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Duration and Booster Effect of Phylactic Response against White Spot Syndrome Virus Infection in Kuruma Shrimp Orally Administrated with Recombinant Viral Proteins, rVP26 and rVP28

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ABSTRACT—White spot syndrome virus (WSSV: a synonym of penaeid rod-shaped DNA virus, PRDV) is the causative agent of white spot disease (WSD: penaeid acute viremia, PAV), one of the most serious diseases affecting decapod crustaceans around the world. Recently, "quasi-immune response" was found in kuruma shrimp *Penaeus japonicus*, wherein individuals that naturally survived from WSD showed protection against a rechallenge with WSSV. The phylaxis against WSSV was also inducible by oral vaccination with recombinant WSSV proteins, rVP26 and rVP28. In the present study, kuruma shrimp orally vaccinated with rVPs were sequentially challenged with WSSV to evaluate onset and duration of phylactic response and booster effect. The phylactic response of shrimp against WSSV-challenge peaked at day 45 after the vaccination with rVP26 (RPS: 100%) and at day 55 with rVP28 (RPS: 93%), and decreased within 10–20 days. The phylaxis against WSSV-challenge was boosted by the secondary vaccination with homologous rVPs, but not by those with heterologous rVPs. The peaks of phylactic responses appeared at day 22 after the secondary vaccination more rapidly than those after the primary vaccination. These results demonstrated that the duration of phylaxis induced by oral vaccination with rVPs was relatively short, but could be extended by booster vaccination with homologous rVPs.

Key words: white spot syndrome virus, WSSV, quasi-immune response, kuruma shrimp, oral vaccination, phylaxis, booster effect, PRDV

White spot syndrome virus [WSSV: a synonym of penaeid rod-shaped DNA virus (PRDV) (Inouye *et al.*, 1996)], a member of the genus *Whispovirus* in the family *Nimaviridae*, is a causative agent of white spot disease [WSD: a synonym of penaeid acute viremia (PAV) (Inouye *et al.*, 1996)], one of the most serious diseases affecting decapod crustaceans in culture industries around the world (Lightner, 1996; Wang *et al.*, 1998). WSSV is ovoid or ellipsoid to bacilliform in shape; it is 120-150 nm in diameter and 270–290 nm in length. The virion consists of an inner, rod-shaped nucleocapsid with a tight-fitting capsid layer and an outer, loose-fitting, lipid-rich trilaminar envelope. The viral nucleocapsid contains a DNA-protein core bounded by a distinctive capsid layer and a single molecule of circular ds-DNA with an approximate size of 300 kbp (van Hulten *et al.*, 2001; Yang *et al.*, 2001). The virions contain at least six major proteins; VP28 and VP19, which are associated with the envelope; VP664 and VP15, which are associated with the nucleocapsid; and VP24 and VP26, locating in a space between the envelope and the nucleocapsid (Chen *et al.*, 2002; van Hulten *et al.*, 2000a; 2000b; Leu *et al.*, 2005; Tsai *et al.*, 2006). Unfortunately, a stable cell line for propagation of WSSV *in vitro* has not been established.

Although the major route of WSSV infection is vertical transmission in seed production facilities for kuruma shrimp *Penaeus japonicus*, stable production of specific-pathogen-free (SPF) shrimp was accomplished by countermeasures for the prevention of WSSV transmission, such as selection of WSSV-free spawners by polymerase chain reaction (PCR), disinfection of eggs with iodine and sterilization of rearing water with ozone (Mushiake *et al.*, 1999; Satoh *et al.*, 1999). In kuruma shrimp farms, horizontal transmission by cannibalism and waterborne routes is also very important among reared shrimp and cohabiting crustaceans in those environments (Mushiake *et al.*, 1999; Wu *et al.*, 2001). Infection of WSSV by oral route is significantly less efficient than those by the immersion and intramuscular

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routes, however, a consequent WSSV infection may be easily established in shrimp farms due to ingestion of a large amount of WSSV by cannibalizing WSD shrimp (Satoh *et al.*, 2008). Thus, it is still difficult to prevent horizontal infection by WSSV at shrimp farms (Maeda *et al.*, 1998; Momoyama 2003).

Recently, a quasi-immune response was found in kuruma shrimp, wherein individuals that naturally survived from WSD showed protection against a rechallenge with WSSV (Venegas *et al*., 2000). The protection of shrimp against WSSV challenge appeared at 3 weeks after primary infection and lasted for 2 months (Wu *et al.*, 2002). Neutralizing activity against WSSV was observed in the hemolymph of the surviving shrimp (Venegas *et al*., 2000). It was also possible to induce phylaxis against WSSV infection by intramuscular injection of formalin-inactivated WSSV or the recombinant WSSV proteins (rVPs), rVP26 and rVP28 (Namikoshi *et al.*, 2004). Moreover, the phylaxis was induced by oral vaccination with rVP26 and rVP28 (Satoh *et al.*, 2008). A similar degree of phylaxis was also inducible in whiteleg shrimp *Litopenaeus vannamei*, black tiger prawn *Penaeus monodon*, and crayfish *Procambarus clarkii*, by the oral and/or intramuscular routes (Witteveldt *et al.*, 2004a, 2004b, 2006; Vaseeharan *et al.*, 2006; Jha *et al.*, 2006).

As mentioned above, cannibalism is one of the most important routes in horizontal transmission of WSSV. It is thus considered that oral vaccine with WSSV rVPs is necessary for the prevention of WSD outbreaks in shrimp aquaculture industry, because oral route is more convenient for shrimp vaccination than intramuscular route in farms rearing a large number of shrimp. However, little is known about duration of shrimp phylaxis against WSSV and booster effect of oral vaccine with WSSV rVPs. In the present study, we performed sequential WSSV challenge of kuruma shrimp orally vaccinated with rVP26 and rVP28 to elucidate a time-dependent change of shrimp phylaxis response against WSSV challenge.

Materials and Methods

Shrimp and WSSV inoculum

The kuruma shrimp (body weight: 0.08−0.92 g) produced at a shrimp farm with no history of WSD in Miyazaki Prefecture were used in the present study. The shrimp were confirmed to be WSSV-free by nested PCR just before using for the following experiments. The shrimp were reared with dechlorinated electrolysed seawater (23 ± 1°C) in a flow-through system inside double-bottomed tanks with sand beds, and fed with a commercial crumbled diet at 3−20% of body weight per day.

The WSSV suspension was prepared by the method reported by Satoh *et al.* (2008), i.e. muscle of moribund WSD shrimp was homogenized with four-time volumes of PBS, and centrifuged at 3,000 × *g* for 10 min at 4°C. The resulting supernatant was stored at −85°C until use as a source of WSSV inoculum for the following experiments.

Preparation of shrimp diet containing rVP26 and rVP28

WSSV rVP26 and rVP28 were produced as described by Namikoshi *et al.* (2004). Briefly, transformed *Escherichia coli* cells, in which rVP26 and rVP28 had been induced by IPTG (isopropyl-1-1-thio-β-D-galactoside), were suspended in TE buffer (50 mM Tris-HCl and 2 mM EDTA; pH 8.0) containing 0.1% (v/v) Triton X-100 and 0.1 mg/mL lysozyme, and incubated at 30°C for 15 min. After sonication to eliminate viscosity, the cell suspension was washed twice by centrifugation (12,000 × *g*, 15 min), and rVP26 and rVP28 were harvested from the insoluble fraction. The resulting pellet containing rVP26 or rVP28 was resuspended in PBS for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Analysis with a software, ImageJ (NIH, USA), for density profiles from digital images of the SDS-PAGE gels revealed that intensities of rVP26 and rVP28 were approximately 30%, and the remaining 70% composed of proteins originated from *E. coli* cells (data not shown). The rVP26 and rVP28 suspensions were reconstituted at 0.5% (v/w) into a commercial dry diet (Maruha, Japan), and were coated with an adhesive agent (SD Tenchaku #1; Schering-Plough Animal Health, Japan) for preparation of oral vaccine, as described by Satoh *et al.* (2008).

Oral vaccination of shrimp and WSSV challenge

Experiment I: Onset and duration of phylaxis against WSSV challenge in vaccinated shrimp

Kuruma shrimp (mean body weight, 0.09 g) were divided into four groups (n = 780), and were fed with 10 μg of rVP26 and rVP28/g shrimp/day, 25 μg of *E. coli* proteins/g shrimp/day (control 1) or PBS (control 2) with the commercial diet for 15 days. The dosage of the vaccine was referred to Satoh *et al*. (2008). As shown in Fig. 1, at days 29, 36, 45, 55, 75, 106, 112, 119, 126 and 135 after the initial vaccination, 20−25 shrimp from each group were challenged with WSSV by immersion (1 h) route, which was the same condition by Satoh *et al*. (2008). After the challenge, shrimp were reared for additional 30 days to observe those mortalities. The WSSV challenge doses were adjusted to approximately 70% of cumulative mortality in naïve shrimp.

In the experimental infection groups, dead shrimp were removed twice a day and stored at −30°C for PCR analysis to confirm the association of mortality with WSSV infection. For the detection of WSSV by PCR, total DNA was extracted from shrimp using the method described by Nonaka *et al*. (1998) and two specific PCR primer sets were used. Primers P1 (5'-ATC ATG GCT

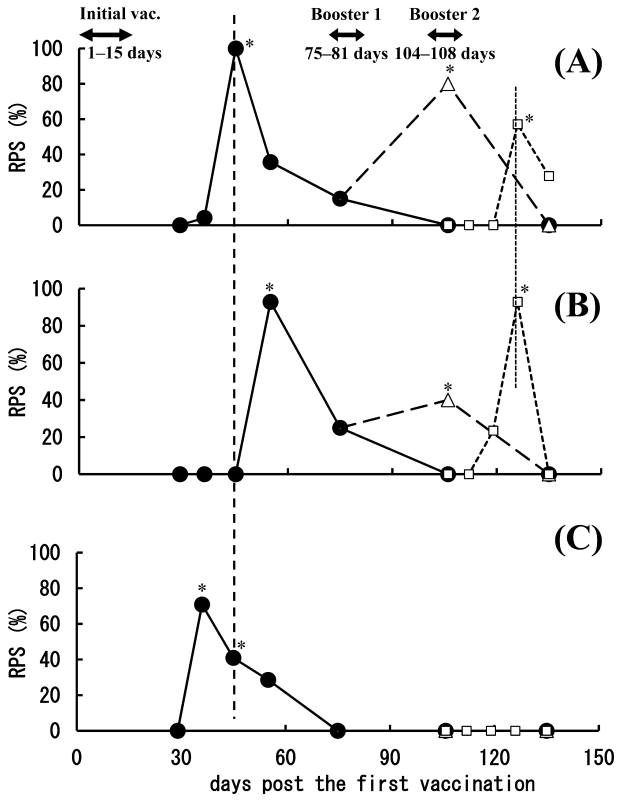

Fig. 1. Time-dependent changes of relative percent of survivor (RPS) against WSSV challenge in kuruma shrimp vaccinated with WSSV rVP26, rVP28 or *E. coli* proteins. (A): rVP26, (B): rVP28, (C): *E. coli* proteins ; RPS of the shrimp with initial vaccination (●), with booster 1 (△), with booster 2 (□). *$p < 0.01$: from the cumulative mortality.

GCT TCA CAG AC-3') and P2 (5'-GGC TGG AGA GGA CAA GAC AT-3') were used for the first-step PCR, and P3 (5'-TCT TCA TCA GAT GCT ACT GC-3') and P4 (5'-TAA CGC TAT CCA GTA TCA CG-3') were used in nested PCR (Kimura *et al*., 1996).

Experiment II: Booster effect of vaccine to maintain shrimp phylaxis against WSSV challenge

At day 75 after the initial vaccination with rVP26, rVP28, *E. coli* and PBS (control) in the experiment I, 65 shrimp of each group were transferred to new aquaria with 210 litres for oral boosting with rVP26, rVP28 or *E. coli* proteins for 7 days. At days 106 and 135 after the initial vaccination (at days 31 and 60 after the booster vaccination, respectively), 20−29 shrimp of each group were respectively challenged with WSSV by immersion for 1 h. The challenged shrimp were reared for additional 30 days to observe mortalities of the shrimp.

Experiment III: Booster effect of vaccine after disappearance of the shrimp phylaxis against WSSV challenge

At day 104 after the initial vaccination, 100 shrimp of each group were transferred to four new aquaria with 150 litres, and fed with rVP26, rVP28, *E. coli* proteins or PBS (control) as booster for 5 days in the same manner as in the experiment II. The shrimp (n = 12 to 25) of each group were challenged with WSSV or PBS (mock challenge) by the immersion route at days 112, 119, 126 and 135 after the initial vaccination (at days 8, 15, 22 and 31 after the booster vaccination, respectively). The challenged shrimp were reared for additional 30 days to observe mortalities of the shrimp.

Experiment IV: Booster effect of vaccination with heterologous rVPs

A new batch of kuruma shrimp (mean body weight, 0.08 g) was divided into three groups (n = 500), and fed with 10 μg of rVP26 or rVP28/g shrimp/day with commercial diet for 15 days. At days 35, 45, 55 and 106 after the initial vaccination, 20 or 25 shrimp in each group were challenged with WSSV in the same manner as in the experiment I to confirm onset and decline of phylactic response against WSSV due to the initial vaccination. At day 106 after the initial vaccination, the shrimp in each group were transferred into two new aquaria with 210 litres, and fed with rVP26 or rVP28 as booster vaccination for 6 days; i.e. there were four vaccination groups; rVP26-rVP26, rVP26-rVP28, rVP28-rVP26 and rVP28-rVP28, respectively. The booster of 10 μg of each rVP/g shrimp/day was given together with commercial diet for 7 days. The shrimp (n = 20) in each group were subjected to experimental challenge with WSSV at day 7 after the booster vaccination (at day 113 after the initial vaccination). The challenged shrimp were reared for additional 30 days to observe mortalities of the shrimp.

Statistical analysis

The mortalities of the experimental versus control groups were analyzed using chi-square test (χ^2) with a significance level of 1%. The relative percentage survival (RPS) values were calculated according to the method of Amend (1981).

Results

Onset and duration of shrimp phylaxis against WSSV challenge (Experiment I)

Cumulative mortalities of the vaccinated shrimp by WSSV challenge and calculated RPS values of each group versus PBS group are shown in Table 1. Also time-dependent changes of RPS in each group are shown in Fig. 1. In the shrimp vaccinated with rVP26 (Experiment I), phylaxis against WSSV challenge appeared at day 36 after the initial vaccination and lasted until day 75. The highest RPS value against WSSV challenge was 100%, which was recorded at day 45 after the initial vaccination. Phylaxis against WSSV challenge of the shrimp vaccinated with rVP28 and *E. coli* proteins was observed between days 55 and 75

Table 1. Cumulative mortality and relative percent survival (RPS) of kuruma shrimp vaccinated with recombinant WSSV (rVP26, rVP28) and *E. coli* proteins in WSSV challenge.

	Initial vaccination with (1–15 days)	Cumulative mortality (%) and relative percent survival (RPS) in challenge at:									
		29 d		36 d		45 d		55 d		75 d	
		Mortality	RPS	Mortality	RPS	Mortality	RPS	Mortality	RPS	Mortality	RPS
Experiment I	rVP26	92	0	92	4	0*	100	45	36	68	15
	rVP28	96	0	96	0	88	0	5*	93	60	25
	E. coli	92	0	28*	71	52*	41	50	29	96	0
	PBS	84	–	96	–	88	–	70	–	80	–
Experiment II	rVP26										
	rVP28									Booster vaccination (75–81 days)	
	E. coli										
	PBS										
Experiment III	rVP26										
	rVP28										
	E. coli										
	PBS										

	Initial vaccination with (1–15 days)	Cumulative mortality (%) and relative percent survival (RPS) in challenge at:									
		106 d		112 d		119 d		126 d		135 d	
		Mortality	RPS	Mortality	RPS	Mortality	RPS	Mortality	RPS	Mortality	RPS
Experiment I	rVP26	60	0							96	0
	rVP28	72	0							84	0
	E. coli	72	0							75	0
	PBS	60	–							70	–
Experiment II	rVP26	15*	80							76	0
	rVP28	45*	40							79	0
	E. coli	75	0							71	0
	PBS	75	–							71	–
Experiment III	rVP26	Booster vaccination (104–108 days)		85	0	90	0	30*	57	65	24
	rVP28			95	0	65	24	5*	93	88	0
	E. coli			75	0	85	0	75	0	92	0
	PBS			65	–	85	–	70	–	86	–

* Significantly different ($p < 0.01$) from the PBS group by χ^2 test.

and between days 36 and 55, respectively. The RPS peak against WSSV challenge was recorded at day 55 after the initial vaccination in the shrimp with rVP28 (RPS 93%) and at day 36 in that with *E. coli* proteins (RPS 71%). These results showed that RPS peak in the shrimp fed with rVP26 appeared 10 days earlier than that in the shrimp with rVP28, but 10 days later than that in the shrimp with *E. coli* proteins (Fig. 1A).

Booster effect of oral vaccination to maintain the shrimp phylaxis against WSSV challenge (Experiment II)

The shrimp vaccinated with rVP26, rVP28 or *E. coli* proteins were given a booster vaccination with homologous rVPs for 5 days from days 75 to 81 after the initial vaccination, and were challenged with WSSV at days 106 and 135 after the initial vaccination. The recorded RPS values of the shrimp with rVP26 or rVP28 booster were 80% and 40% at day 106 after the initial vaccination, respectively. These RPS values were clearly higher than those of the shrimp without booster vaccination in the Experiment I. However, at day 135 after the initial vaccination, the shrimp phylaxis against WSSV challenge disappeared in the boosted groups with rVP26 or rVP28 (Table 1, Experiment II; Fig. 1, Booster 1). No phylaxis against WSSV challenge was observed in the shrimp boosted with *E. coli* proteins at either day 106 or 135 after the initial vaccination (Table 1, Fig. 1).

Booster effect of vaccination after disappearance of the shrimp phylaxis against WSSV challenge (Experiment III)

Cumulative mortalities in shrimp that received booster vaccination at days 104 to 108 after the initial vaccination are shown in Table 1, and the time-dependent changes in RPS are shown in Fig. 1. In the shrimp with rVP26 booster, no phylaxis against WSSV challenge was observed at days 112 and 119 after the initial vaccination; however, RPS values with 57% and 24%

were recorded at days 126 and 135 after the initial vaccination, respectively. Shrimp with rVP28 booster showed no significant phylaxis against WSSV challenge at day 112 and 119 after the initial vaccination; however, RPS value with 93% was recorded at day 126 after the initial vaccination. Phylaxis against WSSV challenge in the shrimp boosted with rVP28 disappeared at day 135 after the initial vaccination. No phylaxis against WSSV challenge was observed in the shrimp boosted with *E. coli* proteins during the experimental periods between days 112 and 135 after the initial vaccination.

Booster effect of vaccination with heterologous rVPs (Experiment IV)

To evaluate the booster effect in shrimp phylaxis induced by oral vaccination, the shrimp vaccinated with rVP26 or rVP28 were newly prepared, and boosted with homologous or heterologous rVPs for 7 days from day 106 to day 112 after the initial vaccination (Table 2). The shrimp vaccinated with rVP26 showed phylaxis against WSSV challenge (RPS 72%) at day 55 after the initial vaccination, but the RPS decreased to 21% at day 106 after the initial vaccination. The rVP26-vaccinated shrimp boosted with homologous antigen (rVP26) showed 100% of RPS at day 113 after the initial vaccination, but those with heterologous antigen (rVP28) showed no significant phylaxis at day 113. On the other hand, the shrimp vaccinated with rVP28 showed phylaxis against WSSV challenge (RPS 59%) at day 55 after the initial vaccination; however, phylaxis disappeared by day 106 after the initial vaccination. The rVP28-vaccinated shrimp boosted with homologous antigen (rVP28) showed 67% of RPS at day 113 after the initial vaccination, but those with booster of heterologous antigen (rVP28) showed no significant phylaxis at day 113 after the initial vaccination.

Discussion

Peaks of phylaxis against WSSV challenge in the shrimp vaccinated with rVP26 and rVP28 were recorded at days 45 and 55 after vaccination, but the phylaxis disappeared at day 106 after the vaccination, suggesting that the phylaxis induced by oral vaccination with rVPs lasted for 20−30 days after its appearance (Fig. 1A, B). Wu *et al*. (2002) reported that protection against WSSV-challenge appeared in survivors of shrimp at 3 weeks after the experimental infection with WSSV and lasted for 2 months. Previously, it was confirmed that kuruma shrimp needed approximately 30 days for appearance of protection against WSSV-challenge after the intramuscular vaccination with rVP26 and rVP28 (Namikoshi *et al*., 2004). Thus, it was considered that the duration of shrimp phylaxis against WSSV infection induced by WSSV rVPs was shorter than that observed in shrimp survived from experimental infection. On the other hand, in *P. monodon* and *L. vannamei* vaccinated orally with rVP28, protection against WSSV infection was observed between days 3 and 21 of vaccination, but peaks of the phylaxis was observed at day 7 after vaccination (Witteveldt *et al.*, 2004a, 2004b). It is thus considered that the period for onset of shrimp phylaxis against WSSV infection could be independently influenced by experimental conditions, such as species and size of shrimp, vaccine materials, vaccination routes, and temperature of rearing water. We believe that shrimp size has an especially important role in the onset of phylaxis against WSD in kuruma shrimp, because the onset of phylaxis against WSSV infection appeared around at day 55 after vaccination in shrimp weighing 0.1 g, at day 45 in shrimp weighing 0.8 g, and at day 36 in shrimp weighing 2.5 g (data not shown). Also the present data demonstrated that the appearance time of shrimp phylaxis against WSSV challenge after the booster vaccination was slightly earlier than that after the primary vaccination; it appeared at 25−30 days after the booster in Experiment II and at 7 days after the booster in Experiment III. Unfortunately, it was not clear that the onset shifting of the shrimp phylaxis against WSSV is due to change of shrimp sizes or booster effect.

Booster effect of the oral vaccination with rVP26 and rVP28 was evaluated in Experiments II and III, because Experiment I showed that duration of the phylaxis induced by the oral vaccination with these rVPs was only 20–30 days. In Experiment II, booster vaccination with rVP26, rVP28 and *E. coli* proteins was given for 7 days from day 75 to day 81 after the initial vaccination, when there was still a low level of shrimp phylaxis against WSSV challenge by the initial vaccination with rVP26 or rVP28 (Table 1, Experiment I, 75 days). RPS values of the shrimp boosted with rVP26 or rVP28 were 80% and 40% at day 106 after the initial vaccination, respectively; these RPS values were clearly higher than those without the booster vaccination. Thus, the observed phylactic ability against WSSV challenge in Experiment II was considered to result from the booster effect.

After complete disappearance of the shrimp phylaxis against WSSV challenge, booster vaccination with rVP26 and rVP28 was given for 5 days from day 104 to day 108 after the initial vaccination (Experiment III). In the groups boosted with either rVP26 or rVP28, the peak of RPS against WSSV challenge was recorded at day 126 after the initial vaccination, which corresponded to 18–20 days after the booster vaccination (Fig. 1). This indicates that appearance of the phylactic response against WSSV challenge by booster vaccination was advanced in comparison with primary vaccination (Experiment I). Unfortunately, no enhancement of phylactic ability against WSSV infection was observed in the present results. It has been previously reported

Table 2. Booster effects of homologous and heterologous recombinant WSSV proteins, rVP26 and rVP28, in shrimp vaccination (Experiment IV).

	Initial vaccination with (1–15 days)	Cumulative mortality (%) and relative percent survival (RPS) in challenge at:								
		45 d		55 d		106 d		Booster vaccination with (106–112 days)	113 d	
		Mortality	RPS	Mortality	RPS	Mortality	RPS		Mortality	RPS
Experiment IV	rVP26	87	0	24*	72	55	21	rVP26	0*	100
								rVP28	30	33
	rVP28	87	0	36*	59	90	0	rVP26	26	42
								rVP28	15*	67
	PBS	80	–	88	–	70	–	PBS	45	–

* Significantly different ($p < 0.01$) from the PBS group by χ^2 test.

that shrimp phylaxis against WSSV challenge was significantly enhanced by giving of twice intramuscular-injections of rVP26 or rVP28 (Namikoshi *et al.*, 2004). The present data demonstrated that booster with WSSV rVPs is useful to extend duration of shrimp phylaxis against WSSV infection. However, the duration of the boosted phylaxis was also relatively short; it disappeared within 30–50 days after the booster vaccination (Fig. 1). Therefore, it was considered that booster vaccination with rVPs should be given to shrimp every 30–40 days to maintain the phylactic level against WSSV infection.

Next, the shrimp initially vaccinated with rVP26 and rVP28 were boosted with homologous or heterologous rVP antigens in Experiment IV. The RPS value of the shrimp vaccinated with rVP26 was 21% just before the booster vaccination (at day 106 after the initial vaccination), and the value increased to 100% after the booster with homologous antigen (at day 113 after the initial vaccination). But, no significant increase of RPS was observed in the shrimp boosted with the heterologous antigen, rVP28 (RPS 33%). A similar tendency was observed in the shrimp initially vaccinated with rVP28; RPS increased from 0% to 67% before and after the booster vaccination with homologous antigen (rVP28-rVP28), but RPS in the shrimp with heterologous antigen (rVP28-rVP26) increased up to 42%, which was lower than that with rVP26-rVP26 (Table 2). Thus, it was concluded that a homologous antigen could be needed for boosted phylactic response in shrimp, suggesting a possibility that shrimp seemed to recognize both rVP26 and rVP28 as different proteins.

In this study, *E. coli* proteins were used as one of controls, because the present rVP suspensions contained *E. coli* proteins equivalent to approximately 70%. Interestingly, shrimp phylaxis against WSSV infection was also induced by the oral administration of *E. coli* proteins (Table 1 and Fig. 1). Shrimp phylaxis induced by oral administration of peptidoglycan, β-1,3-glucan or lipopolysaccharide (LPS) is effective not only against bacterial infections (Sung *et al.*, 1994; Teunissen *et al.*, 1998; Sritunyalucksana *et al.*, 1999) but also against WSSV infections (Itami *et al.*, 1998; Takahashi *et al.*, 2000). In the last decade, β-1,3-glucan and LPS binding protein genes have been cloned from *P. monodon*, *L. vannamei* and *L. stylirostris* (Sritunyalucksana *et al.*, 2002; Romo-Figueroa *et al*., 2004; Cheng *et al.*, 2005; Roux *et al.*, 2002). Lectin genes have been cloned from *P. monodon* (Luo *et al.*, 2006) and *L. schmitti* (Cominetti *et al.*, 2002), and more recently, a Toll receptor gene has been cloned from *L. vannamei* (Yang *et al.*, 2007). In the present data, however, the phylaxis peak (RPS 71%) appeared at day 36 after the initial administration of *E. coli* proteins, which peak appeared 10–20 days earlier than those by the vaccination with rVP26 and rVP28 (Experiment I). Moreover, the shrimp phylaxis induced by the initial administration of *E. coli* proteins was never boosted by the secondary administration (Experiments II and III). Thus, we speculate that the mechanism for the phylactic response in shrimp administrated with *E. coli* proteins may be different from that vaccinated with rVP26 and rVP28. The shrimp with *E. coli* proteins showed 41% and 29% of RPS against WSSV challenge at the days 45 and 55 after the vaccination (Table 1), thus it was considered that the RPS values of the shrimp vaccinated with rVP26 or rVP28 (100% or 93%) might include a phylactic response due to *E. coli* proteins. Therefore, high purification of rVPs may not be required for the oral vaccination, because containing *E. coli* proteins also support induction of shrimp phylaxis against WSSV, especially in the initial vaccination.

Finally, the present study demonstrated booster effect of oral vaccine with rVPs in kuruma shrimp. In vertebrates, booster effect is due to acquired immune responses characterized by memory and specificity. Therefore, we presume that the observed booster effect in shrimp may be an interesting phenomenon suggesting quasi-immunological memory and specificity in invertebrates, although detail analyses for molecule(s) with respect to the quasi-immune response are necessary in our future investigation.

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