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Characterization of the infectious hematopoietic necrosis virus glycoprotein using neutralizing monoclonal antibodies

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ABSTRACT: To study the antigenic nature of the glycoprotein (G protein) of infectious hematopoietic necrosis virus (IHNV), 31 neutralizing monoclonal antibodies (MAbs) were produced against a reference isolate of the virus. The MAbs were compared using a neutralization assay, an enzyme-linked immunosorbent assay (ELISA), and by immunoblotting of the G protein in the native, reduced, and deglycosylated forms. Hybridoma culture fluids of the various MAbs could be diluted from 1:2 to 1:512 and still completely neutralize 1×10^4 plaque-forming units of IHNV. Similarly, the end point dilutions that produced optical density readings of 0.1 or greater in the ELISA were 1:40 to 1:10240. Western blotting showed that all of the MAbs reacted with the G protein in the unreduced (i.e. native) conformation; however, only 9 nine of the MAbs were able to react with the G protein following reduction by 2-mercaptoethanol. Deglycosylation of the protein did not influence the binding ability of any of the MAbs. These data indicate that all the MAbs recognized amino acid sequences on the protein itself and that the IHNV glycoprotein contains linear as well as conformation-dependent neutralizing epitopes. When rainbow trout *Oncorhynchus mykiss* fingerlings were passively immunized with MAbs against either a linear or a conformation-dependent epitope, the fish were protected against challenge with wild-type IHNV.

KEY WORDS: Rhabdovirus · Salmon · Trout · Antigen

INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is a fish rhabdovirus that produces an acute disease (IHN) resulting in destruction of the hematopoietic tissues of the kidney and spleen and in the loss of millions of salmon and trout each year (Wolf 1988). Because IHN is untreatable, control methods have relied on prevention by improved water quality, disinfection of eggs, sanitation of fish culture facilities, and destruction of infected fish populations (Winton 1991).

Several vaccines have been developed and tested for controlling IHN, including killed and attenuated preparations (Leong et al. 1988). Engelking & Leong (1989) showed that the IHNV glycoprotein (G protein) alone was able to induce neutralizing antibodies in rabbits and to immunize salmonids against challenge with the virus. The newest vaccines against IHNV are subunit vaccines developed by cloning regions of the glycoprotein gene of IHNV into *Escherichia coli* (Gilmore et al. 1988, Xu et al. 1991) or baculovirus (Koener & Leong 1990) expression systems. Laboratory and field trials showed that a subunit vaccine could stimulate protective immunity in trout against IHNV (Leong et al. 1992). Similarly, the glycoprotein of viral hemorrhagic septicemia virus (VHSV) expressed in *Escherichia coli* was shown to be immunogenic in trout (Lorenzen et al. 1993).

Monoclonal antibodies (MAbs) have been useful in the study of rhabdovirus glycoprotein structure and function and in defining neutralizing epitopes on rabies virus (Lafon et al. 1983, Benmansour et al. 1991) and vesicular stomatitis virus (VSV; Lefrancois & Lyles

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1982, Luo et al. 1988). Among fish rhabdoviruses, neutralizing MAbs have been developed against isolates of IHNV (Winton et al. 1988, Ristow & Arnzen de Avila 1991) and VHSV (Lorenzen et al. 1988, 1990). In addition to serving as reagents for determining the antigenic diversity among field isolates of IHNV, these MAbs have been used to select variants of the virus that were attenuated in virulence (Roberti et al. 1991), to develop a preliminary epitope map of the IHNV glycoprotein (Xu et al. 1991), and to passively protect fish against VHSV (Lorenzen et al. 1990).

The purpose of this research was to produce a large panel of MAbs against a single reference isolate of IHNV that would recognize the various neutralizing epitopes on the viral glycoprotein to further characterize the nature of these antigenic determinants.

MATERIALS AND METHODS

Cells and virus. Chinook salmon embryo (CHSE-214) cells (Lannan et al. 1984) were maintained in minimum essential medium (MEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 0.3 % tryptose phosphate broth (Difco Lab., Detroit, MI, USA), 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, and 100 µg ml^{-1} gentamicin sulphate (U.S. Biochem. Corp., Cleveland, OH, USA) at pH 7.5. For the neutralization assay, we used epithelioma papulosum cyprini (EPC) cells (Fijan et al. 1983).

We used the Western Regional Aquaculture Center (WRAC) reference isolate of IHNV recovered in 1982 from rainbow trout fry at a commercial facility in the Hagerman Valley, ID, USA. Monolayer cultures of CHSE-214 cells were infected with IHNV at a multiplicity of infection of 0.001 plaque-forming units (PFU) per cell and incubated at 15 °C for 7 d. Culture supernatant was harvested and centrifuged at 10000 × *g* for 20 min at 4 °C to remove the cell debris. Viral purification was performed on discontinuous and continuous sucrose gradients according to the procedures described by Hsu et al. (1986) and Kurath & Leong (1985).

Production of hybridomas and monoclonal antibodies. Adult female BALB/c mice were immunized by intraperitoneal injection with 0.5 ml of a mixture of purified IHNV and an equal volume of Freund's complete adjuvant. A similar inoculation was given 3 wk later using Freund's incomplete adjuvant. After an additional 4 wk, the immunized mice received a final booster by intravenous injection in the tail vein with 0.2 ml of diluted IHNV antigen without adjuvant. The fusion was carried out 3 d after the final injection using methods described by Mishell & Shiigi (1980) and Lane (1985). Briefly, fusion between IHNV-immunized spleen cells and SP2/0 Ag-14 myeloma cells was induced with 50 % polyethylene glycol 1500 and 4000 (4:1). Hybridomas secreting IHNV-specific neutralizing antibody were selected by a micro-neutralization assay (Winton et al. 1988) and positive clones were subcloned twice by limiting dilution and then expanded in culture flasks for *in vitro* production of antibody. MAb class and subclass were determined by enzyme-linked immunosorbent assay (ELISA) using mouse sub-isotyping kit (Bio-Rad Laboratories, Richmond, CA, USA).

Neutralization assay. Neutralizing antibody titers were determined by a modification of the method of Rovozzo & Burke (1973). Briefly, serial 2-fold dilutions of the hybridoma fluids were prepared in 96-well microtiter plates with MEM and reacted with equal volumes of a suspension containing 1×10^4 PFU ml^{-1} of IHNV. After 1 h incubation at 15 °C, titrations were performed using monolayers of EPC cells. The neutralization titer was expressed as the last dilution of the hybridoma culture supernatant completely neutralizing all the input virus in the well.

Enzyme-linked immunosorbent assay. Ninety-six well, Immulon I MicroElisa plates (Dynatech Lab., Alexandria, VA, USA) were coated with 0.5 µg of purified IHNV per well in carbonate buffer, pH 9.6, and allowed to incubate overnight at 4 °C. Plates were washed 3 times with phosphate buffered saline (PBS) containing 0.05 % Tween 20 (PBST), and then 50 µl of test hybridoma supernatant was added. After 2 h incubation at room temperature (RT), the plates were washed 3 times with PBST. Next, 50 µl of diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Ig) (Hyclone Laboratories, Logan, UT, USA) was added to each well, and the plates were incubated for 60 min at RT. After 5 washes, the chromogen, consisting of 1 mM ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] and 0.03 % hydrogen peroxide (H_2O_2) in 0.1 M sodium citrate buffer, pH 4.2, was added and allowed to react for 30 min at RT The absorbance of the samples was monitored at 405 nm in a microplate reader (Bio-Tek Instruments, Winooski, VT, USA), and the ELISA titer was expressed as the last dilution of the hybridoma culture supernatant that produced an optical density (OD) reading equal to or greater than 0.1 OD units.

Preparation of samples for electrophoresis. The structural proteins of IHNV were separated by electrophoresis under either reducing or non-reducing conditions. The viral glycoprotein was deglycosylated by means of an enzyme preparation containing equal activities of endoglycosidase F and glycopeptidase F (ENDO-F; Sigma Chemical Co., St. Louis, MO, USA; Plummer et al. 1984). Purified virus (50 µg ml^{-1}) was incubated overnight at 37 °C with 2 units ml^{-1} of

ENDO-F in 15 % sucrose, 150 mM NaCl, 10 mM EDTA, 10 mM NaH_2PO_4, pH 7.5, and 0.025 % (w/v) sodium dodecyl sulfate (SDS) solution. Samples were subsequently mixed with gel loading solution containing 3 % glycerol, 1 % SDS, and 30 µM bromophenol blue. In samples to be run under reducing conditions, 25 µM 2-mercaptoethanol was included in the solution. All the samples were boiled for 3 min and then subjected to electrophoresis.

Electrophoresis and immunoblotting. Ten percent polyacrylamide gels containing SDS were run according to the method of Laemmli (1970) and transferred by electroblotting onto nitrocellulose sheets (Schleicher & Schuell, Keene, NH, USA), as described by Towbin et al. (1979). Excess binding sites on the membranes were blocked by incubation in 5 % bovine serum albumin for 60 min at 37 °C. The nitrocellulose sheets were cut into strips and reacted with individual MAbs followed by incubation with HRP-conjugated goat anti-mouse Ig (Hyclone). After washing the strips, the reactions were visualized by color development with a substrate solution composed of 10 ml of 0.3 % 4-chloro-1-napthol (Bio-Rad) in methanol and 50 ml of 0.03 % H_2O_2 in PBS.

Purification of immunoglobulin. MAbs in culture supernatants were concentrated about 50-fold by precipitation with 50 % $(NH_4)_2SO_4$. This concentrate was dialyzed against PBS and then purified by affinity chromatography using protein A-Sepharose 4B (Pharmacia Biotech, Piscataway, NJ, USA) according to the procedure described by Ey et al. (1978). The eluate was then concentrated with a Centricon-100 microconcentrator (Amicon, Beverly, MA, USA).

Passive immunization. To determine if selected MAbs could provide passive protection to rainbow trout, we injected MAbs directed against conformation-dependent (MAb 1H8) and linear (MAb 6A7) epitopes. Two hundred juvenile rainbow trout (2 g) were anaesthetized with benzocaine (35 mg l^{-1}) and given an intraperitoneal injection with 50 µg of purified MAb in 50 µl PBS or with 50 µl PBS only. After a 2 h recovery period, duplicate groups of 25 fish that were injected with each of the MAbs and a duplicate group of controls that was injected with PBS were challenged by 1 h bath exposure to 1×10^5 PFU ml^{-1} of wild-type IHNV. A fourth group of duplicate aquaria contained fish injected with PBS but not challenged with IHNV. Fish were maintained in flowing water at 12 °C in aquaria containing 5 l of water. Dead fish were collected daily and examined for visible signs of disease. After 4 wk, the percentage of survivors was calculated and the data tested for significance by chi-square analysis.

Table 1. Properties of 31 neutralizing monoclonal antibodies developed against the Western Regional Aquaculture Center isolate of infectious hematopoietic necrosis virus. Neut. titer: neutralization titer; +: strong reaction; ±: weak reaction; blank: no reaction; ND: not determined

Clone	Isotype[a]	Neut. titer[b]	ELISA titer[c]	Immunoblotting of G protein		
				Native	Reduced	Deglycosylated
1B3	IgM	1:8	1:640	+		+
1D9	IgM	1:2	1:320	ND		ND
1E11	IgG2b	1:128	1:2560	+		+
1H8	IgG2a	1:128	1:5120	+		+
1H10	IgG2a	1:2	1:2560	+		+
1C4	IgG1	1:16	1:1250	+		+
1H12	IgG2a	1:2	1:1280	+	±	+
2C5	IgG2a	1:256	1:1024	+		+
2E9	IgG2a	1:2	1:2560	+		+
2H12	IgM	1:64	1:5120	+	+	+
2D3	IgM	1:2	1:2560	+	±	+
2D5	IgG1	1:2	1:320	+		+
3B12	IgM	1:32	1:2560	+	+	+
3F12	IgG2b	1:2	1:5120	+		+
3G8	IgM	1:2	1:5120	+		+
3H12	IgG3	1:2	1:640	+		+
4D3	IgM	1:2	1:320	+		+
4E3	IgM	1:2	1:80	ND		ND
4E10	IgM	1:2	1:1024	+	+	+
4C7	IgM	1:2	1:40	±		±
5A6	IgG2a	1:2	1:2560	+	+	+
5B4	IgM	1:2	1:160	+		+
5G3	IgG3	1:512	1:1024	+		+
5H2	IgM	1:8	1:2560	+		+
5A7	IgM	1:2	1:1280	+		+
5B2	IgM	1:8	1:2560	+		+
6A7	IgG2a	1:4	1:2560	+	+	+
6D7	IgG2b	1:128	1:2560	+		+
6F2	IgM	1:2	1:160	±		±
6H7	IgG2a	1:2	1:5120	+	+	+
6A12	IgG2a	1:2	1:640	+	±	+

[a] All *k* light chains

[b] Neutralizing titers are expressed as the last dilution of the hybridoma culture supernatant completely neutralizing an equal volume of a suspension containing 1×10^4 PFU ml^{-1} of IHNV

[c] ELISA titers are expressed as the last dilution that provided an absorbance reading ≥ 0.1

RESULTS

Characterization of anti-IHNV MAbs

Thirty-one hybridomas that produced useful levels of neutralizing antibodies against IHNV were selected

and cloned (Table 1). Culture fluids from the 31 hybridomas could be diluted between 1:2 and 1:512 and still completely neutralize infectivity in an equal volume of a suspension containing 1×10^4 PFU ml^{-1} of IHNV. The end-point dilutions to produce an OD reading of 0.1 or greater in the ELISA ranged from 1:40 to 1:10240. The Ig class or subclass and light chain type of each of the 31 MAbs were determined by ELISA. About half of the clones (15 of 31) produced IgM antibodies while the remainder were IgG1 (2), IgG2a (9), IgG2b (3), or IgG3 (2). While there did not appear to be a significant relationship between the class of antibody and the neutralization or ELISA titers, in general, those MAbs with a high neutralizing ability also had a high binding titer.

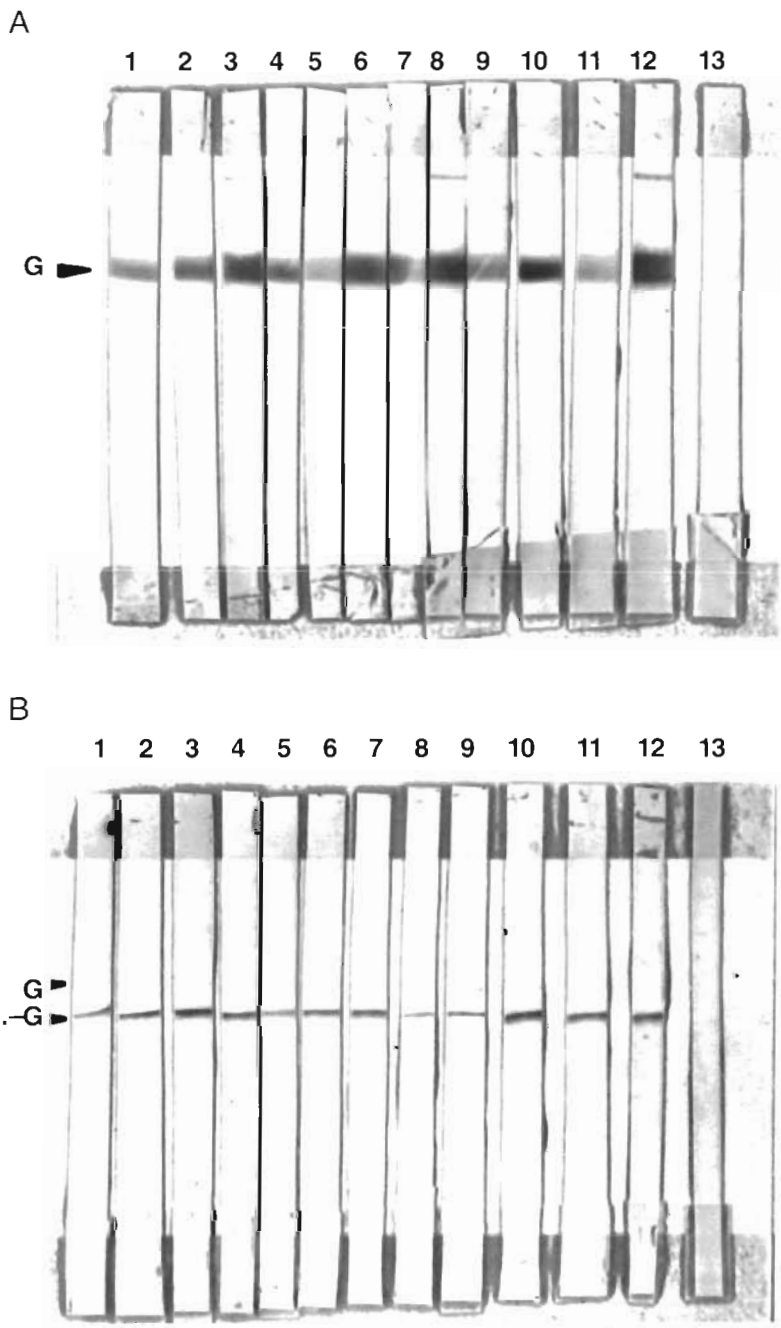

Fig. 1. Immunoblotting analysis of the glycoprotein of infectious hematopoietic necrosis virus (IHNV) using 12 neutralizing monoclonal antibodies (MAbs). All of the MAbs reacted with the glycoprotein in (A) the native (unreduced) conformation or (B) the native conformation following deglycosylation. Strips 1 to 12 were developed using cell culture fluids containing MAbs 1E11, 1H8, 1H10, 2C5, 2E9, 3F12, 3H12, 5A6, 5G3, 6A7, 6D7 and 6H7, respectively. Strip 13 was developed using culture fluid from SP2 myeloma cultures as a control. The positions of the IHNV glycoprotein in the native (G) and deglycosylated (Deg.-G) forms are shown

Immunoblotting of IHNV glycoprotein

The specificity of each MAb for the reduced, unreduced, and deglycosylated forms of the IHNV glycoprotein was determined by immunoblotting. All of the MAbs reacted with the G protein in the unreduced (native) conformation and MAbs 1H12, 2H12, 2D3, 3B12, 4E10, 5A6, 6A7, 6H7, and 6A12 were able to bind to the G protein after reduction of disulfide bonds indicating these nine MAbs were directed at linear determinants on the molecule (Table 1). Treatment of the purified virus with ENDO-F prior to electrophoresis and blotting did not affect the ability of any of the MAbs to bind to the G protein (Fig. 1). As expected, the removal of carbohydrates reduced the molecular weight of the G protein and sharpened the bands (Fig. 1). None of the MAbs reacted with other structural proteins of the virus.

Passive protection of fish

MAbs 1H8 and 6A7 were concentrated and purified from culture supernatants in order to determine the protective ability of these preformed neutralizing antibodies in fish. Both MAbs conferred a significant degree of protection against waterborne challenge (Table 2). The duplicate group of 25 fish that received MAb 1H8 averaged 98 % survivors and the duplicate group receiving MAb 6A7 averaged 94 % survivors. These results were similar to the average survival of fish in the control group injected with PBS but not

Table 2. Protection of juvenile rainbow trout *Oncorhynchus mykiss* by intraperitoneal injection of purified monoclonal antibodies (MAbs) against infectious hematopoietic necrosis virus

Treatment	Group of fish[a]			
	A	B	C	D
Injected with:[b]	PBS	PBS	MAb 1H8	MAb 6A7
Challenged with virus[c]	No	Yes	Yes	Yes
Survivors[d]	94 %	46 %	98 %	94 %

[a]Each group included 2 aquaria with 25 fish in each
[b]Each fish was injected with 50 µl of PBS or 50 µl PBS containing MAb (1 µg µl^{-1})
[c]Fish were challenged by 1 h waterborne exposure to 1 × 10^5 PFU ml^{-1} of wild-type IHNV
[d]Values represent average of 2 aquaria

challenged with IHNV (94 %) and significantly greater ($p < 0.001$) than the average survival among fish in the control group which were challenged with the virus (46 %).

DISCUSSION

In this study, a panel of neutralizing MAbs was produced against a single isolate of IHNV. All the MAbs reacted specifically with the viral glycoprotein, extending the results of Engelking & Leong (1989), who showed that purified IHNV glycoprotein stimulated neutralizing antibody in rabbits and a protective response in fish. Likewise, in rabies virus and VSV, neutralizing MAbs have been shown to be directed against the glycoprotein, and protection was induced by purified G protein (Lefrancois & Lyles 1982, Lafon et al. 1983).

For investigation of the role of disulfide bonds and tertiary structure of the glycoprotein on the ability of neutralizing antibodies to bind to epitopes, the IHNV glycoprotein was reduced by including 2-mercaptoethanol in the electrophoresis sample buffer. Because all MAbs reacted with the IHNV glycoprotein in the unreduced state, but only 9 of the MAbs recognized the protein following reduction of disulfide bonds, we believe that the IHNV glycoprotein contains linear as well as conformational (disulfide bond-dependent) neutralizing epitopes.

In addition to determining the presence of linear neutralizing epitopes, we wanted to know if carbohydrate moieties were integrated into the neutralizing epitopes reacting with these MAbs. Because N-linked oligosaccharide chains represent the only known type of carbohydrate in rhabdovirus glycoprotein (Dubois-Dalcq et al. 1984), and because Engelking & Leong (1989) reported that N-linked, but not O-linked, oligosaccharides were present on the IHNV G protein, we removed the N-linked carbohydrate groups of the protein by ENDO-F (Plummer et al. 1984). Deglycosylation of the IHNV antigen did not abolish the binding ability of any of these MAbs, confirming that these neutralizing MAbs are specific to the G protein structure and recognize carbohydrate-free epitopes. Analogous results have been reported for the glycoprotein of VHSV (Lorenzen et al. 1990). However, glycosylation is known to influence protein folding (Hongo et al. 1986). In the case of VSV, such G protein glycosylation has been found to be essential for the correct formation of disulfide bonds, as well as for intracellular transport (Machamer & Rose 1988, Grigera et al. 1991). The precise role of N-linked glycosylation in the IHNV glycoprotein remains to be investigated.

The linear neutralizing epitopes on the IHNV glycoprotein identified by certain of our MAbs probably represent those epitopes stimulating neutralizing antibodies in fish vaccinated by recombinant IHNV and VHSV glycoproteins expressed in *Escherichia coli* because these recombinant proteins would be neither glycosylated nor folded into the native structure. The presence of 1 or more linear epitopes indicates that a synthetic peptide may also stimulate protective immunity against the disease. A synthetic peptide resembling the structure of a linear, virus-neutralizing epitope of the rabies virus glycoprotein has been demonstrated to be capable of inducing G protein-specific neutralizing antibody *in vivo* (Dietzschold et al. 1990). The use of synthetic peptides representing selected amino acid sequences from the IHNV G protein may offer an alternative approach for development of an effective vaccine.

The protective ability of 2 of our neutralizing MAbs was demonstrated by passive immunization of rainbow trout. Both MAb 1H8, which recognized a conformation-dependent epitope, and MAb 6A7, which recognized a linear epitope, conferred significant protection against waterborne challenge. This result indicates that if neutralizing antibodies could be induced in fish by a synthetic peptide vaccine that contains the amino acid sequence of the linear epitope, such antibodies might have a protective effect *in vivo*.

The MAbs developed in this project have also served as useful diagnostic reagents for confirmation of IHNV. Although they do not recognize all isolates of the virus (Scott LaPatra, Clear Springs Trout Company, ID, USA; Ted Meyers, Alaska Department of Fish and Game, unpubl. data), they are consistent reagents that can be produced in large quantities with little effort or expense. These antibodies have also found an important application in epidemiological studies of IHNV and in examining the antigenic diversity among isolates of

IHNV within commercial trout facilities (S. LaPatra unpubl. obs.).

Our results provide new information about the nature of the epitopes on the IHNV glycoprotein that stimulate neutralizing antibodies. In addition, the MAbs developed in this study will serve as useful tools for more precisely locating these epitopes by sequencing the G protein of IHNV mutants that escape neutralization. More detailed knowledge of the IHNV neutralizing epitope structure will aid in the design of IHN vaccines, which are needed for control of this disease in aquaculture.

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