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 $1 \times 10^4$ 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 1980).
1985). Briefly, fusion between IHNV-immunized with 2 units ml⁻¹ of incubated overnight at 37°C and methods described by Mishell & Plumrner et al. 1984). Purified virus (50 pg ml⁻¹) was fusion was carried out 3 d after the final injection using ENDO-F (Sigma Chemical Co., St. Louis, MO, USA; 0.2 ml of diluted IHNV antigen without adjuvant. The activities of endoglycosidase F and glycopeptidase F booster by intravenous injection in the tail vein with means of an enzyme preparation containing equal additions. The viral glycoprotein was deglycosylated by purifying 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 & Shigi (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 × 10⁶ 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 horse-radish 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 (H₂O₂) 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 NaH₂PO₄, 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 mM 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% H₂O₂ in PBS.

**Purification of immunoglobulin.** MAbs in culture supernatants were concentrated about 50-fold by precipitation with 50% (NH₄)₂SO₄. 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 but not challenged with 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.

## 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 $1 \times 10^4$ 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.

**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
Huang et al.: Glycoprotein characterization of IHNV

Oncorhynchus mykiss (1989) reported that N-linked, but not O-linked, oligo-
mykiss by intraperitoneal injection of purified monoclonal
antibodies (MAbs) against infectious hematopoietic necrosis
virus 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 pro-

A B C D

Injected with: PBS PBS MAb 1H8 MAb 6A7
Challenged with virus: No Yes Yes Yes
Survivors: 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 con-
taining MAb (1 μg μl⁻¹)
Challenged with virus
Survivors
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 pro-
duced against a single isolate of IHNV. All the MAbs
reacted specifically with the viral glycoprotein, ex-
tending the results of Engelking & Leong (1989), who showed that purified IHNV glycoprotein stimulated
neutralizing antibody in rabbits and a protective re-

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-mercapto-
ethanol 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 carbohy-
drate 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, oligo-
saccharides 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 pro-
tein structure and recognize carbohydrate-free epi-

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did not abolisa
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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