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SHORT COMMUNICATION

Cellular aspects of allorecognition in the compound ascidian *Botrylloides simodensis*

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Abstract

When colonies of the compound ascidian *Botrylloides simodensis* contact each other at their cut surfaces, either fusion or rejection occurs. Contact between genetically compatible colonies leads to the complete fusion of their tunics and vasculature within 24 h. Conversely, the rejection reaction between incompatible colonies is characterized by the appearance of a melanic, necrotic band along the contact border. In the case of fusion, limited crowding of cytotoxic morula cells (MCs) was observed in the ampullae near the contact border. In rejection, limited tunic fusion occurred in the contact region and MCs were selectively recruited inside the ampullae near the cut surface: most of them leaked into the tunic where they changed their morphology and contributed to the formation of the necrotic region. Granular amebocytes, like MCs, have granules well stained by eosin and were also seen inside the ampullae involved in the rejection reaction and along the contact border between incompatible colonies. Immunohistochemical analysis using antibodies raised against *Botryllus schlosseri* phenoloxidase (PO) and mammalian IL-1-α and TNF-α indicate that MCs were the only cells recognized by the anti-PO antibody; they resulted immunopositive also to the anti-cytokine antibodies in both fusion and rejection, whereas granular amoebocytes were recognized by the latter antibodies only during the rejection reaction.

Key Words: *Botrylloides simodensis*; ascidians; colony specificity; hemocytes; morula cells

Introduction

Botryllid compound ascidians share the ability of intraspecific colony recognition (colony specificity) which enables contacting colonies to fuse their tunic and circulation when genetically compatible (Taneda *et al*., 1985; Saito *et al*., 1994). In the case of incompatibility, contacting colonies do not fuse and, frequently, a rejection reaction occurs. The latter, typically, manifests itself as a series of necrotic, melanic spots along the contact border (Rinkevich, 1992; Saito *et al*., 1994). In this reaction, the key role of the enzyme phenoloxidase (PO) in the induction of cytotoxicity was clearly established (Ballarin *et al*., 1995, 1998; Shirae and Saito, 2000; Shirae *et al*., 2002; Cima *et al*., 2004). PO is stored inside the vacuoles of morula cells (MCs), an ubiquitous haemocyte type in botryllid ascidians, together with its polyphenol substrata and quinones (Ballarin *et al*., 1995; Shirae and Saito, 2000; Ballarin, 2008). In the course of the rejection reaction, a typical inflammatory reaction takes place, involving the selective recruitment of MCs in the blind endings of the peripheral tunic vasculature, called ampullae, facing the alien colony, their subsequent crossing the epithelium of the ampullar tips and migration into the tunic. Here, the induction of cytotoxicity occurs through MC degranulation and the release of MC vacuolar content (Hirose *et al*., 1990; Sabbadin *et al*., 1992; Shirae *et al*., 2002; Cima *et al*., 2004; Ballarin, 2008). Temporary crowding of MCs inside the facing ampullae was observed also in the course of the interaction between genetically compatible colonies of *B. schlosseri* and *Botrylloides leachi* but, in this case, the amount of MCs is significantly lower than that observed in contacting incompatible pairs and no migration in the tunic occurs (Cima *et al*., 2006; Zaniolo *et al*., 2006; Ballarin and Zaniolo, 2007).

In *B. schlosseri*, MC chemotaxis and cytotoxicity is modulated by cytokines (Cima *et al*., 2004, 2006). These immunomodulatory molecules are recognized by antibodies raised against

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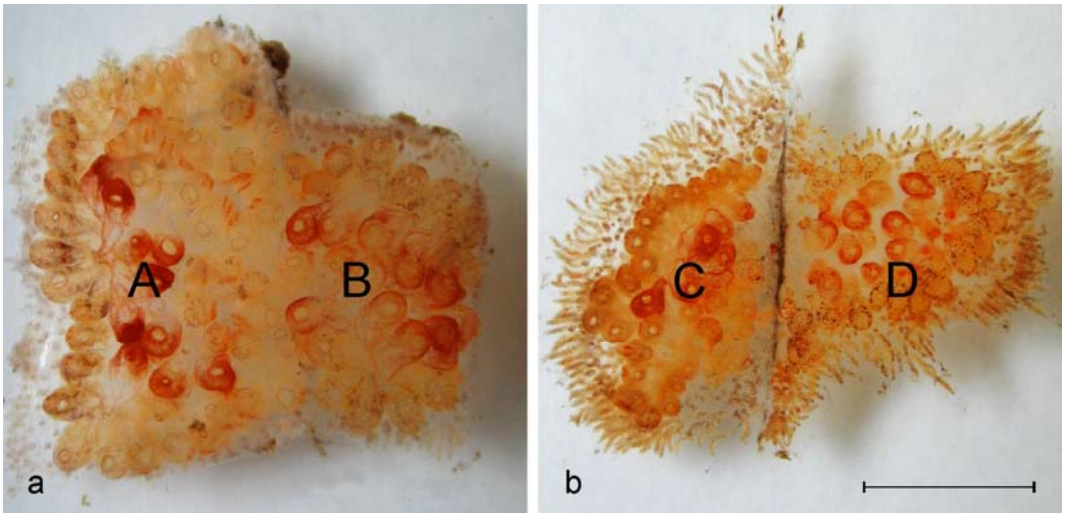

Fig. 1 Colonies of *B. simodensis* after 24 h from the contact at their cut surfaces. a: genetically compatible colonies A, B) showing complete fusion of the tunic; b: incompatible colonies (C, D) with a necrotic, melanic line clearly visible along the contact border. Scale bar: 5 mm.

mammalian IL-1-α and TNF-α and are synthesized and secreted by MCs upon the recognition of non-self molecules (Ballarin *et al*., 2001) and, in addition to MCs themselves, they can influence also the behavior of phagocytes (Menin *et al*., 2005; Menin and Ballarin, 2008). The immunopositivity of MCs to the above-reported antibodies has been observed also in the course of the intensive rejection reaction observed when genetically incompatible colonies of *B. leachi* are brought into contact at their cut surfaces. In this case, also granular cells, considered MC precursors, resulted positive (Ballarin and Zaniolo, 2007).

Botrylloides simodensis is a Japanese ascidian where a clear allorecognition reaction, either fusion or rejection, is observed when colonies contact at their cut surfaces. Even in this case, the pivotal role of MCs and PO in the rejection reaction was clearly demonstrated (Shirae *et al*., 2002).

In the present study, we continued our previous investigations on the cellular events in colony specificity of *B. simodensis*, using a panel of antibodies to compare the behavior of MCs in both fusion and rejection reactions at the contacting cut surfaces, with particular reference to the expression of putative cytokines. Results were compared with what already known in the reference species *B. schlosseri*.

Materials and Methods

Animals

Colonies of *B. simodensis* were collected near Shimoda (Shizuoka Prefecture, Japan). They were attached to glass slides and reared in culture boxes immersed in Nabeta Bay.

Cut surface allorecognition assay

Colonies were cut with a razor blade and pieces of the same size from different colonies were brought into contact at their cut surfaces on a glass slide and left to adhere for 2 h in a moist chamber. Juxtaposed fragments of the same colony were used as reference controls (compatible pairs). Slides were maintained for 24 h in plastic Petri dishes filled with filtered seawater (FSW) and the outcome of the reaction at the contact area was then observed under a binocular microscope.

Immunohistochemical analyses of contacting colonies

Colony pairs were fixed in 4 % formaldehyde in FSW, dehydrated through a butanol series and embedded in paraffin. Deparaffined sections (6 µm) were treated for 30 min with 1 % H_2O_2 in 80 % methanol to block endogenous peroxidase, for additional 30 min in 1 % skimmed powdered milk in phosphate-buffered saline (PBS: 8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 1.15 g/l Na_2HPO_4, pH 7.2) for the reduction of unspecific staining and incubated overnight with 10 µg/ml primary antibody raised against *B. schlosseri* PO (Frizzo *et al*., 1999), human recombinant IL-1-α and human recombinant TNF-α (Santa Cruz Biotech., Santa Cruz, CA, USA), in PBS. Slides were then washed in PBS, incubated for 30 min in 50 µg/ml biotinylated anti-rabbit IgG antibody (Santa Cruz Biotech.) in PBS, washed again and incubated for 30 min in the avidin-biotin-peroxidase complex (ABC, Vector Labs., Burlingame, CA, USA). After thorough washing in PBS, sections were finally incubated for 5 min in a solution of 0.025 % 3,3' diaminobenzidine (DAB) containing 0.004 % H_2O_2 and mounted with Eukitt. Positive sites appeared brown. Reference slides were stained with hematoxylin and eosin.

Results

Fusion and rejection reactions

In the case of compatible pairs, no cytotoxic reaction was observed and complete tunic fusion was observed after 24 h from the contact of the cut

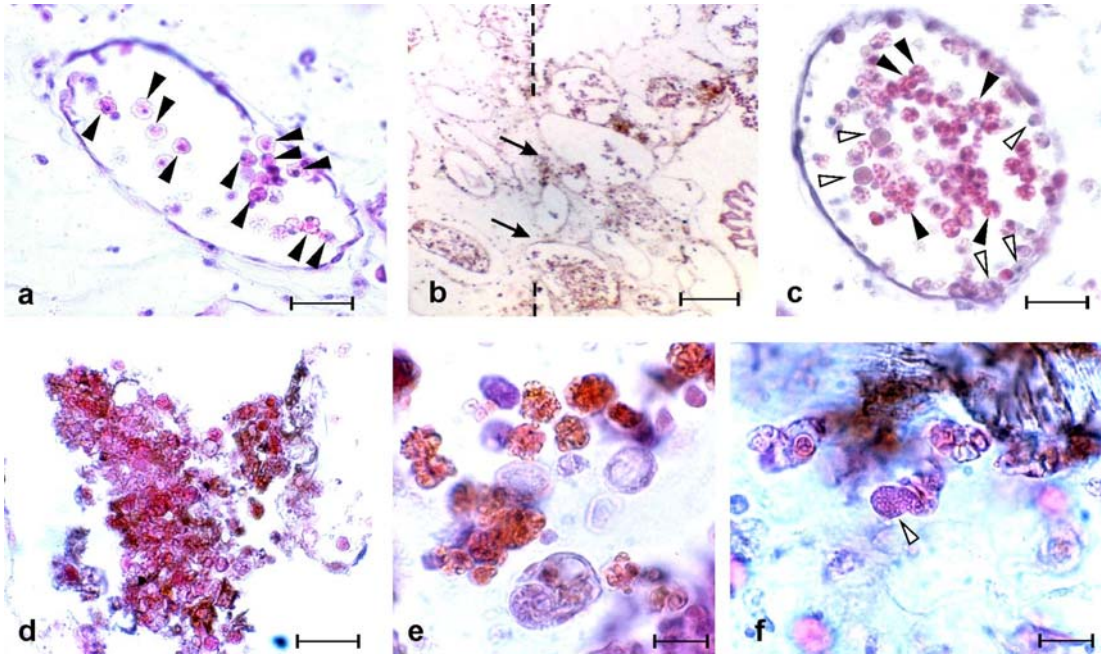

Fig. 2 Paraffin sections of contacting genetically compatible and incompatible colonies of *B. simodensis*. a: lumen of a peripheral ampulla facing a compatible colony. Limited recruitment of eosinophilic MCs (arrowheads) is observable. b: contact region between incompatible colonies (right and left of the dashed lines); arrows indicate the regions of fusion of the contacting tunics. c: lumen of a peripheral ampulla facing an incompatible colony. Selective recruitment of eosinophilic MCs (dark arrowheads) and some granular amoebocytes (open arrowheads) are observable. d-f: contact border between incompatible colonies. Massive leakage of MCs in the tunic (d), where they show altered morphology (e), together with some granular amoebocytes (f) can be observed. Scale bars: 20 μm in e, f; 50 μm in a, c, d; 250 mm in b.

surfaces (Fig. 1a). Limited cell crowding occurred in the ampullae near the contact border: most of them were MCs which, as already reported (Shirae *et al*., 2002; Ballarin and Zaniolo, 2007), had granules well stained by eosin. Cell leakage was never observed (Fig. 2a).

In the case of incompatible pairs, a clear necrotic, melanic band (Fig. 1b) characterized the border between the cut surfaces after 24 h from the contact (Shirae *et al*., 2002). Limited tunic fusion was observed in the contact region (Fig. 2b), as well as selective recruitment of MCs inside the ampullae close to the contact border (Fig. 2c). Many of these cells leaked from the ampullar lumen into the tunic, crowding along the border, where they showed altered morphology and contributed to the formation of the necrotic region (Fig. 2d). Granular amoebocytes like MCs, have granules stained by eosin and also increase their frequency inside the ampullae involved in the rejection reaction, although to a lesser degree than MCs, and in the tunic, close to the contact border (Figs 2c, f).

Immunohistochemical analyses during fusion and rejection reactions

Circulating MCs resulted immunopositive to anti-PO antibodies in both fusing and non-fusing colonies (Figs 3a, b). A similar result was observed with the anti-cytokine antibodies (Figs 3c-f). Conversely, granular amoebocytes inside the ampullar lumen never resulted immunopositive to the assayed antibodies in the case of fusion, whereas a clear positivity was observed during the rejection reaction (Figs 3d, f). No recognition the cells of the ampullar epithelium or of the tunic by the antibodies was observed.

Discussion

Allorejection reaction between contacting, genetically incompatible colonies, has been extensively studied in the compound ascidian *B. schlosseri*, where a typical inflammatory reaction occurs in the colonial vasculature facing the alien colony. In this species, the pivotal role of cytotoxic MCs as effectors of the reaction has been demonstrated (Ballarin *et al*., 1995). These cells, directly involved in immunosurveillance, are able to sense non-self molecules (Ballarin, 2008; Ballarin *et al*., 2001) and, as a consequence of the recognition, they release immunomodulatory molecules recognized by antibodies raised against mammalian pro-inflammatory IL-1-α and TNF-α (Ballarin *et al*., 2001, 2005). In the case of rejection reaction, these molecules, released as a consequence of the recognition of soluble allogeneic factors diffusing

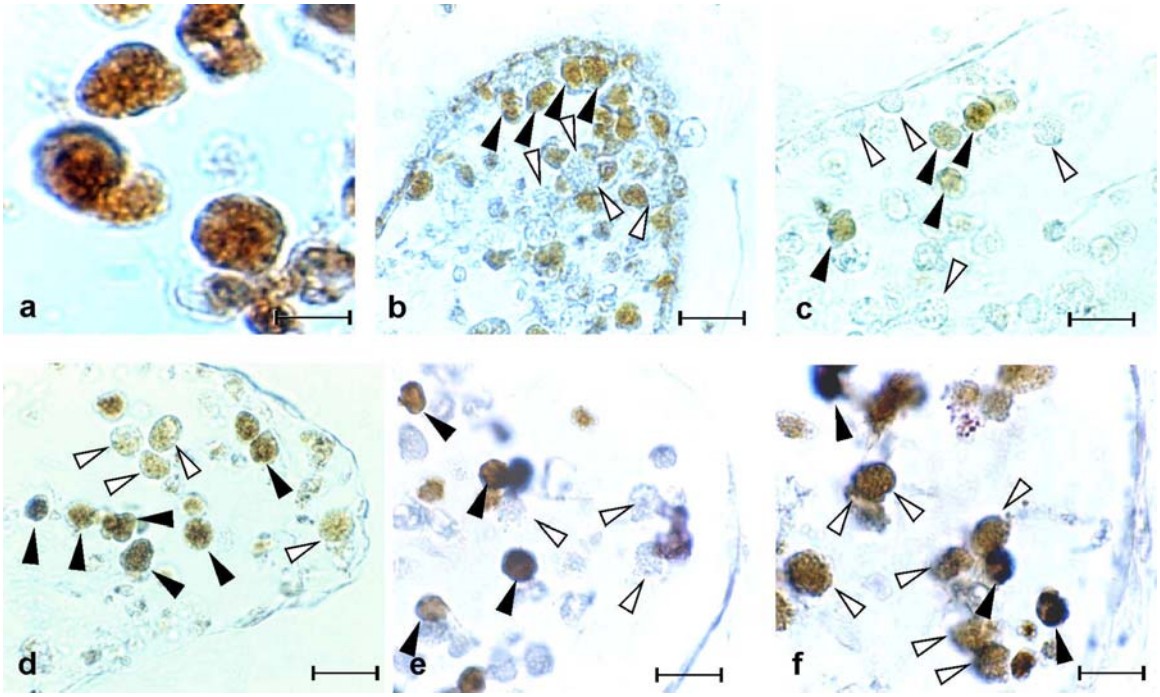

Fig. 3 Immunohistochemical analysis on paraffin sections of facing ampullae in fusion (a, c, e) and non-fusion (b, d, f) reactions between contacting colonies of *B. simodensis*. a, b: anti-PO antibody. Positivity is limited to MCs (dark arrowheads) in both fusion (a) and rejection (b); granular amoebocytes (open arrowheads) are never stained. c, f: anti-IL-1-α (c, d) and anti-TNF-α (e, f) antibodies. MCs (dark arrowheads) are stained in both fusion (c, e) and non-fusion (d, f), whereas granular amoebocytes (open arrowheads) are stained only in the case of rejection (d, f), Scale bars: 10 μm in a; 50 μm in b-f.

from the circulation of the alien colony through the partially fused tunics, are responsible of the selective recruitment of MCs inside the facing ampullae which characterizes the early phases of the rejection reaction (Cima *et al*., 2006). These cells, then, cross the ampullar epithelium and enter the tunic where they degranulate releasing their vacuolar content, mainly the enzyme PO and its substrata (Ballarin *et al*., 1998, 1998; Cima *et al*., 2004) which are responsible of the formation of the melanic, necrotic spots clearly visible along the contact border (Sabbadin *et al*., 1992).

In *B. simodensis*, humoral factors involved in the rejection reaction have been partially characterized from the blood plasma: they are heat labile, resistant to dialysis and divalent cation-dependent (Saito and Watanabe, 1984). In the same species, the rejection reaction between contacting growing edges of genetically incompatible colonies occurs in the form of a so-called “subcuticular rejection”. In the course of this reaction, a few hemocytes leak from the ampullar lumen into the tunic and contribute to the formation of small necrotic regions along the contact border which are scanty visible under the binocular microscope (Hirose *et al*., 1997). However, if colonies were brought into contact at their cut surfaces (cut surface assay), a more intense and rapid cytotoxic reaction can be observed (Hirose *et al*., 1990). In the present work, we performed this latter assay in order to have a clear view of the events occurring during the rejection reaction at the contacting cut surfaces of incompatible colonies of *B. simodensis*.

As reported elsewhere (Shirae *et al*., 2002), in the course of the reaction, MCs selectively crowd inside the ampullae near the contacting cut surfaces and result positive to the cytoenzymatic assay for PO (Shirae *et al*., 2002, present work). The selective recruitment, although less marked, was observed also in the case of fusible pairs. However, in the case of incompatible pairs, MCs leak from the ampullae and enter the tunic where they change their morphology and discharge their vacuolar content (Shirae *et al*., 2002). In both compatible and incompatible combinations, MCs resulted strongly labeled by the antibodies against mammalian cytokines. This supports the idea that, analogously to *B. schlosseri*, MCs of *B. simodensis* release immunomodulatory molecules upon the contact with another colony. In *B. leachi*, which has a behavior similar to *B. simodensis* with regard to allorecognition (*i.e.,* it gives a limited subcuticular rejection at the growing edges and an intense reaction at the cut surfaces), immunopositivity to anti-IL1-α and anti-TNF-α was observed only in incompatible combinations. In *B. simodensis*, contrarily to our expectations, immunopositivity was observed also in the case of contact between compatible pairs, the only difference being represented by the presence of immunopositive granular amoebocytes in allorejection which were not observable in fusible pairs. During the rejection reaction, these cells, considered related to MCs, are recruited inside facing ampullae, migrate into the tunic and secrete molecules recognized by antibodies raised against mammalian proinflammatory cytokines. In *B. schlosseri* and *B.*

leachi, granular amoebocytes are considered the precursors of MCs, on the basis of common staining properties (Cima et al., 2001; Ballarin and Cima, 2005) and, in *B. leachi*, they can be found along the contact surfaces of incompatible pairs (Ballarin and Zaniolo, 2007). As stated before, in *B. schlosseri*, molecules recognized by antibodies raised against mammalian IL-1-α and TNF-α recognize MCs and modulate the rejection reaction. We assume that a similar role is exerted by the same molecules also in our species: this hypothesis is supported by the observation that the above-reported antibodies recognize the MCs involved in the rejection reaction at cut surfaces of incompatible colonies of *B. leachi* (Ballarin and Zaniolo, 2007). Unlike *B. schlosseri* and *B. leachi*, *B. simodensis* granular amoebocytes do not show any detectable PO activity. Therefore, it results that, in *B. simodensis* as well as in *B. leachi*, cytotoxic cells can exert their immunomodulatory role even before their full maturation to morula cells. This resemble the behavior of circulating mammalian monocytes that, although not fully differentiated to functional macrophages, can contribute to the modulation of immune responses (Saha and Geissman, 2011; Patel and Davidson, 2014). Further studies will be directed to a better clarification of the role of granular amoebocytes in *B. simodensis* and their functional relationships with MCs.

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