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Classification of circulating hemocytes from the red swamp crayfish *Procambarus clarkii* and their susceptibility to the novel pathogen *Spiroplasma eriocheiris* in vitro

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ABSTRACT

Hemocytes from the red swap crayfish *Procambarus clarkii* were classified into three subpopulations: hyalinocytes (H), semigranulocytes (SG) and granulocytes (G) by morphologic observation and flow cytometry (FCM). The primary hemocyte culture was then established for studies on the in vitro propagation of *Spiroplasma eriocheiris* isolated from naturally infected *P. clarkii*. Grace's insect medium supplemented with 15% fetal bovine serum (FBS), along with 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, with a final pH of 7.2–7.4, incubated at 28 °C, supported the best survival in primary cultures. However, the granulocytes dehisced rapidly in culture medium, potentially impacting the survival of the other cell types. A two-step density gradient centrifugation with Percoll was developed to separate the hemocytes. When cultured separately, hyalinocytes and semigranulocytes maintained higher viability (>75%) after 16 days of incubation compared with granulocytes, which degraded over 2–6 days. After a challenge with *S. eriocheiris*, cytopathic effects (CPE) of the cultured hemocytes were observed as early as 48 h post-inoculation, and as the infection progressed, CPE became more apparent, with cell debris and cellular exudates in inoculated cultures. Cell lysis was noticeable within 60 h after challenging. A quantitative real-time RT-PCR was conducted to detect the immune responses during the challenging process at 2 h, 4 h, 8 h, 10 h, 24 h, 48 h and 60 h, respectively. Clear time-dependent expression patterns of the peroxinectin gene (referred as *Pcpxin*), recently isolated from the crayfish hemocytes, and heat shock protein 70 (HSP70) gene were observed after *S. eriocheiris* challenge. The results above should be helpful in promoting research with *S. eriocheiris*, including elucidation of pathogenesis, host pathogen interaction and the defense mechanisms, and ultimately lead to prevention of this crustacean disease.

1. Introduction

Red swamp crayfish is a freshwater crustacean of the genus *Procambarus clarkii*, resembling a lobster but considerably smaller. This species, originally from America, was introduced into China and multiplied and proliferated abundantly in the southeast of China, especially in Jiangsu Province. Recently, it is blighted by spiroplasma disease outbreaks, which have been found in many commercially exploited crustaceans including Chinese mitten crab *Eriocheir sinensis*, shrimp *Penaeus vannamei*, prawn *Macrobrachium rosenbergii* and crayfish *P. clarkii*, causing serious diseases and catastrophic economic losses in aquaculture (Liang et al., 2011; Nunan et al., 2005; Wang et al., 2005, 2011). The agent is highly pathogenic to the crayfish *P. clarkii*, leading to weakness, rapid death when removed from traps (Ding et al., 2007; Wang et al., 2005), and it has been formally named as *Spiroplasma eriocheiris* sp. nov. (Wang et al., 2011). It was assumed that crayfish which received *S. eriocheiris* had decreased resistance due to compromised immunity, leading to death. Therefore, understanding the defense mechanism of *P. clarkii* and their immune responses to *S. eriocheiris* has become a priority.

As an invertebrate animal, *P. clarkii*, lacks an adaptive immune system and must rely on relative efficient innate immune systems in which the hemocytes play a key role (Bachere et al., 2004; Jose et al., 2010; Matozzo and Marin, 2010). The hemocytes are directly involved in recognition, phagocytosis, melanization and cytotoxic reactions. Three types of circulating hemocytes are generally recognized in most species of crustaceans, i.e. hyalinocytes (agranular), semigranulocytes (small granule), and granulocytes (large granule) (Lin and Söderhäll, 2011). The morphological classes are essentially based on granule number and size, and on nucleus to cytoplasm ratio by using phase contrast or bright-field light microscopy and transmission electron microscopy. Although the hemocyte classifications have been reported for several crustaceans (Battison et al., 2003; Cheng and Chen, 2001; Giulianini et al., 2003, 2007; Li and Shields, 2007; Roulston and Smith, 2010; Xue

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et al., 2000; Zhang et al., 2006), there have been relatively few studies investigating the hemocytes in crayfish *P. clarkii* at present. In addition to the differing morphological features, each of the three main cell types was suggested to have a main function. Hyalinocytes were considered as phagocytes (Söderhäll et al., 1986), semigranulocytes, which display some phagocytic capacities, would be specialized in particle encapsulation (Persson et al., 1987), and granulocytes would participate in the pro-phenoloxidase (proPO) system that is an important component of the cellular defense reactions (Soderhall and Smith, 1983). Identification and characterization of the hemocytes are fundamental requirements for understanding the immune mechanism of *P. clarkii*.

Primary cultures of crustacean tissue were developed as an infection model for studies on the in vitro propagation of a variety of pathogens. The tissues selected for crustacean cell culture included the lymphoid (Chen and Wang, 1999; Lang et al., 2002), the ovary (Maeda et al., 2003; Shimizu et al., 2001), the hepatopancreas (Ghosh et al., 1995), the heart (Chen and Wang, 1999) and the embryos (Fan and Wang, 2002; Frerichs, 1996). However, no immortal crustacean cell line has yet been developed. In spite of the significance of hemocytes in crustacean immunology, in vitro culture has been attempted only by a few researchers, in *P. vannamei*, *Penaeus aztecus* (Ellender et al., 1992), *Penaeus japonicus* (Itami et al., 1999), *Penaeus chinensis* (Jiang et al., 2006) and the lobster, *Panulirus argus* (Li and Shields, 2007). In the present study, since *P. clarkii* hemocytes were preferentially infected by *S. eriocheiris* under natural conditions (Wang et al., 2005), they represented targets for our in vitro study of the novel pathogen. Hemocytes have been exploited in the previously published research on the host-pathogen responses in penaeid shrimp (Jiravanichpaisal et al., 2006), *Penaeus monodon* (Jose et al., 2010), *Pacifastacus leniusculus* (Watthanasurorot et al., 2010) and the Caribbean spiny lobster, *P. argus* (Li and Shields, 2007). In these studies, most of the crustacean hosts were infected with white spot syndrome virus (WSSV) or PaV1 Virus. Until now, no reports about *P. clarkii* primary hemocyte culture and its infection with spiroplasma have been published. These requirements, and the increasing need to more fully document hemocyte infections, prompted us to characterize the different classes of blood cells in *P. clarkii* and standardize primary hemocyte culture of *P. clarkii* as an in vitro model to assess the susceptibility of hemocytes to the novel crustacean pathogen *S. eriocheiris* and examine morphological and immune gene responses to *S. eriocheiris* challenge.

2. Materials and methods

2.1. Experimental animals

Red swamp crayfish, *P. clarkii*, were obtained from Freshwater Fisheries Research Institute of Jiangsu Province in China. The average length of the crayfish carapace (tip of the rostrum to the posteriomedial edge of the cephalothorax) was 42.5 ± 3.7 mm and the average weight of individual crayfish was about 20 g. After being removed from traps that were placed in the crayfish pond, the crayfish were brought into the laboratory where they were acclimated in a recirculating holding system at about 23–25 °C, pH 7.0–7.2, for 1 week. This holding system had its own filtration unit to maintain adequate water quality. The crayfish were examined initially by a simple and fast PCR detection method (Ding et al., 2007). Healthy crayfish were chosen for the experiments.

2.2. Light and transmission electron microscopy

Hemolymph was drawn with a 1 mL syringe from the juncture of the tail. Prior to bleeding, the sample area was wiped with 70% ethanol. In most cases, hemolymph was collected into a syringe containing an equal volume of anticoagulant ACD-B (4.8 g l^{-1} citric acid, 13.2 g l^{-1} sodium citrate, 14.7 g l^{-1}, glucose).

For light microscopy, 200 μl hemolymph was collected into an equal volume of 4% formaldehyde solution in an eppendorf tube. Then 100 μl of the mixture was sampled, deposited on a glass side, and covered with a cover glass. It was examined with an Olympus BH-2 microscope. Total hemocyte counts (THCs) and differential hemocyte counts (DHCs) were obtained with six crayfish, using a hemacytometer (Neubauer improved, Bright Line; two counts per crayfish).

In preparation for ultrathin sectioning, another 200 μl hemolymph was collected, and dropped on a clean glass slide under ice. After fast coagulation, the hemolymph was first fixed in 2.5% glutaraldehyde prepared in phosphate buffer, and then transferred to 1% osmium tetroxide in the same buffer, followed by serial dehydration with acetone and embedding of specimens in Epon 812. Ultrathin sections with a thickness of 50–80 nm were made using a Reichert-Jung ultramicrotome and double-stained with uranyl acetate and lead citrate. The sections were observed and photographed by a Hitachi 600-2A transmission electron microscopy (TEM).

2.3. Flow cytometry

Five hundred microliter volumes of fresh hemolymph, obtained and subsequently diluted with an anticoagulant solution (1:1), were run through FACSCalibur (Becton-Dickinson USA). The side- and forward-scatter parameters (SSC and FSC) of flow cytometry (FCM) were used for the determination of cell granularity and cell size, respectively.

2.4. Development of primary hemocyte culture

The culture medium used in our study was Grace's insect medium (GIBCO, USA). The medium was adjusted to match the osmolarity of the hemolymph of the crayfish (320–360 mOsM kg^{-1}) by addition of 0.5% NaCl. pH values were controlled between 7.2 and 7.4. 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin were added to minimize potential bacterial contamination. In addition, heat-treated 15% fetal bovine serum (FBS) was supplemented. All media were sterilized though 0.22 μm sterile filter (Millipore, Bedford, MA, USA).

Samples of collected hemolymph were centrifuged at $300 \times g$ for 4 min at 4 °C (Eppendorf 5415R), then resuspended in Grace's insect medium. Aliquots of 0.2 ml of the hemocyte suspension at densities of $\sim 1 \times 10^6$ were seeded into 24-well culture plates (Corning-Costar Corp., Corning, New York, USA) containing an additional 1.0 ml of culture medium per well. Plates were incubated at 28 °C in the presence of 5% CO_2 in CO_2 cell culture incubators (Model: 4150 C, Contherm Manufacturing, New Zealand). Media were refreshed at 2-day intervals. Cultured cells were observed with an inverted microscope (TEU-2000, NIKON Japan). Images were taken with a Nikon DXM 1200 digital camera prior to media refreshment. Mortality rates were calculated by the fluorescent dye propidium iodide (PI) method (Du et al., 2012; Laake et al., 1999).

2.5. Two-step density gradient centrifugation with Percoll

The granulocytes could be enriched from whole hemolymph by density gradient centrifugation on 60% (v/v) Percoll at $300 \times g$ as in Roulston and Smith (2010) and Sperstad et al. (2010). But, the hyalinocytes migrate to a layer close to and immediately above the semigranulocyte band (Fig. 1A). The proximity of the two bands complicates harvesting the cells without cross contamination. To improve purity, a second step separation on Percoll was employed. For this the hyalinocytes together with semigranulocyte band was aspirated from the 60% Percoll and gently layered onto a second gradient (Fig. 1B) of 30% Percoll. This second gradient was then spun at $500 \times g$ for 15 min at 4 °C. The separated cells were harvested from the gradients with fine bore sterile plastic Pasteur pipettes. The commercial Percoll solution (Pharmacia) was adjusted to 320–360 mOsM kg^{-1} by addition of 0.5% NaCl, and the gradients were prepared in Grace's insect medium. For primary cell culture, the enriched cells above were resuspended in Grace's insect medium respectively, and cultured as mentioned above.