Reference: *Biol. Bull.* **201:** 59–64. (August 2001)

Morula Cells as the Major Immunomodulatory Hemocytes in Ascidians: Evidences From the Colonial Species *Botryllus schlosseri*

LORIANO BALLARIN[1,*], ANTONELLA FRANCHINI[2], ENZO OTTAVIANI[2], AND ARMANDO SABBADIN[1]

[1]*Department of Biology, University of Padova, via U. Bassi 58/B, 35100 Padova, Italy; and*
[2]*Department of Biology, University of Modena and Reggio Emilia, via Campi 213/D, 41100 Modena, Italy*

Abstract. Immunocytochemical methods were used to study the presence and distribution of IL-1-α- and TNF-α-like molecules in the hemocytes of the colonial ascidian *Botryllus schlosseri.* Only a few unstimulated hemocytes were positive to both the antibodies used. When the hemocytes were stimulated with either mannan or phorbol 12-mono-myristate, the phagocytes were not significantly changed in their number, staining intensity, or cell morphology. In contrast, stimulated morula cells were intensely labeled, indicating that these cells play an important immunomodulatory role.

Introduction

Phagocytes and morula cells are two types of circulating hemocytes that play a key role in ascidian immunobiology. Phagocytes can easily recognize and ingest non-self cells and particles (Smith, 1970; Anderson, 1971; Fuke and Fukumoto, 1993; Ballarin *et al.,* 1994; Ohtake *et al.,* 1994; Dan-Sohkawa *et al.,* 1995; Cima *et al.,* 1996) and are able to synthesize and release opsonic agglutinins (Coombe *et al.,* 1984; Kelly *et al.,* 1992; Ballarin *et al.,* 1999). Morula cells, a ubiquitous hemocyte type among ascidians, take part in a variety of biological functions of immunological relevance, such as hemolymph clotting, tunic synthesis, and encapsulation of foreign bodies (Endean, 1955b; Smith, 1970; Anderson, 1971; Chaga, 1980; Wright, 1981; Zaniolo, 1981). They are by far the most frequent circulating ascidian cell-type (Endean, 1955a; Andrew, 1961; Smith, 1970; Kustin *et al.,* 1976; Ballarin *et al.,* 1995), and their abundance suggests direct involvement in other important defense reactions. Although most of their roles in ascidian immune responses still remain unclear, morula cells can induce cytotoxicity after recognition of foreign molecules or cells (Parrinello, 1996; Cammarata *et al.,* 1997; Ballarin *et al.,* 1998), and they are also required for phagocytosis (Smith and Peddie, 1992).

Cytokines are soluble molecules that mediate communication among various immunocyte types in vertebrate immune systems. In the last decade, much evidence has accumulated indicating that cytokine-like molecules are also involved in invertebrate immune responses, and their presence has been demonstrated in hemocytes of molluscs, annelids, arthropods, echinoderms, and tunicates (Beck and Habicht, 1991; Ottaviani *et al.,* 1995a,b, 1996; Franchini *et al.,* 1996). Cytokine-like molecules stimulate cell proliferation, increase hemocyte motility and phagocytic activity, and induce nitric oxide synthase (Raftos *et al.,* 1991; Ottaviani *et al.,* 1995b). As regards ascidians, the activities of interleukin-1 (IL-1)- and IL-2- but not tumor necrosis factor (TNF)-like molecules have been revealed in various species, either solitary or colonial (Beck *et al.,* 1989). Tunicate IL-1-like molecules modulate immune responses and are secreted by hemocytes in response to exogenous stimuli (Raftos *et al.,* 1991, 1992, 1998; Beck *et al.,* 1993; Kelly *et al.,* 1993).

Received 18 July 2000; accepted 10 May 2001.

* To whom correspondence should be addressed. E-mail: ballarin@civ.bio.unipd.it

Abbreviations: FSW, filtered seawater; HA, hyaline amoebocytes; IL, interleukin; MLC, macrophage-like cells; PMM, phorbol 12-mono-myristate; TNF, tumor necrosis factor.

We have studied—in hemocytes of the colonial ascidian *Botryllus schlosseri*—the presence and distribution of molecules that are immunoreactive to antibodies raised to human IL-1-α and TNF-α. The results indicate that these immunoreactive molecules are mainly detectable in stimulated morula cells, suggesting that these cells have a role in immunomodulation. Moreover, previous results in other ascidian species are supported (Smith and Peddie, 1992).

Materials and Methods

Animals

Wild colonies of *Botryllus schlosseri* from the lagoon of Venice, Italy, were used. They were kept in aerated aquaria, attached to glass slides, and fed with Liquifry Marine (Liquifry Co., England) and algae.

Hemocyte monolayers

Colonies were rinsed in filtered seawater (FSW), pH 7.5, containing 10 m*M* L-cysteine as anticoagulant. The tunic marginal vessels were then punctured with a fine tungsten needle, and hemolymph was collected with a glass micropipette. Hemolymph was centrifuged at 780 $\times$ *g* for 10 min, and pellets were resuspended in FSW to a final hemocyte concentration of 8–10 $\times$ 10^6 cells/ml. Samples of the hemocyte suspension (50–100 μl) were cytocentrifuged onto slides with a Shandon Instrument Cytospin II running at 500 rpm for 2 min. Hemocytes were then stained with May Grünwald-Giemsa for morphological examination with a Leitz Dialux 22 light microscope.

Hemocyte stimulation

Cell suspensions were placed in 1-ml tubes on a revolving mixer, and hemocytes were stimulated by incubation for 5, 15, 30, and 60 min with mannan at 5 mg/ml or phorbol 12-mono-myristate (PMM) at 20 n*M* in FSW containing 10 m*M* L-cysteine to prevent cell clotting. Mannan, a quite common microbial polysaccharide, is easily recognized by mannose receptors, the presence of which has been indirectly inferred on the surface of *Botryllus* phagocytes (Ballarin *et al.,* 1994). PMM is a well-known activator of protein kinase C that mimics the action of diacylglycerol (Wolfe, 1993). The above-reported concentrations of the two compounds were previously demonstrated as the most effective in stimulating *Botryllus* phagocytes and the related respiratory burst (Ballarin *et al.,* 1994; Cima *et al.,* 1996). FSW was used for controls. The viability of hemocytes, after the incubation, was assessed by the trypan blue exclusion assay (Gorman *et al.,* 1996).

Immunocytochemistry

The immunocytochemical procedure described by Ottaviani *et al.* (1990) was performed. The following two primary antibodies were used: polyclonal anti-human IL-1-α (1:250, 1:500, 1:1000) (Santa Cruz Biotech., USA) and monoclonal anti-human TNF-α (1:25, 1:50, 1:100) (NeoMarkers, USA). Cells were incubated with primary antibodies overnight at 4°C, and reactivity was revealed by immunoperoxidase staining using avidin-biotin-peroxidase complex (Hsu *et al.,* 1981). The best results were obtained with anti-IL-1-α and anti-TNF-α diluted 1:500 and 1:25, respectively. In control preparations, the primary antibodies were either substituted with non-immune sera or absorbed with homologous antigen (*i.e.,* human IL-1-α and TNF-α) before addition to hemocyte monolayers. Moreover, a polyclonal antibody raised against *Botryllus* agglutinin (BA) (Ballarin *et al.,* 2000) was also assayed as a control for specificity. Nuclei were counterstained with hematoxylin. The frequency of positive hemocytes, phagocytes, and morula cells was reported as the percentage of the total hemocyte number, which was determined by counting at least 600 cells in 10 fields under the light microscope.

Statistical analysis

All experiments were repeated in triplicate, and statistical analysis was performed using the chi-square test (χ^2).

Results

Morphology of cytocentrifuged Botryllus *hemocytes*

The main hemocyte types present in *B. schlosseri* hemolymph were identifiable under the light microscope after cytocentrifugation. Lymphocyte-like cells, representing 2%–4% of circulating hemocytes, contain a large round nucleus surrounded by a thin layer of basophilic cytoplasm. Phagocytes, which include hyaline amoebocytes (HA; actively phagocytosing cells) and macrophage-like cells (MLC) (Ballarin *et al.,* 1994), have roundish nuclei and neutrophilic cytoplasm which, in the case of MLC, surrounds one or more vacuoles containing ingested material (Fig. 1a, b). Phagocytes constitute 30%–40% of circulating blood cells. Morula cells, the frequency of which is 30%–50% of total hemocytes, are characterized by the presence of several yellowish-green vacuoles (Fig. 2a, c). Nephro-

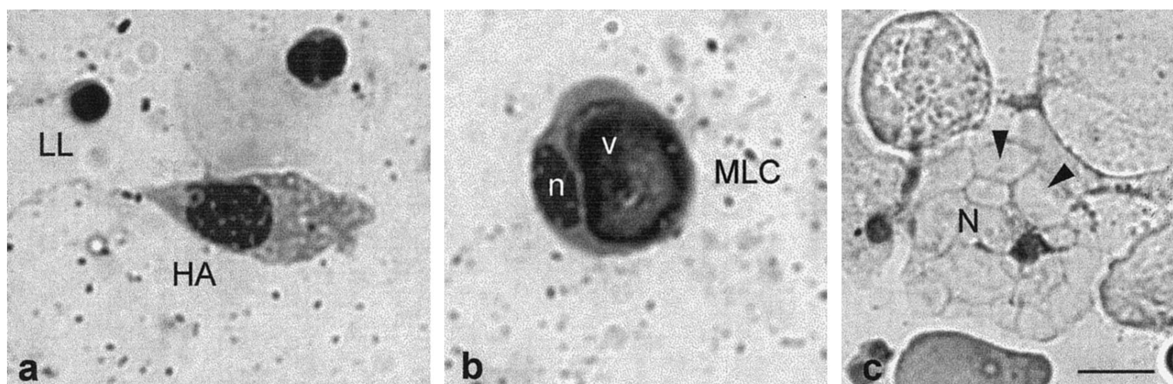

Figure 1. Cytocentrifuged *Botryllus schlosseri* hemocytes stained with May Grünwald-Giemsa solution. (a) Lymphocyte-like cell (LL) and hyaline amebocyte (HA); (b) macrophage-like cell (MLC; n: nucleus; v: vacuole); (c) nephrocyte (N) with several empty vacuoles (arrowheads). Bar = 10 μm.

cytes and pigment cells (6%–10% of circulating hemocytes) were not well preserved after cytocentrifugation; they appeared as giant cells with empty vacuoles (Fig. 1c).

Response of unstimulated hemocytes to anti-cytokine antibodies

Using anti-IL-1-α and anti-TNF-α, only some phagocytes and a few morula cells were labeled after immunoperoxidase staining (Table 1). Thus, most HA, MLC, and morula cells were not immunoreactive with either antibody (Fig. 3). Moreover, no other cell-types stained positively for the two cytokines. No labeling was observed when non-immune sera were used.

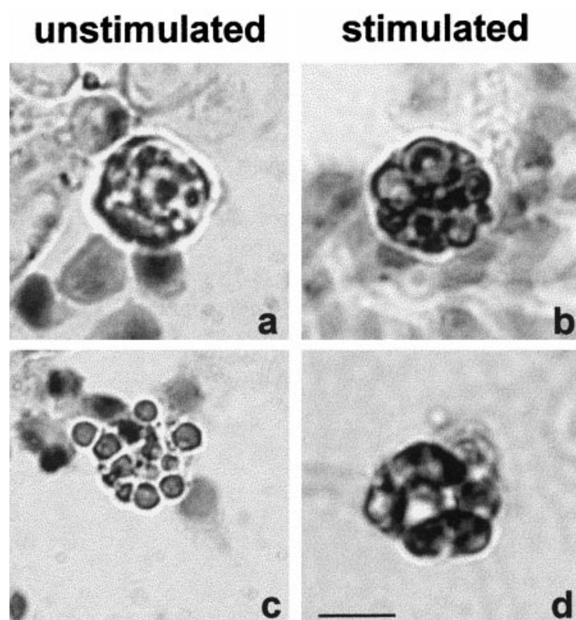

Figure 2. Unstimulated (a, c) and stimulated (b, d) morula cells after immunoperoxidase staining with anti-cytokine antibodies. (a, b) Incubation with anti-IL-1-α antibody; (c, d) treatment with the TNF-α antibody. Bar = 15 μm.

Response of stimulated hemocytes to anti-cytokine antibodies

When monolayers of hemocytes were activated with either mannan or PMM, the number of immunoreactive morula cells and the intensity of their immunoreactivity were progressively augmented with increasing incubation times (Figs. 2, 4). The difference in the number of unstimulated and stimulated reactive morula cells was always significant ($P < 0.001$). In contrast, no significant changes with respect to unstimulated hemocytes were observed in the number, morphology, or stain intensity of positive phagocytes for all the incubation times. In each preparation, more than 95% of hemocytes were viable. Unstimulated and stimulated hemocytes always showed negative results with either non-immune sera or absorbed antibodies. The anti-BA antibody, as previously reported (Ballarin *et al.,* 2000), only recognized amebocytic phagocytes and no morula cells (Fig. 3), supporting the specificity of the anti-cytokine antibodies used.

Table 1

Immunoreactivity of unstimulated Botryllus *hemocytes to antibodies raised to human cytokines*

	Antibodies[a]	
Cell type	Anti-IL-1-α	Anti-TNF-α
Phagocytes[b]	0.4 ± 0.3	0.9 ± 0.4
Morula cells	1.1 ± 0.9	4.5 ± 1.2

[a] Values are percentage of total hemocytes plus or minus the standard deviation.

[b] Phagocytes include hyaline amoebocytes and macrophage-like cells.

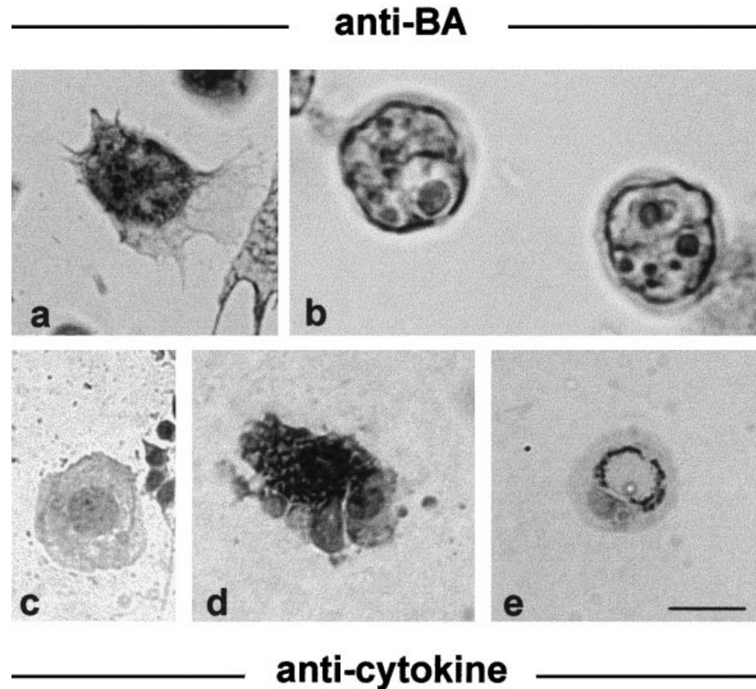

Figure 3. Immunocytochemistry on *Botryllus schlosseri* hemocytes with anti-BA (a, b), and anti-cytokine (c–e) antibodies. (a) Positive HA; (b) negative morula cells; (c) unlabeled, unstimulated HA; (d) stimulated HA positive for IL-1-α; (e) stimulated MLC positive for TNF-α. Bar = 15 μm.

Discussion

In the present work, we demonstrate that molecules recognized by antibodies raised to human IL-1-α and TNF-α are present in immunocytes of the compound ascidian *Botryllus schlosseri.* After stimulation, only morula cells, among all hemocytes, show a marked and significant increase in immunoreactivity. The increase in the number of immunoreactive cells depends on the length of the time of hemocyte incubation with the stimulating agents. In contrast, among unstimulated hemocytes, only some morula cells and a few phagocytes are immunoreactive. Therefore, although the ligands recognized by the antibodies used are unknown and notwithstanding that serological cross-reactivity is not sufficient proof of evolutionary homology between those ligands and vertebrate cytokines, still our data indicate that the morula cells have an important immunomodulatory role in ascidian blood.

We hypothesize that morula cells are the main source of cytokine-like molecules in *Botryllus* hemolymph, which can better explain their abundance in the circulation. Indeed, these cells are able to encapsulate foreign bodies (Anderson, 1971; Wright, 1981; De Leo *et al.,* 1996) and are involved in clotting after blood vessel damage (Vallee, reported by Wright, 1981). In many ascidian species, they can also induce cytotoxicity after recognition of foreign molecules or cells (Parrinello, 1996; Cammarata *et al.,* 1997; Ballarin *et al.,* 1998). All these events can be modulated by cytokine-like molecules produced by activated cells. In agreement with this view, TNF-α-like molecules are involved in insect encapsulation (Franchini *et al.,* 1996), and IL-1-like molecules have been shown to stimulate echinoderm coelomocyte aggregation, which occurs in encapsulation (Beck and Habicht, 1991). Moreover, in vertebrates, both TNF-α and IL-1-α stimulate immune and inflammatory responses, and TNF-α is required for blood coagulation (Abbas *et al.,* 1991).

The induction of cytokine-like molecules in hemocytes after stimulation has already been reported in bivalve molluscs and insects: in all these cases, phagocytes are the immunoreactive cells (Hughes *et al.,* 1990; Franchini *et al.,* 1996). Analogously, in vertebrates, mononuclear phagocytes are the main source of both IL-1-α and TNF-α (Abbas *et al.,* 1991). Nevertheless, the situation in *Botryllus* appears peculiar in that positivity to anti-cytokine antibodies is absent from the majority of phagocytes without significant differences in its distribution between unstimulated and stimulated cells.

Although morula cells have no phagocytic activity, they are reported to promote phagocytosis by ascidian phagocytes (Smith and Peddie, 1992). Thus, the stimulatory effect on phagocytes and the enhancement of phagocytosis by morula cell lysates (Smith and Peddie, 1992) may easily be explained by the immunomodulatory role of the cytokines they produce. This idea is strongly supported by the obser-

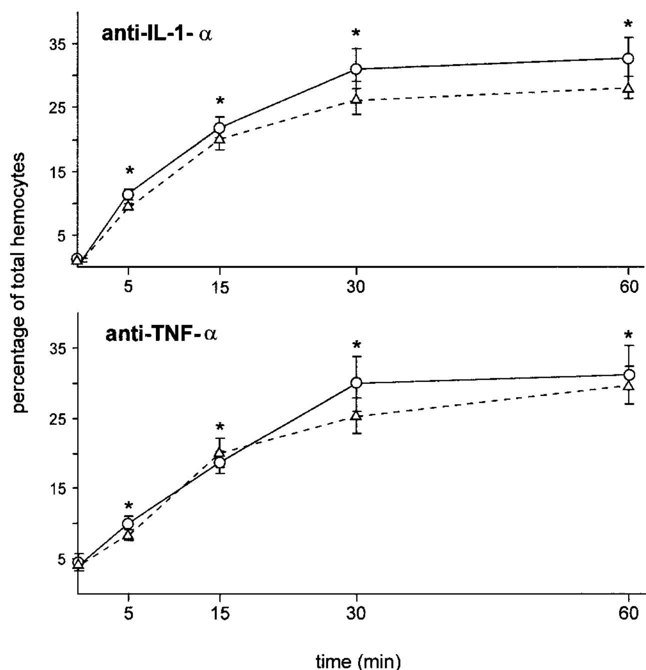

Figure 4. Morula cells positive to anti-IL-1-α and anti-TNF-α, expressed as percentage of total hemocytes, after stimulation with either mannan at 5 mg/ml (circles) or PMM at 20 n*M* (triangles) for 5, 15, 30, and 60 min. $*P < 0.001$ *vs.* control (unstimulated hemocytes, $t = 0$).

vation that the time-dependent increase of immunoreactive morula cells closely resembles the time-dependent increase in the frequency of phagocytizing hemocytes in *in vitro* assays (Ballarin *et al.,* 1997). The opsonic role of tunicate IL-1-like molecules reported by Kelly *et al.* (1993) is in agreement with this view.

Acknowledgments

The authors wish to thank Mr. M. Del Favero, Mr. R. Mazzaro, and Mr. C. Friso for their technical assistance. This work was supported by a grant from the University of Padova to one of us (L.B.).

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