FISH IMMUNOLOGY

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FISH IMMUNOLOGY AND FISH HEALTH

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I. INTRODUCTION

The remarkable progress in the field of fish immunology goes hand in hand with the increasing interest in fish farming. Fish hatcheries and farms are becoming increasingly important in supplementing sport fisheries and food production. However, in most facilities the animals are kept at relatively high densities causing well known problems of stress and disease. It is not surprising that a number of recent meetings were organized dealing with the subjects of fish diseases, health and immunology. A number of valuable proceedings are available today (Ahne, 1980; Anderson and Hennessen, 1981; Oláh et al., 1981; Van Muiswinkel and Cooper, 1982; Anderson et al., 1983). From most studies, it is evident that the problems on the farms can be encountered by measures preventing disease outbreak, or by treatment of the actual disease with drugs or chemicals.

II. EXPERIMENTAL DESIGN AND RESULTS

ANTIBIOTICS

One of the drugs which has been approved for the treatment

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of specific bacterial disease in fish food is the antibiotic oxytetracycline (oxyTC). Initial studies in our laboratory by Rijkers et al., (1980) and Grondel and Boesten (1982) have shown that this drug can be immunosuppressive for thymus-dependent immune responses in carp. Recent experiments in co-operation with the U.S. Fish and Wildlife Service have shown that oxyTC is also immunosuppressive in rainbow trout. Feeding oxyTC-

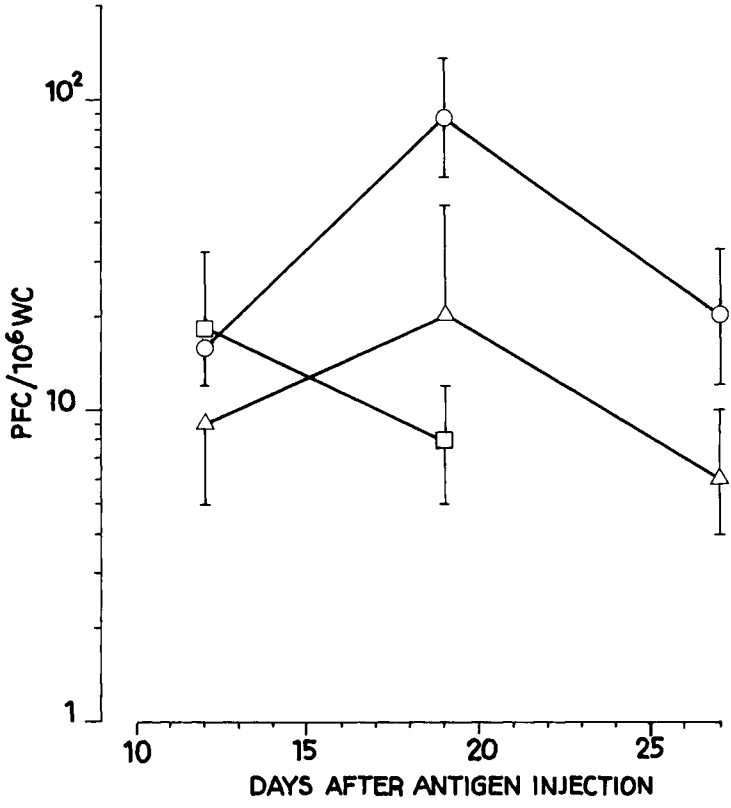

Fig. 1. The number of plaque forming cells (PFC) per 10^6 white cells (WC) in the headkidney of rainbow trout (12.5° C) after i.p. injection of Y. ruckeri O-antigen (5 μg/animal). Control animals were fed normal pellets (-O-) at 1.5% body weight/day. The other groups were fed pellets supplemented with 3333 ppm oxytetracycline during 14 days before (- △ -) or after (- □ -) antigen injection. Each point represents the geometric mean ± 1 S.E. (n=5).

containing pellets before antigen injection reduced the number of antibody-producing cells by 75% on the peak day of the response (Fig. 1). Feeding oxyTC in the period after antigen injection had an even more dramatic effect (90% reduction). It is worthwhile to mention that the antigen used in these studies (O-antigen from Yersinia ruckeri) is regarded as thymus-independent (Anderson and Dixon, 1980).

In the light of these results, it is obvious that we would only recommend a therapeutic use of oxyTC. Prophylactic use should be avoided.

III. VACCINATION

Prevention of diseases by vaccination will provide an alternative to treatment with antibiotics or other drugs. However, vaccination procedures will only be effective when enough information about the basic properties of the defence system is available. In this respect the development of immunological memory is an important aspect. There are some reports on this subject (Avtalion, 1969; Rijkers et al., 1980) but data on the effect of bacterial antigens are scarce (Lamers et al., 1984). Therefore, we studied the primary and secondary response after injection of heat killed Aeromonas hydrophila in carp. It was observed that the height of a standard secondary response, which is regarded as an estimate for memory induction, was dependent on the priming dose (Fig. 2). Interesting enough, an intermediate priming dose of 10^7 bacterial cells gave the best results. The number of antibody-forming cells at the peak day of the secondary response was about 20 x higher than during the primary response. In another study it was shown that both the priming and challenge route (e.g. bath versus injection) played a role in the induction of memory and the evocation of the secondary response (See Lamers and De Hass, this volume). As the next step, challenge experiments with virulent pathogens are needed before successful vaccination under farm conditions can be achieved. The recent reports on this subject (Anderson, et al., 1983) are very promising.

IV. DEVELOPMENT AND TOLERANCE

It is important to know at what age a fish becomes reac-

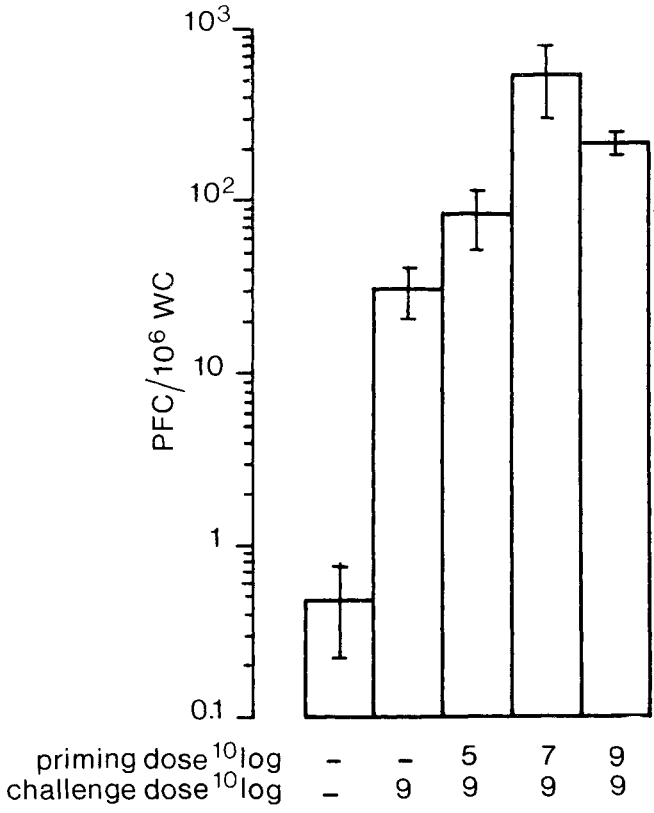

Fig. 2. The number of plaque forming cells (PFC) per 10^6 white cells (WC) in the headkidney of carp (22°C). A priming dose was given by i.m. injection with no (-), 10^5, 10^7 or 10^9 A. hydrophila cells. The response was measured 9 days after a standard second injection of 10^9 bacterial cells (challenge). Each bar represents the arithmetic mean ± 1 S.E. (n = 4).

tive to foreign material. To this end, studies on the ontogeny of the immune system of fish are very valuable (Botham and Manning, 1981). The production of monoclonal antibodies specific for surface determinants of carp thymocytes or serum immunoglobulin provides new possibilities for the recognition of cell subpopulations in young animals (Secombes et al., 1983a, 1983b). Morphologic data from these studies suggest that the immune system of carp is fully developed at 2 - 4 weeks of age. However, when the animals are tested for humoral

immune function, another picture arose. In fact, intramuscular injection of sheep red blood cells (SRBC) at 4 - 5 weeks post-hatch did not result in a plaque-forming cell (PFC) response. A second SRBC injection of the same experimental group 2 - 3 months later also showed the absence of a response (Fig. 3). Control animals of this age did contain normal PFC numbers after SRBC injection (150 PFC/10^6 white cells). At 5 or 13 months after the first injection, a second injection gave a normal

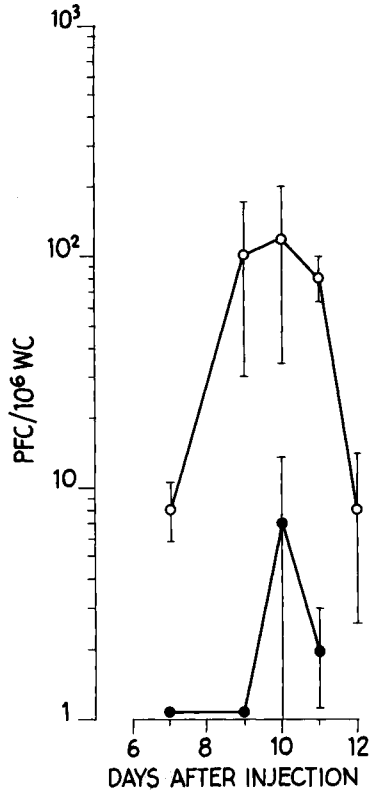

Fig. 3. The number of plaque forming cells (PFC) per 10^6 white cells (WC) in the headkidney of carp at 4 months post-hatch (23°C). Non-primed animals (-O-) received an i.p. injection with 5 x 10^8 sheep red blood cells (SRBC). Primed animals (-●-) were i.m. injected with 5 x 10^6 at 1 month of age and i.p. injected with 5 x 10^8 SRBC at 4 months. Each point represents the arithmetic mean $\pm$ 1 S.E. (n = 4).

"primary" response. The results indicate that contact with SRBC at an early age can induce a temporary state of unresponsiveness or tolerance. However, this phenomenon may be species dependent. Tatner and Horne (1983) have shown that rainbow trout as early as 2 weeks post-hatch are able to raise protective immunity after bath vaccination with Vibrio bacterin.

V. CONCLUSIONS

It seems inevitable that some drugs will be used for medication in fish health problems, notwithstanding the adverse effects like those described for oxyTC. As long as effective prevention can not be achieved for all diseases, the fish farmer has no choice. In the short term, it is important to choose drugs which are not immunosuppressive. It is worthwhile mentioning that preliminary experiments from our own group indicate that potentiated sulfonamide (Ro5-0037) is less harmful to the immune system than oxyTC.

Several bacterial and viral vaccines are available today (Anderson, et al., 1983). It is envisaged that thorough studies on the application of these vaccines in young animals are needed. It can not be excluded that an early contact with a relative high dose of antigen is a disadvantage. Tolerance may make fry nonreactive to certain pathogens.

In the long term, the relationship between fish genetics and immune reactivity is an exciting new area of research. All the present data on fish cell populations (e.g. characterisation by monoclonal antibodies), regulation of the response by factors (see Grondel and Harmsen, this volume), and the major histocompatibility complex (Cohen and Gloudemans, to be published) will be needed to unravel the question why certain fish strains differ in disease resistance.

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SPECIALISATION IN THE TELEOST AND ANURAN IMMUNE RESPONSE: A COMPARATIVE CRITIQUE.

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I. INTRODUCTION

The Teleostei are the dominant fish fauna in marine and freshwater ecosystems today. Although the Anura are not dominant in terrestrial ecosystems, they are, nevertheless, a successful order with many species, well adapted to a variety of habitats. Among the Osteichthyes and the Amphibia respectively, the Teleostei and the Anura are the most advanced and highly evolved living forms, both groups having radiated extensively during their phylogenetic history.

Examination of the phylogenetic trees of the fishes and the ampnibians reveals some interesting comparisons. The Teleostei lie at the end of an evolutionary branch, well away from the main line of evolution via Crossopterygii of the order Rhipidistia to the Tetrapods (Fig. 1). The Anura lie at the end of an evolutionary branch whose point of attachment to the tree is uncertain: the relationships between the modern Lissamphibia to the Palaeozoic Labyrinthodontia, whence derived the Reptilia, remain very problematical. To change the metaphor from trees to streets, one could suggest that the Teleostei and the Anura both lie on evolutionary cul-de-sacs away from the main road of craniate evolution, although the "dead end" analogy is misleading in that our two taxa are alive and well, and are presumably actively evolving.

Both groups have been investigated with respect to their immune phenomena in some depth. The Teleostei are of

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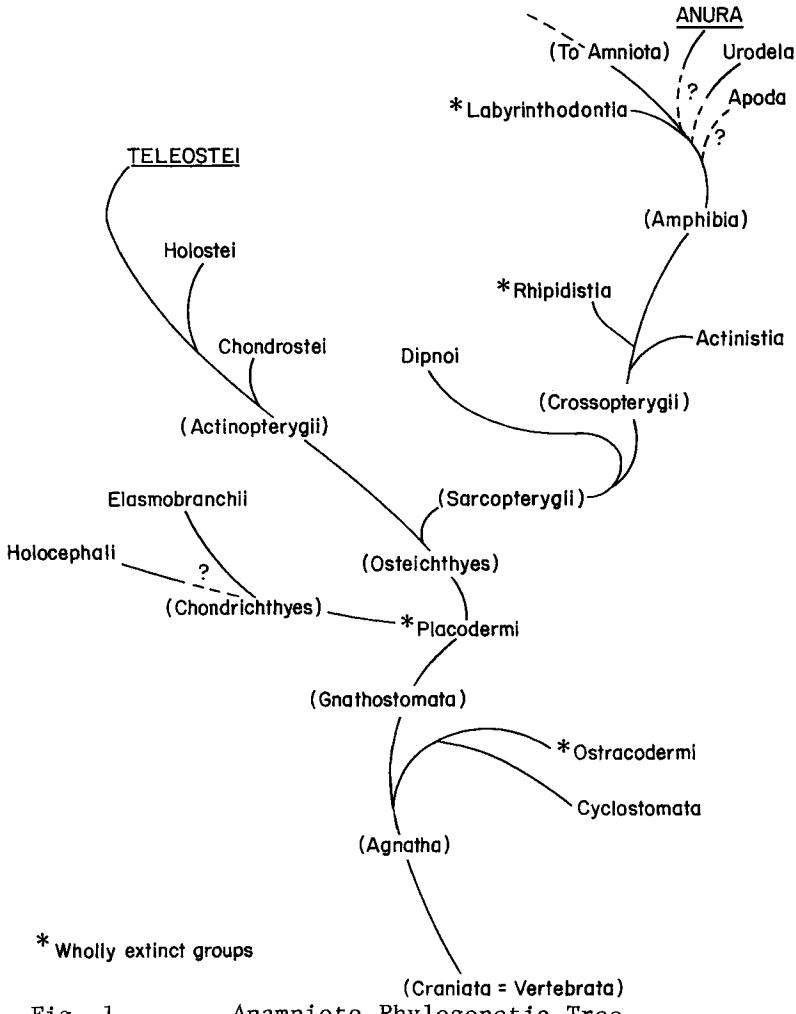

Fig. 1. Anamniote Phylogenetic Tree.

considerable economic importance and an understanding of teleosteans' immune systems, as it pertains to their ability to resist infection, is of great interest to fisheries (van Muiswinkel, 1982). The Anura have been investigated extensively: the adult frog or toad is an easily maintained organism whose general physiology is well understood, and the tadpole represents a free-living, accessible larva open to experimental manipulation. Xenopus laevis has, arguably, the most completely investigated immune system of any poikilotherm (Katagiri, 1978).

If we consider immune phenomena in Teleostei or Anura we find several parallel or convergent specialisations which are not found in other living fishes or amphibians. There are also, of course, some differences between the teleostean and anuran immune responses.

II. CELL-MEDIATED IMMUNITY

In both the Teleostei and the Anura, immune responses are temperature-dependent (as they are in all poikilothermic craniates) (Avtalion et al., 1976). Rejection of grafts of allogeneic skin tissue in advanced Anura such as members of the Ranidae or the Bufonidae, at temperatures of 20°C and above, is "acute", such rejection being characterised by capillary dilation and disintegration, haemostasis and lymphocyte infiltration, followed by destruction of the cells of the foreign tissue. For example, Hildemann and Haas (1959) showed that adult and larval Rana catesbeiana rejected first-set skin allografts acutely at 25°C, the median survival time (MST) being 11-14 days. At 15°C the grafts survived some three times as long. Pipid anurans such as Xenopus laevis and Discoglossids such as Alytes obstetricans are less efficient in their ability to reject allografts, rejection being often described as "sub-acute". At 23°C, Xenopus laevis normally takes 3 weeks to reject 1st-set skin allografts (Horton, 1969); at 9°C rejection takes over 6 months. The Pipidae and the Discoglossidae are considered to be evolutionarily more primitive than the Ranidae and the Bufonidae (Noble, 1931). This acute or sub-acute rejection of allografts (which is usually accelerated when 2nd-set grafts are applied) contrasts with the findings in the Urodela (Cohen, 1971) where allograft rejection is almost always slow or "chronic", accompanied by lymphocytic infiltration followed by a rejection phase marked by haemostasis, haemorrhaging, melanophore destruction and cellular necrosis. MSTs for skin allografts on Diemictylus viridescens viridescens, maintained at 25°C vary strikingly, individual end-points occurring between 7 and 155 days. Total acceptance of allografts is occasionally seen, and the histological events accompanying such variation exhibit a lack of consistency. Similar chronic rejection is seen in the apodan Typhlonectes compressicauda (Cooper and Garcia-Herrera, 1968).

When we turn to the fishes, we again find variety in the kinetics of allograft rejection, most work having been done using scales or skin grafts. Rijkers (1982) reviews

graft rejection in fishes, noting that the cellular reactions which occur at the graft site are not dissimilar to those in mammals: revascularisation of the graft, overgrowth by host tissue with vasodilation at points of graft-host contact, and invasion by lymphocytes and phagocytes. Hosts exhibit donor-specific anamnaesia, with long-lived memory resulting from exposure to living tissue only; isohaemagglutinating antibodies appear not to be implicated in rejection (which is not antigen-dose dependent), and increased genetic distance (e.g. xenografts) and higher temperatures, speed the rejection processes.

Of particular interest to this critique is, however, the corpus of work which universally indicates that Teleostei reject allografts in an acute fashion: MSTs of 14 days or less are usually recorded. Agnatha, Chondrichthyes and Actinopterygii of the Chondrostei infra-class are usually reported to reject grafts in a chronic or sub-acute manner with MSTs of at least 30 days. For example, Hildemann and Haas (1960) found that 1st-set scale allografts in 4 species of teleosts were rejected with MSTs of between 7.2 and 8.6 days, 2nd-set grafts between 4.1 and 6.0 days; Borysenko and Hildemann (1970), by contrast, report a 41.1 day MST for Heterodontis francisci (Elasmobranchii) skin allografts. The borderline is not totally defined since in Lepisosteus platyrhincus, a member of the Holostei, the infra-class of fishes "nearest" to the Teleostei, 1st-set scale allografts are rejected in an acute fashion (McKinney et al., 1981), whereas the "primitive" teleost Osteoglossum bicirrhosum demonstrates sub-acute rejection (Borysenko and Hildemann, 1969). As in the Anura, graft rejection times are very temperature-dependent: Carassius auratus rejects allogeneic scales within 7 days at 25°C but takes 40 days at 10°C (Hildemann, 1957), there being a critical threshold with respect to MST between 20° and 25°C (Hildemann and Cooper, 1963).

The generally acute allograft rejection times seen in Teleostei and Anura suggest the presence of a highly discriminating and effective cell-mediated immune system in these poikilotherms, and the probable presence of the products of a major histocompatibility system (MHS) on the cells. For instance, Bernard et al. (1981) present functional evidence for such an MHS in Xenopus laevis which, as in mammals, controls collaboration between subpopulations of immunocytes: this, and similar evidence is discussed below. Cohen and Collins (1977), reviewing

the occurrence of such markers of MHS activity as acute allograft rejection among the ectothermic craniates, discuss the evolutionary significance of their conspectus: they suggest that the markers evolved independently 4 times in evolution, as the Teleostei, Anura, Aves and Mammalia evolved. Other groups of craniates, such as the Urodela, which reject grafts chronically, would appear on this evidence to lack an MHS (Flaherty and Cohen, 1982).

It would thus appear that two "successful" craniate groups, the Teleostei and the Anura, have separately evolved an MHS. In mammals the MHS can be divided into regions (Klein, 1977). Histocompatibility reactions showing themselves in the form of acute allograft rejection, are a function of the products of the Class I regions. Thus a further postulation from the allograft data from Teleostei and Anura is that these groups have evolved the analogue or homologue of Class I regions in their MHS.

Fishes are capable of exhibiting other cell-mediated immune phenomena including stimulation and transformation of lymphocytes by T cell mitogens, delayed hypersensitivity reactions and mixed lymphocyte reactions (MLRs). With respect to the last named, strong, reproducible MLRs are found in Teleostei (Etlinger, 1975), as are lethal graft-versus-host reactions (GVH) (Wright and Cooper, 1982): both these phenomena are associated with the presence of Class II regions of the MHS (Klein, 1977). Members of other fish taxa lack such strong reactions (Sigel et al., 1973; Cooper and Du Pasquier, 1974).

The more advanced Anura such as the Ranidae and the Bufonidae, and to a lesser extent the Pipidae, also possess lymphocytes capable of reacting strongly in MLRs, and showing strong GVH reactions, again strongly suggesting the presence of a Class II of the MHS. This view is supported by the presence of serologically detectable erythrocyte and lymphocyte alloantigens (Du Pasquier et al., 1975), and cell-mediated lympholysis (Bernard et al., 1979). Bernard et al. (1981) present a convincing case for a Xenopus laevis Class II in the MHS, on the basis of carrier-hapten cell co-operation experiments using genetically defined cell populations. They term the X. laevis MHS "XLA". Flaherty and Cohen (1982) report unpublished experiments suggesting that the X. laevis thymus educates lymphocytes for antigen recognition and cell co-operation.

If these findings are compared with the evidence

obtained from Urodela, we find, as with the evidence from allograft rejection, little support for an MHS. Cohen (1980) reviews MLR studies using 4 urodelan genera: results are only significantly positive about half of the time, and stimulation indices are very low. He notes that the cells involved in allograft rejection in salamanders appear to be T-dependent, and that the explanation for "sporadic" MLRs in salamanders may be that they have an MHS Class II homologue, but that they lack a population of determinant-responding cells. However, the urodeles so far investigated seem to possess thymus-dependent "T" cells different in nature to those in Xenopus laevis: they do not respond to phytohaemagglutinin, for example. On balance there seems to be only doubtful evidence for Class II regions of an MHS in Urodela, and it is unlikely that any MLR locus in salamanders is ancestral to the relevant MHS loci in Anura or homoiotherms.

Cell co-operation in the immune response is another feature associated with the Class II regions of the MHS. Hapten-carrier experiments in Pseudopleuronectes americanus (Stolen and Makela, 1976) and in Carassius auratus (Ruben et al., 1977) would seem to indicate lymphocyte heterogeneity and co-operation in these teleosts. Wishkovsky and Avtalion (1982) present data strongly indicating that helper function and suppressor function can be induced in Cyprinus carpio in response to antigen at high or low temperatures respectively. Thymectomy of young Salmo gairdneri fry results in a reduced lymphoproliferative response in the spleen and kidney to human gamma-globulin (a T-dependent antigen in mammals) (Manning et al, 1982). Intact young S. gairdneri seem to react vigorously to T-independent antigens, but responses to T-dependent antigens are feeble (Etlinger et al., 1979) and develop later in ontogenesis (Manning et al., 1982). The latter group present "tentative evidence" for a thymus suppressor role in C. carpio, on the basis of adult thymectomy causing elevated serum antibody titres to Aeromonas salmonicida (a T-independent antigen). In S. gairdneri no such effect was noted, but heightened responses to human gamma-globulin in Freund's complete adjuvant were seen. Van Loon et al., (1980) suggests that in young C. carpio sheep red blood cells can induce tolerance to the antigen.

From these (selected) experiments the existence of lymphocyte heterogeneity, and the presence of helper and suppressor populations of T cells (or at least functional-T cells) can be inferred in Teleostei. Data

from other fish taxa are more limited, but such evidence for Class II MHS-associated phenomena are lacking.

In Anura, lymphocyte heterogeneity and the presence of functionally discrete subpopulations of thymus-dependent lymphocytes is, as might be expected from other cell-mediated immune phenomena already discussed, strongly implicated. Hapten-carrier experiments in Xenopus laevis (Blomberg et al., 1980) show a requirement for an antigen-specific T-B cell co-operation in response to soluble antigens in vitro. Thymectomy of young X. laevis larvae radically affects such measures of cell-mediated immunity as allograft rejection capacity (Horton and Manning, 1972). Antibody production to most thymus-dependent antigens is abrogated by thymectomy (Horton et al., 1977): production of antibody to Limulus haemocyanin is an exception (Manning and Jurd, 1980). Antibody production to T-independent antigens is unaffected by thymectomy (Tochinai, 1976). Differential responses to B and T cell mitogens are observed in X. laevis (Manning, et al., 1976).

Evidence for lymphocyte heterogeneity in Urodela is rather enigmatic. Ruben and Edwards (1976), using TNP-conjugated horse erythrocytes, suggest that Triturus viridescens does possess interacting, heterogeneous populations of lymphocytes. However, the same authors, later reviewing evidence for cell co-operation using hapten-carrier experiments in Urodela felt that many experiments might be better explained by a polyfunctional specificity of antigen-binding cells (Ruben and Edwards, 1980). Ruben (1983) suggests that the immunocytes in Notophthalmus viridescens may be able to recognise and respond to haptens independently of their carrier. In my own laboratory we have found evidence (using hapten-carrier immunisation and anti-thymocyte sera) for a population of helper T cells in Ambystoma mexicanum (Tahan and Jurd, 1981), although Charlemagne (1982) feels that our experiments would be more happily interpreted in terms of suppressor cells which derive from a population of thymocytes that respond early to hapten. On balance we must conclude that the Urodela probably lack the refinements of lymphocyte heterogeneity and co-operation found in Anura, Teleostei and homoiotherms, although elements of these phenomena may be evolving in, for example, A. mexicanum. Certainly the urodelan data in this context offer little evidence for Class II regions of the MHS in this order, and the Urodela seem similar to the non-teleost fishes (and to the Reptilia) in this respect

(Wright and Cooper, 1982).

Physical markers of lymphocyte heterogeneity support these observations. In mammals, B cells possess surface immunoglobulin (sIg) whereas T cells lack it. Early studies of lymphocytes from Raja naevus (Elasmobranchii) showed 60-80% of lymphocytes to be sIg positive in the spleen, thymus and peripheral blood (Ellis and Parkhouse, 1975); even higher proportions were observed in teleosts from the genus Carassius (Warr et al., 1977). In Xenopus laevis 60-85% of peripheral blood lymphocytes possessed sIg, 47-55% of tadpole thymocytes, 70-75% of metamorph thymocytes and 8-12% of adult thymocytes were sIg positive, as detected by immunofluorescence (Jurd and Stevenson, 1976). Similar findings are reported for Rana catesbeiana (Mattes and Steiner, 1978). Recently doubt has been cast on findings such as these, because some of the anti-Ig sera used to test for surface immunoglobulin have been shown to react with common carbohydrate epitopes found on both sIg and on the cell membrane, unassociated with sIg. Nevertheless other techniques, such as radioimmunoassay, do indicate a surface immunoglobulin or a related receptor molecule on fish thymocytes, and Warr and Marchalonis (1980) do feel that the occurrence and physicochemical properties of surface immunoglobulins warrant the conclusion that subpopulations of lymphocytes exist in the Teleostei, supporting the evidence for a functional lymphocyte dichotomy discussed above. The same authors argue that the teleost sIg is almost certainly a receptor molecule.

Similar studies on the anuran Xenopus laevis by Hadji-Azimi and Schwager (1980) suggest that the previous findings concerning proportions of X. laevis thymocytes with sIg, are also exaggerated because of anti-carbohydrate reactivity in the testing antisera. The authors doubt the existence of thymocyte sIg, although its presence on splenocytes is not questioned; they postulate the existence of separate populations of lymphocytes in X. laevis on the basis of the cells' surface physicochemical properties, the evidence correlating strongly with functional evidence (Hadji-Azimi and Schwager, 1981).

The evolutionary significance of these findings is interesting. Warr and Marchalonis (1980) suggest that a sIg acts as an antigen receptor on all fish lymphocytes, and that as the teleost line diverged from the main line of craniate evolution towards the Tetrapoda, a T/B cell functional dichotomy evolved. A similar process seems to have occurred in anuran evolution, but largely associated

with the loss or modification of sIg on thymocytes, and presumably again in the evolution of Aves and Mammalia. It is noteworthy that in the urodele Pleurodeles waltlii up to 90% of splenocytes exhibit sIg as shown by immunofluorescence; 98% of thymocytes show sIg although the fluorescence is less intense than on spleen cells (Charlemagne and Tournefier, 1975).

III. ANTIBODY-MEDIATED IMMUNITY

No fish group so far investigated has any immunoglobulin (Ig) class other than IgM. Such heterogeneity as does occur concerns heavy chain length, or the degree of polymerisation of the basic 4-chain IgM unit. Serum IgM in Chondrichthyes is pentameric, in Teleostei it is tetrameric. 7S, monomeric IgM is also commonly found in Elasmobranchii (Ambrosius, et al., 1982). Studies of antigenic relationships between Cyprinus carpio IgM and the high molecular weight Igs of other piscine, amphibian, reptilian and mammalian species, together with that from Petromyzon sp. (Agnatha), indicate that the C. carpio μ chain shares epitopes common to μ chains of other craniate taxa. Litman and Marchalonis (1982) present data arguing that IgM arose early in craniate evolution, and that the μ chain sequence has been highly conserved. Teleostei have not diversified their Ig isotypes: Ambrosius et al. (1982) show that the sIg on C. carpio lymphocytes is either 7S IgM, or else a thymocyte membrane Ig which is very cross-reactive with the C-region epitopes of the μ chain, lacks carbohydrate, and probably consists of two heavy chains with molecular weights of 65,000. Both surface IgM and the membrane Ig probably act as antigen receptors.

Thus, despite the relatively advanced immune responses in Teleostei, with several MHS-associated features, there is little evidence of the diversification and compartmentalisation of the immune response seen in successful tetrapod groups (Jurd, 1983). We do have hints of a trend towards the evolution of novel Ig classes in the occurrence in several fish groups of an IgM-derived molecule with a deleted μ chain. Epinephelus itaira (Teleostei) has such an Ig (Clem, 1971), and a similar Ig in Neoceratodus forsteri (Dipnoi), with a sedimentation coefficient of 5.9S, has been termed IgN (Marchalonis, 1969).

Ig classes other than IgM have not been satisfactorily described in Urodela or Apoda, but a 7S non-IgM molecule is well-characterised in Anura (Hadji-Azimi and Schwager,

1981). This 7S Ig has been called IgRAA (Atwell and Marchalonis, 1976), IgY or IgG-like. The heavy chain of this molecule seems to be distant from both the μ chain of mammalian IgG and the heavy chain of avian IgY. Litman and Marchalonis (1982) consider the evolution of this Ig in Anura to be a parallel development to that which has occurred in Aves (and Reptilia), rather than it being an ancestral Ig. In mammals the IgM to IgG switch is under thymic control and is associated with the products of the Class II regions of the MHS: there is evidence to suggest that the synthesis of IgRAA (which supplements, but does not supersede IgM synthesis in Xenopus laevis) is under similar control, although that evidence is somewhat conflicting (Manning and Jurd, 1980).

It is notable that this function of the Class II regions of the MHS is absent in fishes which lack a non-IgM Ig, but that they can nevertheless handle T-dependent antigens adequately. By comparison, the Urodela also seem to cope with T-dependent antigens without recourse to an IgG-like antibody; thymectomy has little effect on anti-Salmonella flagellar H antigen production in Pleurodeles waltlii (Charlemagne and Tournefier, 1977a), and may even enhance anti-horse erythrocyte antibody production in Ambystoma mexicanum by removal of suppressor cells (Charlemagne and Tournefier, 1977b). The evidence for helper T cells in A. mexicanum (Tahan and Jurd, 1981) should not, however, be overlooked.

IV. OTHER IMMUNE AND RELATED PHENOMENA

Our comparative critique of immune phenomena in Teleostei and Anura should not overlook other features of immunity. The fishes have a variety of lymphopoietic tissues (Fange, 1982), the pronephros acting as the main stem cell generator in Teleostei, with the thymus and spleen playing roles as primary and secondary lymphoid organs. Lymphoid and myeloid elements in the blood forming tissues become increasingly separated in the course of piscine evolution. In Anura, stem cells are originally generated in the "intermediate cell mass", mesodermal tissue whence also develops the mesonephros (opisthonephros) (Volpe and Tompkins, 1982). The stem cells populate the thymus whence lymphocytes go to secondary lymphoid organs such as the GALT, spleen and subcapsular layer of the liver. The presence of GALT, and secretory Ig (mainly IgM but also some IgRAA) in body fluids such as bile in Xenopus laevis (Jurd, 1977) is interesting, suggesting the development of

a first line of defence akin to the GALT/IgA system in homoiotherms (although no IgA has been found in any poikilotherm). Advanced anurans possess lymph nodes and also bone marrow, but the latter's role in lymphopoiesis is probably of secondary importance and is more likely to reflect biomechanical considerations of skeletal structure associated with the transition to land rather than a haematological development.

Antigen-trapping in Anura such as Xenopus laevis tends to be more advanced than in Teleostei such as Cyprinus carpio (Secombes and Manning, 1980, 1982): in the former antigen is concentrated in the white pulp of the spleen (although germinal centres are absent). In C. carpio soluble antigen is trapped in the spleen and pronephros; it is found in the ellipsoid sheaths surrounding the splenic capillaries, associated with reticulin fibres in the ellipsoid walls - i.e. it is extracellular. Particulate antigen appears in macrophages. Secombes et al. (1982) note that germinal centres may be associated with an IgM to IgG switch in mammals, so that lack of germinal centres in teleosts may not be surprising: their absence in Anura is more unexpected if IgRAA is analogous to IgG.

"Natural" antibodies (e.g. iso- and hetero-haemagglutinins) are found in Teleostei (Bezkorovainy et al., 1971) and in other fishes, and in Anura (Jurd, 1978), these normally being IgM molecules. Non-Ig defence systems, which may be relics of invertebrate armouries dating from before the evolution of the specific receptor/Ig system, are also found in both taxa. For example, Davies and Lawson (1982) describe α-precipitins in Salmo salar, and Balding and Gold (1976) an α_1-macroglobulin haemagglutinin in Bufo regularis. Other teleost non-specific defences include phagocytes (Grace et al., 1980), lysozyme, and C-reactive protein (Fletcher et al, 1977). Grace et al. (1980) suggest that in early ontogenesis fish fry may need to rely on such mechanisms prior to the development of efficacious immune responses (at about 14-days post-hatch in Salmo gairdneri (Tatner and Manning, 1983). Similar mechanisms and considerations may apply in the Anura: Turner (1969) notes that in stage 48 Xenopus laevis tadpoles, pericardial and peritoneal macrophages alone phagocytose colloidal carbon, suggesting an efficient phagocytosis system. However, there seems to be less reliance on such devices in Anura, and more reliance on the immune system.

Hypersensitivity reactions (which could be described as

the immune system "over-reacting", and are thus manifestations of an effective immune system) are described in both Teleostei and Anura. Ellis (1982) reviews immediate hypersensitivity in fishes, finding some similarities between anaphylactic hypersensitivity in mammals and a variety of findings in fishes, but he notes that fishes lack histamine-associated mast cells, possess low histamine levels, and lack an IgE. C-reactive protein is implicated in Pleuronectes platessa (Fletcher and Baldo, 1974) as may be SRS-A (Baldo and Fletcher, 1975), suggesting that convergent evolution of an anaphylactic--type reaction has occurred in Teleostei in response to similar adaptive pressures, and fulfilling the same (rather uncertain) role. More orthodox anaphylactic hypersensitivity has been described in Rana pipiens with mast cells and histamine implicated (Cohen et al., 1968). Delayed hypersensitivity is seen in the teleosts Ameirurus melas=Ictalurus melas and Salmo gairdneri (Corbel, 1975) and in the anuran Xenopus laevis (Abdul-Salam, pers. comm.), but it is also found in the urodele Ambystoma mexicanum (Tahan and Jurd, 1979), and in the holostean Amia calva (Corbel, 1975).

Finally, complement, associated with the Class III regions of the MHS, is found in all gnathostome groups so far investigated (Gewurz et al., 1966).

V. CONCLUSIONS

In conclusion, what highlights can be seen from an overview of the immune systems of fishes and amphibians, and in particular from those of Teleostei and Anura? (Fig. 2).

Among poikilotherms, Teleostei and Anura alone show acute allograft and xenograft rejection, albeit temperature-dependent: this is a marker of the products of Class I regions of the MHS in mammals and suggests convergent evolution of a homologous (or analogous) system in these taxa. Similarly, markers of the Class II regions of the MHS in Mammalia are also seen in Teleostei and Anura: strong MLRs, lethal GVH reactions, cell cooperation, heterogeneity in mitogen responsiveness and surface physicochemical characteristics of lymphocytes. Such markers seem absent in other fish and amphibian taxa.

Differences between major facets of the teleost and anuran immune responses should not be understressed.

1. "ADVANCED" FEATURES OF IMMUNITY FOUND IN TELEOSTEI AND ANURA

Acute (or sub-acute) allograft rejection (temperature-dependent) (also found in Holostei)	associated with Class I region MHS (in Mammalia)
Strong mixed lymphocyte reactions Lethal graft-versus-host reactions Cell-mediated lympholysis Serologically detectable MHS antigens Cell collaboration (? also in Urodela) Delayed hypersensitivity (also found in Elasmobranchii and Urodela)	associated with Class II region MHS (in Mammalia)
Complement activity (also found in all Craniata and several invertebrate phyla)	associated with Class III region MHS (in Mammalia)

2. MAJOR DIFFERENCES IN IMMUNE PHENOMENA FOUND BETWEEN TELEOSTEI AND ANURA

No non-IgM immunoglobulin found in Teleostei IgRAA (=IgY =IgG-like) in Anura; IgM to IgRAA switch	associated with Class II region MHS (in Mammalia)
Mechanism of anaphylactic hypersensitivity Disposition of lymphopoietic tissue	

3. WAYS IN WHICH TELEOST IMMUNE PHENOMENA ARE MORE PRIMITIVE THAN THOSE OF ANURA

μ-chain containing immunoglobulin only
More reliance by Teleostei on non-immune defence mechanisms (???)
Antigen-trapping mechanisms
Disposition of lymphopoietic tissues, and degree of separation of lymphoid and myeloid elements in blood-forming tissues

Fig. 2. Immune Phenomena in Teleostei and Anura.

Teleosts possess only IgM Ig (although a related lymphocytic receptor molecule is known to exist), whereas in Anura we first meet a non-μ-chain containing IgRAA, the switch from IgM to IgRAA synthesis probably being under thymic control and a product of Class II region MHS activity. This function is obviously absent in the teleosts, but they have very effective antibody responses. Anaphylactic hypersensitivity reactions are mediated in different ways in the two taxa.

In some cases, the anuran phenomena are more advanced than the teleostian: antigen-trapping and disposition of lymphopoietic tissue, for example. Teleosts seem to rely more heavily on non-immunological defence mechanisms than do anurans, such mechanisms presumably being survivors from invertebrate defence mechanisms.

Thus we can observe (? convergent) evolution in the teleosts and the anurans, independently, of a number of indices of an effective immune system, associated with what in mammals would be the Class I and II regions of the MHS. (In the Teleostei we may also be seeing effective IgM-based humoral immunity not associated with Class II regions of the MHS. The presence of other facets of MHS activity such as a complement system, delayed hypersensitivity and arguable cell co-operation in the "lower" fish taxa and in the Urodela should not be forgotten).

In simple terms, we may note that Teleostei and Anura have independently evolved sophisticated and effective immune responses which have many closely parallel features. Many of these may be associated with the independent, convergent evolution of a mammal-like or bird-like MHS. Teleostei and Anura are very successful craniate groups; the question we are left to ponder is: has the advanced immune systems permitted the success of the Teleostei and the Anura, or did success of the groups allow an advanced immune system to evolve?

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PHAGOCYTOSIS IN FISH

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I. INTRODUCTION

Phagocytosis, the cellular ingestion and digestion of particulate matter, is probably the most widely distributed defence reaction occurring in virtually all animal phyla. As shown by primitive organisms, the process is directly related to nutrition as well as defence but with the appearance of metazoan organization, cells specifically dedicated to the recognition and elimination of non-self material emerged. The presence of a vascular system also enhanced the speed with which these phagocytic cells could be mobilized at sites of injury, to dispose of invading bacteria and damaged cells from the organism itself. The phagocytic cells which play an important role in vertebrate defence mechanisms fall into two categories: the granulocytes and the mononuclear phagocytes, the latter represented by tissue macrophages and circulating monocytes (Furth et al., 1972). The phagocytic cells of fish can be generally considered within these groups.

ONTOGENY OF PHAGOCYTES

The ontogeny of phagocytic cells in teleosts has not been described although ontogenic studies on erythropoiesis in rainbow trout (Salmo gairdneri) have shown that cells are initially derived from both intraembryonic cell masses as well as extraembryonic blood pools in the yolk sac and

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thereafter in the kidney and spleen at 1 day pre-hatch (Iuchi and Yamatoto, 1983). In addition, Grace et al. (1981) noted that the kidney of rainbow trout 4 days post-hatch was not appreciably lymphoid yet erythrocytes and macrophages were present. It is therefore interesting to speculate that macrophage production may similarly occur at these different locations during embryogenesis and that these cells represent the first leucocytes to originate.

CELLS DISPLAYING PHAGOCYTOSIS

Various morphologically different types of cells have been described as being phagocytic in teleosts. Blood monocytes and tissue macrophages, which are noted as being avidly phagocytic in mammals, were similarly so for carbon in plaice (Pleuronectes platessa L.) (Ellis, 1976). Boomker (1981), during histological studies of blood cells, found a cell resembling a monocyte with a number of intracellular bacteria in the bream (Sarotherodon mossambicus) while after an intraperitoneal injection of colloidal carbon, tissue macrophages contained ingested carbon in the catfish (Clarias gariepinus). Although Weinreb (1958) did not usually find monocytic/macrophage-like cells in the blood of normal rainbow trout, during bacterial infections these cells were prominent and contained abundant debris. Such cells may have originated from the kidney since macrophages from the pronephros were shown to phagocytose a variety of different particles in vitro (Braun-Nesje et al., 1981). In the gar (Lepisosteus platyrhincus) it was found that during in vitro studies, phagocytosis of yeast particles was performed principally by monocytes and not by granulocytes (McKinney et al., 1977). Although conflicting results exist as to the phagocytic ability of granulocytic neutrophils in fish (Ellis, 1977) we have found that neutrophils in plaice kidney were phagocytic for carbon (Fig. 1) and under inflammatory conditions these cells were actively involved in the uptake of glycogen and bacteria in the peritoneal cavity (MacArthur et al., 1984). Neutrophils in goldfish (Carassius auratus) (Weinreb, 1963) and those of coho salmon (Oncorhynchus kisutch) with bacterial disease, were also phagocytic (Lester and Budd, 1979) while Elarifi (1982) suggested that whiting (Merlangius merlangus L.) neutrophils might also be phagocytic since they contained dark staining bodies. Hoole and Arme (1982) described the neutrophils in tapeworm-infected roach (Rutilus rutilus)

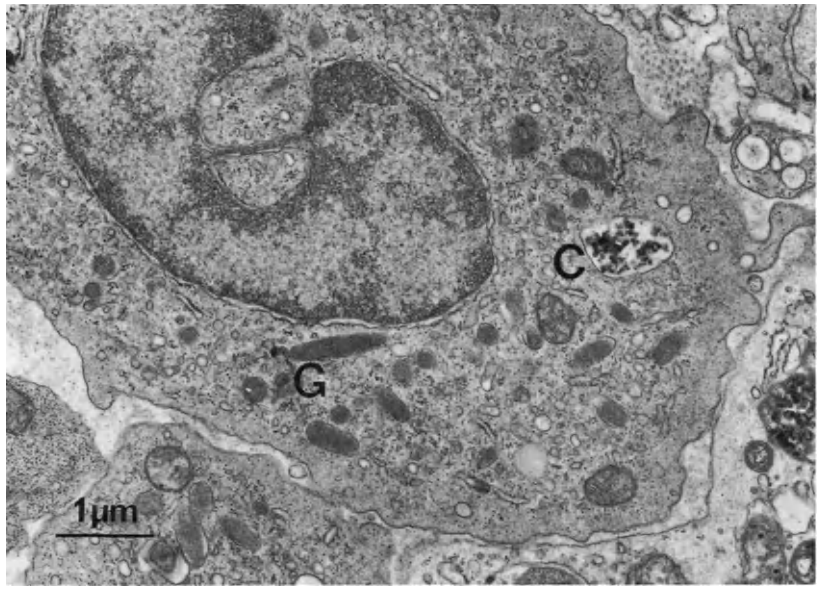

Fig. 1. Neutrophil, in plaice kidney, containing ingested carbon particles (C) and characterised by elongated cytoplasmic granules (G).

as possessing a limited phagocytic capacity while noting that different stages during macrophage development were variably phagocytic. It is worth considering that the conflicting results obtained concerning neutrophil phagocytosis in different species might be attributed to the state of their development, location i.e. blood or tissue, and relative activity at the time the study was performed. In addition to those blood cells already discussed, blood thrombocytes have also been described as phagocytic for carbon in plaice (Ferguson, 1976) and bacteria in coho salmon (Lester and Budd, 1979). Apart from these blood cells, endothelial cells lining blood sinuses and gill pillar cells which were found to phagocytose carbon and latex particles in rainbow trout (Chilmonczyk and Monge, 1980) can be classed as weakly phagocytic when compared with those already described.

CYTOCHEMICAL CHARACTERISTICS

The cytochemical staining of teleost phagocytes has enabled better classification of these cells; however, confusion can arise since immature cells may have different staining intensities. For example, Garavine and Martelli (1981) found that alkaline phosphatase activity appears earlier than peroxidase activity during the development of goldfish neutrophils. The granulocytic cell of which the neutrophil type is either the predominant or only granulocyte, is characterised by its generally reliable staining with Periodic acid Schiff (PAS) and less reliable peroxidase staining which may be attributed to the substrate and fixation procedures employed (Cannon et al., 1980). Both neutrophils and monocytes/macrophages are positive for PAS, which detects the presence of carbohydrate groups. A strong PAS reaction occurred with both whiting (Elarifi, 1982) and plaice neutrophils while macrophages produced a much less intense colouration in plaice (Ellis, 1976). Peroxidase activity has been described in eosinophilic granulocytes of carp (Cyprinus carpio) (Kralj-klobucar, 1982) and neutrophils of plaice (Ellis, 1976), whiting (Elarifi, 1982) and channel catfish (Ictalurus punctatus) (Cannon et al., 1980), and the last authors suggest that this enzyme acts as an excellent marker for granulocytes and strongly indicates antibacterial and phagocytic functions. Esterase staining is regarded as the most reliable cytochemical marker for mammalian macrophages (Kaplow, 1981); however this has not been widely used for fish histochemistry although plaice macrophages showed non-specific esterase activity (MacArthur et al., 1984). Acid phosphatase was also shown in plaice monocytes and macrophages as being concentrated in coarse cytoplasmic granules, while in neutrophils they appeared finer (Ellis, 1976).

PHAGOCYTOSIS

Phagocytosis proceeds through stages of recognition and attachment of the particle, engulfment, degranulation, killing and digestion and is sometimes preceded in vivo by a chemically guided movement of mobile phagocytes towards their target. Nothing is known of this mechanism of chemotaxis in fish, although leucocytes migrate towards foreign material such as glycogen, introduced into the peritoneal cavity of the plaice (MacArthur et al., 1984)

or sites of injury in the rainbow trout (Finn and Nielson, 1971).

The basis of recognition is a central theme of biology and Ruben (1982) has discussed the evolutionary aspects of immune recognition. Fish phagocytes exhibit the discriminatory capacity for their targets common to other vertebrates but the basis for this is ill-understood. Surface phenomena are involved and the role of surface charge and the hydrophilic properties of the phagocyte and particle have been considered (Weir and Ögmundsdóttir, 1977; Van Oss, 1978). It is however well established that coating particles with various serum proteins enhances their ingestion by phagocytes (opsonization). In mammals, the major serum opsonins are antibodies of the IgG class and complement components (Walters and Papadimitriou, 1978). Only IgM-type antibodies have been found in fish and so the lack of a demonstrable Fc receptor on fish phagocytes (Wrathmell and Parish, 1980) could be due to fish antibodies not being opsonic. Recent evidence however does not support this. Griffin (1983) found that specific antibody to the pathogen Yersinia ruckeri exerted a significant opsonic effect on the in vitro rate of ingestion by phagocytic cells from the rainbow trout. The complete absence of serum from the experimental system resulted in a marked reduction in uptake compared with even normal serum, so that a requirement for protein may indicate some surface charge effect. Griffin (1983) also observed that mononuclear phagocytes engulfed greater numbers of bacteria than did neutrophils. Fujii (1981) working with Arctic lamprey (Lampetra japonica) identified phagocytic polymorphonuclear leucocytes showing significantly enhanced phagocytic activity in vitro against sheep erythrocytes, provided they were opsonized with specific lamprey antibody to them. The exclusively IgM-type antibody of lamprey again raises the question of the membrane receptor on the phagocyte. Weissmann et al. (1978) stated that the phagocytic cells of the dogfish (Mustelus canis) possessed surface Fc receptors for heat-aggregated, but not native, immunoglobulins. There is evidence for cytophilic antibody on some fish macrophages (Chiller et al., 1969; Ellis, 1976) which if specific for an invading bacterium, would enhance its binding to the macrophage surface. External opsonins would also not be required if receptors on the phagocyte membrane reacted directly with components of the bacterial surface, such as the lectin-like receptors described on mouse macrophages (Weir and Ögmundsdóttir, 1980). Peritoneal macrophages

elicited by inflammatory stimulation in a Japanese trout (Oncorhyncus rhodurus) expressed receptors for an homologous lectin which could function as an opsonin (Ozaki et al., 1983). Fibronectin is a nonimmune serum opsonin, found in mammals, which could aid in the clearance of non-bacterial debris arising as a consequence of trauma although it is doubtful whether it is involved in the phagocytosis of bacteria (Van De Water et al., 1983). Fibronectin contains several binding sites, including one for gelatin and our unpublished observations would suggest the presence of a fibronectin-like molecule in plaice blood. We observed a significantly more rapid ($p < 0.02$) clearance from the circulation of the plaice, of gelatin-coated carbon particles, with a mean phagocytic index ($x10^{-2}$) $\pm$ S.E. of 2.45 $\pm$ 0.39 (5 fish) compared with 1.07 $\pm$ 0.2 for 5 fish receiving uncoated carbon (4 mg carbon per 100g weight of fish).

Attachment, whatever its means, appears to be a relatively passive phenomenon, whereas ingestion, even

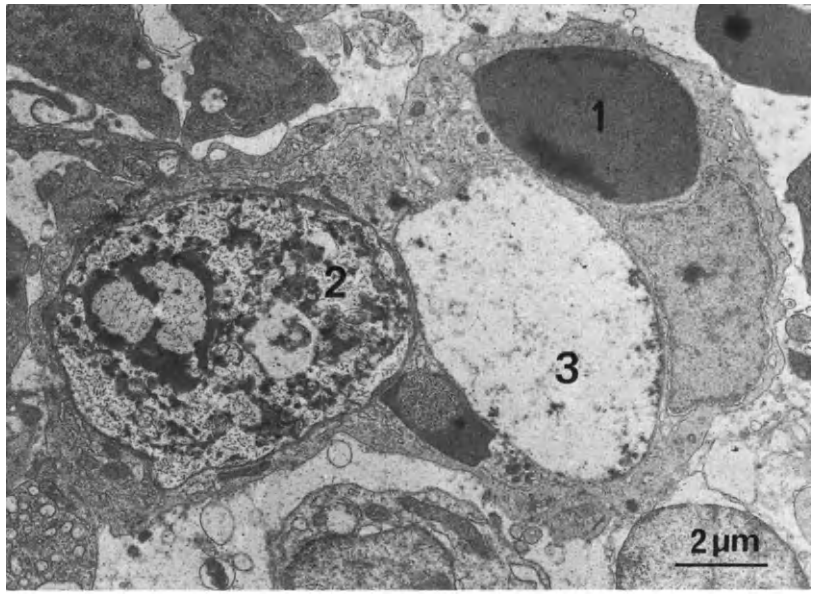

Fig. 2. Mononuclear phagocyte in plaice kidney containing ingested turbot erythrocytes at various stages of digestion; (1) none (2) partial and (3) complete.

when only observed with the electron microscope, appears to be active and presumably energy-dependent. Fujii (1981) observed the cytoplasm of lamprey phagocytes to extend long pseudopodia upon contact with an opsonized erythrocyte. Phagocytosis of yeast particles by monocytes from the gar was usually by engulfment but McKinney et al. (1977) sometimes also observed a unique mechanism of "wrapping" psuedopods around particles. Both mechanisms usually resulted in vacuole formation with the yeast particles undergoing digestion. The digestion of turbot erythrocytes in the vacuoles of a plaice macrophage is shown in Fig. 2. The enzymes, which by analogy with mammalian phagocytosis, are released from neutrophil granules and macrophage lysosomes into phagocytic vacuoles, have not been studied in detail in fish. Information has mostly been derived from histochemical studies where, for example, lysozyme, hydrolytic enzymes and peroxidase have been found in some phagocytes. Little is therefore known of the mechanism of killing the phagocytosed parasites but it is probable that the ability to generate specific oxygen metabolites is involved. Kanner and Kinsella (1983) have extracted myeloperoxidase from the neutrophils of the rainbow trout and shown it to resemble mammalian myeloperoxidase in its properties. It might therefore contribute to the antimicrobial activity of the phagocyte in conjunction with other substances.

KINETICS OF PARTICLE CLEARANCE

The location of phagocytes around the body has resulted in the formation of a phagocytic system capable of removing invading pathogens and effete blood and tissue cells. In mammals the activity of these phagocytes, especially those lining blood capillaries and sinuses, can be quantitatively measured by injecting particulate material into the bloodstream, and has provided a method for assessing the effects of different factors on the functioning of this system. On injecting carbon, bacteria or radiolabelled erythrocytes, the percentage lost from the bloodstream is proportional to that taken up by sinus-lining macrophages and blood phagocytes.

In fish, the clearance of particulate matter has been described as being "biphasic" where an initial rapid component is followed by a slower one. Avtalion (1981) demonstrated that up to 99% of Staphylococcus aureus was cleared from carp blood in 30 min while Ferguson et al. (1982) found 90% of Salmonella pullorum was lost from

rainbow trout blood within 15 min. Carbon and ^{51}Cr-turbot erythrocytes (TRBC) were similarly cleared in a biphasic fashion from plaice blood with 80% and 90% removed respectively, during 30 min (MacArthur et al., 1983). However in comparison to these relatively fast clearances, O'Neil (1980a) found that the bacteriophage MS2 was not eliminated from the circulation of brown trout (Salmo trutta) until two days after inoculation. Clearance could be correlated with a coincident rise in specific antibody, which was responsible for bacteriophage removal.

Since teleosts are poikilothermic animals and thus metabolically influenced by their environmental temperature, it has been shown that at temperatures below their optimum the generation of specific antibody, although not abolished, is slowed down (Avtalion et al., 1973). In consequence, O'Neill (1980b) demonstrated that the time required for MS2 bacteriophage clearance in brown trout, carp and Notothenia rossii was increased when the water temperature was reduced. This delay in antibody synthesis, in fish maintained at below optimum temperatures, is not thought to be the result of impaired phagocytosis but might be an impairment of T and B lymphocyte interactions (Avtalion et al., 1976). In support of their former observation, the in vivo clearance of carbon and ^{51}Cr-TRBC was not significantly different in plaice acclimated at 5, 12 or 19°C for 7 days (MacArthur et al., 1983) or 28 days (unpublished observations). In vitro uptake of S. aureus by carp blood leucocytes was similarly unaffected by temperature; however at 10°C no intracellular killing of the bacteria occurred compared with killing at 25°C (Avtalion, 1981).

Phagocytosis relies upon the cell membrane being sufficiently mobile to allow realignment and subsequent engulfment. Experiments performed on pinfish (Lagadon rhomboides) showed that the fluidity of lymphocyte membranes from 27°C acclimated fish, measured at 27°C, was nearly identical to the membrane fluidity of 17°C acclimated fish, measured at 17°C (Abruzzini et al., 1982). If membrane fluidity is similarly maintained in phagocyte membranes after acclimation, this would explain why no difference was found in the clearance of carbon and ^{51}Cr-TRBC in plaice tested at their acclimation temperatures (MacArthur et al., 1983).

Although non-antibody dependent clearance is unaffected by temperature, subsequent killing, such as that of bacteria by carp leucocytes (Avtalion, 1981) is temperature-sensitive and this may partly explain why fish

are unable to eliminate bacterial infections during winter months.

ORGAN LOCALISATION OF ANTIGENIC MATERIAL

In the teleost, organ uptake studies have revealed the kidney and spleen as being the main areas for carbon localisation in the cunner (Tautogolabrus adspersus) (MacMull and Michels, 1932) and plaice (Ellis et al., 1976) and for human gamma globulin in the carp (Secombes and Manning, 1980). In the kidney, material is initially phagocytosed by the reticuloendothelial cell network within the haemopoietic parenchyma, while in the spleen antigen is trapped extracellularly on reticular fibres in the ellipsoid walls. Later, in both organs, macrophages containing phagocytosed material aggregate in lymphoid areas, often in the presence of melanomacrophages. It is suggested that the concentration of antigenic material at focal sites may represent an ancestral analogue of the germinal centres found in higher vertebrates (Ellis et al., 1976). In addition, endothelial cells in the ventricle of the Amazon molly (Poecilia formosa) heart (Woodhead, 1981) and atrial endocardial macrophages in plaice heart (Ferguson, 1975) also demonstrated carbon phagocytosis.

Quantifying the percentage uptake by organ phagocytes has recently revealed the apparent discriminatory powers of these cells. Ferguson et al. (1982) found that ^{51}Cr-Salmonella pullorum was principally localised in rainbow trout kidney, both on a whole organ and per gram weight basis. In plaice injected with ^{51}Cr-TRBC the kidney contained most counts due to its larger size, but per gram the spleen was more active (MacArthur et al., 1983). In addition more bacteria localised in the gills of rainbow trout than the spleen, while very few radioactive counts were detected in the gills of plaice receiving ^{51}Cr-TRBC. In order to explain these differing results it might be worth considering that the degree of recognition and phagocytosis of these foreign particles (i.e. bacteria and TRBC) by phagocytes in different organs may be related to the amount of exposure these phagocytes normally receive to similar material. Since the spleen is usually involved in removing effete erythrocytes from the circulation, this may explain why TRBC uptake was greatest per gram of spleen. When colloidal carbon was injected into plaice, four times as many carbon-containing leucocytes per gram were located in the kidney compared to

the spleen (unpublished observation), indicating that the spleen does not show the same degree of uptake for carbon as it does for TRBC. Gill macrophages and pillar cells may similarly assume a semi-selective role in phagocytosing bacteria which can enter via this route (Bowers and Alexander, 1981), especially since gill tissue is easily damaged.

STRESS AND ENDOTOXIN EFFECT ON PHAGOCYTOSIS

Farmed fish which are often reared in crowded tanks are susceptible to stress (Perlmutter et al., 1973) and this produces a rise in the teleost stress hormone, cortisol (Wedemeyer and McLeay, 1981). In mammals, the effects of these glucocorticoid hormones on different aspects of the immune response have been well documented (Parrillo and Fauci, 1979). Apart from temperature, the effects of stress on the immune response in fish may be of great importance and for that reason the action of stress on the phagocytic process was considered. In mice, as little as 2.5 mg/kg of cortisone resulted in a depression of phagocytic activity (Heller, 1955) which may be a consequence of membrane receptor modulation (Parrillo and Fauci, 1979).

Both the rate of ^{51}Cr-TRBC (antigen) clearance from plaice blood and its 2 hr organ localisation was measured in fish receiving i.v. cortisol (hydrocortisone-21-phosphate) in the following experiments: a) 1 mg cortisol/300g body weight, 2 hr prior to antigen; (b) 1 mg cortisol/300g body weight daily, for 2 days prior to antigen; (c) 3 mg cortisol/300g body weight daily, for 3 days prior to antigen. No difference in either the rate of clearance or organ uptake of this antigen occurred for any of the experimental groups. However, it was found that when plaice were either environmentally stressed, so that endogenous cortisol levels increased, or they received cortisol as in experiment (c), the accumulation of cells in the peritoneal cavity in response to intraperitoneally injected glycogen was significantly reduced (MacArthur et al., 1984). Therefore, immunologically, one of the most important effects of stress in the teleost is probably its ability to inhibit leucocyte recruitment at sites of tissue injury.

In mammals, endotoxin, the lipopolysaccharide component of Gram negative bacterial cell walls is known to produce an increased stimulation of the phagocytic system which is paralleled by increased spleen and liver weights, due to

the proliferation of macrophages and lymphocytic cells (Stuart, 1970). Liver and spleen localisation of antigen was found to be enhanced in endotoxin-treated mice (Warr and Šljivić, 1974). In consequence it was decided to investigate the effects of E. coli lipopolysaccharide on the 40 min organ uptake of ^{51}Cr-TRBC (antigen) in the following experiments: 500 μg endotoxin/300g body weight given a) 3 hr prior to or b) 24 hr prior to antigen; c) daily for 3 days or d) daily for 5 days prior to antigen. Only group d) fish showed an increase in splenic uptake of ^{51}Cr-TRBC (Table I) which could be the result of the significantly increased ($p < 0.02$) spleen weight. The spleen index, calculated as spleen weight (mg)/weight of fish (g), was 1.04 ± 0.09 for endotoxin-treated plaice and 0.68 ± 0.08 (mean ± S.E.) for normal fish. Endotoxin-treated plaice also produced significantly enhanced numbers of inflammatory cells in response to intraperitoneally injected glycogen (MacArthur et al., 1984) and for this reason endotoxin might be investigated as an immune stimulant in fish.

Table I. Effect of endotoxin on the uptake of ^{51}Cr-TRBC[a] at 40 min.

	n	% cpm of total/gram tissue Kidney	Spleen	Liver
[b]Endotoxin	7	23.7 ± 2.3	*53.3 ± 4.7	0.9 ±0.1
[c]Controls	6	21.1 ± 3.3	*37.0 ± 6.0	0.9 ±0.1

Figures represent the mean ± S.E.

n represent number of fish

* denotes significant difference at $p < 0.05$

a ^{51}Cr-labelled turbot erythrocytes (MacArthur et al., 1983)

b 500 μg E. coli lipopolysaccharide Boivin preparation/300g body weight, i.v., daily for 5 days.

c no prior treatment.

PHAGOCYTOSIS AND DISEASE

Studies on the behaviour of phagocytic cells in fish exposed to pathogens could provide needed information on their effectiveness as defensive cells. A defect in phagocyte recognition, ingestion or intracellular killing could be a contributory factor in the pathogenicity of some organisms. Wolke (1975) commented on the lack of an inflammatory response in fish tissues to Aeromonas salmonicida, but this was found to be due to the destruction of leucocytes and depression of haematopoiesis by bacterial products. Klontz et al. (1966) in a study of sequential pathological changes in rainbow trout inoculated with A. salmonicida, did observe an early inflammatory response but noticeably the macrophages did not phagocytose the bacteria, although they engulfed necrotic debris.

It would seem more usual for the deficiency in the fish to be in the killing of pathogens and not in their uptake. Sporozoan parasites were observed in the macrophages of the turbot (Scophthalmus maximus), which appeared to lack the ability to kill the trophozoites (Ferguson and Roberts, 1975). Host cells from the brook trout (Salvelinus fontinalis) resembling neutrophils, macrophage and pigment cells, are reported to phagocytose the bacterial kidney disease organism. However, the morphological integrity and active cell division of the phagocytosed bacteria would indicate that they were not necessarily being killed by the phagocytes (Young and Chapman, 1978). Results from in vitro studies (Avtalion and Shahrabani, 1975) showed no difference in the uptake of S. aureus by whole blood from both immunized and non-immunized carp, but only cells from immunized fish were effective in killing or inactivating the bacteria. In some instances, the location of the phagocytic cells may be harmful to the organ in which they are sited, if the bacteria are not killed. The active atrial phagocytosis observed with carbon in the plaice, could make the heart vulnerable to pathogens, as pointed out by Ferguson (1975).

Some indication of the importance of phagocytes derives from the results of Miyazaki and Kubota (1981). Japanese eels (Anguilla japonica) pretreated with a bacterial sonicate of Vibrio, 24 hr before exposure to viable bacteria, exhibited an extensive response by phagocytic neutrophils which prevented bacterial dissemination. Phagocytes may have a role in the encapsulation of tissue parasites which are too large to be phagocytosed

(Sommerville, 1981). A fibrous capsule forms around nematode larvae in the liver of the whiting, but initially there is a dense infiltration of apparently phagocytic neutrophils and fewer macrophages, which clear all necrotic debris from the capsule (Elarifi, 1982).

The measurement of chemiluminescence (Scott and Klesius, 1981) has considerable potential for elucidating the mechanism of phagocytosis in fish. Stave et al. (1983) have already applied this technique to a study of the phagocytosis of fish pathogens by cells from the pronephros of striped bass (Morone saxatilis). Bacterial species vary widely in their ability to stimulate chemiluminescence and this may be correlated with their biochemical characteristics. It is only with an understanding of what constitutes defects in the phagocytic system and a knowledge of how the system can be activated, that a true assessment of its importance to fish health can be made.

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AN IN VITRO STUDY OF POLYMORPHONUCLEAR PHAGOCYTOSIS AND THE EFFECT OF TEMPERATURE

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I. INTRODUCTION

There is little doubt that teleosts possess an efficient particulate-antigen trapping and avid phagocytic system within the meshwork of the reticulo-endothelium and macrophages. However, in the higher vertebrates two major types of "professional" phagocyte, the mononuclear phagocyte and the polymorphonuclear leucocyte (PMN), are essential, if effective immunity is to be maintained. The PMN or neutrophil, in contrast to the mononuclear phagocyte, is a circulating cell that forms an important part of the non-specific defence mechanisms, and which will very rapidly migrate to a site of inflammation, phagocytose, and destroy pathogenic organisms. Similar neutrophil-like PMNs are found in teleosts, though reports of their phagocytic function are equivocal (Ellis, 1977). In this preliminary study a method of preparing PMN-rich suspensions, from the blood of brown trout (Salmo trutta L.), was used to observe the ability of these cells to phagocytose a yeast cell, Candida guilliermondii.

II. MATERIALS AND METHODS

EXPERIMENTAL FISH

Yearling brown trout (Salmo trutta L.), 150 - 200g, obtained from the Severn-Trent Water Authority fish farm, Nottinghamshire, were held in 100 L polyethylene aquaria

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with a through-flow of well-aerated and chlorine-free tap water, at a temperature of 15.5 ± 0.5°C. The fish were acclimatised in their holding tanks for at least 14 days before commencing the experiments. A photoperiod of 12h light:12h dark was used and the fish were hand-fed daily on Beta Trout Growers Diet 417, No.5 (B.P. Nutrition). When handled the fish were gently netted and lightly anaesthetised in a 1g:200 L solution of tricaine methanesulphonate (MS222, Sandoz) made up in aquarium water.

POLYMORPHONUCLEAR LEUCOCYTES

PMN-rich suspensions were prepared by a sterile procedure at the temperatures to be used in the subsequent experiments. Blood samples of 1.0 cm^3, obtained by caudal venipuncture, were drawn into 5 cm^3 plastic syringes that contained 600 units of preservative-free heparin (Pularin, Evans Medical) in 1.0 cm^3 sterile teleost saline, pH 7.6 (O'Neill, 1979). 1.0 cm^3 of plasmagel (Laboratoire Roger Bellon) was drawn into each syringe and the contents were gently mixed by rotating the syringes for 10 min. A new 21G 3 cm hypodermic needle, bent through an angle of 120°, was attached to each of the inverted syringes before the red cells were allowed to sediment. The sedimentation time was dependent on the incubation temperature and ranged from 2 - 4h. The PMN-rich supernatants were carefully expelled from the syringes into 1.5 cm^3 polypropylene micro-reaction tubes (Sarstedt), centrifuged at 150 xg for 10 min and resuspended in temperature equilibrated tissue culture medium (TC199, Wellcome) at a concentration of 10^5 PMNs cm^{-3}. The viability of the PMNs, tested by trypan-blue exclusion, was in all cases greater than 95%.

YEAST CELLS

Candida guilliermondii was used for the reasons discussed by El-Maalem and Fletcher (1976). The yeast was cultured overnight at 37°C in 10 cm^3 aliquots of glucose broth, heat shocked for 5 min in a boiling water bath, and twice washed with sterile saline. The washed cells were suspended in 1.0 cm^3 of fresh plasma, pooled from at least 4 brown trout, and incubated at 10°C for 1h to opsonise. After a final wash in sterile saline the yeast cells were

resuspended, with vigorous agitation, in TC199 at a concentration of 10^6 cells cm^{-3}.

PHAGOCYTOSIS

The PMN and yeast suspensions were maintained at the experimental temperatures of 1°, 5°, 10° or 20° ± 0.5°C for a further period of 15 min, then 0.5 cm^3 of each suspension was added to a micro-reaction tube and the contents gently mixed. The tubes were then only agitated prior to the removal of 0.05 cm^3 aliquots at

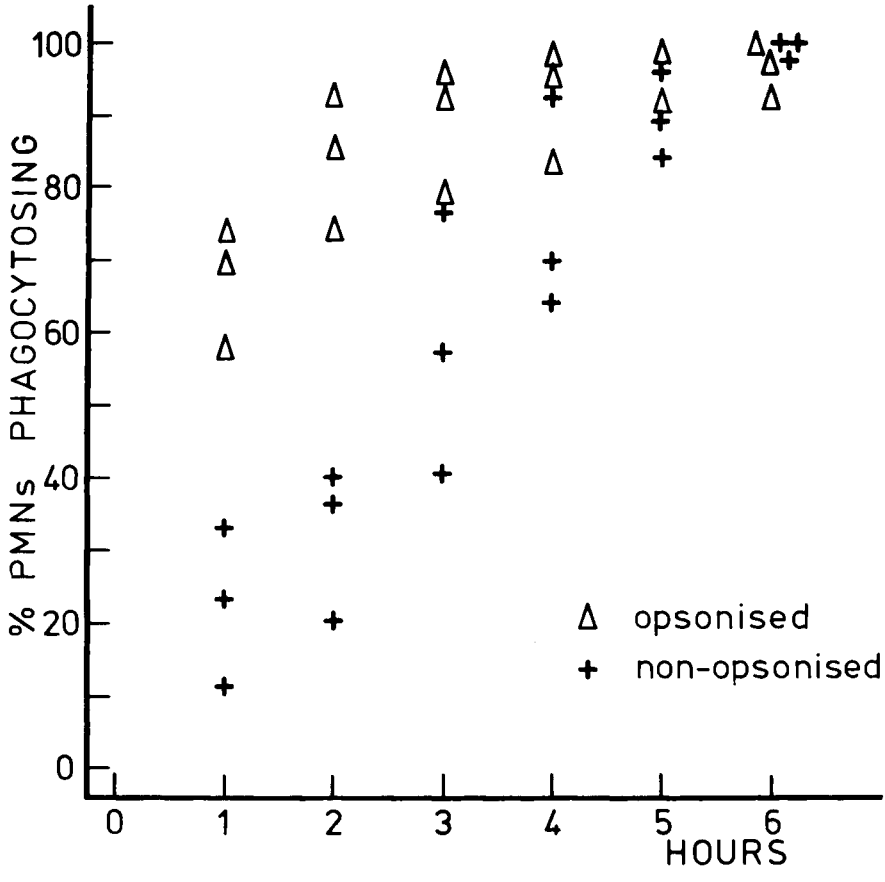

Fig. 1. The phagocytosis of opsonised and non-opsonised Candida guilliermondii by trout PMNs at 10°C. Each point represents the mean of three replicates from one fish.

timed intervals. The aliquots were transferred quickly into 1.0 cm^3 of cold teleost saline, containing 0.006M EDTA, to stop phagocytosis. Microscope slide preparations were made with a cytocentrifuge (Cytospin, Shandon Southern), 1000 rpm for 5 min, and stained with a rapid differential stain (Diff-Quik, Harleco). The yeast cells within 200 PMNs from each time sample were counted and the results of three replicates averaged for each of the three trout used in the study.

Phagocytosis was quantified in two ways, firstly, as the proportion of PMNs that had engulfed yeast cells (% phagocytosis); and secondly, the proportion of yeast cells that had been engulfed (% uptake). In one experiment, at 10°C, the phagocytosis of opsonised yeast cells was compared with that of unopsonised cells.

III. RESULTS

The PMNs of the brown trout were observed to phagocytose C. guilliermondii, albeit the percentage of active PMNs was reduced when non-opsonised yeast cells were used, and at low temperatures. At 10°C the maximum % phagocytosis was reached after 5 - 6h for both the opsonised and nonopsonised yeast cells (Fig. 1). However, 50% of the PMNs were observed to have phagocytosed opsonised yeast cells within 1h, at least 1 - 2h before the non-opsonised cells.

The maximum phagocytic activity was observed at 20°C, below which both the % phagocytosis (Figs. 2, 3) and % uptake (Figs. 4, 5) were decreased, though different temperature dependent relationships were observed between the two. The early kinetics of % phagocytosis and the total number of PMNs activated (Fig. 2) indicated a greater temperature dependence below 10°C, summarised by the 50% phagocytosis plotted in Fig. 3, whereas the % uptake (Fig. 4) and the rate of uptake (Fig. 5) of the yeast cells indicated a direct temperature dependence.

The number of yeast cells phagocytosed by any one PMN ranged from 1 to a maximum of 23 cells. Those PMNs that had ingested large numbers of yeast cells were capable of great distension without rupture. After 12h at 20°C, a number of yeast cells within the PMNs were seen as paler-staining degraded cells or ghosts.

IV. DISCUSSION

The brown trout, like the mammals, would appear to possess a neutrophil-like PMN that can act as a "professional" phagocyte. The demonstration of similar morphological and

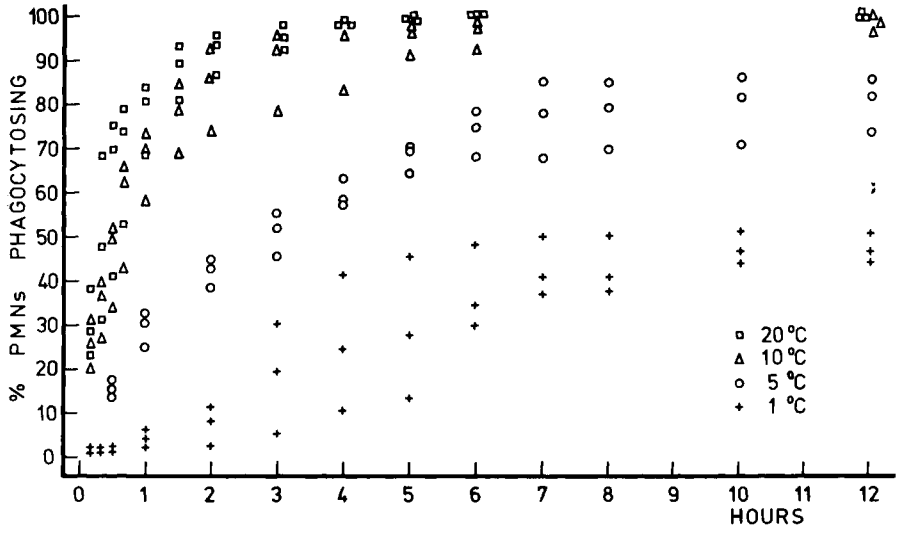

Fig. 2. Temperature and the phagocytosis of opsonised Candida guilliermondii by trout PMNs. Each point represents the mean of three replicates from one fish.

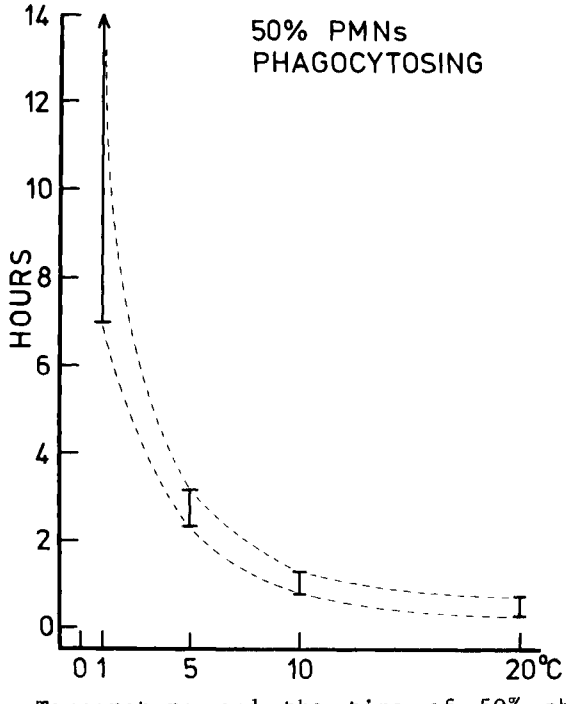

Fig. 3. Temperature and the time of 50% phagocytosis. The vertical bars indicate the range.

histochemical characteristics in trout PMNs to those of mammalian neutrophils has been reviewed by Ellis (1977). He indicated that, although fish PMNs may be a major participant in the leucocyte migration to an inflammatory site, the phagocytic ability of these cells was doubtful.

Trout PMNs were found to possess the non-specific ability to phagocytose non-opsonised as well as opsonised C. guilliermondii. The enhanced ability to phagocytose the plasma treated yeast cells would suggest an opsonin and PMN recognition mechanism, possibly similar to that of C3b recognition by human neutrophils. The ability of the trout PMN to recognise non-opsonised yeast cells could be one of "non-self" recognition, or a microbial surface marker and PMN-receptor mechanism, though opsonins could have been produced by cells in the in vitro PMN suspension. These freshly produced opsonins could have been a contributing factor in the increased PMN activation observed at 3 - 5h with the non-opsonised yeast cells. Also, the production

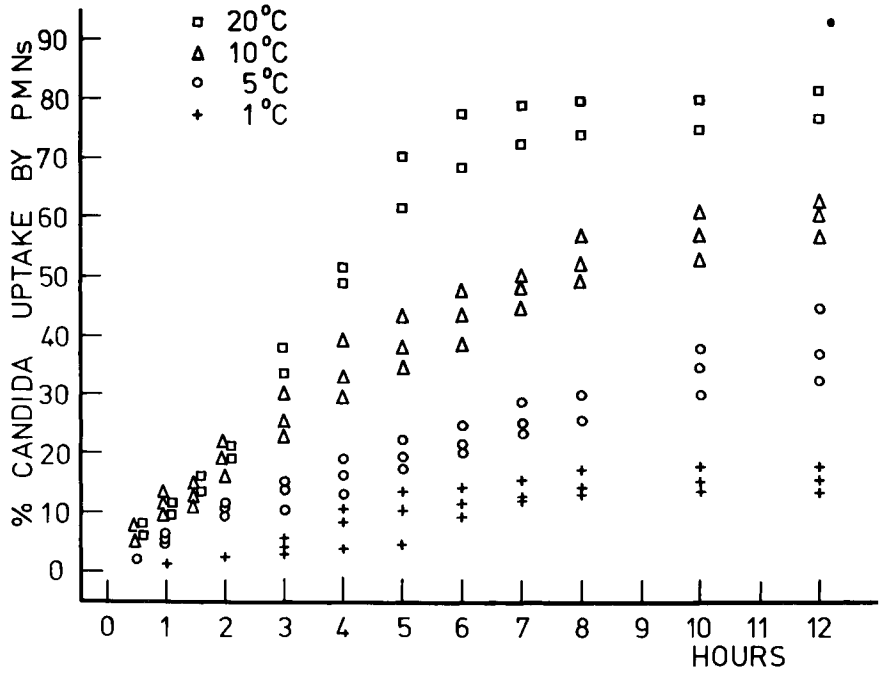

Fig. 4. Temperature and the uptake of opsonised Candida guilliermondii by trout PMNs. Each point represents the mean of three replicates from one fish.

of cross-reacting "natural" antibody opsonins and the recognition of antibody-antigen complexes by the PMNs cannot be ruled out. Antibody opsonisation is essential for the optimal ingestion and killing of micro-organisms by mammalian PMNs. The need for such antibody opsonisation was observed in the in vivo blood clearance of small viral particles in brown trout (O'Neill, 1980b), and an enhanced in vitro response was found for the blood phagocytes of carp immunised with Staphylococcus aureus (Avtalion and Shahrabani, 1975).

Although the trout PMNs were activated quickly, 50% within 1h at 10° and 20°C, the progressive uptake of yeast cells was in contrast slow. Avtalion and Shahrabani (1975) observed that S. aureus uptake in the whole blood of carp reached a maximum at 2-3h, at 25°C, whereas in the trout, 7-8h at 20°C were required to reach the maximum yeast uptake. Apart from the temperature difference, the larger concentration and smaller size of

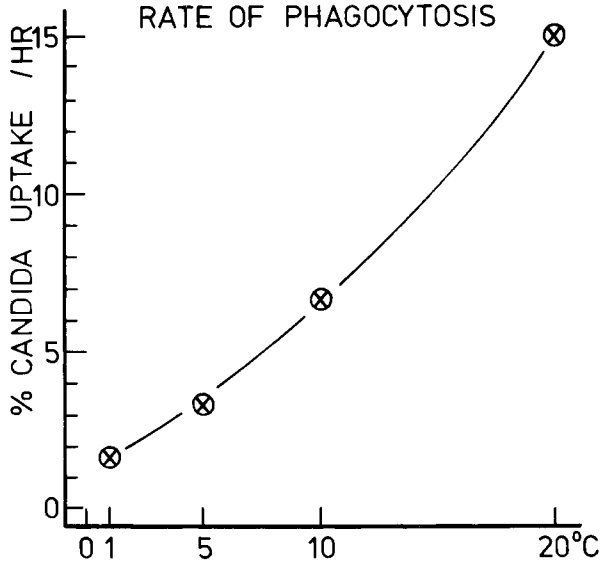

Fig. 5. Temperature and the rate of Candida guilliermondii uptake.

the S. aureus could have contributed to the quantitatively faster uptake. The concentration of yeast cells, approximately 10 yeast cells: 1 PMN, was sufficient to produce several observable surface contacts between the yeast cells and PMNs. Certain PMNs were observed to have been more active than others and ingested large numbers of yeast cells. This range of phagocytic ability within the PMN population was probably an indication of their functional maturity.

Environmental temperature is probably one of the most significant external factors involved in the life processes of fish, affecting all aspects of cell metabolism and activity, therefore the in vitro temperature dependence of phagocytic ability in the trout PMN could be expected. However, the significant decrease in PMN activation which occurred only below 10°, may be an indication, as was observed for humoral immunity (O'Neill, 1980a) and for other body functions (Hazel and Prosser, 1974), that acclimation and adaptation of the phagocytic ability of PMNs could exist in teleosts. Further, the phagocytic ability of trout PMNs at low temperatures has not been observed in mammalian PMNs from hibernating species or by in vitro studies (Prendergast and Proctor, 1981), and, although the ability of the trout PMNs at 20°C was lower than that observed for human PMNs phagocytosing C. guilliermondii at 37°C (El-Maalem and Fletcher, 1976), an extrapolation of the results to 37°C would bring trout phagocytosis within the range of human kinetics.

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STUDIES ON GRANULOCYTE HETEROGENEITY IN ELASMOBRANCHS

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I. INTRODUCTION

The literature on fish leucocytes is extensive and most authors have used the now well-established mammalian terminology to describe these cells. In a recent review by Ellis (1977), however, the often indiscriminate use of mammalian terminology for fish leucocytes was questioned in view of the absence of detailed information on their function and ontogeny. Indeed, although neutrophilic, eosinophilic and occasionally basophilic granulocytes have been identified in fish, little evidence exists for these cells performing the same functions as their mammalian counterparts. Furthermore, no attempt has been made to carry out a detailed examination of the leucocyte characteristics from one group to another within the fish classes. We might expect distinct differences in the leucocyte characteristics between cartilaginous and bony fish particularly in view of the belief of Nelson (1969) and other authors that the latter group is more closely related to the tetrapod line than the cartilaginous fish.

Unfortunately, only a few studies have concentrated on the haematology of cartilaginous fish and almost all of these are limited to the elasmobranchs (Fänge, 1968a,b; Stokes and Firkin, 1971; Morrow and Pulsford, 1980; Hyder et al., 1983). Most of these authors have noted an abundance of eosinophilic granulocytes in the peripheral blood and a lack of neutrophils. Therefore, it appears, at least in the elasmobranchs studied, that eosinophils are the major granulocyte type in cartilaginous fish.

In their survey of vertebrate granulocytes, Kelényi and Németh (1969) produced a very useful list of the main

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morphological characteristics of mammalian eosinophils. These characteristics include:

(a) orange-yellow granules after staining with Giemsa (after methanol fixation);
(b) a high isoelectric point of the specific granules;
(c) selective anisotropic staining and selective fluorochroming by substantive dyes (e.g. Congo red) and fluorochromes (e.g. thioflavine S);
(d) a positive benzidine-peroxidase reaction at pH 11;
(e) the specific granules consist of a matrix and crystalloid surrounded by a single membrane;
(f) affinity of the granule matrix for phosphotungstic acid.

In the present study, we have made a detailed investigation of the granulocytes of three elasmobranch species, namely Scyliorhinus canicula, Raja clavata and R. microcellata to determine if the eosinophilic granulocytes of these fish possess any of the morphological characteristics described by Kelényi and Németh (1969). This study represents part of an ongoing project designed to investigate the structural and functional biology of fish granulocytes.

II. MATERIALS AND METHODS

ANIMALS

S. canicula, R. clavata and R. microcellata were obtained from Carmarthen Bay and maintained in large tanks containing continuously recirculating seawater at a temperature of 15° ± 1°C. The animals were fed weekly on chopped whiting.

BLEEDING TECHNIQUE

The animals were anaesthetised in a 0.04% solution of tricaine methanesulphonate (Sigma [London] Chemical Co. Ltd.). Blood was collected into a 1ml syringe fitted with a 23G needle either by cardiac puncture (rays) or via the caudal vein (dogfish).

BLOOD SMEARS

After air-drying, smears were fixed in 95% methanol and subsequently stained with Giemsa or Wright's stain.

HISTOCHEMICAL TECHNIQUES

Smears fixed in 10% methanol were stained with Congo red according to the method of Puchtler et al. (1962). Lipid was demonstrated using the Sudan Black B test (Sheehan and Storey, 1947) and carbohydrate demonstrated by the PAS technique (modified from McManus, 1946).

ELECTRON MICROSCOPY

Blood was collected into plastic tubes, centrifuged at 500g for 7 min and the overlying serum pipetted off and replaced by an excess of ice-cold 2.5% glutaraldehyde in 0.05M sodium cacodylate buffer (osmolality 1100 mOsmoles kg^{-1}). After fixation for 10 min, the white cell pellet was removed as a disc and cut into smaller pieces using a Sorvall TC-2 tissue chopper and fixed for a further 25 min in fresh ice-cold glutaraldehyde. After washing in cacodylate buffer, the cells were post-fixed for 1 h in 1% osmium tetroxide, dehydrated, and embedded in Araldite. Thin sections were stained with uranyl acetate and lead citrate. Some thin sections were stained with phosphotungstic acid according to the method of Hudson (1966).

ENZYME CYTOCHEMISTRY

A). Light Microscopy

The following enzymes were tested for: (1) peroxidase at pH 6.3 and at pH 11, after the method of Hanker et al. (1977); (2) two esterases, alpha naphthyl acetate esterase and naphthol AS-D chloroacetate esterase using Histozyme kits (Sigma) based on the method of Yam et al. (1971); (3) acid phosphatase after Hayhoe and Flemans (1979); (4) alkaline phosphatase after Hayhoe and Quaglino (1958); (5) β-glucuronidase after Hayashi et al. (1964) and (6) aryl sulphatase after Austin and Bischel (1961). No substrate and inhibitor controls were also used.

B). Electron Microscopy

Acid phosphatase activity was investigated on cells fixed in 1.5% glutaraldehyde for 20 min and then incubated at 37°C with β-glycerophosphate as substrate and lead nitrate as capture agent in Tris-maleate buffer, pH 5.0 (Bainton and Farquhar, 1968).

III. RESULTS

Table I shows the granulocyte types present in the peripheral blood of the three elasmobranchs examined. Several morphologically distinct eosinophils were found in the dogfish and they have been termed G1, G3 and G4 using a similar terminology to that of Morrow and Pulsford (1980). These cells account for 27%, 2.9% and 9% respectively of the leucocyte population. This predominance of eosinophils compares with only 1.2% of the leucocytes recognised as neutrophils (G2) and an absence of basophils (Table I). Basophils and neutrophils were not identified in the blood of the rays, but two morphologically distinct eosinophils are present and we have called these G1 and G2. These two eosinophil types are structurally similar in both species of ray examined.

TABLE I. Granulocyte types present in three elasmobranch species

Species	Granulocyte types and percentage of total leucocytes		
	Neutrophil/Heterophil	Eosinophil	Basophil
Scyliorhinus canicula (Lesser-spotted dogfish)	+ (G2, 1.3%)	(G1, 27.4%) +(G3, 2.9%) (G4, 9.4%)	-
Raja clavata (Thornback ray)	-	+(G1, 7.7%) (G2, 8.3%)	-
Raja microcellata (Small-eyed ray)	-	+(G1, 12.9%) (G2, 25.1%)	-

Figures 1-5 Dogfish granulocytes.

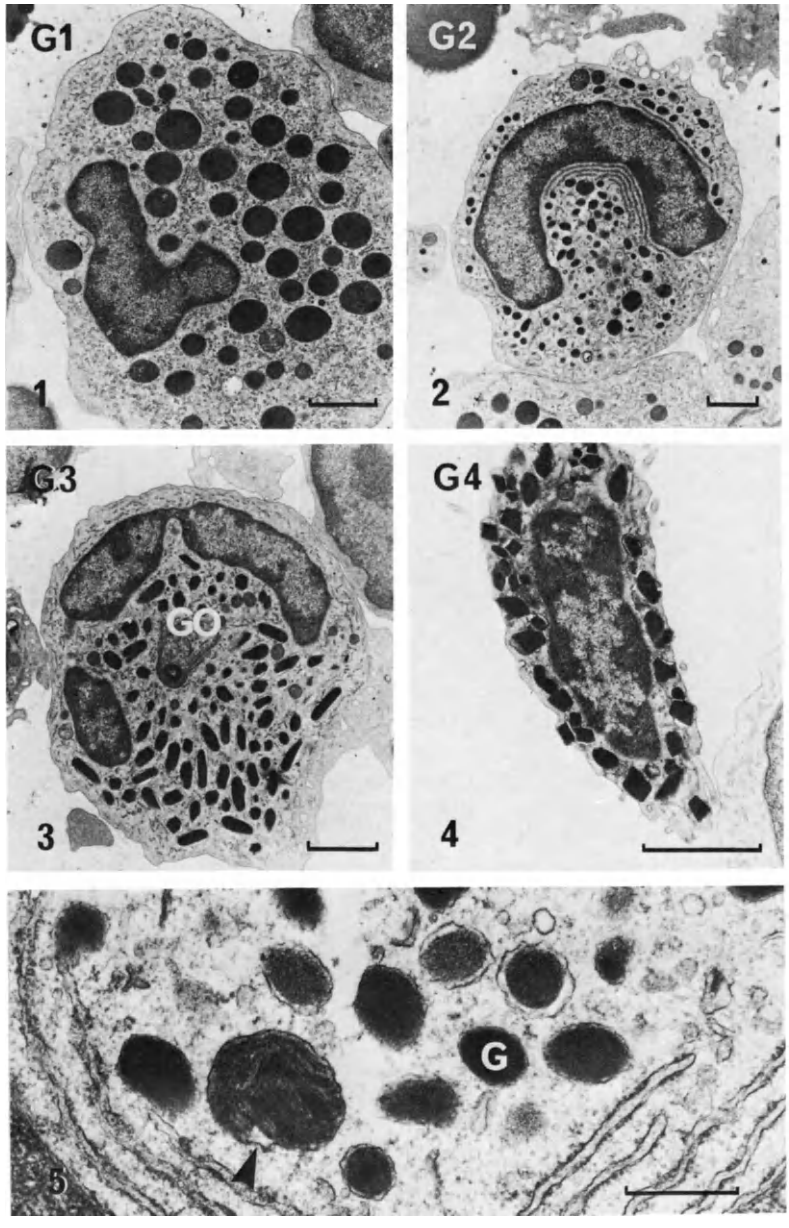
Fig. 1. G1 eosinophil, scale bar = 2 μm.

Fig. 2. G2 neutrophil. Note horseshoe-shaped nucleus, scale bar = 2 μm.

Fig. 3. G3 eosinophil. Note characteristic prominent Golgi complex (GO), scale bar = 2 μm.

Fig. 4. G4 eosinophil, scale bar = 2 μm.

Fig. 5. High power electron micrograph of part of the cytoplasm of a G2 neutrophil. Note small granules (G) and larger granule containing fibrillar material (arrow). Scale bar = 1 μm.


G1
1
G2
2
G3
GO
3
G4
4
G
5

When blood smears of S. canicula, R. clavata, and R. microcellata are examined after staining with Giemsa, the granules of the eosinophils stain orange-yellow or orange-red, which compares with the situation found in mammalian eosinophils. With Congo red, however, only a limited colour reaction is present. This stain is thought to visualise protein-mucopolysaccharide complexes and so there is presumably only a small quantity of this material present in the dogfish and ray eosinophilic granules.

Figures 1-5 show the major ultrastructural characteristics of the dogfish eosinophils and neutrophil. The granules in eosinophils G1, G3 and G4 are all uniformly electron-dense and membrane-bound (Figs. 1,3,4) and this is in contrast to mammalian eosinophils where a crystalline core and surrounding homogeneous matrix are usually present in the granules (Zucker-Franklin, 1980). The granules are, however, structurally dissimilar within the three classes of eosinophils and this, together with differences in nuclear shape, lobation, size etc., suggests that they are part of separate lineages. The dogfish neutrophil (G2) contains two distinct types of granule, the first, which are the most numerous, being small, spherical and 0.3-0.6 μm in diameter, while the second are generally larger (0.5-1.2 μm in diameter) and contain a fibrillar material (Fig. 5). The latter type of granule may represent the remnants of ingested material which has fused with the smaller granules.

The ultrastructure of the eosinophils of R. clavata is shown in Figures 6-11. As mentioned earlier, these cells are very similar to the corresponding cells in R. microcellata and hence, the micrographs presented are from the former species only. Although the granules of G1 and G2 from both ray species often possess a heterogenous content (Figs. 7, 8, 10, 11), this does not resemble the typical mammalian crystalloid and matrix. Within the cytoplasm of immature G1 cells are a single population of granules which are elongate and contain a moderately electron-dense matrix (Figs. 6,9). As the cell matures, the granules become much more elongate and a heterogeneous substructure develops (Figs. 7, 10). At this stage, a second granule population is produced and these are small, spherical, lysosome-like organelles with an electron-translucent zone underlying the bounding membrane (Fig. 7). Mature G1 eosinophils are also characterised by many large vacuoles containing debris (Fig. 7) which possibly suggests that these cells are actively phagocytic. The second eosinophil, G2, has a single granule population, (1-2 μm in diameter) which have a

TABLE II. CYTOCHEMISTRY OF S. CANICULA AND R. CLAVATA GRANULOCYTES

SPECIES AND CELL TYPE EXAMINED		ACID PHOSPHATASE	PEROXIDASE		ARYL SULPHATASE	BETA GLUCURONIDASE	α-NAPHTHYL ACETATE ESTERASE	NAPHTHOL AS - D CHLOROACETATE ESTERASE	ALKALINE PHOSPHATASE	PAS	SUDAN BLACK B
			pH6.3	pH 11							
S. canicula											
Eosinophils	G1	+++	-	-	++	-	+	+	-	+++	-
	G3	+++	-	-	++	-	+++	+++	++	++	-
	G4	+/-	-	-	-	-	+	+	+	-	-
Neutrophil	G2	+++	-	-	++	-	-	+	-	+/-	-
R. clavata											
Eosinophils	G1	+++	+	+	++	-	+	++	-	+++	-
	G2	+++	-	-	++	-	+	++	-	+++	-

+++ Strong reaction product
++ Diffuse reaction product
+ Faint diffuse reaction product
+/- Weak or questionable reaction product
- Negative reaction

heterogeneous substructure (Figs. 8,11). These granules have a rod-shaped core which is generally less electron-dense than the surrounding matrix. The phosphotungstic acid test applied to the heterogeneous granules of R. clavata failed to produce a reaction similar to that seen in the specific granules of mammalian eosinophils.

Table II shows the results of the cytochemical tests carried out on the elasmobranch granulocytes. Peroxidase was found to be absent from the eosinophils and neutrophil of the dogfish when the reaction was carried out at both pH 6.3 and at pH 11. In the thornback ray, however, some peroxidase activity was evident in the G1 eosinophil at both pH levels and the number of positive cells approximately corresponds with the number of mature G1s seen in preliminary ultrastructural investigations. The two esterases tested for are commonly used in mammalian haematology to differentiate granulocytes from monocytes. For example, alpha naphthyl acetate esterase differentiates mammalian lymphocytes and monocytes from other leucocytes (Horwitz et al., 1977), while naphthol AS-D chloroacetate esterase is present only in the granulocytes (Yam et al., 1971). These enzymes do not adequately differentiate any of the granulocytes of the fish tested (Table II) and enzyme activity is also present in the monocytes (results not shown). Lysosomal enzymes such as acid phosphatase and aryl sulphatase produce considerable amounts of reaction product.

As acid phosphatase is known to be an enzyme component of mammalian eosinophils (Wetzel et al., 1967), it was investigated at the ultrastructural level in the

Figures 6-11 Granulocytes of Raja clavata

Fig. 6. Immature G1 eosinophil, scale bar = 1 μm.

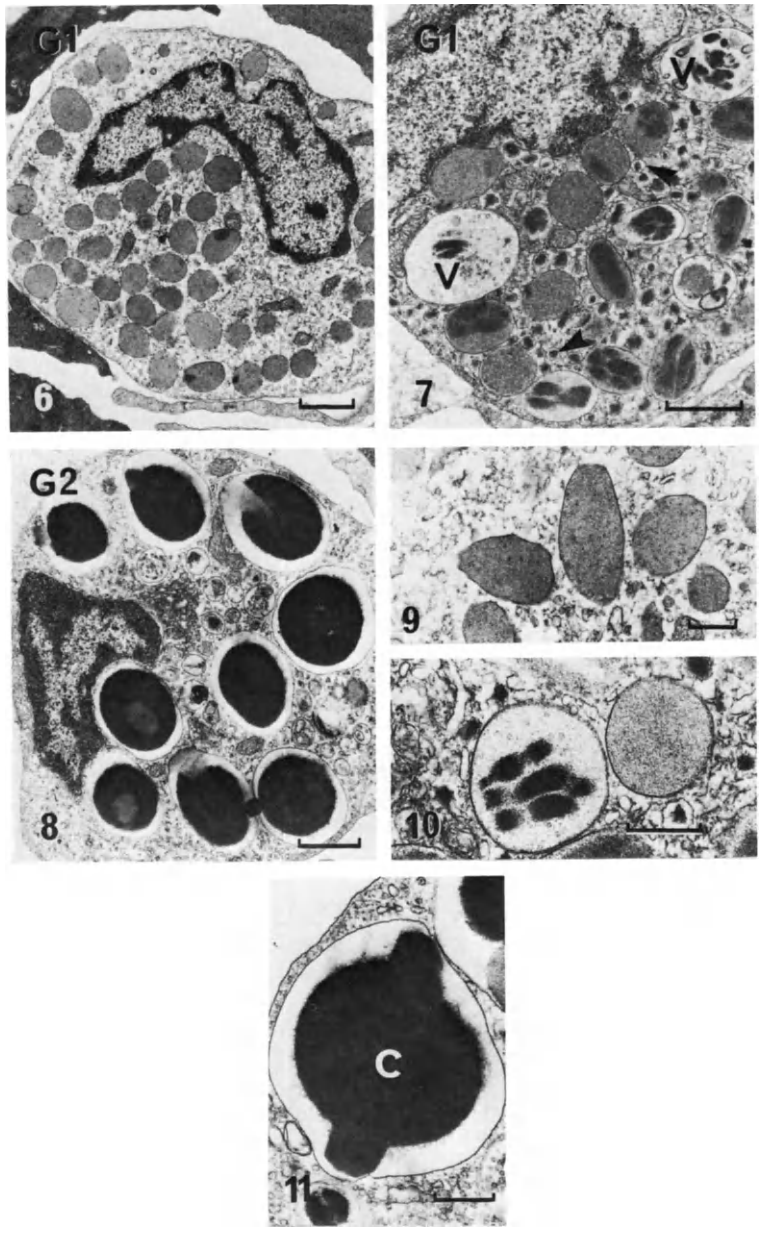
Fig. 7. Mature G1 eosinophil. Note vacuoles (V) containing debris and small lysosome-like granules (arrows). Scale bar = 1 μm.

Fig. 8. G2 eosinophil, scale bar = 1 μm.

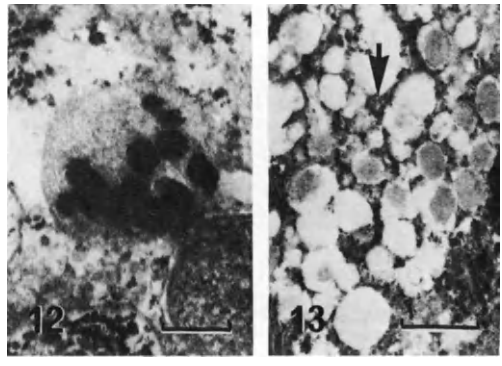
Fig. 9. High power electron micrograph of granules in an immature G1 eosinophil. Scale bar = 0.5 μm.

Fig. 10. High power electron micrograph showing the substructure in a heterogeneous granule of a mature G1 eosinophil. Scale bar = 0.5 μm.

Fig. 11. High power electron micrograph of G2 granule. Note rod-like core (C). Scale bar = 0.5 μm.


G1
6
G1
V
V
7
G2
8
9
10
C
11

granulocytes of S. canicula. Enzyme activity is present in the granules of all three types of eosinophil and in particular G1 (Fig. 12) but absent from the neutrophilic granules (Fig. 13). The distribution of enzyme activity within the eosinophilic granules is apparently uniform which again is dissimilar to the mammalian situation.



Figures 12-13 Acid phosphatase activity in Scyliorhinus canicula granulocytes.

Fig. 12. High power electron micrograph of acid phosphatase activity in G1 eosinophilic granule. Scale bar = 0.25 μm.

Fig. 13. High power electron micrograph of acid phosphatase activity in the cytoplasm of a G2 neutrophil. Note that the activity is apparently localised only in the cytosol (arrow). Scale bar = 0.5 μm.

IV. DISCUSSION

The blood of the elasmobranchs studied is characterised by a predominance of eosinophilic granulocytes, and neutrophils when present, occur as only a small proportion of the total number of leucocytes. This compares with the work of Morrow and Pulsford (1980) who also described the predominance of eosinophils in the blood of S. canicula. Fänge (1968a), described two distinct populations of eosinophils in the blood of a number of species of elasmobranchs including Etmopterus spinax, Squalus acanthias, R. radiata, R. batis and R. lintea which compares with our findings in R. clavata and R. microcellata.

The comparative work of Kelényi and Németh (1969) has revealed a number of differences in the morphological characteristics of mammalian and teleost eosinophils. Similar results were also found in the present study where the eosinophils of S. canicula, R. clavata and R. microcellata possess only a few of the morphological features characteristic of mammalian eosinophils. Their granules stain orange-red or orange-yellow with Giemsa after methanol fixation and occasionally the G1 eosinophil of R. clavata produces a weak peroxidase reaction at both pH 6.3 and pH 11. Peroxidase activity is, however, absent in the dogfish at both pH values tested. The heterogeneous granules of the two rays have a substructure reminiscent of the crystalloid of the mammalian eosinophil but the contents are not crystalline and possess no periodicity. When the granules of the eosinophils of R. clavata are treated with phosphotungstic acid, the staining pattern produced is dissimilar to that seen in mammalian eosinophils. Furthermore, there is extremely limited anisotropic staining of dogfish and ray eosinophils by the substantive dye Congo red.

Mammalian eosinophils are known to be involved in allergy reactions and parasitic infections and a marked eosinophilia is noted in the blood of such animals (Kazura, 1980). The high level of eosinophils in the peripheral blood of S. canicula was suggested by Fänge and Pulsford (1983) to be associated with the level of parasitism by the nematode, Proleptus obtusus. Morrow (1978), however, found no evidence for eosinophil involvement in reactions against this parasite and in the present study with the dogfish and rays, there was no apparent correlation between the degree of parasitism and the levels of eosinophils in the blood.

Due to the large numbers of eosinophils present in both dogfish and ray blood, we feel it is likely that these cells function in a similar way to the neutrophil of mammals. Furthermore, the existence of several morphologically distinct eosinophils in these animals suggests that they probably have separate functions in the cellular defence reactions. Therefore, the use of the term eosinophil in elasmobranch haematology may not have the same significance as in mammals. Obviously functional studies on elasmobranch eosinophils will ultimately decide whether these cells are truly homologous to their mammalian counterparts.

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PHAGOCYTIC CELLS IN THE DOGFISH (SCYLIORHINUS CANICULA L.)

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In fish, the phagocytic system has been defined in very few species. In those species so far studied the phagocytic system comprises "fixed" and "free" cells which, as in mammals, may be mobilised under appropriate conditions (Ellis et al., 1976). As part of a study of the cellular responses in the dogfish, we have been concerned with identifying and characterising the phagocytic cells by morphological, histochemical and functional criteria in order to elucidate their roles in immune phenomena (Parish, et al., in prep.). This paper reports some of the morphological properties of the "free" phagocytic cells in this species and an investigation into the efficiency of such cells in the clearance of particulate material.

We have defined seven distinct leucocyte types:- lymphocytes, monocytes, two types of thrombocytes and three types of granulocytes. Their relative numbers in the peripheral blood are shown in Fig. 1. Lymphocytes and thrombocytes are the commonest cell types, as in other fish species investigated (Pitombeira and Murilo Martins, 1970) while other leucocyte types are highly variable in number, both within and between species. A peripheral blood smear (Fig. 2) shows several of these cell types.

The phagocytic cells were found, by uptake of particulate material, to be the monocyte and type 1 granulocyte. These have been further characterised morphologically (LM, SEM and TEM). Fig. 3 shows a monocyte from a fish injected intravenously with colloidal carbon. These cells possess abundant cytoplasm, which was very

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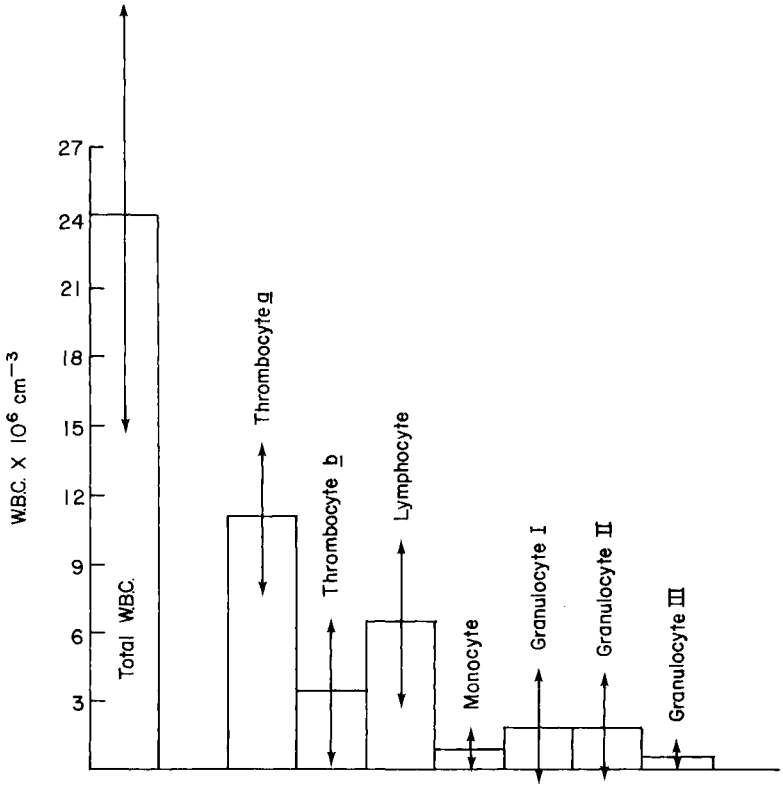

Fig. 1. Differential leucocyte counts in dogfish peripheral blood. (Arrow represents standard deviation).

slightly basophilic and often vacuolated. The nucleus was eccentric and frequently kidney shaped. This was the main blood leucocyte to phagocytose injected colloidal carbon. They would adhere firmly onto glass or tissue culture plates and were not removed by washing or by trypsinisation.

Three main types of granulocyte were evident (Figs. 4, 5 and 6).

Type 1 granulocyte (G1)

Within type 1 there were two types which may represent different developmental forms. One form (Fig. 4a)

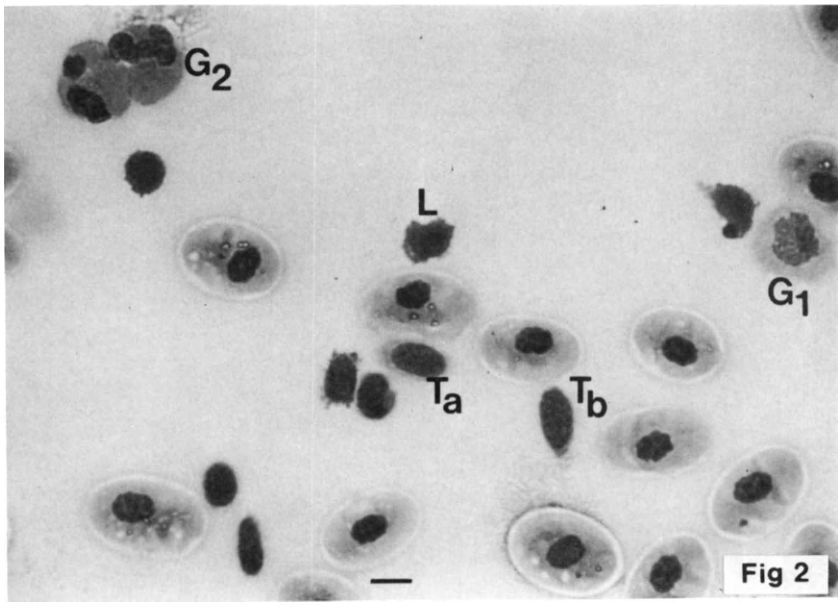

Fig. 2. Dogfish blood smear showing various leucocytes. LM (x573). Giemsa. Bar = 10μ. L-lymphocyte. Ta-thrombocyte type a. Tb-thrombocyte type b. G1 and G2 - granulocyte types.

possessed large, round, eosinophilic granules, with a nucleus which was irregular in shape yet rarely polymorphic. These cells were capable of adhering and spreading on glass or plastic and were often seen in large aggregations with monocytes (Fig. 7). The second form of type 1 granulocyte (Fig. 4b) contained much smaller granules and the granules stained more faintly with Giemsa and were more neutrophilic than those with large granules. The nucleus was not polymorphic. This was the only granulocyte type ever seen with engulfed carbon, and as with the other forms, was capable of adhering to and spreading on glass or plastic. This granulocyte could be considered an analogue of the mammalian neutrophil, and in this study was frequently referred to as a neutrophil.

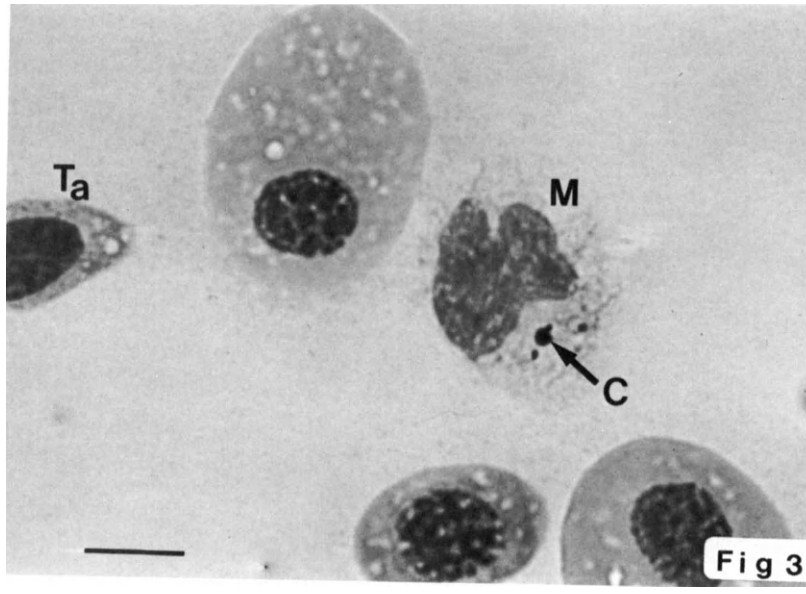

Fig. 3. Dogfish peripheral blood monocyte containing carbon. LM (x1,431). Giemsa. Bar = 10μ. M - Monocyte. C - carbon particles.

Type 2 granulocyte (G2)

These granulocytes had slightly elongated, rod shaped granules. The granules generally stained brighter and pinker than type 1 granulocytes (Fig. 5). The nucleus was invariably polymorphic and eccentric (Fig. 8). They were adherent but were never observed phagocytosing any material. This granulocyte bears some resemblance to the mammalian eosinophil; however, they do not have the crystalline inclusions typical of mammalian eosinophils, but these have rarely been observed in the granulocytes of lower vertebrates.

Type 3 granulocyte (G3)

The remaining granulocyte possessed granules which did not stain at all with Giemsa but appeared as a very bright round cell with an eccentric, dumb-bell shaped nucleus (Fig. 6). Under the electron microscope the cell appeared to be highly vacuolated, the round vacuoles containing small granules which never completely filled the vacuole.

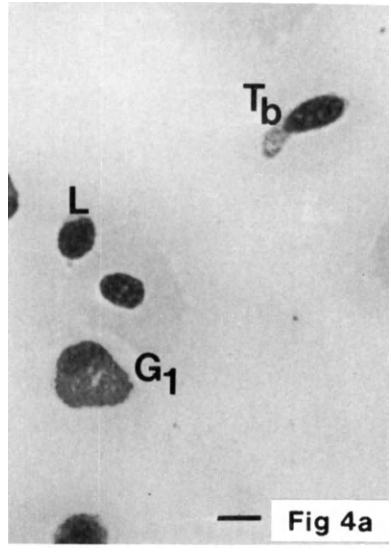

Fig. 4a. Type 1a granulocyte in peripheral blood smear. LM. Bar = 10μ. G1 - Type 1a granulocyte. Tb - thrombocyte. L - lymphocyte.

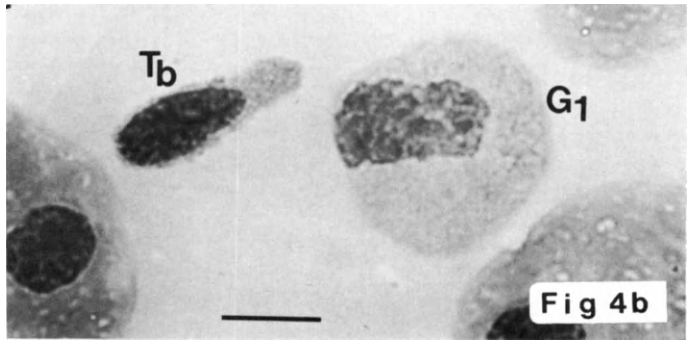

Fig. 4b. Type 1b granulocyte. Peripheral blood smear. LM. Giemsa. Bar = 10μ. G1 - Type 1b granulocyte. tb-thrombocyte.

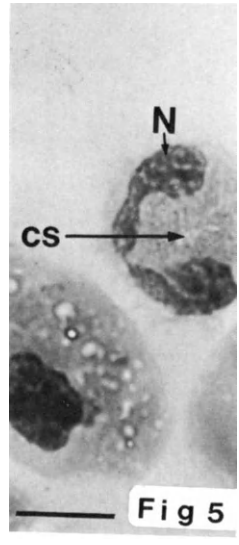

Fig. 5 Type 2 granulocyte. Peripheral blood smear. LM. Giemsa. Bar = 10μ.

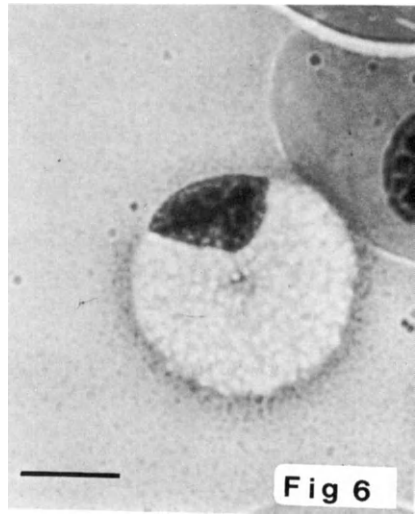

Fig. 6. Type 3 granulocyte. Peripheral blood smear. LM. Giemsa. Bar = 10μ.

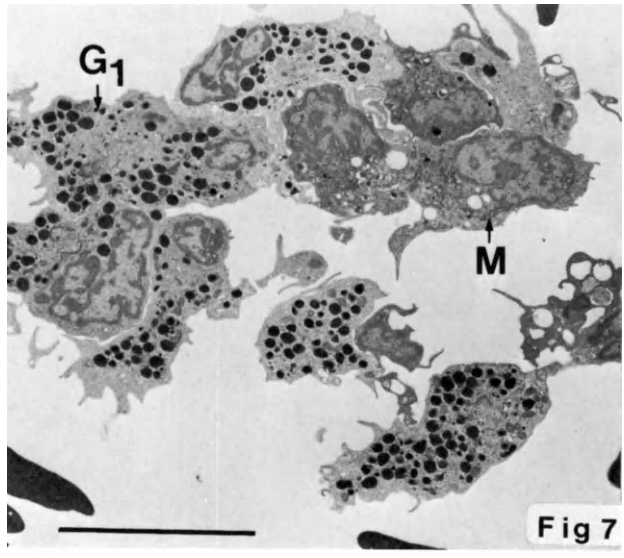

Fig. 7. Type 1 granulocytes and monocytes showing aggregation. TEM. Bar = 10μ. G1 - Type 1 granulocyte. M - monocyte.

A wide variety of antigens and particulate material were taken up by blood leucocytes (Fig. 9). Monocytes appeared to be less discriminatory than type 1 granulocytes but the granulocytes phagocytosed more avidly. However, the granulocytes seemed to become more fragile having undergone phagocytosis and were often observed at various stages of degranulation. Heat killed yeasts and heat-killed bacteria were readily taken up by both monocytes and neutrophils (Figs. 10, 11, 12 and 13). The uptake of bacteria may not necessarily indicate that viable bacteria are endocytosed in the same way. In two cases where bacteria were tested as live preparations, blood cells were not observed with ingested bacteria. Adherent cells, in general, were more avidly phagocytic than those in suspension.

Quantitative studies showed optimum uptake at pH 7 and between 10 and 15°C. The presence of heat-inactivated or whole fresh plasma showed no enhancing effect on yeast uptake. No conclusive evidence has been demonstrated in any fish species regarding the enhancement of phagocytosis by serum factors.

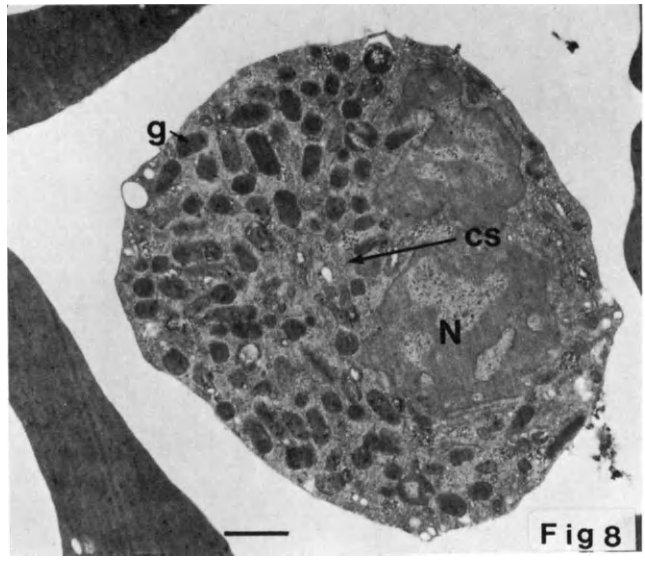

Fig. 8. Type 2 granulocyte with elongated granules. TEM. Bar = 1μ. g-granule. cs-centrosome. N-nucleus.

Although phagocytosis is easily demonstrated in vitro, clearance studies showed that there is no significant depletion of bacterial numbers in vitro or in vivo. In vitro the presence of phagocytes had little or no effect on bacterial numbers and they appeared to take up only non viable cells. In vivo studies also showed that, following an initial sharp reduction in the number of bacteria injected intravenously, the numbers persisted for a considerable period of time. Fig. 14 shows the result of a typical experiment involving 2 fish which were injected with approx. 10^9 S. typhimurium intravenously and maintained for 36 days. After the initial decrease which lasted 8 hours the numbers of bacteria in the blood remained between 10^4 and 10^5 per ml. The fish showed no obvious signs of distress but there was no indication that bacterial numbers were declining. Blockading the system with carbon had no effect on the clearance of bacteria in contrast to mammalian studies.

These studies of antigen clearance suggest that the dogfish has a poorly developed phagocytic system and hence is not well equipped to defend itself against external

\+ = cells observed containing particle or antigen
\- = cells not observed containing particle or antigen
ϕ = phagocytes tested as adherent monolayers, otherwise tested as cell suspensions
* = FITC - conjugated
NT = Not tested

Antigen or particle	Blood Monocytes	Blood Neutrophils
Yeasts:- S. cerevisiae	+ ϕ	+ ϕ
Bacteria:-		
Micrococcus lysodeikticus*	+	+
Bacillus subtilis	+ ϕ	+ ϕ
V. anguillarum UNH 569	+ ϕ	+ ϕ
" " COB 408	+ ϕ	+ ϕ
S. typhimurium*	+	+
Strep. faecalis	+	+
E. coli*	+	+
Proteus vulgaris*	+	+
Staph. aureus*	+	+
Erythrocytes:-		
Sheep	+ ϕ	+ ϕ
Calf	+ ϕ	+ ϕ
Horse	+ ϕ	+ ϕ
Latex:-		
0.81μ	+ ϕ	- ϕ
1.1μ	+ ϕ	- ϕ
Carbon:-	+ also thrombocytes + ve	-
Parasite:-		
Kodoa sp (myxosporidial parasite)	-	-
Soluble Antigen:-		
KLH*	+ ϕ	+ ϕ
BGG*	+ ϕ	+ ϕ

Fig. 9. Endocytosis of various antigens and particles by dogfish phagocytes.

pathogens. However, there are a number of environmental and physiological conditions which should be taken into consideration. For example, the dogfish lives in deep sea water where the temperature rarely exceeds 10°C and so an invasion of bacteria of this magnitude would be unlikely. Those bacteria which did gain entry would

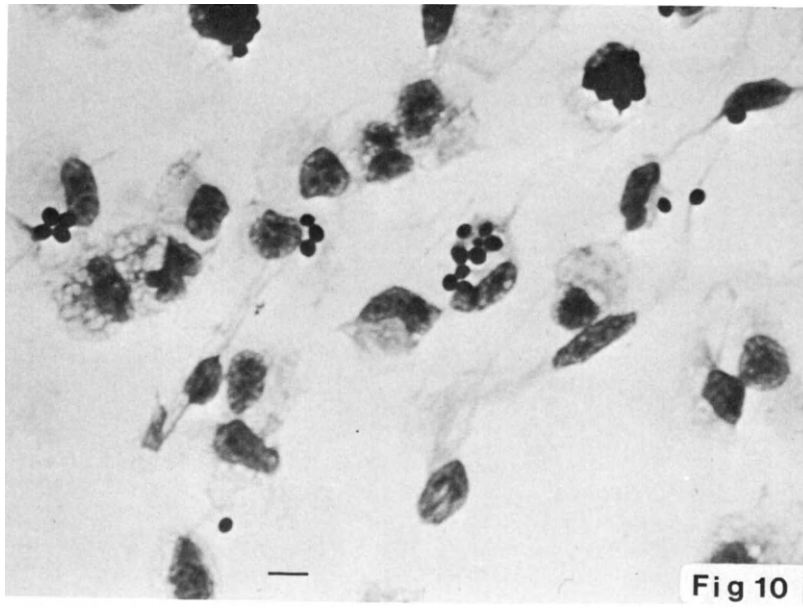

Fig. 10. Dogfish adherent monocytes phagocytosing yeasts. LM . Giemsa. Bar = 10μ.

perhaps be adequately dealt with by the unfavourable internal environment where there are high levels of urea, trimethylamine oxide, salt and natural "antibodies" present in the circulation. In fact the major function of the phagocytic cell may be to "mop up" those organisms already rendered non-viable by other factors.

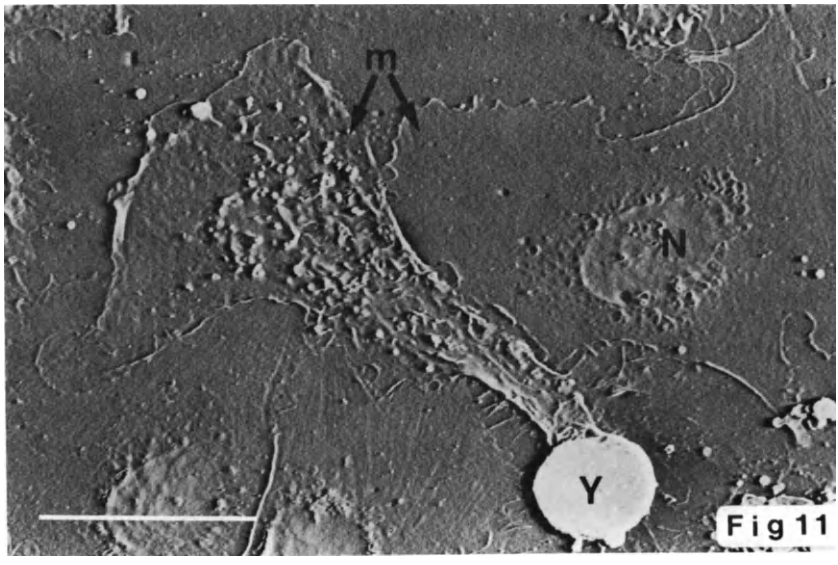

Fig. 11. Dogfish adherent monocyte in the process of engulfing a yeast cell. SEM. Bar = 10 μ. Y-yeast cell. N - nucleus. M- monocyte.

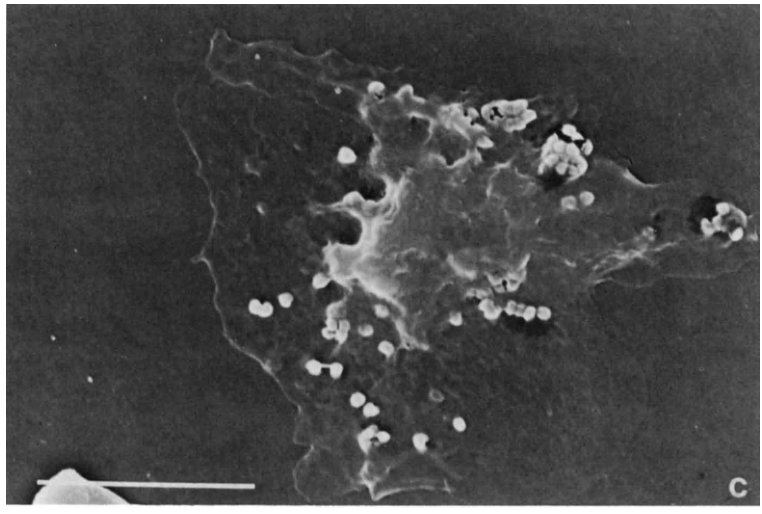

Fig. 12. Dogfish adherent monocyte phagocytosing S. faecalis SEM. Bar = 10μ.

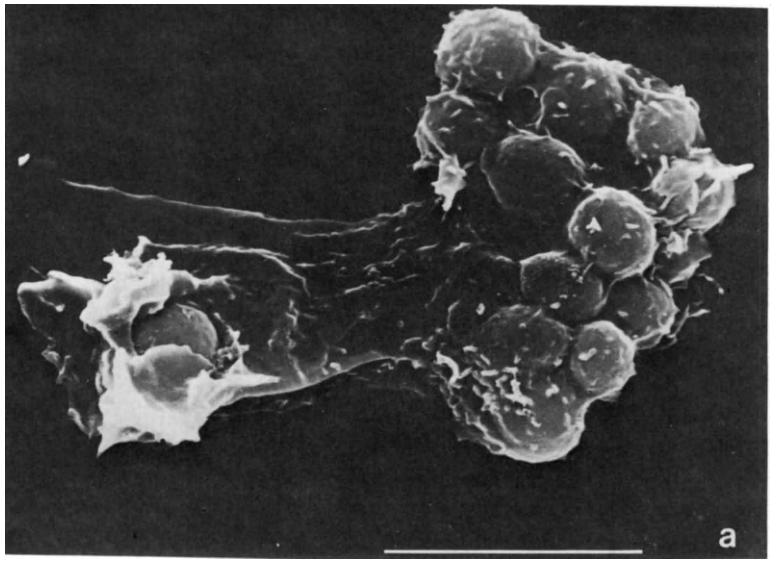

Fig. 13. Dogfish adherent monocytes phagocytosing yeast cells. SEM. Bar = 10μ.

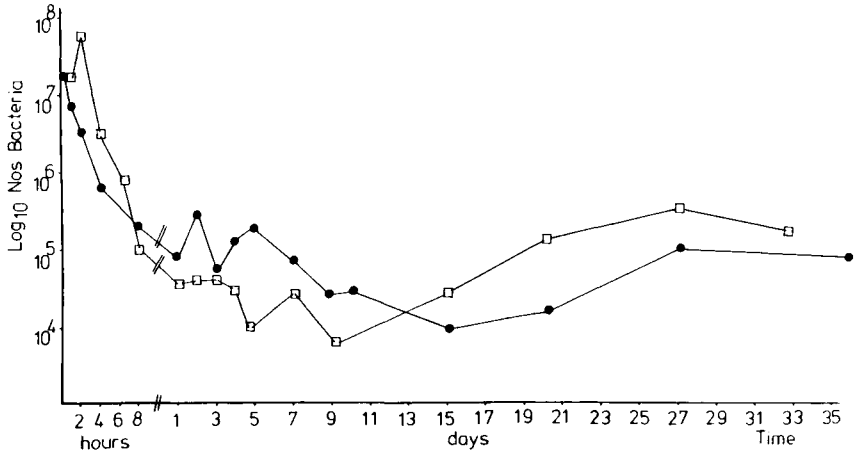

Fig. 14. Long term clearance of S. typhimurium from the circulation of two dogfish. Inoculum = 2.75×10^9. Fish 1, Fish 2.

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THE MELANO-MACROPHAGE CENTRES OF FISH: A REVIEW

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INTRODUCTION AND BRIEF REVIEW OF THE LITERATURE

Pigment-containing cells are a prominent feature of fish haemopoietic tissues. Up until very recently they were largely ignored by histologists and histopathologists in spite of their prolific occurrence under certain pathological conditions. Recently, however, they have come to be recognised as an integral part of the reticulo-endothelial system.

Jolly (1923) was the first to mention these cells when he described them as macrophages containing greenish-brown pigment, representing breakdown products of haemoglobin from degenerated red blood cells. Mackmull and Michels (1932) working with the cunner Tautogolabrus adspersus demonstrated that intraperitoneally injected carbon eventually homed to these sites. This was later confirmed for plaice Pleuronectes platessa (Ellis et al., 1976). Tokumaru and Ferri (1970), combining solubility, bleaching and histochemical tests on the pigments in the spleen, kidney and liver of three freshwater teleost species (Pimelodus maculatus, Prochilodus scrofa and Cyprinus carpio) suggested that they are ferrigenous lipochromes, comparable to the Gandy-Gamma nodules observed in humans suffering from some infections.

The first detailed structural and histochemical descriptions of these structures were published by Ellis (1974) and Roberts (1975). Adopting Edelstein's (1971) definitions of melanins, Roberts (1975) proposed the name

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melano-macrophage centres, because of the ultrastructural resemblance of these cells to macrophages, their high pigment content and their tendency to aggregate to form distinct nodules. Ferguson (1976a, b) in his ultrastructural studies of the spleen of the turbot Scophthalmus maximus described the relationship of the centres to the splenic ellipsoids. Oguri (1976) investigated some histochemical properties of the pigment cells in the rainbow trout Salmo gairdneri kidney and concluded that the pigment granules are melanins. A series of papers published in the last few years by Agius and his colleagues (Agius 1979a, b; 1980; 1981a, b; 1982; Agius and Roberts, 1981; Agius and Agbede, 1983) forms the basis of this review.

THE PIGMENTS

Three pigment types are normally observed, melanin, haemosiderin and the lipid pigment lipofuscin; all three pigment types can be present in one and the same macrophage. Occasionally, under certain pathological conditions the lipid pigment ceroid can also be observed. Lipofuscin is by far the most abundant pigment. Melanin is commonly present although in some cases at very low levels. Haemosiderin can also be abundant in teleost spleens under conditions of starvation and disease (Agius, 1979a, b).

MELANIN

Integumental melanin is synthesised by oxidation of the amino acid tyrosine to 3, 4-dihydroxyphenylalinine (DOPA) and then to DOPA-quinone which polymerizes to form the melanin. Such synthesis takes place on premelanosomes, and the mature pigmented granule, the functional pigmentary organelle, is known as the melanosome. In fish, melanogenesis takes place in the dermis and the pigmentary melanin-containing cells are the melanocytes and melanophores. Melanocytes are immature melanophores actively producing melanin but capable of becoming melanophores and moving up into the functional melanophore layer (Bullock and Roberts, 1974). A second type of melanin known as visceral or neuromelanin has been described from several tissues such as the hepatocytes and Kuppfer cells of the liver and has been associated with various pathological conditions. It appears to be a distinct substance and may not be closely related chemically to the other melanins.

The melanin observed in fish melano-macrophages is generally present as discrete granules that resemble integumental melanosomes. They are mostly uniformly electron dense, although some granules consist of an electron dense shell bounding a less electron dense core. Less frequently, very electron dense granules with electron lucent vesicular spaces within them can be observed. Granules with less regular outlines and irregularly shaped patches of different electron densities can sometimes also be observed within melano-macrophages (Agius and Agbede, 1983). The amount of melanin present in melano-macrophages is very variable, even within the same species, but it is particularly abundant in certain tissues notably the kidneys of salmonid species.

LIPOFUSCIN

Lipofuscin is formed, in part at least, by the oxidation of unsaturated tissue lipids or lipoproteins. Bloom and Fawcett (1970) suggested that it represents the end stage of lysosomal activity associated with normal wear and tear accumulations in the cell. It is commonly referred to as "brown atrophy", "wear and tear" or "waste pigment". In fish, lipofuscin is by far the most widespread pigment, being found in the melano-macrophages of all species. Under the electron microscope, lipofuscin granules are observed to differ greatly in size, shape and electron density. Agius (1979a) and Agius and Agbede (1983) observed that one of the main sources of lipofuscin granules were effete cellular components such as mitochondria. They observed various degenerative stages of these intracellular structures, together with pigment granules and other cellular debris, within the cytoplasm of melano-macrophages in all five species examined; the dogfish Scyliorhinus canicula, eels Anguilla anguilla, plaice Pleuronectes platessa, rainbow trout Salmo gairdneri, and tilapia, Oreochromis niloticus/O. aureus hybrids. Among the cellular debris of melano-macrophages they also observed myelin-like whorls, and in dogfish liver and spleen what they described as multimembraneous bodies. It is thought that all these different bodies contribute significantly to lipofuscin formation.

HAEMOSIDERIN

Haemosiderin is a brown, granular, relatively insoluble pigment containing a protein component (apoferritin) and

an iron (ferric) component. It is derived from haemoglobin, the respiratory pigment, when effete red blood cells start to degenerate. Haemosiderin is the chemical form in which reticulo-endothelial cells store excess ferric iron (Thompson, 1966). In fish melano-macrophages, haemosiderin appears as a fine granular material and has been observed both within the lysosomal apparatus and lying freely in the cytoplasm of pigment carrying macrophages. On occasions it has been observed in very close association with lipofuscin granules (Agius and Agbede, 1983). In teleosts the distribution of haemosiderin was observed to be largely restricted to the melano-macrophages of the spleen. This pigment has not been traced in melano-macrophages of elasmobranch haemopoietic tissues (Agius, 1979b, 1982).

THE PIGMENT-CARRYING CELLS

It has been confirmed by various workers that the pigmented cells under discussion here are macrophages (Ellis, 1974; Roberts, 1975; George, 1978, pers. comm.). Recent ultrastructural studies on rainbow trout revealed that in the kidney of this species, melanocyte-like cells far outnumber melano-macrophages (Agius and Agbede, 1983). The presence of this second pigmented cell type has also been noted by George and Wolke (pers. comm.). While the presence of melanocyte-like cells in the haemopoietic tissues of fishes has been recognised for a long time, it has generally been assumed that the majority of pigmented cells within the spleen and kidney of teleosts are of a phagocytic nature. Moreoever, like the phagocytic cell counterpart, these melanocyte-like cells in the trout kidney increase markedly in numbers during starvation. Their cytoplasm is replete with melanosomes but no other pigment is present and a lysosomal apparatus appears to be lacking. While the significance of this finding remains speculative there is an urgent need to determine how common this phenomenon is. Indeed in a number of teleost species (e.g. Nezumia aequalis) the kidney centres carry mostly melanin pigment while the splenic ones contain mostly lipofuscin and sometimes haemosiderin (Agius, 1980).

In teleosts, the precursor cells of macrophages are thought to reside in the haemopoietic tissue of the kidney (Ellis, 1974). Mature macrophages are known to exist in the peritoneal lining of the abdominal cavity, lining the ellipsoids of the spleen, in the white and red pulp of the spleen, around the portal tracts of the liver, throughout

the haemopoietic tissue of the kidney, and in the intermuscular spaces in the atrium of the heart. In the spleen and kidney of all teleosts, except the salmonids, phagocytes including melano-macrophages aggregate in association with lymphoid elements forming the melano-macrophage centres. Thus, these centres can be considered as an integral part of the reticulo-endothelial system. The centres are usually nodular with a delicate argyrophilic capsule and generally closely applied to vascular channels. Ferguson (1976b) claimed that in turbot spleens, this capsule, which is composed of both cellular and acellular elements, separates the centres from the surrounding lymphoid elements. In some species, notably in turbot Scopthalmus maximus and bluegill Lepomis macrochirus spleens, a distinct collar of lymphocytes surround the centres and the associated ellipsoids (Ferguson 1976b; Agius, 1980). In the kidney, the centres are often associated with peritubular capillaries and vascular sinuses while in the liver they are associated with the portal tracts. The centres are usually periodic acid-Schiff (PAS)-Schmorl's and Ziehl-Neelsen-positive, but DOPA-negative. The splenic centres are sometimes Perls' positive depending on the haemosiderin content.

ONTOGENIC AND PHYLOGENETIC CONSIDERATIONS

According to Ellis (1977) who investigated the ontogenic development of the lymphoid system in Atlantic salmon Salmo salar, it is likely that the pre-feeding salmon larva is immunologically immature, possessing neither humoral nor cell-mediated immunity. Immunological maturity appears to be attained at first feeding. Shortly afterwards, melano-macrophages appear within fish haemopoietic tissues (Agius, 1981a). In clinically normal specimens, they subsequently accumulate steadily with age so that in older fish they are found in considerable quantities. This rate of accumulation of melano-macrophages is markedly accelerated by starvation and in the course of some diseases.

The major evolutionary trends observed in terms of the distribution and cytological organization of the melano-macrophage centres can be summarized as follows:

i. a progressive increase in the abundance of pigment cells;
ii. a structural evolution from the distribution of pigmented macrophages as individual cells, observed in Agnatha and Chondrichthyes, to the organized

centres characteristic of all Osteichthyes (except the salmonids);

iii. a change in organ location of these pigment cells from the liver in Agnatha, Chrondrichthyes and the primitive bony fishes, to the spleen and kidney in the advanced bony fishes.

The increasing level of structural complexity of the centres and their increasing proclivity for the main lymphoid organs have been correlated with the evolution of the lymphoid system (Agius, 1980). This and the observed parallel ontogenic development of the two systems add weight to the suggestion that these centres may represent the primitive analogues of the germinal centres of birds and mammals.

Evidence for this analogy with the lymph nodes found in the neck region of birds, and in the neck, thorax, abdomen and body extremities of mammals has been accumulating (see for example Ellis, 1974, 1980; Roberts, 1975; Ferguson 1976a, b). Cytologically, both germinal centres and melano-macrophage centres consist of aggregations of reticular cells, lymphocytes, macrophages and plasma cells. By means of autoradiographic studies, Ellis and de Sousa (1974) showed that ellipsoids and melano-macrophage centres in plaice are areas through which metabolically active, small lymphocytes migrate, thereby providing suitable sites where circulating lymphocytes could be influenced by immune complexes in a similar way to the formation of germinal centres through the aggregation of antigen-bearing dendritic macrophages, in the spleen of the chicken (White and Gordon, 1970). Furthermore, both germinal centres and melano-macrophage centres contain prominent areas of cellular degeneration and accumulation of indigestible substances (Congdon and Hanna, 1967; Congdon, 1969; Chen et al., 1978; Ferguson, 1976b; Agius and Agbede, 1983).

There are however major differences between the melano-macrophage centres in fish and the germinal centres of birds and mammals which can be summarized as follows:

i. in fish the lymphoid tissue is poorly differentiated. Ellis (1980), however, maintains that the diffuse lymphoid tissue in fish can adequately carry out many of the functions ascribed to germinal centres.

ii. germinal centre formation is dependent on the presence of dendritic cells. The evidence for the presence of such cells in fish is somewhat

conflicting. Ellis (1980) during his studies on the trapping of bovine serum albumin (BSA) in plaice found no evidence for the presence of such cells. Instead antigen was observed to be held extracellularly, most likely as an immune complex, in association with the metalophil reticular fibres in the ellipsoid walls and scattered throughout the peri-ellipsoidal areas and the melano-macrophage centres. In the kidney, antigen uptake was observed to be mainly intracellular within the reticulo-endothelial system (RES) and free macrophages. This localization of BSA differed markedly from that observed with inert particles, such as colloidal carbon, where macrophages which aggregated within melano-macrophage centres were involved (Ellis et al., 1976). Similar studies have been carried out on common carp by Secombes and Manning (1980). Earlier, Ferguson (1976a, b) working on the turbot had described both rounded and dendritic macrophages. He described how melano-macrophage centres arise in the outer part of the wall sheath of ellipsoids and once their phagocytic powers have been expended they migrate to the melano-macrophage centres. During this migration they come into close contact with the immuno-competent cells; cytoplasmic interdigitations of plasma cells with dendritic macrophages were seen under the electron microscope. Stuart (1970) reported that in lungfish Protopterus aethiopicus, the phagocytic cells are also found in two forms; the dendritic and the rounded form but this worker did not elaborate on the possible immunological significance of this finding.

iii. the much higher pigment content of melano-macrophage centres as compared with germinal centres. This is discussed in the next section.

WHY SO MUCH PIGMENT?

An explanation for this phenomenon could lie in the differing abilities of animals to control their body temperature. As they occur in animal tissues, saturated fats are solid at ordinary temperatures and unsaturated fats are liquid, i.e. oils (Lowy and Harrow, 1940). It is therefore imperative for poikilotherms to have high levels of unsaturated fats in their body tissues; it is a well established fact that the fatty acids of living organisms shift towards greater unsaturation under lower environ-

mental temperatures, as a means of keeping protoplasmic viscosity within the range necessary for normal metabolic processes (Lewis, 1962; Cowey and Sargent, 1972, 1977; Hazel and Prosser, 1974; Swan, 1975; Aloia, 1978). To give a specific example, Knipprath and Mead (1968) working with goldfish Carassius auratus demonstrated that low environmental temperatures resulted in increased unsaturation of tissue fatty acids. For this same species, Kemp and Smith (1970) observed that if the rearing temperature of the fish was increased by 20°C, the percentage of polyunsaturated fatty acids in the membrane lipids from the intestinal sub-mucosa was halved, while that of the more saturated acids was nearly doubled.

Studies of this type have been extended to the organelle level. Richardson et al., (1961) analysed the fatty acid contents of mitochondria from chicken liver, beef heart, rat liver, channel catfish liver, common carp liver, salmon liver and salmon heart, and found that the degree of unsaturation of the fatty acids increased in the above order. Comparison of the fatty acid composition of rat liver and catfish liver mitochondria revealed a 15% greater degree of unsaturation in the mitochondrial fatty acids from fish (Richardson and Tappel, 1962). Leslie and Buckley (1976) demonstrated that the fatty acids incorporated into the phospholipids of goldfish liver microsomes were more highly unsaturated at incubation temperatures of 10°C than at 30°C; this indicates that temperature is the main factor determining which fatty acids are inserted into membrane phospholipids. Moreover, Caldwell and Vernberg (1970) reported that the lipids of mitochondria isolated from the gills of cold-acclimatized (10°C) goldfish were more unsaturated than the lipids of warm-acclimatized (30°C) specimens. These results are consistent with those of Irving and Watson (1976) who found the fatty acid composition of tropical fish mitochondrial membranes to be more saturated than in previously reported cold-water fish mitochondrial membranes. According to this same report, while the necessity of enhancing membrane unsaturated fatty acids at low temperatures is obvious, the reverse, that higher temperatures induce formation of fats of higher melting point is not always true.

Lipofuscin formation has been reported in practically every organ in homeotherms, including man (Dubin, 1955; Smith and Jones, 1961; Thompson, 1966) and some of it is eventually transported to and deposited in the lymph nodes. Thus it seems as if a higher rate of formation of

lipofuscin pigment from peroxidation of unsaturated lipids and its eventual accumulation in what are thought to be primitive germinal centre analogues is to be expected in fish. Lipid peroxidation is a free radical chain reaction and once set in motion, can give rise to a widening zone of pathology within the cell. The formation of lipid peroxides in biological membranes is accompanied by an extensive disturbance of their organised structure and loss of many specialised functions such as respiration in mitochondrial membranes (Slater, 1972). By an incompletely known mechanism, Vitamin E inhibits such peroxidation reactions (Lucy, 1972; Tappel, 1972) but according to Dicks (1965; cited in Tappel, 1975) fish have relatively low levels of this anti-oxidant and therefore their tissues are particularly prone to the formation of lipofuscin.

Another important suggestion that could give some insight as to why fish have such prominent areas of phagocytic cells heavily laden with indigestible materials is that intracellular digestive processes of fish macrophages may not be well developed on the evolutionary scale. This hypothesis has been proposed by Corbel (1975) to explain the slow digestion of phagocytosed bacteria in the rainbow trout and by Bach et al. (1978) to explain the resistance of the bacterium Aeromonas hydrophila to intracellular enzymatic digestion in channel catfish fingerlings.

THE POSSIBLE ROLE OF MELANIN IN MELANO-MACROPHAGE CELLS

Melanin within melano-macrophages seems to be derived from that phagocytosed from melanocytes and melanophores. A similar process has been observed to occur in higher animals. In normal human skin, the melanocytes are continuously being renewed and some of the melanin is eliminated by being absorbed by the lymphatics (Thompson, 1966). In this respect, phagocytic macrophages within the dermis and subcutis may phagocytose melanin eliminated by the melanocytes and transport this melanin to the lymphatics. These melanophages, like fish melano-macrophages, do not give a positive DOPA reaction but they may be PAS positive (normal skin melanocytes are DOPA positive and PAS negative, unless they come from albinos). However, there is every possibility that melanin is phagocytosed by macrophages for some specific purpose. This pigment is well-known for its ability to absorb free radicals and cations thereby rendering them inactive and

thus harmless (Edelstein, 1971; Gan et al., 1977). According to Mason et al. (1960), melanin can act as a biological electron exchange polymer and thus protect cells against reducing or oxidising conditions which might release highly reactive free radicals. This property could be of immense value in melano-macrophage centres where extensive lipid peroxidation reactions, and in some cases considerable traffic of iron-containing compounds, can occur. A further complication in this respect is the fact that iron compounds are known to catalyse the peroxidation of lipids in animal tissues, including fish (Castell and Bishop, 1969). The possible mobilisation of melanin in order to neutralise free radical and cation activity could also explain why this pigment is so commonly observed at sites of infection or tissue injury (Edelstein, 1971; MacQueen et al., 1973; Roberts, 1975).

THE ROLE OF MELANO-MACROPHAGES IN HAEMOSIDERIN RECYCLING

It is well established that in higher animals haemosiderin is frequently present at the same sites at which lipogenic pigments are deposited, indicating that deposition of haematogenous and lipogenic pigments may occur concomitantly. The same appears to hold for fish since macrophages laden with iron compounds, melanin and lipofuscin pigments abound, particularly within splenic centres. Erythrocytes, presumably effete, have been observed to lie both singly and in clumps within melano-macrophages in some species such as the golden shiner Notemigonus crysoleucas (George, pers. comm.) and the tilapia Oreochromis niloticus/O. aureus hybrids (Agius and Agbede, 1983). How these erythrocytes are phagocytosed and broken down, as well as the fate of the various components, is still largely unknown. In higher animals the breakdown of effete erythrocytes takes place in lysosomes; proteins, iron and bile pigment precursors derived from the haemoglobin are re-utilised in metabolic processes elsewhere in the body (Muir and Miven, 1935; Essner and Novikoff, 1960). Although it appears that a similar sequence of events occurs within fish melano-macrophages, no one has yet described the mechanisms of such processes. Particularly intriguing is the mechanism whereby material digested in lysosomes and earmarked for re-utilisation is safely transported to the cytoplasm and further. This process has been described in the rat spleen by Edwards and Simon (1970) and in the rabbit spleen by Simon and Burke (1970) and by Daems et al. (1972).

FUNCTIONAL DIVERSITY AMONG MELANO-MACROPHAGES IN DIFFERENT ORGANS

Evidence is accumulating that despite the morphological similarity of the melano-macrophage centres of the kidney, spleen and liver, there are important functional differences between the centres of the three organs. Both in normal and diseased fish, only the splenic centres are normally involved in handling haemosiderin; this appears to hold true for all teleost species (Agius, 1979b). Only when rainbow trout were splenectomised was that role taken over by the kidney (Agius, 1981b). Important differences have also been reported with respect to the involvement of the centres in the immune response. Ellis (1974, 1980) studied the process of antigen trapping within the lymphoid tissues of the plaice and observed important differences in the pattern of distribution of the soluble antigen bovine serum albumin (BSA) and in the associated immunoglobulin production as well as in the persistence of the immune complexes in the centres of the spleen as compared with those of the kidney. Thus, while in the spleen the antigen appeared to be held extracellularly and could be detected within the centres, in the kidney uptake of BSA appeared to be a predominantly intracellular process and antigen was detected in aggregates of lymphocyte-type cells, adjacent to melanin centres but not actually within centres. Also, the extracellularly trapped antigen on dendritic cells within the splenic centres persisted far longer than that in the kidney. Similar studies were carried out by Secombes and Manning (1980) using common carp. They too found important differences between the spleen and kidney in relation to antigen trapping.

These differences must certainly involve other differences in the functioning of the melano-macrophage centre systems of the different organs. In view of this it would be extremely valuable to investigate whether there occurs any exchange of information or material between the centres of different organs, and in particular whether the centres in one organ in some way "control" or "regulate" those in other organs. Moltenius (1970) asserts that the main task of the spleen is to monitor the blood stream in the defence of the organism against bacteria and toxins. Perhaps the least one can say is that in teleosts the splenic centres do appear to carry out a wider range of functions than those in the kidney and liver.

THE CENTRES IN DISEASE PROCESSES

Evidence of active participation of the centres in disease processes has been accumulating. For instance, they have been observed to play a specific role in the pathogenesis of aeromonad diseases of salmonid fish (Klontz et al., 1969; Thorpe and Roberts, 1972). Roberts (1975) reported that in the parasitic infestation of roach (Rutilus rutilus) caused by Myxobolus pseudodispar, the spores localize specifically in the melano-macrophages of the spleen and kidney. In this same report Roberts discussed the involvement of these cells in vibriosis and in chronic skin lesions associated with bacteria. Roberts (1976) also reported the association of pigment cells with lymphocystis lesions in plaice. Ellis and Wootten (1978) observed that melano-macrophages were common in primary and secondary gill lamellae in Ichtyobodo-infected salmon smolts. In some scabbard fish Aphanopus carbo specimens that were heavily infected by an Ichthyophonus-like fungus, melano-macrophage centres were observed in close association with the induced granulomas (Agius, 1978). Huizinga et al. (1979) noted an abundance of centres in the liver, kidney and spleen of red-sore diseased (Aeromonas hydrophila) largemouth bass (Micropterus salmoides). In their histopathological studies on fish that had been exposed to the insecticide endosulfan during tsetse fly control operations in Botswana, Matthiessen and Roberts (1982) reported that melano-macrophage centres disappeared from poisoned livers and were not replaced until at least 6 months later. Dyková et al. (1983) reported that mature oocysts of the coccidian Eimeria branchiphila sp. nov. were observed situated within, or in close proximity to, melano-macrophage centres in the spleen of roach, which were collected in Bulgaria.

It seems as if the involvement of the centres in disease processes could be of three types:

i. direct involvement; for example, the pigments could exert a bactericidal effect as suggested by Edelstein (1971);

ii. due to tissue damage at the sites of infection; peroxidation of unsaturated lipid cellular components to lipofuscin (possibly in association with melanin, and their possible eventual transport to the melano-macrophage centres could result in enlargement of the latter (anaemia also leads to haemosiderin deposition);

iii. due to tissue damage through debilitation; by this

is implied a more widespread form of tissue damage caused, for example, by stressed fish going off food; starvation results in marked enlargement of the centres.

FACTORS AFFECTING ABUNDANCE OF MELANO-MACROPHAGE CENTRES

Agius and Roberts (1981) studied in a quantitative manner the relationship between starvation and increased pigment deposition. The relationship of the centres to various abnormal and disease processes has been reviewed. It would be interesting to try and identify what other factors could stimulate or depress these cells. Bern (1958) studied the effects of a variety of hormonal, dietary and operative procedures on the formation of lipofuscin pigment in the adrenal glands of mice. He observed that both oestrogen administration and hypophysectomy induced the formation of the pigment. It would be interesting to investigate whether similar treatments affect lipofuscin deposition in the melano-macrophage centres of fish.

CONCLUDING REMARKS

Macrophages participate in the defence of the organism in a number of ways. They remove debris during tissue involution and repair, they destroy micro-organisms by phagocytosis, they may take up and process antigens, they may kill antigenically deviant cells, notably neoplastic cells, and they may actively secrete various substances such as lysozyme and interferon. While only further research can establish whether fish melano-macrophages are capable of carrying out all these functions, it is obvious that they do execute a multiplicity of functions. At the two extremes, they have been described as being of a lympho-reticular nature as well as acting as dumps for a wide range of materials. The term "dump", however, needs much qualification in the light of the probable recycling of various substances. It is clear that considerable amounts of materials such as iron compounds are recycled and are only stored in the centres temporarily, presumably more so under stressful conditions. Thus the centres should be regarded as sites where a variety of materials are aggregated, processed, sifted and disposed of in a number of ways rather than regarding them as static areas passively accepting and storing indefinitely any material

that comes their way. Carbohydrate, protein and lipid moieties as well as iron may well be re-utilised. It would seem then that only those compounds that are highly indigestible and which the animal otherwise would have difficulty in handling (such as melanin and lipogenic pigments) acumulate gradually in the centres. It is probable that such pigments, or at least some of them, formed elsewhere in the body, eventually also accumulate in the centres. By analogy, in rats with cirrhotic livers much of the ceroid produced eventually ends up in lymph nodes (Hartroft and Porta, 1965).

However, the possibility that the pigments that accumulate in the centres could be broken down and disposed of in some way cannot be discounted. It is true that their steady accumulation with age and during stressful conditions such as starvation, seems to argue against the presence of an efficient cellular defaecation mechanism but the observation of Drochmans (1967) that in humans, melanin can apparently be destroyed by dermal melanophages shows that this question is still open to investigation and should stimulate biochemists to search for a melanin depolymerase. In this context, De Duve (1964) noted that lower species, notably protozoa, can eliminate pigment through cellular defaecation but higher organisms appear to have lost this ability due to cellular "constipation" acquired in the course of evolution, and thus ageing pigment accumulates. The question as to whether this gradual accumulation of pigment within haemopoietic tissues impairs their functioning in any way needs investigating. Moreover, the potential danger of having large amounts of pigments, that are highly toxic to living cells, released into the surrounding tissues should the centres rupture for some reason or other, must not be overlooked.

With the increasing threat of pollution in recent years, fisheries biologists have been searching for histological indicators in fish of environmental quality and physiological stress (see for example Smith and Ramos, 1976). The involvement of melano-macrophage centres in various disease processes and the changes brought about in them by such factors as starvation, strongly indicate that these centres could provide sensitive indicators of stressful conditions in the aquatic environment. Blois (1965) observed that melanin binds to a large number of compounds including para-aminobenzoic acid-C14 chlorpromazine and a host of others. The possible role of melanin in protecting living cells against various

pollutants was reviewed by Edelstein (1971). It seems possible then that chemical pollutants might be preferentially concentrated within melano-macrophage centres. Much effort has been directed at attempting to utilize haematological parameters in assessing sublethal concentrations of environmental pollution (Blaxhall, 1972). Results, however, have been inconsistent partly because of the lack of species normal values and partly because such values could alter very rapidly depending on such simple phenomena as methods of capturing and transporting the fish (see for example Hattingh and Van Pletzen, 1974). In view of this, it might be useful to try and develop melano-macrophage centres as indicators of the state of health of the fish. In particular, the accumulation of haemosiderin within the splenic centres could be extremely useful.

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FISH SERUM PRECIPITINS TO AEROMONAS SALMONICIDA EXOTOXINS AND PROTEASE-LIPOPROTEIN REACTIONS: A CRITICAL APPRAISAL

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I. INTRODUCTION

Precipitation in agarose and cellulose acetate gels is used routinely to detect the presence of antibodies and antigens and has been used widely in studies of fish immunology. However, this method of detecting antibody production in fish is very unreliable and many workers have found that fish do not in general produce good precipitating antibodies, a positive reaction appearing to be dependent upon the species of fish and the antigen used (Ellis, 1982). Furthermore, formation of a precipitate in Ouchterlony double diffusion gels does not necessarily mean the presence of an antibody/antigen reaction. Baldo and Fletcher (1973) pointed out that precipitates with serum from plaice (Pleuronectes platessa) could be formed by C-reactive protein (CRP) with C-substance present in the extracts of a variety of micro-organisms. CRP precipitates require the presence of Ca^{++} ions, so by incorporating chelating agents into the gel or by washing the gel after diffusion with EDTA or sodium-citrate, the CRP precipitates can be prevented from forming or dissolved after formation and thus be differentiated from antibody/antigen precipitates.

A further precipitating factor in the serum of Atlantic salmon (Salmo salar) has recently been described by Alexander (1980, 1982). This factor was discovered in a proportion of salmon when their sera were diffused against an extract of the necrotic tissue associated with

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ulcerative dermal necrosis (UDN) affected individuals. It was demonstrated that this precipitating factor had an alpha migrating character in electrophoresis, that the precipitate was insoluble in EDTA, and it was suggested that its activity was directed towards carbohydrate moieties such as amylopectin.

During recent work in our laboratory to investigate the toxic components of the extracellular products (ECP) of Aeromonas salmonicida (the aetiological agent of furunculosis), we discovered a precipitating reaction between normal trout serum and the ECP which did not appear to be mediated by any of the mechanisms discussed above and which was dependent upon the proteolytic activity of the ECP (Munro et al, 1980; Ellis et al, 1981). This paper reports on the conditions affecting this precipitation reaction. The results suggest that the reaction is artifactual in the sense that the precipitate is not formed by complexing of reactants, but represents the proteolytic breakdown of serum lipoproteins to insoluble products. The significance of these findings relating to the interpretation of precipitating reactions in gels, particularly using complex antigen mixtures, is discussed.

II. MATERIALS AND METHODS

SERA

Normal serum was obtained by caudal vein puncture with a hypodermic syringe from rainbow trout (Salmo gairdneri), Atlantic salmon (S. salar) and cod (Gadus morhua) which were held in the laboratory aquarium. Normal human serum (CRP negative) and CRP positive human serum were obtained from Wellcome Reagents Ltd. Normal sera from chicken and rabbit were obtained from Sera-Lab, Sussex.

PROTEASES

Trypsin, thermolysin, kallikrein, collagenase and plasmin (Sigma) were used at 4mg/ml 0.04M sodium barbitone buffer pH 9.25. The extracellular products (ECP) of A. salmonicida were prepared as described previously (Munro et al., 1980).

PRECIPITATION IN GELS AND STAINING PROCEDURES

Precipitation reactions between sera and proteases were

performed by the Ouchterlony double diffusion method in 1% agarose or cellulose acetate strips (Cellogel, Whatman) in 0.04M sodium barbitone buffer pH 9.25 (Reeve Angel).

Electrophoresis was carried out in Cellogel using the same buffer. Aliquots of sera (4μl) were run for 50 min at 200 v. Protease (20μ 1) was streaked along the strips after electrophoresis, allowed to diffuse overnight, washed in buffer for 2 h and stained by the recommended procedures for protein, with Acid Black 2CT (Whatman) and for lipid, with Sudan Black B and Fat Red 7B. Glycoproteins were stained by the periodic acid-Schiff (PAS) technique. Staining for protein using Coomassie Brilliant Blue was carried out by the method recommended for immunoprecipitates in agarose by LKB.

PROTEASE INHIBITORS.

Trypsin and ECP were inhibited by incubation with freshly prepared inhibitors at room temperature for 5 min prior to use in the precipitation assay. Soybean trypsin inhibitor (SBTI; Sigma) was incubated with trypsin in a ratio of 2:1. Phenylmethyl-sulfonylfluoride (PMSF;Sigma), 36 mg/ml isopropanol was incubated with trypsin in a ratio of 1:1 w/w. PMSF was also used to inhibit the proteolytic activity of ECP. Inhibition of the proteases was confirmed using casein digestion in agarose plates (Bio-Rad protease detection kit).

LABELLED PROTEASES

^{125}I-labelled trypsin was obtained from CIS Ltd. Trypsin and kallikrein, 14mg in 2.5 ml 0.25M sodium bicarbonate/carbonate buffer pH9.0 was incubated and shaken with 7mg celite fluorescein isothyocyanate (Sigma) at 25^{o}C for 10 min, centrifuged at 8000xg for 5 min and the supernatant passed down a pre-prepared 5cm G25 Sephadex column (Pharmacia) equilibrated with 0.02M phosphate buffer pH6.6. The labelled protease was eluted and collected in the same buffer. Proteolytic activity was checked using casein digestion in agarose plates (Bio-Rad protease detection kit).

Labelled proteases were diffused against electrophoretically separated normal trout serum and the precipitates were stained in Acid Black 2 CT. The presence of ^{125}I-trypsin in the precipitate was analyzed using a radiochromatogram imaging system (AID Ltd.). For analysis of the presence of fluorescein-labelled proteases in the

precipitate, cellogel strips were viewed under a standard bench U.V. lamp.

ISOELECTRIC POINT OF PRECIPITATING FACTOR OF TROUT SERUM

Preparative isoelectric focusing of 3ml trout serum was performed in granulated gels (LKB Multiphor) using pH range 3.5 - 10. Fractions were eluted and collected in isotonic phosphate buffered saline pH 7.2 (Gibco) and protein concentration of each fraction estimated by U.V. absorptiometry at 280nm. Protein concentrations of the fractions were equalized by concentrating fractions with lymphogel, and screening for precipitating activity against ECP was performed in Ouchterlony double diffusion agarose gels.

III. RESULTS

PRECIPITATION IN CELLOGEL

(a) Ouchterlony double diffusion gels stained in Acid Black 2 CT showed a precipitation reaction between sera of rainbow trout, salmon, cod, human and rabbit on diffusion against trypsin and ECP (Fig. 1). Reaction to the latter serum was faint but sera from other individuals gave equivalent reactions to the other species tested. Other proteases were tested against trout and human serum.

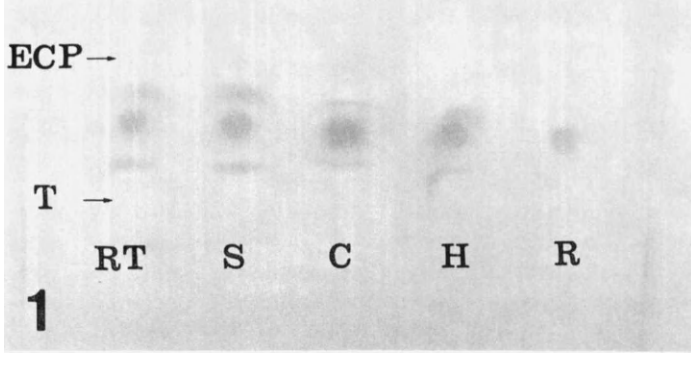

Fig. 1 Serum diffused against ECP and trypsin (T), in cellulose acetate Ouchterlony diffusion, stained with Acid Black 2CT. RT, rainbow trout; S, salmon; C, cod; H, human CRP negative; R, rabbit.

Thermolysin and kallikrein gave intense precipitation lines while plasmin and collagenase gave no precipitation reaction.

(b) Electrophoresis. Sera from human, rabbit and trout were electrophoretically separated and allowed to diffuse against trypsin or ECP (Fig. 2 a, and 2b). Precipitation reactions occurred in the α-migrating region of all sera, though slight species differences in the intensity and area of precipitation could be observed. The reaction to trypsin and ECP also differed slightly. In addition to the α-migrating band of precipitation, an area of β-precipitation could also be observed with human and trout serum.

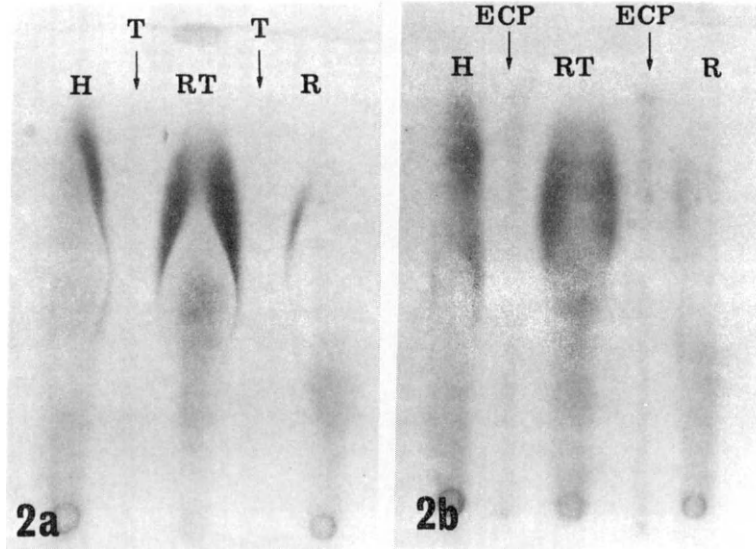

Fig. 2 (a) Electrophoresis of human (H), rainbow trout (RT) and rabbit (R) sera and diffusion against trypsin. Stained with Acid Black 2CT (b) The same sera diffused against ECP.

PRECIPITATION IN AGAROSE

Ouchterlony diffusion of normal trout serum against ECP generally produced several bands of precipitation. An outer and an inner zone of granular precipitate occurred and two or three distinct lines in between. Trout which had been vaccinated with formalin -inactivated ECP with or

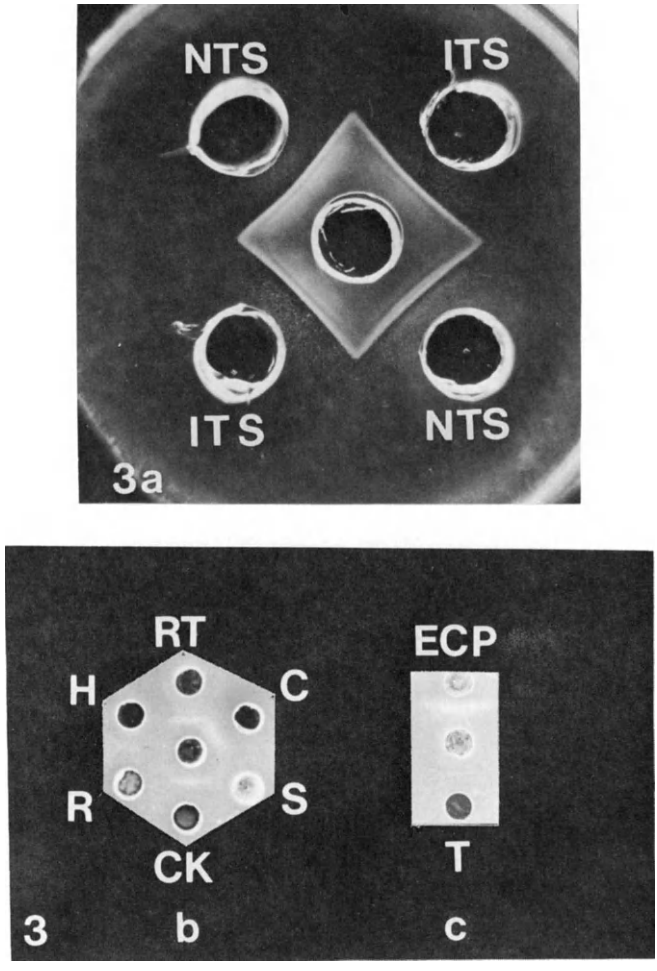

Fig. 3 Ouchterlony double diffusion in agarose (a) ECP in centre well. NTS, normal trout serum; ITS, immune trout serum. (b) ECP in centre well. Sera: RT, rainbow trout; C, cod; S, salmon; CK, chicken; R, rabbit, H, human. (c) Agarose containing 0.1M calcium lactate. Trout serum centre well, diffused against ECP and trypsin (T).

without killed A. salmonicida cells, and which had high agglutinating antibody titres, gave the same precipitating patterns as normal serum which had no agglutinating activity (Fig. 3a).

The reaction of ECP with sera from trout, cod, salmon, chicken, human and rabbit is shown in Fig. 3b. A precipitation line can be seen for all serum samples except for the two mammalian species. When diffused against trypsin, none of the sera resulted in precipitation.

EFFECT OF CHELATING AGENTS, AND CA^{++} ON PRECIPITATION

When EDTA (0.1M) was incorporated in the buffers, precipitation in cellogel and agarose did not occur. When precipitation had occurred in the absence of EDTA and the gels were then washed in buffer containing 0.1M EDTA or 5% sodium citrate for 2 hours, the precipitation lines did not dissolve in cellogel. In agarose, the results were variable depending on the buffer system. Using agarose in 0.9% NaCl the precipitates were stable on washing with EDTA or citrate, however in the barbitone buffer the precipitates almost entirely dissolved in both chelating agents, though traces of precipitates were stable for at least 48 hours.

To determine the effect of Ca^{++} on the precipitation reaction in agarose, the buffer was prepared to include 0.1M calcium lactate. In agarose plates the effect was to intensify the precipitation reactions with ECP and to allow a precipitate to form between trypsin and trout, cod and salmon sera (Fig. 3c). In cellogel electrophoresis of trout and human serum in barbitone buffer with 0.2M calcium lactate, the effect of calcium on the precipitation reaction with trypsin was to greatly intensify the β-migrating precipitate formed with the trout and human serum (Fig. 4).

EFFECT OF TRYPSIN INHIBITORS ON PRECIPITATION

When trypsin was inhibited with PMSF or SBTI precipitating reactions with human and trout serum were prevented. PMSF, which was also determined to inhibit ECP proteolytic activity, similarly abolished precipitation of human and trout serum components by ECP.

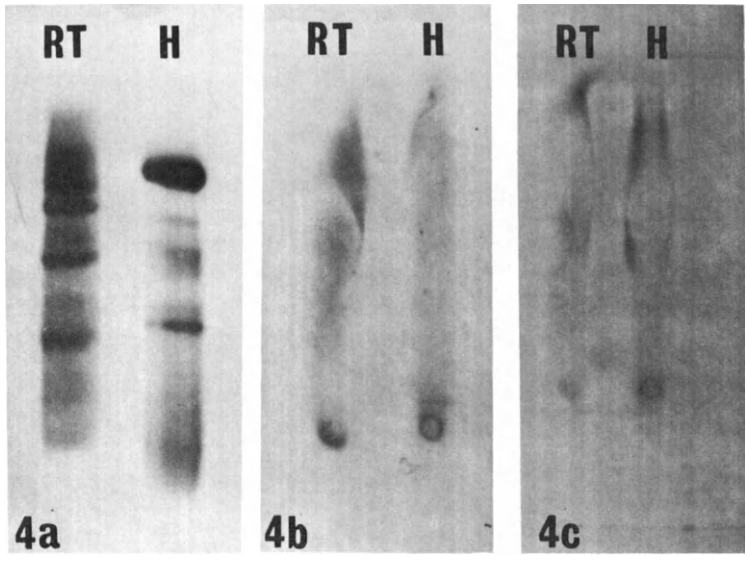

Fig. 4 (a) Electrophoresis of trout (RT) and human (H) serum in cellulose acetate stained with Acid Black 2CT.
(b) Electrophoresis of same sera in barbitone buffer diffused against trypsin. Acid Black, 2CT.
(c) Electrophoresis of same sera in buffer containing 0.1M calcium lactate, diffused against trypsin. Note intensification of the precipitates particularly in the β region. Acid Black, 2CT.

EFFECT OF HEATING SERUM

Trout serum was incubated at 45°C and 60°C for 15 min prior to diffusion against trypsin and ECP in Ouchterlony diffusion tests on cellogel. Heating had no observable effect on the precipitating reaction.

SEPARATION OF THE SERUM PRECIPITATING FACTOR BY ISO-ELECTRIC FOCUSSING

In preparative iso-electric focussing, protein fractions collected within the pH range 4.6 - 5.3 precipitated against ECP, with most intense precipitation reactions occurring in fractions with a pH of 4.9.

STAINING PROPERTIES OF THE PRECIPITATE

Attempts to stain the precipitate in agarose for protein with Coomassie Brilliant Blue, and for lipoproteins with Sudan Black B/Fat Red resulted in very poor staining reactions. However, in cellogel, the precipitate stained well for proteins with Coomassie Brilliant Blue and Acid Black 2 CT and also for lipoproteins. In electrophoresis of human and trout serum and diffusion against ECP and trypsin the precipitate occurred in the area of migration of the serum lipoproteins (Fig. 5 a and 5b). Precipitates between trypsin and trout serum, but not human serum, in cellogel also stained for carbohydrate with the PAS technique.

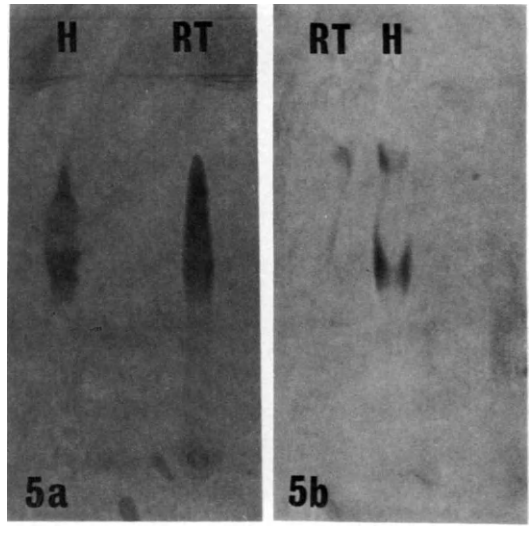

Fig. 5. (a) Electrophoresis of human (H) and trout (RT) sera on cellogel stained for lipoprotein. (b) Electrophoresis of same sera diffused against trypsin in the presence of calcium lactate. Precipitates stained for lipoproteins.

EFFECT OF SATURATING TROUT SERUM WITH FREE FATTY ACID

Normal trout serum was mixed with oleic acid (100 μl and 1 μl respectively), ultrasonicated for 2 min and incubated at 4^{o}C overnight. Electrophoresis of normal trout serum,

serum plus oleic acid, and serum from a trout which had been starved for 28 days prior to bleeding was performed and the separated sera were diffused against trypsin (4 mg/ml barbitone buffer, 0.2 M calcium lactate). The precipitates were stained with Acid Black 2. A very faintly stained precipitate occurred with serum from the starved fish, while an enhanced precipitate, particularly in the fast migrating part of the arc, occurred with the addition of oleic acid to normal serum (Fig. 6).

NATURE OF THE PRECIPITATE

Attempts to investigate if the precipitate was formed by complex formation between a serum protein and the protease were performed using either 125 I-trypsin or fluorescein isothyocyanate conjugated to trypsin and kallikrein. No evidence could be found for the presence of the proteases in the precipitate to trout or human serum in cellogel.

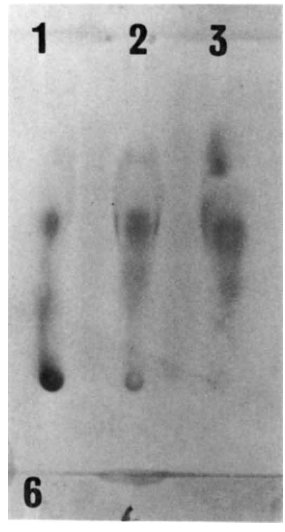

Fig. 6 Electrophoresis in cellulose acetate of sera from rainbow trout; 1, starved fish; 2, normal fish; 3, normal serum saturated with oleic acid; diffused against trypsin. Barbitone buffer with 0.1M calcium lactate. Acid Black, 2CT.

IV. DISCUSSION

Precipitation reactions in gels are widely used to detect the presence of immunoglobulins, C-reactive protein and lectins. Recently, we reported the occurrence of a precipitation reaction between an α-migrating component of rainbow trout serum and the ECP of A. salmonicida (Munro et al., 1980), which in its electrophoretic migration resembled the α-precipitin of S. salar described by Alexander (1980, 1982) and Davies and Lawson (1982). The present study investigated the nature of the precipitating reaction in cellulose acetate and agarose gels.

Certain differences in the precipitating reactions occurred in these gel systems; namely, only the non-mammalian sera precipitated in agarose with ECP and trypsin while all sera tested precipitated in cellulose acetate. Precipitation with trypsin in agarose also required the addition of excess Ca^{++}. Furthermore, the precipitates in agarose did not stain readily with any of the procedures used. The reason for these differences is unknown but differences in the physico-chemical nature of the gels and also of the fish and mammalian serum components may be responsible (see below).

The results suggest that the precipitation reaction was not entirely non-specific. Precipitates were formed between all sera and ECP, trypsin, kallikrein and thermolysin but not with plasmin and collagenase. This indicates that the precipitation reaction was dependent upon the nature of the proteolytic activity of the enzyme which, in addition, must be intact since the presence of enzyme inhibitors prevented precipitation. These observations suggested that precipitation was due to an enzyme-substrate reaction with the formation of insoluble products rather than precipitation of a complex in the manner of true precipitins such as immunoglobulin, C-reactive protein or lectins. The failure to detect radio-labelled or fluorescein labelled proteases in the precipitate is strong evidence that this was the case.

Precipitates occurred in the region of migration of the serum albumin and lipoproteins and the staining properties of the precipitate in cellogel indicated the presence of a lipoprotein. Furthermore, experiments showing a weak precipitation reaction of serum from starved fish, when serum lipid concentrations are low, and an enhanced precipitation when serum was saturated with free fatty acid, indicate that formation of the precipitate was

dependent upon the amount of lipid bound to the lipoproteins. The effect of calcium was illuminating in this respect. The presence of calcium ions enhanced precipitation reactions and their removal, by carrying out the diffusion in the presence of EDTA, abolished the precipitation reaction. However, once formed, the precipitate in cellogel did not dissolve in EDTA or sodium citrate. One possible interpretation of these results is that certain proteases are able to cleave lipoproteins into lipid-polypeptide complexes where the lipid is sufficiently exposed to form insoluble salts with calcium. This would account for the lipoprotein nature of the precipitate and its dependence upon calcium for formation.

Proteolytic digestion of serum lipoproteins has been investigated by various workers. Tayeau and Breton (1947) showed that proteolytic action on serum lipoproteins resulted in the formation of lipid-peptide complexes which possessed a higher lipid-protein ratio than the original lipoprotein. Mihalyi (1978) reviewed work on tryptic digestion of bovine serum albumin with and without associated lipid. Digestion was slower when lipid was associated with the albumin but essentially the fragments were the same and could be grouped into 3 classes with molecular weights of 8,000, 20,000 and 40,000.

The observation that precipitation reactions with ECP were more intense, particularly in agarose, may be relevant here. ECP contains a very active protease (Shieh and MacLean, 1975; Munro et al., 1980) and also an enzyme with phospholipase and glycero-phospholipid cholesterol acyltransferase (GCAT) activity (MacIntyre et al., 1980; Buckley et al., 1982). It is known that phospholipase A is able to cleave the lipid from lipoproteins with the release of free fatty acids (Marinetti, 1965). It seems likely, therefore, that the proteolytic and phospholipase activities of ECP could combine to cause exposure and release of fatty acids associated with lipoproteins and their subsequent precipitation as calcium salts.

The glycoprotein staining properties of the precipitate formed with trout serum is consistent with the interpretation of lipoprotein being the precipitated factor. Fish serum lipoproteins and albumin have not been well characterized but most of the lipid appears to be associated with a fast migrating glyco-lipoprotein with a molecular weight of 160-180,000, which is regarded as the analogue of mammalian serum albumin (Nakagawa, 1978, 1979). Human serum albumin and lipoproteins do not contain carbohydrate moieties and the precipitates obtained with

human serum did not stain with PAS. Fish lipoproteins have greater similarity to their mammalian counterparts than albumin with only minor physico-chemical differences e.g., trout low density lipoprotein (LDL) has a higher density than human LDL (Skinner and Rogie, 1978; Chapman et al., 1978). However, significant differences in the lipid moieties have been reported e.g. trout lipoproteins have a higher concentration of long chain polyunsaturated fatty acids and triglycerides than human lipoproteins and the serum has a higher concentration of LDL and high density lipoproteins (HDL) than human (Fremont et al., 1981). Also, the degree of cholesterol esterification is lower in trout lipoproteins (Perrier et al., 1979). These physical and chemical differences between fish and human albumin and lipoproteins may account for the differences in ease of precipitation by proteases in the cellulose acetate and agarose gels reported here.

Comparison between the results reported here and those of Alexander (1980, 1982) are noteworthy. The precipitating agents used by Alexander were extracts of diseased tissue from UDN infected salmon, Saprolegnia culture supernatants and amylopectin. The former two preparations almost certainly contained proteases. The precipitates were difficult to stain and it was suggested a glycoprotein may be involved. In electrophoresis, α and β serum components were observed to precipitate but no evidence could be obtained to suggest the precipitation was inducible. Furthermore, Davies (1982) reported the α-precipitin to have a molecular weight of 163 - 175,000 which closely resembles that of fish serum albumin (Nakagawa, 1978). Thus, it is possible that the $-\alpha$ precipitin reaction reported by Alexander and Davies and Lawson (1982) may represent the proteolytic digestion products of a serum lipoprotein as suggested here.

It is worth noting that artifactual precipitation of serum lipoproteins have been reported before. Staub and Grabar (1943) commented on the non-specific precipitating action of agar gels on horse and dog sera which could be confused with immunoprecipitates. This non-specific precipitation is caused by the sulphate groups in most agar preparations (Wieme, 1965) and the basis of this reaction has been used to visualize lipoproteins in agarose electrophoresis by precipitating them with polyanions e.g. heparin, sodium dextran sulphate, sodium polyanethol sulphate and long chain inorganic phosphates, in the presence of cations such as Mn^{++}, Ca^{++} or Mg^{++}. In some of these reactions sucrose facilitates the

precipitation (Burstein et al., 1970; Neubeck et al., 1977).

In conclusion, the precipitating reactions of various vertebrate sera to proteases is considered to represent a partial digestion of lipoproteins and albumin-lipid complexes with the formation of polypeptide-lipid fragments which form insoluble calcium salts. These serum components cannot therefore be regarded as 'precipitins' which form insoluble complexes with a ligand. It is recommended that serum precipitation reactions in gels should be interpreted with extreme caution, particularly when complex mixtures of biological components such as microbial culture supernatants or tissue extracts are being used.

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SERUM PRECIPITINS OF THE ATLANTIC SALMON SALMO SALAR, AND THEIR POSSIBLE ROLE IN IMMUNOLOGICAL DEFENCE

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I. INTRODUCTION

Precipitins have recently been described in the sera of Atlantic salmon Salmo salar (Alexander, 1982; Davies and Lawson, 1982). These proteins precipitate in gels when sera are challenged with extracts prepared from diseased tissue of fish suffering fungal infection, extracts prepared from the fungus Saprolegnia diclina and extracts of soluble starch and amylopectin (Wilson, 1976; Hodkinson and Hunter, 1970; Alexander, 1980). Proteins demonstrating this activity fall into two groups, α- and β-precipitins, in terms of electrophoretic mobility and molecular weight (Davies and Lawson, 1982). The α-precipitin has been isolated and partially characterized (Davies and Lawson, 1982).

This paper includes investigation of levels of α-precipitin in two groups of wild fish; a group believed to be suffering from Ulcerative Dermal Necrosis (UDN) and a healthy group showing no external evidence of disease. UDN is a disease of unresolved aetiology in salmonids and breaks out in Atlantic salmon when the fish migrate into freshwaters to spawn (Roberts, 1972). Diseased fish show epidermal ulceration and associated fungal infection, Saprolegnia diclina type I. The aims of this part of the study were to investigate the effects of outbreak of fungal infection, associated with UDN, on levels of α-precipitin in serum.

In addition, a preliminary study was carried out on the

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involvement of α- precipitin in passive cutaneous anaphylactic reactions in an attempt to discover if the precipitin functioned as an inflammatory mediator.

II. MATERIALS AND METHODS

α-PRECIPITIN IN SALMON SERA

(a) Sera. Salmon sera used in the investigation were from two groups of fish (i) a marine/estuarine group of fish which showed no visible signs of disease and which were caught off the coast of Northern Ireland at Torr Head and Carnlough, and (ii) an upstream group which showed evidence of epidermal ulceration and fungal infection, caught on the Rivers Lune and Hodder in Lancashire. Both groups of sera were from fish taken in the same spawning year.

(b) Total serum protein determination. Total serum protein levels were measured on all sera by the Biuret method (Williams and Chase, 1968).

(c) Quantitation of α- precipitin. Levels of α- precipitin were determined by radial immunodiffusion in gel in a method similar to that described by Mancini et al., (1965) using a specific antiserum prepared in rabbits against the α-precipitin. The isolation of the precipitin and the preparation of antiserum have been described previously (Davies and Lawson, 1982).

The antiserum was incorporated at a 3% concentration into a 1% agar gel. Test sera and standards were placed in wells punched into the agar. After diffusion (48 hrs) precipitin rings were formed around the wells where the α- precipitins in the test and standard samples reacted with the specific antisera in the gel. Experimental plates were set up with 10μ l of individual sera at three dilutions (neat, 1 in 20 and 1 in 40) and a control of a 1 in 40 dilution of a standard calibrated serum. In the majority of cases, the diameters of precipitin rings produced in the test sera were measured at the 1 in 40 dilution. However, for a few sera, neat and 1 in 20 dilutions had to be used. Calibration of a serum for use as a standard was necessary to prevent repeated use of fractions of α-precipitin which was of limited availability.

Calibration of a standard serum was achieved by setting up dilutions of a standard serum along with samples of

isolated α- precipitin of known protein concentration (3.2 mg/ml by Biuret method). A standard curve of the diameter squared (D^2) against percentage concentration of a standard serum is illustrated in Fig. 1. The isolated α- precipitin fraction gave a ring equivalent to a 14% concentration of the standard serum and thus the amount of precipitin in the standard (neat) was calculated to be 22.8 mg/ml. Precipitin levels in test sera were read from the standard curve and calculated from the following formula:-

$$C = \frac{A \times 22.8}{100} \times \frac{1}{B} \times \frac{D1^2}{D2^2}$$

Where
C is the concentration of α-precipitin in test serum mg/ml
A is the % concentration of standard serum of corresponding D^2
B is the dilution of test serum
$D1^2$ is the diameter squared of standard on calibration plate
$D2^2$ is the diameter squared of standard on test plate

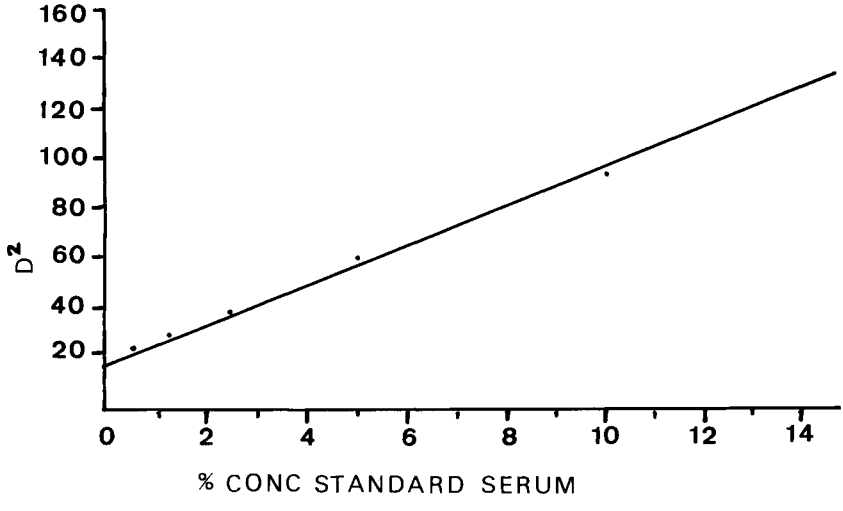

Fig. 1. Standard curve for quantitative immunodiffusion.

PASSIVE CUTANEOUS ANAPHYLAXIS

This was performed using a traditional approach similar to that described by Ovary (1958). An α-precipitin is also present in brown trout, Salmo trutta and these were used as experimental animals rather than salmon. Groups of brown trout were mildly anaesthetized (1 in 20,000 MS 222) and initially given an intradermal/subcutaneous injection (0.2 ml aliquot) of normal trout serum, normal salmon serum, isolated α-precipitin and a saline buffer control respectively. The fish were then left for 4h, after which they were again anaesthetized and injected with a 0.15 ml aliquot of a mixture of 5% amylopectin and 1% Evans Blue dye solution into the caudal vein. The fish were observed after 2 h and sacrificed after 16 h for subcutaneous investigation of the injection site. An antigen control was also tested in which fish were given the subcutaneous injection of α-precipitin but amylopectin was omitted from the intracaudal vein injection.

III. RESULTS

Precipitin levels and total serum protein values for the two groups of fish examined are shown in Table I.

TABLE I. Mean and standard deviation of α-precipitin (α-ppt) concentration, total protein (TP) concentration and 100 x α-ppt/TP for fish in the marine and freshwater groups.

	Marine Group healthy fish n = 55	Freshwater Group diseased fish n = 78
α-ppt conc. mg/ml	65.7 $\pm$ 15.4	37.6 $\pm$ 17.29
TP conc. mg/ml	93.4 $\pm$ 24.8	55.4 $\pm$ 26.6
100 x $\frac{\alpha\text{-ppt}}{\text{TP}}$ (for each fish)	74.0 $\pm$ 29.9	72.0 $\pm$ 36.7

Comparison of the means shows the values of total protein concentration for the marine group and river group to be significantly different at all levels of probability. The same applies to the α - preciptin concentration. The mean percentage of α - preciptin to total protein concentration shows no significant difference between the marine group and freshwater groups. Also, the percentage fall of α - precipitin concentration (42.8%) is similar to that of total protein (41.0%) between the two groups. A scatter plot of the relationship between precipitin concentration to total protein concentration is shown in Fig. 2. It is evident from this that the data falls into two groups, marine fish and river fish. The correlation coefficient for the marine group is 0.36 and that for the river group is 0.42.

Differentiating the sexes of the river fish, the males show a lower α-preciptin concentration but a higher average total protein concentration. The sex difference in the total protein concentration (male 63.4 $\pm$ 26.7 mg/ml and female 51.9 $\pm$ 17.3 mg/ml) is significant at the 5% level but not at 1%. The same applies for the sex

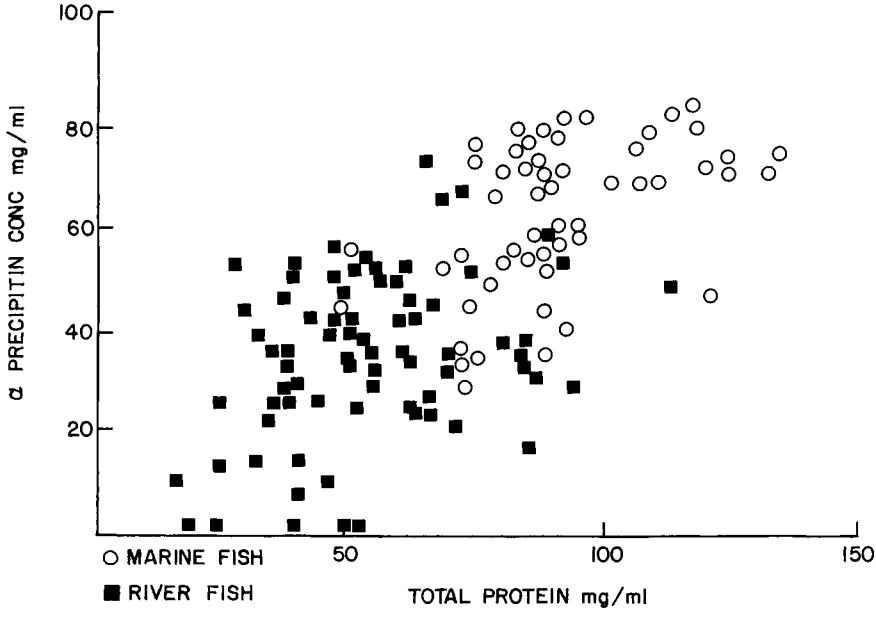

Fig. 2. Scatter diagram of α-precipitin and total protein values for individual sera.

difference in α- precipitin concentration (female 41.9 $\pm$ 17.0 mg/ml and male 30.1 $\pm$ 19.5 mg/ml) but in this case the females have the higher levels. However, the difference in the relative percentage of α- precipitin to total protein is significantly different between the sexes at the 1% level (female 84.1 $\pm$ 37.7 and male 46.7 $\pm$ 28.4).

The results from the PCA tests showed that fish in the groups which received subcutaneous injections of trout sera, salmon sera, and isolated α- precipitin, demonstrated a marked blueing in the tissues around the injection site. This was noticeable externally on most fish in each group after 2 h and 16 h following intravenous injection of amylopectin and Evans' Blue dye. When the injection site was examined internally after 16 h, all fish in these groups showed a positive blueing in the tissues. In comparison, fish in control groups had no such blueing response at either 2 h or 16 h.

IV. DISCUSSION

The specific immune response in fish is now well understood in terms of the nature and timing of the response subsequent to antigenic challenge, the ontogenetic development, and the immunoglobulins produced etc. However, the role of non-specific mechanisms of immunity are less clearly understood in the defence processes of fish to pathogens. These mechanisms depend principally on phagocytic cells and humoral components which could be antimicrobial, or could when activated, elicit some physiological phenomena such as, for example, an inflammatory reaction, leucocyte chemotaxis or phagocytosis.

The α- precipitin in salmon serum appears not to be stimulated when fish become infected with fungus. A comparison of α-precipitin and total protein levels between the healthy and diseased groups of fish, shows that the level of both parameters falls in the diseased group. However, as this group were taken from a freshwater environment this reduction is due largely to haemodilution. The relative percentage values of α- precipitin to total protein is the same in both groups and the percentage falls in total protein and α-precipitin are seen to be similar. Thus, in wild fish populations which become infected with fungus there appears to be no relative increase in α-precipitin levels compared with a healthy group of fish.

This differs from the specific immune system in which

raised levels of immunoglobulin are detected after antigenic challenge (Ingram and Alexander, 1979). Another group of proteins, the lectins, found in plants and invertebrates appear to play an important role in defence mechanisms against potential pathogens. In crustaceans, if agglutinins to pathogens have been lacking, then exposure to pathogen has been fatal (Cornick and Stewart, 1968) while Pauley et al. (1971) found that marine bacteria which were agglutinated by sea hare, Aplysia californica, haemolymph were rapidly cleared from the circulation, in marked contrast to bacteria which were not agglutinated. Agglutinin titres in the sea hare have been greatly depressed following bacterial injections but returned to normal as the bacteria were cleared from circulation. Although secondary bacterial injections were more rapidly cleared than primary, in several invertebrates secondary clearance was not associated with any increase in agglutinin titre (McKay and Jenkin 1970, Pauley et al., 1971, Bayne 1973). However, in other invertebrates such as the earthworm, Lumbricus terrestris, it has been possible to stimulate lectin levels by antigenic challenge (Stein et al., 1982). Thus lectins are considered to function as recognition molecules for foreign materials and in some instances may act as opsonins enhancing phagocytosis. Haemolymph levels appear to either rise or fall on antigenic challenge depending on the species.

C-Reactive Protein (CRP) is yet another protein binding with foreign material and has been detected in fish (White et al., 1981). This protein binds to phosphocholine containing molecules which are widely distributed in nature and Fletcher (1982) has suggested that this might afford protection whilst specific antibodies are being synthesized. In mammals, the amount of circulating CRP increases in inflammatory conditions and White et al. (1981) have found that levels in the serum of plaice, Pleuronectes platessa increase rapidly following exposure to bacterial endotoxins. Changes in levels of α-precipitin subsequent to controlled laboratory exposure to fungal extracts have yet to be determined but there may be an immediate change following challenge and then a return to normal levels. Such changes would not be detectable in the current investigation.

The α-precipitin which on characterization bears no resemblance to immunoglobulins, appears to be associated with lectin like substances in that it reacts with carbohydrate and has a molecule composed of non-covalently linked subunits (Davies and Lawson, 1982). It may function

as a recognition molecule detecting carbohydrate moieties and, like CRP, be involved in defence reactions prior to stimulation of immunoglobulin. However, the specificity of its interaction with carbohydrate has yet to be elucidated. In this interaction, the molecule appears to be involved in production of an inflammatory response. In instances where α-precipitin is introduced subcutaneously, either in whole serum or as an isolated protein, then an inflammatory reaction occurs at the injection site subsequent to intravenous introduction of carbohydrate (amylopectin) with which the protein reacts. However, at present the inflammatory reaction has only been demonstrated by infiltration of the Evans' Blue into the tissues from local blood vessels at the reaction site. The mechanism of this inflammatory reaction has yet to be elucidated to determine if vaso-active amine secreting cells are involved in a similar fashion to mammalian cutaneous anaphylaxis, or if complement components are implicated. Previous attempts to demonstrate skin sensitizing antibody in fish by homologous and heterologous PCA tests have failed (Hodgins et al., 1967; Clem and Leslie, 1969; Harris, 1973). In a recent review of histamine, mast cells and hypersensitivity responses in fish, Ellis (1982) concludes that fish lack typical histamine-associated mast cells, possess low histamine levels and lack IgE. However, typical PCA responses have been demonstrated in some flat fish and CRP has been implicated in this response, but mediators other than histamine have been proposed as the response was blocked by SRS-A antagonists (Fletcher and Baldo, 1974; Baldo and Fletcher, 1975). Whether the inflammatory reaction produced with α-precipitin is blocked by similar antagonists as is the CRP reaction, has yet to be studied. However, it appears that fish may have alternative mechanisms for production of inflammatory reactions than those demonstrated by higher vertebrates.

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NON-IMMUNOGLOBULIN HUMORAL DEFENCE MECHANISMS IN FISH

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Immunoglobulins first appear in the vertebrates and reach maximum development and efficiency in the mammals and birds. In the lower vertebrates, immunoglobulins are relatively slow to develop following an infection, whereas in invertebrates and plants, immunoglobulins are not present at all.

In fish, precipitins are extremely rare. The only immunoglobulin class produced by fish is IgM, and this is much better at agglutination and complement activation than precipitation. We have not been able to stimulate precipitins to carbohydrate antigens (Ingram and Alexander, 1980), only to proteins (Ingram and Alexander, 1976). This could be due to the presence of non-immunoglobulin precipitins in serum (Alexander, 1980).

The serum of vertebrates contains a number of substances, which are not immunoglobulins, that affect the growth of micro-organisms. These substances are predominantly proteins. Many of them probably have their counterparts or precursors in the blood and haemolymph of invertebrates. Most have been referred to as 'non-specific' but are in fact highly specific in that they react with just one chemical group or configuration. However, because the substances with which they react are very common, and because they do not influence the growth of only one micro-organism, they have been called 'non-specific'.

These substances can be classified into groups according to the effect they have on the invading organism:

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1. Substances which inhibit or retard the growth of micro-organisms - 'Anti-growth' substances.
2. Those which neutralise the activity of enzymes - 'Anti-enzymes'.
3. Enzymes which bring about the lysis of cells and organisms - 'Lysins'.
4. Those substances which cause the clumping of cells or molecules and stimulate phagocytosis - 'Agglutinins', 'Precipitins' and 'Opsonins'.

I. ANTI-GROWTH SUBSTANCES

These substances act either by depriving the micro-organism of essential nutrients or by interfering with their cell metabolism. They include transferrin and interferon.

TRANSFERRIN

Transferrin, in its many forms, is the main iron transporting protein in animals and plants. In its unsaturated form, it inhibits the growth of micro-organisms by depriving them of the iron they need (Emery, 1980). Transferrin and related proteins have been found in the serum of all vertebrates, mucous secretions, egg albumin and milk. Transferrin is genetically polymorphic in all the species studied (Putnam, 1975). Whether or not this has a selective advantage in defence is not known. However, the variants differ in their ability to chelate iron and this may be an advantage when transferrin has to compete with the siderophillins of micro-organisms.

INTERFERON

This group of proteins, of which three distinct classes have been identified in mammals, interfere with the replication of viruses by stimulation of the synthesis of enzymes, which either interfere with RNA translation or protein sheath formation (Friedman, 1981). They also increase the activity of macrophages and natural killer cells. The three types are called α, β and γ. Interferon is extremely active. With human interferon only 10 million molecules are required for a positive effect.

Each class of interferon is polymorphic. For example, there are 8 distinct genes for human α-interferon. α-interferon is produced by leucocytes, while fibroblast

cells produce β-interferon. However, β-interferon can be produced by leucocytes and α-interferon by fibroblasts. γ-interferon is produced only by T-cells and is distinct both immunologically and physico-chemically from the other two. It is a lymphokine and plays an important role in the regulation of the immune response. For its production both macrophages and a T-cell-T-cell interaction, mediated by interleukin II, seems to be necessary.

Interferon has been found several times in fish, but to date only one class has been identified (Galabov, 1973). This may be because, when most of the work was carried out, only one class of mammalian interferon had been identified. At the present, it is not known whether fish lymphocytes produce interleukin II or γ-interferon. The interferon that has been identified in fish probably belongs to either, or both of the α-interferon and β-interferon classes.

II. ANTI-ENZYME

Many micro-organisms penetrate the host and obtain their nutrients by extracellular enzymes. The serum of vertebrates contains a number of substances which are anti-enzymes and which probably have the primary function of defending the body against auto-digestion.
Substances in this group include:

i. α2-macroglobulin - a protease inhibitor. A similar substance has been identified in fish by Ellis et al. (1981).
ii. α1-antitrypsin.
iii. α2-antichymotrypsin.
iv. α2-antiplasmin.

None of the latter 3 have yet been identified in fish.

III. SUBSTANCES WHICH CAUSE LYSIS

All of these are enzymes. They may be either single enzymes such as lysozyme and chitinase, or enzyme complexes like complement.

LYSOZYME OR MURAMIDASE

This is ubiquitous (Jolles, 1969). It attacks the cell walls of bacteria and other micro-organisms. It is produced by phagocytic cells and as it works extracellularly this probably accounts for its increase in serum during infection. It also increases during

inflammatory reactions. It is specific in that it attacks the B1-4 link between N-acetylmuramic acid and N-acetylglucosamine. However, whilst some bacteria are very sensitive to this enzyme, most pathogens are not and require the cell wall to be potentiated by other factors such as complement. Lysozyme has been found in the serum and secretions of fish (Fletcher and White, 1973; Fange et al., 1976).

CHITINASE

This enzyme hydrolyses the N-acetyl glucosamine linkages of chitin. Therefore, it is able to attack the cell walls of fungi, nematodes and arthropods. Chitinase has been found in the lymphoid cells of fish (Fange et al., 1976).

COMPLEMENT

Activation of this group of pro-enzymes and other factors results in the production of a phospholipase which digests cell membranes. In mammals there are at least two pathways, which lead to the production of a C5-convertase and so stimulate a common membrane attack sequence. These are the classical, and the alternate pathways. In both, there are three stages - the activation of an initiating substance; the formation of a C3/C5 convertase; and a membrane attack sequence. The classical pathway requires for its initiation that IgM or IgG reacts with an antigen. The alternative pathway can be started by bacterial cell walls, etc. and is probably the more ancient. In higher vertebrates it is very rare for the systems to proceed to completion because the foreign cell is ingested by a macrophage or neutrophil. This is brought about by the opsonising effects of C3b and C5b and the chemotaxic effects of C4a, C3a and C5a on phagocytic cells.

In mammals, there are 12 components, excluding inhibitors, in the classical pathway and either 10 or 11 in the alternative, depending upon whether an initiating factor is in or out of favour.

In nurse sharks, Jensen and Festa (1981) identified 6 components in the classical pathway - C1n, Y, X, Z, C8 and C9. One of the unknowns must be equivalent to C3 and C5. These are essential to both sequences and because they have evolved by gene duplication, it is possible that only one is present in fish. The most likely candidate for this role is X. This would mean that Y is equivalent to C4/C2 and Z to C6/C7.

One factor that is characteristic of the alternate pathway is properdin, a long molecule which stabilises the C5-convertase. Properdin has been found in a number of fish (Day et al., 1970). Kaastrup (pers. comm.) contends that the alternative pathway is the only one present in fish.

IV. AGGLUTININ, PRECIPITINS AND OPSONINS

These can be considered together because agglutinins and precipitins both involve the cross-linking of molecules. In the case of agglutinins, these are still attached to the micro-organisms whilst with precipitins they are in solution. Both groups can then act as opsonins. This group includes the 'natural' antibodies of vertebrates and the lectins and lectin-like substances of animals and plants. They all seem to have one property in common, that is, they react with carbohydrate residues. Even those which have been identified as anti-protein may still be reacting with the carbohydrate part of the molecule, as proteins without carbohydrate attached are very rare.

ANTI-BLOOD GROUP SUBSTANCES

The most commonly identified group of agglutinins are those which react with mammalian blood group antigens, especially those of the ABO(H) system. However, these antigens are ubiquitous and are common carbohydrate constituents of cell walls (Springer, 1970a and b).

C-REACTIVE PROTEIN

CRP reacts with the C-polysaccharide of bacteria and other organisms. Whereas lysozyme and chitinase react to N-acetyl glucosamine containing polysaccharides, CRP precipitates those containing N-acetyl galactosamine. It can be distinguished by the ease with which it can be dissociated from the complex by Ca++ chelating agents and phosphocholine.

CRP can both agglutinate and precipitate. Following its reaction with its substrate, it appears to have an opsonising effect on macrophages, and the ability to activate complement. Whether the latter is due to the CRP part of the complex, or because of the aggregation of the carbohydrate is not clear. CRP has been recorded in fish (Balbo and Fletcher, 1973).

FRUCTOSAN-SPECIFIC PROTEIN

A protein which precipitates laevans and inulin has been found in the nurse shark (Harisdangkul et al., 1972).

α- AND β-PRECIPITINS

In the sera of all healthy vertebrates and in some invertebrates there are a collection of proteins which react predominantly with carbohydrates. These have α and β -globulin electrophoretic mobility, and molecular weights in the range 170,000 and 500,000. The α-precipitin exists in two forms 170,000 and 500,000, whilst the β-precipitin is 500,000. The α-precipitin is the one that has been studied in detail by my group (Alexander, 1976, 1980, 1982; Davies, 1975). It was first discovered by its reaction with extracts of UDN-infected salmon and was later shown to react with extracts of fungi and bacteria. The precipitin has now been found to react with amylose and amylopectin. It is not a single molecular species but a group of molecules with different specificities.

When reacted with extracts of fungus-infected tissue and, to a certain extent, with extracts of fungi, a number of precipitin lines are produced. These are not due to the presence of CRP because, when plaice CRP was run in the plates, treatment with EDTA resulted in the CRP precipitate disappearing within 30 min but not the α-P one.

When the sera were examined against amylopectin, which must have a molecular weight above 1,000,000, one strong and sometimes one weak precipitin lines were produced. Sera which gave reactions with diseased tissue extract did not necessarily react with amylopectin and vice versa (Alexander, 1980). In SRID plates, two circles of precipitation were obtained against amylopectin. The outer ring remained relatively constant in diameter from serum to serum. However, the concentration of the inner ring precipitate increased dramatically in UDN-infected salmon 3 years after the disease was first recorded. As UDN started to decline, then the concentration also decreased.

Davies (1975) measured the concentration in salmon serum of α-P by SRID. It represented about 70% of the serum protein and was relatively constant. However, when I compared the inner ring concentration with total serum protein there was no correlation. This is what one would expect if the material was stimulated by infection (compare with immunoglobulin in mammals). We have now

measured the concentration of α-P in the sera of a much larger sample of salmon sera by ELISA and have found concentrations varying up to 20 g/100 ml. (The serum of salmon caught in the sea can contain well over 25 g/100 ml total protein (Alexander, 1977). These results were obtained using an antiserum to α-P raised in rabbits. We have produced a small quantity of a monoclonal antibody to α-P and hope to use this to compare the concentrations in sera given by the two types of antisera.

When examined by isoelectric focusing, partially purified α-P was found to contain at least 8 or 9 variants. These variants differ in concentration in different animals (Alexander, 1982).

Similar precipitins have been found in the sera of all vertebrates examined. Within the fish, many of them cross react with salmon α-P antiserum. We have not yet been able to raise an antiserum to mammalian α-P probably because of similar cross-reactions. In the mammals and the birds, α-P has undergone a molecular weight change and is present as a component of about 45 to 50,000 daltons (Blower, pers. comm.).

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SEQUENTIAL ANTIGENIC COMPETITION IN TELEOSTS: A BACTERIOPHAGE-AEROMONAS SALMONICIDA INTERACTION

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I. INTRODUCTION

The aquatic environment is ideal for the growth and transport of micro-organisms, both fish pathogens and the ubiquitous non-pathogens of fresh water. Hyperosmotic infiltration techniques have shown that soluble (Amend and Fender, 1976) and particulate (Bowers and Alexander, 1981) antigens can be taken up from the aqueous environment by fish. Recently, Tatner and Horne (1983) have demonstrated the uptake of formalin-treated Vibrio anguillarum in direct immersion experiments with Salmo gairdneri. Indeed, a pre-immersion in a hyperosmotic solution was found not to affect the uptake of the bacterium. The passive penetration of the fish integument by micro-organisms from the environment may thus represent a continuous and multiple species challenge of the piscine immunological mechanisms, although this challenge will be at a low, but significant, level of antigen concentration. Passive immersion techniques have been used successfully in the bath immunisation of large numbers of fish (Egidius and Anderson, 1979), and have thus demonstrated that there is an interaction of water-borne antigens with the immunological mechanisms of fish. Such vaccines may contain attenuated pathogen species, consisting of

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numerous immunogenic components and antigenic determinants, and in the future it may be desirable to immunise fish stocks with two or more vaccines simultaneously or in close sequence. Thus the immunological mechanisms of fishes will be challenged on occasions with a multiplicity of immunogenic components, either naturally from the aquatic environment or from prophylactic immunisation procedures, which may competitively interact.

The fact that the challenge by one antigen may partially suppress the parallel immune response to a second antigen species has been recognised since the beginning of this century. The early literature on antigenic competition in mammals has been reviewed by Taussig (1977). In the first study of antigenic competition in teleosts, made by Richter and Ambrosius (1972), the soluble immunogens bovine and human gammaglobulins were found to compete in Cyprinus carpio. In a later study, particulate immunogens, the bacteriophages MS2 and X174, were found by O'Neill (1980c) to compete in Salmo trutta and C. carpio, and in this case the degree of immune suppression was shown to be dependent on the time interval between the two bacteriophage challenges. The present work was initiated to examine sequential antigenic competition in teleosts challenged with the fish-furunculosis bacterium Aeromonas salmonicida. This bacterium was used as an example of a large and complex particulate immunogen which, as a formalin-killed organism or in the form of a natural infection, could be used to interact with the immune response to the bacteriophage MS2.

II. MATERIALS AND METHODS

EXPERIMENTAL FISH

Brown trout (Salmo trutta L.), 1+ years and 174-237g, and mirror carp (Cyprinus carpio L.), 3+ years and 97-267g, obtained from the Severn-Trent Water Authority fish farm, Nottinghamshire, were held in 100 L polyethylene aquaria, with a through-flow of well-aerated and chlorine-free tap water. To ensure that all the "healthy" fish were free of the furunculosis bacterium, each animal was intra-peritoneally (i.p.) treated with 3mg tribrissen (Wellcome-Cooper, USA) per 100g body weight, made up in sterile teleost saline, pH 7.6 (O'Neill, 1979). No other treatment with antibiotics or fungicides was undertaken.

Individual fish were identified by an opercular tag and randomly distributed between the aquaria. Trout and carp were held in separate tanks. The fish were then acclimatised in the experimental tanks, at a water temperature of 15.5° ± 0.5°C, for a period of two weeks before commencing any immunogen challenge. A photo-period of 12h light: 12h dark was used and the fish were fed daily on Beta Trout Growers Diet 417, No. 5 (B.P. Nutrition).

A group of 1+ year brown trout, harbouring a latent infection of fish-furunculosis and demonstrating no outward sign of disease, were obtained by the Severn-Trent Water Authority. These fish were isolated from other fish stocks and suitable precautions were taken to eliminate any transfer of the disease organism.

At the termination of an experiment all the fish were challenged with 3mg of the synthetic corticosteroid prednisolone (Sigma) and then observed. Precautions were again taken to eliminate any transfer of the disease organism.

HANDLING AND BLOOD SAMPLING

The fish were netted and anaesthetised in a 1g:200 L solution of tricaine methane-sulphonate (MS222, Sandoz) made up in aquarium water. Blood samples of not more than 0.05 cm^3 were obtained by caudal venipuncture and were left to clot at 1°C. The sera were decanted and assayed in duplicate for live MS2 and viral neutralisation activity immediately.

IMMUNOGENS

The bacteriophage MS2 (picornovirus, group 1 RNA-phage) was grown using the Petri-plate and "soft-agar" overlay method of Eisenstark (1967) with Escherichia coli K12 as the host. The agar overlays were homogenised in sterile teleost saline, with the addition of chloroform, and aided by ultrasonic disruption (20 kHz at 6nm), followed by two low-speed (3000 xg for 2 min) and then two high-speed (40,000 xg for 120 min) centrifugations in teleost saline at 4°C. The final suspension was stored at 1°C.

A pigmented and auto-agglutinating strain of the furunculosis bacterium, Aeromonas salmonicida, was obtained from the National Collection of Marine Bacteria, Aberdeen. The bacterium was cultured in 200 cm^3 aliquots

of tryptone soya broth (Oxoid) at 22°C with sterile aeration for 36h. The bacterium was inactivated over-night by the addition of $2cm^3$ formalin. After 3 washes in teleost saline (3000 xg for 20 min) the bacterium was stored at 1°C as a 10^{10} cm^{-3} suspension of whole bacteria.

BACTERIOPHAGE COUNTS AND NEUTRALISATION ASSAY

A semi-micro viral plaque neutralisation assay procedure was modified from the Petri-plate methods of Adams (1959), and the neutralisation antibody titre of the sera was described as the dose of serum required to produce 50% inactivation of the bacteriophage (SD_{50}). Two-fold serial dilutions in teleost saline were prepared in duplicate from 0.025 cm^3 aliquots of sera and were incubated with a known number of viral particles. About 150 - 200 were found to be ideal. By omitting the MS2 aliquot the retention of live bacteriophage in the sera could be detected and titred. The incubation times and temperatures selected for the assay were 30 min at 20° for S. trutta and 2h at 20°C for C. carpio. The full procedure has been described elsewhere (O'Neill, 1979).

IMMUNISATION

Sequential challenge: Serum samples from 9 groups of 5 trout and 5 carp were taken at 12h intervals after a single i.p. inoculation of $6.6 \times 10^9 \pm 1.1 \times 10^8$ plaque forming units (PFU) MS2 in 0.1 cm^3 teleost saline. At intervals of -14, -8, -4, -2, -1, 0, +1, 0, +1, or +2 days from MS2 challenge 8 groups of trout and carp were given a single i.p. inoculation of $1.3 \times 10^7 \pm 2.6 \times 10^5$ A. salmonicida organisms in 0.1 cm^3 teleost saline. The final group of fish was challenged with MS2 only. A further 5 groups of 5 trout and 5 carp were examined for MS2 neutralisation activity at weekly intervals after i.p. challenge with $2.6 \times 10^9 \pm 1.3 \times 10^8$ PFU MS2. Four of the groups were inoculated i.p. with 1.3×10^7 A. salmonicida organisms at 0, 1, 2 or 7 days before the MS2 challenge. Again one group of fish was challenged with MS2 only. All the inoculation schedules were repeated 7 weeks later for both the above experiments.

Latent furunculosis - MS2 interaction: MS2 clearance and neutralisation activity were followed in 5 healthy trout and in 5 trout harbouring a latent furunculosis infection,

after primary and secondary challenge with 2.6 x 10^9 PFU MS2.

A. salmonicida immunisation and repeated challenge: One group of 5 trout and 5 carp were immunised with 2.5 x 10^9 + 1.3 x 10^7 A. salmonicida organisms 14, 4 and 0 weeks before challenge with 6.5 x 10^9 PFU MS2. A similar number of fish were immunised with 10^9 PFU MS2 at 2, 7, and 14 weeks before weekly challenge with 2.5 x 10^9 A. salmonicida organisms. The sera of these fish were examined at weekly intervals for MS2 neutralisation activity.

III. RESULTS

Sequential challenge: The presence of the formalin-killed A. salmonicida was found to suppress the clearance of the live MS2 bacteriophage at all the intervals examined and in both species (Fig. 1). A maximum suppression was observed when the virus was challenged 4 days after the bacterium, at which time the clearance times were increased at least 5-fold. On secondary MS2 challenge the 6 - 7 day clearance time for the virus was found to be unaffected by bacterial challenge. Although the detection of MS2 neutralisation activity in the sera was dependent on the MS2 clearance times, the joint challenge with A. salmonicida did not suppress the primary humoral immune response but enhanced it (Fig. 2). The secondary humoral responses were suppressed in both the trout and carp. The maximum suppression was observed when the bacterium was challenged 1 day before the MS2, and minimal when 1 week separated the inoculations.

Latent furunculosis - MS2 interaction: In the furunculosis infected trout MS2 clearance was increased, from the normal 2.5 days, to 7 days (Fig. 3a), though the secondary clearance was again that expected for control fish, 6 days. Both the primary and secondary humoral immune responses demonstrated an enhanced MS2 neutralisation activity in the infected trout, although the initiation of both responses was suppressed (Fig. 3b).

A. salmonicida immunisation and repeated challenge: In the groups pre-immunised with A. salmonicida no difference was observed in the MS2 clearance times, when compared to the control fish. However, the initial primary humoral response in the trout was retarded and then progressed to enhanced levels of neutralisation activity (Fig. 4a), as

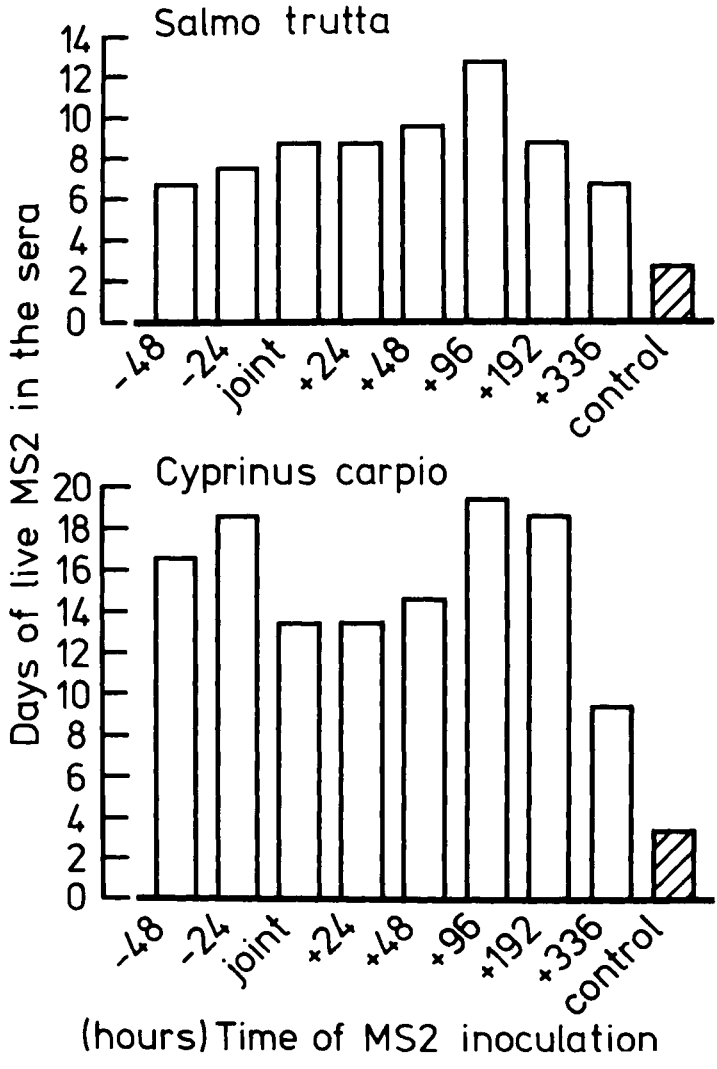

Fig. 1 Blood clearance of primary MS2 challenge, ± 0.5 day, before or after A. salmonicida challenge. Control groups were not challenged with A. salmonicida.

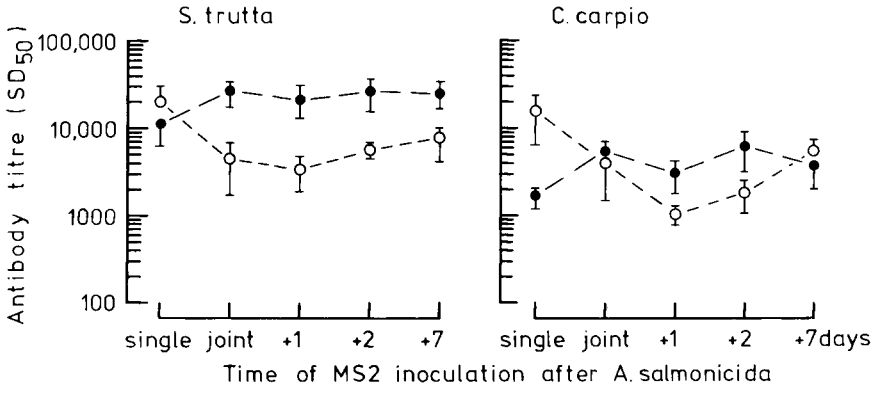

Fig. 2 Peak primary (●) and secondary (O) MS2 neutralisation antibody titres, ± 2 S.E., after A. salmonicida challenge.

was observed in the latent furunculosis infected trout. The carp exhibited a similar response to that observed after single A. salmonicida-MS2 inoculations 1 week apart, and only the humoral response indicated a slight suppression of MS2 neutralisation activity (Fig. 4b). It has been observed that MS2 immunised fish will maintain high levels of viral neutralisation activity. In the trout these levels were not changed by the repeated challenge of A. salmonicida (Fig. 5). The carp also indicated an unchanged response until the fourth weekly challenge of A. salmonicida, at which time a rise in neutralisation activity was observed. Figure 5 also demonstrated the higher level of neutralisation antibody in the trout, compared to that of the carp, produced by the 5° difference in optimum temperature between the two species (O'Neill, 1980a).

The inoculation of prednisolone at the end of the experiments was found to produce no abnormal symptoms in any of the fish, except for those trout harbouring the latent furunculosis infection. The latter fish died within 3 - 5 days of the prednisolone challenge. The pigmentation of the moribund fish was considerably darker than that of the healthy trout and they all exhibited discrete dermal lesions, 3 - 6 mm in diameter. Larger oedematous swellings and haemorrhage were observed on the caudal peduncle and about the vent.

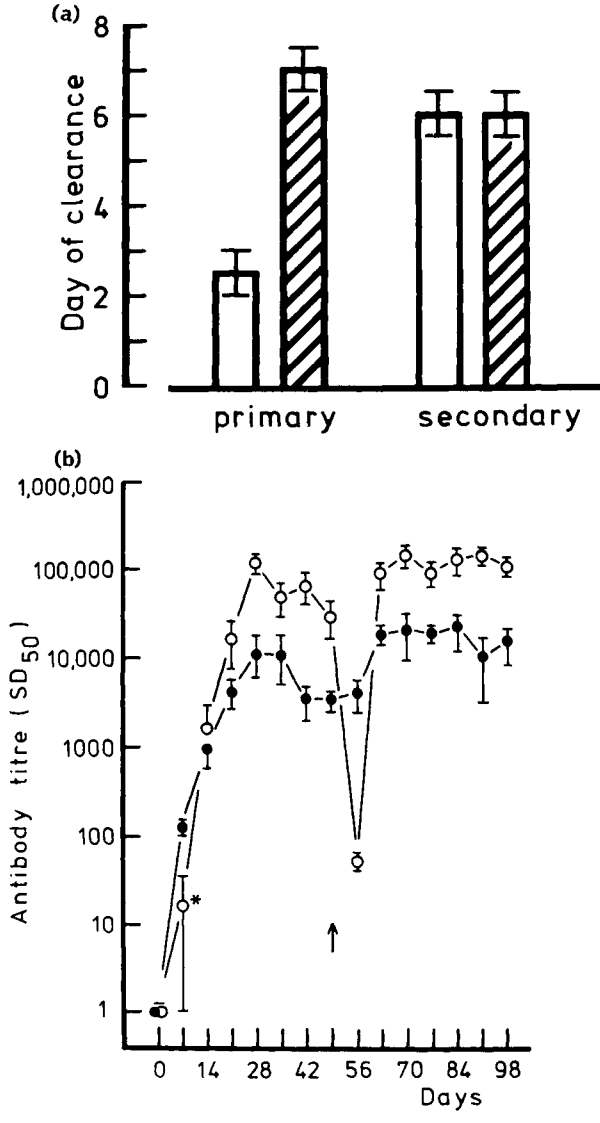

Fig. 3 The response of furunculosis infected trout to MS2.
(a) Blood clearance of primary and secondary MS2 challenge in healthy (open bars) and infected (hatched bars) fish, ± range.
(b) MS2 neutralisation antibody response in healthy (●) and infected (O) fish ± 2SE. (↑) Point of second MS2 challenge. (*) Live MS2 detected in the blood.

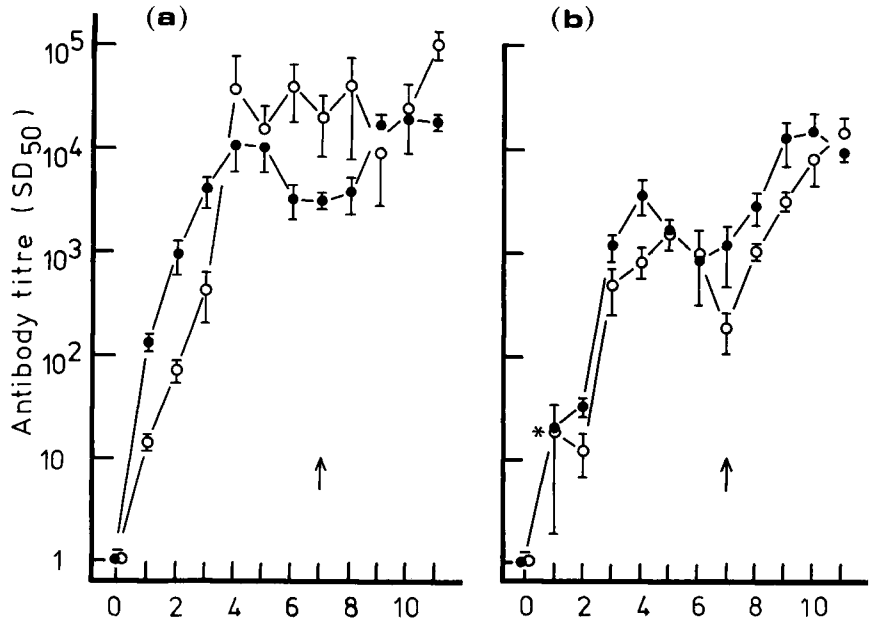

Fig. 4. The MS2 neutralisation antibody response of (a) trout and (b) carp in control fish (•) and fish pre-challenged 14, 7 and 0 weeks before MS2 challenge (o) , ± 2 S.E. (↑) Point of second MS2-A. salmonicida challenge, MS2 only in the controls. (*) Live MS2 detected in the blood.

IV. DISCUSSION

In the interaction with A. salmonicida two aspects of the immune response to the bacteriophage MS2 were examined, firstly, the clearance of the bacteriophage from the peripheral blood and, secondly, the production of viral neutralisation antibody. From the results of earlier work (O'Neill, 1980b) it was concluded that MS2 clearance was

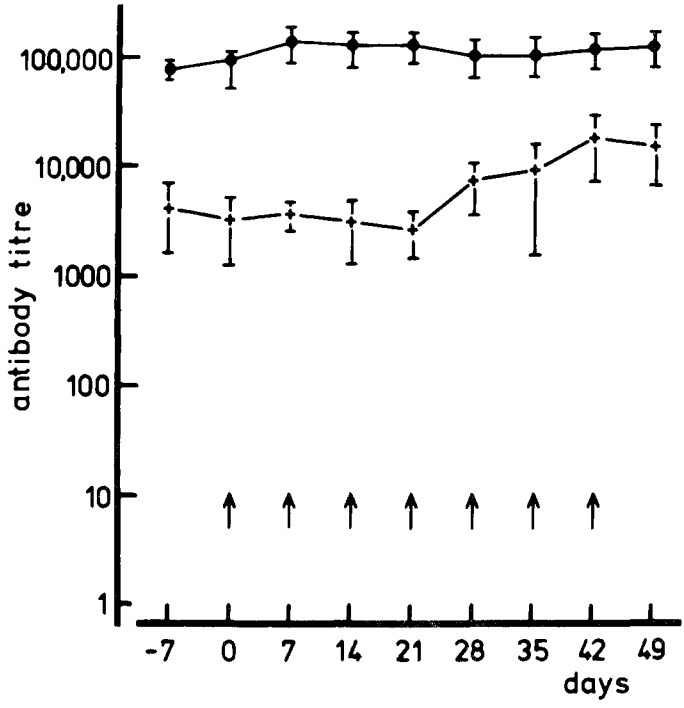

Fig. 5. The effect of repeated A. salmonicida challenge (↑) on the secondary MS2 neutralisation antibody responses, after the onset of the plateau phase, in trout (●) and carp (+). Mean antibody titres, $SD_{50} \pm 2$ S.E.

dependent on the production of antibody and, because the clearance phase was rapid, a precise measurement could be made of the lag, or inductive, phase of the humoral immune response.

In both this study and that of viral:viral interaction (O'Neill, 1980c) the major effect of immunogen interaction was the suppression of humoral antibody induction, and thus viral clearance. The presence of the bacterium produced longer MS2 clearance times than those observed in the viral:viral interaction, 1.5x longer in trout and 2x longer in carp. This could have been the result of a larger stimulation, produced by the greater antigenic mass or complexity of the bacterial challenge. The maximum

suppression of the clearance time was observed, in both trout and carp, when the bacterium was challenged 4 days before the MS2 (Fig. 1), whereas in the viral:viral interaction this was on conjoint challenge. A maximum inhibition was found to occur with a 4 day interval between heterologous erythrocyte challenge in mammals (Radovich and Talmage, 1967; Waterston, 1970). A second challenge of the bacteriophage and bacterium, in all the sequences, was found to produce the delayed 6 - 7 day clearance period that was observed for a single second challenge of MS2. This suppression of the latent period in the secondary response could have been the short-lived result of direct interactions by immune complexes with antibody-producing lymphocytes and associated immune-macrophages, or the activation of suppressor T-like cells (O'Neill, 1980b).

In contrast to the marked suppression observed in the induction of the primary humoral immune response, the subsequent MS2 neutralisation titres were enhanced by the bacterial challenge. The addition of Freund's complete adjuvant (FCA) to the MS2 inoculum, which in this study was challenged in saline only, has been demonstrated to produce similar enhanced neutralisation antibody titres (O'Neill, 1979). It is thus probable that components in the Gram-negative A. salmonicida, similar to those found in the FCA, could promote the expansion of helper T-like cell populations. It is also probable that these mitogenic components could stimulate suppressor populations, and in the secondary humoral immune response a moderate suppression of the MS2 neutralisation titres was observed. The latter suppression was at a maximum when up to 1 day, and not the 4 days observed for MS2 clearance, separated the MS2 and bacterial challenges (Fig. 2). Once the maintained levels of MS2 neutralisation activity were reached in the secondary response (Fig. 5), A. salmonicida challenge had little effect. Only in the carp, after several bacterial challenges, was a small enhancement of activity evident. The plateau phase of the secondary response appeared to have reached a balance in the trout, and in the period prior to the enhanced response in the carp, that was unaffected by any suppressor or adjuvant activity from the bacterial challenge.

Trout furunculosis in the latent form is an infection which, under good farm management, need not present any clinical symptoms (McCarthy and Roberts, 1980). In order to detect these "healthy" carrier fish, it is necessary to advance the infection into a disease state with the use of

a stressor, such as the synthetic corticosteroid prednisolone. However, in this latent state the immunological mechanisms of the trout would appear to recognise the presence of the A. salmonicida bacterium. The primary clearance of MS2 and the initiation of the neutralisation antibody response in both the primary and secondary responses were suppressed, and the subsequent antibody titres were enhanced in the infected fish. A similar effect was observed in trout pre-immunised with formalin-killed A. salmonicida (Fig. 4a), with the exception that MS2 clearance was not suppressed. In specimens of brown trout exhibiting chronic furunculosis symptoms, Ferguson and McCarthy (1978) observed an accumulation of lymphoid cells around the major afferent vessels of the spleen that could have been a mitogenic response to the Gram-negative A. salmonicida. It is thus possible that a natural A. salmonicida infection could activate dual populations of regulatory lymphocytes, suppressor T-like and helper T-like cells, which would be non-specific in their activity. In the latent furunculosis infection it could be expected that there was a maintained stimulation of these suppressor and helper mechanisms. However, the suppression of the humoral antibody producing mechanisms was short-lived in these fish and was not maintained by the continued stimulation of the suppressor mechanism. This may suggest that the life of the suppression was determined by the antibody producing mechanisms, rather than by the suppressor mechanism. Further, the observed sequential antigenic competition in trout and carp would suggest that the degree of suppression was determined by the level of suppressor activity, which increased as the suppressor mechanism developed, and probably exerted its effect at one discrete suppression-sensitive phase in the humoral mechanisms. After the suppressor susceptible phase had been passed the effect of the helper mechanism was then expressed as enhanced antibody titres. Even at this stage a fine balance appears to exist between the suppressor and helper mechanisms. Although the latent furunculosis infection and pre-immunisation with formalin-killed A. salmonicida produced enhanced secondary responses to MS2 in trout, those fish receiving single inoculations of the bacterium, at the time of MS2 challenge, produced a suppressed response. Helper function could in this case need to be enforced to a greater degree in the secondary response, if an enhanced response is to be produced and be dominant over the suppressor mechanism.

The early mammalian literature on antigenic competition, reviewed by Taussig (1977), suggested that antigens competed for receptor sites on a multipotential antigen-sensitive cell. As no antigenic competition was observed in the mammalian secondary response, which was also the case for secondary MS2 clearance in the fish, it was further suggested that the immune memory cells had matured into antigen-sensitive cells of one specificity. Radovich and Talmage (1967) and Waterston (1970) concluded that for this type of competition to occur, there would have to be a conjoint challenge of the two antigenic species. In their experiments, it was found that conjoint challenge did not suppress the immune response, but to do so required the competing antigen to be challenged, within a specific time span, after the first antigen. A humoral inhibitor, released in response to the first antigen, was proposed and this was suggested to be an antibody feedback mechanism. The requirement for activated suppressor T-cells in antigenic competition was demonstrated by Gershon and Kondo (1971) in mice reconstituted with thymocytes. It was this involvement of the T-cell that placed the phenomenon of mammalian antigenic competition as a side effect of the immune regulatory mechanisms.

A sequential antigenic competition was observed in trout and carp, similar to that seen in mammals. This may indicate that similar mechanisms of a short-lived and non-specific T-cell activity, and the possible production of non-specific humoral inhibitors, rather than a competitive act for a multipotential antigen-sensitive cell, do exist in fish. However, suppression was observed in both trout and carp after conjoint viral:bacterial challenge in this study, viral:viral challenge (O'Neill, 1980c) and in carp after soluble antigen challenge (Richter and Ambrosius, 1972). In the case of the viral:viral interaction there was a mutual suppression of the response to the two viral species on conjoint challenge. Such observations may not exclude a competitive act for a multipotential antigen-sensitive cell. A requirement exists, therefore, for the examination of antigenic competition in fish, with the use of T-independent antigens and cell-transfer techniques, to test the involvement of possible T-like lymphocyte populations.

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THE IMMUNE RESPONSE OF BROWN TROUT, SALMO TRUTTA TO SHEEP AND HUMAN 'O' ERYTHROCYTES

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I. INTRODUCTION

Both innate and acquired specific defence mechanisms exist in fish and much work has been undertaken into their immune response (Hennessen, 1981; van Muiswinkel and Cooper, 1982).

The sera of fish, other vertebrates, and invertebrate haemolymph contain naturally-occurring agglutinins, which react to a wide range of cellular antigens (Stein and Cooper, 1982). These molecules, usually proteins or glycoproteins, are often called natural antibodies and may confer immunity against infection. In some cases these substances are immunoglobulins stimulated by environmental pathogens. In other instances they resemble antibody-like compounds or lectins and there is no evidence of previous antigen stimulation (Ingram, 1980). Furthermore, these natural antibody-like molecules present in fish serum differ from immunoglobulins in their physico-chemical, biological and antigenic properties (Gold and Balding, 1976).

This paper presents the results of the humoral immune response of brown trout Salmo trutta, to sheep (SRBC) and human O red blood cells (HRBC), together with changes in serum proteins following injection. Investigations were also performed in an attempt to elucidate whether the natural and stimulated anti-SRBC haemagglutinins (Hagg) were similar molecules.

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II. MATERIALS AND METHODS

FISH EXPERIMENTS

One year old brown trout (length 195 - 255 mm and weight 75 - 154 g) were maintained at 12 + 1°C. Fish were given a single 1 ml intraperitoneal (IP) injection of 20% suspensions of either SRBC or HRBC in phosphate-buffered saline (PBS) pH 7.4. The trout that received SRBC were given a second injection on day 22. Control fish comprised uninjected animals and those injected IP with 1 ml PBS.

The isolation of serum has been described elsewhere (Ingram and Alexander, 1979). Fish that received a single injection were bled at regular intervals between day 3 and day 28 in the case of SRBC, or between days 7 and 70 for HRBC. Trout given a second injection of SRBC were bled between day 25 and day 70. Control animals were bled at random intervals throughout the experiment.

DETECTION OF HUMORAL ANTIBODY AND SENSITISED LYMPHOCYTES

Sera from 20 trout were examined against 17 types of erythrocytes from 14 vertebrate species for the presence of naturally-occurring Hagg by the direct haemagglutination test - DHA (Table I). The sera of control and antigen-stimulated fish and serum fractions were investigated by DHA for antibody activity.

The assays for the enumeration of plaque-forming (PFC) and rosette-forming cells (RFC), in the spleen and pronephros, together with times of sampling have been reported in a previous paper (Ingram and Alexander, 1981). The plaques were developed using salmon serum as the source of complement. The control animals were examined for naturally-occurring PFC and RFC against SRBC.

ABSORPTION SPECIFICITY

Absorption studies were performed to determine the specificity of the agglutinin. Normal and immune sera were absorbed with an equal volume of either SRBC or HRBC. The mixtures were incubated at 30°C for 1 h and centrifuged. The supernatants were removed and the absorption process repeated. The mixtures were again centrifuged and the supernatants examined against either SRBC or HRBC for agglutination activity. Normal trout and anti-SRBC sera with high Hagg titres were separately pooled and fractionated by neutral salts (Na_2SO_4 and

$(NH_4)_2SO_4$) (Ingram, 1978) and gel filtration. The preparation, calibration and running of Sephadex G-200 and Sepharose 6B columns were as reported by Ingram and Alexander, (1979). The fractions were examined by DHA, immunoelectrophoresis (IE) and cellulose acetate electrophoresis (CAE) as previously reported (Ingram and Alexander, 1979, 1980).

PHYSICO-CHEMICAL TREATMENTS

Trout sera and the antibody-active fractions from gel filtration were treated with various concentrations of pronase, papain, trypsin and sodium periodate to determine the nature of the Hagg. The effect of heat and dithiothreitol (DTT) on the Hagg was studied by the method of Ingram and Alexander (1979, 1980). The action of DTT was stopped by the addition of 0.1 M iodoacetamide. In addition, the requirement of Hagg for divalent cations was examined by first treating the material with EDTA, followed by the addition of various molarities of calcium and magnesium ions.

CARBOHYDRATE INHIBITION.

The nature of the receptor on the SRBC surface for the agglutinin molecule was examined with 49 different carbohydrates and 11 glycoproteins. To doubling dilutions of positive trout sera (neat to 1/32), equal volumes of either 0.4 M carbohydrate or 20 $mgml^{-1}$ glycoprotein were added. The mixtures were incubated for 2 h at 37°C, 4% SRBC suspensions were added to each well, further incubated for 1 h at 37°C and examined for agglutination.

TABLE I. Natural agglutinin titres in trout sera against the erythrocytes (RBC) of several vertebrate species.

RBC	No.	Hagg titres (log 2)			
	+	1	2	3	4
Human 'A'	3	2	1		
Human 'B'	1		1		
Human 'AB'	2	1	1		
White Rat	6	6			
Hooded Rat	4	4			
Sheep	5	2	2	1	
Horse	5	4	1		
Rabbit	4	3	1		
Pigeon	17	2	9	6	
Lizard	8	8			
Frog	19	3	4	10	2

III. RESULTS

AGGLUTININS, PFC AND RFC

The results of the natural Hagg titres against the different types of erythrocytes are given in Table I. Hagg were not found against HRBC, cotton rat, multimammate rat, mouse, salmon and trout erythrocytes. Taking into account availability of erythrocytes, a further 95 sera were examined for Hagg against SRBC and HRBC; 14 were positive to SRBC and again, all were negative to HRBC. In the light of these results SRBC and HRBC were used in the injection experiments. SRBC were selected to check whether the natural anti-SRBC Hagg titres could be increased, and HRBC chosen to see if anti-HRBC antibodies could be induced.

After 1 injection of SRBC, stimulated PFC and RFC first occurred after 3 days (pronephros) and 6 days (spleen), reached maximum counts on day 12 and fell to background levels by day 25 (Fig. 1a and 1b). After a second injection on day 22, peak PFC and RFC values were reached on day 32 and then declined to background by day 55. Both PFC and RFC were significantly increased ($P < 0.01$) above

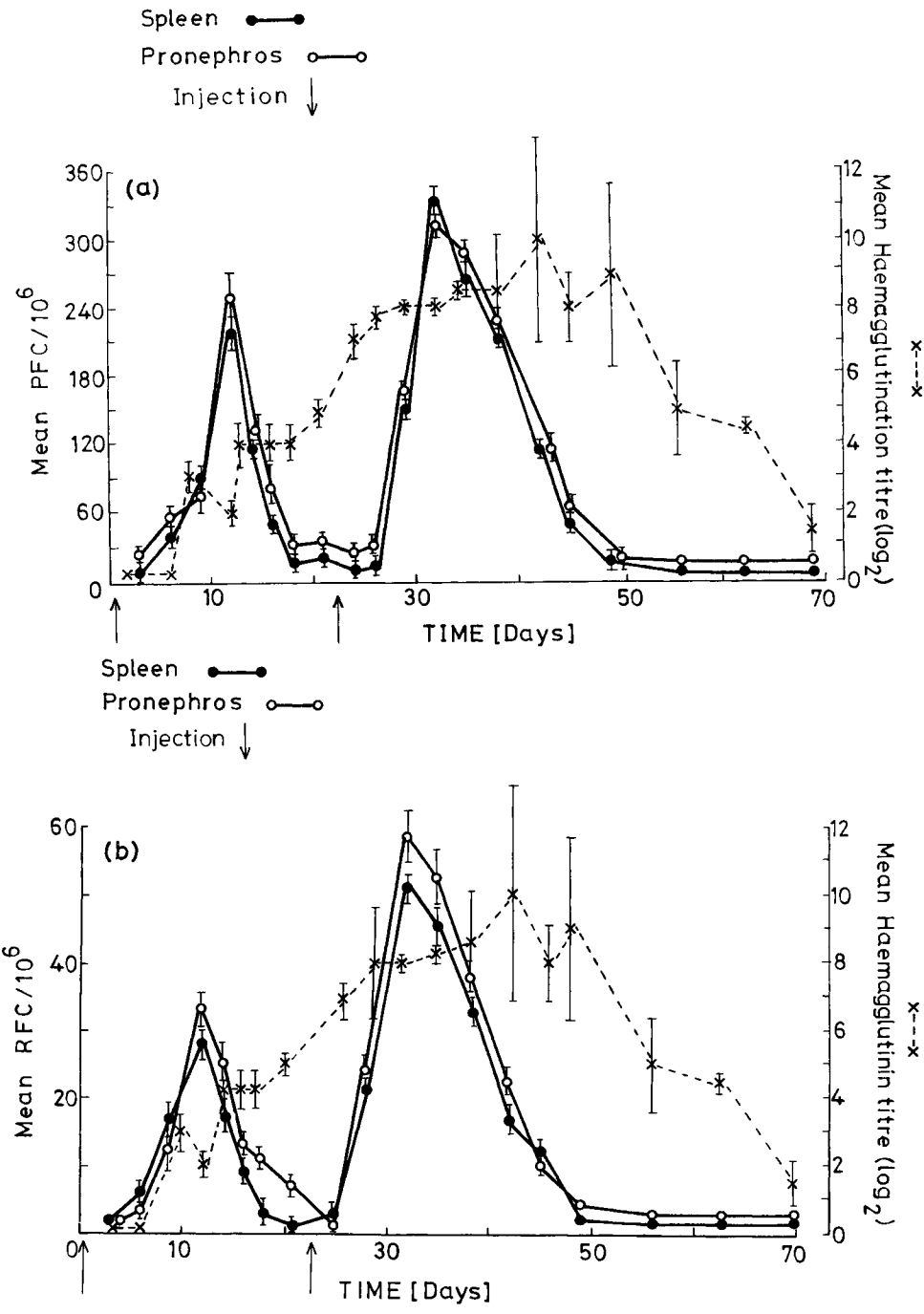

Fig. 1. The primary and secondary kinetics of (a) PFC and (b) RFC responses in the spleen and pronephros of brown trout immunised with SRBC. The mean ± S.E. Hagg titres are also indicated.

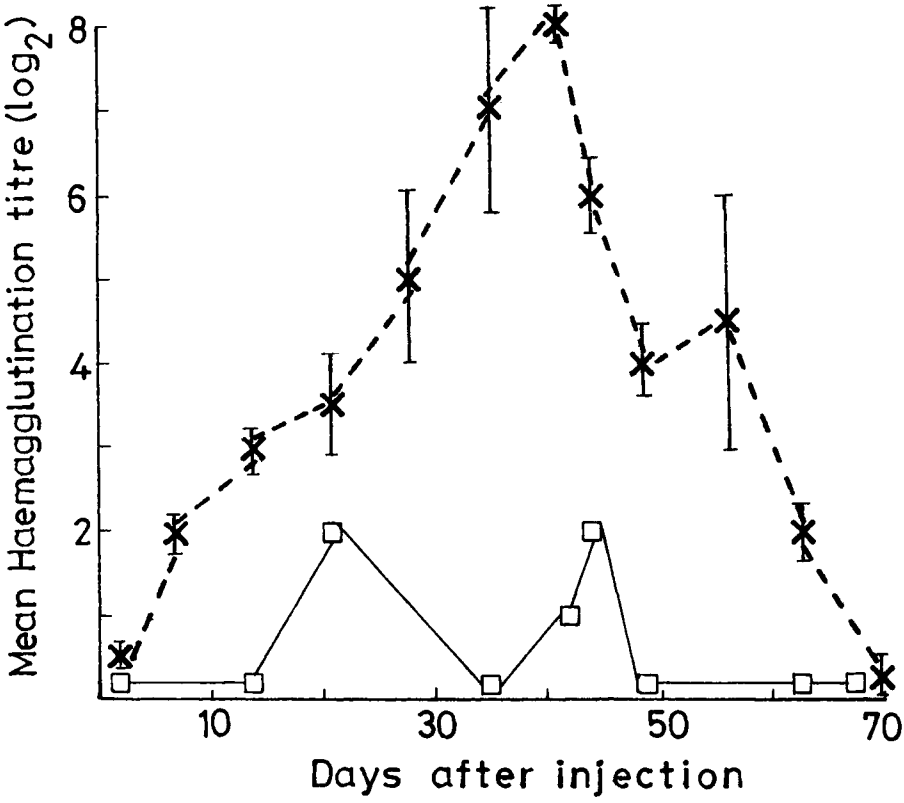

Fig. 2. The primary immune response of brown trout to HRBC (X--X). Each point represents the mean ± S.E. Hagg titre from three fish. PBS-injected control titres are also shown (□——□).

the controls. The pronephros contained more sensitised lymphocytes per 10^6 lymphoid cells than the spleen.

Increases in Hagg titres against SRBC were detected 21 days after the first injection and continued to rise after the second injection to reach a maximum on day 42 and returned to background by day 70. HRBC Hagg were detected 7 days after injection, reached maximum titres on day 42 and declined to background levels by day 70 (Fig. 2).

ABSORPTION AND SPECIFICITY

No agglutination occurred when anti-SRBC sera were titred against HRBC. With anti-HRBC, SRBC gave background titres. These results were not altered when the antisera were absorbed first with the homologous RBC.

None of the glycoproteins inhibited Hagg. Inhibition occurred with 12 of the carbohydrates (D-glucose = D-galactose = amylose = dextrin = cellobiose = starch = 2-deoxy-D-glucose = glycogen = sucrose = maltose = dextran sulphate = chitin).

SERUM FRACTIONATION AND ELECTROPHORESIS

Following treatment of sera with neutral salts, Hagg activity was highest in the 13% sodium sulphate supernatant, 17% precipitate and the 2 M ammonium sulphate precipitate. The fractions were very heterogeneous on IE. After gel filtration, Hagg activity was present in the first peak from Sephadex G200 (Fig. 3) and in the second peak from Sepharose 6B (Fig. 4). On IE these fractions

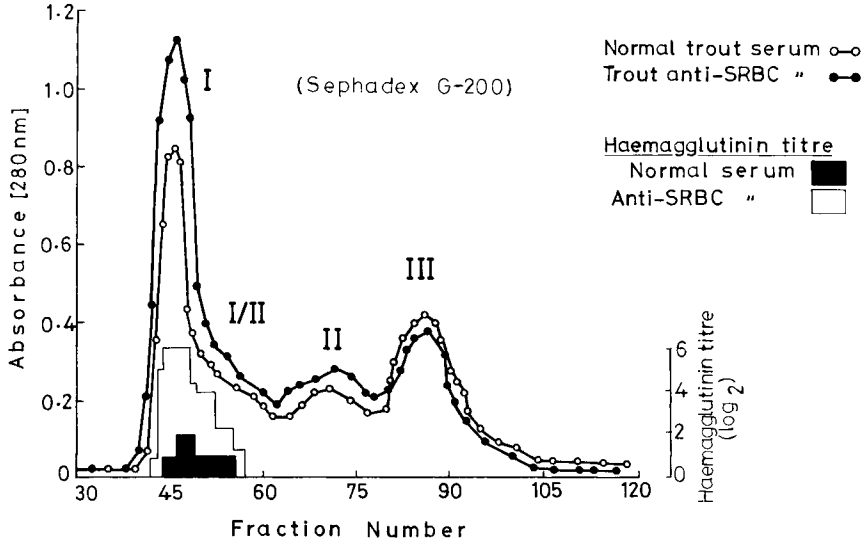

Fig. 3. Elution profiles of Hagg in normal and anti-SRBC trout sera from Sephadex G200. The Roman numerals indicate the fractions into which the eluants were divided.

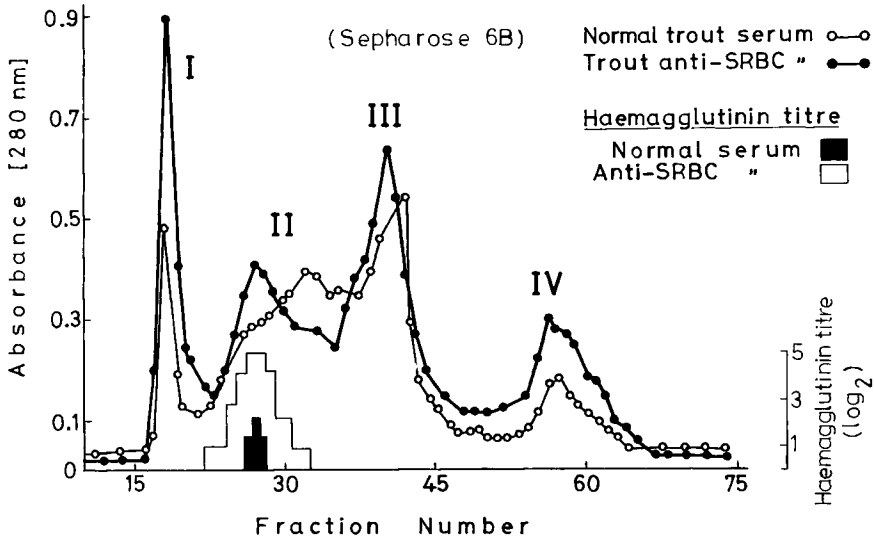

Fig. 4. Elution profiles of Hagg in normal and anti-SRBC trout sera from Sepharose 6B. The Roman numerals indicate the fractions into which the eluants were divided.

contained a single protein with β_2-γ_1 mobility.

On CAE trout sera separated into 10 bands, T1 to T10 in order of decreasing mobility. In comparison to human serum, T1 was pre-albumin, T2 and T3 were similar in migration to albumin, T4 to T7 were α-globulins, T8 and T9 β-globulins and T10 was γ-globulin. In immunised fish, significant increases occurred in T2 and T8 and decreases were found in T1, T7 and T10 ($p < 0.01$, Table II). In DTT- and heat-treated sera, T8 and T10 were reduced and in these sera a β_2-globulin was found to be reduced in IE. When trout anti-SRBC serum was absorbed with SRBC and examined by CAE and IE, similar reductions occurred.

TABLE II. Percentage distribution of protein in the bands separated by cellulose acetate electrophoresis.

Band No.	Control	SRBC-Injected
T1	1.5 ± 0.6* (0 - 7.8)	0.6 ± 0.1 (0 - 2.5)
T2	10.0 ± 0.4 (3.5 - 20.9)	13.8 ± 0.5 (9.1 - 17.6)
T3	11.5 ± 0.6 (0 - 25.8)	12.8 ± 1.5 (0 - 26.2)
T4	11.1 ± 0.6 (0 - 24.5)	12.7 ± 1.1 (0 - 20.8)
T5	12.6 ± 0.9 (0 - 30.2)	9.1 ± 1.4 (0 - 25.9)
T6	7.1 ± 0.8 (0 - 23.2)	7.8 ± 0.8 (0 - 13.0)
T7	1.1 ± 0.3 (0 - 10.6)	0.3 ± 0.2 (0 - 4.6)
T8	20.0 ± 0.6 (9.8 - 33.0)	23.0 ± 1.1 (14.9 - 35.1)
T9	0.9 ± 0.3 (0 - 16.4)	1.0 ± 0.6 (0 - 13.7)
T10	24.2 ± 0.9 (10.5 - 49.1)	18.9 ± 0.7 (13.1 - 26.5)

* Mean ± standard error (range)

PHYSICO-CHEMICAL TREATMENTS

The results are given in Table III.

TABLE III. Final haemagglutination titres (log_2) in normal and immunised trout serum and the gel filtration fractions derived therefrom, after selected physico-chemical treatments. Before treatment the normal serum had a titre of 3; its Sephadex G200 and Sepharose 6B titres were 2 and 2 respectively. For immune serum the initial titres were 8, 6 and 5.

Treatment	Normal Serum			Immune Serum		
	Neat	G200	6B	Neat	G200	6B
$NaIO_4$	1	1	0	5	3	2
Pronase	0	0	0	2	2	1
Papain	1	0	0	3	2	1
Trypsin	1	0	0	4	3	3
EDTA	3	2	1	8	5	5
Ca & Mg ions	3	2	1	7	5	5
DTT	1	1	0	3	3	2
Heat	2	1	1	5	3	3

IV. DISCUSSION

The finding of natural Hagg in brown trout serum against a variety of erythrocytes is consistent with the reports of their presence in other species (Ingram, 1980; Cohen and Vasta, 1982). The injection of trout with SRBC stimulated significant increases in the Hagg titres and induced the production of sensitised lymphocytes. In addition, a second dose of SRBC produced higher Hagg titres and further increased both PFC and RFC counts, the latter reaching maxima quicker and exhibiting a longer response. These findings are similar to those reported for other fish, e.g. carp (Rijkers, 1982) and lamprey (Fujii et al., 1979), although the time taken to reach peak antibody titres appears to be temperature dependent (Lamers and Pilarczyk, 1982; Stolen et al., 1982). In the current study trout were kept at 12°C and this may account for the differences in the times taken to reach maximum antibody titres and sensitised lymphocyte counts reported.

The results of the specificity studies indicate that the induced Hagg was specific for the erythrocyte type and

that no immunological cross-reactivity occurred. However, this does not always appear to be the case since some fish sera possess non-specific heteroagglutinins for the human A, B and O erythrocytes (Baldo and Boettcher, 1970). Brown trout sera did contain heteroagglutinins to A and B, but not against O.

The results of the physico-chemical treatment and electrophoretic analyses of normal and immune anti-SRBC trout sera indicate that the Hagg in both cases were either proteins or glycoproteins, exhibiting β_2 to γ_1 mobility and whose activity was independent of the presence of divalent cations. Furthermore, the gel filtration studies suggest that both types of Hagg are of high molecular weight and elute from columns in the same fractions as human IgM (Ingram, 1978). Similar findings have been reported during the isolation of other teleost immunoglobulins (Ikeda, 1978; Ingram and Alexander, 1979; Fukuda and Kusuda, 1982) and of ratfish IgM-like Hagg (Sanchez et al., 1980). Moreover, the sensitivity to and decrease in Hagg titres following reduction with DTT supports the idea that both the natural and induced Hagg in trout sera are macroglobulins, possibly IgM. Holt and Anstee (1975) described a natural anti-A agglutinin in brown trout serum with properties similar to those reported here. In contrast, natural agglutinins against rabbit RBC in the windowpane flounder, investigated by Kamiya and Shimizu (1980), were heat labile, unaffected by proteases and resistant to reducing agents. Other fish species possess natural agglutinins similar to those of brown trout (Di Conza, 1970; Hazen et al., 1981), but few experiments have been carried out to determine whether the titres could be increased by injection. Nevertheless, the immunoglobulins of hagfish produced after antigenic stimulation were different from the natural anti-SRBC Hagg in hagfish serum (Raison et al., 1978a), whilst in other fish, natural Hagg titres were not increased following immunisation (Ingram, 1980).

Hagg inhibition experiments showed that the brown trout agglutinin was inhibited mainly by polymers of glucose. The strongest inhibition was caused by B-D-glucopyranose, D-galactose and sugars with structures comprising 1->4-linked a- or B-Dglucopyranose residues. Furthermore, slight inhibition occurred with structures composed of al->4- and al->6-linked glucopyranose residues in the same molecule. By comparison, the immuno-dominant sugars recognised by hagfish immunoglobulin and eel natural human O agglutinins, were L-fucose and L-rhamnose respectively

(Springer and Desai, 1970; Raison et al., 1978b). However, none of the carbohydrates used in the present work were shown by Holt and Anstee (1975) to react against the brown trout anti-A agglutinin. It is possible that the natural Hagg present in brown trout sera against SRBC differs in antigenic determinants from the natural anti-erythrocyte antibodies of other fish species in its specific reactivity against carbohydrate receptors on the SRBC surface. Chitin, which is composed of N-acetylglucosamine residues, caused slight inhibition of agglutination of SRBC by trout serum. In mammals, the immuno-dominant sugar recognised by antibodies is N-acetylglucosamine. This suggests that the trout natural Hagg could be an immunoglobulin with a sugar specificity similar to that found in higher vertebrates.

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ABSENCE OF ANAMNESTIC ANTIBODY RESPONSE TO DNP-HAEMOCYANIN AND DNP-FICOLL IN RAINBOW TROUT

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I. INTRODUCTION

The existence of immunological memory in trout is of interest both for fundamental research (study of the phylogeny of the immune response) and also for applied research (seeking for better methods of immunization of fish against pathogens).

Previous investigations into the anamnestic response have produced rather contradictory results depending on the species of fish and the experimental conditions. Ambrosius and Frenzel (1972) and Avtalion (1969) on carp (Cyprinus carpio), and Trump and Hildermann (1970) on goldfish (Carassius auratus), showed a weak memory in cyprinids. Compared to mammals, the difference between the maximum titres of the primary and the secondary response was less important.

In salmonids, Ingram and Alexander (1980) working on brown trout (Salmo trutta L.) showed that when lipopolysaccharide was injected once, the maximum titre was reached after 56 to 63 days, compared to 34 to 40 days after a second or third injection, and that the antibody titres increased significantly after each injection. Only 60% of the fish produced antibodies after the first injection, this number increasing to 91% after 2 or more injections. Control groups were absent but one can suppose that for 31% of fish, the "booster" was in fact a primary injection. O'Neill (1980) with MS_2 bacteriophage, showed that in brown trout, the secondary antibody titres were

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lower than the peak primary titres, contrary to some previous results (O'Neill, 1979).

Anderson et al. (1982), using rainbow trout (Salmo gairdneri Richardson) tested the anti-DNP response, and postulated the existence of a memory (measured by the passive haemolytic plaque assay) when the DNP was grafted onto a thymus-dependent carrier, haemocyanin, and its absence when grafted onto a thymus-independent one, Ficoll (as far as this difference has a significance in fish). However, the number of fish used was not sufficient (3 to 5 fish), to draw any definite conclusions, when we consider the great individual heterogeneity within a trout population (Dunier, 1983).

It would appear, therefore, that no conclusive evidence exist at present for the existence of an anamnestic response in trout. Consequently boosters are often used in vaccination protocols for salmonids without any evidence of a beneficial effect.

One of the classical features of a secondary immune response in mammals, the switch from IgM to IgG occurring during the secondary response, cannot occur in salmonids, since only tetrameric IgM (M.W.670,000) has been detected in trout sera (Ingram and Alexander, 1977; Dorson, 1972, 1981) and in coho salmon sera (Oncorhynchus kisutch Walbaum), (Voss et al., 1980; Cisar and Fryer 1974) as in most teleosts. The exceptions are a few species including the margate, (Haemalon album) a marine teleost fish where (7S) antibodies were found by Clem and McLean (1975), or the giant Grouper (Epinephelus itaira) where 6.4 S immunoglobulins were found by Clem and Small (1970). In both cases, no switch from high molecular weight to low molecular weight IgM was reported, though this has been described in sharks by Suran et al. (1967) and Clem and Small (1967).

In teleosts, if memory cells are responsible for an anamnestic response, this would be characterized by:

- an enhanced secondary response
- an earlier occurrence of antibodies after a secondary injection.

It is well known that fish can reject secondary allografts more quickly (Botham, et al., 1980), thus exhibiting memory for cellular immunity.

In the mouse, a particular category of thymus-independent antigens such as DNP-ficoll, and

DNP-dextran, have been defined, which do not stimulate memory cells (Colle et al., 1983). In this study, two different kinds of antigen, as used by Anderson (1982) were chosen; DNP-haemocyanin (KLH) and DNP-ficoll. The humoral response against DNP, in both cases, was tested by passive haemagglutination. The kinetics of the response were established individually, and a statistical analysis performed to compare groups of 30 fish.

II. MATERIALS AND METHODS

ANIMALS

Yearling rainbow trout (Salmo gairdneri Richardson), average weight 100 g, were purchased from the fish-farm of Neuville St. Gemme (51, France) and brought to our facilities in Thiverval-Grignon. They were held in 140 L plastic tanks supplied with tap water (1 L/min) and recirculated filtered water (10 L/min), varying from 9°C in winter to 16°C in summer.

For the experiments, fish were divided into groups of 30 and were fed with commercial pellets (Trouw, France). They were individually marked by nicking the opercula and severing pelvic fins.

ANTIGENS

Dinitrophenol, coupled to keyhole limpet (Megathura crenulata) haemocyanin (625 DNP groups per molecule) was obtained from Calbiochem-Behring (La Jolla, California). DNP-Ficoll was prepared in the laboratory according to Sharon et al., (1975): 32 units of DNP-lysine were grafted onto Ficoll (average M.W. 400,000), purchased from Pharmacia, Piscataway, New-Jersey.

ADJUVANTS

Freund's Complete and Incomplete adjuvant was obtained from Bio-Merieux laboratories, Charbonnieres Les Bains, France. ST_2 and AE_1 were kindly furnished by IFFA laboratory, Lyon, France.

IMMUNISATION REGIME

In all our protocols 1 mg of antigen was dissolved in 0.2 ml of saline or oily adjuvant (v/v). After a short

anaesthesia using 0.4 ml of phenoxyethanol (Eastman, Kodak) per litre of water, fish were injected intraperioneally (being starved for 24 h previously). The antigen-animal weight ratio was 1 mg per 100g.

SAMPLING PROCEDURE

Every fourteen days, fish were bled (0.2 ml total blood, intracardiac). Sera were obtained after clotting for 24 h at 4°C and stored at -20°C.

ANTIBODY TITRATION

Titres of anti-DNP antibodies were determined individually by passive hemagglutination, according to Rittenberg and Pratt (1969), using sheep red blood cells sensitized with trinitrobenzene-sulfonic acid. Briefly, serial dilutions of the sera were made in Mayer's buffer with a 25 μl microdiluter, in Linbro (Flow laboratories, Mac Lean, Virginia) 96 well plates (conic bottom), and 25 μl of a 1% sensitized sheep red blood cells (SRBC) added to each well. Plates were scored for agglutination after an incubation of at least 2h at room temperature. Aliquots of sera of know titre, stored at -30°C, were used as standards for each titration, and observed titres were corrected according to these standards. Each titration operation involved all the sera from one (or more) bleedings, corresponding to the same experiment. Variation of the standards never exceeded $\pm$ one $\log_2$. Sera of individual fish were checked for the absence of anti SRBC natural agglutinins, and there was consequently no need for absorption with SRBC. Sera were heated (45°C for 30 min) to destroy complement, before titration.

STATISTICAL STUDY

A statistical analysis was undertaken on a WANG mini-computer at the IFFA laboratory, to compare group mean kinetics. For this, arithmetic means, standard errors, and variances were calculated from individual hemagglutinating titres for each blood sample and for each group.

These were used to establish the group mean kinetics and to calculate the area under that curve (integrating all individual areas). An analysis of variance was carried out to compare, by statistical tests, the mean area of one group to another, and to determine if the difference

observed was statistically significant or not.

III. RESULTS

STUDY OF THE SECONDARY RESPONSE TO DNP-KLH INJECTED WITHOUT ADJUVANT.

Figs. 1 and 2, depict the results from four different experiments. 106 to 123 days elapsed between the primary and secondary injection of DNP-KLH in saline, so that anti DNP titres had returned to basal level, before the booster. Each point corresponds to the mean of 30 fish injected at the beginning of the experiment, with at least 25 remaining at the end, after mortalities due to handling or aggressions. On the first curve (Fig. 1a), the primary control, injected conjointly with the booster, allows us to compare the secondary response with the primary one, developing at the same time, and on fish of the same age,

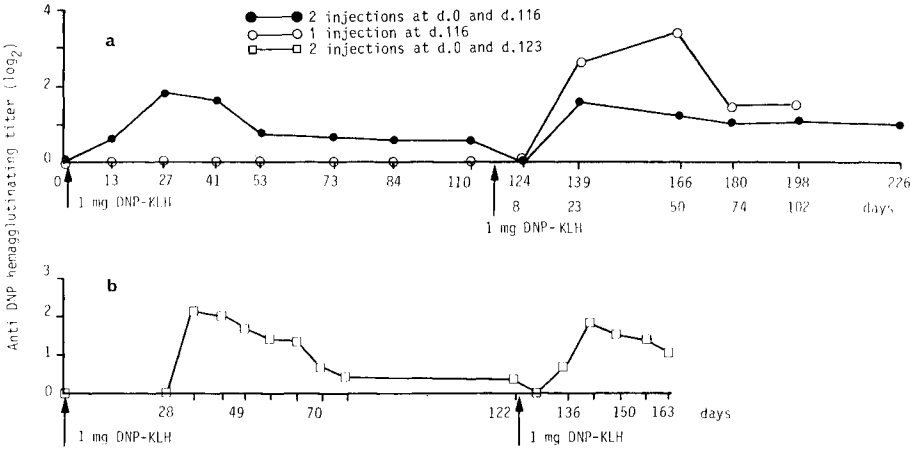

Fig. 1. Comparative study of anti-DNP primary and secondary response without adjuvant
Rainbow trout (30 per group) were injected with 1mg of DNP-KLH per 100g of fish and anti-DNP antibodies tested by passive hemagglutination.
Each point represents the mean of individual titres
a) upper kinetic: d0 temperature : 14°C
d116 temperature : 16°C

b) lower kinetic: d0 temperature : 12°C
d124 temperature : 14°C

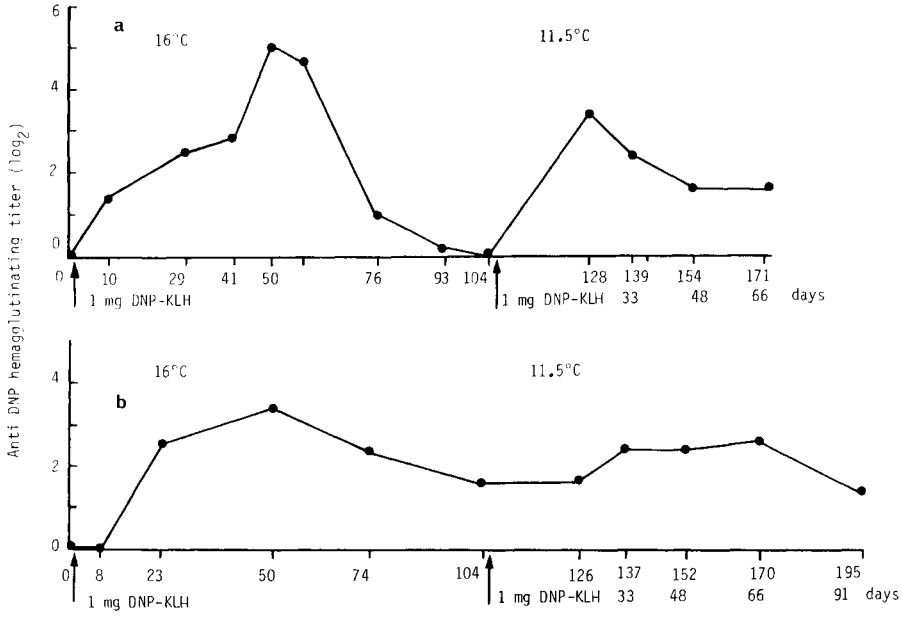

Fig. 2. Comparative study of anti-DNP primary and secondary response without adjuvant.
Same conditions as Fig. 1.
Temperatures at the time of injection are shown on the curves.

size and history and we can see that the antibody titre in the secondary response rises later and peaks lower than the primary one. In Fig. 1b, although the temperature was higher for the secondary response, it developed more slowly than the primary one. In Fig. 2a the secondary response, this time at a lower temperature, was again reduced and did not develop any faster. In the last case (Fig. 2b) the booster was given when the antibody titre was still significant, and here again the secondary peak was lower than the primary one. In the four experiments, the secondary response was similar to or lower than the primary one. No typical anamnestic response was observed.

STUDY OF THE SECONDARY RESPONSE TO A "THYMUS-DEPENDENT" ANTIGEN, DNP-KLH, COMPARED TO A "THYMUS-INDEPENDENT" ANTIGEN, DNP-FICOLL, INJECTED IN FREUND'S COMPLETE ADJUVANT (FCA).

As shown in Fig. 3, experimental groups received 2 injections on day 0 and 246, while controls received only one injection on day 0 or 246. As represented by the mean curves of groups and concluded by statistical analysis, the secondary response was identical to a primary one obtained at day 246. No comparisons could be drawn, in this case, between the initial kinetics of the secondary and the primary response, due to the high level of antibody which was still remaining after primary injection. But it is noteworthy that, for both antigens, as soon as the primary response kinetic, initiated at day 246, rose above the plateau of the first primary one, (initiated at day 0) it was absolutely superimposed on the secondary response kinetic.

These results, obtained with thymus-dependent and

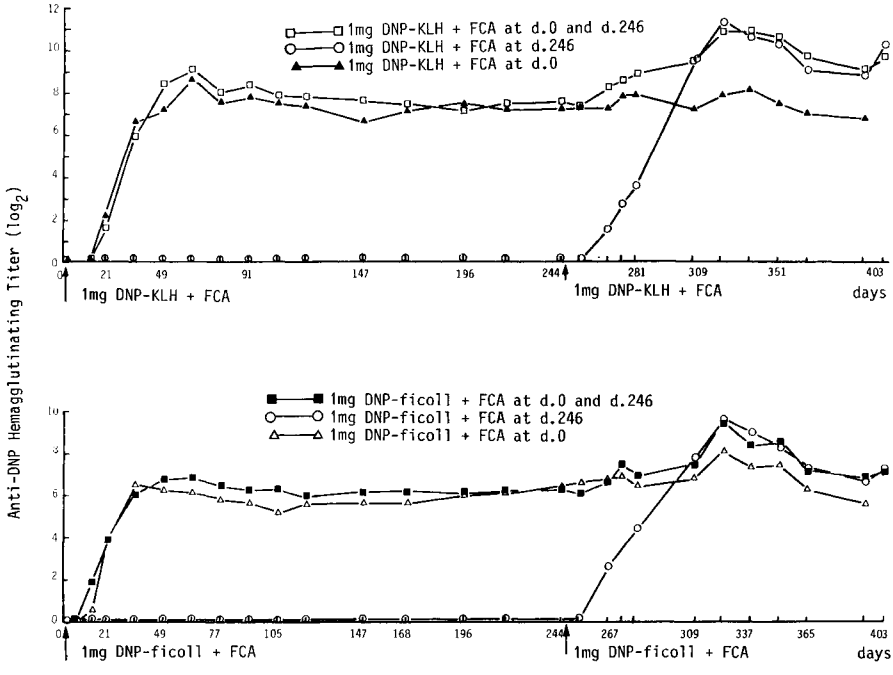

Fig. 3. Primary and secondary response against DNP-KLH or DNP-Ficoll with adjuvant,
Antigens were injected in Freund's Complete Adjuvant (FCA).
First injection for both curves : temperature 16°C.
Second injection for both curves : temperature 10.5°C.

thymus-independent models, confirm the previous results that the criteria for an anamnestic response are not fulfilled.

In this experiment, the high level of anti-DNP antibodies persisted for more than 700 days (data not shown), with adjuvant, compared to less than three months, without adjuvant.

Immunoglobulins found in sera, taken at a late stage in the experiment were exclusively of a high molecular weight, as shown by sucrose density gradient ultracentrifugation (results not detailed here), according to the technique used by Dorson (1972).

STUDY OF THE SECONDARY RESPONSE TO DNP-KLH WITH DIFFERENT ADJUVANTS.

This experiment was undertaken to confirm the previous results, only this time using different adjuvants (AE1 and ST2 oily adjuvant, furnished by the IFFA Laboratory).

The two experimental groups were compared to the control ones which received either 2 injections in saline or one injection on day 116. As has been stressed previously (Fig. 1a), the primary response following the 116th day injection rose above the secondary one. Bearing this in mind, (Fig. 4) the slightly enhanced secondary response observed with AE1 (there was no 116th day primary control with adjuvants in this experiment), has no significance. The statistical analysis confirmed that, in this experiment, as in all previous ones, there was no anamnestic response to DNP.

EFFECT OF REPEATED INJECTIONS OF DNP-KLH IN FREUND'S INCOMPLETE ADJUVANT.

As shown in Fig. 5, and statistically verified, no significant difference occurred between the experimental group, receiving three injections on days 0, 3, and 7, and the control group, receiving only the first injection, on day 0.

IV. DISCUSSION

This work was undertaken to verify the existence of an anamnestic immune response for antibody production in rainbow trout, under conditions allowing reliable results; these included a sufficient number of fish to overcome the great individual heterogeneity observed among trout, and primary response controls which allowed us to balance

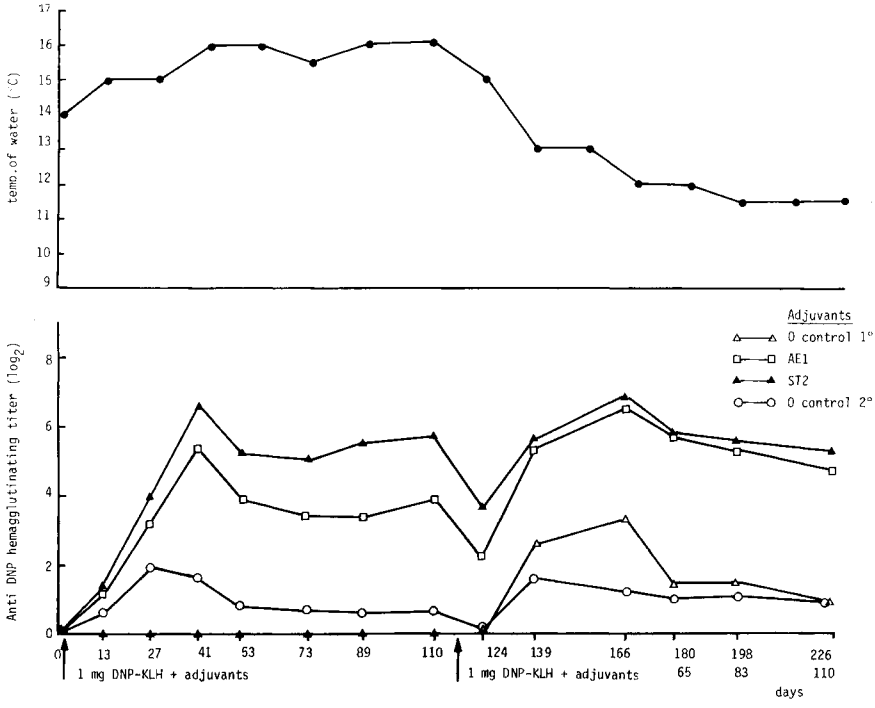

Fig. 4. Secondary response against DNP-KLH with adjuvants. 2 injections at d0 and d116 except "control 1°" group which received only one injection at d116.
First injection : temperature 14°C
Second injection : temperature 16°C
The curve of the water's temperature throughout the experiment is represented.

variations in the immune response due to increasing age and size of fish, or due to seasonal variations (Yamaguchi, et al., 1981). The main feature which was taken into account was the intensity of the response, which in mammals can be enhanced up to 100 times following a secondary injection of a T-dependent antigen. It is clear from our results that, with or without adjuvant, there was no significant difference between primary and secondary kinetics. This is in agreement with the few authors who have reported the absence of an anamnestic response. Devaux and Charlemagne (1981), using goldfish Carassius auratus, pointed out that in cases where an enhanced secondary response has been claimed, the

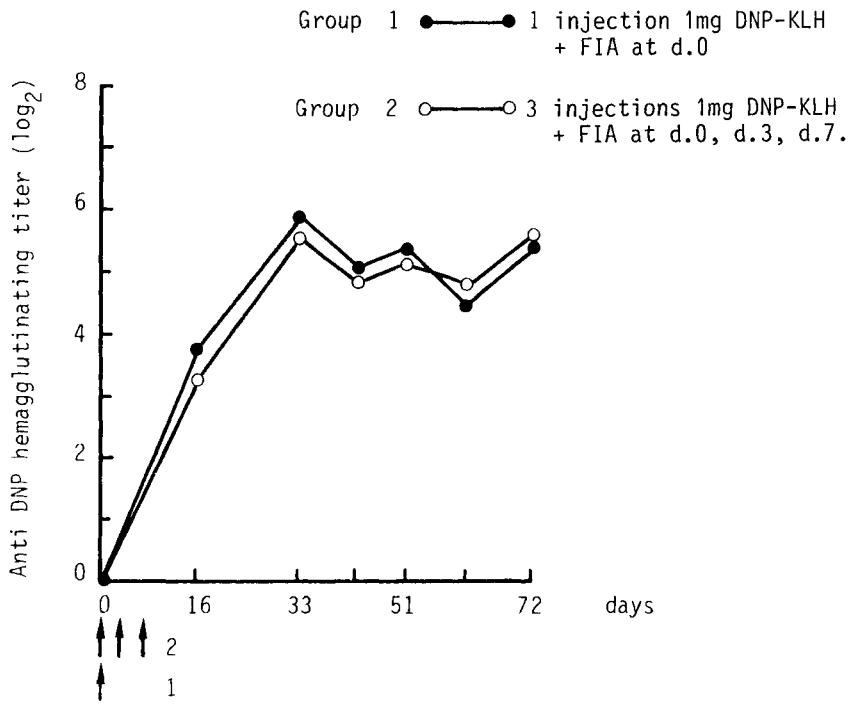

Fig. 5. Primary immune response against DNP after one or three injections of DNP-KLH with Freund's Incomplete Adjuvant (FIA).
Group 1 : 1 injection at d0
Group 2 : 3 injections at d0, d3, and d7.

enhancement rarely reached 10 times; moreover, the number of fish was low, and no primary response controls were used (Avtalion, 1969; Trump and Hildemann, 1970).

In this study, no obvious differences were seen when two different carriers (haemocyanin and Ficoll) were used in the presence of adjuvant. This result is contrary to the results of Etlinger et al., (1979) who observed that DNP-LPS and DNP Ficoll (T-independent antigens in mice) were good immunogens, whereas DNP-KLH, DNP-HGG and DNP-BSA were not. This discrepancy can be explained by differences in the test used (mainly anti-DNP plaque forming cells), the lower doses used by these authors, and perhaps the lower number of DNP groups per KLH molecule. Our results are also in contradiction with the results of Anderson et al., (1982) who claimed that a heightened secondary response (investigated mainly at the level of splenic

antibody producing cells) occurred with DNP-KLH and not with DNP-Ficoll. In this case, the heterogeneity was great among the 5 (or less) fish of each group, and no individual kinetics could be followed since the fish were sacrificed.

In the present study, three injections within one week gave the same results as a single one; no "amplified response" was obtained, in contrast to the results of Devaux and Charlemagne (1981). This could be explained by an inadequate immunization schedule (supraoptimal) doses, insufficient interval between injections or the lack of memory cells in trout. Resolving this question requires repeated experiments with varying doses and immunization calendars. Our results cannot be interpreted as the lack of any immune memory in trout, since accelerated second-set graft rejection seems well established in teleosts (Botham et al., 1980) as well as the hapten-carrier effect (Stolen and Makela 1976; Yocum et al., 1975; Ruben et al., 1977) and delayed hypersensitivity (Bartos and Sommer, 1981). But the lack of any secondary enhancement in our experiments proves at least that immunological memory is not as obvious in trout (and possibly teleosts) in general as it is in mammals, and that the generation of memory cells is more restricted. This could be related to the fact, stressed by Du Pasquier (1982), that lower vertebrates possess fewer possibilities for diversification because of the lower proliferation rate of their lymphocytes. It will be of interest to verify this fact using other models, and other antigens, especially pathogenic viruses and bacteria.

Thanks are due to Jacqueline Bonnal for expert technical help. The statistical study was performed on the WANG mini computer of IFFA laboratory Rhone-Merieux, Lyon, France, which also kindly provided AE1 and ST2 adjuvants. Thanks are due to the Neuville Ste Gemme Fish farm, for providing a year round supply of healthy fish. I am most grateful to Michel Dorson, Director of the laboratory, for pertinent advice in the planning of the experiments and for reading the manuscript.

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ORAL IMMUNISATION AGAINST FURUNCULOSIS: AN EVALUATION OF TWO FIELD TRIALS

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I. INTRODUCTION

Published accounts of furunculosis vaccination show the first reported work was that of Duff (1942), who found prolonged feeding with chloroform-killed Aeromonas salmonicida whole cells gave protection to cutthroat trout (Salmo clarkii), when challenged artificially. In 1949, Snieszko and Friddle reported that the use of heat-killed whole cells did not seem to increase immunity sufficiently to protect brook trout (Salvelinus fontinalis) against an injected challenge, although a natural challenge gave some indication that oral aplication was beneficial.

At about this time it was shown that sulphonamide drugs had good potential for combatting furunculosis (Snieszko and Friddle, 1949) so that during the 1950's their successful application resulted in a decline in efforts at vaccine development. However, towards the end of the decade the emergence of drug-resistant strains of A. salmonicida led to a renewal of interest in oral immunization. Thus, in 1964, Krantz et al. reported that intraperitoneal injection (I.P.) of formalin-killed whole cells in a mineral oil adjuvant gave protective agglutinating antibodies, but a feeding technique was unsatisfactory and was not as effective as injection (1964b). Meanwhile, Spence and Fryer (1965) found that serum from vaccinated rainbow trout (Salmo gairdneri) could passively protect coho salmon (Oncorhynchus kisutch), but oral vaccination of formalin-killed whole cells failed to induce protection in the laboratory or the field.

Klontz et al. (1966) showed that a soluble extract of

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cells gave similar pathological conditions to those of an active infection, and an alum precipitated antigen from the water soluble portion of ultrasonically disintegrated cells was non-toxic to fish and antigenically similar to the toxic extract of intact cells. It also protected brook trout (Salvelinus fontinalis) and coho salmon (O. kisutch) (Klontz, 1967), although this could not be verified by subsequent larger scale trials using a commercially prepared antigen (Klontz, 1969).

Paterson and Fryer (1974) showed that a single parenteral vaccination with formalin-killed A. salmonicida whole cells in Freund's complete adjuvant gave protective immunity against furunculosis, but Hara et al. (1976), using field trials, achieved only slight protection with a soluble antigen administered in the food. However, Antipa and Amend (1977) found that formalin-killed I.P. and hyperosmotic (H.I.) applications did induce production of serum antibodies.

In 1978, Udey and Fryer showed that virulent A. salmonicida strains appeared to have an additional extracellular layer, which was absent from avirulent strains. They also found that three types of orally administered bacterins were not effective in conferring protection in coho salmon (O. kisutch) when naturally challenged. However, parenteral immunization with a whole cell bacterin in Freund's complete adjuvant conferred a high degree of protection against the natural challenge.

More recently, Palmer and Smith (1980) obtained significant protection in Atlantic salmon (S. salar) with H.I. vaccination of formalin-killed whole cells, but not with an I.P. and adjuvant application, although natural challenge was low and it was not easy to determine the response to a heavy infection. At about this time, Smith et al. (1980) found oral vaccination using formalised whole cells gave a greater degree of protection in brown trout (S. trutta) than disrupted cells given by H.I. More importantly, Austin and Rodgers (1981) reported an oral formalised whole cell vaccine gave good protection to brown trout (S. trutta), whereas a formalised toxoid vaccine was toxic to fish, although this was at first only apparent in field trials. Cipriano (1982) showed that a growth product fraction, possibly a glycoprotein, could protect brook trout (Salvelinus fontinalis) by I.P. injection, as could vaccination by immersion in an attenuated strain of A. salmonicida (Cipriano and Starliper, 1982).

The most recent work by McCarthy et al. (1983) indicated that passive immunization was possible using

rabbit serum raised against a virulent strain. Parenteral protection by I.P. injection was not clearly demonstrated, however, since field trials were complicated by escapees, uncontrollable losses and the problems of other diseases affecting the overall mortality pattern. However, it was suggested that protection is associated with virulent strains possessing the extra-cellular layer.

Thus, the work of the past 42 years has failed to produce a successful furunculosis vaccine for field use. With this in mind, and with the current awareness of the role of extracellular products in the pathology and virulence of furunculosis (Fuller et al., 1977; Udey and Fryer, 1978; Trust et al., 1980; Ellis et al., 1981), and the suggestion by other workers (Smith et al., 1980) that there may be a need for more than one strain to be used in whole cell vaccines, further work has been carried out with the aim of producing more effective vaccines, and to evaluate them under field conditions.

II. MATERIALS AND METHODS

The bacterial strains used in these trials were obtained from rainbow trout (S. gairdneri) or brown trout (S. trutta) over the period 1977-1982, using a site at which furunculosis epizootics were a regular summer occurrence. For vaccine preparation, the isolates were grown on Brain Heart Infusion agar (B.H.I. Oxoid) in order to help retain certain morphological properties, namely the presence of rough, virulent colonies, deemed important in vaccine work.

Large square plates (bioassay dishes, Nunc) of B.H.I. were overlayered with sheets of unplasticised cellulose film, and inoculated with the bacterial culture. Following incubation at 22°C for 72 h, cells were harvested using the procedure outlined by Austin and Rodgers (1981). The cells were inactivated with formalin, and incubated at room temperature for 72 h. The supernatant obtained prior to treatment with formalin was dialysed overnight against tap water, then concentrated with polyethylene glycol 6000 (BDH) to 10% of the initial volume. This toxin was treated in one of two ways: either with 5% (v/v) formalin for 14 days at 37°C and 10% (v/v) chloroform for 14 days at room temperature; or with the addition of 0.1M lysine, as a third stage inactivation, for 14 days at 22°C.

The extracellular or 'A' layer was extracted from whole cells by the phenol method of Sutherland and Wilkinson (1971).

The components were shown to be antigenic by an agar

diffusion technique using rabbit whole cell anti-*A. salmonicida* antiserum (titre = 1:16384) and an antiserum prepared against a formalin/chloroform toxoid. The toxoid used in the first trial (Trial A) was found to have more antigens in common with the toxoid antiserum than with the whole cell antiserum, whereas a lipopolysaccharide (LPS) fraction had no antigens in common with the toxoid antiserum. All the components of the second trial (Trial B) were antigenic to both antisera, the only differences being that the 'A' layer fraction contained an extra antigen to the whole cell serum. Once the antigenicity had been verified, the vaccines were formulated.

The formulation for Trial A contained 5 strains of formalin-killed *A. salmonicida* whole cells at a final concentration of 10^6 cells/ml. The second was a toxoid previously treated with formalin and chloroform and used as a vaccine at a concentration of 8 μg/ml, and the third was a combination or "dual" vaccine containing 10^6 cells/ml and 4 μg/ml toxoid.

The results of Trial A indicated that possible improvements were needed, insofar as an *A. hydrophila* septicaemic condition was diagnosed in addition to furunculosis, therefore formalised *A. hydrophila* whole cells and an improved toxoid treated with lysine were included in some formulations for Trial B; all groups (Trial B) contained *A. salmonicida* whole cells (10^8 cells/ml) and in addition:

(a) *A. hydrophila* whole cells (10^4 cells/ml); toxoid (6 μg/ml) - designated S.H.T.
(b) *A. hydrophila* whole cells (10^4 cells/ml); 'A' layer (2.7 μg/ml) - designated S.H.A.
(c) 'A' layer (2.7 μg/ml) - designated S.A.
(d) 'A' layer (2.7 μg/ml); toxoid (6 μg/ml) - designated S.A.T.

Both trials included control groups. In Trial A, each group contained 3,000 fry, with the oral vaccine being fed at a rate of 16 g food/day for 20 days, starting 45 days after hatching. With Trial B, there were 1,500 fish in each group, with vaccination again starting at 45 days post-hatch but the fish were fed 17 g food/day for 30 days.

It should be emphasised that all vaccines were tested before use in the field. Batches were tested by inoculating appropriate media or broth, and the absence of growth after incubation was taken as an indication of sterility. Their safety in rainbow trout was also determined by checking palatability of the oral

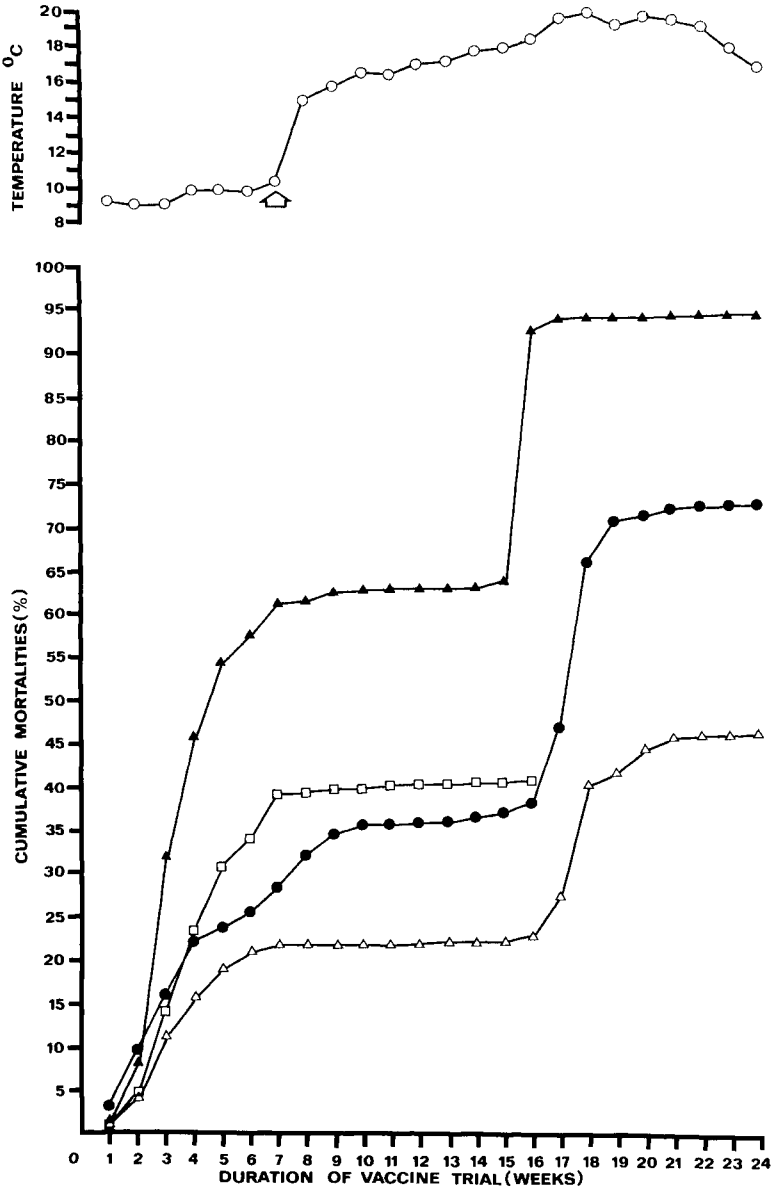


Fig. 1. Field trial A of Aeromonas salmonicida vaccines. △——△ Whole cell vaccine; ▲——▲ toxoid vaccine; □——□ dual vaccine; ●——● control. ⌂ Fish moved to outside tanks. Vaccine administration ceased at week 4.

preparations, and monitoring distress or apparent toxicity for 28 days. Any unused vaccine was returned to the laboratory for disposal by incineration.

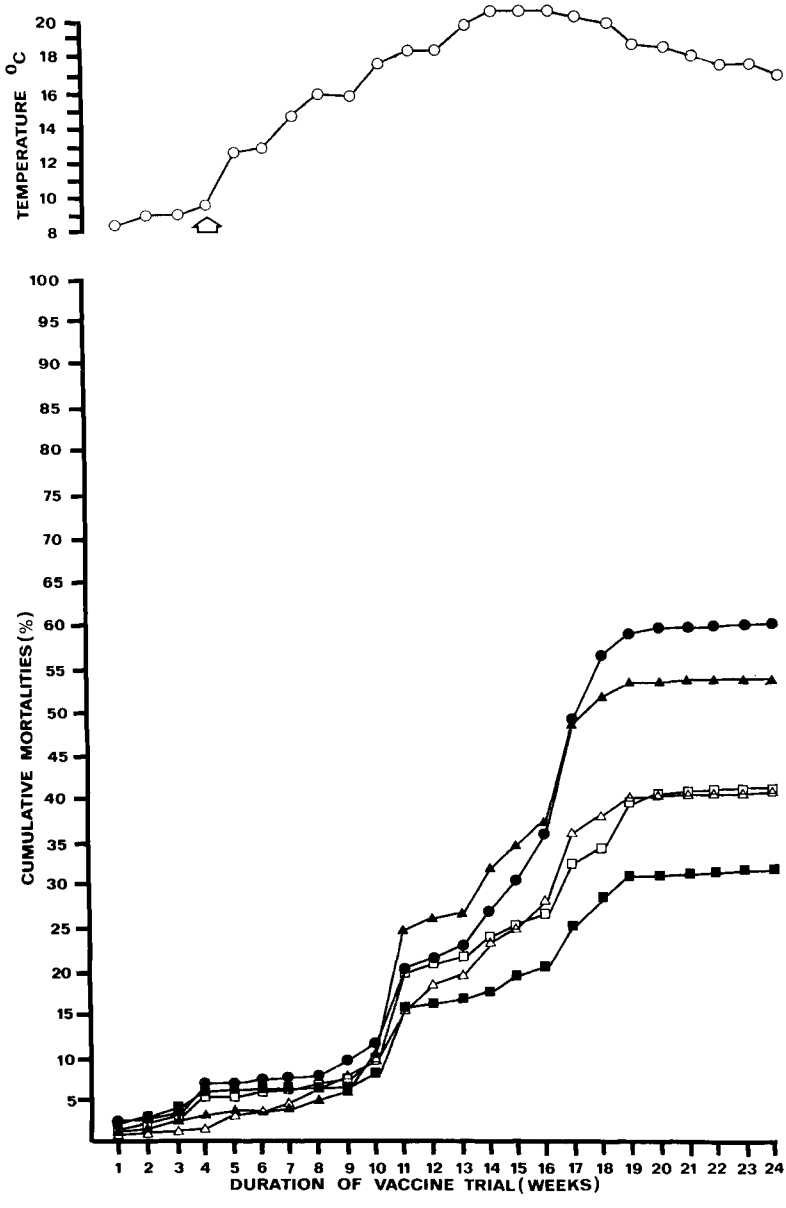
III. RESULTS AND DISCUSSION

In Trial A, the first signs of furunculosis appeared during week 16 of the trial in August when the water temperature reached 18°C (Fig. 1). The presence of A. salmonicida was confirmed by bacteriological examination. At the conclusion of the trial (after 24 weeks) the group vaccinated with polyvalent whole cells had experienced cumulative mortalities of 47%, whereas the controls had losses of 73%. The toxoid vaccine group fared less well, ending with total losses of 94%. Unfortunately, the group receiving the dual formulation developed a marked Ichthyophthirius ("white spot") infection and were so debilitated that many fish succumbed to this and/or the attempted treatment with formalin and malachite green. Consequently, this group was terminated early, at week 17. Although A. salmonicida was not isolated from this group, 60% of the moribund fish had a bacterial septicaemia caused by A. hydrophila.

There was no apparent tissue damage, due to residual toxicity, in either of the toxoid-containing vaccines. In fact, the toxoid was judged to be non-toxic for brown trout gonad (BTG) cells in tissue culture, whereas in contrast, the toxin was highly toxic. However, it was found that toxoid, which had been left at room temperature for 4 months, did possess some toxicity for BTG cells, indicating partial reversal to a toxic form. This may have some bearing on the results obtained with the toxoid vaccine group. The detrimental effect was possibly caused by reversion to the toxic form within the fish. Therefore, in Trial B, the method of toxoiding was modified.

In the second trial (Trial B), the first signs of furunculosis were seen in August, during weeks 10 and 11, as the water temperature reached 18°C (Fig. 2). At the end of the trial (week 24), all vaccinated groups fared better than the controls. The best result was obtained with the S.A.T. formulation which experienced a 31% cumulative mortality as compared to the 60% in the control group. Losses of 53%, 41% and 40% were obtained with S.H.A., S.A. and S.H.T. formulations, respectively. With this trial, there did not appear to be any benefit from incorporating A. hydrophila antigens into the vaccines.

In conclusion, it would seem that whole cell vaccines coupled with toxoided sub-cellular components gave some


TEMPERATURE °C
20
18
16
14
12
10
8
CUMULATIVE MORTALITIES (%)
100
95
90
85
80
75
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65
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55
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45
40
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
DURATION OF VACCINE TRIAL (WEEKS)

protection in rainbow trout against furunculosis. Moreover, there appears to be no significant difference between results obtained with monovalent and polyvalent whole cell vaccines. Further work is necessary to determine the best method of application.

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Fig. 2. Field trial B of Aeromonas salmonicida vaccines. ■——■ A. salmonicida whole cells with 'A' layer and toxoid (SAT); ▵—▵ A. salmonicida whole cells with A. hydrophila whole cells and toxoid (SHT); ▲——▲ A. salmonicida whole cells with 'A' layer (SA); A. salmonicida whole cells with A. hydrophila whole cells and 'A' layer (SHA); ●——● control.
⌂ Fish moved to outside tanks. Vaccine administration ceased at week 4.

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AN INVESTIGATION OF THE SKIN OF RAINBOW TROUT, SALMO GAIRDNERI RICHARDSON, FOR ANTIGEN UPTAKE MECHANISMS FOLLOWING SPRAY VACCINATION

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I. INTRODUCTION

One of the more recent developments in the quest for a simple and efficient means of mass immunizing fish in the aquaculture industry has been spray (shower) vaccination (Gould et al., 1978). Using this delivery method large numbers of fish of all sizes can be vaccinated by the simple administration of bacterin sprayed over the body surface (Itami and Kusuda, 1978; Rosenkvist-Jensen, 1982). Spray vaccination has additional advantages in that it is neither labour intensive nor does it require any particular skill from the operator. More importantly, fish receive only the minimum of handling and are therefore less susceptible to stress (Pickering et al., 1982). Direct immersion or bath immunization (Egidius and Andersen, 1979), is another method that requires less fish handling and indeed, manufacturers of current vaccines now recommend that both bath and spray techniques be used with their products (Wildlife Vaccines Inc., U.S.A.). There are, however, questions to be answered as to how the immunogenic agent in the vaccine is presented to the fish following spray vaccination (Amend and Johnson, 1981). The skin is the first tissue to come into contact with a sprayed vaccine and therefore deserves to be considered as a possible route for antigen uptake. Cellular activity has been seen in the skin of rainbow trout fry following bath vaccination (Smith, 1982), and a report exists of a Langerhans-like cell in the skin of a teleost (Mittal et al., 1980) which suggests that active uptake of antigen

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may occur at the skin surface. The aim of this study was to clarify whether or not vaccine uptake can occur at the skin surface following spray vaccination.

II. MATERIALS AND METHODS

FISH

Rainbow trout Salmo gairdneri Richardson 1836, weighing 100-200g were purchased from a disease free stock at Walkmill trout farm, New Deer, Scotland. The animals were placed in an outdoor stock tank supplied by a copper-free mains water system at ambient temperature. Experimental fish were transferred to a closed recirculating water aquarium system in an 11°C constant temperature room 72h prior to use. Fish were fed three times daily on Ewos-Baker No. 5 food pellets (Westfield, Bathgate U.K.).

ANTIGENS

A soluble antigen human gamma globulin (HGG) and a particulate antigen Vibrio anguillarum were used in this study. Lyophilized HGG, cohn fraction II (Koch-Light Laboratories, U.K.), was reconstituted in phosphate buffered saline (PBS) pH 7.2 and allowed to dissolve without agitation for 30 min immediately prior to use. V. anguillarum, serotype I, was supplied from Unilever Research, Aberdeen. A live culture grown in tryptone soya broth (Oxoid, U.K.) was killed with 1% formalin and stored at 4°C. The stock bacterin solution contained 4.68×10^8 cells per ml.

APPARATUS

A system was designed for directly spraying antigen onto the surface of the skin, whilst the head was isolated from the spray with the gills perfused with oxygenated water. The apparatus 'Salmospray' (Fig. 1) consisted of two boxes, one for the head (Box A) and a spray chamber (Box B) for the region posterior to the pectoral girdle. A rubber membrane set in the partition between the two boxes (D) provided a water-tight seal around the girth of the fish. The head was kept fully submerged in water at 11°C within a flooded compartment (C) and anaesthesia with benzocaine (Laird and Oswald, 1975) was regulated between 17 and 25 ppm, so as to avoid voluntary movements without inhibition of spontaneous ventilation. The posterior

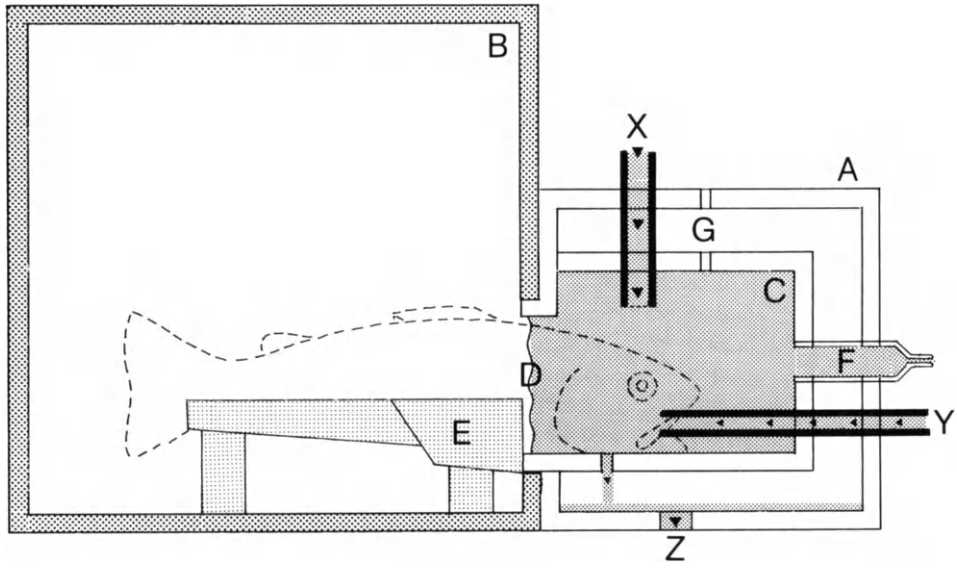

Fig. 1. Detail of the apparatus-SALMOSPRAY.
Key: A-Anaesthetic unit, B-Experimental enclosure, C-Flooded chamber, D-Rubber membrane, E-Cradle, F-Anaesthetic injection site, G-Overflow.
Water enters at X and Y and exits at Z.

region of the fish was supported in air (E) and the enclosure prevented diffusion of antigen containing aerosols into the surrounding laboratory. Antigen was sprayed onto precise areas of skin using a Badger 350-1-M artists' airbrush (Badger Air-Brush Co., U.S.A.). Dosage and area of application could therefore be precisely reproduced on a series of fish and they could be left in the air after spraying for predetermined lengths of time in order to investigate the time course of antigen uptake.

EXPERIMENTS

Fish were subjected to a variety of spray treatments and antigen doses within the apparatus. Passive uptake of sprayed antigen was investigated using an ELISA assay to detect the presence of HGG in the blood of animals sprayed with the antigen. Active mechanisms of antigen uptake were studied at the cellular level using both soluble (HGG) and particulate antigen (V. anguillarum) in conjunction with immuno-fluorescent techniques.

(A) PASSIVE UPTAKE

Fish were sprayed with HGG in 2 ml of carrier (PBS pH 7.2) in doses of 5mg, 10mg, 50mg and 100mg per fish. The spray was applied with the airbrush, usually within the first 30 min of the fish being introduced to the apparatus, but the exact time of application was dependent on the success of the anaesthesia with each individual animal. The areas of skin receiving the spray were:

(i) The whole body surface posterior to the pectoral girdle (WBS).
(ii) The lateral line region in a spray band 10mm wide, centered on the lateral line (LN).
(iii) The whole body surface posterior to the pectoral girdle, not including the lateral line (WBS-LN).

(B) ACTIVE UPTAKE

Three fish were sprayed with 1ml of stock formalized V. anguillarum bacterin on the body surface posterior to the pectoral girdle to include the adipose fin. Three fish were similarly sprayed with HGG using 10mg of antigen in 2ml of carrier (PBS pH 7.2).

ELISA ASSAY

The presence of HGG in the blood of sprayed animals was assayed using the technique described by Lems-Van Kan (1983). A 200 μl blood sample was taken from each fish after exposure to HGG sprays. Serum was collected by centrifugation and stored at -20°C until assayed. Microtitre plates (Nunc, Denmark) were coated, 100 μl per well, with 50ng rabbit anti human IgG/Fc (Nordic, Netherlands) in 0.1M bicarbonate buffer pH 9.6. Before use plates were washed x3 in PBS pH 7.2 containing 0.05% Tween 20 (Sigma, U.S.A.). The outer perimeter of the plates were not used. Sera from HGG sprayed animals and 1.0μg, 2.5μg, 5.0μg, 7.5μg and 10.0μg HGG standards were incubated in the plates for 2h at 37°C, the plates washed x3 in PBS/Tween 20 and 1:1000 diluted goat anti human IgG peroxidase conjugate (Sigma, U.S.A.) incubated for 2 h. The enzyme reaction was performed at room temperature using o-phenylenediamine (Aldrich Chemical Co., U.K.), 40 mg dissolved in phosphate-citrate buffer pH 5.0. Plates were read at 492nm on a Titertek multiscan plate reader (Flow Laboratories, U.K.), 30 min after addition of substrate.

IMMUNOFLUORESCENCE

Adipose fin sprayed with HGG and V. anguillarum was studied using both direct and indirect immunofluorescent techniques, using serial sections cut at 5 μm on a cryostat. The adipose fin was chosen for histology because it was free from scales and conveniently excised without damaging the epidermis. Sections were also taken from the spleen, pronephros and the thymus of HGG sprayed animals.

Sections from HGG sprayed animals were incubated at room temperature in 1:40 diluted sheep anti human IgG fluorescein conjugate (Wellcome, U.K.) in PBS pH 7.2. Adipose tissue from V. anguillarum sprayed animals was first incubated in rabbit anti V. anguillarum antisera (National Fish Health Research Laboratory, Kearneysville, U.S.A.) and then in a second step incubation with sheep anti rabbit IgG fluorescein conjugate (Wellcome, U.K.). All steps involved x3 5 min washes in PBS/Tween 20 pH 7.2, and incubations were for 30min periods. Sections were mounted in glycine-saline buffer pH 8.6 and viewed under U.V. light.

III. RESULTS

(A) PASSIVE UPTAKE

Initially antigen doses of 5mg HGG were applied to the skin surface using WBS (Table 1) and exposure times of between 2-5h were achieved. Antigen doses were then raised to 10.0mg, 50.0mg and 100.0mg with contact exposures all in excess of 2h. Despite a contact exposure of more than 7h using 100.0mg HGG, there was no evidence of passive uptake in any of the WBS treated fish. Similarly, after an application of sprayed antigen directly onto the lateral line (LN), using 100.0mg HGG for an exposure period of 4h, no antigen could be detected in the blood sera. The occlusion of the lateral line in WBS-LN treatment, where only the skin was in contact with antigen, was also negative. However, the ELISA assay was capable of detecting HGG in the blood sera of animals that had received 10.0mg HGG by intraperitoneal injection 24h earlier. Possible cellular events in the application of antigen to the skin were then investigated.

TABLE 1. The treatments and determination of antigen in the blood of HGG sprayed animals using the ELISA assay

Treatment	HGG Dose mg	Contact Exposure min	ELISA
WBS-LN	5	420	(-)
WBS	5	120	(-)
		240	(-)
		330	(-)
	10	150	(-)
		170	(-)
		270	(-)
	50	270	(-)
	100	440	(-)
		450	(-)
LN	100	135	(-)
		240	(-)
IP injection	10	24h	(+)

(B) ACTIVE UPTAKE

Animals were subjected to 10 min, 285 min and 360 min contact exposures to V. anguillarum bacterin. The immunofluorescent technique was capable of visualizing the sprayed bacteria and in all sections studied, no cellular activity within the epidermis was visible. The cuticle was seen to trap the bacterin within its surface, and sloughing of the cuticle containing the sprayed bacteria was observed at all exposure times.

The spraying of the soluble antigen HGG involved contact exposures of 120 min and 360 min. Fish studied

after 120 min showed the antigen to be incorporated in the cuticle (Fig. 2). The outline of sloughing surface epithelial cells could be seen below the cuticle but no HGG could be visualized within the epidermis. After 360 min of contact exposure, HGG could be visualized within the cuticle and also within cells of the epidermis (Fig. 3). This was observed in only a few of the serial sections taken for immunohistochemical analysis and it is likely that the cells were intrusive leucocytes; because of the very long contact exposures required to ellicit this response, no attempt was made to identify the cells. Sections of spleen, pronephros and thymus were negative for immunofluorescence.

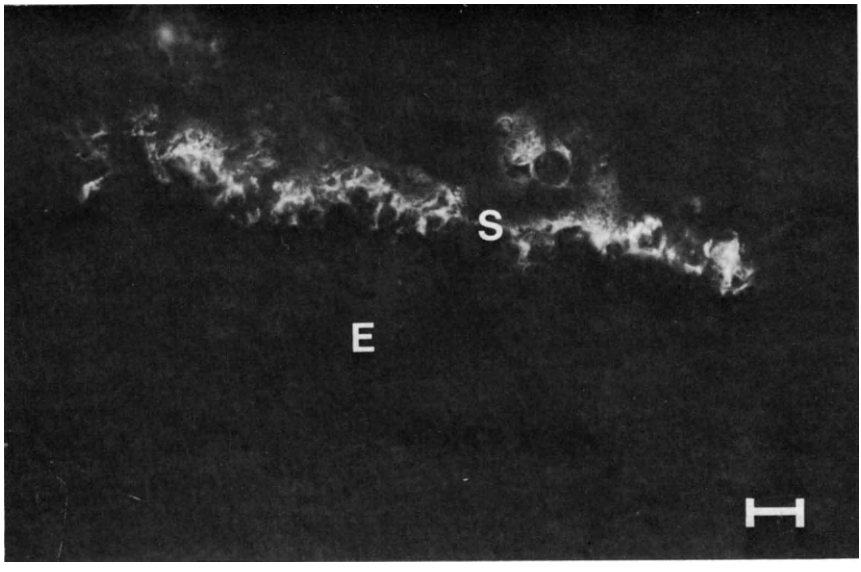

Fig. 2. Cryostat section of skin taken from the adipose fin of a rainbow trout 120 min after a 10mg HGG spray. Positive immunofluorescence can be seen at the skin surface (S) within the cuticle, after incubation with fluorescein labelled sheep anti HGG antiserum. No fluorescence is visible within the epidermis (E). The outline of surface epithelial cells can be seen. Bar = 25 μm.

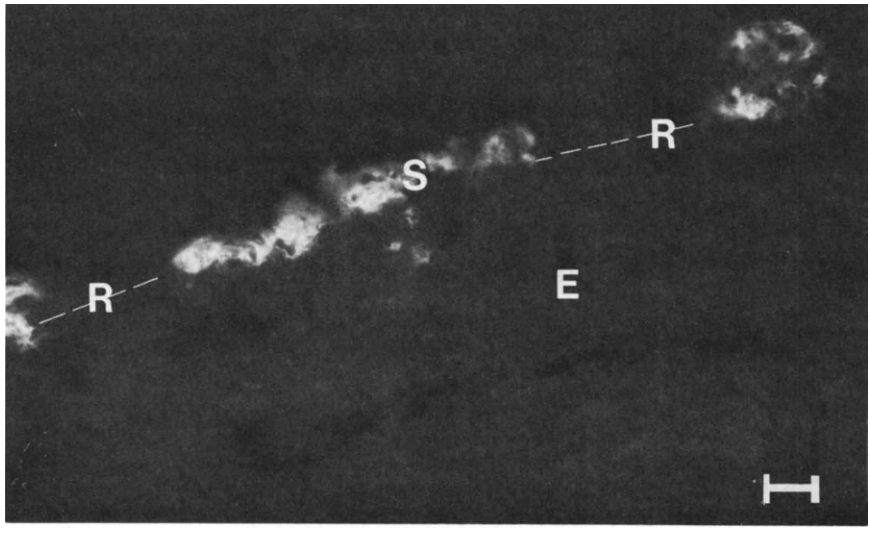

Fig. 3. Cryostat section of skin from the adipose fin of a rainbow trout, 360 min after a 10mg HGG spray. Positive immunofluorescence can be seen within the epidermis after incubation in fluorescein labelled sheep anti HGG antisera. Regions where surface sloughing has taken place (R) are shown by broken line. Bar = 25 μm.

IV. DISCUSSION

In both bath and spray vaccination methods the skin is in direct contact with antigen. To date, there has been very little information available on skin-antigen contact and it is important to clarify whether or not an uptake mechanism exists. This information could help in the future development of vaccines and vaccination methods (Amend and Johnson, 1981).

The passive uptake of bacteria across the skin has been studied by Bowers and Alexander (1982). In an in vitro study, excised skin flaps taken from brown trout Salmo trutta L. were investigated for their permeability to bacteria in hyperosmotic solutions, and no bacteria were seen to permeate across the skin. There were, however, obvious limitations to the conclusions that could be drawn

from such an experiment, as the skin preparations were not subject to circulatory, endocrine or nervous influences. In the present study these limitations were overcome by investigating the skin in vitro. Antigen was allowed to remain in contact with living skin tissue for very long periods of time and in none of the fish tested was there evidence of a passive uptake mechanism. This observation corroborates that reported by Bowers and Alexander (1982) and it seems unlikely, therefore, that the skin is implicated in vaccine uptake via a passive route. The cuticle may, however, have a passive function in the trapping of sprayed antigen on the skin surface. It was visible in the skin biopsies studied using immuno-fluorescent techniques that both soluble (HGG) and particuate (V. anguillarum) antigen was capable of being trapped in the cuticle after spraying (Fig. 2). The surface epithelial cells and the cuticle were, however, seen to slough from the surface of sprayed animals (Fig. 3). The sloughing process is a normal feature of the salmonid epidermis (Roberts et al., 1970) and despite the ability of bacteria to attach to epithelia (Costerton et al., 1978), the continuous shedding of the surface epithelial cells and cuticle prevents further entry of invasive bacteria into the epidermis (Munro, 1982).

The skin was also studied for the existence of cellular uptake. Some cellular activity was seen in the epidermis of HGG sprayed animals after 6h exposures (Fig. 3). Smith (1982) reports that cellular activity in the skin of rainbow trout fry can be seen 4h-8h following hyper-osmotic and bath immersions in bovine serum albumin. The cellular events that were observed in the skin of HGG sprayed animals were very limited and only occurred after very long exposures to antigen. Interestingly, no similar activity to that seen in the HGG sprayed animals was observed using V. anguillarum; the bacterium is highly antigenic and only very small amounts are required to ellicit an immune response in fish (Gould, 1977). It is possible that the cellular events seen in the epidermis of HGG sprayed animals represent a local immunity to the antigen trapped in the cuticle and because of the contact times involved are unlikely to be important in spray vaccination.

It is known that a 5 sec contact with sprayed bacterin is sufficient to ellicit an immune response and give good protection (Gould et al., 1978). Similarly, in a study of immersion (bath) vaccination, it has been demonstrated that a 5 sec dip in bacterin is enough time for the uptake

of an adequate dose of vaccine (Johnson et al., 1982) and that immersions of longer than 10 sec do not significantly increase vaccine uptake (Tatner and Horne, 1983).

It has been shown that the head region is important in the uptake of vaccine following bath immersion (Tatner and Horne, 1983) and that the gills are the likely site of the uptake mechanism (Smith, 1982). Using indian ink as a marker to simulate a sprayed vaccine, it can be seen that the vaccine is immediately subjected to dilution and washing effects once the animal is returned to water (personal observation). As both spray and bath vaccination give similar degrees of protection (Gould et al., 1978), it may be that they share the same mechanism of antigen uptake. In view of the fact that no firm evidence has been found to show the existence of uptake mechanisms in the skin, it is possible that spray vaccination functions by the release of sprayed antigen from the skin surface when the fish is reimmersed in the water, leading to a local bathing effect on the gills and head region. Furthermore, in normal spraying procedures no precautions are taken to protect the head region and gills from direct contact with vaccine.

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NATURAL AND ADAPTIVE IMMUNITY IN MARINE TELEOSTS TO BACTERIAL ISOLATES FROM SEWAGE SLUDGE

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I. INTRODUCTION

Nearly seven million tons of sewage sludge is dumped annually 12 miles off the mid-Atlantic coast of the United States. This is an area which is important for commercial and recreational fisheries. The interaction of fish populations with bacteria found in sewage sludge has not been examined to any extent. These sludge-associated bacteria could be potentially pathogenic to fish as well as to humans.

Fish with serologic evidence of infection with human pathogens have been found in Chesapeake Bay (Janssen and Meyers, 1968). A fresh water species, brown bullhead catfish, Ictalurus nebulosus was found to have sera reactive to Escerichia coli, and Enterobacter cloacae in areas of high coliform counts (Troast, 1975). Studies done in our laboratory have shown that summer flounder (Paralichthys dentatus), winter flounder (P. americanus), tautog (Tautoga onitis), and killifish (Fundulus heteroclitis) produce antibody to sludge bacterial isolates whether they have been injected, bathed, or exposed in situ at the sewage dumpsite (Stolen et al., 1983). We report here on the kinetics, physico-chemical

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properties and specificity of the antibody produced to these isolates. We also show that migratory fish caught far from the dumpsite location show serologic evidence of contact with sludge-associated bacteria, and might in fact be responsible for the dissemination of these pollutants to presumably clean locations.

II. MATERIALS AND METHODS

FISH

Summer flounder, (P. dentatus) 30-40 cm in length, and tautog (T. onitis) 25-30 cm in length, were caught by otter trawl in Sandy Hook Bay, New Jersey, USA. They were allowed to acclimate for two weeks and maintained in 250 gallon flow through salt water tanks. Winter flounder (P. americanus), yellowtail flounder (Limanda ferruginea), and windowpane flounder (Scophthalmus aquosus) were also sampled.

BACTERIAL ANTIGEN

The following organisms were isolated from sediment samples from the municipal sludge dumpsite in the New York Bight. Klebsiella oxytoca, K. pneumonia, Proteus morganii, Aeromonas hydrophila, Enterobacter cloacae, Serratia liquifaciens, and Escerichia coli. The maintenance, storage of bacteria and antigen preparation were as previously reported (Stolen et al., 1983).

"O" ANTIGEN PREPARATION

"O" antigen was extracted from bacteria according to the method of Ewing (1972). The supernatant from the boiled, powdered antigen was used as a source of "O" antigen.

FISH SERUM

Fish were anaesthetised with TMS (tricane methane sulphonate) and were bled from the caudal vein. The serum was isolated, heat inactivated at 50°C for 30 min and stored at -20°C until tests were performed.

2 MERCAPTOETHANOL (2ME)

2ME in the presence of 2M urea was added to the first well

of a microtiter plate with an equal volume of serum. Plates were incubated for 1 h at 37°C and 1 h at room temperature before a microtiter agglutination test was performed.

BACTERIAL AGGLUTINATION

Direct bacterial agglutination was performed as previously described (Stolen et al., 1983).

INDIRECT HEMAGGLUTINATION TEST (IHAT)

Equal volumes of 10% sheep red blood cells (SRBC) and 5 mg/ml "O" antigen were incubated at 37°C for one hour and 4°C overnight. The cells were then washed three times in saline. The microtiter method used was the same as the direct agglutination test.

SEPHAROSE 4B, GEL FILTRATION

The sepharose 4B column used had bed dimensions of 2.0 x 42.0 cm and the void volume (Vo) was 51 ml. The eluant used was 0.05 M phosphate buffer at pH 7.3 (0.05 m Na_2HPO_4, 0.05 m KH_2PO_4, 2.2% Nacl, 0.02% NaN_3). The elution rate was 3.3 $\pm$ .37 $ml/cm^2/h$. A sample volume of 1.5 ml was used. Fractions were examined for antibody activity.

III. RESULTS

Tautog (T. onitis) and summer flounder (P. dentatus) immunized with bacterial isolates produced high titered antibody. The titers were still high after 245 days (Table I). These fish had been injected in September, over-wintered at environmental temperatures which dropped to 6-8°C and bled again in May.

The kinetics of the immune response of summer flounder to A. hydrophila at 20°C showed high agglutination titers, which appeared at 14 days after injection and peaked at 28 days (Fig. 1).

Antibody produced to one bacteria cross-reacted with some of the other bacteria tested but not all others (Tables II, III). Tautog injected with PM also agglutinated AH but not KP and KO. Antibody activity could be absorbed out by incubating with homologous bacteria but not by non-cross reacting bacteria (Table III).

Table I. Antibody response in marine teleosts to bacteria isolated from sewage sludge.

Species	Antigen	Days after first injection $\log_2$ agglutination titers ± standard deviation 0	28	245
T. onitis[1]	P. morganii	2.0 ± 0.77	9.25 ± 0.70	>8
T. onitis	K. pneumonia	1.25 ± 1.20	5.00 ± 1.60	5.5 ± 1.80
P. dentatus	E. cloacae	0.60 ± 1.40	10.40 ± 1.80	>8
P. dentatus	P. morganii	0.60 ± 0.54	>12	>8
P. dentatus	S.liquifaciens	0.00 ± 0.00	10.8 ± 1.90	>8

[1]Fish were injected on day 0 and day 7 with 0.3 ml of a 10% suspension of bacteria in an equal volume of Freunds complete adjuvant (FCA).

Table II. The cross-reactivity between sludge bacterial isolates in summer flounder (Paralichthys dentatus).

Antigen	$\bar{M}$ $\log_2$ agglutination titers 28 days after injection to: PM	SL	AH	Ecl	KO	KP	EC
Ecl #	1.2	9.6	0.2	9.8	10.6	10.4	1.6
PM	11.0	1.2	10.8	1.1	1.6	2.2	2.4
SL	1.3	10.8	0.8	10.9	12.0	10.6	7.0
AH + Ecl	9.7	8.6	9.8	8.8	10.1	9.2	1.8

PM = Proteus morganii; SL = Serratia liquifaciens; AH = Aeromonas hydrophila; Ecl = Enterobacter cloacae; KO = Klebsiella pneumonia; EC = Eschericia coli.

#Fish were injected on day 0 and 7 with 0.3 ml of a 10% suspension of bacteria in FCA and bled 28 days later.

Table III. The cross-reactivity between whole sludge bacterial isolates vs "O" antigen. Absorption studies.

Fish Species	Antigen	Serum absorbed with	$\bar{M}$ $\log_2$ direct agglutination titers KP	KO	PM	AH	Ah-"O"Ag $\log_2$IHAT
Tautog	PM	-	4	3	8	7	-
Tautog	PM	KP	0	0	8	7	-
Tautog	PM	PM	3	2	0	0	-
Tautog	KP	-	7	5	1	0	-
Tautog	KP	PM	6	5	0	0	-
S. flounder	AH	-	-	-	-	9	7
S. flounder	AH	AH-"O"	-	-	-	6	0
S. flounder	AH	PM	-	-	-	-	6
S. flounder	AH	AH	-	-	-	0	0

Proteus morganii (PM) and Aeromonas hydrophila (AH) appear to share common determinants but do not cross-react with the other bacteria tested. This shared antigenic determinant did not seem to be the "O" antigen. Absorption with PM of serum from fish immunized with AH did not reduce titers to "O" antigen in the IHAT test. Absorption of the same serum with "O" antigen of AH reduced the titers against whole bacteria from $\log_2$ 9 to 6 (Table III).

Gel filtration chromatography of summer flounder serum with Sepharose 4B yielded 5-6 peaks (Fig. 2). Peaks II, III, and IV were best resolved in the 21 day elution profile (Fig. 3C). The highest agglutinating activity was found in Peak III with some activity in Peaks II and IV. By 35 days some activity also appeared in Peak I (Fig. 3D). Treatment of the 21 and 35 day peaks with 2ME reduced most of the titers in peaks other than Peak III. Peak III titres were reduced by 25% (Table IV).

Species-specific differences were seen in elution profiles of pooled uninjected summer flounder, windowpane flounder, and tautog (Fig. 2).

The percent of winter flounder (Fig. 4A) and summer flounder (Fig. 4B) with positive agglutination titers to

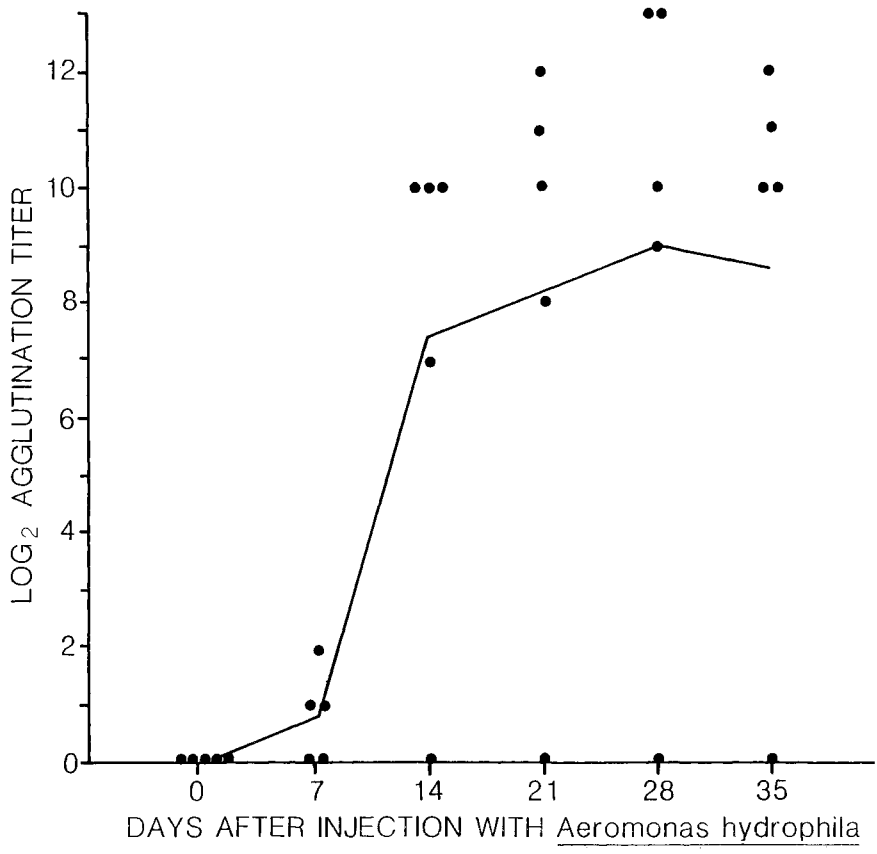


Fig. 1. The kinetics of the immune response of summer flounder to A. hydrophila at 20°C. Fish were injected with 0.3 ml of a 10% suspension of bacteria in FCA.

isolates showed seasonal variation when sampled from Raritan Bay over an extended period of time. Fish with positive agglutination titers to sludge-associated bacteria were found as far as the continental shelf (Fig. 5B, Station 20) off the coast of Maine and off the coast of New Jersey (Fig. 5C, Station 51).

2ME treatment of antiserum from both naturally exposed and injected summer founder showed reduction in titers in all cases but the amount of reduction showed quite a bit of variation (Table IV). The injected fish seemed somewhat more resistant to 2ME treatment.

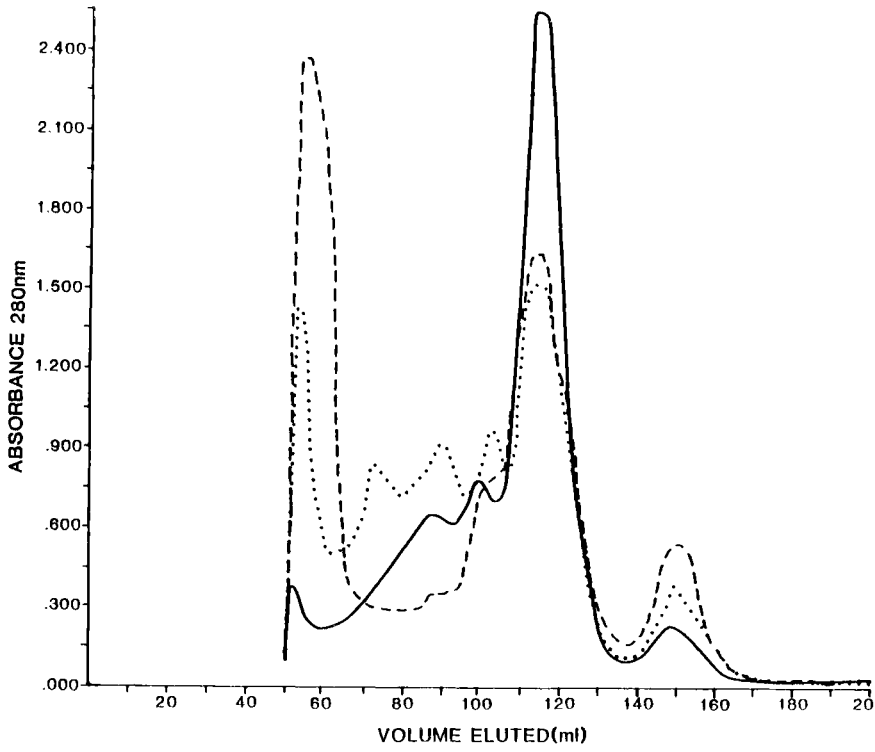
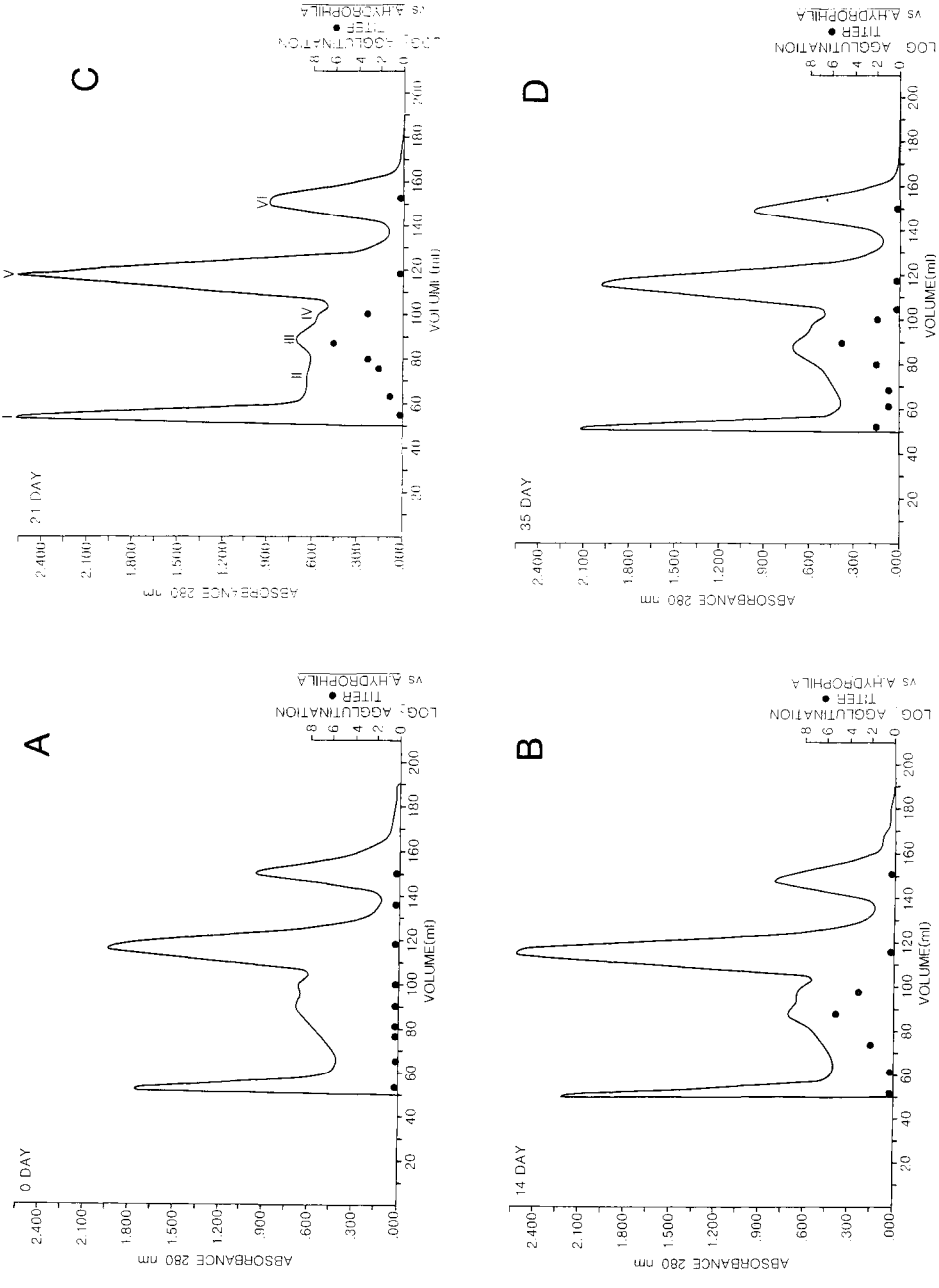


Fig. 2. A comparison of the elution profiles of three species of marine teleost sera; summer flounder (————), windowpane flounder (····), and tautog (- - -), on sepharose 4B.

Fig. 3. Elution profiles of summer flounder serum on sepharose 4B. Fish were injected with A. hydrophila and bled on days 0, 14, 21, and 35. A 1.5 ml pool of the sera of five fish was used. (See page 214.)


A
0 DAY
B
14 DAY
C
21 DAY
D
35 DAY
ABSORBANCE 280 nm
VOLUME(ml)
LOG AGGLUTINATION TITER vs A.HYDROPHILA
I
II
III
IV
V
VI

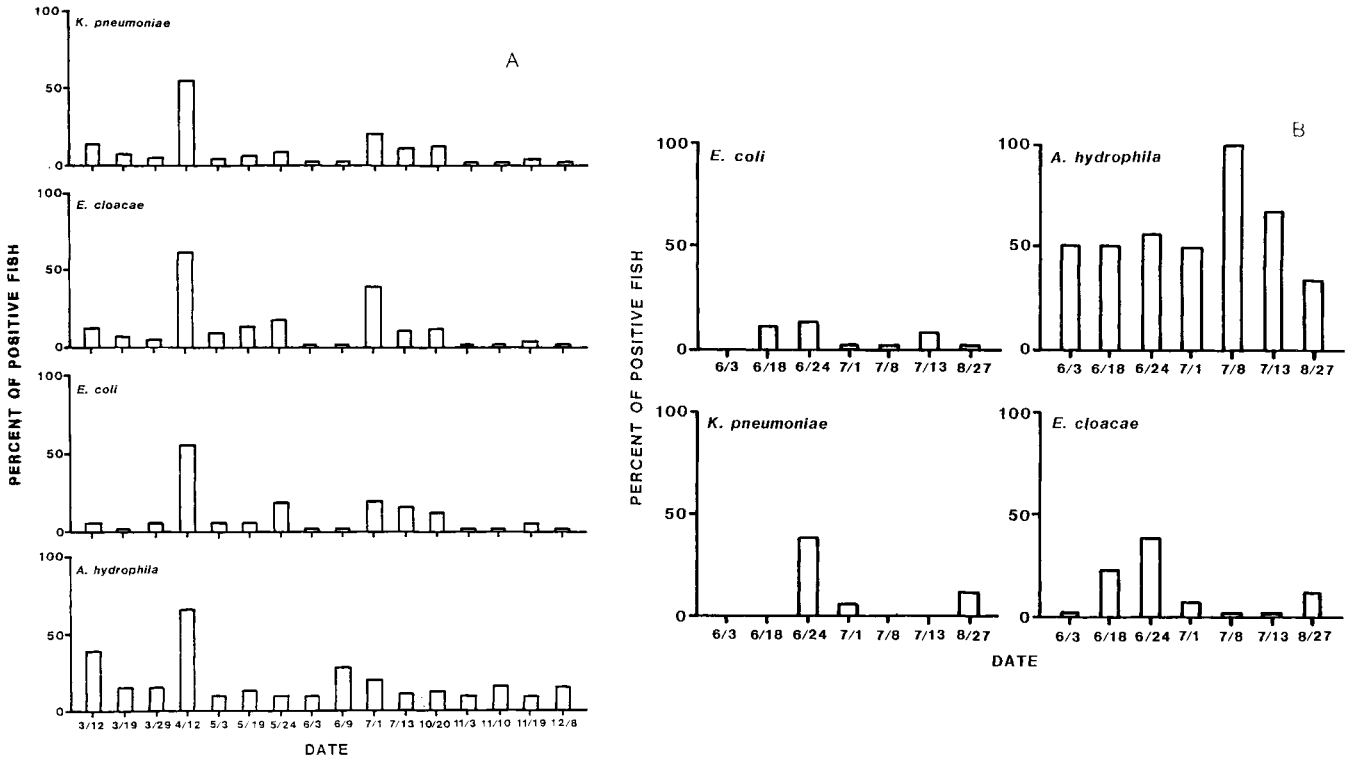


Fig. 4. Antibody profiles to sludge bacterial isolates in winter flounder (A) and summer flounder (B) caught in Raritan Bay, New Jersey. Each bar represents a pool of approximately 20 fish

Table IV. The effect of 2ME on both injected and naturally exposed summer flounder agglutinins to AH

Source	$\log_2$ agglutination titers Untreated Serum	0.2M 2ME 2.0 + M urea	% reduction in titers after treatment with 2ME
N*	4.0	1.5	62.5
N	4.0	3.0	25.0
N	3.0	1.0	67.0
I[a]	8.0	5.5	31.0
I	8.0	6.0	25.0
I	8.0	7.0	13.0
P II (I_{21})	3.0	0	100.0
P III (I_{21})	4.0	3.0	25.0
P IV (I_{21})#	2.5	0	100.0
P I (I_{35})	1.0	0	100.0
P II (I_{35})	3.0	1.0	67.0
P III (I_{35})	4.0	3.0	25.0
P IV (I_{35})	2.0	0	100.0

*Naturally exposed

[a]Injected

#Peak II (injected, 21 day bleeding) from Sepharose 4B column

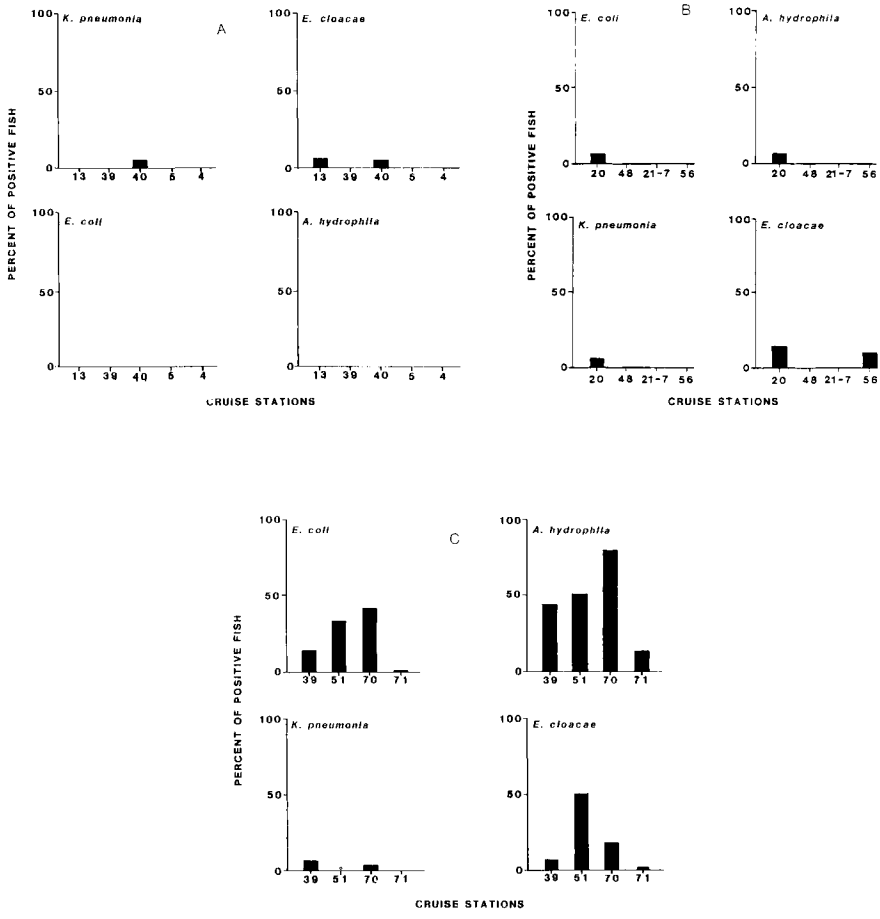


Fig. 5 Antibody profiles of winter flounder (A), yellowtail flounder (B), and summer flounder (C), caught on an offshore cruise in November 1982. The cruise covered an area from the Gulf of Maine to Cape Hatteras along the Atlantic coast of the United States. The stations are numbered from the north (lowest numbers) to the south (highest numbers).

IV. DISCUSSION

Summer flounder and tautog produce antibody to sludge-associated bacteria which is detectable after a

period of over five months, even after wintering over at cold temperatures. Summer flounder have been shown to produce antibody to horse red blood cells for long periods of time. The magnitude of the response was not temperature sensitive, only the time of appearance. It was also shown that once antibody production was underway, the response was temperature-independent (Stolen et al., in press).

Cross-reactivity was seen in certain groups of fish by the direct bacterial agglutination technique using whole bacterial cells. More strain-specificity can be obtained by extracting the "O" antigen of the bacteria to be tested. Proteus morganii and Aeromonas hydrophila cross-react strongly in direct agglutination tests, but serum from AH-injected fish absorbed with PM showed no reduction in titers when tested in IHAT to the "O" antigen of AH. Absorption of antisera to AH with AH "O" antigen reduced the titers from $\log_2$ 9 to $\log_2$ 6 indicating that the "O" antigen is not the only active antigenic determinant on the bacterial cell.

Sepharose 4B fractionation of the serum of AH injected summer flounder yielded six peaks. Peak III had the bulk of the antibody activity although there was some activity in Peaks II and IV. After 35 days some activity appeared in Peak I as well. In most cases Peak II was not clearly resolved. A calculation of molecular weights using standard proteins, indicated that fraction IV was close to a molecular weight of 7×10^5 daltons and Peak III about 1×10^6 daltons. Ingram and Alexander (1979) found activity in Peak II using sepharose 6B with brown trout serum. This was a broad peak and perhaps in the summer flounder using sepharose 4B this is resolved into more than one peak containing activity.

The most 2ME-resistant peak appeared to be Peak III which contained most of the activity. Reduction of antibody activity with 2ME in the presence of 2M urea was also seen in channel catfish, Ictalurus punctatus (Heartwell, 1975). Ingram and Alexander (1979) showed reduced activity to KLH after treatment with dithiothreitol in brown trout, Salmo trutta.

Almost all studies on the immunoglobulins of fish have shown it to be of the IgM class (Marchalonis, 1971; Litman, 1976) and a tetramer (Acton, et al.,1971; Shelton and Smith, 1970). The appearance of agglutinating antibody in several different peaks and differential sensitivity to 2ME might reflect a difference in the polymeric state, the length of the heavy chain, and the binding capacity for the secretory components during the course of

immunization. It has been suggested that the Ig M type produced seem to be adaptations to specific functions (Ambrosius et al., 1982). Antibody to an erythrocyte in the same species, tested under the identical circumstances showed activity only in Peak III (Stolen and Gahn, unpublished results). Different species of teleosts seem to display somewhat different elution profiles. Windowpane flounder showed very distinct Peaks III, II, and IV.

Serological evidence of contact of fish with sludge-associated bacteria have been found in fish caught in various locations along the Atlantic coast of the United States. Contact of fish with bacteria in the water and sediment is sufficient to induce an immune response. Bath or in situ exposure to the sludge-associated bacteria showed an increase in agglutinating titers to the bacteria tested (Stolen et al., 1983). Bath exposure has been studied extensively by many investigators concerned with the vaccination of fish. The main portal of entry into fish during bath exposure seems to be the gills (Alexander et al., 1982).

The goal of the field studies is to utilize these data to serve as a means of monitoring the spread of sludge-associated pollution, as well as assessing the health and suitability for consumption of fish that come in contact with the sewage sludge dumpsites.

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FACTORS INFLUENCING THE EFFICACY OF VACCINES AGAINST VIBRIOSIS CAUSED BY VIBRIO ANGUILLARUM

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I. INTRODUCTION

Vaccines against bacterial diseases are playing an increasingly important role in the management of intensively reared fish. The earliest vaccines to be used on a large scale were those against Enteric red mouth disease caused by Yersinia ruckeri and Vibriosis caused by Vibrio anguillarum. With the help of these vaccines it is now possible to maintain these diseases in check.

The original development work on vaccines was largely empirical, concerned mainly with the causative organisms and not the immune system of the fish since detailed knowledge of this was not available. It is only recently that Busch (1983) and others have shown that acquired immunity to artificial infection with Y. ruckeri goes hand in hand with a rise in serum agglutinins for Y. ruckeri and that stimulation of the gut associated lymphoid tissue by anally administered vaccine will provide effective immunity. Clearly more work needs to be done to identify the protective component(s) of the fish's immune response to bacterial antigens.

In a programme to develop vaccines for fish we have investigated some of the factors likely to affect the immune response and it is these results in relation to vaccines against Vibrio anguillarum which comprise this communication.

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II. MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Rainbow trout Salmo gairdneri (Richardson) obtained from a commercial trout farm were kept in fibre glass tanks supplied with running water at ambient temperature. They were fed on commercial trout pellets at a rate of approximately 2% body weight per day. When fry were required they were hatched in the laboratory and kept until they reached the right size.

VACCINE PREPARATION

Appropriate strains of V. anguillarum were grown in Tryptic soy broth (TSB Difco) + 1% NaCl at 25°C, either in baffled shake flasks or in 2 L glass fermenters.

Cultures were treated as follows to give the various antigens used.

Formalin killed vaccine (FKV).

Whole culture was treated with a final concentration of 0.5% formalin (0.2% formaldehyde) and adjusted to contain 30mg wet weight of organisms ml^{-1}, measured after centrifuging the culture at 10,000g for 10 min.

Perchloric acid extract vaccine (PEV).

Culture was centrifuged at 10,000g and the sedimented cells washed twice in saline. They were extracted at 4°C for 10 min with 10mls of 0.5N perchloric acid per gram wet weight of cells. After centrifuging, the cells were extracted twice more with 0.2N perchloric acid. All three extracts were pooled and neutralized with 10N KOH; the precipitate of KCl O_4 was removed and the extract filtered through an 0.45 μ filter.

ADJUVANTS

When an oil adjuvant was required antigen + 1% Tween 80 was homogenized in the ratio 30:70 by volume with the oil phase. Alum adjuved vaccines were prepared by adding to antigen 25% v/v of 10% potassium aluminium sulphate and adjusting the pH to 6.5. All vaccines were stored at +4°C and shaken well before use.

VACCINE ADMINISTRATION

Intraperitoneal injections (I.P.) were administered under anaesthesia (MS 222) and between 50 and 250 μl injected

depending on the size of the fish. Oral vaccines were prepared by spraying antigen onto food pellets at the rate of 1.5g wet weight of bacteria or the equivalent in extract per kg of food. This treated food was fed on alternate days for 32 days. Direct immersion (D.I.) vaccination was performed by dipping fish for between 20 sec and 2 min in antigen diluted to contain 3mg wet weight of bacteria per ml of bathing fluid.

CHALLENGE

Challenge was performed by intraperitoneal injection of 0.1ml of an overnight culture of the organism used to prepare the vaccine. This volume contained approximately 2×10^7 organisms and killed all control fish in 4 days. Vaccinated fish were watched for 10 days.

III. RESULTS AND DISCUSSION

ROUTE OF ADMINISTRATION

From the point of view of minimizing the work and stress involved in vaccinating fish oral dosing would clearly be the method of choice. We have investigated oral dosing and compared it with direct immersion and intraperitoneal injection to assess them all as possible commercial alternatives (Agius et al., 1983). Results are listed in Table 1 and it is clear that there are differences between the three methods. A major difference is found in the amount of antigen needed to provoke immunity and the number of doses in which it has to be presented. Oral dosing requires a large number of antigen administrations over a period of about 30 days to produce acceptable levels of immunity, whilst direct immersion and intraperitoneal injection require 1 antigen administration only. The data on protection show similar differences; intraperitoneal injection of antigens is effective in protecting in excess of 90% of animals between 2 and 4 weeks after injection; direct immersion is of the order of 70% effective after 4-6 weeks and oral dosing between 50 and 70% effective at 8 weeks, all against a severe intraperitoneal challenge killing control animals in 4 days.

The antibody response of vaccinated fish as measured by the circulating agglutinin titres for heat killed homologous V. anguillarum are listed in Table 1; in

addition we have measured the antibody response by passive haemagglutination using purified V. anguillarum LPS as antigen, and by complement fixation with essentially similar results. They clearly indicate that a circulating antibody response is not necessary for protection. Fish vaccinated by intraperitoneal injection produce good antibody responses but the two non-parenteral routes produce little or no circulating antibody. Amongst these latter fish were individuals which had no circulating antibody as measured by any of the three techniques listed, but which were protected from the severe challenge given. Clearly there is a response to vaccination which we have not measured.

TABLE 1. Agglutination titres and protection levels induced by Vibrio anguillarum vaccine, presented by three different routes.

Route of Administration	Antigen(1)	Dose	Week 2 Titre	Week 2 Protection(2)	Week 8 Titre	Week 8 Protection
Oral	FKV	15 administrations at 1-3mg/dose	0	0	4	50-70
	PEV		0	0	0	10-20
Direct immersion	FKV	1 immersion at 3 mg/ml	0	10		70
Intra-peritoneal	FKV	1 injection of 3-5mg antigen	64	75	ND	91
	PEV		64	100	ND	ND

(1) FKV = Formalin killed vaccine
PEV = Perchloric acid extract vaccine

(2) Protection against challenge with 2 x 10^6 cells I.P.

The role of antibody in protecting fish against Vibriosis was investigated by Harrell et al., (1975) who passively transferred immune fish or rabbit serum to unimmunized fish and produced protection against challenge. Serum absorbed with heat killed V. anguillarum was unable to protect fish, suggesting that protection was mediated through antibodies against the 'O' antigen. The half life of the transferred globulin in these experiments was of the order of 10 days and fish with a titre of < 8 were still protected 65 days after transfer. The half life of I^{125} labelled globulin in coho salmon (Onchorhynchus kisutch) was calculated by Grobert et al. (1979) to be 46 hours, so clearly a discrepancy exists and this work needs to be repeated.

If circulating antibody is only part of the protective

mechanism against vibriosis then other aspects of the immune response must be relevant. Both oral administration of vaccine and direct immersion of fish in vaccine are effectively local applications of antigen to mucus membranes, one to the gastrointestinal tract and the other to the skin and gills. There are no reported investigations into the role of locally produced antibody in vibriosis; Davina et al., (1982) have shown that the number of intra-epithelial leukocytes in the gut of carp is enhanced within 30 minutes of application of Vibrio vaccine but their relevance is unclear and local antibody production was not assessed. If local antibody production is important then antibody in mucus should be relevant. Parenteral immunization would then exert its effect by the transfer of antibody from the serum to the mucus. Such a transfer was confirmed by Ourth (1980) whereas Harrell et al., (1976) showed that transfer does not occur until extremely high circulating titres are obtained (>131,000). Clearly, studies on this aspect, on the role of circulating antibody, of cellular immunity and of the method of pathogenesis of V. anguillarum are needed before these contradictions can be clarified.

USE OF ADJUVANTS

The role of adjuvants in mammalian immunotherapy is well documented. In fish we tested both oil based and mineral gel based adjuvants with similar results. The response to adjuved vaccines was faster than the response to unadjuved antigen. The final level of the response was not, however, enhanced. This applied to both the circulating antibody titre and protective capacity. However, to offset the benefits of a more rapid response there were secondary effects arising from the use of adjuvants which could affect their commercial acceptability.

5 cm fish injected with alum adjuved vaccine presented unacceptable peritoneal lesions including peritoneal fibrosis and scarring with chronic degeneration of the liver and pancreas (Horne et al., 1983); larger fish (20cm) showed proportionally less effect. Over a season's growth it was found that fish given an adjuvant suffered a growth penalty when compared with untreated fish. This amounted to $\pm$10% of the increase in body weight. In the light of the lesions found this is not surprising. We believe, therefore, that the use of an adjuvant in fish vaccines is contraindicated unless it can be shown to confer significant vaccinial advantage.

AGE OF FISH AT VACCINATION

Clearly, the age at which fish can be vaccinated is an important commercial consideration since many diseases are diseases of fry. Knowledge of the ontogeny of the immune system in fish and the more practical aspects of susceptibility to disease and ability to be protected are clearly important in this respect. We have studied the more practical aspects by investigating the age at which trout fry become susceptible to vibriosis and the age at which they can be protected by vaccination (Tatner and Horne, 1983).

Trout from 2 weeks old and weighing from 0.14g were challenged with virulent V. anguillarum by immersion in diluted culture (Table II). Not until they were 6-8 weeks old, and weighing 0.2-0.3g did they become susceptible to vibriosis and even then only 30% of the challenged fish succumbed to the highest dose used, 10^8 V. anguillarum/ml. At 7 weeks old, however, the fry were susceptible with 88% mortality to intraperitoneal injection of 2 x 10^6 organisms. It seems probable that

TABLE II. Susceptibility and protective immunity in rainbow trout fry, to Vibrio anguillarum.

Age of Fish Weeks	Specific Control Mortalities after challenge(1)%		RPS in DI vaccinates(2) after challenge	
	DI	IP	DI	IP
2	0	ND	11	16
4	0	ND	0	17
6	20	ND	ND	70
7	ND	88	ND	ND
8	20	ND	ND	ND
10	ND	94	ND	47(3)
14	ND	100	ND	68

(1) Challenge with 2 x 10^7 cells/ml DI or 2 x 10^6 cells IP.

(2) RPS = Relative percent survival = $100 \left(\frac{\text{Vaccinate mortality}}{\text{control mortality}}\right)$

(3) Fish vaccinated and challenged IP at 10 weeks had RPS = 100

if smaller fish were able to survive the trauma of injection they too would be susceptible to infection and that the results point in part to a protective mechanism in small fish but also to the inadequacies of a bath challenge. These workers also find that on challenge 4 weeks after vaccination there is significant survival of all groups of fish even those vaccinated 2 weeks post hatching. This is interpreted as indicating that even fry of 0.15g are capable of mounting a low level immune response to V. anguillarum vaccine. This protective response increases as the fish grows, reaching commercially meaningful levels of $>60\%$ when the fish are approximately 0.5g.

These results suggest that in the face of an epidemic it would be possible to vaccinate very small fish and expect a degree of protection. It must be said, however, that as the fish increase in size to the threshold level of 0.5g vaccination becomes more efficient.

TEMPERATURE AT VACCINATION

The rate of development of an immune response in fish is in general temperature dependent, reaching a plateau at the normal summer temperature of the species involved (O'Neill, 1980). As temperatures rise, so does the incidence of disease and this produces a situation where fish which are to be vaccinated at the optimum temperature for a good immune response are also subject to challenge. To try to overcome this, fish were vaccinated up to 3 months before the expected spring temperature rise when the water temperature was $<6^{o}C$. All three methods of vaccine presentation were used and the fish were challenged 85 days later by i.p. injection. Protection in the i.p. vaccinated group was 93%, in the DI group 47% and in the oral group 6% (Horne et al., 1982). Considering the severity of the challenge this suggests that commercial vaccination could take place during the colder part of the year to allow fish to develop a protective response in conditions where no challenge exists.

Considering the results above with data on the longevity of protective immunity (Horne et al., 1982), where effective immunity is present up to 330 days after vaccination, it would appear that vaccination on a farm could take place anytime from the autumn of the previous year and good protection should be seen when a natural challenge appears in the following spring/summer.

Vaccination of fish is clearly effective and will

become an accepted husbandry measure in aquaculture. Aspects dealt with in this paper only touch on the problems which exist and until work is done on fundamental aspects of the pathogenicity of organisms causing commercially important diseases, and on functional aspects of the immune response in fish, the preparation and standardisation of vaccines will remain empirical.

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BATH VACCINATION AND DEVELOPMENT OF IMMUNOLOGICAL MEMORY IN CARP (CYPRINUS CARPIO L.)

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Direct immersion and hyperosmotic infiltration are frequently used as methods for fish vaccination. As a result, the animals can be protected from a number of diseases, depending on the vaccines available (Anderson et al., 1983). Testing vaccines can be done by studying the development of immunological memory and the kinetics of the secondary response. Studies following this type of approach after bath immunization are very limited and clear secondary responses still have to be demonstrated (Anderson and Dixon, 1980; Lamers and Van Muiswinkel, 1983).

In our experiments carp were kept at 20-22°C and vaccinated by direct immersion (for 1 h) in a 1:10 diluted culture of heat inactivated Aeromonas hydrophila. One treatment did not result in a primary serum antibody (Ab) response. A second bath given at 1, 3 or 8 months after priming did result in a distinct Ab titer which peaked at day 30 and persisted for at least 90 days. The highest titer was observed when the second treatment was given 3 months after priming. A second bath at 12 months did not result in measurable Ab titers, indicating that memory had faded away. These results are in strong agreement with the temporal character (3-12 months) of the reported protection in salmonids, which were vaccinated against Vibrio anguillarum or Yersinia ruckeri (Johnson et al., 1982). Other groups of bath primed carp were challenged by intramuscular (i.m.) injection with either a high (10^9) or low (10^6) dose of bacterial cells. Responses in these groups did not differ from the primary response

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after i.m. injection.

It is concluded that in carp immunological memory can be induced after direct immersion in A. hydrophila bacterin. However, this memory could only be demonstrated for a restricted time interval (1-8 months after vaccination). Interestingly enough, the route of challenge had to be the same as the priming route. This might be an indication of the existence of a local immune system in fish.

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MATERNAL EFFECTS AND RESISTANCE TO INFECTION BY ICHTHYOPHTHIRIUS MULTIFILIIS IN XIPHOPHORUS MACULATUS

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I. INTRODUCTION

In 1975 the FAO estimated that production from aquaculture could possibly increase five-fold over the next 30 years (Reay, 1979). Two major problems are those of maximising growth and minimising mortality. Thus, for fish farming to reach its fullest potential, it will be essential to further develop techniques for the control and prevention of disease.

Traditional methods of control using chemical treatments are not always effective. They can be costly, cause problems of pollution and of drug resistance in the pathogen, and they may be subject to prohibitive legislation. Newer techniques of immunising fish, some of which are described elsewhere in this volume, are still in early stages of development. At present it is not possible to assess just how important these will become. They may prove to be more applicable to brood stock rather than ongrowing fish, and to intensive systems rather than the more traditional fish farming systems common in tropical countries.

An alternative approach is to try and develop disease resistant strains by artificial selection. Kirpichnikov et al (1976) had some success in selecting for resistance to dropsy in common carp. For selection to be effective the fish stock must exhibit some genetic variation in susceptibility. Occasionally, such variation is under the control of major genes and can be studied by segregation

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analysis, e.g. tumour development in xiphophorin hybrids (Anders et al, 1973) and possibly swimbladder inflammation and epidermal epithelioma disease in common carp (Hines et al, 1974).

More probable, however, is that any genetic influence is due to minor or polygenes. Here, several genes, similar in effect, act in an additive manner without dominance. Environmental effects, dominant genes, gene interactions and gene-environment interactions affecting the phenotype may also be apparent, so that the character exhibits continuous variation (see Falconer, 1981). Instead of segregation analysis, statistical analysis must be employed. The proportion of the total phenotypic variance due to additive genetic variance is the heritability (h^2). Its importance is due to the fact that the response to selection is equal to the heritability multiplied by the selection differential ($R = h^2S$).

Early workers looked at variation in disease susceptibility by examining differences between populations, e.g. Snieszko et al. (1959). This approach may uncover useful sources of genetic variation which can be utilised by intraspecific hybridisation. More recently investigators have begun to measure variation within populations by calculating h^2 (see table 18, Kirpichnikov, 1981). This is usually done by using percentage survival after a number of days as the criterion of disease resistance, and is therefore rather removed from the actual infection process.

In this paper we describe our attempts to develop a model system which would allow for a more precise estimation of the amount of genetic variation which exists among fish in susceptibility to a specific disease. To do this, it is necessary that the degree of infection on a fish can be accurately estimated.

The pathogen chosen was the ciliate protozoan Ichthyophthirius multifiliis which causes the disease ichthyophthiriasis, commonly known as white spot. The parasite causes severe epizootics in freshwater aquaria, hatcheries and ponds as well as in wild fish populations. It is widely distributed, occurring in both tropical and temperate regions. The life cycle, which requires no intermediate host, has been described many times, e.g. Van Duijn (1967). After infection by the parasite, the fish responds by forming a cyst around it. These "white spots" are easily countable.

Xiphophorus maculatus was selected as the host species. This fish, known as the platy, is a livebearing poeciliid from Central America. Fertilisation is internal and

typically 10-50 young are produced after 3-4 weeks gestation (Kallman, 1975). It is easy to maintain in the laboratory and has a short generation time. Different varieties are available from both wild and domesticated stocks.

II. MATERIALS AND METHODS

A domesticated stock of X. maculatus known as the red wagtail variety (Kallman, 1975) was used in all experiments. They were obtained through the retail trade from Singapore and were pure-breeding with respect to the colour pattern. The main aim of this investigation was to determine the heritability of susceptibility to white spot infection in this one stock of fish.

CONTROL AND EXPERIMENTAL PROCEDURES

In developing a standardised technique for infection work, a number of preliminary experiments were conducted. The main information is given below; a fuller rationale can be found elsewhere (Bone, 1983).

All fish used, whether for breeding purposes or experimental infections, had not previously been exposed to white spot. All fish were maintained at 24°C ± 0.5°C under the same day/night regime. The pH was kept between 7.0 and 7.3. These parameters were checked before and after experiments. 20 L perspex aquaria were used in infection experiments, normally with 10 fish per tank. If less than 10 fish were used, water levels were reduced proportionally. Fish were allowed to acclimatise for three days in infection tanks before being exposed to the parasite, although water conditions in these tanks were the same as the maintenance tanks. Fish used in experiments were of similar size and age. Any obviously stunted or unhealthy fish were excluded.

The usual parasite source was infected tropical fish obtained from retailers. Parasites were maintained by serial transmission using healthy fish. In any single experiment, only parasites from a single source were used. Infections were carried out using the trophozoite stage. To obtain these, several infected fish were placed in a small beaker of water. The activity of the stressed fish facilitates vacation of the host by the parasite. Trophozoites were collected after 10 minutes. Only trophozoites of size 0.15 - 0.45mm diameter were used. Twenty trophozoites per fish was the experimental dose in all infections.

Infection levels on the fish were scored after 4 days to ensure that the parasite was in the first cycle of infection. Infections were assessed by counting the number of cysts on the left hand side of the fish excluding the head anterior to the operculum, the gills, the dorsal, anal, and pelvic fins. The remaining regions are easy to score accurately and are considered to give a good estimate of the relative magnitude of infection levels on the fish.

Females are not synchronous in giving birth and in the heritability study it proved necessary to infect fish over a period of time. To check for possible changes in infectivity over this period, 39 fish from a single brood were separated into 4 tanks and infected at intervals over a period of 8 weeks.

HERITABILITY ESTIMATION

Heritability was estimated using a half-sib breeding programme. 60 week old fish were used for breeding. Several males were each placed in tanks with several females. In all, three males and nine females produced progeny. The progeny were infected over a 6 week period when they were between 46 and 54 weeks of age. Between 5 and 20 fish per brood were infected (Table IV). In 5 broods there were more than 10 fish. These were each split into two tanks and infected at different times.

For analysis the data was transformed using a square root transformation. Analysis of variance was undertaken and the total variance (σ_p^2) was partitioned into that due to sires (σ_s^2), dams within sires (σ_D^2), and progeny within dams (σ_w^2) (Table I). The genetic model is shown in Table II. Heritability can be estimated from

III. RESULTS

TEMPORAL VARIATION

Results are shown in Table III. The mean number of cysts per fish varies between 482 and 572 in the four groups. Analysis of variance shows that the difference between these means is not significant ($F = 1.291$; $df = 3, 35$; $p > 0.25$). Thus, there is no evidence for any short-term changes in infectivity of the parasite occurring over this 8 week period.

TABLE I

Analysis of variance for half-sib breeding programme.

Source of variation	df	SS	MS	Expected MS
Among sires	a-1	SS_S	MS_S	$\sigma_w^2 + n\sigma_D^2 + nb\sigma_S^2$
Among dams within sires	a(b-1)	SS_D	MS_D	$\sigma_w^2 + n\sigma_D^2$
Among progeny	ab(n-1)	SS_w	MS_w	σ_w^2
Total	abn-1			

a = number of sires; b = number of dams/sire; n = number of progeny/dam.

TABLE II

Interpretation of observational components of variance in a sib-analysis (modified from Falconer, 1981).

Observational component	Main causal components estimated			
	V_A	V_D	V_{EC}	V_{EW}
Sires σ_S^2	25%			
Dams σ_D^2	25%	25%	100%	
Progenies σ_W^2	50%	75%		100%

V_A = additive genetic variance (breeding value)

V_D = dominance variance

V_{EC} = common environmental variance

V_{EW} = within-group environmental variance

ANALYSIS OF HALF-SIB BREEDING PROGRAMME

Infection levels on fish after exposure to the parasite are given in Table IV. There is considerable variation in mean infection levels between tanks (5.6 - 314.0 cysts/fish). This is mainly accounted for by variation between the dams (brood means 6.1 - 314.0 cysts/fish).

TABLE III

Temporal variation in infectivity of Ichthyophthirius multifiliis on Xiphophorus maculatus at 24°C.

	Fish infected on:			
Code number of fish	19.8.81	31.8.81	8.9.81	2.10.81
	Number of epidermal cysts per fish			
1	598	528	428	778
2	594	697	806	892
3	558	499	521	546
4	314	583	1,227	421
5	348	618	268	637
6	934	594	789	623
7	410	638	491	365
8	370	715	667	554
9	238	487	873	918
10	460	358	427	-
$\bar{x}$	482.4	571.7	649.7	637.1
SD	200.00	107.20	281.56	193.61

Exposure level = 20 trophozoites per fish

TABLE IV

Infection levels in Xiphophorus maculatus produced from half-sib breeding programme and exposed to Ichthyophthirius multifiliis at 24°C.

Sire	Dam	Tank	Number of epidermal cysts per Fish										Mean number of epidermal cysts per fish per:- Tank	Dam	Size
I	A	1	27	70	34	49	0	3	8	9	30	16	24.60	27.95	115.97
		2	17	30	38	23	19	0	20	10	69	87	31.30		
	B	3	237	305	341	287	294	269	317	277	271	324	292.20	292.20	
II	C	4	109	246	127	222	261	203	189	173	171	-	189.00	180.79	142.48
		5	176	224	273	107	236	183	152	129	113	141	173.40		
	D	6	14	10	6	4	2	18	0	8	4	0	6.60	6.10	
		7	1	0	21	6	9	19	0	0	0	0	5.60		
	E	8	53	39	69	56	39	-	-	-	-	-	51.20	51.20	
	F	9	251	358	221	218	189	390	240	314	137	348	266.60	271.74	
		10	255	330	327	324	194	251	353	265	198	-	277.44		
III	G	11	2	0	17	6	3	0	28	11	7	19	9.30	9.30	148.17
	H	12	108	158	137	172	108	89	151	101	91	169	128.40	134.70	
		13	99	112	284	98	136	165	129	157	110	120	141.00		
	J	14	248	351	413	283	260	297	262	378	241	407	314.00	314.00	

Exposure level = 20 trophozoites per fish

There is little variation between the three sires (mean values of 116, 142, and 148). In the five broods where two tanks were used, t-tests show that there are no significant differences ($p > 0.05$) between tank means within broods. Thus, tank effects appear to be negligible. This also provides further evidence against short term changes in parasite infectivity. Coefficients of variation within broods tend to be high (>20%) but the magnitude of variation between broods is such that it is possible to distinguish easily broods with 'high' and 'low' susceptibilities to infection.

Analysis of variance (Table V) confirms the above. When the mean squares are partitioned into their separate components, it is found that the dam component accounts for approximately 95% of the total variation. The estimate of the sire component is negative, indicating no variation between the males. This also indicates that there is no additive genetic variance and that heritability, based on σ^2_S is nil. Estimation of h^2 from σ^2_D would not be meaningful because of the extreme difference between σ^2_S and σ^2_D. This difference is mainly due to common environmental variance (Table II). These are environmental circumstances that cause differences between unrelated individuals but not between members of the same brood. They could be due to tank effects or maternal effects, but evidence has already been presented that tank effects are unimportant. Hence, the differences in susceptibility between different broods is mainly due to the different mothers, i.e. maternal effects.

TABLE V

Analysis of variance of infection levels in Xiphophorus maculatus produced from half-sib breeding programme and exposed to Ichthyophthirius multifiliis at 24°C. Data transformed by square root.

Source	df	SS	MS
Between sires	2	67.0	33.5
Between dams within sires	6	4,528.2	754.7
Between progeny within dams	124	459.8	3.7

IV. DISCUSSION

The system described allows accurate quantification of infection doses and resulting infection levels on the fish. In one red wagtail strain of X. maculatus we found a heritability of nil for susceptibility to white spot infection. There are several possible reasons for this:-

(a) Only a few males were used in the breeding programme. More breeding groups were set up originally, but several failed to produce any offspring.

(b) Inbreeding in producing this strain may have depleted any additive genetic variation. Using electrophoresis, Morizot and Siciliano (1982) have demonstrated a decline in genetic variability following inbreeding in various stocks of X. maculatus.

(c) Natural selection for resistance may also have depleted genetic variation.

(d) Additive genetic variance may never have existed for this trait.

At present it is not possible to say which of these reasons is most probable. It would be worthwhile examining other domesticated varieties, some of which have been maintained in the laboratory for many generations, and also natural populations. Fish from different drainage systems exhibit little gene flow between them. They show genetic differentiation and possibly incipient subspeciation is occurring (Morizot and Siciliano, 1982). In any future heritability estimations, precautions should be taken to ensure a greater number of half-sib groups.

The major point of interest from this investigation is the demonstration of maternal effects affecting susceptibility to infection by white spot. They are the largest maternal effects recorded in fish. They are of particular interest in that their effects are demonstrable in offspring which are a year old and have grown to maturity.

Other workers who have identified maternal effects in fish have been examining eggs or young fry. For example, Ayles (1974) found such effects affecting egg survival and resistance to blue sac disease in splake fry. Kanis et al (1976) found similar effects for egg, alevin, and fry mortality in various salmonids. However, they were unable to determine the magnitude of any tank effects. In our study, tank effects seem to have little relevance.

In viviparous fish, trophic patterns are diverse varying from complete dependence on yolk for nourishment (lecithotrophy), to nourishment by the mother (matrotrophy). In the latter case, the contribution by the mother in diffferent species is very variable (see review by Wourms, 1981). In poeciliids, the primary foetal adaptation is the expanded pericardial sac and its associated portal capillary plexus. This is looped around the neck and is in close association with the ovarian wall of the mother. In Xiphophorus, the pericardial tissue envelops the anterior 1/4-1/3 of the embryo forming a double layered sac (Wourms, 1981). The dry weight of Xiphorphorus embryos remains constant during gestation suggesting a maternal contribution of 30-40% (Scrimshaw, 1945).

What actual evidence is there documenting the passage of organic material into embryos during gestation? Wegman and Gotting (1971) administered an iron-dextran tracer to X. helleri females and were later able to detect it in embryonic tissues. Haas-Andela (1976) studied survival of explanted embryos of X. helleri and X. maculatus. Survival was poor in phosphate buffered saline but good (70%) when organic materials, i.e. glucose, chick embryo extracts, were added to the saline. This therefore indicates a dependency on maternal nutrients during early development. In 1982, Bly was able to detect haemagglutinating activity attributable to antibodies in the eggs of immunised plaice, thus suggesting the possibility of transfer of passive immunity from mother to young in that species. More recently, Bly et al (1983) injected labelled IgM into pregnant X. helleri. After three hours, high molecular weight labelled material was detected in the guts of the developing young.

It seems likely therefore that the young of different female X. maculatus could be subject to variation in prenatal "nutrition", which may have affected their ability to resist white spot infection later in life. Maternal effects could result from either genetic or environmental differences among the mothers causing environmental differences between fish belonging to different broods. It is not known which. It is perhaps worth reiterating that the parents had never been exposed to the pathogen and that the offspring were tested at approximately one year of age.

It is proposed to expand this work on maternal effects and disease susceptibility by investigating other viviparous fish, especially those in the family Goodeidae.

Here complex nutritive and respiratory rectal processes known as trophotaeniae are present. They vary in size and structure in different species so that there is a progressive specialisation during ovarian gestation from strict lecithotropy to extreme matrotrophy (Wourms, 1981). In one species, Ameca splendens, embryonic dry weight increases during gestation by 8,430% (Miller and Fitzsimons, 1971).

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THE SEPARATION AND CULTIVATION OF FISH LYMPHOCYTES

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I. INTRODUCTION

The lymphocyte has over recent years become the centre of interest for immunological and cytogenetic studies. It is easily obtainable from peripheral blood, can be induced to transform into a precursor blast cell which will elaborate various substances such as antibodies and lymphokines, and can also be made to mitose, making chromosomes readily available for karyotype studies. In mammalian immunology, separation procedures are now established and have been well reviewed (Denman, 1973). However, this work is still in its infancy with fish blood.

II. METHODS

LYMPHOCYTE SEPARATION

The aim of any separation technique should be to obtain the maximum recovery of viable lymphocytes in the best condition for subsequent use. The degree of purity required of the suspension depends upon the tests to be performed and in this respect fish blood starts with a significant lead over mammalian blood in respect of the lymphocyte/granulocyte ratio in the peripheral circulation. However, fish lymphocyte markers have not been fully investigated to give a means of characterisation of T and B lymphocytes, comparable to that in humans.

The main separation techniques used are as follows:-

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(1) Centrifugation of diluted blood.
(2) Plasmagel.
(3) Ficoll-Paque gradient centrifugation.
(4) Percoll gradient centrifugation.

Brown trout and carp were used throughout. Blood was collected aseptically from anaesthetized fish by puncture of the dorsal vessel, using a plastic syringe containing a solution of heparin (0.2 ml, containing 500 units heparin). All manipulations were carried out in a Laminar flow cabinet to maintain sterility. 1 ml of blood was diluted with about 4 ml of Hanks balanced salt solution and well mixed before separating by each method below.

(1) Centrifugation of diluted blood.

The diluted blood was placed in a sterile, plastic tube (10 cm x 1.3 cm) and centrifuged at 300-350 rpm, the leucocyte rich supernatant being removed at 5 minute intervals approximately 3 times. It is important to adjust the speed and time of centrifuging to avoid loss of lymphocytes into the erythrocyte layer.

(2) Plasmagel

In this method about 1-2 ml Plasmagel was mixed with the diluted blood before being centrifuged at 40-50 g for about 10 min. The supernatant leucocyte rich plasma was removed and the original tube centrifuged again if necessary. Plasmagel accelerates the sedimentation of the erythrocytes.

(3) Ficoll-Paque gradient centrifugation

The diluted blood was carefully layered on top of 3 ml of Ficoll-Paque in a sterile plastic tube. The sealed tube was then centrifuged at 400 g for 30-40 min in an M.S.E. Chilspin centrifuge, temperature controlled to 8°-15°C. The lymphocytes separate into a thin white band at the plasma/Ficoll interface (Fig. 1). They are then carefully withdrawn with a fine pasteur pipette.

(4) Percoll gradient centrifugation

Percoll was prepared according to the manufacturers instructions to give density layers of 1.042, 1.056, 1.077, 1.084 g/ml. These were formed into a gradient and the diluted blood carefully layered onto it before centrifuging at 450 g for 15 min with an angle head rotor. The lymphocytes separated into two layers as shown (Fig. 2), these being removed with a fine pasteur pipette.

The cells recovered in the last two methods require washing in Hanks solution, prior to use, to free them from either Ficoll or Percoll.

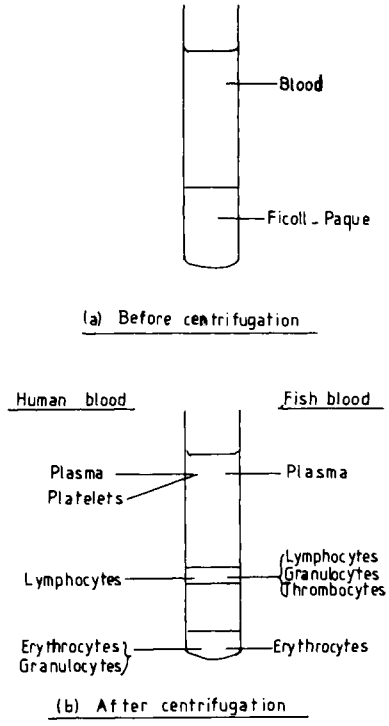

Fig. 1. Lymphocyte separation on a Ficoll-Paque gradient.

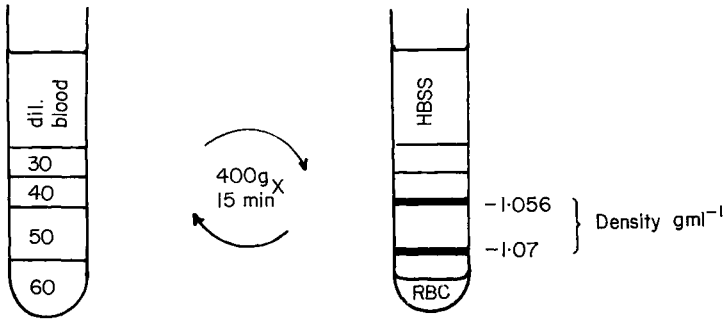

Fig. 2. Lymphocyte separation on a Percoll discontinuous gradient.

LYMPHOCYTE CULTURE

The most suitable procedure for culture was as follows (Blaxhall, 1983):

(a) The blood was mixed with Hanks balanced salt solution (HBSS) in the ratio of 1 vol blood: 3 vols HBSS and the mixing continued over a period of 5-10 min at 4°C.

(b) The lymphocytes were then separated over a Ficoll-Paque gradient.

(c) The cells were washed once in 5 ml HBSS at 8°C for 10 min at not more than 100 g.

(d) After resuspending the cells in 1 ml medium they were counted and their viability assessed by trypan blue exclusion.

(e) The lymphocytes were then added to the complete medium to yield a final concentration of 1-2 x 10^6 ml^{-1} in a 3ml vol in 10 ml sterile, capped plastic tubes.

(f) Addition of phytohaemagglutinin-purified (PHA-P) to yield a final concentration of 0.001 units ml^{-1} for trout and 0.005 units ml^{-1} for carp. (When PHA-M, reagent grade PHA was used, the optimum concentration was 25 μl ml^{-1}.)

(g) The cultures were incubated at 19-20°C for 5 days mixing gently every day. The atmosphere in the tubes was either air or 0.5% CO_2 in air.

The most suitable media for use (in order of preference) was found to be McCoy's 5A (Modified), Medium 199, or Eagles minimum essential medium supplemented with 2% of a 200 mM glutamine solution and 0.2 mgl^{-1} cholesterol. There seemed to be a preference by the cells for media containing Hanks salts and sodium bicarbonate levels of about 350 mg l^{-1}. The addition of 20% serum was essential and was supplied as foetal calf serum; for some unknown reason newborn calf serum was not found to be suitable. Different batches of foetal calf serum were found to vary in ability to support the growth of the lymphocytes.

III. RESULTS

The results of separating lymphocytes from the blood of a limited number of carp (Cyprinus carpio) and brown trout (Salmo trutta) by the first three methods are summarised in Table 1.

Table 1: Viability of the lymphocytes by all the methods was greater than 95% by trypan blue exclusion.

Species	Method	Mean recovery of lymphocytes %	S.D.	Range	Number of samples
Trout	Standard	32.5	21.0	5-98	22
Carp	Ficoll-Paque	26.0	21.4	3-95	18
Trout	Plasmagel	3.2	9.4	0-32	11
Carp		9.0	5.7	0-20	12
Trout	Medium only	36.5	21.6	11-64	7
Carp	(Centrifugation)	42.5	17.9	16-68	8

Table II shows the lymphocyte recovery from brown trout following separation on a Percoll gradient.

Table II: Mean % total lymphocyte recovery following separation on a Percoll discontinuous gradient.

	Lymphocyte count x 10^6/ml			% Total Lymphocyte recovery
	Whole blood	Percoll Density 1.053 g/l	1.07 g/l	
Mean	21.2	2.84	7.2	48.3
S.E.	1.3	0.35	0.5	2.3

IV. DISCUSSION

LYMPHOCYTE SEPARATION METHODS

Many factors have been shown to affect the separation of cells, such as pH, osmolarity of the gradient, cell content, volume and dilution of the sample (Denman, 1973).

The Plasmagel method has the disadvantage of yielding a mixed suspension containing lymphocytes, granulocytes, thrombocytes and a very variable number of erythrocytes. The method needed careful controlling of the centrifugation speed to avoid undue sedimentation of lymphocytes and also great care in aspirating the suspension of cells to avoid gross contamination with erythrocytes. With care, the erythrocyte contamination was generally 2-5%. The method would seem more applicable for use with larger volumes of blood but as the results indicate the recovery of lymphocytes using medium alone is much better. The addition of Plasmagel to fish blood does sometimes result in delayed coagulation, possibly due to the calcium content of the preparation. This is especially so when aspiration of the blood is difficult or delayed. The coagulum resulting from Plasmagel was not always prevented by increasing the amount of heparin used. Another disadvantage is the unsuitability of Plasmagel for use at low temperatures.

The method whereby the blood is simply diluted with medium with no other additives included has certain advantages. These include low centrifugation speeds and no additives which could damage the cells or their receptors. The recovery of lymphocytes by this method from trout blood paralleled that of the standard Ficoll method, while using carp blood it gave a better recovery rate. However, although the recovery rate was high the contamination with erythrocytes was very variable, but with attention to technique this could be reduced to 2%. It does, however, need to be borne in mind that the suspension will contain all types of leucocytes together with thrombocytes, and a variable amount of autologous plasma. This is a simple and efficient separation method but it requires attention to technique in respect of the dilution of the blood, the height of the column of blood-medium mixture, and the speed and duration of centrifugation.

To obtain high yields of relatively pure lymphocytes consistently and easily, the standard Ficoll method is the one of choice. Although the figures in Table 1 show the mean recovery of lymphocytes to be no better than the

previously discussed methods, the range indicates that higher recovery rates are possible with the Ficoll method. This was borne out in practise as the recovery rates rose as experience was gained in the technique involved. This method does have the advantage that the lymphocyte layer is separated from the erythrocytes by the relatively wide layer of Ficoll.

Using the Percoll discontinuous gradient, the fish lymphocytes separated into two sub-populations differing in density. This difference may reflect a difference in function which could be used to assess cell-mediated and humoral immunity as a monitor of infection or pollution (Koller, 1979). Stimulation of these sub-populations with PHA-P (Purified Phytohaemagglutin) showed that the cell layer of density 1.056 g/ml contained more immuno-competent T cells than in the layer of density 1.07 g/ml. The mammalian T and B cell sub-populations can be separated using a Percoll gradient, but it is likely that the divergence between lymphocyte sub-populations in teleosts is at an early stage, since the sub-populations are clearly functionally different but cannot as yet be separated by virtue of density or surface markers. The indications are that fish T cells are found mainly in the 1.056 g/ml density layer and the B cells presumably in the 1.07 g/ml density layer. This appears to be the reverse of that found in the human system (Gutierrez et al., 1979). It is possible that the divergence of two lymphocyte sub-populations in fish has not occurred at this level of evolution and therefore, lymphocytes of differing density separated on Percoll may represent different stages of development of the same cell line. McKinney et al. (1976) suggested that a single lymphocyte type may be responsible for immunological functions in fish.

Transmission electron microscopy indicated that two clearly distinct cell types were present, one being moderately villous lymphocytes and the other smooth lymphocytes. Moderately villous cells predominated in the density layer 1.07 g/ml, with smooth lymphocytes in both layers. An interesting feature of the villous lymphocytes was their higher mitochondrial content compared to the smooth lymphocytes.

LYMPHOCYTE CULTIVATION

The use of peripheral blood culture has been investigated for some species by a number of workers and is well established in medicine for a variety of purposes. A review of the literature for mammalian lymphocyte culture

shows that many factors are important which may affect these techniques. There is also indication that methods may need modifying to suit different animal species.

Factors affecting lymphocyte culture

The cultivation of fish lymphocytes appears to have similar factors affecting it, as in mammals. Although culture conditions can be reasonably controlled, there are a number of variables which make consistency impossible. Many of the factors can have significant effects on the cyclic AMP responses of culture cells and during the past 15 years, the importance of cyclic nucleotides in lymphocyte proliferation and differentiation has become apparent (Hadden, 1977). It has been suggested that cAMP is associated with inhibition of lymphocyte proliferation and that cGMP is involved in induction of lymphocyte proliferation (Hadden, 1978). Calcium has also been reported as important in lymphocyte proliferation (Parker and Lukes, 1971). So changes in intracellular cAMP, cGMP and calcium in the cell have been suggested as the regulatory signals for cell growth and replication (Schonhofer and Peters, 1977). A summary of their action has been given by Berridge (1967).

One source of variability is the fish lymphocyte population itself. So little is known about their types (T and B), responses to mitogens, functions and possible sub-populations that one cannot at present work with a population of known types. The optimum cell density for culturing fish lymphocytes was about 1-2 x 10^6/ml. Although this is the optimum density for economy of cells, the upper limits are more than double this figure. Lowering the cell density has a greater effect in reducing the mitotic index. The round bottomed tubes used, allowed good cell to cell contact without undue overlapping when between 2.5-5.0 x 10^6 cells were contained in a 3 ml culture volume.

The atmosphere required for fish lymphocyte culture differs from that of mammals in that they require either air or about 0.5% CO_2 in air (Fig. 3). 5% CO_2 in air seems to be inhibitory.

The dosage of mitogen, especially PHA, required to obtain optimum mitosis would seem to be about twice that required for mammals when using reagent grade PHA, i.e. about 25 μl/ml (Figs. 4 and 5). When using purified PHA, the optimum is about 0.001-0.01 units/ml, the response showing a plateau effect, not seen with human lymphocytes (Figs. 6 and 7). The reason for the higher dose required

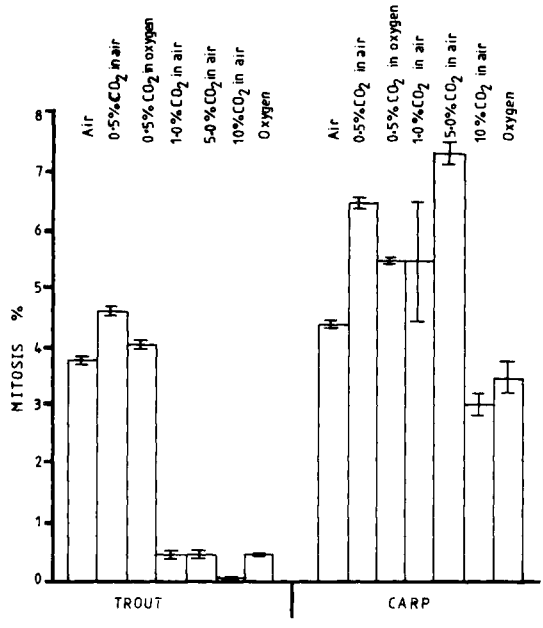

Fig. 3. The atmospheric requirements of trout and carp lymphocytes in culture. The cell concentrations were in the range 1-5 x 10^6/ml, and PHA-M was used at a concentration of 25 μl/ml.

in fish may be a function of the greater number of lymphocytes contained in a unit number of leucocytes than in mammals, thus requiring more PHA to be present for reaction with the increased number of lymphocytes. When using reagent grade PHA with fish lymphocytes it was found impossible to obtain mitosis consistently, although the lymphocytes showed morphological changes indicating that stimulation had commenced. Whereas when purified PHA was used the cells consistently proceeded to mitose, which would indicate that certain impurities were present in the reagent grade, causing an elevation of intracellular cAMP or preventing calcium uptake by the cell. This effect is not so pronounced with mammalian cells.

Possibly as great a variable factor as lymphocyte population is the serum added to the culture medium, which is seemingly essential to growth. Reports of inhibitory substances present in human serum affecting lymphocyte growth are available (Humphrey et al., 1974; Fitzgerald and Hosking, 1976). Serum has also been reported to lower

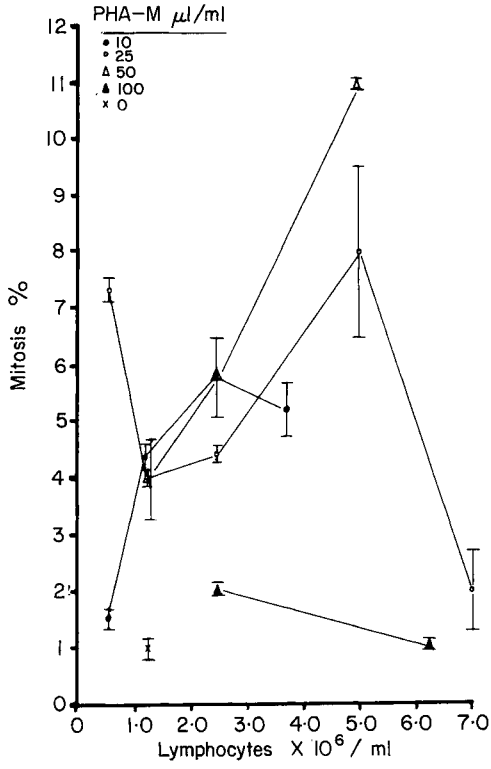

Fig. 4. Mitogen (PHA-M) dose-response curve for trout lymphocytes showing mitosis as a percentage of the total lymphocytes, against lymphocytes in culture at various mitogen doses.

based cAMP levels in a number of mammalian cell culture systems (Chlapowski et al., 1975). Routinely foetal and newborn calf serum are used to enrich cell culture media, though there are few established facts as to what it supplies which seem to be essential. It was found that newborn calf serum was inhibitory, whereas foetal calf serum was not. The effect of serum on fish lymphocytes in culture affects trout to a greater extent than carp. Trout serum added to cultures seemed to be inhibitory, whereas carp lymphocytes seemed to be more tolerant of the serum species. While this inhibition of mitosis with serum from the same species seems difficult to understand, it has been reported with other species of fish (Kang and Park, 1975). It would seem that there may be a close connection

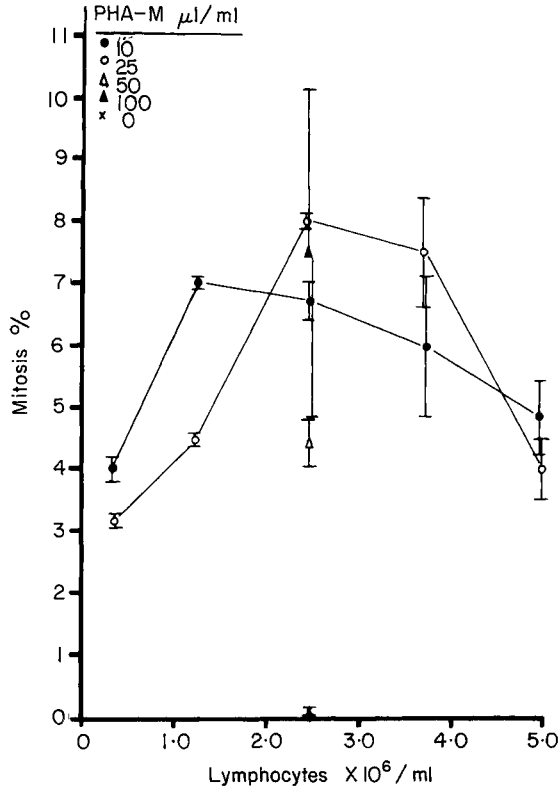

Fig. 5. Mitogen (PHA-M) dose-response curve for carp lymphocytes showing mitosis as a percentage of the total lymphocytes, against lymphocytes in culture at various mitogen doses.

between certain serum factors and the possibility of them stimulating a rise in intracellular cAMP thus preventing mitosis. Work is needed to identify the factors supplied by serum which are necessary for growth and the provision of these in a synthetic form. One such medium devised for use with human lymphocytes is Iscoves modified Dulbecco's medium (Iscove and Melchers, 1978). Although this was tried with fish lymphocytes, no significant improvements were obtained and there are considerable difficulties involved in the preparation of the serum alternative. It is known that serum from very young animals is relatively low in certain types of lipids which accounts for the great variability between different sera. If the medium is unable to supply lipids in a suitable form to maintain the

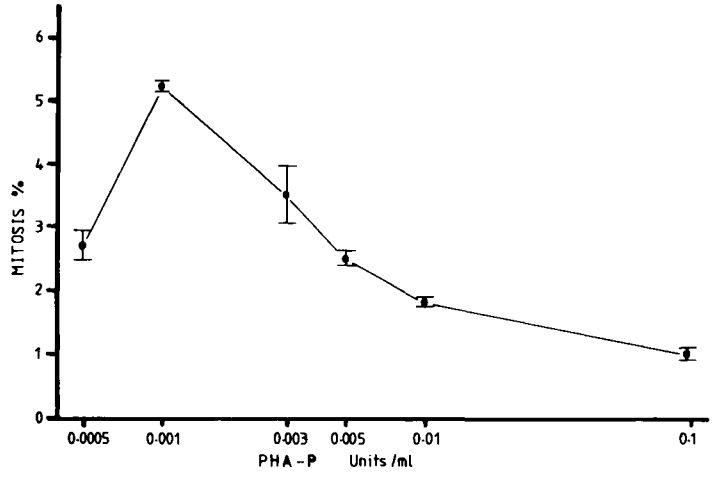

Fig. 6. The effect of PHA-P on trout lymphocytes in culture. Lymphocyte concentrations used were 1-2 x 10^6 ml^{-1}. Mitosis is expressed as a percentage of the lymphocytes in the culture.

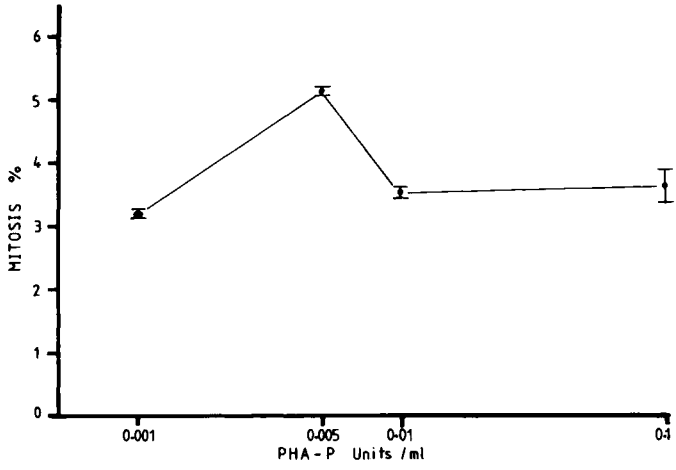

Fig. 7. The effect of PHA-P on carp lymphocytes in culture. Lymphocyte concentrations used were 1-2 x 10^6 ml^{-1}. Mitosis is expressed as a percentage of the lymphocytes in the culture.

integrity of the cell membrane, this would allow the escape of cGMP from the cell as it is formed and possibly arrest the mitotic cycles. It was therefore found advantageous to add cholesterol (0.02 mg/l) to the McCoys medium, in addition to the foetal calf serum (Figs. 8 and 9).

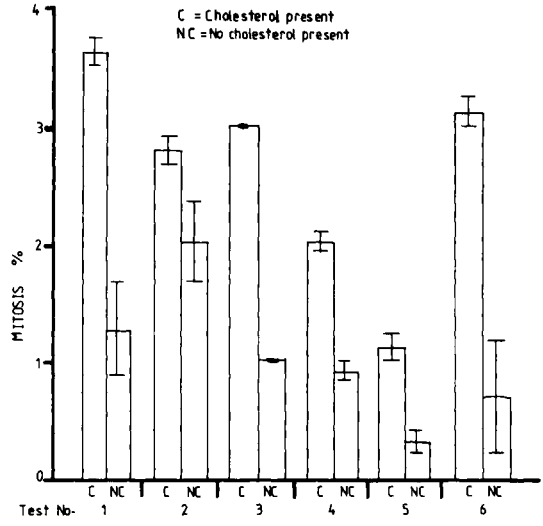

Fig. 8. The effect of cholesterol (0.02 mg l^{-1}) in the medium on the mitotic response of trout lymphocytes in culture. Mitosis is expressed as a percentage of the lymphocytes in the culture. C-Cholesterol present; NC - no cholesterol present.

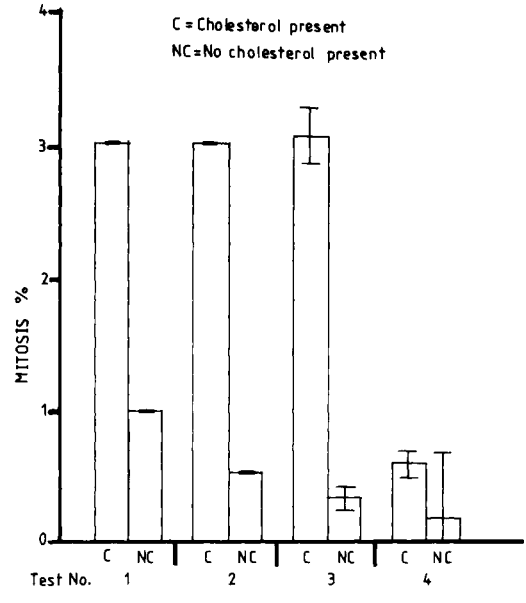

Fig. 9. The effect of cholesterol (0-02 mg l^{-1}) in the

medium on the mitotic response of carp lymphocytes in culture. Mitosis is expressed as a percentage of the lymphocytes in the culture. C - Cholesterol present; NC - no cholesterol present.

V. CONCLUSIONS

The establishment of a fish lymphocyte culture technique giving as consistent results as with mammalian lymphocytes appears to be difficult. However, having examined some of the factors affecting fish lymphocytes in culture, it is possible to establish a technique for the species studied. Other species may require the establishment of different criteria for cultivation. The areas still requiring further investigation would seem to be as follows:

(1) The development of a suitable alternative to serum.

(2) An investigation of the action of cell nucleotides in mitogen stimulated fish lymphocytes, together with their subsequent loss as a result of possible depletion of membrane lipids.

(3) The application of lymphocyte culture techniques in monitoring the effects of environmental pollutants and in fish cytogenetics.

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DO FISH HAVE INTERLEUKINS?

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Regulatory factors play an important role in the immune response. In several mammalian species these regulatory products could be detected in supernatants of mixed lymphocyte cultures as well as mitogen-stimulated leucocyte cultures (Andersson et al., 1979; Gillis et al., 1980). In mammals there are at least two important immunoregulatory factors: interleukin 1 (IL-1) and interleukin 2 (IL-2). The macrophage-derived product IL-1, (Smith et al., 1980), together with antigen or mitogen, triggers the T-helper cell to produce IL-2. Only activated cells are responsive to the latter factor which maintains their proliferative capacity (Farrar et al., 1980).

In fish immunoregulatory factors may play an important role in the immune response as they do in mammals. Recent results confirm the existence of growth-promoting factors which are present in supernatants of PHA-activated pronephric leucocytes from carp (Cyprinus carpio L.) (Grondel and Harmsen, 1984). The mitogenicity of this factor(s) is restricted to purified lymphoblasts. Freshly isolated leucocytes are unresponsive. Furthermore, the growth-promoting activity can be reduced by absorption of the supernatant with mitogen-stimulated blasts.

It is concluded that carp leucocytes can produce and release growth factors in response to mitogen stimulation. The role of these products in the regulation of the immune response in fish and the homology with the mammalian interleukins is being investigated.

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SUPPRESSION BY CORTISOL OF THE MITOGEN-INDUCED PROLIFERATION OF PERIPHERAL BLOOD LEUCOCYTES FROM PLAICE, PLEURONECTES PLATESSA L.

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I. INTRODUCTION

Outbreaks of disease in fishes are frequently associated with environmental stressors, although a causal relationship between stressors and disease is less often established (Wedemeyer, 1970; Snieszko, 1974; Wedemeyer and McLeay, 1981). Since stressors commonly, but not universally, cause increased activity in the hypothalamo-pituitary-adrenocortical system (Donaldson, 1981) it has been proposed that corticosteroids are important mediators of the stressor's action. Treatment of fish with cortisol can cause increased incidence of disease (Robertson et al., 1963; Roth, 1972) although it is questionable whether the plasma levels of steroid in these studies were physiological. Evidence that physiological levels are effective has been provided recently by Pickering and Duston (1983). Using non-stressful methods for steroid administration, they have shown that moderately elevated levels of plasma cortisol do increase the susceptibility of brown trout, Salmo trutta L. to infections of Saprolegnia and Aeromonas salmonicida.

Although evidence from fish is lacking, the disease-related action of corticosteroids in mammals almost certainly includes immunosuppressive mechanisms such as macrophage inhibition, sequestration of lymphocytes and inhibition of lymphocyte function (Ellis, 1981). In mice exposed to stress, lymphocyte proliferation

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in response to mitogens is depressed during the period when plasma cortisol is elevated (Monjan and Collector, 1977). Furthermore, corticosteroids at physiological levels can suppress the mitogen-induced transformation of mammalian lymphocytes (Smith, et al., 1977; Staples et al., 1983).

The present study was undertaken to investigate the effect of cortisol on the mitogen-induced proliferation of peripheral blood leucocytes of plaice.

II. MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Wild plaice Pleuronectes platessa L. were trawled from inshore waters off the coast of Anglesey, Gwynedd and held in a recirculating seawater aquarium for at least two days prior to use. No attempt was made to feed the fish since plaice seldom start feeding until 1-2 weeks after capture. Selection of fish was restricted to immature males and females which had never matured. Fish were 24-31 cm in total length and aged 2-4 years.

PREPARATION OF LEUCOCYTES

Prior to blood sampling the area of skin overlying the caudal vessel was swabbed with absolute alcohol to minimise microbial contamination and to remove traces of mucus. Within 2.5 min of netting the fish, blood was drawn into a sterile syringe containing 2 volumes of sterile, ice-cold balanced salt solution and heparin at a concentration of 500 U ml^{-1}. The samples were mixed and layered on top of a sterile, discontinuous gradient of standard isosmotic Percoll (Pharmacia) diluted to 30, 60 and 100% with balanced salt solution. After centrifugation at 450g for 10min leucocytes separated into two distinct bands, one of density < 1.087 and > 1.075, and one of density < 1.075 and > 1.062. The ratio of lymphocytes: thrombocytes: granulocytes in the more dense band was approximately 8:1:6, and in the less dense band was 60:30:4. Attempts at total separation of lymphocytes from other leucocytes were unsuccessful. In all cases the two bands were pooled to produce a heterogeneous leucocyte mixture free of erythrocytes. After a preliminary cell count by haemocytometry, leucocyte suspensions were diluted with medium to give a final concentration of from 2×10^6 to 5×10^7 cells per 100 μl. 100 μl of cell

suspension were dispensed into round-bottomed wells of sterile microtitre plates (Linbro, Flow Laboratories) containing either 50 μl of medium or 50 μl of mitogen in medium. Sterile covers were placed on the plates which were then incubated in sealed sandwich boxes for 5-7 days in an O_2 atmosphere saturated with water vapour.

CULTURE MEDIUM

RPMl 1640 medium with L-glutamine (Flow Laboratories) was buffered with 17mM l^{-1} of HEPES (Sigma), 9mM l^{-1} HCO_3 and adjusted to pH 7.3. The standard medium was supplemented with Ca, Mg, and Na to mimic the elecrolyte and osmotic concentration (340 mOsm l^{-1}) of plaice plasma, and further supplemented with 10% heat-inactivated foetal calf serum (Wellcome), 5% pooled plaice serum, 0.1g l^{-1} of streptomycin sulphate (Sigma) and 1000,000 Units l^{-1} of Na penicillin G (Sigma).

All glassware with which cells came in contact was siliconised and autoclaved. Balanced salt solution and culture medium were filtered through 0.22 μm Millipore filters. All manipulations except blood sampling and centrifugation were conducted in a laminar flow cabinet.

MITOGENS

Wheat germ lectin (Sigma) and pokeweed mitogen (Flow Laboratories) were made up freshly in medium on each occasion. Mitogen concentrations refer to final concentrations in the wells.

MIXED LEUCOCYTE CULTURE

Pairs of plaice were segregated in separate tanks and whole blood (0.05 ml) was exchanged between the members of each pair. After 10-13 days blood was withdrawn for the coculture of leucocytes from each pair of plaice. Since cells were not irradiated, leucocytes from both members of the pair could respond to the presence of the other. Experimental wells contained 100 μL of cell suspension from each member of the pair (total 200 μL). Two sets of control wells were run, each well containing 100 μL of cells from only one member of the pair.

MEASUREMENT OF CELL STIMULATION

Preliminary attempts at measuring cell stimulation by the

method of ^{3}H-thymidine incorporation were unsuccessful due to a proportion of the cells failing to adhere to the glass fibre filters. Cell proliferation was measured by normal haemocytometry. Wells were counted in triplicate and results were expressed as the stimulation index (number of cells in stimulated cultures ÷ number of cells in non-stimulated cultures). In the case of mixed leucocyte cultures the index was calculated from the expression:- number of cells in experimental wells ÷ Sum of the number of cells in the two control wells.

III. RESULTS

Preliminary experiments showed that, using the haemocytometer method, no proliferation of cells was detectable until 4-7d (mixed leucocyte culture, Fig. 1), 7d (pokeweed mitogen, Fig. 2) and 5-7d (wheat germ lectin, Fig. 3). Furthermore, the magnitude of the response was small and never exceeded a mean stimulation index of 3.

The speed and magnitude of the response to wheat germ lectin was similar at all three concentrations of lectin tested, 6,60 and 600 $\mu g\ ml^{-1}$ (Fig. 4). Within this range of concentrations no dose response relationship was established. Similarly there was no difference in the response of cells when tested with pokeweed mitogen at concentrations of 0.03, 0.3 and 3 $\mu g\ ml^{-1}$ (Fig. 5).

THE EFFECT OF CORTISOL

Leucocytes were cultured with pokeweed mitogen at 2.6 $\mu g\ ml^{-1}$ and with cortisol at concentrations ranging from 0 to 2000 ng ml^{-1}. In the absence of exogenous cortisol or in the presence of very low levels (2 ng ml^{-1}), leucocytes showed a pronounced proliferative response to pokeweed mitogen (Fig. 6). With cortisol levels of 20 and 200 ng ml^{-1} cell proliferation was increasingly suppressed. Further elevation of cortisol levels to 2000 ng ml^{-1} caused no greater suppression.

IV. DISCUSSION

The modest cell response and its slowness may be partly attributable to the method used for its measurement. All leucocytes were counted, but of these only a proportion of the lymphocyte fraction probably responded to mitogens. The diluting effect of the non-responding leucocytes would

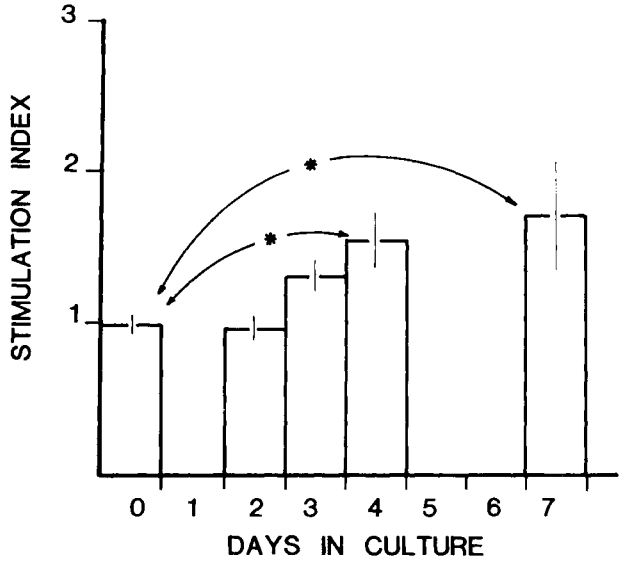

Fig . 1. Mixed leucocyte response of cocultured cells from paired plaice. n equal to 5 pairs. * $p < 0.05$.

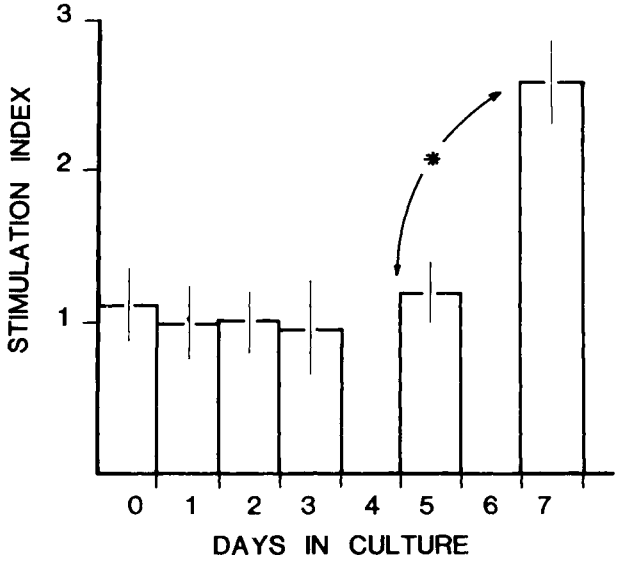

Fig. 2. Leucocyte proliferation in response to pokeweed mitogen at a concentration of 3 $\mu g\ m^{-1}$. n equal to 5 fish, except for day 7 when n equals 4. * $p < 0.05$.

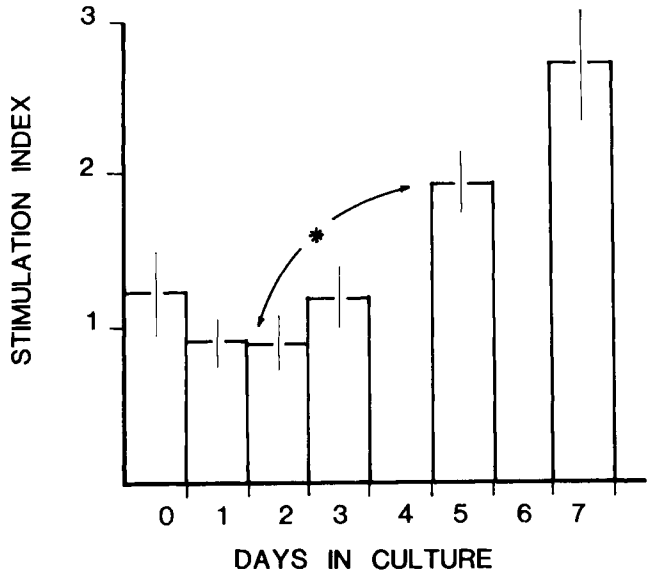

Fig. 3. Leucocyte proliferation in response to wheat germ lectin at a concentration of 600 μg ml^{-1}. n equal to 5 fish. * $p < 0.05$.

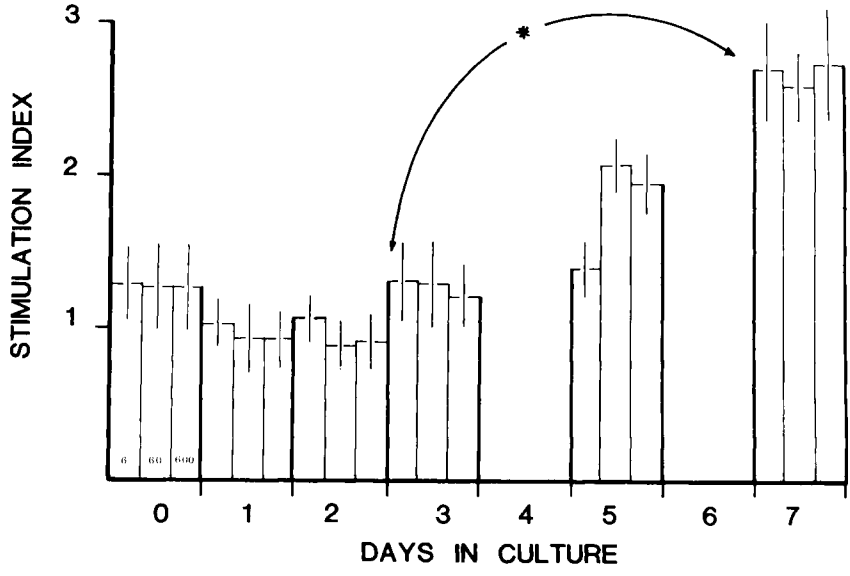

Fig. 4. Leucocyte proliferation in response to wheat germ lectin at concentrations of 6, 60 and 600 μg ml^{-1}. n equals 4 fish. *$p < 0.05$.

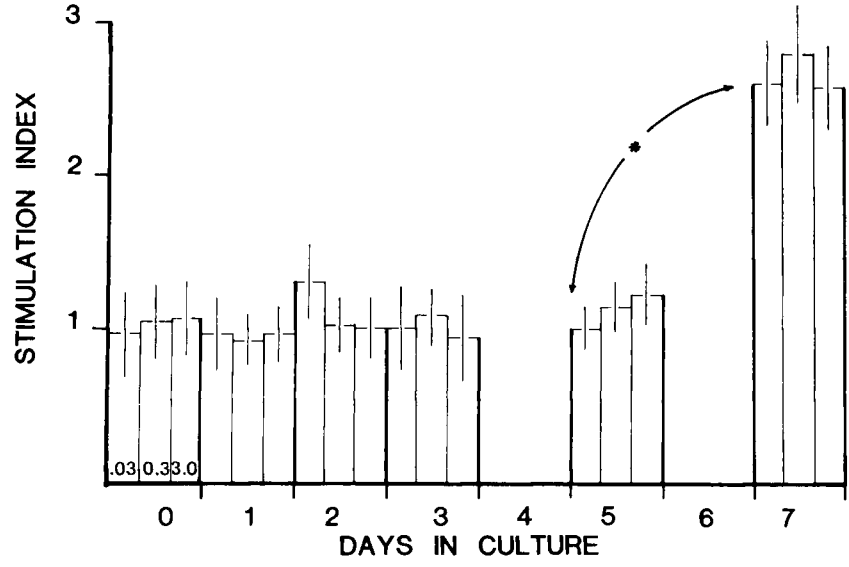

Fig. 5. Leucocyte proliferation in response to pokeweed mitogen at concentrations of 0.03, 0.3 and 3.0 g ml^{-1}. n equals 5 fish except for the 3 μg wells on day 7 when n equals 4. *$p < 0.05$.

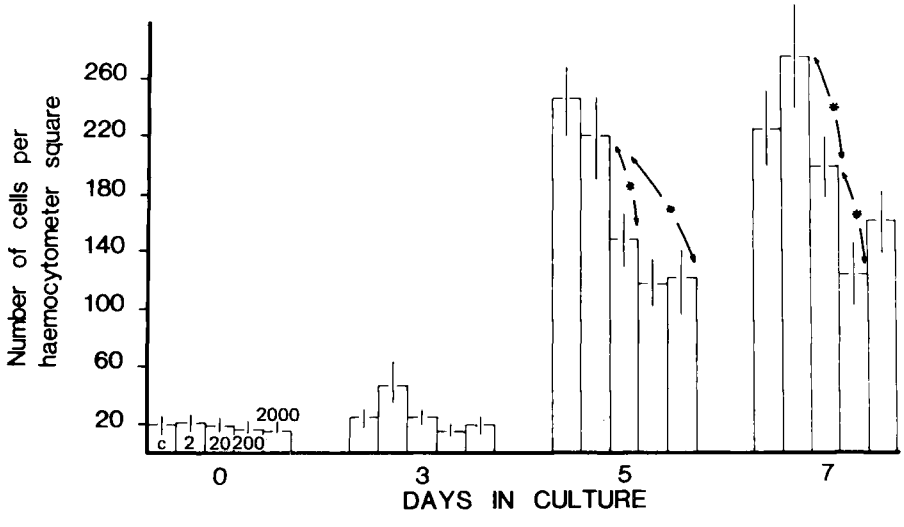

Fig. 6. The effect of cortisol on PWM-stimulated leucocyte proliferation. C - control without cortisol. Numerals 2 to 2000 refer to concentrations of cortisol in ng ml^{-1}. Concentration of PWM - 2.6 μg ml^{-1}. *$p < 0.05$.

explain the weak response which never exceeded a mean of 3, in contrast to stimulation indices of up to 13 for relatively pure lymphocyte preparations used by Smith and Braun-Nesji (pers. comm.). The slowness of the response may be partly due to the same diluting effect but also to the fact that haemocytometry will detect no response until a leucocyte has completed cell division, whereas by using the method of ^{3}H-thymidine incorporation a response will be detected much earlier in the cell cycle.

The absence of a dose response relationship for both mitogens suggests that the levels of mitogen chosen may have been inappropriate for the particular cells and incubation conditions although the levels of pokeweed mitogen chosen were similar to those used in mammalian studies. Further work is required to establish a dose response relationship.

With few exceptions (Smith et al., 1977; Pearson et al., 1978; and Staples et al., 1983) most studies which have demonstrated an inhibitory action of corticosteroids on lymphocyte function have used high pharmacological doses of steroid. The significance of the present work lies in the fact that the inhibitory action of cortisol occurs at levels of hormone which are physiological. Blood levels of cortisol in long-term, tank-adapted plaice range from 1-150 ng ml^{-1} (Dawson, 1978) which is similar to the range within which there is progressive inhibition of proliferation. The fact that physiological levels are suppressive *in vitro* suggests that cortisol may function similarly *in vivo*. It is further suggested that this steroid action may be a significant part of the mechanism by which stressors induce disease. Any such steroid action on lymphocyte function may be complementary to its action on lymphocyte traffic (Slicher, 1961; Pickford et al., 1971; McLeay, 1975; Pearson et al., 1978).

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LAMPREY MELANO-MACROPHAGES: STRUCTURE AND FUNCTION

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INTRODUCTION

Melanin-containing macrophages, usually termed melano-macrophages, have been described in a wide range of fish (Roberts, 1975; Agius, 1979, 1980, 1981). They are often found in haemopoietic/lymphoid tissues and occur either as single cells or in aggregates, termed melano-macrophage centres (Agius, 1979). Many aspects of melano-macrophage biology remain enigmatic. For example, where are these cells formed and what is their relationship to melanin-free macrophages and non-phagocytic melanocytes? Several functions have been ascribed to melano-macrophages including iron storage following starvation (Agius, 1979) and antigen trapping (Ellis, 1980).

In a comprehensive survey on the distribution of melano-macrophages in 72 species of fish (Agius, 1980), these cells were found to be absent from only one species, the river lamprey Lampetra fluviatilis. We recently showed that scattered macrophage-like cells containing a dense brown/black pigment are, in fact, present in adult river lampreys (Page and Rowley, 1984). This pigment is apparently melanin or a related compound as it is bleached by prolonged incubation with hydrogen peroxide, a test claimed to be specific for melanin (Humason, 1972). The present report provides an overview of our studies to date on the structure and functions of lamprey melano-macrophages.

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DISTRIBUTION AND MORPHOLOGY OF LAMPREY MELANO-MACROPHAGES

Melano-macrophages (MMs) are widely distributed in several organs of the adult river lamprey, Lampetra fluviatilis, including the kidney, cavernous bodies of the gills and the supraneural organ. In the liver, although melano-macrophages have not been observed, macrophage-like cells are situated in the sinusoids in a similar position to mammalian Kuppfer cells. In the kidney, both macrophages and MMs are found in the interstitial spaces of the excretory tubules. The largest accumulations of macrophages and MMs are found in the cavernous bodies. These structures are situated at the base of each gill filament and are bordered by a sheath of connective tissue (Nakao, 1978; Yamaguchi et al., 1979; Page and Rowley, 1984). The cavernous bodies form an integral part of the afferent arteries which serve each gill filament and hence blood must pass through a compact filter bed of macrophages and MMs in order to gain access to the secondary lamellae (Fig. 1). Less than 10% of the cavernous body macrophages contain melanin pigment and these cells are scattered throughout the filtration region (Figs. 1,2). Both MMs and macrophages are held in place by collagenous trabeculae which span the cavernous bodies (Figs. 1,2).

The haemopoietic activity of the supraneural organ (=fat column of some authors) is first seen at the end of the larval life when blood cells migrate into this region and infiltrate the resident fat cells (Potter et al., 1982). This process corresponds with the disappearance of the larval protospleen (typhlosole) and it has been suggested that the supraneural organ replaces this structure and serves a similar function during the adult life of the lamprey (Potter et al., 1982). The adult supraneural organ consists of a mass of fat cells, immature and mature neutrophilic granulocytes, fibroblast-like cells, lymphocytes, and erythrocytes as well as macrophages and MMs (George and Beamish, 1974; Kelēnyi and Larsen, 1976; Page and Rowley, 1984). The MMs and macrophages are not randomly distributed throughout the supraneural organ. Instead, they are localised in regions which we term 'vascular areas' (Fig. 3). These vascular areas appear to be the sites where blood permeates through the supraneural organ, as evidenced by the large number of erythrocytes associated with these regions (Fig. 3). Most of the fixed cells in the vascular areas therefore come into contact with 'foreign' material

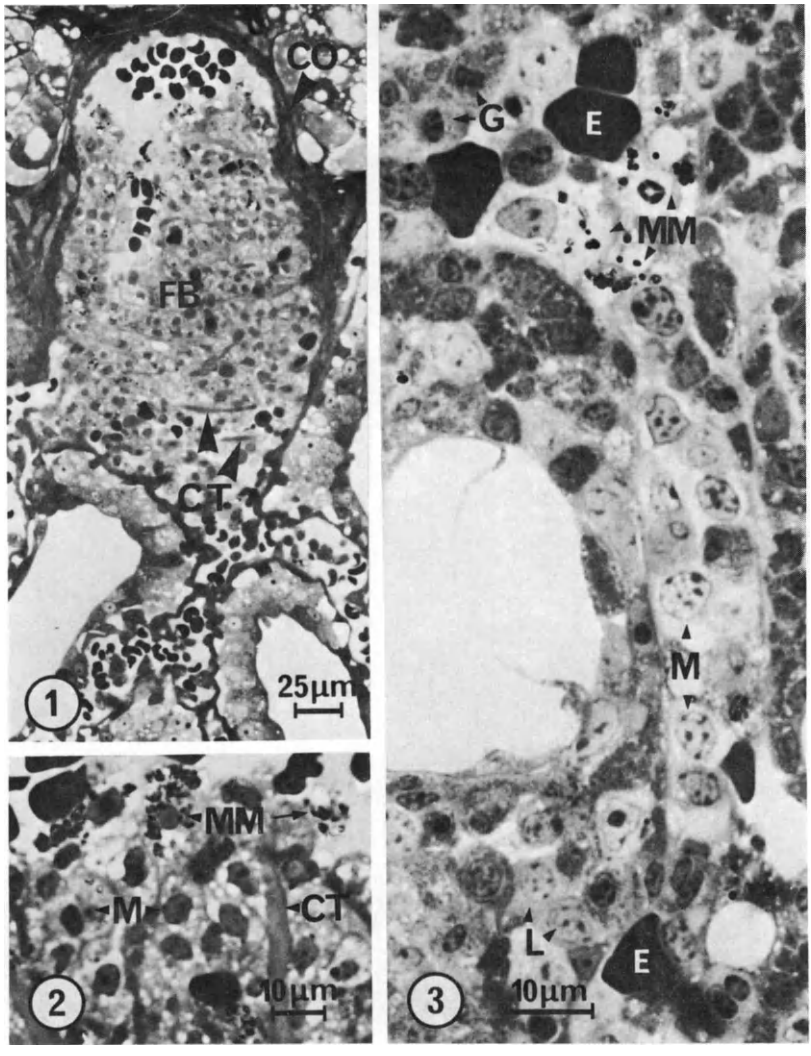

Fig. 1. Gross structure of a cavernous body. Note filter-bed (FB) composed of macrophages and melano-macrophages, collagenous trabeculae (CT) and surrounding connective tissue sheath (CO).

Fig. 2. Thick plastic section through the filter-bed region of a cavernous body showing macrophages (M), melano-macrophages (MM) and collagenous trabecula (CT).

Fig. 3. Thick plastic section through supraneural organ showing vascular region composed of

erythrocytes (E), fixed macrophages (M) and melano-macrophages (MM). In the matrix are neutrophilic granulocytes (G) and lymphocytes (L).

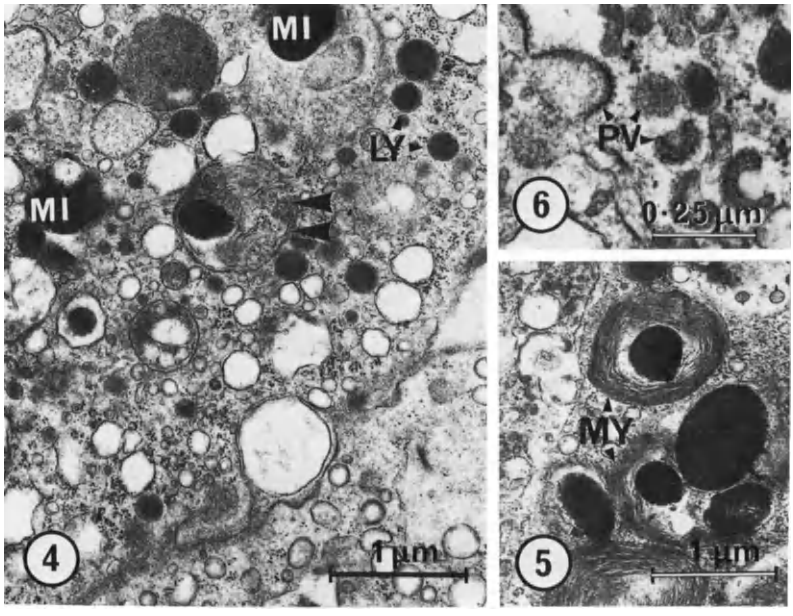

Fig. 4. Electron micrograph of part of a cavernous body melano-macrophage. Note melanin inclusions (MI), primary lysosomes (LY) and secondary lysosome containing a melanin inclusion (arrows).

Fig. 5. Residual bodies characterised by myelin whorls (MY) with associated melanin inclusions.

Fig. 6 Pinocytotic vesicles (PV) associated with the peripheral cytoplasm of a cavernous body macrophage.

passing through this organ.

Melano-macrophages are structurally similar from one tissue to another and this suggests that only a single population exists, developing from the same precursor cells in one haemopoietic centre. Furthermore, apart from the incorporation of melanin inclusions, the MMs differ very little from the normal tissue macrophages. At present, nothing is known concerning the lineage of the

MMs. For example, are these cells formed from a pool of circulating monocytes and which haemopoietic tissues are involved in their production?

Ultrastructurally, the MMs are readily identified by the electron-dense melanin inclusions incorporated within the cytoplasm (Fig. 4). These inclusions are apparently not bounded by a limiting membrane, but the very nature of the electron-dense contents may make visualisation of this membrane difficult. Melanin inclusions are often found associated with the lysosomal system where these structures are seen in both secondary lysosomes (Fig. 4) and residual bodies (Fig. 5). Other organelles within the cytoplasm of MMs (and macrophages) include a moderate amount of rough endoplasmic reticulum, a Golgi complex and numerous primary lysosomes (Fig. 4). The peripheral cytoplasm of MMs is characterised by a large number of pinocytotic vesicles (Fig. 6), consistent with the view that these cells are active in the removal of soluble 'foreign' material from the circulation.

FUNCTIONS OF LAMPREY MELANO-MACROPHAGES

Melano-macrophages are thought to have two main functions, namely, iron storage following erythrocyte destruction (Agius, 1979, 1981) and clearance of soluble and particulate material from circulation (Ellis, 1980). Agius (1979) first demonstrated that iron storage is markedly increased in splenic MMs of many teleosts during a period of starvation, presumably as a result of erythrophagocytosis. Furthermore, if the spleen is surgically removed from these fish, the site of iron storage is assumed by the kidney MMs (Agius, 1981). There has recently been renewed interest in iron storage in lampreys (Macey et al., 1982a, b) and these authors have suggested that at metamorphosis there is extensive breakdown of larval erythrocytes and haemoglobin by the Kuppfer cells and hepatocytes within the liver. Furthermore, during the feeding phase large quantities of iron are likely to be absorbed across the intestine as a result of the blood sucking habit. Also, at the time of spawning, when the animals are non-feeding, extensive erythrophagocytosis is also thought to occur (Percy and Potter, 1976). Hence, iron storage is likely to take place in lamprey macrophages and MMs at several periods of the life cycle. The possible storage of iron in the MMs of spawning-run, sexually immature, river lampreys, L.

fluviatilis has been studied using electron-probe x-ray microanalysis. This technique has several advantages over specific histochemical techniques for iron, including precise localisation of elements even at very low concentrations. The distribution of iron and other elements was determined in the MMs of the kidney, supra-neural organ and cavernous bodies and the results are shown in Figs. 7 and 8. Low levels of iron were found in the MMs from all three tissues (Figs. 7,8). For example, in the cavernous body MMs, similar levels of iron were observed in the melanin inclusions, lysosomes and the cytosol (general cytoplasm), indicating that this element is apparently not stored in any particular area of these

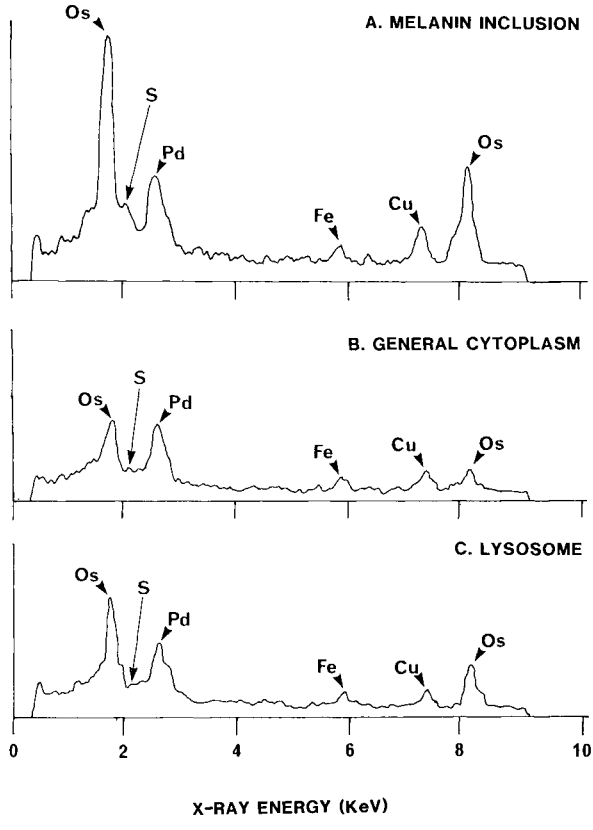

Fig. 7 Elemental spectra of a melanin inclusion, general cytoplasm [cytosol] and lysosome from a cavernous body melano-macrophage. The osmium and palladium peaks originate from the fixative and supporting grid respectively.

cells (Fig. 7). The x-ray spectra also showed significantly higher levels of sulphur in the melanin inclusions than any other region analysed (Fig. 8), perhaps inferring that these organelles contain disulphide linked proteins and/or sulphated compounds. Rather surprisingly, high count rates for copper were also found associated with the melanin inclusions (Figs.7, 8); whether this is an artifact of the method is uncertain but these results were reproducible from one cell to another in all the tissues examined. Hence, our only conclusion from these studies, at present, is that at least during this stage of the life cycle, i.e. pre-spawning, little accumulation of iron occurs in lamprey MMs. In this context, it would be most interesting to elucidate the changes in iron levels within the MMs in post-spawning lampreys in an attempt to correlate this with the reported reduction in the total number of circulating erythrocytes (Ivanova-Berg and Sokolova, 1959).

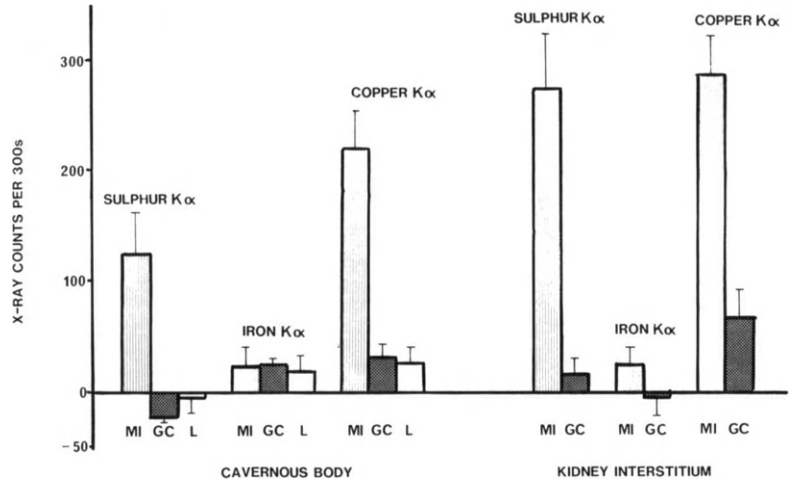

Fig. 8 Number of x-ray counts for sulphur, iron and copper in the melanin inclusions (MI), general cytoplasm (GC) and lysosomes (L) of melano-macrophages from the cavernous body and kidney. The counts given are mean values ±1 S.E., n = 7-14 analyses from 15 cells.

The role of both macrophages and MMs in the clearance of small and large particles has been investigated by the intravascular injection of colloidal carbon and formalin-killed Aeromonas hydrophila NCIB 9240, into adult

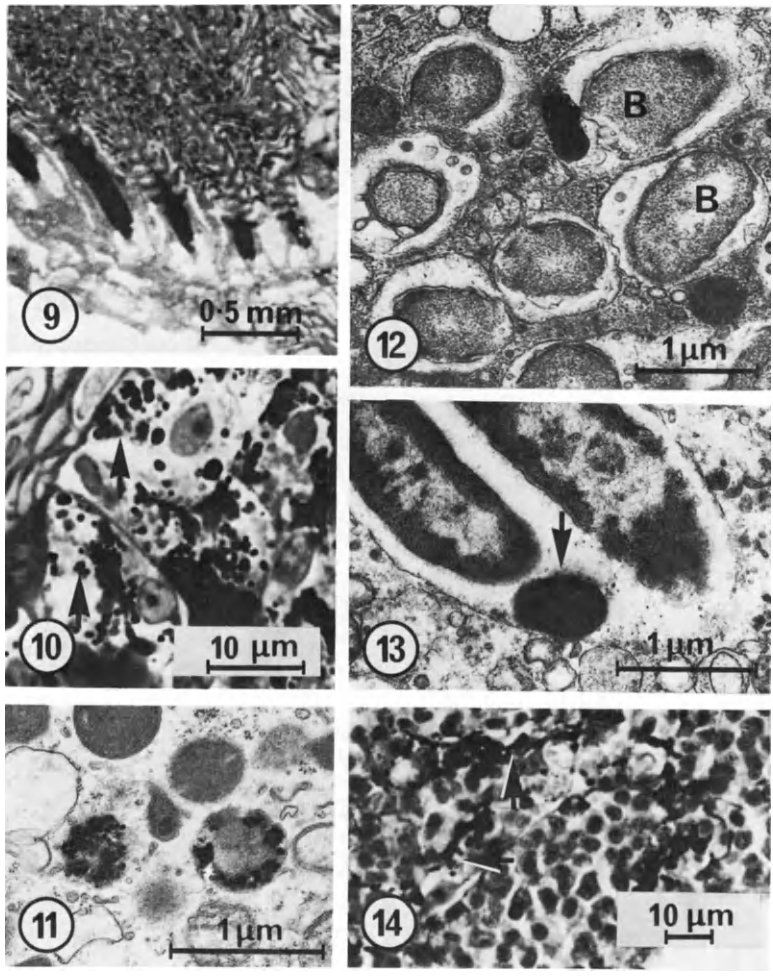

Fig. 9 Low power micrograph showing cavernous bodies replete with carbon 60 min post-injection.

Fig. 10. High power thick plastic section of cavernous body macrophages showing the large amounts of intracellular carbon (arrows). One hour post-injection.

Fig. 11. Electron micrograph of cavernous body melano-macrophage 60 min after the injection of colloidal carbon. Note resultant fusion of lysosomes with carbon-containing phagosomes.

Fig. 12 Cavernous body melano-macrophage laden with A. hydrophila bacilli (B) 24 h after intravascular injection.

Fig. 13 Melanin inclusion (arrow) in a phagosome containing two A. hydrophila bacteria.

Fig. 14 Wax section through the supraneural organ 1 week after the injection of colloidal carbon. Note carbon (arrows) associated with vascular regions.

river lampreys. Both of these particles are rapidly removed from circulation by the macrophages and MMs of the cavernous bodies. For example, 60 min after the injection of colloidal carbon at a dose of approximately 40mg/100g body weight, large deposits of this substance can be found in the cavernous body and a significant proportion of it is already within the macrophages and MMs (Figs. 9, 10). The colloidal carbon is apparently taken up in pinocytotic vesicles and once internalised, fusion of the lysosomes with the carbon-laden phagosomes occurs (Fig. 11). In the case of Aeromonas hydrophila, it is not unusual to find cavernous body macrophages and MMs laden with bacteria (Fig. 12) and occasionally these intracellular micro-organisms are seen in phagosomes enclosing melanin inclusions (Fig. 13). This is an interesting observation particularly as melanin and its by-products are thought to have possible antimicrobial properties (Edelstein, 1971). Far smaller quantities of bacteria and carbon are found in the supraneural organ, kidney and liver macrophages following intravascular injection. This is probably due to the situation where most particles are removed from circulation during the first few encounters with the cavernous body macrophages and MMs, and those remaining are then transported via the vascular system to the reticulo-endothelial centres of the kidney, liver and supraneural organ which finalise the clearance of these substances. In the supraneural organ, both bacteria and carbon come to lie in the macrophages within the vascular areas (Fig. 14) and only limited uptake is achieved by the granulocytes. The arrangement of macrophages in the vascular regions is of significant interest as recent reports have shown that the adult supraneural organ is a major site of antibody synthesis (Hagen et al., 1983) and hence, the macrophages in this organ may well act as areas for the retention of antigen, which then in turn present it to either lymphocytes in the matrix or to those cells in general circulation. The role of the supraneural organ MMs and macrophages in antigen retention/presentation is presently under study.

CONCLUDING REMARKS

Preliminary studies indicate that in the river lamprey, L. fluviatilis, MMs occur as single cells rather than in aggregates ("melano-macrophage centres") as seen in most higher fishes (Agius, 1980). Furthermore, they appear to be both structurally and functionally similar to the normal tissue macrophages, the only difference being that they contain melanin inclusions which may have originated from the pigmented melanocytes. The precise role of melanin in the MMs is at present uncertain, but it may have some antimicrobial properties. It should not be difficult to isolate melanin inclusions from fish MMs/melanocytes to determine whether they possess any antimicrobial/antiparasitic activity.

The arrangement of MMs and macrophages in the cavernous bodies of the gills may have potential phylogenetic interest as similar, though less well-developed (degenerate?) structures are present in some elasmobranchs (Wright, 1973), though they are apparently absent in bony fishes. This may reflect a greater reliance by both lampreys and elasmobranchs on non-specific mechanisms, such as phagocytosis, to deal with pathogenic micro-organisms which use the gills as a site for invasion. In higher fishes, however, with the remodelling of the gills and the development of a more sophisticated immune system, the role of the cavernous bodies may have been adopted by reticulo-endothelial cells associated with the lymphoid tissues where antigen exposure leads to a rapid humoral response. Other aquatic animals, where complex humoral and cell-mediated immune systems are lacking, such as cephalopods and crustaceans, utilise phagocytic cells in the gill region as a mechanism to maintain the animal free from infection (Stuart, 1970; Smith and Ratcliffe, 1980). This similarity with the lampreys is not really surprising, bearing in mind that the gills are a major site for both microbial and macrobial invasion in all aquatic animals and, therefore, part of the immune strategy of such organisms must always be to protect these vital tissues from attack.

We are grateful to Professor J.S. Ryland in whose department this work was carried out and to the University of Wales for a postgraduate studentship to M.P. Part of the equipment used in this study was provided by the Science and Engineering Research Council (Grant No. GR/B/1014.1) and the Royal Society.

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EVOLUTION OF THE THYMUS IN RAINBOW TROUT

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I. INTRODUCTION

Many studies have been devoted to the origin and the description of the fish thymus. The main works were performed at the beginning of this century. Among the various species of fish observed at that time, the thymus of teleostean fish were the most studied (Deanesley, 1927; Lagabrielle, 1928). During recent years immunological studies have renewed interest in the thymus. But it appears that our knowledge of the fish thymus, according to the first descriptions, is insufficient to explain the possible roles played by the thymus in immunological and pathological processes.

With regard to salmonid fish, the histogenesis of the early thymic rudiment was examined by Ellis (1977) and Grace and Manning (1980). Recently, we described the thymus in the rainbow trout fingerling (Chilmonczyk, 1983), and in the present work, we studied its development from hatching to the adult stage.

II. MATERIALS AND METHODS

After hatching in our laboratory the fry were maintained in dechlorinated tap water at 11 $\pm$ 1°C. Developing fish were studied for several months. Sub adults and adults were collected from fish farms.

For histological examination samples were fixed in Bouin's fluid, embedded in paraffin and sectioned serially

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at 5μm.

Tissues for transmission electron microscopy were fixed in 1% glutaraldehyde buffer with Na-cacodylate 0.1M (pH 7.3), post fixed in 1% osmium tetroxyde and embedded in Epon. Thick sections (1 μm) for light microscopy were stained with methyl blue and Azur II. Thin sections were stained with uranyl acetate and lead citrate and observed on a Philips 300 EM under 80KV.

For scanning electron microscopy samples were fixed overnight in 2.5% glutaraldehyde buffer with 0.1 M Na cacodylate. After dehydration samples were dried by a critical point dryer apparatus and coated with gold. Topography of the thymus surface was examined on a Hitachi scanning electron microscope with an accelerating voltage of 15 or 20 KV.

III. RESULTS

HATCHING

At hatching, the thymus is well developed and occupies its adult position. It is a paired organ located in the gill chamber at an angle between the opercular and the head wall (Fig. 1). It is characterized by an homogeneous aspect. The thymocytes are supported by a close network of epithelial cells. At this stage the characteristic intra epithelial position of the gland is obvious (Fig. 2). The organ is non-vascularized and its base is always delineated by the basal lamina of the gill chamber epithelium. The thymus grows inside this epithelium and the thymocytes are separated from the water by a single layer of epithelial cells.

FRY

Thymic vascularization occurs after reabsorption of the yolk-sac. The blood vessels enter the core of the organ from the subepithelial tissue (Fig. 3). They push away the basal lamina of the epithelium and fibrocytes are carried away from the sub epithelial tissue along the blood vessels (Fig. 4). Thus a characteristic epithelio-vascular structure is established: the thymic septum. Each septum is formed by an elongated fold of the sub-thymic zone. This structure penetrates deeply throughout the thymic tissue. Its centre is cystic and contains various cellular types; phagocytic cells, lymphocytes, fibrocytes.

The fact that blood vessels are associated with septa

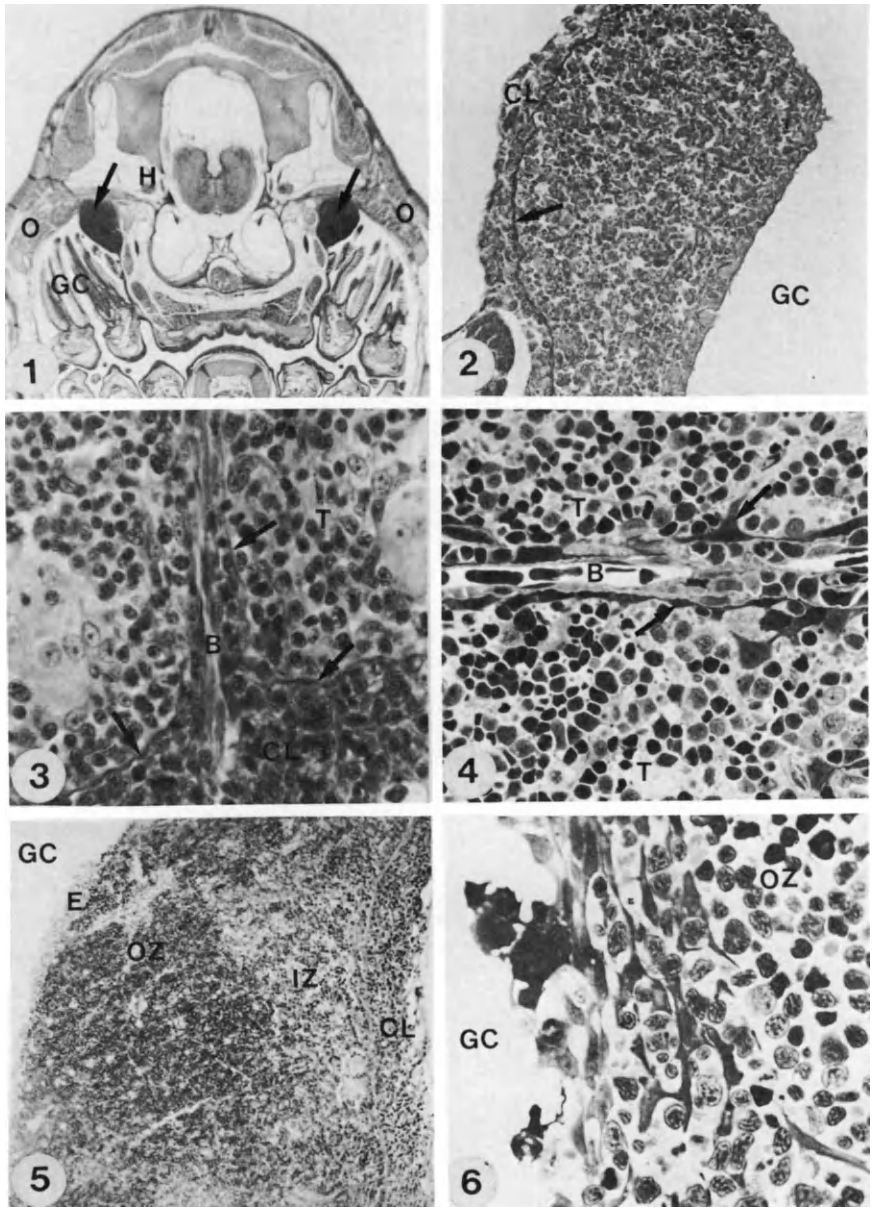
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Fig. 1. Cross section through the head of a rainbow trout fry. The thymus glands (arrows) lie within the gill chambers (GC) in the angle between the operculum (O) and the head (H). (x15)

Fig. 2. Section of the thymus at hatching showing the intra-epithelial localization of the gland. The thymus lies on a connective tissue layer (CL), its base is delimited by the basal lamina (arrow) of the gill chamber (GC) epithelium. (x240)

Fig. 3. Section through the thymus of a rainbow trout fry showing the formation of a thymic septum associated with a blood vessel (B). A fold of the connective tissue layer (CL) pushes away the basal lamina (arrow) of the epithelium and penetrates into the thymic tissue (T). (x540)

Fig. 4. Longitudinal semi-thin section of a septum. A blood vessel (B) occupies the centre of the septum. Thymic tissue (T). Epithelial cells (arrows). (x600)

Fig. 5. Fingerling. Cross section of the thymus. Gill chamber (GC). Epithelial capsule (E). Outer zone (OZ). Inner zone (IZ). Subthymic connective tissue layer (CL). (x480)

Fig. 6. Fingerling. Semi-thin section of the epithelial capsule. The epithelial cell network is more developed than in the outer zone (OZ). Lymphoid cells fill the inter-epithelial spaces and occur in a very apical position. Gill chamber (GC). (x620)

leads to a particular relationship between the blood and the thymic tissue. We described this relationship elsewhere (Chilmonczyk, 1983).

FINGERLINGS AND YOUNG TROUT

We recently described the structure of the thymus corresponding with this stage (Chilmonczyk, 1983). In fingerlings the thymus achieves its complete structure (Fig. 5). The gland lies on a thick sub-thymic connective tissue layer and numerous lymphocytes are dispersed in the fibrocytic network. The thymus itself is composed of 3 adjacent zones; the inner zone, the outer zone, and the

epithelial capsule.

In the outer zone small lymphocytes are the main cellular population and the frame-work of supporting epithelial cells is less developed than in the inner zone.

The epithelial capsule represents the protective zone overlaying the lymphoid tissue. Compared to the fry this layer is enlarged and invaded by leucocytes (Fig. 6). Small groups of leucocytes fill a very apical position and are separated from the water of the gill chamber by a single layer of epithelial cells. We have observed the surface of the thymus and have shown that in fingerlings and young trout, the thymic surface is perforated by many

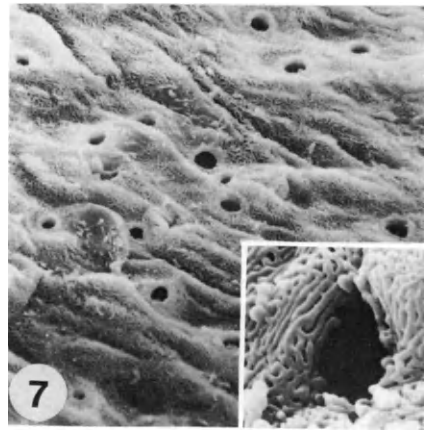

Fig. 7. Fingerling. Scanning electron micrograph of the surface of the thymus showing the characteristic perforated aspect (x960) Insert: a magnification of a hole between 2 microfolded epithelial cells. (x3200)

holes of various sizes (Fig. 7). The maximum size we have measured corresponded to holes of 20 μm diameter.

ADULT

In trout the thymus persists for the whole life of the fish. We never observed involution in 5 year-old rainbow trout. The thymus remains highly lymphoid and its structure is only slightly modified. Neither the outer zone nor the inner zone are clearly delineated. The septa are considerably enlarged (Fig. 8). The septal

peri-vascular space is filled with fibrocytes and collageneous fibrils. The epithelial capsule is thicker and a continuous sheet of mucous cells overlays the organ. Scanning electron microscope observations show a surface devoid of perforations (Fig. 9).

The proliferation of various epithelial components is characteristic of the old trout thymus. In addition to the increase of the epithelial network, histological sections show many mucous cells, myoid cells, and canalicular and ciliated structures, though we have never observed Hassall's corpuscles.

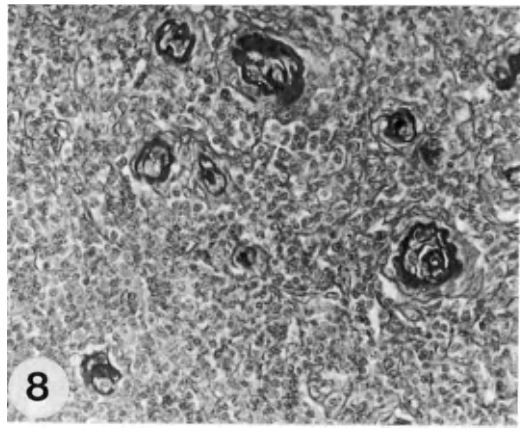

Fig. 8. Adult. Histological section of the thymus. In cross sections the septa appear as deeply stained corpuscles. (x340)

IV. DISCUSSION

The thymus of rainbow trout is a typical lymphoid organ. During the whole life of the fish the thymus fills an intra-epithelial position inside the epithelium of the gill chamber.

The main structural transformations which appear during the growth of the fish are due to the epithelial tissue. The epithelial cells proliferate from the germinal layer and progressively migrate throughout the thymic tissue according to a classical polarity from the base (basal lamina) to the apical part (epithelial capsule) of the organ. This epithelial network is scanty in fry. In fingerlings, it occupies mainly the basal part of the organ delimiting the inner zone. In adults, it reaches the

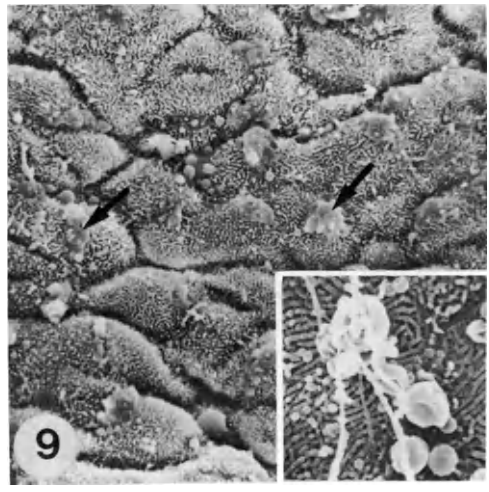

Fig. 9. Adult. Scanning electron micrograph of the thymic surface. The surface is devoided of perforations and coated by mucous secretions (arrows) (x630) Insert: high magnification of mucous droplets and filaments. (x1600)

apical part of the organ (the fingerling's outer zone) so the whole organ presents an homogeneous aspect. The inner and the outer zones are not equivalent to the cortex and medulla of the mammalian thymus. We have demonstrated that in rainbow trout the thymus is sensitive to cortisone treatment (Chilmonczyk, 1982). The reduction in the organ size after cortisone injections is not due to the involution of a particular zone of the thymus, but of many small zones scattered throughout the organ.

The thymus of salmonid fish is devoid of Hassall's corpuscles. This observation is in agreement with other descriptions (Zapata, 1981). In histological preparations, cross sections of septa present a similar appearance to Hassall's corpuscles and may be mistaken for these structures (Fig. 8).

In view of the peripheral localization of the thymus the epithelial capsule may play an important role. In addition to its protective function, the epithelial capsule is probably of importance in the relationships between the thymic tissue and the outside environment. These relationships may differ with the age of the fish. In fry, only a single layer of cells isolates the thymocytes from the water, whereas in fingerlings and

young trout, the perforated surface of the thymus allows a direct contact between the thymic tissue and the water. In the adult thymus, because of the disappearance of the perforations and the increase in the mucous cells at the surface, such exchanges might be more difficult than in the younger fish.

The thymus is usually considered as being well protected against antigenic stimulation. In rainbow trout, the situation may differ. Do the holes at the surface of the thymus allow antigens or pathogens to penetrate into the thymus and sensitize the thymocytes directly? It would be of great interest for future work to deal with this problem.

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THE THYMUS OF THE ANGLER FISH, LOPHIUS PISCATORIUS (PISCES: TELEOSTEI) A LIGHT AND ELECTRON MICROSCOPIC STUDY

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I. INTRODUCTION

In most teleosts, the thymus is situated superficially in contact with the epithelium of the branchial cavity, and undergoes involution at sexual maturity (Hammar, 1908; Lagabrielle, 1938; Tamura, 1978). However, in the teleost Lophius piscatorius, the angler fish, the thymus is remarkably large and situated remote from the branchial cavity (Stannius, 1850; Burne, 1927). The histology was described briefly by Schaffer (1893) and Burne (1927); while Picchio (1933) investigated the same organ in L. budegassa. Because the thymus of the angler fish is somewhat unique in its size and anatomic position we have reexamined it with emphasis on the ultrastructure. Only a few ultrastructural studies of fish thymus, including elasmobranchs (Zapata, 1980; Pulsford, et.al., 1983) and teleosts (Zapata, 1981; Chilmonczyk, 1983; Gorgollon, 1983) have been made, which show a mainly lymphoid organ.

II. MATERIALS AND METHODS

The animal material consisted of about 20 newly caught specimens of Lophius piscatorius, and two specimens of L. budegassa. Only the first species was used for ultrastructural studies.

The animals were killed by pithing, dissected, and weights of fish and dissected thymus tissue recorded. For histology pieces of thymus were fixed in 10% neutral formalin. Paraffin sections were stained with haematoxylin-eosin, Mallory or May-Grunwald-Giemsa (MGG).

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Imprints from freshly cut tissue were stained with MGG after air-drying and 5 min fixation in absolute methanol. For electron microscopy tissues were fixed in 5% glutaraldehyde in teleost ringer solution, rinsed in teleost ringer and post-fixed in 1% osmium tetroxide in teleost ringer. Tissue was then dehydrated and embedded in Spurr resin. Sections were cut on a Reichert OMU3 ultratome at 1.0 μm for light microscopy and 0.1 μm for electron microscopy. Semi-thin sections were stained with 1% toluidine blue and thin sections with lead citrate after mounting on 1 mm formvar-coated slot grids. Sections were examined with a Philips EM 300.

III. RESULTS

ANATOMY

The thymus of L. piscatorious was located behind the gills (Fig. 1A); the anatomical description by Burne (1927) was confirmed by visual observation, although it was not possible to identify with any certainty a "duct" connecting the thymus with the pharyngeal mucosa.

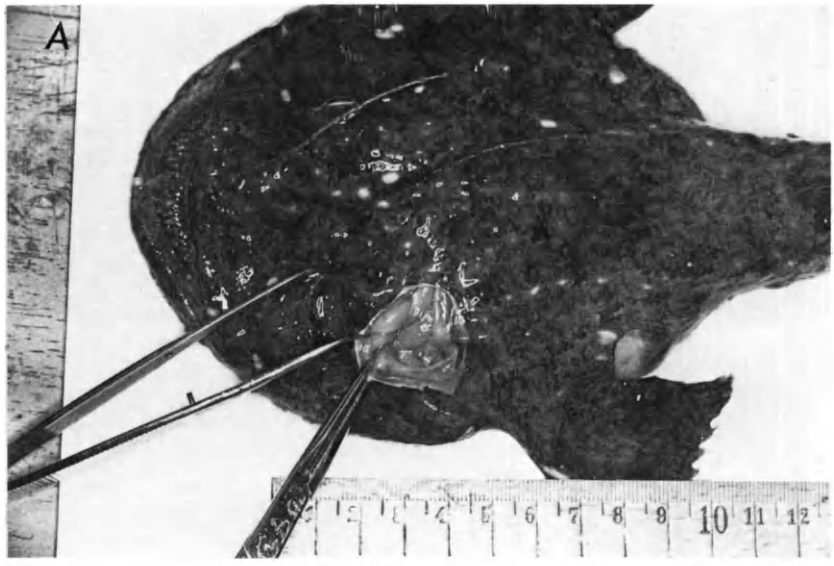
The total weight of the left and right thymus of Lophius piscatorius varied between 0.157 g and 2.822 g. The thymic weights were positively correlated with the body weights ($r = 0.8$) with only little involution (Fig. 2).

In two specimens of the related fish Lophius budegassa, the thymus had the same general appearance as in L. piscatorius.

HISTOLOGY

The thymus was surrounded by a connective tissue capsule from which trabecles projected into the interior of the organ, dividing it into incomplete lobes or compartments. The predominant cells found in histological sections, particularly in young fish, were lymphocytes of varying sizes. A separate cortex and medulla could not be distinguished, but dense cortex-like accumulations of lymphocytes alternated with medulla-like areas in most parts of the organ (Fig. 1B). Mitoses were frequent among large lymphocytes.

Various other cell types became increasingly numerous in older fish. These included macrophages containing various inclusions. Numerous large round myoid cells with an eosinophilic (or weakly basophilic) cytoplasm, containing concentrically arranged bundles of myofibrils



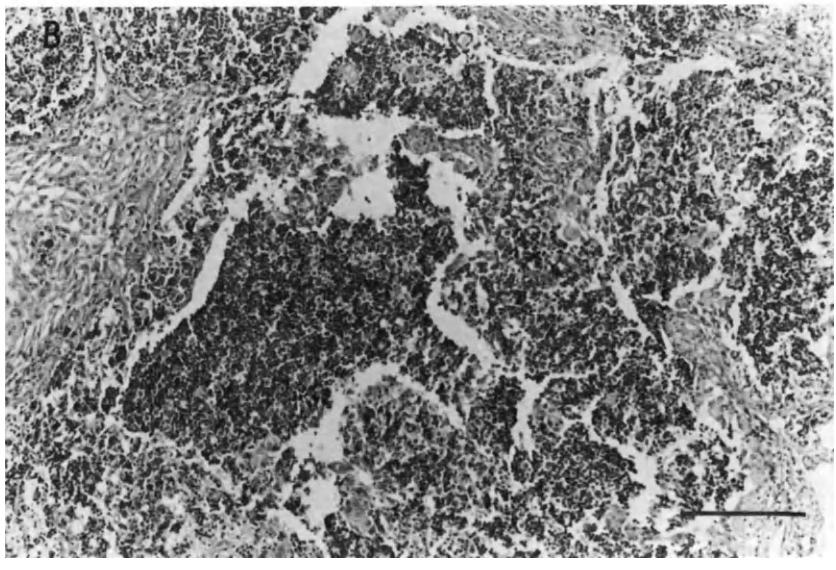


Fig. 1A. Photograph showing the position of the thymus (left side) in a specimen of Lophius piscatorius. Body weight 256.9 g. Dorsal view.

Fig. 1B. Thymus of Lophius piscatorius: histological section stained with haematoxylin-eosin. Low magnification. Scale bar = 250 m.

were present. Mucus cells were also numerous containing vacuoles which stained red-violet with MGG and were unstained with Mallory. In certain parts of the thymus, irregular cysts or cavities were bordered by mucus cells. Large multinucleate bodies or giant cells, possibly enlarged reticular cells, were sparingly distributed within the thymic mass.

ULTRASTRUCTURE

CONNECTIVE TISSUE

The capsule of the thymus consisted of elongated fibrocytes, with extracellular collagen fibrils (Fig. 3A). Within the connective tissue ground substance, other connective tissue cells including melanophores (Fig. 3G) and guanophores (Fig. 3F) were located. Lymphocytes and myoid cells were also found in transit within the connective tissue trabecles.

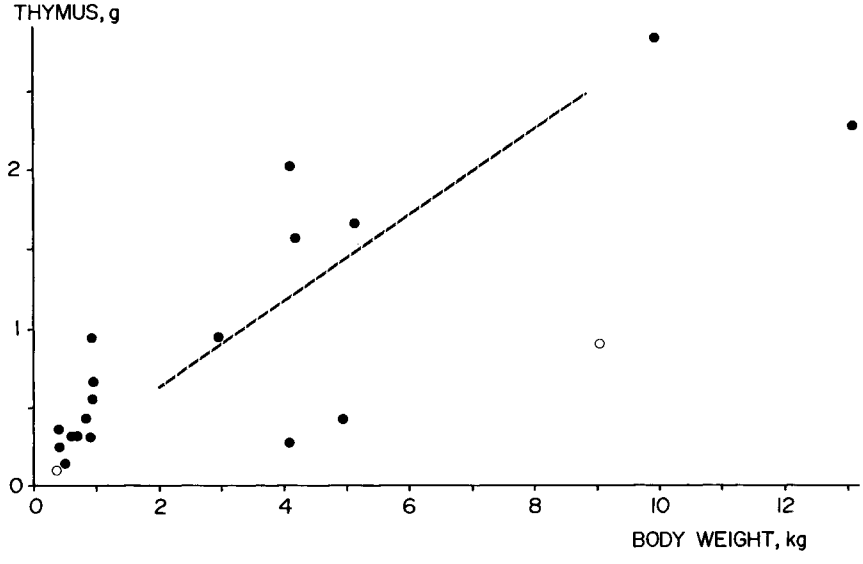
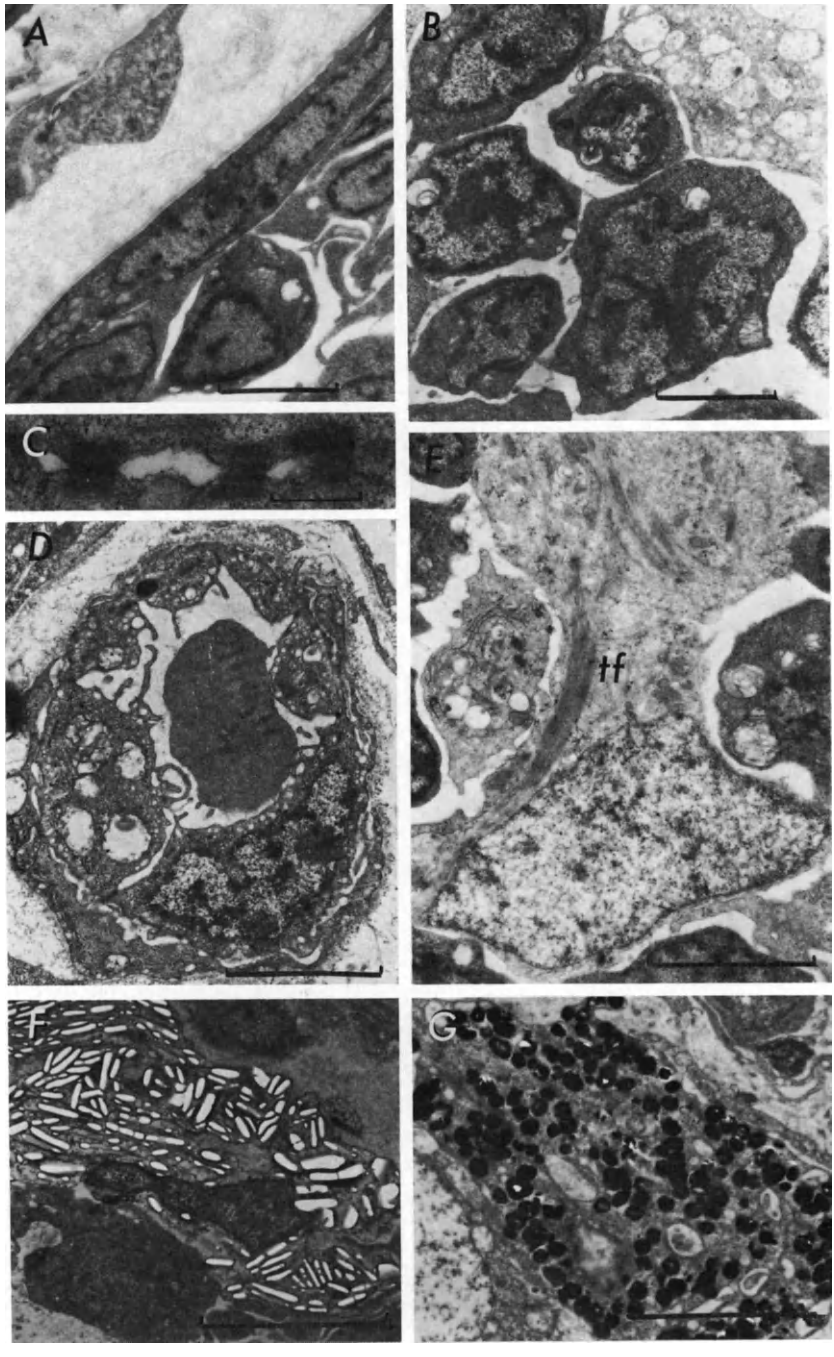


Fig. 2. Weight of the thymus plotted against the body weight of Lophius. Black dots = L. piscatorius, unfilled circles = L. budegassa. Interrupted line = regression line, calculated (L. piscatorius only).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BLOOD VESSELS

Numerous branches of blood vessels penetrated the tissue via the continuous epithelial layer, so that vessels were separated from the surrounding lymphoid tissue by a layer of epithelial cells (Fig. 3D). Capillaries were formed from endothelial cells, often joined by distinct desmosomal junctions (Fig. 3C).

RETICULAR EPITHELIAL CELLS

Epithelial cells formed a reticulum, the meshes of which were filled with mobile cells, including lymphocytes and macrophages (Fig. 3E). Flattened epithelial cells from the inner surface of the connective tissue capsule connected with stellate epithelial cells which formed the framework of the thymic tissue between the connective tissue trabecles.

Fig. 3 Thymus of Lophius piscatorius: connective and reticular tissues.

A. Connective tissue capsule of fibrocytes with extracellular collagen, and the contained small lymphocytes. Scale bar = 2.5 μm.

B. Small lymphocytes and larger lymphoblasts. Scale bar = 2.5 μm.

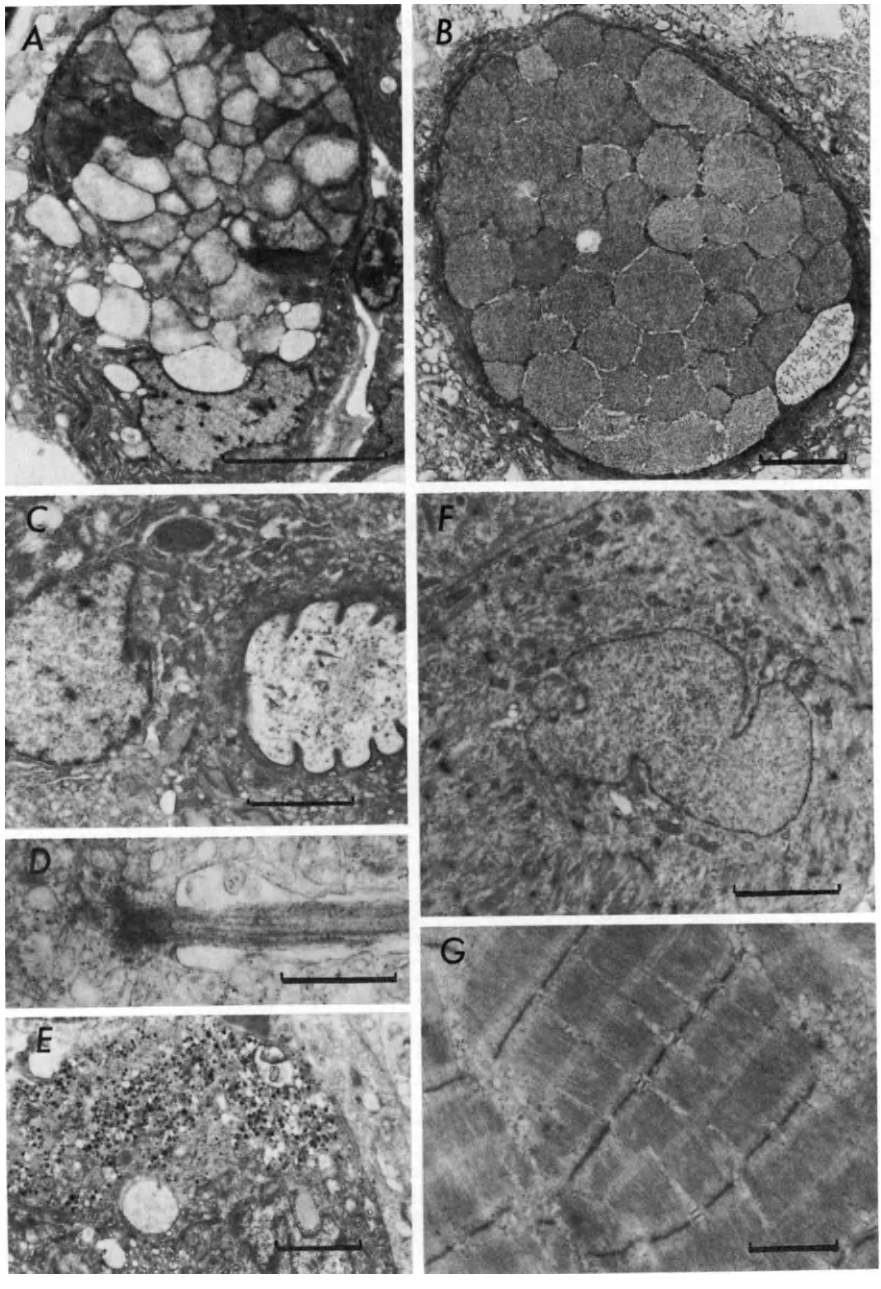
C. Detail of desmosomal junction between the endothelial cells of capillary. Scale bar = 0.2 μm.

D. Transverse section of capillary with erythrocyte in lumen. Capillary surrounded by single layer of epithelial cells. Scale bar = 2.5 μm.

E. Pale reticular epithelial cell with cytoplasmic tonofilaments bundles (tf). Scale bar = 2.5 μm.

F. Guanophore within the connective tissue trabecles which penetrate the thymus. Scale bar = 5.0 μm.

G. Melanophore with contained melanin granules from the connective tissue trabeces. Scale bar = 5.0 μm.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The ovoid to irregular nucleus had a loose chromatin structure often with a prominent nucleolus. The cytoplasm was much paler than that of the lymphoid cells and characterised by bundles of tonofibrils (Fig. 3E).

HYPERTROPHIC EPITHELIAL CELLS

These cells were more rounded in shape and without the cytoplasmic processes characteristic of the reticular epithelial cell. The nucleus was eccentrically located and cytoplasmic vacuoles were bordered by microvilli (Fig. 4C), and often contained homogeneous material or contained single cilia (Fig. 4D). These cells were sometimes found in groups forming intercellular cysts.

Fig. 4. Thymus of Lophius piscatorius: secretory and myoid cells.

A. Mucus cell with intracellular mucus globules filling the cytoplasm, with the nucleus in peripheral position. Scale bar = 5.0 μm.

B. Mucus cyst with thick cell wall and cytoplasm completely filled with mucus droplets. Scale bar = 2.5 μm.

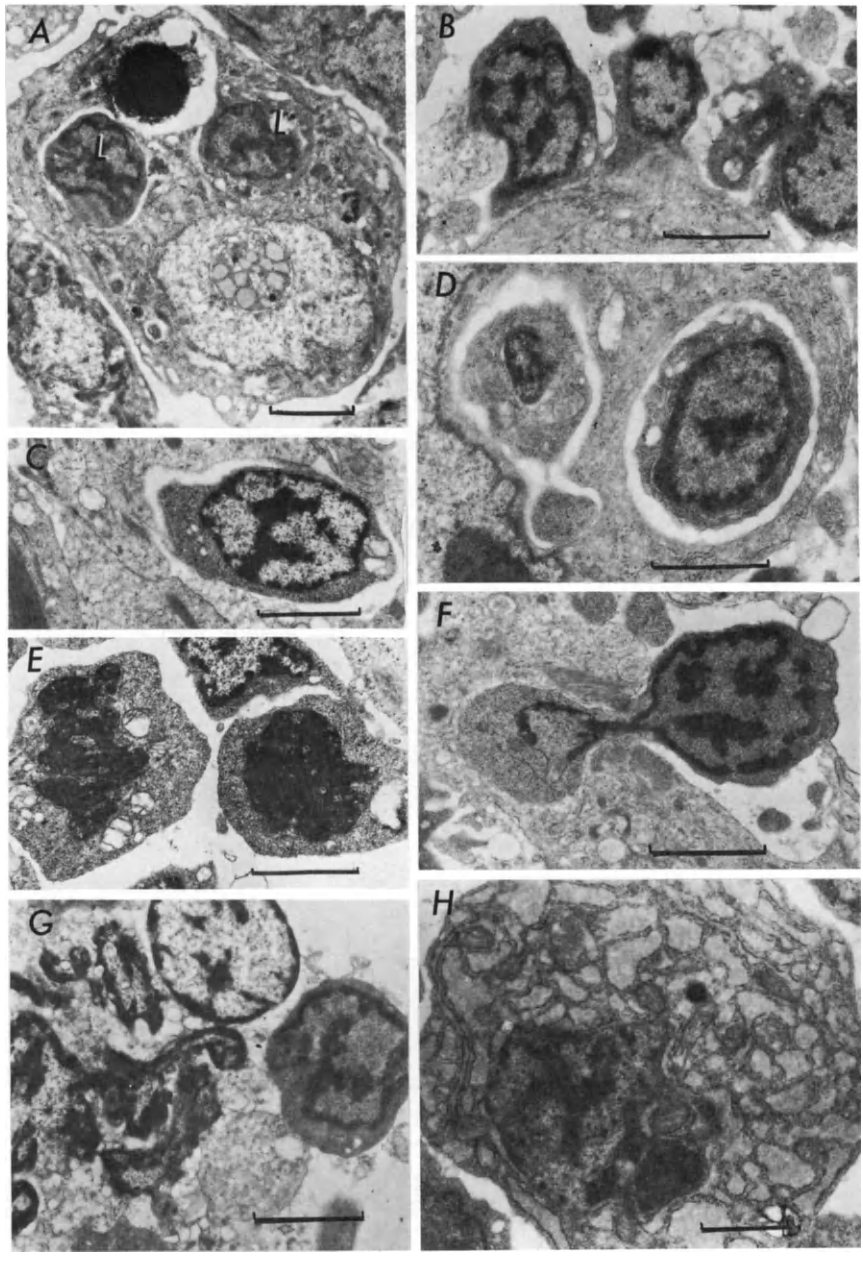
C. Hypertrophic epithelial cell with intracellular cyst lined with microvilli and filled with homogeneous material. Scale bar = 2.5 μm.

D. Intracellular cilium within hypertrophic epithelial cell cytoplasm. Scale bar = 0.5 μm.

E. Granular secretory cell containing numerous small electron dense granules in the cytoplasm. Scale bar = 2.5 μm.

F. Part of a myoid cell with irregular centrally located nucleus with the myofibrils arranged in bundles around the nucleus. Scale bar = 2.5 μm.

G. Detail of the cytoplasm of a more differentiated myoid cell showing the regularly arranged bundles of myofibrils and distinct Z lines. Scale bar = 1.0 μm.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LYMPHOID CELLS

These were the most common cells of the thymus found in groups. Three size forms were observed (Fig. 3B); the larger 10.0 μm diameter lymphoblasts, among which mitotic figures were frequently observed (Fig. 5E), medium sized lymphocytes 7.0 μm diameter, and small lymphocytes of 3.0 μm diameter. All stages were characterised by a large nucleus and narrow rim of cytoplasm containing mitochondria and ribosomes.

LYMPHOCYTE/MACROPHAGE ASSOCIATIONS

Large, medium and small lymphocytes were frequently observed forming rosettes around central macrophages (Fig. 5B). The degree of association of the cytoplasm of the two cell types varied from apposition of the cell membranes to complete fusion of the surfaces of both cells (Fig. 5B).

Fig. 5 Thymus of Lophium piscatorius : lymphocytes.

A. Two small lymphocytes (L) contained within the cytoplasm of macrophage. Scale bar = 2.5 μm.

B. Small lymphocytes closely associated with the surface of a macrophage. Scale bar = 2.0 μm.

C. Small lymphocyte within cytoplasmic vacuole of a macrophage. Scale bar = 2.0 μm.

D. Detail of macrophage containing small lymphocytes within cytoplasmic vacuoles. Scale bar = 2.0 μm.

E. Two small lymphocytes after mitosis. Scale bar = 2.0 μm.

F. Small lymphocyte in transit squeezing between epithelial cells. Scale bar = 2.0 μm.

G. Degenerating lymphocytes with remnants of nuclei. Scale bar = 2.0 μm.

H. Plasma cell. Scale bar = 1.0 μm.

Lymphocytes were also found within cytoplasmic vacuoles of the macrophage, only one or two lymphocytes within each macrophage (Fig. 5A). These macrophages contained much less ingested material but more extensive rough endoplasmic reticulum profiles (Fig. 5D).

MACROPHAGES

These large 12.0 μm diameter cells were commonly found among the lymphocytes. The nucleus was eccentric and the cytoplasm contained ingested material in the process of degradation (Fig. 6C). This often included lymphocytes and myoid cells.

PLASMA CELLS

Plasma cells with a typically eccentric nucleus and extensive cisternae of rough endoplasmic reticulum were rare in the thymus. Dilated cisternae of the endoplasmic reticulum were filled with a homogeneous medium density material. Mitochondria and small electron dense vesicles were the other cellular inclusions (Fig. 5H).

MULTINUCLEATED GIANT CELLS

Certain areas of the thymus contained aggregations of macrophages characterised by numerous, extensive and interdigitating extensions of the plasma membrane. Among these groups of large macrophages some cells often contained numerous nuclear profiles giving a multinucleated cell (Fig. 6D), visible by light microscopy. The nuclear profiles were irregular with the chromatin denser peripherally. The pale cytoplasm contained some rough endoplasmic reticulum profiles, mitochondria and electron dense granules.

GRANULATED CELLS

Large macrophage like cells with the cytoplasm containing numerous homogeneous small, round electron-dense cytoplasmic granules were infrequently found (Fig. 4E). Extensive rough ER cisternae and small vesicles gave these cells a distinct secretory appearance.

MUCOUS CELLS

Unicellular thick-walled mucous cysts were randomly distributed throughout the epithelial cell component of

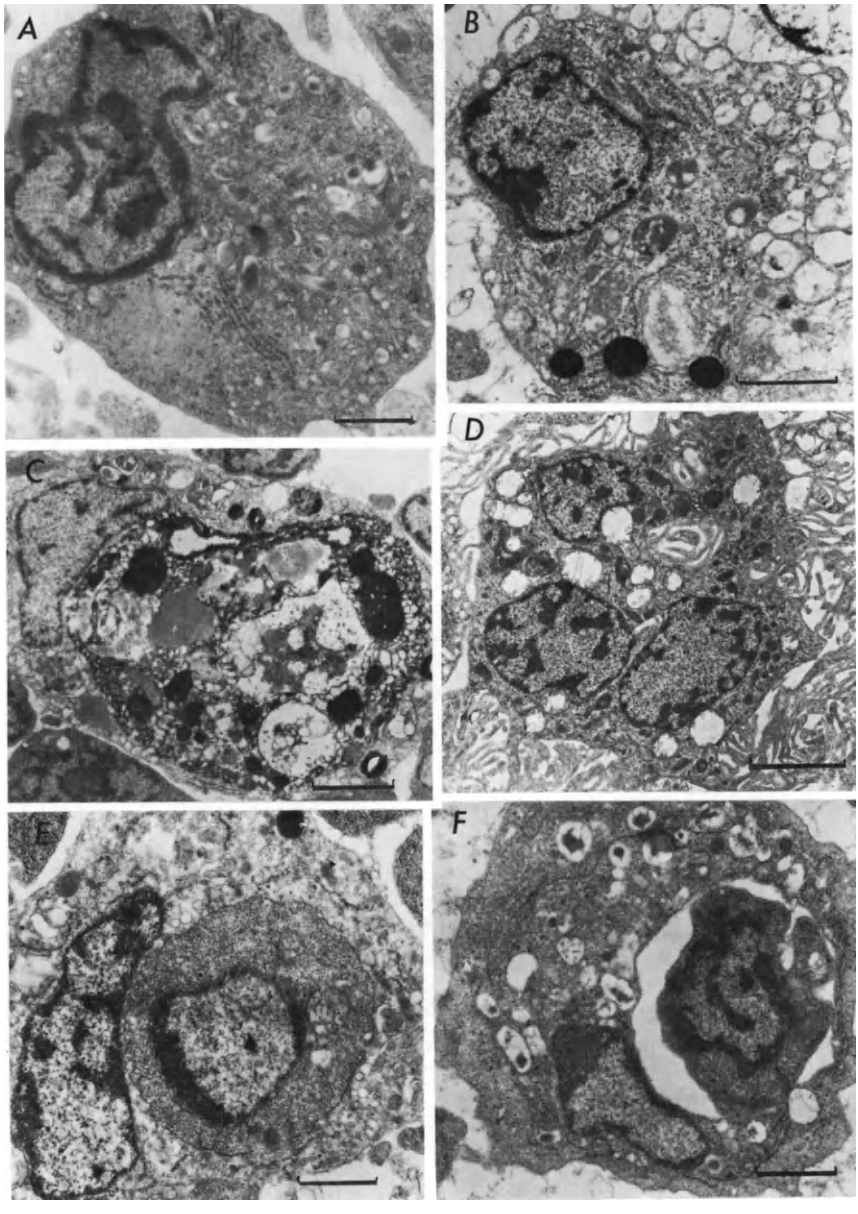
the thymus (Fig. 4B), increasing in older fish. The ultrastructural appearance varied depending on the amount of secretory material present in the cytoplasm. Cells not completely full of secretory material had an eccentrically displaced, bean-shaped nucleus, with the chromatin denser peripherally (Fig. 4A). The marginal cytoplasm without mucous droplets showed Golgi profiles and extensive endoplasmic reticulum, characteristics of secretory cells. The central area of the cell was filled with membrane-bound mucous globules varying in density and ultrastructural appearance (Fig. 4A). In cells filled with secretory material the nucleus was often absent and the wall of the cell thickened (Fig. 4B).

MYOID CELLS

These large cells measured up to 30 μm in diameter and were found either singly as large round cells, or in small groups, in which case the cells were more elongate and strap-shaped. These small groups of myoid cells did not appear to have junctions with adjacent cells, or to be innervated. Ultrastructurally great variations occurred in the cytoplasm of the myoid cells.

Young myoid cells were large, 15.0 μm diameter pale cells with eccentrically located nuclei and the cytoplasm containing numerous small tubular mitochondria but without cytoplasmic myofilaments. Mature myoid cells (30.0 μm) were characterised by the bundles of cytoplasmic myofibrils composed of thick and thin myofilaments with distinct Z lines. Great variations occurred in the arrangement of the myofibrils, some were concentrically arranged around the centrally located nucleus (Fig. 4F), while others were randomly arranged throughout the cytoplasm. In some cells the myofibrillar bundles were arranged in definite sarcomeres with dark Z lines and more electron lucent M lines, the myofibrils filling the entire cytoplasm (Fig. 4G). A sacroplasmic reticulum membrane system penetrated between the myofibrillar bundles (Fig. 4G), and the characteristic mitochondria were interspersed between the bundles of myofibrils and numerous glycogen particles.

Myoid cells were found both within the connective tissue and epithelial component of the thymus and appeared to be mobile within the tissue, as migrating cells were found. Degenerating myoid cells were observed within macrophages.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IV. DISCUSSION

The thymus of the angler fish, Lophius piscatorius contains predominantly lymphocytes, particularly in younger fish. No involution was recorded, but with increasing age numerous non-lymphoid cells became more apparent. Great variation in the onset of involution has been recorded in teleost fishes (Rizkalla, 1969): and seasonal changes also occur in some fish (Tamura and Honma, 1977). As is generally true for fish (Manning, 1981), the thymus of Lophius showed a poor cortical/medullary distinction.

Macrophages were very numerous in the thymus of Lophius appearing to have functions other than the breakdown of effete cells and debris. They were often found in close association with lymphocytes, similar to associations observed in mammalian and elasmobranch lymphoid tissue (Lipsky and Rosenthal, 1973; Pulsford, et al., 1982), which has been suggested to represent antigen presentation by macrophages to immune T lymphocytes (Rosenthal et al., 1975). Lymphocytes were also found within the cytoplasm of

Fig. 6. Thymus of Lophius piscatorius: macrophages.

A. Large macrophage with cytoplasmic granules and granular endoplasmic reticulum. Scale bar = 1.0 μm.

B. Macrophage with eccentric nucleus, lipid inclusions and marginal cytoplasmic vacuoles. Scale bar = 2.0 μm.

C. Macrophage with cytoplasm almost completely full of ingested cellular debris. Scale bar = 2.0 μm.

D. Multinucleated macrophage with three nuclear profiles visible in section and marginal cytoplasm with numerous microvillii. Scale bar = 2.0 μm.

E. Macrophage containing ingested degenerating lymphocyte. Scale bar = 1.0 μm.

F. Macrophage with small lymphocyte within cytoplasmic vacuole. Scale bar = 1.0 μm.

macrophages, similar to observations made on the mammalian thymus (Olah, et al., 1975), and the dogfish thymus (Pulsford, et al., 1983). This may either represent the breakdown of aged lymphocytes or alternatively suggest that certain macrophages are involved in the maturation of lymphocytes to immunocompetent T cells in a similar way to 'nurse cells' from mammalian thymus (Wekerle and Keteisen, 1980; Wijngaert, et al., 1983). However, whether distinct populations of T and B lymphocytes exist in fish is open to question and most reports suggest a less specialised function for fish lymphocytes (McKinney et al., 1976; Manning 1981).

The capillaries within the thymus were surrounded by an epithelial layer similar to that found in other fish (Zapata, 1981; Pulsford, et al., 1983; Chilmonczyk, 1983) and mammals (Raviola and Karnovsky, 1972). This suggests the presence of a blood/thymus barrier; however separation of lymphocytes from blood-borne antigens probably does not occur, as plasma cells were present suggesting that antibody synthesis may be one of the functions of the thymus. These cells have also been reported from the thymus of other teleosts (Hulet, 1971; Zapata, 1981; Fange and Pulsford, 1983); and in vitro production of antibody from fish thymus has also been demonstrated (Ortiz-Muniz and Sigel, 1971). Plasma cells have also been demonstrated in mammalian thymus, although restricted to the connective tissue component (Toro and Olah, 1966; van Haelst, 1967).

One of the most interesting cells found in the thymus of Lophius piscatorius was the myoid cell, which appeared to undergo a cycle of formation, development and degeneration. Various stages of maturation were observed and effete cells were found within macrophages. A similar cycle of formation and degeneration has been recorded from the thymus of the rat (Hanzlikova, 1979). The richness of myofibrils and mitochondria in the cytoplasm of myoid cells suggests a mechanical function; however, groups of myoid cells were not joined by intercellular junctions and there was no evidence of innervation. Some cells were observed migrating through the thymus, and it is possible that they play some role in the release of lymphocytes from the thymus into the circulation. Myoid cells have been found in the thymus of fishes (Hammar, 1908; Hafter, 1952; Rizkalla, 1969; Zapata, 1980), and are morphologically similar to those of other vertebrates (Henry, 1966; Raviola and Raviola, 1967; Canaday, 1968; Ito, et al., 1969; Hanzlikova, 1979). None however have been observed to be innervated (Gilmore and Bridges, 1974)

and apart from their suggested association with the autoimmune disease myasthenia gravis, their function remains largely unknown (Rimmer, 1980).

Various secretory cells were identified, the most prominent and numerous being the mucus cells. Other secretory cells included the hypertrophic epithelial cells with large intracellular vacuoles often lined by microvilli or cilia and sometimes arranged in groups or cysts. The intercellular vacuoles or intercellular cysts contained a homogenous material presumably secretory. Similar intra or intercellular cysts have been found in fishes (Hammar, 1908; Salkind, 1915; Sailendri and Muthukkaruppan, 1975; Zapata, 1981), and mammals (Hoshino, 1961; Raviola and Raviola, 1967). It was suggested by Gorgollon (1983), that these cells might be involved in the secretion of thymic hormone. However, a granular secretory cell was also identified in the thymus of Lophius similar to that associated with hormonal secretion in mammals (Clark, 1966; Schmitt, et al., 1980). The function of this and many of the other cell types found in the thymus of Lophius piscatorius remains to be resolved.

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ANTIBODY RESPONSES OF YOUNG CARP, CYPRINUS CARPIO, AND GREY MULLET, CHELON LABROSUS, IMMUNIZED WITH SOLUBLE ANTIGEN BY VARIOUS ROUTES.

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I. INTRODUCTION

There has been considerable interest in the maturation of the immune system of fish, particularly in relation to immunization in the younger age groups. As in other vertebrates, the ability to phagocytose foreign material appears early in development in fish (Prazdnikov and Mikhailova, reviewed by Finn, 1970; Grace, et al., 1981). Non-specific immunity is, however, soon supplemented by the rapidly developing cell-mediated component of the immune system (Manning, et al., 1982) and second-set alloimmune reactivity can be demonstrated as early as one month post-hatching in carp, Cyprinus carpio, (Botham and Manning, 1981).

The humoral antibody response to certain antigens also matures quite quickly in fish. Juvenile (1.2g) coho salmon, Onchorhynchus kisutch, could produce antibody against Aeromonas salmonicida (Paterson and Fryer, 1974) while rainbow trout, Salmo gairdneri, responded to Aeromonas liquefaciens approximately 23 days after the commencement of feeding (Khalifa and Post, 1976). Rainbow trout could produce antibody against Aeromonas salmonicida vaccine when immunized at three weeks post-hatching (Secombes, 1981) and against DNP-KLH (dinitrophenol - keyhole limpet haemocyanin) at one month post-hatching (Dorson, 1974). On the other hand, injection of the soluble protein antigen HGG (human gamma globulin) into three week old rainbow trout yielded no humoral antibody

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response (Secombes, 1981). Similarly, 4 week old carp failed to respond to sheep erythrocytes (van Loon et al., 1981) or to HGG (M.S. Mughal, unpublished results). HGG and sheep erythrocytes are both thymus dependent antigens. These findings therefore agree with those of Etlinger, et al., (1979) who demonstrated that juvenile rainbow trout give poor responses to thymus dependent antigens at an age when they are capable of good antibody production against antigens which are thymus independent in mammals. Furthermore the experiments of Secombes (1981), van Loon et al., (1981) and M.S. Mughal (unpublished) all indicate that early exposure of the young fish to a thymus dependent antigen results in tolerance or a suppression of the immune response when the fish are challenged with a second injection of the same antigen at an age when normal fish are capable of yielding a humoral antibody response.

In mammals, aspects which determine the outcome of early exposure to antigen include the dose of the antigen, its persistence, its physical state, the level of foreignness, how it is presented, and whether T-cell help is required for antibody production. Given certain of these conditions, premature exposure to antigen can elicit a state of immunological tolerance. In fish, phenomena suggestive of tolerance have been described in the adult by Lopez, et al., (1974), Serero and Avtalion, (1978), Avtalion, et al., (1980), and Manning et al., (1982), and it is necessary to consider this aspect of fish immunology, particularly in any programme of immunization involving immature fish. The present experiments therefore set out to investigate the use of a soluble, thymus-dependent antigen (HGG) administered by various routes. This primary exposure to antigen was followed by a challenge dose of HGG injected intraperitoneally in adjuvant. Antibody levels were determined to see whether the first exposure had elicited a positive secondary humoral response, or whether any suppression of antibody production indicative of immunological tolerance could be detected. The fish used in these experiments were the grey mullet, Chelon labrosus, taken at a time of year when the young mullet (aged approximately 6 months) could conveniently be captured from local shallow waters, and the carp, Cyprinus carpio, aged either 4 weeks or 9-10 weeks at the time the experiments commenced.

The methods used for primary immunization included antigen administration by direct immersion. Direct immersion in an antigen bath has been shown to be an effective method for vaccinating fish (Egidius and

Anderson, 1979; Gould, et al, 1979; Anderson and Dixon, 1980). This method gives good protective immunity to young salmonids immunized with bacterial vaccines at upwards of 1g body weight, the size of the fish being of greater importance than age (Johnson, et al., 1982). Indeed, a measure of protective immunity can be demonstrated in rainbow trout from as early as 2 weeks post-hatching, although before this age the young fish appear to be refractory to bacterial uptake (Tatner and Horne, 1983). The present experiments included the administration of a soluble thymus dependent antigen (HGG) to carp fry starting from an even earlier age (one week post-hatching) to determine the effect of repeated immunization by direct immersion of immature fish on subsequent immunological memory.

II. MATERIALS AND METHODS

ANIMALS

Cyprinus carpio. Carp were obtained from Humberside Fisheries, Skerne, Driffield and from Priory Fisheries, Kerswell, Devon. They were held 10-20 fish per tank of approximate dimensions 15 x 23 x 19 cm, containing aerated tap water at 18-22°C. They were fed daily on powdered Omega No. 2 trout foods (Baker, Sudbury), waste material being removed either using sponge filters or by means of a bed of fine gravel approximately 3cm deep on the bottom of the tank.

Chelon labrosus. Grey mullet weighing approximately 0.5g and believed to be about 6-7 months old were taken in the autumn from a creek near St. John, Cornwall. They were kept in the laboratory under conditions similar to those described for carp except that sea water was used in the tanks.

ANTIGEN AND ANTIGEN ADMINISTRATION

HGG (human gamma globulin, Kabi, Stockholm) was prepared by dissolving 5 mg/ml HGG in 0.85% saline, then diluting to 2.5 mg/ml by adding an equal volume of either saline (termed 'HGG in saline') or Freund's complete adjuvant, FCA, Difco, Detroit (termed 'HGG in adjuvant'). The material was then administered at the rate of 0.01ml/g body weight. OVA (ovalbumin, Sigma, St. Louis) or KLH (keyhole limpet haemocyanin, Calbiochem, La Jolla) were used as alternative antigens in some experiments. HGG

coated on to latex beads was prepared according to the method outlined in Hudson and Hay (1980) using latex beads of approximately 300nm diameter in a 0.8% suspension (w/v) with 2.5mg HGG/ml.

Injections were administered using a glass micrometer syringe (Agla) fitted with a sterile 30 gauge needle. Intraperitoneal injections were made by inserting the needle through the skin into the abdominal cavity from a ventro-lateral position. Intra-muscular injections were delivered into the epaxial musculature of the anterior part of the tail. Administration per os was performed using a truncated hypodermic needle carefully ground down to prevent damaging the fish. The needle was placed into the mouth and passed back into the buccal cavity towards the opening of the oesophagus where the antigen was delivered cautiously. This is termed 'oral administration'. Injection of antigen into the thymus involved lifting up the operculum to gain access to the thymus which is superficially situated in fish. The antigen was then injected into the thymus; both left and right thymus glands were used, half the total dose being placed in each thymus. For direct immersion in antigen, fish were placed for 30 min in a bath containing 5 mg of antigen per litre of standing tap water. Controls were handled in a similar manner and placed in baths of tap water without antigen.

Fish were bled from the caudal vein and serum antibody levels were assayed by passive haemagglutination using the method of Stavitsky (1954). 1mg/ml HGG was used to coat tanned erythrocytes for the test and the method was modified for use with carp antibodies by employing 1% carp serum as the serum diluent.

III. RESULTS

The results shown in Table 1 confirm our earlier finding that the soluble antigen HGG is tolerogenic when injected into 4 week old carp; the fish treated at this age failed to produce antibody after secondary challenge at a time when carp can normally respond to HGG, and when those fish which received either saline or an unrelated antigen (OVA) were able to do so. Furthermore, the use of latex beads as a particulate carrier for the injected antigen failed to prevent this tolerogenic effect. In contrast, when the direct immersion method was used to administer the soluble antigen, there was no effect on antibody production after challenge, the titres being similar to those in fish which

had received no preliminary immersions (Table 2). Even when multiple immersion was started from a very early stage of development, antibody titres remained unaffected

Table 1 Humoral antibody production in young carp, Cyprinus carpio. The fish were 4 weeks old when first injected, 9-10 weeks old when challenged, and 15-16 weeks old when tested for antibody production. Each figure represents the $-\log_2$ anti-HGG passive haemagglutination antibody titre for serum pooled from 6-11 fish. HGG Human gamma globulin. OVA = Ovalbumin i.p. = injected intraperitoneally.

Primary treatment	Challenge injection	Anti-HGG titre	No. of fish
Saline only	Adjuvant alone i.p.	0	8
OVA in saline	" " "	0	7
HGG in saline	" " "	0	8
Latex beads only	" " "	0	7
HGG on latex beads	" " "	0	8
Saline only	HGG in adjuvant i.p.	8,12	17
OVA in saline	" " "	12	8
HGG in saline	" " "	0,0,0	22
Latex beads only	" " "	8,11	15
HGG on latex beads	" " "	0	8

Table 2. Humoral antibody production in young carp, Cyprinus carpio, immunized with a soluble antigen (HGG) by direct immersion. The fish received five immersions in HGG (5mg/Litre) at weekly intervals commencing at 4 weeks old. They were challenged at 10 weeks old with HGG in adjuvant (25 μg HGG/g body weight i.p.) and antibody titres were assessed at 15-16 weeks old. Passive haemagglutination antibody titres expressed as $-\log_2$. Serum pooled from 6-8 fish.

Primary treatment	Challenge injection	Anti-HGG titre	No. of fish
Control immersion	Adjuvant alone	0	6
Control immersion	HGG in adjuvant	6, 7	16
HGG immersion	HGG in adjuvant	5, 6	15
HGG immersion	Adjuvant alone	0. 1	14
HGG immersion	None	0	6

Table 3. Humoral antibody production in young carp, Cyprinus carpio, immunized with a soluble antigen (HGG) by direct immersion. The fish were directly immersed in antigen (5mg/litre) once a week for 15 weeks starting at the age of one week post hatching (Stage 36 of Balinsky, 1948). The challenge injection (25 μg HGG/g body weight i.p.) was given in adjuvant 5 weeks after the last direct immersion. Passive haemagglutination antibody titres expressed as $-\log_2$. Serum pooled from 8-12 fish.

Primary Treatment	Challenge injection	Anti HGG titre	No. of fish
Control immersion	Adjuvant alone	0	10
Control immersion	HGG in adjuvant	7	8
HGG immersion	HGG in adjuvant	8	12

The object of the experiment shown in Table 4 was to select for investigation routes of primary exposure to

Table 4. Humoral antibody production in young carp, Cyprinus carpio. The fish were 9-10 weeks old at the time of first treatment, 13-14 weeks old when challenged, and 20-22 weeks old when tested for antibody production. Each figure represents the $-\log_2$ passive haemagglutination antibody titre for serum from 6-10 fish.

Primary treatment	Challenge injection	Anti-HGG titre	No. of fish
Controls saline i.p., i.m., oral or intrathymic	Adjuvant alone i.p.	0,0,0,0	34
Saline i.p. or oral	HGG in adjuvant i.p.	5,6	14
Unrelated antigen (OVA) in saline i.p.	" "	2	8
" " " i.m.	" "	9	10
" " " oral	" "	1	6
" " " intra-thymic	" "	8,7	17
HGG in saline i.p.	" "	8,8,9	24
" " " i.m.	" "	9	7
" " " oral	" "	7,9	15
" " " intra thymic	" "	10,11	18
HGG in adjuvant i.p.	" "	7,8,9,11	31
Adjuvant in saline i.p.	" "	7	6

antigen which, from mammalian experience, could be potentially tolerogenic in immature animals; the oral route, intra-thymic injection, and the administration of antigen in soluble form without adjuvant, intra-peritoneally or intra-muscularly. In these

Table 5. Humoral antibody production in young grey mullet, Chelon labrosus. The fish were approximately 6-7 months old when antigen was first administered. They were challenged 4 weeks later, and tested for antibody production 7-8 weeks after challenge. Each figure represents the $-\log_2$ anti-HGG passive haemagglutinating antibody titre for serum pooled from 7-12 fish.

Primary Treatment	Challenge injection	Anti HGG titre	No. of fish
Controls: untreated, saline i.p., saline oral, FCA alone	Adjuvant alone i.p.	0,0,0,0,0,0	55
HGG in saline i,p.	" " "	0,0	15
HGG in saline oral	" " "	0,0	18
Controls: untreated or saline i.p.	HGG in adjuvant	0,0,0	33
Unrelated antigen: OVA or KLH in saline i.p.	" " "	0,0	17
Saline oral	" " "	0,5	23
Unrelated antigen: OVA or KLH in saline, oral	" " "	0,0	22
HGG in saline, i.p.	" " "	6,10	22
HGG in saline, oral	" " "	5,9	22
HGG in adjuvant i.p.	" " "	8,10,10,15	41
Adjuvant alone	" " "	5	15

experiments, carried out on 9-10 week old carp, it was found that, regardless of the route of primary exposure, the young fish were still able to produce an antibody response to HGG after challenge. There was, in fact, a small enhancement of the antibody titres, the mean of differences between groups receiving treatment at 9-10

TABLE 6. Humoral antibody production in grey mullet, Chelon labrosus .

A. Adult fish

Adult fish weighing approximately 26g (average length 13.3cm). The fish were injected once with HGG in adjuvant (25 μg HGG/g body weight) intraperitoneally. Haemagglutinating antibody titre $-\log_2$ tested 7-8 weeks later:

Anti-HGG antibody titre = 12.6 $\pm$ (SD) 2.6, n = 5.

B. Juvenile fish

Summary table for fish approximately 6-7 months old taken from Table 5.

Primary treatment	Challenge	Anti-HGG titre
Controls	Adjuvant only	None
Control injections i.p.	HGG in adjuvant	None
Controls oral	" " "	None
Adjuvant only	" " "	Moderate
HGG in adjuvant i.p.	" " "	High
HGG in saline i.p.	" " "	High
HGG in saline oral	" " "	High

weeks old and those receiving the challenge injection only, being 3.5 $\pm$ (SE) 1.1, degrees of freedom 6, $P < 0.01$. In the grey mullet, the antibody titres shown in Table 5 indicate that young fish, unlike the adult mullet (Table 6), fail to respond to a single injection of HGG administered in adjuvant. They did respond, however, when previously primed with the appropriate antigen, both the intra-peritoneal route and the oral route being effective in priming for a good antibody response on challenge.

IV. DISCUSSION

Although carp aged 4 weeks appear to be susceptible to some degree of tolerance induction when injected with thymus-dependent antigens, our present experiments indicate that, by 9-10 weeks old, it is no longer possible to obtain any suppressive influence following the administration of soluble antigen by various routes. Carp primed at this age were capable of normal antibody production on challenge and the effect of the primary immunization was to yield a slight, but not impressive, secondary humoral antibody response. These studies included the use of the intrathymic route in order to discover whether fish thymocytes resemble mammalian thymocytes in being susceptible to tolerance induction by direct exposure to antigens. The results failed to show tolerance induction, the antibody levels attained on challenge being similar to those obtained using the more conventional routes for primary immunization. Similarly, oral vaccination with a soluble antigen, which can be tolerogenic in young mammals (see Silverman, et al., 1982) had no suppressive effect in 9-10 week old carp.

In the grey mullet, the primary exposure to HGG had a beneficial effect on the subsequent immune response. The 6-7 month old mullet, unlike carp of younger age (but similar body weight) could not respond to a single injection of HGG in adjuvant. This reveals a certain immaturity in their humoral immune response when compared with adult mullet. Priming of these young fish, however, enabled them to yield good positive responses on challenge. The immunization was therefore effective in enhancing the antibody producing capabilities of the juvenile mullet.

Direct immersion is a useful method of immunization. In very young fish, the effect of the presence of antigen in the pharynx during the early stages of development is of

special interest (Tatner and Manning, 1982) since the thymus is separated from the pharyngeal lumen by only a single layer of epithelium which, at least in rainbow trout, contains pores in the young fish (Chilmonczyk, this Symposium). In the young carp, however, direct immersion in HGG was ineffective when commenced from one week or from four weeks old; it neither induced tolerance nor elicited enhanced positive responses. Young carp appear to be refractory to stimulation by direct immersion in soluble antigens of this type. In contrast, when a bacterial vaccine (formalin-killed Aeromonas salmonicida) is used, direct immersion of 4 week old carp in antigen can improve the secondary response (unpublished results from this laboratory).

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THE ONTOGENY OF THE IMMUNE SYSTEM IN THE VIVIPAROUS TELEOST ZOARCES VIVIPARUS L.

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I. INTRODUCTION

There are several accounts of the development of the teleost lymphoid system as a whole (Schiffman and Fromm, 1959; Sailendri and Muthukkaruppan, 1975; Ellis, 1977; Grace and Manning, 1980; Manning et al., 1981, and Tatner and Manning, 1983). Other workers have traced the development of single lymphoid organs (Thymus: Deanesly, 1927; Hill, 1935; Hafter, 1952; Zapata, 1981, and Chilmonczyk, 1983. Spleen: Denton and Yousef, 1976).

With few exceptions only oviparous species have been investigated but notable among these exceptions are the studies on the viviparous teleosts, the American surfperch (Hyperprosopon argentium), by Engen (1968), and the Japanese surfperch (Ditrema temmincki) and the guppy (Lebistes reticulatus), by Tamura et al., (1981). In view of the interesting immunological relationship between mother and foetus the present work set out to investigate the ontogeny of the lymphoid organs in the viviparous blenny, Zoarces viviparus L. using light and electron microscopy.

II. METHODS

ANIMALS

Pregnant female specimens of Zoarces viviparus L. were collected from Odense, Denmark; Lowestoft, England; Robin Hood's Bay, England and Oban, Scotland. Fish were maintained in a closed, recirculating, sea water aquarium. Gestation lasts 4 months, during which time fertilised ova

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develop from approximately 20mg weight to embryos of approximately 240mg weight and 44mm in length. For the first month, embryos receive nutrients from a small yolk sac, and thereafter from embryotrophe (ovarian fluid) in which they are bathed. It is thought that the embryos absorb nutrients from the embryotrophe across the wall of a highly enlarged and invaginated hindgut, which reduces back to normal size at birth. The presence of a yolk sac and the development of the hindgut can be used as visual markers for aging the embryos (Table 1).

TABLE 1. Physical indicators used to age embryos.

Age	Physical indications
younger than 3 months pre-parturition (p-p)	<20mm length large yolk sac
3 months p-p	yolk sac smaller hindgut beginning to develop
2 months p-p	yolk sac no longer present very large hindgut
1 month p-p	hindgut still enlarged maximum embryonic size 240mg weight, 44m length
<2 weeks p-p	late January - early February other females giving birth

HISTOLOGY

Tissues from animals at known stages pre-parturition and up to 3 months post-parturition, were fixed for light microscopy using Bouin-Hollande fixative in sea water. Tissues were embedded in paraffin wax, sectioned at 7μm and stained with either Heidenhain's haematoxylin and eosin, or Mallory's Triple Stain. For transmission electron microscopy, tissue samples <1mm^3 were fixed in 3% gluteraldehyde overnight at 4^{o}C, post fixed in 1% osmium tetroxide, stained in uranyl acetate and embedded in Araldite. Silver sections (750Å) were cut using a LKB 2088 Ultratome V, counter stained in lead citrate, mounted on copper grids, coated with amyl acetate and

nitrocellulose mixture, and observed in an AEI transmission electron microscope, Type Corinth 275.

In order to determine the relative volumes of the body organs, outlines of serial sections were traced using a planimeter and measurements calculated using a custom built x, y digitiser interfaced with a DEC PDP-11 computer.

III. RESULTS

THYMUS

The thymus of Zoarces is a paired structure appearing just behind the last gill arch. It lies in the angle between the operculum and the body wall, and is covered by a single layer of epithelium (Fig. 1). It is evident in embryos as early as 3 months pre-parturition (p-p) and by 2 months p-p it has already differentiated into two zones distinguishable by the density of lymphocytes:- a lateral,

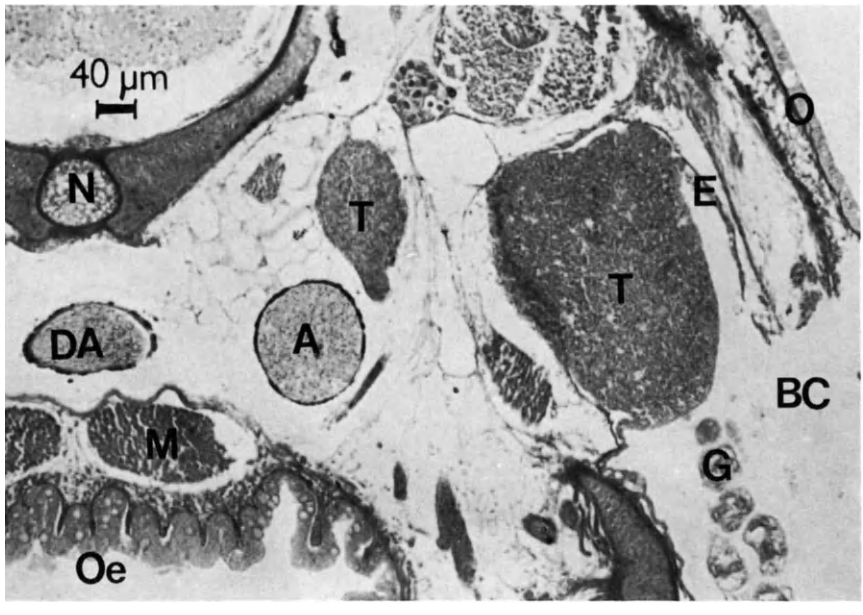

Fig. 1. Cross section through the head to show the position of the thymus (T), thin single cell epithelium (E), operculum (O), branchial cavity (BC), gills (G), notochord (N), dorsal aorta (DA), anterior cardinal vein (A), muscle blocks (M), lumen of the oesophagus (Oe). Heidenhain's haematoxylin and eosin.

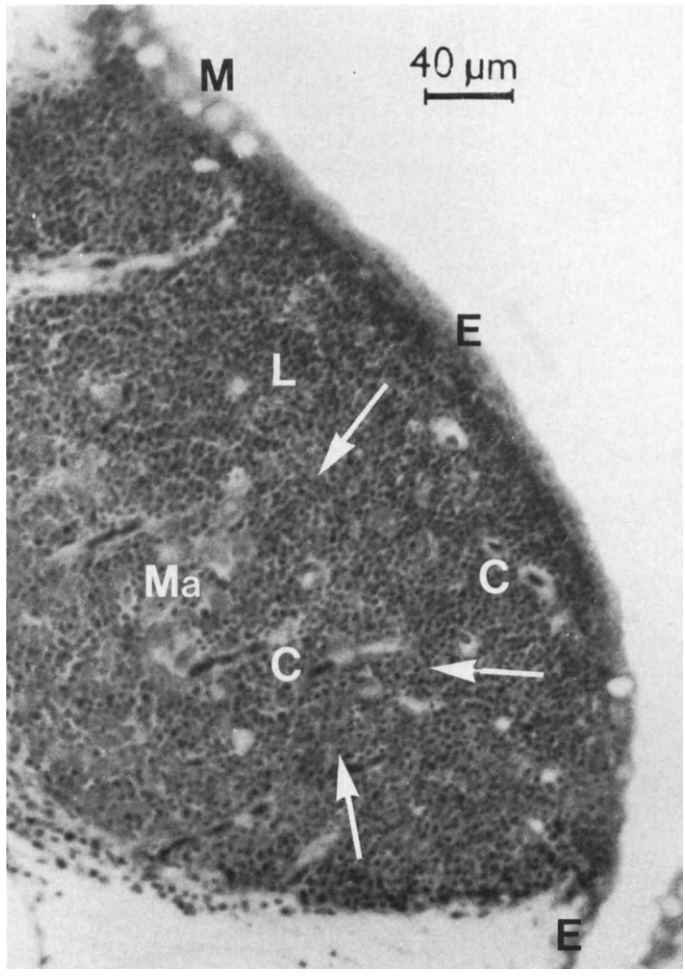

Fig. 2. Cross section of the thymus taken 1 day after birth. Single cell layer of epithelium (E), mucus secretory cells (M), lymphocytes (L), pale staining large macrophages (Ma), sections of capillaries containing erythrocytes (C). Zoning is marked by arrows pointing from the lateral dense zone to the median light zone. Mallory's Triple Stain.

densely stained zone composed mainly of lymphocytes and a few reticular cells, and an inner, median, lightly-staining zone of fewer lymphocytes. There is no distinct boundary between the two zones (Fig. 2). The

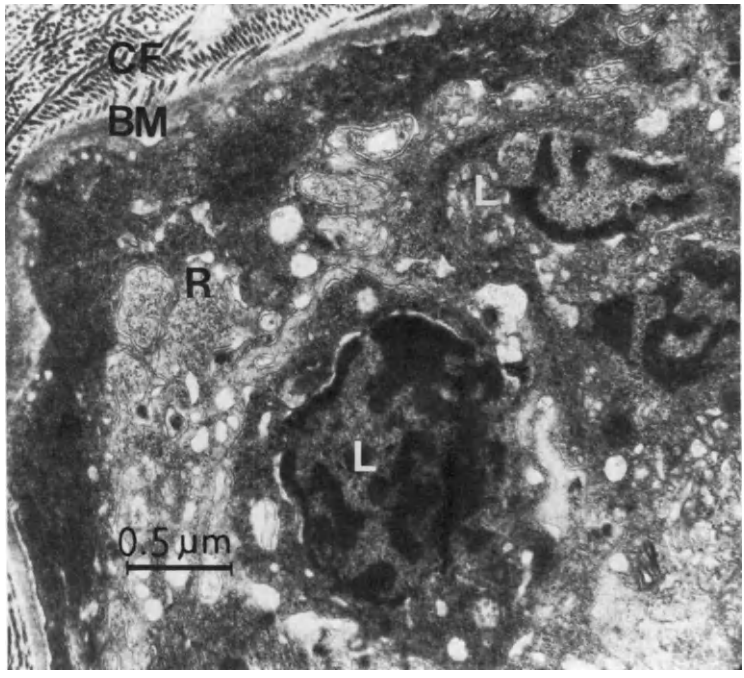

Fig. 3. Electron micrograph of the capsule wall surrounding the thymus. Lymphoctyes (L), reticular epithelial cells (R), basement membrane (BM), collagen fibres (CF).

thymus is bound by a thin connective tissue capsule sitting on a basement membrane which is surrounded by collagen fibres (Fig. 3).

The thymus is composed mainly of lymphocytes though many macrophages are also seen. No myeloid cells are evident. Sections of capillaries contain erythrocytes, but no lymphocytes were seen though they may have been present.

The thymus reaches a maximum size 1 month p-p when it occupies 0.83% of the total body volume. Thereafter it decreases in size and by 3 months post-parturition occupies only 0.048% of the total volume. The thymus at this stage appears to be disorganised and dark, due to the densely staining nuclei of degenerating cells.

HEAD-KIDNEY

The head-kidney is first seen 3 months p-p. At this stage it consists of a few kidney tubules surrounded by a small

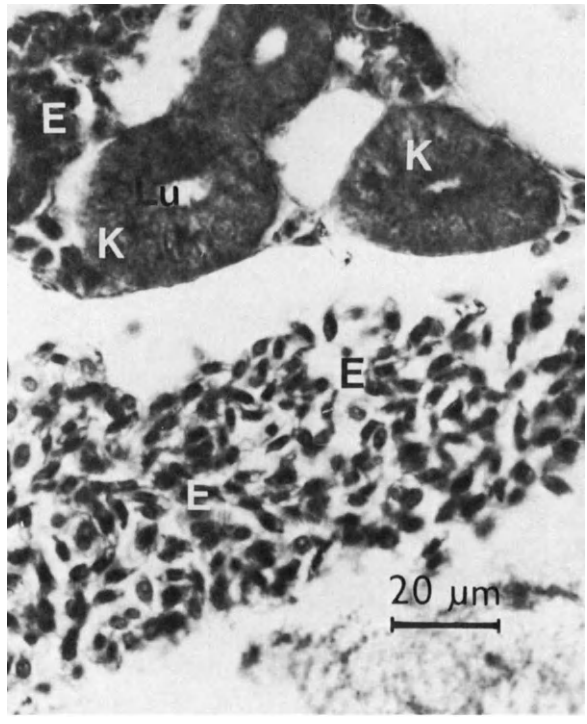

Fig. 4. Cross section of head-kidney 2 months pre-parturition. Kidney tubules (K), lumen of kidney tubules (Lu), erythrocytes in inter-tubular sinuses (E). Heidenhain's haematoxylin and eosin.

number of erythrocytes (Fig. 4). At 1 month p-p the head-kidney contains recognisable lymphoid tissue (Fig. 5). In the cellular matrix granulocytes are seen (Fig. 6) as well as small aggregations of large melanomacrophages containing dark pigment (Fig. 7).

SPLEEN

The spleen is present early in the development of Zoarces embryos, being first seen as a distinct organ 2 months p-p. The organ is in close contact with the pancreas from which the stalk of the spleen arises, being composed of trabeculae which branch out into the spleen as a supportive network (Fig. 8). Differentiation within the spleen is evident at two months post-parturition when two

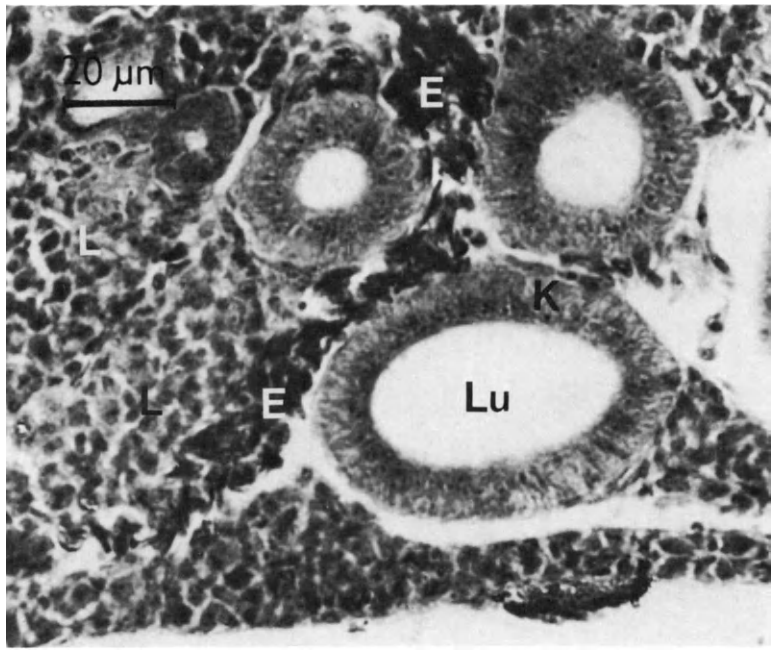

Fig. 5. Cross section of head-kidney 2 weeks pre-parturition. The organ has differentiated into many lightly stained areas of lymphocytes (L), and darkly stained areas of erythrocytes (E). Kidney tubules (K), lumen of kidney tubules (Lu). Malory's Triple Stain.

zones are apparent; lightly-staining lymphoid areas aggregated around the trabeculae, and darkly-staining areas composed mainly of erythrocytes. These two zones may be analogous to the red pulp (erythrocytes) and the white pulp (lymphocytes) of the mammalian spleen (Fig. 9). The paucity of splenic lymphocytes is evident in Fig. 10. As with the thymus and head kidney, the spleen also contains granulocytes and macrophages (Fig.11).

HINDGUT

Red cells of presumed maternal origin were obvious in the embryotrophe of many females, together with decaying debris. Embryos commonly exhibit gulping motions. To ascertain whether such activity was ingestive or simply

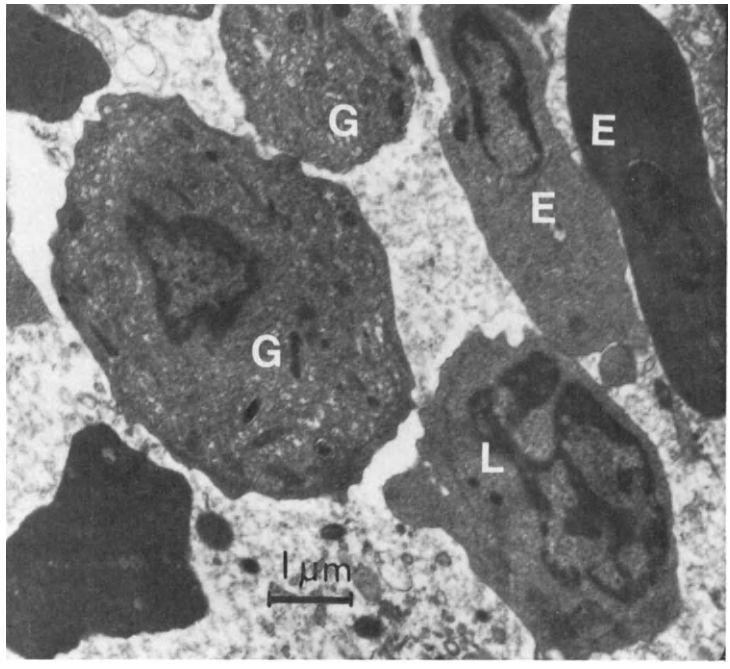

Fig. 6. Electron micrograph of the general cellular content of the head-kidney showing a lymphocyte (L), granulocytes (G), and erythrocytes (E).

respiratory, the gut was scanned for embryotrophe cells and debris. It is of particular interest that maternal red blood cells and tissue debris were seen in many sections of foregut and hindgut (Fig. 12).

IV. DISCUSSION

In comparison with the lymphoid organs of oviparous species, those of the viviparous blenny show a similar sequence of development with the thymus developing first, followed by the head-kidney and lastly the spleen. However, the timing of these events with respect to first contact with water, differs greatly. In oviparous species the thymus becomes lymphoid from as early as 22 days pre-hatch in Salmo salar (Ellis, 1977) to as late as 7-11 days post-hatch in Astyanax (Hafter, 1952). By contrast, differentiation of the thymus in viviparous species occurs much earlier - as early as 3 months preparturition in the

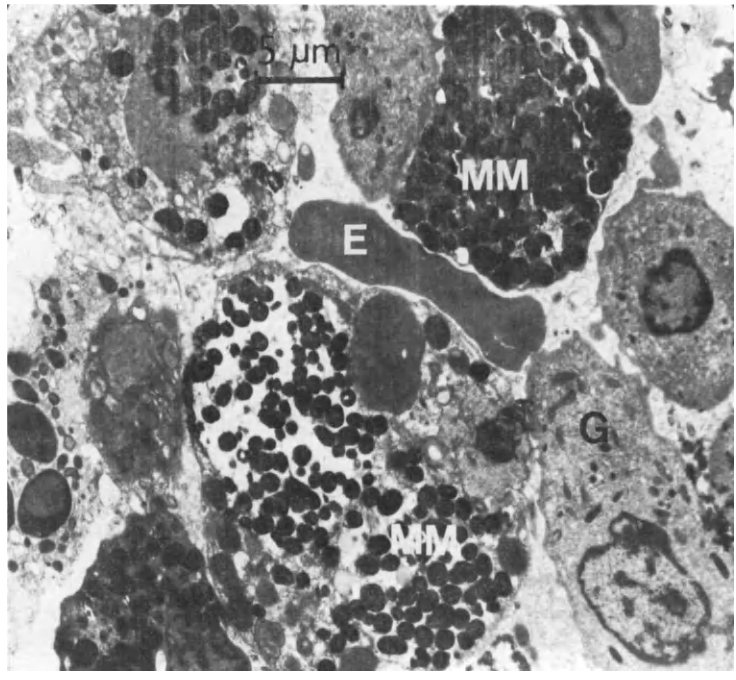

Fig. 7. Electron micrograph of head-kidney to show an accumulation of melanomacrophages containing dark pigment (MM), erythrocytes (E) and a granulocyte (G).

American and Japanese surfperches (Engen, 1968; Tamura et al, 1981). The present work on Zoarces confirms that the thymus of viviparous species develops at a very early stage of embryogenesis.

In the case of the head-kidney, differentiation in oviparous species may occur as early as 14 days pre-hatch in Salmo salar (Ellis, 1977), to 6-8 days post-hatch in rainbow trout (Manning, et al., 1981). By contrast, the head-kidney of Zoarces was seen 3 months p-p and differentiated 1 month p-p. The question of whether the head-kidney lymphocytes are thymic in origin can not be answered because of the absence of any positive identification of lymphocytes in capillaries.

In oviparous species the spleen only develops after hatching. In the viviparous Zoarces, although the spleen develops early, it is thought to play little part in the

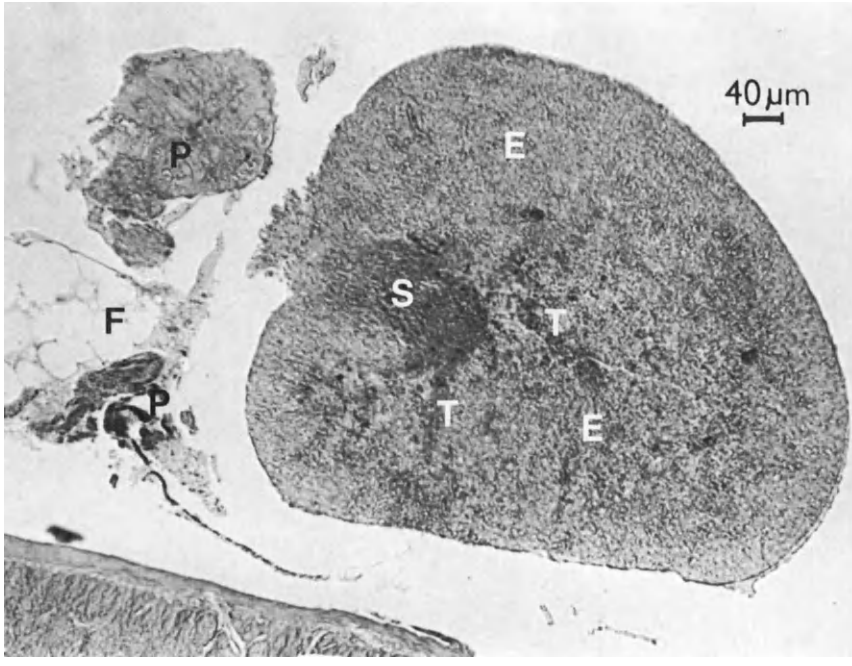

Fig. 8. Spleen, 2 months prior to parturition, not yet fully differentiated. Erythrocytes (E), stalk (S), composed of trabeculae (T) which branch out into the spleen. Pancreas (P), fat cells (F). Heidenhain's haematoxylin and eosin.

ontogeny of the immune system because it remains predominantly erythrocytic throughout embryonic life. The early development of this erythrocytic tissue may have particular significance for Zoarces embryos. Unlike many live bearers, there are no specialised "pseudoplacentae" in Zoarces to aid materno-foetal exchange. The early development of an efficient oxygen transporting system may be particularly advantageous to embryos packed tightly in embryotrophe, which may well have an oxygen tension lower than sea water.

The factors which govern the timing of the development of the lymphoid system in teleosts are unknown, but there are at least two hypotheses; firstly, natural selection will favour individuals in which the development of lymphoid tissues coincides with the first exposure to potential pathogens, i.e. hatching in oviparous species

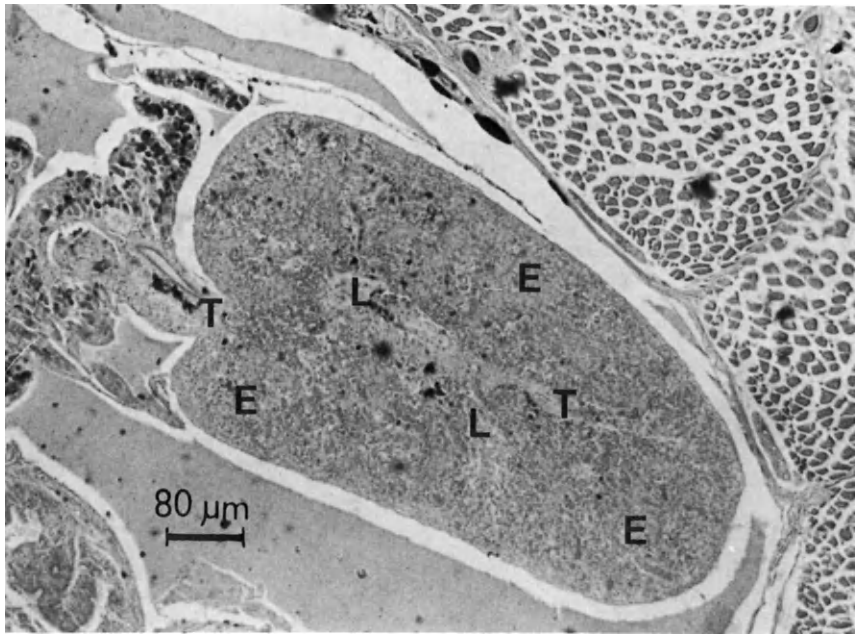

Fig. 9. Spleen, 2 months after birth, differentiated into lightly staining lymphoid zones (L), around the trabeculae (T) and dark outer zones of erythrocytes (E). Mallory's Triple Stain.

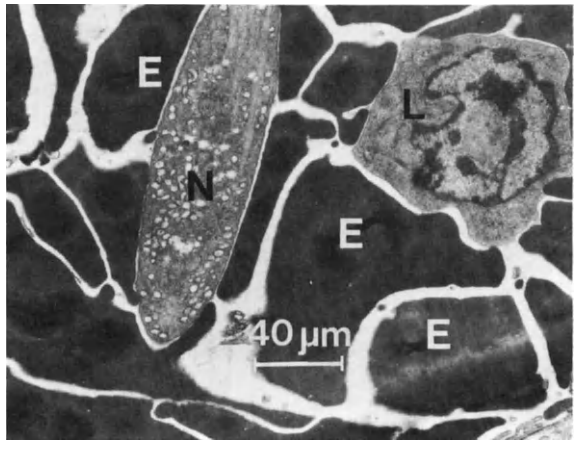

Fig. 10. Electron micrograph to show the cell types within the spleen, 2 weeks pre-parturition. Erythrocytes (E), lymphocyte (L), nerve fibre (N).

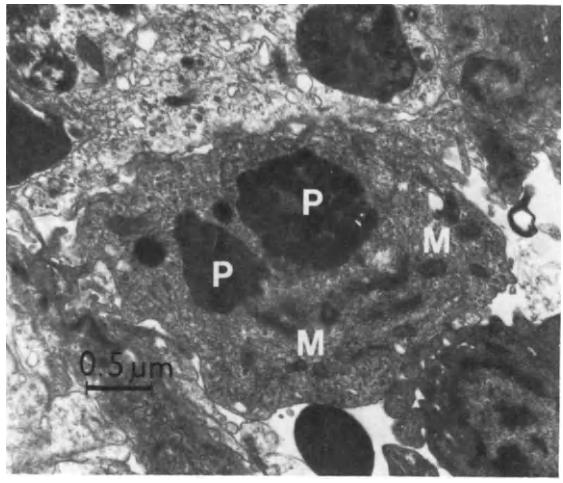

Fig. 11. Electron micrograph of a macrophage in the spleen 2 weeks pre-parturition, showing phagocytosed particles (P), and mitochondria (M).

and parturition in viviparous species. However, the lymphoid system of viviparous species develops very early, long before parturition. It may be highly significant that the gut of Zoarces embryos contains maternal blood components (Fig. 12, and Kristofferson, et al., 1973), and tissue debris probably from resorbed siblings. This strongly indicates that the embryotrophe is not sterile and that it may contain potential antigens of maternal and sibling origin. The development of lymphoid organs at a stage much earlier than parturition would clearly be an advantage to such exposed embryos. Secondly, development of the lymphoid organs may occur at a pre-set time during embryogenesis (Solomon, 1971) once the embryo has reached a certain physiological age, differentiation being completely independent of first exposure to potential pathogens. Further research is required to decide which is the preferred hypothesis.

Finally, the early development of the lymphoid system in Zoarces raises the possibility that an embryo may be capable of producing an immune response to siblings within the ovary.

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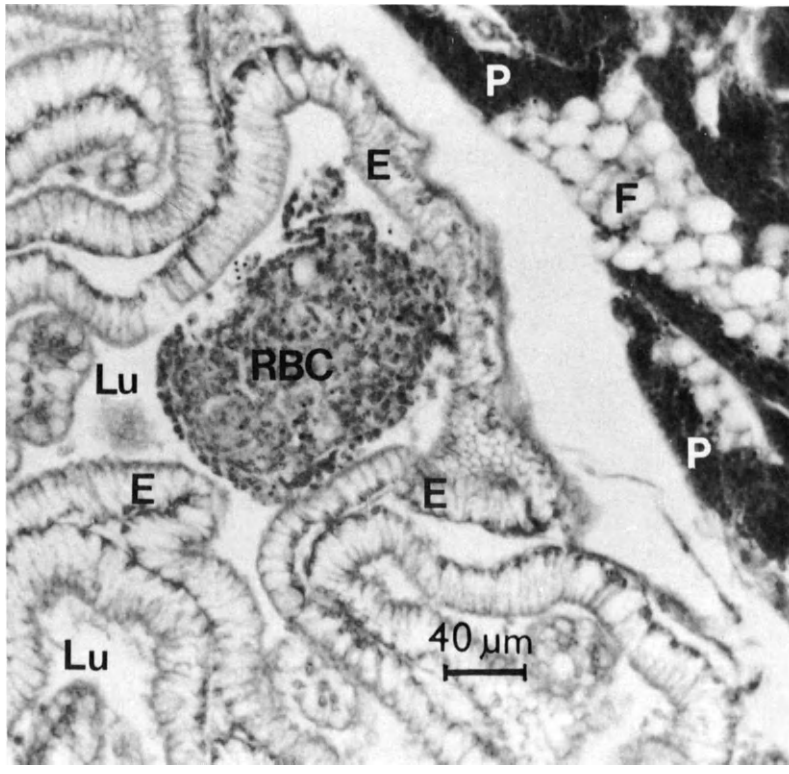

Fig. 12. Cross section of the hindgut of an embryo 2 months prior to parturition showing the presence of maternal red blood cells (RBC) in the lumen (L) of the gut. Epithelial lining of the gut (E), pancreas (P), fat cells (F). Heidenhain's haematoxylin and eosin.

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EXPERIMENTALLY INDUCED IMMUNE REACTIONS TO GONAD IN RAINBOW TROUT (SALMO GAIRDNERI)

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I. INTRODUCTION

The control of maturation in fish is one of many problems still to be solved in aquaculture. Methods now exist which can produce mature rainbow trout at any time of the year, and which can produce all female ova. However, the commercial production of sterile fish for human consumption has still to be achieved. The advantages of sterile fish have been mentioned elsewhere (Laird et al., 1980; Donaldson and Hunter, 1982; Refstie, 1982). Many approaches have been proposed including irradiation, chemo-sterilisation, surgical techniques, hormone administration, hybridisation and chromosome manipulation. These methods have to date proved unsuccessful because they are unreliable, uneconomical or unsuitable when the fish are destined for the table. Recent work at Aberdeen University has been concerned with an immunological approach (Laird et al., 1978; Secombes et al., 1982).

In mammals the immunological approach to fertility regulation is based on the fact that immune responses can be induced not only towards "non-self" antigens, but also towards "self" antigens to which tolerance has not been induced during the early stages of development. The latter are autoimmune responses. The antigens of sperm are good examples because they develop at a late stage of ontogenesis, much later than the stage at which tolerance to them might be developed. Thus sperm-specific antigens have auto- as well as iso-immune properties. Such antigens are normally shielded from the immune system by a

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blood-testis barrier. For reviews see Rümke (1980) and Hogarth (1982).

Using this approach in salmonids, it has already been shown that an intraperitoneal injection of testis homogenate emulsified in Freund's complete adjuvant, can induce both cellular (Laird et al., 1978, 1980) and humoral (Secombes et al., 1982) responses. To what extent these reactions represent a true autoimmune response or adjuvant "side-effect" will now be considered.

II. MATERIALS AND METHODS

ANIMALS

Immature rainbow trout Salmo gairdneri Rich. 1836 were purchased from Tullynessle Trout Farm, Alford, and weighed on average 50g. These fish were kept in an open water system at the ambient temperature 12-17°C. Male broodstock rainbow trout were supplied by the Highlands and Islands Development Board Hatchery at Moniack. These fish weighed approximately 600g and were kept at a constant 11°C.

IMMUNISATION REGIME

Immature rainbow trout were injected either intraperitoneally (i.p.) or intramuscularly (i.m.) during April-August with homogenised allogeneic gonad material (mature testis or ovary plus testis) emulsified in Freund's complete adjuvant (FCA) (Difco, Detroit). Each fish received 0.1 ml of a 50% gonad solution mixed with an equal volume of FCA. Fish receiving an i.p. injection were bled 4 and 8 weeks after immunisation, and were killed for histology on week 10 or 15. Fish receiving an i.m. injection were also bled 4 and 8 weeks after immunisation, but at the second sampling all gonad injected fish were given a second injection of testis in phosphate buffered saline (PBS) and bled after a further 4 and 8 weeks. Some of these fish were killed for histology after each blood sampling.

Male broodstock were injected i.m. in November with 0.4 ml of a 50% testis/FCA solution, and bled at fortnightly intervals for 8 weeks. Blood samples were also taken when these animals were killed for histology.

Controls consisted of animals injected with PBS/FCA or PBS alone. Six to ten fish were bled per group at each sampling time.

HISTOLOGY

Animals killed after immunisation had their tissues fixed in Bouin's fluid, for staining with haematoxylin and eosin (H & E). The gonad, liver, spleen, mesonephros, pronephros, pyloric caecae (including pancreas), heart, brain and muscle (site of i.m. injection) were fixed for histological examination. Wax sections were cut at 5 μm and stained. In addition milt samples taken from male broodstock were smeared and stained with H & E.

ANTIBODY TITRATION

All sera were decomplemented at 50°C for 30 min before use. Sera were serially diluted in PBS in microtitre plates. To each well was added 50 μl of fresh allogeneic trout spermatozoa, adjusted to 4 x 10^7 cells/ml. After 3 hours a sample was taken from each well and examined for sperm agglutination under a microscope. In addition some milt samples from the broodstock were centrifuged and the cell-free supernatant (seminal plasma) tested for its agglutinating activity. Selected sera were also tested against autologous spermatozoa.

III. RESULTS

HISTOLOGY

In fish receiving an i.p. injection of FCA, with or without gonad material, severe adhesions were found on the peritoneal organs, particularly on the pancreas, the gonad and in the gut mesentery. Tissues outwith the peritoneal cavity showed no effects. The most profound response was seen in the gut mesentery, especially in the pyloric caecae region. The pancreas was enclosed in a gross mesenteric adhesion (Fig. 1) consisting of a dense fibrous stroma supporting mainly mononuclear cells. Despite the fact that it interdigitated over the surface of the pancreas, it was invasive in only a few regions where it appeared to have replaced fat cells. Thus the pancreatic tissue was unaffected except in a few isolated areas. However, pancreatic blood capillaries and lymphatics were distended. Further down the gut the mesentery supported gross adhesions. The epithelial lining was replaced by fibrocytes and granulation tissue streamed inwards around the isolated and necrotic fat cells. Such gross adhesions were extensively melanised.

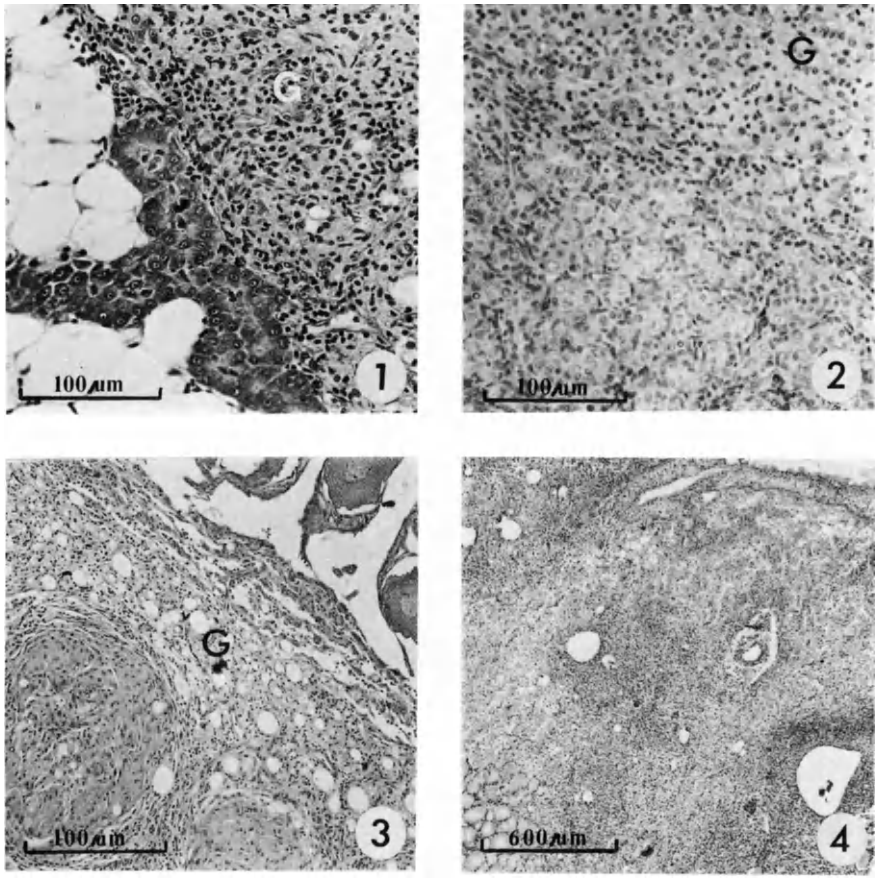

Fig. 1. Granuloma on the surface of the pancreas. H & E.

Fig. 2. Invasive granuloma streaming in between testis lobules. H & E.

Fig. 3. Granuloma on the surface of the ovary. H & E

Fig. 4. Muscle necrosis and invasion of polymorphs at site of intrasmuscular injection. H & E

G = Granuloma

The gonad, whether ovary or testis, was enveloped in a large adhesion. On the testes the bulk of the adhesions were concentrated along the hilum, extending over the organs to completely cover them at their thickened anterior region. They were not invasive. The bulk of the adhesions were dense with a fibrocytic organisation supporting mononuclear cells and the occasional eosinophilic granulocyte. The lesion was permeated by lymph spaces. A few fish injected with testis/FCA showed a more invasive adhesion (Fig. 2). The testis capsule was disrupted and the lesion appeared to be streaming in between the lobules. Necrotic fragments were evident amongst the leading edge of inflammatory tissue. Similarly in the ovaries the ovarian capsules were grossly thickened into large adhesions (Fig. 3). The whole mass had a connective tissue stroma supporting capillaries and lymphatics. The stroma was suffused with mononuclear cells and some melanocytes. Occasionally, encapsulated foci of giant cells and eosinophilic cells were seen in the stroma, with a central necrotic and degenerative zone. The granulomatous tissue was in general non-invasive, but again some animals injected with testis/FCA showed finger-like tracts extending into the ovary from the surface. However the oocytes appeared to be unaffected.

In contrast to the fish receiving an i.p. injection containing FCA, immature fish receiving an i.m. injection showed no adhesions on any tissues. However, they did possess deep muscle abscesses at the injection site (Fig. 4). The main feature of these abscesses was extensive necrosis of muscle bundles. The predominant cell types in the dense inflammatory response packing the abscesses were polymorphs and macrophages, some containing sperm heads. There was early fibrocytic organisation and interspersed amongst the dense matrix of fibrocytes were numerous large lacunae, many of which contained abundant sperm heads. This focal inflammation was most marked during the first two weeks after injection. Interestingly, in fish killed 3 days after injection to examine the muscle abscess, sperm heads could be found in the occasional distended lymphatic in the mesonephros. No other degenerative changes were observed in the tissues of i.m. injected fish, including the gonads.

Male broodstock were stripped periodically and the milt samples examined for autoagglutination. During this routine stripping, the milt samples in 5 out of 10 fish injected at least 8 weeks previously with testis/FCA were found to contain immense numbers of active phagocytes

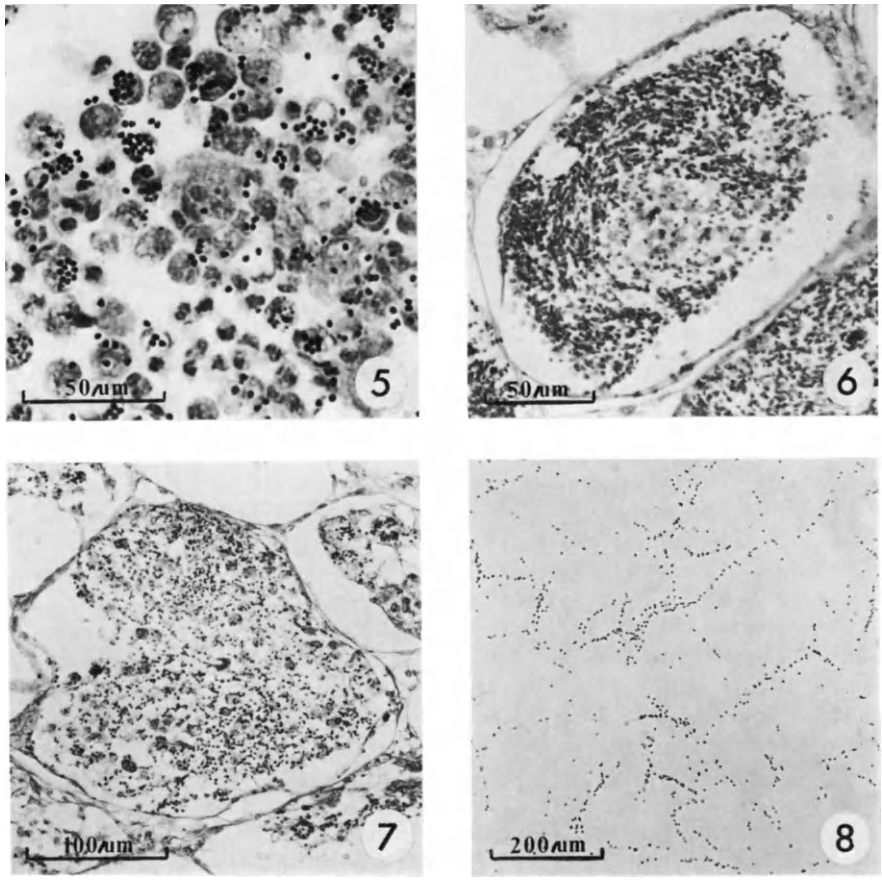

Fig. 5. Smear of milt sample showing active phagocytes engorged with sperm heads. H & E

Fig. 6. Mature testis showing invasion of phagocytes into the seminiferous lobule. H & E

Fig. 7. Mature testis showing seminiferous lobules almost devoid of sperm. H & E

Fig. 8. Agglutination of spermatozoa seen after incubation with serum from testis/FCA injected trout.

engorged with sperm heads (Fig. 5). Most were large cells (15-17 μm), probably macrophages, although some smaller cells (9-11 μm) were also present. Relatively more sperm heads were found in the larger cells. Some free spermatozoa were also present, as was an occasional erythrocyte.

Within a few days of finding the phagocytes it became clear that such fish were far from normal. There was an extreme loss of body colour and the fish were obviously very weak. If the fish were not killed at this time they died within another few days. When examined internally, it was found that these fish possessed intensely inflamed testes, but all other organs were normal. In wax sections of these testes, some regions looked normal (compared with control testes), with the lumen of the seminiferous lobules containing sperm cells. In other areas, however, the lumen appeared to be invaded by large pale staining phagocytes, ingesting the spermatozoa (Fig. 6), and indeed many lobules were almost completely devoid of sperm (Fig. 7). Blood vessels appeared to be distended, but little haemorrhaging had occurred.

Control fish did not contain phagocytes in their milt samples, even late in the season when it became increasingly difficult to strip fish.

ANTIBODY TITRES

Tables I and II show the sperm agglutinating serum antibody titres from immature trout injected i.p. and i.m. respectively. Fish injected with gonad/FCA showed low, but significant, titres, whereas PBS injected fish showed no titres at any time. Fish injected i.p. with FCA also showed some antibody titres. However, only about 50% of these fish responded, compared to almost 100% of gonad injected fish, giving large standard deviations by week 8. This group was thus subdivided into responders and non-responders in the sperm agglutination results.

In contrast to the i.p. injected fish no titres could be detected in any immature trout injected i.m. with FCA alone. When injected i.m. with gonad plus FCA good titres were obtained, going as high as 1:1024 after a booster at week 8. Again PBS injected fish gave no titres. Whether the fish were injected i.p. or i.m. the type of sperm agglutination was the same. Agglutination occurred along the whole length of the sperm (Fig. 8), similar to type E (tangle) agglutination described by Rose et al. (1976) for mammalian anti-sperm auto-antibodies. Head-to-head and tail-to-tail agglutination did not occur.

TABLE I. Mean sperm agglutination titres from immature trout injected intraperitoneally. Titres are expressed as $-\log_2$ titre $\pm$ standard error.

Treatment	Week 4	Week 8
Mixed gonad plus FCA	3.63 $\pm$ 0.68	3.25 $\pm$ 0.94
Testis plus FCA	2.25 $\pm$ 0.41	2.25 $\pm$ 0.75
PBS plus FCA (responders)	2.50 $\pm$ 0.29	4.50 $\pm$ 1.50
PBS plus FCA (nonresponders)	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
PBS	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00

TABLE II. Mean sperm agglutination titres from immature trout injected intramuscularly. Titres are expressed as $-\log_2$ titre $\pm$ standard error. Titres in parentheses were obtained after a booster given at week 8.

Treatment	Week 4	Week 8
Mixed gonad plus FCA	3.50 $\pm$ 1.15 (2.00 $\pm$ 0.95)	3.50 $\pm$ 0.67 (N.T.)
Testis plus FCA	4.10 $\pm$ 0.48 (6.10 $\pm$ 1.09)	4.30 $\pm$ 0.50 (4.67 $\pm$ 0.63)
PBS plus FCA	0.10 $\pm$ 0.10 (0.20 $\pm$ 0.20)	0.00 $\pm$ 0.00 (0.00 $\pm$ 0.00)
PBS	0.00 $\pm$ 0.00 (0.00 $\pm$ 0.00)	0.00 $\pm$ 0.00 (0.00 $\pm$ 0.00)

N.T. = Not Tested.

In male broodstock injected with testis/FCA, no antibody could be detected in the serum during the first 8 weeks after injection or when killed during the following 8 weeks to examine the testes. However, 1 of the 5 fish which were found to contain phagocytes in their milt, did have a titre of 1:16 in the cell-free supernatant. When the water temperature was raised from 11°C to 14°C 19 weeks after immunisation, 2 of the remaining 5 fish without phagocytes did develop serum antibody titres of 1:8 within 2-4 weeks. Such antisera were also positive to the same degree with autologous spermatozoa.

IV. DISCUSSION

It is clear that an i.p. injection of FCA, with or without gonad material, leads to the production of a granuloma on the surface of the gonad and pancreas, and the production of anti-sperm antibodies, in immature rainbow trout. The formation of a granuloma has been taken to represent a cell-mediated autoimmune response (Laird et al., 1980), and indeed, in mammals, injection of FCA alone is known to induce lesions resembling those seen in organ-specific auto-immune disease (Sell, 1980). This could explain why half the population of fish injected with FCA alone produced antibodies to sperm. Approximately half the population would be expected to be males, and contact of testes with FCA in the peritoneal cavity may stimulate an immune reaction. However, in mammals the histopathology of experimental allergic orchitis (EAO) consists of an interstitial mononuclear infiltrate with invasion of macrophages into the seminiferous tubules, followed by degenerative changes in the germinal epithelium (Tung, 1977), and is more characteristic of a delayed hypersensitivity reaction than a granulomatous reaction. Tuberculosis is a classic example of a granulomatous disease, so it seems likely that it is the killed Mycobacterium tuberculosis contained in the FCA which causes such a reaction wherever it lays in the peritoneal cavity. The ability of i.p. injected FCA to induce anti-sperm antibodies further complicates any quantitative studies and so the i.m. route was investigated.

Intramuscularly FCA injected alone gave no detectable antibody response to spermatozoa, and no granuloma formation around any organs in the peritoneal cavity. Similarly, no granuloma or organ destruction was seen after injection of gonad material/FCA i.m. However, clear serum antibody responses were seen. Whether or not these

antibodies are cytotoxic, and whether they cross-react with premeiotic germ cells has still to be ascertained. Spermatozoal autoantigens occur only in postmeiotic stages of spermatogenesis in mammals (Radu and Voisin, 1975; O'Rand and Romrell, 1977). Clearly the presence of sperm agglutinating antibody in this study did not cause germ cell destruction.

The results with mature fish were very interesting. Here, 5 out of 10 fish developed a response resembling in many ways that of EAO. There was a massive invasion of macrophages into the seminiferous lobules with gross ingestion and destruction of spermatozoa.

These events were not detected until late in the reaction when they were beginning to have a detrimental effect on the fish. In mammals, the first signs of delayed hypersensitivity (focal clusters of lymphocytes and macrophages) can be seen histologically 6-9 days after injection, and by 8 weeks or so only some spermatogonia remain (Hogarth, 1982). With rainbow trout, the events seem slower, and this may be related to the relatively low water temperature (11°C) at this time. If there was an initial early influx of lymphocytes this was missed by the late sampling.

In trout where there is an annual cycle of spermatogenesis, it is difficult to demonstrate the occurrence of complete aspermatogenesis and germ cell destruction. Certainly large areas of the testes were devoid of sperm. Perhaps the large size of the testes at this time of year, up to 6.5% of the body weight (van den Hurk et al., 1978) can account for the severity of the reaction. If such a large proportion of the body becomes antigenic this in itself may be fatal. Control fish did not show such a gross reaction at any time, although normal involution of the testes in trout does involve phagocytosis of residual sperm (Henderson, 1962; van den Hurk et al., 1978).

In EAO a complex interplay between humoral antibody and cell-mediated immunity leads to immunopathological reactions. However, in the present study no sperm agglutinating antibodies could be detected in the serum of fish showing phagocytes in the testes. This may indicate that humoral immunity has no role in the above response. Perhaps it cannot develop at these water temperatures or time of year. Alternatively, it may be that the testes are so large that any testis-specific antibodies are absorbed from the serum. In this respect it is interesting that trout immunoglobulin could be detected in the seminiferous

lobules of affected fish using immunofluorescent techniques (personal observation), although it is not known if this was specific immunoglobulin, and one fish had sperm agglutinating activity in its seminal plasma. In mammals, agglutinins are seldom found in the seminal plasma if they are not present in the serum (Rümke, 1980), so perhaps they arise from local synthesis and secretion as occurs with IgA in human seminal plasma (Hjort, 1977). Nevertheless, there remains the possibility that antibody coated spermatozoa could have an opsonic effect on macrophages.

When antibodies were found in mature fish they agglutinated both allogeneic and autologous spermatozoa in an identical manner. So autoantibodies to spermatozoa do exist in fish. This may indicate that the antibodies to allogeneic spermatozoa obtained in the immature fish would be able to agglutinate autologous spermatozoa after or during spermatogenesis. Immature female fish produced antisera that reacted in an identical manner to male antisera, but such antisera would clearly not react against autoantigens. However, studies in humans have shown that female sera containing sperm antibodies can react against the same (auto) antigen as male auto-antisera (Poulsen, 1983).

In mammals disruption of the normal blood-testis barrier appears to be a prerequisite to the induction of EAO (Tung, 1977). As the barrier is less impregnable in the rete testis than in the semniferous tubules themselves, it is usually here that the first signs of immune aggression are apparent. In fish, a blood-testis barrier does exist (Abraham et al., 1980; Marcaillou and Szöllösi, 1980), but there is no corresponding structure to the rete testis. One possibility is that FCA may cause ultrastructural alterations and an increase in the permeability of the blood-testis barrier at the seminiferous lobule as occurs in the guinea pig (Willson et al., 1973). Another possibility is that autoimmune damage of the germinal epithelium is secondary to autoimmune damage of the seminiferous lobule basement membrane. Recent studies using rats have shown that autoimmune orchitis can be induced by injection of seminiferous tubule basement membrane antigens in FCA (Denduchis et al., 1978; Lustig et al., 1982).

Whatever the mechanism by which the blood-testis barrier is breached, clearly an experimentally induced orchitis can be invoked in trout. The extent to which testis autoantibodies are part of this response has still

to be determined. Further research is needed before these findings can be used to devise techniques for the control of maturity in fish.

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IMMUNOSUPPRESSION AND IMMUNOLOGICAL TOLERANCE IN CARP

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I. INTRODUCTION

Immunological memory, immunosuppression and immunological tolerance are all facets of two opposing control mechanisms in immune responses, namely helper and suppressor cells. Helper cells were first demonstrated in fish by means of the hapten-carrier effect (Yocum, et al., 1975; Stolen and Mäkelä, 1975). More recently attention has been drawn to the question of whether suppressor cells exist in fish. Serero and Avtalion (1978) and Wishkovsky and Avtalion (1982, and in preparation) have achieved immunosuppression or tolerance while the fish were exposed to low temperatures. This generation of suppression only seems to occur with high doses of antigen and may be aided by the fact that helper cell maturation is temperature sensitive (Avtalion, 1981). In mammals, suppressor cell activity can be generated by use of self cells which are slightly modified by the presence of hapten molecules. Since Battisto and Bloom's work in 1966, it has been recognised that haptens such as the dinitrophenyl determinant are capable of producing immunosuppression when coupled with syngeneic lymphocytes or erythrocytes. Hamilton and Miller (1974) demonstrated that in mice the ratio of hapten to syngeneic carrier, in

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this case 4 hydroxy-5-iodo-3-nitro-phenyl (NIP) and erythrocytes, was an essential element in whether immuno suppression occurred or not. Complete suppression was only achieved with the highest ratio used. Shearer et al., (1975) showed that under certain conditions the HMS cells could be immunogenic and generate cytotoxic reactions whose effector cells required homology with the modified target cells at the K or D ends of the murine major histocompatibility complex (H_2). This was one of the first hints that the MHC was functionally involved in the response to, and formation of, new antigenic determinants which involve modified self components. So it appeared to Scott and Long (1976) that hapten modified self cells may produce differential B cell tolerance and T cell immunity. Their in vitro experiments confirmed this view. The same preparation of TNP spleen cells was able to block a plaque forming cell (PFC) response whilst simultaneously stimulating a cytotoxic T cell response. Scott and his co-workers in their series of papers on the role of self carriers in the immune response developed the hypothesis that T_S cells or cytotoxic cells (killer cell activity being undiminished) were responsible for tolerance induction (Long and Scott, 1977; Scott, 1978; Jandinski and Scott, 1979; Jandinski et al., 1980; Li and Scott, 1980a and 1980b). Certainly lymphocyte sub-populations respond to (or recognize) the hapten modified self cells differently. Work with athymic nude mice, or cyclophosphamide, showed that T cells are required, but that cytotoxic or delayed hypersensitivity T cells were not involved. Further experiments refined this idea to show that tolerance induction involved Ly 1, 2, 3 positive T cells. It was now recognized that two systems may be involved. Antigen associating with self MHC structures may induce suppression (through T_S cells) whereas those which do not, cause unresponsiveness by direct blockade or deletion. The plaque forming cell response was shown to have the same restriction in recognition as the cytotoxic response, indicating that the K and D region determinants were capable of producing T_S and cytotoxicity. Work in vitro has shown that T_S cells can be generated in this environment in response to modified self. Furthermore heavily haptenated (in this case a mouse thymoma line) were able to induce both tolerance and suppression. Sub-optimally haptenated carriers could induce tolerance but not suppression. This suggests that suppression may be generated primarily in response to modified MHC encoded determinants.

Other similar work has been carried out using a variety of hapten-carrier systems. Ramos et al., (1980) using fluorescein haptenated mouse spleen cells, found that a prerequisite for suppression of a B cell response was preimmunization with non-immunogenic structures. Furthermore, only a medium hapten-carrier ratio was capable of producing an immune response. Higher and lower ratios produced insignificant responses. Using delayed type hypersensitivity, Reiner and Claman (1981) found that there was a wide range of hapten-carrier densities over which suppression could occur. The unresponsiveness could also be dissected into two components. The in vitro proliferative response was more sensitive to the tolerizing signal than the ear swelling response and required lower hapten-carrier ratios. Reiner and Claman therefore suggest that each response is mediated by different cells.

It was with the above experiments in mind that the work reported here was carried out. In order to further investigate the elements of the fish immune response and to establish its similarity or otherwise with the mammalian system, it was necessary to find out whether hapten modified self immunosuppression was possible. As yet there are no inbred lines of carp and so the carrier cells had to be readily available in large enough numbers without sacrificing the experimental animals. Fish erythrocytes were entirely suited to this role, most especially as they have MHC encoded antigens present on the cell surface (mammalian erythrocytes do not) and MHC restriction has been implicated in the response.

II. MATERIALS AND METHODS

ANTIGEN AND IMMUNIZATION

Thrice washed erythrocytes (fish, horse or sheep) were haptenated with picryl sulphonic acid (TNBS) (Sigma) by the method of Rittenberg and Pratt (1969). The fish were injected intramuscularly with 50μl of packed erythrocytes (2×10^9 cells). Three hapten carrier ratios were used:

Low	6mg TNBS in 21ml Cacodylate buffer
Medium	60mg TNBS in 21ml Cacodylate buffer
High	600mg TNBS in 21ml Cacodylate buffer

These ratios have been abbreviated to 6mg TNP, 60mg TNP and 600mg TNP respectively.

FISH

Adult carp (Cyprinus carpio) were purchased from Humberside Fisheries, Skerne, Driffield, North Humberside (4 - 6 inch animals) or obtained from Drake's Reservoir, North Hill, Plymouth, Devon (6 - 8 inch animals). They were maintained in dechlorinated water at 18°C ± 1°C and fed on Mainstream Trout diet, BP Nutrition. The carp were bled from the caudal vein using a 25G needle. The blood was washed three times in a large volume of Hanks balanced salt solution (HBSS) and 5% foetal calf serum (FCS) before use.

HAEMOLYTIC PLAQUE ASSAY

Cell suspensions from the pronephros were prepared by pushing the tissue through a fine steel grid into cold HBSS containing 5% FCS. The haemolytic plaque assay was carried out using a modification of the Cunningham plaque assay described by Rijkers et al. (1980) for carp cells. The complement source used was serum from bream (Abramis brama) absorbed against sheep and horse erythrocytes. The target erythrocytes were haptenated with TNBS to achieve a medium hapten - erythrocyte ratio as described above.

III. RESULTS

PRIMARY RESPONSE

Experimental and control fish were divided in to three groups each and injected on Day 0 as follows:

Experimental	6mg	TNP Self erythrocytes
	60mg	TNP Self erythrocytes
	600mg	TNP Self erythrocytes
Control	6mg	TNP HRBC
	60mg	TNP HRBC
	600mg	TNP HRBC

The haemolytic plaque assay was carried out on Day 19, the day of peak response for carp under the conditions used in this laboratory. The results are shown in Fig. 1. It is quite plain that TNP Self elicits no or very little anti-TNP response from the fish. On the other hand, the anti-TNP response in the control animals was good, most especially in the group given the medium hapten-erythrocyte ratio. There was little or no anti-HRBC

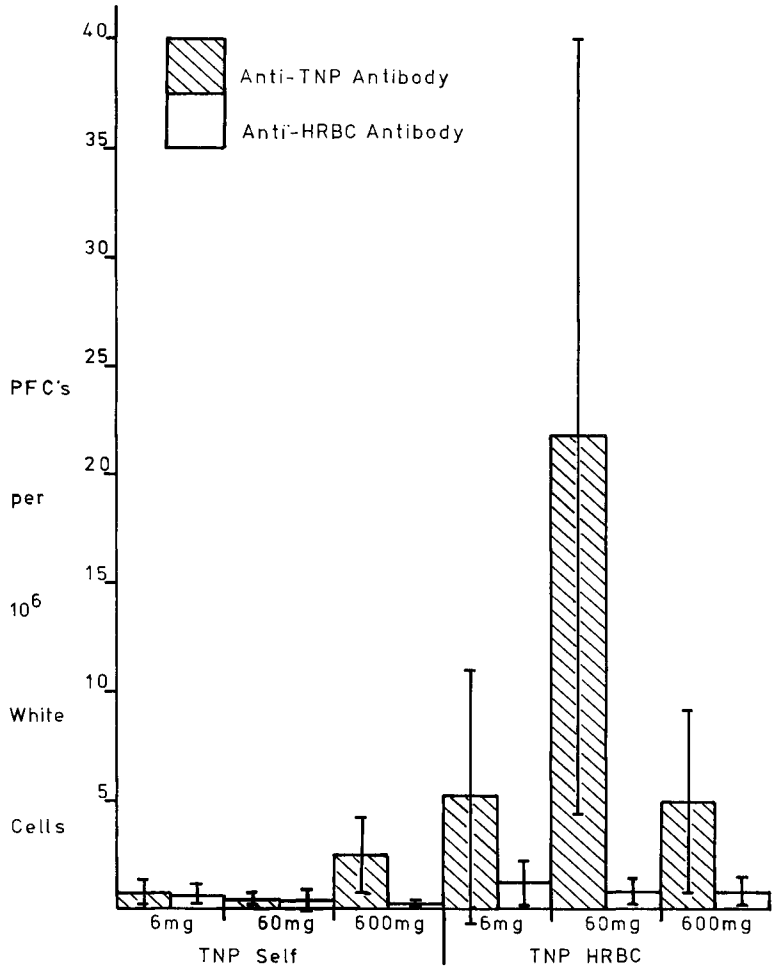

Fig. 1. Primary plaque forming cell response in carp, Cyprinus carpio, following injections of TNP Self, or TNP Horse Erythrocytes.

activity in either the experimental or control groups.

SECONDARY RESPONSE

Primary injections were carried out as for the primary response. On Day 23 all fish were injected with TNP-HRBC of medium hapten-erythrocyte ratio. The results are shown in Fig. 2. Comparison with Fig. 1 shows that the

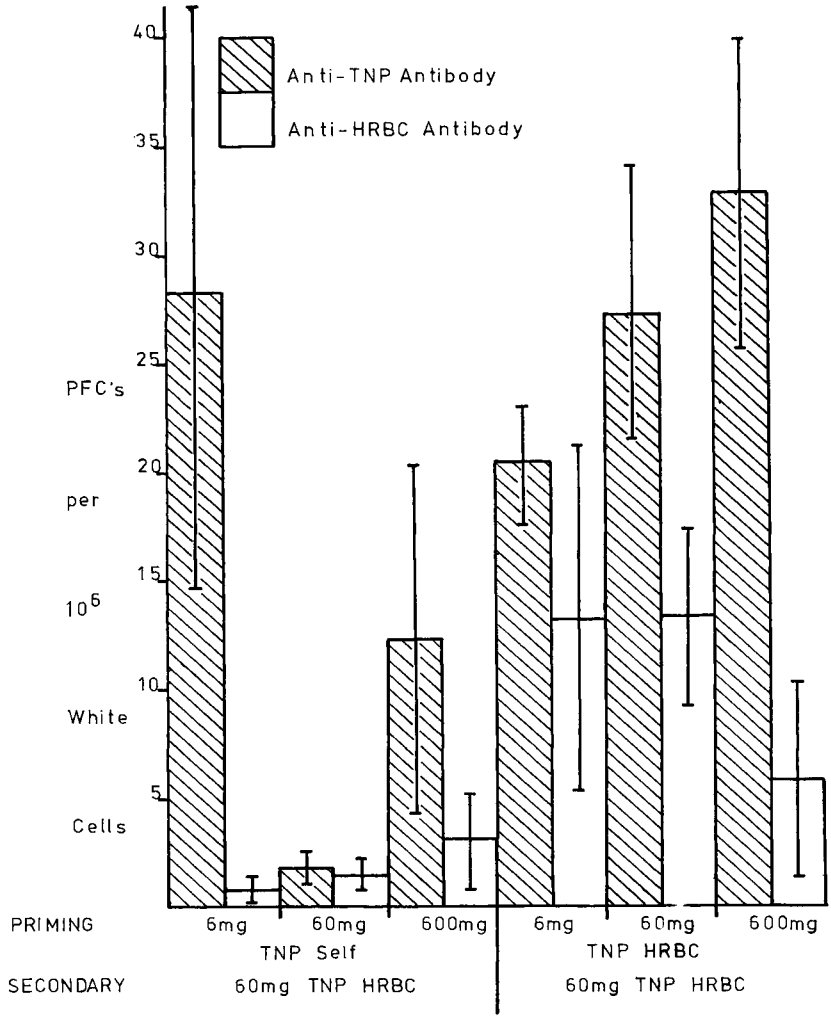

Fig. 2. Secondary plaque forming cell response in carp, Cyprinus carpio, to a secondary injection of TNP-HRBC.

secondary response of the medium ratio TNP Self primed fish is significantly below that of the medium ratio TNP-HRBC injected fish. TNP Self primed fish gave good responses only when immunized at low and high hapten densities. Control fish showed good secondary responses. Anti-HRBC activity was still small in the TNP-Self primed animals but in the control animals it had risen to a

higher level.

IV. DISCUSSION

The work presented here has, principally, two elements. On the one hand it shows that immunosuppression to hapten modified self structures does occur in adult carp, while on the other hand it shows the dependence of this response on the hapten-carrier ratio.

The anti-TNP response of the 60mg TNP Self primed, 60mg TNP HRBC challenged group is significantly depressed when compared with the higher and lower hapten carrier ratio groups. If the priming with 60mg TNP Self had no effect whatsoever, then one would expect the response to be similar to that of the 60mg TNP-HRBC injected group, i.e. a normal primary response. This is clearly not the case and the effect of the 60mg TNP Self priming has been to induce unresponsiveness. Specific tolerance has already been shown in adult carp (Serero and Avtalion, 1978; Avtalion, et al., 1980; Wishkovsky and Avtalion, 1982). However, one third of the animals in these experiments failed to become tolerant. Tolerance in this case was achieved by intracardial injection and although it did not appear to be dose dependent it was dependent upon the route of injection. Injection into the paracardiac space was less effective in producing tolerance. The temperature at which the work is carried out is another important factor. Only high doses were capable of inducing tolerance at low temperatures. Cuchens and Clem (1977) have been able to divide bluegill lymphocytes into two sub-populations based upon their reactivity to mitogens at different temperatures. This may, therefore, point to a T-like cell mediated suppressor function in fish which operates at high ambient temperatures, but which can be activated at low temperatures only by high doses.

T-like cell helper function has been known in fish for some years (Yocum, et al., 1975; Avtalion, et al., 1975; Stolen and Mäkelä, 1975; Avtalion, et al., 1976; Weiss and Avtalion, 1977) as demonstrated by the hapten-carrier effect. Carrier priming at high temperatures will allow humoral anti-hapten responses to occur at low temperatures. Once again this points to a temperature sensitive T-like cell sub-population in fish.

There is a wealth of work in mammals pointing to the involvement of suppressor T cells in the induction of immunosuppression to hapten modified self structures, for instance the papers of Scott and his colleagues (Scott and

Long, 1976; Long and Scott, 1977; Scott, 1978; Jandinski and Scott, 1979; Jandinski et al., 1980; Li and Scott, 1980a and 1980b). Thus it is entirely possible for the immunosuppression observed in the 60mg TNP Self injected fish to have been mediated by T-like suppressor cells.

A single injection of TNP Self yielded little or no reaction, even though a single injection of TNP-HRBC was quite enough to provoke a good response, particularly with the 60mg TNP-HRBC group. It can be surmised that TNP Self is non-immunogenic (although, as has been seen, the fish does react to produce suppressor cells). Ramos et al. (1980) also found that for suppression of a B cell response, preimmunization with non-immunogenic, lightly haptenated cells was a prerequisite.

The variation in response to TNP-HRBC must be due to the hapten-carrier ratio. 60mg TNP-HRBC is the normal ratio used for immunizing animals against TNP as laid down by Rittenberg and Pratt (1969) and so it is not surprising that the fish respond well to this. The density of TNP determinants in the 6mg TNP-HRBC group may have been too low to elicit a significant immune response, hence the relatively low number of plaque forming cells. In contrast, the density of TNP molecules on the surface of the erythrocytes in the 600mg TNP-HRBC group may have been so high as to mask the carrier, which is required for the response to develop. Avtalion and Milgrom (1976) produced a similar effect in carp using high hapten density conjugates (between 1.6 and 30 Penicilloyl groups to each BSA carrier molecule). The fish failed to produce any anti-BSA antibody to the Pen_{30} BSA conjugate.

The TNP Self primed fish were unable to produce a good anti-HRBC response when given 60mg TNP-HRBC, but the response that did occur would appear to be proportional to the hapten-carrier ratio of the TNP Self. Although the effect is small, the experiment has been repeated and the results were identical. It would appear to indicate a form of hapten-carrier effect in which the TNP element acts in the role of carrier, and the HRBC surface molecules as the hapten. The dose response relationship merely indicates that larger amounts of carrier produce greater T-like cell help. The hapten-carrier effect is now well documented in fish (Stolen and Mäkelä, 1975; Yocum, et al., 1975; Avtalion, et al., 1976) despite doubts about the existence of separate T and B cells. Another instance where T-like cell help may be occurring in these experiments lies with the animals primed with the three hapten-carrier ratios of TNP HRBC. The anti HRBC response to this is minimal.

However, on injection with 60mg TNP HRBC the response increases to a reasonable level. The lack of primary response to 60mg TNP HRBC but the subsequent response on secondary injection, indicates that the primary response involves T-like cell help which acts on the second injection. The smaller response to the 600mg TNP HRBC primed group shows, as mentioned previously, that the erythrocyte membrane determinants may be masked by the TNP elements.

Ruben and his colleagues (1977), evoking the hapten carrier effect in fish, found that high dose priming with carrier was ineffective in generating helper activity. In a similar vein, low dose carrier priming produced helper activity but it was a short lived effect. These results would appear to be in agreement with the work reported here. Weiss and Avtalion (1977) believe that for maximal T-like cell help a low hapten density is required. Certainly, too high a hapten-carrier ratio has a deleterious effect on the response.

Experiments conducted on rainbow trout (Salmo gairdneri) using T dependent and T independent carriers have demonstrated a marked difference in the responses to the two groups (Anderson et al., 1982). Immunological memory, and hence helper function, was quite marked when the fish were injected with the T dependent carrier, Keyhole Limpet haemocyanin (KLH). Memory was slight or non-existent when the carrier was the T independent polysaccharide Ficoll. This may demonstrate the heterogeneity of fish lymphocytes and their division into T-like and B-like sub-populations. However, the small amount of memory shown to the T independent carrier cannot be ignored and does agree with the findings of Schott and Merchant (1979). This paper describes the discovery of low levels of immune memory in athymic nude mice, thus indicating that the generation of immune memory does not rest entirely with the T cells. However T cells are the major factor involved. If mice have other cells capable of carrying out traditional T cell functions then it is quite possible that the phylogenetically more primitive animals (e.g. fish) still retain both T and B cell functions in one cell. Considerably more work is required on this problem before the answer is known. To further complicate the phylogenetic aspect of this question, Horton et al. (1979) used three different carriers, SRBC (T dependent), LPS and Ficoll (both T independent) in experiments on thymectomised Xenopus laevis toadlets. The response to TNP with SRBC as the carrier was virtually abolished by

thymectomy, reiterating its thymic dependence. Not surprisingly the presence or absence of a thymus had little effect on the response to TNP-LPS. The response to TNP-Ficoll (by mammalian standards, a T independent antigen) was shown to require the presence of the thymus. TNP-Ficoll is a TI_2 antigen and will stimulate a different B cell sub-set to TI_1 antigens such as TNP-LPS. It would appear therefore that the TI_2 reactive B cell sub-set requires the thymus or thymic factors to be present. They may even mature within the thymus. Once again this indicates a "greyness" in the T cell/B cell distinction in the lower vertebrates when compared with the "black and white" mammals. Suppressor and helper functions do exist in carp lymphocytes, as shown in this paper, but their division into separate populations is still uncertain.

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CONCLUDING REMARKS

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The Fisheries Society of the British Isles sponsors various meetings one of which, each year, is regarded as a major meeting and is devoted to a subject of substance within fish biology. In every other year the major meeting takes the form of an international symposium. This year's meeting, which is devoted to fish immunology, is of the national type although, in fact, the importance of the subject has generated a large international contribution. It follows a similar symposium held in Wageningen in 1981 under the auspices of the Agriculture University, Wageningen.

Two special symposia in the last few years must mean that fish immunology has come of age and now represents a significant branch of science within its own right. The wide ranging subject matter of the present symposium also reflects this maturity and yet, to an outsider like myself, there is still an element of uncertainty in the way in which the participants of this meeting have reacted together, or to be more precise, have not reacted. I cannot recollect a meeting at which so many contrasting results were presented without discussions, challenge or controversy. Is it that fish immunologists still pay undue heed to mechanisms of immunity or defence in mammals and fail to get beyond the purely comparative study? Certainly, almost all the major elements of the mammalian scene were there with roughly equal coverage of the relevance of phagocytes, lymphoid cells, tissue and circulating antibodies and their organs or origin, self-recognition, antigenic memory and so on. Only one

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subject seemed neglected and that was graft rejection.

Agreements were noteworthy too. Phagocytosis was, by general consent, a dominant reaction to injected foreign material as well as the basis of tissue turn over. More striking, was the overall feeling that the thymus was in some way responsible for the soluble components of defence mechanisms. Its close contact with the outside world, early development and association with lymphocytes were all cited as circumstantial evidence but no one appears yet to have looked at thymectomized fish.

The major areas where accord was not obvious were:

a) on the nature and/or production of immunologically relevant precipitins;

b) whether or not a second exposure to antigen boosted antibody production;

c) on the existence of self recognition;

d) on the relevance of non-specific versus specific responses;

e) on the route of entry of antigen.

These are all fundamental aspects of the immune response of higher vertebrates but there seems no reason to suppose that fish will exhibit them all or that all fish will exhibit any one. So the need is not only for more work but for a more healthy and vigorous discourse.

Sufficient detail is apparent of the complexities of the immune mechanisms of fish for the innovators in the field to become emancipated from the overt influence of mammalian concepts. We should, perhaps, look to the international symposium of 1988 for the fuller realisation of this, and Plymouth Polytechnic with its excellent facilities would seem a natural choice of venue.

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