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Short communication

Various cellular responses of different shrimp haemocyte subpopulations to lipopolysaccharide stimulation

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ABSTRACT

Different haemocyte types have been reported to play diverse roles in immune defense of shrimp. To investigate the roles of the three haemocyte types [hyaline cells (HC), semigranular cells (SGC) and granular cells (GC)] of shrimp in immune responses against lipopolysaccharide (LPS), percentage, non-specific esterase activity (EA), reactive oxygen species (ROS) production and nitric oxide (NO) production of the three haemocyte subpopulations were analyzed in LPS-injected *Penaeus monodon* using flow cytometry. Results showed that percentage of HC increased after 3 h injection, and returned to the original level after 48 h. Proportion of SGC and GC reduced after 6–36 h and 3–12 h respectively, and recovered to the initial level after 48 and 24 h respectively. Loss of SGC and GC might be related to degranulation to release proPO system, and degranulation of GC seemed more sensitive to LPS stimulation. EA of both HC and SGC improved after 3–6 h injection, while EA of GC was induced after 3–24 h. No significant effect of LPS injection could be found in ROS production and NO production of HC. Enhanced ROS levels was observed in SGC and GC after 3–24 h and 3–36 h respectively, and NO production of SGC and GC improved after 3–48 h injection. These results demonstrated that SGC and GC possessed strong capabilities for LPS-induced EA, ROS production and NO production, while HC only displayed EA response to LPS, suggesting that GC and SGC play the main role in immune defense of shrimp against Gram-negative bacteria.

1. Introduction

Gram-negative bacteria, such as *Vibrio* species, is one of the main groups of pathogenic microorganism to shrimp. Lipopolysaccharide (LPS), which located in the outer membrane of gram-negative bacteria, is the highly antigenic and cytotoxic substance. Because of the LPS, Gram-negative bacteria would be recognized by pattern recognition proteins lipopolysaccharide and β-1,3-glucan binding protein (LGBP), and the immune responses would be triggered [1]. In decapod crustaceans, previous studies have found that this antigenic substance induced degranulation and prophenoloxidase release of haemocytes [2–5], improved the mRNA expression levels of immune-related genes [3,6]. Meanwhile, negative effects could also be observed after LPS infection. It caused haemocytes depletion, apoptosis and total haemocyte count (THC) decline [2–4,7,8]. Our previous researches using flow cytometry (FCM) demonstrated strong cellular responses of shrimp haemocytes to LPS, such as abundant productions of reactive oxygen species (ROS) and nitric oxide (NO), improved non-specific esterase activity (EA), and the haemocyte proportion alteration [4].

Three morphological haemocyte types were found in decapod crustaceans, including hyaline cells (HC), semigranular cells (SGC) and granular cells (GC). Different haemocyte types have been reported to play different roles in immune responses of crustaceans [9]. In penaeid shrimp, SGC and GC have highly refractive granules which contain components of the prophenoloxidase (proPO) system [9], and they are also reported to be active in phagocytosis [10]. However, roles of these three haemocyte types in cellular responses against LPS have not been demonstrated. FCM is an objective, reproducible, rapid and sensitive technique for individual cell detecting. Morphologic and immunological characterisations of different cell types could be easily analyzed at the same time. In this study, in order to further understand the immune roles of the three haemocyte types of shrimp against gram-negative bacteria, ROS productions, NO productions, non-specific esterase activities, and the proportions of the three haemocyte subpopulations from LPS-infectious *Penaeus monodon* were determined by FCM.

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2. Materials and methods

2.1. Animals

The experimental tiger shrimp *P. monodon* (9.28 ± 1.22 g) were obtained from a commercial shrimp farm in Wenchang, Hainan Province, China. They were maintained in the laboratory with diluted seawater at 25‰, pH 7.7–7.8 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. LPS injection

LPS (from *Escherichia coli* 055:B5, purified by phenol extraction, Sigma) was dissolved in physiological saline solution (0.85% NaCl) to give dose of 2 μg μl^{-1}. Shrimp were randomly divided into two groups (twenty-five shrimp/group in separate aquaria) of different LPS doses: 0 (shrimp injected with the same amount of sterile physiological saline solution were maintained as control) and 8 μg g^{-1} wet weight (this dose of LPS significantly stimulated the cellular responses of total haemocytes according to our previous study [4]). Haemolymph of three shrimp from each group were collected individually at 0, 3, 6, 12, 24, 36 and 48 h after injection. The experiment was repeated three times.

2.3. Preparation of haemocyte suspension

Experiment was performed on individual sample. Haemolymph (about 300 μl) was extracted from each shrimp by a 2.5 ml syringe containing an equal volume of ice-cold 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). Haemolymph was diluted with about four times of AS to obtain a final concentration of about 1×10^6 cells ml^{-1}, and then the haemocyte suspension was used for flow cytometric analysis.

2.4. Flow cytometry

Flow cytometry was performed with a flow cytometer (FC, 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 record was 530 nm (green fluorescence, FL1). For each haemocyte sample, 10,000 events were counted. Cell Quest® software (Becton-Dickinson, San Jose, CA, USA) was used to create distribution histograms and to analyze fluorescence data.

2.5. Percentage of different haemocyte subpopulations

A volume of 200 μl haemocyte suspension was directly analyzed by flow cytometer. Forward scatter (FSC) and side scatter (SSC) indicate the relative size and granularity of haemocytes respectively. Results were expressed as FSC-H/SSC-H dot plot. Haemocytes were divided into three subpopulations (HC, SGC and GC) according to their discrepant values in FSC and SSC as described previously [11]. Percentages of different haemocyte subpopulations were analyzed.

2.6. Non-specific esterase activity (EA) of different haemocyte subpopulations

EA in haemocyte 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 (26 °C) in the dark. Then the FDA fluorescence of haemocytes was recorded on FL1 detector of FC. EA was expressed as mean fluorescence intensity of FDA in arbitrary units (A.U.).

2.7. Reactive oxygen species (ROS) production of different haemocyte subpopulations

The cell-permeant probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma) was used to detect the intracellular ROS production in shrimp haemocytes. 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. ROS production was expressed as mean fluorescence intensity of DCF in arbitrary units (A.U.).

2.8. NO production of different haemocyte subpopulations

The fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA, Sigma) was used to measure intracellular NO production as described previously [10]. 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. NO production was expressed as mean fluorescence intensity of DAF-FM in arbitrary units (A.U.).

2.9. Statistical analyses

All data are presented as means ± standard deviation (SD) of three independent experiments. Normality test of the data was cheched by the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was used to analyze the data and a multiple comparison (Tukey) test was conducted to compare the significant differences using SPSS 18.0 program (SPSS Inc., Chicago, IL, USA). A P value < 0.05 was considered significant.

3. Results

3.1. Percentage of different haemocyte subpopulations

Effect of LPS injection on percentage of different haemocyte subpopulations is presented in Fig. 1. At the beginning, the mean percentages of HC, SGC and GC were about 9.05%, 61.76% and 24.93%, respectively. The shrimp injected with saline solution kept stable haemocyte proportion during the 48 h experiment ($P > 0.05$). Percentage of HC increased after 3 h LPS injection, and reached the highest level (about 47.62%) after 6 h ($P < 0.05$). Then the HC percentage gradually decreased after 12 h, and then returned to the original level after 48 h ($P > 0.05$). SGC proportion significantly reduced in LPS-injected shrimp after 6 h, and reached the lowest level (about 32.74%) after 12 h ($P < 0.05$). After that, the proportion of SGC gradually recovered, and then returned to the initial value after 48 h ($P > 0.05$). Significant reduction in GC proportion could be observed after 3–12 h LPS injection ($P < 0.05$), and then recovered to the initial level after 24 h ($P > 0.05$).

3.2. EA of different haemocyte subpopulations

Effect of LPS injection on EA of different haemocyte subpopulations is presented in Fig. 2. At the beginning, the EA of HC, SGC and GC were about 60.08 A.U., 65.97 A.U. and 134.67 A.U., respectively. The EA of both HC and SGC were induced by LPS injection after 3–6 h ($P < 0.05$), and then decreased to the initial level