.

D.P.M. of each experimental triplicate is shown in shaded bars, with its standard deviation indicated by the error bar. Numbers in brackets denote different batches of supernatants, which were produced by stimulating Histopaque-treated splenocyte populations (5x 10^6 leucocytes / ml) from several outbred *X.laevis* with 1µg/ml ConA for ASN and medium for CSN. All supernatants were (0.1M) ɑmm-treated at the time of collection.

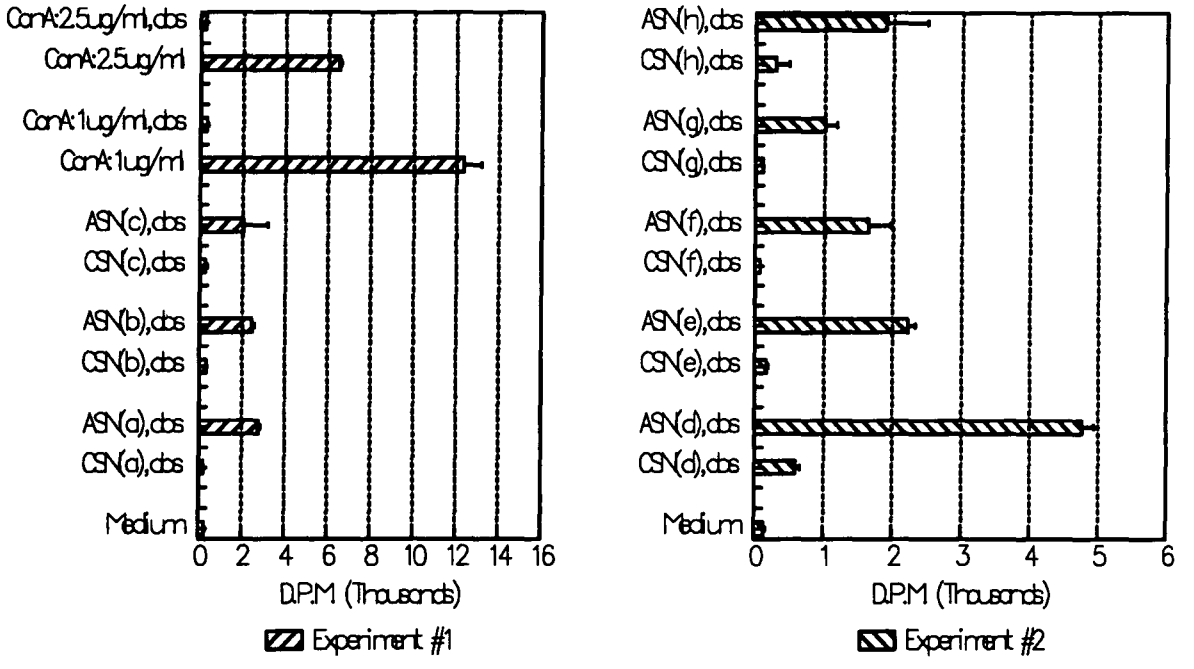

Fig.2.9 Test of Mitogenic Ability of Supernatants on Freshly-Prepared Splenocytes

Splenocytes of J strain *X.laevis* were dispensed (1x 10^5 leucocytes / well) into 96-well flat-bottomed plates in 1% FCS L-15 medium. Individual supernatant was added in at 50µl / well. ConA was added to produce 1 and 2.5µg/ml ' in-well ' concentrations. Their ɑmm-absorbed dilutions were also prepared . Final volume per well was 200µl. Data shown above were 3-day assays. Each well was pulsed with 1µCi ^{3}HTdR after being in culture for 48 hours.

D.P.M. of each experimental triplicate is shown in shaded bars, with its standard deviation indicated by the error bar. Letters in brackets denote different batches of supernatants, which were produced by stimulating Histopaque-treated splenocyte populations (5x 10^6 leucocytes / ml) from several J strain *X.laevis* with 2.5µg/ml ConA for ASN and medium for CSN. All supernatants were (0.1M) ɑmm-treated at the time of collection.

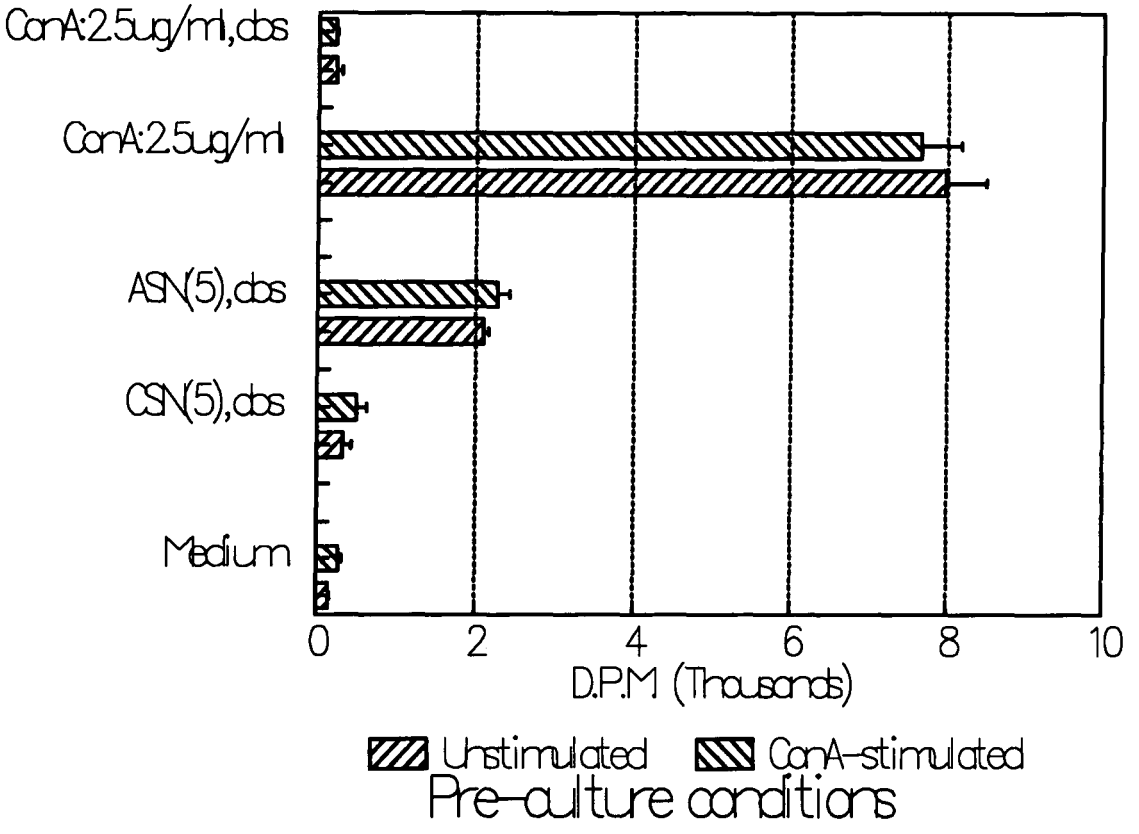

Fig.2.10 Test of Mitogenic Ability of Supernatant on 3 Days Pre-Cultured Thymocytes

X.borealis thymocytes, which had been pre-cultured for 3 days with either 1μg/ml ConA or in medium at 3.75x 10^6 leucocytes / ml in 5% FCS L-15 medium in a 24-well plate, were subsequently dispensed (1x 10^5 leucocytes / well) into a 96-well flat-bottomed plate in 1% FCS L-15 medium.

ConA was added to produce ' in-well ' dilution of 2.5μg/ml; its (0.1M) αmm-treated dose was also prepared in order to confirm the neutralization of ConA presented in the tested supernatants. Each well was pulsed with 1μCi ^{3}HTdR at 48 hours and harvested at 72 hours.

This tested batch of supernatants was ' absorbed ' with 0.1M αmm, as routine, at the time of collection and its ASN was produced by stimulating 5x10^6 leucocytes / ml with 1μg ConA and CSN by using medium.

Shaded bars indicate the mean intensity of radioactivity incorporations of different experimental triplicates, whereas error bars are standard deviations.

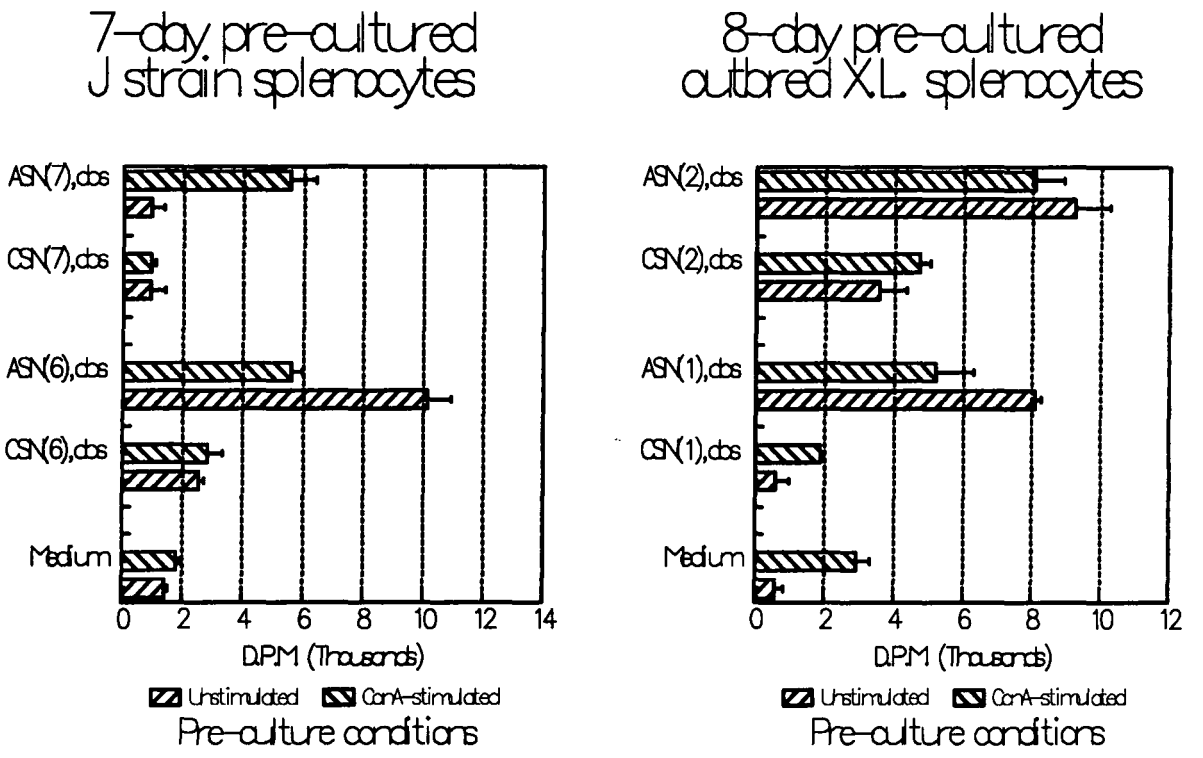

Fig.2.11 Test of Mitogenic Ability of Supernatant on Pre-Cultured Splenocytes

Splenocytes from a J strain ($2x10^6$ leucocytes / ml) and an outbred *X.laevis* ($5x10^6$ leucocytes / ml) were pre-cultured for 7 & 8 days respectively, with either 1μg/ml ConA or medium, in 5% FCS L-15 medium.

These pre-cultured splenocytes were then dispensed into 96-well flat-bottomed plates at $1x\ 10^5$ leucocytes per well in 1% FCS L-15 medium. Supernatants were added to give a final ' in-well ' concentration of 25% SN in a total volume of 200μl. Cultures were set up in triplicate. After 48 hours of culture, each well was pulsed with 1μCi ^{3}HTdR and harvested 24 hours later. Numbers in brackets specify different batches of supernatants, with ASN produced by stimulating Histopaque-treated splenocytes (4 - $5x\ 10^6$ leucocytes / ml) from several outbred *X.laevis* with 1μg/ml ConA; CSN was from medium-cultured cells. Supernatants of batch #1 were tested on the cells that generated them. All supernatants were αmm-absorbed prior to use.

D.P.M. of every experimental triplicate of each cultured cell population is shown in bars in specified shading pattern. Error bars indicate standard deviations.

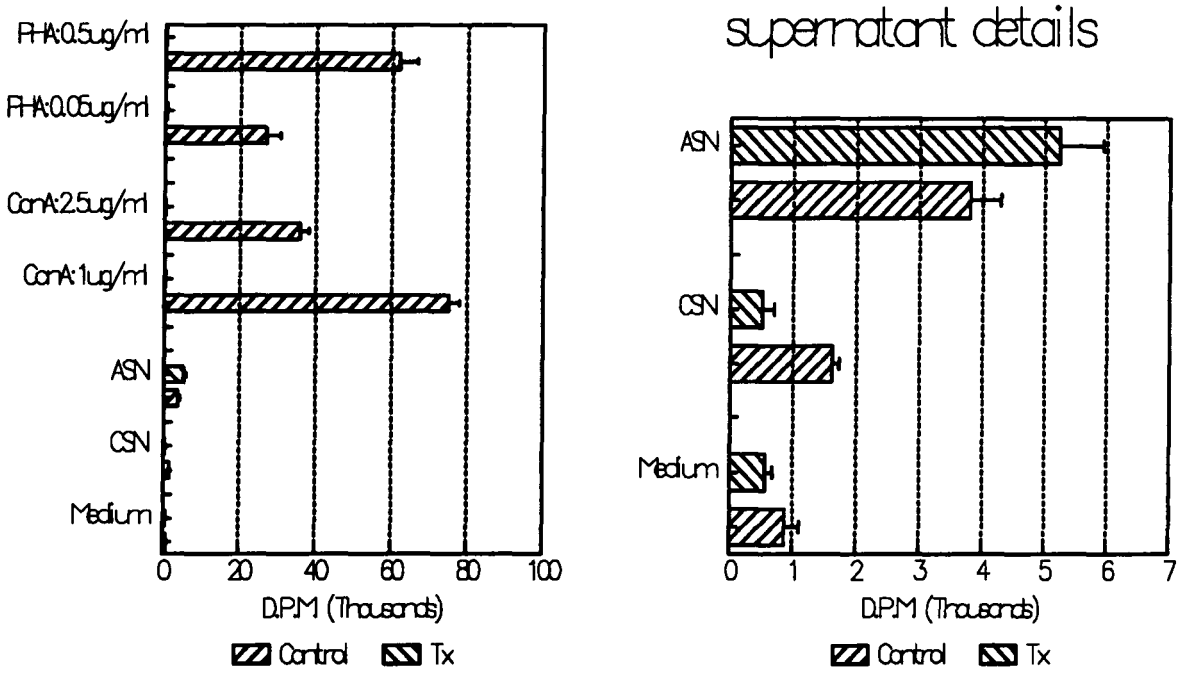

Fig.2.12 Test of Mitogenic Ability of Supernatant and T Cell Mitogen on Splenocytes from Control and Thymectomized Toadlets

A range of ConA (1 and 2.5μg/ml) and PHA (0.05 and 0.5μg/ml) ' in-well ' concentrations were prepared. These were assayed along with supernatants on splenocytes of 4 months old control and 7-day thymectomized post-metamorphic outbred *X.laevis* toadlets. Splenocytes were dispensed at 1x 10^5 leucocytes per well into a 96-well flat-bottomed plate in 1% FCS L-15 medium. Each well was pulsed with 1μCi ^{3}HTdR 24 hours before harvesting at 72 hours.

Diagramme on the right magnifies details of the supernatant section of the original on the left. This batch of supernatant used was an aggregate from several individual batches of supernatants, of which their ASNs shown to have good level of stimulations and CSNs of low (close to background's) counts

Shading patterns indicate type of animals used in this study. Mean D.P.M. of each experimental triplicate is shown in shaded bar and standard deviations in error bar at top.

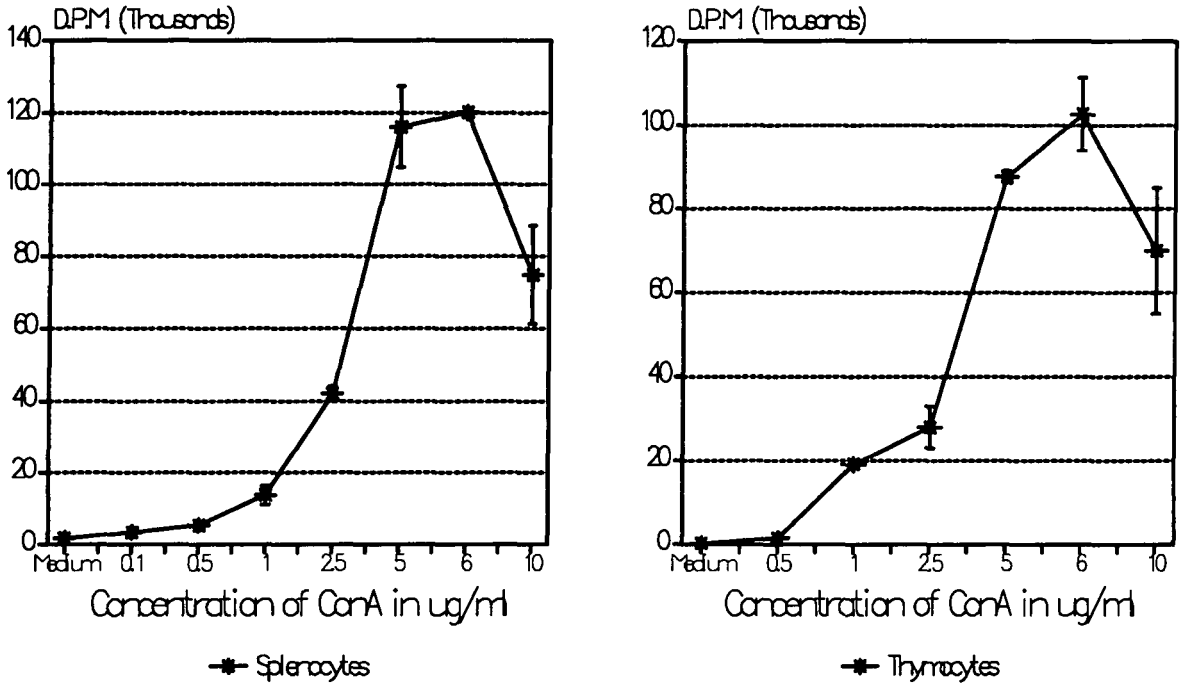

Fig.2.13 Assessment of Optimal ConA Dose for Murine Lymphocytes in RPMI 1640 Medium

Various ' in-well ' dilutions of ConA were prepared as shown. Freshly-prepared and Histopaque-treated splenocytes (2x 10^6 leucocytes / ml) and thymocytes (1x 10^7 leucocytes / ml) from a BALB/c mouse were dispensed in 100µl / well into 96-well V-bottomed plates in 10% FCS RPMI 1640 medium. These lymphocytes were cultured for 3 days at 37°C, with each well pulsed with 1µCi ^{3}HTdR at 48 hours.

Respective dose response curves were plotted with the stated ConA concentrations against D.P.M.. Mean D.P.M. of every experimental triplicate is represented by its co-ordinate, with the error bar indicating its standard deviations.

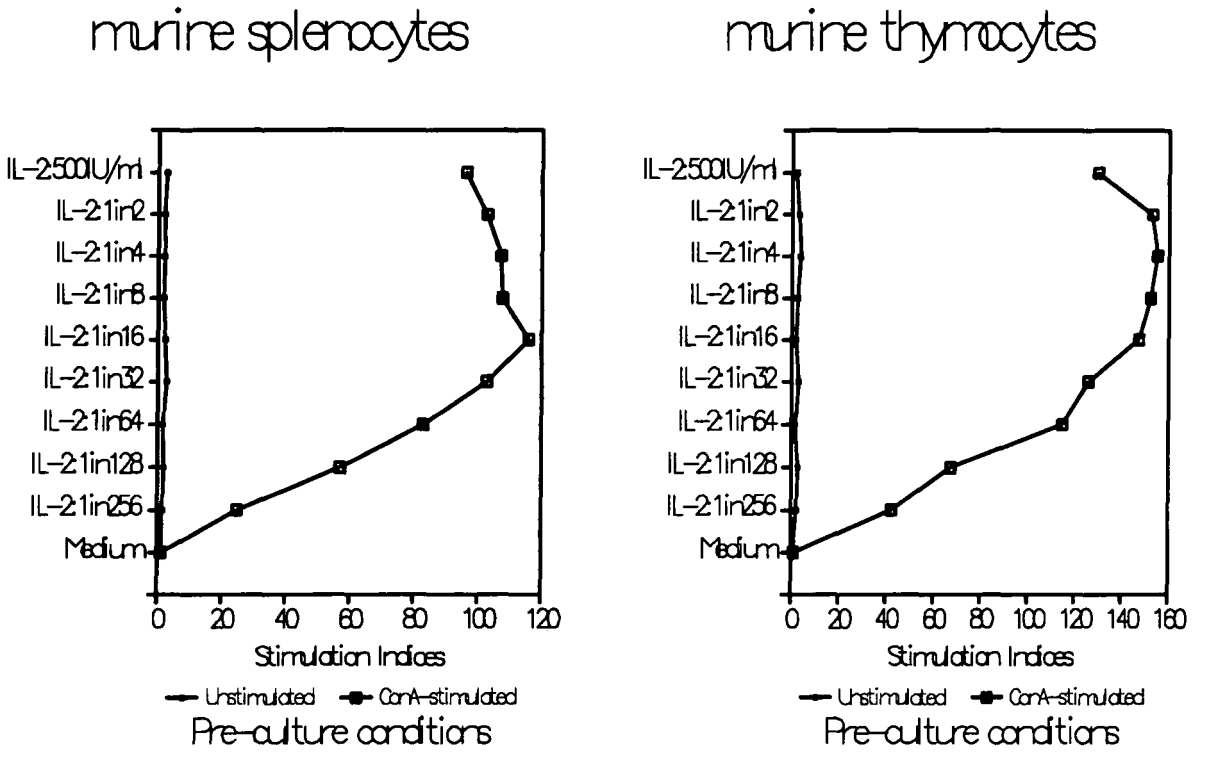

Fig.2.14 Potency of Human Recombinant Interleukin-2 (HrIL-2) Tested on Murine Lymphocytes

Serial dilutions (1 in 2) of HrIL-2 was prepared with an initial ' in-well ' concentration of 500IU/ml at 50µl per well. Splenocytes (2x 10^6 leucocytes / ml) and thymocytes (1x 10^7 leucocytes / ml) of a BALB/c mouse had been pre-cultured for 24 hours with either 2.5µg/ml ConA or medium in 10% FCS RPMI 1640 medium before assaying. Such pre-cultured splenocytes (1x 10^6 leucocytes / ml) and thymocytes (2x 10^6 leucocytes / ml), which had been washed 3 times in RPMI medium containing 25mM ɑmm before plating, were then dispensed at 50µl per well into 96-well flat-bottomed plates. Cultures were set up in triplicate and assayed for 3 days at 37°C. Each well was pulsed with 1µCi ^{3}HTdR at 48 hours.

All data is shown as simulation indices (S.I.).

$$S.I. = \frac{\text{Mean D.P.M. of Culture Treated with a particular HrIL-2 Dilution}}{\text{Mean D.P.M. of Culture Treated with Medium}}$$

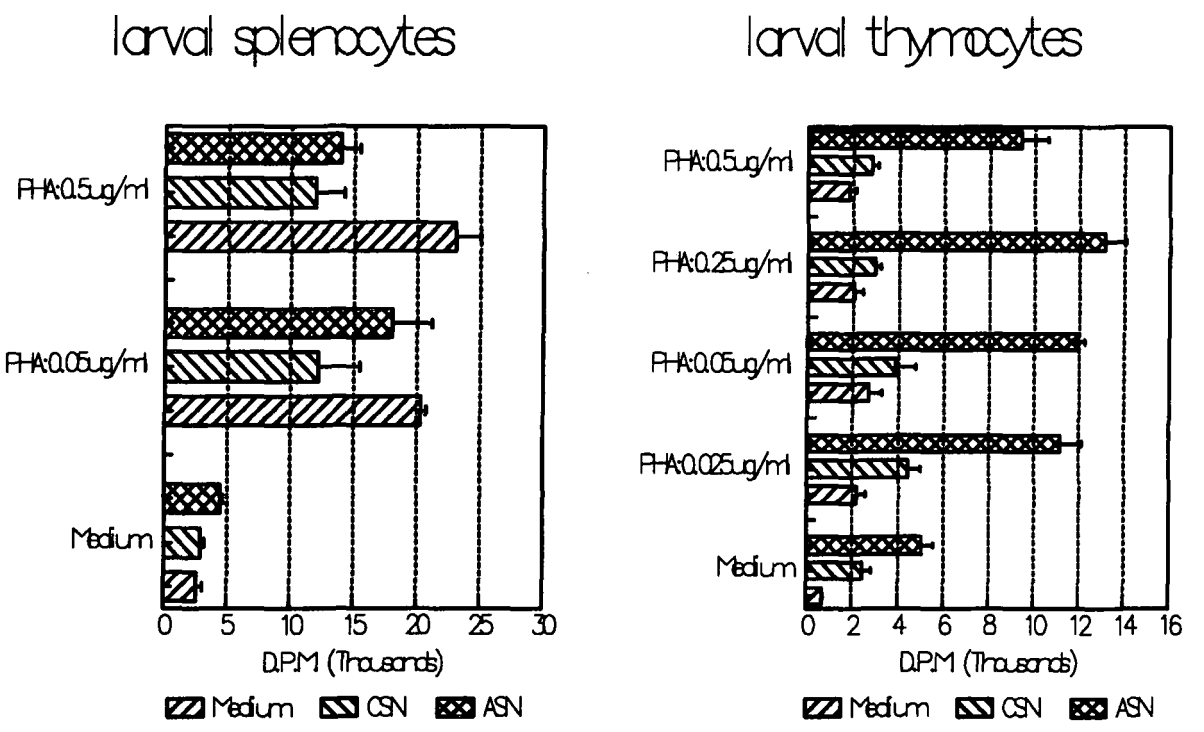

Fig.2.15 Co-Stimulation of PHA plus ASN on Larval Thymocytes but Not on Larval Splenocytes

Freshly-prepared splenocytes (5x 10^5 leucocytes / ml) and thymocytes (2x 10^6 leucocytes / ml) from several outbred *X.laevis* tadpoles (st. 56 - 58) were pooled and dispensed in 100µl per well in 96-well flat-bottomed plates in 1% FCS L-15 medium. 50µl ASN, CSN or medium were added (shown by different shaded boxes) to give a final ' in-well ' concentration of 25%. Volumes were made up to 200µl by the addition of a range of PHA doses or additional 50µl of medium, as shown on the Y-axis. Final concentrations (' in-well') of PHA are shown. After 48 hours of culture, each well was pulsed with 1µCi ^{3}HTdR and harvested 24 hours later.

The tested supernatants were ' pooled ' from several batches of supernatants, which had been ' absorbed ' with 0.1M αmm as routine.

Shaded bars represent the mean intensity of radioactivity incorporations of the experimental triplicates, with error bars indicating the standard deviations.

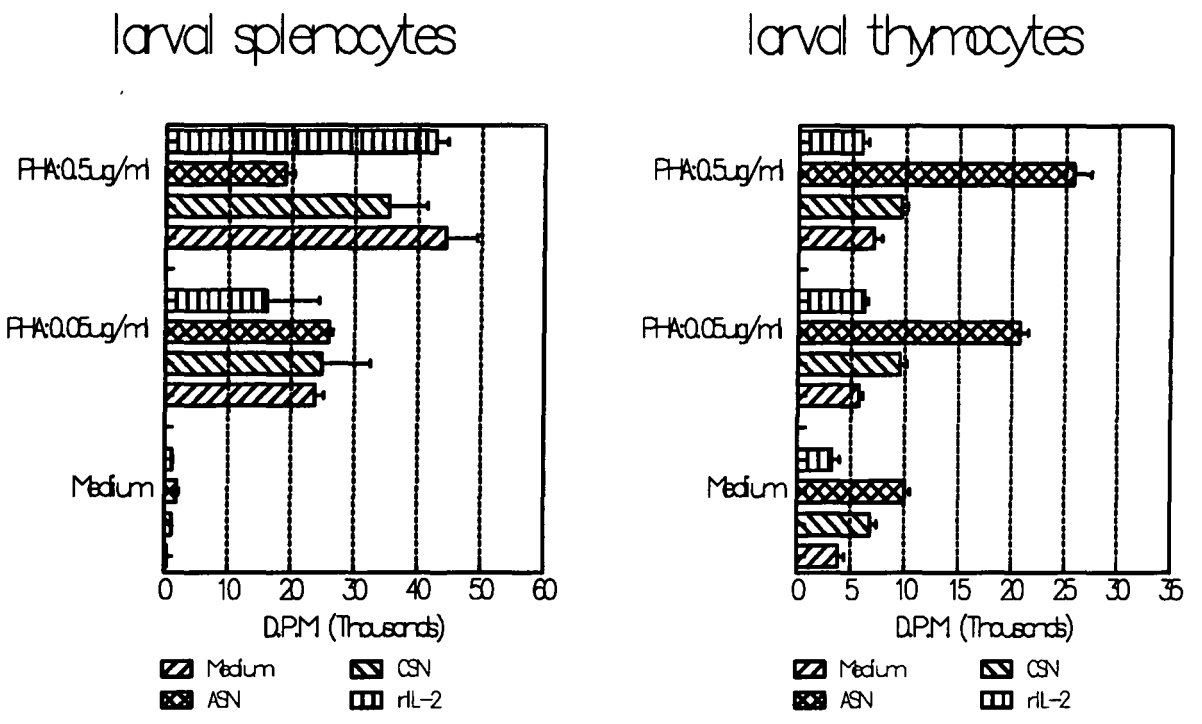

Fig.2.16 Co-Stimulation of PHA with ASN, but Not with Human Recombinant Interleukin-2 on Larval Lymphocytes

Freshly-prepared splenocytes (5x 10^5 leucocytes / ml) and thymocytes (2x 10^6 leucocytes / ml) from several LG15 *X.laevis* tadpoles (st. 57 - 59) were pooled and dispensed in 100µl per well in 96-well flat-bottomed plates in 1% FCS L-15 medium. HrIL-2 was prepared to give an ' in-well' concentration of 15IU/ml. Supernatants or medium were added to give a final ' in-well ' concentration of 25%. Volumes were made up to 200µl by the addition of 50µl PHA or additional medium, as shown on Y-axis. Final (' in-well ') concentrations of PHA are shown. Cultures were set up in triplicate. After 48 hours of culture, each well was pulsed with 1µCi ^{3}HTdR and harvested 24 hours later.

The tested supernatants were ' pooled ' from several other batches of supernatants, which had been ' absorbed ' with 0.1M αmm as routine.

Shaded bars represent the mean intensity of radioactivity incorporations of the experimental triplicates, with error bars indicating the standard deviations

Table 2.2

Supernatant Assay Tested on Cultured Thymocytes

CULTURE STIMULANTS	CELL TYPE	
	ConA Pre-cultured	**Medium Pre-cultured**
ASN *	2271 ± 154 (4.5)	2101 ± 64 (6.3)
CSN *	502 ± 129 (1.0)	334 ± 98 (1.0)
Medium	273 ± 42	155 ± 14

The D.P.M. [mean ± standard deviation] of thymic cells pre-cultured with or without ConA responding to ASN, CSN and medium are shown above. Respective stimulation indices are shown in brackets. In terms of actual counts, thymocytes pre-cultured with ConA displayed higher D.P.M.. However, if the responsiveness of cells is expressed with S.I.s, ASN was more stimulatory to medium pre-cultured cells. Caution then has to be taken in the interpretation of tritiated thymidine incorporation data.

$$\text{S.I.} = \frac{\text{Mean D.P.M. of ASN Absorbed with 0.1M αmm}}{\text{Mean D.P.M. of CSN Absorbed with 0.1M αmm}}$$

* SNs used here was batch #5, which had been produced by stimulating 5x 10^6 splenocytes / ml from a J animal with either 1μg/ml ConA or medium only. These SNs were absorbed with 0.1M αmm at the time of collection.

Table 2.3

Stimulation Indices Effected by Supernatants Using 7-Day Pre-cultured J Strain Splenocytes

CULTURE STIMULANTS	CELL TYPE	
	ConA Pre-cultured	**Medium Pre-cultured**
ASN (6), abs	5606 ± 357 (1.98)	10118 ± 804 (4.02)
CSN (6), abs	2833 ± 478 (1.00)	2515 ± 177 (1.00)
CSN (6)	3187 ± 316	2594 ± 465
ASN (7), abs	5554 ± 859 (5.98)	938 ± 428 (1.02)
CSN (7), abs	928 ± 148 (1.00)	928 ± 484 (1.00)
CSN	1168	989
Medium	1782 ± 135	1419 ± 107

Supernatant batches were specified in brackets. Supernatants (6) were generated using an outbred *Xenopus* whereas supernatants (7) were generated using a J strain *Xenopus laevis*. Both SNs were produced by stimulating 5x 10^6 splenocytes / ml with either 1μg/ml ConA or medium. SNs had been treated or untreated with 0.1M αmm, as specified (abs = absorbed with 0.1M αmm).

$$\text{S.I.} = \frac{\text{Mean D.P.M. of ASN Treated with 0.1M αmm}}{\text{Mean D.P.M. of CSN Treated with 0.1M αmm}}$$

Table 2.4

Potency of Human Recombinant Interleukin-2 (HrIL-2) Tested on Murine Lymphocytes

CULTURE STIMULANTS	CELL TYPE Splenocytes and *Thymocytes*	
	ConA Pre-cultured	**Medium Pre-cultured**
rIL-2: 500IU/ml	86471 ± 11960 (94.6) *44655 ± 5475 (129.8)*	386 ± 56 (2.6) *130 ± 13 (2.0)*
rIL-2: 250IU/ml (1 in 2)	92386 ± 13439 (102.7) *52613 ± 3571 (152.9)*	291 ± 65 (2.0) *197 ± 103 (3.0)*
rIL-2: 125IU/ml (1 in 4)	96433 ± 9740 (107.2) *63346 ± 2758 (155.1)*	294 ± 64 (2.0) *244 ± 43 (3.8)*
rIL-2: 62.5IU/ml (1 in 8)	96820 ± 14382 (107.6) *52408 ± 2764 (152.3)*	255 ± 32 (1.7) *159 ± 12 (2.5)*
rIL-2: 31.25IU/ml (1 in 16)	104334 ± 8546 (115.9) *50672 ± 6011 (147.3)*	340 ± 71 (2.3) *125 ± 25 (1.9)*
rIL-2: 15.625IU/ml (1 in 32)	92457 ± 5652 (102.7) *43326 ± 5059 (125.9)*	324 ± 78 (2.7) *191 ± 27 (2.9)*
rIL-2: 7.8125IU/ml (1 in 64)	74597 ± 14554 (82.9) *39479 ± 5022 (114.8)*	246 ± 49 (1.7) *103 ± 10 (1.6)*
rIL-2: 3.90625IU/ml (1 in 128)	53329 ± 5623 (57.0) *23141 ± 7541 (67.3)*	277 ± 82 (1.9) *127 ± 77 (2.7)*
rIL-2:1.953125IU/ml (1 in 256)	22431 ± 16449 (24.9) *14552 ± 5212 (42.3)*	231 ± 78 (1.6) *150 ± 23 (2.3)*
Medium	900 ± 28 (1.0) *344 ± 57* (1.0)	148 ± 22 (1.0) *65 ± 8* (1.0)

This table shows the actual counts [mean ± standard deviation] of murine splenocytes and thymocytes (in *Italic*), pre-cultured for 24 hours with or without ConA, responding to rIL-2. Respective stimulation indices are shown in brackets.

2.4 Discussion

Preliminary tritiated thymidine incorporation studies carried out in FCS-supplemented medium confirmed that 1μg/ml of ConA is optimal for inducing proliferation of 1X 10^6 *Xenopus* splenocytes / ml and 2X 10^6 thymocytes / ml. The employment of Histopaque 1077 in the preparation of leucocyte-enriched suspensions was also found to be effective in sustaining viability of *Xenopus* splenocytes *in vitro*. Proliferative studies have also revealed the optimal conditions required for the generation of active, ConA-induced *Xenopus* culture supernatants, when BSA supplemented medium is used in order to minimize foreign serum components. The optimum dose of ConA for inducing 5X 10^6 leucocytes / ml into ' maximum ' ^{3}HTdR incorporation in BSA- supplemented medium proved to be 2.5μg/ml. ConA doses less than 2.5μg/ml were insufficient to drive this particular concentration of cells into maximum cell proliferation. On the other hand, any doses of ConA higher than 2.5μg/ml proved not to be desirable, probably because an over-dose of T cell mitogen (in this case ConA) is toxic to cells (Lis & Sharon, 1986).

The preliminary studies further revealed that ConA can readily be neutralized by 0.1M methyl-mannopyranoside (αmm), a carbohydrate complex, which was shown to exhibit binding specificity for this T cell mitogen, but <u>not</u> for PHA. Furthermore, it was shown (Fig.2.8) that the repression of cell proliferation following a ConA over-dose can be reversed, when excess ConA is ' absorbed ' by αmm; this explains why 0.1M αmm-treated 25μg/ml ConA can induce more proliferation amongst 1X 10^6 leucocytes / ml than the untreated 25μg/ml ConA. Watkins, reported in his Ph.D. thesis (1985) that supernatant able to promote mitogenic reactivity could be generated by the addition of 0.8μg/ml ConA to 5X 10^6 splenocytes / ml for 24 hours, with free ConA being removed from supernatant by 0.02M αmm at time of collection.

Following the protocols discussed above, soluble factors (free of any active ConA) with mitogenic ability were routinely generated from ConA-stimulated splenocytes of adult *Xenopus laevis*. The ability of such soluble factors in these ConA-induced culture supernatants to induce proliferation was revealed by the enhanced tritiated thymidine incorporation observed by ASN-treated *Xenopus* splenic and thymic cells. The mitogenic effect of such active supernatants was readily apparent when tested on freshly-prepared adult *Xenopus* splenocytes. There were no significant differences when supernatants were assayed using either medium- or ConA- pre-cultured splenocytes or thymocytes, whereas the best stimulation indices were achieved when freshly-prepared splenocytes were used as assay cells. These observations contradict the published results of Watkins & Cohen (1987*b*), but are consistent with the findings of Turner (1990) and Turner *et al* (1991). The PHA-induced amphibian supernatants generated by Watkins & Cohen (1987) and Cohen, Watkins & Parsons (1987) were only capable of inducing proliferation in T cell mitogen - treated splenic blast cells, but not unstimulated splenocytes (whether freshly-prepared or medium pre-cultured).

Our finding that freshly-prepared splenocytes from control adult *Xenopus* respond positively to active culture supernatant through proliferation may be explained in several ways. The first possibility is that *in vivo* activation of leucocytes of animals used for assay had occurred prior to their use *in vitro*. As a result, expression of cytokine receptors would presumably be expressed and cells would now be ready to interact with the exogenous cytokines added *in vitro*. Such *in vivo* stimulation would be likely when there is chronic infection of a *Xenopus* colony. Thus a recent publication of Haynes & colleagues (1991) identified a naturally-occurring infection of *Xenopus* in their colony, caused by a microorganism (a putative protozoan) which activates the immune system of *Xenopus*. The majority of splenocytes from their infected individuals were reported to exhibit morphological changes seen only with activated cells, i.e., these cells are lymphoblastoid and pseudopodial (Haynes *et al*, 1991). Furthermore, splenocytes

assayed directly from these infected animals (but not from uninfected *Xenopus*) were also able to proliferate in response to TCGF - containing supernatants. However, our findings on responsiveness of ' fresh ' cells to ASNs are not readily explained in this fashion, since there is no sign of this or other ' disease ' prevailing in our *Xenopus* colony; additionally, in contrast to what Haynes *et al* described, ' fresh ' splenocytes prepared for assays in our research appeared to be small and round, i.e., were not active on morphological criteria.

A second, more likely explanation for the ability of ' fresh ' splenocytes to respond well to ASNs may be that cytokines other than IL-2 - like proteins are also present in this ConA-derived supernatant. In mammalian studies, it has been found that in addition to IL-2, a whole array of cytokines, such as IL-1, IL-4, IL-6 (B cell growth factor), IFNγ and other colony stimulating factors, are also produced when lymphocytes were challenged with T cell mitogens (Puré, Inaba & Metlay, 1988; Trinchieri & Perussia, 1985). Since the culture supernatants used throughout the present studies were only in their crude form, i.e., neither concentrated nor purified by any means, it is probable that the ConA-induced amphibian material is also replete with various growth factors which can target various different sub-sets (i.e., Th, Tc, B cells) of lymphocytes (and other cells?), causing them to proliferate. For example, in mammalian models, it is well established that cytokines like IL-1 potentiate activation of T helper cells, by increasing IL-2 release and up-regulation of IL-2 receptor expression, through ' co-stimulation ' of the T cell. IL-1 is also known able to enhance the growth and differentiation of B cells (Hamblin, 1988). It is likely that soluble factor with IL-1 - like reactivity analogous to mammalian IL-1 is present in our ConA-derived ASNs (see above). A factor with IL-1 - like reactivity was produced by Watkins, Parsons & Cohen (1987) with LPS-activated *Xenopus* peritoneal cells. This factor was reported capable of enhancing (co-stimulating) a T cell proliferative response in *Xenopus* in the presence

of a submitogenic dose of PHA, but failed to support long term thymic blast growth (in contrast to TCGF).

Since freshly-prepared adult *Xenopus* splenocytes were able to respond positively to ConA-induced active supernatants, proliferative assays were also carried out using freshly-harvested larval lymphocytes, to explore ontogenetic aspects of this finding. At the stages examined (st. 56 - 59), larval thymocytes showed no response to PHA, whereas larval splenocytes were driven into intense proliferation by this mitogen. This observation is consistent with the published work of Rollin-Smith, Parsons & Cohen's (1984). However, enhanced ^{3}HTdR uptake by freshly-prepared larval thymocytes was achieved following incubation with active supernatant, but not with control supernatant. Further, elevated thymidine incorporation was pronounced when larval thymocytes were ' co-stimulated ' by ASN plus PHA. In contrast to adult (freshly-prepared) splenocytes, larval splenocytes showed only minimal, if any, level of responses to ASN, and the latter failed to achieve co-stimulation when used with PHA. The ' susceptibility ' displayed by larval thymocytes, but not larval splenocytes, in responding to ASNs indicates that freshly-prepared larval thymocytes (but not splenocytes) express receptors for the cytokines found in the ASNs. The inability of larval thymocytes to respond to T cell mitogen alone may therefore represent a failure of larval thymic cells to secrete cytokines such as IL-1 or IL-2 (Watkins, Parsons & Cohen, 1988). When these are provided exogenously, thymocytes can then undertake enhanced proliferation. Presumably this ' lack ' of cytokine production by larval thymus relates to requirements needed by developing T - lineage lymphocytes —— for example to a need for these cells to undergo extensive negative selection at this time (Blackman, Kappler & Marrack, 1990; von Boehmer & Kisielow, 1991; Matis, Hedrick & Bluestone, 1991), to prevent reactivity against self. The main drawback of the co-stimulation assay is its lack of specificity, since either IL-1 (Di Giovine & Duff, 1990) or IL-2 (Smith, 1990) can act as ' co-stimulator '.

The idea that the proliferative capabilities of late larval thymocytes, but not splenocytes, are dampened, seems to be consistent with other observations made from Figs.2.15&16. In Fig.2.15, despite there being only 5X 10^4 splenocytes (in 100µl) per well, these cells gave a D.P.M. of 2617 (± 518) when incubated with medium alone. The assay lymphocytes used in this experiment were pools of lymphocytes from several outbred (potentially MHC-disparate) *X.laevis* larvae (st. 56 - 58). The abnormally high background counts of (yet relatively low numbers of) splenocytes here is probably due to a MLC reaction. The assay cells used in the experiment shown in Fig.2.16 were pooled from several isogeneic LG15 larvae, where no MLR can take place. Here 5X 10^4 splenocytes cultured in medium yielded a D.P.M. of only 408 (± 66). Thymocyte counts (in medium) were actually lower in the experiment (Fig.2.15) involving allogeneic mixtures; the relatively high D.P.M. seen in LG15 thymocytes (Fig.2.16) is not unexpected from the number of thymocytes (2X 10^5 cells per well) cultured. Splenocytes of late larval *Xenopus* appear able to respond to mitogenic (and antigenic) challenges; on the contrary, late larval thymocytes may be immunosuppressed to prevent any allo-immune response. Previously, Du Pasquier (1982*a*) revealed that MLC reaction of larval thymocytes near metamorphosis is suppressed, but no such suppression is seen with splenocytes at metamorphosis (Cohen & Haynes, 1990). Overall, it seems reasonable to assume that, as Cohen, Watkins and Parsons suggested (1987), ' the immune system of the larval *Xenopus* is compartmentalized in the sense that tolerogenic processes occur in the thymus, whereas functional immunity develops in the spleen '.

Perhaps the major findings (Turner *et al*, 1991) confirmed in this Chapter has been that splenocytes from 7-day thymectomized young (4 months old) post-metamorphic *Xenopus* were able to proliferate when treated with ConA-derived supernatants. These splenocytes were capable of being stimulated by ASNs when prepared directly from the animals and required no pre-stimulation. When compared with splenocytes from control animals from the same sibship, splenocytes from

thymectomized animals showed better level of proliferation when incubated with active supernatants. Control *Xenopus* examined here showed a classical proliferative response to T cell mitogens but virtually no response was shown by thymectomized animals. This unresponsiveness of splenocytes from thymectomized animals confirmed the loss of T cells from their immune system. However, there should be no surprise that other cell types in the Tx animals are able to respond to the putative array of cytokines likely to be present in ASNs. A recent publication by Haynes and Cohen (1990) has shown that LPS-stimulated, B lymphocyte - enriched *Xenopus* splenocytes proliferated following incubation with T cell mitogen-induced active supernatant (IL-2 - like material). The identity and nature of the responding splenocytes in Tx animals in our experiments await further experimentation. The functional consequences of stimulating Tx splenocytes with ASNs is probed in the next Chapter.

The final point worth mentioning is that studies presented here indicate that human recombinant IL-2 appears unable to co-stimulate freshly-prepared larval *Xenopus* thymocyte cultures. This finding is consistent with that made by Watkins and Cohen (1987*b*), who tested HrIL-2 on *Xenopus* splenic lymphoblasts *in vitro*. On the other hand, there have been reports (Ruben, Clothier & Balls, 1985; Ruben, 1986; Ruben *et al*, 1987) that HrIL-2 does have an *in vivo* effect on *Xenopus*; furthermore human anti-Tac (IL-2 receptor, p55 chain) antibody recognized a proportion of freshly-prepared splenocytes from non-immunized *Xenopus* (Langeberg *et al*, 1987*a*&*b*), a proportion which increased after PHA stimulation (Ruben *et al*, 1990*b*). Such divergent observations remain to be reconciled with further research.

Chapter 3

Use of Flow Cytometry to Explore Possible Changes in Expression of Lymphocytes Surface Antigens Induced by Culture Supernatants and T Cell Mitogens

3.1 Introduction

T lymphocytes are mediators of cell-mediated immunity, whereas B lymphocytes are the effectors of humoral antibody responses. Although both B and T cells are antigen-specific, T cells functionally differ from B cells in several important ways. Firstly, T lymphocytes not only kill target cells (such as virus-infected cells) directly, but also T cell subsets help regulate activity of both B and T lymphocytes populations. Secondly, since their antigen receptors exist only in membrane-bound form (contrast immunoglobulin of B cells), T cells interact directly with the cells they kill or regulate. Thirdly, T cells recognize foreign antigen only when the latter is presented by a host cell in association with MHC class I or II molecules. T cells recognize only linear determinants defined predominantly by primary amino acid sequences and such antigenic fragments have to be able to form stable complexes with MHC molecules. Both the T cell receptors and the MHC molecules these lymphocytes interact with are, therefore, of prime importance in the antigen recognition mechanism of T lymphocytes.

As reviewed by Du Pasquier and colleagues (1989), the *Xenopus* immune system exhibits many of the hallmarks of the immune system of higher vertebrates: for example, T and B cells (Green-Donnelly & Cohen, 1979; Bleicher & Cohen, 1981), a diversity of immunoglobulin isotopes, existence of ' cytokines ' analogous to interleukin-1 (Watkins, Parsons & Cohen, 1987) and interleukin-2 (Watkins & Cohen 1987*a*&*b*),

and a MHC complex (the XLA, for *Xenopus* lymphocyte antigen —— see Du Pasquier, Chardonnens & Miggiano, 1975). Extensive studies have been carried out to show that T cell responses in a lower vertebrate like *Xenopus* are also MHC controlled. However, the precise role that T cell - specific surface molecules and MHC antigens play in the recognition process of foreign antigens by *Xenopus* still requires further investigation.

It has been demonstrated in the previous Chapter that ConA-induced *Xenopus* ' cytokines ' are capable of inducing lymphocytes from either larval or adult *Xenopus* to proliferate. The work included in this Chapter is designed to investigate the possible up-regulation of various cell surface antigens on *Xenopus* lymphocytes, following mitogenic challenge from either culture supernatants containing putative cytokines or by use of T cell mitogens.

The first part of this Chapter assesses the expression of MHC class II molecules following stimulation of larval thymocytes. According to a recent paper by Harding and colleagues (in press, 1992), MHC class II antigens are up-regulated in adult *Xenopus* splenocytes following T cell mitogen - induced proliferation. It is also known that when incubated with (T cell mitogen - induced) culture supernatants in a co-stimulation fashion with PHA, larval thymocytes are induced to proliferate (see previous Chapter). Since larval thymocytes are considered by Du Pasquier and Flajnik (1990) not constituitively to express MHC class II molecules, the ability to stimulate larval thymocytes in this way appears an appropriate model for probing the possible up-regulation of MHC class II expression on *Xenopus* T cells following mitogenic challenge. Anti- *Xenopus* class II monoclonal antibody, together with flow cytometry are used in these experiments. The second experimental model for investigating possible increase in MHC class II expression following T cell stimulation has employed longer-term *in vitro* culture of splenocytes from young adult *Xenopus*, following initial stimulation with ConA.

The possibility of initiating the appearance of T cell - specific surface markers [for example, the XTLA-1 antigen and the putative CD8 marker (AM22-reactive)] on splenocytes from thymectomized animals is dealt with in the second part of this Chapter. It has been well documented that following such thymectomy on day 7 post-fertilization, splenocytes from such Tx animals respond at best minimally to T cell mitogens. Moreover such spleens have a very high proportion of Ig-positive B lymphocytes (Bleicher *et al*, 1983). However, as described in Chapter 2, splenocytes from thymectomized *Xenopus* could be potentiated to proliferate following incubation with ' cytokine ' - rich culture supernatant. Work by others in this laboratory has suggested that the responding cells are the Ig^{-ve} population (Turner *et al*, 1991). The question asked here was:- would it be possible to induce T cell specific markers on ' non - T ' (and ' non - B ') cells following treatment of these cells with ConA-induced ASNs? This question is addressed by *in vitro* and *in vivo* stimulation of Tx splenocytes with culture supernatant derived from ConA-stimulated splenocytes, followed by staining with anti - T cell specific monoclonal antibodies and flow cytometry.

3.2 Materials and Methods

3.2.1 Animals and Thymectomy

Outbred *X.laevis*, which are MHC undefined, inbred MHC-compatible J strain *X.laevis* (MHC = jj), interspecies hybrid clones, namely LG15 (MHC = ac) and LM3 (MHC = wy), were used in these studies. All animals were reared and bred in this laboratory, as described in **2.2.1.** Ages are given in the Results section; the studies involved larvae, young toadlets and older adults.

The thymectomy operations performed on these animals were as described in **2.2.2.**

3.2.2 Experiments Probing MHC Class II Expression following *in vitro* Stimulation of Lymphocytes from Thymus-intact *Xenopus*

3.2.2.1 Experimental plans

Two experimental approaches were followed, to explore possible changes in surface antigen expression after *in vitro* stimulation. These approaches were as follows.

A] Thymocytes from larvae (st. 56 - 60 , between 6 - 7 weeks) were co-stimulated with ASN plus PHA; after 18 or 48 hours of culture they were harvested, stained with McAbs and prepared for flow cytometry.

B] Attempts were made to develop longer-term T - cell cultures by initial stimulation of splenocytes from 6 - 18 months old toadlets with ConA and, in some instances, with subsequent stimulation with supernatants. Cells were successfully kept for periods up to 2 weeks, at which time they were stained with appropriate monoclonal antibodies and used in flow cytometry.

3.2.2.2 Culture Conditions Used in the 2 Experimental Approaches

A] Thymocytes taken from tadpoles were prepared as described in **2.2.4.** Cell concentration was adjusted to 5X 10^6 leucocytes / ml. 250µl of cells was dispensed into individual wells of 24-well plates, then either 250µl of medium or 125µl of 2µg/ml PHA (i.e., 0.5µg/ml ' in-well ') plus 125µl of ASN (αmm-absorbed) was added. In the first experiment, cells were cultured in 10% FCS-supplemented L-15 and harvested at 18 hours. In the second experiment, cells were cultured in 1% FCS- supplemented L-15 and harvested at 48 hours. Cells were cultured in a 5% CO_2 humidified incubator at 26°C ± 1°C. Fresh thymocytes were taken for comparison in the second experiment and were prepared on the day when cultured cells were collected for FACS analysis.

Some parallel ^{3}HTdR studies were carried out alongside the FACS analyses. Here, 1X 10^6 leucocytes / ml were cultured in 1% FCS L-15 in 96-well plates, either in a supernatant assay (as in **2.2.8**) or mitogen dose response assay (as in **2.2.6**). Cells were pulsed at 48 hours with ^{3}HTdR and harvested at 72 hours for scintillation counting.

B] Assay cells for longer-term *in vitro* culture were adult splenocytes, initially used for ConA- induced supernatant generation (see **2.2.7**). After removing ASN at 24 hours, these cells were subsequently maintained in either 10% FCS supplemented L-15 throughout, or in one instance, split and cultured with supernatants, for various lengths of time. Every 3 - 4 days, they were fed with fresh medium (as in **2.2.8** for pre-cultured lymphocytes). Furthermore, at the end of one complete week, cells were collected from wells, spun down and dead cells eliminated by density gradient centrifugation. Viable cells were then transferred to a fresh 24-well plate for continued culture, with the addition of either 10% FCS L-15 or supernatants.

Cell cultures were always kept in a 5% CO_2 humidified incubator at 26°C ± 1°C.

3.2.3 Experiments Probing T Cell Marker Expression following *in vitro* and *in vivo* Treatment of Control and Thymectomized *Xenopus* with Supernatants

3.2.3.1 Experimental Plans and Culture Conditions

Two experimental approaches were followed, to explore possible changes in surface antigen expression after stimulation with ASN. These approaches were as follows.

A] Splenocytes from control and thymectomized toadlets (14 - 25 weeks) were stimulated *in vitro* with ASN or CSN. They were adjusted to 1X 10^6 leucocytes per ml for all but the last experiment in this series (3X 10^6 leucocytes / ml). Cultures were set up in 96-well flat-bottomed plates, when toadlets were 16 weeks and younger, whereas cells from older animals were cultured in 24-well plates. Depending on the number of cells and availability of SN, the final total ' in-well ' volumes varied. However, the ratio between the volumes of cells, supernatant and medium was always maintained at 2 : 1 : 1. These experiments were initially set up in 1% FCS L-15, but later on 10% FCS- and 5% FCS- supplemented medium were tried, to ascertain the effect of various concentrations of foreign proteins on cultured cells.

Cells were harvested at 72 hours, when some were taken for flow cytometry and others were harvested for scintillation counting, the latter having been pulsed with tritiated thymidine 24 hours earlier. The ^{3}HTdR studies were performed to confirm that ASNs were stimulatory (at least in terms of their ability to induce

lymphocyte proliferation). Some proliferative studies were also carried out on ConA-stimulated cells, so as to confirm the loss of T cells in Tx animals.

B] 6 month old toadlets, either ' Tx ' or ' control ', were injected with either 200µl ASN or CSN, via their dorsal lymph sacs on day 1. Three days later they were similarly injected with the same stimulant and same volume via the intraperitoneal (i.p.) route. Some animals were taken for FACS analysis on day 7. The remaining animals were injected again on day 8 (i.p.) and were sacrificed for FACS analysis on day 14.

3.2.4 Flow Cytometry

3.2.4.1 Preparation of Leucocyte Suspensions

Fresh thymocytes and splenocytes were prepared as described in **2.2.4**, except that flow cytometry amphibian-strength phosphate buffered saline (FC-APBS) was used. This medium was made up with 6.6g NaCl, 1.5g Na_2HPO_4 and 0.2g KH_2PO_4 per litre of double distilled water and adjusted to pH 7.4 (Flajnik; 1983). The FC-APBS was supplemented with 0.1% bovine serum albumin (BSA; Sigma, A7030) as a source of irrelevant protein. 0.01% sodium azide was added to this medium so that microbial growth would be discouraged and cell metabolism inhibited, thereby ' capping ' and subsequent endocytosis or shedding of labelled cell surface antigens could be prevented.

Previously-cultured cells were recovered from either 96-well flat-bottomed or 24-well plates by washing with either 100µl or 1ml (according to well size) of L-15 medium, by repeated pipetting to ensure that as many cells as possible were collected. Cells were transferred to Falcon tubes and then pipetted vigorously (but carefully) so that as many cells as possible could be freed from the ' rosettes ' or clumps

formed during *in vitro* culture. Cells were washed twice in FC-APBS at 300g for 10 mins at 4°C and finally resuspended in FC-APBS. Cell viability and numbers were determined as in **2.2.4.**

All cells, both ' fresh ' and ' cultured ' were adjusted to a concentration of 1X 10^6 lymphocytes per ml for staining with antibodies for flow cytometry.

3.2.4.2 Primary and Secondary Antibodies

Staining with primary antibodies involved the use of several mouse monoclonal antibodies, in the form of hybridoma culture supernatants. Three cell lines were maintained in our own tissue culture laboratory, namely XT-1 (a cell line secreting monoclonal antibodies that label a subset of *Xenopus* peripheral T cells, generously supplied by Dr. S. Nagata), AM20 (anti- MHC class II, gift from Dr. M. Flajnik) and 8E4:57 (anti- *Xenopus laevis* IgM, gift from Dr. R.H. Clothier). XT-1 and AM20 hybridoma cells were cultured in 7% FCS- supplemented RPMI 1640 medium (Flow Labs), which was modified as described in **2.2.10.1**, whereas 8E4:57 hybridoma cells were cultured in Dulbecco's modified Eagles medium (D.M.E.M.). Here 50ml D.M.E.M. (10 times normal strength; Gibco) was diluted with 370ml autoclaved double distilled water and further modified with 10ml L-glutamine, 5ml non-essential amino acids (NEAA: 100 times normal strength), 10ml sodium pyruvate (100mM) , 44.6mM sodium bicarbonate, 1% Penicillin, 1% Streptomycin, 1% Fungizone, 10% FCS (all from Flow Labs) and 100µl β-mercaptoethanol (50mM; BDH). These media were filter-sterilized.

All hybridoma cells were cultured to grow in 25ml plastic tissue culture flasks (Falcon) and cultured at 37°C ± 1°C in a 5% CO_2 humidified incubator. When the hybridoma cells were confluent (which is approximately every fourth day), they were then sub-cultured at a ratio of 1 in 10 in their appropriate fresh media. The

remaining contents of the tissue culture flasks were then aseptically transferred to 10ml sterile polystyrene centrifuge tubes (Sterilin; Cell Cult) and spun at 300g at 4°C for 10 mins to pellet hybridoma cells for the collection of culture supernatants, rich in secreted monoclonal antibodies. The culture supernatants were then stored in autoclaved eppendorfs at 4°C or sterile polythene freezing vials (Nunc; Gibco) at -20°C. 0.02% sodium azide was added into these culture supernatants to curb any microbial growth.

Additional hybridoma supernatants were supplied directly by Dr. M. Flajnik at Miami University. These monoclonal antibodies were AM22 (labels the putative CD8 marker on *Xenopus* cytotoxic T cells) and AM15 (putatively reacts with an Ly-6-like molecule on *Xenopus* lymphocytes). These culture supernatants contained 0.05% sodium azide as preservative.

Secondary antibody used was commercially available fluorescein iso-thiocynate (FITC) - labelled anti- mouse immunoglobulin. Specifically this was a rabbit anti- mouse Ig $F(ab')_2$-FITC (Dako Ltd.). It was stored at 4°C in a light-proof container to avoid ' bleaching ' of the fluorochrome label.

3.2.4.3 Staining Procedure with Primary and Secondary Antibodies: Indirect Immunofluorescence

Each cell suspension ($1X\ 10^6$ leucocytes / ml) was divided into aliquots, with $3X\ 10^5$ leucocytes per tube (Falcon). One aliquot was left completely unstained, to act as a control for cellular autofluorescence; this was the ' unstained control ' and was only set up in preliminary experiments. A second control was routinely prepared; this acted as a control for non-specific binding of the secondary antibody and is known as the ' secondary antibody control ', where cells were incubated with FITC-labelled secondary antibody only. The remaining aliquots were incubated with various primary monoclonal antibodies, followed by subsequent incubation with the secondary antibody.

The primary antibodies which were in the form of culture supernatants, were diluted 1 in 4 with FC-APBS. The secondary antibody was used at 1 in 20, again being diluted with FC-APBS. The secondary antibody had been preadsorbed for about 45 mins on ice with normal *Xenopus* serum (50µl *Xenopus* serum per ml of antibody —— i.e., 1 in 20) to minimize background (2°ab only) staining level.

The indirect immunofluorescence staining procedure began by centrifuging cell suspensions at 1500 r.p.m. for 10 mins at 4°C. After removal of supernatants, cell pellets (3X 10^5 leucocytes) were resuspended in 200µl of the appropriate dilution of primary antibody per tube. Cells (on ice) were then incubated for 45 mins, then resuspended in 2ml of fresh staining medium and then centrifuged at 300g for 10 minutes at 4°C. They then underwent a further cycle of washing and, following the last wash, pelleted cells were resuspended in 150µl per tube of secondary, FITC- antibody (which had previously been adsorbed and spun at 2000g for 10 mins at 4°C to remove any debris) and were incubated for 45 mins on ice. All preparations were kept away from light during and after secondary antibody incubation, so that ' bleaching ' of fluorochrome label could be avoided. Two further cycles of washing and centrifugation followed completion of the second incubation. Cells were finally resuspended in 300µl FC-APBS for transport to the FACS.

3.2.4.4 FACS Analysis of Monoclonal Antibody-labelled Cells

Cell suspensions (on ice) were transported by car to the Department of Surgery, Newcastle University Medical School, for FACS analysis as quickly as possible after indirect immunofluorescent labelling was completed. Cells were analysed on a FACS 420 fluorescence activated cell sorter (Becton Dickinson) which analysed these cell samples using an Argon ion laser with 400mW output at 488nm.

Four parameters are employed to measure every ' event ' or cell counted. They are : FSC (low angle forward light scatter) which is an indirect measurement of cell size; RTO (granularity) which is correlated with the degree of right angle light scatter caused by the cell; FL1 (fluorescence intensity measurement for FITC-labelled cells at wavelength 530nm); FL2 [fluorescence intensity measurement (at 575nm) for propidium iodide (P.I.), which stains dead cells].

Samples could be gated for analysis, by re-setting desired values for low-angle forward light scatter and granularity measurements. The gating parameters used in these studies tended to exclude non-viable cells and cell fragments (low FSC), and also the large, nucleated erythrocytes (high FSC). Gating was of particular importance for the analysis of splenocyte suspensions, which contain relatively high proportions of erythrocytes, if density gradient separation had not been employed prior to fluorescence staining.

For each sample analysed, 10,000 total (ungated) events were recorded and values for different parameters were collected and stored by the equipment's integral computer. Then data was analysed using a BDIS Consort 30 - version F2/88 programme (both from Becton Dickinson).

3.2.4.5 Presentation of Results from FACS Analysis

Several types of graphical read outs of the data were generated. Primarily, histograms were used to display the relationship between FITC fluorescence intensity (on a log10 scale on the X-axis) and relative cell numbers (on a linear scale on the Y-axis). The BDIS Consort 30- version F2/88 programme delegated every ' event ' counted to one of 256 channels from the raw data collected. Log amplifiers had been integrated with the fluorescence detecting channels of the FACS machine and were calibrated in such a way that the full scale (256 channels) was equivalent to a fluorescent intensity of

3 log decades. This greatly extends the scale of measurable fluorescence intensity and condenses various intensities measured into a readable form. Histograms were then generated, which showed the number of cells assigned to each channel.

For each type of cell preparation, at least 2 samples were run through the sorter. The first one being the ' secondary antibody control ' to act as a background staining level. Various experimental samples were compared with this secondary antibody control. If no specific binding of primary antibody occurred, histograms generated from these two print outs should closely resemble each other. A marker was inserted on the secondary antibody histograms, approximately at a position on the X-axis to exclude all but 2% of the background staining. The percentage of specific fluorescence (due to primary antibody staining) in experimental samples relates to this marker - i.e., reflects the proportion of cells with fluorescence intensity to the right of the marker.

Other forms of graphic representation included the contour plot and the dot plot. There are two kinds of contour plot. The ' segmented ' contour plot, which comprised of four quadrants, shows FITC intensity on the X-axis and the intensity of propidium iodide taken up by cells on the Y-axis. 10 - 20µl P.I. (0.25mg/ml) was added to every tube (300µl) of cells immediately prior to flow cytometry. The ' ordinary ' contour plot shows the feature of cells analysed in terms of their RTO and FSC (which are on the X and Y axes respectively). These contour plots could be used to indicate either the ' gated ' population or the total (non-gated) population depending on the user's requirement.

The dot plots, in which each individual dot is a single cell (an ' event ') analysed, shows unique feature of each cell in terms of their RTO (granularity) and FSC (size). The gate shown is set up arbitrarily, depending where usually the majority of relevant ' control ' cells are found. The so-called ' blast ' cells, due to their morphological

enlargement and higher granular internal compositions, are often found outside this gate. The position of the gate can be re-adjusted accordingly.

3.3 Results

3.3.1 Expression of Surface Antigens on Lymphocytes following *in vitro* Stimulation of Cells from Thymus-intact Animals with T Cell Mitogen and / or ConA-induced Supernatants

3.3.1.1 Short-term ' Co-stimulation ' of Larval Thymocytes

In the previous Chapter, enhanced tritiated thymidine incorporation was recorded when larval thymocytes were co-stimulated with PHA and ConA-induced ASN. Similar co-stimulation experiments reported here were conducted to probe if the *in vitro* culture of larval thymocytes with PHA and ASN would result in the induction of MHC class II surface molecules.

Initially, thymocytes taken from several LG15 tadpoles (st. 57 - 58 / 6 weeks old) were pooled and cultured for 18 hours, either in 10% FCS-supplemented L-15 medium alone, or in this medium containing 2μg/ml PHA (i.e., 0.5μg/ml ' in-well ') plus ASN (25% of total in-well volume). Flow cytometric traces revealed no differences in AM20-staining between the medium pre-cultured and PHA/ASN pre-cultured thymocyte populations, with approximately 57% positively-stained cells in both cases (**Fig.3.1**). FACS profiles for unstained and secondary antibody (2°ab) - alone stained aliquots were comparable; it was hence decided to use the latter as background in future FACS studies.

A second experiment investigating MHC class II expression on stimulated larval thymocytes involved outbred *X.l.* larvae (st. 58 - 61 / 7 weeks old). Outbred *X.l.* larvae were used here so as to compare data from different types of *Xenopus*. These thymocytes were cultured for 48 hours, either in 1% FCS-supplemented L-15 medium or PHA/ASN (see above). For comparison, freshly-prepared thymocytes from outbred *X.l.* larvae were also stained with AM20 and taken for FACS analysis. The use of a longer culture period was to increase the likelihood of experimentally-induced changes in MHC expression. The reduced concentration of FCS used in this experiment was an attempt to minimize possible stimulatory effects, initiated by FCS proteins, on the expression of MHC class II molecules.

The FACS profiles on this experiment are shown in **Fig.3.2**. These reveal that, compared with freshly-prepared cells, there was a lower percentage of $AM20^{+ve}$ thymocytes after 48 hours of culture in medium only (18%) or after PHA/ASN incubation (again 18%).

Contour plots of size (forward scatter, FSC) and granularity (side scatter, RTO) indicated a shift towards smaller (dead ?) cells (with low FSC) in the 48 hour pre-cultured populations (**Fig.3.3**). Propidium iodide staining (**Fig3.4**) revealed that 48 hour pre-cultured thymocytes did indeed contain a very high proportion (up to a third) of dead cells ($P.I.^{+ve}$), compared with very few (less than 2%) in the freshly-prepared thymocyte population. This figure also shows that P.I. - excluding (viable) thymocytes represent the major $AM20^{+ve}$ population.

In conclusion, these two culture experiments failed to show any changes in proportion of larval thymocytes expressing MHC class II caused by short term co-stimulation with ASN/PHA. However, the experiments were of interest, since they indicated constitutive MHC class II expression on a proportion of late larval thymocytes, a finding that was unexpected from the previous published FACS data of Du Pasquier & Flajnik (1990).

3.3.1.2 Longer-term Culture of Splenocytes from Adult Animals

Since *in vitro* incubation of larval thymocytes for 48 hours with culture supernatants plus T cell mitogen failed to up-regulate the expression of MHC class II molecules, a second approach involved assaying class II and other cell-surface antigens on adult splenocytes, cultured for prolonged periods following initial mitogenic challenge. Post-metamorphic *Xenopus* were used in these experiments, to provide sufficient cells for long-term culture.

In the first experiment, splenocytes from a 28-week old *Xenopus* LM3 were analysed. These cells were either left ' unstimulated ' in 0.25% BSA-supplemented medium alone for 24 hours, or were initially stimulated in this medium with 2.5µg/ml ConA for 24 hours. They were then resuspended and cultured subsequently in 10% FCS-supplemented L-15 (the culture supernatant collected at 24 hours was used elsewhere). These cells were kept in culture successfully for 2 weeks (see **Methods: 3.2.2.2.C**) and were taken for FACS analysis on day 14. Freshly-prepared LM3 splenocytes from another LM3 animal of the same age were also taken along for FACS analysis on that day.

Fig.3.5 shows flow cytometric traces for splenocytes stained with the McAbs XT-1, AM22 and AM20. The FACS profiles on splenocytes shown from this figure onwards refer to ' gated ' cells, i.e., the profiles exclude the large erythrocytes and small particles (gated out by use of FCS/RTO dot plot as explained in **Materials and Methods**). The proportion of XTLA-1^{+ve} T cells after culture in medium was 43%. More (48%) of the ConA pre-cultured cells were XTLA-1^{+ve}, but this percentage was comparable with that found in freshly-prepared splenocytes (51%). Use of the other T cell marker AM22 (the putative anti-CD8) revealed that cultured cells, especially after initial stimulation with ConA, contain a somewhat elevated proportion of $AM22^{+ve}$ lymphocytes (29% after ConA challenge compared with 21% in freshly-prepared cells).

Proportions of MHC class II^{+ve} (AM20^{+ve}) cells appeared identical in all three splenocyte populations studied, although intensities appeared greater in the cultured cells. Note that constitutive class II expression is found, as expected, on the vast majority of adult splenocytes (see also Du Pasquier & Flajnik, 1990).

A second experiment was carried out using 14-day-old pre-cultured splenocytes from an outbred *X.l.* (1 year old). Unfortunately, splenocytes that were initially pre-cultured in medium alone failed to survive until the time for FACS. The proportion of XTLA-1^{+ve} cells in the ConA pre-cultured splenocytes was approximately 20%, a level that compares well with the proportion of XTLA-1^{+ve} cells found in freshly-prepared *X.laevis* splenocytes (unpublished data form this laboratory). The proportion of ConA pre-cultured splenocytes expressing AM20 (class II) was virtually 100% and the fluorescence intensity was extremely bright (**Fig.3.6**) and required a 4 log decade scale of fluorescence intensity to be set up on the FACS computer! This contrasts the much lower intensity staining of AM20^{+ve} cells seen in freshly-prepared adult *Xenopus* splenocytes (unpublished data from this laboratory).

A third experiment was conducted to probe differences in size and granularity between medium pre-cultured and ConA-treated splenocytes following 9 or 14 days of *in vitro* culture, Following initial mitogenic challenge with ConA, T cells undergo blastogenesis. Blast cells are larger, more granular and pseudopodial compared with resting , unstimulated cells. Size and ' psudopodial ' changes can be visualized directly by eye under a stereo-microscope; whereas size and granularity changes are clearly apparent in the flow cytometric dot plots (**Figs.3.7&8**). It should be noted that after 14 days of culture (**Fig.3.8**) the presence of blast cells was much less obvious when compared with the 9 day-cultured cells (**Fig.3.7**). It is unlikely that these differences reflect the use of different type of *Xenopus* in the 9-day (J strain *Xenopus laevis*) and the 14-day (LM3 *Xenopus*) experiments, since 14-day (ConA- pre-stimulated) cultures of *Xenopus laevis* also showed no obvious blast cell morphology in dot plots (see **Fig.3.6**).

At 9 days, when the usual RTO/FSC gate for splenocytes was set up, while this included the vast majority of medium pre-cultured leucocytes, it excluded a significant proportion of the blast cells found after ConA culture (**Fig.3.7**).

In conclusion, two weeks *in vitro* culture of splenocytes from *Xenopus* adults, especially after initial stimulation with ConA, up-regulates the expression of MHC class II. Proportion of splenic T cells expressing AM22^{+ve} staining may also be increased 2 weeks after ConA stimulation, whereas no obvious changes were noticed at this time with respect to percentages of XTLA-1^{+ve} T cells.

3.3.2 Probing Emergence of T Cell Specific Markers on 7-day Thymectomized Toadlets' Splenocytes following *in vitro* or *in vivo* Stimulation with Culture Supernatants

These experiments were carried out on just one batch of outbred *Xenopus laevis* control and 7-day-thymectomized animals.

3.3.2.1 *In vitro* Stimulation

A] Animals Aged between 14 - 16 Weeks

Thymidine incorporation assays (**Chapter 2, Fig.2.12**) on splenocytes from toadlets thymectomized at day 7 post-fertilization, suggested that T-independent lymphocytes were able to respond through proliferation to ASN treatment. Flow cytometric studies were here carried out to see if incubation with ASN would induce the appearance of ' T cell subset specific markers ' on these T-independent cells.

The expression of XTLA-1 was first investigated in 14 weeks old animals. Splenocytes from both a control and thymectomized toadlets were cultured in 10% FCS L-15 with either ASN or CSN for 72 hours. These cells, together with freshly-prepared splenocytes from additional animals, were then McAb-stained and used in FACS analysis. Those splenocytes from control animals (**Fig.3.9**) cultured in the presence of CSNs and ASNs displayed a somewhat higher proportion of XTLA-1^{+ve} cells than the freshly-harvested cells (16%$^{+ve}$ prior to culture and approximately 23%$^{+ve}$ after 3 days culture). Removal of XT-1^{+ve} T cells following early-thymectomy was demonstrated in FACS profiles of both freshly-prepared and pre-cultured splenocytes (**Fig.3.10**). Overall, it appears that when being compared with CSNs, ASNs do not have a positive impact (within 72 hours) on the induction of XTLA-1 expression on splenocytes from either control or thymectomized animals.

The expression of AM22, another T cell subset marker, was studied in a similar experiment using splenocytes from 16-week old control and thymectomized toadlets. The FACS profiles of control (**Fig.3.11**) and thymectomized (**Fig.3.12**) splenocytes were clearly distinguishable. The proportion of AM22^{+ve} cells in cultured splenocytes of controls (14 - 15%) is noticeably reduced to background levels following thymectomy. It firstly appears that the proportion of AM22^{+ve} cells found in the control toadlet has reduced following *in vitro* culture with either active or control supernatant, when compared with freshly-prepared splenocytes; and secondly with no apparent effect from ASN on the proportion of AM22^{+ve} lymphocytes when compared with those cultured with CSN either. Neither ASN nor CSN had any positive impact on AM22 up-regulation on Tx cells in relation to the expression level found in freshly-prepared cells. **Fig.3.13** shows that AM22^{+ve} populations recorded were P.I. - negative (i.e., viable) cells. Although less than 5% of the cultured splenic lymphocytes from control and Tx animals stained with P.I., it should be pointed out that these cells were the RTO/FSC-gated population. Ungated cultured cells revealed the total population of P.I.$^{+ve}$ ' events '

—— and indicated up to 20% of P.I. - stained ' events ' (including fragmented cells and red blood cells), whereas more than 95% of ' ungated ' freshly-prepared splenocytes were viable.

In view of the negative findings with ASN above, the potency of supernatants to stimulate splenocytes from control and Tx *Xenopus* was checked here, by investigating ^{3}HTdR incorporation, using the same cells (from 16 weeks old animals) as in the FACS experiments above. Both control and Tx splenocytes responded by increased proliferation following 72 hours of stimulation with ASNs only, and not with CSNs (**Table 3.1**). Additional experiments revealed that splenocytes from animals of 9 - 15 weeks of age failed to respond to T cell mitogen stimulation (**Table 3.2**), whereas control siblings mount an excellent response to both ConA and PHA.

B] Animals Aged approximately 6 Months

Flow cytometric analysis on monoclonal antibody - stained, cultured cells was subsequently carried out using splenocytes from 6 months old control and Tx animals (**Figs.3.14&15**). Cells were incubated with either XT-1, AM22, or AM15. The proportions of control cells labelled with these McAbs were similar following either 3 days' culture in ASN or CSN (**Fig.3.14**), with approximately 25 - 29% splenocytes expressing XTLA-1, 18 - 19% expressing AM22 and about 80% were AM15^{+ve}.

At 6 months of age, approximately 6% of CSN-cultured Tx splenocytes now surprisingly expressed XTLA-1 and AM22 (**Fig.3.15**). About 65% Tx splenocytes cultured in CSN expressed AM15. After ASN treatment the proportions of cells stained with each of these antibodies was slightly increased. Thus about 10% Tx splenocytes now expressed the T cell markers and 74% the Ly-6 marker.

The *in vivo* emergence of ' T - like ' cells in thymectomized animals at this age (suggested by the use of McAbs XT-1 and AM22) was confirmed with parallel

^{3}HTdR studies on 3-day ConA stimulated cells (**Table 3.3**). Thymidine incorporation studies carried out on 9 months old control and Tx sibling animals backed up these findings of ' T - like ' cell appearance.

3.3.2.2. *In vivo* Stimulation

Concomitant with the FACS studies following *in vitro* treatment of Tx splenocytes with supernatants, experiments were set up to investigate the capacity of *in vivo* injection of supernatants to stimulate T cell markers. Animals were first injected with ASN or CSN, as described in **3.2.3**, when they were 24 weeks old. **At the time these experiments were set up, we were unaware of the emergence of T-like cells in Tx animals of this age (see above).** Thymus-intact animals were injected with ASN only; Tx toadlets were given either ASN or CSN. FACS traces on splenocytes from thymus-intact animals, 7 days post-injection, are shown in **Fig.3.16**; 19% being XTLA-1^{+ve} and 17% AM22^{+ve}. With respect to the Tx animals (**Fig.3.17**), the Tx toadlet injected with CSN showed background levels of XTLA-1 or AM22 expression on its splenocytes. On the other hand, one of the Tx animals (# 2) injected with ASN displayed a low but distinct positive level of XT-1 staining (8%) and AM22 staining (6 %), whereas the other toadlet showed fluorescence levels comparable to cells stained in 2°ab alone.

The remaining *in vivo* - injected animals (given repeat injections as described in Methods) were taken for analysis on day 14 and the expression of T cell subset and B cell markers was explored with the use of McAbs XT-1 and AM22 for the former and 8E4:57 for the latter. Splenocytes from two non-injected, thymectomized toadlets of the same sibship were also prepared and stained for comparison.

Fig.3.18 reveals the levels of T and B cell markers in two ASN-injected controls. The proportion of cells stained with T cell markers was rather low (maximum

= 17%), whereas sIg^{+ve} B cells represented about 50 - 60% of the splenocyte population. **Fig.3.19** shows the FACS traces of T - and B - cell specific markers expressed on non-injected Tx animals aged 26 weeks. The FACS traces for each cell marker concerned were found to be comparable in the 2 animals under study. Low, but distinct, proportions of XTLA-1^{+ve} cells (approximately 9%) were found in these non-injected Tx toadlets. About another 9% of splenocytes in these two Tx populations stained positively with AM22, whereas the expression level of 8E4:57 was on average about 60%. Splenocytes from two Tx animals injected with CSN (**Fig.3.20**) possessed rather similar percentages of XTLA-1^{+ve} and $AM22^{+ve}$ splenocytes as found in non-injected toadlets. A slightly increased proportion of B cells was suggested (at least 74% being 8E4:57^{+ve}). Of the three ASN-injected Tx animals analysed (**Fig.3.21**), two toadlets (# 1 and # 2) both had only very low percentages of splenocytes positive for XTLA-1 or CD8 T cell markers; B cell levels were about 60%. Splenocytes from the third ASN-injected Tx animal had a much higher proportion of cells expressing the T cells markers (15% for XTLA-1 and 9% for AM22). Approximately 64% of splenocytes from this animal were 8E4:57^{+ve}.

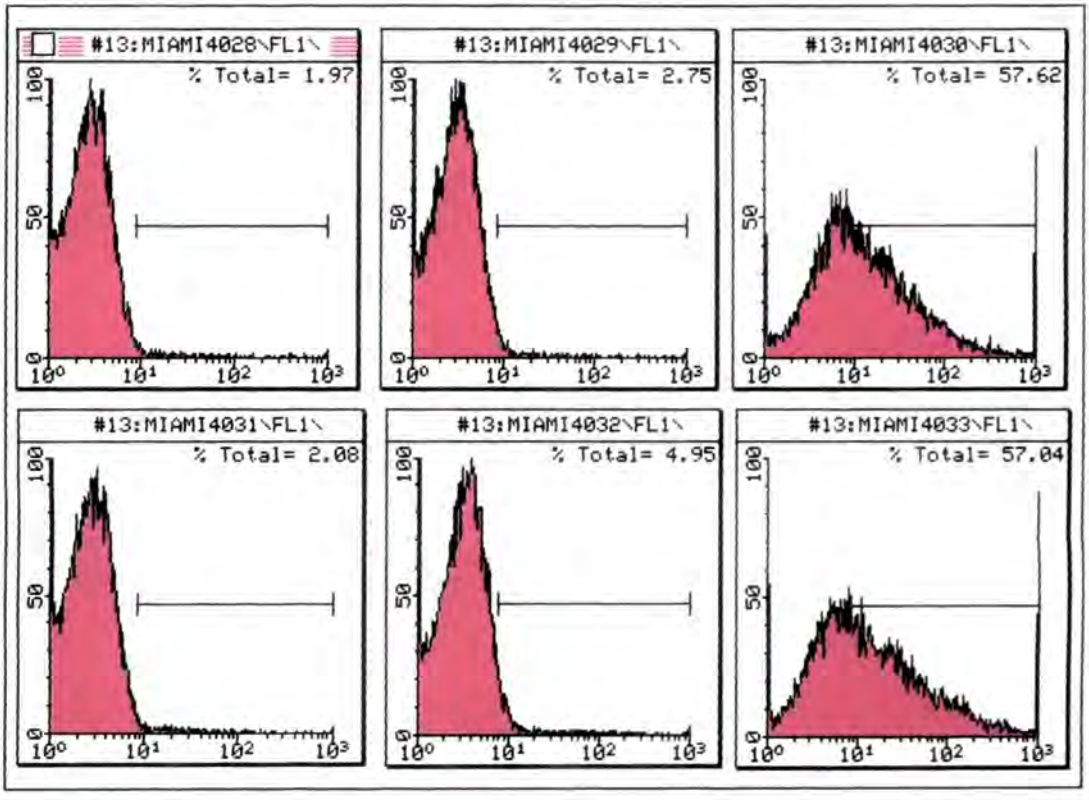

Fig.3.1 Fluorescence Intensity Histogrammes of Larval LG15 Thymocytes, following 18 Hours of ' Co - Stimulation ' with PHA and ASN

Thymocytes from control LG15 larvae (st. 57 - 58 / 6 weeks). Cells had been pre-cultured either in medium alone or PHA/ASN for 18 hours prior to the analysis. FACS-generated fluorescence intensity histogrammes of ' experimental ', and control preparations (all were ungated cell populations) shown have log fluorescence intensity (530nm) on the X-axis, and relative cell number on the Y-axis (10,000 events / sample).

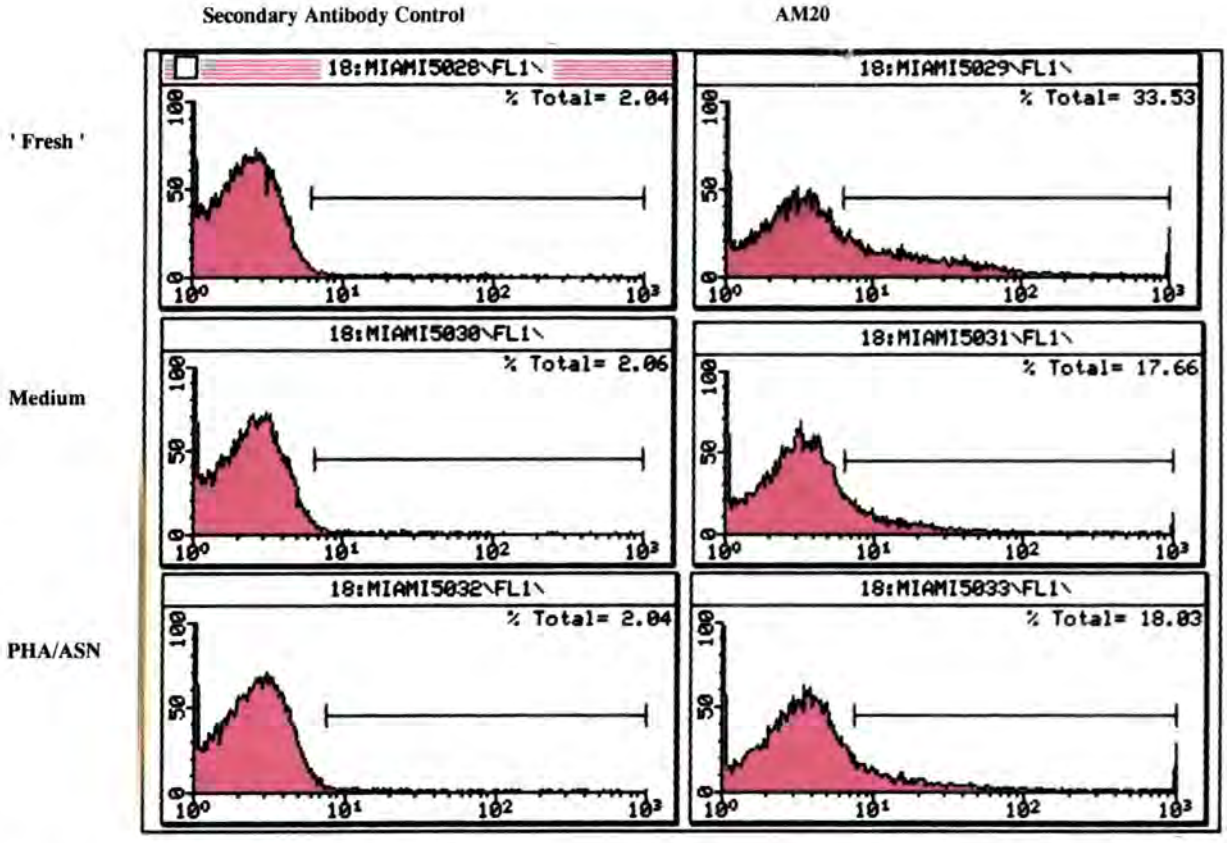

Fig.3.2 Fluorescence Intensity Histogrammes of Outbred Larval Thymocytes, following 48 Hours of ' Co - Stimulation ' with PHA and ASN

Thymocytes from control outbred *Xenopus laevis* (st. 58 - 61 / 7 weeks), freshly-prepared (row 1) or pre-cultured for 48 hours, either in medium alone or PHA/ASN prior to the analysis. FACS-generated fluorescence intensity histogrammes of ' experimental ', and control preparations have log fluorescence intensity (530nm) on the X-axis and relative cell number on the Y-axis (10,000 events / sample).

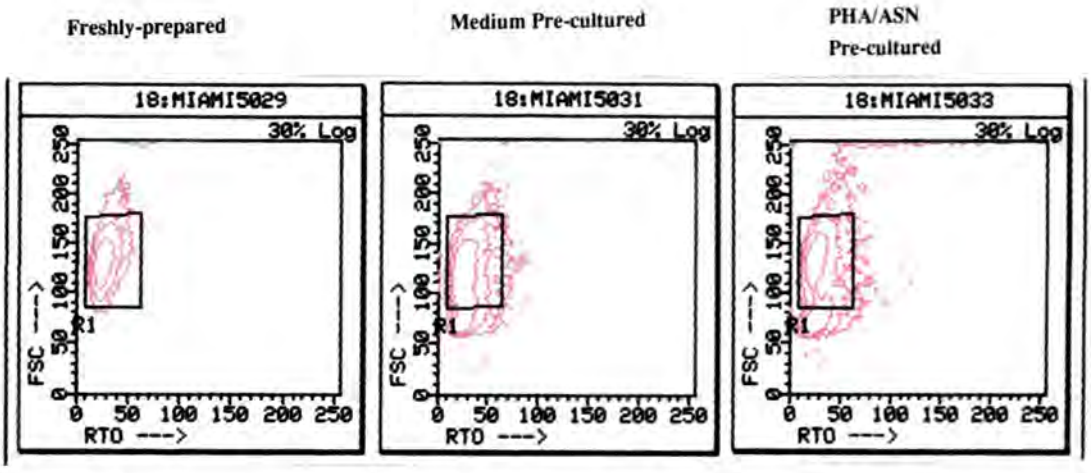

Fig.3.3 Contour Plots showing Changes of 48 Hours Pre-cultured Larval Thymocytes in terms of Granularity (RTO) and Size (FSC)

Freshly-prepared and 48 hours pre-cultured *X.laevis* larval thymocytes are compared in terms of their granularity and size. X-axis: RTO (Granularity); Y-axis: FSC (Size). Each dot is an event and plotted according to its specific RTO/FSC. Contour lines are drawn when the cell numbers become sufficiently high in a particular area of the graph. Contours representing different cell numbers receive different outlines. The gate shown here is set up arbitrarily, depending on where the majority of unstained ' fresh ' cells are found (here 84%, not shown). Lower percentages of cells were found within this gate following *in vitro* culture. Readings of percentages of cells within gate, from left across are: 85.78%, 75.46% & 63.75%.

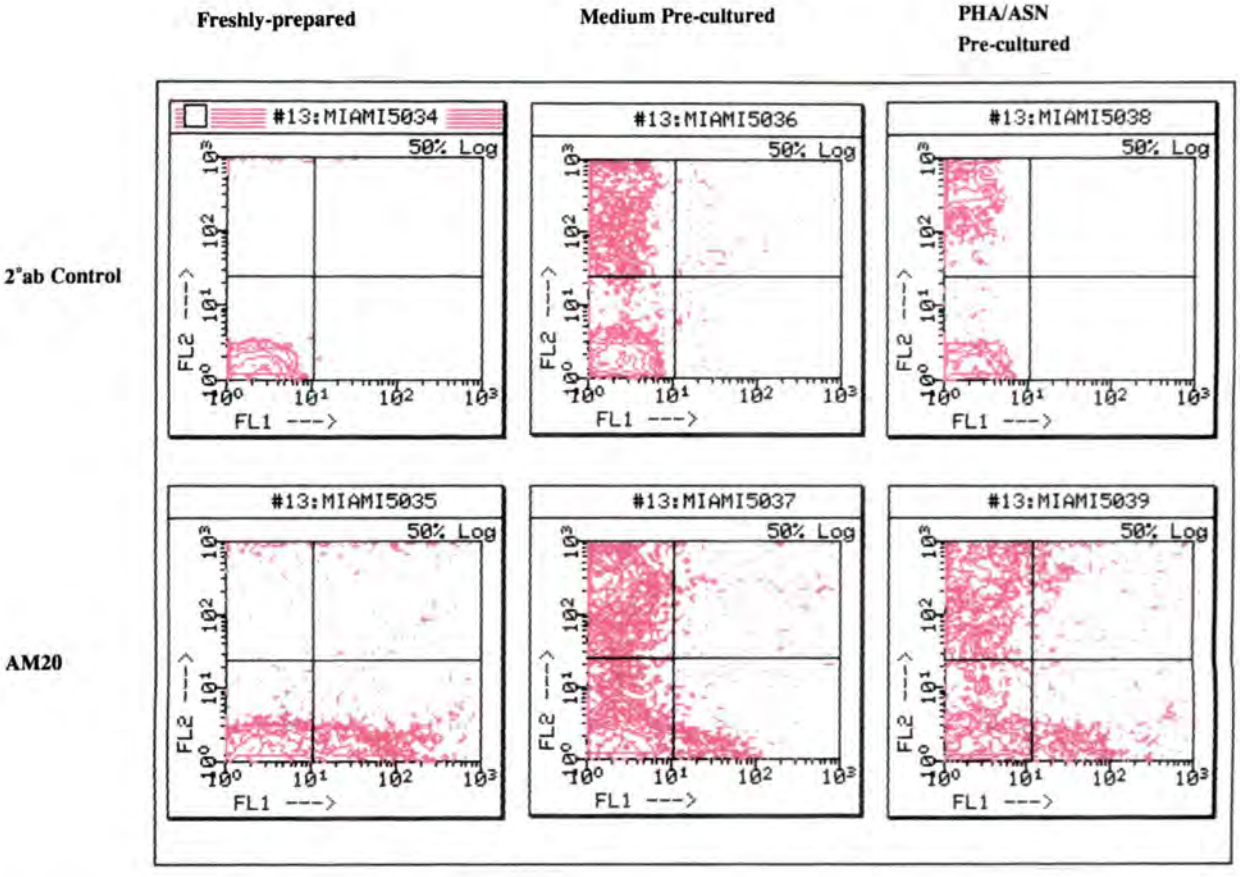

Fig.3.4 Contour Plots showing Propidium Iodide Uptake of *X.laevis* Larval Thymocyte Populations, in relation to AM20-staining

Freshly-prepared and 48 hours pre-cultured thymocytes are compared (thymocytes are left ungated in terms of RTO & FSC). On the X-axis is FL1, which represents the fluorescence intensity of FITC (here $AM20^{+ve}$ cells), whereas the Y-axis is FL-2, which is the log decade scale of propidium iodide.

Thymocytes analysed which were dead (high P.I. intake) and unstained [low fluorescence (1) intensity] are represented in the first quadrant. Cells which were dead but stained positive with AM20 are in the second quadrant. Cells in the third and fourth quadrants are viable, yet the former represents cells unstained and the latter are the $AM20^{+ve}$ population. Each dot is an event recorded with its specific FITC-intensity and P.I. intake and plotted accordingly. Contour lines represent high densities of cells. Quadrant statistics are shown overleaf.

Quadrant Statistics for Fig.3.4

Parameters: FL1/FL2 Quad. Location: 89, 117

Total = 10,000 Gated = 10,000

Sample	Quad	Events	%Gated	%Total
034	1 UL	116	1.16	1.16
	2 UR	43	0.43	0.43
	3 LL	9771	97.71	97.71
	4 LR	70	0.70	0.70
035	1 UL	57	0.57	0.57
	2 UR	135	1.35	1.35
	3 LL	7704	77.04	77.04
	4 LR	2104	21.04	21.04
036	1 UL	2872	28.72	28.72
	2 UR	63	0.63	0.63
	3 LL	7038	70.38	70.38
	4 LR	27	0.27	0.27
037	1 UL	2536	25.36	25.36
	2 UR	166	1.66	1.66
	3 LL	6642	66.42	66.42
	4 LR	656	6.56	6.56
038	1 UL	3489	34.89	34.89
	2 UR	29	0.29	0.29
	3 LL	6428	64.28	64.28
	4 LR	54	0.54	0.54
039	1 UL	4030	40.30	40.30
	2 UR	275	2.75	2.75
	3 LL	4898	48.98	48.98
	4 LR	797	7.97	7.97

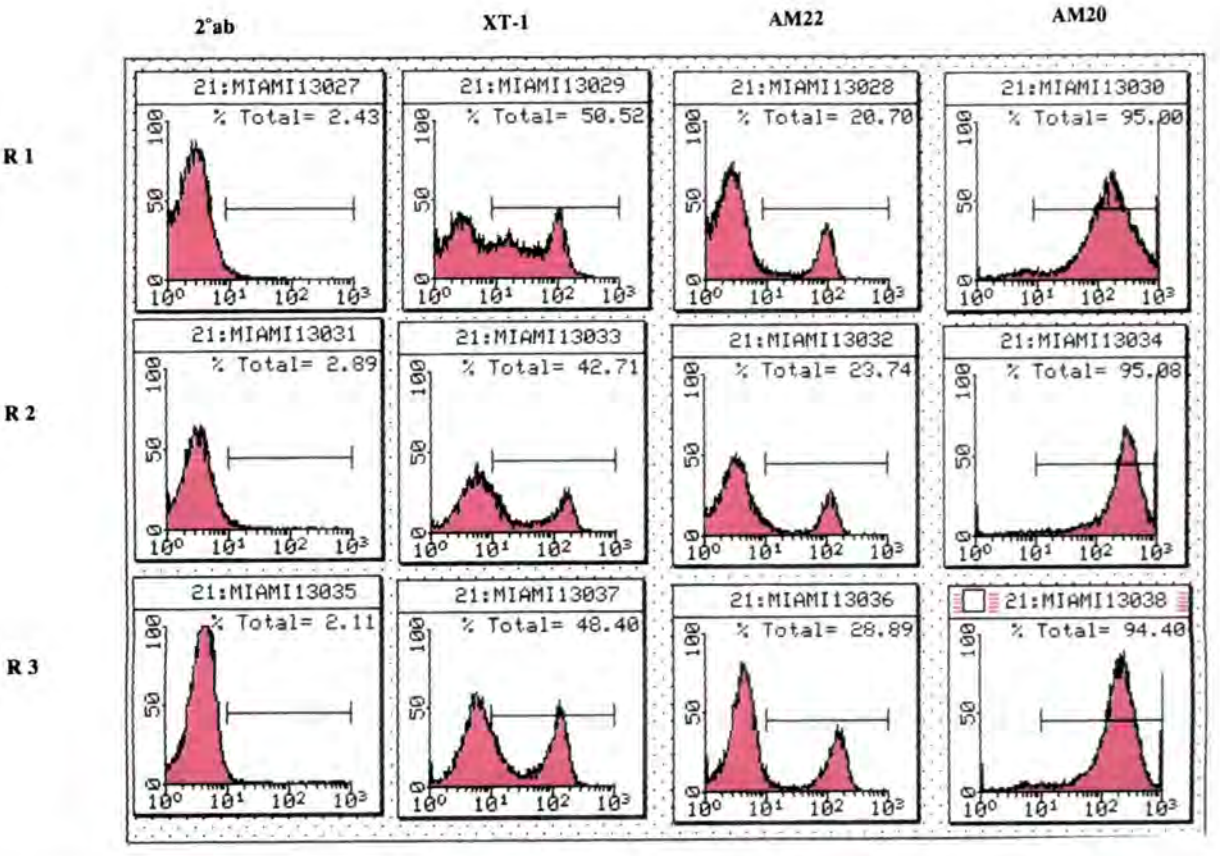

Fig.3.5 FLuorescence Intensity Histogrammes of McAb-stained LM3 *Xenopus*: Comparison of Freshly-prepared and 2 Weeks *in vitro* Culture Splenocytes

X-axis: log fluorescence intensity (530nm); Y-axis: relative cell number.

R 1: Freshly-prepared splenocytes

R 2: Splenocytes cultured in medium throughout

R 3: Splenocytes initially stimulated with 2.5µg/ml ConA, then subsequently in medium

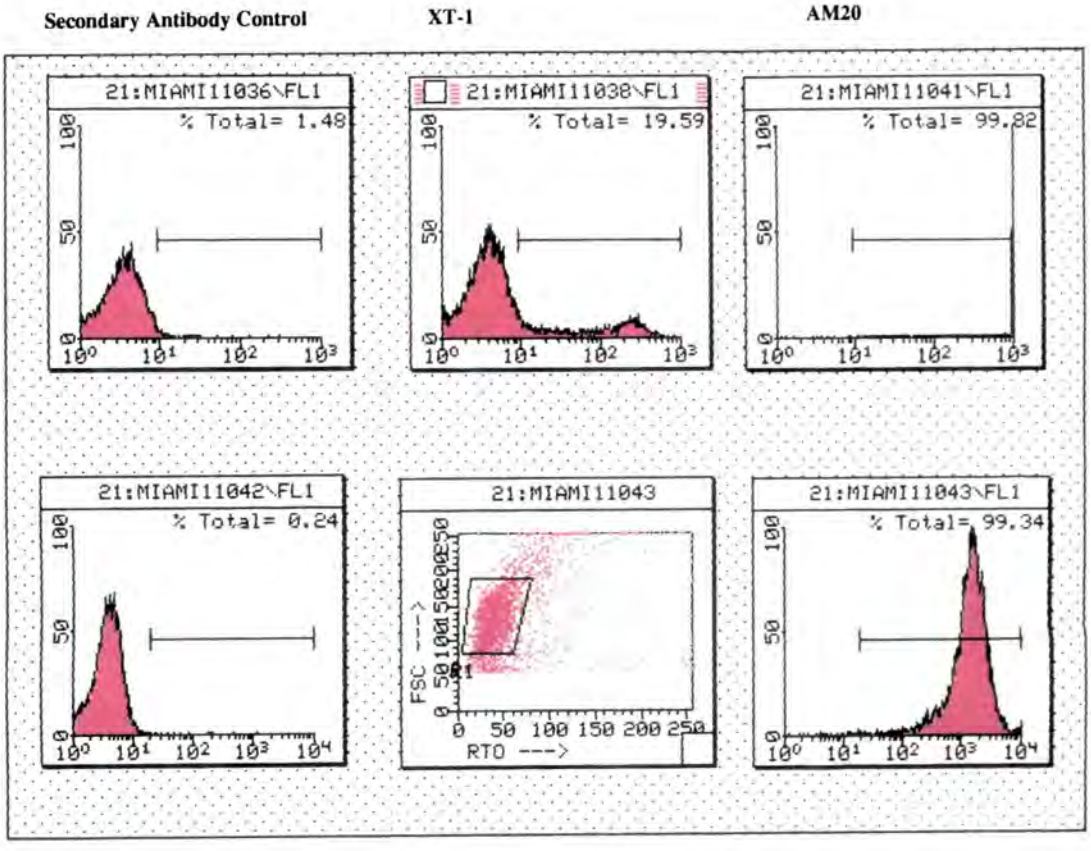

Fig.3.6 Fluorescence Intensity Histogrammes of Outbred *X.laevis* Splenocytes, following 2 Week *in vitro* Culture after Initial ConA Challenge

X-axis: log fluorescence intensity (530nm); Y-axis: relative cell number. Note the extension of the ' revised ' log decade scale on the X-axis. The status of the AM20^{+ve} 2-week ConA pre-cultured splencoytes are shown in the dot plot.

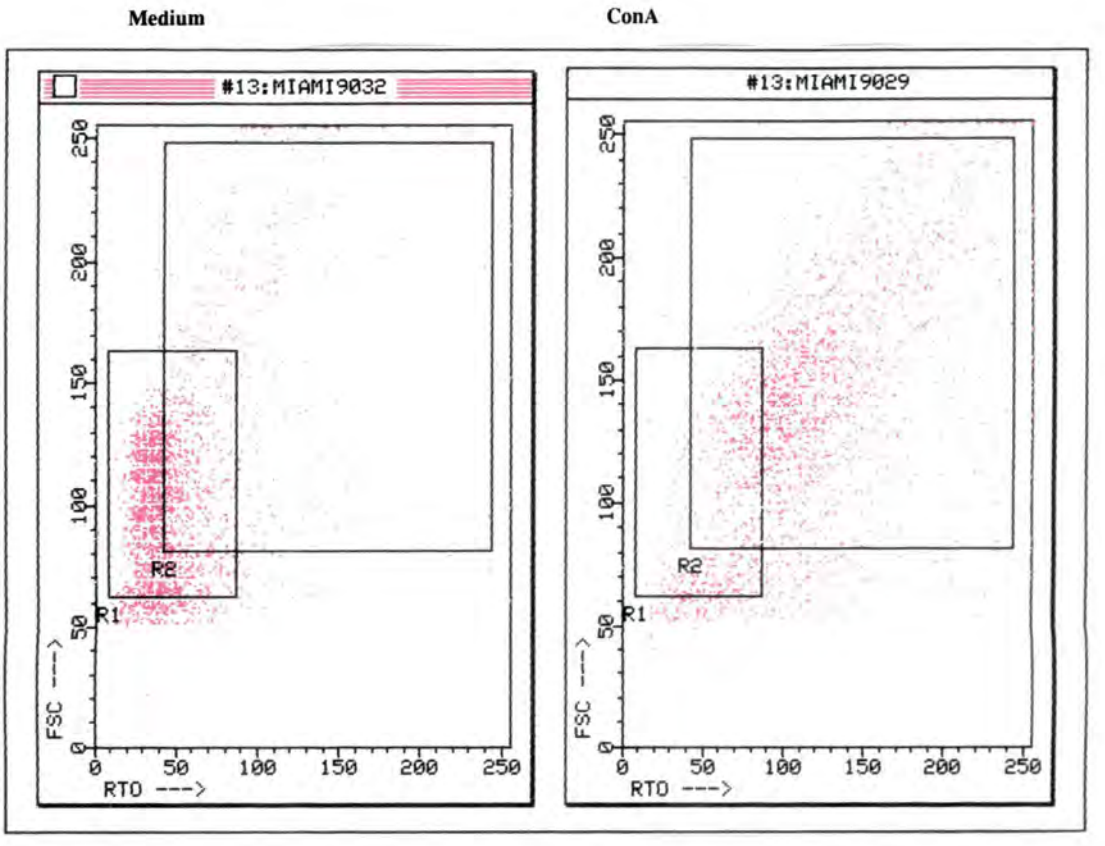

Fig.3.7 Dot Plots showing the Granularity and Size of Medium- and ConA- Pre-cultured Splenocytes following 9 Days *in vitro* Culture: Evidence for Blast Cell Emergence

Splenocytes form a young adult (1 year old) J strain *Xenopus* were maintained in 10% FCS-supplemented L-15 medium for 9 days following initial stimulation with either 0.25% BSA medium or 2.5µg/ml ConA (to 5x 10^6 leucocytes / ml). R1 is the STANDARD gate where usually most lymphocytes are found in a ' control '; whereas R2 is the REVISED gate which includes most blast cells that appear following mitogenic challenge.

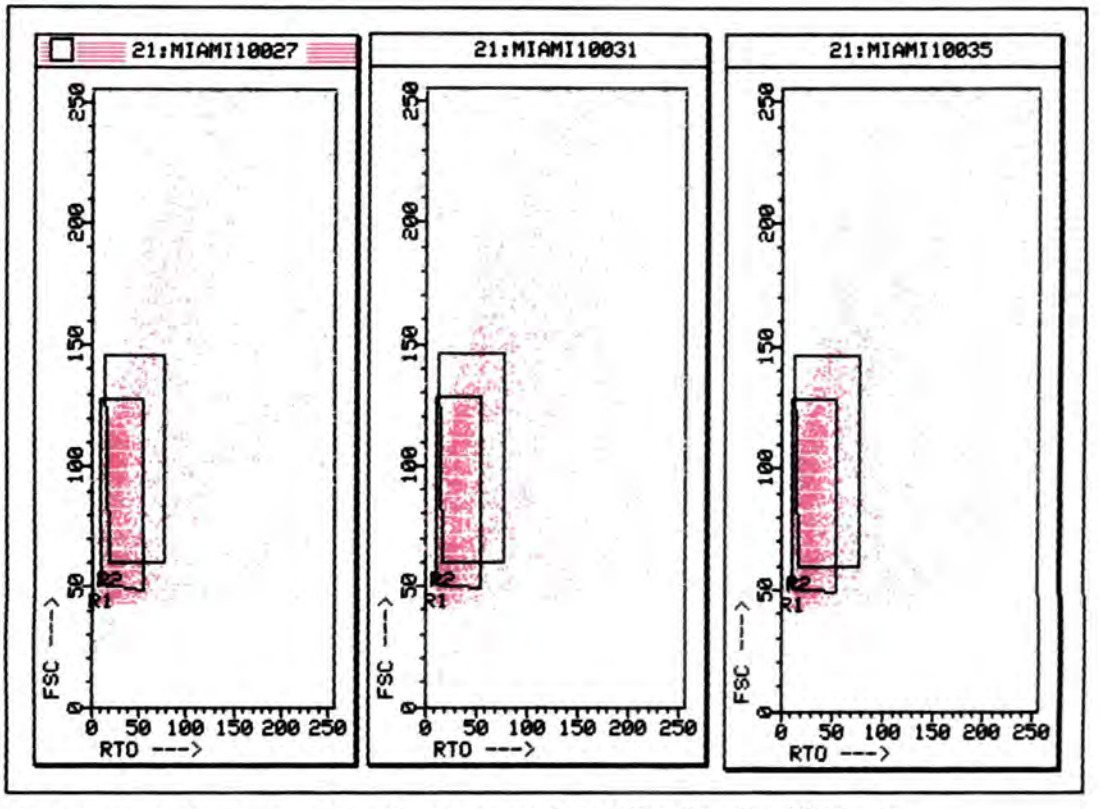

Fig.3.8 Dot Plots showing the Granularity and Size of Freshly-prepared and 14 Days Pre-cultured Splenocytes

Splenocytes from a young adult (30 weeks old) LM3 was maintained in 10% FCS-supplemented L-15 medium for 14 days following initial stimulation with either 0.25% BSA medium or 2.5μg/ml ConA (5x 10^6 leucocytes / ml). Freshly-prepared splenocytes from an animal of the same strain and age was prepared on the day of the FACS analysis. R1 is the STANDARD gate where usually most lymphocytes are found in a ' control '; whereas R2 is the REVISED gate which includes most blast cells that appear following mitogenic challenge.

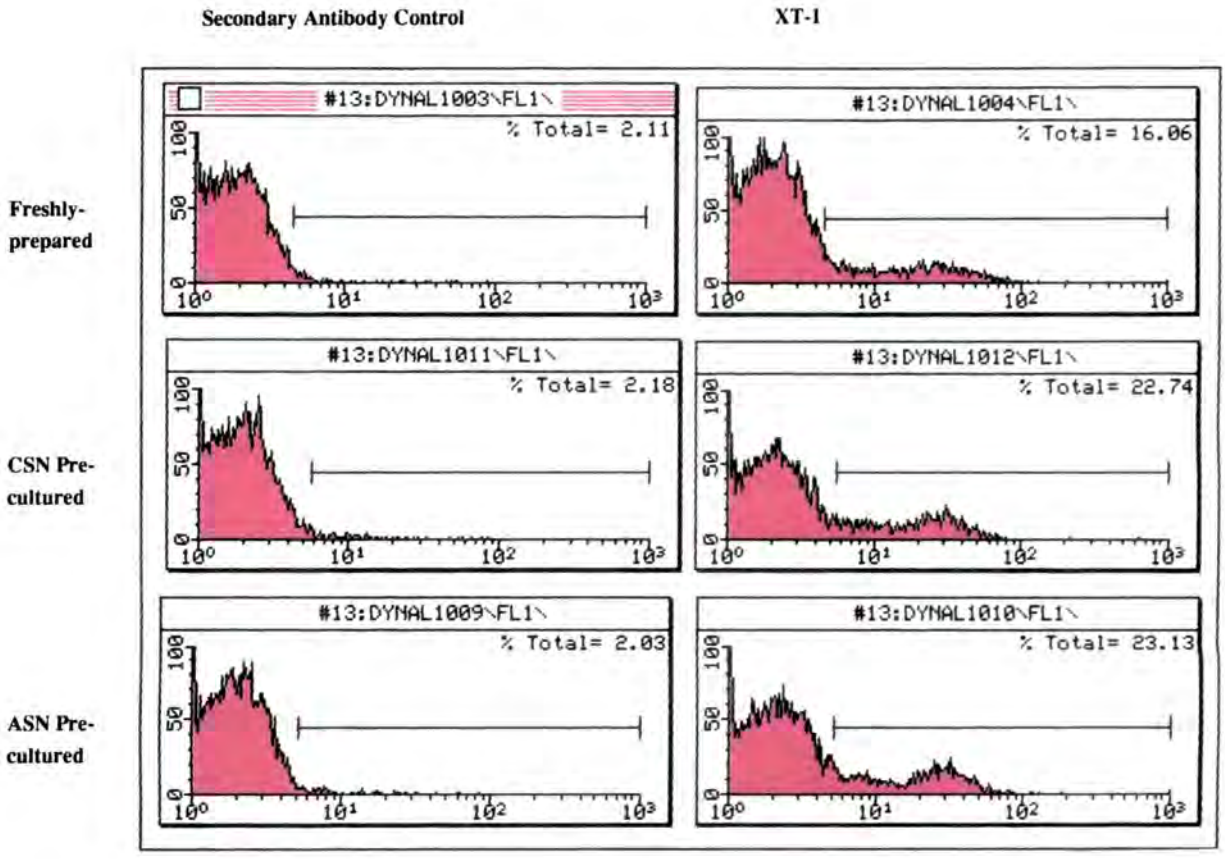

Fig.3.9 XTLA-1 Expression in Splenocytes from Control Outbred *X.laevis* (14 Weeks Old): Effect of *in vitro* Culture

Splenocytes from an individual control animal were cultured for 72 hours in either CSN or ASN. Freshly-harvested splenocytes were prepared from an animal of the same sibship for comparison. X-axis: log fluorescence intensity (530nm); Y-axis: relative cell number.

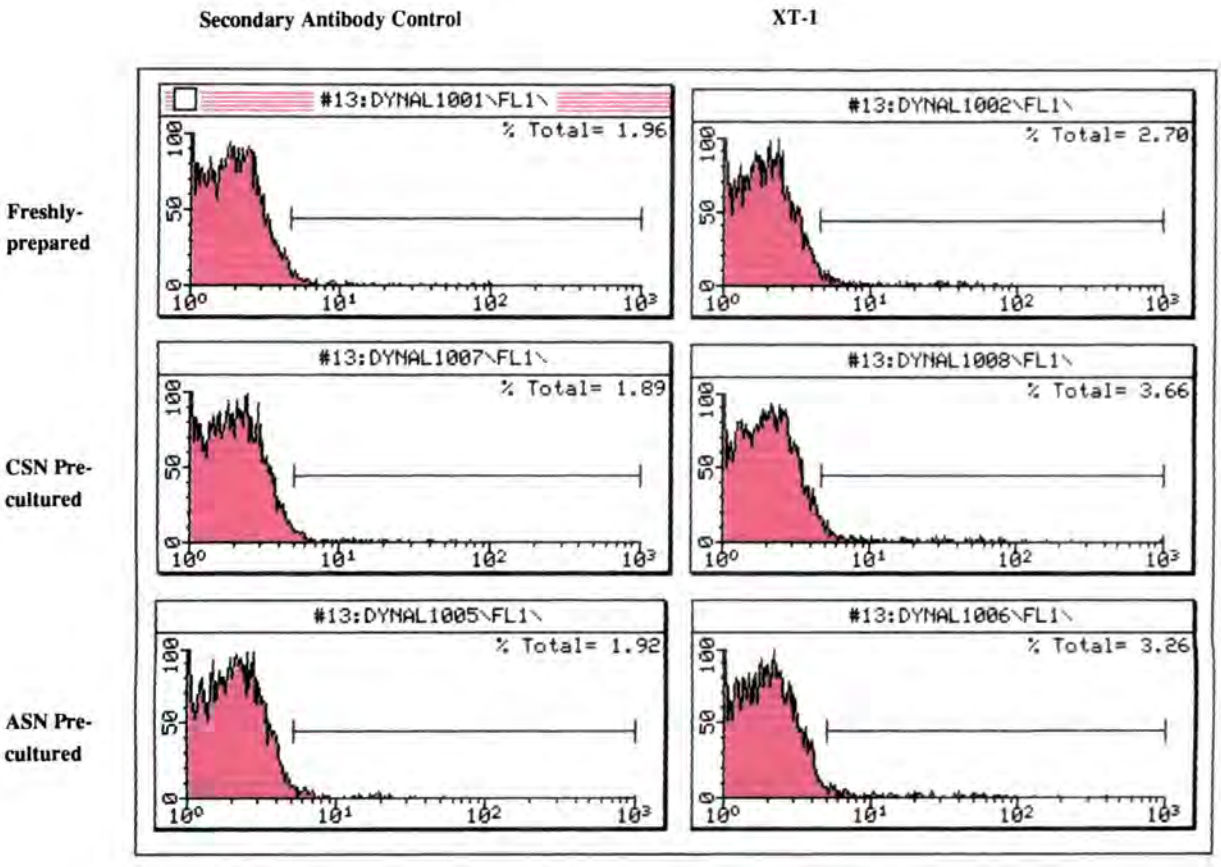

Fig.3.10 The Absence of XTLA-1 Expression in Splenocytes from Thymectomized Outbred *X.laevis* (14 Weeks Old): Effect of *in vitro* Culture

Splenocytes from an individual thymectomized animal were cultured for 72 hours in either CSN or ASN. Freshly-harvested splenocytes were prepared from a Tx animal of the same sibship for comparison. X-axis represents the log fluorescence intensity (530nm) whereas the Y-axis represents the relative cell number.

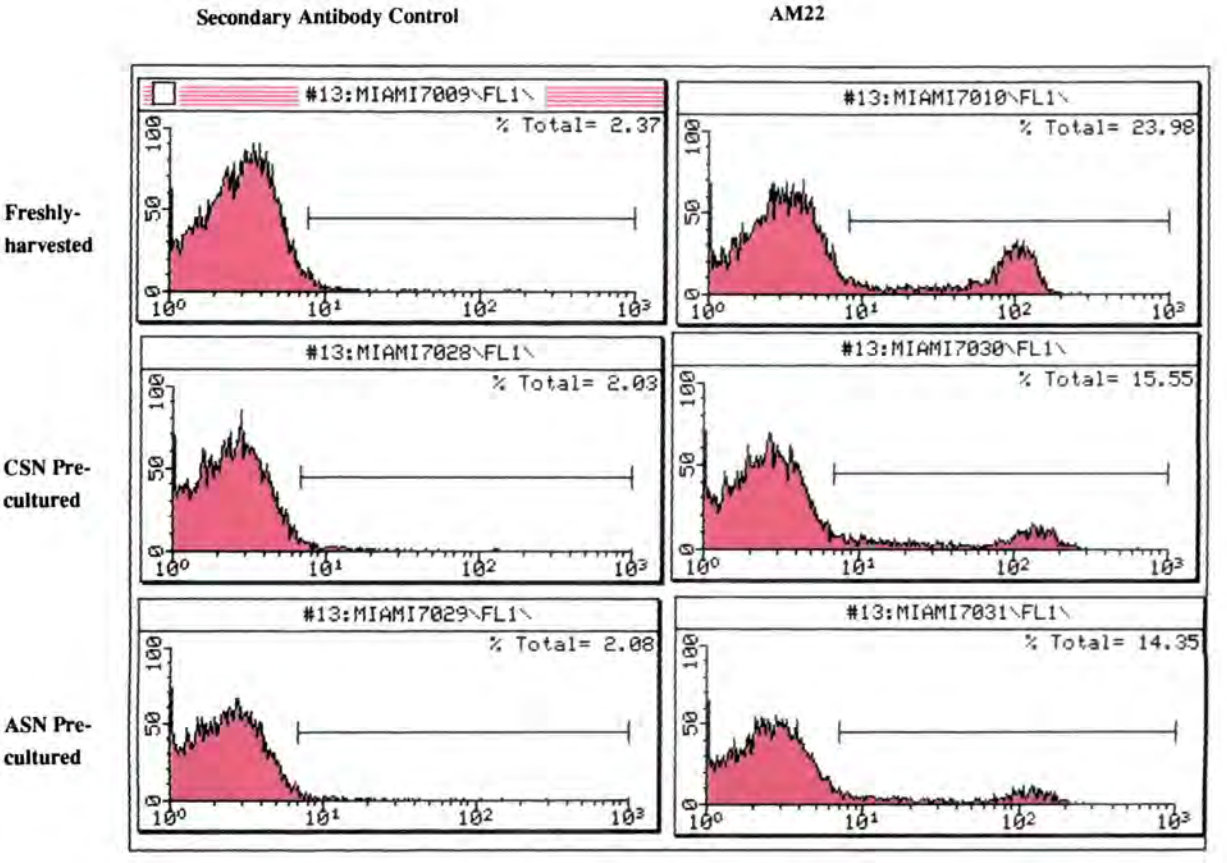

Fig.3.11 AM22 Expression in Splenocytes from Control Outbred *X.laevis* (16 Weeks Old) following 3 days *in vitro* Culture

Splenocytes, taken from an individual control animal, were pre-cultured for 72 hours subject to the conditions shown. Freshly-harvested splenocytes from a sigbling animal were also prepared for comparison. X-axis: log fluorescence intensity; Y-axis: relative cell number.

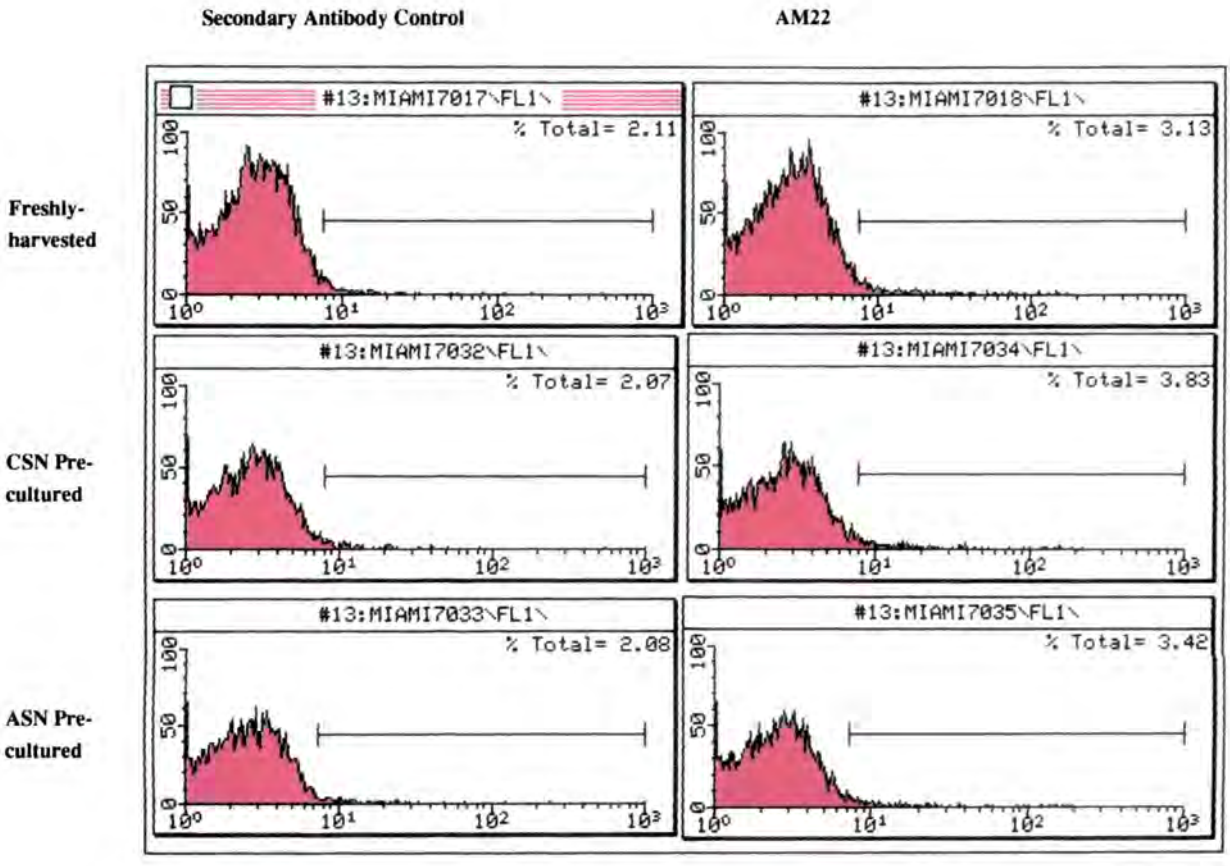

Fig.3.12 The Absence of AM22 Expression in splenocytes from Thymectmized Outbred *X.laevis* (16 Weeks Old) following *in vitro* Culture

Splenocytes from an individual Tx animal were pre-cultured for 72 hours subjected to the conditions shown and compared along with freshly-harvested splenocytes from a sibling animal. X-axis: log fluorescence intensity; Y-axis: relative cell number.

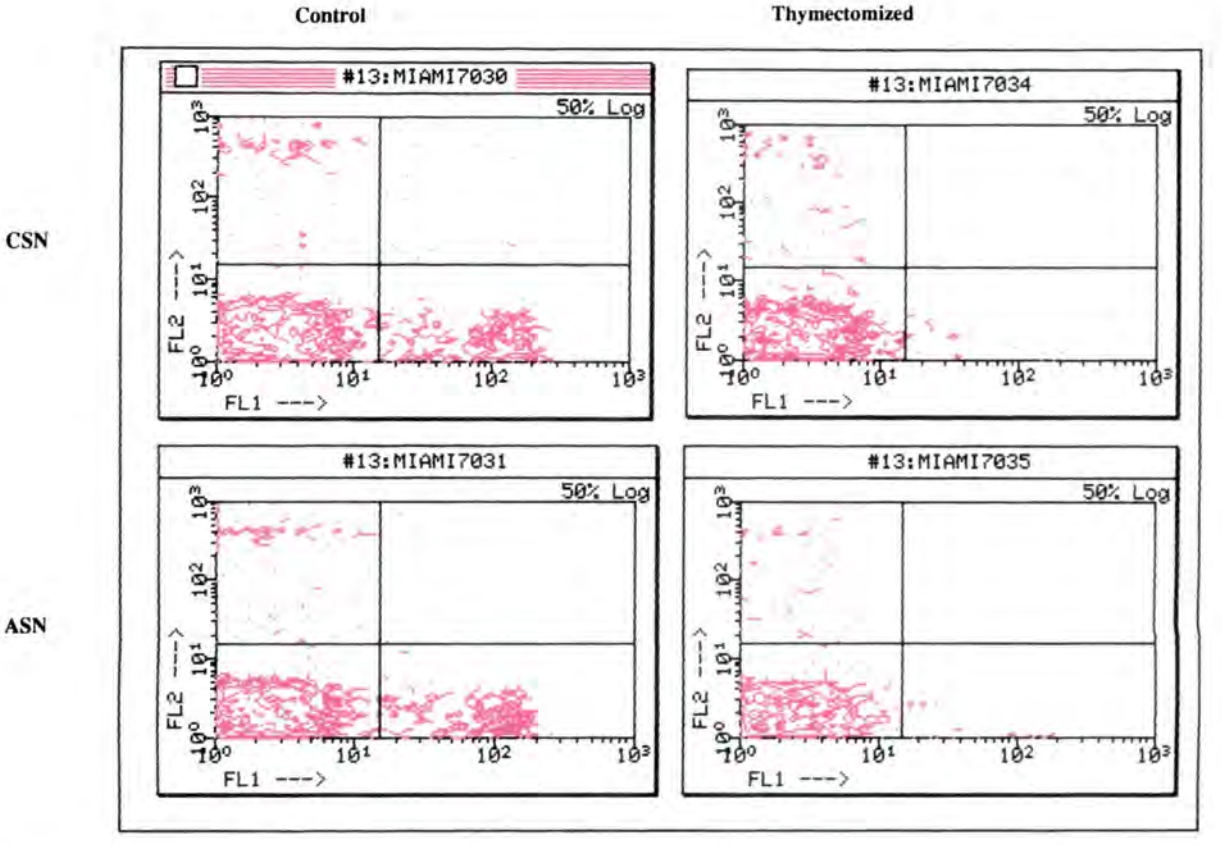

Fig.3.13 Contour Plots showing Pre-cultured Splenocytes from 16 Weeks Old *X.laevis* Toadlets Stained with both the Monoclonal Antibody (AM22) and Propidium Iodide

The above contour plots show FITC fluorescence intensity (AM22) on the X-axis and P.I. fluorescence on the Y-axis of both Tx and control splenocytes (which are the same cell populations as shown in **Fig.3.11&12**), following incubation with either CSN or ASN. Data is from gated cells —— i.e., gated on RTO/FSC to exclude erythrocytes and the majority of dead cells. Quadrant statistics are shown overleaf.

1st (UL) quadrant: dead McAb^{-ve} cells

2nd (UR) quadrant: dead McAb^{+ve} cells

3rd (LL) quadrant: live McAb^{-ve} cells

4th (LR) quadrant: live McAb^{+ve} cells

Quadrant Statistics for Fig.3.13

Parameters: FL1/FL2 Quad. Location: 100, 100

Sample	Quad	Events	%Gated	%Total
Total = 10,000		Gated = 3917		
030	1 UL	108	2.76	1.08
	2 UR	18	0.46	0.18
	3 LL	3307	84.43	33.07
	4 LR	484	12.36	4.84
Total = 10,000		Gated = 3174		
031	1 UL	113	3.56	1.13
	2 UR	10	0.32	0.10
	3 LL	2685	84.59	26.85
	4 LR	366	11.53	3.66
Total = 10,000		Gated = 2456		
034	1 UL	102	4.15	1.02
	2 UR	2	0.08	0.02
	3 LL	2328	94.79	23.28
	4 LR	24	0.98	24
Total = 10,000		Gated = 2450		
035	1 UL	78	3.18	0.78
	2 UR	0	0.00	0.00
	3 LL	2345	95.71	23.45
	4 LR	27	1.10	0.27

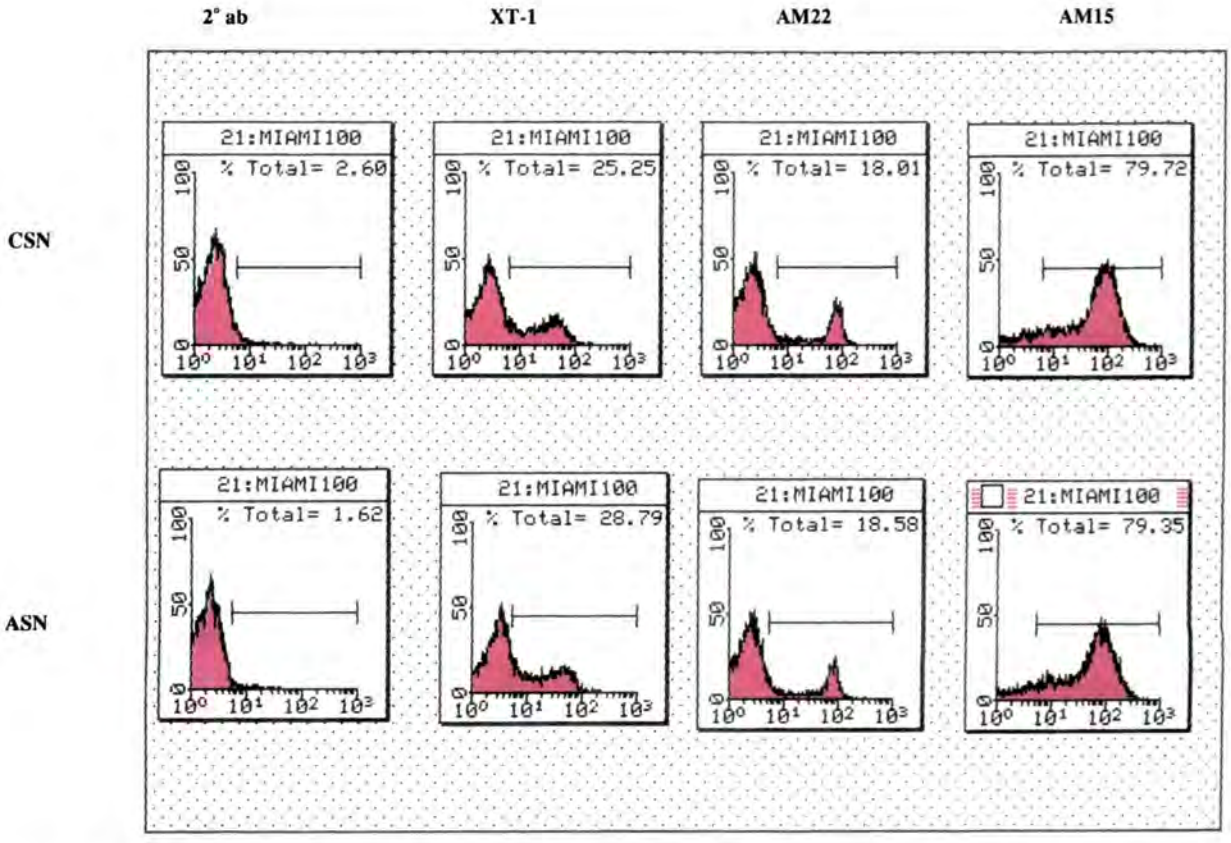

Fig.3.14 Fluorescence Intensity Histogrammes of Splenocytes from a Control 6 Months Old Outbred *X.laevis* Toadlet, following Culture in Supernatants and Staining with Various McAbs

Splenocytes from an individual control animal were cultured for 72 hours in either CSN or ASN, and stained as shown. X-axis: log fluorescence intensity; Y-axis: relative cell number.

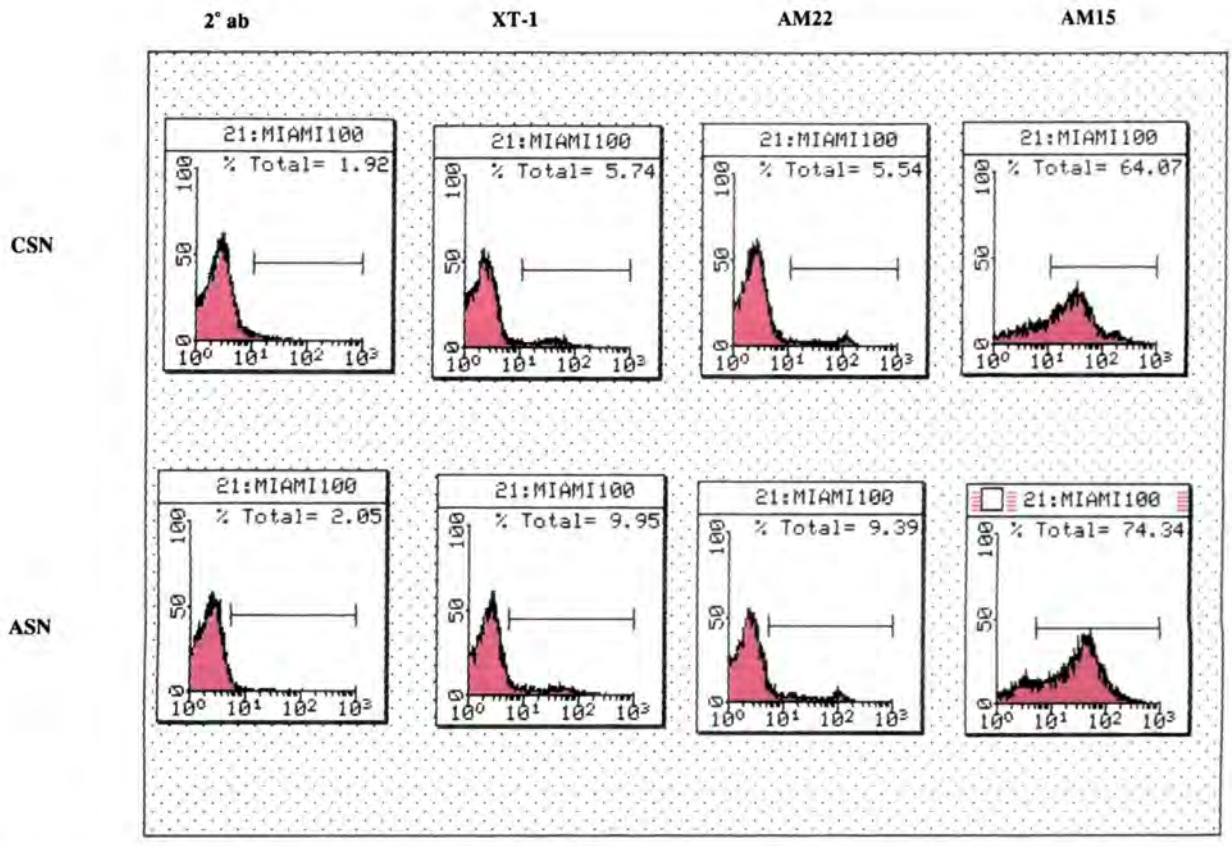

Fig.3.15 Fluorescence Intensity Histogrammes of Splenocytes from a Thymectomized 6 Months Old Outbred *X.laevis* Toadlet, following 3 Days Pre-culture in Supernatants and Stainiing with Various McAbs

Splenocytes from an individual Tx animal were cultured for 72 hours in either CSN or ASN and prepared for flow cytometric analysis. X-axis; log fluorescence intersity; Y-axis: relative cell number.

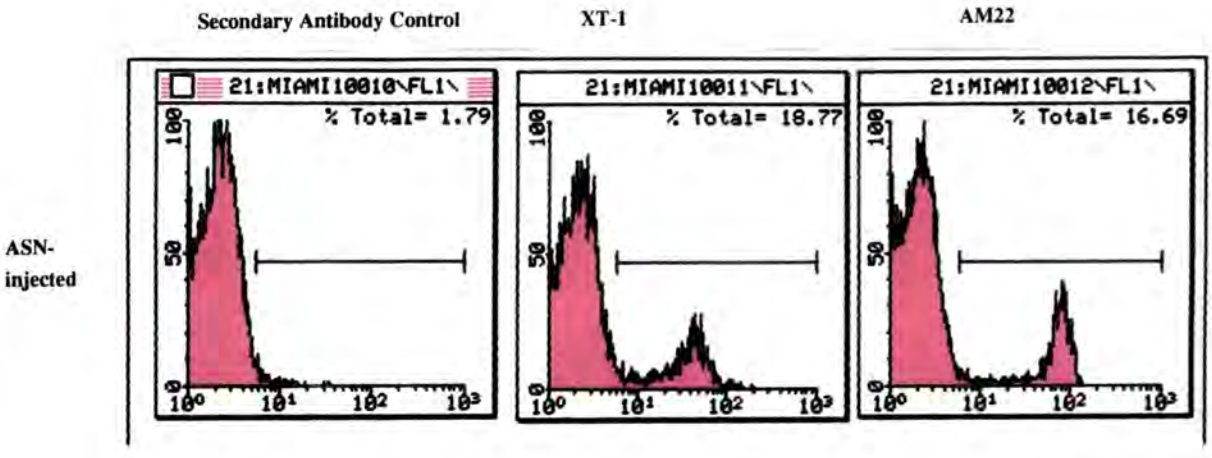

Fig.3.16 Fluorescence Intensity Histogrammes of T Cell Markers on Splenocytes from a Control Outbred *X.laevis* (25 Weeks Old) Toadlet 7 Days Post- *in vivo* Injection with Active Supernatant

X-axis: log fluorescence intensity; Y-axis: relative cell number.

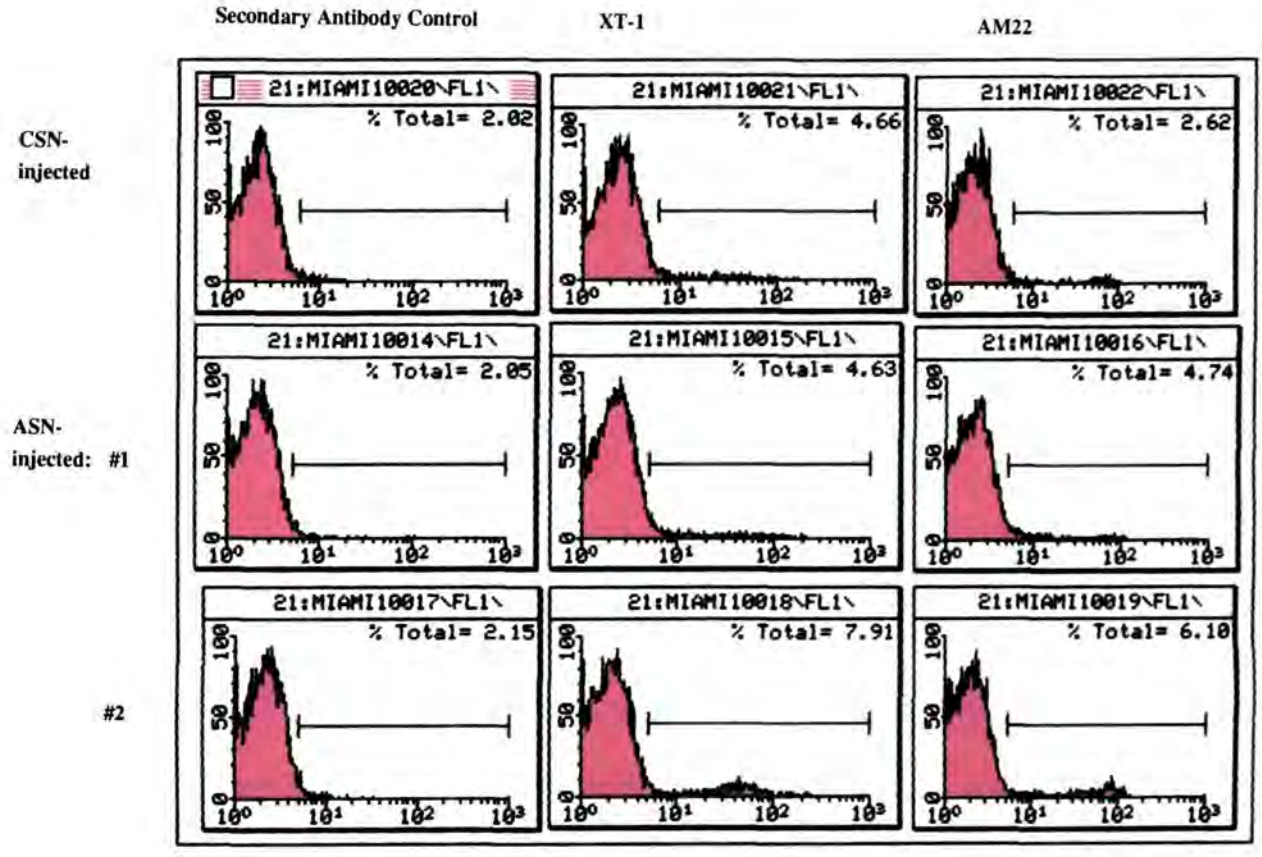

Fig.3.17 Fluorescence Intensity Histogrammes of T Cell Markers on Splenocytes from Thymectomized Outbred *X.laevis* (25 Weeks Old) Toadlets 7 Days Post- *in vivo* Injection with Culture-supernatants

X-axis: log fluorescence intensity; Y-axis: relative cell number.

#1 & #2: number designated to individual animal in the experiment

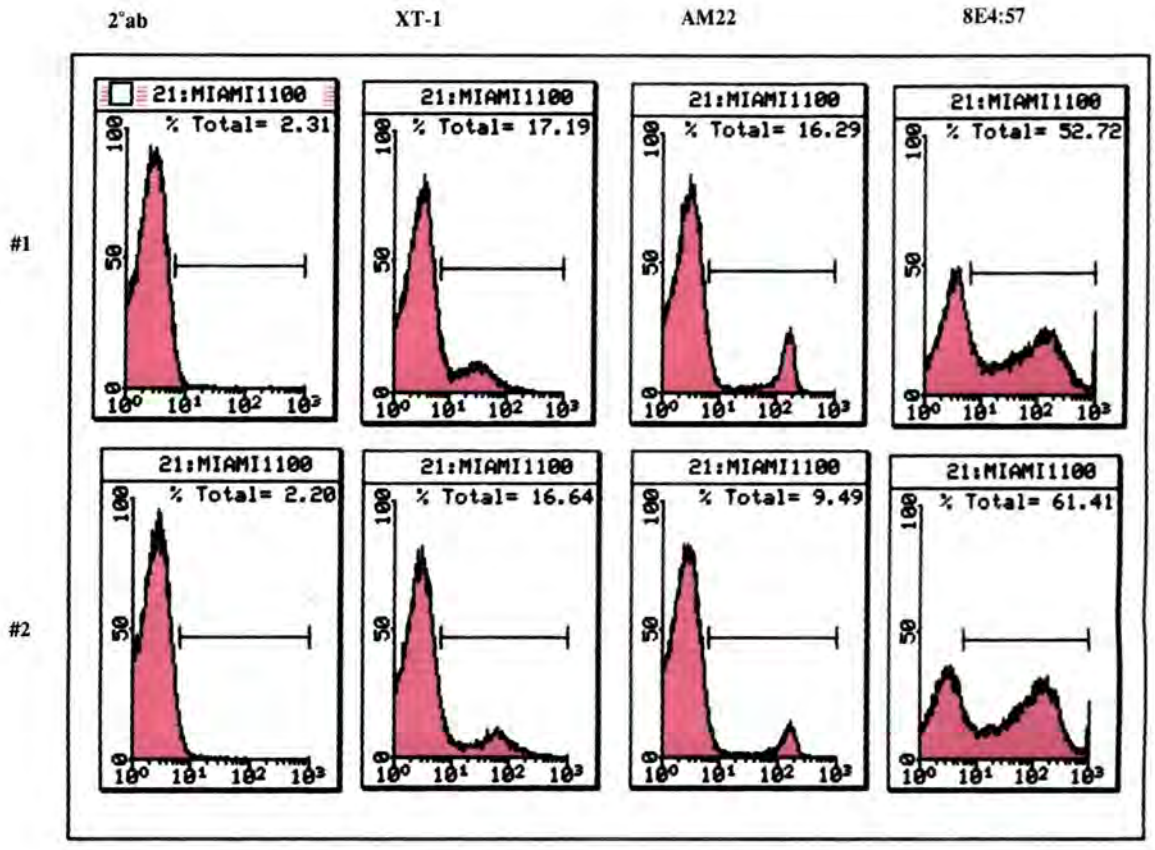

Fig.3.18 Fluorescence Intensity Histogrammes of T and B Cell Markers on Splenocytes from Control Outbred *X.laevis* (26 Weeks Old) Toadlets 14 Days Post- *in vivo* Injection with ASN

X-axis: log fluorescence intensity; Y-axis: relative cell number.

#1 & #2: number designated to individual animal in the experiment

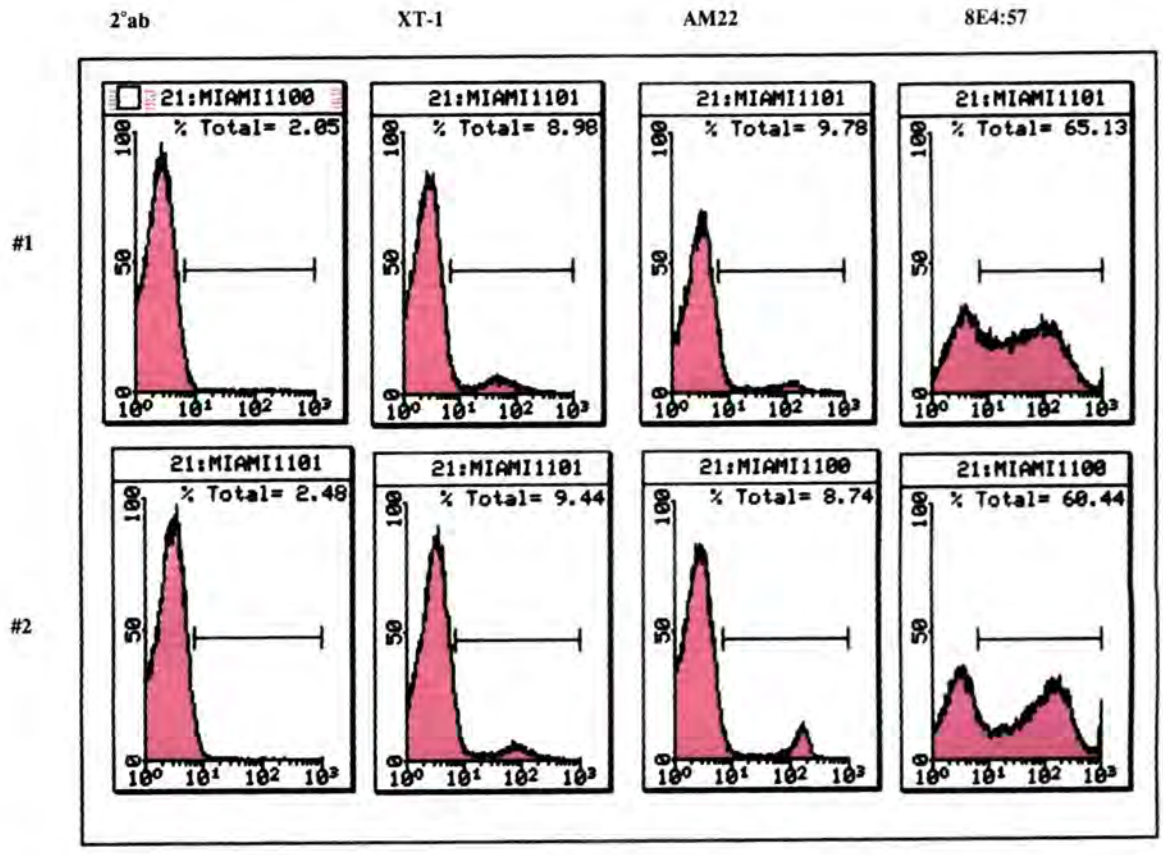

Fig.3.19 Fluorescence Intensity Histogrammes of T and B cell Markers on Splenocytes from 26 Weeks Old Non-injected Thymectomized Outbred *X.laevis* Toadlets

X-axis: log fluorescence intensity; Y-axis: relative cell number.

#1 & #2: number designated to individual animal in the experiment

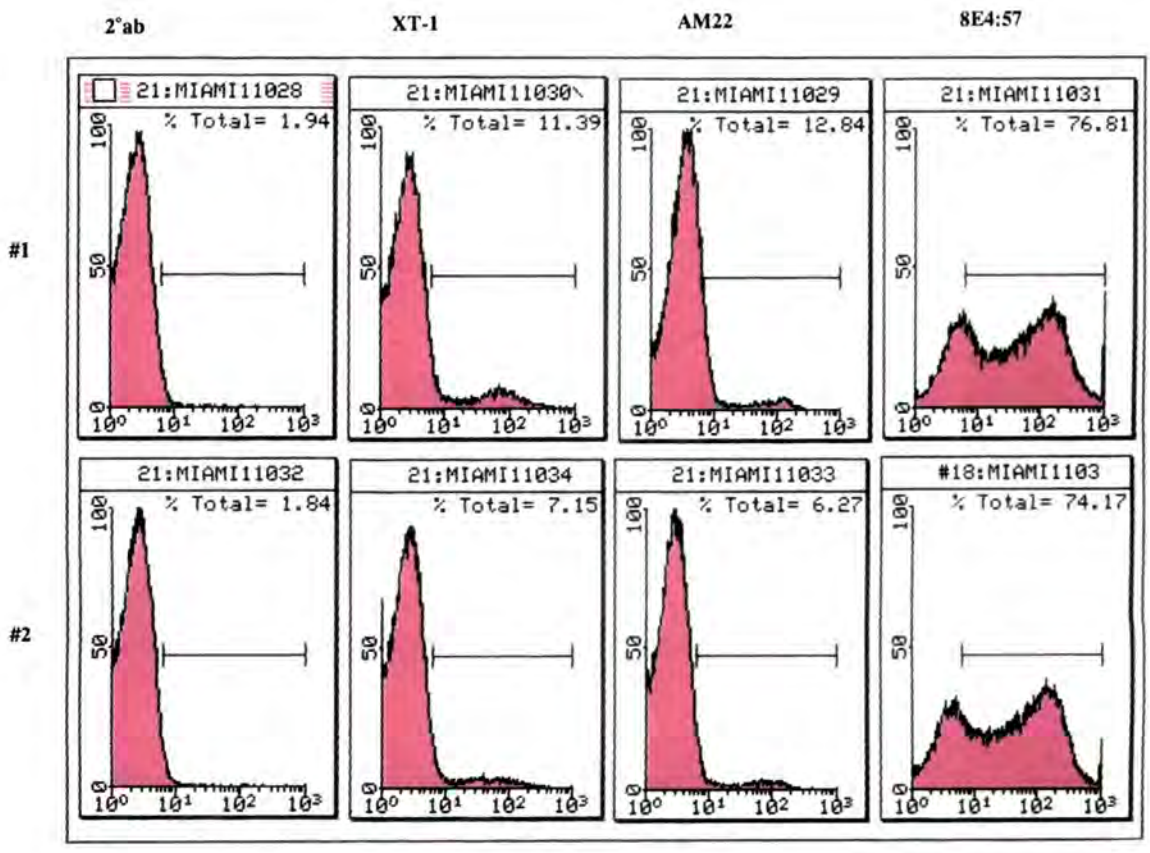

Fig.3.20 Fluorescence Intensity Histogrammes of T and B cell Markers on Splenocytes from 26 Weeks Old Thymectomized Outbred *X.laevis* Toadlets 14 Days Post- *in vivo* Injection with CSN

X-axis: log fluorescence intensity; Y-axis: relative cell number.

#1 & #2: number designated to individual animal in the experiment

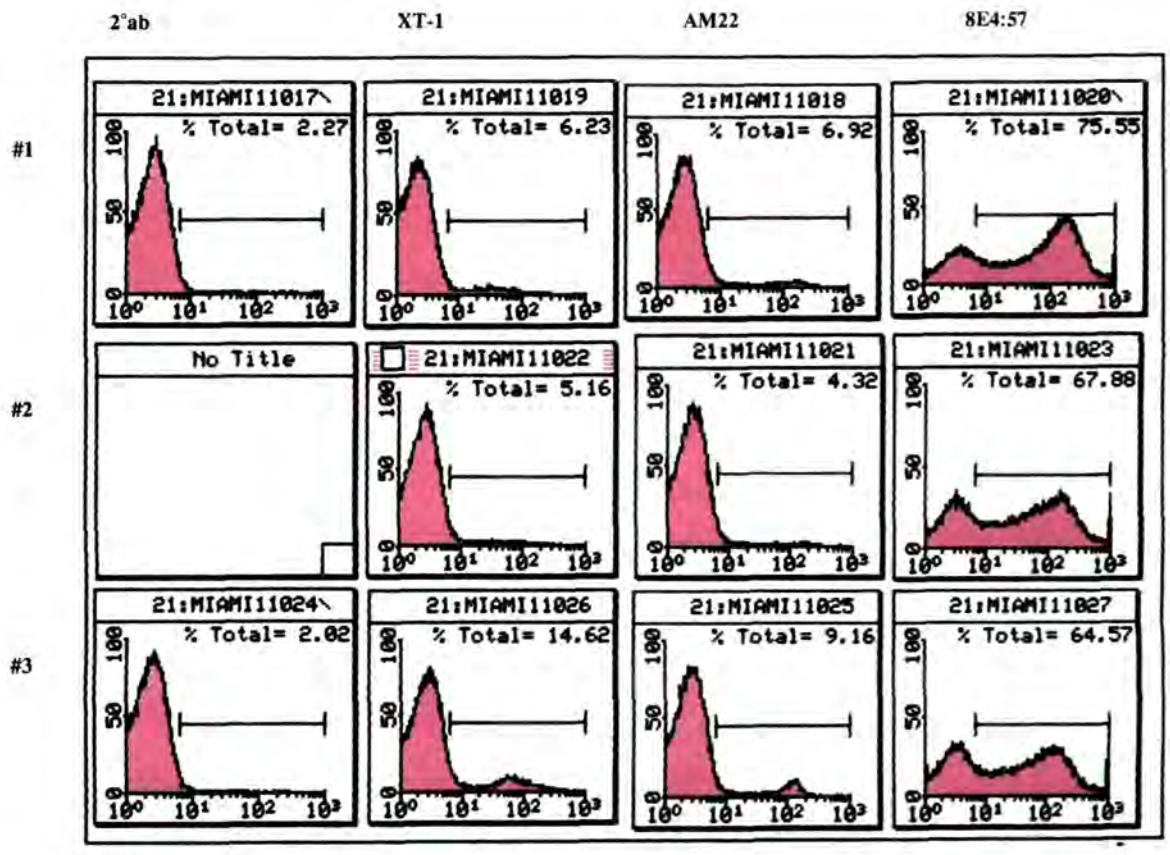

Fig.3.21 Fluorescence Intensity Histogrammes of T and B cell Markers on Splenocytes from 26 Weeks Old Thymectomized Outbred *X.laevis* Toadlets 14 Days Post- *in vivo* Injection with ASN

X-axis: log fluorescence intensity; Y-axis: relative cell number.

#1, #2 & #3: number designated to individual animal in the experiment

Empty window represents data not available.

Table 3.1

Supernatant Assay on 16 Weeks Old *X.laevis* Control and Tx Splenocytes

CULTURE STIMULANTS	CELL TYPE	
	Control	**Thymectomized**
ASN	5248 ± 854 (4.6)	3759 ± 240 (5.4)
CSN	1136 ± 100 (1.0)	700 ± 100 (1.0)
Medium	1800 ± 185	1397 ± 189

The table above compares the proliferative responses of control and Tx toadlets to culture supernatants incubation. Both control and Tx cells here were from the same populations of cells that were used for the FACS study as shown in **Figs.3.11&12** respectively, following 72 hours of culture with supernatants. The same batch of supernatants was used for this proliferative study and the related cytometric analysis.

Table 3.2

Splenocytes from Thymectomized Outbred *Xenopus laevis* Toadlets (9 - 15 Weeks) FAIL to Respond to T Cell Mitogens

a) Animals aged 9 weeks

STIMULANTS	Control	Thymectomized
PHA: 0.5μg/ml	35581 ± 3352 (16.2)	1420 ± 213 (1.8)
PHA: 0.05μg/ml	Not available	538 ± 140 (0.7)
Medium	2193 ± 349 (1.0)	808 ± 259 (1.0)

b) Animals aged 11 weeks

STIMULANTS	Control	Thymectomized
PHA: 0.5μg/ml	61979 ± 4580 (71.2)	354 ± 143 (0.62)
PHA: 0.05μg/ml	26830 ± 3762 (30.8)	556 ± 105 (0.98)
ConA: 2.5μg/ml	35834 ± 2239 (41.2)	349 ± 132 (0.62)
ConA: 1μg/ml	75023 ± 2948 (86.2)	363 ± 120 (0.64)
Medium	870 ± 231 (1.0)	567 ± 143 (1.00)

c) Animals aged 15 weeks

STIMULANTS	Control	Thymectomized
ConA: 1μg/ml	64377 ± 6278 (125.9)	1534 ± 174 (1.38)
Medium	511 ± 75 (1.0)	1109 ± 101 (1.00)

This table shows the responses mounted by splenocytes from control and Tx animals to mitogenic challenges at various time in early post-metamorphic life, following 3 days *in vitro* culture. D.P.M.s are shown in means ± standard deviation, with respective S.I. shown in brackets.

$$\text{S.I.} = \frac{\text{Mean D.P.M. of Mitogen-treated Cells}}{\text{Mean D.P.M. of Medium-treated Cells}}$$

Table 3.3

Splenocytes from Thymectomized Outbred *Xenopus laevis* Toadlets (6 - 9 Months) ABLE to Respond to T Cell Mitogens

a) Animals aged 6 months

	Control		Thymectomized	
	#1	**#2**	**#1**	**#2**
ConA: 1µg/ml	112875 ± 4367 (253.7)	70864 ± 5249 (15.3)	9147 ± 621 (7.4)	8720 ± 315 (8.9)
Medium	445 ± 30 (1.0)	4640 ± 637 (1.0)	1230 ± 248 (1.0)	976 ± 387 (1.0)

b) Animals aged 9 months

	Control		Thymectomized
	#1	**#2**	**#1**
ConA: 1µg/ml	36910 ± 3140 (123.9)	37484 ± 5331 (170.8)	35086 ± 1572 (14.1)
Medium	298 ± 93 (1.0)	395 ± 68 (1.0)	2496 ± 780 (1.0)

The responses of Tx and control splenocytes from 6 and 9 months old toadlets are shown in D.P.M. (mean ± standard deviation) above. Note the low level of proliferation in Tx splenocytes following ConA challenge. Respective S.I. are shown in brackets.

$$\text{S.I.} = \frac{\text{Mean D.P.M. of ConA-treated Cells}}{\text{Mean D.P.M. of Medium-treated Cells}}$$

3.4 Discussion

The present findings indicate that a proportion of late larval (6 - 7 weeks, st. 56 - 61) thymocytes from outbred *X.laevis* or clonal LG15, express surface class II MHC molecules, as detected by flow cytometry of AM20-stained cells. It would be expected that the vast majority of thymocytes examined in the thymocyte cell suspensions would be the cortical thymocytes rather than medullary cell types, since the latter are not readily released when thymi are teased apart (Turpen & Smith, 1986). Co-stimulation in the 6 weeks old larval LG15 thymocytes with ASN and PHA for 18 hours failed to alter surface MHC class II expression , when compared with thymocytes cultured in medium alone. The percentages (57%) of class II positive thymocytes in the LG15 larvae fits well with the findings on non-cultured thymocytes of 4 - 5 weeks old LG15 larvae (Horton *et al*, unpublished observations). However, a somewhat lower percentage (34%) of $AM20^{+ve}$ cells was found in the freshly-prepared thymocytes from 7 weeks old *X.laevis* larvae, perhaps revealing ' strain ' differences with respect to class II expression (see below). The further reduced proportion of MHC class II positive thymocytes after culture for 48 hours in medium or after co-culture in PHA/ASN may, in some way, be associated with poor viability of the cultured cells in medium containing lower concentration of FCS. Nearly 30% of the cultured thymocytes in this experiment stained with propidium iodide, whereas 22% were $P.I.^{+ve}$ in the freshly-prepared thymocytes. It should be pointed out, however, that very few $P.I.^{+ve}$ cells were MHC class II^{+ve}. Interestingly MHC class II was not found prior to metamorphosis (also detected by AM20) on larval F strain *X.laevis* thymocytes (in contrast to larval epithelial cells and antigen presenting cells) in either immuno-histological or flow cytometric studies conducted by Du Pasquier and Flajnik (1990). The reason for the differences between experiments with respect to MHC class II expression on larval thymic

lymphocytes is uncertain, although allelic differences between *Xenopus* strain might also be involved (Varley, 1990; Varley & Horton, 1991).

The experiments involving culture of control, adult splenocytes suggest that initial ConA stimulation leads to blast cell transformation by 9 days. By 14 days increased expression of surface MHC class II molecules was evident. An up-regulation of MHC class II on adult *Xenopus* T cells had also very recently been indicated within 3 days following mitogenic stimulation (Harding, Flajnik & Cohen, 1992). Up-regulation of MHC class II in *Xenopus* had also been observed by Flajnik *et al* (1990), following (3 days) culture of splenocytes in medium containing foetal bovine serum. Such up-regulation involves an increase in the number of MHC class II molecules expressed per lymphocyte, as recorded by increase in fluorescence intensity of anti- class II staining. Modulation of MHC class II may well be effected by T cell - derived cytokines (as occurs in mammals —— see, for example, Hamblin, 1988). The constitutive expression of MHC class II on **adult** *Xenopus* splenocytes observed here corresponds to the published data of Du Pasquier and Flajnik (1990), who indicate class II molecules are found on B cells, APCs **and** T cells after metamorphosis.

In view of the elevated ^{3}HTdR incorporation displayed (Chapter 2) by splenocytes from 7 day thymectomized *Xenopus* toadlets following *in vitro* incubation with ASN, it was interesting to observe the functional consequences of such stimulation, with respect to possible emergence of T cell markers on the sIg^{-ve} lymphocytes that appear to be the ASN-responsive population (Turner *et al*, 1991). Proliferative studies in this Chapter have confirmed the failure of Tx animals aged 9 - 15 weeks to respond to ConA. However, these T cell (functionally) deficient animals responded quite well to ASN. The FACS data on monoclonal antibody - stained splenocytes from 14 - 16 weeks old Tx animals revealed no emergence of T cell markers (XTLA-1^{+ve} and AM22^{+ve}) in either freshly-harvested, CSN-treated cultures or in ASN-treated cultures, despite the confirmed potency of ASN used on these Tx cells. Interestingly a slightly increased

proportion of XTLA-1^{+ve} cells was found in CSN- or ASN- (72 hours) pre-cultured (in comparison to freshly-harvested) control splenocytes. This may be ascribed to an *in vitro* T cell stimulatory effect, or the tendency for B cells to be more susceptible to death than T cells *in vitro* (unpublished observations from this laboratory). Alternatively, as Varley (1990) noted, the variations of XTLA-1 expression seen may simply be attributable to those expected between individual outbred animals.

In contrast to the experiments on younger animals, when thymidine incorporation experiments were performed on 6 months old (and more aged) Tx animals, they routinely showed a positive response of Tx splenocytes to ConA, although this was considerably reduced compared with control splenocytes. Such *in vivo* emergence of a population of ' T - like ' cells with time after thymectomy was confirmed in the FACS experiments with the McAbs XT-1 and AM22.

In the experiments on 6 months old *Xenopus*, somewhat elevated levels of splenocytes expressing these T cell markers recognized by the antibodies XT-1 and AM22 were occasionally noted after ASN treatment, either of splenocytes *in vitro*, or of animals by *in vivo* injection. However, no definite conclusion on possible experimental induction of T cell specific antigens (with ASNs) on splenocytes taken from 6 months old Tx animals can be drawn, due to the low, but distinct proportions of ' T - like ' splenocytes found in CSN-treated Tx animals and splenocyte cultures.

Such emergence of ' T - like ' cells in ' thymusless ' animals has been documented in nude mice of about 9 months of age (see Bell, 1989). It has been suggested that the emergence of T - like cells in these ' athymic ' animals may be achieved by the generation of T cells from extra-thymic sources (Chen *et al*, 1984; Vaessen *et al*, 1986; Lake, Pierce & Kennedy, 1991; Lafrançois, 1991). The absence of thymic remnants in Tx *Xenopus* (contrast the hypoplastic thymus of the nude mammal) and the data presented here on ' T - like ' cell emergence would support this suggestion.

Chapter 4

Concluding Remarks and Suggestions for Future Work

The work presented in Chapter 2 demonstrated that soluble factors are released into the supernatant culture medium when *Xenopus* splenocytes are polyclonally stimulated with concanavalin A. The optimal conditions for the production of these active supernatants with cytokine - like activity were initially investigated. ConA-derived supernatants were able to provide costimulatory signals necessary for larval thymocytes to respond to T cell mitogens. This finding agrees with previously published work (Cohen, Watkins & Parsons, 1987) which suggested that the active supernatant provides the thymocytes with growth factors not produced by the larval thymus itself.

Splenocyte-derived active supernatants were mainly tested on adult splenocytes. ASNs directly induced mitogenesis of freshly-harvested adult *Xenopus* splenocytes equally as well as medium- or mitogen- pre-cultured assay splenocytes. These findings on splenocyte thymidine incorporation support the recent work of Turner (1990) and Turner *et al* (1991) from this laboratory, but contrast the findings of Watkins & Cohen (1987*b*) and those of Haynes *et al* (1991) who find that only splenic blasts (produced experimentally *in vitro* or naturally following *in vivo* immunization) respond to mitogen-induced ASNs. While the latter ASNs were shown in Cohen's laboratory to be rich in TCGF (IL-2 - like) material, the present experiments have not attempted to purify the active factors secreted. Such purification requires large amounts of crude ASNs, which were not available to the author. Future work would, however, do well to characterize the active components present in ASNs. Only then can the nature of splenocyte targets for individual cytokines could then be usefully explored.

Work reported in Chapter 2 also confirmed initial findings from this laboratory (Turner *et al*, 1991) that freshly-harvested splenocytes from post-metamorphic toadlets, thymectomized on day 7 post-fertilization, display enhanced thymidine incorporation in the presence of ASN. Responses to ASNs by these thymectomized animals were possible at early stages of development, when T cell loss could be confirmed by absence of proliferation following challenge with ConA *in vitro*.

Since Turner *et al* (1991) have indicated that the Tx splenocyte population that can respond to ASN is an Ig^{-ve} rather than Ig^{+ve} population, flow cytometric experiments on McAb-stained cells were carried out in Chapter 3 to probe whether ASN treatment can induce ' T - like ' cell emergence. The work showed no clear-cut effect of ASN treatment (either *in vitro* or *in vivo*) on Tx or control splenocytes, but surprisingly revealed the natural *in vivo* emergence of ' T - like ' cells, which were XTLA-1^{+ve} and $AM22^{+ve}$, in 6 months old Tx toadlets. Such animals also possessed splenocytes capable of responding, at a low level, to ConA. Flow cytometric studies also showed increase in MHC class II expression on *in vitro* cultured cells, a phenomenon recently described elsewhere (Harding, Flajnik & Cohen, 1992).

Further studies on the ontogeny of ' T - like ' cell emergence in Tx *Xenopus* are now required, as this model system offers certain advantages over the nude mammal model. The latter animals, which are congenitally hairless, fail to develop a lymphoid thymus and are depleted of T lymphocytes in their blood and thymus dependent areas of the lymph nodes and spleen. However, these nudes do have a persistent, hypoplastic, thymus (Pantelouris, 1973) that could conceivably be responsible for influencing the emergence of T - lineage cells from existing stem cell precursors (Chen *et al*, 1984). In contrast, Tx *Xenopus* appear to be devoid of any thymus remnants and therefore provide an excellent model to explore possible extra-thymic T cell maturation pathways thought to exist (Pardoll *et al*, 1988; Bell, 1989;

Lafrançois, 1991). Now that antibodies are becoming available to *Xenopus* T cell antigen receptor molecules (M.D. Cooper, unpublished observations), the way is open to further explore the nature of ' T - like ' cell emergence in Tx *Xenopus*. Functional studies on ' T - like ' cells in Tx animals are a realistic proposition, since McAbs can be utilized to enrich such lymphocyte populations, through cell sorting (see Turner *et al*, 1991), through enrichment by passing over antibody-coated magnetic beads (current studies in this laboratory), or by ' panning ' using antibody-coated Petri dishes (see, for example, Cohen & Haynes, 1990).

The ability of *Xenopus*-derived cytokines to promote ' T - like ' cell emergence in the absence of a thymus will necessitate studies utilizing purified cytokines on early post-metamorphic, Tx animals, in which such lymphoid cells appear to be lacking. Purification of *Xenopus* IL-2 - like material is in progress (Haynes & Cohen, 1991). Recombinant DNA technology will hopefully lead to the characterization of cytokine genes at this level of evolution. The use of genetically-engineered *Xenopus* cytokines would prove invaluable in further characterizing the immune system of this useful ontogenetic model.

Statistical Calculations

Appendix A1

Notation

X_1 : sample mean D.P.M. of ' 2.5μg/ml ConA ' = 243948
X_2 : sample mean D.P.M. of ' 1μg/ml ConA ' = 239564
μ_1 : population mean D.P.M. of ' 2.5μg/ml ConA '
μ_2 : population mean D.P.M. of ' 1μg/ml ConA '
n_1 : sample size of ' 2.5μg/ml ConA ' = 3
n_2 : sample size of ' 1μg/ml ConA ' =3
S_1 : sample D.P.M. variance of ' 2.5μg/ml ConA ' = 3764
S_2 : sample D.P.M. variance of ' 1μg/ml ConA ' = 2773
S_p : pooled average of sample variances weighted in terms of degrees of freedom
p : probability / significant level

Hypothesis

H_0: 2.5μg/ml ConA and 1μg/ml ConA are equally stimulatory, i.e., $\mu_1 = \mu_2$
H_A: 2.5μg/ml ConA and 1μg/ml ConA are NOT equally stimulatory, i.e., $\mu_1 \neq \mu_2$
i.e., this is a 2-tailed test

Test Statistics

$$t = \frac{(X_1 - X_2) - (\mu_1 - \mu_2)}{S_p \sqrt{1/n_1 + 1/n_2}}$$

$$\text{where } S_p^2 = \frac{(n_1 - 1) S_1^2 + (n_2 - 1) S_2^2}{n_1 + n_2 - 2}$$

Under H_0,

$$\frac{(X_1 - X_2)}{S_p \sqrt{1/n_1 + 1/n_2}} \approx t_{\,n_1 + n_2 - 2} \quad \text{on repeated sampling}$$

Significant Level

Set p = 5% (i.e., 0.05)

Decision Rule

If Calculated t Value is Greater Than or Equal To the Critical t Value, Reject H_0

(Appendix A1, cont'd)

Calculations

$$S_p^2 = \frac{(3-1)(3764)^2 + (3-1)(2773)^2}{3+3-2}$$

and $S_p \approx 4675$

$$\text{then, Calculated } t \text{ value} = \frac{(243948 - 239564)}{3306\sqrt{1/3 + 1/3}}$$

$$\approx 1.148$$

whereas,

at 5%, Critical t value with 4 degrees of freedom, $t_4 = 2.78$

Since Cal. t is less than Crit. t, therefore, FAIL TO REJECT H_0

Statistical Decision

2.5μg/ml ConA and 1μg/ml ConA are equally stimulatory.

Appendix A2

Notation

X_1 : sample mean D.P.M. of ' 6μg/ml ConA ' = 120106

X_2 : sample mean D.P.M. of ' 5μg/ml ConA ' = 116027

μ_1 : population mean D.P.M. of ' 6μg/ml ConA '

μ_2 : population mean D.P.M. of ' 5μg/ml ConA '

n_1 : sample size of ' 6μg/ml ConA ' = 3

n_2 : sample size of ' 5μg/ml ConA ' = 3

S_1 : sample D.P.M. variance of ' 6μg/ml ConA ' = 521

S_2 : sample D.P.M. variance of ' 5μg/ml ConA ' = 11303

S_p : pooled average of sample variances weighted in terms of degrees of freedom

p : probability / significant level

Hypothesis

H_0: 6μg/ml ConA and 5μg/ml ConA are equally stimulatory, i.e., $\mu_1 = \mu_2$

H_A: 6μg/ml ConA and 5μg/ml ConA are NOT equally stimulatory, i.e., $\mu_1 \neq \mu_2$

i.e., this is a 2-tailed test

Test Statistics

$$t = \frac{(X_1 - X_2) - (\mu_1 - \mu_2)}{S_p \sqrt{1/n_1 + 1/n_2}}$$

$$\text{where } S_p^2 = \frac{(n_1 - 1) S_1^2 + (n_2 - 1) S_2^2}{n_1 + n_2 - 2}$$

Under H_0,

$$\frac{(X_1 - X_2)}{S_p \sqrt{1/n_1 + 1/n_2}} \approx t_{\,n_1 + n_2 - 2} \quad \text{on repeated sampling}$$

Significant Level

Set $p = 5\%$ (i.e., 0.05)

Decision Rule

If Calculated t Value is Greater Than or Equal To the Critical t Value, Reject H_0

(Appendix A2, cont'd)

Calculations

$$S_p^2 = \frac{(3-1)(521)^2 + (3-1)(11303)^2}{3+3-2}$$

and $S_p \approx 8001$

$$\text{then, Calculated } t \text{ value} = \frac{(120106 - 116027)}{8001\sqrt{1/3 + 1/3}}$$

$$\approx 0.6244$$

whereas,

at 5%, Critical t value with 4 degrees of freedom, $t_4 = 2.78$

Since Cal. t is less than Crit. t, therefore, FAIL TO REJECT H_0

Statistical Decision

6µg/ml ConA and 5µg/ml ConA are equally stimulatory.

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