Fish and Shellfish Immunology 74 (2018) 62–68

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Short communication

Flow cytometic analysis of *Penaeus monodon* haemocyte responses to poly I:C

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ARTICLE INFO

Keywords:
Penaeus monodon
Haemocyte
Poly I:C
Virus
Flow cytometry

ABSTRACT

This study was aimed at investigating the cellular responses of *Penaeus monodon* haemocytes to poly I:C stimulation using flow cytometric assay. Total haemocyte count (THC), percentages of different haemocyte subpopulations [hyaline cells (HC), semigranular cells (SGC) and granular cells (GC)], non-specific esterase activity (EA), total reactive oxygen species/reactive nitrogen species (ROS/RNS) production, nitric oxide (NO) production, apoptotic haemocyte ratio and plasmic phenoloxidase (PO) activity were determined in poly I:C-injected shrimp. Results showed that poly I:C at a low dose (5 μg shrimp^{-1}) caused obvious increases in THC, GC proportion, ROS/RNS production and NO production, but had no significant effect on EA, apoptosis and PO activity. In the early stage of poly I:C injection at a high dose (20 μg shrimp^{-1}), THC and GC proportion improvements could also be observed, suggesting that GC might be induced to release from hemocytopoietic or other tissues to participate in immune response, and this subpopulation might be the main cell type involved in the cellular defence against virus. In the later period, proportions of both GC and SGC reduced paralleled by THC reduction, indicating that depletion of GC and SGC was mainly contributed to the reduced count of circulating haemocyte. Obvious increases in ROS/RNS production and NO production were induced in haemocyte of shrimp under a high dose of poly I:C stimulation, but only slight rise of EA and suppression of PO activity could be observed in poly I:C-stimulated shrimp, suggesting that ROS/RNS-dependent system was vital in the immune defence of shrimp against virus. On the other hand, increase of apoptotic haemocyte ratio and THC reduction were presented after the drastic increases of ROS/RNS and NO productions, implying that the stimulated ROS/RNS might be excess and harmful, and was the major factor for the haemocyte apoptosis and depletion. THC recovered after 48 h injection, while haemocyte apoptosis also returned to the control level, suggesting that apoptosis might be contributed to eliminate damaged, weak or infected haemocytes to renew the circulating haemocytes, and it could be considered as an important defending strategy against virus.

1. Introduction

Diseases outbreak have caused serious economic losses in shrimp aquaculture all over the world. Virus, such as white spot syndrome virus (WSSV), is one of the major kind of pathogenic microorganism to shrimp. Understanding the immune and pathogenic mechanism of shrimp against virus would help us to prevent viral disease effectively. Previous researches of decapod crustacean found that virus challenge caused decline in total haemocyte count (THC), induced superoxide anion (O_2^-) production, lipid peroxidation and apoptosis, and altered the transcription levels of genes or enzymatic activities [1–6]. Some functional genes of shrimp have been found to involve in immune response against virus [7–12]. Double stranded RNA (poly I:C), which can be used for mimicking of viral infection, is considered as virus-associated molecular pattern [13]. Similar to virus infection, poly I:C stimulation could also triggers some cellular responses in shrimp haemocyte, including THC reduction and increased O_2^- production [14]. In other aquatic animals, poly I:C has been reported to induce a protective antiviral immune state against subsequent challenge with virus [15,16].

Crustacean depends on an innate immune system to defend against microorganisms infection. Haemocyte plays a vital role in the immune defence mechanism. They have found to be active in phagocytosis, encapsulation, cytotoxicity, respiratory burst, storage and release of the prophenoloxidase (proPO) system and other antimicrobial factors [17–19]. Though some cellular responses have been observed in virus-infected or poly I:C-stimulated shrimp [2,6,11,14], the detailed response mechanism and the real roles of these haemocyte responses

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https://doi.org/10.1016/j.fsi.2017.12.045
Received 15 November 2017; Received in revised form 17 December 2017; Accepted 22 December 2017
Available online 28 December 2017

against virus are still not clear. Flow cytometry (FCM) is a rapid and sensitive measurement technique at a cellular level. In the present investigation, in order to further understand the immune roles of shrimp haemocytes against virus, we applied FCM to analyze the cellular responses of *Penaeus monodon* haemocytes to poly I:C.

2. Materials and methods

2.1. Animals

The experimental tiger shrimp *Penaeus monodon* (12.76 ± 1.97 g) were obtained from a commercial shrimp farm in Nansha, Guangzhou, Guangdong Province, China. They were maintained in the laboratory with diluted seawater at 20‰, pH 7.8–7.9 and controlled temperature (24 ± 2 °C), with continuous water circulation. Prior to experimental use, shrimp were acclimated to the laboratory conditions for one week, and fed twice daily with commercial shrimp feed (40% protein, 5.0% fat, 5.0% fiber and 16% ash). Only apparently healthy shrimp in the intermoult stage were used.

2.2. Poly I:C injection

Poly I:C (GE) was dissolved in physiological saline solution (0.85% NaCl) to give concentrations of 0.2 and 0.8 μg μl^{-1}. Shrimps were randomly divided into three groups (twenty-five shrimp per group in separate aquaria). According to the previous study [14] and our pre-experiment, challenge experiment was performed by injecting 25 μl poly I:C solutions (0.2 or 0.8 μg μl^{-1}) into the last abdominal segment of each shrimp to obtain two treatment doses: 5 and 20 μg $shrimp^{-1}$. Shrimp injected with the same amount of sterile physiological saline solution were maintained as control. Each group was set up in triplicates. Haemolymph of each group was collected individually at 0, 3, 6, 12, 24 and 48 h after injection.

2.3. Preparation of haemocyte suspension

Experiment was performed on individual shrimp sample. Haemolymph (400 μl) was extracted from the ventral sinus of each shrimp by a 25 gauge needle and 2.5 ml syringe containing an equal volume of precooled anticoagulant solution (AS, glucose 20.5 g L^{-1}, sodium citrate 8 g L^{-1}, sodium chloride 4.2 g L^{-1}, pH 7.5). Two hundred microliter diluted haemolymph sample was removed for THC measurement, and another two hundred microliter diluted haemolymph was transferred into a separate microcentrifuge tube held on ice for PO activity analysis. The remaining diluted haemolymph was diluted with AS to obtain a final concentration of about 1×10^6 cells ml^{-1}, and then used for other flow cytometric analysis.

2.4. Flow cytometry

Flow cytometry was performed with a FACSCalibur (Becton-Dickinson Immunocytometry Systems, San Jose, CA) equipped with a single argon ion laser with filtered emission at 488 nm. Photomultiplier bandpass filters for fluorescence were 530 nm (green fluorescence, FL1) and 585 nm (yellow/orange fluorescence, FL2). Side scatter (SSC) and fluorescence data were collected on log scale, and forward scatter (FSC) data were collected on linear scales. For each haemocyte sample, 10,000 events were counted. Cell Quest® sofeware (Becton-Dickinson, San Jose, CA, USA) was used to create logical regions and colour gating analyses of fluorescence data.

2.5. Total haemocyte count (THC)

SYBR Green I (Sigma) was used for THC detection. Intact haemocytes and cell particles could be distinguished according to their obvious difference on fluorescent intensity, since intact haemocytes were stained by SYBR Green I with high green fluorescent, whereas cell particles with low content of double-stranded DNA were stained with low fluorescent. Briefly, a volume of 200 μl diluted haemolymph was incubated with 10 x (1/1000 of the DMSO commercial solution) SYBR Green I solution for 60 min in the dark, at room temperature before flow cytometric analysis. The number of intact cells was recorded in 1 min detection with a flow cytometer. The flow rate of the cytometer was measured as described previously [20]. THC is reported as the number of cells ml^{-1} haemolymph.

2.6. Percentages of different haemocyte subpopulations

A volume of 200 μl haemocyte suspension was directly analyzed by flow cytometer. FSC value and SSC value indicate the relative size and granularity of haemocytes respectively. Results were expressed as FSC-H/SSC-H dot plot. Three different haemocyte subpopulations (GC, SGC and HC) were divided according to their morphological difference as described previously [21].

2.7. Non-specific esterase activity

Esterase activity was measured using the non-specific liposoluble substrate fluorescein diacetate (FDA, Molecular Probes). A volume of 200 μl haemocyte suspension was incubated with 5 μM FDA for 30 min at room temperature (22–23 °C) in the dark. Then the fluorescence of cells was recorded on FL1 detector of flow cytometer. Esterase activity was expressed as mean fluorescence of FDA in arbitrary units (A.U.).

2.8. Total reactive oxygen species/reactive nitrogen species (ROS/RNS) production

The cell-permeant probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma) was used for detection of ROS/RNS production as described previously [22]. A volume of 200 μl haemocyte suspension was incubated with 10 μM DCFH-DA for 30 min at room temperature in the dark. Then the DCF fluorescence of cells was recorded on FL1 detector of flow cytometer. ROS/RNS production was expressed as mean fluorescence of DCF in arbitrary units (A.U.).

2.9. NO production

The fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA, Sigma) was used to measure intracellular NO production. A volume of 200 μl haemocyte suspension was incubated with 10 μM DAF-FM DA for 60 min at room temperature in the dark. Then the DAF-FM fluorescence of cells was recorded on FL1 detector of flow cytometer. NO production was expressed as mean fluorescence of DAF-FM in arbitrary units (A.U.).

2.10. Haemocyte apoptosis

Annexin V-FITC/PI apoptosis detection kit (Invitrogen) was used for apoptotic haemocytes detection following the manufacturer's instructions with slight modification [22]. Briefly, 300 μl haemocyte suspension was centrifuged and resuspended in 100 μl 1 × Annexin V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Then the haemocyte suspension was stained with 5 μl of Annexin V-FITC and 10 μl of 50 μg ml^{-1} PI for 15 min in the dark. Then 400 μl 1 × Annexin V binding buffer was added to each tube, and the fluorescence of haemocytes in the FL1 (Annexin V) and FL2 (PI) channels were analyzed immediately by flow cytometry. Results were expressed as Annexin V-FITC/PI dot plot. Cells stained negative with both probes represent live cells (quadrant a). Cells stained positive with Annexin V-FITC and negative with PI are early apoptotic cells (quadrant b). Cells stained positive with both Annexin V-FITC and PI are in the end stage of