

AN ABSTRACT OF THE THESIS OF

Dawn Christine Jordan Hayes for the degree of Master of Science in Microbiology presented on March 2, 2000. Title: Rainbow Trout (*Oncorhynchus mykiss*) Interleukin-1 Beta Plasmid DNA Generates Inflammation *In Vivo* and Demonstrates Adjuvant Potential for Vaccine Against Infectious Hematopoietic Necrosis Virus.

Abstract approved: **Redacted for privacy**

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Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus that causes significant mortality in trout and salmon hatcheries throughout the world. The glycoprotein (G) of IHNV was found to be particularly immunogenic in fish and DNA vaccines constructed from the G gene of IHNV were shown to elicit a protective response in rainbow trout (*Oncorhynchus mykiss*). Although the DNA vaccine for IHNV is efficacious, it is not readily marketable because of several factors including the presence of non-fish genes in the viral vector, the quantity of DNA required, its persistence in the host, and the requirement for injection vaccination. To address the issue of DNA quantity and efficacy we began a study of immunostimulants as vaccine adjuvants.

The expression, function, and adjuvant potential of trout interleukin-1 β (IL-1 β) was examined. Histidine reporter (His) or Green Fluorescent Protein reporter (GFP) genes fused to the trout IL-1 β were used to distinguish between recombinant and endogenous trout IL-1 β in transfected CHH-1 cells. The presence of trout IL-1 β protein in transfected cell media was confirmed as a 15 kD protein by Western blot using goat-anti-mouse IL-1 β antibodies. A second reactive 70 kD protein was found on anti-IL-1 β blots and may be a secretory or repressor protein associated with IL-1 β . The recombinant nature of trout IL-1 β was verified by direct SDS-PAGE detection.

The function of IL-1 β protein generated from plasmid DNA was investigated in two *in vivo* experiments: 1) a plasmid dose-response experiment and 2) a lethal challenge experiment. Trout IL-1 β , transcribed and translated *in vivo* from a plasmid construct, induced acute inflammation above vector control levels in the form of pronounced swelling and hematoma formation at the injection site for at least 72 hours after immunization. Trout IL-1 β DNA caused anorexia at high doses (10 μ g and 1 μ g per 3.8 g fish). Fish injected with either IHNV-G (0.01 μ g) or IL-1 β + IHNV-G (0.03 and 0.01 μ g, respectively) were protected from IHNV (10^4 TCID₅₀/L) challenge. Fish receiving IL-1 β + IHNV-G showed delayed and reduced mortality compared to fish receiving IHNV-G alone for 13 days post challenge. These studies show that recently identified trout IL-1 β is functional *in vivo* and has adjuvant potential in vaccines against IHNV.

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**RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) INTERLEUKIN-1 BETA
PLASMID DNA GENERATES INFLAMMATION *IN VIVO* AND DEMONSTRATES
ADJUVANT POTENTIAL FOR VACCINE AGAINST INFECTIOUS
HEMATOPOIETIC NECROSIS VIRUS.**

by

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CONTRIBUTION OF THE AUTHORS

Dr. Jo-Ann C. Leong was involved in experimental design, data analysis, data interpretation, writing evaluation, and financial support of the work contained in this manuscript. Dr. Tawni Crippen was also involved in experimental design and data analysis and interpretation of the work presented here. Dr. Christopher Secombes and Jun Zou provided the trout IL-1 β gene that was a critical component of this research.

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**RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) INTERLEUKIN-1 BETA
PLASMID DNA GENERATES INFLAMMATION *IN VIVO* AND
DEMONSTRATES ADJUVANT POTENTIAL FOR VACCINE AGAINST
INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS**

**CHAPTER 1
THESIS INTRODUCTION**

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus that causes significant mortality in young salmonid fishes in hatcheries throughout Europe, Asia, and the northwestern United States. High mortality rates due to infectious hematopoietic necrosis (IHNV) result in capital losses for the aquaculture industry and food source losses for the consumer. Killed, attenuated, subunit, and genetic vaccines have been developed but none provide complete protection against mortalities due to IHNV in fish. Therefore, new methods of improving vaccines need to be examined. One way to improve vaccine efficacy is through the use of adjuvants. Typically, bacterial extracts, like Freund's complete adjuvant and lipopolysaccharides have been used to stimulate the immune response in fish. Other types of adjuvants used in aquaculture include oil, glucan, and aluminum-based compounds; vitamins; liposomes; and acrylic acid encapsulation (Anderson 1997). Mammalian vaccine systems include the previously mentioned adjuvants, but also include a plethora of immunomodulators like cytokines, hormones, and leukotrienes. In aquaculture, new adjuvant choices recently became available with the cloning of the first rainbow trout cytokine, interleukin-1 β (IL-1 β) (Secombes et al. 1998; Zou et al. 1999). This thesis will focus on examining the function and adjuvant potential of trout IL-1 β .

Objectives

The objectives of this study were to 1) design reporter vectors containing trout IL-1 β , 2) develop methods to detect recombinant trout IL-1 β , and 3) determine the function and adjuvant potential of trout IL-1 β .

Approach

Reporter vectors containing the trout IL-1 β gene were designed so that production of recombinant IL-1 β could be verified *in vitro* and *in vivo*. Histidine reporter (His) or Green Fluorescent Protein reporter (GFP) genes were used to distinguish between recombinant and endogenous trout IL-1 β . These reporter systems will be used in the future to purify recombinant protein and to detect recombinant IL-1 β in fixed fish tissues. It was also necessary to confirm the presence of the full-length protein produced from the IL-1 β /reporter gene fusions by Western blot using goat-anti-mouse IL-1 β antibodies. The function of IL-1 β protein generated from plasmid DNA was investigated in two *in vivo* experiments: 1) a plasmid dose-response experiment and 2) a lethal challenge experiment to determine the adjuvant potential of trout IL-1 β .

Introduction to Thesis Chapters

Chapter 2 is a literature review that describes the prior literature and background underlying the thesis research. Specific topics include IHNV, IHN, disease prevention and control strategies, and IL-1 β .

Chapter 3 describes the construction of the IL-1 β -GFP fusion vector from the IL-1 β -His fusion vector provided by C. Secombes. This chapter also discusses sequence homology found between rainbow trout IL-1 β and mammalian IL-1 β . The newly constructed plasmids contained the precursor sequence of the IL-1 β gene, so that when the protein was translated, either a C-terminal His-tagged IL-1 β molecule was generated or an N-terminal GFP-IL-1 β molecule was generated. Stable transfection in fish cells and subsequent protein expression was used to monitor the construction of each plasmid. In the case of the GFP plasmid, GFP production was examined.

Chapter 4 describes the detection of recombinant rainbow trout IL-1 β in media samples from stably transfected cells. IL-1 β was detected by Western blot using goat-anti-mouse IL-1 β . This antibody was selected because mouse IL-1 β was shown to have 46% similarity to the amino acid sequence of trout IL-1 β . The detection of recombinant IL-1 β was confirmed with a His-tag SDS-PAGE detection system. Anti-penta and anti-tetra-His antibodies were also used to detect the trout IL-1 β on Western blots, but the antibodies were found to have too much cross-reactivity with other host cell and media antigens.

Chapter 5 describes the assays for trout IL-1 β function *in vivo*. An experiment was carried out with varying doses of IL-1 β -His DNA and vector control DNA in rainbow trout. Fish were measured daily to monitor the level and persistence of swelling at the site of injection near the dorsal fin. These results were used to determine an appropriate dose of IL-1 β DNA to test as an adjuvant in an IHNV trial. Trout IL-1 β was

found to delay mortality and increase efficacy of a DNA vaccine containing the IHNV-G (glycoprotein) sequence.

Chapter 6 is a summary of the findings presented in this thesis. Chapter 6 also briefly discusses the direction of future studies involving trout IL-1 β . The research presented in this thesis forms the foundation for future assays that further evaluate the function and adjuvant potential of trout IL-1 β .

CHAPTER 2 LITERATURE REVIEW

Infectious Hematopoietic Necrosis Virus

Virion Characteristics

Viral Structure and Genome. Standard IHNV particles are bullet-shaped, with a typical length of 160 nm and core diameter of 60-70 nm (Amend and Chambers, 1970). IHNV is a member of the *Rhabdoviridae* family, in the genus *Novirhabdovirus* (Kim and Leong 1999). The negative-sense, 11 kb RNA genome contains six viral genes in the order 3'-N-P-M-G-NV-L-5'. The genes encode the following proteins: a nucleocapsid (N) protein, a phosphoprotein (P), a matrix protein (M), a glycoprotein (G), an RNA dependent RNA polymerase (L), and a non-virion protein (NV) (McAllister and Wagner 1975; Leong et al. 1981; Kurath and Leong 1985; Kurath et al. 1985).

Viral Proteins. IHNV was classified into five types based on SDS-PAGE migration patterns of the nucleocapsid protein and glycoprotein (Leong et al. 1981; Hsu et al. 1986). Virus types also showed differences in virulence between fish species, recognition by monoclonal antibodies, and plaque size (Bootland and Leong 1999; Chen et al. 1990). The N protein is the most abundant viral protein found in the IHNV and it is present as a phosphorylated protein in virus infected cells (McAllister and Wagner 1975; Hsu et al. 1985). The N protein may bind to the viral RNA and regulate the balance between viral transcription and replication (Gillmore and Leong 1988; Bootland and Leong 1999). Because of its abundance and conservation, the N protein has been the

target antigen for diagnostic assays and vaccine development. But, the G protein was actually found to induce protective immunity, in the form of neutralizing antibodies, against all five types of IHNV (Engelking and Leong 1989). The G protein is a membrane-associated protein that forms characteristic, conserved, antigenic spikes on the surface of the mature virus (McAllister and Wagner 1975).

Virus Production

Cell lines and Growth Requirements. IHNV can be produced in a multitude of cell lines, and is most often grown in *epithelioma papulosum cyprini* (EPC) cells (Fijan et al. 1983), chinook salmon embryo (CHSE-214) cells (Lannan et al. 1984), or rainbow trout gonad (RTG-2) cells (Wolf and Quimby 1962). The virus is propagated at physiologic pH between 13-18°C (Wingfield et al. 1969). The virus grows well from 4-20°C (Kim and Leong 1999). Cytopathic effects (CPE) of the virus include thickening of the cellular membrane, the appearance of dark, granular accumulations in the cytoplasm, rounded cells, and detachment of cells from the culture foundation. The first appearance of CPE depends on virulence of the viral strain and susceptibility of the cell line, but can usually be visualized within a few days.

Virus Titer. Virus titer is established by end point dilution in a tissue culture infectious dose (TCID₅₀) assay, or by plaque assay. Typically CPE is observed within 24-48 hours when CHSE-214 cells are used for the assays. Cells are inoculated when 80-90% confluent and incubated at about 15°C for a minimum of 14 days (TCID₅₀) or 7 days (plaque assay) (Amos 1985).

Stability. The titer of IHNV is slightly reduced at prolonged temperatures above 4°C, but the virus titer is relatively stable if stored at -70°C (Pietsch et al. 1977). The virus is susceptible to iodine, temperatures above 24°C, and low pH.

Infectious Hematopoietic Necrosis

Infectious hematopoietic necrosis (IHN) is the clinical syndrome caused by IHNV. It was named by Amend et al. (1969), to describe the necrosis found in the kidney, liver, pyloric caeca, and spleen. The virus also causes necrosis of the skin (petechial hemorrhages). IHN is almost always fatal in fry and results in huge fish losses in hatcheries.

Hosts and Transmission

Distribution and Host Range. IHNV was first identified in sockeye salmon (*Oncorhynchus nerka*) in Washington and Oregon, USA (Rucker et al. 1953; Wingfield et al. 1969). IHNV may have been endemic to Puget Sound, Washington, and then passed to Oregon in contaminated food (Amend and Wood, 1972). Currently, IHNV can be found in fish in Oregon, Washington, Idaho, California, Alaska, and British Columbia (Bootland and Leong 1999). The virus spread to Japan, China, Taiwan, Korea, and Europe through shipments of infected eggs. The *Oncorhynchus* genus, including *O. nerka* (sockeye), land-locked *O. nerka* (kokanee), *O. tshawytscha* (chinook), *O. masou masou* (cherry), and *O. keta* (chum) salmon are considered the primary hosts for IHNV. The virus also targets trout from the *Oncorhynchus* genus including *O. clarki* (cutthroat),

O. masou (yamame) and *O. mykiss* (rainbow) (Kim and Leong, 1999). Several other salmonid species are susceptible as reviewed by Bootland and Leong (1999).

Susceptibility of non-salmonid cell lines and juvenile fishes has also been examined.

IHNV can replicate in a white sturgeon cell line, but the viral titer was 17% of that normally produced in CHSE-214 cells (LaPatra et al. 1995). IHNV can also replicate in the tilapia ovary cell line (TO-2) at room temperature (Simon, personal communication 1999). Tilapia (*Oreochromis* and *Tilapia*), however, are not likely hosts for IHNV because they typically reside in water between 23-30°C, a temperature too high for the propagation of IHNV (American Tilapia Association 2000).

Transmission. IHNV typically causes an acute disease and results in 99% mortality in fry weighing less than one gram. Fish between 1-5 grams may also be infected, but mortality drops significantly with older, larger fish. Large, adult fish may be infected with the virus, but are asymptomatic. Virulence of the virus depends on the viral electropherotype and the susceptibility of the species being infected (Leong et al. 1981; Hsu et al. 1986; Chen et al. 1990; Bootland and Leong 1999). IHNV may be transmitted horizontally through the water depending upon fish density, water flow rate, and temperature. If the temperature exceeds 23°C, viral replication will be inhibited. High flow rates reduce the chance that fish will be infected because virus is “washed away” from infected fish. Increased density in fry results in close contact of fishes, making transmission unavoidable. High stocking density of infected adults in the area may contribute to horizontal transmission. In addition to horizontal transmission, it has been shown that eggs and sperm from infected adults may transmit IHNV to the offspring (Amend 1975; Mulcahy and Pascho 1985).

Disease State

Clinical Signs and Pathogenesis. Infected fry often become lethargic and anorexic, swim in a whirling pattern, have darkened bodies, distended abdomens, scoliosis, and bulging eyes (Amend and Yasutake 1969; Bootland and Leong 1999). These acute symptoms are followed by a sudden increase in mortality. Upon closer examination, fry may also have petechial hemorrhages, pale gills, and opaque fecal casts. During autopsy, kidney, spleen, and liver tissues appear pale and necrotic. The necrosis may only be detectable by histological analysis. General necrosis and virus may be found in kidney, spleen, liver, pancreas, muscle tissue, gills, and gastrointestinal tract during the advanced disease state (Drolet 1994). At the microscopic level, there is a general increase in macrophage number in necrotic tissue and general leukopenia, or reduction in the number of white blood cells. Neutrophils are decreased or absent (Amend and Smith 1975). Neutrophils are primarily responsible for phagocytosis of pathogens. Like macrophages, they also bear Fc and complement receptors important for antibody-dependent cellular cytotoxicity (ADCC). Thus, a reduction in neutrophil populations indicates suppression of a fish's ability to take up and present antigen for the induction of neutralizing antibody production as well as suppression of phagocytic and ADCC-mediated killing.

Diagnosis. IHNV isolated in cell cultures is typically identified by serum neutralization tests using rabbit antiserum or monoclonal antibodies against the IHNV N protein. These tests require about 7-10 days to complete. Therefore, several "quick" diagnostic tests have been developed. One is a direct or indirect fluorescent antibody (IFAT, DFAT) test that can detect any of the five types of IHNV within 10-12 hours after

the addition of virus samples to cell culture (LaPatra et al. 1989). That particular test can also be used to confirm the presence of IHNV in plaque assays (Drolet et al. 1993). Immunoassays can be used to identify virus include enzyme-linked immunosorbent assays (ELISA), dot blots, and Western blots. Electron microscopy identifies IHNV particles from water and tissue samples in a matter of hours. Reverse transcriptase polymerase chain reaction (RT-PCR) is another quick, accurate diagnostic method. For this test, mRNA is extracted from infected tissues and subject to a PCR reaction that amplifies the N gene sequence (Arakawa et al. 1990).

Control and Prevention of IHN

Vaccines and Control Strategies

Control. Hatcheries purchase certified IHNV-free eggs as the primary means to prevent IHNV outbreaks. Outbreaks are also limited by rigorous disinfection of water and supplies associated with rearing fish in hatcheries. The most effective treatment is to subject tanks, hoses, and eggs to iodine treatment, as this will destroy the virus, and limit other infectious agents like bacteria and fungus. Chlorination of outflow water in a hatchery or facility like the Salmon Disease Laboratory at Oregon State University also controls IHNV outbreaks. Unfortunately, most antiviral drugs do not seem to reduce mortalities associated with IHNV. One study reported moderate antiviral effects of 5-fluoropyrimidine nucleosides derived from 3-deoxy-beta-D-ribofuranose type compounds at inhibitory concentrations between 100-500 µg/mL in chinook salmon embryo cells infected with IHNV (Saneyoshi et al. 1995). Although antiviral drugs show slight

promise, they are too expensive to be used as a practical control measure to limit IHNV outbreaks. Therefore, scientists have turned toward vaccine research in hopes of eliminating IHNV from hatcheries and holding facilities.

Vaccines. Several vaccine strategies have been investigated for use against IHNV and these include live attenuated vaccines, inactivated vaccines, protein subunit vaccines, recombinant subunit vaccines, and genetic vaccines (Bootland and Leong 1999). IHNV, inactivated by β -propiolactone or formalin, is very effective as a vaccine only when delivered by intraperitoneal injection (Amend 1976; Nishimura et al. 1985). Effective attenuated IHNV vaccines have been developed, but have not been pursued because of possible reversion of the virus to a more virulent type (Winton 1997). Many IHNV glycoprotein subunit vaccines have also been developed (Engelking and Leong 1989). Engelking and Leong (1989) found that the subunit vaccine was more protective when fish were injected rather than exposed to the vaccine by immersion. Unfortunately, large amounts of viral protein are expensive to produce and purify, so the trend in vaccines turned toward recombinant technologies. Efforts concentrated on expressing the G protein in bacteria, and the first expression system was generated in *Escherichia coli* (Gilmore et al. 1988). Simon et al. (unpublished, 1999) recently generated portions of the G protein in *Caulobacter*. The purified G protein was injected into fish and provided some protection against IHNV when fish were lethally challenged. Although this method of antigen generation and purification is cheap and easy, the antigen does not seem to confer high levels of immunity to fish. This may be due to differences in protein processing between prokaryotic and eukaryotic cells and the fact that only one third of the viral glycoprotein gene was expressed in the *Caulobacter* vector.

Genetic immunization. The next obvious development in IHNV vaccines was a genetic vaccine of naked plasmid DNA. This approach was based on the finding that plasmid DNA injected into mouse muscle tissue *in vivo* resulted in expression of the gene encoded by the plasmid (Wolff et al. 1990). In fish, there is expression of β -galactosidase and luciferase reporter genes after intramuscular injection of plasmid DNA as well (Anderson et al. 1996a). Anderson et al. (1996b) generated the first genetic IHNV vaccine consisting of plasmid DNA encoding the entire IHNV-G gene or IHNV-N gene under control of the cytomegalovirus (CMV) promoter. Rainbow trout (about 1 gram in size) were immunized with the plasmid DNA (10 μ g/fish) containing the N gene, G gene, or both N and G genes. This was based on the earlier discovery that both N and G proteins together provided the greatest level of protection against IHNV (Oberge et al. 1991). Groups immunized with G or G+N genes resulted in only 17% and 15% mortality, respectively when challenged with a lethal dose of IHNV. The N gene alone resulted in 75% mortality and the control, untreated fish resulted in 65% mortality in the same experiment. The presence of IHNV neutralizing antibodies in the groups with the lowest mortalities was confirmed by ELISA (Anderson et al. 1996b).

The advantages of using a genetic vaccine are many (summarized by Winton 1997). DNA vaccines can not revert to virulence because they only generate expression of a single protein. Protein produced from plasmid DNA in eukaryotic systems is made in its native form. Also, genetic vaccines demonstrate near 100% protection, even when given at very low doses. This was determined recently by Corbeil et al. (2000). Rainbow trout immunized with one nanogram of the IHNV-G construct showed an $18 \pm 3.4\%$ mortality compared to vector-immunized control fish that showed $46 \pm 7.5\%$ mortality.

The true disadvantage of the DNA immunization is that it must be injected. Injection administration for very small fish, in this case fry, is just not practical in fish hatcheries. One way to avoid injection, is to design delivery methods based on “suicide bacteria.” In this model, bacteria lacking the ability to synthesize a vital growth requirement carry the plasmid DNA vaccine. The bacteria containing the vaccine DNA are cultured in media supplemented with the particular growth factor(s) to yield large quantities of the “bacterial” vaccine. Fish are immersed in this “bacterial” vaccine which is taken up by phagocytes that ultimately lyse the bacteria to release the plasmid DNA (Darji et al. 1997; Simon, personal communication). The free bacteria can not replicate because fish cells do not supply the necessary growth factor(s).

Even though genetic vaccines are very effective, large quantities of DNA are often injected and efforts to augment their efficacy are useful. Protein and killed virus do not generate consistent levels of protective immunity and it is probable that these vaccines do not induce the cellular immune response. For these reasons, we decided to examine the effects of adjuvants and their potential to enhance the efficacy of IHNV vaccines.

Adjuvants

Traditional adjuvants. Adjuvants are substances that when given in combination with antigen, act to stimulate B-cell and T-cell responses beyond the level of stimulation induced by antigen alone. Types of adjuvants include inorganic salts, delivery systems (liposomes, microspheres, etc.), bacterial products, and natural mediators like cytokines (Roitt et al. 1996). Bacterial extracts like Freund’s complete adjuvant and

lipopolysaccharides have been traditionally used to stimulate the immune response in fish. Other types of adjuvants used in aquaculture include oil, glucan, levamisole, and aluminum-based compounds; vitamins; liposomes; and acrylic acid encapsulation (Anderson 1997).

Cytokines as Adjuvants. Adjuvants enhance the efficacy of vaccines; however, most induce undesirable side effects such as reduced growth rate, fertility problems, and poor carcass quality. The best way to avoid these problems is to employ an intrinsic immunomodulator, like a cytokine, as the adjuvant of choice. Cytokines are potent in small quantities, are nontoxic, and do not promote autoimmune or allergic reactions. Cytokines are also inexpensive to produce when recombinant DNA technologies are employed. This method of production also guarantees high purity and reproducibility. Cytokines can be delivered by several methods. Cytokines have been expressed and delivered by *Salmonella* (Carrier et al. 1992), been delivered in plasmid DNA form (Xiang and Ertl 1995), and been delivered as a fusion plasmid containing a cytokine and ovalbumin (Maecker et al. 1997). It may be most advantageous to give cytokine adjuvant in DNA form because the cytokine may be continuously synthesized for weeks after injection, as compared to the protein form that may only remain active for days.

Fish cytokines. Fish cytokines have not been investigated for their adjuvant potential, because, until recently, few were available for testing. The following is a list of cloned fish immunomediators: transforming growth factor- β A and B from goldfish (Ge et al., 1993); Transforming Growth Factor β from trout (Hardie et al., 1998); granulins 1-3 in carp (Belcourt et al., 1993); CK-1 chemokine from trout (Dixon et al., 1998); and IL-1 β (Secombes et al., 1998). Unfortunately, this list of immunomediators includes mostly

growth factors that act to stimulate cell proliferation, rather than modulators that stimulate differentiation and cell recruitment. The IL-1 β molecule is the exception. Interleukin-1 β acts directly to recruit macrophages and neutrophils, the key cells need for both an innate and cellular immune response.

Fish typically rely on innate responses induced from injury, stress, or infection. Fish do mount cell-based immune responses, as indicated by the production of neutralizing antibodies in response to IHNV infection. The innate and specific immune response systems seem to be similar in both mammals and fish (Iwama et al. 1996). In either system, the key element is the macrophage or neutrophil that can act to take up and present antigen. The antigen presentation may then key the innate response, specifically by triggering the production of oxidative species and by increasing phagocytosis. The cellular response would occur later and would be characterized by cytotoxic T-cell activity and production of antibody. Since IL-1 β has the capacity to stimulate either type of response, it is an ideal cytokine to investigate for its immunoadjuvant potential.

Interleukin-1 β

Molecular Biology and Sequence Conservation

General characteristics. Since little is known about trout IL-1 β , this review will focus on what is known about mammalian IL-1 β . IL-1 β is a proinflammatory cytokine that regulates acute inflammation by activating lymphocytes, up-regulating vascular cell adhesion molecules, stimulating macrophages, and inducing the production of acute phase proteins. The cytokine has been reviewed several times, most recently by

Dinarello (1997, 1998). It is a member of the IL-1 gene family which also includes, IL-1 α and IL-1 receptor antagonist (IL-1Ra). IL-1 β is synthesized as an active, 31 kD precursor protein, lacks a signal peptide, and is primarily composed of β -pleated sheets. The 17 kD mature form of IL-1 β is produced when the molecule is targeted by IL-1 β converting enzyme (ICE or caspase-1) (Thornberry et al. 1992). However, this cleavage may occur by other mechanisms in fish, because the IL-1 β sequence lacks an ICE cleavage site (Secombes et al. 1998, Zou et al. 1998). Cleavages may be induced by trypsin, elastase, chymotrypsin, mast cell chymase, and other proteases found in inflammatory fluid (Dinarello 1998). After cleavage, the pro-segments of IL-1 β (14-17 kD) are also released and it is not known if these fragments possess any biological activity. IL-1 β molecules are secreted from cells by an unknown mechanism, but it is postulated that ATP binding cassettes are crucial to secretion (Hamon et al. 1997). Under stimulated conditions in cell culture, levels of IL-1 β mRNA rise within 15 minutes and stay elevated for about four hours due to regulation by transcriptional repressor and/or a decrease in mRNA half-life (Fenton et al. 1988). However, if IL-1 β is added to the system, levels of mRNA stay elevated. In short, IL-1 β is autoregulatory.

IL-1 Receptors. IL-1 β binds to either of two receptors, IL-1RI (80 kD) or IL-1RII (68 kD). IL-1 receptors show no sequence homology to the IL-1 family, including IL-1 α , IL-1 β , and IL-1Ra. IL-1RI and IL-1RII are found in both membrane bound and soluble forms. The type I receptor binds IL-1 and transduces a signal whereas the type II receptor does not transduce a signal. The IL-1 β /IL-1RI complex must also bind to the IL-1 receptor accessory protein (IL-1R-AcP) in order to transduce the IL-1 β signal. When the IL-1 β signal is transduced, it activates a series of other pathways inducing cytokine

synthesis and secretion, nitric oxide release, macrophage and neutrophil recruitment, and fever (Dinarello 1998). The type II receptor acts to “block” the signal from IL-1 β and essentially down-regulate IL-1 β action. Once bound to IL-1RII, IL-1 β (proform) can not be processed or recognized by IL-1RI (Symons et al. 1995). Likewise, if either receptor binds to IL-1Ra, both the binding and signal cascade caused by IL-1 α and IL-1 β are down-regulated.

Sequence Homology. IL-1 β genes are conserved across species. Carp, chicken, African clawed frog, and guinea pig IL-1 β amino acids share >48% sequence similarity to trout IL-1 β (Table 1). Of cloned and commercially available IL-1 β proteins, human, rat, and mouse sequences share >44% similarity with trout IL-1 β at the amino acid level (Table 1). In addition, cross reactivity of mammalian cytokines in fish systems have been reported for IL-1, transforming growth factor, and tumor necrosis factor (Jang et al. 1994, 1995; Hardie et al. 1998). Cross reactivity of mammalian antibodies to trout IL-1 β has also been reported, but later discredited due to high background and problems with experimental design (Ahne 1993). This cross reactivity can be better examined now that production of recombinant trout IL-1 β is feasible. Since IL-1 is one of the more conserved cytokines known and since the innate response of fish parallels that of mammals, it is highly likely that this cytokine is a key player in the trout proinflammatory response.

TABLE 1. Amino acid sequence similarity for IL-1 β across species. This table was generated using the Blast Search program "blastp" available at the NCBI website. Default parameters were used. (A) examines the sequence similarity between trout and carp, chicken, African clawed frog, guinea pig, horse, rat, mouse, bovine, or human IL-1 β . (B) examines the sequence similarity between bovine and human, mouse, or rat IL-1 β .

TABLE 1

Organism	Accession Number	Identities with Blast Sequence	Similarities	Gaps
A. Blast Against Trout IL-1 Beta (CAA11684.1)				
<i>Cyprinus carpio</i>	BAA24538.1	88/269 (32%)	145/269 (53%)	27/269 (10%)
<i>Gallus gallus</i>	CAA75239.1	78/224 (34%)	110/224 (48%)	18/224 (8%)
<i>Xeonopus laevis</i>	CAB53499.1	71/239 (29%)	120/239 (49%)	32/225 (13%)
<i>Cavia porcellus</i>	AAD38502.1	75/225 (33%)	109/225 (48%)	28/225 (12%)
<i>Equus caballus</i>	AAC39256.1	76/250 (30%)	114/250 (45%)	18/250 (7%)
<i>Rattus norvegicus</i>	M98820	48/132 (36%)	66/132 (49%)	8/132 (6%)
<i>Mus musculus</i>	CAA28637	69/213 (31%)	103/218 (46%)	15/218 (6%)
<i>Bos taurus</i>	M35589	41/237 (29%)	111/237 (45%)	28/237 (11%)
<i>Homo sapiens</i>	CAA39567	71/237 (29%)	107/237 (44%)	17/237 (7%)
A. Blast Against Bovine IL-1 Beta (M35589)				
<i>Homo sapiens</i>	CAA39567	163/269 (60%)	199/269 (73%)	5/269 (1%)
<i>Mus musculus</i>	CAA28637	157/270 (58%)	198/270 (73%)	7/270 (2%)
<i>Rattus norvegicus</i>	M98820	157/269 (58%)	196/269 (72%)	6/269 (2%)

IL-1 β Biology

Function. IL-1 β is produced in many cell types including macrophages, monocytes, NK cells, endothelial cells, neutrophils, and fibroblasts. IL-1 β affects almost all cell types, and induces localized inflammation and fever. In endothelial cells, IL-1 β acts to upregulate the expression of vascular adhesion molecules and secretion of chemokines to facilitate mononuclear infiltration (Rollins 1997). IL-1 β induces expression of itself in newly recruited monocytes (Dinarello et al. 1987). IL-1 also acts in conjunction with other cytokines like interleukin-12 to induce interferon- γ production (Hunter et al. 1995). Interferon- γ (IFN γ) is known to be important in the immune response against viruses. Fish probably have interferon (IFN α , IFN β , or IFN γ) as indicated by the discovery of the IFN-induced molecule, Mx (Stacheli et al., 1989; Trobridge and Leong, 1995). A sequence for an interferon-like molecule was discovered in Japanese flatfish (*Paralichthys olivaceus*) by Tamai et al. (1992, 1993) but the sequence is controversial at the present time.

IL-1 β may have deleterious effects on animals when it is present at high levels. The IL-1 family causes anorexia in mice and rats (Moldawer et al. 1988; Hellerstein et al. 1989; Plata-Salaman et al. 1998). Specifically, administration of recombinant IL-1 β decreased food intake in mice by 10-15% (Moldawer et al. 1988). It was also found that IL-1 β given by intraperitoneal injection at ≥ 4.0 $\mu\text{g}/\text{kg}$ reduced food intake in rats. It was hypothesized that this was due to the production of prostaglandins in response to IL-1 β (Hellerstein et al. 1989). The anorexic effect was completely inhibited by ibuprofen treatment. Prostaglandin E₂ production is a well-established effect of IL-1 β and has been

used as a feature to detect IL-1 β activity in cell culture (Burch et al. 1988, 1989, 1992; Kim et al. 1998). Prostaglandin synthesis as a result of IL-1 β treatment is easily blocked with non-steroidal anti-inflammatory drugs (NSAID), such as ibuprofen, thus explaining Hellerstein's results (Otterness et al. 1988). Unfortunately, NSAID agents do not act specifically to block IL-1 β or any other cytokines' function. To block IL-1 β function completely, anti-IL-1 β neutralizing antibodies or soluble IL-1 β receptor must be used.

Adjuvant potential. The adjuvant potential of IL-1 β has been tested in many mammalian systems. Recombinant human IL-1 β improved vaccine effectiveness in a murine lung cancer model system (McCune and Marquis 1990). IL-1 β and irradiated murine lung tumor cells were given to tumor-bearing mice as a vaccine. The vaccine eliminated tumors in 70-100% of the mice receiving the treatment, as compared to elimination of tumors in 0-20% of the mice receiving irradiated tumor cells alone (McCune and Marquis 1990). IL-1 β also generated nonspecific protection against Sendai virus infection in mice (Azuma et al., 1992). Species-specific recombinant IL-1 β has been used successfully as an adjuvant in calves for respiratory virus vaccines (Reddy et al. 1990, 1993). Recombinant bovine IL-1 β also increased efficacy of a vaccine against bovine herpesvirus-1 by upregulating mucosal specific IgA levels as compared to vaccine alone (Gao et al. 1995).

These adjuvant effects were also seen in studies that used an immunopotentiating portion of the IL-1 β protein as an adjuvant. In one study, mice were immunized with a bioactive fragment (163-171) of human IL-1 β linked to HIV peptides. The IL-1 β fragment lacked pyrogenic and inflammatory effects of the native IL-1 β . Mice receiving the IL-1 β peptide and HIV antigen showed significantly higher levels of antibody

production (detected at 1:8000 dilution) than mice immunized with HIV antigen alone (detected at 1:25 dilution) (Gokulan and Rao, 1997). Using the IL-1 β fragment for vaccines eliminates possible adverse effects including a systemic pyrogenic response. In a separate study, synthetic human IL-1 β peptide containing the immunostimulatory and receptor binding sequences enhanced protective antibody responses in mice when delivered in combination with a commercial hepatitis B vaccine (Manivel and Rao 1991).

It is the intent of this thesis to demonstrate that trout IL-1 β functions similarly to mammalian IL-1 β . Specifically, it will show that trout IL-1 β , transcribed and translated *in vivo* from a plasmid construct, induces acute inflammation, causes anorexia at high doses, and has the potential to act as an adjuvant in combination with the IHNV-G genetic vaccine. The cross reactivity of anti-murine-IL-1 β antibodies to recombinant trout IL-1 β will also be demonstrated.

CHAPTER 3 CONSTRUCTION OF VECTORS AND GENERATION OF RECOMBINANT TROUT IL-1 β

Introduction

In order to determine if trout IL-1 β could be expressed and function *in vitro* and *in vivo*, it was necessary to construct appropriate reporter plasmids. It was then necessary to determine if those plasmids were capable of expressing IL-1 β *in vitro* before *in vivo* experiments were performed.

We constructed plasmids containing trout IL-1 β and green fluorescent protein (GFP) or histidine tag (His) fusion proteins. NIH3T3 and CHH-1 cell lines were transfected with the IL-1 β plasmids and an attempt was made to select stably transfected cells expressing recombinant trout IL-1 β by using neomycin resistance selection.

NIH3T3 cells did not survive prolonged neomycin selection, possibly due to acute trout IL-1 β toxicity or lack of appropriate cleavage or secretion mechanisms for trout IL-1 β . CHH-1 cells did survive prolonged neomycin selection, maintained GFP expression, and were tested for presence of recombinant trout IL-1 β (Chapter 4).

Materials and Methods

Cell lines

CHH-1. Chum salmon (*Oncorhynchus keta*) heart fibroblasts (ATCC, CRL-1680) were grown to confluency in 75 cm² tissue culture flasks and maintained in

complete MEM (autoclaved minimal essential media (GibcoBRL #11700-010) supplemented with 10% Rehatuin fetal bovine serum (FBS) (Intergen #1020-01) and 1% L-glutamine (200mM, GibcoBRL #25030-081). The media was buffered with sterile 7.5% sodium bicarbonate solution so that pH was maintained at 7.2-7.4. For transfections, cells were grown to approximately 80% confluency in 6-well tissue culture plates in complete MEM.

NIH3T3. NIH/Swiss mouse (*Mus musculus*) fibroblasts were grown to confluency in 75 cm² tissue culture flasks and maintained in Dulbecco's Modified Eagle Medium (D-MEM, GibcoBRL #12100-046) supplemented with 10% normal calf serum (NCS) (Intergen # 1100-90). The media was pH adjusted to 7-7.2 and then sterilized through a 0.22 μ m filter. Cells were grown to approximately 80% confluency in 6-well plates for transfections. NIH3T3 cells (ATCC, CRL-1658) were obtained from Dr. Teresa Filtz, Department of Pharmacy, Oregon State University.

Plasmid construction

Subcloning of IL-1 β in pCR2.1. The precursor and mature forms of IL-1 β in pcDNA3 vector were obtained from C. Secombes, Aberdeen, Scotland, UK. The precursor form of IL-1 β encodes the full protein, including the leader peptide targeted by an ICE-like enzyme. The mature form of IL-1 β encodes the shortened protein, post cleavage. The precursor sequence starts at nucleotide 98 and ends at nucleotide 880; the mature sequence begins at position 377 and ends at position 880 in the nucleotide sequence (Fig.1A). Primers were designed to anneal to the precursor mRNA such that a *NheI* site was added to the 5' end and a *SmaI* site was added to the 3' terminus (Fig. 1C).

FIGURE 1. Rainbow trout IL-1 β cDNA, amino acid, and primer sequences. The complete cDNA sequence of rainbow trout IL-1 β retrieved at GenBank accession #AJ223954 is shown in (A). The precursor IL-1 β start and stop codons are boxed, as well as the beginning of the hypothesized mature IL-1 β (A). The corresponding amino acid sequence for the precursor form of trout IL-1 β is shown in (B). The primers and restriction sites designed for the cloning of IL-1 β into the pEGFP vector are shown in (C). The start codon and mutated stop codon of precursor IL-1 β are boxed.

A

1 acaatttagg attcacaaga actaaggact gaatacaaga caactgctac ctacctacaa

IL-1 beta start →

61 acttcaaaac aactcattaa gatctaacta cacagac[atg] gattttgagt caaactacag
 121 ttttaataaag aacacctctg aaagtgcagc atggagctcc aagctgcctc agggctctgga
 181 tctggaggta tcccatcacc ccatcaccat gcgtcacatt gccaacctca tcatcgccat
 241 ggagagggta aaggggtggcg aggggggttac catgggaacc gagttcaagg acaaggacct
 301 gctcaacttc ttgctggaga gtgctgtgga agaacatata gtggttgaggt tggagtcggc

Mature IL-1 beta →

361 acccccagcg agcagg[agg] cagcagggtt cagcagtaca tcacagtatg agtgtagcgt
 421 cactgactct gagaacaagt gctgggtcct gatgaatgag gctatggagc tgcacgccat
 481 gatgctccag ggaggcagca gctaccacaa agtgcatttg aacctgtctt cgtacgtcac
 541 gcccgctccc attgagactg aagccagacc tgtagcccta ggcataaagg gatccaacct
 601 ctacctgtcc tgctccaaat cgggaggcag gccaccctg cacctagagg aggttgccgga
 661 caaggatcag ctgaagtcca tcagccagca gagcgacatg gtgcgtttcc ttttctacag
 721 acggaacacc gggggtgaca tcagtacctt ggagtctgcc agtttcagga actggttcat
 781 cagcagggac atgcagcagg actacaccaa accggtagac atgtgtcaga aggcagcccc

IL-1 beta stop

841 caaccgcctt accaccttca ccatccagcg ccacaad[taa] gatggccgca aacactgaca
 901 caacaccagt caggaggcca aggcctccaa tagagatcct ctacaattca tgtgattcta
 961 ttgggcctct acgatcagga ctgggttgcc actgtgggtg cagcacatc aacagattta
 1021 agtgtattgc ttgtaagaca caagaatttc atgaggttta gagtatgtat cgagtacagc
 1081 atgttaccat tggggtgtat tgacgtagtc aatattgtgg taagcgcccc tgatataatc
 1141 aaaatattgct taaaacgaaa actaagtcac tattgttcta ctatttatta atatatctat
 1201 aattcagtaa tttatactta tttatcaatg tatttatttg gctctgttga tgttgccat
 1261 atccattgat acatgcaaac atttaaaatt cttatgtgct ctttttaata aacatattta
 1321 aatgaaaaaa aaaaaaaaaa aaa

B

1 MDFESNYSLI KNTSESAAWS SKLPQGLDLE VSHHPITMRH IANLIAMER

51 LKGEGVMTMG TEFKDKDLLN FLLESAVEEH IVLELESAPP ASRRAAGFSS

Potential Cleavage Site

101 TSQYECSVT[D]S[ENK]CWLMLN EAMELHAMML QGGSSYHKVH LNLSSVTPV

151 PIETEARPVA LGIKGSNLYL SCSKSGRPT LHLEEVADKD QLKSISQQSD

201 MVRFLFYRRN TGVDISTLES ASFRNWFIST DMQQDYTKPV DMCQKAAPNR

251 LTTFTIQRHN

FIGURE 1

C

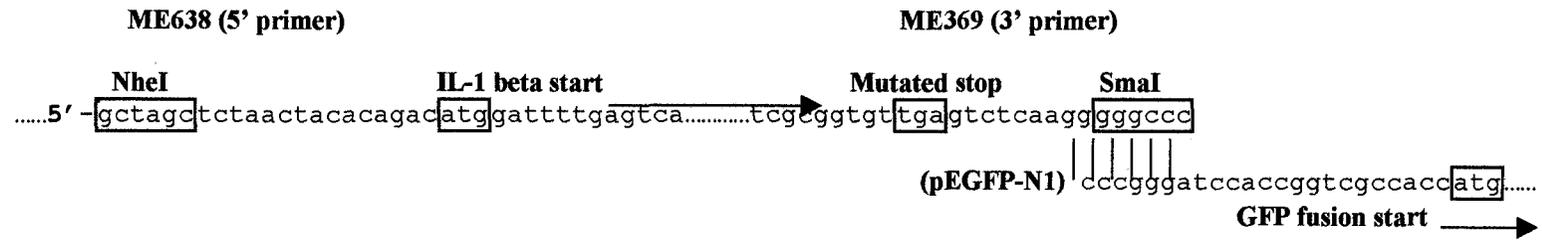


FIGURE 1 (continued)

The primer sequences used were:

ME638 (5' primer): 5'-GCTAGCTCTAACTACACAGACATGGATTTTGAGTCA-3'

ME639 (3' primer): 5'-TCGCGGTGTTGAGTCTCAAGGGGGCCC-3'

A mutation was created in the 3' primer to alter the stop codon so that a GFP gene fusion could be created (Fig. 1C). The stop codon UAA was changed to ACU, which codes for serine, a relatively inert amino acid.

Polymerase chain reaction (PCR) was performed using the designed primers and the precursor IL-1 β sequence as a template. PCR components were added according to the protocol provided in the *Platinum Taq Hifi* system (GibcoBRL #11304-011). The *Platinum Taq Hifi* system contains Taq DNA polymerase, *Pyrococcus* species GB-D thermostable polymerase, and *Platinum Taq* antibody. The *Pyrococcus* polymerase has 3' to 5' exonuclease activity that increases the fidelity of PCR reactions approximately 6 times over that of Taq polymerase alone. The *Platinum* antibody reduces non-specific binding of Taq polymerase. The PCR reaction was run for 30 cycles as follows: 95°C denaturation for 10 sec, 55°C annealing for 1 min, and 68°C elongation for 1 min. Resulting DNA was electrophoresed through a 1.5% agarose gel at 250V for 8min in the *RAGE*TM electrophoresis chamber (Cascade Biologicals, Inc., Model #RGX9707-60). An 819 bp band was isolated and gel purified using the QIAGEN[®] QIAquick Gel Extraction Kit (#28704). Briefly, the QIAquick Gel Extraction Kit solubilizes agar to release DNA that is bound to a column and eluted by changes in pH and salt concentration. The purified IL-1 β gene was then subcloned into pCR2.1 and then the subclone was transformed into One ShotTM cells according to the protocol provided (Invitrogen, Original TA Cloning[®] Kit #K2000-01, Version E, p. 9-13). The pCR2.1 plasmid

containing IL-1 β was isolated using the QIAGEN[®] QIAprep Spin Miniprep Kit (#27106). The QIAGEN[®] plasmid purification systems bind DNA extracted from bacteria by alkaline lysis. The anion exchange resin in the QIAGEN[®] columns binds DNA under low salt and low pH conditions and DNA is eluted when salt and pH are increased. The subcloned IL-1 β was cut from the vector using the *NheI* and *SmaI* restriction sites. The DNA was gel purified as described previously.

GFP and His-tag constructed plasmids. The *NheI*, *SmaI* restricted, gel purified IL-1 β gene was subcloned into the pEGFP-N1 vector (Clontech #6085-1) using standard ligation techniques. The His-tag construct (precursor IL-1 β) was obtained from C. Secombes. Transformations were done in *Escherichia coli* strain JM109. DNA was extracted with the QIAGEN[®] kit as before. Both constructed plasmids were sequenced on the ABIPrism[®] 377 to confirm the presence and accuracy of the IL-1 β gene.

Plasmid amplification and purification. The pcDNA3 plasmid backbone (Invitrogen) and pcDNA3 containing IHNV-G were obtained from laboratory frozen stocks. Transformations with these plasmids, and the His-tagged IL-1 β plasmid, were performed in *Escherichia coli* strain JM109. Transformed colonies were picked and amplified at 37°C overnight in 5 mL of selective media (Luria broth with ampicillin). These cultures were used to inoculate 500 mL overnight cultures from which DNA was extracted using a QIAGEN Plasmid Mega Kit (#12181) according to the protocol provided. The supercoiled form of all plasmids was verified by electrophoresing uncut samples on a 1.5% agarose gel. Plasmid composition was confirmed by restriction analysis on a 1.5% agarose gel.

Transfection of NIH3T3 Cells and CHH-1 Cells

NIH3T3. NIH3T3 cells were transfected according to the protocol provided with the LipofectAMINE PLUS™ Reagent (GibcoBRL #10964-013). Briefly, the PLUS reagent from LipofectAMINE PLUS™ is pre-complexed to the DNA prior to transfection. Once the DNA is coated in a PLUS-liposome, the LipofectAMINE cationic reagent is added to facilitate liposome + DNA entry into cells. The serum-free media used was OptiMEM (GibcoBRL #11058-021). Transfections were performed with both the GFP and His-tagged trout IL-1 β constructed plasmids. An attempt was made to select stably transfected cells expressing the recombinant trout IL-1 β by using Neomycin/G418 (Geneticin, GibcoBRL #11811-023) resistance selection. The attempts were unsuccessful despite the fact that neomycin resistant cells were obtained at a low level.

CHH-1. Since IL-1 β may be processed differently in fish, the CHH-1 cell line was also transfected with both trout IL-1 β constructed plasmids using the LipofectAMINE PLUS™ Reagent by the protocol provided by Gibco. Serum-free media steps were performed with serum-free MEM. Neomycin resistance was used as a selection criteria to establish a stably transfected cell line on two separate occasions.

Results and Discussion

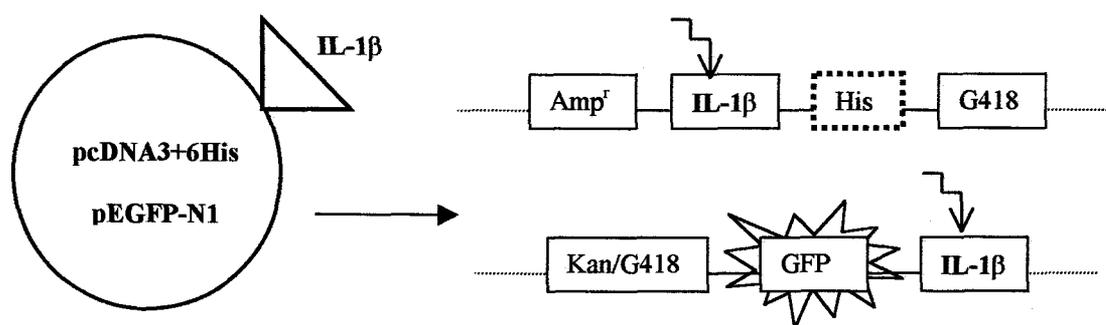
The full-length cDNA sequence of rainbow trout IL-1 β was retrieved from GenBank, accession number *AJ223954* (Fig. 1A). The precursor form of IL-1 β , from nucleotide 97 to 880 encodes the full-length protein, including the leader peptide targeted

by an ICE-like enzyme. The mature form of IL-1 β encodes the shortened protein, post cleavage. The mature sequence is speculated to begin at position 377 and end at position 880 (Secombes, unpublished). It has not been determined if precursor rainbow trout IL-1 β is cleaved, and a consensus ICE-cut site is not present; therefore the speculated sequence of the mature form may be incorrect (Secombes et al. 1998; Zou et al. 1998). Cleavage of mammalian IL-1 β occurs at the N-terminus of the protein at an aspartic acid (D) residue, resulting in the generation of a 116 aa pro-segment and 153 aa mature segment (Thornberry et al. 1992). If cleavage were to occur near the same position in trout, a 110 aa pro-segment and a 150 aa mature segment should be generated (based on the aspartic acid residue present at amino acid 110, Fig. 1B). The expected size of the mature trout IL-1 β protein would then be near that of its mammalian counterparts, 17 kD. Since it is impossible to determine an exact cut site for an ICE-like enzyme, the pro-sequence of IL-1 β was used to generate all constructed plasmids in this thesis.

The GFP and His-tag reporter systems were selected because they permit the easy purification, detection, and assay of recombinant proteins produced from the IL-1 β constructed plasmids *in vitro* and *in vivo*. These tags also provide a means to distinguish between recombinant and native IL-1 β .

The 6xHis tag is normally used as a tag because it is smaller than most affinity tags, is uncharged at physiological pH, rarely alters antigenicity of tagged proteins, and does not interfere with protein secretion. This was an important consideration during the *in vivo* studies because the His-tag had little chance of being cleaved from the IL-1 β protein. The His-tag is located on the C-terminus of the protein, opposite the anticipated ICE-like cleavage site (Fig. 2).

FIGURE 2. Structure of trout IL-1 β expression plasmids and resulting fusion proteins. The arrows indicate the approximate site of post-translational cleavage by an ICE-like enzyme or other proteases, which in turn generates mature IL 1 β .

**FIGURE 2**

GFP, cloned from the jellyfish *Aequorea victoria*, is an easily detectable fluorescent reporter. GFP is maximally excited at a wavelength of 395 nm, with a secondary peak at 475 nm; it emits at a single peak of 509 nm. Green light emission from the reporter protein can be detected by fluorescence microscopy, FACS, or by fluorescence under the microscope. GFP constructed plasmids allowed for localized, non-damaging detection for IL-1 β expression in transfected cells. The GFP fusion was generated as an N-terminal fusion with the IL-1 β protein (Fig. 2). This was advantageous because the GFP-IL-1 β protein was synthesized as a single pro-form molecule, but the GFP portion was cleaved off during IL-1 β export, facilitated by the ICE-like enzyme. The plasmid construction was valuable in two ways. Since N-terminal GFP was cleaved from mature IL-1 β by an ICE-like mechanism, the function of mature IL-1 β was not altered. Cleavage of IL-1 β by some unknown mechanism was also confirmed in tissue culture because GFP expression was only maintained intracellularly in transfected cells. In this model, we assumed that IL-1 β cleavage would occur intracellularly, prior to mature IL-1 β secretion.

Transfections were initially performed in mammalian cell lines because they can be transfected with DNA at high efficiencies. The NIH3T3 murine fibroblast cell line is an easily transfected cell line and has the ability to produce and respond to IL-1 (Burch et al. 1988, 1989). Therefore, it was chosen as an ideal cell line to use as a positive control for monitoring the binding and function of recombinant trout IL-1 β . The transfection efficiency was near 80%, but the cells began to die rapidly after a few days of selection. Protein accumulated within the cells as noted by the formation of dark inclusion bodies and localization of GFP in "pockets" within the cells. Also, the His-tag could not be

detected in media collected from NIH3T3 transfectants; IL-1 β detection was never attempted. It was suspected that trout IL-1 β was essentially toxic to the NIH3T3 cell line due to its accumulation in the cytoplasm. This evidence reaffirms the theory that trout IL-1 β is cleaved and secreted by a different mechanism than mammalian IL-1 β .

To test that theory, the CHH-1 salmonid cell line was transfected. This cell line probably has the ability to respond to IL-1, and may produce it. Transfection efficiency was approximately 5-10%, a good transfection efficiency for a fish cell line. A low number of cells persisted during the first few weeks of selection, but stable transfection was achieved after nearly two months of selection. Low cell numbers prohibited detection of recombinant IL-1 β simply because there was not much protein being made. Clonal expansion of stable transfectants by limiting dilution of cells was attempted several times without success. This may have been due to the lack of growth factors present in the media. Also, stable transfectants began to revert after several months of chronic selection. Some cells may have acquired antibiotic resistance through mutation and eventually out-competed the transfected cells. An image of transfected CHH-1 cells is provided in Fig. 3. Image A is a digital phase contrast image of cells under bright field illumination. Image B is a digital image of the same cells excited by UV light. The cells shown in image B expressed high levels of GFP.

Recombinant protein expression was verified in all CHH-1 transfected cells by monitoring GFP expression, maintenance of selective pressure, and identification of recombinant His-tag-IL-1 β which is discussed in Chapter 4.

FIGURE 3. Digital images of CHH-1 cells transfected with pEGFP-N1-IL-1 β plasmid DNA at 650X total magnification. (A) Phase contrast image. (B) Fluorescent image. Images were taken by Drew Sellers, P. McFadden Lab, Oregon State University.

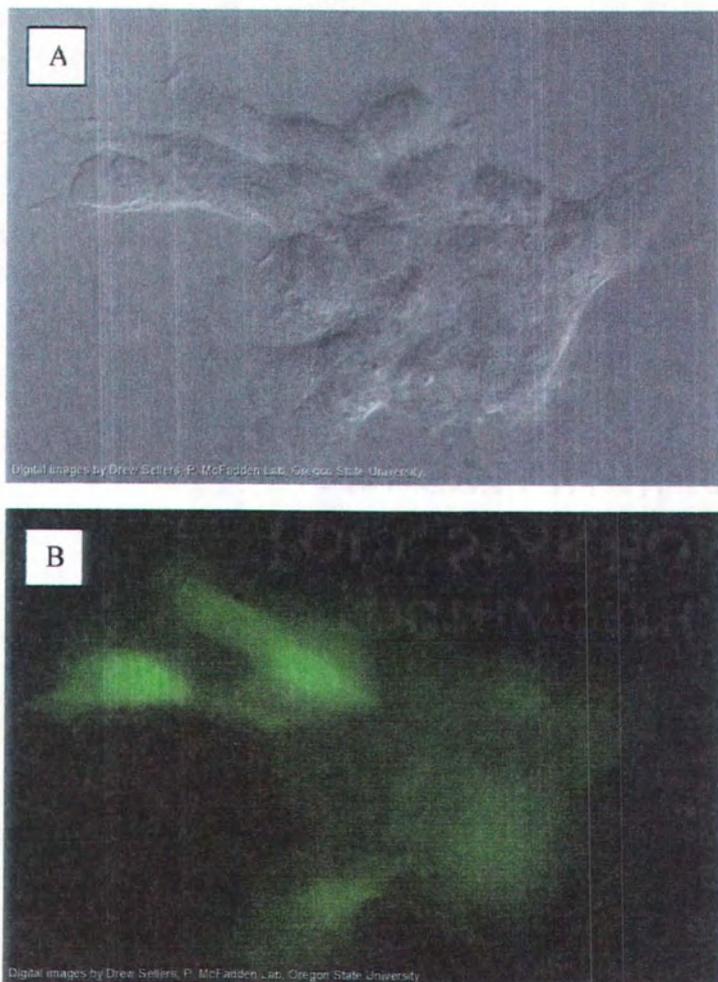


FIGURE 3

CHAPTER 4

DETECTION OF RECOMBINANT TROUT IL-1 β

Introduction

Recombinant trout IL-1 β expression was initially monitored in transfected CHH-1 cells by maintenance of neomycin resistance and GFP expression. However, we needed to confirm the production of IL-1 β species.

Trout IL-1 β was detected by Western blot using polyclonal goat-anti-mouse-IL-1 β antibodies. Western blot with anti-tetra-His antibodies and direct SDS-PAGE detection of the histidine tag were also used to determine if the protein produced by the transfected CHH-1 cells was derived from the recombinant plasmid DNA.

Two reactive proteins, 15 kD and 70 kD, were detected by anti-IL-1 β Western blots. Our results suggested that the 15 kD protein was trout IL-1 β and that the 70 kD protein was most likely a cross-reactive media component. We verified the recombinant nature of trout IL-1 by direct SDS-PAGE detection, and found that a 10 kD species was also detected during SDS-PAGE analysis. We determined that the 10 kD protein was most likely a cleavage product of the preprocessed IL-1 β protein. The 10 kD segment of IL-1 β was cleaved from a 29 kD precursor IL-1 β molecule most probably by some ICE-like enzyme.

Materials and Methods

Western Blots

Collection and Preparation of IL-1 β . IL-1 β samples were collected from transfected cells that had been under G418 selective pressure for approximately two months. Media on transfected cells was replaced approximately every 7-10 days and the “old” media was kept at -20°C for short storage or -80°C for long term storage until assay. Samples were prepared under reducing conditions and were electrophoresed in 15% SDS-PAGE gels and subsequently electro-blotted onto PolyScreen brand polyvinylidene fluoride (PVDF) membranes (NEN Life Science Products, Inc. #NEF1002). Benchmark Prestained Protein Ladder (GibcoBRL #10748010) was used as a molecular weight marker in all Western blots and SDS-PAGE analyses.

Anti-IL-1 β Blots. Blots were blocked overnight at 4°C in 2X Tris buffered saline (TBS) with 2% bovine serum albumin (BSA). Primary antibody was preabsorbed against a PVDF membrane blocked with MEM containing 10% FBS to reduce background. The primary antibody used was polyclonal biotinylated goat-anti-mouse-IL-1 β (R&D Systems #BAF-401) at $0.2\ \mu\text{g}/\text{mL}$. The secondary treatment was ExtrAvidin Peroxidase (Sigma #2886) at a 1:2000 dilution. Primary and secondary incubations were carried out at room temperature for one hour, while staining containers were shaken on the Lab-Line Orbit Shaker (Lab-Line Instruments, Inc., model #3520) at room temperature. All washes were performed three times for 10 minutes each, with 1X TBS-0.1% Tween 20-1%BSA.

Anti-His Blots. Blots were blocked overnight at 4°C in 2X TBS with 2% BSA and 5% nonfat dry milk. The primary antibodies used were murine-anti-tetra-His at 2 µg/mL (Qiagen #34670) or murine-anti-penta-His at 0.8 µg/mL (Qiagen #34660). T. Crippen generously donated a sample of anti-penta-His antibody for use in this study. The secondary antibody used was blotting grade affinity purified goat-anti-mouse-IgG-HRP (BioRad #170-6516). The secondary antibody was used at a 1:3000 dilution. All antibody incubations were carried out at room temperature for one hour with rocking. All washes were performed three times for 10 minutes each, with 1X TBS-0.1% Tween 20-1% BSA.

Detection. Proteins reacting on Western blots were detected by Supersignal West Pico Chemiluminescent Substrate Kit (Pierce #34080) and exposure to Kodac X-Omat Blue XB-1 film (NEN Life Science Products, Inc. #NEF586).

SDS-PAGE His-Detection. His-tag IL-1β was also detected directly in 15% SDS-PAGE gels. The Pierce GelCode® 6xHis Protein Tag Staining Kit (#24570) was used and the protocol provided with it was followed exactly. Positive bands were detected by exposure to a UV-light box. Total protein was detected by GelCode Blue Stain Reagent (Pierce #24590).

Results and Discussion

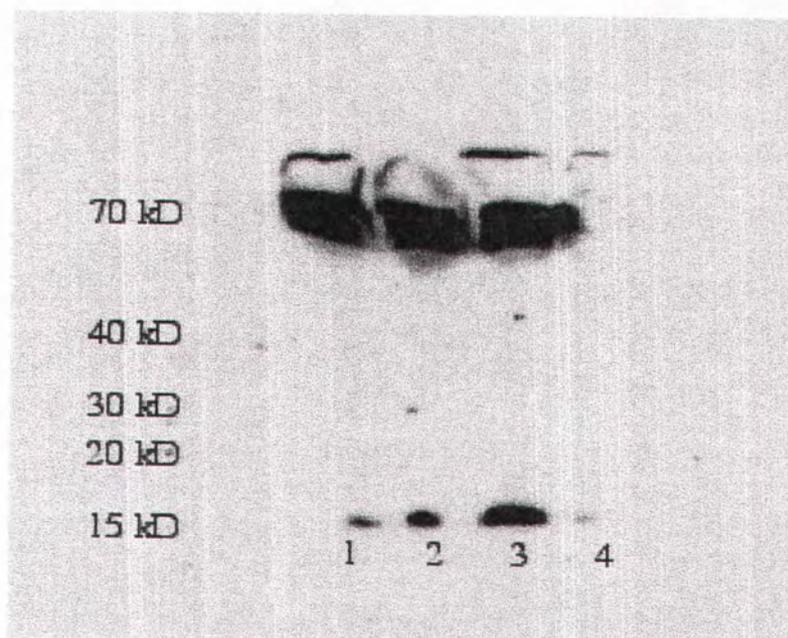
Polyclonal goat-anti-murine-IL-1β antibodies were selected for the detection of trout IL-1β because of the 31% amino acid sequence identity shared by murine and trout IL-1β proteins. The amino acid sequence comparison shows conservation throughout the sequences (Fig. 4), where amino acids listed between trout and mouse IL-1β sequences

show sequence identity and plus signs show sequence similarity. Secondary “ β -trefoil” structure is also preserved in both mammalian and trout IL-1 β proteins (Secombes et al. 1998). The recombinant anti-murine-IL-1 β antibodies were produced based on the particular sequence shown in Fig. 4 (R&D Systems), and we felt that cross-reactivity of the antibody to trout IL-1 β was likely. Murine IL-1 β shared 31% sequence identity and 46% sequence similarity with trout IL-1 β (Table 1). Table 1A compares the trout IL-1 β sequence with mammalian IL-1 β sequences including those for which anti-IL-1 β antibodies are commercially available. Table 1B compares bovine IL-1 β with the commercially available mammalian sequences compared to trout IL-1 β in Table 1A. This was done because bovine IL-1 β was thought to be a potentially cross-reactive species in the tissue culture media. As indicated, murine IL-1 β shared the least sequence homology, of IL-1 β genes compared in Table 1B, with bovine IL-1 β at 58% identity.

It was evident from the results presented in Fig. 5, that goat-anti-murine IL-1 β antibodies recognized two species of proteins, ~70 kD and ~15 kD, in tissue culture media from transfected and untransfected CHH-1 cells. Since mature mammalian IL-1 β species are approximately 17 kD in size, and the predicted molecular weight of trout IL-1 β was 17 kD, we concluded that the ~15 kD reactive band on anti-IL-1 β blots was mature IL-1 β . Mature murine IL-1 β , the positive control protein, was also analyzed by Western blot (results not shown) and found to migrate at 17 kD.

FIGURE 4. Alignment of mouse and trout IL-1 β amino acid sequences. This alignment was generated using default settings of the Blast Search program "blastp" available at the NCBI website. The parameters included match: 1, mismatch: -2, gap open: 5, and gap extension: 2. Identical amino acids are shown as capital letters between the mouse and trout sequences. Similar amino acids are shown as + between the mouse and trout sequences.

FIGURE 5. Western blot using polyclonal goat-anti-murine-IL-1 β antibodies. 30 μ L of protein sample was diluted in 6X SDS-sample buffer and heated for 5 minutes at 95°C. Each lane was loaded with 30 μ L of prepared sample. Lane 1 contained media alone. Lane 2 contained media harvested from confluent, untransfected CHH-1 cells from a T75 flask. Lane 3 contained media from one well of pcDNA3-trout IL-1 β transfected cells (2.5×10^5 cells/well). Lane 4 contained media from one well of pEGFP-N1-trout IL 1 β transfected cells (3.9×10^2 cells/well).

**FIGURE 5**

The 15 kD and 70 kD species were both present in complete media alone (lane 1), media from untransfected CHH-1 cells (lane 2), and media from IL-1 β transfected CHH-1 cells (lanes 3 and 4) (Fig. 5). Considering that the media contained 10% FBS, that itself contains IL-1 (Hida et al. 1995), it was not surprising that we detected a 15 kD IL-1 β molecule in media alone. In fact, murine and bovine IL-1 β share 58% sequence identity and 73% sequence similarity (Table 1B), which explains why anti-murine-IL-1 β antibodies detected bovine IL-1 β . It was obvious that trout IL-1 β -His transfected CHH-1 cells were producing high levels of recombinant IL-1 β -His because of the size and intensity of the band present in lane 3 (Fig. 5). The recombinant trout IL-1 β -His produced from 2.5×10^5 cells was significantly greater than the bovine IL-1 β endogenous to the complete culture media. High levels of recombinant trout IL-1 β -GFP were not detectable because there were only 3.9×10^2 viable transfected cells capable of producing protein.

Although the primary anti-IL-1 β antibody had been preabsorbed against complete media, there was still cross reactivity of the antibody with a 70 kD protein as shown in Fig. 5. The 70 kD protein appeared to be a media component only, as the band appeared to be the same size in lane 1 as it was in lanes 2 and 3. The band appeared mostly absent in lane 4, but this was due to poor trimming of the membrane prior to blocking. However, Ellaesser and Clem (1994) reported that in Western blots of channel catfish peripheral blood monocyte supernatant screened with anti-human IL-1 β sera, a reactive 70 kD protein was detected. They suggested that some of the protein in the large 70 kD band may be associated with catfish IL-1 β . Perhaps the same is true for rainbow trout IL-1 β , although the size and resolution of the 70 kD protein in Fig. 5 make this

impossible to determine. Efforts to minimize the amount of serum protein in the samples were made, including protein concentration through size exclusion filters and reducing the serum concentrations in the media. Unfortunately, the CHH-1 cells were not easily maintained under reduced serum concentrations (1% or no serum) for longer than a few days. Within this period of reduced serum, Western blots of media from CHH-1 cells produced no detectable trout IL-1 β or the 70 kD cross reactive protein. This suggested that trout IL-1 β production might have depended on a serum component. This finding is different from that of McKenzie et al. (1990) who found that an 85 kD FBS protein actually inhibited IL-1 β activity.

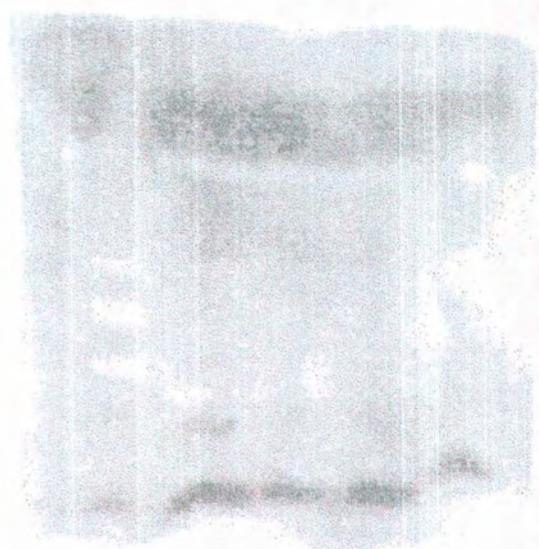
The cross reactive 70 kD protein may have been an excess of bovine serum albumin, or any number of alternative proteins. One alternative was that the 70 kD protein was actually an aggregate, polymer, or chain of active 15 kD IL-1 β molecules that were associated so tightly that the bonds were not broken under typical reducing and denaturing conditions (Ellaesser and Clem 1994). Ellaesser and Clem discovered that the catfish 70 kD protein did have IL-1 β -like function and caused proliferation in assays with monocyte depleted channel catfish peripheral blood lymphocytes or catfish thymocytes in the presence of ConA. Another possibility was that the 70 kD protein was actually the 68 kD type II IL-1 receptor, IL-1RII. It was possible that increased IL-1 β synthesis in transfected CHH-1 cells induced synthesis of soluble IL-1 β receptors to block excess IL-1 β activity. In that scenario, IL-1RII might have bound to IL-1 β so tightly that the bonds were not broken under typical reducing and denaturing conditions, so that IL-1 β and IL-1RII comigrated through the SDS-PAGE gel. It was unlikely that anti-IL-1 β antibodies detected IL-1RII protein directly because the antibodies were screened for cross

reactivity to human and mouse IL-1RII (R&D Systems). Also, no significant sequence homology was found during a two sequence blast between trout IL-1 β and rat IL-1RII precursor (P43303), mouse IL-1RII precursor (P27931), mouse IL-1RII (CAA42440), or human IL-1RII (CAA42441) (GenBank accession numbers). If IL-1 β had comigrated with IL-1RII, then fetal bovine serum must have also possessed high levels of IL-1RII. This is highly unlikely because FBS is taken from healthy cows that should not be expressing elevated levels of IL-1 β receptors. The 70 kD protein may also be an unidentified protein possibly responsible for directing IL-1 β secretion or activity.

To confirm the presence of recombinant IL-1 β , rather than native IL-1 β , in the media samples from transfected CHH-1 cells, anti-histidine Western blots and direct SDS-PAGE analyses were performed. Anti-tetra-His antibodies were initially used for Western blots due to lower specificity that would account for loss of one or more of the histidine residues present at the C-terminal end of trout-IL-1 β . Figure 6 shows a blot prepared with the same samples shown on the anti-IL-1 β Western blot in Fig. 5. Surprisingly, the anti-tetra-His antibodies recognized a ~10 kD protein that was not present in the anti-IL-1 β blot. There might have been slight reactivity with a 15 kD protein in lanes 1 and 3 (Fig. 6), but bands were not intense. The 10 kD species was present in complete media alone (lane 1), media from untransfected CHH-1 cells (lane 2), and media from IL-1 β transfected CHH-1 cells (lanes 3 and 4). The apparent molecular weight shift in lane 4 was due to a bent wire in the electrophoresis apparatus. This time, there was only slight cross reactivity with the 70 kD protein. Since detection levels were so low, the same blot was performed using the higher specificity anti-penta-His antibody. The same results were obtained (data not shown).

FIGURE 6. Western blot using mouse-anti-tetra-His antibodies. 30 μ L of protein sample was diluted in 6X SDS-sample buffer and heated for 5 minutes at 95°C. Each lane was loaded with 30 μ L of prepared sample. Lane 1 contained media alone. Lane 2 contained media harvested from confluent, untransfected CHH-1 cells from a T75 flask. Lane 3 contained media from one well of pcDNA3-trout IL-1 β transfected cells (2.5×10^5 cells/well). Lane 4 contained media from one well of pEGFP-N1-trout IL 1 β transfected cells (3.9×10^2 cells/well).

70kD
40kD
30kD
20kD
15kD
10kD



1 2 3 4

FIGURE 6

It was puzzling that the new reactive 10 kD species seemed to be detected at the same levels in media alone and in media from transfected and untransfected cells. To determine if differences existed between media controls and media from transfected cells, a third recombinant detection method was employed to confirm the presence of His-tagged trout IL-1 β from transfected cell culture. In this technique, recombinant His-tagged proteins are detected directly in SDS-PAGE gels by development with a fluorescent substrate. The results are presented in Fig. 7. The 10 kD protein was only detected in media from untransfected cells (lane 2) and transfected cells (lanes 3 and 4; Fig. 7). No reactive proteins were present in lane 1, containing complete media alone. The IL-1 β -His transfected CHH-1 media (lane 3) obviously contained 2-3 times the amount of reactive protein than did the untransfected or IL-1 β -GFP transfected CHH-1 cells (lanes 2 and 4, respectively), thus confirming the presence of recombinant histidine-tagged protein.

The appearance of a 10 kD reactive species can be best explained by examination of the trout IL-1 β sequence (Fig. 1). Histidine residues are present at amino acid numbers 32, 33, and 39 at the N-terminus of the protein. Cleavage of precursor IL-1 β into mature IL-1 β and a pro-segment occurs at the N-terminus. So, it is possible that the direct histidine detection kit is detecting the three proximal histidine residues at the N-terminus of the pro-segment of trout IL-1 β . The pro-segment of trout IL-1 β , based on the hypothesized cleavage at amino acid number 110, should be 12.1 kD. This would suggest that the pro-segment of trout IL-1 β is detectable by direct SDS-PAGE analysis.

FIGURE 7. Direct SDS-PAGE detection of histidine tagged protein. 30 μ L of protein sample was diluted in 6X SDS-sample buffer and heated for 5 minutes at 95°C. Each lane was loaded with 30 μ L of prepared sample. Lane 1 contained media alone. Lane 2 contained media harvested from confluent, untransfected CHH-1 cells from a T75 flask. Lane 3 contained media from one well of pcDNA3-trout IL-1 β transfected cells (2.5×10^5 cells/well). Lane 4 contained media from one well of pEGFP-N1-trout IL 1 β transfected cells (3.9×10^2 cells/well).

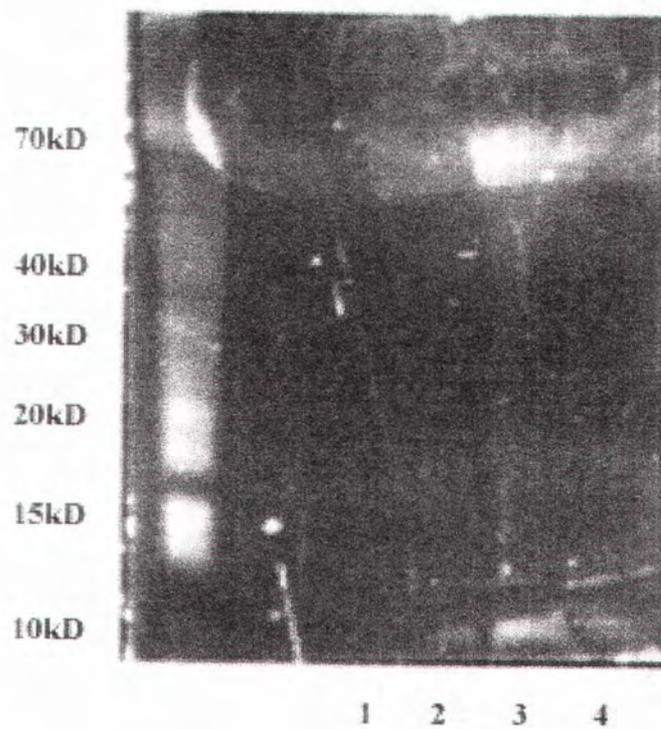


FIGURE 7

Concurrently, bovine IL-1 β does not contain three proximal histidine residues within its pro-segment, validating the observation that bovine IL-1 β is not detected by direct SDS-PAGE analysis.

We concluded that recombinant trout IL-1 β was detected by Western blot, as a 15 kD protein. It is also probable that a 10 kD pro-segment of trout IL-1 β is detectable by direct SDS-PAGE analysis. We confirmed the presence of recombinant trout-IL-1 β by the high expression levels in IL-1 β -His transfected CHH-1 cells, compared to untransfected CHH-1 cells in both anti-IL-1 β Western blot and direct SDS-PAGE analysis. There are probable explanations for the lack of detectable histidine tag on the 15 kD mature peptide. The histidine residues may be cleaved off during IL-1 β processing or secretion. Transcription or translation of the IL-1 β -His recombinant may also be truncated for some reason, thus yielding untagged recombinant IL-1 β molecules.

CHAPTER 5

FUNCTION AND ADJUVANT POTENTIAL OF TROUT IL-1 β IN VIVO

Introduction

The functional expression of the recombinant DNA derived trout IL-1 β in rainbow trout was examined by measuring the inflammatory response in fish as a function of dose and by determining whether the trout IL-1 β would act as an adjuvant for the IHNV DNA vaccine.

A dose-response experiment was set up to observe general inflammation and toxic effects of IL-1 β -His plasmid. Physical measurement, visual examination, and histological analysis of the treatment groups were used to determine inflammatory properties of trout IL-1 β -His. We determined from the dose-response data, that a ratio of 0.066 μ g DNA/ gram fish gave the best inflammatory response, with the least toxicity and used that dose as the standard for the ensuing experiments. We vaccinated rainbow trout fry with pcDNA3, PBS, IHNV-G, IHNV-G + IL-1 β -His, and IL-1 β -His plasmid constructs and challenged these fish 30 days after immunization with a lethal dose of IHNV. Mortality was used as an endpoint to establish a level of protection for each treatment.

Briefly, we found that trout IL-1 β -His plasmid DNA caused significant inflammation and hematoma formation in rainbow trout, but only high doses caused toxic side effects. We determined the optimal dose of IL-1 β -His DNA adjuvant, 0.065 μ g DNA/ gram fish tissue. Results from the lethal IHNV experiment indicated that IL-1 β

acts as an adjuvant to IHNV-G vaccine to delay mortality in fish challenged with 10^4 TCID₅₀/L IHNV.

Materials and Methods

Cell line

CHSE-214. Chinook salmon (*Oncorhynchus tshawytscha*) embryo cells were grown to confluency in a 12-well tissue culture plate (for plaque assay) with autoclaved minimal essential media (MEM, GibcoBRL #11700-010) supplemented with 10% Rehatuin FBS (Intergen, #1020-01) and 1% L-Glutamine (200mM, GibcoBRL #25030-081). The media was buffered with sterile 7.5% sodium bicarbonate solution so that pH was maintained at 7.2-7.4. Cells were grown in 24-well tissue culture plates for TCID₅₀ assay. Cells were grown and maintained in 150 cm² tissue culture flasks for virus propagation. Cells were obtained from the laboratory stocks, but may also be ordered from ATCC, CRL-1681.

Virus

IHNV. IHNV, Clear Springs isolate (220-90, Batch 17, 3/19/97) was propagated at multiplicity of infection of 0.007 in CHSE-214 cells at 20°C for 5 days, when complete cytopathic effects were observed. The cellular debris was removed by centrifugation at 2900 rpm for 10 minutes. Virus was aliquoted into cryovials and stored at -80°C until titration and challenge experiments were performed.

TCID₅₀. Virus titer was determined by tissue culture infectious dose₅₀ (TCID₅₀) assay. CHSE cells were grown to confluency in a 24 well plate. Ten-fold dilutions of virus, in a final volume of 100 µL/well were plated in quadruplicate and incubated at ambient temperature for one hour. The volume in each of the wells was brought up to a final volume of one milliliter with the complete MEM. The plates were incubated for 7 days at 20°C, when the presence or absence of CPE was noted as positive or negative, respectively. Titer was determined to be 3×10^7 TCID₅₀/mL by the Spearman-Kärber method.

Tissue IHNV Isolation. Whole rainbow trout fry were diluted 1:10 (w/v) in Anti-Inc media (Hank's balanced salt solution, GibcoBRL # 61200-036, containing 10% antibiotic-antimycotic, GibcoBRL #15240-062) and homogenized using a Stomacher 80 from Colworth. The homogenate was allowed to settle and samples were filtered by syringe fitted with a sterile 0.2 µm Acrodisk filter (Gelman Sciences, #4192). Virus samples were collected and assayed on the same day.

Plaque Assay. Samples from whole rainbow trout fry were diluted in 10-fold steps (10^1 - 10^4) in Anti-Inc media. One hundred microliters of each dilution was added to one well of a 12-well plate, each well containing CHSE cells in confluent monolayers, and virus was adsorbed to the cells for one hour at room temperature with periodic rocking. Viral dilutions were aspirated and methylcellulose overlay was added to the monolayers. Cells were incubated for 10 days at 20°C and then stained with crystal violet: formalin overnight. The stained plates were rinsed with tap water, air-dried, and plaques were counted. Virus titers were calculated as plaque forming units (pfu)/g of tissue.

In Vivo Experiments

Experimental Animals. Rainbow trout, *Oncorhynchus mykiss*, were obtained from Scott LaPatra (Clear Springs Foods, Buhl, ID) and held at the Salmon Disease Laboratory, Oregon State University, Corvallis, OR. Fish were acclimatized for one week prior to vaccination in 100 L tanks supplied with 13°C specific pathogen free well water and fed daily *ad libitum* with Starter #3 trout pellets (Life Stage Diets for fish, Bio-Oregon).

Dose-Response Experiment. Rainbow trout (mean weight of 3.8 g) were anesthetized by immersion in a 50 mg/L concentration of 3-aminobenzoic acid ethyl ester (MS-222) (Sigma #A-5040). Each fish was given an intramuscular injection at the base of the dorsal fin with 50 µL of DNA diluted in phosphate buffered saline (PBS). For the dose-response experiment, five fish per group were immunized with pcDNA3 (vector control, 10 µg) or IL-1β His (10 µg, 1 µg, 0.25 µg, or 0.03 µg). Three additional fish per group were similarly immunized with the same vaccines and placed in separate tanks to be used for histological analysis. Each group of fish was kept in 25 L tanks supplied with 13°C specific pathogen free water. The histology groups were sacrificed in 200 mg/L MS-222 at 72 hours post immunization. The dorsal region was dissected from the whole fish (approximately a 1 cm² section) and placed in 10% buffered-formalin for seven days. Histology sections were prepared as described by Drolet (1994). The dose-response fish were monitored for seven days post immunization. Photographs of each treatment group were taken daily and the general behavior of the fish was recorded. Trout were measured daily in cross-section at the injection site, to the nearest 0.5 mm with calipers.

Immunization and Lethal Challenge. Clear Springs rainbow trout (mean weight of 0.46 g) were anesthetized by immersion in 50 mg/L concentration of MS-222. All injections were given intramuscularly at the base of the dorsal fin. Trout were immunized with 10 μ L of the following DNA vaccines diluted in PBS: IHNV-G (0.01 μ g), IL-1 β His (0.03 μ g), and IHNV-G (0.01 μ g) + IL-1 β His (0.03 μ g). Control groups were similarly immunized with PBS and pcDNA3 (vector backbone of each construct). Three hundred fish were immunized for each treatment. An additional 175 fish were immunized with IL-1 β His (0.03 μ g) due to a malfunction of one repeat-pipettor during the injection of the first 300 fish in that treatment regime. All six treatment groups were kept separately in 100 L tanks supplied with 13°C specific pathogen free water for 29 days. Fish were fed daily at approximately 0.03-0.04 g food per trout.

IHNV Challenge. Six treatment groups of 75 rainbow trout (25 fish/triplicate) per group were exposed to 10^5 and 10^4 TCID₅₀/L IHNV diluted in PBS (Clear Springs, 220-90) by static immersion (13°C) for 5 hours at the Salmon Disease Laboratory, Corvallis, OR. Negative control fish, also 25 fish/triplicate, were mock-challenged with PBS. Just prior to challenge the six treatment groups were randomly divided 5 fish at a time into nine 25 L challenge tanks. The nine tanks were distributed such that three tanks were placed within 10^5 TCID₅₀/L, 10^4 TCID₅₀/L, and PBS challenge regions designated by letters A-I (Fig. 8). The tank destination for each group of 5 was decided by random card draw until 5 fish were placed into each region, A-I. At this point, the cards were shuffled and fish were again placed 5 at a time into each of the nine tanks. At the end of the entire treatment group distribution there were 25 fish in each tank.

FIGURE 8. Map of challenge randomization. Treatment groups were as follows 1) PBS, 2) IHNV-G, 3) pcDNA3, 4) IHNV-G + IL-1 β -His, 5) IL-1 β -His (225), and 6) IL-1 β -His (125). Distribution groups were A, B, C, D, E, F, G, H, and I. Challenge doses of 10^3 or 10^4 TCID₅₀/L, and a mock challenge with PBS, were used during the trial.

A	A	A	A	A	A
6	5	4	3	2	1

B	B	B	B	B	B	C	C	C	C	C	C
1	2	3	4	5	6	1	2	3	4	5	6
E	E	E	D	D	D	D	D	D	E	E	E
4	5	6	1	2	3	4	5	6	1	2	3

F	F	F	F	F	F	G	G	G	G	G	G
1	2	3	4	5	6	1	2	3	4	5	6
I	I	I	H	H	H	H	H	H	I	I	I
4	5	6	1	2	3	4	5	6	1	2	3

Treatment Groups:

- 1 PBS
- 2 IHNV-G
- 3 pcDNA3
- 4 IHNV-G + IL-1beta His
- 5 IL-1beta His (225)
- 6 IL-1beta His (125)

Distribution Groups:

A, B, C, D, E, F, G, H, I

IHNV Doses:

High (10 ⁵)
Low (10 ⁴)
PBS

FIGURE 8

The additional 175 fish immunized with IL-1 β (0.03 μ g) alone were not mock challenged with PBS, thus accounting for empty tanks marked by asterisks in Fig. 8. Excess fish from the six immunized groups were maintained in separate 100 L tanks for use in later experiments.

The average trout mass at challenge was 0.70 g. Fish were fed at the same time daily with trout pellets. Flow rate was adjusted to 500-600 mL/min per tank. Mortalities were counted, collected, and stored at -80°C daily for 30 days post challenge. Approximately 12% of the fish were assayed for IHNV by plaque assay; the rest were divided and stored at -80°C or autoclaved and discarded. Mortalities also displayed classic symptoms of IHN: distended abdomens, darkened body coloration, vertical drifting, and “popeye.”

Results and Discussion

Production of recombinant trout IL-1 β had been verified in tissue culture. Now it was possible to determine if trout IL-1 β could be produced *in vivo* and function as a potent cytokine. In mammals, IL-1 β functions as a pro-inflammatory cytokine that regulates acute inflammation by activating lymphocytes, stimulating macrophages and inducing the production of acute phase proteins. IL-1 β acts to upregulate the expression of vascular cell adhesion molecules and the secretion of chemokines to facilitate mononuclear infiltration. IL-1 β also induces expression of itself in those monocytes (Dinarello et al. 1987; Rollins 1997). In short, recombinant trout IL-1 β produced *in vivo* from plasmid DNA, should result in visible inflammation at the injection site.

It was important to determine the dose of trout IL-1 β that generated long-lasting inflammation without harmful side effects such as anorexia. The appropriate dose was important because some doses of IL-1 β could induce type II receptor up-regulation that would “block” the activity and signal transduction processes of IL-1 β (Symons et al. 1995).

A dose-response experiment was set up so that general inflammation and the toxic effects of IL-1 β -His plasmid could be monitored. Physical measurement (Fig. 9), visual examination (Fig. 10), and histological analysis of the treatment groups were used to determine inflammatory properties of trout IL-1 β -His. Striking differences were noted between the types of inflammation occurring in the control and IL-1 β treated fish. Within the first 24 hours after injection, all fish in the treatment groups showed some swelling and discoloration at the injection site. After 48 hours, fish immunized with 10 μ g of pcDNA3 control plasmid showed virtually no signs of inflammation (Fig. 10A). Swelling in control fish was reduced and no discoloration was present at the injection site. However, all fish immunized with varying doses of trout IL-1 β -His plasmid (10 μ g, 1 μ g, 0.25 μ g, and 0.03 μ g) showed signs of chronic inflammation well past 72 hours post immunization. Fig. 10B shows the characteristic hematoma and swelling induced by 10 μ g of IL-1 β -His 72 hours after immunization. The swelling and hematoma formation, although not as prominent, was even occurring in trout immunized with as little as 0.03 μ g of IL-1 β -His 72 hours after immunization (Fig. 10C).

FIGURE 9. Determination of swelling at dorsal injection site by caliper measurement. Trout were measured across the dorsal injection site to the nearest 0.5 mm daily for seven days, post injection with plasmid DNA.



FIGURE 9

FIGURE 10. Swelling and hematoma formation in trout immunized with pcDNA3 or pcDNA3 containing trout IL-1 β -His. Fish are shown 48 hours after immunization with 10 μ g pcDNA3 (A), 72 hours after immunization with 10 μ g of IL-1 β -His (B), and 72 hours after immunization with 0.03 μ g of IL-1 β -His (C). Note the darkened hematoma formation and swelling designated by arrows in (B) and (C).

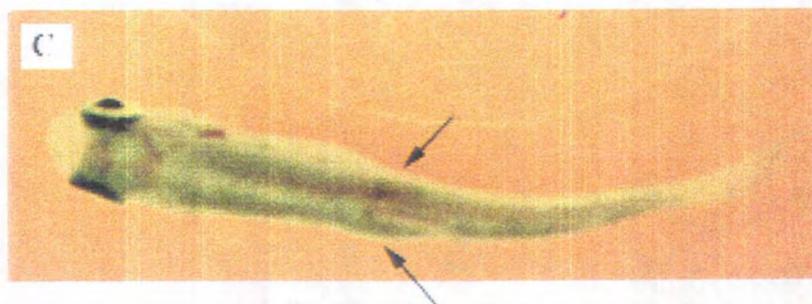
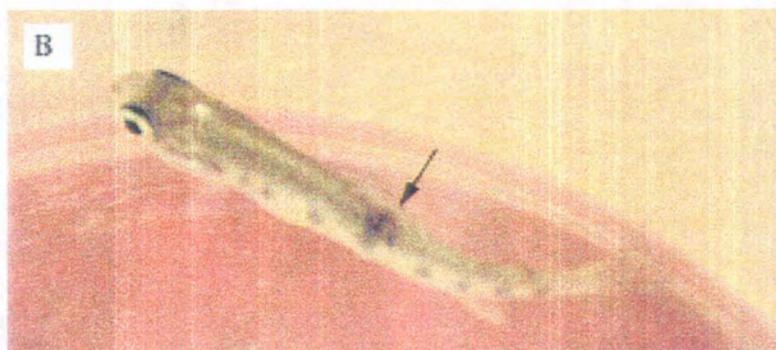
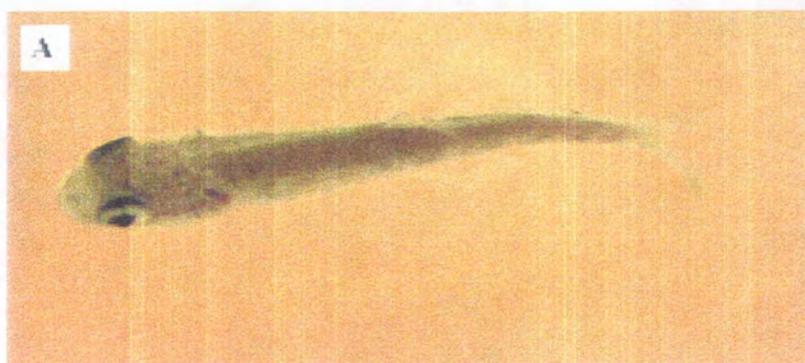


FIGURE 10

Behavior was different between treatment groups at 72 hours post injection.

Trout in the groups immunized with pcDNA3 (10 μ g) or IL-1 β -His (0.25 and 0.03 μ g) ate heartily, but trout immunized with 10 μ g and 1 μ g of IL-1 β -His ate approximately 80-90% less than the other fish. This suggests that the amount of recombinant trout IL-1 β produced *in vivo* was enough to cause anorexia in fish immunized with 10 μ g and 1 μ g of IL-1 β -His plasmid DNA. This theory is supported by previous results that show IL-1 β induces anorexia in mice and rats (Moldawer et al. 1988; Hellerstein et al. 1989; Plata-Salaman et al. 1998). Fish regained their appetites by 120 hours post immunization.

All five trout from each treatment group were measured daily to determine the level and persistence of inflammation at the dorsal injection site. The inflammation for fish immunized with 10 μ g of pcDNA3 peaked at 24 hours with 0.6 mm average swelling (Fig. 11A). The peak inflammation for fish immunized with 10 μ g trout IL-1 β -His occurred at 72 hours (0.6 mm) and inflammation persisted longer than in control fish (Fig. 11A). Although inflammation in IL-1 β -His immunized fish leveled off after 96 hours, the opposite was true for fish immunized with pcDNA3. This data correlates with the observation that pcDNA3 immunized fish ate heartily throughout the experiment, while IL-1 β -His immunized fish did not. Since pcDNA3 immunized fish ate well throughout the time course, and mean dorsal width dropped after the initial inflammatory response, the second dorsal width increase was most likely due to weight gain. The second peak in dorsal width was not seen in IL-1 β -His immunized fish because appetite was not restored until 120 hours after injection.

FIGURE 11. Inflammation induced by various doses of trout IL-1 β -His plasmid DNA and control plasmid, pcDNA3. All vaccines were prepared in sterile PBS. Comparison of swelling generated by 10 μ g doses of pcDNA3 or IL-1 β -His DNA is shown in (A). Swelling generated by 1 μ g, 0.25 μ g, and 0.03 μ g doses of IL-1 β -His DNA as compared to swelling caused by 10 μ g of pcDNA3 is shown in (B).

FIGURE 11A

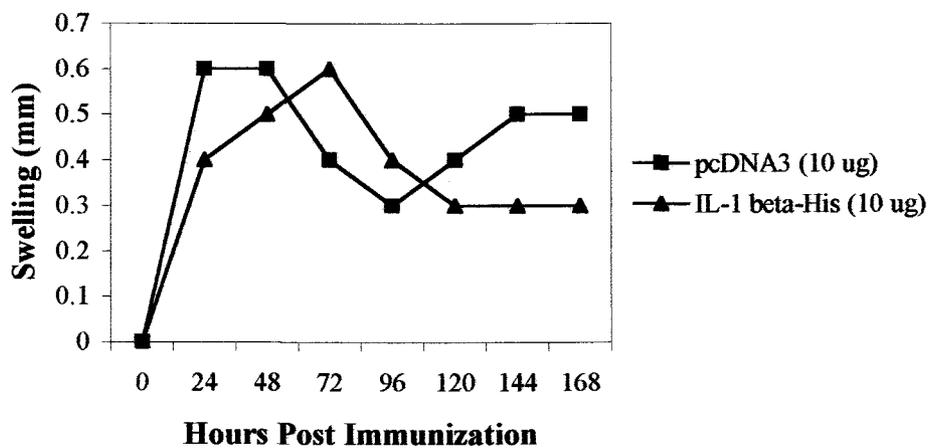
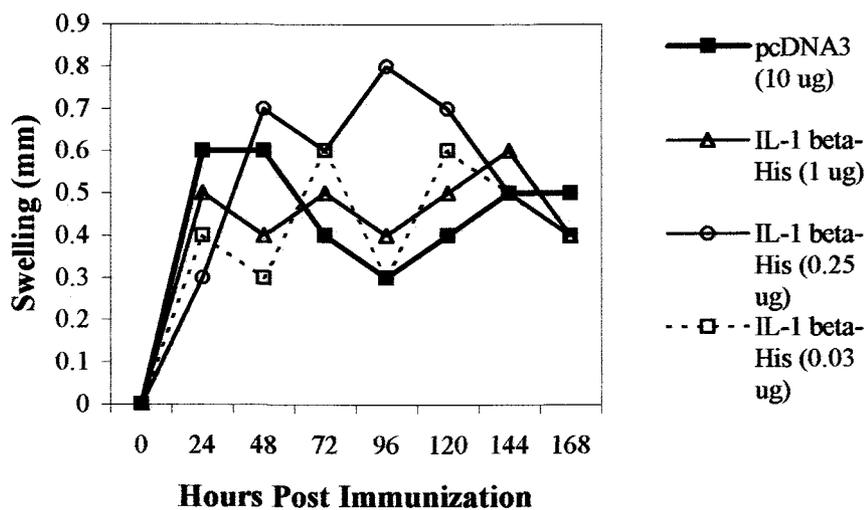


FIGURE 11B



Although the 10 μg dose of IL-1 β -His did stimulate significant inflammation, the swelling only persisted for 24-48 hours longer than in the control group. This may have been due to upregulation of IL-1RII. Thus, it was important to study the effects of lower doses of IL-1 β -His on trout. The 1 μg dose caused peak swelling of 0.6 mm 144 hours post injection (Fig. 11B). The swelling seemed persistent throughout the 168-hour experiment, and anorexia was again induced. The dose of 0.25 μg of IL-1 β -His DNA induced a peak swelling at 0.8 mm and swelling persisted above 0.4 mm throughout the experiment. IL-1 β -His at 0.03 μg also induced swelling to a peak of 0.6 mm. Inflammation fluctuated within this treatment group, but the measured inflammation remained at or above inflammation observed in the control group. Neither the 0.25 or 0.03 μg IL-1 β -His treatments induced anorexia.

Fish were sacrificed at 72 hours post injection for use in histological studies. Standard methods of immunohistochemistry (Vector Laboratories) and Wright-Giemsa staining were employed. Unfortunately, no distinct differences could be detected between the IL-1 β -His and pcDNA3 immunized fish (data not shown).

Fish of mean weight 3.8 g were used for the dose-response experiment, and 0.25 μg of IL-1 β -His DNA was found to induce the highest levels of non-harmful inflammation. Since IL-1 β -His was given at a dose of 0.066 μg DNA per gram of fish tissue, we used this ratio of 0.066 μg DNA/ gram fish as the standard dose for the ensuing experiments. Fish of mean weight 0.46 g were given 0.03 μg of IL-1 β -His DNA during vaccination (0.065 μg DNA/ gram fish tissue).

An efficacious DNA vaccine for IHNV composed of the G gene has been developed (Anderson et al. 1996b). A dose of 10 μg of IHNV-G resulted in a relative

percent survival (RPS) of 75% after challenge with IHNV. Recently, Corbeil et al. found that a single dose of as little as 1 to 10 ng of plasmid encoded IHNV-G protected trout fry against IHNV challenge. In 0.8-1.8 g rainbow trout fry, a dose of 10 ng of IHNV-G DNA resulted in an RPS of 83.3% (Corbeil et al.). In fry under 1 g, a dose of 10 ng of IHNV-G DNA typically resulted in ~75% RPS (LaPatra, personal communication). Since we wanted to determine adjuvant effects of IL-1 β after challenge with IHNV, we used a dose of 10 ng IHNV-G DNA in 0.7 g mean weight rainbow trout fry.

Fish immunized with IHNV-G or IHNV-G + IL-1 β -His plasmids were significantly protected against waterborne challenge with high concentrations of IHNV (Fig. 12 and Table 2). IHNV was verified, by plaque assay, to be the cause of death in 12% of the total number of mortalities occurring in both challenges. Less than 0.4% of the total mortalities were due to extraneous causes. No significant mortalities occurred in PBS mock challenged fish groups. Individual tank variation in the number of mortalities within IHNV-G and IHNV-G + IL-1 β -His treatment groups was not found to be statistically significant ($p \geq 0.5$, Fisher's exact test); therefore tank observations were disregarded and treatment groups of 75 fish per group were examined.

Fish were exposed to 10^4 TCID₅₀/L of IHNV, a dose that killed 50.7% to 68% of the control fish that received PBS, pcDNA3, or IL-1 β -His treatment (Table 2). Of fish receiving either IHNV-G or IHNV-G + IL-1 β -His vaccines, only 17.3% and 14.7% respectively, succumbed to lethal virus infection (Table 2).

FIGURE 12. Mortality rate of fish injected with plasmid DNA and subsequently challenged with IHNV. Rainbow trout at an average weight of 0.7 g were exposed to 10^4 TCID₅₀/L IHNV (A) or 10^5 TCID₅₀/L IHNV (B) 30 days after immunization with PBS, pcDNA3, IHNV-G, IHNV-G + IL-1 β His, or IL-1 β -His. Additional fish from each treatment group were mock challenged with PBS, although no significant mortality occurred (data not shown).

FIGURE 12A

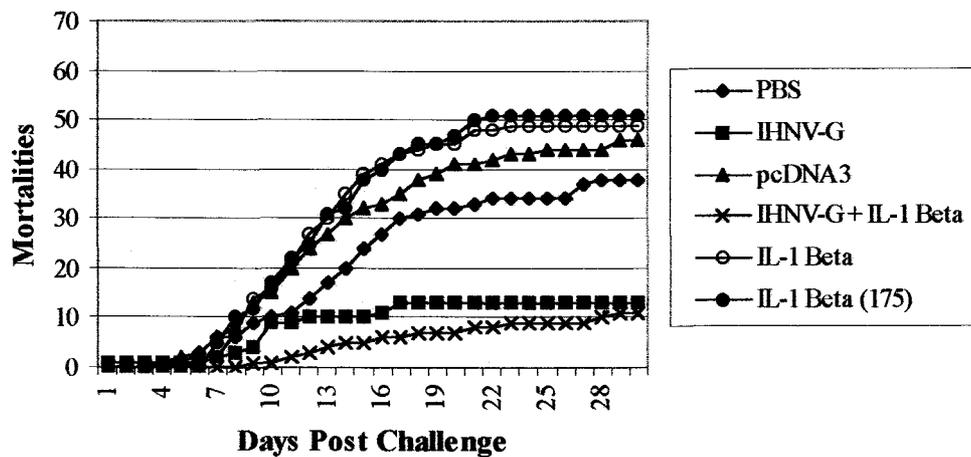


FIGURE 12B

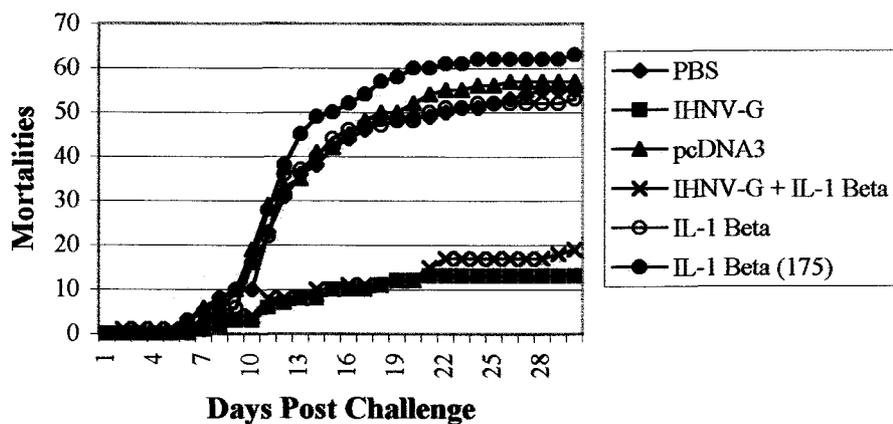


TABLE 2. Survival of fish challenged with IHNV after vaccination with plasmid DNA. Rainbow trout at an average weight of 0.7 g were exposed to 10^4 TCID₅₀/L IHNV (A) or 10^5 TCID₅₀/L IHNV (B) 30 days after immunization with PBS, pcDNA3, IHNV-G, IHNV-G + IL-1 β His, or IL-1 β -His.

TABLE 2

A. IHNV (10⁴) Challenge				
Treatment	Number of fish per group	Total number of deaths	Percentage mortality	RPS*
PBS	75	38	50.7	
pcDNA3	75	46	61.3	-20.9
IHNV-G	75	13	17.3	65.8
IHNV-G + IL-1beta	75	11	14.7	71.1
IL-1 beta	75	49	65.3	-28.8
IL-1 beta (175)	75	51	68	-34.1
B. IHNV (10⁵) Challenge				
Treatment	Number of fish per group	Total number of deaths	Percentage mortality	RPS*
PBS	75	55	73.3	
pcDNA3	75	57	76	-3.7
IHNV-G	75	13	17.3	76.4
IHNV-G + IL-1beta	75	19	25.3	65.4
IL-1 beta	75	53	70.7	3.6
IL-1 beta (175)	75	63	84	-14.6

*RPS indicates relative percent survival, calculated as described by Johnson et al. (1982):

$$\text{RPS} = [1 - \% \text{ mortality of vaccinated fish} / \% \text{ mortality of control fish}] \times 100$$

Fish were also exposed to 10^5 TCID₅₀/L of IHNV, a dose that killed 70.7% to 84% of the control fish that received PBS, pcDNA3, or IL-1 β -His treatment (Table 2). Of fish receiving either IHNV-G or IHNV-G + IL-1 β -His vaccines, only 17.3% and 25.3% respectively, succumbed to lethal virus infection (Table 2).

Due to the unexpectedly similar mortality rates in IHNV-G and IHNV-G + IL-1 β -His vaccine groups, it was necessary to examine the relationship between the number of mortalities in each group with respect to time. We analyzed the total number of mortalities at time points 10, 13, 16, and 21 days to compare the efficacy of the IHNV-G and IHNV-G + IL-1 β -His treatments. The time points were selected because they represent the exponential death phase for trout infected with IHNV in Fig. 12. In Fig. 12A, death occurred by day 10 post challenge (10^4 TCID₅₀/L IHNV) for fish treated with IHNV-G, but death occurred in fish treated with IHNV-G + IL-1 β -His approximately 13 days post challenge. Delayed mortality exhibited by IHNV-G + IL-1 β -His immunized fish was found to be significant on days 10 and 13 post challenge ($p < 0.02$ and $p < 0.10$ respectively). Differences in mortality counts between IHNV-G and IHNV-G + IL-1 β -His treatment groups were not significant at days 16 and 21 post challenge ($p > 0.18$). Differences in mortality counts between IHNV-G and IHNV-G + IL-1 β -His treatment groups were not significant at any time point examined for the 10^5 TCID₅₀/L IHNV challenge dose (Fig. 12B).

We verified the results obtained by Corbeil et al. by showing that 0.01 μ g doses of IHNV-G vaccine protect rainbow trout fry against IHNV challenge. We also concluded that IL-1 β acts as an adjuvant to IHNV-G vaccine to delay mortality in fish challenged with 10^4 TCID₅₀/L IHNV, but not when challenged with 10^5 TCID₅₀/L IHNV. A

possible explanation for this phenomenon is the difference in immunologic response of individual fish to IHNV. Studies have shown that neutralizing antibody titers from fish immunized with IHNV-G plasmid DNA vary between individual fish, especially 10 to 14 weeks after immunization (Anderson et al. 1996b; Corbeil et al.). Co-injection with IL-1 β -His plasmid DNA may have delayed IHNV mortality in our experiment by temporarily increasing the levels of neutralizing antibodies in some individual fish. Previous studies have shown that IL-1 β increased antibody titer in conjunction with hepatitis B vaccine and in conjunction with bovine herpesvirus-1 glycoprotein vaccine (Manivel and Rao 1991; Gao et al. 1995). However, antibody levels were apparently not elevated enough to protect fish from 10^5 TCID₅₀/L IHNV challenge in our study. Antibody titer probably decreased over time in fish challenged with 10^4 TCID₅₀/L IHNV because mortality rates between IHNV-G and IHNV-G + IL-1 β -His treatment groups were not statistically different after day 16 post challenge.

Another account for delayed mortality in IHNV-G + IL-1 β -His treated fish hinges on the induction of the cellular immune response generated by IL-1 β . IL-1 β was required for IL-12 release to induce production of interferon- γ (IFN- γ) in T-cell independent mechanisms for resistance against intracellular pathogens, *Toxoplasma gondii* and *Listeria monocytogenes* (Hunter et al. 1995). IL-1 β was also required for IL-12 release to induce production of interferon- γ (IFN- γ) when used as an adjuvant in an ovalbumin (OVA) vaccine model (Maecker et al. 1997). IFN- γ , produced by T-cells and Natural Killer (NK) cells, is responsible for generation of a T_H1 response by activation of macrophages, NK cells, and CD8⁺ T-cells; it also has some antiviral activity. This

response could trigger antigen elimination via ADCC, cytotoxic T-cells, phagocytosis, or release of nitric oxide intermediates (Roitt et al. 1996).

Therefore, we hypothesized that adding IL-1 β -His as an adjuvant to the IHNV-G vaccine may have generated both an increased neutralizing antibody response and an increased cellular immune response in rainbow trout fry when compared to the immunity conferred by giving the IHNV-G vaccine alone. Since both arms of the immune response were stimulated, rainbow trout may have also generated a stronger memory response to IHNV. Type of immune response conferred and immunological memory resulting from giving IL-1 β as adjuvant with the IHNV vaccine will be investigated in later studies.

We concluded that all doses of IL-1 β -His plasmid DNA caused significant inflammation and hematoma formation in rainbow trout, but only high doses caused anorexia in fish. We determined the optimal dose of IL-1 β -His DNA adjuvant, 0.065 μ g DNA/ gram fish tissue. Results from the lethal IHNV experiment indicated that IL-1 β acts as an adjuvant to IHNV-G vaccine to delay mortality in fish challenged with 10^4 TCID₅₀/L IHNV, but not when challenged with 10^5 TCID₅₀/L IHNV. We verified earlier results indicating that 0.01 μ g doses of IHNV-G vaccine protect rainbow trout fry against IHNV challenge.

CHAPTER 6 THESIS SUMMARY

Production of recombinant trout IL-1 β *in vitro*

Plasmids containing the trout IL-1 β gene and GFP or His fusion genes were used to transfect NIH3T3 and CHH-1 cell lines. An attempt was made to select stably transfected cells expressing recombinant trout IL-1 β by using neomycin resistance selection. NIH3T3 cells did not survive prolonged neomycin selection, possibly due to acute trout IL-1 β toxicity or lack of appropriate cleavage or secretion mechanisms for trout IL-1 β . CHH-1 cells did survive prolonged neomycin selection, maintained GFP expression, and CHH-1 media supernatants were assayed for presence of recombinant trout IL-1 β protein (Chapter 4).

Detection of recombinant trout IL-1 β in transfected CHH-1 cells

Production of recombinant trout IL-1 β was detected by Western blot using polyclonal goat-anti-mouse-IL-1 β antibodies. Results indicated that there were two reactive proteins, 15 kD and 70 kD, detected by anti-IL-1 β Western blots. We concluded that the 15 kD protein was trout IL-1 β and that the 70 kD protein was most likely a cross-reactive media component. Due to the high IL-1 β expression levels in IL-1 β -His transfected CHH-1 media samples, compared to untransfected CHH-1 media samples, we were able to verify the recombinant nature of trout IL-1 β by direct SDS-PAGE detection and anti-His Western blot.

We found that only a 10 kD protein was detected during SDS-PAGE and anti-His Western blot analysis. It is probable that the 10 kD protein was the pro-segment of IL-1 β cleaved from the 29 kD precursor IL-1 β by an ICE-like enzyme. The 10 kD pro-segment of IL-1 β was not histidine tagged, but might have cross-reacted during His-tag detection due to the close proximity of three histidine residues within the pro-segment. There are probable explanations for the lack of detectable histidine tag on the 15 kD mature peptide. The histidine residues may have been cleaved off during IL-1 β processing or secretion. Transcription or translation of the IL-1 β -His recombinant may have also been truncated for some reason, thus yielding untagged recombinant IL-1 β molecules.

Trout IL-1 β plasmid DNA is functional *in vivo* and has adjuvant potential when combined with IHNV-G genetic vaccine

A dose-response experiment was set up to observe general inflammation and toxic effects of IL-1 β -His plasmid. Physical measurement, visual examination, and histological analysis of the treatment groups were used to determine inflammatory properties of trout IL-1 β -His. We determined that a ratio of 0.066 μ g DNA/ gram fish gave the best inflammatory response, with the least toxicity and used that dose as the standard for the ensuing experiments. We found that trout IL-1 β -His plasmid DNA caused significant inflammation and hematoma formation in rainbow trout, but only caused temporary anorexia in fish in high doses (1 and 10 μ g).

We vaccinated rainbow trout fry with pcDNA3, PBS, IHNV-G, IHNV-G + IL-1 β -His, and IL-1 β -His plasmid constructs and challenged these fish 30 days after immunization with a lethal dose of IHNV. Mortality was used as an endpoint to establish

a level of protection for each treatment. Fish injected with either IHNV-G (0.01 μg) or IL-1 β + IHNV-G (0.03 and 0.01 μg , respectively) were protected from IHNV (10^4 TCID₅₀/L) challenge. Fish receiving IHNV-G + IL-1 β -His showed significant ($p < 0.1$) delayed and reduced mortality compared to fish receiving IHNV-G alone for 13 days post challenge. The RPS values for fish immunized with IHNV-G and IHNV-G + IL-1 β -His were 65.8% and 71.1% at 29 days post IHNV challenge. These studies showed that recently identified trout IL-1 β was functional *in vivo* and showed adjuvant potential when combined with the DNA vaccine for IHNV. We also verified results of Corbeil et al. (2000) by establishing that 0.01 μg doses of IHNV-G vaccine protect rainbow trout fry against IHNV challenge.

Future studies

Future studies will focus on identifying the type of immune response generated by using IL-1 β plasmid DNA as an adjuvant to the genetic IHNV vaccine. These studies will provide the foundation for typing disease responses most enhanced by IL-1 β so that appropriate vaccines may be developed. The ability of IL-1 β plasmid DNA to generate a longer lasting, memory response to IHNV will also be investigated. Purification of recombinant trout IL-1 β will allow for confirmation of the 15 kD size of trout IL-1 β identified here by Western blot. Pure recombinant trout IL-1 β will also be used to determine IL-1 β function *in vitro* by quantitating Prostaglandin E2 production in stimulated fibroblasts. Recombinant trout IL-1 β protein could also be assessed for its adjuvant effect in vaccine trials.

The most exciting result of these studies was the evidence that trout IL-1 β plasmid DNA delayed and slightly reduced mortality in fish challenged with 10^4 TCID₅₀/L IHNV. It will be interesting to see how the addition of IL-1 β as an adjuvant to other fish vaccines reduces mortality rates worldwide.

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