Morphology and Immunological Roles of Hemocytes and Fixed Phagocytes in Black Tiger Shrimp *Penaeus monodon*

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ABSTRACT—Morphology and immunological roles of the three distinct types of hemocytes, hyaline, small granular and large granular cells in black tiger shrimp *Penaeus monodon* were studied. The study of functions of these hemocytes in the elimination of injected yeast (*Saccharomyces cerevisiae*) or *Vibrio harveyi* as foreign bodies showed a rapid response against those particles. Together with fixed phagocytes, the blood cells removed the yeast through the process of phagocytosis, nodule formation and encapsulation, which occurred at almost all parts of the body. The injection of the yeast caused a marked reduction in the blood cell counts in the hemolymph during the first 1 h. *V. harveyi* was efficiently removed within 3 h after injection. The granular cells (small granular and large granular hemocytes) were the major blood cells that are associated with phenoloxidase activity. The role of hemocytes and fixed phagocytes on defense mechanism in the shrimp were discussed.

Key words: *Penaeus monodon*, hemocyte, fixed phagocyte, phagocytosis, immune parameter, phenoloxidase, black tiger shrimp

The black tiger shrimp *Penaeus monodon* is aquatic species of economic potential for Thailand and several other countries around the world. The intensive culture system of the shrimp currently practiced has led to a series of problems, for example, the degradation of environment, wide spread of several diseases which seriously affect the production efficiency and the harvests (Kautsky *et al.*, 2000). The scientists from various research disciplines have worked to solve the problems in order to maintain the sustainability of the marine shrimp culture. The significance of the study of immune system and blood components in black tiger shrimp is the utilization of the information in the rectification of the problems that arise. The information are particularly important as indicators for the black tiger shrimp

health. The information also serves the research needs in other relevant research areas. The current study was carried out to present the information on various blood cell types that are associated in the elimination of foreign bodies that indicates the immune system of the shrimp.

Materials and Methods

Test Animals

Live black tiger shrimp with average weight of 10–15 g from the farms with no record of diseases were used in the study. The shrimp showed transparent and intact exoskeleton with no symptoms of diseases. The slight infestation of *Zoothamnium* sp. was, however, noted in some shrimp. Shrimp samples were examined for bacterial infections by a standard microbiological method and also examined for white spot syndrome

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virus (WSSV) and yellow-head virus (YHV) infections by PCR and RT-PCR, respectively (Takahashi *et al.*, 1996; Tang and Lightner, 1999). They were free from bacterial and viral infections. The juveniles were acclimated in 5 m³-concrete ponds with aeration at the Aquatic Animal Health Research Center (AAHRC), Faculty of Natural Resources, Prince of Songkla University. Fifty juveniles were stocked in each pond where the water salinity was 20 part per thousand and water temperature ranged 28–30°C. The juveniles, prior to stocking were treated with 35 ppm formaldehyde to eradicate the external parasites on the individuals. The feed was given 4 times daily, settled particles were removed and water was exchanged every day during the 5–7 days of acclimation period.

Hemocyte structure and their roles in the elimination of foreign bodies

The hemocytes were isolated from blood taken from the above mentioned shrimp by centrifugation at $3,600 \times g$ at 4°C using 4% L-cysteine as an anticoagulant (Supamattaya *et al.*, 2000a). The isolated blood cells were divided into two portions, first of which was cultured on the glass slide in 24-well plate and the cell monolayer was fixed with 0.125% glutaraldehyde for 10 min and stained with Giemsa for the study of cell types and external structures using a light microscope. In order to study the cell surface, the cell monolayer was also fixed with 0.125% glutaraldehyde and post-fixed with 1% OsO₄ for 30 min. The sample was dehydrated through an alcohol series and completely dried with a critical point dryer. The sample was then coated with gold particle and viewed under a scanning electron microscope (JSM-35 CF, Jeol). The second portion of blood sample was employed for the study of internal structures of the cells using a transmission electron microscope. Cell suspensions were centrifuged and cell pellet was fixed in 2.5% cacodylate buffered glutaraldehyde pH 7.4 at 4°C for 1 h and post-fixed with 1% OsO₄ in the same buffer for 1 h. After dehydration with ethyl alcohol and propylene oxide, they were embedded in synthetic resin (Epon-812). Semi- and ultrathin sections were stained with toluidine blue and safranin, and uranyl acetate and lead citrate, respectively, and were examined by a light microscope and an electron microscope (TEM 100 CX II, Jeol) at 80 KV acceleration.

The cells and tissues were monitored for their capacity in the elimination of yeast as a foreign body. Powder of baker yeast (*Saccharomyces cerevisiae*) was dissolved in phosphate buffer at 1% w/v, 0.1 mL of which was injected into the tail muscle of each experimental juvenile. Phosphate buffer alone was injected into the juvenile in control. The injected shrimp were kept in tanks under the same conditions as acclimation period, and the blood samples were collected at 1 h-interval for the first 12 h period. Subsequently, the

blood samples were collected at 24 h-intervals for 96 h (4th day) and on the 7th, 14th and 28th days for the blood cell counts using a hemacytometer. The blood cell counts were performed at 1, 6, 9, 12, 48 h, 7th and 28th days for the control. The tissue samples were also collected at each blood sampling and preserved in Davidson's fixative (Bell and Lightner, 1988). The tissues were processed for the preparation of permanent slides with hematoxylin and eosin (H & E) stain for the microscopic examinations.

Immune parameters in black tiger shrimp

The blood samples were collected from 20 juveniles, and different types of blood cells were isolated from blood samples by continuous gradient centrifugation (Supamattaya *et al.*, 2000a). Sixty percent of Percoll solution (P-1644, Sigma) supplemented with 2.5% NaCl was prepared by centrifuged at $8,500 \times g$ for 30 min at 4°C using a fixed angle rotor. The shrimp blood with 4% L-cysteine was gently overlaid and then centrifuged at $85 \times g$ for 10 min at 4°C using a swing-out rotor. Each band of blood cells was separated and washed three times. Hemocyte lysate (HLS) was prepared in cacodylate buffer pH 7.4 using Vibracell™ sonicator at 30 amplitude for 10 s (Supamattaya *et al.*, 2000a). The isolation of serum was carried out in the blood sampling without anticoagulant and centrifuged at $3,590 \times g$ for 2 min at 4°C. The HLS and serum were analyzed for phenoloxidase activity by the method modified from Söderhäll and Hall (1984). Analyses of the phagocytic activity and superoxide ion (O₂⁻) formation of the blood cells were conducted using sampling blood from 20 juveniles. Each blood sample was mixed with anticoagulant and washed in K-199 solution (Itami *et al.*, 1994) by $3,590 \times g$ centrifugation for 2 min at 4°C. The phagocytic activity of the blood cells was determined by the method modified from Paterson and Stewart (1974). The hemocyte monolayer was prepared for the determination of superoxide production by the modified method of Bell and Smith (1993). The clearance ability to remove bacterial cells from blood circulation of shrimp was studied using the method modified from Martin *et al.* (1993), by injecting *Vibrio harveyi* as foreign particles at a dose of 1.2×10^4 CFU/g shrimp body weight.

Results

Hemocyte structure

Under a compound microscope, the black tiger shrimp possesses three types of blood cells: Hyaline cells of minute dimension, darkly stained, with lesser proportion of granules in the cytoplasm or entirely absent. The small granular cells are of longer dimension and greater proportions of eosinophilic cytoplasmic granules and having ability to adhere to glass surface. The large granular cells are of largest dimen-

sion with greater abundance of eosinophilic cytoplasmic granules (Fig. 1).

The scanning electron microscopy revealed the different patterns of the surface of the three types of blood cells as follows:

1) Hyaline cells: They are smallest-sized blood cells of flattened, rounded shape with smooth surface. Occasionally, they are of fusiform or crescent shape.

Fig. 1. Light micrograph of three types of black tiger shrimp hemocytes adhered on glass. HC: hyaline cell, SGH: small granular cell, LGH: large granular cell. Giemsa stain, Bar = 10 μ m.

Fig. 2. Scanning electron micrograph of black tiger shrimp hemocytes. a) a hyaline cell exhibiting discoid shape on a spreading small granular cell. b) A cluster of large granular cells with abundant cytoplasmic granules (arrows). Bar 2a = 1 mm, 2b = 10 μ m.

There exists no microvilli or pseudopodia on the cell surface. The cells are 6.4–8.3 μ m in diameter (in rounded cell) or 2.5–3.6 μ m in width and 6.8–13.9 μ m in length for the crescent or fusiform shaped cells (Fig. 2a).

2) Small granular cells: The cells have large number of cell processes and pseudopodia. There appeared small number of microvilli on the cell surface. The cells are 4.2–6.8 μ m in width and 9.0–14.2 μ m in length.

Fig. 3. Transmission electron micrograph of black tiger shrimp hemocytes. a) a hyaline cell containing large nuclei with small ratio of cytoplasm (arrowhead). b) a small granular cell (SGH) contained small number of cytoplasmic granules while a large granular cell (LGH) have abundant of granules in the cytoplasm Bar 3a = 1 mm, 3b = 5 μ m.

Fig. 4. The changes in shrimp hemocytic counts of *Penaeus monodon* after injection with yeast cells (*Saccharomyces cerevisiae*).

There are small number of minute cytoplasmic granules with the size $0.6\text{--}1.0\text{ }\mu\text{m}$, not obvious when observed from the cell surface by SEM (Fig. 2a).

3) Large granular cells: They are of larger dimension with similar appearance as that of small granular cells, except the great number of large cytoplasmic granules. The granules are $0.7\text{--}1.0\text{ }\mu\text{m}$ in diameter and are obviously observed from cell surface. The pseudopodia or cell processes can be distinctly noted. The cells are $8\text{--}10\text{ }\mu\text{m}$ in diameter, $12.2\text{--}14.6$

μm in length and $7.2\text{--}7.8\text{ }\mu\text{m}$ in width (Fig. 2b).

The transmission electron microscope revealed that the hyaline cells were of smallest sizes with $5\text{--}6\text{ }\mu\text{m}$ diameter, with high nucleocytoplasmic ratio. Numerous free ribosomes or electron dense deposits were noted in the cytoplasm with small number of mitochondria and Golgi body, and rough endoplasmic reticulum (RER) or smooth endoplasmic reticulum (SER) were also seen. Few cytoplasmic granules were found in the cytoplasm (Fig. 3a). The small granular cells were of larger dimension, $7\text{--}10\text{ }\mu\text{m}$. There appeared $0.5\text{--}1\text{ }\mu\text{m}$ diameter of cytoplasmic granules scattered in the cells (Fig. 3b). The large granular cells were of largest dimension with large number of $1\text{--}1.5\text{ }\mu\text{m}$ diameter granules were seen in the cytoplasm (Fig. 3b).

Roles of hemocytes in elimination of yeast

Upon the injection of yeasts to study the shrimp's responses to the foreign bodies, there was a sharp decline in the total blood cell counts within 1 h of injection. After that the cell counts tended to recover slowly, however, remained at a low level until 9 h. The blood cell counts gradually increased and recovered to normal condition within 24 h, while there were small changes in the blood cell counts for the control (Fig. 4).

The histological examination showed the scattered blood cells among the intercellular spaces and blood sinuses. The phagocytic cells were accumulated in the gills, heart, lymphoid organ and connective tissues. They were capable of performing phagocytic activity. To lesser extent, the phagocytic cells were noted in the muscular and nervous tissues. The compound microscopy showed the presence of yeasts in the blood cells as well as in the fixed phagocytes within 1 h of the injection of yeast. The process of phagocytosis recognized in the fixed phagocyte or blood cells began with the encroachment of single cells and larger number of blood cells encircled the foreign bodies forming a nodule within

Fig. 5. a) Large encapsulation of yeast by hemocytes and fixed phagocytes occurring in the lymphoid organ of black tiger shrimp at 7th day post-injection with yeast. b) Small nodule formation observed in the blood lacuna of gill filament. c) Yeast cells trapped by fixed phagocyte (arrow) in heart tissue. b, shrimp blood cell; c, cuticle; y, yeast cell. H & E stain Bars = $50\text{ }\mu\text{m}$.

Fig. 6. Transmission electron micrograph of a fixed phagocyte in the heart of black tiger shrimp N, nucleus of a fixed phagocyte; H, heart muscle; M, mitochondria of heart muscle. Note autophagic vacuole (arrowhead) Bar = $5\text{ }\mu\text{m}$.

Table 1. Immune defense parameters of *Penaeus monodon* hemocytes

Parameters	Sample sources	Average*
1. Phenoloxidase activity (unit/min/mg protein)	Hyaline cells	199.0 $\pm$ 78.8
	Granular cells	1,249.5 $\pm$ 313.4
	Serum	32.2 $\pm$ 6.0
2. Phagocytic activity (%)	Hemocytes	26.8 $\pm$ 12.7
3. Superoxide activity (unit)	Hemocytes	25.4 $\pm$ 8.3
4. Clearance ability (%)	Blood	98.2

* Mean $\pm$ SD, n = 20.

3–9 h. The capsulation occurred between 24–48 h or longer where there were large number of blood cells surrounding the foreign bodies (Fig. 5a). Both the blood cells and fixed phagocytes were present in various tissues such as lymphoid organ, hepatopancreas, heart and gills (Figs. 5b–c). The electron microscopy indicated the absence of cytoplasmic granules in the fixed phagocytes, instead there existed large vacuoles, debris, and myelin figure that suggested the phagocytic activity and the formation of autophagic vacuoles (Fig. 6). The cells scattered in various tissues like heart, gills and digestive tract.

Immune parameters in black tiger shrimp

The 60% Percoll in 2.5% saline solution was used in the separation of two bands of blood cells for the determination of phenoloxidase activity. The upper band of the blood cells comprising mainly hyaline cells showed lower phenoloxidase activity, while the lower band formed by large number of granular cells showed high phenoloxidase activity. The phenoloxidase activity of serum was 32.2 ± 6.0 unit/min/mg protein. The juveniles maintained in the laboratory had the phagocytic activity of the blood cells of $26.8 \pm 12.7\%$. The level of superoxide formed by the blood cells during the same period was 25.4 ± 8.3 unit for healthy individuals. The study of clearance ability of blood cells and serum in the circulating system indicated that the injected bacteria showed a sharp reduction in their density within 3 h post-injection. With the injection of *V. harveyi* 1.2×10^4 CFU/g shrimp body weight into the tail muscle of healthy shrimp, the bacterial density in the blood dropped to about 2.2×10^2 CFU/g shrimp which accounted for 98.2% clearance ability. The results were concluded in Table 1.

Discussion

Morphology and types of blood cells in black tiger shrimp have been reported (Tsing *et al.*, 1989; Sung *et al.*, 1999; Van de Braak *et al.*, 1996, 2000), although the ultrastructure of the hemocyte types have scarcely been studied. Recently it has been suggested that the hyaline cells are the young and immature hemocytes of both the large granular hemocytes and small granular hemocytes that are produced in the hematopoietic tissue

and released into the hemolymph (Van de Braak *et al.*, 2002a). The black tiger shrimp's blood cells are differentiated into three cell types in the present study based on morphology, cell dimensions, structure of cell surface, sizes of nuclei and other cell components. However, no indication of the hyaline cells as a stem cell for the small granular cells and the large granular cells was proven in this study.

The small granular and large granular cells were well capable of pseudopodia formation which was of lesser extent for the hyaline cells. In a prolonged period, the process of degranulation or exocytosis occurred in the granular cells in which the granules totally disappeared. Hemocytes of black tiger shrimp were smaller than those found in kuruma prawn (Kondo *et al.*, 1998) and similar in size with others. The ultrastructures studied were similar in those three types of hemocyte reported in kuruma prawn and other species (Tsing *et al.*, 1989), for example, the electron dense deposited in the cytoplasm found in hyaline cells of black tiger shrimp was also observed in kuruma prawn (Bachere *et al.*, 1995, Kondo *et al.*, 1998). This structure was not found in small and large granular cells as well as fixed phagocytes.

With the injection of yeast cells into the shrimp, a marked reduction in the blood cell counts was noted within 1 h from the injection as a response against the invasion of foreign bodies. The histopathological examination suggested that the reduction in the blood cell counts were the result of the phagocytic activity that eventually cause the formation of nodules and capsules which will be eliminated from the body. Upon the elimination of foreign bodies from the individuals, freshly produced blood cells are recruited into the system by 24 h after the injection.

Fixed phagocytes located at various organs also played an important role to eliminate the foreign bodies, thus it can be said that the tissues of almost all parts of the shrimp are capable of eliminating the foreign bodies. The histological studies revealed that there were several processes through which the foreign bodies were eliminated. Such processes are phagocytosis, nodule formation and encapsulation. Three types of blood cells and fixed phagocytes are key components in all these processes. Fixed phagocytes in kuruma prawn were identified into 2 types, one located in the

lymphoid organ and the other in the heart tissue (Kondo *et al.*, 1998). In *P. monodon* injected with *Vibrio*, electron microscopy revealed the presence of many phagocytic cells that morphologically resemble small granular hemocytes (Van de Braak *et al.*, 2002a). In the present study fixed phagocytes were not identified in detail but they were found through all organs but very little and with less activity in the muscle and nerve tissue.

Phenoloxidase activity from the hemocytes of black tiger shrimp was abundantly found in the granular hemocytes and less activity in hyaline cells and serum. This finding coincides to those reported in black tiger shrimp studied by Sung *et al.* (1998) and other penaeid shrimp such as *Sicyonia ingentis*, *P. paulensis* and *P. japonicus* (Hose *et al.*, 1992; Sequeira *et al.*, 1995; Perrazzolo and Barracco, 1997; Kondo *et al.*, 1998) as well as in other crustaceans, *Astacus astacus*, *Procambarus clarki*, *Pacifastacus leniusculus* and *Macrobrachium rosenbergii* in which the phenoloxidase activity was found in the granular hemocytes (Smith and Söderhäll, 1983; Johanson and Söderhäll, 1985; Lanz *et al.*, 1993; Sung *et al.*, 1996).

The phagocytic activity may vary from species to species as reported by Paterson and Stewart (1974) who noted the phagocytic activity of 20% in lobsters and 26% in black tiger shrimp. The black tiger shrimp's blood also showed 98.2% clearance ability against *V. harveyi* within 3 h in healthy shrimp or the shrimp from under proper management of the farms. Van de Braak *et al.* (2002b) also reported the hemocytes and lymphoid organ plays an importance role to remove the bacterial cell from blood circulation in black tiger shrimp (*P. monodon*). Sritunyaluksana (1995) reported similar results in black tiger shrimp that the whole blood had higher clearance activity with over 80% in the elimination of bacteria while the separated blood cells had lower of 46% and even less of 5% in separated serum. It indicated that the blood components including humoral and cellular components as well as fixed phagocytes had an important roles in the phagocytic activity. In kuruma prawn it was also found that serum enhance the phagocytic activity (Kondo *et al.*, 1992). The phagocytic activity, however, also depends on shrimp culture condition, for example shrimp exposed to low oxygen level and infectious disease exhibited low phagocytic activity (Supamattaya *et al.*, 2000b).

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