Fish and Shellfish Immunology 72 (2018) 459–469

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

# Hemocytes of the mud crab *Scylla paramamosain*: Cytometric, morphological characterization and involvement in immune responses

Yi-Lian Zhou, Wen-Bin Gu, Dan-Dan Tu, Qi-Hui Zhu, Zhong-Kai Zhou, Yu-Yin Chen*, Miao-An Shu**

*College of Animal Sciences, Zhejiang University, Hangzhou 310058, China*

ARTICLE INFO

*Keywords:*
*Scylla paramamosain*
Hemocyte
Classification
Immune response
Primary culture
Transfection

ABSTRACT

Hemocytes play essential roles in the innate immune system of crustaceans. Characterization of hemocytes from estuary mud crab *Scylla paramamosain* was performed by flow cytometry and morphological studies such as cytochemical staining and electron microscopy. The hemocyte subsets were further separated using a modified Percoll density gradient centrifugation method. Based on the morphological characteristics of the cells, three distinct categories of hemocytes were identified: granulocytes with abundant large granularity representing 5.27 ± 0.42%, semigranulocytes with small or less granularity representing 76.03 ± 3.34%, and hyalinocytes (18.70 ± 3.92%) which were almost no granularity. The total hemocyte cell count and the percentage of hemocyte subsets varied after pathogen infection, including *Vibrio alginolyticus* and the viral double-stranded RNA analog Poly (I:C). The phagocytic process is of fundamental importance for crustaceans' cellular immune response as well as development and survival. The results of the *in vitro* phagocytosis assays analyzed by flow cytometry demonstrated that granulocytes and semigranulocytes had significantly higher phagocytic ability than hyalinocytes. A primary culture system, L-15 medium supplemented with 5–10% fetal bovine serum, was developed to further investigate the immune function of hemocytes. Furthermore, adenovirus can be utilized to effectively transfer GFP gene into hemocytes. Overall, three hemocyte sub-populations of *S. paramamosain* were successfully discriminated, moreover, their response to pathogen infections, phagocytic activity and adenovirus mediated transfection were also investigated for the first time. This study may contribute to a better understanding of the innate immune system of estuary crabs.

## 1. Introduction

The mud crabs (*Scylla* species) are broadly distributed through coastal areas of Indo-Pacific region [1], and *Scylla paramamosain* is a commercially important marine fishery species in China [2]. In recent years, due to over-fishing and sea water pollution, infectious diseases broke out frequently causing large economic losses [3]. The need to reduce the mortality and debilitating effects of pathogens is stimulating the investigations of the defense mechanisms and immune systems of crustacean [4].

As a crustacean animal, *S. paramamosain* lacks adaptive immunity and only relies on a rapid and efficient innate immune system to defend themselves against potential pathogens [5,6]. Hemocytes play essential roles in both cellular and humoral immune reactions in crustaceans, such as recognition, phagocytosis, clotting, prophenoloxidase (proPO) system activation and antimicrobial peptides synthesis [7–10]. The classification of crustacean hemocytes is mostly based on the presence of granules in cytoplasm, in general, they are divided into three distinct cell types and named as hyalinocytes (no granules in cytoplasm), semigranulocytes (a few granules in cytoplasm) and granulocytes (plenty of granules in cytoplasm), and each cell type is active in immune reactions [7,11]. In crayfish, hyalinocytes are chief cells involved in phagocytosis, semigranulocytes are more active in encapsulation, whereas granulocytes mainly participate in storage and release of the proPO system and cytotoxicity [7]. Furthermore, P. Jiravanichpaisal et al. demonstrated that the functions of hemocytes in crustacean and *Drosophila* were quite similar [9]. Hemocyte classifications have been reported in several crustacean species [4,11–17], however, there are no studies describing the classification and immune response of hemocytes in estuary mud crab *Scylla paramamosain*.

Cell culture technique is widely used in biological researches. Since no established cell line has been reported in crustaceans to date,

* Corresponding author.
** Corresponding author.
*E-mail addresses:* chenyy@zju.edu.cn (Y.-Y. Chen), shuma@zju.edu.cn (M.-A. Shu).

https://doi.org/10.1016/j.fsi.2017.10.055
Received 20 June 2017; Received in revised form 17 October 2017; Accepted 28 October 2017

primary culture of crustacean tissues have gained much attention [18,19]. Hemocytes of crustaceans are both the targets of pathogens and efficient immune participators [20], accordingly, the cultured hemocytes may serve as an *in vitro* immune system model to investigate the immune response against pathogen infection. However, there remains controversy over a standard culture method to culture crustacean hemocytes [20–28]. Especially, the optimal culture conditions for hemocytes from estuary *S. paramamosain* still remain obscure. Additionally, adenovirus is one of the most efficient vehicles for transferring foreign genes into mammalian cells [29], however, whether adenovirus can be utilized as an effective transfection approach on crustacean cells still need to be investigated.

In this study, hemocytes from mud crab *S. paramamosain* were firstly characterized and classified into three types: hyalinocytes, semigranulocytes and granulocytes. Meanwhile, hemocytes were challenged with bacteria *V. alginolyticus* and virus-analog Poly (I:C) to investigate their immune response against pathogen infection *in vivo*, and the *in vitro* phagocytosis of bacteria by different sub-populations was also identified. Additionally, a modified primary culture and adenovirus-mediated transfection were also performed on hemocytes, which might provide an *in vitro* platform for further study of innate immunity and specific gene functions in crustaceans.

## 2. Materials and methods

### 2.1. Experimental animals

Eighty healthy *S. paramamosain* weighing 151.3 ± 20.8 g were obtained from Sanmen Bay, Zhejiang province (P. R. China). Crabs were acclimated in filtered artificial seawater (salinity 18 ± 1 ppt, pH 7.8 ± 0.1, temperature 26 ± 1 °C) at least seven days before an experiment. Animals were fed with fresh manila clam *Ruditapes philippinarum* daily and water was changed every day. During the acclimatization, the molt stages of the mud crabs were determined by observing the third maxilliped according to the method described by Xu et al. [30], and only the mud crabs in the intermolt stage were selected and used in this study.

### 2.2. Hemocytes collection

The hemolymph of crabs was aseptically collected from the non-sclerotized membrane of cheliped leg by using 5 mL sterile syringe loaded with pre-chilled anticoagulant ACD-B solution (4.8 g $L^{-1}$ citric acid, 13.2 g $L^{-1}$ sodium citrate, 14.7 g $L^{-1}$ glucose, 1.2 g $L^{-1}$ sodium chloride) at the ratio of 1:1, then immediately centrifuged at 200 g at 4 °C for 10 min. The cell pellets were washed twice with PBS in the same centrifuged condition to collect the hemocytes.

### 2.3. Morphological classification of hemocytes

#### 2.3.1. Cytochemical staining of hemocytes

For cytochemical analysis, 20 μL hemolymph was dropped onto a glass slide and fixed for 5 min at room temperature for completely dryness. Then the smears were stained by hematoxylin-eosin (H & E) method and Giemsa method, separately. Stained hemocytes were observed for cell morphology under a light microscope (Nikon Eclipse E100).

#### 2.3.2. Electron microscopy observations of hemocytes

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were employed to provide more details of inside structure and outside appearance of hemocytes. For TEM, the hemocyte pellets were first fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for more than 4 h and then covered by agar. After washed three times with 0.1 M phosphate buffer, the pellets were postfixed with 1% $OsO_4$ for 1–2 h. A grade series of ethanol and aceton was used to dehydrate after washing three times, and then the pellets were transferred in resin mixture. The ultrathin sections were spliced by an ultramicrotome (Leica EM UC7), after stained by uranyl acetate and alkaline lead citrate, sections were observed in a transmission electron microscope (Hitachi Model H-7650). For SEM, hemocyte pellets were fixed with 2.5% glutaraldehyde and then 1% $OsO_4$ in 0.1 M phosphate buffer (pH 7.0). After a graded series of dehydration, samples were transferred to iso-amyl acetate and finally dehydrated with liquid $CO_2$. The dehydrated sample was observed under a scanning electron microscope (Hitachi Model TM-1000).

#### 2.3.3. Flow cytometry analysis of hemocytes

Hemocyte pellets were collected as mentioned above, and then were resuspended in 500 μL PBS. Forward scatter and side scatter parameters (FSC and SSC) were adopted to determine the relative cell size and cytoplasmic granularity of hemocytes. Flow cytometric analysis was conducted by FACS Calibur (Becton Dickinson, US) and data was analyzed by the BD Cell software. At least 20,000 cells were recorded for each sample.

### 2.4. Separation of hemocyte sub-populations

Percoll (Solarbio®) gradient centrifugation was used to isolate hemocyte sub-populations. Commercial Percoll solution was adjusted to appropriate osmotic pressure by adding NaCl solution to a final concentration of 0.45 M [13]. Hemolymph was collected as mentioned above, followed by being loaded onto the top of a modified Percoll gradient composed of 80%, 60% and 30% (v/v) Percoll. After centrifugation at 1400 g for 30 min, the hemocytes were separated into three layers. The hemocyte sub-populations of each layer were collected, washed twice with PBS, and morphologically checked using a confocal laser microscope (Zeiss LSM 780) after DAPI [4′, 6-diamidino-2-phenylindole] staining.

### 2.5. Cytometric characterization of hemocyte sub-populations during bacterial and viral stimulation

*S. paramamosain* were immune challenged by injection with 100 μL $10^7$ CFU *Vibro alginolyticus* (an anaerobic Gram-negative bacillus), 100 μL 1 mg/mL Poly (I:C) (Sigma) and 100 μL PBS respectively. After 0 h, 24 h, 48 h, 72 h and 96 h of injection, 500 μL hemolymph was collected and washed twice with PBS, then resuspended in L-15 medium (Sigma). Aliquots (30 μL) from hemocyte suspensions were used for total hemocyte counts (THC) by a hemocytometer, and each crab was counted three times. The remaining suspensions were analyzed by flow cytometry FACS Calibur (Becton Dickinson, US) to observe the proportion change of different sub-populations [31]. Each treatment had five crabs and PBS group served as the control group.

### 2.6. Determination of phagocytosis by hemocytes

Separated hemocytes were resuspended in L-15 medium, and hemocyte numbers were counted with a hemocytometer. FITC-labeled *V. alginolyticus* were added into hemocyte suspensions at a ratio of 20:1 and incubated for 30 min at 28 °C. Hemocytes pretreated with Cytochalasin D was served as the negative control. The suspensions were centrifugated to harvest hemocytes and washed three times with PBS to remove extra *V. alginolyticus* [32]. Hemocytes were fixed with 4% paraformaldehyde for 25 min and detected by flow cytometry (Cytomic FC 500MCL).

### 2.7. Development of primary hemocyte culture

Separated hemocyte sub-populations were resuspended in L-15 medium (0.2 mM NaCl, 100 U/mL penicillin and 100 μg/mL streptomycin, pH 7.4–7.6; filtered through a 0.22 μm syringe filter)