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MINIREVIEW

# Ascidian cytotoxic cells: state of the art and research perspectives

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## Abstract

Ascidian cytotoxic cells are multivacuolated cells, variable in morphology, abundantly represented in the circulation, playing important roles in ascidian immunosurveillance. Upon the recognition of foreign molecules, they are selectively recruited to the infection site where they release the content of their vacuoles. Their cytotoxic activity closely linked to the activity of the enzyme phenoloxidase (PO), a copper-containing enzyme widely distributed in invertebrates, contained inside their vacuoles together with its polyphenol substrata. Recent molecular data indicate that ascidian PO shares similarities with arthropod proPO but, unlike the latter, do not require enzymatic cleavage by extracellular serine proteinases for their activity. Possible ways of ascidian PO activation are discussed.

**Key Words**: tunicates; ascidians; cytotoxic cells; phenoloxidase

## Introduction

In recent years, the interest towards invertebrate immunity has considerably raised driven by comparative, evolutionary and ecological studies. Despite their relying only on innate immunity, invertebrates are capable of complex cell-mediated and humoral responses able to guarantee the survival of individuals and, consequently, of the species. Two main immunocyte types are shared by invertebrates: professional phagocytes and cytotoxic cells which coordinate their efforts to cope with potentially pathogenic microbes having entered the organism.

This paper will focus on ascidian cytotoxic cells, whose importance in immune surveillance, although still not completely clear, is progressively emerging thanks to the efforts of relatively few research groups focusing on a limited number of species.

### *Ascidians: a renewed phylogenetic interest*

Since the publication of the paper by Delsuc *et al.* in 2006, changing their phylogenetic position within the phylum Chordata to the rank of vertebrate sister group, tunicates have experienced a period of scientific renown testified by the great increase in the interest of scientists towards this group of marine, filter-feeding organisms. Ascidians represent the best known and richest in species class of tunicates. Embryos give rise to free swimming tadpole-like larvae with a real notochord in their muscular tail, ventral to the neural tube which are replaced, at metamorphosis, by sessile, barrel-shaped adults sharing, beyond the external tunic, a fissured pharynx, which occupies most of the volume of the organism, and a ventral endostyle able to secrete the mucous net useful in removing suspended particles from seawater passing through the pharyngeal slits (Ballarin and Burighel, 2002).

Adult ascidians also have a well-defined tubular heart and an open circulatory system with hemolymph flowing within lacunae and sinuses inside internal tissues and, in most species, entering the tunic inside vessels derived from the epidermis. Hemocytes or blood cells represent an important constituent of the hemolymph, as they are involved in a variety of important biological processes such as wound repair, nutrient mobilization and transport, asexual reproduction and regeneration, waste accumulation, tunic synthesis, allorecognition and, last but not least, immune responses (Goodbody, 1974; Wright, 1981).

### *The ascidian professional killer cells and their poorly known history*

As invertebrates, ascidians rely only on innate immunity to cope with potentially pathogenic microbes or molecules having entered their organisms and their immune responses are mainly based on the activity of circulating immunocytes which include professional phagocytes, able to

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secrete a variety of immune-relevant molecules (Fuke and Fukumoto, 1993; Ballarin, 2008; Franchi *et al.*, 2011), and a peculiar cytotoxic hemocyte-type which exert its biocidal activity through the enzymatic oxidation of polyphenol substrata and the induction of molecular damage consequent to the production of quinones and highly reactive oxygen species (ROS) (Cammarata *et al.*, 1997; Ballarin and Cima, 2005).

In most cases, these cells, which can collectively be called phenoloxidase (PO)-containing cells (POCC), assume a typical berry-like shape which justifies the name "morula cells" (MCs) used to indicate them and usually constitute one of the most abundant circulating haemocyte type (sometimes representing more than 50 % of the hemocytes). MCs are characterized by large diameters (10-15 μm) and cytoplasms filled by many vacuoles where the enzyme PO resides (Chaga, 1980; Smith and Peddie, 1992; Arizza *et al.*, 1995, Cammarata *et al.*, 1997; Frizzo *et al.*, 2000; Shirae and Saito, 2000; Shirae *et al.*, 2002; Parrinello *et al.*, 2003; Cammarata *et al.*, 2008).

Until the end of the 1980s, POCCs were simply one of the numerous circulating hemocyte types reported in scientific papers, whose function was not well defined (Wright, 1981). They were considered too differentiated to be able to exert any important biological function and represented the research subject of relatively few investigators interested to the unusual acidic content of their vacuoles, which did not share any apparent relationship with the lysosomal machinery, and to their supposed ability to store metals such as vanadium (in most cases) and iron, hence the old names of vanadocytes and ferrocytes with which these cells were also known (Endean, 1960; Overton, 1966; Smith, 1970; Pirie and Bell, 1984; Milanesi and Burighel, 1978). This assumption resulted not true in the case of vanadium and the term vanadocytes today applies to cells different from MCs (Michibata *et al.*, 1990; Nette *et al.*, 1999; Yamaguchi *et al.*, 2006).

In 1980, in a paper written in Russian (which, unfortunately, limited its diffusion), Chaga reported, for the first time, that ascidian MCs have PO activity, a fundamental step towards the comprehension on the cytotoxic role of POCCs, but ten additional years were required for the first clear demonstration of the involvement of MCs in the induction of toxicity and the decisive advancement was made using botryllid ascidians as model organisms and one of the outcome of allorecognition, *i.e.*, the rejection (nonfusion) reaction between contacting, genetically incompatible colonies of the same species resulting in the formation of a series of pigmented necrotic spots along the borders of the facing colonies, as the reference phenomenon (Hirose *et al.*, 1990). Although known for many years, botryllid allorecognition was studied mainly in terms of morphology and formal genetics (Oka and Watanabe, 1957, 1960; Sabbadin, 1962; Mukai and Watanabe, 1974; Taneda *et al.*, 1985) and papers dealing with the importance of hemocytes as mediators of the reaction appeared only from 1970s (Tanaka and Watanabe, 1973; Taneda *et al.*, 1982a, b); some observations suggesting a role of MCs in the rejection reaction were published in 1980s (Taneda *et al.*, 1982a; Scofield and Nagashima, 1983). The paper by Hirose *et al.* (1990) was the first histological analysis of the rejection reaction which unequivocally indicated MCs played a decisive role in the considered phenomenon. Today, we know that, during this reaction, MCs are selectively recruited from the general circulation to the lumen of the peripheral ampullae (the blind, sausage-like termini of marginal vascular vessels) facing the alien colony, then they leave the ampullar tips, cross the ampullar epithelium and enter the tunic where they degranulate and release their vacuolar content which, as reported below, is responsible of the induction of the observed cytotoxicity, a series of events sharing many features with vertebrate inflammation (Sabbadin *et al.*, 1992). In *B. schlosseri*, where the nonfusion reaction has been particularly studied, MCs are activated by the recognition of soluble factors diffusing from the alien colony and express chemotactic molecules recognized by antibodies raised against mammalian proinflammatory cytokines, such as IL1α and TNFα, responsible of their selective recruitment inside the ampullar lumen and migration to the tunic (Cima *et al.*, 2006; Ballarin, 2008). The selective recruitment of POCCs and their role in inflammatory and cytotoxic reactions consequent to graft transplantation or injection of nonself molecules was confirmed also in solitary ascidians (Cammarata *et al.*, 1995, 1997, 2008; Parrinello, 1996; Parrinello *et al.*, 1996).

The cytotoxic role of POCCs, suggested by research on colonial and solitary ascidians, helped to insert in the same frame apparently dissimilar phenomena such as botryllid allorecognition, graft rejection and inflammatory reactions in solitary ascidians, contact reaction in mixed cultures of solitary species hemocytes, all induced by nonself recognition and involving POCCs as effector cells (Parrinello, 1996). Today, we can consider these cells as a sort of immunological sentinel able to recognize foreign molecules and quickly respond with the induction of cytotoxicity (Ballarin *et al.*, 2001, 2005).

*Phenoloxidase (PO): the ascidian molecular weapon against nonself*

POs are copper-containing enzymes, widely distributed in invertebrates, with orthodiphenoloxidase (catecholase) activity, able to convert phenolic substrata to quinones which, then, polymerize to form melanin. They exert a role in immune responses related to the induction of cytotoxicity, once released by specific hemocytes, through the production of ROS and semiquinones which can either induce oxidative stress or rapidly react with biomolecules altering their functionality. Quinones and melanin themselves are toxic: the former can undergo oxidation and generate reactive oxygen species (Nappi and Vass, 1993; Nappi and Ottaviani, 2000) or react with -SH groups on biomolecules (Kato *et al.*, 1986), whereas the deposition of the latter in nodules of non-self material enveloped by host immunocytes contributes to the formation of the so-called brown bodies which prevent the survival of foreign

organisms in their interior (Nappi and Ottaviani, 2000).

The role of PO in ascidian cytotoxicity has been clearly demonstrated in both solitary and colonial species with the use of specific inhibitors in appropriate *in vitro* assays (Akita and Hoshi, 1995; Cammarata *et al.*, 1997, 2008; Ballarin *et al.*, 1998, 2005; Hata *et al.*, 1998; Parrinello *et al.*, 2003). In *B. schlosseri*, during the rejection reaction, cytotoxicity is the consequence of a severe oxidative stress consequent to the depletion of reduced intracellular thiols (Ballarin *et al.*, 2002). The involvement of the enzyme in the nonfusion reaction of other colonial species was also demonstrated (Shirae and Saito, 2000; Shirae *et al.*, 2002). Cytochemical analyses indicate that MC vacuoles also contain polyphenols, probably the PO natural substrata (Cammarata *et al.*, 1997; Frizzo *et al.*, 2000; Ballarin and Cima, 2005).

*Ascidian PO: certainties and uncertainties*

The regulation of PO activity and its role in immune defense has been particularly studied in crustaceans, where the enzyme is usually stored, as inactive zymogen (proPO), inside the granules of PO-containing hemocytes, the morphology of which varies greatly among the different species (Aspán *et al.*, 1995; Kawabata *et al.*, 1995). The activation of proPO to PO requires the degranulation of PO-containing cells, the release of the zymogen in the extracellular milieu and its conversion to active PO by extracellular serine proteases which are the last components of a finely regulated series of events, collectively called the proPO activating system, triggered by the recognition of foreign molecules on the surface of microbial cells (Cerenius and Söderhäll, 2004; Cerenius *et al.*, 2008).

Recent molecular investigations indicate that the primary, secondary and tertiary sequences of ascidian PO show many similarities with those of arthropod hemocyanins and proPOs, including the organization in three domains and the conservation of the histidines at both the copper-binding sites (Immesberger and Burmester, 2004).

Similarly to arthropod POs, having a molecular weight ranging around 70 - 80 kDa (Decker *et al.*, 2007), the molecular weight of ascidian POs ranges between 60 and 90 kDa (Hata e *et al.*, 1998; Frizzo *et al.*, 1999; Parrinello *et al.* 2003; Cammarata *et al.*, 2008): these values agree molecular data predicting a molecular weight of 92 and 87 kDa for *Ciona intestinalis* POs (Immesberger and Burmester, 1994). However, unlike arthropod POs, which assemble *in vivo* to form hexamers (Decker *et al.*, 2007), electrophoretic data indicate that ascidian PO monomers interact each other to form dimers (Frizzo *et al.*, 1999; Parrinello *et al.*, 2003; Cammarata *et al.*, 2008).

In addition, despite the absence of incontrovertible data, there is a general consensus that, like arthropods, a proPO activating system is present in all the invertebrates species in which PO activity has been demonstrated (Smith and Söderhäll, 1991; Cerenius and Söderhäll, 2004), ascidians included (Smith and Söderhäll, 1991; Jackson *et al.*, 1993; Arizza *et al.*, 1995; Ballarin *et al.*, 1998; Shirae and Saito, 2000; Shirae *et al.*, 2002; Parrinello *et al.*, 2003; Cammarata *et al.*, 2008).

However, some doubts that this holds true also for ascidians come from the observation that the activation by exogenous serine proteases is never required for detecting the activity of ascidian PO in both hemocyte monolayers and hemolymph or hemocyte lysates (Smith and Söderhäll, 1991; Jackson *et al.*, 1993; Arizza *et al.*, 1995; Ballarin *et al.*, 1994, 1998; Ballarin and Cima, 2005; Parrinello *et al.*, 2003; Cammarata *et al.*, 2008). Moreover, PO-positive MCs are easily observed in histoenzymatic assays on paraffin sections of colonial ascidians (Frizzo *et al.*, 2000; Shirae *et al.*, 2002), implying that either the enzyme is constitutively active, at least in part, inside MC vacuoles or it can be easily activated during hemocyte collection or colony manipulation. The reported increase in enzyme activity observed after treatment with exogenous serine proteinases such as trypsin and chymotrypsin (Smith and Söderhäll, 1991; Ballarin *et al.*, 1994, 1998; Arizza *et al.*, 1995; Cammarata *et al.*, 2003, 2008; Parrinello *et al.*, 2003) which also leads to an increase in electrophoretic mobility of the enzyme (Parrinello *et al.*, 2003), interpreted as evidences in favor of the presence of a proPO in ascidians, can be the result of unspecific effects of the exogenous proteinases which, acting on some cleavage sites and through the removal of enzyme fragment(s), decrease the protein molecular weight, with the consequent increase of its mobility during electrophoresis, and render the copper-binding sites more accessible to the substrate which results in the observed increment of enzyme activity.

Since, as reported above, also the polyphenol substrata are contained inside the vacuoles of POCCs (Cammarata *et al.*, 1997; Ballarin and Cima, 2005), a control of the enzyme activity inside POCC vacuoles is required which can be achieved in at least two possible ways: i) the inhibition of the enzyme inside POCC vacuoles, and ii) the masking of the polyphenol substrata through chemical modifications.

**Conclusions and perspectives**

The two possibilities suggested above are supported by the fact that it is known that POCC vacuoles contain reducing compounds such as thiols (Ballarin *et al.*, 1995) and tunichromes (Bruening *et al.*, 1986; Oltz et al., 1988; Martoja et al., 1994) having inhibitory effects on PO (Ballarin *et al.*, 1998; Hata *et al.*, 1998). Tunichromes, in turn, are polyphenolic compounds (Bruening *et al.*, 1986; Oltz *et al.*, 1988) and, therefore, good candidates as natural substrata of PO. The modulation of PO activity by polyphenol substrata is an interesting point worth of deeper investigations. As for the second point, it is known that MCs contain sulfur in the form of bound sulfates inside their vacuoles (Bell *et al.*, 1982; Scippa et al.,1985; Frank et al., 1987; Ballarin et al., 1995). The presence of the enzyme arylsulfatase inside *Botryllus* MC vacuoles (Ballarin *et al.*, 1993) suggests that sulfates may be bound to the aromatic rings of polyphenols. Sulfates exert inhibitory effect on ascidian PO (Hata et al., 1998)

and the action of the enzyme may be important in detaching them thus rendering phenols available to PO.

In conclusion, the importance of ascidian POCCs in immune responses is, today, definitively established; however, the regulation of the activity of PO, their main cytotoxic tool, and the relationships between polyphenols and PO remain controversial and require future investigations for a better clarification of these points.

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**References**

Akita N, Hoshi M. 1995. Hemocytes release phenoloxidase upon contact reaction, an allogeneic interaction, in the ascidian *Halocynthia roretzi*. Cell Struct. Funct. 20: 81-87.

Arizza V, Cammarata M, Tomasino MC, Parrinello N. 1995. Phenoloxidase characterization in vacuolar hemocytes from the solitary ascidian *Styela plicata*. J. Invertebr. Pathol. 66: 297-302.

Aspán A, Huang TS, Cerenius L, Söderhäll K. 1995. cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation. Proc Natl Acad Sci USA 92: 939–943.

Ballarin L. (2008) – Immunobiology of compound ascidians, with particular reference to *Botryllus schlosseri*: state of art. Inv. Surv. J. 5: 54-74.

Ballarin L., Burighel P. (2002) – Tunicata and Cephalochordata. In: "Biological and Medical Sciences", G. Contrafatto and A. Minelli eds., in: "Encyclopedia of Life Support Systems (EOLSS)", Eolss Publishers, Oxford, UK, http://www.eolss.net.

Ballarin L. Cima F. 2005. Cytochemical properties of *Botryllus schlosseri* haemocytes: indications for morpho-functional characterisation. Eur J Histochem, 49: 255-264.

Ballarin L, Cima F, Sabbadin A. 1993. Histoenzymatic staining and characterization of the colonial ascidian *Botryllus schlosseri* hemocytes. Boll Zool 60: 19-24.

Ballarin L, Cima F, Sabbadin A. 1994. Phenoloxidase in the colonial ascidian *Botryllus schlosseri* (Urochordata, Ascidiacea). Anim Biol 3: 41-48.

Ballarin L., Cima F., Sabbadin A. 1998. Phenoloxidase and cytotoxicity in the compound ascidian *Botryllus schlosseri*. Dev. Comp. Immunol., 22: 479-492.

Ballarin L., Franchini A., Ottaviani E., Sabbadin A. (2001) - Morula cells as the main immunomodulatory haemocytes in ascidians: evidences from the colonial species *Botryllus schlosseri*. Biol. Bull., 201: 59-64.

Ballarin L., Cima F., Floreani M., Sabbadin A. (2002) - Oxidative stress induces cytotoxicity during rejection reaction in the compound ascidian *Botryllus schlosseri*. Comp Biochem Physiol 133C: 411-418.

Ballarin L, Menin A, Franchi N, Bertoloni G, Cima F. 2005. Morula cells and non-self recognition in the compound ascidian *Botryllus schlosseri*. Invertebr. Survival J, 2: 1-5.

Bell MV, Pirie BJS, McPhail DB, Goodman BA, Falk-Petersen IB, Sargent JR. 1982. Contents of vanadium and sulphur in the blood cells of *Ascidia mentula* and *Ascidiella aspersa*. J Mar Biol Ass UK 62: 709-716.

Bruening RC, Oltz EM, Furukawa J, Nakanishi K, Kustin K. 1986. Isolation of tunichrome B-1, a reducing blood pigment of the sea squirt, *Ascidia nigra*. J Nat Prod 49: 193–204.

Cammarata M, Candore G, Arizza V, Caruso C, Parrinello N. 1995. Cytotoxic activity of *Styela plicata* hemocytes against mammalian cell targets: II. Properties of the *in vitro* reaction against human tumour cell lines. Anim Biol 4: 139-144.

Cammarata M, Arizza V, Parrinello N, Candore G, Caruso C. 1997. Phenoloxidase-dependent cytotoxic mechanism in ascidian (*Styela plicata*) hemocytes active against erythrocytes and K562 tumor cells. Eur J Cell Biol, 74: 302-307.

Cammarata M, Arizza V, Cianciolo C, Parrinello D, Vazzana M, Vizzini A, Salerno G, Parrinello N. 2008. The prophenoloxidase system is activated during the tunic inflammatory reaction of *Ciona intestinalis*. Cell Tissue Res 333: 481-492.

Cerenius L, Söderhäll K. 2004. The prophenoloxidase-activating system in invertebrates. Immunological Rev 198: 116-126.

Cerenius L, Lee BL, Söderhäll K. 2008. The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol. 29: 263-271.

Chaga OY. 1980. Ortho-diphenoloxidase system of Ascidians. Tsitologia 22: 619-625.

Decker H, Schweikardt T, Nillius D, Salzbrunn U, Jaenicke E, Tuczek F. 2007. Similar enzyme activation and catalysis in hemocyanins and tyrosinases. Gene 398: 183-191.

Delsuc F, Brinkmann H, Chourrout D, Philippe H. 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature 439:965-968.

Endean R. 1960. The blood-cells of the ascidian, *Phallusia mammillata*. Quart J Microsc Sci 101: 177-197.

Franchi N.; Schiavon F.; Carletto M.; Gasparini F.; Bertoloni G.; Tosatto S.C.E.; Ballarin L. (2011) - Immune roles of a rhamnose-binding lectin in the colonial ascidian *Botryllus schlosseri*. Immunobiology, 216: 725-736.

Frank P, Hedman B, Carlson RMK, Tyson TA, Roe L, Hodgson KO. 1987. A large reservoir of sulfate and sulfonate resides within plasma cells from *Ascidia ceratodes*, revealed by X-ray absorption near-edge structure spectroscopy. Biochemistry 26: 4975-4979.

Frizzo A, Guidolin L, Ballarin L, Sabbadin A. 1999. Purification and characterisation of phenoloxidase from the colonial ascidian *Botryllus schlosseri*. Mar Biol 135: 483-488.

Frizzo A, Guidolin L, Ballarin L., Baldan B, Sabbadin A. 2000. Immunolocation of phenoloxidase in

vacuoles of the compound ascidian *Botryllus schlosseri* morula cells. Ital J Zool, 67: 273-276.

Fuke M, Fukumoto M. 1993. Correlative fine structural, behavioral, and histochemical analysis of ascidian blood cells. Acta Zoologica (Stockholm) 74: 61–71.

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

Hata S, Azumi K, Yokosawa H. 1998. Ascidian phenoloxidase: its release from hemocytes, isolation, characterization and physiological role. Comp Biochem Physiol, 119B: 767-776.

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

Immesberger A, Burmester T. 2004. Putative phenoloxidase in the tunicate *Ciona intestinalis* and the origin of the arthropod hemocyanin superfamily. J Comp Physiol 174B: 169-180.

Jackson AD, Smith VJ, Peddie CM. 1993. *In vitro* phenoloxidase activity in the blood of *Ciona intestinalis* and other ascidians. Dev Comp Immunol 17: 97-108.

Kawabata T, Yasuhara Y, Ochiai M, Matsuura S, Ashida M. 1995. Molecular cloning of insect pro-phenol oxidase: a copper-containing protein homologous to arthropod hemocyanin. Proc Natl Acad Sci USA 92: 7774-7778.

Michibata H, Uyama T, Hirata J. 1990. Vanadium-containing blood cells (vanadocytes) show non fluorescence due to the tunichrome in the ascidian *Ascidia sydneiensis samea*. Zool Sci 7: 55-61.

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

Mukai H, Watanabe H. 1974. On the occurrence of colony specificity in some compound ascidians. Biol Bull 147: 411-421.

Nette G, Scippa S, Genovese M, de Vincentiis M. 1999. Cytochemical localization of vanadium(III) in blood cells of ascidian *Phallusia mammillata* Cuvier, and its relevance to hematic cell lineage determination. Comp Biochem Physiol 122C: 231-237.

Oka H., Watanabe H., 1957. Colony-specificity in compound ascidians as tested by fusion experiments. A preliminary report. Proc. Imp. Acad. Japan, 33: 657-659.

Oka H., Watanabe H., 1960. Problems of colony-specificity in compound ascidians. Bull. Mar. Stat. Asamushi, Tohoku Univ., 10: 153-155.

Oltz EM, Bruening RC, Smith MJ, Kustin K, Nakanishi K. 1988. The tunichromes. A class of reducing blood pigments from sea squirts: isolation, structures, and vanadium chemistry. J Am Chem Soc; 110: 6162-6172.

Overton, J. 1966. The fine structure of blood cells in the ascidian *Perophora viridis*. J Morphol 119: 305–326.

Parrinello N. 1996. Cytotoxic activity of tunicate hemocytes. In: B. Rinkevich, WEG Müller (Eds.) Invertebrate immunology. Springer-Verlag, Berlin, Heidelberg, New York, pp. 190-217.

Parrinello N, Cammarrata M, Arizza V. 1996. Univacuolar refractile hemocytes from the tunicate. *Ciona intestinalis* are cytotoxic for mammalian. erythrocytes *in vitro*. Biol Bull 190: 418-425.

Parrinello N, Arizza V, Chinnici C, Parrinello D, Cammarrata M. 2003. Phenoloxidases in ascidian hemocytes: characterization of the pro-phenoloxidase activating system. Comp Biochem Physiol, 135B: 583-591.

Pirie BJS, Bell MV (1984) The localization of inorganic elements, particularly vanadium and sulphur, in haemolymph from the ascidians *Ascidia mentula* (Müller) and *Ascidiella aspersa* (Müller). J Exp Mar Biol Ecol 74: 187-194.

Sabbadin A. 1962. Le basi genetiche della capacità di fusione fra colonie di *Botryllus schlossen* (Ascidiacea). Rend. Accad. Naz. Lincei, 32: 1031-1035.

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

Scippa S, Botte L, Zierold K, de Vincentiis M. 1985. X-ray microanalytical studies on cryofixed blood cells of the ascidian *Phallusia mammillata*. I. Elemental composition of morula cells. Cell Tissue Res 239: 459-461.

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

Shirae M, Saito Y. 2000. A comparison of hemocytes and their phenoloxidase activity among Botryllid Ascidians. Zool Science, 17: 881-891.

Shirae M, Ballarin L, Frizzo A, Saito Y, Hirose E. 2002. Involvement of quinones and phenoloxidase in the allorejection reaction in a colonial ascidian, *Botrylloides simodensis*: histochemical and immunohistochemical study. Mar Biol, 141: 659-665.

Smith M J. 1970. The blood cells and tunic of the ascidian *Halocynthia aurantium* (Pallas). II. The histochemistry of blood cells and tunic. Biol Bull 138: 379–388.

Smith VJ, Söderhäll K. 1991. A comparison of phenoloxidase activity in the blood of marine invertebrates. Dev Comp Immunol 15: 251-262.

Smith VJ, Peddie CM. 1992. Cell cooperation during host defense in the solitary tunicate *Ciona intestinalis* (L). Biol Bull 183: 211-219.

Tanaka K., Watanabe H., 1973 - Allogeneic inhibition in a compound ascidian, *Botryllus primigenus* Oka. I. Processes and features of «nonfusion» reaction. Cell Immunol., 7: 410-426.

Taneda Y., Watanabe H., 1982a - Studies on colony specificity in the compound ascidian, *Botryllus primigenus* Oka.I. Initiation of «nonfusion» reaction with special reference to blood cell infiltration. Devel. comp. Immunol., 6: 43-52.

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

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

Wright RK. 1981: Urochordates. In “invertebrate blood cells”. NA Ratcliffe and AF Rowley Eds., Academic press. Vol 2, 565-626.

Yamaguchi N, Amakawa Y, Yamada H, Ueki T, Michibata H. 2006. Localization of vanabins, vanadium-binding proteins, in the blood cells of the vanadium-rich ascidian, *Ascidia sydneiensis samea*. Zool Sci 23: 909-915.