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Research paper

Interaction of the attenuated recombinant rIHNV-Gvhsv GFP virus with macrophages from rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

One of the most important threats to the salmonid aquaculture industry is infection caused by novirhabdoviruses such as infectious haematopoietic necrosis virus (IHNV) or viral haemorrhagic septicaemia virus (VHSV). Using reverse genetics, an avirulent recombinant rIHNV-Gvhsv GFP strain was generated, which was able to replicate as effectively as wild type IHNV in a fish cell line and in macrophages. Although this recombinant virus induced protective responses against IHNV and VHSV, the response did not involve the production of antibodies or modulate the expression of some antiviral genes. To determine the immune mechanisms underlying the protection conferred by the rIHNV-Gvhsv GFP virus, different immune parameters (NO production, respiratory burst activity and the induction of apoptosis) were assessed in the macrophage population. The results obtained in the present work may indicate that the Nv protein could be important in the modulation of NO and ROS production. rIHNV-Gvhsv GFP did not appear to have a clear effect on nitric oxide production or apoptosis. However, an increased respiratory burst activity (with levels induced by the recombinant virus significantly higher than the levels induced by the wild type virus), suggests a stimulation of the macrophage population, which could be related to the protection against virulent viruses.

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1. Introduction

Vaccination has an important role in large-scale commercial fish farming and has been a key reason for the success of salmonid fish cultivation. This culture has been highly affected by infections caused by the infectious haematopoietic necrosis virus (IHNV) and viral haemorrhagic septicaemia virus (VHSV), the two major causes of mass mortality (Bootland and Leong, 1999; Smail, 1990). Although a lot of research is being done for the control of these diseases, only one live attenuated vaccine for VHSV is available in Germany (Enzmann, Tübingen), and Novartis Animal Health (Switzerland) commercialised a DNA vaccine against IHNV (Apex®-IHNV) for use in Canada.

Although alternative methods to the traditional formulations such as live virus vaccines (Sommerset et al., 2005), recombinant DNA vaccines (Lorenzen et al., 2002) or live recombinant virus (Biacchesi et al., 2002; Romero et al., 2008) have been tried to generate vaccines, their development is limited by safety concerns for the consumer and for the environment. Using reverse genetics methodology, a new line of live recombinant IHNV strains were developed by Biacchesi et al. (2000a,b, 2002) and tested in vaccination trials (Romero et al., 2005, 2008; Novoa et al., 2006). Two of the six IHNV genes were modified in the recombinant virus used in the present study (rIHNV-Gvhsv GFP). One of them was the G glycoprotein gene which encodes the G protein, involved in viral pathogenicity and capable of eliciting protective antibody production against various IHNV

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strains (Engelking and Leong, 1989). It was replaced with the gene from VHSV. The amino acid homology between the G protein of IHNV and VHSV indicates a high degree of structural and functional similarity between the two fish rhabdovirus glycoproteins (Lorenzen et al., 1993). Although the G-VHSV protein is able to induce nonspecific protection against IHNV in experimental challenges in fish (Lorenzen et al., 1998; Kim et al., 2000) the production of specific antibodies is restricted by the epitope structure of VHSV (Engelking and Leong, 1989; Lorenzen et al., 1990). The other gene modified was the small non-virion protein Nv, which has been proven to be nonessential for recombinant IHNV, although its deletion affects replication in cell culture (Thoulouze et al., 2004). Therefore, the Nv gene can be used as a site of insertion for foreign genes and can serve as vector for expressing additional antigens. This non-structural Nv gene was replaced with the green fluorescent protein (GFP). We have previously demonstrated that this recombinant virus was apathogenic for zebrafish and rainbow trout. Moreover, vaccination trials showed that it was able to induce protective responses against experimental infections with IHNV or VHSV in both species (Novoa et al., 2006; Romero et al., 2008). However, we observed that the non-specific protective response analysed by measuring the gene expression level of some antiviral genes, and the specific immune response evaluated through the antibodies production, did not appear to be involved in this protection, and it was suggested that other immune mechanisms could be responsible for the protection conferred by the rIHNV-Gvhsv GFP virus (Novoa et al., 2006; Romero et al., 2008).

Leukocytes are target cells for the replication of IHNV (Chilmonczyk and Winton, 1994). The viral replication occurs fast in cell culture being detected the first ultra-structural changes of the cytoplasm as early as 24–36 h post-infection by electron microscopy (Björklund et al., 1997; Kazachka et al., 2007). Moreover, the viral titre of IHNV peaked at 2 days post-infection in rainbow trout leucocytes (Chilmonczyk and Winton, 1994). The cycle of infection occurs by series of well described events in the following order: adsorption, penetration and uncoating, transcription, translation, replication, assembly and budding (Bootland and Leong, 1999). The morphogenesis and replication cycle of the recombinant virus rIHNV-Gvhsv GFP was analysed using transmission electron microscopy (TEM) and compared with wild type IHNV to assess if the changes introduced in the viral genome modified the efficacy of viral replication.

Leukocytes constitute an important part of the cellular defence against bacterial and viral infections in fish (Secombes, 1994) by secreting reactive oxygen and nitrogen intermediates and by their phagocytic capacity (Marletta et al., 1988; Nathan and Hibbs, 1991; Secombes and Fletcher, 1992). Taking this into account, we analysed in primary cell cultures enriched with kidney macrophages different immune mechanisms triggered by the viral infection (IHNV or rIHNV Gvhvs GFP), such as NO production, respiratory burst activity and also the induction of apoptosis, to better understand the mechanisms underlying the protection induced by the rIHNV-Gvhsv GFP virus.

2. Materials and methods

2.1. Virus titration

IHNV (French isolates 32/87) (Laurencin, 1987) and the recombinant viruses rIHNV-Gvhsv GFP, rIHNV GFP (Novoa et al., 2006) and rGvhsv (Romero et al., 2005) were propagated in the fish epithelial cell line EPC, which is derived from common carp (*Cyprinus carpio*) (Tomasec and Fijan, 1971). EPC cells were cultured in Eagle's minimum essential medium (MEM Invitrogen, GIBCO) supplemented with 10% foetal bovine serum (FBS Invitrogen, GIBCO), penicillin (100 IU/mL) (Invitrogen, GIBCO), and streptomycin (100 μg/mL) (Invitrogen, GIBCO), and buffered with 7.5% sodium bicarbonate (Invitrogen, GIBCO), and were incubated at 20 °C. The viruses were inoculated on EPC cells grown in MEM with antibiotics and 2% FBS at 15 °C. When the cytopathic effect (CPE) was complete, the supernatants were harvested and centrifuged to eliminate cell debris. Viruses were then titrated according to Reed and Muench (1938).

2.2. Primary cell cultures enriched with kidney macrophages

Primary cell cultures enriched with macrophages from rainbow trout (mean weight 22 g) were obtained (Secombes, 1990). Briefly, the anterior kidney was removed aseptically and passed through a 100-μm nylon mesh using Leibovitz medium L-15 (Invitrogen, GIBCO) supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL), heparin (10 U/mL) (Invitrogen, GIBCO) and 2% FBS. The resulting cell suspension was placed on a 34–51% Percoll density gradient (GE Healthcare) and centrifuged at $500 \times g$ for 30 min at 4 °C. The interface cells were collected and washed twice in L-15 containing 0.1% FBS, spinning at $500 \times g$ for 5 min. The viable cell concentration was determined by trypan blue exclusion. Cells were resuspended in L-15 with 0.1% FBS and dispensed into 24-well plates at a concentration of 10^6 cells/mL. Adherent cells were attached to the bottom of the wells by incubating 3 h at 18 °C. After this period supernatants and non-adherent cells were removed. All the animal experiments were reviewed and approved by the CSIC National Committee on Bioethics.

2.3. Cell infections

Primary cell cultures enriched with kidney macrophages were infected with the rIHNV-Gvhsv GFP virus or wild type IHNV at a multiplicity of infection of 1 (MOI 1). After 30 min of adsorption, cells were washed and incubated at 15 °C in L-15 medium supplemented with 2% FBS. Confluent EPC cell cultures were also infected with IHNV or rIHNV-Gvhsv GFP virus as positive control groups. Infected cell cultures were sampled at 24, 48 and 72 h post-infection (p.i.). Samples were frozen and thawed twice to release the viral particles inside the cells. After centrifugation at $12{,}000 \times g$ for 5 min, the supernatants were stored at −80 °C until use. Titration of supernatants was measured in triplicate according to the protocol

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