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RESEARCH REPORT

The hemocytes of *Polyandrocarpa mysakiensis*: morphology and immune-related activities

L Ballarin[1], K Kawamura[2]

[1]*Department of Biology, University of Padua, Padua, Italy*
[2]*Laboratory of Cellular and Molecular Biotechnology, Faculty of Science, Kochi University, Japan*

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Abstract

A preliminary study of the hemocytes of developing buds of the compound ascidian *Polyandrocarpa misakiensis* was carried out at the light microscope level for a better understanding of their biological role. Similarly to other ascidians, *P. misakiensis* immunocytes are represented by phagocytes and morula cells. Phagocytes include hyaline amoebocytes and round, giant phagocytes, the former the probable precursors of the latter. Hyaline amoebocytes showed high macropinocytotic activity in the presence of bacteria, whereas yeast cells were ingested by phagocytosis. Morula cells contain the enzyme phenoloxidase inside their vacuoles, probably stored as pro-enzyme, which is released upon the recognition of non-self. Together with macrogranular leukocytes, morula cells were the most abundant hemocyte-types which stresses the importance of these cells in *Polyandrocarpa* biology. Macrogranular leukocytes are frequently found inside the vacuoles of phagocytes and were recognized by a polyclonal antibody raised against an opsonin purified from the colonial ascidian *Botryllus schlosseri*, which suggests that a similar lectin can be involved in the interaction between these cells and phagocytes.

Key Words: *Polyandrocarpa misakiensis*; colonial ascidians; hemocytes; morphology; immunity

Introduction

Invertebrate chordates or protochordates are represented by cephalochordates and tunicates, the latter being the sister group of vertebrates (Delsuc *et al*., 2006). The peculiar phylogenetic position of tunicates explains the increasing interest towards their biology, in particular, developmental biology and immunobiology. The majority of tunicates are represented by ascidians, sessile filter-feeding marine animals which include both solitary and colonial species.

Many types of hemocytes are found in ascidians. Their morphology has been described by many authors (Pérès, 1943; Endean, 1955; Sabbadin, 1955; Andrew, 1961; Overton, 1966; Smith, 1970a, b; Milanesi and Burighel, 1978; Scippa *et al*., 1982; Burighel *et al*., 1983; Schlumpberger *et al*., 1984; Sawada *et al*., 1991, 1993; Zhang *et al*., 1992; Azumi *et al*., 1993; Dan-Sohkawa *et al*., 1995; Arizza and Parrinello, 2009) and various classification criteria have been proposed (Goodbody, 1974; Wright, 1981; Rowley *et al*., 1984; De Leo, 1992; Burighel and Cloney, 1997). However, uncertainties still exist on their functions, mutual relationships and differentiation pathways.

Polyandrocarpa misakiensis (Fig. 1) is a polystyelid compound ascidian, common along the coasts of the temperate regions of Japan, which can reproduce asexually through continuous budding from parental zooids (Kawamura and Fujiwara, 1994; Kawamura *et al*., 2008). The morphological, biochemical and molecular events occurring during bud differentiation and maturation have been widely studied (Kawamura and Fujiwara, 1994; Hisata *et al*., 1998; Kawamura and Sugino, 1999; Kamimura *et al*., 2000; Matsumoto *et al*., 2001; Sunanaga *et al*., 2007; Kawamura *et al*., 2006, 2008). Nevertheless, few data are available on *Polyandrocarpa* hemocytes: their morphology has been studied at the electron microscope, but scanty data are available for light microscopy. In addition, the abundance of the different hemocyte types and their possible roles in immune defense have been little investigated.

In order to fill this gap, we carried out a preliminary investigation aimed to a better characterization of *Polyandrocarpa* hemocytes at the

Corresponding author:
Loriano Ballarin
Department of Biology
University of Padua
Via U. Bassi 58/B, 35100 Padua, Italy
Email: loriano.ballarin@unipd.it

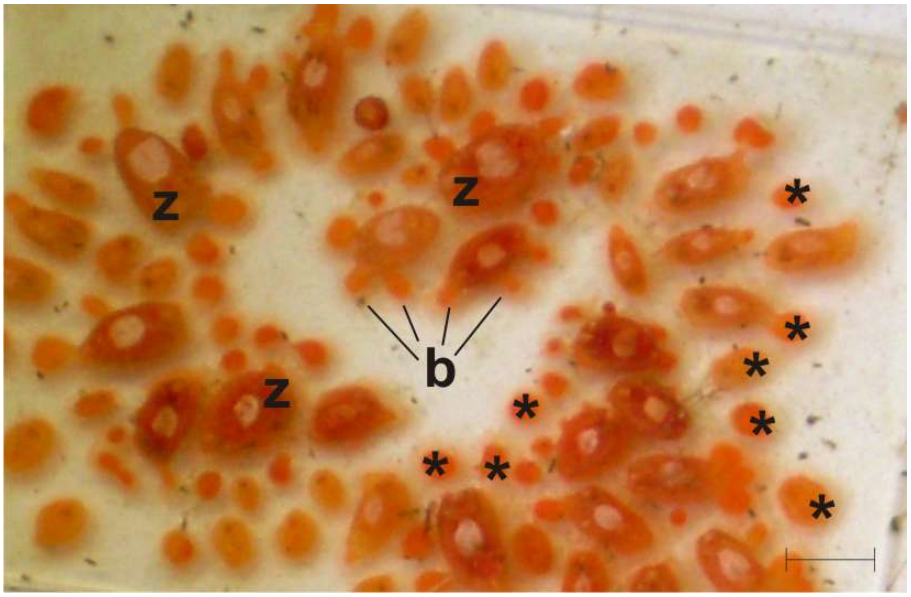

Fig. 1 Colony of *P. misakiensis*. Adult zooids (**z**) bear many buds (**b**). Developing buds are indicated by asterisks. Bar = 0.5 mm.

light microscope, with particular reference to immunocytes, a well-defined class of circulating hemocytes responsible of both cellular and humoral (through their secretions) immune responses. Results confirm the presence of phagocytes, able to quickly ingest foreign particles, through phagocytosis and macropinocytosis, and morula cells which, like many other compound ascidians, are probably involved in cytotoxic immune reactions. In addition, granular leukocytes, which are circulating trophocytes, are recognized by a polyclonal antibody, raised against a lectinic opsonin from *Botryllus schlosseri*, which represents a good and specific marker for this cell type.

Materials and methods

Animals

Colonies of *Polyandrocarpa misakiensis* were reared in the field, attached to glass plates, near the USA Marine Biological Institute, Kochi University, Japan. When required, they were brought in the laboratory of Cellular and Molecular Biotechnology of the Faculty of Science and kept at room temperature for few days before their use in a 20-l aerated aquarium filled with seawater.

Hemocyte collection and culture

Hemolymph was collected from developing buds (Fig. 1) of colonies, previously immersed for few min in 0.38 % Na-citrate in artificial seawater (ASW), pH 7.5, in order to prevent cell clotting and then blotted dry. Buds, already detached from the parent zooid, were punctured with a fine tungsten needle and hemolymph was collected with a glass micropipette and centrifuged at 700x*g* for 10 min at 4 °C; the pellet was re-suspended in ASW to a final concentration of 10^6 cells/ml. Fifty μl of hemocyte suspension were placed in the center of a glass coverslip, previously coated with poly-L-lysine (50 μg/ml), and left to adhere, for 60 min, at room temperature (RT) in a moist chamber. Hemocytes were then observed under the light microscope or, alternatively, fixed in 1 % glutaraldehyde in ASW containing 1 % sucrose, washed in phosphate-buffered saline (PBS: 8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 1.15 g/l, pH 7.4) and stained for 5 min in 10 % Giemsa's solution. Coverslips were then mounted on microscope slides with 80 % glycerol. Hemocytes from at least 10 different colonies were observed and counted.

Blood plasma and hemocyte lysate preparation

Freshly collected hemolymph was centrifuged at 700x*g* for 10 min at 4 °C. The resulting supernatant was referred to as blood plasma (BP), whereas the pellet was re-suspended in an equal volume of distilled water, subjected to sonication for 20 s at 0 °C in a Braun Labsonic U sonifier at 50 % duty cycles and centrifuged at 10,000x*g* for 10 min in order to get hemocytes lysate (HL) as supernatant.

Phagocytosis and macropinocytosis assays

After adhesion, hemocytes were incubated at RT, in a moist chamber, with 50 μl of a yeast (*Saccharomyces cerevisiae*) suspension in ASW (yeast/hemocyte ratio = 10:1) for 60 min, and the uningested yeast was then removed by dipping the coverslips repeatedly in a large volume of ASW. Living hemocytes were then observed under the light microscope. Alternatively, monolayers were fixed and stained as described above before their observation.

In another series of experiments, hemocytes were incubated for 60 min with a suspension of living *Escherichia coli* in ASW (10^9 cells/ml).

Coverslips were then washed by repeated dipping in ASW and cells were fixed and stained as described above before their observation under the light microscope.

Immunocytochemical analysis

Fixed hemocytes were incubated for 30 min in 1 % H_2O_2 to block endogenous peroxidase activity, washed in PBS, treated with 2 % powdered milk for 30 min and then incubated for 1 h with 50 μg/ml of purified polyclonal antibody raised against *B. schlosseri* rhamnose-binding lectin (BsRBL; Ballarin *et al*., 2000; Gasparini *et al*., 2008) in a moist chamber; pre-immune serum was used in controls. After washing in PBS containing 0.1 % Tween 20, they were incubated for 30 min in 1 μg/ml of horseradish peroxidase-labelled mouse anti-rabbit secondary antibody (Vector Laboratories), washed in distilled water and treated with True Blue (KPL), which stains positive sites blue, according to the manufacturer's instructions.

Assay for phenoloxidase (PO) activity on hemolymph, BP, HL and hemocyte incubation medium

Twenty μl of hemolymph, collected as described above, BP or HL were added to 180 μl of a saturated solution of dihydroxyphenyl-L-alanine (L-DOPA) in PBS in the wells of flat bottomed, 96-well microtiter plates and the time course of the reaction was read at 490 nm for 5 min with a BioRad iMark microplate reader. Five mM Na_2SO_3, a PO inhibitor (Kong *et al*., 2000; Cong *et al*., 2005), were added to the hemolymph in negative controls. One relative unit (RU) of PO activity was defined as the increase in absorption of 0.001/min in the reaction mixture (Söderhäll and Smith, 1983). Protein concentrations of the supernatants were determined according to Lowry *et al*. (1951) and results were expressed as RU/mg protein.

In another series of experiments, 100 μl of hemocytes suspension (10^6 cells/ml) were incubated for 60 min in ASW or, alternatively in yeast-containing ASW (yeast:hemocyte ratio = 10:1). The supernatants were then collected by centrifugation at 700x*g* at 4 °C and assayed for PO activity as described above.

Cytoenzymatic assay for PO activity

After fixation, hemocytes were incubated for 1h in saturated dihydroxy-L-phenylalanine (L-DOPA) solution in PBS in the presence or in the absence of 5 mM Na_2SO_3, washed in distilled water, and mounted in Acquovitrex. Positive cells converted L-DOPA to dopachrome and stained blackish-brown.

Statistical analysis

Each experiments was repeated at least three times. PO activity data were compared with the Student's *t* test.

Results

The hemocytes of P. misakiensis

The following main cell-types could be recognized: i) undifferentiated cells, 4-6 μm in diameter, with a high nucleus-cytoplasm ratio. They amounted to 4.4 ± 0.7 % of circulating cells (Figs 2a, b); ii) hyaline amoebocytes, 6-12 μm in length, have a variable shape, homogeneous cytoplasm with various cytoplasmic protrusions (pseudopods), and a roundish nucleus (Figs 2c, d). They represent 4.4 ± 0.4 % of the hemocytes; iii) round phagocytes, or macrophage-like cells, 8.1 ± 2.9 % of the hemocytes. They have a spheroidal shape, 10-15 μm in diameter; their cytoplasm can be stained metachromatically by Giemsa's dye, and shows one or few large vacuole(s) containing ingested material which occupy most of the cell volume (Figs 2h-k); iv) microgranular leukocytes, 15.3 ± 3.5 % of the total hemocytes, 4-6 μm in diameter. They frequently show an amoeboid form and are characterized by the presence of small granules in their cytoplasm which frequently assume a metachromatic red color with Giemsa's dye (Fig. 2e); v) macrogranular (granular) leukocytes, 10-15 μm in size, one of the most abundant circulating cell type in *Polyandrocarpa*, representing 33.9 ± 3.1 % of the hemocytes. They are giant round cells with the nucleus usually found at the periphery of the cell and the cytoplasm filled with many granules, of variable size (up to 2-3 μm in diameter), which appear translucent in living cells and almost empty after fixation (Figs 2i, l, m); vi) morula cells (MCs), 10-15 μm in diameter, 32.9 ± 4.4 % of the circulating cells. They are also round cells characterized by the presence of many vacuoles, variable in size, which appear yellowish in living cells and acquire a green color after aldehyde fixation (Figs 2i, q) and vii) pigment cells,1.2 ± 0.2 % of the total hemocytes number, 10-15 μm in size. They are characterized by the presence of many small vacuoles filled with red pigment and a nucleus located at the periphery of the cell. Their morphology is rarely preserved in fixed samples (Fig. 2l).

Polyandrocarpa phagocytes behave differently in the presence of yeast and bacteria

When hemocytes were incubated in the presence of yeast, phagocytes, mainly round cells, filled with yeast cells were frequently found after 60 min of incubation (Figs 2h, k). No changes in MC morphology were observed. Conversely, in the presence of bacteria most of hyaline amoebocytes showed heterogeneous macropinocytotic vesicles inside their cytoplasm which appeared empty under the light microscope (Fig. 2f). Most of the microbes resulted agglutinated outside the cells (Fig. 2f) and in few cases they were visible inside phagocytes (Fig. 2g).

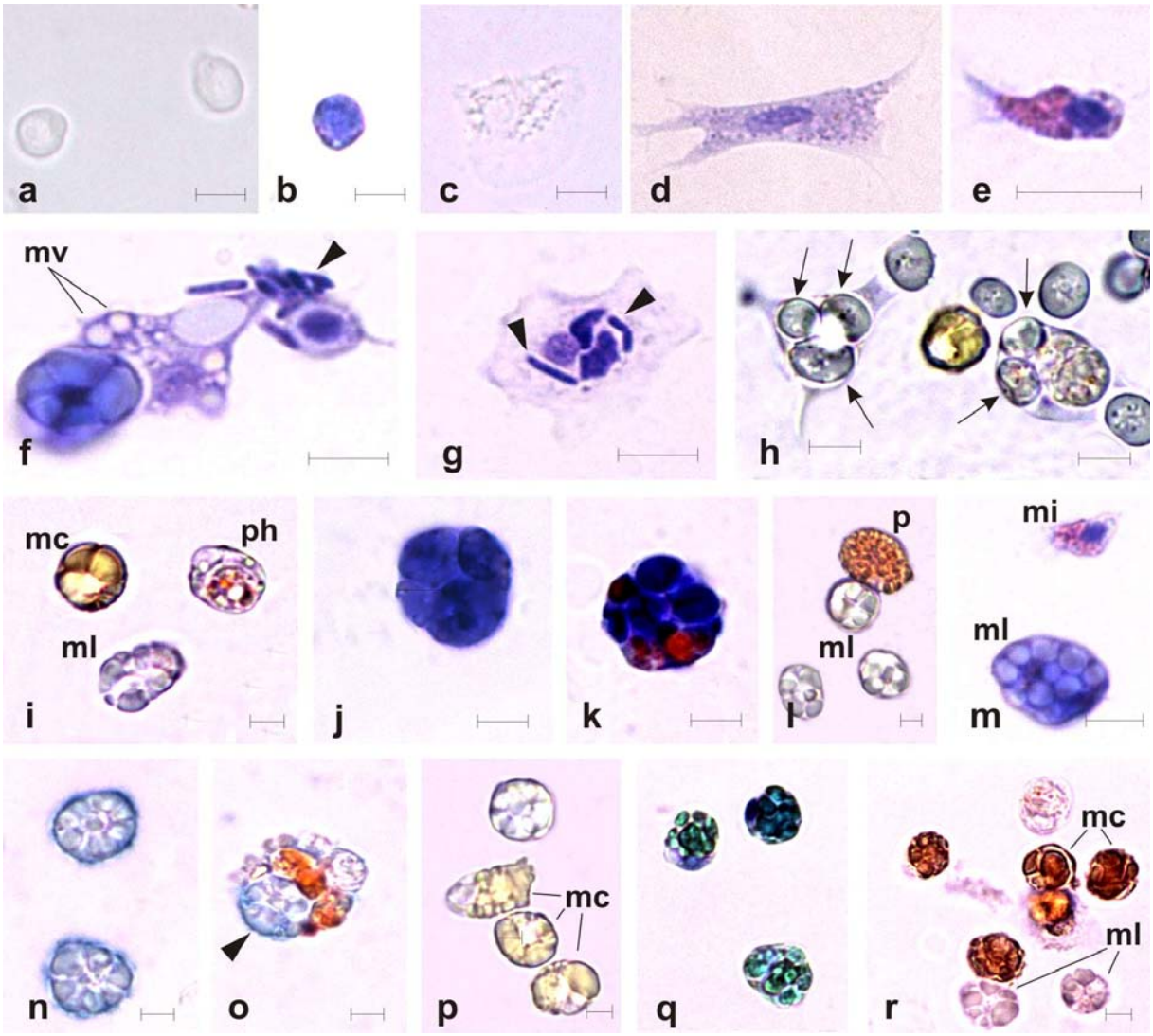

Fig. 2 Hemocytes of *P. misakiensis*. **a**, **b**: living (**a**) and Giemsa-stained (**b**) undifferentiated cells. **c**, **d**: living (**c**) and stained (**d**) hyaline amebocytes. **e**: microgranular leukocyte fixed and stained with Giemsa. **f**: fixed and stained hyaline amebocyte after exposure to *E. coli*; many micropinocytotic vesicles (**mv**) are visible as well as agglutinated bacteria (**arrowhead**) outside the cell. **g**: fixed and stained hyaline amebocyte with ingested bacteria (**arrowheads**). **h**: living phagocytes with ingested yeast cells (**arrows**). **i**: living round phagocyte (**ph**), macrogranular leukocyte (**ml**) and morula cell (**mc**). **j**, **k**: fixed and stained round phagocytes with vacuoles containing ingested materials (yeast cells in **k**). **l**: living macrogranular leukocytes (**ml**) and pigment cell (**p**). **m**: fixed and stained macrogranular (**ml**) and microgranular (**mi**) leukocytes. **n**: fixed macrogranular leukocytes showing immunopositivity to anti-BsRBL antibody on their surface. **o**: round phagocyte having ingested a immunopositive to anti-BsRBL antibody (**arrowhead**). **p**: living macrogranular leukocyte (**ml**) and morula cells (**mc**). **q**: aldehyde-fixed (unstained) morula cells: vacuoles assume a green color. **r**: fixed hemocytes treated with L-DOPA: stain for dopachrome production is evident in morula cells (**mc**) but not in macrogranular leukocytes (**ml**). Bar = 5 µm.

Macrogranular leukocytes are recognized by the anti-BsRBL antibody

The anti-BsRBL antibody recognized specifically the surface of macrogranular leukocytes (Fig. 2n). In developing buds, these cells are frequently found inside phagocyte vacuoles and, in some cases, we could observe labeled cells inside round phagocytes (Fig. 2o).

PO activity is located in MCs

When the PO activity of whole hemolymph (WH) and BP were compared, the former resulted more than three times higher than the latter, suggesting that the majority of the enzyme was present, in normal conditions, inside the hemocytes. This was confirmed by the higher enzyme activity (3 times that of the hemolymph) of HL. The addition of

Table 1 PO activity of whole hemolymph (WH), blood plasma (BP) and hemocyte lysate (HL)

PO source	PO activity (RU/mg protein)
WH	1239.2 ± 53.1
WH + 5mM Na_2SO_3	0.7 ± 0.2 ***
BP	374.3 ± 40.4 ***
HL	3720.5 ± 99.3 ***

*** $p < 0.001$ with respect to WH

Na_2SO_3 to the hemolymph completely abolished the oxidation of L-DOPA (Table 1).

After 60 min of incubation of hemocytes in ASW, the PO activity of the culture medium amounted to 29.8 ± 4.0 RU/mg protein. Conversely, when blood cells were incubated in a suspension of yeast in ASW, the resulting enzyme activity of the medium was significantly ($p < 0.001$) increased and reached the value of 63.3 ± 10.9 RU/mg protein.

Cytoenzymatic analysis in the presence of L-DOPA clearly showed that the only MCs, and no other cells type, were labeled in the presence of L-DOPA (Fig. 2r).

Discussion

Colonies of the ascidian *P. misakiensis* continuously form new buds as outgrowths of the parental body which soon separate so that morphogenesis occurs without any contact with the parental organism. For these reasons, this species is considered an excellent model organism for the study of stem cell differentiation during asexual reproduction and regeneration (Kawamura and Fujiwara, 1994; Hisata *et al*., 1998; Kawamura and Sugino, 1999; Kamimura *et al*., 2000; Matsumoto *et al*., 2001; Sunanaga *et al*., 2007; Kawamura *et al*., 2006, 2008).

Hemocytes have been claimed to take part in *Polyandrocarpa* development to adulthood as both a source of undifferentiated cells (Kawamura *et al*., 1991, 2008) and a reservoir of nutrients required for the completion of bud morphogenesis when the young individuals are not yet feeding (Kawamura and Nakauchi, 1986; Kawamura *et al*., 1991, 1992). However, despite their importance, there are few data in the literature describing *Polyandrocarpa* blood cells, their abundance and behavior (Kawamura *et al*., 1992; Sugino *et al*., 1993). In the present work, we carried out a light microscope morphological study on *P. misakiensis* hemocytes as a further contribution for better understanding the biological roles of these cells.

Undifferentiated hemocytes represent less than 5 % of the circulating cells, in agreement with what found in other compound ascidians (Cima *et al*., 2001; Ballarin *et al*., 2005). The presence of circulating undifferentiated cells, or hemoblasts, involved in asexual reproduction is a common feature of colonial ascidians. Analogously to what described in stolonal budding of *Perophora* (Freeman, 1964) and in vascular budding of botryllid (Oka and Watanabe, 1957, 1959; Sabbadin *et al*., 1975; Rinkevich *et al*., 1995; Rinkevich *et al*., 2007; Voskoboynik *et al*., 2007), these cells exert a fundamental role in *Polyandrocarpa* bud morphogenesis (Kawamura and Nakauchi, 1991; Kawamura *et al*., 1991).

Hyaline amebocytes and round phagocytes have been previously included in the same category of hyaline leukocytes, involved in phagocytosis (Sugino *et al*., 1993). Indeed, like in botryllid ascidians, they are probably different morphs of a single phagocyte type which can actively move towards foreign cells or particles by ameboid progression and, upon the ingestion of non-self material, withdraws its cytoplasmic projections assuming a globular shape (Cima *et al*., 2001; Ballarin and Cima, 2005). Hyaline amebocytes of the compound ascidian *Botryllus schlosseri* are capable of constitutive macropinocytosis (Ballarin and Burighel, 2006): the same process is probably responsible of the abundance of hollow vesicles observed in the cytoplasm of *Polyandrocarpa* hyaline leukocytes (Sugino *et al*., 1993). A clear macropinocytotic activity was observed in the presence of bacteria, in accordance with the general view of macropinocytosis as a process generally responsible of the ingestion of bacteria and necrotic material (Krysko *et al*., 2003). The presence of agglutinated bacteria outside the cells suggests the release of agglutinins, likely lectins, by activated phagocytes. A similar agglutinating ability towards some bacterial strains have been recently demonstrated for BsRBL (unpublished data). Conversely, no increase in macropinocytotic activity was observed when yeast cells were used as foreign particles: in this case, analogously to what described in other colonial ascidians (Ballarin *et al*., 1994), phagocytosis occurred and phagocytes turned to large, round cells filled with yeast-containing vacuoles.

MCs represent one of the most abundant circulating hemocyte types. In botryllid ascidians, their frequency ranges from 20 to 60 % (Ballarin, 2008) and they are important mediators of the response to non-self as both effectors of allorejection between contacting, genetically incompatible colonies (Hirose *et al*., 1990; Ballarin *et al*., 1995, 1998; Rinkevich *et al*., 1998; Shirae *et*

al., 1999, 2002; Cima *et al*., 2004), and sites of synthesis of cytokine(s), able to influence phagocyte activity, in response to non-self recognition (Ballarin *et al*., 2001; Menin *et al*., 2005, Menin and Ballarin, 2008). In *B. schlosseri*, one of the first event consequent to the recognition of foreign molecules by MCs is their degranulation and the release of their vacuolar content, mainly PO and its polyphenol substrates. Like botryllid ascidians, in *Polyandrocarpa*, PO is contained inside hemocytes, probably as a precursor which can be readily activated by cell manipulation (Ballarin *et al*., 1998), and MCs are the only cell type showing the enzyme activity. In addition, the enzyme can be released in the medium upon the recognition of foreign cells such as yeast cells. Botryllid PO is involved in the induction of cytotoxicity consequent to the recognition of non-self in both allorejection (Ballarin *et al*., 1995, 1998; Shirae *et al*., 2002) and responses towards foreign cells or particles (Ballarin *et al*., 1998, 2005): as no allorecognition phenomena are known in *Polyandrocarpa*, we can hypothesize that, in this species, MCs exert a general immunosurveillance role, being capable to induce cytotoxicity through the release of their vacuolar content, both PO (present data) and peroxidase (Kawamura *et al*., 1992), upon the recognition of non-self, even if no apparent signs of degranulation are visible in blood smears.

Macrogranular leukocytes constitute the other most common circulating cell-type in *Polyandrocarpa* developing buds. Their abundance suggests an important role for these cells and, indeed, there is a general agreement on the fact that they are trophocytes, involved in the uptake and storage of nutrients useful for bud morphogenesis (Kawamura and Nakauchi, 1986; Kawamura *et al*., 1992); the alkaline phosphatase activity associated with their plasma membrane could be involved in this process (Kawamura *et al*., 1992). This is in agreement with the observation that, in developing buds, they are frequently found inside phagocytes. The antibody raised against BsRBL recognized specifically the surface of macrogranular leukocytes suggesting the presence of molecules sharing some degree of similarity with BsRBL. A lectin with galactose specificity has already been demonstrated in these cells (Kawamura *et al*., 1991). Since the lectin from *B. schlosseri* has been demonstrated to exert an opsonic role through the coating of foreign cells before their phagocytosis and immunopositive macrogranular leukocytes are sometimes present in vacuoles of round phagocytes, we can hypothesize: i) that the molecule recognized by the anti-BsRBL antibody can have a role in the recognition of trophocytes by phagocytes and ii) that in *Polyandrocarpa* asexual development, like in *Botryllus* take-over (Lauzon *et al*., 2002; Cima *et al*., 2003, 2009; Ballarin *et al*., 2008), phagocytosis is the main process which render available nutrients stored in trophocytes and dying cells, respectively.

Collectively, our results indicate or suggest some possible biological roles for *Polyandrocarpa* hemocytes. However, many questions remain unanswered, such as the molecular mechanism of trophocyte recognition by phagocytes in order to render nutrients available to developing buds, or the role of MC and PO in a species where allorecognition phenomena are not known, or the influence of MC on phagocyte activity. Future research will help in clarifying the above points.

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