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RESEARCH REPORT

Insights on cytotoxic cells of the colonial ascidian *Botryllus schlosseri*

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Abstract

Morula cells (MCs) represent the most abundant circulating hemocyte of the compound ascidian *Botryllus schlosseri*. They are cytotoxic cells involved in the rejection reaction between contacting, genetically incompatible colonies. Upon the recognition of foreign substances, they degranulate and release their content, which contribute to the cell death along the contact borders. A major role in MC-related cytotoxicity is exerted by the enzyme phenoloxidase (PO) that converts polyphenol substrata to quinones which, then, polymerize to form melanins. During this reaction, reactive oxygen species are formed which are the cause of MC-related cytotoxicity. Here, we carried out new analyses to investigate further the nature of MC content and its role in cytotoxicity. Results confirm that PO is located inside MC vacuoles together with arylsulfatase, iron and polyphenols/quinones, the latter probably representing ready-to-use cytotoxic molecules, deriving from the oxidation of DOPA-containing proteins. In addition, small DOPA-containing peptides, called tunichromes, are also present inside MCs. MC degranulation and PO-mediated cytotoxicity are prevented by secretion inhibitors and by H89 and calphostin C. The observation that PO activity is always detectable in MCs in the absence of protease treatment, and its inhibition by sulfites and sulfates, suggest a non-classical pathway of PO modulation in botryllid ascidians.

Key Words: *Botryllus*; ascidians; morula cells; phenoloxidase; immune response; cytotoxicity

Introduction

Phenoloxidase-containing cells represent an ubiquitous hemocyte-type in ascidians, constituting one of the most abundant circulating cell-type, featuring the presence of cytoplasmic vacuoles containing the enzyme phenoloxidase (PO) in an inactive form. These cells are involved in various biological processes, such as wound healing, tunic synthesis and cytotoxicity (Goodbody, 1974; Wright, 1981; Oltz *et al*., 1987; Cammarata *et al*. 1997; Ballarin and Cima, 2005). In many ascidian species, phenoloxidase-containing cells are known as morula cells (MCs), for the berry-like morphology they assume after fixation (see Ballarin, 2012 for references). According to various authors (Smith and Söderhäll, 1991; Jackson *et al*., 1993; Arizza *et al.,* 1995; Ballarin *et al*., 1998; Shirae and Saito, 2000; Shirae *et al*., 2002; Parrinello *et al.,* 2003; Cammarata *et al.,* 2008), PO is stored, analogously to arthropods (Söderhäll and Cerenius, 1998), as a pro-enzyme, although a clear demonstration is still lacking (Ballarin, 2012).

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In the cosmopolitan compound ascidian *Botryllus schlosseri*, colony specificity allows the discrimination between contacting genetically compatible and incompatible colonies. In the case of compatibility, colonies can fuse into a large chimeric colony with common tunic and circulation, whereas, when incompatible colonies are involved, they give rise to a non-fusion or rejection reaction characterized by the appearance of a series of necrotic foci along the contact area (Rinkevich, 1992; Sabbadin *et al.,* 1992; Saito *et al.,* 1994). The non-fusion reaction is characterized by: i) partial fusion of the tunics along the contact borders, ii) crowding of a selected immunocyte population, represented by MCs, inside the lumen of the facing, sausage-like blind termini of the colonial vasculature, called ampullae, iii) fenestration of the apical ampullar epithelium and its crossing by MCs which degranulate and induce the observed cytotoxicity (Sabbadin *et al.,* 1992; Ballarin *et al.,* 1995, 1998).

MCs are the effectors of the non-fusion reaction of botryllid ascidians and, once activated by the recognition of soluble unknown factors diffusing from the facing colony, they synthesize and release chemotactic molecules recognized by anti-mammalian-cytokine antibodies (Cima *et al.,* 2006).

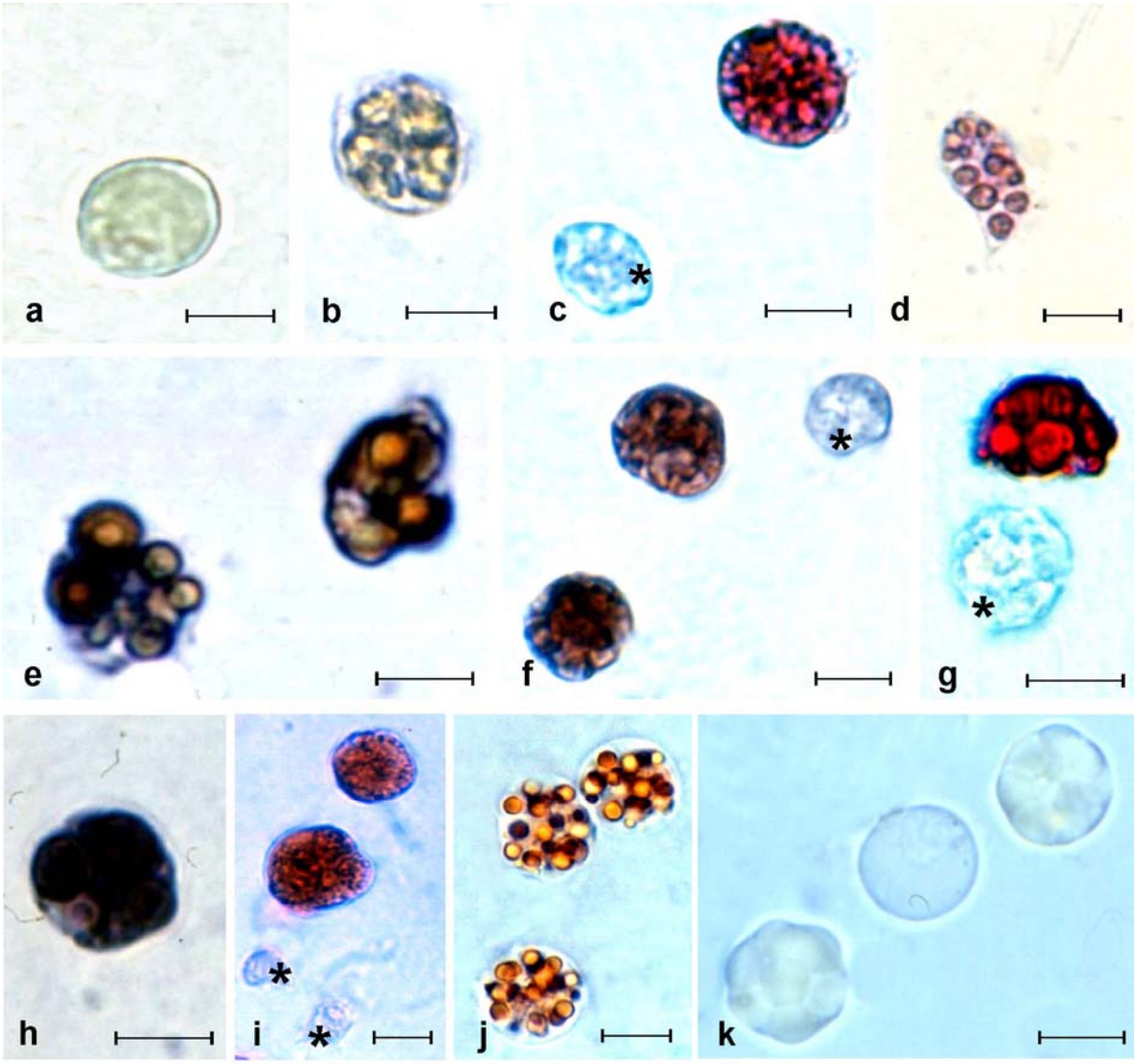

Fig. 1 MC under various experimental conditions. **a**: living cell; **b**: untreated, aldehyde-fixed cell; **c**: after eosin staining; **d**: after May-Grünwald-Giemsa staining; **e**: stained for arylsulfatase; **f**: stained for PO; **g**: MBTH-stained cells: **h**: redox-cycling stain for quinoproteins/DOPA-containing proteins; **i**: immunopositivity to anti-tunichrome antibody; **j**: living cells degranulated in the presence of heterologous CFH; **k**: living cells in the presence of incompatible CFH and monensin. Asterisks: unstained phagocytes. Scale bar: 10 µm.

As previously stated, they contain the enzyme PO inside their vacuoles (Frizzo *et al.,* 2000) which is responsible of the cytotoxicity observed in the rejection reaction (Ballarin *et al.,* 1998, 2002; Shirae and Saito, 2000; Shirae *et al.,* 2002). In addition, simple cytochemical techniques suggest the presence, inside MC vacuoles, of PO polyphenol substrata (Ballarin *et al.,* 1995; Ballarin and Cima, 2005), but the nature of these substances remains largely unknown.

In the present paper, we report the results of new analyses on MCs to investigate further the nature of the vacuolar content. Results obtained confirm that PO is located inside MC vacuoles together with the enzyme arylsulfatase, iron, polyphenols/quinones and DOPA-containing proteins/quinoproteins from which DOPA-containing peptides, also present inside MC vacuoles and called tunichromes (Oltz *et al.,* 1987; Taylor *et al.,* 1997), probably derive. The enzyme activity is inhibited by sulfites and sulfates; PO is released upon MC degranulation as inhibition of exocytosis prevents the PO-related cytotoxicity.

Materials and Methods

Animals

Colonies of *Botryllus schlosseri* from the Lagoon of Venice were used. They were kept in aerated aquaria, attached to glass slides and fed with Liquifry marine (Liquifry Co., Dorking, England) and baker's yeast (*Saccharomyces cerevisiae*).

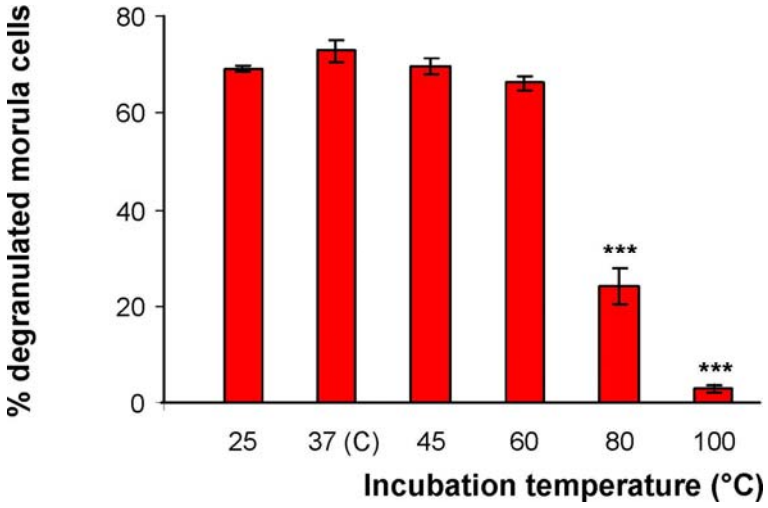

Fig. 2 Percentage of degranulated MCs at various incubation temperatures. Asterisks mark significant differences with respect to the exposure in FSW or, when indicated, between different temperatures. **: $p < 0.01$; ***: $p < 0.001$.

Hemocyte collection

Hemolymph was collected with a glass micropipette after puncturing, with a fine tungsten needle, the tunic marginal vessel of colonies previously rinsed in 0.38 % Na-citrate in filtered seawater (FSW), pH 7.5, to prevent clotting. It was then centrifuged at 780x*g* for 10 min and the pellet was re-suspended in FSW at the final concentration of $5x10^6$ cells/ml. Sixty µl of hemocyte suspension were placed in the centre of culture chambers, prepared as described elsewhere (Ballarin *et al*., 1994), and left to adhere to coverslips for 30 min at room temperature.

Cytochemical and immunocytochemical assays on hemocytes

Staining with eosin and May Grünwald-Giemsa

Hemocyte monolayers were fixed for 30 min at 4 °C with a solution of 1 % glutaraldehyde, 1 % sucrose and 1 % caffeine, to prevent the release of the vacuolar content (Ballarin and Cima, 2005), in FSW. They were then stained with 2 % eosin for 5 min and rinsed in phosphate-buffered saline (PBS: 0.8 % NaCl, 0.02 % KCl, 0.02 % KH_2PO_4, 0.115 % Na_2HPO_4, pH 7.2). Alternatively, hemocytes were stained with commercial May Grünwald solution (Merck) for 2 min, followed by 5 min in 10 % Giemsa solution in distilled water. Coverslips were finally mounted on glass slides with aqueous medium (Acquovitrex, Carlo Erba, Italy) and observed under the light microscope (Leitz, Dialux 22) at the magnification of 1250x.

Arylsulfatase

Fixed hemocyte monolayers were immersed in 0.1 M sodium acetate buffer (AB) containing 1 % sucrose, pH 5.2, for 5 min. Cells were then incubated for 60 min at 37 °C in a reaction mixture prepared by adding 8 mg/ml of p-nitrocatechol sulfate to a solution made by AB, water and a 8 % lead nitrate in volumetric ratio of 6:2:2 (Goldfischer, 1965). After thorough washing in distilled water, hemocytes were treated with 1 % ammonium sulfide for 1 min and washed repeatedly in water. Coverslips were finally mounted as described before and observed under the light microscope. Positive sites appeared dark.

Phenoloxidase (PO)

Fixed hemocyte were incubated for 60 min in a saturated solution of L-DOPA (Fluka) or 2 mM catechol in PBS. After a final washing in PBS, coverslips were mounted on glass slides with Acquovitrex. Positive sites appeared dark brown (Hose *et al*., 1987).

Polyphenols/quinones

Living hemocytes were incubated in a 2-mM solution of 3-methyl-2-benzothiazolinone hydrazone chloride (MBTH) in FSW, containing 0.4 % dimethylformamide for 5 min. The compound reacts with polyphenols and quinones giving a red product (Gasparič *et al*., 1977; Winder and Harris, 1991; Ballarin *et al*., 1995). Cells were then washed in FSW and coverslips were placed, upside down, on a teflon ring (15 mm internal diameter, 1 mm thick), glued on a siliconized glass slide, smeared with vaseline and observed under the light microscope.

DOPA-containing proteins/quinoproteins

After the adhesion to coverslips, hemocytes were fixed as described above, washed in PBS and incubated in a solution of 0.24 mM nitroblue tetrazolium (NBT; Sigma) in 2-M potassium glycinate buffer (15 % glycine and KOH 2 M to pH 10.0) and 20 mM sodium benzoate (Flückiger *et al*., 1995). Cells were then washed in PBS and coverslips mounted with Acquovitrex. Positive sites featured a dark blue color.

Tunichrome

Hemocytes were fixed for 30 min in a solution of 4 % paraformaldehyde and 0.1 % glutaraldehyde in 0.4 M sodium cacodylate buffer, pH 7.4, containing 1 % caffeine. They were then washed in PBS, incubated 30 min in 3 % H_2O_2 in methanol to block endogenous peroxidase activity, washed again, and kept 30 min in a solution of 3 % powdered milk in PBS. Cells were then incubated overnight in a rabbit polyclonal antibody raised against the tunichrome of the solitary ascidian *Phallusia mamillata* (Bayer *et al*., 1992; Taylor *et al*., 1997), a generous gift of Prof. S. Scippa, University of Naples, diluted 1/200 in PBS, washed and incubated in secondary, anti-rabbit IgG antibody conjugated with biotin (Calbiochem), diluted 1/100 according to the manufacturer's instructions, for 30 min. Hemocyte monolayers were washed again, and treated with avidin-biotin-peroxidase complex (ABC, Vector) for 30 min. Positivity was revealed by incubation, for 5 min, in a solution of 0.025 % 3-3' diaminobenzidine (DAB, Sigma) in PBS. The primary antibody was omitted in control slides. After a final washing, coverslips were mounted on glass slides with Acquovitrex.

Evaluation of the cytotoxic effect of cell-free hemolymph (CFH) under various conditions

Hemolymph was collected as described above, from colonies which were previously blotted dry with filter paper, and centrifuged at 780x*g* for 10 min. The supernatant was then collected, referred to as CFH and used as incubation medium for both autologous (*i.e.,* from the same colony) and heterologous (*i.e.,* from incompatible colonies) hemocytes, prepared as described above, in the presence or in the absence of the secretion inhibitors 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS; Korchak *et al*., 1980) and monensin (Kuivaniemi *et al*. 1986), at the concentration of 1 mM and 1 µM, respectively, and of H-89 (10 µM) and calphostin C (1 µM), specific inhibitors of protein kinase A (PKA; Chijwa *et al*., 1990) and protein-kinase C (PKC; Tamaoki, 1991), respectively. After 60 min of incubation, cells were washed with FSW, incubated in a solution of 0.25 % Trypan Blue in FSW for 5 min at room temperature and observed *in vivo* under a Leitz Dialux 22 light microscope (LM) at 1250x. Dying hemocytes, with altered plasma membrane permeability, assumed a blue color. The percentage of blue cells was expressed as the cytotoxicity index. In another series of experiments, heterologous CFH was incubated for 30 min at 37, 45, 60, 80 °C and for 10 min at 100 °C before its use as incubation medium for hemocytes and the effects of the incubation at different temperatures on MC degranulation was then evaluated with the Trypan Blue assay.

Hemolymph collection and SDS-PAGE

Hemolymph from large colonies was collected and centrifuged as described above. The pellet was resuspended in lysis buffer (Tris-HCl 50mM, pH 7.8, sucrose 0.25 M, SDS 1 %, EDTA 5 mM, pepstatin 1 lg/ml, leupeptin 1 lg/ml, sodium orthovanadate 2 mM, NaF 0.01 M, Nonidet P-40 0.1 %, N-ethylmaleimide 5 mM, phenylmethylsulfonylfluoride 0.04 mg/ml). Cell suspensions were then sonicated 2 min with a Braun Labsonic sonifier at 50 % duty cycles at 0 °C. Cell lysates were boiled for 5 min, centrifuged at 12,000x*g* for 15 min and supernatants were diluted with an equal volume of sample buffer (0.5 M Tris-HCl, 2 % SDS, 10 % glycerol) and loaded in the wells of 12 % Tris-glycine SDS-PAGE slab gels. Each well of the gel received a volume of supernatant equivalent to 20 µg of protein. Electrophoresis was run at the constant current of 10 mA/gel and gels were stained with the redox-cycling method for DOPA-containing proteins according to Paz *et al*. (1991).

Spectrophotometric assay for PO activity

Hemolymph was collected as described above in the absence of anti-clotting agents. It was centrifuged as described above and the pellet was re-suspended in PBS. It was then subjected to sonication, as already described, for 1 min and centrifuged at 10,000x*g* for 10 min to obtain cell lysate. Fifty µl of lysate were incubated with 950 µl of a saturated solution of L-DOPA in PBS in the absence (control) or in the presence of Na_2SO_3, Na_2SO_4, reduced glutathione, 2-mercaptoethanol and dithiothreitol (DTT), at 1 mM concentration, and the reaction was followed for 5 min at 490 nm. One unit (U) of PO activity determines an increase of 0.001 absorbance units/min at 25 °C (Söderhäll and Smith, 1983). Results are expressed as percentage of inhibition of the PO activity with respect to control.

Quantitative microanalysis for iron in epoxy sections

Colonies, fixed in 1.7 % glutaraldehyde buffered with 0.2 M sodium cacodylate plus 1.7% NaCl at pH 7.4, were dehydrated and embedded in epoxy resin. Thick sections (1 µm) of *B. schlosseri* blood lacunae were obtained with a LKB Ultrotome. Chemical analyses were done on sections with a Cameca-Camebax electron microprobe with a fine-focused beam (1 µm diameter) operating in the wavelength-dispersive (WDS) mode using ferrosilite as a synthetic end-member mineral standard for Fe. Operating conditions were 15 kV accelerating voltage and 25 nA beam current; counting times were 20 s for peaks and 20 s for backgrounds.

Statistical analysis

Each experiment was carried out in triplicate. Data are expressed as mean ± SD. They were compared with the χ^2 test.

Results

Morphology of Botryllus schlosseri *MCs*

MCs are the most abundant circulating cells, their concentration amounting to 40 - 60 % of total hemocytes, depending on the colony, without significant variation in the course of the colonial blastogenetic cycle. They have a diameter of 10 - 15 µm and they assume, after aldehyde fixation, a typical berry-like morphology (Figs 1a - b) due to the presence of many vacuoles, about 2 µm in diameter, which occupy most of the cell volume. The vacuolar content assume a yellowish color after aldehyde fixation (Fig. 1b).

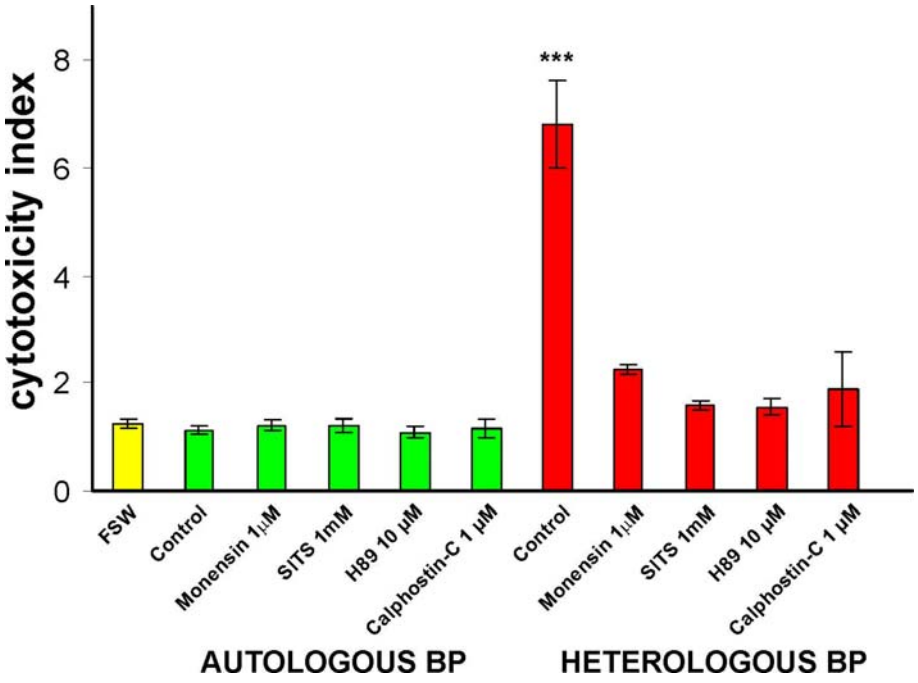

Fig. 3 Cytotoxicity index for hemocytes exposed to FSW, autologous and heterologous CFH. In the last two cases, the effects of the addition of monensin, SITS, H89 and calphostin were also evaluated. Significant differences with respect to the exposure to FSW were marked by asterisks. ***: $p < 0.001$.

Cytochemical and immunocytochemical properties of MCs

MCs assume a pinkish color when stained with eosin or May Grünwald-Giemsa(Figs 1c, d). They resulted positive to the arylsulfatase assay and the reaction product was located inside the vacuoles (Fig. 1e). In addition, the vacuolar content of MCs was able to oxidize polyphenol substrates such as L-DOPA and catechol (Fig. 1f). MC also reacted with MBTH: in this case, vacuoles acquired a red color (Fig. 1g). At alkaline pH and in the presence of potassium glycinate and NBT, vacuoles of most MCs assumed a dark blue color; no other cell-type showed positivity to these assays (Fig. 1h). A similar result was obtained with anti-tunichrome antibody which recognized only MCs (Fig. 1i).

Iron in hemocytes

The electron microprobe, when focused on MC vacuoles, revealed the presence of iron at a concentration up to 90,000 times that of seawater (0.012 mg/l), with a mean value of 687 ± 28 mg/l. No detectable iron was evidenced when the beam was directed towards other cell types.

Effects of incubation of hemocytes in CFH from incompatible colonies

When hemocytes were exposed to CFH from heterologous colonies, living MCs changed their morphology: they degranulated and vacuoles reduced to small vesicles with a brown pigmentation due to residual PO activity (Fig. 1j). The level of MC degranulation observed at 25 °C was significantly ($p < 0.001$) decreased by exposure of CFH at temperatures higher than 80 °C (Fig. 2). In the presence of monensin, SITS, H89 and calphostin C, no degranulation was observed (Fig. 1k).

A significant ($p < 0.001$) increase in the frequency of blue hemocytes, having assumed Trypan Blue as a consequence of altered membrane permeability, with respect to cells exposed to FSW or autologous CFH, was observed in hemocytes challenged with heterologous CFH. This increase was abolished when monensin, SITS, H89 or calphostin C were added to heterologous CFH (Fig. 3).

Electrophoretic analysis of hemocyte lysate

When hemocyte lysate was subjected to SDS-PAGE analysis, and the gel was stained with NBT in potassium glycinate buffer, at alkaline pH, two bands of approximately 48 and 37 kDa appeared (Fig. 4).

PO activity of hemocyte lysate

Hemocyte lysate had a PO activity of 0.044 ± 0.002 U. Na_2SO_3, Na_2SO_4, reduced glutathione, 2-mercaptoethanol and DTT completely inhibited the PO activity when added to the incubation mixture (data not shown).

Discussion

Invertebrate immune responses rely only on natural immunity and, in many cases, the recognition of non-self molecules triggers the

degranulation of circulating cells and the release of PO. PO is a copper enzyme able to convert polyphenols to quinones which, in turn, polymerise to melanin. It can induce cytotoxicity through either the production of reactive oxygen species and the induction of oxidative stress or the rapid conjugation of quinones with -SH groups on essential molecules (Kato *et al*., 1986; Passi *et al*., 1987; Halliwell and Gutteridge, 1999).

In ascidians, PO-containing cells (known as MCs in most cases) are involved in cytotoxic reactions against foreign cells or molecules, in both solitary and colonial species (Akita and Hoshi, 1995; Cammarata *et al*., 1997; Ballarin *et al*., 1998; Hata *et al*., 1998; Shirae and Saito, 2000; Shirae *et al*., 2002; Parrinello *et al*., 2003; Ballarin *et al*., 2005).

MCs play a key role in the rejection reaction between contacting, incompatible colonies of botryllid ascidians (Taneda and Watanabe, 1982; Ballarin *et al*., 1995; Hirose *et al*., 1997; Shirae and Saito, 2000; Shirae *et al*., 2002; Cima *et al*., 2004). Although it has been clearly demonstrated that PO is directly involved in the induction of the cytotoxicity observed along the contact border (Ballarin *et al*., 1995, 1998), uncertainties still persist on the sequence of molecular events controlling PO activation in these organisms. Cytoenzymatic assays demonstrate that the enzyme is located inside MC (Frizzo *et al*., 2000, this report) and is released in the medium as MCs degranulate. Degranulation is mediated by ion fluxes as demonstrated by its inhibition in the presence of the monovalent ionophore monensin and the anion-channel-blocking agent SITS, in agreement with results obtained in human fibroblasts (Uchida *et al*., 1979; Kuivaniemi *et al*., 1986), human neutrophils (Korchak *et al*., 1980), mouse pituitary cells (Heisler and Jeandel, 1989) and pollen tube (Speranza and Calzoni, 1992). In addition, degranulation appears to be mediated by transduction pathways triggered by activated G protein-coupled receptors, such as those involving PKA and PKC (Gomberts *et al*., 2002), as indicated by the inhibition of degranulation by H89 and calphostin C. The *in vitro* inhibition of cytotoxicity in the presence of the above-reported inhibitors is likely related to the inhibition of the release of PO and its consequent absence in the surrounding medium.

Immunocytochemical analysis indicates the presence of tunichromes in MC vacuoles. Tunichromes are polyphenol compounds of low molecular weight, with reducing activity, present inside MC vacuoles of various ascidians (Oltz *et al*., 1987; Bayer *et al*., 1997). Similar molecules, from insect and ascidian hemolymph, were demonstrated to exert antimicrobial activity (Meylears *et al*., 2002; Cai *et al*., 2008). According to Taylor *et al*. (1997), tunichromes represent fragments of larger DOPA-containing proteins which likely are the natural source of PO substrates. These molecules and their quinone derivatives have been effectively revealed inside MC vacuoles by redox-cycling in NBT-glycinate buffer and are responsible of the red color observed inside vacuoles of MCs after the treatment with MBTH. The presence of quinones inside circulating cells has been already reported in other invertebrate species, such as echinoderms

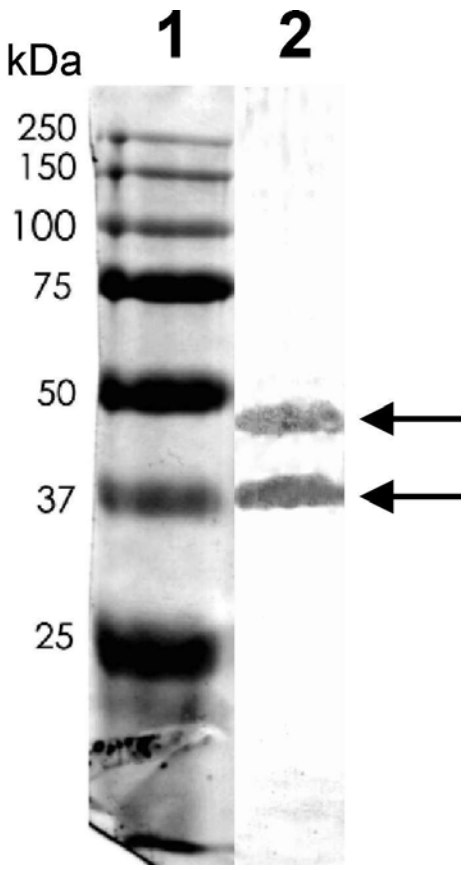

Fig. 4 SDS-PAGE of hemocyte lysates. Lane 1: molecular markers stained with Coomassie blue; lane 2: staining for quinoproteins.

(Kennedy, 1979). The role of these molecules is not clear: they probably act as ready-to-use cytotoxic molecules able to induce an oxidative stress after their release through their reaction with biological molecules or polymerization to melanin (Nappi and Vass, 1993). In some arthropods, a role of secreted quinones in defence against predators has been reported (Machado *et al*., 2005).

The rejection reaction of *B. schlosseri* shares many features with vertebrate inflammation, including the selective recruitment of a hemocyte type, its extravasation and its degranulation with the induction of cytotoxicity (Sabbadin *et al*., 1992). We already demonstrated that the recognition of soluble factors diffusing from the contacting heterologous colony through the partially-fused tunics, activate the synthesis and the release, by MCs, of molecules able to induce their chemotaxis and crowding inside the ampullae facing the contacting colony, their migration through the ampullar epithelium into the tunic and their degranulation with the consequent release of PO which is responsible of the observed cytotoxicity (Cima *et al*., 2004, 2006). The PO-dependent cytotoxicity is also observed *in vitro*, upon the exposure of hemocytes to the incompatible factors contained in heterologous CFH (Ballarin *et*

al., 2002, 2005). Our results indicate that these factors are relatively thermostable and are active in inducing degranulation when stored at a temperature range from 37 to 80 °C. A similar optimum storage temperature range was reported for the CFH of the Japanese colonial ascidian *Botrylloides simodensis* which is capable to induce non-fusion reaction when injected in recipient incompatible colonies (Saito and Watanabe, 1984).

Previous results indicate that PO-driven cytotoxicity is due to the induction of oxidative stress, related to the production of superoxide anions in the course of the oxidation of polyphenols (Ballarin *et al*., 2002). The production of reactive oxygen species is greatly increased in the presence of iron which acts as a catalyser of enzymatic reactions leading to the production of peroxides and hydroxyl radicals (Halliwell and Gutteridge, 1999). Our analysis confirm the presence of iron inside MC vacuoles, already stressed by previous results (Milanesi and Burighel, 1978; Ballarin *et al*., 1995), at a concentration much higher than that in the environment, indicating an active accumulation of the metal that is probably released during the degranulation event and facilitates the induction of the oxidative stress and the consequent cell death.

It is generally assumed that ascidian POs, analogously to arthropod POs (Söderhäll and Cerenius, 1998), derive from the proteolysis of a zymogen (Smith and Söderhäll, 1991; Jackson *et al*., 1993; Arizza *et al*., 1995; Ballarin *et al*., 1998; Shirae and Saito, 2000; Shirae *et al*., 2002; Parrinello *et al*., 2003; Cammarata *et al*., 2008). However, in our cytoenzymatic assays, no previous treatments with proteases are required to detect PO activity as the enzyme is already active, at least in part, inside MC vacuoles. A recent analysis of the aminoacidic sequence of *Botryllus* PO failed to reveal the presence of a prodomain and a cleavage site recognized by serine proteases, analogous to those found in arthropods, in agreement with the idea that some other mechanisms, different from the enzymatic cleavage, should be considered to explain the state of low activity of PO inside MC vacuoles (Ballarin *et al*., 2012).

PO activity is completely inhibited by reducing substances, such as reduced glutathione, 2-mercaptotethanol and dithiothreithol, which probably prevent the oxidation of the polyphenol substrata: this raise the possibility that polyphenols themselves can act as reducing substances preventing or limiting PO activity. In addition, they can be present in a “masked” form inside MC vacuoles, linked to sulfites or sulfates, that inhibit PO activity (present results), already demonstrated inside MCs (Ballarin *et al*., 1995). Sulfates or sulfites can be removed by arylsulfatase, also found inside MC vacuoles, upon cell activation thus making the substrates available to PO. Alternatively, the acidic pH of MC vacuoles, revealed by their pinkish staining by eosin and May Grünwald-Giemsa, can inhibit PO activity which becomes evident when, once degranulation has occurred, the enzyme is released in the medium characterized by a higher pH. These points need further investigations. Therefore, future studies will be directed towards a better understanding of the relationship between PO, arylsulfatase, and DOPA-containing proteins/quinoproteins in *Botryllus* cytotoxicity.

Acknowledgements

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