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REVIEW

Immunobiology of compound ascidians, with particular reference to *Botryllus schlosseri*: state of art

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Abstract

The phylogenetic position of invertebrate chordates closely related to vertebrates explains the increasing interest towards tunicate immunobiology. Most of the tunicates are ascidians which, like all other invertebrates, rely only on innate immunity for their defense. Compound ascidians differ from solitary species for the presence of colony specificity, i.e. the ability for intraspecific non-self recognition. The immunobiology of compound ascidians has been particularly studied in *Botryllus schlosseri*, which is an emerging model organism for this kind of studies. In *B. schlosseri* and related species, immunocytes are represented by phagocytes and cytotoxic morula cells, the former able to ingest foreign cell and particles, the latter representing the effectors of the inflammatory reaction which follows the contact between genetically incompatible colonies. Activated phagocytes release lectins with opsonic activity and are involved in the clearance of apoptotic cells during the colonial generational change. Morula cells recognize the presence of foreign molecules as well as allogeneic soluble factors diffusing from an alien colony and as a consequence they: i) release cytokines in the medium which have chemotactic activity and activate phagocytes; ii) degranulate and release phenoloxidase which induces necrotic cell death by oxidative stress. A better knowledge of *Botryllus* genome will allow a deeper insight into open problems in immunobiology of compound ascidians.

Key words: colonial ascidians; *Botryllus*; immunobiology; immunocytes; allorecognition; phagocytosis

Introduction

Deuterostomes of the phylum Chordata feature: i) the presence of a notochord, permanent or temporary, which prevents shortening of the body when longitudinal muscles contract; ii) a dorsal nerve cord, in the form of a hollow tube, enlarged to some extent at the front end to form a brain; iii) a ventral digestive tract, which expands anteriorly to form a pharynx, provided with gill slits or pharyngeal pouches and a ventral gland secreting iodoproteins (endostyle or thyroid). Both the notochord and the neural tube extend to the tail, the muscular post-anal part of the body.

Tunicates and cephalochordates, collectively named protochordates, represent the invertebrate relatives of *Vertebrata* (Schubert *et al.*, 2006), the major chordate subphylum with nearly 50,000 species. Unlike vertebrates, which radiated on lands, freshwater and seawater and are active predators and/or grazers, protochordates are marine filter-feeding animals, most with a sedentary life-style.

Tunicates (or urochordates) owe their name to the tunic or test, the peculiar tissue which embeds the larval and adult body. Although of epidermal origin, it resembles connective tissue in having an amorphous matrix in which tunicine fibres, similar to cellulose in composition, and interspersed ameboid cells are found. The results of a recent phylogenetic study, based on analysis of many nuclear genes, suggest that tunicates are the closest living relatives of vertebrates (Delsuc *et al.*, 2006), thus increasing interest towards these organisms.

The majority of tunicates are represented by ascidians or sea squirts, sessile animals widespread all over the world with approximately 3,000 species, both solitary and colonial. Today, the genome of some solitary ascidian species (*Ciona intestinalis*, *Ciona savignyi*, *Halocynthia roretzi*) has been fully or partially sequenced (Dehal *et al.*, 2002; Yokobori *et al.*, 2003) which renders these organisms important

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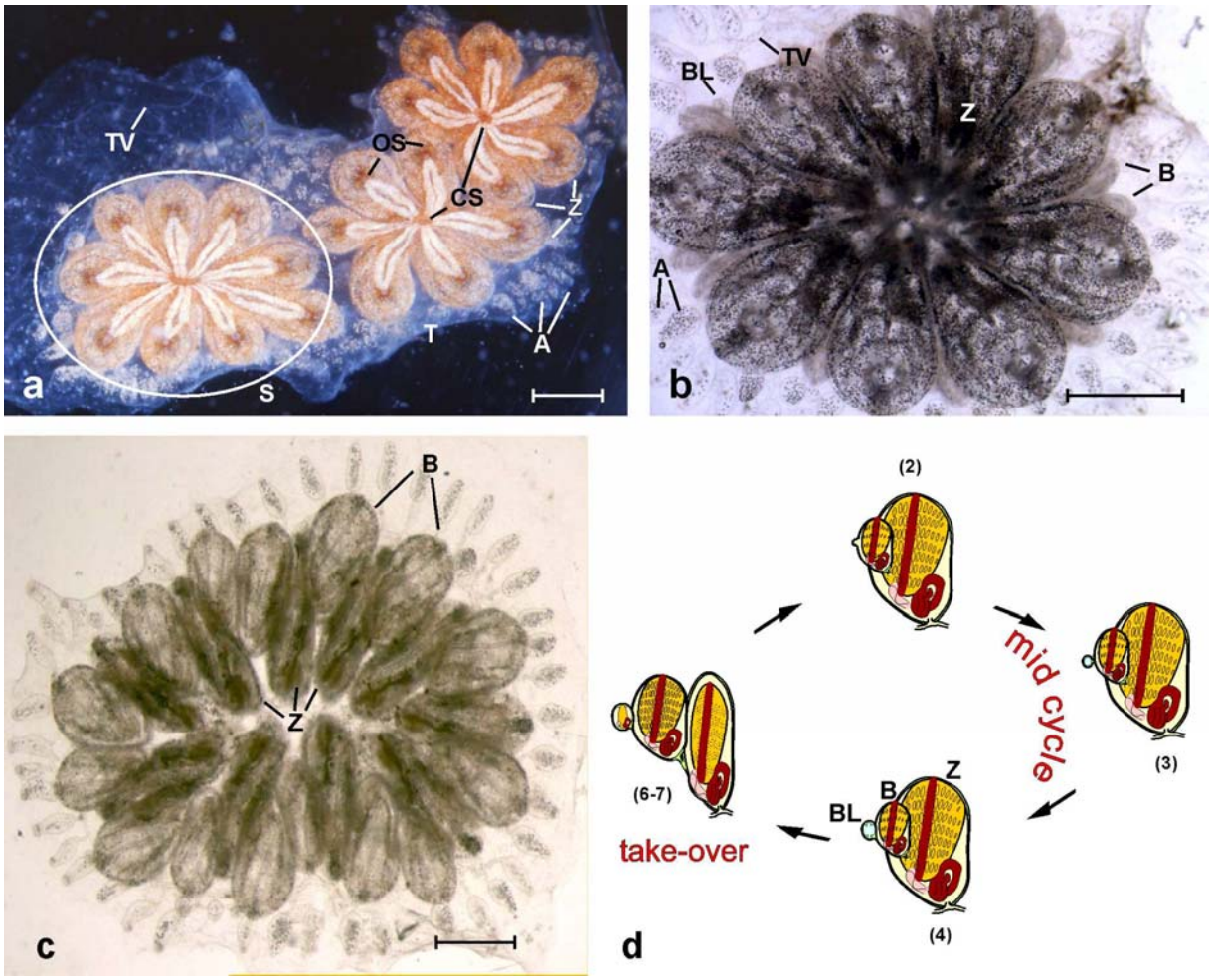

Fig. 1 **a**: Dorsal view of a *B. schlosseri* colony; **b**: colonial system showing buds and budlets; **c**: colony at generational change or take-over; **d**: schematic representation of the main stages of *B. schlosseri* colonial blastogenetic cycle. Days from the beginning of the cycle are in brackets. A: ampullae; B: buds; BL: budlet; CS: cloacal siphon; OS: oral siphon; S: system; T: tunic; TV: tunic vessel; Z: zooids; Bar = 1 mm.

models in studying the molecular control of embryogenesis and differentiation (Nishida, 2002a, b; Oda-Ishii *et al.*, 2005; Passamaneck and Di Gregorio, 2005; Satoh and Levine, 2005; Dufour *et al.*, 2006).

Immunity in ascidians

The evolutionary relationships of tunicates with vertebrates explain the increasing interest in the immunobiology of ascidians. Unlike vertebrates and like all invertebrates, tunicates rely only on innate immunity, characterized by neither somatic recombination nor long-term immune memory, low discriminative power, and a limited array of effector responses. Nevertheless, precursors of vertebrate immune components have been described in ascidians, e.g. proteins of the lectin pathway of the complement activation system (Nonaka *et al.*, 1999; Nair *et al.*, 2000; Nonaka, 2001; Sekine *et al.*, 2001; Raftos *et al.*, 2002; Pinto *et al.*, 2003), orthologues of the C-type lectin-like receptor CD94 (Khalturin *et al.*, 2003; Zucchetti *et al.*, 2007) and other genes involved in innate immunity (Azumi *et al.*, 2003; Shida *et al.*, 2003; Oren *et al.*, 2007)

The immunobiology of solitary ascidians has been studied in the past, mostly in relation with inflammation, cytotoxicity, encapsulation, phagocytosis and allorecognition (Parrinello *et al.*, 1977, 1984, 1993; Parrinello and Patricolo, 1984; Raftos *et al.*, 1987a, b; Raftos, 1991; Peddie and Smith, 1993, 1994; Ohtake *et al.*, 1994; Cammarata *et al.*, 1995; Dan-Sohkawa *et al.*, 1995; Lipari *et al.*, 1995). This kind of research has been improved by the availability of the genomes of *Ciona* and *Halocynthia* (Azumi *et al.*, 2003; Shida *et al.*, 2003), which allowed to study the molecular control of immune responses. A review on the immunity of solitary ascidians is presented elsewhere in this journal.

Although less well studied at the molecular level, compound ascidians have some advantages with respect to solitary species. For instance, several developmental pathways (embryogenesis, blastogenesis, and regeneration) leading to adult, filter-feeding zooids may be compared in the same

organism and at various levels (morphological, biochemical, molecular).

Immunity in compound ascidians

Growing interest in the defense reactions of compound ascidians has been stimulated by the presence of colony specificity, i.e. the ability for intraspecific non-self recognition (allorecognition), and consequent attempts to understand the biological basis of the phenomenon, which frequently results in an inflammatory response (see below).

Most research has been carried out in botryllid ascidians, mainly in the cosmopolitan species *Botryllus schlosseri*, which is emerging as a model organism for studying immunobiology (Manni *et al.*, 2007): i) it is easily found in the field; ii) it can grow and reproduce in laboratory conditions; iii) colonies are embedded in a soft and transparent tunic which allows direct observation of biological processes; iv) large colonies can be cut into clonal fragments which are able to grow and reproduce, so that subclones of the same colony can be used in control and experimental series; v) colonies undergo recurrent generation changes, with diffuse cell death by natural apoptosis in zooid tissues and efferocytosis by circulating phagocytes; vi) colonies have a well-defined tunic circulatory system and hemocytes can easily be collected with glass micropipettes after the tunic vessels have been punctured with a fine tungsten needle. This review, therefore, focuses mainly on this model organism as an important reference species. When available, various data from studies on other compound ascidians are reported.

The colony of *B. schlosseri*

Three blastogenetic generations are usually present in a colony of *B. schlosseri* (Fig. 1a): i) adult, filter-feeding zooids, 1.5-2 mm in length, are grouped in star-shaped systems of 10-12 individuals and oral siphons are located in the anterior part of each zooid (i.e. towards the periphery of the system), whereas the central cloacal siphon connects the cloacal chamber, into which individual atrial siphons open, with the exterior; ii) primary palleal buds on zooids are able to replace the parental generation, and iii) secondary buds (budlets) on buds grow to buds and, lastly, to zooids (Fig. 1b). Budding occurs continuously, in an orderly and synchronized way, as well as the cyclical change of adult generations or take-over (Fig. 1c). One of the first report of budding and generational changes in *Botryllus* was that of Spallanzani in 1784 (in Gibin, 1997). After that, *Botryllus* blastogenesis was described by several researchers (Metschnikow, 1869; Della Valle, 1882; Oka, 1892; Hjort, 1893; Pizon, 1893; Ärnbäck-Christie-Linde, 1923; Berrill, 1941a, b; Watterson, 1945; Sabbadin, 1955a; Milkman, 1967; Izzard, 1973).

The time interval between one generation change and the next is called a blastogenetic cycle: its length is temperature-dependent and lasts one week at 19 °C (Fig. 1d). At take-over, old zooids close their siphons, cease filtering, contract (Fig. 1c), undergo massive, diffuse apoptosis of their tissues (in parallel with some necrosis in the digestive tube), and are gradually resorbed by either wandering professional or fixed occasional phagocytes (Burighel and Schiavinato, 1984; Lauzon *et al.*, 1993, Cima *et al.*, 2003). They are replaced by primary buds, which open their siphons 24–36 h after the beginning of take-over; in the meantime, secondary buds become primary buds and give rise to a new budlet generation (Sabbadin, 1955a, 1958; Manni *et al.*, 2007). Therefore, the life-span of a zooid, from its appearance as a budlet primordium to its resorption at take-over, covers approximately three weeks. As colonies cannot feed until the new adult generation opens its siphons, they rely on recycling of the components of dying zooids, which are used for growth of the developing buds (Sabbadin, 1956; Lauzon *et al.*, 2002).

Each colony is a clone, as it derives from a founder oozoid, which is the outcome of the metamorphosis of a tadpole-like, swimming larva (Fig. 2a) deriving from sexual reproduction. Oozoids (Fig. 2b) bear a palleal bud on their right side, which matures into a filter-feeding blastozooids (Fig. 2c), replacing the parental zooid and producing two or more palleal buds on each side of the body, able to produce budlets and grow to mature blastozooids (Sabbadin, 1969). Zooids, buds and budlets are embedded in a common tunic and interconnected by

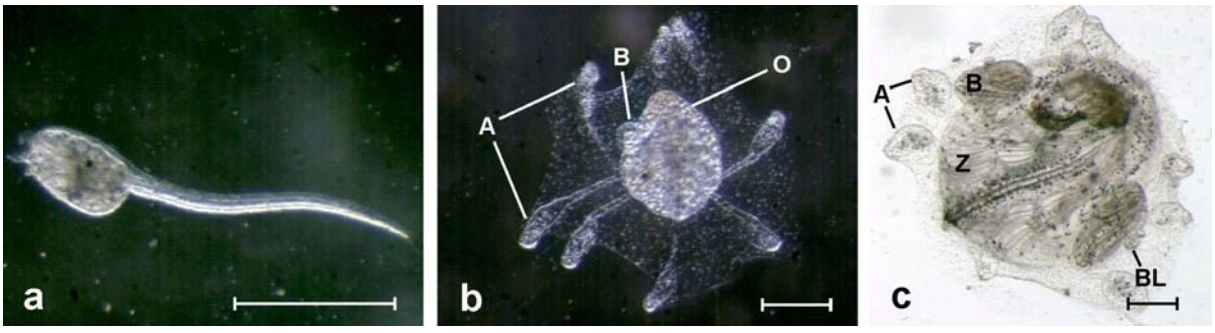

Fig. 2 Swimming larva (**a**), oozooid (**b**) and first blastozooids (**c**) of *B. schlosseri*. A: ampullae; B: bud; BL: budlet; O: oozooid; Z: blastozooids. Bar = 0.5 mm.

a network of vessels of epidermal origin crossing the tunic and joined to a colonial marginal vessel which runs along the contour of the colony and gives rise to many sausage-like blind endings, known as ampullae, where blood cells are stored (Brunetti and Burighel, 1969; Fig. 3). Since adult zooids are cyclically resorbed and replaced by their growing buds, a healthy colony can be formed of hundreds or thousands of zooids and buds, synchronized in their development by the common vascular system.

The development of buds and zooids in *Botryllus* is highly coordinated (Berrill, 1941a, b; Watanabe, 1953; Sabbadin, 1955a; Milkman, 1967) so that the developmental stages of a colony during the blastogenetic cycle can be univocally defined by the developmental stages of its blastogenetic generations. Stages more than one day from the preceding or the following take-over are called mid-cycle stages (Lauzon *et al.*, 1992).

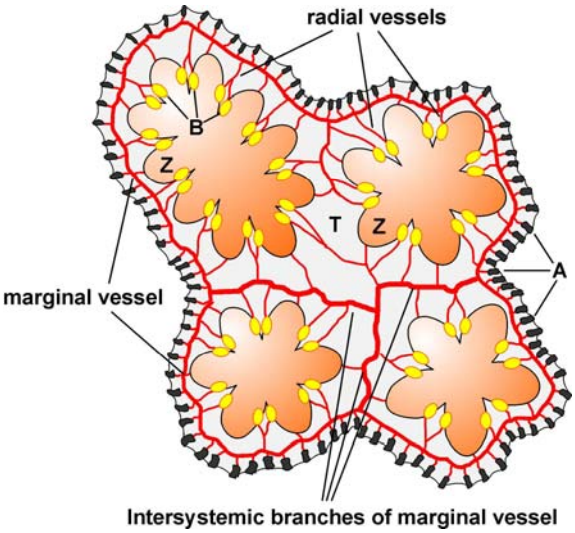

Fig. 3 Schematic drawing of the tunic circulatory system of a *B. schlosseri* colony (modified after Bunetti and Burighel, 1969). A: ampullae; B: bud; Z: zooid; T: tunic.

Immunocytes of compound ascidians

Invertebrate immunocytes represent the key component of immunity, responsible for cell-mediated immune reactions and, through their secretions, of most humoral responses. They are wandering cells, active in immunosurveillance which, in ascidians, represent a well-defined class of circulating hemocytes (Ballarin and Cima, 2005).

Like all other ascidian species, many types of circulating hemocytes are found in colonial ascidians. The morphology of both living and fixed cells has been widely described by many authors (Pérès, 1943; Sabbadin, 1955b; Andrew, 1961; Schlumpberger *et al.*, 1984; Cima *et al.*, 2001; Ballarin and Cima, 2005), as well as their ultrastructure (Overton, 1966; Fujimoto and Watanabe, 1976; Milanesi and Burighel, 1978; Burighel *et al.*, 1983; Hirose and Mukai, 1992; Sugino *et al.*, 1993; Cima *et al.*, 2001; Hirose *et al.*, 2003) and various attempts have been made to find unifying classification criteria (e.g. De Leo, 1992; Burighel and Cloney, 1997). However, doubts still exist about their functions, mutual relationships, and differentiation pathways.

In colonial botryllid ascidians, especially in the species *Botryllus schlosseri*, hemocytes have been particularly investigated for their role in immunity (Ballarin *et al.*, 1993, 1995; Cima *et al.*, 1996, 2001; Ballarin and Cima, 2005). In this species, circulating hemocytes are grouped into three main categories: i) undifferentiated cells; ii) immunocytes; iii) storage cells (pigment cells and nephrocytes). Immunocytes are represented by cytotoxic morula cells (MCs) and phagocytes, the latter including hyaline amebocytes and macrophage-like cells.

Morula cells

MCs represent the most abundant circulating hemocyte type in botryllid ascidians, their frequency ranging from 20 to 60 %. They have a diameter of 10-15 µm, contain many vacuoles of uniform size, approximately 2 µm in diameter (Fig. 4a), and, after treatment with aldehydes, assume a yellowish color and shrink (Fig. 4b), thus conferring the typical berry-like morphology on fixed cells (Sabbadin, 1955b; Ballarin *et al.*, 1993; Ballarin and Cima, 2005). The content of MC vacuoles has reducing properties, as demonstrated by its capability to reduce osmium, and shows positivity for peroxidase, phenoloxidase (PO) and arylsulfatase (Ballarin and Cima, 2005). In addition, it can be stained with eosin (Shirae *et al.*, 2002), gives positive cytochemical reactions for polyphenols, quinones and DOPA-containing proteins (Ballarin *et al.*, 1995; Ballarin and Cima, 2005) and is recognized by antibodies raised against tunichromes of solitary ascidians (Ballarin, 2008). The precursors of MCs are probably granular amebocytes, with which they share similar cytochemical properties and enzymatic content (Ballarin and Cima, 2005). MCs are abundant inside the lumen of the ampullae of the colonial growing edge (Cima *et al.*, 2006a), have specific homing sites such as the lacunae inside the tentacles of the oral siphon (Rinkevich *et al.*, 1998; Rinkevich, 2005), and their frequency inside the contacting ampullae increases abruptly in early stages of the non-fusion reaction (see below).

MCs can sense foreign molecules and, on recognizing them, degranulate (Fig. 4c) and release their vacuolar content (Ballarin *et al.*, 1995, 1998, 2005) consequently inducing cytotoxicity in neighbouring cells. This effect is directly related to the presence in the medium of active PO, as demonstrated by the significantly lower cytotoxicity with respect to controls in the presence of PO inhibitors such as sodium benzoate, phenylthiourea and dimethyldithiocarbamate (Ballarin *et al.*, 1998, 2005).

B. schlosseri PO has been purified and partially characterized: the monomeric form has a molecular

weight of 80 kDa and can polymerize to larger complexes (Frizzo *et al.*, 1999). Using a polyclonal antibody against the purified protein, it was possible to confirm the enzyme location inside MC vacuoles (Frizzo *et al.*, 2000).

The observation that serine protease inhibitors can decrease PO activity suggests that the enzyme is stored, at least partly, as a proenzyme (pro-PO) which, as in arthropods, is converted to active PO through the removal of a short peptide by serine proteases (Söderhäll and Cerenius, 1998). Polyphenol substrata are probably represented by tunichromes, small peptides containing DOPA or TOPA (Oltz *et al.*, 1987; Smith *et al.*, 1991), present inside ascidian MCs (Dorsett *et al.*, 1987; Azumi *et al.*, 1990; Ballarin *et al.*, 1995; Taylor *et al.*, 1995;. Bayer *et al.*, 1997). They are probably present in a masked form inside MC vacuoles, with sulfates, also revealed in MCs, bound to the aromatic ring, and made available to PO by the action of the enzyme arylsulfatase which detaches sulfates from phenols (Ballarin *et al.*, 1995).

Phagocytes

In all metazoans, phagocytes can recognize and ingest foreign cells or particles entering the organism, thus ensuring their clearance. In *B. schlosseri*, circulating professional phagocytes are represented by hyaline amebocytes (Fig. 4d) and macrophage-like cells (Fig. 4e), which represent two diverse morphologies of the same hemocyte type (Sabbadin, 1955b; Ballarin *et al.*, 1993). The former represent cells active in phagocytosis which, upon ingestion, withdraw their pseudopods and turn to the globular morphology of macrophage-like cells (Ballarin *et al.*, 1993, 1994; Ballarin and Cima, 2005). The two hemocyte types have similar cytochemical properties and common contents of lysosomal and antioxidant enzymes (such as phosphatases, 5'-nucleotidase, β-glucuronidase and esterases), share the same surface carbohydrates, as demonstrated by their labelling with *Narcissus pseudonarcissus* agglutinin, and are immunopositive to anti-CD39 antibody (Ballarin and Cima, 2005). Similar results were obtained in *Botrylloides leachi* (Cima *et al.*, 2001). The release of acid phosphatase in the medium has been observed in *in vitro* phagocytosis by *B. schlosseri* hemocytes (Cima *et al.*, 1996), due to leakage of the enzyme by phagocytes during phagosome formation (Davies and Bonney, 1980).

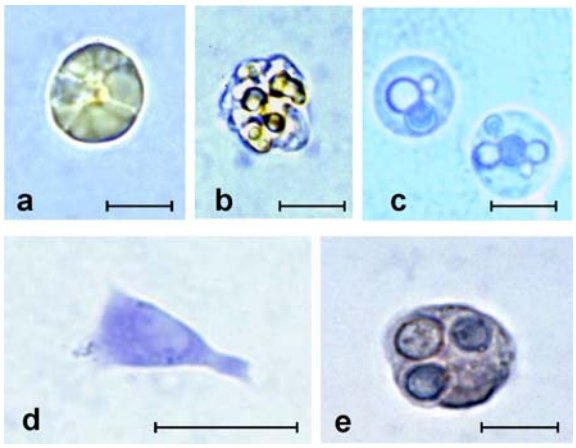

Fig. 4 *B. schlosseri* hemocytes. **a**, **c**: living MCs incubated in the absence (**a**) or in the presence (**c**) of foreign molecules or cells (from Ballarin *et al.*, 2005); **b**: aldehyde-fixed MCs; **d**, **e**: fixed *B. schlosseri* phagocytes (from Menin and Ballarin, 2006). **d**: hyanine amebocyte; **e**: macrophage-like cell. Bar = 10 μm.

Tunic and external immunocytes

The ascidian tunic contains numerous cells deriving from the hemocytes which leave the circulation and colonize the test matrix (Burighel and Cloney, 1997). A common tunic cell type is represented by spreading cells or amebocytes which closely resemble circulating hyaline amebocytes. In *B. schlosseri* three distinct types of cells were recognized: fusiform, fibrocytic and vacuolated cells, the latter deriving from morula cells migrated through the epidermis into the tunic (Zaniolo, 1981). A role in immune defense has been postulated for some of these cells, confirmed by the observation that, in both solitary and colonial ascidians, they have phagocytic activity (Smith, 1970; Hirose *et al.* 1994a; Burighel and Cloney, 1997). In addition, as demonstrated in solitary ascidians, hemocytes can infiltrate the tunic in response to the presence of non-self cells or particles, where they contribute to form a capsule (Parrinello and Patricolo, 1984). In botryllid ascidians, massive infiltration of immunocytes into the tunic is associated with the non-fusion reaction between contacting, genetically incompatible colonies (see below).

In the colonial ascidian *B. schlosseri*, we recently reported the presence of amebocytes, completely exposed to seawater, attached to the tunic of siphonal regions. These cells share many cytochemical features with circulating phagocytes, respond to the same lectins and antibodies used as markers of the phagocytic cell line, and can also engulf carmine particles. All these results suggest that these cells guard the entrances to the branchial and atrial chambers, and trigger a systemic defense response toward non-self particles or cells (Cima *et al.*, 2006b).

Non-self recognition

Phagocytosis

Invertebrate phagocytes recognize non-self molecular patterns through a series of poorly known receptors. In *B. schlosseri*, indirect evidence suggests the involvement of a mannose receptor in yeast recognition (Ballarin *et al.*, 1994); in addition, yeast-matched hemocytes express molecules cross-reacting with antibodies raised against mammalian Toll-like receptor (TLR)-2 and -4, located in phagocytes (Menin and Ballarin, 2007), which suggests the involvement of TLRs in the recognition of foreign cells.

Vertebrate phagocytosis implies a zipper-like mechanism involving a close adhesion, mediated by

integrins, of phagocyte projections with the surface of foreign particles or cells (Griffin *et al.*, 1975a, b) In *B. schlosseri*, the interaction with yeast cells requires the activation of phagocyte integrins after recognition of the Arg-Gly-Asp (RGD) motif on the surface of target particles, for both pseudopod formation and cell spreading, as suggested by the fact that the soluble antagonist tetrapeptide Arg-Gly-Asp-Ser (RGDS) significantly inhibits both yeast phagocytosis and cell spreading and disrupts cytoskeletal organization (Ballarin *et al.*, 2002a).

In *B. schlosseri*, the occurrence of the respiratory burst after the interaction of phagocytes with non-self particles has been clearly documented in the case of yeast phagocytosis, in which an increase in the production of cytotoxic superoxide anions, peroxides and hypochlorite by phagocytes has been reported (Ballarin *et al.*, 1994; Cima *et al.*, 1996). An increase of nitrite in the culture medium was also observed when hemocytes were incubated with yeast cells (Cima *et al.*, 1996), suggesting that, as in vertebrates (Hibbs *et al.*, 1987), the activation of inducible nitric oxide synthase (NOS) in *Botryllus* phagocytes occurs on recognition of foreign cells and, as a consequence, nitric oxide (NO) with microbicidal activity is produced. This hypothesis may be strengthened by the immunocytochemical assay on phagocytes with anti-NOS antibodies.

Non-self recognition by *B. schlosseri* phagocytes triggers various signal transduction pathways. The observation that H89 and calphostin, inhibitors of protein kinase A (PKA) and protein kinase C (PKC), respectively, can decrease the ingestion of yeast cells by phagocytes (Menin and Ballarin, 2006), suggests the involvement, in phagocytosis, of transduction pathways activated by cAMP and diacylglycerol, respectively, and controlled by trimeric G proteins (Gomperts *et al.*, 2002). PKC activation follows activation, by trimeric G proteins, of a membrane-associated phospholipase C which produces inositol triphosphate (IP3) and diacylglycerol (DAG), the former increasing the cytosolic Ca^{2+} concentration, the latter acting on PKC. A transient rise in cytosolic Ca^{2+} concentration is required for ingestion to occur, as demonstrated by the inhibition of phagocytosis in the presence of EDTA or when hemocytes and target cells are incubated in the presence of either thimerosal, which depletes intracellular calcium stores, or inhibitors of the calmodulin-dependent Ca^{2+}-ATPase, such as thapsigargin, pimozide or tributiltin (TBT) chloride, which cause a sustained increase in cytosolic Ca^{2+} concentration (Cima *et al.*, 1995; Ballarin *et al.*, 1997; Cima and Ballarin, 2000). Manumicin, an inhibitor of monomeric G proteins, decreases the fraction of *in vitro* phagocytosing cells, indicating the involvement of these proteins, also suggested by the expression, revealed by immunohistochemical and immunoblot analysis, of molecules recognized by anti-pan-Ras antibodies in yeast-matched phagocytes (Menin *et al.*, 2007). We recently demonstrated that various MAPK inhibitors, such as PD98059 (ERK pathway), SP600125 (JNK pathway) and SB202190 (p38 pathway) can decrease the frequency of yeast-ingesting hemocytes, and an increase in the expression of various activated MAPKs, such as p38, ERK, SAPK/JNK, was observed in immunoblot analysis of yeast-matched hemocyte lysates (Menin *et al.*, 2007). Phosphatidylinositol-3-kinase (PI3K) is also involved in phagocytosis, and its role seems to be related to the cytoskeletal re-organization required for pseudopod formation (Ballarin *et al.*, 2002a). Immunopositivity to anti-NF-kB antibodies is located in the cytoplasm of unstimulated phagocytes, whereas it migrates into the nucleus of yeast-activated cells (Ballarin, 2008) suggesting the involvement of this transcription factor in phagocytosis.

Efferocytosis, i.e. phagocytosis of apoptotic cells (de Cathelineau and Henson, 2003; Gardai *et al.*, 2005), occurs massively during take-over and is performed by professional, circulating phagocytes massively recruited in the senescent tissues (Fig. 5a), and by occasional phagocytes such as cells of the digestive epithelium (Tiozzo *et al.*, 2006). During the generational change, there is a significant increase in the frequency of circulating macrophage-like cells with respect to mid-cycle stages (Fig. 5b), paralleled by a decrease in the fraction of circulating hyaline amebocytes (Fig. 5c) (Cima *et al.*, 2003; Ballarin *et al.*, 2008), matching the hypothesis that the two hemocyte types represent different stages of the same cell. As a result of intense phagocytosis, there is a significant increase in the fraction of hemocytes showing positivity for acid phosphatase which, as stated above, is a phagocyte marker, and in both the activity of acid phosphatase and the concentration of peroxides in the blood plasma (Cima *et al.*, 1996). *Botryllus* phagocytes recognize phosphatidylserine on the surface of effete cells and corpses, as soluble phospho-L-serine can inhibit their ingestion. In addition, clearance of senescent cells requires the expression of molecules recognized by anti-mammalian-CD34 antibodies on the phagocyte surface, the expression of which increases at take-over (Cima *et al.*, 2003).

Phagocytes of *B. schlosseri* are also capable of constitutive macropinocytosis, a process generally responsible of the ingestion of bacteria and necrotic material (Krysko *et al.*, 2003), at sites of membrane ruffling along their leading edge. This activity is enhanced by the presence, on the substrate, of molecules containing the RGD motif, such as fibronectin or fibrinogen. This suggests that, as in mammals (Meier *et al.*, 2002), integrins can also regulate macropinocytosis. The increase in fluid-phase endocytosis is associated with a rise in both oxygen consumption, as indicated by the higher production of reactive oxygen species, and increased cytochrome oxidase activity, related to the synthesis of ATP (Ballarin and Burighel, 2006).

Allorecognition

As stressed by Buss (1987), clonal organisms are characterized by colony specificity or allorecognition, i.e. the ability for intraspecific non-self recognition. Colony specificity of botryllid ascidians has been known since the pioneer observations by Bancroft (1903). It was further investigated in Japanese species (Oka and Watanabe, 1957a, 1960; Oka, 1970; Mukai and Watanabe, 1974) and in *B. schlosseri* (Karakashian

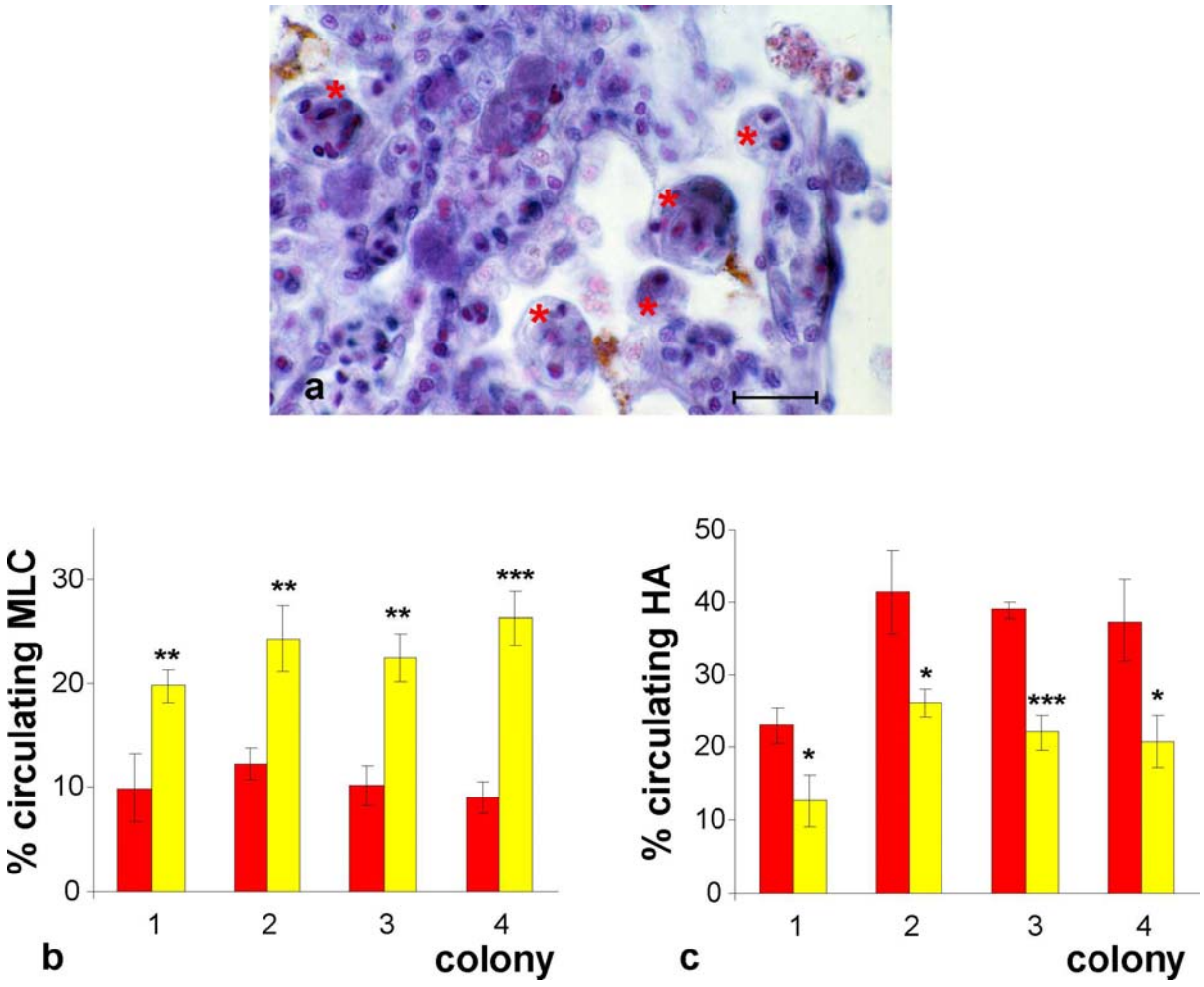

Fig. 5 a: tissues of senescent zooid during take-over. Infiltrated macrophage-like cells having ingested apoptotic cells and corpses are marked by asterisks. Bar = 15 µm. **b**, **c**: frequencies of circulating macrophage-like cells (MLC) and hyaline amebocytes (HA) in 4 colonies, at mid-cycle (red bars) and take-over (yellow bars). Significant differences between mid-cycle and take-over in the same colony are marked by asterisks. *p < 0.05; **p < 0.01; ***p < 0.001.

and Milkman, 1967; Sabbadin, 1962). Colony specificity manifests itself as either fusion or non-fusion of genetically compatible or incompatible colonies, respectively, contacting each other at their growing edges. The phenomenon has been studied in 14 species of botryllid ascidians (Sabbadin, 1962, 1982; Tanaka, 1975; Taneda *et al.*, 1985; Rinkevich, 1992, 2005; Saito *et al.*, 1994; Hirose, 2003), particularly in the Japanese species *Botryllus primigenus* and the cosmopolitan species *B. schlosseri*, where the outcome of the contact is genetically controlled by a highly polymorphic Fu/HC gene, the alleles of which are co-dominantly expressed (Oka and Watanabe, 1957a, 1960; Sabbadin, 1962; Oka, 1970). In *B. schlosseri*, the Fu/HC locus also controls the vascularization and the completion of development of secondary buds grafted from a colony in the tunic of an alien colony, which occur only if the two colonies are fusible (Sabbadin, 1982; Sabbadin *et al.*, 1991).

The great majority of botryllid colonies in nature are heterozygous at the Fu/HC locus and fusion occurs when at least one allele is shared by the two colonies, otherwise non-fusion is observed. Fusion implies the formation of a chimeric colony, with anastomosis of tunic and circulation (Katow and Watanabe, 1980; Zaniolo *et al.*, 2006); non-fusion requires a partial fusion of the facing tunics with the local disappearance of the limiting cuticles (Tanaka and Watanabe, 1973; Taneda *et al.*, 1985; Sabbadin *et al.*, 1992; Saito *et al.*, 1994; Hirose, 2003; Rinkevich, 2005; Zaniolo *et al.*, 2006) and is usually characterized by the appearance of a series of cytotoxic foci (rejection reaction), called points of rejection, along the contact border (Oka and Watanabe, 1957a, 1960; Sabbadin, 1962; Oka, 1970; Rinkevich, 1992, 2005). The growth of contacting ampullae to unusually large size (megaloampullae) during allorecognition has been reported in *Botrylloides leachi* (Rinkevich *et al.*,

1994; Zaniolo *et al.*, 2006). As a consequence of the non-fusion reaction, a change in vectorial growth of the contacting colonies (retreat growth) occurs (Rinkevich and Weissman, 1988).

Both cells and soluble factors are involved in *Botryllus* allorecognition. In *B. primigenus* and *B. schlosseri,* the partners of a chimeric colony separated after a fusion of at least four days show altered fusibility (Mukai, 1967; Sabbadin and Astorri, 1988). In *B. schlosseri*, Sabbadin and Astorri (1988) demonstrated that this alteration is related with the persistence, in a colony, of cells from the partner with which it was previously fused. Tanaka (1973, 1975), working with colonies of *B. primigenus* of defined genotype at the Fu/HC locus, showed that fusion conferred to the chimera AC-BC the ability to reject a colony BD when contacting the BC partner. The fusibility of *B. primigenus* colonies was altered by the previous exposure to X-rays which reduces circulating undifferentiated cells, stressing the role of blood cells in allorecognition (Taneda and Watanabe, 1982c).

Non-fusion reaction is irreversible after the fusion of the tunic cuticles occurred, even if one colony is removed after the contact, suggesting the involvement of diffusible (circulating) humoral factors from one colony to the other through the fused tunics (Tanaka, 1975; Watanabe and Taneda, 1982). This is supported by the observation that, in early stages of non-fusion reaction, an increase in permeability of the ampullar epithelium occurs (Taneda and Watanabe, 1982a) and that the reaction can be mimicked by the injection of whole blood and blood plasma from a colony into the vessels of a genetically incompatible host (Taneda and Watanabe, 1982b; Saito and Watanabe, 1984).

The observation of a simultaneous occurrence of fusion and non-fusion reactions between contacting colonies fits the hypothesis of the recognition of soluble histocompatibility factors diffusing from one colony by effector hemocytes of the facing partner (Taneda, 1985).

Humoral factors involved in the non-fusion reaction have been partially characterized from blood plasma of *Botrylloides simodensis*: they are resistant to dyalisis heat-labile and require divalent cations for their activity (Saito and Watanabe, 1984).
In *B. primigenus* and *B. schlosseri*, the non-fusion reaction shares many characteristics with vertebrate inflammation, such as cell recruitment by chemotaxis, extravasation, cell degranulation, and induction of cytotoxicity. As stated before, it is preceded by partial fusion of the contacting tunics, in front of the facing marginal ampullae, which allows the diffusion of soluble factors from one colony to the next. This event induces selective crowding of MCs inside the ampullae of the growing edge, their migration through the epithelium of the ampullar tips into the tunic, and their final degranulation, with the consequent release of their vacuolar content (Taneda and Watanabe, 1982a; Sabbadin et al., 1992; Saito *et al.*, 1994; Rinkevich *et al.*, 1998; Cima *et al.*, 2006c), in particular, the enzyme PO and its polyphenol substrata, which contribute to inducing the observed cytotoxicity (Fig. 6; Ballarin *et al.*, 1995, 1998; Cima *et al.*, 2004, 2006c).

Analogous behavior, although restricted to a very limited region in front of the facing ampullae (subcuticular rejection), has been described in *B. simodensis*, *Botrylloides fuscus* and *Botrylloides violaceus* (Hirose *et al.*, 1988, 1990, 1997; Shirae *et al.*, 2002) and *B. leachi* (Zaniolo *et al.*, 2006; Ballarin and Zaniolo, 2007).

In *B. schlosseri*, MC degranulation and the induction of cytotoxicity may be mimicked *in vitro* by exposing hemocytes to the blood plasma of incompatible colonies (Ballarin *et al.*, 1995; Cima *et al.*, 2006c) and prevented by the above-reported PO inhibitors (Ballarin *et al.*, 1998). Cytotoxicity is related to the induction of oxidative stress, as indicated by the reduction of nitroblue tetrazolium when added to incompatible blood plasma in *in vitro* assays, and the observation that scavengers of reactive oxygen species, such as superoxide dismutase, catalase and sorbitol, can suppress cell death both *in vitro*, when hemocytes are incubated with incompatible blood plasma, and *in vivo*, in colony allorecognition, although they have no effects on MC degranulation or PO activity (Ballarin *et al.*, 1998, 2002b). The role of NO in the induction of cell death is suggested by the *in vitro* production of nitrite ions when hemocytes are exposed to non-self molecules and the decrease of *in vitro* cytotoxicity in the presence of the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (Cima *et al.*, 2004). The necrotic masses at rejection points share several chemical and cytochemical properties with MC content (Ballarin *et al.*, 1995), thus strengthening the assumption that MCs infiltrated into the tunic play a major role in their formation. Various properties of the pigment deposited at rejection points are also indicative of its melanic nature (Ballarin *et al.*, 1995), and melanins are the end-product of PO activity in metazoans (Waite, 1992).

Despite the well-defined role of MCs as effectors of non-fusion reactions in most botryllid ascidians, even phagocytes take part in rejection reactions:

i) in *B. schlosseri*, fusion between allogeneic colonies, sharing only one allele at the Fu/HC locus, usually leads to the resorption of one of the partners during take-over of one of the blastogenetic cycles of the chimeric colony following fusion (Rinkevich and Weissman, 1987, 1992; Sabbadin and Astorri, 1988; Rinkevich, 2002, 2005). Colony resorption, therefore, mimicks zooid resorption at take-over, implying the involvement of phagocytes in the elimination of tissues of the “losing” partner;

ii) a more direct role of phagocytes in allorejection has been described in *Botryllus scalaris*, which is the only botryllid species reported so far in which phagocytes and not MCs are involved in allorecognition between contacting, incompatible colonies. In this case, the rejection reaction starts after fusion of the ampullae of the facing growing edges and the beginning of blood exchange through the fused vessels. Phagocytes crowd inside the fused ampullae and stimulate the aggregation of hemocytes into large clusters which are finally encapsulated by other phagocytes. In this way, hemocyte clusters plug the lumen of the fused ampullae and blood flow is interrupted in a few

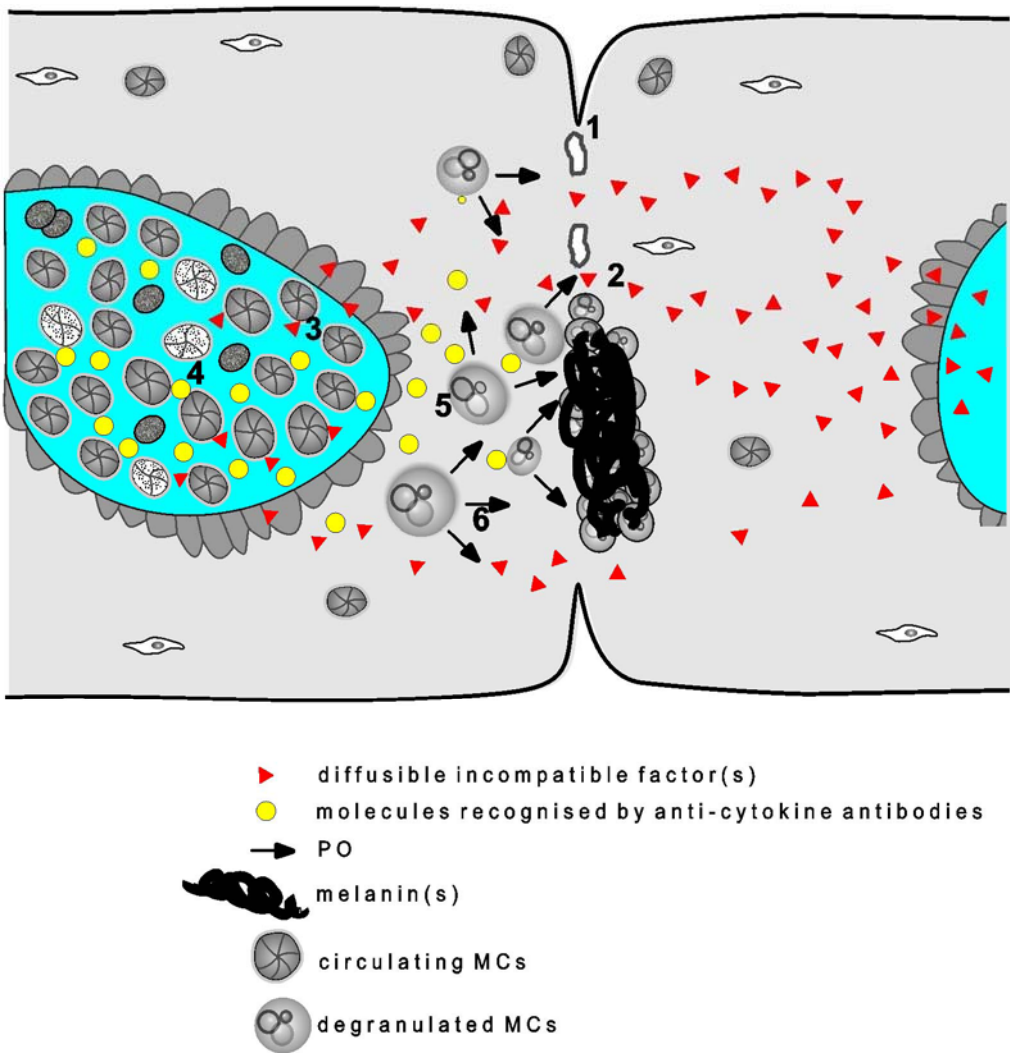

Fig. 6 Schematic representation of the rejection reaction in *B. schlosseri*. For sake of simplicity, the main steps are indicated only on the left colony. **1**: fusion of the contacting tunics after the local disappearance of the cuticles; **2**: diffusion of histocompatible factor(s) through the fused tunics; **3**: recognition of alien factors by MCs inside the tips of ampullae; **4**: release of cytokines by activated MCs enhancing recruitment; **5**: extravasation of MC and their degranulation in the tunic; **6**: release of PO; **7**: cytotoxicity and melanin formation at points of rejection. Modified after Hirose (2003).

minutes. Differently from other botryllid species studied so far, no signs of selective recruitment or degranulation of MCs were observed (Shirae *et al.*, 1999).

When incompatible colonies of botryllid ascidians are artificially brought into contact at their cut surfaces (cut surface allorecognition assay), an intense rejection reaction is observed in ovoviviparous species (Rinkevich, 1992, 2005; Saito *et al.*, 1994; Hirose, 2003; Zaniolo *et al.*, 2006; Ballarin and Zaniolo, 2007). However, fusion of tunics and blood vessels (surgical fusion) always occurs in the case of the viviparous species studied so far, suggesting that the hemocytes of these species have lost the ability for allorecognition, which persists in tunic cells (Saito *et al.*, 1994; Hirose *et al.*, 1994b; Okuyama *et al.*, 2002; Hirose, 2003; Rinkevich, 2005). This may be in relation with the necessity to prevent the immune system from attacking the brooded embryos which shares only one allele at the Fu/HC locus with the mother colony and might undergo rejection/resorption as they are exposed to the circulation for more than a week (Hirose, 2003). In partial confirmation of the above statement, the PO activity of the hemolysate of viviparous species is much lower than that of ovoviviparous ones (Shirae and Saito, 2000; Okuyama *et al.*, 2002).

Humoral factors

Lectins

It is well-known that ascidian hemolymph contains lectins, often revealed by their

hemagglutinating activity (hence the name hemagglutinins), with various carbohydrate specificities (Vasta *et al.*, 1982; Coombe *et al.*, 1984a; Parrinello, 1995). A role in immune defense has been postulated for some of them, but clear involvement in cell proliferation after non-self recognition or in the modulation of phagocytosis has been demonstrated in a few cases (Coombe *et al.*, 1984b; Kelly *et al.*, 1992; Pearce *et al.*, 2001). In compound ascidians, the presence of soluble agglutinins has been reported in various species of the genera *Amaroucium*, *Aplidium*, *Botrylloides*, *Botryllus*, *Didemnum*, *Diplosoma, Clavelina* and *Polyandrocarpa* (Vasta *et al.*, 1982, 1986; Coombe *et al.*, 1984a; Suzuki *et al.*, 1990; Parrinello, 1995).

In *B. schlosseri*, we demonstrated that yeast-activated phagocytes can synthesize and release, through apocrine secretion, lectins with specificity for β-galactosides, which can agglutinate rabbit erythrocytes and yeast cells (Ballarin *et al.*, 1999; Fig. 7) and which were previously thought to be members of the galectin family on the basis of their Ca^{2+}-independence (Ballarin *et al.*, 2000). Polyclonal antibodies against these molecules recognize the surface of erythrocyte or yeast cells clumped by exposure to affinity-purified lectins (Ballarin *et al.*, 1999). These lectins can improve yeast phagocytosis by acting as opsonins and promoting interactions between target cells and phagocytes (Ballarin *et al.*, 1999, 2000). Recently, in a full-length cDNA library from *Botryllus* colonies, we identified five transcripts homologous to known rhamnose-binding lectins (RBLs). Their predicted amino acid sequences exactly match the sequences of the tryptic fragments previously obtained from the above agglutinins purified by affinity chromatography. They probably represent different isoforms of a novel RBL, called *B. schlosseri* RBL (BsRBL), with a molecular weight of approximately 11 kDa. They contain the eight cysteines which characterize RBLs, form four disulfide bonds, and have a single carbohydrate recognition domain; through non-covalent interactions they can form multivalent complexes able to act as bridges between target particles and the phagocyte surface and enhance phagocytosis (Gasparini *et al.*, 2008).

Antiviral, antimicrobial and antitumoral factors

Various bioactive molecules, with antiviral or cytotoxic activities against microbial or tumoral cells have been described in compound ascidians. Most of these compounds were isolated from didemnid ascidians. Ulithiacyclamide and ulicyclamide are cyclic peptides from *Lissoclinum patella* showing antineoplastic and antiviral activity (Ireland and Sheuer, 1980; Ireland *et al.*, 1982; Wasylyk *et al.*, 1983). In the same species, a lactone, named lissoclinolide, with antibacterial and antitumoral activity has been isolated (Davidson and Ireland, 1990; Richardson and Ireland, 2004). Lissoclibadins are cytotoxic and antimicrobial alkaloids from the Indonesian *Lissoclinum* cf. *badium* (Nakazawa *et al.*, 2007). Didemnins and eudistomins are antiviral molecules isolated from whole extracts of colonies of *Tridemnum* sp. and *Eudistoma olivaceum*, respectively. The former are cyclic peptides, able to inhibit the replication of various RNA and DNA viruses,

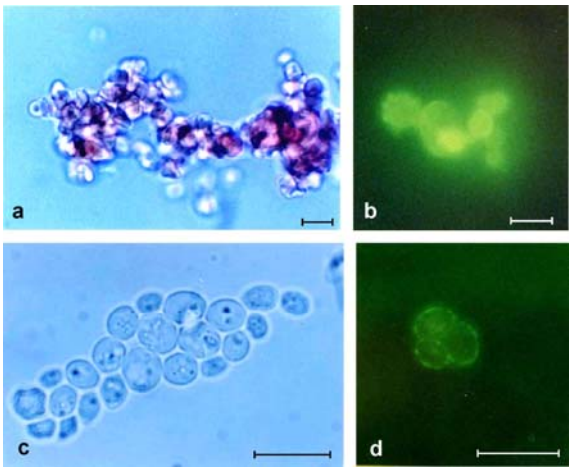

Fig. 7 Agglutination of rabbit erythrocytes (**a**) and yeast cells (**c**) in the presence of purified BsRBL. Immunostaining with polyclonal anti-BsRBL antibodies reveal the presence of the lectin on the surface of clumped erythrocytes (**b**) or yeast cells (**d**). Bar = 10 μm.

which also exert antitumor activity (Rinehart *et al.*, 1981a, b), the latter are β-carboline derivatives containing bromine, active against herpex simplex virus (Rinehart *et al.*, 1984). Lepadins D, E and F are decahydroquinoline derivatives from *Didemnum* sp. with antiplasmodial and antitrypanosomal activity (Wright *et al.*, 2002). Anticancer hydroquinones derivatives with cytotoxic properties have been described in *Aplidium californicum* (Howard and Clarkson, 1979; Cotelle *et al.*, 1991). In the Mediterranean *Aplidium albicans*, the cytotoxic peptide aplidin induces perturbations of the cell cycle and apoptosis of human leukaemia cells (Erba *et al.*, 2002). Another antitumoral peptide, vitilevuamide, isolated from *Didemnum cuculiferum* and *Polysyncraton lithostrotum*, was able to inhibit tubulin polymerization required for the organization of the mitotic spindle (Edler *et al.*, 2002). Metabolites inducing apoptosis of human cancer cells were recently isolated from the Japanese ascidian *Diplosoma virens* (Ogi *et al.*, 2008).

The enzyme PO, as stated above, is also involved in preventing microbial infections through its cytotoxic activity and *B. schlosseri* MCs degranulate and release cytotoxic active PO in response to the recognition of yeast cells or bacterial spores (Ballarin *et al.*, 2005).

Cytokines

The presence of soluble immunomodulatory molecules, cross-reacting with antibodies raised against mammalian IL-1, has been reported in ascidians since the pioneering work by Beck *et al.* (1989). *B. schlosseri* and a member of the genus *Didemnum* were among the tunicate species investigated, showing the presence of lymphocyte activation factors, the activity of which was neutralized by anti-IL-1 antibodies.

In *B. schlosseri*, MCs are the main source of molecules recognized by antibodies raised against the mammalian pro-inflammatory cytokines IL-1-α and TNF-α: when hemocytes are exposed to non-self molecules, such as mannan or incompatible blood plasma, or particles, such as yeast cells or microbial spores, MCs acquire immunopositivity to the above antibodies (Ballarin *et al.*, 2001, 2005). Immunopositive MCs are also observed inside the facing ampullae of contacting incompatible colonies (Cima *et al.*, 2004) in early stages of the non-fusion reaction. In *B. leachi*, MCs are recognized by anti-IL-1-α antibodies, whereas positivity to anti-TNF-α antibodies has been unexpectedly observed in another type of hemocytes which, according to Cima *et al.* (2001), were classified as granular cells (Ballarin and Zaniolo, 2007). In *B. schlosseri*, the above antibodies inhibit both the rise in cell death observed when hemocytes are incubated with incompatible blood plasma (Cima *et al.*, 2004) and MC chemotaxis (Cima *et al.*, 2006c), suggesting that the recognized molecules stimulate both the recruitment of MCs inside the tips of facing ampullae and their degranulation with the consequent release of PO (Fig. 7).

The supernatants from cultures of *B. schlosseri* hemocytes, matched with non-self particles such as yeast cells or zymosan (conditioned media) enhance the phagocytosis of yeast cells (Menin *et al.*, 2005). These effects were abolished by the addition of anti-IL-1-α or anti-TNF-α antibodies, suggesting that molecules recognized by the above antibodies are involved in immunomodulation and may be considered as cytokines in the broad sense of the term. Using cell fractionation by density gradient centrifugation in Ficoll 400, we obtained four hemocyte bands, with different immunocyte distribution, from which we prepared four different conditioned media, one for each band. In this way, we could demonstrate that the enhancing effect on phagocytosis was present in conditioned media derived from hemocyte bands enriched in MCs, but that it was not present in conditioned media from bands rich in phagocytes (Fig. 8), in agreement with previous results indicating MCs as the source of molecules cross-reacting with anti-mammalian cytokine antibodies (Menin *et al.*, 2005).

Immunoblot analysis of the supernatant from zymosan-matched hemocytes showed a 60 kDa band cross-reacting with both anti-IL-1-α and anti-TNF-α. In addition, a 37 kDa band, recognized by anti-BsRBL antibodies, was detected in the above conditioned media, and the presence of the lectins was confirmed by hemagglutinating assay (Menin and Ballarin, 2008).

All the above results fit a scenario in which MCs are the main organism sentinel cells which sense non-self molecules, being recruited as a consequence of the recognition and, on the basis of the nature of the foreign molecules, they are able to trigger a cytotoxic response or stimulate phagocytes to ingest foreign cells and release agglutinins which potentiate their activity.

Immunity and xenobiotics

B. schlosseri is one of the most diffuse encrusting organisms in the Lagoon of Venice, where it characterizes the relative climax of the ecological succession of hard-substratum macrobenthos (Cima *et al.*, 2006c). As colonial ascidians have been reported to be very susceptible to antifoulants (Henderson, 1986), we studied the effects of short-term exposure of *B. schlosseri* hemocytes to biocides such as organotin compounds (tributyltin, triphenyltin) and new organic compounds used in antifouling paint formulations after the ban on tin-based antifoulants, on cell functions in order to reveal the cellular targets of these molecules (Cima *et al.*, 1995, 1997, 1998, 2008; Cima and Ballarin, 1999, 2000, 2004).

Organotin compounds alter the morphology of phagocytes, their capability to ingest target yeast cells, and to induce the respiratory burst in a dose-dependent manner, and these changes are related to disruption of cytoskeletal components (Cima and Ballarin, 2000, 2008; Cima *et al.*, 1995, 1997, 1998). Tributyltin can interact with calmodulin and alter the activity of calmodulin-dependent Ca^{2+}-ATPase (Cima *et al.*, 2002a) or react directly with cytosolic thiols (Cima and Ballarin, 2004). As a consequence, they alter cytosolic calcium and thiol homeostasis which cause the observed alterations in cell morphology (Cima *et al.*, 1995, 1997), inhibition of hydrolytic, detoxifying and mitochondrial enzymes (Cima *et al.*, 2002b), and eventually lead to apoptosis (Cima and Ballarin, 1999), probably due to the severe oxidative stress following the reduction of cytosolic thiols (Cima *et al.*, 2004).

Among the new antifoulants, we assayed Sea-Nine™ (4,5 dichloro-2-n-octyl-4-isothiazoline-3-one), Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile), Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) and TCMS pyridine (2,3,5,6-tetrachloro-4-(methylsulfonyl)pyridine). Both Sea-Nine™ and Chlorothalonil have a negative effect on the phagocyte cytoskeleton, altering cell morphology and severely hindering phagocytosis. Both compounds decrease intracellular reduced glutathione, inducing oxidative stress which is probably the cause of the observed cell death by apoptosis (Cima *et al.*, 2008), and perturb the mitochondrial respiratory chain, whereas only Sea-Nine™ disrupts cytosolic calcium homeostasis (Cima *et al.*, 2008).

Diuron and TCMS pyridine inhibit phagocytosis and cell spreading in a dose-dependent manner, suggesting cytoskeletal alteration, and can induce apoptosis at the higher concentrations assayed (100 and 20 μm, for Diuron and TCMS pyridine, respectively). The two biocides did not have any negative effect on esterase or cytochrome-*c* oxidase activities, or on the homeostasis of cytosolic calcium (Menin *et al.*, 2008).

Conclusions and perspectives

Today, the study of ascidian immunobiology is a topical subject and it is a widespread opinion among scientists that protochordates can greatly

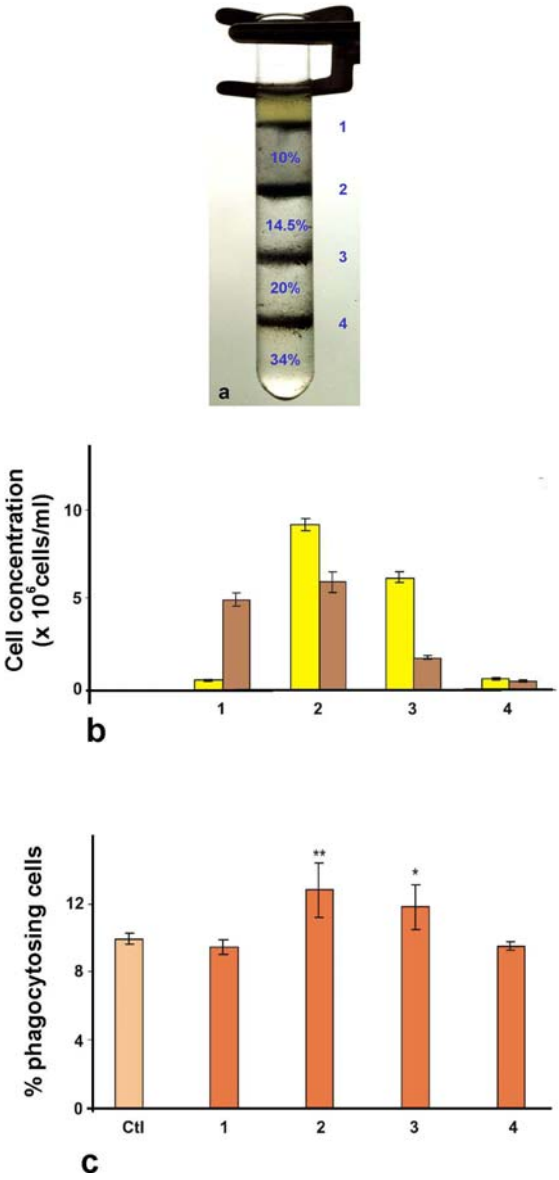

Fig. 8 Density gradient centrifugation of *B. schlosseri* hemocytes. **a**: enriched fractions of cells obtained after centrifugation in discontinuous gradient of Ficoll 400. **b**: concentration of MCs (yellow bars) and phagocytes (brown bars) in each fraction. **c**: percentage of phagocytizing cells in hemocyte cultures exposed to a suspension of yeast cells in seawater (control) or in the supernatants from cultures of hemocytes of each band obtained after density gradient centrifugation; significant differences with respect to the control are marked by asterisks. *p < 0.05; **p < 0.01. Modified after Menin *et al.* (2005).

contribute to answering the still unresolved problems of the origin of the highly sophisticated and complex vertebrate immune system. Among compound ascidians, *B. schlosseri* is diffuse and studied worldwide, and represents an optimal animal model for immunobiological studies, despite the size of its zooids, thanks to good knowledge of the biology of its immunocytes (Manni *et al.*, 2007). In addition, this kind of study has been frequently motivated by interest in understanding the cellular basis of allorecognition. As in other invertebrates, immune responses in ascidians rely mainly on circulating immunocytes which act as the effector arms of immunity, and can ingest or kill non-self cells or release opsonins and immunomodulatory molecules which enhance or reinforce cellular responses. As reported, two distinct immunocyte differentiation lines are present in botryllid ascidians: phagocytes and cytotoxic, phenoloxidase-containing morula cells. Nevertheless, despite the abundance of data gathered in the last two decades, unlike solitary ascidians of the genus *Ciona* and *Halocyntyhia* (Dehal *et al.*, 2002; Yokobori *et al.*, 2003), compound ascidians and *B. schlosseri* in particular still lack an adequate molecular approach to the study of their genomes. Few genes other than the BsRBL genes reported above (Gasparini *et al.*, 2008), which might be involved in non-self recognition, have been isolated. Examples are a CD94 orthologue of the vertebrate CD94 receptor on NK cells, expressed on a subset of *B. schlosseri* blood cells, probably phagocytes, mainly in the contacting ampullae (Khalturin *et al.*, 2003), and genes for putative homologue of C-type lectin (Pancer *et al.*, 1997), human EB1 (Pancer *et al.*, 1996a), FK506-binding protein (Pancer *et al.*, 1993), vertebrate receptor for antigens (Pancer *et al.*, 1996b) and HSP70 (Fagan and Weissman, 1996). Recently, a subtraction cDNA library of contacting, genetically incompatible colonies of *B. schlosseri* allowed the identification of more than 100 genes differentially expressed and related to immunity (Oren *et al.*, 2007). For deeper insight into the immunobiology of compound ascidians, better knowledge of the genome of these organisms, at least of *Botryllus*, on which many of the studies have been concentrated, is required. In addition, there are still some open questions regarding basic biological processes related to immunity, which represent good challenges for future research on compound ascidians. Some of them are listed below.

Origin and differentiation hemocytes (and immunocytes)

Ascidian hemocytes derive from the embryonic mesenchyme (Cowden, 1968; Sala, 1973; Sabbadin *et al.*, 1999). Undifferentiated cells are known as hemoblasts and are characterized by high nucleocytoplasmic ratio, a well-defined nucleolus and a basophilic cytoplasm whereas the term lymphocyte has been used either as a synonymous of hemoblast or to indicate a cell type thought to represent an immature differentiating hemocyte (Pérès, 1943; Sabbadin, 1955b; Ermak, 1976; Kawamura *et al.*, 1988). However, the real nature of the lymphocyte and the differentiation pathways of hemocytes are not clear and require further investigations.

It is known that, in *B. schlosseri*, almost 30 % of hemocytes die by apoptosis at take-over and are replaced by new hemoblasts entering the circulation

at the same stage of the blastogenetic cycle (Ballarin *et al.*, 2008). However, their origin is still unknown as no structures comparable to the branchial hemopoietic nodules, reported by Ermak (1977) in solitary species, have been described in compound ascidians. In certain experimental conditions, mitosis figures have been observed in circulating hemocytes of (Cima and Ballarin, 2007). Undifferentiated blood cells are directly involved in asexual reproduction by stolonal (Freeman, 1964) or vascular budding (Oka and Watanabe, 1957b). The latter occurs spontaneously in *B. primigenus* (Oka and Watanabe, 1957b) and in dormant colonies of *B. leachi* (Burighel *et al.*, 1976) and can lead to the re-building of a whole colony from small colony fragments, in *Botrylloides violaceus* (Oka and Watanabe, 1959) and *B. leachi* (Rinkevich et al., 2007), or from the colonial matrix deprived of zooids and buds, in *B. schlosseri* (Milkman, 1967; Sabbadin *et al.*, 1975). Both blood and epithelial cells can be cultured *in vitro* in appropriate conditions, and this represents an interesting tool in studying cell differentiation (Sala, 1973; Rinkevich and Rabinowitz, 1993, 1994; Rabinowitz and Rinkevich, 2003).

Allorecognition: old problems, new questions

i) Some Japanese authors, in comparing the non-fusion reactions of various botryllid ascidians, hypothesized the key role of tunic cells and the epithelium of the facing ampullae in allorecognition (Taneda *et al.*, 1985; Saito *et al.*, 1994; Hirose, 2003). The Fu/HC gene of *B. schlosseri* has recently been characterized (De Tomaso *et al.*, 2005), as well as that of the putative receptor involved in allorecognition (Nyholm *et al.*, 2006). The receptor is expressed in facing ampullae: intense labelling is observed in the epithelium of the ampullar tips but not in MCs (Nyholm *et al.*, 2006). In addition, the epithelium of the ampullar tips express high levels of BS cadherin in early stages of the non-fusion reaction (Rosner *et al.*, 2007). Indeed, the ampullar epithelium plays a fundamental role in *Botryllus* allorecognition in allowing the diffusion of non-self factors from the alien colony into the ampullar lumen, where they can alert MCs. During the non-fusion reaction, it is highly fenestrated and increases its permeability in both *B. primigenus* and *B. schlosseri* (Taneda *et al.*, 1982a; Sabbadin *et al.*, 1992), which suggests that it perceives signals which are absent in the fusion reaction. As all these events precede MC activation, represent prerequisites for the following inflammatory reaction and are worthy of study.

ii) In botryllid ascidians, in addition to allorecognition, the Fu/HC gene also controls the recognition between sperm and egg. It has been reported that the sperm of the Japanese species *Botryllus primigenus* cannot fertilize eggs when it shares a Fu/HC allele with the diploid, maternally-derived, egg envelope (Oka, 1970; Saito *et al.*, 1994). This limitation also seems to exist in some populations of *B. schlosseri* (Scofield *et al.*, 1982) but not in others (Sabbadin, 1971, 1982; Grosberg 1987). However, at least in the population from the Lagoon of Venice, high levels of inbreeding depression have been reported (Sabbadin, 1971). This offers the possibility of investigating relationships among allorecognition, fertilization and inbreeding depression, and their implications in the ecology of natural populations.

iii) As stated before, in laboratory conditions, resorption of one partner in the chimera usually follows colony fusion (Rinkevich and Weissman, 1987; Rinkevich, 2002, 2005). In natural populations, multichimeras form as the result of kin recognition, controlled by the Fu/HC gene, allowing larvae to settle near parental of genetically compatible adult colonies with which, once metamorphosed, they can fuse (Grosberg and Quinn, 1986; Grosberg, 1987; Ben-Shlomo *et al.*, 2008). This natural multichimerism gives colonies greater fitness in terms of faster growth rate, maximum size reached, better competition for the substrate, earlier achievement of sexual maturity and increased genetic diversity (Sabbadin, 1994; Rinkevich and Shapira, 1999; Paz and Rinkevich, 2002; De Tomaso, 2006). In addition, stem cells can be maintained and proliferate for a long time within a chimeric colony, although the corresponding soma has been resorbed (Sabbadin and Zaniolo, 1979; Sabbadin and Astorri, 1988; Stoner *et al.*, 1999; Laird *et al.*, 2005; De Tomaso, 2006). According to Sabbadin (1994) chimerism has the important role of maintaining the genetic structure of a population allowing the preservation of the genomes of fusible colonies, even in the case of their resorption by a partner, and seems to be diffuse in colonies collected from the field (Ben-Shlomo *et al.*, 2008). For this reason, *Botryllus* is an interesting model for the study of the evolution of microchimerism (Rinkevich, 2001), which seems to be responsible for various human pathologies (Adams and Nelson, 2004), and its relationships with the immune system.

Apoptosis and efferocytosis at take-over

Despite the abundance of *in vitro* model systems, mainly represented by selected cell lines, there is an increasing need for reliable models for *in vivo* investigations of the biological role of apoptosis in organisms. With its spontaneous and recurrent apoptosis of zooid tissues at the end of each blastogenetic cycle, *B. schlosseri* is an interesting model organism for the study of apoptosis and its genetic control. Two genes changing their expression during take-over have already been described (Lauzon *et al.*, 1996), but we do not know the nature of the cyclical signal inducing weekly cell death. In addition, phagocytes have been reported to play an important role in regulating the clearance of senescent cells, the extent of growth of buds and their budding activity (Voskoboynik *et al.*, 2004), but the molecular mechanisms underlying phagocyte recruitment in senescent tissues and recognition of effete cells are still largely unclear.

Tunicate cytokines: what is their relationship with their vertebrate counterparts?

Vertebrate cytokines are immunomodulatory proteins secreted by activated immunocytes after the recognition of foreign molecules, and they take part in several immunological processes such as inflammation, apoptosis, clearance of effete cells

and corpses, cytotoxicity and phagocytosis. In addition, they guarantee fine cooperation between sentinel cells, which recognize non-self molecules, and effector cells, which can mount active responses towards foreign cells or particles, in order to ensure the health and survival of individuals (Abbas *et al.*, 2000).

In tunicates, molecules recognized by antibodies raised against the mammalian pro-inflammatory cytokines IL-1 have been reported in various ascidian species (Beck *et al.*, 1989; Raftos *et al.*, 1991, 1992; Ballarin *et al.*, 2001, Cima *et al.*, 2004; Parrinello *et al.*, 2007). They have been partially characterized and their molecular weights range between 12 and 59 kDa, as resolved by SDS-PAGE and gel chromatography (Beck *et al.*, 1989; Raftos *et al.*, 1992; Parrinello *et al.*, 2007). It is common opinion that invertebrate cytokines share no homologies with their vertebrate counterparts (Beck, 1998; Beschin *et al.*, 2001, 2004) and this may explain the absence of orthologues of vertebrate pro-inflammatory cytokine genes in the genome of *Ciona instestinalis* (Azumi *et al.*, 2003). However, it has been reported that various vertebrate cytokines have a lectin domain and can bind carbohydrates (Cebo *et al.*, 2002; Beschin *et al.*, 2004) and this probably represents the evolutionary link between tunicate and vertebrate cytokines (Beschin *et al.*, 2001, 2004). The availability of the sequenced genome of *Botryllus* may allow us to clarify the evolution of vertebrate cytokines.

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