Cytochemical properties of Botryllus schlosseri haemocytes: indications for morpho-functional characterisation

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In the present study, we carried out a detailed light microscopy investigation of the cytochemical properties of the haemocytes of the colonial ascidian *Botryllus schlosseri*, using new cytochemical stains and enzymatic markers, a panel of antibodies and lectins as probes to characterise *Botryllus* blood cells further.
Results indicate that lymphocyte-like cells are circulating undifferentiated cells recognised by anti-CD34 antibody and there are at least two defined haemocyte differentiation pathways: i) phagocytes, represented by hyaline amoebocytes and macrophage-like cells, which share similar staining properties, the same hydrolytic enzyme content as well as the presence of detectable cytochrome-c-oxidase activity, recognition by anti-CD39 and *Narcissus pseudonarcissus* agglutinin; ii) cytotoxic cell line, represented by granular amoebocytes and morula cells which have vacuoles stained by Ehrlich's stain and Neutral Red; DOPA-containing protein are present inside morula cell vacuoles. Pigment cells and nephrocytes are involved in catabolite storage but their relationships with other cell types are less clear.

Key words: botryllus, haemocytes, cytochemistry, classification.

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Many morphologically different circulating cell types are present in the blood of ascidians. They have been widely described by many authors who focussed their attention on the morphology of both living and fixed haemocytes (Pérès, 1943; Endean, 1955; Sabbadin, 1955; Andrew, 1961; Smith, 1970a, b; Schlumpberger *et al.,* 1984; Sawada et al, 1991, 1993; Dan-Sohkawa *et al.,* 1995) or their ultrastructure (Overton, 1966; Milanesi and Burighel, 1978; Scippa *et al.,* 1982; Burighel *et al.,* 1983; Zhang *et al.,* 1992; Azumi *et al.,* 1993; Sawada *et al.,* 1993; Sugino *et al.,* 1993; Cima *et al.,* 2001; Hirose *et al.,* 2003), and various attempts have been made towards an unifying classification scheme (Goodbody, 1974; Wright, 1981; Rowley *et al.,* 1984; Sawada *et al.,* 1991; De Leo, 1992; Radford *et al.,* 1998). However, in spite of the abundance of studies aimed at better defining the properties and functions of ascidian haemocytes, doubts still exist about their mutual relationships and differentiation pathways.

In the colonial ascidian *Botryllus schlosseri*, according to our previous morpho-functional data (Ballarin *et al.,* 1993, 1994, 1995), blood cells can be grouped into at least four categories: i) lymphocyte-like cells; ii) phagocytes; iii) cytotoxic morula cells (MC) and their precursors; iv) storage cells (pigmented cells and nephrocytes).

Circulating immunocytes are represented by phagocytes and MC. Phagocytes include hyaline amoebocytes (HA) and macrophage-like cells (MLC): the former have an amoeboid shape and appear to be active in phagocytosis; MLC are round and have cytoplasm with vacuoles (one or more) containing cell debris and other ingested material (Ballarin *et al.,* 1994). During the colonial life-cycle, circulating HA change their morphology and decrease their frequency as colonies approach the take-over phase; at the same time, MLC increase their number as old zooids are progressively resorbed (Cima *et al.,* 2003). This behaviour and

the sharing of a common panel of hydrolytic enzymes in their vacuoles led us to suggest that they represent different functional stages of the same cell type (Ballarin *et al.,* 1993, 1994; 1996). MC are the most abundant circulating cell type, their frequency ranging from 20 to 60%, and they represent *Botryllus* phenoloxidase-containing cells (Ballarin *et al.,* 1995), the presence of which has been demonstrated in most of the ascidians studied so far (Chaga, 1980, Smith and Söderhäll, 1991; Jackson *et al.,* 1993; Arizza *et al.,* 1995). Cytoenzymatic analysis carried out on both *B. schlosseri* and *Botrylloides leachi* indicate the close relationship of these cells with granular amoebocytes (GA), which presumably represent the precursors of MC (Ballarin *et al.,* 1993; Cima *et al.,* 2001).

In the present paper, we extended our investigations using new cytochemical stains and enzymatic markers, as well as a panel of commercial antibodies and lectins, as probes to characterise *Botryllus* haemocytes further.

MATERIALS AND METHODS

Animals

Botryllus colonies were collected from the Lagoon of Venice and kept in aerated aquaria, attached to glass slides, filled with filtered sea water (FSW) replaced every other day. They were reared at 19°C and fed with Liquifry (Liquifry Co., England) and algae (*Dunaliella sp.*).

Haemocyte collection and culture

Blood cells were obtained by puncturing, with a thin tungsten needle, the marginal vessels of colonies previously rinsed with a solution of 10 mM L-cysteine in FSW, pH 7.2, to prevent cell aggregation. They were collected with a glass micropipette in a 1.5-ml tube, centrifuged at 780 x g for 10 min, and pellets were resuspended in FSW to obtain a final concentration of 10^7 cells/mL. Sixty µl of the cell suspension were put in the centre of a culture chamber made by glueing Teflon rings (15 mm internal diameter, 1 mm thick) to siliconised glass slides. Coverslips were gently pressed down over the Teflon rings, previously smeared with Vaseline, to touch the drop of cell suspension. Culture chambers were kept upside down for 30 min to let the haemocytes settle and adhere to the coverslips.

Cytochemical assays

After adhesion to the coverslips, cells were fixed for 30 min in a solution of 1% glutaraldehyde and 1% sucrose in FSW containing 1% caffeine, to prevent leakage of phenols from MC vacuoles (Müller and Greenwood, 1978), washed in 0.1 M phosphate-buffered saline (PBS: 8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH2PO4, 1.15 g/L Na_2HPO_4) and stained according to the cytochemical methods reported below. The coverslips were finally mounted on microscope slides with a water-based mounting medium (Acquovitrex, Carlo Erba) and observed under a Leitz Dialux 22 light microscope (LM) equipped with a fluorescence apparatus.

Giemsa's dye

Haemocytes were stained for 5 min in a 10% Giemsa's solution (Fluka) in FSW and then washed in distilled water. Nuclei appeared blue and cytoplasm light blue or violet, due to metachromasia.

Ehrlich's triacid mixture

Haemocytes were incubated in Ehrlich's triacid mixture (Mazzi, 1977: 12 vol. saturated Orange G aqueous solution, 8 vol. saturated acid fuchsin aqueous solution, 10 vol. saturated methyl green aqueous solution, 30 vol. distilled water, 18 vol. absolute ethanol and 5 vol. glycerine) for 15 min and then washed in distilled water. Basophilic granules were light green, neutrophilic violet, and acidophilic copper-red.

Neutral Red dye

After adhesion of haemocytes to coverslips, the FSW of the culture chambers was substituted with 60 µl of Neutral Red (Merck) solution (8 mg/L) in FSW. Living haemocytes were directly observed. This dye specifically stains acid compartments (e.g., lysosomes or acid vacuolar contents) of living cells (Mazzi, 1977).

Periodic acid Schiff (PAS) reaction for polysaccharides

Fixed haemocytes were incubated in 1% periodic acid for 10 min, rinsed in tap water and stained with Schiff's reagent for 30 min at 37°C. Coverslips were then dipped in a solution of 0.6% sodium metabisulphite in 0.02 M HCl for 6 min, washed in tap water for 10 min, and then rinsed in distilled water. Positive sites appeared primary red (Mazzi, 1977).

Sudan Black for lipids

After adhesion of haemocytes, coverslips were dipped in 70% ethanol for 30 sec and stained with a saturated solution of Sudan Black (Sigma) in 70% ethanol for 15 min at 70°C. They were then rinsed in 70% ethanol and washed in distilled water. Black spots revealed the presence of lipids.

Assay for DOPA-containing protein

Fixed haemocytes were incubated in the dark for 60 min in 0.24 mM nitroblue tetrazolium (NBT, Sigma) and 20 mM Na-benzoate (as a phenoloxidase inhibitor; Sigma) in potassium glycinate buffer (15% glycine in distilled water adjusted to pH 10 with KOH 2 N; Flückiger *et al.,* 1995). Cells were then washed in PBS and coverslips were mounted in Acquovitrex (Carlo Erba) and observed under the LM at 1250x. Positive sites appeared dark blue.

Cytoenzymatic assays

In order to detect cytochrome-c-oxidase activity, fixed haemocytes were washed in 0.1 M Na-acetate buffer, pH 5.5, and incubated for 4 h at 37°C, in 0.2% 3-3' diaminobenzidine (DAB; Sigma) in 0.1 M Na-acetate buffer containing 0.1% MnCl2 and 0.001% H_2O_2 (Novikoff and Goldfisher, 1969). Coverslips were then washed in distilled water, mounted in Acquovitrex and observed under the LM. Positive sites (mitochondria) stained brown.

Immunocytochemistry

After adhesion to coverslips, haemocytes were incubated in FSW in the presence or absence (controls) of *Bacillus clausii* spores (200 x 106/ml) for 60 min and fixed for 30 min at 4°C in 4% paraformaldehyde (Serva) in isotonic marine invertebrate solution (ISO: 2.82 g/l Tris, 29.25 g/l NaCl, pH 7.5) (Edds, 1985). They were then washed in PBS, immersed for 30 min in a PBS solution containing 5% powdered milk to block aspecific reactions, and washed again in PBS. Haemocytes were incubated in anti-CD34 mouse monoclonal antibody (10 µg/mL in PBS; Cymbus Biotechnology Ltd), for 60 min at room temperature. They were then washed in PBS and incubated for 30 min in 10 µg/ml fluorescein isothiocyanate (FITC)-labelled goat anti-mouse-immunoglobulin antibody (Sigma). Lastly, they were washed in PBS and mounted in Vectashield (Vector) before observation under the fluorescence microscope equipped with I2/3 filter block for FITC (450–490 nm). Haemocyte autofluorescence, restricted to vacuolar contents of MC and GA, was easily distinguishable from FITC, due to its yellow emission.

In another series of experiments, after fixation, haemocytes were permeabilized with 0.1% Triton X-100 (Merck), incubated for 60 min in one of the following antibodies (10 µg/mL in PBS): anti-CD39 (Cymbus Biotechnology Ltd), anti-CD57 (Cymbus), anti-glutathione-S-transferase (GST) (Sigma) or anti-Se-dependent-glutathione peroxidase (GPX) (MBL), washed in PBS, and incubated for 30 min in 10 µg/ml biotin-conjugated goat anti-mouse-immunoglobulin. They were washed again in PBS and incubated for 30 min in avidin-biotin-peroxidase solution (ABC, Vector) in PBS. After further washing, they were incubated for 5 min in a solution of 0.5 mg/ml DAB in PBS containing 0.04% H2O2, washed again, and mounted in Acquovitrex and observed under the LM.

Lectin cytochemistry

A panel of lectins, both FITC-labelled (Ulex europaeus agglutinin-I (UEA-I, specific for L-fucose), *Datura stramonium* lectin (DSL, specific for N-acetyl-β-D-glucosamine and N-acetyl-lactosamine), *Ricinus communis* agglutinin-I (RCA, recognising β-D-galactosides), wheat germ agglutinin (WGA, specific for N-acetyl-β-D-glucosamine), Helix pomatia agglutinin (HPA, recognising N-acetyl-α-D-galactosamine)) and biotin-conjugated (Arachis hypogaea agglutinin (PNA, specific for galactosyl (β-1,3) N-acetyl-galactosamine), concanavalin A (ConA, recognising α-D-glucopyranosides and α-D-mannopyranosides), Vicia villosa agglutinin (VVA, specific for N-acetyl-D-galactosamine), Narcissus pseudonarcissus agglutinin (NPA, specific for α-D-mannosyl carbohydrate residues)), was assayed on haemocyte monolayers. Lectins were purchased from Sigma (RCA, WGA, ConA) and Vector.

After fixation, haemocytes were incubated for 30 min in PBS containing 5% powdered milk, washed three times for 10 min in PBS, and incubated for 60 min in a 50 µg/ml lectin solution in PBS containing 0.1 mM CaCl2. Incubation with FITC-labelled lectins was followed by extensive washing in PBS, and coverslips were finally observed under the fluorescence microscope after being mounted in Vectashield (Vector). Conversely, after incubation with biotin-conjugated lectins, haemocytes were

washed in PBS, incubated for 30 min in ABC (Vector) in PBS, washed in PBS for 30 min, incubated for 5 min in a 0.5 mg/ml DAB in PBS containing 0.04% H2O2, mounted in Acquovitrex and finally observed under the LM.

Results

We confirm the presence of four main haemocyte categories in the blood of B. schlosseri, identified as follows. Their cytochemical properties are listed in Table 1.

Lymphocyte-like cells (LLC)

These have a diameter ranging from 4 to 6 μm, with a central nucleus containing one or two basophilic nucleoli, surrounded by a thin layer of hyaline cytoplasm stained light blue by Giemsa's dye (Figure 1a). Their frequency ranges from 2 to 4% of circulating haemocytes, independently of the colonial life-cycle, and their surface is recognised by WGA, RCA and ConA. In addition, they are the only circulating cells labelled by the anti-CD34 antibody (Figure 1b; Table 1).

Phagocytes

Phagocytes include hyaline amoebocytes and macrophage-like cells. HA, 6-12 μm in length, have a variable shape, with various cytoplasmic protrusions (pseudopods), and have a roundish nucleus with homogeneous cytoplasm which appears pinkish-violet after Giemsa's staining (Figure 1c). Lysosomes and sometimes a few small vacuoles containing ingested material, able to accumulate Neutral Red and positive to the PAS reaction, are observable. The abundance of HA in the blood is related to the colonial life-cycle stage and ranges from 12-25% during the take-over, to 23-42% in mid-cycle stages (Cima *et al.,* 2003).

MLC, 10-15 μm in diameter, have a spheroidal shape, their cytoplasm is stained metachromatically by Giemsa's dye, and shows one or few large vacuole(s) containing ingested material which occupy most of the cell volume (Figure 1d). They are positive to the PAS reaction (Figure 1e), and can accumulate Neutral Red (phagolysosomes). Their frequency varies during the colonial life-cycle and ranges from 10-12% in mid-cycle stages, to 20-30% during the take-over (Cima *et al.,* 2003).

Both these cell types have a cytoplasm stained by basic fuchsin (Ziehl Nielsen's reaction) and share common contents of hydrolytic enzymes; they also show the presence of cytochrome-c-oxidase activity (Figure 1f). Their vacuoles are stained by Ehrlich's triacid mixture (pinkish-blue), Sudan Black, and NPA (Figures 1g, h); their cytoplasm is labelled by anti-GST- and anti-GPX-antibodies (Figures 1i, j). In addition, their surface is recognised by anti-CD39 (Figure 1k), WGA, RCA and ConA (Figure 1l).

Granular amoebocytes and morula cells

GA (7-10 μm in length) have amoeboid morphology and appear very motile in culture (Figure1m). Their cytoplasm contains many small (≤ 1 μm) vacuoles with homogeneous pale-yellow contents, which turn green after Ehrlich's staining (Figure 1n). Their frequency ranges from 2.5 to 5% of total circulating cells and is not influenced by the colonial life-cycle.

MC (10-15 μm) are large haemocytes, spheroidal in shape with a cytoplasm filled with many round vacuoles uniform in diameter (2 μm), with pale yellow contents, as in the case of GA (Figure 1q) but turning red after Ehrlich's staining (Figure 1r).

Both GA and MC vacuoles acquire a yellowish-green colour after aldehyde fixation, accumulate Neutral Red (Figure 1o). They turn black after exposure to OsO4 vapours, and brown after exposure to either chromium or silver ions (chromaffin and Masson-Fontana's reactions, respectively). In addition, they show positivity for arylsulphatase, peroxidase and phenoloxidase activities. Only MC vacuoles are stained by Lison's and Reeve's reactions for para-(2-4) diphenols and polyphenols, respectively, and in the assay for DOPA-containing protein. Both GA and MC can bind WGA, RCA and ConA (Figure 1l); GA are recognised by DSL (Figure 1p), MC by PNA (Figure 1t). Stimulated MC were recognised by anti-CD57 antibody (Figure 1u).

Storage cells

These include pigment cells (PC) and nephrocytes (N). They are large (10-15 μm) haemocytes, their volume almost entirely occupied by a few, large vacuoles containing pigment granules in Brownian movement, which appear orange or blue in living PC and yellowish in living N.

Pigment cell frequency ranges from 3 to 10% of total circulating haemocytes and their granules turn

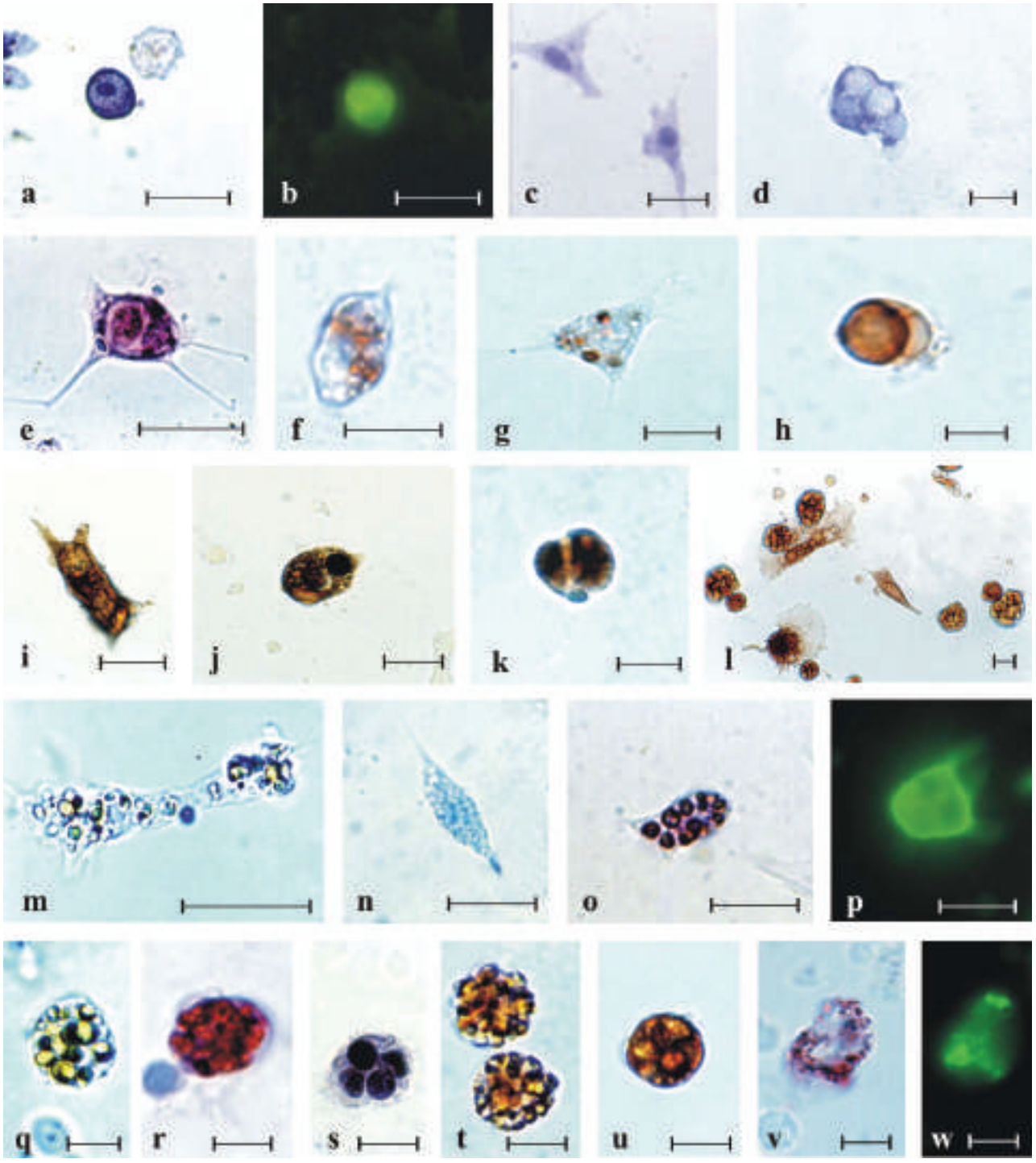

Figure 1. ***Botryllus schlosseri*** **haemocytes. a: LLC, Giemsa's stain; b LLC, immunopositivity to anti-CD34 antibody; c: HA, Giemsa's stain; d: MLC, Giemsa's stain; e: HA, PAS reaction; f: HA, cytochrome-c-oxidase activity; g: HA, recognition by NPA ; h: MLC, recognition by NPA; i: HA, immunopositivity to anti-GST antibody; j: MLC, immunopositivity to anti-GPX antibody; k: MLC, immunopositivity to anti-CD39 antibody; l: haemocytes recognised by ConA; m: GA, Giemsa's stain; n: GA, Ehrlich's stain; o: GA, Neutral Red; p: GA: recognition by DSL; q: MC, Giemsa's stain; r: MC, Ehrlich's stain, s: MC, stain for DOPA-containing proteins; t: MC: immunopositivity to anti-CD57 antibody; u: MC, recognition by PNA; v: PC, Giemsa's stain; w: PC: recognition by UEA-I. Scale bar: 10 µm.**

red after aldehyde fixation (Figure 1v). N seem to be as abundant as PC, but are difficult to preserve as fixed cells. They are recognised by WGA, RCA and ConA. PC are the only cells recognised by UEA-I (Figure 1w).

Table 1. Cytochemical properties, enzymatic activities and lectin affinity of Botryllus schlosseri haemocytes.

	LLC	HA	MLC	GA	MC	PC	N
Cytochemical assays							
Giemsa's dye	+	+	+	–	–	–	–
Ehrilch'smixture	-	+	+	+	–	–	
NeutralRed	–	+	+	+	+	–	–
SudanBlack	–	+	+	–	–	–	–
PASreaction	–	+	+	–	–	–	–
ZiehlNielsen'sreaction[a]	–	+	+	–	–	–	–
Chromaffinreaction[a]	–	–	–	+	+	–	–
Osmiumtetroxidevapours[a]	–	–	–	+	+	–	–
Masson-Fontana'sreaction[a]	–	–	–	+	+	–	–
Reeve'sreaction[a]	–	–	–	–	+	–	–
Lison'sreaction[a]	–	–	–	–	+	–	–
AssayforDOPA-containingproteins	–	–	–	–	+	–	–
Hydrolyticenzymes							
Acidphosphatase[b]	–	+	+	–	–	–	–
Alkalinephosphatase[b]	–	+	+	–	–	–	–
5'-nucleotidase[b]	–	+	+	–	–	–	–
β-glucuronidase[b]	–	+	+	–	–	–	–
β-N-acetylglucosaminidase[b]	–	+	+	–	–	–	–
Non-specificesterase[b]	–	+	+	–	–	–	–
Chloroacetylesterase[b]	–	+	+	–	–	–	–
Acidesterase[b]	–	+	+	–	–	–	–
Arylsulfatase[b]	–	–	–	+	+	–	–
Oxidativeenzymes							
Peroxidase[b]	–	–	–	+	+	–	–
Phenoloxidase[b]	–	–	–	+	+	–	–
Cytochrome-c-oxidase	–	+	+	–	–	–	–
Antioxidantenzymes							
Glutathioneperoxidase	–	+	+	–	–	–	–
Glutathione-S-transferase	–	+	+	–	–	–	–
CDs							
CD34	+	–	–	–	–	–	–
CD39	–	+	+	–	–	–	–
CD57	–	–	–	–	+	–	–
Lectins							
WGA	+	+	+	+	+	+	+
RCA	+	+	+	+	+	+	+
ConA	+	+	+	+	+	+	+
DSL	–	–	–	+	–	–	–
PNA	–	–	–	–	+	–	–
NPA	–	+	+	–	–	–	–
UEA-I	–	–	–	–	–	+	–
HPA	–	–	–	–	–	–	–
VVA	–	–	–	–	–	–	–

[a]*: from Ballarin et al.,1995;* [b]*: from Ballarin et al.,1993.*

Table 2. Comparative classification of Botryllus schlosseri haemocytes.

This report	Previous reports
Undifferentiated cell	Haemoblast,[2] Lymphocyte,[4] Lymphocyte-like cell[5,6]
Phagocytic line	
Hyaline amoebocyte	Microgranular amoebocyte[2,4,6,] Hyaline amoebocyte[1,5]
Macrophage-like cell	Vacuolated phagocyte1 Macrophage2,4,5,6, Signet-ring cell4,5
Cytotoxic line	
Granular amoebocyte	Granular amoebocyte[1,4,5,] Macrogranular amoebocyte[2,4,6]
Morula cell	Morula cell[1,2,4-6]
Storage cells	
Pigment cell	Granular pigment cell4, Pigment cell[3,5,6]
Nephrocyte	Purinic cell,[1] Nephrocyte[2,4,5]

[1]Sabbadin 1955; [2]Milanesi and Burighel 1978; [3]Burighel et al. 1983; [4]Schlumpberger et al. 1984; [5]Ballarin et al. 1993; [6]Rinkevich and Rabinowitz 1993

Discussion

In the present study, we extended our previous investigation (Ballarin *et al.,* 1993) and used new cytochemical assays and molecular probes (antibodies and lectins) to better characterise *B. schlosseri* blood cells and offer new indications for their classification. Cytochemical analysis is a useful tool which offers the possibility of better understanding or defining the biological functions of specific cell types. In particular, cytoenzymology can give indications about the presence of known enzyme activities, whereas immunocytochemistry and lectin cytochemistry can reveal the cellular location of molecules or molecular components.

As a first result, we confirm the presence of four main haemocyte categories, as hypothesized in a previous paper (Ballarin *et al.,* 1993). Table 2 lists the possible correspondence of our categories with *Botryllus* cell types described by other authors.

LLC have a high nucleocytoplasmic ratio, a feature typical of undifferentiated cells, and show no detectable enzyme activity; the presence of nucleoli indicates that they represent the haemoblast described by other authors (Wright, 1981; Rowley *et al.,* 1984; De Leo, 1992). With all other cell type they share positivity to WGA, RCA and Con A, indicating the presence of mannosyl, galactosyl, glucosyl and acetylglucosaminyl carbohydrate residues on the surface of *Botryllus* blood cells. Schlumpberger *et al.,* (1984) obtained similar results with WGA, but they reported that only LLC can bind ConA: in our opinion, this and some other discrepancies with our results are related to the high ionic strength of the incubation medium used by the above authors, made isotonic with *Botryllus* blood, since they carried out their assays on living cells. The lack of haemocyte labelling with VVA and HPA indicates the absence of N-acetylgalactosaminyl residues on the surface of *Botryllus* blood cells. As reported for another botryllid species, Botrylloides leachi (Cima *et al.,* 2001), LLC were immunopositive to anti-CD34 antibody. This indicates that they represent circulating undifferentiated cells able to differentiate into other cell types, since CD34 is a transmembrane protein known to be expressed by mammalian haemopoietic cells (Furukawa, 1998) and well-conserved in metazoan evolution, as demonstrated by its presence in undifferentiated cells of the ray fish Torpedo marmorata (Pica *et al.,* 2000), the freshwater gastropod *Planorbarius corneus* (Franceschi *et al.,* 1991), the clam *Tapes philippinarum* (Cima *et al.,* 2000), and the compound ascidian *Botrylloides leachi* (Cima *et al.,* 2001).

As regards immunocytes, two differentiation pathways were identified, one leading to phagocytes, the other to cytotoxic cells. Phagocytes are represented by HA and MLC which, on the basis of functional assays and similar contents of hydrolytic enzymes, were considered as two different functional stages of the same cell type, able to move rapidly by means of pseudopods and to ingest non-self cells or particles in the form of HA, and rapidly withdrawing their cytoplasmic protrusions and turning to globular MLC when filled with engulfed material (Ballarin *et al.,* 1993, 1994). The results presented here strengthen this assumption: we show that phagocytes also share the presence of detectable cytochrome-c-oxidase activity - a key enzyme in cellular respiration, the abundance of which is explained by the high ATP demand for phagocytosis. In addition, their vacuoles stain with Neutral Red (which usually accumulates inside acid

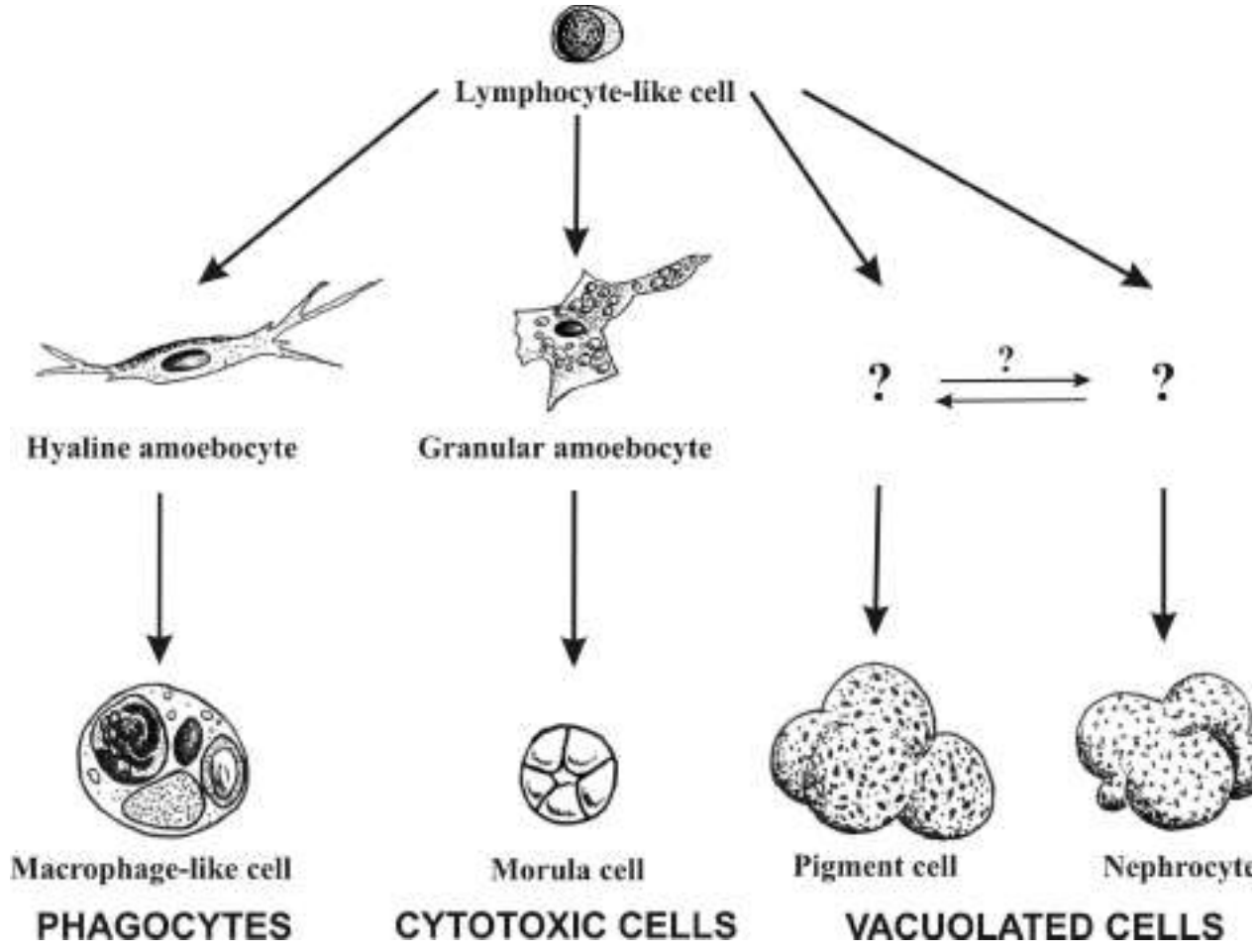

Figure 2. Postulated differentiation pathways of *Botryllus schlosseri* haemocytes..

compartments such as lysosomes or secondary phagosomes), Sudan Black (which reacts with lipids), Ehrlich's triacid mixture (indicating a slightly acid environment) and the PAS reaction (revealing the presence of carbohydrates). HA and MLC vacuoles are also recognised by NPA, which indicates the presence of mannosyl residues in both cell types. Their cytoplasm is also immunopositive to anti-GPX and anti-GST antibodies: these enzymes act as scavengers of cytotoxic reactive oxygen molecules produced during the respiratory burst associated with phagocytosis, as previously reported (Ballarin *et al.,* 1994, 1996). Furthermore, HA and MLC are the only cell types recognised by the anti-CD39 antibody, which is raised against an ectonucleotidase of human endothelial cells and macrophages (Mulero *et al.,* 1999; Goepfert *et al.,* 2001) involved in the modulation of inflammatory cytokine release (Imai *et al.,* 2000): this points to the possibility of similar enzymes in *Botryllus* phagocytes. It is worth emphasizing that we use the term *signet-ring cells* to indicate *Botryllus* univacuolar MLC (Ballarin *et al.,* 1993, 1994). In Enterogonid ascidians, signet-ring cells have similar morphology, due to evolutionary convergence, but a different function, as they are not phagocytes and accumulate vanadium inside their single, large vacuole (see Michibata (1996) for a review). *Botryllus* MC represent cytotoxic immunocytes able to sense the presence of foreign molecules or cells and to give a twofold response as a consequence: i) they can synthesise putative immunoregulatory molecules recognised by antibodies raised against mammalian inflammatory cytokines (Ballarin *et al.,* 2001; Cima *et al.,* 2004); ii) they degranulate and release their vacuolar content, i.e. the precursors of the cytotoxic enzyme phenoloxidase and its substrates (Ballarin

et al., 1995, 1998; Cima *et al.,* 2004). On the basis of similarities in both the cytochemical properties of their vacuoles and their cytoplasmic enzyme contents, GA and MC were ascribed to the same differentiation pathway in both B. schlosseri and B. leachi (Ballarin *et al.,* 1993, 1995; Cima *et al.,* 2001), with GA as precursors of MC. The latter are involved in the formation of the necrotic areas along the facing borders of contacting, genetically incompatible *Botryllus* colonies, through the enzyme phenoloxidase, responsible for the cytotoxicity observed (Ballarin *et al.,* 1995, 1998). The difference in vacuolar staining with Ehrlich's triacid mixture of GA and MC, indicating a shift from basophilic to acidophilic contents, may be related to a change in vacuolar contents as GA become mature MC. This difference is stressed by the absence, in GA, of positivity to the reaction with NBT in K-glycinate buffer which, conversely, is present in MC vacuoles. In addition, this result indicates that the polyphenols present inside MC, as indicated by Reeve's and Lison's reactions (Ballarin *et al.,* 1995), are complexed to proteins, to form DOPA-containing proteins. This results fits previous data indicating the presence of proteins or peptides containing DOPA or DOPA derivatives inside ascidian MC (Oltz *et al.,* 1988; Azumi et al. 1990a, b; Bayer *et al.,* 1992; Taylor *et al.,* 1997). GA maturation also requires a change in surface molecules, as indicated by their ability to bind DSL and their inability to bind PNA. The former lectin, specific for N-acetyl-glucosaminyl and N-acetyl-lactosaminyl residues, does not react with MC; the latter, able to recognise galactosyl-N-acetylgalactosaminyl residues, unlike the situation reported by Schlumpberger *et al.,* (1984), can recognise MC surfaces. In our opinion, this discrepancy in results may again be explained by both differences in incubation medium and the use of living cells. Interestingly, MC are immunopositive to anti-CD57 monoclonal antibody recognising an antigen expressed by a subset of mammalian peripheral blood mononuclear cells with natural killer activity (Abo and Balch, 1981). This suggests the presence of CD57-like molecules in MC, in agreement with the proposed cytotoxic role of these cells (Ballarin *et al.,* 1993, 1995, 1998). Immunopositivity to anti-CD57 antibody has also been reported in NK-like cells of the leech Hirudo medicinalis (De Eguileor *et al.,* 2000).

No enzymatic activities or specific cytochemical reaction products were observed in storage cells. PC were the only cell type recognised by UEA-I, indicating the presence of fucosyl residues: in our conditions, this lectin behaves differently from what has been reported elsewhere (Schlumpberger *et al.,* 1984). The few data available do not facilitate the identification of a differentiation pathway for these two cell types.

In conclusion, in our species, hydrolytic enzymes, NPA and anti-CD39 can be used as markers of the phagocytic differentiation line, whereas the presence of PO and peroxidase activities, and DOPA-containing proteins characterise mature cytotoxic cells. A proposal for *Botryllus* haemocyte differentiation pathways is shown in Figure 2.

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