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Interferon type I responses to virus infections in carp cells: *In vitro* studies on Cyprinid herpesvirus 3 and *Rhabdovirus carpio* infections

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ABSTRACT

Interferons (IFNs) are secreted mediators that play a fundamental role in the innate immune response against viruses among all vertebrate classes. Common carp is a host for two highly contagious viruses: spring viraemia of carp virus (*Rhabdovirus carpio*, SVCV) and the Cyprinid herpesvirus 3 (CyHV-3), which belong to *Rhabdoviridae* and *Alloherpesviridae* families, respectively. Both viruses are responsible for significant losses in carp aquaculture. In this paper we studied the mRNA expression profiles of genes encoding for proteins promoting various functions during the interferon pathway, from pattern recognition receptors to antiviral genes, during *in vitro* viral infection. Furthermore, we investigated the impact of the interferon pathway (stimulated with poly I:C) on CyHV-3 replication and the speed of virus spreading in cell culture.

The results showed that two carp viruses, CyHV-3 and SVCV induced fundamentally different type I IFN responses in CCB cells. SVCV induced a high response in all studied genes, whereas CyHV-3 seems to induce no response in CCB cells, but it induces a response in head kidney leukocytes. The lack of an IFN type I response to CyHV-3 could be an indicator of anti-IFN actions of the virus, however the nature of this mechanism has to be evaluated in future studies. Our results also suggest that an activation of type I IFN in CyHV-3 infected cells can limit the spread of the virus in cell culture. This would open the opportunity to treat the disease associated with CyHV-3 by an application of poly I:C in certain cases.

1. Introduction

Interferons (IFNs) are secreted mediators that play a fundamental role in the innate immune response against viruses among all vertebrate classes [1,2]. In fish, the virus-induced interferons form a group of numerous genes, that, according to their biological and structural properties plus their cognate receptors, appear to be analogues of mammalian type I IFNs [3] and are assigned as IFN type I, or IFN Φ [1,4,5]. The type I IFN are produced and secreted by most types of infected cells and act like alarm proteins. Their antiviral effect is exerted through the binding of interferons to specific cell-surface receptors and triggering a signal transduction through the JAK-STAT signal transduction pathway. This leads to the induction of a wide array of interferon stimulated genes (ISGs), some of which encode for antiviral proteins [1]. Cyprinids express at least four different IFN type I (IFN Φ) genes (often duplicated), assigned as IFN-a1 (IFN Φ1), IFN-c1 (IFN Φ2), IFN c2 (IFN Φ3), and IFN-d1 (IFN Φ4) and which according to their cellular receptors and molecular sequences can be classified into two groups [3,4,6]. In common carp (*Cyprinus carpio*), only 3 sequences of IFN type I genes have previously been described, and in this article they will be referred to as IFN-a1, IFN-a2 [7] and IFN-a1S [8]. We adopted the gene names presented by Zou and Secombes 2011 [4] and based our nomenclature on phylogenic analyses (presented as a Supplementary File 1), which showed that all common carp genes are homologues of zebrafish (*Danio rerio*) IFN-a.

In teleosts, IFN type I responses were primarily analysed in salmonid fishes, which are mostly infected with RNA viruses, such as pancreas necrosis virus (IPNV) [9,10], infectious haemorrhagic necrosis virus (IHNV) [11], viral haemorrhagic septicaemia virus (VHSV) [12] and the infectious salmon anaemia virus (ISAV) [13]. In

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Cyprinids, IFN type I responses were studied during infections with the RNA virus: spring viraemia of carp virus (SVCV, *Rhabdovirus carpio*) in zebrafish [6] and common carp [14]. However, very little information is available about responses to alloherpesviruses, which are becoming a growing threat in aquaculture [15,16].

Common carp is a host for two highly contagious viruses: spring viraemia of carp virus and the Cyprinid herpesvirus 3 (CyHV-3), which belong to *Rhabdoviridae* and *Alloherpesviridae* families, respectively. Both viruses are responsible for significant losses in carp aquaculture, and have a major impact on the production and trade of farmed populations of common carp and its ornamental (koi) varieties in many countries worldwide [17,18]. SVCV is a bullet-shaped RNA virus which belongs to the *Rhabdoviridae* family and causes Spring viraemia of carp (SVC) disease. SVCV can cause significant mortalities of up to 20% in common carp, but it has also been reported in grass carp (*Ctenopharyngodon idella*), bighead carp (*Hypophthalmichthys nobilis*), silver carp (*Hypophthalmichthys molitrix*), and crucian carp (*Carassius carassius*). SVCV has spread all over the world, and is now a serious epidemiological threat to carp populations in Asia and Northern America [18–20]. The Cyprinid herpesvirus 3 (CyHV-3), which is also known as Koi herpesvirus (KHV) is a member of the genus *Cyprinivirus* [21] in the family *Alloherpesviridae* and the order *Herpesvirales* [22]. The genome of CyHV-3 is a 295-kbp, linear, double-stranded DNA molecule and encodes for 156 potential protein-coding open reading frames (ORFs) [23]. Since the first isolations in Israel, USA and Germany in the late 1990's [17,24] CyHV-3 has spread over many countries worldwide and may cause mass mortalities (up to 100%) in populations of common and ornamental (koi) carp [17,24,25]. This virus is one of the fastest spreading viruses in aquaculture and therefore is considered as one of the most dangerous carp pathogens since different fish species can be carriers [26,27].

The current knowledge of the innate immune response of common carp to these two viruses, and particularly to CyHV-3 is limited [14,28]. In this article we present an analysis of interferon type I responses during an *in vitro* infection of carp fibroblast-like cells derived from the common carp brain (CCB) cell line with CyHV-3 and SVCV viruses. The mRNA expression profiles of genes encoding for proteins with various functions during the interferon pathway, from pattern recognition receptors to antiviral genes, are described. This includes (i) toll like receptor 9 (TLR9) and TANK-binding kinase 1 (TBK1), (ii) the interferon regulatory factors (IRF3 and IRF7) [29], (iii) the interferon genes IFN-a1, IFN-a2, IFN-a1S (iv) the antiviral genes: viperin (Vig1 – VHSV-induced gene), RNA-specific adenosine deaminase (Adar), myxovirus resistance protein 3 (Mx3), protein kinase R (PKR), which is known also as eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2). Furthermore, we investigated the impact of the interferon pathway (stimulated with poly I:C) on the CyHV-3 titre and expression levels of CyHV-3 genes encoding for the following virus proteins; thymidine kinase (TK), capsid triplex protein (CTP), major capsid protein (MCP), DNA polymerase (DP) and DNA helicase (DH).

To the best of our knowledge this is the first approach describing and comparing the *in vitro* IFN type I system responses in teleosts to CyHV-3 and SVCV viruses. This approach can provide more insight into IFN responses in *Teleostei* against different virus classes, as these responses will be proven to be completely different in this manuscript.

2. Materials and methods

2.1. Cell cultures

Common carp brain (CCB) cells [24] were cultured in minimum essential medium (MEM) with Earle's salts (Sigma, Germany) supplemented with Non-Essential Amino Acids (NEAA; Sigma, Germany), 10% foetal bovine serum (PAA, Germany), 0.35% glucose, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma, Germany). Fathead minnow (FHM) cells [30] were cultured in MEM with Earle's salts supplemented with NEAA, 10% foetal bovine serum and 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cultures from both cell lines were incubated at 25 °C in a humidified atmosphere containing 2% CO_2.

2.2. Isolation and cultivation of head kidney leukocytes (HKL)

Specific pathogen free common carp (*C. carpio* L.) (52 ± 14 g) were lethally anesthetised in a bath with 0.5 g l^{-1} Tricaine (Sigma, Germany), the blood was collected from the caudal vein and the head kidney was removed and placed in wash medium (Roswell Park Memorial Institute, RPMI 1640 medium with 10 IU ml^{-1}Heparin, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin; Sigma, Germany). Cells were forced trough a polyethylene mesh (50 μm mesh size), and suspended in wash medium. After two washing steps, the cells were re-suspended in culture medium (RPMI 1640 with 2 mM L-glutamine, 1% heat inactivated carp serum, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin; Sigma, Germany) and cultured in 96 well plates at a concentration of 1×10^6 cells per well at 25 °C in a humidified atmosphere containing 2% CO_2 for 24 h.

2.3. Virus cultivation

CyHV-3 strain KHV I [17], was re-isolated from the skin of carp, which had been infected by intraperitoneal injection with the virus, according to a standard protocol. The virus was propagated in CCB cells for two passages. On day 5 post-inoculation (p.i.) of the virus in the cell culture, when a cytopathic effect (CPE) was clearly visible, the culture was mechanically lysed and the cellular debris was removed by centrifugation for 15 min at 3000× *g*. The infectivity of the virus was quantified in terms of a 50% tissue culture infective dose ($TCID_{50}$) assay, which was performed in 96 well plates with CCB monolayers according to the method described by Reed and Muench [31]. The virus-infected plates were incubated in a humidified atmosphere with 2% CO_2 at 25 °C and monitored daily for the appearance of CPE up to day 14 p.i. The virus stock suspension was divided into aliquots and stored at −80 °C until further use.

SVCV (isolate 56–70) was propagated in FHM cells for 3 consecutive passages and incubated at 20 °C. Releasing the virus from infected cells was conducted in the same manner as was described for CyHV-3.

2.4. Effect of poly I:C stimulation of CCB cells on CyHV-3 infectivity and replication

CCB cells were plated in 96-well plates ($n = 9$) and treated with 10 μg ml^{-1} of polyinosinic polycytidylic acid (poly I:C; Invivogen, USA) for 6 h or were left untreated as controls. After pre-incubation, CCB cells were infected with 1.5×10^2 $TCID_{50}$ of CyHV-3 in medium with or without poly I:C in a final concentration of 10 μg ml^{-1}. Nine plates, with one of each treatment, were used to determine the titre of the virus sample using the $TCID_{50}$ method at day 14 p.i.

2.5. Effect of a poly I:C stimulation of CCB cells on CyHV-3 infection dynamics

CCB cells were grown in sixty 25 cm^2 tissue culture vessels for 1 day until a monolayer was formed. On the following day, 21 culture vessels were treated with poly I:C in a concentration of 10 μg ml^{-1}