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Development of two haemocyte culture systems (in attachment and in suspension) for shrimp immunity studies

J.J. Dantas-Lima [a,*], M. Corteel [a], D.T.H. Oanh [c], P. Bossier [b], P. Sorgeloos [b], H.J. Nauwynck [a]

[a] Faculty of Veterinary Medicine, Department of Virology, Parasitology and Immunology, Ghent University, Belgium
[b] Laboratory of Aquaculture & Artemia Reference Center, Faculty of Bioscience Engineering, Department of Animal Production, Ghent University, Belgium
[c] Department of Aquatic Biology and Pathology, College of Aquaculture and Fisheries, Cantho University, Viet Nam

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ABSTRACT

For studying shrimp immunity, *in vitro* haemocyte cultures are essential. Despite various reported attempts, well-described and reproducible culture techniques are lacking. The current work aimed to establish two *in vitro* haemocyte culture systems for *Penaeus* (*Litopenaeus*) *vannamei*. Haemocyte suspensions were either seeded in conventional Nunc® Nunclon™Δ Surface 24-well cell culture plates with glass cover slips (haemocytes in attachment) or in Nunc® Hydrocell Surface 24-well cell culture plates (haemocytes in suspension). The culture medium was based on L-15 (Leibovitz), and was further supplemented with L-glutathione and protease inhibitors in an attempt to improve haemocyte survival. Parameters such as number of living adherent and non-adherent single cells, number and average diameter of clusters and survival of cells inside clusters were evaluated. Additionally, live-cell imaging videos were recorded. It was found that haemocytes cultured for 1 h on glass coverslips in Nunc® Nunclon™Δ Surface plates could be separated in two cell fractions: adherent or non-adherent. Shrimp haemocytes cultured in Nunc® Hydrocell Surface plates remained in suspension and over time formed cell clusters which melanised. L-glutathione supplementation clearly improved haemocyte survival up to 48 h and delayed clustering and melanisation; addition of protease inhibitors did not. To validate the system, the phagocytic and antibacterial activities of adherent haemocytes towards *Vibrio campbellii* were evaluated. After 1 h of co-culture, 11.5 ± 0.14% of haemocytes showed phagocytosis with an average of 2.4 ± 0.1 bacteria internalised per haemocyte. Furthermore, haemocytes clearly demonstrated an antibacterial activity. It was concluded that these systems were reproducible and could keep haemocytes functionally active during the time required for the study of innate immune processes. Consequently, these techniques represent powerful tools for studying a variety of cell-mediated and humoral immune responses of shrimp *in vitro*.

1. Introduction

Research on crustacean immunity has received high priority during the past two decades, mainly triggered by the need of controlling disease outbreaks in shrimp farms. Invertebrates do not possess an acquired immunity. Instead they rely on innate, non-adaptive immune mechanisms. Despite this restriction, shrimp defence has the ability to effectively control most microbial challenges within hours after they occur (Jiravanichpaisal et al., 2006; Liu et al., 2009). The innate immune system consists of a humoral and a cellular branch, with the haemocytes occupying a central position in both. These immune effector cells are known to be involved in all major immune processes such as the prophenoloxidase (proPO) system, clotting, antimicrobial action, opsonisation, phagocytosis, cell agglutination and nodulation/encapsulation of foreign material (Jiravanichpaisal et al., 2006; Johansson et al., 2000). Of these processes, the proPO activation cascade and the clotting system are the most extensively studied in decapod crustaceans and this knowledge even serves as a research model (reviewed by Cerenius et al., 2008; Jiravanichpaisal et al., 2006). There is however still a long way to go in unveiling the mechanisms of other important immune reactions and the role of haemocytes in these reactions.

Decapod crustacean haemocytes are traditionally divided into three morphologically distinct subpopulations: hyalinocytes or hyaline cells, semigranulocytes or small granule cells and granulocytes or large granule cells (Giulianini et al., 2007; Hose et al., 1990; Martin and Graves, 1985; Söderhäll and Smith, 1983). In order to initiate any immune response, it is necessary for haemocytes to identify a target as non-self. In homology with the innate immunity of vertebrates, they use pattern-recognition receptors (PRRs) to identify pathogen-associated molecular patterns (PAMPs) specific for microorganisms like bacteria and fungi (Fearon, 1997). The invertebrate

* Corresponding author at: Faculty of Veterinary Medicine, Department of Virology, Parasitology and Immunology, Ghent University, Salisburylaan 133 9820 Merelbeke, Belgium. Tel.: +32 92 64 73 38; fax: +32 92 64 74 95.
E-mail addresses: joao.lima@ugent.be, joao.dantas.lima@gmail.com (J.J. Dantas-Lima).

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immune system can also very efficiently recognise abiotic targets such as plastic and glass as non-self (Nardi et al., 2006). This feature demonstrates its broad immune reactivity. The innate immune response in vertebrates is initiated with the phagocytosis of pathogens by macrophages. This process is characterised by the uptake of large particles by an actin-dependent mechanism (pathogens or other non-self structures and cell debris) by cells for their further degradation (Aderem and Underhill, 1999). In crustaceans, the cells and processes by which phagocytosis is carried out are still poorly understood and subject of debate in literature. In previous publications, besides the phagocytic cell type differed or was not mentioned, the percentage of cells performing phagocytosis was greatly variable (Deachamag et al., 2006; Hose et al., 1990; Itami et al., 1998; Li et al., 2008; Smith and Ratcliffe, 1978). Interestingly, invertebrates exhibit another peculiar cell-mediated immune reaction described as nodulation or encapsulation. This function appears to be a very important response against invading microorganisms (Auffret and Oubella, 1997; Johansson, 1999; Lavine and Strand, 2002; Nardi et al., 2005; Ratcliffe and Rowley, 1979; Söderhäll et al., 1984). The phenomenon consists of haemocytes identifying a target as being non-self, followed by adhesion of newly attracted haemocytes, forming overlapping layers of cells around the foreign entity. While nodulation immobilises large amounts of biotic targets with the size of bacteria or fungal spores, encapsulation is directed to bigger targets such as parasites (Ratcliffe and Gagen, 1976, 1977) or even abiotic targets such as plastic beads (Pech and Strand, 1996). Nodulation and encapsulation appear to be the same process but directed against different targets (Lavine and Strand, 2002). The process normally ends with the melanisation of the nodule/capsule and the killing of the invader by asphyxiation and action of toxic products (Nappi et al., 1995). Although all factors involved in the capsule formation in arthropods are not completely understood, it is known that after the identification of the foreign target, haemocytes have to pass from a non-adherent to a strongly adherent state, at which point they activate their immune functions (Nardi et al., 2006; Schmidt et al., 2001). Some of the most crucial immune effectors are the proPO system, antimicrobial peptides and reactive oxygen species (ROS). Especially the latter pathway, if not properly balanced by antagonists (enzyme inhibitors, antioxidants), can comprise a serious threat to the host (Johansson and Soderhall, 1989). The major antioxidant molecule present in animal cells is glutathione (GSH), which is a very effective actor in preventing the damage to important cellular components by ROS. Moreover, GSH is also involved in preventing the toxic effects of quinones, which are produced in the proPO cascade (Monks and Lau, 1992; Söderhäll et al., 1994; Uhlig and Wendel, 1992).

Throughout the advancement of crustacean immunity research, methodologies for culturing mature haemocytes and *in vitro* differentiated haematopoietic stem cell have been reported (Cardenas et al., 2004; Chisholm and Smith, 1992; Gargioni and Barracco, 1998; Gollas-Galván et al., 1997; Hose and Martin, 1989; Jiang et al., 2006, 2007; Johansson and Söderhäll, 1988; Jose et al., 2010; Lee et al., 2001; Muñoz et al., 2000; Rodriguez et al., 1995; Smith and Söderhäll, 1983; Söderhäll and Smith, 1983; Söderhäll et al., 2003, 2005; Sun et al., 2010; van de Braak et al., 2000; Vargas-Albores et al., 2005a; Vázquez et al., 1997; Vidya et al., 2007). Although some were properly designed, most of these studies did not take into account or mention important parameters such as the animal's moult stage and age and did not control cell survival and clustering activity over time. Due to poor methodological descriptions, most experimental protocols are difficult to reproduce.

The current work aimed to establish and compare two *in vitro* haemocyte culture systems, in attachment and in suspension, for a representative penaeid shrimp species, the marine *Penaeus* (*Litopenaeus*) *vannamei*. The ultimate goal was to provide reproducible and well-characterised haemocyte culture systems for use in shrimp immunological research and at the same time to provide a solid base for its adaptation to other shrimp species.

2. Materials and methods

2.1. Experimental animals

Specific pathogen-free (SPF) *P.* (*Litopenaeus*) *vannamei* were imported from Piti Syaqua Farm, Syaqua Siam Co. Ltd., Thailand. The shrimp were certified to be SPF for the major shrimp viruses by the Phuket Coastal Fisheries Research and Development Centre of the Thai Department of Fisheries. Batches of postlarvae were reared in a recirculation system at the Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium (water temperature at 27 ± 1 °C, pH at 7.8–8.1 and salinity at 35 ± 1 g l^{-1}). Adult shrimp of 12 months old with a mean body weight of 40 ± 5 g were used in this study. All were selected when they were in inter-moult (C) stage (Corteel et al., 2012).

2.2. Haemocyte medium

Osmolality and pH of *P. vannamei* haemolymph were determined (903 ± 13 mOsmol kg^{-1}, pH 7.5 ± 0.2). Haemocyte medium (HM) and anticoagulant were adapted to these parameters. L-15 medium (Leibovitz; Sigma-Aldrich) was prepared at double strength (2xL-15) and used as basal medium. HM was composed of 2xL-15, 10.5% (v/v) Chen's salts (CS) (Chen and Wang, 1999a), 10% (v/v) foetal calf serum (FCS) and penicillin/streptomycin (P/S: 100 units ml^{-1}/100 μg ml^{-1}) (pH 7.5; 900 mOsmol kg^{-1}). In survival improvement experiments, L-glutathione (GSH) (Sigma Aldrich) and EDTA-free protease inhibitor cocktail (Roche) were added individually to HM at a final concentration of 0.1% (w/v) and 1×, respectively. The media were filtered through a 0.20 μm filter before use.

2.3. Haemolymph collection and haemocyte isolation

Marine Anticoagulant (MA) (Söderhäll and Smith, 1983) with the pH adjusted to 5.4 was used as anticoagulant solution. Haemolymph was taken from the ventral sinus in the second abdominal segment using a 2 ml syringe with a 20G hypodermic needle filled with MA (1:2 with haemolymph). Haemolymph was poured into eppendorfs and centrifuged at $250 \times g$ during 5 min at 4 °C. Supernatant was discarded and the pellet was immediately resuspended in HM. Cell concentration was evaluated using the Bürker-Türk counting chamber. All the materials and solutions were pre-chilled and kept on ice during the whole procedure to prevent clotting and haemocyte activation.

2.4. Haemocyte culture systems and experimental setup

2.4.1. Haemocyte culture in attachment

Freshly collected haemocyte suspensions were seeded into Nunc® Nunclon™Δ Surface 24-well cell culture plates. Heat-sterilised (180 °C for 2 h) round glass cover slips were brought in each well. Cells were seeded at 3×10^5 cells $well^{-1}$ in a total volume of 400 μl. Plates were incubated for 1 h at 27 °C in order to allow cell attachment to the cover slips. Subsequently, non-adherent/weakly adherent haemocytes were resuspended by pipetting gently up and down six times throughout the well. This washing procedure was repeated twice and the wells were finally filled with 400 μl of medium. The cells that remained attached to the glass cover slip were designated as “adherent cell fraction”. The supernatant from each well was recovered in a new well also supplied with a glass cover slip. The cells in this supernatant were designated as “non-adherent cell fraction”. The number of living individual haemocytes in time was determined for both adherent and non-adherent cell fractions. Additionally, the number of living cells from the adherent cell fraction that detached from the glass cover slip in time was evaluated. Samples were collected at 1, 3, 24, 48, 72, 96 and 120 hours post seeding (hps). The cells were kept at 27 °C during the entire experimental period.