Separation of *Penaeus vannamei* haemocyte subpopulations by iodixanol density gradient centrifugation

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**A B S T R A C T**

Methodologies for separation of immune cell subpopulations are essential tools in immunology studies. Up to date, only one methodology for separating crustacean haemocyte subpopulations using Percoll density gradient centrifugation has been described.

In the present work, a new methodology to separate *Penaeus vannamei* haemocyte subpopulations was developed, using a two-step iodixanol density gradient centrifugation. *P. vannamei* haemolymph was collected with anticoagulant and centrifuged through a first gradient (densities from 1.063 to 1.109 g/ml) for 10 min at 2000 g. Three bands were formed: two bands with lower density close together, and a third band with higher density. The first two were collected together whilst the third band was collected separately. The volume fraction in-between these bands contained dispersed cells and was also collected. The suspension containing the mixture of the first two bands was centrifuged through a second gradient (densities from 1.047 to 1.087 g/ml) for 15 min at 2000 g. Two bands were formed and collected individually. All the cell suspensions were used for in vitro culture (cell survival evaluation) and for evaluation of cell morphology by flow cytometry and light microscopy. Each of the three bands contained a major cell type with distinct morphology and behaviour. The dispersed cell fraction contained a mixture of different cell types, which were distinct from the cell types in the bands. By order of appearance from the top of the gradient, the cell types were named: subpopulations (Sub) 1 (band 1), Sub 2 (band 2), Sub 3 + 4 (dispersed cells) and Sub 5 (band 3). The purity level (percentage of the major cell type) of Sub 1, 2 and 5 was 95.0 ± 1.0%, 97.7 ± 1.2% and 99.4 ± 0.8%, respectively. Cells of Sub 2 showed the best survival time in vitro (up to 96 h) followed by cells from Sub 1, Sub 3 + 4 and Sub 5. Phagocytic activity was detected in Sub 1 and 4.

This methodology allowed the separation and characterization of five morphologically distinct and physiologically active *P. vannamei* haemocyte subpopulations, from which three were isolated with a very high degree of purity. Therefore, we consider this methodology a valuable alternative for the traditional crustacean haemocyte separation procedure in Percoll.

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1. Introduction

Haemolymph (blood of invertebrates) is composed of a liquid fraction called plasma and a cellular fraction solely composed of haemocytes, the shrimp immune cells. Haemocytes are key players in invertebrate immunity since they mediate, directly or indirectly, all known invertebrate immune reactions. Crustacean haemocytes are traditionally divided into subcategories or subpopulations according to their morphological characteristics and/or functionality. Three morphologically distinct subpopulations have been described: (i) hyalinocytes or hyaline cells (ii) semi-granulocytes or semi-granular cells and (iii) granulocytes or granular cells (Li and Shields, 2007; Söderhäll and Smith, 1983; van de Braak et al., 1996; Vargas-Albores et al., 2005). When stained with histological dyes, hyalinocytes display a spindle/ovoid shape and few small basophilic and eosinophilic granules. Semi-granulocytes have an ovoid shape and contain several eosinophilic granules. Granulocytes have a spherical shape and have many large eosinophilic granules (Roulston and Smith, 2011; Smith, 2010). When exposed to foreign environments or substances, hyalinocytes and semi-granulocytes are known to present strong adherence to the substrate and acute spreading behaviour. Adherence appears to be more limited in the granular cells (Roulston and Smith, 2011; Vargas-Albores et al., 2005). However, there is still some inconsistency in the description of the morphology, functionality and proportion of each of these cell types. This may be due to differences between different species, differences in analytical methodologies and the somewhat subjective classification of semi-granulocytes.

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A protocol to separate crustacean haemocytes using a Percoll density gradient was developed by Söderhäll and Smith (1983), and was later adapted to other invertebrate species (Falwell et al., 2011; Hammond and Smith, 2002; Li and Shields, 2007; Liu et al., 2005; Pipe et al., 1997; Roulston and Smith, 2011; Smith and Söderhäll, 1983, 1991; Sperstad et al., 2010; Sritunyalucksana et al., 2001; Vargas-Albores et al., 2005). This methodology allowed the separation of haemocyte subpopulations with no apparent deleterious effects since the cell functionality was preserved in most of the cases. Nevertheless, only granulocytes were isolated efficiently probably due to the density that is intrinsic to each cell type.

With the aim of improving the separation of the haemocyte populations, we turned our attention to iodixanol as an alternative separation medium to Percoll. Although both media share suitable characteristics for an efficient separation of cells, cell organelles, and other subcellular structures, iodixanol possesses some advantages over Percoll. Percoll has a very low osmolality (Pertoft et al., 1978) and, as such, often requires sucrose for the preparation of stock solutions. Iodixanol has an osmolality of 290 mOsmol/kg (Solomon, 2005), making the preparation of isosmotic solutions easier. Percoll is light scattering at all wavelengths (Jenkins et al., 1979) and, thus, needs to be removed prior to most flow cytometrical and spectrophotometrical analyses. Iodixanol on the other hand, only exhibits absorbance in the UV range (Jacobsen, 2000). Another advantage of iodixanol is the formation of linear continuous gradients by allowing passive diffusion of pre-formed discontinuous gradients. This excludes the need of ultracentrifugation as is the case of Percoll self-forming gradients. The shape of iodixanol continuous gradients can be customized by manipulating the concentration and volume of the initial gradient fractions and diffusion times (Axis-Shield, 2012). On the other hand, self-forming Percoll gradients present non-linear, S-shaped gradients with two steep density profiles on the top and on the bottom of the gradient and a shallow zone in-between (Amersham Biosciences, 2001). These gradients have limited manipulation possibilities. Furthermore, it has also been reported that cells can become damaged during centrifugation in Percoll (Juan et al., 2012; Oliveira et al., 2011), whilst no such reports on iodixanol were found.

To the best of our knowledge, the present work described for the first time a procedure to efficiently separate highly pure Penaeus vannamei haemocyte subpopulations using iodixanol density gradient centrifugations. The high purity of the subpopulations was obtained by centrifuging the haemocytes through a sequence of two centrifugations. The high purity of the subpopulations was obtained by centrifuging the haemocytes through a sequence of two centrifugations. The high purity of the subpopulations was obtained by centrifuging the haemocytes through a sequence of two centrifugations. The high purity of the subpopulations was obtained by centrifuging the haemocytes through a sequence of two centrifugations. The high purity of the subpopulations was obtained by centrifuging the haemocytes through a sequence of two centrifugations.
2.4.3. Light microscopy of fixed haemocytes

Fixed haemocytes were cytospined (Shandon Cytospin 3, Thermo Scientific, USA) at 700 rpm for 5 min onto glass slides. After drying, cells were stained with haematoxylin and eosin (H&E) in an automatic staining machine (Sakura Linear Stainer II, The Netherlands). Slides were dipped in each staining bath for 105 s. The bath sequence was: 1× distilled water (DW), 2× haematoxylin, 2× DW, 3× eosin, 2× DW, a dehydration series of 50%, 70%, 80%, 94%, 100% ethanol and finally 2× in xylene. After drying, the slides were mounted with DPX mounting medium. Cells were observed under light microscope (Olympus BX61, USA) and pictures were taken. The size and morphological characteristics of the cells were evaluated.

2.5. Haemocyte in vitro culture and survival evaluation

Haemocyte survival evaluation was done as previously described by Dantas-Lima et al. (2012). Cell bands were collected from the gradient and immediately diluted with haemocyte medium (HM; 2 × L-15 medium, 10.5% Chen's salts, 10% FCS, 1% penicillin/streptomycin; pH 7.5; 900 mOsmol/kg). Cells were seeded in 24-well plates (Nunc®Nunclon™ Δ Surface, Thermo Scientific, USA) in which round glass coverslips were previously brought in each well. A volume of 400 μl of cell suspension was seeded in each well. Samples were taken at 0, 1, 3, 6, 12 and 24 h and every 24 h after until the end of each experiment. Cells were stained with ethidium monoazide bromide (EMA) dye for 30 min and with Hoechst dye for 10 min. At the end of the procedure, haemocytes were fixed and mounted on glass slides. Survival was evaluated by fluorescence microscopy and expressed as total number of living cells per well over time. Each experiment was repeated 3 times and the average values were calculated.

2.6. Validation of the functionality of separated haemocyte subpopulations

2.6.1. Production of inactivated GFP-labelled bacterial stocks

GFP-labelled *Vibrio campbellii* (LMG 21363) was obtained as previously described by (Dantas-Lima et al., 2012). Briefly, *V. campbellii* was transfected with a plasmid containing Green Fluorescent Protein (GFP), which was carried by *Escherichia coli* DH5α. After transfection,
colonies of GFP-labelled *V. campbellii* were isolated based on their antibiotic resistance and subsequently grown in marine broth, washed and stored at −80 °C in 20% glycerol.

2.6.2. Detection of phagocytic activity

*GFP*-labelled *V. campbellii* were sub-cultured twice in HM containing selective antibiotics (20 μl of bacterial suspension in 20 ml of HM for 12 h and 14 h at 27 °C). Suspensions were washed as described above. The concentration of bacteria in the suspension was determined by optical density at 600 nm (OD600) and by the conversion formula CRU/ml = (10 × OD600−1) × 10⁶.

Cultures of separated haemocyte subpopulations (150,000 cells well⁻¹) were inoculated with 100 bacteria haemocyte⁻¹ at 1 h after seeding. Samples were taken at 0 and 1 h after inoculation. Before sampling, wells were washed 2× with HM. At the moment of sampling, cells were fixed with 4% PF for 10 min, permeabilized with 0.1% Triton X-100 for 5 min and stained with Texas Red-labelled phalloidin (Invitrogen, Life Technologies) diluted in PBS (4 units ml⁻¹) for 1 h at 37 °C. Ten minutes before the end of this staining, Hoechst (0.01 mg ml⁻¹) was added. After, cells were washed and mounted on glass slides. The detection of phagocytic activity (haemocytes uptaking bacteria) was made using confocal microscopy. Sequential confocal pictures in three different wavelength emission channels (Hoechst: 461 nm; Texas Red: 615 nm; and GFP: 509 nm) were taken from the cell base to its apex. This was made in 10 cells that presented signs of phagocytosis.

3. Results

3.1. Separation of haemocytes in iodixanol density gradients

After 18 h of incubation at 4 °C, the iodixanol gradients became nearly linear. The density profile gradient 1, from the top to the bottom of the tube was: 1.063, 1.063, 1.064, 1.067, 1.070, 1.075, 1.080, 1.084, 1.089, 1.093, 1.095, 1.100, 1.105, 1.110, and 1.109 g/ml (Fig. 5A). After centrifugation, three cell bands were clearly formed with 0.1% Triton X-100 for 5 min and stained with Texas Red-labelled phalloidin (Invitrogen, Life Technologies) diluted in PBS (4 units ml⁻¹) for 1 h at 37 °C. Ten minutes before the end of this staining, Hoechst (0.01 mg ml⁻¹) was added. After, cells were washed and mounted on glass slides. The detection of phagocytic activity (haemocytes uptaking bacteria) was made using confocal microscopy. Sequential confocal pictures in three different wavelength emission channels (Hoechst: 461 nm; Texas Red: 615 nm; and GFP: 509 nm) were taken from the cell base to its apex. This was made in 10 cells that presented signs of phagocytosis.

3.2. Morphological characterization

3.2.1. Light microscopy and live-cell imaging of haemocyte cultures

Fig. 2 and live-cell imaging videos provided details of the morphology and behaviour of the haemocytes in culture. Cells from band 1 adhered very strongly to the glass by means of pseudopod-like projections, which resulted in a high degree of cell spreading. Cells from band 2 on the other hand, presented a very limited spreading and adherence to the glass. These cells were easily re-suspended by gentle pipetting. Cells from band 3 adhered strongly to the glass with moderate spreading. The fraction of dispersed cells contained 2 cell types: small and big cells with morphological characteristics similar to the cells from bands 2 and 3, respectively. From then on, the different haemocytes were classified as subpopulations (Sub); Sub 1 (band 1), Sub 2 (band 2), Sub 3 (small cells from the fraction of dispersed cells), Sub 4 (big cells from the fraction of dispersed cells) and Sub 5 (band 3). The purity (percentage of the major cell type) of Sub 1, Sub 2 and Sub 5 was 95.0 ± 1.0%, 97.7 ± 1.2% and 99.4 ± 0.8%, respectively. Since Sub 3 and Sub 4 were mixed in the dispersed cell fraction, the purity level could not be evaluated. Starting from 24 h of culture, it was common to observe cellular breakdown due to cellular over-spreading in Sub 1. In Sub 2 and Sub 3, cell lysis was observed after 1 h of culture and clustering activity after 24 h of culture. Cells of Sub 4 and Sub 5 started to show signs of degranulation and deterioration (cell fragmentation) after 2 h of culture, which continuously increased up to 24 h.

The live-cell imaging videos revealed differences in cellular motility and morphology and confirmed the purity levels of the subpopulations. Sub 1 cells adhered and spread strongly over the glass and displayed active movement. In contrast, Sub 2 and Sub 3 cells remained mostly rounded and exhibited very limited pseudopod-like projections and adherence. The movement displayed by these cells was mainly caused by brownian motion. Cells of Sub 4 and Sub 5 demonstrated more intense activity than the other subpopulations, both by projection of pseudopodia and cytoplasmatic granules displacement.

3.2.2. Flow cytometry

For each subpopulation, the values of forward and side scatter were related with the cell diameter and granularity, respectively. Cells of Sub 1 had a small average diameter (126.4 ± 4.6) and presented the lowest granularity (2.3 ± 0.1) (Fig. 2 and Table 1). Sub 2 contained the smallest cells (107.2 ± 4.5) which were more granular (3.7 ± 0.5) than the cells of Sub 1. The cells of Sub 3 were slightly bigger (118.2 ± 9.4) and less granular (3.4 ± 0.6) than the Sub 2 cells. Sub 4 cells were bigger (171.5 ± 16.3) but less granular (6.0 ± 2.9) than the cells of Sub 5. These latter were big cells (166.2 ± 6.0) with the highest granularity (10.6 ± 2.9).

3.2.3. Light microscopy of fixed haemocytes

The H&E staining of fixed cell cytospins provided morphological details of the separated haemocytes (Fig. 2). The average cell diameter was the smallest in Sub 2 (7.5 ± 1.3 μm) followed by Sub 3 (7.8 ± 0.9 μm), Sub 1 (8.6 ± 0.8 μm), Sub 5 (9.9 ± 1.0 μm) and finally Sub 4 (10.5 ± 1.5 μm). The nucleus:cytoplasm ratio was high in Sub 1 and Sub 3 and very high in Sub 2. In Sub 4 and Sub 5 this ratio was low. The cytoplasm was eosinophilic in all the cells but with a more intense staining in Sub 4 and Sub 5. The granularity content increased from the cells on top of the gradient (Sub 1) to the ones at the bottom (Sub 5). These granules were always basophilic (when present) in Sub 1, Sub 2 and Sub 3. Sub 4 presented a high number of granules that were predominately eosinophilic with sporadic appearance of basophilic ones. This situation was the same in Sub 5, although the number of granules and their staining intensity was higher. The nuclei of cells in Sub 1 and Sub 4 were in general large with dispersed chromatin (euchromatin). The nuclei of cells in Sub 2 and Sub 3 were small, folded and with very condensed chromatin (heterochromatin). Sub 5 cells had in general small and condensed nuclei. Table 1 presents a summary of the parameters described above.
3.3. Survival evaluation

The survival evaluation for each haemocyte subpopulation is presented in Fig. 3. The cells of Sub 2 showed the best survival performance, followed by the cells of Sub 1, Sub 3 + Sub 4 and finally Sub 5. Living cells were detected up to 120 h in Sub 1 and Sub 2, and up to 24 h in Sub 3 + Sub 4 and Sub 5. In the first 12 h of culture, all the subpopulations presented viability over 50%.

3.4. Detection of phagocytic activity

After 1 h of co-culture with GFP-labelled *V. campbellii*, phagocytosis was only detected in Sub 1 and Sub 4. The remaining subpopulations did not show any uptake (internalization) of bacteria (Fig. 4).

4. Discussion

The centrifugation of *P. vannamei* haemocytes through iodixanol density gradients allowed the isolation and collection of three visibly distinct haemocyte bands. Additionally, the cells collected from the interphase in-between those bands (dispersed cells) also presented morphological differences with the cells from the neighbouring bands.

Since bands 1 and 2 were too close to each other to be collected without cross-contamination (Fig. 1), we composed a new gradient (gradient 2) especially designed to promote the physical separation of these two bands. The principle was to compose a gradient with a narrower density range (gradient 1: 10 –20% iodixanol; gradient 2: 7 –16% iodixanol) in a higher total volume (gradient 1: 7.5 ml; gradient 2: 10 ml).
Fig. 3. *In vitro* survival of separated *P. vannamei* haemocyte subpopulations as determined by ethidium monoazide bromide staining.

Fig. 4. Phagocytosis of *V. campbellii* by separated *P. vannamei* haemocyte subpopulations at 1 h post inoculation (hpi). The images in A provide a general view of the cultures at 1 hpi. The image sequences presented for Sub 1 (B) and Sub 4 (C) are a magnification of the images presented in A. These images are a sequence of confocal microscopy pictures taken from the cell base (1) to its apex (4). This illustrates the process of bacteria uptake and therefore proves that cells from Sub 1 and Sub 4 express phagocytic activity. F-actin fibres are stained with phalloidin–Texas Red (red), the nucleus is stained with Hoechst (blue) and GFP-labelled *V. campbellii* exhibit green fluorescence. Scale bars: A = 15 μm; B = 8 μm; C = 5 μm.
gradient 2: 10 ml). This created a gradient density profile curve with a smaller slope (narrower density range per unit of volume), when compared with gradient 1 (Fig. 5). Although the average density of the cells of Sub 1 and Sub 2 was similar (1.075 and 1.078, respectively) this strategy promoted their physical separation (Figs. 1 and 4) and consequently their collection with a high purity level was possible.

The analysis of the haemocyte bands revealed the existence of 5 haemocyte morphotypes or subpopulations. Two of them were easily identified based on existing literature (reviewed by Jiravanichpaisal et al., 2006). Sub 1 exhibited all the characteristics typically attributed to hyalinocytes and Sub 5 were clearly granulocytes. The classification of Sub 2, Sub 3 and Sub 4 was not that straightforward. Sub 4 resembled typical semi-granulocytes. The classification of the haemocytes of Sub 2 and Sub 3 was very difficult. These cells were smaller but more granular than hyalinocytes and interestingly did not adhere to the glass and presented folds in the nucleus. These characteristics suggested that Sub 2 could be classified as small hyaline cells (Rodriguez et al., 1995), small granule haemocytes or lymphocyte-like hyalinocytes (Hose et al., 1987; Vargas-Albores et al., 2005) and prohaemocytes or immature haemocytes (Roulston and Smith, 2011).

The objective of the present work was to develop a system to efficiently separate biologically-active haemocyte subpopulations. The efficiency was proven by the identification of 2 currently undescribed P. vannamei haemocyte subpopulations, the high degree of purity of the separated subpopulations and the reproducibility of the procedures. The experiments on the in vitro cell viability and phagocytic activity proved that the isolated cells were biologically-active. However, in order to make a clear classification of these cell types, the performance of detailed histochemical and functional studies will be necessary.

The density profiles of the iodixanol gradients used in this study and several Percoll gradients are presented in Fig. 5. The Percoll self-forming gradients traditionally used to separate crustacean

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**Fig. 5.** Iodixanol density gradients used in the present work (A and B) and self-forming Percoll gradients using several initial concentrations (C) (adapted from Amersham Biosciences, 2001). The iodixanol gradients display a continuous and nearly linear density profile. The vertical bars represent the haemocyte bands indicating their position in the gradients. They also indicate the approximate buoyant density of each cell type. Percoll gradient density curves represent self-forming gradients with starting concentrations of stock isotonic Percoll from 20% to 90% in 0.15 M NaCl. Running conditions were: 23° angle-head rotor 30,000 g for 15 min. The vertical bars represent the approximate positions of the bands in the gradient according to the work of Liu et al. (2005) on P. vannamei haemocyte separation. The 70% gradient profile was created using similar conditions to the ones this author used.
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