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Classification and phagocytosis of circulating haemocytes in Chinese mitten crab (*Eriocheir sinensis*) and the effect of extrinsic stimulation on circulating haemocytes *in vivo*

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ABSTRACT

Eriocheir sinensis (Henri Milne Edwards 1854) is one of the most important aquaculture species in China. In this investigation, we characterised the different types of haemocytes of *E. sinensis* using light and electron microscopy combined with cytochemical analysis and determined the *in vivo* phagocytic ability of different haemocyte types by injecting polystyrene beads. The haemocytes of *E. sinensis* were divided into three types: hyalinocytes, semigranulocytes and granulocytes. The hyalinocytes had no or few cytoplasmic granules; the semigranulocytes contained abundant small granules and a few large refractile cytoplasmic granules; and the granulocytes contained numerous large refractile cytoplasmic granules. The hyalinocytes were demonstrated to be the most abundant circulating haemocytes and the most avid phagocytic haemocytes, accounting for approximately 88.7% of the total phagocytes. The haemocyte-containing granules displayed limited phagocytic ability, with approximately 5.0% of granulocytes and 6.3% of semigranulocytes displaying positive phagocytic ability against the invading polystyrene beads *in vivo*. After injection with *Aeromonas hydrophila*, *Bacillus subtilis* and different concentrations of lipopolysaccharide for 0.25, 0.5, 1, 2, 4, 6 and 8 h, all three types of haemocytes experienced dramatic decline and then rapid recovery to their initial levels. A high concentration of lipopolysaccharide and *A. hydrophila* were extremely toxic to the crabs, as they induced a more serious loss of haemocytes compared with a low concentration of lipopolysaccharide and *B. subtilis*. Overall, the results obtained in this study indicate that a small proportion of the haemocytes of *E. sinensis* contributed to the phagocytic process, and the migration of haemocytes and haemocyte lysis were most likely a prominent pathway for pathogen elimination.

1. Introduction

The Chinese mitten crab (*Eriocheir sinensis*) is one of the most important aquaculture species in China. With the development of intensive aquaculture and environmental deterioration, it frequently suffers from various diseases caused by bacteria, fungi, viruses and rickettsia-like organisms, which results in catastrophic economic loss [1].

As an invertebrate, *E. sinensis* lacks adaptive immunity and greatly relies on a relatively efficient innate immunity to defend invading pathogens. The haemocytes play a key role in its innate immunity by being directly involved in the recognition of invading pathogens and in their elimination by phagocytosis, encapsulation, melanisation, nodule formation and cell agglutination [2]. In most crustaceans, three types of circulating haemocytes have been generally recognised: hyalinocytes (hyaline haemocytes), semigranulocytes (small granule haemocytes) and granulocytes (large granule haemocytes) [3–8]. Each type of haemocyte is suggested to perform various immunity functions [9]. Phagocytosis, the process by which haemocytes engulf large particles in inner environment, is vitally important for the elimination of invading pathogens and apoptotic cells [10]. Controversial results concerning the roles of each haemocyte type in the immune system have suggested that the type of haemocytes involved in phagocytosis are distinct among

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different crustacean species [11–13]. In the freshwater crayfish *Astacus leptodactylus*, all three types of haemocyte are involved in the phagocytic response in *in vivo* phagocytosis assays (Giulianini et al., 2007). In the tiger shrimp *Penaeus monodon*, the hyalinocytes are the only haemocytes involved in latex bead phagocytosis *in vitro* [14]. In contrast, Gargioni and Barracco suggested that semigranulocytes and granulocytes, instead of hyalinocytes, were the main types of haemocytes phagocytising yeast particles *in vitro* in *Macrobrachium acanthurus* and *P. monodon* [15]. The phagocytic capability of haemocytes varies among diverse crustaceans, even though the same organisms display different phagocytic capability when encountering different pathogens [12]. In addition, in *in vivo* phagocytosis assays, invertebrates with large quantities of hemolymph tend to present a low proportion of phagocytes [16].

Previous studies indicate that the total haemocyte counts (THC) and differential haemocyte counts (DHC), which are commonly used as the functional parameters to evaluate the cellular immunity, are easily affected by intrinsic alteration and extrinsic stimulation [17]. Experimental injection of a lipopolysaccharide (LPS), fungal cell wall preparation or of a β-1,3-glucan causes a rapid decrease in the number of free haemocytes, followed by a slow recovery [9]. The time to reach the maximum depletion of haemocytes (haemocytopenia) and the rate of decrease is species specific [18].

Understanding the roles of haemocytes of *E. sinensis* is necessary for a better assessment of the relative contributions of environmental factors, pathogenic infections and culture-related stresses in mortality outbreaks. However, little is known about the roles of haemocytes of *E. sinensis* in immune responses. In this study, we attempted to characterise the type of haemocytes in *E. sinensis* and evaluate the proliferation reaction and phagocytosis response of haemocytes to the extrinsic stimulation.

2. Materials and methods

2.1. Experimental crabs

In total sixty-five Chinese mitten crabs (106.52 ± 14.03 g), obtained from a commercial farm in September in Hangzhou, Zhejiang province, China, were used in the whole experiment. Crabs were group in five and acclimated in 40-L plastic tank with thoroughly aerated freshwater at 16–18 °C and a natural photoperiod condition for a week. The crabs were fed once a day before the experiment and their water was refreshed every morning. Each tank with five crabs were fed with 50 g of a commercial crab diet.

2.2. Light microscopy

2.2.1. Haemocyte collection

An approximately 500 μL hemolymph sample was collected from the cheliped root of each crab into a 1.0 mL plastic syringe containing an equal volume of anticoagulant solution of citrate buffer/EDTA [12]. The haemocyte pellet was fixed with an equal volume of fixative (4% paraformaldehyde) after 2000 rpm centrifugation for 3 min.

2.2.2. Phase contrast microscope and cytochemical staining

For the phase contrast microscopy, one drop of haemolymph sample was added onto a glass slide and settled for 10 min at room temperature. Haemocytes were observed and photographed using a phase contrast microscope (Zeiss LSM 780). For the cytochemical analysis, 10 μL of haemolymph sample was added onto a glass slide and fixed for 5 min at room temperature for thorough dryness. The smears were rinsed in distilled water and dried again after staining with Wright's-Giemsa solution for 10 min. The slides were finally observed and photographed using an optical microscope.

2.2.3. Haemocyte count and diameter measurement

The THC and DHC, obtained, respectively, from 10 female and 10 male *E. sinensis*, were performed using a haemocytometer, with each crab count repeated three times. The nucleus/cytoplasm (N/C) ratio of each type of haemocyte was measured from 50 cells.

2.3. Transmission electron microscopy (TEM)

Haemocyte pellets were collected using the same procedure as described in Section 2.2.1. The haemocyte pellets were fixed with an equal volume of 2.5% glutaraldehyde in 0.1 M PBS buffer (pH 7.4) for 12 h. The pellets were washed in 0.1 M PBS buffer (pH 7.4) for 15 min and then transferred to a 1% osmium tetroxide solution with the same pH buffer for 1 h, followed by serial dehydration with ethanol and acetone and embedding of specimens in Epon 812. Ultrathin sections with a thickness of 50–80 nm were sliced using a Leica A-1170 ultramicrotome and double-stained with uranyl acetate and lead citrate. These sections were observed and photographed with a Philips Tecnai 10 transmission electron microscope.

2.4. Lipopolysaccharide and bacteria injection effects on haemocyte counts

Totally thirty-five crabs were used throughout the experiment and divided into seven groups. Five crabs were selected for each group and injected, respectively, with a 26-gauge needle containing 100 μL of (1) different concentrations of LPS (100 μg mL^{-1}, 250 μg mL^{-1} and 500 μg mL^{-1}), (2) *Aeromonas hydrophila* (1×10^8 mL^{-1}) suspended in saline (NaCl 0.21 M, KCl 13.6 mM, H_3BO_3 8.6 mM, NaOH 4.75 mM, $MgSO_4 \cdot 7H_2O$ 20 mM, pH 7.2) and (3) *Bacillus subtilis* (2.5×10^9 mL^{-1}) suspended in saline. Two groups were designed as the controls: one received an injection of 100 μL of saline, and another was bled only at each sampling time. After 0.25, 0.5, 1, 2, 4, 6 and 8 h, 200 μL of haemolymph from each crab was collected, and both the THC and DHC of each crab were measured in triplicate.

2.5. In vivo phagocytosis assay

Red-fluorescent carboxylate-modified polystyrene beads, 1 μm in diameter (Fluospheres, cat. No. 8821, Invitrogen), were chosen as the target for an *in vivo* phagocytosis assay. Three crabs received an injection of 100 μL of bead solution (approximately 2.7×10^9 beads) via pericardial sinus, and another three crabs were set as controls and each received an injection of 100 μL of saline. After 1, 2, 4, 6 and 8 h, 200 μL of haemolymph sample from each crab was collected and fixed in 100 μL of 4% paraformaldehyde after being centrifuged at 2000 rpm for 2 min for the phagocytic ratio analysis. The phagocytic ratio was expressed as the percentage of phagocytised haemocytes in total haemocytes, which was estimated by a haemocytometer with each crab counted in triplicate. In addition, the phagocytic indices and the proportion of different type of haemocytes involved in phagocytosis were counted in triplicate, with at least 100 phagocytes measured for each crab.

2.6. Statistical analysis

The results in the figures are presented as the mean ± SD. SPSS software (11.0) was used for statistical analyses. The effects of treatments were statistically analysed using analysis of variance (one-way ANOVA, LSD and Duncan analysis). Differences were considered significant at $P < 0.05$.