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Hemocytes and hemocytic responses in the mole crab *Emerita emeritus* (Linnaeus 1767)

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ABSTRACT

The mole crab, *Emerita emeritus*, collected from the sandy shores of a Chennai beach, was investigated for cellular immune responses based on the morphology and defensive reactions of the circulating haemocytes. Three haemocyte morphotypes were identified using light and electron microscopy, and separated in a discontinuous percoll gradient. A phagocytosis study using human B erythrocyte as a target cell under phase-contrast optics showed that granular and semi-granular haemocytes were phagocytic, and this response was enhanced by using serum (opsonin)-coated human B erythrocyte in unfractionated and fractionated haemocytes. Observation of TEM image of phagocytosis revealed that the initial recognition and binding of the target cell was restricted to granular and semigranular haemocytes, which were lacking with hyaline cells. However, the encapsulation of DEAE Sepharose CL 6B beads, either untreated or coated with serum (opsonin), was restricted to hyaline cells. This suggests the occurrence of two cell lines in haemocytes, based on the differences observed in the response of haemocytes to bind target cells for phagocytosis or encapsulation. The present study also differentiated the activation of PO in the plasma, serum, and haemocyte lysate supernatant (HLS).

1. Introduction

The innate immune system in crustaceans comprises humoral and cellular immune components with diverse defensive mechanisms of recognition and elimination across the species (Iwanaga and Lee, 2005). The circulating haemocytes play a crucial role in the defensive responses against invasive microbes, via phagocytosis, encapsulation, and nodulation (Sung et al., 2000a,b; Jin et al., 2013), as well as phenoloxidase activity with melanisation (Jiravanichpaisal et al., 2006; Mangkalanan and Sanguanrat, 2014), and prevent viral replication (Wu et al., 2015).

The crustacean haemocytes are generally classified into three types—hyaline, semigranular, and granular—based on the nucleus-to-cytoplasmic ratio and the number or size of the cytoplasmic granules (Bauchau, 1981). However, the classification of haemocytes and their types vary among the crustacean species, based on the cellular characteristics chosen for identification (Cheng and Chen, 2001; Battison et al., 2003; Giulianini et al., 2003, 2007; Zhang et al., 2006). Flow-cytometric analysis based on cell size and granularity (Sequeira et al., 1995; Cárdenas et al., 2004), and molecular identification techniques using monoclonal antibodies also help distinguish the haemocyte types (Rodriguez et al., 1995; Sung and Ryan, 2002).

Consistent with the morphological variations, the functions of the various haemocyte types also show species-specific differences. The hyaline cells have been reported as phagocytic in the crab *Carcinus aestuarii* (Matozzo and Marin, 2010), and the Chinese mitten crab, *Eriocheir sinensis* (Lv et al., 2014). However, in freshwater prawns, *Macrobrachium rosenbergii*, *Macrobrachium acanthurus*, and tiger shrimp *Penaeus monodon*, phagocytosis has been observed in semigranular and granular haemocytes, but not in the hyaline cells (Gargioni and Barracco, 1998). The encapsulation immune response involves cell-to-cell interaction by the haemocytes to eliminate the invasive agents. The large and small granular haemocytes have been reported to show encapsulation the in ridgeback prawn, *Sicyonia ingentis* (Hose and Martin, 1989), the crayfish, *Astacus* (Smith and Söderhäll, 1983a,b), and *Astacus leptodactylus* (Persson et al., 1987).

Moreover, humoral factors regulate and mediate the cellular immune reactions (Vázquez et al., 1997). The serum agglutinins as well as opsonin serve as recognition molecules that enhance the phagocytosis of invading microbes by haemocytes (Raman et al., 2008; Denis et al., 2015). The initial process of phagocytosis includes recognition and binding of pathogens, which can vary among the different haemocyte types, probably owing to their inherent structure and function (Martin et al., 1993; Chaga et al., 1995; Van de Braak et al., 2002a,b).

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The mole crab, *Emerita emeritus*, called the "master of the shore break", survives in the constantly shifting intertidal zone as a burrower and filter feeder including microbial challenge. Its innate immune response resists microbial invasions, and the present study focused on the structure and functions of its haemocyte morphotypes. The cellular immune responses of phagocytosis and encapsulation in unfractionated and fractionated haemocytes implicated a differential immune response among the haemocyte morphotypes. This was significant, as it reflected modulations in the crustacean cellular immune response. In addition, the serum as opsonin in facilitating the cellular immune functions provided an insight into the interaction of serum factors with haemocytes.

2. Material and methods

2.1. Experimental animal

The mole crab, *E. emeritus*, was collected from Besant Nagar, Chennai, Tamil Nadu, India (12 °59′56″N, 80 °16′19″E). In the laboratory, the animals were maintained in fibreglass tanks with a sandy bottom and seawater at a salinity of 35 ± 1‰, with continuous aeration and room temperature of 29 ± 2 °C (representative of tropical regions).

2.2. Haemolymph collection

The anomuran crab shows modulation in sex ratio and size at sexual maturity, based on aspects of moulting and reproduction, including neoteny and protandric hermaphroditism (Gunamalai and Subramoniam, 2002). In order to minimize the variability induced by reproduction or the moulting cycle, only female crabs, measuring 22–24 mm in carapace length at the intermoult stage, were taken for the study. Haemolymph was collected from the haemocoel of the crabs by inserting a 23 g needle through the arthrodial membrane, and was pooled to a final volume of 1 mL from 5 to 10 animals. The collected haemolymph was allowed to clot at room temperature (RT) and centrifuged (400*g*, 5 min at 4 °C) to obtain the serum as the supernatant. The osmolality of the serum was measured using a cryoscopy osmometer (Osmomat 030, Gonotec, Germany).

The haemolymph (50 µL) for the haemocyte study was collected in a clean, sterile syringe containing 950 µL of ice-cold tris buffer saline (TBS I: 50 mM tris, 50 mM dextrose, 5 mM potassium chloride, 3 mM magnesium chloride, 400 mM sodium chloride, pH 7.5, 950 *mOsm*) with 2 mM l-cysteine as an anticoagulant, mixed, and immediately transferred to a pre-chilled polypropylene tube.

2.3. Identification of haemocyte morphotypes

Haemolymph (1 mL) was collected in tris buffer with an anticoagulant (2 mM l-cysteine) and spread on a clean, dry glass slide in a moist chamber at 23 °C to obtain a haemocyte monolayer, and the viability of the haemocytes was determined by trypan blue dye exclusion staining (Garvey et al., 1979).

Haemolymph (1 mL), placed on a haemocytometer (Neubauer), was observed under phase-contrast optics at 40× magnification (Carl Zeiss Axio lab microscope) to determine the total haemocyte count (number of cells counted × dilution × 10^5/number of quadrates counted). Differential counts of haemocyte morphotypes were also assessed by counting the number of the individual morphotypes, and calculated in the same way as the total haemocyte count. The mean of 10 determinations of triplicate repeats was taken as the total or differential count of haemocytes.

For Transmission electron microscopy (TEM), the haemocytes in the haemolymph were fixed in 3% glutaraldehyde (1 h at 4 °C), pelleted (10,000*g*, 5 min at RT), and washed in 0.1 M sodium cacodylate buffer. The cells were post-fixed in 1% osmium tetroxide and washed in 0.1 M sodium cacodylate buffer. Next, they were further dehydrated in an ascending series of graded alcohol concentrations (50–100%), cleared with propylene oxide, infiltrated by propylene oxide and epoxy resin to be embedded in siliconized rubber mould with epoxy resin, and kept in an incubator at 60 °C for 48 h, then allowed to cool and divided into blocks. Thin sections (1-µ thickness), obtained using an ultra-microtome (Leica ultracut UC7) with a glass knife, were stained in toluidine blue and examined under a light microscope. Ultrathin sections (< nm thickness), obtained using an ultra-microtome (Leica) with a diamond knife (Diatome), were taken on a copper grid and stained (Double metallic) with uranyl acetate and Reynold's solution (sodium citrate + lead nitrate). They were then examined under an electron microscope (Phillips Technai T12 spirit by Netherland) and photographed.

For flowcytometry analysis, the haemolymph collected in tris buffer with an anticoagulant, as described earlier, was processed in a flow cytometer (BDFAC5 JAZZ) with a 480-nm laser beam with Side and Forward scatter parameters.

2.4. Phagocytosis assay

The human B erythrocyte (HB) of length 9 ± 0.24 µm was selected, based on its ability to be phagocytosed by the haemocytes of *E. emeritus*, and was fixed in 2.5% glutaraldehyde following Nowak et al. (1976). The serum, diluted to a sub-agglutinating concentration with TBS II (50 Mm tris, 100 mM sodium chloride, pH 7.5, 290 *mOsm*), showing a titre value of 4 with HB, served as opsonin for phagocytosis. The coating of opsonin on HB involved incubation of HB with sub-agglutinating concentration of serum for 30 min at 26 °C and washing (400*g*, 5 min at 4 °C) for suspension in TGS II buffer at 1.5% v/v.

Haemocyte monolayers were overlaid with HB suspension and incubated in a moist chamber for 30 min at 23 °C. The monolayers were then gently washed with isoosmotic buffer to eliminate the non-ingested erythrocytes and a glass cover slip was placed on it; the phagocytic haemocytes were then examined under a phase-contrast microscope (40×). The haemocytes with engulfed erythrocytes were counted as phagocytic, and the percentage of phagocytosis was calculated based on 200 haemocytes taken for evaluation in random microscopic fields in each monolayer. The mean value of 10 determinations each of triplicate repeats was reported as the percentage of phagocytosis.

The protocol for opsonin-facilitated phagocytosis *in vitro* assay was the same as that of phagocytosis, except that the serum-coated HB were used as target cells and a control was set up at the same time with untreated target cells.

For the study of phagocytosis in TEM, the opsonized target HB erythrocyte was incubated with the collected haemolymph in the anticoagulant-containing buffer and processed for TEM as described earlier.

In flowcytometry using FITC annexin V, stained HB erythrocytes were incubated with the diluted haemolymph (1:9 ratio) in TBS II in polypropylene tubes for 10 min. The fluorescence obtained with a 480-nm beam of an argon-ion laser was computationally evaluated and the percentage of phagocytosis calculated.

2.5. Encapsulation assay

Aliquots of the suspension of Sepharose beads, Sepharose CL-6B (neutral), CM-Sepharose CL-6B (negatively charged), and DEAE-Sepharose CL-6B (positively charged), in 25 µL of TBS were mixed gently with 25 µL of washed haemocyte suspension and observed for association with haemocytes. Among the tested bead types, only DEAE Sepharose CL 6B beads were found to attach at least 15 haemocytes, covering 50% of each bead surface under view, and were thus, selected for the encapsulation assay.

Haemocyte pelleted from 25-µL haemolymph (1500*g* at 4 °C) suspension in isoosmotic buffer was mixed with 25 µL DEAE Sepharose CL 6B bead suspension (100–150 beads) in the wells of V-bottom