Immunosuppression in Atlantic salmon by an extracellular protein of *Aeromonas salmonicida*

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To my parents

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SUMMARY

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The extracellular products of *Aeromonas salmonicida* inhibited the antibody response of Atlantic salmon to a second antigen, namely phage MS2. During the characterisation of the humoral immunosuppressive factor (HIF) evidence suggested that the suppressor was the 64-kD serine protease of *A. salmonicida* . Before any further work could be undertaken it was imperative to establish that the immunosuppressive action of the protease was not merely due to proteolytic degradation of the antibody molecule. This was shown not to be the case by both *in vivo* and *in vitro* experiments. Further, it was also illustrated that MS2 phage used as the second antigen was also not degraded by the protease and that the antibody response elicited in fish was specific for the phage.

The effect of the protease on the proliferation of leucocytes from peripheral blood and anterior kidney was investigated and it was shown that the protease caused a dose related inhibition of the cell stimulation in response to the B cell mitogen, LPS. This effect was enhanced when the cells were preincubated with the protease before activation with LPS suggesting that the protease may be interfering with the lymphocyte commitment to blastogenesis.

The involvement of prostaglandins was implicated by *in vivo* experiments which showed that indomethacin, an inhibitor of prostaglandin synthesis, blocked the immunosuppressive activity of the protease when administered to fish. Subsequently, PGE_2 concentration in serum samples of fish injected with indomethacin with or without protease was measured and it was clear that the protease caused an enhancement of PGE_2 concentration. This result was also duplicated in *in vitro* experiments using leucocytes from both peripheral blood and anterior kidney which demonstrated increased levels of PGE_2 in response to the protease.

Furthermore, the protease stimulated an increase in the concentration of intracellular cyclic AMP of salmon leucocytes. It remains to be determined whether

there is a relationship between increased levels of PGE_2 and cyclic AMP stimulated by the protease. These observations indicate that the inhibitory effect of the serine protease of *A. salmonicida* on the humoral response of Atlantic salmon occurs via a prostaglandin-dependent pathway probably involving cyclic AMP as a secondary messenger.

Although it is tentatively concluded that the suppressive factor is the 64-kDa serine protease, conclusive proof is still required. Many roles have been attributed to this protease in the pathogenesis of *A. salmonicida* during furunculosis in Atlantic salmon. However, its involvement in immunosuppression has not been reported before.

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ABBREVIATIONS

AA	Arachidonic acid
Ab	Antibody
Ag	Antigen
ANOVA	Analysis of variance
BHI	Brain-heart infusion
cyclic AMP	cyclic adenosine monophosphate
cm	Centimetre
Con A	Concanavalin A
d.f.	Degrees of freedom
DMSO	Dimethyl sulphoxide
ECP	Extracellular products
EDTA	Ethylenediamine tetra-acetic acid
EPA	Eicosapentaenoic acid
FCA	Freund's complete adjuvant
FCS	Foetal calf serum
FIA	Freund's incomplete adjuvant
g	Gravity
GCAT	Glycerophospholipid: cholesterol acyltransferase
h	Hour(s)
HA	Haemagglutination
HIF	Humoral immunosuppressive factor
HL	Haemolysin
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IDM	Indomethacin
IROMP	Iron-regulated outer-membrane protein(s)
kDa	Kilodalton

LPS	Lipopolysaccharides
M	Molar
MAF	Macrophage activating factor
mA	Milliamperes
min	Minute
ml	Millilitre
mM	Millimolar
MTT	Tetrazolium dye (3-4, 5-dimethylthiazoyl-2-yl)-2, 5-diphenyltetrazolium bromide
O.D.	Optical density
OMP	Outer membrane protein
PBS	Phosphate buffered saline
PBL	Peripheral blood lymphocyte
PGE_2	Prostaglandin E_2
PMSF	Phenylmethylsulphonylfluoride
PSB	Phage storage buffer
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sp	Species
subsp.	Subspecies
TCA	Trichloracetic acid
TEMED	N, N, N', N'-tetramethyl-ethylenediamine
$TGF\beta_1$	Transforming growth factor$_1$
Tris	Tris(hyroxymethyl)aminomethane
Tris-HCl	Tris hydrochloride
w/v	Weight/volume ratio

INTRODUCTION

1.1 Aquaculture

1.1.1 - General background

Aquaculture, which includes fish farming, fish culture, mariculture and sea ranching, can be defined as the human cultivation of organisms in water, whether this is fresh, brackish or marine. The production process in aquaculture is determined by biological, technological, economic and environmental factors. Many aspects of the production process can be brought under human control, the genetic make-up of the fish can be manipulated to improve yields and harvesting can be timed to ensure continuous supplies of fresh product (Pillay, 1993).

From an economic point of view the most significant criterion to define an aquaculture system is intensity (Bjorndal, 1990). Measures of intensity include stocking density, production by area, feeding regimes and input costs. One of the objectives of commercial aquaculture is to produce the maximum yield of fish in a given volume of water in the shortest time and at the lowest possible cost. Achieving this objective requires not only the management and the optimization of growth of the farm stock but also monitoring for the occurrence of disease.

Amongst the six commercially important salmon species the Atlantic salmon,*Salmo salar* L, is native to the Atlantic Ocean whereas the other five, belonging to the genus *Oncorhynchus*, are from the Pacific Ocean (Heen *et al.*, 1993). Salmon are anadromous fish whereby eggs are spawned and hatched in fresh water and the fry remain there until smoltification. During this process they adapt to saltwater life and migrate to sea. The 'salinity' of the blood of fish is intermediate between that of fresh water (0.2mM sodium chloride) and sea water (500mM sodium chloride). This means that fish are constantly fighting against an increase in the salinity of their internal environment in sea water and against a decrease in fresh water.

After spending 1-4 years at sea (depending on species) the wild fish return to their native river to spawn. After spawning they either die (Pacific salmon) or their quality is reduced so sharply (Atlantic salmon) that they are unfit for consumption. Hence, in aquaculture, the fish must be slaughtered prior to maturity.

Based on the lifecycle of wild salmon the biological process in salmon aquaculture consists of the following steps (Shearer 1992; Heen *et al.* 1993) (Figure 1):

1. Production of broodstock and roe - this was originally from wild fish but stocks have become domesticated over time. Broodstock females are stripped of eggs which are fertilized and transported to a hatchery.

2. Production of fry - after an incubation period of about two months, yolk-sac larvae are hatched and feeding subsequently starts about one month later. This is a delicate stage of the biological process when there is often a high mortality, particularly for Atlantic salmon.

3. Production of smolts - Pacific salmon generally smoltify 4-6 months after hatching whereas Atlantic salmon fry take longer (16 months-2 years). The smoltification process is very sensitive to slight environmental changes and high mortalities result if temperature and water salinity are not accurately controlled. Domestic smolts are subsequently placed in sea pens.

4. Production of farmed fish - the fish must be harvested before spawning. Generally, Atlantic and Chinook salmon mature about 28 months after smoltification while for Coho this occurs after only 16 months. However, these figures can vary between stocks.

On salmon farms the continual monitoring of fish throughout their life cycle is crucial for disease control. The two particularly critical stages are, firstly, the period after new smolts are placed in salt water and, secondly, prior to sexual maturation.

1.1.2 - Current production of salmon

The total supply of salmon has increased markedly through the mid 1980s as a result of the dramatic surge in the salmon aquaculture industry (Table 1). Norway has long been the world leader in farmed salmon (Table 2) and current production there exceeds 300 000 tonnes p.a. (Rackham, 1995). Salmon farming in Scotland began in the 1960s, but the most rapid growth in production took place in the 1980s (Table 2) and current production is approximately 60 000 tonnes p.a. (Rackham, 1995). The main regions for salmon farming

Figure 1. Life cycle of Atlantic salmon

Source: Shearer, 1992.

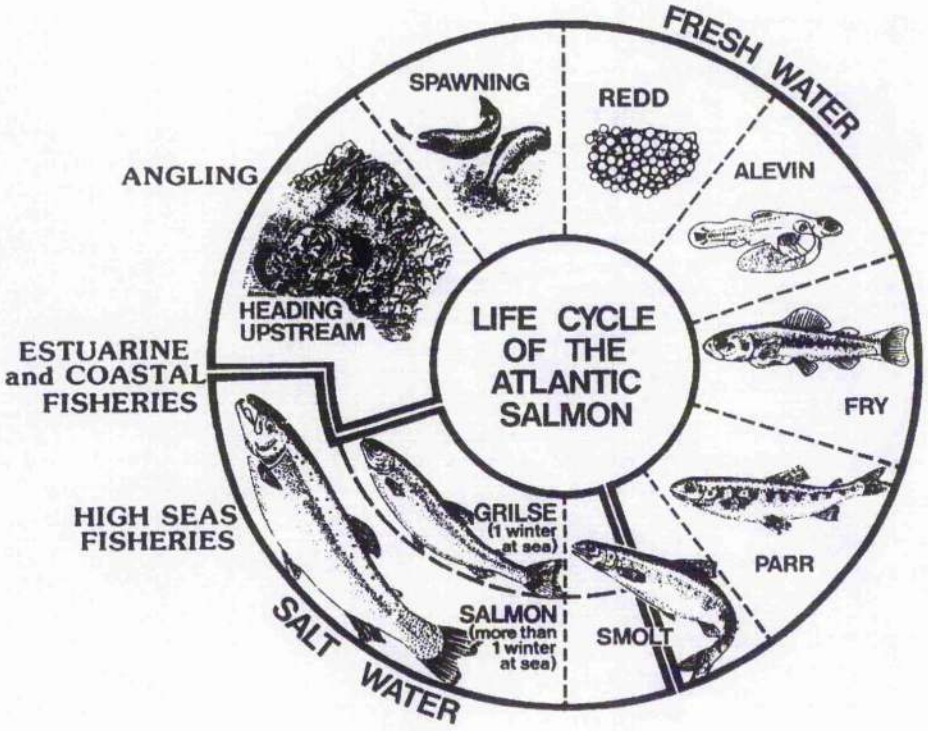

FRESH WATER
SPAWNING
REDD
ALEVIN
ANGLING
HEADING UPSTREAM
LIFE CYCLE OF THE ATLANTIC SALMON
ESTUARINE and COASTAL FISHERIES
FRY
HIGH SEAS FISHERIES
GRILSE (1 winter at sea)
PARR
SALMON (more than 1 winter at sea)
SMOLT
SALT WATER

Table 1. Salmon production and landings in 1980 - 1987

	Landings of wild salmon x 10^3 tonnes	Production of farmed salmon x 10^3 tonnes	Total production x 10^3 tonnes	Farmed as a % of total
1980	573.4	4.8	578.2	1
1981	649.0	11.6	660.6	2
1982	557.2	16.5	573.7	3
1983	678.7	24.6	703.3	3
1984	624.1	32.6	656.7	5
1985	793.5	47.1	839.6	6
1986	675.0	70.0	745.0	9
1987	650.2	87.5	737.6	12

(Source: Bjorndal, 1990).

Table 2. Production of farmed salmon, by country

Country	1987	1988	1990
Norway	47.4	80.3	150.0
Scotland	12.7	18.0	40.0
Ireland	2.2	4.1	10.0
Faroe Islands	2.5	3.1	10.0
Iceland	0.5	1.0	2.0
Canada			
British Columbia	1.2	6.5	14.0
Eastern Canada	1.4	3.2	5.0
Chile	1.8	3.5	15.0
Japan	13.0	16.6	25.0
USA	1.5	3.8	8.0
New Zealand	1.3	1.5	3.0
Others	2.0	3.0	4.0
Total Production	87.5	144.6	286.0

(Source: Bjorndal, 1990)

in Scotland are on the north and northwest coasts as well as on all the major islands (Anon, 1989).

1.1.3 - Factors limiting production of Atlantic salmon

Several factors have constrained the growth of salmon aquaculture in Scotland. These include the availability of only a limited number of suitable sites, consumer demand and market prices, concerns about the damage to the environment, and losses due to infectious diseases. There is also concern about the possible adverse interaction of salmon aquaculture on wild fish stocks (Windsor and Hutchinson, 1995).

The number of inshore aquaculture sites is limited by considerations of hydrography and environmental impact (Anon, 1989); indeed, significant future expansion will require the development of offshore sites or those on land (Blakstad, 1993). There are several concerns regarding the effect of aquaculture on the environment; pollution of the sea bed below cages can occur due to deposition of faeces and uneaten feed and this may result in anoxic sediments (Anon., 1989). Also, the widespread use of antibiotics could lead to transfer of drug resistance to other organisms and potential pathogens (Inglis *et al.*, 1991).

A large number of infectious diseases have been recognized in farmed fish, some of which have profoundly affected the commercial production of fish.

1.2 Furunculosis

1.2.1 - General features

Numerous bacterial diseases of cultured fish have been reported (Munro and Hastings, 1993) and many of these infections occur as secondary infections in association with viral diseases (Austin, 1993). These bacterial diseases have a world-wide distribution and occur in both tropical and temperate climate aquaculture.

Of the bacterial diseases of salmonid fish, furunculosis has proved to be one of the most important in economic terms. The disease in salmonids is caused by typical strains of *Aeromonas salmonicida* but atypical strains of *A. salmonicida* cause ulcerative diseases in a

variety of fish species such as goldfish, carps, eels and salmonids (McCarthy and Roberts, 1980). Although the disease can affect a wide variety of both farmed and feral teleost fish, the principal impact has been in the intensive culture of salmonids in which the organism can inflict severe losses (Hastings, 1988). The disease has almost worldwide distribution being found in Europe, North America, Japan, Korea, Australia and South Africa. In contrast, the disease has not apparently been isolated in South America (Munro and Hastings, 1993).

The name furunculosis is derived from the furuncles that occur in some cases of infected fish (Austin and Austin, 1993). However, this is not a sure sign of the disease as furuncles can occur in other types of infections, and in the acute form of furunculosis furuncles are not present. It is not agreed how the causative organism gains entry to the circulation, the most likely routes are probably via the intestine, gills or through skin damage. Recent work by Cipriano *et al.* (1992; 1994; 1996) indicates that healthy fish may carry *A. salmonicida* in surface mucus. Once it overcomes the leucocytic cell defences of the blood (Klontz *et al.*, 1966) the organism is free to be transported through the vasculature and become localised in any of the organs.

In chronic cases the slow progression of infection results in a greater degree of localisation in visceral organs, commonly kidney, spleen, blood vessel walls, intestines, liver and gills. It is, however, in the skeletal muscle that the characteristic swollen lesions are produced. The chronic condition manifesting the archetypal furuncles is most often seen in older fish, some of which manage to recover from the disease. Furunculosis may manifest itself in wild populations in rivers or in farmed stocks in fresh and sea water.

1.2.2 - Latent or asymptomatic infections

In salmonids, furunculosis can appear either as a latent, acute or a chronic infection. From the work of McCarthy (1980) on furunculosis in brown trout, *Salmo trutta* , it is probable that latency is the normal state which results from the interaction of *A. salmonicida* with salmonids. Acute infections have a rapid onset, with septicaemia, high mortality and few if any external signs in moribund fish (Hastings, 1988). However, in a chronic infection

characteristic furuncles develop in the muscles, mortality is generally low and those fish which survive an outbreak of furunculosis may become asymptomatic carriers of *A. salmonicida* (McCarthy, 1980). Scallon and Smith (1985) concluded that up to 100% of some populations of Atlantic salmon smolts carried *A. salmonicida* in a latent state. Such symptomless carriers may develop characteristic furunculosis under natural stress. Stressing factors such as crowding, poor water quality, fright, high temperature and trauma are all important in precipitating the disease. Furunculosis may be induced experimentally in carrier fish by injection of an immunosuppressive corticosteroid and maintenance at an elevated temperature (Bullock and Stuckey, 1975). Although the fraction of asymptomatic carrier fish in a population decreases with time, fish which survive furunculosis have no demonstrable acquired immunity to *A. salmonicida* (Evenberg *et al.*, 1986).

The incubation period for acute cases is probably 2-4 days, but in chronic cases the period may be extended by several weeks at lower temperatures. Furunculosis is usually seasonal, with peak incidence during the mid-summer months of July and August (Pillay, 1993).

1.2.3 - Pathology of furunculosis

The pathological features of furunculosis have been extensively reviewed by Munro and Hastings (1993). Briefly, fish dying of an acute infection most often show few external signs, whereas in chronic cases one or more of the following may be present; darkening, lethargy, loss of appetite, petechiation at fin bases and sometimes furuncles which may ulcerate to release necrotic tissue debris and bacteria. The gills become very pale and in sea-reared fish, extensive haemorrhage from the gill is common (Miyazaki and Kubota, 1975; Bruno *et al.*, 1986).

1.2.4 - Properties of the causative agent of furunculosis

A. salmonicida is a gram-negative, facultatively anaerobic, non-motile rod-shaped organism with a size in the range of 1.3 - 2 by 0.8 - 1.3 μm (Popoff, 1984). The optimum

growth temperature of the organism is between 22° and 25°C (Popoff, 1984), but it has been reported that growth can occur at 37°C leading to changes in properties such as the loss of pigment production (at temperatures above 30°C), loss of the ability to produce catalase and to degrade aesculin, DNA, elastin and gelatin when cultured at 37°C (McIntosh and Austin, 1991).

The species *Aeromonas salmonicida* is divided into four subspecies; *salmonicida, achromogenes, masoucida* and *smithia* according to Austin *et al.* (1989), and Holt *et al.* (1994). The subspecies *salmonicida,* referred to as the typical strain, is usually only isolated from salmonids and appear to be a homogeneous group with regard to many genotypic and phenotypic characteristics. The other *A. salmonicida* subspecies, often termed atypical strains, differ from typical strains in a number of biochemical and physiological properties, and are within themselves more diverse. The characteristics of the organism and criteria used for subspecies identification are described in Austin *et al.* (1989).

Although a specific selective medium for the isolation of *A. salmonicida* has yet to be developed strains are usually recognisable from their small colony size, production of oxidase and a diffusible brown pigment (Munro and Hastings, 1993). Udey and Fryer (1978) showed that fresh isolates of *A. salmonicida* from clinical cases of furunculosis contain a regular surface protein layer and that this can be lost on subculture. In broth cultures, such freshly isolated cells autoagglutinate and rapidly settle out from the medium, whereas non-agglutinating smooth variants remain in suspension. Possession of the surface layer, termed the A-layer (Udey and Fryer, 1978) is correlated with strong auto-agglutinating activity and virulence, as the A-layer-negative strains are relatively avirulent.

1.2.5 - Survival of *Aeromonas salmonicida* outside its host

It was originally thought that *A. salmonicida* was an obligate pathogen and capable of surviving for only a limited time in water in the absence of a carrier fish, but there are conflicting reports on this (Allen-Austin *et al.,* 1984; Morgan *et al.*, 1991; Rose *et al.,* 1990). More recently Morgan *et. al.* (1993) demonstrated that *A. salmonicida* persisted in

sterile lakewater in a nonculturable but viable state (NCBV) over a 21-day study. However, they failed to revive the cells from this state probably due to a lack of knowledge of appropriate revival media. The existence of such a state may explain how outbreaks of furunculosis can occur in fish populations which apparently have not come into contact with the pathogen. In other studies, *A. salmonicida* was shown to survive and retain pathogenicity in pondbottom mud for up to 9 months (Michel and Dubois-Darnaudpeys, 1980). Effendi and Austin (1994) reported that *A. salmonicida* survived up to 15 days in sterile seawater and 4 days in non-sterile marine water. The authors of both papers concluded that the organism could survive in water long enough to infect other fish. Recently, Wooster and Bowser (1996) indicated that dissemination of *A. salmonicida* in aerosols was a possible route for the spread of the pathogen.

Positive diagnosis of furunculosis has previously been based on isolation and identification of the causative agent on standard bacteriological media. However, detection of *A. salmonicida* DNA in fish is now possible with the development of polymerase chain reaction (PCR) assays (Gustafson *et al.*, 1992; Mooney *et al.*, 1995; Miyata *et al.*, 1996; Hoie *et al.*, 1996). It should be noted that detection of bacterial DNA in fish tissue does not prove the presence of live infective bacteria, and detection of bacterial DNA in fish that have been vaccinated could give rise to false positives when screening for infection (Hoie *et al.*, 1996).

1.3 Virulence factors of *Aeromonas salmonicida*

A number of potential virulence determinants of *A. salmonicida* have been identified (reviewed by Ellis, 1991; Toranzo and Barja, 1993). These include the A-layer, two proteases, leucocytotoxin(s), glycerophospolipid cholesteryl acyl transferase (GCAT) and lipopolysaccharide (LPS), some of which have been shown to be responsible for the pathological features of the disease in fish. Specific antigens capable of conferring protective immunity in fish have not been fully identified, thus, an understanding of the mode of interaction between the bacterium and its host is of crucial importance.

1.3.1 - Components of the bacterial cell surface

1.3.1.1 - A layer.

The A layer is the best studied virulence factor of *A. salmonicida.* It is composed of a 49 kDa protein which forms a regular tetragonal surface array, present in both typical and atypical isolates and which contributes to the ability of the bacteria to colonize fish and cause disease Trust (1986, 1993). Properties reported for A^+ strains include production of adherent "rough" colonies (Ishiguro and Trust, 1981), autoaggregation (Ishiguro and Trust, 1981; Evenberg and Lugtenberg, 1982; Sakai and Kimura, 1985; Olivier, 1990), adherence to cells (Ishiguro and Trust, 1981; Evenberg and Lugtenberg, 1982; Sakai and Kimura, 1985), binding of the dyes Coomassie brilliant blue and Congo red (Olivier, 1990; Ishiguro *et al.*, 1985; Evenberg *et al.*, 1985), hydrophobicity (Trust *et al.*, 1983; Parker and Munn, 1984; Van Alstine *et al.*, 1986), resistance to phage attachment (Ishiguro *et al.*, 1981; Ishiguro *et al.*, 1985), resistance to proteolytic digestion and also to the bacteriolytic activity of serum (Sakai and Kimura, 1985; Munn *et al.*, 1982; Sakai, 1985; Johnson *et al.*, 1985).

It has been widely accepted that there is a correlation between virulence and the possession of the A-layer because isogenic mutants lacking this cell surface protein array have been reported to be avirulent (Ishiguro *et al.*, 1981). However this correlation is not absolute, as shown by the isolation of A^- strains that autoaggregate (Johnson *et al.*, 1985), A^- strains that are virulent (Ward *et al.*, 1985; Adams *et al.*, 1988) and A^+ strains that are avirulent (Santos *et al.*, 1991; Olivier, 1990; Adams *et al.*, 1988; Cipriano and Blanch, 1989).

The cell envelope of *A. salmonicida* consists primarily of the A-layer interspersed between the repeating O-antigen subunits of bacterial LPS (Evenberg *et al.*, 1985), which are required to anchor the A layer to the surface of the cell (Belland and Trust, 1985).

1.3.1.2 - Lipopolysaccharide

The second main cell surface antigenic determinant of *A. salmonicida* is a smooth layer of LPS, possessing O-polysaccharides of homogeneous lengths which are conserved antigenically (Chart *et al* ., 1984). A small proportion of O-polysaccharide chains penetrate the A-layer but the majority of LPS molecules remain hidden beneath the A-layer (Chart *et al.*, 1984; Evenberg *et al.*, 1985).

In their study, Hastings and Ellis (1988) found that the antibodies induced in rainbow trout by LPS of *A. salmonicida* were specific for the O-side chain of LPS and not to the core or lipid A region.

The O-chains contribute resistance to the bactericidal activity of complement and also appear to tether the A-layer to the cell surface (Munn *et al.*, 1982; Belland and Trust, 1985), as mutants lacking O-chains release assembled A-layer into the culture supernatant (Garduno *et al.*, 1995). Therefore, expression of both A-layer and LPS O-chains is crucial to the ability of *A. salmonicida* to produce disease in fish.

1.3.1.3 - Iron-regulated outer membrane proteins

Iron-limitation, in a number of bacterial diseases, has been shown to be a strategy employed by the host to limit growth of the causative microorganism (Weinberg, 1978). Both typical and atypical strains of *A. salmonicida* have the capacity to express high affinity iron-uptake mechanisms which can scavenge iron from host iron-binding glycoproteins such as transferrin and lactoferrin, as well as from the low molecular mass iron chelator ethylenediamine di(*o* -hydroxyphenylacetic acid) (EDDA) (Chart and Trust, 1983; Hirst *et al.*, 1991).

Two main strategies for acquiring iron by typical strains of *A. salmonicida* were identified; one is by an inducible siderophore-mediated iron uptake mechanism involving the production of a 2,3-diphenolcatechol siderophore (Chart and Trust, 1983; Hirst *et al.*, 1991). The other, a siderophore-independent mechanism, in which evidence of direct

contact between the cell surface and an iron-binding protein was noted by Chart and Trust, (1983).

Siderophore production has not been identified in atypical strains although Hirst *et al.*, (1991) demonstrated that both typical and atypical strains were capable of utilizing siderophores present in iron-restricted culture supernatant. The authors proposed that the iron-regulated outer-membrane proteins described by Chart and Trust (1983) and Aoki and Holland (1985) may have a role in the acquisition of iron by *A. salmonicida* .

Growth of *A. salmonicida* under iron limitation resulted in the increased synthesis of several high molecular weight outer membrane proteins in the 70-90 kDa range (Chart and Trust, 1983; Aoki and Holland, 1985; Neelam *et al.*, 1993). These iron-regulated outer membrane proteins have also been shown to confer significant protection in Atlantic salmon *Salmo salar* L against a natural or experimental challenge with *A. salmonicida* (Hirst and Ellis, 1994).

An additional iron-uptake system in pathogenic bacteria, involving proteolytic degradation of transferrin to release free iron was described by Griffiths (1987b). Hirst and Ellis (1996) examined the possible occurrence of a similar system in *A. salmonicida* as both typical and atypical strains produce extracellular proteases which are considered important virulence factors in the pathogenesis of *A. salmonicida* infection (Ellis, 1991). The authors demonstrated that ECP of atypical strains containing proteolytic activity was able to release iron from transferrin and salmon serum. Although the serine protease of typical strains was found to degrade transferrin, it was not established whether the degradation resulted in making iron available for uptake.

With respect to the above suggestion, the observation of Neelam *et al.* (1993) that production of the 70 kD protease by a typical strain of *A. salmonicida* was enhanced in iron-restrictive conditions may be significant in the possible release of stored iron from transferrin.

1.3.2 - Extracellular products of *A. salmonicida*

The extracellular products (ECP) of *A. salmonicida* contains a large number of extracellular proteins, many of which probably have significance as virulence factors in allowing the bacterium to survive and reproduce within the host's tissues. The components of the ECP of typical strains include proteases (Shieh and McLean, 1975; Mellergaard, 1983; Hastings and Ellis, 1985; Fyfe *et al.*, 1986; Price *et al.*, 1989), haemolysins (Titball and Munn, 1981, 1983, 1985; Hastings and Ellis, 1985; Fyfe *et al.*, 1988; Nomura *et al.*, 1988), glycerophospholipid-cholesterol acyltransferase (GCAT) (Buckley *et al.*, 1982; Lee and Ellis, 1990) and leucocytolysin (Fuller *et al.*,1977).

Present evidence indicates that the 64 kDa serine protease and the GCAT complexed with LPS are the most important factors responsible for the lethal toxicity and pathology of the ECP of typical *A. salmonicida* strains. Ellis (1991) suggested that all the membrane damaging haemolytic and cytotoxic activities were properties of the single entity, GCAT/LPS.

1.3.2.1 - Glycerophospholipid:cholesterol acyltransferase

This lethal toxin has been shown by SDS-PAGE to contain a single protein of 24 kDa. There is evidence that in the native state, GCAT exists as a complex with LPS with a MW of over 2000 kDa although small proportion occurs as a free monomeric polypeptide with the same specificity (Lee and Ellis, 1990). GCAT/LPS has cytotoxic, haemolytic and phospholipase activities (Lee and Ellis, 1990). LPS confers enhanced stability to heat and proteolytic degradation to GCAT. The enzyme activity of GCAT is restricted to glycerophospholipids on which it acts as a phospholipase A2.

While GCAT has extremely high haemolytic activity towards salmonid erythrocytes *in vitro*, there is no evidence for *in vivo* haemolysis (Lee and Ellis, 1991a) where it mainly produces a coagulative necrosis of muscle fibres. More recent studies (Lee and Ellis, 1991b) have demonstrated that GCAT/LPS interacts with salmonid serum lipoproteins and results in disturbances of lipid metabolism and activation of inflammatory mediators.

1.3.2.2 - Serine protease

This enzyme is considered to be one of the two most important extracellular virulence factors of *A. salmonicida* . The cloning and determination of the nucleotide sequence of the serine protease gene, *asp A* , (Whitby *et al.*, 1992) was a significant advance in the study of this protein.

A number of other bacterial species have been shown to produce extracellular serine proteases, of which the best known is subtilisin BPN', secreted by *Bacillus amyloliquefaciens* , for which a three-dimensional model is available showing the arrangement of all the amino acids (Wells and Estell, 1988). The amino acid sequence of the serine protease of *A. salmonicida* showed a high degree of homology with that of other bacterial serine proteases (Coleman and Whitby, 1993) which, with molecular masses of approximately 30 kDa, are less than half its size.

Although commonly referred to as the 70 kDa serine protease from estimates of its molecular weight by SDS-PAGE, gene sequencing studies indicated that the mature protein has a MW of 64173 Da (Whitby *et al.*, 1992).

This protease is caseinolytic, gelatinolytic and collagenolytic, and causes extensive tissue liquefaction in fish. Whereas Sakai (1985) concluded that the protease was essential for virulence of the organism by providing amino acid nutrients from host proteins, questions were raised by several authors concerning the indispensable function of the protease in furunculosis when some virulent strains of *A. salmonicida* which did not produce protease under standard culture conditions were isolated (Ellis *et al.*, 1988; Hackett *et al.*, 1984). However, using specific antiserum, the protease was detected in furuncle fluids indicating that, although these strains were apparently protease deficient *in vitro*, they did produce the protease *in vivo*. This illustrates that while certain virulence factors may be absent or present only in undetectable amounts during growth in standard culture media they are expressed normally within the appropriate host.

The protease is resistant to most of the anti-proteases present in the blood and tissue fluids of salmonids. Ellis (1987) demonstrated that the only anti-protease capable of

inhibiting the 64 kDA protease is α–2 macroglobulin, which constitutes less than 10% of the total serum anti-protease activity. In contrast, Salte *et al.* (1992), on the basis of studies with human antithrombin, suggested that the protease was inhibited by antithrombin as well as α–2 macroglobulin.

A further characteristic of the protease is that it has a specificity similar to that of thrombin (Price *et al.*, 1990) and activates the blood clotting cascade in salmonids (Salte *et al.*, 1991). Like thrombin, the protease markedly reduced the clotting time of trout blood but was unable to polymerize bovine fibrinogen suggesting that activation of the clotting cascade by the protease was different from thrombin (Price *et al.*, 1990).

1.3.2.3 - Metalloprotease

Sheeran and Smith (1981) reported the existence of a second protease in relatively low amounts in the ECP of typical strains of *A. salmonicida.* This collagenolytic and gelatinolytic enzyme has a MW of 20 kDa (Price *et al.*, 1989) and differs from the serine protease in that it is a metalloprotease and lacks caseinase activity (Sheeran and Smith, 1981). The potential role of this protease in infection has so far not been described but has been shown to produce lesions in trout upon injection (Sheeran *et al.* 1984).

1.3.2.4 - Haemolysins

Two haemolytic activities were identified by Titball and Munn (1981): one that lysed trout erythrocytes and termed T-lysin, and another that was specific for horse erythrocytes, termed H-lysin. The MW of the T-lysin was estimated to be 56 kDa, and the H-lysin was synthesized as an inactive 42.3 kDa precursor which, on proteolytic cleavage, yielded an active 29.5 kDa hemolysin (Fyfe *et al.*, 1987). Titball and Munn (1983, 1985) reported that T-lysin required the 64 kDa caseinase to cause complete lysis of trout erythrocytes. More recent work suggests that the T-lysin is in fact GCAT (Lee and Ellis, 1990) but the nature of the H-lysin is still uncertain, since molecular cloning studies indicated that clones with T-lysin activity also contained GCAT activity (Munn and Gilpin, personal communication).

1.3.2.5 - Leukocytolytic factor

The leucocytolytic factor (LCL) regarded as a possible virulence factor was identified by Fuller *et al.* (1977) who reported it to consist of one major protein component closely associated with carbohydrates.The MW of the LCL was 100-300 kDa; both virulent and avirulent strains produced identical LCL but the activity of the LCL produced by virulent strains was ten times greater than that of the avirulent preparations. When purified LCL factor was injected on its own into fish it did not cause any deaths but LCL factor supplemented with a low dose *A. salmonicida* cells resulted in a significant increase in mortalities (Fuller *et al.* 1977). There have been no further reports to clarify the nature of this toxin.

1.3.2.6 - Salmolysin

This heat-labile protein of about 200 kDa, purified and characterized by Nomura *et al.* (1988) was reported to exist as a glycoprotein complex containing 68% carbohydrate. It caused the complete lysis of salmonid erythrocytes and was inactivated by subtilisin and trypsin. The properties of this factor are consistent with those of the GCAT/LPS complex characterised by Lee and Ellis (1990) and it is probable that they are one and the same.

1.3.3 - Interaction of virulence factors

The mechanism whereby LPS enhances the haemolytic and toxic activity of GCAT is not known, however, Ellis (1991) speculated that since LPS can readily penetrate phospholipid monolayers composed of unsaturated fatty acids (Kabir *et al.*, 1978) it facilitates the delivery of GCAT to the precise position where the optimal substrates for the enzyme are present.

Purified GCAT-LPS complex was unable to lyse trout erythrocytes completely, in contrast to the complete solubilization of erythrocyte membranes by the ECP (Lee and Ellis, 1990). This incomplete haemolysis is similar to that of the T_1 lysin (Titball and Munn, 1981; 1985). When GCAT-LPS was mixed with the purified 64 kDa serine protease complete lysis

of trout erythrocytes occurred, confirming similarity of GCAT-LPS with T_1 lysin activity (Lee and Ellis, 1990).

The work of Salte *et al.* (1992) suggested that the 64 kDa protease and GCAT-LPS interact with each other in thrombus formation by entering the coagulation systems of salmonid fish at two different levels. GCAT-LPS liberates thromboplastic material into the bloodstream through its haemolytic activity, thereby activating the extrinsic coagulation system, whereas the protease acts as activated coagulation factor X, the significance being that circulatory failure occurs.

The pathogenesis of furuncle formation appears to be due to a combined effect of the protease and GCAT-LPS. When injected intramuscularly purified protease produces a much less severe lesion than ECP containing the same protease activity (Fyfe *et al.*, 1986). In another study (Lee and Ellis, 1991), GCAT-LPS administered alone produced coagulative necrosis of muscle fibres but with little haemorrhaging, whereas a mixture of protease and GCAT-LPS produced an extensive lesion that was liquefactive and haemorrhagic, typical of that induced by ECP.

From the evidence presented above it seems that the presence of the serine protease is required to enhance the activity of other virulence factors of *A. salmonicida* , probably allowing the organism to successfully invade and infect its host.

1.4 Humoral immunity in fish

A knowledge of the immune response elicited in fish is of prime importance in understanding the progression of infectious diseases as well as for the development of suitable vaccines. The efficacy of most anti-bacterial vaccines has been tested by challenging immunised fish with live organisms and/or monitoring the humoral antibody response induced. However, studies on salmonids vaccinated against *A.salmonicida* have shown a poor correlation between antibody response and protection (Michel and Faivre, 1982; Ellis, 1988; Moyner *et al.*, 1993; Aakre *et al.*, 1994). Both specific and non-specific immune

mechanisms are operative in fish but only the mechanisms of specific, adaptive immunity will be reviewed here.

Fish are a heterogeneous group of vertebrates and even the most primitive fishes, such as the hagfish and lamprey, are capable of mounting an antibody response (Kaattari, 1992). In recent years, much has been learned about the antibodies and the genes which encode them in teleost fish (Warr, 1992). Upon immunization, fish produce antibodies with specificity and measurable affinity for the eliciting antigen, and biological properties such as agglutination, precipitation, complement fixation and opsonization.

1.4.1 - Structure of fish immunoglobulin

Fish produce antibodies of a single immunoglobulin class which is equivalent to the mammalian IgM. It consists of ~70k relative molecular mass (Mr) heavy chains (μ) and ~25k Mr light chains (L) in equimolar amounts. The basic repeating structural unit is μ_2L_2, referred to as the monomeric form, which contains two antigen-binding sites (Fig. 2). The IgM molecule in Atlantic salmon isolated and purified by Havarstein *et al.* (1988) occurs as a tetramer containing eight heavy chains and eight light chains, $(\mu_2 L_2)_4$. Analyses of the amino acid sequences of the heavy chains of fish IgM (Kokubu *et al.*, 1988; Amemiya and Litman, 1990; Ghaffari and Lobb, 1989*a,b*) do not indicate a particularly close relationship of the fish μ chain to the heavy chains of the prototypical mammalian IgMs (about 20-30% homology). Nevertheless, they are apparently of similar size (Acton *et al.*, 1971; Shelton and Smith, 1970) and exhibit similarities in the organization of genes encoding the μ chains of both fish and mammals (Wilson *et al.*, 1990).

1.4.2 - The antibody response

Antibodies appear not only in blood but also in secretions such as bile and mucus. Thus, "mucosal" immunity may often be more readily elicited by exposure of the fish to immunizing agents in the aqueous environment, than by injection (Fletcher and White, 1973; Lobb, 1971). Antibody responses of fish are generally influenced by temperature, and low

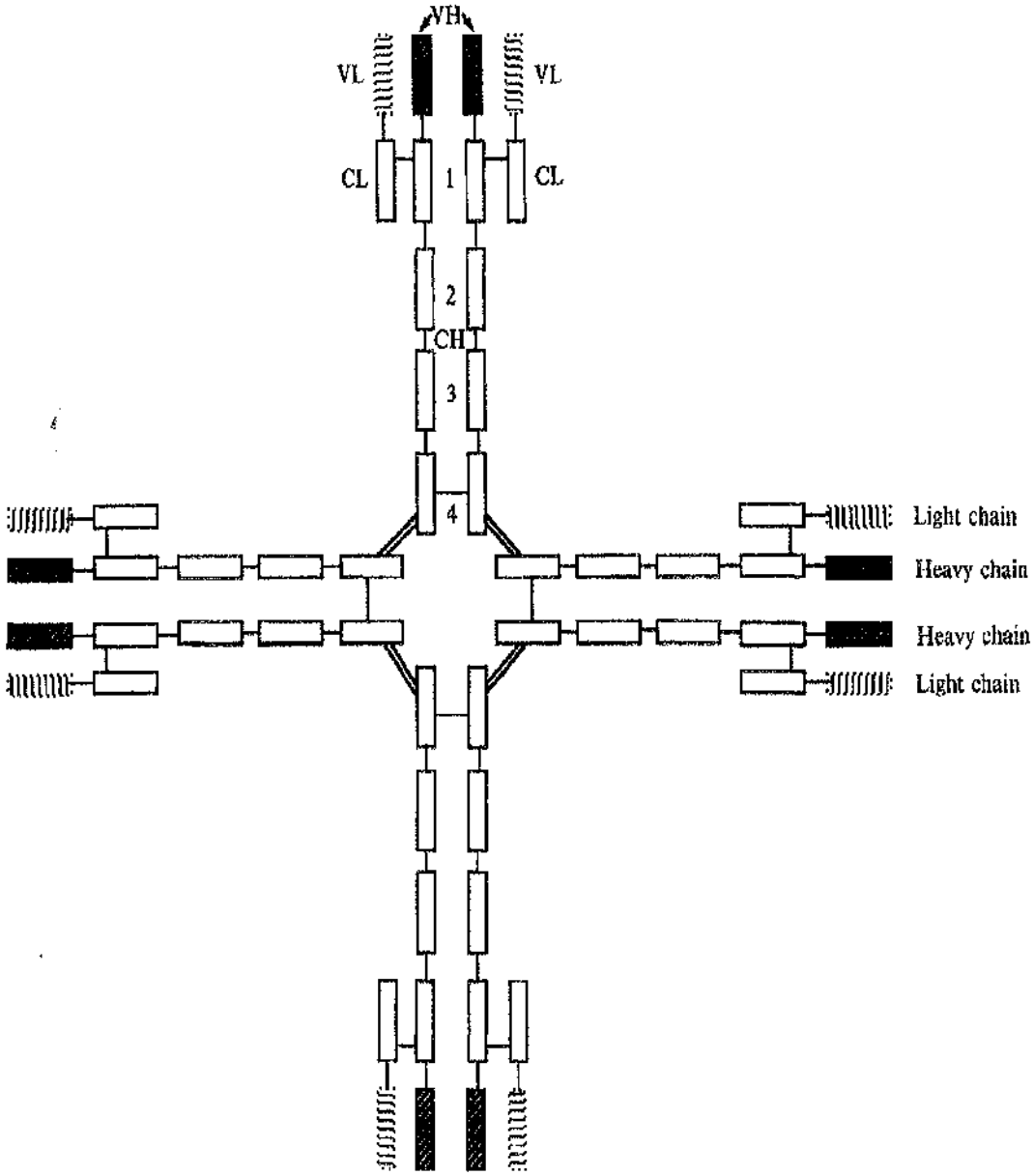
environmental temperatures can suppress antibody formation. Although the mechanism of such immunosuppression is not clear, it has been postulated that the low-temperature-sensitive event in fish involves an inhibition of carrier-specific helper cells (Avtalion, 1981). From their investigation, Miller and Clem (1984) proposed that fish T cells are more susceptible to lower temperatures than are B cells, which may be based on the existence of inherent differences in the membrane compositions (and fluidities) of the two cell types.

Elucidation of the mechanisms of fish B cell induction by antigen have relied heavily upon three technologies, all of which were originally developed for mammalian species: (a) defined hapten-carrier systems (Stolen and Makela, 1976; Ruben *et al.*, 1977; Miller and Clem, 1984b); (b) *in vitro* culture systems for fish leucocytes (Kaattari *et al.*, 1986; Miller and Clem, 1984a; DeKonig and Kaattari, 1991); and (c) cell partitioning techniques (Secombes *et al.*, 1983; Sizemore *et al.*, 1984; Graham and Secombes, 1990). Using monoclonal antibodies for isolation of both B (sIg^+) and T (sIg^-) lymphocytes, these investigators established the cellular requirements for B cell induction. As for mammals, interaction of B cells with both T cells and monocytes (or macrophages) is necessary in fish to produce an antibody response to T-dependent (TD) antigens (Miller *et al.*, 1985). This TD response initially requires proteolytic processing and presentation of the protein antigen by the macrophages (Vallejo *et al.*, 1990; 1992). Such a response is genetically restricted via the major histocompatibility complex-class II antigens (MHC-II) (Vallejo, 1992). The response to T-independent (TI) antigens, such as LPS, appears to only require the presence of the macrophage-derived interleukin 1 (IL-1) (Miller *et al.*, 1985; Clem *et al.*, 1985). Activated macrophages are presumed to produce IL-1, which appears to be required for both the TD and TI responses (Clem *et al.*, 1985). During a TD response, presentation of processed antigen stimulates T cells to produce an activator of the antigen-specific B lymphocytes (Yang *et al.*, 1989) (Fig. 3). In his review, Kaattari (1992) concluded that the intercellular cooperativity in the induction of B cell responses to different antigenic forms in fish is very similar in form to that seen in mammals. Although not all the possible lymphokines participating in this reaction have been identified, however, γ-IFN and

Figure 2. Diagrammatic representation of the tetrameric IgM of the channel catfish, *Ictalurus punctatus*.

The illustrated covalent structure is based on the studies of Lobb (1985), Lobb and Clem (1983) and Ghaffari and Lobb (1989*a,b*). J chain is not shown. Abbreviations: VH, heavy chain variable region domain; CH, heavy chain constant region domain; VL, light chain variable region domain; CL, light chain constant region domain.

(Source: Wilson and Warr, 1992).


VH
VL
VL
CL
1
CL
2
CH
3
4
Light chain
Heavy chain
Heavy chain
Light chain

macrophage-activating lymphokines have been elicited from salmonid leucocytes (Smith and Braun-Nesje, 1982; Graham and Secombes, 1990*a,b*). IL-2-like activity has also been demonstrated in teleosts (Caspi and Avtalion, 1984; Grondel and Harmsen, 1984).

1.4.3 - Generation of immunological memory

The definition of immunological memory in fish has been a matter of some dispute. The characteristics common to both mammalian and fish memory are the generation of enhanced antibody titres (Avtalion, 1969; Trump and Hildemann, 1970; Miller and Clem, 1984; Tatner, 1986; Arkoosh and Kaattari, 1991), accelerated antibody responses (Avtalion, 1969; Desvaux and Charlemagne, 1981), and increased sensitivity to antigen (Arkoosh and Kaattari, 1991).

Direct examination of the secondary B cell response has revealed that the number of antibody-forming cells increased (Sailendri and Muthukkaruppan, 1975; Rijkers *et al.*, 1980; Miller and Clem, 1984; Arkoosh and Kaattari, 1991) which indicated that the heightened antibody titres are not simply due to increased antibody secretion from a given set of B cells but that the number of specific antibody-producing B cells must increase during memory induction. Kaattari (1992), proposed that the quantitative increase in antigen-specific B cells may be due to the simple increase of the antigen-sensitive precursor pool or by a physiological change of the primary B cells resulting in more proliferation, and thus larger clone sizes. Past work with rats has revealed that both precursor pools and clone sizes increase after initial antigen priming (Brooks and Feldbush, 1981). In trout, the increased ability to produce antibody-forming cells during memory is due strictly to an increase in the precursor pool and no differences in the clone size have been observed (Arkoosh and Kaattari, 1991) (Fig. 4).

1.4.4 - Isotype switching and affinity maturation

The processes of isotype switching (Killie *et al.*, 1991; Lobb and Olson, 1988) and affinity maturation do not seem to occur to the degree observed in mammals. The

Figure 3 Stimulation of antibody production by T cell independent and T cell dependent antigens

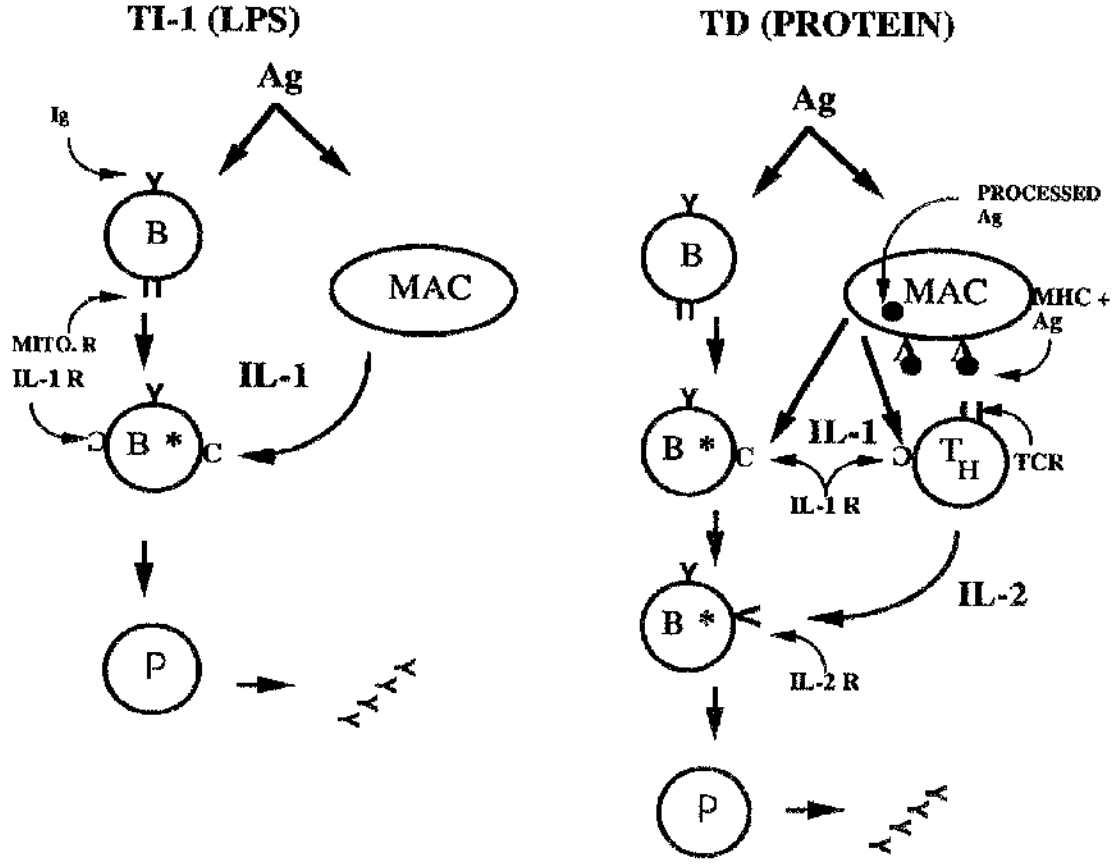
T cell independent (TI-1) antigen such as LPS require only the assistance of an IL-1 secreting cell (MAC). Primary induction of the B cell may occur by antigen binding the Ig receptor, possibly in conjunction with the antigen binding of the mitogenic receptor (MITO. R). This induction may result in the expression of an IL-1 receptor (IL-1R). Interaction of IL-1 with this receptor results in clonal proliferation and secretion of antibody.

Stimulation of B cells by T cell dependent (TD) antigens requires the participation of both an antigen presenting cell (MAC) and T cell (T_H). It appears certain that the T_H cells require both interaction with processed and presented antigens and IL-1. The T_H cell then elaborates IL-2, which is required for B cell differentiation and production of antibody. At this point in time it is uncertain whether there are distinct populations of TI-1 and TD responsive B cells, or if the apparently different combinations of antigenic and lymphokine signals are different in form but comparable in function. The latter situation would enable either antigen to activate the same cellular population of B cells.

B^* - stimulated B cell.

P - plasma cell.

(Source: Kaattari, 1992).


TI-1 (LPS)
Ag
Ig
B
MAC
MITO. R
IL-1 R
IL-1
B *
C
P
TD (PROTEIN)
Ag
PROCESSED
Ag
B
MAC
MHC +
Ag
B *
C
IL-1
T_H
TCR
IL-1 R
IL-2
B *
IL-2 R
P

mechanisms involved in the expression of isotypes await a more thorough analysis of the immunoglobulin heavy chain constant and associated regions. Data from some studies (Ghaffari and Lobb, 1989; Amemiya and Litman, 1990) suggest that teleosts have the potential for a tandem arrangement of genes on the constant region of the heavy chain, similar to that found in mammals. Although shifts of discernible isotypes have been observed during an immune response (Lobb and Olson, 1988) a switch characteristic of IgM to IgG has not been observed. Indeed, there is no evidence for a second immunoglobulin class in fish.

In general terms the process of affinity maturation is one in which the overall affinity of the serum antibodies is found to increase over time after immunization. The lack of, or weak expression of, affinity maturation in salmon (Voss *et al.*, 1978) as well as in many other ectothermic species has been attributed to the possession of a relatively limited antibody repertoire (Du Pasquier, 1982).

1.4.5 - Lymphocyte proliferation

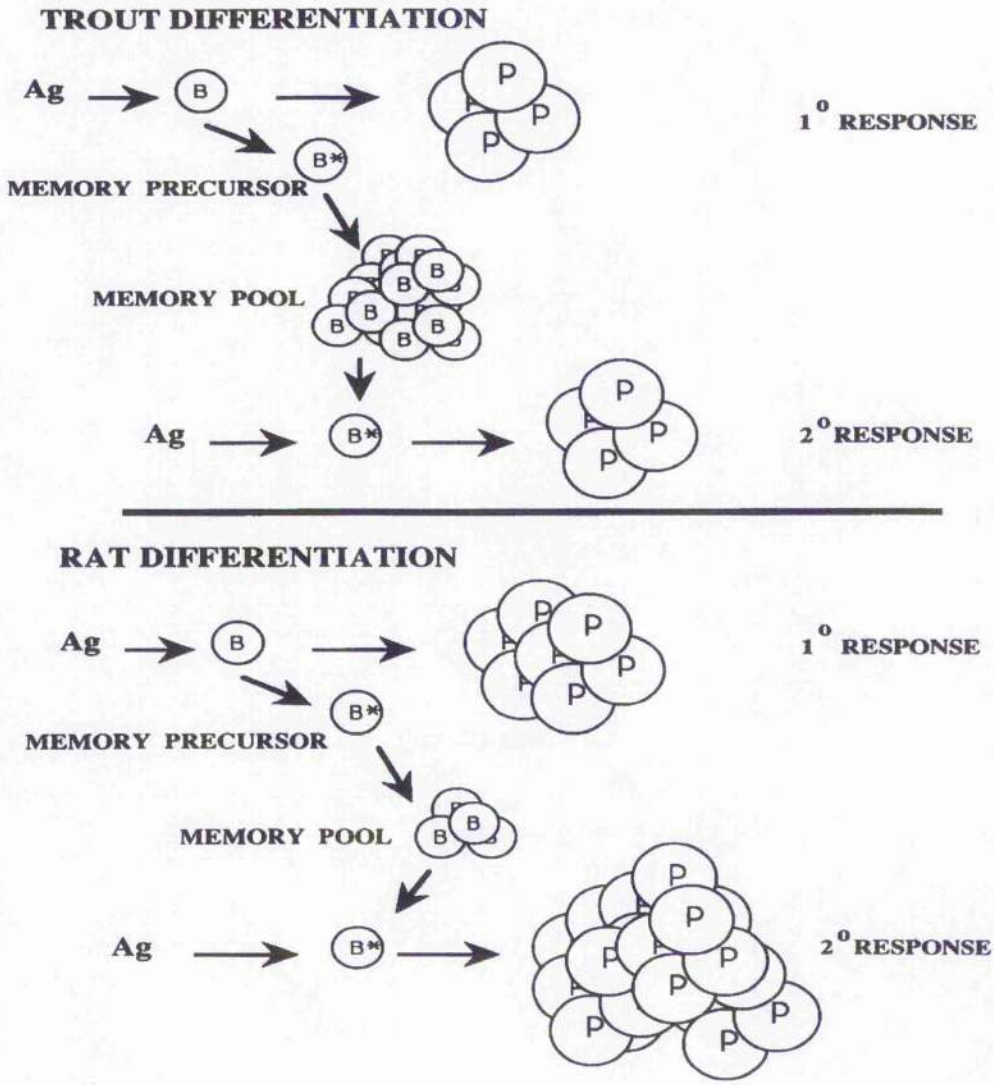
The presence of antibody-producing cells, cells showing membrane-immunoglobulin determinants (Marchalonis 1977; Ellis 1977a; Warr *et al.*, 1979), mitogen-responsive cells (Etlinger *et al.*, 1976,1977, 1978) and phagocytic cells (Avtalion and Shahrabani 1975; Weissmann *et al.*, 1975; McKinney *et al.*, 1977) have been clearly demonstrated in leucocyte suspensions from all the main lymphoid organs in fish. In mammals, lymphocyte activation by specific B cell mitogens, such as lipopolysaccharide (LPS), and T cell mitogens, such as phytohaemagglutinin (PHA) and Concanavalin A (Con A), are widely used for studying general immunocompetence (Urbaniak *et al.*, 1986).

Stimulation of lymphocyte cultures with antigens can be used to determine whether an individual has been sensitised to a given antigen by measuring lymphocyte blastogenesis and/or production of specific antibodies (Ljungman *et al.*, 1985).

In fish, lymphocyte stimulation tests have been performed with varying success in species such as channel catfish (Sizemore *et al.*, 1984), carp (Rosenberg-Wiser and

Figure 4. Development of memory B cell populations

In the trout, primary or secondary (memory) antigenic exposure results in the elaboration of comparable clone sizes of plasma cells (P) from individual precursors (B^*). The increased response results from the greatly enlarged memory pool. In the rat, this memory pool does not appear to be much larger than the naive pool. However, both the naive and memory precursors have greater capacity to produce plasma cells. The memory precursors also possess much greater capacity to proliferate than do the naive precursors.
(Source: Kaattari, 1992).


TROUT DIFFERENTIATION
Ag
B
B*
MEMORY PRECURSOR
P
1° RESPONSE
MEMORY POOL
Ag
2° RESPONSE
RAT DIFFERENTIATION
Ag
B
B*
MEMORY PRECURSOR
1° RESPONSE
MEMORY POOL
Ag
2° RESPONSE

Avtalion, 1982), rainbow trout (Etlinger *et al.*, 1976; Warr and Simon, 1983; Tillit *et al.*, 1988; Thuvander, 1989), Atlantic salmon (Smith and Braun-Nesje, 1982), and chinook and coho salmon (Yui and Kaattari, 1987). From these studies it is evident that fish lymphocytes respond both to mitogens designated as B- and as T-cell mitogens in mammals. In addition, antigen-induced blastogenesis of fish lymphocytes has been demonstrated *in vitro* to antigens from *A. salmonicida* (Tatner, 1990; Reitan and Thuvander, 1991, 1994; Marsden *et al.*, 1994, 1995) and leucocyte culture systems have been used to induce specific antibody production to model antigens (Miller and Clem, 1984; Miller *et al.*, 1985; Kaattari *et al.*, 1986).

Reitan and Thuvander (1991) demonstrated, from *in vitro* studies of lymphocyte proliferation, that the cells from Atlantic salmon and rainbow trout generally showed very similar mitogenic responses and displayed the same culture requirements. These authors also showed that thymocytes from both salmonid species responded to PHA, but not to LPS, indicating the presence of responding T cells, but not B cells. This observation agreed with that reported by Etlinger *et al.* (1976) but contradicted the findings of Warr and Simon (1983) who reported a small but significant response to LPS in cells from the thymus of rainbow trout.

The work of several authors indicated that leucocytes from fish primed with whole cells of *A. salmonicida* showed a higher proliferative response to the homologous antigen than cells from unvaccinated fish (Thuvander, 1989; Reitan and Thuvander, 1991; Marsden *et al.* 1995). However Tatner (1990) only observed an enhanced proliferative response of primed leucocytes in response to ECP antigens and not whole cells.

Detection of measurable amounts of antibody produced *in vitro* to *A. salmonicida* by splenocytes and peripheral blood lymphocytes (PBL) from both immunized and non-immunized fish (Reitan and Thuvander, 1991) was an indication of the differentiation and maturation of B lymphocytes. This culture system therefore provides the potential for studying induction of cellular activity and for measuring on-going immune responses, which should prove valuable in vaccine research.

1.5 Inter-relationship between cytokines, eicosanoids and the immune system

The mammalian immune system is a complex multifactorial process involving the interaction of cytokines, eicosanoids and various leucocyte types. Eicosanoids have been demonstrated to play a central role in immune regulation in mammals, both by their direct effects on cells such as macrophages and lymphocytes and, indirectly, via cytokines. The fish immune system has been shown to contain many of the factors and cell types characteristic of its mammalian counterpart.

1.5.1 - Cytokines

It is generally accepted that factors with cytokine activity play an important role in coordinating leucocyte responses of fish as in other vertebrates (reviewed by Secombes *et al.* 1996). However, the lack of purified fish reagents make firm conclusions difficult.

Cytokines are simple polypeptides or glycoproteins of less than 30 kDa, that act as signalling molecules within the immune system (Thomson, 1994; Callard and Gearing, 1994). Considered to be products of T lymphocyte, cytokine production is triggered as a result of new gene transcription following cell stimulation. Cytokines usually function as local chemical mediators (Hamblin, 1993) although, in some instances they can be carried via the blood stream to target cells where they exert their effect via high-affinity specific factors. They often act synergistically (Vilcek and Le, 1994), both with other cytokines and pathogen-derived molecules such as LPS. Interaction with other signals can augment leucocyte responses, as seen between human recombinant tumor necrosis factor (rTNFα) and mitogens on lymphocyte proliferation (Hardie *et al.*, 1994). However, Jang *et al.* (1994) demonstrated the inhibitory effects of mammalian transforming growth factor β_1 (TGFβ_1) on fish macrophage activating factor (MAF)-containing supernatants and of rTNFα on trout macrophage respiratory burst activity thus highlighting the fact that cytokines can also act antagonistically.

Activities similar to interferons, interleukins, chemokines, macrophage migration inhibition factor (MIF), (MAF) and colony stimulating factor have been described in fish, as

although to date, none have been fully characterised. In the search for fish cytokines biological cross reactivity was shown to occur with IL-1 in channel catfish (Clem *et. al.*, 1991; Ellsaesser and Clem, 1994) and in carp (Verburg van Kemenade *et al.*, 1995).

Cytokine release from mammalian T helper cells is a crucial aspect of specific immune responses, thus, it is important that effective stimulation and release of cytokines occurs from these cells following vaccination. Priming of MAF release has been demonstrated from rainbow trout leucocytes by vaccination against *A. salmonicida* (Marsden *et al.*, 1994). This has importance in the activation of non-specific defences where relatively crude vaccines are used and protective epitopes are ill-defined.

1.5.2 - Eicosanoids

Eicosanoids are oxygenated derivatives of polyunsaturated fatty acids formed by the metabolism of membrane phospholipids by phospholipases. The principal substrate is arachidonic acid (AA; 20:4, *n*-6) with a 20-carbon backbone, but both eicosapentaenoic acid (EPA; 20:5, *n*-3) and docosahexaenoic acid (DHA; 22:6, *n*-3) are also important substrates in fish due to their abundance in membrane phospholipids of these organisms (Henderson and Sargent, 1985).

There are two main pathways involved in eicosanoid generation. The first is catalysed by lipoxygenases which yield a range of mono- and di-hydroxy fatty acids such as 5-hydroxy-eicosatetraenoic acid (5-HETE), leukotrienes (LT), and lipoxins (LX) (Fig. 5).

The second main pathway of eicosanoid biosynthesis is mediated by the rate-limiting enzyme, cyclooxygenase (COX), also known as prostaglandin H synthase (PGHS) and leads to the generation of prostaglandins (PG), prostacyclin (PGI) and thromboxanes (TX). This enzyme is comprised of a cyclooxygenase, that catalyzes the oxygenation of arachidonic acid to PGG_2, and a peroxidase, that reduces PGG_2 to PGH_2 (Pace-Asciak and Smith, 1983). Two prostaglandin synthases exist, a constitutive form, prostaglandin synthase-1 (PGHS-1); and an inducible form, prostaglandin synthase-2 (PGHS-2). Induction of PGHS-2 was demonstrated in monocytes/macrophages (O'Sullivan *et al.*, 1992; Lee *et al.*, 1992) as well as in various other cells and tissues and its expression has been shown to be

1992) as well as in various other cells and tissues and its expression has been shown to be regulated by growth factors, cytokines, and phorbol esters (Raz *et al.*, 1989; Fu *et al.*, 1990; O'Banion *et al.*, 1991).(Fig. 6). Cyclooxygenase activity can be blocked irreversibly by aspirin, and reversibly by non-steroidal anti-inflammatory compounds such as indomethacin or ibuprofen.

Since eicosanoids have short half-lives *in vivo*, they are unlikely to be transported around the body. Thus the source of eicosanoid generation for immune regulatory activity are probably fixed cells in lymphoid organs present at the site of inflammation and immunological reactions. Such cells include macrophages, reticular, dendritic and endothelial cells although free leucocytes must also contribute to the total eicosanoids generated (reviewed by Rowley *et al.*, 1995).

Stimulated macrophages and monocytes produce PGE_2 in relatively higher amounts than any of the other leucocytes. Lymphocytes have been reported to make small amounts of PGE_2, but it was suggested that contamination with monocytes accounted for most or all of the PG formed (Goldyne and Stobo, 1979; Kennedy *et al.*, 1980). Cellular sources of LTB_4 are mononuclear phagocytes (Ferreri *et al.*, 1986; Laviolette *et al.*, 1988) and B cells (Claesson *et al.*, 1993).

In previous studies of rainbow trout unfractionated peripheral blood leucocytes and head kidney macrophages, both were shown to generate significant amounts of lipoxins, 12-HETE, LTB_4, LTB_5 and PGE_2 (Pettitt *et al.*, 1989a,b; Pettitt *et al.*,1991; Rowley *et al.*, 1994).

Increases in PGE secretion occur in response to a variety of stimuli including zymosan, bacteria, antigen-antibody complexes, (Snider *et al.*, 1982), IL-1, IL-6, TNF, LPS, complement components, and cross-linking of Fc receptors (Bernheim, 1986; Frey *et al.*, 1986; Mitchell *et al.*, 1991; Lehmmann *et al.*, 1988; Hsueh *et al.*, 1984).

Eicosanoids can regulate the synthesis and expression of receptors for various interleukins, while in turn these cytokines can affect the biosynthesis of eicosanoids by a number of different mechanisms, hence providing a feedback loop.

Figure 5. Eicosanoids likely to be formed in fish from 20 carbon and 22 carbon fatty acids

Lipoxygenases (LO), hydroxyeicosatetraenoic acids (HETE) and hydroxyeicosapentaenoic acids (HEPE).

(Source: Rowley, 1992).

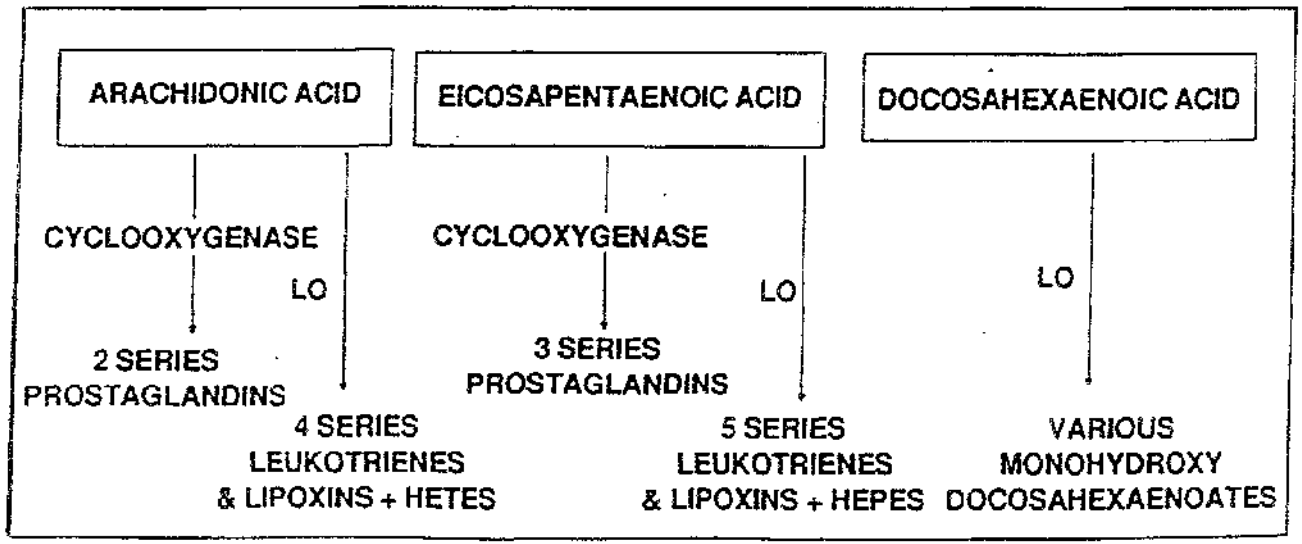
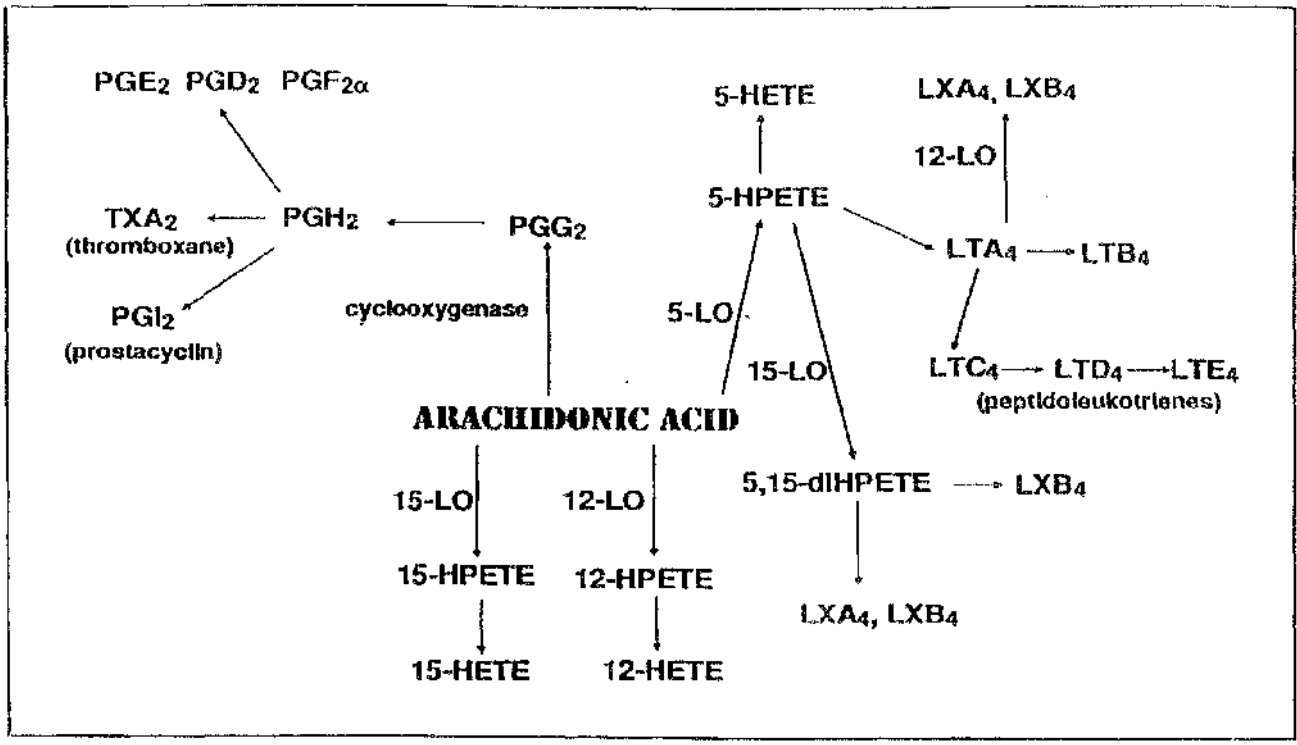

ARACHIDONIC ACID
EICOSAPENTAENOIC ACID
DOCOSAHEXAENOIC ACID
CYCLOOXYGENASE
LO
CYCLOOXYGENASE
LO
LO
2 SERIES PROSTAGLANDINS
4 SERIES LEUKOTRIENES & LIPOXINS + HETES
3 SERIES PROSTAGLANDINS
5 SERIES LEUKOTRIENES & LIPOXINS + HEPES
VARIOUS MONOHYDROXY DOCOSAHEXAENOATES

Figure 6. Pathways of biosynthesis of eicosanoids from arachidonic acids

Cyclooxygenase products include prostaglandins (PG), thromboxanes (TX) and prostacyclin. Lipoxygenases (LO) give rise to a large number of products including lipoxins (LX), leukotrienes (LT), mono- and di-hydroxyeicosatetraenoic acids (HETEs and diHETEs) via unstable hydroperoxyeicosatetraenoic acids (HPETEs). Not all eicosanoids are shown and the pathways are not all present in a single cell.

(Source: Rowley, 1992).


PGE2 PGD2 PGF2α
TXA2
(thromboxane)
PGH2
PGG2
PGI2
(prostacyclin)
cyclooxygenase
5-HETE
LXA4, LXB4
12-LO
5-HPETE
LTA4
LTB4
5-LO
15-LO
LTC4
LTD4
LTE4
(peptidoleukotrienes)
ARACHIDONIC ACID
5,15-diHPETE
LXB4
15-LO
12-LO
15-HPETE
12-HPETE
LXA4, LXB4
15-HETE
12-HETE

1.5.3 - Immune regulation in fish

Eicosanoids have been shown to modify the immunological activities of both macrophages and lymphocytes. For example, PGE_2, inhibits T- and B-cell proliferation (Simkin *et al.*, 1987; Gualde and Goodwin, 1982), T cell IL-2 production and IL-2 receptor expression, B cell activation events, and IgM and IgG3 synthesis (Chouaib *et al.*, 1985; Simkin *et al.*, 1987; Roper and Phipps, 1992), but also has a number of stimulatory immunomodulatory activities, including IL-4 and IL-10 induced proliferation of human B cells (Garrone *et al.*, 1994) and the IL-4 induced class switching in B cells (Phipps *et al.*, 1991). Similarly, LTB_4 enhances the activation, proliferation and differentiation of human B lymphocytes (Yamaoka *et al.*, 1989), stimulates the expression of IL-2 receptors on $CD56^+$ and $CD8^+$ leucocytes (Stankova *et al.*, 1992) and inhibits the mitogen induced proliferation of unfractionated T cells and purified T-helper cells (Gualde *et al.*, 1985). Information on the immunoregulatory roles of lipoxins is limited, although LXA_4 and LXB_4 have been shown to suppress the cytotoxic activity of human natural killer cell *in vitro* (Ramstedt *et al.*, 1985).

The first report suggesting that eicosanoids might influence the immune system of fish was that of Laudan *et al.* (1986). They indicated that PGE_2 was involved in the immunosuppressive activity of a microsporidan parasite, *Glugea stephani*, in the winter flounder, *Pseudopleuronectes americanus*. Their work showed that the immunosuppressive activity was reversed by administration of the cyclooxygenase inhibitor, indomethacin.

Later Knight and Rowley (1995) demonstrated that PGE_2 and PGE_3 had an inhibitory effect on the specific humoral immune response in *O. mykiss* to foreign erythrocytes and *A. salmonicida*. Of the lipoxygenase products tested, only LXA_4 and not LTB_4 had a stimulatory effect on the generation of plaque forming cells to sheep red blood cells in trout splenocyte cultures. This was in contrast to the report by Secombes *et al.*, (1994) who observed that LTB_4 and LTB_5 significantly stimulated the proliferative response of trout leucocytes from the head kidney to phytohaemagglutinin-P.

Thus, as in mammals, prostaglandins and the cyclooxygenase pathway are important in the regulation of the piscine humoral immune response. However the role for lipoxygenase products in salmonids has not yet been resolved

1.5.4 - Role of cyclic AMP in B cell activation

Parker, (1979) suggested that prostaglandins appeared to exert their influence on the cell through membrane bound cyclic nucleotides. Prostaglandins stimulate cAMP which serves as a 'second messenger'. A rise in cAMP in the lymphocyte is associated with inhibition of mitogenesis, reduced production of lymphokines, inhibition of lymphocyte mediated cytolysis and inhibition of T-lymphocyte rosette formation (Stenson and Parker, 1982). The activity of cAMP within the cell is thought to involve ionic calcium and to be effective through regulation of protein phosphorylation. Calcium acts as a 'membrane to nucleus signal' and is required for the activation of phospholipase A_2 which is responsible for making arachidonic acid available for PG synthesis at the cell membrane (Ninnemann, 1984).

It has been observed that PGE_2, cholera toxin and dibutyryl cAMP all cause increases in intracellular cAMP levels in B lymphocytes and that cholera toxin and dibutyryl cAMP mimic PGE regulation of IgM, IgE and IgG1 synthesis (Roper *et al.*, 1990; Phipps *et al.*, 1990; Roper and Phipps, 1992). Using RpcAMP, a competitive inhibitor of cAMP-dependent protein kinase (PKA) Roper *et al.*, (1994) reported that PGE signalling occurs through intracellular cAMP levels in B lymphocytes. These results indicated that the inhibitory action of PGE_2 on B lymphocyte activation was mediated via the second messenger, cAMP. The authors also identified putative cAMP- and PGE-inducible regulatory proteins (PIRP) and implicated their involvement in the regulation of B cell activation and class switching.

OBJECT OF RESEARCH

The antibody response of Atlantic salmon, *Salmon salar* L, was inhibited after intraperitoneal injection of the extracellular products of *A. salmonicida* subsp. *salmonicida* .

The main objectives of this study were :

i)- To identify, purify and characterise the humoral immunosuppressive factor.

ii)- To determine the mode of action of the suppressor.

MATERIALS & METHODS

2.1 - Bacterial strains

A. salmonicida strain 80628 used in this study was a fresh isolate from an outbreak of furunculosis in Scotland. This strain, which produced A layer, protease and GCAT, was obtained from the culture collection of the Division of Infection & Immunity, University of Glasgow.

E. coli strain 12435 was from the American Type Culture Collection.

2.2 - Growth conditions and media

A. salmonicida was cultivated in brain heart infusion broth (BHI, Oxoid) and *E.coli* in Luria broth (L-broth; 1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v sodium chloride, Oxoid). When cultivating bacteria in broth, cultures were agitated in an orbital shaker at 130 rpm for *A. salmonicida* strains and 100 rpm for *E. coli* strains in dimpled conical flasks.

Cultures of *A. salmonicida* were incubated at 20° C and *E. coli* strains were incubated at 37° C for 24 h.

2.3 - Maintenance of bacterial cultures

Organisms were maintained on slopes of BHI agar *(A. salmonicida)* and L-agar *(E. coli)* , subcultured monthly on fresh medium and stored at 4° C. For long storage, bacterial strains were maintained in 25% (v/v) glycerol and kept at -20° C. Organisms were routinely checked for purity by gram staining.

2.4 - Bacteriophage MS2

Bacteriophage MS2 (ATCC 15597) was routinely propagated using *E. coli* (ATCC 12435); plaque assays and preparation of high titre purified stocks of bacteriophage were carried out as described by Sambrook *et al.* (1989) and stored at 4° C. An overnight culture of *E. coli* in L-broth was used to set up a 5-hour culture in exponential phase. Stock MS2 bacteriophage was diluted in phage storage buffer (0.01 M Tris, 0.01M $MgCl_2$ $6H_2O$, 0.1M NaCl , pH 7.4) to contain approximately 10^6 pfu / ml.

E. coli suspension (0.2 ml) was added to 0.1ml of phage dilution and incubated at room temperature for 5 minutes. 5ml of molten L-agar (L-broth +1.2% w/v Technicial agar No.3) was then added and the mixture was layered onto dried L-agar plates and incubated at 37° C overnight.

Newly cultured phage was washed off the plates using PSB and the suspension was centifuged at 2000*g* (Sorvall RC-5B) using an SS-34 fixed angle rotor for 15 minutes to pellet bacterial cells. The supernatant was centrifuged at 31000*g* (Sorvall RC-5B) for 2 hours and the phage pellet was resuspended in 1ml of PSB. Subsequently, serial ten-fold dilutions were made and phage quantified by the addition of 0.1 ml of each phage dilution to 0.2 ml *E.coli* cell suspension as described above. After overnight incubation, plaques were counted to determine the number of plaque forming units (pfu) per ml of suspension.

2.4.1 - Bulk production of MS2 phage

An overnight culture of *E.coli* (5 ml) was added to 500 ml of L-broth in a 2 litre dimpled flask and incubated at 37° C on a shaker until the optical density of the culture at 600 nm ($O.D_{600}$) was 0.1. 50 µl of a suspension of MS2 with a titre of 10^{10} pfu / ml was added and the culture was re-incubated until the O.D = 0.2 (absorbance value increases to about 0.4 before falling down to 0.2). The bacterial cells were lysed by adding 1 ml of chloroform and further incubating at 37° C for 30 min after which the mixture was left to cool to room temperature. Bacterial DNA and RNA were digested by the addition of deoxyribonuclease (DNase) and ribonuclease (RNase) to a final concentration of 0.5 mg of each enzyme per lysed cell suspension and allowed to react for 30 min at room temperature. 0.5M sodium chloride was dissolved in the lysate and left at 4° C overnight to allow cell debris to precipitate. Cellular debris was pelleted by centrifugation at 11000*g* for 10 min at 4° C (Sorvall). The phage was precipitated by the addition of polyethylene glycol (PEG 6000, Fluka) to the supernatant at a 10 % concentration (w/v) and left on ice for 2hr after which the suspension was centrifuged at 11000*g* for 10 min at 4°C. The phage pellet was washed in

20ml PSB and collected by centrifugation at 20000*g* for 2 hr at 4° C. Titration of phage was carried out by the standard plaque assay.

2.4.2 - Bacteriophage neutralisation assay

This assay was based on that described by O'Neill (1979) where a series of dilutions (1/10, 1/50, 1/100, 1/500 and 1/1000) of salmon serum with sterile saline were set up. 0.1 ml of each dilution was then added to an equal volume of diluted phage suspension containing approximately 150 pfu of MS2. After incubation for 30 min at 20° C, 0.1 ml of a late exponential phase culture of *E. coli* was added and left to incubate for 5 min at room temperature, as described before except that 0.7 ml of molten L-agar was used to overlay the phage / bacterial suspension onto 3 ml of L-agar set in a 6-well tissue culture plate (Nunclon). Plates were incubated overnight at 37° C and plaques counted. The neutralisation titre was defined as the reciprocal of the dilution of serum which reduced the plaque count to 50% of the control value, and was determined by interpolation.

2.5 - Production and purification of serine protease

A 250ml baffled Erlenmeyer flask containing 50 ml of brain heart infusion (BHI) broth was inoculated with a colony of *A. salmonicida* 80628 from a plate culture on BHI agar and incubated at 20° C for 18 hours in a controlled environment incubator shaker (New Brunswick Scientific) operating at 170 rpm. The overnight culture (10ml) was inoculated into each of five 2 litre Erlenmeyer flasks with baffles, containing 500ml BHI broth. After 24 h incubation in a shaker at 20° C, cultures were centrifuged at 400*g* for 45 min at 4° C in a refrigerated centrifuge (Sorvall RC-5B) to remove cellular material. The supernate was concentrated overnight at 4° C using a Millipore "Minitan" ultrafiltration system with a membrane having a cut-off size of 30 kDa. The filtrate was further concentrated to 100 ml by dialysis against crystals of solid polyethylene glycol (PEG 20000, British Drug Houses, Poole, UK) followed by overnight dialysis against 4 litres of 1% glycine at 4°C.

2.5.1 - Partial purification by isoelectrofocusing

Isoelectrofocusing (IEF) of extracellular proteins was carried out by mixing 5 ml of 1% Ampholines (pH range 3.5-10; Pharmacia) to 95 ml of concentrated culture supernate. 2.5 grams of washed Sephadex G-200 (Pharmacia) granulated gel was gradually added to obtain uniform consistency. Electrode solutions used were 0.1M NaOH for the cathode and 0.1M H_3PO_4. Strips of Whatman's filter paper (0.4 cm wide) were dipped in electrode buffers and placed along the cathode and anode sides of the plate. The gel slurry was evenly spread on the glass plate and electrophoresed overnight at 1°C in an LKB-Pharmacia Multiphor II electrofocusing system at maximum voltage and current settings and a wattage setting of 8. After electrofocusing for 18 hours or until the final voltage output reached 1000 volts, the resulting 30 fractions were assayed for A_{280nm}, pH and subsequently tested in fish for immunosuppression.

2.5.2 - Purification by gel filtration

Partially purified IEF fractions in the pH range 5.7 to 6.1, previously shown to contain most of the immunosuppressive activity, were pooled; concentrated to a volume of 1 ml using solid polyethylene glycol (PEG 20000, BDH, Poole,UK) and dialyzed overnight against the elution buffer, Tris buffered saline (TBS; 20mM Tris-HCl containing 0.9% NaCl), pH 7.2 at 4° C. The dialysed sample (~1.5 ml) was applied to a column of Sephacryl S-200 Superfine (Pharmacia) gel matrix and proteins eluted at a constant flow rate of 20 ml h^{-1} using a peristaltic pump (Pharmacia). Fractions (2 ml) were collected in an automatic fraction collector (Pharmacia) and tested for haemolytic, haemagglutinating and humoral immunosuppressive activity. The MW of eluted fractions was estimated from a calibration curve of the following standards: alcohol dehydrogenase, bovine serum albumin, ovalbumin, trypsinogen and α-lactalbumin (Sigma) with the corresponding respective molecular weights of 150, 66, 45, 24 and 14 kDa. Estimation of molecular weights from gel filtration were confirmed by SDS-PAGE analysis.

2.5.3 - Preparation of toxoided immunosuppressive factor

Partially purified (IEF fractions) immunosuppressive factor was toxoided by the addition of 1.25% glutaraldehyde suspension (final concentration). The mixture was left at 4°C for 7 days in the dark after which the treated sample was dialysed in TBS (50 mM Tris/HCl, pH 6.5, 0.9% NaCl) for 3 days. Protein content of the sample was estimated using Bradford's assay (Bradford, 1976) and the toxoided preparation was tested in fish for immunosuppression.

2.6 - Fish

Atlantic salmon (*Salmo salar*) with an average weight of 25 grams were maintained in running fresh water at ambient temperature (8° - 14°C) in 5 m diameter tanks at the Marine Harvest Laboratory, Lochailort, Scotland, and fed on proprietary pelleted food.

2.6.1 - Immunization

For immunization, fish were anaesthetized by addition of benzocaine (15 ppm w/v final concentration) to the tank water. Unless otherwise stated, fish were injected intraperitoneally with a total volume of 0.2ml of test fraction containing MS2 phage @10^9 pfu in 0.1ml of phage storage buffer (PSB) and either 0.1ml PBS or 0.1ml of protease in Tris-buffered saline (TBS) @1µg protein per gram of fish body weight. Fish were marked for identification by Pan-jet injection of 4% Alcian blue dye solution (Sigma).

2.6.2 - Sampling

At weekly intervals, or as required, fish were killed by a blow on the head and blood samples were collected from the caudal vein. For immunised fish, blood collected, was allowed to clot at 20°C and sera stored at -70°C until required. Blood for the isolation of leucocytes was collected into heparinised vacutainer tubes and subsequently added to 8 ml of Leibovitz (L-15) medium (Life Technologies, Paisley, Scotland) containing heparin (10 units/ml, Sigma) and 5% FCS (Life Technologies, Paisley, Scotland). Anterior kidneys

from individual fish were removed aseptically and placed in 8 ml of ice-cold L-15 medium containing heparin and 5% FCS. Cell suspensions were prepared by teasing the kidney apart in 2 ml of cold L-15 medium containing heparin and 5% FCS then passing through a stainless steel sieve and finally allowing large aggregates to settle out for a few minutes on ice.

2.7 - Isolation and culture of leucocytes

7 ml of diluted blood or cell suspensions obtained from kidneys was dispensed in plastic universals. Using a plastic syringe and filler tube 9 ml of 51% Percoll (Pharmacia Biotech, St. Albans, England) was carefully layered under the cell suspension. The gradient was centrifuged at 400*g* for 20 min at 10° C to remove erythrocytes. Leucocytes at the 51% interface and within the Percoll layer were collected and washed by centrifugation at 400*g* for 10 min at 10°C in L-15 medium containing 5% FCS. Viable cell counts were determined using trypan blue exclusion and leucocytes were resuspended in L-15 medium plus 5% FCS and adjusted to approximately 2 x10^6 cell ml^{-1}.

2.7.1 - Mitogens

The following mitogens were used to stimulate leucocyte cultures: (a) LPS from *Vibrio cholerae* Serotype Inaba 569B (Sigma). (b) Concanavalin A from *Canavalia ensiformis* (Sigma). Both mitogens were reconstituted in L-15 medium supplemented with 5% FCS.

2.7.2 - Cell proliferation assay

Aliquots of 100 µl L-15 medium containing 10% FCS and 2 x 10^5 leucocytes were added to sterile 96 well round-bottomed Nunc plates (Nunclon) in the presence of a further 100 µl of L-15 medium containing the appropriate mitogen. Cell cultures were incubated at 18°C for 4 days in a humidified atmosphere.

2.7.3 - Cell proliferation quantified by tritiated thymidine

Twenty four hours before harvesting, 0.5 µCi of [^{3}H]-thymidine with a specific activity of 5.0 Ci mmol^{-1} (Amersham, U.K) was added to each well. The cells were harvested using a Betaplate harvester (Pharmacia Wallac, UK) transferring their DNA onto glass fibre filter mats (102 x 258 mm, Pharmacia Wallac) which were subsequently air dried and heat-sealed in individual sample bags (Pharmacia Wallac) with 10ml scintillant (Ecoscint A, National Diagnostics, USA). Counts per minute (cpm) were recorded in a scintillation counter (Pharmacia Wallac). All cpm values had the background cpm values of non-stimulated cultures subtracted. Results were expressed either as total counts per minute or as a stimulation index (SI), which was calculated according to the formula:

$$SI = \frac{\text{cpm of stimulated culture}}{\text{cpm of non-stimulated culture}}$$

The resultant SI values were analysed statistically by one-way analysis of variance using Minitab Statistics package on an Apple Macintosh computer.

2.7.4 - Quantification of cell proliferation by MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide) (Sigma Chemical Co., USA) was dissolved at 5 mg/ml in PBS and used essentially as previously described by Mossman (1983). Briefly, 10µl of MTT solution were added to each well of a 96 well flat-bottomed plate (Nunclon) containing 100µl of leucocytes and appropriate mitogen in L-15 medium. The plate was then incubated at 37°C for 4 h to allow hydrolysis of MTT by live cells to produce MTT formazan. After incubation, 100µl or acidified isopropanol (0.04 N HCl in isopropanol) were added to the cultures and mixed thoroughly using a multichannel pipettor to dissolve the dark blue crystals of formazan. Formazan quantification was performed using an automatic plate reader (Anthos reader 2001, Anthos labtec instruments, Austria) with a 570 nm test wavelength and a 690 nm reference

wavelength. Data were expressed as mean absorbance value of triplicate samples ± standard error of the mean.

2.7.5 - Flow cytometric analysis of leucocytes

Nuclei for flow cytometric DNA analysis were prepared and stained according to the method of Vindelov *et al.* (1983). Briefly, 0.1ml of peripheral blood leucocytes (10^5) were centrifuged at 13 000*g* for 2 min to form a cell pellet which was resuspended in 0.2 ml of citrate buffer. 0.1 ml of this cell suspension was treated with 0.9 ml of trypsin for 10 min at room temperature followed by a further 10 min incubation with 0.75 ml of trypsin inhibitor and RNAase. Finally, nuclei were stained by the addition of 0.75 ml of an ice-cold solution containing propidium iodide (as the DNA fluorochrome) and spermine tetrachloride, and incubating on ice for 15 min in the dark. DNA content of samples was analysed on a FACScan flow cytometer (Becton Dickinson).

2.8 - Biochemical Assays

2.8.1 - Caseinase assay

The assay was carried out using azocasein (Sigma) as substrate for caseinase; test samples were prepared by adding 100µl of culture supernatant to 100µl of BHI broth and 100µl of 1% (w/v) azocasein in 0.1 M phosphate buffer pH 7.5 in sterile microfuge tubes. The contents were mixed, tubes were incubated at 37°C for 30 min in a temperature-controlled water bath, after which time 800µl of 5% (w/v) cold trichloroacetic acid (Sigma) was added to stop the reaction. The mixtures were centrifuged at 13000*g* for 15 min at room temperature using a Heraeus Sepatech Biofuge, pellets were discarded and 800µl of supernatants were transferred into fresh microfuge tubes containing 800µl of 0.5N NaOH and mixed to develop the colour. OD readings were carried out in a Shimadzu UV-240 spectrophotometer; the blank was prepared by adding an equivalent volume of sterile BHI broth instead of culture supernatant. When protease activity of IEF or gel-filtration fractions was analysed the blank was prepared by substituting culture supernatant with PBS.

Absorption readings were at 440 nm and protease activity was determined by interpolation from a standard graph of several dilutions of freshly prepared trypsin (0-30μg/ml).

2.8.2 - Conductimetric enzyme assay of the phospholipase activity of GCAT

Assay of GCAT activity was performed using the conductimetric enzyme assay (Lawrence, 1971), which measures the change in conductance, between two electrodes, due to the activity of GCAT acting upon the substrate. Culture supernatant (48 h) was used as a crude enzyme preparation, dioctanoylphosphatidylcholine (DPC, 40 mg/ml provided by Dr. T.H. Birkbeck), was used as the substrate; the assay was carried out in 10 mM triethanolamine-HCl buffer pH 8.0. Cells of the apparatus were washed three times with 70% (v/v) ethanol and rinsed with sterile distilled water, then rinsed with buffer before 2 ml buffer was loaded into each cell and left for about 5 min. to achieve thermal equilibration; then, 2 ml of DPC substrate solution were added. Immediately, 40 ml of fresh culture supernatant was loaded into the cell and results were recorded, using a dedicated computer, as percentages of conductance change were expressed as mM of fatty acid released using standard curves already established for the experiment.

In order to differentiate the GCAT and phospholipase C activities which could be contained in culture supernatants of *A. salmonicida*, an inhibition test using EDTA and a stimulation test using $CaCl_2$ was carried out by the method described above. DCP (2 ml) was added to 2 ml triethanolamine buffer pH 8.0, 5 ml (1 mg/ml) of commercial phospholipase C (type IX *Clostridium perfringens*, Sigma) was added and reaction was left to proceed for few seconds; 20 ml (100 mM) of $CaCl_2$ (Sigma) was added to induce stimulation, similarly 20 ml (100 mM) of EDTA was added to another cell of the apparatus containing equivalent ingredients, to inhibit the activity of phospholipase C.

2.9 - Haemolysin and haemagglutinin titration

The haemolytic titre of T-lysin and agglutinating titre of the serine protease were determined by making serial doubling dilutions of fractions purified by isoelectrofocusing

followed by gel filtration. Fresh salmon blood was washed three times in sterile phosphate-buffered saline (PBS) pH 7.2 and resuspended to 2% (v/v) in PBS. 100 ml volumes of appropriate fractions were added to 100 ml of 2% blood suspension in microtitre plates (Nunclon) and incubated at 20°C for 3 h. Haemolytic and agglutinating titres were defined as the dilution factors which gave visibly complete lysis or agglutination of salmon blood.

2.10 - SDS-polyacrylamide gel electrophoresis of proteins

The method was based on that described by Laemmli (1970) using a vertical slab gel tank. The stock solutions and recipes for gel and buffer preparation are shown in the Appendix.

The separating (lower) and stacking (upper) gels contained 12.5% (w/v) and 4.5% (w/v) acrylamide respectively. The gel was formed between two glass plates of 17 cm x 19 cm x 0.3 cm with spacers of 1.5 mm thickness. Gel and electrophoresis (running) buffers contained 0.1% (w/v) SDS.

2.10.1 - Preparation of samples

Protein concentration of samples (culure supernatant, partially purified protease fractions and bacteriophage MS2) was determined by the method of Bradford (1976). Undiluted preparations of samples were collected in sterile microfuge tubes and an equal volume of solubilizing buffer (see Appendix) was added to each sample and heated at 100°C for 5 min in a boiling water bath.

To estimate the molecular weight of separated polypeptides, a mixture of polypeptides of known molecular weight was used (SDS-7, Sigma); this contained a mixture of the following seven proteins: α-lactalbumin (14200 Da), trypsin inhibitor (20100 Da), trypsinogen (24000 Da), carbonic anhydrase (29000 Da), glyceraldehyde-3-phosphate dehydrogenase (36000 Da), egg albumin (45000 Da), and bovine albumin (66000 Da). Protein markers were also incubated at 100°C for 5 min, prior to electrophoresis.

2.10.2 - Electrophoresis of protein samples

50 ml samples were loaded onto the gel and electrophoresis was performed at room temperature at a constant current of 30 mA, using a Shandon VoKam SAE 2761 power unit, until the tracking dye had reached the bottom of the gel. Where subsequent analysis of samples by immunoblotting was required, duplicate samples were loaded onto each half of the gel. Following electrophoresis, one half was stained using an appropriate staining method and the other was subjected to western blotting.

2.11 - Analysis of gels after electrophoresis

2.11.1 - Coomassie blue staining

Gels were stained using the method of Weber and Osborn (1969). Gels were soaked in Coomassie blue staining solution (see Appendix 5) overnight at room temperature, then destained the following day in destaining solution (see Appendix 5) with three changes of solution. Finally, gels were soaked in distilled water and stored at 4°C in heat-sealed cellophane bags.

2.11.2 Silver staining of proteins

This method was adapted and modified from the procedure of Oakley *et al.* (1980). Gels were prefixed in a solution containing 50% (v/v) absolute alcohol and 10% (v/v) acetic acid in distilled water, overnight at room temperature; the fixing solution was removed and replaced by a solution containing 10% (v/v) glutaraldehyde (BDH) in distilled water. After gentle shaking for 30 min gels were then rinsed in a large volume of distilled water overnight, then in fresh distilled water for 30 min. Gels were soaked in a freshly prepared solution containing 5 mg/ml of dithiothreitol (Sigma) in distilled water for 30 min, the solution was then discarded and replaced by 0.1% (w/v) silver nitrate solution (BDH) for 30 min. Gels were rinsed once in distilled water and then twice rapidly in developer (50 ml of 37% formaldehyde in 100 ml of 3% (w/v) sodium carbonate solution) before finally leaving the image to develop in 100 ml of developer. When the desired level of staining was reached

the developer was neutralised by adding 5 ml of 2.3M citric acid solution and mixed for 10 min. Finally, gels were washed in water, then soaked in 0.03% (w/v) sodium carbonate solution for 10 min and stored at 4°C in heat-sealed cellophane bags.

2.12 Immunological techniques

2.12.1 - Western blotting

Protein transfer from SDS-PAGE onto nitrocellulose filters (Hybond C, Amersham) was carried out according to the method of Towbin *et al.* (1979) except that 80 mA constant current was applied to electroblot the membrane overnight following electrophoresis using a 12% acrylamide gel. "Sandwiches" were assembled between single layers of 3 MM thick filter paper (Whatman) and transfers were carried out in a pre-cooled transfer buffer (see Appendix) at 80 mA overnight with cooling in a Bio-Rad "Trans-blot" transfer apparatus. Following transfer, blots were soaked in TTS buffer (see Appendix 6) overnight and then in TTS buffer containing 2% (w/v) bovine serum albumin (BSA, Sigma) for 1h; filters were immersed in 100ml TTS buffer containing antiserum (diluted 1/100) for 90 min. Filters were subsequently washed 5 times in fresh TTS buffer for 45 min and immersed in fresh TTS buffer containing 1/500 anti-rabbit horseradish peroxidase conjugate (HRP) (Scottish Antibody Production Unit) and incubated for 90 min at room temperature. The next step was to wash the filters in TBS buffer (TTS buffer without Tween 20) for 10 min, then in TBS containing 0.05% (v/v) Nonidet P40 for 10 min and washed once in TBS only for 10 min. The substrate (3,3 Diaminobenzidine tetrahydrochloride dihydrate, Aldrich Chemical Co.) (Appendix) was added and when the desired level of staining had been reached the reaction was stopped by immersing the developed filters in distilled water for 10 min. Finally, the filters were rinsed in distilled water, dried and stored.

2.12.2 - Ouchterlony gel diffusion test

The procedure was adapted from the method described by Ouchterlony (1958). 1% (w/v) agarose (type I: low EEO, Sigma) in Tris-HCl buffer pH 7.5 was prepared and poured

onto microscope slides. Samples were applied to 3 mm diameter wells and slides were incubated in a moist atmosphere at 37° C overnight. Slides were washed at room temperature in sterile saline for 1 day with three changes of solution, rinsed in distilled water, pressed, air-dried and stained with 0.1% Amido Black stain for 1 min; slides were destained in 3% (v/v) acetic acid until the precipitin lines were clearly visible, washed in distilled water, and then heat-sealed in cellophane bags.

2.12.3 Purification of salmon immunoglobulin

Salmon immunoglobulin was purified by a modification of the method used by Magnadottir (1990). A partially purified globulin fraction was obtained by applying 10 ml of salmon serum to a CM Affi-Gel blue affinity column (Bio-Rad) and eluting with K_2HPO_4 buffer (10 mM containing 0.15 M NaCl, pH 7.25). The eluted globulin fractions were pooled and concentrated using solid PEG 20000 (BDH) and re-applied to the Affi-Gel column under the same conditions as the first run, followed by ammonium sulphate precipitation (50% saturation) as an additional purification and concentration step to obtain a globulin fraction free of protease and serum complement proteins.

The ammonium sulphate precipitated proteins were dialysed against PBS and analysed by SDS-PAGE to determine which fractions contained immunoglobulin; the appropriate fractions were pooled, concentrated using PEG 20000 crystals, dialysed against elution buffer, TBS (10 mM Tris/HCl, pH 8.0 containing 15 mM NaCl), and the concentrate (1 ml) applied to a gel filtration column of Sephacryl S-400 (Pharmacia). Fractions were eluted at a flow rate of 36 ml h^{-1} using a peristaltic pump (Pharmacia) and assayed for absorbance at 280 nm. The final product was considered to be salmon immunoglobulin based on the elution profile from Sephacryl S-400 and the presence of only two protein bands on SDS-PAGE gels corresponding to approximately 70 kDa (heavy chains) and 24 kDa (light chains).

2.12.4 - Immunization of rabbits with protease or salmon IgM

Antiserum to serine protease of *A. salmonicida* or salmon IgM was raised in rabbits by injecting the hind legs intramuscularly (i.m) with 1 ml of an emulsion of equal volumes of either purified protease (180µg protein/ml) or purified IgM (80µg protein/ml) with complete Freund's adjuvant. Further injections (1ml) of protease or IgM preparation in incomplete Freund's adjuvant (1:1 ratio) were administered on days 14 and 44 i.m; rabbits were bled 4 weeks later. Blood was allowed to clot at room temperature and serum collected.

2.12.5 - Purification of anti-protease or anti-salmon IgM from rabbit serum

Rabbit antiserum against protease or salmon IgM was purified and concentrated by sodium sulphate precipitation. Equal volumes of sodium sulphate and antiserum were mixed to obtain a final concentration of 32% (w/v) and left to stir at room temperature for 15 minutes. The suspension was centrifuged at 10 000*g* for 10 minutes, the pellet resuspended in a small volume of distilled water and the precipitation procedure repeated. The precipitate was re-dissolved in the minimum volume of distilled water and dialysed against PBS.

2.13 Assay of prostaglandin E_2 concentration

2.13.1 - Background

Prostaglandin E_2 (PGE_2) concentration in all cells was determined with a PGE_2 [^{125}I] radioimmunoassay kit (Du Pont, NEN Research Products, USA). The kit is based on the use of an iodinated analogue of PGE_2 as labelled antigen of high specific activity and rabbit anti-PGE_2 as the specific antibody. The basic principle of this radioimmunoassay is competitive binding, where a radioactive antigen competes with a non-radioactive antigen for a fixed number of antibody binding sites. When unlabelled antigen from standards or unknown samples and a fixed amount of tracer (labelled antigen) are allowed to react with a constant and limiting amount of antibody, decreasing amounts of tracer are bound to the antibody as the amount of unlabelled antigen is increased.

2.13.2 - Sample preparation

Polypropylene tubes and pipette tips were used at all times to minimize non-specific adsorption of PGE_2 to the sides of tubes and tips. About 2 ml of blood was collected in pre-chilled polypropylene tubes coated with a solution of 4.5mM EDTA containing indomethacin, a prostaglandin synthetase inhibitor (10mg/ml). At this concentration, indomethacin was claimed by the manufacturers not to interfere with the assay and yet be effective at preventing any further synthesis of PGE_2. Serum was isolated from whole blood immediately after collection and stored at -80°C until use.

2.13.3 - Assay procedure for prostaglandin E_2 determination

The assay buffer (0.9% NaCl, 10mM EDTA, 0.3% bovine g-globulin, 0.005% Triton-X-100, 0.05% sodium azide, 25.5mM $NaH_2PO_4.H_2O$, 24.5mM $Na_2HPO_4.7H_2O$, pH 6.8) was used to prepare a series of dilutions (0.25 - 25 pg/0.1 ml) of the unlabelled PGE_2 standard supplied as a solution containing 100 ng/ml of PGE_2 in acetonitrile. The dilutions were used to prepare a standard curve to determine PGE_2 concentration in the unknown samples.

The tracer concentrate containing < 2 mCi of [^{125}I]-PGE_2, in 0.75 ml acetonitrile was diluted 1:20 (v/v) in assay buffer. Lyophilized rabbit anti-PGE_2 antibody was reconstituted with 13 ml of assay buffer. Samples were set up as shown below, all volumes were in microlitres.

	Tube no.	Buffer	Standard	Samples	Tracer	Antibody
Total counts	1 - 2	-	-	-	100	-
Blank	3 - 4	200	-	-	100	-
"0" Standard	5 - 6	100	-	-	100	100
Standards	7 - 20	-	100	-	100	100
Sample		-	-	100	100	100

The contents of the tubes were mixed by vortexing thoroughly for 2 -5 seconds and all tubes were incubated overnight at 4° C. After incubation, tubes were placed in an ice bath and 1 ml of cold precipitating reagent (16% polyethylene glycol (PEG 6000) and 0.05% sodium azide in 50mM phosphate buffer, pH 6.8) was added to all tubes except for tubes 1 and 2 (total counts), and contents vortexed for 2 - 5 seconds. The tubes were incubated in an ice bath at 4°C for 30 min to allow precipitation of antigen-antibody complexes which were subsequently collected by centrifugation at 2000*g* for 30 min at 4°C. The supernatant containing unbound antigen was decanted (except for tubes 1 and 2) and residual liquid was allowed to drain on absorbent paper for about 1 min. Each pellet containing antigen-antibody complexes was counted in a gamma counter for 1 min. Results obtained for the standards were used to construct a standard (dose-response) curve from which the unknowns were read by interpolation.

2.13.4 Procedure for calculating unknown values

The average of counts per minute (cpm) for each set of duplicates was taken after correction for background counts. Average NET counts for all standards and samples were then calculated by subtracting from each the average blank counts (tubes 3 and 4). The normalized percent bound (% B/B_0) for each standard and sample was determined using the following equation:

$$\% \, B/B_0 = \frac{\text{Net cpm of standard or sample}}{\text{Net cpm of "0" standard}} \times 100$$

Using semi-logarithmic graph paper, % B/B_0 for each standard was plotted against the corresponding amounts of PGE_2 added in picogrammes (pg). The concentration of PGE_2 in each sample was determined by interpolation from the standard curve and corrected for the volume and dilution used in the assay.

2.14 Assay of intracellular cyclic AMP concentration

2.14.1 - Background

The receptor protein binding displacement assay was used to determine intracellular cyclic AMP concentration in cells. It is based on competition for protein binding sites between radiolabelled cyclic AMP and the unlabelled cyclic AMP to be quantified. This can be considered as a radioimmunoassay-like system where antibody is replaced by binding protein. Use of a naturally occurring binding protein preparation (usually a crude preparation of the regulatory subunit of cyclic-AMP-dependent protein kinase) which interacts with cyclic AMP with high affinity results in a comparatively simple, but highly sensitive and specific assay. The method used here is essentially a modification of those developed by Gilman (1970) and Brown *et al.* (1972).

2.14.2 - Preparation of cyclic AMP binding protein

This procedure was carried out as described by Brown *et al.* (1972). Briefly, bovine adrenal glands (approximately 30) were obtained and transported to the laboratory on ice. After removal of excessive fat, the glands were hemisected and the medulla removed. Cortical tissue was then scraped from the gland capsule, pooled and transferred into a pre-cooled Waring blender along with 1.5 volumes of ice cold homogenization buffer (0.25M sucrose, 25mM KCl, 5mM $MgCl_2$ and 50mM Tris HCl, pH 7.4). After homogenization at maximum speed the tissue was transferred to centrifuge tubes on ice and centrifuged at 2000*g* for five minutes at 4°C. The supernatant was decanted then re-centrifuged at 6000*g* for 15 minutes at 4°C and the final supernatant fraction pooled, aliquoted as a homogenous mixture and stored at -20°C until use.

2.14.3 - Sample preparation

Leucocytes obtained from salmon kidneys (as described before) were suspended in 200ml of serum-free L-15 medium to contain 10^6 cells and stimulated with serine protease (final concentration 20 mg/ml). IBMX (3-isobutyl-1-methylxanthine) dissolved in dimethyl

sulphoxide (DMSO) (final concentration of 1mM) was included to inhibit the breakdown of cAMP by the action of phosphodiesterases. The mixture made up to a final volume of 400ml with L-15 medium was incubated at 20°C for 0, 15, 30 and 60 min. After stimulation of the cells, cyclic AMP was extracted by adding 100ml of the incubation mixture to 100ml of 4% ice-cold perchloric acid and incubated on ice for 15 min. Cellular debris was removed by centrifugation at 13000*g* for 2 min at 4°C. The supernatant fraction was neutralised with 0.5M triethanolamine in 2M KOH using universal indicator. The precipitate at this stage was pelleted by centrifuging as above and the supernatant used as sample for the binding protein assay.

2.14.4 - Assay procedure for intracellular cAMP determination

The incubation buffer used in the assay was 50mM Tris HCl, pH 7.4, containing 4mM EDTA. Using this, various dilutions (0-320 pmol/ml) of unlabelled cyclic AMP were prepared giving corresponding values in the assay of 0.06, 0.12, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 pmol / 50ml. These were used to prepare a standard curve for unknown cyclic AMP determination by incubating with a fixed concentration of labelled cyclic AMP and binding protein, also used for unknown samples as shown below.

[5',8-^{3}H]-cAMP was diluted in assay buffer to give approximately 500 000 cpm/ml. Binding protein was diluted 1:30 in assay buffer for use. Samples were set up as shown, all volumes were in microlitres:

	Sample	Buffer	^{3}H-cAMP	Binding protein
Blank	-	200	100	-
Total bound	-	100	100	100
Standards	50	50	100	100
Unknowns	50	50	100	100

The incubation was commenced by the addition of binding protein, hence this was always added last. Samples were mixed then allowed to reach equilibrium by incubation at 4°C for two hours. At this time the reaction was terminated by adding 250 ml of a well mixed suspension of 2% (w/v) Norit-GSX charcoal and 1% (w/v) BSA in ice-cold assay buffer. Tubes were rapidly vortexed then centrifuged for 4 min at 13 000*g* and 4°C to sediment the charcoal containing cyclic AMP which had not bound to binding protein during the incubation. 0.3ml of each supernatant was removed for counting in 2ml of Ecoscint A (National Diagnostics, USA) for 4 min in a liquid scintillation counter. Using count per minute values of the known samples, a standard curve was constructed manually from which the amount of unlabelled cAMP in the unknown samples was determined.

2.14.5 - Procedure for calculating unknown values

The cAMP bound in the absence of unlabelled cAMP (C_O) was calculated by subtracting the average count per minute (cpm) of the blank from the average cpm of the total bound. The average blank cpm was also subtracted from the average cpm for each pair of duplicates of standards and unknown samples (C_X). C_O / C_X was calculated for each standard and used to plot a standard curve against pmoles of unlabelled cyclic AMP / assay on linear graph paper.The C_O / C_X value of the unknowns were used to determine the number of pmoles of cyclic AMP in each sample by interpolation from the standard curve.

2.15 - Statistical analysis

Analysis of variance (ANOVA), Fisher's and Dunnet's comparisons were performed using the Minitab statistical package and students' t-tests determined by Statswork.

RESULTS

3.1 Identification of immunosuppressive activity

3.1.1 - Bacteriophage neutralisation assays

When this project began, measurement of immune responses in salmon to test antigens was considered using ELISA. However, the MS2 phage neutralisation assay described by O'Neill (1979) was chosen as this phage had been shown to be a good immunogen in fish and the neutralisation assay provided a simple and reliable assay for antibodies directed against a defined antigen. The effect of particular virulence factors on the humoral response could therefore be measured indirectly by the influence on levels of neutralising antibodies produced against MS2.

3.1.2 - Kinetics of the humoral immune response of Atlantic salmon to MS2 bacteriophage

The kinetics of the immune response of Atlantic salmon to MS2 were determined by collecting weekly serum samples from fish injected intraperitoneally with 10^9 p.f.u. of MS2 phage. In initial experiments the neutralisation assay was done using five dilutions of each serum sample (1/10, 1/50, 1/100, 1/500, 1/1000) to which an equal volume of phage suspension containing approximately 250 p.f.u. MS2 was added. After a fixed time, the fraction of MS2 not neutralised was determined by plaque assay on lawns of *E. coli.*

The dilution of serum which neutralised 50% of the added plaque forming units (ND_{50}) was determined by plotting % neutralisation against $\log_{10}$ serum dilution (Figure 7). As the individual ND_{50} values within a group of fish were log normally distributed (Figure 8) the geometric mean of the ND_{50} values was taken to be equal to the average antibody titre of that group. Although the above method generated reliable data it was laborious for analysis of large numbers of sera. Therefore a less time-consuming kinetic method was developed which was based on determining the time required by a fixed dilution of serum to neutralise 50% of the added plaque forming units (Figure 9). This assay was more suited to analysis of large numbers of samples and gave similar results, in that samples with higher

Figure 7. Neutralisation of MS2 phage by salmon antiserum

Antiserum raised to MS2 in Atlantic salmon was diluted in sterile saline and an equal volume (0.1 ml) of MS2 phage was added. After incubation for 30 min at 20° C the fraction of MS2 not neutralised was detected by plaque assay using *E. coli* . The number of MS2 used was shown to yield approximately 150 plaques when PBS was used as a 0% neutralisation control instead of antiserum. Neutralising titres of antiserum (ND_{50}) were calculated from the graph of % neutralised phage against $\log_{10}$ serum dilution.

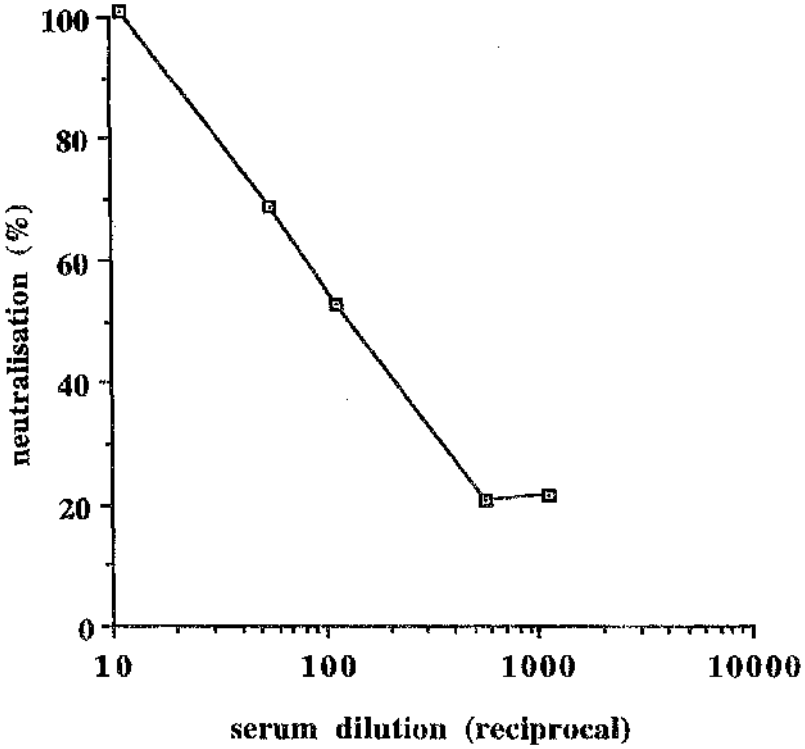

100
80
60
40
20
0
neutralisation (%)
10
100
1000
10000
serum dilution (reciprocal)

Figure 8. Rankit plot of neutralising antibody titres of a group of seven Atlantic salmon injected with phage MS2.

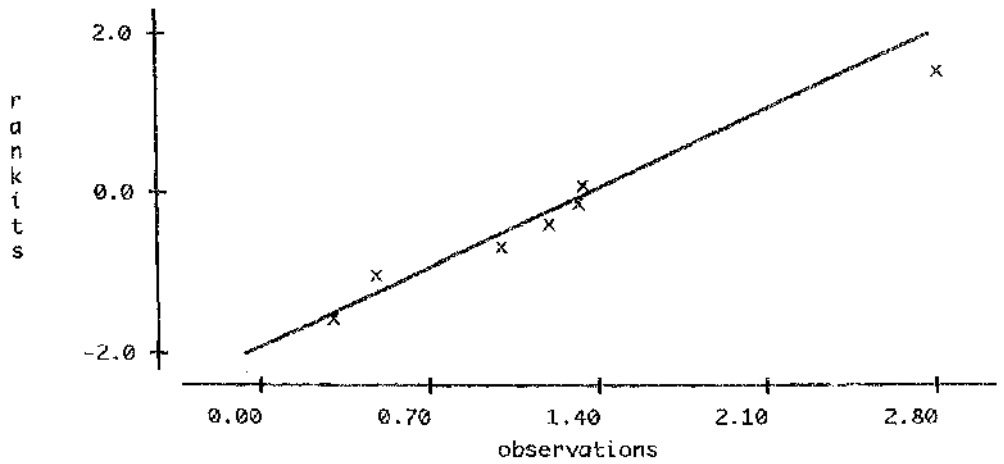
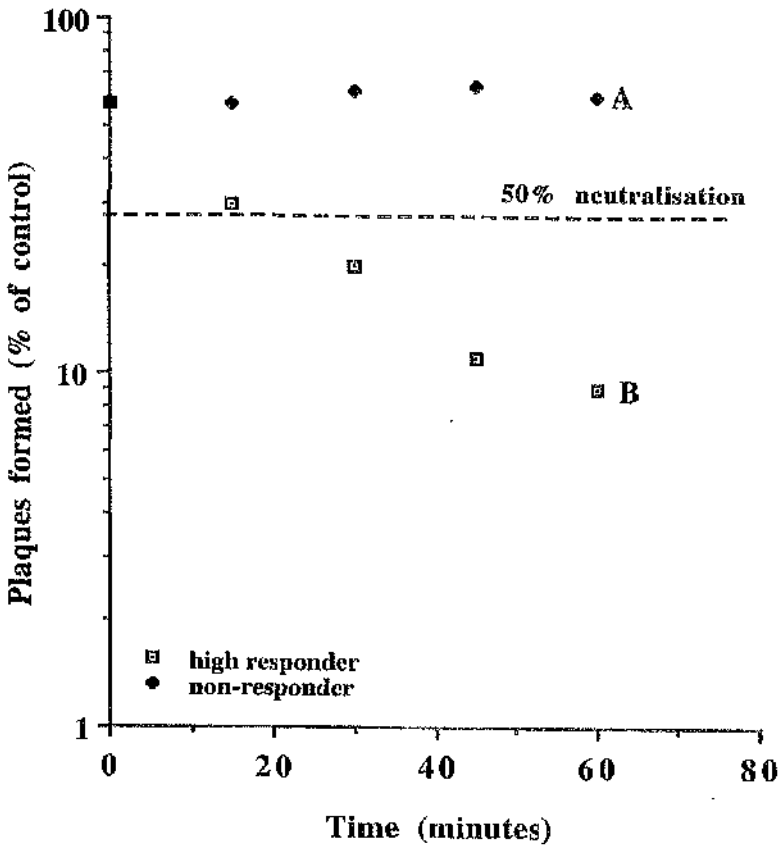

2.0
0.0
-2.0
ranks
0.00
0.70
1.40
2.10
2.80
observations

Figure 9. Kinetic assay for phage neutralisation

A single dilution of salmon serum against MS2 phage was mixed with phage suspension containing 10^4 pfu / ml and incubated at room temperature. At appropriate times aliquots were removed and assayed for residual phage to determine the % neutralised by the serum.

The graph shows the result of serum samples from two different fish ;
effective neutralisation is illustrated by serum B where 50% of phage was neutralised in < 20 min, indicating a high titre of anti-MS2 antibody, whereas, serum A showed no significant neutralisation.
This assay was used only for the experiment in Section 3.5.1 on page 99.


100
10
1
Plaques formed (% of control)
A
50% neutralisation
B
high responder
non-responder
0
20
40
60
80
Time (minutes)

neutralising antibody titres gave more rapid neutralisation than those with low Ab titres. The kinetic assay was used only for the experiment in Section 3.5.1 (page 99).

Antibodies to MS2 were detected as early as fourteen days post-immunisation and continued to increase until peak titres were reached after four to six weeks (Figure 10; this data is considered in more detail later in Figure 13), the time being dependent on the temperature at which fish were maintained.

During the development of the immune response active phage were detected in the serum of fish until a humoral response was detected (C.Mackie, personal communication). In some instances, at lower temperatures, this took as long as four weeks and the humoral response was correlated with the clearance of MS2 from serum.

3.1.3 - Suppression of the primary humoral response of Atlantic salmon by a component of the extracellular products of *A. salmonicida*

Preliminary experiments (C. Mackie, personal communication) had established that *A. salmonicida* ECP diminished the humoral response of salmon to phage MS2. To identify the humoral immunosuppressive factor (HIF) of ECP, thirty fractions from an isoelectric focusing separation were pooled into six groups and injected with MS2 into salmon. An initial experiment indicated that immunosuppression was associated with fractions 16 - 30 from isoelectric focusing. Therefore, in a subsequent experiment six groups of fish were injected with a mixture consisting of 100µl of sample containing 40µg protein ml^{-1}(estimated by Bradford's assay) and 100µl of MS2 phage (10^9 pfu), as shown, and the antibody response monitored over seven weeks :

Group	Injected with
1	MS2 + PBS
2	MS2 + ECP
3	MS2 + IEF fractions 16 - 20
4	MS2 + IEF fractions 21 - 25
5	MS2 + IEF fractions 26 - 30
6	MS2 + toxoided IEF fractions 16 - 30

A glutaraldehyde-toxoided preparation of IEF fractions 16 - 30 (Group 6) was also included in this experiment to determine whether this treatment removed the immunosuppressive activity.

Due to technical problems encountered in the delivery of sera from Marine Harvest, Lochailort, some samples, including all those from week 6 and some from week 4, were not received. However, there was sufficient data for week 4 and week 5 to establish that the immunosuppressive component of ECP was present in IEF fractions 16 - 20, as only basal antibody levels were detected in both Group 2 (MS2 + ECP) and Group 3 (MS2 + fractions 16 - 20) (Figure 11). The antibody titres of Group 2 and Group 3 for week 5 were significantly lower ($P = 0.001$, one-way analysis of variance (ANOVA)) than Groups 1, 4, 5 and 6 and this was confirmed by one-way Dunnett's statistical comparison (Appendix1). Antibody titres of Group 4 (MS2 + fractions 21 - 25) and Group 5 (MS2 + fractions 26 - 30) were comparable to the positive control Group 1 (MS2 + PBS) whereas the toxoided preparation showed a slight enhancement of the humoral response at week 5.

Neutralising antibodies were not detected during the first three weeks. At week 4, with the limitation of missing data for Group 1 (MS2 + PBS) and Group 5 (MS2 + fractions 26 - 30), no statistical difference was shown between any of the groups. However, it was clear from Figure 11 that antibody titres of Group 2 (MS2 + ECP) and

Figure 10. The primary humoral response of Atlantic salmon to an intraperitoneal injection of MS2 phage.

Fish with a mean weight of 13 grammes were immunized with 10^{10} plaque forming units of phage and groups of 10 fish were sacrificed at weekly intervals to provide sera for neutralising antibody titration. Each point is the mean +/- SEM of antibody titres of ten fish.

Only antibody titres of > 10 were regarded as a specific response.

This figure also forms part of Figure 13 shown later.

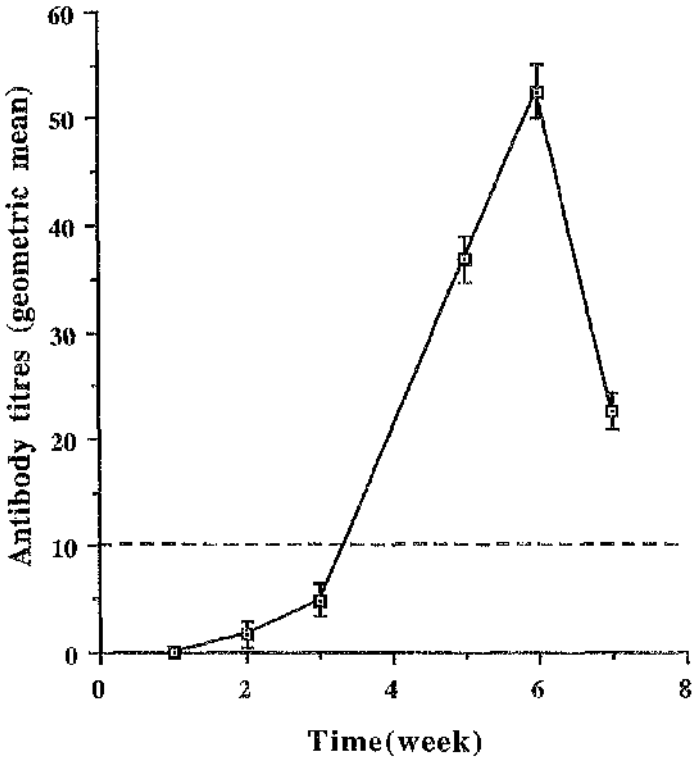
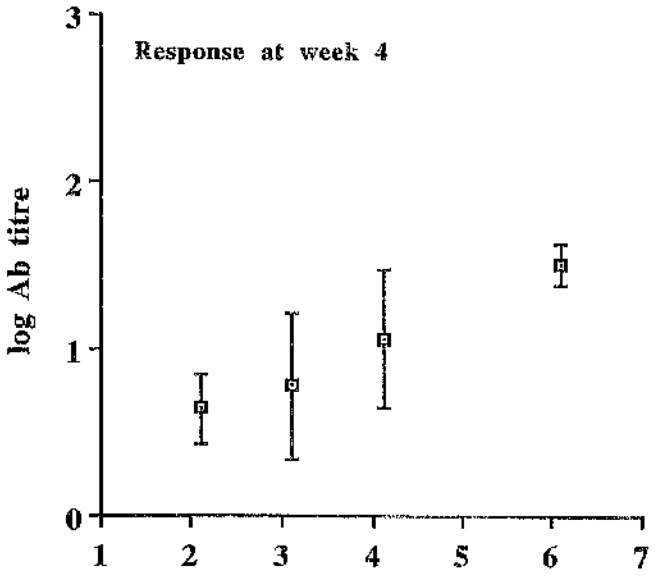
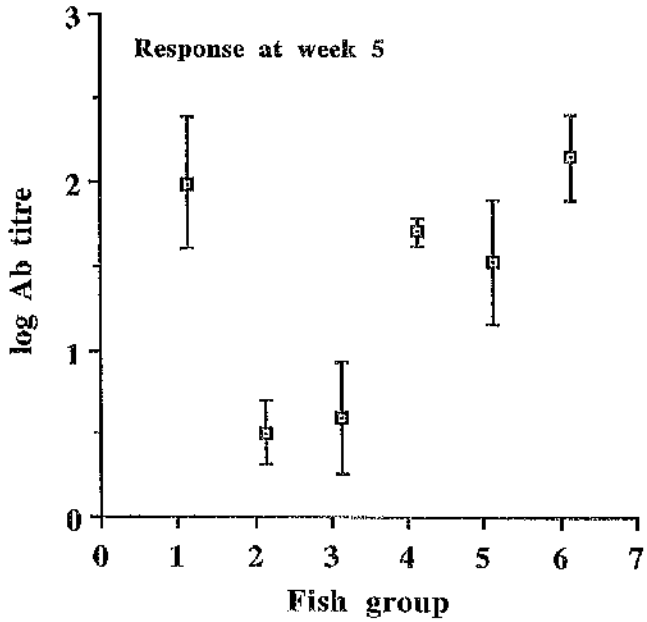

Antibody titres (geometric mean)
60
50
40
30
20
10
0
0
2
4
6
8
Time(week)

Figure 11. Identification of the immunosuppressive component of *A. salmonicida* extracellular products.

Neutralising antibody titres against MS2 of serum samples collected weekly from groups of fish (listed on p.59) were compared at weeks 4 and 5 post-immunization. Each point on the graph represents the average ± SEM of antibody titres from five fish except for groups 1 and 2 where the average was based on measurements from ten fish.


Response at week 4
log Ab titre
3
2
1
0
1
2
3
4
5
6
7


Response at week 5
log Ab titre
3
2
1
0
0
1
2
3
4
5
6
7
Fish group

Group 3 (MS2 + fractions 16 - 20) were lower than those in Group 4 (MS2 + fractions 21 - 25), and Group 6 (MS2 + toxoid).

To minimise the number of fish used, the same control groups, MS2+PBS and MS2+ECP, were used to generate data for both the primary response and secondary response (Sec1.4) experiments, the trials being carried out together.

3.1.4 - The prolonged effect of ECP on the humoral immune response of Atlantic salmon

A secondary immune response in Atlantic salmon was indicated in previous work (C. Mackie) and to confirm this observation a more extensive experiment was carried out over thirteen weeks to include the effect of ECP on the secondary immune response to MS2. Groups of 130 fish were immunized with either 100µl MS2(10^9 pfu) + 100µl PBS or 100µl MS2 + 100µl ECP(62.5µg protein ml^{-1}), ten fish from each group were sacrificed weekly and sera collected. At week eight the two groups were further divided into two sub-groups, each of which received a booster dose (equivalent to the primary dose) of either MS2 + PBS or MS2 + ECP (Figure 12). All sera were examined for anti-MS2 antibody using the phage neutralisation assay. The data for the primary response of the two groups of fish over 6 weeks has been presented previously (Figure 10) but is shown again in Figure 13. ECP suppressed the primary response of salmon to MS2, whereas a primary response was detected in control fish. Antibody titres of the two groups were significantly different at week 5 ($P < 0.05$), week 6 ($P < 0.005$), and week 7 ($P < 0.005$) when analysed statistically by one-way ANOVA (Figure 14) and Appendix 2. Fish from Group 1.2 exhibited a normal immune response after receiving a primary injection of PBS+MS2, however administration of a secondary injection of ECP+MS2 completely inhibited the response. This indicated the potency of ECP in preventing a secondary response taking place even though the fish had previously been exposed to MS2.

A secondary response was noted in fish which received PBS + MS2 (Group 1.1) as both primary and secondary injection. In contrast to the response typically found in

response. This may be explained by the observed level of circulating Ab remaining at the end of the primary response which probably neutralised some of the phage in the booster injection thus lowering the dose of antigen administered. The long-term effect of ECP was evident in fish given an initial injection of MS2 + ECP and boosted with MS2 + PBS (Group 2.1). No response to MS2 was detected in these fish over 13 weeks illustrating the extent of damage caused by ECP rendering the fish unable to recover and mount an immune response to a booster injection of MS2 + PBS.

It was observed that the antibody titres of control fish which received MS2 + PBS as the primary and secondary injection dropped rapidly after the maximum level had been reached during "the secondary" response, again, contrasting with the situation typically found in mammals.

3.1.5 - Other activities associated with the humoral immunosuppressive component of ECP

The immunosuppressive factor, established as being present in IEF fractions 16 - 20 was further analyzed. In titrations to detect haemolytic activity, the immunosuppressive fractions were found to contain both haemolytic (HL, 160 units ml^{-1}) and haemagglutinating (HA, 640 units ml^{-1}) activities against salmon erythrocytes.

3.1.6 - Purification of the agglutinating and haemolytic components

Separation of HA from HL was attempted by adsorption of HA to washed salmon erythrocytes and elution at low pH. However, this was unsuccessful due to non-specific binding of several proteins to the fresh erythrocytes used. Furthermore, when glutaraldehyde-fixed salmon erythrocytes were used, adsorption did not occur, possibly because the erythrocyte surface receptors had been denatured by glutaraldehyde treatment. In a further attempt to purify HA via its affinity for salmon red blood cells a range of sugars was tested for inhibition of agglutination to determine whether HA had lectin-like binding properties. None of the sugars tested inhibited agglutination of salmon erythrocytes indicating that the interaction was not likely to involve the sugar residues listed in Table 3.

Figure 12. Flow chart of the protocol used to determine the effect of the extracellular products of *A. salmonicida* on the primary and secondary antibody response of Atlantic salmon.

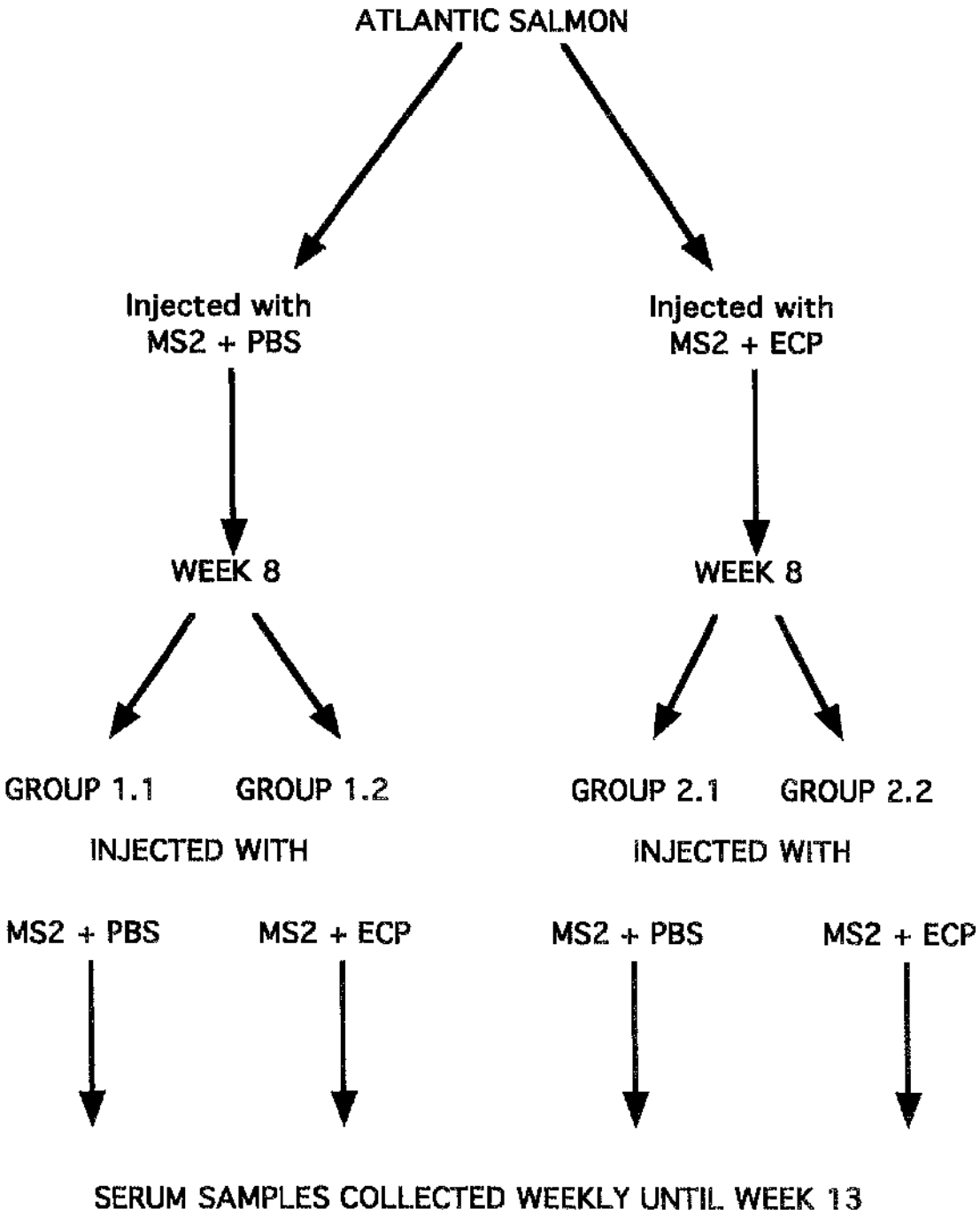

ATLANTIC SALMON
Injected with MS2 + PBS
Injected with MS2 + ECP
WEEK 8
WEEK 8
GROUP 1.1
GROUP 1.2
GROUP 2.1
GROUP 2.2
INJECTED WITH
INJECTED WITH
MS2 + PBS
MS2 + ECP
MS2 + PBS
MS2 + ECP
SERUM SAMPLES COLLECTED WEEKLY UNTIL WEEK 13

Figure 13. Effect of the extracellular products of *A. salmonicida* on the primary and secondary humoral immune response of Atlantic salmon.

Groups of 130 fish were initially immunized with MS2 + PBS or MS2 + ECP and serum samples, collected weekly by sacrifising 10 fish from each group, were used to measure anti-MS2 titres. To investigate the existence of immunological memory, the remaining fish were given a booster injection of either MS2 + PBS or MS2 + ECP at week 8 as described in Figure 12.

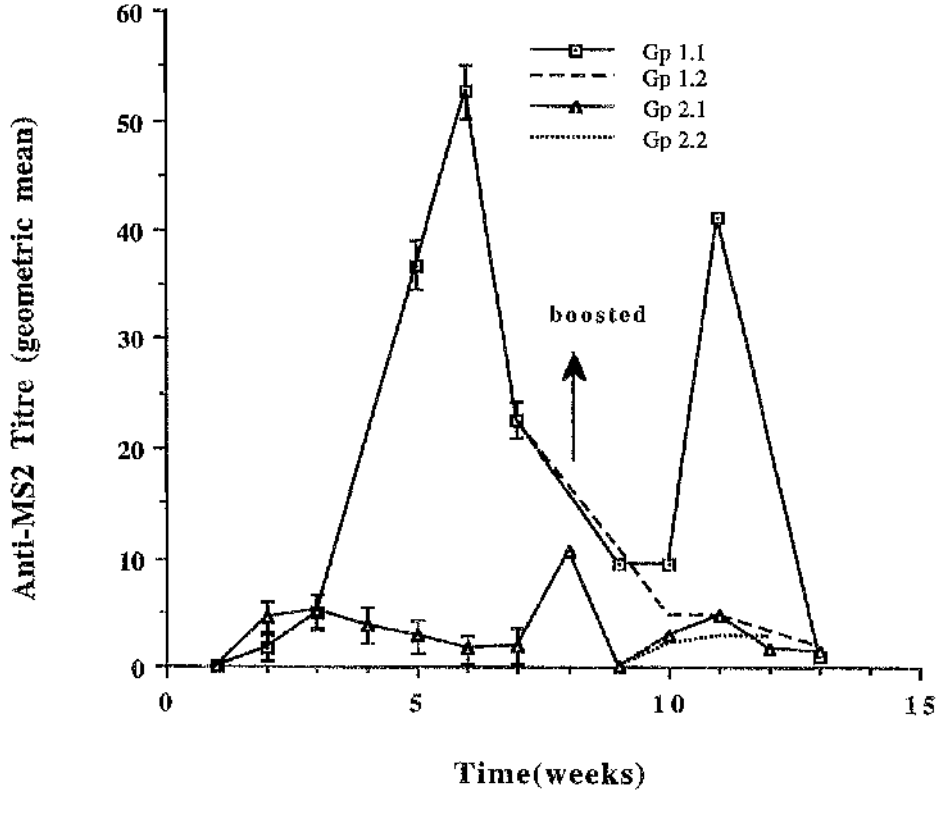

Anti-MS2 Titre (geometric mean)
60
50
40
30
20
10
0
0
5
10
15
Time(weeks)
Gp 1.1
Gp 1.2
Gp 2.1
Gp 2.2
boosted

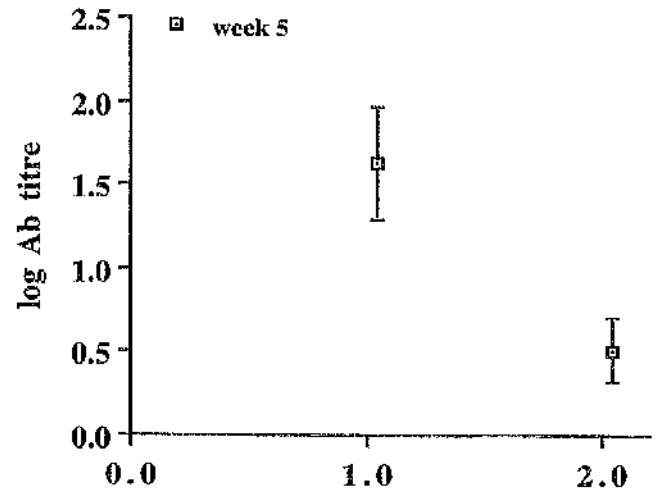
Figure 14. Quantitative analysis of inhibition of the primary antibody response of salmon by extracellular products of *A. salmonicida*.

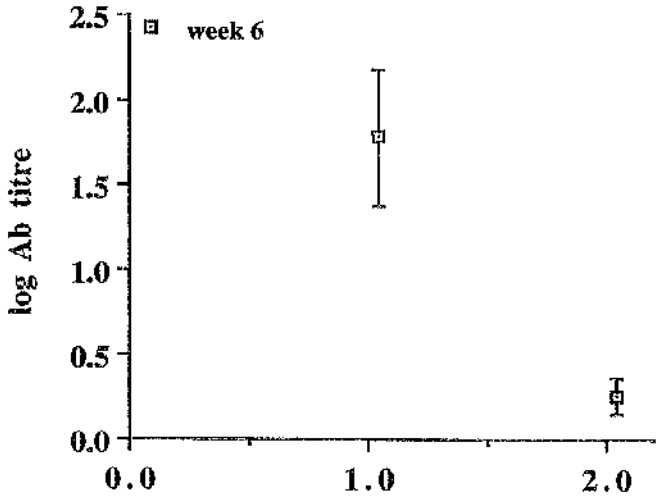
Antibody titres of fish injected with MS2 + ECP were significantly lower than those in control fish consistently at weeks 5, 6 and 7 ($P < 0.001$, ANOVA). This is an alternative illustration of data already presented in Figure 13.

Each point is the mean of ten fish ± SEM.

Group 1: MS2 + PBS

Group 2 : MS2 + ECP


week 5
log Ab titre
2.5
2.0
1.5
1.0
0.5
0.0
0.0
1.0
2.0


week 6
log Ab titre
2.5
2.0
1.5
1.0
0.5
0.0
0.0
1.0
2.0

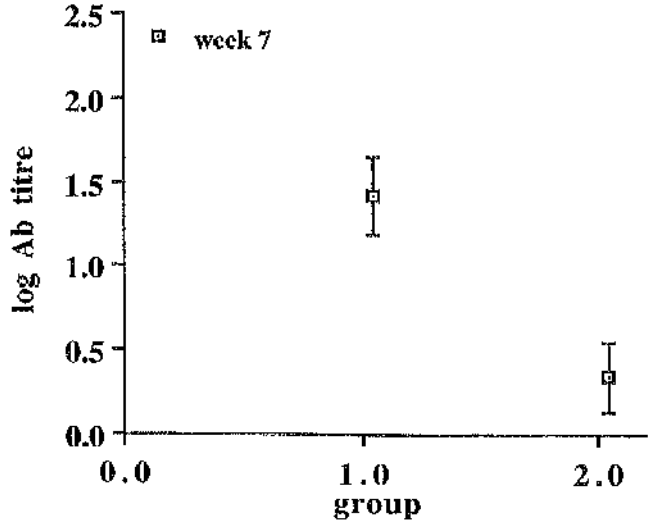

week 7
log Ab titre
2.5
2.0
1.5
1.0
0.5
0.0
0.0
1.0
2.0
group

Table 3. **Sugars used to inhibit agglutination of salmon erythrocytes by the haemagglutinin**

Sugar	Agglutination
D-mannose	+
D-galactose	+
D-glucose	+
D-fucose	+
L-rhamnose	+
D-xylose	+
Trehalose	+
D-fructose	+
L-arabinose	+
D-melibiose	+

Doubling dilutions of all sugars over the range of 2.0 - 0.125M were tested.

Therefore, the immunosuppressive fractions from isoelectric focusing were further purified by gel filtration using Sephacryl S-200. Haemolytic and HA activities were resolved as two distinct peaks (Figure 15) with apparent molecular weights of >200kDa (HL) and 70kDa (HA) respectively. Analysis of the peaks by SDS-PAGE showed that the HL fractions contained a polypeptide of Mr. approximately 25kDa and HA activity was associated with a polypeptide of Mr. approximately 66kDa (Figure 16).

3.1.7 - Effect of the purified haemagglutinin and haemolysin on the salmonid antibody response to MS2

Toxicity of HA and HL were not tested in fish prior to this experiment as it was not anticipated that they would have a deleterious effect on fish. However, 50% of fish injected with 25µg HA per fish died and this corresponded to an LD_{50} of approximately 2500ng g^{-1} of fish. The following results are based on the surviving population. Whereas HA suppressed the response to MS2 (Figure 17), the haemolysin induced a slight increase in antibody titres. There was a significant difference between the effects caused by HA and HL at week 3 ($P < 0.05$, ANOVA). Fisher's pairwise comparison also illustrated a significant difference between the group injected with HA and the control group (MS2 + PBS) (Appendix 3).

Although the difference in the antibody titres between Group 2 (MS2 + HL) and Group 3 (MS2 + PBS) was not significantly different, Figure 18 shows that titres of Group 2 were consistently higher than the titres of Group 3. The relatively low antibody titres of the control group (MS2 + PBS) could not be explained by obvious parameters such as the number of responders, which was comparable to previous observations; there was nothing unusual about the fish to fish variance within the group at any of the sampling times and the temperature was within an acceptable range (7-12°C) throughout the experiment.

3.1.8 - Identification of the haemolysin

The HL possessed phospholipase activity when tested by the method of Djebara & Birkbeck (1996). Therefore, because of the haemolytic and phospholipase activities, the

Figure 15. Purification of haemolytic and haemagglutinating activities present in the extracellular products of *A. salmonicida* cultures

The fraction of *A. salmonicida* culture supernatant which suppressed the humoral immune response of Atlantic salmon contained haemolytic and haemagglutinating activities. Purification of the activities was carried out by applying 1ml of extracellular products containing approximately 1mg protein (estimated by Bradford's assay) to a gel filtration column of Sephacryl S-200. A_{280}nm, haemolysin, and haemagglutinating activities of fractions are shown.

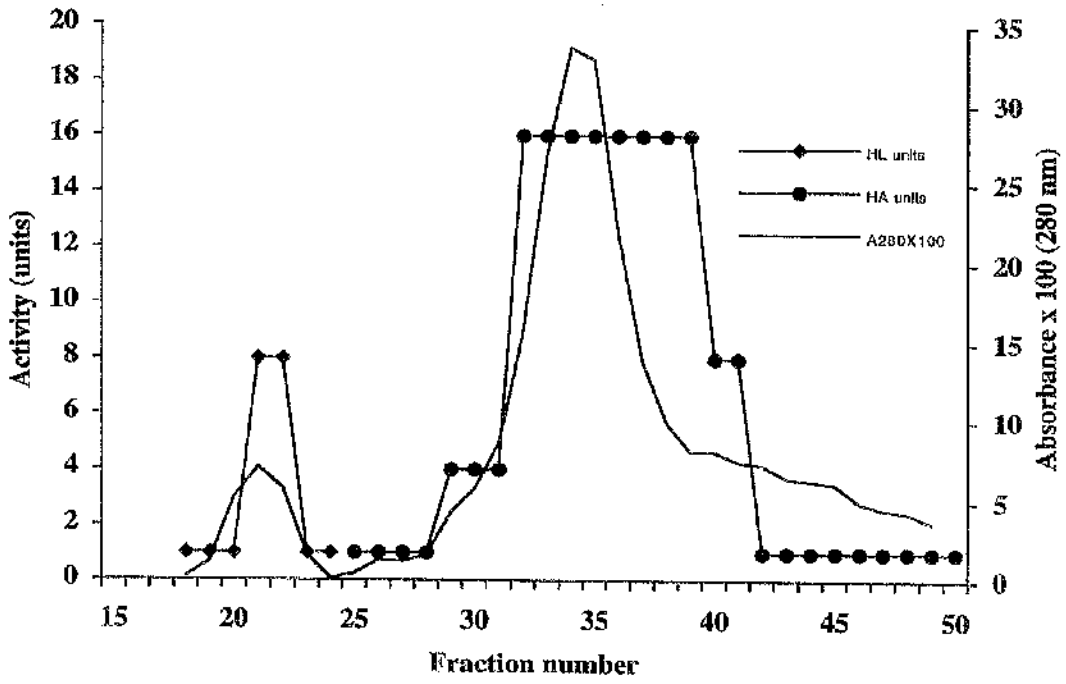

Activity (units)
Absorbance x 100 (280 nm)
Fraction number
HL units
HA units
A280X100
0
2
4
6
8
10
12
14
16
18
20
0
5
10
15
20
25
30
35
15
20
25
30
35
40
45
50

Figure 16. SDS-PAGE analysis of haemolysin and haemagglutinin separated by gel filtration chromatography.

Gel filtration fractions of the two activities from a crude sample containing 1mg protein per ml were separated on Sephacryl S-200 and analyzed by SDS-PAGE. The 12.5 % gel was silver stained following electrophoresis. The MW standard was SDS-7 (Sigma).

Gel A - Purification of haemolysin:

Lanes 1-7 were loaded with 50µl of gel filtration fractions 17-23 (see Figure 15).

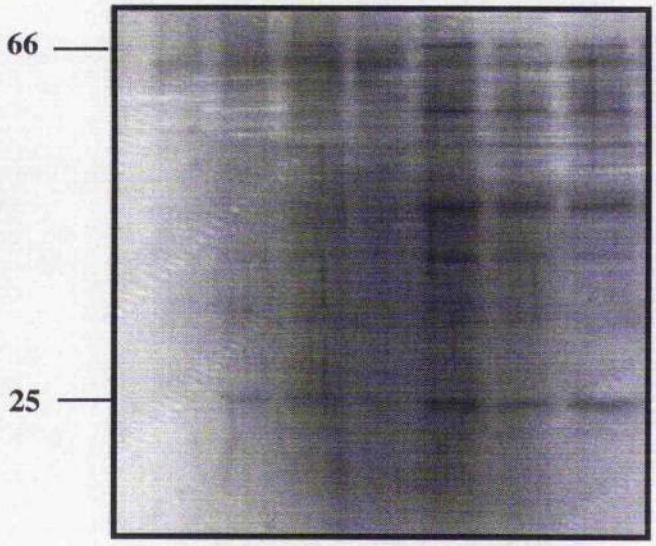
The numbers at the side refer to the MW of the protein bands.

Gel B - Purification of haemagglutinin:

lane 1 - MW standard SDS-7

lanes 2 - 8 were loaded with 50µl of gel filtration fractions 29-35 (see Figure 15).

A



B

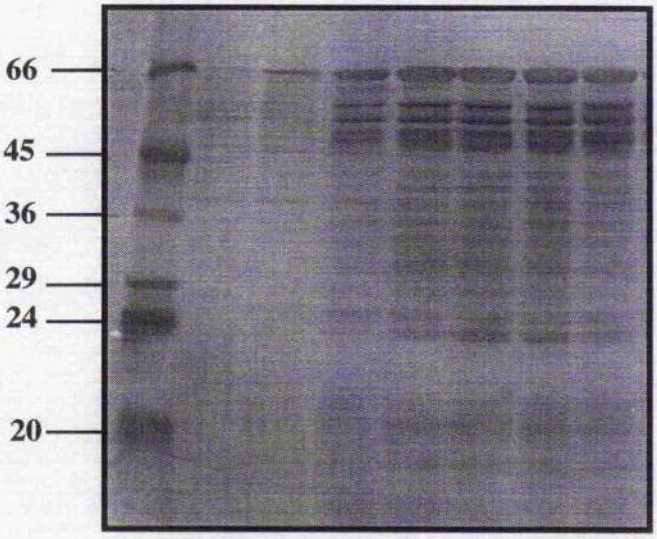


Figure 17. Effect of haemolysin and haemagglutinin on the antibody response of salmon to phage MS2

Three groups of fish were injected with; MS2 + HA (Group 1), MS2 + HL (Group 2) and MS2 + PBS (Group 3). The anti-MS2 antibody response was monitored for 6 weeks by sacrifising 10 fish from each of Groups 2+3 weekly and 5 fish from Group 1 (as a result of half the population of Group 1 fish dying on injection). Serum samples were collected and antibody titres were determined by plaque neutralisation assays.

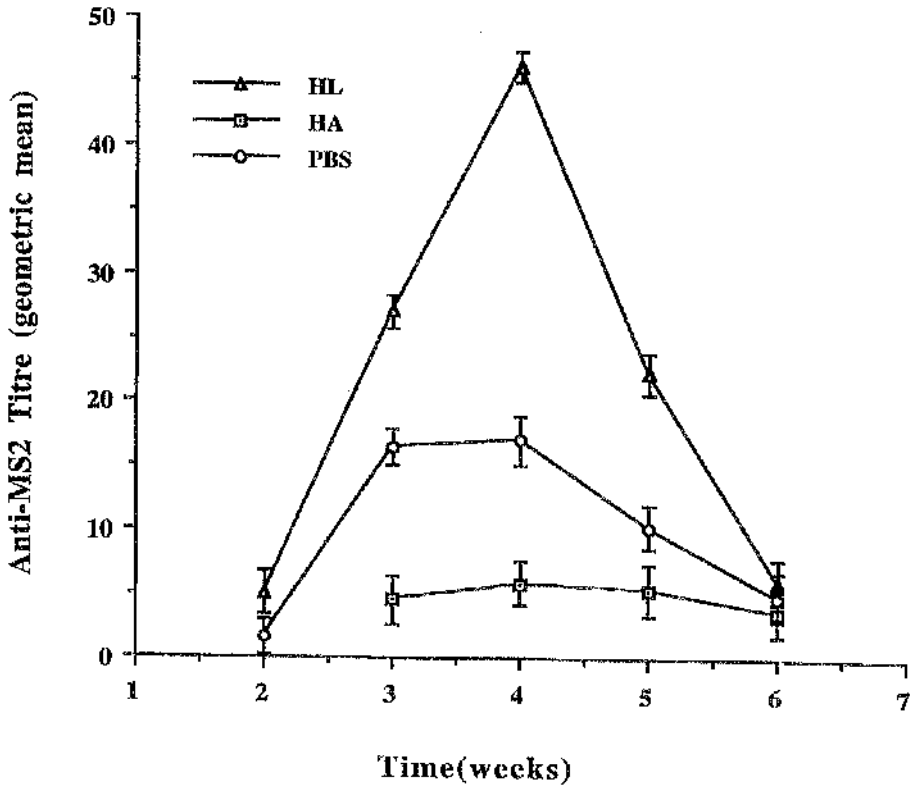
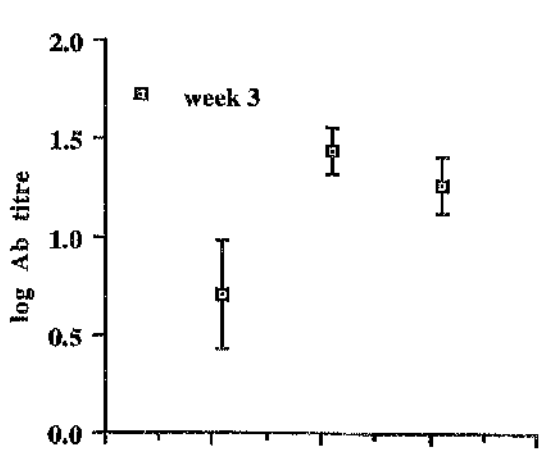
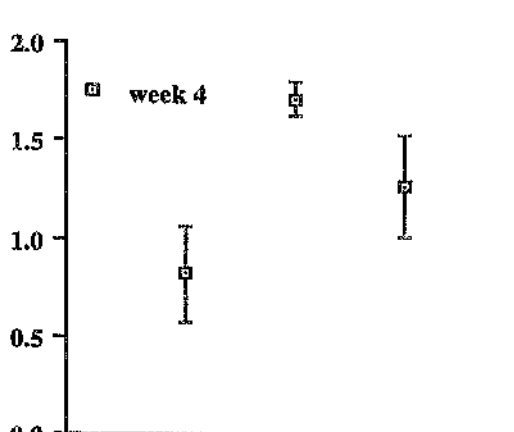

Anti-MS2 Titre (geometric mean)
50
40
30
20
10
0
HL
HA
PBS
1
2
3
4
5
6
7
Time(weeks)

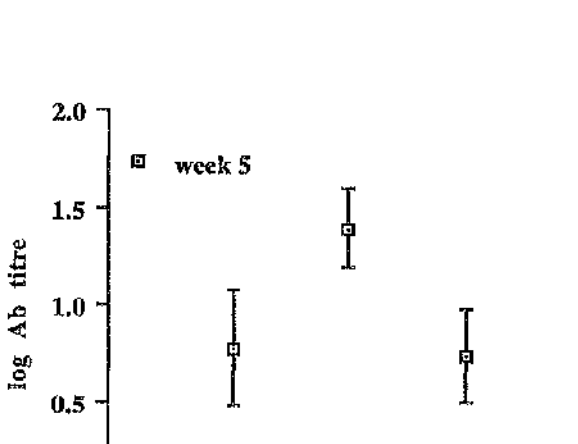
Figure 18. Comparison of the effect of haemagglutinin and haemolysin on the salmon antibody response to phage MS2

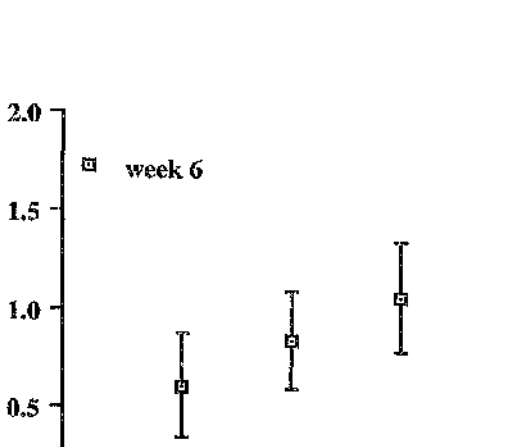
Three groups of fish were immunized with MS2 + HA (Group 1), MS2 + HL (Group 2) or MS2 + PBS (Group 3). At weekly intervals fish from each group were sacrificed and the anti-MS2 neutralising antibody titres of sera were determined.

Each point represents the mean antibody titre ± SEM of 10 fish except for Group 1 where each point represents the average of 5 fish. This is an alternative illustration of data already presented in Figure 17.


week 3
log Ab titre
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4


week 4
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4


week 5
log Ab titre
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
Group no.


week 6
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
Group no.

25kDa band on SDS-PAGE gels and the association of activity with a high molecular weight complex, probably LPS, it was concluded that the HL was the GCAT of *A.salmonicida.*

3.2 Characterization of the humoral immunosuppressive factor

3.2.1 - Identification of the proteinaceous nature of HA

Agglutinating activity of HA was lost when preparations were heated at 56°C for fifteen minutes indicating that it was probably a protein. Further evidence of this was provided by the observation that agglutinating activity (128 units ml^{-1}) of HA was abolished by treatment with glutaraldehyde.

3.2.2 - Identification of serine protease

The first indication of the possible nature of HA arose in the trial for Section 3.1.7 when 30 out of 60 fish died within two days following injection of 25µg HA/fish. The LD_{50} of approximatey 2.5µg HA per gram of fish and the molecular weight of 66kDa on SDS-PAGE indicated that HA was possibly the serine protease of *A. salmonicida.* Using azocasein as the substrate, HA was shown to have strong protease activity, equivalent to approximately 600 µg ml^{-1} as determined by interpolation from a standard curve of protease activity of trypsin (Figure 19). All further references to the units of protease activity of HA are expressed as µg ml^{-1} trypsin equivalent.

Caseinolytic activity of HA was detected by the formation of distinct zones of clearing on casein agar and the addition of >1mM phenylmethylsulphonyl fluoride (PMSF) to a culture of *A. salmonicida* caused inhibition of HA (Figure 20). These observations led to the conclusion that HA was probably the serine protease of *A. salmonicida.*

3.2.3 - Properties of antisera to the haemagglutinin

3.2.3.1 - Immunodiffusion test

When an immunoglobulin fraction of the antiserum raised in rabbits against purified haemagglutinin was tested by immunodiffusion, the major precipitin arc formed between the

Figure 19. Standard graph of Protease assay

Haemagglutinin purified by isoelectrofocusing followed by gel filtration, was assayed for protease activity using azocasein as enzyme substrate. The activity was determined by interpolation from a standard graph of trypsin concentration against the corresponding OD_{440nm}.

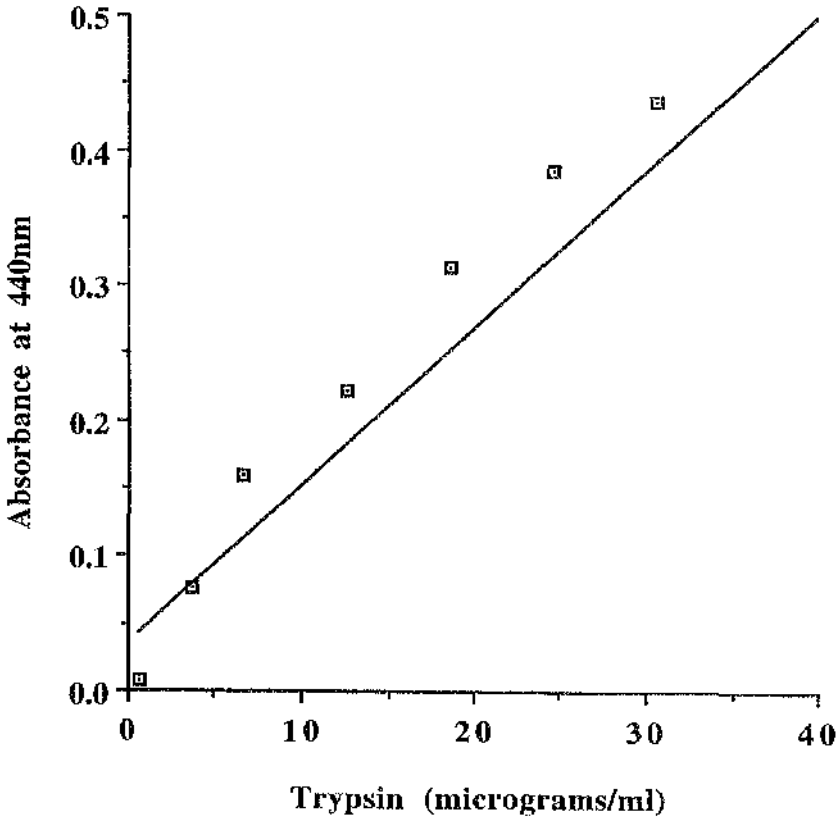

0.5
0.4
0.3
0.2
0.1
0.0
Absorbance at 440nm
0
10
20
30
40
Trypsin (micrograms/ml)

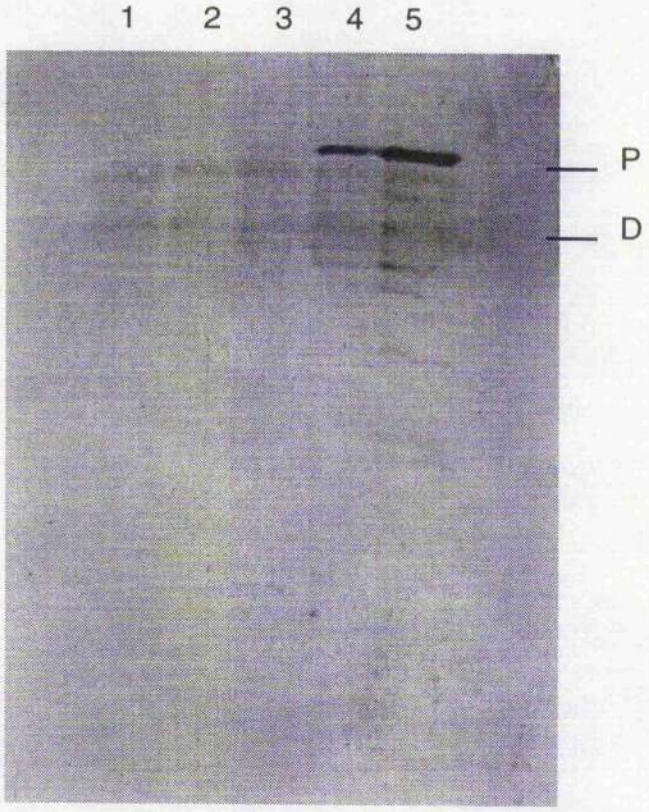
Figure 20. Inhibition of haemagglutinin by PMSF

A culture of *A. salmonicida* was incubated overnight at 20°C with a range of PMSF concentrations (0, 1, 5, 10, 20 mM). The cellular fraction was discarded after centrifugation and supernate samples were subjected to SDS-PAGE and Western blotting with rabbit antiserum raised against the serine protease. The immunoblot shows that haemagglutinin was inhibited by > 1mM PMSF.

Lane	Cultures + PMSF (mM)
1	20
2	10
3	5
4	1
5	0

P - protein band corresponding to protease (66kDa)

D - degradation products


1
2
3
4
5
P
D

antiserum and HA and that between antiserum and crude cell supernatant was continuous, showing a reaction of antigenic identity (Figure 21).

3.2.3.2 - Neutralisation of protease activity

A range of two-fold serial dilutions (1/2 - 1/32) of antiserum to HA (protein content of undiluted antiserum estimated by Bradford's assay to be 15 mg ml^{-1}) was added to a fixed concentration of HA (0.34 mg ml^{-1} protein) and incubated for 1 hour at 20°C. The protease activity of HA was neutralised by the antiserum in a dose-related manner (Figure 22). Anti-HA specifically neutralised protease activity of both HA and trypsin but no decrease in activity of either was seen to occur by the negative control, anti-ovalbumin serum, used at concentrations of up to 50 mg ml^{-1}.

3.2.4 - Determination of the time of optimum protease production in the growth cycle of *A. salmonicida*

The time of optimum yield and the stability of the protease was briefly investigated. The mean generation time of a culture of *A. salmonicida* grown in brain heart infusion broth at 20°C was 2.5 hours. Protease production occurred between the second stage of the exponential phase and the beginning of the stationary phase, the maximum level of protease activity occurring at 52 hours (Figure 23). After storage at -20°C for six weeks protease retained 68% of original activity.

3.3 Mode of action of serine protease

Having determined that the humoral immunosuppressive factor of *A. salmonicida* was the serine protease it was important to determine whether it was truly suppressing the salmonid immune response or merely degrading immunoglobulins and/or antigenic epitopes of the virus. Therefore, a number of different experimental approaches were undertaken to investigate the role of the protease in immunosuppression.

Figure 21 Immunodiffusion reaction of culture supernatant of *A. salmonicida* with antiserum to purified haemagglutinin.

Well 1 : extracellular products (ECP)

Well 2 : purified haemagglutinin (HA)

Well 3 : ECP

Wells 4 and 5 : antiserum to HA

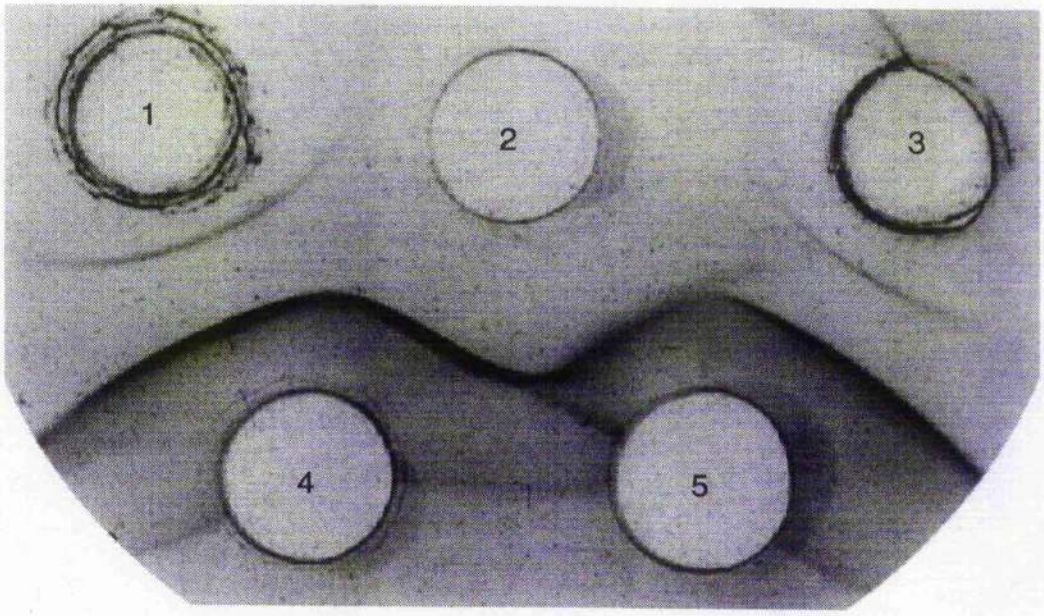

1
2
3
4
5

Figure 22. Neutralisation of protease activity by antisera to haemagglutinin

Purified haemagglutinin was mixed with a series of two-fold dilutions of antiserum to HA and incubated for 1 hour at 20°C. Any remaining protease activity was determined by the protease assay using azocasein as substrate. Antisera to ovalbumin was used as a negative control.

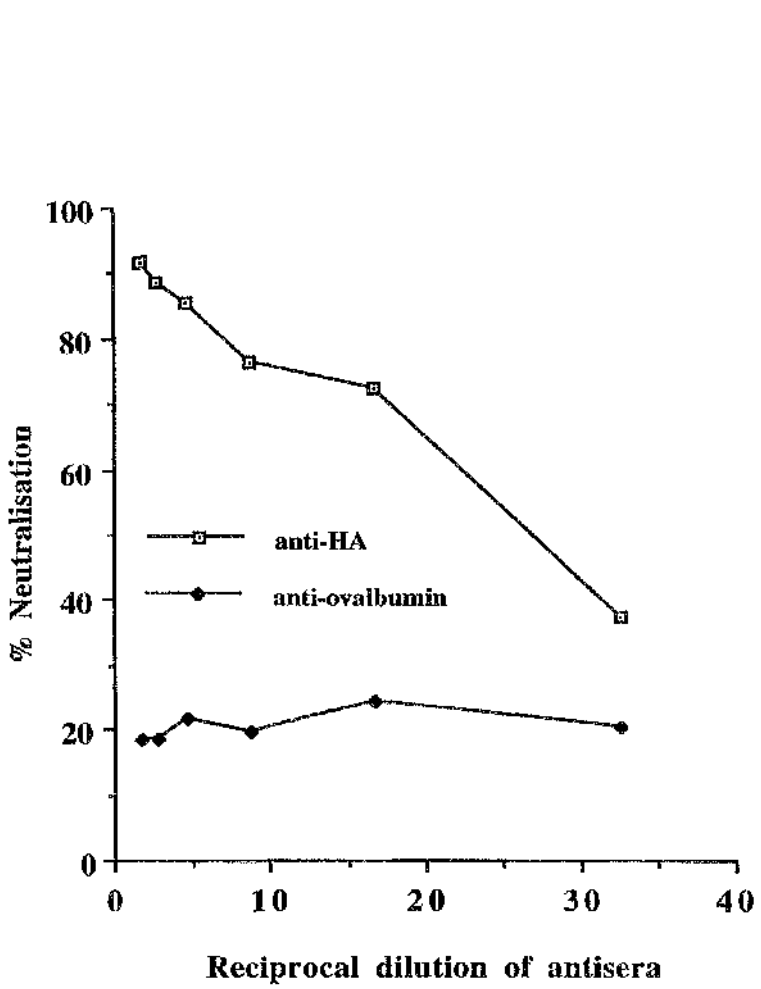
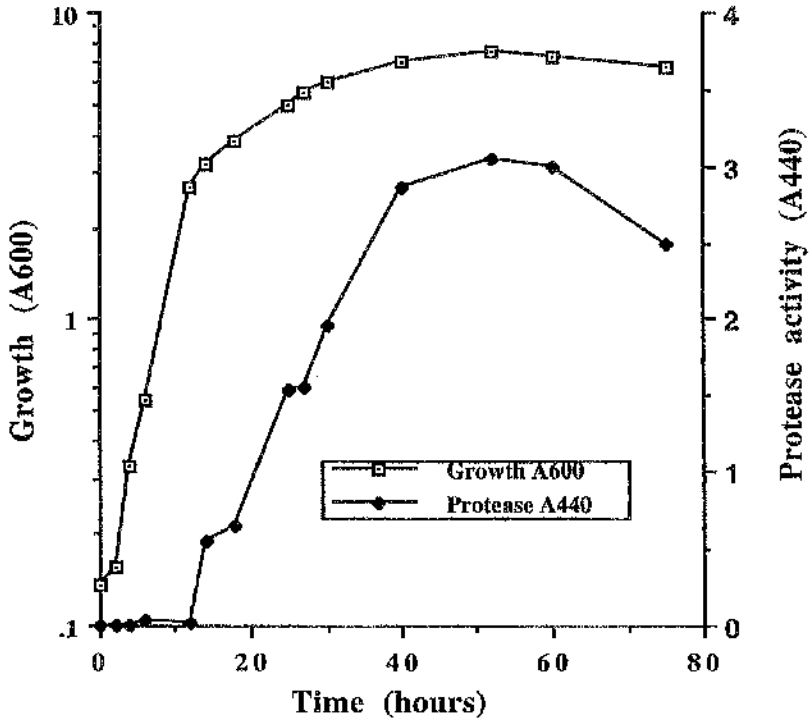

100
80
60
40
20
0
% Neutralisation
anti-HA
anti-ovalbumin
0
10
20
30
40
Reciprocal dilution of antisera

Figure 23. Protease synthesis in relation to growth of *A. salmonicida*

Growth of *A. salmonicida* in brain heart infusion at 20°C was monitored by measuring the absorbance at 600 nm. Protease activity in the cell supernate was determined by the change in absorbance at 440 nm caused by degradation of the substrate, azocasein.


10
1
.1
Growth (A600)
4
3
2
1
0
Protease activity (A440)
0
20
40
60
80
Time (hours)
Growth A600
Protease A440

3.3.1 - Effect of protease on phage infectivity

3.3.1.1 - Stability of phage *in vitro*

To determine whether MS2 was degraded by the protease 100µl of phage suspension (10^4 pfu/ml) was incubated with 100µl protease (770µg ml^{-1} trypsin equivalent) or with 100µl trypsin (300µg/ml) for 18h at 37°C. The reaction was terminated by the addition of 200µl soyabean trypsin inhibitor (600µg/ml). The residual plaque numbers indicated that phage infectivity was reduced by 48 % by the protease but MS2 incubated with phage storage buffer or trypsin lost 51 % and 40 % of initial activity respectively under the same conditions. These losses were not regarded as significantly different and could be accounted for by non-specific factors, such as adsorption of MS2 to the tube.

3.3.1.2 - Stability of phage *in vivo*

In groups of fish immunized with MS2+PBS or MS2+protease (as in Sec 2.6.1), the fraction of fish with infective MS2 remaining in circulation for at least two weeks post-immunisation, determined by plaque assay, was not significantly different ($P > 10\%$, χ^2-test) (Table 4). Thus, there was ample time for MS2 phage to be monitored by the immune system.

3.3.2 - Effect of protease on phage epitopes

Although phage infectivity was unaffected by the serine protease it was possible that epitopes, against which neutralising antibodies were directed, were degraded. To determine whether MS2 retained intact epitopes after treatment with protease, untreated MS2, and MS2 treated with protease (concentration and titres as in 3.3.1.1) for 16 hours were subsequently mixed with different dilutions of salmon serum containing anti-MS2 antibodies. Phage neutralisation reactions in both cases were similar (Figure 24), indicating that protease treatment did not affect the epitopes to which neutralising antibodies were directed against.

Further evidence that MS2 surface proteins were not sensitive to the protease was obtained by SDS-PAGE analysis of MS2 (10^{11} pfu/ml) before and after protease treatment (400µg ml^{-1} trypsin equivalent). The Coomassie stained gel illustrated that purified MS2 yielded two polypeptide bands corresponding to the coat protein (Mr = 18 kD) and A-protein

Table 4. Effect of protease on the infectivity of MS2 phage.

	Proportion of fish with infective MS2 remaining in serum	
	MS2 + PBS	MS2 + Protease
week 1	10/10	5/5
week 2	6/10	5/5

$P > 10\%$, 1 d.f., Chi-square analysis of the total number of fish for weeks 1 and 2.

Figure 24. Effect of protease on phage epitopes

After incubation with protease for 16 hours, MS2 was mixed with soyabean trypsin inhibitor followed by the addition of salmon anti-MS2 antiserum. Neutralisation of protease-treated MS2 by antiserum to untreated MS2 was compared with that for untreated MS2 by plaque assay.

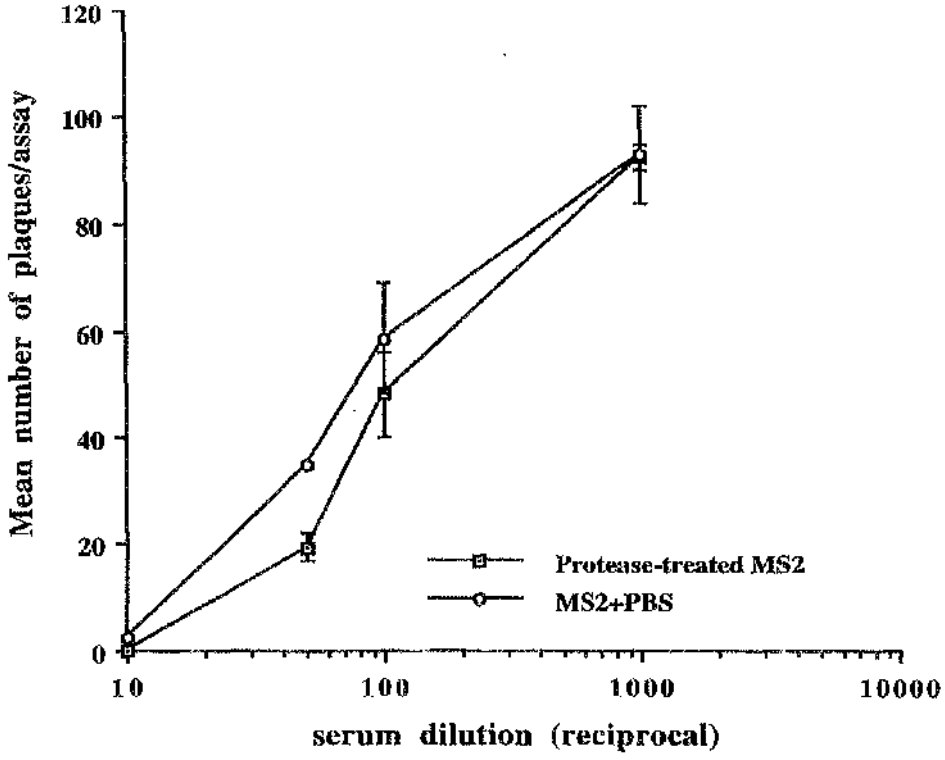

120
100
80
60
40
20
0
Mean number of plaques/assay
Protease-treated MS2
MS2+PBS
10
100
1000
10000
serum dilution (reciprocal)

Figure 25. Resistance of MS2 surface proteins to degradation by protease

SDS-PAGE (12.5% acrylamide) analysis of protease-treated MS2 phage. The gel was stained with Coomassie Blue.

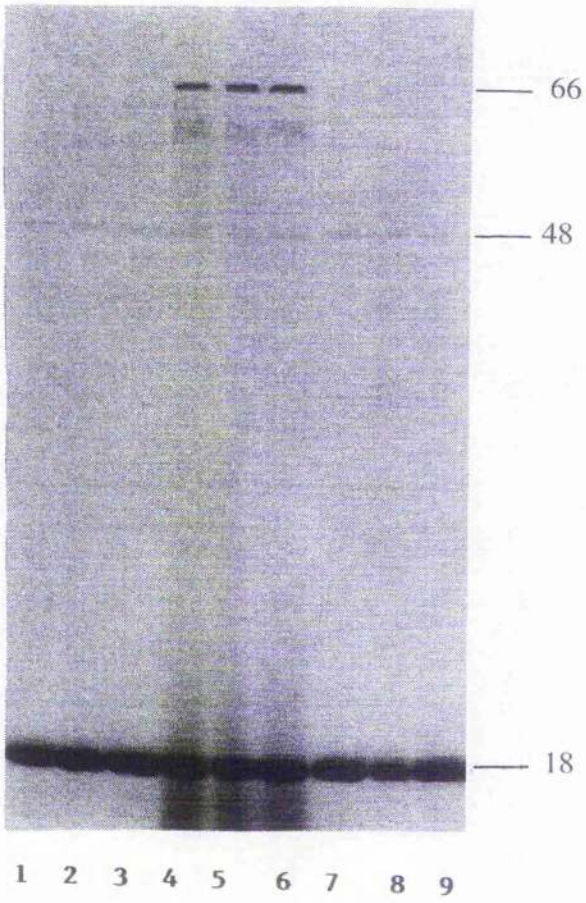
Lane	Time of incubation with protease or PBS
1	MS2 + PBS - 0 hr
2	MS2 + PBS - 4 hr
3	MS2 + PBS - 24 hr
4	MS2 + protease - 0 hr
5	MS2 + protease - 4 hr
6	MS2 + protease - 24 hr
7	MS2 + trypsin - 0 hr
8	MS2 + trypsin - 4 hr
9	MS2 + trypsin - 24 hr

After the appropriate incubation time reactions were terminated by boiling samples in solubilising buffer.

66 kDa band refers to the protease of *A. salmonicida.*

48 kDa band refers to the A protein of phage MS2.

18 kDa band refers to the coat protein of phage MS2.


66
48
18
1 2 3 4 5 6 7 8 9

(Mr = 48 kD) (Figure 25). The intensity of the A-protein band was much weaker than that of the coat protein as the two proteins are present in the ratio 1:180 in the MS2 virion (Fiers *et al.*, 1976). MS2 treated with protease for 24h had no discernible effect on the polypeptide pattern produced on SDS-PAGE (Figure 25). Thus, it was concluded that bacteriophage MS2 epitopes were not destroyed by serine protease either *in vivo* or *in vitro*.

3.3.3 - Interaction of salmon immunoglobulin with protease

A further simple explanation for the suppression of the humoral response could be via degradation of immunoglobulin (Ig). Purified from serum by the method described by Magnadottir (1990), 100µl salmon Ig (80µg protein/ml) was incubated with 100µl protease (400µg ml^{-1} trypsin equivalent) for 2, 4 and 18h. SDS-PAGE analysis followed by immunoblotting with rabbit antiserum to salmon IgM demonstrated that the heavy chains of salmon immunoglobulin were progressively degraded on prolonged incubation (up to 18h) with protease, but light chains were not affected (Figure 26).

3.3.4 - *In vivo* protection of salmon immunoglobulin from injected protease

The above results indicated that the serine protease could degrade purified salmon immunoglobulin. In order to assess whether there was significant degradation of the circulating immunoglobulin molecule *in vivo*, sera collected from two groups of fish one week after immunisation with either MS2 + PBS or MS2 + protease were compared on SDS-PAGE. This revealed that both the heavy and light chains of salmon immunoglobulin remained intact (Figure 27).

Comparison by densitometry of the intensity of staining of the heavy chain polypeptide bands seen on SDS-PAGE confirmed that there was no significant difference between the band intensity values of control sera or sera from fish injected with protease (Figure 28). Thus, the extent of degradation of immunoglobulin *in vivo* appeared very limited and unlikely to affect the neutralising antibody titres significantly.

Figure 26. ***In vitro*** **degradation of salmon immunoglobulin**

Purified salmon IgM was incubated with protease for up to 18 hours. Samples were subjected to SDS-PAGE, transferred to nitrocellulose and detected by immunoblotting with rabbit antiserum to salmon IgM and horseradish peroxidase (HRP) labelled goat anti-rabbit IgG.

Lane	Sample, and time of incubation with protease or PBS
1	protease
2	IgM + protease - 18 h
3	IgM + protease - 4 h
4	IgM + protease - 2 h
5	IgM + protease - 0 h
6	IgM + PBS - 4 h
7	IgM + PBS - 0 h

The non-specific reaction of the protease with either the rabbit anti-salmon IgM or HRP-labelled anti-rabbit IgG is visible in lanes 1 - 5. The positions of IgM heavy chain (H), serine protease (S), IgM light chain (L) and degradation products of the heavy chain (D) are indicated by arrows.

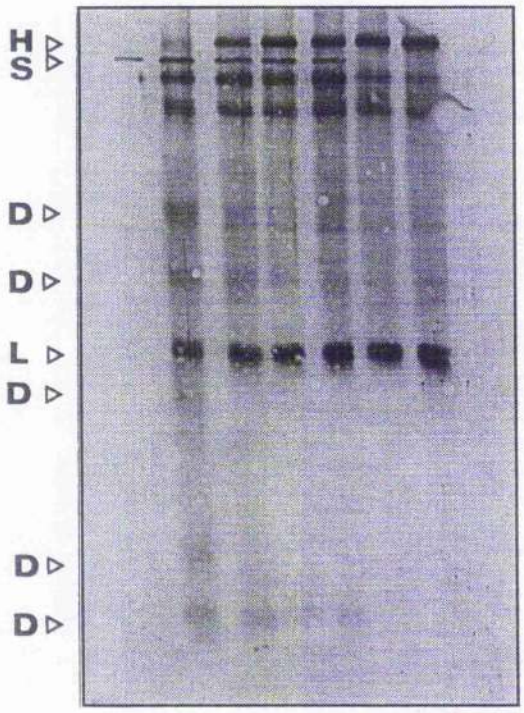

H
S
D
D
L
D
D
D
1 2 3 4 5 6 7

Figure 27. Resistance of salmon IgM to degradation by protease *in vivo*

SDS-PAGE analysis of sera which were collected one week after fish had been immunized with either MS2 + PBS or MS2 + protease (as in Section 2.6.1). Both the heavy and light chain protein bands of IgM appeared to be intact.

Lanes 1 - 4 : each lane consists of serum from individual fish injected with MS2 + Protease

Lanes 5 - 7 & 10: each lane consists of serum from individual fish injected with MS2+PBS

Lane 8 : serum from naive (unimmunized) fish

Lane 9 : IgM purified from naive serum

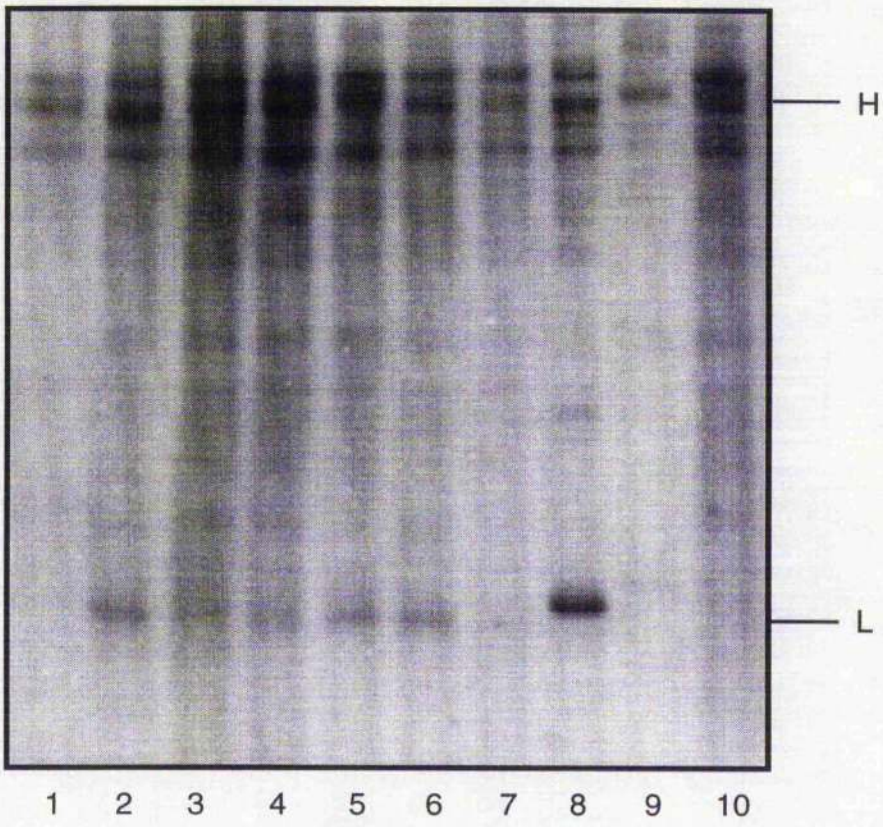

H
L
1
2
3
4
5
6
7
8
9
10

Figure 28. Measurement of the IgM heavy chain band intensity

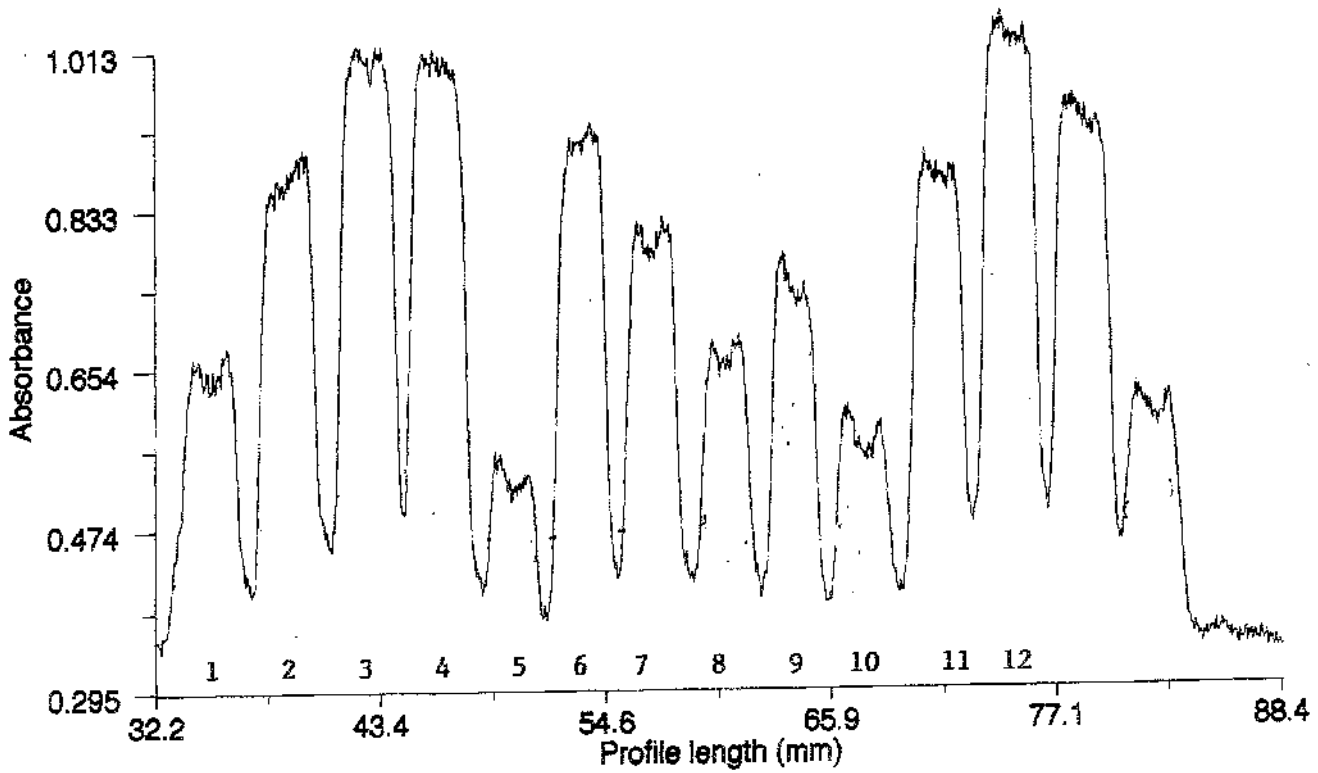
To compare the intensities of the IgM heavy chain polypeptide band from sera of fish immunized with MS2 + Protease and those from fish injected with MS2 + PBS an SDS-PAGE gel of the individual samples was scanned using a densitometer.

Lanes 1 - 5 : MS2 + Protease

Lanes 6 - 9 : MS2 + PBS

Lane 10 : Purified salmon IgM from naive serum

Lanes 11 - 12 : Naive salmon serum


1.013
0.833
0.654
0.474
0.295
Absorbance
1
2
3
4
5
6
7
8
9
10
11
12
32.2
43.4
54.6
65.9
77.1
88.4
Profile length (mm)

3.4 Effect of serine protease on lymphocyte proliferation

As the serine protease appeared to cause a true immunosuppressive effect in Atlantic salmon, attempts were made to determine which stage of the immune response was being targetted.

Following uptake and processing of antigen by antigen-presenting cells, epitopes are presented to B lymphoctes in the presence of T helper cells, inducing differentiation of the B cells to form a clone of antibody producing immunoblasts. *In vitro* culture techniques are important for functional studies of leucocytes. In mammals, lymphocyte activation by specific B [lipopolysaccharide, (LPS)] and T [phytohaemagglutinin (PHA), Concanavalin A (ConA)] cell mitogens are widely used for studying general immunocompetence (Urbaniak *et al.*, 1986).

3.4.1 - Standardisation of lymphocyte proliferation assay

To further investigate immunosuppression by the protease, *in vitro* studies were carried out using the mammalian lymphocyte proliferation system as a model. Initially, it was necessary to adapt the system and standardize experimental conditions for fish leucocytes. Therefore, several factors were evaluated.

3.4.1.1 - Determination of the time required for cell stimulation

Leucocyte suspensions (10^6/100µl L15 medium) were incubated with an equal volume of mitogen, *Salmonella typhimurium* LPS (100µg/ml) at 20°C. At days 0, 1, 2, 3 and 4 the cells were labelled as in Materials and Methods. As this method measures cell proliferation on the basis of incorporation of radioactivity into DNA of cells during DNA synthesis (S-phase) the sequence of cell cycle events in relation to time was investigated by flow cytometry as described by Vindelov *et al.*, (1983). Labelled DNA was stained with propidium iodide, a selective dye which intercalates quantitatively into double-stranded nucleic acid and stained nuclei were detected by a Fluorescence Activated Cell Sorter (FACS). Over 80% of cells reached S phase after four days incubation (Table 5) and this was used as the incubation time for cell stimulation by mitogens.

Table 5. Cell cycle activity of salmon leucocytes stimulated by *S. typhimurium* LPS

Time (days)	% Cell cycle statistics		
	G_1 phase	S phase	G_{2+M}
0	93	5	2
1	91	6	3
2	98	2	0
3	96	3	1
4	14	81	5

The sequence of stages referred to in the table are defined as :

G_1 phase - immediately after mitosis

S phase - period of DNA synthesis

G_2 phase - prior to mitosis and cell division

M - mitosis

3.4.1.2 - Quantitation of cell proliferation

In order to avoid the use of radioactive compounds, several attempts were made to substitute the commonly used radio-labelling method with the colorimetric MTT-formazan assay (Mossman, 1983) to quantify cell proliferation. However, this was abandoned due to inconsistencies in the solubilization of formazan crystals which was influenced by the protein concentration in the culture medium. Therefore, apart from a few early experiments with MTT-formazan, the amount of tritiated thymidine incorporated by cellular DNA was used to quantify lymphocyte proliferation.

3.4.1.3 - Incubation temperature

To determine the optimum temperature range for the proliferation assay four different temperatures; 10, 15, 20 and 37°C, were used with *S.typhimurium* LPS (100μg ml^{-1}) as the mitogen . Cells responded satisfactorily over this temperature range (Figure 29) and 20°C was chosen as the incubation temperature for subsequent assays.

3.4.1.4 - Analysis of fish-fish variation

In initial experiments lymphocyte preparations from individual fish were tested in proliferation assays. However, to be able to investigate a number of the variable factors in the assay more lymphocytes were required than could be obtained from individual fish. Also, there was a wide variation in the Stimulation Indices (SI) in experiments from week to week, for reasons which could not be identified. This was similar to the findings of other workers with the same fish stocks, preparation methods and mitogen (M. Barratt, Unilever Research, personal communication).

The variation in response of lymphocytes from individual fish and for a pool of lymphocytes from each individual was investigated. When *S. typhimurium* LPS (100μg/ml) was used as the mitogen there was a two-fold range in SI for 3 individual fish, and the SI for the pooled lymphocyte preparations was close to the range of these values (Figure 30). As there did not

Figure 29. Optimum temperature for proliferation of salmon lymphocytes

5×10^5 leucocytes purified from peripheral blood of three individual fish were stimulated with *S.typhimurium* LPS ($100\mu g\ ml^{-1}$). Proliferation was quantified by the MTT-formazan assay.

Background absorbance of unstimulated cells = 0.08

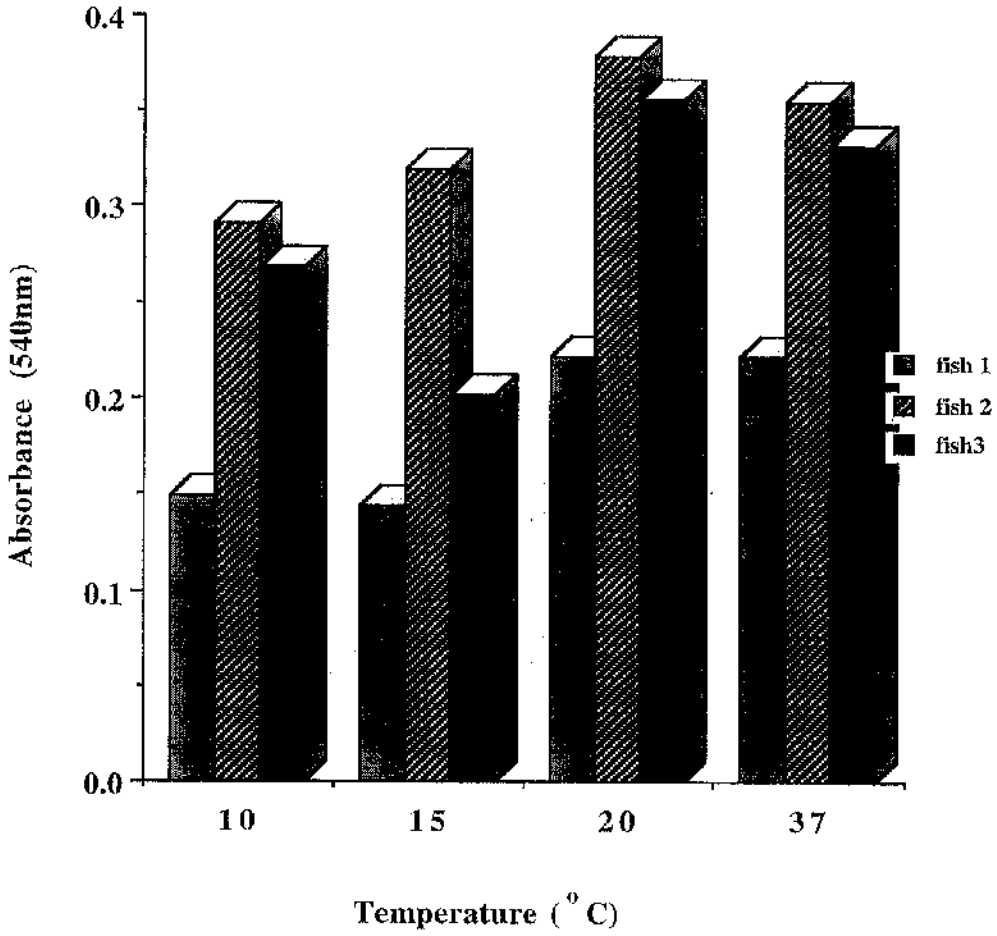

0.4
0.3
0.2
0.1
0.0
Absorbance (540nm)
10
15
20
37
Temperature (° C)
fish 1
fish 2
fish3

Figure 30. Effect on lymphocyte proliferation of pooling cells from individual fish

Mitogen used : *S. typhimurium* LPS (100 µg ml^{-1}).

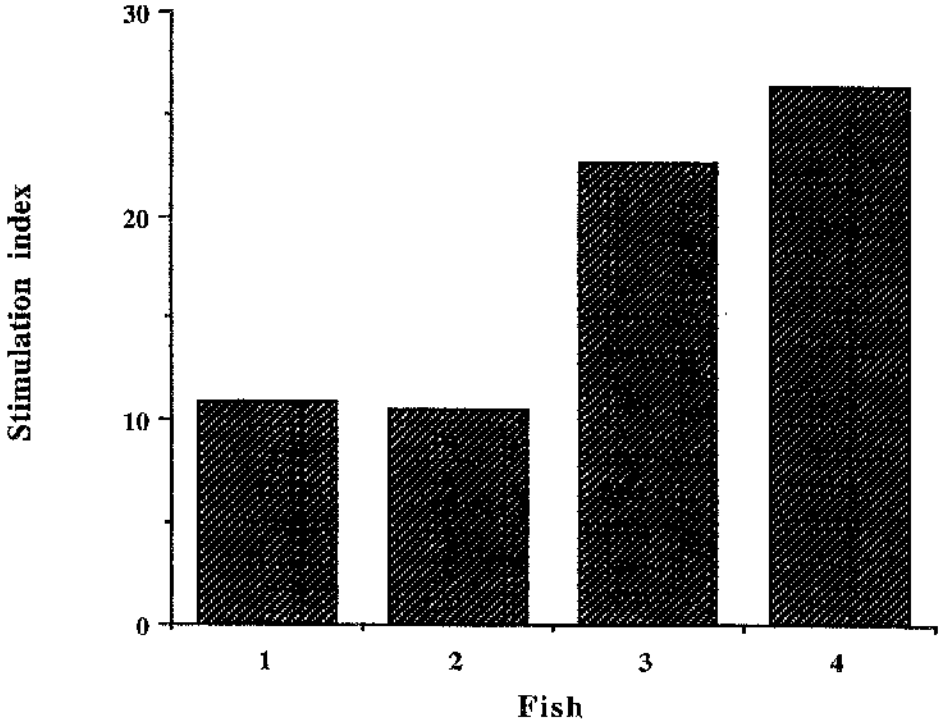
Proliferation was quantified by tritiated-thymidine incorporation.

Average count of unstimulated cells = 355 counts per minute.

Stimulation index was calculated using the following equation:

$$SI = \frac{\text{counts per minute of stimulated cells}}{\text{counts per minute of control cells}}$$

Fish 4 refers to a pooled fraction of lymphocytes from the three individual fish.


30
20
10
0
Stimulation index
1
2
3
4
Fish

seem to be any adverse reaction by pooling lymphocytes from different fish nor was there any evidence of activation due to a mixed lymphocyte reaction. Thus pooled lymphocyte preparations were normally used in further experiments.

3.4.1.5 - Stimulation with other mitogens

A number of mitogens were tested for their ability to stimulate salmonid lymphocytes; these included LPS of *A.salmonicida, S.typhimurium and Vibrio cholera,* Con A and an outer membrane protein (OMP) fraction of *A.salmonicida.* Relatively low stimulation of peripheral blood lymphocytes was achieved with Con A, even when tested over a wide concentration range (maximum SI ~ 5) (Figure 31). In contrast, LPS gave much higher proliferation responses, the greatest stimulation being induced by *V. cholerae* LPS. The stimulatory effect of *V. cholerae* LPS was dose dependent (Figure 32), and this mitogen was used in subsequent experiments. The OMP fraction (100µg/ml) of *A.salmonicida* was not mitogenic for salmon lymphocytes.

3.4.2 - Effect of serine protease on the activation of lymphocytes

The protease at dilutions > 1/32 (≤ 12.5 µg ml^{-1} trypsin equivalent) did not enhance or inhibit the proliferation response of salmon lymphocytes in the absence of added mitogen (Figure 33), thus it appeared that the protease was not inherently mitogenic.

However, when protease was added to lymphocytes at the same time as *V. cholerae* LPS the proliferative response was markedly reduced (Figures 34). Proliferation was reduced in a dose-dependent manner and 50% reduction occurred in the presence of a 1/200 dilution of the serine protease (2µg ml^{-1} trypsin equivalent) and the inhibitory effect was still detectable at a dilution of 1/2000.

To determine the timing of the effect of protease on lymphocytes, protease (6 µgml^{-1}) was added to lymphocytes at different times; 24h or 4h before (-24h and -4h), at the same time as (0h), or 4h after mitogen activation. Inhibition of LPS-induced lymphocyte response was significantly higher when protease was added 24hours before activation in comparison to inhibition of the response when both protease and mitogen were added simultaneously ($P < 0.05$) (Figure 35). The reduced response to LPS was not due to increased cell death as the

Figure 31. Dose-response of Concanavalin A stimulated leucocytes

Peripheral blood leucocytes (10^6 per 0.1ml) were stimulated with an equal volume of Con A dissolved in L15 medium for four days at 20°C. Proliferation was quantified by tritiated thymidine incorporation as described in Materials and Methods.

Background counts (unstimulated cells) = 250 counts per minute.

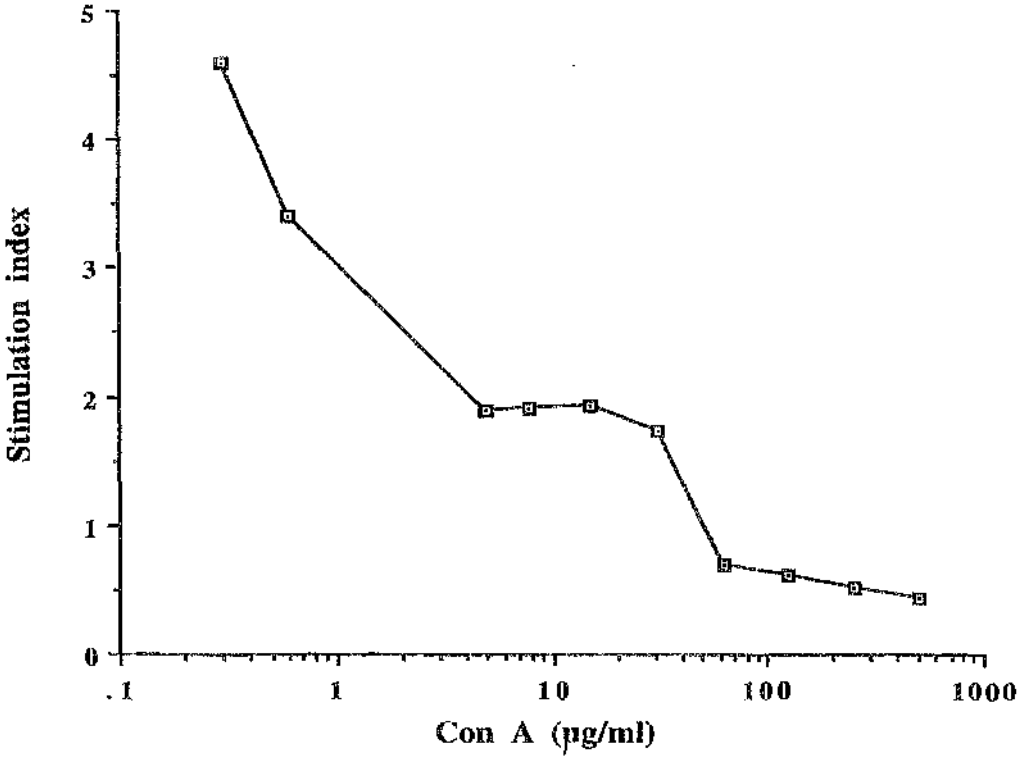

Stimulation index
5
4
3
2
1
0
.1
1
10
100
1000
Con A (µg/ml)

Figure 32. Dose-response of LPS-stimulated leucocytes

Peripheral blood leucocytes (10^6 per 0.1ml) were stimulated with an equal volume of *V. cholerae* LPS in L15 medium for four days at 20°C.

Background count of unstimulated cells = 292.

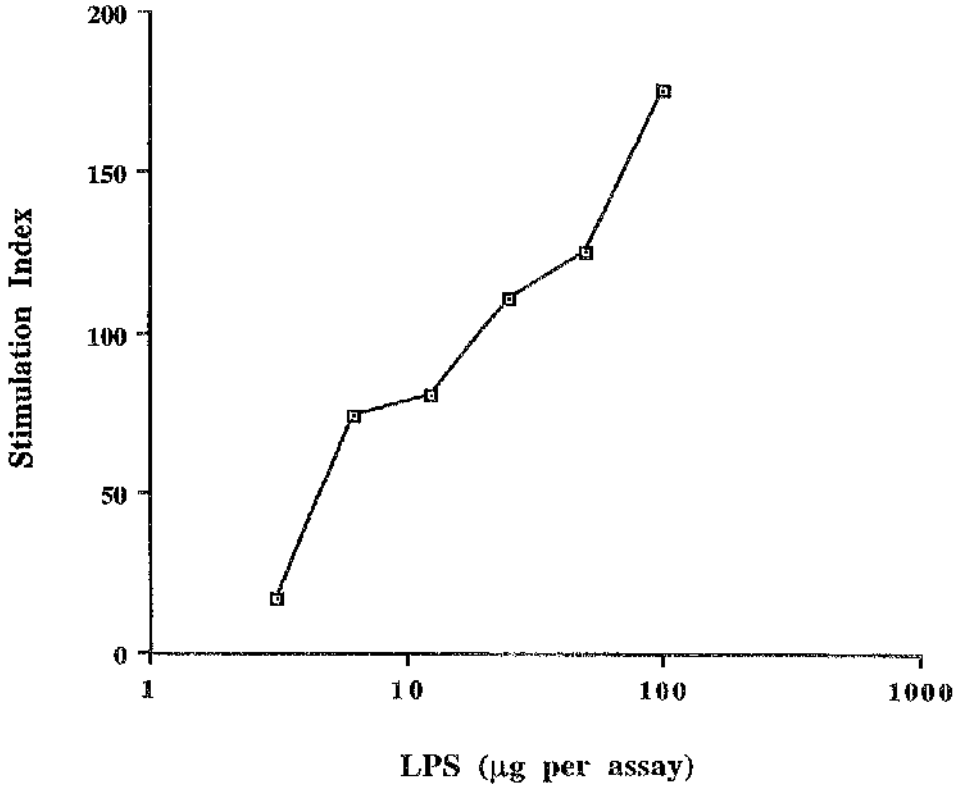

200
150
100
50
0
Stimulation Index
1
10
100
1000
LPS (μg per assay)

Figure 33. Effect of protease on unstimulated salmon leucocytes

Peripheral blood leucocytes (10^6 per 0.1ml) were added to an equal volume of various protease dilutions and incubated for four days at 20°C. Cells were subsequently labelled with tritiated thymidine overnight and counted.

Undiluted protease = 400 μg trypsin equivalent per ml.

Background counts of cells only = 335 counts per minute.

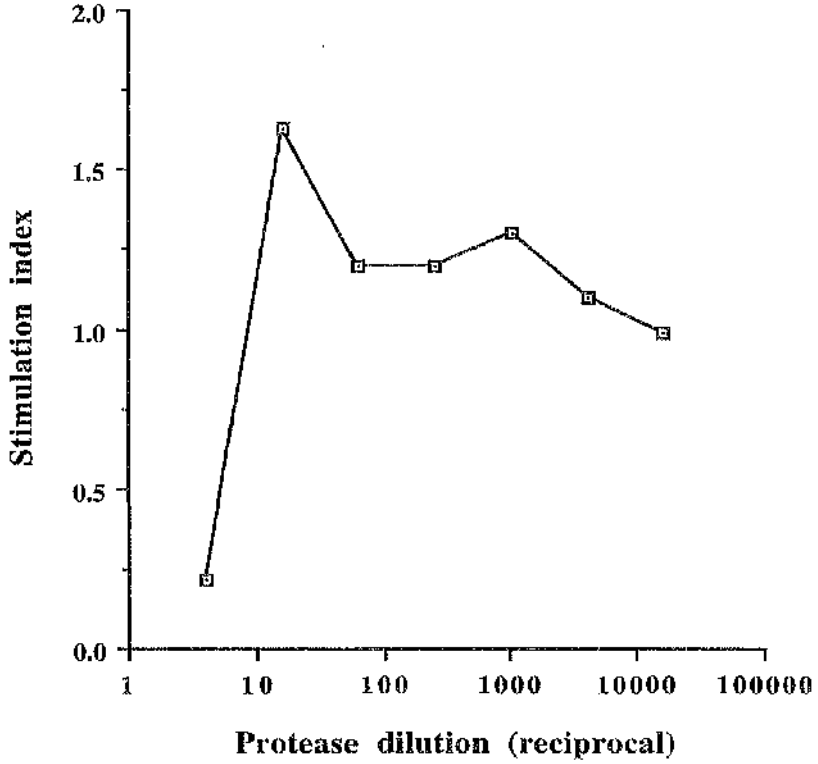

2.0
1.5
1.0
0.5
0.0
Stimulation index
1
10
100
1000
10000
100000
Protease dilution (reciprocal)

Figure 34. Inhibition by protease of LPS-induced lymphocyte proliferation

0.1ml of different dilutions of protease (stock = 400µg trypsin equivalent ml^{-1}) were added to 10^6 peripheral blood leucocytes in 0.1ml L15 medium four hours before, after and at the same time as 0.1ml of *V. cholerae* LPS (100µg ml^{-1}) and incubated for four days at 20°C. The control was LPS-stimulated leucocytes without the addition of protease.

Stimulation index of control = 38.6

Background counts of unstimulated cells = 381.

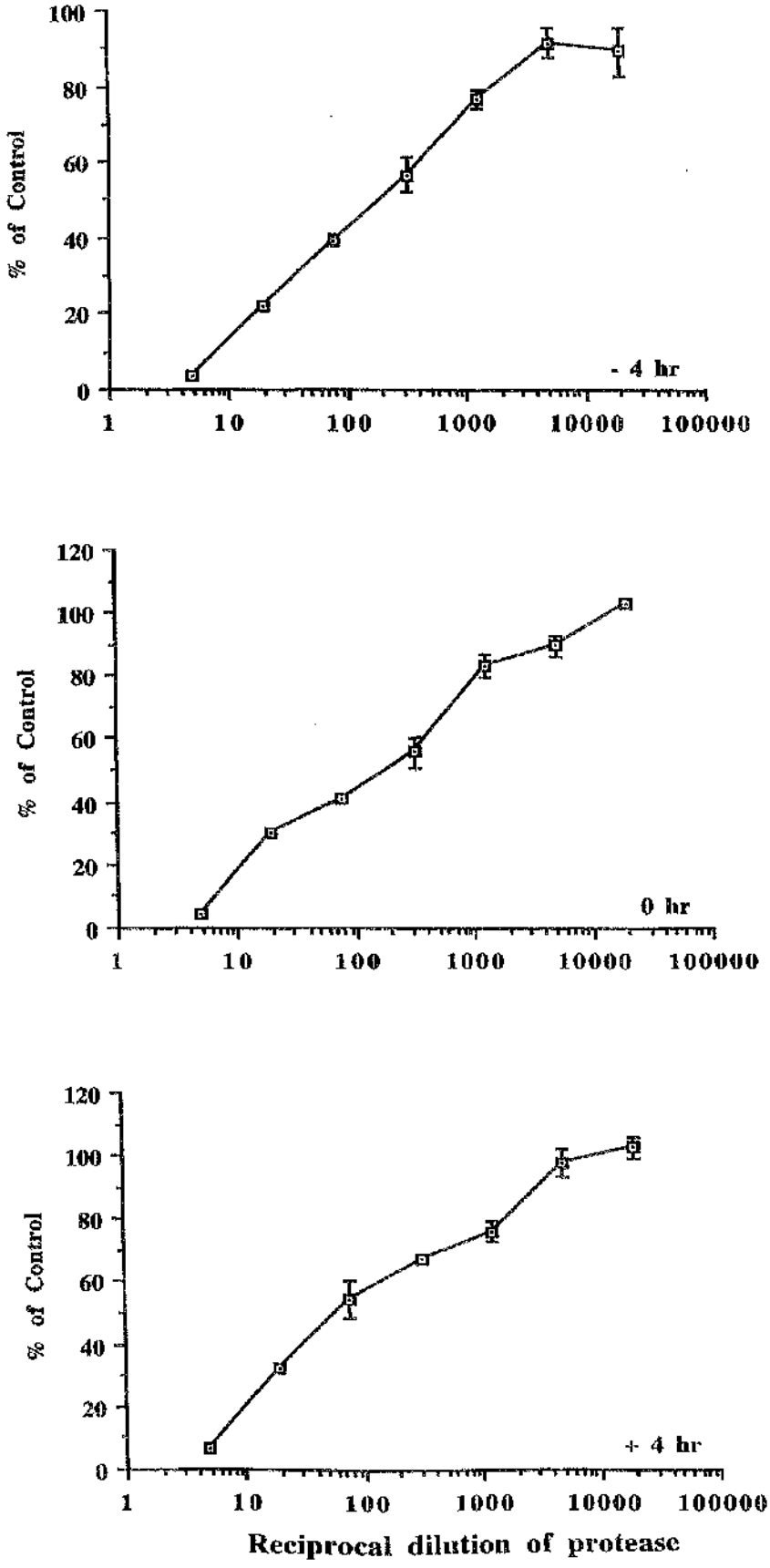

% of Control
100
80
60
40
20
0
- 4 hr
1
10
100
1000
10000
100000
% of Control
120
100
80
60
40
20
0
0 hr
1
10
100
1000
10000
100000
% of Control
120
100
80
60
40
20
0
+ 4 hr
1
10
100
1000
10000
100000
Reciprocal dilution of protease

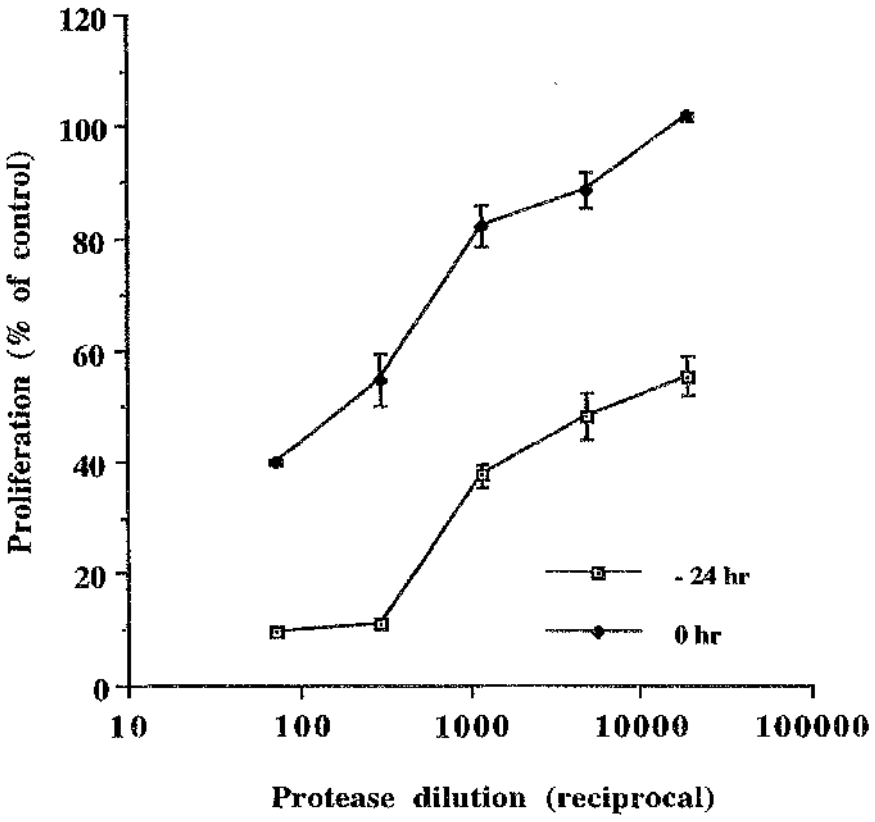
Figure 35. Inhibition by the protease of the early stages in lymphocyte activation

0.1ml of different dilutions of protease (stock = 400μg trypsin equivalent ml^{-1}) were added to 10^6 peripheral blood leucocytes in 0.1ml L15 medium twenty-four hours before and at the same time as 0.1ml of *V. cholerae* LPS (100μg ml^{-1}) and incubated for four days at 20°C.

The control was LPS-stimulated leucocytes without the addition of protease.

Stimulation index of control = 38.6

Background counts of unstimulated cells = 381.


120
100
80
60
40
20
0
Proliferation (% of control)
10
100
1000
10000
100000
Protease dilution (reciprocal)
- 24 hr
0 hr

protease, at sub-lethal levels, only exerted an inhibitory effect on proliferating cells and did not affect cell viability as no change in leucocyte morphology was observed in the presence of protease (photographs not shown). Although there appeared to be slight differences in the proliferative responses obtained when protease was added 4h before, at the same as mitogen or 4h later,these differences were not statistically significant (Student t-test, $P > 0.1$). Thus, it was tentatively assumed that the protease may be blocking the early stages of lymphocyte commitment to blastogenesis.

3.5 Immunomodulation by prostaglandins

The results obtained so far indicated that the protease interferes with the humoral response of Atlantic salmon directly or indirectly by its effect on leukocytes. Cells of the immune system are subject to control by interleukins, and also respond to leukotrienes and prostaglandins (see Introduction), which generally act to enhance or suppress immune responses, respectively. To determine whether serine protease might act via an effect on prostaglandins, two experimental approaches were used, firstly, by blocking the effect of prostaglandins *in vivo* and, secondly, by direct measurement of prostaglandin levels in fish serum.

3.5.1 - Inhibition of protease immunosuppressive activity by indomethacin

To determine whether indomethacin, an inhibitor of prostaglandin synthesis, blocks the effect of the protease 160 fish were divided into four groups of 40 fish and injected with; MS2+PBS, MS2+protease, MS2+indomethacin and MS2+protease+indomethacin as shown below:

Group 1	MS2 + PBS (control)	Day 0 : 0.1ml MS2 (10^9 pfu) + 0.1ml PBS
Group 2	MS2 + protease	Day 0 : 0.1ml MS2 (10^9 pfu) + 0.1ml protease (1µg per gram fish body weight)
Group 3	MS2 + PBS	Day 0 : as for Group 1
Group 4	MS2 + protease	Day 0 : as for Group 2

Table 6 (a). Blocking of the inhibition of Ab response by indomethacin

	Week 5			
	Time (minutes) required to neutralize 50% of MS2 ($t_{1/2}$)			
Fish number	Group 1	Group 2	Group 3	Group 4
	MS2+PBS	MS2+P	MS2+IDM	MS2+P+IDM
1	22	100	100	58
2	50	100	100	30
3	100	100	68	63
4	22	100	100	100
5	100	100	100	100
6	57	100	100	100
7	38	100	100	35
8	100	100	45	40
9	100	100	94	100
10	100	100	67	100
number of responders with $t_{1/2} < 100$ min	5/10	0/10	4/10	5/10

P : Protease

IDM : Indomethacin

The results presented in Tables 6 (a-c) were obtained using the kinetic phage plaque assay (see Figure 9).

Table 6 (b).

	Week 6			
	Time (minutes) required to neutralize 50% of MS2 ($t_{1/2}$)			
Fish number	Group 1 MS2+PBS	Group 2 MS2+P	Group 3 MS2+IDM	Group 4 MS2+P+IDM
1	28	100	100	86
2	100	100	58	28
3	52	100	83	45
4	100	100	74	40
5	100	100	32	45
6	47	100	50	34
7	9	100	70	33
8	26	100	60	33
9	30	100	100	30
10	100	18	-	54
number of responders with $t_{1/2} < 100$	6/10	1/10	8/9	10/10

P : Protease

IDM : Indomethaciu

Table 6 (c)

Sampling time	Neutralisation response index			
	Group 1	Group 2	Group 3	Group 4
week 5	30	0	13	38
week 6	41	8	30	57

The response index was calculated by taking the average of the neutralisation times of each group and this value was deducted from 100 (as this will give zero if all were negative).

Indomethacin was injected (ip) as 1μg per gram fish body weight in 15% gelatin solution in PBS on days 1, 4, 8, 11, 15 and 18 to groups 3 and 4. At the same time 0.1ml gelatin solution was administered to groups 1 and 2 (based on the method described by Laudan *et al.*, 1986). Serum samples collected from 10 fish per group on weeks 3, 4, 5 and 6 were analysed by the kinetic assay to determine antibody titres to MS2 (Table 6(a-c)). Those sera which gave a neutralisation time of > 100min (value obtained by extrapolation) were considered to contain little or no neutralising antibodies and for further analysis of the data these values were considered as 100. Thus, the data was analysed in two ways, by considering the number of fish per group which responded to MS2 with a neutralisation time of < 100 min and, secondly, by comparison of the mean neutralisation times.

The serine protease-treated group showed the expected inhibition of the humoral response (Group 2) with 0/10 fish responding at week 5 (Table 6a) and only 1/10 at week 6 (Table 6b). Indomethacin had no significant effect on the humoral response of fish to MS2 (Group 3) but when administered with serine protease and MS2 (Group 4), indomethacin blocked the inhibitory effect of the protease (Tables 6a & 6b), with a significant increase in the proportion of fish with neutralising antibody to MS2 at week 5 and week 6 post immunization and in the mean neutralisation titres (Table 6c). (ANOVA, $P < 0.01$). Therefore, it appeared possible that the serine protease exerted its inhibitory effect through prostaglandins.

3.5.2 - *In vivo* stimulation of PGE_2 by protease

To determine whether prostaglandin concentrations in serum were elevated following administation of protease, two groups of fish were injected with MS2+protease or MS2+PBS (as in Section 2.6.1). Serum samples were collected over seven days and PGE_2 production was measured using a radioimmunoassay kit (as in Section 2.13). Although the concentration of PGE_2 in many of the serum samples was greater than the measurable range of the standard curve, a significantly higher proportion of fish injected with protease demonstrated values >250pg ml^{-1} PGE_2 than those fish which were not injected with

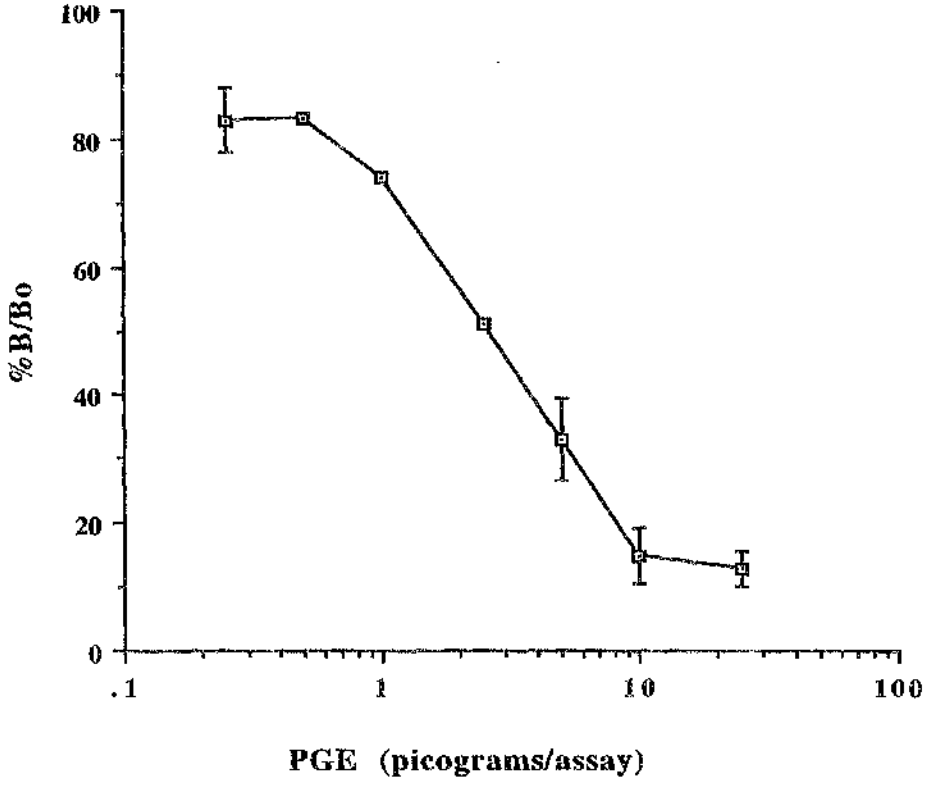
Table 7. Induction of PGE_2 by protease

Time (days)	Proportion of samples with $PGE_2 > 250$ pgml^{-1}	
	Group 1 MS2+PBS	Group 2 MS2+protease
10 hours	3/7	2/9
1	4/7	8/8
2	2/10	6/8
4	2/9	8/10
7	5/9	7/9

Chi-square analysis on the total number of fish in each group over the 7-day sampling period: $P < 1\%$, 1 d.f.,

Figure 36. Standard curve of PGE_2 assay kit

PGE_2 [^{125}I] radioimmunoassay kit (NEN Research Products) was used.


100
80
60
40
20
0
%B/Bo
.1
1
10
100
PGE (picograms/assay)

protease (Table 7) (Chi-square analysis, $P < 0.01$, 1 d.f.).

3.5.3 - Quantification of PGE_2 induced *in vivo*

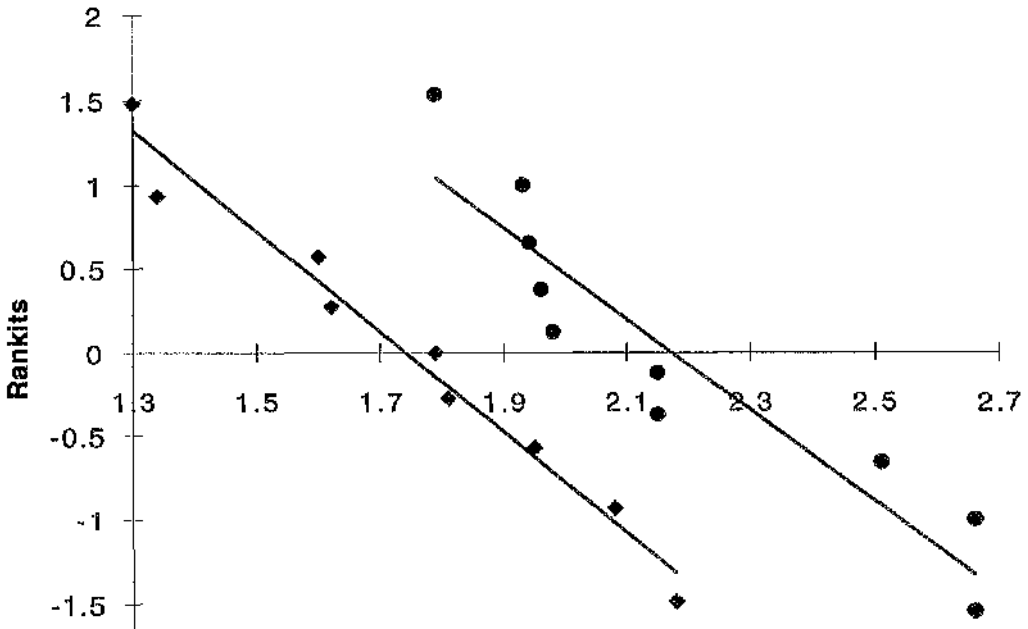
In further experiments, serum samples were diluted 1/10 and 1/100 so that they would fall within the measurable range of the standard curve (Figure 36) and permit more accurate measurement of PGE_2 concentration. Two groups of 20 fish were injected (as in Section 2.6.1) with MS2 + PBS (Group 1) and MS2 + protease (Group 2), and 10 fish from each group were sacrificed at 24 and at 48 hours post immunization. The basal level of PGE_2 measured in uninjected fish was 150 ± 2.9 picogram ml^{-1} of serum but in fish injected with MS2+PBS, concentrations were significantly higher at both 24 and 48 hours (Student t-test, $P < 0.01$) probably resulting from handling of the fish. Fish treated with serine protease + MS2 produced even higher amounts of serum PGE_2 than those fish given MS2 alone. The data was log-normally distributed, as shown in the Rankit plots in Figure 37. At both sampling times (24 and 48 hours) the elevation of PGE_2 concentration was statistically significant (Student t-test, $P < 0.05$), with a 2.4-fold increase in PGE_2 concentration at 24 hours and a 2.9-fold increase at 48 hours.

3.5.4 - Source of PGE_2

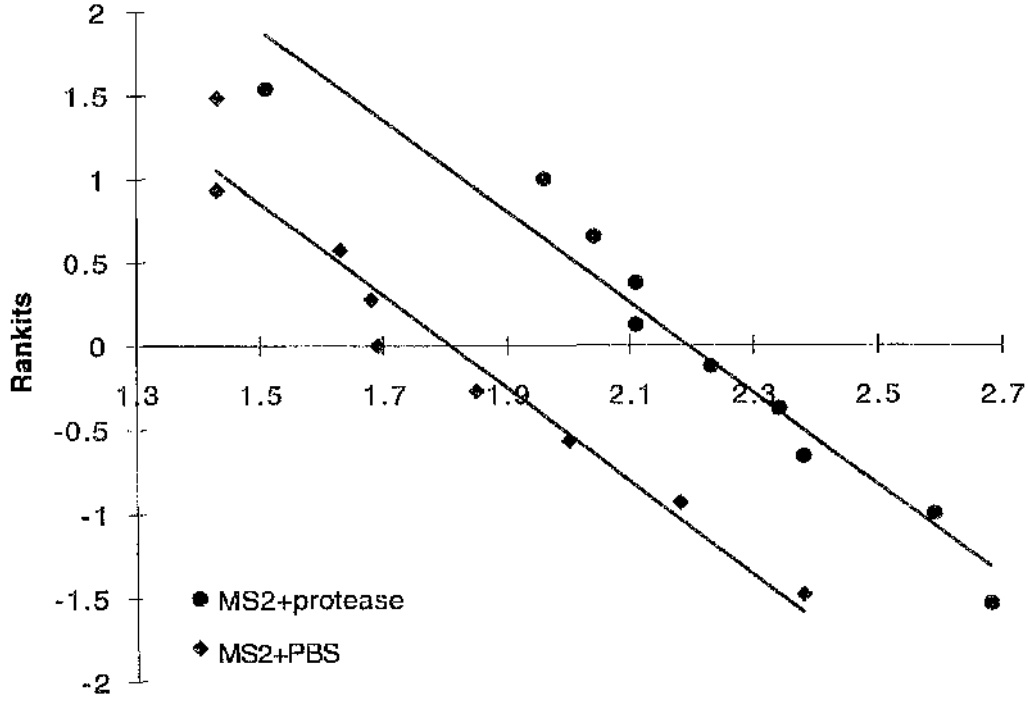
In humans, macrophages and monocytes are the main source of PGE_2 production (reviewed by Parker, 1986), therefore, the effect of serine protease on PGE_2 synthesis by salmon kidney leucocytes was tested in an *in vitro* system. Macrophage-enriched cultures were prepared by allowing cells to adhere to the plastic surface of 25 cm^3 tissue culture flasks (Rowley, 1991). Non-adherent cells were removed by washing with L-15 medium. Adhered cells were incubated with protease (6µg ml^{-1} trypsin equivalent) for 1, 4 and 24 hours at 20°C. After each incubation time samples were quickly frozen in liquid nitrogen and stored at -70°C until further analysis. PGE_2 concentration was measured using a radioimmunoassay. As shown in Figure 38 the protease induced a significant rise in PGE_2 concentration after 24h (Student t-test, $P = 0.01$) compared to untreated cells. Although not

Figure 37. Enhancement of PGE_2 release by protease

Forty fish were divided into two groups which were injected with either MS2+PBS or MS2+Protease (as described in Section 2.6.1). Serum samples were collected from 10 fish per group at 24 and 48 hours post-immunization. PGE_2 production was measured by a radioimmunoassay. Data is expressed as rankits to illustrate log normal distribution.



24 hours

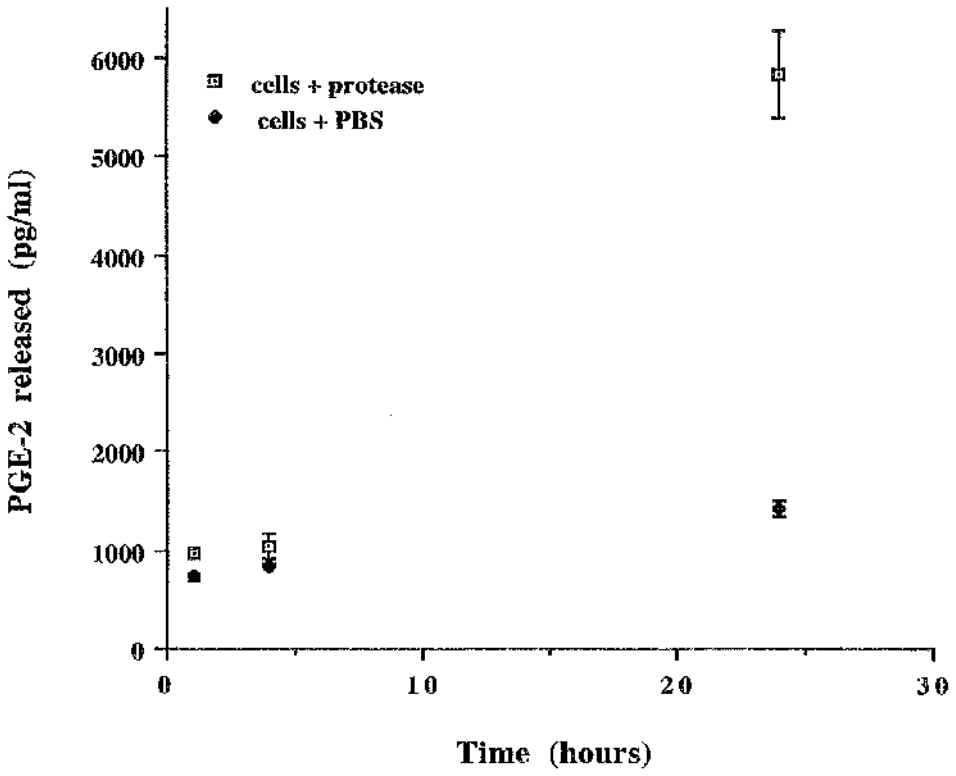


48 hours

Figure 38. *In vitro* induction of PGE_2 in macrophage-enriched cultures

A layer of macrophages from a culture of anterior kidney leucocytes ($2x10^6$ cells) was formed by allowing them to adhere to the surface of a plastic flask. After treatment of cells with protease (6 µg trypsin equivalent per ml) for 1, 4, and 24 hours PGE_2 levels were measured by radioimmunoassay.

Each point represents the mean ± SEM of 3 individual observations.


cells + protease
cells + PBS
PGE-2 released (pg/ml)
6000
5000
4000
3000
2000
1000
0
0
10
20
30
Time (hours)

statistically significant, PGE_2 concentrations induced by the protease at the earlier sampling times (1h and 4h) were both higher than the control values.

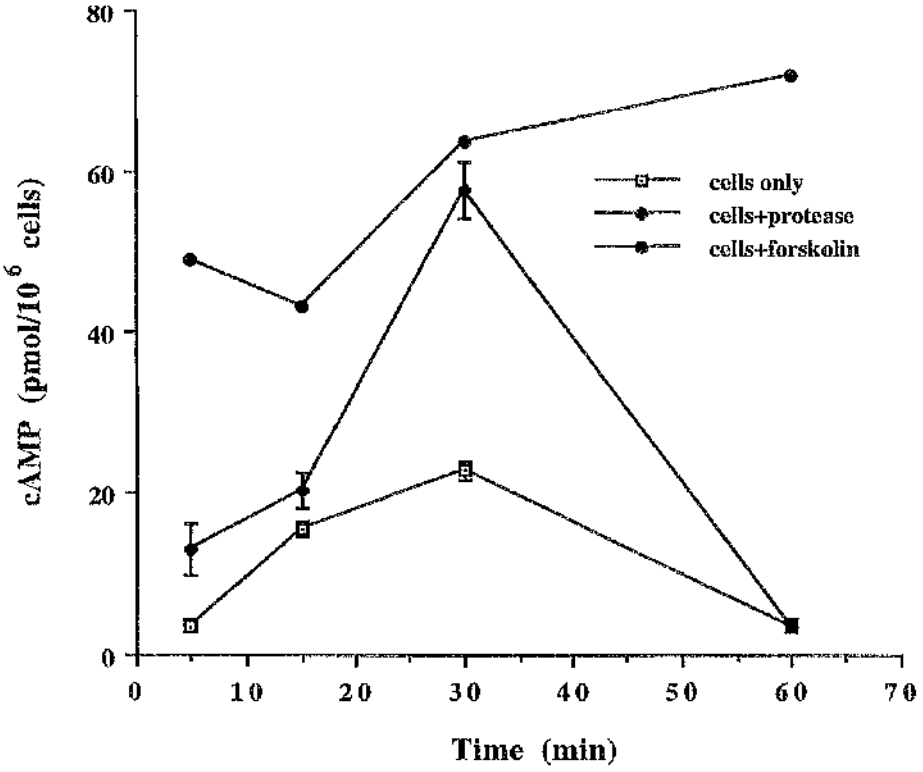
3.5.5 - Elevation of intracellular cyclic AMP

The possible role of intracellular cyclic AMP in the regulation of PGE_2 synthesis was investigated by analyzing protease-stimulated leucocytes for intracellular cyclic AMP concentrations. A suspension of 10^6 leucocytes from kidneys was prepared in serum-free L-15 medium and incubated with protease (6µg ml^{-1} trypsin equivalent) at 20°C. After incubation for 5, 15, 30 and 60 minutes intracellular cyclic AMP of leucocytes was measured by a receptor protein binding displacement assay. Forskolin, a non-specific activator of adenylate cyclase, was used at 10^{-5} M as a positive control. Cells incubated without the addition of protease were used as the negative control. In order to inhibit degradation of cyclic AMP by the action of phosphodiesterases, 1mM 3-isobutyl-1-methylxanthine (IBMX) dissolved in dimethyl sulphoxide (DMSO) was incorporated in the incubation mixtures.
As illustrated in Figure 39 the protease induced a highly significant increase in the amount of intracellular cyclic AMP released at 30 minutes after stimulation (ANOVA, $P = 0.001$).

Figure 39. Elevation of intracellular cyclic AMP

Intracellular cyclic AMP concentration in leucocytes (10^6 per assay) stimulated with protease (6µg ml^{-1}) at 20° C was analysed by a competitive binding assay. Forskolin at a concentration of 10^{-5} M was used as a positive control.

Each point represents the mean ± SEM of 3 observations.


80
60
40
20
0
cAMP (pmol/10^6 cells)
0
10
20
30
40
50
60
70
Time (min)
cells only
cells+protease
cells+forskolin

DISCUSSION

4.1 Properties of the serine protease

The major role of the serine protease is widely believed to be in providing a source of free amino acids for survival and growth (Sakai, 1985*b*). However, several other functions have been reported for the enzyme, such as enhancing the toxic activity of GCAT (Lee & Ellis, 1989) in Atlantic salmon. It is clear from the work of Fyfe *et al.* (1986; 1988) that the pathogenesis of furuncle formation requires the combined effects of the protease and GCAT/LPS; this observation was confirmed by Lee & Ellis (1991) who demonstrated that GCAT/LPS alone produced coagulative necrosis of muscle fibres but with little haemorrhaging, whereas a mixture of protease and GCAT/LPS produced an extensive lesion that was liquefactive and haemorrhagic, typical of that induced by ECP. This is paralleled by the *in vitro* haemolytic effect of GCAT/LPS on salmonid red blood cells where the presence of protease is required for complete solubilization of erythrocyte membranes (Lee & Ellis, 1990).

A further characteristic of the protease is its ability to reduce markedly the clotting time of trout blood (Price *et al.*, 1990). It was shown that both the GCAT/LPS and protease were involved in thrombus formation by entering the coagulation cascade at two different levels (Salte *et al.*, 1991; 1992), the significance being that circulatory failure is probably the major cause of death in acute furunculosis. Although Ellis (1987) reported that the serine protease was only inhibited by α_2-macroglobulin, Salte *et al.* (1992) noted that the protease is also inhibited by antithrombin which is apparently a major serine protease inhibitor in man as well as in the lower vertebrates (Carrell *et al.*, 1987). Salte *et al.* (1992) also proposed that administration of such plasma inhibitors of protease would be a means to prevent furunculosis independently of vaccines.

4.2 Evidence of immunosuppression by protease

The initial work of this study confirmed that the culture supernatant of *A. salmonicida* suppressed the humoral immune response in Atlantic salmon. Suppression of the humoral response was measured using the method described by O'Neill (1979) who

showed that *A. salmonicida* suppressed the antibody response to the bacteriophage MS2 and increased the clearance time of the antigen; it was further concluded that suppression was related to the initial phases of the primary and secondary responses.

On initial separation of culture supernatant proteins by isoelectric focusing, immunosuppressive activity was found to be associated with fractions with an isoelectric point (pI) in the range 5.7 to 6.5.

As pooled fractions in this pI range contained a haemagglutinin (HA) specific for salmon red blood cells, and which failed to react with sheep or horse erythrocytes, the HA appeared to be a candidate for the immunosuppresive factor. This was initially considered to be via a lectin-like activity, as lectins are carbohydrate-binding proteins that agglutinate cells or precipitate glycoconjugates through interaction with glycoproteins or glycolipids (Boyd and Shapleigh, 1954), and the modulating effect of lectins, e.g. concanavalin A, on the immune system are well recognised. Also, as the agglutinating activity was lost after heating at 56° C for 15 minutes the HA was presumed to be a protein. Agglutination was not observed with glutaraldehyde-fixed salmon rbc indicating that the HA was probably binding to protein receptors on the surface of rbc.

The inhibitory effect of *A. salmonicida* ECP on the salmonid humoral response to MS2 continued for thirteen weeks after the initial injection even though the fish were boosted with a preparation of MS2 phage and PBS at week 8. The failure of the fish to recover after the booster injection and exhibit an increase in the antibody response to MS2 was an indication that the suppression was a result of a block in the early stages of the induction of the humoral response.

Generally, in any of the *in vivo* experiments undertaken five or more fish were used per group in order to take into account the problem of non-responding fish. The occurrence of non-responding fish has been noted previously by Warr & Simon (1983) and Tatner (1990) but, as yet, this phenomenon is not clearly understood.

Enhanced antibody titres have been reported during the secondary response in various fish by several authors (Miller & Clem, 1984*a* ; Avtalion, 1969; Trump &

Hildemann, 1970; Tatner, 1986; Arkoosh & Kaattari, 1991). However, in this study, the antibody titres during the secondary response did not rise above the level observed in the primary response (see Figure 13). The likeliest minor explanation for this is may be due to a drop in temperature resulting in a lowered immune response in the fish (Miller & Clem, 1984*b*). Another possible reason could have been due to the fish becoming increasingly stressed by the handling during booster injections as well as being in the confines of the containment tank for over 8 weeks being subjected weekly to the threat of being captured as the weekly proportion of fish were taken out of the tank. It has been demonstrated that stress can affect the immune response in fish (Anderson *et al.*, 1982; Stave and Roberson, 1985). However a recent report by Espelid *et al.* (1996) concluded that although a short-term negative effect of exogenous cortisol (used to simulate stress) on immune cells *in vitro* and *in vivo* occurred, there was no evidence that repeated handling stress induced suppression of the immune functions vital to antibody production and immunological protection.

In the purification of the haemolytic and haemagglutinating activities by gel filtration the haemolytic peak resolved at an apparent MW of >200kDa whereas on SDS-PAGE the haemolysin produced a band of MW of approximately 25kDa. This was consistent with the properties of GCAT being a polypeptide of 25kDa which associates in solution with LPS to form complexes of >200kDa (Lee and Ellis, 1989).

Partially-purified GCAT, when administered to Atlantic salmon, induced a slight enhancement of the humoral response to a mean level which was higher than the response of the control group. It should be noted that the response of the control group was unusually low, thus the response induced by GCAT may not in fact be an enhancement in real terms. However, low concentrations of membrane-damaging toxins have been reported to stimulate the activity of phagocytic cells (Gemmell *et al.*, 1982). The GCAT readily associates with LPS (Lee & Ellis, 1990) and it is possible that the enhanced immune response to MS2 in the presence of GCAT may have been due to LPS in the GCAT preparation (Lee & Ellis, 1990). When both GCAT and serine protease were present together in the culture supernatant the

slight stimulatory effect seen with the GCAT preparation may have been totally overwhelmed by the suppressive effect of protease.

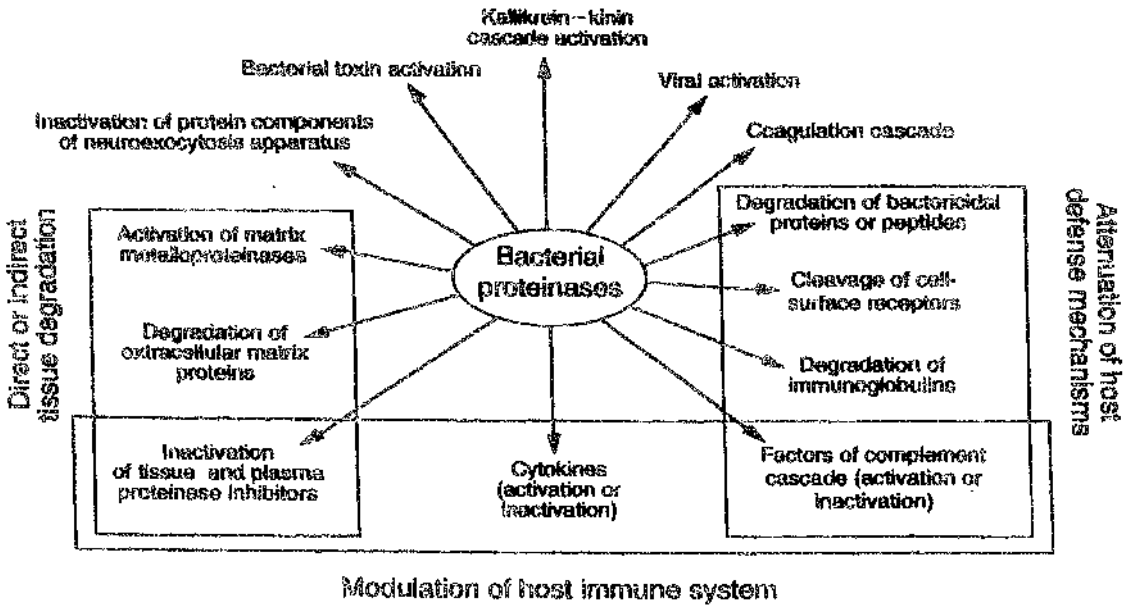
Properties of the humoral immunosuppressive factor; strong caseinase activity (600 μg ml^{-1} trypsin equivalent), MW of 66kDa on SDS-PAGE and LD_{50} of approximately 2.5 μg protein per gram fish weight were identical to those of the 64kDa serine protease of *A. salmonicida*. Earlier work (Evenberg *et al.*, 1986) reported suppression of the immune system of carp by culture supernatants of atypical strains of *A. salmonicida*, but the factor responsible was not identified. The authors also noted that fish treated with *A. salmonicida* culture supernatant were susceptible to secondary bacterial infections, illustrating further evidence for the ability of this organism to interfere with normal anti-bacterial defences.

The pathogen responsible for bacterial kidney disease in salmonids, *Renibacterium salmoninarum* , was reported to suppress *in vitro* antibody production by lymphocytes (Turaga *et al.*, 1987) via a surface haemagglutinin (MW 57kDa). Although the mechanism of suppression was not established, subtilisin-like activity has been ascribed to both the *R. salmoninarum* hemagglutinin (Griffiths & Lynch, 1991) and to the serine protease of *A. salmonicida* (Coleman & Whitby, 1993). Thus, it is possible that both these pathogens may cause suppression by a similar mechanism. The various routes by which bacterial proteinases may be involved in the pathogenesis of infectious diseases (Figure 40) has recently been reviewed by Travis *et al.*, (1995).

Furunculosis occurs in latent, acute or chronic forms and the mechanisms underlying these different disease states is not known. Protease is produced in furuncles (Ellis *et al.*, 1981) and reproduces some of the major symptoms of furunculosis when injected intramuscularly into fish (Ellis, 1991); however, it is not known whether switching of gene expression (Simon *et al.*, 1980) can occur *in vivo* to produce different disease states. Latent carriage of *A. salmonicida* by fish is an important means of maintenance of disease within a population and it is possible that protease is involved in maintaining this state. One possible candidate for the site of *in vivo* carriage of the organism are macrophages which fail to kill the ingested pathogen.

Figure 40. Multiple functions of bacterial proteinases

(Source: Travis *et al.*, 1995)


Kallikrein–kinin cascade activation
Bacterial toxin activation
Viral activation
Inactivation of protein components of neuroexocytosis apparatus
Coagulation cascade
Direct or indirect tissue degradation
Activation of matrix metalloproteinases
Degradation of extracellular matrix proteins
Bacterial proteinases
Degradation of bactericidal proteins or peptides
Cleavage of cell-surface receptors
Degradation of immunoglobulins
Attenuation of host defense mechanisms
Inactivation of tissue and plasma proteinase inhibitors
Cytokines (activation or inactivation)
Factors of complement cascade (activation or inactivation)
Modulation of host immune system

4.3 Possible mode of action of the serine protease

The ways in which the protease could exert its immunosuppressive activity were explored and amongst the more obvious ways in which this could occur were via its proteolytic activity in destruction of surface epitopes of MS2 phage or by degradation of serum immunoglobulin. However, MS2 phage was extremely resistant to degradation by protease and showed no significant loss of infectivity both *in vivo* and *in vitro*. That, little or no degradation of MS2 occurred *in vivo* was evident from the slow clearance of injected phage by the fish with high titres of viable phage were recovered from the serum of fish at least 1 week after injection. At this time there was no difference between control and protease-treated fish in the frequency of finding viable phage in serum or in its titre. Thus, under experimental conditions *in vivo* the phage did not lose its activity. Similarly, phage which had been treated overnight with the protease lost very little infectivity and the virus was still neutralised by salmon anti-MS2 antisera to the same extent as control phage; therefore, the epitopes of MS2 which induce neutralising antibodies appeared to be unaffected. It seems that the phage epitopes, at least in their native configuration in the virion, were protected from the action of the protease either by the primary, secondary or tertiary structure of the molecule.

Salmon immunoglobulin represents a second possible target for the protease and it was clear that heavy chains of salmon immunoglobulin were degraded by the protease after overnight incubation but the light chains remained unaffected (Figure 26). Such degradation is characteristic of the action of many proteases on the Fc region of mammalian immunoglobulins (Porter, 1959). However, despite the sensitivity of immunoglobulin to protease, there was no evidence of degradation *in vivo* of immunoglobulin in sera from fish immunized with MS2+protease. In addition, there was no significant difference in the concentration of circulating immunoglobulin between fish immunized with MS2+protease and those with MS2+PBS.

In salmonids, the major host defence against proteases is α_2-macroglobulin (Ellis, 1987) and it is probable that the bulk of the protease was neutralised by α_2-macroglobulin, leaving insufficient active protease to cause significant degradation of serum proteins.

The cell mediated immune response to MS2 was not affected by the protease (C. Mackie-personal communication) indicating that the process of antigen presentation was not altered even though different epitopes would be involved.

4.4 Effect of protease on lymphocyte blastogenesis

The lymphocyte proliferation assay was used as a model to study the *in vitro* effects of protease on the humoral response. Leucocytes from blood and kidneys were stimulated with *Vibrio cholerae* LPS and it was demonstrated that the protease inhibited this mitogenic proliferative response. Attempts to stimulate salmon lymphocytes by Concanavalin A resulted in much lower levels of proliferation (maximum stimulation index = 4.6) than that induced by LPS. In contrast, Warr & Simon, (1983) found that there was a clear response to both Con A and LPS by thymus, spleen, anterior kidney and peripheral blood lymphocytes of rainbow trout.

Due to the limitations in the yield of cells from any one fish, cultures of leucocytes from up to five fish were pooled for the proliferation assay experiments. In doing so the response of any non-responding fish was counter-balanced by the leucocytes from other responding fish. On the other hand no evidence of cell activation was observed as a result of mixed lymphocyte reactions.

There was a wide variation in the stimulation indices in experiments from week to week. This was also observed by other workers with the same fish stocks and lymphocyte preparation methods (M. Barratt, Unilever Research, personal communication) as well as other authors working on rainbow trout (Secombes *et al.*, 1994). One of the contributing factors may be the temperature that the blood or kidneys were subjected to during transit from Marine Harvest Fish Farms, Invernesshire. Data have shown that immune responses of teleosts are influenced by environmental temperatures (reviewed by Bly & Clem, 1992). It is

of economic importance that lower environmental temperatures within the physiological range of the particular species tend to inhibit immune responses in ectotherms (reviewed by Avtalion, 1981). The immunologically 'non-permissive' temperature for salmonids was established as ~4° C (Fryer *et al.*, 1976). Initial studies of Clem *et al.*, (1984) indicated that *in vitro* catfish T cell proliferation to Con A was suppressed at the low 'non-permissive' temperature (17° C) while B cell responses to LPS were not affected. These authors also determined that the block in low temperature-induced immunosuppression in catfish occurred relatively early in cell activation and involved the specific inhibition of virgin T helper cell generation/activation rather than T suppressor cells or suppressor factors, or the induction of tolerance.

The concept of homeoviscous adaptation, a process whereby ectotherms can compensate for the rigidifying effects of lower temperatures by changing their cellular membrane composition to result in more optimal membrane viscosities and hence function at such low temperatures was observed by Hazel (1984). Bly & Clem (1988) disputed the earlier suggestion that catfish T cells were less able than B cells to undergo homeoviscous adaptation thus making their plasma membranes too rigid to function properly at lower temperatures. The authors claimed that T cells did achieve homeoviscous adaptation albeit more slowly than B cells.

The cellular changes that occur during homeoviscous adaptation involve major changes in plasma membrane fatty acid composition (Bly *et al.*, 1986). These compositional changes were associated with the ability to develop helper T cell functions at normally 'non-permissive' temperatures (Clem *et al.*, 1984; Miller & Clem, 1984*b*).

Although several authors have investigated blastogenesis of fish leucocytes in response to whole-cell antigen preparations of *A. salmonicida* (Tatner, 1990; Reitan & Thuvander, 1991, Marsden et al., 1995) there have been no reports of the immunosuppressive effect of purified serine protease in Atlantic salmon.

The serine protease caused inhibition of LPS-induced lymphocyte proliferation and its effect was more pronounced when the cells were pre-incubated with the protease before

activation. This suggested that the protease exerted its effect during the early stages (between 4 and 24 hours) of B lymphocyte stimulation. A greater inhibition of the LPS-induced response was seen when the protease was added to the cells 24 hours before the mitogen than when it was added at the same time as or 4 hours before or after the mitogen was added. The reduced response to LPS was not due to increased cell death as the sub-lethal concentration of protease used (6μg ml^{-1} trypsin equivalent) only exerted an inhibitory effect on proliferating cells (Figure 33) and did not affect cell viability (microscopic examination of cell morphology in the presence of protease).

4.5 Immune regulation

As fish share many of the characteristics of the mammalian immune system such as immunoglobulin synthesis, major histocompatibility complex molecules and anamnestic responses (Faisal & Hetrick, 1992) the mammalian model was used to investigate the role of PGE_2 in immune regulation.

PGE inhibit numerous immunologic events including B and T lymphocyte proliferation, T cell IL-2 production and IL-2 receptor expression, B cell activation events, and IgM and IgG3 synthesis (from Roper *et al.*, 1994). PGE is induced by IL-1, IL-6, TNF, LPS, complement components, and cross-linking of Fc receptors (from Roper *et al.*, 1992).

Little is known of the potential role of eicosanoids in immunoregulation in non-mammalian vertebrates such as fish, however, studies have demonstrated that piscine leococytes synthesize a wide range of prostaglandins, leukotrienes and lipoxins following ionophore stimulation (Pettitt *et al.*, 1991; Rowley, 1991).

Evidence of the protease modulating PGE_2 production came from the observation that indomethacin, inhibitor of prostaglandin synthesis, blocked the immunosuppressive activity of the protease. Administration of indomethacin to fish which had been injected with MS2+protease resulted in a significant increase in antibody titres to MS2.

In vivo quantification of PGE_2 revealed that there was a significant increase above the basal level (152 picogram ml^{-1}) in control fish at both 24 and 48 hours post injection with MS2+PBS ($P < 0.01$ and $P = 0.01$ respectively). Fish immunized with MS2+protease demonstrated even higher levels of PGE_2 than the control fish again at both sampling times ($P < 0.05$).

In vitro protease-induced release of PGE_2 was successfully carried out using macrophage-enriched cultures from the anterior kidney of Atlantic salmon. Measurement of the concentration of PGE_2 in the culture medium revealed a significant rise in PGE_2 levels 24h after the addition of protease. Protease also caused an increase in the amount of PGE_2 released at the earlier times (1h and 4h) but the difference was not statistically significant from the control cells without protease added.

To obtain a clearer understanding of eicosanoid involvement in Atlantic salmon an extensive investigation of other possible cyclooxygenase and lipoxygenase metabolites generated and their respective roles is required.

A recent study by Knight & Rowley, (1995) demonstrated the inhibitory effect of PGE_2 on the specific humoral immune response of rainbow trout injected intraperitoneally with either sheep erythrocytes or formalin-inactivated *A. salmonicida* in the presence or absence of the stable PGE_2 analogue, 16,16-dimethyl-PGE_2. However, the authors did not specify which virulence factor was responsible for the suppression.

Another recent study by Secombes *et al.* (1994) has shown that proliferation of rainbow trout head kidney leucocytes to the mitogen phytohaemagglutinin-P was modulated by the presence of exogenous eicosanoids and inhibitors of their biosynthesis. The observed effects on lymphocyte proliferation were that PGE and lipoxins were inhibitory, the former being more potent, whereas the addition of leukotrienes was stimulatory. However, only PGE was detectable in supernatants from parallel cultures.

Regulation of PGE_2 production was further investigated and it was demonstrated, in this study, that the protease induced a rise in the level of intracellular cAMP which peaked at

30 minutes. This preliminary work indicates that the sequence of events during immunosuppression may involve cAMP as a second messeger.

In many systems, elevation of cAMP leads to a dampening of certain cellular and humoral events (Phipps *et al.*, 1991). Several investigators showed that PGE_2 as well as other cAMP elevating agents enhance immunoglobulin classs switching, inducing the synthesis of IgG1 and IgE while inhibiting IgM (from Phipps *et al.*, 1991). In addition, PGE_2 was reported to promote synthesis of the cytokine, Il-4 which influences Ig class switching.

The possible involvement of prostaglandins in the pathogenesis of cholera emerged many years ago. To this end, (Peterson *et al.*, 1990) further investigated the molecular mechanism of action of cholera toxin (CT) on Chinese hamster ovary cells and observed that the release of PGE_2 and accumulation of cAMP were dependent on the dose of CT and confirmed the earlier observation of (Kimberg *et al.*, 1971) that PGE_2 and CT stimulated intestinal mucosal adenyl cyclase.

Recently (Roper *et al.*, 1994) proposed that PGE could affect B cell Ig production by either interfering with the elicitation of T cell help for Ig synthesis or by direct action of PGE on B cells. In addition, the ability of B cells to present antigen to T lymphocytes may block T cell activation and their ability to produce and respond to cytokines (from Roper *et al.*, 1994). The dependence of T cell responses on B cells has been shown in B cell depleted mice, where T cell cytotoxicity and proliferative responses were blocked (Schultz *et al.*, 1990).

It was previously found that PGE_2, PGE_1, cholera toxin, and dibutyryl cAMP which all cause increases in intracellular cAMP have similar suppressive effect on B lymphocyte activation (Roper *et al.*, 1994). Since all effects of PGE_2 examined on B lymphocyte activation were inhibited by RpcAMP, a competitive inhibitor of cAMP-dependent protein kinase (PKA), it was concluded that PGE_2 was acting via a cAMP-dependent mechanism in inhibiting B cell activation events (Roper *et al.*, 1994).

Although *in vivo* and *in vitro* measurements of PGE_2, in this study, convincingly showed PGE_2 to be involved in inhibition of antibody synthesis more work has to be done to establish the effect of PGE_2 in B cell activation by studying the effect of exogenously added PGE_2 on leucocyte proliferation. The involvement of cAMP could be confirmed by determining whether dibutyryl cAMP can mimic PGE regulation of the humoral response. In addition, the use of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) which does not induce a cAMP response and does not regulate B cells would yield useful information.

To further characterize the role of cAMP in PGE signalling an investigation of cAMP-dependent enzymes will be required. (Roper *et al.,* 1994) suggested phosphorylation of certain proteins by PKA may either block activation signals from being received by B cells or change the ability of the lymphocytes to respond to the signal.

The effect of eicosanoids on the immune system in mammals is closely associated with changes in cytokine generation and the distribution of their receptors on leucocytes (from Knight & Rowley, 1995). The source of protease-induced PGE_2 in head kidney leucocyte cultures was shown to be the macrophages. As many studies in human and mouse models have shown down-regulation of IL-2 synthesis by PGE_2 it will be interesting to see if this is also the case in salmon. The T cell lymphokine, IL-2, plays a pivotal role in mammalian immune responses by stimulating antigen-activated B lymphocytes to progress through the cell cycle and to differentiate into antibody-secreting cells. The limitation here, of course, is the unavailability of fish cytokines and antibodies to them. However, IL-1 and IL-2-like activity has been reported in channel catfish carp (Ellsaesser & Clem, 1994; Caspi & Avtalion, 1984). Once available purified cytokines will confirm the relevance of cytokine production to disease resistance in fish.

It is known that PGE_2 and LTB_4 derived from arachidonic acid (AA) are more potent than PGE_3 and LTB_5, derivatives of eicosapentaenoic acid (EPA), which is the major C_{20} PUFA in fish. In mammals, dietary fatty acid manipulation has been shown to affect immune reactivity principally via alterations in the pattern of eicosanoid generation (Leitch *et al.*, 1984; Lee *et al.*, 1985). Secombes *et al.*, (1994) suggested that altering the type of

eicosanoids released by dietary means may have a potential in influencing lymphocyte clonal expansion by vaccines supplemented with appropriate lipids. It should, however, be noted that AA was found to be the preferred substrate for leukotriene biosynthesis in plaice neutrophils despite the general abundance of EPA in this species of fish.

Further to this, a study of the mechanism of AA release from membrane phospholipids and the enzymes involved in fish would enable the possibility of controlled manipulation of eicosanoid generation by inhibiting specific enzymes of the pathway.

In conclusion the multi-functional role of the serine protease illustrates that it is an important virulence factor. Although it has been argued that protease-deficient mutants are still virulent (Hackett *et al.*, 1984; Ellis *et al.*, 1988). These strains when injected into fish produced typical furunculosis lesions and protease was detectable in the furuncle fluid (Hastings *et al.*, unpublished results from Ellis 1991). This illustrates the importance of differentiating the expression of virulence factors *in vitro* and *in vivo*. Due to lack of appropriate host model systems reports on antigen expression in vivo are scarce. Many questions remain to be answered, however, with the advancement of recent molecular studies in fish a greater understanding of the intricacies of the complex interactions that contribute to an effective immune response and thus protection from disease should be elaborated.

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APPENDICES

Appendix 1.

Computer printout of the statistical analysis showing that the humoral immunosuppressive factor was present in IEF fractions 16-20.

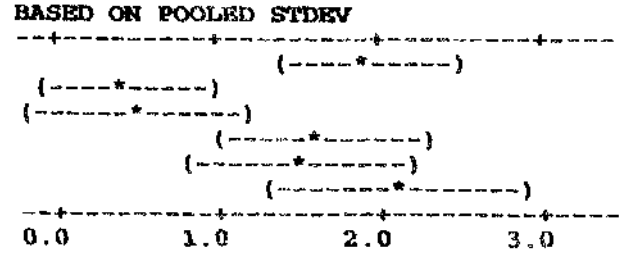
ANALYSIS OF VARIANCE ON wk5logA

SOURCE	DF	SS	MS	F	p
ToxinA	5	15.235	3.047	5.42	0.001
ERROR	29	16.318	0.563		
TOTAL	34	31.553			

LEVEL	N	MEAN	STDEV
1	8	1.9362	1.1004
2	8	0.4468	0.5413
3	5	0.5329	0.7415
4	5	1.6461	0.1768
5	5	1.4657	0.8259
6	4	2.0898	0.4960

POOLED STDEV = 0.7501

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV



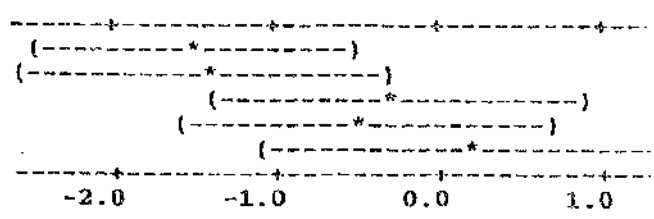
Dunnett's intervals for treatment mean minus control mean

Family error rate = 0.0500
Individual error rate = 0.0117

Critical value = 2.69

Control = level 1 of ToxinA

Level	Lower	Center	Upper
2	-2.4984	-1.4894	-0.4805
3	-2.5536	-1.4033	-0.2529
4	-1.4404	-0.2901	0.8603
5	-1.6209	-0.4705	0.6798
6	-1.0821	0.1536	1.3893



Appendix 2.

Statistical comparison of antibody titres in the presence or absence of extracellular products at weeks 5, 6 and 7 post-immunization.

'log C9' 'log C24'.

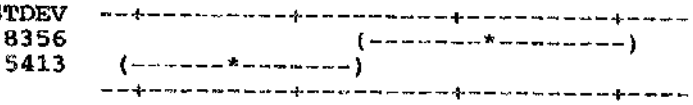
ANALYSIS OF VARIANCE

SOURCE	DF	SS	MS	F	p
FACTOR	1	4.292	4.292	9.29	0.010
ERROR	12	5.542	0.462		
TOTAL	13	9.834			

LEVEL	N	MEAN	STDEV
log C9	6	1.5656	0.8356
log C24	8	0.4468	0.5413

POOLED STDEV = 0.6796

INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV



'log C11' 'log C26'.

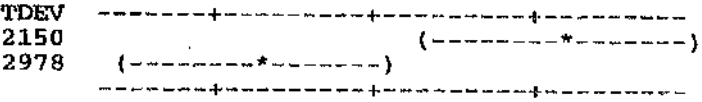
ANALYSIS OF VARIANCE

SOURCE	DF	SS	MS	F	p
FACTOR	1	9.796	9.796	11.82	0.004
ERROR	15	12.430	0.829		
TOTAL	16	22.225			

LEVEL	N	MEAN	STDEV
log C11	9	1.7211	1.2150
log C26	8	0.2003	0.2978

POOLED STDEV = 0.9103

INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV



'log C7' 'log C28'.

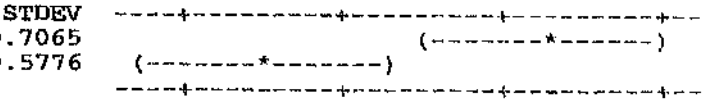
ANALYSIS OF VARIANCE

SOURCE	DF	SS	MS	F	p
FACTOR	1	5.090	5.090	11.93	0.003
ERROR	16	6.828	0.427		
TOTAL	17	11.918			

LEVEL	N	MEAN	STDEV
log C7	10	1.3550	0.7065
log C28	8	0.2848	0.5776

POOLED STDEV = 0.6532

INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV



Key :

Data for week 5 : log C9, log C24

Data for week 6 : log C11, log C26

Data for week 7 : log C7, log C28

Appendix 3.

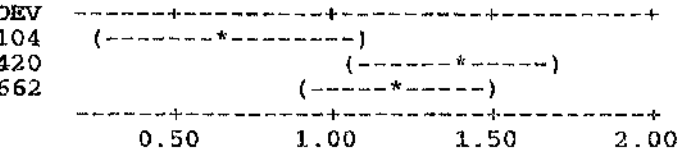
Difference in the antibody titres induced by haemagglutinin and haemolysin at week 3 post-immunization.

SOURCE	DF	SS	MS	F	p
Gps	2	1.696	0.848	3.98	0.035
ERROR	20	4.265	0.213		
TOTAL	22	5.961			

LEVEL	N	MEAN	STDEV
1	5	0.6560	0.6104
2	8	1.3825	0.3420
3	10	1.2160	0.4662

POOLED STDEV = 0.4618

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV



Fisher's pairwise comparisons

Family error rate = 0.118
Individual error rate = 0.0500

Critical value = 2.086

Intervals for (column level mean) - (row level mean)

	1	2
2	-1.2757 -0.1773	
3	-1.0876 -0.0324	-0.2904 0.6234

N : number of observations

Level 1 : MS2 + haemagglutinin

Level 2 : MS2 + haemolysin

Level 3 : MS2 + PBS

Appendix 4.

Statistical analysis of the induction of intracellular cyclic AMP by the protease of *A. salmonicida*.

```
MTB > AOVOneway 'C-30' 'C+HIF 30'.

ANALYSIS OF VARIANCE
SOURCE      DF        SS        MS         F        p
FACTOR       1    1858.6    1858.6     88.00    0.001
ERROR        4      84.5      21.1
TOTAL        5    1943.0

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL       N      MEAN     STDEV    ----------+---------+---------+------
C-30         3    22.800     2.078    (----*----)
C+HIF 30     3    58.000     6.158                        (----*----)
                                      ----------+---------+---------+------
POOLED STDEV =     4.596              30        45        60
MTB > AOVOneway 'C-5       'C+HIF 5'.

ANALYSIS OF VARIANCE
SOURCE      DF        SS        MS         F        p
FACTOR       1     116.2     116.2      7.22    0.055
ERROR        4      64.3      16.1
TOTAL        5     180.5

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL       N      MEAN     STDEV    ----+---------+---------+---------+--
C-5 pm/c     3    4.400     1.386     (---------*---------)
C+HIF 5      3   13.200     5.499                (---------*---------)
                                      ----+---------+---------+---------+--
POOLED STDEV =     4.010              0.0       6.0      12.0      18.0
MTB > AOVOneway 'C-15' 'C+HIF 15'.

ANALYSIS OF VARIANCE
SOURCE      DF        SS        MS         F        p
FACTOR       1     54.00     54.00      5.92    0.072
ERROR        4     36.48      9.12
TOTAL        5     90.48

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL       N      MEAN     STDEV    ----------+---------+---------+------
C-15         3   15.200     1.833     (---------*---------)
C+HIF 15     3   21.200     3.857               (---------*---------)
                                      ----------+---------+---------+------
POOLED STDEV =     3.020              15.0      20.0      25.0
MTB > AOVOneway 'C-60' 'C+HIF 60'.

ANALYSIS OF VARIANCE
SOURCE      DF        SS        MS         F        p
FACTOR       1     1.50      1.50      1.14    0.346
ERROR        4     5.28      1.32
TOTAL        5     6.78

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL       N      MEAN     STDEV    -+---------+---------+---------+-----
C-60         3    3.200     1.249     (-----------*-----------)
C+HIF 60     3    4.200     1.039           (-----------*-----------)
                                      -+---------+---------+---------+-----
POOLED STDEV =     1.149              1.5
```

C : control cells, C + HIF : cells + protease

The numbers following the above abbreviations refer to the time of incubation in minutes.

Appendix 5. Polyacrylamide gel electrophoresis stock solutions

Acrylamide / Bis:

Acrylamide	30 grams
N,N-bis-methylene acrylamide	0.8 grams
Distilled water	100ml

Lower buffer:

Tris	18.1 grams
SDS	0.4 grams
Distilled water	70 ml

Adjust pH to 8.8 with concentrated HCl and make up the final volume to 100 ml with distilled water.

Upper buffer:

Tris	6.06 grams
SDS	0.4 grams
Distilled water	70 ml

Adjust pH to 6.8 with concentrated HCl and make up the final volume to 100 ml with distilled water.

Solubilising buffer:

Glycerol	10 ml
2-mercaptoethanol	5 ml
SDS	3 grams
Bromophenol blue	0.01 grams
Upper buffer (1 in 8 dilution)	100 ml

Appendix 5 (ctd)

Running buffer:

Tris	6.06 grams
Glycine	28.8 grams
SDS	2 grams
Adjust pH to 8.3 with concentrated HCl	
Distilled water	2000 ml

Coomassie blue staining solution:

Coomassie blue R250 (BDH)	1.25 grams
50 % (v/v) methanol	454 ml
Glacial acetic acid	46 ml

Destaining solution:

Methanol	50 ml
Glacial acetic acid	75 ml
Distilled water	875 ml

Slab-gel preparation:

Lower separating gel - 12.5 %:

Lower buffer	10 ml
Distilled water	13.4 ml
Acrylamide/bis	16.6 ml
After degassing the following were added	
Ammonium persulphate	200 µl
(10 % freshly prepared in distilled water)	
Temed (undiluted)	20 µl

Appendix 5 (ctd)

Upper stacking gel - 4.5%:

Upper buffer	2.5 ml
Distilled water	6 ml
Acrylamide / bis	1.5 ml
Ammonium persulphate (10%) freshly prepared	30 µl
Temed	20 µl

Appendix 6. Western blotting buffers

Transfer buffer:

Tris	7.5 grams
Glycine	36 grams
Methanol	500 ml
Distilled water up to	2500 ml

TTS buffer:

1 M Tris-HCl (pH 7.2)	2 ml
Tween 20	1 ml
Saline	97 ml

Blot developing substrate solution:

3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB)	0.05 grams
Cobalt chloride 1% (w/v) in dist. water	2.0 ml
PBS pH 7.4	98.0 ml
Hydrogen peroxide (30%) added immediately before use.	0.1 ml