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Christopher J. Bayne

The acute phase response (APR) includes an early response of the innate immune system to an inflammatory agent. Acute phase proteins (APP) are produced at increased rates in the liver during this response. The APR has been well characterized in mammals, but it is unclear to what degree teleosts mount a similar response. This thesis describes research conducted to characterize this response in rainbow trout. We further developed and used a Tandem Crossed Immunoelectrophoresis Technique (TCIEP). With this we were able to determine that concentrations of at least three trout plasma proteins were higher several days after trout had been injected with Freund's Complete Adjuvant. One of these proteins was isolated and partially characterized, using electrospray mass spectrometry (ESI-MS), and tentatively identified as transferrin. Due to the limited amount of any protein that could be obtained from T-CIEPs, we switched to the use of 2D gel electrophoresis based on isoelectric focusing and SDS PAGE. When comparing such 2D gels containing trout plasma from before and after treatment, at least 5 of approximatly 200 protein spots were increased in size. An N-terminal sequence was obtained from three of the increased spots, and degenerate primers constructed from one of those sequences; 5' and 3' RACE PCR revealed a 188 amino acid long polypeptide. It was 29% identical at the amino acid level with precerebellin from rat. The function of precerebellin is unknown but it appears likely that it is part of the innate immune system since it is upregulated in trout

injected with Vibrio bacterin. This thesis has begun the characterization of the acute phase proteins in rainbow trout, but much work remains to be conducted.

ACUTE PHASE PROTEINS IN RAINBOW TROUT (*Oncorhynchus mykiss*).

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Lena Gerwick, Author

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CONTRIBUTION OF AUTHORS

Dr. Christopher Bayne was the major professor, involved in encouraging and discussing ideas, helping with design of experiments, writing of manuscripts and providing laboratory space, supplies and equipment. Wendy Reynolds shared her knowledge and supplies for the cloning and sequencing of precerebellin. Dr. Nora Demers helped with experimental designs, sample collecting and preparation of the manuscript.

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I dedicate this thesis to my "Moster" Eivor and to all the patients who I could not help and who made me understand the importance of increasing our knowledge of immunology.

ACUTE PHASE PROTEINS IN RAINBOW TROUT (*Oncorhynchus mykiss*)

CHAPTER 1

INTRODUCTION

Functioning immune systems are crucial for the survival of the species but their importance is sometimes forgotten when matters of evolution are discussed. All living plants and animals must be equipped with defense mechanisms against potential pathogens. For example, the unicellular amoeba produces anti-microbial peptides (Lieppe, 1999) and many antibacterial and antifungal compounds have been isolated from terrestrial plants (Grosvenor et al, 1995) and algae (Singh et al, 1999). Invertebrates rely on innate (natural) immune systems whereas vertebrates have an adaptive system as well. The origin of the adaptive system is thought to have occurred with a serendipitous insertion of a recombination-activating gene (RAG) transposon into the genome of an ancestor to the jawed fishes before the divergence of cartilaginous fishes (Agrawal et al, 1998). Much research has been conducted on adaptive immune systems, in particular the mammalian system. However, in recent years more researchers have taken an interest in the innate system and the interactions that occur between the two different systems.

Teleosts are good models for studying the innate immune response and its interactions with the adaptive immune system since they probably rely more on the innate system, as compared to mammals, but still have interactions with the

adaptive arm. In order to achieve a comprehensive understanding of the immune system of any named species, the different components of the system need to be known. Since the so-called acute phase proteins include several mediators and effectors of innate defenses, a project has been started to describe these in the rainbow trout (*Oncorhynchus mykiss*). This thesis describes some of acute phase proteins that are upregulated in the liver of this species.

THE INNATE IMMUNE SYSTEM

The innate immune system can be divided into first and second lines of defense. The first line takes the form of anatomical and physiological barriers. Examples of anatomical barriers in mammals are skin and mucosal membranes. Skin, being heavily keratinized and low in moisture content, is hard for pathogens to penetrate. This difficulty is also enhanced by the low pH of 3-5 that is created by lactic acid and certain fatty acids that are produced in the sebaceous glands. Microbial penetration of mucosal membranes is also difficult. Abundant mucus secretions can simply wash the pathogens away, and the cilia that line the airways of mammals beat constantly and transport the trapped pathogens so that they can be cleared.

The mucus that is released onto the body surface of teleosts also works as an anatomical and physiological barrier by making it hard for parasites, bacteria and fungi to enter or to start growing on the skin itself.

Antimicrobial proteins and peptides.

The second line of defense includes a wide range of molecules that inhibit the growth and spread of microbes by direct killing, or by facilitation of receptor-mediated phagocytosis. The skin and mucosal membranes produce antimicrobial proteins and peptides. Lysozyme, a hydrolytic enzyme that cleaves the peptidoglycan layer in the cell wall of Gram-positive bacteria, is found in tears and in the respiratory mucosa and is also produced in the Paneth cells of the intestinal mucosa of mammals. Lactoferrin, secreted in the mammalian respiratory system, also shows antimicrobial activity. Its activity seems to be confined to the first 25 amino acids of the N-terminus and this fragment gets cleaved off to form the active peptide (Lehrer and Ganz, 1999). Antimicrobial peptides are also produced from the N-terminal of pepsinogenin from the bullfrog, *Rana catesbeiana* (Minn et al, 1998). Other antimicrobial peptides have been isolated, mainly from mammals and *Drosophila,* and they can be divided into α and β defensins depending on if their secondary structures mainly consist of an α-helix or a β-sheet.

The *Drosophila* antibacterial and antifungal peptides include cecropins, attacin, diptericin, drosocin and drocomycin (Hetru et al, 1998). They are secreted from the fat body, analogous to the liver in mammals. The intracellular signaling that leads to transcription of the relevant genes is in part conducted by *relish*, which is homologous to the mammalian NF-κB transcription factor (Hedengren et al, 1999). This factor is responsible for activation of many of the acute phase proteins in mammals. A couple of the α-defensins, human defensin-5 (HD-5) and HD-6,

are constitutively expressed in the Paneth cells of the intestine; HD-5 is also secreted constitutively into the mucus of the vagina and cervix (Quayle et al, 1998).

Many antimicrobial peptides are cytotoxic to both Gram negative and Gram-positive bacteria, and some peptides, such as Metchnikowin from *Drosophila* (Levashina et al, 1995), have both antifungal and antimicrobial activity. While some are expressed constitutively, other peptides are inducible (Lehrer and Ganz, 1999). Antimicrobial peptides are also found in neutrophil granules and recently they have been found to interact with the adaptive immune system by attracting dendritic cells and T-cells to sites of infection (Yang, 1999).

Several antimicrobial peptides have been isolated from the skin secretions of different fishes. One example is Parasin I, found in catfish, which is nineteen amino acids long and appears to be derived from the N-terminal of the histone H2A (Park et al, 1998). A second is Pleurocidin, a dermal peptide isolated from winter flounder (Cole et al, 1997).

The hepatic acute phase response.

Humoral components of innate immunity are not limited by constitutive expression at fixed rates. Indeed increases and decreases in protein synthesis in the liver during the early phase of an infection or immediately following a traumatic event help protect the individual from opportunistic pathogens. These so-called acute phase proteins (APPs) are secreted into the plasma. Those that increase in

quantity are considered to be positive APPs whereas those that decrease are negative APPs.

More than thirty-five APPs have been identified in mammals (Steel and Whitehead, 1994). There are three major mammalian APPs: C-reactive protein (CRP), Serum Amyloid P (SAP) and Serum Amyloid A (SAA). Others include complement components, proteinase inhibitors, coagulation factors and metal binding proteins. CRP and SAP are both pentraxins, proteins that form pentameric structures containing five identical subunits (Pepys and Baltz, 1983). Comparison of sequence data and gene organization of the CRP and SAP pentraxins implies that they evolved from the same ancestral gene and that a gene duplication event took place 200-250 million years ago, after the branching of amphibians and mammals (Benton, 1990). This correlates well with findings in Atlantic salmon (Jensen, 1997), in which evidence was found for just one pentraxin gene. In mammals only one pentraxin is up-regulated during an acute phase response; humans up-regulate CRP while mice up-regulate SAP. CRP is a multifunctional protein, binding phophorylcholine residues on the cell membranes of bacteria and damaged autologous cells. CRP can opsonize with or without complement. It also induces the secretion of TNF-α, IL-1β and IL-6 from human monocytes (Ballou et al, 1992). Furthermore, CRP stops neutrophils from adhering to epithelial cells (Zouki et al, 1997). It has also been shown that mice, transgenic for rabbit C-reactive protein, can better withstand endotoxemia (Xia et al, 1997). Recently, a great interest has been taken by the medical community in using CRP concentrations for

early diagnosis of acute infection (Hatherill et al, 1999) and severity of coronary artery disease (Rohde et al, 1999).

SAA, another major acute phase reactant, is an apolipoprotein; it binds to high-density lipoproteins during inflammatory events (Banka et al, 1995). It may also have some chemotactic properties and may cause inflammation in vessels due to oxidation of low-density lipoproteins (Berliner et al, 1995) but more research is needed for a comprehensive understanding of the function(s) of SAA.

The complement components are also acute phase reactants. Most of the thirty or so proteins are synthesized in the liver, however the first complement component (C1) appears to be synthesized in macrophages. The complement components work together to create the membrane attack complex (MAC), which forms a pore in the cell membrane of the target cell and thus kills it. Complement components can also exert effects working alone. C3b, containing a reactive thiolester, readily binds to free amino or hydroxyl groups. C3b bound to a membrane serves as an opsonin, marking the target for recognition by the C3b receptor which is expressed on phagocytic cells. C1q, by itself, also functions as an opsonin (Mitchell et al, 1999). A receptor (C1qR) for C1q, found on phagocytic cells, has been cloned and sequenced. The receptor also recognizes mannose binding lectin and pulmonary surfactant protein (Nepomuceno et al, 1997).

Many of the complement components have been found and fully or partially sequenced in rainbow trout (Bayne, personal communication), as well as in some other teleost species (Sunyer et al, 1997); (Nakao and Yano, 1998). Unlike the

situation in mammals, at least some teleosts have several isoforms of C3 (Sunyer et al, 1997). The various isoforms of C3 evidently have distinctive affinities for different molecular structures, leading to the speculation that diversity of C3 isoforms may be a means of increasing the diversity of antigen recognition (Sunyer and Lambris, 1998).

Other categories of acute phase proteins include protease inhibitors, for example α2-macroglobulin, α1-antitrypsin, α1-antichymotrypsin and α2-antiplasmin, and metal-binding proteins like haptoglobin (Fe), hemopexin (hemegroups with Fe) and ceruloplasmin (Cu). Protease inhibitors will limit tissue damage caused by one's own proteases or by proteases secreted by pathogens. Metal-binding proteins, on the other hand, salvage free metal-ions for reuse in metabolic processes. However, the entire heme group is salvaged by hemopexin, and this prevents oxidative damage (Tolosano et al, 1999). Haptoglobin inhibits chemotaxis and phagocytosis (Rossbacher et al, 1999).

While many of the acute phase proteins have been described, it is probable that some of their function(s) have not. It appears that several of the proteins have more than one function as is the case with CRP and haptoglobin. This suspected multifunctionality makes the study of these proteins more fascinating, and potentially more rewarding, and at the same time more challenging.

Cells in innate immunity

Granulocytes (neutrophils, eosinophils and basophils), dendritic cells, monocytes and tissue macrophages are important components of the secondary line of defenses in innate immune systems. Most are phagocytic and can secrete molecules (interleukins) that orchestrate the inflammatory response. Of the granulocytes, neutrophils and eosinophils can phagocytose but basophils cannot. The granules in neutrophils stain with both acidic and basic dyes, eosinophil granules stain with acidic dyes and the granules in basophils, as the name implies, stain with basic dyes.

Neutrophils, or polymorphonuclear leukocytes (PMLs) as they are also called, are important in the diagnosis of acute infections in mammals, since their numbers increase rapidly early in an infection. In mammals, the neutrophil granules contain collagenase and other proteolytic enzymes, lysozyme, lactoferrin, peroxidase, and bactericidal/permeability-increasing protein, as well as several α-defensins. These granules fuse with phagosomes and help in the digestion of engulfed particles. Neutrophils also produce different oxygen and nitrogen radicals that are highly damaging to pathogens (Cohen et al, 1988; Abu-Soud and Hazan, 2000).

Eosinophils are thought to be involved in defense against parasites but the roles of basophils are not well defined. However, both eosinophils and basophils, by their release of histamines and other vasoactive substances, are part of the pathology seen in allergies and asthma.

The composition of fish granulocyte subpopulations varies among species. Catfish for example have all three types of granulocytes but neutrophils and basophils are less numerous than eosinophils (Rowley et al, 1988). This is in contrast to the salmonids and mammals in which neutrophils are the most abundant. Several of the components from fish neutrophil granules have been isolated and characterized, for example collagenase (Noya et al, 1999) and peroxidase (Afonso et al, 1998). Fish neutrophils also possess a strong oxidative burst response (Plytycz et al, 1989; Ainsworth and Boyd, 1998).

Macrophages and dendritic cells (DC) have many functions. They are capable of opsonin-dependent and opsonin-independent phagocytosis. Secondly, they produce cytokines after they are "activated" by phagocytosed particles and thirdly, they degrade captured particles and present some of the resulting peptides, in the context of MHC II, on the cell surface. The antigen, held within a grove in the MCH molecule, is then recognized by T-helper cells as foreign. This event, which involves several signals, eventually leads to expansion of the specific T-cell clones. These T-cells trigger B-cells to produce antibody. Monocytes circulate in the blood and, when needed outside the vasculature, become activated and migrate into the affected tissue as macrophages. Monocytes, alternatively, differentiate into dendritic cells as a result of stimulation by LPS or the CD40 ligand (Palucka et al, 1998; Zhou and Tedder, 1995), or when cultured with TNF and IL-1 (Palucka et al, 1998). DCs occur in the epidermis (Langerhans cells), in the interstitium and in

lymphoid tissue. The Langerhans cells and interstitial DCs are of myeloid origin whereas the lymphoid DCs are of lymphoid origin (Palucka, 1999).

Teleost monocytes and macrophages have been studied extensively. However, it is uncertain if teleosts have dendritic cells. Alvarez (1993 as cited in Zapata, 1996) suggests that some interdigitated cells occur in the thymus of brown trout but their identity has not been confirmed by specific DC surface markers.

ADAPTIVE IMMUNITY

Lymphocytes, B and T-cells, clonal expansion, memory, self-recognition, antibody diversity, primary and secondary responses are hallmarks of the adaptive immune system. B-cells, like macrophages and DCs, are antigen-presenting cells (APCs) with the main difference between the cell types being that B-cells can differentiate into plasma cells that secrete antibodies. A large diversity of antibody specificities can be achieved by rearranging immunoglobulin gene segments. The ability of a particular antibody to recognize a specific determinant (epitope) can depend on the substitution of one amino acid for another. A very large number of epitope specificities present in the T and B-cell repertoires allow for recognition of very rare antigens. This is in contrast to some of the so-called pattern recognition molecules of the innate system, for example mannose-binding protein, that will recognize a certain motif expressed by many microbes.

T- cells consist of T-helper and T cytotoxic cells. The T helper cells interact with B-cells via a complicated set of signals to create a memory response. In the course of a primary response to specific antigen, some memory lymphocytes are produced. These facilitate a qualitatively superior and quantitatively more productive response if the antigen is encountered again. This so-called secondary response makes a re-infection by the same pathogen more difficult since the antibody titer becomes much higher and peak titer is achieved much faster than during the primary response. In addition, the immunoglobulins of the secondary response bind their antigens with much higher affinity. Parish and O'Neill (1997) even suggest that the main reason the adaptive immune system developed was to combat re-infection.

Cytotoxic T-cells (CTL) are involved in cell-mediated immunity. On recognizing a foreign cell, CTLs will kill it using cytolytic peptides (perforin and granulysin). The cell-mediated response, like its humoral counterpart, is more rapid upon re-exposure to the antigen, indicative of a memory function.

Teleosts B cells produce antibody that is IgM-like. Whereas IgM is a pentamer in mammals, it is a tetramer in fishes. In both cases, the monomers are linked with disulfide bonds but the placement of the disulfide bonds varies considerably between different teleost species (Lobb, 1981, Warr, 1983, Lobb, 1983, Ghaffari, 1989 and Sanchez, 1991). The intermolecular disulfide bonding seen in teleosts may make the tetrameric molecule more flexible than the pentameric mammalian molecule (Kattari, 1996). This could make the fish Ig more

versatile in the way that it binds antigen. Other mechanisms may exist in fish for increasing the antibody affinity for a certain antigen but there is no evidence of class switching (Ross, 1998) or affinity maturation of Ig molecules in fish (Arkoosh, 1991), as occurs in mammals.

INTERACTIONS BETWEEN THE INNATE AND ADAPTIVE IMMUNE SYSTEMS

In recent years there has been an increased interest in the interactions between the innate and adaptive immune systems. It has been known that adjuvants are needed for the priming of the adaptive immune system when vaccines are given. Janeway (1989) called the use of adjuvants "the immunologist's dirty little secret" and he went on to suggest that adjuvants stimulate the activation of the innate system which is needed to activate the adaptive one. Recent studies have shown that C3 binding to APCs (Kerekes et al, 1998) increases the number of antigen-specific T-helper cells that are generated. The same increase in antigen specific T-cell clonal expansion was seen when human neutrophil peptide (HNP) defensins were introduced intranasaly together with an antigen. Furthermore, the T-cells exposed to HNP produced more interferon γ, interleukin (IL)-5, IL-6 and IL-10 (Lillard et al, 1999). Yang et al 1999 showed that β-defensins are chemotactic for dendritic cells and memory T-cells, thus drawing these cells into the site of infection.

In addition to their roles as effectors of innate immunity, dendritic cells and macrophages are important in initiating primary antibody responses. Initial recognition of antigen is facilitated by soluble innate factors (complement, mannose binding lectin, defensins etc.) during a pathogen exposure. This is followed by endocytosis of the pathogen, proteolysis of the engulfed proteins, and antigen (peptide) presentation in the context of MHC II and CD1. The "activation", caused by the phagocytosed pathogens, of the dendritic cells or macrophages increases the production of co-stimulatory molecules and cytokines important for initiation of the adaptive immune response (Palucka and Banchereau, 1999). The CD1 receptor presents microbial lipoglycan antigens to T-cells. It has been shown that in order for that to happen, the pathogen has to be taken into the DC via the mannose receptor (Ernst et al, 1998).

In summary, the innate immune system is ancient. Some forms of defense against potential parasites and pathogens can be found in all organisms. The evolution of the defense system depended on the duplication and mutation of already existing genes. The adaptive immune system arose from the innate system some time between the origin of the jawless fishes and the divergence of the jawed fishes from the sharks and rays. The innate immune system is complex. Its interactions with the adaptive immune system are poorly understood. In order to fully understand subtle modulations of immune responses, we need to know the different components. This thesis contributes to the basic knowledge of the innate

immune system by identifying and characterizing some of the acute phase proteins in rainbow trout.

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CHAPTER 2

AN ACUTE PHASE REACTANT OF RAINBOW TROUT ISOLATED USING TANDEM CROSSED IMMUNOELECTROPHORESIS

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ABSTRACT

The acute phase response (APR) includes an early response of the innate immune system to an inflammatory agent. Acute phase proteins (APP) are produced at altered rates in the liver during this response. The APR has been well characterized in mammals, but it is unclear to what degree teleosts mount a similar response. In an effort to characterize the APPs of rainbow trout we used Tandem Crossed Immuno-Electrophoresis (T-CIEP) to compare the proteins in plasma collected from the same fish before and after injection with Freund's Complete Adjuvant (FCA). This technique allows a side-by-side comparison of the samples, thus avoiding the "run to run" variability seen with standard 2D Crossed Immuno-Electrophoresis in which each gel contains a single sample. Using this technique we have identified at least three plasma components that were either up or down regulated following the FCA injection. Individual immuno-precipitates of APPs and rabbit antibody were subsequently extracted from the agarose gel and electrophoresed on SDS PAGE gels, then blotted on to a membrane, stained for determination of approximate molecular weight, and submitted for amino acid sequencing or electrospray mass spectrometry. The data obtained from the sequencing and electrospray mass spectrometry indicated that one of the proteins might be transferrin. This approach allows the revelation of APPs without fore-knowledge of their identities.

INTRODUCTION

The acute phase response (APR) is a complex and early response of the body to a traumatic insult or to infection. It is an important normal physiological process that precedes specific T and B cell responses. In mammals lipopolysaccaride (LPS), turpentine, mast cell degranulation and degradation products of opsonins can stimulate macrophages and monocytes to release factors that initiate this response (Baumann and Gauldie, 1994). Heavy metals (Sinha and Mandal, 1996) and polyaromatic hydrocarbons (Winkelhake et al, 1982) can also initiate the response. Interleukin-1 (IL-1), Interleukin-6 (IL-6) and Tumor Necrosis Factor-α (TNF-α) appear to be the cytokines that are most important for eliciting the part of the response that involves the production of the so-called Acute Phase Proteins (APP) in the liver. An APP is a plasma protein that is up or down regulated more than 25% during the APR (Kushner and Mackiewics, 1993). However, the major APPs, Serum Amyloid A (SAA), C-reactive protein (CRP) and Serum Amyloid P (SAP), can increase as much as 1000 fold within 24hrs after the body has been exposed to a pathogen or subjected to other physical trauma (Steel and Whitehead, 1994).

While the production, chemistry and functions of the APPs have been studied in several mammalian species in considerable detail, questions remain unanswered regarding kinetics and functions.

CRP is known to work as an opsonin, because it binds to bacterial cell wall components and to chromatin from both self and nonself and thereby enhances

clearance. SAP probably has similar functions to those of CRP since these proteins are structurally related and belong to the family of proteins called pentraxins. The functions of SAA remain obscure.

In rainbow trout (*Oncorhynchus mykiss),* exposure to the Gram negative pathogen *Aeromonas salmonicida* induces transcription of SAA mRNA, and in work reported by Jensen et al, (1997) it increased about 40 times from its resting level after six days. This is the best evidence we have so far that trout possess an APR and that a homologue of at least one mammalian APP is present. Following much effort to identify CRP (Murata, 1995) and SAP from trout it appears that SAP exists (Jensen et al, 1997), but it is still not clear if it is up or down regulated during the APR.

Transferrin is known to be up regulated during the inflammatory response in rats and rabbits (Mackiewics et al, 1988; Schreiber et al, 1989). One function is to bind free iron that has been released from free hemoglobin, reducing its availability to microbes, but other functions may exist.

Alpha2-macroglobulin, together with several of the complement proteins, belongs to the thiol-ester family. It is a proteinase inhibitor, and is constitutively expressed in humans but is a major acute phase reactant in rats. Rainbow trout α2-macroglobulin has been isolated and partially characterized (R.C.Bender, personal communication).

Rainbow trout is likely to be a good model for studies of the APR because it is a lower vertebrate with a less developed adaptive immune response and,

consequently, it may rely more on the innate arm of the immune system than do higher vertebrates. In such an organism, the function of the APPs should be easier to elucidate than in more derived species such as mammals.

We have sought to more fully define the APR in trout and have evaluated a variety of elicitors that effectively induce it. Using Tandem Crossed Immuno-electrophoresis (T-CIEP) we have found that several plasma proteins are either up-regulated or down-regulated after injection with LPS, turpentine or Freund's Complete Adjuvant (FCA). One protein was consistently up-regulated (average 51.6%, eight days after treatment) in trout injected with FCA. Some attributes of the molecule imply that it might be transferrin. Alpha2-macroglobulin, an acute phase protein in rats, did not appear to be consistently up- or down-regulated in trout.

MATERIALS AND METHODS

Animals

Trout (*Oncorhynchus mykiss*), Shasta strain, ranging in size from 200g - 650 g, were kept at 12°C in large circular tanks with constant water flow at the Oregon State University Food Toxicology & Nutrition Laboratory. One week before treatment 7 fish were moved from a stock tank into a separate tank. On Day 0, all the fish were anesthetized with 0.025% 2-phenoxyethanol, and bled (1 ml) from the caudal vein, using a heparinized syringe. The fish were then injected in the dorsal musculature with 0.1 ml Freund's Complete Adjuvant; . Individual fish

were marked by a needleless intradermal injection of 0.1% Alcian blue dye (Panjet, Wright Dental Group, Dundee, Scotland), so that individuals could be subsequently identified. The fish were anesthetized with 2-phenoxy ethanol and subsequently bled using heparinized syringes, on Day 1, Day 2, Day 6 and Day 8 after the injection. Samples were immediately placed on ice, transported to the laboratory and centrifuged at 800g for 10 minutes. The plasma was collected, aliquoted into microfuge tubes and stored at -80C.

Production of antisera

Six New Zealand white rabbits, kept at the Laboratory Animal Resources Facility, Oregon State University, were injected subcutaneously with O.8 mL trout plasma emulsified in Freund's Complete Adjuvant (distributed between four sites). The plasma was from trout injected with 0.5 mL turpentine 24 hrs before the bleed. The rabbits were given a booster injection every four weeks, for 20 weeks; each was 0.4 mL of trout plasma emulsied in Freund's Incomplete Adjuvant. After an initial prebleed (before treatment), the rabbits were bled every four weeks, two weeks after each booster injection. The rabbits were bled a total of six times. A total of 500mL antisera were obtained. The antibody responses of the different rabbits were checked using rocket immunoelectrophoresis. All the rabbits responded with adequate antibody production to the trout plasma proteins. The antisera were pooled, aliquoted and stored at -80°C until used for Crossed Immunoelectrophoresis.

Tandem Crossed Immunoelectrophoresis

To prepare the first dimension gel, 0.7 g of Agarose (Seakem, FMC) was dissolved by heating in 70mL of 0.12 M Barbital buffer (pH 8.2) and poured on a 26 cm x 12 cm glass plate. Two 4mm holes were punched at the bottom left corner, one centimeter apart along the cathode anode axis. One sample (10-15 μl),using the same buffer, was loaded in each well and electrophoresed at 5°C on a horizontal LKB Multiphor apparatus for 4hrs at 10V/cm. The gel was cut and much of it was removed, leaving a 2.5 cm wide strip containing the samples. To prepare the second dimension gel, 45mL of 1% agarose in Barbital buffer containing 14% antiserum, was mixed and heated to 56°C. This was poured onto the 26cm x 12 cm glass plate where much of the former gel had been removed. The second dimension gel was electrophoresed perpendicular to the first dimension at 2V/cm overnight (Kroll, 1976)(Figure 2.1.).

In such gels, precipitate peaks form when the trout plasma proteins and the rabbit antibodies are at their zones of equivalence, a greater amount of antigen results in a precipitin line that is larger. The peaks were visualized by Coomassie staining and the relative difference of the peaks between the two samples was determined by measuring the area under the peaks (Pharmacia, Imagemaster Software), and calculating the percent change. Change in peak size indicates that the antigens differ in concentration in samples taken before and after injection of FCA.

Identification of trout α2-macroglobulin and trout immunoglobulin

Identification of the α2-macroglobulin peak was done by using purified α2-macroglobulin. One gel contained only purified α2-macroglobulin in the first dimension. Another gel had one of the regular plasma samples "spiked" with 5μg of the purified protein. This gel was then compared to a gel in which nothing was added to the plasma samples. The electrophoretic conditions were the same as previously used. Identification of the immunoglobulin (Ig) peak was attempted by running purified Ig on the first dimension gel and using the rabbit anti-trout plasma protein antisera to precipitate trout antigens.

Extraction of protein

To visualize the peaks of interest while not rendering them insoluble, and to avoid "fixing" the proteins, the gel was stained in 5% Coomassie Blue in PBS (phosphate buffered saline pH 7.0) for two hours followed by destaining in PBS overnight. The peaks of interest were excised using a clean scalpel and the excised pieces of agarose containing the precipitated antigen were placed in microfuge tubes each containing 200 μl of extraction buffer: 2% SDS and 10% 2-mercaptoethanol in PBS. Extraction occured in the tubes placed on a rocker at 37°C for two hours and overnight at room temperature. The fluid was then separated from the agarose pieces by use of a micro spin filter (0.45μM)(Lida

Manufacturing Company, Kenosha, WI). Tubes containing the filters were centrifuged at 800g for 10 minutes, and the buffer with the extracted protein was collected.

SDS - PAGE of extracted protein

The extraction buffer, containing the protein of interest and the rabbit antibody, was loaded and electrophoresed on a 7.5% SDS - PAGE minigel (Biorad), and the gel was blotted onto a PVDF membrane using a semi dry blotter (Biorad, Transblot SD). The proteins were visualized on analytical blots using Colloidal Gold Stain (Biorad) or, for the preparative blots, Coomassie Blue stain. At least four preparations of peaks cut from four different gels were pooled and then concentrated by using a "Speedvac" (Savant) for 2.5h. The remaining solution contained enough protein to be visualized after electrophoresis by Coomassie Blue stain either by direct staining of the gel or after blotting of proteins on to a membrane (PVDF, Millipore). Regions of the gel containing the selected protein were cut out and sent for electrospray mass spectometry (Keck Laboratory, Yale University) and membrane pieces were sent for N-terminal sequencing to the University of Oregon's protein facility.

RESULTS

Plasma samples from five fish collected before and after FCA injection were compared by means of T- CIEP. Figure 2.1 shows the result obtained with one fish. Two peaks (Peak 1 and 2) were consistently higher in the samples taken after the FCA injection, and one (Peak 3) was consistently lower. Peak 1 was up-regulated an average of 51.6%±3% (SE) on the eighth day after FCA injection. This immunoprecipitate was cut from the gel (Figure 2.2) using the method descibed above. On SDS PAGE analysis it appeared to be a heterodimer and the peptide components, under reducing conditions, had masses of 76 and 86 kDa as determined by the positions of the bands relative to the molecular weight markers (Figure 2.3). Visualization of these peptides required the use of Colloidal Gold stain, which is sensitive enough to detect nanograms of protein. In order to prepare enough of the protein for conventional Edman degradation sequencing or electrospray mass spectrometry, the same peak was cut out from four gels, and the extracted proteins were concentrated on a Speedvac. Proteins visualized with Coomassie stain were sent for sequencing or electrospray mass spectrometry. To prepare the protein for N-terminal sequencing, gels containing the proteins were blotted onto a PVDF membrane and stained with Coomassie Blue. The stained membranes were sent to the University of Oregon's protein facility for sequencing. The sequence attempt was unsuccessful, probably due to insufficient amounts of

protein or possibly due to N-terminal blockage. Pieces of gel containing the 76 and 86 KDa proteins were sent for internal sequencing or electrospray mass spectrometry (Keck laboratory, Yale University). Identification was hampered by the small quantity of the 76 and 86 kDa proteins, which were estimated to be only 250 femtomoles or less, based on the HPLC absorbance profiles of trypsin digested protein. It was deemed impossible to do Edman sequencing with such low amounts of protein. Using electrospray mass spectrometry we determined that two of the peptides from the 86 kDa protein had approximately the same mass as peptides from Coho salmon transferrin (Fig 2.5).

The α2macroglobulin peak was identified on a CIEP gel by "spiking" a plasma sample with 10 μg of purified α2-macroglobulin and comparing that gel to a gel in which a sample was not "spiked" (Figure 2.4). The relative concentrations of α2-macroglobulin were determined by measuring the areas under the peaks in plasma samples taken before and after treatment. The % change in these varied after treatment from a decrease of 14% to an increase of 50%. The mean change was an increase of 15.7%±11.6(SE). When purifed trout immunoglobulin was used to run a T-CIEP gel, no precipitate was seen.

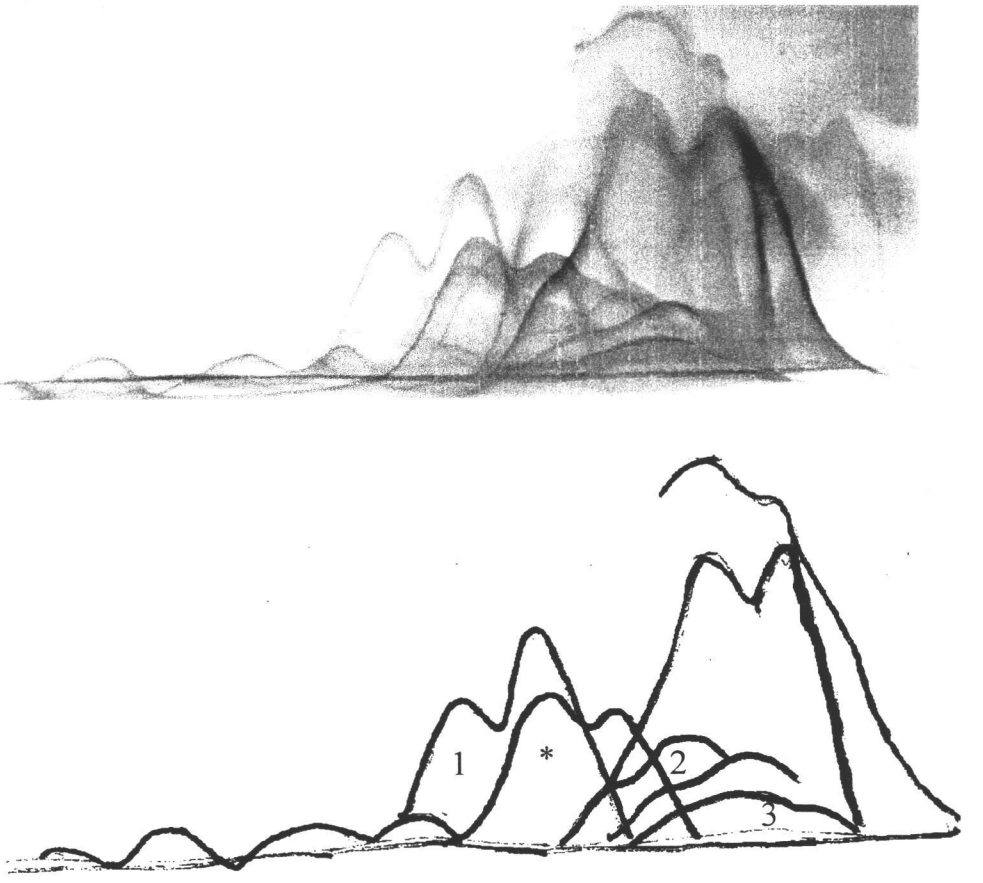

Figure 2.1. Tandem Crossed Immunoelectrophoresis (T-CIEP) analysis of trout plasma proteins. Immunoprecipitates form when the trout plasma proteins and the rabbit antibody are at the zone of equivalence. The peaks are visualized by Coomassie Blue staining and the sizes of the peaks in the two samples are determined. Differences in peak area indicate that the antigen differs in concentration in samples taken before and after injection of FCA. As seen in the hand drawn tracing of the gel, peaks 1 and 2 increased in concentration after treatment. Peak 3 decreased. The double α2-macroglobulin peak is marked with *.

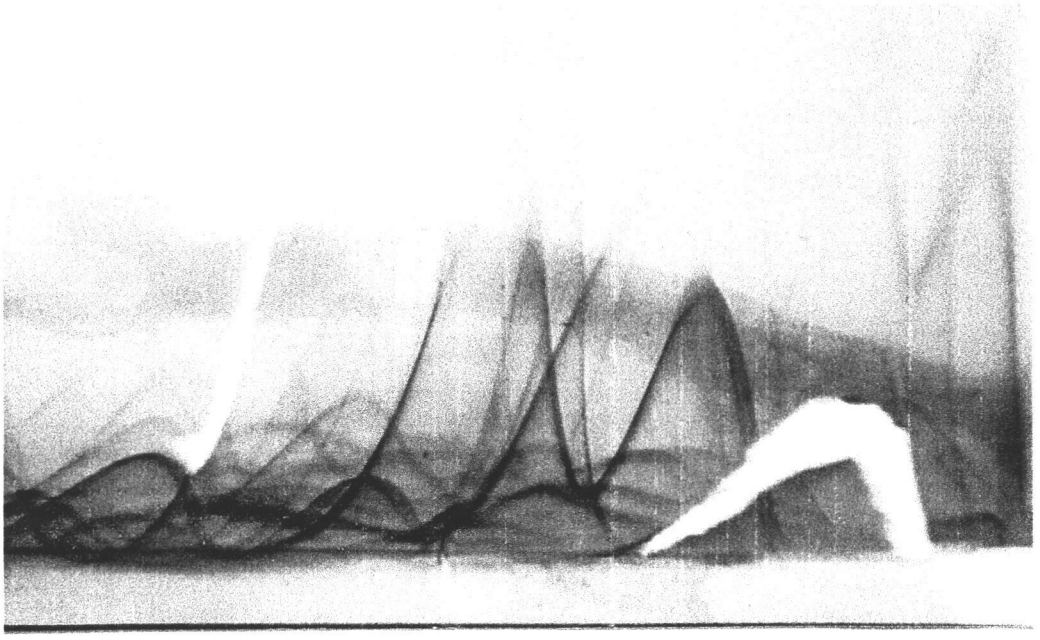

Figure 2.2. A gel from which peaks of interest have been excised with a clean scalpel and extracted in a buffer containing SDS and 2-mercaptoethanol.

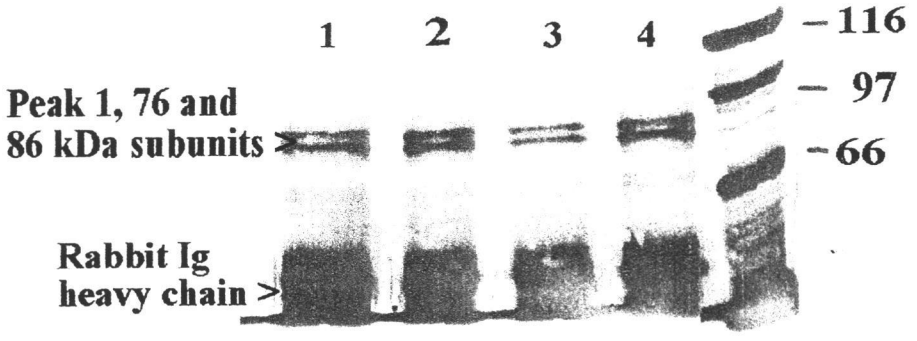

Figure 2.3 SDS-PAGE of extracted protein. Lanes 1-4 show the extracted protein from peak 1 cut from the TCIEP. Lane 5=molecular weight markers.

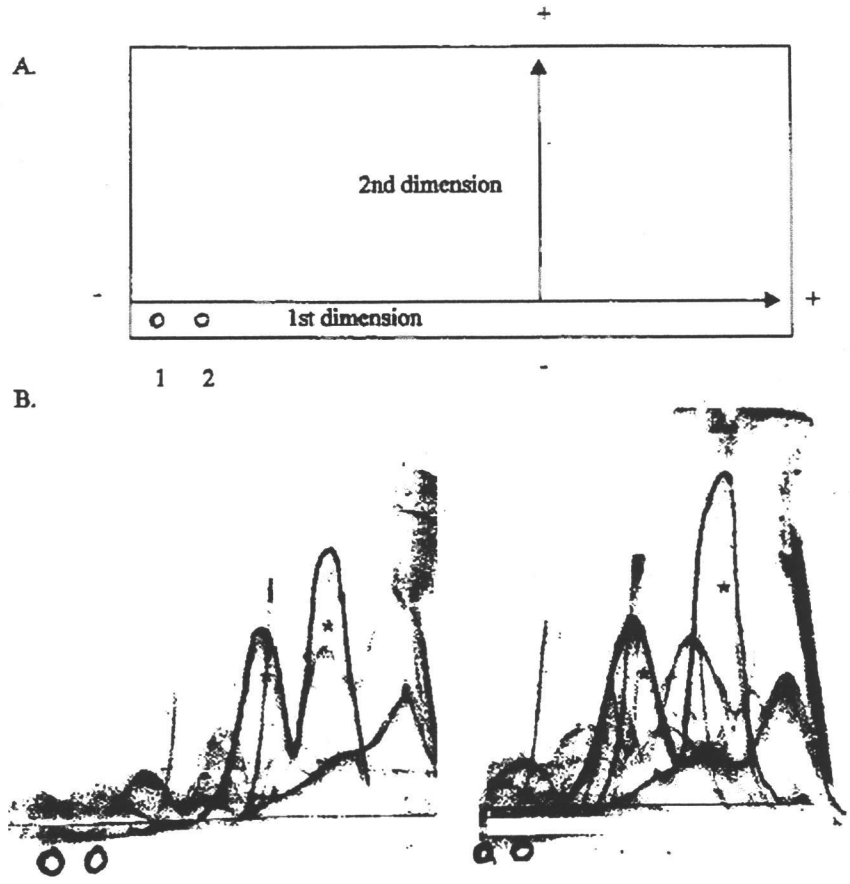

Figure 2.4. A. The diagram shows the two wells used and the direction of electrophoresis for the first and second dimensions on a T-CIEP gel. B. In the T-CIEP gel to the right, the plasma sample was "spiked" in the second well with 10μL of purified α2-macroglobulin. The α2-macroglobulin peaks are marked with *. The second α2-macroglobulin peak in the right gel (spiked sample) is increased relative to the first peak and to the second peak of the gel to the left.

A. **Peptide 1: 1849.7 Da**

Coho salmon (*Oncorhynchus kisutch*) (K)EADAIAVDGGEVFTAGK (1850.7 Da)

Rainbow trout (*Oncorhynchus mykiss*) (K)EADAIAVDGGEVFTAGK (1850.7 Da)

Peptide 2: ca.1180 Da

Coho salmon (*Oncorhynchus kisutch)* (K)NLLFKDSTK (1192.7)
Rainbow trout (*Oncorhynchus mykiss*) (K)NSLFKDSTK (1166.6)

B.

```
  1 mkllvsall gclatvyaap aegmvrwcvk sdkelqkchd laanvaqfsc vrrdnsleci
 61 qaikreeada itldggdiyi aglhnynlqp iiaedygeds dtcyyavava kkgtdfgfln
121 lrgkkschtg lgksagwnip igtlvtvgqi qwagiedrpv esavsdffna scapgankds
181 klcqlckvdc srshnepyyd yagafqclkd gagevafikh ltvpaaekas yellckdntr
241 apidsyktch lsrvpahavv srknpelanr iysklmaven fnlfssdgya aknlmfkdst
301 qnlvqlpmtt dsflylgaey mstirsltka qatgatsrai kwcavghnek vkcdawtins
361 ftdgdsriec qdaptvdeci kkimrkeada iavdggevft agkcglvpvm veqydavqcs
421 apgeassyfa vavakkgsgv twntlqgkrs chtglgrtag wnipmglihk etnncdftty
481 fskgcapgfe vdspfcaqck ggggsvggdr arciasseeq yygytgafrc lvegagdvaf
541 ikhtivpent dgsgpvwaqd lkssdfellc qdgttqpvtk frdchlakvp ahavitrpes
601 rgevvsille qqarfgssgs dssfnmfqsd lgknslfkds tkclqeipsg tkfqdflgee
661 ymiamqslre csnstsdlek actfhscqqk k
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Figure 2.5. A. The masses and proposed amino acid sequences of the two peptides, that were identified by means of electrospray mass spectrometry aligned below the same region of Coho salmon transferrin (Lee et al 1996). Trout has a serine in the place of leucine. B. The sequence of the complete rainbow trout transferrin (Tange, 1997) is shown with the peptides underlined.

DISCUSSION

Tandem Crossed Immunoelectrophoresis (T-CIEP) has proven to be a suitable means to obtain data on the relative concentrations of some of the plasma proteins that are involved in the APR of trout. The advantages of T-CIEP are that it is sensitive and cheap, and yields data on relative concentrations simultaneously

for a large number of proteins. One of the proteins that was found to be up-regulated at the protein level is a heterodimer of 76 and 86 kDa polypeptides. When samples from Day 8 after treatment were compared to those from non-treated fish, the peak area increased an average of 51.6%±3(SE). The two polypeptides were subjected to trypsin digestion while still in the gel, and the peptides were separated on a microbore HPLC column before being injected in to an electrospray mass spectrometer. The masses of two of the peptides from the 86kDa polypeptide yielded significant hits when the Swissprot database was searched. The peptides were nearly identical to masses of two peptides from Coho salmon transferrin (Fig 2.5). The peptides ended with the amino acid lysine which is expected for cleavage products of trypsin digestion.

Since the completion of our electrospray mass spectrometry analysis, the deduced amino acid sequence for rainbow trout transferrin has been published (Tange et al, 1997). The amino acid sequences of the two peptides that shared similar masses to the peptides in Coho salmon were completely conserved, except for one substitution of serine for leucine, in the rainbow trout sequences. Under reducing conditions on an SDS PAGE gel the isolated protein/proteins appeared as two bands with masses of 76 and 86 kDa. This could due be to the presence of two isoforms of transferrin differing slightly in amino acid composition or to differences in glycosylation. Transferrin is an acute phase reactant in rats and rabbits (Schreiber, 1989). If the up-regulated protein we see in the T-CIEPs is indeed transferrin then it is clearly an acute phase reactant in rainbow trout.

According to our data, α2-macroglobulin does not appear to be an acute phase protein in rainbow trout since no consistent pattern of up or down regulation was seen in our collection of plasma samples (Day 8 after treatment of five FCA-injected fish). This is in contrast to the rat, in which α2-macroglobulin is a major positive acute phase reactant (Okubo, 1981).

Purified trout immunoglobulin did not form a precipitate peak with the antisera. One reason could be that trout Ig is not immunogenic when injected into a rabbit. However, the more likely explanation could be that Ig migrates towards the cathode, thus disappearing off the gel to the left of where the sample was applied.

The second dimension gels work basically as an affinity chromatography step, and proteins are recovered in a highly purified state. However, only small amounts of protein can be recovered at one time and the extraction process is less efficient than desired. In other species, two other techniques have been used for comparison of samples from before and after different treatments. These are differential display (Liang & Pardee, 1992) and 2D electrophoresis using isoelectric focusing in the first dimension and SDS -PAGE in the second dimension. There are advantages and disadvantages to each of these approaches. With T-CIEP only proteins that were immunogenic will be precipitated in the gel. Successful visualization of a single protein with an electrophoresed sample requires both that it is immunogenic and that it moves to an appropriate place on the gel during 2^{nd} dimesional electrophoresis. Because of these requirements, an unknown number of proteins in a mixture may go undetected.

In conclusion, Rainbow trout, a teleost, is capable of mounting an Acute Phase Response following injection with FCA. We have found that transferrin appears to be an acute phase protein in trout wheras α2-macroglobulin appears not to be. Some proteins were consistently up or down regulated as evidenced by the T-CIEP analysis. In view of phylogenetic considerations, this model has the potential to reveal attributes not yet known of the primordial vertebrate immune system, and we will continue to investigate this aspect of its immunophysiology.

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CHAPTER 3

ISOLATION AND IDENTIFICATION OF RAINBOW TROUT PLASMA PROTEINS BY MEANS OF 1D AND 2D ELECTROPHORESIS, SEQUENCING AND ELECTROSPRAY IONIZATION MASS SPECTROMETRY

Lena Gerwick and Christopher J. Bayne.

ABSTRACT

In an effort to characterize the acute phase response in Rainbow trout (*Oncorhynchus mykiss),* we have injected trout with Vibrio bacterin emulsified in Freund's Incomplete Adjuvant. Blood samples were taken before injection and 3, 5, 7, 10, 14 and 21 days after the injection. We then used 1D and 2D electrophoresis to isolate some of the acute phase proteins. The maximum change in the plasma protein profile was seen around day 10-14. One band that was isolated from a 1D gel of a sample from day 14 contained a mixture of three proteins, and three N-terminal sequences were obtained. These sequences could not be satisfactorily aligned with anything in the databases. From a membrane blot of a 2D gel, three sets of protein spots were cut out and the membrane pieces were sent for sequencing. Three N-terminal sequences were obtained. Two of the proteins had the same N-terminal sequence and this matched the N-terminal of the iron binding protein transferrin. The third N-terminal sequence did not match anything in the database. The two proteins (approximately 47 and 76 kDa) identified as transferrin were also immunoreactive with an antibody to human transferrin. Five distinct protein spots were cut out from other 2D gels and subjected to trypsin digestion electrospray mass spectrometry. The resulting peptide masses did not match with any peptides in the databases. Before the techniques used here can be fruitful, it will be necessary that the organism under study has been described well at the molecular level. In the absence of such knowledge the identities of the proteins in question cannot be confidently assigned.

INTRODUCTION

Acute phase proteins are expressed during the early stages of the inflammatory response. They have been well described in some mammals but little is known about them in lower vertebrates. In order to describe the acute phase response in a non-mammalian vertebrate, we have been attempting to characterize some of these proteins in rainbow trout.

Two-dimensional electrophoresis is an excellent means of revealing differential protein expression in the same tissue at different times or during pathological states. Following electrophoretic separation, individual components can be further examined, such as by mass spectrometry and/or sequencing. The process from start to finish has been termed "Proteomics" (Figure 3.1) to distinguish it from "Genomics" a process that yields nucleotide sequence data. This technique has been well developed using human and mouse tissues (Frey et al 1996; Jungblut et al, 1999).

In two-dimensional electrophoresis, proteins are first separated by isoelectric focusing then by SDS PAGE in the second dimension. The first dimension separates the proteins by charge and the second step does this by size. The "spotty" pattern that appears after staining can then be compared to the control gel. Gels can also be compared to the Swiss-2D databank (http://expasy.nhri.org.tw/ch2d/) in which the identities have been assigned to many of the protein spots. The characterization of the different proteins is done by

sequencing or by mass spectrometry. Two mass spectrometry techniques have been developed: matrix-assisted laser desorbtion/ionization (MALDI-MS), and electrospray ionization (ESI-MS) (reviewed by Jungblut et al, 1996). In short the MALDI-MS technique allows an accurate determination of the molecular mass of the intact polypeptide whereas ESI-MS is performed on the protein after an enzyme digestion; micro-bore HPLC is used to separate the different fragments which are then analyzed in the mass spectrometer to determine the masses of the peptides. The masses of the different fragments can then be matched up with masses of peptides obtained by virtual tryptic digestion of proteins in the database. Nanospray ESI-MS, which measures peptide fragments with extreme sensitivity, can also measure a further fragmentation of the peptide so that individual amino acids cleaved off can be accounted for (Jensen, 1999). Amino acid sequences can be deduced by subtracting the different fragment masses, generated from the same peptide, from each other to yield the mass of the amino acid that was cleaved off. This is done sequentially to obtain an amino acid sequence. ESI-MS is more likely to lead to an identity if the protein has already been sequenced and is available in the databases.

Individual proteins in 2D gels can be isolated by cutting out the region of the gel containing the protein. Alternatively, proteins in a gel can be blotted onto a PVDF membrane and the spots on the membrane can be cut out. In the first case, the protein in the gel is subjected to enzyme digestion (trypsin digestion is common), and the resulting peptides are separated on a Microbore HPLC column.

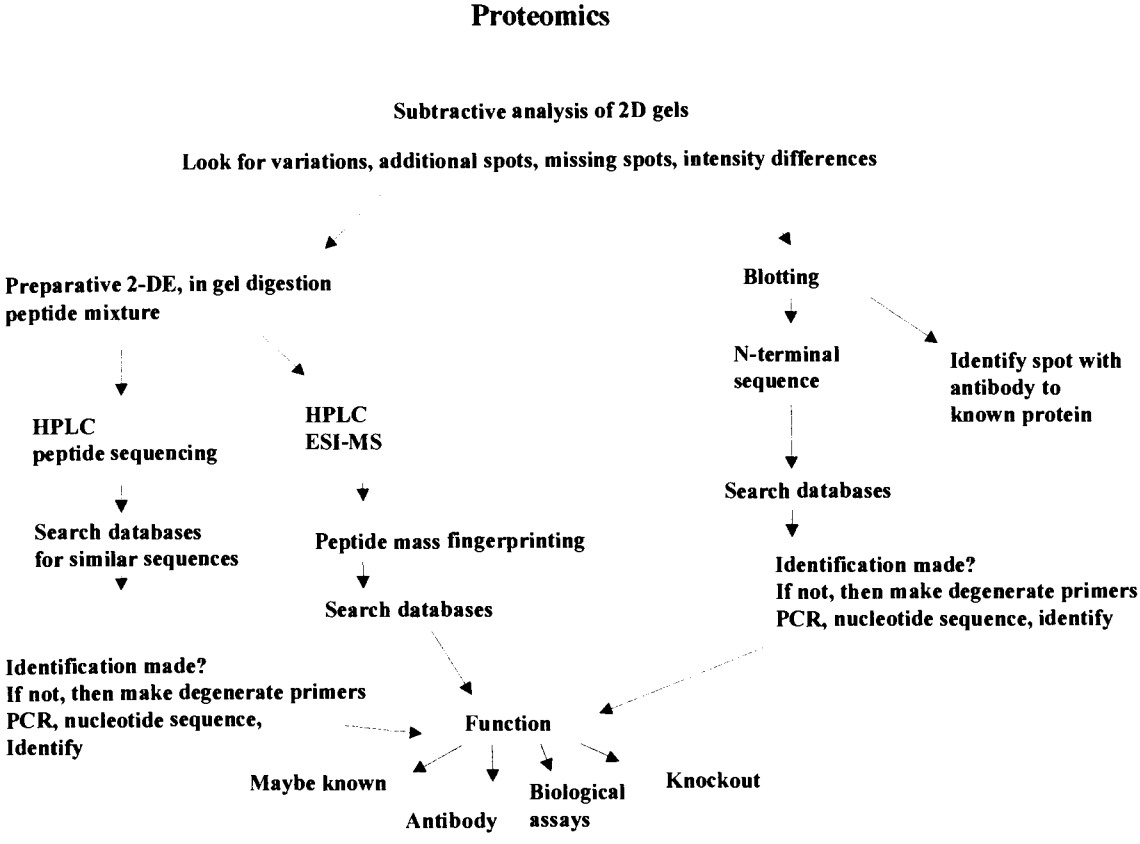

Figure 3.1. The flow chart shows the alternative paths that can be taken for identification of a particular protein when 2D gels are used for separation of a mixture of proteins. This approach to isolation and characterization has been named proteomics.

The individual peptides are sequenced using Edman degradation. In the second case, the membrane piece(s) can be used for N-terminal sequencing.

We have run 2D gels comparing the plasma protein profiles of rainbow trout (*Oncorhynchus mykiss)* from the same individual fish before and after induction of the acute phase response. Several protein spots were cut out and subjected to trypsin digestion and subsequent ESI-MS. Three gels were blotted

onto a PVDF membrane and three spots were cut out and sent for N-terminal sequencing. To further develop a 2D map of trout plasma proteins, antisera to two known plasma proteins were also used to detect their antigens on blots of 2D gels.

MATERIALS AND METHODS

Animals

Shasta strain rainbow trout (*Oncorhynchus mykiss*) were bred and raised at the Food Toxicology Laboratory, Oregon State University. The trout, 250-350 grams, were maintained on a 12 hour light/dark cycle with constantly flowing, single pass well water at approximately 12°C and fed three times weekly with Oregon Moist Pellet.

Bacterin

The bacterin for injection was made by the addition of formalin (0.4%) to a suspension of *Vibrio anguillarum* (scraped off one "lawn covered" plate) in PBS, followed by autoclaving to ensure the absence of surviving cells of this contagious salmonid pathogen.

Treatment

Seven fish were taken from a large holding tank, anesthetized with 2-phenoxyethanol (0.04%), bled (Day 0 control), marked by using a needleless injector loaded with Alcian Blue (0.1%) (Panjet, Wright Dental Group, Dundee, Scotland) and injected intraperitoneally (ip) with 0.2mL of *Vibrio anguillarum* bacterin emulsified in an equal volume of FIA. The fish were anesthetized and bled from the caudal vein on Days 3, 5, 7, 10, 14 and 21 after injection. A pre-bleed was done to provide a reference plasma sample from Day 0. The bleeds were done using heparinized syringes, and blood was stored on ice (about 30min) before being centrifuged 5min at 500g and 4°C. Plasma was aliquoted and stored at -80°C.

1D SDS PAGE

One sample of trout plasma taken before injection of Vibrio bacterin in FIA and five samples from fourteen days after treatment were loaded onto the 16 x 16 cm SDS PAGE gel (12% acrylamide). Each sample contained 173 μg protein, as calculated from the total protein content per milliliter (Coomassie Plus Total protein Assay, Pierce). The gels were electrophoresed on a Hoefer SE 600 electrophoresis apparatus (Pharmacia) at 100 V for 4-5h, followed by blotting onto a PVDF membrane (Immobilon-P^{SQ}) using a semidry blotter (Multiphor II, Pharmacia). One band, approximately 24kD, was cut out from four of the day 14 lanes. This band was absent in the Day 0 lane. The membrane pieces were sent for

N-terminal sequencing (Protein Core Laboratory, University of Texas Medical Branch, Galveston, Texas.)

2D gel electrophoresis

Immobiline isoelectric focusing strips (Pharmacia), 180 mm in length and with non-linear pH gradients from 3-10, were loaded with 500μg of plasma proteins, as determined by Coomassie Plus total protein assay (Pierce), and used for the isoelectric focusing step on a Pharmacia Multiphor II at 500V and 1 mA for 5 hours followed by 3500V and 1mA for 14.5 hours. Twelve percent polyacrylamide gels were used for the second dimension.

Three gels were blotted onto Immobilon-P^{SQ} transfer membrane (Millipore) using the Multiphor II semidry blotter (Pharmacia) and stained with Coomassie Blue. Three spots, from 2Dgels, that appeared to be present in larger amounts in the acute phase sera were sent for N-terminal sequencing (Protein Core Laboratory, University of Texas, Medical Branch, Galveston, Texas).

Trypsin digest and mass spectrometry:

Five spots and one blank piece of gel were cut out from a 2D polyacrylamide gel. The volumes of the gel pieces were calculated by measuring the sides of the piece and multiplying that number (area) by 0.5 mm (the gel

thickness). We followed the protocol developed by the Keck laboratory, Yale University (Papov et al, 1997). Briefly, the gel pieces were placed in tubes prewashed with 0.1% triflouro acetic acid (TFA) mixed in 60% acetonitrile. The pieces were washed in 50% acetonitrile (CH_3CN) in a 200mM ammonium bicarbonate (NH_4HCO_3) solution. A final solution of 1mM of the reducing agent DTT was added and the pieces incubated for 30 minutes followed by methylation of the free sulfhydryl groups with methyl 4-nitrobenzene sulfonate. To be able to get the trypsin into the gel pieces they were spun on a Speedvac (Savant) to complete dryness. Modified trypsin (Promega) at a concentration of 0.5μg (stock solution 0.1mg/mL) per 15 mm^3 and 15μl of 200 mM ammonium bicarbonate per 15 mm^3 was added to the dry gel pieces; this was followed by an incubation at 37°C for 24hrs. The digested peptides were extracted by adding 0.1% of TFA and 60% CH_3CN and shaking at room temperature for 60 minutes. The extraction buffer containing the peptides was collected into prewashed tubes. The samples were spun dry on a Speedvac (Savant) and the residue was dissolved in 20μl of 0.05% TFA in 25% CH_3CN aqueous solution. Solubilized this way, the samples were ready for HPLC and mass spectrometry, which was performed by Dr. C. Maier (Oregon State University, Mass Spectrometry facility).

Identification of 2D gel spots

In order to identify specific antigens in 2D gels, antibodies to trout α2-macroglobulin (C.J. Bayne) and human transferrin (Sigma)were used to probe

membrane blots. The proteins in the gels were transferred using a semidry blotter (Biorad) onto a PVDF membrane (Millipore). The membranes were washed in Tris buffered saline with 0.2% Tween and incubated with 1:500 dilution of the α2-macroglobulin antibody, or 1:1000 of the transferrin antibody. Secondary antibodies with conjugated alkaline phosphatase were used with the α2-macroglobulin and transferrin antibodies. These were developed with the colorimetric method using Nitro Blue Tetrazolium (NBT) and Bromochloro-3-indoyl-phosphate (BCIP).

To further confirm the location of these two proteins, purified trout α2-macroglobulin (from R.C. Bender) was run on a 2D gel and the spots that immuno-reacted with the transferrin antibody were cut out and sent for N-terminal sequencing.

RESULTS

One dimensional gels comparing the protein profiles in samples before and after treatment with Vibrio bacterin emulsified in FIA revealed several changes in the banding pattern. Two bands at approximately 22 and 23 kDa were first evident on day 7 (Figure 3.2). These bands were consistently upregulated in all five fish. To prepare samples for sequencing, four samples from day 14 were electrophoresed, and the gel was blotted onto a PVDF membrane. Only one of the two anticipated bands could be seen on the blot. Membrane containing this band

was cut out and sent for sequencing. The membrane pieces, not surprisingly, contained a mix of three proteins. The probable N-terminal sequences were assembled by looking at the relative concentrations of the peaks coming off the sequencer. The three sequences are: 1. APAPELAGGHLVKLLAQEDQHTIFGK, 2. MIRGTDXPVVAWQAMVYLXKXMAG, 3. DQVKNHXHDKKPIXVT. When performing a Blast search using BLASTP no significant hits were found.

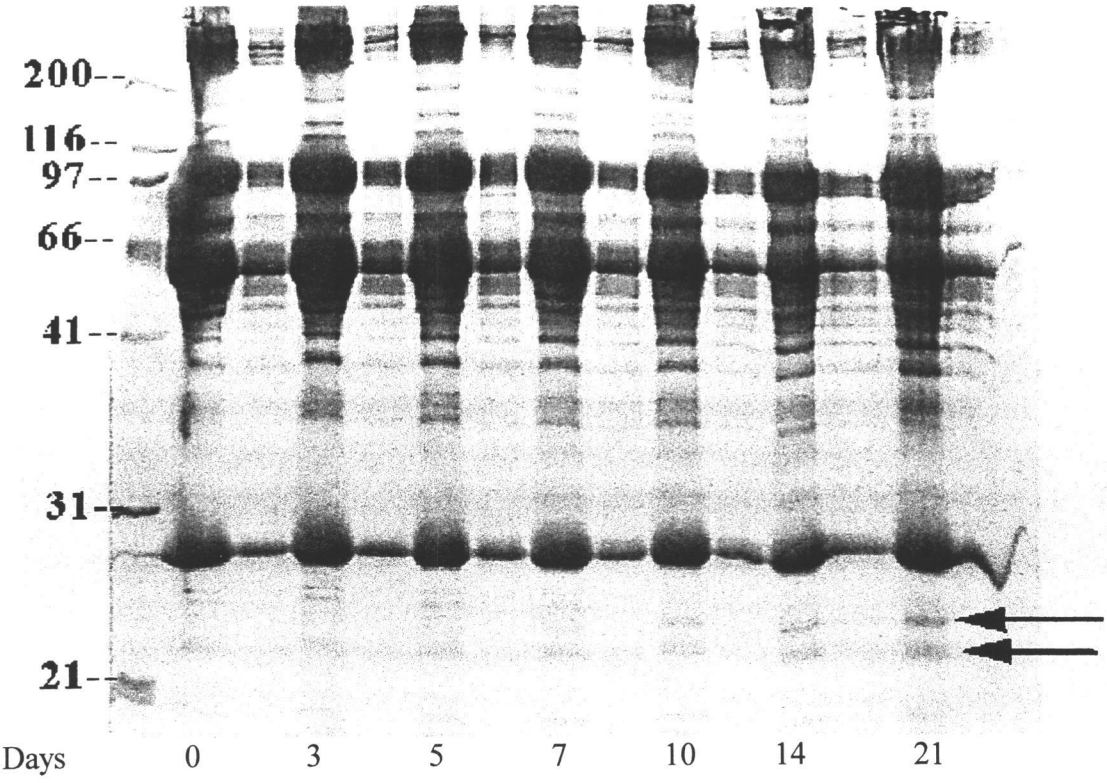

Figure 3.2. A 1D SDS PAGE showing the two upregulated bands (indicated with arrows) that, when blotted onto a PVDF membrane, appeared as one band. That band was sent for N-terminal sequencing and three different sequences were obtained.

When 2D gels were used to compare plasma samples before and after treatment, at least 4 spots of approximately 200 were larger on the gels containing plasma samples collected after the Vibrio bacterin treatment. After electrotransfer of the contents of a 2D gel to a PVDF membrane and staining with Coomassie Blue, one spot at approximately 28kD and with a PI of 4.5 to 5 was cut out and sent for sequencing (Figure 3.4). To obtain enough protein for sequencing, three identical gels were run and blotted. The membrane pieces contained one dominant protein. A second protein was present but only in trace amounts. The N-terminal sequence of the dominant was DEPKPQLEQLTDAFXXT. A BLASP search revealed no significant hits.

The peptides obtained by trypsin digestion were separated on a 0.3X300 mm Vydac 218TP C18 protein/peptide HPLC column and automatically sent through the ionization mass spectrometer (Perkin Elmer Sciex API III+ triple quadrupole Ionspray mass spectrometer). Spectra were analyzed using the MacSpec 3.3 program. We were able to see reliable peptide peaks (Figure 3.3). However, when the masses of these peptides were analyzed with the PeptIdent mass finger printing program (http://expasy.nhri.org.tw/tools/peptident.html) no significant hits were found.

The mapping of a rainbow trout 2D gel was started by electrophoresing purified α2-macroglobulin and by probing blotted 2D gels with antibodies to α2-macroglobulin, and human transferrin. The α2-macroglobulin was located as a horizontal string of proteins at around 100 kDa. The human transferrin antibody

recognized four different spots, two discrete spots parallel to each other around 50kDa and two at about 45kDa. The four spots make the corners of a square (Figure 3.4). In addition to these four spots, the transferrin antibody also reacted with a set of proteins forming a horizontal string around 76 kDa. This string of proteins had already been identified as a possible acute phase reactant since the set of spots was increased in size on the 2D gels containing plasma from day 10 after treatment. When the set of protein spots was measured (pixel count, Pharmacia Imagemaster) on eight gels representing four individual fish, before and after treatment with Vibrio bacterin, upregulation was calculated to be 1.4, 3, 39 and 1.1 fold.

The spot in the lower left corner of the four spots that formed a square was cut out from two PVDF membranes and five of the spots from the horizontal string were cut out from the same membranes and sent for N-terminal sequencing. Both sequences were confirmed to be transferrin (Figure 3.4).

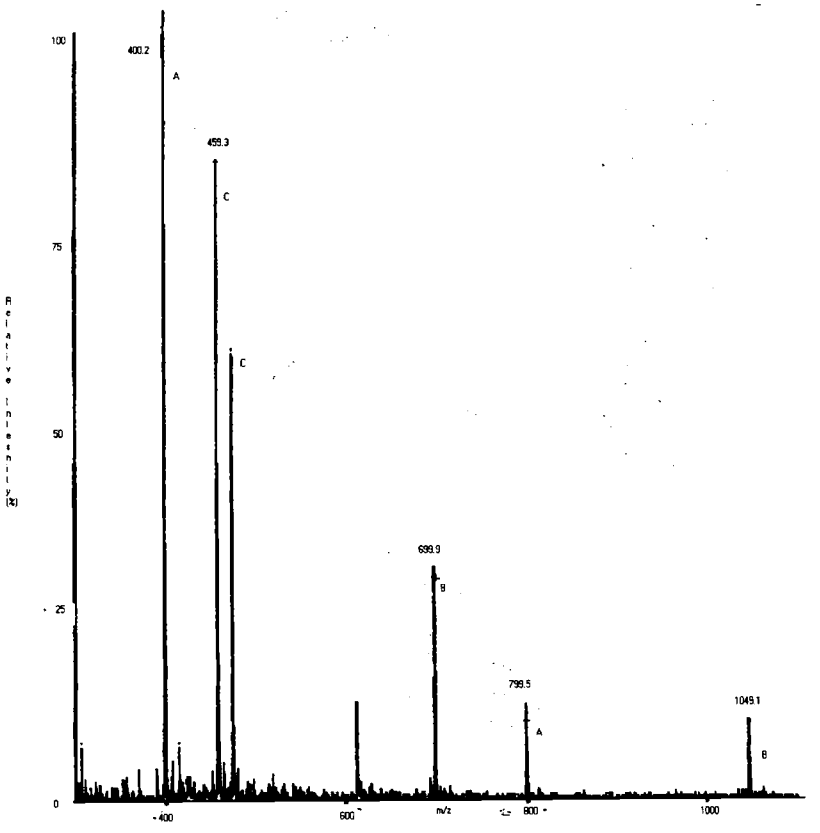

Figure 3.3. The m/z scan of two of the peptide fragments from one of the proteins subjected to trypsin digestion and ESI mass spectrometry. A= the same peptide, but the peak to the left carries a 2+ charge and the one to the right a 1+ charge. This peptide therefore has a mass of 799.5 Da. B= a second peptide; the peak to the right has a charge of 3+ and the peak to the right a 2+ charge. This gives a mass for the peptide of 2096.4 Da. C= peaks found in the blank also. The blank represents a piece of acrylamide gel that does not contain any protein spot.

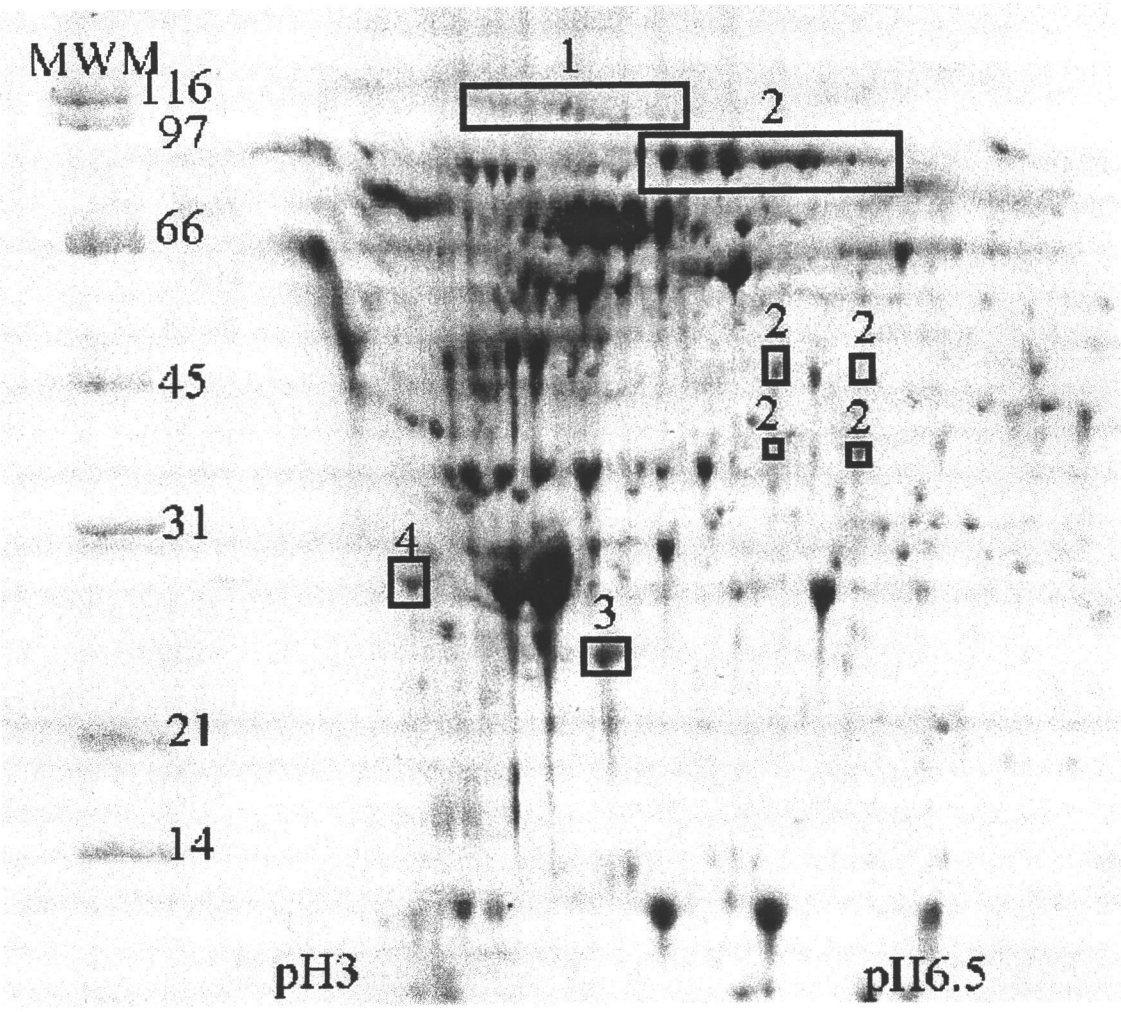

Figure 3.4. Plasma proteins from Rainbow trout had been injected with Vibrio bacterin 10 days prior to bleeding. Separation was achieved with 2D electrophoresis. Boxed regions indicate the location of 1. α2- macroglobulin, 2. Transferrin, 3. Precerebellin-like protein (see chapter 4) and 4. protein with N-terminal sequence: DEPKPQLEQLTDAFXXT.

DISCUSSION

By comparing the concentrations of specific proteins in plasma samples taken before and after injection with an inflammatory stimulus we have determined that rainbow trout change their plasma protein profile after elicitation with FCA (see Chap. 2 of this thesis) and Vibrio bacterin emulsified in FIA. As mentioned in the methods and materials ` By N-terminal sequencing of one band (visible only after treatment) cut from a 1D gel, we obtained sequences for three proteins. Since this band was evident only after elicitation with an inflammatory agent and not changed on gels with plasma from fish that were bled but not injected, at least one of the N-terminal sequences we obtained must be from an upregulated protein. At this point we are not able to determine which one. This clearly demonstrates the shortcomings of using 1D gels for isolation of proteins. The added resolution that is achieved using 2D gels, in which proteins are separated on the basis of charge as well as size, should produce cleaner samples. However, the membrane spots cut out from a 2D membrane also contained two proteins, one being at a lower concentration.

The sequences we obtained from the proteins isolated from 1D and 2D gels are probably correct since the proteins were present in different concentrations so that peaks coming off the sequencer could be grouped into different sequences. However, the subjectivity of this method leaves room for improvement. It is not surprising that the N-terminal sequences did not line up with likely homologs in the databases because the sequences were in general short (26-17 aa) and many trout

proteins are yet not sequenced, much less entered into the databases. Under these circumstances, the short N-terminal sequences have to line up with homologs in another species. In view of the possibiltity that the N-terminal might not be highly conserved, the matches would seldom be precise. More sequence data could, however, be obtained by PCR using degenerate forward primers based on the N-terminal sequences and, since all the proteins isolated are 25kD or less, an oligo dT reverse primer (Frohman, 1990) could be used.

Trypsin digestion, followed by HPLC and electrospray mass spectrometry of the resulting peptides, yielded some clear peptide peaks when the spectra were analyzed and precise mass data were obtained. However, use of such data to identify proteins requires that the sequence first be present in the databases so that exact matches can be obtained. As mentioned before, just a small fraction of the proteins in trout, and in particular plasma proteins, have been sequenced. The chance of finding the right match is therefore remote. Two dimensional electrophoresis followed by ESI-MS has been successfully used with organisms in which most of the genome has been sequenced and entered into the database, such as *E.coli*, mice and humans (Wilkins et al, 1998; Frey et al , 1996; Jungblat et al, 1999).

We were able to identify three of the more than 200 spots on our 2D gels (Figure 3.4). Alpha2-macroglobulin appeared as "pearls on a string", indicating that it contains several isoforms, either due to differential glycosylation or to amino acid substitutions (Aramburo, 1991).

Transferrin appears to have three mass isoforms and at least ten PI isoforms. At least six of these appear as "pearls on a string" at around 76kDa, however some of these spots are shifted slightly towards a higher molecular weight. This compares well to the data obtained in chapter 1 of this thesis, where two bands were seen on a 1D gel. The molecular masses obtained from the 1D gel, 76 and 86 kDa, were about the same as those seen on the 2D gel (76 kDa). When the mass of the published trout transferrin sequence was calculated using the program Peptidemass (http://expasy.nhri.org.tw/tools/peptide-mass.html) the molecular weight for rainbow trout transferrin was 75kDa. The other spots that were recognized by the antibody to human transferrin, and were confirmed as transferrin by N-terminal sequencing, have an estimated mass of 50kDa. Transferrin sequences of two different lengths (517aa and 672aa) have been found in Coho salmon (*Oncorhynchus kisutch*) (Ford, 1999); these two different length polypeptides could correspond to the different mass isoforms that were seen in rainbow trout. Early reports (Suzumoto, 1977) indicated that different transferrin genotypes in Coho salmon seemed to confer different levels of resistance to bacterial kidney disease suggesting that the iron scavenging ability of transferrin could be important for resistance. The many different glyco- and mass isoforms that we see in trout plasma, and that others have identified in human plasma (http://expasy.nhri.org.tw/cgi-bin/map2/def?PLASMA_HUMAN) could be a result of selective pressure in the arms race against pathogens. Some pathogens are known to have transferrin receptors which bind transferrin so that the iron bound to

transferrin can be utilized. If the host produces a variety of transferrins this may enable it to out-compete the potential pathogen for iron. Ford (1999) compared mutation rates of transferrin in five different teleost species and concluded that there is a selective pressure on this protein and that it appears to be forced by pathogens.

Transferrin was found to be inconsistently up regulated (1.4, 3, 39 and 1.1 fold), in four trout ten days after treatment. Two of the four show good upregulation but two are only marginally upregulated or not at all. This is in contrast to the findings in Chapter 2, where five fish injected with FCA all showed upregulation seven days after treatment. The differences could be due to the agent injected or the different techniques used. However, it does appear that transferrin is an acute phase protein in trout.

In conclusion, we have used 1D and 2D gel electrophoresis followed by either sequencing and/or ESI-MS to help us identify acute phase plasma proteins in rainbow trout plasma. Six N-terminal sequences were obtained and two of these were identified as transferrin. At this point sequencing should be the prefered route. ESI-MS will be a more reliable technique when more trout plasma proteins have been entered into the databases since that method requires exact masses for the peptide fragments to be matched.

ACKNOWLEDGMENTS

We like to thank the Food Toxicology Laboratory at Oregon State University for supplying the rainbow trout and the laboratory of Virginia Weis for help in developing the 2D protocol. We also like to thank the EHSC for financial support (NIEHS grant #T32 ES07060) and for use of the mass spectrometry facility. We especially like to thank Dr. Claudia Meier for separating the trypsin-digested peptides and then running the ESI-MS experiments.

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CHAPTER 4

A PRECEREBELLIN-LIKE PROTEIN IS PART OF THE ACUTE PHASE RESPONSE IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*.

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24(6-7): 597-607

Abbreviations: aa = amino acids, FIA = Freund's Incomplete Adjuvant, RACE = rapid amplification of cDNA ends, ip = intraperitoneally, kD = kilodalton, APR = acute phase response, APP = Acute Phase Protein, pcb = precerebellin-like protein, LPS = lipopolysaccharide, TGF-α = transforming growth factor-α, TGF-β = transforming growth factor-β, IL-1 = interleukin 1, IL-6 = interleukin-6, ORF= open reading frame

ABSTRACT

The acute phase response (APR) has a long evolutionary history, but it remains to be characterized fully in lower vertebrates. To study the acute phase proteins of a teleost, rainbow trout (*Oncorhynchus mykiss)*, we induced an APR by injecting *Vibrio* bacterin emulsified in FIA. In samples taken over the next 3 weeks, the total plasma protein profile changed consistently as seen in one and two-dimensional SDS PAGE. One 18.1kDa upregulated protein was isolated from 2D gels and an N-terminal sequence obtained. Using reverse transcriptase-PCR, a 700 bp cDNA sequence was amplified. The sequence is 53% similar at the amino acid level with rat precerebellin (regions aa 42-184 from trout and aa 89-224 precerebellin), and 46% similar with the globular portion of the human B chain of the first complement component C1q. However, it lacks the collagen portion of C1q with its characteristic Gly-X-Y repeats. The isolated protein seems to be involved in the inflammatory response but its physiological function is unknown.

INTRODUCTION

The acute phase response is an early and complex protective response of the body to a traumatic insult. Its attributes include increases in the quantities of some plasma proteins (positive APPs) and decreases of others (negative APPs). Bacterial LPS (lipopolysaccharide), TGF-ß (transforming growth factor-ß), mast cell degranulation, turpentine and degradation products of opsonins can stimulate the release of initiating factors for this response from macrophages and monocytes in mammals (Baumann and Gauldie, 1994). IL-1 (Interleukin-1), TNF- α (tumor necrosis factor-α) and IL-6 (Interleukin-6) appear to be the factors most important for the initiation of the acute phase response since they start the process that leads to leukocyte migration into the affected tissue, increase the permeability of the capillary bed, and induce the liver to alter the transcription of the acute phase proteins (APP).

Several of the mammalian positive acute phase proteins have been identified, but it is anticipated that more are still unknown because the response is complex and has received only marginal attention. The identified proteins include fibrinogen, C- reactive protein (CRP), α-2 macroglobulin, serum amyloid A (SAA), serum amyloid P (SAP), mannose-binding protein A (also known as mannose binding lectin) (Sastry et al, 1991) and complement component 3 (C3). Several additional complement components may be positive APPs, since the transcription of mRNA for complement components 2 and 4 (C2 and C4), which occurs in the liver, increases with LPS stimulation (Colten, 1991).

The physiological functions of several of these proteins are only partially determined. Fibrinogen is involved in the clotting process, C3 is an opsonin and

initiator of complement-mediated lysis, and α2-macroglobulin is an anti-proteinase, possibly with additional transport functions. CRP can bind to bacterial cell wall components and chromatin from "self" and "non-self", and mannose-binding lectin binds to glycans on the surfaces of microorganisms. However, for several of the other proteins (for example serum amyloid A) the functions are still relatively unknown. It is interesting to note that in mammals SAA, CRP or SAP can increase 1000-fold within hours of an insult and that they remain elevated for only a few days (Steel and Whitehead, 1994) suggesting that, whatever their functions are, they seem to be critical during a brief period (Baumann and Gauldie, 1994).

Several of the proteins considered to be APPs in other species have been isolated from salmonids and characterized, including a pentraxin (Jensen et al, 1995), C3 (Sunyer et al, 1997) and α_2-macroglobulin (C. Bayne's lab, OSU, 1994). C1 has also been isolated from a teleost (carp) (Nakao and Yano, 1998) but no sequence data were obtained, and from nurse shark (Dodds et al, 1993). The isolated shark C1q was N-terminally sequenced (Smith, 1998).

Salmonids possess an acute phase response as illustrated by the 1D and 2D gels run in our laboratory. Furthermore, there is a substantial increase in mRNA levels of SAA in Arctic char after exposure to *Aeromonas salmonicida* (Jensen et al, 1997). This suggests that the acute phase response in rainbow trout, and potentially all teleosts, resembles the mammalian response. However, questions regarding the functions, kinetics and sites of production, and total changes in concentration of the various acute phase proteins remain to be answered.

In this study, we isolated an upregulated protein from a 2D gel of rainbow trout acute phase plasma. One region of the protein, from aa 42-184, has 29% and 28% identity, at the amino acid level, with the region from aa 89-224 of rat

precerebellin, and with the globular region of the human C1q B chain. As determined by the cDNA sequence obtained, the protein lacks the GLY-X-Y repeats in the collagen region that characterize the C1q B chain in other species. It is also synthesized in the liver, which is in contrast to the site of synthesis in mammals (macrophages). Upregulation is detected at the protein level three days after treatment with autoclaved *Vibrio anguillarum* bacterin emulsified in FIA. The protein remains elevated in concentration even 21 days after treatment. Comparison of its N-terminal sequence with that of the putative C1q chain from nurse shark (Smith, 1998) revealed no similarity at the protein level. However, a 44% similarity could be seen when the N-terminal from the globular region of the shark protein was compared to that of the precerebellin-like protein of rainbow trout.

MATERIALS AND METHODS

Animals

Shasta strain rainbow trout (*Oncorhynchus mykiss*) were bred and raised at the Food Toxicology Laboratory, Oregon State University. The trout, 250-350 grams, were maintained on a 12 hour light/dark cycle with constantly flowing, single pass well water at approximately 12°C and fed three times weekly with Oregon Moist pellet.

Bacterin

The bacterin for injection was made by the addition of 0.4% formalin to overnight cultures of *Vibrio anguillarum*, followed by autoclaving to ensure the absence of surviving cells of this contagious salmonid pathogen.

Treatment

Seven fish were taken from a large holding tank, anesthetized with 2-phenoxyethanol (0.04%), bled (Day 0 control), marked by using a needleless injector loaded with Alcian Blue (0.1%) (Panjet, Wright Dental Group, Dundee, Scotland) and injected intraperitoneally (ip) with 0.2mL of *Vibrio anguillarum* bacterin emulsified in an equal volume of FIA. The fish were anesthetized and bled from the caudal vein on Days 3, 5, 7, 10, 14 and 21 after injection. A pre-bleed was done to provide a control from Day 0. The bleeds were done using heparinized syringes, and blood was stored on ice (about 30 min) before being centrifuged at 500g and 4°C. Plasma was aliquotted and stored at -80°C.

1D SDS PAGE

Total protein concentrations were determined using the Coomassie Plus Assay (Pierce) and the appropriate dilutions calculated to allow 173 μg and 39.6 μg of each plasma sample from Day 0 (before injection) and Days 3, 5, 7, 10,14 and 21 after injection to be loaded onto the 16 x 16 cm SDS PAGE gel (12%

acrylamide). The high (173 μg) and low (39.6μg) concentrations from the same sample were run in lanes next to each other. The gels were electrophoresed on a Hoefer SE 600 electrophoresis apparatus (Pharmacia) at 100 V for 4-5h, followed by staining with Colloidal Coomassie (Neuhoff et al, 1990), photographed digitally, and analyzed using Kodak 1D analysis software for absence or presence, and increase or decrease of band intensities between the different time points.

2D gel electrophoresis

Immobiline isoelectric focusing strips (Pharmacia), 180 mm in length and with non-linear pH gradients from 3-10, were loaded with 300μg of plasma proteins, as determined by Coomassie Plus total protein assay (Pierce), and used for the isoelectric focusing step on a Pharmacia Multiphor II at 500V and 1 mA for 5 hours followed by 3500V and 1mA for 14.5 hours. Prepoured gels (Pharmacia Excelgel XL 12-14 %, 245 x 180 x 0.5 mm) were used for the gels that were subjected to image analysis (Pharmacia, Image Master). These gels contained individual plasma samples from 5 fish that were bled before and 10 days after treatment. We poured our own gels (12%, 0.5 mm thick) for isolation of the precerebellin-like protein. Gels were fixed with 12% trichloroacetic acid and stained with Colloidal Coomassie (Neuhoff et al, 1990).

Peptide and N-terminal sequencing

Several proteins were identified that increased in concentration following the treatment. One of these proteins was excised from several identical gels after the second dimension separation, and sent for trypsin digestion and peptide sequencing (Protein Core Laboratory, University of Texas Medical Branch, Galveston, Texas). Four peptide sequences, each 8-9 residues long, were obtained. Three additional gels were blotted onto Immobilon-P^{SQ} transfer membrane (Millipore) using the Multiphor II semidry blotter (Pharmacia) and the spot of interest was sent for N-terminal sequencing (Protein Core Laboratory, University of Texas, Medical Branch, Galveston, Texas). A sequence of twenty-four amino acids was obtained (ESEVDGLLRELTARVEKLESKPKV).

Degenerate primers

Two degenerate primers, from The Great American Gene Company (Ramona, CA), were made from the N-terminal sequence ESEVDGLLRELTARVEKLESKPKV (primer regions are underlined) (Table 4.1), using inosine as a neutral base for the degenerate codons.

PCR

Five rainbow trout (20-60 grams) were injected ip with 0.2 mL of *Vibrio* bacterin in FIA and the livers and head kidneys were harvested five days after the treatment. Total RNA was isolated from both liver and head kidney using RNA Stat-60 (Tel-Test "B" Incorporated, Friendswood, Texas) and mixed in a 1:1 ratio. Five μg of total RNA was used for cDNA production using an oligo (dT) primer and the Gibco BRL Superscript Preamplification system. A PCR product within the predicted size range was obtained using 5 μM oligo (dT) primer and 100 μM of the degenerate Nterm 1a+1b (ESEVDG N-terminal primer) (Table 4.1), denaturing for 3 minutes at 94°C, annealing for 45 seconds at 49°C and extending for 2 min and 30 seconds at 72°C. The thermocycler (PTC-100, MJ research, Inc.) was run for 35 cycles.

Nested PCR was performed using the degenerate primer constructed from the sequence TARVEK (2nTerm). This forward primer was used at a concentration of 2.5 μM and the reverse oligo (dT) primer at 0.25μM. The previous PCR product was used as the template at a dilution of 1:200, and the annealing temperature was 51°C for 35 cycles. The PCR product, from the PCR reaction mixture, was cloned into the pCR 2.1 vector using the TA-cloning kit (Invitrogen). Three of the clones were sent for forward and reverse sequencing, performed by the Oregon State University Central Services Laboratory, Corvallis, Oregon.

5' Rapid Amplification of cDNA Ends (5' RACE)

In order to obtain the nucleotide sequence upstream from the N-terminus, a 5'RACE reaction was performed using a protocol modified from Frohman et al, (1990). Two new reverse primers were made from the midsection of the known nucleotide sequence (from previous nested PCR):
5'CCGCCGTGGTAAATACCGG3' (SGI rev primer) and
5'AATGTGAAGTAAGTAGGCCCCT3' (GAY rev primer). cDNA was made from total liver RNA using the SGI reverse primer (Gibco Superscript preamplification kit). A poly-G tail was added to the 5'end using Terminal Deoxynucleotidyl Transferase (Gibco). An initial PCR reaction used a poly-C primer and the SGI reverse primer both at final concentrations of 0.125 μM and an annealing temperature of 52°C for 50 cycles. The nested reaction was run with the GAY rev and RCC (poly C) primers at final concentrations of 0.025 and 0.0125 μM respectively and an annealing temperature of 56°C for 35 cycles.

The resulting PCR product was ligated into the pCR 2.1 vector using the Invitrogen TA cloning kit. Three clones were sequenced by the Oregon State University Central Services Laboratory.

Phylogenic analysis

The phylogenetic analysis was performed with the Phylip distance program using the neighbor joining method.

Primer name	Nucleotide position	Nucleotide Sequence and Amino Acid sequence (where appropriate)
Nterm1a	114-131	5'GA(A,G)TC(C,T)GA(A,G)GTIGA(C,T)GG3' E S E V D G
Nterm1b	114-131	5'GA(A,G)AG(C,T)GA(A,G)GTIGA(C,T)GG3' E S E V D G
2nTerm	148-165	5'AC(C,T)GCI(A,C)GIGTIGA(A,G)AA3' T A R V E K
Oligo dT	Downstream of 700	5'CTCTAGAACTAGTCTTTTT3'
RCC	Upstream of 1	5'TGTGGACGAATTCCCCCCCCCCCC3'
SGI rev (antisense)	510-529	5'CCGCCGTGGTAAATACCGG3' G G H Y I G S
GAY rev (antisense)	394-414	5'AATGTGAAGTAGTAGGCCCCT3' F T F Y Y A G

Table 4.1. Names, nucleotide positions and sequences of all the primers used. Amino acid sequences are shown, when appropriate.

RESULTS

1D gels

Plasma samples from Day 0, 3, 5, 7, 10, 14 and 21 from five fish injected with autoclaved *Vibrio anguillarum* in FIA were electrophoresed in one dimension. The protein banding patterns at the different time points were analyzed using the Kodak 1D analysis program (Figure 4.1). The strongest evidence of upregulated proteins was seen in samples from Day 10 and 14. The samples from Day 10 were used initially for the 2D electrophoresis. Two samples from Day 3 were also used for 2D gels.

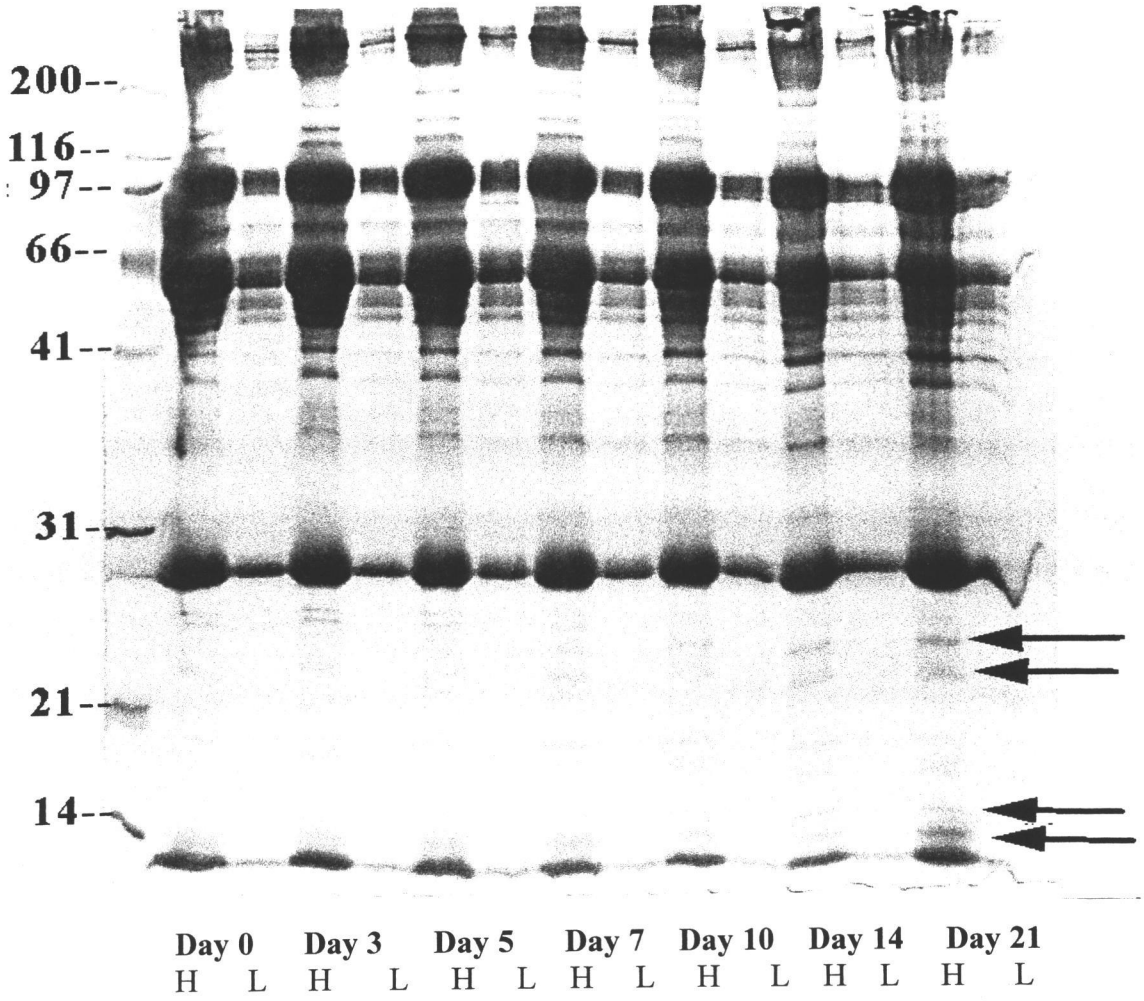

Figure 4.1. One-dimensional non-reduced 12% SDS PAGE gel showing plasma samples from the same trout taken at Day 0, before treatment, and Days 3, 5, 7, 10,14 and 21 after treatment. Total protein concentration was determined from the Coomassie Plus Assay (Pierce) and 173 μg (H=heavy load) and 39.6 μg (L=light load) of each sample were loaded onto the gel. The arrows indicate some of the proteins that increased in concentration following injection of *Vibrio* bacterin into the fish.

2D gels

A prominent spot could be seen at approximately 24 kDa and PI of 5-6 in all five of the 2D gels containing plasma from treated fish. It was also visible in both of the samples from fish bled on Day 3 and in five of the fish bled on Day 10 ("A" Figure 4.2). The protein spot, as measured by image analysis, increased from 0% (absent) to 1.25% ± SE 0.19 (N=5) of the total pixels representing all Coomassie-stained proteins on the gel (Table 4.2). The relative amounts of the protein on Day 10 were calculated by multiplying the % of pixels representing the spot by the total protein concentration (determined by Coomassie Plus method) for the different samples. This calculation method is only an approximation since some proteins on the gel are not visible with the Coomassie stain (Table 4.2).

Initial and nested PCR

The first PCR reaction utilized the most N-terminal degenerate primer (mixture of Nterm 1a+1b) and an oligo (dT) primer (Table 4.1) (Figure 4.3) using as template a mixture of cDNA made from liver and head kidney total RNA. The resulting reaction yielded several bands as visualized on a 1% agarose gel with ethidium bromide. A gene-specific PCR product was obtained by performing a nested PCR with the second N-terminal (2nTerm) degenerate primer down stream from the first primer and an oligo (dT) primer as the reverse primer, resulting in an approximately 500bp PCR product. Three clones were sequenced. The sequences

were aligned using the GCG10 program and a consensus sequence was constructed. An Advanced Blast search of the predicted peptide sequence revealed 29, 30, 28% identities, and 53, 43, and 46% similarities with a portion of rat precerebellin and with the globular portion of the complement component 1q B chain of mouse and human respectively. All the sequences from the previously sequenced internal peptides could be found within the full length deduced peptide sequence, further supporting the correctness of the sequence.

The PCR reactions to this point had been performed on a mixture of cDNA from head kidney and liver. Subsequent PCR reactions with separate cDNA from the two sources revealed a band in the cDNA from liver and no band in the head kidney reaction. The following 5'RACE reactions were subsequently performed only on liver cDNA.

Since the precerebellin-like protein is a plasma protein and therefore secreted from the cell, we wanted to determine the sequence upstream from the N-terminal that would be lost during transport and secretion. Thus a 5'RACE experiment was performed and it revealed a 20 aa signal peptide with 85, 80 and 76% similarity respectively to signal peptides found in equine herpes virus, tobacco hornworm (*Manduca sexta*) and mouse. The putative signal peptide was also analyzed using the SignalP program (Nielsen et al, 1997) which confirmed it as a signal peptide. This cloned and sequenced precerebellin-like cDNA contains 700bp, comprised of an ORF consisting of a 60 bp (20 aa) long signal sequence and a 498 bp (166 aa) (GenBank submission number: AF192969) sequence forming the

secreted protein (Figure 4.4). Its estimated mass and PI, using the ProtParam program, were 18.1 kD and 5.9 respectively. Furthermore, a possible N-glycosylation site was identified at position 69 (Asn) using the Prosite protein motif program (Hofman et al, 1999).

Fish Identification code	Day0, before, treatment	Day10, after treatment (% of pixels)	Day10, approx. conc. of pcb in plasma(ug/mL)
H	n.d *	1.38	235
I	n.d	0.75	150
J	n.d	1.08	351
K	n.d	1.14	447
L	n.d	1.88	300
Average	n.d	1.25±0.19(SE)	320±70(SE)

Table 4.2. Two-dimensional gels were run using plasma samples taken from the same fish before and ten days after they were injected (ip) with *Vibrio* bacterin emulsified in FIA. Protein spots were quantified by measuring the numbers of pixels representing the spot and the total pixel count for all the protein spots, using the analysis program Imagemaster (Pharmacia). The approximate concentration of the precerebellin-like protein was calculated using the total protein concentration of the plasma sample. Concentration pcb (pcb μg/mL) = % total pixels of spot X total protein concentration (μg/mL). Pcb= precerebellin-like protein. *not detected.

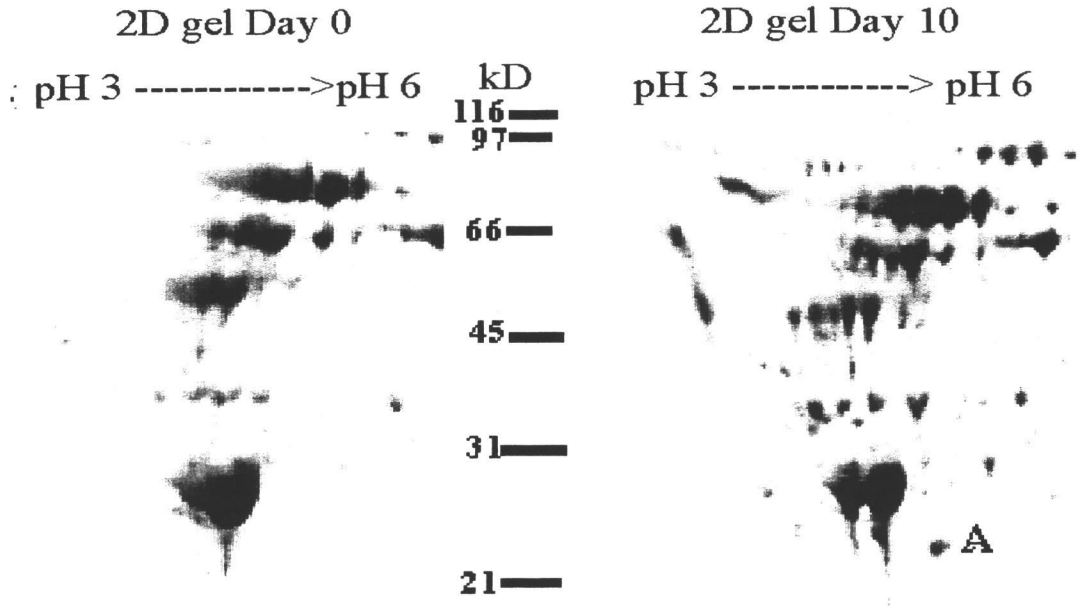

Figure 4.2. Two 2D gels, of plasma from one individual trout, showing the different patterns of spots before (left) and ten days after treatment (right). The precerebellin-like protein is marked with an A.

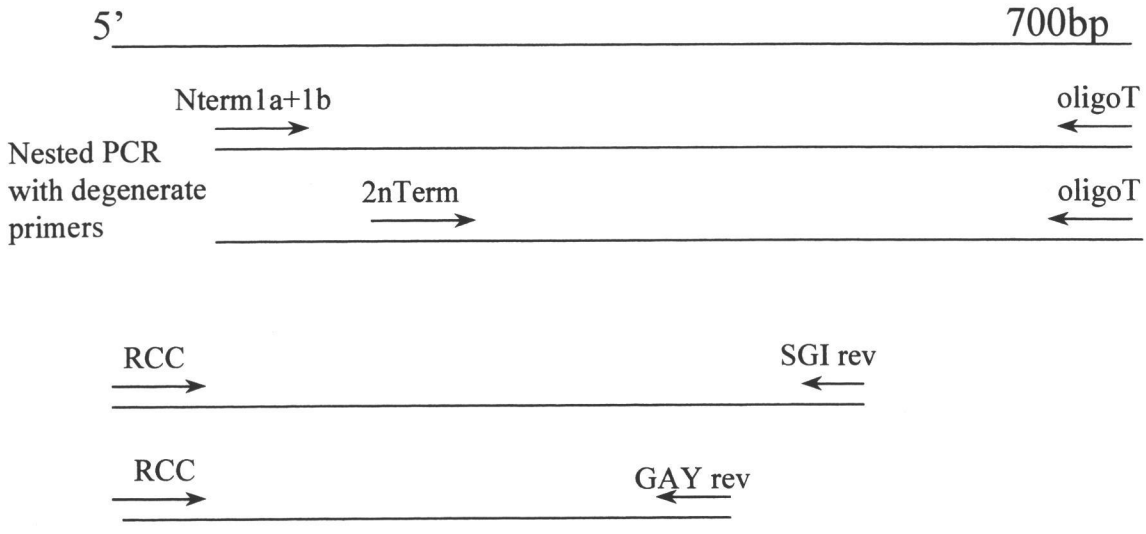

Figure 4.3. The different primers, and their placement relative to each other, used for regular, nested and the 5'RACE PCR.

```
   1  GCAGTCAGACATCTCAGCAAACCTCTCCCACCCGGTTCGTTGAAAGCCCT

  51  GCGATGGAGTCTGTTTTGGCAGTGGTGGTGTTGCTTTGCTGTTGTATGGT
        M  E  S  V  L  A  V  V  V  L  L  C  C  C  M  V
 101  TGAGACTCAGACGGAGAGTGAGGTTGACGGGCTGCTGAGGGAACTGACAG
       E  T  Q  T  E  S  E  V  D  G  L  L  R  E  L  T  A
 151  CCCGGGTGGAGAAACTGGAGAGTGAGTATAATGGCAAACCAAAAGTGGCC
         R  V  E  K  L  E  S  E  Y  N  G  K  P  K  V  A
 201  TTCTCCGCTTCCTTTATGATCAATGATGATCATTGTCACTTAGGACCTTT
       F  S  A  S  F  M  I  N  D  D  H  C  H  L  G  P  F
 251  CGGCGAGAACACCACTGTGGTGTATGAAAAGGTCATTACAAACATCGGTG
        G  E  N  T  T  V  V  Y  E  K  V  I  T  N  I  G  E
 301  AAGCATATAACCCAGATACAGGTGTCTTTACTGCACCTGTGAGAGGGGCC
         A  Y  N  P  D  T  G  V  F  T  A  P  V  R  G  A
 351  TACTACTTCACATTCACTTGCAATGTTGGGAATTCAGGGAAGGCGAATGC
        Y  Y  F  T  F  T  C  N  V  G  N  S  G  K  A  N  A
 401  AGCGTTGCTTAAGAACGATGTGAAAATGGTTGCCATCTATGAAACTGCCA
         A  L  L  K  N  D  V  K  M  V  A  I  Y  E  T  A  N
 451  ACCCAAGATCCGGTATTTACCACGGCGGGGCCAATGGAGTCACACTAGAC
         P  R  S  G  I  Y  H  G  G  A  N  G  V  T  L  D
 501  CTGGTGAAAGGAGACAAAGTCAATGTGGTTCTCTGGAGAGGCAGTAGCAT
        L  V  K  G  D  K  V  N  V  V  L  W  R  G  S  S  M
 551  GTTTGACAACAGCAGAATTAACATGTTCAGTGGTTACCTTCTATTTCCTA
         F  D  N  S  R  I  N  M  F  S  G  Y  L  L  F  P  I
 601  TCACTACTAAGTAATAGTAAAAGTCTGATATTTACATGGCAGACTTAGCA
         T  T  K  stpstpstp
 651  AGAATTGGAAATGCTTATTTCAACGTTTCATTAAAGGAATAATCCACTCA
```

Figure 4.4. The precerebellin-like full-length cDNA with corresponding amino acid sequence. **Bold** = potential N-glycosylation site, ***bold Italics*** = signal peptide, stp = stop codon, underline = the position of cerebellin in the human precerebellin. GenBank submission number: AF192969.

Figure 4.5. Identities and similarities between, trout precerebellin-like protein, rat precerebellin, and C1q B chain from human. Black = identical amino acids and Gray = conservative substitutions.

DISCUSSION

In an effort to map the plasma proteins of rainbow trout, and in particular to identify those that are increased in concentration after induction of a general inflammatory response, we have isolated an upregulated protein, determined its N-terminal sequence, made degenerate probes, performed RT-PCR and sequenced a full length cDNA. The protein shows 53 % similarity at the amino acid level to the precerebellin protein of rat and 46% similarity to the globular region of the human C1q B chain (Figure 4.5). Its estimated mass by SDS PAGE gel was approximately 24kD, however the ProtParam estimation program indicated a calculated molecular mass of 18.1 kD. This difference could be due to glycosylation, which can cause a protein to migrate more slowly on SDS PAGE gels.

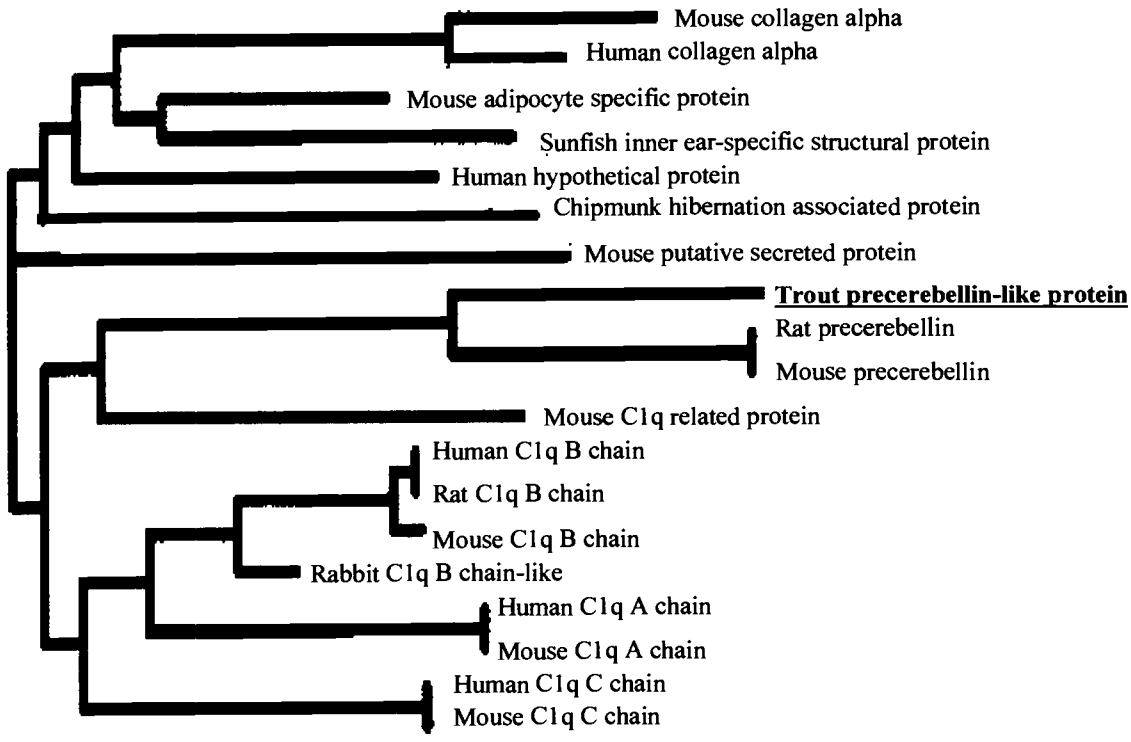

Figure 4.6. A phylogenetic tree (Phylip distance program using the neighbor joining method) showing the relationship of trout precerebellin-like protein to other members of the C1q globular domain family (Kishore and Reid, 1999).

The protein spot on 2D gels remained in the same location under non reducing and reducing conditions. This indicates that the precerebellin-like protein is probably not covalently attached to other subunits, but it does not rule out the possibility that it forms non-covalent multimers with itself or other subunits.

Precerebellin has previously been mainly located in the brain. It is thought to be the precursor protein to cerebellin, a sixteen amino acid long peptide, that has been shown to increase in concentration during early development (Urade et al, 1990). Cerebellin also increases the secretion of catecholamines when applied to adrenal gland slices (Mazzocchi et al, 1999). Furthermore, the rat precerebellin gene is expressed in the adrenal gland and the spleen (Wada and Ohtani, 1991). However, it might be alternatively spliced in these tissues. These authors suggested a putative transmembrane spanning region as determined by the amino acid sequence, suggesting that precerebellin might be a membrane-associated protein. However, the protein that we have isolated is a soluble plasma protein, making it unlikely that it would possess a transmembrane region.

The high similarity of this newly identified teleost protein to precerebellin raises some interesting questions. Does precerebellin itself have functions additional to those of cerebellin? Why make a 224 aa (rat) protein when only 16 aa are cleaved off to form the active cerebellin? This resembles the situation seen with the proopiomelanocortin (POMC) gene, in which several biologically active peptides are produced by cleavage of a propeptide. We speculate that precerebellin peptides outside of the known cerebellin peptide might be physiologically active.

However, since the whole protein occurs intact in trout plasma, an alternative explanation could be that, extraneuronally, it has a function distinct from that of cerebellin. This alternative is supported by a report of an antibody to cerebellin that recognizes a protein of higher molecular weight in peripheral tissues (Burnet et al, 1988).

The precerebellin-like protein has 28-30% identity and 43-46% similarity to the globular region of the C1q B chain of mouse and human. Several pieces of evidence suggest that the isolated and cloned protein is not a functional homologue of the C1q B chain. While most APPs are synthesized in the liver, in mammals the subunit C1q of the first complement component (C1) is mainly synthesized in macrophages (Trinder et al, 1995). We find that the trout precerebellin-like protein is synthesized in the liver. Secondly, the isolated protein lacks the collagen-like region (GLY-X-Y repeats) which is characteristic of mammalian C1q B chain, as well as the A and C chains. This repeat is required for the interaction between these three proteins

When comparing the precerebellin-like molecule to the N-terminal sequence of the putative nurse shark C1q (Smith, 1998) no similarity was evident. However, the N terminal of the globular region of this putative shark C1q has 18.5% identity and 44% similarity with the isolated protein. The most interesting part of this similarity is a stretch of 3 aa at position 29-31; this sequence (AFS) is conserved in all known C1q chains and is part of the cerebellin peptide in mammals.

A phylogenetic analysis of most of the proteins belonging to the C1q – globular domain family as described by Kishore and Reid (1999) indicated a close relationship between the trout precerebellin–like protein and the precerebellin protein sequences from rat and mouse (Fig.4.6).

The function of precerebellin has yet to be determined completely but it would appear that the precerebellin-like protein of trout is involved in the innate immune response since it is upregulated shortly after the injection of an inflammatory agent, *Vibrio* bacterin in FIA. C1q has been reported to protect against glomerulonephritis without the involvement of C3 (Mitchell et al, 1991), and C1q, by itself, binds a C1q receptor which increases the rate of phagocytosis and the oxidative burst *in vitro* (Nepomuceno et al,1998). Further studies with the purified protein will have to be conducted to determine if the molecule we have characterized is involved in similar functions in rainbow trout. Furthermore, it would be of interest to determine if humans, mice and rats also upregulate the precerebellin protein extra-neuronally during an acute phase response, as this could be indicative of a defensive function.

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CONCLUSION

In the search for identification of acute phase proteins in Rainbow trout we have obtained five N-terminal sequences and one full length deduced amino acid sequence of up-regulated proteins. To achieve this, we exploited both Proteomic and genomic approaches. Starting with the second chapter of this thesis we describe the further development and the use of Tandem Crossed Immunoelectrophoresis (T-CIEP). Tandem crossed immunoelectrophoresis has been used since the middle of the seventies (Kroll, 1976). This technique allowed us to compare rainbow trout plasma samples from before and after injection with Freund's Complete Adjuvant. We were able to identify two proteins that were consistently up regulated and one that was consistently down regulated. One of these proteins was isolated from the gel and determined by electrospray ionization mass spectrometry to be transferrin. T-CIEP makes it possible to clearly visualize the proteins that react with the antisera, but from these gels it is difficult to isolate enough protein for subsequent analysis. We therefore adopted 2D gel electrophoresis. In this approach the sample is isoelectric focused in the first dimension and separated on a SDS PAGE gel during the second dimension.

In order to determine the kinetics and the relative consistency of the acute phase response in rainbow trout, we obtained plasma samples from before and 3, 5,

7, 10, 14 and 21 days after treatment with vibrio bacterin emulsified in Freund's Incomplete Adjuvant (FIA). These samples were first examined by means of 1D gel electrophoresis in order to identify the samples with the largest change in the plasma profile as compared to the sample taken before injection of the inflammatory stimulus. Samples taken on day10 were determined to have the maximum changes. One band of an up-regulated protein from a 1D gel was sent for sequencing, however the band was found to be a mixture of three proteins, since three N-terminal sequences could be deduced from the data.

Five sets of samples from day 0 and day 10 were analyzed by 2D gel electrophoresis and at least three proteins were found to be up-regulated. One of these proteins has been identified by N-terminal sequencing as transferrin. Two other N-terminal sequences were obtained. One of these was used to make degenerate primers and these were used to successfully obtain a full length cDNA sequence by using 5' and 3' RACE PCR (Chapter 4). This protein is a homologue of the precerebellin protein found in humans, mice and rats. Its function is not known. Precerebellin and the precerebellin-like trout protein belong to the same family of proteins as the C1q A, B and C chains. The other N-terminal sequence obtained did not align with anything in the databases. In order to obtain a full length sequence, it will be necessary to prepare degenerate primers and conduct 5' and 3' RACE reactions, as was done to identify the precerebellin protein.

We have shown that Rainbow trout are capable of up-regulating or down-regulating several acute phase proteins during an acute phase response. Transferrin

and precerebellin-like protein (Pcb) were identified as up-regulated. Alpha2-macroglobulin, an acute phase protein in rats, did not appear to be consistently up or down regulated, and is not an acute phase protein in trout.

Transferrin binds free iron that is released in plasma during normal and pathological hemolysis. In rainbow trout, transferrin has six different glycoforms and three different mass isoforms. We speculate that these different forms have different affinities for iron binding and this is needed to be able to sequester iron away from pathogenic bacteria that are dependent on iron for their growth and division.

As mentioned above, the function of precerebellin-like protein is unknown. Functional studies, for example opsonization and phagocytosis assays, will be conducted when the intact protein has been isolated in enough quantity to conduct such studies. We are in the process of making antibodies to two peptides from the precerebellin-like protein and these will be used for affinity chromatography. A suppressive subtractive hybridization library (SSH) from liver cDNA, from a pool of five trout before and after treatment with Vibrio in FIA, was made recently. When 220 clones were sequenced, pcb was found to be one of the identified sequences. More interesting, however, another sequence showed 45% identity over 73 of pcp's 186 amino acids. This other protein has an N-terminal amino acid sequence that matches exactly the sequence for a plasma protein reported by Murata et al- (1995), which was isolated by using a C-polysaccharide affinity

column. Studies need to be conducted to determine if pcb also binds to C-polysaccharide, a common component of bacterial walls.

As mentioned above, a SSH library was made from trout liver cDNA and this library has yielded sequences of 26 or more immune related proteins in trout (Bayne et al, 2000). The knowledge gained from this thesis work and from the sequence information obtained from the SSH library has markedly increased the number of reagents that can be used for studies of the innate immune system of rainbow trout. Full length sequences need to be obtained, proteins expressed, antibodies made and functional studies conducted in order to determine the function of some of the proteins. This added knowledge will be useful when asking questions about the interactions between the innate and adaptive arms of the immune system as well as evaluating the evolution of the system. Furthermore, efforts to restore populations of endangered salmonid species will be helped by an increased knowledge of the actions and interactions of the components of the innate immune system.

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APPENDIX:

MODULATION OF STRESS HORMONES IN RAINBOW TROUT BY MEANS OF ANESTHESIA, SENSORY DEPRIVATION AND RECEPTOR BLOCKADE

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ABSTRACT

Sympathetic activation leading to increased levels of blood catecholamines, and stimulation of the hypothalamic-pituitary-inter-renal axis leading to increased cortisol, are difficult to avoid when handling animals. Yet, in research on effects of acute stress, elicitation of such responses must be minimized in the control groups. The work examines means to achieve a minimally disturbed state in rainbow trout (*Oncorhynchus mykiss).* Level of arousal was determined by adrenaline and cortisol concentrations-in plasma, and by the spleen: somatic index. Fish were prepared for bleeding by rapid capture and concussion, by infusion of anesthetic into the undisturbed home tank, by confinement in black boxes, or by being fed α- and β-receptor antagonists. Even when done quickly, netting and concussion yielded fish with ca. 200-pmol adrenaline/ml plasma. Cortisol was elevated to > 10 ng/ml within 30 s of stress initiation. Surreptitious infusion of anesthetic 2-phenoxyethanol (PE) into tanks yielded fish with lower adrenaline levels (means 19.34 and 19.58 pmols/ml in home tank and black boxes, respectively). Among fish given phentolamine and propranolol, spleen: somatic indices and plasma adrenaline were higher than in diet controls, whether undisturbed or stressed, indicative of successful receptor blockade. Since careful infusion of 2-PE yielded the lowest adrenaline levels, and requires no special apparatus, it is the method of choice for obtaining minimally stressed fish.

INTRODUCTION

The disruptive effects of stressors on homeostasis have been of concern to biologists for over a century (Bernard, 1973; Cannon, 1929). Although a great deal is now known about both the mechanisms and the effects of stress (Selye, 1950; Selye, 1973; Wedemeyer et al, 1990) consensus has been hampered by the general lack of precision in terminology. Furthermore, immediate and acute effects have been less the focus than the longer- term consequences of stressors. Though commonly stated, the view that stress is harmful is not universally held (Maule et al, 1989). In the aquaculture industry, fish are subjected to a variety of stressful handling protocols. While such stress levels are considered undesirable, and efforts are made to minimize them, they are also considered largely unavoidable, and their negative effects are tolerated.

The types of stress that are clearly harmful tend to be chronic and inescapable. Acute stressors that are brief but sufficient to elicit the fight-or-flight response, such as unexpected encounters with dangerous predators, do not always have negative physiological consequences. The fight-or-flight response, associated with activation of the sympathetic nervous system, involves altered states in multiple organ systems: sensory perceptiveness increases, cardiovascular functions change, and the status of innate immune effector systems is also changed (Demers and Bayne, 1997; Kiecolt-Glaser et al, 1992; Matthews et al, 1995; Naliboff et al,

1995). In vertebrate species, these changes are coordinated centrally when appropriate sensory information, channeled through the hypothalamus, elicits action potentials in adrenergic fibers of the sympathetic nervous system. The end effects of this coordination are contraction of smooth muscle in the spleen and hair follicles, and the release of adrenaline from the adrenal medulla (chromaffin cells of the pronephros in teleosts). While catecholamines mediate the most immediate somatic responses, a surge in plasma adrenaline is usually followed by a surge in plasma corticosteroid (Thomas, 1990).

What, if any, selective advantage is there in the release of stress hormones? Immunologists, for example, have explored the possible beneficial effects of cortisol and adrenaline, and a number of inferences have been drawn from experimental data (Demers and Bayne, 1997; Schreck, 1996). In general, cortisol is immunosuppressive whereas catecholamines (adrenaline and noradrenaline) seem to have pleiotrophic effects. Feedback between these two hormonal systems, and differential effects on shared target cells complicate interpretations. In vitro, fish neutrophils react instantaneously to catecholamines, with altered metabolism of reactive oxygen species (Bayne and Levy, 1991a; Bayne and Levy, 1991b). In vivo, sympathectomy increased lymphocyte responses to antigen (Flory, 1989; Flory, 1990). Furthermore, blood clotting and killing of phagocytosed yeast were accelerated in trout that had experienced brief stressors (Ruis and Bayne, 1997), and several plasma proteins increased in concentration within minutes of the initiation of a stressful experience (Demers and Bayne, 1994; Demers and Bayne,

1997). To facilitate further investigation of the effects of acute stressors on innate immunity, a means was needed to obtain blood and other tissue samples from fish which were minimally stressed or alarmed. Reliance on blood vessel cannulation (Gamperl et al, 1994) was rejected as the surgical trauma was considered likely to induce an acute phase response that would complicate interpretation of immunological data. As corticosteroids have generally been found to respond over a time of several minutes (Pottinger et al, 1993) and to have delayed (transcription-dependent) effects, whereas catecholamines respond within seconds and tend to have 'instantaneous' membrane receptor-mediated effects, it was chosen to focus on adrenergic events, while monitoring corticosteroid levels in the plasma. The work addresses concerns of any physiologist for whom the reduction or elimination of stress responses are desirable. Several protocols were used in an effort to reduce the catecholamine surge, including surreptitious anesthesia in the home tank, concussion, acclimation in dark boxes, and the addition of α- and β-adrenergic receptor blockers to the diet. Efficacy was determined by the concentrations of catecholamines in the plasma, and by spleen: somatic indices. It was found that the appropriate administration of a selected anesthetic offers an easy means to minimize sympathetic activation and its consequences.

MATERIALS AND METHODS

Animals

Shasta strain rainbow trout (*Oncorhynchus mykiss)* were bred and raised at the Food Toxicology Laboratory, Oregon State University. The trout were maintained on a 12 h light/dark cycle with constantly flowing, single-pass well water at 12°C and fed three times weekly with Oregon Moist Pellet.

Handling stress treatment

Ten fish (150-300 g) were stocked in each of three 400 L tanks 1 week prior to experiments to allow for acclimatization. Food was withheld on sampling days. Samples were taken between 08:00 and 10:00 h to minimize possible temporal variations of the hormone levels. On each day of sampling, an initial fish was quickly netted, killed by cerebral concussion and bled from the caudal vein using a heparinized syringe. Four more fish, from the same tank, were then netted and held in the air for 30 s. One fish was concussed and bled immediately and the other three were placed in a bucket (30 cm in diameter) containing water that was only half as deep as the dorso-ventral axis of the fish. After 30, 60 and 90 s individual fish were recaptured from the bucket, concussed and bled. This was repeated in each tank on 2 separate days, 1 week apart. Blood samples were kept on ice for

approximately 30 min until centrifuged at 500g and 4°C. Plasma was aliquoted and stored at - 80°C until assayed for catecholamines and cortisol levels.

Anesthesia in the home tank

Ten fish were stocked in each of five 400 L tanks and allowed to acclimate for 2 weeks. On each of 5 separate days of the experiment, 100 ml of 2-phenoxyethanol (2-PE) (Sigma, Saint Louis, MO), mixed by shaking with 100 ml of water, was infused over 30 seconds into the tank via the water inflow to avoid unnecessarily disturbing the fish. This was calculated to yield 0.025% of 2-PE. The authors worked quietly- and did not lift the tank lid until 2 min after the introduction of 2-PE. Each fish was then bled (approx. 1ml) from the caudal vein using heparinized syringes and placed in a separate net either in the tank with the anesthetic (resting group), or, after being revived in clean water, the fish were held in the air for 30 s, then placed in separate buckets containing shallow, fresh, clean water (stressed group). Fish were re-bled 10 min later. Samples were stored on ice until centrifuged. Plasma aliquots were frozen at -80°C until assayed for levels of catecholamines and cortisol.

Anesthesia in black box

Two black Plexiglas boxes (L 40 cm x W 30 cm x D 7 cm), with six slots each and a black lid covering the entire box (Gamperl et al, 1994), were stocked with one trout (100-150 g each) per slot, one week prior to experiment. Each box was supplied with constantly flowing (4 L/min) 12°C well water. On the day of sampling, the 12 fish were anesthetized; each box received 7 ml of 2-PE mixed with 13 ml of water infused over 30 s. One minute later, the water flow was turned off so that the fish would remain anesthetized when blood samples were taken for catecholamine determination. After 1 more min, the fish were bled from the caudal vessel. The blood samples were stored on ice then centrifuged at 500*g* at 4°C; plasma was aliquoted and frozen at - 80°C for subsequent analysis of adrenaline concentrations.

Effects of α- and β-adrenergic antagonists administered in the diet

Ten fish were stocked in each of four tanks (400 1) and allowed to acclimatize for 1 week. Food was withheld for the last 2 days to improve the appetite of the fish before the treatment diet was given. On the day of the treatments, fish in two tanks (treatment resting and treatment stressed) were fed propranolol (β-receptor antagonist)(Sigma) and phentolamine (α-receptor antagonist) (Sigma) mixed into their regular food at a concentration of 10 mg of each drug/1.5 g of food. The control group was fed drug-free diet. The food

(control and treatment) was supplied at a rate of 3% of the total estimated body weight of the fish in the tank. Four hours after feeding, the fish were anesthetized and bled. The fish in *the resting* groups (control and drug-treated, ten fish in each group) were anesthetized with 2-PE using the tank treatment described above. Fish in the two *stressed* groups (control and drug-treated) were netted and held in the air for 8 min, then killed by cerebral concussion. Spleens were quickly removed, carefully dissected free of any extraneous tissue, and placed in pre-weighed tubes on ice for subsequent weighing and calculation of the spleen: somatic index - see below. After the removal of the spleen, the fish were bled from the caudal vein with a heparinized syringe. The blood was placed on ice, centrifuged, and the plasma was aliquoted and stored at - 80°C until assayed for catecholamines and cortisol levels. This experiment was repeated once.

Catecholamine analysis

Catecholamine levels (noradrenaline and adrenaline) in plasma were measured on a Shimadzu HPLC system with an electrochemical detector. Plasma (100 μL) was mixed with 1 ml of 0.1 M perchloric acid. Then 3,4-dihydroxybenzylamine (DHBA) was added as an internal standard (50 μL of 4.5 nmol/mL aqueous solution). After being vortexed, samples were centrifuged (8000g, 4°C), then 1 ml of the deproteinized supernatants were transferred to a tube containing 10 mg of acid washed alumina (Sigma), 25 μL of 5 mM sodium

metabisulfite (Sigma) and 25 μL of 10% ethylene diamine tetra-acetic acid (EDTA) (wt:v) (Sigma). Tris buffer 600 μL, 2.0 M, (pH 8.6) was added and samples were vigorously vortexed. The tubes were then placed on a rocker and the catecholamines were allowed to adsorb onto the alumina for 30 min. This was followed by a centrifugation at 2000g and the alumina was subsequently washed three times with 1 ml of chilled Nanopure water. The catecholamines were then eluted with 100 μL 0.1 M perchloric acid and stored at - 80°C until analysis of 10 μL aliquots on the HPLC. Adrenaline and noradrenaline levels were calculated by the relative areas of their peaks compared to the peak area for the internal standard (DHBA).

Cortisol analysis

Cortisol levels were determined by Radio Immuno Assay (Redding et al, 1984) in the laboratory of Carl Schreck (Department of Fisheries and Wildlife, Oregon State University).

Spleen index

Tubes containing spleens were pre-weighed, and the spleen weights were determined by the difference. Each fish was weighed, and its spleen: somatic index (SSI) was calculated by the formula SSI =spleen weight/ body weight x 100.

RESULTS

Handling stress treatment

This was designed to mimic a handling stress typically encountered by trout in hatcheries and in research laboratories. Short-term changes of catecholamine and cortisol levels were measured. The stress of netting and 30 s air exposure elicited increases of both adrenaline from 196+22.26 to 395 $\pm$77.68 pmols/ml and cortisol from 2.37 $\pm$1.69 to 16.83 $\pm$10.08 ng/ml (Fig. 1). When the fish were placed in a bucket of shallow water, both the adrenaline and cortisol concentrations initially decreased, to 229 $\pm$29.17 pmols/ml for the adrenaline and 1.80 $\pm$0.79 ng/ml for the cortisol. However, the levels started climbing again; after being captive 30 s more in the shallow bucket, adrenaline reached its highest level in this experiment, 582 $\pm$98.31 pmols/ml. After an additional 30 s in the bucket the adrenaline level was decreased to 445 $\pm$75.45 pmols/ml. The cortisol levels followed a similar trend, increasing to 13.58 $\pm$8.6 ng/mL at 90 s after the initial netting and 2.53 $\pm$10.45 ng/ml after 120 s. t. There were significant increases of the adrenaline levels after 30s ($p<0.05$) and the cortisol levels 120s after the stressor was initiated, using a randomized block design and analysis of variance (ANOVA).

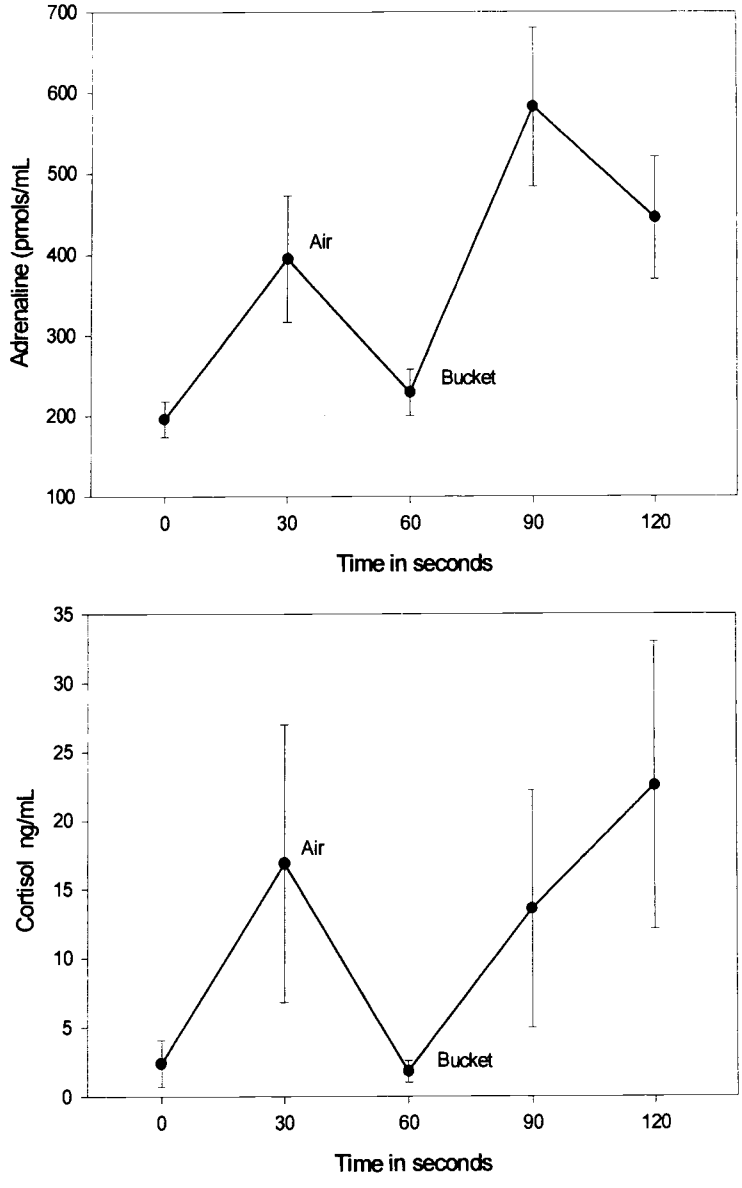

Figure.1. The kinetics of change in cortisol and adrenaline levels in trout undergoing a "handling stress". The fish were netted (time=0), held in the air for 30s (time=30) and bled after cerebral concussion, or placed in a bucket with shallow water for 30, 60, or 90 additional s (time=60,90,120) and subsequently recaptured and bled. N=5 for each time point. There were significant increases of the adrenaline levels after 30 s and the cortisol level after 120s ($p<0.05$) after the stressor was initiated, using a randomized block design and ANOVA.

Anesthesia in the home tank

Adrenaline and cortisol levels were determined in plasma from control trout as well as trout exposed to a 30 s air stress followed by 10 min in a bucket with shallow water. The average resting adrenaline level was 19.39 SE$\pm$2.69 pmols/ml (n = 25) (Fig. 2), which is similar to the adrenaline level seen in the black box treatment (see below). The values for cortisol and lysozyme levels in these fish have also been reported (Demers and Bayne, 1997): the resting cortisol levels ranged from 1.25 to 17.60 ng/ml (mean = 7.88 $\pm$3.55).

Black box treatment

The adrenaline levels measured in the black box treatment experiment (Fig. 2) remained low in all 12 fish, with an average of 19.58 $\pm$5.65 pmols/ml.

Effects of α and β-antagonists administered in the diet

Among the fish that were not intentionally stressed ('resting'), those given phentolamine and propranolol had significantly higher adrenaline levels 379.9 $\pm$ 82.77 pmols/ml, (n = 12) than those that were drug-free 63.25 $\pm$9.74 pmols/ml, (n = 13) ($p < 0.001$, Mann- Whitney Rank Sum Test) (Fig. 3). Cortisol levels were also higher in these resting drug-treated fish 66.32 $\pm$24.22 ng/ml) than in the

resting drug-free controls (7.5 $\pm$4.84 ng/ml). However, the difference was not significant using the Mann-Whitney rank sum test.

Both adrenaline and cortisol values increased with stress. Among drug-free fish, cortisol increased with stress (to 24.5 $\pm$4.6 ng/mL), but in the drug-treated fish, cortisol (already high - see above) was not increased further as a result of stress (59.9 $\pm$15.6 ng/mL). However, among this stressed group there was high variance in the data (S.D. = 58.5 ng/ml). Adrenaline increased to 1555 $\pm$366 pmols/ml in the drug-free fish when stressed, and to 1601 $\pm$146 pmols/ml in the drug- treated fish when stressed. The SSI were significantly different ($p < 0.001$ using the Mann-Whitney Rank Sum Test) between the control (0.084 $\pm$0.01 0) and drug-treated (0.124 $\pm$0.009) resting groups, as well and in the stressed state 0.065 $\pm$ 0.005 in controls, and 0. 119 $\pm$0.008 in drug treated fish; ($p = 0.0003$) (Fig.4).

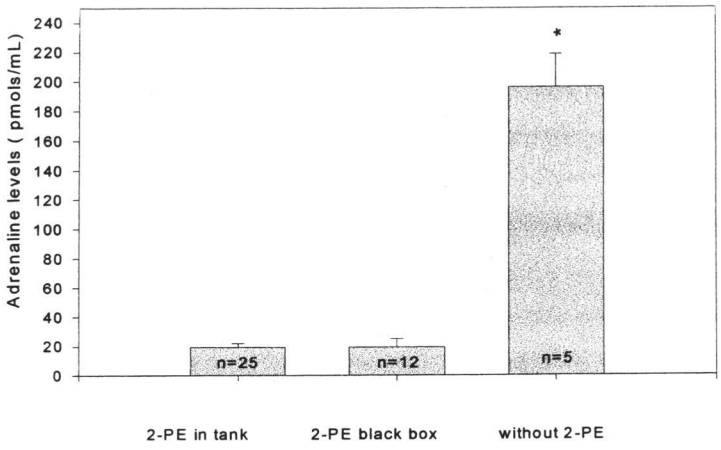

Fig.2. Adrenaline levels in trout plasma following different treatments: anesthesia in home tank, anesthesia in a black box, and netting and concussing. Left and middle bars: 2-phenoxyethanol (PE) was surreptitiously infused into the inflow water and the trout were left undisturbed for 2 min until they were anesthetized. Right bar: fish were captured in a 400 L tank without the use of anesthetic, concussed and bled quickly (generally less then one minute).

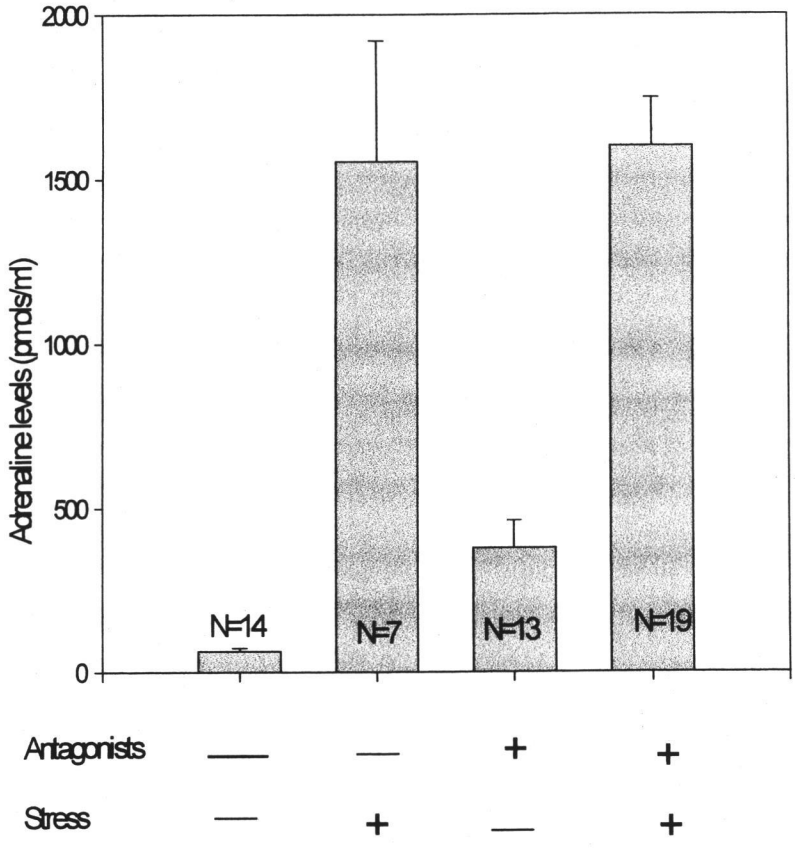

Fig.3. Effects of α and β- adrenergic receptor antagonists supplied in the diet, on adrenaline levels in trout plasma.

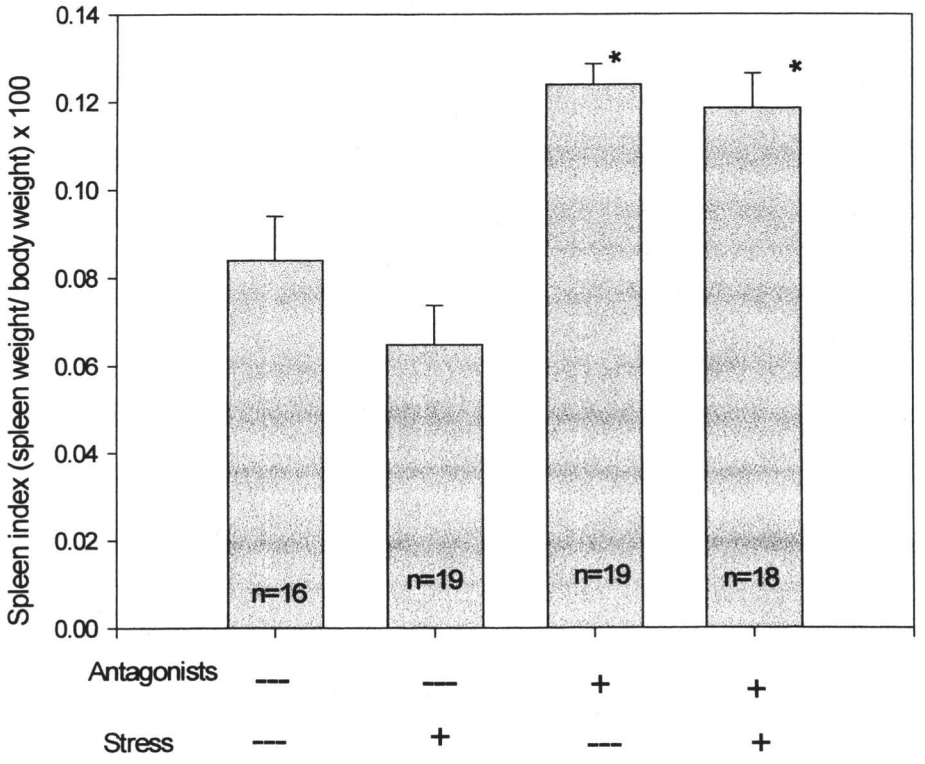

Fig.4. The influences of dietary α- and β-antagonists on spleen: somatic indices (SSI). The antagonists blocked the sympathetic response evidenced by a reduced contraction of the spleen in fish fed the treatment diet as compared to fish fed a regular diet.

DISCUSSION

A common means of preparing fish for experimental handling is to capture them and transfer them to a smaller container containing anesthetic, commonly MS- 222 or buffered benzocaine (Iwama et al, 1988). This procedure exposes the fish to the stress of being chased, netted, passed through the air, put into a small, unfamiliar container frequently crowded with other individuals, before the anesthetic begins to take effect. In rainbow trout, this procedure, even when executed efficiently, is clearly sufficient to elicit sympathetic activation. It was satisfying, therefore, to note the markedly lower levels of plasma adrenaline that were obtained by the surreptitious infusion of 2-PE into the home tank prior to capturing the fish. While use of this drug is not permitted in fish destined for human consumption, its property of minimally eliciting sympathetic arousal (Iwama et al, 1988) makes it an important drug for fish physiological research.

However, lower levels of catecholamines have been measured in cannulated fish, but for our purposes cannulation was not an option because of the risk of activating the immune system. The observation of reduced levels of both cortisol and adrenaline at one minute, after increased levels at 30 s, was surprising. The speed of the initial rises certainly implies the existence of presynthesized pools of both adrenergic and steroid hormones, or their precursors. It remains unclear whether the subsequent transient decreases were due to a relief that was perceived on return to water, whether these were due to natural depletion of the putative

presynthesized pools and metabolism of the released hormones, with a delay before de novo synthesis could re-supply the molecules, or that the free cortisol had time to bind to the cortisol binding protein that circulates in plasma.

Others have used black boxes, together with cannulation of the fish, as means to obtain quiescent animals (Gamperl et al, 1994). The work confirmed the efficacy of black box confinement. Cannulation has been avoided, since the ultimate goal is to obtain fish without activating the immune system, and invasive surgery invariably induces an acute phase response. An advantage of confinement in black boxes may be the need for significantly reduced amounts of anesthetic: six fish could be anesthetized with 7.0 ml 2-PE in the black boxes, while 100 ml was needed to anesthetize ten fish in the home tanks.

However, since levels of stress hormones in the plasma were no lower in fish held in black boxes than in those anesthetized in their home tanks, the simpler system (home tank anesthesia) is preferred.

The elevated plasma levels of adrenaline that were observed in resting fish fed with α and β-antagonists are taken to imply homeostatic feedback pathways in these fish. By inhibiting adrenergic receptors, these drugs may have elicited an increased need for the native ligands, leading to the values we observed. Alternatively, clearance may have been slowed due to receptor occupation by the antagonists. It is clear from the SSI for these fish that the drugs were effective as used. When effective, adrenergic receptor blockers bind to adrenergic receptors on target tissues, antagonizing the normal physiological responses (such as the

contraction of smooth muscles in the spleen) to the relevant ligands (adrenaline and noradrenaline). Thus, the larger SSI seen in the drug-treated fish indicated that the antagonists blocked the normal physiological response. The antagonists had the anticipated effect on SSI, presumably resulting from a reduction of smooth muscle contraction. The fact that SSI values reported here are lower than those reported by others (Pearson and Stevens, 1991) suggests that room exists for further refinement of the regimen: both dose and time need to be optimized if adrenergic receptor blockers are to be effectively administered per os.

This work has demonstrated an easy and practical means to prepare aquatic vertebrates for experimental manipulations in cases where the perception of stress, and the stress response, should be kept to a minimum. Both the anesthetic (2-PE) and the manner of its administration are likely important for the efficacy of the treatment.

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