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Adjuvant effects of poly I:C and imiquimod on the immunization of kuruma shrimp (*Marsupenaeus japonicus*) with a recombinant protein, VP28 against white spot syndrome virus

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ABSTRACT

The adjuvant effects of poly I:C and imiquimod during immunization with a recombinant protein, rVP28 derived from white spot syndrome virus (WSSV) was investigated in kuruma shrimp (*Marsupenaeus japonicus*). Shrimps were injected intramuscularly with different doses of rVP28, poly I:C and imiquimod, and combined rVP28 + poly I:C or imiquimod, and challenged with WSSV. Expression of innate immune-related genes was examined in the heart and lymphoid organ of combined rVP28 + poly I:C or imiquimod immunized shrimps at 1, 3 and 7 days after WSSV challenge. Shrimps which received rVP28 + poly I:C and rVP28 + imiquimod had significantly higher survivals of 52 and 58%, respectively compared to the rVP28 alone or PBS injected control groups ($P < 0.05$). A significant up-regulation of innate immune-related genes, such as Rab7, lysozyme, penaeidin, crustin, Toll and TNF was noticed in combined rVP28 + poly I:C or imiquimod immunized shrimps. Our results indicate that injection administration of poly I:C or imiquimod + sub-unit protein (rVP28) provides a significant protection and induces immune response in kuruma shrimps against WSSV. Therefore, poly I:C and imiquimod have potentials to be used as adjuvants or immunostimulants in shrimp immunization.

Statement of relevance: Major contributors to economic losses in shrimp aquaculture are viral diseases, of which white spot syndrome virus (WSSV) is the most important one due to its rapid spread and economic impact. In this paper, we present an immunization method towards prevention of WSSV disease in kuruma shrimp using adjuvants such as poly I:C or imiquimod along with a sub-unit protein (rVP28).

1. Introduction

The global crustacean aquaculture industry with shrimp as a major product is worth in excess of US$30 billion annually (FAO, 2014), but continues to be beset by endemic viral diseases (Johnson et al., 2008). Farmed shrimps are susceptible to a wide variety of pathogens, including viruses, bacteria, fungi and protozoa. Losses which resulted from diseases have had a devastating impact on shrimp aquaculture during its 30-year lifespan. The main contributors to these losses were viral diseases (Lotz, 1997), of which white spot syndrome virus (WSSV) is the most important one due to its epizootic spread and economic impact. Kuruma shrimp (*Marsupenaeus japonicus*), the highest priced shrimp species among the farmed crustaceans and widely cultured in Japan, China, Australia and Southeast Asian countries (Rosenberry, 2001) is also infected by WSSV including other penaeid shrimps, such as black tiger shrimp (*Penaeus monodon*), Chinese shrimp (*Fenneropenaeus chinensis*), crayfishes, crabs and lobsters (Otta et al., 1999; Supamattaya et al., 1998). Control of viral diseases is, therefore, of paramount necessity in sustaining this important primary production industry.

Adjuvants are helper substances that are added to vaccine formulations in order to improve immune response to the incorporated antigen (Bowden et al., 2003). In the conventional vaccines, adjuvants are used to elicit an early, high and long-lasting immune response. Numerous compounds are under evaluation as immunological adjuvants and peptide-carriers to improve the immune response. The double-stranded synthetic RNA, poly I:C has been widely used as a viral mimic to examine immune responses in vertebrates (Huang et al., 2006). In shrimps, antiviral immunity is induced by double-stranded RNA (dsRNA) that confers protection against WSSV infection (Robalino et al., 2004). Although the exact mechanism of action is unknown, imiquimod, a nucleoside analog of imidazoquinoline is an agonist for

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toll-like receptors (TLRs) 7 and 8 (Schön and Schön, 2007). In mammalian models, imiquimod acts on induction of cytokines, such as interferon alpha (IFN-α), interleukin-12 (IL-12), and tumor necrosis factor alpha (TNF-α) (Gupta and Khandelwal, 2004). The desire for new and improved adjuvants stems not only from the need to make existing inactivated vaccines more potent, but also to gain features such as antigen-spreading ability, stimulation of T-cell immunity, and longer-lasting protective immunity. Therefore, the benefits of incorporating any adjuvant into vaccines must be balanced against any increased risk of adverse reactions.

Modern practices in finfish aquaculture include highly effective routine vaccinations against multiple pathogens that have dramatically reduced impact of diseases. It has been widely assumed that true adaptive systems do not exist in invertebrates (Kimbrell and Beutler, 2001), thus vaccines have not been routinely developed and used in shrimp aquaculture. In particular, invertebrate immune response to viruses is poorly understood. Within the last decade, a body of literature has been building that indicates that shrimp and other crustaceans can be immunized with either inactivated virus or protein "sub-unit" vaccines and thereby protected from mortality induced by WSSV (Johnson et al., 2008). Recently, the protective efficacy of oral delivery and injection of a recombinant protein, rVP28 derived from WSSV envelope and synthesized using wheat germ cell-free technology was demonstrated in kuruma shrimp (Kono et al., 2014).

The aim of the present work was to investigate the adjuvant effects of poly I:C and imiquimod during immunization with rVP28 protein in kuruma shrimp and to use them as conjugative carriers of viral proteins.

2. Materials and methods

2.1. Experimental shrimps

Healthy kuruma shrimps (mean body mass 10 ± 1 g) without any overt disease symptoms were obtained from Matsumoto Fisheries, Miyazaki, Japan. Shrimps were maintained in an indoor system facilitated with running artificial seawater at 20 °C and fed with a commercial diet (Higashimaru, Tokyo, Japan) once a day. Before challenge test, shrimps were sampled randomly and screened for WSSV by PCR (Kim et al., 2007).

2.2. Preparation of rVP28 sub-unit protein

Recombinant VP28 (rVP28) was synthesized using the ENDEXT Wheat Germ Expression H Kit (CellFree Sciences, Ehime, Japan). Protein synthesis and purification were performed as described in our previous report (Kono et al., 2014). Total protein, wheat-GST-rVP28 was used for shrimp immunization. The concentration of rVP28 in total protein was measured by SDS-PAGE using α-lactalbumin (Sigma-Aldrich, St. Louis, MO, USA) as standard.

2.3. Immune challenge and sample collection

In the first experiment, the effective dose of the rVP28 to protect the shrimp against WSSV disease and the onset of protection were determined. Individual shrimp from three groups was immunized with an intramuscular (i.m.) injection of 100 μg of total protein containing 5.0, 2.5 or 1.0 μg of rVP28 dissolved in 100 μL phosphate buffered saline (PBS). A control group received an i.m. injection of 100 μL PBS. Injection was made in the second abdominal segment. An artificial challenge with WSSV was carried out by immersion method described previously by Kono et al. (2009) 7 days post-injection. Challenged shrimps from each group were distributed in 3 replicate tanks (n = 20) and maintained in seawater flow-through system. Shrimp survival from each tank was recorded until mortality stopped.

In the second experimental trial, the protective effect of poly I:C and imiquimod against WSSV in kuruma shrimp was investigated. Shrimps were i.m. injected with 100 μg of poly I:C (Sigma-Aldrich) or imiquimod [1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4 amine] (LKT Laboratories, Inc., St. Paul, MN, USA) dissolved in 100 μL PBS. A control group was injected with 100 μL PBS. WSSV artificial challenge was carried out by immersion at 7 days post-injection and challenged shrimps from each group were distributed in 3 replicate tanks (n = 20) facilitated with seawater flow-through system. Survival from each group was monitored until mortality stopped.

In the third experimental trial, the efficiency of using rVP28 + adjuvant (poly I:C or imiquimod) in protecting kuruma shrimp against WSSV after injection was determined. Shrimps divided into four groups (n = 90) were i.m. injected with 100 μg of total protein containing 2.5 μg rVP28 only, 2.5 μg rVP28 + 100 μg poly I:C or imiquimod dissolved in 100 μL PBS. A control group had an i.m. injection of 100 μL PBS. WSSV artificial challenge was carried out by immersion at 7 days post-injection and challenged shrimps from each group were distributed in 3 replicate tanks (n = 30). Survival from each group was recorded daily for 11 days. For expression analysis of innate immune related genes, nine shrimps were used from each group having three individuals from each replicate. The heart and lymphoid organ (LO) from shrimps were collected at 1, 3 and 7 days after WSSV infection for RNA extraction and cDNA synthesis.

2.4. Total RNA isolation and cDNA synthesis

Total RNA was extracted from the heart and LO of kuruma shrimp using ISOGEN (Nippon Gene, Osaka, Japan) in accordance with the manufacturer's instructions. The amount of nucleic acid in each total RNA sample was determined by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer, ND-1000 (Thermo Scientific, Wilmington, DE, USA). The purity of each total RNA sample was assessed by measuring the ratio of O.D. 260 nm/O.D. 280 nm. cDNA was synthesized from 1.0 μg of total RNA using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) following the manufacturer's instructions, and this cDNA was used as a template for polymerase chain reaction (PCR).

2.5. Expression analysis of innate immune-related genes by semi-quantitative RT-PCR

Expression of innate immune-related genes in the heart and LO of WSSV challenged shrimps injected with combination of rVP28 and poly I:C or imiquimod from the third trial was determined. PCR was conducted with the primer combinations according to the protocol of Kono et al. (2014). The immune-related genes (Mj Rab7, Mj penaeidin, Mj crustin, Mj lysozyme, Mj TNF and Mj Toll), the internal control gene β-actin, and their respective primers with GenBank accession numbers are presented in Table 1. PCR products were separated by 1.5% agarose

Table 1
Primers used for expression analysis in this study.

Name	Sequence (5′ → 3′)	Length (mer)	Accession no.
Mj Rab7 Fw[a]	CTCGCAAGAAGATTCTCCTG	20	AB379643
Mj Rab7 Rv[b]	CTTCGTTGATACCGCCCTAT	20	
Mj lysozyme Fw	TCCTAATCTAGTCTGCAGGGA	21	AB080238
Mj lysozyme Rv	CTAGAATGGGTAGATGGA	18	
Mj penaeidin Fw	GCTGAACCCACTATAGTCTTT	21	AU175636
Mj penaeidin Rv	CTACCATGGTGATGAAACAAA	20	
Mj crustin Fw	CATGGTGGTGGCTTAGGAAA	19	AB121740
Mj crustin Rv	GTAGTCGTTGGAGCAGGTTA	20	
Mj Toll Fw	TCTTTCTGGTGTTTTAGCTACTGTAA	26	AB333779
Mj Toll Rv	TTTGATGAGAGCACGACAATC	21	
Mj TNF Fw	AAGAAAACCCCCAGGAAGAA	20	AB385697
Mj TNF Rv	AACCAGTGTGCACTCCAGA	19	

[a] Fw = Forward.
[b] Rv = Revers.