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Fish immunization using a synthetic double-stranded RNA Poly(I:C), an interferon inducer, offers protection against RGNNV, a fish nodavirus

Toyohiko Nishizawa[1,*], Ikuo Takami[2], Yuki Kokawa[1], Mamoru Yoshimizu[1]

[1]Faculty of Fisheries Sciences, Hokkaido University, Hakodate, 041-8611, Japan
[2]Nagasaki Prefectural Institute of Fisheries, Taira, Nagasaki Prefecture 851-2213, Japan

ABSTRACT: Viral nervous necrosis (VNN), caused by a fish nodavirus, is one of the most serious fish diseases worldwide. Here we report a unique vaccination method in sevenband grouper *Epinephelus septemfasciatus* using a synthetic double-stranded RNA polyinosinic polycytidylic acid (Poly(I:C)), an interferon inducer, followed by challenge with a live fish nodavirus. Fish injected with Poly(I:C) at 200 µg fish^{-1} were highly protected from artificial challenge with red-spotted grouper nervous necrosis virus (RGNNV) (relative percentage survival, RPS: 100%), and specific antibodies against RGNNV were detected in sera from survivors. Moreover, the surviving fish were protected from re-challenge with RGNNV (relative percent survival RPS: 100%). Thus, it was confirmed that specific immunity against RGNNV was established in sevenband grouper by injection with live RGNNV following Poly(I:C) administration. Antiviral state was induced in fish by injection with Poly(I:C) at ≥50 µg fish^{-1}, but no toxic response was observed in the fish even if Poly(I:C) was injected at a dose of 200 µg fish^{-1}. In fish injected with Poly(I:C) at 200 µg fish^{-1}, a high level of antiviral state of > 90% RPS against RGNNV challenge lasted for at least 4 d after Poly(I:C) injection. However, no curative effect by Poly(I:C) injection was observed in fish already infected with RGNNV. It is considered that the present immunization method using Poly(I:C) followed by a live virus injection could offer protection against various viral infections in a broader range of fish species.

KEY WORDS: Poly(I:C) · Immunization · Fish nodavirus · Viral nervous necrosis · VNN · Sevenband grouper · Vaccine · Antiviral state

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INTRODUCTION

Viral nervous necrosis (VNN) is one of the most serious diseases of teleost fishes, because it causes high mortality in more than 20 species of cultured marine fishes worldwide (Munday et al. 1992, 2002, Muroga et al. 1998, Muroga 2001). Fish nodaviruses (genus *Betanodavirus*, family *Nodaviridae*), causative agents of VNN, are non-enveloped and spherical in shape (25 to 30 nm in diameter), and consist of a single coat protein and 2 molecules of positive sense single-stranded RNA (ssRNA) (Mori et al. 1992, Nagai & Nishizawa 1999, Schneemann et al. 2005). Based on nucleotide sequences, 4 genotypes of fish nodaviruses are known: striped jacked nervous necrosis virus (SJNNV), tiger puffer NNV (TPNNV), barfin flounder NNV (BFNNV) and red-spotted grouper NNV (RGNNV) (Nishizawa et al. 1995, 1997). Moreover, new nodavirus isolates from turbot *Scophthalmus maximus* were reported as a fifth genotype (Johansen et al. 2004). VNN-affected fish showed abnormal swimming behavior such as spiralling and darting, and were also characterized histopathologically by vacuolation and necrosis in the central nervous system (Munday et al. 2002). In general, losses due to VNN tend to be very high at larval and/or juvenile stages. However, in recent years sig-

*Email: jjnishi@fish.hokudai.ac.jp

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nificant mortalities have also occurred in adult and harvest-size fish, especially in groupers *Epinephelus* spp., European sea bass *Dicentrarchus labrax* and Atlantic halibut *Hippoglossus hippoglossus* (Munday et al. 2002). Selection of specific pathogen-free (SPF) spawners is important to prevent vertical transmission of fish nodavirus in seed production (Mushiake et al. 1994, Mori et al. 1998, Watanabe et al. 2000), but there are still problems at grow-out and adult stages in sea cages due to horizontal transmission of fish nodavirus though the rearing water sourced from the environment. Under these circumstances, several kinds of fish nodavirus vaccines have been developed, such as formalin-inactivated vaccine, subunit vaccine with a recombinant coat protein, virus-like particle vaccine, etc. (Húsgarð et al. 2001, Tanaka et al. 2001, Yuasa et al. 2002, Sommerset et al. 2005, Yamashita et al. 2005, Liu et al. 2006, Thiéry et al. 2006).

Recently, co-infections in Japanese flounder *Paralichthys olivaceus* with aquabirnavirus (ABV) and viral hemorrhagic septicemia virus (VHSV) or fish nodavirus have been reported (Nguyen et al. 1994, Takano et al. 2001, Watanabe et al. 2002). Furthermore, primary infection with ABV in Japanese flounder and sevenband grouper *Epinephelus septemfasciatus* involved protection of fish from other pathogenic viruses, such as VHSV and fish nodavirus (Pakingking et al. 2003, 2005). It was also reported that protection from VHSV and infectious hematopoietic necrosis virus (IHNV) was induced in rainbow trout *Oncorhynchus mykiss* by pre-exposure to infectious pancreatic necrosis virus (IPNV), aquareovirus or a picornavirus (de Kinkelin et al. 1992, Hedrick et al. 1994, La Patra et al. 1995, Byrne et al. 2008). Protection from secondary viral infection was due to interferon (IFN) activity induced in fish by the primary viral infection (Pakingking et al. 2004, Das et al. 2007, McBeath et al. 2007). More recently, Kim et al. (2009, this issue) reported that immunity against IHNV in rainbow trout was induced by injection of live IHNV following IPNV injection. Moreover, it was shown that a synthetic double-stranded RNA with polyinosinic and polycytidylic acid (Poly(I:C)) could substitute IPNV. Poly(I:C) is well known as a toll-like receptor (TLR) agonist, which results in production of alpha/ beta IFNs. Subsequently, IFNs induce an antiviral state within multiple cell types. The antiviral mechanisms of IFNs and IFN-inducers have been reviewed previously (De Clercq 2006) and prophylactic treatment with Poly(I:C) offers protection in different fish models of virus infection (Jensen et al. 2002, Lockhart et al. 2004, Purcell et al. 2004, Plant et al. 2005, Saint-Jean & Pérez-Prieto 2007, Fernandez-Trujillo et al. 2008).

In the present study, we demonstrated that immunity against RGNNV in sevenband grouper was inducible by injection of live RGNNV following Poly(I:C) administration. Moreover, the minimum dosage of Poly(I:C) necessary for induction of an antiviral state and duration period of the antiviral state were investigated in sevenband grouper infected with RGNNV.

MATERIALS AND METHODS

Fish and virus. SPF sevenband grouper weighing from 60 to 140 g, reared in Nagasaki Prefectural Institute of Fisheries (NaPIF), and RGNNV (SgNag05) from VNN-affected sevenband grouper from Nagasaki Prefecture in 2005 (Kokawa et al. 2008, Nishizawa et al. 2008) were used. The SPF fish were reared under conditions for the prevention of vertical transmission of fish viruses (Mori et al. 1998, Watanabe et al. 2000), using UV-treated seawater in a separate facility of NaPIF. Moreover, samples of the fish were inspected for presence of viruses by culture isolation using SSN-1 cells and PCR to confirm SPF status, prior to the experiments. RGNNV was cultured at 25°C in SSN-1 cells maintained using Leibovitz L-15 medium (Gibco) with 10% (V/V) fetal bovine serum (FBS, Gibco), 150 IU ml^{-1} penicillin G and 100 µg ml^{-1} streptomycin. After centrifugation (12 000 × *g*, 10 min, 4°C), viral culture supernatants were subdivided into small quantities and stored at –80°C until use. Titration of viral infectivity was performed using 96-well microplates seeded with SSN-1 cells. After 14 d of culture, appearance of CPE (cytopathic effect) was evaluated to determine the 50% tissue culture infectious dose ($TCID_{50}$).

Injection of Poly(I:C) and primary challenge with RGNNV. The experimental design scheme of Poly(I:C) injection and RGNNV challenges is shown in Fig. 1. Poly(I:C) was dissolved in diethylpyrocarbonate (DEPC)-treated water (Sigma) at predetermined concentration before use in each experiment, and the remaining Poly(I:C) solution was discarded. A total of 160 SPF sevenband grouper with 115 ± 25 g mean body weight (MBW) were reared in 4 aquaria (n = 40 in each) with 40 l flowing UV-sterilized seawater at 25°C (24 cycles d^{-1}). Poly(I:C) was injected intramuscularly at a dose of 200 µg 100 μl^{-1} $fish^{-1}$ into fish in 2 of the aquaria, and 100 µl $fish^{-1}$ of DEPC-treated water (control) was injected intramuscularly into fish of the remaining 2 aquaria. After 2 d, fish injected with Poly(I:C) or DEPC water in 1 out of 2 aquaria were challenged intramuscularly with RGNNV at a dose of $10^{4.3}$ $TCID_{50}$ 100 μl^{-1} $fish^{-1}$ (Poly(I:C)-RGNNV and Control-RGNNV groups, respectively), while fish of the remaining 2 aquaria were mock challenged with 100 µl $fish^{-1}$ of L-15 medium (Poly(I:C)-Mock and Control-Mock groups, respectively). The challenged fish were reared without feeding for an additional 21 d,

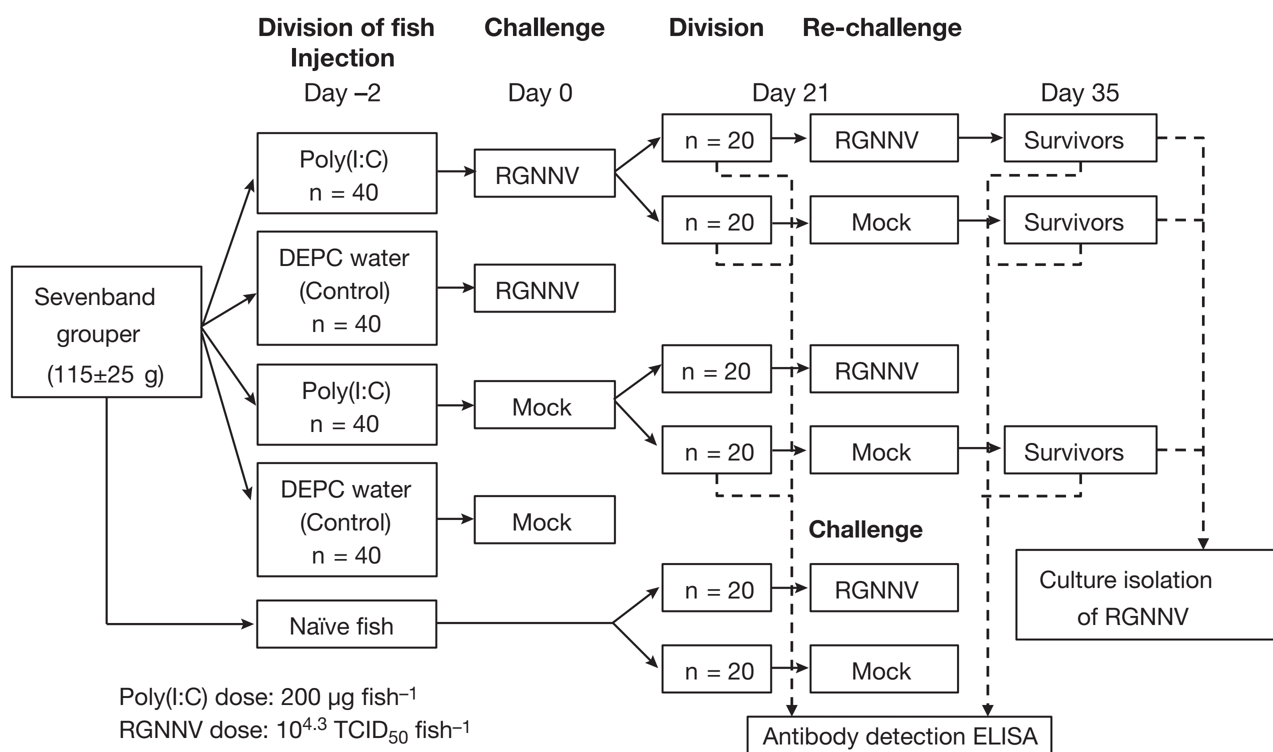

Fig. 1. Experimental design scheme of polyinosinic polycytidylic acid (Poly(I:C)) injection, red spotted grouper necrosis virus (RGNNV) challenges and collection of sera for antibody detection ELISA. DEPC: diethylpyrocarbonate. Dashed lines: collection of fish sera and brain tissues from survivors for the antibody detection ELISA and culture isolation of RGNNV, respectively

and mortality was monitored daily. Relative percent survival (RPS) values were calculated according to the method of Amend (1981). At the end of the experiment, sera were collected from 10 survivors of each group to be tested for RGNNV-specific antibodies using ELISA.

Secondary challenge of fish surviving the primary challenge with RGNNV. After a total of 3 wk the primary challenge with RGNNV, the surviving fish in each group, i.e. Poly(I:C)-RGNNV and Poly(I:C)-Mock, were divided into 2 aquaria with 20 fish each (Fig. 1). Fish in 1 out of 2 aquaria for Poly(I:C)-RGNNV and Poly(I:C)-Mock groups were challenged intramuscularly with RGNNV at a dose of $10^{4.3}$ $TCID_{50}$ 100 µl^{-1} fish^{-1} (Poly(I:C)-RGNNV- RGNNV and Poly(I:C)-Mock-RGNNV groups), while fish in the remaining aquaria of each group were mock challenged with L-15 medium (Poly(I:C)-RGNNV-Mock and Poly(I:C)-Mock-Mock groups). For positive and negative controls, naïve fish were reared in 2 other aquaria with 20 fish each, and were challenged with RGNNV at the same dosage of RGNNV or L-15 medium (Naïve-RGNNV and Naïve-Mock, respectively). Fish in each group were reared without feeding for an additional 14 d and mortality was monitored. After the experiment, brain tissues and sera of the survivors were collected and subjected to titration of RGNNV and RGNNV-specific antibody detection using ELISA, respectively.

Antibody detection using ELISA. Detection of specific antibodies against RGNNV was performed using the ELISA system according to a modified method of Kim et al. (2007). Briefly, culture fluids of RGNNV with $10^{8.3}$ $TCID_{50}$ ml^{-1} were diluted 10 times with distilled water, 50 µl placed in ELISA plate wells, and viral antigen was fixed by drying overnight at 37°C. Sera were diluted 1:40 with 5% skim milk solution and incubated at 25°C for 1 h, then placed in duplicate wells of the ELISA plate and incubated at 25°C for 1 h followed by 3 rinses with PBS containing 0.05% Tween 20 (T-PBS). Fish IgMs captured by RGNNV antigen were detected using rabbit (secondary) antiserum against sevenband grouper IgM, horseradish peroxidase (HRP)-conjugated swine serum against rabbit IgG (Dako) and substrate (100 mM Na_2HPO_4, 50 mM citric acid, 1.0 mg ml^{-1} *o*-phenylenediamine, 0.03% H_2O_2). After colour development the reaction was stopped with 2 N H_2SO_4 and absorbance at an optical density of 492 nm (OD_{492}) was read using a microplate reader (MTP-300, Corona). The ELISA values among challenged groups were analyzed statistically using Tukey-Kramer multiple comparisons test at a significance level of 1%.

Minimum concentration of Poly(I:C) for induction of antiviral state. SPF sevenband grouper (71±10 g MBW) were divided into 7 aquaria with 12 fish each. In 6 of the aquaria, fish were injected with 2-fold diluted

Poly(I:C) at 6 doses ranging from 200 to 6.3 µg 100 µl^{-1} fish^{-1} (see Fig. 4). In the seventh aquarium (Control), fish were injected with 100 µl fish^{-1} of DEPC-treated water. The fish were challenged intramuscularly with RGNNV at a dose of $10^{4.3}$ $TCID_{50}$ 100 µl^{-1} fish^{-1} 2 d after Poly(I:C) injection and mortality was monitored for 14 d.

Curative effect and duration of antiviral state by Poly(I:C)-injection. SPF sevenband grouper (78 ± 15 g MBW) were divided into 8 aquaria with 20 fish each. All fish were injected with Poly(I:C) (200 µg 100 µl^{-1} fish^{-1}) and challenged intramuscularly with RGNNV at a dose of $10^{4.3}$ $TCID_{50}$ 100 µl^{-1} fish^{-1}. Fish in 2 aquaria were challenged with RGNNV at 2 and 4 d, respectively, before Poly(I:C) injection to evaluate any curative effect of Poly(I:C). In the other aquaria, fish were injected with Poly(I:C) and then challenged with RGNNV at 0, 1, 2, 4, 7 and 14 d after injection to evaluate duration of antiviral state induced by Poly(I:C). The fish were reared for an additional 2 wk and mortality was monitored.

RESULTS AND DISCUSSION

A time-dependent change of survival rate in sevenband groupers after RGNNV challenge following Poly(I:C) injection is shown in Fig. 2A. The fish without Poly(I:C) began to die with typical VNN signs on the 4th day after RGNNV challenge, and all fish died within 8 d (Control-RGNNV group). No mortality was observed in fish challenged with RGNNV following Poly(I:C) injection (Poly(I:C)-RGNNV group). There was no mortality in mock challenged fish regardless of Poly(I:C) injection (Poly(I:C)-Mock and Control-Mock groups), except that one fish died accidentally in the Poly(I:C)-Mock group. Sera from 10 fish each of the survivors in the Poly(I:C)-RGNNV, Poly(I:C)-Mock and Control-Mock groups were assayed for RGNNV-specific antibodies (Fig. 3A). ELISA absorbance values (OD_{492}) of sera from survivors of the Poly(I:C)-RGNNV group ranged from 0.20 to 0.86 (average: 0.33), but those from mock challenged survivors of the Poly(I:C)-Mock and Control-Mock groups were all less than 0.08. Distribution of ELISA values for sera from the RGNNV-challenged group was statistically different from those of the mock challenged groups ($p < 0.01$). Thus, it was demonstrated that fish injected with Poly(I:C) were highly protected from RGNNV challenge, and that antibodies against RGNNV were produced in the surviving fish through RGNNV challenge following Poly(I:C) injection. These results were consistent with those observed in rainbow trout with Poly(I:C) injection and IHNV challenge (Kim et al. 2008). Also, Japanese flounder and sevenband grouper

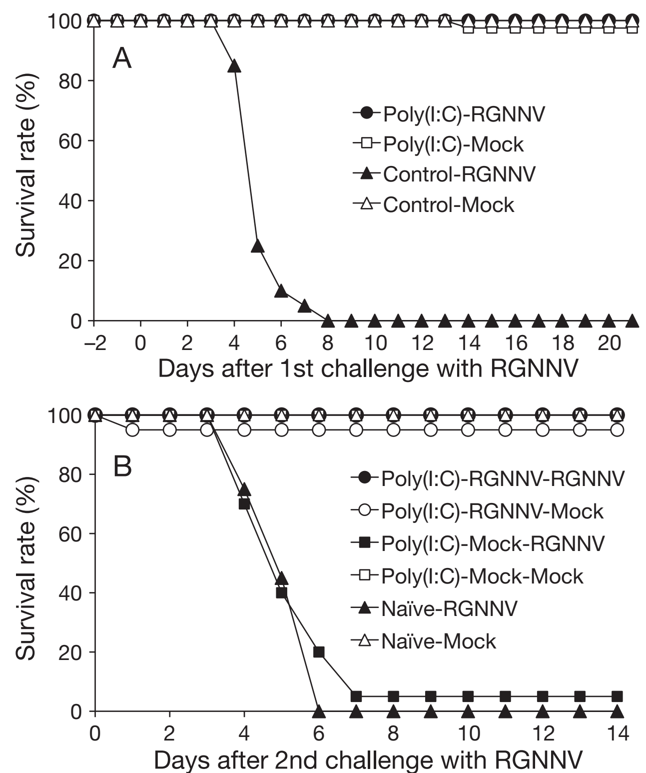

Fig. 2. *Epinephelus septemfasciatus.* Survival rate of fish challenged with red spotted grouper nervous necrosis virus (RGNNV) following polyinosinic polycytidylic acid (Poly(I:C)) injection. (A) Fish challenged intramuscularly with RGNNV ($10^{4.3}$ $TCID_{50}$ fish^{-1}) or L-15 (mock challenge) 2 d after intramuscular injection with Poly(I:C) at a dose of 200 µg fish^{-1} or diethylpyrocarbonate (DEPC) water (Control). (B) Survivors from the primary RGNNV- and mock challenges following Poly(I:C)-injection re-challenged with RGNNV ($10^{4.3}$ $TCID_{50}$ fish^{-1}) or L-15 (mock challenge); naïve fish were also challenged in the same manner

pre-infected with ABV were protected from VHSV and/or RGNNV infection, and in that study it was confirmed that fish *Mx* gene, an indicator of IFN, was expressed after the ABV injection (Pakingking et al. 2004, 2005). Kim et al. (2009) demonstrated that an antiviral state was established by injecting rainbow trout with Poly(I:C), as a substitute for IPNV. Furthermore, induction of antiviral states against pathogenic viruses by Poly(I:C) injection has been reported in several fish species (Jensen et al. 2002, Plant et al. 2005, Saint-Jean & Pérez-Prieto 2007, Fernandez-Trujillo et al. 2008). Thus, it was considered that the sevenband grouper injected with Poly(I:C) could be in an antiviral state due to IFN induction by Poly(I:C), a TLR agonist.

A time-dependent change in survival rate in fish after the secondary challenge, as well as those from the positive and negative controls (Naïve-RGNNV and Naïve-Mock) is shown in Fig. 2B. The fish of the

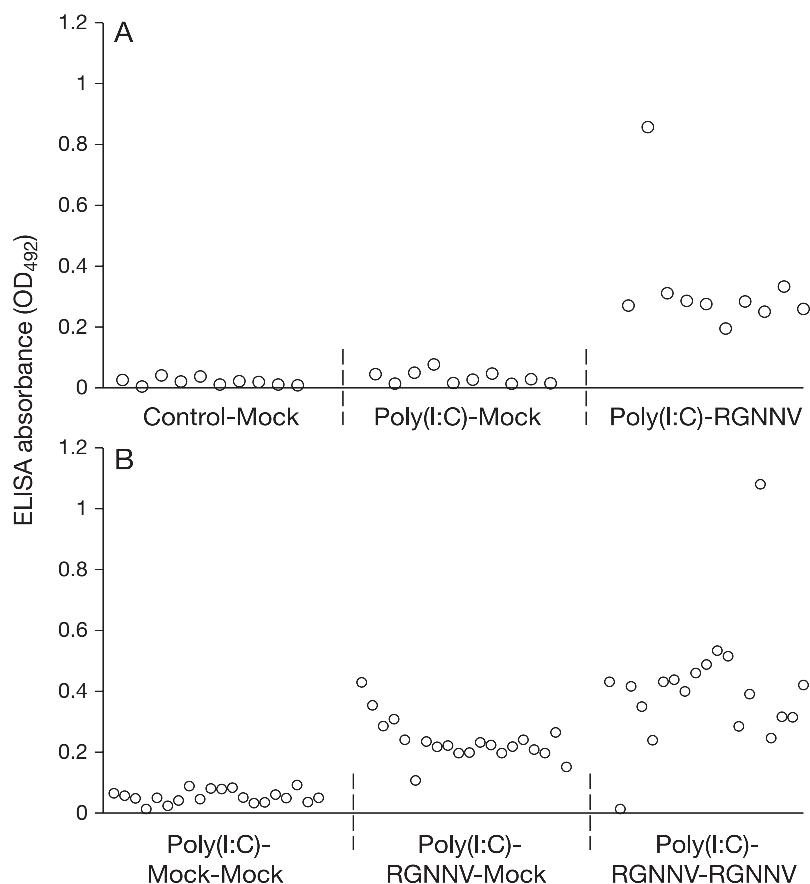

Fig. 3. *Epinephelus septemfasciatus.* Detection of specific antibodies against red-spotted grouper necrosis virus (RGNNV) from sera of the survivors in the experiment shown in Fig. 2. (A) Fish sera obtained from survivors 3 wk after the primary RGNNV challenge. (B) Sera obtained from survivors 5 wk after the primary RGNNV challenge (corresponding to 2 wk after the secondary RGNNV challenge). OD_{492}: optical density at 492 nm

Poly(I:C)-Mock group showed 5 % of survival rate after re-challenge with RGNNV (Poly(I:C)-Mock-RGNNV group), and all naïve fish died within 6 d after RGNNV challenge (Naïve-RGNNV group). However, no mortality from the re-challenge was observed in the Poly(I:C)-RGNNV group (Poly(I:C)-RGNNV-RGNNV). In the fish with the secondary mock challenge (Poly(I:C)-RGNNV-Mock, Poly(I:C)-Mock-Mock and Naïve-Mock groups), there was no mortality, except that one fish died due to an accident in the Poly(I:C)-RGNNV-Mock group. Sera from the survivors were assayed for RGNNV-specific antibodies (Fig. 3B). ELISA absorbance values of the Poly(I:C)-RGNNV-RGNNV group ranged from 0.01 to 1.08 (average: 0.41), which were statistically different from those of the survivors of the Poly(I:C)-RGNNV-Mock group (from 0.11 to 0.49, average: 0.28, $p < 0.01$). No antibody against RGNNV was detected from the survivors with 2 mock challenges following Poly(I:C) injection (Poly(I:C)-Mock-Mock, ELISA absorbance <0.09). RGNNV titers in brain tissues of the survivors in the Poly(I:C)-RGNNV-RGNNV group were all under the detection limit ($<10^{1.8}$ $TCID_{50}$ g^{-1}), and were clearly lower than those from the dead fish of the Poly(I:C)-Mock-RGNNV and Naïve-RGNNV groups (from $10^{8.3}$ to $10^{9.1}$ $TCID_{50}$ g^{-1}). No RGNNV was detected in the survivors of the Poly(I:C)-Mock-Mock groups. From these results, it was demonstrated that the surviving fish from the primary challenge with RGNNV following Poly(I:C) injection had acquired immunity against RGNNV. It is noted that RGNNV titers in the survivors were under the detection limit even though $10^{4.3}$ $TCID_{50}$ of RGNNV had been injected, suggesting that the injected RGNNV could be eliminated from fish by induction of the antiviral state following Poly(I:C) injection and establishment of RGNNV immunity by injection of RGNNV. It was thus considered that fish immunized by injection with live RGNNV following Poly(I:C) administration may not become RGNNV carriers. However, the fish should be monitored for a long time until these fish become spawners.

After injection of Poly(I:C) at different concentrations, sevenband grouper were challenged with RGNNV to determine the minimum concentration of Poly(I:C) required for induction of the antiviral state (Fig. 4). The fish injected with Poly(I:C) at a dose of 50 µg $fish^{-1}$ or more showed more than 90 % survival rate following RGNNV challenge, while the fish with Poly(I:C) at a dose of 25 µg $fish^{-1}$ showed 50 % survival rate. All of the fish injected with Poly(I:C) at doses of 12.5 µg $fish^{-1}$ or less died within 16 d after RGNNV challenge. It was thus considered that doses of Poly(I:C) greater than 50 µg $fish^{-1}$ are needed for induction of the antiviral state in these fish. It has been reported that Poly(I:C) induces some side effects in humans, including renal failure and hypersensitivity reactions (Robinson et al. 1976). As a non-toxic analog with similar IFN induction capacities, Poly(I:C12U), which consists of one strand of polyriboinosine (poly I) hybridized to a complementary strand of polyribocytosine containing a uridine residue statistically at every 13th monomer (poly C12U) in a RNA polymeric linkage, was derived by investigators at Johns Hopkins University (Carter et al. 1972). However, in the present study, no toxic response appeared in the sevenband grouper injected with Poly(I:C) at a

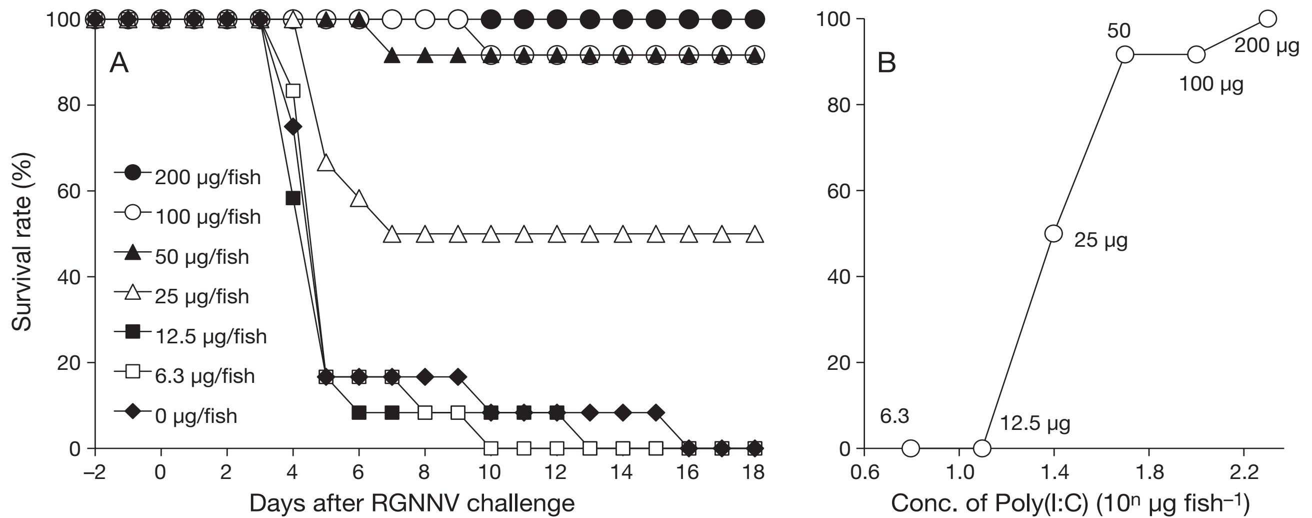

Fig. 4. *Epinephelus septemfasciatus*. Minimum concentration of polyinosinic polycytidylic acid (Poly(I:C)) for induction of an antiviral state. (A) Time-dependent change of survival rate of fish challenged with red-spotted grouper nervous necrosis virus (RGNNV) following injection of Poly(I:C) at different concentrations. The fish were challenged intramuscularly with RGNNV at a dose of $10^{4.3}$ $TCID_{50}$ fish^{-1} 2 d after Poly(I:C) injection. (B) Final survival rate of fish following challenge with RGNNV versus concentration of injected Poly(I:C)

dose of 200 µg fish^{-1}, although more detailed tests for side effects should be carried out in the future.

In human medicine, IFN therapy is used for treatment of hepatitis C virus (HCV) infection. Thus, in the present study, sevenband grouper were injected with Poly(I:C) at 200 µg fish^{-1} before and after the RGNNV challenge to evaluate any curative effect and the duration of the antiviral state by Poly(I:C) injection (Fig. 5). The fish challenged with RGNNV at 2 and 4 d before the Poly(I:C) injection showed a 0 and 5% survival rate, respectively, demonstrating that no curative effect by Poly(I:C) injection was observed in the fish pre-infected with RGNNV. In the present experiments, typical signs of VNN were observed at 2 d after the challenge (data not shown) and almost all of the challenged fish died within a few days after RGNNV challenge (Figs. 2, 4 & 5). Thus, we speculate that the present challenge dose of RGNNV ($10^{4.3}$ $TCID_{50}$ fish^{-1}) may be too high to observe some curative effect of Poly(I:C) against RGNNV infection.

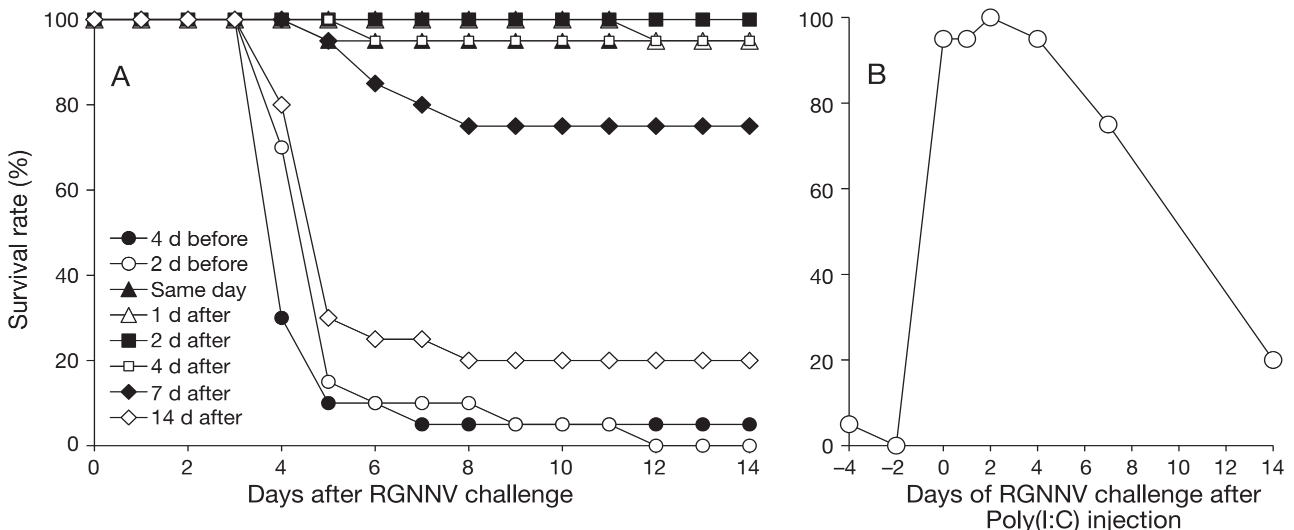

Fig. 5. *Epinephelus septemtasciatus*. Curative effect and duration of antiviral state induced by polyinosinic polycytidylic acid (Poly(I:C)) injection. Fish were challenged intramuscularly with red-spotted grouper nervous necrosis virus (RGNNV) at 2 and 4 d before Poly(I:C) injection to evaluate curative effect of Poly(I:C), and challenged at 0, 1, 2, 4, 7 and 14 d after the Poly(I:C) injection to evaluate the duration of the antiviral state. (A) Time-dependent change of survival rate of fish. (B) Final survival rate of fish following challenge with RGNNV before/after Poly(I:C) injection

The fish challenged with RGNNV 0, 1, 2 and 4 d after the Poly(I:C) injection showed 95% or more survival rate, while the fish challenged 7 and 14 d after the Poly(I:C) injection showed 75 and 20% survival, respectively. It was thus considered that the antiviral state appeared just after Poly(I:C) injection and lasted for at least 4 d, but subsequently decreased and disappeared within 14 d. These results agree with previous experiments in salmonids and other fish species in which the fish *Mx* gene response following Poly(I:C) administration peaked 1 d after the injection, and subsequently disappeared within 15 d (Lockhart et al. 2004, Purcell et al. 2004, Plant et al. 2005, Saint-Jean & Pérez-Prieto 2007, Fernandez-Trujillo et al. 2008). It was noted that a high survival rate (95%) was observed in the fish receiving both Poly(I:C) injection and RGNNV challenge at the same time (Fig. 5), which would reduce stress on the fish and and the cost of fish immunization.

In conclusion, the present study demonstrated that fish were able to acquire RGNNV immunity via injection of live RGNNV following Poly(I:C) administration. There are some advantages in this immunization method in comparison with other known fish immunization schemes against pathogenic viruses: (1) The injected Poly(I:C) does not remain in fish tissues for a long time because Poly(I:C), a synthetic RNA, is unstable like other RNAs, indicating that there may be no problem with immunized fish as food sources and also in the fish-rearing environment. (2) Neither attenuation nor inactivation of virus is necessary, and high levels of immunity can be expected using this method. (3) No recombinant DNA technology is needed, suggesting a relatively low cost for vaccine development, and the method would be more acceptable to consumers with an antipathy towards foods produced with recombinant DNA technology. It was confirmed that the present immunization method was also useful for protection of Japanese flounder against VHSV (I. Takami, T. Nishizawa & M. Yoshimizu unpubl. data) and rainbow trout against IHNV (Kim et al. 2009). Thus, it is expected that the present method will be applicable to wide range of fish species and to other viruses including those not yet identified or culture-isolated. However, as mentioned by Kim et al. (2009), more detailed experiments would be required prior to application of the present method to open aquaculture, because some risks, such as re-induction of the live virus used for immunization under stress conditions such as spawning, and the spread of the virus used for immunization beyond the target fish, require investigation.

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