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Short Communication

Ribavirin stimulates the immune response of Atlantic salmon

A. Rivas-Aravena[a,b,*], S. Guajardo[c], B. Valenzuela[d], J. Cartagena[a], M.I. Imarai[d], E. Spencer[a], A.M. Sandino[a,c]

[a] Laboratorio de Virología, Centro de Biotecnología Acuícola, Universidad de Santiago de Chile, Santiago, Chile
[b] Departamento de Aplicaciones Nucleares, Comisión Chilena de Energía Nuclear, Santiago, Chile
[c] Activaq S.A. General del Canto 460, Santiago, Chile
[d] Laboratorio de Inmunología, Centro de Biotecnología Acuícola, Universidad de Santiago de Chile, Santiago, Chile

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ABSTRACT

Ribavirin is a synthetic nucleotide analog capable of inhibiting or even preventing some viral infections in mammals and also in fish. It has been seen by others that ribavirin by itself is able to stimulate the immune system of mammals, causing a differentiation of T-cells to T helper 1 cells (Th)-1. In this work, we evaluated the immune effect of ribavirin *in vitro* on kidney cells from Atlantic salmon and *in vivo* by oral administration of ribavirin to Atlantic salmon. For this purpose, the transcripts of immune molecules Tbet, GATA3, CD8, CD4, IFNα, IFNγ, IL-4/13, IL-10, IL-12, IL-15 and TGF-B were quantified. The results show that ribavirin administered orally in food to Atlantic salmon increased IFNγ and CD4 transcripts in the *in vivo* assays and, in addition, increased IL-12, IL-15 and CD8 in the *in vitro* analyses, indicating that the treatment stimulates a Th1 type response in salmon.

1. Introduction

Ribavirin is a synthetic nucleotide analog used as inhibitor of a large number of viruses in mammals and other vertebrates (Beaucourt and Vignuzzi, 2014). In fish, it has been reported that some viral infections are inhibited by ribavirin. In particular, infections with viral hemorrhagic septicemia virus (VHSV) (Marroqui et al., 2007), chum salmon reovirus (CSV) (DeWitte-Orr and Bols, 2007), infectious pancreatic necrosis virus (IPNV) (Jashés et al., 1996; Migus and Dobos, 1980), infectious hematopoietic necrosis virus (IHNV) (Hudson et al., 1988) and infectious salmon anemia virus (ISAV) (Rivas-Aravena et al., 2011) have been shown to be dramatically reduced by ribavirin.

Ribavirin inhibits viral replication by several mechanisms. Inhibition of cellular inosine monophosphate dehydrogenase (IMPDH) by ribavirin reduces the intracellular pool of guanosine mono-, di- and triphosphate (GMP, GDP and GTP) (Leyssen et al., 2005; Oxford, 1975; Streeter DG et al., 1973; Sun et al., 2007), decreasing the rate of viral and cellular RNA synthesis (Jashés et al., 1996; Leyssen et al., 2005) and the capping of mRNAs (Bougie and Bisaillon, 2004; Goswami et al., 1979). Poliovirus polymerase 3Dpol can incorporate ribavirin triphosphate (RTP) as either GTP or ATP analog, resulting in several mutations on the gRNA in the progeny, which inhibit the infectivity of the poliovirus (Crotty et al., 2000, 2001). Incorporation of RTP into nascent genomic RNA could also cause an early termination of RNA synthesis or the misincorporation of cytidine and uridine, instead of guanidine or adenine producing a "lethal mutagenesis" in viral progeny (Crotty et al., 2000; Vignuzzi et al., 2005). Moreover, some viral

* Corresponding author at: Laboratorio de Virología, Centro de Biotecnología Acuícola, Facultad de Química y Biología, Universidad de Santiago de Chile, Avenida Libertador Bernardo O'Higgins 3363, Santiago, Chile. Tel.: +56 2 7181115; fax: +56 2 6812108.
E-mail address: andrea.rivas@usach.cl (A. Rivas-Aravena).

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polymerase (Cassidy and Patterson, 1989; Eriksson et al., 1977; Fernandez-Larsson et al., 1989; Prochaska et al., 1993; Severini et al., 1995; Wray et al., 1985), transcriptase (Rankin et al., 1989) and guanylyltransferase (Goswami et al., 1979) may be inhibited directly by ribavirin.

It has been documented that the administration of interferon-alpha (IFNα) and ribavirin can modulate the immune system in mammals. In fact, the combinatory therapy of IFNα and ribavirin in HCV patients caused higher CD4 proliferation than the administration of only IFNα and promoted a Th1/Th2T helper differentiation (Marinho et al., 2004). Furthermore, *in vitro* treatment with ribavirin alone in human T-cells could modulate the expression profile of some cytokines, increasing IL-2, IFNγ and TNFα and suppressing IL-4/13, IL-5 and IL-10, allowing the differentiation to Th1 cell (Tam et al., 1999b). This effect of ribavirin on the modulation of cytokines was also shown *in vivo* in a mouse model (Tam et al., 1999a). Other groups have also reported the Th1 polarization caused by ribavirin in humans and mice (Langhans et al., 2012; Martin et al., 1998; Ning et al., 1998; Trapero-Marugan et al., 2006).

Although ribavirin has inhibitory effects on some fish viruses, its effect on the immune system had not been evaluated yet. Knowledge on the immune system of fish is limited, but increasing numbers of cytokines and immune molecules, and also some T-cell markers in fish, have been identified (Reyes-Cerpa et al., 2012). The major transcription factors involved on Th1 and Th2 cell differentiation, Tbet and GATA3, respectively, were described on salmonids (Kumari et al., 2009; Wang et al., 2010). Besides, cytokines and immune molecules associated with Th1 (IFNγ, TNFα, IL-12), Th2 (IL-4/13) and regulatory T-cells (Treg; IL-10, TGF-β1) have been described in fish (Boschi et al., 2011; Castro et al., 2011; Liu et al., 2008; Moore et al., 2009). However, the development of antibodies against fish immune molecules has been scarce, and no commercial antibodies against salmon cytokines are available.

Usually, vaccines against viruses in aquaculture are not efficient enough to prevent disease. The lack of knowledge about the functioning of the immune system hinders the efficient design of vaccines. The major concern is that generally there is no correlation between the antibody response elicited by the vaccination and the vaccine protection against the pathogen (Cuesta et al., 2010; de las Heras et al., 2010; Dorson et al., 1978; Gomez-Casado and Coll, 2011; Koellner and Kotterba, 2002). Therefore, it is important to progress in the search for strategies that allow stimulating cellular immune response in fish mimicking the pathogen infection, thereby reinforcing the action of vaccines.

The present study investigated the effect of the administration of ribavirin in food to Atlantic salmon (*Salmo salar*). In order to clarify whether ribavirin exerts an effect on the immune response of fish, we analyzed the expression of immunological markers of T-cell differentiation *in vitro* and *in vivo*. Our results show that oral administration of ribavirin to Atlantic salmon can promote an immune response related to Th1 cells. These results gave us valuable information on the immune response of fish and on the use of ribavirin as an antiviral and immunostimulant.

2. Materials and methods

2.1. Treatment of Atlantic salmon with ribavirin

Healthy post-smolt Atlantic salmon with an average weight of 100 g were kept in 200 L tanks with 25 ppt of salinity at a density of 22 kg/m^3, a temperature of 14–18 °C and oxygen flow rate of 5.8–7.1 mg/L. Samples of fish were taken at this point to evaluate the initial levels of transcripts (day 0). Then, 168 fish were treated with 6.5 μmol of ribavirin/kg of fish for 10 days in the feed, in duplicate farming tanks as described (Rivas-Aravena et al., 2011). After that, the fish were fed without ribavirin. At days 4, 10, 20, 30 and 40 after treatment with ribavirin started, the spleens of 6 fish of each tank (12 fish in total) were extracted for posterior analysis. Analysis of the cytokine expression was performed using day 0 (before administration of ribavirin) as a control.

2.2. Treatment of kidney cells of Atlantic salmon with ribavirin in vitro

Atlantic salmon weighing 50 g were obtained from a local farm and were maintained at the immunology laboratory facilities at 15 °C in tanks at a biomass of 22 kg/m^3 with continuous oxygen support and fed daily with a commercial diet. After 2 weeks of acclimation, the kidneys of five fish were separately and aseptically removed and disaggregated in RPMI-1640 medium (Gibco), supplemented with 10% FCS, 4 mM L-glutamine (Hyclone), 40 μM 2-mercaptoethanol (Gibco) and 50 μg/mL gentamycin (USB Biological). Each cell suspension was filtered through a 100-mesh membrane filter, centrifuged at 400 × *g* for 10 min and the pellets were suspended in supplemented RPMI-1640.

To determine the effect of ribavirin on T-cell differentiation, 1×10^6 kidney cells per well obtained from each fish separately were seeded in 96-well plates in RPMI complete medium and incubated with 0, 0.5, 1 and 5 μg/mL of ribavirin for 48 h at 15 °C. Control cells were cultured without ribavirin. At the end of the experiment, the RNA was extracted and the cytokine expression levels were compared in treated and control cells.

2.3. Determination of cytotoxicity of ribavirin on cells of kidney

About 1.8×10^8 cells from the kidney were incubated with 0, 0.1, 0.5, 1, 5 and 7 μg/mL of ribavirin. After 48 h, cells were washed two times in PBS and incubated with 1 μg/mL of propidium iodide for 10 min. Cells were washed with PBS, and the percentage of viable cells was determined in an FACS CANTO II, BD (Becton Dickinson) flow cytometer using the FL-2 channel. The cytotoxicity was evaluated from the dependence on the number of cells treated with ribavirin, relative to the number of control cells.