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Granulocytes of the red claw crayfish *Cherax quadricarinatus* can endocytose beads, *E. coli* and WSSV, but in different ways

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ABSTRACT

The hemocytes of the red claw crayfish *Cherax quadricarinatus* are classified by morphologic observation into the following types: hyalinocytes (H), semi-granulocytes (SG) and granulocytes (G). Density gradient centrifugation with Percoll was developed to separate these three subpopulations of hemocytes. Beads, *Escherichia coli*, and FITC labeling WSSV were used to investigate the characteristics of granulocytes by using scanning electron microscope, transmission electron microscope, and laser scan confocal microscope. Results showed that granulocytes could phagocytose beads and *E. coli* by endocytic pathways. WSSV could rely on caveolae-mediated endocytosis to mainly enter into granulocytes. These results could elucidate the mechanism of the innate immunity function of granulocytes, and it also showed the mechanism by which WSSV invaded granulocytes in the red claw crayfish.

1. Introduction

In vertebrates, innate and adaptive immunities function to protect hosts from infections (Iwasaki and Medzhitov, 2010). However, invertebrates lack an adaptive immune system (Cerenius et al., 2008; Jiravanichpaisal et al., 2006). Innate immunity plays an essential role in responding to infectious pathogens. The innate defense system of invertebrates, including crustaceans, is grouped into humoral and cellular immunities (Li and Xiang, 2013). At present, reactions of humoral immunity are well understood in insects. Cellular immune reactions, however, are not extensively explored yet (Ribeiro and Brehélin, 2006). Cellular immune responses are mediated by the circulating hemocytes (blood cells) in invertebrate and play a key role in immobilizing or destroying invasive pathogens.

Engulfment and destruction of invading microorganisms by phagocytosis, which is one of cellular endocytic pathways, are critical components of cellular immune responses. Other cellular endocytic pathways that are mainly involved in the entry of foreign particles into target cells are as follows: (1) clathrin-mediated endocytosis, (2) caveolae-mediated endocytosis, and (3) macropinocytosis (Bhattacharyya et al., 2010). Phagocytosis is an actin-based endocytic mechanism, which is defined as the receptor-mediated engulfment of large ($\geqslant$0.5 μm) particles, such as bacteria, into plasma membrane-derived vacuoles called phagosomes (Botelho and Grinstein, 2011). Phagocytosis is mediated by cup-like membrane extensions that are used to internalize large particles (Mayor and Pagano, 2007).

In drosophila, three main classes of hemocytes are distinguished. They are plasmatocytes, crystal cells, and lamellocytes, which play different roles in phagocytosis, melanization, and encapsulation, respectively (Honti et al., 2014; Meister and Lagueux, 2003; Ribeiro and Brehélin, 2006). In crustacean, hemocytes greatly vary in response to environmental stress, struggle, endocrine activity during molting cycle, and infection (Lorenzon et al., 1999). The types of hemocytes in marine crustaceans are mainly based on granule number and size, and the ratio of nucleus to cytoplasm. The types can also be classified into hyalinocytes (H), semi-granulocytes, (SG), and granulocytes (G) (Giulianini et al., 2003, 2007; Li and Shields, 2007; Lin and Söderhäll, 2011; Roulston and Smith, 2011; Zhang et al., 2006). However, compared with drosophila, crustacean hemocytes of immunity functions are still poorly understood. Some studies did not mention the phagocytic cell type and the percentage of cells performing phagocytosis was greatly variable (Dantas-Lima et al., 2012).

Both bacteria and virus can be dangerous pathogens of shrimp by harassing the healthy shrimp aquaculture development in China (Li and Xiang, 2012). Over a decade, however, researchers cannot

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precisely illustrate which among the type cells have endocytic abilities and white spot syndrome virus (WSSV) that can enter into the host cell in crayfish or shrimp. Nowadays, various investigations opt to use the red claw crayfish (*Cherax quadricarinatus*) to study invading pathogens because of its advantages, such as having a bigger unit and being easy to breed. In preliminary experiments, WSSV is found to be capable of infecting *C. quadricarinatus*. In the present study, classification and characterization of hemocytes are fundamental requirements to further understand the innate immune mechanism of crayfish. Separated granulocytes from *C. quadricarinatus* are used as experimental model to observe the process of cellular endocytic pathways for different foreign particles. Results explain how granulocytes perform innate immunity function for foreign particles and how WSSV adopts with endocytosis pathway to enter the granulocytes in *C. quadricarinatus*.

2. Materials and methods

2.1. Experimental animals

C. quadricarinatus were purchased from Xiamen in Fujian, China and were maintained in a flow-through fresh water aquarium at IOCAS laboratory at 24 °C to 26 °C. The average length and weight of the crayfish were 15 cm ± 2 cm and 120 g ± 15 g, respectively. Only the healthy ones were chosen for experiments. They were acclimated for at least 2 weeks and fed one time every day with chopped mussels.

2.2. Classification of the hemocytes

Hemolymph was drawn from the abdomen with a 2.5 ml syringe. Prior to bleeding, the sample area was wiped with 70% ethanol. In most cases, hemolymph was collected directly into an ice-cold 0.22 μm filtered citrate-EDTA anticoagulant (Söderhäll and Smith, 1983) to produce a 1:1 dilution and then centrifuged at 800–1000*g* for 5 min at 4 °C. Supernatant was discarded and the pellet was washed with crayfish phosphate buffer saline (CPBS: 10 mM Na_2HPO_4; 10 mM KH_2PO_4; 150 mM NaCl; 10 μM $CaCl_2$, and 10 μM $MnCl_2$; pH 6.8) to remove the remaining serum by spinning down at 1000 g for 5 min at 4 °C. For light microscopy, 200 μl suspension was placed on a clean cell culture dish, dyed with Wright's stain (Nanjing, Jiancheng) according to the kit instructions, and examined with a NIKON TS100 microscope. Accordingly, hemocytes were categorized based on morphological observation, including cell size, cell shape, and granularity (Li et al., 2008).

2.3. Transmission electron microscopy

For transmission electron microscopy (TEM), the hemocytes were first fixed in a 2.5% glutaraldehyde that was prepared in phosphate buffer (pH 7.4). Then, they were stored overnight at 4 °C and transferred to 1% osmium tetroxide in the same buffer for 2 h. Hemocytes were subsequently washed in double distilled water and were dehydrated in ethanol, propylene oxide, and serial dehydration with acetone. They were then embedded in Epon 812 resin (TAAB, UK). Ultrathin sections (approximately 60 nm thick) were cut by using a Reichert–Jung ultramicrotome and were double-stained with uranyl acetate and lead citrate. The sections were observed and photographed by a Hitachi H-7000 transmission electron microscope.

2.4. Enrichment of different cell types with Percoll

The hemocytes were re-suspended and adjusted to a density of 2–5 × 10^5 cells/ml by CPBS. Percoll solution was adjusted by adding 0.85% of NaCl (Sperstad et al., 2010). Granulocytes were enriched from hemocytes by density gradient centrifugation on 65% (v/v) Percoll (Pharmacia, 1.130 g ml^{-1}) at 320*g* for 30 min at 4 °C (Fig. 1). However, H migrated to a layer close to and immediately above the SG band. Proximity of the two bands was complicated for harvesting the cells without cross contamination. To enrich two other types of hemocytes, another separation with Percoll was performed, in which H with SG band were aspirated from 65% Percoll and gently layered onto a second gradient of 35% Percoll. This second gradient was then spun at 600 g for 15 min at 4 °C. Separated cells were harvested from gradients with Pasteur pipettes (Eppendorf). To prevent cross contamination, the interlayer were discarded after centrifugation. The entire process was described as shown in Fig. 1.

For primary cell culture and other assessments, the enriched cells mentioned above were re-suspended in modified L-15 medium (Liu et al., 2011) and cultured in 12-well plates (Corning).

2.5. Assessment of endocytosis

Assay for phagocytosis was performed in a cover glass (NEST Biotechnology). Granulocytes were incubated at 26 °C with fluorescent beads (Fluoresbrite Yellow Green Microspheres, Polysciences) at a cell/beads ratio of 1:10. *Escherichia coli* was cultured overnight in LB broth at 37 °C and centrifuged at 4000*g* for 5 min to harvest the bacteria. Then, *E. coli* was washed three times with phosphate-buffered saline (PBS, pH 7.4). Granulocytes were incubated for 2 h at 26 °C with *E. coli* at a cell/bacteria ratio of 1:10. Non-ingested beads and *E. coli* were removed by washing granulocytes three times with CPBS. Samples were fixed overnight at 4 °C with 2.5% glutaraldehyde in 0.1 M PBS buffer (pH 7.4). After fixation, the samples were dehydrated in a graded series of ethanol and replaced by isoamyl acetate. Samples were then critical point dried, mounted, sputtered, and coated with gold. They were observed with Hitachi S-3400 scanning electron microscope.

2.6. Viral infection and entry

To prepare purified WSSV, a gill homogenate from the WSSV-infected ridge tail white shrimp, *Exopalaemon carinicauda*, was purified by sucrose-gradient ultracentrifugation (van Hulten et al., 2001). Intact WSSV virions were purified as previously described (Xie et al., 2005). Purified WSSVs were injected into healthy crayfish, with 1 × 10^5 virions each. Crayfish was fed only once a day. Hemolymph was drawn with a 2.5 ml syringe from the crayfish abdomen during the 7 days post-infection. These cells were centrifuged at 5500*g* for 5 min at 4 °C and then fixed in 2.5% glutaraldehyde in PBS (pH 7.4) at 4 °C for 24 h. The pellet was post-fixed in 1% osmium tetroxide for 1 h and was washed again with the same PBS. Other preparations for observation with TEM were done as mentioned as above. To study how WSSV enters into hemocyte, harvested granulocytes by density gradient centrifugation in 65% (v/v) Percoll were incubated at 26 °C with purified WSSV at a cell/virion ratio of 1:50 for more than 2 h. Samples were washed and carefully fixed for SEM. Other preparations for observation with SEM were done as mentioned above.

2.7. FITC-labeled WSSV and inhibition assay

WSSV infection status can be detected by labeling virions with fluorescein isothiocyanate (FITC, Sigma). This analysis is in reference to other papers on some virus, such as Sendai virus (Hoekstra and Klappe, 1986), human influenza virus (Korte et al., 1999), *Autographa californica* multicapsid nucleo-polyhedro virus (Dong et al., 2010; Wang et al., 1997), and WSSV (Li et al., 2007; Liu et al., 2009). Proliferation and preparation of intact WSSV