Morphofunctional characterization and antibacterial activity of haemocytes from *Octopus vulgaris*

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This study focused on the morphological and functional characterization of the haemocytes from *Octopus vulgaris* as the first agents responsible for innate immunity. Three major haemocyte types were identified by light microscopy based on nucleus/cytoplasm ratio and the presence or absence of cytoplasm granules: haemoblast-like cells, hyalinocytes and granulocytes. The presence of three haemocyte populations was also confirmed by flow cytometry. Cytochemical characterization suggests that they perform different activities during humoral responses. Using the plate radial diffusion method we demonstrated the bactericidal activity of haemocytes in the presence of different bacteria strains. To investigate the presence of soluble biotic compounds responsible for antibacterial activity, using a disc diffusion method and the minimum inhibitory concentration, we have also tested the methanolic acid extract from these cells. Our results pave the way for the development of potent antibacterial drugs that could lead to several applications.

**Keywords:** *Octopus vulgaris*; primary culture; haemocytes; antimicrobial activity

**Introduction**

The marine environment comprises complex ecosystems, and many organisms are known to possess bioactive factors as a common means of self-defence or for the protection of eggs and embryos (Nair et al. 2011). In invertebrate organisms, immunity involves both cell-mediated and humoral systems that synergistically provide protection from invading pathogens. They have an immune defence primarily based on the activation of antibacterial mechanisms recruited by the innate immune system, which in turn is primed by the invading organism itself (Söderhäll 2010).

The cellular defences in these organisms are carried out by haemocyte-mediated responses (in the case of open circulatory systems) or coelomocyte-mediated responses (in the case of semi-open circulatory systems), such as phagocytosis, nodule formation, encapsulation, degranulation and the production of reactive oxygen intermediates (Pipe 1992; Hegaret et al. 2003). The humoral responses include also the activation of proteins constitutively present in the haemolymph, such as the prophe- nol oxidase and coagulation cascades, as well as the activation of different intracellular signalling pathways that stimulate production of different defence proteins, such as antimicrobial peptides (Iwanaga and Lee 2005). Many of these innate immune

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reactions are highly evolutionarily conserved and are found throughout the animal kingdom (Vorbach et al. 2006).

The economic and phylogenetic importance of molluscan species, mainly bivalves and cephalopods, has led to an increasing number of investigations of their immune defence mechanisms (Pipe and Coles 1995; Iglesias et al. 2000; Vaz-Pires et al. 2004; Domingues et al. 2010). Bivalve haemocytes are responsible for cell-mediated immunity through phagocytosis and various cytotoxic reactions, such as lysosomal enzymes, antimicrobial peptide release and production of reactive oxygen intermediates. Two cell types, hyalinocytes and granulocytes, both of which are capable of phagocytic activity, have been found in many bivalve species including Anodonta cygnea, Dreissena polymorpha, Mytilus edulis, Scrobicularia plana, Ruditapes philippinarum, Mercenaria mercenaria, Meretrix lusoria and Crassostrea gigas (Moore and Eble 1977; Giamberini et al. 1996; Cima et al. 2000; Soares-da-Silva et al. 2002; Wootton and Pipe 2003; Delaporte et al. 2007; Le Foll et al. 2010). In many of these species, granulocytes are the most numerous cell type and have been subclassified as eosinophilic, basophilic and neutrophilic (McCormick-Ray and Howard 1991; Fryer and Bayne 1996; Leclerc 1996; Lopez et al. 1997). Moreover, bivalve haemocytes show chemotactic activity towards pathogens or their products (Prieur et al. 1990; Canesi et al. 2002). In Crassostrea virginica and Mercenaria mercenaria, haemocytes are able to migrate towards peptidic attractants produced by both Gram-positive and Gram-negative bacteria (Fawcett and Tripp 1994).

Among cephalopods, the association between the squid Euprymna scolopes and the bioluminescent bacterium Vibrio fischeri has been used as a model system to study the involvement of the innate immune system in establishing and maintaining such specific symbiotic relationships. Several pathways of innate immune response activation have been recognized in Euprymna, such as pattern recognition receptors, peptidoglycan recognition proteins, lipopolysaccharide-binding proteins, as well as other innate immune factors such as thioester-containing proteins, which play an important role in innate immune response as members of a complement-like system (Nyholm and Graf 2012).

Only one immune cell type (granulocytes) was observed in the haemolymph of three species of Octopoda (Bidder et al. 1989; Kondo and Tomonaga 2003). Under the electron microscope, two distinct morphologies among circulating haemocytes of Octopus vulgaris were identified by Novoa et al. (2002). However, these authors indicated that their results were not conclusive and more work should be done to determine whether these two morphologies correspond to different states of activation or if they constitute two different cell types (Novoa et al. 2002). Kondo and Tomonaga (2003) described only granulocytes in O. vulgaris, and classified them on the basis of their abundance, dimension and eosinophilic or basophilic properties.

More recently, many bioactive compounds have been extracted, purified and characterized from various marine animals like sponges, corals, molluscs and tunicates (Rajasekharan Nair et al. 2011). Previous studies on cephalopods demonstrated the presence of antimicrobial, lysozyme and antiprotease activity in the haemocytes and haemolymph of O. vulgaris, Eledone cirrhosa, Sepioteuthis lessoniana and Euprymna scolopes (Malham et al. 1998a; Novoa et al. 2002; Rajasekharan Nair et al. 2011; McFall-Ngai et al. 2012). However, the only studies investigating cephalopods that identified bioactive peptides with antimicrobial activity (antimicrobial
peptides) were those conducted on squids and cuttlefishes. These animals leave their eggs to fend for themselves and interestingly they do not get any infections. Their eggs release polypeptides, sexual pheromones and antimicrobial peptides trapped in the egg capsule, mainly expressed by female accessory sex glands, which confer on them an efficient protection against microorganisms (Laurencin et al. 2012).

The only data related to *O. vulgaris* concern haemagglutination activity in cell-free haemolymph (Rogener et al. 1985; Fisher and Dinuzzo 1991) and an antiprotease activity belonging to the α-macroglobulin family detected in the haemolymph (Thogersen et al. 1992). Therefore, the detailed identification of the cellular components and the characterization of defence mechanisms in *O. vulgaris* have received little attention. This species, as other cephalopods but unlike other molluscs, has a closed circulatory system and the blood consists of haemolymph, haemocyanin and haemocytes (Novoa et al. 2002). This characteristic makes cephalopods a particularly good model to investigate aspects of immune responses similar to vertebrates.

In this study we obtained, for the first time, primary haemocyte culture by developing a novel growth medium. Furthermore, we characterized *O. vulgaris* haemocyte populations based on their morphological, cytofluorimetric and functional features.

**Material and methods**

**Animals**

Male (*n* = 2) and female (*n* = 2) *O. vulgaris* Cuvier, 1797 (bodyweight in the range 0.8–1 kg) were captured from the Bay of Naples. Animals were transported in the dark in a fish bag containing seawater (about 5 ml seawater per 1 g of animal) and maintained in aquarium tanks (80 × 60 × 50 cm³) with circulating seawater until the beginning of the experiments. Water temperature was 16°C and photoperiodicity was maintained according to local conditions.

Animals were identified and examined for bodyweight, gender and general conditions (regeneration of arms, skin lesions or scars).

**Animal care**

There are no specific legal or ethical regulations related to experimental work with octopuses in Italy. However, our research using octopuses conforms to the ethical principles of Reduction, Refinement and Replacement (Russell and Burch 1959). Specific attention was paid to avoiding and minimizing any suffering in accordance with Directive 2010/63/EU. Animals were properly anaesthetized and sacrificed (Motschaniwskyj et al. 2007; Polese et al. 2012).

**Haemolymph collection and haemocyte primary cultures**

When the octopuses were totally relaxed the ventral mantle was partially folded backwards to expose the branchial hearts for blood sampling (Malham et al. 1998b). The haemolymph was collected at 4°C using a 2.5-ml sterile syringe with 30G needle, and mixed immediately with an equal volume of marine anticoagulant solution (0.1 M Glucose, 15 mM trisodium citrate, 13 mM citric acid, 10 mM ethylene diamine...
tetraacetic acid, 0.45 M NaCl, at pH 7.0 and 1000 mOsm; Barcia et al. 1999). The serum was obtained by centrifugation of the whole haemolymph at 200 g for 10 min at 4°C, sterilized through a 0.22-µm pore filter and stored at –20°C. The haemocytes were counted using a Neubauer chamber and plated on round dish (ibiTreat plates by Ibidi GmbH) in Leibovitz L-15 medium (Sigma, St Louis, MO, USA) containing 20.2 g/l NaCl, 0.54 g/l KCl, 0.6 g/l CaCl₂, 1 g/l MgSO₄, 3.9 g/l MgCl₂, 20.8 g/l glucose, and 100 units/ml penicillin G, 100 g/ml of streptomycin, 0.1 g/ml of gentamycin and 0.10 g/ml of amphotericin B (pH 7.0 and 1000 mOsm) at a concentration of 1.5 × 10⁶ to 2.0 × 10⁶ cells/ml, modifying the classical growth medium used for bivalve haemocytes (Lebel et al. 1996). Cells were incubated at 16°C for 30 min to allow cell attachment. Non-adherent haemocytes were removed by washing with sterilized artificial seawater. Haemocyte monolayers were supplemented with haemolymph serum and kept at 16°C. Cell viability was assessed by trypan blue 0.1% exclusion assays and monitored every day. In these conditions the cultures survived for up to 3 months.

Live cell analysis
Haemocyte motility and morphology were analysed with a Nikon eclipse ti-4200 microscope equipped for epifluorescence and differential interference contrast.

Differential haemocyte counts were made to calculate the relative percentage of different haemocyte types; 20 random fields of view from five culture plates were analysed by time-lapse photomicroscopy experiments (one image every 30 s for 90 min). The images were analysed using the imaging software NIS-Elements (Nikon Imaging Software). The data obtained from the differential counts were analysed using analysis of variance and Tukey’s test (p < 0.05).

Cytological analysis
Cultured haemocytes were stained with the following protocols and observed with a Nikon eclipse E400 equipped with Nikon CoolPix E4500. All reagents and buffers were from Sigma-Aldrich (Castle Hill, NSW, Australia). The filtered seawater (FSW) was obtained from the Bay of Naples, Italy, and sterilized by filtration with Millipore Durapore™ (cut-off 0.22 µm).

Toluidine blue stain
Samples were fixed in paraformaldehyde at 4% [weight/volume (w/v)] dissolved in phosphate-buffered saline (PBS) with 2% NaCl (w/v) for 30 min; native haemocytes were stained with toluidine blue to observe the staining of granules. The stain solution was prepared dissolving toluidine blue at 0.1% (w/v) in FSW; haemocyte samples were stained for 15–30 min at room temperature and washed with FSW. Samples were mounted and analysed under light microscopy.

Giemsa/May–Grünwald stain
Haemocytes were stained with Romanowsky’s Giemsa/May–Grünwald solution to characterize basic cellular morphology (Aladaileh et al. 2007). The haemocytes were
fixed in paraformaldehyde at 4% (w/v) dissolved in PBS with 2% NaCl (w/v) for 30 min and then covered with May–Grünwald stain for 6 min. After several washes in PBS, the plates were covered with Giemsa stain for 30 min before being washed in PBS and air dried. Plates were mounted and analysed under light microscopy.

**Flow cytometry**

Cell cycle analysis was carried out by fluorescence-activated cell sorting: $5 \times 10^6$ cells were fixed in 70% ice-cold ethanol for 4 h at 4°C and washed three times in PBS. Cell pellet was resuspended and stained for 30 min at room temperature in 0.1% Triton X-100, 0.2 mg/ml DNase-free RNaseA and 20 mg/ml propidium iodide. Fluorescence was determined by using the FACScalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Experiments were performed in triplicate. Data were acquired using CELLQuest software (Becton Dickinson) and analysed with FlowJo software (using the Dean–Jett–Fox model) to define the G1, S, and G2 phases of the cell cycle.

**Enzymatic histochemistry analysis**

Cultured haemocytes were treated to detect the presence of two specific enzymatic activities and observed with a Nikon Eclipse E400 equipped with Nikon CoolPix E4500. All reagents and buffers were from Sigma-Aldrich (Castle Hill, NSW, Australia).

**Peroxidase activity**

The cultured haemocytes were fixed in formaldehyde (4% in FSW) for 10 min, washed in PBS and treated according to Cima et al. (2001).

** Phenol oxidase activity**

Thirty microlitres of phenol oxidase solution was applied to non-fixed cultured haemocytes for 20 min and treated according to Cima et al. (2001).

**Phagocytosis assay**

The phagocytic ability of *O. vulgaris* haemocytes was detected by Congo red stained yeast, *Saccharomyces cerevisiae* (Sigma-Aldrich). The plates were viewed under a Nikon Eclipse Ti-4200 microscope equipped for epifluorescence and differential interference contrast.

**Method**

Five milligrams yeast was suspended in 5 ml FSW and 5 ml of filtered Congo red (Sigma-Aldrich) at 0.8% in FSW. The suspension was autoclaved at 120°C for 15 min, and then washed twice and centrifuged at 1300 $g$ for 5 min. The pellet was suspended in 10 ml FSW and stored at 4°C.
To measure phagocytic activity, 30 μl of fresh haemolymph, adjusted with FSW to $1 \times 10^5$ cells/ml, were placed on ibiTreat plates and the haemocytes were allowed to adhere for 30 min at room temperature in a moist chamber. The adherent cells were then washed five times with FSW, covered with 100 μl of Congo red stain yeast ($7.0 \times 10^5$ cells/ml) and incubated for 30 min at room temperature (25°C). Non-phagocytosed yeast cells were removed by dipping plates in FSW 10 times. Phagocytic activity was calculated by counting the number of granulocytes and hyalinocytes that had phagocytosed one or more yeasts. This experiment was conducted in triplicate.

**Microbial strains and culture conditions**

Seven species of bacteria were used as test organisms: three Gram-positive (Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus) and four Gram-negative (Escherichia coli D31, Pseudomonas aeruginosa, Shigella sp., Salmonella sp.). All the bacteria strains were obtained from the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures). Microbial strains were cultured in Luria–Bertani broth (Difco Laboratories, Sparks, MD, USA) at 37°C and 200 g in an orbital incubator. Stocks were maintained on Luria–Bertani agar (1.5%; w/v) (Sambrook and Russel 2001).

**Killing assay**

**Bactericidal activity**

Killing assays were performed as described by Volety and Chu (1995), in the absence of antibiotics. Experiments were performed in triplicate and absorbance values were corrected by subtracting the background absorbance of the FSW only. The percentage of bacteria killed (KI) was calculated from absorbance values obtained after reduction of [3 - (4,5-dimethylthiazol-2-yl) -5 - (3-carboxymethoxyphenyl) -2 - (4-sulfophenyl)-2H-tetrazoliumand] - [phenylmethasulfazone] MTS-PMS in soluble formazan as follows: $KI = [(AH + B−AH)/AB] \times 100$, where AH and AB are the absorbance value obtained for haemocytes (H) and bacteria (B), respectively. The bactericidal activity is expressed as the mean ± standard error (SE) of killing percentage. The statistical analysis was performed using analysis of variance and Student’s t-test.

**Disc diffusion method**

The antibacterial activity of haemocytes was determined according to Bauer et al. (1996) in culture medium (Müller–Hinton agar). The experiments were conducted in triplicate.

**Preparation of haemocyte methanolic acid extract**

The fresh haemolymph was centrifuged at 160 g for 10 min to separate haemocytes. Haemocytes were incubated with a methanolic acid solution (methanol 96% containing HCl 1 M) at 4°C for 72 h. The cell extract was centrifuged at 10,000 g for 60 min at 4°C. The surnatant was neutralized with NaOH 10 M and concentrated under vacuum in a rotary evaporator at 30°C. The pellet was suspended in
3-(N-morpholino)propanesulphonic acid (MOPS) (Fluka). The concentration of haemocyte methanolic acid extract (HMAE) was calculated with the Bradford method (Sambrook and Russel 2001). The bactericidal activity was evaluated with a growth curve of bacterial strain *E. coli* in the presence of different concentrations of HMAE (0.2, 0.4 and 0.8 μg/ml), using ampicillin (100 μg/ml) as positive control and MOPS buffer (50 mM at pH 5.0) as negative control.

**Results**

**General remarks**

Using a combination of several methods, we have characterized morphofunctionally the haemocytes from *O. vulgaris*. Three types of haemocytes were identified: haemoblast-like cells, hyalinocytes and granulocytes (Figure 1A–C), and they exhibited a heterogeneous distribution (Figure 1D). In culture medium, living haemocytes showed the ability to adhere spontaneously to culture plates, and also to form pseudopodia.

**Haemoblast-like cells**

Haemoblast-like cells (Figure 1A) are the smallest cells observed in the haemolymph, with 8.0 μm in diameter. They have a spherical shape with a high nucleus/cytoplasm ratio and represent 30 ± 3% (Figure 1D) of the total haemocyte populations. These cells are not motile and they require about 60 min to adhere to culture plates.

This haemocyte type stained with toluidine blue, resulting in a deep blue nucleus (Figure 2A). After staining with Giemsa/May–Grünwald dye, haemoblast-like cells appeared light acidophilic in both the nucleus and cytoplasm (Figure 3A, arrowhead). These cells do not show pseudopodia. They have a phenol oxidase activity as pink to red granular deposits (Figure 4B).

**Hyalinocytes**

Hyalinocytes (Figure 1B) vary in size within a range of 5–10 μm, having an average diameter of 7.5 ± 1.0 μm, and represent 60 ± 6% of the total haemocyte populations (Figure 1D). Hyalinocytes have the ability for amoeboïd movement and to form pseudopodia. These cells have round or oval nuclei and adhere to culture plates within 15–20 min. Their cytoplasm contains no or few granules and is filled with vacuoles of varying sizes. Some hyalinocytes maintain a round shape when attached to glass slides and spread only slightly, forming some small pseudopodia (Figure 1B). When stained with toluidine blue, this haemocyte type shows a blue round nucleus and a cytoplasm containing few blue granules (Figure 2B). The hyalinocytes show a basophilic nucleus and blue cytoplasm when stained with Giemsa/May–Grünwald (Figure 3B). They exhibit a phenoloxidase activity as shown in Figure 4C.
Figure 1. Differential interference contrast micrographs showing different types of haemocytes in culture plate: (A) haemoblast-like cell without pseudopodia; (B) two hyalinocytes connecting each other with pseudopodia; (C) well-attached granulocyte showing dendritic pseudopodia formation; (D) graph showing the ratio among the three haemocyte types. Scale bar = 5 μm.
Figure 2. Light microscopy micrographs of haemocytes stained with acidic toluidine blue revealing their metachromatic property: (A) in vivo haemoblast-like cell; (B) in vivo hyalino-ocytes containing blue granules; (C) in vivo granulocyte containing red and blue granules; (D) in vivo haemocytes aggregate; (E) fixed haemocytes connected by their filopodia [hyalinocytes (arrowheads) and granulocytes (arrows)]. Scale bar = 5 μm.

Figure 3. Light microscopy micrographs of haemocytes stained with Giemsa/May–Grünwald stain: (A) acidophilic haemoblast-like cells (arrowheads) and granulocytes with acidophilic cytoplasm and cells (arrows); (B) hyalinocytes with basophilic nucleus and cytoplasm filled with vacuoles and few granules; (C) granulocytes cells with basophilic cytoplasm. Scale bar 5 μm.
**Granulocytes**

Granulocytes (Figure 1C) represent 10 ± 1% of the total haemocyte populations (Figure 1D). They vary in size from 5 to 10 \( \mu \text{m} \), with an average diameter of 8.5 ± 0.3 \( \mu \text{m} \). These cells show the ability to adhere to the plate within 15–20 min, and they are highly amoeboid after adhesion. They also have the ability to form many long filopodia and are characterized by eccentric oval nuclei and numerous cytoplasmic granules. Their granules vary in size and are concentrated in the endoplasmic area. The ectoplasm does not contain granules (Figure 1C). The granulocytes show a blue oval eccentric nucleus and a cytoplasm containing many red and blue mixed granules when stained with toluidine blue (Figure 2C). Moreover, hyalinocytes and granulocytes revealed the ability to form mixed aggregates, connected to each other by filopodia (Figure 2D, E). When stained with Giemsa/May–Grünwald, granulocytes could be classified into cells with basophilic (Figure 3C) and acidophilic (Figure 3A, arrows) cytoplasm. Both basophilic and acidophilic cells show the ability to form many long filopodia and are characterized by numerous cytoplasmic granules. Granulocytes show a peroxidase activity, localized as dark brown deposits in 7 ± 1% of the population (Figure 4A), and a phenol oxidase activity, as pink to red granular deposits (Figure 4D). Furthermore, granulocytes are capable of ingesting yeast cells. Individual cells can phagocytose up to 20 yeast particles. They engulf foreign particles by cell surface invagination or by forming very short pseudopodia (Figure 5).

**Flow cytometry**

Five populations could be identified by flow cytometry light scatter plots (Figure 6A, left panel). “A” represents the most abundant population (33.8 ± 3%), containing cells with medium size (intermediate forward scatter) and moderate granularity (intermediate 90° light scatter). “B” (4.9 ± 0.5%) represents a population with small size and low granularity (low forward and low side-angle light scatter). Population “C” (21.5 ± 2%) has high forward and 90° light angle scatter, consistent with large size and high granularity; this population includes some aggregates and large proliferating cells (see below). Population “D” (2.1 ± 0.2%), containing cells with medium size and low granularity, consists of small proliferating cells (probably derived from “B” population). “E” represents a very heterogeneous population with small size and high granularity (low forward and high 90° light scatter), consisting of cell debris.

After staining with propidium iodide and removal of doublets, cell fragments and other artefacts that contain misleading amounts of DNA, three populations could be identified (Figure 6A, centre and right plots). Population R1 has low forward and side angle light scatter, containing small, resting cells with low granularity. R2 represents a population of resting cells with medium size and moderate granularity. R3 has high forward scatter and high 90° light scatter, indicative of a population of large granular cells; this population is the only one that is actively proliferating as demonstrated by the analysis of DNA content (Figure 6B, right plot).
The results show that bacterial mortality is significantly elevated \((p < 0.05\%\) in the presence of \(O.\ vulgaris\) haemolymph, and that the mortality of Gram-positive bacteria is significantly higher than that of Gram-negative bacteria (Figure 7).

**Killing assay**

**Bactericidal activity**

The results demonstrate an inhibition of bacterial growth proportional to the concentration of haemolymph added (Table 1). In all concentrations (10 µg, 100 µg and 1 mg), the haemolymph shows a greater inhibition of bacterial growth of *Staphylococcus aureus*, *Lysteria monocytogenes* and *Bacillus cereus* with respect to...
Escherichia coli D31, Pseudomonas aeruginosa and Shigella sp. The lowest antibacterial activity was detected in the presence of Salmonella sp. at the same concentrations.

The bactericidal activity of haemocyte methanolic acid extract

The HMAE at 0.2 μg/ml concentration dramatically reduced normal bacterial growth, while inhibiting it completely at 0.8 μg/ml (Figure 8).

Discussion

Octopus vulgaris is an edible species of high economic importance not only for Mediterranean countries. Currently, despite many studies concerning neurophysiology and behaviour (De Lisa et al. 2012a, 2012b), very little information has been reported about the immunity of this species (Novoa et al. 2002; Kondo and Tomonaga 2003). The haemopoiesis of molluscs has not been fully understood. Several theories have been proposed to explain it, but the most credited is Hine’s (1999) theory. This theory suggests that one blast type might give origin to

Figure 5. Micrograph of Octopus vulgaris cultured haemocytes showing phagocytic activity. Arrows indicate the phagocytosed yeast particles. Scale bar = 10 μm.
hyalinocytes that would further mature into granulocytes. The results obtained in our study tend to support this theory. Indeed, the haemoblast-like cell showed no granules or vacuoles in the cytoplasm, the haemocyte populations of *O. vulgaris* appearing as a gradient of differentiation stages. The haemopoietic organ of cephalopods is believed to be a soft organ rounded by the optic nerve called the "white body" (Stuart 1968; Cowden 1972; Bolognari et al. 1980). Novoa et al. (2002) have found, at the electron microscopy level, two distinct cell morphologies localized both in the white body and in the haemolymph of *O. vulgaris*. The first type has a kidney-shaped nucleus and a high number of granules; the second possesses a round nucleus and few granules. Our results, from fixed and native cells, identified three distinct populations: haemoblast-like cells, hyalinocytes, and granulocytes. On the basis of their N/C ratio, relative percentage distribution and proliferating ability, the cell type herein classified as hyalinocytes is consistent with the second type described by Novoa et al. (2002), while granulocytes correspond to the first type. Moreover, based on our results, we propose, in accordance with Hine's theory and the current classification of haemocytes in bivalves and gastropods (Donaghy et al. 2010; Le Foll et al. 2010), that the

Figure 6. Flow cytometric light scatter plots, DNA content and cell cycle analysis of haemocytes from *Octopus vulgaris*. (A) The left plot shows flow cytometric forward-angle light scatter (FSC) versus side-angle light scatter (SSC) plot on "ungated" sample. The percentage of each population is indicated. The centre plot shows pulse width (PI-W) versus area (PI-A) plot of the PI (propidium iodide) channel used to distinguish between single cells and aggregates. Single cells (G0/1 or G2/M) will have similar pulse width (transit time) values. Aggregates will have larger width values and can be easily seen on the plot to the right of the single cell region. Single cells have been gated (left plot). The right scatter plot shows the FSC versus SSC plot of PI-gated cells (single cells). Populations and percentage are indicated; (B) FL2–Area histograms and determination of the cell cycle of each population. The Dean–Jett–Fox model was used to define the G1, S, and G2 phases of the cell cycle. RMS refers to Root Mean Square.
haemoblast-like cells (classified as R1 and with percentage distribution of 12.8 ± 1%) might differentiate into hyalinocytes. This latter cell type still shows central or slightly eccentric nucleus, few vacuoles and was classified as R2, expressing a percentage distribution of 71.2 ± 7%. Hyalinocytes might mature into granulocytes, which are fully mature haemocytes, with numerous cytoplasmic granules, corresponding to the R3 population, expressing a percentage distribution of 16.0 ± 1%, and comprising the only cell type that shows the ability to proliferate.

Table 1. Diameters of the inhibition zone of the haemolymph determined by radial diffusion test on Müller–Hinton agar plate (Ø mm). Results are expressed as mean ± SD. The experiments were conducted in triplicate.

| Bacterial strain       | Haemolymph (10 µg) Ø mm | Haemolymph (100 µg) Ø mm | Haemolymph (1 mg) Ø mm |
|------------------------|-------------------------|--------------------------|------------------------|
| *E. coli*              | 1.69 ± 0.4              | 2.0 ± 0.2                | 2.65 ± 0.7             |
| *S. aureus*            | 1.64 ± 0.2              | 2.5 ± 0.4                | 4.7 ± 0.4              |
| *L. monocytogenes*     | 1.61 ± 0.7              | 3.8 ± 0.5                | 5.0 ± 0.6              |
| *B. cereus*            | 1.35 ± 0.5              | 4.5 ± 0.3                | 5.7 ± 0.6              |
| *P. aeruginosa*        | 1.54 ± 0.2              | 2.2 ± 0.1                | 3.3 ± 0.2              |
| *Shigella* spp.        | 1.49 ± 0.3              | 1.89 ± 0.5               | 3.2 ± 0.2              |
| *Salmonella* spp.      | 0.97 ± 0.1              | 1.2 ± 0.2                | 1.3 ± 0.6              |
To characterize the cellular components of the *Octopus vulgaris* immune system, our results on phagocytosis activity exhibited by haemocyte populations pointed out that both granulocytes and hyalinocytes contribute to phagocytosis activity, but granulocytes seem to be the most avid phagocytes to incorporate microorganisms, up to 20 yeast cells. The role played by granulocytes in phagocytosis is linked to their capability for intracellular killing. Furthermore, this population expressed high levels of phenol oxidase enzymatic activity, as well as the ability to form peroxidase. Even though hyalinocytes were not so avid in phagocytotic activity, they could play a crucial role in haemocyte aggregation processes, as shown herein in cytological analyses with toluidine blue.

A classification scheme based upon cellular activities is in accordance with previously suggested morphological classifications, which consider haemoblast-like cells as immature haemocytes. Unlike hyalinocytes and granulocytes, haemoblast-like cells did not seem to contribute to the defensive response, lacking both phagocytotic activity and the intracellular enzyme system associated with host defence (Hine 1999; Cima et al. 2001). This opens a new scenario about the possibility of these cells to behave like stem cells from which the other two populations, identified according to the characterization and classification proposed based on several invertebrates (Cima et al. 2000), differentiate.

The other objective of the current study was to further elucidate the functions of the *O. vulgaris* immunity system identifying novel and characteristic antibacterial activities. A first appreciable antimicrobial activity against most of the clinically isolated human pathogens, such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli* D31, *Pseudomonas aeruginosa*, *Shigella* sp.,
Salmonella sp. has been recognized. This result revealed that the effect of haemolymph was different for each bacterial strain, showing that the mortality of Gram-positive bacteria is significantly higher than that of Gram-negative bacteria, and this is in accordance with most of the screening studies of antibacterial activity conducted across many invertebrate phyla, including molluscs (Casas et al. 2011).

In recent years, great attention has been paid to the study of antimicrobial activity from marine organisms because of its pharmacological potential. Many of these organisms are molluscs such as oyster (Crassostrea virginica), mussels (Mytilus edulis and Geukensia demissa), muricid snail (Dicathais orbita), sea hare (Dolabella auricularia) and cuttlefish (Sepia officinalis) (Gunthorpe and Cameron 1987; Anderson and Beaven 2001; Benkendorff et al. 2001; Johnston et al. 2001). In most of these species the haemolymph, egg masses (Laurencin et al. 2012) or the whole body (Ramasamy et al. 2011) have been tested for antimicrobial activity.

Unlike previous studies conducted with cephalopods, where antimicrobial activity was detected in the cuttlebone and in the methanolic extract of body tissues of some species of cuttlefish and octopus (Ramasamy et al. 2011), we evaluated the antimicrobial activity from *O. vulgaris* haemocyte crude methanolic acid extracts. We assayed the antimicrobial activity against a selected Gram-negative human pathogen (*E. coli*). The results show that a very low concentration of this extract (0.8 μg/ml) was able to completely inhibit bacterial growth. *Octopus vulgaris* haemocytes are clearly involved in the specific host immune response against bacterial infection.

In conclusion, the characterization of the immune system of *O. vulgaris* is necessary to further assess responses to environmental, anthropogenic and pathological stresses, and, no less important, to identify the best practice of husbandry in relation to the ethical principles defined in Directive 2010/63/EU and in new welfare laws for cephalopods expected to be implemented next year. Furthermore, the state of knowledge on how microorganisms affect *O. vulgaris* biology is deficient (Oestmann et al. 1997; Shaw et al. 1999; Di Cosmo 2012). The identification of bioactive compounds could lead to many applications in fields such as ecology (sexual pheromones), fisheries, animal feeding (antimicrobial peptides in pet food and aquaculture pellets), and health care (antimicrobial peptides).

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