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Humoral and cytokine responses in giant groupers after vaccination and challenge with betanodavirus

Yuan-Kai Cheng [a], Yu-Chi Wu [a, b, **], Shau-Chi Chi [a, *]

[a] Department of Life Science, National Taiwan University, Taipei 10617, Taiwan
[b] Institute of Molecular Medicine, National Taiwan University, Taipei 10002, Taiwan

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ABSTRACT

Giant groupers were immunized with two dosages (V_{high} and V_{low}) of inactivated nervous necrosis virus (NNV) and subsequently challenged with NNV at 4 weeks post vaccination (wpv). Several indicators were used to analyze the protective effects of the NNV vaccine. The neutralizing antibody titer of fish serum mostly corresponded to the survival rate of immunized fish in the NNV challenge test. Extravascular IgM^+ cells were detected in the brains of both NNV-infected and noninfected groupers. After NNV infection, CD8α and IgM gene expression increased in the brains, indicating $CD8\alpha^+$ and IgM^+ lymphocyte infiltration. Moreover, the NNV load was not the highest in dead grouper brains, suggesting that this load in the brain was not the key factor for the death of groupers. However, the brains of dead fish showed the highest expression of the interleukin (IL)-1β gene, a neurotoxic factor in the brain. Therefore, IL-1β overexpression is likely to be associated with the death of NNV-infected groupers.

1. Introduction

Giant grouper (*Epinephelus lanceolatus*), a warm-water fish with a high commercial value in Asia, is susceptible to viral nervous necrosis (VNN), resulting in mass economic losses. VNN is caused by nervous necrosis virus (NNV), which is classified into the *Betanodavirus* genus of the *Nodaviridae* family. NNV is a non-enveloped virus, with a diameter of 20–34 nm, and contains two single-stranded, positive-sense RNAs (Mori et al., 1992). RNA1 encodes RNA-dependent RNA polymerase, and RNA2 encodes the capsid protein. A subgenomic transcript derived from RNA1, named RNA3, is present in infected cells during NNV infection and encodes the B2 protein (Sommerset and Nerland, 2004). B2 suppresses RNA silencing and results in the accumulation of viral RNA (Fenner et al., 2006; Iwamoto et al., 2005). Moreover, B2 is suggested to trigger mitochondria-mediated cell death (Su et al., 2009, 2014).

During disease outbreak, VNN-induced mass mortality is typically observed in larval and juvenile fish, the clinical signs of which include anorexia, lethargy, darker or paler skin depending on species, loss of equilibrium, and abnormal swimming behavior such as darting and spiral swimming. Moreover, the pathological characteristics of VNN are vacuolation and necrosis of the brain, retina, and spinal cord (Munday et al., 2002).

Various NNV vaccines have been developed to immunize different fish species, including bath immunization with inactivated vaccine in groupers (Kai and Chi, 2008), intraperitoneal injection with inactivated vaccine in groupers and Asian sea bass (Pakingking et al., 2009, 2010; Yamashita et al., 2005; Yamashita et al., 2009), intraperitoneal injection with subunit vaccine in turbot and Atlantic halibut (Husgag et al., 2001), oral subunit vaccine for groupers (Lin et al., 2007), intramuscular injection with subunit vaccine in groupers and European seabass (Liu et al., 2006; Tanaka et al., 2001; Thiery et al., 2006), and intramuscular injection with DNA vaccine in Asian sea bass (Vimal et al., 2016). All the vaccines induce high NNV-specific antibody titers and confer desirable protection with high survival rates. Moreover, the neutralizing antibody titer was suggested as a measure of vaccine

* Corresponding author.
** Corresponding author. Department of Life Science, National Taiwan University, Taipei 10617, Taiwan.
E-mail addresses: yuchiwu@ntu.edu.tw (Y.-C. Wu), shauchi@ntu.edu.tw (S.-C. Chi).

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efficacy against VNN in sevenband groupers (Yamashita et al., 2009). For evaluating the inactivated infectious pancreatic necrosis virus (IPNV) vaccine in Atlantic salmon, the antibody level has been suggested as a marker of vaccine-induced protection, and interferon (IFN)-α and Mx genes have been used as biomarkers of IPNV infection progression (Munang'andu et al., 2013). Therefore, analyzing the humoral response and immune gene expression after vaccination and virus challenge will facilitate the determination of the indicators of vaccine efficacy and identification of immune genes involved in the vaccine-induced immune response and viral disease progression.

The effects of different NNV vaccines on immune genes have been reported in different fish species. Immunization of virus-like particles of NNV upregulated immune genes associated with humoral, cellular, and innate immunity in the liver, spleen, and head kidney of orange-spotted groupers (Lai et al., 2014). Moreover, vaccination with the recombinant NNV capsid protein in Atlantic halibut increased the expression of cytokine genes in the spleen, including IL-6, IL-1β, and IFN-γ genes (Overgard et al., 2013). An inactivated NNV vaccine, developed in our laboratory, exerted desirable protection on grouper larvae against NNV (Kai and Chi, 2008). Our previous study focused on the immune gene expression of grouper larvae after bath and oral immunization with inactivated NNV (Kai et al., 2014). In this study, juvenile giant groupers were subjected to intraperitoneal immunization with two dosages of the inactivated NNV vaccine and intramuscular challenge. The humoral response and expression of several immune genes were analyzed, namely IFN-γ, Mx, IL-1β, and tumor necrosis factor (TNF)-α, CD8α and IgM genes. We discussed the association of these immune biomarkers with the survival rate of immunized fish after NNV infection and identified which immune responses were closely related to the death of NNV-infected fish.

2. Materials and methods

2.1. Fish, cell line and virus

Giant groupers (*Epinephelus lanceolatus*) with average body weight of 18.22 g were obtained from a hatchery farm in Xinying, Tainan, Taiwan. The groupers were reared at the salinity of 15 ppt at 26 °C. Six fish were randomly sampled for detecting NNV RNA2 in the brain by real-time PCR (Section 2.8), and the batch of groupers was confirmed to be NNV-free.

GF-1 cells were derived from the fin tissue of grouper, *Epinephelus coioides*, and maintained in Leibovitz's L-15 medium (Invitrogen) with 5% FBS (GIBCO) and incubated at 28 °C (Chi et al., 1999).

The NNV used for vaccine preparation and challenge was HGNNV strain, which was isolated from humpback grouper (*Cromileptes altivelis*) (Chi et al., 2003) and propagated in GF-1 cells. The NNV solution from culture supernatant of NNV-infected GF-1 cells was collected, titrated, and stored at −80 °C.

2.2. Vaccine preparation

The inactivated NNV vaccine was prepared by the method described in our previous report (Kai and Chi, 2008). NNV with titer at 10^{10} $TCID_{50}$ ml^{-1} was inactivated by 4 mM binary ethylenimine (BEI) at 25 °C for 72 h. The inactivation of virus was confirmed via three passages in GF-1 cells without appearance of any cytopathic effect (CPE). The inactivated NNV was diluted to the final concentration of 5×10^9 and 5×10^8 $TCID_{50}$ ml^{-1} as virus infectious titer before inactivation, and respectively mixed with an oil adjuvant AS for the ratio of 1:1 to obtain high- and low-dose NNV vaccines. The AS adjuvant was developed and provided by Dr. Hong-Jen Liang, Department of Food Science, Yuanpei University, Taiwan.

2.3. Vaccination

The groupers (N = 264) were anaesthetized by 100 mg L^{-1} tricaine methanesulfonate, MS-222 (Sigma) and divided into three groups (N = 88 fish per group). Two groups were intraperitoneally (i.p.) injected with high- and low-dose inactivated NNV vaccines (V_{high} and V_{low} groups: 2.5×10^8 and 2.5×10^7 $TCID_{50}$ per fish as virus infectious titer before inactivation), and the third group was i.p. injected with PBS (control group). The fish in each group were distributed into 3 tanks and reared at 15-ppt salinity at 26 °C. At 1, 2 and 4 weeks post vaccination (wpv), six fish from each group were randomly sampled. Another 5 fish without any injection were also sampled as noninjected group. The blood was collected from caudal vein, clotted at room temperature for 1 h, and then stored at 4 °C for overnight to shrink the blood clots. Sera were obtained by centrifugation at 10,000×*g* for 10 min, and then stored at −80 °C for detecting the titers of NNV-neutralizing antibodies and NNV-specific antibodies (Section 2.5 and 2.6). Afterwards, the fish were sacrificed by using MS-222 overdose. The head kidneys, spleens and brains were collected from the sacrificed fish, and stored at −80 °C for detecting immune gene expression (Section 2.8).

2.4. NNV challenge

Before NNV challenge, three groups of fish were acclimated to the salinity at 30 ppt within 7 days by increasing 5 ppt every two days, and then intramuscularly (i.m.) injected with HGNNV at a dose of 1.0×10^6 $TCID_{50}$ at 4 wpv. The fish in each group (N = 65 per group) were distributed into 3 tanks and reared at 30-ppt salinity at 28 °C. The survival rates of fish were recorded for 19 days and generated by using Kaplan-Meier method. The significant difference between groups was analyzed by Log-rank test. Five alive fish at 3 and 5 days post infection (dpi) and surviving fish at 19 dpi of each group were randomly sampled for collecting blood as described above. Afterwards, the fish were sacrificed, and the brains were removed. Half part of each brain was fixed with 3.7% formalin in PBS for immunohistochemistry staining (Section 2.7), and another half was stored at −80 °C for detecting immune gene expression (Section 2.8). The dead fish of V_{high}, V_{low} and control groups were collected at 8–12 dpi, and the brains of dead fish and non-challenged fish were also sampled for detecting immune gene expression (Section 2.8).

2.5. Neutralization test

Beta neutralization test (constant virus plus serially diluted antibodies) was modified for detecting NNV-neutralizing antibody titer in fish serum. Serum of each sample was 32-fold diluted, and then serially 4-fold diluted with L-15 medium. Each diluted serum was mixed with equal volume of viral supernatant containing 2000 $TCID_{50}$ ml^{-1} HGNNV. The mixtures were incubated at room temperature for 1 h, and added into four wells (0.1 ml $well^{-1}$) of the 96-well cell culture plate (Nunc) which was pre-seeded with GF-1 cells (5×10^3 cells per well). After viral adsorption at 28 °C for 1 h, the solution in wells was removed and replaced with fresh L-15 medium supplemented with 1% FBS. The plates were incubated at 28 °C for 6 days, and the CPE of cells was recorded. The 50% neutralization dose (ND_{50}) was defined as the inverse of the highest dilution factor of serum that results in half of the infected cells showing CPE. The

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