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Jo-Ann C. Leong

Recent advances in DNA vaccine technology has brought about a promising strategy for the control of viruses that contain surface membrane glycoproteins. This type of vaccine involves the intramuscular injection of a bacterial plasmid containing a gene encoding a viral protein. The strategy uses eukaryotic processing of the protein as would naturally occur during a viral infection. In this study, plasmid DNA encoding the glycoproteins of infectious hematopoietic necrosis virus (pcDNA3-IHNV-g), snakehead rhabdovirus (pcDNA3-SHRV-g), or spring viremia of carp virus (pcDNA3-SVCV-g) was injected into the skeletal muscle of rainbow trout fry. At 30 days post-vaccination, fish were challenged with IHNV. Protection against IHNV was observed among all DNA vaccinated groups. Fish injected with plasmid pcDNA3-IHNV-g, pcDNA3-SHRV-g, or pcDNA3-SVCV-g had relative survival rates of 93.2%, 98.3% and 94.9%, respectively.

The mechanisms for the viral mediated resistance induced by these glycoprotein based DNA vaccines is unknown. A parallel study conducted by Dr. Carol Kim on the production of Mx proteins in these fish indicates that the observed protection might be a consequence of the stimulation of interferon.

DNA Vaccines Encoding the Glycoprotein Genes of Spring Viremia of Carp Virus, Snakehead Rhabdovirus, or Infectious Hematopoietic Necrosis Virus Induce Protective Immunity in Rainbow Trout (*Oncorhynchus mykiss*) Against an Infectious Hematopoietic Necrosis Virus Lethal Challenge

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John D. Drennan

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Approved:

Redacted for Privacy

Major Professor, representing Microbiology

Redacted for Privacy

Chair of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

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John D. Drennan, Author

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CHAPTER 1
INTRODUCTION

Recent advances in DNA vaccine technology has brought about a promising strategy for the control of viruses that contain surface membrane glycoproteins. This type of vaccine involves the intramuscular injection of a bacterial plasmid containing a gene encoding a viral protein. The strategy uses eukaryotic processing of the protein as would naturally occur during a viral infection.

Our lab has developed a glycoprotein based DNA vaccine for rainbow trout against infectious hematopoietic necrosis virus (IHNV) (Anderson, E.D. *et al.*1996). The vaccine is composed of the bacterial plasmid pcDNA3 and the complete gene sequence for the glycoprotein of IHNV. As would occur during a normal viral infection, the glycoproteins are synthesized and incorporated into the plasma membrane. Part of the glycoprotein projects out of the infected cell, appearing as spikes observed through electron microscopy.

Immunization experiments with this DNA vaccine followed by IHNV challenge produced relative survival rates of 75 percent or greater, even in fish as small as 0.3-0.4 g average weight (Anderson, E.D. *et al.*1996). These findings were very encouraging and we assumed that protection was based on neutralizing antibodies specific for IHNV. However, studies within the last decade in mammalian systems have shown that viral

glycoproteins can stimulate the production of interferon which could be the basis for the protection (Ito, Y. 1994; Ito, Y. *et al.* 1994; Ito, Y. & Y. Hosaka, 1983). The results of the mammalian studies led to a model where viral glycoproteins, in general, can act as mitogens to stimulate lymphoid cells into producing interferon and subsequently leading to non-specific protection. This stimulation is based on a membrane-membrane interaction between lymphoid cells and cells containing the protruding transmembrane glycoproteins.

On the basis of this model, we sought to examine the specificity of the IHNV response induced by the IHNV DNA vaccine by treating fish with several different plasmid vectors containing glycoprotein genes from different rhabdoviruses. In this study, we present evidence that glycoprotein-based DNA vaccines induce an immune response that protects trout through production of non-specific factors. All of the glycoprotein vaccines in the test group provided protection against IHNV infection. Plasmid DNA encoding the glycoproteins of IHNV (pcDNA3-IHNV-g), SHRV (pcDNA3-SHRV-g), or SVCV (pcDNA3-SVCV-g) was injected into the skeletal muscle of rainbow trout fry. After 30 days, the fish were challenged with IHNV. Results clearly indicated that the glycoproteins of these different viruses all induced a protective immune response in fish. The mechanisms for the viral mediated resistance induced by these DNA vaccines is unknown, but a parallel study conducted by Dr. Carol Kim, who examined the production of Mx proteins in these fish, suggests that the protection observed might be a consequence of the stimulation of interferon.

CHAPTER 2
LITERATURE REVIEW

Rainbow trout, *Oncorhynchus mykiss,* is a member of the family *Salmonidae* and is an important aquaculture food product worldwide. Europe, Japan, South America, and the United States are the major producers of farmed reared trout (Parsons, J. 1998). In 1997, the United State Department of Agriculture reported $78 million worth of trout sold (Aquaculture Magazine Buyer's Guide 1998). There are 18 states that commercially farm trout and in 1997, Idaho was the leading producer with 75% of the United States production (Trout Production report, 1997)

The success of trout farming can be attributed to the ease in which trout can be cultured in a variety of captive environments. But as in any situation where animals are placed in high-density regimes, susceptibility to infectious diseases is a devastating problem. In the United States, approximately 5 million pounds of trout during 1997 were lost to disease (Aquaculture Magazine Buyer's Guide 1998). Clearly, the trout industry must develop control strategies for disease at trout rearing facilities. Efforts to develop an understanding of the trout immune system and to use this understanding to develop better vaccines are important for the industry.

DNA Vaccines for Fish Aquaculture

The goal of any vaccine is the stimulation of an immune response that resolves infection and provides long lasting protection. In mammals, this primarily involves the activity of B and T lymphocytes and subsequently, the production of antibodies (humoral

response). In addition, vaccines prime immune cells such as cytotoxic T lymphocytes, natural killer cells, and macrophages (cellular response).

For the past 100 years, vaccine development has been limited to vaccines consisting of live-attenuated virus, killed virus, or purified viral protein (subunit vaccines). Most recently, advancements in vaccine technology introduced the DNA vaccine. This type of vaccine involves the intramuscular injection of a bacterial plasmid containing a gene of an appropriate viral protein. This strategy enables eukaryotic processing of the protein as would naturally occur during a viral infection. The advantage of DNA vaccines is the stimulation of both humoral and cellular immune responses (Donnelly, J.J. *et al.* 1997). DNA vaccines not only induce the production of antibodies but also stimulate cytotoxic T cells and helper T cells, which can be regarded as necessary components of a truly effective vaccine (Leung, K.N. & Ada, G.L. 1982; Taylor, P.M. & Askonas, B.A. 1986).

With regard to fish aquaculture, the ability of a vaccine to provide protection is not sufficient. Other factors such as route of administration, protection against heterologous strains, safety, and expense contribute to the difficulties in the development of effective vaccines for high-density fish farming. Cost effectiveness is the number one issue. Typically, the production of a viral vaccine is expensive and any requirement for the administration of the vaccine to individual fish can dramatically heighten costs to the fish farmer. In many cases, the expense of replacing the diseased fish is less costly than administrating a vaccine.

If DNA vaccines are to become a viable alternative in aquaculture it is necessary to overcome the cumbersome task of individual inoculations, which is labor intensive and

difficult to administer to small fish. Although laboratory experiments show that DNA vaccines are very effective and rather inexpensive to produce, unless an easy vaccination procedure is developed, the adoption of a fish DNA vaccine is uncertain.

Fish Immune System

Our understanding of the immune system in fish is not as extensive as it is for the mammalian immune system. Nevertheless, we can point out the important differences between the immune response in fish versus mammals. One of the most conspicuous differences is the tissue location of the progenitor cells. In mammals, all of the cellular elements of blood arise from hematopoietic stem cells in the bone marrow. There is no bone marrow in fish and the hematopoietic stem cells are believed to reside in the anterior portion of the kidney. Also, fish do not possess lymph nodes. The spleen, liver, and kidneys are the primary organs in fish where substances are eliminated from the blood. Regardless of this difference, the immune components in the circulating blood of fish are basically the same as in mammals. Granulocytes and macrophages are present in fish and function in a similar manner. In addition, fish contain sub-populations of "T" and "B" lymphocytes, although specific markers for these distinct cell types have not been identified. Components of the non-specific defense system such as complement and interferon have also been described in fish. These components appear to perform the equivalent functions as those described in mammals.

Fish Antibody

In comparison to mammals, the fish acquired immune response is quite restricted. Fish do produce an antigen-specific antibody response; however, the response is directed towards fewer epitopes on complex molecules and there does not seem to be a progression in the antibody response to yield antibodies with tighter binding affinities. There appears to be a secondary antibody response in fish, which would confirm the presence of memory cells (Kaattari, S.L. 1994). The magnitude of the secondary response is not logarithmic and is dramatically lower than in mammals. There is also no substantive evidence for isotype switching. (Lobb & Olson, 1988; Killie *et al.*, 1991) The absence of affinity maturation is striking and may be due, in part, to the fact that fish only produce a single class of tetrameric IgM like immunoglobulin. The humoral response in fish is also considerably slower than in mammals and is temperature dependent.

Within the last decade, researchers have identified the cDNA sequences for major histocompatibility complex (MHC) class II β, class II α, and β_2 microglobulin (β_2m) genes in teleost fish (Godwin, U.B. *et al.* 1997; Antao, A.B. *et al.* 1997). Additional studies have detected β_2m on periphereal blood lymphocytes (PBL) as well as on clonal B and T cell lines of catfish (Antao, A.B. *et al.* 1997). Also, the T cell receptor in teleost fish has been described (Hordvik, I. *et al.* 1996). These discoveries would indicate that although there are some structural differences in the components of the fish immune system, the function and immunological pathways leading to protection are similar to that in the mammalian system.

Fish Cellular Immune Response

The key cells in the non-specific cellular immune response in fish are granulocytes and monocytes/macrophages. In mammals, granulocytes are comprised of three distinct cell types: eosinophils, neutrophils, and basophils. Neutrophils are present in teleost fish, but whether the other two cell types are present has not been clearly determined (Ellis, A.E. 1977; Ainsworth, A.J. 1992). The functional role of mammalian macrophages is the elimination of invading microorganisms by phagocytosis, and this role is no different in fish. These macrophages are located in most tissues and are present as monocytes in circulating blood. Macrophages also play an enormous role in the specific cellular response by regulating T and B cell function (Seljelid R. & T. Eskeland, 1993). One population of cells that are regulated by macrophage activity is the cytotoxic T cells. These cells are involved in killing targeted cells in a specific manner by recognizing antigen in the context of MHC class I. Even though these cells have been well documented in fish, the nature and development of these cells is undefined because of the difficulties in efficiently thymectomizing fish at early developmental stages (Partula, S. *et al.* 1995).

Interferon

The host responds in many ways to a virus infection. One response is the production of interferons (IFN). Interferons are proteins that inhibit virus replication by inducing a series of antiviral and other proteins which can block the spread of virus to uninfected cells by controlling cellular regulation (White, D. 1994) (Janeway, C. 1996).

The interferon released from a virus-infected cell binds to specific receptors on the plasma membrane of neighboring cells and its original host cell and up-regulates the expression of over 20 cellular genes called interferon-regulated proteins. These proteins can directly or indirectly interfere with viral replication. In mammals, there are two types of interferon (Stewart, W.E. 1980). Both function in a similar manner, but are structurally and genetically dissimilar and bind to different receptors. Type I interferons includes IFN-α and IFN-β. These interferons are produced by leukocytes and fibroblasts (Alexander, J.B. and G.A. Ingram 1992). Type II interferon is called IFN-γ and is produced by T-lymphocytes (Kiener, P.A. & Spitalny, G.L. 1987). This type of interferon, which is considered a cytokine, activates immune cells such as natural killer cells and macrophages. IFN-γ is also known to control the expression of MHC class I and class II genes and was discovered to be a macrophage activating factor, (MAF) (Alexander, J.B. & Ingram, G.A. 1992).

Even though the gene for any interferon protein has not been successfully cloned from fish, there have been many studies indicating that fish cells have interferon activity. The first demonstration of interferon activity in fish was shown in 1965 when Gravell and Malsberger observed the inability of infectious pancreatic necrosis virus to replicate in a fathead minnow (*Pimephales promelas*) culture cell line (Gravell, M. & Malsberger, R.S. 1965). Many other studies have been conducted on rainbow trout that were experimentally infected with viral hemorrhagic septicemia virus (VHSV) (De Kinkelin, P. & Dorson, M. 1973; Dorson, M. *et al.* 1975) and on rainbow trout cell lines (DeSena, J. & Rio, G.J., 1975; Okamoto, N. *et al.* 1983; Graham, S. & Secombest, C.T. 1988).

It has not been possible to precisely determine what types of interferons are produced by fish. DNA hybridization experiments using probes from human DNA interferons suggest that fish possess IFN-β but not necessarily IFN-α (Wilson, V. *et al.* 1983). Also, experiments using rainbow trout leukocytes stimulated with the mitogen concanavalin A secrete a soluble MAF which is characteristic of IFN-γ (Graham, S. & Secombes, C.J. 1990). Until a fish interferon gene is cloned, there can be no definitive statement on fish interferons.

Viral Pathogens

In 1898, Martinus Beijerinck, who repeated the experiments of Dimitrii Ivanovsky, showed that a filterable agent separable from bacteria could cause disease, and introduced the term "virus". Since that first discovery, the number of viruses that have been described has increased. The viruses have since been classified into two main groups, DNA and RNA viruses. Viruses have been discovered and isolated from infected hosts of every Kingdom, and have been shown to infect nearly every cell type within multi-cellular organisms. In the late 1950's it was discovered that viruses were important pathogens of fish, and IHNV and IPNV were described. Since then, over 75 different viruses that infect fish have been isolated and partially characterized. Of these, one important family is Rhabdoviridae.

Taxonomy

The family Rhabdoviridae is comprised of viruses that infect invertebrates, plants, and animals. Included in the family is an important human pathogen, rabies. The taxonomic structure of the family consists of 5 genera: *Vesiculovirus* (Vesicular stomatitis virus), *Lyssavirus* (Rabies serogroup), *Ephemerovirus, Cytorhabdovirus, Nucleorhabdovirus*, and a newly designated genus, *Novirhabdovirus*. The classification is based on serological cross reactivity and N gene similarity.

Rhabdoviruses of fish

Infectious hematopoietic necrosis virus (IHNV), Spring viremia of carp virus (SVCV), Viral hemorragic septicemia virus (VHSV), Hirame rhabdovirus (HIRRV), and Snakehead rhabdovirus (SHRV) are the only fish rhabdoviruses for which sequence information is available.

Previously, SVCV had been considered as most closely related to vesiculoviruses and VHSV and IHNV as most closely related to lyssaviruses (Hill, B.J. 1975). More recent studies comparing the glycoprotein sequence from SVCV, IHNV, VHSV, and HIRRV with other rhabdoviruses indicated that the relationships might be different. While SVCV is more closely related to vesiculoviruses, the other three fish rhabdoviruses are most closely related to each other and do not group with any of the mammalian virus glycoprotein genes (Bjorkland, H.V. *et al.* 1996). This and other genetic similarities based on the NV gene have led the Rhabdoviridae study group to petition the International Committee on the Taxonomy of Viruses (ICTV) for the adoption of a new

genus, *Novirhabdovirus* (Walker, P. 1998). The genus will contain HIRRV, IHNV, and VHSV, and the prototypic member will be IHNV.

Genomic Structure

A rhabdovirus virion contains a single-stranded, negative sense RNA genome that makes up approximately 2% of the virion weight. The genome typically encodes five structural proteins: nucleoprotein (N), polymerase (L), phoshoprotein (P), matrix protein (M), and glycoprotein (G). However, there are several genera in the family Rhabdoviridae that have genomes encoding six and seven genes (Bjorkland, H.V. *et al.* 1996). IHNV, for example, contains an additional gene that encodes a nonstructural protein (NV) whose function remains unknown. Similarly, the *Ephemeroviruses* also contain an additional gene, which is nonfunctional.

Morphology

The virion of most rhabdoviruses has an average size of 170 x 70nm and a distinct bullet shape morphology that is hemispherical at one end and planar at the other end. The virion is enclosed by a lipid bilayer envelope, which is obtained when the virus buds through the plasma membrane of the infected host cell. Glycoprotein peplomers surround the helical wound nucleocapsid and protrude through the lipid envelope. The matrix proteins are located on the inner side of the envelope and have been shown to interact with both the N and G proteins.

Infectious hematopoietic necrosis virus

IHNV is a viral pathogen of salmonid fishes. It is enzootic to Western North America but has spread throughout countries of the Northern Pacific Rim and Europe by the movement of infected fish and eggs (Winton, J.R. 1991). Young fish are the most susceptible to disease and resistance increases with age, where adults tend to be lifelong carriers. Extensive mortality is seen in hatchery reared fry where losses due to disease can exceed 90% (Leong, J.C. *et al.* 1988).

The first report of this viral disease was in 1953 in sockeye salmon (*Oncorhynchus nerka*) at two fish hatcheries in the state of Washington, USA (Rucker, R.R. *et al.* 1953). In 1958, a similar viral disease was isolated from juvenile sockeye salmon at the Oakridge Salmon Hatchery, Oregon. The virus was propagated in cell culture and was given the name Oregon Sockeye Virus (OSV). In the same year, chinook salmon (*Oncorhynchus tshawytscha*) fry at hatcheries along the Sacramento River in California were infected with a similar viral disease and was named Sacramento River Chinook Disease (Ross, A.J. *et al.* 1960; Parisot, T.J. & J. Pelnar 1962). Finally in 1967, rainbow trout and sockeye salmon in British Columbia, Canada were reported to have a viral disease, which revealed extensive necrosis of the hematopoietic tissue of the anterior kidney and spleen. This disease was named infectious hematopoietic necrosis disease (Amend, D.F. *et al.* 1969). The viruses isolated from all of these geographic regions showed the same bullet shape morphology as seen by electron microscopy, the same mortality rate and pathology in diseased fish, and the same cytopathic effect on cultured cell lines. It was proposed that the same virus caused these geographically distinct diseases and Amend and Chambers declared the common name, IHNV, in1970.

Salmonids Susceptible to IHNV are sockeye salmon, chinook salmon, rainbow/ steelhead trout and Atlantic salmon (*Salmo salar*), while coho salmon (*Oncorhynchus kisutch*) appear to be resistant (Fryer, J.L. *et al.* 1996). Brown trout (*Salmo trutta*) and cutthroat trout (*Oncorhynchus clarki*) have been experimentally infected. The route of infection is probably through the gills, skin, (Yamamato *et al.* 1992; Drolet *et al.* 1995; Lapatra *et al.* 1989) and gastrointestinal tract. Transmission of IHNV occurs both horizontally and vertically. Traxler in 1993, demonstrated transmission in water from infected spawning adults to uninfected fry. Also, IHNV has been demonstrated to have high affinity binding for salmon sperm (Mulcahy, D. & R.J. Pascho, 1984) and that IHNV-infected cells are present in the ovarian fluid (Mulcahy & W.N. Batts, 1987).

Like most virus diseases, there is a characteristic pathology. External clinical signs of disease include abdominal distention, exopthalmia, and darkened coloration of the body, hemorrhaging throughout the musculature and at the base of fins, fecal casts, and lethargy. Internally, the liver, spleen, and kidney appear pale and the stomach and intestine are filled with a milky or watery fluid (Fryer, J.L *et al.* 1996)

Spring viremia of carp

Spring viremia of carp (SVC) is a disease among cultured cyprinid fishes. The virus *Rhabdovirus carpio* or more commonly referred to in the literature as SVC virus (SVCV) causes this disease. The disease is most serious in Europe and the Soviet Republics where cyprinid aquaculture is intense. Carps are the primary fishes that are infected. This includes crucian carp (*Carassius carassius*), grass carp (*Ctenopharyngon idella*), bighead carp (*Hypophthalmichthys molitrix*), and silver carp (*Aristichthys nobilis*)

(Fijan, N. 1993). Natural infection and subsequent mortality have also been observed in other fish such as the fry of sheatfish (*Silurus glanis*). Also, experimental infections induced by injection or immersion have been shown in pike (*Esox lucius*) and guppies (*Poecilia sp*). SVCV is not pathogenic for rainbow trout (Haenen, OLM, & A. Davidse 1993) and goldfish (*Carassius auratus*) appear to be resistant to infection.

Both juvenile and adult fish are susceptible to disease but once infected, fish are not subject to reinfection (Fijan *et al.* 1971; Wolf, 1988). Ambient water temperature plays an important role in the incubation and progression of the disease (Avtalion, R.R. 1969; Cone, R.E. & J.J. Marchalonis, 1972). Outbreaks of disease primarily occur during April through June when water temperatures are commonly between 11 and 17°C. It is thought that the lower water temperatures have a marked effect on antibody production, which increases the susceptibility to SVCV (Cone & Marchalonis, 1972).

Clinical signs of fish affected with SVCV include exopthalmia, petechial hemorrhages of skin and gills, abdominal distention, and trailing pseudofecal casts (Fryer, J.L *et al.* 1996)

Snakehead rhabdovirus

SHRV was first isolated in 1986 from Snakehead fish (*Ophicephalus striatus*) in Thailand (Wattanavijarn, W. 1986). The virus is enzootic to Southeast Asia and is associated with, but is not necessarily the causative agent of an ulcerative disease in both wild and cultured Snakehead fish (Boonyaratpalin, S. 1989). Even though the rhabdovirus has been frequently isolated from snakehead that show clinical signs of the

ulcerative disease, laboratory challenge with a characterized SHRV isolate did not induce disease (Frerichs *et al.* 1993).

SHRV has bacilliform morphology, which is more characteristic of plant rhabdoviruses (Kasornchandra, J. *et al.* 1992). On the other hand, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein profiles closely resemble that of IHNV, VHSV, HRRV, and rabies (Kasornchandra, J. *et al.* 1992).

Rhabdovirus Glycoproteins

The glycoproteins (G protein) of rhabdoviruses, are homotrimeric trans-membrane-associated proteins that appear as spikes on the surface of the virion (Gaudin, Y. *et al.*1992). These spikes are responsible for the initiation of infection, facilitate the fusion of the endocytic vesicle membrane with the viral envelope, and effect the process of budding and release of the virion by exocytosis (Burger, S.R. *et al.* 1991; Gaudin, Y. *et al.*1992; Coll, J. 1997;Xiang, Z. *et al.* 1994).

Glycosylation is important for proper folding, transport, and function of the G protein (Burger, S.R. *et al.* 1991). Synthesis of the G protein in the host cell follows the typical pathway of cellular membrane proteins via exocytosis. The addition of sugar moieties is N-linked and in rabies accounts for 10% of the final mass of the protein (Reading, C.L. *et al.* 1978). Failure to properly glycosylate the G protein inhibits transport to the plasma membrane of the infected cell. Rabies has three possible acceptor sites for glycosylation of which two are glycosylated (Wunner, W.H. *et al.* 1985). This pattern of glycosylation is typical of Rhabdoviruses. Studies on VSV indicate that glycosylation at both sites is necessary for transport (Bergmann, J.E. *et al.* 1981). In all

rhabdoviruses studied, the basic structure of the G protein is highly conserved (Coll, J.M. 1995).

Rhabdoviral glycoproteins initiate infection

Rhabdoviral glycoproteins act as the ligand that targets cell receptors for the initiation of infection. Previous studies have shown that certain phospholipids inhibit the attachment function of rabies and VSV (Conti, C. *et al.* 1988; Mastromarino, P. *et al.* 1987). The phospholipid, phosphatidylserine, strongly inhibits the attachment and infection of VSV and VHSV by binding to a site on the amino terminal end of the glycoprotein (Coll, J. 1997). The phosphatidylserine-binding domain has also been shown on the glycoproteins of IHNV as well. Studies on the rabies virus indicate that an amino acid substitution at arginine-333 of the glycoprotein abrogates virulence (Dietzschold *et al.* 1983), indicating that the glycoprotein is an important controlling factor for the initiation of infection.

The effects of pH on Rhadboviral glycoprotein

Rhabdovirus glycoproteins are profoundly affected by pH. In order for the virus nucleocapsid to enter the cytoplasm of the target cell, it is necessary for the virion to be endocytosed where the glycoprotein and membranes of the virion and the endocytic vesicle fuse. It is well established that this interaction is highly dependent on pH (Gaudin, Y. *et al.* 1995; Brown, J.C. *et al.* 1988). During acidification in the endocytic vesicle of a rabies infection, a pH $\leq$ 6 changes the conformation of the glycoprotein of

rabies to an activated state, which initiates the fusion process (Gaudin, Y. *et al.* 1993; Konieczko, E.M. *et al.* 1994). Studies on VSV show that a lower pH aggregates the glycoproteins to the ends of the virion, which may be the preformed sites for the fusion process (Brown, J.C. *et al.* 1988). Unlike other viral families, the fusion properties of rhabdoviruses appear to be reversible where the loss of fusion activity at low pH can be reinstalled by bringing the pH to 7 (Gaudin, Y. *et al.* 1995).

Rhabdoviral glycoproteins augment budding

During the final stages of the replication cycle of rhabdoviruses, the transmembrane glycoprotein spikes target the host plasma membrane where the N-terminus protrudes outside the membrane and the C-terminus is located on the inside as an anchor. Matrix proteins of the viral core target the glycoproteins in the plasma membrane and budding occurs (White, D.O., and F.J. Fenner 1994; Durrer, P. *et al.* 1995). This process is called exocytotic budding. The induction of the budding process is totally dependent on the glycoproteins for hepadnaviruses and alphaviruses (Bruss, V. and D. Ganem 1991). The glycoprotein of rabies was also believed to have a similar role but in 1996 Mebatsion showed that the rabies virus was able to bud from infected cells in the absence of the spike glycoproteins (Mebatsion, T. *et al.* 1996). These studies indicated that the budding process of rhabdoviruses is not dependent on the presence of the glycoproteins. Even though the glycoproteins do augment the ability to bud, they are not absolutely dependent on the spike glycoproteins. Currently, it appears that the budding process for rhabdoviruses is more dependent on the interactions of the M protein of the virus with the plasma membrane proteins of the infected target cell.

Viral glycoproteins induce neutralizing antibodies

The glycoproteins of rhabdoviruses are also capable of stimulating an immune response in the organisms they infect. Glycoproteins of all rhabdoviruses, whose immunization properties have been examined, are the only major structural protein that stimulates neutralizing antibodies (Benmansour, H. *et al.* 1991; Kelly, J. *et al.* 1972). Immunoblotting and enzyme-linked immunosorbant assays (ELISAs) of antisera from rainbow trout surviving an infection of IHNV show that these fish produce binding antibodies to the glycoprotein. They also produce neutralizing antibodies to the glycoprotein, which subsequently helps protect fish from the lethal effects of IHNV infections (Ristow, S.S. & J. Arnzen de Avila 1991; Lapatra, S. *et al.* 1993; Engelking, M. & J.C. Leong 1989). Similarly, rainbow trout injected with plasmid vectors containing the glycoprotein of IHNV generated glycoprotein specific antibodies that neutralized IHNV (Anderson, E.D. *et al.* 1996). Research on rabies also shows virus-neutralizing antibodies to the rabies glycoprotein. When rabbits and mice are injected with a vaccinia-rabies glycoprotein recombinant virus, neutralizing antibodies in the sera are detected and these animals are protected from subsequent challenge of rabies (Wiktor, T. *et al.* 1984). In addition to neutralizing antibodies, research from Ertl's group has shown that intramuscular injection of a plasmid vector containing the glycoprotein gene of rabies in mice induces glycoprotein-specific cytolytic T-cells and lymphokine-secreting T-helper cells (Xiang, Z. *et al.* 1994). The glycoprotein of VHSV is also the target of neutralizing antibodies in fish. Neutralizing antibodies specific for VHSV glycoprotein are detected from sera of rainbow trout that are injected intraperitoneally

with a VHSV-glycoprotein recombinant subunit vaccine (Lecocq-Xhonneux, F. *et al.* 1994).

The glycoproteins of viruses also induce stimulation or inhibition of host cellular functions. When purified VSV glycoprotein is placed onto Syrian hamster kidney (BHK21-F) cell monolayers, an early inhibition of macromolecular synthesis was observed. Specifically, there was a decline in the incorporation of nucleosides into cellular DNA and RNA (McSharry, J.J. & P.W. Choppin 1978). Similarly, an inhibition of cellular RNA synthesis was observed in murine lymphoma cell lines (EL4) in the presence of isolated glycoproteins of disrupted mumps virus (Yamada, A. *et al.* 1984).

Glycoproteins induce interferon

As described earlier, interferon is an antiviral protein that is produced in animals and cells when there is a viral infection. It is well known that virus nucleic acid, especially double-stranded RNA, induce the production of interferon. Furthermore, studies mimicking virus nucleic acid by using synthetic homopolymers such as polyriboinosinic-polyribocytidylic acid (poly I:C), has confirmed the production of an interferon-like cytokine in fish (Congelton, J. & B. Sun, 1996; Eaton, W.D. 1990;).

While dsRNA is the best-understood inducer of interferon, it has been shown that viral glycoproteins also induce interferon. Many studies by Yasuhiko Ito have described this phenomenon. In one experiment, mouse spleen cells that were cocultured with primary monkey kidney cells (PMK cells) infected with formaldehyde treated human parainfluenza type 4A (HPIV-4A) virus produced interferon. This finding suggests that a double-stranded RNA intermediate of virus infection was not required to induce

interferon production. Similarly, a recombinant plasmid containing the sequence of hemagglutinin-neuraminidase (HN) glycoprotein transfected into COS7 cells and cocultured with mouse spleen cells produced interferon (Ito, Y. *et al.* 1994). In another experiment, Ito showed, *in vitro*, that contact of isolated HN glycoproteins of Sendai virus with the cell surface of mouse spleen cells stimulated the production of interferon. Each of these experiments provides evidence that viral glycoproteins are capable of stimulating lymphoid cells into synthesizing interferon. The model for this stimulation of interferon production is a membrane-membrane interaction between the lymphoid cells and the cells protruding glycoproteins. Ito proposes that virus glycoproteins act as lectins similar to concanavalin A and phytohemagglutinin, which have mitogenic properties that induce interferon in lymphoid cells (Ito, Y. 1994).

Mx proteins

One class of regulated proteins that are induced by interferon is the Mx proteins. All Mx proteins contain a conserved guanosine triphosphate (GTP)- binding domain, which is essential to their functional activity (Bourne, H.R. *et al.* 1991; Nakayama, M. *et al.* 1991; Pavlovic, J. *et al.* 1990). Located either in the nucleus or the cytoplasm, they appear to act directly by GTP- binding activity on the viral polymerase and/or indirectly by modifying cellular functions along the replication pathway of some viruses during an infection (Pavlovic, J. *et al.* 1993; Horisberger, M.A. 1995). In mice, the mRNA synthesis of influenza virus is inhibited by Mx1 within the nucleus of infected cells (Staeheli, P. 1986). Similarly in humans, MxA proteins located in the cytoplasm inhibit the cytoplasmic replication steps of VSV at the transcription level (Horisberger, M.A.

1992). The conferred resistance is thought to be the result of the direct interaction of GTPase activity on the viral polymerase (Pavlovic, J. *et al.* 1993).

These proteins have also been shown to have intracellular anti-viral activity in other vertebrate cells. For example, expression of murine Mx1 inhibits the orthomyxoviruses, Thogoto virus, and Dhori virus (Haller, O. *et al.* 1995; Thimme, R. *et al.* 1995). The MxA in humans has antiviral activity against bunyaviruses, phleboviruses, and hantaviruses (Frese, M. *et al.* 1996). Mx-like proteins have been identified in a number of vertebrates as well as fish (Rothman, J.H. *et al.* 1990)(Trobridge, G.D. *et al.* 1997). Our lab has previously described the presence of Mx proteins in rainbow trout injected with poly I:C. Under these conditions, the synthesis of Mx is induced following IHNV vaccination or infection. This induction could be an indicator that an interferon-like protein is produced in fish.

CHAPTER 3

DNA Vaccines Encoding the Glycoprotein Genes of Spring Viremia of Carp Virus, Snakehead Rhabdovirus, or Infectious Hematopoietic Necrosis Virus Induce Protective Immunity in Rainbow Trout (*Oncorhynchus mykiss*) Against an Infectious Hematopoietic Necrosis Virus Lethal Challenge

John D. Drennan and Jo-Ann Leong

Department of Microbiology and the Center for Salmon Disease Research,
Oregon State University, Corvallis, Oregon 97331, U.S.A.

Introduction

The recent advances in DNA vaccine technology allows for a great strategy in the control of viruses that contain surface membrane glycoproteins. This type of vaccine involves the intramuscular injection of a bacterial plasmid containing a gene encoding a viral protein. This strategy enables eukaryotic processing of the protein as would naturally occur during a viral infection. The advantage of DNA vaccines is the stimulation of both humoral and cellular immune responses (Donnelly, *et al.* 1997). DNA vaccines not only induce the production of antibodies but also stimulate cytotoxic T cells and helper T cells, which can be regarded as necessary components of a truly effective vaccine (Leung, K.N. & G.L. Ada, 1982; Taylor, P.M. & B.A. Askonas, 1986). There have been many recent studies that show glycoprotein based DNA vaccines stimulate these kinds of immune responses in the hosts that receive them (Anderson, E.D. *et al.*1996; McClements, W.L. *et al.* 1996; Xiang, Z.Q. *et al.* 1994; Manickan, E. *et al.* 1995; Xu, D. & F.Y. Liew, 1995; Cox, G.J.M. *et al.* 1993; Bourne, N. *et al.* 1996).

Our lab has developed a glycoprotein based DNA vaccine for rainbow trout against IHNV (Anderson, E.D. *et al.* 1996). Immunization experiments followed by IHNV challenge produced relative survival rates of 75 percent or greater, even in fish as small as 0.3-0.4 g average weight. These findings were very encouraging and we assumed that protection was specific for IHNV. Earlier studies by Anderson *et al.* 1996 had shown that fish injected with the DNA vaccine do produce neutralizing antibodies to IHNV at eight weeks post vaccination. However, Yasuhiko Ito had shown that viral glycoproteins could stimulate the production of interferon, which could be the basis for the protection. According to Ito, viral glycoproteins, in general, can act as a mitogen to

stimulate lymphoid cells into producing interferon. This stimulation is based on a membrane-membrane interaction between the lymphoid cell and the cell containing the protruding transmembrane glycoprotein (Ito, Y. 1994; Ito, Y. *et al.* 1994; Ito, Y. & Y. Hosaka, 1983). We sought to examine the specificity of the IHNV response induced by the IHNV DNA vaccine by treating fish with several different plasmid vectors containing glycoprotein genes from different rhabdoviruses.

In this study, we present evidence that glycoprotein based DNA vaccines injected into rainbow trout induce an immune response which is similar to that described by Ito. All of the tested glycoprotein vaccines provided non-specific protection against IHNV infection. Plasmid DNA encoding the glycoproteins of IHNV (pcDNA3-IHNV-g), SHRV (pcDNA3-SHRV-g), or SVCV (pcDNA3-SVCV-g) was injected into the skeletal muscle of rainbow trout fry. After 30 days, the fish were challenged with IHNV. Results clearly indicate that the glycoproteins of these different viruses all induced a protective immune response in fish. The mechanisms for the viral mediated resistance induced by these DNA vaccines is unknown, but a parallel study conducted by Dr. Carol Kim, who examined the production of Mx proteins in these fish, suggests that the non-specific protection observed might be a consequence of the stimulation of interferon.

Materials and Methods

Cell line and virus

The chinook salmon embryo (CHSE-214) cell line was obtained from J.L. Fryer, Oregon State University, Corvallis. These cells were propagated in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic (GibcoBRL; 10,000 units/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate and 25 μg/ml of amphotericin B), 1% 200 mM L-glutamine, and buffered with 7.5% sodium bicarbonate to pH 7.4.

The IHNV isolate (IHNV-RA) used in this study was obtained from Rangen Research Laboratories, Idaho, USA. Virus was propagated in CHSE-214 cells grown in a ten-layer cell factory (Nunc Company) at a multiplicity of infection of 0.01 at 16°C. At Day 7 complete cytolytic effect (CPE) was confirmed by microscopy and the culture fluid was harvested and centrifuged in a sorvall GS-3 rotor at 10,825 x g for 30 minutes at 4°C. The Supernatant was then filtered through a 0.2 μm bottle top filter (Schleicher & schvell), alloquoted, and stored in -80°C.

Plaque assay

Virus titer was determined by plaque assay (Burke, J.A. and D. Mulcahy 1980). A sub sample taken from -80°C was thawed and 10 fold serial dilutions were performed in MEM media. Dilutions were plated onto CHSE-214 monolayers in six well plates and incubated for one hour by hand rocking the plates every ten minutes. After incubation, 3

mls of methylcellulose were placed over the monolayers and incubated for 10 days in 6% CO_2 at 16°C. At day 10, cell monolayers were fixed and stained with crystal violet in formalin (25% formalin, 10% ethanol, 5% acetic acid, 1% w/v crystal violet) for 24 hours. Cell monolayers that contained between 25-250 plaques were used to determine virus titer. IHNV-RA strain was determined to be 1.24×10^8 pfu/ml.

Plasmid constructs

Plasmid vectors encoding the glycoprotein gene sequences of IHNV, SHRV, and SVCV were constructed for this experiment. All of these glycoprotein gene sequences were previously cloned in our laboratory. The gene for the glycoprotein of IHNV (IHNV-g) was originally cloned into the plasmid pCMV. The glycoprotein genes of SHRV (SHRV-g) and SVCV (SVCV-g) were originally cloned into the plasmid pcDNA3. IHNV-g was removed from pCMV by restriction enzyme digest and inserted into pcDNA3 by ligation. DNA sequencing of the first 400 nucleotides verified correct nucleotide sequence and orientation. *In vitro* translation of the plasmids pcDNA3-IHNV-g, pcDNA3-SHRV-g, and pcDNA3-SVCV-g determined the correct protein product.

Amino acid sequence analysis

Glycoprotein amino acid sequences of IHNV, SHRV, and SVCV were compared for homology. The sequences were imported into a genetic data environment where the initial amino acid alignments were created using CLUSTAL. Conserved amino acids

were identified among these three glycoproteins and were used to determine percent amino acid identity.

Purification of plasmids

The resulting Plasmids, pcDNA3, pcDNA3-IHNV-g, pcDNA3-SHRV-g, and pcDNA3-SVCV-g were grown in transformed *Escherichia coli*, JM109 competent cell strain, in the presence of Ampicillin. Using the Qiagen purification plasmid kit according to the manufacturer's protocol, large-scale purification of each plasmid was performed. The concentration of purified plasmids was determined by optical density (OD) at 260 nm.

Plasmid injection of rainbow trout

Rainbow trout used in this research were a cross between Klamath strain (Klamath hatchery, Oregon) and Cape cod strain (Roaring river hatchery, Oregon). Both strains were spawned at their corresponding hatcheries and then fertilized at the Oregon State University, Salmon Disease Laboratory, Corvallis, Oregon. Fry were held in 100 liter tanks until fish mean weight was 0.5 grams.

The previously described plasmids, pcDNA3-IHNV-g, pcDNA3-SHRV-g, pcDNA3-SVCV-g, pcDNA3 and phosphate saline buffer (PBS) were used in the injection. Plasmid DNA concentrations were adjusted to 10 μg/25μl by diluting in PBS, pH 7.4 and confirmed by spectrophotometry. Five groups of 200 fish (.56 grams mean wt) per group were anesthetized in 0.1% Tricane methanesulfonate (MS-222, Finquel)

and injected intramuscularly with the corresponding plasmid or PBS and placed in 25 liter tanks, 50 fish per tank. A total of twenty tanks were used: four for pcDNA3, four for pcDNA3-IHNV-g, four for pcDNA3-SHRV-g, four for pcDNA3-SVCV-g, and four for PBS injected fish. Tanks were interspersed amongst each other to eliminate possible tank effects. The injection of PBS was the positive control for the injection procedure and the injection of pcDNA3 was the negative control of the immune response to the plasmid without the glycoprotein insert.

Challenge of Rainbow trout to IHNV

At 30 days post injection fish were challenged, by immersion, with IHNV-RA strain. Mean fish weight was 1.33g. Fish were challenged with two viral doses: 10^3 and 10^5 pfu/ml. In both challenge doses, two tanks (fifty fish per tank) of each group were exposed to IHNV-RA in two liters of water at 15°C for 5 hrs. Post-challenge fish mortality from each tank was removed and recorded daily for 30 days.

Results

Plasmid Construction

The different plasmids used in the study are shown in Figure 1. Each plasmid contains a glycoprotein gene from each of three rhabdoviruses. The plasmid pcDNA3-IHNV-g contains the gene encoding IHNV glycoprotein. Similarly, the plasmid pcDNA3-SHRV-g contains the gene encoding SHRV glycoprotein and pcDNA3-SVCV-g contains the gene encoding SVCV glycoprotein.

DNA sequence analysis, restriction enzyme analysis, and *in vitro* translation confirmed each plasmid construct. The sizes of the proteins produced by *in vitro* translation correlate well with the expected size for a non-glycosylated protein for each plasmid (Figure 2). These findings indicated that the viral glycoprotein genes used in the study were complete, in the correct orientation, and contained no nucleotide sequence changes.

Figure 1. Plasmid vectors encoding the glycoprotein gene sequences of IHNV, SHRV and SVCV. Restriction enzymes used in ligation and analysis are shown.

Figure 1.

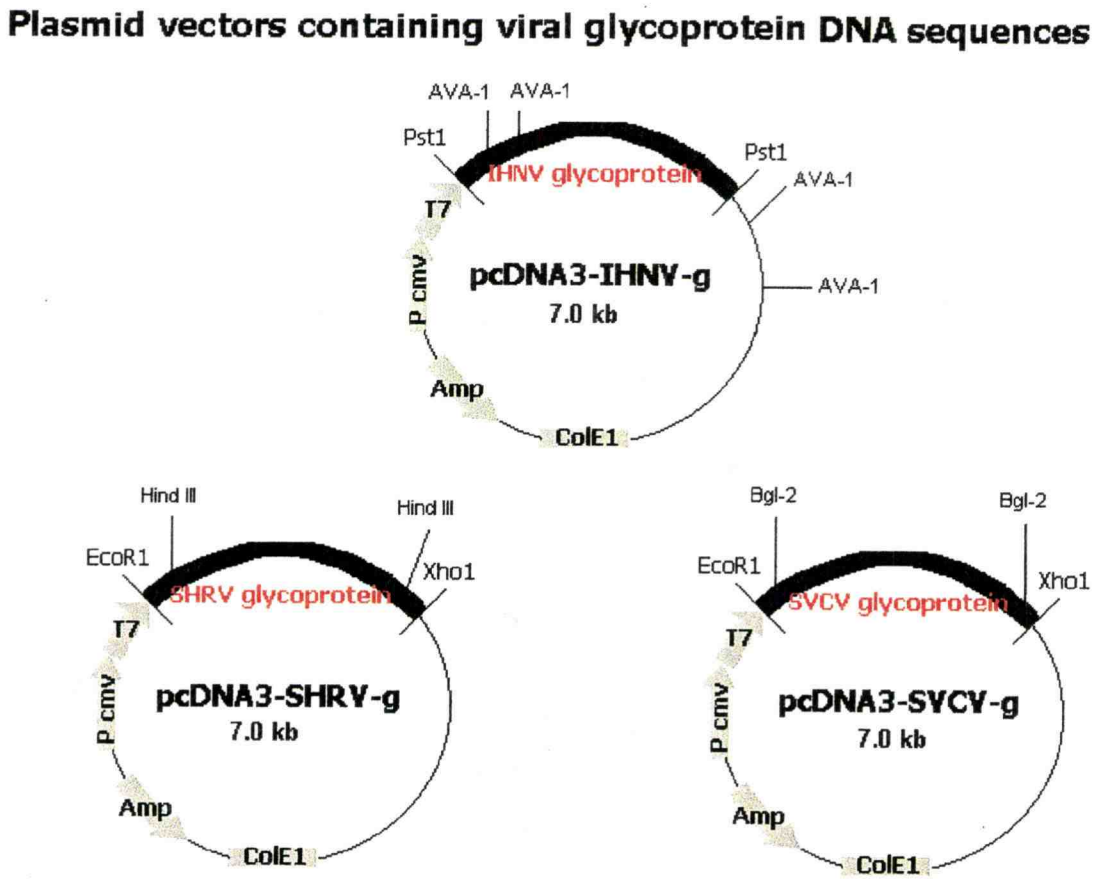
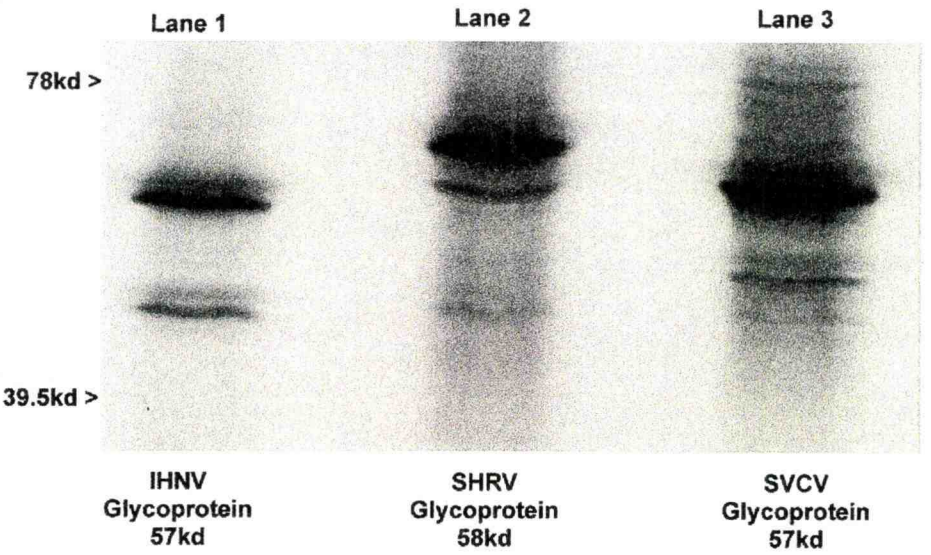

Plasmid vectors containing viral glycoprotein DNA sequences
AVA-1
AVA-1
Pst1
IHNV glycoprotein
Pst1
AVA-1
T7
P cmv
pcDNA3-IHNV-g
7.0 kb
AVA-1
Amp
ColE1
Hind III
Hind III
EcoR1
SHRV glycoprotein
Xho1
T7
P cmv
pcDNA3-SHRV-g
7.0 kb
Amp
ColE1
Bgl-2
Bgl-2
EcoR1
SVCV glycoprotein
Xho1
T7
P cmv
pcDNA3-SVCV-g
7.0 kb
Amp
ColE1

Figure 2. Autoradiograph of the proteins produced *in vitro* translation reactions of RNA synthesized from pcDNA3-IHNV-g, pcDNA3-SHRV-g, and pcDNA3-SVCV-g. The glycoproteins were produced using TNT® coupled reticulocyte lysate system. T7 polymerase was used to drive transcription from the T7 promoter. The proteins synthesized off those transcripts were labeled by incorporating [^{35}S] methionine. Products were electrophoresed on a 10% denaturing SDS PAGE gel. Lane 1, pcDNA3-IHNV-g, lane 2, pcDNA3-SHRV-g, and lane 3, pcDNA3-SVCV-g. Molecular weights for the glycoproteins of IHNV, SHRV, and SVCV correlate well with the expected size for non-glycosylated g-proteins. (Autoradiograph Performed by Marc Johnson, 1998)

Figure 2.


Lane 1
Lane 2
Lane 3
78kd >
39.5kd >
IHNV
Glycoprotein
57kd
SHRV
Glycoprotein
58kd
SVCV
Glycoprotein
57kd

Glycoprotein amino acid sequence analysis

Glycoprotein amino acid sequence analysis of IHNV, SHRV, and SVCV were aligned with each other to determine homology. The alignment shows that there are no large conserved amino acid motifs among the three glycoproteins (Table 1). Furthermore, only a single motif exists in which three consecutive amino acids are conserved. A total of 61 sites of a possible 560 are identical between the three glycoproteins, resulting in an eleven percent amino acid identity.

Table 1. Amino acid sequence analysis of the glycoproteins of IHNV, SHRV, and SVCV. Proteins were aligned using CLUSTAL (GDE version 2.1) protein alignment program. Highlighted, are the amino acids conserved among all three proteins.

Table 1.

Amino acid sequence analysis of the glycoproteins used in this study

```
SVCV-  MSIIS-----YIAFLLLIDSNLGIPIFVPSGR--------NISWQPVIQPFDYQCPIH---
SHRV-  MTLPNMKPKRIVLFLVFLNAWVSNAQVTHKPRPDSIVEYSE-EWENPIYTTPSHCFEDTFA
IHNV-  MDTTITTP--LILILI------TCGANSQTVKPDTASESDQPTWSNPLFTYPEGCTLDKLS
                                                                    4

SVCV-  ----GNLP-------NTMGLSATKLTIKSPSVFSTD---KVSGWICHAAEWKTTCDYRWYG
SHRV-  PVKPEKLRCPHIFDDQNLGITASKAKILHMDLKPEDTHFEAKGRLLHKVTYQVLCSTGFFG
IHNV-  KVNASQLRCPRIFDDENRGLIAYPTSIRSLSVGNDLGDIHTQGNHIHKVLYRTICSTGFFG
                                                                   11

SVCV-  PQYITHSIHPISPTIDECRRIIQRIASGTDEDLGFPPQSCGWASVTTVSNTNYR-VVPHSV
SHRV-  GRTVTRKVLETPMGDNE----AQAYKAVDREFPYFPEPLCFWLRDNVAAERVFHFSTPKTV
IHNV-  GQTIEKALVEMKLSTKE----AGAYDTTTAAALYFPAPRCQWYTDNVQNDLIFYYTTQKSV
                                                                   17

SVCV-  HLEPYGGHWIDHEFNGGECREKVCEMKGNHSIWITEETVQHECAKHIEEVEGIMYGNVPRG
SHRV-  TVDLYSRKYISPDFVGGQCAKSPCPTHWPNVYWVGETQSP-ECPS-IDTEGGHIFTKKDTH
IHNV-  LRDPYTRDFLDSDFIGGKCTKSPCQTHWSNVVWMGDAGIP-ACDS-SQEIKGHLFVDKISN
                                                                   25

SVCV-  DVMYANNFIIDRHHRVYRFGGSCQMKFCNKDGIKFARGDWVEKTAGTLTTIHDN-------
SHRV-  RITKAVVH----GHHPWGLTKACQIQFCNEQ--------WIRTDLGDLIRIEPNDGTSSLT
IHNV-  RVVKATSY----GHHPWGLHQACMIEFCGKQ--------WIRTDLGDLISVEYNSGAEILS
                                                                   33

SVCV-  VPKCVDGTLVSGHRPGLD----LIDTVFNLENVVEYTLCEGTKRKINKQEKLTSVDLSYLA
SHRV-  LPKCKENVV--QMRGNLDDFPYLNHAIVNMAQRSE---CLEAHSSIVAQQKVSPYLLSKFR
IHNV-  FPKCEDKTV--GMRGNLDDFAYLDDLVKASESREE---CLEAHAEIISTNSVTPYLLSKFR
                                                                   44

SVCV-  PRIGGFGSVFRVRNGTLERGSTTYIRIEVEGPIVDSLNGTDPRTNASRVFWDDWELD-GNI
SHRV-  PPHPGLGKAHYLQNNTIMRG--DCIYEGVAEISENRTTYRNLKGE-----WKKWSLSRGGK
IHNV-  SPHPGINDVYAMHKGSIYHG--MCMTVAVDEVSKDRTTYRAHRATS----FTKWERPFGDE
                                                                   48

SVCV-  YQGFNGVYKGKDGKIHIPLNMIESGIIDDELQHAFQADIIPHPH----YDDDEIREDDIFF
SHRV-  --GYDGMTVGN--KIVIPDLEKYQSIYDNGMFIPKLLGEVPHPSIVITYNQTDSIETGIF-
IHNV-  WEGFHGLHGNN--TTIIPDLEKYVAQYKTSMMEPMSIKSVPHPSILALYNETDVSGISIR-
                                                                   57
```

```
SVCV-   DNTGENGNPVDAVVEWVSGWGTSLKFFGMTLVALILIFLLIRCCVACTYLMKRSKRPATES
SHRV-   ----TDGKLLNMGVNW-TLWPS---LSGISLFTVASLILIWYCCC---RVTPQALNYSIPM
IHNV-   ----KLDSFDLQSLHW-SFWPT---ISALGGIPFALLLAVAACCCWSGRRRTPSASQSIPM
                                                                    61

SVCV-   HEM--RSL-V
SHRV-   HTITSRGVEI
IHNV-   YHLANRS---
                                                                    61
```

Total alignment between the three glycoproteins = 61
560 amino acids : 61 alignments

(61/560) x 100 = 11% alignment relationship

Effect of DNA immunization against challenge to IHNV

Immunized and control (PBS and pcDNA3) fish were challenged by immersion in water containing different concentrations of IHNV (Rangen isolate). The fish were observed daily for signs of disease and dead individuals were removed. Figure 3 shows the effect of pcDNA3-IHNV-g, pcDNA3-SHRV-g, or pcDNA3-SVCV-g immunization on survival against a challenge dose of 10^5 pfu/ml. Significant protection was observed with relative percent survivals of 93.2% (pcDNA3-IHNV-g), 98.3% (pcDNA3-SHRV-g), and 94.9% (pcDNA3-SVCV-g) (Table 2). The control fish that received either PBS or pcDNA3 had percent cumulative mortalities of 55% and 57%, respectively. The results observed for fish challenged with 10^3 pfu/ml are similar to that observed at 10^5 pfu/ml, i.e. all glycoprotein-vaccinated fish were protected as compared to the controls (Figure 4). As expected, the mortalities that were observed in the 10^3 pfu/ml challenge dose was lower (Table 3).

Figure 3. The effect of intramuscular DNA immunization on survival against IHNV (10^5 pfu/ml). DNA vaccines consisted of pcDNA3-IHNV-g, pcDNA3-SHRV-g, and pcDNA3-SVCV-g. Trout in the control groups were injected with PBS and pcDNA3.

Figure 3: Percent cumulative mortality of fish injected with DNA and challenged with IHNV (10^5 pfu/ml)

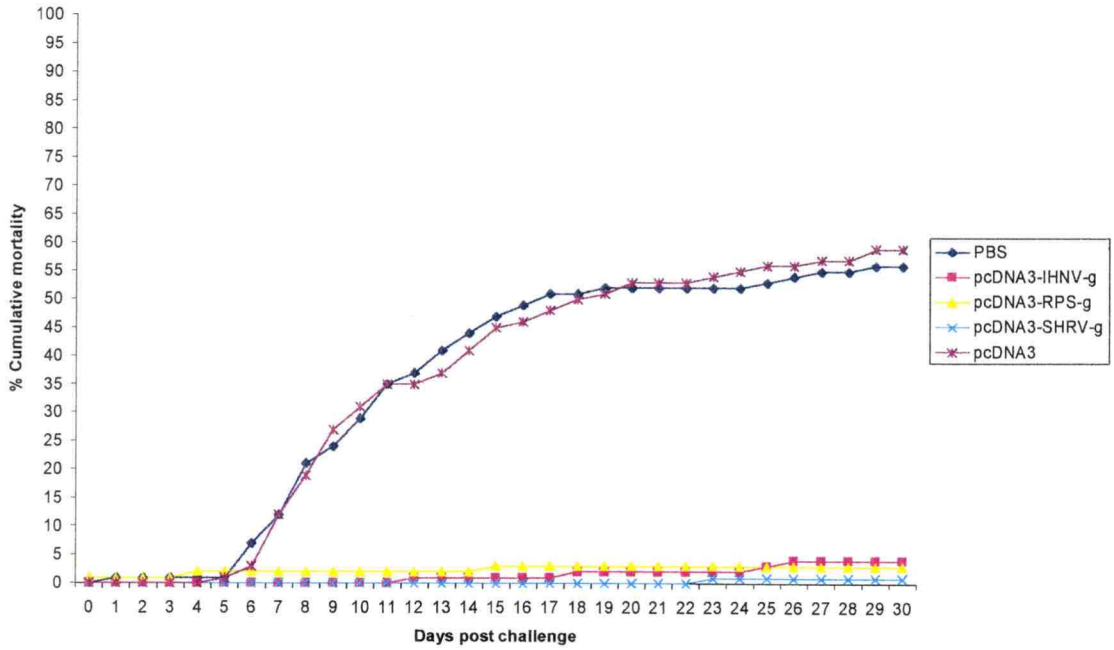


Table 2. Survival of fish challenged with IHNV (10^5 pfu/ml) after injection with plasmid DNA. 100 fish per group was obtained by the sum of 50 fish in duplicate for each treatment. Dead fish were removed from each tank and recorded daily for cumulative mortality results. Percent cumulative mortality is based on the 100 fish from both tanks (50 per tank) for each treatment group.

Table 2. Survival of fish challenged with IHNV (10^5 pfu/ml) after injection with plasmid DNA.

Treatment	No. of fish per group	Total no. of deaths	% Cumulative mortality	RPS*
PBS	100	56	56	
pcDNA3	100	59	59	
pcDNA3-IHNV-g	100	4	4	93.2
pcDNA3-SHRV-g	100	1	1	98.3
pcDNA3-SVCV-g	100	3	3	94.9

Fish (mean wt .5g) were injected the phosphate-buffered saline (PBS) or each respective plasmid as indicated. 30 days after injection, fish (mean wt. 1.13g) were challenged with 10^5 pfu/liter of IHNV (Rangen). The challenge trial was conducted for 30 days.

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

RPS = [1-(% mortality of vaccinated fish/ % mortality of control fish, pcDNA3)] x 100.

Figure 4. The effect of intramuscular DNA immunization on survival against IHNV (10^3 pfu/ml). DNA vaccines consisted of pcDNA3-IHNV-g, pcDNA3-SHRV-g, and pcDNA3-SVCV-g. Trout in the control groups were injected with PBS and pcDNA3.

Figure 4: Percent cumulative mortality of fish injected with DNA and challenged with IHNV (10^3PFU/ML)

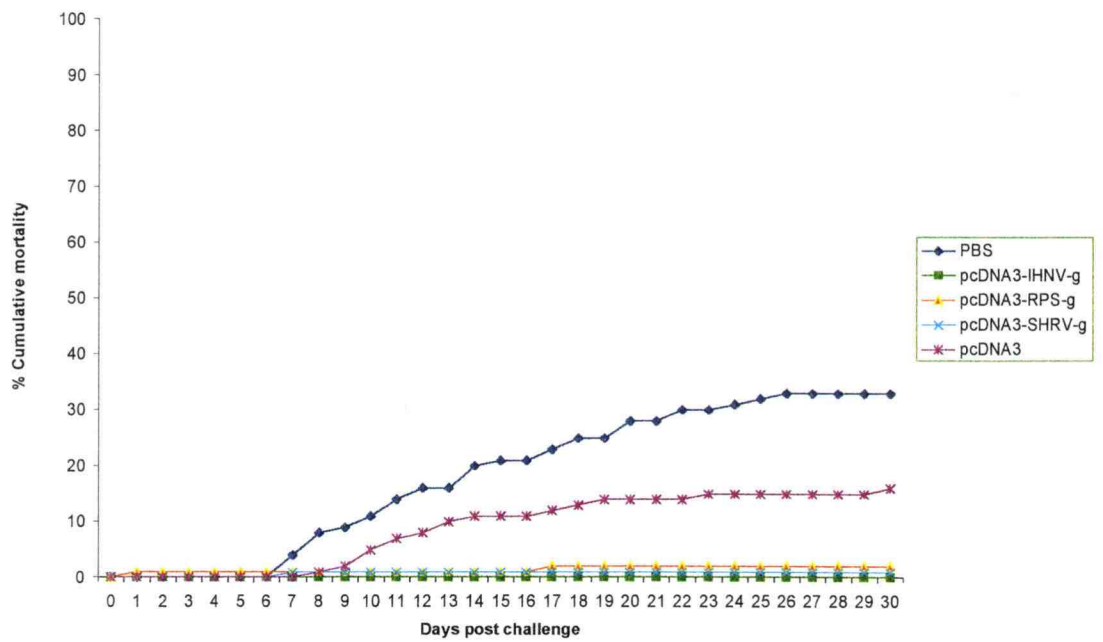


Table 3. Survival of fish challenged with IHNV (10^3 pfu/ml) after injection with plasmid DNA. 100 fish per group was obtained by the sum of 50 fish in duplicate for each treatment. Dead fish were removed from each tank and recorded daily for cumulative mortality results. Percent cumulative mortality is based on the 100 fish from both tanks (50 per tank) for each treatment group.

Table 3. Survival of fish challenged with IHNV (10^3 pfu/ml) after injection with plasmid DNA.

Treatment	No. of fish per group	Total no. of deaths	% Cumulative mortality	RPS
PBS	100	33	33	
pcDNA3	100	16	16	
pcDNA3-IHNV-g	100	0	0	100
pcDNA3-SHRV-g	100	1	1	93.8
pcDNA3-SVCV-g	100	2	2	87.5

Fish (mean wt .5g) were injected the phosphate-buffered saline (PBS) or each respective plasmid as indicated. 30 days after injection, fish (mean wt. 1.13g) were challenged with 10^3 pfu/liter of IHNV (Rangen). The challenge trial was conducted for 30 days.

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

RPS = [1-(% mortality of vaccinated fish/ % mortality of control fish, pcDNA3)] x 100.

Summary of statistical findings

In the challenge dose of 10^5 pfu/ml, there is strong evidence that the mean mortalities of fish in the DNA vaccinated groups differ from that of the PBS control group (two-sided *p*-value < .0001, t-test). Conversely, There is no evidence that the mortalities of fish in the pcDNA3 control group are different from that of the PBS control group (two-sided *p*-value = .30, t-test).

In the challenge dose of 10^3 pfu/ml, there is similar evidence that mortalities between the DNA vaccinated groups and the PBS control group are different (two-sided *p*-value < .001, t-test). However, there is no evidence that the PBS control group and the pcDNA3 control group are the same (two-sided *p*-value < .001, t-test). All statistical findings were based on the comparison of means using a pooled estimate of the standard deviation and five degrees of freedom (Table 4 and Table 5).

Table 4. Data from fish challenge dose 10^5 pfu/ml used to derive statistical significance between treatment groups. The data represents the actual mortalities in each tank.

Table 4. Data for statistical findings for Challenge dose 10^5 pfu/ml.

Treatment group	fish per tank	Mortalities	Average mortalities within group	Standard deviation
PBS	50	30	28	2.83
PBS	50	26		
pcDNA3	50	30	29.5	0.71
pcDNA3	50	29		
pcDNA3-IHNV-g	50	2	2	0
pcDNA3-IHNV-g	50	2		
pcDNA3-SHRV-g	50	0	0.5	0.71
pcDNA3-SHRV-g	50	1		
pcDNA3-SVCV-g	50	2	1	0.71
pcDNA3-SVCV-g	50	1		

Pooled estimate of the standard deviation (Sp) = 1.90

$Sp^2 = [(n_1-1)s_1^2 + (n_2-1)s_2^2 + \ldots + (n_I-1)s_I^2]/ [(n_1-1) + (n_2-1) + \ldots + (n_I-1)]$

Degrees of freedom = 5

Table 5. Data from fish challenge dose 10^3 pfu/ml used to derive statistical significance between treatment groups. The data represents the actual mortalities in each tank.

Table 5. Data for statistical findings for Challenge dose 10^3 pfu/ml.

Treatment group	fish per tank	Mortalities	Average mortalities within group	Standard deviation
PBS	50	18	16.5	2.12
PBS	50	15		
pcDNA3	50	8	8	0
pcDNA3	50	8		
pcDNA3-IHNV-g	50	0	0	0
pcDNA3-IHNV-g	50	0		
pcDNA3-SHRV-g	50	0	0.5	0.71
pcDNA3-SHRV-g	50	1		
pcDNA3-SVCV-g	50	2	1	1.41
pcDNA3-SVCV-g	50	0		

Pooled estimate of the standard deviation (Sp) = 1.40

$Sp^2 = [(n_1-1)s_1^2 + (n_2-1)s_2^2 + \ldots + (n_I-1)s_I^2] / [(n_1-1) + (n_2-1) + \ldots + (n_I-1)]$

Degrees of freedom = 5

Discussion

In this study, we show that DNA vaccines encoding the glycoproteins from different fish viruses stimulate a protective immune response to IHNV challenge. Injection of plasmid vectors containing the glycoprotein gene sequences of IHNV (pcDNA3-IHNV-g), SHRV (pcDNA3-SHRV-g), or SVCV (pcDNA3-SVCV-g) into the skeletal muscle of rainbow trout induced protection against an IHNV lethal challenge.

The viral glycoproteins used in this experiment belong to viruses that are members of the family *Rhabdoviridae*, and are serologically distinct. Amino acid sequence identity indicates that SVCV is similar to vesicular stomatitis virus (VSV), which is the prototype virus of the *Vesiculovirus* genus (Bjorklund, H.V. *et al.* 1996). The protein profile of IHNV is more similar to the *Novirhabdovirus* genus (Bjorklund, H.V. *et al.* 1996). Sequence homology for the SHRV glycoprotein also indicates that this virus is a member of the *Novirhabdovirus* genus, but is distinct from IHNV (Johnson, M. unpublished results, 1997). In addition to these differences in protein structure, cross neutralization experiments show that these viruses are not related serologically. Eight fish rhabdoviruses were examined for cross neutralization (Kasornchandra, J. *et al.* 1992). These tests using polyclonal antibodies derived from mice and rabbits injected with IHNV, SHRV, and SVCV, were negative according to the alpha method of Rovozzo and Burke (Rovozzo, G.C. and C.N. Burke 1973).

The non-specific protection observed in these fish could be attributed to the production of interferon by immune cells such as macrophages and nonspecific cytotoxic cells or by other lymphoid cells. When a DNA vaccine is injected into the skeletal muscle of fish, the plasmid DNA is delivered inside the cells by a mechanism that is still

unknown. Once inside the cell, the cDNA of the viral glycoproteins, gets transcribed, translated, glycosylated, and then transported to the plasma membrane. The glycoproteins protrude through the membrane, comparable to a normal viral infection, and are detected by an immune cell (Figure 5). Ito's hypothesis is that lymphoid cells detect these glycoproteins by membrane-membrane interactions and produce interferon (Ito, Y. 1994). Thus, the rainbow trout injected with the glycoprotein based DNA vaccines thirty days prior to virus challenge remain in an antiviral state upon challenge with IHNV or possibly any other virus. Subsequently, these fish became less susceptible to the lethal effects of virus infection. Production of a Mx protein was observed in a parallel study of these fish conducted by Dr. Carol Kim. Preliminary results showed that Mx was detected in fish injected with the DNA vaccines but not in the control fish (data not published). Mx is an interferon-regulated protein that is stimulated by the induction of interferon in mammals (Staeheli, P. 1990; Sammuel, C.E. 1991). Collectively, these observations support Ito's model that glycoproteins induce interferon synthesis.

There are many arms of the immune system in fish and undoubtedly there is a specific immune response to the glycoproteins, which would induce neutralizing antibodies specific for epitopes on the glycoproteins. One could imagine that either the plasmid vectors themselves are transfected into macrophages or alternatively, that the glycoproteins are phagocytosed and processed. Either would be presented in the context of MHC class II for the stimulation of antibody production. Previous experiments using the glycoprotein of IHNV as a DNA vaccine provides evidence that neutralizing antibodies are produced (Anderson, E.D. *et al.* 1996). In addition to the detection of Mx in the treatment groups upon injection, the very opposite occurs in fish vaccinated with

pcDNA3-IHNV-g upon challenge with IHNV. The loss of the ability to detect Mx in this group might be accountable by the stimulation of other arms of the immune system leading to neutralizing antibodies and cytotoxic cells. With the stimulation of interferon production as the first line of defense against virus infection, followed by the synthesis of antibodies specific for the IHNV glycoprotein and the activation of cytotoxic T cells, the virus is cleared before the infection process can be amplified. Roers *et al.* 1994 studying the induction of Mx proteins in vaccinated and unvaccinated individuals upon infection with the 17-D strain of yellow virus, reported that unvaccinated individuals produced 50-fold more Mx protein than vaccinated individuals. They suggested that the vaccinated individuals had enough circulating antibody to clear the virus before interferon was induced. The same model might account for the clearance of IHNV in fish injected with pcDNA3-IHNV-g. The virus is reproductively inhibited by interferon, neutralized and opsonized by antibodies, and cytotoxic T cells destroy infected cells leading to virus clearance. However, the fish vaccinated with pcDNA3-SHRV-g and pcDNA3-SVCV-g most likely produced specific immune responses against their respective glycoproteins, which may not have influenced the protection against IHNV that was observed.

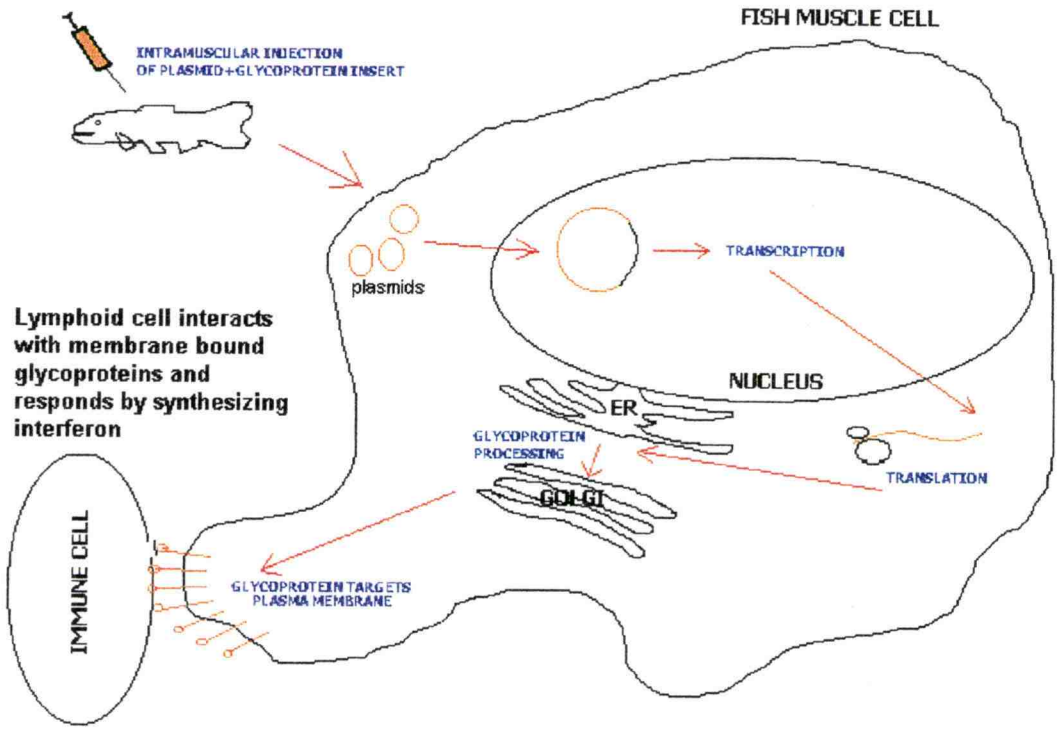
Virus-specific immune responses other than neutralizing antibodies also provide protection. Xiang, *et al.* 1994, has characterized the protective immunity in mice vaccinated against rabies with a plasmid vector carrying the rabies glycoprotein. This study showed that cytotoxic T leukocytes were activated specifically to the rabies glycoprotein expressed by target cells in the context of MHC class I (Xiang, Z.Q. 1994). Although specific immune responses have been observed for rabies, there may still be an alternative explanation for protection. Xiang, Z.Q. 1994, did not consider non-specific

immune factors. We show in this report that all three unrelated viral glycoproteins induced protection to IHNV and that the DNA induced a non-specific protective response.

Although numerous questions remain to be answered about the specific mechanisms that are involved with the induction of the observed protection, this study has shown that non-specific, non-humoral protective immune responses in rainbow trout plays an important role in controlling an IHNV infection. Understanding the relationship between viral glycoproteins and the stimulation of a non-specific immune response will provide important information for the development of vaccines in rainbow trout fry, which will focus on cell-mediated immunity. It is generally accepted that the humoral response in trout fry is relatively poor or non-existent and is not the controlling factor leading to protection. This study reinforces the notion that glycoprotein based DNA vaccines stimulate non-specific immune responses, which leads to sufficient protection during the time when trout fry are most susceptible to IHNV. The use of this technology will improve our understanding of the immune system of trout, viral pathogenesis of IHNV, and may subsequently improve the development of vaccines.

Figure 5. Proposed illustration on the pathway of glycoprotein based DNA vaccines leading to the stimulation of interferon by a lymphoid cell. When the DNA vaccine is injected into the muscle of trout, the plasmids are transfected into muscle cells. This results in the production of transmembrane glycoproteins and a membrane-membrane interaction between the lymphoid cell and the muscle cell containing the protruding glycoproteins.

Figure 5.


FISH MUSCLE CELL
INTRAMUSCULAR INJECTION
OF PLASMID+GLYCOPROTEIN INSERT
plasmids
TRANSCRIPTION
NUCLEUS
ER
GLYCOPROTEIN
PROCESSING
GOLGI
TRANSLATION
Lymphoid cell interacts
with membrane bound
glycoproteins and
responds by synthesizing
interferon
IMMUNE CELL
GLYCOPROTEIN TARGETS
PLASMA MEMBRANE

CHAPTER 4
SUMMARY

Intramuscular injection of glycoprotein-based DNA vaccines into rainbow trout induces protective immunity against an IHNV lethal challenge. This study shows that the different plasmid vectors encoding the gene sequences of serologically distinct glycoproteins from different fish rhabdoviruses all protect trout fry against IHNV. The differences between the DNA vaccinated treatment groups and the control groups are statistically significant and clearly show that some kind of protection mechanism is induced by the DNA vaccines. Although the exact mechanism is unknown, this study coupled with the additional studies by Dr. Kim suggest that interferon may be contributing to the protective immune response. Collectively, these two studies suggest that the model developed by Ito regarding the synthesis of interferon by viral glycoproteins can be applied to teleost systems as well.

Undoubtedly, this study presents an important finding in understanding the of immune response in fish and much more research will have to be done in characterizing the mechanism(s) involved with this type of observed protection. Cloning of trout interferon will be a major advancement for this type of research. Once cloned, research can be directed to how interferon is stimulated, how much interferon is needed to ascertain biochemical functions leading to protection, and what other components of a viral infection leads to the production of interferon.

Even though there are important differences between the immune response in fish versus mammals, much of the immune components and functions are similar. In mammalian and teleost systems, the paradigm in developing vaccines for controlling viral

pathogens has been focused on stimulating the humoral immune response for the production of neutralizing antibodies. Within the last decade, researchers have come to an understanding that although neutralizing antibodies play an important role, a truly effective vaccine also needs to induce the cellular-mediated immune response, especially during the early stages of an infection. The results of this study are not only important in furthering our understanding of the trout immune system, but also provides the development of a future vaccine strategy for the use in trout aquaculture. In these high density-rearing facilities, fish at early life stages are most susceptible to viral pathogens and mortality can be extremely high. It is generally accepted that the humoral response in trout fry is relatively poor or non-existent. Thus, the development of a vaccine that stimulates other arms of the immune system that leads to protection is of great importance.

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