Full length article

# IFN-adjuvanted DNA vaccine against infectious salmon anemia virus: Antibody kinetics and longevity of IFN expression

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## ARTICLE INFO

## ABSTRACT

Plasmids expressing interferon (IFN) have recently been shown to function as adjuvants in Atlantic salmon when co-injected with a DNA vaccine encoding hemagglutinin-esterase (HE) from infectious salmon anemia virus (ISAV). In this work we have compared the antibody kinetics and the systemic Mx/ISG15 response of fish vaccinated with HE-plasmid using either IFNa plasmid (pIFNa) or pIFNc as adjuvants over a longer time period, i.e. 22 weeks post vaccination (pv). The results showed that the antibody response against ISAV with pIFNa as adjuvant arose earlier (7 weeks pv) than with pIFNc as adjuvant (10 weeks pv), peaked at week 10 and declined at week 22. The antibody response with pIFNc as adjuvant peaked at 16 weeks and kept at this level 22 weeks pv. Fish injected with pIFNc alone expressed high levels of Mx and ISG15 in liver throughout the 22 week period. In contrast, fish injected with pIFNc together with HE-plasmid expressed high levels of Mx and ISG15 in liver for the first 10 weeks, but at week 16 this response was absent in two of three fish at week 16 and was absent in all tested fish at week 22 pv. This suggests that cells expressing HE and IFNc are intact at week 10 pv, but are eliminated by adaptive immune responses after week 10 due to recognition of HE. The longevity of the Mx/ISG15 response in pIFNc treated fish is likely due to the fact that IFNc is a self-antigen of salmon and is not attacked by the adaptive immune system.

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## 1. Introduction

DNA vaccination of farmed fish against virus is a promising method for protecting farmed fish against virus diseases. A DNA vaccine against infectious hematopoietic necrosis (IHN) virus has been used in farmed Atlantic salmon in Canada since 2005 and there has since apparently been no reports of IHN outbreaks in vaccinated populations [1]. On the other hand, DNA vaccines against other virus types have shown much less protection than DNA vaccines against rhabdoviruses [1]. However, recent work has demonstrated that plasmids expressing type I interferon (IFN) function as adjuvants when co-injected with a DNA vaccine against infectious salmon anemia virus (ISAV) and strongly increase the adaptive immune response against ISAV [2]. The ISAV DNA vaccine was a plasmid expressing the virus surface antigen hemagglutinin-esterase (HE). Atlantic salmon presmolts vaccinated with an IFN expression plasmid together with an HE-plasmid, displayed elevated IgM antibody levels against ISAV and showed higher survival after infection compared to presmolts vaccinated with the HE-plasmid alone 10 weeks post vaccination. In the present work we have studied the antibody kinetics of DNA vaccine with HE as antigen together with either IFNa plasmid (pIFNa) or IFNc plasmid (pIFNc) as adjuvant over a longer time period, i.e. until 22 weeks pv. In addition, we wanted to measure Mx and ISG15 expression in liver of fish vaccinated with pIFNc alone or pIFNc + pHE because this might indicate the longevity of the DNA vaccine constructs at the muscle injection site. Vaccination of salmon with IFN plasmids alone has previously shown that pIFNc, but not pIFNa, induced systemic expression of antiviral proteins such as Mx and ISG15, which lasted at least 10 weeks [3].

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## 2. Materials and methods

### 2.1. Ethics statement

All handling of fish was performed in accordance to the Norwegian "Regulation on Animal Experimentation" (FOR-2015-06-18-761) and all fish experiments were submitted to and approved by the Norwegian Animal Research Authority (NARA) before initiation.

### 2.2. Fish

Atlantic salmon (*Salmo salar* L.) presmolts (35–45 g) of the strain Aquagen standard (Aquagen, Kyrksæterøra, Norway) were kept at Tromsø Aquaculture Research Station, Norway in 300 l tanks supplied with fresh water at 10 °C and were fed commercial dry food. Before treatments, the fish were anesthetized with 0.005% benzocaine (ACD Pharmaceuticals, Norway). Fish groups harvested at 7 and 10 weeks after vaccination were labelled by tattooing (2% alcian blue, Panjet inoculator) and kept in separate tanks while fish groups harvested at 16 and 22 weeks after vaccination were labelled by intraperitoneal implantation of PIT-tag-numbers using a tag applicator and kept in the same tank. Tag numbers were entered in an Excel sheet (Microsoft Excel™) using a handheld ISO Reader ARE H5 (AEG Identifikasionssysteme GmbH, Ulm, Germany) remotely connected to a computer. Before harvest of organs, fish were killed by an overdose of benzocaine (0.01%).

### 2.3. Plasmids used for intramuscular injection

Plasmids encoding the open reading frame (ORF) of Atlantic salmon IFNa, IFNc and ISAV hemagglutinin-esterase (HE) were available from a previous study [2]. A re-ligated pcDNA3.3 plasmid without insert was used as control. Plasmids were transformed and grown in One Shot TOP10 *E. coli* (Invitrogen) and purified by EndoFree plasmid purification kit (Qiagen).

### 2.4. DNA vaccination and sampling

Presmolts were injected intramuscularly (i.m.) approximately 1 cm below the dorsal fin with one or two 15 µg plasmids in 50 µl sterile phosphate-buffered saline (PBS) at pH 7.4 or with PBS only. Treatment groups are shown in Table 1. Blood and liver were harvested from fish in all groups at 7, 10, 16 and 22 weeks post vaccination. Sera were prepared from blood and kept at −20 °C and used for antibody measurements. Livers were stored at −80 °C and used for immunoblotting of Mx and ISG15.

### 2.5. Measurement of antibody against ISAV by ELISA

Sera were diluted 500-fold and used for measuring ISAV-specific

antibodies by ELISA using ISAV as coating antigen as described [2]. All sera from each time point were run on the same 96-well plate and were tested in duplicate plates. Optical density (OD) was measured at 450 nm and the mean OD value of duplicates from each fish was calculated. The mean OD value from each fish group was then plotted as a column, using box and whiskers graph (GraphPad Prism vision 6.01 for Windows, San Diego). The box represents 25%–75% of the observation (lower and upper quartile), and the whiskers represent the total variation from min to max. Significant differences were analysis by two-tailed *t*-test with Mann-Whitney method (GraphPad Prism vision 6.01).

### 2.6. Measurement of Mx and ISG15 protein expression in liver

Livers were sampled to measure systemic expression of Mx and ISG15 proteins by immunoblotting. Extraction and electrophoresis of proteins was performed as described [3]. Blotting, antibody incubations and development of blots were done as described [4]. Blots were developed with polyclonal antibodies against Atlantic salmon Mx protein (1:3000) or against salmon ISG15 protein (1:3000) or against actin from Sigma (1:1000) [5,6]. MagicMark XP (Invitrogen) was used as molecular weight protein standard. Three individuals were used from each group except for Mx from fish injected with pIFNc and pHE at 7 weeks p.v. from which two individuals were used. As control we used a pool of protein extracts from liver of three fish injected with pcDNA3.3 harvested 7 weeks pv.

## 3. Results and discussion

### 3.1. Kinetics of the antibody response with IFNa and IFNc as adjuvants

Serum antibody against ISAV was not detectable in fish injected with the single plasmids pcDNA3.3 (vector without insert), pIFNa, pIFNc or even pHE at any of the time points (Fig. 1). In contrast, fish injected with pHE together with pIFNa or pIFNc showed elevated antibody levels at all time points except at 7 weeks post vaccination where only fish injected with pHE + pIFNa contained significant anti-ISAV antibody levels. Interestingly, pIFNa and pIFNc used as adjuvants resulted in different antibody kinetics. Serum antibody concentration against ISAV in fish injected with pIFNa + pHE
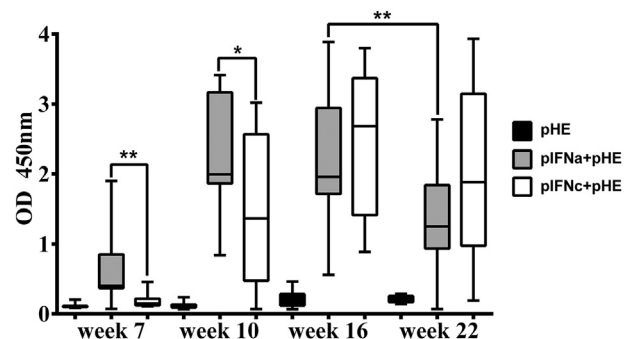


**Fig. 1.** Antibody response against ISAV in fish vaccinated with HE-plasmid in combination with IFNa- or IFNc-plasmids as adjuvants. Groups of presmolts (n = 15) were injected with pIFNa alone, pIFNc alone, pHE alone, pHE + pIFNa or pHE + pIFNc. Sera were harvested after 7, 10, 16 and 22 weeks, diluted 500 fold and analysed for antigen specific IgM antibodies by ELISA using purified ISAV as coating antigen in the wells of the ELISA-plates. The antibody responses of the groups (OD450) are shown as box and whiskers graphs where the horizontal line shows the mean of the group, the box represents 25%–75% of the observations (lower and upper quartile), and the whiskers represent the total variation. Significant differences between groups at each time point are shown as *(p < 0.05) and ** (p < 0.005).

**Table 1**
Vaccination groups and sampling time points.

| Fish groups | Injected plasmids | Tank 1 W 7 | Tank 2 W 10 | Tank 3 W 16 and 22 |
|---|---|---|---|---|
| 1 | pcDNA3.3 | 15 | 15 | 30 |
| 2 | pIFNa | 15 | 15 | 30 |
| 3 | pHE | 15 | 15 | 30 |
| 4 | pIFNa + pHE | 15 | 15 | 30 |
| 5 | pIFNc | 15 | 15 | 30 |
| 6 | pIFNc + pHE | 15 | 15 | 30 |

The table shows number of fish in each treatment group and time for sampling post vaccination (W = week).

started to increase at week 7, reached a peak at week 10 and then declined, but was still readily detectable at week 22 pv. In fish injected with pIFNc + pHE, antibody against ISAV was first detected at week 10 pv and reached a peak at week 16, which lasted at least through week 22. It is uncertain why the antibody response is more delayed with pIFNc than with pIFNa as adjuvant. The systemic induction of ISGs by pIFNc may possibly have a delaying effect on the adaptive immune response.

Similar antibody kinetic studies have previously apparently not been performed in DNA vaccinated salmonids. However, high titres of neutralizing antibodies were found in rainbow trout after multiple DNA vaccinations against VHSV and IHNV 38 and 45 days pv [7]. In another study, neutralizing antibodies were observed in trout 3 months after DNA vaccination against IHNV, but not at later time points although protection was observed 3—25 months after vaccination [8].

In carp, DNA vaccination with a β-galactosidase construct showed appearance of antibodies against β-gal at day 7 pv, which increased until day 14 pv and kept at this level throughout the 70 day trial period [9]. The earlier antibody response in carp may be due to the higher temperature (22 °C) used for this fish species.

### 3.2. Longevity of DNA vaccine constructs measured by IFNc-induced expression of Mx and ISG15 in liver

We have previously shown that pIFNc induces systemic induction of Mx and ISG15 in salmon while IFNa plasmid did not [3]. A possible explanation for this is that IFNc is more stable than IFNa and thus survives longer in circulation [3]. Because of this, we reasoned that expression of Mx and ISG15 proteins in internal organs might be used to monitor longevity of cells that are transfected with pIFNc alone or co-transfected with pIFNc and pHE. Accordingly, we measured expression of Mx and ISG15 protein in liver of fish groups in the study of antibody kinetics described above (Figs. 2 and 3). pIFNa groups were omitted since vaccination with pIFNa does not induce Mx [3]. As shown in Fig. 2A, Mx protein expression in liver of fish injected with pIFNc alone was strong and slightly increasing during the whole 22 week trial period. This suggests that cells transfected with pIFNc are expressing IFNc at a similar level throughout 22 weeks and may express IFNc for an even longer time period. Longevity of IFNc expression is probably due to the fact that IFNc is a self-antigen in salmon. Cells transfected with pIFNc are thus not likely to be attacked by the adaptive immune system of salmon.
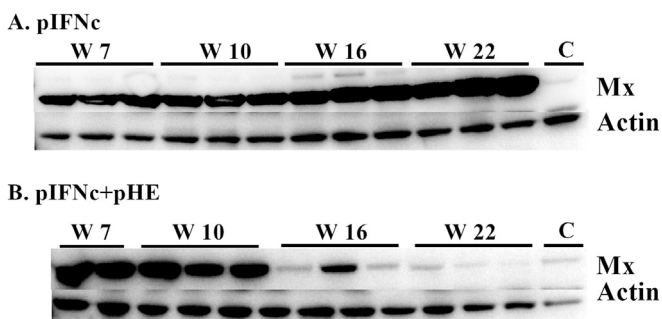


**Fig. 2.** Immunoblotting of Mx protein in liver of presmolts after i.m. injection of IFNc plasmid alone (A) or together with HE plasmid (B). Liver tissues were harvested after 7, 10, 16 and 22 weeks and analysed for Mx protein by immunoblotting. Each lane was loaded with 10 μg protein from one individual except in the last lane labelled C, which represents pooled protein extracts from four fish injected with control plasmid harvested 7 weeks pv. Blots were stained with antibody against Mx protein or actin (control for equal loading of protein). Protein bands from 3 individuals per time point are shown, except for pIFNc + pHE at 7 weeks pv where 2 individuals were used.
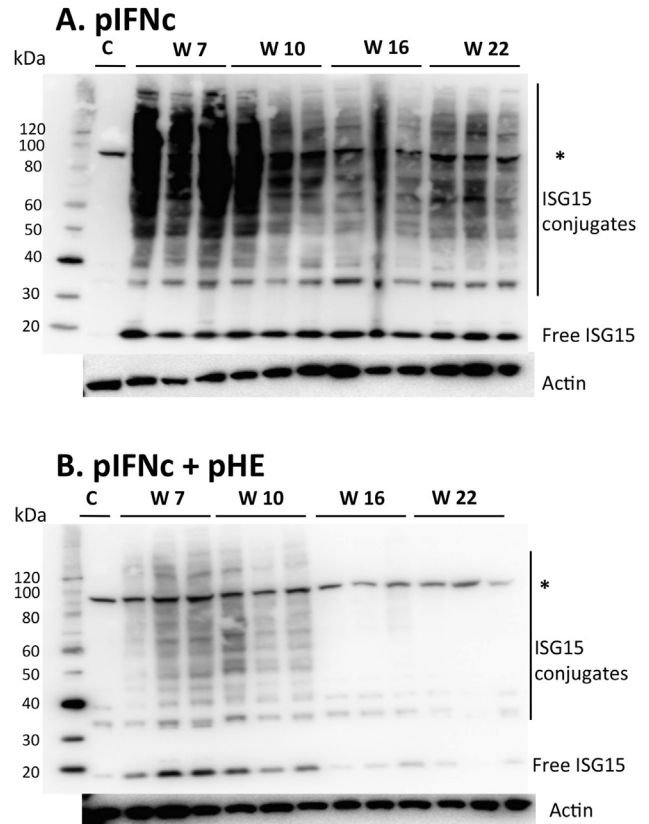


**Fig. 3.** Immunoblotting of ISG15 protein in liver of presmolts after i.m. injection of IFNc plasmid alone (A) or together with HE-plasmid (B). Sampling and loading of gels as in Fig. 2. Blots were stained with antibody against ISG15 protein or actin (protein loading control). Lane 1 shows the protein standard. Non-specific binding with the anti-ISG15 antibody was observed with a 90 kDa protein band (indicated with *).

Long-lasting expression of a transgene after DNA vaccination has also been shown for luciferase in Atlantic salmon, glass catfish and mice [10—12]. In salmon, luciferase activity in muscle was highest at day 10 pv, then declined, but was still detectable 535 days pv [10]. The longevity of luciferase in plasmid-injected fish and mice suggests that cells expressing luciferase are not attacked by the immune system due to poor immunogenicity of luciferase or due to its intracellular localization [13].

Expression of Mx proteins in liver of fish co-injected with pIFNc and pHE was strikingly different compared to fish injected with pIFNc alone during the 22 week trial period (Fig. 2B). During the first 10 weeks, Mx expression in liver was similar for fish injected with pIFNc or pIFNc + pHE. However, at week 16 pv two of three fish vaccinated with pIFNc + pHE showed no expression of Mx in liver while the third fish showed weak expression. At week 22 pv liver of fish vaccinated with pIFNc and pHE showed no expression of Mx. As it was possible that the durability of Mx protein in liver might be due to the stability of Mx aggregates in the cytoplasm such as described for human MxA [14], we analysed expression of ISG15 in liver as well (Fig. 3). In fish treated with pIFNc alone free ISG15 was expressed at similar levels at all time points (Fig. 3A). High levels of ISG15 conjugated cellular proteins were also observed at all time points, but seemed to occur at higher levels at week 7 pv compared to later time points (Fig. 3A). In fish vaccinated with pHE together with pIFNc, expression of free ISG15 and ISG15 conjugates followed a similar pattern as with Mx protein, being expressed at elevated levels at week 7 and 10 while only background levels were detected at week 16 and 22 pv (Fig. 3B). Taken

together, this suggests that transfected cells have taken up both pIFNc and pHE and have become destroyed by the adaptive immune response directed against HE-expressing cells at about week 16 after immunization. Another possibility would be that IFNc activated natural killers cells, which attacked HE-expressing cells. However, the disappearance of Mx/ISG15 expression should then have happened much earlier and be visible at week 7 in the present experiment since HE is expressed in the fish from week 1 pv [2].

Immune mediated destruction of antigen-expressing myocytes has been shown both in rainbow trout and mice following DNA vaccination [13,15]. DNA vaccination of trout with the G-protein from the surface of viral haemorrhagic septicaemia virus (VHSV) led to strong protection against infection while DNA vaccination with the nucleocapsid protein N gave little or no protection [16,17]. Co-injection of G-protein and luciferase encoding plasmids into the muscle of rainbow trout resulted in a rapid decrease of luciferase activity compared to fish injected with luciferase-plasmid and control plasmid [17]. The G-protein was expressed on the membrane of myocytes and these cells were most strongly stained between day 16–23 post vaccination [15]. The number of G-protein positive myocytes then declined and positive cells were hardly detectable 31–38 dpv [15]. The rapid decline in luciferase activity in trout co-injected with G-plasmid and luciferase plasmid and the disappearance of G-protein positive myocytes in trout injected with G-plasmid is likely to be mediated by adaptive immune mechanisms [15,17]. Destruction of cells expressing the antigen in DNA vaccines could be caused by attack by cytotoxic T-cells, but mechanisms involving antibodies may also be involved. Infiltration of inflammatory cells positive for MHC II, IgM and C3 was observed concomitant with peak expression of G-protein, and was suggested to cause the disappearance of G-protein positive myocytes [15]. Immune mediated destruction of antigen-expressing myocytes in mice has been shown after DNA vaccination with the hepatitis B virus antigen (HBsAG) [13]. Elimination of HBsAg expressing myocytes was found to be dependent on MHC II restricted CD4$^+$ T cell activation, but was not mediated solely by MHC I restricted or perforin-mediated lysis [18]. Destruction of HBsAg -expressing myocytes was suggested to be antibody dependent since HBsAg was expressed on the cell surface and since myocyte destruction was associated with a HBsAg specific antibody response [18]. Antibody-directed complement-mediated cytotoxicity and Fc receptor mediated antibody-dependent cytotoxicity were suggested as mechanism [19,20]. On the other hand, clearance of expression of the plasmid DNA vaccine antigen SIV GAG AL11 was mediated through the Fas/FasL pathway and CD4 T lymphocytes were found to be involved [21,22]. In that case, however, the antigen was expressed internally and not on the cell surface such as HBsAg.

The HBsAg mouse studies leads to the hypothesis that DNA vaccination of Atlantic salmon with ISAV HE is associated with an antibody dependent process since HE is expressed on the surface of cells and since cessation of Mx-expression in fish vaccinated with pIFNc + pHE occurred concomitant with peak antibody levels. At present little is known about induction of cytotoxic T-cell responses upon vaccination of salmonids and other fish species. However, cell-mediated cytotoxicity against VHSV-infected cells was detected among peripheral blood leukocytes from rainbow trout that had received multiple injections of a plasmid expressing the VHSV G-protein [23]. A long lasting, but slow T cell response in trout vaccinated with a plasmid encoding the G-protein of VHSV was also observed by using spectratyping methodology to study the modifications of rainbow trout TCRβ repertoire [24]. The role of T-cells in DNA-vaccination against ISAV has to await development of adequate cytotoxic T-cell assays for Atlantic salmon.

The disappearance of the HE/IFNc construct occurred much later than the reported disappearance of G-protein in DNA vaccinated rainbow trout. Whether this difference is due to difference in methods or the antigens is not known. However, the longevity of G-protein expression in trout has been based on staining of G-protein expressing muscle cells at the injection site [15], or measurement of luciferase activity in muscle after co-injection of G-protein plasmid together with a plasmid expressing luciferase [17]. These methods are dependent on hitting the exact place of injection after several weeks, which may be difficult. The present method is, however, independent of hitting the injection site.

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