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NOTE

Immunogenicity of a recombinant infectious hematopoietic necrosis virus glycoprotein produced in insect cells

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ABSTRACT: A recombinant infectious hematopoietic necrosis virus (IHNV) glycoprotein (G protein), produced in *Spodoptera frugiperda* (Sf9) cells following infection with a baculovirus vector containing the full-length (1.6 kb) glycoprotein gene, provided very limited protection in rainbow trout *Oncorhynchus mykiss* challenged with IHNV. Fish were injected intraperitoneally (i.p.) with Sf9 cells grown at 20°C ($RecG_{low}$) or 27°C ($RecG_{high}$) expressing the glycoprotein gene. Various antigen (Ag) preparations were administered to adult rainbow trout or rainbow trout fry. Sera collected from adult fish were evaluated for IHNV neutralization activity by a complement-dependent neutralization assay. Anti-IHNV neutralizing activity was observed in sera, but the percent of fish responding was significantly lower ($p < 0.05$) in comparison to fish immunized with a low virulence strain of IHNV (LV-IHNV). A small number of fish immunized with $RecG_{low}$ or $RecG_{high}$ possessed IHNV G protein specific antibodies (Abs) in their serum. Cumulative mortality (CM) of rainbow trout fry (mean weight, 1 g) vaccinated by i.p. injection of freeze/thawed Sf9 cells producing $RecG_{low}$ was 18% in initial trials following IHNV challenge. This level of protection was significant ($p < 0.05$) but was not long lasting, and neutralizing Abs were not detected in pooled serum samples. When trout fry (mean weight, 0.6 g) were vaccinated with supernatant collected from sonicated Sf9 cells, Sf9 cells producing $RecG_{low}$, or Sf9 cells producing $RecG_{high}$, CM averaged 46%. Protection was enhanced over negative controls, but not the positive controls (2% CM), suggesting that in the first trial soluble cellular proteins may have provided some level of non-specific protection, regardless of recombinant protein expression. Although some immunity was elicited in fish, and $RecG_{low}$ provided short-term protection from IHNV, Ab-mediated protection could not be demonstrated. The results suggest that recombinant G proteins produced in insect cells lack the immunogenicity associated with vaccination of fish with an attenuated strain of IHNV.

KEY WORDS: IHNV · Baculovirus · Recombinant glycoprotein · Immunization · Rainbow trout

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus which causes acute disease primarily in juvenile sockeye salmon *Oncorhynchus nerka*, chinook salmon *O. tshawytscha* and rainbow trout *O. mykiss* (Wolf 1988). Infectious hematopoietic necrosis (IHN), the disease caused by this virus, results in large economic losses to public and private salmonid aquaculture operations. Control of IHN is achieved primarily through avoidance and no licensed commercial vaccines aimed at mass immunization of juvenile salmonids are available. Fish surviving infection with IHNV are generally resistant to re-infection and often possess IHNV neutralizing antibodies (Abs) (LaPatra et al. 1993, Ristow et al. 1993). The ability of the virus to elicit an immune response in salmonids suggested that vaccine development may be feasible and provide an effective method of disease control (Amend 1976). Experimental vaccines that provide varied levels of protection have been developed, but adaptation of these for large-scale use in commercial facilities has proven difficult (Gilmore et al. 1988, Leong & Fryer 1993).

This study evaluates the potential of a baculovirus derived recombinant IHNV vaccine to elicit humoral immune responses in rainbow trout and provide protection against IHNV challenge. The expression of genes in insect cells using baculovirus vectors has been extensively utilized as a means of producing full-length functionally active proteins. Unlike bacterial cells, insect cells have the ability to glycosylate proteins in a eukaryotic manner (Luckow & Summers 1988, Luckow 1991, King & Possee 1992). The glycoprotein (G protein) of IHNV is important in eliciting a protective immune response in fish (Engelking & Leong 1989), and it is thought that a glycosylated G protein may enhance immunity over non-glycosylated

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products. Expression of the glycoprotein gene of IHNV in insect cells was first achieved by Koener & Leong (1990), but the ability of that antigen (Ag) to elicit immunity to IHNV was not reported. Lecocq-Xhonneux et al. (1994) found that rainbow trout injected with insect cell lysates producing a baculovirus derived recombinant viral hemorrhagic septicaemia virus (VHSV) glycoprotein developed neutralizing Abs and were protected from lethal challenge.

Initial characterization of the baculovirus product developed for this study has revealed interesting temperature-dependent properties (Cain et al. 1998). The recombinant glycoprotein, produced in insect cells *Spodoptera frugiperda* (Sf9) at 20°C ($RecG_{low}$), was shown to be transported and localized to the cell surface. In contrast, production at 27°C ($RecG_{high}$) resulted in a protein which remained internalized within the cells. The results suggest that low temperature production may produce a more native product and agree with similar findings associated with the glycoprotein of VHSV (Lorenzen 1997). The objective of this study was to evaluate the ability of fish to elicit neutralizing antibodies and/or protective immunity following immunization with a baculovirus derived IHNV G protein produced in insect cells at 20 or 27°C.

Materials and methods. Fish stocks were obtained from Clear Springs Foods Inc., Buhl, Idaho, USA, and all immunizations and challenges were conducted at their research facility. Adult rainbow trout with no history of an IHNV infection were obtained from research lots of fish, which had been maintained in outside raceways prior to being moved into the research laboratory. Juvenile rainbow trout were hatched and reared in ultra-violet (UV) light treated specific pathogen free (SPF) spring water prior to vaccination trials. All experiments were conducted in tanks supplied with UV-treated SPF spring water with an ambient temperature of 15°C.

IHNV and assays: Monolayers of *Epithelioma papulosum cyprini* (EPC) cells (Fijan et al. 1983) were grown in minimal essential media (MEM, Gibco) supplemented with 10% fetal calf serum (FCS). An isolate of IHNV (220-90; LaPatra et al. 1991) and a low virulence IHNV strain (039-82; LaPatra et al. 1991) were propagated and assayed in EPC cells. Stocks of virus were obtained by harvest of cell culture lysate 6 to 8 d post-infection. A plaque assay (Burke & Mulcahy 1980) was performed on a sub-sample of the lysate and aliquots were stored at −75°C. Low virulence IHNV (LV-IHNV) was used for immunizations of fish while isolate 220-90 was utilized for virus challenges and complement dependent neutralization assays (LaPatra et al. 1993). Purified IHNV (Round Butte I isolate; previously described in Hsu et al. 1986, Ristow & Arnzen de Avila 1991) was used for immunoblotting analysis, while recombinant baculovirus stocks and Sf9 cells were maintained as previously described (see 'Materials and methods', Cain et al. 1998).

Immunization: Adult fish were tagged for individual identification prior to immunization. Simultaneously, serum was obtained from individual fish with no history of IHN and confirmed negative (titer < 20) for IHNV neutralization titers by the complement-dependent neutralization assay (LaPatra et al. 1993). In Trials I-1 and I-2, 8 treatment groups were established, and 60 adult rainbow trout were immunized in each trial (Table 1). In Trial I-1 , fish (mean weight, 1400 g) were anesthetized with MS-222 (Argent chemical) and immunized by i.p. injection with 2 ml of Ag. For those treatment groups receiving Freund's complete adjuvant (FCA), a 1:1 mixture consisting of 1 ml of each preparation and 1 ml of FCA was emulsified just prior to injection. Immunization Trial I-2 was designed similar to the first trial but fish were smaller (mean weight, 400 g) and only 1 ml of each preparation with or without FCA was injected. In both trials, fish were maintained in 1140 l circular tanks. Serum was collected from individual fish in each trial at 3 wk intervals and analyzed for IHNV neutralization titers. Fish were fasted 48 h prior to immunization or sera collection.

Vaccination and challenge: Vaccination trials (V-1 and V-2) were set up to evaluate protective immunity to IHNV conferred to rainbow trout fry following injection of preparations of Sf9, $RecG_{high}$ and $RecG_{low}$ cells. Treatments were identical in the 2 trials; however, Ag

Table 1. *Oncorhynchus mykiss*. Experimental design for adult rainbow trout immunized in trials (I-1 and I-2). Treatment groups received either freeze/thawed cells or supernatant from an equal concentration of cells subjected to sonication followed by removal of cellular debris by centrifugation (10 000 × *g*) for 5 min. LV-IHNV served as the positive control Ag in both immunization trials, while negative controls included phosphate-buffered saline (PBS) and/or Sf9 cells

Treatment groups ($RecG_{high}$; I-1)[a]	N	Treatment groups ($RecG_{low}$; I-2)[b]	N
Freeze/thawed cells	10	Freeze/thawed cells	10
Freeze/thawed cells/FCA	10	Freeze/thawed cells/FCA	10
Supernatant	10	Supernatant	10
Supernatant/FCA	10	Supernatant/FCA	10
LV-IHNV	5	LV-IHNV	5
LV-IHNV/FCA	5	LV-IHNV/FCA	5
PBS	5	Sf9/FCA	5
PBS/FCA	5	PBS/FCA	5

[a]Cells producing $RecG_{high}$ were harvested at various times post-infection with recombinant baculovirus and pooled cells were adjusted to a final concentration of 3×10^6 cells ml^{-1}

[b]Cells were adjusted to a final concentration of 1×10^7 cells ml^{-1} in PBS

Table 2. *Oncorhynchus mykiss*. General experimental design for IHNV challenge of vaccinated juvenile rainbow trout

	Vaccination trial (V-1)	Vaccination trial (V-2)
Initial fish size (g)	1.0	0.6
Size at challenge (g)[a]	2.8 (3.8)	0.9 (1.9)
Fish/tank	25	25
Tanks	15	15
Treatments[b]	Cells (supernatant)	Supernatant
Length of study (weeks)[c]	6 (9)	6 (9)

[a]Duplicate IHNV challenges were performed. Fish size at the second challenge given in parentheses

[b]Treatments (Ags) administered in each trial included: Sf9 cells; $RecG_{low}$; $RecG_{high}$; rabbit sera containing anti-IHNV Ab titers (fish were passively immunized 24 h prior to challenge), and tissue culture media (injected 24 h prior to challenge as a negative control). Antigen preparation in Trial V-1 included freeze/thawed cells for the initial immunization followed by a booster immunization 4 wk later with supernatant from sonicated cells (shown in parentheses). Fish in Trial V-2 received only supernatant from sonicated cells both initially and as a booster immunization

[c]Length of the study is given as weeks following initial immunization to time of first challenge. Number in parentheses indicates time repeat challenge performed on identically treated fish 5 wk post-booster

preparation differed (Table 2). Fish were anaesthetised with MS-222 and injected with 50 µl of Ag for primary and booster immunizations. Treatment groups (200 fish $tank^{-1}$) were maintained in 116 l tanks. Fish were held in the system for 6 wk following initial vaccinations (2 wk post-booster), at which time a portion of the fish were removed and challenged with IHNV. Remaining fish were challenged 3 wk later (9 wk post-primary immunization). Duplicate groups of approximately 25 fish were challenged by waterborne exposure to 1×10^5 plaque forming units (pfu) ml^{-1} of IHNV and transferred to separate 20 l aquaria. Single 25 fish groups from each treatment were also mock challenged with phosphate-buffered saline (PBS). Following IHNV challenge, fish were monitored for mortality for 21 d. Positive controls consisted of fish passively immunized by i.p. injection of 50 µl of polyclonal rabbit anti-IHNV serum (1:25 in MEM-2)/fish 24 h prior to IHNV challenge, and negative controls received only tissue culture media. The use of passive immunization provides Ab-mediated protection. Although this tells little of the immunocompetence of the animals, the use of a low virulence or attenuated virus vaccine as a positive control may have caused residual mortality due to subclinical infection of such small fish. For ease of interpretation, vaccination trials were designated as V-1a and V-1b (corresponding to replicate challenges for Trial V-1) and V-2a and V-2b (corresponding to replicate challenges for Trial V-2).

Prior to virus challenge in Trial V-1, 10 fish were removed from each of the 116 l stock tanks, and pooled serum samples were obtained for analysis of IHNV neutralization titers. Due to the small size of the fish, serum samples were not collected in Trial V-2. Virus isolation from dead fish in each trial was performed with kidney-liver-spleen homogenates collected from a minimum of 20% of the daily mortalities using a standard plaque assay (LaPatra et al. 1989).

Statistical analysis: The percentage of fish in Trials I-1 and I-2 which had positive IHNV neutralization titers was analysed by a 2-way analysis of variance (ANOVA). Treatment groups exhibiting statistical significance were isolated by a multiple comparison procedure. Cumulative percent mortality resulting from rainbow trout fry vaccinations followed by IHNV challenge was analysed using chi-square analysis. Values in all cases were considered significantly different at p values ≤ 0.05.

Results and discussion. In initial experimental trials, adult rainbow trout exhibited IHNV neutralizing Abs in sera from a low number of fish immunized with preparations of either $RecG_{low}$ or $RecG_{high}$ (Table 3), but the frequency of positives were not significantly different from control fish injected with PBS or Sf9 cells emulsified in FCA. Neg-

Table 3. *Oncorhynchus mykiss*. Anti-IHNV neutralizing activity in serum samples collected from fish immunized with $RecG_{high}$ (I-1) and $RecG_{low}$ (I-2) up to 9 wk post-immunization. Percent of fish testing positive (titer ≥20) by complement dependent neutralization (LaPatra et al. 1993, Ristow et al. 1993) are given out of the total number of fish sampled (in parentheses). Mortalities or tag loss accounts for variation in number of fish sampled. Treatment groups in immunization Trials I-1 and I-2 are represented, and significant treatment differences ($p < 0.05$) are indicated by an asterisk

Treatment group (I-1)	3 wk	6 wk	9 wk
Freeze/thawed cells	0% (9)	0% (9)	22% (9)
Freeze/thawed cells/FCA	10% (10)	10% (10)	10% (10)
Supernatant	10% (10)	0% (9)	0% (9)
Supernatant/FCA	22% (9)	11% (9)	11% (9)
LV-IHNV*	100% (5)*	100% (5)*	100% (4)*
LV-IHNV/FCA*	80% (5)*	80% (5)*	60% (5)*
PBS (negativ control)	0% (5)	0% (5)	0% (5)
PBS/FCA	20% (5)	20% (5)	20% (5)
Treatment group (I-2)	**3 wk**	**6 wk**	**9 wk**
Freeze/thawed cells	0% (10)	20% (10)	10% (10)
Freeze/thawed cells/FCA	13% (8)	38% (8)	25% (8)
Supernatant	0% (9)	11% (9)	22% (9)
Supernatant/FCA	0% (7)	0% (7)	14.3% (7)
LV-IHNV*	80% (5)*	80% (5)*	40% (5)*
LV-IHNV/FCA*	100% (2)*	100% (2)*	100% (2)*
Sf9/FCA	0% (4)	25% (4)	25% (4)
PBS/FCA	0% (5)	20% (5)	0% (5)

ative controls immunized with PBS alone (Trial I-1) showed no serum neutralization activity in sera throughout the study, whereas fish injected with a low virulence strain of IHNV (LV-IHNV) seroconverted at a significant level.

The ability of serum Abs to bind to the viral or recombinant G protein was evaluated by Western blot. Serum from a single fish immunized with $RecG_{high}$ emulsified in FCA that exhibited a neutralization titer of ≥160 showed that G specific Abs were elicited and capable of recognizing reduced and non-reduced viral G protein (Fig. 1). The lower band identified in the blot of the reduced virus is likely an unglycosylated G protein as it migrates at a similar molecular weight (MW). Serum tested by Western blot from fish immunized with preparations of $RecG_{low}$ also possessed Abs with the ability to recognize the IHNV G protein (data not shown). Lorenzen et al. (1993) demonstrated similar Ab development to the VHSV G protein following immunization with a recombinant VHSV glycoprotein produced by bacterial expression. Serum collected from a fish immunized with LV-IHNV/FCA contained

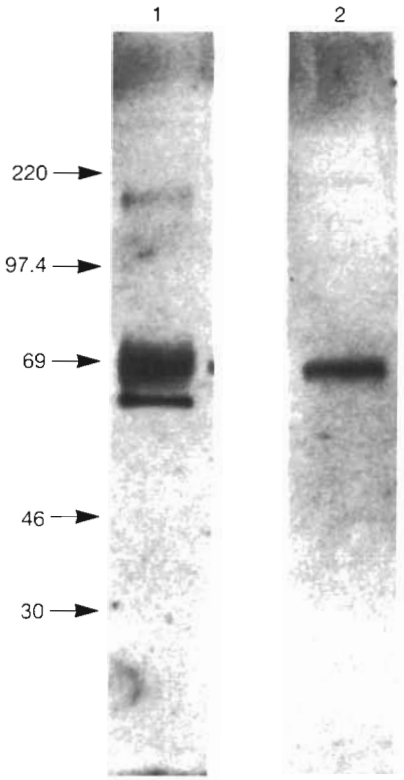

Fig. 1 Immunoblot of IHNV proteins. Nitrocellulose strips containing IHNV proteins were incubated with a 1:100 dilution of serum from a fish immunized with $RecG_{high}$. Strip 1 shows Ab recognition of wild-type IHNV G protein electrophoresed under reduced conditions, and strip 2 shows Ab recognition of a non-denatured IHNV G protein

Abs that only weakly recognized the recombinant G proteins, $RecG_{low}$ and $RecG_{high}$ (data not shown). Although Western blot results indicated that fish developed Abs to the G protein, the low percentage of fish seroconverting suggested that the antigenicity of recombinant preparations was low. It should be recognized that the use of a low virulence strain of virus would have established a subclinical infection in these fish resulting in continued stimulation of the immune system. Alternative positive controls for this experiment may have been a killed whole virus or purified viral G protein. An IHNV virus with low viruluence was chosen as a control in this study principally because it readily elicits a humoral response measured by neutralizing Abs to IHNV and it is capable of producing protection upon challenge.

It was demonstrated that immunization by i.p. injection with freeze/thawed Sf9 cells producing $RecG_{low}$ provided significant ($p < 0.05$) but short-term protection from IHNV challenge. Cumulative mortality in this treatment group was 18%, and the degree of protection observed compares with similar studies of rainbow trout immunized by i.p. injection of Sf9 cells producing a recombinant VHSV G protein emulsified in adjuvant and challenged by waterborne exposure to VHSV (Lecocq-Xhonneux et al. 1994). Although results from this initial trial appeared promising, identically treated fish challenged 5 wk post-booster (V-1b) were susceptible to IHNV (Table 4). Additionally, neutralizing Abs were not detected in pooled serum samples collected from vaccinated stocks. The protection observed suggested that low temperature expression of the G protein was responsible for specific protection. Since a control consisting of Sf9 cells grown at 20°C was not included in this first vaccination trial (V-1a and V-1b), it cannot be ruled out that some property induced by the low temperature cultivation of cells (without baculovirus infection) may have been responsible for enhanced protection. Virus was re-isolated from 93 and 90% of mortalities sampled in the first trial and mean virus titers were 4.8×10^6 pfu ml^{-1} and 2.6×10^6 pfu ml^{-1}, respectively. Since long-term immunity is a critical requirement in the development of an efficacious vaccine (Leong & Fryer 1993), our initial results raised concern.

Additional trials (V-2a and V-2b) to evaluate the ability of the 'sonicated' Ag to provide protection from virus challenge were conducted. Sonication of cells was considered important since the development of an immersion-based vaccine would likely require the cellular dissociation and solublization of the G protein. It is known that when the G protein of IHNV is stripped from the surface of the virus by treatment with Triton X-100 it remains immunogenic to fish (Engelking & Leong 1989). Sonication allowed the dissociation of Ag

Table 4. *Oncorhynchus mykiss*. Protection of rainbow trout fry following IHNV challenge. Trials designated as V-1a and V-2a indicate data were collected from IHNV challenge 2 wk post-booster, while V-1b and V-2b indicate data were collected following IHNV challenge of identically treated fish 5 wk post-booster. N: Replicate groups. Significant differences ($p < 0.05$) in cumulative percent mortality are indicated by different letters in subscript. Relative percent survival (RPS, Johnson et al. 1982) of vaccinated over control fish was determined by the formula: RPS = [1 – (% loss of vaccinated fish/% loss of controls)] × 100. Note: Positive control fish were passively immunized with 50 µl of a 1:25 dilution of rabbit anti-IHNV antiserum 24 h prior to challenge, while negative control fish received only tissue culture media

Trial	Treatment	Booster	N	Mortality (%)	RPS
V-1a	Freeze/thawed cells ($RecG_{high}$)	Supernatant	27+25	33_a	18
"	Freeze/thawed cells ($RecG_{low}$)	Supernatant	27+30	18_b	56
"	Freeze/thawed cells (Sf9)	Supernatant	25+25	46_a	–
"	(+) control (rabbit anti-IHNV)	Supernatant	25+24	6_b	85
"	(–) control (tissue culture media)	Supernatant	25+25	40_a	–
V-1b	Freeze/thawed cells ($RecG_{high}$)	Supernatant	24+23	58_a	–
"	Freeze/thawed cells ($RecG_{low}$)	Supernatant	26+24	52_a	–
"	Freeze/thawed cells (Sf9)	Supernatant	25+25	58_a	–
"	(+) control (rabbit anti-IHNV)	Supernatant	26+26	10_b	78
"	(–) control (tissue culture media)	Supernatant	26+26	44_a	–
V-2a	Supernatant ($RecG_{high}$)	Supernatant	24+24	46_a	45
"	Supernatant ($RecG_{low}$)	Supernatant	25+25	48_a	43
"	Supernatant (Sf9)	Supernatant	24+20	43_a	49
"	(+) control (rabbit anti-IHNV)	Supernatant	24+24	2_b	98
"	(–)control (tissue culture media)	Supernatant	25+25	84_c	–
V-2b	Supernatant ($RecG_{high}$)	Supernatant	24+23	68_a	–
"	Supernatant ($RecG_{low}$)	Supernatant	26+24	31_a	21
"	Supernatant (Sf9)	Supernatant	25+25	44_a	–
"	(+) control (rabbit anti-IHNV)	Supernatant	26+26	8_b	78
"	(–) control (tissue culture media)	Supernatant	26+26	39_a	–

from the cell membrane as demonstrated by use of G specific MAbs in Western and dot blot analysis of sonicated supernatant (data not shown). In vaccination Trial V-2a, fish immunized with supernatant from sonicated Sf9 cells, Sf9 cells producing $RecG_{high}$, or $RecG_{low}$ exhibited cumulative mortalities of 43, 46, and 48%, respectively. Although this initial level of protection was significant ($p < 0.05$) when compared to mortalities in the negative control groups, it was not comparable to positive control groups which exhibited cumulative mortalities of only 2% (Table 4). Since protection in this trial was associated with all groups immunized with insect cells, regardless of recombinant protein expression, it is possible that non-specific components of the immune system were stimulated which enhanced cell-mediated immunity over negative control fish. When the challenge was repeated at 5 wk (V-2b), no protection was observed in fish from any of the vaccinated groups, but positive control groups again exhibited significantly lower ($p < 0.05$) mortality than negative controls. In Trials V-2a and V-2b, virus was re-isolated from 95 to 96% of mortalities sampled, and ranged in mean titer from 3.7×10^6 pfu ml^{-1} to 5.1×10^6 pfu ml^{-1}, respectively.

The results of this study provide evidence that a recombinant IHNV G protein produced in insect cells may elicit limited virus neutralizing Abs and may provide short duration protection in rainbow trout; however, these responses appear limited, and further analysis and purification of these recombinant products is needed. The short-term protection conferred by immunization in the first trial with $RecG_{low}$ appeared interesting, but in retrospect, immunogenicity of $RecG_{high}$ and $RecG_{low}$ appears minimal. The properties of both G proteins tested here differ from the native IHNV glycoprotein (Cain et al. 1998), which may explain the lack of immunostimulatory properties observed. Temperature manipulations during expression of recombinant proteins could prove crucial in future vaccine development, and as more information is obtained about the G protein of IHNV, a better immunogen may be developed. Results presented in this study emphasise the need for a greater understanding of the complex interactions of the fish immune system with the glycoprotein of IHNV.

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