MARINE BIOTECHNOLOGY

Analysis of DNA-Vaccinated Fish Reveals Viral Antigen in Muscle, Kidney and Thymus, and Transient Histopathologic Changes

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Abstract

A highly efficacious DNA vaccine against a fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV), was used in a systematic study to analyze vaccine tissue distribution, persistence, expression patterns, and histopathologic effects. Vaccine plasmid pIHNw-G, containing the gene for the viral glycoprotein, was detected immediately after intramuscular injection in all tissues analyzed, including blood, but at later time points was found primarily in muscle tissue, where it persisted to 90 days. Glycoprotein expression was detected in muscle, kidney, and thymus tissues, with levels peaking at 14 days and becoming undetectable by 28 days. Histologic examination revealed no vaccine-specific pathologic changes at the standard effective dose of 0.1 μg DNA per fish, but at a high dose of 50 μg an increased inflammatory response was evident. Transient damage associated with needle injection was localized in muscle tissue, but by 90 days after vaccination no damage was detected in any tissue, indicating the vaccine to be safe and well tolerated.

Key words: Fish DNA vaccine — infectious hematopoietic necrosis virus (IHNV) — biodistribution — persistence — histopathologic effects — vaccine safety

Introduction

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus that infects salmon and trout and causes substantial economic loss to aquaculture and resource hatcheries in North America. A considerable amount of research has been conducted to develop an effective vaccine for control of this pathogen (reviewed in Leong and Fryer, 1993; Winton, 1997). Despite these efforts, no commercial vaccines against this virus are available today. However, one approach that has proved highly protective against IHNV is the use of DNA vaccines.

The first application of a DNA vaccine against IHNV was reported by Anderson et al. (1996a). Since then, a modified version of this vaccine has been developed containing the glycoprotein gene of the Western Regional Aquaculture Consortium (WRAC) reference strain of IHNV (Corbeil et al., 1999). In an initial minimum dose study, this vaccine, denoted pIHNw-G, showed nearly complete protection in rainbow trout fry (1.0–2.0 g) vaccinated with a single dose of as little as 10 ng, and a standard effective dose of 0.1 μg per fish was selected for further work (Corbeil et al., 2000b). The exceptional efficacy of pIHNw-G at this low 0.1-μg dose has been well documented in numerous trials under various conditions including different IHNV challenge strains and different host species (Corbeil et al., 2000b; Garver et al., 2004). Additionally, the frequency and strength of the humoral immune response to pIHNw-G vaccination has been well characterized, both in rainbow trout fry (Corbeil et al., 2000a, 2000b) and subadults (LaPatra et al., 2000), and in sockeye and Chinook salmon fry (Garver et al., 2004).

A DNA vaccine approach has also proved effective against another aquatic rhabdovirus, viral hemorrhagic septicemia virus (VHSV). N. Lorenzen et al. (1999) used a DNA vaccine based on the glycoprotein gene of VHSV to protect rainbow trout against lethal infection with VHSV. The protection against fish rhabdovirus challenge elicited by the IHN and VHS DNA vaccines has been shown not

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only to be long lasting (Corbeil et al., 1999; E. Lorenzen et al., 2000), but also to be established as early as 4 to 7 days after vaccination (LaPatra et al., 2001; N. Lorenzen et al., 2002). Furthermore, at early time points after vaccination these vaccines are cross-protective in that the IHNV DNA vaccine protects against VHSV challenge (LaPatra et al., 2001) and vice versa (N. Lorenzen et al., 2002). However, with both IHNV and VHSV DNA vaccines, the nature of protection at later time points switches from cross-protective to highly specific (LaPatra et al., 2001; N. Lorenzen et al., 2002)

Although the efficacy of the rhabdoviral DNA vaccines has been well established and the humoral responses have been characterized, little is known about the fate of the plasmid DNA vaccine following intramuscular injection in fish. Studies of the VHSV DNA vaccine detected plasmid and the expressed glycoprotein in the muscle at the site of inoculation (Boudinot et al., 1998; N. Lorenzen et al., 1998). These reports indicate the importance of the muscle as a site of plasmid uptake and expression, but they did not investigate the role of other fish tissues in DNA immunization. Therefore the extent to which the plasmid vaccine and its expressed antigen are distributed throughout fish tissues remains largely unknown.

In this report a detailed and systematic evaluation was performed to investigate the fate of intramuscularly administered plasmid DNA expressing the glycoprotein gene of IHNV. The trafficking of DNA after administration was assessed by polymerase chain reaction (PCR) in different tissues and at various time points from 10 minutes to 90 days after vaccination. Additionally, in similar tissues and at similar time points, glycoprotein expression was evaluated by immunohistochemistry, and histopathology was used to assess any pathologic effects of the intramuscularly administered plasmid in order to address important vaccine safety issues.

Materials and Methods

Plasmid Constructs. The plasmid constructs of the IHNV DNA vaccine (pIHNw-G) and the luciferase control vaccine (pLuc) have been described previously (Corbeil et al., 1999, 2000b). Briefly, to construct plasmid pIHNw-G, the full-length G gene of IHNV was placed downstream of the immediate-early enhancer-promoter sequences of human cytomegalovirus (CMV) of pCDNA 3.1 vector (Invitrogen). The G gene sequence in pIHNw-G was from the WRAC strain, also referred to as isolate 039-82, ATCC VR-1392 (LaPatra et al., 1994; Morzunov et al., 1995). For plasmid pLuc, the luciferase gene from the pGL3 vector (Promega) was cloned into the pCDNA 3.1 vector. All constructs were amplified in *Escherichia coli* strain DH5α cells grown in Luria broth supplemented with 100 μg/ml ampicillin. Subsequently, plasmid DNA was purified to produce high-quality DNA for use as a vaccine following the ammonium acetate protocol of Saporito-Irwin et al. (1997). Plasmid DNA was resuspended in Tris-EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and stored at −20°C until use. All plasmid DNA used had a DNA-to-protein ratio (A_{260}/A_{280}) of 1.8:2.0.

Fish husbandry. Specific-pathogen-free rainbow trout *Oncorhynchus mykiss* (mean weight, 0.3 g; provided by Clear Springs Foods) were maintained at the Western Fisheries Research Center in a 120-L circular tank filled with 15°C sand-filtered and UV-treated lake water at a flow of 3.78 L/min. Fish were reared for 2 months before being used in experiments and fed daily at 1.5% of their body weight, a semi-moist pelleted diet 1.0 mm in size (Bioproducts).

Fish Vaccination by Intramuscular Injection. For vaccination, trout were first anesthetized by immersion in a 100 μg/ml solution of tricaine methane sulfonate (MS-222; Argent Chemical Laboratories). The specified dose of IHNV DNA vaccine or control vaccine in 50 μl Tris-EDTA buffer (TE) was injected into the left epaxial muscle below the dorsal fin with a 27G 3/4-inch needle.

Distribution and Persistence of pIHNw-G DNA in Fish. To assess the tissue distribution and persistence of pIHNw-G DNA after vaccination, 178 rainbow trout (mean weight, 2.5 g) were each injected with 0.1 μg of pIHNw-G. A second group of 178 uninjected rainbow trout served as controls.

Tissue Collection and DNA Extraction. Multiple tissues including thymus, kidney (posterior and anterior), liver, spleen, gill, heart, intestine, pancreas, pyloric ceca, left epaxial muscle (surrounding the injection site), and right epaxial muscle (opposite the injection entry site) were dissected at 15 minutes and 1, 2, 7, 14, 28, and 90 days after vaccination from 5 fish each in the vaccinated and control groups. To avoid cross-contamination during collection of tissues, a clean set of instruments was used for each fish dissected. Different instruments were used for each tissue or tissue pool. Direct contact between fingers and any tissues to be excised was avoided, and the injection site muscle was removed last to prevent contamination of other tissues. The muscle tissue samples were assayed separately, while the kidney, liver, and spleen from a

given fish were analyzed as a pooled sample. Likewise, the gill, heart, intestine, pancreas, and pyloric ceca were analyzed as a pooled sample. Tissue pools or muscle samples ranged in weight from 30 to 100 mg. All tissues were collected into microfuge tubes, frozen in liquid nitrogen, and stored at −70°C until DNA isolation. Tissues were placed into tubes containing a 1/4-inch ceramic sphere and garnet matrix and subsequently homogenized using the FastPrep instrument (Bio101 Systems). DNA was extracted from homogenates using the FastDNA Kit (Bio 101 Systems).

Blood was collected from 5 or 6 fish each from the pIHNw-G-injected group and from the control group at 1 and 15 minutes, 6 and 12 hours, and 1, 2, and 7 days after vaccination. Fish were bled from the caudal vein into 1.5-ml microfuge tubes stored on ice. Total DNA, including both plasmid and cellular DNA, was extracted from the blood immediately upon collection using the DNeasy tissue kit (Qiagen) following the protocol for the isolation of DNA from whole nucleated blood as provided by the manufacturer.

PCR Analysis. DNA isolated from fish tissue samples taken at each time point was assayed by nested PCR for the presence of plasmid pIHNw-G. The nested PCR analysis was performed using two primer pair sets that are specific for IHNV glycoprotein sequences. The first-round PCR primer sequence was (forward) 5′-AGAGATCCCTACACCAGAGAC-3′ (reverse) 5′-GGTGGTGTTGTTTCCGTGCAA; and the second-round sequence was (forward) 5′-TCACCCTGCCA GACTCATTGG-3′ (reverse) 5′-ATAGATGGAGCCTTTGTG CAT-3′. The first set of primers amplifies a 692-bp fragment in the middle of the glycoprotein gene that extends from base 3515 to 4207 (sequence numbering as in GenBank L40883). The second set of primers amplifies a 482-bp fragment that extends from base 3575 to 4057 of the glycoprotein gene. Each PCR contained 4 μl of sample DNA combined with 1 μl of 20 pmol/μl of each first-round primer, 5 μl of 25 mM $MgCl_2$, 5 μl of 10× PCR buffer (Promega), 1 μl of dNTPs (10 mM each), and 2.5 U *Taq* to produce a 50-μl reaction. Two microliters of the first-round PCR was then utilized as template for the second-round PCR. Both PCR amplifications were performed with a MJ Research PTC-100 thermocycler programmed with the following conditions: 95°C for 2 min, 30 amplification cycles (95°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute), and 72°C for 7 minutes. Included in every set of PCRs was a ''no DNA'' control reaction to verify the absence of reagent contamination. Second-round PCR products were analyzed by agarose gel electophoresis in the presence of ethidium bromide. The products of amplification were visualized with a UV transilluminator. A 482-bp band indicated the presence of pIHNw-G in a tissue sample.

Sensitivity of the PCR Assay. To determine the sensitivity of the PCR procedure, 10-fold dilutions of purified pIHNw-G ranging from 0.1 to 120,000 copies (1 ag to 1 pg) were added to PCR mixtures directly or used to spike unvaccinated control fish tissues. Tissues that were spiked with vaccine were then homogenized, and total DNA was extracted and analyzed by PCR as previously described. The limit of sensitivity was determined for each different tissue or tissue pool type, as well as for reactions containing only purified pIHNw-G DNA. All sensitivity limits were validated by at least 3 independent experiments.

Tissue Distribution of IHNV G Protein and Histopathology. Rainbow trout fry (mean weight, 2.5 g) were separated into 4 treatment groups of 50 fish each: (1) fish injected with 0.1 μg of pIHNw-G; (2) fish injected with 0.1 μg of pLuc; (3) fish injected with Tris-EDTA buffer; and (4) noninjected fish. Fish were vaccinated as described above. Five fish were sampled from each group immediately after vaccination and at 1, 2, 7, 14, 28, and 90 days after vaccination. In a separate higher vaccine dose experiment, the distribution of G protein was investigated in 26 rainbow trout vaccinated with 50 μg of pIHNw-G and in 23 fish vaccinated with 50 μg of pLuc. A minimum of 3 fish were sampled per group at 0, 1, 2, 7, 14, and 28 days after vaccination. On the basis of the results of this experiment, an additional 20 fish were vaccinated with 50 μg of pIHNw-G and sampled 14 days after vaccination.

At each sampling time point, fish were euthanized by an overdose of MS-222, opened ventrally to expose the internal organs, and then fixed in 10% neutral buffered formalin. After fixation, the gill, thymus, liver, heart, spleen, kidney, skin, and epaxial muscle surrounding the injection site, skin and epaxial muscle opposite the injection site, pyloric ceca, pancreas, upper intestine, and lower intestine were collected and subjected to routine tissue processing and paraffin embedding. Serial 5-μm transverse sections were taken from each tissue except the gill, thymus, liver, heart, spleen, and kidney, which were sectioned longitudinally along the frontal plane, collected on positively charged glass slides, and stored at 4°C. For routine histopathologic examination, tissue sections were deparaffinized in Hemo-De xylene substitute (Scientific Safety Solvents), rehydrated through graded

alcohol, and stained with Gill's hematoxylin and eosin.

Immunohistochemical staining was performed on deparaffinized, rehydrated tissue sections that were enzymatically digested in 0.05% protease XIV (Sigma Chemical Company) diluted with 0.05 M Tris buffered saline (TBS, pH 7.4). Sections were incubated at 37°C for 15 minutes followed by immersion in 4°C TBS. Immunostaining was performed according to the manufacturer's instructions supplied with the EnVision+ polymer-based, biotin-free detection kit (Dako Corporation). Briefly, endogenous peroxidase blocking solution (0.03% hydrogen peroxide) was applied to sections, which were incubated for 5 minutes, then rinsed thoroughly with TBS. Tissue sections were then incubated for 40 minutes in a 1:100 dilution of 3 pooled mouse monoclonal antibodies (IH8, 5A6, and 6A7; Huang, 1994; Huang, 1996) directed against the G protein of the WRAC strain of IHNV. A horseradish peroxidase-labeled goat antimouse immunoglobin secondary antibody was then applied and incubated for 40 minutes. The reaction products were subsequently visualized with 3-amino-9-ethylcarbazole (AEC) and counterstained with Mayer's hematoxylin.

Incubations were performed at room temperature in a humidified chamber unless otherwise noted, and each incubation was followed by 2 rinses in TBS. Tissues taken from rainbow trout infected with IHNV were used as positive controls. For negative controls, nonimmune mouse serum was substituted for the primary antibody.

Results

Determination of Sensitivity Limits of PCR Assays. In our PCR assays we consistently detected fewer than 10 copies of plasmid DNA per reaction when analyzing purified pIHNw-G. However, for the purpose of this study, the more relevant sensitivity measure was the limit of plasmid detected in the presence of tissue samples. PCR analyses of DNA from tissue samples spiked with pIHNw-G rarely detected fewer than 40 copies of plasmid DNA per reaction. Inconsistency in the limit of detection was demonstrated with control reactions in which single 150 μl PCR mixtures, spiked with the equivalent of 4, 40, 400, 4000, or 40,000 copies of plasmid pIHNw-G per 50 μl volume, were aliquoted into 50 μl volumes for PCR, and resulted in nonequivalent amplification among 3 replicates (data not shown). Such inconsistency has been reported by others (Martin et al., 1999), and should be considered when defining the limit of sensitivity. Therefore we used as a conservative measure the quantity of plasmid that was reproducibly detected in 3 out of 3 PCR reactions for each tissue type. For muscle tissue and blood, this was 48 copies (10 fg) and 75 copies (15.6 fg), respectively, while the conservative detection limit in either of the tissue pools (kidney, liver, and spleen or gill, intestine, pyloric ceca, and heart) was 240 copies (50 fg) per PCR reaction.

Distribution and Persistence of pIHNw-G DNA in Fish. To determine the fate of pIHNw-G plasmid DNA in fish, a tissue distribution study employing nested PCR analysis was conducted following a single intramuscular injection. The intramuscular route is the most commonly used efficacious route of administration (Corbeil et al., 2000a). For this study two experimental groups were evaluated: one vaccinated with pIHNw-G and the other an unvaccinated negative control group. An equal number of tissues from both groups were processed and analyzed at the same time. All tissues analyzed from the unvaccinated group were found to be free of plasmid as determined by the absence of any amplification products, thereby validating that the tissue collection and PCR analysis was free of contamination. To verify that the PCR analysis was able to detect plasmid DNA, a positive control of spiked tissue was included in each assay.

Immediately after intramuscular injection (1–15 minutes), pIHNw-G plasmid DNA was detected in all tissues analyzed, including blood (Table 1). At this early time plasmid DNA was detected in all fish in the injection site (left side) muscle, and in the blood. Plasmid DNA was also detected, albeit less frequently, in the right side muscle opposite the injection site and in the two tissue pools. In the blood the prevalence of plasmid DNA was 100% up to 6 hours after injection, and it then decreased at 12 and 24 hours. No plasmid was detectable in blood of any fish tested 2 or 7 days after vaccination.

Plasmid DNA detection in other tissues was largely limited to muscle by 1 to 2 days after vaccination. In the injection site (left side) muscle, plasmid DNA persisted at a high prevalence throughout the 90-day period examined. Muscle opposite the injection site (right side) also had detectable DNA in 1 to 2 out of 5 fish throughout the 90-day study. In contrast, the two tissue pools had only sporadic positives from 1 to 7 days, and no plasmid was detected in any fish sampled 14, 28, or 90 days after vaccination (Table 1).

Glycoprotein Detection. In the first experiment the presence of the IHNV glycoprotein (G) encoded by pIHNw-G was evaluated in 12 different tissues of

Table 1. Summary of pIHNw-G Plasmid DNA Distribution in Vaccinated Rainbow Trout Fry[a]

	Time point									
Tissue samples	*1 min*	*15 min*	*6 h*	*12 h*	*1 d*	*2 d*	*7 d*	*14 d*	*28 d*	*90d*
Injection site muscle (left side)	ND	5/5	ND	ND	5/5	5/5	4/5	5/5	5/5	4/5
Opposite injection site muscle (right side)	ND	2/5	ND	ND	1/5	1/5	1/5	1/5	2/5	1/5
Spleen, liver, kidney	ND	3/5	ND	ND	2/5	2/5	0/5	0/5	0/5	0/5
Gill, intestine, pyloric ceca, pancreas, heart	ND	3/5	ND	ND	0/5	0/5	1/5	0/5	0/5	0/5
Blood	6/6	5/5	5/5	4/5	2/5	0/5	0/6	ND	ND	ND

[a]All unvaccinated fish tissues at all time points were negative. ND indicates not determined.

each fish at various time points following intramuscular injection of 0.1 μg of pIHNw-G. At this low vaccine dose, G-protein-specific staining by immunohistochemistry revealed only one positive detection out of 420 tissue samples representing 35 pIHNw-G-vaccinated fish. The single positive result occurred 1 day after vaccination in the epithelium of a few renal proximal tubules of the posterior kidney of a fish in the pIHNw-G-vaccinated group (Figure 1A). All negative control fish were negative for immunohistochemical staining in all tissues analyzed.

Owing to the low level of detection of glycoprotein in the fish vaccinated with 0.1 μg pIHNw-G, a second experiment was conducted using a higher dose of 50 μg pIHNw-G per fish. At the higher vaccine dose, glycoprotein expression was much more readily observed, with the first detection occurring at 7 days after vaccination in the muscle tissue at the site of injection (Table 2). At 14 days, glycoprotein was evident in muscle tissue from both the left and right sides of the majority of fish sampled (Table 2; Figure 1B). By 28 days, glycoprotein expression was not detected in any fish.

To expand on these results, a third experiment was conducted focusing on the day 14 time point with multiple tissues from 20 fish (Table 2). Analysis showed that glycoprotein was detected at a high rate (65%) in the muscle on the left side at the site of injection as well as in the muscle on the right side opposite the injection entry point (75%). Other tissues that stained positive for glycoprotein were the thymus (Figure 1C) and posterior kidney in 50% of the fish and in the anterior kidney in 15% of the fish (Figure 1D). Fish in which glycoprotein was detected in the anterior kidney also showed positively stained cells in the thymus.

In the muscle the glycoprotein staining occurred peripherally in some cells and within the sarcoplasm in others (Figure 1B), and was visible in both red (slow or steady-swimming oxidative) muscle and white (fast or burst-swimming glycolytic) muscle. The staining observed in the thymus was localized in the inner zone, where cells are predominantly large immature lymphocytes and lymphoblasts, but phagocytic cells also occur (Figure 1C). In the anterior kidney the glycoprotein staining was frequently observed in the cytoplasm of cells associated with melanomacrophage aggregates (Figure 1D). The specific staining observed in the posterior kidney largely occurred in the cytoplasm of the proximal renal tubule epithelium (Figure 1A).

Histopathology. Histopathologic analyses of fish that were injected intramuscularly with 0.1 μg of pIHNw-G, 0.1 μg of pLuc, or TE buffer revealed lesions only in tissues surrounding the injection sites (Table 3). No abnormalities associated with vaccination or control injections were observed at any sample time in the gill, thymus, liver, heart, spleen, kidney, gonad, pancreas, or gastrointestinal tract tissues of these fish, and no lesions were observed in noninjected fish. Histopathologic changes in injected fish encompassed an area that included the needle track and adjacent tissues on the left side of the fish where the needle was inserted, and frequently extended into the epaxial muscle on the right side of the fish where some of the injected material would have been discharged. In some fish the injection track was not apparent, but the lack of histopathologic changes in these fish may have represented a plane-of-section artifact rather than the absence of a response of the fish to injection.

Early changes around the injection site, including hemorrhage, tissue compression, degeneration and necrosis of muscle cells, and infiltration of leukocytes, were observed in red and white muscle tissues of some fish in all injected groups within 1 day after vaccination (Table 3; Figure 2). Areas of inflammation associated with injection often extended into the connective tissue myosepta between myomeres, and in a few fish inflammation was observed in epidermis and dermis of the skin, or surrounding the nervous tissue near the spinal cord or lateral line. Inflammation persisted in the majority of fish examined in all injected groups up to 28 days after injection (Figure 3).

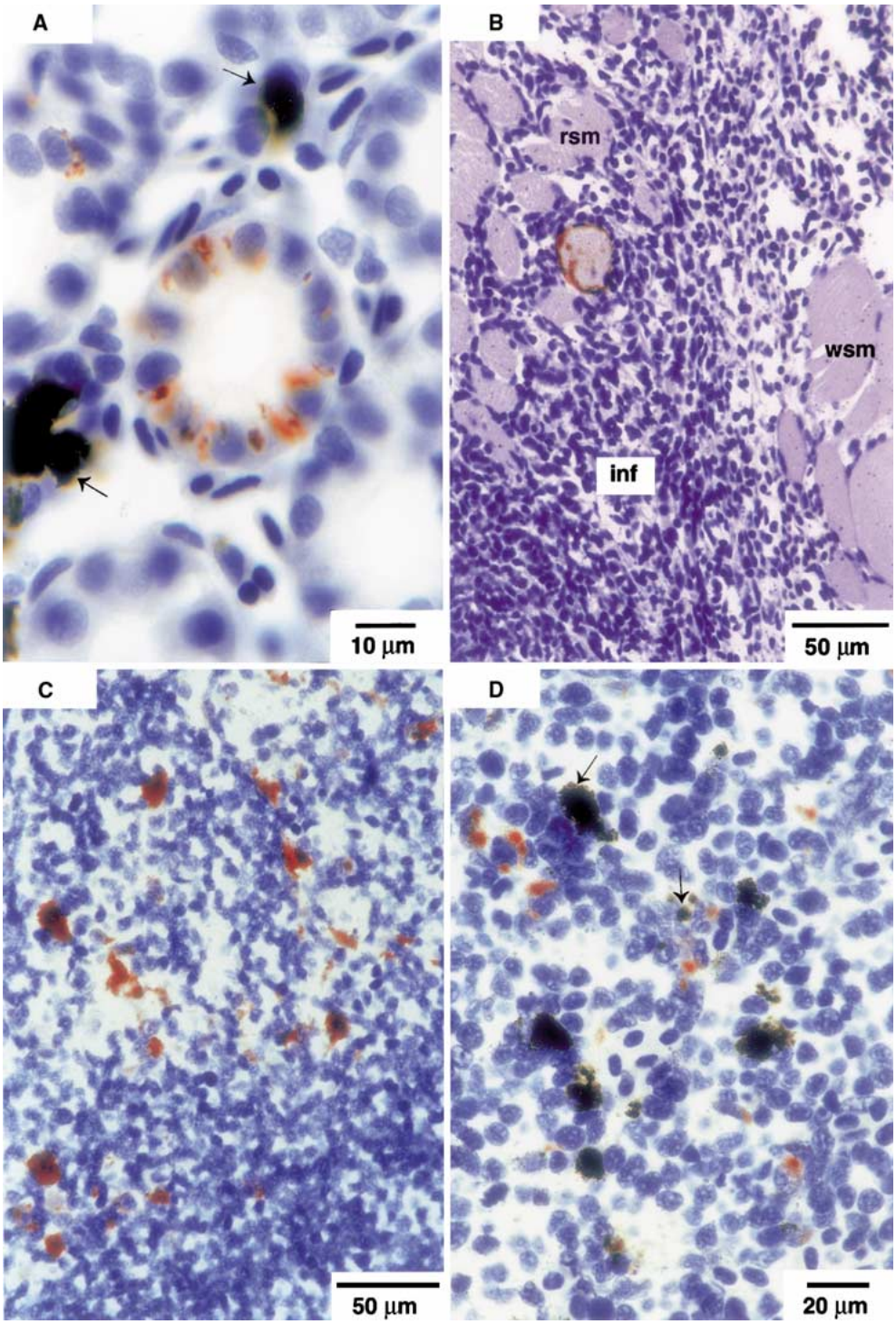

Fig. 1. Immunohistochemical staining for IHNV G protein in rainbow trout vaccinated with pIHNw-G plasmid DNA; cells staining positive for IHNV G protein show red-orange coloration. **A:** Positive staining for IHNV G protein in the cytoplasm of proximal renal tubule epithelial cells 1 day after vaccination in a fish vaccinated with 0.1 μg pIHNw-G. Brown-black material (arrows) is melanin granules in melanomacrophages and does not represent positive immunohistochemical staining. **B:** Positive staining for IHNV G protein visible both peripherally and within the sarcoplasm of a red (oxidative) muscle cell of a fish 14 days after vaccination with 50 μg pIHNw-G. Both red (rsm) and white glycolytic (wsm) skeletal muscle cells are visible in the injection site, surrounded by a marked infiltrate of inflammatory cells (inf) **C:** Positive staining for IHNV G protein in the inner zone of the thymus of a fish 14 days after vaccination with 50 μg pIHNw-G. **D:** Positive staining for IHNV G protein in the anterior (hematopoietic) kidney of a fish 14 days after vaccination with 50 μg pIHNw-G. Cells staining positive for IHNV G protein were frequently located in areas of melanomacrophage aggregates (arrows).

Table 2. Immunohistochemical Distribution of IHNV G Protein in Rainbow Trout Vaccinated with 50 μg pIHNw-G[a]

	Sample time points (after vaccination)		
	Experiment 2[b]		*Experiment 3*[c]
Tissue samples	*7 d*	*14 d*	*14 d*
Epaxial muscle, injection site (left side)[d]	2/3	3/3	13/20
Epaxial muscle, opposite side (right side)[e]	0/3	2/3	15/20
Kidney, posterior, proximal tubule epithelial cells[f]	0/3	0/3	10/20
Kidney, anterior[g]	0/3	0/3	3/20
Thymus[g]	0/3	0/3	10/20
Spleen, liver, kidney	0/3	0/3	0/20
Gill, pyloric ceca, pancreas	0/3	0/3	0/20
Upper intestine, lower intestine	0/3	0/3	0/20

[a]Rainbow trout fry (mean weight, 2.5 g) were vaccinated intramuscularly with 50 μg of either pIHNw-G (treatment group) or pLuc (control group). G-protein-specific staining was not detected in tissues collected 0, 1, 2, and 28 days after vaccination with pIHNw-G or at any time point in the control group. Values are presented as number of positive fish per total examined.
[b]Fish were vaccinated as described above, and a minimum of 3 fish per treatment were sampled at 0, 1, 2, 7, 14, and 28 days after vaccination.
[c]After Experiment 2, an additional 20 fish were vaccinated with 50 μg pIHNw-G and sampled at the 14-day time point.
[d]Mean no. of positive muscle cells per fish (±SD) sampled at 7 and 14 days (Exp. 2) and 14 days (Exp. 3), respectively: 5 (± 3), 6 (± 6), and 3 (± 2).
[e]Mean no. of positive muscle cells per fish (± SD) sampled at 14 days (Exp. 2 and 3, respectively): 4 (± 4) and 3 (± 2).
[f]Mean no. of positive proximal tubules per total proximal tubules (± SD): 20 (± 18)/606(± 136).
[g]Mean no. of positive cells per fish (± SD) in anterior kidney and thymus, respectively: 55 (± 14) and 7 (± 4).

Polymorphonuclear leukocytes were the predominant cells in the inflammatory infiltrate during the early inflammatory response (1–7 days postinjection; Figure 2), whereas macrophages predominated during the later phase of inflammation (14–28 days postinjection; Figure 3). In addition, lymphocytes were frequently present in the inflammatory foci at all stages of the inflammatory process. Macrophages (and occasionally polymorphonuclear leukocytes) were often observed within degenerating and necrotic muscle bundles (Figure 3), and fibroblasts became prevalent in the surrounding connective tissue within 14 days after injection. By 14 and 28 days after injection, regeneration of skeletal muscle in the injection track was evidenced by the presence of small-diameter muscle fibers (Figure 4). By 90 days after injection, the skeletal muscle regeneration appeared complete as no injection track could be discerned in any fish. In general, the observed histopathologic changes occurred equivalently in the 3 injected treatment groups, such that no changes specifically associated with delivery of the pIHNw-G vaccine could be identified at the 0.1 μg dose.

Among fish injected with the high dose of 50 μg of pIHNw-G or 50 μg of pLuc, histopathologic changes in the injection site muscle were examined at time points up to 28 days after vaccination (Table 4). The sequence and timing of histopathologic changes in the integument and skeletal musculature were similar to those observed in fish injected with 0.1 μg of pIHNw-G or 0.1 μg of pLuc. However, the magnitude of the inflammatory response, as evidenced by the infiltration of numerous leukocytes into the injection area, was particularly pronounced 14 days after vaccination in fish injected with the high-dose pIHNw-G (Figures 1 B, and 5). A moderate to severe inflammatory response was observed at this time point in fish injected with 50 μg of pIHNw-G, in comparison with a mild to moderate inflammatory response observed at this same time point in fish injected with either 50 μg of pLuc, 0.1 μg of pIHNw-G, or 0.1 μg of pLuc. By 28 days after injection, the inflammatory response had subsided to a minimal to moderate level in all fish injected with either the low or high dose of the test or control vaccine (Figure 4). A foreign-body-type response characterized by the presence of multinucleate giant cells was evident in some fish injected with the high dose of pIHNw-G at 14 and 28 days after injection, and in some fish injected with pLuc at 28 days (Table 4; Figure 6). These cells were not observed in fish injected with 0.1 μg of either pIHNw-G or pLuc.

Discussion

This study was conducted to analyze tissue distribution, persistence, expression patterns, and histopathologic effects of the pIHNw-G vaccine. The results of this study revealed that immediately after injection, plasmid DNA was distributed throughout multiple fish tissues, whereas at later time points DNA persisted only within muscle tissue. The finding that plasmid DNA pIHNw-G was rapidly

Table 3. Histopathological Changes Observed in Epaxial Muscle Tissue of 2.5 Rainbow Trout Vaccinated Intramuscularly with 0.1 μg of pIHNw-G or 0.1 μg of Control Vaccine pLuc, Mock-Vaccinated with TE Buffer, or Unvaccinated (Not Injected)[a]

Injection group by days after vaccination	*Hemorrhage*	*Muscle degeneration/ necrosis*	*Inflammation*	*Muscle regeneration*
Time 0				
pIHNw-G	2	0	0	0
pLuc	0	0	0	0
TE buffer	0	0	0	0
Not injected	0	0	0	0
1 Day				
pIHNw-G	4	3	2	0
pLuc	3	3	1	0
TE buffer	5	5	3	0
Not injected	0	0	0	0
2 Days				
pIHNw-G	4	4	4	0
pLuc	3	3	1	0
TE buffer	2	3	3	0
Not injected	0	0	0	0
7 Days				
pIHNw-G	5	5	5	0
pLuc	5	5	3	0
TE buffer	5	5	5	0
Not injected	0	0	0	0
14 Days				
pIHNw-G	1	1	3	1
pLuc	2	0	3	2
TE buffer	3	0	5	3
Not injected	0	0	0	0
28 Days				
pIHNw-G	0	1	5	3
pLuc	0	0	3	4
TE buffer	0	0	3	4
Not injected	0	0	0	0
90 Days				
pIHNw-G	0	0	0	0
pLuc	0	0	0	0
TE buffer	0	0	0	0
Not injected	0	0	0	0

[a]Values are presented as number of positive fish (of 5 fish examined per group).

detected systemically and later found primarily at the injection site is consistent with other reports of direct introduction of DNA by intramuscular injection of mice (Parker et al., 1999) and sheep (Mena et al., 2001). Therefore it is conceivable that the mechanism of plasmid dispersal is similar for fish and mammals. Studies with mice, rabbits, and sheep have indicated that the circulatory system is a possible route for the dispersal of plasmid DNA after intramuscular vaccination (Parker et al., 1999; Mena et al., 2001). Likewise, the dispersal of DNA plasmids in fish may well occur via the blood. In our study, plasmid DNA was detected in the blood immediately (1 minute) after injection and persisted up to 1 day, indicating this as a means of distributing plasmid to distal tissues. Alternatively, the initial systemic dispersal of the DNA vaccine may be a consequence of the injection procedure. Because a relatively large volume (50 μl) of vaccine was delivered into the small fish (2.5 g) in these experiments, the vaccine may have been perfused into the bloodstream and all tissues.

Plasmid DNA persisted in the muscle tissue as long as 90 days (the last sampling time); however, in all other tissues analyzed no plasmid DNA was detected beyond 7 days after vaccination with the 0.1-μg vaccine dose, suggesting that the plasmid was either absent or below the detection limit of 240 copies. The persistence of plasmid in various tissues is undoubtedly dependent on the route of administration, nature of the expressed antigen, and vaccine dose (Lee et al., 2000; Rahman and Maclean, 1992; Sudha et al., 2001). It may be that DNA persisted longer in tissues other than muscle after the high 50-μg vaccine dose used to facilitate detection of G protein, but this was not assessed.

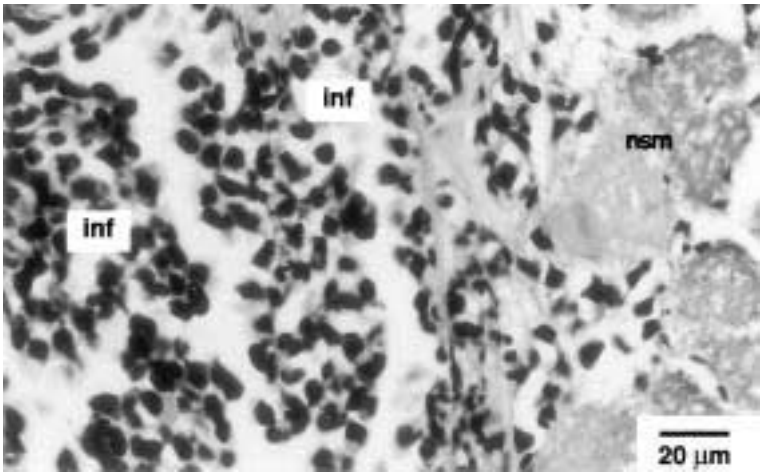

Fig. 2. Inflammatory response (inf) in injection site of a rainbow trout 1 day after vaccination with 0.1 µg pIHNw-G. Responding cells in the early inflammatory infiltrate are predominantly polymorphonuclear leukocytes. Degenerating and necrotic skeletal muscle tissue (nsm) is visible.

By immunohistochemical staining, glycoprotein expression was seldom detectable in fish vaccinated with the low standard vaccine dose that is known to elicit strong protection under these conditions. By using the high vaccine dose to enhance detection, glycoprotein was observed mainly in the muscle at the site of injection; however, it was also detected in the thymus and kidney (anterior and posterior). To our knowledge this is the first report to describe a pathogen antigen expressed in fish tissues not associated with the site of DNA vaccine injection. It is not clear from our data whether the G protein present in the thymus and kidney resulted from direct transfection, from transfected cells migrating to these organs, or from trafficking of the G protein either alone or in phagocytic cells. Because the G protein is transported to the plasma membrane after synthesis (McAllister and Wagner, 1975), it is readily accessible to antigen-presenting cells and marks transfected cells as targets for macrophage destruction. This has been shown by positive immunostaining for VHS glycoprotein in inflammatory cells infiltrating the injection site in fish vaccinated with the VHSV DNA vaccine (Boudinot et al., 1998). Our work suggests that subsequently these cells may migrate to the thymus. Although the inner zone of the rainbow trout thymus where the specific staining was detected is largely populated by large lymphocytes and lymphoblasts, it also contains macrophages and phagocytic epithelial cells (Chilmonczyk, 1983).

The localization of specific IHNV G protein staining within the anterior kidney near melanomacrophage aggregates (Figure 1D) may also have resulted from transportation of the antigen itself from the site of DNA vaccine injection. Lamers (1985) reported that antigen from an *Aeromonas hydrophila* bacterin intraperitoneally injected into fish is transported, processed, and localized in areas adjacent to melanomacrophage aggregates in the kidney, spleen, and liver. Because the cell population of the anterior (hematopoietic) portion of the salmonid kidney is comprised of diverse cell types in various stages of maturation, further immunohistochemical staining studies would be required to

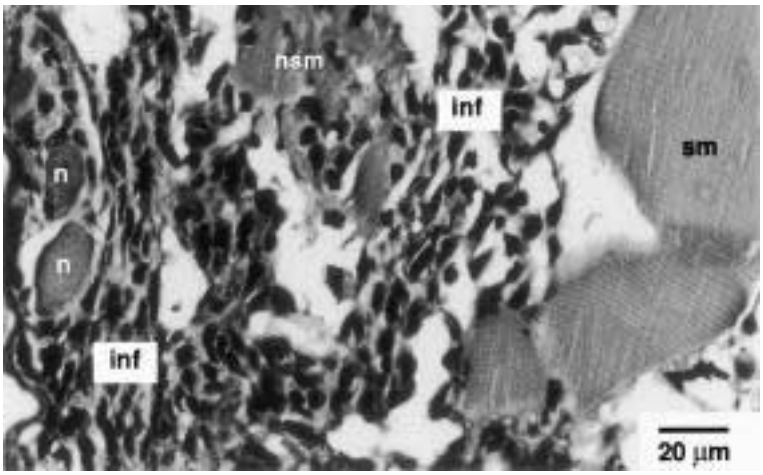

Fig. 3. Inflammatory response (inf) in injection site of a rainbow trout 28 days after vaccination with 0.1 µg pIHNw-G. The inflammation surrounds nervous tissue (n) near the spinal cord; predominant cells in the inflammatory infiltrate at this time point are macrophages. Inflammatory cells are visible within necrotic skeletal muscle (nsm); intact skeletal musculature (sm) showing characteristic striations is visible at right.

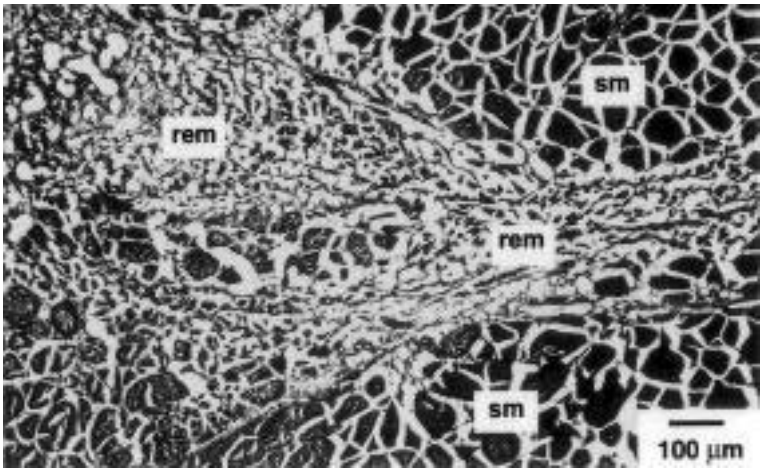

Fig. 4. Regenerating skeletal muscle tissue (rem), evidenced by the presence of small-diameter muscle fibers in comparison with mature skeletal muscle fibers (sm), in a rainbow trout 28 days after vaccination with 50 μg pIHNw-G. Infiltration of inflammatory cells is minimal at this time point.

definitively identify the cell types that stained positive for IHNV G protein.

Although some specific staining was detected in the anterior kidney in our study, specific staining was most frequently observed in the cytoplasm of epithelial cells of proximal renal tubules in the posterior kidney. The proximal renal tubule is the principal site of reabsorption of glomerular filtrate by endocytosis, and is also the main site of secretion of ions and organic substances (Elger, 2000). In the first segment of the proximal tubule, the reabsorption and digestion of macromolecules within lysosomes occurs (Bonga, 1973). In our study the morphologic features of the tubule epithelial cells and the location of the specific immunostaining within the cytoplasm of these cells indicated that IHNV G protein was being concentrated within the lysosomal system of the epithelium of the first proximal tubule segment, presumably for subsequent degradation.

Glycoprotein was most readily detected in the left epaxial muscle at the site of injection and in the right epaxial muscle opposite the injection site. It is most probable that the occurrence of glycoprotein within the muscle tissues is not due to trafficking of glycoprotein throughout muscle tissue but rather is a consequence of the injection procedure. Histopathologic analyses of injected fish muscle clearly showed needle tracks extending from the site of injection on the left side of the fish into the right side of the fish, thereby permitting the simultaneous delivery of the DNA vaccine to the distant muscle tissues.

The peak detection of glycoprotein in the muscle tissue (Table 2) correlated temporally with muscle regeneration in vaccinated fish at 14 days after vaccination (Tables 3 and 4). This observation is not surprising because studies with mice have indicated that muscle regeneration induced by myotoxic agents enabled higher levels of gene expression from plasmid DNA (Wells and Goldspink, 1992; Davis and Jasmin, 1993; Danko et al., 1994; Vitadello et al., 1994). The high regenerative capacity of fish skeletal muscle reported previously by others (Anderson and

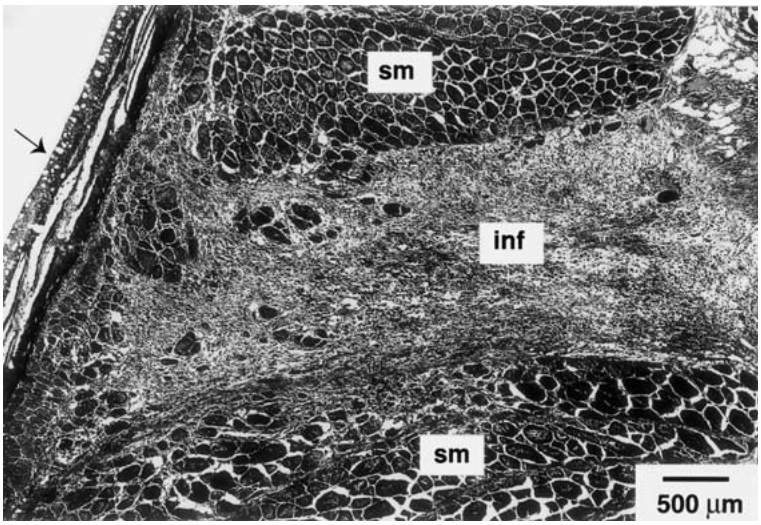

Fig. 5. Severe inflammation (inf) in the injection track of a rainbow trout 14 days after vaccination with 50 μg pIHNw-G. Intact skeletal muscle (sm) is visible on either side of the track, and the integument (arrow) has regenerated.

Table 4. Histopathological Changes Observed in Epaxial Muscle Tissue of 2.5 g Rainbow Trout Vaccinated Intramuscularly with 50 μg of pIHNw-G or 50 μg Control Vaccine pLuc[a]

Injection group by days after vaccination	*Hemorrhage*	*Muscle degeneration/necrosis*	*Inflammation*	*Muscle regeneration*
Time 0				
pIHNw-G	5/5	0/5	0/5	0/5
PLuc	3/5	0/5	0/5	0/5
1 Day				
pIHNw-G	7/7	7/7	7/7	0/7
PLuc	5/6	5/6	5/6	0/6
2 Days				
pIHNw-G	3/3	3/3	3/3	0/3
pLuc	3/3	3/3	3/3	0/3
7 Days				
pIHNw-G	2/3	3/3	3/3	0/3
pLuc	3/3	3/3	3/3	0/3
14 Days				
pIHNw-G	23/23	19/23	23/23[b]	21/23
pLuc	3/3	1/3	3/3	3/3
28 Days				
pIHNw-G	3/5	2/5	5/5[c]	5/5
pLuc	1/3	0/3	3/3[d]	3/3

[a]Values are presented as number positive per total number examined.
[b]Multinucleate giant cells observed in areas of inflammation in 6 of 23 fish.
[c]Multinucleate giant cells observed in 1 of 5 fish.
[d]Multinucleate giant cells observed in 2 of 3 fish.

Roberts, 1975; Dutta and Rai, 1994; Mittal and Munshi, 1974; Unguez and Zakon, 1998) and observed in this study may promote gene expression. Moreover, studies investigating expression of foreign genes following direct injection of plasmid DNA into carp muscle revealed that younger and fast-growing fish had much higher levels of chloramphenicol acetyltransferase (CAT) activity than older fish (Hansen et al., 1991). In contrast to mammals, in which muscle fiber number is fixed at, or shortly after birth (Goldspink, 1977), the fiber number in many fish species may increase throughout life, and the increase is especially pronounced in young fish when growth rates are high (Johnston, 1982).

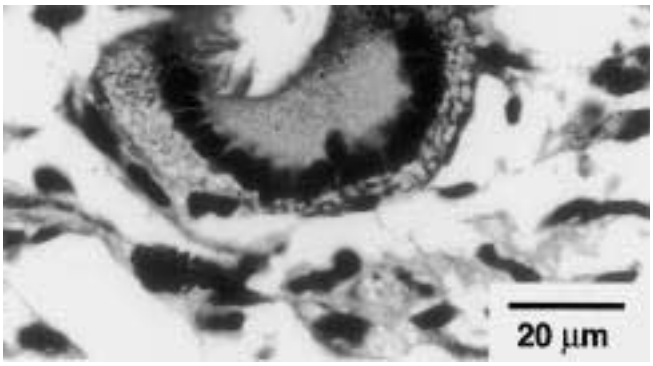

Fig. 6. Multinucleate foreign-body-type giant cell (syncytium formed by fusion of macrophages) in connective tissue associated with the injection site of a rainbow trout 28 days after vaccination with 50 μg pIHNw-G. No giant cells were observed in fish vaccinated with 0.1 μg pIHNw-G.

Although G protein was rarely detected by immunohistochemistry in the fish vaccinated with 0.1 μg pIHNw-G, it has been well established that this vaccine dose elicits strong protection under these conditions (Corbeil et al., 1999, 2000a, 2000b). It is likely that the level of G protein expressed in fish vaccinated with the low dose (0.1 μg) was below the level detectable by the immunohistochemistry methodology used in this study. This may in part explain why the kidney, a site where xenobiotic substances may be concentrated for secretion or degradation, was the only tissue that showed positive G protein staining in the fish vaccinated with the low dose of pIHNw-G (Figure 1A).

Similar to reports describing an increase in luciferase activity with increasing plasmid concentration (Gomez-Chiarri et al., 1996; Heppell et al., 1998), a dose effect was evident in our study as glycoprotein expression increased in proportion to the plasmid concentration between 0.1 and 50 μg. However, the duration of glycoprotein expression in rainbow trout is quite different from the duration of luciferase activity. Glycoprotein expression was first detected at 7 days in the muscle following intramuscular injection of rainbow trout with 50 μg DNA. Expression peaked by 14 days, when it was

detected in muscle, thymus, and kidney, and by 28 days there was no detectable gycoprotein. In contrast, expression of luciferase activity in the injection site muscle has been reported as early as 2 days and as long as 115 days after injection (Anderson et al., 1996b). One study investigating luciferase expression in glass catfish reported activity 2 years after injection (Dijkstra et al., 2001). Some of this difference in kinetics of expression is likely due to the greater sensitivity of the luciferase detection methods relative to the detection of glycoprotein by immunohistochemistry. However, luciferase is known to be a relatively poor immunogen, and this may allow for a longer duration of expression (Wolff et al., 1990; Heppell et al., 1998; Jones, 2001). Heppell et al. (1998), showed that co-injection of the VHSV G protein and luciferase genes in fish muscle resulted in an accelerated decrease of the luciferase activity over time in comparison with the injection of the luciferase gene alone, suggesting that fish raised a cellular immune response to the G protein. Similarily, the disappearance of β-galactosidase (β-gal) expression in mice (Davis et al., 1997) and goldfish (Russell et al., 2000) was shown to correlate with increased levels of anti-β-gal antibodies in serum. Therefore it is probable that the absence of IHNV glycoprotein detection after 14 days in our study is the result of an immune response directed against the expressed antigen. Alternatively, the drop in G protein expression may have been due to inactivation of the CMV promoter, as has been reported for other DNA vaccines (Manthorpe et al., 1993; Hartikka et al., 1996; Herweijer et al., 2001).

Histologic examination of the low dose pIHNw-G vaccinated fish for 90 days revealed no persistent pathologic changes. The transient histopathologic changes observed in the epaxial muscle of the low-dose fish appeared to be solely a consequence of needle injection, as similar lesions were observed in all injected groups. Despite the presence of both pIHNw-G DNA and glycoprotein in multiple tissues of vaccinated fish, the lack of pathologic changes in tissues other than the epaxial muscle indicated that the introduction of the foreign DNA and its expressed protein was well tolerated.

In contrast to the results from the low-dose (0.1-μg) pIHNw-G and pLuc injection groups, which revealed no discernible differences in the inflammatory responses of fish between the two groups, fish injected with the high dose (50 μg) of pIHNw-G showed a greater infiltration of inflammatory cells into the injection site than fish injected with 50 μg of pLuc. Similarly, N. Lorenzen et al. (2002) noted a prolonged and marked inflammatory response in the injection sites of rainbow trout vaccinated intramuscularly with high doses (≥ 10 μg) of a VHSV DNA vaccine encoding the G protein in comparison with fish injected with a nonprotective construct encoding the N protein, the vector without the insert, or with low but protective doses (≤ 1 μg) of the VHSV DNA vaccine. Multinucleate giant cells, observed in some fish in both the high-dose pIHNw-G and pLuc groups, are a common component of the chronic inflammatory response in fish, and are more frequently observed in fish than in mammals (Secombes, 1985; Timur et al., 1997). As in mammals, these cells are derived from macrophages, and in rainbow trout they exhibit some phagocytic capabilities (Secombes, 1985).

In summary, we have used sensitive techniques to follow the distribution, persistence, and expression of an intramuscularly injected IHNV DNA vaccine. The IHNV plasmid DNA, despite being immediately distributed to multiple tissues, was rapidly cleared from the peripheral sites and only retained in muscle tissue without any associated muscle toxicity or damage. The absence of histopathologic changes at the 90-day time point is a positive indication for the safety of this vaccine in fish. Although transfected muscle tissues are a major source of glycoprotein production, the presence of antigen in kidney and thymus demonstrates that antigen trafficking, direct transfection of distal tissue cells, or transfection of migrating antigen-presenting cells also occurs. This antigen distribution and/or efficient muscle regeneration in fish may in part explain why the IHNV DNA vaccine is efficient at stimulating an extremely strong protective immune response.

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