THE IMMUNE RESPONSE OF THE DOGFISH SCYLIORHINUS CANICULA L.

by

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A thesis presented for the degree of
Doctor of Philosophy
in accordance with the regulations of C.N.A.A.

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August, 1978.

DECLARATION

I hereby certify that this work has not been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree other than that of Doctor of Philosophy for the Council for National Academic Awards.

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Date 6th October 1978

Director of Studies J.E. Adams

Date 10.10.78

Acknowledgements

I am indebted to everyone who has helped, advised and assisted me during the course of this work. Particular credit must go to the following staff of Plymouth Polytechnic:

To Dr. Jack Harris for supervising the project and providing encouragement and moral support when needed.

To Dr. Tony Matthews for many useful discussions and suggestions regarding fish parasitology.

To Dr. Peter Glynn for advice on separation techniques and biochemical methodology.

To Sara Ford and Sue Murray for providing invaluable technical assistance.

I should also like to thank:

Dr. Dave Davies of the University of Salford, for acting as external supervisor and for giving meticulous instruction in immunochemical procedures.

Dr. Ann Pulsford of the Marine Biological Association of the U.K., Plymouth, for help with electron microscopy.

Professor Ragnar Fänge of the University of Göteborg, Sweden, for stimulating discussions regarding the function and existence of lymphomyeloid tissue in fish.

The Marine Biological Association of the U.K., Plymouth, for generously providing fish holding facilities during the course of the project.

"Results obtained through studies on lower animals can be viewed as new support for certain basic ideas of cellular pathology."

Elias Metchnikoff. 1884.

CONTENTS

Aims of the Study

The overall object of the study was to examine the immune response of the dogfish and thereby gain expertise in a number of immunological and biochemical techniques and training in scientific thought and method.

Specific aims were:

i) To examine the humoral response of the fish and purify and characterise the factors involved.

ii) To survey the tissues associated with immunological function.

iii) To study a variety of cell mediated immune reactions *in vitro* and *in vivo*, particularly from a phylogenetic viewpoint.

iv) To examine the immunological relationship between the fish and its helminth parasite fauna.

ABSTRACT

A number of aspects of the humoral and cellular immune responses of the common dogfish Scyliorhinus canicula L. were examined in order to define the immunological status of the animal in relation to its phylogenetic origins.

The dogfish was shown to have the ability to produce specific antibody in response to a variety of soluble and particulate protein antigens. The existence of several non-specific defence mechanisms was demonstrated including a well established reticulo-endothelial system capable of rapidly clearing injected foreign material from the blood and also the bacteriolytic enzyme, lysozyme.

Specific antibody from the fish had marked agglutinating properties which could be destroyed with reducing agents but which could not be demonstrated to form precipitin lines in agar gels. It could also fix complement. Isolation and characterisation of the antibody molecule showed it to have virtually neutral electrophoretic mobility, a molecular weight of approximately 800,000 daltons and a sedimentation coefficient of 18.6S. On reduction the molecule was shown to comprise heavy and light polypeptide chains with molecular weights of 76,000 and 18,000 daltons, respectively. On the basis of these findings the antibody was considered to be of the IgM class of immunoglobulin found in higher vertebrates. This was the only immunoglobulin found in the dogfish although an antigenically identical protein with a molecular weight of 160,000 daltons was also demonstrated and this was considered to be a monomeric form of an IgM pentamer.

A survey of lymphoid tissues revealed that the fish had a well developed spleen. This organ was also shown to be directly involved in immunological reactions. Conversely, the thymus was poorly developed and involuted during an early stage in the life of the fish. Its immunological contribution was not determined. Two other tissues, the organ of Leydig and the epigonal tissue were found to comprise a high proportion of poorly differentiated lymphoid cells and these were considered to be similar to the stem cells of the bone marrow found in mammals. Ultrastructural examination of peripheral blood leucocytes showed that they comprised populations of cells analagous, to mammalian lymphocytes, plasma cells, granulocytes, and monocytes. Using a specific antiserum and an immunofluorescence technique, a high proportion of leucocytes were revealed to have immunoglobulin determinants on their surface.

In a number of experiments to investigate cellular functions *in vitro* it was found that dogfish leucocytes could be stimulated by a variety of mitogenic substances but these results were insufficient to provide evidence for lymphocyte heterogeneity. Evidence that the dogfish was capable of weak recognition of histocompatibility antigens was provided *in vitro* by mixed lymphocyte cultures and *in vivo* by reaction to injected allogeneic leucocytes.

Although several different techniques were used no evidence of immediate hypersensitivity reactions could be demonstrated. Delayed hypersensitivity was shown, however, by the leucocyte migration inhibition test and possibly by local skin reactions.

The immunological relationship of the dogfish to an intestinal helminth parasite, *Proleptus obtusus*, was also

investigated. The fish was found to be capable of producing antibody specific for the cuticle of the nematode. The parasite could also invoke several cellular reactions including the stimulation of leucocytes *in vitro* and delayed but not immediate hypersensitivity.

CHAPTER 1

INTRODUCTION

Over the past decade a considerable research effort has taken place in the field of comparative immunology, with a large number of non-mammalian models being studied including birds, reptiles, amphibia and fish. Investigations have also been made on the immune capabilities of invertebrates, particularly annelids and molluscs. Extensive reviews have been made by Cooper (1976a), Marchalonis (1976), Manning and Turner (1976).

Comparative immunology has been largely stimulated by the enormous medical and scientific interest in organ transplantation and tumour immunology. Besides pure academic interest, phylogenetic investigation contributes to contemporary immunology: studies on the relatively simple defence mechanisms of lower animals may enable a greater understanding of the more sophisticated immunology of advanced vertebrates by acting as model systems.

Interest in fish immunology has also been stimulated, apart from the reasons stated, by recent progress in fish farming methods. World shortage of protein has accelerated the development of aquaculture and like all other intensive rearing situations which necessitate a high population density, commercially reared fish are particularly prone to infection. Notable are the bacterial epizootics, furunculosis and vibriosis caused by Aeromonas salmonicida and Vibrio anguillarum respectively (Anderson, 1974). Also a disease of unknown aetiology known as ulcerative dermal necrosis (UDN) has decimated the economically important salmonid populations of British and European waters in recent years (Roberts, 1971). Consequently, much interest has been taken in the pathology of fish diseases, in the hope that a better understanding of defence mechanisms may facilitate their control.

Many theoretical studies on fish immunology have been carried out, recent reviews include Anderson (1974) and Corbel (1975). Most of these investigations concern immunochemical studies of immunoglobulins, with elasmobranch species being extensively researched (Clem & Leslie, 1969). Relatively little progress has been made regarding cellular immunology. This is surprising in view of the current interest in mammalian leucocyte heterogeneity and function; however, notable contributions to this field have been made by Emmrich, Richter & Ambrosius (1975), Ellis & Parkhouse (1975), Ellis (1976), Warr & Marchalonis (1977) and Clem, Mclean, Shankey & Cuchens (1977) regarding lymphocyte surface determinants.

This study set out to investigate the immune response of the common dogfish, Scyliorhinus canicula L. This model was chosen as it was readily available in Plymouth, easy to keep given good water quality, and relatively large quantities of blood could be obtained without difficulty. These fish are interesting phylogenetically, as they are more primitive but on a parallel evolutionary line with the more commonly studied Osteichthyes.

It was intended that the project should be broad so that a number of current techniques used in mammalian immunology could be used.

The major topics investigated were:

a) Humoral responses.

The responses of the animal to a variety of antigens was investigated. Partial characterisation of humoral antibody was made. A brief investigation of non-specific humoral factors was also included.

b) Lymphoid Organisation.

Few recent descriptions of the cells and tissues involved in the immune response are to be found. Virtually no histological studies of shark leucocytes exist. Thus it was considered that an examination of lymphoid and lymphomyeloid tissues was of primary importance and should be carried out before embarking on further studies on cellular immunology.

c) Cellular Immunology.

It was decided that in view of the relatively few reports of cellular immune responses in fish, a significant part of the project should be devoted to investigations on cell-mediated immunity. Thus, a number of reactions attributed to cell-mediated immunity in mammals were studied. These examinations included hypersensitivity and leucocyte migration inhibition. Attempts were made to establish evidence for lymphocyte heterogeneity by differential mitogenic stimulation and demonstrating the presence of cell surface immunoglobulin.

d) Parasitology.

Increasing concern is being expressed by commercial fisheries regarding the incidence of helminth disease in marine fish (Williams & Jones, 1976). Dogfish examined in Plymouth showed 100% infection with Proleptus obtusus, an intestinal spiruroid nematode. Thus, with the availability of a convenient model, the opportunity was taken to make a brief examination of the host-parasite relationship, using serological and cellular techniques, developed at other stages in the project.

LITERATURE REVIEW

Since the work of Metchnikoff at the turn of the century, it has been known that fish are capable of responding, immunologically, when challenged with injected, 'non-self', material (Metchnikoff 1892, 1901, 1905). However, the work was not followed up until the last two decades, when, for reasons already described, there has been an upsurge of interest in the immunology of lower vertebrates and a large number of reports have been published on the subject.

Phylogenetic Emergence of the Immune System

Many invertebrate animals are capable of cellular recognition and have the ability to make a primitive immune response. Tartar (1964, 1970) has demonstrated cellular incompatability in ciliated protozoa. Wilson (1907) showed a cellular recognition system in the Porifera; dissociated sponge cells, when mixed together, aggregate with like kind.

The ability of annelids, particularly the earthworms Lumbricus spp. and Eisenia spp. to reject skin grafts has been well documented (Cooper, 1965, 1966, a, b, 1968, a, b; Du Pasquier & Duprat, 1968; Chateaureynaud-Duprat 1970, 1971). Cameron (1932) noted that coelomocytes (amoebocytes) of Lumbricus terrestris were phagocytic. Humoral factors have also been shown to be active against foreign bacteria (Chateaureynaud-Duprat, 1973) although these do not have the physico-chemical properties of mammalian immunoglobulin molecules.

Results from transplantation reaction experiments in molluscs have been inconclusive although it would seem that this is due to surgical and other technical difficulties. The

humoral systems of these animals, however, have been well studied; the oyster Crassostrea virginica has large protein molecules (33S) in its haemolymph which will agglutinate sheep erythrocytes. The molecule could be dissociated to form polypeptide chains of 20,000 daltons (Acton, Bennett, Evans & Schrohenloher, 1969). Crassostrea has also been shown to clear a secondary injection of bacteriophage more rapidly than a primary one (Acton & Evans, 1968) but this response was found to be relatively non-specific.

Marchalonis and Edelman (1968a) isolated a similar haemagglutin from horsehoe crab haemolymph. This protein had a sedimentation coefficient of 13.5S and dissociated into polypeptide chains of molecular weight 22,500. Graft rejection experiments in the arthropoda have given conflicting results but it is clear that animals of this phylum are capable of non-self recognition, although the specificity of this response is variable (Cooper, 1976).

In the colonial tunicate Botryllus primigenus there is evidence of allogeneic recognition but there appears to be a higher degree of tolerance than in vertebrates (Freeman, 1970).

The Immune Response in Fish

Fish show great diversity as a group and the evolutionary and phylogenetic relationships are depicted overleaf (Fig. 1.1).

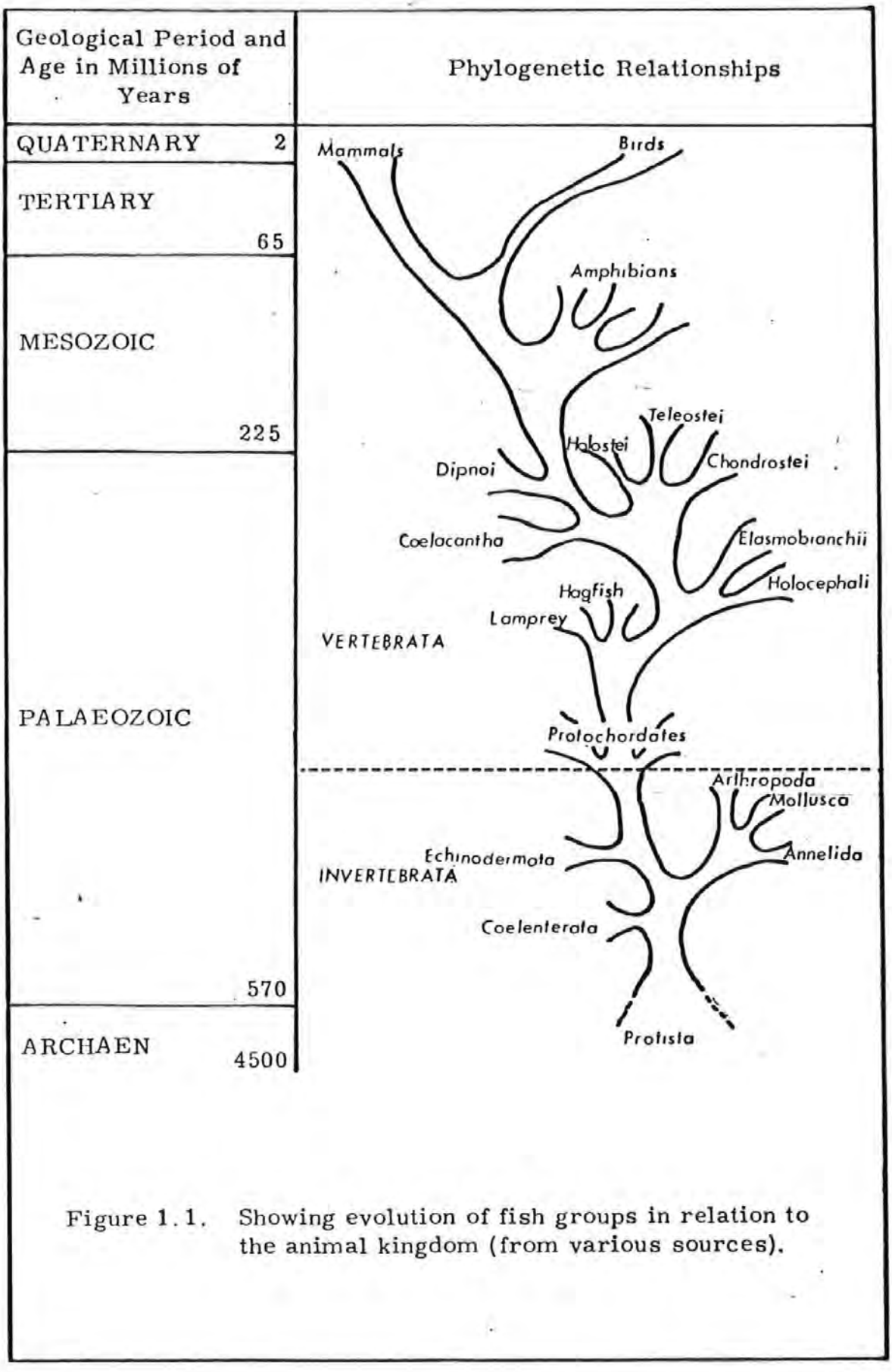


Figure 1.1. Showing evolution of fish groups in relation to the animal kingdom (from various sources).

I. Lymphoid Cells and Tissues

An understanding of the basic mechanisms of cell-mediated immune reactions has been rapidly advanced, in recent years, as a result of studying lymphoid tissue, particularly *in vitro*. To date, relatively few studies have been made on fish, with the exception of histological examinations. This review examines current trends in fish cellular immunology and attempts to relate them to contemporary immunology. Classical descriptions of lymphoid organisation are also included.

Leucocytes

In the primitive agnathan, *Eptatretus stoutii* (the Pacific hagfish) Hildemann & Theones (1969) identified leucocytes with similar morphology to mammalian granulocytes, macrophages and lymphocytes. Good & Papermaster (1964) concluded that the hagfish was below the phylogenetic level of specific immunocompetence. They claimed that the fish did not have an equivalent of the plasma cell and they could not elicit a detectable immune response. However, Theones & Hildemann (1969) conclusively demonstrated that, if the husbandry was adequate, the hagfish would make antibody to a specific antigenic challenge. This finding implied that a plasma cell equivalent existed in the fish. These authors also noticed and identified macrophages, granulocytes and lymphocytes around the sites of immune reactions.

In the lamprey *Petromyzon marinus*, cells of the lymphoid series were noted in the spleen, thymus and blood (Good & Finstad, 1964; Good & Papermaster, 1964; Finstad, Papermaster & Good, 1964).

Good & Papermaster (1964) described lymphoid cells, similar to those found in mammals, in elasmobranchs;

chondrostean and holostean fish. Other morphological aspects of fish leucocytes have been well reviewed (Duthie, 1939; Catton, 1951; Jakowska, 1956 and Hawkins & Mawdesley-Thomas, 1972).

Ellis (1976) described the leucocyte cell series of the plaice, Pleuronectes platessa and Ferguson (1976) detailed the ultrastructure of monocytes, lymphocytes, neutrophils and thrombocytes of the same fish.

Very little information is available on lymph re-circulation in fish but Wardle (1971) described the position and anatomy of a neural lymphatic duct. Ellis & de Sousa (1974) suceeded in cannulating this duct and carried out lymphocyte homing experiments with radiolabelled cells that had been returned to the fish. The labelled cells returned to the spleen and anterior kidney, but not to the thymus.

The salmonid leucocyte response in relation to infection has been described by Conroy (1972) and Klontz (1972). Hines & Spira (1973) gave details of the inflammatory response in carp, Cyprinus carpio, infected with the protozoan parasite Ichthyophthiriasis multifillis.

The eosinophilic cellular response of the white sucker fish, Catostomus commersonnii, to lesions made by parasitic leeches and experimental incision was described by Lester & Daniels (1976).

It has long been established that two major sub-populations of lymphocytes exist in mammals.

i) The T-lymphocyte (T-cell) which undergoes processing in the thymus and plays a role in cell mediated immunity.

ii) The B-lymphocyte (B-cell) which is transformed in the Bursa of Fabricius (in birds) or bursa equivalent tissue in mammals and is concerned with the synthesis of circulating antibody molecules.

One of the characteristics of the mammalian B-lymphoctye is the presence of immunoglobulin determinants on the cell surface (reviewed by Greaves, Owen & Raff, 1975). Recent studies using specific anti-immunoglobulin sera and fluorescent labelling has shown that fish leucocytes also have immunoglobulin on their surface: Emmrich et al. (1975) for carp; Ellis & Parkhouse (1975) for skate, Raja naevus, Ellis (1976) for plaice and Warr & Marchalonis (1977) for goldfish Carassius auratus. Each of these authors also described the characteristic 'capping' as the stained leucocytes endocytosed the fluorochrome bearing immunoglobulin molecules. These authors also showed subsequent regeneration of these surface determinants in a manner similar to mammalian lymphocytes. In each case the number of leucocytes bearing surface immunoglobulin was 65% or more, a figure nearly three times higher than that of equivalent mammalian cells.

Weinreb (1958) injected the salmonid, Salmo gairdneri irideus with cortisone; this treatment caused lymphocyte and thrombocyte depletion. Injections of adrenocorticotropic hormone (ACTH) produced heterophilia. Turpentine injections induced heterophilia and lymphopenia.

Ezzat, Shabana & Farghaly (1974) observed seasonal changes in the numbers of leucocytes in the blood of the cichlid teleost Tilapia zilli. They noted eosinophils, basophils, lymphocytes and monocytes, but could not demonstrate the presence of polymorphonuclear leucocytes.

Sailendri & Muthukkurappan (1975b) carried out morphological studies on Tilapia mossambica. They noted cells similar to those described by Ezzat et al. (1974) and again could not demonstrate the presence of polymorphonuclear leucocytes.

Clawson, Finstad & Good (1966) carried out a study, by electron microscopy, of plasma cells and lymphoid tissue of the paddlefish Polydon spathula. They described cells similar to the mammalian equivalent of small, medium and large lymphocytes, eosinophils, neutrophils and plasma cells. They also speculated that differences in cytoplasmic ribosomal configuration might be criteria for population heterogeneity.

Blaxhall (1972) and Blaxhall & Daisley (1973) reviewed haematological procedures that could be used as diagnostic procedures in fish pathology.

As mentioned earlier, heterogeneity of mammalian lymphocyte populations has long been established. Apart from the functional differences, the two classes of lymphocytes display differential in vitro responses to various mitogenic substances. It is generally understood that concanavalin (Con A) and phytohaemagglutinin (PHA) only induce mitosis in T-cells, whereas Pokeweed mitogen (PWM) stimulates both B and T cells. Bacterial lipopolysaccharide (LPS) and a purified protein derivative (PPD) from Mycobacterium tuberculosis give selective mitogenic stimulus to B-cells alone. These and other properties of B and T cells have been fully reviewed by Greaves et al. (1975).

It is not known whether separate classes of B and T cells exist among fish, but recent studies using mitogens indicates a possible heterogeneity in the lymphocyte

populations of several species. Lopez, Sigel & Lee (1974) centrifuged nurse shark (Ginglymostoma cirratum) peripheral blood on a Ficoll-Isopaque density gradient and obtained two separate bands of cells, only one of which would respond to PHA. Both bands responed to Con A. This differential mitogen responsiveness is similar to that described by Stobo & Paul (1973) in the mouse.

Etlinger, Hodgins & Chiller (1976) reported differential responses of rainbow trout (Salmo gairdneri) lymphoid tissues to Con A, LPS and PPD mitogens. Thymocytes reacted strongly to Con A, but not to LPS or PPD. In contrast, leucocytes from the lymphoid anterior kidney were stimulated with LPS but not with Con A or PPD. Cells from the spleen and peripheral blood were stimulated by each mitogen. In each case, the degree of stimulation was measured by the uptake and incorporation, by the cells, of tritiated thymidine.

Bogner (1975) carried out in vivo experiments in the plaice, using LPS, Con A, PWM and PHA mitogens and again noted differential stimulation.

Lymphocyte transformation can be induced when allogeneic cells are cultured together (reviewed by Wilson, 1971; Sorensen, 1972). The lymphocytes undergo transformation similar to that of mitogen induced blastogenesis and again this can be measured by uptake of radiolabelled thymidine. The phenomenon is called the mixed lymphocyte reaction (MLR). Hildemann & Reddy, quoted by Cohen (1975) have claimed that hagfish can recognise allogeneic lymphocytes and undergo MLR transformation. Sigel, Ortiz-Muniz, Lee & Lopez (1973) failed to observe positive MLR's in leucocytes taken from various species of sharks. Sigel & Lee (1973) also failed to demonstrate MLR with marine teleost leucocyte cultures.

Irradiation is a commonly used form of immunosuppression in experimental procedures. Most cells will not die until the first or second dose of gamma or x-irradiation (50-1000R) after mitosis (mitotic death) whereas small lymphocytes are rapidly destroyed before entering the mitotic cycle (interphase death) (Trowell, 1952). Neale & Chavin (1974) subjected goldfish to whole body irradiation. The small lymphocytes were most susceptible to the treatment. Immunisation prior to irradiation provided immunological protection, an effect noted in mammals by Keuning, Van der Meer, Niewenhuis & Oudendijk (1963) and Schrek & Stefani (1964).

Spleen

The hagfish does not have a definitive spleen but a possible primitive equivalent exists as foci of haemopoietic tissue along the length of the lamina propria of the gut (Good & Papermaster, 1964). These authors did not find lymphoid accumulations anywhere in the fish. In the higher agnathan, the lamprey, splenic tissue is located in an invagination of the anterior stomach. Lymphocytes were noted in this tissue as well as haemopoietic and granulopoietic areas (Good, Finstad, Pollara & Gabrielsen, 1966).

The spleen exists as a discrete organ in elasmobranchs and higher vertebrates and is divided into areas of red and white pulp. After antigenic stimulation, plasma cell equivalents are detectable. Follicular development is not as marked as in vertebrates (Good & Papermaster, 1964).

Recently, Sailendri & Muthukkaruppan (1975a,b) have studied the spleen of the teleost, Tilapia mossambica, particularly in relation to antigenic challenge. The normal spleen consisted of areas of white pulp in a larger mass of red pulp. The white pulp areas comprised poorly developed reticular centres around blood vessels. The

presence of granulocytes, plasma cells, lymphocytes and monocytes was recorded. After challenge by BSA and sheep red blood cells, an increase in the number of lymphocytes, particularly in the reticular areas, was observed.

Ellis, Munro & Roberts (1976) described the spleen of the plaice; mature erythrocytes were found to be the most common cellular element. Small numbers of thrombocytes were noted. The white pulp areas were not extensive and comprised mostly lymphocytes forming a cuff around blood vessels. Arterial capillaries, termed 'ellipsoids' were described and had a flattened endothelial lining of macrophage cells. Pigmented cells, similar to macrophages and previously called 'melano-macrophage' by Roberts (1974) were also observed.

Ferren (1967) carried out experiments with marine teleosts and elasmobranchs, and noted no impairment of the immune response to injected influenza A virus in splenectomised fish.

There have been several independent reports of the existence of high-rate antibody forming cells in fish spleen. Smith, Potter & Merchant (1967) reported such cells in the teleost Lepomis macrochirus. Ingram (personal communication) described similar cells in the brown trout, Salmo trutta. Sailendri & Muthukkaruppan (1975a) made comparable observations for Tilapia mossambica. Each group of workers used a modification of the haemolytic plaque technique (Jerne & Nordin, 1963) to show the presence of antibody secreting cells.

Chiller, Hodgins Chambers & Weiser (1969) demonstrated antibody producing cells by the immunocytoadherence (rosette) test (Storb & Weiser, 1967) and also described the nature of the leucocytes concerned by using both light and electron microscopy techniques. Five classes of splenic leucocytes

were shown to be capable of forming rosettes: lymphocytes, plasma cells, blast like cells, macrophages and eosinophils.

Thymus

The thymus of poikilotherms has been studied for over 100 years, although most reports have been concerned with the morphology of the organ. The literature has been reviewed by Good, Finstad, Pollara & Gabrielsen (1966) and Cooper (1973).

Salkind (1915) and Good & Papermaster (1964) described a primitive epithelial thymus in the lamprey. The ability of these fish to reject skin grafts, a capability mediated by thymus derived lymphocytes in mammals, suggests that it has a functional thymus at some stage in its development.

The primitive elasmobranch, the guitarfish Rhinobatis productus, has a thymus derived from the pharyngeal pouches (as do mammals). It is born with the thymus present as a fully developed, encapsulated lymphoid organ, located dorsally between the second and the fifth gill arches. It has a cortex and medulla and contains primitive Hassall's corpuscles (Good et al., 1966).

Higher elasmobranchs studied include the leopard shark (Triakis semifasciata) and the nurse shark (Ginglymostoma cirratum). Both of these fish were observed to have a discrete lymphoid thymus separated into cortex, medulla and epithelial accumulations similar to Hassall's corpuscles (Good et al., 1966). The chondrostean, Polydon spathula, has a well developed thymus organised into lobes and lobules; Hassall's corpuscles are also present (Good et al., 1966).

A more recent report concerning thymus morphology is that of Sailendri & Muthukkaruppan (1975b) regarding Tilapia mossambica. As with mammals, the thymus involutes with age in both teleosts (Good et al., 1966) and elasmobranch fish (R. Fänge, personal communication).

Other Lymphoid and Lymphomyeloid Structures

In teleosts, the anterior kidney, or pronephros is a discrete lymphoid organ (Good *et al.*, 1966). Smith *et al.* (1967) showed that cells in the organ were capable of antibody production by using the haemolytic plaque method. Ingram (1974) Chiller *et al.* (1968, 1969) using the same technique, demonstrated similar cells in brown and rainbow trout respectively. The latter authors found that after antigenic challenge, antibody was first produced in the pronephros, and later in the spleen. They also identified rosette forming cells of similar morphology to those found in the spleen.

Ortiz-Muniz & Sigel (1968) made organ cultures of antigen stimulated pronephros and spleen taken from the grey snapper, *Lutjaneus griseus*, and found that these tissues would produce IgM-like antibody for up to three months.

Sailendri & Muthukkaruppan (1975a, b) studied the pronephros of *Tilapia mossambica* and described two zones, one being a lymphoid area consisting of tightly packed lymphoid follicles and the other, a non-lymphoid area containing granulocytes and islands of erythrocytes. After immunisation, large numbers of spherical bodies were found in the lymphoid zone.

The non-excretory part of the elasmobranch kidney contains too few cells of the leucocyte series to be considered a lymphoid organ, Good *et al.* (1966); (R. Fänge, personal communication).

Elasmobranch fish possess large tracts of lymphomyeloid tissue. Leydig's organ and epigonal tissue, associated with the oesophagus and gonads respectively, have been observed in several species of sharks and rays. A study of the ultrastructure of these bodies has shown they comprise mostly large granular lymphoid-like cells and eosinophils. The occasional plasma cell has also been noted, Fänge & Pulsford (1978). These authors also suggested that these organs were primitive haemopoietic sites or bone marrow equivalents and the majority of cells within were undifferentiated stem cells.

In mammals there are areas of unencapsulated lymphoid tissue in the respiratory, alimentary and genito-urinary tracts; this includes the tonsils, small intestinal Peyer's patches and appendix. All these regions show similar separation of lymphocytes into T- and B- dependent areas (reviewed by McConnell, 1976). Both elasmobranchs and teleosts have patches of gut-associated lymphoid tissue (GALT) but the function of this is largely unknown (Good et al., 1966).

II Antibody Production in Fish.

a) Immunochemistry

The ability of fish to make a specific humoral immune response has been recognised since the turn of the century (Babes & Riegler, 1903) but with a few exceptions, little attention was given to the subject until the early 1960's.

Agnatha

Good & Papermaster made an initial examination of the Pacific hagfish in 1964, followed by Good et al. (1966). The Atlantic hagfish, Myxine glutinosa was also examined by Finstad & Fichtelius (1965) and Finstad & Good (1966). None of these workers could demonstrate an inducible immune response and it was concluded that the hagfish was phylogenetically too primitive to be capable of specific immunocompetence.

After improving conditions of husbandry, Hildemann & Theones (1969) demonstrated immunological memory and the ability to reject allogeneic skin grafts in the Pacific hagfish. Further studies reported the production of antigen-specific precipitating and agglutinating antibodies (Acton, Weinheimer, Hildemann & Evans, 1969, 1971; Linthicum & Hildemann, 1970 and Theones & Hildemann, 1970). The latter authors stimulated antibodies against keyhole limpet haemocyanin (KLH) and purified them. They were found to be macroglobulins with a sedimentation coefficient of 28S, and a slight anodic electrophoretic mobility.

Acton et al. (1971) partially characterised the hagfish bactericidal antibodies: they were heat labile and totally excluded from Sephadex G-200. The antibody could not be reduced to show heavy and light chains. They did not fix complement.

Pollara, Finstad & Good (1966) and Pollara, Litman, Finstad, Howell & Good (1970) partially characterised the anti-Brucellosis abortus agglutinating antibody from the lamprey, Petromyzon marinus. It had a molecular weight of 900,000 and eluted in the second peak from Bio-Gel A15. Its sedimentation coefficient was 9S and its electrophoretic mobility was in the α-globulin region.

Dissociation into heavy and light chains after treatment with reducing agents was not shown. These results are contradictory to the work of Gerwuz, Finstad, Muschel & Good (1966) who described the agglutinating antibody of the same fish to have a cathodic migration in starch gel, and was excluded from Sephadex G-200.

Marchalonis & Edelman (1968b) recorded the presence of 6.6S and 14S neutralising antibodies in the serum of lampreys immunised with T2 bacteriophage. The antibodies had distinct heavy and light chains. These sub-units were non-covalently bonded.

Chondrichthyes

The immunochemistry of the elasmobranch group is the best documented aspect of fish immunology. The ability of sharks to respond to antigenic challenge has been demonstrated (Clem & Sigel, 1963; Sigel & Clem, 1963, 1965, 1966 and numerous other workers).

The antibody molecules of several elasmobranchs have been characterised by several groups: the lemon shark, Negaprion brevirostris (Clem & De Boutaud, 1967; Clem & Small, 1967); the leopard shark, Triakis semifasciata (Suran, Tarail & Papermaster, 1967); the smooth dogfish, Mustelus canis (Marchalonis & Edelman, 1965, 1966); the nurse shark, Ginglymostoma cirratum (Clem, De Boutaud & Sigel, 1967); the stingray, Dasyatis americana (Johnston, Acton, Weinheimer, Niedermeier, Evans Shelton & Bennett, 1971); the thorn-back ray, Platyrhinoides triseriata (Thomas, Sanders & Wiley, 1972).

A feature of the immune response in sharks is the ability to produce two species of immunoglobulin. An antibody molecule with a sedimentation coefficient of 18-19S is initially produced followed by a 7S response. The phenomenon was noted in antisera from immunised lemon

sharks (Clem & Small, 1967) nurse sharks (Clem, et al., 1967) and leopard sharks (Suran & Papermaster, 1967; Suran, et al., 1967). The 7S antibodies were detectable by haemagglutination and precipitation techniques and were thus presumed to be multivalent. In a relatively short term experiment, Marchalonis & Edelman (1965) could demonstrate 18S antibody, only, in the smooth dogfish.

Antibody macroglobulins from elasmobranch sera have been purified using combinations of two separation techniques, one based on molecular size separation and the other according to electrical charge. Both the 18S and 7S immunoglobulin species of the sharks described comprised disulphide linked heavy (H) and light (L) polypeptide chains. The H & L chains accounted for approximately 75% and 25% respectively of the mass of the molecule. Calculations based on the molecular weights of the whole molecule, the component polypeptide chains and the mass ratios of these chains show that shark 7S immunoglobulin comprises 2 heavy and 2 light chains. Similarly, the 18S component has been calculated to be a pentameric molecule composed of disulphide linked subunits. The complete molecule has 10 heavy and 10 light chains. Therefore, each monomeric form has 2H and 2L chains. The immunochemistry of elasmobranchs has been reviewed in detail by Clem & Leslie (1969).

An equivalent of the mammalian 'J' (joining) chain has been found in leopard shark 18S macromolecules, but not in the 7S form (Klaus, Halpern, Koshland & Goodman, 1971; Klaus, Nitecki & Goodman, 1971; Klapper & Clem, 1972).

The criteria for classifying elasmobranch immune macroglobulins as a mammalian class equivalent have been

discussed by Clem & Leslie (1969). The major problem is the absence of data regarding amino acid sequences for the fish antibody molecule. Similarities in the sequences of fish and human heavy chains leaves little doubt as to the class designation of the fish antibody. Tryptic digests and antigenic comparisons have failed to show significant similarities between lemon shark and rabbit H (γ, α, μ) chains. Thus fish immune macroglobulins (both 18S and 7S forms) have been classified as IgM molecules on the basis of their gross architecture.

Clem & Leslie (1969) also reported antigenic dissimilarity between the H chains of nurse and lemon sharks, but similarity in the L chains. Nurse and lemon sharks are, phylogenetically, far apart and this observation lead the authors to suggest that the L chain cistron is, in terms of evolution, more stable than the H chain cistron and thus selective pressure on the latter may have given rise to multiple immunoglobulin classes.

Klapper, Clem & Small (1971) digested lemon shark 7S IgM with proteolytic enzymes to yield core fragments similar to the 6S $F(ab)_2^{\mu}$ and 3.7S Fabμ fragments described by Miller & Metzger (1966) on studies on human IgM myeloma (Waldenström) protein.

Gitlin, Pericelli & Gitlin (1973) studied the immunoglobulins of 16 species of shark and found 4 classes of immunoglobulin by antigenic analysis. Two species of immunoglobulin were found having both 19S and 7S forms in general which evolved between 130 and 138 million years ago.

Several studies have been made on the ontogeny of elasmobranch immunoglobulins. Fidler, Clem & Small (1969) demonstrated the virtual absence of antibody in neo-natal

nurse sharks and the initial production of the 19S molecule was followed by 7S IgM. Both these molecular forms reached approximately half their adult concentration after 42 days of life. Suran & Papermaster (1967) and Suran et al. (1967) reported similar results for the leopard shark.

Osteichthyes

Chondrostei

The best studied fish of this group is the paddlefish, It has a well developed lymphoid system (Clawson et al., 1966) and is capable of a vigorous humoral antibody response (Finstad & Good, 1966; Legler, Weinheimer, Acton, Dupree & Russel, 1971). Pollara, Chartrand & Good (1968) and Pollara, Suran, Finstad & Good (1968) purified and partially characterised the immune macroglobulin. The molecule had a molecular weight of 870,000 and could be dissociated with the reducing agents guanidine and urea, to give heavy and light chains with molecular weights of 75,300 and 23,500 respectively. The terminal amino-acid sequences of the H and L chains were similar to the sequences of mammalian kappa chains.

Holostei

The gar, Lepisosteus platyrhynchus, has been the focus of studies on holostean fish. Bradshaw, Clem & Sigel (1969, 1971) described the immunoglobulin as having a molecular weight of 650,000 and a sedimentation coefficient of 14S. The molecule was tetrameric and had β electrophoretic mobility. Reduction and alkylation demonstrated H and L chains with molecular weights of 70,000 and 22,000 respectively.

Acton, Weinheimer, Dupree, Russell, Wolcott, Evans, Schrohenloher & Bennett (1971) confirmed the tetrameric conformation of the immunoglobulin molecule by electron microscopy.

Litman, Frommel, Finstad & Good (1971a, b) isolated immunoglobulins from the bowfin, Amia calva. Species with molecular weight of 643,000 and 146,000 and sedimentation coefficients of 14S and 6.5S were present. Bradshaw, Richard & Sigel (1971) showed that both forms were antigenically identical and that the 14S species was a tetrameric form of the 6.5S molecule.

Teleostei

Antibody production in teleost fish has been the subject of numerous studies over the past decade, and is the subject of several reviews including Clem & Leslie (1969), Snieszko (1970), Finn (1970), Carton (1973a, b) and Corbel (1975). Many of the investigations have examined the response to bacterial and viral pathogens that are important in aquaculture epizootics.

Uhr, Finkelstein & Franklin (1962) reported that goldfish were capable of producing an antibody response to the bacteriophage ØX 174. The antibody like mammalian IgM was sensitive to 2-mercaptoethanol (2-ME) and had γ-electrophoretic mobility. After prolonged immunisation antibody production shifted from the 19S to predominantly 7S form. This was the first successful demonstration of a conformational change in immunoglobulin species in fish. Marchalonis (1971) showed that the goldfish macroglobulin was analagous to the mammalian IgM antibody class.

References describing similar physico-chemical characteristics of teleost fish antibody include reports by Hodgins, Weiser & Ridgway (1967) for rainbow trout, Harris (1973a) for the dace Leuciscus leuciscus and Cisar & Fryer (1974) for the coho salmon, Oncorhynchus kisutch.

Heartwell (1975) described the immune response of the channel catfish, Ictalurus punctatus, to the naturally occurring pathogens Chondrococcus columnaris and channel catfish virus, using agglutination and complement fixation assay systems. Both primary and secondary responses to these antigens were noted. Physico-chemical analysis showed the molecule to be analogous to the mammalian IgM class.

Sailendri & Muthukkaruppan (1975a) reported a strong humoral response to injected bovine serum albumin and sheep erythrocytes in the cichlid Tilapia mossambica. Immunological reactivity of the serum was abolished after treatment with 2-mercaptoethanol, thus implicating the presence of IgM antibody.

Antibody production in fish has been shown to be a temperature dependent process. Nybelin (1935) could not detect a response in the European eel Anguilla anguilla, to a challenge of Vibrio anguillarum at low temperature, when kept at 8°C. Fish kept at 18°C produced antibody.

Cushing (1942) noted that lower temperatures slowed the rate of antibody production.

Avtalion (1969) reported that antibody synthesis in carp was temperature dependent. Fish held at 25°C responded, immunologically, to injected challenges of BSA but a response could not be detected in those held at 12°C and 14°C after 63 days.

Harris (1973a) compared the immune response of dace at 2°C, 5°C, 10°C and 18°C and found that antibody production decreased with lower temperature.

Other reports on the temperature dependent nature of the immune response in fish include: Bisset (1946, 1947, 1948) Bradshaw & Sigel (1968) and Paterson (1972). Although antibody production is less vigorous at lower temperatures it would appear that the kinetics of the response are largely related to the normal environmental range of the individual species.

b) Vaccination

The greatest application derived from an improved knowledge of fish immunology has been in the field of aquaculture, particularly in producing vaccines for economically important freshwater teleost species.

As early as 1942, Duff showed that the salmonid Salmo clarkii could be protected against furunculosis by oral immunisation. Klontz (1966, 1967, 1968) successfully vaccinated salmonids against furunculosis, experimentally, although the degree of protection likely to be afforded in a commercial situation was uncertain.

Fujihara (1969) and Fujihara & Nakatani (1971) succeeded in protecting rainbow trout and coho salmon against infection from Chondrococcus columnaris.

It appears that a higher degree of protection can generally be obtained by administering antigens parenterally rather than orally but this method is obviously impractical for large numbers of fish. Recently a novel method of vaccination has been described (Amend & Fender, 1976) in which juvenile fish are placed in a hyperosmotic saline solution containing antigen, or in successive baths containing salt and antigen. This method has been reported as providing effective protection in coho and chinook salmon against Vibrio anguillarum and Aeromonas salmonicida bacterial infections (Antipa & Amend, 1977). This latter technique may be the most useful as it does not involve undue handling stress and ensures a consistent degree of antigenic input.

Fish immunisation has been reviewed in detail by Klontz & Anderson (1970), Finn (1970) and Snieszko (1970).

III The Humoral Immune Response of Fish to Naturally Occurring Parasitic Infection.

Despite the large volume of information available on the principles of fish immunology, there are relatively few reports concerning the response of fish to parasitic infection. Bauer (1953), Beckert & Allison (1964), Putz (1964) and Hines & Spira (1973) reported that fish which had recovered from infections of the protozoan parasite Ichthyophthiriasis multifillis were resistant to subsequent re-infection.

Hines & Spira (1974) showed that serum from parasite sensitised fish could immobilise free swimming stages of I. multifillis. A maximum serum immobilisation titre of 1:1024 was recorded between 10 and 22 days after infection. Protection was also found to be associated with the epidermal mucus.

Harris (1972) studied the response of the chub, Leuciscus cephalus, to the acanthocephalan parasite, Pomphorhynchus laevis. Infected fish had serum precipitins to parasite extracts. The precipitins were also found in the intestinal mucus. Physico-chemical tests showed that the antibody from both sources was of the IgM type. The parasite antigen appeared to be of an excretory-secretory nature.

Harris & Cottrell (1976) reported that the plaice serum contained a precipitin that would react with a helminth antigen associated only with the dogfish, Scyliorhinus canicula. The authors originally thought that a non-specific factor such as C-reactive protein could be involved, but characterisation revealed that the component was a specific immunoglobulin related to antibody of the IgM class.

Cottrell (1977a) examined the response of plaice to infestations of metacercariae of two digenean parasites Cryptocotyle lingua and Rhipidocotyle johnstonei. The fish produced precipitating antibody to both parasites. Immunochemical investigations showed that the antibodies were macroglobulins resembling mammalian IgM. The rate and magnitude of antibody production was determined by ambient temperature. Cottrell (1977b) also found circumstantial evidence for an immune response in plaice directed against marine trypanosome parasites.

IV Non-Specific Cellular responses and Hypersensitivity

Non-specific cellular responses in fish have been the subject of many studies since the work of Metchnikoff (1892, 1905) who observed that goldfish were capable of phagocytosing injected guinea pig red blood cells.

The elasmobranch, Mustelus canis, has been reported to have a cellular response to injections of turpentine (Reznikoff & Reznikoff, 1934). Experimentally induced inflammation in the perch, Perca flavens, leads to heterophilia and lymphopenia (Yokoyama, 1947). Janssen & Waaler (1967) injected cod, Gadhus morhua, with silica particles. This gave rise to an inflammatory response involving infiltration of lymphocytes, macrophage and fibroblasts into the site of injection.

Post (1963) described an inflammatory response in rainbow trout after giving injections of Aeromonas hydrophilia and Freund's complete adjuvant.

Tubercle formation has been recorded in a number of cases, particularly following injection of acid fast mycobacteria (Wood & Ordal, 1958; Van Duijn, 1961; Reichenbach-Klinke & Elkan, 1965) although these organisms did not always cause an immune reaction.

Klugh (1965) observed granulomata in fish infected with Flavobacteria spp. and Wood & Yasutake (1956) noticed similar reactions in salmonids with Dee disease.

Finn & Nielsen (1971) compared the inflammatory response of rainbow trout to that of mice after both groups had been challenged with Staphylococcus aureus in Freund's complete adjuvant. In both animals there was infiltration

of polymorphonuclear leucocytes and macrophages into the injection sites.

Klontz (1966) described leucocyte infiltration into the muscle of rainbow trout after injection with Aeromonas salmonicida.

It is uncertain whether viral agents cause an inflammatory reaction, probably because of their very diverse effects on cellular immune mechanisms (Finn, 1970).

Roberts, Shearer, Munro & Elson (1969) could not demonstrate a response to fungal infection in salmonids. Stuart & Fuller (1968) reported similar results, although Wilson (1975) characterised a response to Saprolegnia in salmon.

Several attempts have been made to relate inflammatory responses to mammalian hypersensitivity reactions. Attempts were made to induce anaphylaxis in several species of teleosts by Arloing & Langeron (1922). Repeated injections of horse serum failed to cause any abnormal behavibur in the fish although Dreyer & King (1948) in similar experiments reported that sensitisation resulted in fish showing behaviour patterns symptomatic of physiological stress. Lukyanenko (1967) failed to detect anaphylactic responses in three species of teleost fish after repeated stimulation with horse serum.

Clem & Leslie (1969) attempted to demonstrate passive systemic anaphylaxis in the margate, Haemulon album, using bovine serum albumin as an antigen but without success.

Harris (1973b) using 2 species of cyprinid fish, the chub and the dace, tried to show hypersensitivity by direct sensitisation and by heterologous and homologous passive cutaneous anaphylaxis. All results were negative.

Fletcher & Baldo (1974) reported immediate type hypersensitivity responses in the plaice and the flounder, Platichthyes flesus. These authors used fungal antigens that would precipitate with human C-reactive protein. The response could not be shown in species that did not have calcium dependent serum precipitins to the fungi used in the skin reactions.

Hypersensitivity can be demonstrated, in vitro, by the macrophage migration inhibition test (reviewed by Vernon-Roberts, 1972). Three brief reports exist describing a comparable phenomenon in fish: Carlisle (1975) observed epidermal papillomas of the Atlantic salmon and showed that extracts of this growth would inhibit leucocyte explants of the fish. Kirmse (1975) showed that unnamed protozoan parasites causing a proliferative disease of farmed turbot, Scopthalmus maximus, caused migration inhibition in leucocyte cultures. Timur (1975) described similar results in the plaice.

V Non-Specific Factors

A number of factors playing a role in non-specific immunity have been described:

The anti-viral agent, interferon, has been demonstrated in cell cultures by Beasley, Sigel & Clem (1966) and by Oie & Loh (1969). The latter authors reported interferon production in fathead minnow cells to Reovirus.

De Kinkelin & Dorson (1973) showed in vivo production of interferon by rainbow trout challenged with Egtved virus.

The enzyme lysozyme (muraminidase) is widely distributed throughout the animal kingdom including fish. After initial reports by Lukyanenko (1965), Fletcher & Grant (1968) reported the presence of the enzyme in secretions from plaice. Fletcher & White (1973) attempted to localise the origins of these secretions, with limited success. Murray & Fletcher (1976) using immunochemical methods showed that the enzyme was associated with neutrophils, peritoneal macrophages, gill cartilage, epithelium, spleen, kidney and mucus. Fänge, Lundblad & Lind (1976) carried out a survey of lysozyme distribution in a number of marine fish and observed that activity was not restricted to teleosts, but was also present in elasmobranchs and holocephalans. In the same report, the authors also found that the enzyme chitinase was also widely distributed throughout the various groups of fish.

Various non-specific precipitins have been found in fish sera. Fletcher & Grant (1969) and Baldo & Fletcher (1973) found a component of plaice serum that would precipitate with fungal and parasite extracts and that had α_2 electrophoretic mobility, thus behaving in a similar fashion to the human acute phase protein, C-reactive protein. Davies (1975) isolated and biochemically characterised a similar substance from the serum of Atlantic salmon that had been infected with ulcerative dermal necrosis (UDN).

Fish also have serum components resembling mammalian complement. Gerwurz, Finstad, Muschel & Good (1966) showed that complement activity was abolished by heat and inhibited by EDTA in both chondrostean and elasmobranch fish. Legler, Evans & Dupree (1969) demonstrated complement activity in a number

of fish including the channel catfish, the bowfin, Amia calva, the paddlefish and the sturgeon, Acipenser spp. and made partial biochemical analysis of it. Similarly, Jensen, Sigel & Ross (1968) partially purified the first component of complement from the nurse shark.

The exact role of these non-specific factors, especially C-reactive protein, is uncertain but they may be very important in defence against pathogenic micro-organisms, since the classical immune mechanisms in fish are not so sophisticated as those of mammals.

CHAPTER 2

MATERIALS AND METHODS

I GENERAL MATERIALS

A) Biological Material

i) Fish - maintenance and handling

Dogfish were supplied by the Marine Biological Laboratory, Plymouth, and they were kept at this institute in concrete tanks measuring approximately 2m x 1.5m x 1.5m supplied by a flow-through sea water system and were fed daily on chopped, fresh fish.

A daily record of water temperature was kept and where appropriate, is given in the experimental details. Salinity was also monitored and fell within the range of 33-36 parts per thousand.

Where necessary, fish were identified by a coloured, trailing, plastic flag tag (M.A.F.F., Lowestoft) attached to the dorsal fin by 80 lb breaking strain monofilament nylon or a number of small scissor clips from the trailing edge of the pectoral and or pelvic fins.

During these studies adult dogfish of either sex weighing between 500g-900g and between 50cm-70cm in length were used.

Anaesthetic was only employed in non-routine handling procedures such as transportation and bleeding by cardiac puncture. In such cases MS222 (methane tricaine sulphonate) was used at a concentration of approximately 1 part in 10,000.

ii) Parasites

All dogfish examined in Plymouth were heavily infected with the nematode parasite Proleptus obtusus Dujardin 1845. Parasites were removed from the intestinal lumen of the fish, washed in elasmobranch saline (Table 2.1) and stored at -20°C. Antigen extracts were also prepared for certain experiments by treating worms in a Teflon-glass hand homogeniser. Following the removal of debris by centrifugation at 2000g for 5 minutes, the homogenate was diluted with saline and soluble protein was measured by the Folin-Ciocalteu method (Lowry, Rosebrough, Farr & Randall, 1951). The extract was then stored at -20°C until required.

B) Chemicals

All reagents were obtained from the Sigma Chemical Co. or BDH Chemicals Ltd. and were of the purest grade or of Analar quality unless stated otherwise. Water was distilled once from glass.

II PARENTERALLY INJECTED ANTIGENS

In this study, several antigens were used and were prepared as follows:

A) Bovine Serum Albumin (BSA)

Cohn fraction V (Koch-Light Laboratories Ltd.) was dissolved in saline at a concentration of 40 mg ml^{-1}.

B) Keyhole Limpet Haemocyanin (KLH)

Lyophillised KLH was obtained from Calbiochem Ltd. and reconstituted in saline at a concentration of 20 mg ml^{-1}.

C) Horse Serum

Inactivated horse serum (Wellcome Reagents Ltd.) was used undiluted.

D) Salmonella typhi

Formalin-killed Salmonella typhi H antigen 'a' (Difco Laboratories Ltd.) were used at a concentration of 10^8 cells ml^{-1}.

E) Sheep Red Blood Cells (SRBC)

Sheep erythrocytes were obtained from Tissue Culture Services Ltd. and used within one week of delivery.

F) Purified Protein Derivative of Mycobacterium tuberculosis (PPD)

Freeze dried PPD was supplied by the Central Veterinary Laboratories, Weybridge and made up to 20 mg ml^{-1} in saline.

G) Other Antigens

Injections of other materials were occasionally made. These are described in the relevant experimental details.

III PRODUCTION OF SPECIFIC ANTISERA

A) Rabbits

Antigens were prepared in 0.85% physiological phosphate buffered saline (PBS) at pH 7.2. Soluble antigens were emulsified in Freund's complete adjuvant (FCA) (Difco

Laboratories Ltd.) in a 1:1 ratio. Sub-cutaneous injections were given at weekly intervals for 28 days.

B) Fish

Soluble antigens were made up in elasmobranch saline (see Table 2.1) emulsified with FCA in a 1:1 ratio and unless otherwise stated, injected intra-muscularly (IM) behind the dorsal fin. Insoluble antigens were injected without adjuvant at the same site. Injections made occasionally via other routes e.g. intra-venous (IV) and intra-peritoneal (IP) are described where appropriate.

Constituents	g litre^{-1}
Na Cl	16.38
K Cl	0.89
Ca Cl_2	1.11
Na HCO_3	0.38
Na H_2 PO_4	0.06
Urea	21.6
Dextrose	1.0

Table 2.1 Elasmobranch saline - after Hale (1965). The saline was autoclave sterilised and stored at 4^{o}C until required.

IV BLOOD COLLECTION

A) Rabbits

Blood samples were taken by venesection from the marginal ear-vein after pre-treatment with xylene. A

lignocaine spray (Xylotox - Pharmaceutical Manufacturing Co.) was used as a local anaesthetic. Blood was collected in 25 ml universal containers and a suitable anticoagulant was added if required.

B) Fish

Fish were bled routinely from the caudal sinus on the ventral side, approximately 1 cm behind the anal fin, using a 25 gauge needle and a 1 ml syringe. Anaesthetic was only employed when large samples were taken by cardiac puncture. A 21 gauge needle was used for this purpose.

If serum from either animal was required, blood was allowed to clot in a collecting vial for 1 hour at room temperature and then left overnight at $4^{o}C$. The sample was then centrifuged at 200g for 5 minutes in a bench centrifuge. The separated serum was then decanted.

To obtain plasma, two methods were employed to stop the blood clotting process: either a few grains of solid EDTA were added to the collecting vial to act as an anticoagulant or the vials were first rinsed with a heparin solution (1000 IU's per ml). The latter method was preferred.

All serum and plasma samples were stored at $-20^{o}C$ in 2.5 ml auto-analyzer vials (Becton Dickinson Ltd.).

V IMMUNOLOGICAL METHODS

A) Direct Agglutination

In both methods described below the tests were performed using a microtiter system (Cooke Engineering Co.). The tests were carried out in disposable microtiter trays. The capillary transfer was 25 µl in each case.

i) Bacterial agglutination

Twofold dilutions of serum from dogfish immunised with S.typhi were made to a concentration of 1:4096 using PBS as a diluent. Equal aliquots of Salmonella typhi suspension (10^8 cells ml^{-1}) were added to each well. Sera from non-immunised dogfish and PBS alone were used as controls. The trays were sealed with adhesive cellulose acetate tape and incubated at 55°C. The plates were examined after 2 hours, left at room temperature and examined after a further 18 hours. The agglutination titre was taken as the reciprocal of the lowest dilution to exhibit macroscopic bacterial agglutination.

ii) Haemagglutination

Doubling dilutions of sera from fish immunised with SRBC were made as for bacterial agglutination after heat-inactivation at 56°C for 30 minutes. Sheep erythrocytes less than 7 days old were washed with PBS and equal aliquots were added to each well containing antiserum. The trays were sealed and incubated at 37°C for 2 hours and 18 hours at room termperature; the results were noted after each period. The end point was taken as the lowest dilution that would exhibit visible agglutination. Sera from non-immunised fish and PBS alone were used as controls. No naturally occurring agglutinins or lysins to SRBC were observed in dogfish sera.

B) Passive Agglutination (Latex Fixation)

Latex particles, 0.81 μ diameter (Difco Laboratories Ltd.) were incubated with an equal volume of 5% (w/v) antigen solution (BSA, KLH or Proleptus extract) at 37°C for one hour. The suspension was filtered, under partial pressure, through a membrane filter with a pore size of 0.45 μ (Millipore Ltd.). After filtration the membrane with the impinged latex was cut into small strips and made up to the original volume with 0.01M glycine buffered saline at pH 8.2 (Severin, 1974). The tube was then vigorously agitated on a rotary mixer to remove the beads from the membrane. The latex particles were stored at 4°C until ready for use and would remain stable for over six months. Doubling dilutions of antiserum from immunised fish were made. The dilutions were transferred to a ruled black glass slide (Behring Diagnostics) as individual 25 μl drops. To each drop an equal aliquot of sensitised latex was added. The slide was gently rocked for several minutes and then examined for agglutination. Controls of non-sensitised latex and PBS were made with each test. The specificity of the technique was checked using sera from non-immunised fish, normal rabbit serum and horse serum. End point criteria were the same as for the direct agglutination test, Fig. 2.1.

C) Agar Gel Precipitation Studies

i) Double Diffusion

The method described by Ouchterlony (1948) was used as a semi-quantitative test for antibody activity. A 1% (w/v) agarose suspension was made up in PBS. Sodium azide (0.01% w/v) was added as a preservative. Occasionally 3% (v/v) polyethylene glycol was added to enhance precipitin lines. The suspension was dissolved by boiling and poured into plastic petri dishes in 15 ml aliquots. A variety of well-cutter template patterns were used according to the antigen-antibody system being investigated.

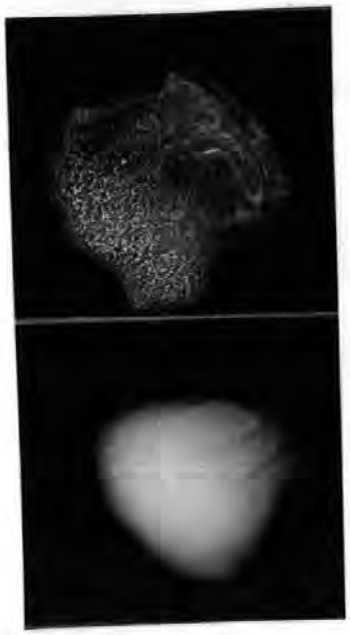


Fig. 2.1. Latex Agglutination Test

Latex beads sensitised with a single component protein antigen rapidly agglutinate in the presence of a specific antiserum.

The top slide shows strong agglutination, indicating a positive test.

The lower slide shows a negative control.

N.B. The photograph is of a model reaction using BSA coated beads and rabbit anti-BSA antiserum. Similar reactions using dogfish antiserum to beads coated with various antigens were identical in appearance.

ii) Quantitative Single Radial Immuno Diffusion (SRID)

The technique is based on the gel diffusion method as originally described by Mancini, Carbonara & Heremans (1965). Petri dishes containing a layer of agarose were made up in a similar way to the double-diffusion technique except that a 2% (v/v) specific antiserum was incorporated into it. Serum samples (10 µl) were placed in cylindrical wells and 18 hours later the circular area of precipitated antigen/antibody complex was measured with vernier calipers and related to a calibration curve.

iii) Immunoelectrophoresis (IEP)

The micro-immunoelectrophoresis technique of Scheidegger (1955) was used employing equipment manufactured by Shandon Scientific Ltd. Standard microscope slides (76mm x 25mm x 10mm) were cleaned in absolute alcohol and mounted in a perspex tray and covered with a thin film of 1% (w/v) agarose in a 100 mM barbitone acetate buffer, pH 8.6, ionic strength 0.1 (Oxoid Ltd.) Sodium azide (0.01% w/v) was added as a preservative. The surface of the agarose was levelled while still molten and then allowed to gel. Patterns of the 'two-hole, one trough' configuration were cut and the excess agar was removed with a pasteur pipette connected to a vacuum pump. Serum samples were loaded into the wells using pasteur pipettes drawn out in a bunsen flame to give a very fine capillary. Bromophenol Blue was used as a migration marker. The tank buffer was also 100 mM barbitone acetate buffer, pH 8.6. Contact between the tank buffer and the gel was maintained using moistened filter paper wicks. Electrophoresis was carried out at 150V for approximately 90-100 minutes after which time the troughs were filled with antiserum and diffusion was allowed to take place.

After 24 hours, the slides were examined and photographed using background illumination. Slides were washed in PBS and several changes of distilled water to remove any non-complexed protein, stained with 1% (w/v) Coomassie Brilliant Blue in 7% (v/v) acetic acid for 18 hours and destained in 7% (v/v) acetic acid and washed again in distilled water. Finally, the slides were dried between strips of lint-free paper (Gelman Hawksley Ltd.).

iv) Rocket Electrophoresis

The procedure based on that of Laurell (1966) was used. Agarose was made up as for immunoelectrophoresis except that a 2% (v/v) specific antiserum was incorporated into it.

An 81 mm square glass slide (Kodak Ltd.) was cleaned with absolute alcohol and 15 ml of the agarose solution cooled to 56^{o}C in a waterbath, was pipetted onto it. This quantity was sufficient to give an even layer of gel approximately 2 mm thick over the surface of the glass. A series of wells 4 mm in diameter were cut and loaded with a 10 µl of sample. The plate was placed in an electrophoresis tank and a potential difference of 250V-280V applied for 4 hours, by which time the sample had migrated and had been completely complexed by the antiserum in the agarose, thus forming rocket shaped precipitin lines (Fig. 2.2). The height of the rocket was measured with vernier calipers and the quantity of component being measured was calculated by relation to a standard calibration curve.

v) Counter-Current Immunoelectrophoresis

The procedure described by Culliford (1964) was used.

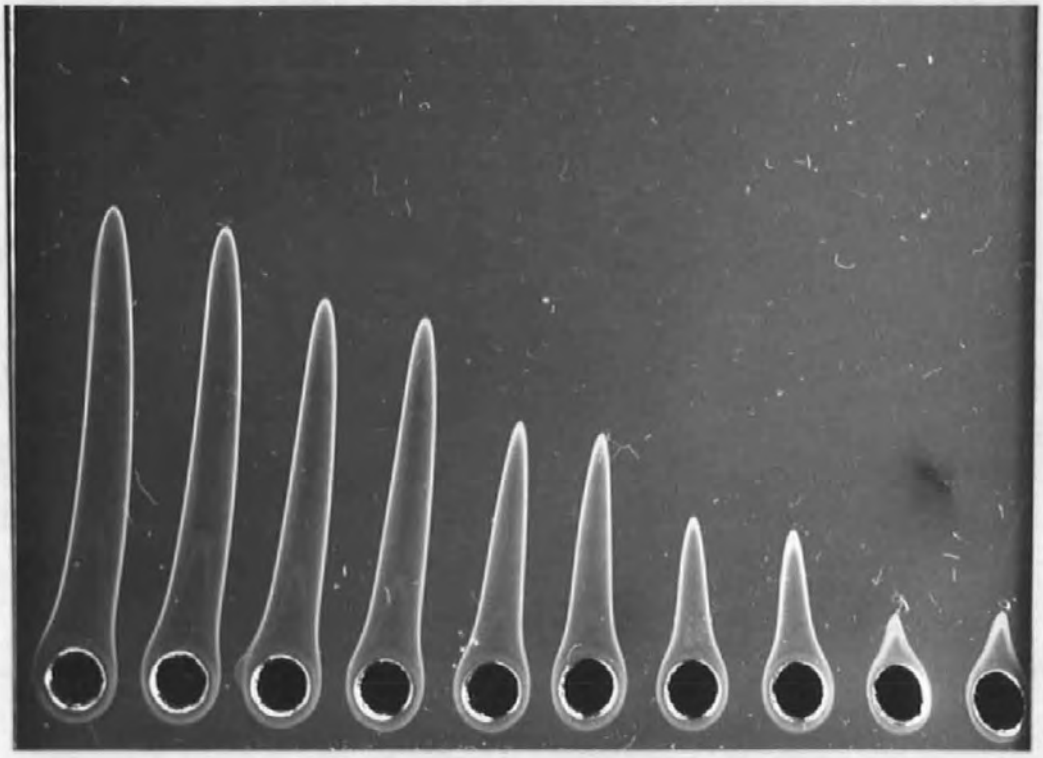


Fig. 2.2. Rocket Immunoelectrophoresis

Showing precipitin "rockets" of differing heights relating to various concentrations of a BSA solution after electrophoresis in an agarose gel containing 2% (v/v) specific antiserum. Reading from left to right, paired wells contained 500, 400, 250, 150 and 50 $\mu g\ ml^{-1}$ of BSA.

N.B. Rabbit antiserum was used in all such experiments.

An 81 mm glass slide was prepared in a similar manner to that described for rocket electrophoresis except that the specific antiserum was omitted. Paired wells, 1.5 mm diameter and 3 mm apart, were cut using a specially constructed template and a gel punch (Shandon Scientific Ltd.). The slide was placed in an electrophoresis tank as previously described. Samples of antigen and antiserum were loaded into the wells with a fine glass capillary and a potential of 150V applied for 30 minutes. After this period the plate was removed and examined for the presence of precipitin lines.

D) Cellular Techniques

i) Cell Viability Assay

Leucocyte viability was estimated by the Trypan Blue exclusion test. One part of 0.5% (w/v) Trypan Blue was mixed with 9 parts of cell suspension and examined in a haemocytometer. Cells excluding the dye were considered viable (Fig. 2.3). Occasionally cells that appeared badly damaged were seen to exclude Trypan Blue, these were considered non-viable.

ii) Assay for High-Rate Antibody Producing Cells

In order to demonstrate cells actively synthesising humoral antibody, a modification of the haemolytic plaque technique (Cunningham & Szenberg, 1968) was used. The technique was performed as follows: a dogfish, sensitised with SRBC was killed and the spleen rapidly removed and placed in 10 ml of tissue culture medium comprising: Eagle's minimum essential medium with Hank's balanced salts and supplemented with 0.25% (w/v) sodium bicarbonate, 350 mM urea, 1% (w/v) sodium chloride, 1000 IU's ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin. The organ was

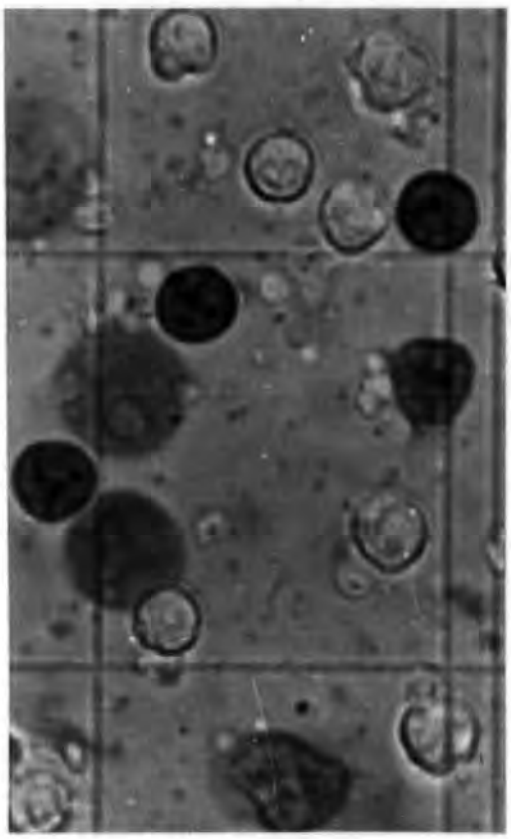


Fig. 2.3. Trypan Blue Viability Test

Micrograph of a spleen cell suspension, treated with Trypan Blue, in a haemocytometer chamber. Dark cells have taken up the stain and are non-viable. Cells that have excluded the Trypan Blue are viable.

N.B. The photograph depicts dogfish splenocytes treated with Trypan Blue.

teased out and disrupted with a large pair of forceps. A portion of the cloudy cell suspension was decanted off, a viability assay made and a reaction mixture comprising 1 part complement, 1 part target SRBC (15%-20% packed cell volume) and 4 parts cell suspension (containing approximately 10^6 viable splenic leucocytes ml^{-1}) made up. Lyophilised guinea pig serum was initially used as a complement source but proved to be unsatisfactory as it did not cause lysis of target SRBC in the presence of specific dogfish antibody so fresh dogfish serum was used. This was found to contain a haemolytic factor (presumably complement) in the presence of inactivated (dogfish) anti-SRBC antiserum. An optimum complement dilution was estimated by a standard complement titration assay. Dogfish sera was not found to have naturally occurring lytic factors for SRBC.

The reaction mixture (70 µl) was introduced as a monolayer between two standard glass microscope slides (76mm x 25mm) that had been pre-cleaned in alcohol. The slides were stuck together with 3 strips of double-sided adhesive tape, approximately 6mm wide, to give 2 chambers in a slide bilayer. After the reaction mixture had been introduced into the slide chambers, the edges were sealed with molten wax maintained at 55°C. The slides were incubated at 37°C for 60 minutes and the number of haemolytic plaques (Fig. 2.4) were scored. Control cultures were made using splenocytes from non-immunised fish.

iii) Leucocyte Migration Inhibition

A modification of the method described by Bendixen & Søberg (1970) was employed. Both peripheral blood cells and peritoneal exudate cells were used in the tests.

a) Peripheral Blood Cells - Blood samples (1 ml) were taken from the caudal sinus of fish (sensitised with either KLH, BSA or PPD or infected with Proleptus) and transferred to heparinised vials. Migration inhibition tests were carried out in Mackaness-type chambers (Leucocyte Migration Plate, Sterilin Ltd.). Whole blood was drawn into

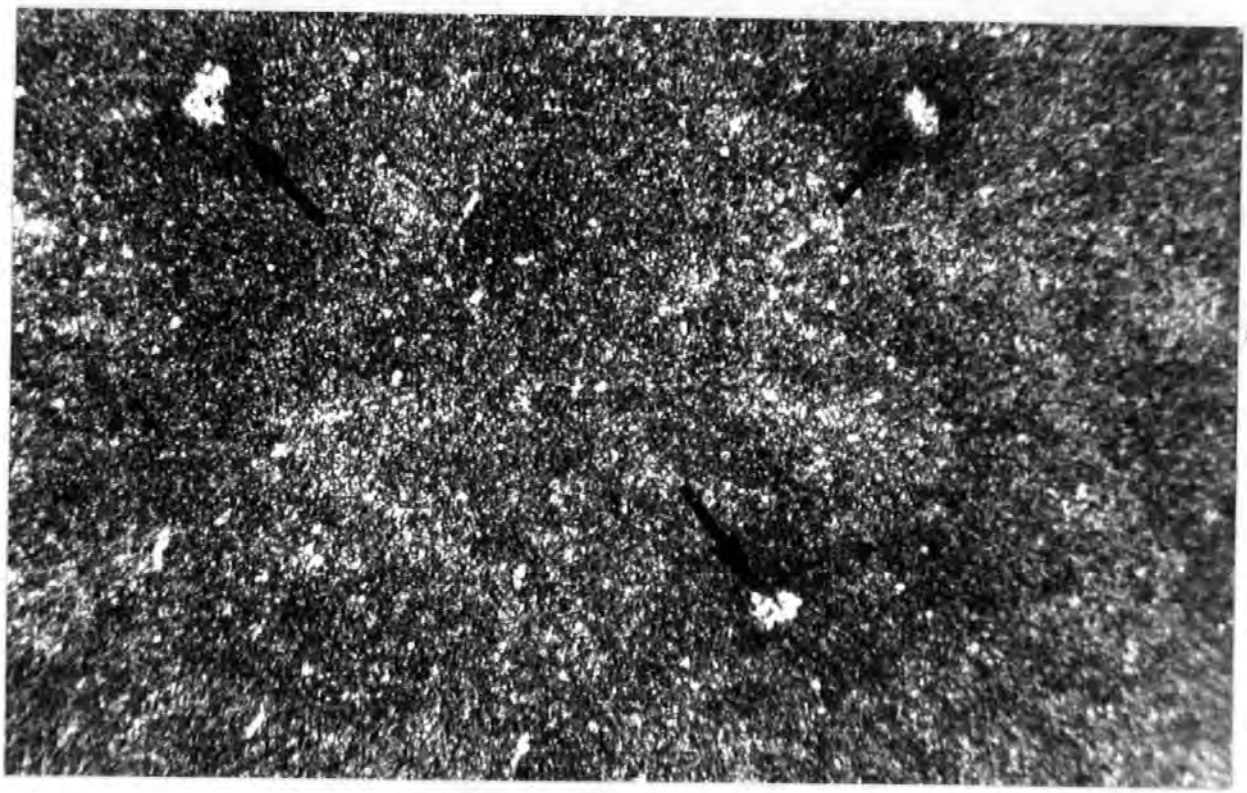


Fig. 2.4. Haemolytic Plaque Technique.

Spleen cells, taken from a fish previously sensitised with sheep red blood cells cause plaque formation (arrowed) of target SRBC when incubated in a slide chamber in the presence of complement.

Fig. 2.5. a) Leucocyte migration in the absence of antigen.

b) Inhibition of leucocyte migration in the presence of antigen.

N.B. Cultures of dogfish peripheral blood leucocytes incubated in the presence or absence of Proleptus antigen extract are shown in these photographs.

heparinised capillary tubes (Gelman Hawkesley Ltd.) which were then flame-sealed at one end. The cell suspension was packed by spinning for 5 minutes, at 3,500 r.p.m. in a hematocrit centrifuge. The tubes were then cut at the plasma/buffy layer interface and fixed in the chambers of a leucocyte migration plate with a spot of vaseline. The wells were filled with 450 µl of supplemented Eagle's medium. Appropriate antigen was added to the test cultures at a suitable concentration. Suitable controls (without antigen) were also made. The tops of the chambers were lightly smeared with vaseline and sealed with a coverslip. At least 5 replicates were made of each sample. Cultures were incubated for 18 hours at room temperature.

b) Peritoneal Exudate Cells - these were taken from fish sensitised with PPD or infected with Proleptus. They were induced by injecting 10 ml of technical white oil (Marcol 82, Esso Petroleum Ltd.) into the peritoneal cavity of a dogfish through a needle (19G). Two days later the fish was killed and 30 ml of tissue culture medium was injected into the peritoneal cavity and the body kneaded gently. The cells were then collected by making a small incision into the body wall and withdrawing the cell rich fluid with a pasteur pipette. The cells were washed 3 times by centrifuging at 1500g for 5 minutes and resuspending in tissue culture medium. The cells were drawn into capillary tubes and set up in chambers as described for peripheral blood leucocytes.

After incubation, the areas of migration of cells from the end of the tubes were measured by planimetry. The migration index (MI) was calculated as follows:

$$Ml = \frac{Mx}{Mo}$$

where Mx = area of leucocyte migration in culture containing antigen.

Mo = area of leucocyte migration in control cultures without antigen extract.

Using this calculation, control cultures always have a value of unity. If a figure for test cultures was less than this, inhibition of migration had occurred (Fig. 2.5).

iv) Leucocyte Cultures and Mitogen Responsiveness

The effect of mitogens on fish leucocyte cultures was measured using the method of Sigel, McKinney & Lee (1973). The same technique was also used for mixed lymphocyte cultures (MLC) and also to determine the effect of parasite antigens on leucocytes. The following basal medium was used for these cell cultures. Medium 199 (Tissue Culture Services Ltd.) supplemented with heparin (10 IU's ml^{-1}) 0.01M HEPES buffer (Calbiochem Ltd.) 0.2M NaCl, 350 mM urea, penicillin 1000 IU's ml^{-1}, and streptomycin, 100 ug ml^{-1}. For final cell suspension and growth, 15% of filter sterilised homologous serum was added.

All washing procedures were carried out using basal medium containing double the antibiotic concentration.

a) Preparation of Peripheral Blood Leucocytes

Blood samples from normal fish were taken and transferred to sterile, stoppered, tissue culture tubes (Tissue Culture Services Ltd.) that had been rinsed with a sterile heparin solution. The blood was allowed to sediment for

1-2 hours and the leucocyte rich plasma was aseptically removed and transferred to another tube. The cells were washed twice with medium, centrifugation being carried out at approximately 200g for 10 minutes. The leucocytes were then resuspended in normal medium at an appropriate concentration.

b) Leucocyte Stimulation Assay

To each culture tube 0.9 ml of culture medium and 0.05 ml antigen or mitogen (in various concentrations) were added. Leucocyte viability was determined by the Trypan Blue exclusion method and 10^6 cells were added to the culture in 0.1 ml of medium. The cultures were incubated at 25°C for approximately 60 hours after which time 2 µCi ^{3}H-thymidine (specific activity 25 Ci/mMol, Radiochemical Centre, Amersham) was added in a 50 µl inoculum. Sixteen hours later the experiment was terminated. Viability counts were made, the cell suspensions were washed twice in elasmobranch saline and then subjected to 2 freeze-thaw cycles. To each tube 25 µl of calf serum was added and acid insoluble material was precipitated by adding 2 ml of 5% (w/v) trichloracetic acid (TCA). The precipitates were washed once with 5% (w/v) TCA and the resulting pellets were dissolved in 0.5 ml hyamine hydroxide. The tubes were sealed with parafilm and incubated overnight at 37°C. Finally, 10 ml of Unisolve II scintillation fluid (Koch Light Laboratories Ltd.) was added to each sample and was measured by counting in a Phillips scintillation counter. Cell cultures that had not been mitogen stimulated were used as controls.

E) Immunofluorescence

The immunofluorescence methods described were carried out using the "sandwich" technique. This is a double layer technique and is summarised in Fig. 2.6 below.

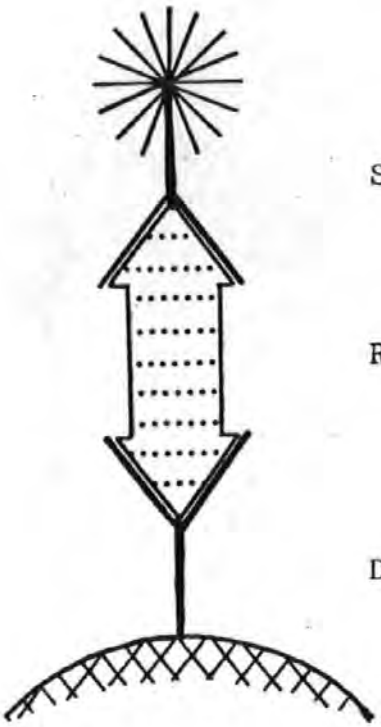


Fig. 2.6. Schematic diagram to show double layer 'sandwich' indirect immunofluorescence technique.

i) Staining for Lymphocyte Surface Immunoglobulin

The method of Taylor, Duffus, Raff & de Petris (1971) was used to demonstrate the presence of immunoglobulin molecules on the surface of peripheral blood leucocytes. Dogfish leucocytes obtained as previously described were washed 3 times with elasmobranch saline and the suspension (approximately 10^7 cells ml^{-1}) was incubated for 30 minutes with rabbit anti-dogfish

immunoglobulin (1:10 dilution) that had previously been absorbed with a large volume of dogfish erythrocytes and de-complemented by heating at 56°C for 30 minutes. The cells were washed 3 times with elasmobranch saline and incubated with a 1:20 dilution of sheep anti-rabbit immunoglobulin conjugated with fluorescein isothiocyanate (FITC) (Wellcome Reagents Ltd.) for a further 30 minutes. Finally the cells were washed again with elasmobranch saline smeared on a microscope slide and examined.

(ii) Staining for Dogfish Immunoglobulin on the Parasite Surface

Washed Proleptus were placed on a specimen chuck and snap frozen with compressed CO_2. Sections were cut at 8 μ using a Slee cryostat and placed on coverslips. Where necessary the sections were stored in stoppered tubes at -70°C. The sections were stained in a similar manner to the leucocytes.

Stained material was examined with a Vickers fluorescence microscope using an HBO 200 mercury vapour bulb (Osram) and with a UG2 primary filter.

F) Hypersensitivity Techniques

The ability of the dogfish to produce skin-sensitising antibody was tested by homologous and heterologous passive cutaneous anaphylaxis (PCA). The method employed for heterologous PCA was identical to that of Harris (1973) modified from Ovary (1958) and Campbell, Garvey, Cremer & Sussdorf (1964). The technique was carried out in white albino guinea pigs weighing over 400g. The flanks were partially shaved and 3 intradermal injections were given:

i) 0.2 ml of undiluted antisera (from fish that had been antigen sensitised).

ii) 0.2 ml fish antisera diluted 1:1 with normal saline.

iii) 0.2 ml saline as a control.

After either 3 or 72 hours the animals were anaesthetised with Nembutal (Abbott Laboratories Ltd.) and an injection comprising 0.2 ml of antigen in a 2% (w/v) solution of Evans Blue dye (Brocklehurst, 1967) was given by cardiac puncture. After 20 minutes the animals were killed and the skin around the site of the intradermal injection was examined from the sub-cutaneous surface for signs of the characteristic 'blueing' reaction.

Homologous PCA was carried out directly in fish. Three intradermal injections were given on the ventral surface of the fish. The injections were identical to those given to guinea pigs except that elasmobranch saline was introduced as a normal control. Antigen in 2% (w/v) Evans Blue was given intravenously, via the caudal sinus, after 4 or 72 hours. The intradermally injected sites were examined after 20 minutes as with the guinea pigs.

VI TESTS FOR NON-SPECIFIC FACTORS

A) Lysozyme

Lysozyme (Muraminidase) EC 3.2.1.17, was assayed semi-quantitatively according to its ability to lyse the bacterium Micrococcus lysodeiktus. Freeze-dried cells of this organism were incorporated into 1% (w/v) agar cooled to 55°C, to give suspensions of 1 mg ml^{-1}. Aliquots (15 ml) were dispersed into petri dishes and allowed to set. Four wells, 4 mm in diameter were cut in each plate and 25 µl of test or standard lysozyme solution were placed into each.

The plates were incubated at 25°C for 24 hours and then examined. Areas of bacterial lysis were measured using vernier calipers. A calibration curve of area of lysis against $\log_{10}$ enzyme concentration was constructed and test results were calculated from this curve.

B) C-Reactive Protein (CRP)

Attempts were made to qualitatively assay CRP. Assays were made by the double diffusion method: sera were tested against sonicated homogenates (containing between 25-50 mg ml^{-1} soluble protein) of:

Bacillus subtilis

Candida albicans

Proleptus obtusus

Saprolegnia spp. (taken from infected carp)

Ascaris lumbricoides

Plates were examined for the presence of precipitin lines after 1, 3, 7 and 14 days. To determine if a precipitate was due to CRP or a naturally occurring antibody, the agar gels containing precipitin lines were immersed in the following solutions for 7 days.

5% (w/v) sodium citrate

0.1M EDTA

Elasmobranch saline as a control

CRP precipitin lines are calcium-dependent and chelating agents such as citrate and EDTA will dissolve them.

VII PROTEIN SEPARATION

A) Electrophoresis

i) Cellulose Acetate Electrophoresis (CAE)

Serum samples were applied to cellulose acetate slides (Millipore Ltd.) in 0.075% (w/v) veronal buffer and a potential of 100V applied for 20 minutes. The slides were then stained and fixed in Ponceau-S (Millipore Ltd.) and then cleared using an ethyl acetate/ glacial acetic acid mixture (30 vols. : 70 vols.) and then dried. The stained bands of protein on each strip were quantified using a Phoroscope densitometer (Millipore Ltd.) and the percentage of each serum component calculated.

ii) Polyacrylamide Gel Electrophoresis (PAGE)

The technique was carried out in gel slabs. Sodium dodecyl sulphate (SDS) was incorporated in a modification of the method of Weber & Osborn (1969) for calculating relative molecular weights. When the technique was used as a purely comparative method, the SDS was omitted.

a) Sample Preparation

Samples of serum protein (approximately 20mg in total) were each placed in a vial, mixed with an equal volume of solubilising solution (Table 2.2) and incubated for 2 hours at 37^{o}C.

b) Gel Preparation

A 12% (w/v) acrylamide gel was made up as in Table 2.2. The ammonium persulphate initiator was freshly made up each time a gel was cast. On addition of the initiator, the solution was poured into a gel plate (Shandon Scientific Ltd.) layered with distilled water in order to prevent a meniscus forming and allowed to polymerise.

Gel Buffer

8.8g $Na\ H_2\ PO_4.2H_2O$

25.5g $Na_2HPO_4.2H_2O$

(2g SDS)

Distilled Water to 1000 ml

Electrolyte Buffer

Gel buffer diluted 1:1 with distilled water

Acrylamide Solution

22.2g acrylamide

0.6g bis-acrylamide

Distilled Water to 100 ml

Acrylamide Gel

15 ml gel buffer

13.5 ml acrylamide solution

1.5 ml ammonium persulphate solution (15 mg ml^{-1})

0.045 ml N,N,N',N' tetramethylethylenediamine (TEMED)

Solubilising Solution

0.01M Sodium phosphate buffer pH 7.0

1% 2-mercaptoethanol (2-ME)

(1% SDS)

Table 2.2. Table to show composition of solutions necessary for PAGE. A 12% acrylamide gel is formed using the ratios given above.

When the gel had set, the water was removed, well formers were placed in position and a 3% (w/v) capping gel poured around them. After allowing 20 minutes for polymerisation the well formers were carefully removed, the wells were washed with distilled water and blotted dry with narrow strips of filter paper.

c) Sample Application

One drop of Bromophenol Blue and one drop of glycerol was mixed with 500 µl of sample incubation mixture. Between 10 µl - 30 µl of sample from each mixture was placed in the wells.

c) Electrophoresis

Separations were carried out at approximately 200V for 4-5 hours. The gel was removed, rinsed in distilled water and stained and fixed overnight in a solution containing 7% (v/v) acetic acid containing 0.2% (w/v) Coomassie Brilliant Blue. Destaining was carried out in 7% (v/v) acetic acid containing 30% (v/v) methanol.

ii) Preparative Agarose Electrophoresis

This technique was performed as follows:

100 ml of 1% (w/v) agarose in barbitone buffer was poured into a slab gel mould (Shandon Scientific Ltd.) measuring 5 cm x 12 cm and allowed to set. A section 0.5 cm wide was cut across the width of the gel, approximately 4 cm from one end and the slab was placed in a Kohn electrophoresis tank. Electrophoresis was carried out for 4 hours at 200V at $4^{o}C$.

The differential electrophoretic mobility of the sample protein components was determined by cutting a longitudinal strip of the gel and staining in solution

containing 7% (v/v) acetic acid and 0.2% (w/v) Coomassie Brilliant Blue. Destaining was carried out in 7% (v/v) acetic acid.

Recovery of proteins in the gel was achieved by slicing out the required section, macerating it, eluting with buffer and centrifuging at 2,600g for 20 minutes.

B) Chromatography

i) Column Chromatography (Gel Filtration)

Gel filtration is a technique extensively used in immunochemistry in order to separate serum proteins according to their molecular dimensions. Two types of gel were used in conventional column chromatography and are described below:

a) Sephadex G-200 (Pharmacia Fine Chemicals)

Sephadex is a cross-linked dextran gel and was first described by Porath & Flodin (1959). Sephadex G-200 will fractionate globular proteins with molecular weights in the range 10^4 - $3x10^5$ daltons.

b) Sepharose 6B (Pharmacia Fine Chemicals)

Sepharose is an agarose gel (Polson, 1961) and has larger pore sizes than the Sephadex gel and will therefore separate proteins of a higher molecular weight. The fractionation range of Sepharose 6B, for globular proteins is between 1.5×10^4 - 1.5×10^6 daltons.

Gels were poured, allowed to pack and equilibrated with 20 mM phosphate buffer, pH 8.0, containing 1% (w/v) sodium chloride and 0.01% (w/v) sodium azide. Ascending flow was used to avoid gel compaction and was carried

out using a peristaltic pump (Watson Marlow Ltd.). Flow rates used for various gels and column sizes are given in Table 2.3. The homogeneity of the gel and the void volume were calculated using blue dextran (Pharmacia Fine Chemicals). The column effluent was continuously monitored at 280 nm in a Uvicord II detector unit (LKB Ltd.) in conjunction with a chopper bar chart recorder. A fraction collector (LKB Ltd.) was also used to collect samples of specific volume size. All operations were carried out at $4^{o}C$.

GEL	COLUMN SIZE	SAMPLE SIZE	FLOW RATE	FRACTION SIZE
SEPHADEX G-200	85 cm x 1.5 cm	2 ml	6 ml hr^{-1}	50 drops (3.4 ml)
SEPHAROSE 6B	85 cm x 1.5 cm	2 ml	10 ml hr^{-1}	50 drops (3.4 ml)
SEPHAROSE 6B	85 cm x 2.5 cm	5 ml	24 ml hr^{-1}	130 drops (8.85 ml)

Table 2.3 Showing the conditions for running Sephadex and Sepharose in different column sizes.

ii) Thin Layer Gel Chromatography (TLG)

In this technique a layer of swollen gel is spread on a glass plate and when connected to a buffer reservoir and tilted can be used to separate proteins of differing molecular weights in a similar manner to conventional column gel filtration. The advantage of the technique is that very small quantities of protein (as little as 0.5 µg) can be

used (Rydon & Smith, 1952). The method was carried out as follows: Sephadex G-150 superfine (Pharmacia Fine Chemicals) was pre-swollen in gel filtration buffer and applied to a glass plate 20 cm x 20 cm. The gel slurry was levelled to an even 1.0 mm thickness with a gel spreader (Pharmacia Fine Chemicals). Once spread, the plate was put in a TLG chamber (Pharmacia Fine Chemicals) for equilibration. The gel layer was connected to eluant resevoirs at both ends of the tank by filter paper bridges. Equilibration was carried out overnight with the plate tilted at an angle of 20^{o}. Samples and molecular weight markers (at an approximate concentration of 10 mg ml^{-1} in gel buffer) were applied along the upper edge of the plate in 5 µl quantities. With the plate tilted at an angle of 20^{o} a flow rate of about 3 cm $hour^{-1}$ was achieved. Flow across the plate was maintained for 3-4 hours. Proteins were detected on the plate by the replica staining method (Radola, 1968). A piece of filter paper (Whatman 3MM) was cut to 20 cm x 20 cm and rolled over the gel and left in contact for about 1 minute. The paper was then removed and fixed and stained in 0.25% (w/v) Coomassie Brilliant Blue in methanol and glacial acetic acid (90:10, v/v) for 5 minutes and rinsed in methanol-glacial acetic acid-water (50:10:50, v/v). The molecular weight of the sample was calculated by relating to a calibration curve of the molecular weight markers in which migration distance was plotted against $\log_{10}$ molecular weight.

C) Analytical Ultracentrifugation

An estimate of the sedimentation coefficient of the dogfish immune macroglobulin was made by the following method. Protein from the first elution peak of Sephadex G-200 (containing the active antibody fraction) was pooled, dialysed against distilled water and lyophilised. The

protein was reconstituted at a concentration of 15 mg ml^{-1} in PBS and the sedimentation coefficient of the molecule (the major component of this fraction) was calculated by ultracentrifugation at 52,400 r.p.m. and at 20°C in a Spinco model E analytical ultracentrifuge. The sedimentation of the protein was viewed by Schlieren optics The sedimentation coefficient was calculated from the following formula:

$$S = \frac{2(x_2 - x_1)}{(x_2 + x_1)\, w^2 (t_2^2 - t_1)}$$

where S = the sedimentation coefficient in Svedberg units.

x_1 = distance protein peak has moved after t_1 (seconds)

x_2 = distance protein peak has moved after t_2 (seconds)

w = the angular velocity in radians per second.

The value obtained was not corrected for concentration.

VIII MICROSCOPICAL STUDIES

A) Light Microscopy

This technique was used to examine the following: spleen, epigonal tissue, Leydig's organ and whole juvenile fish. Specimens were fixed in Bouin's fluid, dehydrated in alcohol and embedded in paraffin wax. These procedures were varied according to specimen size. Sections were cut at 6 μ - 8 μ on a Jung rotary microtome and stained with either May-Grünwald/Giemsa, Haematoxylin and Eosin (H & E) or Methyl Green Pyronin Y according to Pearse (1968). The sections were examined with a Zeiss photomicroscope.

B) Electron Microscopy (EM)

Electron microscopy was used to examine dogfish leucocytes. An osmotically balanced fixative was used which has been found to be particularly effective for elasmobranch tissues (A. Pulsford, personal communication).

Reagents

i) Phosphate Buffer

0.3M $NaH_2PO_4.2H_2O$ pH 4.9
0.3M Na_2HPO_4 pH 9.0

add 3 ml $NaH_2PO_4.2H_2O$
+ 17 ml Na_2HPO_4

pH 7.5

ii) Salt Solution

4.5 ml KCl 0.54M
3.75 ml $CaCl_2.6H_2O$, 0.36M
6.0 ml Na_2SO_4 0.44M
4.5 ml $MgCl_2$ 0.36M
8.25 g NaCl
200 ml distilled water

iii) Fixing Agent

18% (v/v) glutaraldehyde

The sum of the osmolalities of the phosphate buffer (220 mOs) and the salt solution (420-440 mOs) was calculated and urea was added so that an equal mixture of the two solutions was of the same osmotic strength as sea water.

The fixing solution was made from equal volumes of phosphate buffer, salt solution and glutaraldehyde.

Fixation of the material was carried out by treating with 18% (v/v) glutaraldehyde for 1-2 hours, rinsing with a mixture of salt solution/phosphate buffer/distilled water in equal volumes and a final treatment of 2% (w/v) osmium tetroxide for 1 hour. The sample was then dehydrated in alcohol and placed in embedding resin (TAAB Laboratories). Silver or gold sections (between 600 Å - 800 Å) were cut on an L.K.B. ultramicrotome and stained with lead citrate and uranyl acetate (Reynolds, 1963). The sections were mounted on grids and examined in a Phillips EM 301 transmission electron microscope.

CHAPTER 3

HUMORAL RESPONSES OF THE DOGFISH

I Specific Humoral Responses

A series of experiments were designed to investigate aspects of the primary humoral responses of the dogfish to a variety of injected antigens.

Experiment 1.1 Determination of the Humoral Immune Response

In this experiment 4 antigens were used, namely, Salmonella typhi H (flagellar) antigen a, BSA, KLH and horse serum, in order to demonstrate a primary immune response.

Groups of 5 fish were injected with each antigen as described in Chapter 2. Various doses were used:

Salmonella	5×10^7 cells in 0.5 ml inoculum.
KLH	10 mg in 1 ml inoculum.
BSA	25 mg in 1 ml inoculum.
Horse Serum	0.5 ml in 1 ml inoculum.

In all cases a second identical injection was given after 14 days. Blood samples (0.5 ml) were taken at weekly intervals and antibody levels in the serum were evaluated. The following responses were noted:

a) Response to Salmonella -

Micro-agglutination tests on the sera showed a significant antibody titre after 21 days, rising to a maximum of 56 days when the experiment was terminated (see Fig. 3.1).

b) Response to KLH -

Levels of antibody activity were evaluated by the latex agglutination test. A response was noted after 14 days rising to a maximum titre of 4,096 after 35 days. At the end of the experiment this level had declined. The fish displayed great variation in their response to this antigen, some recording barely significant antibody titres and others extremely high levels (Fig. 3.2). Low antibody levels in 2 fish were detected shortly after the initial injection and these remained consistently low throughout the experiment. Four of the five test fish had a detectable titre of naturally occurring anti-KLH antibody at the beginning of the experiment. The sera were tested for precipitin activity by double diffusion and crossed-over immunoelectrophoresis techniques. All these tests proved negative.

c) Response to BSA -

BSA appeared to be a much weaker immunogen than KLH. Low titres of latex agglutinating antibody were detected after 14 days (Fig. 3.3). The maximum antibody titre, recorded after 42 days, was 64. After this antibody activity declined. Two fish failed to respond to this antigen. Naturally occurring antibody was also noted. All serum samples failed to give a positive precipitin test.

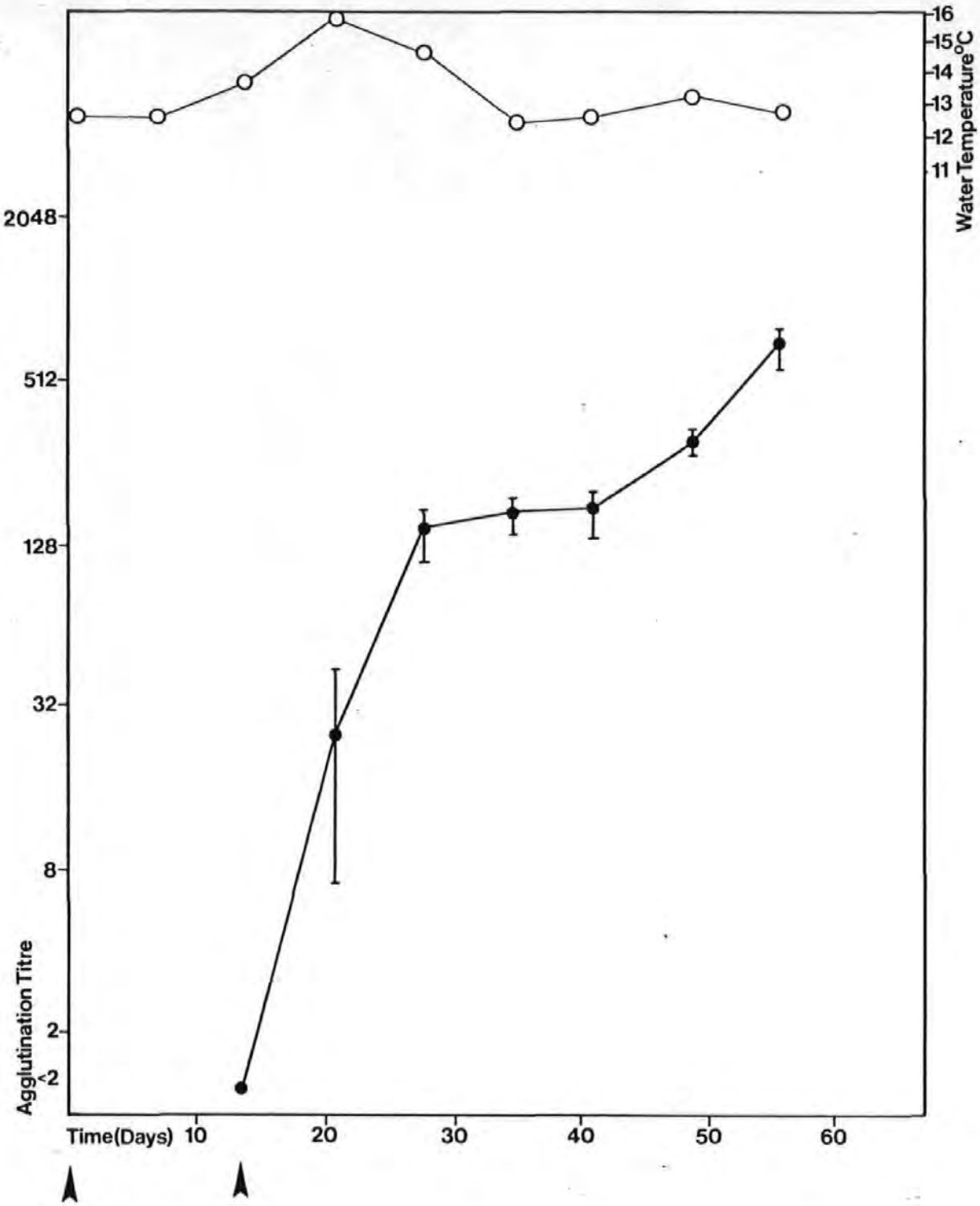


Fig. 3.1. The immune response of 5 adult dogfish to intra-muscularly injected Salmonella. Vertical bars represent standard error. Arrows indicate when injections were made. Open circles show water temperature.

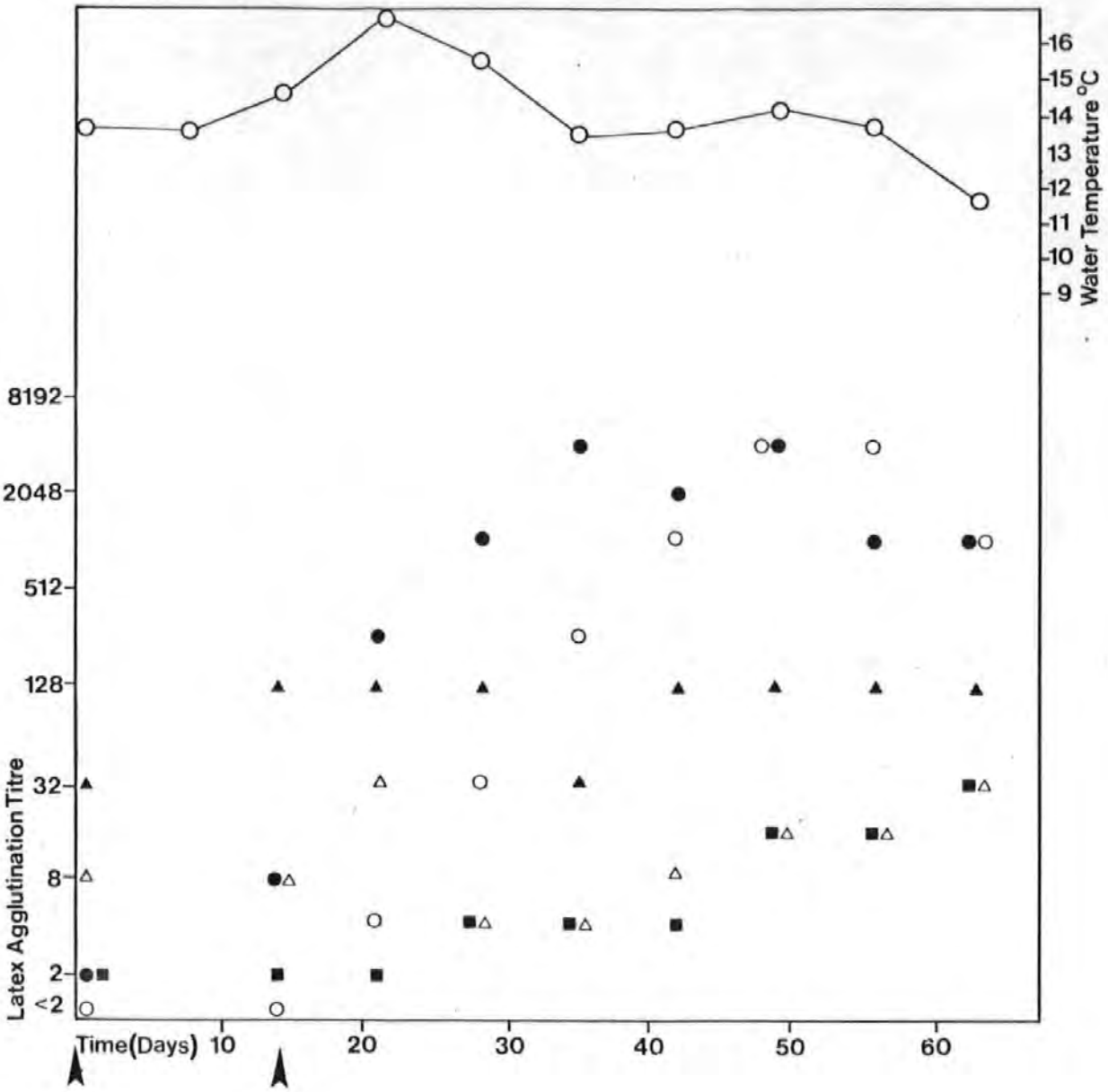


Fig. 3.2. The immune response of 5 adult dogfish to intra-muscularly injected KLH. Each symbol represents the latex agglutination titre from an individual fish. Arrows indicate when injections were made. Large open circles show water temperature.

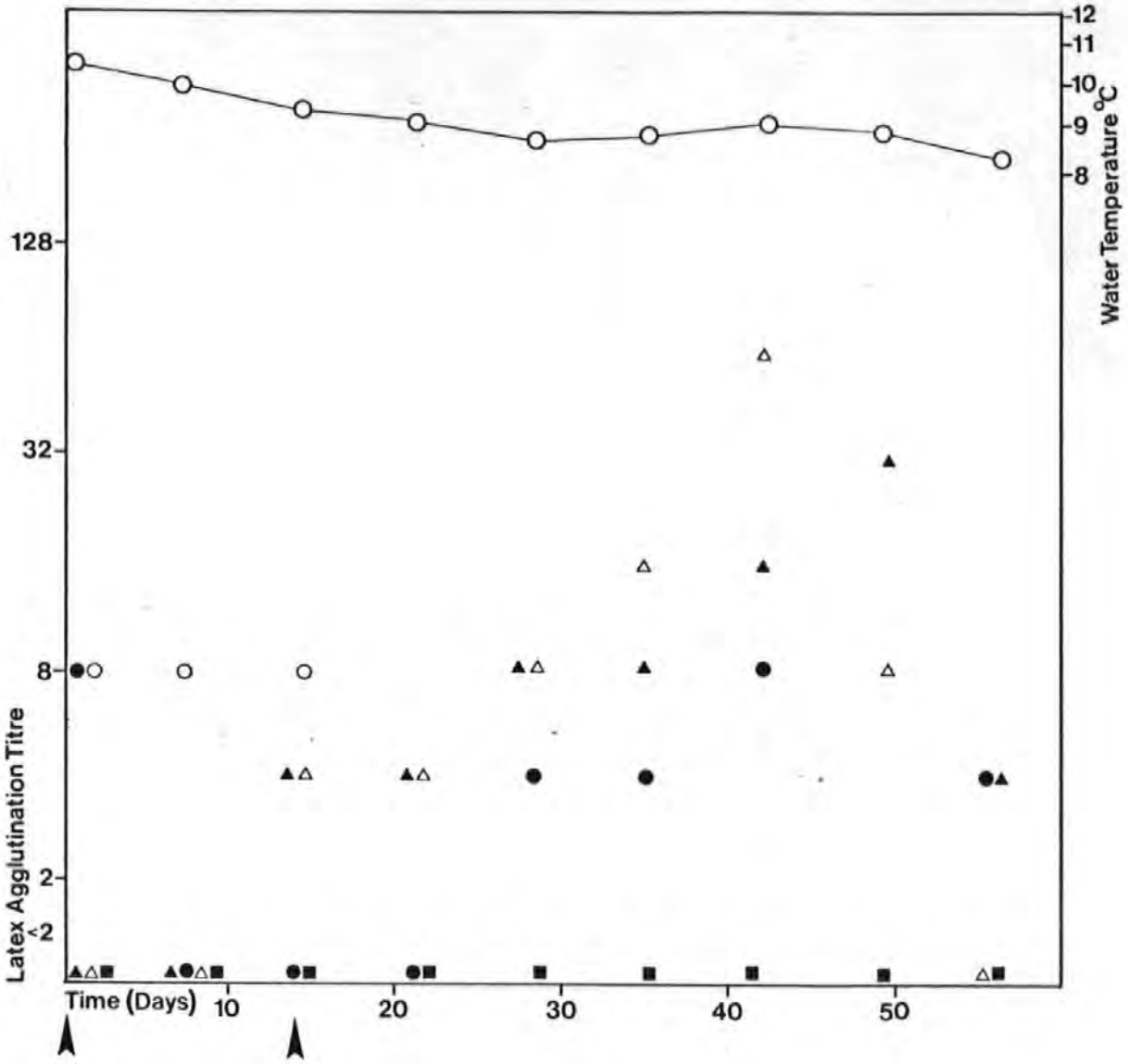


Fig. 3.3. The immune response of 5 adult dogfish to intra-muscularly injected BSA. Each symbol represents the latex agglutination titre from an individual fish. Arrows indicate when injections were made. Large open circles show water temperature. Note that the fish represented by small open circles died after 14 days.

d) Response to Horse Serum -

Only precipitin tests were used to attempt to demonstrate antibody production to this antigen. It was felt that to adsorb a multi-component antigen onto the surface of a latex particle was invalid as selective coating could occur. Three methods were tried: the double and single radial immunodiffusion tests and the crossed-over immunoelectrophoresis technique. All methods gave negative results.

Experiment 1.2 Antigen Clearance

The purpose of this experiment was to observe rates of expression and clearance, in the blood, of an intra-muscularly injected antigen and also to comment on the ability of fish either to retain immunological memory or reflect enhanced antigen processing.

Eight fish were injected with 50 mg of BSA in a 1.0 ml inoculum of elasmobranch saline. Four of the fish had previously been immunised with BSA, 70 days before the experiment, the others had not. The experiment was conducted at a water temperature of 12^{o}C. Blood samples were taken at intervals over a 72 hour period and the quantity of BSA in the sera was estimated by rocket electrophoresis. The rates of expression and clearance for the immunised and non-immunised fish were virtually identical (Fig. 3.4). The antigen reached a maximum concentration in the blood after 2 hours. This peak declined rapidly at first and then more gradually. No antigen was detected after 30 hours.

II Non-Specific Responses

Sera and tissues of fish were examined for the presence of non-specific defence factors.

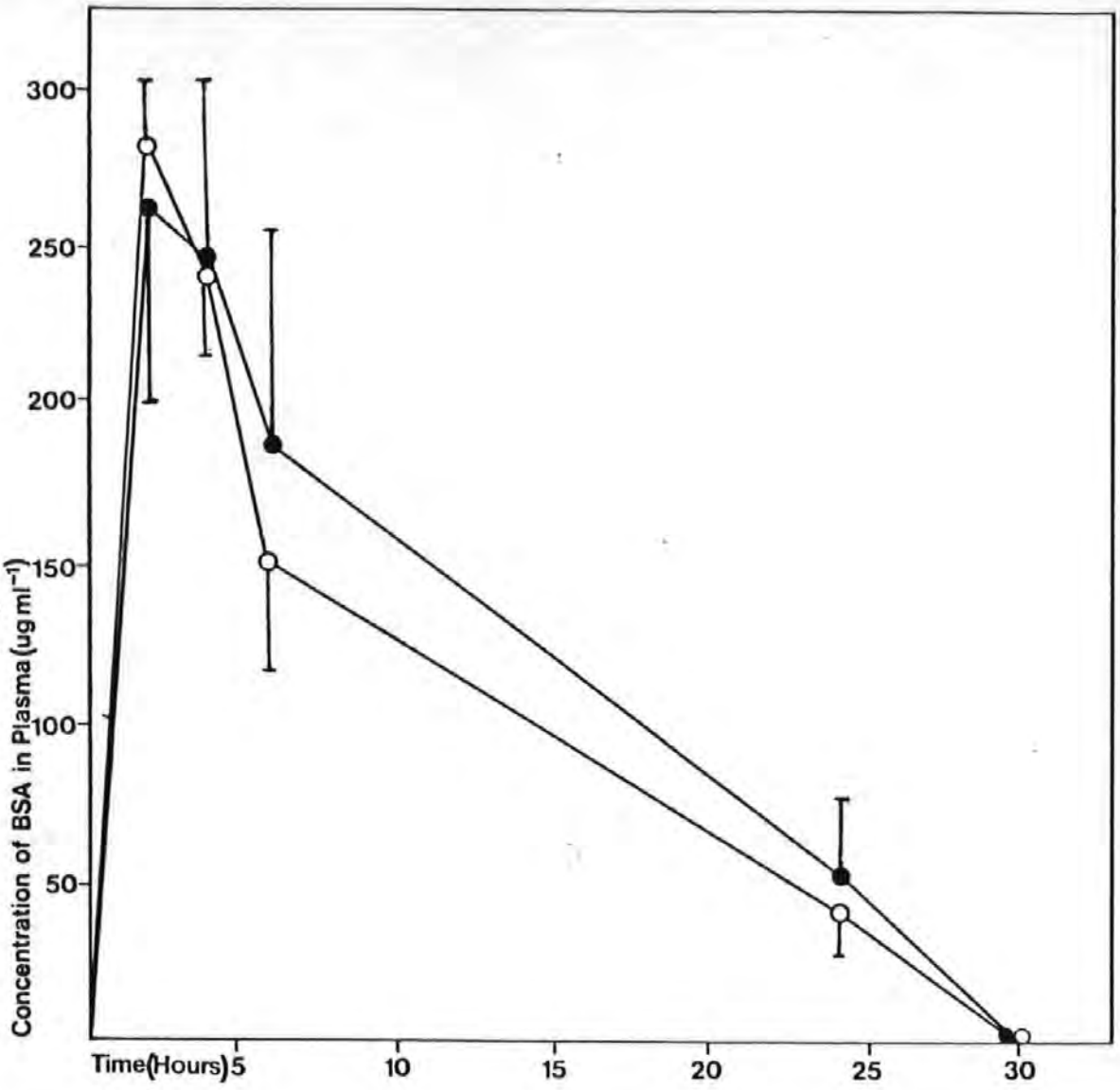


Fig. 3.4. The expression and clearance of intramuscularly injected BSA from the bloodstream of adult dogfish. Each point represents the mean of 4 fish. Open circles represent primed fish, solid circles represent fish that had not been previously immunised. Vertical bars represent standard error.

Experiment 1.3 Tissue Localisation of Lysozyme

Sera and sonicated extracts of dogfish tissues were examined for the presence of lysozyme according to their ability to lyse Micrococcus lysodeiktus as described. Owing to the relative insensitivity of the test and the low number of fish examined (4) a scale of + - +++ was adopted to show relative instead of absolute quantities of the enzyme. The distribution found is shown in Table 3.1.

Source	Lysozyme Concentration
Spleen	+
Epigonal tissue	++
Peripheral blood leucocytes	Not detectable
Liver	Not detectable
Serum	+++

Table 3.1. Localisation of lysozyme in dogfish tissues.

Experiment 1.4 The Presence of C-Reactive Protein in Dogfish Serum

Qualitative CRP tests were made using sonicated extracts of bacteria, fungi and nematode parasites. Sera from 10 normal fish were examined. Additionally, sera from 5 fish stressed with an immunosuppressive regime of 200 mg of cyclophosphamide (Koch-Light Laboratories Ltd.) injected intravenously twice weekly for 2 weeks, were tested. All tests proved negative after 14 days.

Discussion

This series of experiments confirms that the dogfish, like other elasmobranchs, is capable of an immune response to specific antigenic challenge. Clem & Sigel (1963) made the first report of antibody production in a marine fish. They found that lemon sharks would produce high titres of neutralising antibody when injected with the PR8 strain of influenza virus. A subsequent series of investigations by this group firmly established that elasmobranchs, principally the nurse shark and the lemon shark were capable of mounting a primary immune response to a variety of antigens including both live and formalised PR8 virus, equine influenza virus, chicken erythrocytes and BSA (Sigel & Clem, 1966). Experiment 1.1 clearly demonstrates the capability of the dogfish to produce high titres of agglutinating antibody. A mean titre of 1:7$\frac{1}{2}$6 was recorded for anti-*Salmonella* activity and an higher titre (1:4096) for anti-KLH was measured in one fish in a group of 5. These results bear resemblance to those obtained by Sigel & Clem (1966) in that the agglutinin titre increased (relatively slowly in comparison with mammals) following multiple injections of antigen. Similarly, antibody levels were either still increasing or at least showing no significant decrease at the time the experiment was terminated (63 days after initial challenge). Direct comparison between data is not possible owing to differences in immunisation regimes, antibody detection methods and temperature. The response elicited differed between antigen and also between fish. The anti-*Salmonella* response showed relatively little variation within the group of fish immunised with this antigen, unlike the groups immunised with KLH and BSA. Both of these antigens produced a variable response in the challenged fish: some responded with very high titres of antibody, some only slightly and others not at all. These differences could be accounted for in a number of ways:

i) The difference between latex fixation titres of KLH and BSA injected fish probably reflects the difference in immunogenicity between these antigens. KLH is a very high molecular weight protein (approximately 3,000,000 daltons) and is a powerful immunogen. BSA has a much lower molecular weight (approximately 67,000 daltons) and appears not to elicit such a vigorous antibody response in mammals (Mitchison, 1971). Sigel & Clem (1966) challenged lemon sharks with 3 x 25 mg of BSA and noted a similar heterogeneity in the response with differences in agglutinin titres as high as 16 fold between individual fish. The highest haemagglutination titre recorded in an individual fish was 1:2560, much higher than the titres recorded for the dogfish (the highest latex fixation titre was 1:64) however this may be because latex fixation is a less sensitive technique than the passive haemagglutination method (Varela-Diaz, Coltorti, Prezioso, Lopez-Lemes, Guisantes & Yarzabal, 1975).

Variable reports of anti-BSA in teleosts exist. Sailendri & Muthukkaruppan (1975a) measured haemagglutination titres of 1:10240 and 1:81920 in Tilapia after primary and secondary challenge injections. Harris (1973a) could obtain a haemagglutination titre of 1:256 in dace repeatedly challenged with BSA and it seems clear that there is much variation in the ability of fish, even those within the same group, to mount a response to the same antigen although the extent to which this can be attributed to differences in experimental conditions is very hard to assess.

Little information regarding the humoral response of fish to KLH exists. Finstad & Good (1966) recorded a haemagglutination titre of 1:1600 in horned shark 21 days after immunisation with haemocyanin. Harris (1973) demonstrated precipitating antibody to KLH in the dace but did not measure the agglutinin titre, however, the

precipitin titres were higher than those of the other antigens (BSA and horse serum). Ingram & Alexander (1976) measured complement-fixing antibodies to KLH in the brown trout after a single or multiple injection challenge and recorded very high levels of antibody. Therefore, it would appear that KLH is highly immunogenic to fish and able to induce a very vigorous response, whereas BSA does not seem to have the same effect.

ii) The apparently poor responses of some fish to these antigens may be due to them individually being poor immunologic responders. McDevitt & Benacerraf (1969) reported that various strains of mice responded very differently to an identical antigenic challenge and it is now generally believed that the immune response to thymus(T)-dependent antigens (that require the co-operation of thymus derived cells in order to provide immunity) is under genetic control by the immune response or Ir-genes. It is possible that, in the absence of syngeneic strains, high and low responder fish comprised the outbred population examined in this study, thus accounting for the heterogeneity of response to antigenic challenge. Both BSA and KLH are T-dependent antigens in mammalian terms but cannot be defined as such in fish until the role of the thymus in these animals is more clearly defined. Nevertheless, the possibility of high and low responders in such a population seems likely. Salmonella is considered to be a T - independent antigen (only requires B-cells to elicit an immune response) and the apparent similarities in agglutinating titres could be explained as such: Ir genes do not control the response to T-independent antigens and the marked heterogeneity, as seen with BSA and KLH, does not occur.

iii) The phenomenon of tolerance may also account for the variation in response of individual fish to the same antigen. Relatively large quantities of antigen were used to sensitise the fish (2 x 10 mg and 2 x 25 mg injections for KLH and BSA respectively) and it is possible that such doses may have made the fish unresponsive by inducing "high-zone" tolerance described by Mitchison (1968). It must be emphasised that these explanations are of a speculatory nature: too little data regarding fish immune responses exists and there is a danger of indiscriminate borrowing of terms such as "Ir-gene" and "tolerance" from contemporary mammalian immunology. Until more information is available, particularly regarding responses of inbred lines, it will not be possible to relate such phenomena to lower vertebrates.

An immune response to horse serum could not be detected, however, only techniques to demonstrate precipitating antibody were used. The failure of double diffusion, SRID and crossed-over electrophoresis to detect precipitins in all animals examined would appear to indicate the absence of such antibody. Precipitins could not be detected in sera that had high agglutinating titres of anti-Salmonella, KLH and BSA antibody. To date only one report of such antibody in an elasmobranch has been made (Suran, et al., 1967). These workers demonstrated antibodies to KLH in challenged leopard sharks using a microprecipitation method and ^{125}I-labelled antigen. This method is considerably more sensitive than the gel precipitation methods used in this study and it is probable that low quantities of precipitating antibody could be detected by this technique. Numerous reports of precipitins in teleost fish exist including Hodgins et al. (1967), Harris (1970, 1972), Harris & Cottrell (1976) and Ingram & Alexander (1976). No reason can be offered as to why teleosts should be more capable of producing precipitating antibody than elasmobranchs when they both

apparently have the same "IgM-like" antibody class (see Chapter 4).

The rate of appearance of antibody in challenged fish was relatively slow in comparison to other investigations. Serum antibody was not detectable for about 14-28 days after the initial immunisation. It is probable that this is due to the temperature at which the fish were maintained. Several workers have clearly established that the humoral immune response in fish is temperature dependent (Cushing, 1942; Bisset, 1946, 1947, 1948; Bradshaw & Sigel, 1968; Avtalion, 1969; Harris, 1973a). The dogfish used in these studies were generally maintained at a water temperature between 10°C and 14°C which is significantly less than those of other studies on elasmobranchs: most reports from American groups describe environmental temperatures of 22°C to 30°C and this appears to reflect the vigorous humoral responses seen in their fish (Sigel & Clem, 1966; Suran et al., 1967; Small, Klapper & Clem, 1970; Johnston et al., 1971). In fact the apparently weak response of the dogfish to BSA may be partly due to a temperature dependent immune system. The vigorous responses to KLH and Salmonella were achieved with water temperatures around 12°C - 16°C. The group challenged with BSA were maintained at 8°C - 10°C and these lower environmental temperatures may have decreased the rate of antibody production.

Experiment 1.2 was an attempt to test memory capability by comparing the ability of fish primed with BSA and an unprimed group to clear a challenge of the antigen from their blood. The clearance curves were virtually identical for both groups of fish. This result possibly indicates that only a weak immunologic memory exists in these fish. Biozzi, Benacerraf, Halpern, Stiffel & Hillemond (1958) demonstrated that clearance of albumin from the blood of man was principally effected by the

phagocytic cells of reticulo-endothelial (RE) system. Classic antigen clearance occurs by RE cells trapping antigen-antibody complexes and then processing this material. In this experiment it was expected that the primed animals with circulating antibody would quickly complex with antigen which would facilitate more rapid processing than the unprimed control group. The fact that there was little difference between the two groups could be for several reasons.

i) There was insufficient antibody in the circulation of the primed group, either because the initial levels had declined or too little was produced (although these fish were used in Experiment 1.1 and seven days before this experiment had demonstrable, though somewhat low, levels of anti-BSA antibody) and therefore did not allow enhanced processing by the RE system.

ii) The large dose (50 mg) of BSA injected into the fish swamped the relatively low levels of specific antibody in the serum and therefore did not allow significant enhancement of antigen clearance by the RE system.

iii) The BSA was cleared by an alternative antibody-independent antigen-trapping mechanism such as those used in the removal of carbon and gold colloids (Rabinovitch, 1970).

Nelstrop, Taylor & Collard (1968a) showed that clearance of a secondary dose of T_1 bacteriophage from the circulation of goldfish, lamprey and shore crab was more rapid than the clearance of a primary dose of phage. This phenomenon occurred in the absence of any detectable humoral antibody in a manner similar to that described for rabbits (Nelstrop *et al.*, 1968b). These workers could not, however, demonstrate a similar effect in the dogfish and concluded

that some sort of immunological blockade or paralysis occurred in these animals. Sigel, Acton, Evans, Russell, Wells, Painter & Lucas (1968) demonstrated that unimmunised lemon sharks would clear T_2 bacteriophage from their circulation within 4 days whereas immunised fish with detectable levels of neutralising antibody cleared the virus almost immediately after injection. Sigel & Clem (1966) reported that the lemon shark produced no significant secondary humoral antibody response (defined as a more rapid and higher magnitude of antibody production) to further challenges of myxoviruses, chicken erythrocytes or BSA. Finstad & Good (1966) demonstrated rapid clearance of ^{131}I-labelled BSA from the circulation of the horned shark in the absence of serum antibody.

From these reports it would appear that elasmobranchs have a limited immunologic memory in terms of an enhanced capacity for antigen clearance and increased secondary antibody production. Nevertheless, the fish are still capable of efficiently clearing antigen from their blood even in the apparent absence of circulating antibody. No comment can be made on the role of serum antibody in relation to antigen clearance in the dogfish as this was not measured during the experiment.

The presence of lysozyme in the serum, spleen and epigonal tissue is very similar to the findings of Fänge et al. (1976) who found relatively high levels of the enzyme in the oesophageal lymphomyeloid tissue of the elasmobranchs the velvet belly (Etmopterus spinax) and the starry ray (Raja radiata). Slightly lower levels of lysozyme were found in the spleens of both these fish. Surprisingly, the enzyme was not detectable in the plasma of the ray; the plasma of the velvet belly was not examined. In fact very few fish these authors studied had lysozyme activity in their plasma including the following teleosts:

the haddock (Melanogrammus aeglefinus), the cod (Gadhus morhua) and the pollack (Pollachius pollachius). Fletcher & White (1973) demonstrated lysozyme in the serum of plaice but could not show its presence in cod sera.

The findings of Experiment 1.4 demonstrate that lysozyme exists in the tissues of the dogfish but because of the lack of general background information regarding the function, presence and distribution of this enzyme in lower vertebrates, very few conclusions can be made, although its presence in epidermal mucus (Fletcher & White, 1973) has led to suggest that this proteolytic enzyme might be important in the innate immunity of fish. Non-specific defence mechanisms may compensate for the relative inefficiency of humoral reactions in fish i.e. temperature dependency, relatively slow response and restricted antibody class and thus it can be suggested that the immune response has evolved to deal primarily with intrinsic factors.

In experiment 1.4 C-reactive protein was not detectable in the sera of normal and cyclophosphamide stressed fish despite using techniques similar to those described by Baldo & Fletcher (1973) who found this substance in plaice sera and little further comment can be made. The exact role of this acute-phase protein is ill-defined but is found in sera of humans suffering from infections, inflammatory conditons and tissue damage and thus is thought to act as a mediator of non-specific immunity (Raffel, 1961). The selective pressure for the existence of this protein in the blood of either lower or higher vertebrates is unknown.

CHAPTER 4

ISOLATION AND CHARACTERISATION OF DOGFISH ANTIBODY

I Comparative Serum Studies

Before isolating and characterising dogfish antibody a general comparison of its serum components with human serum was made using several electrophoretic techniques.

Experiment 2.1 Cellulose Acetate Electrophoresis

Human and dogfish sera were separated by CAE and the components were quantified in a densitometer. The profiles obtained were compared (Fig. 4.1). The CAE technique was found to give relatively poor resolution and only provided an approximate idea of the nature of dogfish serum components: there appeared to be much less albumin in the fish serum and most proteins did not migrate as far as human material, either to the anode or cathode. A larger proportion of dogfish serum had cathodic mobility than the human serum.

Experiment 2.2 Polyacrylamide Gel Electrophoresis

Human and dogfish sera were separated by PAGE (Fig. 4.2). Approximately 5 bands could be resolved for dogfish serum, relatively few in comparison with the 10-12 resolved from human serum. Albumin, the serum component with the fastest anodic electrophoretic mobility was very obvious in the human samples but as with the CAE, a comparable band could not be detected in the dogfish.

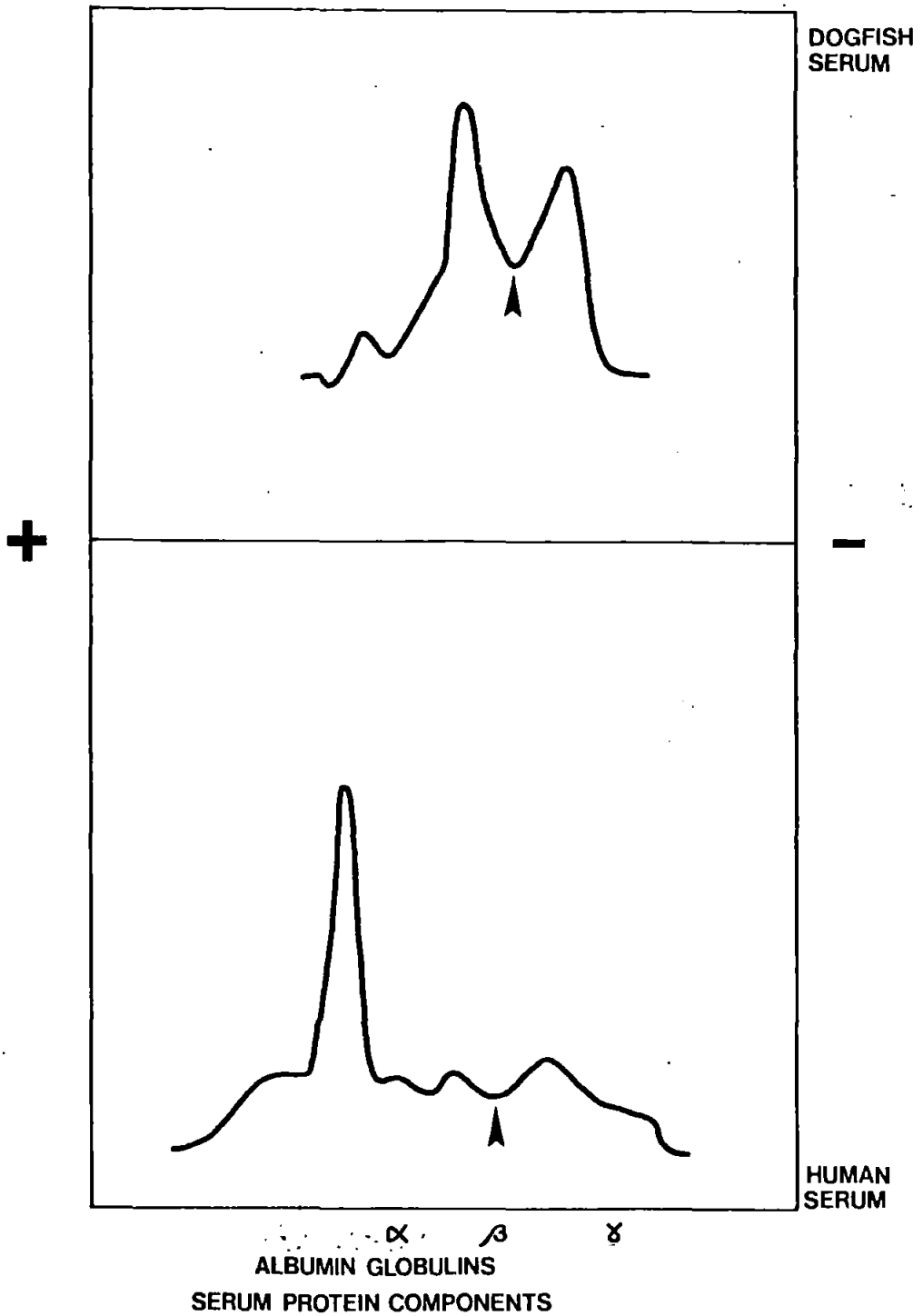


Fig. 4.1. Showing densitometer profiles of dogfish and human serum proteins after separation by cellulose acetate electrophoresis. Arrows represent the points of sample application. The positions of the major electrophoretic fractions are also marked.

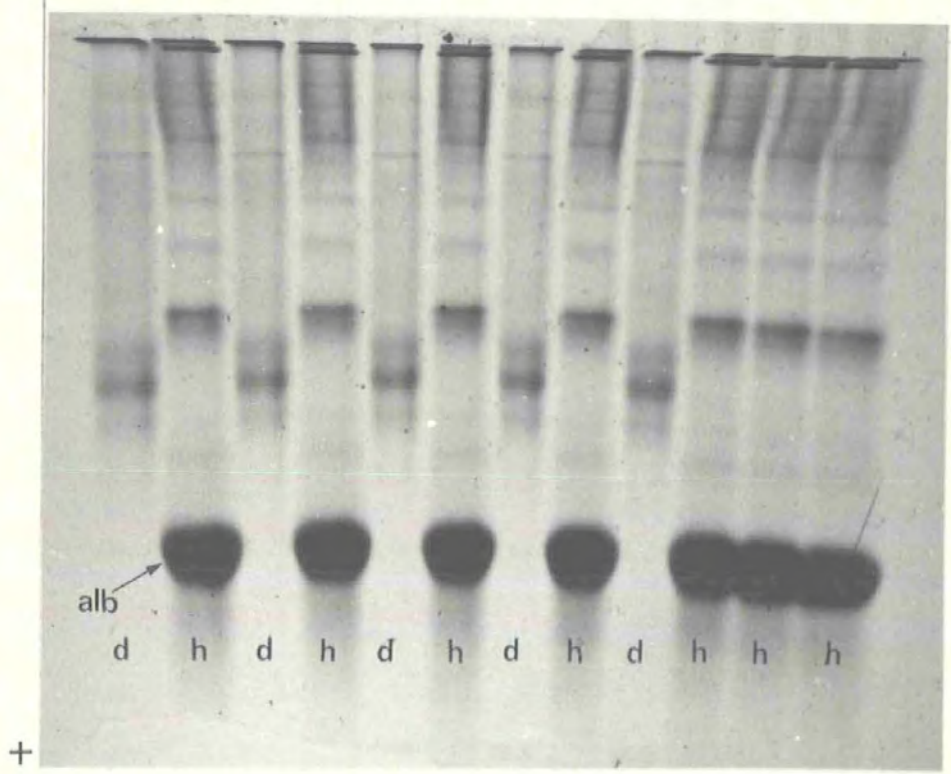


Fig. 4.2. Comparative polyacrylamide gel electrophoresis of dogfish (d) and human (h) serum. Dogfish serum lacks the very prominent albumin (alb) component of human serum.

Experiment 2.3 SDS-Polyacrylamide Gel Electrophoresis

This procedure was similar to Exp. 2.2 except that the samples were electrophoresed in the presence of sodium dodecyl sulphate. For comparison of relative molecular weights, protein markers were run alongside the fish serum. These were:

BSA	MW	=	67,000
Ovalbumin	MW	=	45,000
Chymotrypsinogen	MW	=	25,000
Lysozyme	MW	=	14,000

The results of this experiment can be seen in Fig. 4.3. SDS treatment gives proteins a constant charge-to-mass ratio and migration is due to the size of the protein molecules alone (separation being effected by the pore size of the gel). SDS-PAGE apparently gives a better resolution than the standard PAGE method (which separates molecules according to their size and charge). Approximately 17 bands were detected by SDS-PAGE.

Experiment 2.4 Immunoelectrophoresis

An immunoelectrophoretic analysis was made (Fig. 4.4). Again the dogfish serum appeared to have relatively few serum components. No precipitin lines formed in the region corresponding to human albumin. Approximately four arcs were anodic: one migrating as an α_1-globulin, and 3 as α_2-globulins. Another four components had β mobility and one precipitin arc was formed in the γ-globulin region. In contrast, human serum had a marked albumin content, approximately 10 components in both the α_1 and α_2 globulin regions and one β and one γ-globulin arcs.

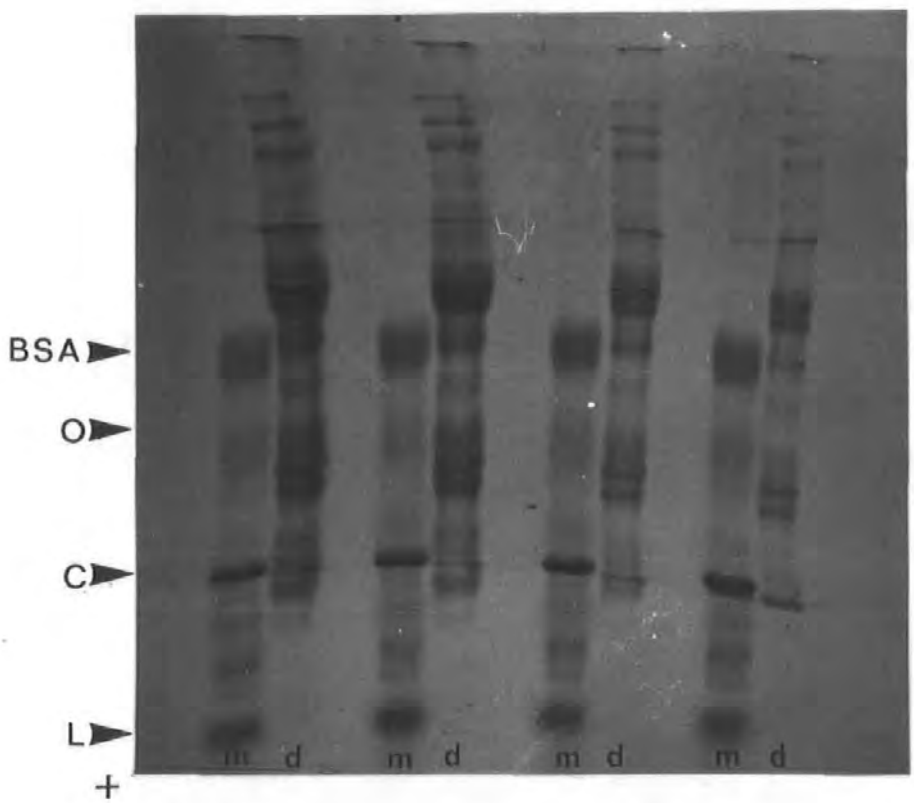


Fig. 4.3. SDS-polyacrylamide gel electrophoresis of dogfish serum (d) and protein markers (m). The markers are:

Bovine serum albumin (BSA)	MW = 67,000
Ovalbumin (O)	MW = 45,000
Chymotrypsinogen (C)	MW = 25,000
Lysozyme (L)	MW = 14,000

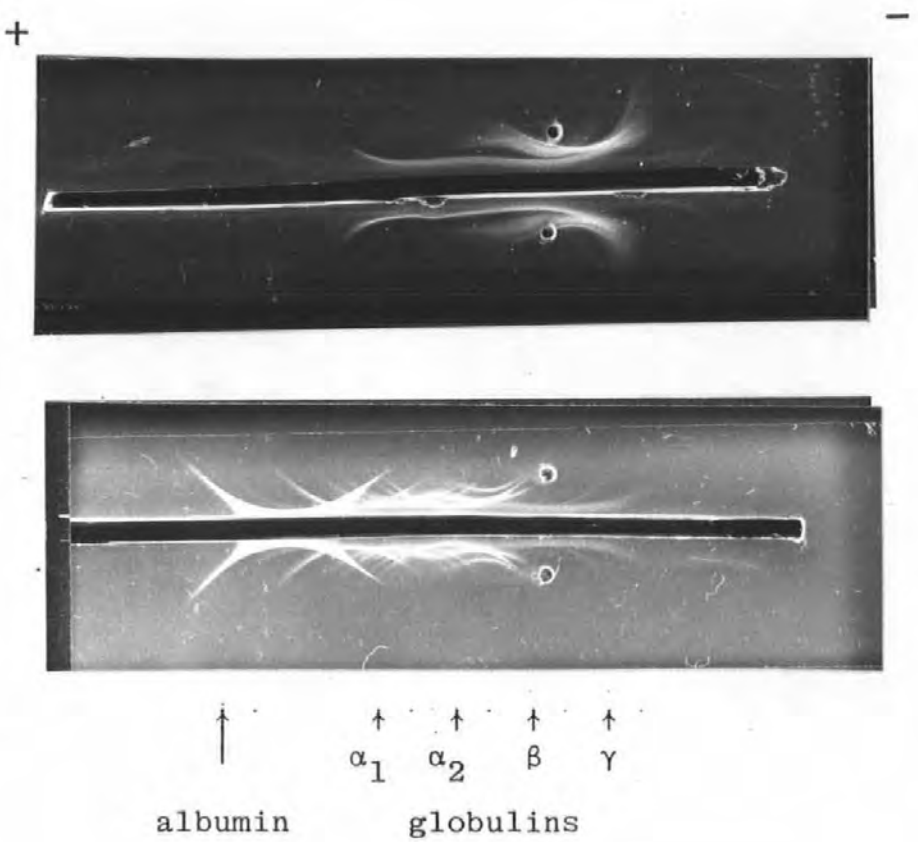


Fig. 4.4. Agarose gel immunoeletrophoresis of dogfish serum (upper slide) and human serum (lower slide). Appropriate rabbit antiserum was applied to the central trough in both instances. The positions of the major electrophoretic fractions are shown.

II Isolation of Dogfish Immunoglobulin

Experiment 2.5 Separation of Serum on Sephadex G-200

A column of Sephadex G-200, 1.6 cm x 85 cm was prepared and 2 ml of dogfish anti-KLH serum were applied to it. The various fractions were concentrated 4x in Minicon-S125 concentrators (Amicon Ltd.) and tested for latex agglutinating antibody. The fractions containing agglutinin activity were totally excluded from the gel (Fig. 4.5): they were found to be contained in the same effluent volume as blue dextran, indicating a molecular weight greater than 800,000. The elution profile of dogfish serum and the relative position of anti-KLH antibody is shown in Fig. 4.5. Immunoelectrophoresis of the concentrated samples was carried out in order to analyse the serum components of the various fractions. As can be seen from Fig. 4.6 the principal antibody containing fraction (fraction 24) comprised approximately five different precipitin arcs. Therefore serum components other than immunoglobulin were also totally excluded from Sephadex G-200. As the presence of these other proteins would make further purification of the immunoglobulin (by an electrophoretic method) more difficult, it was decided to use another gel with a higher fractionation range. Gel filtration using Sephadex G-200 alone was used to partially purify an antibody preparation for ultra-centrifugal analysis.

Experiment 2.6 Separation of Serum on Sepharose 6B

Dogfish anti-KLH antiserum was accordingly separated on Sepharose 6B and on this occasion the immunoglobulin-containing fraction was not eluted with blue dextran and therefore not totally excluded from the gel. Fraction 21 was found to contain the highest anti-KLH activity (Fig. 4.7). Immunoelectrophoresis of this fraction revealed only 2 major components (Fig. 4.8) one with little electrophoretic

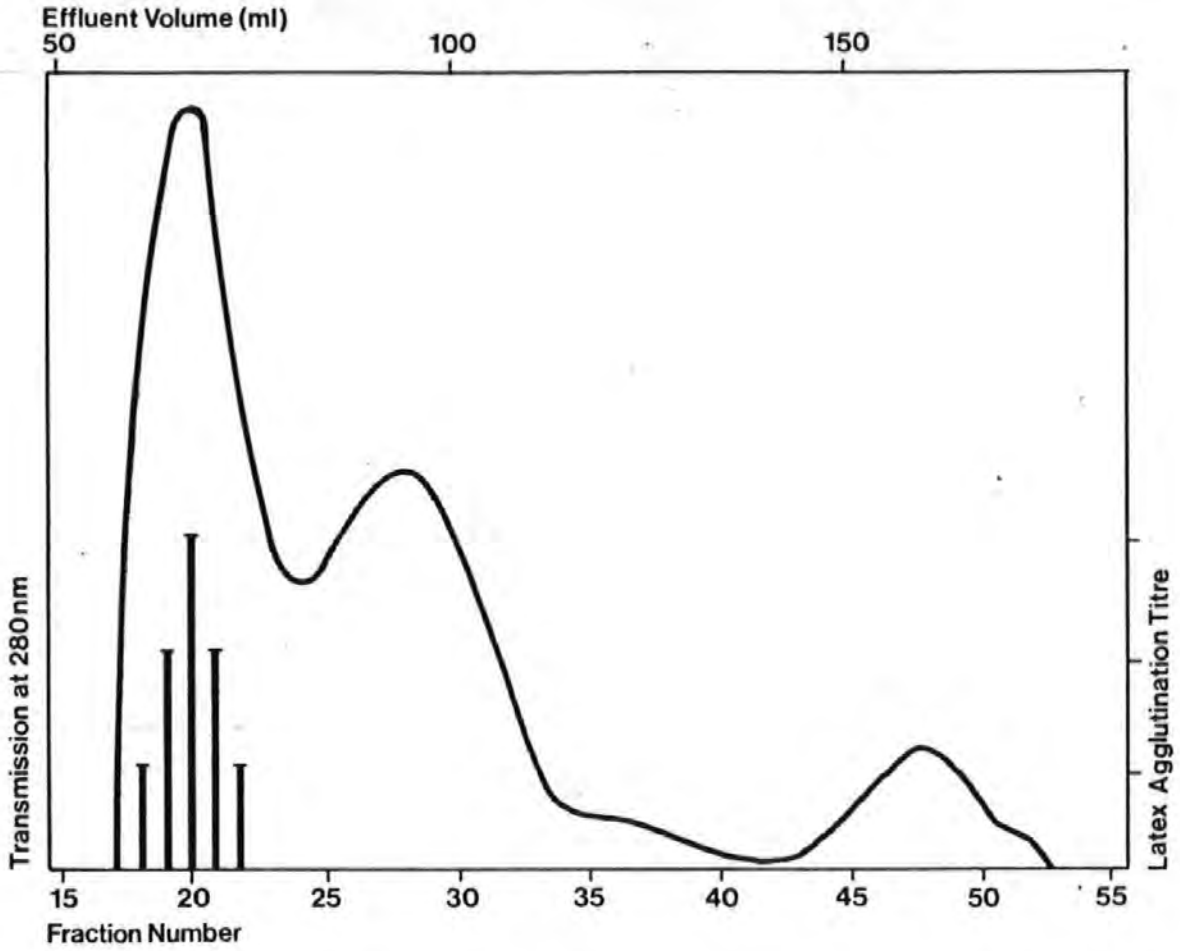


Fig. 4.5. Elution profile of dogfish anti-KLH serum separated on Sephadex G-200. Fractions with antibody activity are marked with vertical bars.

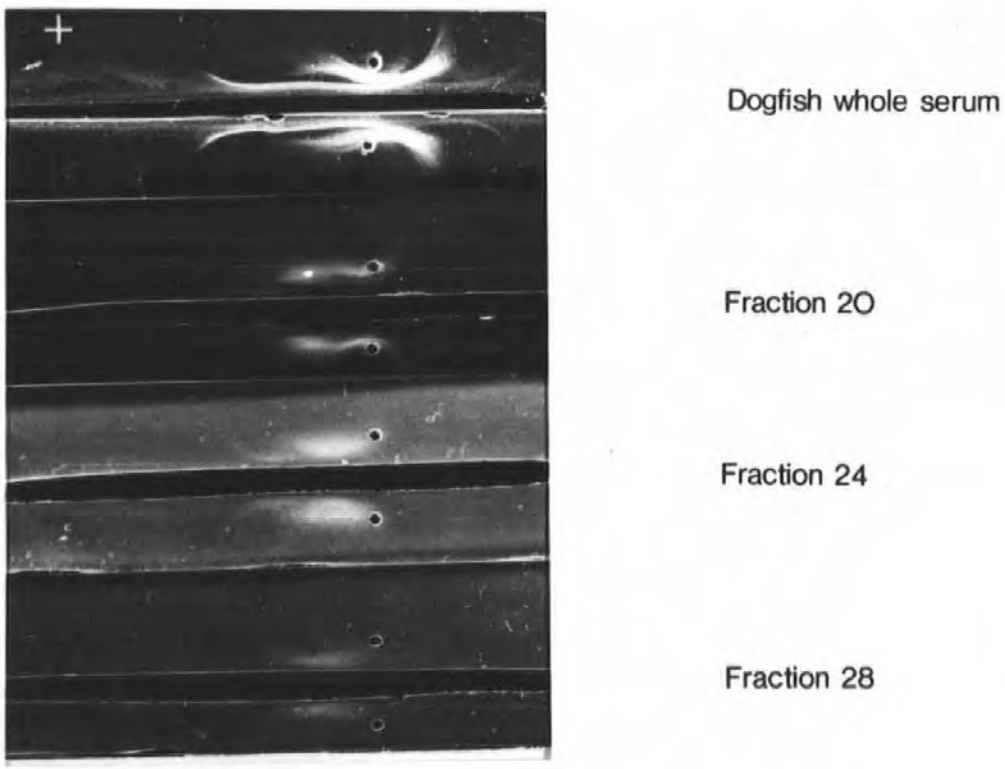


Fig. 4.6. Immunoelectrophoretic patterns of dogfish anti-KLH serum fractionated on Sephadex G-200. Precipitin arcs were not visible beyond Fraction 28.

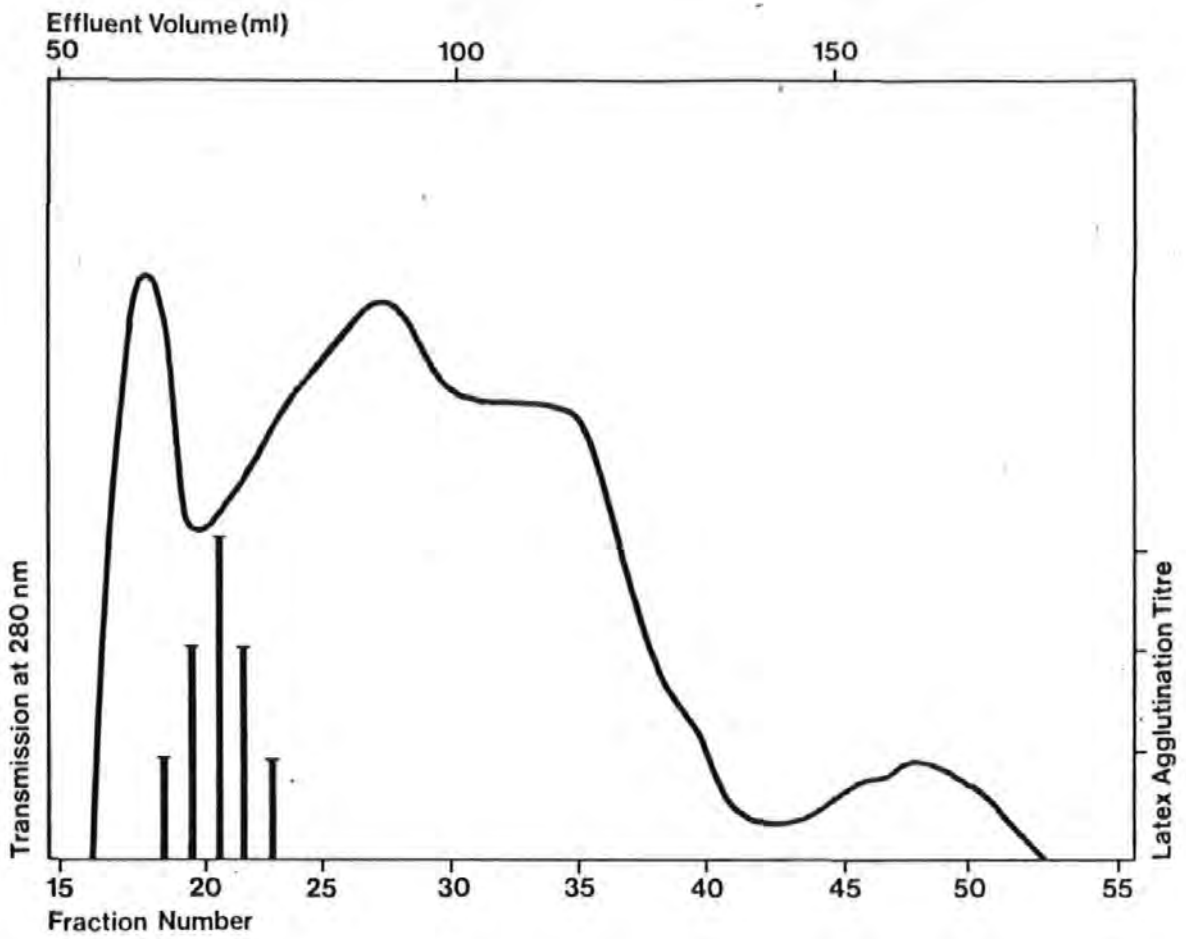


Fig. 4.7. Elution profile of dogfish anti-KLH serum separated on Sepharose 6B. Fractions with antibody activity are marked with vertical bars.

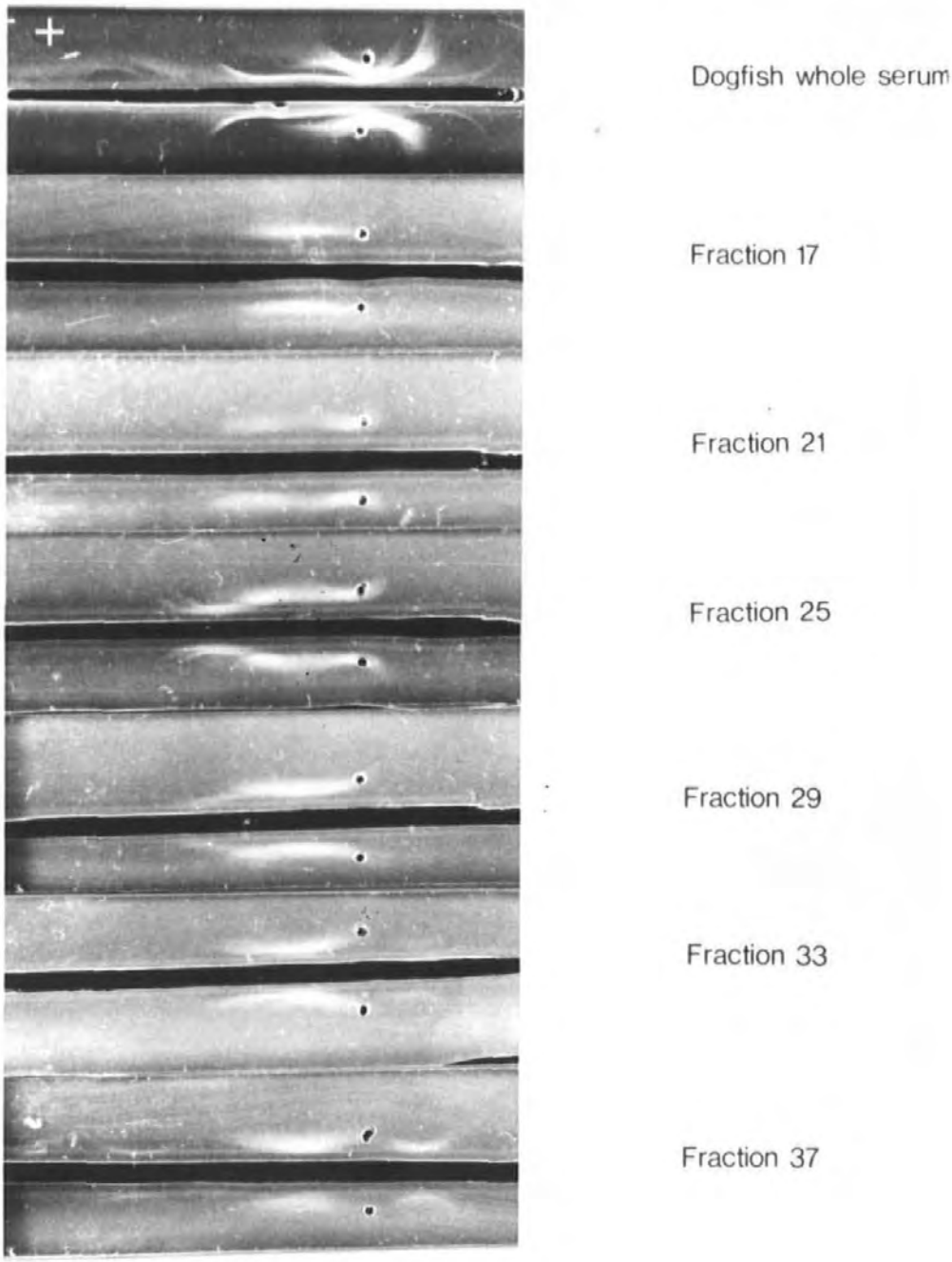


Fig. 4.8. Immunoelectrophoretic patterns of dogfish anti-KLH serum fractionated on Sepharose 6B.

mobility, the other with relatively fast anodic migration. It was decided that further separation of this fraction was feasible and a large volume of the material was prepared using a 2.5 cm x 85 cm column of Sepharose 6B.

Experiment 2.7 Preparative Agarose Gel Electrophoresis of Fractionated Dogfish Serum

Pooled antibody fraction from Exp. 2.6 was concentrated approximately 10x by dialysis in 33% (w/v) Aquacide III (Calbiochem Ltd.) and then further separated by agarose block electrophoresis. The two protein components were eluted separately from the block, further concentrated (approximately 4x) in Aquacide III and checked for antibody activity. Only the slower migrating component contained anti-KLH agglutinins. Immunoelectrophoresis of this purified protein confirmed its slight anodic electrophoretic mobility (Fig. 4.9b). This purified antibody was then used to immunise a rabbit in order to produce an anti-dogfish immunoglobulin.

III Characterisation of the Immunoglobulin Molecule

Experiment 2.8 Electrophoretic Mobility of Dogfish Immunoglobulin

Using the antiserum prepared to the purified antibody (Exp. 2.7) the electrophoretic mobility of the dogfish immunoglobulin was determined. After IEP of whole fish serum only a single precipitin arc was formed in the β globulin region which extended into the α region (Fig. 4.9c).

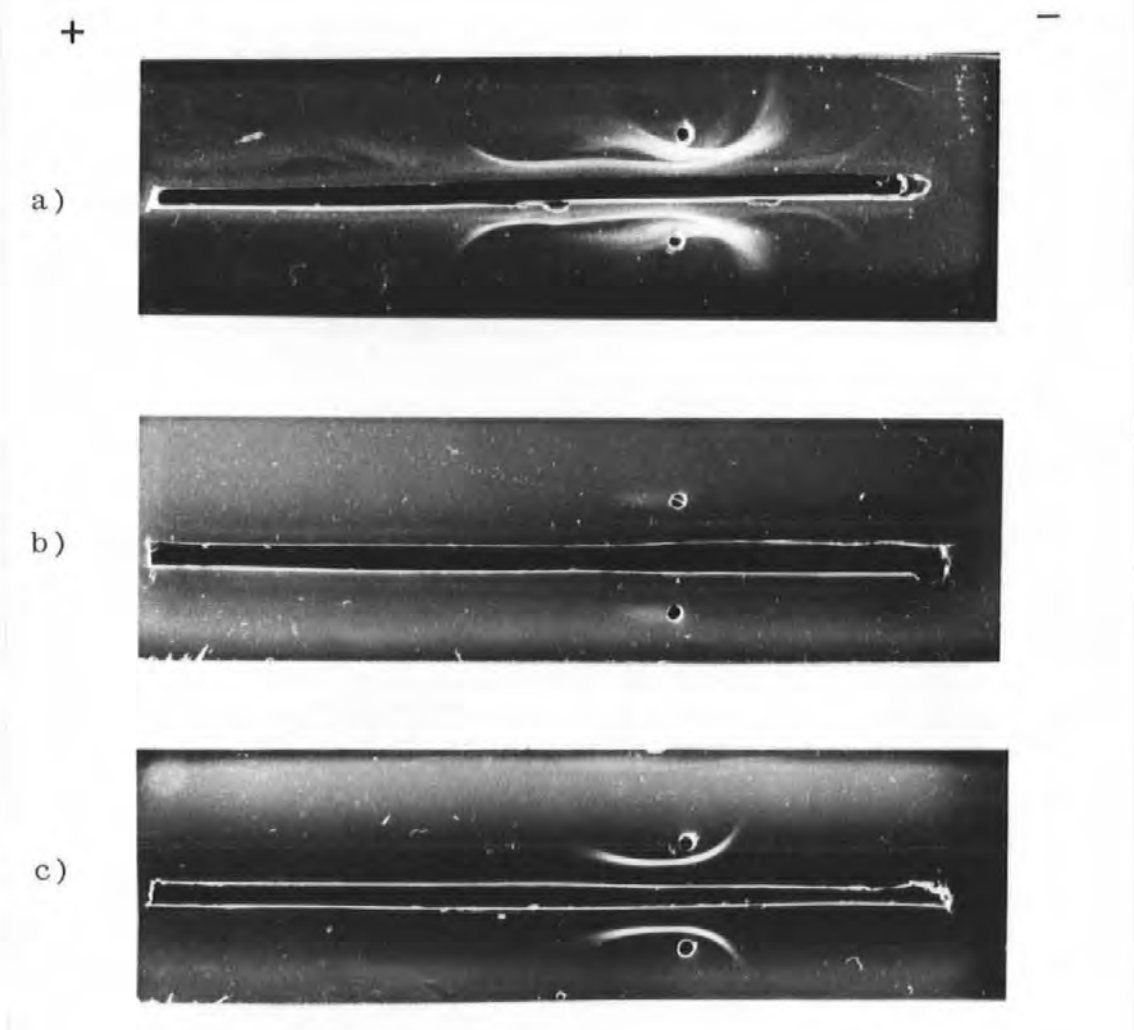


Fig. 4.9. Immunoelectrophoresis patterns of dogfish whole serum (a) and the antibody containing fraction after purification on Sepharose 6B and agarose slab electrophoresis (b).

Rabbit anti-whole dogfish serum was applied to the central trough in both instances.

Rabbit antiserum was prepared to the purified fraction and its specificity against dogfish whole serum was examined (c).

Experiment 2.9 Inactivation of Antibody Activity

This experiment was carried out using whole dogfish serum.

i) Reduction with 2-mercaptoethanol (2-ME)

Samples of anti-Salmonella and anti-KLH sera with strong agglutinating activity for their respective antigens were dialysed against 0.2M 2-ME for 12 hours followed by 24 hours dialysis against several changes of elasmobranch saline. After this treatment all sera tested failed to agglutinate KLH-coated latex or Salmonella cells.

ii) Mild Reduction with Dithiothreitol (DTT) and Alkylation with Iodoacetic Acid (IAA)

Anti-Salmonella and anti-KLH antisera were reduced and alkylated by a method modified after that described by Miller & Metzger (1965). Briefly, samples were dialysed against 10 mM DTT (made up in 0.5 M Tris and 0.2 M NaCl buffer, at pH 8.0) for 1 hour in the dark. The samples were then alkylated to prevent re-association of the reduced monomers to a polymeric form. The reduced samples were alkylated with 2x re-crystallised 120 mM IAA and allowed to stand at room temperature, in the dark for 1 hour. This was followed by an addition of another volume of IAA. Finally the samples were dialysed for 24 hours against several changes of elasmobranch saline. Again, this treatment abolished all agglutinin activity of previously high titre antisera.

Experiment 2.10 Determination of the Sedimentation Coefficient of Dogfish Immunoglobulin

Dogfish immunoglobulin, partially purified on Sephadex G-200 (Exp. 2.5) was desalted by dialysis against several changes of distilled water and then freeze-dried. The material was re-constituted at a concentration of 15 mg ml^{-1} in PBS and analysed by ultracentrifugation. Only one major peak was seen using Schlieren optics and this was presumed to be the immunoglobulin (Fig. 4.10). Its sedimentation coefficient was calculated to be 18.6S. This value was not corrected for concentration.

Experiment 2.11 Molecular Weight Determination and Transformation of Dogfish Immunoglobulin

The molecular weight of dogfish immunoglobulin was estimated by measuring its elution volume from a column of Sepharose 6B and relating this to a calibration curve of elution volumes of proteins with a range of molecular weights.

A group of 10 dogfish were given similar intra-muscular injections of KLH (10 mg ml^{-1}) in FCA. Initially 2 injections 7 days apart were given followed by an identical injection at 3 monthly intervals for up to 18 months. Blood samples (2 ml) were taken from each fish prior to initial immunisation and then one week after booster injections. Sera from these samples were pooled. The pooled sera were fractionated on Sepharose 6B and the fractions checked for anti-KLH agglutinating activity. One month after injection, anti-KLH antibody was demonstrated in the sera and could be detected for the following 18 months. The anti-KLH activity was only found to be associated with first protein peak (fraction 20) eluted from Sephadex G-200 and fraction 21 associated with

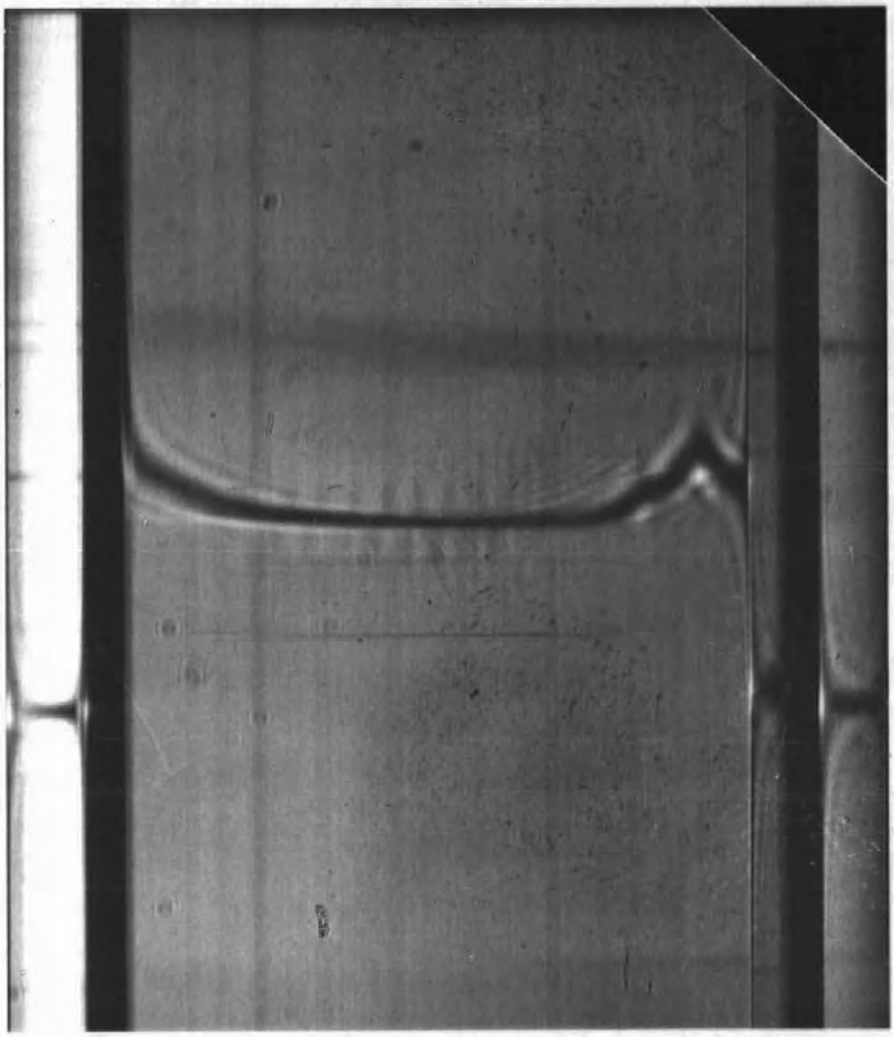


Fig. 4.10 Schlieren peak of dogfish immune macroglobulin obtained by analytical ultracentrifugation at 52,400 r.p.m.

FRACTION NO.	15	16	17	18	19	20	21	22	23	24
ELUTION VOLUME (ml)	51.0	54.4	57.8	61.2	64.6	68.0	71.4	74.8	78.2	81.6
L.A. TITRE (+ - +++)	ND	ND	ND	ND	+	++	+++	++	+	ND
SRID (RING DIA, m.m.)	<3.0	<3.0	<3.0	<3.0	3.6	4.7	6.5	5.2	4.6	4.4
FRACTION NO.	25	26	27	28	29	30	31	32	33	34
ELUTION VOLUME (ml)	85.0	88.4	91.8	95.2	98.6	102.0	105.4	108.8	112.2	115.6
L.A. TITRE	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SRID	4.1	3.8	3.6	3.6	3.2	3.1	<3.0	3.3	4.9	4.7
FRACTION NO.	35	36	37	38	39	40	41	42	43	44
ELUTION VOLUME (ml)	119.0	122.4	125.8	129.2	132.6	136.0	139.4	142.8	146.2	149.6
L.A. TITRE	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SRID	4.6	4.4	4.2	4.1	3.8	3.6	3.3	3.2	3.1	<3.0

Table 4.1. Detection of antibody in dogfish anti-KLH serum separated on Sepharose 6B using latex agglutination and SRID techniques. The SRID results are also approximately representative for normal sera separated in the same way. Note that maximum agglutinin activity occurred in fraction 21 which also corresponded with a peak level of immunoglobulin as measured by SRID. Immunoglobulin was also detected, by SRID, at a relatively high concentration in fraction 33. ND: no detectable latex agglutination titre.

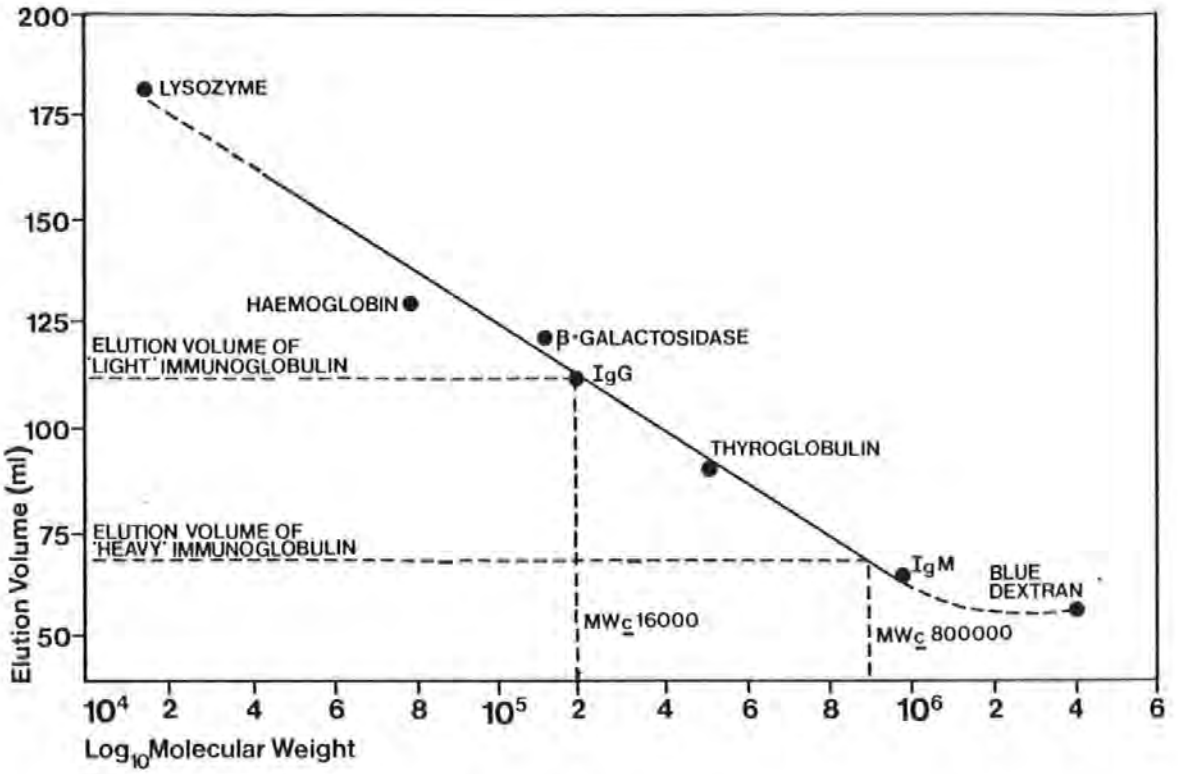


Fig. 4.11. Molecular weight determination of dogfish immunoglobulin on a column of Sepharose 6B, 1.6 cm x 85 cm, calibrated with proteins of known molecular weight. The presence of immunoglobulin in the effluent was detected using specific antiserum and SRID.

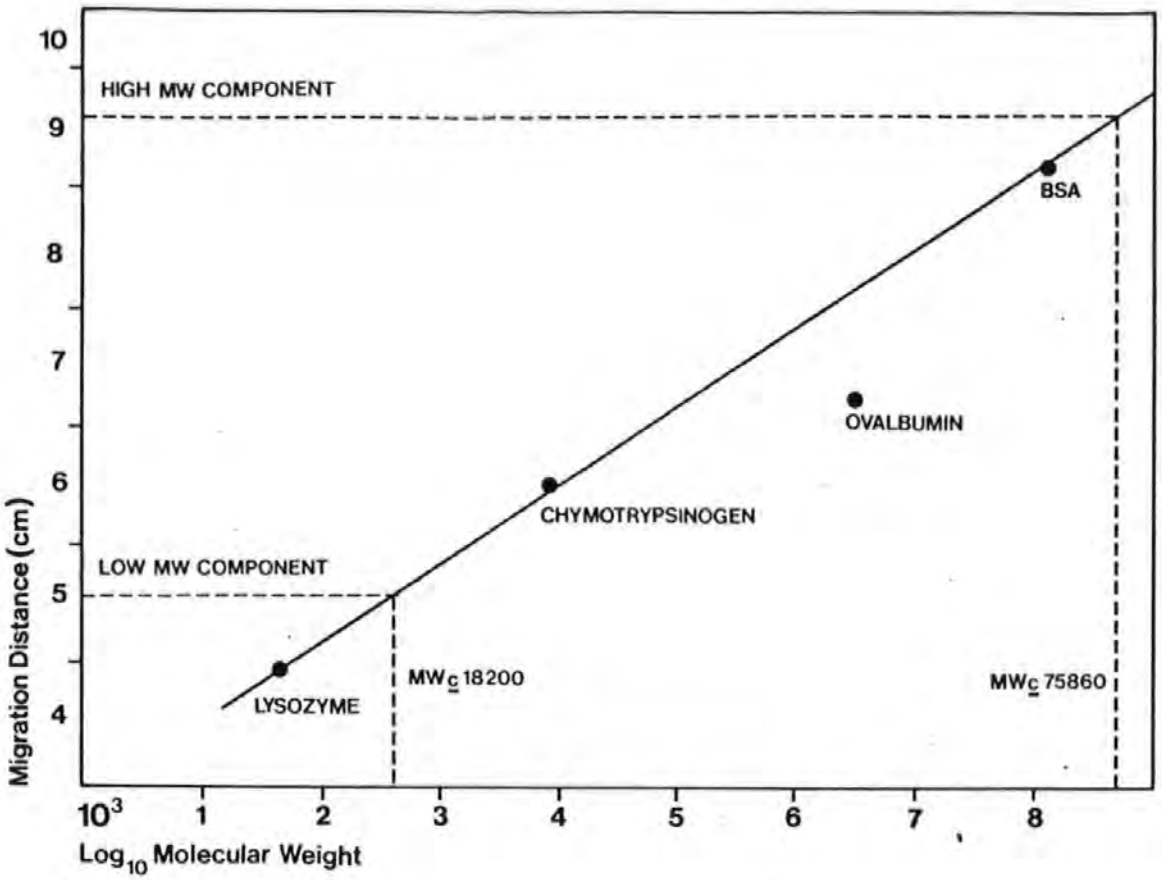


Fig. 4.12. Molecular weight analysis of reduced and alkylated dogfish immunoglobulin on a thin layer gel of Sephadex G-150 Superfine. Two components with molecular weights of approximately 18,200 and 75,860 were revealed.

Sepharose 6B.

Serum samples from normal and long term immunised fish were separated on Sepharose 6B and the fractions were tested by SRID using the anti-dogfish immunoglobulin serum prepared in Exp. 2.7. Precipitin rings were formed with serum fractions corresponding to a molecular weight of approximately 800,000 (Table 4.1 and Fig. 4.11). Surprisingly, precipitin arcs corresponding to a protein with a molecular weight of 160,000 were detected in both immunised and normal fish.

Experiment 2.11. Polypeptide Chain Structure of Dogfish Immunoglobulin

In order that a more positive characterisation of the antibody molecule could be made, an attempt was made to estimate the molecular weights of the polypeptide chains. Purified immunoglobulin was reduced with 2-ME and alkylated with IAA and then fractionated on Sephadex G-150 superfine using a TLG plate. Replica staining revealed that the molecule comprised two major components with molecular weights of approximately 18,200 and 75,860 respectively (Fig. 4.12). A third component was noted on the plate: it migrated across the plate at the same rate as blue dextran and thus was totally excluded from the gel. This component may have been a contaminant not revealed by IEP or a partially reduced polymeric form of the immunoglobulin.

Discussion

The comparative electrophoretic studies show that the dogfish has relatively few serum protein components in comparison with human serum.

The five bands detected in Exp. 2.2 shows that the dogfish has fewer serum proteins than the 10-12 detected in human serum. In fact this is also rather less than most teleost fish which have between 4-9 bands although protein bands analagous to mammalian albumin are present in all fish studied from this latter group (Harris 1971, 1974). The dogfish apparently has no albumin but marked γ-globulin components. Pollara, Finstad & Good (1966) studied a number of fish by paper and agar gel immunoelectrophoresis and related these findings to sera from invertebrates, normal and agammaglobulinaemic humans. The elasmobranch fish(es) examined proved to have fewer components than the teleost and human sera and lacked the fast anode-migrating albumin band present in both humans and the higher fish. They also noted that all lower vertebrates capable of expressing a specific antibody response had serum γ-globulin components. Some initial reports concerning the electrophoretic mobility of fish serum proteins were of a conflicting nature in that some species were thought to be agammaglobulinaemic. Clem & Sigel (1963) examined the serum of elasmobranchs and marine teleosts and noted that only the former species produced γ-globulin zones on gel electrophoresis. Post (1966) could not detect γ-globulins in the serum of rainbow trout. It is now well established, however, that immunoglobulins from various animal species do not necessarily have cathodic mobility, for example, mouse IgA has α-globulin electrophoretic mobility. (Hudson & Hay, 1976).

The other experiments described in this section were designed primarily to isolate and characterise the fish immunoglobulin using standard immunochemical procedures. Clem & Leslie (1969) reviewed the information regarding fish immunoglobulins and concluded that the antibody molecule in sharks and higher fish was similar to mammalian IgM. IgM usually displays the following physicochemical parameters:

i) A molecular weight of approximately 900,000.

ii) A sedimentation coefficient of approximately 19S.

iii) The molecule has a pentameric structure comprising 5 identical components each made up of 2 heavy and 2 light polypeptide chains and joined by disulphide bands.

iv) It will agglutinate particulate antigen but has poor precipitin activity.

v) The agglutinin activity can be destroyed by reducing agents such as 2-mercaptoethanol.

vi) It will strongly fix complement.

vii) It has slight cathodic electrophoretic mobility.

Gel filtration studies (Expts. 2.5 and 2.11) clearly demonstrate that dogfish immunoglobulin is totally excluded from Sephadex G-200 and has a molecular weight of approximately 800,000 as determined on Sepharose 6B. These results compare with those of numerous workers and have been extensively reviewed (Clem & Leslie, 1969; Corbel, 1975; Davies, 1975).

Similarly, the finding that the sedimentation coefficient of the molecule is 18.6S (Exp. 2.10) agrees with many similar findings that the antibody molecule in elasmobranchs and higher fish is a macroglobulin (Clem & Leslie, 1969; Anderson, 1974).

Classically, mammalian IgM is reduced to monomeric 7S components by reagents such as 2-mercaptoethanol. The reduced immunoglobulin molecules do not have agglutinin

activity even though their combining sites are apparently unaltered. The dependence of agglutinin activity on more than two sites could be due to low intrinsic affinity (per site) or to unknown stearic factors that interfere with the function of the 7S sub-unit. Whatever the reason, IgM can be distinguished from IgG by loss of agglutinin activity after incubation with 2-ME (Eisen, 1974).

Like mammalian IgM, the dogfish antibody activity was also inactivated by 2-ME, thus fulfilling another criterion for analogy to that mammalian class. Sensitivity to reducing agents, like molecular weight and sedimentation coefficients, are characteristics that have been described by many groups studying immunochemical aspects of immune responses in fish and have been extensively reviewed by Clem & Leslie (1969) and Corbel (1975). Mild reduction with dithiothreitol followed by alkylation with iodoacetic acid does not subject the molecule to such harsh conditions as 2-ME and will only reduce the disulphide bands joining the 5 monomeric immunoglobulin components. The IAA alkylates exposed sulphydryl groups on the Fc portion of the molecule and thus prevents spontaneous re-association of the reduced 7S monomers to the polymeric form. As with 2-ME treatment, DTT abolished agglutinin activity in the immune dogfish sera and it would appear that the fish immunoglobulin is reduced in a similar manner to mammalian IgM, however, as no attempt was made to detect reduced 7S subunits, this is still a matter of some conjecture.

Separation of the dogfish antibody was achieved by a two stage process:

i) gel filtration which separated serum components according to molecular weight and

ii) preparative electrophoresis which further separated molecules according to their electric charge.

This process appeared to give a single component antigen with neutral to slight anodic mobility when characterised by IEP. Many workers have described elasmobranch immunoglobulins as having neutral or γ-mobility including Suran et al. (1967) for the leopard shark, Gitlin et al. (1973) who described 16 species of sharks and Ellis & Parkhouse (1975) for the skate. The slight anodic mobility of the dogfish immunoglobulin may be attributed to the following:

i) Differences in the conditions of electrophoresis i.e. the pH and ionic strength of the electrolyte buffer causes proteins to have a slightly different mobility. Indeed there are variations in the electrophoretic mobilities of the elasmobranch immunoglobulins described by the above authors. Gitlin et al. (1973) described antibody with marked γ-mobility, Ellis & Parkhouse (1975) purified immunoglobulin from the skate and found it to have only slight cathodic migration, whereas Suran et al. (1967) found that the leopard shark immunoglobulin remained at the point of application. Each of these workers varied their IEP procedure.

ii) The separated immunoglobulin may have been contaminated by another protein with similar molecular weight and electrophoretic characteristics. The presence of an additional band on TLG that may have been a protein other than partially reduced heavy and light polypetide chains would appear to substantiate this explanation.

Immunoglobulins have been isolated from many species of fish. To date virtually all reports describe a two-stage method combining separations made according to

charge and molecular weight. Porath, Axen & Ernbach (1967) described how proteins could be co-valently coupled to an agarose matrix such as Sephadex and suggested that the method could be used to make immunoadsorbents. The technique has been further described by Cuartrecasas (1970) and Hudson & Hay (1976). This method is now extensively used for affinity purification of mammalian immunoglobulin and it is surprising that it has not been applied to fish immunology as it is a very simple one-step procedure that yields highly purified antibody.

The finding of a 18.6S protein that will agglutinate particulate antigen with a molecular weight of approximately 800,000, is susceptible to reducing agents and with nominally neutral electrophoretic mobility is indicative of the presence of an "IgM-like" antibody in dogfish serum. This is substantiated by the ability of immune sera to strongly agglutinate particulate antigens without concomitant precipitin activity (Chapter 3) and to fix homologous complement in the formation of haemolytic plaques (Chapter 6).

The final criterion for describing the antibody as "immunoglobulin-like" in structure is the presence of heavy and light polypeptide chains. TLG analysis shows that the purified antibody comprises two identifiable protein components of 18,200 and 75,860 daltons. This finding is approximately in agreement with those of other groups (Clem & Leslie, 1969) who have shown that elasmobranch immunoglobulin is made up of heavy (MW = 70,000) and light (MW = 22,000) polypeptide chains. If sufficient material had been available, analysis of polypeptide chain structure would have been made by the much more accurate SDS-PAGE technique and results more comparable with other works may have been obtained (TLG operates within an accuracy of ± 10%). Nevertheless, the findings in this study leave

little doubt that the dogfish antibody molecule, like that of other elasmobranchs, is a macroglobulin resembling IgM of higher vertebrates.

In mammals an IgM (19S) response to antigenic challenge preceeds an IgG (7S) response of the same (or slightly higher) specificity. Many elasmobranchs have been shown to first produce 19S antibody but after prolonged immunisation a 7S immunoglobulin can be detected. This light antibody is antigenically identical to the 19S macromolecule and thus is not a separate class of antibody but merely a monomeric form of the 19S IgM pentamer. Clem & Leslie (1969) reviewed this data and concluded that the two molecular forms of shark IgM were functional mimetics of mammalian IgG and IgM.

Experiment 2.11 set out to substantiate these findings by demonstrating a similar phenomenon in the dogfish, however the findings were somewhat different from those reported elsewhere. Agglutinin activity was detected only in the 19S fractions and initially it was thought that either the fish did not produce 7S antibody or that the latex fixation test was inadequate to demonstrate it because of either i) its inherent insensitivity or ii) the monomeric antibody was not multi-valent and would not agglutinate latex particles. When the anti-dogfish immunoglobulin serum was produced it was decided to use this as an additional detection method. Surprisingly the antiserum detected protein of 160,000 daltons (corresponding exactly with 7S monomeric IgM) in fractionated sera from long term immunised and non-immunised fish. There may be several reasons for this finding:

i) The antiserum may not have been monospecific and precipitated with other proteins. This does not appear

likely, however, for if non-specific reactions were occurring it seems incredibly coincidental that they should exactly correspond with the molecular weight of monomeric IgM.

ii) That a switching mechanism may have already been involved. All fish examined in Plymouth were infected with the nematode parasite Proleptus obtusus (see Chapter 7) which provided a constant antigenic challenge to the fish. This may have provided the animal with the necessary stimulus to activate the 19S - 7S IgM switch and hence the finding of the monomeric form of the antibody in "normal" fish that had not been on an artificial long term immunisation schedule. No reports exist regarding the presence of parasites in fish and the production of 7S antibody.

Whether or not monomeric IgM is a mimetic of IgG is debatable. Humans suffering with trypanosomiasis produce 7S IgM (Mattern, Klein, Radema & van Furth, 1967) and reports also exist of low molecular weight IgM in systemic lupus erythematosus (SLE) (Stobo & Tomasi, 1967) and rheumatoid arthritis (Stage & Mannik, 1971 and Theofilopoulos, Burtonboy, Lo Spalluto & Ziff, 1974). Therefore, although repeated challenges of injected antigen may lead to the production of 7S IgM in fish, the conclusion that this is a form of the classic IgM-IgG switch may be incorrect and that this antibody species is simply monomeric IgM and produced after repeated antigenic challenge as with parasite infections and autoimmune disease in man.

CHAPTER 5

LYMPHOID AND LYMPHOMYELOID TISSUES OF THE DOGFISH

Before undertaking studies on the immunocompetence of the various leucocytes and tissues of the dogfish, they were examined at the light and electron microscope level. The locations of the lymphoid and lymphomyeloid tissues examined are shown in Fig. 5.1.

I. Lymphoid Organs

These are tissues in which the predominant cells are lymphocytes. In the dogfish the spleen and thymus were considered to be the primary lymphoid organs. Other tissues such as lymph nodes, Peyer's patches, tonsils and adenoids which are traditionally termed 'lymphoid' in mammals have not been shown to exist in fish.

Experiment 3.1. Structure of the Spleen

The dogfish spleen is a relatively large organ measuring 7 cm - 9 cm in length and weighing between 2.5g - 3.5g in adult (650g - 950g) fish. It is deep red in colour and lies along the right hand (ventral) surface of the intestine (Fig. 5.1). It is drained by the hepatic portal vein.

Spleens from normal fish were removed and examined by light microscopy. A well defined splenic capsule was evident and it appeared to comprise a network of collagen strands (Fig. 5.2). A network of fibres extended throughout the organ, frequently forming trabeculae (Fig. 5.2): these are reticular areas often observed supporting blood vessels. Arterial and venous blood

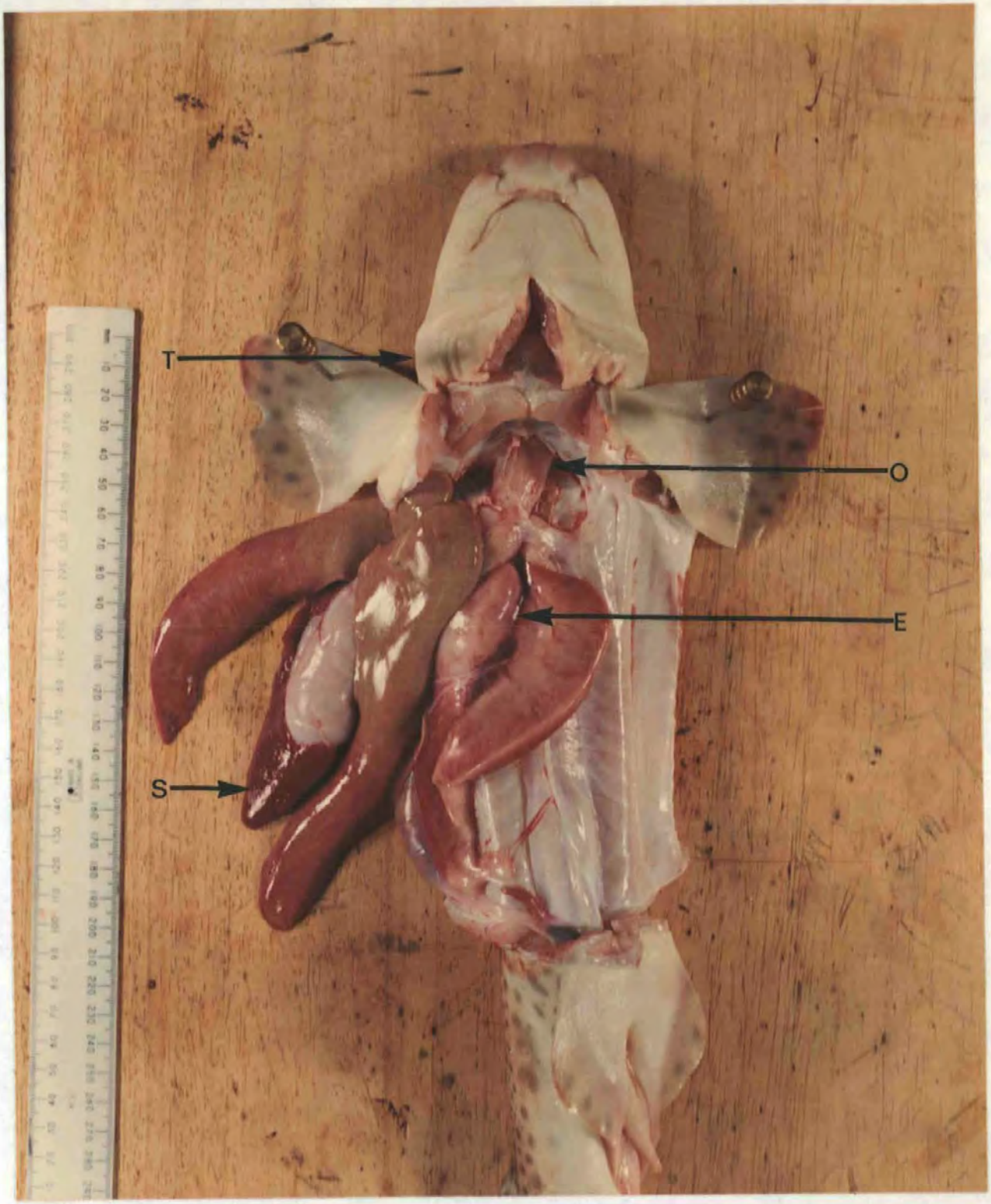


Fig. 5.1. Dissection of a male dogfish to show the location of lymphoid and lymphomyeloid tissues.

T - Thymus (not visible, located above gill arch)
S - Spleen
E - Epigonal Tissue
O - Organ of Leydig.

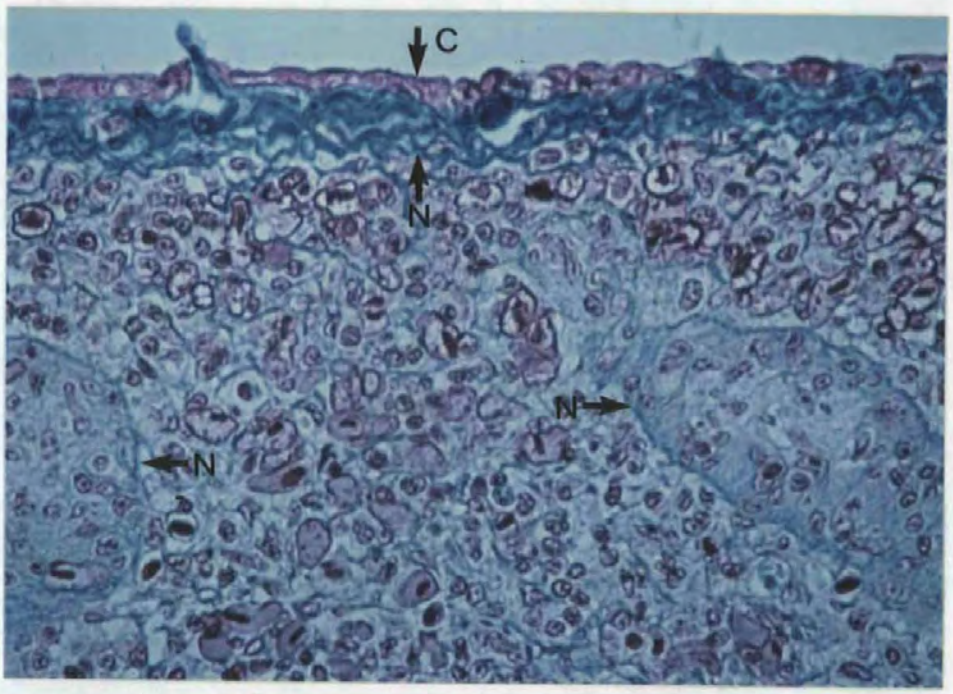


Fig. 5.2. Spleen (X 345). Mallory's triple stain. Showing capsule (C) and collagen network (N).

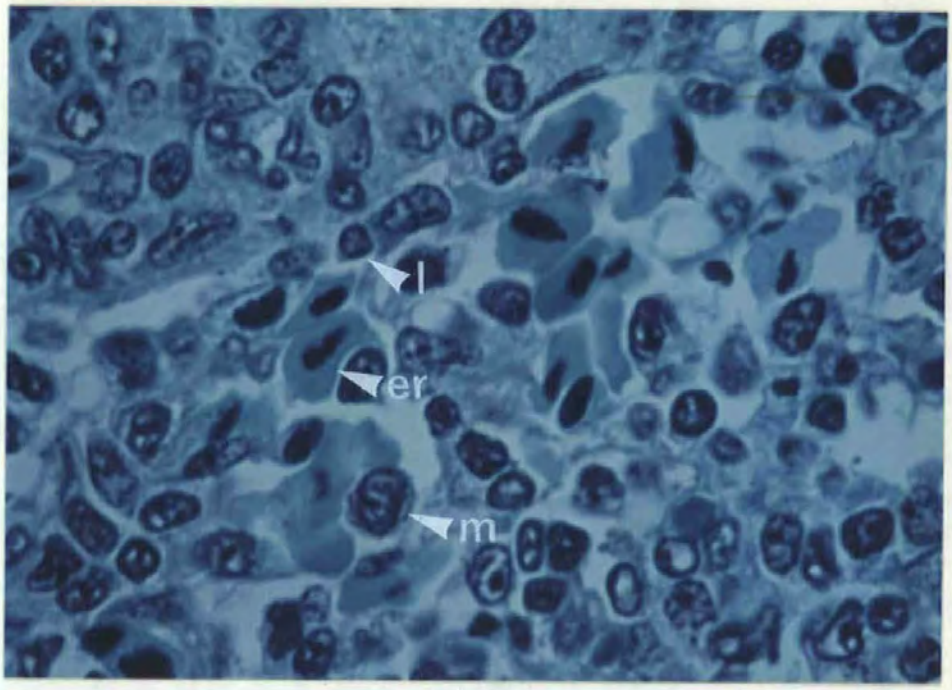


Fig. 5.3. Spleen cells (X 864). May-Grünwald Giemsa. er - erythrocyte, m - macrophage, l - lymphoid cell.

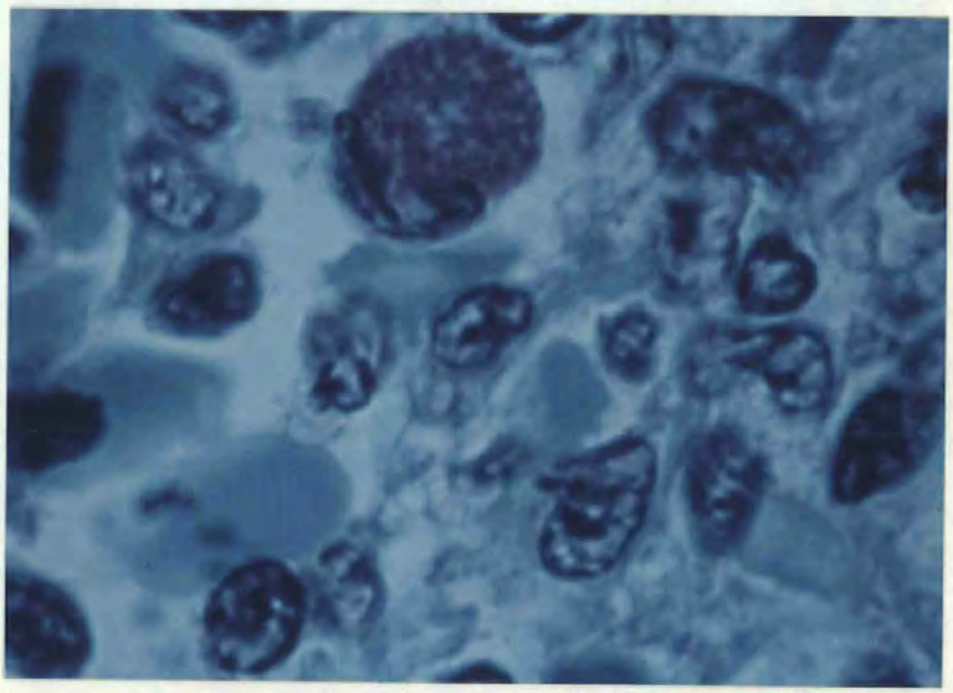


Fig. 5.4. Eosinophil (top centre) in spleen (X 2160). May-Grünwald Giemsa.

vessels were well represented (Chapter 6, Figs. 6.2, 6.3).

Large areas of erythroid pulp were not as clearly defined as in higher animals although many red blood cells could be seen scattered throughout the organ (Fig. 5.3). Areas exclusively comprising white cells were more clearly marked after antigen stimulation (see Chapter 6, Figs. 6.2, 6.3). Small lymphoid cells were most frequently observed although larger eosinophils and macrophages were also noted (Figs. 5.3 and 5.4). Representative sections from all parts of the spleen were examined and no distinct inter-regional variations were observed.

Experiment 3.2 Structure of the Thymus

The dogfish thymus is a very diffuse organ which apparently involutes with age, making it virtually impossible to detect in adult fish. In order to locate this organ, the head of a juvenile fish (3 weeks old) was serially sectioned and examined for the presence of thymic tissue. The thymus was found to lie superior to the gill slits and consisted of irregular masses of tissue (Fig. 5.5). These were arranged in a series of lobes running parallel to the gill slits.

Areas of connective tissue were found to surround whorls of densely packed cells which resembled small lymphocytes (thymocytes) (Fig. 5.6) although owing to the extremely fibrous nature of the tissue only thick (7μ-8μ) sections could be cut and this made precise identification of the cells type difficult. Islands of epithelial tissue were also observed. There was no apparent demarcation into cortex and medulla as in higher animals or structural equivalents of Hassal's corpuscles. Extensive networks of blood capillaries were not seen.

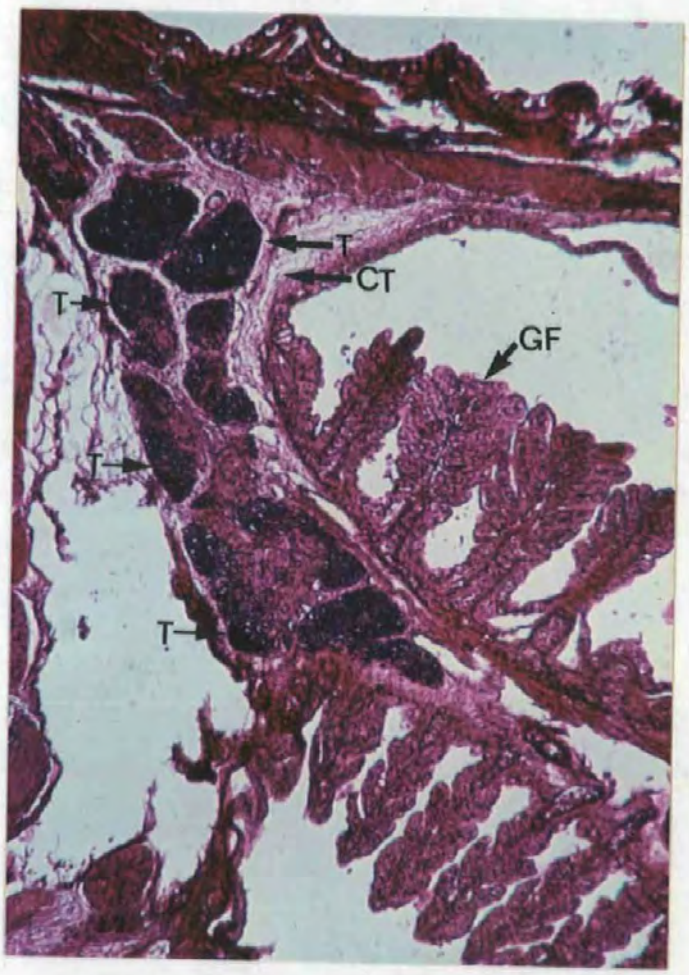


Fig. 5.5. Low power plan of dogfish thymus (X 57). Haematoxylin and Eosin. GF - gill filament, CT - connective epithelial tissue, T - thymocytes.

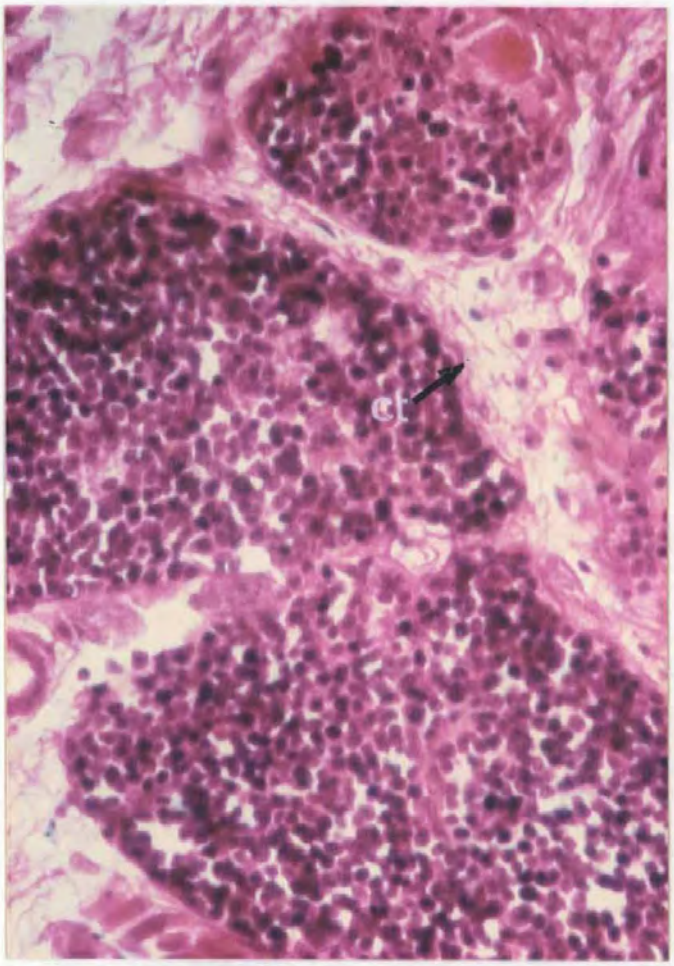


Fig. 5.6. Thymus (X 345). Mallory's triple stain. Showing thymocytes enclosed by a network of fibrous connective tissue (ct).

Experiment 3.3 Structure of the Anterior Kidney

Serial sectioning of the excretory and non-excretory regions of the kidney revealed these to be non-lymphoid zones, unlike the pro-nephric area of teleost fish. The organ principally comprised dense connective tissue although the occasional lymphocyte was visible. However, these were insufficient in number to classify it as a lymphoid organ.

II. Lymphomyeloid Tissue

In mammals lymphomyeloid tissues are considered to comprise multipotential stem cells which have the ability to differentiate into both lymphoid or myeloid (granular leucocyte) cell types. In adult mammals and birds, these cells are located in the bone marrow (Greaves et al., 1974). Fish do not have bone marrow, although sharks have two organs which are possibly functional equivalents of this tissue, i.e. the organ of Leydig and epigonal tissue.

Experiment 3.4 Structure of the Organ of Leydig

This organ appears as a whiteish mass of cells and is situated in the oesophageal mucosa (Fig. 5.1). Serial sectioning revealed that the organ had an ill-defined structure. There was no boundary of connective tissue, although, like the thymus, cells were arranged in whorls surrounded by reticular fibres (Fig. 5.7). Small lymphoid-like cells with irregularly staining nuclei and cytoplasm comprised the bulk of this tissue (Fig. 5.8) although large numbers of eosinophil-like cells were frequently seen grouped together (Fig. 5.9). Extensive vascularisation was not obvious.

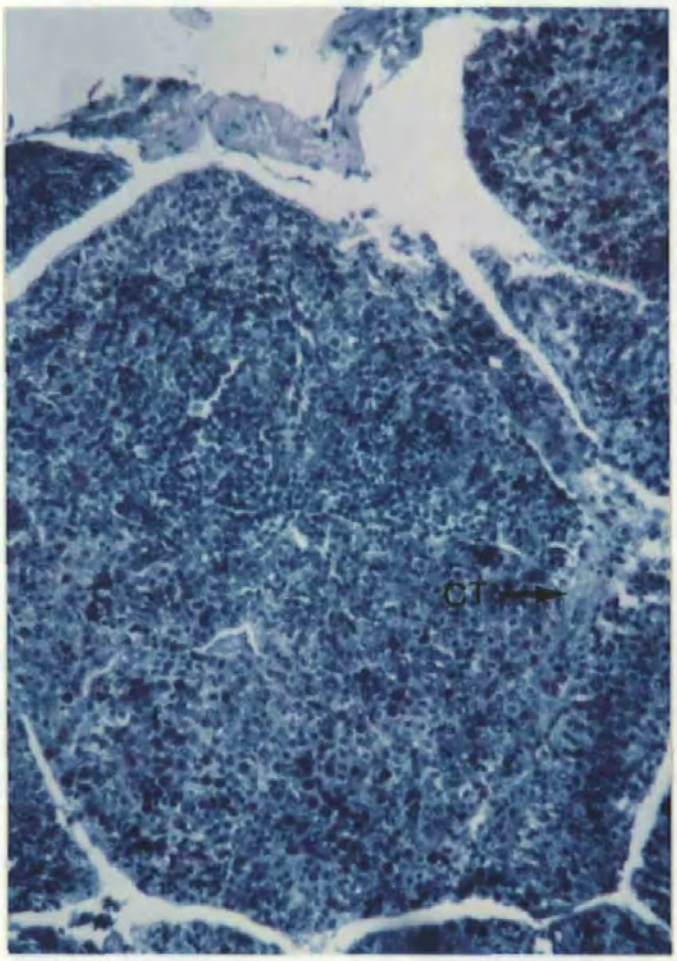


Fig. 5.7. Organ of Leydig (X 137). May-Grünwald Giemsa. Connective tissue is clearly visible.

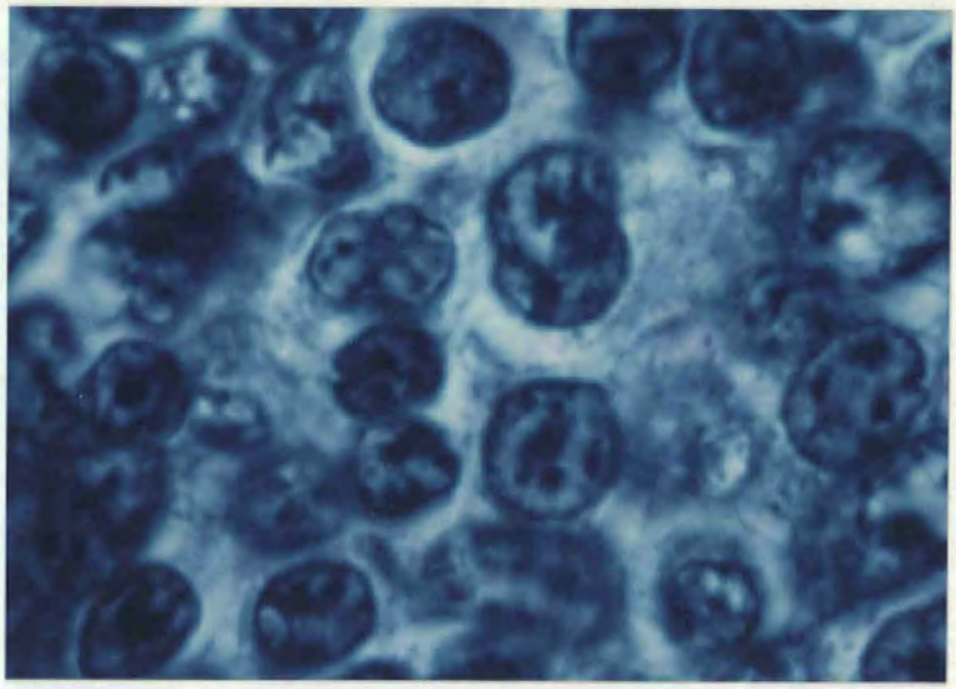


Fig. 5.8. Organ of Leydig (X 2160). May-Grünwald Giemsa. Showing granular, poorly differentiated lymphoid cells.

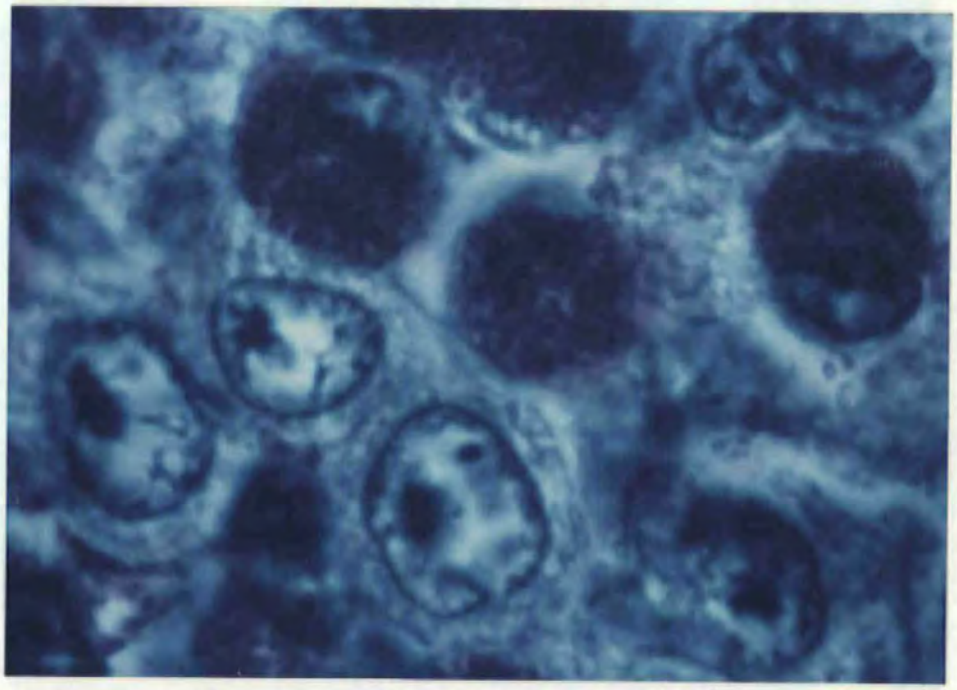


Fig. 5.9. Organ of Leydig (X 2160). May-Grünwald Giemsa. Showing eosinophils (centre and centre left).

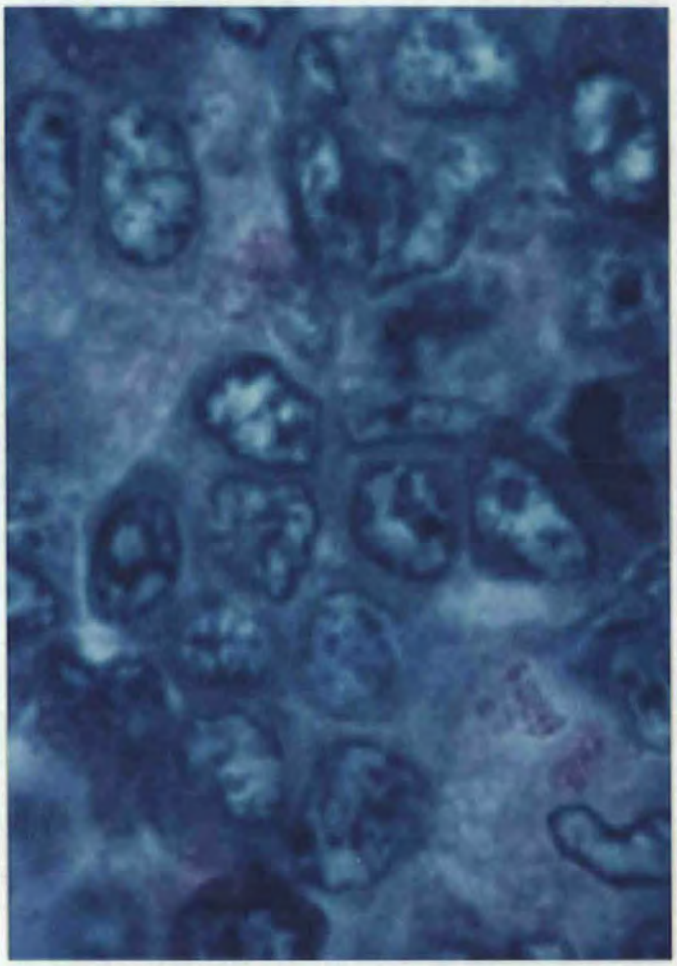


Fig. 5.10. Epigonal tissue (X 2160). May-Grünwald Giemsa. Showing undifferentiated, granular, lymphoid cells.

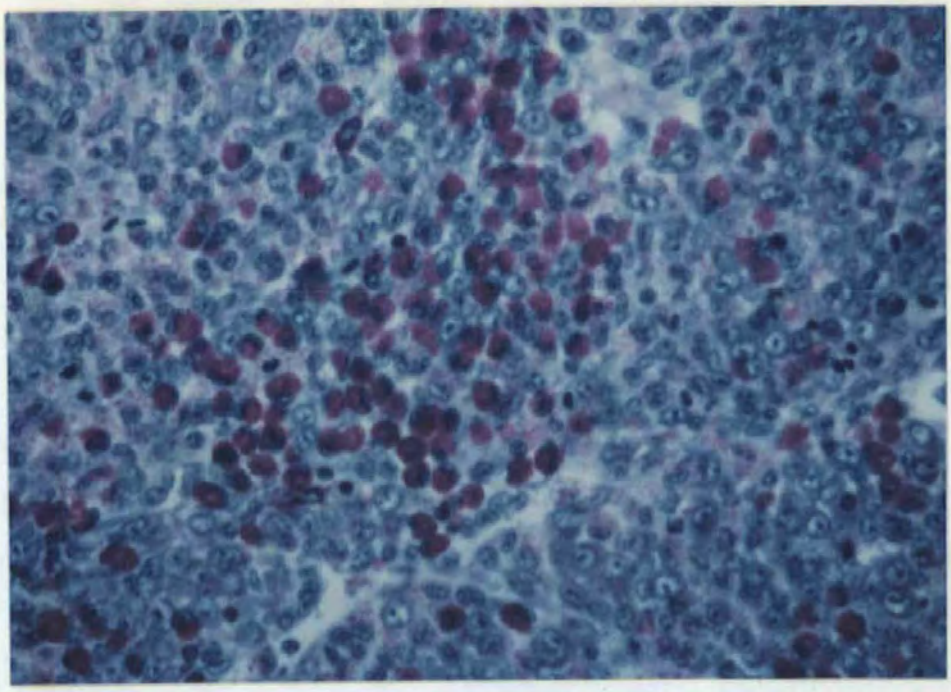


Fig. 5.11. Epigonal tissue (X 345). May-Grünwald Giemsa. Showing eosinophil accumulation.

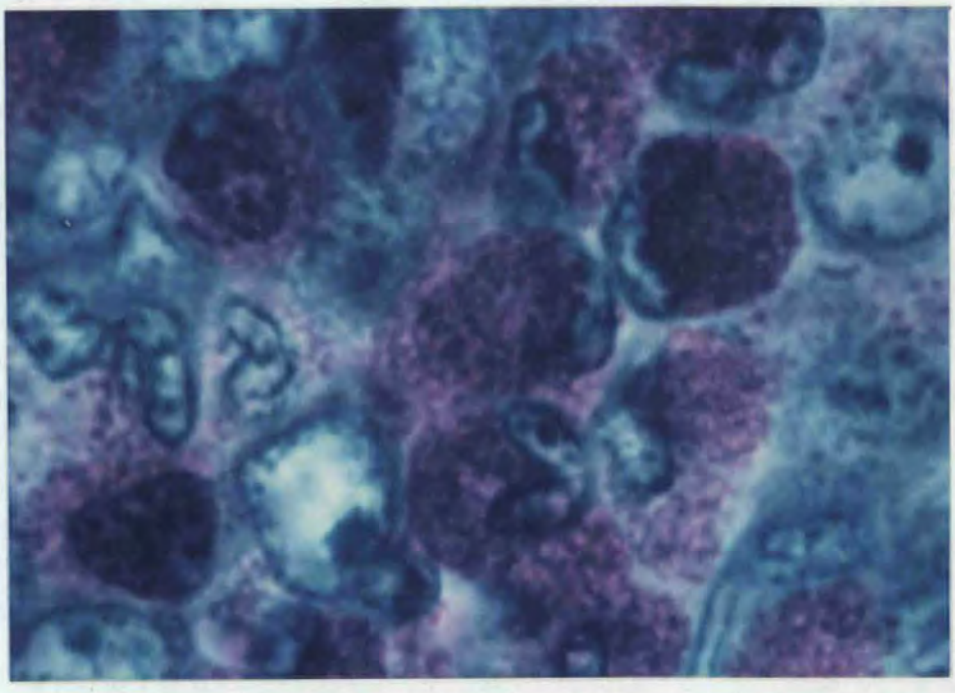


Fig. 5.12. Epigonal tissue (X 2160). May-Grünwald Giemsa. Showing eosinophils.

Experiment 3.5 Structure of the Epigonal Tissue

In male fish this tissue is located at the end of the testes (Fig. 5.1) and in females it is associated with the ovaries. The tissue is very similar in microscopical appearance to that of Leydig's organ. The tissue was sectioned and was found to comprise mainly poorly differentiated, irregularly stained lymphoid-like cells in a matrix of connective tissue (Fig. 5.10). Epigonal tissue was also distinguished by dense eosinophil foci (Figs. 5.11, 5.12).

III Peripheral Blood Leucocytes (PBL)

Despite numerous attempts to identify dogfish leucocytes by conventional blood smears and Romanowsky staining procedures, none succeeded. On each occasion cells lysed after fixation and large crystalline structures appeared in and around them. This phenomenon was attributed to the presence of a relatively high concentration of urea (0.35M) in dogfish sera (and therefore cell cytoplasm). Crystalline structures appeared in the cells after air dried films had been made and this was presumed to be intra-cellular urea crystallising and causing destruction of the leucocytes. All attempts to dialyse urea from cell suspensions failed. Examination of "live" unstained preparations did not reveal sufficient structure of the cell by which identification could be made. Thus leucocytes were examined using electron microscopy as the fixation process would not be affected by the presence of urea.

Experiment 3.6 Ultrastructure of Dogfish Peripheral Blood Leucocytes

In order that dogfish PBL could be partially classified according to phagocytic capabilities as well as gross morphological characteristics, fish were challenged with

colloidal carbon. Carbon-in-water colloid (Reeves Ltd.) was supplemented with 0.5% (w/v) gelatin (Ferguson, 1976) and autoclave-sterilised. Approximately 15 mg of carbon in a 0.5 ml inoculum was injected intravenously into the fish via the caudal sinus. Twenty four hours later, blood samples (2 ml) were taken from the fish, transferred into a plastic centrifuge tube without anti-coagulant and centrifuged at 400g for 10 minutes. The serum was removed and replaced with isotonic fixative. After further fixing with osmium tetroxide and dehydration with alcohol, the buffy coat was sufficiently hard to be cut from the centrifuge tube and the preparation completed.

After examination of the sections, the following cells were classified according to their morphological and (to a lesser extent) functional similarities with mammalian PBL.

A) Lymphocytes

These cells were of varying sizes and were distinguished by a high nuclear to cytoplasmic ratio (Fig. 5.13, 5.14). The nucleus was always approximately circular in outline and had heavy chromatin condensation around the periphery. The thin rim of cytoplasm was non-vacuolated and comprised many small granules. A few mitochondria were noted (Fig. 5.13). The cytoplasm occasionally extended to form small pseudopodia. The cells appeared to have no phagocytic properties and no endocytosed carbon was noted in any of the lymphocytes examined.

B) Plasma Cells

Fig. 5.15 shows a cell virtually identical to a mammalian plasma cell. These cells were distinguished by a considerable content of rough endoplasmic reticulum (RER)

Fig. 5.13. Small lymphocyte (X 17,200). Mitochondria (M) are clearly visible.

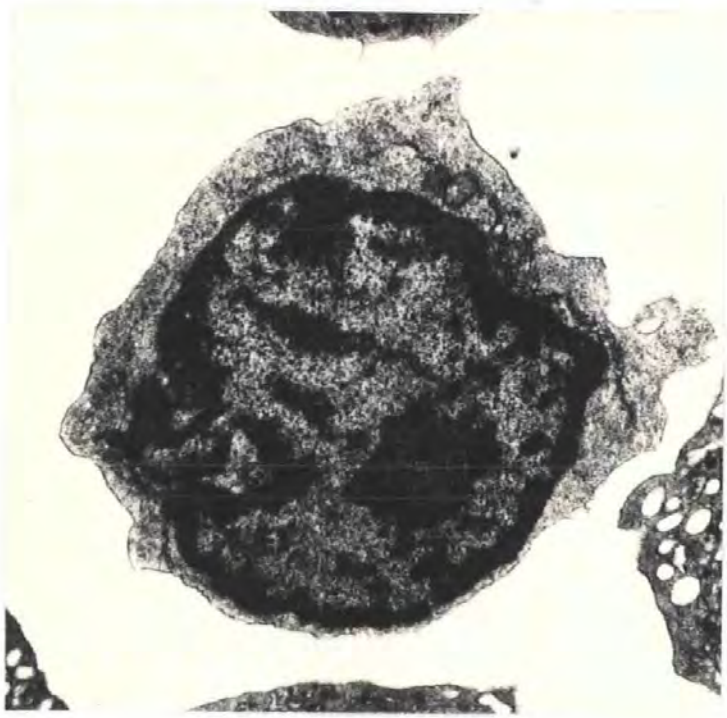


Fig. 5.14. Large lymphocyte (X 17,200).

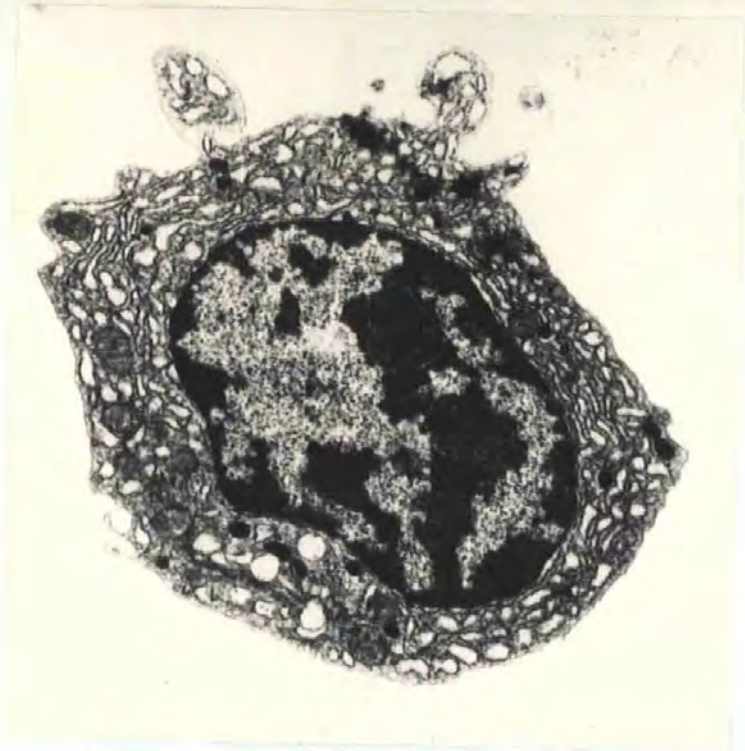


Fig. 5.15. Plasma cell (X 10,600).

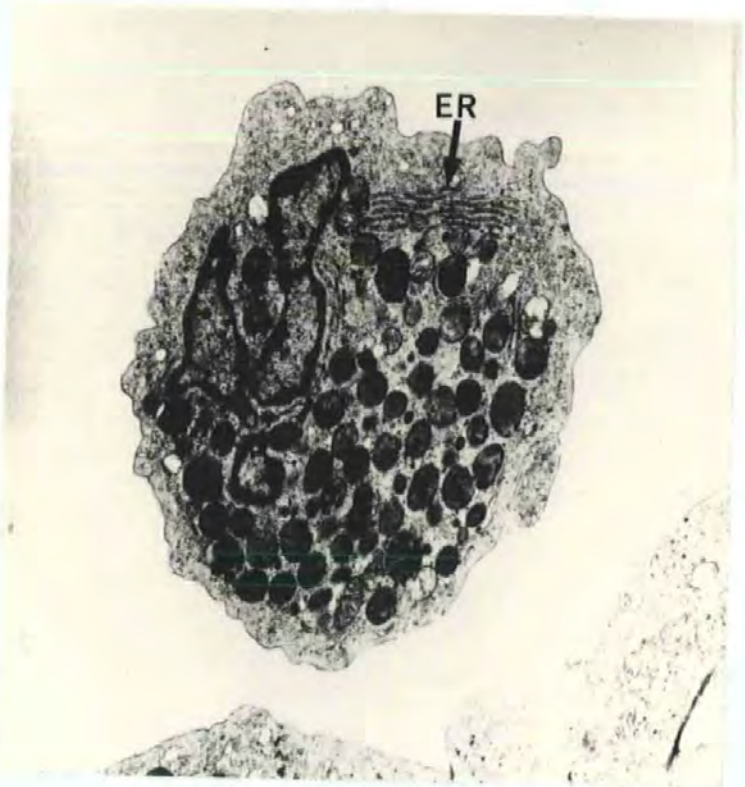


Fig. 5.16. Granulocyte. Type I (X 7,900).

Endoplasmic reticulum (ER) is clearly visible and also large, electron dense granules.

and large, slightly epicentric nuclei containing condensed chromatin. The sacs of RER were arranged around the nuclei in a concentric pattern. A few mitochondria were apparent and several electron dense granules were randomly distributed throughout the cytoplasm. These cells were the most rarely encountered in the sections examined.

C) Granulocytes

These were the most commonly occurring in the sections examined. Two principle types were noted:

TYPE I. These were large cells with lobed nuclei and were very similar to electron micrographs of mammlaian eosinophils. The cytoplasm contained many large, circular, electron dense granules. Regions of endoplasmic reticulum were noted. The cells were approximately circular in shape and had no pseudopodia or phagocytic properties (Fig. 5.16, 5.17).

TYPE II. These cells were rather smaller than the Type I granulocyte but differed principally in the size and shape of their cytoplasmic granules (Figs. 5.18, 5.19). These were smaller, more numerous and varied in electron density although they were rather more opaque than those described above. In other respects these cells were similar to the Type I group. Some of the cells appeared to be phagocytic as ingested carbon particles were seen within the cytoplasm. It is possible that these cells were an equivalent of the mammalian polymorphoncuclear leucocyte (neutrophil) or an immature form of the Type I population.

D) Monocytes

These were relatively large cells with non-lobed epicentric nuclei composed of varying condensed chromatin. The cytoplasm had a coarse appearance but was agranular. Mitochodria and RER were clearly visible in the cytoplasm.

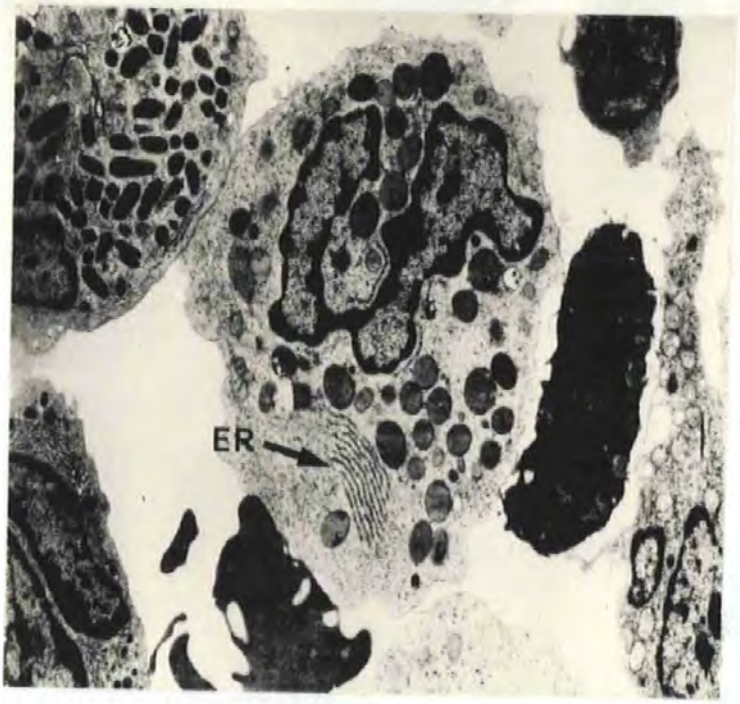


Fig. 5.17. Granulocyte. Type I (X 6,500).

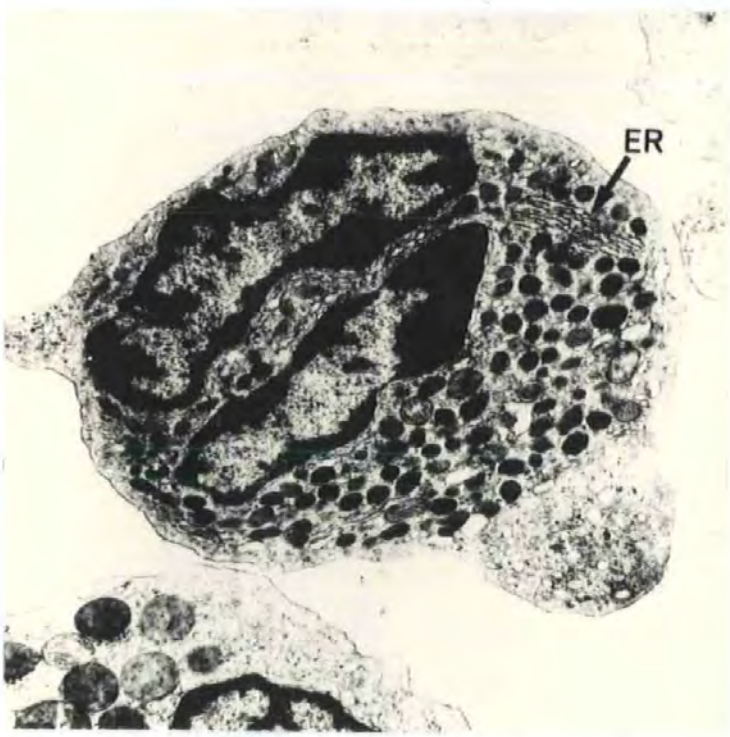


Fig. 5.18. Granulocyte. Type II (X 10,600).

Showing endoplasmic reticulum and many small granules of varying electron density.

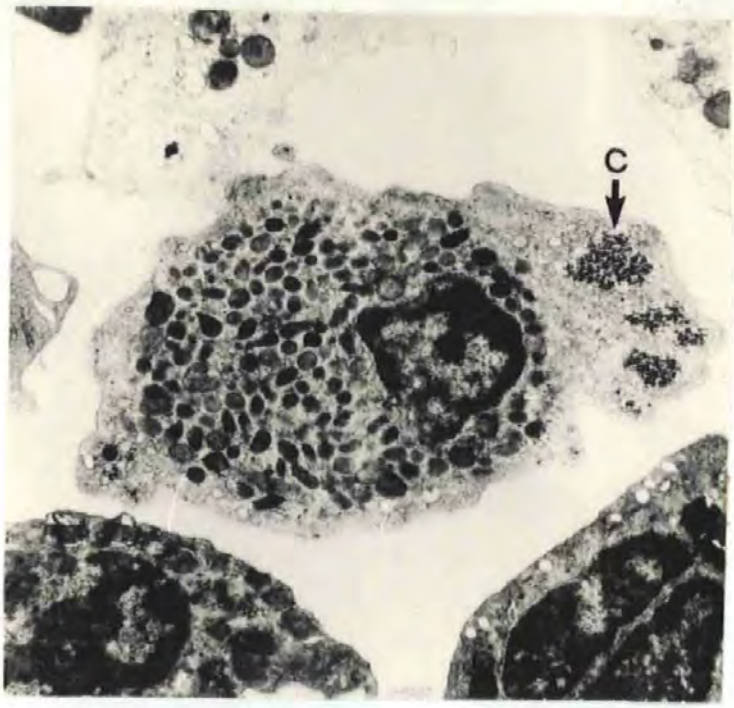


Fig. 5.19. Granulocyte. Type II (X 10,600).
Ingested carbon particles (C) can be seen in the cytoplasm.

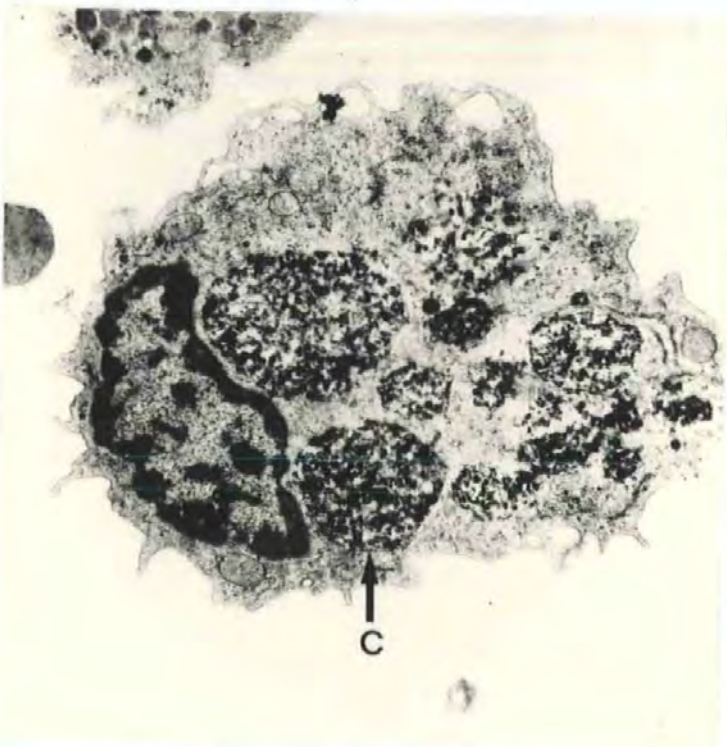


Fig. 5.20. Monocyte (X 10,600).
Large accumulations of ingested carbon are clearly visible.

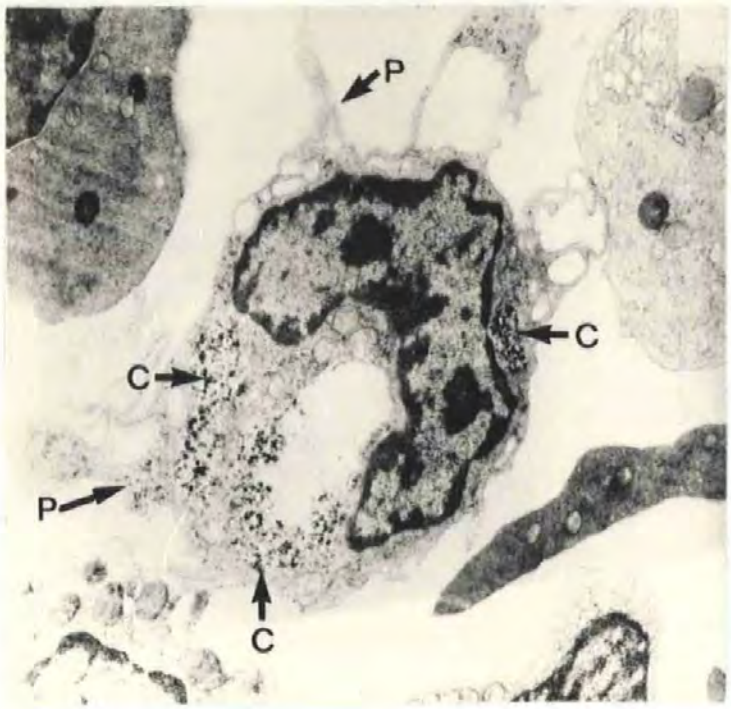


Fig. 5.21. Monocyte (X 7,900)

Ingested carbon can be seen in the phagolysomes. Pseudopodia (P) are also visible.

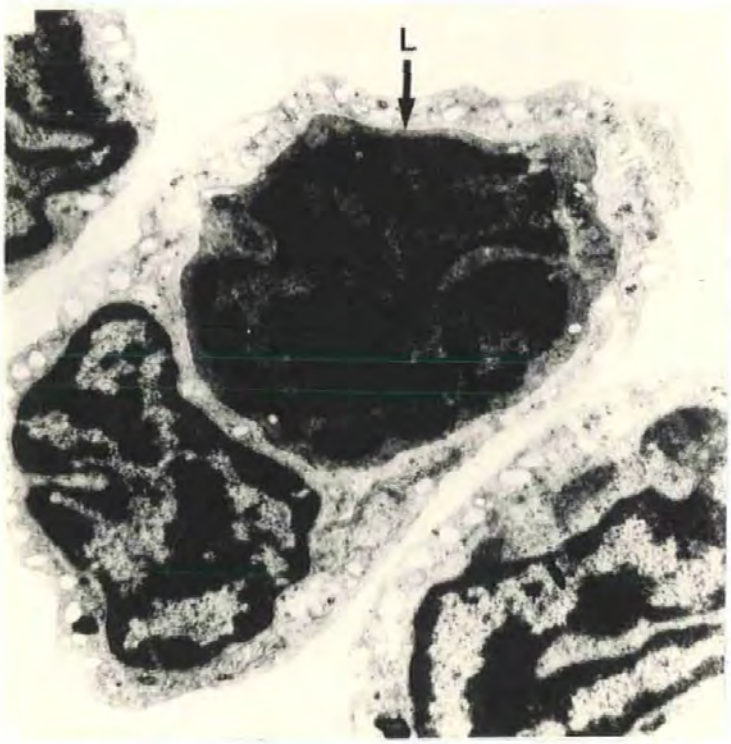


Fig. 5.22. Monocyte (X 7,900)

This cell apparently contains a whole, undamaged lymphocyte (L).

Golgi apparatus was not noted. The cells exhibited pseudopodia and were actively phagocytic, ingested carbon being visible within the phagolysosomes (Fig. 5.20, 5.21). On several occasions monocytes were seen to have engulfed whole cells, apparently without causing them damage (Fig. 5.22). It is possible that this was a transient phase with one cell merely "passing through" the cytoplasm of another.

E) Thrombocytes

Very few of these cells were observed, either because differential sedimentation occurred during the initial part of the experiment and the region of the buffy layer examined did not contain many thrombocytes. An alternative explanation is that cells may have been destroyed during the early stages (pre-fixing) of the experiment. The cell type thought to be a thrombocyte was ovoid and with an elongated and slightly indented nucleus (Fig. 5.23). The cells were approximately the same size as circulating lymphocytes. The gross morphology of this cell was virtually identical to those identified as thrombocytes from "live" unstained smears examined by light microscopy (Fig. 5.24). The cytoplasm was slightly vacuolated and some electron opaque granules were visible. Large vesicles were noted and it appeared that the cells had some phagocytic properties as carbon particles were visible within these, although insufficient cells were observed to make a positive statement regarding this phenomenon.

Discussion

The dogfish spleen contains many cells similar to those traditionally associated with antibody production and other immune functions in mammals. In Chapter 6 the ability of splenic leucocytes to synthesise antibody is described, thus establishing the immunocompetence of this organ. The

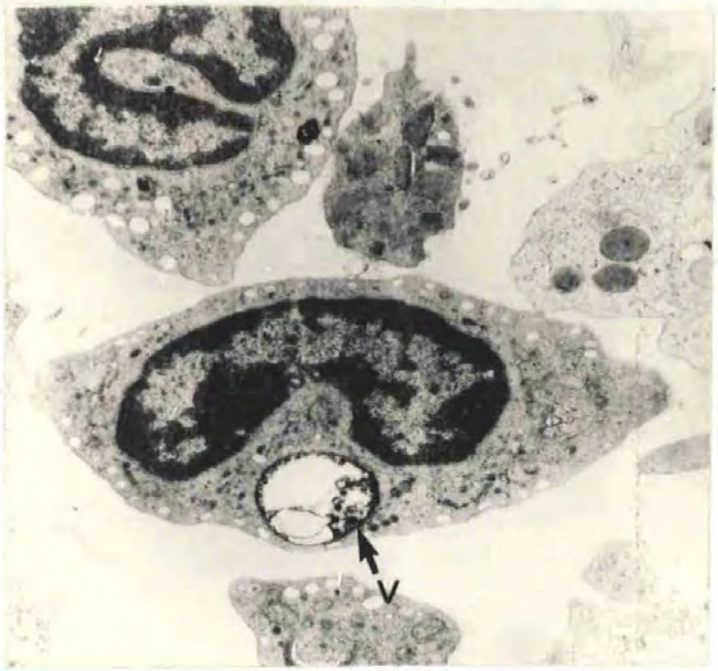


Fig. 5.23. Thrombocyte (X 10,600).
Vesicle (v) appears to contain carbon particles.

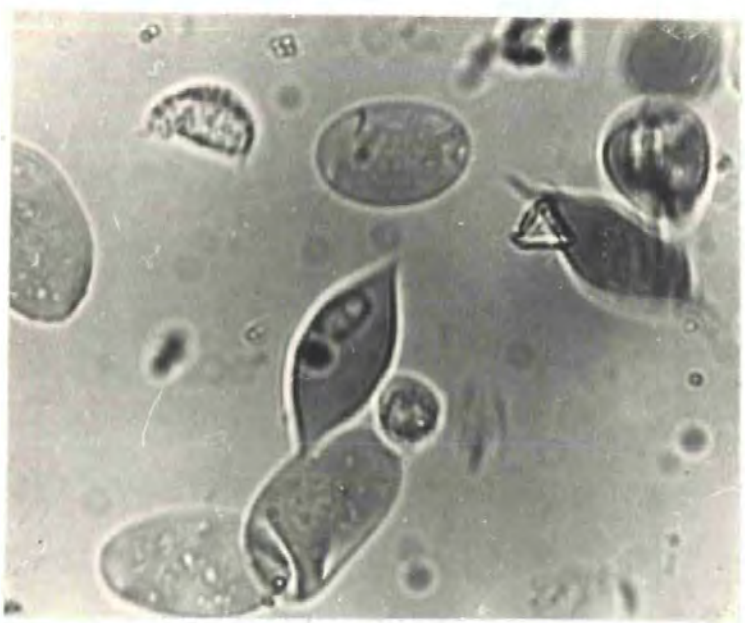


Fig. 5.24. Phase contrast micrograph of an unfixed, unstained smear of dogfish PBL. The ovoid cells (centre and centre right) are very similar in appearance to the thrombocyte in Fig. 5.23.

haemolytic plaque assay (described in the next chapter) did not identify a cell type responsible for antibody secretion and plasma cells were not identified by light microscopy although EM studies have revealed that this cell is present in the dogfish spleen (A. Pulsford, personal communication).

The function of other splenic leucocytes, the mononuclear phagocytes and granulocytes is, presumably, like their mammalian equivalents, to remove unwanted material that has been filtered from the blood e.g. effete erythrocytes and particulate foreign or antigenic material.

Although many individual red blood cells were observed in the spleen no discrete erythroid areas were seen and the site of erythropoiesis in these fish is unknown. It is presumed that the major function of the dogfish spleen is immunological. These findings differ from those of Good et al. (1966) who described the spleens of leopard and nurse sharks as comprising discrete areas of red and white pulp; these authors offered no photographic evidence to substantiate this claim and direct comparisons cannot be made. These is a little literature regarding the structure of elasmobranch lymphoid tissue and few other comments can be made regarding the spleen. No information is available relating to the presence of structures similar to the peri-arteriolar sheath found in mammalian splenic tissue although leucocyte aggregation around blood vessels was observed in spleens removed from antigen stimulated fish (Chapter 6).

There can be no doubt that the dogfish possesses a thymus during the early stages of its life although whether it functions in a similar way to the mammalian organ is very doubtful. The organ is very small and involutes at a

relatively early though, undetermined, stage in the development of the animal and it could not be found in any of the adult fish examined. Whether this small and apparently short lived structure is capable of providing the fish with a population of circulating lymphocytes analagous to the mammalian T-cell is unkown. As yet no evidence for the existence of T-lymphocytes in fish has been demonstrated either by using functional assays such as antibody independent cytotoxic killing or by surface markers and until further investigations have been made, the role of the fish thymus will be a matter of speculation. The dogfish thymus did not have structures resembling Hassal's corpuscles and there was no delineation of cortex and medullary areas although Good et al. (1966) claimed to be able to clearly differentiate between these areas in leopard and nurse sharks.

No attempt was made to look for gut-associated lymphoid tissue and to serially section the intestine would have been a formidable task. Good et al. (1966) made brief reference to this tissue in the horned shark and the guitar fish. The anterior kidney clearly has no similarities to the pronephros of teleosts and higher fish and this organ is thought to be a functional equivalent of the mammalian lymph node and is a more recent phylogenetic development. The teleost pronephros is discussed further in Chapter 6.

Fish lack bone marrow which is the source of undifferentiated stem cells in mammals. No attempts have been made to determine the sites of equivalent tissue in fish. Ellis (1976) considered the kidney to be the primary site of erythropoiesis in the plaice. He also considered it to be the major site of origin for lymphocytes and granulocytes although these cells could also be traced back to the spleen. Fänge (1968a,b), Fänge & Sundell (1969) and more recently Fänge & Pulsford (1978) have described lymphomyeloid-like tissue

in a variety of elasmobranch fish. These tissues comprised small cells with poorly differentiated structure resembling the bone marrow cells of higher vertebrates. These reports also described the presence of eosinophils in these tissues; an observation also made in this study. If the kidney is the site of granulopoiesis in teleost fish it seems likely that the lymphomyeloid tissue particularly the epigonal region associated with the testes and kidney is the origin of similar cells the elasmobranchs, however, until more complete studies have been carried out no further comment can be made.

The descriptions of dogfish peripheral blood leucocytes made in this study have virtually been confined to making analogies with mammalian cells by referring to anatomical similarities. This classification is somewhat unsatisfactory for several reasons:

i) it necessarily adopts a nomenclature assigned to mammalian cells and there is a tendency to fit cells into the various classes with a resulting loss of objectivity.

ii) a nomenclature founded on a descriptive basis seems, in the light of contemporary immunology, to be a dated method. With current interest in immunology centred around cellular capabilities and a system which classifies cells according to morphology and function is to be preferred.

Although this latter method ideally gives a more complete characterisation of leucocytes it presents some practical problems. Understanding of fish immunology is still in its infancy and although many species have been examined for immunologic function, the technology for applying immuno-cytological techniques has been poorly

developed and to date virtually no reports exist in which cells have been classified according to immunologic markers, morphology and cellular function although Ellis (1976) has attempted to do this for plaice leucocytes including surface immunoglobulin, phagocytosis, general and histochemical staining as well as gross morphology (at light microscope level) as criteria for assessing cell types.

Only fleeting references to shark lymphocytes have been made to date: Fänge (1968b) published some camera lucida drawings of leucocytes from various elasmobranchs but no photographs of any shark white blood cells have been found. This is surprising in view of the immunological interest taken in these fish. Dogfish leucocytes appeared to be very similar to those of mammals and other fish when examined under the electron microscope. The four cell types described here are similar to the findings of Ferguson (1976) who described lymphocytes, monocytes, thrombocytes and neutrophils in the plaice. The thrombocytes and monocytes had phagocytic properties and were seen to contain carbon particles that had been injected into the bloodstream in colloidal form. Dogfish monocytes were noted to be phagocytic and some carbon was identified in the few thrombocytes seen. Carbon particles were only seen in one of the two types of granulocyte observed. Ferguson (1976) made no comment on the phagocytic capabilities of granular leucocytes: he called these cells neutrophils but had reservations about this terminology particularly as these cells were not strictly polymorphonuclear. The cells were finally classified as neutrophils according to the appearance of their granules which were similar to those of mammalian neutrophils.

The dogfish granular leucocytes were not termed either neutrophil or eosinophil; the term granulocyte being preferred. Two distinct populations of these cells were

noted, each bearing certain similarities to the mammalian types. Traditionally, mature eosinophils have larger and fewer granules then the neutrophil. Their granules also have crystalline inclusions. The cells are not phagocytic (Cawley & Hayhoe, 1973). The Type I granulocyte described here is virtually identical to the mammalian eosinophil. The neutrophil has smaller granules of varying electron density and showing a large degree of heterogeneity. The cell is also phagocytic. The Type II granulocytes seen in dogfish peripheral blood appeared very similar to this cell and was also phagocytic. It was felt that arbitary titles such as neutrophil and eosinophil were not valid in the absence of information regarding the staining properties of these cells. No cells bearing ultrastructural resemblances to the mammalian basophil were observed. Conflicting reports regarding the presence of this cell type in other fish (Ellis, 1977a) and its existence in the Pisces group has yet to be firmly established.

Circulating, non-granular phagocytic cells were observed which were essentially similar to mammalian monocytes as described by Cawley & Hayhoe (1973). Ferguson (1976) described similar cells in the plaice. These cells have been termed monocytes and it would seem reasonable to expect to find a cell that is involved in non-specific defence mechanisms throughout the animal kingdom in the dogfish.

A population of dogfish PBL was identical to lymphocytes of mammals, plaice and the paddlefish (Clawson et al., 1966) and it would seem that this cell has changed very little during the evolution and development of animal species for it to be found in such diverse phyla and presumably it is fundamental to immune processes. The plasma cells seen were also similar to those described in mammals by Cawley & Hayhoe (1973) and the paddlefish by

Clawson et al. (1966). It is now well known that elasmobranchs are capable of making a specific antibody response (Clem & Leslie, 1969). The plasma cell has been well established as being capable of a high rate of antibody synthesis (Greaves et al., 1974) and their appearance at a relatively early stage of phylogenetic development reinforces the ideas that these cells are essential for the production of a specific antibody response.

The mammalian platelet or thrombocyte is a small cell, approximately 3μ in diameter, that is involved in the blood clotting process. It is frequently elongated or oval in shape, anucleate and is derived from the megakaryocyte of the bone marrow. The cell thought to be an equivalent of the thrombocyte in the dogfish was elongated or with an ovoid shape. Unlike the mammalian cell, the fish cell was nucleated and approximately the same size as circulating lymphocytes. Too few cells were examined by EM to make positive conclusions but they appeared to contain some ingested carbon. Several reports exist stating that these cells are phagocytic (Yokoyoma, 1960; Fänge, 1968a and Ferguson, 1976) although no data can be found regarding this phenomenon in mammals. Ellis (1977a) considered that this was unlikely to be phagocytosis but a process in which particulate matter was mechanically trapped by cytoplasmic labyrinthine vesicles. The principal reason for regarding these cells as platelets was their overall shape, although this may be a false assumption. The oval cell seen in Fig. 5.23 may not be the same as that seen in Fig. 5.24, a "live" cell from a wet smear. In fact the ovoid cell of Fig. 5.23 may have been an artefact from the preparative process although this is considered unlikely as although these cells were comparatively rare, others of this type were observed. Ferguson's work on this does little to clarify the situation other than show that in the plaice the thrombocyte is a cell line distinct from the lymphocyte.

This tentative classification according to certain similarities with mammalian cells is a slight advancement on some of the vague descriptions of leucocytes that are at present confounding fish haematological studies. If the immune response of the dogfish is to be investigated further a more detailed examination of its PBL will be necessary although there may be problems in deciding on definite functional criteria for the classification of fish leucocytes.

This problem reflects the whole state of leucocyte studies on fish and the available literature is sparse and contradictory with unsatisfactory attempts being made to examine, describe and classify cells. Fish immunology is now stimulating much interest but its progress is being hampered by poor or incomplete studies of lymphoid tissues. Leucocytes are fundamental to all immune reactions and they should be studied carefully before extravagant assumptions are made regarding their functions and capabilities.

CHAPTER 6

CELLULAR IMMUNE RESPONSES OF THE DOGFISH

I Responses of the Spleen to Antigenic Challenge

Having established that the dogfish was capable of producing specific antibody to injected antigen (Chapter 3) and that the spleen appeared, histologically, to be the major functional lymphoid organ (Chapter 5) the following experiments were performed in order to establish its immunocompetence.

Experiment 4.1 Production of Antibody by Spleen Cells

In order to show antibody production at the cellular level, the following experiment was carried out. Twenty fish were inoculated with 1 ml of SRBC, as described in Chapter 2, given a similar injection after 5 days and sacrificed at intervals so that assays of haemolytic plaque forming cells in the spleen could be made. Complement, as fresh serum, was taken from a single fish throughout the experiment. The optimum complement dilution was found to be 1:10 by titration. Serum samples from test fish were also taken and the SRBC agglutinating antibody activity was measured in inactivated serum.

The kinetics of the response are shown in Fig. 6.1. After 5 days an elevated level of plaque forming cells (PFC) was noted. The number of PFC reached a peak after 10 days and then dropped sharply. After 27 days the numbers had fallen to the level of the non-immunised controls. Circulating antibody was present at very low levels at first but by the end of the experiment had reached significant levels. It was noted that a threshold number of PFC was always present in control fish.

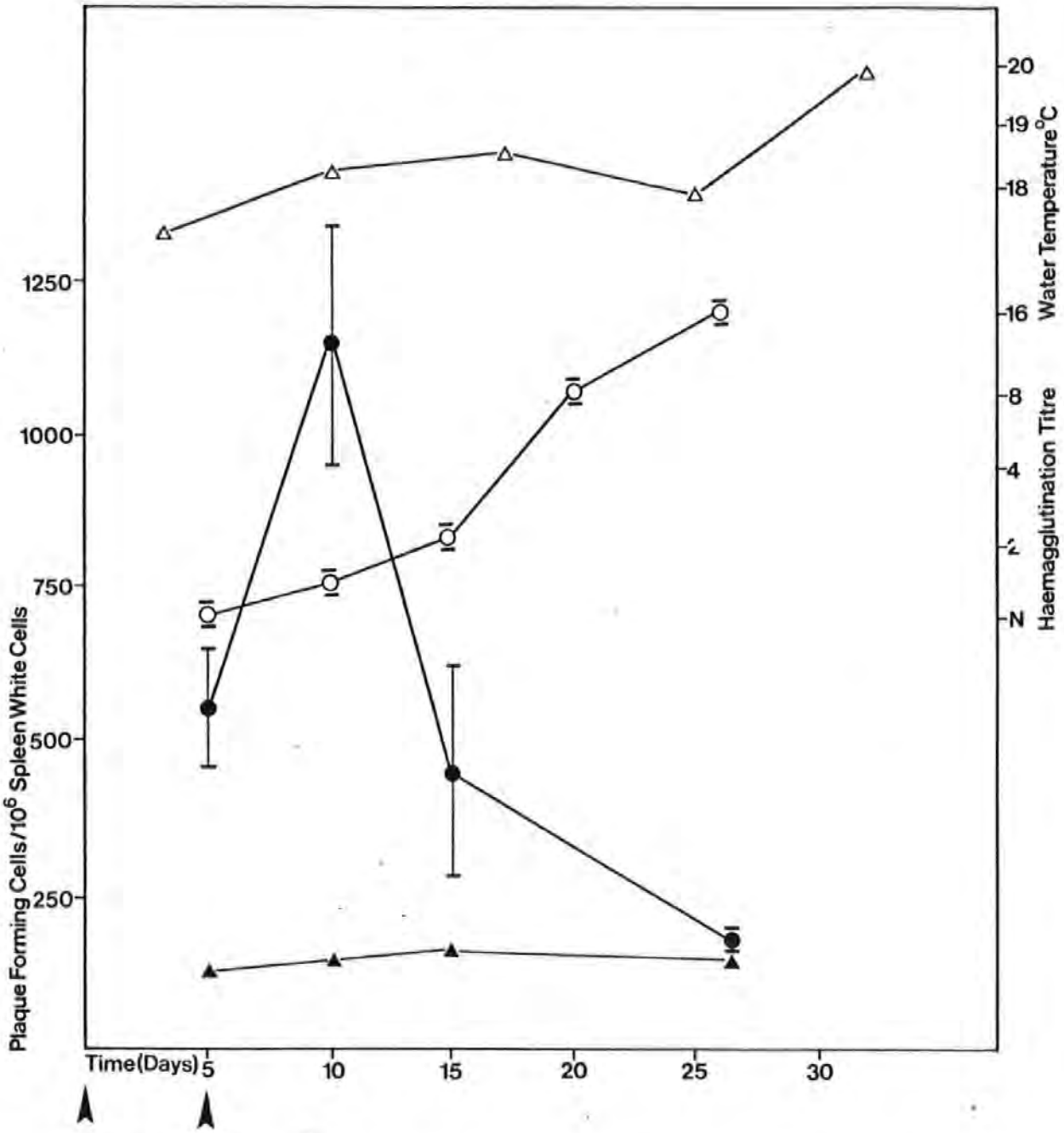


Fig. 6.1. The kinetics of the plaque forming cell response in dogfish immunised with SRBC (solid circles). The humoral immune response is also shown (open circles). Each point represents the mean of 3 fish and vertical bars indicate the standard error. Spleen assays of non-immunised controls (solid triangles) are an average of 2 fish. Open triangles show mean mid-weekly water temperature. Arrows indicate when injections were made.

Experiment 4.2 Histological changes in the Spleen after Antigenic Challenge

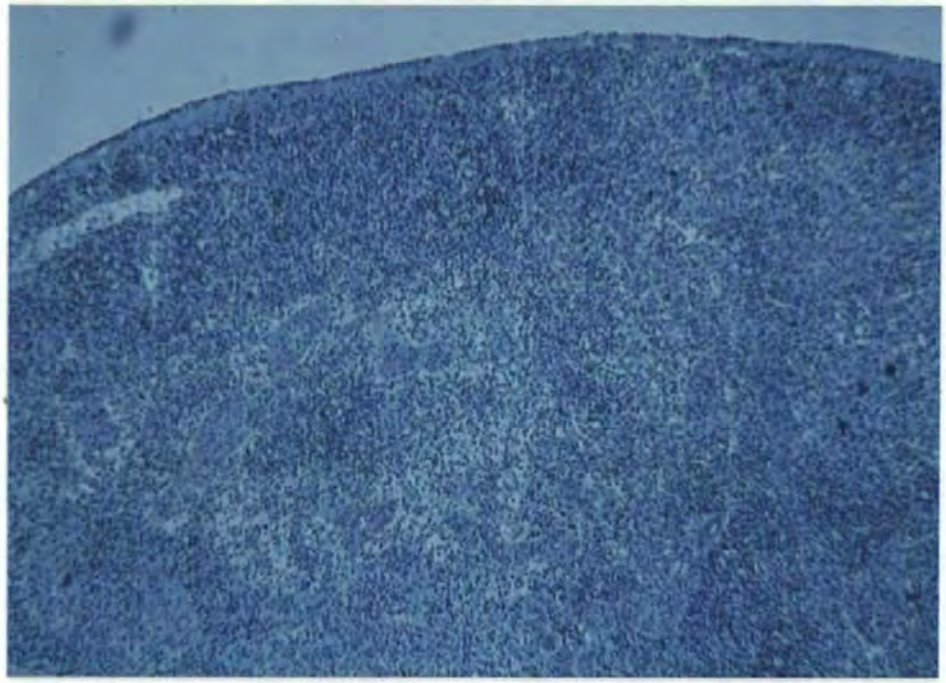
Six fish were given an intra-muscular injection of KLH (10 mg in 1 ml elasmobranch saline) without adjuvant. An identical injection was given after 5 days. The fish were sacrificed at 5, 10, 15, 20, 25 and 30 days after the initial injection and their spleens were then removed, fixed and wax embedded. Sections were stained with May-Grünwald/Giemsa or Methyl Green Pyronin Y and examined. Spleens from three unimmunised fish were used as controls.

After 5 days areas of densely staining cells became prominent, particularly surrounding arterial and venous blood vessels (Figs. 6.2, 6.3). These areas almost exclusively comprised lymphocytes and were thought to be areas of white pulp that could not be differentiated in normal spleens. These areas became more prominent and increased in size and number for 15-20 days. At 25 days, however, the response had diminished considerably and after 30 days no difference could be detected between spleens from the test and control fish.

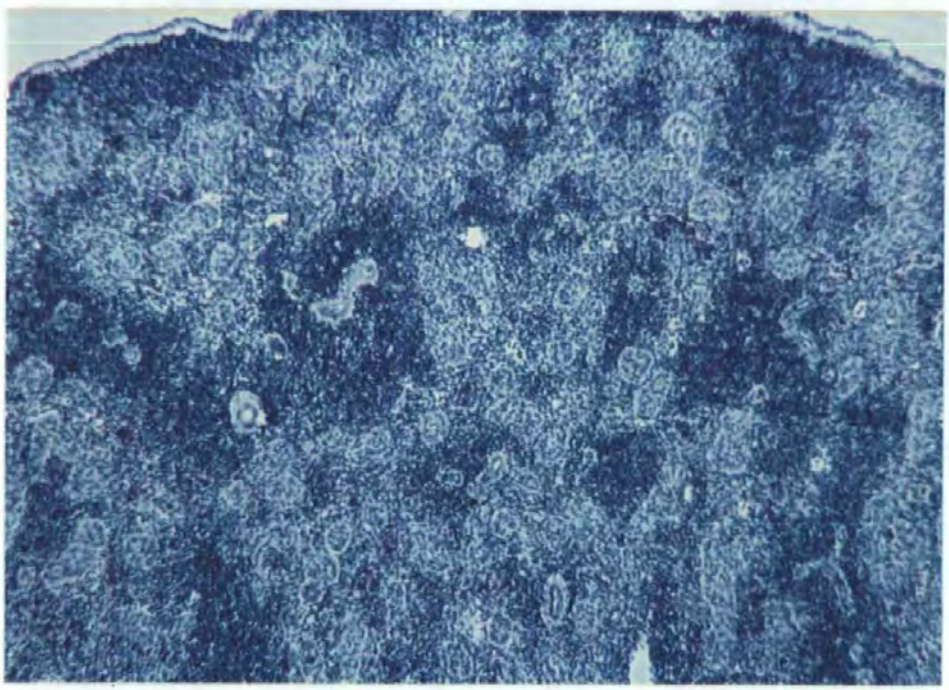
II Leucocyte Surface Determinants and Reactivity

The following experiments were made in an attempt to characterise dogfish leucocytes according to physical characteristics and also by monitoring various *in vitro* and *in vivo* reactions.

A) The Presence of Immunoglobulin on the Leucocyte Membrane



(a)



(b)

Fig.6.2 a) Spleen from normal fish (x57). May-Grünwald Giemsa.

b) Spleen from KLH immunised fish (x57) May-Grünwald Giemsa. Showing areas of cellular aggregation.

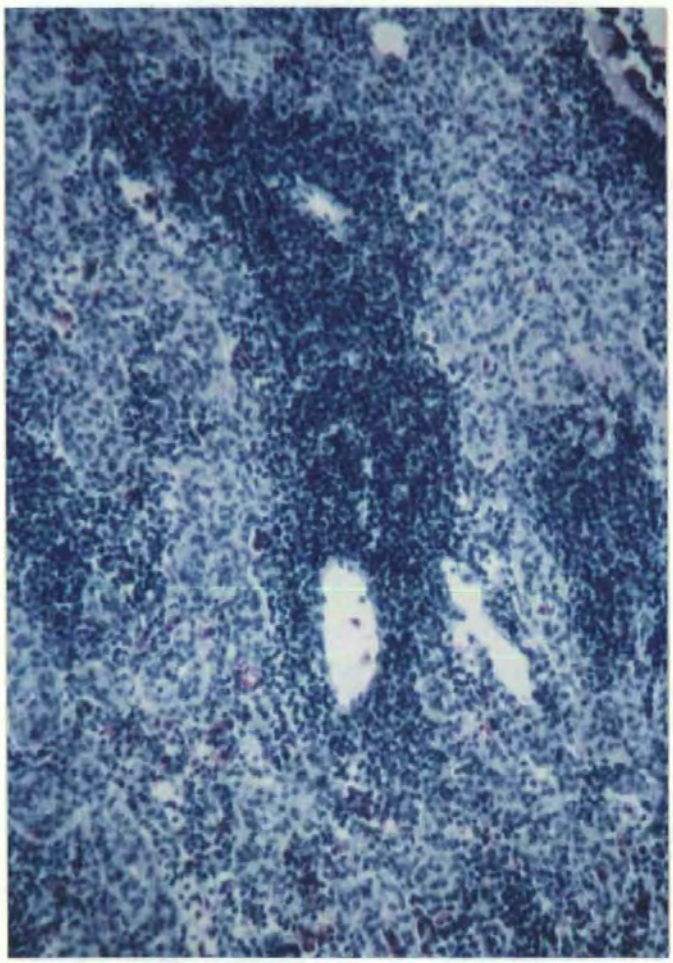


Fig. 6.3. Spleen from KLH immunised fish. (x137) May-Grünwald Giemsa. Showing large lymphoid accumulation around blood vessels.

Experiment 4.3 Detection of Surface Immunoglobulin by the Indirect Immunofluoresence Test

Blood samples (1 ml) were taken from several fish, allowed to settle in a heparinised centrifuge tube for 2-3 hours and the leucocyte rich plasma was withdrawn and the cells were stained for surface immunoglobulin by the indirect fluorescent antibody test. The following controls were made:

i) Cells incubated with FITC alone.

ii) Cells incubated with normal rabbit serum and FITC.

iii) Cells that had not been incubated with either reagent (to check for autofluorescence).

PBL from 4 fish were examined by this technique. At least 200 cells from each fish were scored and the percentage that stained positive for surface immunoglobulin are shown in Table 6.1.

FISH NO.	STAINED CELLS RINGS (%)	CAPS (%)	UNSTAINED CELLS (%)
1	51	5	44
2	80	9	11
3	68	5	27
4	75	10	15

Table 6.1. Immunogloblin on the surface of dogfish leucocytes after staining by the indirect immunofluorescence technique.

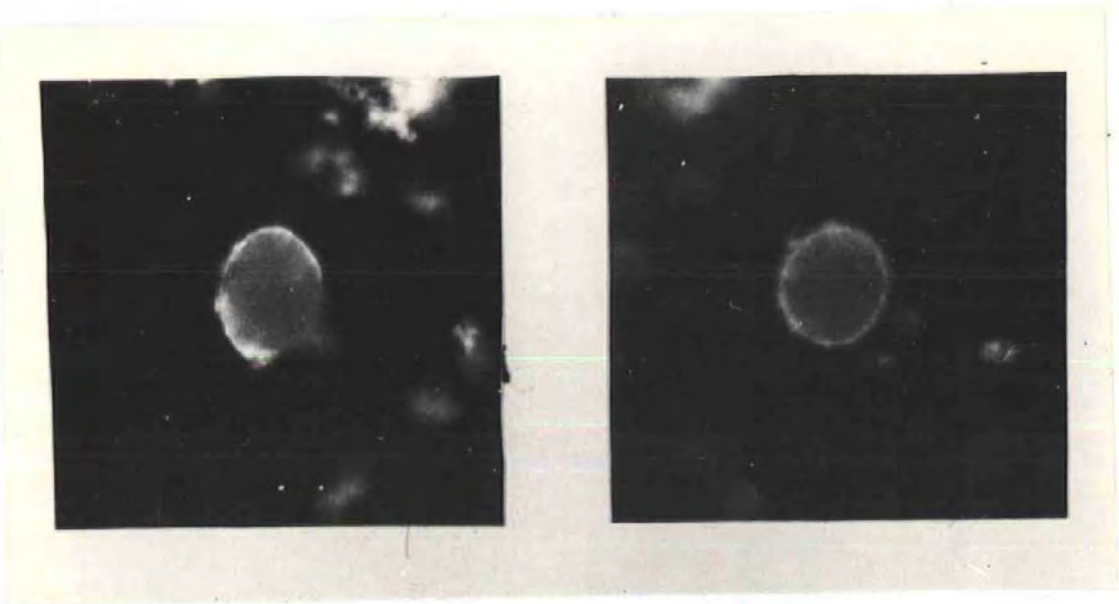


Fig. 6.4. Dogfish leucocytes stained by the indirect fluorescent antibody technique to show surface immunoglobulin determinants.

Immunoglobulin positive cells either stained with a ring of fluorescent specks around the circumference (Fig. 6.4) or as a cap where peripheral fluorescence was present around half or less of the cell circumference. Some cells fluoresced all over their surface and these were considered to be non-viable. The identity of immunoglobulin-positive cells was not established although small lymphocyte-like cells appeared to comprise most of the stained population although large, granular cells were also seen. No fluorescent-stained thrombocytes were observed.

Polarisation of surface fluorescence (capping) and subsequent endocytosis was not observed as a continuous process, at least over a short period. Stained cell suspensions, maintained at room temperature were examined for 3-5 hours during which time there was no noticeable increase in cells with cap fluorescent patterns or decrease in the numbers of stained cells. Fluorescence was lost overnight when preparations of stained cells were incubated at either room temperature or $4^{o}C$. In both instances virtually all fluorescence from viable cells disappeared but subsequent re-staining once again showed the presence of immunoglobulin on the cell, thus indicating that the stain was either being shed from the cell surface or being endocytosed by a slow capping process. Sodium azide 1% (w/v) was added to a stained preparation of cells which were re-examined after 18 hours incubation at room temperature. No change in the total of cells that stained positively for surface immunoglobulin or fluorescent staining patterns was recorded after this time.

B) Sensitivity of Dogfish Leucocytes to Mitogenic Stimuli

The following experiments were made using duplicate leucocyte cultures from each of 3 fish and using 5, 10, 25

and 50 μg ml^{-1} of appropriate mitogen in the culture medium. Duplicate cultures from 7 fish were made in the absence of mitogen and used as controls. Estimates of cell viability were made by the Trypan Blue exclusion method at the beginning and end of each experiment and ^{3}H-thymidine incorporation expressed as counts per minute (cpm) per 10^{6} viable leucocytes. Stimulation indices (SI) were calculated from the following formula:

$$SI = \frac{\text{Mean cpm of mitogen stimulated cultures}}{\text{Mean cpm of control cultures}}$$

Experiment 4.4 Response of Dogfish Leucocytes to Concanavalin A.

In 2 of the cultures 10 μg ml^{-1} of mitogen gave a maximum response, the third culture achieved a maximum SI using 25 μg ml^{-1} Con A. The maximum stimulation indices were 2.25, 115 and 7.1 (Fig. 6.5).

Experiment 4.5 Response of Dogfish Leucocytes to Pokeweed Mitogen

Again 2 of the cultures achieved a maximum response using a concentration of 10 μg ml^{-1} PWM. The third culture (Fish A) appeared to respond only slightly and even with 50 μg ml^{-1} PWM achieved a maximum SI of 1.6. Fish B and C recorded stimulation indices of 74 and 4.75 respectively (Fig. 6.6).

Experiment 4.6 Response of Dogfish Leucocytes to Phytohaemagglutinin

All maximum stimulation indices were obtained using 10 μg ml^{-1} PHA in the culture medium: these were 5.25, 95 and 8.5 (Fig. 6.7).

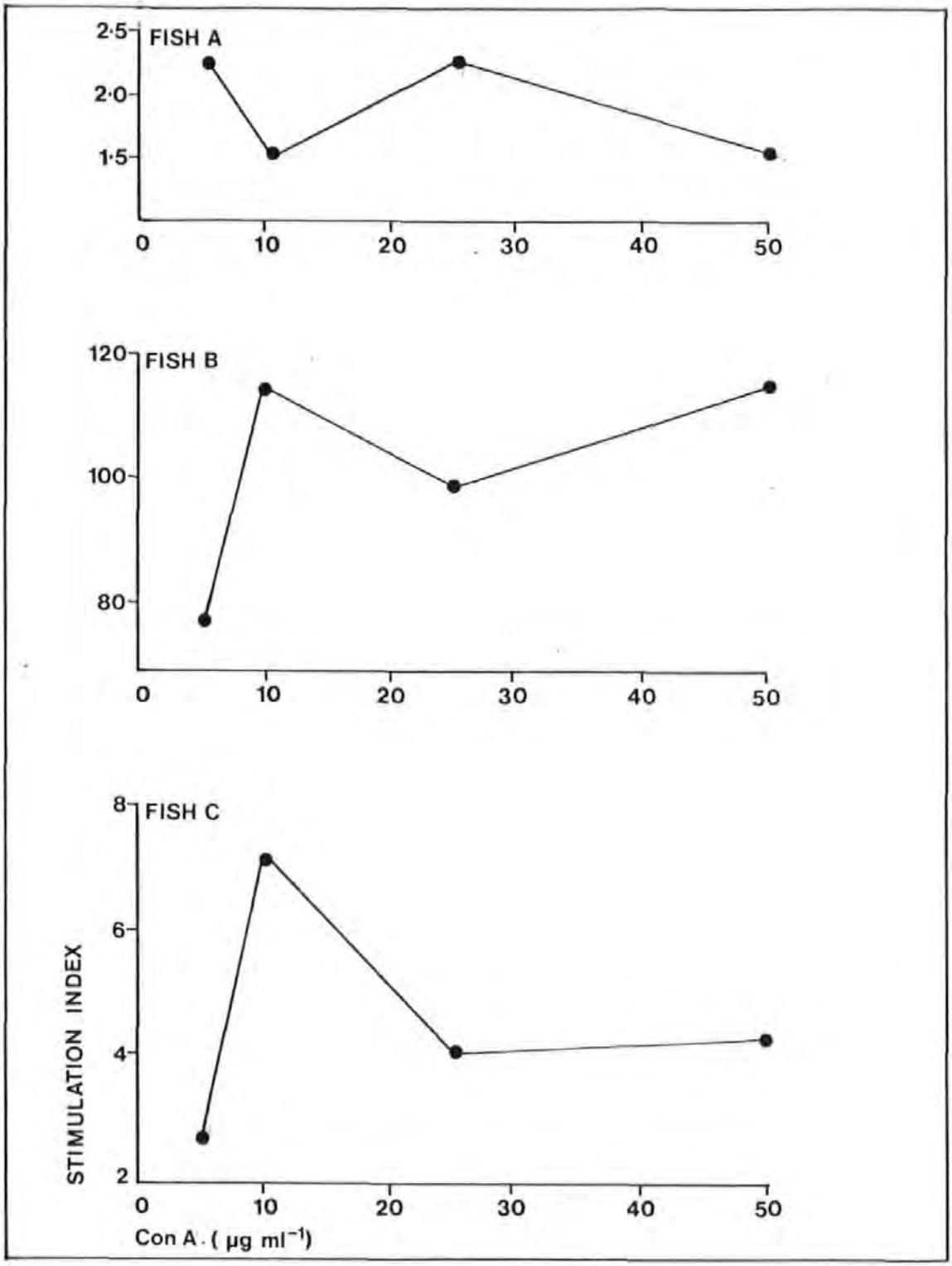


Fig. 6.5. The effect of varying concentrations of Concanavalin A on peripheral blood leucocyte cultures from three dogfish.

With the exception of Exp. 4.8 (mixed lymphocyte reactions) Exps. 4.4, 4.5, 4.6, 4.7 and 5.4 were carried out simultaneously and used the same control cultures to calculate the stimulation indices. The controls comprised leucocytes from 7 different fish cultured in growth medium alone.

Fish		Duplicates	Mean
1	(A)	270 116	193
2	(B)	181 69	125
3	(C)	164 330	247
4	(D)	244 326	285
5	(E)	248 178	213
6	(F)	163 365	264
7	(G)	469 337	406

The mean and standard deviation of c.p.m. obtained for these cultures was : 248 $\pm$ 87.

Exp. 4.4: Response to Concanavalin A

FISH A

$\mu g\ ml^{-1}$ Con A	c.p.m.	Mean	SI
5	634 496	565	2.3
10	407 361	384	1.5
25	673 457	565	2.3
50	421 333	377	1.5

FISH B

$\mu g\ ml^{-1}$ Con A	c.p.m.	Mean	SI
5	24338 14052	19195	77.4
10	37159 19861	28510	115
25	27860 20996	24428	98.6
50	32516 24028	28272	114

FISH C

$\mu g\ ml^{-1}$ Con A	c.p.m.	Mean	SI
5	749 551	650	2.6
10	1963 1561	1761	7.1
25	1086 898	992	4.0
50	1529 555	1042	4.0

Concanavalin A was obtained from Calbiochem Ltd.

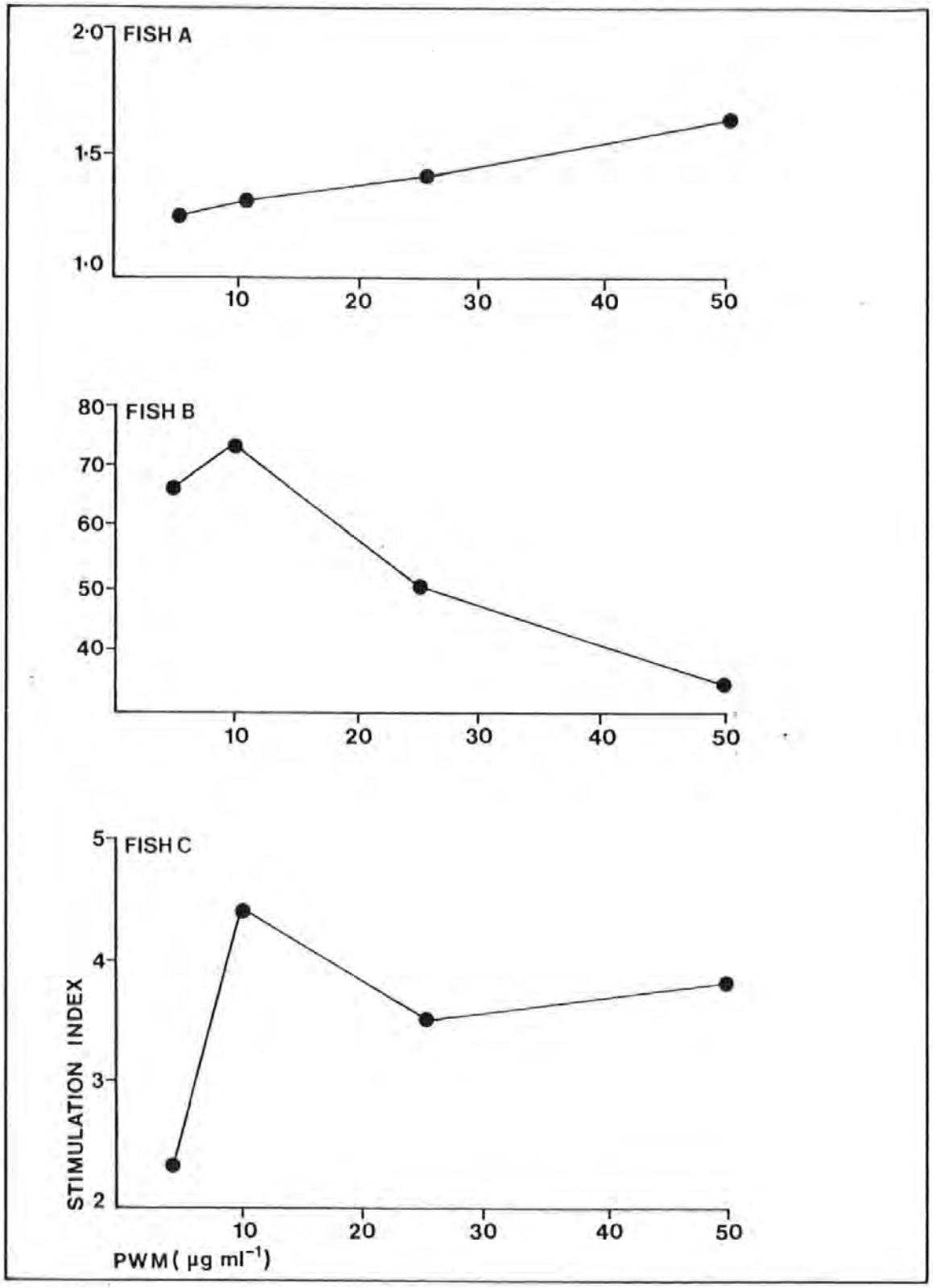


Fig. 6.6. The effect of varying concentrations of Pokeweed mitogen on peripheral blood leucocyte cultures from three dogfish.

Exp. 4.5: Response to Pokeweed Mitogen

FISH A

$\mu g\ ml^{-1}$ PWM	c.p.m.	Mean	SI
5	411 219	315	1.27
10	366 278	322	1.3
25	263 437	350	1.4
50	561 267	414	1.67

FISH B

$\mu g\ ml^{-1}$ PWM	c.p.m.	Mean	SI
5	11960 21550	16755	68
10	15040 21704	18372	74
25	12369 13415	12467	50
50	5857 11429	8643	35

FISH C

$\mu g\ ml^{-1}$ PWM	c.p.m.	Mean	SI
5	617 523	570	2.3
10	825 1357	1091	4.4
25	1129 607	868	3.5
50	1016 868	942	3.8

Pokeweed mitogen was obtained from the Sigma Chemical Co.

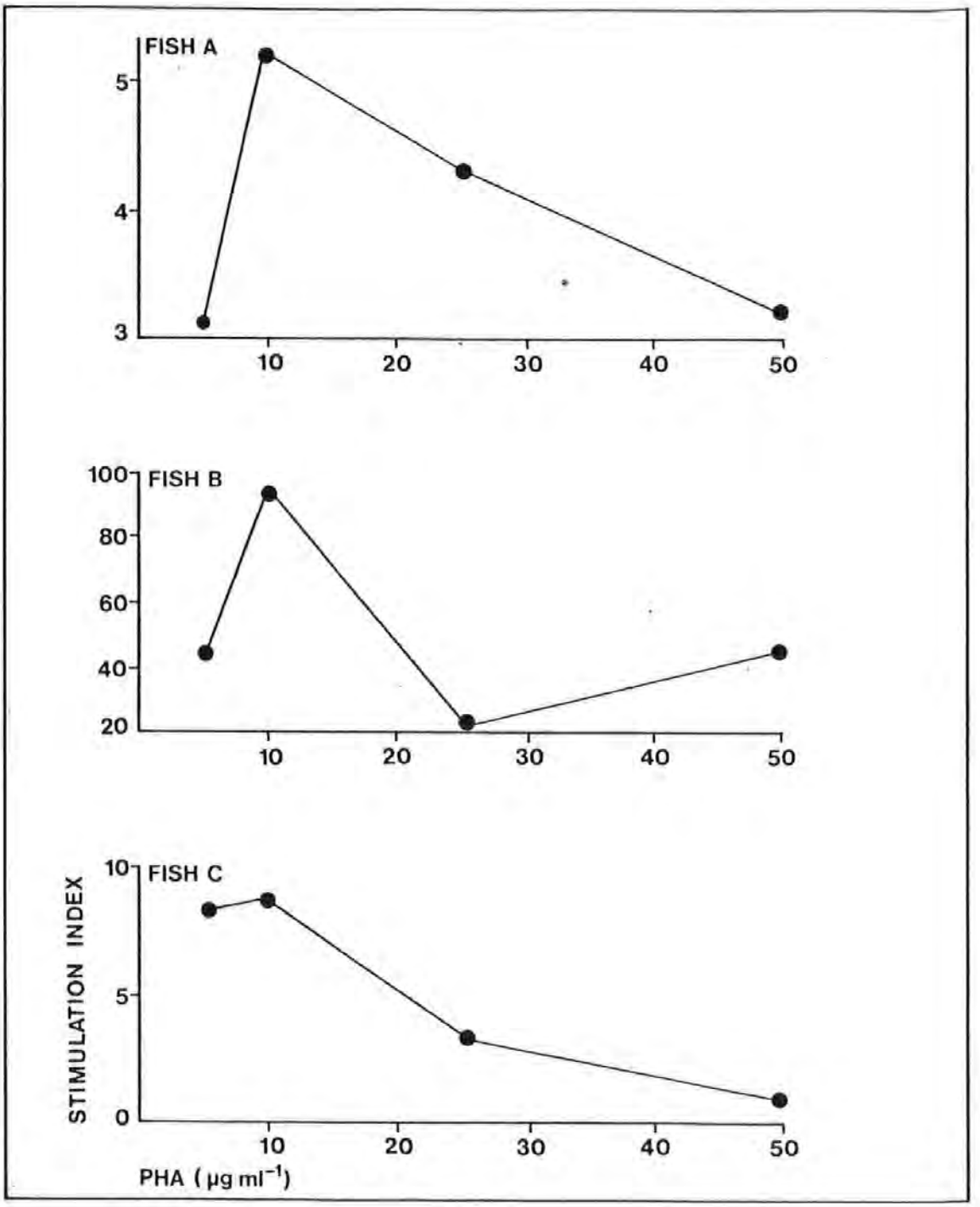


Fig. 6.7. The effect of varying concentrations of Phytohaemagglutinin on peripheral blood leucocyte cultures from three dogfish.

Exp. 4.6: Response to Phytohaemagglutinin

FISH A

$\mu g\ ml^{-1}$ PHA	c.p.m.	Mean	SI
5	855 683	769	3.1
10	1042 1538	1290	5.2
25	742 1390	1066	4.3
50	885 703	794	3.2

FISH B

$\mu g\ ml^{-1}$ PHA	c.p.m.	Mean	SI
5	13155 8473	10813	43.6
10	15951 30573	23262	93.8
25	6082 4086	5084	20.5
50	13565 8607	11086	44.7

FISH C

$\mu g\ ml^{-1}$ PHA	c.p.m.	Mean	SI
5	2161 1955	2058	8.3
10	1853 2412	2133	8.6
25	915 721	818	3.3
50	172 374	273	1.1

Phytohaemagglutinin was obtained as PHA-P from Wellcome Ltd.

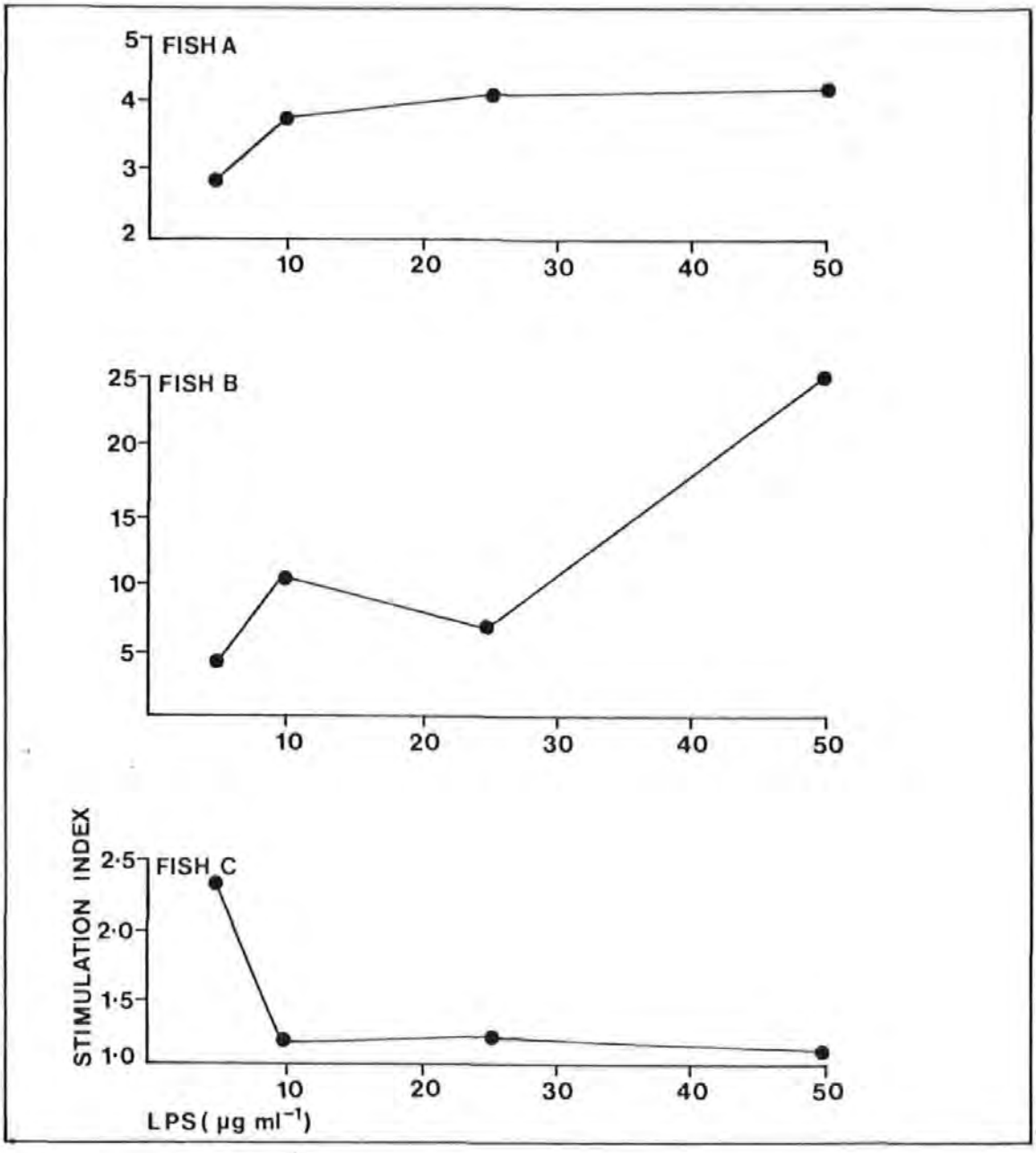


Fig. 6.8. The effect of varying concentrations of bacterial lipopolysaccharide on peripheral blood leucocyte cultures from three dogfish.

Exp. 4.7: Response to Lipopolysaccharide

FISH A

$\mu g\ ml^{-1}$ LPS	c.p.m.	Mean	SI
5	882 556	719	2.9
10	967 869	918	3.7
25	781 1253	1017	4.1
50	785 1299	1042	4.2

FISH B

$\mu g\ ml^{-1}$ LPS	c.p.m.	Mean	SI
5	1128 906	1017	4.1
10	2079 2931	2505	10.1
25	1208 2264	1736	7.0
50	7354 5046	1154	25.0

FISH C

$\mu g\ ml^{-1}$ LPS	c.p.m.	Mean	SI
5	637 519	578	2.3
10	440 156	298	1.2
25	224 372	298	1.2
50	237 309	273	1.1

Bacterial (E. coli)lipolysaccharide was obtained from the Sigma Chemical Co.

Experiment 4.7 Response of Dogfish Leucocytes to Bacterial Lipopolysaccharide

An obvious response pattern to this mitogen was not seen, either in similarity of the dose required for maximum stimulation or in terms of the response profiles obtained. Maximum SI were 4.2, 25 and 2.3 obtained with 25, 50 and 5 μg ml^{-1} mitogen respectively. It is perhaps of note that the response profiles obtained from fish A and C reached a plateau after the 10 μg ml^{-1} LPS and were not enhanced by further addition of this mitogen (Fig. 6.8).

C) Recognition of Histocompatibility Antigens

Experiment 4.8 Mixed Leucocyte Culture in the Dogfish

Leucocytes from 3 fish (A,B and C) were mixed together in various combinations, cultured and their uptake of ^{3}H-thymidine measured. Each culture contained approximately 2 x 10^6 cells (10^6 from each fish). Controls were cultures containing 2 x 10^6 cells from each individual fish. The SI for each cell culture is given in Table 6.2.

COMBINATION OF LEUCOCYTES CULTERED	STIMULATION INDEX
A/B	2.2
A/C	1.7
B/C	1.8

Table 6.2. Stimulation indices obtained from mixed leucocyte cultures made in combination with cells taken from 3 animals, A, B and C.

Exp. 4.8: Mixed Leucocyte Cultures

Cultures	c.p.m. 2×10^6 cells
FISH A	672
	424
FISH B	780
	576
FISH C	750
	582
	mean = 631

Culture	c.p.m.	Mean	SI
A/B	1592	1388	2.2
	1184		
A/C	1261	1073	1.7
	885		
B/C	1497	1136	1.8
	775		

N. B. These animals are not those used in Exps. 4.4, 4.5, 4.6, 4.7 & 5.4.

Experiment 4.9 Graft Versus Host Reaction in the Dogfish

This experiment was based on the procedure of Borysenko & Tulipan (1973) in which allogeneic spleen cells were injected into a non-immunosuppressed animal.

Two groups of 5 fish were used. The first group was injected intraperitoneally with 10^7 allogeneic spleen cells in sterile elasmobranch saline. The second group was given 2 injections of allogeneic cells: one intraperitoneally and the other intravenously. Each of these injections comprised 10^7 cells. Ths fish were examined every day for 5 weeks for obvious respiratory distress, abnormal behaviour patterns, rashes, erythema or other signs of malaise. No ill effects were observed. After this period the fish were killed. No gross abnormalities were noticed in the major internal organs (spleens, liver and kidney) and there were no signs of lesions on them or in the peritoneal cavity. Spleen weights were recorded and related to a curve showing total body weight against spleen weight for normal fish (Fig. 6.9). Tentative evidence of splenomegaly was seen in the test group. Histological examination of spleens from this group revealed cellular aggregation around venous and arterial blood vessels (Fig. 6.10) similar to those in antigen stimulated fish (Experiment 4.2) and it would appear that some immunological reaction had occurred.

III Hypersensitivity Reactions in the Dogfish

A series of *in vivo* and *in vitro* experiments were made in order to assess the ability of the animal to make immediate and delayed hypersensitivity responses.

Experiment 4.10 Assessment of Active Systemic Anaphylaxis

A group of 5 fish were each given intramuscular

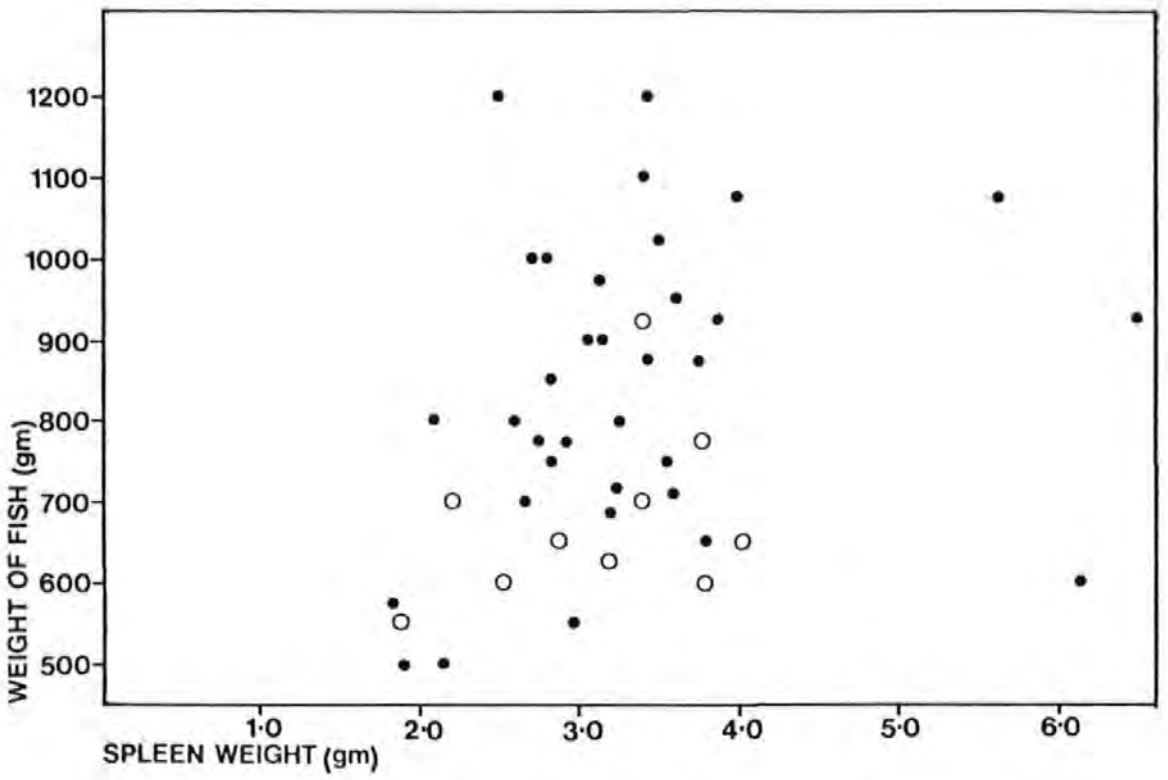
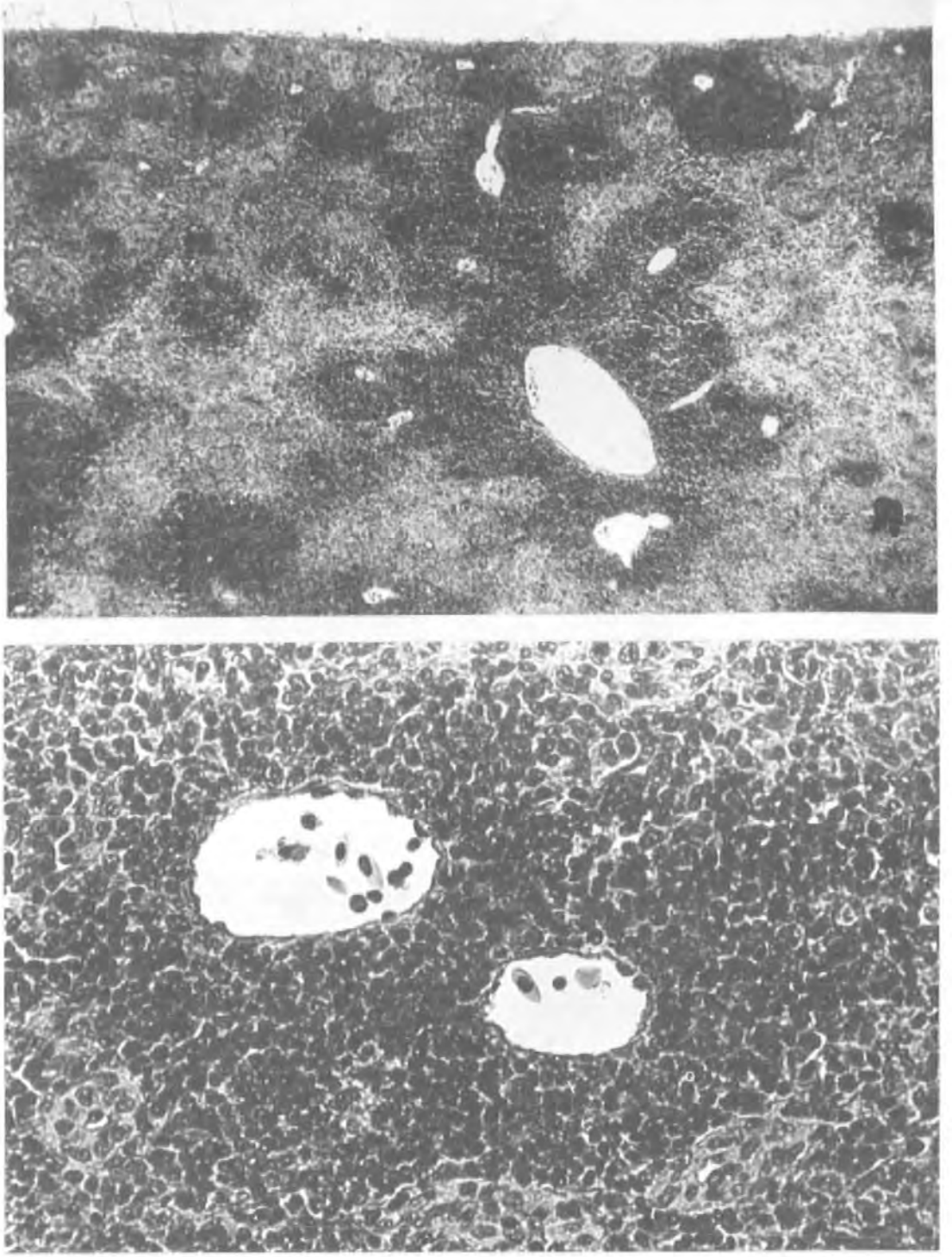


Fig. 6.9. Relationship of total body weight/spleen weight in normal fish (solid circles) and in fish injected with allogeneic spleen cells (open circles).



(a)

(b)

Fig. 6.10. Micrographs of spleens from fish injected with allogeneic spleen cells. Stain: Methyl Green Pyronin Y.

a) Spleen (x48) from fish taken 20 days after treatment with 10^7 allogeneic spleen cells. Highly defined areas of white pulp are clearly visible.

b) Spleen (x212) from the same animal showing aggregation of lymphocytes around blood vessels.

injections comprising 0.25 ml horse serum emulsified in 0.25 ml FCA, identical injections were administered 5 days later and challenge injections comprising 0.5 ml horse serum alone given at 14, 21 and 28 days.

The fish were examined for 30 minutes after each challenge. No apparent respiratory distress, abnormal behaviour or other ill effects indicative of anaphylactic shock were observed at any time.

Experiment 4.11 In Vitro Assessment of Anaphylaxis

These fish were sensitised with one intramuscular injection comprising 10 mg PPD in FCA. After 21 days the fish were sacrificed and several portions of gut including the oesophageal lymphomyeloid tissue (organ of Leydig) were cut from each fish and suspended in an organ bath attached to a kymograph. The organ bath was filled with supplemented Eagle's medium and was constantly aerated (Hudson & Hay, 1976). After allowing the tissue to relax for 20 minutes various quantities of PPD were added. Addition of antigen to the bath in various concentrations ranging from 1 $\mu g\ ml^{-1}$ to 250 $\mu g\ ml^{-1}$ failed to elicit contraction in any portion of intestinal muscle from any of the sensitised fish.

Experiment 4.12 Homologous Passive Cutaneous Anaphylaxis

Sera from a group of fish sensitised with horse serum (Experiment 4.11) were pooled. A group of 4 unsensitised fish were given intradermal injections on their ventral surface. Three such injections were administered to each fish, two of antiserum (0.25 ml and 0.1 ml) and one of elasmobranch saline (0.15) ml). An intravenous injection comprising 0.5 ml horse serum and 2% (w/v) Evans Blue was given to 2 fish 4 hours later. The other 2 fish were given the antigen/dye injection after

72 hours in an attempt to demonstrate delayed PCA. Two fish that had been initially given an injection of elasmobranch saline were used as controls. After an injection of antigen/dye the injected sites were examined for signs of blueing. No heavy deposition of dye was noted around these sites in any of the fish although they were kept under observation for up to 6 hours.

Experiment 4.13 Heterologous Passive Cutaneous Anaphylaxis

A group of 4 guinea pigs were given 3 injections in their flanks comprising 0.1 ml and 0.2 ml dogfish anti-horse serum and 0.15 ml PBS. Four hours later a challenge injection of 0.25 ml horse serum in 2% (w/v) Evans Blue was given intracardially to 2 of the animals. One guinea pig was sacrificed after 20 minutes and the sub-cutaneous surface of the skin was examined for blueing. The second animal was killed after 4 hours and examined similarly. The antigen/dye injections were delayed for 72 hours after the initial intradermal injections in the other 2 animals. Both these guinea pigs were killed after 30 minutes and examined for sub-cutaneous blueing. No immediate or delayed PCA reactions were observed in any of the animals tested.

Experiment 4.14 Assessment of Delayed Hypersensitivity by Direct Skin Testing

Sixteen fish were sensitised with one intramuscular injection consisting of 10 mg PPD in FCA. Half this group were placed on an immunosuppressive regime consisting of twice weekly injections of 200 mg cyclophosphamide (Koch-Light Laboratories Ltd.) in 1 ml of elasmobranch saline (to which a few drops of absolute alcohol were added to aid solubility). These were administered intraperitoneally. Challenge injections comprising 10 mg PPD in 0.5 ml in FCA were given 2 weeks later. These injections were made intradermally in the ventral surface of the fish. Half of the

immunosuppressed group (4 out of 8) showed haemolytic lesions after 3-4 weeks indicative of a delayed hypersensitivity response (Fig. 6.11). Some of these animals developed lesions at the original (sensitising) injection site. Only 2 out of the 8 non-immunosuppressed fish developed lesions.

Experiment 4.15 Leucocyte Migration Inhibition

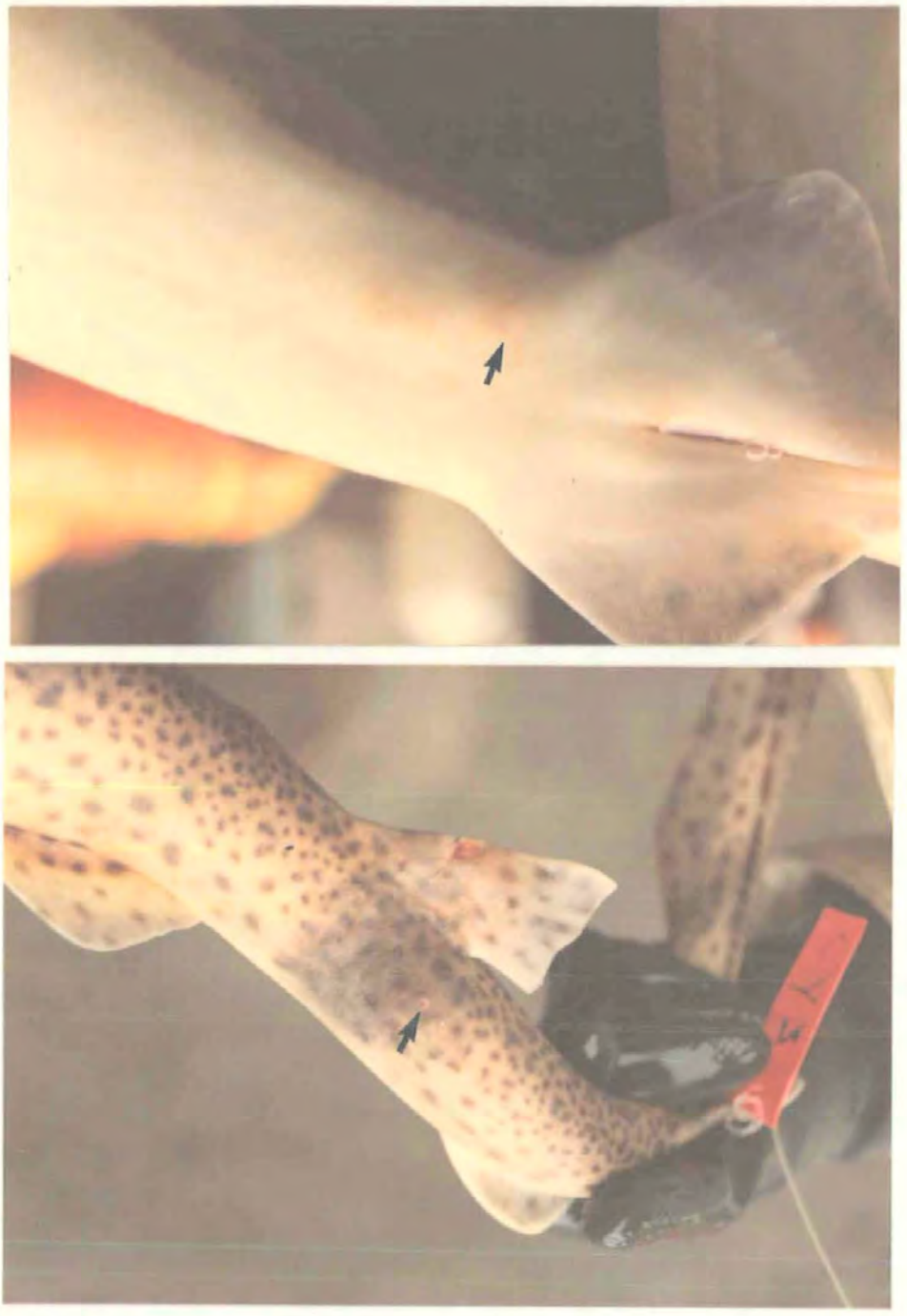
An attempt to demonstrate delayed hypersensitivity reactions in vitro was made using the leucocyte migration inhibition test.

i) Initially 2 groups of dogfish, each comprising 3 animals, were sensitised with BSA and KLH respectively. Standard LMI tests were set up using various concentrations of antigen in the culture medium (40, 100, 200 $\mu g\ ml^{-1}$). The specificity of the response was examined by testing the sensitised leucocytes with the reciprocal antigen e.g. KLH-sensitised cells were challenged with BSA and vice versa. In each case the response was shown to be specific. The results were shown in Table 6.3.

CONCENTRATION OF ANTIGEN ($\mu g\ ml^{-1}$)	40	100	200
ANTIGEN	MIGRATION INDEX		
BSA	0.41	ND*	0.71
KLH	0.62	0.51	0.55

ND* - not done

TABLE 6.3. Effect of antigen dose on migration inhibition. Each migration index represents the mean of a group of 3 fish.



(a)

(b)

Fig. 6.11. a) Erythema at the site of a PPD challenge injection given to a previously sensitised fish.

b) Haemorraghic adjuvant lesion at a primary injection site.

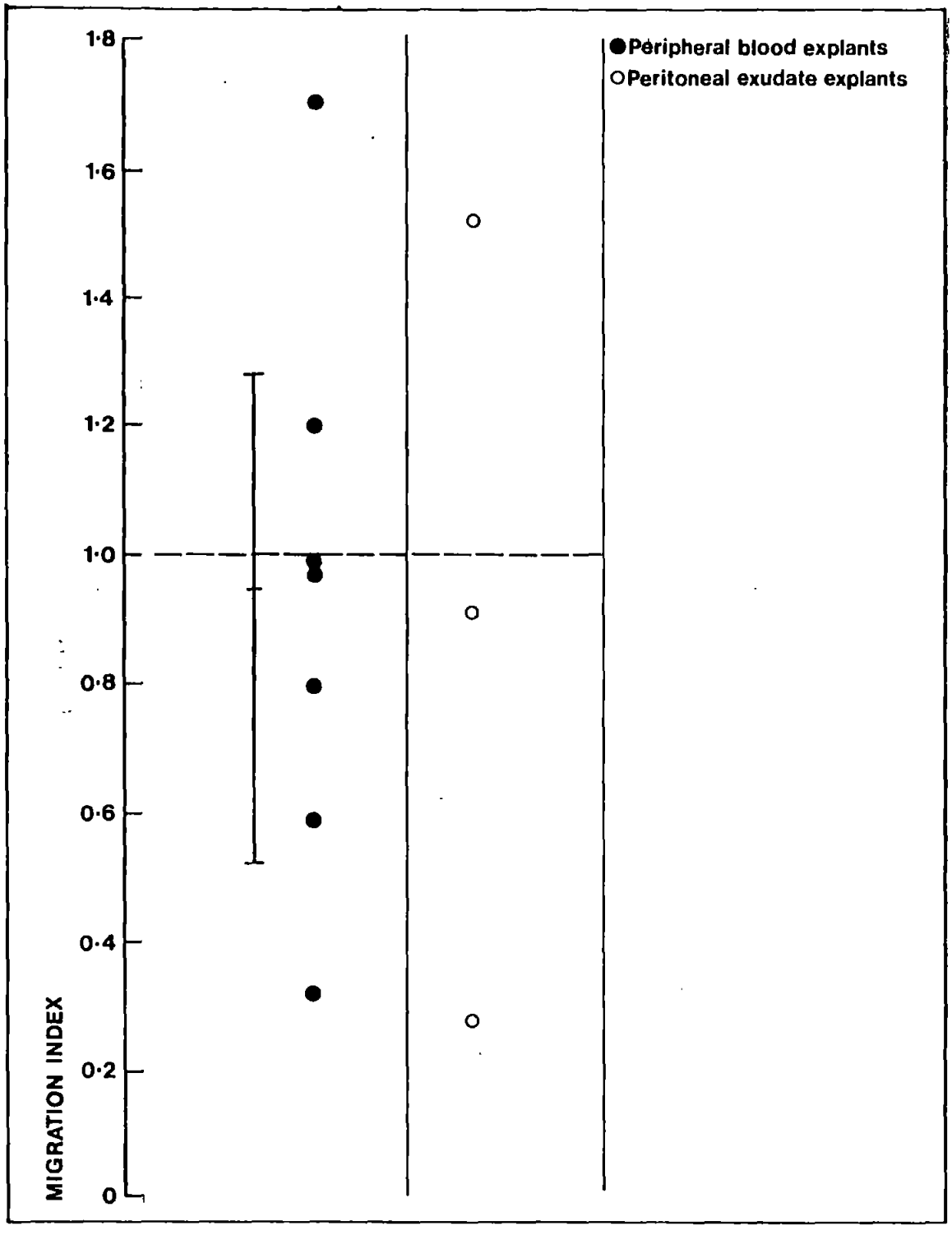


Fig. 6.12. Migration inhibition indices for leucocyte cultures from sensitised fish incubated in the presence of PPD. A value of less than one indicates migration inhibition. Each point represents a single fish. The vertical bar represents standard deviation.

ii) Having established that the response was apparently specific, a larger experiment was carried out. Eight fish were sensitised with PPD (two intramuscular injections each of 10 mg in FCA, the second injection being given after 7 days). One month after the initial injection the fish were bled and the ability of PPD to cause leucocyte migration inhibition was examined. Most cultures demonstrated some inhibition (Fig. 6.12) although this reaction was not as marked as that observed in the leucocytes cultured from fish sensitised with BSA or KLH.

An attempt to use oil induced peritoneal exudates from the same group of fish was unsuccessful. Half of the fish (4) were given intraperitoneal injections of oil. After 3 days one fish had died. Leucocyte cultures from the remaining animals were inconclusive: both inhibition and negative inhibition being observed (Fig. 6.12).

Discussion

To date, there have been no reports regarding the site of antibody producing cells in an elasmobranch fish. The findings from Experiment 1.3 demonstrates that the spleen is a major site of antibody synthesis and that plaque forming cells can be detected in this organ before the presence of circulating immunoglobulin.

Since Jerne & Nordin (1963) described the haemolytic plaque assay, there have been several reports regarding the site of antibody production in teleost fish. Smith et al. (1967) recognised cells producing haemolytic antibody from the spleen and pronephros of the bluegill. The pronephros contained larger numbers of antibody secreting cells than the spleen, a finding which the authors attributed to the more prominent lympho-reticular morphology of the organ. Histological examination showed marked similarities between the cellular structure of the pronephros of the bluegill and mammalian lymph nodes. They also found

that antibody producing cells appeared in the pronephros and (to a lesser extent) the spleen before the appearance of serum antibody, a comparable finding to the present study where spleen cells capable of producing antibody were detected before serum antibody of the same specificity. Similarly they noted that the assay would only function in the presence of homologous complement.

Sailendri & Muthukkaruppan (1975a) demonstrated PFC in the pronephros, spleen and thymus of Tilapia. Again the appearance of PFC preceded the presence of serum antibody and also that fresh homologous serum was necessary for haemolysis to take place, guinea pig complement again proving to be unsatisfactory.

Thus it would appear that the spleen is the major site of antibody synthesis in the dogfish. The pronephros was not examined as it was found to be a non-lymphoid organ (see Chapter 5). The thymus can only be found in neonatal fish and the other possible sites, Leydig's organ and the epigonal tissue appear to be too poorly differentiated to be capable of producing antibody (in the sense that it did not contain lymphocytes and plasma cells traditionally associated with this function - see Chapter 5) although their possible immunological contribution cannot be ignored. To assess this contribution might pose technical difficulties, however, due to the distribution and diffuse nature of these tissues.

The inability of the dogfish and the other fish described to fix mammalian complement may reflect on the phylogenetic differences in these proteins. Borsos & Rapp (1965) showed some forms of antibody fix complement much less efficiently. The reasons for this are uncertain but it seems reasonable to speculate that the initial antibody-complement binding does not take place due to fundamental

differences in either the complement binding sites on the antibody molecule (the CH_2 region) or the complement molecule itself (the Clq part of the first complement component the Clqrs complex).

Histological changes in the spleen also seem to reinforce the idea that this organ is a site of antibody synthesis. The appearance of dense lymphoid accumulations around blood vessels was parallel to the kinetics and time course of spleen PFC, with a maximum response being recorded after 10-15 days. Little information exists on antigenic stimulation of lymphoid tissue in lower vertebrates. Sailendri & Muthukkaruppan (1975a) reported that spleen imprints from immunised Tilapia showed a considerable increase in lymphoid cells, particularly in the reticular areas. Increased vascularisation was also consistently observed after immunisation.

Borysenko (1976) described similar changes in the spleens of snapping turtles (Chelydra serpentina) immunised with KLH. The reaction followed a slightly faster time course to that observed in the dogfish with maximum enlargement of the white pulp areas occurring after 8-12 days followed by a return to normal after 15-20 days. This experiment was carried out at 30°C and this relatively high temperature probably caused this more rapid response. Lymphoid and lymphoblast cells were primarily associated with these enlargements.

Over the past 2-3 years there have been a number of studies made on leucocyte membrane determinants of fish including the presence of surface immunoglobulin. This work is summarised in Table 6.4.

AUTHOR	SPECIES STUDIED	CELL LINE	Ig^+ CELLS
Emmrich et al. 1975	Carp	Lymphocytes	30-58%
		Splenocytes	25-45%
		Thymocytes	60-70%
Ellis & Parkhouse 1975	Skate	PBL	60-80%
		Splenocytes	63-80%
		Thymocytes	44-82%
Ellis, 1976	Plaice	Lymphocytes	100%
Warr, et al. 1976	Goldfish	Splenocytes	68-80%
		Thymocytes	82-99%
Etlinger et al. 1976	Rainbow Trout	Lymphocytes	>90%
		Splenocytes	>90%
		Thymocytes	>90%
		Anterior Kidney Cells	>90%
Clem et al. 1977	Bluegill	Lymphocytes	>90%

TABLE 6.4. Summary of reports describing the presence of immunoglobulin determinants on various fish lymphoid tissues.

The results obtained from the studies on the dogfish appear to agree with the findings of these authors in that a high proportion of lymphoid cells appear to have immunoglobulin on their surface. The actual numbers of these cells differs slighly from the findings of other investigators but this discrepancy probably just reflects the lack of standardisation of cell purification procedures and variable specificity of the antisera used. It seems certain, however, that the numbers of cells that stain positively for immunoglobulin is much higher than mammalian leucocyte preparations, for example only 10%-20% of mouse lymphocytes stain in this way (Greaves et al., 1974).

Warr & Marchalonis (1977) reacted specific anti-immunoglobulin antiserum with radio-iodinated and detergent solubilised surface membranes from goldfish splenocytes. Analysis by SDS-polyacrylaimide gel electrophoresis was carried out and comparisons were made with purified and radiolabelled goldfish and mouse serum immunoglobulins. They found that the antiserum specifically detected polypeptides with molecular weights approximately corresponding to the heavy and light chains of goldfish serum IgM, however, the heavy (μ) chain of the splenocyte IgM differed from that of the serum IgM by some 10,000 daltons.

Using similar techniques, Clem et al. (1977), showed that membrane bound immunoglobulin from lymphocytes, splenocytes, thymocytes and head kidney cells from the bluegill closely resembled the heavy and light chain components of its serum IgM. They did express concern, however, that these immunoglobulin determinants may have strong physical association with molecules that did not resemble immunoglobulins and that this may have hindered the interpretation of their results.

Fiebig, Scherbaum & Ambrosius (1978) made a careful immunochemical study on membrane bound immunoglobulin in carp lymphoid tissue and demonstrated that not only did thymocytes have immunoglobulin determinants on their surface but that these molecules differed from both serum and lymphocyte surface immunoglobulin. The constant (C) regions of these molecules were found to have common determinants. These authors hypothesised that at an earlier evolutionary stage the immunoglobulin heavy chain was separated into a μ_B and μ_T chains, long before the appearance of a non-μ isotype.

Thus there is convincing evidence that immunoglobulin is clearly expressed on a high percentage of fish lymphoid cells as demonstrated by immunofluorescent and electrophoretic methods. Because of this the immunofluorescent staining technique will probably not be a very useful tool for differentiating between lymphocyte sub populations as cell surface immunoglobulin is not a specific marker of B-cells as it is in mammals.

The attempts to distinguish leucocyte populations according to their mitogen responsiveness were largely unsuccessful for a number of reasons. All the mitogens tested stimulated in vitro cultures of the cells. This is a relatively crude method as the experiments did not take into account possible regulatory effects (either suppressor or helper) that may be exerted by cells other than lymphocytes or the possible uptake of ^{3}H-thymidine by these cells themselves. Ideally, lymphocytes only should have been used but dogfish lymphocytes are hard to identify and quantitate quickly (Chapter 5) and attempts to purify them on Ficoll-Triosill gradients were completely unsuccessful. The observation that dogfish leucocytes can respond to mitogenic stimuli is useful in that it can form the basis of further investigations, however, as none of

these substances was obviously more efficient at promoting increased uptake of ^{3}H-thymidine, a sub-population of cells was not identified on the basis of a differential response.

The magnitude of the response is of interest because of the great difference between fish. Generally the response profiles were found to be similar for each mitogen but the stimulation indices were highly variable between individual fish. One animal (Fish B) consistently gave a very high response in terms of isotope uptake and incorporation and on the other hand Fish A always responded only slightly. As the cultures were not apparently contaminated with bacterial infections, these differences were probably due to genetic variation: some fish behaving as high and others as low responders and this serves to reflect the difficulties in working with a such an outbred population.

Lopez et al. (1974) reported that only a sub-set of nurse shark lymphocytes would respond to PHA stimulation. When the cells were applied to a Ficoll-Isopaque gradient 2 bands and a sediment were obtained. All these bands responded to Con A but only the bottom layer of cells responded to PHA. Leucocytes that had not been fractionated did not respond to PHA and these authors attributed this phenomenon to the presence of suppressor cells. This explanation is perhaps presumptuous for the presence of cells which can control mitogenesis does not necessarily mean they can act as immunological suppressors and further experimental work is necessary before such a population of cells can accurately be identified.

The work of Etlinger et al. (1976) shows that rainbow trout leucocytes respond differentially to the mitogens Con A, LPS and PPD. Thymocytes were stimulated by Con A (a mammalian T-cell mitogen) but not by LPS or PPD

(B-cell stimulators). Leucocytes from the anterior kidney (a B-cell like area - Chapter 5) responded only to LPS and PPD and peripheral blood and splenic leucocytes responded to all 3 mitogens. To date this is the most convincing evidence to show that populations of fish lymphoid cells can behave similarly to mammalian B and T cells in terms of mitogen responsiveness. Until the methodology of fish cell culture techniques has been more fully investigated, for example, the physical nature of the culture vessels, the effects of homologous and heterologous sera and CO_2 on cell growth, all conclusions must be made with caution.

The phylogenetic origins of self-non-self immune recognition in vertebrates are uncertain. The needs for such discrimination are self evident and gross manifestations of immunoincompatability have been observed in very primitive species. Raison, Hull & Hildemann (1976) described allograft rejection in marine corals. Annelids have also been shown to be capable of recognising and rejecting skin allografts and xenografts (reviewed by Cooper, 1976 a,b,c). In fish, rejection of scale transplants has been demonstrated in several species including the lamprey stingray and goldfish (Hildemann, 1970) and recently, the rosy barb (Barbus conchonius) Rijkers & Van Muiswinkel (1978).

Demonstration of the in vitro mixed leucocyte reaction (MLR) or mixed leucocyte culture (MLC) in fish has met with limited success. McKinney et al. (1976) consistently failed to obtain a positive MLR in cell cultures from snappers, gars and sharks. Salmonid fish, however, do seem to be capable of such a response. Ellis (1977b) reported a positive MLR in salmon fry and described the ontogeny of this response. The maximum stimulation index (approximately 2) was relatively small but nevertheless can be considered

positive. A stimulation index of 2-6 is commonly found for mouse cultures (Röllinghoff, Pfizenmeier, Trostmann & Wagner, 1975) and is usually much less than those obtained for mitogen stimulated cells.

Etlinger, Hodgins & Chiller (1977) also obtained a positive response with peripheral blood leucocyte cultures taken from rainbow trout. These workers obtained SI values in test cultures approximately 8 times higher than the control cultures.

The stimulation indices obtained in this study from MLR cultures appear to be comparable with the results achieved with salmonid fish leucocyte cultures but are at variance with those of McKinney et al. (1976) and therefore must be regarded with caution although the results of the in vivo graft-vs.-host reaction go some way to substantiate these findings.

The GVH reactions were not vigorous like those reported by Borysenko & Tulipan (1973) in the snapping turtle probably because the reaction was carried out in adult fish and thus major physiological manifestations such as growth impairment would not have become obvious. Nevertheless histological changes were noted in the spleens of challenged fish and there appeared to be some evidence of splenomegaly although to make a definite conclusion about this latter result would have entailed examination of very much larger groups of test and control fish.

The experimental procedure was based on that of Borysenko & Tulipan (1973) and more dramatic results may have been obtained if the splenic leucocytes had been injected into fish that had been immunosuppressed either by X-irradiation or, more practically, had been treated with a lymphocytotoxic agent such as cyclophosphamide.

It would appear that the dogfish is capable of recognising histocompatability antigens although the response to them is apparently weak. However, like other experiments carried out at the cellular level in fish, complete measurement and interpretation of such reactions is limited until the experimental methodology is more fully understood.

The current literature regarding hypersensitivity reactions in fish is very sparse and mainly only attempts to induce anaphylactic and skin sensitising reactions (the immediate, Type I, hypersensitivity reactions as defined by Coombs & Gell, 1975) have been made.

All attempts to induce such reactions in the dogfish failed. Several groups of workers have tried to demonstrate Type I hypersensitivity in fish and with only two exceptions none succeeded. Failure to induce systemic anaphylaxis in teleost fish was reported by Arloing & Langeron (1922), Lukyanenko (1967) and Harris (1973). All these groups used repeated injections of horse serum in order to induce this reaction. The inability to cause direct anaphylaxis in the dogfish is consistent with the findings of these workers.

Dreyer & King (1948) made the only report of a positive anaphylactic reaction in 4 species of teleost fish challenged with ovalbumin and horse serum. These investigators observed gross behavioural abnormalities. changes in operculum movement and general weakness in the antigen-challenged fish. As these results have not been repeated by any workers, despite meticulous attempts to reproduce the experimental conditions described by Dreyer & King there is an element of doubt as to the validity of their results and their findings may have been due to environmental conditions rather than an allergic state of the fish.

Attempts to show immediate hypersensitivity reactions by passive cutaneous anaphylaxis have also failed. Clem & Leslie (1969) could not induce PCA in margates sensitised with BSA; Hodgins, et al., (1967) failed using both homologous and heterologous PCA in trout using anti-KLH sera. Harris (1973) could not obtain a positive reaction from dace or chub using horse serum and antigen extracts of acanthocephalan parasites (with which the fish were infected). Both heterologous and homologous PCA techniques were employed. Again these findings are consistent with the failure of this study to demonstrate any sort of skin-sensitising reactions in the dogfish.

Fletcher & Baldo (1974) were successful in demonstrating an immediate reaction in the plaice using fungal extracts that would precipitate with human C-reactive protein. Immediate hypersensitivity (characterised by rapidly appearing erythema reactions following sub-dermal antigen injections) could be induced in a non-reactive species after injection with serum from a reactive species. The factor was not destroyed after heating at 56^{o}C but was only found in species where calcium-dependent "CRP-like" precipitins were present so whether or not this was a true Type I reaction mediated by an effector protein such as an IgE-like molecule is uncertain. In the light of these findings the inability to show the presence of CRP in the dogfish (Chapter 3) may, to some extent explain its apparent inability to produce immediate skin reactions.

Type I reactions are classically initiated by an antigen which reacts with IgE (reaginic antibody) bound to mast cells or basophils. This leads to the degranulation of these cells and the release of vasoactive amines. Although only an IgM antibody class has been reported in fish, it can exist in monomeric, tetrameric and pentameric form

and is thought that it possibly acts as a functional mimetic for the classes of antibody found in higher vertebrates (IgG, IgA & IgM). To date there have been no reports of an IgE-like molecule in fish and this reinforces the argument that these animals are incapable of Type I (IgE mediated) hypersensitivity reactions, however, the concentration of IgE in the serum of animals tends to be very low (10-130 μg 100 ml^{-1}) and requires the use of radioimmunoassay for its detection as normal serological techniques are too insensitive. Thus the possibility of such an antibody in fish cannot entirely be ruled out.

A further reason for the inability of Scyliorhinus and other fish species to produce anaphylactic reactions is the absence of mast cells and basophils. In mammals these are the cells from which histamines are released. The literature describing these cells is confused and conflicting and it is uncertain whether they exist in fish (Ellis, 1977a). Basophils were not detected in the peripheral blood leucocyte population of the dogfish (Chapter 5). Since these cells are necessary for anaphylaxis (in vertebrates) their apparent absence in fish possibly precludes this type of reaction.

Type IV hypersensitivity is the cell-mediated, delayed type of reaction. In humans a variety of clinical manifestations can occur but all are due to cellular infiltration around the site of antibody challenge.

Finstad & Good (1966) reported a delayed hypersensitivity (DH) reaction in sharks. Horned sharks, after receiving a single injection of bovine gamma globulin in FCA developed chronic necrotising lesions and 100% mortality occurred after 30 days. These authors also reported 100% mortality for guitarfish and 80% mortality in the lamprey,

when similarly treated. They claimed that these reactions were a delayed allergy, although it is possible that deaths could have been due to the physical or toxic effects of adjuvant. The reaction was not true DH in the classical sense as a primary challenge injection was not given. Certainly such devastating reactions were not observed in dogfish immunised with antigen in FCA, particularly after only one injection.

Skin testing in sensitised dogfish revealed that the fish is apparently capable of DH reactions although it is not clear whether the lesions that appeared at the secondary challenge sites were entirely due to immunologic stimulus or the physical nature of the antigen itself. It is uncertain whether the reaction could be modulated by the use of immunosuppressives. In mammals cyclophosphamide treatment destroys a population of cells that are either B or T helper cells. After treatment with CY, the reaction caused by a challenge injection in a sensitised animal is greatly enhanced and this has been attributed to the loss of regulatory effect from this population of cells (Turk & Parker, 1973). Half the CY treated fish developed skin reactions and although this provides some evidence of a DH reaction, observations on a rather larger sample are necessary before positive conclusions can be made. It is perhaps of note that lesions appeared at the original injection site in 2 of the non-immunosuppressed fish and it is therefore important that the physical effects of adjuvant alone are assessed in future experiments of this type. Some further comments relating to parasite induced DH skin reactions are made in Chapter 7.

The phenomenon of leucocyte migration inhibition mediated by lymphokines such as macrophage migration inhibition factor (MIF) has been well documented in mammals (David, 1966; George & Vaughan, 1962; Remold & David, 1971)

although there are few reports of its occurrence in lower vertebrates. The lizard, Calotes versicolor, when sensitised to skin allografts showed in vitro migration inhibition in the presence of donor antigen (Jayaraman & Muthukkaruppan, 1977). McKinney et al. (1976) reported leucocyte migration inhibition in the gar. These authors described LMI using the lectins PWM, Con A and PHA, however as these substances (particularly phytohaemagglutinin) are agglutinins the migration inhibition observed in these experiments may have only been a non-specific "clumping" of the cells. In the absence of data regarding puromycin treatment to block lymphokine production (and therefore remove non-specific effects) such results must be regarded with caution. Similarly, Carlisle (1975) showed that extracts of an epidermal papilloma taken from Atlantic salmon would induce LMI but again made no attempt to control non-specific agglutination.

Surprisingly, the results obtained from the pilot experiment using BSA and KLH primed fish gave very good LMI indices and yet PPD, an antigen commonly used in DH experiments gave only slight inhibition. No explanation can be offered for this result. Very marked inhibition of migration was observed when parasite antigens were used and these results and further comments including remarks on the effect of puromycin on LMI are more fully discussed in Chapter 7. The use of oil to stimulate macrophages/ monocytes in fish peritoneal exudates does not appear to be a useful procedure for several reasons:

i) the deleterious effect of the oil (supposedly non-toxic) on the fish - high mortality occurred following the injection of oil into the peritoneal cavity and this procedure is obviously impractical.

ii) injection of substances such as oil, proteose-peptone or starch into the peritoneum can lead to anomalous results either because the induced cells do not function

normally after this challenge or, like mice, there is the possibility that eosinophils may be collected and the time course of the reaction must be carefully monitored in order to harvest the required cell line (B. Cottrell, personal communication).

CHAPTER 7

IMMUNE RESPONSES OF THE DOGFISH TO THE NEMATODE PARASITE Proleptus obtusus

Proleptus obtusus Dujardin 1845 is a spiruroid worm that parasitises the intestinal lumen of the dogfish (Fig. 7.1). Every fish examined in this study was found to be infected. The worm causes a marked inflammatory reaction (Schuurmans Steckhoven & Botman, 1932) and it was decided to make a brief investigation of the immunological relationship between the fish and the worm utilising a number of techniques already employed in this study.

I Humoral Responses of the Dogfish to Proleptus obtusus

Experiment 5.1 The Presence of Circulating Antibody to Proleptus obtusus

The sera of 20 fish were examined for the presence of anti-Proleptus antibody by the latex fixation technique. Such circulating antibody was demonstrated in 50% of the fish examined (Table 7.1).

Experiment 5.2 Specificity of the Anti-Proleptus Response

i) In order to examine the antigenic determinants of Proleptus, a number of worms were washed 3 times in elasmobranch saline, placed on a cryostat chuck, snap frozen, sectioned and stained by the indirect immunofluorescent technique in order to detect dogfish antibody. Appropriate controls were made as described previously. A ring of apple-green fluorescence could be detected around the body wall of the parasite by this procedure (Fig. 7.2) indicating

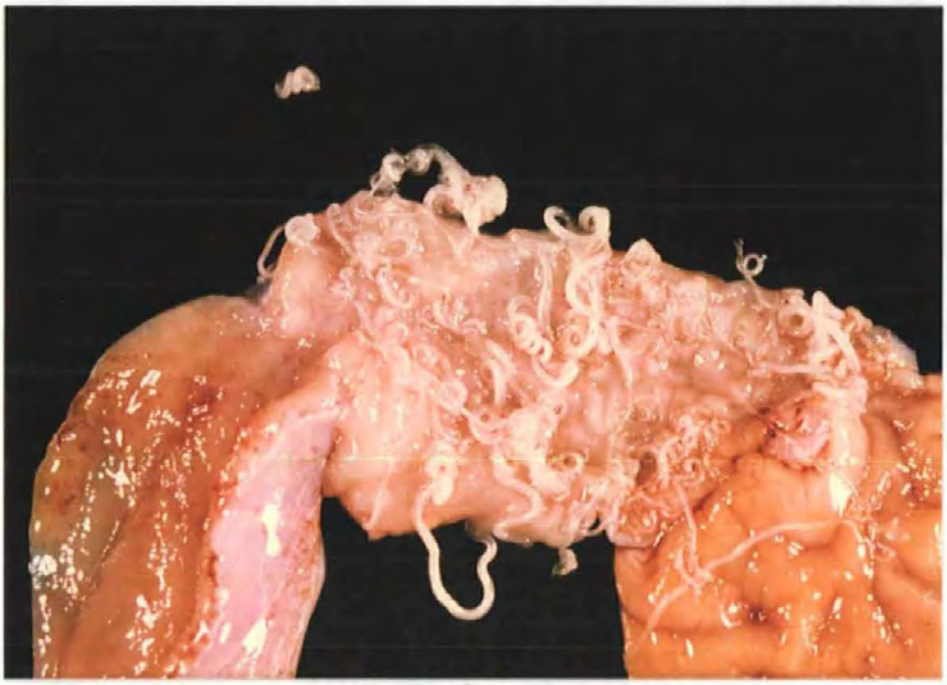
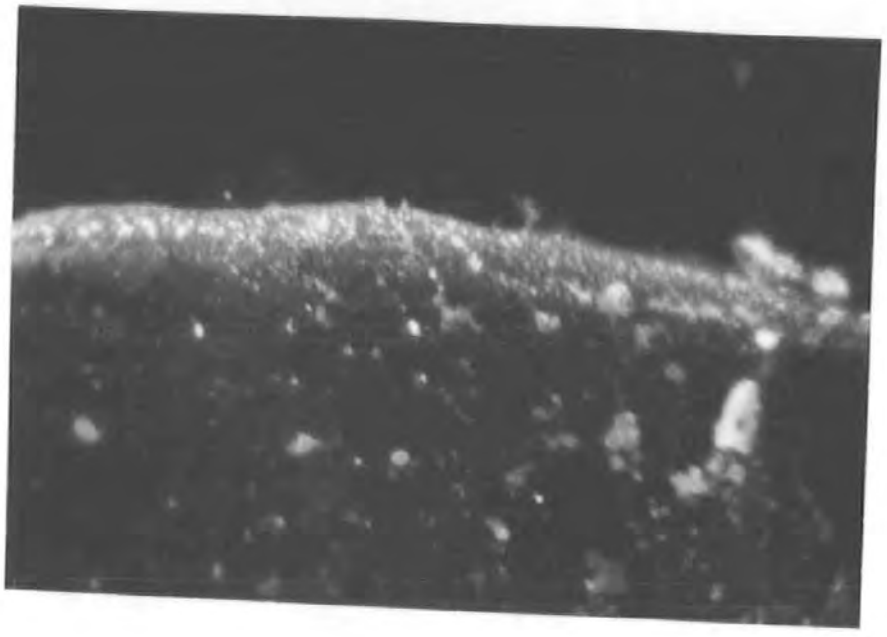
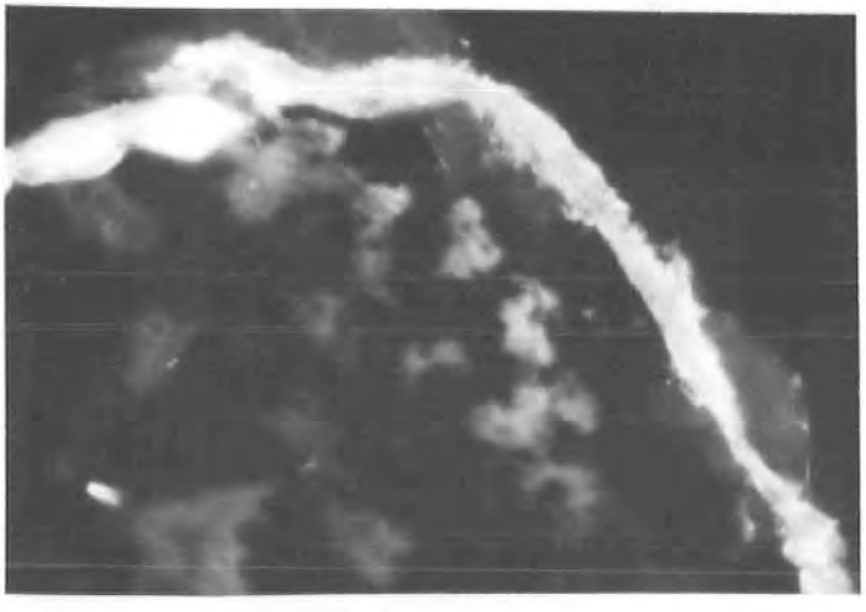


Fig. 7.1. Dogfish spiral intestine parasitised by the nematode Proleptus obtusus.



(a)



(b)

Fig. 7.2. Proleptus cuticle stained by the indirect fluorescent antibody technique to show the presence of dogfish immunoglobulin. a) x 825; b) x 1320.

FISH NO.	L.A. TITRE	FISH NO.	L.A. TITRE
1	N.D.	11	1:128
2	N	12	N.D.
3	N	13	N.D.
4	1:4	14	N
5	N.D.	15	N
6	N.D.	16	N.D.
7	N.D.	17	1:8
8	N.D.	18	1:32
9	N.D.	19	N.D.
10	1:16	20	1:4

TABLE 7.1. Latex agglutination titres of sera from 20 fish infected with Proleptus obtusus.

N indicates agglutination with undiluted serum.
N.D. indicates no detectable agglutination.

that this is a major antigenic determinant.

ii) Live worms were cultured in elasmobranch saline within the wells of a double diffusion agar plate. Dogfish sera were placed in adjacent wells in an attempt to detect excretory/secretory antigens by precipitin formation. This method is based on that of Harris (1970). All tests proved negative.

Experiment 5.3 Host-Parasite Cross Reacting Antigenic Determinants

The possibility of shared antigenic determinants between the dogfish and Proleptus (which may enable the worm to disguise itself as "host") was investigated. Rabbit anti-dogfish whole serum was tested against a Proleptus homogenate by IEP. A faint precipitin line with anodic electrophoretic mobility appeared after 2 days (Fig.7.3) The converse experiment did not show a positive result: no precipitin lines appeared when dogfish serum was separated electrophoretically and rabbit anti-Proleptus serum was allowed to diffuse against it.

The possibility that the precipitin line that formed with the Proleptus homogenate/rabbit anti dogfish serum may have been due to rabbit CRP was investigated and IEP slides were incubated with:

0.1M EDTA
5% (w/v) Sodium citrate
PBS control.

The slides were regularly examined for 5 days. After this period none of the precipitin lines had disappeared and the reaction was not considered to be non-specific.

II Cellular Responses of the Dogfish to Proleptus obtusus

Experiment 5.4 Stimulation of Dogfish Leucocyte Cultures by Proleptus obtusus

Extracts of Proleptus were prepared by blending a number of worms and a small quantity of elasmobranch saline in a teflon-glass hand homogeniser. The soluble material was filter-sterilised and added in a range of concentrations to leucocyte cultures from 3 fish. The stimulation indices are shown in Table 7.2.

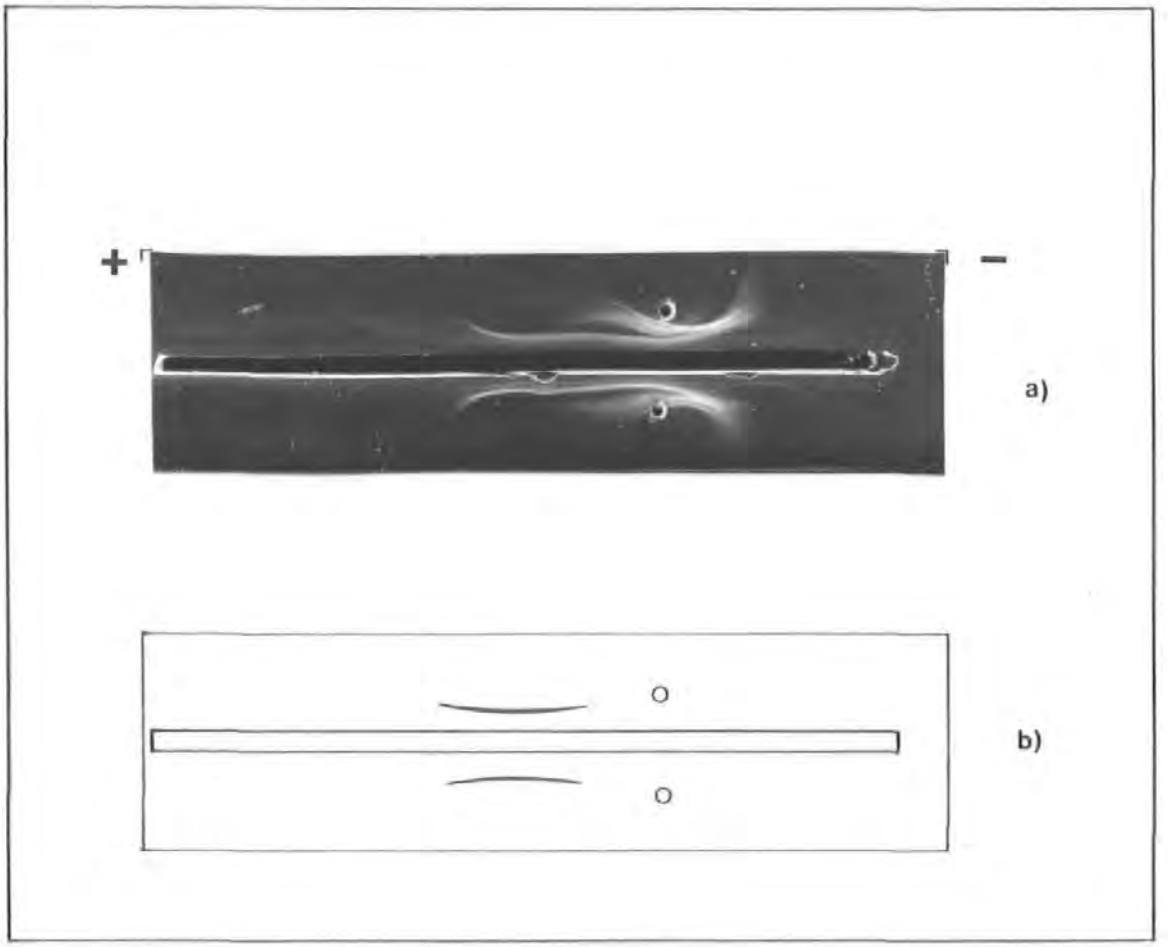


Fig. 7.3. Showing partial antigenic similarity between dogfish serum proteins and a Proleptus extract by IEP. Parasite extract was placed in the wells of slide (b) and after electrophoresis the central trough was filled with rabbit anti-dogfish antiserum : a single line developed. Slide (a) is for reference and shows standard IEP pattern obtained with dogfish serum and the same rabbit-anti dogfish serum used in (b).

FISH	A	B	C
CONCONTRATION PROLEPTUS IN CULTURE ($\mu g\ ml^{-1}$ soluble protein)			
88	1.9	2.6	14.9
8.8	2.2	1.1	5.4

Table 7.2. Stimulation indices obtained from leucocyte cultures from 3 fish incubated with various concentrations of Proleptus extract.

Exp. 5.4: Effect of Proleptus Extract on Leucocyte Cultures

FISH A

μg (protein) ml^{-1} Proleptus extract	c.p.m.	Mean	SI
88	352 590	471	1.9
8.8	589 503	546	2.2

FISH B

μg (protein) ml^{-1} Proleptus extract	c.p.m.	Mean	SI
88	568 722	645	2.6
8.8	211 335	273	1.1

FISH C

μg (protein) ml^{-1} Proleptus extract	c.p.m.	Mean	SI
88	5216 2174	3695	14.9
8.8	1611 1067	1339	5.4

Experiment 5.5 Eosinophilic Adherence

In higher vertebrates, eosinophils have been shown to play an important role in immunity to helminths and in this experiment an attempt was made to demonstrate the involvement of these cells in the immune response of the fish to Proleptus. Dogfish leucocytes were incubated for 24 hours at room temperature in the presence of the parasite in two ways:

i) Leucocytes were separated by differential sedimentation washed 3 times and resuspended in elasmobranch saline and incubated in a tissue culture vial.

ii) Leucocytes were separated as above and incubated with the worm in the presence of dogfish plasma (and therefore immunoglobulin).

The experiment was carried out with leucocytes from four fish. After 24 hours no leucocytes were seen to be in intimate contact with the parasites.

Experiment 5.6 Homologous Passive Cutaneous Anaphylaxis

The experimental protocol was similar to that in Experiment 4.12. Sera from Proleptus infected fish were pooled. Four fish were each given three intradermal injections on their ventral surfaces. These comprised one of 0.25 ml serum, one of 0.1 ml serum and one of 0.15 ml elasmobranch saline. An intravenous injection of 0.5 ml parasite extract (88 mg ml^{-1}) soluble protein) and 2% (w/v) Evans Blue was given to two fish 4 hours later. The other two fish were given the antigen/dye injection after 72 hours in an attempt to demonstrate delayed PCA. A further two fish that had initially received an injection of elasmobranch saline alone were used as controls. Additionally, further controls included two animals that were injected

with Evans Blue alone. After injection of the antigen/dye mixture the sites were examined for signs of blueing. As with the identical experiment carried out with horse serum, no heavy blueing reaction was noted around the intradermal injection sites of any fish after 6 hours of observation. Post-mortem examination did not reveal any sub-cutaneous deposits of Evans Blue.

Experiment 5.7 Heterologous Passive Cutaneous Anaphylaxis

The experimental protocol was identical to the heterologous PCA experiment using horse serum (Experiment 4.13). Four guinea pigs were each given three injections in the flank of 0.2 ml and 0.1 ml pooled dogfish anti-Proleptus serum and one of 0.15 ml PBS. A challenge injection of 0.25 ml Proleptus extract (88 mg ml^{-1} protein) in 2% (w/v) Evans Blue in PBS was given intracardially to two of the animals 4 hours later. One guinea pig was killed after 20 minutes and the sub-cutaneous surface of the skin was examined for blueing. The second guinea pig was killed after 4 hours and examined similarly. The antigen/dye injections were delayed for 72 hours after the initial intradermal injections in the other 2 animals. Both these guinea pigs were killed after 30 minutes and examined for sub-cutaneous blueing. As observed with horse serum no immediate or delayed PCA reactions were seen in any of the animals tested.

Experiment 5.8 Leucocyte Migration Inhibition Induced by Proleptus Extracts

The effect of Proleptus homogenate on LMI was examined. Antigen extract (100 µg mg^{-1} protein) was added to replicate leucocyte cultures taken from eight fish. Paired control cultures without antigen were also made. Puromycin was added to another group of test cultures at a concentration of 2 µg ml^{-1}. Fig. 7.4 shows that Proleptus antigen caused a very marked inhibition of leucocyte migration. Puromycin partially blocked this reaction, indicating that it is lymphokine mediated.

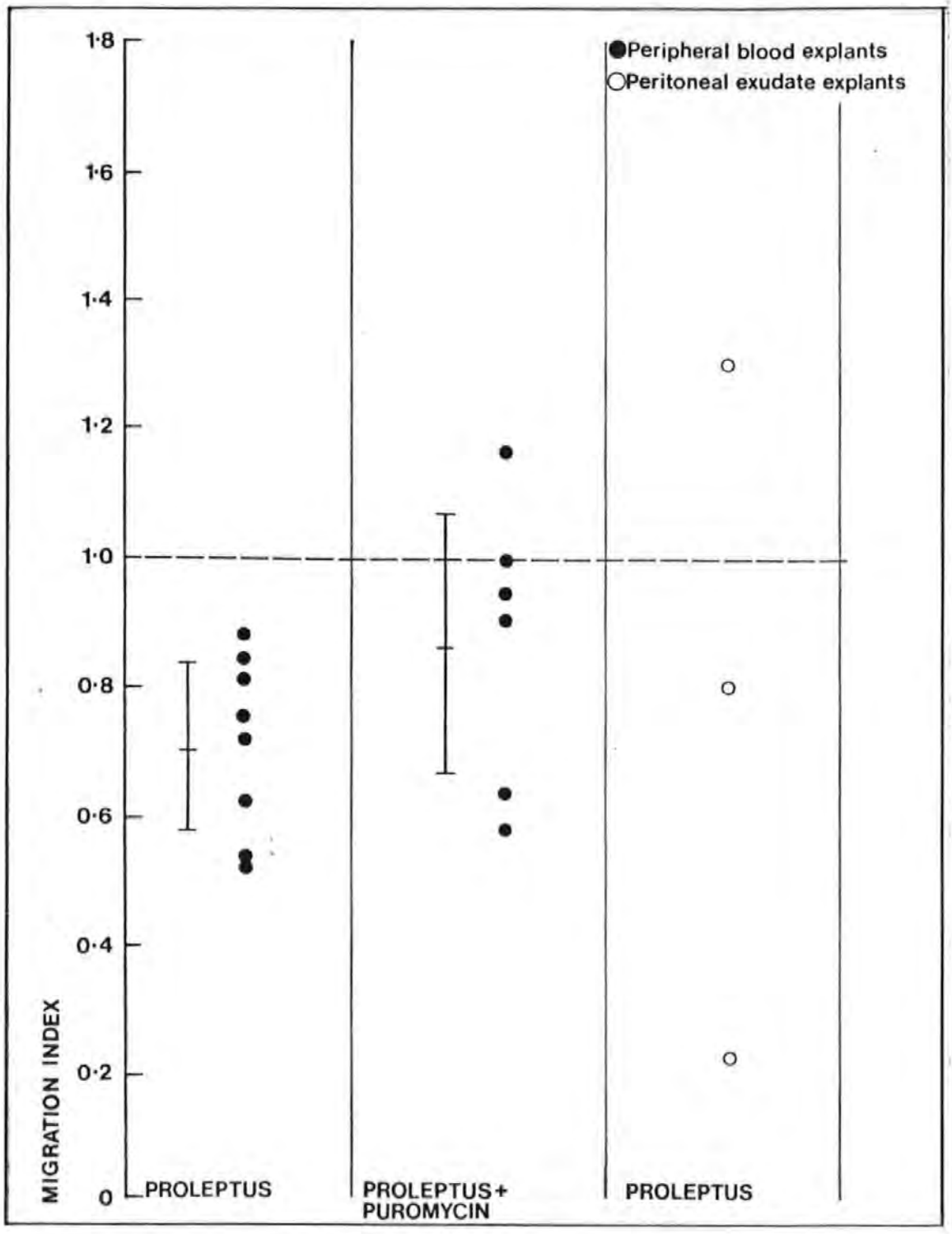


Fig. 7.4. Migration inhibition indices for leucocyte cultures from infected fish incubated in the presence of Proleptus extract. A value of less than one indicates migration inhibition. Each point represents a single fish. Vertical bars represent standard deviation.

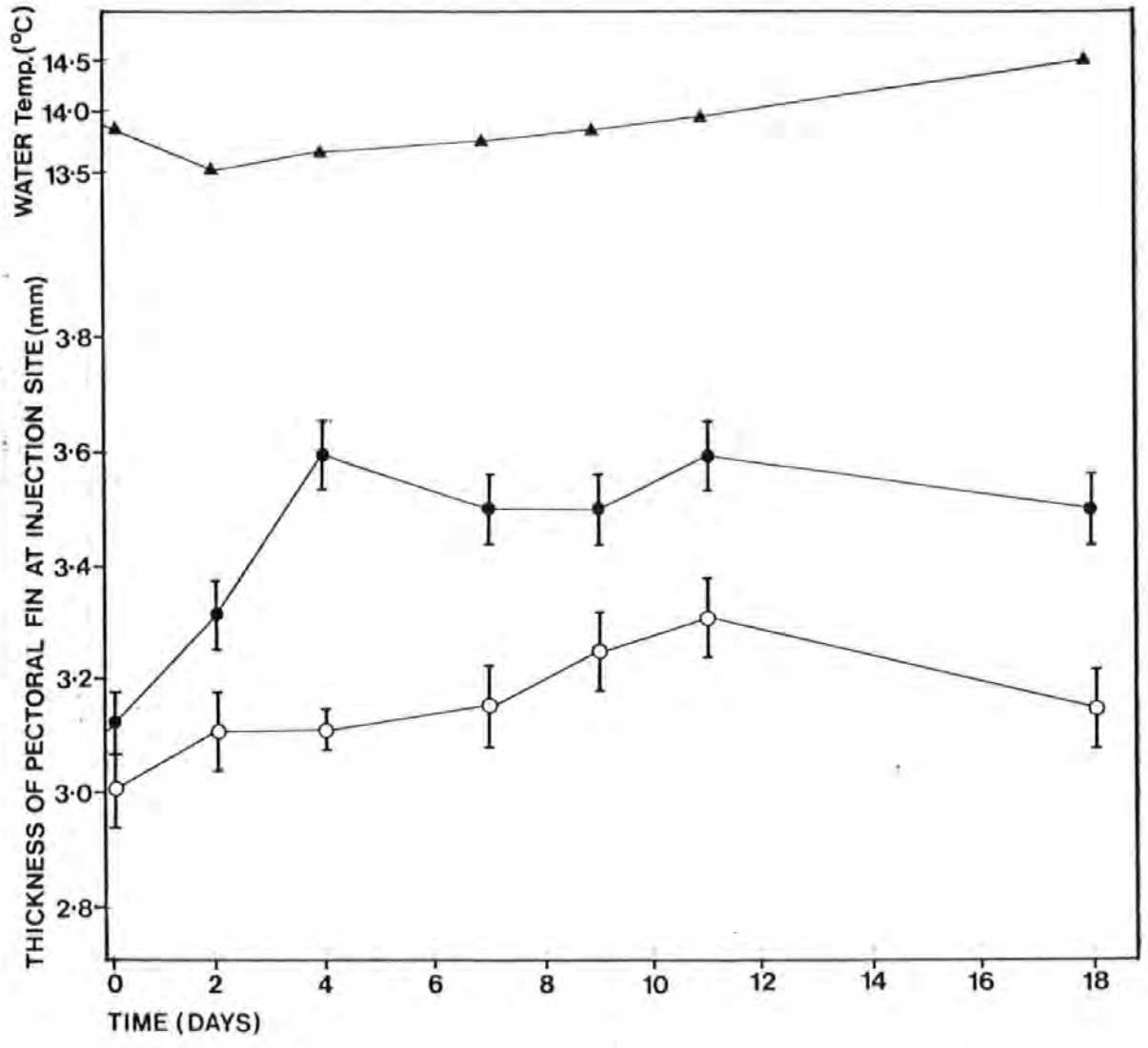


Fig. 7.5. Variation in pectoral fin thickness after injection of Proleptus extract (solid circles). Control fins, injected with saline alone are also shown (open circles). Each point represents a mean of 8 fish. Vertical bars indicate standard error. Water temperature is also shown (triangles).

Experiment 5.9 Delayed Hypersensitivity Measured by Fin Thickening

Eight fish were each given a 0.25 ml sub-cutaneous injection of filter sterilised Proleptus homogenate (88 mg ml^{-1} protein) in the centre of the right pectoral fin. At the same site on the opposite fin a second injection of 0.25 ml saline was given. The thickness of each fin at the injection site was measured with a micrometer and was monitored over a period of 18 days. The fins that had received an injection of antigen became thicker. This response reached a maximum after 4 days. The reaction then declined. In each case the test fins were much thicker than those of the saline injected controls (Fig. 7.5).

Discussion

Although many hundreds of reports of the existence of parasites in fish exist, very few investigations of the immunological interactions between the host and the parasite have been made. These experiments have examined some aspects of the relationship between Proleptus and the dogfish and established that the parasite is capable of invoking the hosts humoral and cellular immune mechanisms and also reflect the immunological status of the fish from a phylogenetic viewpoint.

It is not surprising to find that 50% of the fish examined had low levels of antibody to the parasite because anti-parasite serum antibody can be found in the majority of infections (Cohen & Sadun, 1976) although its functional significance is unknown. The antibodies do not seem to be capable of eliminating the worm as virtually all fish examined were infected. This may be because although some antigenic sites on the cuticle of the worm are present, as detected by immunofluorescent studies, many other epitopes

may be masked because there is antigenic identify between dogfish serum proteins and various components of Proleptus. Whether or not these were body wall components was not established, although this explanation does seem feasible since various mammalian parasites are known to disguise themselves with host protein, for example Schistosoma mansoni (a parasite of man) carries host-like antigenic determinants on its surface (Damian, 1967; Capron, Biguet, Rose & Vernes, 1965). The masking of antigenic determinants may also explain why serum antibody was only found in 50% of infected fish.

Although not detected, it is conceivable that Proleptus may release excretory or secretory (ES) antigenic products and the antibody may act as a neutralising agent for this material which may otherwise prove harmful to the fish. Harris (1970, 1972) showed that the chub produced precipitating antibodies to antigen extracts of the acanthocephalan parasite Pomphorhynchus laevis and also to its ES products. The presence of this antibody in the serum and intestinal mucus did not seem to confer any resistance on the fish as those with serum precipitins often carried heavy worm burdens. Moss (1971) also found that high levels of antibodies to the cestode Hymenolepis microstama did not seem to protect infected mice.

Cottrell (1977b) found that the percentage of '0' group plaice infected with the haemoflagellate Trypanosoma platessae declined throughout the year and this was concomitant with elevated levels of serum β-globulins and suggested this was associated with an antibody response against the parasite and the possible cause of its rejection.

Apart from the report of Harris (1973) mentioned previously, the only report of the nature of the antigenic determinants of fish parasites is that of Cottrell (1975)

who used immunofluorescence techniques to demonstrate that the cell wall of the digenean parasite Cryptocotyle lingua (which infects plaice) was the site of antibody deposition.

There is a lack of information regarding specific cellular immune responses of fish to parasites and the immunological reactions of the dogfish to Proleptus must generally be discussed in terms of mammalian parasitology.

There seems little doubt that components of Proleptus homogenates can act as mitogens and cause blast transformation of dogfish leucocytes although the specific component responsible for this is unknown. The immunological significance of this observation is not clear despite the ability of the helminth to induce a vigorous mitogenic response. This does not seem to confer any resistance on the host and it is possible that if the parasite cell wall contains the mitogenic material, the in vivo response is limited to a local inflammatory reaction. Again it is possible that ES antigens are the agents capable of producing this effect in vitro but in vivo they are neutralised by serum antibody. Cottrell, Playfair & De Souza (1978) demonstrated pronounced blastogenesis in mice spleen cell cultures incubated with malarial antigens. The magnitude of blastogenesis was associated with animals vaccinated with B. pertussis compared to those vaccinated with adjuvant alone.

Despite extensive testing, no evidence could be found of eosinophil (or leucocyte) attachment to the parasite, either in the presence or absence of antibody molecules. Butterworth, David, Franks, Mahmoud, David, Sturrock & Houba (1977) demonstrated that eosinophils attach and are cytotoxic to schistosomes and that this reaction is mediated

by antibody via Fc receptors on the cells. It is not known whether such a receptor exists on fish leucocytes and the absence of such a site may preclude the attachment of cells to the parasite surface.

As with soluble protein antigens, no skin sensitising reactions were observed using parasite extracts. This finding reinforces the accepted hypothesis that fish in general are incapable of such reactions because they lack the IgE equivalent antibody class. IgE has been shown to be induced by various parasitic infections (Oglivie & Jones, 1969; Ogilvie, 1970) and the nearly universal capacity of helminths to stimulate Type I hypersensitivity reactions has been widely used in immunodiagnosis (Zvaifler, 1976). Thus it would seem that the apparent inability of dogfish to produce skin reactions, despite having marked helminth infections, indicates either the absence of homocytotropic antibody or the effector cell (basophil) or both of these components that are normally considered necessary for immediate hypersensitivity reactions.

Experiments described in Chapter 6 indicated that the dogfish was capable of a delayed hypersensitivity response as indicated by the leucocyte migration inhibition test. Proleptus homogenates appeared to induce marked migration inhibition which was possibly blocked by the presence of puromycin. The ability of puromycin to prevent this reaction implies that it is dependent on the production of a soluble protein such as MIF (puromycin classically inhibits MIF). Kirmse (1975) demonstrated that an unidentified protozoan parasite of the turbot would cause migration of leucocyte cultures from the fish, however, no attempt was made to show that this was not a non-specific agglutination phenomenon. Parasites of mammals can cause inhibition of migration and Tremonti & Walton (1970)

reported positive results in leucocyte cultures taken from guinea-pigs infected with Toxoplasma. Gaines, Aranjo, Krakenbuhl & Remington (1972) developed the test for humans infected with the same parasite.

The fact that Proleptus can cause inhibition of leucocyte migration in a relatively primitive fish would appear to indicate that delayed hypersensitivity perhaps appeared earlier, phylogenetically, than has yet been demonstrated and possibly evolved from an ancient cellular defence mechanism. The test may have practical applications in the diagnosis of latent disease states in fish.

That Proleptus extracts could cause a slow thickening of the (fin) tissues into which it was injected is further evidence that the dogfish is capable of delayed hypersensitivity. The reaction was virtually identical to the footpad swelling (caused by infiltration of mononuclear cells) in antigen challenged animals and has been demonstrated with parasite antigens for example Boros & Warren (1971) and Colley (1972) noted footpad and ear swelling in mice challenged with Schistosoma mansoni. In a modification of this technique, Cottrell et al. (1978) demonstrated homing of radio-labelled bone marrow cells to the ears of mice challenged with malarial antigens. *

The dogfish/Proleptus model was useful in demonstrating a number of characteristics of the immune system of the fish but did not indicate its possible protective role against the parasite. It is possible that the fish has adapted to the presence of the worm in a manner comparable to that described by Sprent (1959) who proposed a theory of "adaptive tolerance". He suggested that new hosts react very vigorously to parasitic invasion but as both the parasite and host became adapted to each other the latter gradually loses immunologic activity. This may be achieved

*Although a phenomenon similar to the tissue thickening hypersensitivity responses in mammals was demonstrated in the dogfish, the antigen specificity of this reaction was not shown.

by the selection of parasites whose antigenic structure is similar to that of the host and thus the parasite disguises itself as host material and evades the immune system. Therefore it is possible that during the course of evolution Proleptus and Scyliorhinus have become well adapted and the immune response of the latter has become tolerant, through lack of recognition, of the helminth.

CHAPTER 8

CONCLUSIONS

This study has established that the dogfish is capable of a variety of immune reactions. It can respond to antigenic challenge with specific and non-specific serum factors. It is also capable of a variety of specific and non-specific cellular responses. Despite having a relatively unsophisticated immune system compared with mammals, the fish is nevertheless capable of dealing with naturally occurring pathogens and some groups were kept for over 18 months without suffering infections. This is also emphasised by the high agglutinin titres raised in response to naturally occuring antigen suçh as Salmonella.

The immune system of the dogfish (and all other fish species) is still poorly understood and this work merely provides a starting point for further studies. Some suggestions for future investigations along with their attendant problems are outlined below.

The greatest problems facing fish immunologists are the diversity of species being studied and also the lack of inbred lines. It is of no surprise that such a large number of species have been the subjects of investigations bearing in mind the very great evolutionary diversity found amongst the fishes and also the considerable economic importance of certain species : therefore studies have been initiated from both academic and commercial interests. Now that a basic understanding of the fundamentals of fish immunology exist, more sophisticated research can be made but the current lack of inbred strains precludes such procedures as cell transfer and many transplantation

experiments. The problems associated with establishing and maintaining such a line are considerable but nevertheless not insurmountable.

Ideally a few standard models should be established e.g. rainbow trout, carp or Tilapia for freshwater representatives and plaice and a shark species for marine fish. The use of such models could provide a pool of information of much greater depth then the somewhat scattered reports that now exist.

Leucocytes are the basis of all immune reactions and thus an understanding of these cells (both their morphology and basic functions) is fundamental to the study of fish immunology. In studies made to date, too much reliance has been placed on the assumption that the cells are counterparts of mammalian lymphoid cells. Therefore a careful investigation of the white blood cell system of any species examined is vital to the understanding of the immune system of the animal.

A major difference in the studies of immune mechanisms in higher and lower vertebrates is that the experimental technology associated with the former group is well established; with the latter group such methodology first has to be created before any investigation can be carried out and this has often proved to be a considerable hurdle. There also appears to be a certain reluctance however to use certain techniques which are routinely used in mammalian studies and which would lend themselves ideally to investigations of fish immunology.

Some suggestions for future investigations are:

a) Amino acid sequencing homologies of purified molecules with those of higher (and lower) vertebrates and also between serum and lymphocyte immunoglobulin from the same

fish species may provide further evidence for the presence of antibody classes and subclasses.

b) The demonstration of idiotypic variation, especially amongst the more primitive fish species would do much to support contemporary theories of immunological regulation, particularly the Network Theory proposed by Jerne (1973). The recent work of Machulla, Richter & Ambrosius (1978) describing idiotype expression in carp has gone some way to substantiate this.

c) Studies on non-specific defence mechanisms are also necessary, particularly on the complement systems of fish. To date all that is really understood is that many fish species possess a (haemo) lytic factor in their serum. The biological function, activation sequence and physical nature of these substances is not known. To date the only reports of the anti-viral agent, interferon, have been made regarding teleost fish and to demonstrate its presence and specificity in more primitive groups would provide valuable information on the phylogenetic origins of this substance.

d) The possibilities for investigations at the cellular level are considerable. First of all these can be examined at the level of the cell surface. Experimental data on the nature of immunoglobulin on leucocyte membranes is now being published and these findings will do much to demonstrate the heterogeneity of fish lymphocyte populations. Other cell surface markers worthy of consideration are the Fc and complement receptors. The discovery of a surface determinant such as the θ antigen which is used to identify T-cell populations in certain strains of mice or the sheep erythrocyte receptors of human T-cells would be invaluable in a fish species but before such investigations can be made, lymphocyte sub-populations must first be positively identified according to their function.

Various classifications can be made according to differential lectin responsiveness and the use of mitogens as tools to identify fish leucocyte sub-populations must be seriously evaluated. It must be stressed, however, that results derived from such studies must be treated with caution as there is no reason why fish leucocytes should react in a similar manner to the B and T cells of higher vertebrates. Other cellular function tests which may prove more useful than the use of lectins are those concerning cytotoxic mechanisms. To demonstrate a similar phenomenon to the antibody-dependent cell-mediated cytotoxic reaction of higher vertebrates or the functional equivalent of K (killer) cells at a relatively early stage of phylogenetic development would give a better understanding of the origin and evolution of such mechanisms.

(i) investigations into methods of control of fish disease are essential of the aquaculture industry.

(ii) host-pathogen relationships can provide good "natural" models for immunological mechanisms.

At present the pathogenesis of bacterial disease is being relatively well investigated although fish viruses are less well understood although like their mammalian counterparts these are hard to study because of their size. Studies on fish parasites, which can provide ideal immunological models, have surprisingly been neglected. Parasite infections make good model systems because of the relatively large size of the pathogen and the fact that the host can survive for an indefinite period with such infections : these two factors make parasitic infections relatively easy to study. Greater research efforts in this direction may provide valuable information of both academic

and practical interest.

Little is known or understood about the immunology of neoplasms or autoimmune syndromes in fish. There have been many reports of tumours in various fish species but all are concerned with histopathological studies. Immunological investigations of malignant growths is of considerable theoretical interest but the lack of knowledge of fish cytotoxicity mechanisms and the generally poor understanding of tumour immunology (in higher vertebrates) itself make this problem difficult to approach. Similar comment can be applied to the problems of autoimmune disease; although it would be intriguing to know whether such diseases affected lower vertebrates and such knowledge would do much to enable an understanding of the origins of the immune system. The intrinsic problems posed by such a study make work in this direction hard to pursue, at least for the present time.

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