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RESEARCH REPORT

The days after: cellular events following the inflammatory allorejection reaction in the colonial ascidian *Botryllus schlosseri*

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Abstract

Allorecognition between contacting, genetically incompatible colonies of the ascidian *Botryllus schlosseri* represents a typical inflammatory reaction. It leads to the formation of a series of cytotoxic, necrotic spots along the contact border known as points of rejection (PORs). This is the consequence of the selective recruitment of morula cells (MCs), a peculiar granular, cytotoxic cells present in botryllid ascidians, in the lumen of the ampullae (the peripheral blind termini of the colonial vasculature) facing the alien colony, their migration into the tunic and their degranulation. The released material includes the enzyme phenoloxidase, the activity of which is responsible for the observed cytotoxicity. In the present work, we studied the cellular events in the facing ampullae for 9 days following the initial contact of the colonies. Data confirm that MCs gather inside the lumen of facing ampullae already at day 1 from the contact and start to leak into the tunic at day 2, when the first PORs appear. MCs then decrease progressively in the following days, probably because most of them leave the circulation and enter the tunic whereas, at day 5 from the contact, round phagocytes increase significantly inside facing ampullae, likely deriving from spreading phagocytes having ingested MC corpses in order to prevent a diffuse inflammation in the circulatory network. After the allorejection reaction, colonies orient their growth towards opposite directions and ampullae previously involved in the allorecognition remain in a region of old tunic which is progressively released by the colonies.

Key Words: *Botryllus*; colonial ascidians; allorecognition; inflammation; immunocyte dynamics; phagocytosis

Introduction

Inflammation is a complex biological response to injuries or harmful stimuli. Its basic features are: i) change in vascular flow; ii) selective recruitment of inflammatory immunocytes in the infection area and their migration through the vessel epithelium into the surrounding tissues; iii) degranulation of the infiltrated cells and iv) induction of cell necrosis as a consequence of the release of cytotoxic molecules by degranulation. All these events are modulated by a variety of humoral factors (cytokines and chemokines) that exert chemotactic activities and induces changes in the surface of both the vessel epithelial cells and immunocytes. (Bellanti, 2012).

The cosmopolitan colonial ascidian *Botryllus schlosseri* relies on innate immunity for its defense from potentially pathogenic microorganisms (Franchi *et al.*, 2017) and immunocytes represent the majority of the circulating haemocytes. They include phagocytes and cytotoxic morula cells (MCs). Hyaline or spreading phagocytes are amoeboid cells that, upon the ingestion of foreign material, withdraw their cytoplasmic projections and assume a spheroidal form (round phagocytes). Conversely, MCs are provided with many granules, uniform in size (around 2 µm in diameter), containing the enzyme phenoloxidase (PO). They degranulate upon the recognition of foreign molecules and induce an inflammatory reaction (Ballarin and Cima, 2005).

In *B. schlosseri*, a typical inflammatory reaction is observed when genetically incompatible colonies converge and contact each other. Here, a partial fusion of the tunics is followed by the allorejection reaction leading to the formation of a series of dark, cytotoxic foci in the contact area called points of rejection (PORs; Sabbadin, 1982; Scofield and Nagashima, 1983; Rinkevich, 1992; Sabbadin *et al.*, 1992; Ballarin *et al.*, 1995, 1998). From a cellular point of view, the reaction is characterized by the selective recruitment of MCs inside the blind termini of the vasculature, called ampullae, facing the alien

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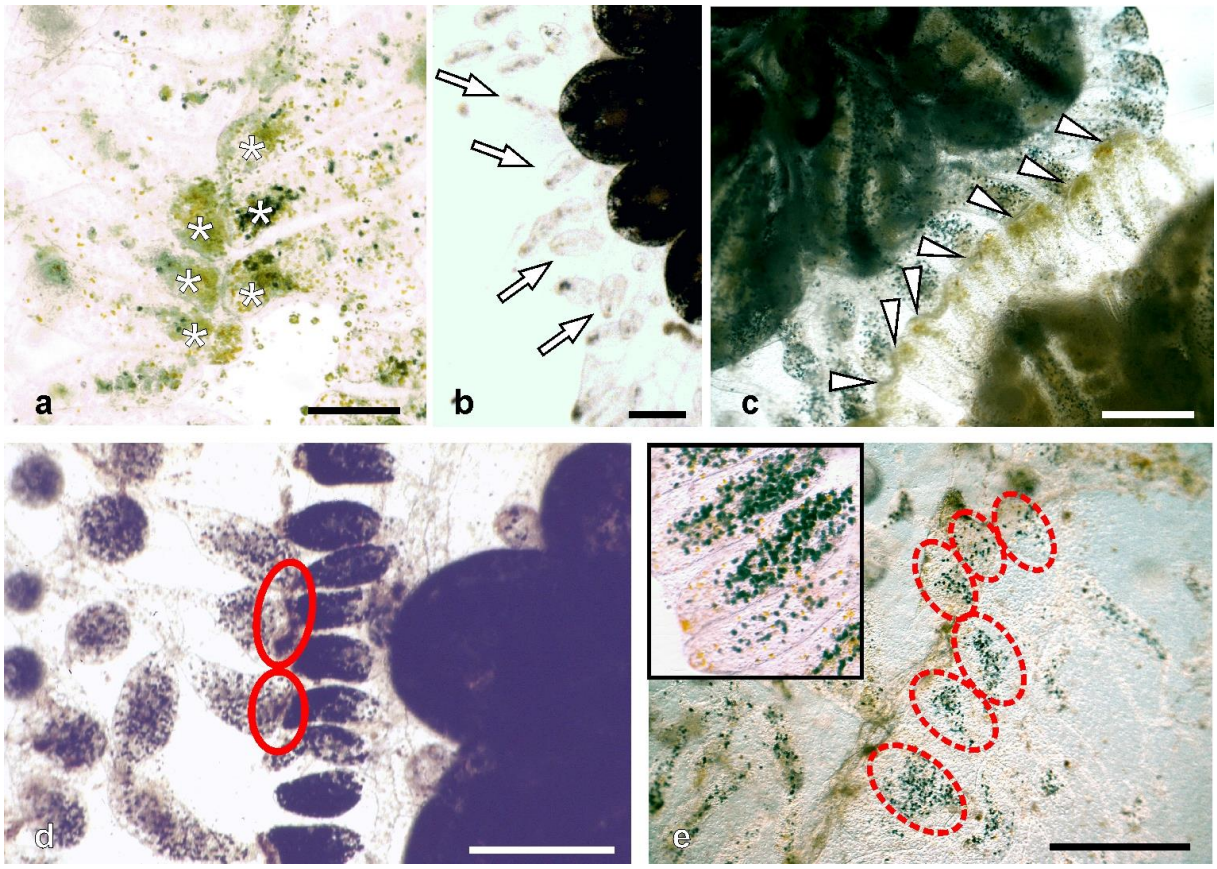

Fig. 1 Living contacting, genetically incompatible colonies (dorsal side). a: facing ampullae at day 1. Asterisks mark the selective recruitment of MCs inside the tips of contacting ampullae. b: lateral ampullae at day 1. White arrows indicate the lateral ampullae appearing pale as they lack the high concentration of hemocytes inside their lumen. c: contact region at day 2. Arrowheads indicate the close contacts between facing ampullae. d: contact region at day 3. The red ellipses show the PORs. e: the same region at day 9. The dotted red ellipses show the abundance of blue pigment cells (dark spots) inside the facing ampullae. A magnification of the ampullae of the contact region, with blue pigment cells inside, is visible in the inset. Scale bar: a, e: 0.2 mm; b-d: 0.5 mm

colony (Sabbadin *et al.*, 1992; Franchi *et al.*, 2017). These cells, once gathered inside the facing ampullae, migrate into the tunic (the colony outer envelope) through the ampullar epithelium where, following the recognition of still unknown soluble factors diffusing from the alien colony, degranulate, and dye. Most of the MCs, once left the ampullae, dye in front of them thus contributing to the formation of the cup-shaped melanic PORs (Cima *et al.*, 2004; Franchi *et al.*, 2019; Fig. 1). Death occurs through a necrotic process (Cima *et al.*, 2004), probably related to ferroptosis (Dixon *et al*., 2012) or oxytosis (Tan *et al*., 2001). Indeed, PO, by acting on polyphenol substrata also contained inside MC granules, catalyzes their oxidation to quinones that then polymerize to melanin. In the course of the reaction, reactive oxygen species (ROS), a by-product of PO activity, induce oxidative stress in the surrounding cells and their death by necrosis (Ballarin *et al.*, 2002). However, in advanced stages of the allorejection reaction, part of MCs degranulate and dye inside the ampullar lumen (Cima *et al*., 2004).

The disposal of cell corpses and cellular debris is required to maintain the homeostasis of organisms (Westman *et al*., 2020): inflammatory signals promote the recruitment of professional phagocytes (Cooke, 2019) that recognize damage-associated molecular patterns (DAMPs) released by necrotic cells (Westman *et al*., 2020). However, necrotic debris are potent inducers of inflammation (Westman *et al*., 2020) and sustained inflammation delays tissue regeneration (Wang *et al*., 2022). Therefore, in *Botryllus*, MC remains have to be removed from the ampullar lumen in order to prevent the persistence of inflammation in the circulation that could be harmful to colonies. So far, how MC debris are removed from the ampullar tips has not been investigated properly. Therefore, with the present study, we aim to fill this gap and elucidate the role of circulating phagocytes in the clearance of cell debris in the ampullar lumen following the allorejection reaction.

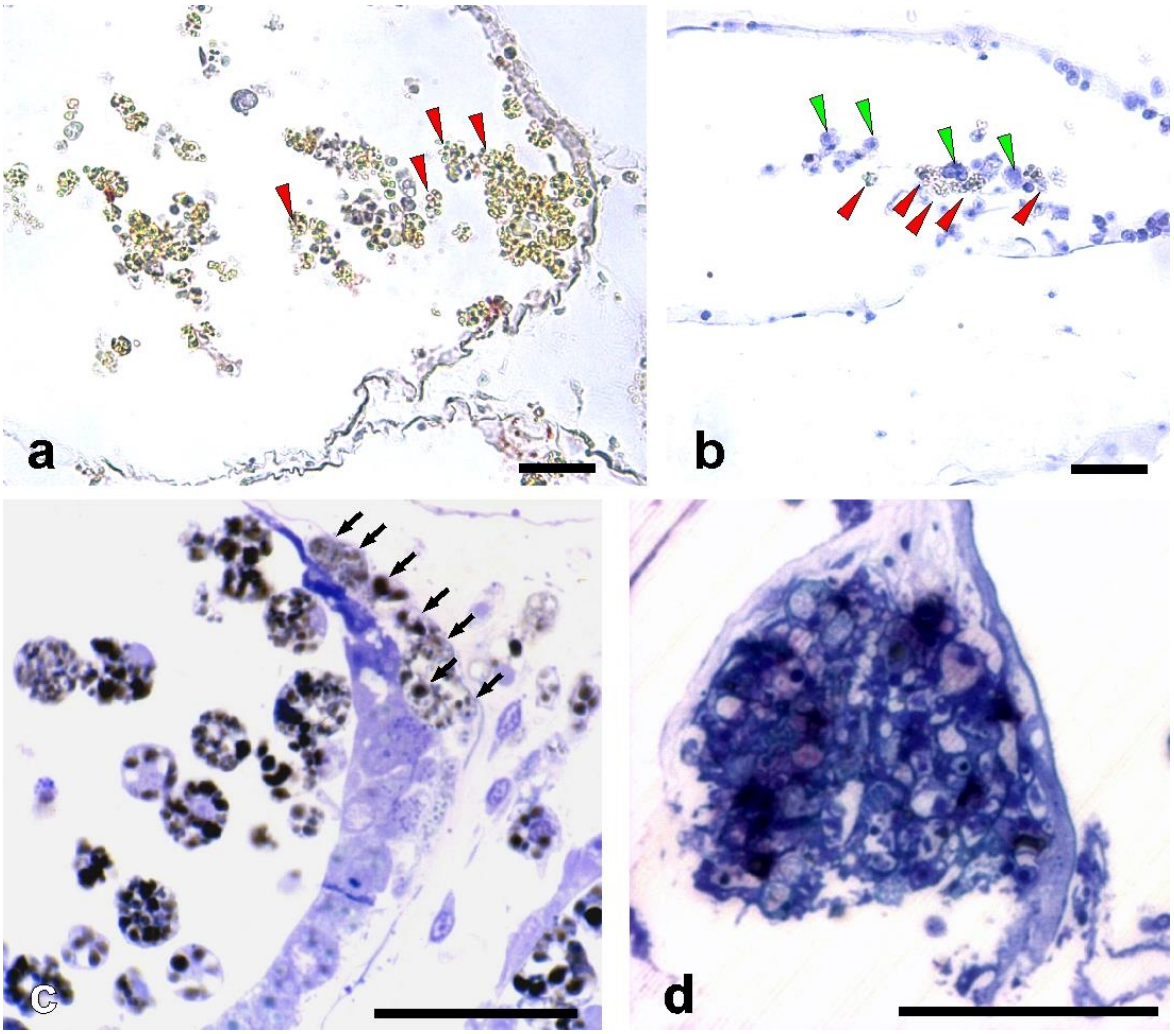

Fig. 2 a, b: Histology of the contacting (a) and lateral (b) ampullae at day 1 from the contact; red arrowheads: MCs; green arrowheads: phagocytes. c: semithin section of a facing ampulla at day 2 from the contact showing a POR, with MCs in the tunic (arrows). d: magnification of a POR composed of cell debris and deposits of melanin. Scale bar: 50 µm

Material and methods

Animals

The Department of Biology, University of Padua, is authorized by the port authority to collect marine animals for scientific purposes. Colonies of *B. schlosseri* were collected in the Southern Lagoon of Venice, near the Marine Station of the Department of Biology, University of Padua, in Chioggia. They were carefully detached from the substrate with a razor blade, transferred to a glass slide where they adhered, and kept in aerated aquaria filled with filtered seawater (FSW) at a salinity of 33 ppm, in thermostated rooms, at 20 °C, at 12h:12h light:dark cycle, for at least two weeks before their use in the experiments described below.

Colony specificity assay

Colonies were cut in subclones of 1-2 systems and let to grow for one week. They were then transferred on a supporting glass slide and pairs of different subclones were faced with their leading edges, at a distance of 1-2 mm. They were kept in a moist chamber for 30 min before being returned to the aquarium. Within 24-48 h, the facing ampullae of the colonies extended and contacted each other. Colonies can either fuse or reject, depending on the sharing of at least one allele at the fusibility/histocompatibility (Fu/HC) locus (Oka and Watanabe, 1957, 1960; Sabbadin, 1962; Oka, 1970; Mukai and Watanabe, 1974; Scofield *et al.*, 1983; Taneda *et al.*, 1985; Rinkevich, 1992; Sabbadin *et al.*, 1992; Saito *et al.*, 1994; De Tomaso *et al.*, 1998; De Tomaso, 2006). Colonies were daily observed under the stereomicroscope: fusible pairs were not considered and we focused our attention to the allorejection reaction, as indicated by the appearance of a series of melanic, necrotic PORs along the contract border. Four pairs of genetically incompatible colonies were used in our

experiments: from each colony we got six subclones that were paired so to trigger the allorejection reaction. Pairs were fixed, as described below, at the beginning of the ampullar contact and after 1, 2, 3, 5, 7 and 9 days from it.

Histology

Colony pairs were fixed in Bouin's solution (saturated solution of picric acid in FSW, acetic acid and formaldehyde at the volumetric ratio of 15:5:1; Bancroft and Gamble, 2008) for 30 min, dehydrated in an increasing ethanol series, transferred to xylene and embedded in Paraplast plus (Sigma Aldrich). They were then cut to 7-µm frontal sections with a Reichert-Jung 2040 microtome. Sections, once dewaxed, were stained for 1.5 min with Mayer's hematoxylin (Sigma Aldrich), kept for 15 min in tap water, dehydrated and mounted with Eukitt (Electron Microscopy Sciences). Sections of at least ten ampullae of the leading edge, facing the alien colony (facing ampullae), and 10 ampullae of the other sides of the colony (lateral ampullae) were considered for each colony of a colony pair. The number of immunocytes, classified according to Ballarin and Cima (2005), inside them were counted under a Leitz Dialux 22 light microscope at the magnification of 100x and expressed as percentage of the total number of hemocytes visible in the ampullar section.

As an alternative, colonies, fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.2 M sodium cacodylate buffer (CB; pH 7.4) containing 1.7% NaCl and 1% sucrose, were postfixed in 1% OsO_4 in 0.2M CB, dehydrated and embedded in Epon 812 (Electron Microscopy Sciences) according to manufacturer's instructions. Serial semithin sections (1 µm), obtained with a Leica EM UC7 ultramicrotome, were hot stained with 1% toluidine blue and 1% borax in distilled water and observed under the light microscope.

Statistical analysis

Data are expressed as means ± SD. The Kruskal-Wallis test was used to evaluate the presence of significant differences among the percentages of the various haemocyte types at different days. Means were compared with the χ^2 test.

Results

Day 1 from the contact

As already described, the first sign of the interaction between colonies was the recruitment of cytotoxic MCs, the effectors of the reaction, inside the lumen of the faced ampullae (Fig. 1a) that appear opaque. In sections (Fig. 2), MC were easily identified as they contain many granules that acquire a yellow-green color after fixation with aldehydes. MC frequency amounted to more than 80% of the haemocytes in the lumen (Fig. 2a), significantly ($p < 0.001$) differing from that in lateral ampullae. The latter appeared pale (Fig. 1b) as spreading phagocytes prevailed inside the lumen, representing more than 50% of the total haemocytes (Fig. 2b), a percentage significantly ($p < 0.001$) different from that in facing ampullae (Table 1).

Day 2

At day 2 from the contact, the interaction between the ampullae of the two colonies was well established (Fig. 1c). MCs started to cross the epithelium of the ampullar tips and migrate into the tunic (Fig. 2c), where they form the PORs that characterize the allorejection reaction, so that their amount inside the ampullar lumen resulted lower than before although still significantly ($p < 0.001$) different from that in the lateral ampullae. Conversely, the amount of spreading phagocytes increased inside the facing ampullae, reaching a value higher than 60%, comparable to that of lateral ampullae (Tab. 1).

Day 3

The PORs between genetically incompatible colonies were clearly visible in front of the contacting ampullae of the leading edge (Fig. 1d, 2c,d). The fraction of MCs in the lumen of facing ampullae was still significantly ($p < 0.01$) higher than what observed in the lateral ampullae. The percentage of spreading phagocytes remained high (around 70%) in both facing and lateral ampullae. (Tab. 1).

Day 5

The content of MCs in facing ampullae reduced to less than 15%, a value not significantly different from that of lateral ampullae. The frequencies of spreading phagocytes in facing and lateral ampullae were not significantly different whereas the amount of round phagocytes in facing ampullae reached a value higher than 5%, significantly ($p < 0.01$) different from that of lateral ampullae (Tab. 1).

Day 7

The fraction of MCs remained lower than 15% in both facing and lateral ampullae. The percentage of spreading phagocytes decreases to 45% in both facing and lateral ampullae, whereas round phagocytes almost disappeared from them (Table 1).

Day 9

MCs represented only a small fraction of haemocytes (around 1%) in both facing and lateral ampullae. Spreading phagocytes remained the most abundant cell type in both facing and lateral ampullae (more than 40%) with a significant ($p < 0.01$) difference between facing and lateral ampullae. Round phagocytes decreased to 1% in facing ampullae, a value comparable to that of lateral ampullae (Table 1). A general increase in the content of blue pigment cells in facing ampullae, as dark blue spots inside the ampullar lumen, was observed (Fig. 1e). Ampullae remain embedded in old tunics as colonies create new leading edges and start to growth towards opposite directions. Fig. 3 summarizes the behavior of the circulating immunocytes in facing and lateral ampullae.

Discussion

Inflammation is an innate immune response characterized by the rapid recruitment of immunocytes in the infection site via the circulation,

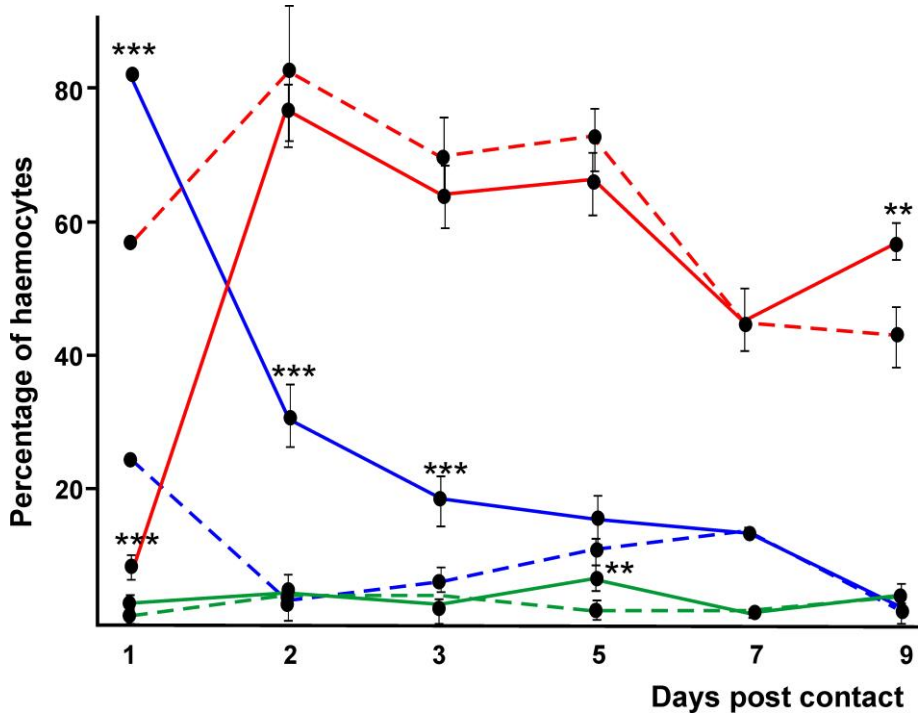

Fig. 3 Variation of the percentage of spreading phagocytes (red lines), round phagocytes (green lines) and morula cells (blue lines), in the days following the initial contact between genetically incompatible colonies. Full lines: facing ampullae; dashed lines: lateral ampullae. Asterisks mark significant differences between the specific hemocyte content in facing and lateral ampullae. **: $p < 0.01$; ***; $p < 0.001$

their extravasation and degranulation, the latter leading to local cytotoxicity (Bellanti, 2012). In the present work, we continued our previous studies on the allorejection reaction of the colonial ascidian B. *schlosseri*, a typical inflammatory phenomenon occurring when genetically incompatible colonies converge and contact each other at the level of their leading edges (Rinkevich, 1992; Franchi and Ballarin, 2017). We do not have analogous data on the first few days following the contact of genetically compatible colony. The only data on hemocyte behavior during fusion come from a previous study on *Botrylloides leachii*. In this species, phagocytes and pigment cells increase their concentration inside the lumen of paired ampullae after 48 h from the contact (Zaniolo *et al*., 2006). Conversely, both in *B. leachii* and *B. schlosseri*, the circulating immunocytes responsible for the inflammatory response are granular cells, known as MCs, storing the enzyme PO and their phenol substrates inside their granules (Ballarin *et al*., 1995; Zaniolo *et al*., 2006). From the ampullar lumen, MCs migrate into the tunic crossing the ampullar epithelium and degranulate. Their granular content is responsible of the cytotoxicity that appeared, as a series of necrotic spots (POR) in front of the facing ampullae, 48 h from the contact (Taneda and Watanabe, 1982; Hirose *et al.*, 1990; Sabbadin *et al.*, 1992; Ballarin *et al.*, 1995; Shirae *et al.*, 2002; Cima *et al.*, 2004; Franchi *et al.*, 2017, 2019; Rosental *et al*., 2018). PORs are due to the accumulation, in front of the ampullar tip, of cell debris, melanins and lipofuscins (Ballarin *et al.*, 1995), all related to the activity of the enzyme PO, released by degranulating MCs (Ballarin *et al.*, 1995, 1998; Franchi and Ballarin, 2017; Ballarin, 2023). Previous experiments (Ballarin et al., 1995, 1998) supported this conclusion demonstrating that PO released by MCs, is responsible for cytotoxicity observed when hemocytes are incubated *in vitro* in the presence of cell-free hemolymph from genetically incompatible colonies, consequent to the recognition of nonself molecules in the alien hemolymph (Ballarin et al., 1995; 1998). This observation is further corroborated by the results by Rosental *et al* (2018) who observed an increase in cytotoxicity when hemocytes from two incompatible colonies were mixed together, a situation resembling the “contact reaction” observed by Fuke (1980) in the solitary ascidian *Halocynthia roretzi*, also related to the activation of PO (Akita and Hoshi, 1995).

Although the events associated with the formation of the PORs are well known (Cima *et al.*, 2004; Franchi and Ballarin, 2017), scarce attention has been devoted to the fate of the facing ampullae and their content in the days following the appearance of the PORs. Here, we intend to fill this gap and give a first description of what happens, at the morphological and cellular level, in facing ampullae following the allorejection reaction. After the well-described increase in the content of MCs inside the lumen of facing ampullae, observed one day from the contact, when their number exceed 80% of the haemocytes, their frequency, although

Table 1 Percentages of immunocytes (spreading and round phagocytes and morula cells) in facing and lateral ampullae of contacting colonies at various days from the contact. Asterisks mark significant differences in the content of specific cell types at the same days. **: $p < 0.01$; ***: $p < 0.001$

Facing ampullae

Day	% spreading phagocytes	% round phagocytes	% morula cells
1	**7.19 ± 0.36% *** **	1.49 ± 1.06%	**84.06 ± 2.67% *** **
2	62.93 ± 22.88%	1.45 ± 1.07%	**31.40 ± 11.25% *** **
3	79.44 ± 11.27%	2.08 ± 1.64%	**17.34 ± 9.98% ** **
5	60.05 ± 12.55%	**5.40 ± 3.03% ** **	14.68 ± 5.69%
7	47.74 ± 7.11%	0.56 ± 0.41%	12.28 ± 4.73%
9	**58.13 ± 7.82% ** **	1.56 ± 1.12%	1.11 ± 0.99%

Lateral ampullae

Day	% spreading phagocytes	% round phagocytes	% morula cells
1	58.73 ± 3.72%	0.10 ± 0.01%	23.90 ± 8.50%
2	84.95 ± 5.40%	2.94 ± 2.64%	1.81 ± 1.10%
3	71.82 ± 24.41%	2.78 ± 1.81%	4.76 ± 4.25%
5	74.93 ± 6.81%	0.58 ± 0.15%	9.91 ± 5.96%
7	45.60 ± 6.68%	0.10 ± 0.05%	13.04 ± 5.59%
9	43.34 ± 10.66%	2.78 ± 2.24%	0.84 ± 0.49%

still significantly different from that in the lateral ampullae, abruptly decreases to around 30% at day 2 from the contact and at less than 20% at day 3. This is likely due to their migration into the tunic where they degranulate and contribute to form the necrotic masses that characterize the allorejection reaction. Conversely, the number of phagocytes, in the form of spreading phagocytes, very low at day 1 inside facing ampullae, increases to a value around 80% at day 3 and slowly decreases to values around 50% in the following days. As for round phagocytes, i.e., phagocytes with ingested material, their frequency significantly increases in facing ampullae, with respect to lateral ampullae, at day 5. We can argue that the abundance of spreading (hyaline) phagocytes and the increase of round phagocytes at day 5 is related to necessity of the disposal of MC corpses, the presence of which in the circulation might induce a persistent status of inflammation in the circulation. Indeed, part of MCs degranulate inside the ampullar lumen upon the recognition of humoral factors diffusing from the alien colony through the partially fused tunics (Cima *et al.*, 2004; Franchi and Ballarin, 2017). As for the late increase of pigment cells inside the lumen of facing ampullae at the end of the process (day 9), it represents a sign of ageing that, progressively, leads to the final exclusion of the ampullae from the circulation and the re-orienteering of the colony towards another direction with a new leading edge. It is well-known, indeed, that, aged colonies show an increase of pigment cells in their circulation (Chadwick-Furman and Weissman, 1995; Riodriguez *et al.*, 2021). Collectively, the above-reported data indicate that, in the allorejection reaction, subsequently to MC recruitment and degranulation, phagocytes enter the lumen of facing ampullae and change their morphology from spreading to round, suggesting an active activity of engulfment, likely to clear MC corpses. Future efforts will be directed to a better comprehension of the cellular events in the rejection area after the formation of the PORs with particular reference to the biochemical pathways activated in phagocytes.

Acknowledgements

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