ISJ 3: 125-136, 2006 ISSN 1824-307X

Research Report

Colony specificity in *Botrylloides leachi*. I. Morphological aspects

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Accepted December 20, 2006

Abstract

We studied colony specificity in the colonial ascidian *Botrylloides leachi* which, as in other botryllid ascidians, leads to either fusion or non-fusion between contacting colonies. Fusion requires the prior disappearance of contacting tunic cuticles and contact between facing ampullar epithelia. The epithelial cells of the ampullar tip show “pad regions” rich in ribosomes, which contribute to the synthesis of new tunic and cuticle. Blood cells, mainly phagocytes and pigment cells, increase their concentrations inside the ampullar lumen and phagocytes can cross the ampullar epithelium and enter the tunic, where they can contribute to the digestion of tunic cuticles and cells of the ampullar epithelium in order to establish a common circulation. Non-fusion reaction, as studied in the colony allorecognition assay, resembles the subcuticular rejection described in Japanese *Botrylloides*, characterised by limited tunic fusion, hemocyte leakage, and necrotic spots. Conversely, in the cut surface assay, a more intense cytotoxic reaction is observed along the contact border. In this case, morula cells crowd massively inside the facing ampullae, enter the tunic, and release their vacuolar contents which are probably required for the formation of necrotic spots.

Key words: *Botrylloides*; ascidians; colony specificity; allorecognition

Introduction

The term allorecognition defines the capability of intraspecific non-self recognition; in clonal organisms, it is known as colony specificity and has been described in many species of compound ascidians, in which it leads to either fusion of facing, genetically compatible colonies into a larger chimerical colony, or non-fusion, when contacting colonies are genetically incompatible (Taneda *et al.*, 1985).

Botryllid ascidians are a group of compound tunicates, characterised by palleal budding, with colonies formed of many zooids embedded in a common tunic containing a vascular network, which connects and synchronises all the zooids. A marginal vessel runs along the border of the colony, from which many sausage-like blind endings, known as ampullae, sprout. Ampullae sited at the growing edge allow the adhesion of the colony to the substratum and have a columnar epithelium on their tips, provided with pad cells (Katow and Watanabe, 1980; Rinkevich *et al.*, 1998).

The outcome and intensity of the non-fusion reaction in botryllid ascidians depends on the tissues involved in the recognition. According to Rinkevich (1992) and Saito *et al.* (1994), allorecognition can occur:

i) after the disappearance of most of the contacting cuticular layers, and consequent fusion of the tunics. In this case, the ampullae penetrate the opposite tunic and face the basal side of alien ampullae (tip-to-side interaction); massive crowding of hemocytes inside the ampullar tips then occurs, followed by leakage of cells into the tunic, contributing to the formation of a clear necrotic area. The ampullar tips then shrink, are amputated, and new tunic cuticles are formed by the two colonies to isolate the necrotic region along the contact border. This is typical of *Botryllus primigenus* (Taneda and Watanabe, 1982);

ii) after partial fusion of the tunic, restricted to regions in front of the facing (tip-to-tip) ampullae, which then can (Woods Hole *Botryllus schlosseri*) or

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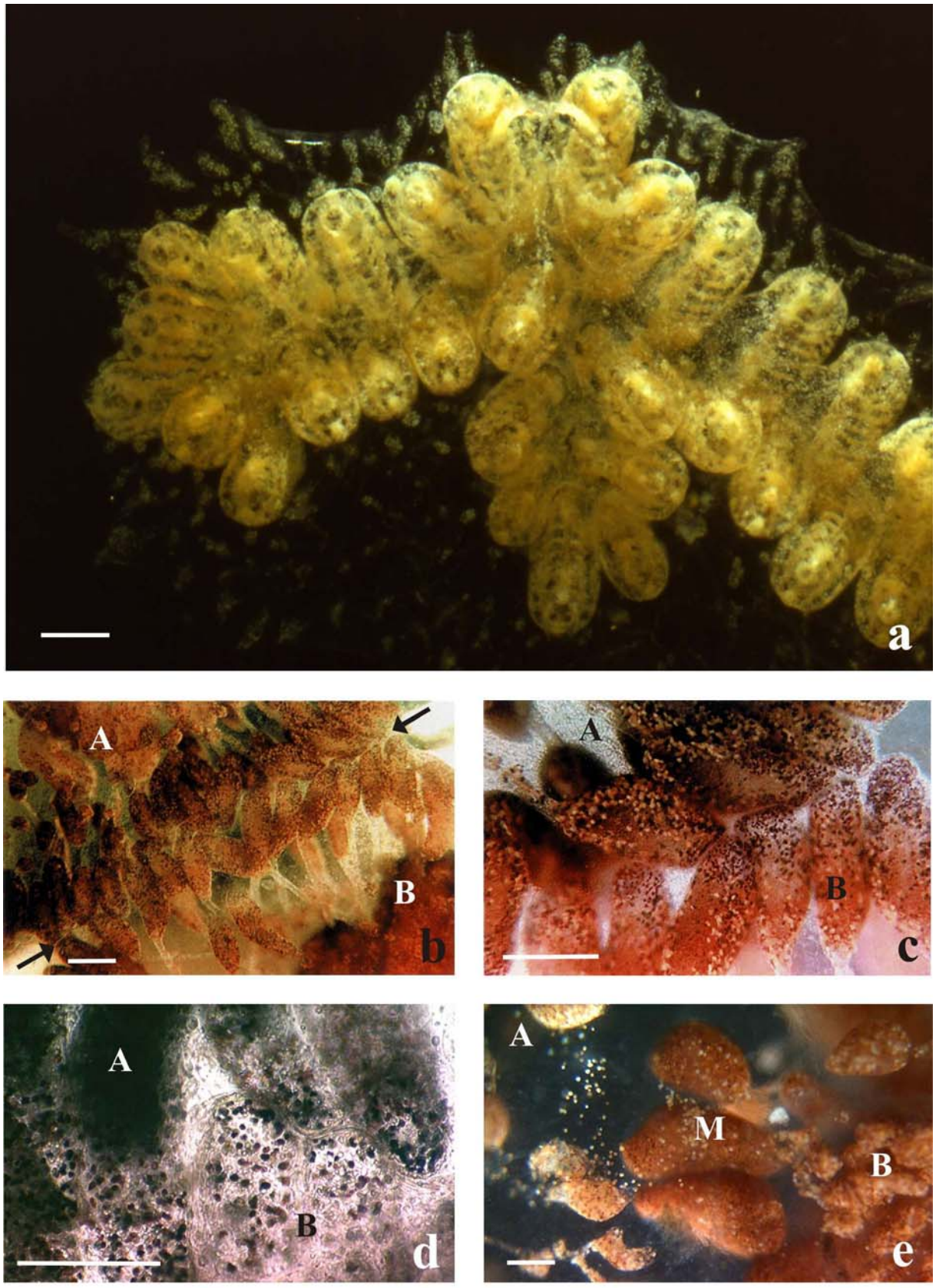

Fig. 1 a) living colony of *Botrylloides leachi* from the dorsal side. Zooids and buds are embedded in the common tunic, crossed by vessels with terminal ampullae. b) interacting ampullae of fusible colonies (A, B). Arrows indicate the contact region. c-d) contacting ampullar tips of fusible colonies (A, B). The ampullar tips can contact either the tip or the apical side of the facing counterparts. Bar = 150 µm. e) megaloampullae (M) in one of the contacting colony. Bars = a, 1 mm; b-e, 150 µm.

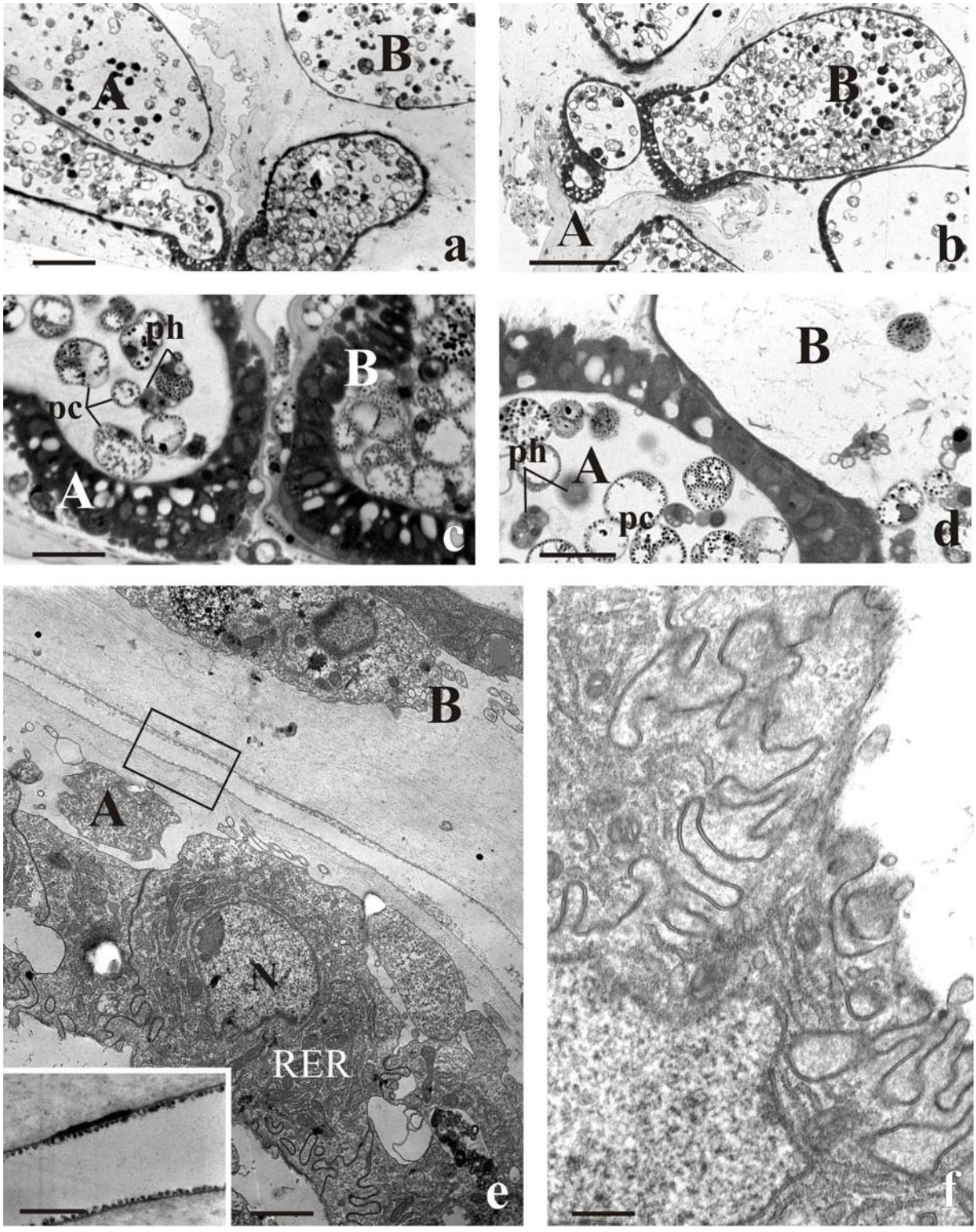

Fig. 2 Interacting ampullae of contacting fusible colonies (A, B). a, c) contacting tips, semithin sections. Bar = 50 and 15 µm, respectively. b, d) contact between tip and ampullar apical side. Bars = 50 and 15 µm, respectively. e) contact between fusible colonies, ultrathin section. The two cuticles show numerous papillae (inset) and are separated by a narrow space; cells of the ampullar epithelium are cylindrical, with large nuclei (N) and well-developed rough endoplasmic reticulum (RER). Bar = 2 µm (inset: 0.6 µm). f, highly folded plasma membrane in baso-lateral region of epithelial cells of the ampullar tip. ph, phagocytes; pc, pigment cells. Bar = 2 µm.

cannot (Monterey and Mediterranean *B. schlosseri*) enter the facing tunic. Cells crowd inside the ampullar tips, leak into the tunic, and contribute to the formation of a series of necrotic spots along the contact border. The ampullae then shrink and withdraw; in the Woods Hole population, their tips are amputated near the colonial boundary (Boyd *et al.*, 1990; Sabbadin *et al.*, 1992);

iii) at the outer part of the tunic, just underneath the cuticle (subcuticular), which dissolves in very narrow regions in front of the facing ampullae. A few hemocytes leak from the ampullar lumen into the tunic, and contribute to the formation of small necrotic regions, which are not recognisable under the binocular microscope, along the contact border. This is reported in *Botrylloides simodensis*, *Botrylloides fuscus* and *Botrylloides violaceus* (Hirose *et al.*, 1988, 1997);

iv) after the fusion of the facing ampullae and an initial blood exchange. This characterises the non-fusion reaction of *Botryllus scalaris* (Saito and Watanabe, 1982; Shirae *et al.*, 1999).

Non-fusion reaction has been widely investigated in *B. schlosseri*, in which morula cells (MC), representing the majority of the circulating hemocytes, massively crowd inside the tips of facing ampullae before crossing the ampullar epithelium and entering the tunic, where they degenerate and contribute to the formation of the necrotic masses observed along the contact border (Ballarin *et al.*, 1995, 1998). These cells degranulate and release the enzyme phenoloxidase, which is responsible for the cytotoxicity observed, due to the induction of oxidative stress (Ballarin *et al.*, 2002).

Botrylloides leachi is a compound ascidian, common in the Mediterranean, and it can easily grow and reproduce in laboratory conditions. Unlike from the sympatric species *B. schlosseri*, in which zooids are grouped in star-shaped systems and colonies are characterised by high chromatic polymorphism, *B. leachi* is easily distinguishable, thanks to the organisation of zooids in ladder-shaped systems and their orange to brown colonies (Fig. 1a) .

In spite of its abundance, colony specificity has been poorly investigated in this species (Rinkevich *et al.*, 1994; Rinkevich, 1995) To fill this gap, we carried out a detailed investigation, at morphological and cellular level, of fusion and non-fusion in *B. leachi*, using both colony allorecognition assay (CAA) and cut surface assay (CSA) as defined by Rinkevich (1992). Results are discussed in the context of colony specificity in botryllid ascidians, and cellular events compared with those known in *B. schlosseri*.

Materials and Methods

Animals

B. leachi colonies, collected in the Lagoon of Venice, were allowed to adhere to glass slides (5 x 5 cm) and reared in aerated aquaria, filled with filtered seawater (FSW), at a temperature of 19 °C. They were fed with Liquifry Marine (Liquifry Co., Dorking, England) and water was changed every other day.

Colony specificity assays

In colony allorecognition assay (CAA), colonies were juxtaposed on a supporting glass slide at a distance of 1-2 mm, their growing edges facing each other, in a moist chamber for 30 min, before being returned to the aquarium. Within 24-48 h, the colonies extended towards each other and their facing ampullae contacted. Cut surface assay (CSA) was carried out to better investigate the non-fusion reaction: in this case, colonies were cut with razor blades and pieces of the same size from different colonies were brought into contact at their cut surface on a supporting glass slide and allowed to adhere for 30 min in a moist chamber. They were then returned to the aquarium. In each case, colonies were observed under a binocular microscope for up to 48 h, until either fusion or non-fusion occurred.

Light microscopy

Contacting colonies, at various stages of both fusion and non-fusion, were fixed for 2 h in 4 % paraformaldehyde plus 1 % glutaraldehyde in saline buffer (0.2 M Na-cacodylate buffer, pH 7.4, plus 1.7 % NaCl and 1 % sucrose), rinsed in saline buffer, dehydrated and embedded in Paraplast X-Tra (Oxford Labware). Sections (7 µm thick) were obtained with a Leitz 1212 microtome and stained with either haematoxylin/eosin or 2 % eosin G (Shirae *et al.*, 2002).

Electron microscopy

Fusing and non-fusing colonies were fixed for 2 h in 1.5 % glutaraldehyde in saline buffer, post-fixed for 1 h in OsO_4 in saline buffer, dehydrated and embedded in Epon. Thick sections (1 µm) were obtained with a LKB 2128 ultratome, stained with a hot solution of 1 % toluidine blue and 1 % borax in distilled water, and observed under the light microscope. Thin (60 nm) sections were collected on copper grids, stained with uranyl acetate and lead citrate, and observed under a Hitachi H 600 transmission electron microscope.

Results

Fusion

Interacting colonies extend the ampullae of their growing edges towards those of the alien colony to a close contact, in which ampullar tips are separated only by a thin layer of tunic (Figs 1b-d). The ampullar tips can contact either the tips (Figs 1c, d, 2a, c) or the apical sides (Figs 1c, d, 2b, d)) of the alien counterpart. In both cases, facing tunic cuticles of early contacting colonies appear separated by a narrow space (Figs 1c, d, 2c-e); they present numerous protruding papillae, 90 nm in height (Fig. 2e (inset)). The tunic matrix appears rich in fibres, and tunic cells can be observed in the contacting region (Figs 2c, e). The cells of the epithelium of the ampullar tips have a central nucleus, many mitochondria and a well-developed rough endoplasmic reticulum (RER), organised in a series of overlapping cisternae (Fig. 2e); the basolateral plasma membranes are tightly folded (Fig. 2e). The epithelium appears cylindrical in shape,

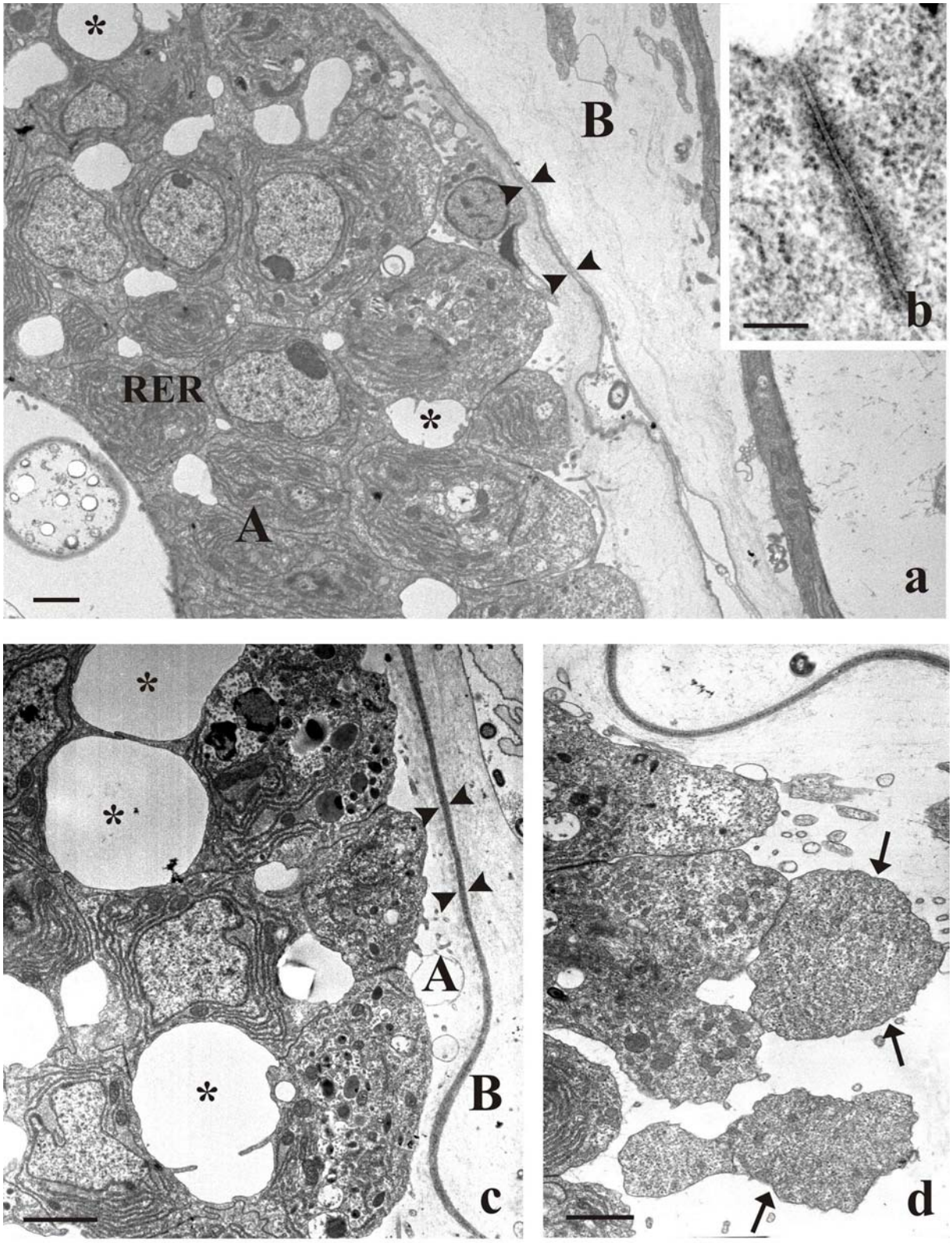

Fig. 3 Interacting ampullae of contacting fusible colonies (A, B), ultrathin sections. a) tight contact between cuticles. The epithelium appears fenestrated (asterisks) and cells show tight junctions in their apical region. b) magnification of a tight junction of the ampullar epithelium. c) pad regions (arrows) in epithelial cells of the ampullar tips. RER, rough endoplasmic reticulum. Bars = 2 µm in a, c, d; 0.2 µm in b.

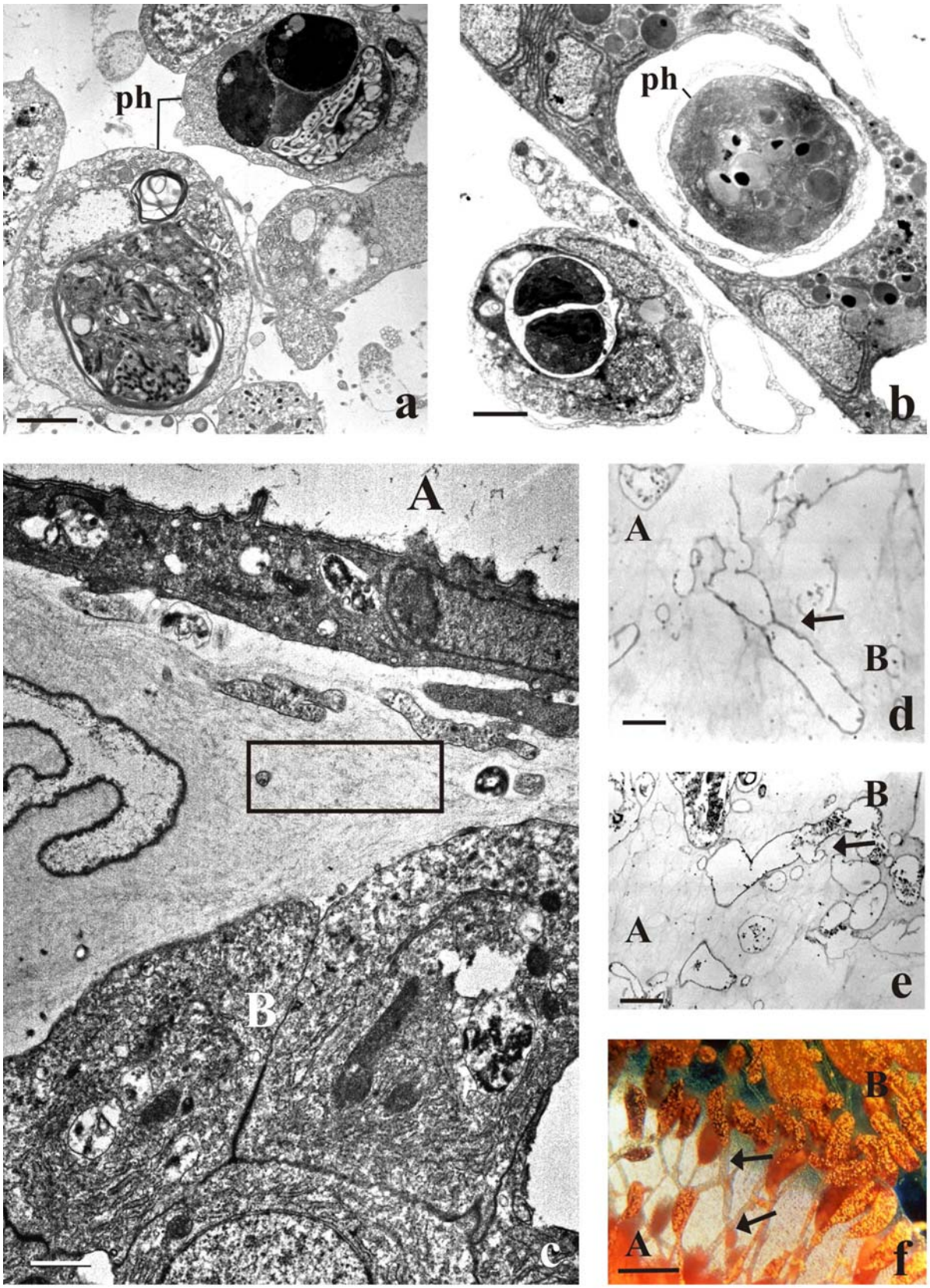

Fig. 4 Interacting fusible colonies. a-c) electron micrographs of the contact region between fusible colonies showing phagocytes (ph) in the lumen of facing ampulla (a), a phagocyte crossing the ampullar epithelium (b) and the complete fusion of contacting tunics of contacting colonies A and B in the framed region (c). Bars = 3 µm for a and b; 1 µm for c. d-f) light micrographs of the interacting region between fusible colonies showing close contact between the tips of two facing ampullae (d) and formation of a new vessel allowing blood exchange between the two colonies A and B (e, f; arrows). Bar = 250 µm.

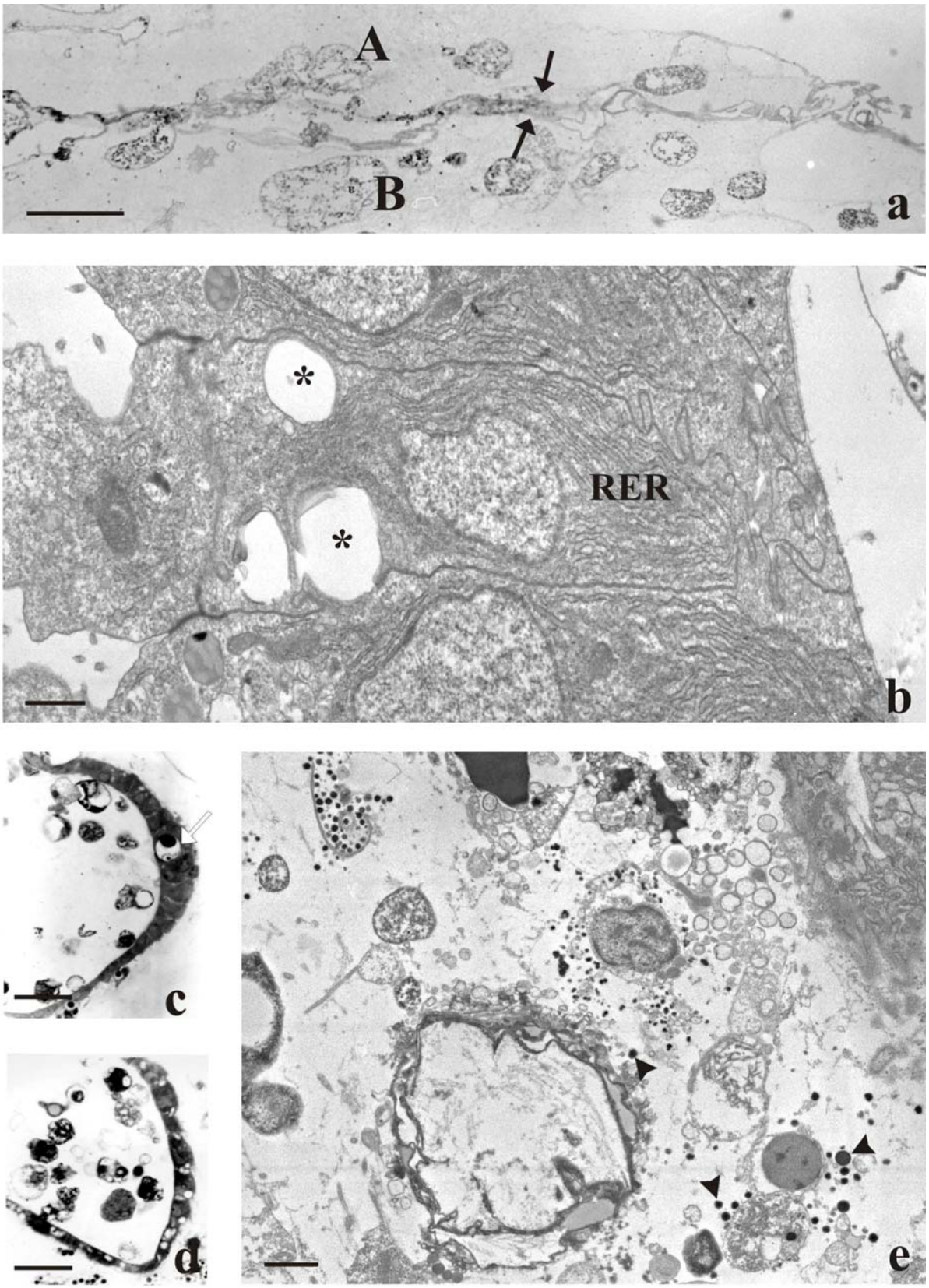

Fig. 5 a) light micrograph of the contact region between non-fusible colonies in CAA. Arrows indicate the region of close adhesion between the cuticles. Bar = 0.5 mm. b) electron micrograph of the tip epithelium of interacting ampullae, cylindrical, rich in RER, and fenestrated (asterisks). Scale bar: 1.5 µm. c-d) semithin sections of interacting ampullae showing crossing hemocytes (c; arrow) and fenestrated epithelium (d). Bar = 20 µm. e) degenerated cells in the common tunic in front of interacting ampullae. Electron-dense granules leaked from morula cells are indicated by arrowheads. Bar = 2 µm.

lying on a thick basal lamina and provided with wide tight junctions in the apex region of the cells (Figs 2e, 3a, b). Once contact is established, the ampullae can grow to form megaloampullae (Fig. 1e), as defined by Rinkevich *et al.* (1994). No "wavy" epithelium, as described by Rinkevich *et al.* (1994) in *Botrylloides* from the Mediterranean coast of Israel, was observed in interacting ampullae. Within 24-48 h from the first touch, a tight contact between the cuticles is observed (Figs 3a, c). The epithelium of the ampullar tips is always continuous, although it now appears fenestrated (Figs 3a, c); cells form irregular expansions at their apex (pad regions, according to Katow and Watanabe (1980)), containing ribosomes, finely granular material, and some small vesicles (Fig. 3d). An increase in the concentration of various hemocytes is observed inside the facing ampullar tips (Figs 2a-d), mainly represented by phagocytes and pigment cells (Figs 2c, d, 4a). Blood cells, particularly phagocytes, can be seen crossing the epithelial cells towards the tunic (Fig. 4b).

Within 24-48 h of contact, the cuticles disappear, and tunic fusion is attained along the whole contact border (Fig. 4c). Tunic fusion is followed by fusion of the ampullar tips, so that blood can now flow from one colony to the other (Figs 4d-f).

Non-fusion, CAA

At the beginning of the reaction, the tunic cuticles are well developed and separate the two contacting colonies (Fig. 5a). The morphology of the ampullar tip epithelium is comparable to that observed in fusing colonies. Megaloampullae may form (Fig. 6a). In later stages, the contact between tunic become tighter, and the cuticles disappear in a narrow region in front of the facing ampullae where fusion of the tunic occurs; crowding of hemocytes is observed inside the facing ampullae. At about 24 h from contact, hemocytes begin to cross the ampullar tip epithelium (Fig. 5c), which appears fenestrated (Figs 5b, d) and, on entering the tunic, they degenerate and contribute to the formation of small, dark cytotoxic spots (Fig. 6b). Some of these cells are easily recognisable (e.g., phagocytes and MC; Figs 5c, d); others appear degenerated, and granules with electron-dense contents are visible in the tunic (Fig. 5e). Leakage of cells is followed by the withdrawal of ampullae.

Non-fusion, CSA

In early stages of CSA (Fig. 6c), tunics are still separated, and several ampullae are observable in the region pushing towards the contact border. Their lumen are crowded with hemocytes, particularly MC (Figs 6d; 7a), which are easily recognisable due to their morphology and eosinophily, and are characterised by the presence of many small vacuoles, 2 µm in size, which give them a typical mulberry shape. The epithelium of the ampullar tips is flattened (Figs 6d; 7a-c), with large nuclei and a well-developed RER (Fig. 7b). Within 24 h, tunic fusion occurs in limited regions and, over the next 24 h, many blood cells, mainly MC, leak from the ampullae (Figs 7b, c) and crowd in the tunic of the contact region (Fig. 6e), together with some granular cells (Fig. 6f), as defined by Cima *et al.* (2001). In this phase, MC alter their morphology and show empty vacuoles of larger size (Fig. 6g). Filamentous eosinophilic material is visible around the MC (Fig. 6h). In advanced stages of the non-fusion reaction, MC aggregate along the contact border and contribute to the formation of a series of clearly visible, pigmented necrotic spots (Fig. 6i).

Discussion

As compound organisms, botryllid ascidians share the ability for intraspecific recognition, observable when colonies come into contact, leading to either fusion or non-fusion of genetically compatible or incompatible colonies, respectively (Saito *et al.*, 1994). The genetic bases of allorecognition have been deeply studied in *B. schlosseri* and *B. primigenus*, in which colonies can fuse when they share at least one allele at a highly polymorphic Fu/HC locus; the absence of common alleles results in non-fusion (Oka and Watanabe, 1957, 1960; Sabbadin, 1962; Oka, 1970;). Other botryllid species seem to follow the same kind of genetic control (Saito *et al.*, 1994).

We studied fusion and non-fusion reactions between contacting colonies of the compound ascidian *B. leachi*. Both tip-to-tip and tip-to-side interactions occur between the ampullae of the growing edges, although they never enter the opposite tunic in advanced stages of the reactions. A typical feature of this species is the frequent enlargement of contacting ampullae to form megaloampullae, already described in the case of non-fusion (Rinkevich *et al.*, 1994), and now described also for fusion.

In the case of fusion, vascular anastomosis is preceded by the disappearance of the contacting cuticles and fusion of the thin layer of tunic covering the apex of growing-edge ampullae, which begins in front of the facing ampullae and extends rapidly to the whole contact border. Blood cells, especially phagocytes, crowd inside the ampullar lumen and, after crossing the epithelium, enter the tunic. They can contribute to the digestion of tunic cuticles and ampullar tips, in order to allow the establishment of a common circulation. The epithelium of the ampullar tips changes its morphology, as reported by Katow and Watanabe (1980) in *B. primigenus*: numerous fenestrae appear between cells which remain closely adherent through well-developed tight junctions, and their apexes form pad regions which may contribute to the synthesis of new tunic and cuticle (Katow and Watanabe, 1980).

The non-fusion reaction has been widely investigated in *B. schlosseri*: in this species incompatible colonies lead to limited fusion of the tunic in front of the ampullae facing tip-to-tip. Soluble factors diffusing from the alien tunic and activated hemocytes attract blood cells, mainly MC, which crowd inside the apex of the facing ampullae, before crossing the epithelium of the ampullar tips and entering the tunic. During this process, MC degranulate, release their vacuolar contents, and contribute to the formation of a series of necrotic spots scattered along the contact border (Sabbadin *et al.*, 1992; Ballarin *et al.*, 1995, Cima *et al.*, 2006). According to our results, the non-fusion reaction in

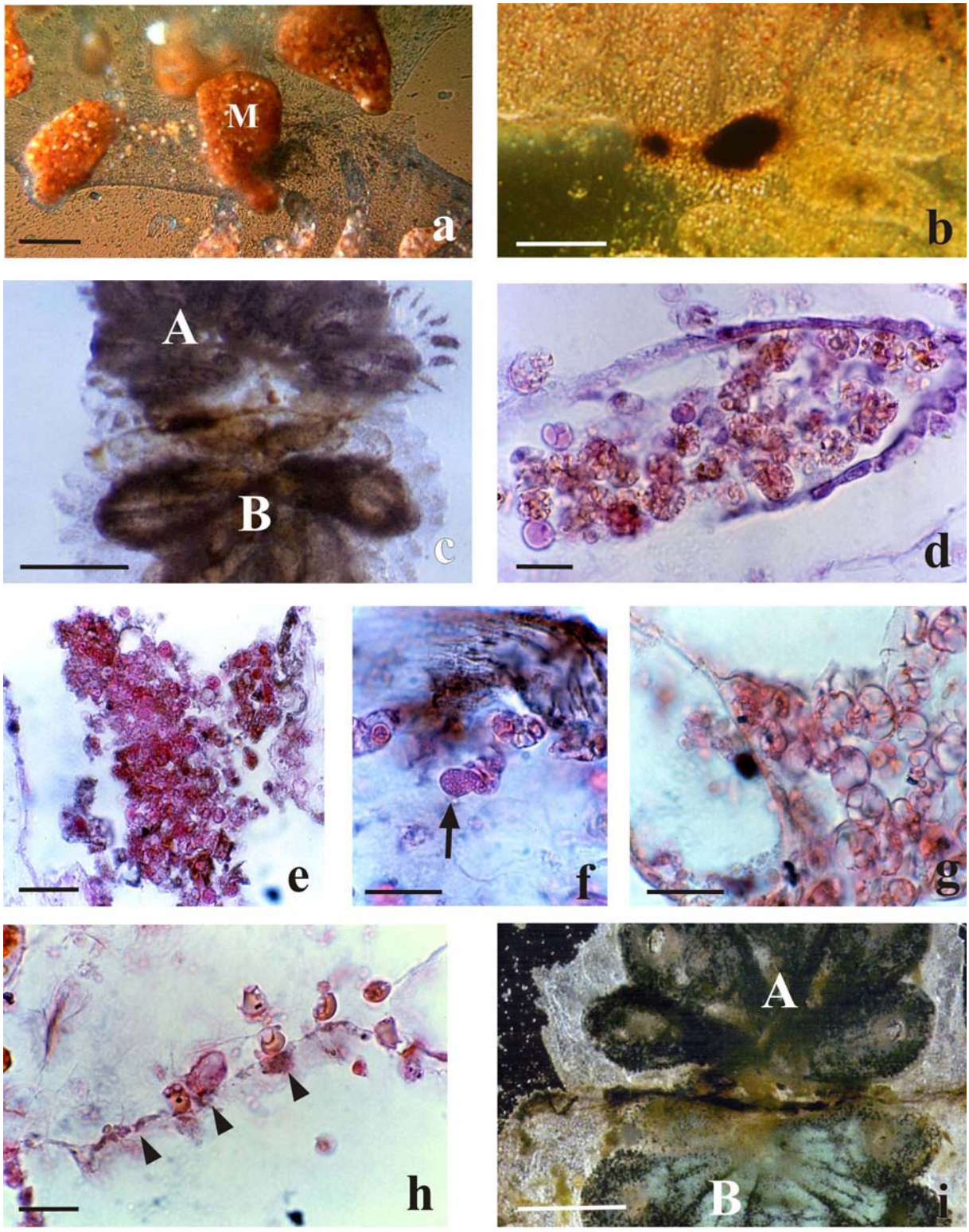

Fig. 6 a-b) CAA. Megaloampullae (M) in contact region between non-fusible colonies (a) and dark, cytotoxic spots in the contact area (b). Bars = 250 and 150 µm, respectively. c-I) CSA between two colonies (A, B) showing several ampullae in the contact region (c). The lumen of the ampullae in the contact area is filled with hemocytes, mainly morula cells (d), which leak in the tunic and crowd in the contact area (e), together with some granular cell (f, arrowhead), where morula cells degranulate, changing their morphology and showing large, empty vacuoles (g). Eosinophilic material (arrowheads) is visible around morula cells (h). In advanced stages of the non-fusion reaction, diffuse necrotic regions are visible along the contact area (i). Bars = 1 mm for c and i, 25 µm for d, f, g; 50 µm for e.

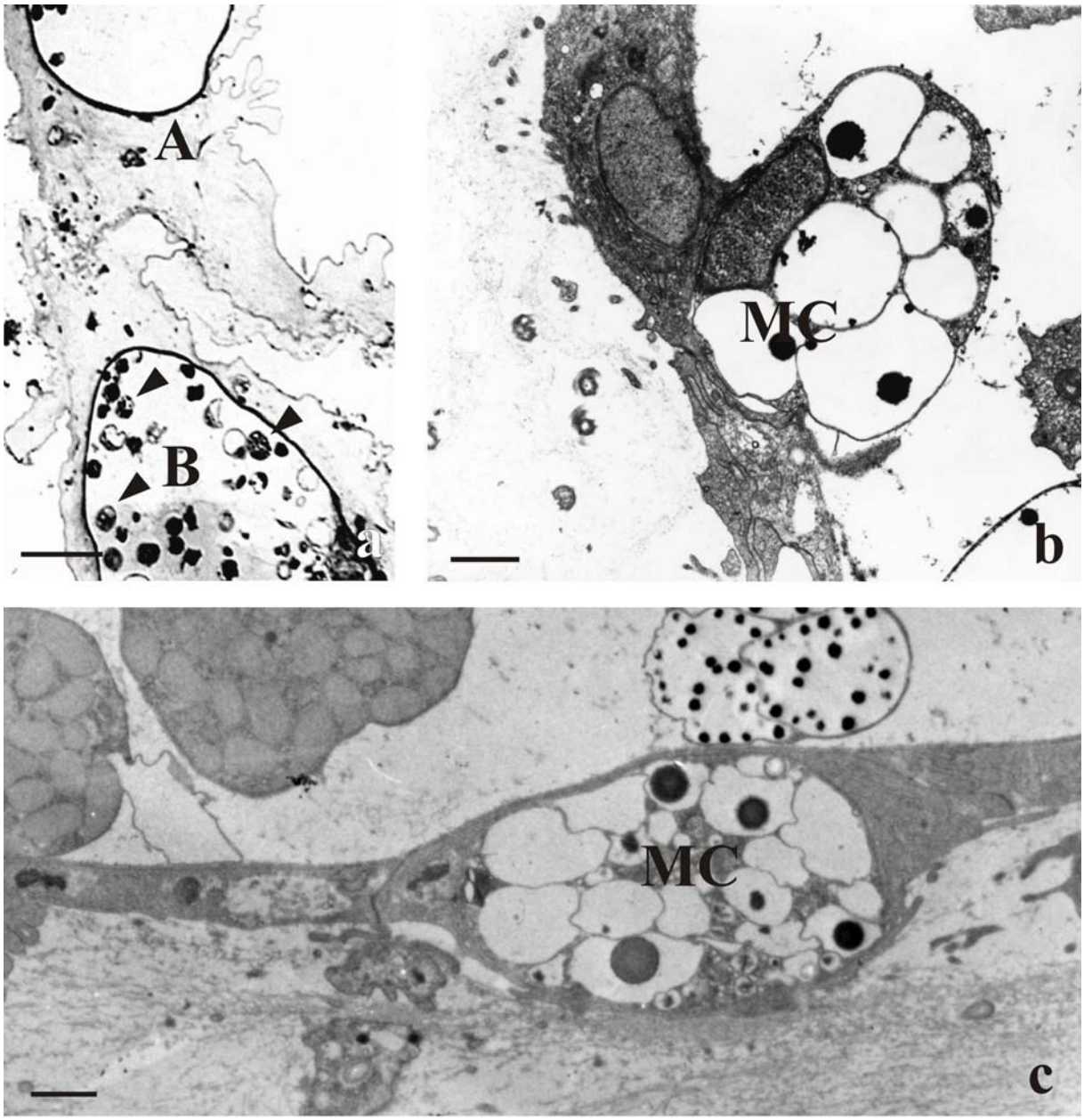

Fig. 7 Interacting ampullae in CSA. a) semithin section of ampullae with flattened epithelium and blood cells, many morula cells (arrowheads) are visible inside their lumen. Bar = 50 µm. b-c) electron micrographs of a morula cell (MC) interacting with the epithelium of the ampullar tip (b) and crossing it (c). Bar = 1 µm

B. leachi, as observed in CAA, is characterised by limited tunic fusion and blood cell leakage from facing ampullae which do not penetrate the opposite tunic. Giant ampullae sometimes, appear, but the outcome of the reaction seems to be similar in both the presence and absence of megaloampullae: hemocytes crowd inside the facing ampullae, and later move into the tunic through the fenestrated epithelium of the ampullar tips. The ampullae then, withdraw, and the colonies change their preferential direction of growth. In any case, the extent of the leakage of hemocytes and the size of the necrotic spots are very small when compared with the case in *B. schlosseri* (Sabbadin, 1982; Scofield and Nagashima, 1983; Sabbadin *et al.*, 1992), and the reaction resembles the subcuticular rejection described in Japanese species of the genus *Botrylloides* (Hirose *et al.*, 1988, 1997), in which a few blood cells leak through the ampullar epithelium in front of the ampullar tips, and cytotoxic foci are poorly visible. In order to study better the role of blood cells in the non-fusion reaction, we used CSA, which gives a more intense cytotoxic reaction in those species characterised by subcuticular rejection in CAA (Hirose *et al.*, 1990, 1997, 1998). With this assay, we were able to demonstrate that MC are involved in the non-fusion reaction in *B. leachi*. Similarly to what occurs in *B. schlosseri* (Ballarin *et al.*, 1995, 1998; Rinkevich *et al.*, 1998; Cima *et al.*, 2006), MC selectively accumulate inside

facing ampullae. This suggests chemotactic activity by soluble factors from the alien colony, which may be reinforced by the release of chemotactic chemokines by activated hemocytes. Similar behaviour by activated MC has recently been demonstrated in *B. schlosseri* (Cima *et al.*, 2006). Once inside the ampullae, MC leak into the tunic, together with some granular cells, and change their morphology: the latter event is probably related to the release of vacuolar contents which, as in *B. schlosseri* (Ballarin *et al.*, 1995, 1998), may be responsible for the induction of cytotoxicity. The presence of filamentous material, sharing staining affinity with the contents of morula cell vacuoles in the tunic surrounding morula cell aggregates, fits the above hypothesis.

The intense dark pigmentation along the contact region suggests that phenoloxidase is involved in the induction of cytotoxicity in CSA, as reported for *B. schlosseri* and other ascidian species (Ballarin *et al.*, 1995, 1998; Shirae and Saito, 2000; Shirae *et al.*, 2002). Future investigations will be directed towards both better comprehension of the role of phenoloxidase in the non-fusion reaction of *B. leachi* and the search for the immunomodulatory molecules, secreted by activated hamocytes, involved in the process.

Acknowledgements

This work was supported by the Italian MIUR. The authors wish to thank M Del Favero for technical help.

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