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New data on phagocytes and phagocytosis in the compound ascidian *Botryllus schlosseri* (Tunicata, Ascidiacea)

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ABSTRACT

In the compound ascidian *Botryllus schlosseri*, phagocytes change their morphology and frequency during the colonial blastogenic cycle. In the regression phase, characterized by intense phagocytosis, zooids of the old generation are progressively resorbed, the frequency of hyaline amoebocytes falls abruptly, and the frequency of macrophage-like cells increases significantly. Moreover, the hyaline amoebocytes engulf foreign materials, withdraw their cytoplasmic projections, and change their shape from flat and fusiform to spherical or ovoidal: all these processes suggest that hyaline amoebocytes represent the precursors of macrophage-like cells. During regression, a significant increase in blood levels of hydrogen peroxide and acid phosphatase is observed, in agreement with data obtained in *in vitro* experiments showing an increase in reactive oxygen metabolite production, nitrite ion release, and acid phosphatase secretion associated with phagocytosis.

KEY WORDS: Ascidians - *Botryllus* - Phagocytosis.

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INTRODUCTION

Ascidian hemocytes are the subject of vast production in the scientific literature, mainly addressed towards describing their morphology (see Goodbody, 1974, Wright, 1981, and Rowley *et al.*, 1984, for a review), although many papers provide evidence of their involvement in various biological processes such as defense reactions (Anderson, 1971; Wright & Cooper, 1983; Parrinello & Patricolo, 1984; Parrinello *et al.*, 1984), allorecognition (Fuke, 1980; Taneda & Watanabe, 1982; Sabbadin *et al.*, 1992), tunic synthesis (Endean, 1955; Smith, 1970; Chaga, 1980; Zaniolo & Trentin, 1987), catabolite storage (Azéma, 1937; Das, 1948; Sabbadin & Tontodonati, 1967), asexual reproduction (Oka & Watanabe, 1957; Freeman, 1964; Fujimoto & Watanabe, 1976), and synthesis and excretion of humoral factors (Parrinello & Arizza, 1988; Beck *et al.*, 1989; Azumi *et al.*, 1990; Raftos *et al.*, 1991; Kelly *et al.*, 1993).

In the colonial ascidian *Botryllus schlosseri*, hemocytes have been described by Azéma (1929), Sabbadin (1955a), Milanesi & Burighel (1978), Burighel *et al.* (1983), and Schlumpberger *et al.* (1984). Following a widely accepted classification scheme, they may be grouped into four categories, i.e. lymphocyte-like cells, granulocytes (including both hyaline and granular amoebocytes and macrophage-like cells), vacuolar cells (signet-ring, compartment and morula cells), and storage cells (nephrocytes and pigmented cells (Milanesi & Burighel, 1978; Burighel *et al.*, 1983; Ballarin *et al.*, 1993).

On the basis of their abundance in lysosomal hydrolytic enzymes, we suggested that three hemocyte types of *B. schlosseri*, i.e. hyaline amoebocytes, signet-ring cells and macrophage-like cells, are involved in phagocytosis (Ballarin *et al.*, 1993). This hypothesis has recently been confirmed by a series of *in vivo* and *in vitro* experiments (Ballarin *et al.*, 1994). Following pulse and chase experiments with test particles, we also proposed that the three above-mentioned cell types are different functional states of a single cell type involved in phagocytosis which, after rapid and active ingestion of foreign particles as a hyaline amoebocyte, withdraws its cytoplasmic projections and changes its shape from flat and fusiform to spherical, thus assuming univacuolar signet-ring-like or multivacuolar macrophage-like morphology.

In the course of the present work, we studied the frequency of hemocytes during the colonial weekly life-cycle in order to provide further evidence supporting the above hypothesis. In addition, we extensively examined events associated with phagocytosis: the previously reported presence of a respiratory burst was confirmed and the increased production of various reactive oxygen metabolites was demonstrated as well as the release of reactive nitrogen species and lysosomal enzymes as a consequence of test particle ingestion.

MATERIALS AND METHODS

Animals

Colonies of *B. schlosseri* from the Venetian lagoon or produced in the laboratory were reared in aerated aquaria, attached to glass slides and fed with Liquifry Marine (Liquifry Co., Dorking, England) and algae. Some of them were observed daily and the phases of the blastogenic cycle were staged according to Sabbadin (1955b), each stage being represented by a triplet of numbers indicating the developmental steps of the adult zooids and of the primary and secondary buds, respectively.

Blood smear preparation

Hemocytes were obtained by puncturing the marginal vessel of colonies previously rinsed in filtred sea water (FSW) containing 10 mM L-cysteine to prevent clotting. They were transferred with glass micropipettes to glass slides coated with poly-L-lysine, and left to adhere for 30 min at room temperature. Blood cells were then fixed for 30 min at 4° C with 1% glutaraldehyde in FSW containing 1% sucrose, stained with 10% Giemsa for 10 min and observed under a Leitz Dialux 22 light microscope. They were then counted at a magnification of 1250×, at least 300 cells per coverslip, in order to evaluate hemocyte frequencies at different stages. Hemocytes were classified according to their morphology.

Collection of blood plasma

To obtain blood plasma, colonies were blotted dry, and marginal vessels were lacerated with fine tungsten needles; blood was then centrifuged at 780 × g for 15 min and the supernatant collected. The protein concentration of blood plasma was determined according to Bradford (1976), using bovine serum albumin as standard.

Hemocyte cultures

Hemocytes, collected as described above, were centrifuged at 780 × g for 15 min and resuspended in FSW. Sixty µl of hemocyte suspension were placed in the centre of the wells, made by glueing teflon rings (15 mm internal diameter, 1 mm thick) on siliconized glass slides. Washed coverslips were gently pressed down over the teflon rings, previously smeared with vaseline, to touch the drop of cell suspension. Culture slides were kept upside-down for 30 min to allow the cells to settle and adhere. Dirty FSW was then discarded and replaced with equal volumes of either a suspension of yeast in FSW (yeast:hemocyte ratio = 10:1) or a solution of one of the following substances: 20 µM phorbol 12-mono-myristate (PMM; Sigma), 0.5% mannan (Sigma), 1% polygalacturonic acid (Sigma).

Production of metabolites and enzyme activity assays

Superoxide anion

Hemocyte cultures, treated as described above, were washed, incubated with 60 µl of 0.3% nitroblue tetrazolium (NBT; Sigma) in FSW for 30 min, washed again and dipped in absolute methanol for 2 min before being air-dried. Eighty µl of a solution of 2 M KOH and DMSO (ratio 6:7) were added to the monolayers to dissolve formazan precipitates and recovered after 5 min: their absorbance at 620 nm was then read with a microplate reader (modified after Song & Hsieh, 1994). Yeast was omitted in controls; 25 µg of superoxide dismutase (SOD; Sigma, ca 6000 U/mg) were added to the incubation medium as a control for specificity.

Hydrogen peroxide, hypochlorite and nitrite ions

To measure hydrogen peroxide, collected hemocytes were centrifuged at 780 × g for 15 min and resuspended in a freshly prepared hydrogen peroxide reaction mixture (200 µl of 1% phenol red and 200 µl of 200 U/mg peroxidase (grade II, Boehringer Mannheim) in 9.6 ml of FSW; Pick, 1986) containing yeast, PMM or carbohydrates. At the end of the incubation period, suspensions were centrifuged at 780 × g for 15 min, 100 µl of 1 N NaOH were added to the supernatants and their absorbance at 620 nm was read with a microplate reader.

In the case of hypochlorite, washed hemocytes were resuspended in 1.87% taurine (Sigma) and yeast in FSW. After 30 or 60 min, 0.5 µl of a 50 mg/ml solution of catalase (Sigma) were added to stop the reaction and suspensions were centrifuged at 780 × g for 15 min. KI, to a final concentration of 20 mM, was added to the supernatants and absorbance at 350 nm was read with a Kontron Uvikon 930 UV/Vis spectrophotometer (Gressier *et al.*, 1994); the thiol-containing antioxidant sodium 2-mercaptoethane sulfonate (MESNA; Sigma) was added to the incubation medium at a concentration of 10 mM as a control for specificity.

To reveal nitrite ions, washed hemocytes were resuspended in yeast-containing FSW and, at the end of the incubation period, they were centrifuged at 780 × g for 15 min, and 100 µl of supernatants were incubated for 10 min with 100 µl of Griess reagent (equal volumes of 0.1% naphtylethylene diamine (Sigma) in distilled water and 1% sulfanilamide (Sigma) in 5% H_3PO_4). The absorbance at 550 nm was then read with a microplate reader (Ding *et al.*, 1988, modified after Shen *et al.*, 1994). A precalibrated standard curve, with $NaNO_2$ as standard, was used to calculate nitrite concentrations. Yeast was always omitted in controls.

The amount of hydrogen peroxide in blood plasma was measured by mixing, in the wells of a microplate, 10 µl of the hydrogen peroxide reaction mixture with 10 µl of the supernatants obtained from the centrifugation (780 × g for 15 min) of blood repeatedly collected from colonies at different stages. After 10 min, 16 µl of 1 N NaOH were added and the absorbance at 620 nm was read with a microplate reader. Blood plasma was substituted with FSW in controls.

Acid phosphatase

Cytochemical assay. Smears of hemocytes collected at different stages of the blastogenic cycle were incubated for 60 min at 37° C in a solution of hexazonium p-rosaniline, buffered with 0.1 M sodium acetate, pH 5.2, containing 40-50% of naphtol AS-BI phosphate (Sigma) according to Lojda *et al.* (1979). They were then washed and observed under a light microscope: sites of positivity for acid phosphatase were marked by a red colour.

Spectrophotometric assay. The release of acid phosphatase, as a reference lytic enzyme, was measured according to Absolom (1986) in both culture medium and blood plasma. Collected hemocytes were resuspended in yeast-containing (yeast:hemocyte ratio = 10:1) isotonic salt solution (ISO: 20 mM Tris, 0.5 M NaCl, 10 mM $CaCl_2$, pH 7.5). At the end of incubation, they were centrifuged at 780 × g for 15 min, and 100 µl of the acid phosphatase reaction mixture (4.5 mM p-nitrophenyl phosphate and 0.5% triton X in 0.1 M acetate buffer, pH 4.5) were added to the supernatants. After 30 min, 100 µl of 2 N NaOH were added to stop the reaction and the absorbance at 405 nm was read with a microplate reader. Yeast was omitted in controls.

To assay plasma acid phosphatase, 25 µl of reaction mixture were incubated with 2.5 µl of blood plasma, collected at different stages, for 30 min at room temperature before the addition of 25 µl of 2 N NaOH. The absorbance at 405 nm was then read with a microplate reader.

Phenoloxidase

One µl of blood plasma was incubated with 50 µl of 0.01 M Na-cacodylate buffer (CAB) containing 5 mM $CaCl_2$ and 50 µl of a saturated solution of dihydroxyphenyl-L-alanine (DOPA) in CAB, and the absorbance at 492 nm was recorded at 1-min intervals with a microplate reader. In both cases, FSW was used instead of blood plasma in controls; results are expressed as relative units (RU)/mg of protein content. 1 RU is defined as a change of 0.001 absorbance units/min.

Statistical analysis

All experiments were repeated three times; data are expressed as mean ± SD. Hemocyte frequencies were subjected to angular transformation and their variability analysed by ANOVA performed with the SAS statistical package (SAS Institute Inc., Cary, NC); means were compared using Duncan's test. Absorbances were compared with Student's *t*-test.

RESULTS

Determination of the length of colonial developmental stages

Botryllus schlosseri colonies can be reared in the laboratory for years. Their life-cycle consists of a sequence of blastogenic cycles involving feeding adult zooids and differentiating primary and secondary buds. A blastogenic cycle begins with the maturation of the primary buds, which replace the regressed zooids of the preceding cycle, and the production of a new blastogenic generation by the secondary, now primary, buds. The end of the cycle is marked by the crucial step of regression and resorption of the adult zooids (Fig. 1). As the stages of the different blastogenic generations are closely related, the stage of the whole colony can be indicated by a series of three numbers, referring to the developmental stage of adult zooids, and of primary and secondary buds, respectively (Berrill, 1941; Sabbadin, 1955b). The blastogenic cycle begins with stage 9,7,1, followed by stages 9,8,2 to 9,8,5, the latter preceding regression. Stage 9,6 marks the transition between the end of regression and the beginning of a new blastogenic cycle. A detailed description of the regression and resorption stage is given in Table I.

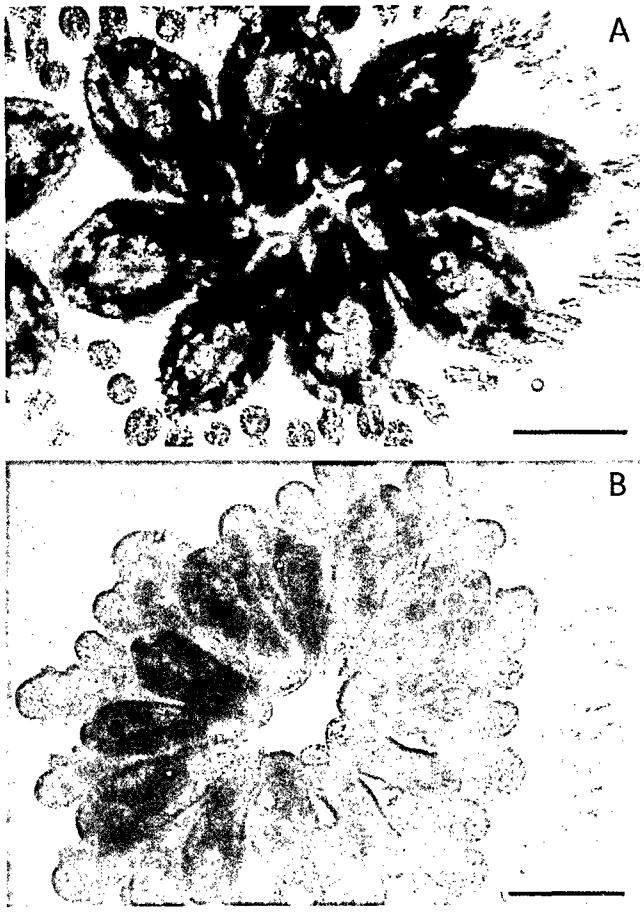

Fig. 1 - *Botryllus schlosseri* colonies in mid-cycle stages (**A**) and during the regression phase (**B**). Bar length: 1.5 mm.

TABLE I - *Events associated with regression of zooids during blastogenic cycle of* Botryllus schlosseri *colonies.*

Substage	Description
11a	Contraction of adult zooids.
11b	Marked contraction of zooids and closing of oral and anal siphons.
11c	Closing of cloacal openings. Each contracted zooid maintains an elongated shape; stomach and gut are still distinguishable.
11d	Zooids appear as round dark masses at centre of each system. Heart is still beating.
11e	Heart stops beating. Since zooid remains persist for a long time in colonies, opening of oral siphons of new adult generation was designated as end of regression and beginning of a new blastogenic cycle.

Stage 11 of the regressing zooids, is further subdivided into five substages (11a-e).

Hemocytes and blood plasma during the blastogenic cycle

Morphology of phagocytes during the colonial life-cycle – Hyaline amoebocytes, macrophage-like cells and signet-ring cells are the blood cells able to ingest foreign particles, both *in vivo* and *in vitro*. Their morphology changes during the blastogenic cycle. Hyaline amoebocytes are fusiform in shape and have homogeneous cytoplasm in stages far from regression (Fig. 2A); during regression they progressively accumulate ingested particles, withdraw their cytoplasmic projections and change their shape from fusiform to spherical or ovoidal (Fig. 2B, C). Similarly, macrophage-like and signet-ring cells show empty vacuoles and reduced size in stages far from regression (Fig. 3A, D). During the latter stage, they increase in size as they accumulate large amounts of ingested material inside their vacuoles (Fig. 3B, E) which, at the end of regression, still contain finely dispersed material (Fig. 3C, F).

Frequency of hemocytes during the colonial life-cycle – During the blastogenic cycle, significant differences ($P < 0.05$ to $P < 0.001$) with respect to stage 9,8,2, far from

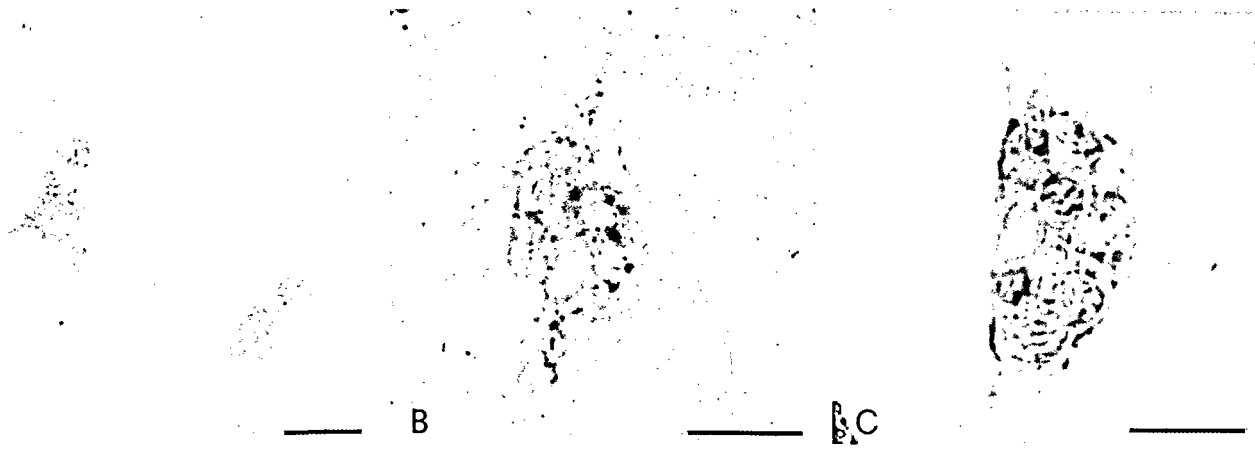

Fig. 2 - Morphological changes in hyaline amoebocytes during blastogenic cycle of *Botryllus schlosseri* colonies. **A**: stage 9,8,2, far from regression; **B**: stage 11a,8,5, beginning of regression; **C**: stage 11d,8,5, end of regression. Bar length: 5 μm.

regression, are evident only for macrophage-like cells and hyaline amoebocytes. As summarized in Figure 4, the frequency of hyaline amoebocytes significantly decreases during regression, in parallel with a significant increase in the frequency of macrophage-like cells, which reaches its maximum at the end of the blastogenic cycle.

Frequency of acid phosphatase-positive hemocytes – The frequency of cells showing positivity for acid phosphatase changes significantly ($P < 0.05$) in the course of the blastogenic cycle: it increases as regression approaches, and peaks at the end of this stage (Table II).

Enzyme activities and hydrogen peroxide in blood plasma – Changes in the activity levels of acid phosphatase and phenoloxidase in blood plasma in the course of the blastogenic cycle were examined in three different colonies: the activity of acid phosphatase, as compared to stages far from regression (9,7,1 - 9,8,2) significantly ($P < 0.05$ or 0.01) increases in stages close to regression and during this latter stage (Fig. 5). Conversely, the course of phenoloxidase activity does not reveal any significant change throughout the whole blastogenic cycle.

As regards the level of hydrogen peroxide in blood plasma, a significant ($P < 0.001$) increase in phenol red oxidation is observed during regression (relative H_2O_2 concentration = 19.97 ± 0.91) with respect to stage 9,8,2 (relative H_2O_2 concentration = 10.05 ± 0.69).

TABLE II - *Frequency of hemocytes showing positivity for acid phosphatase during blastogenic cycle.*

Stage	Hemocyte frequency (%)
9,7,1	24.6 ± 6.1
9,8,2	14.8 ± 2.4
11d,8,5	16.5 ± 2.8
9,6	32.0 ± 10.0 *

*: significant difference with respect to the stage 9,8,2, far from regression. $P < 0.05$.

In vitro *phagocytosis*

Production of reactive oxygen metabolites (ROM) – Phagocytosis by *Botryllus* hemocytes is associated with a respiratory burst which leads to increased production of ROM. A significant increase ($P < 0.001$) in superoxide anion production can be observed after 5 min of incubation of hemocytes with yeast, and a plateau is reached after 15 min. No significant differences with respect to controls are seen after the addition of SOD to the incubation medium. A significant increase in superoxide production can also be obtained after 30 min incubation of hemocytes with 20 μM PMM, 0.5% mannan or 1% polygalacturonic acid ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively) (Fig. 6). Hydrogen peroxide release shows similar behaviour, its concentration in the medium being significantly increased ($P < 0.01$) after only 5 min of incubation with yeast and reaching a plateau after 15 min. Incubation of hemocytes with 0.5% mannan in FSW for 30 min also gives a significant ($P < 0.05$) enhancement of hydrogen peroxide release in the incubation medium as compared with controls (Fig. 6). The extracellular concentration of hypochlorous acid rises to significance ($P < 0.05$) only after 30 min of incubation with yeast; although MESNA does not influence phagocytosis, addition of the drug significantly ($P < 0.05$) decreases the release of hypochlorous acid in the medium (Fig. 7).

Production of nitrite ions – A significant ($P < 0.05$) increase in nitrite ion concentration with respect to controls is already observed 5 min after the addition of yeast to hemocyte cultures (relative nitrite concentration = 2.04 ± 0.2). No significant changes are thereafter seen up to 60 min of incubation.

Secretion of acid phosphatase – The incubation of hemocytes with yeast leads to significantly ($P < 0.05$) in-

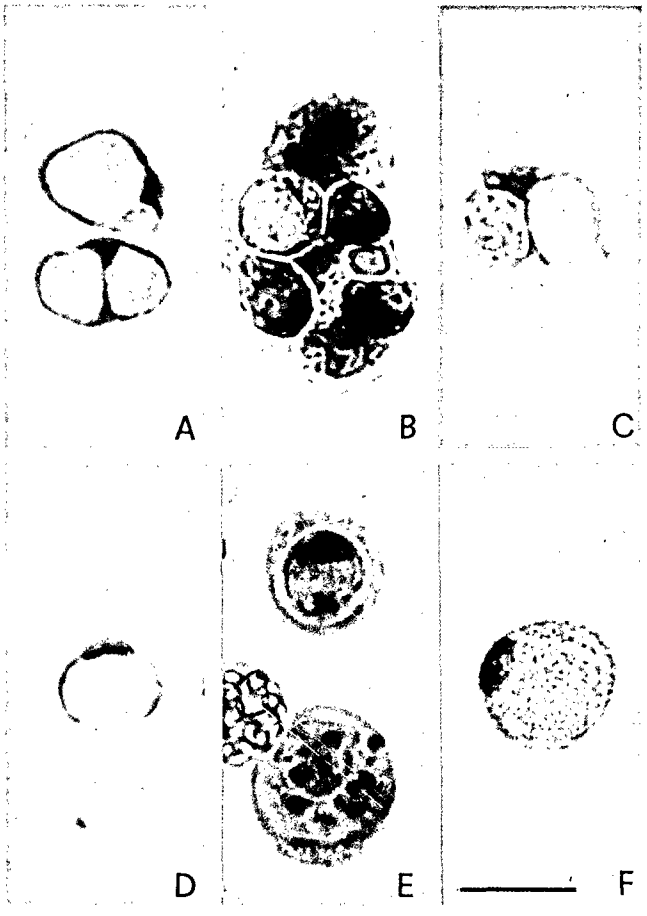

Fig. 3 - Morphological changes in vacuolar phagocytes during blastogenic cycle of *Botryllus schlosseri* colonies. **A-C**: macrophage-like cells; **D-F**: signet-ring cells. **A, D** stage 9,8,2, far from regression: cells have empty vacuoles; **B, E**: stage 11,8,5, regression: vacuoles are filled with ingested materials; **C, F**: stage 9,7,1, beginning of a new blastogenic cycle: vacuoles still contain finely dispersed material. All photographs have the same magnification; bar length: 5 μm.

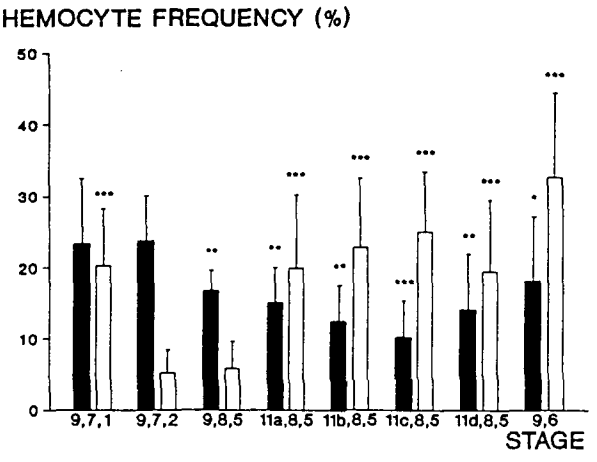

Fig. 4 - Changes in frequency of hyaline amoebocytes (dotted bars) and macrophage-like cells (open bars) during blastogenic cycle of *Botryllus schlosseri* colonies. Asterisks: significant differences with respect to frequency of stage 9,8,2, far from regression. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

creased release of acid phosphatase in the culture medium as compared to controls (relative acid phosphatase release = 1.23 ± 0.09) after 5 min of incubation. The quantity of the released enzyme does not significantly differ for longer incubation times.

DISCUSSION

Phagocytosis is an endocytotic process which is widespread among animals and which plays an important role in immune defence. In the colonial ascidian *B. schlosseri*, three hemocyte types are involved in phagocytosis, i.e., hyaline amoebocytes, macrophage-like, and signet-ring cells. We have previously suggested that they represent different functional aspects of a single blood cell type involved in phagocytosis (Ballarin *et al.*, 1994). For more evidence supporting the above hypothesis, we focused our attention on the last stage of the blastogenic cycle, characterized by progressive resorption of zooids and intense phagocytosis of apoptic material (Burighel & Schiavinato, 1984). Over most of the cycle, hyaline amoebocytes contain homogeneous cytoplasm, appear flat and fusiform, and are the most abundant phagocyte type, their frequency being about four times that of macrophage-like cells. During the short period of regression, lasting less than one day, they withdraw their cytoplasmic projections, engulf foreign materials and change their shape to spherical or ovoidal; at the same time, their frequency decreases significantly, in parallel with a four-fold increase in the frequency of macrophage-like cells. The vacuoles both of macrophage-like and signet-ring cells, looking empty in stages far from regression, appear to be filled with materials, probably phagocytized cells and cell debris, and their size increases. All these processes fit the hypothesis that hyaline

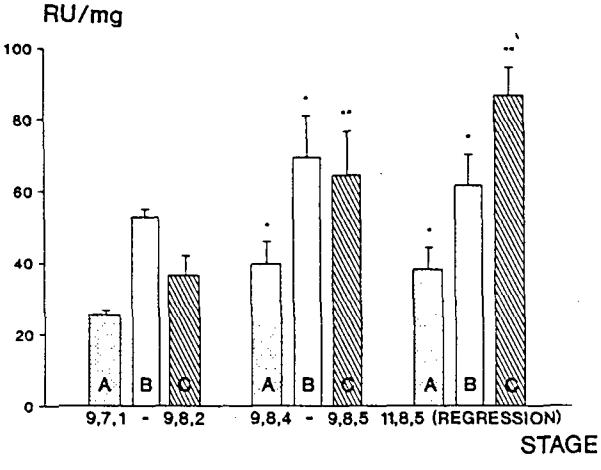

Fig. 5 - Changes in relative activity of blood plasma acid phosphatase during the blastogenic cycle of three different colonies (A, B and C). Asterisks: level of significance with respect to stages 9,7,1 - 9,8,2 far from regression. *: $P < 0.05$; **: $P < 0.01$.

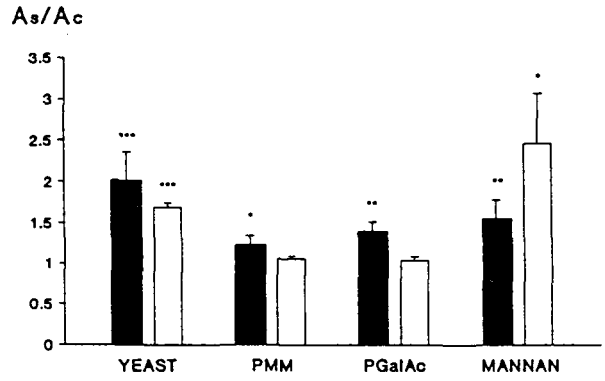

Fig. 6 - Relative production of superoxide anion (dotted bars) and hydrogen peroxide (open bars) by *Botryllus schlosseri* hemocytes incubated for 30 min with various stimuli. A_s/A_c = absorbance at 620 nm of stimulated hemocytes / absorbance at 620 nm of control hemocytes. Asterisks: significant differences with respect to controls. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

amoebocytes are the precursors of macrophage-like cells which, after phagocytosis, change their shape and form vacuoles containing ingested materials. They do have a common enzyme content (Ballarin *et al.*, 1993), and the number of amoebocytes falls abruptly as the frequency of macrophage-like cells rapidly increases.

The frequency of signet-ring cells does not change significantly during the blastogenic cycle. Nevertheless, they share many features with macrophage-like cells, such as common enzyme content (Ballarin *et al.*, 1993), increase in size, filling of vacuoles during regression, and finely dispersed material inside their vacuoles in stages immediately following regression: this material probably represents undigested matter which is subsequently discharged. Therefore, in our hypothesis, they represent a class of univacuolar macrophage-like cells, differing

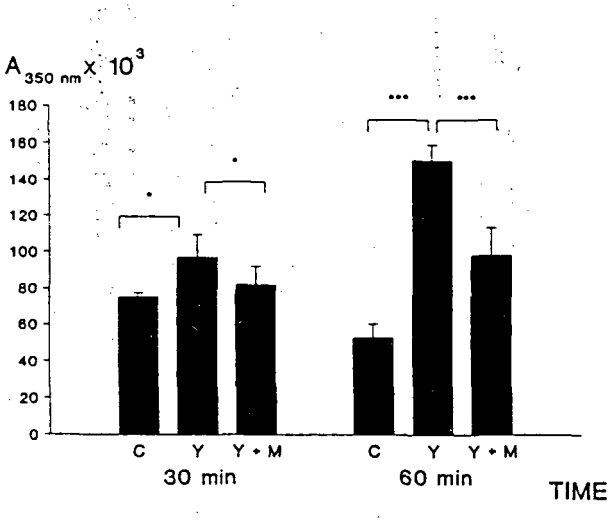

Fig. 7 - Release of hypochlorite ions by yeast-stimulated *Botryllus schlosseri* hemocytes as compared with controls and with hemocytes incubated in the presence of 10 mM sodium 2- mercapto ethane sulfonate (MESNA). Significant differences are marked by asterisks. *: $P < 0.05$; ***: $P < 0.001$. C: FSW (control); Y: FSW + yeast; Y + M: FSW + yeast + 10 mM MESNA.

from typical macrophage-like cells in the number of vacuoles which, at least in *B. schlosseri*, cannot be considered as the precursors of morula cells, as suggested by Schlumpberger *et al.* (1984) on the basis of a common response to some lectins.

Phagocytosis in *Botryllus* is associated with a respiratory burst, as demonstrated by the increased production of ROM associated with this process. Significant increase in the blood level of hydrogen peroxide is observed during regression. Moreover, significant amounts both of superoxide anion and hydrogen peroxide are measured after only 5 min of incubation with yeast. The retarded release of hypochlorous acid is probably related to its derivation from hydrogen peroxide (Gressier *et al.*, 1994). The increase in superoxide anion and hydrogen peroxide production can also be induced after treatment of hemocytes with phorbol esters or polysaccharides. The effect of immunostimulant agents such as phorbol esters in activating plasmalemmal NADPH-oxidase is well known in mammals (Absolom, 1986). Polysaccharides probably act by activating membrane lectins involved in the recognition of foreign microorganisms; in particular, the observation that mannan can promote the production both of superoxide anion and hydrogen peroxide to levels comparable with those obtained with yeast is in good agreement with our previous suggestion that a Ca-dependent membrane lectin with specificity for mannan is involved in the recognition of yeast by *Botryllus* hemocytes (Ballarin *et al.*, 1994).

Measurement of superoxide anion, as the first product of the respiratory burst, is considered to be an accurate method quantifying the intensity of increased oxidative metabolism (Secombes, reported by Song & Hsieh, 1994). Our data, showing an intracellular superoxide concentration in yeast-treated hemocytes amounting to 1.5 - 2.5 times that of controls, as detected by NBT reduction, are comparable with those obtained with tiger shrimp hemocytes stimulated with phorbol esters or zymosan (Song & Hsieh, 1994) and with phorbol ester-stimulated rainbow trout macrophages (Chung & Secombes, 1988). Human macrophages respond to stimulation with a 8-9-fold increase in the intracellular concentration of superoxide anion (Johnston *et al.*, 1976). According to Song & Hsieh (1994), this discrepancy may be explained by a difference in the structure or number of phagocyte receptors, with invertebrate and anamniotes on one hand and amniotes on the other.

Phagocytosis in *Botryllus* also causes a significant increase in the release of nitrite ions, produced through the activity of an L-arginine-dependent nitric oxide synthase (Hibbs *et al.*, 1987; Sies & Groot, 1992), even after 5 min of incubation with yeast: ROM, nitric oxide and nitrite ions are known to act as antimicrobial and antitumoral toxins in mammals (Nathan, 1987). The swift increase in nitrite ion secretion requires early activation of nitric oxide synthase: this differs from the behaviour of mammalian macrophages, in which reactive nitrogen intermediates can be measured only several hours after ex-

posure to the appropriate stimulus (Shen *et al.*, 1994), but is similar to the situation in molluscan hemocytes, in which measurable nitric oxide synthase activity has been demonstrated after 20 min of incubation with test particles (Franchini *et al.*, 1995).

Since the studies of Cohn (1970), it has been well established that stimulation of phagocytosis in mammals leads to a dramatic increase in the synthesis and cellular levels of lysosomal enzymes. *Botryllus* hemocytes show similar behaviour, as the frequency of cells showing positivity for acid phosphatase increases significantly during regression, as compared with mid-cycle stages, and peaks at the beginning of a new blastogenic cycle in coincidence with the frequency peak of macrophage-like cells.

Phagosome formation during phagocytosis causes the secretion of a certain amount of lysosomal hydrolytic enzymes, due to an increase in the fusion of lysosomes with the cell membrane: these enzymes also play an important microbicidal role (Davies & Bonney, 1980; Absolom, 1986). A similar process has been reported in some invertebrates (Cheng & Rodrick, 1974; Cheng, 1992). Using acid phosphatase as a lysosomal reference enzyme, we demonstrated a significant increase in extracellular enzyme concentration after *in vitro* incubation of *B. schlosseri* hemocytes with yeast: activity reaches significance in 5 min and remains constant up to 60 min of incubation. This result may explain the significant increase in acid phosphatase activity measured in the blood during the regression phase of the blastogenic cycle: in this period, programmed cell death leads to progressive resorption of old zooids (Lauzon *et al.*, 1992) and intense phagocytosis of apoptic material occurs (Burighel & Schiavinato, 1984). Therefore, the release of the enzyme in the blood cannot be ascribed to cell rupture subsequent to necrosis, but to phagocyte secretion. In accordance with this view, the activity of a non-lysosomal reference enzyme, such as phenoloxidase, does not significantly change during the entire colonial weekly life-cycle.

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