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Aquabirnavirus-induced Protection of Marine Fish against Piscine Nodavirus Infection

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ABSTRACT—Experimental dual-infections with a non-lethal aquabirnavirus (ABV) and a lethal betanodavirus (redspotted grouper nervous necrosis virus: RGNNV) were carried out in Japanese flounder *Paralichthys olivaceus* and sevenband grouper *Epinephelus septemfasciatus*. In the dual-infection group, ABV was intramuscularly (IM) injected into fish seven days before the IM-injection with RGNNV. In the experiments with flounder, a high expression of an Mx gene, a molecular marker for type I interferon(s) (IFN) production, occurred in the head kidneys and brains at Day 7 post-ABV injection. Although no mortality was found not only in the dual-infected group but also in the single infection group with RGNNV (control group), the infective titers of RGNNV in the tissues of the dual-infected group were significantly lower at any sampling times than those in the control group. In the experiments with grouper, the preceding ABV infection resulted in complete protection against RGNNV infection. The infective titers of RGNNV in the tissues were also lower in the dual-infected group than in the control group throughout the experiments, and finally the virus disappeared from the head kidneys and brains of the dual-infected group at Day 14 and Day 56 post-injections, respectively. These results suggest that an ABV-induced IFN(s) effectively suppresses the progression of secondary betanodavirus infection.

Key words: aquabirnavirus, betanodavirus, viral nervous necrosis, interferon, Mx protein, *Epinephelus septemfasciatus, Paralichthys olivaceus,* VNN

A variety of marine fish species for aquaculture in the world have been devastatingly affected by outbreaks of viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) caused by piscine nodaviruses (the Betanodavirus, Nodaviridae) (Muroga, 2001; Munday *et al.*, 2002; Office International des Epizooties, 2003). The disease occurs mostly at early developmental stages (larvae and juveniles) and is characterized by various neurological abnormalities such as erratic swimming behavior and extended vacuolation of the central nervous tissues and retina. Betanodaviruses have two positive-sense ssRNAs; RNA1 (3.1 kb) encodes the viral replicase and RNA2 (1.4 kb) encodes the coat protein (Mori *et al.*, 1992; Ball *et al.*, 2000) and currently are divided into four genotypes (striped jack nervous necrosis virus-type; SJNNV, tiger puffer nervous necrosis virus-type; TPNNV, redspotted grouper nervous necrosis virus-type; RGNNV, and barfin flounder nervous necrosis virus-type; BFNNV) based on the RNA2 sequences (Nishizawa *et al.*, 1997). Furthermore, the viruses are classified into three serotypes (A, B, C) according to the virus neutralization assay using the antiserum against each of the four viruses (Mori *et al.*, 2003). Among these viruses, RGNNV belonging to serotype C has been most frequently isolated from diseased warm-water marine fishes (Nishizawa *et al.*, 1997; Iwamoto *et al.*, 1999; Chi *et al.*, 2003; Thiery *et al.*, 2004) and the infection experiments proved that various marine fish species are particularly susceptible to RGNNV (Tanaka *et al.*, 2003, Peducasse *et al.*, 1999).

In Japan, aquaculture of sevenband grouper *Epinephelus septemfasciatus* has been severely damaged by VNN because this species is highly susceptible to RGNNV even at grow-out stages. As in VNN of striped jack *Pseudocaranx dentex* (Mushiake *et al.*,

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1994), elimination of virus-carrying broodfish by a PCR-based method and disinfection of fertilized eggs and rearing water by ozone proved to be effective for controlling the disease in larval and juvenile sevenband grouper (Tsuchihashi *et al.*, 2002). However, there are no means to prevent the disease in older fish during net-pen culture, though the protective effects of the recombinant coat protein or inactivated virus vaccines were experimentally demonstrated (Tanaka *et al.*, 2001; Yamashita *et al.*, 2005).

We previously demonstrated through dual-infection experiments with Japanese flounder *Paralichthys olivaceus* that primary infection with a non-lethal aquabirnavirus (ABV) could create a resistant state in fish against the subsequent infection with a lethal viral hemorrhagic septicemia virus (VHSV) (Pakingking *et al.*, 2003, 2004). The nonspecific protection lasted for at least 14 days post-ABV infection with the maximal protection occurring within 7 days post-ABV infection. Additionally, the nonspecific protection was substantiated by the presence of antiviral activity in the sera and the expression of an Mx gene, a molecular marker of type 1 interferon (IFN) production, in the head kidneys of flounder infected with ABV.

In order to find an alternate control method for VNN at grow-out stages in marine fishes, we investigated the protective effect of the IFN-like substance(s) induced by non-pathogenic ABV against betanodavirus infection, in two fish models; Japanese flounder and sevenband grouper.

Materials and Methods

Viral strains

Aquabirnavirus: A strain (FBV) of ABV originally isolated from Japanese flounder and used in our previous studies (Pakingking *et al.*, 2003, 2004) was employed as the primary agent in the present dual-infection experiments. ABV was propagated at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$/cell in rainbow trout gonad-2 (RTG-2) cell line maintained at 20°C in Eagle's minimum essential medium (MEM; Nissui, Japan) supplemented with 10% (V/V) fetal bovine serum FBS (= MEM_{10}), 150 IU/mL penicillin G and 50 μg/mL kanamycin sulfate. When the cytopathic effect (CPE) was extensive, the supernatant was harvested, clarified by centrifugation, and stocked at −80°C prior to use. In order to determine the viral inoculum dose injected to fish, a portion of the virus stock was titrated in 96-well plates seeded with RTG-2 cells and the virus infective titer (log_{10} $TCID_{50}$/mL) was quantified according to Reed and Muench (1938).

Nodavirus: A strain (SGWak97) of RGNNV originally isolated from diseased sevenband grouper (Iwamoto *et al.* 1999) was used as the secondary agent in the dual-infection experiments. RGNNV was propagated (MOI: 0.01 $TCID_{50}$/cell) in E-11 cells (Iwamoto *et al.*, 2000), maintained at 25°C in Leivovitz's L-15 medium (Gibco BRL, USA) supplemented with 5% FBS. The harvested culture supernatant was processed and stocked as in the case of ABV.

Experimental fish

Healthy Japanese flounder and sevenband grouper, both with an average body weight of 12 g, were produced and reared at Kamiura Sea-Farming Center, Fisheries Research Agency (formerly Japan Sea-Farming Association). Fish were acclimatized in the laboratory for 10 days and maintained in tanks supplied with sand-filtered and flow-through seawater at 20°C for flounder or 25°C for grouper. The absence of ABV and betanodaviruses in acclimatized flounder (n = 10) and grouper (n = 20) was ascertained by cell culture isolation using RTG-2 and E-11 cell lines.

Infection experiments

Japanese flounder: One hundred and twenty acclimatized Japanese flounder were randomly divided into two groups. One group was intramuscularly (IM) injected with ABV at a dose of $10^{6.6}$ $TCID_{50}$/fish and the other group was IM-injected with MEM_{10}. After kept in 100 L tanks with flow-through system at 25°C for 7 days, each five fish were sampled for Mx gene expression experiments and the remaining fish of both groups received IM-injection of RGNNV at a dose of $10^{8.6}$ $TCID_{50}$/fish. These fish were then divided into two groups, one consisting of 20 fish for mortality observation and the other of 35 for the kinetics of RGNNV in the organs, and were kept at 20°C. In the virus kinetics experiment, fish were examined at Days 3, 7, and 14 post-RGNNV infections for the infective titers of RGNNV in the head kidney and brain tissues of 10 or 15 individual fish.

Sevenband grouper: To examine the infectivity of ABV in sevenband grouper, 15 acclimatized fish were IM-injected with ABV at a dose of $10^{7.1}$ $TCID_{50}$/fish and maintained in a 100 L aquarium at 25°C. At 1, 2 and 3 weeks post-infection, ABV titers in the head kidney and brain tissues of each five randomly sampled fish were determined by using RTG-2 cells. The dual-infection experiment was carried out as in Japanese flounder described above. One hundred acclimatized grouper were equally divided into two groups; one was IM-injected with ABV at a dose of $10^{7.1}$ $TCID_{50}$/fish and the other was IM-injected with MEM_{10}. At Day 7 post-ABV or MEM_{10} injection, both groups were IM-injected with $10^{5.1}$ $TCID_{50}$/fish of RGNNV. Each group was then divided into two subgroups; 20 fish for mortality and the others for the kinetics of RGNNV in the organs, and were kept at 25°C. In the virus kinetics experiment, fish were examined at Days 3, 7, 14, 21, 28, and 56 post-RGNNV infection for the infective titers of RGNNV in the head

kidney and brain tissues of five individual fish (three fish for Day 14 as an exception).

Virus titrations

RGNNV and ABV infective titers in dual-infected fish were quantified following a protocol adapted from our previous study (Pakingking *et al.*, 2004). Briefly, RGNNV titration was carried out in 96-well plates seeded with E-11 cells after the sample homogenate was treated with a diluted rabbit antiserum (1:100) against yellowtail ascites virus (YTAV) to neutralize ABV infectivity. No residual ABV infectivity was present in the treated sample as ascertained by the absence of CPE in RTG-2 cells inoculated with a portion of the treated homogenate. The head kidney and brain tissues of fish solely infected with RGNNV were titrated using the same cell line without YTAV-neutralizing process. Since RTG-2 cells do not support RGNNV multiplication, the filtered homogenates were directly inoculated into RTG-2 cells to quantify ABV titers. Virus multiplication in E-11 or RTG-2 cells, that produced CPE, was confirmed by an indirect fluorescent antibody technique using rabbit antiserum against RGNNV or YTAV.

Mx gene expression

The detection of Japanese flounder Mx (JFMx) gene expression in the head kidney and brain tissues of Japanese flounder at Day 7 post-ABV infection was conducted following a method described previously (Pakingking *et al.*, 2004). Briefly, total RNA was extracted from the tissues of individual flounder (n = 5) using ISOGEN (Nippon Gene, Japan) according to the manufacturer's instructions and dissolved in diethylpyrocarbonate-treated water. The following specific primers were used for amplification (557 bp) of the Japanese flounder Mx gene (Lee *et al.*, 2000): JFMx-F, 5'-TATGAGGAGAAGGTGCTGCCCTGCAT-3' and JFMx-R, 5'-TTCAAGGCCTCTGTGGTTGCTATGTC-3'). The Japanese flounder β-actin gene-specific primers (JFbAct-F, 5'-GACAGAAGGACAGCTACGTG-3' and JFbAct-R, 5'-CATGTAGTCAGTCAGATCTC-3') were also used to amplify a 426-bp fragment as internal control. The PCR products were separated on 1% agarose gel and stained with ethidium bromide.

Statistical analyses

Effects of the single and dual infection on the mortalities of sevenband groupers were compared using the χ^2 test at 5% confidence level. Statistical differences in the RGNNV titers between the single and dual-infected groups of flounder and sevenband grouper were determined using the Student's *t* test at 5% level of significance.

Results

Dual-infection in Japanese flounder

Mortality of fish: Mortality or abnormal behavior was not found either in the dual challenges with ABV and RGNNV or in fish challenged with RGNNV alone.

Kinetics of RGNNV in fish (Fig. 1): At Day 7 post-ABV injection when fish were challenged with RGNNV, ABV was detected in all the head kidneys (n = 5) at titers of $10^{4.8-6.0}$ $TCID_{50}$/g but only one of five brains was positive for ABV ($10^{4.4}$ $TCID_{50}$/g). Thereafter, the titers of ABV in dual-infected fish were not examined. RGNNV was isolated from all kidney and brain samples of fish with single (MEM_{10} + RGNNV) and dual infection (ABV + RGNNV) throughout the experiments. RGNNV infective titers in the kidneys of singly infected fish (control) ranged $10^{6.9}$~$10^{8.8}$ $TCID_{50}$/g at Day 3, slightly increased to $10^{7.1}$~$10^{9.8}$ $TCID_{50}$/g at Day 7, and decreased to $10^{6.2}$~$10^{8.0}$ $TCID_{50}$/g at Day 14. On the contrary, the titers of RGNNV in the kidneys of dual-infected fish was significantly lower than those in the control; $10^{6.1}$~$10^{6.8}$, $10^{5.6}$~$10^{6.9}$, and $10^{4.4}$ ~$10^{6.2}$ $TCID_{50}$/g at Day 3, Day 7 and Day 14, respectively. Similarly, RGNNV was detected

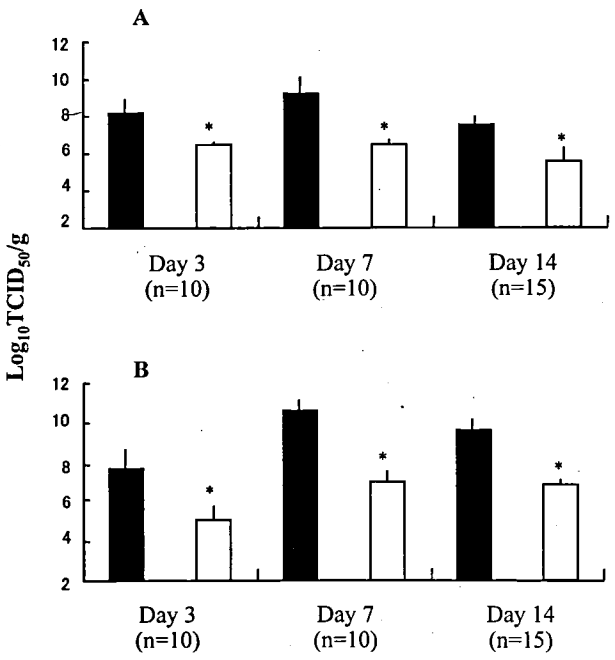

Fig. 1. RGNNV titers in the head kidneys (A) and brains (B) of Japanese flounder.
Fish were intramuscularly injected with RGNNV seven days after ABV inoculation (open bar) or without preceding ABV inoculation (closed bar: control) and the virus infective titers were examined at Days 3, 7, and 14 post-RGNNV inoculation. Data are given as mean titer of each group with standard deviation. n: number of fish examined. * The titer is significantly ($p < 0.05$) lower than that in control.

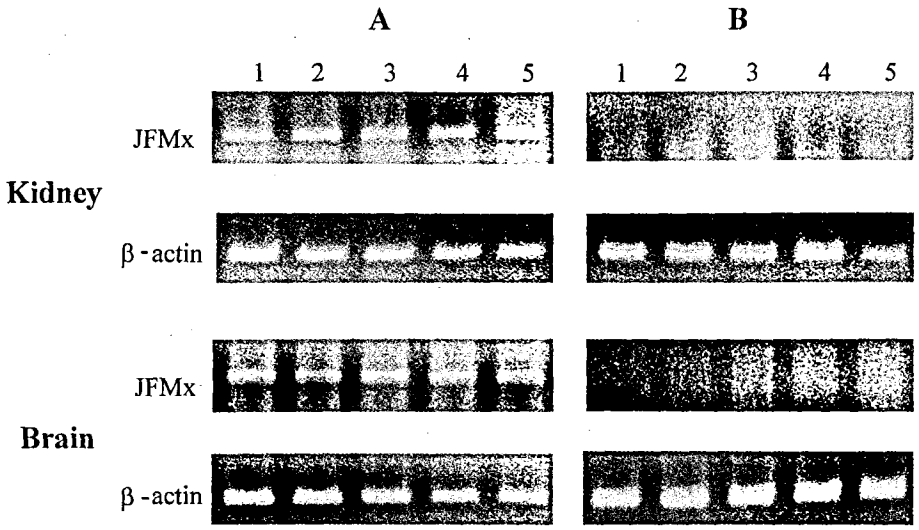

Fig. 2. JFMx gene expression in the head kidneys and brains of Japanese flounder.
Fish were examined for JFMx gene expression seven days after ABV inoculation (A) or MEM10-inoculation (B). The upper gel shows the 557-bp RT-PCR product specific to JFMx mRNA. β-actin gene expression (426-bp) was used as internal control in the lower gel. Lanes 1–5 represents individual fish examined.

from the brains of dual-infected fish at significantly lower titers than those of the control at any sampling time. The titers were $10^{3.1}$~$10^{5.6}$, $10^{6.1}$~$10^{7.1}$, and $10^{6.0}$~$10^{7.2}$ $TCID_{50}$/g for dual-infected fish and $10^{5.9}$~$10^{8.1}$, $10^{10.0}$~$10^{11.1}$, and $10^{8.1}$~$10^{10.3}$ $TCID_{50}$/g for the control fish at Day 3, Day 7, and Day 14, respectively.

JFMx gene expression in Japanese flounder

Expression of a JFMx gene was confirmed in the head kidneys and brains of all fish examined at Day 7 post-ABV infection but not in all MEM_{10}–injected fish (Fig. 2).

Infectivity of ABV in sevenband grouper

ABV was detected with titers ranging from $10^{5.0}$~$10^{6.3}$ $TCID_{50}$/g in the head kidneys of five fish examined at Day 7 post-infection, but at Day 14 only one out of five fish examined was positive for ABV at a titer of $10^{3.5}$ $TCID_{50}$/g and all were negative at Day 21. In contrast, ABV was not isolated in either of the brains examined. No mortalities or abnormal signs in fish were noted throughout the experiment.

Dual-infection in sevenband grouper

Mortality of fish: No mortality and abnormal behavior were observed in fish challenged with RGNNV at Day 7 post-ABV injection. In contrast, a significantly higher mortality (80%) was recorded in the control fish challenged with RGNNV without preceding injection of ABV (Fig. 3). The affected fish showed anorexia and abnormal swimming behavior, and loss of balance on the tank bottom at Days 4~5 post-infection. Thereafter, some of these fish had distended swim bladders and floated at the water surface with loss of balance.

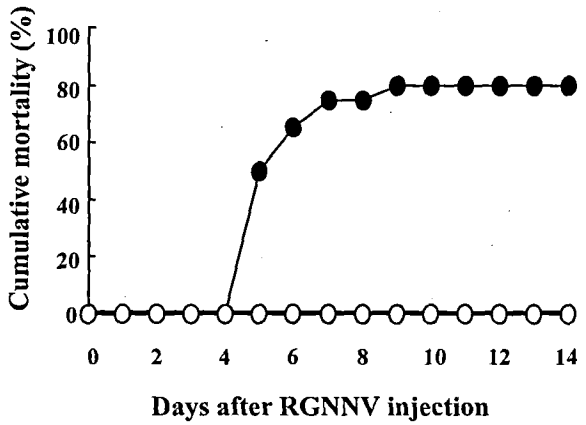

Fig. 3. Cumulative mortalities in sevenband grouper.
Fish were intramuscularly injected with RGNNV seven days after ABV inoculation (○) or without preceding ABV inoculation (●).

Kinetics of RGNNV and ABV: Fig. 4 shows the kinetics of RGNNV in the head kidneys and brains of sevenband grouper after RGNNV-challenge. The virus titers in the kidneys of the control fish were $10^{6.4}$~$10^{7.8}$ $TCID_{50}$/g at Day 3 and $10^{7.1}$~$10^{7.9}$ $TCID_{50}$/g at Day 7, and $10^{5.0}$~$10^{6.3}$ $TCID_{50}$/g at Day 14, while those in the kidneys of dual-infected fish were significantly lower, i.e. $10^{3.8}$~$10^{4.2}$ $TCID_{50}$/g at Day 3, $10^{3.4}$~$10^{6.0}$ $TCID_{50}$/g at Day 7, and thereafter under detectable level (less than $10^{2.8}$ $TCID_{50}$/g). Similarly, RGNNV were detected in the brains of the control fish, i.e. $10^{9.7}$~$10^{10.2}$ $TCID_{50}$/g at Day 3, $10^{9.4}$~$10^{10.7}$ $TCID_{50}$/g at Day 7, and $10^{8.2}$~$10^{9.9}$ $TCID_{50}$/g at Day 14. These titer levels were significantly higher than those in the dual-infected fish, i.e. $10^{4.4}$~$10^{6.8}$, $10^{5.3}$~$10^{7.3}$, $10^{4.6}$~$10^{6.8}$ $TCID_{50}$/g at Days 3, 7, and 14, respectively. The viruses in the brains then decreased

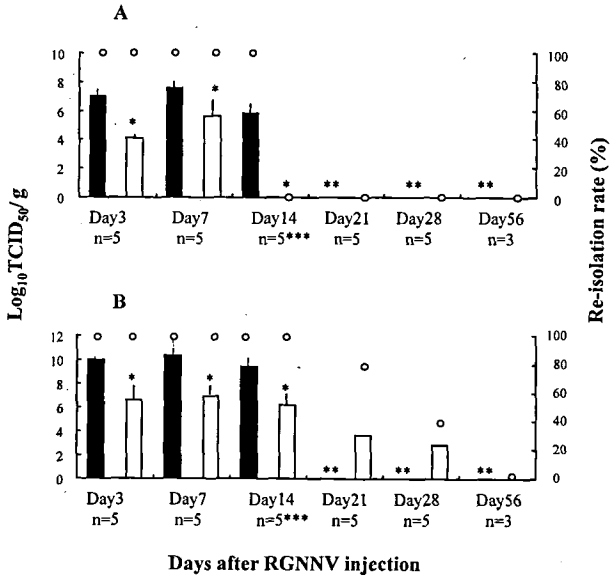

Fig. 4. RGNNV titers in the head kidneys (A) and brains (B) of sevenband grouper.
Fish were intramuscularly injected with RGNNV seven days after ABV inoculation (open bar) or without preceding ABV inoculation (closed bar: control) and the virus infective titers were examined at Days 3, 7, 14, 21, 28, and 56 post-RGNNV inoculation. Data are given as mean titer of each group with standard deviation. n: number of fish examined. * The titer is significantly lower ($p < 0.05$) than that in control. Small open circles indicate reisolation rates of RGNNV from the tissues of surviving fish. ** no available surviving fish. *** n = 4 for the control

and completely disappeared at Day 56.

ABV was detected in all the head kidneys of dual-infected fish at Day 3 post-RGNNV infection with virus titers of $10^{3.9}$~$10^{4.7}$ $TCID_{50}$/g. However, only one fish was positive ($10^{3.3}$ $TCID_{50}$/g) for ABV at Day 7 and none of five fish at Day 14 was positive. ABV was not isolated from the brains of any dual-infected fish throughout the experiment.

Discussion

The present dual-infection experiments revealed the potentiality of non-pathogenic aquabirnavirus (ABV) as an effective modulator of IFN-induced antiviral substance(s) that suppresses betanodavirus (RGNNV). The current effort was prompted by our previous work on the ability of the ABV to induce protection against VHSV in Japanese flounder (Pakingking *et al.*, 2003, 2004). There have been also extensive *in vivo* and *in vitro* studies on the induction of non-specific protection by IFN-like activity in salmonids, in which a number of fish-pathogenic double-stranded or negative-stranded RNA viruses like infectious pancreatic necrosis virus (IPNV), VHSV, infectious hematopoietic necrosis virus (IHNV), and infectious salmon anemia virus (ISAV) were used in addition to dsRNA polyinosinic polycytidylic acid (poly I:C) (Eaton, 1990; Rogel-Gaillard *et al.*, 1993; Congleton *et al.*, 1996; Jensen *et al.*, 2002; Jensen and Robertsen, 2002). To our knowledge, this is the first report to document the *in vivo* effect of IFN-like substance(s) against a positive-sense ssRNA virus; betanodaviruses.

Unexpectedly, RGNNV (SGWak97 strain) produced no mortality in Japanese flounder (12 g in body weight) though the inoculation dose was 1,000 times higher than that for sevenband grouper. As juvenile Japanese flounder less than approximately 20 mm in total length or 0.1 g in body weight are so susceptible to betanodavirus isolates (RGNNV) in experimental and natural infections (Nguyen *et al.*, 1994; Tanaka *et al.*, 2003), this fish species seemingly has a strongly age-dependent protection against betanodavirus infection. Following our previous report (Pakingking *et al.*, 2004), we tested the antiviral activity in the sera of ABV-inoculated flounder against RGNNV by an *in vitro* cell protection assay employing E-11 cells. However, the *in vitro* assay with this cell line could not be utilized because the flounder sera were heavily cytotoxic to E-11 cells (data not shown). The homologous Japanese flounder embryo (HINAE) cell line (Kasai and Yoshimizu, 2001) that we previously used in the antiviral assay for VHSV was not available for the present study because it did not support the multiplication of RGNNV.

Thus, JFMx gene expression in the kidneys and brains of ABV-infected flounder sampled prior to the secondary nodavirus infection was instead examined to indicate the appearance of the IFN-induced antiviral substance(s). As expected, JFMx gene expression was confirmed in both the organs of all fish examined. To further substantiate the suppressor activity of ABV against RGNNV, we monitored the multiplication kinetics of RGNNV in the kidneys and brains of flounder with single (RGNNV only) and dual-infection (ABV + RGNNV) sampled at scheduled time intervals. The results of virus titration revealed that RGNNV multiplication was significantly suppressed in dual-infected fish compared with those single-infected fish. However, the effect of the IFN-induced antiviral substance(s) was not so profound for nodavirus infection, compared with that for VHSV infection where VHSV almost disappeared from the kidneys, one of the target organs of aquabirnaviruses, at three day post-challenge (Pakingking *et al.*, 2004). Whether this difference in the antiviral effect of IFN-induced antiviral substance(s) between VHSV and nodavirus infections is owing to the difference in target organs for these viruses or owing to the constitutional difference in sensitivity to IFN(s) of these viruses is left for future studies.

A promising finding of the preset study is that the preceding ABV injection conferred complete protection against RGNNV infection on sevenband grouper, one of the most susceptible species to betanodaviruses. The

lower multiplication kinetics of RGNNV in previously ABV-infected fish can explain this protection. However, the supporting data are only limited *in vivo*, due to the cytotoxicity of flounder serum to E-11 cells and a lack of sevenband grouper Mx gene available, as already mentioned.

Among the affected species of VNN or VER, groupers and other fish species are severely affected even in older fish up to harvest size (Fukuda *et al.*, 1996; Le Breton *et al.*, 1997; Bovo *et al.*, 1999). Recent studies demonstrated that not a small population of wild fish carried betanodaviruses, and suggested that these wild fish can be a persistent potential source of the virus for cultured fish (Barker *et al.*, 2002; Gagne *et al.*, 2004; Gomez *et al.*, 2004). This fact means that net-pen culture of fish species like groupers highly susceptible to betanodaviruses is not successful without development of specific prophylactic method, i.e. vaccination system. The previous vaccination studies revealed that the recombinant coat protein or formalin-inactivated virus induced high and long duration of virus-neutralizing antibodies and protection against virus challenges (Húsgard *et al.*, 2001; Tanaka *et al.*, 2001; Yamashita *et al.*, 2005). However, it takes 2 or more weeks to the onset of specific immunity against nodaviruses and thus there is a room for vaccine improvement. Although a more detailed experiments on the onset and duration of the ABV-induced protection against betanodavirus infection was not conducted in the present study, our previous study using Japanese flounder and VHSV (Pakingking, 2004) demonstrated that the non-specific protection conferred by ABV infection commenced within 3 days and persisted at least for 2 weeks. Therefore, if non-pathogenic ABV is combined with a vaccine candidate, it is expected to protect fish from virus infection(s) before induction of the specific immunity. ABV contained in the vaccine may act as "immunomodulator" of the innate immune response. The present strong protection against a subsequent nodavirus infection at Day 7 post-ABV infection indicates that non-specific protective components in fish defense system, such as IFN(s), can be effective against virus multiplication and should be considered an essential component in the overall protective response of fish. Furthermore, it should be also taken into account that the antiviral activity of IFN is generally not virus-specific. These seemingly support the use of aquabirnavirus as an immunomudulator or immuno-adjuvant in the vaccine.

Aquatic birnaviruses are the most ubiquitous microorganisms in aquatic species (nearly 80 species), though information on the virulence is very limited except for IPNV, as reviewed by Reno (1999). ABV isolates from wild Japanese flounder with no diseased signs, which were serologically identified as YTAV, were not pathogenic to the homologous host in the experimental infection (Isshiki *et al.*, 2001; Takano *et al.*, 2001). As a consequence of persistent infection of these non-pathogenic ABV ubiquitous in natural environments, fish may acquire non-specific protection against pathogenic viruses. In order to verify this speculation, however, further intensive ecological and pathological studies will be required.

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