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Morphologic, cytometric and functional characterization of the common octopus (*Octopus vulgaris*) hemocytes

S. Castellanos-Martínez [a], M. Prado-Alvarez [b,c], A. Lobo-da-Cunha [b,c], C. Azevedo [b,c], C. Gestal [a,*]

[a] Instituto de Investigaciones Marinas, Consejo Superior de Investigaciones Científicas, Eduardo Cabello, 6, 36208 Vigo, Spain
[b] Interdisciplinary Center of Marine and Environmental Research, University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal
[c] Institute of Biomedical Sciences Abel Salazar, University of Porto, Rua Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal

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ABSTRACT

The hemocytes of *Octopus vulgaris* were morphologically and functionally characterized. Light and electron microscopy (TEM and SEM), and flow cytometry analyses revealed the existence of two hemocyte populations. Large granulocytes showed U-shaped nucleus, a mean of 11.6 μm ± 1.2 in diameter with basophilic granules, polysaccharide and lysosomic deposits in the cytoplasm. Small granulocytes measured a mean of 8.1 μm ± 0.7 in diameter, and have a round nucleus occupying almost the entire cell and few or not granules in the cytoplasm. Flow cytometry analysis showed that large granulocytes are the principal cells that develop phagocytosis of latex beads (rising up to 56%) and ROS after zymosan stimulation. Zymosan induced the highest production of both ROS and NO. This study is the first tread towards understanding the *O. vulgaris* immune system by applying new tools to provide a most comprehensive morpho-functional study of their hemocytes.

1. Introduction

Cephalopods are the subject of important fisheries and biomedical studies, serving as host for parasites like *Anisakis* sp. that can infect humans (Hochberg, 1990). In addition, cephalopods have a great potential as aquaculture species (Iglesias et al., 2007). These molluscs possess a close circulatory system, where the hemolymph is restricted to blood vessels and capillaries (Cowden and Curtis, 1981). The leukopoietic organ of cephalopods is known as the white body, located behind the eyes in the orbital pits of the cranial cartilages (Cowden, 1972). Studies performed by light and electron microscopy on *Octopus vulgaris* (Cowden, 1972), *Octopus briareus* (Cowden and Curtis, 1973) and *Sepia officinalis* (Bolognari, 1949; Claes, 1996) described the morphology of precursor stages of cells developed in the white body before maturation and release to the circulating hemolymph.

Hemocytes are the circulating cells of the hemolymph. They are involved in several functions such as wound repair, nutrient digestion, transport and excretion. In addition, they play an important role in the internal defense (Cheng, 1975; Chu, 2000). The general agreement differentiates two types of cells in bivalves and gastropods (granulocytes and hyalinocytes) according to the presence/absence of cytoplasmic inclusions (Cheng, 1975; Donaghy et al., 2010). A single type of hemocyte round in shape, with several cytoplasmic inclusions and U-shaped nucleus resembling mammalian monocytes has been identified in cephalopods to date (Cowden and Curtis, 1981; Malham and Runham, 1998). However, their morphological characterization has not been fully studied, and much less attention has received their functionality (Ford, 1992).

The non-adaptive immune system of invertebrates, composed by cellular and humoral components, works in a combined action to control infections and diseases. The main cellular defensive mechanism, phagocytosis, was detected in the cephalopods *Eledone cirrhosa* and *O. vulgaris* using traditional light microscopy techniques (Malham et al., 1997; Novoa et al., 2002; Rodríguez-Domínguez et al., 2006). Linked to phagocytosis is the release of oxidative chemicals such as the reactive oxygen species (ROS), named respiratory burst; and the reactive nitrogen species (RNS) including the nitric oxide (NO), both acting as killing agents (Tiscar and Mosca, 2004).

Conventional methods for measuring ROS, as reduction of nitroblue tetrazolium, that measures intracellular radicals (Anderson, 1994) or cytochrome-C to measure extracellular oxygen radicals (Wootton et al., 2003) have been used in cephalopods. Intracellular ROS was measured in *E. cirrhosa* hemocytes after the octopus was air exposed (Malham et al., 2002) and extracellular ROS was observed in *O. vulgaris* hemocytes following stimulation with zymosan. The same stimuli was effective to induce significant NO production, whereas *Escherichia coli* lipopolysaccharide (LPS) induced a weak reaction (Novoa et al., 2002).

* Corresponding author. Tel.: +34 986 231 930; fax: +34 986 292 762.
E-mail address: cgestal@iim.csic.es (C. Gestal).

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Different methods for hemocyte characterization in molluscs could rise to disparities in the results. Therefore, automated methods that can remove bias are valuable for further application in this field of research (Ashton-Alcox and Ford, 1998). Flow cytometry is used to compliment time consuming techniques based on physical separation of cells. This methodology provides information about different characteristics including their size and granularity, valuable parameters used to characterize cell populations in bivalves and gastropods (Ashton-Alcox and Ford, 1998; Allam et al., 2002). In addition, flow cytometry has been used to measure cellular defense parameters such as phagocytosis, respiratory burst and production of nitric oxide in *Mytilus galloprovincialis* (García-García et al., 2008), *Haliotis tuberculata* (Travers et al., 2008), *Crassostrea ariakiensis* (Donaghy et al., 2009), *Haliotis discus discus, Turbo cornutus* (Donaghy et al., 2010) and *Ruditapes decussatus* (Prado-Alvarez et al., 2012).

Since *O. vulgaris* is an important mollusc for commercial fisheries worldwide and aquaculture, the understanding of the immune system is required to assess the effect of environmental factors and natural pathogens. The purpose of the present study is the morphological and functional characterization of the circulating hemocytes of *O. vulgaris* by light and electron microscopy complemented with flow cytometry for the first time.

2. Materials and methods

2.1. Biological material and hemolymph collection

O. vulgaris from 65–220 mm mantle length were collected by traps, an artisanal gear used by local fishermen, from the Ria of Vigo, Spain (24°14′N, 8°47′W) and transported to the laboratory. Octopuses were placed in culture tanks of open seawater system at 15 °C for 24 h to acclimate before experimentation.

Following ethical procedures (Directive 2010/63/EU) octopuses were anesthetized using 7.5% magnesium chloride ($MgCl_2$) according to Messenger et al. (1985). Hemolymph was withdrawn from the cephalic aorta using a disposable syringe (0.50 × 16 mm Ø, 1 ml) containing different solutions depending on the procedure. Hemolymph from each octopus was used immediately or transferred into a vial and kept on ice until use.

2.2. Morphological characterization of O. vulgaris hemocytes

2.2.1. Light microscopy: fresh and fixed hemocytes

2.2.1.1. Cells in suspension. Prefilled-syringes with 0.22 μm filtered seawater (FSW), 4% paraformaldehyde solution (in 0.1 M phosphate-buffered saline (PBS), pH 7.4) or 2.5% glutaraldehyde (in 0.2 M sodium cacodylate buffer pH 7.4) were used to dilute (1:1) the hemolymph. A subsample of freshly drawn hemolymph (without dilution and without anti-aggregating solution) was centrifuged for 5 min at 300*g* and 4 °C). The hemocytes were then re-suspended in Squid Ringer Solution (SRS: 530 mM NaCl, 10 mM KCl, 25 mM $MgCl_2$, 10 mM $CaCl_2$ and 10 mM HEPES buffer, pH 7.5). Preparations of 100 μl of SRS re-suspended hemocytes and diluted hemolymph samples were immediately observed at light microscopy DM2500 (Leica) equipped with Nomarsky interference contrast to enhance the contrast of fresh non-stained hemocytes. Hemocyte diameters were measured (at least 200 cells) using Leica Application Suite software v4.

2.2.1.2. Hemolymph cell monolayer. Hemolymph cells monolayer were prepared by cell adhesion and cyto-centrifugation. For both cases freshly drawn hemolymph, hemolymph diluted 1:1 in FSW and cells re-suspended in SRS were used. In order to perform spontaneous cell adhesion, 100 μl of each hemocyte solution was settling onto a glass slide and allowed to adhere for 20 min in a moist chamber at 15 °C. Cyto-centrifugation was carried out with 100 μl of hemocyte solution at 200*g* for 5 min in a Cytospin 4 cytocentrifuge (Thermo Scientific). Hemolymph cell monolayers were fixed in 100% ethanol for 1 min and stained for 1 min with each of the two solutions included in the rapid Hemacolor® kit (Merck). Glass slides were gently washed in distilled water and mounted in DPX resin (BDH, Chemicals). Hemocyte diameters were measured as previously mentioned.

2.2.2. Electron microscopy analysis

Freshly drawn hemolymph (100 μl) was fixed for 4 h in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) at 4 °C and washed for 30 min in the same buffer for scanning electron microscopy (SEM) study. Samples were then dehydrated in an ethanol series, critical point dried in CO_2 using a Polaron E3000 and sputter-coated in a Polaron SC 500 using 60% gold–palladium. Hemocytes were examined with a Philips XL 30 scanning electron microscope operated at 5 kV.

Transmission electron microscopy (TEM) was applied to circulating hemocytes. Freshly draw hemolymph was fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer pH 7.4 after 4 h at 4 °C. Hemocytes were washed for 12 h at 4 °C in 0.2 M sodium cacodylate buffer pH 7.4 and post-fixed in buffered 2% OsO_4 for 3 h at the same temperature. Hemocytes were pelleted and pre-embedded in 4% agar at 40 °C. Small agar pieces containing fixed hemocytes were dehydrated in a graded ethanol series and embedded in Epon resin. Semi-thin sections obtained by diamond knife were stained with methylene blue. Ultrathin sections were double stained with uranyl acetate and lead citrate, and visualized using a JEOL 100CXII TEM operated at 60 kV.

2.2.3. Cytochemical characterization of circulating hemocytes

2.2.3.1. Periodic acid Schiff (PAS). Periodic acid Schiff (PAS) technique was applied for detection of polysaccharides and glycoproteins in semi-thin sections obtained from Epon resin embedded pieces (see above). Oxidation of sections was carried out with 1% periodic acid for 10 min. Hemocyte sections were washed with water, stained with Schiff reagent during 20 min and a final washed was done. Periodic acid was avoided for controls (Loboda-Cunha et al., 2010).

2.2.3.2. Tannic acid-uranyl acetate staining. For glycogen detection by TEM, ultrathin sections were treated with a 5% solution of tannic acid for 10 min, briefly washed in water, stained with 2% uranyl acetate for 10 min and finally washed in water (Sannes et al., 1978).

2.2.3.3. Arylsulphatase. Samples were obtained as previously mentioned for TEM assays (see above). For lysosomal enzymes detection, circulating hemocytes were fixed for 1 h at 4 °C in 2.5% glutaraldehyde (diluted in 0.2 M cacodylate buffer pH 7.4). Samples were washed in acetate buffer 0.2 M pH 5.0 for 30 min, incubated during 60 min at 35 °C in medium containing $BaCl_2$, p-nitrocatechol sulphate in acetate buffer 0.1 M pH 5.0 (Hopsu-Havu et al., 1967). For controls, p-nitrocatechol sulphate was omitted. Post-fixation was done during 2 h at room temperature in 1% OsO_4 plus 1% potassium ferrocyanide in cacodylate buffer. Samples were dehydrated and embedded as reported above, and the ultrathin sections were observed without further staining.

2.2.4. Flow cytometry (FCM) analysis

Hemocyte population was determined in freshly drawn or FSW diluted hemolymph using a FACScalibur flow cytometer (Becton Dickinson; Franklin Lakes, NJ) equipped with cell-sorting and cell-concentrator modules. Hemocyte population was discrimi-