RSIVはPoly(I:C)投与により誘導される一過性自然免疫の影響をおそらく受けない

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RSIV is Probably Insensitive to the Transient Innate Immune Response Induced by Administration of Poly(I:C), a Synthetic Double-Stranded RNA

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ABSTRACT—Red seabream iridovirus (RSIV) causes significant mortality in many marine fishes. Polyinosinic-polycytidylic acid [Poly(I:C)] immunization with a live virus confers protection of fishes from viral infection. Thus, we applied this immunization with live RSIV to rock bream Oplegnathus fasciatus, red seabream Pagrus major and yellowtail Seriola quinqueradiata. No significant difference was observed in mortalities due to RSIV infection between the fishes that did or did not receive Poly(I:C), indicating that fishes administered Poly(I:C) were not protected from RSIV infection. It was confirmed that the Mx gene, an indicator of induced interferon, was well expressed in rock bream that received Poly(I:C). The results suggest that RSIV is probably insensitive to the transient innate immune response induced by Poly(I:C).

Key words: red seabream iridovirus, megalocytivirus, interferon, Poly(I:C), RSIV, innate immune response

Red seabream iridovirus (RSIV) infection causes significant mortality in more than 30 cultured marine fish species in many Asian countries (Kawakami and Nakajima, 2002). In particular, serious economic losses due to RSIV infection have occurred in fingerlings and market-sized fishes including rock bream Oplegnathus fasciatus in Korea (Jung and Oh, 2000; Kim et al., 2002), and red seabream Pagrus major and yellowtail Seriola quinqueradiata in Japan (Matsuoka et al., 1996; Kawakami and Nakajima, 2002). Fish infected with RSIV are lethargic and exhibit severe anemia, petechiae of the gills, and an enlarged spleen (Inouye et al., 1992). Typical histopathological signs of RSIV-infected fish are an enlargement of cells and necrosis of renal and splenic hematopoietic tissues (Inouye et al., 1992). RSIV belongs to the genus Megalocytivirus in the family Indoviridae, representing a type species of infectious spleen and kidney necrosis virus (Inouye et al., 1992; Kawakami and Nakajima, 2002; Jancovich et al., 2012). Megalocytiviruses, including RSIV, are icosahedral with a 200–240 nm diameter, a single linear dsDNA molecule of 111 kbp and a genome encoding 124 putative open reading frames in sizes from 40 to 1,208 amino acids (He et al., 2001; Do et al., 2004; Lü et al., 2005; Nakajima and Kurita, 2005; Jancovich et al., 2012). A formalin-inactivated RSIV vaccine has been commercialized and is effective for protecting red sea bream, yellowtail and other fishes from RSIV infection (Nakajima et al., 1997, 1999, 2002); however, protection of fish belonging to the genus Oplegnathus by vaccination is difficult (O. I. E., 2012). Although efficacious subunit and DNA vaccines with the RSIV major capsid protein (MCP) gene have been developed experimentally (Park et al., 2005; Caipang et al., 2006), it seems too early for Asian consumers to accept a vaccine created from recombinant DNA technology.

It was recently reported that polyinosinic-polycytidylic acid [Poly(I:C), a synthetic double-stranded RNA] immunization with a live pathogenic viruses confers protection of fishes from infections of homologous viruses, such as nervous necrosis virus (NNV) infection in sevenband grouper Epinephelus septemfasciatus, viral hemorrhagic septicemia virus infection in olive flounder Paralichthys olivaceus and infectious hematopoietic necrosis virus infection in rainbow trout Oncorhynchus mykiss (Kim et al., 2009; Nishizawa et al., 2009, 2011a, 2011b; Takami et al., 2010; Oh et al., 2012a, 2012b). This method involves Poly(I:C) immunization of fish with a pathogenic live virus following administration of Poly(I:C), which induces a transient, non-specific antivi-
ral state. As a result, fish in an antiviral state survive the initial immunization with the live virus, and the surviving fish are able to mount a specific protective immune response against the injected pathogenic virus. It has been confirmed that Poly(I:C) immunization is possible by the immersion route using live virus (Nishizawa et al., 2011a, 2011b). Thus, it is conceivable that Poly(I:C) immunization of fish with live pathogenic viruses will eventually be applicable to a wide range of fish species and other viruses.

As mentioned above, rock bream economic losses due to RSIV infection are serious in Korea (Jung and Oh, 2000; Kim et al., 2002), but protection of fish belonging to the genus Oplegnathus by vaccination is difficult (O. I. E., 2012). Thus, in the present study, we applied Poly(I:C) immunization to RSIV infection not only in rock bream but also in red seabream and yellowtail.

Materials and Methods

Virus and fishes

We used the RSIV RBHad09 isolate, which was recovered from moribund rock bream that were infected with RSIV at Hadong, Korea in 2009. In separate phylogenetic analyses based on the MCP, ATPase, and PstI fragment genes, it was confirmed that RBHad09 was classified as a M1a/P1a type of genogroup I (data not shown), as defined by Kwon et al. (2011). To prepare the RSIV, RBHad09 was injected into rock bream to obtain spleen tissue from fish that died due to RSIV infection. The spleen tissue was homogenized with nine volumes of Leibovitz’s L-15 medium (Gibco), centrifuged (3,000 xg, 10 min, 4°C), and the resulting supernatants were subdivided and stored at -80°C until use.

Pathogenicity of the present RSIV isolate

Fifty red seabream with a mean body weight (MBW) of 61.2 g were reared in five aquariums (n = 10 fish each) with 40 L of seawater at 28°C, whereas 120 rock bream with a MBW of 13.9 g were reared in six aquariums (n = 20 fish each) with 10 L of seawater at 28°C. Fish in each aquarium were intramuscularly inoculated with 100 μL of the RSIV stock solution diluted 103-fold with L-15 [Poly(I:C)-RSIV (diluted 104) and control-RSIV (diluted 105), respectively]. Fish in the remaining two aquariums were injected with the same volume of L-15 [Poly(I:C)-mock and control-mock, respectively]. The fish were reared and fed once per week for an additional 15–22 days to monitor mortality.

A total of 100 rock bream with a MBW of 13.9 g were reared in five aquariums (n = 20 fish each) with 10 L of seawater at 28°C. Fish in two aquariums were intramuscularly injected with Poly(I:C) at 200 μg/100 L/fish, whereas fish in the remaining three aquariums were injected with DEPC water at 100 μL/fish (control). On the second day of the Poly(I:C) administration, fish in one of the two aquariums that was administered Poly(I:C) and in one of the two control aquaria were intramuscularly injected with 100 μL of the RSIV stock solution diluted 104-fold with L-15 [Poly(I:C)-RSIV (diluted 105) and control-RSIV (diluted 106), respectively]. Fish in the remaining two aquariums were injected with the same volume of L-15 [Poly(I:C)-mock and control-mock, respectively]. The fish were reared for an additional 22 days to monitor mortality.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay to determine the fold change in Mx gene expression in fish administered Poly(I:C)

For rock bream with MBW of 23.7 g were reared in an aquarium with 20 L of seawater at 28°C. Just before and 3 hours, 1, 3, 5, 7, and 14 days after administering Poly(I:C) at 200 μg/100 L/fish, four fish were sacrificed to obtain spleen tissue for RT-qPCR assay targeting the Mx and β-actin genes (internal control genes).

Total RNAs of the spleen tissue were prepared with an RNA-extraction kit (Isogen; Nippon Gene), according to the manufacturer’s instructions and were quantitated by spectrophotometry. cDNAs were synthesized from 1 μg of extracted RNA with the oligo(dT)18 primer and the Transcript First Strand cDNA Synthesis kit (Roche). Briefly, after denaturing at 65°C for 10 min, the RNA was incubated at 50°C for 60 min in 20 μL of cDNA synthesis buffer containing 50 pmol oligo(dT)18 primer, 20 U RNase inhibitor, 2.5 mM dNTPs, and 10 U reverse transcriptase. After an incubation at 85°C for 5 min, a
RSIV is insensitive to innate immune response

In the present qPCR assay, the primers Rb-Mx-F (5'-TCC GCT ATC AGA TGT TGC AGG AGG AG-3') and Rb-Mx-R (5'-TCT TGC TGC CAA AGT CAA AGT CC-3'), designed based on the nucleotide sequence of the Mx gene in our cDNA library (GenBank accession number: JX145038), were used for targeting the Mx gene, whereas the primers Rb-β-actin-F (5'-TCA TCA CCA TCG GCA ATG AGA GGT-3') and Rb-β-actin-R (5'-TGA TGC TGT AGG TGG TCT CGT-3') (Umasuthan et al., 2011) were used for targeting the β-actin gene. The RT-qPCR assays were carried out in an Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer) using SYBR Green AccuPower Greenstar qPCR PreMix (Bioneer), according to the manufacturer's instructions with the following program; one cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 20 s and 58°C for 40 s. Specifications of the qPCR reaction were analyzed via melting curve analysis. Baseline was determined automatically by the Exicycler analysis software (Bioneer), and Mx gene transcription fold-changes relative to the mock control were calculated by the 2- ΔΔCT method of Livak and Schmittgen (2001).

**Statistical analysis**

The statistical analysis was performed using GraphPad PRISM 5.04 (GraphPad Software Inc.). Gene expression at various sampling times was compared by one-way analysis of variance with Dunnett's multiple comparison test. P-values < 0.05 were considered significant.

**Results and Discussion**

Prior to the main experiments, we evaluated pathogenicity of the RSIV stock solution in red seabream and rock bream. Mortality of the fishes infected with different doses of RSIV are shown in Fig. 1. Red seabream infected with the RSIV stock solution diluted 10^3, 10^4, 10^5, and 10^6-fold began to die 6–12 days after the RSIV inoculation, and their cumulative mortality rates were

![Fig. 1. Pathogenicity of red seabream iridovirus (RSIV) to red seabream and rock bream. A) Mortalities in red seabream, and B) rock bream.](image-url)
90%, 90%, 70%, and 40%, respectively (Fig. 1A). Rock bream infected with RSIV at 10³, 10⁴, 10⁵, and 10⁶-fold dilutions of the stock solution began to die 9–12 days after RSIV inoculation, and their mortality rates were 100%, 100%, 95%, 20%, and 0%, respectively (Fig. 1B). Based on these results, the calculated 50% lethal dose (LD₅₀) of the RSIV stock solution against red seabream and rock bream was an approximate 10³.₅-fold dilution. Thus, we used 10⁵–10³-fold diluted RSIV stock solution for subsequent experiments.

The changes in red seabream and yellowtail mortality following infection with RSIV and Poly(I:C) administration are shown in Figs. 2A and B. Red seabream infected with RSIV showed the same mortality curve regardless of Poly(I:C) administration (Fig. 2A, Poly(I:C)-RSIV (diluted 10³) and control-RSIV (diluted 10³); fish began to die from day 8 of RSIV infection, and all fish had died by day 11. No mortality was observed in the Poly(I:C)-mock and control-mock groups. The same tendency was observed in the yellowtail experiments (Fig. 2B); fish infected with RSIV began to die on day 9–9 of RSIV infection regardless of Poly(I:C) administration, and cumulative mortalities of the Poly(I:C)-RSIV (diluted 10³) and control-RSIV (diluted 10³) groups were 90% and 95%, respectively. No mortality was observed in the Poly(I:C)-mock and control-mock groups, except one fish died accidentally in the Poly(I:C)-mock group (Fig. 2B).

![Fig. 2](imageURL)  
Fig. 2. Mortality change due to red seabream iridovirus (RSIV) infection in red seabream, yellowtail, and rock bream that were or were not administered Poly(I:C). A) Red seabream administered a 10³-fold dilution of the RSIV stock solution, B) yellowtail administered a 10³-fold dilution of the RSIV solution, C) rock bream administered 10³- or 10²-fold dilution of the RSIV solution.
viruses but the present results revealed that it was not applicable to RSIV infection. Economic losses of rock bream due to RSIV infection are serious in Korea (Jung and Oh, 2000; Kim et al., 2002). Moreover, protection of fish belonging to the genus Oplegnathus by vaccination is difficult (O. I. E., 2012). Thus, a novel strategy may be necessary for protecting rock bream from RSIV infection in a future study.

Acknowledgements

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References


Fig. 3. Mx gene transcript fold-change in rock bream administered Poly(I:C) determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR) targeting the Mx and β-actin genes. *: a significant difference (p < 0.05).


RSIV は Poly(I:C) 投与により誘導される一過性自然免疫の影響をおそらく受けない

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Poly(I:C) を投与したインシダイ、マダイおよびブリを RSIV で攻撃したところ、何れの魚種においても RSIV による死亡率は対照区と差が出認められなかった。これは、Poly(I:C) を投与しても魚を RSIV 感染から防御することが出来ないことを示す結果である。しかし、Poly(I:C) を投与したインシダイでは、インターフェロン誘導の指標である Mx 遺伝子が十分に発現されていた。したがって、RSIV は、おそらく Poly(I:C) 投与により誘導される魚の一過性自然免疫の影響を受けないと考えられた。

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