Fish & Shellfish Immunology 33 (2012) 174–185

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Oral immunization of rainbow trout to infectious pancreatic necrosis virus (Ipnv) induces different immune gene expression profiles in head kidney and pyloric ceca

Natalia A. Ballesteros [a], Sylvia S. Rodriguez Saint-Jean [a], Paloma A. Encinas [b], Sara I. Perez-Prieto [a], Julio M. Coll [b,*]

[a] Centro de Biologia Molecular CSIC, 28040 Madrid, Spain
[b] Instituto Nacional Investigaciones Agrarias (INIA), Departmento Biotecnologia, crt. Coruna km 7, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 25 January 2012
Received in revised form
14 March 2012
Accepted 15 March 2012
Available online 10 April 2012

Keywords:
IPNV
Microarray
Oral vaccination
Alginates

ABSTRACT

Induction of neutralizing antibodies and protection by oral vaccination with DNA-alginates of rainbow trout *Oncorhynchus mykiss* against infectious pancreatic necrosis virus (IPNV) was recently reported [1]. Because orally induced immune response transcript gene profiles had not been described yet neither in fish, nor after IPNV vaccination, we studied them in head kidney (an immune response internal organ) and a vaccine entry tissue (pyloric ceca). By using an oligo microarray enriched in immune-related genes validated by RTqPCR, the number of increased transcripts in head kidney was higher than in pyloric ceca while the number of decreased transcripts was higher in pyloric ceca than in head kidney. Confirming previous reports on intramuscular DNA vaccination or viral infection, *mx* genes increased their transcription in head kidney. Other transcript responses such as those corresponding to interferons, their receptors and induced proteins ($n = 91$ genes), VHSV-induced genes ($n = 25$), macrophage-related genes ($n = 125$), complement component genes ($n = 176$), toll-like receptors ($n = 31$), tumor necrosis factors ($n = 32$), chemokines and their receptors ($n = 121$), interleukines and their receptors ($n = 119$), antimicrobial peptides ($n = 59$), and cluster differentiation antigens ($n = 58$) showed a contrasting and often complementary behavior when head kidney and pyloric ceca were compared. For instance, classical complement component transcripts increased in head kidney while only alternative pathway transcripts increased in pyloric ceca, different β-defensins increased in head kidney but remained constant in pyloric ceca. The identification of new gene markers on head kidney/pyloric ceca could be used to follow up and/or to improve immunity during fish oral vaccination.

1. Introduction

Oral delivery is considered the most desirable way to vaccinate both humans and animals [2,3]. Nevertheless, despite many efforts to find suitable fish oral vaccination methods, there are yet few reports describing their successful use. However, recent reports using either pathogen-coding DNA in trout [1] and in Japanese flounder (*Paralichthys olivaceus*) [4] or pathogen recombinant proteins in salmon [5,6], suggest fish oral vaccination might be possible in the future.

Oral vaccination methods are needed because present oil-adjuvanted vaccines delivered by intraperitoneal injection have important side effects on fish welfare [7–9], while the present licensed DNA salmon vaccines in Canada still require fish-to-fish intramuscular injection [10]. Furthermore, small immunocompetent fish to be vaccinated cannot be injected. However, oral vaccines have many difficult-to-obtain requirements such as to be protected from stomach digestion by some antigen-encapsulation method, adhere to fish guts, avoid induction of immune tolerance or induce immune responses in both local epithelial surfaces and internal organs. Because most of those necessary immune responses are not yet known, we have focused this study on trying to clarify those. In order to do that, the recently reported successful oral immunization of rainbow trout *Oncorhynchus mykiss* against infectious pancreatic necrosis virus (IPNV) with a DNA vector coding for the VP2 capsid gene of infectious pancreatic necrosis virus (IPNV), has been used. Alginate microspheres protected the DNA, which was expressed early and late in different organs of the vaccinated trout, induced neutralizing antibodies and protected 80% of the vaccinated trout [1,11].

IPNV is an economically important *Birnaviridae* which causes severe acute lethal infections in young salmonid fish, remaining one of the most widespread causing-disease virus in aquaculture

* Corresponding author.
E-mail address: juliocoll@inia.es (J.M. Coll).

1050-4648/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2012.03.016

[12,13]. The IPNV genome consists of two double-stranded RNA segments (A and B) that encode VP1 (a RNA-dependent RNA polymerase), VP2 (the major antigenic capsid protein and type-specific antigen with a great antigenic diversity), VP3 (an internal capsid protein and group-specific antigen), NS and VP5 (non structural proteins). The accepted serotyping of IPNV includes two serogroups, the first containing nine serotypes from fish (A_1–A_9) and the second containing a single serotype (B_1) [14]. Because following a disease outbreak, surviving fish may become asymptomatic carriers for life; broodstock carriage is considered an important source of IPNV for lethal infection of hatchery-reared fry. The development of an effective vaccine is a necessity to secure the future of salmonid (salmon and trout) farms.

To study the transcriptional profile of rainbow trout organs after successful oral immunization, we used a newly designed 60-mer oligo microarray enriched in immune-related genes. We studied not only one of the most important fish internal organs involved in fish responses to infection (head kidney) but also one of the entry sites of the vaccine: the pyloric ceca (mucosal immunity). The head kidney was selected as the target internal organ because other reports have demonstrated the presence of DNA at different times after oral vaccination in Atlantic salmon [15] or brown and rainbow trout [1,11]. On the other hand, pyloric ceca could offer a novel approach to study the immunity response at the gut mucosa, which might be especially important for oral vaccination. Results confirmed the induction of *mx1* transcripts in head kidney reported earlier for this and for other immunization methods. More immune-related transcripts increased in head kidney than in pyloric ceca after oral immunization. The study contributed to clarify the immune response to oral vaccination and allowed the identification of novel genes which can be used as markers to improve oral vaccination in fish.

2. Materials and methods

2.1. Preparation of the IPNV-VP2 DNA vector

The plasmid DNA vector (pcDNA-VP2) was prepared as described previously [1]. Briefly, the IPNV-VP2 gene was cloned into the pcDNA3.1/V5/His-TOPO plasmid (pcDNA) (Invitrogen) under the control of the immediate-early CMV promoter and amplified in *Escherichia coli* TOP10. The plasmid DNA was isolated with the Endofree Plasmid Maxi purification Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA concentration was measured in a spectrophotometer before it was aliquoted and conserved at −20 °C. The pcDNA was used as control plasmid.

2.2. Preparation of microspheres and formulation of the oral vaccine

The procedure for the preparation of the microspheres was described previously [1]. Briefly, 2.5 mL of 3% (w/v) of sodium alginate were mixed with 1.5 mL of 1 mg/ml of pcDNA-VP2 and the mixture stirred at 500 rpm for 10 min. This solution was then added to an Erlenmeyer flask containing 100 mL of paraffin oil and 0.5 mL Span 80, and the mixture was emulsified for 30 min at 900 rpm. Microspheres were prepared by drop-by-drop adding 2.5 ml of 0.15 M $CaCl_2$ to the emulsion and stirring for 2 h at 900 rpm. Microspheres were then collected by centrifugation at 1000*g* for 10 min, and were washed twice with 70% ethanol, lyophilized and stored at 4 °C.

2.3. Oral vaccination of rainbow trout

Rainbow trout (*O. mykiss*) of a mean weight of 1 g (mean size of 3.5 cm) were purchased from a spring water local farm with no history of viral disease. Two pools of 5 fish were tested by standard methods to confirm the absence of IPNV or any other salmonid virus by isolation using BF cells [16]. The trout were acclimatized for 2 weeks and kept under a 12/12 h light/dark regime at 15 °C in 350 L closed re-circulating water tanks (Living Stream, Frigid Units Inc., Ohio) at the "Centro de Investigaciones Biológicas" (CSIC, Madrid, Spain). Groups of 20 trout were maintained in separate 45 L tanks supplied with non-chlorinated water using exterior carbon filters (Eheim) and additional aeration. The trout were fed daily with a diet of commercial pellets. Trout were obtained from a unique farm but from 4 different trout populations, one population per group. Each of the 4 groups was divided into 2 subgroups of 6 trout each. First subgroup was orally vaccinated with 10 μl of suspension of the vaccine microspheres each containing 10 μg of pcDNA-VP2 diluted in 10 μl of PBS, while second subgroup received similar amounts of microspheres with pcDNA. Vaccination was performed with an automatic pipette with a 20 μl tip which was introduced into the mouth of each trout, supporting the tip end at the entrance of the esophagus. The water-quality parameters were maintained at optimum levels and the conditions in all tanks were equal.

The trout were anaesthetized by immersing in 50 mg/mL tricaine-ethanesulfonate (MS-222, Sigma, Madrid, Spain) buffered in PBS prior to handling. After decapitation, the head kidney and pyloric ceca were harvested from each trout. The organs were immediately immersed in RNAlater (Ambion, Austin, USA) and kept at 4 °C overnight before being frozen at −70 °C until processed. Experimental protocols were performed with the approval of the CSIC ethical committee.

2.4. RNA extraction of trout head kidney and pyloric ceca

RNA was extracted from each individual trout head kidney and pyloric ceca after sonication (1 min × 3 times at 40 W in ice) in the RTL buffer and by using the RNAeasyPlus kit (Qiagen, Hilden, Germany). RNA concentrations were estimated by nanodrop and the presence of 18 and 28 S bands confirmed by denaturing RNA agar electrophoresis (Sigma, Che.Co, MS, USA). Stringent RNA quality control was performed prior to hybridization. For each group, 4 of the best quality RNA per group were pooled and further analyzed.

2.5. Design of oligo microarrays enriched in rainbow trout immune-related genes

To design the immune-related gene enriched microarray used in these experiments, rainbow trout sequences were selected from both GenBank and Agilent's EST-derived oligo microarray (ID16271) [17]. The immune-related genes were retrieved by using the following keywords: interferon, chemokine, interleukin, cytokine, defensin, macrophage, lymphocyte, antimicrobial, neutrophil, leukocyte, cytotoxic, natural killer, antiviral, antibacterial, LPS, Vig, antigen, histocompatibility, phagocyte, viral, Mx, complement, immunoglobulin, hepcidin, IgG, IgM, Toll, T cell, B cell, dendritic, presenting, TNF, perforin, MHC, NK, transcription, chaperone, stress, Hsp, Hsp70, Hsp90, tlr, flagellin, keratinocyte, cathepsin, NOD, IRF, IKK, JBK1, TRAM, TAK, TAB, JNK, P38, AP-1, TIRAP, IgT, IgH and high mobility. To simplify the analysis of results, the probes were classified according to 16 groups: AM, antimicrobial peptides; C, complement components; CD, cluster differentiation antigens; CK, chemokines; HSP, heat shock proteins; IFN, interferons; IG, immunoglobulins; IL, interleukins; MA, macrophage; MHC, major histocompatibility; MX, interferon-inducible proteins mx; TCR, T cell receptors; TLR, toll-like receptors; TNF, tumor necrosis factor; TR, transcription factors; and VIG, VHSV-induced genes. The resulting list of retrieved gene accession numbers and/or genes and sequences was formatted in excel, duplicates eliminated and the