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Recombinant infectious hematopoietic necrosis viruses induce protection for rainbow trout *Oncorhynchus mykiss*

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ABSTRACT: Infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicaemia virus (VHSV) are rhabdoviruses that infect salmonids, producing serious economic losses. Two recombinant IHN viruses were generated by reverse genetics. For one (rIHNV GFP) the IHNV NV gene was replaced with the green fluorescent protein (GFP) gene. In the other (rIHNV-Gvhsv GFP) the G gene was also exchanged for that of VHSV. No mortalities, external signs or histological lesions were observed in experimental infections conducted with the recombinant viruses. Neither the rIHNV GFP nor rIHNV-Gvhsv GFP was detected by RT-PCR in any of the examined tissues from experimentally infected fish. In order to assess their potential as vaccines against the wild type viruses, rainbow trout were vaccinated with the recombinant viruses by intraperitoneal injection and challenged 30 d later with virulent IHNV or VHSV. The GFP viruses provided protection against both wild type viruses. None of the recombinant viruses induced antibody production, and the expression of interferon (IFNαβ) and interferon induced genes such as Mx protein and ISG-15 was not different to that of controls. The rIHNV-Gvhsv GFP did not inhibit cellular apoptosis as it was observed in an IHNV inoculated fish cell line. These studies suggest that the recombinant rIHNV-Gvhsv GFP is a promising candidate as a live recombinant vaccine and also provides a good model to further study viral pathogenicity and the molecular basis of protection against these viral infections.

KEY WORDS: IHNV · VHSV · Recombinant virus · Vaccine

INTRODUCTION

Infectious hematopoietic necrosis (IHN) and viral hemorrhagic septicaemia (VHS) are serious viral diseases of salmonids with high negative impacts on aquaculture (Wolf 1988, Winton 1991). Epizootics have been detected in wild fish populations (Grischkowsky & Amend 1976, Williams & Amend 1976, Traxler & Rankin 1989, Olson & Thomas 1994, Follett & Burton 1995), and outbreaks are common at hatcheries in North America, Europe and Asia, where the losses among juvenile fish can reach 90% of production. The Idaho trout industry estimated that the annual losses caused by IHNV are about 3 million USD (Bootland & Leong 1999). The causative agents of IHN and VHS are 2 distinct viruses of the *Rhabdoviridae* family, IHNV and VHSV respectively. They have a single-stranded RNA genome that encodes 5 structural proteins (Morzunov et al. 1995, Schütze et al. 1995) and a non-virion protein NV (Kurath & Leong 1985, Schütze et al. 1996).

Due to the lack of effective control strategies, IHNV and VHSV infections usually cause high mortality rates particularly in young fish. Although vaccination is the most suitable control method, there is only one available live attenuated vaccine for VHSV licensed in Germany since 1996 (Enzmann et al. 1998) and a DNA IHNV vaccine licensed in Canada (Novartis). However, highly efficient IHNV and VHSV DNA vaccines have been developed (Anderson et al. 1996,

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Heppell et al. 1998) and are being tested in field trials.

Reverse genetics has been recently used to generate modified rhabdoviruses that could be potential live vaccine candidates. Reverse genetics consists of the recovery of recombinant viruses from vaccinia-infected cells that are transfected with an expression plasmid coding for the full length antigenomic cDNA together with plasmids coding for the nucleoprotein (N), the phosphoprotein (P) and the RNA polymerase (L). This system has been applied to several rhabdoviral models (Schnell et al. 1994, Lawson et al. 1995, Whelan et al. 1995, Biacchesi et al. 2000a,b, Johnson et al. 2000, Ito et al. 2001).

The recombinant viruses used in the present study had 2 of the 6 IHNV genes modified. One encodes the G protein, which is involved in viral pathogenicity and capable of eliciting protective antibody production against various IHNV strains (Engelking & Leong 1989a,b). The other encodes the NV protein, the function of which is not well understood but it is able to enhance viral growth in cell culture (Kurath & Leong 1985). Changes in the viral genome might modify viral characteristics such as virulence and tissue tropism.

The IHNV genome has been shown to be extremely flexible and can accommodate heterologous structural protein genes. The complete IHNV G gene has been replaced by the VHSV G gene, recovering a recombinant virus expressing VHSV G protein instead of IHNV G protein (Biacchesi et al. 2002). This recombinant virus has been shown to efficiently replicate in cell culture and to be virulent for rainbow trout by bath infection. The replacement of the IHNV G protein by VHSV G protein (rIHNV-Gvhsv) did not modify the tropism or the lesions induced by viral infection (Romero et al. 2005).

Kim et al. (1994), using a monoclonal antibody directed against the IHNV G protein, selected a G-mutant strain, RB-1, which was particularly attenuated and the authors related the loss of virulence to G protein amino acid changes at positions 78 and 218 and probably to other changes in the viral genome (Romero et al. 2005). Since it was demonstrated by successful recovery of recombinant virus expressing foreign genes instead of the NV gene, the NV protein is not essential for viral replication and a viral construction was created in which the entire NV open reading frame was deleted and replaced by the green fluorescent protein (GFP) encoding gene (Biacchesi et al. 2000a). Using these mutants, Novoa et al. (2006) demonstrated the use of zebrafish as a suitable animal model to study VHSV infection and immune (innate and adaptive) responses and as a vaccination model for viral diseases of fish. Reverse genetics is a novel and highly useful tool to generate attenuated viruses that may allow the study of the basic protective response mechanisms.

In order to evaluate the potential of recombinant viruses as live attenuated vaccines, 2 recombinant IHN viruses were tested in a vaccine trial. The recombinant virus rIHNV GFP, described by Biacchesi et al. (2000a), has never been tested in experimental infections and vaccine trials. In addition, a new recombinant virus, rIHNV-Gvhsv GFP, similar to the rIHNV-Gvhsv described by Biacchesi et al. (2002), in which the NV gene was replaced by the GFP gene, was assayed for the first time.

To our knowledge, this is the first report describing changes in the viral cycle, pathology and tissue tropism of both recombinant viruses. The protective response was characterised by determining antibody production, expression of several antiviral genes and apoptosis induction.

MATERIALS AND METHODS

Viruses and cell cultures. Recombinant rIHNV GFP and rIHNV-Gvhsv GFP (Biacchesi et al. 2000a,b, 2002), were used in the present study. The non-structural NV gene was replaced with the green fluorescent protein (GFP) gene and the G protein gene was changed for that of VHSV in the rIHNV-Gvhsv GFP. The wild type virus isolates used were IHNV, strain 32/87 and VHSV, strain 07-71 (Laurencin 1987).

Viruses were replicated in the epithelioma papulosum cyprini (EPC) fish cell line (Tomasec & Fijan 1971) and in the the rainbow trout gonadal (RTG-2) cell line (Wolf & Quimby 1962). Viral replication was carried out at 15°C and the viral titer was calculated as described by Reed & Müench (1938). RTG-2 and EPC cell line stocks were cultured in Eagle's minimum essential medium (MEM, Gibco) supplemented with 10% foetal bovine serum (FBS), penicillin (100 IU ml^{-1}), streptomycin (100 µg ml^{-1}), buffered with 7.5% sodium bicarbonate and incubated at 20°C.

Fish. Rainbow trout *Oncorhynchus mykiss* L. from a commercial fish farm were acclimatised to laboratory conditions for 2 wk. Experiments were performed in 50 l closed circuit tanks with aeration and water temperature maintained at 14°C. Fish were fed daily with a commercial dry pellet food.

Experimental infections. Six experiments were performed to estimate the virulence of the IHNV recombinants by intraperitoneal (i.p.) injection. For each treatment, 270 fish (weight in the range 4 to 7 g) were inoculated (in each trial) with 100 µl of a 10^6 pfu ml^{-1} dilution of the different recombinants. As a positive control group, IHNV and VHSV wild types were included and a negative control group was injected with culture medium. External signs (nature and intensity)

and mortalities were recorded every day for 30 d after virus exposure. The work was conducted following Good Laboratory Practice guidelines. Statistical analysis comparing infected and control groups were undertaken using the *t*-test.

Histology. To study any histopathology induced by the recombinant viruses, rainbow trout (weighing 4 to 7 g) were distributed into 30-fish groups in 50 l tanks and inoculated (ip) with 100 µl (5×10^6 pfu ml^{-1}) of either of the 2 IHNV recombinants or with wild type IHNV as a positive control group. Fish in the negative control group were injected with culture medium only. Histological lesions were evaluated on 4 fish from each group sampled at Days 1, 3, 7, 14 and 28 post-infection (p.i.). Muscle, kidney, liver, spleen and brain were sampled and processed for histology. Transverse sections of approximately 5 mm thickness from the organs were excised, fixed in 4% buffered formalin, embedded in paraffin and stained with haematoxylin and eosin. The severity of the lesions was recorded using a grading system established for the most frequent kidney and liver lesions (Romero et al. 2005).

Virus replication *in vitro*. Virus replication assay was undertaken using cultures of EPC and RTG-2 cell lines. Confluent monolayer cultures in 25 cm^2 tissue culture flasks (Falcon) were infected using a 0.2 ml suspension of the recombinant viruses (MOI of 1). After allowing 30 min for viral adsorption, cells were covered with 10 ml culture medium containing 2% FBS. The appearance of cytopathic effect (CPE) and morphological changes were observed. To ensure that the cellular changes were induced by the viruses, UV fluorescent microscopy (Nikon Eclipse TS100) was used to detect viruses with the GFP (rIHNV GFP and rIHNV-Gvhsv GFP).

Viral distribution *in vivo*. The detection of the viral replication *in vivo* was assayed by a semi-nested reverse-transcriptase polymerase chain reaction (RT-PCR). Trout groups (n = 15 per group, with a mean total weight of 5.5 g) were injected (ip) with 100 µl of either the wild IHNV (positive control group) or with the different recombinants (2×10^6 pfu ml^{-1}). At 1 and 3 d p.i., the kidney, liver and spleen were sampled from 3 fish in each group. RNA extraction was performed using Trizol Reagent (Invitrogen) following the manufactureris instructions. The RT-PCR tests were performed following the procedure described by Miller et al. (1998).

Immunization and virus challenge. Three trials were performed to study the protection induced by each recombinant virus against experimental challenges with pathogenic IHNV and VHSV. Trout groups (n = 80 per group, with a mean weight of 5.5 g) were inoculated (i.p.) with 100 µl of either recombinant (10^6 pfu ml^{-1}). An additional negative control group was mock infected with phosphate-buffered saline (PBS). Fish were challenged (i.p.) with 100 µl containing 2×10^6 pfu ml^{-1} of a pathogenic wild type virus, IHNV or VHSV, 30 d after vaccination. Cumulative mortality was recorded 30 d p.i.. The vaccine efficacy was expressed as the fraction of the mortality that was prevented by the vaccination up to the end of the experiment referred to as the relative percent of survival (RPS = 1 – (% mortality in vaccinated group/% mortality in control group) × 100) (Jarp & Tverdal 1997). A number of *t*-tests (significant difference indicated by $p < 0.05$) were used to compare the results of the vaccinated and control groups.

Antibody production. To determine the ability of the different IHN recombinant viruses to induce antibody production, rainbow trout (mean weight 22 g) were injected (ip) with a 250 µl suspension containing 10^3 pfu ml^{-1} virus. Positive control groups were injected with IHNV or VHSV wild types. A non-lethal infection (low viral dose) was used to avoid killing the fish but was sufficient to induce antibody production before the end of the experiment. Blood was drawn from the caudal vein after 4, 7 and 10 wk of exposure to the viruses, allowed to clot overnight and serum, obtained by centrifugation for 15 min at 500 × *g* in a refrigerated centrifuge, was stored at –20°C until used for quantification of antibody levels by an enzyme-linked immunosorbent assay (ELISA) described previously (Romero et al. 2005).

For the ELISA, 96-well plates were coated overnight at 4°C with a suspension of IHNV (2×10^6 pfu ml^{-1}, 50 µl $well^{-1}$) that was diluted in coating buffer (Na_2CO_3 0.0015 M, $NaHCO_3$ 0.03 M, pH 9.6). All unbound sites were then blocked for 1 h at 37°C with PBS-T (PBS + 1% Tween-20) with 3% bovine serum albumin (BSA). Serum dilutions were performed in PBS-T + 1% BSA and added in duplicate to precoated and blocked ELISA plates. After 1 h of incubation at 37°C, plates were washed twice with PBS-T and incubated for 2 h at room temperature with a polyclonal anti-trout Ig (1G7) (Sánchez et al. 1991) diluted 1:500 in PBS-T, followed by an 1 h incubation with a peroxidise-conjugated anti-IgG antibody diluted 1:1000 in PBS. After 2 PBS-T rinses, the enzyme substrate, orthophenylendiamine (OPD) (Sigma) was added and the reaction was stopped 20 min later with the addition of 3 M sulphuric acid. The reaction intensity was measured by reading the optical density at 492 nm and the antibody titer was calculated according to Shapiro et al. (1997). We took the serum from 1 positive control fish infected with IHNV or VHSV with the highest antibody titer as a standard sample and we compared it with all the other serums obtained from fish infected with recombinant viruses or treated with PBS (negative control group).

The antibody titer was represented as Units per ml. In the case of samples taken from fish inoculated with

rIHNV-Gvhsv GFP, an additional ELISA was performed to quantify antibodies against VHSV using the same protocol except that the plates were initially coated with a suspension of VHSV.

Host gene expression. To study gene expression related to antiviral immune response, new experimental infections were performed with the wild type IHNV and VHSV (positive control group) and the recombinant viruses rIHNV GFP and rIHNV-Gvhsv GFP. Fish were injected (ip) with 100 µl of the recombinant viruses (10^6 pfu ml^{-1}) or PBS (negative control group) and kidney and liver were sampled at 24, 48 and 72 h p.i. from 6 fish of each treatment. RNA extraction was performed using Trizol Reagent (Invitrogen). After DNAase treatment of RNAs (5 µg per sample), reverse transcription was performed with SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Specific primers were designed using the Primer3.cgi v 0.2a software with available sequences in the GeneBank database for IFNαβ and the interferon induced genes, Mx protein and ISG-15 protein (accession numbers: AF483529, AY788890, U47945). ISG-15 primers (F: TCA GGT GTC AAT GGG AAC AA. R: TTT GGA CCT TGG CTT TGA AC), IFNαβ primers (F: GTC CTT TTC CCA ACA TCA CTT TA. R: TGC ACT GTA GTT CAT TTT TCT CA) and Mx primers (F: GGA CCC CCT GAA GAC CCA AAA G. R: ACT AAC TCT CCC TCC TCC AAC TCT). The β-actin gene was amplified as standard internal control with primers designed at our laboratory (accession number: AF157514. Primer F: AAA ACC AAA AGT TCA AAA TGG AAG. Primer R: GTT GAA GGT CTC AAA CAT AAT CTG G). The cycling conditions used were: 94°C for 5 min, 40 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min, followed by 72°C for 7 min. The sizes of the amplified fragments were 204, 285 and 584 bp, respectively. The PCR products were analysed in a 1% agarose gel and stained with ethidium bromide. Mx, IFNαβ and ISG-15 band intensity was determined by image analysis (Kodak software, 1D 2.02) and divided by the respective β-actin band intensity to obtain a gen/β-actin ratio band intensity for each individual sample. Results are expressed as the mean value of the ratios of the gen/β-actin band intensity ± standard deviation. Comparisons between the vaccinated and negative control groups were made using *t*-tests ($p < 0.05$).

Apoptosis assay. EPC confluent monolayers in 24-well tissue culture plates (Falcon) were infected with 1 ml of 10^6 pfu ml^{-1} of the recombinant viruses to study the induction of apoptosis. A negative control group and positive control group were included. The negative control group was treated with culture medium only, while the positive control group was treated with UV light (288 nm) for 30 min. The exposure to UV light induces cellular apoptosis by producing helix-distorting lesions in DNA (Aragane et al. 1998). Samples were taken at 24 and 48 h post treatment and measured by Annexin V-PE/7AAD flow cytometry. Briefly, cells were washed twice with PBS and resuspended in 1× Binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 5 µl of Annexin V-PE and 5 µl of 7-aminoactinomycin D (7-AAD). Cells were incubated for 15 min at room temperature in the dark and measured in a FACscan flow cytometer using Cell Quest software (BD Biosciences).

7-AAD (7-aminoactinomycin D) is a fluorescent DNA dye that selectively binds to GC regions of the DNA. During the cell death process, the plasma membrane is progressively altered and becomes permeable to the dye. The necrotic cells were recorded by the 7-AAD emission, detected in the FL-3 channel (>650 nm). During the apoptotic process the plasma membrane asymmetry is lost. The phosphatidylserine (PS) normally presented in the inner side of the membrane translocates to the outer side of the plasma membrane exposing it to the external cellular environment. The Annexin V-PE is a 35–36 kDa phospholipid-binding protein with high affinity for PS. The apoptotic cells were recorded by their PE emission, detected in the FL-2 channel (585 nm). The fluorescence emitted by the viral GFP protein was measured in the Fl-1 channel (530 nm). This parameter recorded the percentage of infected cells. This technique not only provides information about the percentage of apoptotic and necrotic cells, but also estimates GFP recombinants infected cells. To compare groups, *t*-tests were conducted.

RESULTS

Experimental infections

The pathogenicity of the recombinant viruses (rIHNV GFP and rIHNV-Gvhsv GFP) was compared to that of wild type IHNV and VHSV by experimental infection of juvenile rainbow trout. Both wild type viruses (IHNV and VHSV) produced 100% cumulative mortality by the end of the experiment (30 d); mortalities began at Day 1 p.i. and increased quickly to reach the maximum (100%) at Days 21 and 24, respectively. Infection with the recombinant viruses induced low mortality rates that were similar to negative control groups (both less than 5%) (Fig. 1). Thus, mortality rates (100%) due to infection with wild type viruses were significantly different from the mortality induced by the recombinant viruses (*t*-tests, all p's < 0.05).

No external clinical signs were observed in fish treated with the recombinant viruses, while signs typical of IHN and VHS (dark coloration, distended vis-

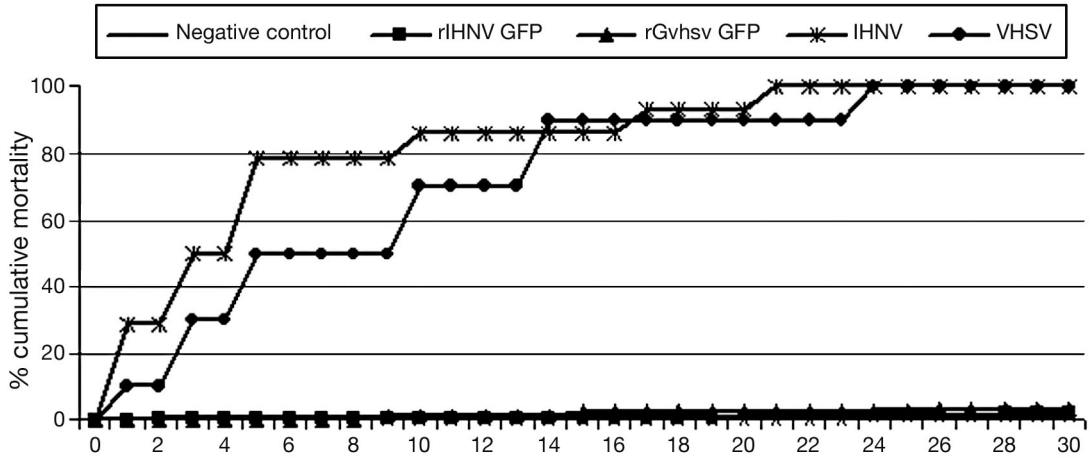

Fig. 1. *Oncorhynchus mykiss*. Cumulative mortality from experimental infections (representative results from one experiment chosen from the 6 conducted)

ceral cavity, exophthalmia, pale gills and petechial haemorrhages at the base of the fins and abdomen) were observed in fish infected with the wild type IHNV virus (positive control group) (Fig. 2) as well as those infected with wt VHSV.

Histology

Histological analysis was performed to compare the lesions associated with each recombinant virus in different tissues. Although, as already described for IHNV wild type, the kidney and the liver are the main target organs, significant lesions were also found in tissues from fish infected with the recombinant viruses. The kidney showed small haemorrhages in fish treated with rIHNV GFP (at Day 7 p.i.) and with rIHNV-Gvhsv GFP (at Day 1 and 3 p.i.), while the liver was only affected by the rIHNV-Gvhsv GFP at Day 3 p.i. The spleen and brain were not affected by the infection and some erythrocytes were observed between the muscular fibres (Fig. 3).

Virus replication *in vitro*

The first morphological changes in cell cultures infected with the recombinant viruses were detected as soon as 48 h p.i. with rIHNV-Gvhsv GFP infected cells and resembled those induced by IHNV wild type (positive control group). Enlarged cells with their nuclei displaced to one side were observed in IHNV wild type and rIHNV-Gvhsv GFP infected cells (Fig. 4B). The cells in the infected cultures clustered together with a 'bunch of grapes' appearance typical of IHNV infections (Fig. 4C). Association between the damaged cells and the appearance of fluorescence was observed by UV microscopy in rIHNV-Gvhsv GFP

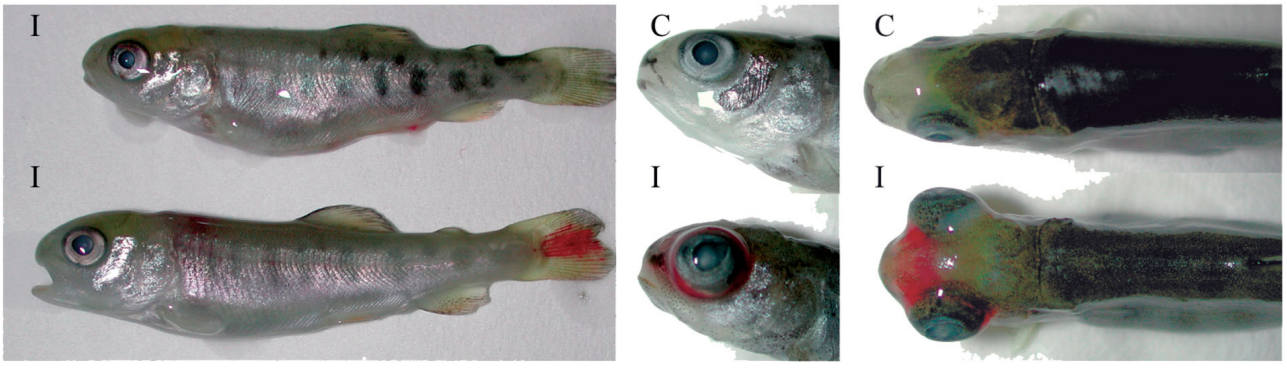

Fig. 2. *Oncorhynchus mykiss*. Clinical signs observed in fish infected with IHNV wild type. Distended visceral cavity, petechial haemorrhages at the base of the fins and abdomen and exophthalmia were observed in fish infected with IHNV (I). Control uninfected fish and fish treated with the recombinant viruses did not show any clinical signs (C)

infected cells. Total CPE was observed at Day 6 p.i. (Fig. 4D). Although the cells infected with rIHNV GFP showed light green fluorescence in the cytoplasm, neither morphological changes nor a clear cytopathic effect was observed.

Viral distribution *in vivo*

The viral replication *in vivo* was assayed by RT-PCR. No positive signal was found in samples from fish infected with recombinant viruses (rIHNV GFP and

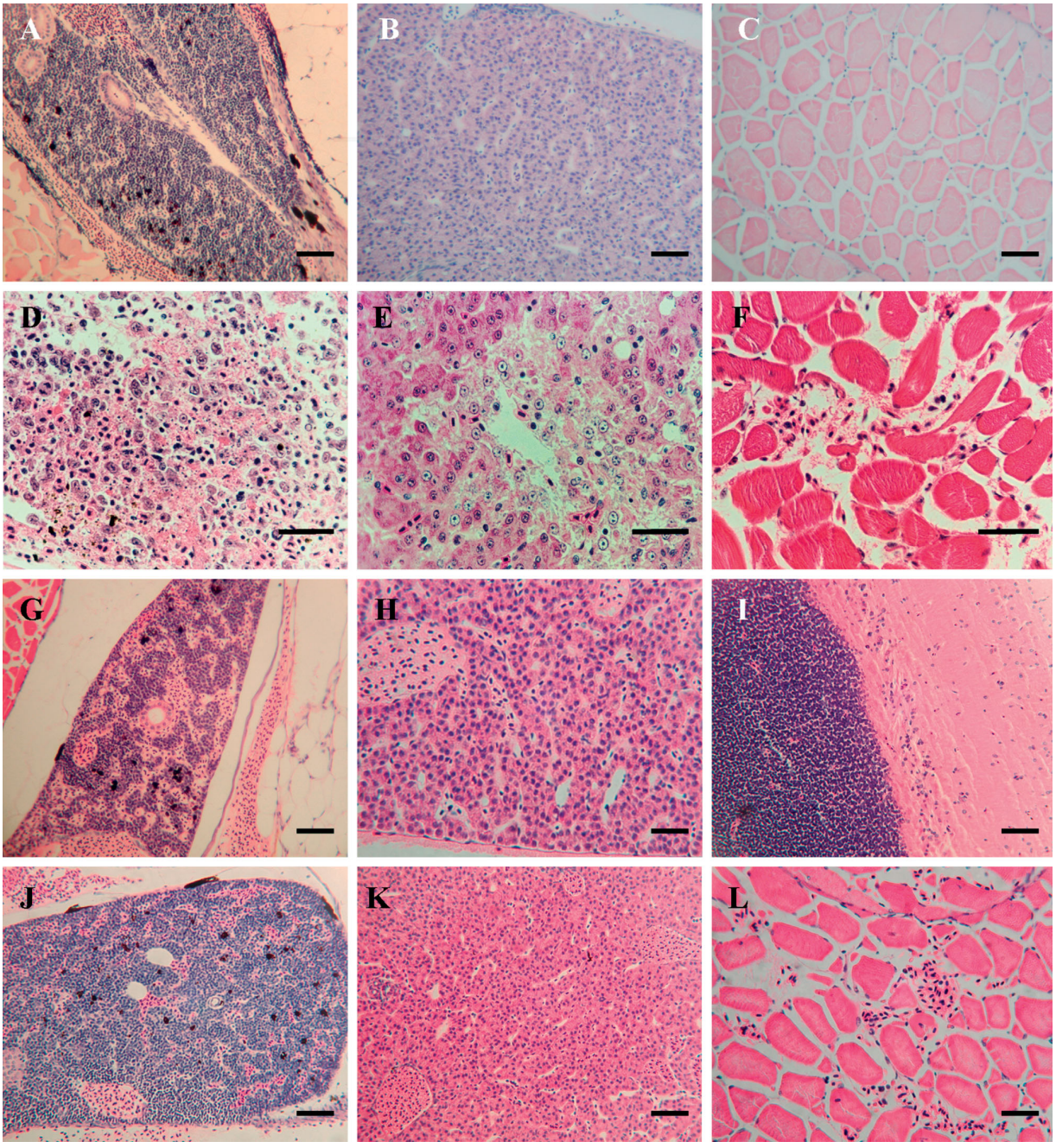

Fig. 3. *Oncorhynchus mykiss*. Main lesions observed in fish infected with recombinant viruses. (A, B, C) The kidney, liver and muscle respectively from negative uninfected fish. (D, E, F) Lesions induced in fish experimentally infected with IHNV (positive control group). (D) Kidney and (E) liver showed extended haemorrhages and necrotic areas mainly at Day 4 to 7 post-infection (p.i.). (F) Some fish showed histological changes in the muscle at Day 7 p.i.. (G, H, I) lesions observed in rIHNV GFP infected fish. (G) Small haemorrhages were observed in the kidney mainly at Day 7 p.i.. (H) No lesions were observed in the liver and (I) brain. (J) rIHNV Gvhsv GFP induced small haemorrhages in the kidney and (K) liver at the beginning of the infection and occasionally, (L) erythrocytes between the muscular fibres were observed at the end of the infection. H&E staining. Scale bars = 40 µm

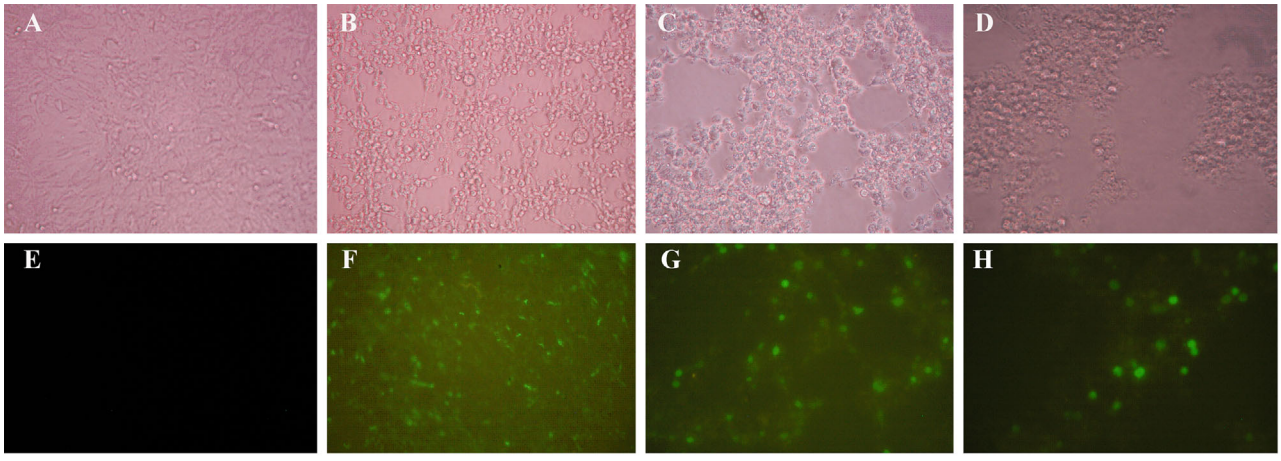

Fig. 4. (A, B, C, D) Cytopathic effect and morphological changes in EPC cell cultures infected with recombinant viruses and revealed by light microscopy and (E, F, G, H) UV microscopy. (A) and (E) show the negative control group without CPE by light and UV microscopy. (B, C, D) show morphological changes in the cell culture at 1, 3 and 6 d p.i. with rIHNV-Gvhsv GFP. No morphological changes or clear cytopathic effect was observed in rIHNV GFP infected cell lines. Similar results were obtained with RTG-2 cell cultures

rIHNV-Gvhsv GFP). Faint bands were observed in rIHNV GFP infected fish kidney at Day 1 p.i., but were absent at Day 3 p.i. (Table 1). IHNV was clearly detected in all IHNV infected fish samples (positive control group).

Protection experiments

Vaccination of fish with rIHNV GFP and rIHNV-Gvhsv GFP recombinant viruses resulted in a decrease in the cumulative mortality in comparison with the mortality recorded in the negative control group (treated with PBS) challenged with IHNV or VHSV 30 d after vaccination (Fig. 5A & B respectively)

Mortality kinetics in the negative control group infected with IHNV or VHSV were similar to those described earlier (see section 'Experimental infections'). Mortalities in the experimental groups vaccinated with recombinant viruses began at Day 3 p.i. and increased slowly until the end of the experiment. IHNV induced a 97.3% (± 4.16) mortality in unvaccinated fish, while only 29% (± 18.3) and 36% (± 4) mortality was recorded in the rIHNV GFP and rIHNV-Gvhsv GFP treated groups, respectively. The mortalities recorded in both vaccinated groups against IHNV were significantly different from the control group ($p = 0.006$ and 0.00006, respectively). By using rIHNV GFP and rIHNV-Gvhvs GFP, the RPS values (percentage of protection) against IHNV were 70% and 62% respectively.

Similar results were obtained in the vaccination experiments against VHSV. Of the unvaccinated fish, 91% (± 7.8) died by the end of the experiment while only 47.5% (± 3.5) (using rIHNV GFP) and 27% (± 17.2) (using rIHNV-Gvhsv) of the recombinant-treated fish died. In this case the mortalities recorded in both vaccinated groups were also significantly different from the control group ($p = 0.005$ and 0.004, respectively). The VHSV RPS values (percentage of protection) were 49% when using rIHNV GFP and 61% when rIHNV-Gvhsv GFP was used. No mortalities were detected in the negative non-infected control groups before the challenge.

Table 1. *Oncorhynchus mykiss*. Detection of wild-type IHNV and recombinant viruses by RT-PCR. Each symbol represents the result obtained in organs from different fish. (±) light band observed in the agarose gel. (+) positive sample and (–) negative detection

	Liver		Kidney		Spleen	
	D1	D3	D1	D3	D1	D3
IHNV	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
rIHNV GFP	– – –	– – –	± ± –	– – –	– – –	– – –
rIHNV-Gvhsv GFP	– – –	– – –	– – –	– – –	– – –	– – –

Antibody production

In order to establish whether antibody production could be correlated with the protection induced by the recombinant viruses against IHNV and VHSV, ELISA assays were performed (Fig. 6). The recombinant viruses induced an antibody production against the G protein included

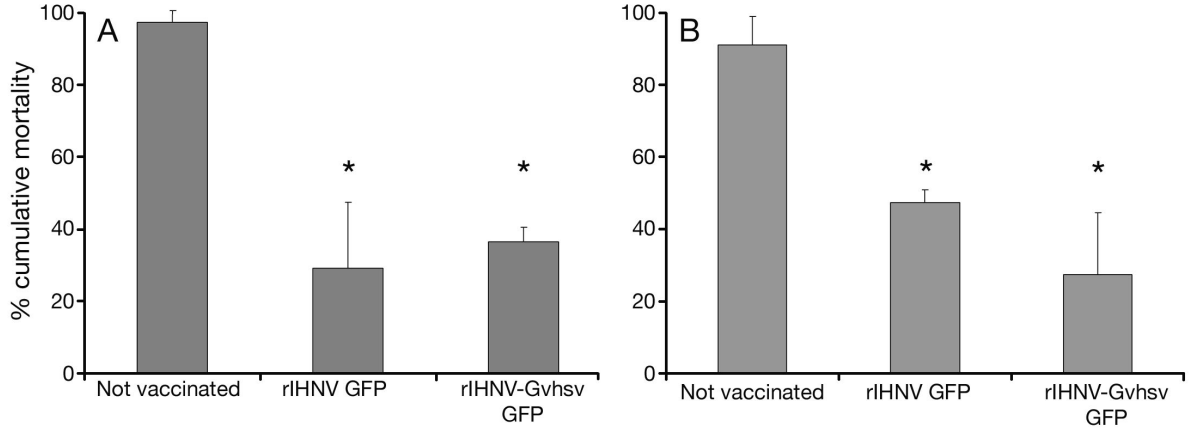

Fig. 5. *Oncorhynchus mykiss*. Cumulative mortality obtained after 30 d post-vaccination with rIHNV GFP and rIHNV-Gvhsv GFP and subsequent challenge with (A) IHNV and (B) VHSV. * *t*-test, $p < 0.05$. Error bars = SD

in their genomes. The rIHNV GFP induced a low antibody response to IHNV that did not increase with time as was observed with the IHNV wild type (positive control group). No antibodies against VHSV were detected. The kinetics of antibody production against VHSV in fish infected with rIHNV-Gvhsv GFP was similar to the kinetics induced by VHSV wild type (positive control group). At Week 10 the antibody titer was as high as the titer induced by VHSV.

Host gene expression

Interferon (IFNαβ) gene induction in fish experimentally infected with recombinant viruses (rIHNV GFP and rIHNV-Gvhsv GFP) was studied and compared with the levels induced in fish treated with wild type viruses, IHNV and VHSV (positive control group), and with a negative control group (Fig. 7).

The infection of fish with IHNV induced an increase in the IFNαβ, Mx and ISG-15 expression levels in the liver and an increase in the Mx expression levels in the kidney. The infection of fish with VHSV induced an increase of the expression levels of the same genes in kidney and only an increase in the IFNαβ expression levels in liver. The kinetics of IFNαβ, Mx and ISG-15 expression were the same in fish treated with one of the 2 wild type viruses. A rapid increase of expression was observed from Day 1 to Day 2 p.i., followed by a decrease at Day 3 p.i. IHNV induced maximum levels of these genes in the liver, while VHSV induced them in the kidney.

Variations were observed in gene expression levels in the negative control group. The GFP recombinant viruses did not induce increased gene expression in the kidney or in the liver. Although up-regulation of IFNαβ expression was observed in the kidney and liver from rIHNV-Gvhsv GFP inoculated fish, it was always lower than that induced by IHNV and VHVS. The induction levels recorded in rIHNV GFP infected fish were always as low as the levels detected in the negative control group.

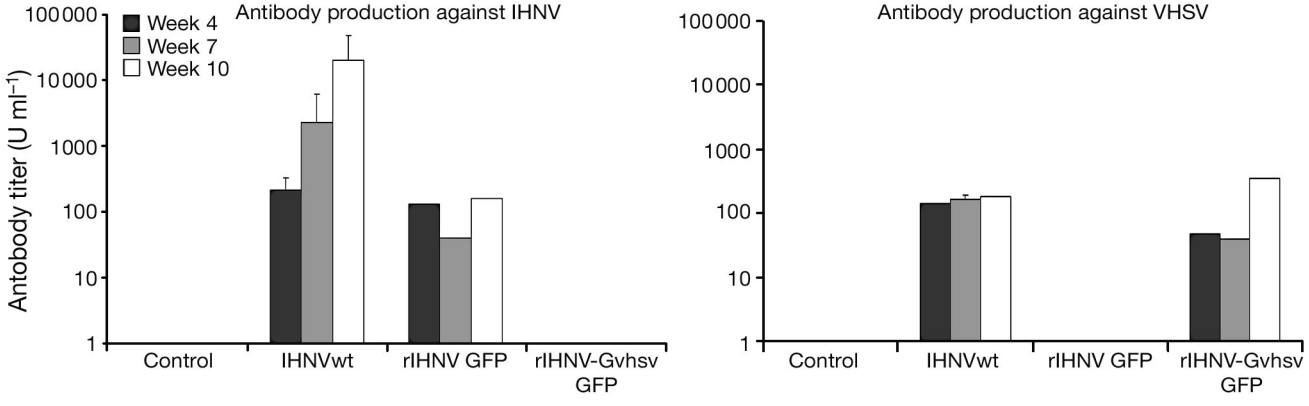

Fig. 6. *Oncorhynchus mykiss*. Antibody production against the different recombinant viruses. No production was detected in the control group against IHNV and VHSV. The recombinant viruses induced antibody production against the G protein. Each bar represents the mean and SD of 4 serum samples

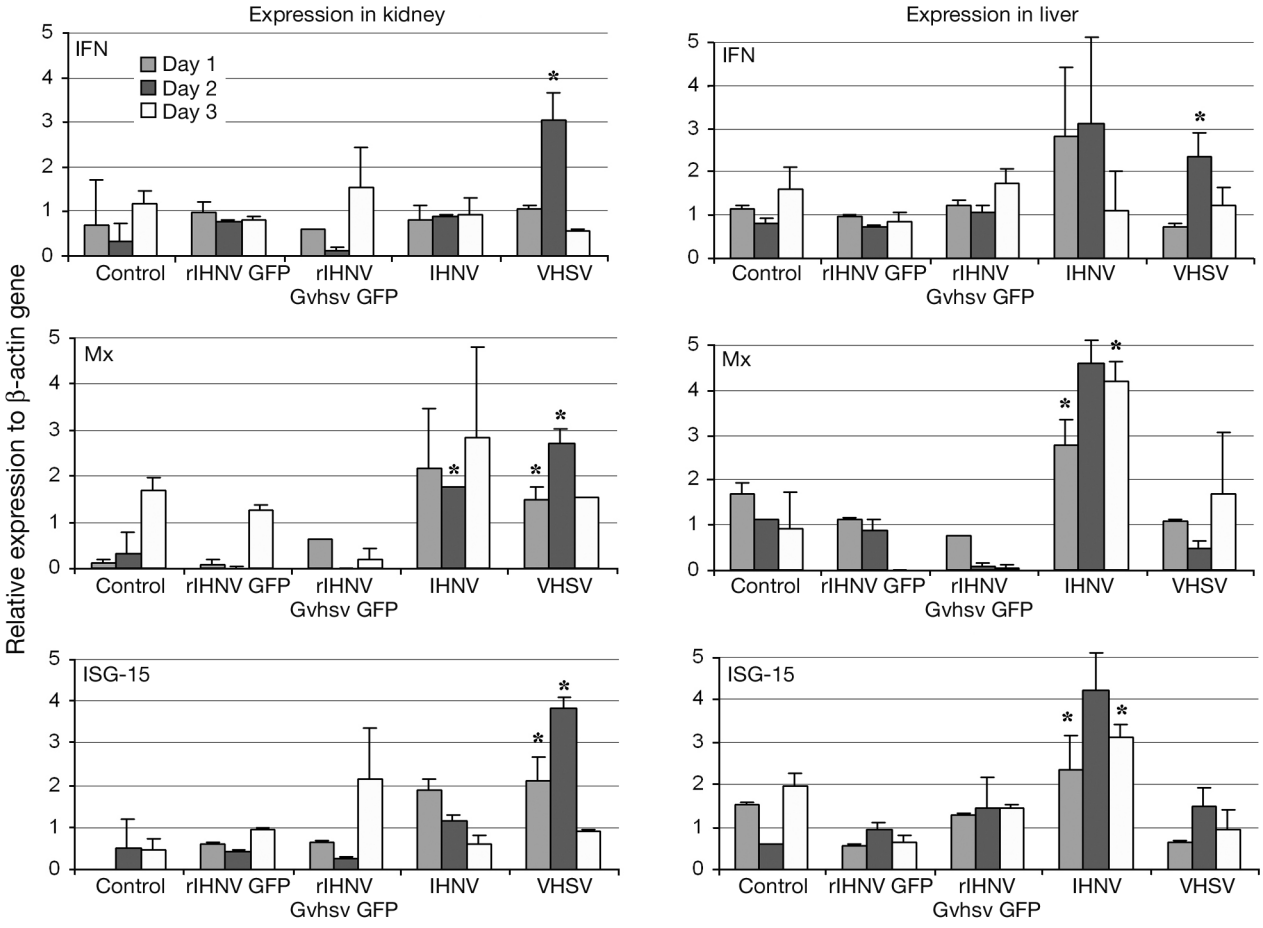

Fig. 7. *Oncorhynchus mykiss*. Expression of antiviral genes in kidney and liver after 24, 48 and 72 h post-treatment with rIHNV GFP, rIHNV-Gvhs GFP and the wild type viruses, IHNV and VHSV. Each bar represented the mean and SD of 9 fish. *t-test, $p < 0.05$

Apoptosis assay

The levels of early apoptosis were different in cells treated with the rIHNV-Gvhsv GFP, IHNV wild type and UV light. Using PE-Annexin V / 7-AAD 2-parameter flow cytometry, apoptotic, necrotic and living cells could be distinguished. Viable cells excluded 7-AAD and were negative for PE-Annexin V. Early apoptotic cells could be distinguished from necrotic cells by the lack of counterstaining with 7-AAD. These cells excluded 7-AAD and bound PE-Annexin V to phosphatidyl serine exposed on the cell surface. In contrast, necrotic cells were labelled with 7-AAD which stains dead cells independent of the cause of cell death (apoptosis or any other cause) (Fig. 8).

UV light induced the highest apoptosis levels at 24 and 48 h (16.5% and 13% of apoptotic cells respectively) in comparison with the negative control group (less than 2%). Cells infected with the apathogenic rIHNV-Gvhsv GFP had low apoptosis levels at 24 h but showed around 13% of apoptosis, similar to UV-treated cells, at 48 h. It is important to point out that apoptosis levels induced by wild type IHNV were significantly lower than those induced by the recombinant virus (less than 5%). The percentage of necrotic cells induced by IHNV was similar to the percentage induced by rIHNV-Gvhsv GFP (around 15%) but lower than the positive control (27%). Using the FL-1 channel, it was possible to detect 5% of cells infected with rIHNV-Gvhsv GFP at 48 h p.i. The percentage of infected cells treated with wild type IHNV was always lower than 0.5%.

DISCUSSION

The pathogenicity of rIHNV GFP and rIHNV-Gvhsv GFP recombinant viruses was assessed by determining cumulative mortalities, clinical external signs and histopathology induced by experimental infection of juvenile rainbow trout. Fish infected with wild type viruses (IHNV or VHSV) showed external signs (dark

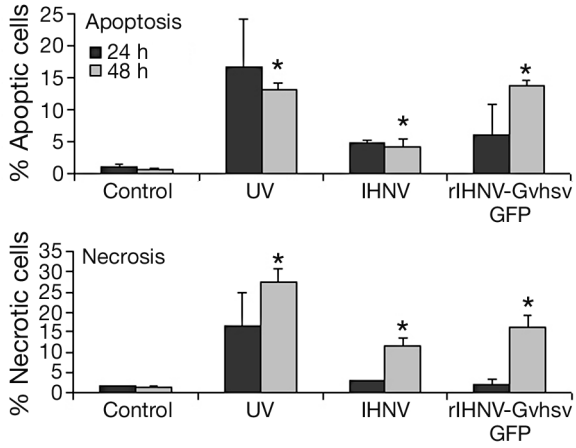

Fig. 8. Percentage of early apoptotic cells (Annexin-V +/7-AAD –) and necrotic cells in EPC cell line (Annexin-V –/7-AAD +) with different treatments. *t-test, $p < 0.05$

coloration, distended visceral cavity, exophthalmia and petechial haemorrhages at the base of the fins and abdomen) (Bootland & Leong 1999, Smail 1999) and histopathological changes (Amend et al. 1969, Yasutake & Amend 1972, Wolf 1988) typical of IHN and VHS. The mortality rates in fish groups infected with recombinant viruses were similar to the negative uninfected controls and the few dead fish did not show external clinical signs or histopathological changes. The lack of mortalities and histopathological changes in rIHNV-Gvhsv GFP infected fish had been previously described in the zebrafish by Novoa et al. (2006). We have previously shown that modifications induced in the viral G protein gene can modify mortality kinetics and viral pathogenesis (Romero et al. 2005). Kim et al. (1994), analyzed a G-mutant strain, RB-1, that was particularly attenuated and its tissue distribution was markedly different. Although the NV gene is not necessary for completing the viral cycle (Biacchesi et al. 2000a,b) its exchange for the GFP gene could modify some viral characteristics. In our experiments, cell cultures infected with rIHNV GFP or rIHNV-Gvhsv GFP showed green fluorescence in the cytoplasm, but only those treated with rIHNV-Gvhsv GFP developed a clear cytopathic effect. Results obtained using cell cultures experimentally infected with rIHNV GFP are in agreement with previous results obtained by Biacchesi et al. (2000a) who detected low levels of GFP expression in rIHNV GFP infected cells by UV-light microscopy. However, these authors did not describe the appearance of CPE and morphological changes in the cell line. Further investigation is required to determine the cause of the observed difference in the replication capacity of the 2 recombinant viruses.

IHNV route of entry and progression has been studied in depth (Yamamoto 1990, Yamamoto & Clermont 1990, Drolet et al. 1994) and it has been demonstrated that IHNV infection progresses from the gills to the circulatory system and from the oral region into the gastrointestinal tract and circulatory system. By inoculation (ip) of different virulent recombinant IHN viruses (rIHNV and rIHNV-Gvhvs) Romero et al. (2005) detected virus in the liver, kidney and gills as early as 3 d p.i.. In the present study, rIHNV GFP and rIHNV-Gvhsv GFP were not detected in infected fish in the kidney or liver by RT-PCR. This result suggests that tissue tropism or levels of infection might be affected by the introduced changes in the recombinant viruses described here.

It is not evident if the introduction of the GFP gene or the substitution of the NV gene were responsible for the changes observed in virulence and pathogenicity. It is important to point out that NV gene elimination was shown not to be required for pathogenesis of a novirhabdovirus (Alonso et al. 2004).

High levels of protection were conferred by vaccination with the GFP recombinant viruses against IHNV and VHSV. The heterologous protection has been previously described in vaccination trials against IHNV using different vaccination approaches. Engelking & Leong (1989a,b), using purified RB-1 IHNV strain glycoprotein, recorded cross-protective immunity against 5 types of IHNV. DNA vaccines encoding the viral G protein of different rhabdoviruses (IHNV, VHSV, SHRV and SVCV) elicited protective immunity against IHNV and VHSV (Kim et al. 2000, Lorenzen et al. 2002). The results obtained here with rIHNV GFP and rIHNV-Gvhvs GFP in the vaccination trials also demonstrate heterologous protection against both IHNV and VHSV.

The timing (30 d post-vaccination) of the induced protection with the recombinant viruses is interesting. IHNV vaccines based on DNA plasmids elicited high protection levels against this rhabdovirus but protection was transient, and often did not persist past 30 d post-vaccination (LaPatra et al. 2001). It might be that the recombinant viruses provide prolonged heterologous protection because they replicate after vaccination (although not by much, as shown by the data presented in this paper), while the DNA vaccines do not.

Antibody production is one of the mechanisms involved in the effectiveness of a vaccine. LaPatra et al. (1993) and Lorenzen & LaPatra (1999) showed that rainbow trout can produce specific and highly functional antibodies that are able to neutralise IHNV *in vitro* as well as *in vivo*. The kinetics of antibody production following an experimental infection with the recombinant viruses were not similar to those previously described for IHNV. The rIHNV GFP was able to induce specific IHNV antibodies but the titer did not increase with time. As expected, the rIHNV-Gvhsv

GFP was able to stimulate VHSV antibody production but the titer did not increase with time. The highest antibody titer was recorded at Week 10. As shown in the results, the recombinant viruses did not induce high antibody production that could explain the level of protection obtained. It is likely that other immune mechanism(s) are involved in the observed protection.

A correlation between high antibody production, high levels of antiviral cytokine expression and protection has been described using several fish vaccination models (Boudinot et al. 1998, McLauchlan et al. 2003). Kim et al. (2000) using a DNA vaccine encoding the viral glycoprotein, correlated an early, non-specific antiviral protection with high levels of Mx protein in the kidney and liver, and they proposed that the initial IFNαβ induction was the basis of the protection. The critical role for a type I interferon-like response in early anti-viral defence against IHNV has been confirmed by using quantitative real-time reverse transcriptase PCR methodology (Purcell et al. 2004, Acosta et al. 2005, Overturf & LaPatra 2006). The treatment with the GFP recombinant viruses used here, did not induce significant antiviral gene expression in the kidney or in the liver and the levels recorded were always as low as the levels obtained in the control group. Similar expression profiles were obtained by Novoa et al. (2006) for rIHNV-Gvhvs GFP infected zebrafish. Our results support the hypothesis that the IFNαβ pathway is not implicated in the cross-protection observed 30 d after vaccination with the GFP recombinant viruses (rIHNV GFP and rIHNV-Gvhsv GFP) and that other immune mechanism(s) are likely to be involved.

Recent studies have shown that apoptosis may play an important role in many viral infections (Thoulouze et al. 1997, Gadaleta et al. 2002, Blaho 2003, 2004). Cell death due to rhabdovirus infection is thought to result from necrosis following cell membrane damage caused by the budding virions (Wolf 1988). In addition, apoptosis appears to be involved in cell death caused by rhabdovirus infection in cell lines (Björklund et al. 1997, Chiou et al. 2000, Du et al. 2004) and in tissues (Eléouët et al. 2001). Recent studies have indicated that highly virulent viruses may use one or more strategies to inhibit apoptosis, including inhibition of transcription factors and caspase activation to complete their viral cycle before programmed cell death (Fazakerley & Allsopp 2001, Liacarta & Harty 2003). Morimoto et al. (1999) compared the pathogenicity, and the ability to induce apoptosis, of 2 variants of rabies virus in primary neuron cultures. It was shown that the less pathogenic variant had a high level of G protein expression and induced significantly more apoptosis in infected cells than the more pathogenic variant. The down-regulation of G protein expression in neuronal cells contributed to rabies virus pathogenesis by preventing apoptosis. Our results would support this phenomenon. rIHNV-Gvhsv GFP induced higher apoptosis levels than the IHNV wild type. It is possible that the protection conferred by rIHNV-Gvhsv GFP against IHNV and VHSV could be related to an increase in apoptosis which reduces spread of the virus. The modifications in the recombinant viruses explains their limited capacity to replicate in fish tissues.

Our results indicate that reverse genetics is a powerful tool for the generation of modified viruses that could be used as vaccines. Based on the results obtained in challenge experiments, the recombinant virus rIHNV-Gvhsv GFP represents a promising live vaccine against fish rhabdoviruses. On the other hand, the low antibody titer and antiviral cytokine expression cannot explain the high level of protection obtained following experimental challenge. Further research is needed to clarify the mechanisms involved which may include not only immune responses but also other cellular responses such as apoptosis.

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