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Histoenzymatic staining and characterization of the colonial ascidian *Botryllus schlosseri* hemocytes

LORIANO BALLARIN
FRANCESCA CIMA
ARMANDO SABBADIN
Dipartimento di Biologia, Università di Padova,
via Trieste 75, I - 35121 Padova (Italy)

ABSTRACT

The distribution pattern of some hydrolytic and oxidizing enzymes among *Botryllus schlosseri* hemocytes has been investigated histochemically. Signet-ring cells, macrophages, and some hyaline amoebocytes were found to stain positively for phosphatases, glycosidases, and esterases, and morula cells and some granular amoebocytes for arylsulfatase, peroxidase, and phenoloxidase. Our results suggest two differentiation pathways for these blood cell types: one involves hyaline amoebocytes, signet-ring cells, and macrophages and leads to phagocytic cells; the other involves granular amoebocytes and morula cells and leads to cells responsible for inflammatory responses.

KEY WORDS: Ascidian hemocytes - Enzyme histochemistry - Tunicates - Immune responses.

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INTRODUCTION

Circulating hemocytes play a key role in immune responses in ascidians. They can phagocytize or encapsulate invading pathogens or foreign materials (Anderson, 1971; Wright & Cooper, 1975; Wright, 1981; Wright & Cooper, 1983) and are strictly involved in allorecognition (reviewed by Taneda *et al.*, 1985; Sabbadin *et al.*, 1992). Yet, a great variety of blood cell types are present and doubts still exist about their classification, mutual relationships, and functions (Goodbody, 1974; Wright, 1981).

In *Botryllus schlosseri*, blood cells have been studied morphologically by Sabbadin (1955), Milanesi & Burighel (1978), Burighel *et al.* (1983), and Schlumpberger *et al.* (1984). At least eight cell types, grouped in four different categories, have been distinguished, namely: (i) lymphocyte-like cells, (ii) amoebocytes (hyaline and granular, differing from each other in nuclear staining and size of cytoplasmic granules) and macrophages, (iii) vacuolar cells (signet-ring, compartment, and morula cells), and (iv) storage cells (pigment cells and nephrocytes). The role of some of these cell types in allorecognition has been emphasized by Mukai (1967) and Taneda & Watanabe (1982a, b) for *Botryllus primigenus*, and by Scofield & Nagashima (1983) and Sabbadin & Astorri (1988) for *B. schlosseri*. Nevertheless, much less is known about their involvement in other immune responses, such as phagocytosis and encapsulation; also, scanty cytochemical data are available.

Blood cells of several species of invertebrates have been characterized on the basis of enzymatic properties (Bayne *et al.*, 1979; Sminia & Barendsen, 1980; Granath & Yoshino, 1983; Hose *et al.*, 1987). As this characterization was shown to be useful for a better understanding of the blood cells differentiation pathways and functions, we also studied the distribution of some hydrolytic and oxidizing enzymes in *B. schlosseri* hemocytes.

MATERIALS AND METHODS

Chemicals

Ethylenediaminetetraacetic acid (EDTA), naphthol AS-BI phosphate, naphthol AS-BI ß-glucuronide, naphthol AS-BI ß-N-acetylglucosaminide, naphthol AS-D chloroacetate, 1-naphthyl acetate, L-cysteine, mucic acid, levamisole, adenosine-5'-monophosphate (AMP), and Na-diethyldithiocarbamate (DECA) were purchased from Sigma; 3,3'-diaminobenzidine (DAB), p-nitrocatechol sulphate, dimethyl-formamide (DMF), and 3-(3,4-dihydroxyphenul)-L-alanine (L-DOPA) from Aldrich; fast blue B, BB, VRT and fast violet B from Fluka Chemie.

Blood smear preparation

Hemocytes punctured from laboratory-cultured colonies (previously kept in sea water containing 5 mM EDTA for 5 min to prevent clotting) were left to adhere to poly-L-lysine-coated slides for 30 min, fixed for 30 min at 4° C with 1% glutaraldehyde in sea water containing 1% sucrose, washed with appropriate buffer, and incubated for revealing enzyme activities. After glutaraldehyde

fixation, blood smears were observed under a Leitz Dialux 22 light microscope.

Cytolocalization of enzyme activities

The following hydrolytic and oxidizing enzymes were tested. Hydrolytic enzymes: acid phosphatase, alkaline phosphatase, 5'-nucleotidase, ß-glucuronidase, ß-N-acetylglucosaminidase, non-specific esterases, acid esterase, chloroacetyl esterase, and arylsulfatase. Oxidizing enzymes: peroxidase and phenoloxidase.

Simultaneous azo-coupling detection methods were used for acid and alkaline phosphatases, esterases, and glycosidases with the diazonium salts hexazonium-p-rosaniline, fast blue B, BB, VRT, and fast violet B as coupling reagents. Metal salt procedures were employed for 5'-nucleotidase and arylsulfatase, the diaminobenzidine method for peroxidase, and oxidizing polymerization for phenoloxidase.

Phosphatases

Acid and alkaline phosphatases were tested according to Lojda (1962) and Burstone (1962), respectively. Samples were incubated for 1 h at 37° C in a solution of hexazonium-p-rosaniline, or other diazonium salts containing 40-50% of naphthol AS-BI phosphate, previously dissolved in DMF and 0.1 M Na-acetate buffer at pH 5.2 (acid phosphatase) or 0.1 M Tris-HCl at pH 9.0 (alkaline phosphatase).

5'- nucleotidase was revealed by the method of Wachstein & Meisel (1957). Samples were incubated, for 2 h at 37° C, in a reaction mixture containing 40% AMP as substrate, 6% lead nitrate as capture agent, and 10% magnesium sulphate in 0.1 M Tris-maleate buffer at pH 7.2. Slides were subsequently placed in 1% ammonium sulphide for 2 min to reveal enzyme-active sites.

Glucosidases

To detect ß-glucuronidase activity according to Hayashi *et al.* (1964), samples were kept, at 37° C for 2 h, in 20% naphthol AS-Bi ß-glucuronide (dissolved in DMF) in buffered hexazonium-p-rosaniline (0.1 M Na-acetate buffer, pH 5.0). The method of Hayashi (1965) was, instead, used for detecting ß-N-acetylglucosaminidase. Samples were incubated for 3 h, at 37° C, in a solution of 20% naphthol AS-BI- ß-N-acetylglucosaminide (dissolved in DMF) in hexazonium-p-rosaniline buffered at pH 5.0 with 0.1 M Na-acetate.

Esterases

Nonspecific esterases were detected according to Davis & Ornstein (1959). The incubation was carried out at 4° C for 3 h, in 2% 1-naphthyl acetate (previously dissolved in acetone) in hexazonium-p-rosaniline, or other diazonium salts, buffered at pH 7.2 with 0.1 M phosphate buffer. The acid esterase activity was revealed by incubating slides, for 16 h at 4° C, with 15% 1-naphthyl acetate (dissolved in acetone) in hexazonium-p-rosaniline containing 0.1 M citric acid-phosphate buffer, at pH 5.5 (Lojda, 1977). The procedure of Moloney *et al.* (1960) was followed for chloroacetyl esterase. Consistently, 30% naphthol AS-D chloroacetate (dissolved in DMF) and 0.1% fast blue B or BB buffered with 0.1 M phosphate buffer, pH 6.5, were used.

Arylsulfatase

Cells were incubated, for 1 h at room temperature, with 0.8% p-nitrocatechol sulphate and 1.6% lead nitrate in 0.02 M Na-acetate buffer, pH 5.2; subsequently, they were transferred into 1% ammonium sulphide, for 2 min, to reveal arylsulfatase activity (Goldfischer, 1965).

Peroxidase

To assay peroxidase activity, slides were immersed, for 10 min at room temperature, in 0.5 mg/ml of DAB in 0.1 M phosphate buffer, at pH 7.2 (Graham & Karnovsky, 1966).

Phenoloxidase

To reveal phenoloxidase activity, incubation of cells was carried out for 16 h at 4° C, in L-DOPA-saturated-cacodylate buffer (0.1 M, pH 7.0) (Hose *et al.*, 1987).

Controls

Substrates were omitted in controls. To be sure that reaction products were not due to artifacts, hemocytes were preincubated for 5 min in appropriate buffers with different concentrations of the following inhibitors (which were also added to the reaction mixtures): sodium fluoride for acid phosphatase and esterases; sodium sulphate for arylsulfatase; mucic acid for ß-glucuronidase; L-cysteine, or levamisole, for alkaline phosphatase; DECA for phenoloxidase. Levamisole was used also in the 5'-nucleotidase test to block alkaline phosphatase activity.

RESULTS

The principal blood cell-types of *B. schlosseri*, except storage cells, are shown in Figure 1. Lymphocyte-like cells appear spherical in shape, 4-5 µm in diameter, and with a large nucleus containing one or two nucleoli. In addition, they are surrounded by a thin layer of hyaline cytoplasm. Amoebocytes, with a diameter ranging from 5 to 10 µm, manifest a strong tendency to spread on glass surfaces. Cells of the hyaline type show long and thin pseudopodia and a cytoplasm filled with numerous small granules which can hardly be detected with light microscope. On the other hand, cells of the granular type contain distinct granules, up to 1 µm in diameter. Macrophages appear as granular cells, 10-15 µm in size, with some giant vacuoles usually containing cellular debris. Signet-ring cells, about 10 µm in diameter, contain a unique vacuole which apparently pushes cytoplasm and nucleus towards the cell periphery. Morula cells, ranging from 10 to 15 µm, contain several globular vacuoles of about 2 µm in diameter; after fixation, their appearance changes from berry-like to spherical and the vacuoles turn pale yellow.

As reported in Table I, the hemocytes of *B. schlosseri* showed two distribution patterns of the enzyme activities tested. Vacuoles of macrophages and signet-ring cells and granules of some of the hyaline amoebocytes stained for the different enzymes of the phosphatase, glycosidase, and esterase type (Fig. 2 a and c-f). Vacuoles of morula cells and granules of some granular amoebocytes stained for arylsulfatase, peroxidase, and phenoloxidase (Fig. 2 b and g-i).

The inhibition tests for different concentrations of inhibitors gave the results shown in Table II. NaF inhibited both acid phosphatase and acid esterase at 10 mM concentration, whereas 100 mM concentration was required to suppress nonspecific esterases; 10 mM levamisole inhibited both alkaline phosphatase and 5'-nucleotidase: this last enzyme activity was also suppressed when 10 mM L-cysteine was used; ß-glucu-

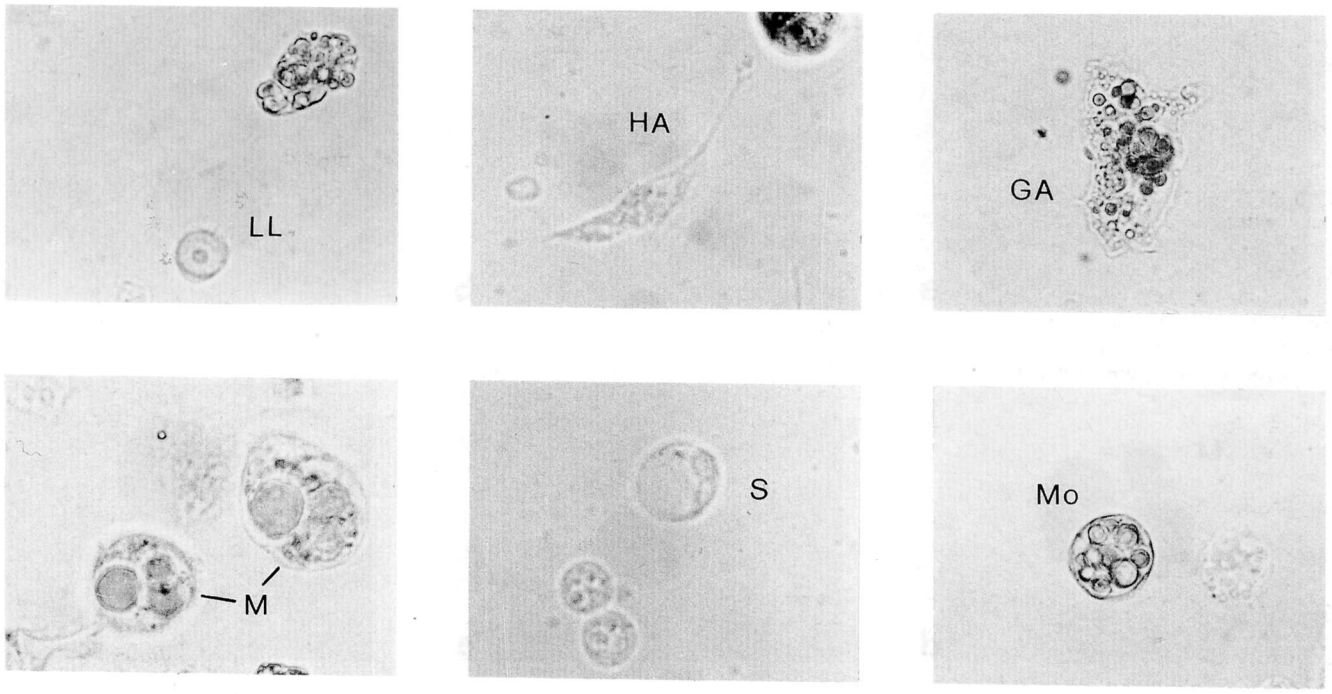

Fig. 1 - Blood smears of some unstained *Botryllus schlosseri* hemocytes after glutaraldehyde fixation. LL, lymphocyte-like cell; HA, hyaline amoebocyte; GA, granular amoebocyte; M, macrophage; S, signet-ring cell; Mo, morula cell. ×700.

TABLE I - *Distribution of enzymatic activities among* Botryllus schlosseri *hemocytes.*

	L	HA	GA	M	S	Mo
HYDROLYTIC ENZYMES						
Phosphatases						
Acid phosphatase	–	+/–	–	+	+	–
Alkaline phosphatase	–	+/–	–	+	+	–
5'-Nucleotidase	–	+/–	–	+	+	–
Glycosidases						
ß-Glucuronidase	–	+/–	–	+	+	–
ß-N-Acetylglcosaminidase	–	+/–	–	+	+	–
Esterases						
Nonspecific esterases	–	+/–	–	+	+	–
Acid esterase	–	+/–	–	+	+	–
Chloroacetyl esterase	–	+/–	–	+	+	–
Sulfatases						
Arylsulfatase	–	–	+/–	–	–	+
OXIDIZING ENZYMES						
Peroxidase	–	–	+/–	–	–	+
Phenoloxidase	–	–	+/–	–	–	+

L: lymphocyte-like cells; HA: hyaline amoebocytes; GA: granular amoebocytes; M: macrophages; S: signet-ring cells; Mo: morula cells.

ronidase activity was completely inhibited by 100 mM mucic acid; arylsulfatase was suppressed by 1 mM sodium sulphate, peroxidase by 1.5% H_2O_2, and phenoloxidase by addition of 0.01 mM DECA in the reaction mixture.

DISCUSSION

It is generally assumed that animal leukocytes are directly involved in immune responses, more in particular in the recognition of non-self and the establishment of a defense reaction, such as phagocytosis or encapsulation of foreign bodies, or inflammation after allorecognition (Anderson, 1981; Millar & Ratcliffe, 1989). In these processes hydrolytic and oxidizing enzymes are largely involved; the former degrade ingested material inside lysosomes, whereas the latter exert antibacterial and lytic activities through the production of toxic compounds (e.g., oxygen-free radicals by peroxidase or quinones by phenoloxidase).

The results shown indicate that the hemocytes of the compound ascidian *B. schlosseri* contain both hydrolytic and oxidizing enzymes. The majority of these activities, with the exception of arylsulfatase and the two oxidizing enzymes, are shared by macrophages, signet-ring cells, and some hyaline amoebocytes. Conversely, arylsulfatase, peroxidase, and phenoloxidase have been localized in morula cells and some granular amoebocytes.

The presence of hydrolytic enzymes within *B. schlosseri* hyaline amoebocytes, macrophages, and signet-ring cells is indicative of a role of these cell types in phagocytosis. Indeed, all of them can assume carmine or latex particles, *E. coli* cells, and zymosan (our unpublished data); it is not rare to find one or more cells inside the vacuoles of macrophages and signet-ring cells. Furthermore, in *Halocynthia aurantium*, Smith (1970) reported that hyaline amoebocytes can phagocytize carbon particles.

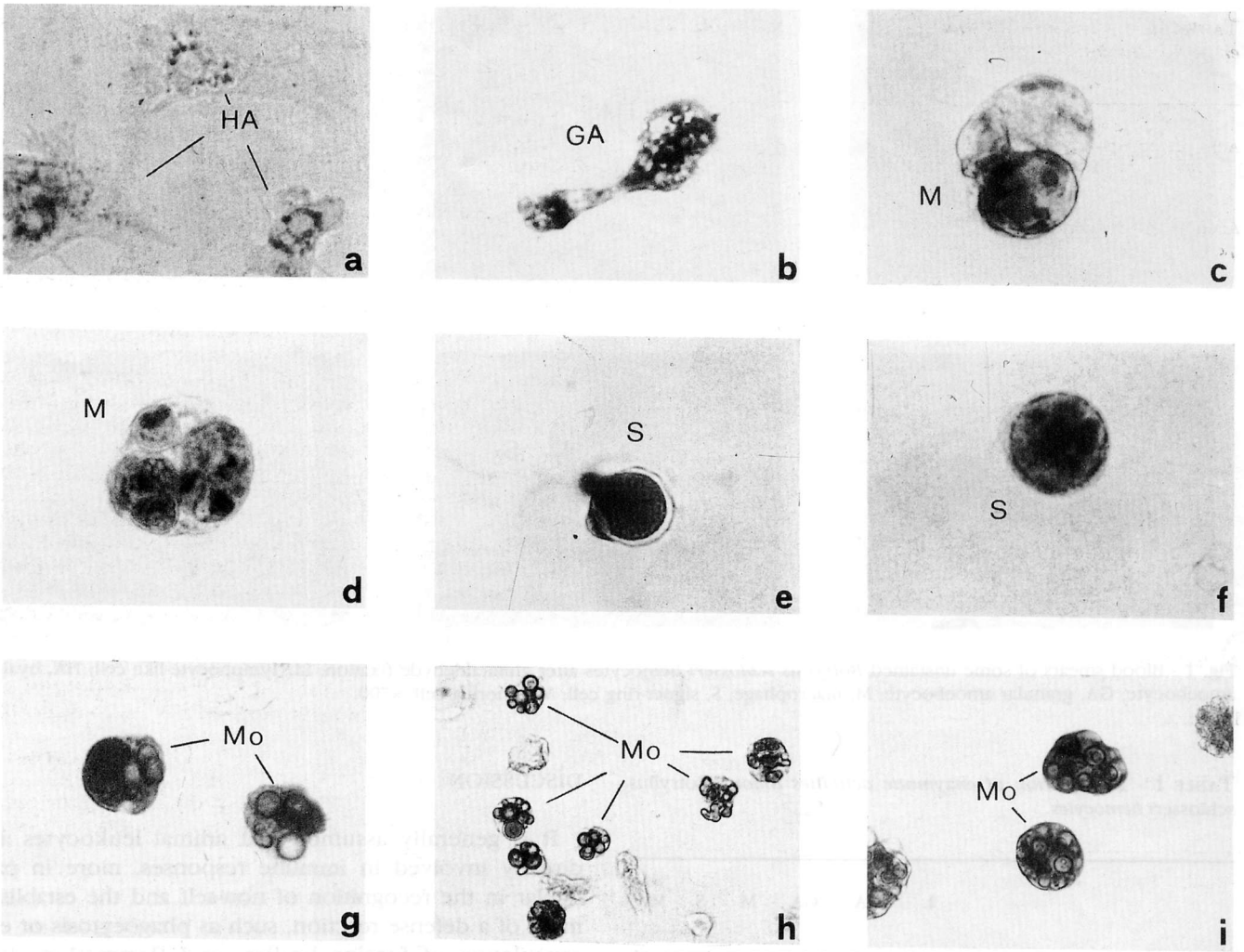

Fig. 2 - Histoenzymatic staining properties of *Botryllus schlosseri* hemocytes: **a, e**, alkaline phosphatase; **b, i**, phenoloxidase; **c**, 5'-nucleotidase; **d**, acid phosphatase; **f**, nonspecific esterases; **g**, peroxidase; **h**, arylsulfatase. HA, hyaline amoebocyte; GA, granular amoebocyte; M, macrophage; S, signet-ring cell; Mo, morula cell. a-g, i: ×700; h: ×400.

With regard to morula cells, they are known to be involved in the rejection reaction between contacting non-fusible colonies of *B. schlosseri* (Scofield & Nagashima, 1983; Sabbadin *et al.*, 1992) and *Botrylloides simodensis* (Hirose *et al.*, 1990). They infiltrate into the tunic through the ampullar epithelium; they degenerate, release their contents, and contribute to the formation of the necrotic mass. The enzymatic content reflects their function as mediators of allorecognition: arylsulfatase plays an initial role in human cell lysis (Zucker-Franklin *et al.*, 1983), whereas peroxidase and phenoloxidase convert their substrates into toxic products with a high lytic activity.

On the basis of morphological studies, Endean (1955) and Kalk (1963) proposed that signet-ring cells are the precursors of morula cells. Consistently, Schlumpberger *et al.* (1984) observed a common behaviour of *B. schlosseri* signet-ring, compartment, and morula cells in response to some lectins and anti-hemocyte monoclonal antibodies. However, this view is not in agreement with results obtained by Uyama *et al.* (1991), who described a cross-reaction of an anti-*Ascidia sydneiensis samea* signet-ring cell monoclonal antibody with small granular amoebocytes of *Halocynthia roretzi*; in addition, no labelling was observed when the same antibody was tested on morula cells of the immunizing species.

The data presented here suggest a close relationship between hyaline amoebocytes, macrophages, and signet-ring cells which appear to be unrelated to morula cells. This conclusion is also supported by the observation that some monoclonal antibodies raised against *B. schlosseri* hemocytes were capable of reacting with the three cell types, but not with morula cells (our unpublished data). Based on cytological and cytochemical observations, Scippa *et al.* (1982) suggested that morula cells originate from an amoebocyte-like precursor. In accordance with this hypothesis, we think that the granular amoebocytes positive for arylsulfatase, peroxidase, and phenoloxidase are the best candidates for this role.

In conclusion, two differentiation pathways may be

TABLE II - *Effect of different concentrations of inhibitors on enzyme activities.*

Acid phosphatase	—		+
	NaF	1 mM	±
	NaF	10 mM	–
Alkaline phosphatase	—		+
	L-cysteine	1 mM	+
	L-cysteine	10 mM	–
	Levamisole	1 mM	+
	Levamisole	10 mM	–
ß-Glucuronidase	—		+
	Mucic acid	5 mM	+
	Mucic acid	10 mM	±
	Mucic acid	100 mM	–
Non specific esterases	—		+
	NaF	1 mM	+
	NaF	10 mM	+
	NaF	100 mM	–
Acid esterase	—		+
	NaF	1 mM	±
	NaF	10 mM	–
Arylsulfatase	—		+
	Na_2SO_4	1 mM	–
Peroxidase	—		+
	H_2O_2 (30%)	5%	–
Phenoloxidase	—		+
	DECA	0.01 mM	–

– Null; ± Partial; + Total.

postulated for *B. schlosseri* hemocytes: one directed to cells with phagocytic activity, i.e. hyaline amoebocytes, macrophages, and signet-ring cells; the other to morula cells involved in inflammatory responses, through a granular amoebocytic stage.

Further studies are now necessary to understand better the relationships among phagocytic cells and to localize, in specific intracellular structures, the enzymatic activities detected here.

REFERENCES

Anderson R. S., 1971 - Cellular responses to foreign bodies in the tunicate *Molgula manhattensis* (DeKay). Biol. Bull., *141*: 91-98.

Anderson R. S., 1981 - Comparative aspects of the structure and function of invertebrate and vertebrate leucocytes. *In*: N. A. Ratcliffe & A. F. Rowley (eds), Invertebrate blood cells. Vol. II. Academic Press, New York, London, pp. 629-641.

Bayne C. J., Moore M. N., Carefoot T. H., Thompson R. J., 1979 - Hemolymph functions in *Mytilus californianus*: the cytochemistry of haemocytes and their responses to foreign implants and hemolymph factors in phagocytosis. J. Invertebr. Pathol., *34*: 1-20.

Burighel P., Milanesi C., Sabbadin A., 1983 - Blood cell ultrastructure of the ascidian *Botryllus schlosseri* L. II. Pigment cells. Acta Zool., *64*: 15-23.

Burstone M. S., 1962 - Enzyme histochemistry and its application in the study of neoplasms. Academic Press, New York - London.

Davis B. J., Ornstein L., 1959 - High resolution enzyme localization with a new diazo reagent, «hexazonium pararosaniline». J. Histochem. Cytochem., *7*: 297-298.

Endean R., 1955 - Studies of the blood and tests of some Australian ascidians. I. The blood of *Pyura stolonifera* (Heller). Aust. J. Mar. Freshwat. Res., *6*: 35-59.

Goldfischer S., 1965 - The cytochemical demonstration of lysosomal arylsulfatase activity by light and electron microscopy. J. Histochem. Cytochem., *13*: 520-523.

Goodbody I., 1974 - The physiology of ascidians. Adv. mar. Biol., *12*: 1-149.

Graham R. C., Karnovsky M. J., 1966 - The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem., *14*: 291-302.

Granath W. O., Yoshino T. P., 1983 - Characterization of molluscan phagocyte subpopulations based on lysosomal enzyme markers. J. exp. Zool., *226*: 205-210.

Hayashi M., 1965 - Histochemical demonstration of N-acetyl-ß-glucosaminidase employing N-acetyl-ß-glucosaminide as substrate. J. Histochem. Cytochem., *13*: 355-360.

Hayashi M., Nakajima Y., Fishman W. H., 1964 - The cytologic demonstration of ß-glucuronidase employing naphthol AS-BI glucuronide and hexazonium pararosanilin; a preliminary report. J. Histochem. Cytochem., *12*: 293-297.

Hirose E., Saito Y., Watanabe H., 1990 - Allogeneic rejection induced by cut surface contact in the compound ascidian, *Botrylloides simodensis*. Invertebr. Reprod. Dev., *17*: 159-164.

Hose J. E., Martin G. G., Nguyen V. A., Lucas J., Rosenstein A. T., 1987 - Cytochemical features of shrimp hemocytes. Biol. Bull., *173*: 178-187.

Kalk M., 1963 - Intracellular sites of acidity in the histogenesis of tunicate vanadocytes. Q. J. microsc. Sci., *104*: 483-493.

Lojda Z., 1962 - Detection of acid phosphatase. Paper delivered at the 1st Conference of the Czechoslovak Histochemical Committee on 28th September 1962, (in Czech).

Lojda Z., 1977 - Studies on glycyl-proline naphthylamidase. I. Lymphocytes. Histochemistry, *54*: 299-309.

Milanesi C., Burighel P., 1978 - Blood cell ultrastructure of the ascidian *Botryllus schlosseri*. I. Hemoblast, granulocytes, macrophage, morula cell and nephrocyte. Acta Zool., *59*: 135-147.

Millar D. A., Ratcliffe N. A., 1989 - The evolution of blood cells: facts and enigmas. Endeavour, *13*: 72-77.

Moloney W. C., McPherson K., Fliegelman L., 1960 - Esterase activity in leukocytes demonstrated by the use of naphthol AS-D chloroacetate substrate. J. Histochem. Cytochem., *8*: 200-207.

Mukai H., 1967 - Experimental alteration of fusibility in compound ascidians. Sci. Rep. Tokyo Daigaku, B, *13*: 51-73.

Sabbadin A., 1955 - Studio sulle cellule del sangue di *Botryllus schlosseri* (Pallas) (Ascidiacea). Arch. ital. Anat. Embriol., *60*: 33-67.

Sabbadin A., Astorri C., 1988 - Chimeras and histocompatibility in the colonial ascidian *Botryllus schlosseri*. Dev. comp. Immunol., *12*: 737-747.

Sabbadin A., Zaniolo G., Ballarin L., 1992 - Genetic and cytological aspects of histocompatibility in ascidians. Boll. Zool., *59*: 167-173.

Scippa S., Botte L., De Vincentiis M., 1982 - Ultrastructure and X-ray microanalysis of blood cells of *Ascidia malaca*. Acta Zool., *63*: 121-131.

Schlumpberger J. M., Weissman I. L., Scofield V. L., 1984 - Separation and labeling of specific subpopulations of *Botryllus* blood cells. J. exp. Zool., *229*: 401-411.

Scofield V. L., Nagashima L., 1983 - Morphology and genetics of rejection reactions between oozoids from the tunicate *Botryllus schlosseri*. Biol. Bull., *165*: 733-744.

Sminia T., Barendsen L., 1980 - A comparative morphological and enzyme histochemical study on blood cells of the freshwater snails *Lymnaea stagnalis, Biomphalaria glabrata*, and *Bulinus truncatus*. J. Morph., *165*: 31-39.

Smith M. J., 1970 - The blood cells and tunic of the ascidian *Halocynthia aurantium* (Pallas). I. Hematology, tunic morphology, and partition of cells between blood and tunic. Biol. Bull., *138*: 354-378.

Taneda Y., Watanabe H., 1982a - Studies on colony specificity in the compound ascidian, *Botryllus primigenus* Oka. II. *In vivo* bioassay for analyzing the mechanism of «nonfusion» reaction. Dev. comp. Immunol., *6*: 243-252.

Taneda Y., Watanabe H., 1982b - Effects of X-irradiation on colony specificity in the compound ascidian, *Botryllus primigenus* Oka. Dev. comp. Immunol., *6*: 665-673.

Taneda Y., Saito Y., Watanabe H., 1985 - Self or non-self discrimination in ascidians. Zool. Sci., *2*: 433-442.

Uyama T., Nishikata T., Satoh N., Michibata H., 1991 - Monoclonal antibody specific to signet ring cells, the vanadocytes of the tunicate, *Ascidia sydneiensis samea*. J. exp. Zool., *259*: 196-201.

Wachstein M., Meisel E., 1957 - Histochemistry of hepatic phosphatases at a physiologic pH with special reference to the demonstration of bile canaliculi. Am. J. clin. Pathol., *27*: 13-23.

Wright R. K., 1981 - Urochordates. *In*: N. A. Ratcliffe & A. F. Rowley (eds), Invertebrate blood cells. Vol. II. Academic Press, New York, London, pp. 565-626.

Wright R. K., Cooper E. L., 1975 - Immunological maturation in the tunicate *Ciona intestinalis*. Am. Zool., *15*: 21-27.

Wright R. K., Cooper E. L., 1983 - Inflammatory reactions of the protochordata. Am. Zool., *23*: 205-211.

Zuckèr-Franklin D., Grusky G., Yang J. S., 1983 - Arylsulfatase in natural killer cells: its possible role in cytotoxicity. Proc. Natl. Acad. Sci., *80*: 6977-6981.