Analyses of Hemolymph Immunoparameters in Kuruma Shrimp Infected with Penaeid Rod-shaped DNA Virus

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The present study was conducted to examine the changes provoked by penaeid acute viremia (PAV) in hemolymph immunoparameters of peptidoglycan (PG)-fed kuruma shrimp Penaeus japonicus. The shrimps were fed a commercial diet containing PG for 14 days prior to the challenge with PRDV, causative agent of PAV, by the water-borne method. A control group fed PG-free diet was also challenged. Hemolymph obtained from infected and control shrimps was analyzed for total hemocyte count, plasma protein concentration, plasma Mg concentration, phenoloxidase activities in the hemocyte lysate and cell protein concentration. The total hemocyte count declined over 5 days after challenge in both groups. The total hemocyte count values of the PG-fed group were higher than those of the control group at every sampling point, but no significant differences were observed. A significant increase in plasma Mg concentration was found toward 3rd day of post-challenge for the control group (p < 0.05). The control group had significantly increased values in plasma protein concentration in 1st and 3rd days of post-challenge, but these changes were mitigated in PG-fed group. The survival rates after 10 days of the challenge were 70% and 35% for the PG-fed group and control group, respectively. These results indicate that PG can mitigate physiological changes during PRDV infection.

Key words: PRDV, PAV, hemolymph parameter, Penaeus japonicus, white spot syndrome

The penaeid rod-shaped DNA virus (PRDV), also known as white spot baculovirus (WSB) (Durand et al., 1996) and a variety of other names (Chou et al., 1995; Huang et al., 1995; Wang et al., 1995; Wongteerasupaya et al., 1995), has caused serious damage to cultured penaeid shrimps in Asia (Takahashi et al., 1994, 1996).

Our previous studies revealed that the oral administration of peptidoglycan (PG) derived from Bifidobacterium thermophilum is a potential prophylactic agent for the control of penaeid acute viremia (PAV) which is caused by PRDV (Itami et al., 1998). The present study was conducted to examine the changes provoked by an experimental PRDV infection in selected hemolymph immunoparameters of PG-fed kuruma shrimp.

Materials and Methods

Experimental shrimps

Healthy juveniles of Penaeus japonicus (average body weight: b. w., 11.6 g) were provided by a local farm. Upon arrival, the shrimps were confirmed to be PRDV-free by 2-step PCR (Maeda et al., 1998).

Feeding protocol

The shrimps were divided in two groups after a 2-week acclimation period. They were fed a commercial diet either with peptidoglycan (PG) derived from B. thermophilum (PG-fed group) or without it (control group) for 14 days before challenge with PRDV. PG was administered at a concentration of 0.2 mg/kg. b. w./day. The PG feeding was maintained after the virus challenge.

Experimental infection

Three heads of PRDV-infected P. japonicus (12 g b. w.) were minced after the carapace had been removed,
and homogenized with 40 ml of sterile seawater. The homogenate was centrifuged (1,600 × g, 5°C, 15 min) and diluted 20 times with sterile seawater (stock virus suspension). All the steps were performed on ice.

After the 14-day feeding period, all the shrimps from PG-fed and control groups were immersed for 2 h in the virus solution, which contained 2.5 ml of the stock virus suspension per liter of seawater. Following the immersion, the shrimps were placed in 8 aquaria. Four of the aquaria containing 17 shrimps each were used for hemolymph sampling to analyze the immunoparameters. The other four aquaria received 10 shrimps each for a challenge trial to monitor mortality. All of the aquaria had the same feeding protocol and water exchange rate (30% per day). The dead shrimps were collected every day for 10 days after the challenge with PRDV.

**Hemolymph sampling**

Hemolymph samples were collected using the following schedule: one day prior to the challenge (D-0), and one day (D-1), three days (D-3) and five days (D-5) after the challenge. Ten shrimps were used from each group every sampling day, except on D-5 when 7 shrimps were sampled from each group. Hemolymph was withdrawn from the ventral sinus into a 1-ml disposable syringe containing 0.4 ml of Tris-HCl buffer with an anticoagulant (TC buffer, 0.01 M Tris-HCl, 0.25 M sucrose, 0.1 M sodium citrate; pH 7.6). Only intermolt shrimps were sampled.

Hemolymph was analyzed for cell and plasma parameters. Cell parameters included total hemocyte count (THC), and phenoloxidase activities (PO) and cell protein concentration (CP) in the hemocyte lysate. Plasma parameters were plasma protein concentration (PP) and plasma Mg concentration (PMg).

**Examination for hemolymph immunoparameters**

Hemolymph samples were centrifuged (1,100 × g, 5°C, 10 min) and the precipitated cells were used for PO and CP determination. The supernatants were used for PP and PMg analyses. All parameters were assayed in duplicate.

**THC:** From the collected hemolymph, a sub-sample was diluted with TC buffer containing 5% formalin, and the hemocytes were counted using a hemocytometer.

**PO activity assay:** One milliliter of PO reaction buffer (5 mM CaCl2, 5 mM MgCl2, 50 mM HEPES, 3% NaCl; pH 7.0) was added to each sample tube containing 1 × 106 cells. The tubes were then frozen at −80°C. After 24 h, cell suspensions were thawed, ultrasonicated, centrifuged (6,500 × g, 5°C, 10 min) and filtered through a 0.20-μm membrane filter. L-DOPA (Sigma) solution (2.9 mg/ml of PO reaction buffer) was filtered through 0.20-μm membrane filter. A mixture containing 0.1 ml of the L-DOPA filtrate, 0.8 ml of PO reaction buffer and 0.1 ml of the filtered hemocyte lysate was incubated at 60°C for 1 h. The absorbance was measured at 490 nm.

**Protein assay:** The Bio-Rad Protein Assay (Bio-Rad Laboratories) was used for measuring both PP and CP. The filtered hemocyte lysate was used for CP analyses.

**Plasma magnesium assay:** Plasma magnesium was measured using the Xyldyl Blue method (Magnesium B Kit, Wako Pure Chemical).

**Results and Discussion**

**THC**

The THC declined after the PRDV challenge for both control and PG-fed groups (Fig. 1). The THC of control and PG-fed groups on D-3 and D-5 were significantly lower than their own values measured at D-0 (p < 0.05).

The THC values of PG-fed group were higher than those of the control group at every sampling point: The THC of PG-fed group was 1.22 times higher than those of the control group on D-0, 1.27 times higher on D-1, 1.36 times higher on D-3, and 1.53 times higher on D-5. However, no significant differences were found between two groups on the same sampling day.

**Fig. 1.** The total hemocyte count (THC) of kuruma shrimp, *Penaeus japonicus*, one day prior to the challenge (D-0), and one day (D-1), three days (D-3) and five days (D-5) after the challenge with PRDV. Each value represents a mean ± S.D. ■: PG-fed group, □: control group

*: Significantly different from respective D-0 values (p < 0.05).
The THC of the shrimps upon arrival at the laboratory was $1.5 \times 10^7$ cells/ml. This value of PG-fed group on D-0 was $2.07 \times 10^7$ cells/ml which is 1.38 times higher than the initial value, after feeding PG for 2 weeks, in contrast to $1.7 \times 10^7$ cells/ml of the control group (1.13 times higher).

A THC decline after PRDV infection was also reported by Maeda et al. (1997). In our preliminary experiment, the average THC of shrimps ($n = 20$) was $3.2 \times 10^7$ cells/ml before the virus injection, but it decreased to $1.6 \times 10^7$ cells/ml and $2.3 \times 10^6$ cells/ml, 24 h and 48 h after injection, respectively (unpublished data). These results that THC was rapidly decreased by injection with PRVD indicated that the virus is directly responsible for the decrease in THC. This decline was thought to be provoked by the shrimp immune system which attempted to eliminate the infected cells by the attachment to host tissues by which virus-infected hemocytes are removed from circulation (Beckage, 1996). Another possibility is that the THC decline could be due to cell burst resulting from budding of the virus, or by virus-induced apoptosis, since this type of cell "suicide" may be induced or repressed during a viral infection (Cohen, 1993).

**PO activity**

No difference in the PO activity was observed either between groups or among the sampling days. D-5 was the only exception with a 2-fold increase for both groups (Fig. 2).

**Protein**

Changes of CP and PP showed a similar pattern, increasing on D-1, having the highest values at D-3 and decreasing at D-5 (Figs. 3 and 4). The main difference between CP and PP patterns was found on D-5 when CP values were still higher than those at D-0, and PP values were slightly lower than those at D-0. No differences

**Fig. 2.** Pheloloxidase activity (PO) of kuruma shrimp, *Penaeus japonicus*, one day prior to the challenge (D-0), and one day (D-1), three days (D-3) and five days (D-5) after the challenge with PRDV. Each value represents a mean ± S.D. ■: PG-fed group, □: control group *: Significantly different from respective D-0 values ($p < 0.05$).

**Fig. 3.** Plasma protein concentration (PP) of kuruma shrimp, *Penaeus japonicus*, one day prior to the challenge (D-0), and one day (D-1), three days (D-3) and five days (D-5) after the challenge with PRDV. Each value represents a mean and ± S.D. ■: PG-fed group, □: control group *: Significantly different from respective D-0 values ($p < 0.05$).

**Fig. 4.** Cell protein concentration (CP) of kuruma shrimp, *Penaeus japonicus*, one day prior to the challenge (D-0), and one day (D-1), three days (D-3) and five days (D-5) after the challenge with PRDV. Each value represents a mean and ± S.D. ■: PG-fed group, □: control group *: Significantly different from respective D-0 values ($p < 0.05$).
in CP and PP were observed between PG-fed and control groups.

The PP of the control group on D-1 and D-3 were significantly higher than the values on D-0 ($p < 0.05$). The PP of PG-fed group on D-3 were significantly higher than those on D-0 ($p < 0.05$).

Since both PP and CP increased after the virus infection, this increase of protein might be owing to the increase in the amount of the virus in the hemolymph. Harwood et al. (1994) reported the presence of an abundant, virus protein in the hemolymph of *Manduca sexta* larvae infected with polydnavirus. Another possibility for the increase in PP is the fact that baculoviruses encode a variety of proteases and other enzymes that "melt" the tissues (Beckage, 1996), and that the cytoplasmic protein of the "melted" cells would be incorporated into the shrimp hemolymph.

**Plasma magnesium**

No significant difference in PMg of either group was found between D-0 and D-1. The PMg of both groups increased on D-3. This increase was significant, however, only for the control group ($p < 0.05$). On D-3, the control group values were also significantly higher than those of PG-fed group ($p < 0.05$). The PMg on D-5 decreased from the D-3 values for both groups but the D-5 values were still higher than the values on D-0 and D-1 (Fig. 5).

The PMg concentration in the hemolymph is regulated by an active excretion process (Sartoris and Porter, 1997) and since shrimp are hypotonic in full-strength seawater ([Mg] = 126.8 mg/dl), an increase in the plasma Mg concentration would occur during an osmoregulatory malfunction (Boglio, 1995). Many studies have been conducted on the osmoregulatory capacity of penaeid shrimp, and it has been shown that this capability is affected by stress (Cochard et al., 1992; Chamantier and Soyez, 1994). Therefore, increases in PMg were taken as an indicator of osmoregulatory disorder provoked by the virus infection. PMg failed to show any abnormalities during early infection (D-1), hence PMg is thought to be unsuitable as a tool for measuring the health status of *P. japonicus* in relation to a PRDV infection. On the other hand, the PMg analyses were useful for revealing that the PG-fed group could maintain a better osmoregulatory capacity than the control group after the virus infection.

**Survival rate**

The mean survival rates 10 days after the challenge with the virus were 70% and 35% for PG-fed and control groups, respectively (Fig. 6). Similar results were obtained in previous studies done by Maeda et al. (1997) and Itami et al. (1998), in which it was found that PG-fed groups had significantly higher survival rates than the control ($p < 0.05$).

In conclusion, the efficacy of an oral administration of peptidoglycan derived from *B. thermophilum* was confirmed by the survival rates of *P. japonicus* after a PRDV infection shown in the present study. The over-

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**Fig. 5.** Plasma magnesium concentration (PMg) of kuruma shrimp, *Penaeus japonicus*, one day prior to the challenge (D-0), and one day (D-1), three days (D-3) and five days (D-5) after the challenge with PRDV. Each value represents a mean and ± S.D. ■: PG-fed group, □: control group *: Significantly different from respective D-0 values ($p < 0.05$).

**Fig. 6.** Survival rate of kuruma shrimp, *Penaeus japonicus*, during 10 days after the shrimps were challenged with PRDV. ■: PG-fed group, ▲: control group (n = 10 for each repetition)
all results present a better understanding of the physiological changes provoked by PRDV infection in the hemolymph of *P. japonicus* and show that oral administration of PG can mitigate these changes. More studies are needed to reveal possible interactions among those hemolymph parameters as well as how the environmental factors, molting cycle and size of the shrimp can affect them. This information could be used as a tool for analyzing health status of shrimp.

**References**


