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Evaluation of the immune response against immature viral particles of infectious pancreatic necrosis virus (IPNV): A new model to develop an attenuated vaccine

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ABSTRACT

Infectious pancreatic necrosis virus (IPNV) is a worldwide problem affecting both freshwater and seawater fish.

Vaccines developed against IPNV are not as efficient in the field as they are in tests. Moreover, research in the development of vaccines against IPNV has often shown that vaccines can stimulate the immune response of fish antibodies but do not protect efficiently against IPNV. In fact, sometimes dead infected fish show high antibody titers against IPNV. This suggests that the magnitude of total antibodies stimulated by the vaccine is not necessarily related to the level of protection against IPN, suggesting that a new method is needed to evaluate vaccine stimulation of the immune system. We propose in vitro evaluation of the non-specific cytotoxic cells (NCC) of the innate immune response, in addition to humoral specific response.

Moreover, it is necessary to develop innovative methods to improve fish vaccines. In this work, IPNV replicative intermediaries (provirus) were used to inject rainbow trout fry, which is the most vulnerable state to IPNV. To evaluate the immune response triggered by this vaccine, NCC and total and neutralizing antibodies against IPNV and the provirus were determined. Results indicated that NCC activity in rainbow trout fry is triggered by IPNV infection. Both IPNV and the provirus stimulate humoral and NCC immune response in rainbow trout fry. Although the total antibodies triggered by the provirus were half of that triggered by IPNV infection, the number of neutralizing antibodies was similar in the two treatments. This suggests that the ratio of neutralizing antibodies is higher among the antibodies stimulated by provirons than among those stimulated by IPNV infection. Thus, immature provirus is sufficient to activate immune response and is a good candidate as an attenuated vaccine in rainbow trout fry. In addition, neutralizing antibodies, together with non-specific cytotoxic activity, are a more suitable strategy to evaluate new vaccines than humoral immune response alone.

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1. Introduction

Infectious Pancreatic Necrosis (IPN) is a viral disease affecting several fish species worldwide [1–5]. IPNV infects salmonids in both freshwater and seawater, typically when fish are under stress. Indeed, fry are more susceptible shortly after hatching, reaching mortalities of 10–90%. Post-smolt fish are more susceptible after transfer to seawater, where mortalities can be around 10–50% [6]. In addition to the mortality caused directly by viral infection, the virus also causes immunosuppression in fish, making them more vulnerable to other pathogens [7–10]. Viral transmission takes place both vertically (fish to eggs) and horizontally (fish to fish, 11). In addition, surviving fish become asymptomatic carriers of the virus, disseminating it in the environment [6,11].

The causative agent of IPN belongs to the *Birnaviridae* family and has a naked icosahedral capsid of 60 nm in diameter [12,13] that encloses two segments of double-stranded RNA (dsRNA), denominated as segments A and B [14]. Segment A contains a large open reading frame (ORF) that codifies for a polyprotein. The polyprotein is the precursor of capsid proteins VP2 and VP3 and the protease VP4. VP4 processes the polyprotein allowing the releases of viral proteins and the maturation of the virus [15,16]. Segment B encodes VP1 protein that can be found free as RNA-dependent RNA-polymerase [17–19] or covalently linked to each the 5′-end of

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both genomic segments, where it is denominated VPg, and acts as a primer of RNA replication [20].

During the assembly of the virus in the infective cycle, genomic RNA is encapsulated in a large low-density particle (particle A or provirus), which is immature and non-infective. Its capsid contains both processed and unprocessed viral polyproteins. These provirus can be visualized as open particles by electron microscopy [21]. Upon maturation, particle A yields mature particle B through the proteolytic cleavage of the remaining viral precursors catalyzed by viral protease VP4, leading to the compaction of the viral particle and viral maturation. The maturation process can be blocked by iodoacetamide (IAA), which inhibits VP4 and other Cys-proteases by covalently binding to the thyol group of catalytic cysteine residue [22]. In fact, it has been reported that in vitro IAA treatment causes an accumulation of provirus in viral preparation [21].

Vaccines developed against IPNV are not fully protective and are often aimed at juvenile to post-smolt salmonids, leaving younger fish vulnerable to the disease [23]. Vaccines against IPNV seek to stimulate immune response by producing neutralizing antibodies in rainbow trout [24], often using the most immunogenic protein, VP2 [25,26]. In fact, IPNV can be neutralized by antibodies in the serum of infected fish, such as: rainbow trout [24,27], Atlantic salmon [28], brook trout [29] and striped bass [30]. However, vaccines based on recombinant VP2 or the complete segment 2 are not sufficiently protective against IPNV infection [31]. Likewise, neither the viral-like particle (VLP) vaccine, which resembles a viral capsid [32,33], nor DNA vaccines, which provoke viral protein expression within the cell [34], elicit protective immunity against IPNV, although they stimulate antibody response.

Studies indicated above suggest that the stimulation of antibodies by the vaccine in fish is not necessarily indicative of the ability of the vaccine to protect against the pathogen. Thus, it is necessary to develop a new system to evaluate in vitro the immune system triggered by vaccines to predict their effectiveness.

Studies conducted until now have shown that the immune response of fish shares some characteristics with mammalian immune response in that both show humoral and cellular immune responses (reviewed in [35]. IgM was the first and is the prevailing antibody isotype identified in teleosts [36–39]. Recently, IgD [40–43] and IgT have been described [44–46]. Antibody response in teleostei displays low affinity maturation and there is no evidence of isotype switching [36,47]. This could be why available vaccines that are able to trigger an antibody response against some viruses have not been able to prevent new outbreaks [48].

The cellular immune response is present in fish, as in higher vertebrates. Both elasmobranchii and teleostei show non-specific cytotoxic cells (NCC) that kill allogeneic (from the same species), xenogeneic (from a different species) and virus-infected target cells through the release of granzyme and perforins. Although fish NCC have different morphological characteristics from mammalian natural killer (NK) cells, such as being smaller and agranular [49], it has been proposed than NCC are the equivalent in fish to mammalian NK [50]. The NCC population in channel catfish is heterogeneous at the level of receptor expression: some NCC expresses both TCR and NK markers. The NCC population present in organs, but not in blood, expresses a surface protein receptor termed non-specific cytotoxic cells (NCCRP-1, 49) in channel catfish, zebrafish, tilapia, gilthead seabream and common carp (reviewed in 50). NCCRP-1 functions in both protein recognition and NCC toxicity (reviewed in 51). Moreover, markers such as FcμR, causes that antibodies can increase the ability of fish NCC to kill target cells [51]. In rainbow trout, the NK cell enhancement factor (NKEF) is the only identified marker of its NK-like cell [52].

Although NCC mediated response against viral infection is important in higher vertebrates, there have been only a few studies of cytotoxic cell activity against virus-infected cells in fish [53–55]. For example, NCC cells represent the first cellular innate immune response against the hemorrhagic septicaemia virus (VHVS) in fish [56]. In crucian carp (*Carassius carassius*) it has been shown that a very small fraction of leukocytes can lyse syngeneic cells infected with IPNV [57]. There is evidence that IPNV immunosuppresses NCC activity in kidneys and peripheral blood of juvenile rainbow trout [10], but there are no studies of NCC activity in younger fish.

As stated above, vaccines developed against IPNV are not effective, probably because they are designed to stimulate only the specific antibody response. Therefore, we changed the strategy of vaccine design: This study evaluates the immunostimulatory capacity of an IPNV intermediary that could activate innate antiviral immune response at the level of non-specific cytotoxic cells.

Therefore, provirions, resembling an attenuated vaccine, were used to stimulate the immune response of rainbow trout fry which is mostly affected by IPNV that later stages of development of the fish. Total and neutralizing antibodies and nonspecific cytotoxic activity of treated fish were determined and compared to that of IPNV infected fish.

2. Material and methods

2.1. Virus and cell culture

The IPNV VR299 strain was cultured in CHSE-214 cells in minimal essential medium (MEM, Gibco) supplemented with 2% fetal bovine serum (FBS, Gibco), 50 μg/mL gentamycin (Gibco) and 25 U/mL nystatin (Gibco), at a multiplicity of infection levels (moi) of 1 plaque forming units (PFU)/cell. The virus was adsorbed for 1 h with back and forth mixing at 15 °C. After adsorption, cells were washed three times with phosphate-buffered saline (PBS: NaCl 137 mM, $Na_2HPO_4 \times 2H_2O$ 10 mM, KCl 2.7 mM, KH_2PO_2 2 mM pH of 7.4, Winkler), and MEM supplemented with 2% FBS and antibiotics was added. This point is defined as 0 infection time. Infected cells were cultured until the cytopathic effect was visible, at 24–48 h post-infection (hpi). The supernatants of culture were then tittered by plaque formation assay, as described by [58], aliquoted and stored at −20 °C.

To obtain provirus, cells were infected as described above, but at 12 hpi cells were partially disrupted with Tris–HCl 3 mM pH 8; $MgCl_2$ 0.5 mM and NaCl 3 mM (Sigma), and 3 mM of iodoacetamide (IAA, Sigma) was added. Cells were homogenized with a Dounce homogenizer, returned to the culture bottle and incubated in MEM 2% FBS until 24 hpi, at which point cells were subjected to 3 freeze–thaw cycles. Cell debris was then removed by centrifugation at 5000 × *g* for 10 min at 4 °C. Viral particles were concentrated by ultracentrifugation at 130,000 × *g* for 2 h at 4 °C, suspended in ice-cold 50 mM Tris–HCl (pH 8.0), and then partially purified by ultracentrifugation through a 25% sucrose (Sigma) in 50 mM Tris pH 8 cushion at 145,000 × *g* for 4 h at 4 °C. Provirions were suspended in PBS and proteins were quantified by Biuret assay.

To detect proviral and viral particles by electrophoresis, IPNV infected CHSE-214 was pulsed at 4 hpi with 25 μCi/mL of [^{35}S] methionine (Perkin Elmer) for 4 h in methionine-deficient MEM (Gibco). At 12 hpi infected cells were treated or not with 3 mM of IAA, as described above, and allowed to complete 24 hpi. To ensure that the disruption treatment did not interfere with the viral replicative cycle, metabolically labeled cells were treated with lysis buffer in the Dounce homogenizer in absence of IAA, and then incubated until 10, 12, 14, 16, 18 and 24 hpi.

For analysis with Tris–glycine agarose (TGA) electrophoresis, radioactive labeled viral particles were suspended in the TGA loading buffer containing Tris–HCl 3 mM pH 8, NH_4Cl 66 mM (Sigma), Magnesium acetate 3 mM (Sigma), potassium acetate

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