A study of lymphocyte heterogeneity in the rainbow trout, *Oncorhynchus mykiss*.

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Submitted for the Degree of Doctor of Philosophy in the University of Stirling.

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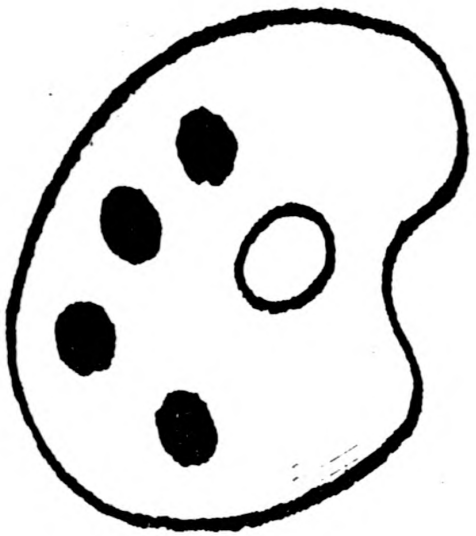
March, 1994

NUMEROUS ORIGINALS IN COLOUR



Declaration

I, Cameron Findlay hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial/complete fulfilment of any other degree or qualification.

Signed Cameron Findlay Date 17/7/94

Acknowledgements

Three years of postgraduate research at Stirling have seen the accumulation of a large number of friends and colleagues, without whose help I would never have completed this thesis. Thanks must go first to my supervisor, Dr Mary Tatner, who has given continuous encouragement and advice throughout my project. I would also like to thank Dr Tatner for allowing me to register for a PhD while working as a research assistant. I would also like to give special thanks to my mother, who constantly encouraged me throughout my studies, without her support I would probably never have started a postgraduate degree.

I gratefully acknowledge all members of the Aquatic Vaccine Unit (Dr Kim Thompson, Dr Ruth Cambell, Dr Sandra Adams and Carlos Farious) for their advice and friendship througout my study, and I will miss working in the lab tremendously. I would also like to give a special thanks to Niall Auchinachie for all his advice and help with fish husbandry, but more importantly for all the laughs that he provided.

The financial support of the NERC for this study is gratefully acknowledged.

Abstract:

Lymphocyte *in vivo* migration pathways were investigated in rainbow trout using the tracer sample method. Peripheral blood lymphocytes were separated into sIg+ and sIg- cells and injected back into the same fish. After 24 hours, following a passage through the thymus, the distribution of sIg+ cells between the spleen and kidney was approximately equal, but more sIg- cells localised in the kidney. When unseparated lymphocytes were injected into non-histocompatible recipients 42% of the total cells were in the spleen after 72 hours. When organ specific sIg+ and sIg- lymphocytes were injected into non-histocompatible recipients, it was found that sIg- cells (from the spleen, thymus and kidney) migrated preferentially to the spleen. It was concluded that the phenomenon of ecotaxis occurs in trout.

A comparative study of rainbow trout T and B lymphocytes following their separation by nylon wool adherence and lectin agglutination techniques, was carried out. The results revealed that nylon wool separation produced an adherent and a non-adherent population of cells. The adherent population showed B cell properties, based on mitogenic responses and enzyme and immunocytochemical staining profiles, whereas the non-adherent population showed T cell properties. The lectin Soybean agglutinin was used to separate lymphocytes into an agglutinated and a non-agglutinated population. A mitogenic and immunocytochemical study of these subpopulations revealed that they were unrelated to T and B cells.

In vitro studies were carried out to determine the effect of thymocyte coculture on the plaque forming cell response of spleen leukocytes from fish given a primary *in vivo* injection of DNP-KLH, SRBC, TNP-SRBC and TNP-LPS. Significant suppression was observed (when compared to spleen alone responses) in both allogenic and autogenic thymus-spleen cocultures from the

DNP-KLH, SRBC and TNP-SRBC immunised fish, but not the TNP-LPS immunised fish. Cyclophosphamide treatment, pre-*in vitro* transfer, abolished the observed suppression in the DNP-KLH, SRBC and TNP-SRBC immunised autogenic cocultures, and in some cases enhanced the PFC response as compared to the spleen alone cultures. No clear effects of NMU treatment, pre-*in vitro* transfer, were observed. The results showed clear evidence for a cyclophosphamide sensitive thymus derived suppressor cell population

The effect of adult thymectomy on the *in vivo* antibody response to DNP-KLH, SRBC and *A.salmonicida* was examined. Two month adult thymectomy had no measurable effect on antibody response to any of the antigens, when compared to intact controls. Eight month adult thymectomy led to a significantly reduced secondary response to SRBC and DNP-KLH, which was partly restored when the fish were reconstituted with their own cryopreserved thymocytes one week before secondary immunisation. In contrast eight month adult thymectomy produced a significantly elevated secondary antibody response to *A.salmonicida*, which was partly returned to control levels by reconstitution of the fish's autogenic cryopreserved thymocytes one week prior to secondary immunisation.

Electrophoretic analysis of whole cell leukocytes, lysed using non-ionic detergent lysis buffers, revealed organ specific protein bands in the thymus and kidney. Con A and LPS stimulation of leukocyte cultures produced clear changes in the SDS-PAGE protein patterns in silver stained and periodic acid-silver stained gels. A polyclonal antibody was produced to a 38kD thymocyte specific protein band, which stained 22% of thymocytes using the immunoperoxidase method.

Table of Contents

Table of Contents.

Table of Contents.

Table of Contents.

List of Tables.

Chapter 2.

Chapter 3.

Chapter 4.

List of Tables.

Chapter 5.

Chapter 6.

List of Tables.

Chapter 6.

Chapter 7.

Appendices.

List of Tables.

Appendix.

List of Figures.

Chapter 2:

Chapter 3.

List of Figures.

Chapter 4.

Chapter 5.

List of Figures.

Chapter 6.

List of Figures.

List of Figures

List of Plates.

List of plates.

Chapter 7.

Chapter 1 Introduction

All higher vertebrates possess both cellular and humoral immunity, which is mediated by interactions between subpopulations of cells. The higher vertebrate immune system is composed of lymphocytes, macrophages, monocytes, basophils, mast cells, eosinophils and neutrophils (Rowley and Ratcliffe, 1988). The most important dichotomy of lymphocyte types in mammals is the major division into T and B lymphocytes, and accessory cells. Mammalian B cells are antigen specific lymphocytes which are most readily identified by the presence of surface immunoglobulin (sIg) and their ability to undergo clonal expansion and differentiation into antibody-secreting cells in response to the proper stimuli. T cells are also antigen-specific, clonally expandable lymphocytes but, in contrast to B cells, do not exhibit sIg and develop in the thymus during ontogeny to differentiate into specialised subpopulations, capable of subsequently exhibiting either suppressor, cytotoxic or helper activities. In the higher vertebrate species studied in detail, each of these subpopulations of T cells exhibit distinctive cell surface markers (Bierer et al, 1989). Accessory cells such as macrophages and monocytes are generally considered to be antigen-nonspecific and to function primarily by secreting interleukin-1 (IL-1, a cytokine involved in T-cell activation) and participating in antigen processing and presentation (Unanue, 1984). In addition, the immune responses in higher vertebrates can be divided into two general categories, based on the types of lymphocytes involved: thymus-dependent (TD) responses which require T-cell help or participation and thymus independent (TI) responses which do not. It is important to note that in higher vertebrates the T and B lymphocytes do not only show functional differences but they also show distinct developmental differences, with the T cells developing in the thymus whilst the B cells remain in the bone marrow during development.

Fish are not only the descendants of the earliest vertebrates to emerge, but also represent the most primitive animals to posses an immune system characterised by lymphocytes and secretory immunoglobulins (Marchalonis, 1977). It is therefore of some interest to determine whether or not the immune system of fish possesses the complexity of cell types and interactions typical of higher vertebrate immunity. For fish to show true lymphocyte heterogeneity, the T and B cells must be embryologically distinct as well as functionally distinct.

In fish, lymphocytes are the most commonly observed leukocyte type present in the blood, and can account for as much as 85% of the total leukocyte population (Rowley and Ratcliffe, 1988) Lymphocytes are small cells ~5-8 μm in diameter with a high nuclear to cytoplasmic ratio and a basophilic cytoplasm (Ellis, 1977a). Ultrastructurally they are easily identifiable from other leukocyte types such as thrombocytes due to the central nucleus with dense patches of heterochromatin and an occasional nucleolus, with a poorly developed rim of cytoplasm containing a few vesicles, mitochondria, RER and occasional golgi complex (Ferguson, 1976). The lifespan of fish lymphocytes has received little attention, but in mammals the circulating pool of small lymphocytes is composed of short-lived cells with a lifespan of only 3-4 days, and long-lived cells surviving for up to 90 days (Everett and Tyler, 1967). More recently Freitas and Rocha (1993) reviewed the current data, and suggested that 30-40% of mammalian peripheral immunocompetent T and B cells were renewed every 3 days, and that most B cells are renewed within 10 days.

There is growing evidence that both cellular (T cell-mediated) and humoral (B cell-mediated) acquired immunity are present in all teleost fish so far studied (Manning and Turner, 1976; Ellis, 1982). This is based on evidence from several sources including:

(a) Behaviour of lymphocytes during separation studies

To study lymphocyte function a suitable method for lymphocyte separation must be found. As a first step in the enrichment process, centrifugation of peripheral blood or cell suspensions from the spleen, thymus or kidney over a Ficoll-sodium diatrizoate column was able to separate fish leukocytes from erythrocytes (Faulmann et al, 1983). Centrifugation over a continuous or discontinuous gradient of percoll has been successfully used in the purification of leukocyte subpopulations. Gutierrez et al (1979) used a discontinuous gradient of percoll for the purification of human T and B cells. They separated peripheral blood lymphocytes into three distinct bands, in which the top layer (fraction I) contained mainly B cells based on mitogen responses, and fractions II and III contained two different T cell populations. This technique has also been employed on fish lymphocytes (Blaxhall and Sheard, 1985; Blaxhall and Hood, 1985; Bayne, 1986; Waterstat et al, 1988). Blaxhall and Sheard (1985) separated brown trout lymphocytes into two fractions on a percoll discontinuous gradient. The lower density lymphocytes (band 1.056gml^{-1}) contained both moderately villous lymphocytes and smooth lymphocytes, whereas the higher density band (1.07 gml^{-1}) were predominately moderately villous lymphocytes. The moderately villous lymphocytes had a higher mitochondrial content than the smooth lymphocytes, which they suggested indicated higher metabolic activity of possible B cells. The lower density band lymphocytes were more responsive to phyto-haemagglutinin(PHA, T cell mitogen). Blaxhall and Hood (1985) showed that cells from the two fractions showed equal enzyme activity when stained for acid esterase and acid phosphatase. About 70% of the lymphocytes gave a positive enzyme reaction, which if the reaction is comparable with mammalian lymphocyte cytochemistry would indicate that they were T cells. Therefore it

appears that brown trout lymphocytes cannot be separated into T and B like cells on a percoll gradient. Nylon wool separation has been found to enrich T cells in fish (Ruben et al, 1977; Sakai, 1989). Sakai (1989) separated out circulating lymphocytes in the rainbow trout into nylon wool adherent (NW+) and non-adherent (NW-) cells. The author found that NW- cells were proliferative with PHA but not with lipopolysaccaride (LPS, B cell mitogen), and NW+ cells were proliferative with LPS but not PHA. This suggests that it is the B cell population which binds to the nylon wool and the T cell population which is eluted.

Another important separation technique used in fish studies is the 'panning' method (Sizemore et al, 1984; Thuvander et al, 1990). This method uses monoclonal antibodies to coat plastic cell culture plates, which binds to antibody positive cells and leaves the non-adherent cells free in the supernatant. Thuvander et al (1990) used mouse monoclonal anti-trout IgM coated plates to separate out the sIg+ and sIg- cells from the spleen, thymus and peripheral blood. They found that the proportion of sIg+ bearing cells (B cells) in the thymus, spleen and blood was 2%, 28% and 35% respectively. Miller et al (1985) used the panning sytem to separate channel catfish leukocytes into sIg+ and sIg- cells and accessory cells. They showed that the response to thymus independent antigens (TNP-LPS) required sIg+ (B cells) and macrophages, whereas the response to thymus dependent antigens (TNP-KLH) required sIg+, macrophages and sIg- (T cells). Miller et al (1987) attempted to produce a monoclonal antibody that would react only with channel catfish T cells. They tried a variety of commercially available anti-human and mouse T cell reagents, but all of these failed to react with channel catfish lymphocytes. Therefore they developed a monoclonal antibody which reacted with channel catfish sIg- lymphocytes, but not sIg+ lymphocytes. This

monoclonal, when used in panning experiments was able to isolate those channel catfish lymphocytes which provide helper activity for antibody synthesis to a thymus dependent antigen (TNP-KLH). In addition, this antibody was observed to react with most thymocytes, neutrophils, thrombocytes, and with some brain cells. They concluded that this antibody reacted with a relatively high molecular weight antigen on channel catfish T lymphocytes and that it may be an anti-T cell reagent possibly akin to the Thy-2 antigen of mammalian lymphocytes.

(b) Monoclonal antibody analysis

In mammals it has been accepted that only B lymphocytes readily display immunoglobulin, where it acts as a receptor. Previously it was thought that most, if not all, fish lymphocytes posses abundant surface immunoglobulin which was demonstratable by membrane immunofluoresence using heterologous polyclonal antisera to fish Ig (Emmrich et al, 1975; Warr et al, 1976; Clem et al, 1977; Warr et al, 1979). Later work (Yamaga et al, 1979) suggested that this may have been due to carbohydrate cross reactivity of the polyclonal antisera. This problem of cross reactivity was overcome by the use of monoclonal antibodies. Lobb and Clem (1982) assayed channel catfish peripheral blood and splenic lymphocytes for surface immunoglobulin using 15 mouse monoclonal antibodies to catfish serum immunoglobulin. These studies showed that this battery of monoclonal antibodies did not show significant Ig on all lymphocytes. Unlike polyclonal antisera, which detected nearly 100% sIg+ cells, these monoclonals detected ~40% sIg+ lymphocytes. More recently DeLuca et al (1983) obtained a monoclonal antibody to trout serum IgM. This was tested by imunofluoresence analysis with lymphocytes from the thymus, spleen and head kidney. They showed that the antibody

reacted with only a subpopulation of lymphocytes: 5.2% thymus cells, 30.3% of kidney cells and 12.4% in the spleen. They extended this work, by using the monoclonal anti-IgM antibody to deplete head kidney cells positive for sIgM. This was found to significantly reduce the mitogenic response to LPS (B cell mitogen) but not to concanavalin A (Con A; T cell mitogen). The relative numbers of Ig positive lymphocytes from the organs in trout were confirmed by the work of Sanchez et al (1993) who produced a series of monoclonal antibodies to the heavy and light chains of trout Ig. Thuvander et al (1990) produced a battery of monoclonal antibodies to rainbow trout IgM, which reacted with 35% and 46% of spleen and peripheral blood lymphocytes respectively, but reacted with less than 2% of thymocytes. Navarro et al (1993) produced monoclonal antibodies to gilthead seabream serum Ig, and found that 22%, 23% and 3% of head kidney, spleen and thymus stained positive for surface Ig. Immunogold labelling of living cells showed that Ig was frequently present in small clusters at the external membrane of Ig-bearing and Ig-containing cells. This work was important since it seems to support the existence of distinct subpopulations of fish lymphocytes.

Recently van Diepen (1991) used monoclonal antibodies against carp serum immunoglobulin and thymocytes. These were characterised at the ultrastructural level for their reactivity with leukocytes from carp head kidney, and for the corresponding distribution of the cell membrane molecules recognised by these antibodies. With the anti-serum Ig monoclonal 20-30% of the lymphocytes, probably B lymphocytes, and the majority of plasma cells appeared to be Ig-immunoreactive. It was found that membrane Ig mainly occured in clusters on the B cell membrane. The anti-thymocyte monoclonal reacted with all leukocytes present and the monoclonal recognised molecules that were regularly distributed over the whole cell membrane. Therefore these

surface immunoglobulin studies show that there appears to be at least two subpopulations of lymphocytes which appear to correspond to T-like and B-like lymphocytes.

(c) *In vitro* Functional assays

The majority of evidence supporting the presence of T and B lymphocytes in fish has come from the use of various *in vitro* functional assays. One of the most widely used *in vitro* functional assays, for the study of fish lymphocyte heterogeneity, has been the proliferative responses of different lymphocyte populations to mitogens. A mitogenic response to the lectins concanavalin A (Con A) and phytohaemaglutinin (PHA) is specific for T cells in mammals and birds, and lipopolysaccaride is considered a B cell mitogen in mammals and birds (Weber, 1973).

Most of the work done on the mitogenic responses of fish lymphocytes has been done on teleosts, however some workers have looked at other classes of fish. Cooper (1971) showed that lymphocytes from the larval lampreys responded to PHA *in vitro* by the production of blast cells, which may suggest the existence of T-like lymphocytes. Sigel et al (1978) found that after density gradient centrifugation of nurse shark lymphocytes, 3 layers were obtained and the cells from all bands responded to high concentrations of Con A, but only cells from the bottom responded to PHA. These results only indicated the presence of T cells. However Petty and McKinney (1981) separated leukocytes from the nurse shark based on their adherence to glass, into an adherent and non-adherent population. Only the adherent population showed cytotoxic reactions following stimulation with Con A, PHA and LPS and formed erythrocyte-rosettes, while the non-adherent cells had surface associated immunoglobulin. These results by Petty and McKinney (1981)

pointed towards a T-cell B-cell dichotomy.

By far the greatest amount of work on mitogen responses of fish lymphocytes has been done using teleost fish (Etlinger et al, 1976; Cuchens and Clem, 1977; Warr and Simon, 1983; Caspi et al, 1984; Clem et al, 1984; Sizemore et al, 1984; Tillit et al, 1988; Bly et al, 1990; Flory and Bayne, 1991). One of the earliest studies was by Etlinger et al (1976), who looked at the response of rainbow trout lymphocytes from different lymphoid organs to Con A, LPS and PPD (purified protein derivative). They found that thymocytes responded to Con A but not to LPS or PPD. In contrast lymphocytes from the anterior kidney were stimulated with LPS but not Con A or PPD, and cells from the spleen and peripheral blood were stimulated with all three mitogens. The degree of stimulation at optimally stimulatory concentrations of each mitogen was distinctive. These results were important since they suggested that lymphocyte heterogeniety existed in trout and that it had a unique tissue distribution i.e. T cells in the thymus and B cells in the kidney. However Cuchens and Clem (1977) were unable to find a similar distributional dichotomy when working with the blue gill *Lepomis macrochirus*. They isolated the lymphocytes from the anterior kidney, thymus and spleen. The cells from the thymus responded to LPS, PHA and Con A unlike those of the rainbow trout. Some heterogeniety was however observed using cells from the anterior kidney, with one population responding to PHA and Con A but not to LPS, while the other responded to LPS only. These subpopulations were further investigated by incubating lymphocytes from the anterior kidney with rabbit antiserum to blue gill brain tissue. The idea behind this approach was that murine T lymphocytes and brain cells exhibit the surface Thy-1 antigen in common, and so by raising antiserum to these brain cells and incubating this with complement and lymphocytes, the T cell population was destroyed. In

blue gill, lymphocytes from the anterior kidney treated with antiserum to brain tissue failed to respond to the T cell mitogen PHA, whereas the LPS response was enhanced compared with untreated lymphocyte cultures from the anterior kidney. Hence the PHA sensitive lymphocytes from the blue gill share a common antigenic determinant with brain tissue in a way similar to murine T cells. Further evidence for lymphocyte heterogeniety in the blue gill was that the PHA and Con A sensitive cells did not form stable rosettes with rabbit erythrocytes, while the LPS reactive lymphocytes did.

Further work by Warr and Simon (1983) on the properties of lymphocytes from the rainbow trout, looked at the mitogenic potential of lymphocytes from the thymus, spleen, anterior kidney and peripheral blood. The results showed that lymphocytes from all sources tested were capable of some degree of blastogenic response to both Con A and LPS. These results suggest the organ compartmentalisation of mitogen responsiveness in rainbow trout resembles that reported for the blue gill, in contrast to that previously described for the rainbow trout by Etlinger et al (1976). More recently Tillit et al (1988) worked on optimization of conditions for mitogen responses in rainbow trout, and during these studies they found that peripheral blood lymphocytes responded better to Con A stimulation than LPS, PHA or pokeweed mitogen (PWM) stimulation. Thuvander et al (1990) found that rainbow trout peripheral blood and kidney lymphocytes responded to both PHA and LPS, but thymocytes responded to PHA only.

A great deal of work has been done on the mitogenic responses of channel catfish lymphocytes (Sizemore et al, 1984; Clem et al, 1984; Bly et al 1990). These authors used separation and enrichment techniques to isolate three distinct leukocyte populations present in channel catfish peripheral blood. Surface immunoglobulin positive (sIg+) and negative (sIg-)

lymphocytes were separated by the panning technique employing monoclonal antibodies to catfish Ig. A third cell population composed of macrophages was isolated by adherence to baby hamster kidney cell exudate coated surfaces. They assessed the functional features of these three populations by looking at the responses to Con A and LPS. The results obtained indicated that the sIg+ cells responded only to LPS regardless of the presence or absence of macrophages. However the sIg- subpopulation responded to neither LPS or Con A unless macrophages were present, in which case responses were obtained to both mitogens.

The migration inhibition factor (MIF) assay is another *in vitro* functional assay that has been used to provide evidence for functional lymphocyte heterogeniety in fish, by measuring cell mediated immunity (T cell activity). The MIF response to sheep red blood cells (SRBC; Jayaraman et al, 1979) and to non-self alloantigens (McKinney et al, 1980) has been studied in fish. Jayaraman et al (1979) demonstrated the MIF response in *Tilapia mossambica* to be antigen specific. MIF responses were found to be inversely proportional to the amount of antigen injected, as were PFC (plaque forming cell) responses which were a measure of the humoral immune response. They found that cyclophosphamide administered immediately after immunization completely abrogated both PFC and MIF responses. In contrast, injection of formalised SRBC resulted in greater elevation of MIF levels accompanied by the absence of PFC production. Formalised SRBC have been shown to activate T-helper cells specifically without the generation of PFC in mice (Dennert and Tucker, 1972). Jayaraman et al (1979) suggested that in fish the enhanced cell mediated immune (MIF) response they had seen was accompanied by a low B cell response (PFC). They also suggested that the activation of B cells by large doses of SRBC might lead to antigen-antibody

complexes which might lead to inhibition of T cell activity (lower MIF response with higher SRBC doses), as occurs in mice. In the gar, MIF reactions were found to occur with kidney and peripheral blood cells (McKinney et al, 1980). They found that stimulation with the lectins PHA, Con A and PWM also evoked MIF reactions. Blazer et al (1984) looked at the cellular immune responses of rainbow trout to SRBC. The primary and secondary responses were measured using the MIF, antigen binding cell (ABC) and PFC assays. The kinetics of these three responses, at 16°C, were examined by sampling fish over an 18 day period for the primary response and a 10 day period for the secondary response. The peak MIF response occurred 2 days after injection, while the peak PFC response occured 14 days post injection. Two ABC peaks occured in the primary response, one at 4 days and one at 10 days post injection. In the secondary response the peak ABC response was observed at 4 days and peak PFC response at 6 days post injection. They suggested that the first peak in ABC response was T cells (probably T helper cells), while the latter PFC peak was B cells. An increase in PFC was concurrent with a decrease in ABC. Ingram and Alexander (1981) studied the ABC and PFC responses of brown trout to SRBC. They observed only one peak in ABC, 12 days after injection. However, they used a higher dose of SRBC. It has been shown in the lizard that after high doses of SRBC, a single late ABC peak is observed (Subramonia et al, 1977). These authors suggested that higher doses of SRBC may elicit suppressor cell activity, rather than helper cell activity. This explanation could also explain the differences in ABC responses found in fish immunised with different doses of SRBC.

Another *in vitro* assay for cellular immunity in fish which points towards different lymphocyte sub-populations in fish is the mixed lymphocyte reaction (MLR). The MLR is an *in vitro* response in which lymphocytes

proliferate as a consequence of appropriate allogenic stimulation (non-self antigens controlled by the major histocompatibility complex: MHC). This reaction is widley present among vertebrates, having been observed among all the higher vertebrate groups and in the lower groups including fish (Cuchens and Clem, 1977; Etlinger et al, 1977; Caspi and Avtalion, 1984a; Miller et al, 1986; Kaastrup et al, 1988). There are some cases however where a MLR reaction has not been succesfully obtained, for example sharks, snappers and gars (McKinney et al, 1976, 1980). Etlinger et al (1977) showed that mixtures of rainbow trout peripheral blood or kidney leukocytes obtained from 2 individual fish revealed marked proliferative responses. Maximum response was observed on day 6 with the kidney cells and on day 7 with the peripheral blood lymphocytes. Cuchens and Clem (1977) showed that in the blue gill a positive mixed lymphocyte reaction was obtained with head kidney cells at 32°C but not at 22°C. One of the most comprehensive studies on the MLR was by Miller et al (1986) who established the cellular requirments for the MLR in the channel catfish. They showed that vigorous MLR's were obtained using peripheral blood leukocytes when equal numbers of responder and stimulator cells ($5x10^5$ cells each) were cocultured. The use of 2000 rads of X-irradiation was sufficent to block subsequent proliferative responses of the stimulator cells. The cellular requirements for the MLR response was assessed by using three functionally distinct leukocyte subpopulations isolated from peripheral blood: sIg+ and sIg- lymphocytes were isolated by panning against a monoclonal anti-catfish Ig and a third population, monocytes, were isolated by adherence to baby hamster kidney cell exudate coated surfaces. The results clearly indicated that the sIg- lymphocytes were the responding cells and that monocytes were required as assessory cells. Furthermore , although each of the three cell types could serve as stimulators, monocytes and sIg+

lymphocytes were significantly more efficient stimulators than the sIg- lymphocytes.

(d) Study of cell-cell cooperation using the hapten-carrier effect

The use of *in vitro* assays such as mitogen responsiveness as the sole indicator of fish lymphocyte heterogeniety is tenuous. In higher vertebrates a lot of information regarding the interactions of the lymphocyte subpopulations has come from the study of cellular cooperation using Hapten-carrier conjugates. Haptens are small molecules such as trinitrophenyl (TNP) which on their own are unable to evoke an immune response, but if linked to a carrier molecule such as BSA (bovine serum albumin) or KLH (keyhole limpet haemocyanin), these complexes evoke antibody production against the hapten. The reaction has been shown to be carrier specific i.e. if the primary immunisation is with TNP-KLH and the rechallenge occurs with the same hapten-carrier complex then an elevated secondary response takes place to TNP. If however a different carrier molecule is linked to TNP for the rechallenge then the elevated response is lacking. This carrier effect is explained by the cells involved in the antibody response recognising two parts of the antigenic complex, with T cells reacting to the carrier while B cells recognise the hapten. The carrier primed T cells involved in this response are T helper cells. Such experiments have been carried out in mammals (Turk and Parker, 1977; Goodman, 1975).

A hapten-carrier effect has been demonstrated in the winter flounder (Stolen and Makela, 1980), the goldfish (Ruben et al, 1977) and the channel catfish (Miller and Clem, 1984a; Miller et al, 1985). Ruben et al (1977) looked at the anti-hapten TNP response in goldfish and found it to be specifically enhanced by carrier priming. The helper function of carrier-stimulated cells

was short lived and greatest when low dose carrier priming was used. They also found that the proportion of carrier stimulated thymus and head kidney antigen binding cells was considerably enriched by passage through a nylon-wool column.

Considerable work went into the development of an *in vitro* culture system which would allow the *in vitro* responses of individual subpopulations of fish leukocytes to be investigated. Miller and Clem (1984a) developed an *in vitro* culture system for the channel catfish, to study the generation of anti-hapten antibody secreting cell (PFC) responses to hapten-carrier conjugates known to be either thymus dependent (TD) or independent (TI) in mammals. They found that *in vitro* secondary PFC responses to the TD antigens (TNP-KLH) exhibited a strong hapten-carrier effect. The nature of the hapten and carrier specificities was also tested by depletion of antigen-reactive sIg+ and/or sIg- lymphocytes. The results clearly demonstrated that carrier specificity was limited to sIg- cells which probably acted in a similar way to T helper cells.

Another important finding from these studies by Miller and Clem (1984b) showed that low temperature suppressed antibody formation following immunisation with these hapten carrier conjugates. They found that the magnitude of primary responses to the TI TNP-LPS and secondary responses to the TD DNP-KLH were relatively independent of *in vitro* culture temperature. In contrast, the magnitudes of primary responses to TD DNP-KLH was suppressed at lower *in vitro* temperatures. Furthermore, it was observed that some of the low temperature suppression of the primary response to TD antigens could be removed by appropriate low temperature *in vivo* acclimation. These findings support the principle that low temperatures suppress the production of an immune response and that elevation to higher

temperatures can abrogate this suppression (Bly and Clem, 1992). The immunosuppressive effect is probably mediated via the T helper cells. Temperature may have its specific effect on the T helper cells due to differences between T and B cells in cell membrane fluidity at low temperatures (Bly and Clem, 1992). Recent work by Bly et al (1990) has looked at the differential effects of temperature and exogenous fatty acids on mitogen induced proliferation in channel catfish T and B lymphocytes. They found that at permissive *in vitro* temperatures (27°C) high concentrations (>240*u* m) of all fatty acids used were inhibitory. However, at lower concentrations (80-160*u* m) differences were noted in the ability of some fatty acids to modulate mitogen responses. While palmitic acid (16:0) and linoleic acid (18:2) had little effect on LPS induced B cell or Con A induced T cell proliferation, stearic acid (18:0) suppressed while oleic acid (18:1) enhanced T cell responses only. Oleic acid (18:1) was used to successfully rescue ~60% of the Con A induced T cell proliferation normally inhibited at nonpermissive *in vitro* temperatures (17°C). It was proposed that 18:1 enhances T cell responses at high permissive temperatures and rescues suppressed T cell responses at low non permissive temperatures by increasing membrane fluidity. The authors suggested that the catfish T cells may naturally have fewer 18:1 fatty acids in their membranes.

(e) Adoptive transfer experiments

A novel approach to the study of lymphocyte interactions in fish was carried out by Sakai (1982, 1991) who used adoptive transfer experiments to demonstrate the existence of two lymphocyte populations and their cell-cell interactions. He looked at two populations of lymphocytes, one sensitive to intraperitoneal (I.P.) cyclophosphamide injection (400-500 mg/kg) and another

that was resistant to its effects. He showed that when lymphocytes isolated from the blood and spleen of GFRBC (goldfish red blood cell) primed, cyclophosphamide treated rainbow trout were transfered intravascularly into unprimed cyclophosphamide treated rainbow trout, the antibody titres of the recipients were low or undetectable. This suggested that the transfer of the cyclophosphamide-resistant cells from the donor was ineffective and the lack of cyclophosphamide-sensitive cells in the recipient was fatal to the anti-GFRBC immune response of the latter. When lymphocytes including cyclophosphamide- resistant and cyclophosphamide-sensitive cells, separated from GFRBC-primed donors were transfered to immunological non-responders as recipients (which were cyclophosphamide treated), the antibody response of the recipients was restored to the same level as in normally immunised trout. The restoration due to this adoptive transfer suggested two things: that an antibody response to GFRBC in rainbow trout clearly requires both the cyclophosphamide sensitive and cyclophosphamide resistant lymphocyte population to be present, and that a cell-cell cooperation of the two cell types is essential for the production of antibody to GFRBC.

(g) Soluble factors such as Lymphokines and Cytokines

Further evidence for cell-cell cooperation and heterogeniety in fish lymphocytes has come from the study of regulatory factors produced by the leukocytes. In mammalian systems these regulatory factors can be detected in the supernatants of mixed lymphocyte cultures (Anderson et al, 1979; Gillis et al, 1980). In mammals these non-immunoglobulin products secreted by T cells, collectively called lymphokines, are mediators of cellular cooperation. Many of the lymphokines characterised in mammals serve to locally activate non-specific immune responses, in which the macrophage plays a key role.

Once activated, these macrophages produce a number of secretory products including complement factors, interferons and interleukin 1 (IL-1) and can stimulate other cells, such as T lymphocytes, to synthesise a range of products (e.g. IL-2 and B cell growth factor). There is now mounting evidence for a similar interaction between lymphocytes and macrophages in fish (Smith and Braun-Nesje, 1981; Miller et al, 1985; Secombes, 1987; Graham and Secombes, 1990). Therefore it seems likely that factors such as IL-2 are produced during this process. Caspi and Avtalion (1984b) found evidence for the existence of an IL-2 like lymphocyte growth promoting factor in carp. IL-2 is a T helper cell derived growth promoting factor, necessary for the proliferation of activated T cells. They found activity promoting growth of carp T like cells in the supernatants of Con A and alloantigen (MLR) stimulated carp leukocyte cultures. They also found that the activity level in culture supernatants was elevated by phorbol myristate acetate (PMA), similar to the IL-2 of mammals (Farrar et al, 1982). Proliferation of carp T like lymphoblasts was also promoted in the presence of IL-2 containing supernatants of mammalian origin. The ability of carp T like cells to recognise and utilise mammalian IL-2 for proliferation would seem to indicate a great deal of conservation of structure during evolution. It has yet to be elucidated if this IL-2 production in fish is brought about by a macrophage-T cell interaction, as in mammals , but this seems likely.

There are also a few studies which have looked at IL-1 production in fish (Sigel et al, 1986). In mammals, IL-1 acts on T and B cells, increasing the expression of their IL-2 receptors on B cells and stimulates T helper cells to produce IL-2 which leads to proliferation of B cells. Activated T cells also release lymphokines, some of which act on macrophages (macrophage activating factor [MAF]) stimulating these in turn to produce IL-1 (Bernheim

et al, 1980; Dinarello, 1981). The work of Sigel et al (1986) showed that catfish peripheral blood lymphocytes recognise and respond to human IL-1 and that an epithelial cell line from hypertrophic skin lesions of carp produces a factor with functional similarities to mammalian IL-1. However they were unable to tell whether the cell type involved in the production of IL-1 in catfish was a mononuclear phagocyte and whether these cells could stimulate lymphocytes to produce IL-2. More recently Secombes (1987) looked at lymphokine release from rainbow trout leukocytes stimulated with Con A and its effects on macrophage spreading. They found that rainbow trout blood and head kidney leukocytes could be stimulated with Con A to release a factor that increased macrophage spreading and adherence. Con A present in control cultures was also shown to have this effect and so removal of Con A was essential to investigate the action of potential lymphokines. The Con A was removed by absorption with Sephadex G-10; this was found to succesfully remove activity from control supernatants whilst having no effect upon the lymphokine containing supernatants. The authors suggested that the lymphokine which increased macrophage spreading was probably a kind of macrophage activating factor (MAF). Recently Graham and Secombes (1990) looked at the ability of different subpopulations of rainbow trout leukocytes to produce MAF following stimulation with Con A/PHA. This was assessed by the amount of NBT (nitroblue tetrazolium) reduction in target macrophages. The effect of varying lymphocyte or macrophage number on MAF production in the presence of a constant number of macrophages or lymphocytes respectively, showed that in both cases MAF activity initially increased with increasing cell number and then reached a plateau. Macrophages alone did not produce MAF, whereas some MAF activity was produced with macrophage depleted lymphocytes, although significantly lower than in the presence of

macrophages. Separation of leukocytes into sIg+ and sIg- cells, by panning with an anti-trout IgM monoclonal, showed that only sIg- lymphocytes (T cells) could produce MAF and that macrophages were necessary as accessory cells, similar to the situation found in mammals.

Miller et al (1984a) and Miller et al (1985) found that monocytes or macrophages were required for the antibody production towards both TI and TD antigens *in vitro* and for the MLR assay. This prompted studies to investigate if the monocyte accessory function could be attributed to a soluble factor, similar to IL-1 in mammals. Clem et al (1985) found that culture supernatants of isolated catfish peripheral blood monocytes stimulated with LPS could replace the need for monocytes in mitogenic responses to Con A as well as PFC responses to both TD (TNP-KLH) and TI (TNP-LPS) antigens.

(g) Antigen processing and presentation

As mentioned earlier, when Miller et al (1986) were looking at the cellular requirements for the MLR response, they found that monocytes were required as accesory cells by the sIg- peripheral blood lymphocytes, and Miller et al (1985) found that monocytes were required for *in vitro* anti-hapten PFC responses to both TD (TNP-KLH) and TI (TNP-LPS) antigens. This leads to the question of antigen processing in fish. In mammals it is accepted that processing of TD antigens by accessory cells (or antigen presenting cells [APC]) is required for the induction of immune responses (Yewdell and Bennick, 1990). The end result of such processing is the modification of the antigen into a form capable of interacting with self MHC molecules and subsequently being re-expressed on the surfaces of APC, where it is then recognised by the T cell receptor of the T helper cell.

Recent work has looked at the cellular pathways of antigen processing

in fish (Vallejo et al, 1990, 1991, 1992). Vallejo et al (1990) showed that IL-2 production by sIg- cells was required for a response by sIg+ cells to the TD KLH antigen. Vallejo et al (1991) looked at whether or not antigen processing was an important process in channel catfish *in vitro* secondary immune responses elicited with structurally defined proteins (pigeon heart cytochrome C [pCytC], hen egg lysozyme, and horse myoglobulin). They found that the use of *in vitro* antigen pulsed and fixed (in 0.5% paraformaldehyde for 15 minutes) B cells or monocytes as APC resulted in autologous peripheral blood leukocytes (PBL) responding with vigorous proliferation and antibody production *in vitro*. In addition several long term catfish monocyte lines were found to function as efficient APC with autologous but not allogenic responders. This work demonstrated that the generation of catfish secondary *in vitro* immune responses involves steps akin to antigen processing and presentation in mammals (Vallejo et al, 1990), and that both B cells and monocytes were effective APC. The use of antigen-pulsed and paraformaldehyde fixed APC in the production of *in vitro* proliferation and antibody formation, suggested that these responses must be due to antigen that became cell associated during pulsing and was presumably processed intracellularly. Subsequent separation of the responding PBL into sIg- (T cell) and sIg+ (B cell) subpopulations showed that both underwent proliferative responses to antigen pulsed and fixed APC. Also, allogenic cells used as APC were found to induce only strong MLR reactions without specific *in vitro* antibody production. This suggests that fish alloantigens are present on fish cells, although as has been mentioned their relationship to the mammalian MHC is not known at present.

Vallejo et al (1992) looked at the effects of different *in vitro* temperatures during cellular processing of TD antigens by these channel

catfish APC. The protocol involved pulsing long term monocyte lines (used as APC) with antigen (KLH, pCytC, horse myoglobulin) at different temperatures for various periods of time and then fixing them (as above) and coculturing them with PBL as responders at permissive temperatures (27°C). They showed that APC incubated with antigen at a low permissive temperature (11°C and 17°C) still elicited secondary proliferative responses by autologous PBL. However, responses elicited with APC pulsed at 11°C and 17°C required longer exposure to antigen prior to fixation. Further, they found that there was sufficient cell associated antigen during a short pulsing period at both 11°C and 17°C to provide efficient presentation after subsequent incubation of APC at 27°C for an additional 10 hours before fixation. These results suggested that the previously observed suppression of primary T cell responses in fish at low temperatures (Bly and Clem, 1992) was not due to impaired antigen processing and presentation. The results regarding antigen processing and presentation in fish taken as a whole further emphasise the mammalian like cellular diversity present in fish responses.

Recently Vallejo et al (1993) looked at the fine specificity changes in channel catfish immune repertoires to the structurally defined protein cytocrome C. Previously they had found that species variants of cytochrome C were cross stimulatory to peripheral blood leukocytes (PBL) from catfish immunised with the pigeon variant (Vallego et al, 1991). They used data base analyses to reveal the existence of overlapping epitopes that defined the specificity of a family of closely related antigens. They showed that peptide 81-104 and intact cytochrome C were stimulatory to PBL from fish previously immunised with the intact molecule. In contrast, PBL from fish previously primed with the peptide 81-104 responded only to immunising peptide as well

as to some of the variants of peptide 81-104. The differences in the stimulatory capacities of the peptide variants was correlated with amino acid substitutions at various positions of the peptide and changes in their predicted secondary structures. In mammals single amino acid changes have been shown to result in a complete change of immune specificity, that is, the peptide becomes amenable for presentation by another MHC allotype (Carbone et al, 1987). Therefore the T cell repertoire is expanded to include a new previously unknown specificity, a phenomenon often called heteroclitic. The authors suggested that the observed differences in catfish PBL stimulatory capacities to cytochrome C peptides may be a form of heteroclitic response.

Therefore when taken collectively the results so far can be interpreted as providing strong evidence to support the theory that teleost fish have separable B cells and T cells which are functionally equivalent to their counterparts in higher vertebrates. On one hand, the sIg+ lymphocytes seem to fulfill the functional criteria for being designated B cells i.e. they exhibit hapten specificity in the hapten carrier responses, differentiate into antibody secreting cells, respond to LPS but not Con A, and do not respond in the MLR, and seem to be present in only very small numbers in the thymus. On the other hand, the sIg- lymphocytes can be designated T cells as they exhibit carrier specificity in providing help for antibody production toTD antigens, but are not required for the antibody production toward TI antigens. They also respond well to Con A and only very poorly to LPS, and respond in the MLR. In higher vertebrate systems the T cells are further divided into suppressor/helper cells and cytotoxic cells which all show distict functional and developmental patterns (Bierer et al, 1989).

(h) T suppressor cells

As mentioned earlier Miller et al (1987) obtained a monclonal antibody that was reactive with sIg- populations from the peripheral blood and thymus and isolated a population of cells that provided helper activity in the antibody response against TD antigens. There have been other studies which have suggested that the helper and suppressor activities that are often seen in teleost fish may be separable, suggesting that they may be due to distinct T cell subpopulations. At present there is little direct evidence for the existence of T suppressor cells in fish, although much indirect evidence suggests their presence (Manning et al, 1982a; Kaattari et al, 1986; Nakanishi, 1986; Tatner et al, 1987). Manning et al (1982a) obtained some evidence for a suppressive role for the thymus in antibody formation, in a series of experiments in which adult rainbow trout and carp were thymectomised and then immunised 4 weeks later. Adult thymectomy in carp caused elevated serum antibody titres at day 7 after immunization with *Aeromonas salmonicida* but the response returned to normal levels later. In one year old trout , thymectomy had no effect on antibody production to *A.salmonicida* but with human gamma globulin (HGG) administered in adjuvant, humoral antibody levels were elevated in the thymectomised fish. The authors speculated that this elevated response to HGG (a TD antigen in mammals) may have been due to the removal of suppressor cells by thymectomy. However Tatner et al (1987) found that adult rainbow trout, thymectomised 5 months previously, responded poorly to HGG and no secondary response was seen, and unlike the elevated response mentioned above, found that the titres in the thymectomised fish were slightly lower than that of the intact controls, although this was not significant. However they used HGG at the much lower dose of 0.008 mg/g fish compared to the 0.025 mg/g used by Manning et al (1982). It has been shown that the amount

of antigen given in the priming injection can effect the level of both the primary and secondary antibody responses (Lamers et al, 1985). Strong evidence for the existence of a suppressor cell population in rainbow trout was provided by Kaattari et al (1986), who found that low *in vitro* doses of X-irradiation (100-600 rads) enhanced the PFC response of spleen leukocytes to both the TD antigen TNP-KLH and TI antigen TNP-LPS. Nakanishi (1986) found evidence that the suppressor activity seen in the marine teleost *Sebasticus marmoratus* may reside within the thymus by looking at the effects of combinations of X-irradiation (dose of 2000 rads) and one month adult thymectomy on the antibody response to SRBC and the rate of allogenic scale graft rejection. The author found that in non-thymectomised and irradiated fish, injected with SRBC one week after irradiation, antibody production was completely suppressed and they required twice the amount of time to reject scales, from an allogenic donor, transplanted 3 days after irradiation. On the other hand, fish which were irradiated 4 days after thymectomy and injected one week after irradiation, showed a fairly high level of antibody production but no differences were seen in the allograft rejection rates. The results suggest that suppressor cells, which are resistant to X-irradiation, are abundant in the thymus of *Sebasticus marmoratus*. The existence of an X-irradiation resistant suppressor cell population has been reported in mammals (Lobo and Spencer, 1974). Evidence that a suppressor cell population also resides within the T cell population of the channel catfish was provided by Clem et al (1991) when they mixed various combinations of X-irradiated (2000 rads) peripheral blood sIg- (T cells) and/or sIg+ (B cells), isolated by panning from TNP-KLH immunised fish, with equal numbers of non-irradiated T and/or B cells from the same fish and assayed for secondary *in vitro* PFC responses to the TD TNP-KLH antigen. The results clearly indicated that cultures containing

X-irradiated sIg- cells and unirradiated sIg+ cells exhibited enhanced PFC responses when compared to unirradiated sIg- and sIg+ mixtures, suggesting that irradiation of the sIg- population removed a suppressor cell population. This X-irradiation sensitive suppressor cell function is opposite to the X-irradiation resistant suppressor cell population suggested for the marine teleost *Sebasticus marmoratus*, using a similar X-irradiation dose of 2000 rads (Nakanishi, 1986). This enhanced response was not seen in every case, especially when the unfractionated controls showed high PFC responses, which the authors suggested meant that only low levels of suppressor cells were present. In spite of some differences in results there is fairly strong circumstantial evidence for a T suppressor cell population, located in the sIg- (T cell) population in teleosts, although at the present time there is no surface marker, such as the CD8 38 kD protein marker on mammalian suppressor lymphocytes (Blann, 1987), which can be used to positively select for this cell population in fish.

(i) Cell mediated cytotoxicity in fish

Cell mediated cytotoxicity has been well documented in a variety of mammalian and avian species. As has been previously mentioned the cell-mediated immune responses in fish have been shown to have many similarities with that of higher vertebrates, and the evidence of cytotoxic T cell immunity that has been found in fish further emphasises this mammalian like cellular diversity. Some fish species have been shown to produce cytotoxic T cell immunity (Hayden and Laux; 1985 Verlhac et al, 1990). Hayden and Laux (1985) found that lymphoid cell populations from the peripheral blood, thymus and head kidney, but not the spleen, showed cytotoxic activity in the rainbow trout. They found that the addition of PHA (T cell mitogen) to the reaction

mixture resulted in markedly enhanced cytotoxic reactivity, which suggested that the cytotoxic effector cells are capable of mediating lectin-dependent cell mediated cytotoxicity (LDCC) in the presence of PHA. In the presence of PHA lysis was readily detectable at 4 hours (^{15}Cr-release assay). The ability to cause lysis in a 4 hour assay is significant since previous studies on mammalian systems have shown that LDCC occurs in a 4 hour assay only if the effector cells have been activated prior to the cytotoxicity assay. In mice such reactivity is mediated by activated cytotoxic T cells (Davignon and Laux, 1978). Whether the effector cells which mediated cytotoxicity in trout in the absence of PHA represent the same population which mediated cytotoxicity in the presence of PHA was not shown. However the authors suggested that the ability to detect significant levels of LDCC in a 4 hour assay indicates that trout possess effector cells with cytotoxic potential.

Recently Verlac et al (1990) investigated the effect of fish acclimatisation at low, intermediate and high temperatures (9°C, 18°C and 26°C respectively) on the cellular cytotoxicity of carp kidney lymphocytes against TNP-modified autologous cells. The results showed that the highest cytotoxic activity was obtained following acclimatisation of the fish at 18°C and with an effector:target cell ratio of 100:1. Acclimatisation at 9°C significantly reduced the rate of lysis for the ratio of 100:1, but not for the ratio of 50:1. A significant decrease in cytotoxicity, from both the 50:1 and 100:1 effector:target ratios, was obtained with a 10:1 effector:target cell ratio regardless of acclimatization temperature. These results suggest that selective changes in proliferation or differentiation of cytotoxic cells might occur during the *in vivo* acclimation period in the carp. Miller and Clem (1984b) found no change in PFC responses at higher acclimation temperatures, suggesting that

such changes were not occuring in the PFC forming cells (B cells).

Verlac et al (1990) also looked at a genetic restriction phenomenon at this phylogenetic level of cytotoxic response, based on the model reported by Shearer (1974), who showed that mammalian T cells can be sensitised *in vitro* to hapten (TNP) modified syngeneic cells and that the specificity of the cytotoxic reaction is determined by both the hapten and MHC class I antigens on the modified spleen cells. They looked at the percentage of lysis as a function of the nature of the target cell, and the effector to target cell ratio was tested in carp following their acclimatisation to 18°C. They found that the percentage of lysis for autologous hapten modified cells was considerably higher than that obtained for the allogenic modified cells. These results suggest that a genetic restriction phenomenon is involved in this specific cytotoxic activity. Such cytotoxic activity in mammals is mediated by T cells, which recognise antigen in association with MHC (class I) molecules (Zinkernagol and Doherty, 1975), a process refered to as haplotype restriction. Although the existence of the MHC system (a cluster of closely linked genes, which give rise to a group of cell membrane glycoproteins divided into class I [found on cytotoxic T cells] and class II [found on B cells and antigen presenting cells])) has not been fully characterised in fish (Stet and Egberts, 1991), the authors suggested that these results showed that the specific cellular immune responsiveness to haptenated autologous target cells is mediated by a subpopulation of lymphocytes similar to cytotoxic T cells in mammals.

(i) Memory responses in fish

In mammals, after initial contact with antigen, memory cells are produced which are responsible for a rapid recall of response on second

contact with the antigen. One of the most obvious differences that distinguish a mammalian memory response from a primary response is the logarithmic increase in the concentration of antibody produced (Roitt, 1988). There is also the process of affinity maturation (thought to be associated with increased somatic cell mutation during proliferation) and a switching from mainly IgM type antibody production during the primary response to IgG during the secondary response, known as isotype switching. In fish it was previously assumed that there was a general lack of antibody diversity, which was taken as an explanation for the absence of any evidence of isotype switching in fish (DuPasquier, 1982). It was previously thought that a single isotype of the tetrameric IgM-like immunoglobulin was present in teleosts (Voss et al, 1980; Dorson, 1981), although a second Ig class was found in the skate (Tomonaga et al, 1984, 1985; Tomonaga and Kaboyashi, 1985). However it is now becoming evident that antibody isotype differences also occur in teleost fish. Sanchez et al (1993) produced a battery of 14 monoclonal antibodies which recognised determinants on heavy and light chains of trout immunoglobulins. Experiments using immunoprecipitation and immunostaining of lymphoid cells carried out using these monoclonals, suggest the existence of antigenic variants within the trout immunoglobulin. It was suggested that these variants were isotypes , as they were present in each of 30 sera samples taken from individual rainbow trout. However to date there has been no evidence of isotype switching in either elasmobranchs or teleost fish (Lobb and Olson, 1988; Killie et al, 1991), nor has there been any studies of somatic cell mutation during the secondary response, as is thought to be associated with mammalian antibody isotype switching (Nahm et al, 1992).

Previous reports on evidence for an anamnestic response in teleost fish have produced some rather contradictory results. Ingram and Alexander

(1980) gave brown trout a second I.P. injection of LPS, and found that the maximal titre was reached after 34 to 40 days, instead of 56 to 63 days after a primary injection. In contrast, O'Neill (1980) found a secondary antibody response inferior to the primary response, following immunisation with MS_2 bacteriophage. These results however contradicted his previous findings, when he did obtain a heightened secondary response using the same antigen (O'Neill, 1979). Anderson et al (1982), testing anti-DNP antibody responses in rainbow trout, postulated the existence of memory (measured by PFC response) when the TD antigen DNP-KLH was used, and its absence when the TI antigen DNP-Ficoll was used. Dunier (1985) used the same two antigens and showed that the secondary response was never enhanced when compared to the primary one. Dunier (1986) immunised rainbow trout with DNP-KLH and again found the secondary response was not enhanced compared to the primary, but in contrast, a noticeable secondary response was observed with the bacterial antigen *Yersinia ruckeri* (10^8 formalised cells were injected). In contrast, Anderson and Dixon (1980) failed to show a memory response to the O-antigen of *Yersinia ruckeri* administered by flush exposure. Tatner et al (1987) found no significant memory response in rainbow trout immunised with HGG but a good secondary response was obtained when the fish were immunised with *Aeromonas salmonicida*. Therefore there is no clear evidence for a consistent anamnestic response in teleosts. Some of the variation in results obtained from different studies could be explained by the differences in immunisation schedules and doses of antigen administered. For example Lamers et al (1985) showed that the height of both the primary and secondary response to the bacterin *A.hydrophila* was directly proportional to the dose of antigen given in the priming injection. The length of time between the primary and secondary injections also effected the

height of the secondary response.

One of the most comprehensive studies of memory in fish was carried out by Arkoosh and Kaattari (1991). They found an accelerated *in vivo* antibody response and a higher concentration of antibody activity in the secondary *in vivo* response following TNP-KLH (TD antigen) immunization. They also looked at the B cell precursor frequency and the clone sizes of primed (left for 1 year to rest following priming) and unprimed spleen cells responsive to TNP-LPS, using the technique of limiting dilution analysis. The assay using leukocytes from primed fish, showed an increase in the average number of splenic B cell precursors of 1 precursor in 4324 leukocytes compared to 1 precursor in 61210 leukocytes in unprimed controls, but there was no increase in the B cell clone size as occurs in mammals (reviewed by Nahm et al, 1992). They found that in TNP-KLH immunised fish there was no significant change in the affinity of serum antibodies to TNP, between the primary and secondary responses. However they did find a distinct shift in the fine specificity profiles of the antibodies (to various inhibitor molecules) during the secondary response. Fiebig et al (1977) determined that while only minor intrinsic affinity shifts occured in carp IgM, tremendous functional affinity shifts were noted. Functional affinity is a measure of the avidity of the entire multimeric molecule as opposed to the intrinsic affinity of a single binding site. A small change in the intrinsic affinity of the binding site is greatly magnified when the entire multimeric molecule is considered. Therefore a very small shift in intrinsic affinity could result in a very large increase in functional affinity. Hence in fish, a magnification in functional affinity may occur, instead of isotype switching, by small changes in the intrinsic affinity of the binding sites within the multimeric IgM (Kaattari, 1993). Arkoosh and Kaattari (1991) also looked at the ability of rainbow trout lymphocytes to demonstrate a memory

response *in vitro* to both the TD (TNP-KLH) and TI (TNP-LPS) antigens. An *in vitro* memory reponse to the TI antigen was found after only one *in vivo* priming injection, whereas an *in vitro* memory response to the TD antigen was not observed after one *in vivo* priming injection, but after two priming injections. The authors suggested that these results, taken as a whole, suggest that memory in trout may be due to a simple expansion in the antigen-specific precursor pool, without many of the qualitive changes in antibody or B cell function (affinity maturation, isotype switching, somatic variation, and a considerably greater clonal proliferation) seen in mammalian systems.

(k) Ontogenic development of immunity in fish

There have been several morphological and functional studies describing the ontogeny of the immune system in teleosts (Ellis, 1977b; Grace and Manning, 1980; Manning et al, 1982b; Tatner and Manning, 1983, 1985; Bly, 1985; O'Neill, 1989; Razquin et al, 1990). These have shown that during teleost development, the thymus is the first lymphoid organ to appear and shortly after lymphocytes make their appearance in the kidney followed by the spleen (Grace and Manning, 1980). These observations suggest that the thymus may seed the kidney and spleen with lymphocytes (T cells). Indeed, rainbow trout, which were injected intrathymically with tritiated thymidine showed a migration of radiolabelled cells (lymphocytes) to the spleen and kidney (Tatner, 1985), though the majority went to the spleen. Secombes et al (1983b) showed that mouse anti-carp thymocyte monoclonal antibodies which appear to identify a population of thymocytes in the thymus in carp at 4 days post-fertilization, also label lymphocytes in the pronephric kidney 3 days later. Using another clone of the monoclonal, thymocytes stained positive on day 7 after fertilization, and the pronephros lymphocytes stained positive on day

12 -16 post fertilization, which the authors suggested showed that different surface determinants on thymocytes appear at different times during ontogeny. They suggested that this may reflect the appearance of distinct subpopulations during ontogeny, or the sequential acquisition of differential determinants.

The kidney is thought to be the main antibody-producing organ in fish. It has been shown to contain a generalised haemopoietic tissue rich in lymphocytes and plasma cell (Ellis, 1977b). The kidney of fish differentiates ontogenetically in two phases. The first to develop is the pronephros, or head kidney, which loses its excretory function as the opisthonephros, which contains the excretory tubules, develops. Both parts of the kidney contain a generalised haemopoetic tissue rich in lymphoid cells and granulocytes (Ellis, 1976), which it is suggested resembles the bone marrow in many respects (Zapata, 1980). However, unlike bone marrow, the kidney tissue also contains many antibody producing cells and phagocytes (Rijkers et al, 1980a) and following antigenic stimulation many pyronophilic cells appear, often in large clusters (Secombes, 1981). These authors suggested that the kidney tissue performs functions associated with both mammalian bone marrow and lymph nodes.

It has been shown that in salmon, rainbow trout and the antartic teleost, *Harpagifer antarticus* , the spleen is the last organ to become lymphoid during ontogeny and develops a lymphoid population about the time of first feeding (Ellis, 1977a; van Loon et al, 1981; Grace Manning, 1980; O'Neill, 1989). The spleen contains fewer haemopoietic and lymphoid cells than the kidney, being composed mainly of blood held in sinuses (Ellis, 1977a). However it has been shown that it contains specialised capillary walls called ellipsoids which are composed of reticulum fibres and macrophages (Ellis et al, 1976). The latter are highly phagocytic while the reticulin fibres

network is involved in trapping immune complexes (Ellis, 1980). The spleen also contains some antibody-producing cells (Rijkers et al, 1980a) and following antigen stimulation, clusters of pyroninophilic cells appear in the ellipsoid walls. Observations suggest that these clusters may develop into melanomacrophage centres (Secombes, 1981).

As has been mentioned, there seems to be increasing evidence through the use of monoclonal antibody analysis, that anti-IgM monoclonals mark a subpopulation of lymphocytes which show B cell like properties (Egberts et al, 1983; DeLuca et al, 1983; Thuvander et al, 1990). There have been a few studies which have looked at the ontogenic appearance of anti-IgM positive cells (van Loon et al, 1981; Razquin et al, 1990). Razquin et al (1990) looked at the ontogenic appearance of IgM-producing cells in the lymphoid organs of the rainbow trout using anti-trout IgM monoclonal antibodies and lymphocyte enzyme marker techniques. They found that the first IgM positive cells occurred at days 4-5 post-hatching in the renal lympho-haemopoeitic tissue. By 1 month after hatching IgM positive cells also appeared in the spleen and thymus. This agrees with the findings of Secombes et al (1983b) who found that in carp IgM positive cells appeared in the spleen and thymus one month after hatching. More recently Castillo et al (1993) looked at the ontogeny of IgM and IgM-bearing cells in the rainbow trout. They found that lymphocytes showing cytoplasmic IgM were first observed in embryos at 12 days before hatching, but cells positive for surface IgM were not observed until 8 days before hatching. They also found that unfertilized trout eggs contained detectable amounts of IgM, indicating that transfer of IgM from mother to embryo can occur in salmonids.

It should be noted however that the presence of a lymphoid organ does not necessarily mean that the fish is immunologically mature, therefore

these histological observations have to be compared with studies on the ontogenic appearance of certain immunological functions. Work has been done on the ontogenic appearance of mixed leukocyte reactivity (MLR; Ellis, 1977b), allograft rejection (Botham et al, 1980; Botham and Manning, 1981;Tatner and Manning, 1983), and humoral antibody production (Paterson and Fryer, 1974; Tatner, 1986).

Tatner and Manning (1983) looked at the stage which rainbow trout became immunocompetent with respect to allograft rejection. They showed that fry became immunocompetent with respect to the allograft response as early as 14 days post hatch. The ability to respond was found to be correlated with the presence of a morphologically mature lymphoid population in the thymus and the kidney, and the presence of circulating lymphocytes. Botham et al (1980) showed that adult rainbow trout rejected skin grafts in 19-21 days. First-set grafts were invaded by large numbers of lymphocytes on day 5 post grafting followed by maximal infiltration on day 9. However in 26 day old trout fry, lymphocytes were not present in the graft before 7 days, reaching a maximum at day 12. However, the total range of rejection times fell within that of adults. So a cellular immune response appeared to be present in these very young fish.

Several studies have been performed which provide evidence that fish, at an early stage in their development can mount a humoral immune response to various antigens (Paterson and Fryer , 1974; Tatner, 1986). However in some cases tolerance induction has been reported against antigens which are thymus dependent. van Loon et al (1981) showed that in carp, tolerance could be induced to sheep red blood cells, which are considered a TD antigen in mammals. Etlinger et al (1979) showed that in rainbow trout, the ability to respond to TI antigens preceded that to TD

antigens. In these studies the antigen was injected. Tatner (1986) looked at the ontogeny of amoral immunity in rainbow trout fry using a TI (*Aeromonas salmonicida*) and TD (human gamma globulin; HGG) antigen given by immersion, at known ages/weights from 7 days post hatch, and at 1, 2,3 and 4 months post hatch. The results indicated that the fry were capable of mounting a humoral response very early in ontogeny. There was a period of unresponsiveness which lasted for longer periods against HGG than against *A.salmonicida*, though the author suggested that this was not tolerance as such. Memory was detected to HGG in fry given a first immunisation at 2 months. These results were then compared with experiments in which the fry were first thymectomised 4 weeks before the first immunisation. In fry thymectomised at one week post hatch, and tested for primary and secondary responses at 2 and 3 months, the primary response to HGG was unaltered, but the secondary response was reduced. Both the primary and secondary responses to *A.salmonicida* were unaltered. When thymectomy was performed later, the earlier effect on the secondary response to HGG was no longer seen, but the primary response to *A.salmonicida* was slightly lower. These results provided evidence for the presence of T helper and suppressor cells in trout.

(I) Temperature effects on the immune response in fish

Numerous external factors have been shown to effect the immune response of fish such as temperature (Bly and Clem, 1992), stress (Maule and Schreck, 1990; Peters et al, 1991) and dietary additives such as vitamin E (Hardie et al, 1990). The best studied modulator of the fish immune response has been temperature. Temperature has been shown to play an important role in fish immune responses both *in vivo* and *in vitro* (Avtalion et al, 1980;Rijkers

et al, 1980a; van Ginkel et al, 1985). Most warm water fish species have optimal immune responses at temperatures between 20 and 30°C, whereas cold water species respond best at temperatures between 10 and 15°C (Avtalion, 1969, van Ginkel et al, 1985). It is also generally accepted that higher environmental temperatures or higher *in vitro* assay temperatures, within the physiological range, enhance immune responses i.e. the production of antibody is faster and of a higher magnitude (Rijkers et al, 1980a; Ellis, 1982; Secombes et al, 1991), and that lower environmental and *in vitro* assay temperatures tend to slow down the immune responses. Studies on the effects of temperature on the mitogenic responses of channel catfish sIg+ (B cells) and sIg- (T cells) showed that the two subpopulations were differentially affected by both *in vitro* and *in vivo* temperatures (Clem et al, 1984). On the one hand, the magnitude of T cell responses to Con A were significantly suppressed, although this suppression could be partly abrogated by appropriate *in vivo* acclimation to lower temperatures. On the other hand the magnitude of the B cell proliferative responses to LPS was relatively independent of both *in vitro* culture temperature and *in vivo* acclimation temperature. Further *in vitro* temperature shift experiments indicated that catfish T cell responses to Con A could be obtained at nonpermissive temperatures following an initial (~18 hours) *in vitro* exposure to Con A at permissive temperatures. The converse was not true in that proliferative responses at permissive temperatures were not seen after initial stimulation with Con A at nonpermissive temperatures. Miller and Clem (1984b) looked at the effects of temperature variations on the secondary *in vitro* PFC response to TD (TNP-KLH) and TI (TNP-LPS) antigens. They found that the response to TD, but not TI, antigens was suppressed at nonpermissive temperatures. Furthermore, it was observed that this suppression of TD responses could be

abrogated by appropriate *in vivo* acclimation of the donor fish at low temperature. These results suggest that low temperature suppression was occuring in the sIg- (T cell) population only. As mentioned earlier in this review the work of Bly et al (1990) and Clem et al (1991) suggests that this suppression of T cell responses at nonpermissive temperatures could be due to a reduction in the cell membrane fluidity of T cells at low temperatures, due to the low number of 18:1 PUFA in the T cell membrane when compared to the B cell membrane. Again this work provides further evidence for at least two (sIg+ and sIg-) lymphocyte populations in fish.

Therefore it can be seen that there is an extensive literature on fish lymphocyte responses. One of the major questions in comparative immunology today is the level at which the dichotomy of the immune system into a thymus dependent and thymus independent system occurred in vertebrate immunity. It can be seen, from the work mentioned here, that there is at present fairly strong evidence that teleost fish exhibit at least a degree of lymphocyte heterogeniety, based on the subdivision into sIg+ (B cells) and sIg- (T cells). However there is less evidence available to suggest that the teleost T cell population is divided into separable helper/suppressor subpopulations as in the CD4/CD8 system in mammals (Bierer, 1989). It is one of the major aims of this study to provide further evidence for the existence of not only T and B cells, but also the existence of suppressor and helper subsets of T cells, in the teleost fish, the rainbow trout.

Chapter 2

Materials and methods:

2.1. Maintenance of experimental fish:

All fish were maintained in 400 litre fiber glass tanks located in the departmental aquarium. The tanks were maintained with a constant throughflow of water, which was filtered and dechlorinated by a Elgamat AC-4 carbon filter. Unless otherwise stated, the water was maintained at ambient environmental temperature and a flow rate of 10-15 litres min^{-1}. The fish were maintained on a daily diet of EWOS (Livingstone, Scotland) trout pellets.

2.2. Leukocyte Isolation:

Rainbow trout leukocytes were separated from whole blood, spleen, kidney and thymus using a development of the method described by Boyum (1968) for the isolation of mammalian mononuclear cells from circulating blood and bone marrow. Briefly, one ml of blood was removed from the caudal vein of the trout using a 25 gauge needle and 1 ml syringe, and diluted 1:5 in calcium and magnesium free hanks balanced salt solution (CMF-HBSS; Sigma, Dorset, UK) containing 20iu of heparin (Sigma). The anterior kidney, spleen and thymus were gently pressed through a nylon mesh into 5 ml of CMF-HBSS, and the samples were spun at 600g for 10 mins, at 4°C. The supernatants were discarded and the pellets resuspended in 5 mls of CMF-HBSS containing heparin. The diluted blood and organ suspensions were then carefully layered onto 7ml of lymphocyte separation medium (LSM; Flow Labs, Herts, UK) and centrifuged at x1100g for 40mins, at 4°C. The buffy coat layer at the medium-LSM interface, containing the leukocytes was carefully removed using a sterile Pasteur pipete. The cells were then washed three times in CMF-HBSS, before being prepared and resuspended at the appropriate concentration for experimental use. Separation of the leukocytes from the blood and lymphoid organs using this method produced a

heterogeneous mixture of all the major types of leukocytes.

2.3 Leukocyte cell culture:

The separated leukocytes were cultured in Leibovitz L-15 medium (Sigma; with sodium bicarbonate) containing 5% heat inactivated foetal bovine serum (FBS; Sigma) and supplemented with penicillin/streptomycin (100 units of penicillin and 50ug of streptomycin per ml of medium; Sigma) and L-glutamine (2mM; Sigma). No Hepes buffer was added to the medium as this was found to adversely effect rainbow trout leukocyte viability. The cell cultures were incubated in an ordinary air mixture, in airtight and sterile moist chambers at 15°C. The numbers of leukocytes in the cultures was determined using a haemocytometer as described by Doyle and Morris (1991), and the viability of the cells was assessed using the trypan blue exclusion test (Mishell and Shiigi, 1980). Only cells with a viability of >95% were used in culture experiments. The cell number per ml of culture medium varied depending on the experimental use i.e. mitogen assays ($5x10^6$ ml^{-1}), plaque assays ($1x10^6$ ml^{-1}).

2.4 Nylon wool separation:

This technique was based on the method described by Trizio and Cudkowiez (1974). All incubations were carried out at 20°C using 20ml syringes packed with 0.6g of nylon wool (Polysciences Inc, Warrington, USA). The non-adherent cells, passing through the column, were collected into sterile petri dishes. The adherent cell population was recovered by adding ice cold HBSS to the column and subsequent collection into sterile petri dishes (Sterilin Ltd, Hounslow, UK). Cell suspensions were washed three times in

HBSS and resuspended in L-15/5% FBS at a concentration of $5x10^6$ cells/ml.

2.5 Separation of Lymphocytes into surface Ig+ and sIg- populations using the panning technique:

This was based on the method described for separation of mammalian lymphocyte subpopulations (Mage et al, 1977), and developed for separation of teleost lymphocytes (Sizemore et al, 1984; Graham and Secombes, 1990). Six well petri dishes were coated overnight at 4°C, with 8 ml of monoclonal anti-trout IgM ascites fluid (this clone reacts only with the heavy chain of trout serum IgM), diluted 1:200 in phosphate buffered saline pH7.4 (PBS; appendix 1) containing 5% FBS. The anti-trout IgM ascites fluid was a gift from Dr C.J. Secombes, Aberdeen. The plates were then washed twice in sterile PBS, and 8 ml of L-15/10% FBS added to each well. The plates were incubated at room temperature for 1 hour. The separated leukocytes (section 2.3.) were then panned twice for 1 hour, at $1x10^7$ cells per well. After removal of the non-adherent cells (sIg-) from the coated plates, an intermediate wash with L-15 was carried out before removal of the adherent cells (sIg+) by vigorously pipetting medium into the wells.The two batches of sIg+ lymphocytes were pooled before use.

2.6. Separation of Rainbow trout Leukocytes using Lectins

Two lectins were used to separate rainbow trout leukocytes: soybean agglutinin (SBA) was used to separate rainbow trout thymus, spleen, kidney and peripheral blood leukocytes into two populations, and peanut agglutinin (PNA) was used to separate thymocytes into two main populations.

2.6.1. Soybean Agglutinin separation:

Soybean agglutunin (SBA) separation of rainbow trout leukocytes was

developed from the method described by Reisner et al (1976) for the separation of mouse T and B lymphocytes. Briefly, a single cell leukocyte suspension was prepared (as in section 2.2) in HBSS of approximately $5x10^7$ cells ml^{-1}. Soybean agglutinin was used at a concentration of $2mg/ml^{-1}$ in HBSS. A one ml aliquot of the leukocyte suspension was then mixed with one ml of the SBA solution and incubated at 20°C for 30 mins. This mixture was then carefully layered onto 20ml of HBSS containing 2% Bovine Serum Alubumin (BSA; Sigma) and incubated at 20°C for 45 mins. The agglutinated cells fall to the bottom of the column and the unagglutinated cells remained at the interface at the top of the column. Subsequently, the unagglutinated and agglutinated cells were aspirated using a pasteur pipette and incubated for 10-15 mins in 20 ml of 0.2M D-galactose (Sigma) in HBSS. The cells were then washed three times using large volumes of HBSS/2% foetal bovine serum (FBS) and resuspended in L-15/5% FBS. The viability of the cell subpopulations was subsequently checked using the trypan blue exclusion test (Mishell and Shiigi, 1980).

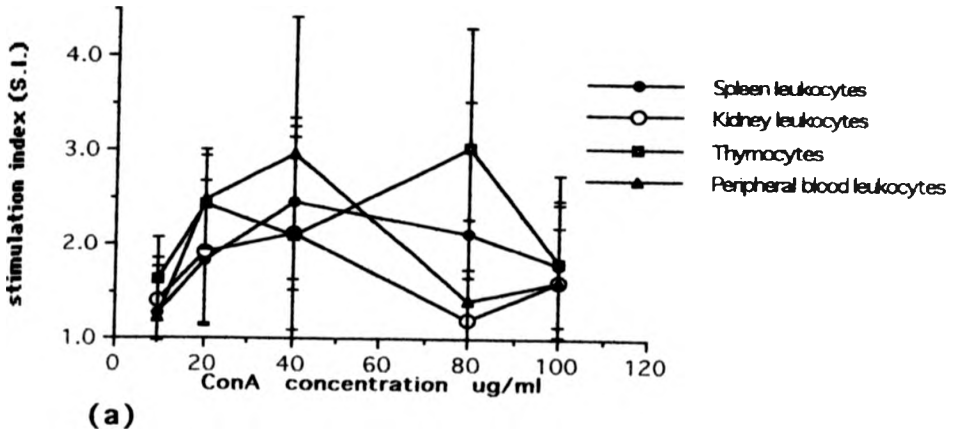
2.6.2. Peanut agglutinin separation:

This was based on the method described by Reisner et al (1976). A single cell thymocyte suspension of approximately $5x10^7$ cells ml^{-1} was prepared. The PNA (Sigma) was resuspended in HBSS at a concentration of 1mg/ml. A one ml aliquot of the thymocyte suspension was added to one ml of the PNA solution and incubated at 20°C for 45 mins. The agglutinated cells fell to the bottom of the column and the unagglutinated cells remained at the interface at the top of the column. A suspension of the agglutinated and non-agglutinated cells was incubated at 20°C for 10-15 mins in 20ml of 0.2M D-galactose (in HBSS). Subsequently the cells were washed three times in

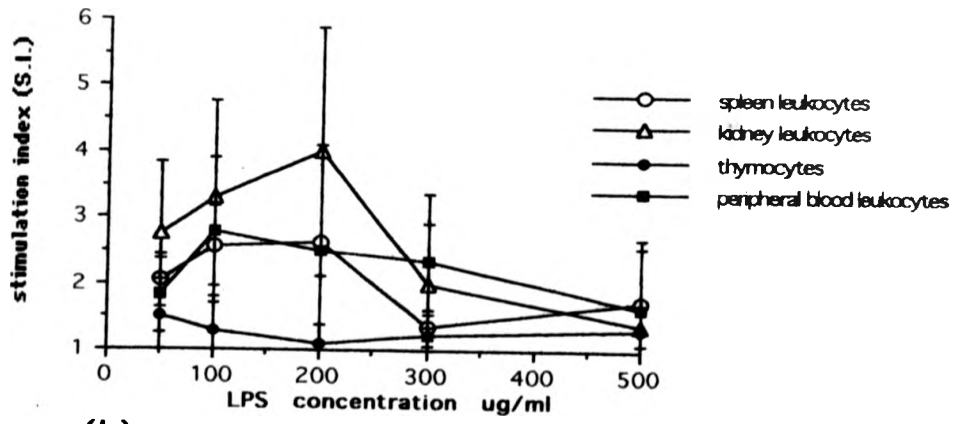
HBSS/2% FBS and resuspended in L-15/5% FBS at a concentration of $5x10^6$ cells ml^{-1}. The viability was checked as before.

2.7 Mitogen assays:

Mitogen assays were carried out according to the methods described by Tillitt et al (1988) and Reitan and Thuvander (1991), with some variations. Concanavalin A (ConA from *Canavalia ensiformis* : Sigma) and Lipopolysaccaride (LPS from *E.coli* 0111:B4 : Sigma) were the mitogens used. They were reconstituted using HBSS and then diluted to the optimal concentrations of 40ug/ml-1 for ConA (Fig 2.1) and 200ug/ml-1 for LPS (Fig 2.2), in L-15/5% FBS containing penicillin/streptomycin (100 units penicillin and 50iu streptomycin per ml of medium) and L-glutamine (2mM). One hundred ul aliqouts of the leukocyte cell suspensions were added to each well of a 96 well round bottomed microtitre plate (Nunclon, Paisley, UK), followed by 100ul of either ConA, LPS or L-15/5% FBS diluent only, giving a final volume of 200ul/well. The plates were incubated at 15°C in moist chambers for 72 hours, followed by addition of ^{3}H-thymidine (Amersham, Bucks, UK: 0.5uCi per well). After 24 hours the contents of each well were harvested onto filter paper discs (Skatron, Berks, UK) using a Titertex cell harvester (Flow Laboratories, Herts, UK), and prepared for scintillation counting. The filter discs were placed in 5ml vials (Canberra-Packard, Pangbourne, UK) with 300ul of Soluene-350 (Canberra-Packard) and incubated at 60°C for 1 hour. The vials were allowed to cool down to room temperature before 2ml of Ecoscint scintillation fluid (Canberra-Packard) was added. The rate of ^{3}H-thymidine uptake was assayed using a scintillation counter (Canberra-Packard, Tricarb). Mitogen assays were performed using triplicate wells for each mitogen. The



(a)



(b)

Fig 2.1: *In vitro* stimulation of rainbow trout leukocytes with various concentrations of ConA (from *Canavalia ensiformis* ; Fig 2.1(a)) and LPS (from *E.coli* 0111:B4; Fig 2.1(b)). The responses are given as stimulation indexes (S.I.). Each point represents the mean (+ standard deviation) of three fish.

degree of stimulation for each mitogen was calculated using the stimulation index (S.I.):

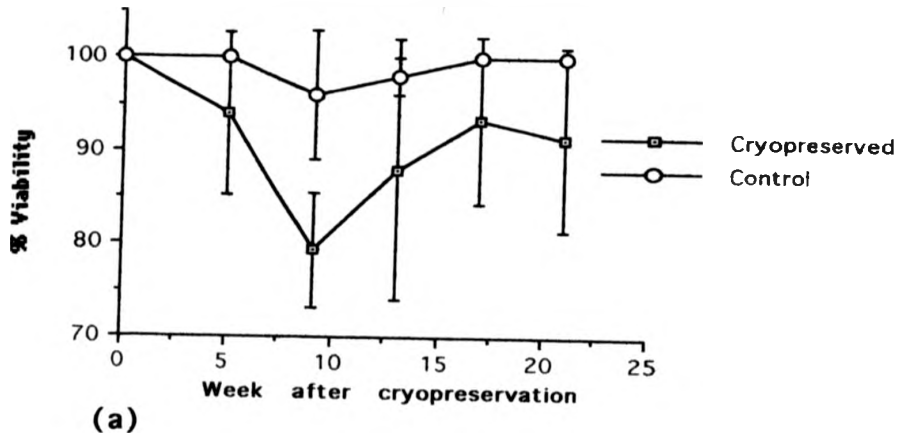
$$S.I. = \frac{\text{mean counts per minute for stimulated cultures}}{\text{mean counts per minute for unstimulated cultures}}$$

2.8 Cryopreservation of rainbow trout leukocytes:

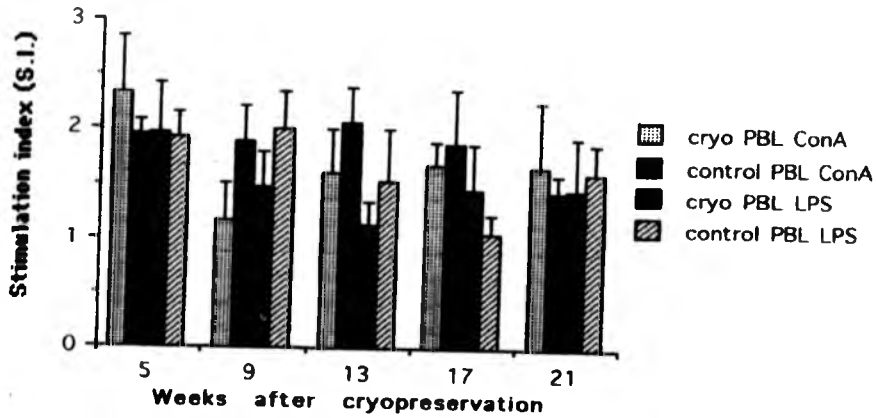
The essential requirements for efficient cryopreservation of animal cells are a slow freezing rate i.e. a rate between 1°C and 3°C per minute, and fast thawing, which is usually achieved by placing the ampoules in a water bath at the required temperature . The techniques used for mammalian lymphocytes (Cachiero et al, 1985; Ichino and Ishikawa, 1985) were modified slightly for use with fish (Tatner and Findlay, 1992). In this instance the modified technique was used to cryopreserve peripheral blood leukocytes after separation from whole blood samples taken from the caudal vein, although the same protocol would be used for cryopreservation of splenocytes, thymocytes and anterior kidney leukocytes.

2.8.1. Cryopreservation of peripheral blood leukocytes.

The peripheral blood leukocytes were separated from whole blood samples as described (2.2). Viable cell counts were performed using the trypan blue exclusion assay, as outlined by Mishell and Shiigi (1980). It is important to note that only healthy cell populations were cryopreserved i.e. populations where the cell viability was >95%. The separated leukocytes were then frozen as described by Tatner and Findlay (1992). Briefly, after washing three times in Hanks balanced salt solution (Sigma) the cells were resuspended in 50% Leibovitz L-15 medium/ 50% heat inactivated foetal bovine serum (FBS,Sigma) to $5x10^7$ cells ml^{-1}, in cryovials (Greiner labs, UK). These were kept on ice until addition of an equal volume of 40% L-15/ 10%



(a)



(b)

Fig 2.2: The effect of cryopreservation on the viability (a) and mitogenic responses (b) of peripheral blood leukocytes. The viability was measured using the trypan blue exclusion test. The mitogens used were ConcanavalinA (ConA, at a concentration of 40ug ml^{-1}) and Lipopolysaccaride (LPS, at a concentration of 100ug ml^{-1}).

dimethlysulphoxide (DMSO, Sigma)/ 50% FBS giving a final cell concentration of $2.5x10^7$ ml^{-1}. The vials were then immediately put into the programmable freezing unit (Planer Biomed Kryo 10 Series Controller Model 10-20, Planer, UK) and frozen at a rate of -2°C per minute to 2.0°C, then -1°C per minute to -55°C and finally -5°C per minute down to -80°C. At this point the cryovials were immediately transfered to a dedicated liquid nitrogen storage (Gallenkamp) vessel.

2.8.2 Thawing of cryopreserved leukocytes:

At various time intervals after cryopreservation, the vials containing the peripheral blood leukocytes were removed from the liquid nitrogen storage vessel and placed in a 22°C water bath to facilitate rapid thawing. Just as the last ice crystals were melting, the ampoule was wiped with a tissue soaked in ethanol and the contents transfered to a 15 ml centrifuge tube using a sterile Pasteur pipette. Two mls of L-15/5%FBS was added slowly, using a Pasteur pipette, and the tube spun x400g. This part of the procedure was carried out in a sterile laminar flow cabinet. The cell pellet was then resuspended in 8ml of L-15/5% FBS and washed two times in the same mixture. Cell viabilty counts and mitogen assays (section 2.5) were then carried out on the thawed peripheral blood leukocytes (Fig 2.2). The results show that there is no loss of viability or function of the cryopreserved peripheral blood leukocytes when compared with their matched controls, for up to 21 weeks in liquid nitrogen storage. Thymocytes, kidney leukocytes and spleen leukocytes were all cryopreserved using the same protocol.

2.9. Development of Enzyme-Linked Immunosorbent Assays (ELISA) for the detection of antibodies to DNP-KLH, sheep red blood cells (SRBC) and *Aeromonas salmonicida*, in rainbow trout serum samples.

The antibody activity of the serum samples from fish immunised with DNP-HKLH, SRBC and *A.salmonicida* was determined using three ELISA assays, each of which was based on the procedure described by Arkoosh and Kaattari (1990) for quantification of fish antibody to a specific antigen.

2.9.1. Development of ELISA to quantify serum antibody titres to *A.salmonicida*

a) *Aeromonas salmonicida* 1102 (a non-virulent non-auto agglutinating strain) was grown in brain-heart infusion broth (BHIB; Gibco LTD, Paisley, Scotland) for 24 hours in a shaking incubator at 20°C. Formalin (0.05% of v/v 50%) was added for 24 hours, before the bacteria were washed three times in 0.85% saline to remove any traces of formalin and resuspended in PBS pH 7.4 (appendix 1). The absorbance of the bacterial suspension was determined spectrophotometrically at 610nm with an LKB Ultraspec II spectrophotometer, and the absorbance adjusted to 0.75, which was shown to correspond to $1x10^8$ bacterial cells ml^{-1} (Fig 2.3) using a standard curve prepared previously using the plate count method (Collins and Lyne, 1976).

b) ELISA plates (medium binding; Dynatech labs, Sussex, UK) were pre-coated with Poly-L-Lysine (10ug/ml in PBS; Sigma) at 50ul $well^{-1}$ for 2 hours at room temperature. The plates were then washed three times in low salt wash (appendix 2) and 50ul of $1x10^7$ cells ml^{-1} *A.salmonicida* 1102 was added to each well. This was shown to be the optimal bacterial coating concentration (Fig 2.4)The plate was incubated at room temperature for 45

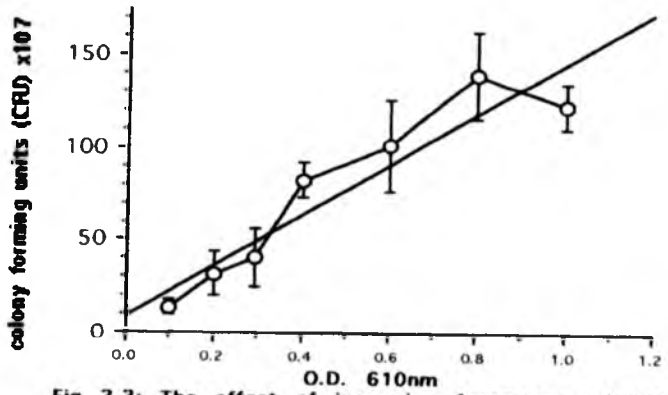


Fig 2.3: The effect of increasing Aeromonas salmonicida cell number (suspended in 0.85% saline) on the optical density read at 610nm. This was constructed using the plate count method. Each point represents the mean CFU from 3 agar plates.

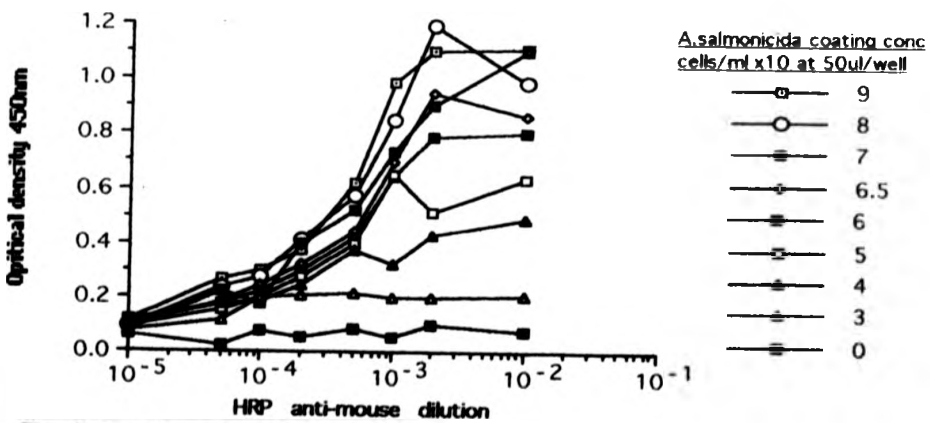


Fig 2.4 : Determination of the optimal coating concentration of A.salmonicida 1102 antigen for ELISA assay.

minutes, then 25ul of 0.25% gluteraldehyde (Sigma) was added to each well, without removing the supernatant, for 10 mins at room temp.

c) The plates were washed three times in low salt wash and 200ul well^{-1} of 5% Marvel dessicated milk, in PBS pH7.4 containing 0.05% NaN_3, was added and the plates incubated at 4°C overnight. The plates were then washed three times in low salt wash, and 50ul of PBS pH7.4 was added to each well. The trout anti-*A.salmonicida* sera was added to the top well of each row (50ul), and serially diluted down the plate. The trout sera was left on the plate for 3 hours at 15°C.

d) The plates were then washed three times in low salt wash, followed by addition of 50 ul of undiluted mouse anti-trout IgM monoclonal antibody culture supernatant (Thuvander et al, 1990; this clone reacts only with the heavy chain of trout serum IgM) to each well. The plate was incubated at room temperature for one hour.

e) The plate was washed three times in low salt wash, followed by the addition of HRP anti-mouse IgG whole molecule polyclonal antibody (Scottish Antibody Production Unit: SAPU), at 50 ul per well. The HRP anti-mouse conjugate was diluted 1:1000 in PBS/5% FBS, which was shown to be the optimal conjugate dilution (Fig 2.5). The plate was then incubated at room temperature for 1 hour.

f) The plate was washed three times in high salt wash (appendix 2), followed by the addition of 50ul of substrate to each well. The TMB dihydrochloride/Citric acid substrate buffer system was used (appendix 2). The substrate was allowed to develop for 15 mins at room temperature before the reaction was stopped by addition of 25ul of 2M H_2SO_4 stop solution per well. The optical density of each well was read using an MR5000 ELISA plate reader (Dynatech, Sussex, UK) at 450nm.

g) The negative controls within the assay included omission of fish sera, anti-

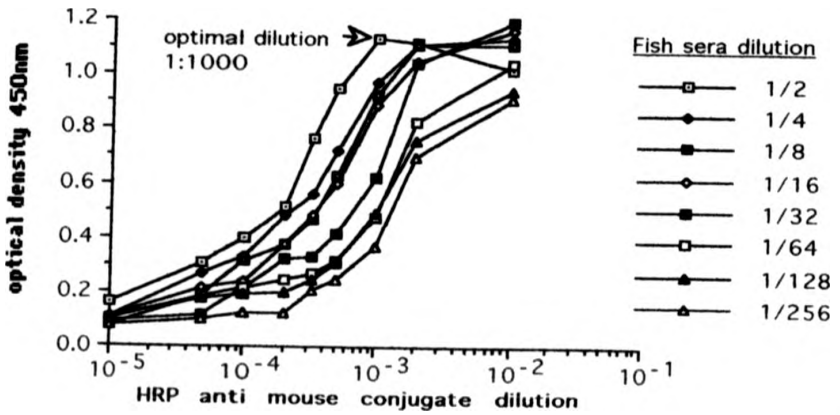


Fig 2.5 : Determination of the optimal HRP anti-mouse IgG conjugate dilution in the A.salmonicida ELISA assay

trout IgM monoclonal or HRP conjugate. Every time the assay was run a well incubated with unimmunised trout sera was included to check that the background level remained constant. The endpoint of the ELISA was taken as 0.15 OD units.

2.9.2. Development of an ELISA to quantify serum antibody activity against SRBC.

The SRBC ELISA was carried out using the same basic protocol as described for the *A.salmonicida* assay. Plates were prepared as above, and coated with 50ul per well of 0.5% SRBC (SAPU) in PBS pH 7.4, which was found to be the optimal coating concentration (Fig 2.6) using a checkerboard ELISA as described by Campbell (1984). The fish sera and mouse anti-trout monoclonal antibody were added to the plate as above. In this case however an alkaline phosphatase anti-mouse IgG whole molecule monoclonal antibody conjugate (Sigma) was used, instead of the HRP conjugate, to overcome the problem of endogenous peroxidase activity in the SRBC. This was added at 50 ul per well, at a dilution of 1:1000 in PBS/5% FBS (Fig 2.7), for one hour at room temperature. After washing in high salt wash, 50ul of *p*-nitrophenyl-phosphate in diethanolamine buffer (Dynatech Labs; appendix 2) substrate solution was added, and left for 25 mins at room temperature, which was found to be the optimal incubation period (Fig 2.7.). The optical density was then read at 405nm. The endpoint and negative controls were as in the *A.salmonicida* assay.

2.9.3. Development of an ELISA to quantify serum antibody activity against DNP-KLH.

As DNP-KLH is a soluble antigen the protocol was different from the two cellular assays above. DNP-BSA (Calbiochem, Lajolla, USA; 44 DNP/molecule BSA) was dissolved in bicarbonate coating buffer (appendix 2)

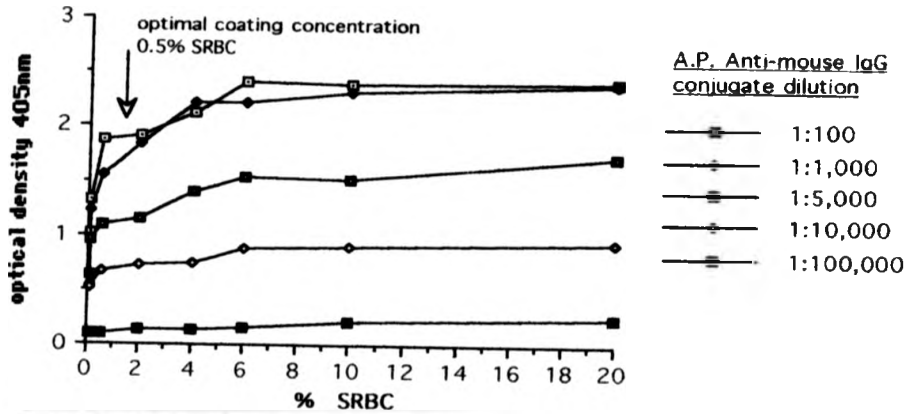


Fig 2.6: Determination of optimal SRBC coating concentration in the SRBC ELISA (fish sera diluted 1/2)

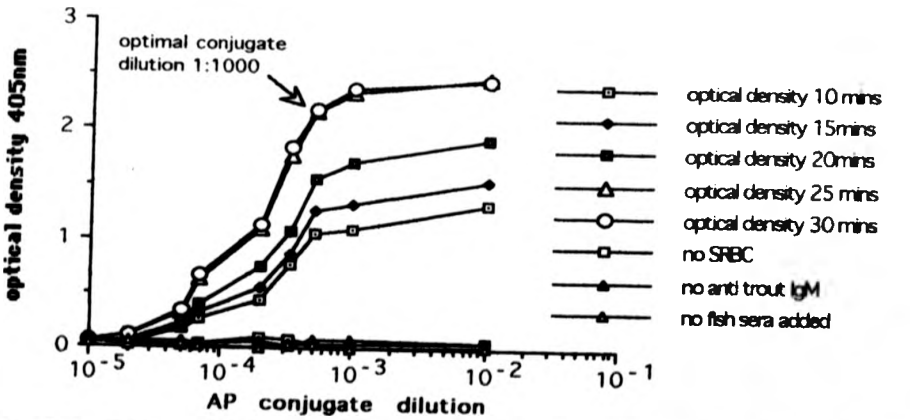


Fig 2.7: Determination of optimal alkaline phosphatase anti mouse IgG conjugate dilution in the anti-SRBC ELISA (fish sera diluted 1/2)

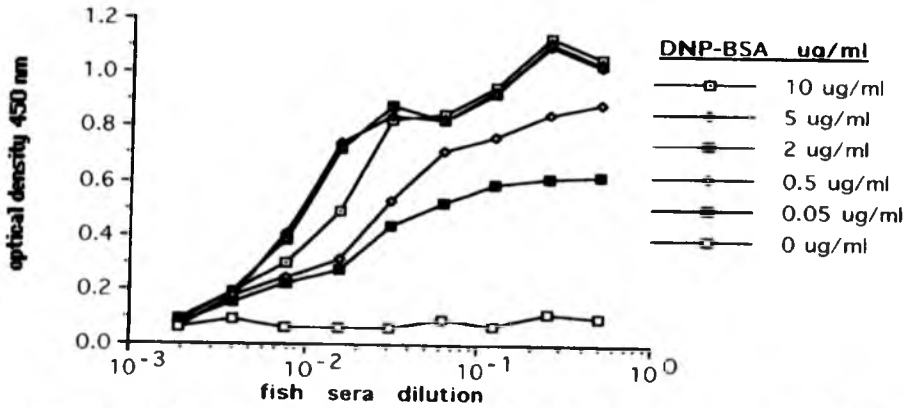


Fig 2.8: The optimization of DNP-BSA coating concebtration in the DNP-KLH ELISA (HRP anti-mouse IgG used at 1:1000)

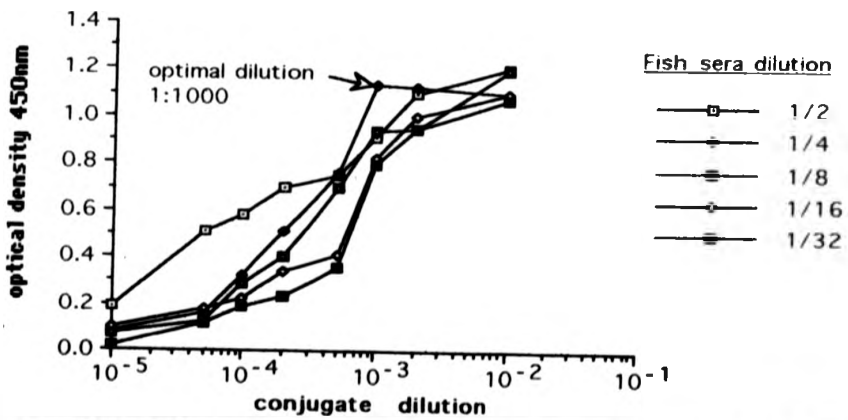
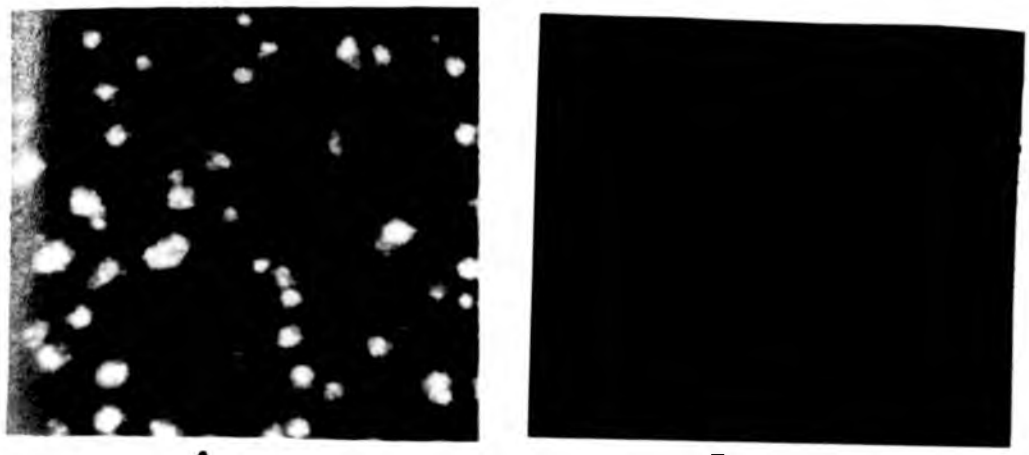


Fig 2.9: Optimization of HRP anti-mouse IgG conjugate dilution in the DNP-KLH ELISA.



A B

Plate 2.1: Haemolytic plaques obtained using the microwell assay. A trout was immunised with 0.1ml of 5% SRBC in 0.85% saline, mixed with an equal volume of Freunds complete adjuvant, eight weeks before *in vitro* transfer of the spleen leukocytes, at a concentration of $5x10^6$ cells ml^{-1} in L-15/5% foetal calf serum, at 200ul per well. After five days in culture the spleen leukocytes were assayed for haemolytic plaques using 5%SRBC/10% complement as described in the text. A-represents $1x10^6$ spleen cells assayed with 5% SRBC/10% complement. The plaques,which are clearly visible, vary in size depending on the rate of secretion by the antibody forming cell. At this cell concentration the plaques begin to overlap and individual plaques become difficult to distinguish. Therefore for use in the *in vitro* thymus-spleen cocultures (Chapter 6) a cell concentration of $2.5x10^5$ cells per well was used. B-represents $1x10^6$ spleen cells assayed with 5% SRBC without complement.

to a concentration of 2ug/ml-1, which was found to be the optimal coating concentration, using a checkerboard ELISA (Fig 2.8). The plate was left at 4°C overnight. The plate was washed three times in low salt wash, and the plate was then processed with the fish sera and mouse anti-trout IgM monoclonal as in the *A.salmonicida* assay. The HRP anti mouse IgG conjugate system was used, at an optimal dilution of 1:1000 in PBS/5% FBS (Fig 2.9). The plate was processed as in the *A.salmonicida* assay. The end point was taken as five times the background level (optical density reading obtained when the fish sera was omitted), and controls were the same as in the SRBC and *A.salmonicida* assays.

2.10. Optimisation of the microwell haemolytic plaque assay using kidney, spleen and peripheral blood leukocytes from adult rainbow trout injected with sheep red blood cells (SRBC).

The microwell technique of Kappler (1974) was optimized for use with rainbow trout leukocytes. Kidney, spleen and peripheral blood leukocytes were removed from adult rainbow trout as described in section 2.2. The cells were adjusted to $1.25x10^5$, $1.25x10^6$ and $2.5x10^6$ cells ml^{-1} in L-15/5% foetal bovine serum and aliquoted at 200ul $well^{-1}$ into 96 well flat bottomed microtitre plates (Gallenkamp, Loughborough, UK), giving *in vitro* concentrations of $2.5x10^4$, $2.5x10^5$ and $5x10^5$ cells $well^{-1}$. The plate was cultured overnight at 15°C, and the next day plaque assays were set up as follows:

1) SRBC (Scottish Antibody Production Unit, Carluke, UK) were washed three times in Hanks balanced salt solution (HBSS, Sigma) and resuspended to 5%. A mixture of 5% SRBC and either 2%, 5%, 10% or 20% complement solution was prepared. The complement was obtained by collecting serum from non-

immunised adult rainbow trout, which was absorbed against SRBC before use (see section 6).

2) The plates containing the leukocytes were centrifiged at 250g for 10 mins at 4°C (MSE chilled centrifuge), at an angle so that the cells pelleted at the sides of the wells. The plates were then inverted and given a brisk shake to expell the culture medium. The plates were very gently shaken on a vortex mixer to redistribute the leukocytes.

3) The SRBC/complement mixture was dispensed at 100ul well^{-1} into the microtitre plates and the contents gently mixed on a vortex mixer. The trays were then incubated at 4°C for one hour to allow the cells to settle and form a monolayer.

4) The plates were then incubated in a moist box at 4°C, 15°C or 20°C for four hours to allow the plaques to develop. The plaques were examined using an inverted microscope.

5) The plaques appeared as clear spots in the red blood cell background (Plate 2.1) and were enumerated, under a dissecting microscope at x10 magnification, and expressed as the number of plaques per $2.5x10^5$ leukocytes.

6) The complement used in the assays was absorbed against SRBC to remove naturally ocurring antibodies in rainbow trout serum to SRBC, otherwise general lysis would occur (Mishell and Shiigi, 1980). The complement used throughout consisted of non-pooled serum obtained from periodic bleeding of adult rainbow trout (500g) over a period of weeks, and stored at -70°C. The serum was absorbed as follows:

i) A volume of SRBC, equal to 1/3-1/2 of the complement to be absorbed, was removed directly from the suppliers bottle and washed three times in

HBSS.

ii) After the last wash the serum was poured over the packed SRBC and the contents very gently mixed. The suspension was then transfered to an autoclaved glass flask which was buried up to the neck in ice. The mixture was incubated on ice for thirty minutes, with periodic turning to ensure the cells remained in suspension.

iii) The cell suspension was transfered into a centrifuge tube and spun at 400g for ten minutes at 4°C. The serum was then removed and the centrifugation repeated to remove any remaining SRBC. The serum was then stored at -70°C in 0.5 ml aliquots.

7) The effects of the different complement concentrations and incubation temperatures are shown in Tables 2.1, 2.2 and 2.3. Table 2.1 shows that by increasing the temperature of incubation of the spleen leukocyte/SRBC/ complement mixture from 4°C up to15°C there is a general increase in the number of plaque forming cells (PFC), and by increasing the temp from 15°C up to 20°C there is a slight increase in the number of PFC or it remains much the same. When the complement concentration was increased from 2% up to 10% there was a general increase in the number of PFC, but from 10% up to 20% there was only a very slight increase, or in some cases a decrease in the number of PFC. Table 2.2 shows that when kidney leukocytes were used, 15°C again appears to be the optimal temperature of incubation. Increasing the complement concentration up to 10% increased the number of PFC. Increasing the complement concentration up to 20% does not significantly increase or decrease the number of PFC. These results also held true when peripheral blood leukocytes were used (Table 2.3). Accordingly, it was decided that the plaque assays would be carried out using 10% complement and an

incubation temperature of 15°C The standard deviations produced when $2.5x10^4$ cells $well^{-1}$ were used in the culture plates were very high due to the fact that very few plaque forming cells were produced, with some wells failing to show any PFC, and when the figures were expressed as PFC per $2.5x10^5$ cells, the resultanting variation between wells became very large. The wells cultured with $5x10^6$ leukocytes per well produced so many PFC that in some cases it became difficult to count them due to overlapping plaques (Plate 2.1). The optimal concentration of rainbow trout leukocytes for the microwell plaque assays was found to $2.5x10^5$ cells $well^{-1}$. Therefore, future experiments using the microwell plaque assay to study rainbow trout leukocyte PFC responses were carried out in L-15 with 10% complement, at $2.5x10^5$ cells per well and with the plates cultured at 15°C.

2.11. Optimization of *in vivo* to *in vitro* transfer of trout spleen leukocytes for analysis of the Plaque Forming Cell response (PFC) to SRBC.

The optimal time after *in vivo* immunization for the spleen to be exised and the leukocytes held *in vitro* to detect a PFC immune response against SRBC was determined. This would allow the *in vivo* immunised leukocytes to be further tested for the effects of *in vitro* SRBC challenge in autogenic and allogenic thymus leukocyte/ spleen leukocyte cocultures (Chapter 6). To determine this, thirty fish were immunised with 0.1 mls of 5% SRBC in 0.85% saline mixed with an equal volume of Freunds complete adjuvant (FCA, Sigma). At various time intervals after immunisation, the spleens were removed from the trout and the leukocytes separated (section 2.2) and transfered *in vitro* as described before (section 2.8). The cells were then assayed for the PFC response to SRBC as in section 2.8. The results of the

Table 2.1 : The PFC response to SRBC of spleen leukocytes at various temperatures and complement concentrations.

Temp	% complement	PFC per $2.5x10^5$ cells		
		Leukocyte cell number ml^{-1} (200 ul added per well)		
		$2.5 x10^5$	$2.5x10^6$	$5x10^6$
4^oC	2%	10.0 ±17.5	7.4 ±5.1	5.3 ±3.23
4^oC	5%	12.5 ±17.7	3.4 ±2.2	6.6 ±3.4
4^oC	10%	20.0 ±25.8	11.4 ±3.65	12.3 ±3.83
4^oC	20%	20.0 ±19.7	14.8 ±8.5	15.4 ±3.89
15^oC	2%	10.0 ±17.4	11.8 ±6.9	12.8 ±5.2
15^oC	5%	12.5 ±17.7	19.8 ±9.4	15.1 ±4.6
15^oC	10%	22.5 ±24.8	26.4 ±10.4	21.9 ±7.53
15^oC	20%	30.0 ±25.8	22.8 ±9.3	22.6 ±7.1
20^oC	2%	17.5 ±16.9	10.6 ±4.0	11.9 ±5.83
20^oC	5%	22.5 ±36.2	21.4 ±8.43	13.7 ±4.1
20^oC	10%	10.0 ±17.5	39.2 ±8.33	23.0 ±6.56
20^oC	20%	12.5 ±24.2	28.0 ±10.6	25.6 ±7.03

Table 2.2 : The PFC response of Kidney leukocytes at various temperatures and complement concentrations.

Temp	% complement	PFC per $2.5x10^5$ cells		
		cell number per ml^{-1} (200ul added per well)		
		$2.5x10^5$	$2.5x10^6$	$5x10^6$
4°C	2%	7.5 ±12.1	3.8 ±2.6	2.1 ±1.7
4°C	5%	2.5 ±7.9	14.2 ±9.82	10.9 ±6.5
4°C	10%	25.0 ±20.4	20.4 ±9.37	4.4 ±1.5
4°C	20%	5.0 ±15.8	20.0 ±12.8	24.3 ±7.0
15°C	2%	10.0 ±6.95	10.2 ±6.95	12.5 ±11.5
15°C	5%	17.6 ±16.7	23.2 ±18.0	14.0 ±9.6
15°C	10%	65.0 ±35.7	68.6 ±23.1	48.6 ±19.5
15°C	20%	50.0 ±39.1	29.4 ±14.6	26.1 ±14.0
20°C	2%	35.0 ±21.2	20.6 ±14.0	26.0 ±8.2
20°C	5%	15.0 ±12.9	32.4 ±16.6	12.4 ±2.41
20°C	10%	25.0 ±26.4	46.4 ±11.84	12.4 ±2.41
20°C	20%	30.0 ±15.8	36.2 ±14.8	18.3 ±4.2

Table 2.3: The PFC response of peripheral blood leukocytes to SRBC at various temperatures and complement concentrations.

Temp	% complement	PFC per $2.5x10^5$ cells		
		Leukocyte cell number ml-1 (200 ul added per well)		
		$2.5x10^5$	$2.5x10^6$	$5x10^6$
4°C	2%	32.5 ±29.0	5.2 ±3.15	6.3 ±2.91
4°C	5%	12.5 ±17.7	17.8 ±10.2	16.5 ±5.4
4°C	10%	12.5 ±31.7	14.2 ±8.4	11.6 ±4.7
4°C	20%	12.5 ±14.3	11.5 ±3.1	19.8 ±8.87
15°C	2%	15.0 ±12.91	11.0 ±6.0	15.7 ±4.3
15°C	5%	27.5 ±21.75	18.6 ±7.83	13.1 ±4.79
15°C	10%	30.0 ±28.4	21.0 ±8.23	20.4 ±4.52
15°C	20%	27.5 ±29.9	26.8 ±10.6	23.6 ±4.9
20°C	2%	15.0 ±24.15	14.4 ±3.2	16.6 ±4.2
20°C	5%	7.5 ±12.1	12.4 ±5.6	8.8 ±4.3
20°C	10%	17.5 ±16.9	28.0 ±11.15	19.5 ±7.4
20°C	20%	30.0 ±32.9	16.6 ±8.7	17.6 ±4.6

in vivo to *in vitro* transfer experiments are shown in Table 2.4. This shows that the optimal PFC response after SRBC challenge *in vitro*, was 12-15 days post *in vivo* immunization, and by eight weeks post immunization the response had started to decline. Therefore, in future experiments the spleen was transfered *in vitro* eight weeks after immunization with the test antigen, when the primary response had begun to decrease.

2.12. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE):

SDS-PAGE was used in the characterisation of of rainbow trout leukocyte lysate antigens. All SDS-PAGE techniques were carried out as described by Davies and Brown (1987), using the Hoeffer Mighty Small minigel vertical slab electrophoresis system (Hoeffer Scientific Instruments, California, USA). All protein separations were carried out under reducing conditions, using 2-*B*-mercaptoethonal (Sigma), and the buffers and reagents used are described in appendix 3.

Table 2.4: The kinetics of appearance of splenic plaque forming cells (PFC), from trout injected with SRBC, after *in vivo* to *in vitro* transfer of splenic leukocytes.

Days after *in vivo* immunization*	mean number of PFC per $2.5x10^5$ spleen cells**		
	fish1	fish2	fish3
0	2.05 ±1.64	2.52 ±1.92	1.00 ±1.94
3	8.50 ±1.73	6.50 ±4.79	12.25 ±5.38
6	10.75 ±8.63	21.50 ±5.26	10.00 ±3.56
9	13.00 ±6.48	14.06 ±6.16	9.75 ±6.5
12	23.75 ±5.25	15.25 ±5.21	18.00 ±2.16
15	16.75 ±7.13	20.75 ±8.37	12.25 ±1.89
18	15.75 ±2.21	11.75 ±7.0	7.75 ±4.64
35	13.84 ±6.43	14.55 ±4.32	12.04 ±4.98
42	8.88 ±5.95	6.75 ±4.57	9.04 ±3.96
49	4.23 ±3.92	3.37 ±3.31	7.49 ±5.06

* The fish received an *in vivo* intramuscular injection of 0.1 mls of 5% SRBC, emulsified in an equal volume of FCA.

** Three fish were used in the experiment with five microtire plate wells, at $2.5x10^5$ cells $well^{-1}$, counted for each fish. The mean of the five wells (± standard deviation) is shown.

Chapter 3:

Lymphocyte migration and localisation patterns in rainbow trout, studied using the tracer sample method.

3.1. Introduction:

Mammalian lymphocytes continuously circulate through the blood and lymph to the peripheral lymphoid organs. Gowans and Knight (1964) demonstrated this capacity for recirculation by isolating lymph from the thoracic duct of a rat and radiolabelling the lymphocytes *in vitro,* and transfusing them back into the donor animals. By monitoring the location of the labelled cells at various time intervals, they found that the lymphocytes spent 2-12 hours in the blood before appearing in the lymph or lymphoid organs. Work on the destination of radioisotopically labelled thoracic duct, thymus, spleen and lymph node lymphocytes to the lymphoid organs of autogenic recipients showed that each cell population had a distinct distribution pattern. Thoracic duct lymphocytes and thymocytes homed preferentially to the thymus dependent areas, whereas spleen lymphocytes distributed themselves over all the various compartments of the peripheral lymphoid organs (Parrott , 1967; Parrott and deSousa, 1969). deSousa (1971) extended this work to look at the difference in migration pathways between thymus and bone marrow derived lymphocytes labelled *in vitro* with ^{3}H-adenosine, following intravenous injection into autogenic recipients. The thymus cells were found to home predominately to the thymus dependent areas in the recipients peripheral lymphoid organs, whereas the majority of the bone marrow cells were found in the thymus independent sites. The author suggested that the cell populations behaved as if they had the ability to distinguish and home towards what was probably their usual environment, and termed this non-random migration 'ecotaxis'.

The capacity of mature lymphoid cells to migrate and organise themselves in clearly defined microenvironments in the peripheral lymphoid organs is a reflection of the high degree of specialisation of the mammalian

lymphoid system, both in terms of structure and function. Whether this occurs at the phylogenic level of fish is not known, as there has been very little work done on lymphocyte migration pathways in fish. A study by Ellis and deSousa (1974) looked at the fate of lymphocytes, from the plaice (*Pleuronectes platessa*), collected by drainage of the neural duct and labelled *in vitro* with ^{3}H-uridine, before being reintroduced to the donor fish. The migration of the labelled lymphocytes was measured over time using scintillation counting. The characteristics of lymphoid cell migration observed in the plaice were very similar to those described in higher vertebrates. No significant numbers of labelled cells penetrated the thymus, with the majority found in the spleen and kidney. The autoradiographic study revealed that the population found in the white pulp of the spleen and kidney consisted mainly of small lymphocytes, whereas the labelled large lymphocytes were found in the red pulp of the spleen and kidney. Tatner (1985) looked at the migration of thymocytes in rainbow trout, following *in situ* thymic labelling using ^{3}H-thymidine. The migration of the labelled thymocytes to the peripheral lymphoid organs was followed using scintillation counting and autoradiography, and it was shown that twice as many thymocytes migrated to the spleen, as to the kidney.

Until recently, the subdivision of fish lymphocytes into surface immunoglobulin positive (sIg+ or putative B cells) and surface negative (sIg- or putative T cells) was not possible, and the lack of histocompatible strains of fish made such cell transfer experiments problematic. The tracer sample method described by Gowans and Knight (1964) has been used extensively in mammalian immunology to study all aspects of lymphocyte traffic. In this method, a population of marked lymphocytes is introduced into the bloodstream of a histocompatible recipient. After a lapse in time, the numbers of lymphocytes in various anatomical compartments of the recipient are

estimated. The most commonly used method of marking lymphocytes has been radioactive labelling *in vitro*, but chromosome markers, antigenic markers and even functional markers, such as dependence of a secondary antibody response on memory cells, have been used. Ford and Smith (1982) described some pitfalls of the tracer sample method, but with careful experimental design in the choice of sampling times and compartments studied, a great deal of useful information can be obtained from these studies.

There are no histocompatible lines of rainbow trout available for immunological studies, therefore the present work involved injecting labelled homologous cells back into the original donors. Lymphocytes were divided into sIg+ and sIg- populations (based on panning with a monoclonal antibody against trout serum IgM), and their localisation after 24 hours was determined. In addition, two further experiments were performed in which unseparated lymphocytes from one fish were injected into non-histocompatible recipients and studied over time, and secondly the localisation of organ derived separated lymphocytes from one fish was studied after injection into non-histocompatible recipients.

3.2. Materials and Methods:

3.2.1. Experiment 1: The migration of sIg+ and sIg- peripheral blood lymphocytes injected intravenously into the donor fish.

(a) Separation of lymphocytes into sIg+ and sIg- populations - Six well petri dishes were coated overnight at 4°C, with 8 ml of a monoclonal anti-trout IgM, diluted 1:200 in sterile PBS. The monoclonal antibody, supplied as ascites fluid, reacted with the heavy chain of trout serum IgM (a gift from Dr Secombes, Aberdeen, UK). The leukocytes were removed from the peripheral blood as

described in section 2.2. The leukocytes were then separated into sIg+ and sIg- cells as described in section 2.5.

(b) Labelling of lymphocytes *in vitro* - The lymphocytes were counted using a haemocytometer and their viability assessed using the trypan blue exclusion test (section 2.3.) After centrifugation, the cells were resuspended in L-15/10% foetal bovine serum (FBS), with 100iu of penicillin/streptomycin and L-glutamine (2mM), to $1x10^6$ cells ml-1, and seeded at 100ul ($1x10^5$ cells) per well in a 96-well microtitre plate. After incubation at 18°C for 2 days, the cell cultures were pulsed with 0.5 *u* Ci of ^{3}H-thymidine (Amersham, Bucks, UK), and harvested 24 hours later. After washing three times, the pooled cell populations were counted using a haemocytometer, their viability rechecked, centrifuged and resuspended in sterile saline to $1x10^6$ cells ml^{-1}. The original donor fish then received 0.1 ml ($1x10^5$ cells) of their own sIg+ or sIg- cells, injected intravenously into the caudal vein. The experiment was repeated six times; three fish received their own sIg+ or sIg- blood cells, and three fish were injected with unlabelled lymphocytes, for background counts per minute (C.P.M.) levels.

(c) Fish sampling : The fish weighed on average 50g and were maintained at 12°C. At exactly 24 hours after an injection of leukocytes, the fish were killed and the thymus, spleen and kidney removed, and weighed. Each organ was macerated in 1 ml of distilled water in a Stomacher apparatus (Packard) for 2 minutes, and three 0.2 ml samples were removed and pipetted onto filter paper discs in glass scintillation vials. After air drying, 0.3 mls of Soluene-350 (Tissue solubiliser, Packard) was added to each vial, and the vials were incubated at 50°C for 30 mins. After cooling to room temperature, 5 ml of Ecoscint Scintillation Fluid was added to each vial, the contents thoroughly

agitated, and after standing for 1 hour, the vials were counted on a Packard liquid scintillation counter.

Experiment 2 : The migration of unseparated lymphocytes from one donor injected intravenously into non-histocompatible recipients: A time course study.

In this experiment, one adult rainbow trout (100g) served as the source of lymphocytes. The cells from the peripheral blood, spleen and kidney were isolated on lymphocyte separating medium, cultured and labelled, as described above, with the spleen and kidney first being teased apart in 1 ml of sterile saline. All the cells were then pooled and injected intravenously at a dose of 10^5 cells, into a group of recipient 50g fish. Three fish were sacrificed at 1.5, 3, 24 and 72 hours after injection, the thymus, spleen and kidney removed and weighed, and the organs processed for scintillation counting as described for experiment 1.

Experiment 3: The migration of organ derived sIg+ and sIg- lymphocytes from one donor fish injected intravenously into non-histocompatible recipients.

For this experiment, one fish served as the source of lymphocytes from peripheral blood, thymus, kidney and spleen. The three organs were teased apart in petri dishes containing 1 ml of sterile saline to release the cells, which were then layered onto lymphocyte separation medium. The lymphocytes so obtained were separated into sIg+ and sIg- cells by panning, as described in section 2.5. After labelling with ^{3}H-thymidine *in vitro* as above, the cells were injected intravenously into groups of fish (three fish were used for each type of lymphocyte from each organ). Twenty-four hours later, the fish were bled out to reduce red cell contamination and then killed, and the thymus, spleen, and kidney removed, weighed, and processed for scintillation counting as before. For thymus cells, there was a 78% recovery after panning, and all cells were

sIg-. For kidney, 61.3% of the cells were recovered, of which 69.5% were sIg+ and 30.5% sIg-. The corresponding figures for the spleen were 72% recovery, (45% sIg+, 55% sIg-).

Data Analysis:

The results were analysed using the Student's *t*-test.

3.3. Results:

Experiment 1: Localisation of sIg+ and sIg- peripheral blood lymphocytes in the original donors.

The results of the migration and localisation of sIg+ blood lymphocytes, 24 hours after injection into their original donors are shown in Table 3.1. The aliquots of 0.1 ml (10^5 cells) which were injected into fish numbers 1, 2, and 3 had C.P.M.'s of 13 455, 10 376 and 14 155, respectively. Mean background levels of C.P.M. in the organs of three fish injected with unlabelled lymphocytes were 54 (thymus), 49 (spleen) and 31 (kidney). Table 3.1 shows that based on the uptake of cells per organ, very few sIg+ cells entered the thymus, less than 0.6% of the injected dose. However, this is simply a reflection of the small size of the thymus gland compared to other lymphoid organs. On an uptake of labelled cells per gram organ weight, there was a significantly higher number of sIg+ cells entering the thymus (3.95 ± 1.19) compared to the spleen or kidney (2.23 ± 0.48; 2.07 ± 0.24; $P>0.05$), at the 24 hour sample period. As a percentage of the total cells injected, approximately equal numbers of the sIg+ cells migrated to the spleen and kidney.

The data with regard to the sIg- lymphocytes are shown in Table 3.2. The aliquots of 0.1 ml (10^5 cells) which were injected into these fish had

Table 3.1: Localisation of radiolabelled sIg+ lymphocytes, 24 hours after injection into the original donors.

Fish number	organ	organ weight (g)	*corrected C.P.M.	uptake cells per organ x10^3	uptake cells per gram x10^4	% total cells injected
1	Thymus	0.0141	83	0.62	4.40	0.62
	Spleen	0.286	915	7.04	2.46	7.04
	Kidney	0.217	505	3.88	1.79	3.88
2	Thymus	0.0183	89	0.89	4.86	0.89
	Spleen	0.318	813	8.13	2.56	8.13
	Kidney	0.273	608	6.10	2.23	6.10
3	Thymus	0.0108	41	0.29	2.60	0.29
	Spleen	0.152	364	2.56	1.68	2.56
	Kidney	0.168	520	3.66	2.18	3.66
mean	Thymus	0.0144 ±0.0037	71 ±26	0.60 ±0.30	3.95 ±1.19	0.60 ±0.30
	Spleen	0.252 ±0.088	697 ±293	6.03 ±3.03	2.23 ±0.48	6.03 ±3.03
	Kidney	0.219 ±0.052	544 ±55	4.55 ±1.35	2.07 ±0.24	4.55 ±1.35

*Corrected C.P.M. (counts per minute) has mean background figure from fish injected with unlabelled lymphocytes subtracted

Table 3.2: Localisation of radiolabelled sIg- lymphocytes, 24 hours after injection into the original donors.

Fish number	organ	organ weight	*corrected C.P.M.	uptake cells per organ x10^3	uptake cells per gram x10^4	% of total cells injected
1	Thymus	0.0093	124	1.36	1.40	1.30
	Spleen	0.287	384	4.01	1.40	4.01
	Kidney	0.198	415	4.34	2.20	4.34
2	Thymus	0.0121	81	0.65	5.37	0.65
	Spleen	0.319	185	1.50	0.47	1.50
	Kidney	0.263	1203	9.70	3.69	9.70
3	Thymus	0.0113	55	0.89	7.90	0.89
	Spleen	0.217	345	5.61	2.58	5.61
	Kidney	0.235	598	9.72	4.13	9.72
Mean	Thymus	0.0113 ±0.0014	86 ±34	0.95 ±0.33	4.89 ±3.30	0.95 ±0.33
	Spleen	0.274 ±0.052	304 ±105	3.71 ±2.10	1.48 ±1.06	3.71 ±2.10
	Kidney	0.232 ±0.032	738 ±412	7.92 ±3.10	3.34 ±1.01	7.92 ±3.10

*Corrected C.P.M. (counts per minute) has mean background figure from fish injected with unlabelled lymphocytes subtracted.

C.P.M.'s of 9574, 12 355 and 6152 respectively.

On a cell per gram weight basis, significantly more sIg- cells entered the thymus gland (4.89 ±3.30) than the spleen or kidney (1.48 ±1.06; 3.34 ±1.01; P>0.05) within the first 24 hours after injection. As a percentage of the total cells injected, there was a significant difference in the localisation of sIg- cells to the kidney compared to the spleen (P>0.05).

Experiment 2: Migration of unseparated lymphocytes from one donor, injected into non-histocompatible recipients.

The results of the time course pattern of localisation of unseparated lymphocytes are shown in Table 3.3, and illustrated graphically in Fig 3.1. The mean C.P.M. of the aliquots (10^5 cells) which were injected into the recipient fish was 3922. Background C.P.M. levels from fish injected with non-labelled lymphocytes were 39 (thymus), 41 (spleen), and 34 (kidney).

Based on C.P.M. per gram weight values, the numbers of labelled cells entering the thymus were significantly higher than the spleen or kidney during the first 24 hours (134 ±18.4, compared to 46.6 ±28.7 and 12.3 ±3.25, respectively, P>0.05). By 72 hours the numbers in the thymus had remained high (1.25 ±0.91), but with a corresponding increase in the spleen (84.6 ±60.8) and kidney (66.87 ±9.23) (P>0.01), with the spleen sequestering the largest percentage of total cells injected (37.8%, Fig 3.1)

Experiment 3: Migration of organ-derived, separated lymphocytes from one donor, injected into non-histocompatible recipients.

The results of this experiment are shown in Table 3.4, and illustrated graphically in Fig 3.2. The injected cells (0.1 ml aliquots of 10^5 cells) had C.P.M. values of 13 007 (Kidney sIg-), 28 782 (kidney sIg+), 95 444 (spleen sIg-), 10 405 (spleen sIg+), 8595 (blood sIg-), 11634 (blood sIg+), 95 444 (spleen sIg-), 10 405 (spleen sIg+), 8595 (blood sIg-), 11 634 (blood sIg+) and

Table 3.3: Migration and localisation of unseparated allogenic lymphocytes over a 72 hour incubation period.

Time (hours)	Fish	Organ	Organ weight (g)	*Corrected C.P.M.	Cells per organ x10^3	Cells per gram x10^4	% of total cells injected
1.5	1	Thymus	0.0058	112	2.85	121	2.85
	2	Thymus	0.0031	25	4.11	132	4.11
	3	Thymus	0.0035	40	1.29	36.8	1.29
Mean ±SD			0.0041±0.0014	59.0 ±46.0	2.75 ±1.41	96.6 ±52.0	2.75 ±1.44
1.5	1	Spleen	0.0633	107	2.72	4.3	2.72
	2	Spleen	0.0533	110	1.82	3.4	3.9
	3	Spleen	0.0619	56	1.81	3.6	1.81
Mean ±SD			0.0595 ±0.0054	91.0 ±30.0	2.11 ±0.52	3.76 ±0.47	2.81 ±1.04
1.5	1	Kidney	0.0670	152	3.87	5.78	3.87
	2	Kidney	0.0619	51	8.36	13.50	8.4
	3	Kidney	0.0615	54	1.75	2.82	1.75
Mean ±SD			0.0634 ±0.003	85.0 ±57.0	4.66 ±3.37	7.36 ±5.5	4.67 ±3.39
3	1	Thymus	0.0039	23	0.75	19.3	0.75
	2	Thymus	0.0029	35	0.73	25.4	0.73
	3	Thymus	0.0057	37	1.04	18.2	1.04
Mean ±SD			0.0041 ±0.0014	31.0 ±7.5	0.84 ±0.17	20.9 ±3.87	0.84 ±0.17

Table 3.3: continued.

Time (hours)	Fish	Organ	Organ weight (g)	*Corrected C.P.M.	Cells per organ x10^3	Cells per gram x10^4	% of total cells injected
3	1	Spleen	0.0543	133	4.31	8.08	4.31
	2	Spleen	0.0679	253	4.88	7.19	4.88
	3	Spleen	0.0439	46	1.28	2.92	1.28
Mean ±SD			0.055 ±0.012	144.0 ±103	3.49 ±1.93	6.06 ±2.65	3.49 ±1.91
3	1	Kidney	0.0412	59	1.94	4.78	1.94
	2	Kidney	0.0497	101	1.94	3.91	1.94
	3	Kidney	0.0529	57	1.58	2.98	1.58
Mean ±SD			0.0479 ±0.006	72 ±24.0	1.82 ±0.20	3.89 ±0.90	1.82 ±0.20
24	1	Thymus	0.0042	202	5.12	121	5.12
	2	Thymus	0.0064	370	9.43	147	9.43
Mean ±SD			0.0053 ±0.0015	286.0 +119.0	7.28 ±3.04	134.0 ±18.4	7.28 ±3.04
24	1	Spleen	0.0651	670	17.10	26.3	17.1
	2	Spleen	0.0497	1302	33.20	67.0	33.2
Mean ±SD			0.00574 ±0.01	986 ±447	25.1 ±11.3	46.6 ±28.7	25.15 ±11.3
24	1	Kidney	0.0483	190	4.84	10.0	4.84
	2	Kidney	0.0623	357	9.10	14.6	9.10
Mean ±SD			0.0553 ±0.010	273 ±118.0	6.95 ±3.04	12.3 ±3.25	6.95 ±3.01

Table 3.3: continued.

Time (hours)	Fish	Organ	Organ weight (g)	*Corrected C.P.M.	Cells per organ x10^3	Cells per gram x10^4	% of total cells injected
72	1	Thymus	0.0049	204	5.20	106.0	5.2
	2	Thymus	0.0041	324	8.26	201	8.26
Mean ±SD			0.0045 ±0.0005	264.0 ±84.0	6.73 ±2.16	153 ±67.2	6.73 ±2.16
72	1	Spleen	0.0487	795	20.27	41.60	20.27
	2	Spleen	0.0443	2167	55.25	127.6	55.25
Mean ±SD			0.0465 ±0.003	1481 ±970.2	37.8 ±24.7	84.6 ±60.8	37.8 ±24.7
72	1	Kidney	0.0537	1273	32.40	60.34	32.40
	2	Kidney	0.0518	1247	31.80	73.40	31.80
Mean ±SD			0.0527 ±0.001	1260 ±18.38	32.1 ±0.42	66.87 ±9.23	32.1 ±0.42

* Corrected C.P.M. (counts per minute) has mean background figure from fish injected with unlabelled lymphocytes subtracted.

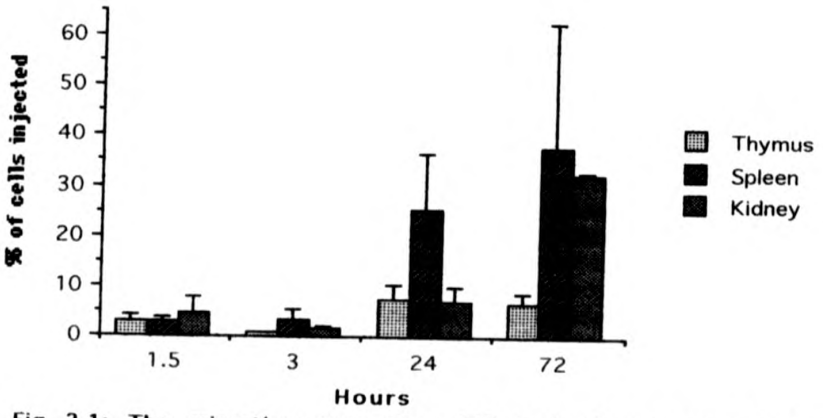


Fig 3.1: The migration over time (72 hrs) of unseparated lymphocytes from one donor, injected into non-histocompatible recipients.

8794 (thymocytes). Mean background C.P.M. values from control fish injected with unlabelled cells were 30 (thymus), 46 (spleen) and 21 (kidney).

At 24 hours sIg+ cells from the blood, spleen and kidney had their highest C.P.M. per gram in the thymus. However, in terms of the percentage of cells injected, the majority of the sIg+ cells were in the spleen, irrespective of the organ source of the cells.

The sIg- cells from the blood, spleen and kidney again appeared briefly in the thymus in relatively high numbers based on the C.P.M. per gram values. In terms of the percentage of the cells injected, however, there appeared to be a preferential homing of sIg- cells to the spleen, irrespective of their organ source (more sIg- kidney cells, and most of the thymocytes [sIg-] localised in the spleen). However most of the sIg- peripheral blood lymphocytes were detected in the kidney.

Taking the results of all three experiments together, the following points emerge:

(1) Using sIg+ or sIg- peripheral blood lymphocytes injected intravenously into their original donors, it was found that the distribution of sIg+ cells between the spleen and kidney was approximately the same, but more sIg- peripheral blood cells localised in the kidney than in the spleen.

(2) A time course study of the localisation of unseparated lymphocytes showed that high levels migrated through the thymus in the first 24 hours, but by 72 hours the spleen had sequestered the majority of the cells (37.8%).

(3) Using organ derived separated lymphocytes injected into non-histocompatible donors, the source of the cells was not found to have an effect on their migration and localisation pattern. More of the sIg- peripheral blood lymphocytes were detected in the kidney than in the spleen, as found in experiment 1. However, sIg- cells from the spleen, kidney and thymus

Table 3.4: Localisation of sIg+ and SIg- lymphocytes from the organs of one donor fish, 24 hours after injection into non-histocompatible recipients.

Source of cell	Organ	Organ weight (g)	*Corrected C.P.M.	Cells per organ x10^3	Cells per gram x10^4	% of total cells injected
Blood sIg+	Thymus	0.0039	77	7.4	19.0	0.74
	Thymus	0.0036	163	1.6	44.0	1.6
	Spleen	0.0548	956	9.2	17.0	9.2
	Spleen	0.0583	853	8.2	14.0	8.2
	Kidney	0.0561	325	2.8	4.99	2.8
	Kidney	0.0529	154	1.5	2.8	1.5
	Kidney	0.0627	471	4.51	7.2	4.5
Blood sIg-	Thymus	0.0023	94	0.99	43	0.99
	Spleen	0.0511	281	2.95	5.8	2.95
	Spleen	0.0641	243	2.8	4.4	2.82
	Kidney	0.0539	181	1.4	3.5	1.4
	Kidney	0.0486	678	7.9	16.0	7.9
Spleen sIg+	Thymus	0.0033	535	4.6	140	4.6
	Thymus	0.0037	173	1.5	40.4	1.5
	Thymus	0.0019	239	2.1	110	2.1
	Spleen	0.0598	3315	28.5	48.0	28.5
	Spleen	0.0579	568	4.9	8.5	4.9
	Spleen	0.0397	1077	9.3	23.3	9.3

Table 3.4: continued.

Source of cell	Organ	Organ weight (g)	Corrected C.P.M.	Cells per organx10^3	Cells per gram x10^4	% of total cells injected
Spleen sig+	Kidney	0.0639	817	7.05	11.0	7.0
	Kidney	0.0712	1066	9.2	13.0	9.2
	Kidney	0.0475	219	1.9	4.0	1.9
Spleen sig-	Thymus	0.0037	957	10.1	272.0	10.1
	Thymus	0.0039	417	4.39	110.0	4.39
	Spleen	0.0673	2099	22.4	32.8	22.4
	Spleen	0.0493	2430	25.6	52.0	25.6
	Kidney	0.0617	1424	15.0	24.0	15.0
	Kidney	0.0477	678	7.1	15.0	7.2
Kidney sig+	Thymus	0.0036	185	0.65	18.0	0.65
	Thymus	0.0028	242	0.84	30.3	0.85
	Thymus	0.0031	89	0.31	10.1	0.3
	Spleen	0.0591	734	2.57	4.35	2.57
	Spleen	0.0495	1515	5.31	10.6	5.31
	Kidney	0.0624	876	3.1	4.9	3.1
	Kidney	0.0518	321	1.1	2.27	1.1
	Kidney	0.0613	415	1.45	2.37	1.45
Kidney sig-	Thymus	0.0024	180	1.4	58	1.4
	Thymus	0.0034	91	0.7	21	0.7
	Thymus	0.0041	217	1.7	41	1.7

Table 3.4: continued.

Source of cell	Organ	Organ weight (g)	Corrected C.P.M.	Cells per organ x10^3	Cells per gram x10^4	% of total cells injected
Kidney sig-	Spleen	0.0544	2210	17	31.2	17
	Spleen	0.0673	561	4.3	6.4	4.3
	Spleen	0.0563	3965	30.5	54.0	30.5
	Kidney	0.0697	721	5.6	7.9	5.6
	Kidney	0.0739	280	2.1	2.9	2.1
	Kidney	0.0494	1093	8.4	17.0	8.4
Thymocytes	Thymus	0.0023	137	1.6	68.0	1.6
	Thymus	0.0027	219	2.5	92.6	2.5
	Thymus	0.0041	97	1.1	27.0	1.1
	Spleen	0.0543	1678	19.1	35.0	19.1
	Spleen	0.0519	2099	24.0	46.0	24.0
	Spleen	0.0639	1503	17.0	27.0	17.0
	Kidney	0.0479	314	3.5	7.5	3.5
	Kidney	0.0639	121	1.4	2.3	1.4
	Kidney	0.0691	679	7.7	11.2	7.7

* Corrected C.P.M. (counts per minute) has mean background figure from fish injected with unlabelled lymphocytes subtracted.

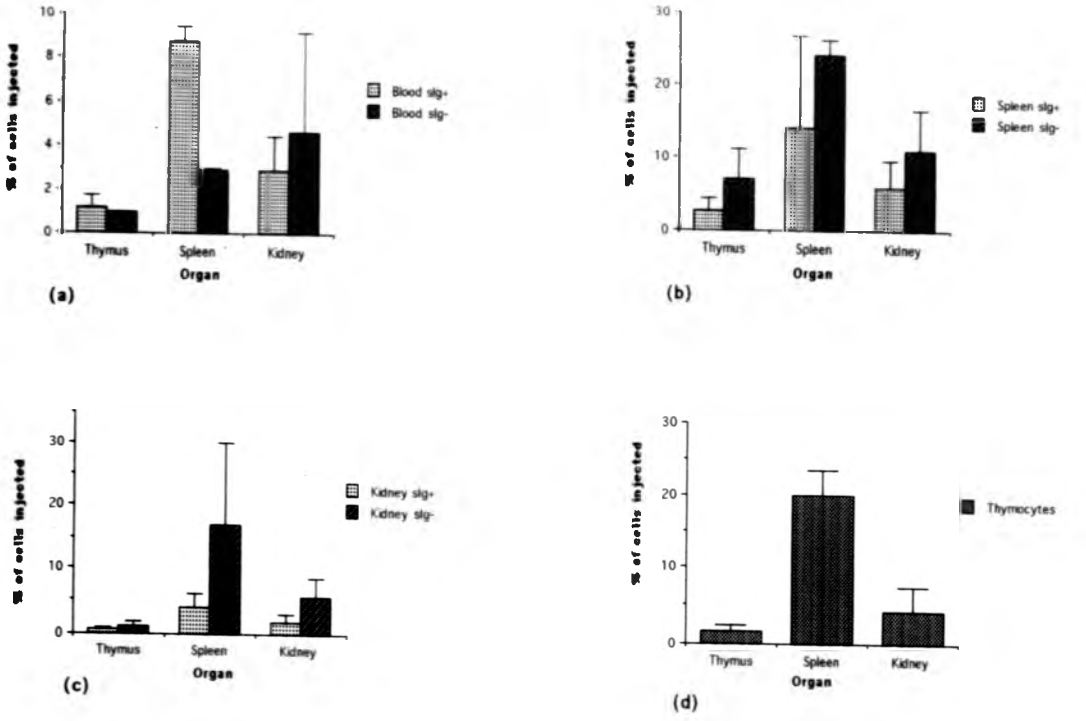


Fig 3.2: The localisation of organ specific slg+ and slg- lymphocytes, 24 hours after injection into non-histocompatible recipients. (a) localisation of slg+ and slg- lymphocytes from the blood. (b) Localisation of slg+ and slg- lymphocytes from the spleen. (c) Localisation of slg+ and slg- lymphocytes from the kidney. (d) Localisation of slg- lymphocytes from the thymus.

migrated preferentially to the spleen.

3.4. Discussion:

The selective migration and localisation of subpopulations of lymphocytes within different peripheral lymphoid organs is known as ecotaxis (deSousa, 1971). Many experiments in mammals on the tissue distribution of labelled lymphoid cells other than thoracic duct lymphocytes have followed the experimental design first adopted by Gowans and Knight (1964). It was found that, irrespective of source, T cells migrated to the thymus dependent areas of the spleen, lymph nodes, Peyers patches and appendix, and B cells migrated to the thymus independent areas (deSousa, 1981).

The choice of tritiated thymidine to label the fish cells selected a population of dividing cells, which by analogy with mammalian studies were short lived, had slightly different migration patterns from small recirculating lymphocytes collected from the lymph, and which also had lower recovery rates. A previous report on this aspect of fish lymphocyte traffic by Ellis and deSousa (1974), in which they collected neural lymphatic duct lymphocytes from plaice labelled them with (5-^{3}H) uridine. After reinjection, the labelled cells localised in the spleen and kidney, with no significant numbers entering the thymus. This contrasts with the present study, in which the dividing cell populations labelled here appeared to migrate through the thymus, and then to the peripheral lymphiod organs (spleen and kidney).

If the distribution of the cells was entirely random, all the tissues would have had a similar C.P.M. per gram. This was not found to be the case. There was a preferential migration of sIg- cells from the spleen, kidney or thymus to the spleen; though peripheral blood sIg- cells localised preferentially in the kidney.

The finding that the spleen appears to be the 'home' for organ-derived sIg- cells (especially thymus cells) leads to speculation that it is through this organ that ' T 'cells recirculate, once in the periphery. Early thymus removal in the trout has been shown to significantly deplete the number of cells in the spleen, whilst those in the kidney were unaffected (Grace, 1981).

The cells selected as sIg+ in this study can be safely assumed to be lymphocytes, but the non-selected sIg- cells from the spleen and kidney may have contained haemopoietic cells. Thus, the possibility exists that the differences in organ localisation between sIg+ and sIg- cells is a reflection of the behaviour of the sIg- non-lymphocyte cells. However, the sIg- spleen and kidney cells acted similarly to the sIg- thymus cells in preferentially homing to the spleen, which suggests that the cell populations involved are all lymphocytes.

The sIg- blood cells, on the other hand, are almost certainly lymphocytes as in the blood the other leukocytes are all end cells, and as they do not divide further, would not have incorporated the tritiated thymidine during the *in vitro* labelling process. The fact that these cells migrate preferentially to the kidney, and not the spleen, may be of significance, though the reasons for this are unclear at the present time.

One disadvantage with using 3-H thymidine as a label is that free label can be taken up by other dividing cells in the organs, and thus the C.P.M. values obtained are not due exclusively to the localisation of the migrating labelled cells within that organ. This could be checked by performing autoradiographs of the organs studied, and precautions taken against the reutilization of label by simultaneously injecting the fish with cold thymidine. However, it was felt that during the short time span of the experiments, the cells labelled *in vitro* would not release significant amounts of their label when

injected *in vivo*. The similar results obtained using non-histocompatible recipients, and those using autologous cells suggest that there was no rejection of injected cells by the non-histocompatible recipients, and no cytotoxicity of the cells, which would also have resulted in a loss of label. Sakai (1991) found that lymphocytes from rainbow trout, immunised with goldfish red blood cells, transplanted into non-histocompatible recipients, were able to maintain their haemolytic activity for more than 4 weeks after transfer.

In summary, the present results strongly suggest a non-random migration of a subpopulation of lymphocytes in the peripheral lymphoid organs in trout, in that sIg- cells migrate preferentially to the spleen, and it is concluded that the phenomenon of ecotaxis occurs in fish.

This work has been published: Tatner M F and C Findlay (1991). Lymphocyte migration and localisation patterns in rainbow trout, *Oncorhynchus mykiss*, studied using the tracer sample method. *Fish and Shellfish Immunology* 1 p107-117.

Chapter 4:

A comparative study of T and B lymphocytes in rainbow trout, following their separation by nylon wool adherence and lectin agglutination techniques.

4.1.Introduction :

One of the most valuable methods for studying teleost lymphocyte heterogeniety is the use of specific cell separation techniques coupled with relevant *in vitro* immunological assays. To date the most commonly used tools for separating teleost lymphocyte populations have been monoclonal antibodies (Miller et al, 1987; Ainsworth et al, 1990). For example, the most common monoclonal antibodies used to separate fish lymphocytes are anti-surface IgM monoclonal antibodies (DeLuca et al, 1983; Thuvander et al, 1990). DeLuca et al (1983) used a monoclonal antibody to serum IgM to deplete head kidney of cells positive for surface IgM using the panning technique, and found that the mitogenic response to lipopolysaccaride, but not concanavalin A, was significantly reduced suggesting that the sIg- population showed T cell properties. Recent literature also cites the production of monoclonal antibodies to a range of other teleost leukocyte populations, including non-specific cytotoxic cells (Jaso-Friedman et al, 1988), neutrophils (Bly et al, 1990) and T cells of the channel catfish (Ainsworth et al, 1990), and the leukocytes of the carp (Egberts et al, 1983; Secombes et al, 1983a; van Diepen et al, 1991)

There are other separation methods which have been succesfully used in mammalian systems. For example, the application of lectins (a class of proteins which bind and cross-link specific cell surface carbohydrate residues) in the separation of murine and human lymphocyte subpopulations is well established (Reisner et al, 1976; Nakano et al, 1980; Reisner and Sharon, 1980). Reisner et al (1976) used soybean agglutinin (SBA) to separate mouse splenic lymphocytes into T and B cell populations. The two cell fractions obtained were characterised by surface antigens and by their response to various mitogens. They found that B cells but not T cells were agglutinated by

SBA.

Reisner and Sharon (1980) showed that peanut agglutinin (PNA) could be used to separate mouse thymocytes into two subpopulations. The immature (cortical) thymocytes bound PNA readily, while the mature (medullary) thymocytes bound the lectin poorly. Nakano et al (1980) showed that PNA-agglutinable mouse splenic cells were able to exert a significant suppressor effect on the primary antibody response to sheep red blood cells, whereas PNA non-agglutinable cells had no effect on this antibody response. These findings suggested that suppressor lymphocytes were being agglutinated by the PNA.

It has also been shown that the passage of human and murine lymphocytes over a nylon wool column as described by Julius et al (1973) is an effective means of obtaining a population enriched with T cells. B cells, plasma cells and some accessory cells preferentially adhere to the column, while T cells pass through the column (Trizio and Cudkowicz, 1974; Tada et al. 1978). Little work has been done on the separation of teleost lymphocytes using these methods. The aim of the present study was to compare the use of nylon wool and SBA separation of rainbow trout lymphocytes, and to characterise the cell populations thus obtained on the basis of their mitogenic responses and cytochemical characteristics. The PNA separated thymocyte subpopulations were tested for any *in vitro* cyclophosphamide toxicity, as it has been shown that mammalian T suppressor cells are susceptible to cyclophosphamide treatment (Chan et al, 1983). Fish thymus derived suppressor cells have also been shown to be susceptible to *in vivo* cyclophosphamide treatment (Nakanishi, 1986).

4.2. Materials and methods:

4.2.1. Fish: Adult rainbow trout (approximate weight 150g) were used throughout this study. The fish were maintained in aquaria, in filtered and dechlorinated water at 12-15°C.

4.2.2. Soybean agglutinin (SBA) separation: SBA separation of thymus, spleen, kidney and peripheral blood leukocytes were carried out as described in section 2.6. The separated leukocyte populations were then washed three times in large volumes of hanks balanced salt solution (HBSS; Sigma)/2% foetal bovine sera (FBS; Sigma) and resuspended to $5x10^6$ cells ml^{-1} in L-15/5% FBS containing penicillin/streptomycin (100units of penicillin and 50ug of streptomycin per ml of medium; Sigma) and L-glutamine (2mM; Sigma).

4.2.3. Peanut agglutinin (PNA) separation: PNA separation of thymocytes was carried out as described in section 2.6. The separated thymocyte populations were resuspended to $5x10^6$ cells ml^{-1} in L-15/5% FBS.

4.2.4. Nylon wool separation: The nylon wool separation of thymus, spleen, kidney and peripheral blood leukocytes was carried out as described in section 2.4. The separated populations were resuspended to $5x10^6$ cells ml^{-1} in L-15/5% FBS.

4.2.5. Mitogen assays: Mitogen assays on the SBA and nylon wool separated subpopulations were carried out as described in section 2.7.

4.2.6. Acid phosphatase staining: Three slides of each separated cell

population from the spleen, kidney thymus and peripheral blood were prepared using a Cytospin centrifuge (Shandon, Berks, UK). The slides were allowed to air dry at room temperature for 30 minutes before being fixed in methanol for 5 minutes. The cytospin preparations were then processed for acid phosphatase staining as described by Blaxhall and Hood (1985).

4.2.7. Staining for cell associated IgM: Slide preparations of cells were made and fixed as described in 4.2.6. Mouse anti-trout IgM monoclonal antibody clone 4C10 (Thuvander et al, 1990: this antibody reacts only with the heavy chain of trout serum IgM) culture supernatant was diluted 1:5 in Tris buffered saline (TBS) containing 2% FBS before being applied to the slides, and incubated at room temperature for 45 minutes. The slides were then washed using TBS and horseradish peroxidase (HRP) conjugated goat anti-mouse whole IgG antibody (Scottish antibody production unit : [SAPU], Carluke, UK) was added at a dilution of 1:100 in phosphate buffered saline (PBS) containing 2% FBS, for 45 minutes at room temperature. The slides were washed using TBS before being immersed in Diaminobenzidine substrate solution (DAB) for 5 minutes. The slides were removed and washed using TBS, before being counterstained using Haematoxylin. As a control, the anti-trout IgM primary antibody was omitted.

4.2.8. Cyclophosphamide microcytotoxicity assay: The PNA separated thymocyte subpopulations were adjusted to $5x10^6$ cells ml^{-1} in L-15 containing 5% FBS and dispensed into the wells of a 96 well flat bottomed microtitre plate (Nunclon, Paisley, UK) to give a concentration of $5x10^5$ cells/well. Cyclophosphamide (Sigma) was dissolved in PBS pH7.4 (appendix 1) and the stock solution (10mg/ml^{-1}) was diluted in L-15 medium to provide

concentrations ranging from 1000ug/ml^{-1} to 1ug/ml^{-1}. The different cyclophosphamide concentrations were added to the microtitre plate (100ul/well: triplicate wells for each cyclophosphamide concentration), and the whole plate was incubated in a moist chamber at 15°C for 72 hours. The cells from each well were removed, the triplicates pooled and washed three times in HBSS. The cells were counted using a haemocytometer (section 2.3.) and their viability assesed by trypan blue exclusion.

4.2.9. Calculations and statistics: Mitogen assays were performed using triplicate wells for each mitogen. The degree of stimulation for each mitogen was measured using the stimulation index (S.I.):

$$S.I. = \frac{\text{mean counts per minute for stimulated cultures}}{\text{mean counts per minute for unstimulated cultures}}$$

To test whether there was any significant differences between the different groups of cells to the different mitogens, a T-test for matched pairs was carried out

4.3. Results:

When leukocytes from rainbow trout were separated using nylon wool, two populations were obtained (adherent and non-adherent). The cell recoveries were in general low, ranging from 21.6%, 22.8%, 25.1% and 27.4% of the original leukocytes from the spleen, kidney, peripheral blood and the thymus respectively. After incubation with SBA, leukocytes were separated into two main cell populations by sedimentation. These were classified as agglutinated and unagglutinated cells. Again cell recoveries were low, ranging from 17%,22.6% and 27.3% recovery for the peripheral blood, thymus and spleen respectively, and 41.8% recovery from the kidney leukocytes. Two

Table 4.1: The immunocytochemical and mitogenic properties of soybean agglutinin (SBA) and nylon wool separated leukocytes.

leukocyte source	mean C.P.M.* ± S.D.	stimulation index ConA	stimulation index LPS	% of cells +ve for acid phosphatase	% of cells +ve for anti trout IgM
unseparated spleen	**1. 797.3 ±250.4 2. 1874.8 ±641.8 3. 1971.9 ±695.8	2.31 ±0.45	2.45 ±0.93	81.0 ±5.3	16.7 ±4.04
unseparated blood	1. 739.7 ±211.9 2. 2409.6 ±846.5 3. 1919.6 ±713.7	3.25 ±0.91	2.59 ±0.21	88.0 ±7.9	9.3 ±1.5
unseparated kidney	1. 740.5 ±109.0 2. 1414.3 ±438.7 3. 3219.4 ±462.6	1.91 ±0.87	4.33 ±1.13	52.0 ±10.5	21.7 ±11.0
unseparated thymus	1. 423.3 ±62.7 2. 1748.5 ±509.3 3. 480.0 ±80.2	4.12 ±0.65	1.13 ±0.20	0	2.33 ±2.08
spleen nylon wool adherent	1. 924.3 ±273.7 2. 1118.2 ±275.6 3. 2668.2 ±905.1	1.20 ±0.33	2.87 ±0.46	28.0 ±14.5	21.1 ±6.3
spleen nylon wool non-adherent	1. 889.1 ±317.4 2. 2305.7 ±951.7 3. 1360.3 ±406.6	2.60 ±0.20	1.53 ±0.21	68.0 ±12.6	13.7 ±6.4
blood nylon wool adherent	1. 879.1 ±219.4 2. 1494.5 ±643.7 3. 2601.6 ±921.7	1.70 ±0.46	2.96 ±0.36	24.0 ±8.3	19.5 ±8.2
blood nylon wool non-adherent	1. 942.6 ±48.3 2. 2310.3 ±721.0 3. 1498.7 ±770.4	2.45 ±0.43	1.59 ±0.23	74.0 ±8.0	15.4 ±4.1
kidney nylon wool adherent	1. 771.1 ±118.0 2. 1341.7 ±519.7 3. 1896.9 ±454.0	1.74 ±0.10	2.46 ±0.15	35.0 ±31.3	24.6 ±9.3
kidney nylon wool non-adherent	1. 873.6 ±55.7 2. 2079.9 ±274.9 3. 1324.1 ±121.3	2.38 ±0.33	1.51 ±0.12	23.0 ±17.0	19.2 ±2.2
thymus nylon wool non-adherent	1. 892.8 ±99.2 2. 2928.4 ±677.5 3. 1705.2 ±392.4	3.26 +0.86	1.91 +0.32	0.0	1.6 +1.4
spleen SBA agglutinated	1. 771.8 ±160.9 2. 1237.8 ±276.8 3. 1323.0 ±316.4	1.60 ±0.26	1.71 ±0.46	77.0 ±9.16	15.7 ±6.11
spleen SBA unagglutinated	1. 847.9 ±159.8 2. 2358.7 ±396.1 3. 1892.2 ±547.4	2.78 ±0.57	2.23 ±0.53	81.0 ±15.6	17.0 ±7.21
blood SBA agglutinated	1. 802.8 ±233.2 2. 1553.2 ±302.9 3. 1492.1 ±277.2	1.93 ±0.31	1.86 ±0.18	89.6 ±12.8	11.7 ±5.1

Table 4.1: continued

leukoctye soarce	mean C.P.M.* ± S.D.	stimulation index ConA	stimulation index LPS	% of cells +ve for acid phosphatase	% of cells +ve for anti-trout IgM
blood SBA unagglutinated	1. 928.6 ±292.6 2. 1883.6 ±391.8 3. 2179.7 ±413.9	2.03 ±0.11	2.34 ±0.33	83.0 ±5.03	13.0 ±4.0
kidney SBA agglutinated	1. 766.3 ±120.0 2. 1329.1 ±233.6 3. 2638.3 ±595.8	1.74 ±0.27	3.46 ±0.38	17.3 ±9.5	22.7 ±6.51
kidney SBA unagglutinated	1. 826.4 ±135.6 2. 1313.5 ±343.8 3. 2500.8 ±467.5	1.59 ±0.14	3.08 ±0.60	12.5 ±7.3	24.0 ±6.24
thymus SBA agglutinated	1. 848.2 ±92.03 2. 2736.3 ±661.8 3. 1144.5 ±308.0	3.25 ±0.43	1.35 ±0.09	0.0	2.1 ±1.91
thymus SBA unagglutinated	1. 869.4 ±189.8 2. 3263.2 ±1188.7 3. 1034.6 ±344.3	2.76 ±0.17	1.19 ±0.20	0.0	1.8 ±1.75

* the C.P.M. (counts per minute) values are the means of three fish with each fish done in triplicate.

** 1. control unstimulated cultures.
2. Concanavalin A stimulated cultures.
3. Lipopolysaccaride stimulated cultures.

populations of PNA separated thymocytes were obtained (agglutinated and unagglutinated). The recovery of PNA separated thymocytes was 50% of the original cells put onto the column.

Table 4.1 shows the results of the mitogen stimulation assays from nylon wool and SBA separated subpopulations. When non-separated control leukocytes were used there were no significant differences ($P>0.05$) between spleen and peripheral blood cell responses to ConA. However, the blood lymphocytes responded significantly more to ConA than the kidney lymphocytes ($P>0.05$). The thymus derived lymphocytes responded significantly more to ConA than the spleen, kidney and peripheral blood lymphocytes ($P>0.05$). Against LPS, the kidney derived lymphocytes had a significantly higher response than the other three organs, and both the spleen and peripheral blood lymphocytes responded significantly more to LPS than the thymus derived lymphocytes ($P>0.05$).

The splenic nylon wool non-adherent cell population responded significantly more to ConA ($P>0.05$) than the adherent population (Table 4.1). However the splenic adherent population responded significantly more to LPS than did the non-adherent population. A similar response pattern was obtained with peripheral blood and kidney lymphocytes. The thymus non-adherent cells responded significantly more to ConA than to LPS ($P>0.05$). There were insufficient adherent thymocytes to perform mitogen stimulation assays.

With SBA separated cell populations, there was no significant difference between the agglutinated and unagglutinated cell population in the response to ConA or LPS, in any of the organs or tissues tested. An important consideration when considering when assessing the mitogenic response of the SBA separated populations is whether or not the SBA lectin itself exerts a large mitogenic effect on the leukocytes. To investigate this, leukocyte

populations were exposed to varying concentrations of SBA in the same tritiated thymidine assay as before. It was found that there was only a very small mitogenic effect (maximum S.I. of between 1.1-1.2.) seen in the SBA cultures at any of the concentrations tested (Fig 4.1). This combined with the fact that the SBA separated populations were thoroughly washed in HBSS containing 0.2M D-galactose would have ensured that any mitogenic effect being exerted by SBA did not interfere with the ConA and LPS assays.

The results of the cytochemical staining of the unseparated and separated leukocyte populations are shown in Table 4.1. The thymocytes did not stain positive for acid phosphatase, which can be visualised as a red focal dot close to the cell membrane at one end of the cell only (Plate 4.1). The lymphocytes from all of the other sources showed some acid phosphatase staining lymphocytes. The macrophages and monocytes also stained positive for acid phosphatase, but thrombocytes did not. The lymphocytes from the spleen and peripheral blood showed significantly higher numbers of positive lymphocytes than from the kidney ($P>0.05$). There was no significant differences in the level of acid phosphatase positive lymphocytes between SBA agglutinated and unagglutinated populations from the spleen, kidney or peripheral blood. There were significantly more acid phosphatase positive lymphocytes in the nylon wool non-adherent population than in the adherent population, from both the spleen and kidney. Table 4.1 shows that all organs and tissues tested contained some lymphocytes that stained positive with the anti-trout IgM monoclonal antibody (Plate 4.2); ranging from 2.33% in the thymus up to 21.7% in the kidney. There was no significant difference in the number of IgM positive lymphocytes in any of the SBA and nylon wool subpopulations from all of the organs and tissues tested.

The two PNA separated thymocyte subpopulations were tested for

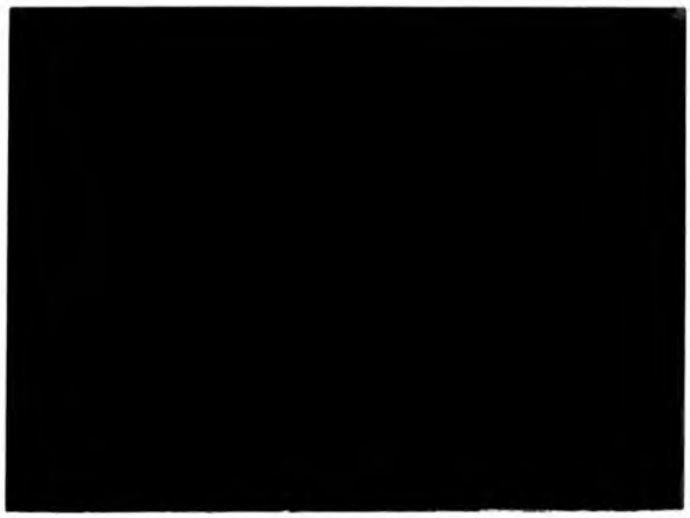


Plate 4.1: Rainbow trout peripheral blood leukocytes showing acid phosphatase activity as a red focal dot, marked by the arrows (►), present close to the cell membrane, at one end of the cell only. The thrombocyte (⟶) does not show this focal dot. Geimsa counterstain (x1000 magnification).

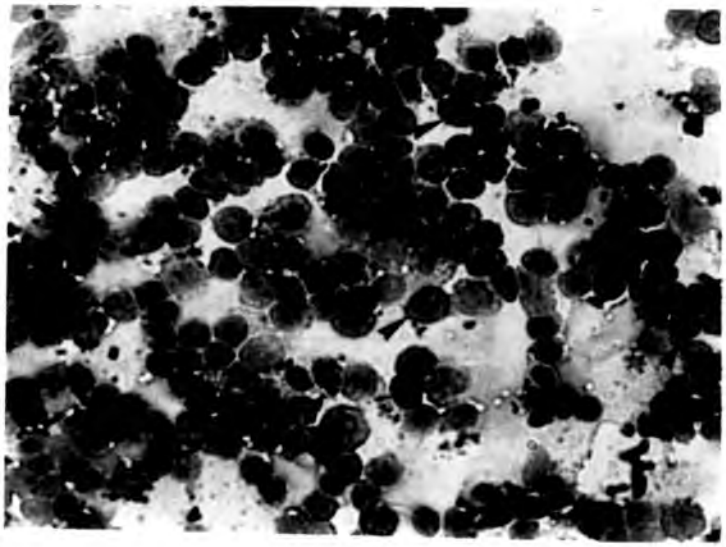


Plate 4.2: Mouse anti-trout IgM monoclonal antibody staining of rainbow trout spleen leukocytes. The HRP anti-mouse IgG was added as the secondary antibody, and a DAB substrate was added for 5 minutes. The slides were then counterstained using haematoxylin. Some of the positive leukocytes can be visualised by the arrows. (x400 magnification)

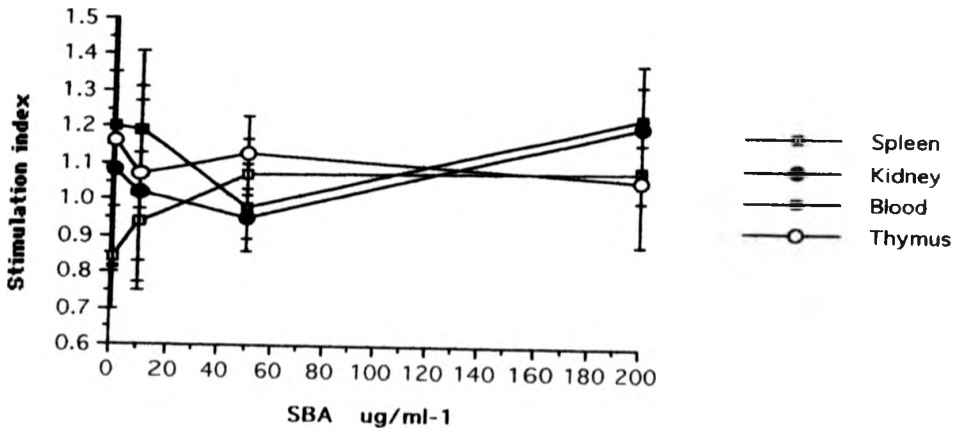


Fig 4.1: The mitogenic response of thymus, spleen, kidney and peripheral blood leukocytes to increasing *in vitro* concentrations of the lectin soybean agglutinin (SBA). The mitogen assays were carried out using the same protocol described for Con A and LPS assays.

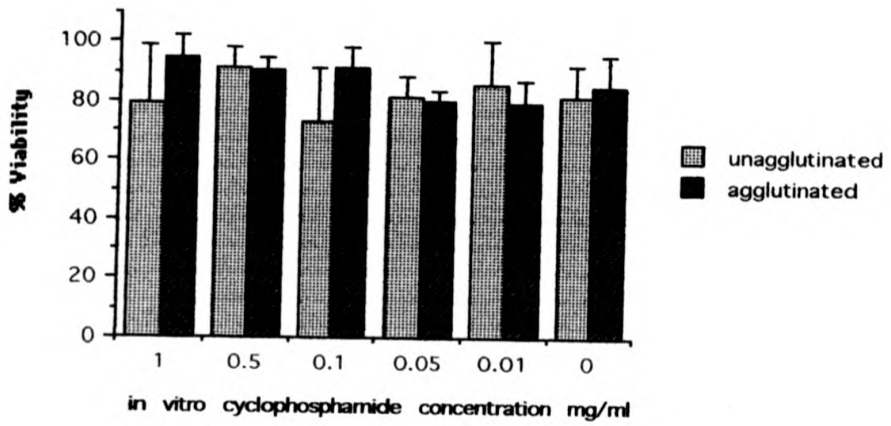


Fig 4.2: The *in vitro* toxicity of cyclophosphamide on peanut agglutinin (PNA) separated thymocytes. The viabilty was measured, after 72 hours in culture, using the trypan blue exclusion test.

in vitro cyclophosphamide cytotoxicity to see if either one was more susceptible. Fig 4.2 shows that cyclophosphamide did not appear to have any *in vitro* cytotoxicity to either of the two PNA separated thymocytes.

4.4. Discussion:

This study has shown that when lymphocytes isolated from rainbow trout are tested for their capacity to respond to the mitogens ConA and LPS, the cells from the different lymphoid organs display unique responses. These results confirm those of Etlinger et al (1976) on the differential mitogenic responses of lymphocytes from the different lymphoid organs in rainbow trout. These results suggest rainbow trout thymocytes represent a cell population that is restricted to T-like cells (ConA responsive lymphocytes) while kidney lymphocytes contain a larger proportion of B-like cells (LPS responsive lymphocytes). The splenic and peripheral blood lymphocytes responded well to both mitogens, suggesting that they contain a significant number of both cell types.

The use of enzyme markers for the identification of mammalian lymphocyte populations has demonstrated that acid phosphatase is of T lymphocyte origin (Tomaoki and Essner, 1969; Catovsky et al, 1974; Stein and Muller-Hermelink, 1977). Acid phosphatase has also been shown in fish lymphocytes (Blaxhall and Hood, 1985), with approximately 70% of peripheral blood lymphocytes staining positive for the enzyme. In the present study it was found that 88% and 81% of the blood and splenic lymphocytes stained positive, with only 51% of the kidney lymphocytes staining positive. If, in fact, acid phosphatase is a T cell marker in fish then these results correspond fairly well with the mitogenic study, where the splenic and peripheral blood lymphocytes show a greater response to Con A. Kidney lymphocytes, on the

other hand, respond significantly more to LPS and show significantly lower levels of acid phosphatase staining than the splenic and peripheral blood cells, further suggesting a higher number of B-like cells in this population. However, rainbow trout thymocytes showed no acid phosphatase activity unlike human thymocytes where Basso et al (1980) found that acid phosphatase activity was present in a high percentage of foetal and post-natal thymocytes and circulating T lymphocytes. It is possible that in rainbow trout, acid phosphatase activity is restricted only to mature circulating lymphocytes, but further work is necessary to substantiate this hypothesis.

The passage of murine lymphocytes over a nylon wool column leads to a T cell enriched non-adherent population (Trizio and Cudkowicz, 1974). In the current study, fish lymphocytes were separated into two subpopulations, using nylon wool columns, which differed significantly in their responses to LPS and Con A. The non-adherent cell populations from the spleen, kidney and peripheral blood responded significantly better to Con A than to LPS. However the peripheral blood non-adherent cell population had a significantly lower response to Con A than the peripheral blood control lymphocytes, which were untreated with nylon wool. Also, the splenic non-adherent cell population did not differ significantly from the unseparated control cells in their response to Con A. If the non-adherent cell population are enriched in T cells then an increased response to Con A would have been expected (compared to the unseparated population). However, it has been found that some T cell subpopulations in mammals may be retained in the nylon wool column (Tada, 1977; Tada et al, 1978). This might also be the case with fish lymphocytes, with some of the T cell population involved in the response to mitogens being retained. The fact that up to 35% of the kidney adherent cell population still contain lymphocytes that stain positive for acid phosphatase suggests that

there is considerable retention of T-like cells in the nylon wool column. However, significantly more T-like cells pass through the nylon wool column (based on acid phosphatase staining) suggesting that some T cell enrichment has occured. Since there was no difference in the numbers of cells staining positive with the anti-trout IgM monoclonal antibody, which together with the mitogenic response to LPS of the non-adherent cell populations from the splenic, kidney and peripheral blood, suggests that some B cells are also passing through the column. Hence it would appear that the two nylon wool cell subpopulations show some T (non-adherent) and B (adherent) cell properties but with some cross-contamination. Further in vitro characterisation of the cell subpopulations will be needed before any further conclusions can be drawn regarding the nature of the two subpopulations.

When rainbow trout lymphocytes are separated using soybean agglutinin (SBA), two main subpopulations are obtained which (unlike the nylon wool-separated populations) do not show any significant differences in their response to the mitogens Con A and LPS. The subpopulations also do not show any significant differences in staining patterns for acid phosphatase or IgM. These data suggest that the two populations fractionated by SBA are unrelated to T and B cell subsets. Further in vitro analysis , such as response to thymus dependent and independent antigens will be required to further clarify these findings.

In murine systems it has been shown that separation of thymocytes using PNA produces a population of agglutinated cells, consisting of immature cortical thymocytes and a population of mature medullary cells which bind the lectin poorly (Reisner and Sharon, 1980). The unagglutinated layer contained cells which were similar to cyclophosphamide resistant mature thymocytes or splenic T cells. In the current study, neither of the two populations obtained

using PNA showed any significant *in vitro* cyclophosphamide sensitivity. However, before any further conclusions can be drawn, *in vitro* analysis such as the mixed lymphocyte response (MLR) of the two subpopulations must be carried out.

In conclusion, the present study has shown that it is possible to separate rainbow trout leukocytes into two subpopulations using soybean agglutinin and nylon wool. The results suggest that the best method for obtaining enriched populations of T and B cells is nylon wool separation (based on the maximal response to ConA and LPS, and acid phosphatase staining patterns). While SBA separates leukocytes into two populations, these are unrelated to T and B cells.

N.B. Part of this chapter has been accepted for publication in Comparative Haematology International: Findlay, C and Tatner, M F. A comparative study of T and B lymphocytes in rainbow trout (*Oncorhynchus mykiss*) following their separation by nylon wool adherence and lectin agglutination techniques.

Chapter 5:

The effect of reconstitution with cryopreserved lymphocytes on the antibody response to sheep red blood cells, *Aeromonas salomonicida*, and DNP-KLH, in adult long term thymectomised rainbow trout.

5.1. Introduction: One of the problems concerning the relevance to teleost fish of designating antigens as thymus dependent (TD) or thymus independent (TI) is that the role of the thymus in the control of antibody production is not fully understood. There has been very little work on the role of the thymus in the immune response of fish. Although thymectomy is the best approach to study the involvment of the thymus in immunological processes, this method has unfortunately received very little attention. This is probably due to the difficulties involved in performing a reliable ablation of the thymus (Tatner, 1990). However thymectomies performed mostly by microsurgery or microcauterisation have been attempted in carp (Manning et al, 1982a), trout (Tatner, 1986; Tatner, 1987; Tatner et al, 1987) and the rockfish (Nakanishi, 1986). A thymectomy-like effect was described in the goldfish by Desvaux and Charlemagne (1983), where acute thymic depletion was induced by X-ray irradiation focused on the thymic area. The effect of thymectomy on cellular immunity has been studied by skin grafting. It was shown that removal of the thymus from two month old rainbow trout delayed allograft rejection (Manning et al, 1982a). Skin grafts were rejected by day 14 in the non-thymectomised control fish, but rejection in the thymectomised fish was still incomplete at the end of the experiment on day 20. Adult thymectomy had no effect on the allograft rejection (Sailendri and Muthukkaruppan, 1975; Manning et al, 1982a).

The effects of thymectomy on the humoral response are controversial. Manning et al (1982a) provided tentative evidence for a suppresive role for the thymus in antibody formation, by thymectomising adult carp, and then immunising with *Aeromonas salmonicida* four weeks later. This resulted in elevated serum antibody titres at day 7 after immunization, returning to normal levels later in the response. In one year old trout, thymectomy had no effect on

antibody production to *A.salmonicida*, but with human gamma globulin in adjuvant, humoral antibody levels were elevated in the thymectomised fish. Nakanishi (1986) showed that short term thymectomy of 2-3 year old adult rockfish, *Sebastiscus marmoratus*, coupled with X-irradiation enhanced antibody levels to sheep red blood cells. Tatner (1986) showed that in rainbow trout fry thymectomised for 1-2 months, the primary response to human gamma globulin (HGG) was unaltered, but the secondary response was reduced. When thymectomy was performed later, the effect on the secondary response to HGG was no longer apparent, but the primary response to *A.salmonicida* was slightly reduced. Using adult trout Secombes (1981) found that thymectomy had no effect on the response to *A.salmonicida*, but both the primary and secondary responses to HGG were elevated. Tatner et al (1987) investigated the antibody response to HGG and *A.salmonicida* in control and long term thymectomised rainbow trout. There was no difference in the response between control fish and five month thymectomised fish to HGG, however nine month thymectomised fish had lower titres than the controls. There was no difference in the response to *A.salmonicida* between the controls and five and nine month thymectomised fish. The authors suggested that the reduced response to HGG could be due to a reduction in the level of T-helper cells in the periphery due to thymectomy. Clearly the role of the thymus and the existence of helper and suppressor cell equivalents in the fish humoral immune response needs further investigation in both adult and young stage fish.

In the present study, the effect of two month and eight month thymectomy on the *in vivo* antibody response to sheep red blood cells (SRBC), *A.salmonicida* bacteria and dinitrophenylated keyhole limpet heamocyanin (DNP-KLH) antigens was investigated. SRBC and DNP-KLH

have both been shown to be thymus dependent antigens in mammals (Miller and Mitchell, 1969; Feldman et al, 1974). *A.salmonicida* is a particulate, putative thymus independent antigen. One way of clearly showing that the thymus is involved in the antibody response to a particular antigen, is to remove the thymus, and once an immune dysfunction has been established, to return autogenic thymocytes and show restoration of the immune function. Hence a particular function could be undisputably assigned to the thymus. For example Horton and Horton (1975) looked at the effects of transplantation of thymus into early thymectomised (5 days post-hatching) *Xenopus laevis*, on allograft immunity. Thymectomy greatly increased the time required for *Xenopus* to completely reject the allografts. The fact that the absence of the thymus was specifically related to the defect in first-set alloimmunity was confirmed by implanting allogenic thymus into 2 week thymectomised recipient toads. The donor thymus remained healthy and restored the allograft response, in contrast to allogenic spleen which did not restore the normal allograft response, and itself often underwent destruction. Tochinai et al (1976) thymectomised *Xenopus laevis* larvae 5 days after fertilisation. The 1-1.5 year old thymusless toads, thus obtained, were implanted with histocompatible thymus from 1 year old toads and 4 weeks later were given skin allografts or rabbit red blood cells (RRBC) to determine their immune response. In contrast to the perfectly tolerant thymusless toads, the implanted animals rejected skin grafts after only slightly longer times than the non-thymectomised controls. Accelerated rejection times of second-set skin grafts were also seen in the implanted animals. Similarly , the capacity of antibody production against RRBC was restored to the thymus implanted animals to almost the nonthymectomised control level. In humans, a number of immune deficiency diseases result from the lack of a functioning thymus (Taussig, 1984), and in a

limited number of cases, thymus transplantation is used as a clinical treatment (Cachiero et al, 1985), although, in the absence of a compatible donor, such therapy is severely limited. The use of cultured thymus fragments, which depletes the donor lymphocytes, has allowed some allogenic grafts to be accepted, although complete imunological restoration is not readily achieved. The lack of inbred lines is also the major problem associated with the use of thymus transplantation to investigate the effects of long term thymectomy in fish. This study aims to overcome the lack of inbred lines, in rainbow trout, by using the technique of cryopreservation of thymocytes.

The technique of cryopreservation has been successfully applied to murine and human thymocytes, to show a direct causal relationship between certain cell types and a distinct immunological function (Cachiero et al, 1985; Ichino and Ishikawa, 1985). Cachiero et al (1985) showed that transplantation of a single cryopreserved 18-20 day old foetal or 1-2 day old neonatal congenic thymus into an athymic nude mouse (from a homozygous phenotypically normal hairy litter mate), resulted in the thymus grafted animal's spleen cells proliferative capacity, to the T cell mitogens concanavalin A and phytohaemagglutin, and the generation of plaque forming cells to sheep red blood cells, being greatly increased (up to 10 times) when compared to non-grafted control athymic mice, and only slightly below the responses of the normal hairy mice. Ichino and Ishikawa (1985) have shown that human lymphocyte viability and natural killer (NK) activity are unaffected following cryopreservation. Fish leukocyte viabilty and proliferative responses, to both T and B cell mitogens, have also been shown to be largely unaffected following cryopreservation (Tatner, 1988; Tatner and Findlay, 1992; and section 2.8).

In the present study the effects of reconstitution of cryopreserved thymocytes, into long term thymectomised adult rainbow trout, on the *in vivo*

antibody response to thymus dependent (SRBC and DNP-KLH) and thymus independent (*A. salmonicida*) antigens was assessed. The cryopreserved thymocytes were thawed and returned to the original donor at the desired time via the caudal vein, which has previously been shown to be a viable route for reintroducing cells back into the circulation (Tatner and Findlay, 1991).

5.2. Materials and Methods:

5.2.1. Fish : One year old rainbow trout (approximate weight 200-250g), supplied by College Mill Trout Farm, were used througout this experiment. They were kept in aerated and dechlorinated water, within a temperature range of 7-12 ^{o}C. The fish were fed daily with EWOS No4 trout pellets.

5.2.2. Thymectomy, cryopreservation and reconstitution: Fish were thymectomised as described by Tatner (1990). For each antigen, four groups of fish were used: intact controls , thymectomised controls, secondary response controls and thymectomised fish which had had their thymocytes cryopreserved as described in section 2.8. This last group of fish were marked individually using a panjet (Wright Dental Group, Dundee, U.K.) loaded with 1% alcian blue (using the same mark given to the cryovial containing their cells). One week before the secondary immunization the thymocytes were thawed as described in section 2.8, resuspended in 0.1 mls of sterile PBS, and injected into the fish via the caudal vein using a 1ml syringe with a 26 gauge needle. Three antigens were used in this study: sheep red blood cells (SRBC), *Aeromonas salmonicida* 1102, and dinitrophenylated keyhole limpet heamocyanin (DNP-KLH). Two groups of thymectomised fish were used for each antigen, one group which was thymectomised for two months and the other group which was thymectomised for eight months before primary

immunisation. The experimental protocols for each group of fish are described in Fig 5.1-Fig 5.5. The thymectomised fish were examined regularly throughout the dormant period to ensure that there was no regeneration of thymic tissue. The numbers of cells frozen and the viabilty of the thymocytes before freezing and after thawing, for each group of fish are shown in Appendix 4.

5.2.3. Antigens : Dinitrophenylated Keyhole limpet Haemocyanin (DNP-KLH) was purchased from Calbiochem (Lajolla, California, USA: 49 DNP groups per protein molecule). The sheep red blood cells (SRBC) were obtained from the Scottish Antibody Production Unit (SAPU). These were supplied in 0.01% Alseviers solution, hence the cells were washed three times in sterile 0.85% saline before use. The *Aeromonas salmonicida* strain 1102 (a non-virulent non auto-agglutinating strain) was obtained from the Institutes own bacterial stocks. The bacteria were grown in 100 mls of brain heart infusion broth (Difco, East Mosley, U.K.) for 24 hours at 20°C, and formalin added at 0.6% for 48 hours. The cells were washed three times in 0.85% saline and resuspended to 10^9 cells ml^{-1}, using the same technique as in section 2.9.1.

5.2.4. Immunizations :

(a) DNP-KLH:

The rainbow trout were immunized by intraperitoneal (i.p.) injection of 100ug of DNP-KLH in 100ul of phosphate buffered saline (appendix 1) emulsified in an equal volume of Freunds complete adjuvant (FCA, Sigma). The final volume injected into the fish was 0.2 mls. Where applicable, the fish were given a second injection of 50ug DNP-KLH (in 0.1 mls PBS) emulsified in an equal volume of Freunds incomplete adjuvant (FIA, Sigma). Also at this time, a

further group of control fish were given a primary injection of 50ug DNP-KLH emulsified in an equal volume of FIA (Secondary response controls) in 0.2 mls (Fig 5.1).

(b) Sheep red blood cells (SRBC):

The SRBC groups (Fig 5.2 and Fig 5.3) were given a primary i.p. injection of 0.1mls of 5% SRBC in 0.85% saline emulsified in an equal volume of FCA. The final volume injected was 0.2 mls. Where applicable, the fish were given a secondary injection of 0.1 mls Of 0.25% SRBC emulsified in an equal volume of FIA, and a further group received this as their primary injection (secondary response controls).

(c) *Aeromonas salmonicida* 1102:

The *Aeromonas salmonicida* group (Fig 5.4 and Fig 5.5) received a primary injection of 10^8 cells of *A.salmonicida* suspended in 0.1 mls of 0.85% saline, emulsified in an equal volume of FCA. The secondary injection consisted of 10^5 *A.salmonicida* cells suspended in 0.1 mls of 0.85% saline emulsified in an equal volume of FIA, and this was also given to the secondary response control group, injected at the same time.

5.2.5 Sera collection: Blood samples were collected fortnightly from the caudal vein using a 1 ml syringe, fitted with a 26 gauge needle.Between 0.2 and 0.3 mls of blood was withdrawn from each fish. If any fish showed signs of becoming anaemic, it was omitted from the next sampling, thus ensuring minimal loss of experimental fish. The samples were added to 0.5 ml epindorf tubes and allowed to clot overnight at 4°C. The next day the tubes were spun x3000g in a MSE microcentrifuge and the serum removed from the clot using a Pasteur pipette, and frozen at -20°C. Serum samples were thawed once only.

DNP-KLH immunized, eight month thymectomised group

Intact control	8 month thymectomised control	8 month thymectomised reconstituted	secondary response control
week 0			
primary injection 0.2 mls of 100ug DNP-KLH/FCA	primary injection 0.2 mls of 100ug DNP-KLH/FCA	primary injection 0.2 mls of 100ug DNP-KLH/FCA	
		week13 thymocytes reconstituted	
week 14 secondary injection 0.2 mls of 50ug DNP-KLH/FIA	secondary injection 0.2 mls of 50ug DNP-KLH/FIA	secondary injection 0.2 mls of 50ug DNP-KLH/FIA	primary injection 0.2 mls of 50ug DNP-KLH/FIA

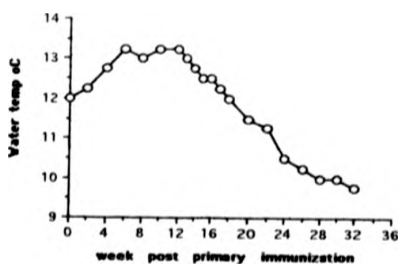


Fig 5.1: The immunization schedule used in the DNP-KLH eight months thymectomised group. The water tank temperature variation throughout the sampling period is also shown.

SRBC immunised two month thymectomised group

Intact controls	2 month thymectomised controls	2 month thymectomised reconstituted	Secondary response controls
week 0			
primary injection 0.2 mls of 5% SRBC/FCA	primary injection 0.2 mls of 5% SRBC/FCA	primary injection 0.2 mls of 5% SRBC/FCA	
		week 15 thymocytes reconstituted	
Week 16 secondary injection 0.2 mls of 0.25% SRBC/FIA	secondary injection 0.2 mls of 0.25% SRBC/FIA	secondary injection 0.2 mls of 0.25% SRBC/FIA	primary injection 0.2 mls of 0.25% SRBC/FIA

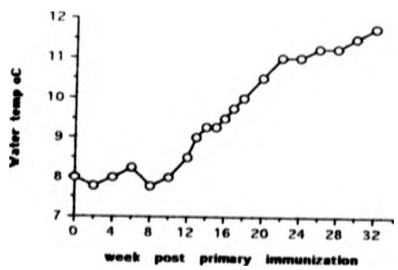


Fig 5.2: The immunization schedule used in the SRBC two month thymectomised group. The water temperature variation throughout the sampling period is also shown.

SRBC immunised, eight month thymectomised group

Intact controls	8 month thymectomised controls	8 month thymectomised reconstituted	Secondary response controls
Week 0			
primary injection 0.2 mls of 5% SRBC/FCA	primary injection 0.2 mls of 5% SRBC/FCA	primary injection 0.2 mls of 5% SRBC/FCA	
		week 13 thymocytes reconstituted	
week 14 secondary injection 0.2 mls of 0.25% SRBC/FIA	secondary injection 0.2 mls of 0.25% SRBC/FIA	secondary injection 0.2 mls of 0.25% SRBC/FIA	primary injection 0.2 mls of 0.25% SRBC/FIA

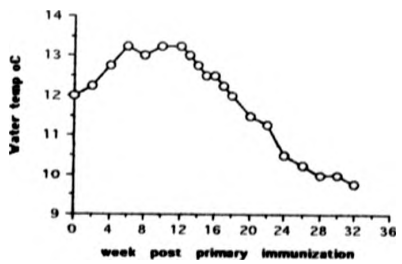


Fig 5.3: The immunization schedule used in the SRBC eight month thymectomised group. The water temperature variation throughout the sampling period is also shown.

A.salmonicida 1102 immunised, two month thymectomised group

Intact controls	2 month thymectomised controls	2 month thymectomised reconstituted	Secondary response controls
week 0			
primary injection	primary injection	primary injection	
0.2 mls of 10^8 cells	0.2 mls of 10^8 cells	0.2 mls of 10^8 cells	
in saline/ FCA	in saline/FCA	in saline/FCA	
		week17	
		thymocytes reconstituted	
week 18			
secondary injection	secondary injection	secondary injection	primary injection
0.2 mls of 10^5 cells	0.2 mls of 10^5 cells	0.2 mls of 10^5 cells	0.2 mls of 10^5 cells
in saline/FIA	in saline/FIA	in saline/FIA	in saline/FIA

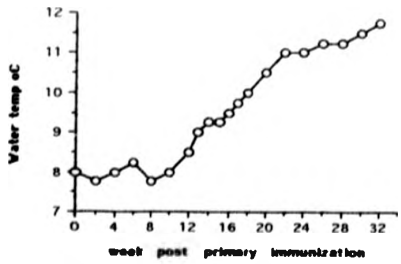


Fig 5.4: The immunization schedule used in the *Aeromonas salmonicida* 1102 two month thymectomised group. The water temperature variation throughout the sampling period is also shown.

A.salmonicida 1102 immunised, eight month thymectomised group

intact controls	8 month thymectomised control	8 month thymectomised reconstituted	secondary response control
week 0			
primary injection	primary injection	primary injection	
0.2 mls of 10^8 cells	0.2 mls of 10^8 cells	0.2 mls of 10^8 cells	
in saline/FCA	in saline/FCA	in saline/FCA	
		week 15	
		thymocytes reconstituted	
week 16			
secondary injection	secondary injection	secondary injection	primary injection
0.2 mls of 10^5 cells	0.2 mls of 10^5 cells	0.2 mls of 10^5 cells	0.2 mls of 10^5 cells
in saline/FIA	in saline/FIA	in saline/FIA	in saline/FIA

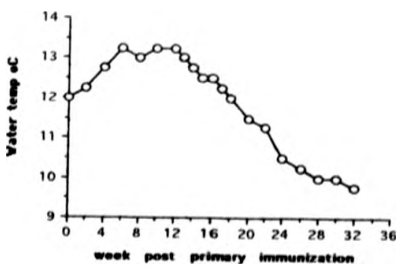


Fig 5.5: The immunization schedule used in the *A. salomonicida* 1102 eight months thymectomised group. The water temperature variation throughout the sampling period is also shown.

5.2.6. Determination of antibody activity: The antibody levels in the serum samples were determined by an ELISA procedure . The assays used to detect antibodies to each of the antigens used are described in section 2.9.

5.2.7. Analysis of results: The results were analysed using Minitab on a Apple Macintosh computer. To test whether differences in titres between groups were significant, a two-sample *t*- test was used. To test for secondary responses, the memory factor (Nossal et al, 1965) was calculated as follows:

$$\text{memory factor} = \frac{S(x) - S(o)}{P(x)}$$

Where S(x) is the titre of fish sampled at day x (peak response) after secondary injection; S(o) is the residual titre, resulting from priming, present at the day of secondary injection, and P(x) is the titre of fish sampled at day x after primary injection.

Results :

The antibody responses of the intact control, two month thymectomised control and reconstituted groups to SRBC are shown in Table 5.1. During the primary response, the fish responded well to this antigen, with the highest titre at week 2 post primary immunization in the intact control and thymectomised control groups, and at week 10 in the thymectomised cryopreserved group (Fig 5.6). The variation in response was fairly low througout the primary response. There was no significant difference in the primary response titres between any of the three groups at any time tested. After a second injection, the titres did increase slightly and reached a peak at week20, for all the three groups. All three groups showed significantly higher titres ($P>0.05$) than the secondary response controls throughout the secondary response. For the intact control

Table 5.1: The *in vivo* antibody response to SRBC in control, two month thymectomised and two month thymectomised reconstituted rainbow trout,as measured by ELISA.

week post primary immunization	mean anti-SRBC titre (-log2+1)[1] Intact control	thymectomised control	thymectomised reconstituted	secondary response control	significance (differences were considered significant if P>0.05)
	n=15	n=15	n=15	n=15	
2	5.59 ±0.77	5.80 ±0.61	5.36 ±0.98	-	NS
4	4.69 ±0.73	4.83 ±0.58	5.06 ±0.69	-	NS
6	5.52 ±1.08	5.11 ±1.43	5.29 ±0.84	-	NS
8	5.66 ±1.16	5.62 ±1.46	5.57 ±0.90	-	NS
10	5.85 ±0.98	5.21 ±1.44	5.61 ±1.33	-	NS
12	4.35 ±1.11	4.83 ±1.03	5.01 ±1.20	-	NS
14	3.98 ±0.87	3.60 ±1.73	4.21 ±1.24	-	NS
15	-	-	thymocytes reconstituted	-	
16	secondary immunization	secondary immunization	secondary immunization	primary immunization	
18	8.11 ±1.35	8.77 ±1.89	8.48 ±1.03	3.37 ±2.26	All higher (P>0.001) than secondary response controls.
20	8.72 ±1.82	9.13 ±1.95	9.40 ±2.11	3.09 ±2.34	All higher (P>0.001) than secondary response controls.
22	8.62 ±1.74	8.99 ±1.86	8.42 ±1.41	4.01 ± 1.98	All higher (P>0.005) than secondary response controls.

Table 5.1: continued.

weeks post primary immunization	mean anti-SRBC titre (-log2+1) Intact control	thymectomised control	thymectomised reconstituted	secondary response controls	significance (differences were considered significant if P>0.05)
24	7.88 ±1.50	8.58 ±1.47	7.47 ±1.71	4.14 ±1.17	All higher (P>0.001) than the secondary response controls.
26	7.19 ±1.37	6.82 ±1.50	7.27 ±2.01	3.68 ±1.85	All higher (P>0.001) than the secondary response controls.
28	6.77 ±1.70	6.13 ±1.11	5.98 ±1.89	3.08 ±1.05	All higher (P>0.005) than the secondary response controls.
30	5.23 ±1.79	4.62 ±1.17	4.91 ±1.33	3.22 ±1.57	All higher (P>0.005) than the secondary response controls.

N.S. means no significant difference between titres. (N.B. differences were considered significant if P>0.05)

1. SRBC ELISA were done using a PBS blank in this case. It was found that non-immunised fish sera had a natural antibody titre against SRBC. This was found to have a mean titre of 2.84 ±0.56. A control of pooled (7 weeks post primary immunisation) non-immune sera was included in each ELISA.

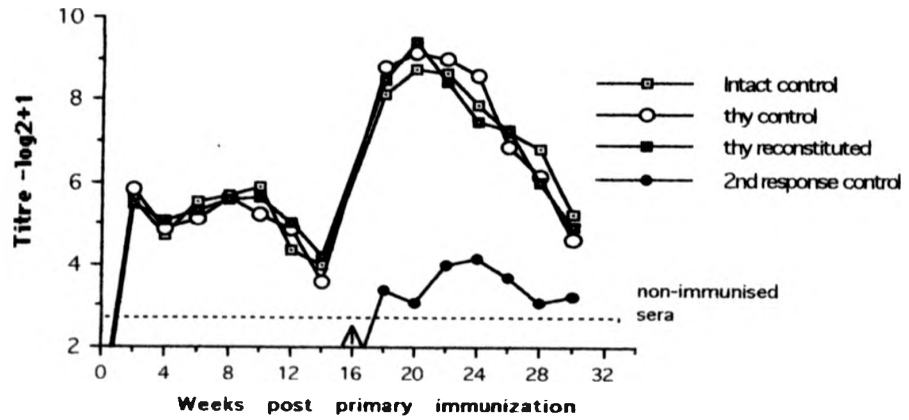


Fig 5.6: The effect of two month adult thymectomy on the *in vivo* primary and secondary antibody response to SRBC, as measured by ELISA. The arrow (^) represents the time of secondary immunization. It was found that non-immune sera had a natural antibody titre of 2.86 +0.56. This is represented in the graph as the broken line.

group, the peak titre, in the secondary response was 8.72 on week 20 (Fig 5.6), which produced a memory factor of 1.03 (Table 5.6). The thymectomised control group had a peak secondary response titre of 9.13 on week 20, producing a memory response of 1.14. The thymectomised reconstituted group reached a peak secondary response of 9.40 on week 20, producing a memory factor of 1.03.

The *in vivo* antibody responses of the intact control, eight month thymectomised control and thymectomised reconstituted fish to the SRBC antigen are shown in Table 5.2. There was a prominent primary response in all of the groups, with only one peak in the response at week 6 for the intact and thymectomised controls, and at week 8 for the thymectomised cryopreserved group (Fig 5.7). There was no significant difference in the titres between any of the groups at weeks 2, 4, 6, 8 and 12. However the intact control titres were significantly higher ($P>0.05$) than the thymectomised control titres at week 10. After the secondary injection there was a significant secondary response in the intact control and thymectomised reconstituted groups, with memory factors of 1.36 and 1.10 respectively (Table 5.6). There was a negative memory factor of 0.89 in the thymectomised control group. During the secondary response all three groups had only one peak in the response at week 18 (Fig 5.7). At weeks 18 and 20 the intact control group titres were significantly higher ($P>0.01$ and $P>0.005$ respectively) than the thymectomised controls. The intact control, thymectomised control and reconstituted groups all had titres significantly higher ($P>0.01$) than the secondary response control group at weeks 16, 18 and 20. On week 22, only the intact control titres were significantly higher ($P>0.05$) than the secondary response controls. By week 24 there was no significant difference in titres between any of the groups.

Table 5.3 shows the antibody response of the intact control, two

Table 5.2: The *in vivo* rainbow trout anti-SRBC response in intact control, eight month thymectomised and eight month thymectomised reconstituted groups, as measured by ELISA.

Week post primary immunization	mean anti-SRBC titre($-\log_2+1$)[1] Intact control n=20	thymectomised control n=15	thymectomised reconstituted n=15	secondary response control n=20	significance (differences were considered significant if P>0.05)
2	2.16 ±1.93	2.31 ±2.11	1.83 ±1.4	-	NS
4	3.91 ±0.86	4.55 ±1.89	3.77 ±1.39	-	NS
6	5.29 ±1.46	4.97 ±1.89	4.39 ±1.27	-	NS
8	4.21 ±0.97	4.36 ±0.68	4.74 ±0.63	-	NS
10	3.43 ±1.62	2.98 ±1.54	2.39 ±0.87	-	Intact control higher (P>0.05) than thy control.
12	1.51 ±0.97	1.72 ±1.16	2.12 +1.64	-	NS
13	-	-	thymocytes reconstituted	-	
14	secondary immunization	secondary immunization	secondary immunization	primary immunization	
16	3.22 ±1.04	3.84 ±1.03	3.82 ±1.27	1.25 ±0.57	All higher (P>0.01) than secondary response controls.
18	6.82 ±1.06	5.78 ±0.97	6.26 ±0.89	1.94 ±1.08	Intact control higher (P>0.01) than thy controls. All higher (P>0.01) than secondary response controls.

Table 5.2 : continued

weeks post primary immunization	mean anti-SRBC titre -log2+1 Intact control	thymectomised control	thymectomised reconstituted	secondary response control	significance (differences were considered significant if P>0.05)
20	5.47 ±0.86	4.29 ±1.45	5.16 ±1.36	1.73 ±1.01	thy control lower (P>0.005) than intact controls. All higher (P>0.001) than secondary response controls.
22	3.21 ±1.43	2.56 ±1.42	2.37 ±1.33	2.14 ±0.93	Intact control higher (P>0.05) than secondary response controls.
24	1.69 ±1.12	2.78 ±1.68	1.89 ±1.43	1.88 +1.11	NS

NS means no significant difference between titres.
1. The SRBC ELISA was done using pooled non-immunised sera as negative controls. Since it was found that non-immunised sera had a natural titre of 2.84 ±0.36 this was subtracted from all of the immunised sera ELISA titres.

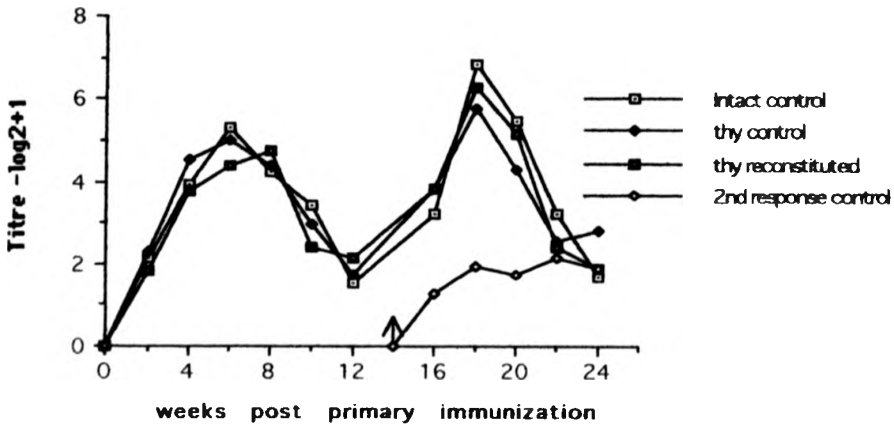


Fig 5.7: The effect of eight month thymectomy on the *in vivo* primary and secondary antibody response to SRBC, as measured by ELISA. The arrow (^) represents the the time of secondary immunization.

month thymectomised control and thymectomised reconstituted groups, to *A.salmonicida*. All fish responded well to this antigen during the primary response. The intact control and thymectomised cryopreserved groups had two peaks in the primary response, at weeks 6 and 10 and 4 and 8 respectively (Fig 5.8). The thymectomised control group showed only one peak in the primary response, at week 10. There was no significant difference in the primary response titres between any of the groups. After a second injection, there was also no significant increase in the titres in any of the groups tested. Table 5.6 shows that there was a negative memory factor of 0.88 and 0.85 in the intact control and thymectomised control groups respectively. The thymectomised reconstituted group showed a very slight positive memory response of 1.01. However all three groups had secondary response titres that were significantly higher ($P>0.05$) than the secondary response controls up to week 26, but only the thymectomised control and reconstituted groups showed titres that were significantly higher ($P>0.01$) at week 28 of the secondary response.

Table 5.4 shows the *in vivo* antibody response of intact control, eight month thymectomised controls and reconstituted groups to *A.salmonicida* 1102.There was a good primary response, with one peak in the primary response, at week 10 , in the thymectomised groups and two peaks , at weeks 6 and 10, in the intact control group (Fig 5.9). The thymectomised control titres were significantly ($P>0.01$) higher than the intact control titres at week 4, though there was no significant difference between any of the groups for the rest of the primary response. There was no real increase in the titres, after secondary injections, in any of the three groups, with memory factors of 0.72, 0.91 and 0.79 for the intact control, thymectomised control and thymectomised reconstituted groups respectively (Table 5.6). There was only

Table 5.3: The *in vivo* anti-*Aeromonas.salmonicida* 1102 antibody response in control, two month thymectomised and two month thymectomised reconstituted groups, as measured by ELISA.

weeks post primary immunization	mean anti-*A.salmonicida* titre (-log2+1) Intact control n=15	thymectomised control n=15	thymectomised reconstituted n=15	secondary response control n=15	significance (differences were considered significant if P>0.05)
2	3.06 +1.37	2.79 +1.14	2.74 +1.10	-	NS
4	3.40 +1.13	3.84 +1.23	3.80 +1.70	-	NS
6	4.17 +0.86	3.94 +0.97	3.71 +1.22	-	NS
8	3.52 +1.75	4.54 +2.24	4.31 +1.99	-	NS
10	4.45 +2.24	4.59 +1.15	4.26 +2.04	-	NS
12	2.55 +1.61	3.43 +1.79	3.20 +1.41	-	NS
14	2.79 +1.47	2.84 +1.21	2.36 +1.69	-	NS
16	2.22 +1.08	2.86 +1.43	2.10 +1.11	-	NS
17			thymocytes reconstituted.		
18	secondary immunization.	secondary immunization.	secondary immunization.	primary immunization.	
20	4.91 +1.55	5.23 +1.31	4.86 +1.77	1.46 +1.91	All higher (P>0.005) than secondary response controls.
22	4.23 +1.19	5.14 +1.38	4.86 +1.25	1.69 +1.36	All higher (P>0.005) than secondary response controls.
24	3.39 +1.81	4.12 +0.86	4.03 +1.20	0.98 +1.04	All higher (P>0.001) than secondary response controls

Table 5.3 : continued.

week post primary immunization	mean anti-*A.salmonicida* titre (-log2+1) Intact control	thymectomised control	thymectomised reconstituted	secondary response control	Significance (differences were considered significant if P>0.05).
26	3.89 +1.29	4.17 +0.97	3.80 +1.09	2.24 +1.01	All higher (P>0.005) than the secondary respopnse controls
28	3.06 +1.94	3.43 +1.16	3.52 +0.97	2.06 +0.98	Thy control and reconstituted groups higher (P>0.01) than secondary response controls.
30	2.41 +0.92	2.14 +1.04	2.40 +1.01	1.95 +1.76	NS
32	1.91 +0.85	1.49 +1.79	1.62 +0.45	1.60 +0.51	NS

NS means that there are no significant differences between titres.

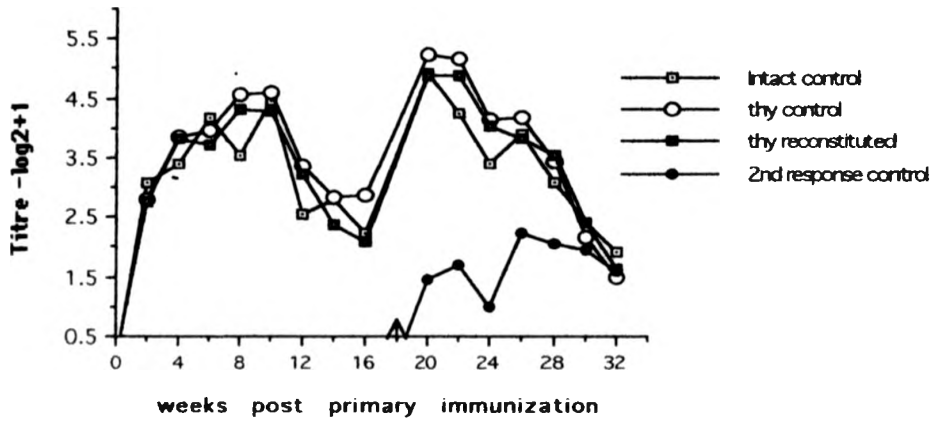


Fig 5.8: The effect of two month adult thymectomy on the *in vivo* primary and secondary antibody response to *A. salmonicida* 1102, as measured by ELISA. The arrow (^) represents the time of secondary immunization.

one peak in the secondary response at week 20 for the intact control, and at week 22 for the thymectomised control and reconstituted groups (Fig 5.9). Thymectomised control titres were significantly higher (P>0.05) than the intact control titres at week 22. There was no significant difference between the intact control, thymectomised control and thymectomised reconstituted groups at any other point during the secondary response. At weeks 18, 20, 22, 24 and 26 all three groups had titres that were significantly (P>0.01) higher than the secondary response controls, but at week 28 only the intact control and thymectomised reconstituted groups had titres that were significantly higher (P>0.05). At week 30 only the thymectomised reconstituted titres were significantly higher (P>0.05) than the secondary response controls.

The effect of eight month thymectomy on the *in vivo* antibody response to DNP-KLH is shown in Table 5.5. The fish showed a good primary response, with the thymectomised control group having only one peak in the response at week 4 (Fig 5.10), whereas the intact control group showed two peaks in the primary response at weeks 2 and 6. At week 4 the thymectomised control titres were significantly higher (P>0.01) than the intact control titres. There was a clear increase in antibody titres after the secondary injection on week 14, with a memory factor of 2.45 for the intact control group, 1.32 for the thymectomised reconstituted group and 1.17 for the thymectomised control group (Table 5.6). The peak in the secondary response occured on week 18 for all of the three groups. At week 16 the intact control titres were significantly higher (P>0.05) than the thymectomised control group, and on week 20 the thymectomised control and thymectomised reconstituted groups had significantly lower (P>0.05 and P>0.005 respectively) titres than the intact control group. At all points throughout the secondary response, the secondary response controls were significantly (P>0.05) lower than the other three

Table 5.4: The *in vivo* anti-*A.salmonicida* 1102 response in control, eight month thymectomised and eight month thymectomised reconstituted groups, as measured by ELISA.

weeks post primary immunization	mean anti-*A.salmonicida* titre (-log2+1) Intact control n=20	thymectomised control n=20	thymectomised reconstituted n=15	secondary response control n=20	significance (differences were considered significant if P>0.05)
2	3.03 ±0.61	2.92 ±0.94	2.87 ±1.15	-	NS
4	2.89 ±0.69	3.59 ±0.49	3.20 ±0.49	-	thy control higher (P>0.01) than intact controls.
6	3.82 ±1.03	3.59 ±0.61	3.72 ±1.13	-	NS
8	3.44 ±0.68	3.96 ±0.80	4.05 ±0.68	-	thy reconstituted group higher (P>0.05) than intact controls.
10	4.11 ±0.76	4.07 ±0.85	4.61 ±1.09	-	NS
12	3.22 ±0.95	3.57 ±1.37	3.52 ±0.79	-	NS
14	2.16 ±1.10	1.99 ±0.86	1.86 ±1.26	-	NS
15	-	-	thymocytes reconstituted	-	
16	secondary immunization	secondary immunization	secondary immunization	primary immunization	
18	3.54 ±1.29	4.36 ±1.07	3.80 ±1.66	1.14 ±0.99	All higher (P>0.001) than secondary response controls.
20	4.24 ±0.88	5.15 ±1.46	4.59 +1.26	1.98 +1.04	All higher (P>0.005) than secondary response controls.

Table 5.4: continued.

weeks post primary immunization	mean anti-*A.salmonicida* titre (-log2+1) Intact control	thymectomised control	thymectomised reconstituted control	secondary response	significance (differences were considered significant if P>0.05)
22	4.15 ±0.85	5.25 +1.53	4.79 ±1.03	1.99 +0.72	Thy control higher (P>0.05) than intact control. All higher (P>0.001) than secondary reponse controls.
24	3.47 ±1.10	3.96 ±1.43	3.31 ±1.54	1.13 ±1.49	All groups higher (P>0.01) than secondary response controls.
26	3.16 ±1.47	3.47 ±1.35	3.68 ±0.94	1.63 ±1.09	All groups higher (P>0.01) than secondary response controls.
28	2.46 ±1.34	1.91 ±1.71	2.25 ±0.84	1.24 +0.88	Intact control and thy reconstituted groups higher (P>0.05) than secondary response controls.
30	1.91 ±1.25	2.02 ±1.05	1.68 ±0.99	1.08 ±0.91	thy control higher (P>0.05) than secondary response controls.

NS means there was no significant differences between titres.

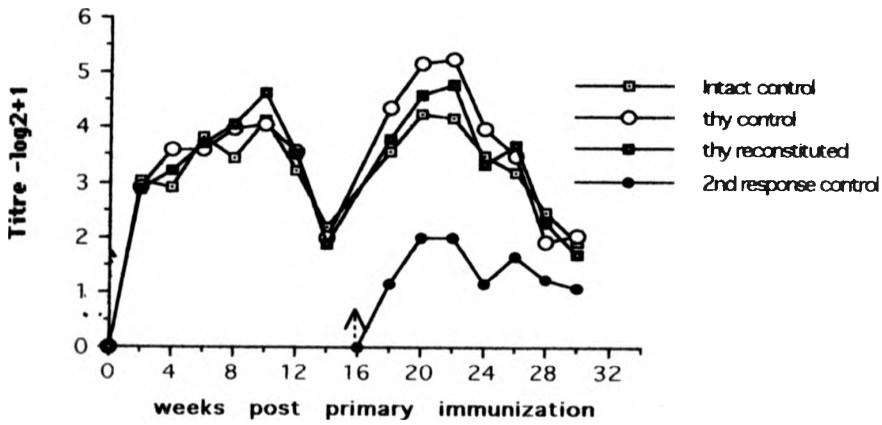


Fig 5.9: The effect of eight month adult thymectomy on the *in vivo* primary and secondary antibody response to *A.salmonicida* 1102, as measured by ELISA. The arrow (^) represents the time of secondary immunization.

groups.

Discussions : This study has shown that rainbow trout are able to mount an immune response against SRBC, *A.salmonicida* and DNP-KLH. The fish responded best to SRBC and had the lowest titres to DNP-KLH. SRBC and DNP-KLH have been described as thymus dependent (TD) antigens in mammals, and *A.salmonicida* is considered thymus independent (TI). Etlinger et al (1979) showed that trout responded poorly to TD antigens, but in this study the thymus dependent SRBC appeared to produce the greatest response. This could have been due to a dose effect. For example Lamers et al (1985) looked at the humoral antibody response and memory formation in the carp, following injection with *Aeromonas hydrophila* bacterin. They found that the height of the secondary response was positively correlated with the amount of antigen given in the priming dose.

Miller and Clem (1984a) have shown a primary response to both TD and TI antigens, using an *in vitro* microsystem for channel catfish. Miller et al (1985), using the same system, demonstrated that the response to TI antigens required the presence of B cells (sIg+) and macrophages, whereas the response to TD antigens required B cells, macrophages and sIg- cells (T helper cells). In the present study, both the control and two month thymectomised trout responded equally well to SRBC (Table 5.1). There was a good primary response in all groups. After the secondary injection, there was a poor secondary response, with no significant difference between the thymectomised and intact control groups. However there was a trend of slightly higher titres in the thymectomised control group for the first eight weeks of the secondary response (Fig 5.6).

In the eight month thymectomised SRBC immunised group, there was

Table 5.5 : The *in vivo* anti DNP-KLH response in control, eight month thymectomised and eight month thymectomised reconstituted group, as measured by ELISA.

week post primary immunization	mean anti DNP titre (-log2+1) Intact control n=20	thymectomised control n=15	thymectomised reconstituted n=15	secondary response control n=20	significance (differences were considered significant if P>0.05)
2	1.84 ±1.26	1.58 ±0.92	1.44 ±0.83	-	NS
4	1.13 ±0.82	2.20 ±1.22	1.49 ±0.82	-	thy control titres higher than the intact control (P>0.01).
6	2.11 ±0.58	1.72 ±0.56	1.68 ±0.96	-	NS
8	1.33 ±0.87	1.17 ±0.88	1.58 ±0.88	-	NS
10	1.40 ±0.75	1.29 ±0.82	1.13 ±0.82	-	NS
12	1.24 ±0.77	0.82 ±1.01	1.35 ±0.70	-	NS
13	-	-	reconstitution of thymocytes	-	
14	secondary immunization	secondary immunization	secondary immunization	primary immunization	

Table 5.5: continued.

Week post primary immunization	mean anti-DNP titre (-log2+1) Intact control	thymectomised control	thymectomised reconstituted	secondary response control	significance (differences were considered significant if P>0.05)
16	3.08 ±1.05	2.32 ±0.82	2.89 ±0.76	0.79 ±0.87	Intact control titres higher thy control (P>0.05). All higher than secondary response controls (P>0.001).
18	4.01 ±1.13	3.40 ±0.90	3.31 ±0.93	1.23 ±0.82	All higher than the secondary response controls(P>0.001).
20	3.91 ±1.02	2.99 ±0.82	2.36 ±1.06	1.24 ±0.77	thy control and thy reconstituted lower (P>0.05, P>0.005) than intact control. All higher (P>0.001) secondary response controls.
22	2.98 ±1.04	2.29 ±0.78	2.43 ±0.81	1.06 ±0.91	All higher (P>0.001) than secondary response controls.
24	2.09 ±0.83	1.52 ±1.03	1.82 ±1.16	0.68 ±0.86	All higher than the secondary response controls (P>0.05).

NS means that there was no significant differences between titres.

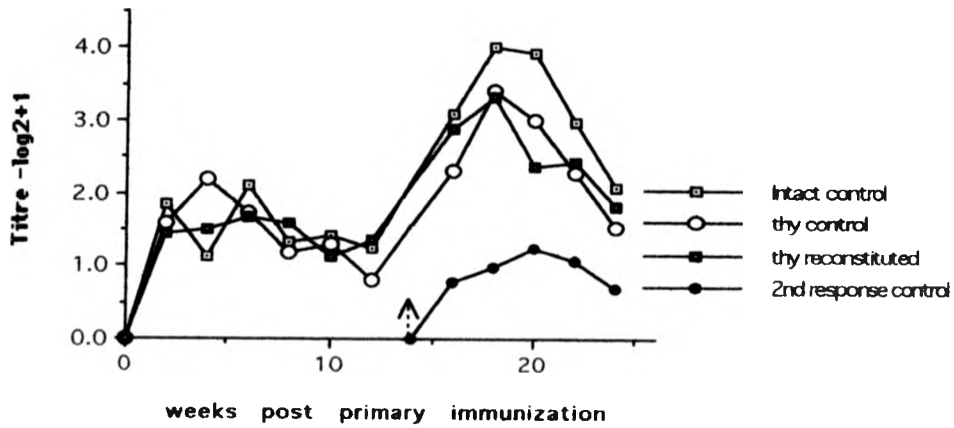


Fig 5.10: The effect of eight month adult thymectomy on the *in vivo* primary and secondary antibody response to DNP-KLH, as measured by ELISA. The arrow (^) represents the time of secondary immunization.

Table5.6: The effect of two month and eight month adult thymectomy on the memory responses of SRBC, *A.salmonicida* 1102 and DNP-KLH immunized rainbow trout.

antigen administered	Memory factor* Intact control	thymectomised control	thymectomised reconstituted	Week of peak secondary response**
SRBC 2 month thymectomised	1.03	1.14	1.03	20, 20 and 20 weeks.
1102 2 month thymectomised	0.88	0.85	1.01	20, 20 and 22 weeks.
SRBC 8 month thymectomised	1.36	0.89	1.10	18, 18 and 18 weeks.
1102 8 month thymectomised	0.72	0.91	0.79	20, 22 and 22 weeks
DNP-KLH 8 month thymectomised	2.45	1.17	1.32	18, 18 and 18 weeks

* The memory factor is calculated by taking the peak in the secondary response (Mean from all the fish tested) and the corresponding mean response from the corresponding week in the primary response (Lamers et al, 1985).

** The values given are for the intact control, thymectomised control and thymectomised reconstituted groups respectively.

a good primary response, though on week 10 post primary immunization, the thymectomised control group titres were significantly lower than the intact control group (Table 5.2). During the secondary response, the thymectomised control group also had lower titres, than the inact control, at weeks18 and 20. This suppressed response to SRBC seemed to be partly compensated for by the reconstitution of the thymocytes in the thymectomised reconstituted group, since this group's titres were never significantly lower than the intact control group. This may suggest that the introduced thymocytes were in some way correcting the immune dysfunction seen in the thymectomised control group. However it should be noted that during the primary response this group of fish did not have significantly lower titres than the intact control group, unlike the thymectomised controls. It could simply be that the thymectomised reconstituted group had higher titres throughout the whole response. One reason for this could be perhaps that some of the fish in this group still had thymic remnants left over from incomplete thymectomies, but this seems unlikely due to the care taken to check for thymic remnants or regeneration througout the dormant periods between thymectomy and primary immunization. The data suggests that SRBC is a thymus dependent antigen in trout, and that removal of the thymus for up to eight months leads to a decrease of a thymus derived cell population in the periphery, which may result in the observed decline in antibody levels. This may be an equivalent to a T-helper cell. These results are similar to those of Gruenewald and Ruben (1979) who found that in adult *Xenopus*, thymectomised for up to six months, there was a very large decrease in the helper activity directed towards TNP-SRBC.In mammals, adult thymectomy has also been shown to suppress the antibody response to erythrocyte antigens. Kerbel and Eidinger (1972) found that there was a suppressed antibody response to goose red blood cells (GRBC), but not

to sheep red blood cells or rat red blood cells (RRBC), after four months adult thymectomy. They suggested that this was due to a small decline in the T helper cell population, and the fact that SRBC and RRBC did not induce a suppressed response was due to the fact that they were less thymus dependent than the GRBC. Conversely, Charlemagne (1979) found that forty day thymectomised adult axolotls showed an enhanced response to SRBC when compared to non-thymectomised controls. The author suggested that SRBC could be a TI antigen in this animal and that the enhanced response to SRBC was due to the removal of a short lived T suppressor population that was present in the thymus. Tournefier (1982) also found enhanced antibody production to SRBC in short term thymectomised axolotl. These results, however, could equally be interpreted as suggesting that the SRBC were in fact TD but that the T helper cell population was longer lived than the suppressor cells. The fact that there was no enhancement of the SRBC response in two month thymectomised trout (Table 5.1) does not necessarily imply that suppressor cells do not exist in trout. It could be that they have a lifespan in the periphery of more than two months, and that by eight months there was a decrease in the helper cell population, which more than offset any enhancement in antibody level that would be due to the loss of T suppressor cells. The fact that there was a decrease in the memory response when the fish were thymectomised for eight months (Table 5.6) again could be interpreted as the reduction of a peripheral T-helper cell population that is required for the production of antibodies to SRBC. Also the fact that reconstitution of the cryopreserved thymocytes partly offsets the reduction in memory factor produced by eight months thymectomy (Table 5.6) suggests that the reconstitution may help to restore the depleted peripheral helper cell population, though the data are not conclusive.

Similar results observed for SRBC are also seen when DNP-KLH is used as the antigen (Table 5.5). The thymectomised control group titre was significantly lower than the intact control titre, in both the primary and secondary response. If DNP-KLH is a strongly TD antigen in fish, these results could be explained by a reduction in a peripheral helper cell population, leading to reduced antibody production to the TD antigen. There was a positive memory factor, of 2.45 in the intact control group (Table 5.6), with a decrease to 1.17 in the thymectomised control group. It should be noted that the use of Nossal's formula for calculating memory factors can be misleading, such as in the case of the DNP-KLH group. The reason for the highest memory factor of 2.45 in the intact control group (Table 5.6) was almost certainly due in a large part to the dip in the primary response at week 2 (Fig 5.10), as compared to the peak in primary response at week 2 of the thymectomised control group, giving a memory factor of only 1.17. If the week of peak titre in the primary response is substituted for P(x) (section 5.2.7.) in Nossal's formula, then the memory factors work out as 1.31, 1.17 and 1.17 for the intact control, thymectomised control and thymectomised reconstituted groups respectively. Therefore it is worth noting that the way in which the memory factor is calculated can dramatically effect the final value obtained. The data from the DNP-KLH and SRBC experiments do, however, provide some evidence for the existence of a T helper cell population in rainbow trout.

All groups of fish responded well to the *A.salmonicida* antigen (Tables 5.3 and 5.4). However there was only a very small increase in the secondary response peaks, when compared to the primary response (Fig 5.8 and Fig 5.9). There was no significant differences in the titres obtained from the two month thymectomised group and the intact controls in either the primary response or the secondary response, though they were all higher than the

secondary response control group for weeks 20, 22, 24 and 26 of the secondary response. This was probably due to the much lower antigen dose given as a primary injection to the secondary response control group, and does not necessarily point to a significant memory response. Thymectomy in the eight month thymectomised control group led to a very small increase in memory factor (Table 5.6), but it still remained negative. Two month thymectomy seemed to have very little effect on antibody titres although during the secondary response the thymectomised control group tended to have higher titres than the intact control (Fig 5.8), although not significantly. However, during the secondary response of the eight month thymectomised group, the thymectomised controls had higher titres than the intact controls, which were significantly higher at week 6 post secondary injection. One explanation for this enhanced response in the thymectomised group, could be the removal of a suppressor cell population present in the thymus, which acts on the B cell clones directed towards the TI antigen. The reconstitution of the cryopreserved thymocytes seemed to have a significant effect on the level of suppression, as the thymectomised reconstituted group did not have higher titres than the intact controls at any point during the response. This could suggest that the reconstitution of thymocytes led to some restoration of suppressor cell levels in the periphery. The fact that this enhanced effect was not seen in the two month thymectomised experimental group further suggests that the suppressor cells have a lifespan in the periphery of more than two months, though possibly not much longer as a slightly enhanced response was seen in the two months thymectomised group though this was not significant (Fig 5.8).

Enhanced responses to thymus independent antigens have been reported in adult thymectomised mammals (Kerbel and Eidinger, 1972). These

authors found that mice injected with polyvinyl-pyrrolidone (PVP), showed an elevated response after only three months adult thymectomy. They suggested that this could be due to the removal of a suppressor cell population acting on the B cell clones. A similar explanation could explain the enhanced response to the thymus independent *A.salmonicida*, in rainbow trout. Tatner et al (1987) found that nine month adult thymectomised rainbow trout showed no elevated response to *A.salmonicida*. However they measured the *in vivo* response to the antigen using the agglutination method, which has been shown to be a much less sensitive method of measuring antibody titres than ELISA (Yoshimizu et al, 1992). Therefore any differences between controls and thymectomised fish may not have been apparent using an agglutination detection system.

In general the memory factors, calculated using the formula of Nossal et al (1965) throughout these experiments were low (Lamers et al, 1985). The best memory response was seen against the TD antigens SRBC and DNP-KLH, with the later producing the highest memory factor using Nossal's formula, although as already mentioned this can be misleading. This is in contrast to the work of Cossarini-Dunier (1986) who found no memory to DNP-haemocyanin or DNP-ficoll, with or without adjuvant, but a significant secondary response to *Yersinia ruckeri*, which is regarded as a thymus independent antigen in fish. Conversely Anderson et al (1982) found a positive memory response in trout to DNP-KLH but not to DNP-ficoll, and more recently Arkoosh and Kaattari (1991) found a strong memory response to TNP-KLH in rainbow trout. Many factors can influence whether a positive memory effect is seen in a given experiment. Low temperatures inhibited memory in carp to SRBC (Rijkers et al, 1980b), and the dose of antigen and the time interval between the primary and secondary injections can effect the

magnitude of the memory response observed (Rijkers et al, 1980b; Lamers and Van Muiswinkel, 1984; Lamers et al, 1985). Lamers et al (1985) found that a high primary dose, of *A.hydrophila*, and a long time interval between priming and boosting maximised the secondary response. In the present study the water temperature throughout the studies was fairly low (Table 5.1-Table 5.5); perhaps a higher temperature at the time of secondary injection of the eight months thymectomised fish would have made the observed differences between the intact and thymectomised groups even more acute than those observed. Recently Arkoosh and Kaattari (1991) looked at memory responses to TD and TI antigens in the rainbow trout. They found there was an accelerated *in vivo* antibody response following immunisation with TNP-KLH (a TD antigen). They found no evidence of isotype switching and no increase in B cell clone sizes in TNP-LPS immunised fish, but they found an increase in the average number of B cell precursors. They did find changes in the fine specificity profiles of the antibodies (to various inhibitor molecules) during the secondary response, which was suggested may lead to a large increase in the functional affinity of the antibodies. The authors suggested that memory in trout was due to a simple expansion in the antigen-specific precursor pool, without many of the qualitive changes in antibody or B cell function, such as isotype switching and considerably greater clonal proliferation, seen in higher vertebrates.

The results from these experiments clearly show that in adult rainbow trout, the thymus still plays an important role in the immune response to both thymus dependent and thymus independent antigens, and have confirmed the thymus dependency or independency of these three antigens commonly used in fish studies. This is important , since most workers have tended to assume this to be the case based on extrapolation from mammalian studies.

Several reports on thymic growth indicate that histological changes occur as the fish age. These structural modifications (decrease in size, reticular cell infiltration) are not necessarily signs of a forthcoming involution. Many reports agree that the thymus persists in older fish (Zapata, 1980; Matisson and Fange, 1986 and Bijgaj et al, 1987). McArdle and Roberts (1974) noted a reduction in thymocyte numbers with increasing age of rainbow trout, but it did not involute. It is clear that the thymus still plays an important role in the immune response in fish up to one year old (150-200 grams in weight). The data in Chapter 3 has shown that there is still lymphoid cell movement to and from the thymus in adult rainbow trout. The data presented here provides some evidence for the existence of both suppressor and helper cell populations and, using classic thymic ablation and reconstitution studies, that they are both intimately associated with the adult thymus in rainbow trout.

Chapter 6

The effects of thymus coculture and drug treatment on the *in vitro* splenic plaque forming cell response to SRBC, TNP-SRBC, DNP-KLH and TNP-LPS in rainbow trout (Oncorhynchus mykiss).

6.1 Introduction:

Mammalian humoral immune responses are regulated by a complex array of genetically restricted and unrestricted interactions between cells and soluble factors (Tada and Okumura, 1979). Thymus-derived T cell subsets modulate antibody levels generated by sensitised antibody producing cells. This modulation may be accomplished by indirect or direct effects on antibody producing cells. Populations of amplifying or helper T cells (Falkoff and Kettman, 1972; Davies et al, 1976) and suppressor T cells (Kerbel and Eidinger, 1972) have been identified in mammals. T helper and suppressor cells have also been identified in some species of lower vertebrates, such as the amphibian *Xenopus leavis* (Gruenewald and Ruben, 1979; Clothier et al, 1989).

In contrast to the situation in higher vertebrates, the existence of functionally different interacting T-cell subpopulations (suppressor, cytotoxic, and helper cells) in teleost fish has not been definitively demonstrated, though there is strong circumstantial evidence for their presence. Miller et al (1987) obtained a monoclonal antibody, designated 13C10, which did not react with channel catfish sIg+ lymphocytes (B cells) but defined a lymphocyte population which provided the requisite helper function for *in vitro* PFC responses to thymus dependent TNP-KLH. However, although the antibody isolated a population of catfish cells with helper activity, it also reacted with all sIg- (T cell) lymphocytes and thus, by inference, with all T cells and not specifically a unique subset. There are, however, some studies which indicate that suppressor and helper activities may be separable in fish. For example, Serero and Avtalion (1978) have shown that intravenous injection of high doses of deaggregated soluble antigen can result in antigen-specific suppression of antibody formation, possibly due to antigen specific suppressor

cells. The results of another study, involving adult thymectomy in rainbow trout, showed that thymectomised animals developed higher antibody titres to *A.salmonicida* , after *in vivo* immunization, than did sham-operated controls. It was speculated that this phenomenon may be due to the removal of a source of suppressor cells by thymectomy (Manning et al, 1982b). A similar notion regarding putative suppressor cells in trout was consistent with the findings that low (100-600 rads) *in vitro* doses of X-irradiation augmented the *in vitro* plaque forming cell (PFC) responses to TNP-LPS and TNP-KLH (Kaattari et al, 1986). Evidence that such radiosensitive suppressor cells may actually reside in the T cell population was obtained with the channel catfish (Clem et al, 1991). The protocol involved mixing various concentrations of X-irradiated (2000 rads) T and/or B cells, isolated by panning from previously immunised fish, with equal numbers of T and/or B cells from the same fish and assaying for secondary *in vitro* PFC responses to the thymus dependent DNP-KLH. The results clearly indicated that cultures containing X-irradiated T cells and unirradiated B cells exhibited greatly enhanced responses when compared to unirradiated T and B cell mixtures or unfractionated controls. The authors noted however that this *in vitro* irradiation-induced augmentation phenomenon was not routinely seen in all cases. For example, it was not observed in the mixed cell cultures when unfractionated controls showed high-level PFC responses, i.e. presumably when only low levels of the putative suppressor cells were present. Based on these results it seems likely that fish have T cells with radiosensitive suppressor function similar to the situation found in mammals (Chan and Henry, 1976). However Nakanishi (1986) found evidence for a suppressor T cell population which was resistant to X-irradiation (2000 rads) in the rockfish, *Sebasticus marmoratus*, by studying the effects of X-irradiation and thymectomy on the immune response to sheep red

blood cells (SRBC). It seems likely that a population of suppressor and helper cells exists in teleost fish, and most data suggest that they are radiosensitive. A great deal of work remains to be done to better define the specificity and the required conditions for the development of this type of suppression, in addition to the isolation of the cellular subsets involved.

In amphibians, much of the work done to investigate the characteristics of *in vitro* thymus suppression to TD and TI antigens involved using an *in vitro* coculture system (Ruben et al, 1980,1983 and 1984; Clothier et al, 1989). Ruben et al (1983) using *Xenopus leavis* thymus-spleen coculture systems, were able to show that using allogenic and autogenic thymus-spleen combinations, significant suppression occurred compared to spleen cultures alone, with regard to haemagglutinin titres secreted into the culture medium. The results suggested that thymus suppression of a TD (horse red blood cells) response in this species was antigen inducible, and that depending on the antigen, specific or unspecific suppression was generated, with both functioning across allogenic boundries. They found that responses to thymus independent antigens (TNP-LPS) were not subject to thymus suppression. Ruben et al (1984) found that N-methyl-N-nitrosourea (NMU), which destroys the thymic cortex of *Xenopus* (Clothier et al, 1980), had no effect on suppressor function, whereas cyclophosphamide removed thymic suppression. In mammals, cyclophosphamide has been used to deplete T-suppressor cells (Chan et al, 1983). They found that the MHA hamster did not normally respond to a footpad injection of Pichnide virus by swelling (a measure of cell mediated immunity). However, if animals were injected with cyclophosphamide two days prior to virus injection, an immunosuppressive epitope which acts by specifically stimulating these cells can no longer operate in such animals, and a response is seen, compared to untreated

control animals Therefore the work of Clothier et al (1980) and Ruben et al (1984) on the drug sensitivity of the thymus suppression in *Xenopus* indicates that the suppressor population resides within the thymus medulla.

The aim of this study is to investigate *in vitro* thymic suppression of plaque forming cell (PFC) response to both thymus dependent antigens (SRBC, TNP-SRBC and DNP-KLH) and a thymus independent antigen (TNP-LPS) in rainbow trout utilising the technique of thymus-spleen cocultures. In this case, dissociated splenocytes and thymocytes were used, instead of spleen and thymus fragments. Previous work has shown that fish leukocytes can mount a plaque forming cell response *in vitro* to both thymus dependent and independent antigens (Miller and Clem, 1984; Kaattari et al, 1986). The effects of allogenic and autogenic thymocytes on the PFC response of spleen leukocytes were investigated as well as the effect of allogenic thymocyte coculture from fish immunised with different antigens from the autogenic spleen leukocytes. The sensitivity of the suppression to the immunosuppressive drugs NMU and cyclophosphamide was investigated. The data presented strongly supports the existence of a suppressor cell population located in the thymus of rainbow trout.

6.2 Materials and Methods

6.2.1. Fish:

Adult rainbow trout (approximate weight 150-200g) were used throughout this study. They were kept in filtered and dechlorinated water at a temperature of 10.0-12.0°C. They were fed daily using EWOS No4 trout pellets.

6.2.2. Antigens and Immunization:

Sheep red blood cells were supplied by the Scottish Antibody Production Unit (SAPU), in 0.01% Alseviers solution. The cells were washed three times with sterile 0.85% saline, and resuspended to 5% in this same solution. The fish were given an intraperitoneal (I.P.) injection of 0.1mls of 5% SRBC emulsified in an equal volume of Freunds complete adjuvant (FCA). The fish were not used as sources of cells for secondary response *in vitro* studies until at least eight weeks after immunization, i.e, when serum antibody titres were declining (section 2.11).

The DNP-KLH (Calbiochem, California, USA: 494 DNP groups per protein molecule) was resuspended in Hanks balanced salt solution (HBSS, Sigma) to 2mg/ml. The fish were given an I.P. injection of 0.1 mls of the DNP-KLH solution emulsified in an equal volume of FCA. The fish were not used as sources of cells for secondary *in vitro* responses until at least eight weeks after immunization.

TNP-LPS was prepared according to the method of Jacobs and Morrison (1975). Briefly 50mg of lipopolysaccaride (LPS, from *E. coli* serotype 0111.B4; Sigma) was dissolved in 2.5mls of 0.28M cacodylate buffer (appendix 1) and the pH adjusted to 11.5. A solution of 30mg of 2,4,6-trinitrobenzene sulfonic acid (TNBS, ICN Flow) in 2.5ml of 0.28M cacodylate buffer was added dropwise to a test tube containing the LPS solution. The test tube was wrapped in foil and placed in an orbital incubator at 23°C for two hours. After coupling the TNP-LPS solution was exhaustively dialysed against three changes of 0.077M phosphate buffered saline pH7.4. In the case of the *in vitro* immunogen, the TNP-LPS was given a final dialysis against L-15 medium. For use as an *in vivo* immunogen, the TNP-LPS was dialysed against the 0.077M PBS only. These solutions were then pasteurised

for 30 mins in a 70°C water bath and stored in a sterile stoppered glass vial. The substitution ratio was not determined. Eight weeks weeks before *in vitro* transfer, the fish received an I.P. injection of 0.1ml of 2.5mg ml^{-1} TNP-LPS in PBS,emulsified in an equal volume of FCA. A fourth group of fish received an I.P. injection of 5%TNP-SRBC in PBS eight weeks before *in vitro* transfer.

The fish were challenged *in vitro* with unmodified SRBC in the case of SRBC immunised fish, or TNP modified SRBC in the case of DNP-KLH,TNP-SRBC or TNP-LPS immunized fish. It has previously been shown that fish immunized with the DNP hapten can be challenged *in vitro* using TNP modified SRBC (Miller and Clem, 1984a). The SRBC were modified with TNBS using the method described by Mishell and Shiigi (1980). Briefly 20 mg of TNBS was dissolved in 7 mls of 0.28M cacodylate buffer, pH 6.9. The SRBC were washed three times in glucose-phosphate buffered saline (G-PBS, appendix1) pH7.6 , at 400g for 10 mins. After the last wash 1 ml of the packed SRBC was added dropwise to the TNBS/cacodylate buffer mixture, and the mixture was tumbled at room temperature for 10 mins. The reaction mixture was covered in foil throughout to prevent photodecomposition. The modified cells were then washed in cold G-PBS containing 1% heat inactivated foetal bovine serum, until the supernatants were colourless (a minimum of four washes). The modified SRBC cells were used at 5% in the haemolytic plaque assay.

Five fish were used for each antigen, for the experiments on *in vitro* thymus suppression , except for the TNP-LPS group where only three fish were used. The cyclophosphamide and NMU experiments were carried out on three test fish for each antigen. The PFC values were calculated using the mean of five wells.

6.2.3 Measurement of plaque forming cells:

The microwell plaque assays were carried out as described in section 2.10. The spleen cells, separated as described in section 2.2, were resuspended to $2.5x10^6$ or $1.25x10^6$ cells ml^{-1} in L-15/5% foetal bovine serum. To measure the PFC response of spleen cells alone 200ul of the $1.25x10^6$ cells ml^{-1} suspension was added per well, giving a concentration of $2.5x10^5$ cells per well. To measure the thymus suppression on the PFC response of spleen leukocytes, 100ul of a $2.5.x10^6$ cells ml^{-1} thymocyte suspension was added to the wells containing 100ul of the $2.5x10^6$ spleen cell supension, giving a total cell number of $5x10^5$ cells per well. To investigate the PFC response of thymocytes alone the thymocytes were resuspended to $1.25x10^6$ cells ml^{-1}, and 200ul was added to each well giving a cell concentration of $2.5x10^5$ cell per well. Each fish was tested with autogenic and allogenic thymocytes from immunized and non-immunised fish injected with each of the test antigens.

The cells *in vitro* were challenged with 0.05% TNP-SRBC or SRBC for 5 days in culture. After the cells were challenged for 5 days, the plates were spun at 400g, the supernatant discarded, and the PFC response was measured as described in section 2.10. To remove the SRBC indicator cells, 200ul per well of L-15/10% anti-SRBC trout sera (pooled sera from week 4 post secondary immunization fish) was added for one hour at 15°C. Once the indicator cells were lysed the plates were spun down at 400g, the cells were resuspended and fresh L-15/5%foetal bovine serum was added at 200ul per well.

6.2.4 Cyclophosphamide and N-Methyl-N-Nitrosourea (NMU) treatment :

Fish were anaesthetised in 25 p.p.m. benzocaine (Sigma), and given an I.P. injection of 30mg of cyclophosphamide (Sigma; mean 150ug per gram body weight) in PBS pH 7.4 two days before *in vitro* transfer. Three fish were used for each cyclophosphamide injected group injected with each of the three antigens. . The cells were also cultured in L-15/ 5% FBS containing cyclophosphamide at 50ug/ml, as a control.The allogenic thymocytes used in the cyclophosphamide groups came from non cyclophosphamide treated fish.

Fish to be treated with NMU (Sigma) were given an I.P. injection of 30mg dissolved in phosphate buffer pH5.1, eight days before *in vitro* transfer. The allogenic thymocytes used in the NMU groups came from non-NMU treated fish.

6.2.5 Statistical analysis:

To test whether any differences in PFC response between autogenic and allogenic cocultures were significant, the students *t* -test was employed. These tests were carried out using the Minitab programme (Minitab, Birmingham, UK) on an apple macintosh computer. Differences were considered significant if $P>0.05$.

6.3. Results:

(a) DNP-KLH immunised fish: Fig 6.1-Fig 6.14 show that fish immunised *in vivo* with the DNP-KLH antigen are successful in eliciting a significant *in vitro* anti-hapten response using spleen leukocytes. They also show that addition of autogenic or allogenic thymocytes, from fish immunised with the same antigen, can significantly effect the plaque forming cell (PFC) response of the spleen leukocytes *in vitro*. These effects are summarised in Table 6.1.

Fig 6.1 shows that for fish 1 of the DNP-KLH immunised group, the spleen+autogenic thymus cocultures and the spleen+allogenic thymus cocultures, from a DNP-KLH immunised donor, had significantly lower ($P>0.01$) PFC responses than the spleen alone cultures, after 5 days in culture. After 10 days in culture these differences were no longer significant.

In fish 2 of the DNP-KLH immunised group (Fig 6.2) the spleen alone cultures showed a higher PFC response than the spleen+autogenic thymus ($P>0.01$), spleen+allogenic thymus (DNP-KLH; $P>0.01$), spleen+allogenic thymus (SRBC; $P>0.05$) and the spleen+allogenic thymus (TNP-LPS; $P>0.01$) cocultures, after 5 days in culture. These differences were no longer significant after 10 days in culture.

Fig 6.3 shows that for fish 3 of the DNP-KLH group, the spleen+autogenic thymus and spleen+allogenic thymus (DNP-KLH) cocultures had a lower PFC response ($P>0.01$) than the spleen alone cultures after 5 days in culture. After 10 days in culture only the spleen+allogenic thymus coculture, from a DNP-KLH immunised donor, had a PFC response that was significantly lower ($P>0.05$) than the spleen alone cultures, but the spleen+autogenic thymus coculture showed a significantly higher ($P>0.005$) PFC response than the spleen alone.

Fig 6.4 shows that for fish 4 of the DNP-KLH immunised group the spleen alone cultures showed a higher ($P>0.01$) PFC response than the spleen+autogenic thymus and the spleen+allogenic thymus cocultures, from a DNP-KLH immunised donor, after 5 days in culture. After 10 days in culture only the autogenic culture maintained a significantly lower ($P>0.01$) PFC response than the spleen alone cultures.

Fish 5 of this group (Fig 6.5) had no significant differences in PFC responses, between any of the groups, after 5 days in culture. After 10 days in

culture the spleen+autogenic thymus coculture showed a higher (P>0.05)PFC response than the spleen alone cultures. There were no significant differences in PFC responses, between any of the groups, when the responses of all 5 test fish are combined (Fig 6.6), after 5 or 10 days in culture. However when the results of all five fish are examined on an individual basis, we can see that 4/5 fish showed suppression of the spleen alone PFC responses by both the spleen+autogenic thymus and the spleen+allogenic thymus cocultures (from a DNP-KLH immunised donor fish), after 5 days in culture. After 10 days in culture only 1/5 fish had spleen+autogenic thymus cocultures which showed a lower PFC response than the spleen alone, but 2/5 fish showed a higher PFC response in the autogenic coculture, when compared to the spleen alone. None of the spleen+allogenic thymus (DNP-KLH) cocultures maintained the suppression of spleen alone PFC responses seen after 5 days culture, after 10 days in culture.

The effects of cyclophosphamide treatment, 2 days before *in vitro* transfer, on the suppression seen in the spleen+autogenic thymus cocultures are shown in Fig 6.7-Fig 6.10. The suppression seen in the autogenic cocultures seems to have been abolished, and in fact 2/3 fish showed a significantly higher (P>0.05) PFC response, in the autogenic cocultures when compared to spleen alone , after 5 days in culture, and 1/3 fish after 10 days in culture (Table 6.1). There was still significant (P>0.05) suppression seen in the spleen+allogenic thymus (DNP-KLH) cocultures, compared to the spleen alone, in 2/3 fish after 5 and 10 days in culture. When the responses of all 3 cyclophosphamide treated fish are combined (Fig 6.10) there was no significant differences between any of the groups, after 5 or 10 days in culture. However when examined individually it seems as if the cyclophosphamide treatment has removed the autogenic thymus induced suppression of spleen

Table 6.1: The effects of coculturing with autogenic or allogenic thymocytes, and the effects of drug treatment, on the *in vitro* PFC response of spleen leukocytes to TNP-SRBC, following *in vivo* immunization with DNP-KLH.

Leukocyte Cocultures	PFC response to TNP-SRBC after 5 days in culture					after 10 days in culture				
A- no drug treatment										
spleen alone	S1	S2	S3	S4	S5	S1	S2	S3	S4	S5
Spleen+autogenic thymus	-	-	-	-	NS	NS	NS	+	-	+
*Spleen+allogenic thymus (DNP-KLH)	-	-	-	-	NS	NS	NS	NS	NS	NS

Comment: Evidence of autogenic thymus induced suppression in 4/5 fish after 5 days and 1/5 after 10 days. Evidence of allogenic thymus induced thymus suppression in 4/5 fish after 5 days in culture.

B- pretreatment with cyclophosphamide.

Spleen alone	S1	S2	S3	S1	S2	S3
Spleen+autogenic thymus	NS	+	+	NS	+	NS
*Spleen+allogenic thymus (DNP-KLH)	-	NS	-	-	NS	-

Comment: Cyclophosphamide treatment seems to abolishes any autogenic thymus induced suppression, and in fact 2/3 fish show higher responses in autogenic cocultures after day 5 and 1/3 after day 10 inculture. There is evidence of allogenic thymus induced suppression in 2/3 fish after 5 and 10 days incubation.

Table 6.1: Continued.

leukocyte coculture	PFC response to TNP-SRBC after 5 days in culture			After 10 days in culture		
C-pretreatment with NMU						
Spleen alone	S1	S2	S3	S1	S2	S3
Spleen+autogenic thymus	NS	NS	-	NS	NS	NS
*Spleen+allogenic thymus (DNP-KLH)	NS	-	-	NS	NS	NS

Comment: No clearcut evidence of NMU effects, with 1/3 fish showing evidence of autogenic thymus induced suppression after 5 days culture. Evidence of allogenic thymus induced suppression in 2/3 fish after 5 days inculture.

Key

* Allogenic thymus donor fish immunised with the same antigen as the spleen leukocyte donor fish.

S(+number of test fish) represents the in vitro PFC response of spleen alone cultures.

+ indicates a significant increase in PFC response of coculture, when compared to spleen alone cultures.

- indicates a significant decrease in PFC response of cocultures, when compared to spleen alone cultures.

NS indicates no significant difference in PFC responses between spleen alone cultures and the thymus-spleen cocultures.

NB. Differences in PFC responses were considered significant if $P>0.05$.

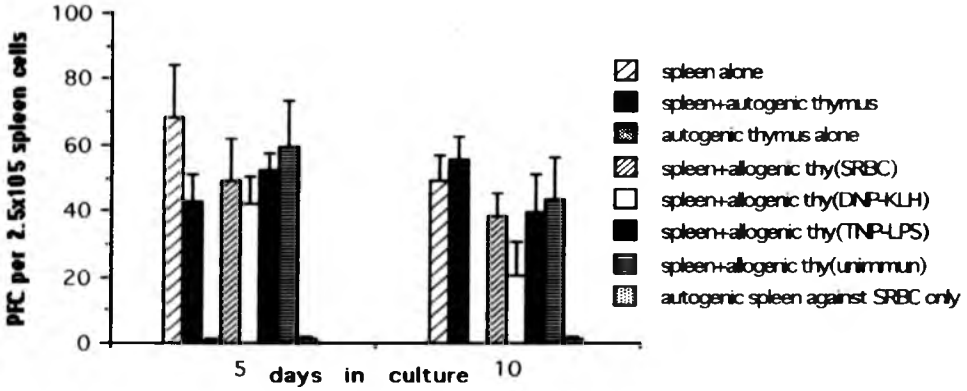


Fig 6.1: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to DNP-KLH, in fish 1.

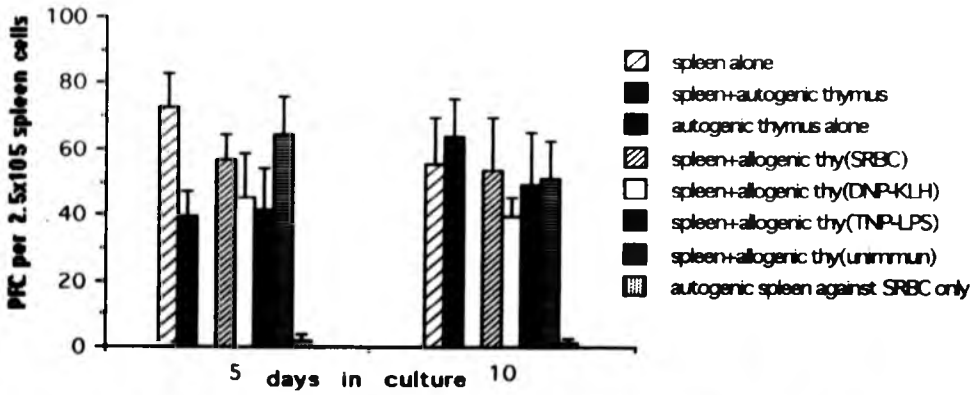


Fig 6.2: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to DNP-KLH, in fish 2.

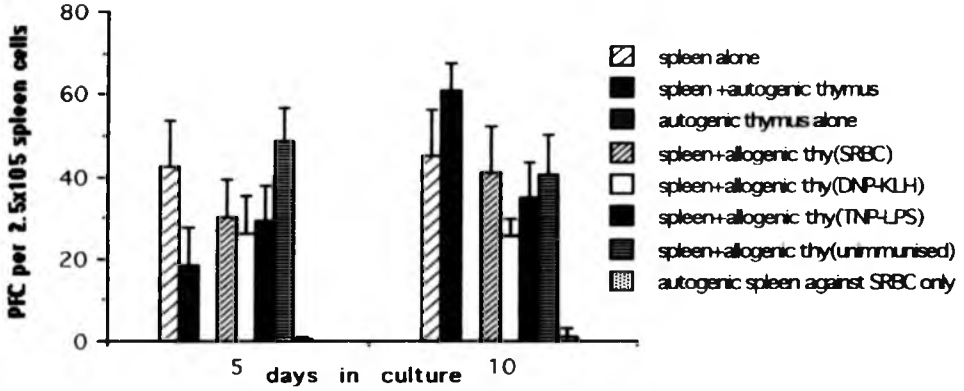


Fig 6.3: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to DNP-KLH, in fish 3.

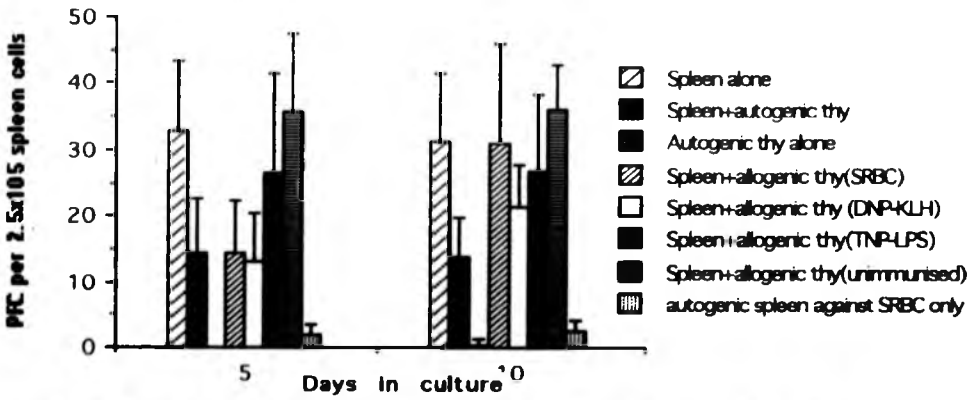


Fig 6.4: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to DNP-KLH, in fish 4.

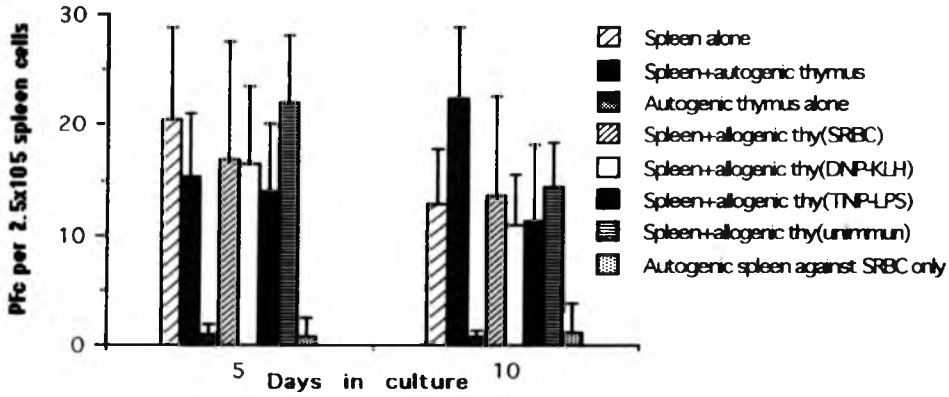


Fig 6.5: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to DNP-KLH, in fish5.

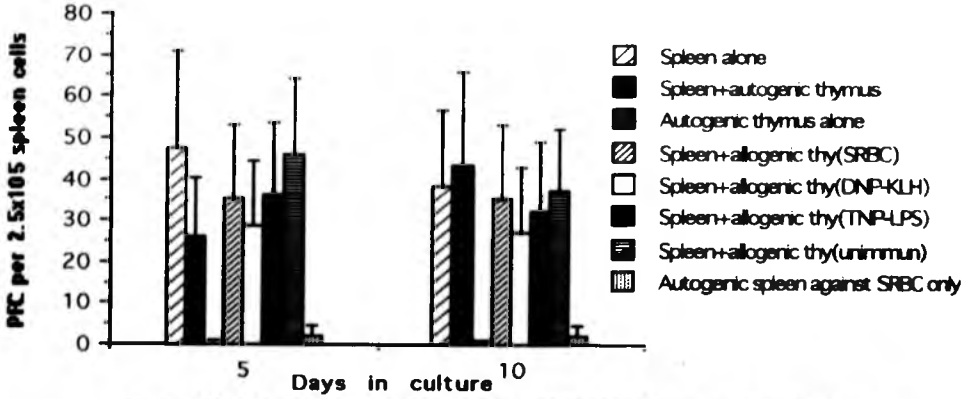


Fig 6.6: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to DNP-KLH, the means of all five fish tested

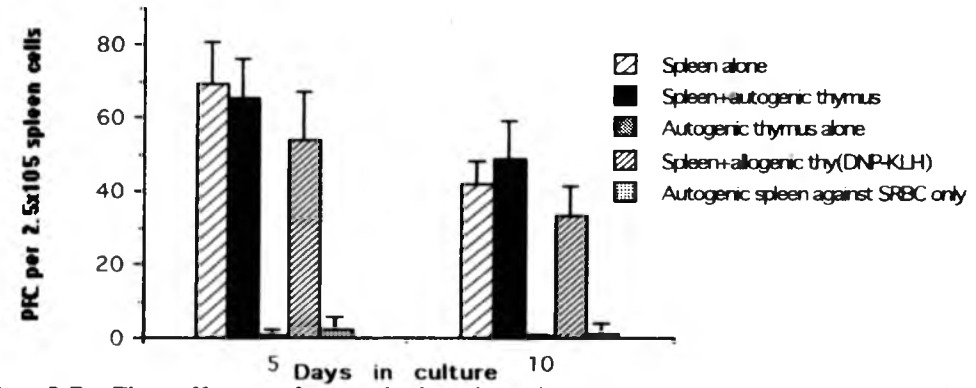


Fig 6.7: The effects of cyclophosphamide treatment pre-in vitro transfer of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to DNP-KLH, in fish1

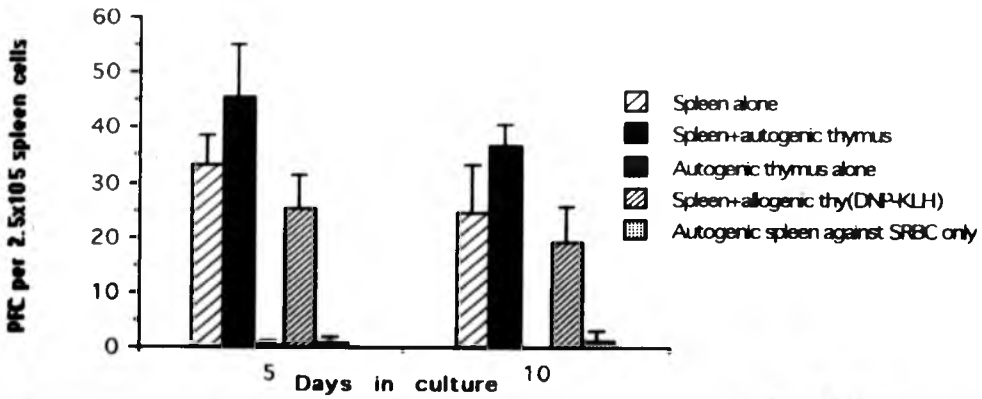


Fig 6.8: The effects of cyclophosphamide treatment pre-in vitro transfer of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to DNP-KLH, in fish2

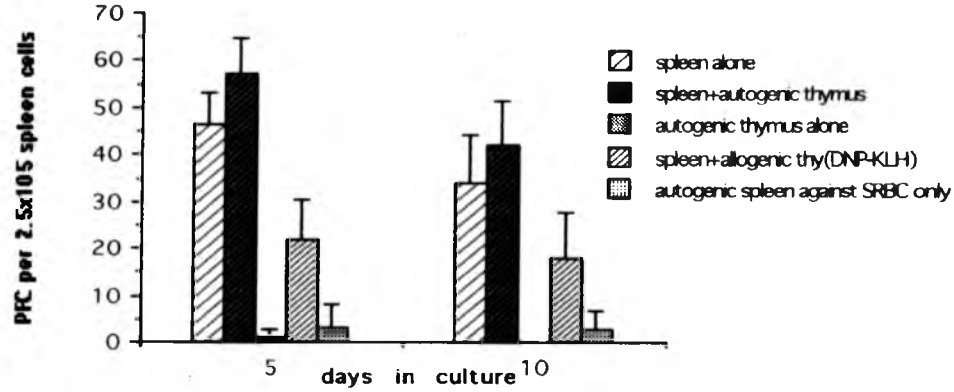


Fig 6.9: The effects of cyclophosphamide treatment pre-in vitro transfer of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to DNP-KLH, in fish3

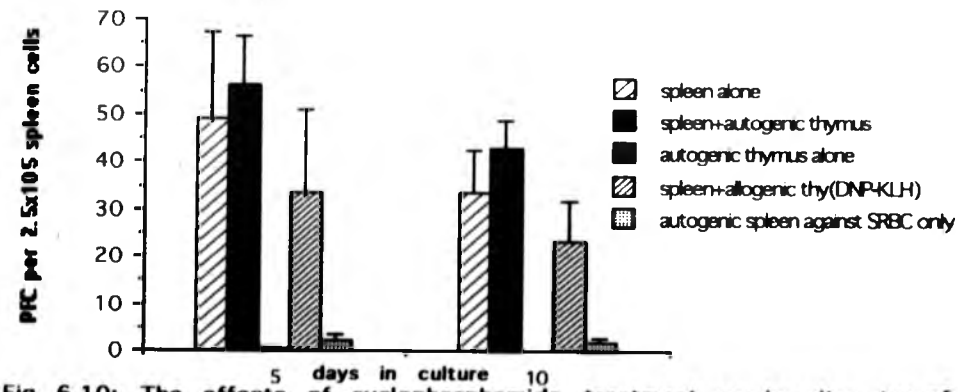


Fig 6.10: The effects of cyclophosphamide treatment pre-in vitro transfer of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to DNP-KLH, means of all three fish tested.

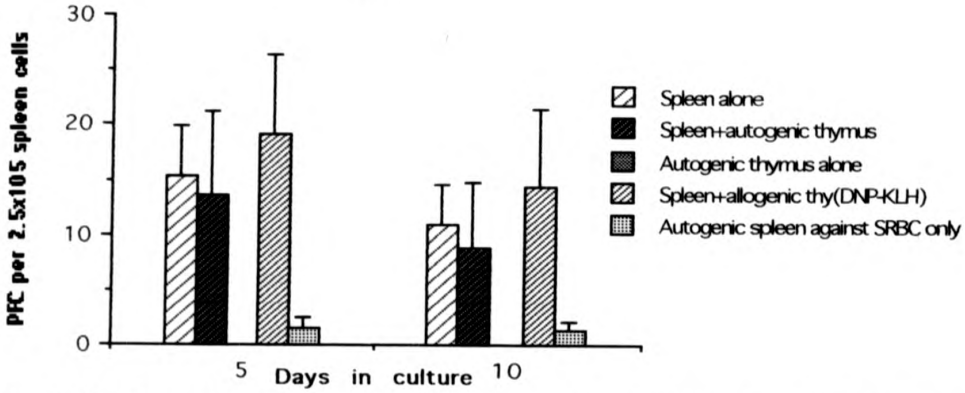


Fig 6.11: The effects of NMU treatment, pre-in vitro transfer, of the autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to DNP-KLH, in fish1

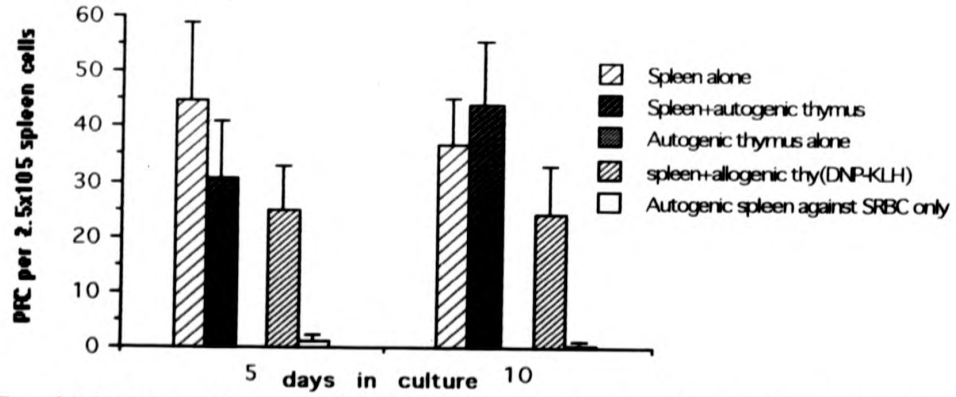


Fig 6.12: The effects of NMU treatment, pre-in vitro transfer, of the autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to DNP-KLH, in fish2

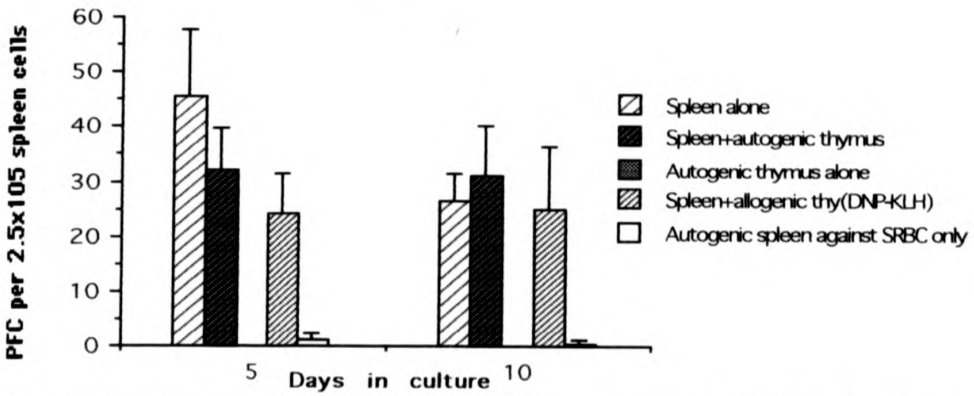


Fig 6.13: The effects of NMU treatment, pre-in vitro transfer, of the autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to DNP-KLH, in fish3.

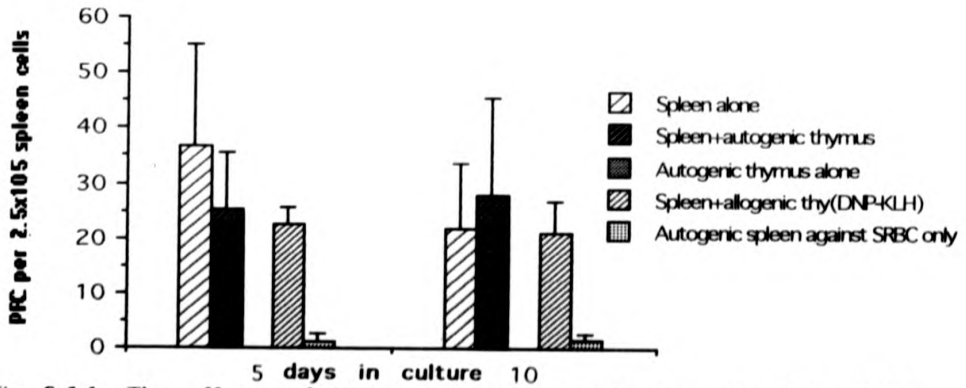


Fig 6.14: The effects of NMU treatment, pre-in vitro transfer, of the autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to DNP-KLH (means of all fish tested).

alone PFC responses seen after 5 days in culture in the untreated fish (Table 6.1), and the PFC responses of the autogenic cocultures are restored to similar levels, or show significantly higher PFC responses than the spleen alone cultures.

The effects of NMU treatment pre-*in vitro* transfer, on the DNP-KLH immunised fish are shown in Fig 6.11-6.14. The effects are summarised in Table 6.1. Fish 1 showed no significant differences in PFC responses, between any of the groups, at either 5 or 10 days in culture (Fig 6.11).

Fish 2 (Fig 6.12), of the NMU treated and DNP-KLH immunised group, showed a lower ($P>0.05$) PFC response in the spleen+allogenic thymus (DNP-KLH) than in the spleen alone cultures, after 5 days incubation. There were no significant differences after 10 days in culture. Fish 3 of the NMU treated group (Fig 6.13) showed a higher spleen alone PFC response than in the spleen+autogenic thymus ($P>0.05$) and the spleen+allogenic thymus (DNP-KLH; $P>0.01$) cocultures, after 5 days in culture, but these differences were no longer apparent after 10 days incubation.

When the PFC responses of the three NMU treated fish are combined (Fig 6.14), there were no significant differences, between any of the groups, after 5 or 10 days in culture. Pre-*in vitro* transfer NMU treatment did not have any clear effects on PFC responses of the spleen alone or allogenic and autogenic cocultures; however it did seem to decrease the level of autogenic thymus induced suppression, when compared to spleen alone, with only 1/3 of NMU treated fish showing autogenic thymus induced suppression compared to 4/5 of the untreated fish (Table 6.1). Unlike cyclophosphamide treatment there was never any enhancement of the autogenic coculture PFC response, compared to spleen alone, after 5 days in culture.

Table 6.2: The effects of coculturing with autogenic and allogenic thymocytes, and the effects of drug treatment, on the *in vitro* PFC response of spleen leukocytes to TNP-SRBC, following *in vivo* immunization with TNP-LPS.

Leukocyte coculture	PFC response to TNP-SRBC after 5 days in culture			after 10 days in culture		
A-no drug treatment						
Spleen alone	S1	S2	S3	S1	S2	S3
Spleen+autogenic thymus	NS	NS	NS	NS	NS	NS
*Spleen+allogenic thymus	NS	NS	NS	NS	NS	NS

Comment: No evidence of thymus induced suppresion.

B- after cyclophosphamide treatment						
spleen alone	S1	S2	S3	S1	S2	S3
Spleen+autogenic thymus	NS	NS	NS	NS	NS	NS
*Spleen+allogenic thymus	NS	NS	NS	NS	NS	NS

Comment: No clear cut evidence of any effects due to cylophosphamide treatment.

Key

* Allogenic donor fish were immunised with the same SRBC antigen as the autogenic spleen donor fish.

S(+number of test fish) represents the *in vitro* PFC response of spleen alone cultures.

Table 6.2: continued.

Key

+ indicates a significant increase in PFC response of coculture, when compared to spleen alone cultures.

- indicates a significant decrease in PFC response of coculture, when compared to spleen alone cultures.

NS indicates no significant difference in PFC responses between cocultures and spleen alone cultures.

NB. Differences in the PFC responses were considered significant if $P>0.05$.

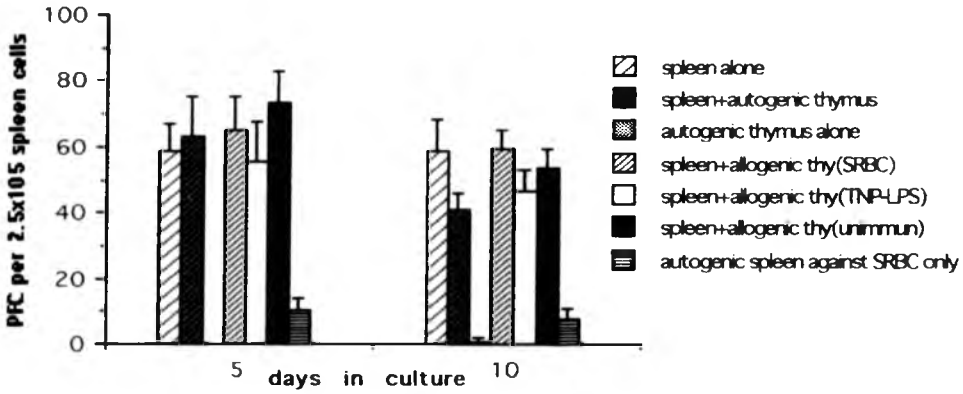


Fig 6.15: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to TNP-LPS, in fish 1.

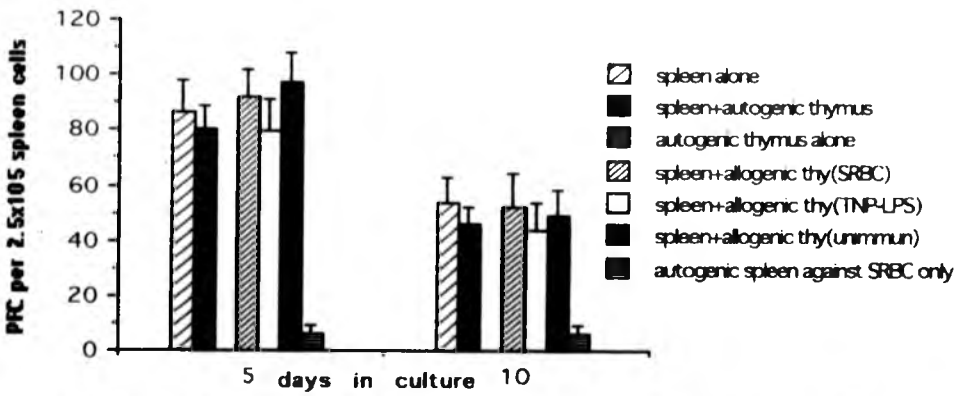


Fig 6.16: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to TNP-LPS, in fish 2.

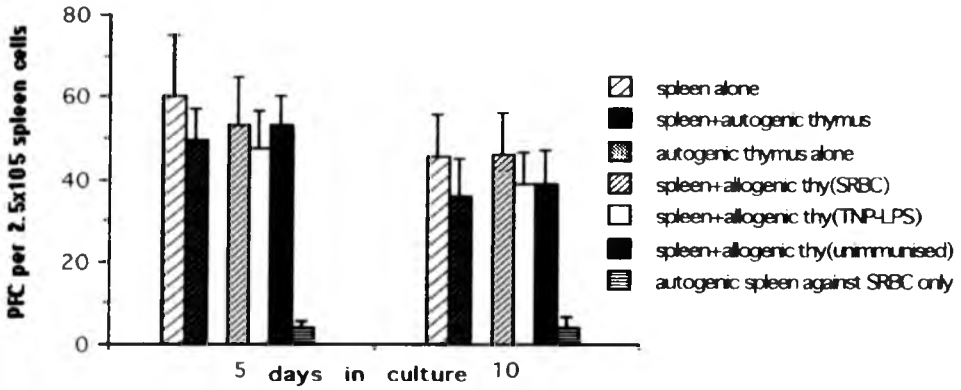


Fig 6.17: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to TNP-LPS, in fish3

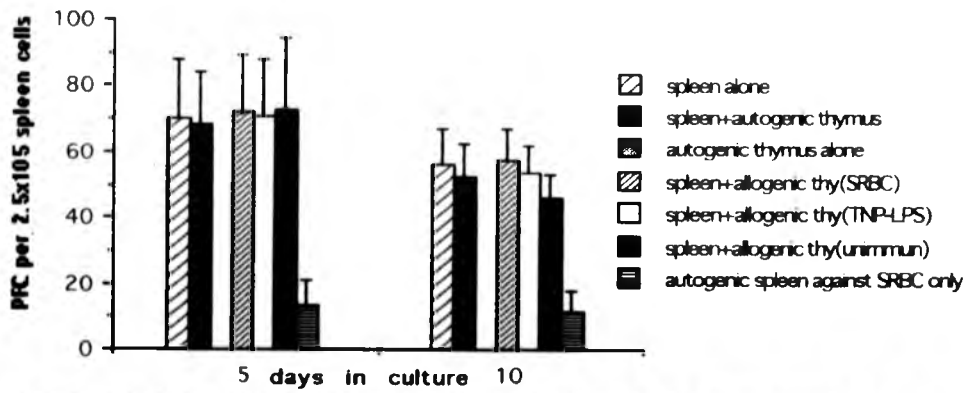


Fig 6.18: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to TNP-LPS, the means of all three fish tested.

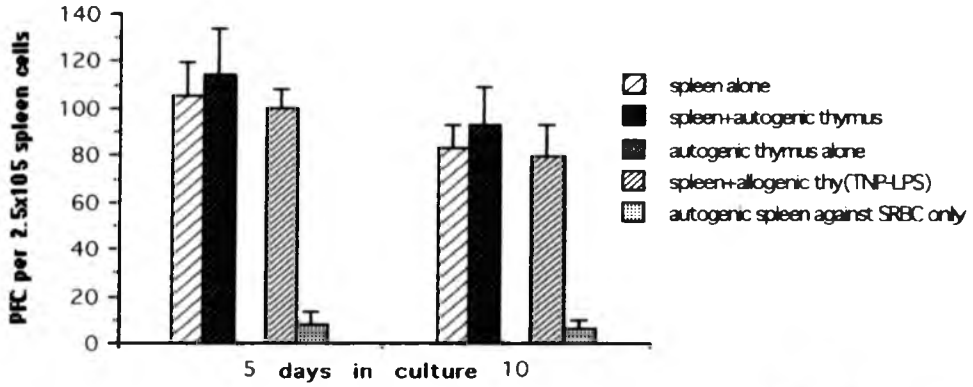


Fig 6.19: The effects of cyclophosphamide treatment pre-in vitro transfer of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to TNP-LPS, in fish 1.

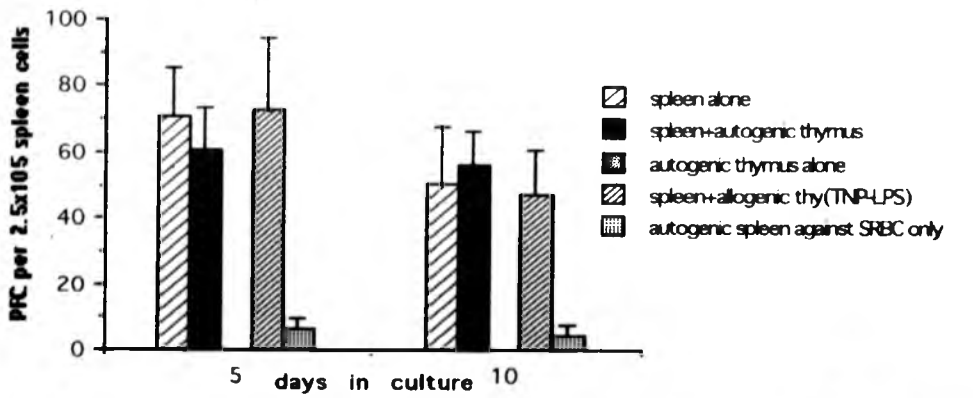


Fig 6.20: The effects of cyclophosphamide treatment pre-in vitro transfer of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to TNP-LPS, in fish2.

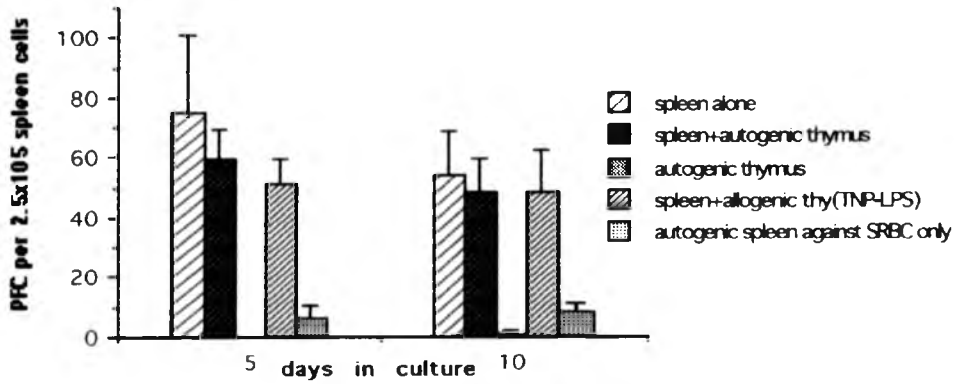


Fig 6.21: The effects of cyclophosphamide treatment, pre-in vitro transfer of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultutres to TNP-LPS, in fish3.

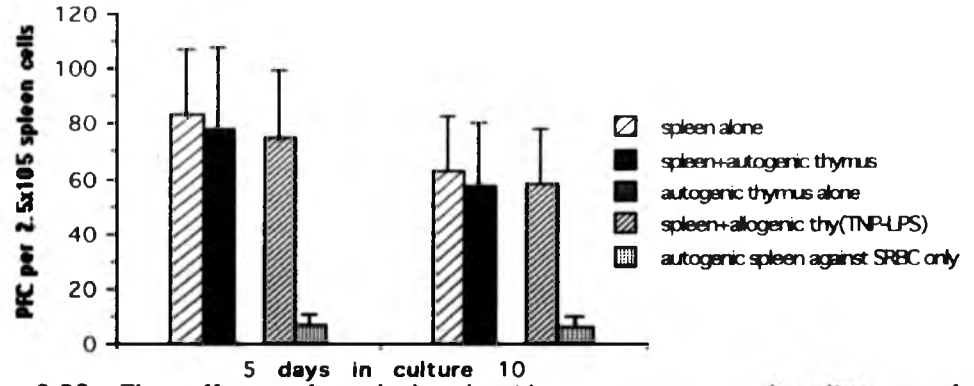


Fig 6.22: The effects of cyclophosphamide treatment, pre-in vitro transfe of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to TNP-LPS, the means of all three fish tested

(b) TNP-LPS immunised fish: Fig 6.15-Fig 6.18 show that TNP-SRBC elicits a strong *in vitro* PFC response from fish immunised *in vivo* with TNP-LPS. The results are summarised in Table 6.2. There were no significant differences in PFC responses, between any of the different groups in the 3 untreated test fish, after either 5 or 10 days in culture. Therefore it seems that there was no significant evidence of either autogenic or allogenic thymus induced suppression of spleen alone PFC responses, when fish are immunised with the TNP-LPS antigen.

The effects of treatment of the TNP-LPS autogenic donor fish with cyclophosphamide 2 days before *in vitro* transfer are shown in Fig 6.19-Fig 6.22. There were no significant differences in PFC responses, between any of the groups, after either 5 or 10 days in culture in any of the three fish tested. Therefore there was no obvious effect of cyclophosphamide treatment, on the PFC responses of spleen alone or autogenic and allogenic cocultures. In all of the TNP-LPS immunised fish the PFC responses, of all groups, to TNP modified SRBC were significantly higher ($P>0.0001$) than responses of autogenic spleen alone cultures against unmodified SRBC. This showed that the PFC responses of the spleen leukocytes were hapten specific.

(c) TNP-SRBC immunised fish: When fish immunised *in vivo* with TNP-SRBC are challenged *in vitro* with the same antigen, a significant anti-hapten response is observed in all cases (Fig 6.23-Fig 6.36). Fig 6.23-Fig 6.28 show that addition of autogenic thymocytes or allogenic thymocytes, from fish immunised with the same antigen, to the spleen leukocytes can have a significant effect on the PFC response. The results of the TNP-SRBC immunisation experiment are summarised in Table 6.3.

Fig 6.23 shows that in fish 1 of the TNP-SRBC immunised group, the

spleen alone cultures showed a higher (P>0.05) PFC response than the spleen+autogenic thymus and spleen+allogenic thymus (TNP-SRBC) cocultures after 5 days in culture. After 10 days in culture only the spleen+allogenic thymus (TNP-SRBC) coculture showed a lower (P>0.01) PFC response than spleen alone.

Fish 2 of the TNP-SRBC immunised group (Fig 6.24) showed a lower (P>0.05) PFC response in the spleen+allogenic thymus (TNP-SRBC) coculture, than in the spleen alone after 5 days in culture. After 10 days there were no significant differences in PFC responses.

There was no significant differences in PFC responses, after either 5 or 10 days in culture, in fish 3 and fish 5 of the TNP-SRBC immunised group (Fig 6.25 and Fig 6.27). However with fish 4 of this group (Fig 6.27) the spleen+allogenic thymus (TNP-SRBC) and the spleen+allogenic thymus (SRBC) cocultures showed lower (P>0.05) PFC responses than the spleen alone, after 5 days incubation. After 10 days the spleen alone cultures showed a higher (P>0.05) PFC response than the spleen+autogenic thymus and the spleen+allogenic thymus (TNP-SRBC) cocultures.

When the PFC responses of all 5 TNP-SRBC immunised fish are combined (Fig 6.28) there were no significant differences, between any of the groups. However when examined individually there is some evidence of autogenic thymus induced suppresion of spleen alone PFC responses in 1/5 fish , after 5 days and 10 days in culture. There is evidence of allogenic thymus (from TNP-SRBC immunised donor) induced suppression in 2/5 fish after 5 days, and 3/5 fish after 10 days in culture. There was only 1 fish (Fig 6.26) which showed evidence of allogenic thymus induced suppression , from a fish immunised with a different antigen than the autogenic spleen donor, of spleen alone PFC responses.

Treatment of the TNP-SRBC immunised fish with cyclophosphamide, two days before *in vitro* transfer, had significant effects on the PFC responses of spleen+autogenic thymus cocultures, when compared to spleen alone cultures (Table 6.3). Fish 1 of the cyclophosphamide treated fish (Fig 6.29) showed no significant differences between any of the cocultures and the spleen alone, after 5 or 10 days in culture.

Fig 6.30 shows that fish 2 of the cyclophosphamide treated group had significantly higher PFC responses in the spleen+autogenic thymus cocultures, compared to the spleen alone, after 5 days ($P>0.005$) and after 10 days ($P>0.05$) in culture. There was no suppression or enhancment of spleen alone PFC response by the allogenic coculture, after 5 or 10 days in culture.

For fish 3 of the cyclophosphamide treated TNP-SRBC immunised group (Fig 6.31) the spleen alone PFC response was higher ($P>0.01$) than the spleen+allogenic thymus (TNP-SRBC) after 5 days in culture, but not after 10 days. After 10 days in culture the spleen+autogenic thymus coculture showed a higher ($P>0.001$) PFC response than the spleen alone.

When the PFC responses of all three cyclophosphamide treated fish are combined (Fig 6.32) no significant thymus induced suppression or enhancement of spleen alone responses is evident. Taken individually however, 1/3 fish after 5days and 2/3 fish after 10 days showed significantly elevated PFC responses in the spleen+autogenic thymus cocultures, when compared to spleen alone. The cyclophosphamide seems to be removing any autogenic thymus induced suppression and replacing it with enhancement of PFC responses.

The effects of NMU treatment pre-*in vitro* transfer on the TNP-SRBC immunised fish are shown in Fig 6.33-Fig 6.36. For fish 1 of the group (Fig 6.33) there were no significant differences after 5 or 10 days in culture. In fish 2

Table 6.3: The effects of coculturing with autogenic and allogenic thymocytes, and the effects of drug treatment, on the *in vitro* PFC response of spleen leukocytes to TNP-SRBC, following *in vivo* immunization with TNP-SRBC.

Leukocyte coculture	PFC response to TNP-SRBC after 5 days in culture					after 10 days in culture				
A-no drug treatment										
spleen alone	S1	S2	S3	S4	S5	S1	S2	S3	S4	S5
Spleen+autogenic thymus	-	NS	NS	NS	NS	NS	NS	NS	-	NS
*Spleen+allogenic thymus (TNP-SRBC)	-	-	NS	-	NS	-	-	NS	-	NS

Comment: Only evidence of suppression, in 1/5 fish, in the autogenic cocultures after 5 and 10 days inculture. Allogenic thymus induces suppression in 2/5 fish after 5 day culture and 3/5 fish after 10 days culture.

B- after pretreatment with cyclophosphamide						
Spleen alone	S1	S2	S3	S1	S2	S3
Spleen+autogenic thymus	NS	+	NS	NS	+	+
*Spleen+allogenic thymus (TNP-SRBC)	NS	NS	-	NS	NS	NS

Comment: No clear pattern, but elevated PFC response of autogenic cocultures, when compared to spleen alone, in 1/3 fish after 5 days in culture and 2/3 fish after 10 days in culture.

C- after pretreatment with NMU						
spleen alone	S1	S2	S3	S1	S2	S3
Spleen+autogenic thymus	NS	-	-	NS	NS	-

Table 6.3: Continued

Leukocyte coculture	PFC response to TNP-SRBC after 5 days in culture			after 10 days in culture		
*Spleen+allogenic thymus (TNP-SRBC)	NS	-	-	NS	-	-

Comment: Autogenic thymus induced suppression in 2/3 fish after 5 days, and 1/3 fish after 10 days in culture. Allogenic thymus induced suppression 2/3 fish after 5 days and 10 days culture.

Key

* Allogenic thymus donor fish immunised with the same TNP-SRBC antigen as the autogenic spleen.

S(+number of test fish) represents the in vitro PFC response of spleen alone cultures.

+ indicates a significant increase in PFC response of coculture, when compared to spleen alone cultures.

- indicates a significant decrease in PFC response of coculture, when compared to spleen alone cultures.

NS indicates no significant difference in PFC responses between cocultures and spleen alone cultures.

NB. Differences in the PFC responses were considered significant if $P>0.05$.

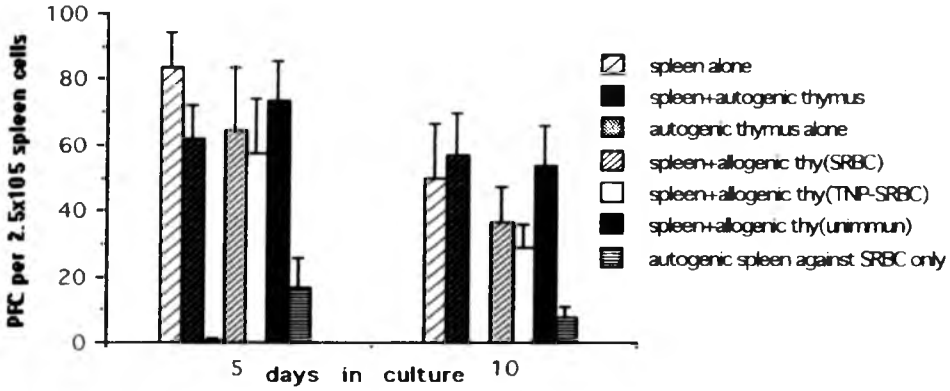


Fig 6.23: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to TNP-SRBC, in fish1

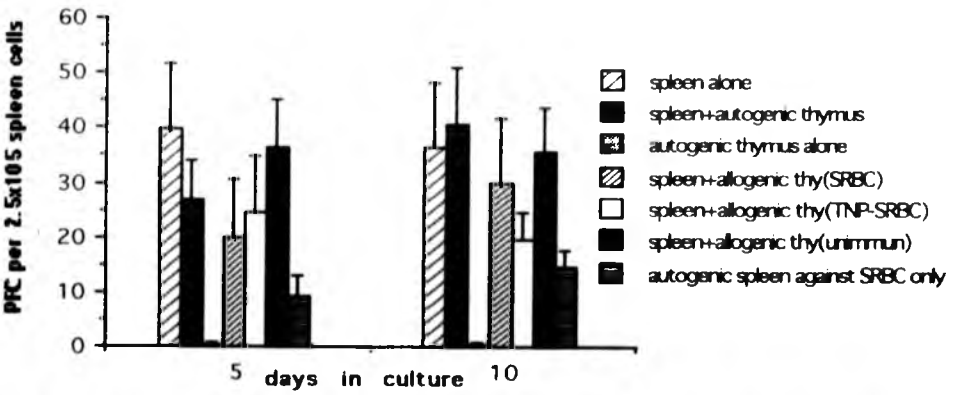


Fig 6.24: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to TNP-SRBC, in fish2.

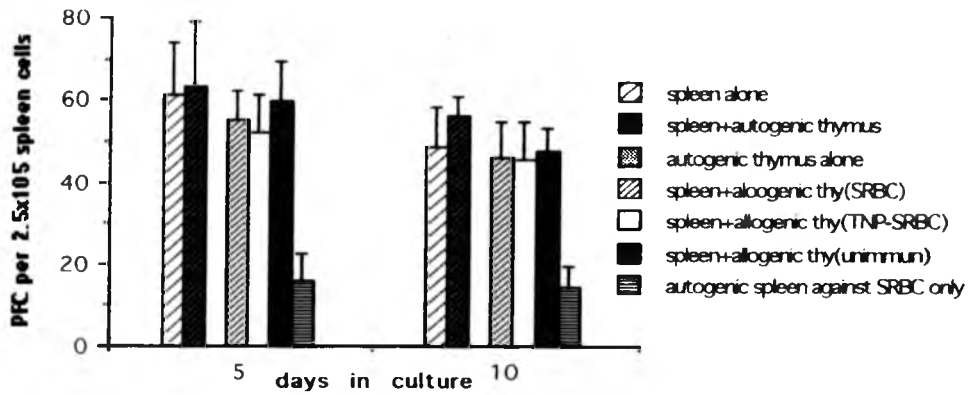


Fig 6.25: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to TNP-SRBC, in fish3.

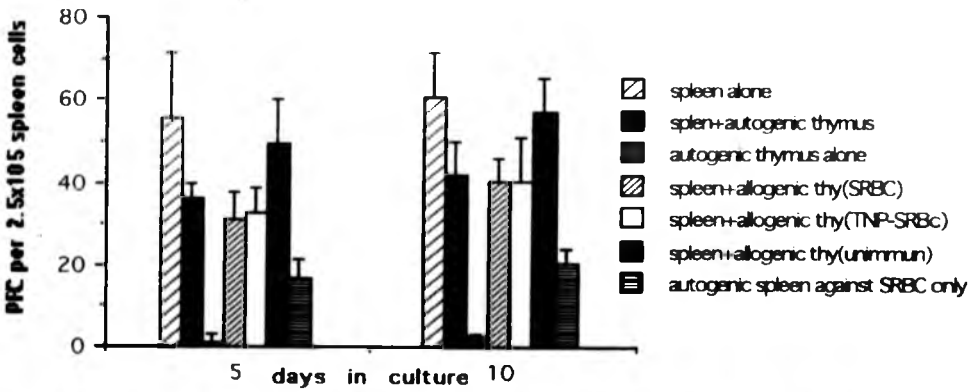


Fig 6.26: The effect of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to TNP-SRBC, in fish 4.

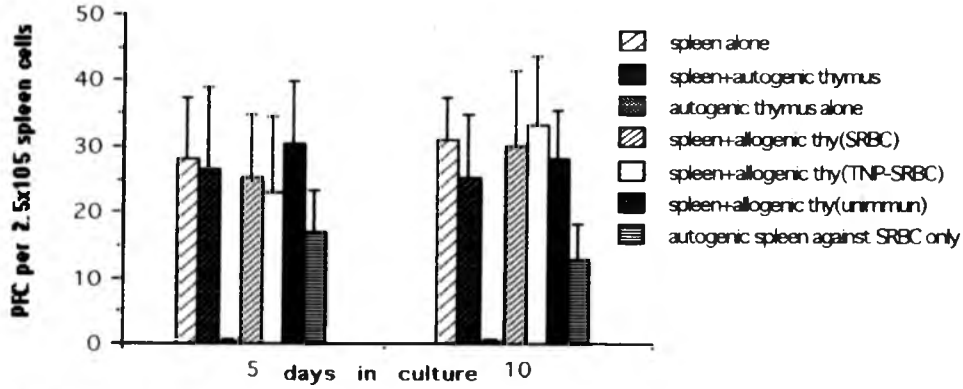


Fig 6.27: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to TNP-SRBC, in fish5.

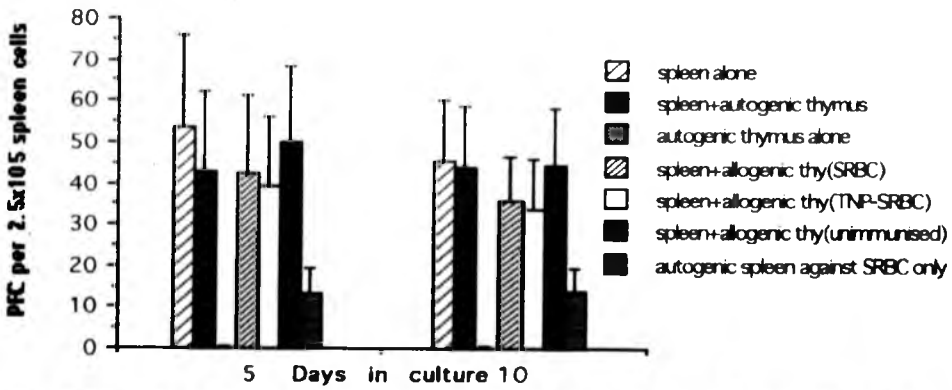


Fig 6.28: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to TNP-SRBC, the means of all five fish tested.

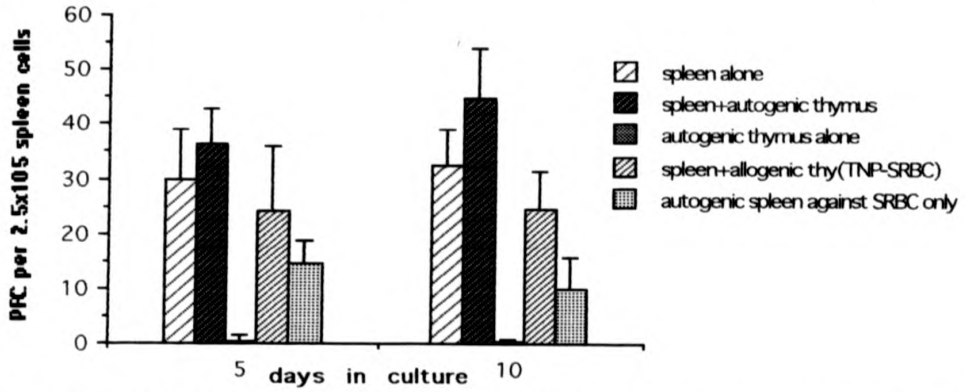


Fig 6.29: The effects of cyclophosphamide treatment, pre-in vitro transfer of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to TNP-SRBC, in fish1.

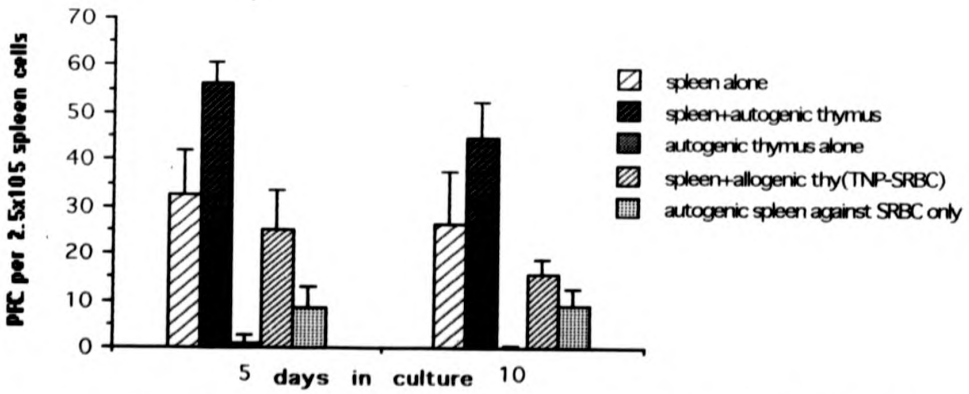


Fig 6.30: The effects of cyclophosphamide treatment, pre-in vitro transfer of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to TNP-SRBC, in fish2.

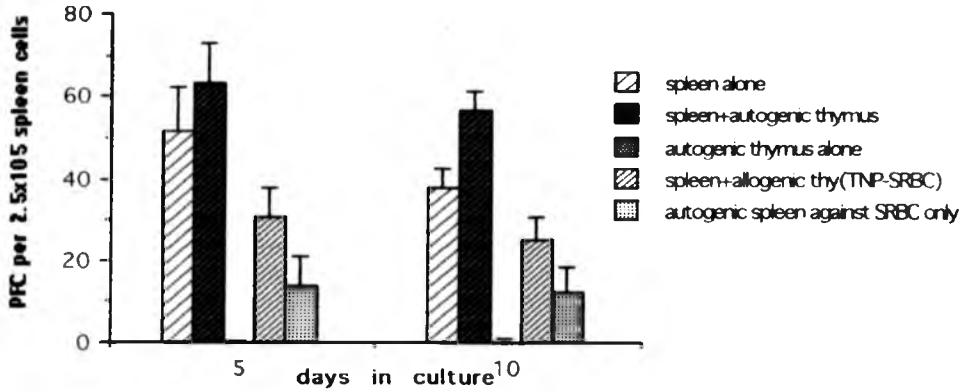


Fig 6.31: The effects of cyclophosphamide treatment, pre-in vitro transfer of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to TNP-SRBC, in fish3

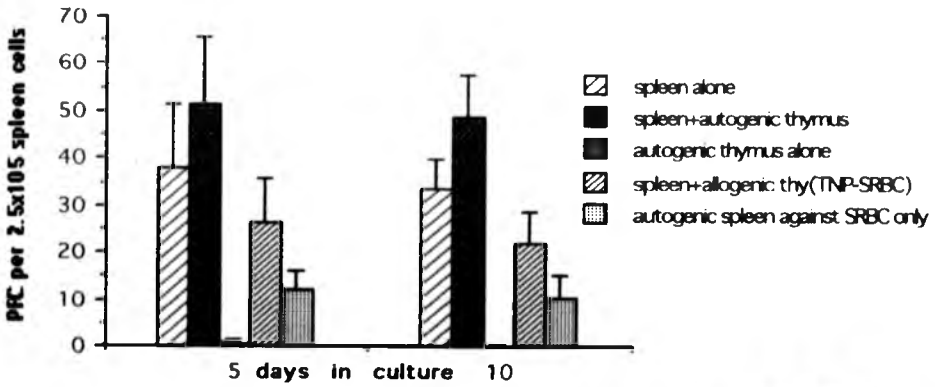


Fig 6.32: The effects of cyclophosphamide treatment, pre-in vitro transfer, of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures (the mean PFC responses of all three fish tested).

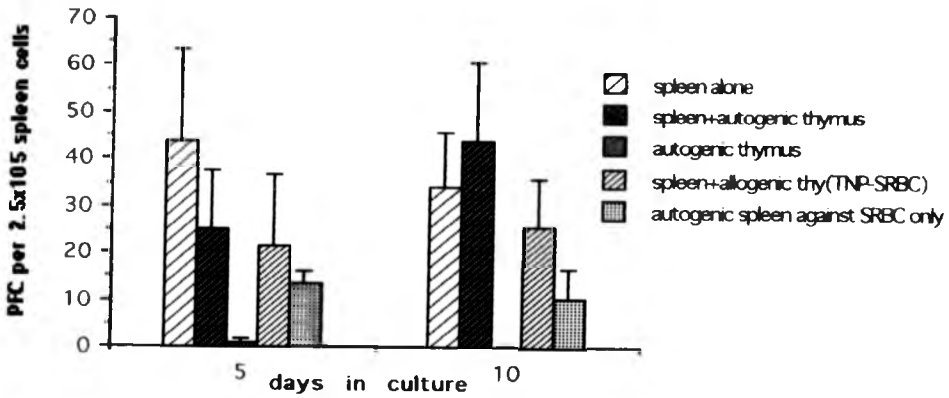


Fig 6.33: The effects of NMU treatment, pre-in vitro transfer, of autogenic thymus and spleen leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures, in fish1.

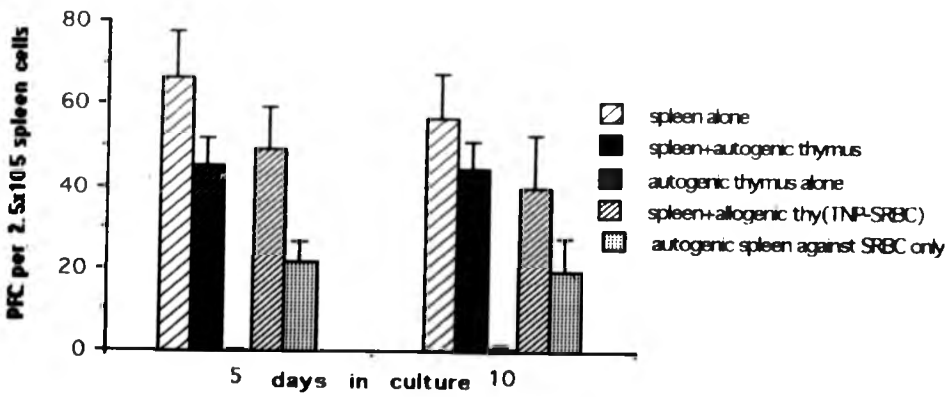


Fig 6.34: The effects of NMU treatment, pre-in vitro transfer, of autogenic thymus and spleen leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures, in fish 2

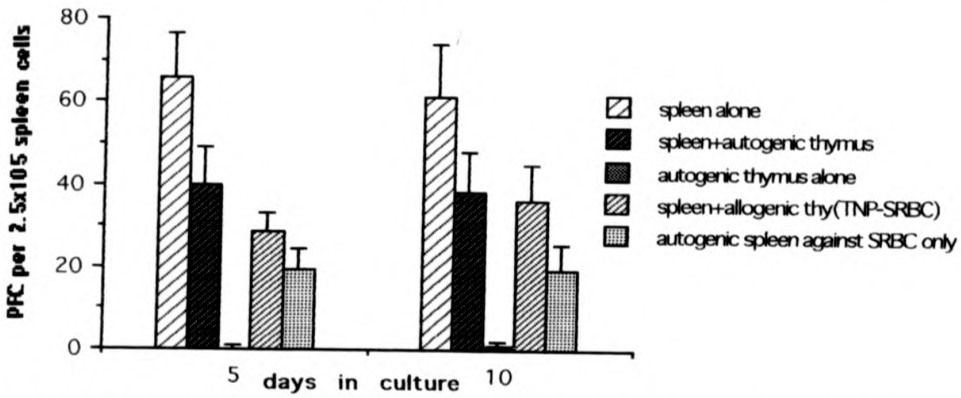


Fig 6.35: The effects of NMU treatment, pre-in vitro transfer, of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures, in fish 3.

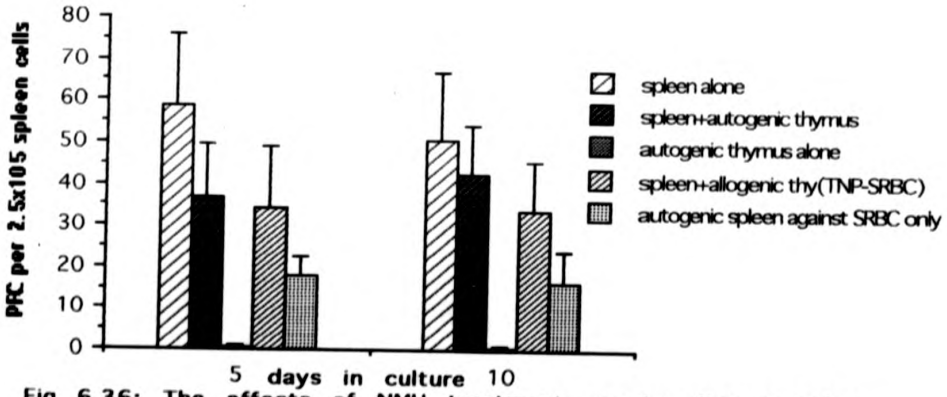


Fig 6.36: The effects of NMU treatment, pre-in vitro transfer, of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures (the means of all three fish tested).

of the NMU treated group however (Fig 6.34) the spleen alone PFC responses were higher ($P>0.05$) than both the spleen+autogenic thymus and the spleen+allogenic thymus (TNP-SRBC) cocultures after 5 days, but after 10 days in culture only the spleen+allogenic thymus (TNP-SRBC) showed lower ($P>0.05$) PFC responses than the spleen alone.

The third fish of the TNP-SRBC immunised, NMU treated group (Fig 6.35), had a lower ($P>0.005$) PFC response in the spleen+autogenic thymus and the spleen+allogenic thymus (TNP-SRBC; $P>0.001$) cocultures than the spleen alone cultures, after 5 days incubation. After 10 days in culture the spleen alone cultures still showed a higher PFC response than the spleen+autogenic thymus ($P>0.05$) and the spleen+allogenic thymus (TNP-SRBC; $P>0.01$) cocultures.

When the results of all 3 NMU treated, TNP-SRBC immunised, fish are combined (Fig 6.36) there were no significant differences between any of the groups after 5 or 10 days incubation. However taken individually 3/3 fish show lower PFC responses in the autogenic coculture than in the spleen alone, although this was only significant in 2/3 fish after 5 days in culture and 1/3 fish after 10 days in culture. There was significant allogenic thymus (taken from a TNP-SRBC immunised donor) induced suppression, of spleen alone PFC responses, in 2/3 fish after 5 and 10 days in culture. There was no clear effect of NMU treatment, although it seems as if it may increase autogenic thymus induced suppression of spleen alone responses, as untreated fish only showed 1/5 fish with significant autogenic coculture suppression, as compared to 2/3 in the NMU treated fish. In all cases the PFC response of autogenic spleen alone cultures against TNP-SRBC were significantly higher ($P>0.01$) than against unmodified SRBC alone.

(d) SRBC immunised fish: SRBC challenge of autogenic spleen leukocytes alone ,and autogenic and allogenic spleen-thymus cocultures, from SRBC immunised fish produced a fairly strong *in vitro* PFC response, but with great variability between fish (Fig 6.37-Fig 6.50). The results are summarised in Table 6.4. Fish 1 of the untreated SRBC immunised group (Fig 6.37), showed a higher PFC response in the spleen alone cultures than in the spleen+autogenic thymus ($P>0.05$) and the spleen+allogenic thymus (SRBC; $P>0.01$) cocultures, after 5 days in culture. After 10 days in culture there were no longer any significant differences between any of the groups.

In fish 2 (Fig 6.38) of the SRBC group, the spleen alone showed a higher ($P>0.01$) PFC response than the spleen+autogenic thymus coculture, after 5 days incubation. This difference was no longer apparent after 10 day culture. Fish 3 (Fig 6.39) showed a higher ($P>0.05$) PFC response in the spleen alone when compared to the spleen+allogenic thymus (SRBC) coculture after both 5 and 10 days in culture.

Fig 6.40 shows that in fish 4 of the SRBC immunised group, the spleen alone cultures showed a higher ($P>0.05$) PFC response than the spleen+allogenic thymus (SRBC) and the spleen+allogenic thymus (TNP-LPS) cocultures, after 5 days in culture. After 10 days in culture only the spleen+allogenic thymus (SRBC) showed a lower ($P>0.05$) PFC response than the spleen alone.

For fish 5 of the SRBC immunised group there were no significant differences, between any of the groups, after 5 or 10 days in culture (Fig 6.41). This was also the case when the PFC responses of all 5 SRBC immunised fish are combined (Fig 6.42). When the overall results are analysed individually there was evidence of autogenic thymus induced suppresion, of spleen alone PFC responses, in 2/5 fish after 5 days in culture, but no fish showed any

evidence of this after 10 days in culture. There was strong evidence of allogenic thymus induced suppression (from a donor fish immunised with the same antigen as the autogenic spleen donor) in 4/5 fish after 5 days and 2/5 fish after 10 days in culture. Allogenic thymus induced suppression, from a donor immunised with a different antigen (TNP-LPS) than the autogenic spleen, occurred in 1/5 fish, after 5 days incubation, only.

The effects of cyclophosphamide treatment 2 days pre-*in vitro* transfer on the SRBC immunised fish are shown in Fig 6.43-Fig 6.46. Fish 1 (Fig 6.43) showed no significant differences in PFC responses at any time. However fish 2 (Fig 6.44) showed a higher ($P>0.01$) PFC response in the spleen+autogenic thymus coculture than in the spleen alone cultures, after 5 days incubation. After 10 days incubation there were no significant differences in PFC responses. Fish 3 of the cyclophosphamide treated group (Flg 6.45) showed a higher ($P>0.05$) PFC response in the spleen alone cultures than in the spleen+allogenic thymus (SRBC) after both 5 and 10 days in culture.

When the PFC responses of all three cyclophosphamide treated fish are combined there are no overall significant differences in PFC responses. Examination of the results individually showed that there was no evidence of the autogenic thymus induced suppresion which was seen in 2/5 of the untreated fish after 5 days incubation(Table 6.4), and in fact 1/3 of the cyclophosphamide treated fish showed a higher PFC response in the autogenic coculture after 5 days incubation. Therefore once again cyclophosphamide treatment seems to remove any suppression due to the autogenic thymus, and can in fact enhance PFC responses of the autogenic cocultures. There was evidence of allogenic thymus induced suppression , taken from a SRBC immunised donor, in 1/3 of the cyclophosphamide treated fish.

The effects of NMU treatment, 8 days before *in vitro* transfer, on the SRBC immunised fish are shown in Fig 6.47-Fig 6.50. NMU treated fish 1 (Fig 6.47) cultures, after 5 and 10 days incubation, showed no significant differences in PFC responses between either the allogenic or autogenic cocultures and the spleen alone.

Fig 6.48 shows that in fish 2 of the NMU treated group there were no significant differences, between any of the groups, after 5 days in culture. However after 10 days in culture the spleen alone cultures showed a significantly lower ($P>0.05$) PFC response than the autogenic spleen-thymus coculture. There was no evidence of allogenic thymus induced suppression after 5 or 10 days in culture.

After 5 days incubation the third fish in the NMU treated group (Fig 6.49) showed significantly lower PFC responses in both the spleen+allogenic thymus ($P>0.01$) and spleen+autogenic thymus ($P>0.05$) cocultures when compared to the spleen alone cultures. There were no significant differences after 10 days incubation. No significant differences were seen after 5 or 10 days incubation when the PFC responses of all 3 NMU treated, SRBC immunised fish, were combined (Fig 6.50). Examination of the overall results individually showed that 1/3 fish show autogenic thymus induced suppression, of spleen alone PFC responses after 5 days in culture, and 1/3 show autogenic thymus induced enhancement of spleen alone responses after 10 days in culture. Allogenic thymus induced suppression occurred in 1/3 fish, after 5 days culture only. It is difficult to see any clear effects of the NMU treatment. However unlike cyclophosphamide treatment, NMU treatment does not completely remove all evidence of autogenic thymus induced suppression. These results are summarised in Table 6.4.

An interesting point to note is that allogenic thymocytes from

Table 6.4: The effects of coculturing with autogenic and allogenic thymocytes, and the effects of drug treatment, on the *in vitro* PFC response of spleen leukocytes to SRBC, following *in vivo* immunisation with SRBC.

Leukocyte coculture	PFC response to SRBC after 5 days in culture					after 10 days in culture				
A-no drug treatment										
Spleen alone	S_1	S_2	S_3	S_4	S_5	S_1	S_2	S_3	S_4	S_5
Spleen+autogenic thymus	-	-	NS	NS	NS	NS	NS	NS	NS	NS
*Spleen+allogenic thymus (SRBC)	-	-	-	-	NS	NS	NS	-	-	NS

Comments: Evidence of autogenic thymus induced suppression in 2/5 fish after 5days culture. Allogenic thymus induced suppression in 4/5 fish after 5 days and 2/5 fish after 10 days in culture.

B- after pretreatment with cyclophosphamide

spleen alone	S_1	S_2	S_3	S_1	S_2	S_3
spleen+autogenic thymus	NS	+	NS	NS	NS	NS
*Spleen+allogenic thymus (SRBC)	NS	NS	-	NS	NS	-

Comment: No clear effect of drug, however there is no evidence of autogenic thymus induced suppression after 5 or 10 days culture, but in 1/3 fish autogenic coculture showed significantly higher PFC response, after 5 days culture, than spleen alone. Allogenic thymus induced suppression in 1/3 fish after 5 and 10 days in culture.

Table 6.4: Continued.

Leukocyte coculture	PFC response to SRBC after 5 days in culture			after 10 days in culture		
C- after pretreatment with NMU						
Spleen alone	S1	S2	S3	S1	S2	S3
spleen+allutogenic thymus	NS	NS	-	NS	+	NS
*Spleen+allogenic thymus (SRBC)	NS	NS	-	NS	NS	NS

Comment: No clear effect of NMU. Autogenic thymus induced suppression in 1/3 fish after 5 days culture , and significantly enhanced autogenic coculture PFC response in 1/3 fish after 10 days in culture. Allogenic thymus induced suppression in 1/3 fish after 5days in culture

Key

* Allogenic donor fish were immunised with the same SRBC antigen as the autogenic spleen donor fish.

S(+number of test fish) represents the *in vitro* PFC response of spleen alone cultures.

+ indicates a significant increase in PFC response of coculture, when compared to spleen alone cultures.

- indicates a significant decrease in PFC response of coculture, when compared to spleen alone cultures.

NS indicates no significant differnce in PFC responses between cocultures and spleen alone cultures.

NB. Differences in the PFC responses were considered significant if P>0.05.

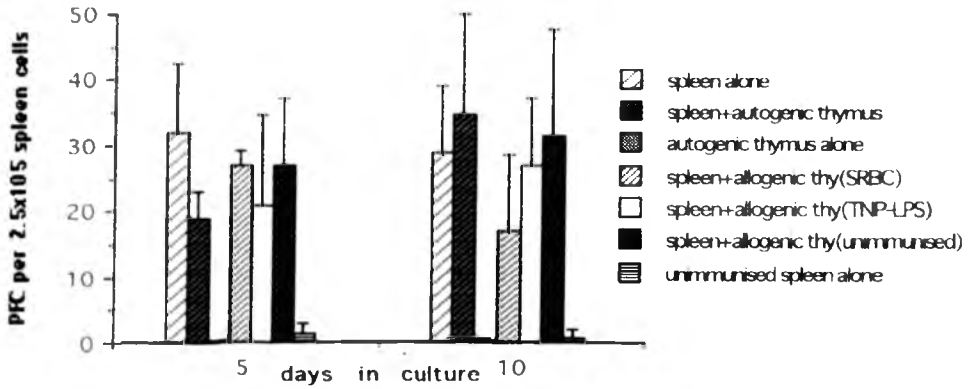


Fig 6.37: The effect of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to SRBC, in fish 1.

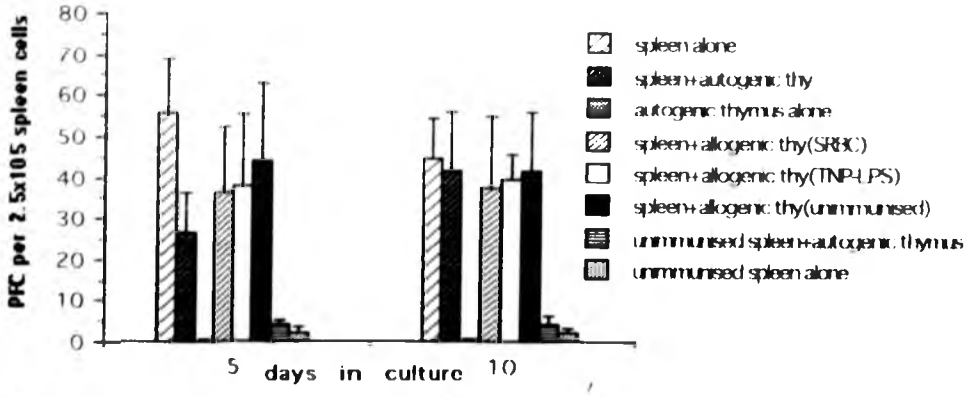


Fig 6.38: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to SRBC, in fish2.

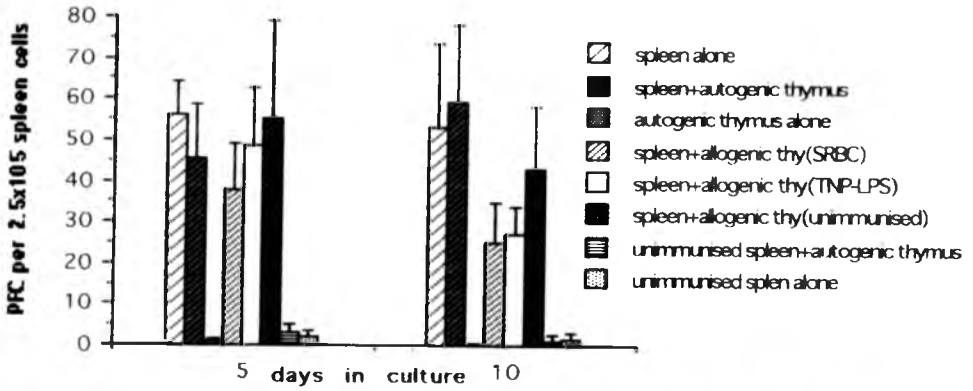


Fig 6.39: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to SRBC. in fish3.

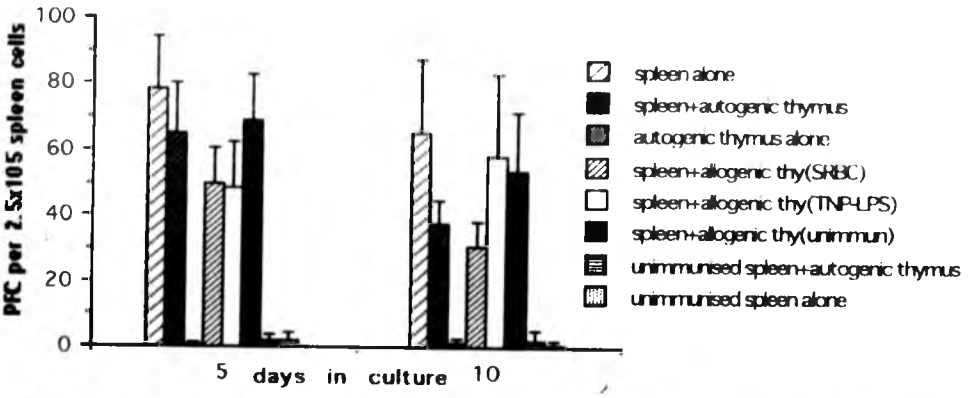


Fig 6.40: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to SRBC. in fish4.

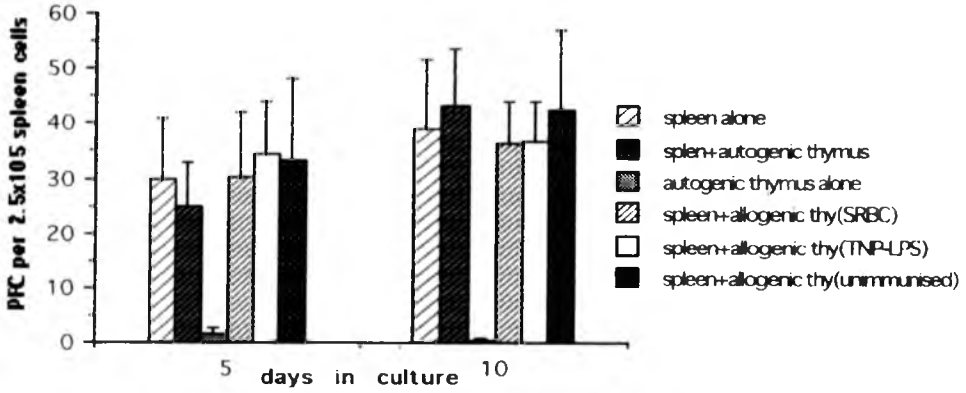


Fig 6.41: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to SRBC, in fish5.

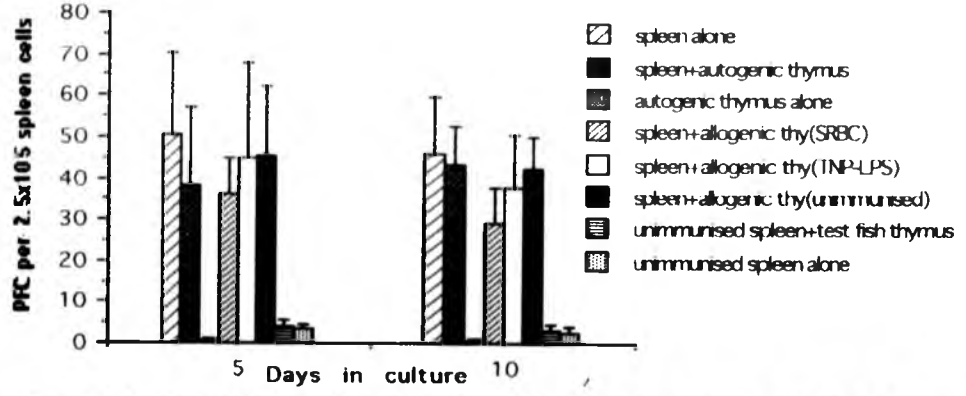


Fig 6.42: The effects of autogenic and allogenic thymus coculture on the PFC response of spleen leukocytes to SRBC, the means of all five fish tested.

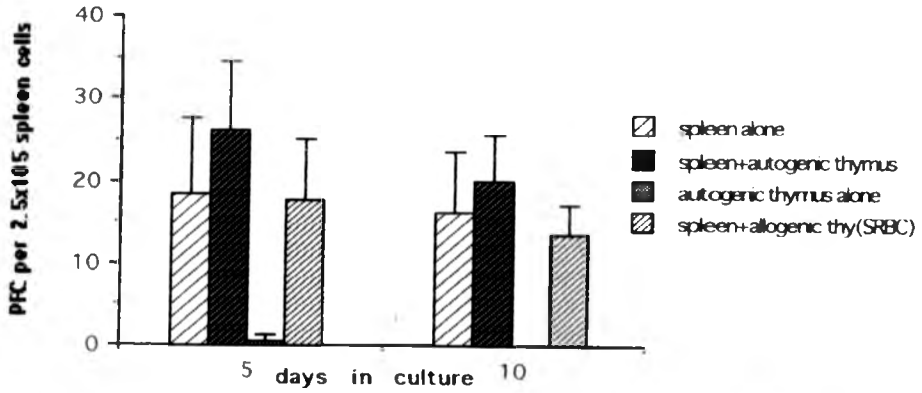


Fig 6.43: The effects of cyclophosphamide treatment, pre-in vitro transfer, of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to SRBC, in fish1

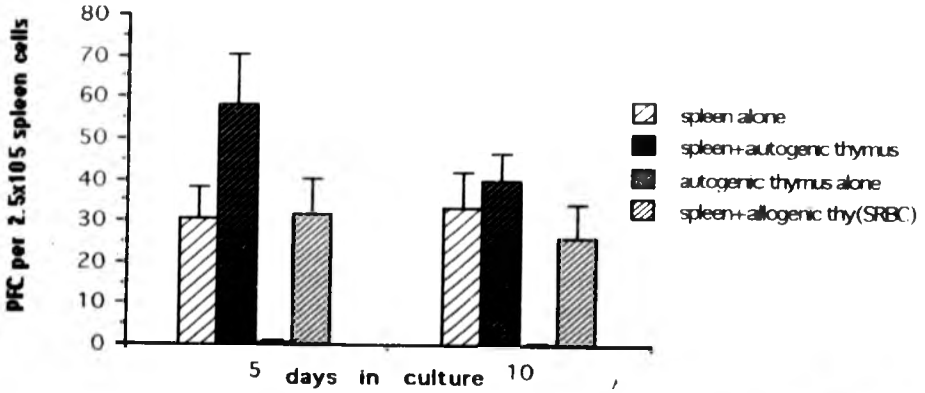


Fig 6.44: The effects of cyclophosphamide treatment, pre-in vitro transfer, of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to SRBC, in fish 2

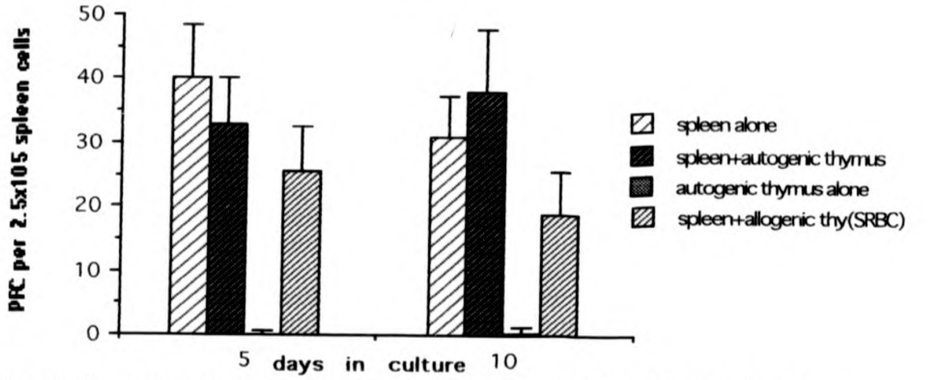


Fig 6.45: The effects of cyclophosphamide treatment, pre-in vitro transfer, of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to SRBC, in fish3.

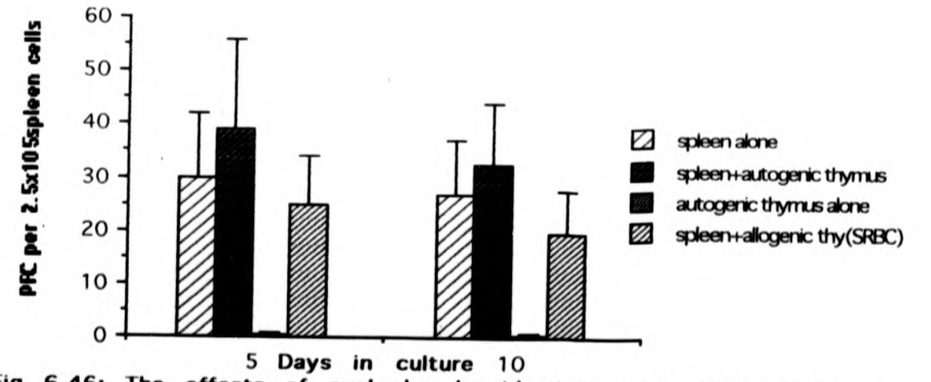


Fig 6.46: The effects of cyclophosphamide treatment, pre-in vitro transfer, of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to SRBC (the means of all three fish tested).

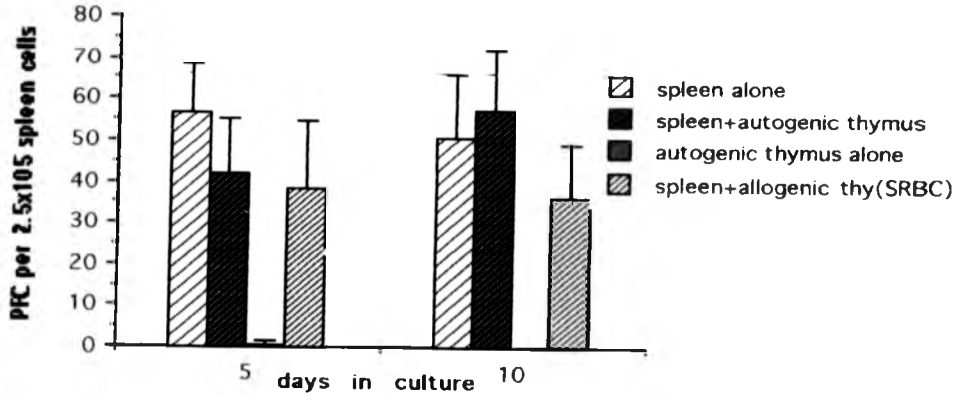


Fig 6.47: The effects of NMU treatment, pre-in vitro transfer, of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to SRBC, in fish 1.

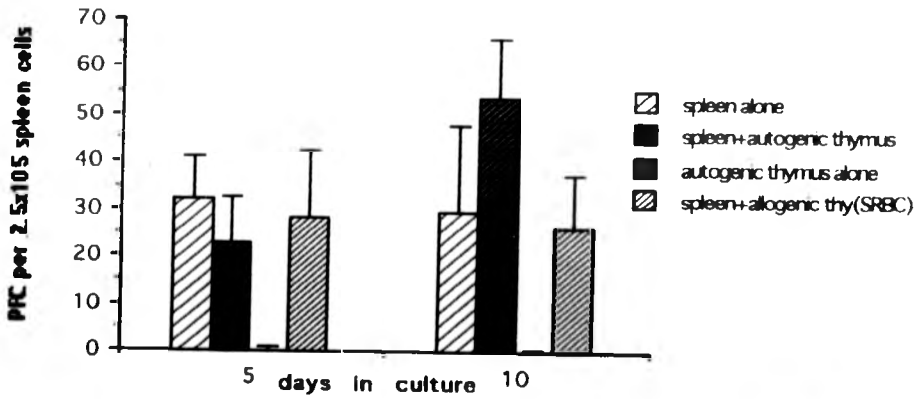


Fig 6.48: The effects of NMU treatment, pre-in vitro transfer, of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to SRBC, in fish 2.

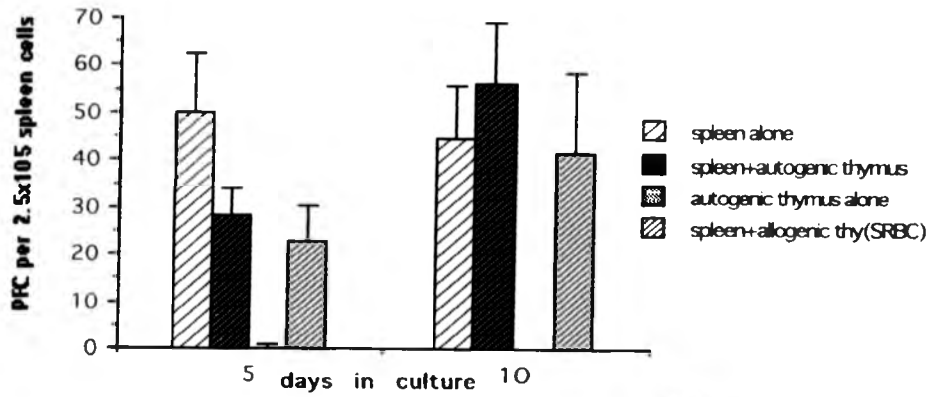


Fig 6.49: The effects of NMU treatment, pre-in vitro transfer of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to SRBC, in fish3.

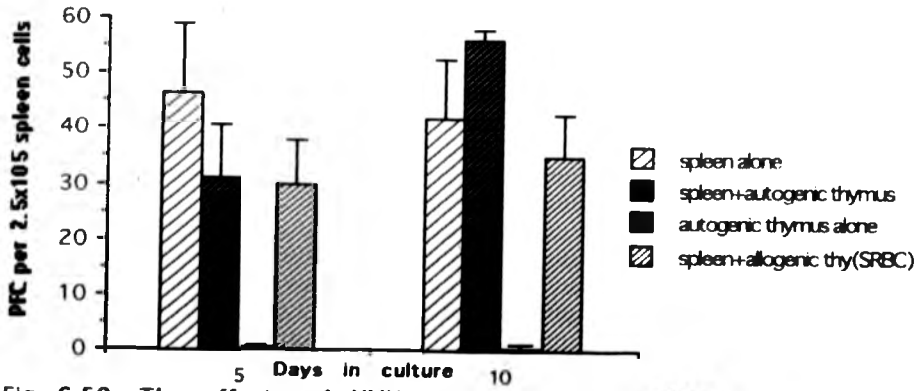


Fig 6.50: The effects of NMU treatment, pre-in vitro transfer, of autogenic spleen and thymus leukocytes, on the PFC response of autogenic and allogenic thymus-spleen cocultures to SRBC (means of all three fish tested).

unimmunised donors had no ability whatsoever to produce any suppressive effects, in any of the experimental assays tested. There were also only a very small number of cases (1/5 DNP-KLH immunised fish and 1/5 SRBC immunised fish) where the allogenic thymus, taken from a donor fish immunised with a different antigen from the autogenic spleen donor, was able to exert any suppressive effect, strongly suggesting that the thymus induced suppression that was seen was in fact antigen specific. There were no cases where allogenic thymocytes were able to produce an enhancement of the spleen only PFC responses, which was sometimes seen in the autogenic cocultures.

6.4. Discussion:

The present study provides strong evidence for the existence of T suppressor cells in the rainbow trout. In all the cultures, with all of the antigens, there were negligible PFC responses by the autogenic thymus alone, which suggests that the thymus lacks a significant number of antibody secreting cells. In the case of the DNP-KLH, SRBC and the TNP-SRBC immunised fish, there was significant suppression in the spleen+autogenic thymus cocultures, after 5 days incubation compared to the spleen alone group. In the DNP-KLH group, 4 out of the 5 test fish showed significant suppression in the spleen+autogenic thymus cocultures. In the TNP-SRBC immunised group, all 5 test fish showed lower PFC responses in the spleen+autogenic thymus cocultures, when compared to spleen alone, though this was only significant in 1 out of the 5 fish. In the SRBC immunised group again, all five test fish showed spleen+ autogenic thymus cocultures with lower PFC responses than the spleen alone cultures, though this was only significant in 2 out of the 5 fish. After 10 days in culture the autogenic thymus induced suppression of PFC responses was

present in only 1 out of the 5 test fish, and in fact the suppression was replaced by significant enhancement in 2 out of the 5 test fish, when compared to spleen alone cultures. In the SRBC immunised group, 4/5 fish showed suppression of the PFC response in spleen+allogenic thymus cocultures (2/5 in spleen+autogenic thymus cocultures) compared to spleen alone cultures after 5 days, though this effect had largely disappeared by 10 days. Therefore in trout , there is strong evidence of an initial suppression of the spleen leukocyte PFC response by coculture with both autogenic and allogenic thymocytes which is however reduced or diminished in the second half of the culture period. Only the DNP-KLH immunised fish showed any evidence of significant autogenic thymus induced amplification of spleen alone PFC responses. This type of amplification of response, after an initial suppression, has been seen in the amphibian *Xenopus leavis* after immunisation with erythrocyte antigens (Ruben et al, 1983), although the amplification observed there was on a much larger scale than evident in the present study. The authors suggested that this amplification was due to a balance between helper and suppressor cells, with the suppressor cells being reduced during the second half of the culture period. In trout after 10 days in culture the initial level of suppression seen was reduced or in some cases completely abolished. This could be due to the decline of suppressor cells due to the continuing absence of antigen, other than at the time of challenge, while helper cells may persist for longer periods in the absence of antigen.

In the case of the fish immunised with TD antigens, the PFC response of the splenocytes cocultured with allogenic thymocytes from donors immunised with the same antigen as the autogenic spleen donor, was mostly suppressed (although not always significantly so) when compared to the spleen alone cultures, and in general this suppression carried on into the

second half of the culture period (more so than with the autogenic cocultures). Allogenic thymocytes, from fish immunised with a different antigen than the autogenic spleen donor, were able to produce some suppression of spleen alone responses, but this was only in a very small number of fish (3 in total). These results strongly suggest that the suppression observed in the present study is antigen specific and is able to operate across genetic barriers.

The allogenic thymus induced suppression persisted into the second half of the culture period, and in general with a lower level of reduction in suppression, than was seen with the autogenic thymus induced suppression, which in fact sometimes led to a significant amplification of the spleen alone PFC responses. Sakai (1991) found that it was possible to transplant lymphocytes from a rainbow trout immunised with goldfish red blood cells, into non-immunised recipient trout, and to detect high haemolysin titres in the recipient fish for up to 4 weeks after transfer. This suggested that the allogenic helper cells were able to function in the recipient fish, thus producing the high haemolysin titres. If the amplification of the splenocyte PFC responses, seen in the second half of the culture period in some of the present fish, was restricted to autogenic cocultures, then it suggests that T helper cell activity in rainbow trout is genetically restricted. T helper cell activity in mammals is considered to be genetically restricted in that recognition of both antigen and compatible 'self' glycoproteins, which are products of the genes located on the major histocompatibility complex (MHC), is required (Klein, 1986). In amphibian systems, helper cells have also been shown to be genetically restricted while suppressor cells are not (Bernard et al, 1981). It could be that a similar process occurs in trout, however at present there has been very little work done on the degree of homology between the principle regulatory mechanisms of the MHC in mammals and fish (Stet and Egberts, 1991).

Another interesting point about the thymus suppression observed in the present study was that although allogenic thymocytes from fish immunised with antigens different from the autogenic splenocytes produce some suppression, allogenic thymocytes from unimmunised donor fish never produced suppression in any of the fish, immunised with any of the antigens. This is important because it shows that any possible effects of a mixed lymphocyte reaction (MLR) generated in the allogenic coculture system would not markedly influence the results as no suppression was noted in the non-immunised thymocyte-splenocyte cocultures. This also shows that the *in vitro* thymus suppression observed is antigen inducible as only thymocytes from immunised fish were able to produce any suppression of the splenocyte PFC response. Since only allogenic thymocytes, taken from a donor fish immunised with the same antigen as the autogenic splenocyte donor, were consistently able to produce suppression, the suppressor cells seem to act in an antigen-specific manner.

The suppression seen in the spleen+autogenic thymus combinations appeared to be cyclophosphamide sensitive, since in all of the TD antigen immunised fish there was an absence of any significant suppression in the cyclophosphamide treated autogenic cocultures, and in fact the PFC responses of some of these were significantly higher than cyclophophamide treated spleen alone responses. There was, however, still significant suppression in these fish when the autogenic spleen from cyclophosphamide treated fish was cocultured with allogenic thymocytes from untreated fish. This suggests that the cyclophosphamide sensitive suppressor function lies within the thymus in rainbow trout. This situation is also found in the mammalian immune system (Chan et al, 1983), where cyclophosphamide has been used to deplete mammalian T-suppressor cells. In the amphibian *Xenopus leavis*, it

has been shown that suppression induced by the haptenated antigens, TNP-SRBC and TNP-LPS, is cyclophosphamide sensitive (Clothier et al, 1989). In general, the PFC responses of the cyclophosphamide treated splenocytes alone cultures seemed to be slightly higher than the untreated cultures, though not significantly so. This could perhaps be due to the removal of any autogenic suppressor cells present within the splenocyte population.

Unlike cyclophosphamide, NMU treatment, pre-*in vitro* transfer, did not seem to have any consistent effect on either the magnitude of PFC responses of the splenocyte alone cultures to the TD antigens, or on the degree of suppression seen in the autogenic splenocyte-thymocyte cocultures. In mammals it has been shown that a single dose of NMU temporarily reduces the white blood cell count and temporarily suppresses cell-mediated immunity and humoral immune responses in rats and mice (Wayneforth and Magee, 1974). NMU has also been shown to remove the thymic cortex, suppress allograft rejection and remove the ability to mount a carrier primed, helper T cell mediated hapten specific response in *Xenopus leavis* (James et al, 1982). In this study, a similar dose was used as in the amphibian studies, but it had no consistent effect on the PFC response of splenocytes or autogenic cocultures. It may be that fish leukocytes are more resistant to NMU and that some changes might have been seen if a higher dose had been used.

Autogenic and allogenic coculture did not have the same level of effect on TNP-LPS immunised fish leukocytes that it had on the response to the TD antigens . There was also no evidence of thymus induced suppression against the TNP-LPS antigen, in either allogenic or autogenic cocultures in the present study. In the amphibian coculture experiments carried out by Ruben et al (1983), also there was no suppression observed in TNP-LPS immunised

cocultures. However Kaattari et al (1986) found that low doses of X-irradiation applied to *in vitro* cultures of rainbow trout leukocytes led to an increased PFC response to both the TD antigen TNP-KLH and the TI antigen TNP-LPS, suggesting that suppressor cells were acting against both TD and TI responses. Cyclophosphamide treatment of the autogenic leukocyte donor fish two days prior to *in vitro* transfer had no effect on the PFC response of the TNP-LPS immunised leukocytes. There were no significant differences between any of the cultures in any of the fish tested at either five or ten days incubation. There was no enhancement of PFC responses in the TNP-LPS immunised fish following cyclophosphamide treatment, as seen in the autcgenic cocultures in some of the TD antigen immunised fish. Hence, the present results indicate that no thymus induced suppression occurred against TNP-LPS, under the experimental conditions used.

In conclusion, the data presented further strengthens the argument that suppressor cells exist in rainbow trout, that the suppressor function is centered in the thymus and that it is directed mainly, if not exclusively, to TD antigen responses. The suppression observed also appears to be genetically unrestricted, as it functions across both allogenic and autogenic boundaries.

Chapter 7:

Electrophoretic analysis of rainbow trout lymphocyte whole cell proteins

7.1. Introduction :

The lack of a range of surface markers to characterise lymphocyte subpopulations is the single biggest obstacle to rapid and significant advances in the study of fish immunology (Kaattari, 1992). The availability of such markers would place the study of fish immunology on a par with that of higher vertebrate groups, in addition to increasing our knowledge of the evolution of the immune system. There is increasing evidence that teleost fish exhibit some degree of lymphocyte heterogeneity, based on monoclonal antibody analysis (Deluca et al, 1983), lymphocyte migration pathways (Tatner and Findlay, 1991), responses to mitogens (Etlinger et al. 1976; Caspi et al. 1984; Reitan and Thuvander, 1991), and cellular co-operation in the response to hapten-carrier complexes (Miller and Clem, 1984; Miller et al, 1985). While these studies strongly indicate the presence of T and B lymphocytes, final proof has yet to be obtained. Antibodies to surface markers need to be produced which could then be used for specific cell separations, coupled with immunologically relevant *in vitro* assays, to investigate the function of defined lymphocyte subpopulations.

At the present time, fish lymphocytes can be broadly separated into 'T' and 'B' cells, by use of anti surface-IgM (sIg) monoclonal antibodies. DeLuca et al (1983) have shown that sIg- pronephric lymphocytes from rainbow trout respond well to Concanavalin A (Con A) but poorly to Lipopolysaccaride (LPS) mitogen, while the sIg+ population respond well to LPS, but less well to Con A. More recent work on the channel catfish has demonstrated that there is a requirement for putative T cells (sIg-) as well as B cells (sIg+) for antibody production to known mammalian thymus dependent antigens (Miller and Clem, 1984b; Miller et al, 1985). This evidence suggests that the sIg+ population exhibits B cell properties, and the sIg- population, T cell properties.

There has been some work on the development of markers for teleost T lymphocytes. Secombes et al (1983a, b) obtained a monoclonal antibody that reacted with thymocytes but not with serum IgM. Miller et al (1987) produced an antibody that reacted with channel catfish T lymphocytes, and when used in panning experiments, isolated a lymphocyte population that provided helper activity for antibody synthesis to a thymus dependent antigen. More recently, Ainsworth et al (1990) produced two clones which identified a lymphocyte population which reacted more to Con A than to LPS, suggesting T lymphocyte properties. However, none of the T cell markers so far produced have been able to distinguish sub populations of T lymphocytes.

In amphibians, T and B cells can be clearly differentiated by the presence or absence of surface Ig. In addition, some as yet not fully characterised T cells exhibit a seemingly unique cell surface 120 kD glycoprotein (Nagata, 1985, 1986a, 1986b, 1988). Recently, Guillet et al (1990) identified a 26 kD protein which was present only in the cytoplasm and nucleus of hydrocortisone sensitive thymocytes, and suggested that this protein was a T cell development protein.

It was the aim of the present study to try to obtain markers that could be used to positively select for the T lymphocyte population as a whole, and any T lymphocyte sub populations, such as helper and suppressor cells, that exist in rainbow trout. Rainbow trout leukocytes were treated with the non-ionic detergents NP-40 and Triton X-100, which solubilises the proteins without denaturing them. Both of these detergents have been widely used in the study of mammalian (Trowbridge et al, 1975; Chambers et al, 1986) and amphibian (Guillet et al, 1990) lymphocyte antigenic determinants. In the present study the production of rainbow trout leukocyte NP-40 and triton X-100 whole cell lysates permitted use of the technique of polyacrylamide gel electrophoresis

(PAGE) to look for any proteins that were specific for, or occurred in larger amounts, in leukocytes from each of the major lymphoid organs. In addition, the effects of various *in vivo* drug treatments on the resultant PAGE protein patterns of the leukocyte NP-40 or triton X-100 lysates were examined. *In vivo* hydrocortisone (HC) treatment has been shown to produce a profound lymphocytopenia in the thymus (83% HC sensitive) and the spleen (50% HC sensitive) of the amphibian axolotl (Tournefier, 1982), and in the mouse results in selective depletion of cortical thymocytes (Papiernik et al, 1977).In the axolotl, HC treatment has been shown to have the same effect as adult thymectomy, in enhancing anti-sheep red blood cell antibody responses. It was suggested that both exerted an effect on the T-suppressor cell population (Charlemagne, 1979). One aim of this study was to look at the effects of *in vivo* HC treatment and adult thymectomy on the PAGE protein patterns of rainbow trout leukocytes, to investigate whether any proteins, in the NP-40 and triton X-100 lysates, were enhanced or reduced due to these treatments. The effects of stimulation of the rainbow trout leukocytes with Concanavalin A (Con A), which is a mammalian T cell mitogen, and lipopolysaccaride (LPS), which is a mammalian B cell mitogen, on the PAGE protein patterns of triton X-100 leukocyte lysates were also investigated.

7.2. Materials and Methods

7.2.1. Fish :

Adult rainbow trout (150-250g) were used throughout this study. They were maintained as described in section 2.1. All fish used as lymphoid organ donors were killed using an overdose of Benzocaine (Sigma) at a concentration of 30 ppm.

7.2.2. Preparation of 0.1% NP-40 leukocyte lysates :

Spleen, kidney, thymus and peripheral blood leukocytes were treated with 0.1% NP-40 lysis buffer as described by Davies and Brown (1987). Briefly leukocytes from spleen, kidney, thymus and peripheral blood were separated as described in section 2.2. The cells were washed twice in hanks balanced salt solution (HBSS; Sigma) and adjusted to $2x10^7$ cells ml^{-1}. One ml aliqouts of the cells were then pelleted, in a sterile tissue culture test tube (Sterilin), and resuspended in 50ul of HBSS. The tube containing the pelleted cells was stored on ice before addition of 1 ml of NP-40 lysis buffer (10 mM Tris-HCl buffer pH7.4, 0.1% NP-40 (w/v), 150 mM NaCl, 1 mM diethylenediamine tetraacetic acid (EDTA)) and 1ul of 1M phenylmethylysulphonyl fluoride (PMSF; Sigma) in acetone. The contents of the tube were gently mixed and left on ice for 30 mins. The tubes were then spun x800g at 4°C to pellet the nuclei. The supernatant was then removed using a Pasteur pipette, and spun at x100000g for 60 mins at 4°C, in a Beckman ultracentrifuge. After centrifugation the supernatant was removed and stored at -70°C. The supernatants from three fish were pooled for each organ and for each treatment before addition of the sample buffer.

7.2.3. Preparation of 1% triton X-100 leukocyte lysates :

The 1% triton X-100 spleen, kidney, thymus and peripheral blood lysates were prepared as in 7.2.2, with the 0.1% NP-40 lysis buffer being replaced with 1% Triton X-100 lysis buffer (phosphate buffered saline pH 7.4 [appendix 1] containing 1% triton X-100 [Sigma], 0.1% SDS, 1mM PMSF and 5mM EDTA [Sigma]). As before the supernatants from three fish were pooled before the samples were run on the gel.

7.2.4. Hydrocortisone treatment :

Three fish received a single I.P. injection of Hydrocortisone sodium hemisuccinate (HC; Sigma) at a dose of 0.5 g/kg body weight (Tournefier, 1982). The HC was dissolved in phosphate buffered saline (PBS) pH 7.4 and each fish was injected with 0.2 mls containing the appropriate dose.

7.2.5. Thymectomy:

Twelve adult rainbow trout were thymectomised as described by Tatner (1990). Each fish was left for 6 months between thymectomy and removal of lymphoid organs for analysis. The lysis buffer treated leukocyte supernatants from three fish were pooled before running on the gel.

7.2.6. Determination of protein concentration in leukocyte lysates :

The protein concentrations of the thymus, spleen, kidney and peripheral blood lysates was measured using the Biorad Protein assay Kit (Biorad Laboratories, Herts, UK). The Biorad protein assay is based on the observations of Bradford (1976), that the absorbance maximum for an acidic solution of Coomassie blue G-250 shifts from 465nm to 595nm when binding to protein occurs. A standard curve was set up using bovine serum albumin (BSA; Sigma) as the protein standard. The BSA was dissolved in either 1% triton X-100 or 0.1% NP-40 lysis buffer, and not PBS, to control for the effects these detergents have been shown to have on the optical density of the Biorad protein dye reagent (Bradford, 1976).

7.2.7. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE was carried out as described in section 2.12. All gels were

carried out using the Hoeffer Labs Mighty Small II electrophoresis system, under reducing conditions. The 0.1%NP-40 and 1% triton X-100 lysates were dissolved 1:1 in SDS sample buffer (appendix 3) to give a protein concentration of 5 mg/ml^{-1} when the gel was to be stained using 0.2% Coomassie blue, or 3 mg/ml^{-1} when the gel was to be stained using the silver staining method. The gel was loaded with 10ul of lysate/sample buffer mixture per well, giving a protein concentration of 50ug or 30ug per well.

The gels were stained using 0.2% Coomassie (appendix 2) for 1 hour at room temperature, and then destained overnight using up to four changes of destain solution (appendix 2). When gels were stained using the silver staining method a Biorad Silver Stain Kit was used, which is based on the technique described by Switzer et al (1979). Some of the gels were also stained using the Periodic acid-Silver stain method described by Dubray and Bezard (1982).

7.2.8. Polyclonal rabbit antisera to 38 kD 0.1% NP-40 lysis buffer soluble thymocyte specific protein.

The 38 kD thymocyte specific polypeptide was cut out from the gel using a scalpel and the gel slices added to 1 ml of PBS pH 7.4. The gel slices were then macerated using a glass stirring rod until there were no large fragments present. The solution was then repeatedly passed through a 26 gauge needle using a 1 ml syringe until a paste was formed. This was incubated overnight at 4°C, and then injected subcutaneously into two sites in the rabbit. The rabbit was given three further subcutaneous immunisations before being bled out four days after the final injection. The corresponding control serum was obtained just before the first immunization.

7.2.9. Immunocytochemical staining using rabbit anti-38 kD polypeptide antiserum.

Slide preparations were made for spleen, thymus, kidney and peripheral blood leukocytes using a cytospin centrifuge (Shandon, Berks, UK). The slides were allowed to air dry at room temperature for 30 mins before being fixed in methanol for 5 minutes. The rabbit anti-38 kD polypeptide was then added to the slide, diluted 1:100 in TBS pH 7.4/2% foetal bovine serum, for 1 hour at room temperature. The slide was washed using tris buffered saline pH 7.6 (TBS; appendix 1) and HRP anti-rabbit IgG (whole molecule; SAPU), diluted 1:100 in TBS/2% foetal bovine serum, was added to the slide for 1 hour at room temperature. The slides were then washed using TBS and the Diaminobenzidine substrate solution (DAB; Sigma) was added for 5 minutes. The slides were washed using TBS, before being counterstained using haematoxylin. Non-immune rabbit serum was used as a control.

7.2.10. Mitogen assays using Con A and LPS:

The mitogen assays on the spleen, thymus, kidney and peripheral blood leukocytes were carried out as described in section 2.7. Con A was used at a concentration of 40ug/ml^{-1} and LPS was used at a concentration of 200ug/ml^{-1}. The cells were incubated for 4 days before being harvested. The T and B cell blasts were then treated with 1% triton X-100 lysis buffer as described in section 7.2.3.

7.3. Results :

Treatment of rainbow trout leukocytes with 0.1% NP-40 lysis buffer produced two different fractions following ultracentrifugation, an insoluble fraction and a soluble fraction. The vast majority of the protein was in the

Table 7.1 : The protein content of 0.1% NP-40 and 1% Triton X-100 lysis buffer treated leukocytes and untreated whole cell leukocytes, as determined by the Biorad protein assay, using a bovine serum albumin standard curve.

Leukocyte treatment	*ug protein ml^{-1} Spleen	Thymus	Kidney	Peripheral blood
Untreated whole cells ($1x10^6$ cells)** from intact fish.	132.17 ±18.21	149.41 ±29.40	125.80 ±40.52	123.71 ±18.67
Whole cells from thymectomised*** fish ($1x10^6$ cells)	132.97 ±22.35	-	138.67 ±30.88	119.20 ±13.39
0.1% NP-40**** soluble fraction	28.01 ±14.57	19.67 ±12.54	25.70 ±12.69	17.87 ±6.22
0.1% NP-40 insoluble fraction	6.09 ±2.31	3.63 ±1.28	4.82 ±1.39	4.99 ±0.81
1% Triton X-100 soluble fraction	46.23 ±11.61	35.15 ±8.15	38.25 ±10.43	40.08 ±16.79
1% Triton X-100 insoluble fraction	3.84 ±2.53	2.98 ±1.18	3.18 ±0.53	2.64 ±1.38

*All protein concentrations given are the mean (± standard deviation) of three fish.

** Whole cell leukocytes were suspended in PBS pH 7.4 (appendix 1) to $1x10^7$ cells ml^{-1}.

*** The fish were thymectomised for 6 months before use.

**** The leukocytes were suspended in the 0.1% NP-40 and 1% Triton X-100 lysis buffers to $5x10^7$ cells ml^{-1}.

soluble fraction, with an average of 4.73 ±0.85 more protein in the soluble fraction compared to the insoluble fraction in lysates from all the lymphoid organs used (Table 7.1). Treatment of leukocytes with 1% triton X-100 lysis buffer again produced an insoluble and a soluble fraction, with an average of 12.76 ±1.62 times more protein in the soluble fraction than in the insoluble fraction. Both of these values are considerably smaller than the amount of protein associated with the whole cells, both the intact and thymectomised lysates. The NP-40 treated leukocyte soluble fractions, from all the lymphoid organs used, contained an average of 30.16 ±7.22 times less protein than the whole cells taken from the corresponding lymphoid organ. The triton X-100 treated leukocyte soluble fraction, from all the lymphoid organs used, contained an average of 16.85 ±3.06 times less protein than whole cells from the corresponding untreated lymphoid organ. The difference could be due to the removal of the nuclei from the NP-40 and triton X-100 lysates before ultracentrifugation (section 7.2.3.).

Plate 7.1. shows the effect of 6 month adult thymectomy on the SDS-PAGE profiles of thymus, kidney and peripheral blood 0.1% NP-40 treated leukocyte lysates. There is a 16.5 kD protein band which is present in the intact peripheral blood lysate and the thymus lysate, but absent from the 6 month thymectomised peripheral blood lysate and from the intact and thymectomised kidney lysates. There is also a 13.5 kD protein band that is heavily present in the thymus and intact peripheral blood lysates, but stains less intensely in the intact kidney and the thymectomised kidney and peripheral blood lysates. Plate 7.2 shows the effect of 6 month thymectomy on the SDS-PAGE profiles of thymus, intact and 6 month thymectomised spleen NP-40 lysates. Here, the 16.5 kD protein band is heavily present in both the thymus and intact spleen lysates but stains less intensely in the

thymectomised spleen lysates. There is also a 200 kD protein band which is only present in the intact spleen and peripheral blood NP-40 lysates (Plates 7.1 and 7.2).

The effects of an I.P. injection of hydrocortisone sodium hemisuccinate on the SDS PAGE profiles of spleen and thymus leukocyte NP-40 lysates are shown in Plate 7.3. The 7% gel clearly shows a 38 kD protein band that is present only in the thymus lysate, and appears to be a surface protein (Plate 7.10). This 38 kD protein band is absent from kidney and peripheral blood lysates (not shown). The band appears to be HC sensitive, in that it stained only very lightly in the 2 days post HC treatment lysate, but returned to a similar staining intensity as the untreated control 4 days post HC treatment.

The SDS PAGE protein profiles of 1% triton X-100 treated spleen, kidney, thymus and peripheral blood leukocytes are shown in Plate 7.4. A 38 kD protein band that stains very intensely is present in the kidney lysate, present but less intensely stained in the spleen and peripheral blood, and absent from the thymus lysate profile. The thymus lysate has a 45 kD protein band that stains very intensely, and is present in much smaller amounts in the spleen and peripheral blood, but appears to be absent from the kidney profile. The thymus lysate also has a 18 kD protein band that is absent from the spleen, kidney and peripheral blood lysate profiles.

When spleen and kidney leukocytes are separated using nylon wool two populations of leukocytes are obtained, an adherent and non-adherent population (see Chapter 3). Plate 7.5 shows the effect of nylon wool separation on the SDS PAGE profile of spleen leukocytes, when the gel is stained with 0.2% Coomassie blue. There is a 73 kD protein band that is present in both the intact control and adherent leukocyte lysates but appears to be enriched in the non-adherent spleen lysate. A 51 kD protein band is

present in both separated lysates, but only shows very faint staining in the intact control lysate. A 27.5 kD protein band is present in both separated leukocyte lysates, but is absent in the unseparated control. When the nylon wool separated spleen and kidney leukocyte lysates are stained using the silver staining method there are obvious differences between the separated leukocyte lysates (Plate 7.6). A 65 kD protein band is enriched in both the spleen and kidney non-adherent lysates, and a 59 kD protein band is enriched in the spleen adherent lysate. There is also a group of four protein bands, with molecular weights of 29, 31, 34 and 37 kD, that are present in the unseparated controls and both nylon wool separated lysates from the spleen and kidney, but they seem to be enriched in the adherent lysates when compared to the non-adherent lysates in both types of leukocytes.

Plate 7.7 (a) and (b) show the effect of Con A stimulation on the triton X-100 treated leukocyte lysates, when the gels are stained using the silver staining method. A 67 kD protein band is heavily enriched in the thymus, spleen and peripheral blood Con A stimulated lysates, when compared to the unstimulated control lysates. However the 67 kD band is not enriched to the same extent in the Con A stimulated kidney lysate. There is also a group of protein bands with molecular weights ranging from 38-51 kD, whose presence is greatly enhanced in the Con A stimulated thymus, spleen and peripheral blood lysates, but in the kidney Con A stimulated lysate only the 41 and 47 kD protein bands are enhanced. A group of 3 main proteins, with molecular weights of 29, 31 and 34 kD, are greatly reduced in the Con A stimulated lysates of spleen, thymus and peripheral blood, but remain major components of the kidney Con A stimulated lysate. The unstimulated thymus, spleen, kidney and peripheral blood lysates contain a group of four protein bands, with molecular weights of 21, 22.5, 24 and 26 kD, but they only remain a major

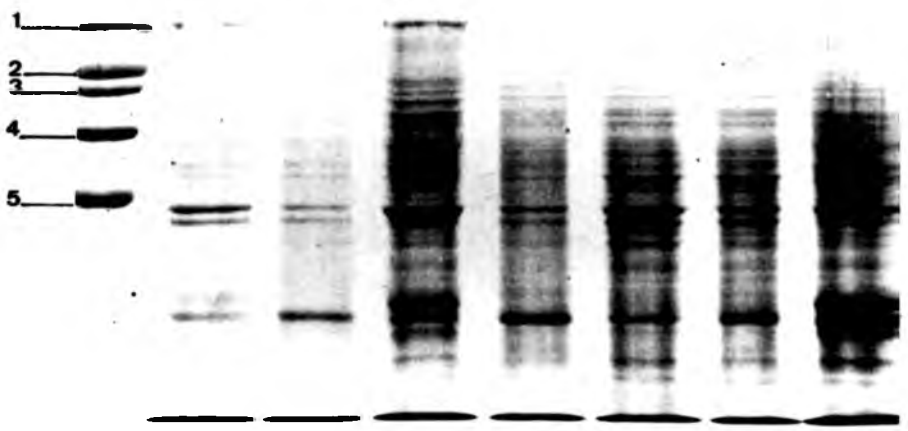


Plate 7.1: The effect of six month adult thymectomy on the protein patterns of peripheral blood and kidney leukocytes treated with 0.1% NP-40 lysis buffer. The 12% gel was run under reducing conditions, and is stained with 0.2% Coomassie blue:

Track 1 : Biorad high molecular weight standards (1=200 kD, 2=116.25 kD, 3=97.4 kD, 4=66.2 kD, 5=45 kD) .

Track 2 : Six month thymectomised peripheral blood leukocyte lysate.

Trach 3 : Six month thymectomised peripheral blood leukocyte lysate.

Track 4 : Peripheral blood intact control leukocyte lysate.

Track 5 : Six month thymectomised peripheral blood leukocyte lysate.

Track 6 : Six month thymectomised kidney leukocyte lysate.

Track 7 : Six month thymectomised kidney leukocyte lysate.

Track 8 : Intact control kidney leukocyte lysate.

The arrows ( ) point to two protein bands (13.5kD and 16.5kD) which are heavily present in the thymus and intact control blood lysates, but not the thymectomised blood and kidney or the intact control kidney lysates (50 ug of protein was applied to each of wells 2-8).

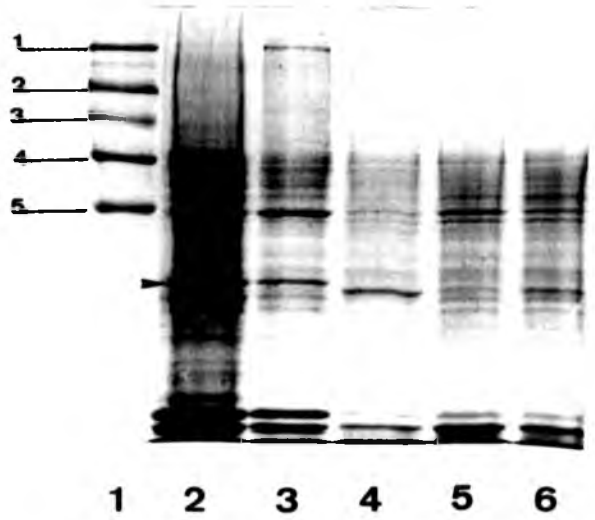


Plate 7.2 : The effect of six month adult thymectomy on the protein patterns of spleen leukocytes treated with 0.1% NP-40 lysis buffer. The 12% gel was run under reducing conditions, and is stained with 0.2% Coomasie blue:

Track 1 : Biorad high molecular weight standards (1=200 kD, 2=116.25 kD, 3=97.4 kD, 4=66.2 kD, 5=45 kD).

Track 2 : Thymus leukocyte lysate control.

Track 3 : Spleen leukocyte intact control.

Track 4 : Six month thymectomised spleen leukocyte lysate.

Track 5 : Six month thymectomised spleen leukocyte lysate.

Track 6 : Six month thymectomised spleen leukocyte lysate.

The arrow (►) represents a protein band (16.5kD) which is heavily present in the thymus and intact control spleen lysates, but appears to be absent in the thymectomised spleen lysates (50ug of protein was applied to each of wells 2-6).

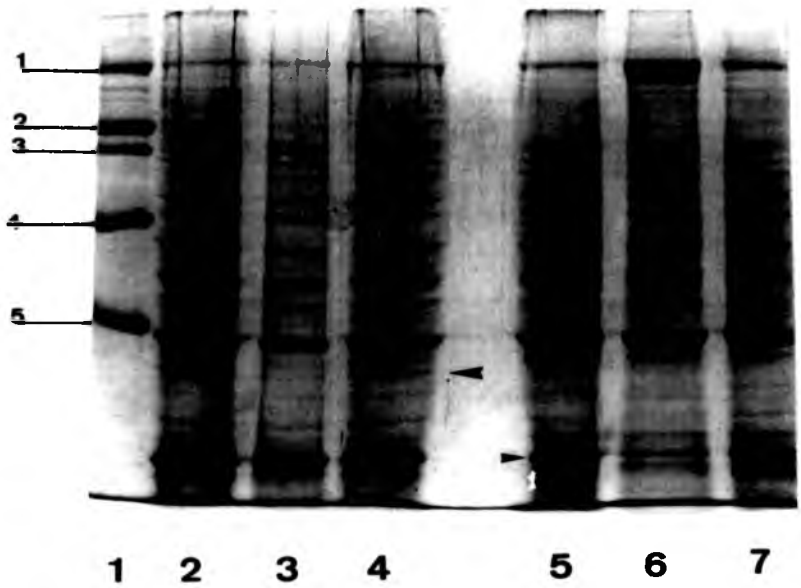


Plate 7.3 : The effects of an I.P. injection of hydrocortisone sodium hemisuccinate on the protein patterns of spleen and thymus leukocytes treated with 0.1% NP-40 lysis buffer. The 7% gel was run under reducing conditions and stained with 0.2% Coomassie blue:

Track 1 : Biorad high molecular weight standards (1=200 kD, 2=116.25 kD, 3=97.4 kD, 4=66.2 kD, 5=45 kD).

Track 2 : Thymus leukocyte lysate control.

Track 3 : Two days post-hydrocortisone injection thymus leukocyte lysate.

Track 4 : Four days post-hydrocortisone injection thymus leukocyte lysate.

Track 5 : Spleen leukocyte lysate control.

Track 6 : Two days post-hydrocortisone injection spleen leukocyte lysate.

Track 7 : Four days post-hydrocortisone injection spleen leukocyte lysate.

There is a protein band (38kD), marked by the large arrow (►), which is present in the thymus lysates but not the spleen lysates. The intensity of this band is reduced in the 2 days post-hydrocortisone injection thymus lysate. (50ug of protein was applied to each of wells 2-7).

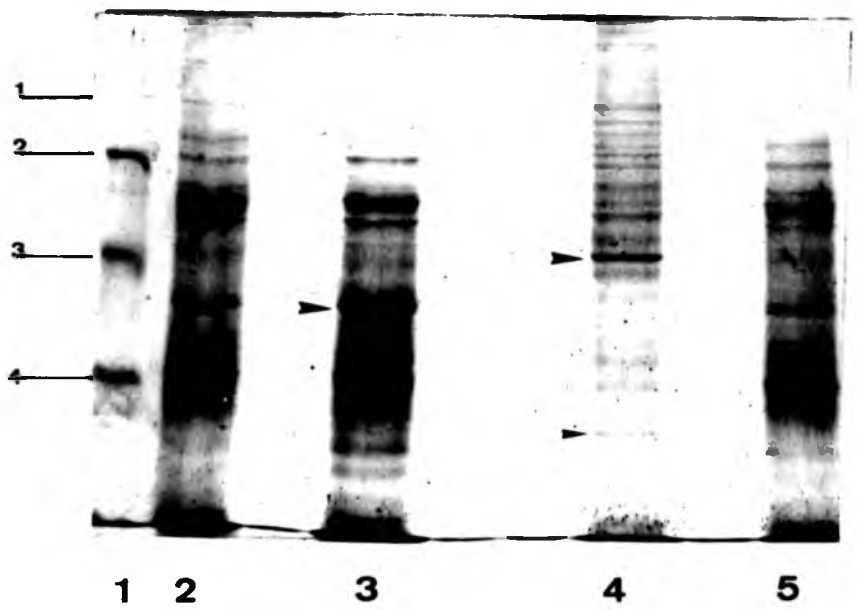


Plate 7.4: The SDS-PAGE protein patterns from spleen, thymus, peripheral blood and kidney leukocytes treated with 1% triton X-100 lysis buffer. The 12% gel was run under reducing conditions and stained with 0.2% Coomassie blue.

Track 1 : Pharmacia molecular weight standards (1=94 kD, 2=67 kD, 3=43 kD, 4=30 kD).

Track 2 : Spleen leukocyte lysate.

Track 3 : Kidney leukocyte lysate.

Track 4 : Thymus leukocyte lysate.

Track 5 : Peripheral blood leukocyte lysate.

The arrow in lane 3 points to a protein band (38kD) that is heavily present in kidney lysate, but less so in the spleen and blood lysates and is absent from the thymus lysate. The two arrows in lane 4 point to two protein bands, one has a molecular weight of 45kD ( ) and the other 18kD ( ), that are present in the thymus lysate but not in any of the other organ lysates. (50ug of protein was applied to wells 2-5).

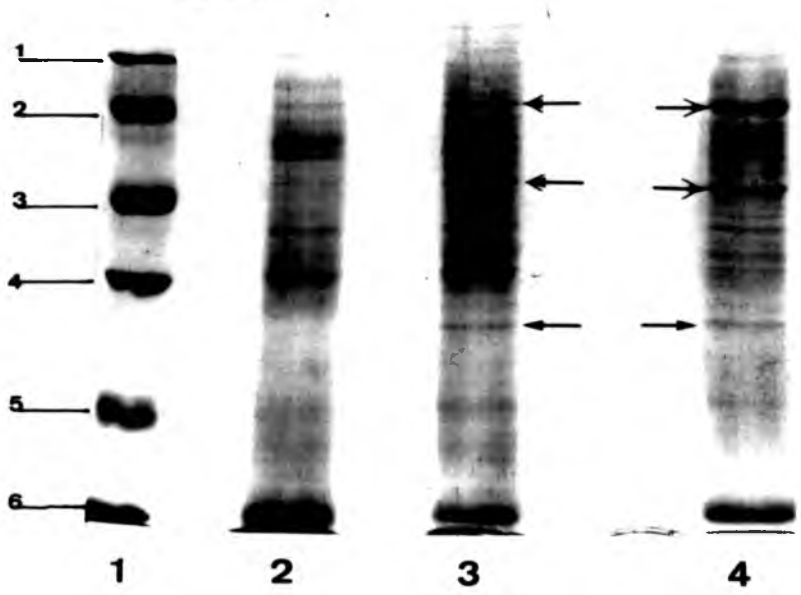


Plate 7.5 : The SDS-PAGE protein patterns of nylon wool separated and unseparated spleen leukocytes treated with 1% triton X-100 lysis buffer. The 12% gel was run under reducing conditions and stained with 0.2% Coomassie blue.

Track 1 : Pharmacia molecular weight standards (1=97 kD, 2=67 kD, 3=43 kD, 4=30 kD, 5=20.1 kD 6=14.4 kD).

Track 2 : Unseparated spleen leukocyte lysate.

Track 3 : Nylon wool adherent spleen leukocyte lysate.

Track 4 : Nylon wool non-adherent spleen leukocyte lysate.

The three arrows in tracks 3 and 4 point to three proteins that were enriched in the nylon wool separated lysates. The top arrow represents a protein (73.5 kD) that is enriched in the non-adherent lysate (track 4) compared to the unseparated and adherent lysates. The second arrow represents a protein (51 kD) that is present only in the two nylon wool separated lysates. The third arrow points to a protein (27.5 kD) that again is present in both the nylon wool separated lysates, but absent in the unseparated lysate (track 2).Fifty ug of protein was added to tracks 2-4.

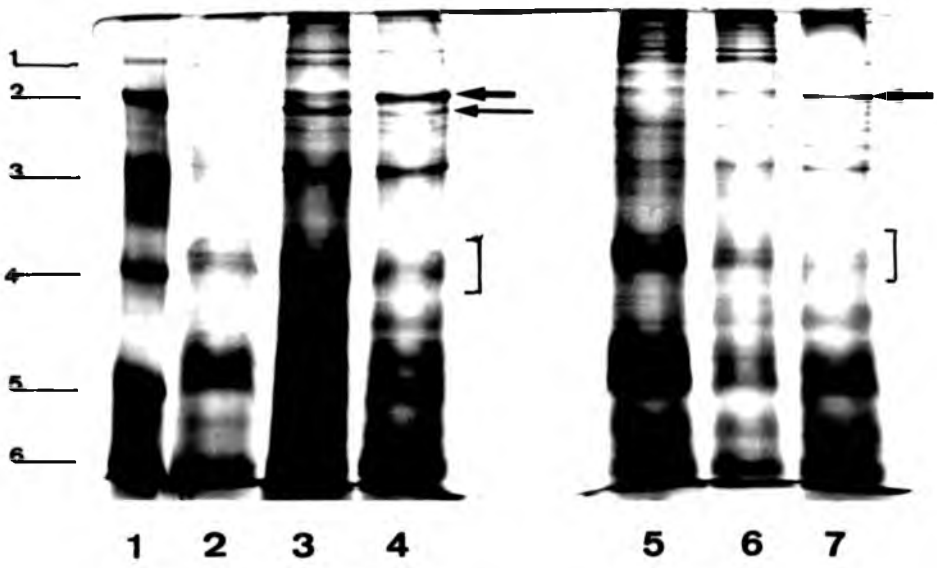


Plate 7.6 : The SDS-PAGE protein patterns of nylon wool separated and unseparated spleen and kidney leukocytes treated with 1% triton X-100 lysis buffer. The 12% gel was run under reducing conditions and stained using the silver staining method.

Track 1 : Pharmacia protein standards (1=97 kD, 2=67 kD, 3=43 kD, 4=30 kD, 5=20.1 kD, 6=14.4 kD).

Track 2 : Unseparated spleen leukocyte lysate.

Track 3 : Nylon wool adherent spleen leukocyte lysate.

Track 4 : Nylon wool non-adherent spleen leukocyte lysate.

Track 5 : Unseparated kidney leukocyte lysate.

Track 6 : Nylon wool adherent kidney leukocyte lysate.

Track 7 : Nylon wool non-adherent kidney leukocyte lysate.

The large arrow in lanes 4 and 7 (⟶) points to a protein band (65 kD) that is enriched in both the spleen and kidney non-adherent lysates. The longer arrow (⟶) in lane 4 points to a protein (59 kD) that is enriched in the spleen adherent lysate, but not the kidney. There is also a group of four proteins (29, 31, 34 and 37 kD) marked by the square brackets that are present in greater

amounts in the adherent lysates of both spleen and kidney (30 ug of protein was applied to each of wells 2-7).

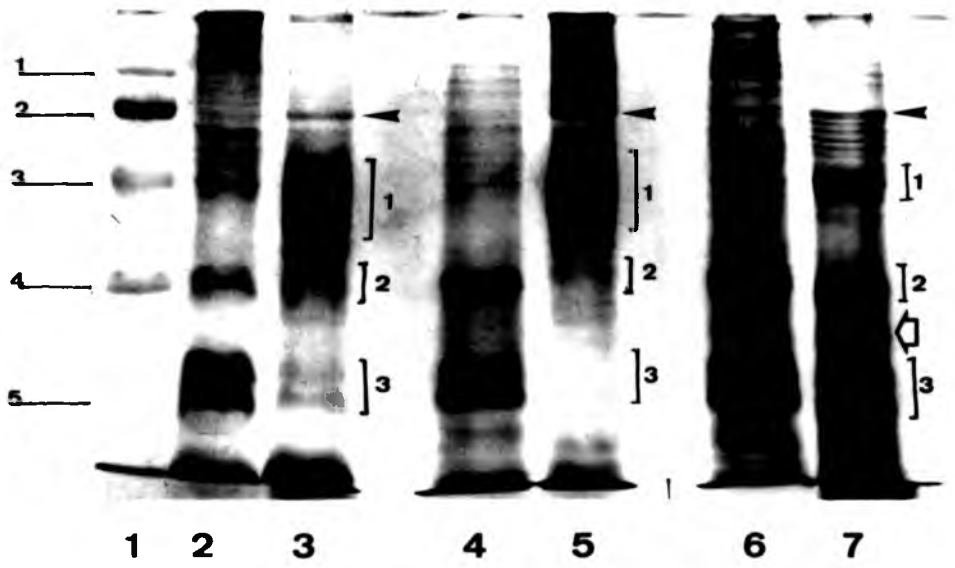


Plate 7.7 (a) : The SDS-PAGE protein patterns of Con A stimulated and unstimulated thymus, spleen and kidney leukocytes treated with 1% triton X-100 lysis buffer. The 12% gel was run under reducing conditions and stained using the silver staining method.

Track 1 : Pharmacia protein standards (1=97 kD, 2=67 kD, 3=43 kD, 4=30 kD, 5=20.1 kD).

Track 2 : Unstimulated thymus leukocyte lysate.

Track 3 : Con A stimulated thymus leukocyte lysate.

Track 4 : Unstimulated spleen leukocyte lysate.

Track 5 : Con A stimulated spleen leukocyte lysate.

Track 6 : Unstimulated kidney leukocyte lysate.

Track 7 : Con A stimulated kidney leukocyte lysate.

In track 3 and 5 there is a protein band (67 kD), marked by the arrow (➡), which is more heavily present in the Con A stimulated thymus and spleen lysates than the unstimulated lysates, but not to the same extent in the case of the kidney stimulated and unstimulated lysates. The number 1 square brackets

enclose a group of proteins (38-51 kD) that are vastly more heavily present in the Con A stimulated, than the unstimulated lysates, of the thymus and spleen, and to a much lesser extent in the Con A stimulated lysate from the kidney , where only two proteins (41 and 47 kD) appeared to have increased. The number 2 square brackets enclose a group of 3 main proteins (29, 31 and 34 kD) present in all three unstimulated lysates, that are much less evident in the thymus and spleen Con A stimulated lysates, but are still heavily present in the kidney Con A stimulated lysate. The number 3 square brackets show a group of four main proteins (21, 22.5, 24.5 and 26 kD) that are heavily present in all three unstimulated lysates, but only remain heavily present in the kidney Con A stimulated lysate. The arrow (⇨) points to 2 proteins (27 and 28.5 kD) that are only very faintly present in the unstimulated spleen and kidney lysates, but are heavily present in the Con A stimulated kidney lysate. They are absent from the thymus stimulated and unstimulated lysates.

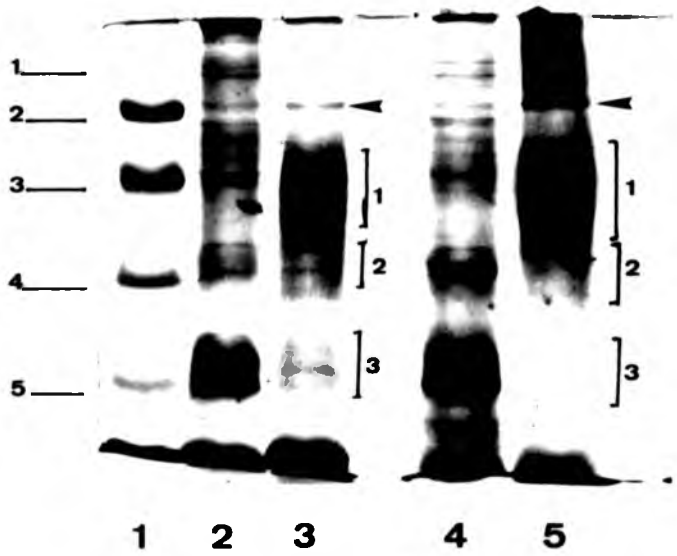


Plate 7.7 (b) : The SDS-PAGE protein patterns of Con A stimulated and unstimulated thymus and peripheral blood leukocytes treated with 1% triton X-100 lysis buffer. The 12% gel was run under reducing conditions and stained using the silver staining method.

Track 1 : Pharmacia protein standards (1=97 kD, 2=67 kD, 3=43 kD, 4=30 kD, 5=20.1 kD).

Track 2 : Unstimulated thymus leukocyte lysate.

Track 3 : Con A stimulated thymus leukocyte lysate.

Track 4 : Unstimulated peripheral blood leukocyte lysate.

Track 5 : Con A stimulated peripheral blood leukocyte lysate.

The protein band (67 kD), marked by the arrow (➤), is present in enhanced quantities in the Con A stimulated lysates from both thymus and peripheral blood. The number 1 enclosed brackets enclose a group of proteins (38-54 kD)whose presence was greatly enhanced, in both thymus and peripheral blood lysates, after Con A stimulation. The number 2 square brackets enclose

a group of 3 main protein bands (30, 32 and 34 kD) that are present in both unstimulated lysates, but are present in smaller quantities in the thymus and peripheral blood Con A stimulated cultures. The number 3 square brackets enclose a group of four protein bands (21, 23, 24.5 and 26 kD) that are present in large quantities in both the unstimulated thymus and peripheral blood lysates, but are present in much smaller amounts in the Con A stimulated thymus lysate and absent from the Con A stimulated peripheral blood lysate.

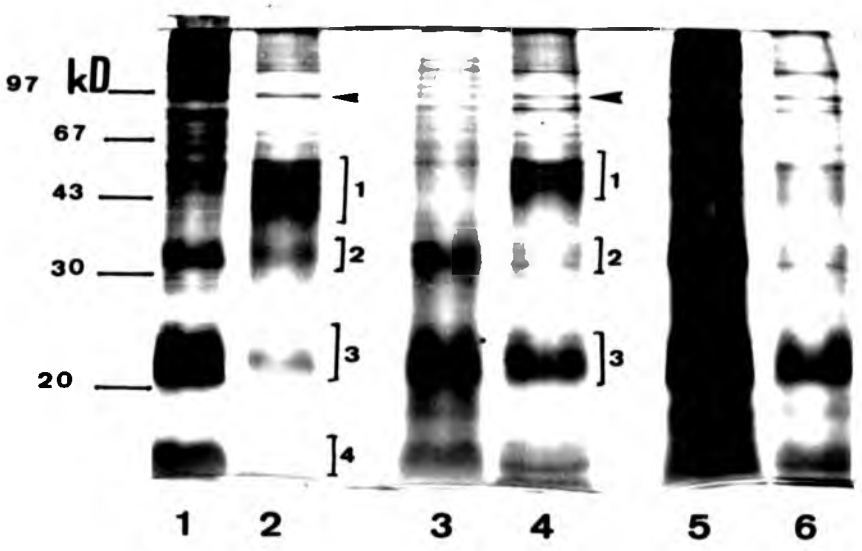


Plate 7.8 : The SDS-PAGE protein patterns of LPS stimulated and unstimulated thymus, spleen and kidney leukocytes treated with 1% triton X-100 lysis buffer. The 12% gel was run under reducing conditions and stained using the silver staining method.

Track 1 : Unstimulated thymus leukocyte lysate.

Track 2 : LPS stimulated thymus leukocyte lysate.

Track 3 : Unstimulated spleen leukocyte lysate.

Track 4 : LPS stimulated spleen leukocyte lysate.

Track 5 : Unstimulated kidney leukocyte lysate.

Track 6 : LPS stimulated kidney leukocyte lysate.

The arrow (➤) points to a protein band (67 kD) that appears to increase in staining intensity in the stimulated spleen lysate (track 4), but not in the thymus or kidney lysates. The number 1 brackets in track2 point to a group of 7 protein bands (38-51 kD) that increase in staining intensity in the three LPS stimulated thymus lysate. The spleen LPS activated lysate, enclosed in number 1 brackets, also shows an increase in staining intensity of the same 7

protein bands as the thymus, but it is most evident in 4 main bands (45-51 kD). The staining intensity of the LPS stimulated kidney lysate in this area seems to be restricted to a 50 kD protein band, although the other six bands are still in evidence. The number 2 brackets enclose a group of three main protein bands (29, 31 and 34 kD) that are present in all three unstimulated lysates, and remain present in the stimulated lysates, but appear to show a reduced staining intensity. The four main protein bands (21, 23, 24.5 and 26 kD), enclosed by the number 3 brackets, are present in all three unstimulated lysates and retain a similar staining intensity in the stimulated spleen and kidney lysates, whereas the thymus stimulated lysate shows a reduced staining intensity.

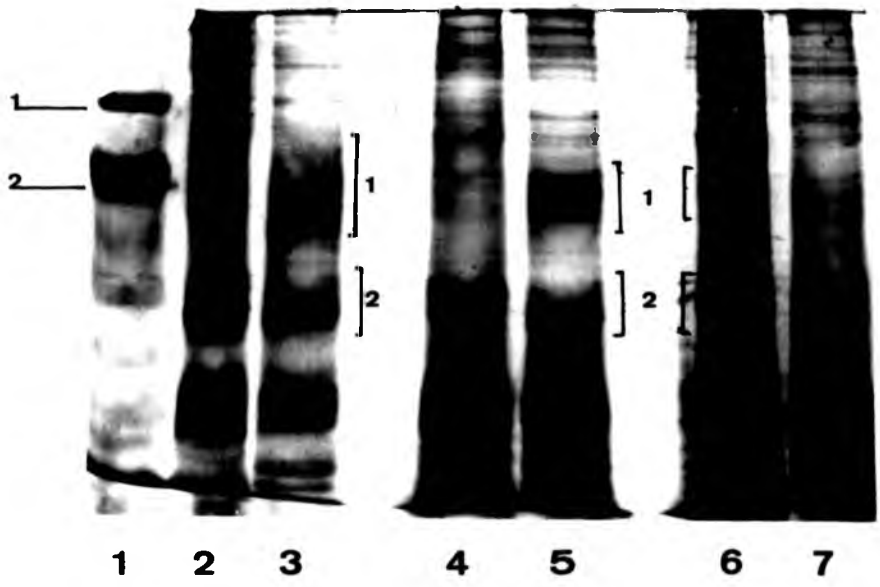


Plate 7.9 (a) : The SDS-PAGE protein patterns of Con A stimulated and unstimulated thymus, spleen and kidney leukocytes treated with 1% triton X-100 lysis buffer. The 12% gel was run under reducing conditions and stained using the Periodic Acid-Silver Stain for glycoproteins.

Track 1 : Pharmacia protein standards (1=67 kD, 2=43 kD).

Track 2 : Unstimulated thymus leukocyte lysate.

Track 3 : Con A stimulated thymus leukocyte lysate.

Track 4 : Unstimulated spleen leukocyte lysate.

Track 5 : Con A stimulated spleen leukocyte lysate.

Track 6 : Unstimulated kidney leukocyte lysate.

Track 7 : Con A stimulated kidney leukocyte lysate.

The number 1 square brackets enclose a group of 4 protein bands (41-49 kD) in the unstimulated thymus, spleen and kidney lysates and in the Con A stimulated spleen and kidney lysates (Track 2,4,5,6 and 7), and a group of 8 protein bands (38-51 kD) in the Con A stimulated thymus lysate (Track 3) The number 2 square brackets enclose a group of 2 main protein bands (34 and

36 kD) in the unstimulated spleen (Track 4) and kidney (Track 6) lysates and 4 main protein bands (29, 31, 34 and 36 kD) in the unstimulated thymus lysate (Track 2). After Con A stimulation the number 2 brackets enclose a group of 4 protein bands (29, 31, 34 and 36 kD) in the thymus, spleen and kidney lysates (Tracks 3, 5 and 7), and in all cases the 29 and 31 kD bands stain more intesely than the 34 and 36 kD bands. The number 3 square brackets enclose a group of heavily staining protein bands (21-24 kD) in all three unstimulated lysates. These proteins remain in all three Con A stimulated lysates, although staining intensity is reduced in the Con A stimulated thymus lysate.

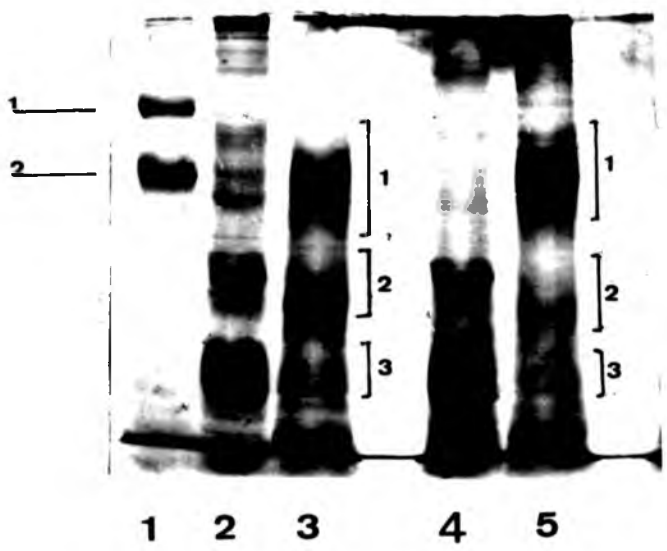


Plate 7.9 (b) : The SDS-PAGE protein patterns of Con A stimulated and unstimulated thymus and peripheral blood leukocytes treated with 1% triton X-100 lysis buffer. The gel was run under reducing conditions and stained using the Periodic Acid-Silver Stain for gylcoproteins.

Track 1 : Pharmacia protein standards (1=67 kD, 2=43 kD).

Track 2 : Unstimulated thymus leukocyte lysate.

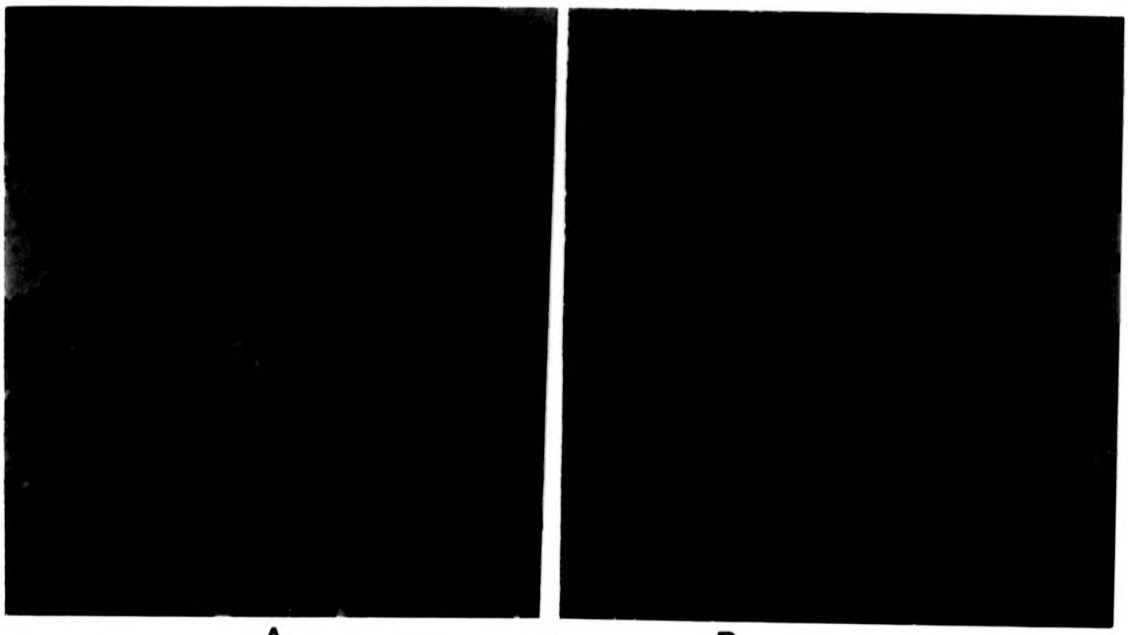
Track 3 : Con A stimulated thymus leukocyte lysate.

Track 4 : Unstimulated peripheral blood leukocyte lysate.

Track 5 : Con A stimulated peripheral blood leukocyte lysate.

The number 1 square brackets enclose a group of 4 protein bands (41, 44, 47 and 49 kD) stained in the unstimulated thymus and peripheral blood lysates, and a group of 8 main protein bands (38-51 kD) that stain positively in the Con A stimulated thymus and peripheral blood lysates. The number 2 square brackets enclose a group of four main proteins (27, 29, 31 and 34 kD) present in the thymus unstimulated lysate, with the 31 kD protein band staining very

heavily. The Con A stimulated thymus lysate has only two main protein bands in this area (27 and 29 kD), which stain up much more intensely than in the unstimulated lysate, with the 31 and 34 kD proteins staining very faintly. The number 2 square brackets enclose 2 main protein bands (31 and 34 kD) in the unstimulated peripheral blood lysate. The Con A stimulated peripheral blood lysate shows only very faint traces of the 31 and 34 kD bands, but there is an area positive staining where the 27 and 29 kD protein bands are present in the thymus lysates. The number 3 square brackets enclose a group of four protein bands (21, 22, 24 and 26 kD) which are heavily present in both of the unstimulated lysates, but show a greatly reduced staining intensity in the two Con A stimulated lysates. (30ug of protein was apllied to each well).



A B

Plate 7.10: **A**- Cytospin preparation of rainbow trout thymocytes stained with a polyclonal rabbit anti-38 kD 1% NP-40 lysis buffer soluble protein antibody. The antibody was diluted 1:100 in PBS pH7.4/2% foetal bovine serum. The slides were counterstained using haematoxylin. The positive staining cells can be visualised by the brown peroxidase ring round the cell membrane (x200 magnification). **B**- Control cytospin preparation of thymocytes, in this case with the rabbit anti-38 kD protein band omitted, but all other steps in the staining procedure carried out (x200 magnification).

component of the kidney Con A stimulated lysate. A 27 and a 28.5 kD protein band appears to be enriched in the Con A stimulated kidney lysate, when compared to the unstimulated kidney, but appear to be absent from the thymus, spleen and peripheral blood unstimulated and Con A stimulated lysates.

The effect of stimulation of thymus, spleen and kidney leukocytes with LPS, on the SDS PAGE protein profiles is shown in Plate 7.8. A group of 7 protein bands are present, with molecular weights ranging from 38-51 kD, that show enhanced staining intensity in the thymus and spleen LPS stimulated lysates, but the increase is restricted mainly to 4 bands, with molecular weights of 45, 47, 48 and 51 kD, in the spleen lysate. The increased staining intensity of the kidney lysate, in this area, seems to be restricted to a 50 kD protein band, although the other 6 bands are still evident in the activated kidney lysate. These protein bands are in the same area of the gel as the increased staining intensity seen after Con A stimulation (Plate 7.7(a) and (b)), although the increase was much more intense after Con A stimulation. There are also three main protein bands, with molecular weights of 29, 31 and 34 kD, present in the unstimulated thymus, spleen and kidney lysates whose staining intensity is decreased in the LPS stimulated lysates, but to a much lesser extent than with the Con A stimulated lysates (Plate 7.7 (a) and (b)). The four protein bands with molecular weights of 21, 23, 24.5 and 26 kD, that were greatly reduced in staining intensity in Con A stimulated thymus, spleen and peripheral blood lysates remain a major component of the LPS stimulated spleen and kidney lysates, but were reduced slightly in the LPS activated thymus lysate.

When Con A stimulated lysates are stained for glycoprotein content, using the Periodic acid-Silver stain method, some quite obvious differences

are seen in the SDS-PAGE protein profiles when compared to unstimulated controls (Plate 7.9 (a) and (b)). The most obvious difference is that in the unstimulated spleen, kidney and peripheral blood there are 2 gylocoprotein bands (34 and 36 kD), but in the same area of Con A stimulated spleen, kidney and peripheral blood lysates there are 4 glycoprotein bands (29, 31, 34 and 36 kD), with the 29 and 31 kD bands staining most intensely. In the unstimulated thymus lysate all four of the glycoprotein bands are present with the 34 kD band making up the largest component, but in the Con A stimulated lysate the 29 and 31 kD bands stain more intensely and the 34 and 36 kD bands almost disappear. The four glycoprotein bands (21, 22, 24 and 26 kD) are greatly reduced in staining intensity in the Con A stimulated thymus and peripheral blood lysates, but remain major components of the Con A activated spleen and kidney lysates.

When cytospins of spleen, kidney, peripheral blood and thymus leukocytes were stained using the polyclonal antibody to the 38kD thymocyte specific band (Plate 7.3), only the thymocytes showed any positive staining. It was found that 22.3 $\pm$12.8 % of thymocytes stained positive, which showed up as an intense brown ring round the membrane only (Plate 7.10).

7.4. Discussion:

The aim of this comparative electrophoretic analysis of rainbow trout thymocyte, splenic, kidney and peripheral blood leukocyte NP-40 and triton X-100 lysate protein profiles was to identify some possible marker proteins that could be used for the analysis of functional lymphoid subpopulations. It has been shown in mammalian systems that T and B lymphocytes have characteristic surface proteins (Trowbridge et al, 1975; Gahmberg et al, 1976). More recently the use of cloning technology combined with monoclonal

antibody production, has allowed the production of a large number of monoclonal antibodies which recognise different molecules on the surface of T cells and which are used in the definition of mammalian T lymphocyte subpopulations, based on the CD1/2/3/4 and 8 nomenclature (reviewed by Blann, 1987). The results of the present study have shown that resting leukocyte whole cell lysates from different organs of intact and thymectomised fish have different SDS PAGE protein profiles, as do nylon wool separated and mitogen stimulated leukocyte whole cell lysates.

In the present study, a NP-40 lysis buffer soluble 38 kD polypeptide was found to be a major protein expressed by ~22 % of thymocytes, but not by spleen, kidney or peripheral blood leukocytes. The immunocytochemical study has shown that this protein seems to be restricted to the thymocyte outer membrane. In SDS-PAGE, fish sampled two days after *in vivo* hydrocortisone (HC) treatment showed reduced staining intensity of the 38kD band, but this was not seen in fish sampled 4 days after HC treatment, where the staining intensity returned to pre-HC treatment levels. It has been shown that *in vivo* HC treatment in the axolotl induces a rapid thymus depletion, with 60% of cells lost 8 days after treatment (Tournefier, 1982). No signs of regeneration were observed in the axolotl 16 days after HC treatment, in contrast to mice where thymus regeneration began 4-5 days after HC treament (Blomgren and Anderson, 1971). In the trout, if the 38 kD protein band is associated with a HC sensitive thymocyte population then regeneration is very rapid, as staining intensity of this band returned to normal levels after only 4 days. It could be that trout thymocytes are more HC resistant than those of the axolotl, and so the dose used was suboptimal for complete removal of this population. Tournefier (1982) found that axolotl antibody production and allograft rejection were not impaired by HC treament, which the author suggested could mean

that the more mature thymus cell subpopulations and the mature peripheral T cell populations were resistant to corticosteriods. It could be that a similar situation occurs in the fish thymus and that the 38 kD protein is expressed as a developmental protein in an immature, HC sensitive thymus subpopulation only. More work needs to be done to resolve this issue.

Evidence for thymus subpopulations, helper and suppressor cells in fish comes from the use of thymectomy and X-irradiation (Manning et al, 1982a; Nakanishi, 1986; Tatner et al, 1987). Manning et al (1982a) found that an elevated antibody response to the thymus dependent (TD) antigen human gamma globulin (HGG), in one month thymectomised adult rainbow trout suggesting the presence of suppressor cells in the thymus. Tatner et al (1987) found no such elevation in antibody response to HGG in 9 month thymectomised trout, and in fact found a slight decrease in antibody response when compared to non-thymectomised controls. The authors suggested that this was due to a decrease in T helper cells in the periphery of the thymectomised fish. Nakanishi (1986) looked at the effects of one month thymectomy and X-irradiation on the humoral response to the TD antigen sheep red blood cells, in *Marmoratus sebasticus*. The author found evidence of a X-irradiation resistant suppressor cell population residing within the thymus. In the present study, 6 month adult thymectomy had a significant effect on the SDS PAGE protein profiles of spleen, kidney and peripheral blood NP-40 lysates. Most obviously, a 16.5 kD protein band that was heavily present in the intact spleen and peripheral blood lysates, was absent or present in much smaller amounts in the thymectomised spleen and peripheral blood lysates. Both the intact control and thymectomised kidney lysates failed to show any evidence of large amounts of the 16.5 kD protein band, but it was very heavily present in the thymus lysate. It could be possible that the 16.5 kD protein is a

protein or part of a protein that is specific for a T suppressor or other T cell subpopulation. The fact that the kidney shows only very small traces of this 16.5 kD protein band may be due to the fact that this organ has a large number of B cells compared to T cells, based on anti-surface IgM monoclonal antibody staining (Deluca et al, 1983) and mitogen responses (Etlinger et al, 1976; Tillit et al, 1984). An antibody against the protein could be produced for use in immunocytochemical and cell separation studies to confirm this.

It has been shown that passage of mammalian lymphocytes over a nylon wool column as described by Julius et al (1973) is an effective means of obtaining a population enriched with T cells, as B cells preferentially adhere to the column and T cells do not (Tada et al, 1978). There is strong evidence, from the present study, to suggest that a similar process occurs with rainbow trout lymphocytes (Chapter 3). When the nylon wool adherent (NW+) and nylon wool non-adherent (NW-) rainbow trout leukocyte lysates were run on SDS-PAGE there were obvious differences in protein profiles. The most striking difference was a 65 kD protein band that was enriched in the NW- spleen and kidney lysates (Plate 7.6). This protein band may be the same as that enhanced in the Con A stimulated spleen, thymus and peripheral blood lysates. If so, then this protein could prove to be a strong candidate as a representative protein of T cells. The protein does not seem to be a glycoprotein since it failed to stain in in the periodic acid-silver stained gels (Plate 7.9 (a) and (b)). There was a second protein band with a molecular weight of 59 kD (PLate 7.6) which was enriched in the spleen NW+ (B cell) lysate, which could make it a candidate for a B cell specific protein. However, it should be noted that this band was not a major component of either intact or NW+ kidney lysates.

It has been shown that mammalian lymphocytes stimulated with the

mitogens Con A and LPS show different surface glycoproteins. Gahmberg et al (1976) showed that mouse T lymphocytes stimulated with Con A showed an increased presence of 77-86 kD glycoproteins when compared to unstimulated controls. B lymphocytes stimulated with LPS showed a similar change. T blasts produced by mixed lymphocyte cultures showed slightly different glycoprotein profiles, with an increase in the presence of 58-86 kD glycoproteins. In the present study there were major changes in the SDS- PAGE profiles of both Con A and LPS stimulated leukocyte lysates. In the Con A stimulated thymus, spleen and peripheral blood lysates there was a very large increase in a group of protein bands, with molecular weights between 38-51 kD. Most of these proteins seemed to be glycoproteins since they stained in the periodic acid-silver treated gels (Plate 7.9). It could be that these are membrane glycoproteins, as in mammals it has been shown that most of the exposed plasma membrane proteins are glycoproteins (Warr et al, 1984). It could be that in the trout these 38-51 kD glycoproteins are involved as receptors, for lectins such as Con A, in the plasma membrane. The fact that there was only an incease in a 41 and a 47 kD protein band in the Con A stimulated kidney lysate, is not suprising since kidney lymphocytes have been shown to respond poorly to Con A when compared to thymus, spleen and peripheral blood (Tillit et al, 1988). There were four glycoproteins with molecular weights of 21, 22.5, 24.5 and 26 kD, that disappeared or were greatly reduced components of thymus, spleen and peripheral blood Con A stimulated lysates, but remained a major component of the Con A stimulated kidney lysate, and all of the LPS stimulated cultures. This suggests that these four glycoproteins warrant further investigation as possible B cell specific proteins. There was also a change in glycoproteins from predominately 34 and 36 kD bands in the unstimulated lysates to predominately 29 and 31 kD in the

Con A stimulated spleen, blood, and kidney lysates. The fact that the 29 and 31 kD proteins were also present in the unstimulated thymus suggests that they may be associated with activated T cells. Whatever the case, it is obvoius that both the T and B blasts have altered SDS PAGE protein profiles, when compared to their unstimulated controls, and suggests that there are probably major surface changes occuring after initiation of proliferation, as occurs in mammals (Gahmberg et al, 1976).

The results of this study clearly show that there are obvious differences in SDS PAGE protein patterns of both unseparated and nylon wool separated resting leukocytes, and of mitogen stimulated leukocyte populations. Several proteins have been found that could possibly be useful as T cell or B cell markers, although this is clearly only very preliminary work, and more work is required regarding their usefulness as lymphocyte subpopulation markers. Nevertheless, it does provide some encouraging results, suggesting that it might be possible to obtain lymphocyte subpopulation specific protein markers using this SDS PAGE technique with whole cell lysates.

Chapter 8: General Discussion.

The present study has provided further evidence to support the hypothesis that teleost fish have functionally separate T and B lymphocyte subpopulations. It should be noted that this only applies to teleost fish and not necessarily to any of the lower classes of fish, which have not been studied in as much depth. The class Pisces is extremely heterogeneous, and spans an enormous distance in the evolutionary tree (McFarland et al, 1985), from the jawless fish of the Agnatha (with ~30 species of lamprey and hagfish), the cartilaginous fish of the Chondrichthyes (incuding the holocephalans with ~25 species and the elasmobranchs represented by over 600 species of sharks, rays and skates) and the bony fish of the Ostreichthyes (represented by a few species like the sturgeon), the Holosteans (represented by a few species such as the gar) and finally the teleosteans which include some 20,000 species ranging from salmon and trout to catfish and perch. Clearly these animals live in very different environments and are subjected to very different selection pressures. Therefore, from an evoloutionary point of view it is of great interest to know at which level during phylogeny the dichotomy of the immune system into T and B lymphocytes occurred, as fish are phylogenetically the most primitive of the vertebrates.

By far the most important group of fish economically are the teleosts (Ellis, 1988). It is important to expand our existing knowledge of their immune system as the aquaculture industry expands worldwide and the need for more environmentally acceptable methods of disease control (vaccines rather than chemical treatments) becomes more pressing.

As shown in chapter 1 there is a large amount of functional evidence for the existence of separable T and B lymphocytes in teleost fish. A large part of this functional evidence has been obtained using anti-surface IgM

monoclonal antibodies, to produce sIg- (T cells) and sIg+ (B cell) subpopulations (DeLuca et al, 1983; Miller and Clem, 1984 a and b, Miller et al,1985; Graham and Secombes, 1990; Thuvander et al, 1990; Reitan and Thuvander, 1991). The most comprehensive part of this work has been done using the channel catfish (Miller et al, 1984a and b, 1985, 1987), which is a phylogenetically advanced warm water teleost. In the present study, strong evidence has been provided for T and B lymphocyte dichotomy in the rainbow trout, which again is important from a phylogenetic point of view as the rainbow trout is a more primitive cold water species, compared to the channel catfish.

The present study has expanded on the evidence for functional T and B cell heterogeniety in the rainbow trout (DeLuca et al, 1983; Graham and Secombes, 1990; Reitan and Thuvander, 1991), by showing that the sIg- and sIg+ subpopulations also have distinct *in vivo* migratory pathways. Chapter 3 showed that autogenic sIg- peripheral blood lymphocytes, reinjected into the original donor, migrated preferentially to the kidney after 24 hours. The kidney has been shown to have a large population of sIg+ lymphocytes when compared to the thymus, spleen and peripheral blood (Table 4.1). In contrast to this it was found that when sIg+ and sIg- lymphocytes were injected into non-histocompatible recipients, the sIg- lymphocytes from the spleen, thymus and kidney migrated preferentially to the spleen. This corresponds with the higher number of acid phosphatase (mammalian T cell specific) positive lymphocytes in this organ (Table 4.1). The reason for the different migratory pathways between the histocompatible and non-histocompatible sIg- lymphocytes is unknown. However it should be noted that the non-histocompatible peripheral blood lymphocytes also migrated preferentially to the kidney. The significance of this is unknown, although there is clearly a

major blood borne sIg- (labelled) population that migrates preferentially to the kidney. The data presented in Chapter 3 provides strong evidence for ecotaxis occurring in the rainbow trout. This phenomenon of ecotaxis is important as it is characteristic of the high degree of specialisation of the higher vertebrate immune system (deSousa, 1981). This raises the question of whether rainbow trout lymphocytes also have the equivalent of the higher vertebrate lymphocyte homing receptors, and the other specialised structures associated with the distinct migratory pathways of mammalian T and B lymphocytes (reviewed by Duijvestijn and Hamann, 1989; deSousa et al, 1991).

Chapter 4 has expanded the functional evidence for T and B cell heterogeniety in rainbow trout by showing that nylon wool separation can be used as an alternative to the sIgM panning method, to obtain T and B cell enriched populations. The results (Table 4.1) showed that the nylon wool adherent population showed T cell like functional and cytochemical properties, whereas the non-adherent population showed B cell like properties. This again shows a similarity with mammalian lymphocytes, which can also be separated using nylon wool (Julius et al, 1973), suggesting that the trout and mammalian adherent lymphocytes share a common receptor. This is not the case however with the lectin soybean agglutinin, suggesting that unlike mammalian B cells the D-galactose residues on trout B cells are not exposed (SBA is specific for D-galactose), or that both T and B cells have exposed D-galactose residues, resulting in a heterogeneous agglutinated population. The results of the nylon wool separation do however provide further functional evidence for the existence of distinct T and B cells in rainbow trout.

A characteristic of the higher vertebrate immune system is the interplay of different subpopulations of helper and suppressor cells in the cellular immune response to TD antigens. The work described in Chapter 6 provided

strong evidence that rainbow trout possess a genetically unrestricted suppressorcell population which is centred in the thymus, which like mammalian suppressor cells are cyclophosphamide sensitive (Chan et al, 1983). The results in chapter 6 showed that there was no significant *in vitro* suppression when the TD antigen TNP-LPS was used. This contrasts with the work in chapter 5, where 8 month adult thymectomy led to an enhanced *in vivo* antibody response to the TI antigen *A. salmonicida*. This suggests that there are two types of suppression occuring in the trout, one against the TD antigens, which is clearly visible *in vitro*, and the other which is operating directly on the B cell, which does not show up clearly in *in vitro* systems. Recently van Ginkel et al (1992) showed that channel catfish *in vitro* antibody responses to the TD antigen TNP-KLH, were very similar to *in vivo* antibody responses, in terms of antibody isotype produced and in their functional affinity. It may be that in the rainbow trout this anti-B cell suppression is weaker than that observed against TD antigens, and so is not as clearly visible *in vitro*. The work in Chapter 6 also provided tentative evidence for a T helper cell population, at least when DNP-KLH was used as antigen (the evidence of Chapter 5 and 6 suggested that DNP-KLH was probably the most thymus dependent antigen used in this study), as during the second half of the culture period the suppression of PFC, evident in the autogenic thymus-spleen cocultures during the first half of the culture period, was replaced by a slightly enhanced PFC response (as compared to the spleen alone cultures). This suggested an interaction between suppression and enhancement that is again characteristic of the higher vertebrate immune system (Sercarz and Krzych, 1991; Dorf et al, 1992). However, further work in fish is required to show that these suppressor and helper functions belong to two distinct types of T lymphocytes.

As mentioned in Chapter 5, a great deal of information regarding the role which the thymus and T lymphocyte populations play in the immune response, comes from the use of the classical thymus ablation and reconstitution experiments. However this has never previously attempted in fish due to the lack of inbred lines for immunological research. This study attempted to overcome this problem by using the technique of cryopreservation of thymocytes for later reconstitution into the original donors (Chapter 5). It was found that two month adult thymectomy had no measurable effect on antibody response to any of the antigens used. However eight month adult thymectomy led to a significantly reduced secondary response to the TD antigens SRBC and DNP-KLH, which was partly restored when the fish were reconstituted with their own cryopreserved thymocytes one week before secondary immunisation. In contrast eight month adult thymectomy produced a significantly elevated secondary antibody response to *A.salmonicida*, which was returned to control levels by reconstitution of the fish's autogenic cryopreserved thymocytes one week prior to secondary immunisation. The results from this work provided evidence for the existence of a short lived suppressor population and a longer lived helper population, which again is similar to the situation seen in higher vertebrates (reviewed by Freitas et al, 1993). These results are important as they provide *in vivo* evidence to support the much greater amount of *in vitro* evidence for the existence of T cell subpopulations (Kaattari et al, 1986; Miller et al, 1987; Clem et al, 1991) in teleosts.

The results presented in Chapters 3 to 6 in this thesis strongly suggest that rainbow trout exhibit a lymphocyte heterogeniety remarkably similar to that seen in higher vertebrates. As the rainbow trout is a primitive cold water teleost this may suggest that further study on the phylogenetic origins of the putative

primordial multipotential lymphoid cell would be better directed at fish lower down in the phylogenetic tree, such as hagfish or sharks. The point should always be made, and emphasised, that conclusions reached with regard to lymphocyte heterogeniety in one class of fish may possibly be invalid for another class, especially considering the extremely wide spectrum of different species.

However it is clear that at present there are still considerable gaps in our knowledge of the immune cells and their interactions in teleost fish. For example it should be noted that for teleost fish to show true lymphocyte heterogeniety, akin to that seen in higher vertebrates, these lymphoid cell subpopulations must show not only distinct functional heterogeniety, as appears to exist, but also distinct developmental pathways as well. As was mentioned in Chapter 1 there has been some work done on the ontogenic development of sIg+ lymphocytes (Razquin et al, 1990; Castillo et al, 1993), however no markers are availible to study the development of T lymphocyte subpopulations, as the T cell markers so far obtained are unable to distinguish particular T cell subpopulations (Secombes et al, 1983; Miller et al, 1987; Ainsworth et al, 1990). In mammals there is a whole series of markers for T cell subpopulations such as helper, suppressor and cytotoxic cells (reviewed by Blann, 1987). The identification and characterisation of these mammalian cell surface molecules specific for particular lymphocyte subpopulations has been facilitated in the last 10-15 years by the use of monoclonal antibody technology (Galfre and Milstein 1981; Barclay and Williams, 1986). The results presented in chapter 7 show that electrophoretic analysis of whole cell leukocytes, lysed using non-ionic detergent lysis buffers, revealed organ specific protein bands in the thymus and kidney. Con A and LPS stimulation of leukocyte cultures produced clear changes in the SDS-

PAGE protein patterns in silver stained and periodic acid-silver stained gels, a situation which also occurs in mammalian lymphocytes (Gahmberg et al, 1976). Nylon wool enriched subpopulations also showed clear differences in SDS-PAGE profiles. A polyclonal antibody was produced which stained 22% of thymocytes using the immunoperoxidase method. This antibody could be a marker for a T cell developmental protein, similar to the one found in amphibians (Guillet et al, 1990), which could prove useful in the study of T cell development. Therefore this study reveals that there are clear differences in protein composition between different lymphocyte populations, as well as the functional differences already mentioned.

In conclusion this thesis strongly suggests that rainbow trout possess functionally distinct and separable lymphocyte subpopulations that are remarkably similar to mammalian T and B cells. The reconstitution (Chapter 5) *in vitro* thymus suppression (Chapter 6) experiments also provided evidence for functionally distinct T cell populations. However there can be no conclusive proof of this until T cell surface markers can be found (akin to the CD4/CD8 system in mammals), which would allow separation studies and *in vitro* assays on the subpopulations obtained to be carried out. Only then could the diverse activities associated with the rainbow trout T cell be assigned to a single multipotential cell type or functionally distinct subpopulations as in higher vertebrates.

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Appendices.

Appendix 1: Buffers.

(a) Phospate buffered saline (PBS) pH 7.4.

$NaH_2PO4.2H_2O$	0.876g/litre
$Na_2HPO_4.2H_2O$	2.56g/litre
NaCl	8.77g/litre

Dissolve the above in 950 ml of dH_2O, and adjust to pH7.4 using 1M NaOH. Make up to 1 litre with dH_2O.

(b) Glucose-Phosphate buffered saline (G-PBS) pH 7.4.

PBS pH 7.4 (as above)	10ml
0.14M NaCl (8.2g/litre)	1000ml
Dextrose	10g

(c) 0.28M Cacodylate buffer pH 6.9.

38.6 g of cacodylic acid (Sigma) was dissolved in 900ml of dH2O, and the pH adjusted to 6.9 using 10M NaOH (40g/100 ml), using about 22 ml. The volume was then brought to 1 litre with dH_2O.

(d) Tris buffered saline (TBS) pH 7.6.

2.42 g of Trizma base (Sigma) was added to 58.48g of NaCl and made up to 1 litre using dH_2O. The pH was adjusted to 7.6 using 1M HCL.

Appendix 2: ELISA reagents.

(a) Bicarbonate coating Buffer pH9.6.

Na_2CO_3	1.59g/litre
$NaHCO_3$	2.93g/litre
NaN_3	0.2g/litre

These were mixed and dissolved in 950 mls of nanopure water, and the PH adjusted to 9.6 using 1M HCL. This could then be stored at 4°C for up to 1 month.

(b) Low salt wash.

$NaH_2PO_4.2H_2O$	0.876g/litre
$Na_2HPO_4.2H_2O$	2.56g/litre
NaCl	22.2g/litre

(c) High salt wash.

$NaH_2PO_4.2H_2O$	0.876g/litre
$Na_2HPO_4.2H_2O$	2.56g/litre
NaCl	29.22g/litre

(d) Substrate buffer for use in horseraddish peroxidase (HRP) labelled antibody systems.

0.1M Citric acid	19.2g/litre
0.1M Sodium acetate (anhydrous)	8.2g/litre

The pH was adjusted to 5.4 using 1M NaOH. This could then be stored at 4°C for up to one month. Immediately before use 33.3 ul of H_2O_2 was added to 100mls of the above solution.

(e) TMB dihydrochloride/acetic acid chromagen for use in HRP antibody systems.

1. Prepare 5 ml of a 2M acetic acid solution and add 42 mM TMB dihydrochloride (Sigma; 0.0658g/5 ml of 1M acetic acid).
2. Store at 4°C in a foil covered bijou.

(f) p-Nitrophenyl phosphate substrate for alkaline phosphatase labelled antibody systems.

Add one p-nitrophenyl phosphate tablet (KpL Labs Inc, Maryland USA) to 10 mls of diethanolamine buffer pH9.8 (see below) and allow to dissolve at room temperature for 30 mins.

(g) 0.05M Diethanolamine buffer pH9.8.

101mg of $MgCl_2$ (BDH) was dissolved in 800mls of dH_20. When this was dissolved 97ml of diethanolamine (BDH) and the pH adjusted to 9.8 with concentrated HCL. This was then made up to 1 litre with dH_2O and 200mg of NaN_3 was added. This could be stored in a foil covered bottle for up to 2 months.

Appendix 3 SDS PAGE stock solutions and reagents:

(a) Acrylamide/bis.

Acrylamide (Biorad) - 29.2g/100ml of nanopure water

N',N'- bis-methylene acrylamide (Biorad) - 0.8g/100ml of nanopure water.The above reagents were acurately weighed out into separate chemically clean containers. The bis was dissolved in ~30 ml of nanopure water, and this solution was transfered to the acrylamide. The bis container was rinsed out with several small aliquots of nanopure water and this was made up to 100 ml with nanopure water. This solution was then filtered through Whatman No4 filter papers and stored in a foil covered bottle at 4°C.

(b) 1.5M Tris-HCl buffer pH 8.8.

Trizma base (Sigma) - 18.15g/100 ml of nanopure water.

The 18.15g of trizma base was dissolved in ~80ml of nanopure water and the pH adjusted to 8.8 using 1M HCl. The solution was then made to 150 ml using nanopure water and stored at 4°C.

(c) 0.5M Tris-HCl buffer pH6.8.

Trizma base - 6g/100 ml of nanopure water.

The 6g of Trizma base was dissolved in ~60 ml of nanopure water and the pH adjusted to 6.8 using 1M HCL. The solution was then made up to 100 ml and stored at 4°C.

(d) 10% Sodium dodecyl sulphate (SDS).

10g of SDS (Biorad) was dissolved in 100 ml of nanopure water with gentle stirring and stored at room temperature.

(e) SDS-Sample buffer:

nanopure water	4.0 ml
0.5M Tris-HCL pH 6.8	1.0 ml
Glycerol	0.8 ml
10% SDS	1.6 ml
2-*B*-mercaptoethanol	0.4 ml
0.05% (w/v) Bromophenol blue	0.2 ml
	8.0 ml in total

This solution had to be made up fresh daily and stored at room temperature.

(f) 5x Electrode (Running) Buffer pH 8.3.

Trizma base	15g/litre of nanopure water
Glycine	72g/litre " " "
SDS	3g/litre " " "

The pH should already be 8.3 and so should not be adjusted. This solution was stored at 4°C for up to one month.

(g) 10% Ammonium persulphate (APS):

100 mg of APS was dissolved in one ml of nanopure water. This solution has to be prepared fresh daily.

(h) Coomassie blue stain:

0.2% (w/v) Brilliant blue (Biorad)

450 ml of methanol

450 ml of nanopure water

100 ml of glacial acetic acid (Sigma).

The brilliant blue was dissolved in the methanol first and then the acetic acid and water. This solution was then filtered and stored at room temperature.

(i) Separating gel preparations- 0.373M Tris, pH8.8.

	12%	10%	7%
nanopure water	3.35 ml	4.0 ml	4.85 ml
1.5M Tris-HCl pH 8.8	2.5 ml	2.5 ml	2.5 ml
10% SDS	100 ul	100 ul	100 ul
Acrylamide/bis	4.0 ml	3.35 ml	2.5 ml
Ammonium persulphate	50 ul	50 ul	50 ul
TEMED (Biorad)	5 ul	5 ul	5 ul

The water, Tris-HCl, SDS and Acrylamide/bis solutions were added first and degassed for 15 mins at room temperature. The APS and TEMED solutions were then added to acheive polymerisation.

(j) Stacking gel preparation - 4.0% gel, 0.125M Tris, pH 6.8.

nanopure water	6.1 ml
0.5M Tris-HCl pH 6.8	2.5 ml
10% SDS	100 ul
Acrylamide/bis	1.3 ml
10% APS	50 ul
TEMED	10 ul

The water, Tris-HCl, SDS and Acrylamide/bis were added first and the solution was degased for 15 mins, before the APS and TEMED were added to initiate polymerisation.

Appendix 4: The cell number and viability of thymocytes from DNP-KLH, SRBC and *A.salmonicida* 1102 immunised fish (Chapter 5).

Table 1: The viability and cell number of the cryopreserved thymocytes from SRBC (two months thymectomised) immunized fish.

Panjet[1] mark	Date frozen	Viabilty on[2] freezing	Date thawed	Viability on[3] thawing	Number of cells frozen
ILA	5.10.92	100%	6.3.92	61%	$5.98X10^7$
ILM	5.10.92	100%	6.3.92	71%	$6.27X10^7$
ILP	5.10.92	100%	6.3.92	64%	$4.75X10^7$
IRA	5.10.92	100%	6.3.92	59%	$5.94x10^7$
IRM	5.10.92	100%	6.3.92	67%	$6.90x10^7$
IRP	5.10.92	100%	6.3.92	73%	$6.01x10^7$
2LA	5.10.92	100%	6.3.92	71%	$4.40x10^7$
2LM	5.10.92	100%	6.3.92	69.5%	$5.61x10^7$
2LP	5.10.92	100%	6.3.92	70.5%	$6.40x10^7$
2RA	5.10.92	100%	6.3.92	76%	$7.21x10^7$
2RM	5.10.92	100%	6.3.92	57%	$4.46x10^7$
2RP	5.10.92	100%	6.3.92	69%	$5.11x10^7$
IRA/IRM	7.10.92	100%	6.3.92	65%	$4.74x10^7$
IRM/IRP	7.10.92	100%	6.3.92	71%	$5.22x10^7$
ILA/ILM	7.10.92	100%	6.3.92	83%	$7.78x10^7$

1. Fish were individually marked using a panjet loaded with 1% alcian blue. The thymocytes from the individual fish were then frozen in a vial identified with this same mark.

2. Viability of the cells was measured, using the trypan blue exclusion test, before addition of the DMSO freezing mixture.

3. The viability of the thawed cells was measured, using the trypan blue exclusion test, after the cells were removed from the freezing mixture and washed three times in HBSS.

Table 2: The viability and cell number of the cryopreserved thymocytes from the *A. salmonicida* (two months thymectomised) immunised fish.

Panjet[1] mark	Date frozen	Viability on[2] freezing	Date thawed	viability on[3] thawing	number of cells frozen
ILA	6.10.91	100%	6.3.92	65%	4.41×10^7
ILM	6.10.91	100%	6.3.92	67%	5.97×10^7
ILP	6.10.91	100%	6.3.92	86%	6.3×10^7
IRA	6.10.91	100%	6.3.92	73%	7.4×10^7
IRM	6.10.91	100%	6.3.92	74%	5.3×10^7
IRP	6.10.91	100%	6.3.92	54%	4.8×10^7
2LA	6.10.91	100%	6.3.92	69%	6.3×10^7
2LM	6.10.91	100%	6.3.92	63%	6.85×10^7
2LP	6.10.91	100%	6.3.92	71%	5.82×10^7
2RA	6.10.91	100%	6.3.92	69%	8.13×10^7
2RM	6.10.91	100%	6.3.92	64%	6.94×10^7
2RP	6.10.91	100%	6.3.92	67%	8.1×10^7
ILA/ILM	7.10.91	100%	6.3.92	78%	5.60×10^7
ILM/ILP	7.10.91	100%	6.3.92	82%	6.3×10^7
IRA/IRM	7.10.91	100%	6.3.92	76%	7.7×10^7

1. Fish were individually marked using a panjet loaded with 1% alcian blue. The thymocytes from the individual fish were then frozen in a vial identified with this same mark.

2. Viability of the cells was measured, using the trypan blue exclusion test, before addition of the DMSO freezing mixture.

3. The viability of the thawed cells was measured after the cells were removed from the freezing mixture and washed three times in hanks balanced salt solution.

Table 3: The viability and cell number of cryopreserved thymocytes in the DNP-KLH (eight months thymectomised) immunized fish.

panjet mark[1] of fish	number of cells frozen	viability on[2] freezing	date frozen	viabilty[3] on thawing	date of thawing
1LP	$3.7x10^7$	100%	7/12/92	79%	15/7/93
2LA	$6.5x10^7$	100%	7/12/92	84%	15/7/93
2LM	$8.1x10^7$	100%	7/12/92	91%	15/7/93
2RP	$4.9x10^7$	100%	7/12/92	87%	15/7/93
1LA/1LM	$4.4x10^7$	100%	7/12/92	69%	15/7/93
1LA/1LP	$5.9x10^7$	100%	7/12/93	93%	15/7/93
1LM/1LP	$7.2x10^7$	100%	7/12/93	90%	15/7/93
1RM/1RP	$6.1x10^7$	100%	7/12/92	81%	15/7/93
3LA	$3.8x10^7$	100%	7/12/92	85%	15/7/93
3LP	$6.8x10^7$	100%	7/12/92	86%	15/7/93
3RA	$9.0x10^7$	100%	8/12/92	88%	15/7/93
3RM	$8.5x10^7$	100%	8/12/92	79%	15/7/93
3RP	$7.3x10^7$	100%	8/12/92	77%	15/7/93
2LM/1LP	$7.7x10^7$	100%	8/12/92	91%	15/7/93
2LM/1RA	$6.5x10^7$	100%	8/12/92	86%	15/7/93

1. Fish were individually marked using a panjet loaded with 1% alcian blue. The thymocytes from the individual fish were then frozen in a vial identified with this same mark.

2. Viability of the cells was measured, using the trypan blue exclusion test, before addition of the DMSO freezing mixture.

3. The viability of the thawed cells was measured after the cells were removed from the freezing mixture and washed three times in hanks balanced salt solution.

Table 4: The viability and cell number of cryopreserved thymocytes in the *A salmonicida* 1102 (eight months thymectomised) immunized group.

Panjet mark[1] of fish	number of cells frozen	viability on[2] freezing	date frozen	viability[3] on thawing	date of thawing
1LP	$7.1x10^7$	100%	6/12/92	86%	15/7/93
2LA	$6.0x10^7$	100%	6/12/92	81%	15/7/93
2RA	$5.3x10^7$	100%	6/12/92	80%	15/7/93
1LA/1LM	$6.2x10^7$	100%	6/12/92	86%	15/7/93
1LA/1LP	$8.6x10^7$	100%	6/12/92	88%	15/7/93
1RA/1RM	$4.1x10^7$	100%	8/12/92	78%	15/7/93
1RM/1RP	$3.8x10^7$	100%	8/12/92	81%	15/7/93
1RA/1RM	$9.5x10^7$	100%	8/12/92	86%	15/7/93
1LA/1RA	$6.7x10^7$	100%	8/12/92	89%	15/7/93
1LM/1RM	$5.5x10^7$	100%	8/12/92	69%	15/7/93
2LA/1RM	$7.7x10^7$	100%	8/12/92	87%	15/7/93
2RM/1RP	$7.4x10^7$	100%	7/12/92	78%	15/7/93
2RP/1LP	$8.3x10^7$	100%	7/12/92	90%	15/7/93
1LA/1LP	$8.1x10^7$	100%	7/12/92	93%	15/7/93

1. Fish were individually marked using a panjet loaded with 1% alcian blue. The thymocytes from this individual fish were then frozen in a vial marked with this identifying mark.
2. Viability of the cells was measured, using the trypan blue exclusion test, before addition of the DMSO freezing mixture.
3. The viability of the thawed cells was measured after the cells were removed from the DMSO freezing mixture and washed three times in hanks balanced salt solution.

Table 5 : The viability and cell number of cryopreserved thymocytes in the SRBC (eight month thymectomised) immunized fish.

Paniet mark[1] of fish	number of cells frozen	viability on[2] freezing	date frozen	viability[3] on thawing	date of thawing
1LM	$5.2x10^7$	100%	4/12/92	81%	15/7/93
1LP	$9.6x10^7$	100%	4/12/92	89%	15/7/93
1RA	$7.5x10^7$	100%	4/12/92	90%	15/7/93
1RM	$6.9x10^7$	100%	4/12/92	96%	15/7/93
1RP	$6.5x10^7$	100%	4/12/92	85%	15/7/93
2LP	$5.0x10^7$	100%	4/12/92	87%	15/7/93
2RA	$8.3x10^7$	100%	8/12/92	79%	15/7/93
2RP	$8.7x10^7$	100%	8/12/92	87%	15/7/93
1LA/1LM	$4.8x10^7$	100%	8/12/92	91%	15/7/93
1LM/1LP	$4.5x10^7$	100%	8/12/92	83%	15/7/93
1LA/1LP	$8.0x10^7$	100%	8/12/92	74%	15/7/93
1RM/1RP	$7.1x10^7$	100%	8/12/92	85%	15/7/93
1LA/1RM	$7.8x10^7$	100%	8/12/92	69%	15/7/93
1LM/1RM	$8.3x10^7$	100%	8/12/92	86%	15/7/93
2LA/1RA	$6.6x10^7$	100%	8/12/92	93%	15/7/93

1. Fish were individually marked using a panjet loaded with 1% alcian blue. The thymocytes from that fish was then frozen in a vial marked with this identifying mark.

2. Viability of cells before freezing was measured before addition of the DMSO containing freezing solution, using the trypan blue exclusion test.

3. The viability of the thawed cells was measured after the cells were removed from the freezing mixture and washed three times in hanks balanced salt solution.

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