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Morula Cells and Histocompatibility in the Colonial Ascidian *Botryllus schlosseri*

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ABSTRACT—The role of morula cells (MC) in the formation of necrotic regions characterizing the rejection reaction between incompatible (i.e. nonfusible) colonies was investigated in the colonial ascidian *Botryllus schlosseri*. These blood cells share several chemical and histochemical properties with the pigment of the necrotic masses: both of them show strong reducing activities and contain high quantities of sulphur and iron. Sulphur is present mainly as sulphates and thiols in MC and as sulphates and disulphides in necrotic regions; iron is in the form of ferrous iron in MC and as ferric iron in the necrotic regions. Inside MC vacuoles phenoloxidase (PO) activity and polyphenol substrata are present: the latter are oxidized by PO to quinones, which then polymerize to form the melanin-like substances of dark-brown colour of the necrotic regions. When hemocytes are incubated with heterologous incompatible blood plasma (BP), MC change their morphology, and a significant increase in PO activity is found in the recovered medium as compared with the activity of untreated plasma. No increase in PO activity is observed after incubation with autologous or heterologous compatible blood plasma. These results are consistent with the hypothesis of a humoral factor diffusing from incompatible colonies which is recognized by MC and leads to their degranulation, with the consequent release of the content of their vacuoles, mainly oxidative enzymes, responsible for the localized cell death in necrotic regions.

INTRODUCTION

Ascidian blood contains several types of hemocytes whose functions and differentiation pathways are still controversial. They are traditionally grouped in four categories, i.e. i) lymphocyte-like cells, ii) amoebocytes of both hyaline and granular type and macrophage-like cells, iii) vacuolated cells, including signet-ring, compartment and morula cells, and iv) storage cells, namely nephrocytes and pigmented cells [21, 44, 65]. The subject has recently been critically reviewed by De Leo [14].

Of the vacuolated cells, morula cells (MC) represent an ubiquitous cell-type among ascidians. They are characteristic for their mulberry-like shape, due to the presence of several vacuoles about 2 μm in diameter filled with a yellow-green substance. Electron microscope analysis reveals homogeneous masses of strongly electron-dense material inside these vacuoles when the MC are postfixed in OsO_4 [32, 37, 65]. These cells are involved in clotting after blood vessel damage (Vallee, reported by Wright [65]), encapsulation of foreign bodies [3] and tunic synthesis [13, 17, 55, 66]. They have been reported to accumulate iron in Pleurogona [16, 32] and vanadium in Enterogona [9, 21, 36, 39, 49, 65]. Since the early work of Henze [24], they are known to contain high levels of sulphur, mainly as sulphate, in both Enterogona and Pleurogona [9, 11, 17, 18, 20, 29, 31, 39, 43, 50, 51, 63]. However, thiols have recently been revealed in *Ascidia ceratodes* blood cells [20]. Sulphur inside cells has been claimed to be coordinated with metal ions as sulphate or sulphonate and therefore required for their storage [8, 19, 20], and to be necessary for the maintainance of a reducing environment inside vacuoles as thiols [31]. Other reducing polyphenol compounds have been described in the MC of several ascidians: they have been named "tunichromes" in *Ascidia nigra, Ascidia ceratodes, Molgula manhattensis* [10, 23, 36] and *Phallusia mammillata* [8] and "halocyamines" in *Halocynthia roretzi* [4]. In addition, DOPA-containing peptides other than halocyamines have been found in the MC of *Halocynthia roretzi* [5] and a DOPA-containing protein, called "ferreascidin", has been reported in the blood cells of *Pyura stolonifera* [15]. The abundance of reducing substances justifies the presence of iron in the reduced form inside the MC of iron-accumulating Pleurogona [1, 17]. Furthermore, phenoloxidase activity has been revealed in the MC of all ascidians so far tested [6, 7, 13, 27, 56], and arylsulphatase and peroxidase have been demonstrated in the MC of the colonial ascidian *Botryllus schlosseri* [6].

The MC of botryllid ascidians are involved in the rejection reaction between incompatible colonies which leads to the formation of necrotic regions along the contact border: they have been seen to accumulate at the apices of contacting ampullae, cross the ampullar epithelium, and infiltrate into the fused tunics where they finally degenerate, discharge the contents of their vacuoles and contribute to form necrotic masses [25, 46, 47, 52, 53, 61].

As we are interested in the role of MC in histocompatibility in the colonial ascidian *B. schlosseri*, we undertook the present study to acquire information about the chemistry and cytochemistry of this cell-type in our ascidian species. We

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Abbreviations used in the text: BP, blood plasma; CAB, Na-cacodylate buffer; DOPA, dihydroxyphenyl-L-alanine; FSW, filtered sea water; MBTH, 3-methyl-2-benzothiazolinonehydrazone hydrochloride; MC, morula cells; PO, phenoloxidase; TEM, transmission electron microscope

also performed *in vitro* experiments to elucidate the mechanisms responsible for MC degranulation and lysis in the rejection reaction.

MATERIALS AND METHODS

Animals

Colonies of *B. schlosseri* from our laboratory cultures and wild colonies from the Venetian lagoon were used. They were kept in aerated aquaria attached to glass slides. Sea water was renewed every other day, and the animals were fed with Liquifry Marine (Liquifry Co., Dorking, England) and algae.

Blood smear preparation

Hemocytes were obtained by puncturing the marginal vessels of colonies previously rinsed in filtered sea water (FSW) containing 10 mM L-cysteine to prevent clotting. They were collected with a glass micropipette, transferred to a glass slide coated with poly-L-lysine, and left to adhere for 30 min at room temperature. Blood smears were fixed for 30 min at 4°C with 1% glutaraldehyde in FSW plus 1% sucrose and rinsed in appropriate buffer containing 1% sucrose before being subjected to chemical and histochemical analysis. Hemocytes (at least 200 cells per coverslip in 10 fields) were examined and counted under a Leitz Dialux 22 light microscope, at a magnification of 1250×.

Histological preparations

Rejecting colonies were fixed for 30 min at 4°C in 2% glutaraldehyde buffered with 0.2 M Na-cacodylate buffer (CAB) containing 1.7% NaCl, pH 7.0, dehydrated and embedded in paraffin. 7-μm sections were used for chemical and histochemical analysis.

For transmission electron microscope (TEM) studies, colonies were fixed in 2% glutaraldehyde in CAB, plus 1% caffeine, to prevent leakage of phenols from MC vacuoles into the cytoplasm [33]; they were then dehydrated and embedded in Epon. Ultrathin sections, briefly stained with uranyl acetate, were examined under a HITACHI H 600 E.M.

Chemical and histochemical analysis

Phenoloxidase. To reveal phenoloxidase (PO) activity, glutaraldehyde-fixed blood smears were incubated for 16 hr at 4°C with L-DOPA-saturated 0.01 M CAB plus 5 mM $CaCl_2$ (CAB-Ca), pH 7.0 (modified after Hose *et al.* [26]): a dark product, due to the formation of the melanin polymer appears in the MC. To locate PO activity on ultrathin sections, fixed colonies were incubated overnight at 4°C in L-DOPA-saturated CAB-Ca and for 1 hr at 37°C in the same renewed medium, before dehydration and embedding. Post-fixation in OsO_4 was omitted because of the high reducing activity inside morula cell vacuoles which causes osmium precipitation (see below) and impedes the detection of PO-positive sites. In contact regions between rejecting colonies PO activity was put in evidence by incubating the fixed colonies for 2 hr at room temperature in a mixture containing CAB-Ca plus 4% N,N'-dimethylformamide, L-DOPA-saturated CAB-Ca and 20.7 mM 3-methyl-2-benzothiazolinonehydrazone hydrochloride (MBTH) in the ratio of 4.9:2:2.9 (modified after Winder and Harris [64]). In these experimental conditions MBTH reacts with dopaquinones forming a pink product clearly visible under the light microscope.

Iron. Perl's reaction with Prussian blue [38] and Lillie and Fullmer's reaction with Turnbull blue [12] were used to reveal ferric and ferrous iron respectively on hemocyte monolayers and paraffin sections of rejecting colonies. In both cases, preincubation in H_2O_2 (with a few drops of carbonate buffer 0.1 M, pH 9.5) for 30 min for monolayers and 24 hr for sections was needed to detect iron.

Sulphur. The methods of Geyer for sulphates, of Chèvremont and Frédericq for thiols and of Gabe for disulphids [38] were followed to reveal sulphur in both blood smears and paraffin sections.

Polyphenols. The following assays were tested on blood smears and colony sections to characterize the phenol content of MC vacuoles and necrotic regions of rejected colonies: staining with Nile-blue sulphate after Lillie, staining with basic fuchsin after Ziehl-Nielsen [38], staining for polyphenols according to Reeve [41], staining for ortho- (1–2) and para- (2–4) diphenols according to Lison [30], U.V. and blue fluorescence (340 nm and 490 nm filters respectively), solubility in organic solvents (xylene, absolute ethanol, acetone), bleaching in H_2O_2.

Reducing activity. The reducing activity of hemocytes and necrotic regions was revealed with the following methods [38]: reduction of silver nitrate after Masson-Fontana (argentaffin reaction), reduction of potassium ferricyanide after Schmorl, reduction of potassium bichromate (chromaffin reaction), reduction of OsO_4 after incubation with OsO_4 vapours. Blood smears were treated with 0.1 M sodium iodoacetate in 0.1 M NaOH for 20 hr at 37°C, in order to block the thiols and evaluate residual silver-reducing activity.

Other histochemical techniques. The P.A.S. reaction for polysaccharides [38] was tested on both hemocyte smears and paraffin sections of the necrotic regions.

Preparation of blood plasma and blood cells

Colonies of about 800 zooids were used to get high quantities of hemocytes: they were blotted dry and marginal vessels were lacerated with fine tungsten needles. Blood was collected with a glass micropipette and centrifuged at 780×g for 15 min. The supernatant is referred to as blood plasma (BP); blood cells in the pellet were resuspended in filtered sea water (FSW) and washed again by centrifugation before being incubated as described below.

Phenoloxidase release assay

Blood cells from large colonies Bonies were resuspended in FSW to a final concentration of about 30×10^6 hemocytes/ml. 60μl aliquots were centrifuged and cells resuspended in the same volume of one of the following media: FSW, autologous (i.e. from same donor) blood plasma and heterologous (i.e. from different donors) blood plasmas from both fusible and nonfusible colonies (compatible and incompatible BP respectively). After 30 min incubation at room temperature suspensions were centrifuged and supernatants collected. The phenoloxidase activity of each supernatant was measured by addition of 1 μl of supernatant to 50 μl of CAB-Ca, pH 7.0, and 50 μl of L-DOPA-saturated CAB-Ca in 96-well microplates. FSW and blood plasmas kept 30 min at room temperature were used as controls. The increase in absorbance at 492 nm was recorded every minute for 5 min using a 96-well microplate reader.

Results are expressed as increase in absorbance/min. Five independent experiments with different colonies were carried out; in four of them the heterologous plasma was from incompatible colonies and in the fifth experiment BP from a compatible donor was used. Each experiment was replicated three times. Data are expressed as means ± SD. Differences between mean values were compared with Student's *t*-test.

Evaluation of cell viability and morula cell degranulation

Hemocyte monolayers were incubated for 30 min at room temperature with FSW, autologous and heterologous BP, from both fusible and nonfusible colonies, collected as described above from our laboratory cultures. Cells were then washed in FSW, and their morphology observed under a phase contrast light microscope (Leitz Dialux 22). Viability was assessed by trypan blue exclusion after 30, 60 and 120 min of incubation. Alternatively, after incubation, blood smears were incubated with L-DOPA-saturated CAB-Ca or with OsO_4 vapours, as described above, to reveal PO and reducing activities respectively. Hemocytes were counted at a magnification of 1250× (at least 200 cells per slide in 10 fields). Results are expressed as percentages of positive cells. Absolute values were compared using the χ^2 test.

RESULTS

Morula cells: general morphology, chemistry and cytochemistry

The morphology of *Botryllus* MC does not differ from that of ascidian MC described in the literature [21, 44, 65]. They are large cells, 10–15 μm in diameter, containing several yellowish vacuoles about 2 μm in diameter. After fixation with aldehydes, their shape changes from berry-like to spherical and the content of their vacuoles turns intense yellow-green. The number of MC ranges from 15 to 60% of the total hemocyte number in different colonies and does not change significantly during the life-cycle of the colony.

The results of our cytochemical analysis on MC are reported in Table 1. The content of MC vacuoles has strong reducing properties, stains for polyphenols and ortho(1–2) diphenols, is insoluble in organic solvents, does not produce a positive PAS reaction, and is bleached after treatment with

TABLE 1. Physico-chemical and staining properties of *Botryllus* morula cells and intercolonial necrotic regions

Reaction	Morula cells	Necrotic region
Reduction of $AgNO_3$ (Masson-Fontana)	+	+
Reduction of ferricyanide (Schmorl)	+	+
Reduction of OsO_4	+	+
Reduction of $K_2Cr_2O_7$ (Chromaffin reaction)	+	+
Staining with Nile blue sulphate (Lillie)	+	+
Staining for polyphenols (Reeve)	+	+
Staining for ortho-(1–2) diphenols (Lison)	+	n.t.
Staining for para-(2–4) diphenols (Lison)	−	n.t.
Staining with basic fuchsin (Ziehl-Nielsen)	−	+
U.V. fluorescence (340 nm)	+	−
Blue fluorescence (490 nm)	+	−
Bleaching by H_2O_2	+	+
Perl's reaction (ferric iron)	−	+
Lillie & Fullmer's reaction (ferrous iron)	+	−
Solubility in organic solvents	−	−
PAS reaction (polysaccharides)	−	−
Geyer's reaction (sulphates)	+	+
Chèvremont & Frédericq's reaction (thiols)	+	−
Gabe's reaction (disulphides)	−	+

+ and −, positive and negative reactions; n.t., not tested.

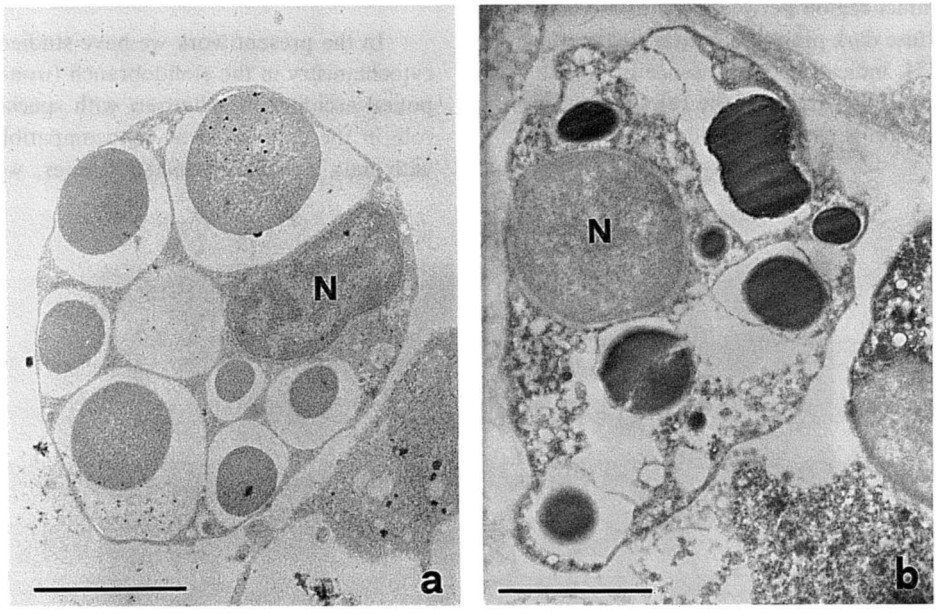

FIG. 1. TEM micrographs of *Botryllus schlosseri* morula cells from colonies fixed in glutaraldehyde and caffeine, without postfixation in OsO_4. a: cell from an untreated control colony; b: cell from a colony incubated 16 hr at 4°C in L-DOPA-saturated CAB-Ca: reaction product is located only inside vacuoles. N: nucleus. Scale bar: 4 μm.

H_2O_2. It is positive to Lillie and Fullmer's reaction for ferrous iron (the reaction is visible only after prior treatment with H_2O_2) and fluoresces when excited with U.V. and blue light: the latter property is enhanced after fixation with glutaraldehyde. MC are also positive to Geyer's reaction for sulphates and Chèvremont and Frédericq's reaction for thiols, but negative to Gabe's reaction for disulphides. The above reducing activity also occurs after blocking of thiols with iodoacetate. Together with their amoebocytic precursors MC are the only blood cells positive for phenoloxidase [6]: TEM images clearly show that enzyme activity is confined within MC vacuoles and no labelling is visible in cell cytoplasm in caffeine treated cells (Fig. 1).

Necrotic regions: general morphology, chemistry and histochemistry

Necrotic masses form along the contact border of colonies which do not share any allele at the histocompatibility locus [34, 45]. They mark the site of morula cell infiltration and subsequent death [25, 46, 52]. They appear as dark brown regions inside the common tunic (cuticles have locally disappeared), between facing marginal ampullae. Cell debris and tunic fibres are closely intermingled and no intact cells can be seen.

The results of chemical and histochemical analysis on necrotic regions between rejected colonies are reported in Table 1. Like the content of MC vacuoles, the pigment deposited in the necrotic regions has strong reducing properties, stains for polyphenols, is insoluble in organic solvents, has a negative PAS reaction, and is bleached after treatment with H_2O_2. It is positive on Perl's reaction for ferric iron: in this case too the reaction is visible only after prior treatment with H_2O_2. The necrotic regions are also positive on Geyer's reaction for sulphates and Gabe's reaction for disulphides. The contact region between nonfusible colonies shows an intense diffuse dark pink colour after treatment with L-DOPA and MBTH, indicating the presence of diffuse PO activity, even at the very beginning of the rejection reaction, when pigment deposition is not yet visible (Fig. 2).

PO activity of BP significantly increases after incubation with heterologous hemocytes fron incompatible colonies

The PO activities of BP from different colonies show great variability, as the frequency of MC does in the blood. However a significant increase ($P<0.05$) in the enzyme activity of BP, as detected spectrophotometrically, is always seen after 30 min incubation at room temperature with hemocytes from incompatible donors, as compared with controls (without hemocytes). No significant differences with respect to controls are observed when hemocytes are incubated in FSW, autologous BP or heterologous BP from a compatible donor (Table 2).

Increased PO activity is to be ascribed to morula cell degranulation

The viability of hemocytes incubated for 30 min in autologous or heterologous blood plasmas shows no significant differences but a significant ($P<0.001$) increase in the mortality of cells is observed after an incubation of 60 and 120 min in heterologous incompatible BP with respect to both autologous and heterologous compatible blood plasmas (Fig. 3). The morphology of MC is greatly modified after a 30 min incubation in heterologous incompatible BP with respect to heterologous compatible or autologous BP: they become irregular in shape and the vacuoles reduce to small vesicles (Fig. 4). As for their reducing and PO activities, the percentage of positive cells is significantly ($P<0.001$) decreased after incubation in heterologous incompatible BP (Table 3). A diffuse granular PO-positive material is often seen around MC in blood smears treated with heterologous incompatible BP but not in those incubated in FSW, autologous BP or heterologous compatible BP.

DISCUSSION

In the present work we have studied MC chemistry and cytochemistry in the stolidobranch (iron-accumulating) compound ascidian *B. schlosseri* with special reference to their role in the expression of histocompatibility. In agreement with data on other ascidian species, we demonstrated the

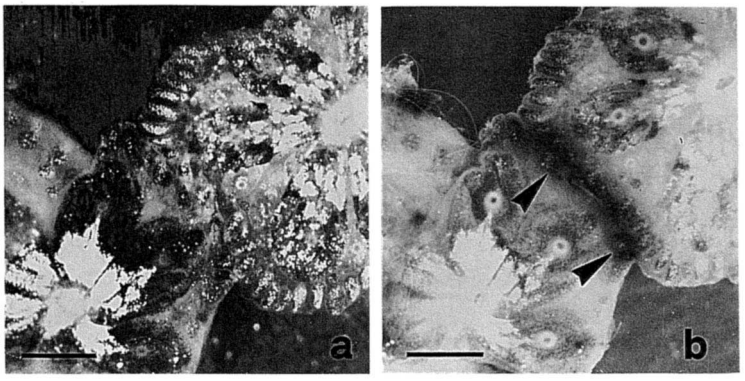

FIG. 2. PO activity in the contact borderline between nonfusible colonies as revealed with the L-DOPA-MBTH assay after fixation in 1% glutaraldehyde. a: untreated colonies (control); b: treated colonies: arrowheads indicate the site of enzyme activity. Scale bar: 1 mm.

TABLE 2. Phenoloxidase activities of filtered sea water (FSW) and blood plasma (BP) after 30 min incubation at room temperature

Treatment activity	PO activity (ΔA_{492}/min×1000) −hemocytes	+hemocytes
Experiment 1		
FSW	0.8±0.6	2.9±1.3
Autologous BP	35.2±2.1	32.7±1.3
Heterologous incomp. BP (A)	2.7±0.6	18.0±3.9**
Heterologous incomp. BP (B)	18.2±1.5	29.8±2.0**
Experiment 2		
FSW	0.5±0.3	2.3±1.8
Autologous BP	48.8±4.0	43.5±2.1
Heterologous incomp. BP (A)	5.3±1.2	28.3±1.5***
Heterologous incomp. BP (B)	20.0±4.6	44.0±1.7**
Experiment 3		
FSW	10.3±3.8	14.3±4.7
Autologous BP	28.7±1.5	28.8±4.2
Heterologous incomp. BP (A)	32.7±2.5	50.7±6.4*
Heterologous incomp. BP (B)	0.7±0.6	58.7±3.2***
Experiment 4		
FSW	1.7±0.6	3.3±1.2
Autologous B	7.3±1.5	10.5±2.1
Heterologous incomp. BP (A)	5.7±1.4	32.0±1.0***
Heterologous incomp. BP (B)	5.5±0.7	23.7±4.2**
Experiment 5		
FSW	1.2±0.3	0.9±0.4
Autoloous BP	9.7±0.5	10.3±0.7
Heterologous comp. BP	25.3±3.1	22.5±1.4

In each experiment plasmas were incubated without hemocytes or with equal quantities of hemocytes from a single colony. Autologous and heterologous blood plasmas were used. Unlike the heterologous compatible combinations, the incompatible ones show significant increases in PO activity of BP as compared with untreated (without hemocytes) BP. Significance levels are indicated by asterisks: *; $P<0.05$; **; $P<0.01$; ***; $P<0.001$. Incomp. and comp.; incompatible and compatible BP respectively.

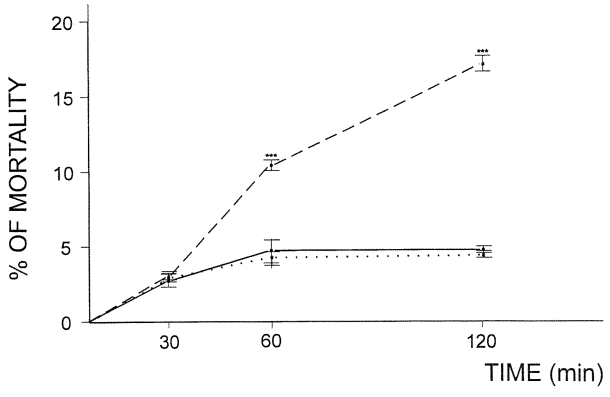

FIG. 3. Effect of incubation in autologous (solid line), heterologous compatible (dotted line) and heterologous incompatible (broken line) blood plasma (BP) on hemocyte viability. Blood cells were incubated 30, 60 and 120 min at room temperature. Significant differences with respect to autologous BP are shown by asterisks. ***: $P<0.001$.

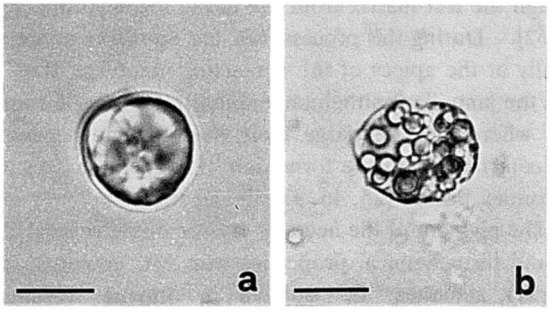

FIG. 4. Morphology of living morula cells from blood smears incubated for 30 min in autologous (a) or heterologous incompatible (b) blood plasma. Scale bar: 10 μm.

presence in these cells of sulphur, in the form of both sulphate and thiols, and that of polyphenol compounds which, together with thiols, may explain the reducing environment found inside their vacuoles. In addition, MC do not stain with the PAS reaction, as reported by Smith [55] in *Halo-*

Table 3. Percentage of morula cells showing positivity for phenoloxidase activity and osmium-reducing activity after 30 min incubation in different media

Treatment (30 min)	% of Positive cells PO	Osmium
FSW	18.6±1.4	16.8±1.3
Autologous BP	19.5±1.7	15.2±2.0
Heterologous compatible BP	20.4±0.5	14.8±1.5
Heterologous incompatible BP	1.4±0.1***	4.5±0.8***

Significant differences with respect to autologous blood plasma (controls) are indicated by asterisks. ***; $P<0.001$

cynthia aurantium. The presence of iron inside the MC of the compound ascidian *Botryllus schlosseri* was demonstrated by X-ray microanalysis by Milanesi and Burighel [32]. Our results indicate that in the MC of *Botryllus* iron is present in its reduced form; the need for severe chemical attack (preincubation in H_2O_2) to reveal the metal suggests that it is firmly bound as "masked iron" to intravacuolar components [38]. High PO activity also occurs in the MC of *B. schlosseri* as well as of other ascidian species [6, 7, 13, 27, 56]. MC and some granular amoebocytes are the only hemocytes showing positivity for PO in *Botryllus*. Therefore, on the basis of their enzyme content, MC seem to be related to granular amoebocytes and not to signet-ring cells, as claimed by several authors [8, 17, 21, 28, 44, 65]. Conversely, the latter show an enzyme content and behaviour which relates them to phagocytes [6].

As pointed out by several authors, both cellular and humoral components are involved in colony specificity in botryllid ascidians [42, 47, 57, 59, 60, 62]. In *Botryllus*, rejection occurs after the fusion of the tunics of colonies in contact [52] and is triggered by the interaction between the blood cells of one colony and a humoral factor diffusing through the test matrix from the facing incompatible colony [60, 62]. During this process, MC are known to accumulate initially at the apices of the contacting ampullae; they then cross the ampullar epithelium and infiltrate into the common tunic where they discharge their vacuoles and degenerate, thus contributing to the formation of necrotic masses along the contact border [25, 46, 47, 52, 53, 61].

The pigment of the necrotic masses shares several chemical and histochemical properties with MC contents: it has reducing activities, is insoluble in organic solvents, is bleached by H_2O_2, and does not stain with the PAS reaction. Unlike Harp *et al.* [22], we did not detect fluorescence in the necrotic areas. The latter have a negative reaction for thiols, but are positive for sulphates and disulphides, and iron is present as ferric ion.

Since necrotic regions are mostly formed of infiltrated MC which undergo cell death, as a first step in allorejection MC must be able to recognize allogeneic humoral factors from incompatible colonies and consequently move chemotactically towards the ampullar tips, as suggested by Hirose *et al.* [25], and infiltrate into the tunic where they release the content of their vacuoles thus leading to the formation of necrotic masses.

According to our hypothesis [7], morula cell phenoloxidase is a key enzyme in the formation of necrotic regions: by acting on polyphenol substrata present inside the MC, it oxidizes them to quinones which may be responsible for localized cell death and which polymerize to form melanin-like substances. In agreement with this hypothesis an intense diffuse PO activity has been revealed in the contact area between nonfusible colonies. In addition a significant increase in cell death is observed in blood smears after 60 and 120 min of incubation in heterologous incompatible BP with respect to heterologous compatible or autologous BP. Moreover the physico-chemical properties of the pigment deposited in contact regions between nonfusible colonies, its staining for polyphenols and bleaching by peroxides are indication of its melanic nature [38, 40]. The unreactivity of cellular PO is probably due both to its presence in an inactive form as a proenzyme (an increase in enzyme activity after preincubation of BP with serine proteases has been demonstrated by our group [7]) and to the unavailability of the poliphenol substrate which can be present in a "masked" form. Actually the arylsulphatase activity which characterizes *Botryllus* MC [6] suggests that sulphates may be bound to the aromatic ring of polyphenols: the action of the enzyme may be important in detaching them from phenols, thus making the latter available to PO. Oxidation of iron from ferrous to ferric ions and of thiols to disulphides suggests the involvement of other oxidative activities in the rejection reaction. In agreement with this assumption, we demonstrated the presence of a peroxidase inside *Botryllus* MC [6], and peroxidase activity has also been shown in the tunic around rejecting ampullae [52].

The release of vacuolar content by MC may be consequent to recognition of humoral factors diffusing from incompatible colonies. To test this hypothesis, we mixed *in vitro* blood cells and blood plasmas from wild colonies and from fusible laboratory colonies in autologous and heterologous combinations and measured PO activity of BP as an indicator of MC degranulation. Wild colonies are usually heterozygous at the highly polymorphic histocompatibility locus [35, 54], so that the probability of fusion between two different colonies in the natural environment is very low [58]. A spontaneous release of PO usually occurs and the great variability of the "basal" level of PO activity in plasmas from different colonies may reflect the variability in the frequency of MC in different colonies which ranges from 15 to 60% of the total hemocyte number. However, our results, showing significantly increased PO activity in BP after incubation with heterologous incompatible hemocytes are indicative of recognition of heterologous incompatible humoral factors by MC, inducing a degranulation response. This conclusion is supported by the observed change in the morphology of MC after incubation of blood smears with heterologous incompatible BP. In addition, in such blood smears there is a reduction in

the number of cells showing positivity for PO and osmium-reducing activities inside their vacuoles and a diffuse granular PO-positive material is frequently visible around the MC, representing the just released content of morula cell vacuoles sticking to the glass slide. The above hypothesis is in good agreement with the recent report of Sawada and Ohtake [48] of an alloreactivity reaction in *Halocynthia roretzi*, evidenced *in vitro* in nonself hemolymph mixtures as intensive discharge of hemocyte contents, precipitation of cell clusters, and general darkening of the media and that of Akita and Hoshi [2] who demonstrated the release of PO during contact reaction between allogeneic hemocytes in the same species.

Future research will be directed towards better clarification of the role of MC and their enzymatic content in colony specificity in botryllid ascidians, with particular attention to events leading to the activation of PO following the recognition of incompatible humoral factors.

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