Research Report

Morula cells and non-self recognition in the compound ascidian *Botryllus schlosseri*

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Abstract

In the ascidian *Botryllus schlosseri*, we studied the effects of hemocyte incubation with foreign cells, such as bacteria, bacterial spores and yeast. In the presence of yeast and bacterial spores, morula cells, a common cell type in botryllid ascidians, changed their morphology, release phenoloxidase in the medium, thus causing an increase in cytotoxicity, and express molecules recognised by anti-IL-1-α- and anti-TNF-α-antibodies. These effects were not observed when hemocytes were incubated with both gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria. Considering that morula cells are the main source of molecules recognised by anti-cytokine-antibodies we suggest an immunosurveillance role of these cells, which may influence immune responses such as phagocytosis.

Key words: ascidians; *Botryllus;* cytotoxicity; morula cells; phenoloxidase; immunomodulation

Introduction

Two types of circulating immunocytes are present in the colonial ascidian *Botryllus schlosseri*: phagocytes and morula cells (MC). Phagocytes include hyaline amoebocytes and macrophage-like cells. The former are amoeboid cells, 5-10 μm in size, capable of active movements and phagocytosis, whereas the latter are larger (10-15 μm in diameter) roundish cells with at least one vacuole containing ingested material, deriving from hyaline amoebocytes having engulfed foreign cells or particles (Ballarin *et al*., 1993, 1994). MC are large, vacuolated cells which represent the most abundant blood cells, their frequency ranging from 20 to 60% of circulating hemocytes (Ballarin et al., 1995).

They are the mediators of the inflammatory

(rejection) reaction which occurs, in the form of a series of necrotic spots, along the contacting borders, when genetically incompatible colonies are juxtaposed. MC induce cytotoxicity in the contacting tissues through the release of the enzyme phenoloxidase (PO), probably stored as a pro-enzyme inside their granules (Ballarin *et al*., 1995, 1998).

We have recently demonstrated that, in *B. schlosseri*, MC synthesise molecules recognised by antibodies raised against the mammalian inflammatory cytokines IL-1-α and TNF-α upon the recognition of soluble foreign molecules, such as mannan (Ballarin *et al*., 2001) and allogeneic humoral factors diffusing from one colony into the blood of the alien colony (Cima *et al*., 2004) in the course of the rejection reaction.

In an attempt to better understand the role of MC in *Botryllus* immunobiology, we studied the behaviour of MC when hemocytes are incubated *in vitro* with foreign cells, such as bacteria, bacterial spores and yeast. Results indicate that MC can recognise bacterial spores and yeast cells and, as a consequence of this recognition, degranulate and release the enzyme PO that, in turn, increases *in vitro* cytotoxicity among hemocytes. In addition, MC acquire

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immunopositivity to anti-IL-1-α- and anti-TNF-α-antibodies. Such responses are not observed when hemocytes are incubated with bacteria. We suggest that the synthesis and release of molecules immunopositive to anti-cytokine-antibodies by MC can influence phagocyte activity.

Materials and methods

Animals

Colonies of *B. schlosseri* from the Lagoon of Venice were used. They were kept in aerated aquaria, attached to glass slides and fed with Liquifry Marine (Liquifry Co., Dorking, England).

Hemocyte collection and culture

Blood samples were collected with a glass micropipette by puncturing, with a fine tungsten needle, the tunic marginal vessels of colonies previously rinsed in 0.38 % Na-citrate in filtered sea water (FSW), pH 7.5, as anti-clotting agent. They were then centrifuged at 780 x g for 10 min and pellets were finally resuspended in FSW to give a concentration of 5 x 10^6 cells/ml. Sixty µl of hemocyte suspension were placed in the centre of culture chambers prepared as described elsewhere (Ballarin *et al*., 1994) and left to adhere to coverslips for 30 min at room temperature (RT).

Morula cell morphology

After the adhesion of hemocytes to the coverslips, the debris-containing FSW was discarded and replaced with an equal volume of one of the following cell suspensions in FSW: *Escherichia coli* (200 x 10^6 cells/ml), *Staphylococcus aureus* (50 x 10^6 cells/ml), *Bacillus clausii* spores (200 x 10^6 spores/ml) or ordinary baker's yeast (50 x 10^6 cells/ml) in FSW (FSW alone in controls). After 60 min of incubation at RT, the morphology of living MC was observed under a Leitz Dialux 22 light microscope at a magnification of 1200 x.

Immunocytochemistry

After the incubation with foreign cells, hemocyte monolayers were fixed for 30 min at 4°C in 4 % paraformaldehyde plus 0.1% glutaraldehyde in saline buffer (SB: 0.2 M Na-cacodylate buffer, pH 7.4, plus 1.7% NaCl and 1% sucrose), rinsed in phosphate-buffered saline (PBS: 1.37 M NaCl, 0.03 M KCl, 0.015 M KH_2PO_4, 0.065 M Na_2HPO_4, pH 7.2) and permeabilised with 0.1% Triton X-100 in PBS for 5 min. Immunoreactivity against rabbit polyclonal anti-human-IL-1-α (Santa Cruz Biotech) and goat polyclonal anti-human TNF-α (Santa Cruz Biotech) (1 µg/ml, according to manufacturer's suggestions) was revealed by immunoperoxidase staining using the avidin-biotin-peroxidase complex method for signal enhancement (Hsu *et al*., 1981) and 3,3'-diaminobenzidine (Fluka) as substrate. Endogenous peroxidase was blocked by incubation for 30 min in a solution of 6 % H_2O_2 in methanol. In control preparations, primary antibodies were either substituted with non-immune sera or absorbed with homologous antigen, i.e., recombinant human IL-1-α and TNF-α (Peprotech). Moreover, a rabbit polyclonal anti-*Botryllus* agglutinin (BA) antibody (Ballarin *et al*., 2000) was used for specificity control. Cells were observed under the light microscope to determine the frequencies of positive haemocytes.

PO assay

In each experiment, 400 µl of hemocyte suspensions (5 x 10^6 cell/ml) were incubated, in 1.5 ml vials, with bacterial spores (final concentration: 200 x 10^6 spores/ml), bacterial cells (final concentration: 200 x 10^6 and 50 x 10^6 cells/ml for *E. coli* and *S. aureus*, respectively), yeast cells (final concentration: 50 x 10^6 cells/ml) or FSW (reference control) at RT for 60 min. Cells were then centrifuged at 780 x g for 10 min, and the PO activity of supernatants was determined spectrophotometrically. Briefly, 20 µl of supernatant were added to 440 µl of PBS and 440 µl of a saturated solution of dihydroxyphenyl-L-alanine (L-DOPA) in PBS and the time course of the reaction was read at 490 nm for 5 min. One relative unit (RU) of PO activity was defined as an increase in absorption of 0.001/min in the reaction mixture (Söderhäll and Smith, 1983). Protein concentrations of the supernatants were determined according to Bradford (1976) and results were expressed as RU/mg protein.

Cytotoxicity assay

Cytotoxicity among hemocytes was assessed by Trypan Blue exclusion, after 60 min of incubation of hemocytes with foreign cells or spores. Blood cells were exposed to 0.25 % Trypan Blue in FSW for 5 min. The cytotoxicity index, i.e., the percentage of haemocytes positive for Trypan Blue staining, was then calculated. In the experiments with yeast, the effects of the addition of the PO inhibitors Na-benzoate, phenylthiourea (PTU) and tropolone (20 mM, 1 mM and 2 mM, respectively) to FSW were also evaluated.

Statistical analysis

At least 300 cells, in ten optical fields at a magnification of 1250 x, were counted in each type of experiment. Each experiment was carried out in triplicate. Data are expressed as means ± SD and were compared with the c^2 test. PO activities were compared with Duncan's test.

Results

MC change their morphology in the presence of foreign cells

In unexposed hemocyte, living MC appeared as large, slightly mulberry-shaped cells, with the cytoplasm almost completely occupied by round, uniform vacuoles, 2 µm in diameter (Fig. 1a). After aldehyde fixation, the granules reduced in size and show a yellowish content (Fig. 1b). In the presence of either bacterial spores or yeast cells, living MC changed their morphology: some of their granules reduced their size, others coalesced into a few, large granules which appeared devoid of their contents (Fig. 1c). No changes the morphology of MC were observed in the presence of either *E. coli* or *S. aureus*.

MC acquire immunopositivity to anti-cytokine antibodies in the presence of foreign cells
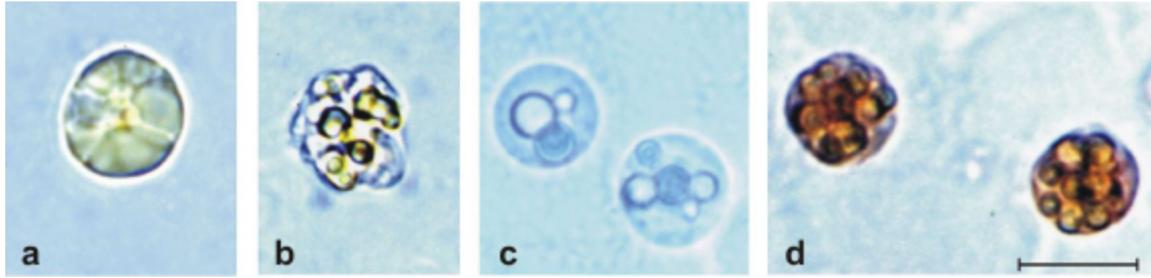
Following incubation with bacterial spores or

Fig. 1 Morula cell morphology in various experimental conditions. (**a**, **c**) living cells incubated in absence (**a**) or presence (**c**) of foreign cells; (**b**, **d**) aldehyde-fixed cells, immunostained for anti-cytokine antibodies, previously incubated in absence (**b**) or presence (**d**) of foreign cells. Scale bar: 15 μm.

yeast cells, a significant increase ($p < 0.001$) in the amount of cells showing immunopositivity to anti-IL-1-α- and anti-TNF-α-antibodies was observed. Most of the immunopositive cells were MC, accounting for more than 95% of labelled cells. Immunopositivity was located in the cytoplasm of MC (Fig. 1d) and the frequency of MC recognised by anti-cytokine antibodies shifted from about 10 % in unexposed cultures to more than 80 % in the presence of either microbial spores or yeast cells. No significant increase of labelled MC was observed when haemocytes were incubated with *E. coli* or *S. aureus* (Fig. 2). No labelling of MC was observed with anti-BA antibodies.

Influence of foreign cells on PO activity in the culture medium and cytotoxicity

When hemocytes were incubated in the presence of either *Bacillus* spores or yeast cells, PO activity in the medium significantly increased ($p < 0.001$ and $p < 0.01$, respectively) with respect to controls. No significant increase was reported in the presence of either *E. coli* or *S. aureus* cells (Table 1).

A significant increase in the cytotoxicity index was observed when haemocytes were incubated in the presence of bacterial spores or yeast cells ($p < 0.05$ and $p < 0.001$, respectively). In experiments with yeast, no significant increase in cytotoxicity was measured when Na-benzoate, PTU or tropolone were added to FSW. *E. coli* and *S. aureus* did not induce any significant increase in cytotoxicity (Table 2).

Discussion

Many different types of circulating hemocytes are present in the blood of the colonial ascidian *B. schlosseri* (Ballarin et al., 1993). Among them, MC are the most frequent cells and play a key role as effectors of the rejection reaction which occurs when genetically incompatible colonies of the ascidian *B. schlosseri* contact each other. In the course of this reaction, MC crowd inside the facing ampullae (blind, sausage-like endings of the marginal vessels) and cross the ampullar epithelium to reach the tunic (Sabbadin *et al.*, 1992; Rinkevich *et al.* 1998). In the meantime, they degranulate and release the contents of their vacuoles, mainly PO and its polyphenol substrata (Ballarin *et al.*, 1998; Cima *et al.*, 2004). The enzyme, in turn, induces cytotoxicity that appears in the form of a series of necrotic spots along the contact

border. This sequence of events is triggered by recognition of allogeneic humoral factors that diffuse through the partially fused tunics from one colony to the alien one and it can be mimicked *in vitro* when hemocytes are exposed to blood plasma from incompatible colonies (Ballarin *et al.,* 1994, 1998, 2002).

Here, we demonstrate that a change in the morphology of MC occurs in response to the recognition of foreign cells, such as bacterial spores or yeast cells: it is similar to what observed when hemocytes are incubated with blood plasma from genetically incompatible colonies (Ballarin *et al*., 1998) and, therefore, ascribable to a degranulation event. This indicates that MC can readily "sense" the presence of particular foreign molecules or cells in the environment (both *in vivo* and *in vitro*) and activate themselves upon their recognition, thus acting as "sentinel" cells which degranulate as a consequence of their activation.

MC degranulation is followed by an increase in both PO activity in the culture medium and hemocytes cytotoxicity among hemocytes. The two events are closely related as demonstrated in previous reports (Ballarin *et al*., 1998) and in the present paper, since the addition of PO inhibitors prevents cytotoxicity.
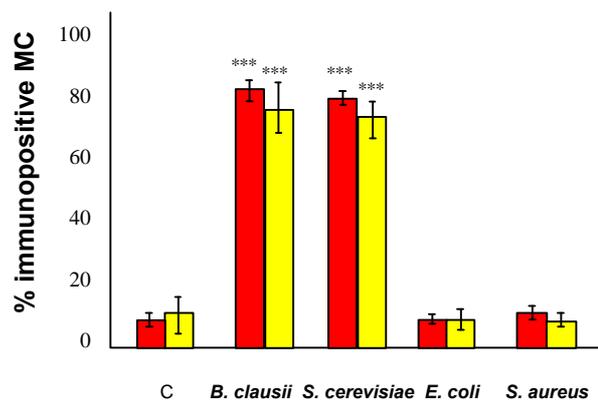

Fig. 2 Percentage of MC showing immunopositivity to anti-IL-1-α (dark grey) and anti-TNF-α (light grey) antibodies after incubation of hemocyte cultures in FSW without (**C** = control) or with foreign cells. Asterisks: significant differences with respect to controls. *** $p < 0.001$.

Table 1 PO activity in culture medium of hemocytes incubated in FSW in presence of *B. clausii* spores, *S. cerevisiae*, *E. coli* and *S. aureus* with respect to controls (incubation in FSW alone). Significant differences are marked by asterisks. *** $p < 0.001$; * $p < 0.05$.

	PO activity (RU/mg protein)
Control (FSW)	10.73 ± 0.96
B. clausii spores	16.89 ± 0.67 ***
S. cerevisiae cells	15.73 ± 1.55 *
E. coli cells	11.83 ± 0.66
S. aureus cells	9.73 ± 0.45

Table 2 Cytotoxicity index in hemocyte cultures incubated in FSW, in absence and presence of foreign cells. Yeast cells were also incubated in the presence of PO inhibitors. Asterisks mark significant differences with respect to controls. *** $p < 0.001$; * $p < 0.05$.

	Cytotoxicity index
Control (FSW)	3.93 ± 1.11
B. clausii spores	6.02 ± 1.60 *
S. cerevisiae cells	7.17 ± 1.69 ***
S. cerevisiae cells + Na-benzoate	5.07 ± 2.00
S. cerevisiae cells + PTU	5.00 ± 1.10
S. cerevisiae cells + tropolone	3.44 ± 1.07
E. coli cells	3.33 ± 1.44
S. aureus cells	3.69 ± 1.19

We can, therefore, state that, through the release of PO, MC induces an increase in hemocyte cytotoxicity, which represents a first, relatively rapid response towards cells non-self cells. The above assumption is further supported by the finding that bacterial cells do not induce any MC degranulation: the absence of MC activation is associated with the lack of both the increase in PO activity in the culture medium and hemocyte cytotoxicity.

MC cannot recognise *E. coli* and *S. aureus* cells: this probably reflects the difference in cell surface with respect to bacterial spores and yeast cells and the absence of molecules able to activate MC and trigger a degranulation event. The observation that *E. coli* LPS does not induce any degranulation of MC (our unpublished data) fits the above hypothesis. Analogously, Hata *et al*. (1998) found that PO is not released by hemocytes of the solitary ascidian *H. roretzi* in the presence of β1-3 glucan.

Recent evidences indicate that activated MC synthesise molecules recognised by anti-mammalian-cytokine antibodies. This occurs when hemocytes are exposed to either soluble microbial surface molecules or allogeneic blood plasma (Ballarin *et al*., 2001; Cima *et al*., 2004). The above-presented results indicate that

MC can synthesise these molecules even as a consequence of the recognition of non-self particulate material, such as foreign cells. The presence of a low percentage of immunopositive MC in controls is probably to be ascribed to their activation during blood collection. Again, the absence of a significant increase in immunopositive MC in the presence of bacteria, is the consequence of the lack of recognition of these cells by MC. It is our opinion that these molecules can modulate immune responses.

Therefore, in our hypothesis, MC act as "surveillance" cells able to readily recognise foreign molecules or cells and, as a consequence of this recognition, they: i) release PO and induce cytotoxicity; ii) synthesise and release cytokine-like molecules which induce, in a paracrine way, either the activation of other MC or phagocyte stimulation. Our preliminary (unpublished) data indicate that activated MC release factor(s) able to stimulate phagocytosis. This could explain why *E. coli* cells that do not activate MC, are engulfed less actively than yeast cells by *B. schlosseri* phagocytes (Ballarin *et al.,* 1994). In addition, agreement with the above *roretzi*, PO

activity in the culture medium and phagocytosis are closely related (Hata *et al*., 1998). Moreover, in *C. intestinalis*, lysates of MC from hemocyte cultures previously exposed to yeast cells can positively influence phagocytosis (Smith and Peddie, 1992) This observation was considered as evidence of the synthesis of opsonins by ascidian MC, but the absence of data directly proving the synthesis of opsonins by MC and the evidence of the production of such molecules by phagocytes (Ballarin *et al*., 2000) fit the idea that it can be the consequence of the synthesis of cytokine-like molecules by activated MC, able to enhance phagocytosis. Further investigations are in progress to better elucidate this point.

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