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Naked DNA vaccination of Atlantic salmon *Salmo salar* against IHNV

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ABSTRACT: A naked plasmid DNA encoding the glycoprotein (pCMV4-G) of a 1976 isolate of infectious hematopoietic necrosis virus (IHNV) obtained from steelhead *Oncorhynchus mykiss* was used to vaccinate Atlantic salmon *Salmo salar* against IHNV. Eight weeks post-vaccination the fish were challenged with a strain of IHNV originally isolated from farmed Atlantic salmon undergoing an epizootic. Fish injected with the glycoprotein-encoding plasmid were significantly ($p < 0.05$) protected against IHNV by both immersion and cohabitation challenge. Survivors of the first challenges were pooled and re-challenged by immersion 12 wk after the initial challenge. Significant ($p < 0.05$) protection was observed in all of the previously challenged groups including those receiving the complete vaccine. Fish injected with the glycoprotein-encoding plasmid produced low levels of virus-neutralizing antibodies prior to the first challenge. Neutralizing antibodies increased in all groups after exposure to the IHNV. Passive transfer of pooled sera from pCMV4-G vaccinates and IHN survivors provided relative survivals of 40 to 100% compared to fish injected with sera collected from fish immunized with control vaccines or left unhandled. In this study, DNA vaccination effectively protected Atlantic salmon smolts against challenges with IHNV.

KEY WORDS: DNA · Vaccine · G protein · Atlantic salmon · IHNV

INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is enzootic in salmonid species along the Pacific coast of North America. The virus causes significant losses among both wild and cultured fish, usually at the juvenile life stages (Williams & Amend 1976, Mulcahy et al. 1980, Traxler & Rankin 1989). However, in intensive culture, losses of larger fish have been reported (Busch 1983). Over the past decade the aquaculture industry in British Columbia, Canada, has grown to produce 36 000 t of salmon in 1997 (B.C. Salmon Farmers Assoc.). Atlantic salmon *Salmo salar* account for 80% of the total production with chinook *Onchorhynchus tshawytscha* and coho *O. kisuch* salmon making up the remaining 20%. In 1992, infectious hematopoietic necrosis (IHN) was diagnosed in Atlantic salmon at a sea pen site on the east coast of Vancouver Island (Armstrong 1993, Traxler et al. 1993). Over the next several years IHNV caused clinical disease in Atlantic salmon at 11 additional sites all within the same geographic region located on the east coast of central Vancouver Island. Losses of pen-reared Atlantic salmon at affected sites ranged from 20% in the larger fish (1 to 3 kg) up to 80% in the smaller fish (100 to 300 g). The severe economic losses suffered by the fish farming industry due to IHNV has prompted investigations into the development of vaccines to control losses. In British Columbia, autogenous vaccines consisting of killed virus have had limited success in reducing the losses attributed to IHNV. There have been reports of IHNV in vaccinated fish (S. St. Hilarie pers. comm.).

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A new approach to disease prevention in aquaculture has been the application of genetic immunization or DNA-based vaccination (Anderson et al. 1996a, Heppell et al. 1998). The first demonstration of the efficacy of a DNA vaccine in fish was in rainbow trout *Oncorhynchus mykiss* immunized against IHNV (Anderson et al. 1996b). Subsequently, DNA vaccination studies by Lorenzen et al. (1998) showed protective immunity in rainbow trout when challenged with viral hemorrhagic septicemia virus (VHSV), another important rhabdovirus pathogen of fish. A combined DNA vaccination experiment using VHSV and IHNV glycoprotein was shown to induce a protective antibody response measured by passive immunization (Boudinot et al. 1998).

In this study the efficacy of a DNA vaccine prepared with the gene encoding the IHNV glycoprotein administered to Atlantic salmon pre-smolts and smolts was tested for its ability to prevent IHN in fish challenged by immersion and cohabitation. The aquaculture industry in British Columbia immunizies fish while in fresh water prior to smolting. We were interested in knowing whether genetic DNA vaccination of Atlantic salmon against IHNV was effective when administered before and after seawater entry. Fish were challenged in seawater by cohabitation with IHNV injected fish and by waterborne exposure to IHNV. Survivors of the first challenge were rechallenged by immersion. The humoral immune response prior to and after challenge was determined by measuring the neutralizing antibody activity in serum against IHNV and by determining the protection afforded to naive fish after passive transfer of serum containing neutralizing antibody.

MATERIAL AND METHODS

Cell culture and virus. The *epithelioma papillosum cyprinii* (EPC) cell line was used for the propagation of IHNV (Fijan et al.1983). The virus used in this study was isolated from clinically infected Atlantic salmon in sea pens in 1992 (Armstrong et al. 1993, Traxler et al. 1993) and confirmed as IHNV by serum neutralization. Infected monolayers were cultured at 15°C in minimal essential media supplemented with 10% fetal bovine serum (Gibco BRL). The virus was passed in cell culture twice, pooled and aliquoted, and stored at −80°C prior to use. Fish dying from challenges were assayed for IHNV by aseptic removal anterior kidney tissue, dilution of the tissue samples 1:10 with Earle's balanced salt solution and homogenizing with a Polytron generator (Brinkman Instruments Co., Rexdale, Ontario, Canada). Homogenates were centrifuged for 10 min at 2000 × *g* and 10-fold dilutions of the supernatants were inoculated on preformed monolayers of EPC cells. The cultures were fixed and stained after 7 to 10 d incubation at 15°C. The virus was quantified by counting plaques and titers were calculated as plaque forming units (pfu) ml^{-1} of fluid or gram of tissue (Burke & Mulcahy 1980).

DNA preparation. The plasmid DNAs used in this study have been previously described (Anderson et al. 1996a,b). Plasmid pCMV4-G contains the complete IHNV glycoprotein cDNA of a 1976 isolate fused to the human cytomegalovirus immediate early promoter (hCMV-IEP) and pCMVL contains the firefly *Photinus pyralis* luciferase cDNA adjacent to the hCMV-IEP.

The plasmids were grown in *Escherichia coli* DH5α at 37°C with Luria-Bertani broth and agar plates supplemented with 50 mg ml^{-1} ampicillin (Sambrook et al. 1989). The plasmids used for vaccination were prepared from 250 ml overnight cultures using an ammonium acetate purification protocol (Saporito-Irwin et al. 1997). The purified DNAs were stored in 10 mM Tris, 1 mM EDTA, pH 7.4, at 2 mg ml^{-1}. For vaccination DNA was diluted to 250 µl ml^{-1} in PBS (0.14 M sodium chloride, 10 mM sodium phosphate, pH 7.4).

Fish. Atlantic salmon (Mowi strain) were moved from a hatchery with no previous occurrence of IHN to the Pacific Biological Station, Nanaimo, British Columbia. Fish were held in 9°C pathogen-free fresh water for 2 wk to acclimate. The fish were then randomly divided into 2 lots; one group was acclimated to seawater (smolted) by increasing the seawater:freshwater ratio by ¼ every 4 to 5 d, the other group remained in fresh water.

Vaccination. Two weeks after the first group of Atlantic salmon was smolted, both groups were vaccinated and moved to the quarantine laboratory where all subsequent vaccination and challenge experiments were conducted at 10°C. Both groups of Atlantic salmon, pre-smolts (mean weight, 57 g) and smolts (mean weight, 73 g), were anesthetized in 100 µg l^{-1} of tricaine methane sulfonate and injected immediately anterior to the dorsal fin with 100 µl PBS containing 25 µg of DNA (Anderson & Leong 1999). Four groups of 180 pre-smolts and 4 groups of 130 smolts were placed in eight 750 l tanks supplied with flowing fresh and seawater, respectively. In each group of smolts and pre-smolts there were 4 sub-groups including a pCMV4-G-injected group, and 3 negative control groups including a pCMVL (luciferase)-injected group, a PBS (phosphate buffered saline)-injected group, and an uninjected group. After 4 wk the 4 groups of pre-smolts were also acclimated to seawater using the same procedure previously described. The group receiving the vaccine prior to smoltification was designated pre-smolts and those receiving the vaccine after smoltification were desig-

nated as smolts. Both groups of fish were challenged 8 wk post-vaccination.

Challenge protocol. The first virus challenges of each of the 4 groups of pre-smolts and smolts were conducted in duplicate by cohabitation and bath exposure. Groups of 50 pre-smolts or 40 smolts were placed in 750 l tanks supplied with 5 l min^{-1} of flowing water. Ten untreated fish from the same stock were injected with 4.9×10^3 pfu of IHNV, marked by clipping the adipose fin and placed in each of the 16 tanks. For the immersion challenge 20 fish from each treatment were challenged in duplicate by immersion in 20 l of aerated seawater for 1 h. The initial viral titer of the challenge suspension was 5.9×10^3 pfu ml^{-1}, which decreased to 4.6×10^3 pfu ml^{-1} after 1 h. All groups of fish challenged by immersion were held in 50 l tanks supplied with flowing water at 2 l min^{-1}. The fish were fed a commercial ration and dead fish were removed daily. Cumulative percent mortality, mean days to death and relative percent survival were calculated for each group.

Ten weeks after the first challenge, surviving fish were anesthetized, marked with numbered operculum tags and bled by caudal peduncle puncture to obtain serum. Fish were then pooled according to whether they were initially challenged by bath or by cohabitation, and whether they were immunized as pre-smolts or smolts. An effort was made to equalize the number of fish contributed from each group. A similar number of naive fish from the original stock that had not been immunized or challenged was added to each group prior to challenge to serve as controls. At 12 wk after the initial challenge, each of the 4 groups (pre-smolts/immersion, pre-smolts/cohabitation, smolts/immersion and smolts/cohabitation) were re-challenged by bath exposure to 5×10^4 pfu ml^{-1} of IHNV for 1 h. The fish were placed in 750 l tanks supplied with 5 l min^{-1} flowing seawater at 10°C. Each day for 30 d, fish were fed and mortalities were removed and recorded. For both challenges, accumulated mortalities were recorded and random samples were examined virologically, except for fish receiving the pCMV4-G vaccine where all dead fish were assayed for the titer of IHNV present in the internal tissues. Cumulative percent mortality of the replicate tanks from the first challenge and the re-challenged groups was analyzed by analysis of variance (ANOVA) on arcsin $\sqrt{percentage}$ transformed data (Snedecor & Cochran 1967).

Neutralizing antibody. Fish were bled and serum was collected from representative numbers of fish prior to vaccination and from fish at 8 wk post-vaccination just prior to exposure to IHNV. Survivors of the first challenge experiment were also bled for serum collection and marked, with numbered operculum tags, prior to the second challenge. Sera was also collected from the survivors of the second challenge. The presence and titer of neutralizing antibodies was determined using a plaque reduction method (LaPatra et al. 1993). Two-fold serial dilutions of each serum sample in Hanks' balanced salt solution were tested for neutralizing activity by reacting with a standard amount of IHN virus for 1 h at 17°C. Complement in unheated serum collected from disease-free rainbow trout was added to test wells and allowed to incubate an additional 1 h at 17°C. The antibody titer was reported as the reciprocal of the highest dilution that resulted in a 50% reduction in the average number of plaques when compared to the negative controls.

Passive immunization. Serum collected from groups of unhandled, pre-immune, vaccinated and from vaccinated survivors with detectable and non-detectable neutralizing activity was pooled by treatment group. For passive immunization tests, rainbow trout (mean weight, 1 g) were anesthetized and injected intraperitoneally with 50 µl of test or control serum. Groups of 25 fish were challenged in duplicate with 10^4 pfu ml^{-1} by standard procedures 24 h after passive transfer (LaPatra et al. 1994b). At least 20% of the fish that died on any given day were tested for virus. Quantification of virus used in the challenge or isolated from dead fish was performed by plaque assay procedures previously described (LaPatra et al. 1991). The mean cumulative percent mortality of each group was calculated. Comparisons between treatments were made on replicates by ANOVA on transformed data.

RESULTS

First challenge

When fish were challenged 8 wk after vaccination, a significant ($p < 0.05$) level of protection was seen in all groups receiving the vaccine containing the pCMV4-G plasmid (Table 1). The groups of fish injected with pCMVL and PBS also showed some increased but not significant survival when compared to the uninjected groups; however, this survival was considerably less than that observed in the pCMV4-G-vaccinated groups. Among all of the different groups exposed to IHNV, only 5/260 fish receiving the pCMV4-G plasmid died. Losses in the immersion challenged fish began at 7 to 9 d post-exposure, while those in the cohabitation challenged groups began 15 to 21 d post-exposure. Injected fish serving as a source of virus in the cohabitation experiments began dying 4 to 7 d post-injection and the mean time to death for the injected groups was 19 d. Virus was detected in 93% (141/152) of the dead Atlantic salmon examined. Virus concentrations determined on these fish were all $\geq 10^4$ pfu g^{-1} of tissue.

Table 1. *Salmo salar*. Mortality of Atlantic salmon challenged with IHN virus 8 wk post-vaccination. RPS: relative percent survival

Treatment	No. of fish		Percent mortality		Mean days to death		RPS[a]
	Tank 1	Tank 2	Tank 1	Tank 2	Tank 1	Tank 2	
Immersion[b]							
Pre-smolts uninjected	20	20	70	65	22	19	–
Pre-smolts pbs	20	20	35	55	23	18	34
Pre-smolts pCMV-L	20	20	45	55	24	27	26
Pre-smolts pCMV4-G*	20	20	0	5	–	14	96
Cohabitation[c]							
Pre-smolts uninjected	50	50	44	38	43	45	–
Pre-smolts pbs	50	50	20	32	44	40	37
Pre-smolts pCMV-L	50	50	20	32	41	40	37
Pre-smolts pCMV4-G*	50	50	0	0	–	–	100
Immersion							
Smolts uninjected	20	20	50	50	20	21	–
Smolts pbs	20	20	55	45	30	38	0
Smolts pCMV-L	20	20	30	40	24	32	30
Smolts pCMV4-G*	20	20	5	5	20	18	90
Cohabitation							
Smolts uninjected	40	40	28	45	47	45	–
Smolts pbs	40	40	43	28	35	34	3
Smolts pCMV-L	40	40	30	28	35	38	19
Smolts pCMV4-G*	40	40	3	3	33	72	93

[a] $RPS = 1 - \left(\frac{\text{cumulative \% mortality of vaccinated fish}}{\text{cumulative \% mortality of control fish}} \right)$

[b] Fish challenged by immersion were exposed to 5.9×10^3 plaque forming units (pfu) ml^{-1} for 1 h

[c] Fish were challenged by cohabitation by adding 10 fish injected with 4.9×10^3 pfu of IHN virus to each tank

* Significantly different from all other groups ($p < 0.05$)

Second challenge

Surviving fish from the first challenges, both by immersion and cohabitation, were significantly protected compared to naive fish from the original stock when exposed to a high concentration of IHNV by bath. In this study, the fish that were exposed to 5×10^4 pfu ml^{-1} of IHNV for 1 h, a dose that killed 80 to 95% of the control fish, resulted in a loss of 5% or less of the fish vaccinated with pCMV4-G. The losses among the other control groups that had been exposed to IHN virus from either immersion or cohabitation ranged from 0%, in a pCMVL-vaccinated group, to 15% in an uninjected group (Table 2). Virus was detected in 97% (67/70) of the dead Atlantic salmon examined. No virus was detected in 30 fish surviving the second immersion challenge.

Neutralizing antibodies

Specific IHNV neutralizing antibodies were determined from representative fish from each group 8 wk post-vaccination. Only the group injected with the pCMV4-G vaccine developed virus-neutralizing antibody (Table 3). Serum collected from smolts and pre-smolts 8 wk post-immunization with pCMV4-G had a seroprevalence of 44% (4/9) and 27% (4/15) and mean titers of <20 and <10, respectively. After challenge by immersion or cohabitation all groups of pre-smolts and smolts developed a high frequency of seropositive fish. Neutralizing titers were high in the seropositive fish after challenge with over half of the fish having titers of 160 or higher.

Passive immunization

Passive immunization of rainbow trout with serum from Atlantic salmon immunized with pCMV4G provided some protection against IHNV challenge (Table 4). Rainbow trout immunized with serum from Atlantic salmon receiving the glycoprotein vaccine exhibited a cumulative mortality of 30% (15/50) compared to 48 to 60% of the fish receiving serum from unhandled and other control groups. Rainbow trout injected with serum obtained from surviving Atlantic salmon of a cohabitation challenge with IHN virus showed significant ($p < 0.05$) protection. Fish passively immunized with serum from Atlantic salmon exhibit-

Table 2. *Salmo salar*. Mortality of Atlantic salmon survivors re-challenged by immersion in seawater containing 5×10^4 plaque forming units (pfu) ml^{-1} for 1 h, 12 wk after initial challenge. Naive groups were fish from the original stock that had not been previously immunized or challenged. RPS: relative percent survival; ns: not sampled. Number in parentheses in final volumes indicates number of fish

Treatment	No. of deaths/ total no.	Percent mortality	RPS[a]	IHNV neutralization titers of fish that died after re-challenge[b]	
				No. of fish	Antibody titer
Tank 1: Immersion					
Naive	19/20	95	–		–[c]
Pre-smolts uninjected	1/9	11	88	1	≥160
Pre-smolts PBS	2/18	11	88	2	≥160
Pre-smolts pCMV-L	2/17	12	87	2	<20
Pre-smolts pCMV4-G*	0/21	0	100	0	–
Tank 2: Cohabitation					
Naive	36/40	90	–		
Pre-smolts uninjected	6/39	15	83	6	(4) <20, 40, ≥160
Pre-smolts PBS	6/39	15	83	6	<20, 40, (4) ≥160
Pre-smolts pCMV-L	0/40	0	100	0	–
Pre-smolts pCMV4-G*	1/38	3	97	1	20
Tank 3: Immersion					
Naive	16/20	80	–		
Smolts uninjected	1/19	5	94	1	≥160
Smolts PBS	1/16	6	92	1	40
Smolts pCMV-L	0/19	0	100	0	–
Smolts pCMV4-G*	0/21	0	100	0	–
Tank 4: Cohabitation					
Naive	34/40	85	–		
Smolts unhandled	4/39	10	88	4	ns, (3) ≥160
Smolts PBS	4/40	10	88	4	(2) <20, (2) ≥160
Smolts pCMV-L	4/40	10	88	4	(2) 80, (2) ≥160
Smolts pCMV4-G*	2/39	5	94	2	ns, ≥160

[a] $RPS = 1 - \left(\frac{\text{cumulative \% mortality of vaccinated fish}}{\text{cumulative \% mortality of control fish}} \right)$

[b] Neutralizing titers were determined by sampling fish prior to challenge. The titer indicates the reciprocal of the highest dilution that resulted in a 50% reduction in the number of plaques detected in the negative control wells

[c] Antibody titers of 30 naive fish tested prior to challenge were all <20

* Significantly different from all other groups ($p < 0.05$)

ing low neutralizing activity (titer, 20) exhibited a combined mortality of 10% (5/52), whereas those receiving serum with high neutralizing activity (titer ≥160) were completely protected. Among the groups of rainbow trout challenged with IHNV, virus was recovered from 91% (78/86) of the dead fish that were examined. The mean IHNV concentration was $10^{6.7}$ pfu g^{-1} (range $10^{2.9}$ to $>10^{7.3}$) in combined liver-spleen-kidney samples.

DISCUSSION

Viral diseases of fish have to date been controlled by avoidance including the use of virus-free water supplies, broodstock screening and selection, and iodophor disinfection of eggs. In the aquaculture industry, where fish are raised under intensive culture conditions, the opportunity to immunize fish by vaccination has led to the development of several commercially available vaccines. Atlantic salmon introduced to British Columbia in 1985 for aquaculture, now represents the main species of salmon grown by salmon farmers. Atlantic salmon, at all sizes, appear highly susceptible to IHN virus and the industry has experienced severe economic losses. As previously mentioned, numerous IHN injectable vaccines have been developed and tested with limited success, including recently, the use of killed autogenous IHNV vaccine at several net pen sites in the area of British Columbia experiencing the most severe losses to IHN. The promising results of DNA vaccines developed against the rhabdoviruses VHSV and IHNV, when tested in rainbow trout fry, prompted this investigation into the efficacy of the technology when applied to larger Atlantic salmon pre-smolts and smolts.

Table 3. *Salmo salar*. IHN virus neutralizing antibodies present in Atlantic salmon at 8 wk post-vaccination (prior to challenge) and 12 wk after challenge by immersion or cohabitation

Treatment	No. positive/ no. tested	Percent positive	Neutralizing titer mean/range[a]
8 wk post vaccination, prior to challenge			
Uninjected	0/23	0	0
PBS	0/21	0	0
pCMV-L	0/18	0	0
pCMV4-G	8/24	33	11/ 0–80
12 wk post-challenge pre-smolts/immersion challenge			
Pre-smolts uninjected	14/14	100	160/ 160
Pre-smolts PBS	19/21	90	138/ 0–160
Pre-smolts pCMV-L	17/19	89	133/ 0–160
Pre-smolts pCMV4-G	19/22	86	131/ 0-160
12 wk post-challenge pre-smolts/cohabitation challenge			
Pre-smolts uninjected	28/42	66	97/ 0-160
Pre-smolts PBS	29/42	69	95/ 0–160
Pre-smolts pCMV-L	36/42	86	124/ 0–160
Pre-smolts pCMV4-G	37/42	88	104/ 0-160
12 wk post-challenge smolts/immersion challenge			
Smolts unhandled	17/20	85	115/ 0–160
Smolts PBS	17/19	84	136/ 0–160
Smolts pCMV-L	20/22	91	138/ 0–160
Smolts pCMV4-G	19/22	86	118/ 0–160
12 wk post-challenge smolts/cohabitation challenge			
Smolts unhandled	30/42	72	104/ 0–160
Smolts PBS	31/42	73	108/ 0–160
Smolts pCMV-L	35/42	83	117/ 0–160
Smolts pCMV4-G	37/42	88	119/ 0–160

[a]Neutralizing titer indicates the reciprocal of the highest dilution that resulted in a 50% reduction in the number of plaques detected in the negative control wells. Upper detection limits were dilutions of 160; means were calculated using values of 160 when upper detection limits were exceeded

This study demonstrates that a single intramuscular injection of Atlantic salmon with 25 µg DNA vaccine pCMV-G results in significant protection against an IHNV challenge. The quantity of vaccine used and vaccination method was based on *in vivo* challenge studies using rainbow trout (mean weight <15 g) (Anderson et al. 1996b, Lorenzen et al. 1998). The results extend the findings of earlier studies and demonstrate that viral DNA vaccines are effective in larger size fish (70 to 80 g) and that Atlantic salmon, another economically important farmed fish species, can be included in the species that elicit a protective immune response following injection with a DNA vaccine. Recent studies have revealed that DNA vaccine amounts of 1 µg effectively protect rainbow trout against IHNV (Corbeil et al. unpubl.). Thus future work will focus on determining the minimum effective dose needed to confer protection so as to minimize both the potential safety risks and cost associated with the DNA vaccine.

Protection of DNA vaccinated fish was observed when fish were exposed to the virus in high levels for short duration by waterborne challenges or by exposure to low levels of virus over a long period of time by cohabitation with infected fish. The latter challenge method was designed to more accurately simulate the conditions found in sea cages prior to an IHN epizootic. Importantly, we found no significant difference in relative percent survival of fish vaccinated as pre or post-smolts when challenged with the virus in seawater using either method. Thus the physiological stress of smoltification (Maule et al. 1987) appears not to diminish the efficacy of the DNA vaccine.

Fish can be exposed to IHNV throughout their life cycle. Because of this there is concern that DNA vaccinated fish after recovering from an IHN challenge could become subclinical carriers (Amend 1975, Drolet et al. 1994) and thus be a source of IHNV shedding or be more susceptible to IHN upon re-exposure. When survivors of the first challenge were again exposed to virus, the DNA-vaccinated groups were protected to the same degree as mock vaccinated virus-exposed Atlantic salmon. We tested survivors of the second challenge from all treatment groups,

Table 4. *Oncorhynchus mykiss*. Mortality of rainbow trout fry after passive immunization with 50 µl of Atlantic salmon sera from groups after vaccination and after surviving an IHNV cohabitation challenge. Replicate groups of 25 fish (mean weight, 1 g) were challenged with 10^4 plaque forming units ml^{-1} IHNV for 1 h

Treatment	Cumulative mortality Tank 1	Tank 2	Mean percent mortality	Pooled sera titer
Unhandled	12/25	12/25	48	<20
Pre-immune serum	12/26	13/24	50	<20
PBS	11/23	16/24	57	<20
pCMV-L	14/26	16/24	60	<20
pCMV4-G	9/25	6/25	30	<20
Low titer survivors*	3/26	2/26	10	20
High titer survivors*	0/23	0/25	0	160

*Significantly different from all other groups ($p < 0.05$)

including the DNA-vaccinated fish, for virus by standard cell culture methods and found no evidence of infectious virus (data not shown). It is possible that the use of more sensitive IHNV detection methods would reveal the presence of latent viral infection (Drolet et al. 1995). However, given that infectious virus was not present in the DNA vaccinated survivors and that they were also significantly protected upon re-exposure to IHNV, there is reason to believe that the DNA vaccine did not result in establishment of a carrier state.

The IHNV G protein has been shown to be the only viral protein capable of eliciting neutralizing antibodies and stimulating protective immunity in young fish (Engleking & Leong 1989). However, in recent years there appears to be increasing serological variation among IHNV isolates (LaPatra et al. 1994a). The pCMV-G vaccine used in this study has the G gene from a 1976 isolate obtained from steelhead *Oncorhynchus mykiss* from the Deschutes River in Oregon, USA. Although this isolate is temporally and geographically distinct from the more recent isolate obtained from large sea pen-reared Atlantic salmon, the vaccine was still able to confer significant protection. This supports previous results that indicated that fish serum with neutralizing activity that protected against one antigentic variant of IHNV would cross-protect against the antigenic variants and that a vaccine against a single type of IHNV may be efficacious against all IHNV isolates (LaPatra et al. 1994a).

In this study, there was a slight trend in fish vaccinated with pCMV-G to seroconvert at a greater prevalence after being challenged with IHNV compared to the other treatment groups. Although this observation was not consistent in the 4 challenges that evaluated pre-smolts and smolts by immersion and cohabitation challenges, it did suggest that the pCMV-G vaccine may potentially enhance seroconversion. Similar results have been observed using other DNA vaccine model systems including vaccines for lyssaviruses (Bahloul et al. 1997), avian influenza (Fynan et al. 1993) bovine herpesvirus 1 (Cox et al. 1993), and human immumodeficiency virus type 1 (Wang 1993). In these studies it was shown that the DNA vaccine primed an antibody response that was dose and booster dependent. Further, in the absence of neutralizing antibody the challenge virus was responsible for complete seroconversion. If a similar mechanism occurs with pCMV-G vaccinated fish, it will be interesting to determine the rate at which seroconversion occurs following virus exposure. The mechanism would require rapid priming and seroconversion since IHNV results in systemic infection that leads to mortality as early as 5 d after viral exposure (Drolet et al. 1995). The low seroconversion but significant immunoprotection could also indicate that the DNA vaccine elicited antigen-specific cellular immunity or non-specific anti-viral factors that were responsible for conferring protection at early stages of viral infection (Bahloul et al. 1997, Krieg et al. 1998, Whitton et al. 1999).

Previous studies have also shown that passive transfer of serum from juvenile or adult rainbow trout and sockeye salmon with detectable IHNV neutralization activity and titers as low as 20 provides significant protection for rainbow trout against waterborne challenges compared to that of serum from naive groups injected with saline or from fish left unhandled (LaPatra et al. 1993, 1994b, Traxler et al. 1997). Pooling of sera from all 24 pre-smolts and smolts sampled at 8 wk post-immunization with pCMV-G, so that a large volume of serum for the passive transfer studies could be attained, resulted in a neutralization titer below detectable limits (<20). However, relative survivals of 38 to 50% were consistently observed compared to control groups. Possible explanations include the presence of virus-neutralizing activity below detectable limits and/or the presence of other factors within the serum that enhanced or provided for a protective immune response (Bachman et al. 1997). Although the overall seroprevalence in pCMV-G vaccinated fish prior to challenge was only 33% with a mean titer of approximately 10, significant protection was observed and almost 90% of the surviving fish seroconverted. Future studies will attempt to define the mechanism of protection provided by this DNA vaccine for enhancement of the efficacy of this potent biologic.

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