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Research Report

Separated hemocyte populations from the ascidian *Ciona intestinalis* contain and release *in vitro* opsonizing Ca^{2+}-independent and β-galactoside specific lectins

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Abstract

Cytosolic lectins, Ca^{2+}-independent and β-galactoside-specific, were determined to be contained in hemocyte and pharynx lysate supernatants of *Ciona intestinalis,* as revealed by hemagglutination assay with trypsinized rabbit erythrocytes. Ca^{2+}-independence and decreasing β-galactosides inhibitory capacity (TDG > LacNAc ≥ Lactose > Galactose) have been considered properties typical of galectins. These lectins can be promptly released by hemocytes maintained *in vitro* suggesting their involvement in defense responses including inflammatory reactions. Both cell lysate supernatants and hemocyte culture medium presented β-galactoside-inhibitable opsonizing activity versus yeast. Although a Percoll density gradient separation method showed that several hemocyte types contain and release β-galactoside-specific molecules, results suggest that hyaline and granular amoebocytes are the primary source of these molecules.

Key words: hemocyte lectins; hemagglutinins: β-galactosides; phagocytosis; opsonization; hemocytes; tunicates; *Ciona intestinalis*

Introduction

Animal lectins, usually revealed by their hemagglutinating activity, are components of a well-conserved protein-carbohydrate recognition mechanism that function in a variety of biological systems (Feizi, 2000; Sharon and Lis, 2003, 2004). Functions include intra- and extracellular transport processes, sensor branches of innate immunity and recognition of foreign glycans, induction and suppression of effector release, regulation of cell-cell/cell-matrix adhesion or migration, positive/negative growth control with implication for differentiation and malignancy (Kilpatrick, 2000, 2002; Gobius *et al.,* 2002; Sharon and Lis, 2003). Matching glycan diversity with the lectin presence and various glycan epitopes identified as ligands, immune functions appear to be based on the sugar code. In vertebrates and invertebrates lectin families have been established (Cooper and Barondes, 1999; Cooper 2002; Kilpatrick, 2002; Vasta *et al.*, 1999, 2004a), and, among them, Ca^{2+}-independent soluble lectins, generally characterized for a typical carbohydrate recognition domain (S-CRD) with affinity for β-galactosides (S-type/galectins), can be pro-inflammatory (Brewer, 2002; Rabinovich *et al.,* 2002; Vasta *et al.,* 2004b). Of critical importance for galectin characterization is the binding specificity of the basic unit of recognition as shown by the relative inhibitory efficiency of key oligosaccharides α-lactose, N-acetyl-D-lactosamine (Lac-NAC) and thiodi-galactoside (TDG) (Cooper and Barondes, 1999; Dodd and Drickamer, 2001; Sharon and Lis, 2003).

In invertebrates, the defence responses are mainly based on hemocyte types that release humoral factors, including lectins, lysins, clotting and antibacterial proteins (Loker *et al.,* 2004), or display cell-linked activities (Parrinello, 1996; Parrinello *et al.,* 2003). Cellular recognition has been attributed to a protein-carbohydrate molecular mechanism located at the cell surface. Sugar-binding proteins have been found on hemocyte surface (Amirante *et al.*, 1978; Vasta *et al.*, 1984; Parrinello and Arizza, 1988) and they are present in the hemolymph of all

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the examined species, probably involved in non-adaptive immune recognition. In many cases, an opsonic function has been demonstrated (Cheng *et al.,* 1984; Renwrantz and Stahmer, 1983).

In ascidians, considered a key group in chordate phylogenesis (Hori and Osawa, 1987; Field *et al.*, 1988; Swalla *et al.*, 2000; Zeng and Swalla, 2005), multiple naturally occurring or inducible galectins have been found in cell-free hemolymph or bound to hemocyte surface (Parrinello 1995; Nair *et al.,* 2001; Vasta *et al.,* 2001; Green *et al.,* 2003; Quesemberry et al. 2003; Vasta *et al.,* 2004a). In the colonial ascidian *Botryllus schlosseri*, Ballarin *et al.* (1999, 2000) purified from the hemocyte lysate supernatant a D-galactose specific humoral opsonin with galectin properties. In *Styela clava* a C-type humoral lectin with opsonin properties has been purified from the hemolymph (Kelly *et al.,* 1992). In addition, lectins could be released from hemocytes cultured *in vitro* (Arizza *et al.,* 1991; Cammarata *et al.,* 1993) and were contained and released from pharynx explants (Raftos *et al.,* 1990; Arizza *et al.,* 1991*,* 1997).

Previous papers reported that *Ciona intestinalis* serum hemolymph contains Ca^{2+}-dependent (C-type) and Ca^{2+}-independent lectins (Wright, 1974; Parrinello and Patricolo, 1975). A recent report showed that serum galectins could be enhanced in inflammatory responses (Parrinello *et al.,* 2007), whereas C-type lectins may be responsible of complement activation (Pinto *et al.,* 2003). In this respect *C. intestinalis* genome-wide analysis revealed that several C-type lectin and galectin genes have been annotated in the genome (Hori and Hosawa, 1987; Cooper and Barondes 1999; Dehal *et al.,* 2002). However, few data exist on the functional role and tissue distribution of lectins of this ascidian. To ascertain the lectin defence role, tissue localization compatible with internal defence are additional requirements to support the involvement of these molecules in immune protection. In this sense, circulating hemocytes assume particular interest as well as pharynx that represents the main route of pathogen entry. In *C. intestinalis*, several hemocyte types have been described (De Leo, 1992), including stem cells, hyaline and granular amoebocytes, unilocular refractile granulocytes, signet ring cells, morula cells, small and large compartment cells. Of these cell types, only hyaline and granular amoebocytes are capable of phagocytosis *in vitro* (Rowley, 1981), and several granular and vacuolated hemocytes appeared to be mainly responsible of the inflammatory responses *in vivo* (Parrinello, 1981; Parrinello *et al.,* 1984; Parrinello and Patricolo, 1984; Parrinello *et al.,* 1990).

In the present paper we show that soluble lectins are contained in *C. intestinalis* hemocyte and pharynx lysate supernatants, as revealed by β-D-galactoside inhibition of rabbit erythrocytes agglutination. In addition, these lectins are promptly released by hemocytes *in vitro*. Both cell lysate supernatants and hemocyte short-term culture medium presented opsonizing activity versus yeast, revealing the involvement of β-galactoside specific lectins as opsonins. A Percoll density gradient separation method displayed that several hemocyte types, that have been shown to be involved in distinct phase of the inflammatory response, contain and release these lectins, whereas hyaline and granular amoebocytes, that posses *in vitro* phagocytic activity, appear to be rich in cytosolic lectins and are the main source of their release.

Materials and methods

Tunicates, hemolymph collection

Ascidians (7-10 cm long) were collected from Mazara del Vallo Harbor (Italy), held in refrigerated (18 °C) and aerated sea water (60 L aquaria) and fed every second day with a marine invertebrate filter feeding diet (Kent Marine Inc. Wi USA).

The animals were blotted dry to remove any excess of seawater, and bled by removal of the tunic and puncture of the heart. To collect hemocytes, hemolymph was harvested into a fourfold excess of ice cold sterile artificial sea water without $CaCl_2$ and $MgCl_2$ (FSW: 9 mM KCl, 29 mM Na_2SO_4, 2 mM $NaHCO_3$, 0.5 M NaCl, pH 7.4) containing 10 mM ethylenediaminetetracetic acid (FSW-EDTA) as an anticoagulant. After centrifugation at 850xg (10 min, 4 °C), pooled hemocytes (10 ascidians/experiment) were washed twice with FSW-EDTA and, finally, suspended in sterile FSW adjusted for osmolarity with the hemolymph (1,090 mOsm kg^{-1}). Hemocyte mortality, estimated by Trypan blue (0.05 % in FSW) exclusion test, was lower than 5 %.

Pharynx explants were surgically excised with sterile scissors and washed three times with sterilized FSW-EDTA. The same amount of tissue (about 1 gr) was used for every preparation.

All media were sterilized through a 0.22 µm filter (Millipore, Millex).

Preparation of hemocyte and pharynx lysate supernatants (HLS, PhLS)

Hemocytes from pooled hemolymph (15 ascidians for every preparation) were pelleted by centrifuging at 850 xg for 10 min at 4 °C. After two washings in FSW, hemocytes (10 x 10^6 cells/ml) were suspended in diluted ice-cold medium (1:5 in d.w.) to be sonicated at 4 °C for 60 seconds (Branson, model B15, Danbury, CT, USA). The cell lysate was spun (27,000 xg, 20 min, 4 °C), and the resulting supernatant (designed HLS) was dialyzed against TBS (Tris HCl 50 mM, NaCl 0.15 M, pH 7.4), and used for the hemagglutination assay.

Pharynx explants (1 gr tissue) dried with filter paper, frozen at -80 °C and homogenized on ice, were sonicated at 4 °C for 60 seconds. After centrifuging at 27,000xg for 20 min at 4 °C, the supernatant (designed PhLS 0.6 – 0.7 mg/ml protein content) was extensively dialyzed against TBS. For hemagglutination and opsonization assays, samples were 100 times diluted.

To examine the possible effect of cytosolic proteases, in previous experiments, a protease inhibitor cocktail (Sigma, St. Louis, USA) was added (0.1 % final concentration) into the medium just before HLS preparation.

Hemocyte culture and supernatant preparation

Details of the method have been reported elsewhere (Cammarata *et al.,* 1993). Unfractionated or enriched hemocyte populations were suspended in sterile isosmotic artificial sea water (SW) (FSW containing 12 mM $CaCl_2.6\ H_2O$ and 26 mM $MgCl_2.6\ H_2O$). Osmolarity was adjusted to 1090 mOsm kg^{-1}. Hemocytes ($3x10^6$ in 200 µl medium) were put into each well of sterile flat-bottomed culture plates (Nunc, Denmark) and maintained at 4, 10, or 18 °C. In each experiment, cell-free medium from 10 cultures (designed HeS) was pooled and dialyzed against TBS prior to be assayed.

Percentage of dead cells was evaluated with the trypan blue exclusion test. Cell viability of hemocytes cultured for 1.5 h at 10 °C in SW was evaluated with neutral red vital stain (Borenfreund and Puerner, 1984). Values lesser than 3 ± 0.5 % dead cells, and more than 97 ± 1.1 % viable cells were found.

Preparation of rabbit and sheep erythrocyte suspensions and hemagglutination assay

Rabbit erythrocytes (RE) and sheep erythrocytes (SE) were obtained from "Istituto Zooprofilattico della Sicilia" (Palermo). The erythrocyte pellet was washed with PBS (PBS: 6 mM KH_2PO_4, 30 mM Na_2HPO_4, 0,11 M NaCl, pH 7.4) and centrifuged at 500xg for 10 min at 4 °C, then resuspended in TBS to obtain a 1 % suspension.

As previously reported (Parrinello and Canicattì, 1982), hemagglutinating activity (designed HA) was determined in 96-well round bottom microtiter plates using TBS containing 0.1 % gelatin as a dilution medium (serial two-fold dilutions), and an equal volume of 1 % RE or SE in TBS. The microplate was incubated at 37 °C for 1 h, and 2 h at 4 °C. To increase the erythrocyte sensitivity to the hemagglutination assay, trypsin-treated erythrocytes (try-RE, try-SE) were prepared by suspending erythrocyte pellet, from 1.0 ml blood, into 6 ml TBS containing 300 µg trypsin (stock solution prepared in 10 mM HCl). The reaction mixture was incubated at 37 °C for 15 min, and, trypsinized erythrocytes, washed with TBS, were resuspended (1 %) in the same medium. To verify the role of Ca^{2+}, the hemagglutination assay was carried out in the presence of 20 mM EDTA or 10 mM $CaCl_2$.

The titre of hemagglutinating activity (designed HT) was expressed as the reciprocal of the highest dilution giving unequivocal agglutination judged by eye or with a low power binocular microscope. The HT values, expressed as $\log_2$, were recorded as the average ± SD of 10 different assays. Controls consisted of TBS samples in which serum was not added.

To verify the role of Ca^{2+}, lysate supernatants were dialyzed against TBS in the presence of 20 mM EDTA or 10 mM $CaCl_2$, and erythrocytes were suspended in this medium for hemagglutination assay.

Formaldehyde-fixed rabbit erythrocytes (f-RE) were prepared according to the Csizmas's method (1960), and suspended in TBS.

Yeast preparation, opsonization, and in vitro *phagocytosis assay*

A *Saccharomyces cerevisiae* (baker's yeast, type II) suspension was prepared in distilled water at 0.25 % w/v (approx. $1x10^8$ yeast cells/ml), autoclaved for 15 min, washed twice by centrifuging at 2,000xg (5 min, 4 °C), and finally incubated for 2 h at 20 °C with a solution of eosin-Y at 0.05 % final concentration (Cammarata and Arizza, 1994). After repeated washing, yeast were suspended at 0.125 % final concentration in sterile calcium- and magnesium-free SW (FSW), and used immediately.

For opsonization**,** yeast were incubated with lysate supernatant (0.125 % w/v) for 1.5 h at 20 °C, washed in FSW (3 times), and finally suspended in the same medium at the initial concentration. After this treatment, yeast appeared to be agglutinated, forming small clumps, but they were easily re-suspended by washing with FSW.

To verify the role of divalent cations in opsonin-yeast binding, opsonization was carried out in the presence of 20 mM EDTA. Furthermore, the effect of added Ca^{2+} or Mg^{2+} was estimated with FSW containing 10 mM $CaCl_2$ or $MgCl_2$.

For the phagocytosis assay, 200 µl hemocyte suspension ($1x10^6$ cells in SW, indicated as He) was mixed with 100 µl of yeast preparation (10:1 yeast:hemocyte ratio), and incubated in 1 ml test plastic tubes with gentle stirring for 90 min at 20 °C. Then, 50 µl of a quenching solution (2 mg/ml trypan blue, 2 mg/ml crystal violet in 0.02 citrate buffer, pH 4.4, containing 33 mg/ml NaCl) was added. A drop of this suspension was smeared onto slides and examined under a light microscope equipped with a Nomarsky differential interference contrast optic (Diaplan, Leica, Wetzlar, Germany). Hemocytes (about 1000 for every assay, and at least 200/slide) were counted at 800X magnification. Lysate supernatant opsonizing capacity was expressed as percent hemocytes showing ingested yeasts. Results were compared to percent phagocytes in a reaction mixture in which hemocytes from ascidians were mixed with non-opsonized yeasts.

The phagocytic index was calculated according to the following formula: total number of ingested yeasts/total number of counted phagocytes

Absorption with erythrocytes

To absorb agglutinins or opsonins, fRE or fSE were used in the reaction mixture containing packed f-erythrocytes and lysate supernatant or culture medium (v/v). The mixture was incubated for 1 h at room temperature and overnight at 4 °C with occasional shaking, centrifuged at 800xg, and the supernatant assayed with try-RE and try-SE. To control the effect of experimental conditions on the hemagglutinating activity, no absorbed samples were treated as the absorbed ones.

Inhibition of hemagglutinating and opsonizing activities

The supernatants (HLS, PhLS, HeS) were incubated for 60 min at 20 °C with decreasing sugar concentrations (starting from 100 mM final concentration), avoiding sample dilution. The

treated sample was then assayed for hemagglutinating activity.

For inhibiting the opsonizing activity, yeasts were maintained for 1.5 h in lysate or culture supernatant preparations containing decreasing sugar concentrations, and then washed (2 times) with FSW before the phagocytosis assay.

The last sugar concentration (mM) giving an unequivocal inhibitory activity was recorded. D-galactose, α-lactose, D-mannose, L-rhamnose, D-glucose, L-fucose, N-acetyllactosamine (LacNAc) and thio-digalactoside (TDG) were assayed.

To examine the effect of sugar added to the yeast suspension, 25 µl of opsonizing serum containing 100 mM sugar was mixed on a slide with 25 µl yeast suspension and observed after 1.5 h incubation in a wet chamber. Observations with a microscope equipped with Nomarski differential interference contrast optics (Leica) did not show any yeast clamps. To verify changes in yeast sensitivity to phagocyte, sugar treated yeasts were assayed, after washing, in a phagocytosis assay.

Identification and separation of the hemocytes through a Percoll discontinuous density gradient

The hemocytes were classified according to the most popular terminology (Wright, 1981; De Leo, 1992). In the hemolymph several hemocyte types have been recognized. Lymphocyte-like cells are small stem cells. Hyaline amoebocytes contain in their cytoplasm granules of uniform size; granular amoebocytes contain small or large granules; signet ring cells present a single large vacuole; compartment cells contain a variable number of large round and angular vacuoles distributed at the periphery of the cell; morula cells that, when allowed to stand, may assume a berry-like or morular appearance; unilocular refractile granulocyte (URG), characterized by an unique large granule that occupies the cytoplasm and appears to be refractile when observed under a light microscopy.

The hemocyte populations were separated using the method described by Parrinello *et al.* (1996). Briefly, freshly collected hemocyte suspension (approximately $6x10^7$/ml in 4 ml) diluted with FSW-EDTA was spun through a discontinuous gradient of equilibrated Percoll (Pharmacia Fine Chemicals Uppsala, Sweden) (dialyzed against hemocyte isosmotic FSW-EDTA). A gradient was performed with isosmotic medium to obtain decreasing densities (1.105, 1.098, 1.090, 1.079, 1.071, and 1.060 g/ml) into a 10 ml tube. The tube was centrifuged in a swing-out rotor (850xg, 15 min, 7 °C). Bands of cells were gently removed by aspiration from the gradients and washed twice before suspension in FSW. The total population of hemocytes was portioned into six distinct, discrete bands (B1-B6). Dead cells lower than 5 % were found, and viable cells were higher than 95 %. Although each band was mainly enriched for certain hemocyte types, homogeneous populations could not be separated.

For microscopy observations, the cells were removed from the gradients, washed twice in FSW. To check for the hemocyte types contained in each band, 200 µl of the cell suspension was layered on a slide soaked with the poly-L lysine, fixed (30 min) with 1 % saccharose and 1 % glutaraldeyde in FSW, and stained with hematoxilin-eosin (5 min). Differential count of the hemocytes from each band was performed (at least 200 cells/slide).

To prepare HLS the correspondent bands from different Percoll gradients were pooled to reach $1.0x10^7$ cells/ml.

Protein content determination

Protein content was measured by the Bradford method (1976). Bovine serum albumin was used as standard.

Statistical analysis

Data were from five distinct experiments, and each assay repeated three times. Hemagglutinin titres, recorded as $\log_2 \pm$ SD, were examined by the Student *t*-test. Differences were considered significant at $P < 0.05$.

2.9 Chemicals

Unless otherwise reported, all chemicals were purchased from Sigma.

Table 1 Hemagglutinating activity of hemocyte lysate supernatant (HLS), pharynx lysate supernatant (PhLS) and supernatant from cultured hemocytes (HeS) cultured for 1.5 h in SW at 18 °C, assayed against trypsinized rabbit (*try*-RE) or sheep erythrocytes (*try*-SE), in the presence or absence of divalent cations

Medium	Hemagglutinating activity ($\log_2 \pm$ SD, n=5)					
	HLS		PhLS		HeS	
	try-RE	*try*-SE	*try*-RE	*try*-SE	*try*-RE	*try*-SE
TBS	5.2 ± 0.4	--	4.8 ± 0.8	--	3.8 ± 0.4	--
TBS-10 mM $CaCl^2$	5.4 ± 0.5	--	4.6 ± 0.8	--	3.8 ± 0.8	--
TBS-10 mM $MgCl^2$	4.8 ± 0.8	--	4.8 ± 0.4	--	3.8 ± 1.0	--
TBS-20 mM EDTA	4.8 ± 1.0	--	4.4 ± 0.5	--	3.6 ± 0.8	--
SW	4.6 ± 0.8	--	5.6 ± 0.5	--	3.6 ± 0.8	--
F-SW-EDTA	4.6 ± 0.8	--	5.6 ± 0.8	--	3.6 ± 0.5	--

Results

HLS and PhLS agglutinated rabbit erythrocytes in the absence of Ca^{2+}

Preliminary hemagglutination assays with RE of five distinct HLS (20-25 µg/ml protein content), and PhLS in 100 times diluted samples preparations (20-25 µg/ml) showed 3.0 ± 0.8 HT, whereas higher titres (HT: 5.0 ± 0.5 and 4.8 ± 0.5, respectively) were found with *try*-RE as targets. Consequently, trypsinized erythrocytes were used for the next agglutinin titration. No activity was found against SE or *try*-SE (Table 1). The presence of a protease inhibitor cocktail in lysate preparations did not enhance the hemagglutinating titre compared to that of inhibitor-free samples (data not shown).

The addition of 10 mM (final concentration) $CaCl_2$, $MgCl_2$ or 20 mM EDTA into the medium did not affect the hemagglutinating activity of HLS and PhLS samples (Table 1).

Hemocytes release in vitro *Ca^{2+}-independent hemagglutinins*

Supernatant from hemocyte cultures (HeS) agglutinated *try*-RE but not *try*-SE. The hemocytes cultured at 4 °C for 1.5 h released a low amount of lectins into the cell-free medium (HT: 2.2-2.4), whereas highest levels (HT: 3.6-3.8) were found at 10 °C (Table 1). Higher temperatures (up to 18 °C) did not significantly enhance the agglutinin release.

The presence of 20 mM EDTA as well as the addition of $CaCl_2$ or $MgCl_2$ in the hemagglutination medium (TBS) did not affect the activity *vs* both the erythrocyte types (Table 1).

To compare the activity of supernatants from unseparated hemocyte culture with HLS, $15x10^6$ hemocytes/ml were divided in two groups, one of the two was homogenized, and the other one was cultured (1.5 h, 10 °C). In four distinct experiments, the HLSs presented the highest activity (HT: 6.7 ± 0.9) compared with the hemocyte culture supernatants (HT: 4.0 ± 0.15).

Protein content of unseparated hemocyte culture supernatants from twelve distinct experiments ranged from 8 µg/ml to 20 µg/ml. A significant proportionality between protein content and hemagglutinin titre was not observed.

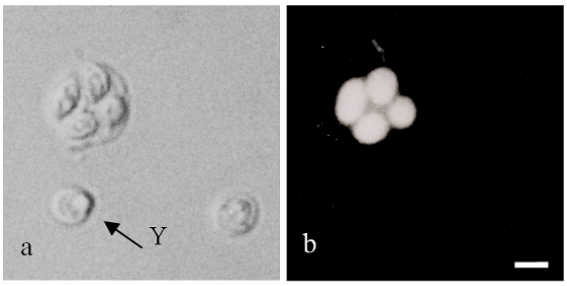


Fig. 1 A *Ciona intestinalis* phagocyte with ingested eosin-y treated yeasts, as shown by Nomarski contrast interference observation (a), or UV–light observation (b). Y: eosin-y treated yeast. Bar = 5 µm

The culture medium composition did not affect the agglutinin-release. In preliminary experiments the same HTs were recorded by assaying supernatants from hemocytes cultured in M199 enriched medium or SW. Therefore, in the next experiments hemocytes were cultured in SW.

Erythrocyte specificity

Absorption experiments with fRE or fSE showed that the hemagglutinating activity of HLS and PhLS was lost after absorption with fRE, whereas it was maintained after treatment of HLS and PhLS with fSE (HT: 4.6-5.6, respectively). The same effect was exerted by fRE on HeS.

HLS, PhLS and HeS opsonize yeast

Table 2 shows that 15 % (mean value) of hemocytes spontaneously ingested non-opsonized yeast (Fig. 1). When the targets were opsonized with HLS, PhLS or HeS, a greater number of hemocytes ingested yeast, and the percentage significantly increased up to 20-24 % ($P < 0.05$). The phagocytic index significantly increased as an effect of the opsonization.

Table 2 Opsonizing activity, in the absence or presence of EDTA (20 mM), of hemocyte lysate supernatant (HLS), pharynx lysate supernatant (PhLS), and supernatant from hemocytes (HeS) cultured for 1.5 h in SW at 18 °C, assayed against yeast cells. Percent (%) phagocytes with ingested opsonized yeasts were compared with % phagocytes assayed with non opsonized (SW) targets. Values are expressed as mean percentage of hemocyte containing yeasts ± SD (n=5). Phagocytic index = total number of ingested yeasts/total number of counted phagocytes. ** $P < 0.01$; ***$P<0.001$

Yeasts treated with			10 mM EDTA	
	Phagocytes (%)	Phagocytic index	Phagocytes (%)	Phagocytic index
SW	15.3 ± 1.2	1.9 ± 0.2	14.6 ± 0.9	1.7 ± 0.3
HLS	24.2 ± 3.2 (**)	2.6 ± 0.3 (**)	22.1 ± 2.3 (**)	2.9 ± 0.3 (**)
PhLS	23.9 ± 1.2 (**)	2.4 ± 0.2 (**)	22.8 ± 1.4 (**)	2.6 ± 0.3 (**)
HeS	20.2 ± 2.1 (**)	3.3 ± 0.3 (***)	19.7 ± 1.8 (*)	3.2 ± 0.2 (***)

Table 3 Sugar inhibition of the hemagglutinating and the opsonizing activity of hemocyte lysate supernatant (HLS), pharynx lysate supernatant (PhLS) and supernatant from hemocytes (HeS) cultured for 1.5 h in SW at 18 °C, assayed against trypsinized rabbit erythrocytes and yeasts respectively. The lowest sugar concentration (mM) that abolished the hemagglutinating activity of the sample against rabbit erythrocytes, or giving significant inhibitory activity of yeast opsonization was recorded. 100 mM starting sugar concentration

Compound	Sugar inhibitory concentration (mM ± SD, n=5)					
	Hemagglutinating activity (1)			Opsonizing activity (2)		
	HLS	PhLS	HeS	HLS	PhLS	HeS
D-Galactose	21.6 ± 9,8	10.6 ± 4,6	5.25 ± 1.7	25.0 ± 5.6	25.0 ± 8.8	25.0 ± 6.3
α-Lactose	21.6 ± 9,8	16.0 ± 0	12.5 ± 1.0	50.0 ± 12.9	50.0 ± 11.7	50.0 ± 11.3
Lactulose	44.0 ± 19,0	27.3 ± 9,8	25.0 ± 1.5	50.0 ± 10.4	50.0 ± 7.5	25.0 ± 10.9
LacNac	25.0 ± 6.8	25.0 ± 4.8	1.5 ± 1.0	50.0 ± 11.7	25.0 ± 3.8	25.0 ± 3.2
TDG	1.5 ± 0.5	1.5 ± 0.5	0.5 ± 1.0	50.0 ± 10.1	25.0 ± 5.1	12.5 ± 9.7
L-Fucose	N.I.	N.I.	N.I.	N.I.	N.I.	N.I.
D-Mannose	N.I.	N.I.	N.I.	25.0 ± 3.7	25.0 ± 6.3	25.0 ± 8.7
D-Glucose	N.I	N.I.	N.I.	N.I.	N.I.	N.I.
NANA	N.I.	N.I.	N.I.	N.I.	N.I.	N.I.
NAG	N.I.	N.I.	N.I.	N.I.	N.I.	N.I.
Laminarin (%)	0.006 ± 0.002	0.012 ± 0.04	0.015 ± 0.001	0.5 ± 0.09	0.05 ± 0.001	0.5 ± 0.05

(1) Hemagglutination assay against trypsinized-rabbit erythrocytes.
(2) Supernatant opsonising activity *vs* yeast examined in a hemocyte phagocytosis assay.
N.I.: No inhibition

The Ca^{2+}-independence of the opsonizing activity was shown by treating yeast with HLS, PhLS or HeS in the presence of 20 mM EDTA, 10 mM $CaCl_2$, or $MgCl_2$. No differences were observed as an effect of opsonization medium composition (Table 2).

Finally, opsonins were absorbed by treating samples with packed fRE whereas was unchanged after fSE absorption.

Galactosides inhibit the hemagglutinating and opsonizing activities

The sugar-lectin binding of both agglutinins and opsonins was shown by sugar inhibition assay. The hemagglutinating activity of HLS and PhLS was abolished by D-galactose, α-lactose, lactulose, LacNAc, at various mM concentrations (Table 3). Except lactulose, lower concentrations (ranging from 1.5 mM LacNAc to 12.5 mM lactose) of these sugars inhibited hemocyte culture supernatant. TDG was the most effective saccharides in inhibiting hemagglutination activity of HLS, PhLS and HeS (ranging from 0.5 to 1.5 mM). In all cases, the hemagglutinating activity was not affected by 100 mM D-glucose, L-fucose, D-mannose (Table 2).

The above reported active sugars inhibited the opsonizing activity of HLS, PhLS and HeS (Table 3) even if higher concentrations were needed, whereas mannose inhibited the yeast opsonization. The presence of high sugar concentration in the medium used for preparing yeast did not affect their sensitivity to phagocytes, and, after washing, they were phagocytosed as the untreated ones.

Hemagglutinating activity of HLS and culture supernatants from hemocyte populations enriched through a Percoll discontinuous density gradient

The cells removed from density gradient separated bands were examined and identified for their morphology. Differential count of the hemocytes from each band was performed (at least 200 cells/slide). B1 mainly contained hyaline amoebocytes (~ 76 %) and, to a lesser extent, stem cells (~ 9 %) and signet ring cells (~ 11 %); B2 was mainly enriched in hyaline amoebocytes (~57 %), but also contained lymphocyte-like cells (~ 5 %), granular amoebocytes (~ 20 %), signet-ring cells (~ 9 %); B3 consisted primarily of ~ 71 % granular amoebocytes, ~ 22 % hyaline amoebocytes and ~ 8 % signet ring cells; B4 was composed of granular amoebocytes (~ 45 %) and morula cells (~ 52 %); B5 contained morula cells (~ 59 %) and univacuolar refractile granulocytes (~ 41 %); finally, B6 was largely (~ 84 %) made up of morula cells. Hemocyte types present in each band at very low percentage were not reported.

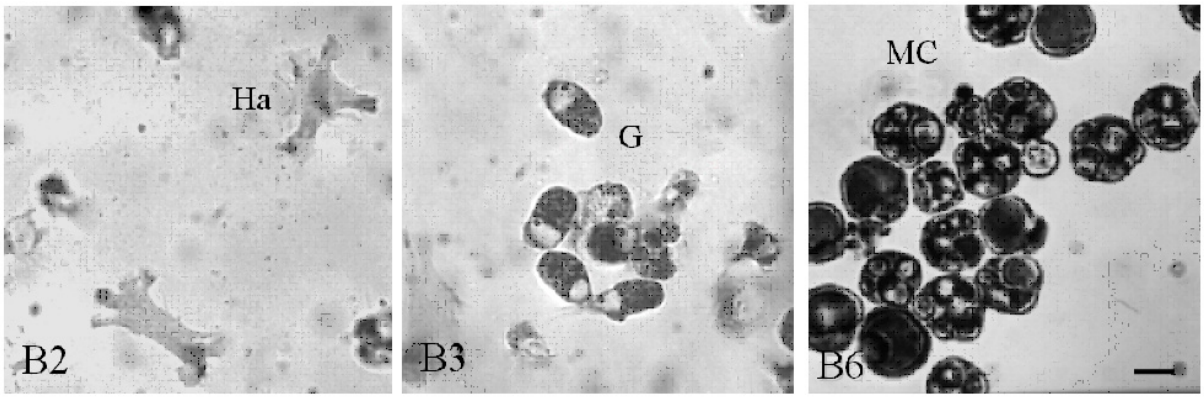


Fig. 2 *Ciona intestinalis* main hemocyte types in B2, B3 and B6 bands separated through a discontinous Percoll density gradient. Hyaline amoebocytes (Ha), Granulocytes (G); Morula cells (MC). Bar = 10 μm

In Fig. 2 hyaline amoebocytes, granular amoebocyte, univacuolar refringent granulocytes and morula cells contained in the separated bands are shown.

To identify the hemocytes that contained anti-RE lectins, HLSs from Percoll-gradient separated hemocytes were assayed for their activity. As shown in Fig. 3, HLS from enriched hemocyte populations, standardized at a same hemocyte number ($10x10^6$/ml), presented various levels of hemagglutinating activity revealing that B2-HLS and B3-HLS reached titres higher (HT: 8.2 and 5.9, respectively; $P < 0.01$) than HLS from the whole hemocyte preparations (4.2 HT). Lower levels were associated with B1 and B6 (HT: 4.0 and 3.6, respectively), whereas the lowest ones (HT: 1.4-2.0) were recorded in B4 and B5 HLSs.

To identify the lectin-releasing hemocytes, the density-gradient enriched hemocyte populations were cultured at 10 °C for three hours, and, then, cell-free medium from pools of each separated band was assayed. The hemagglutination titres of B2 cell-free culture medium, compared with the culture medium of the remaining bands or unseparated hemocytes (HT: 1.3), showed the highest hemagglutinating titre (HT: 3.4), whereas 1.7 HT was found for B1 (Fig. 3). Very low HTs were recorded for B3-B6 cell free culture medium.

Discussion

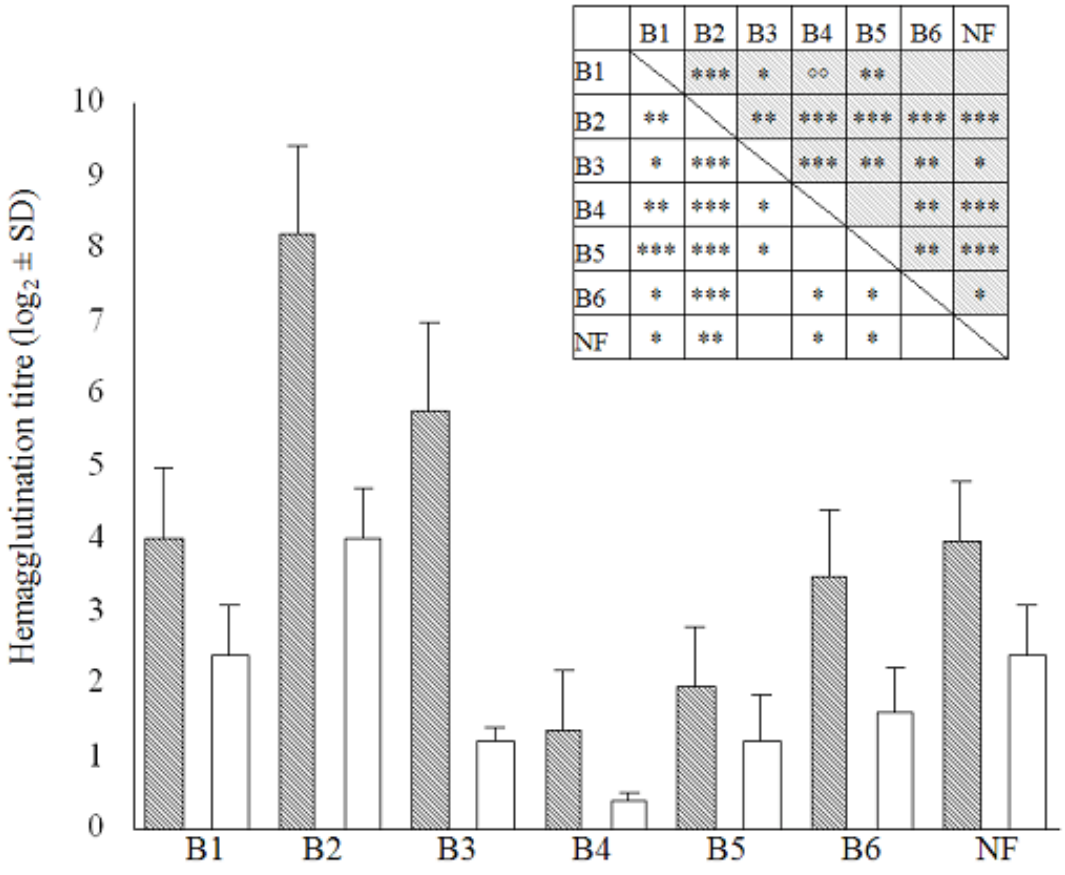
The serum hemolymph of *C. intestinalis* contains naturally occurring lectins that agglutinated rabbit and sheep erythrocytes (Wright, 1974; Parrinello and Patricolo, 1975). In the present paper we show that hemocytes and pharynx lysate supernatants agglutinated rabbit erythrocytes, whereas they were inactive against sheep erythrocytes revealing a certain range of specificity in discriminating target membrane sugar components. Accordingly, erythrocyte trypsinization of sheep erythrocytes that exposed glycosylated components of the membrane outer layer did not affect the hemagglutination of sheep erythrocytes whereas increased rabbit erythrocytes sensitiveness, and higher hemagglutinin titres were recorded. The anti-*try*RE agglutinin titres (HT: 4-5) revealed in the HLS and PhLS were higher than those (HT: 1-3) reported by Parrinello and Patricolo (1975) for the hemolymph serum. Hemocytes and pharynx tissues cannot be directly compared in their agglutinin titres, in fact pharynx blood vessels can contain various amounts of hemocytes.

These agglutinins, are cytosolic being identified in water-soluble extracts from hemocyte and pharynx preparations. The sample preparation method, carried out on ice followed by a prompt separation of the supernatant at 4 °C, presumably avoided any effect of cytosolic proteases as shown by the unchanged hemagglutinin titres after the addition of an anti-protease inhibitor cocktail.

Hemagglutinins can be promptly released by hemocytes maintained viable *in vitro* for a short time (1-3 h). Such a release was temperature-dependent as it decreased with low temperature and reached the highest values at 10-18 °C. We do not know if lectins are secreted or a non classical secretory mechanism may externalize lectins confined to cytoplasm as reported for some galectins (Sato *et al.*,1993; Sato and Hughes, 1994).

The anti-RE agglutinins contained in hemocytes and pharynx as well as released by hemocytes are Ca^{2+}-independent, D-galactoside specific lectins that showed a relative inhibitory activity. TDG, an analog of lactose, and LacNAc, an analog of lactose with a hydroxyl substituted with an acetamide group, were more active than lactose in inhibiting the agglutination. TDG, LacNAc, lactose and galactose were effective at higher concentrations. A such lectin-binding affinity of β-galactosides (TDG > LacNAc ≥ Lactose > Galactose) and the Ca^{2+}-independence have been considered properties typical of galectins (Vasta *et al.*, 2004b).

Accordingly to the defence role, these β-galactoside-specific lectins showed opsonic properties *vs* yeast, and hemocytes could release them *in vitro* presumably as an effect of cell activation due to the experimental procedures. A low percentage of phagocytes internalized non opsonized yeasts, presumably due to mannose receptors on the phagocyte membrane, whereas the opsonization of the targets with supernatants from hemocyte and pharynx lysates, and hemocyte culture medium enable a significantly major number



	B1	B2	B3	B4	B5	B6	NF
B1		***	*	○○	**		
B2	**		**	***	***	***	***
B3	*	***		***	**	**	*
B4	**	***	*			**	***
B5	***	***	*			**	***
B6	*	***		*	*		*
NF	*	**		*	*		

Fig. 3 (▨) Hemagglutinating activity of hemocyte lysate supernatant from non fractioned hemocytes (NF), or enriched hemocyte populations separated through a discontinous Percoll gradient as discrete bands B1, B2, B3, B4, B5 and B6. (□) Hemagglutinating activity of supernatant from cultured NF (1.5 h at 18 °C), or enriched hemocyte populations separated through a discontinous Percoll gradient as discrete bands B1, B2, B3, B4, B5 and B6 and supernatant from 1.5 h hemocyte colture from cell populations enriched in bands B1-B6 through a discontinous Percoll density gradient.
Inset: Student *t*-test comparison between the various groups. *** P<0.001, **P<0.01, *P<0.05

of phagocytes to engulf opsonized targets, also showing an increased phagocytic index. The possibility exists that, as reported for mammalian galectin 10 (Swaminthan *et al.*, 1999), some CRD variants could be contained in *C. intestinalis* β-galactoside-specific lectins that could interact with mannose.

Although pure hemocyte populations could not be separated, in an attempt of correlating lectin content and release with hemocyte types we examined cell populations separated on Percoll discontinuous density gradient. Lysate supernatants from the separated hemocyte bands, prepared from a constant cell number, presented a various degree of hemagglutination titres as compared with the unfractionated hemocytes. Hemocytes from the separated bands appeared to be mixed cell populations therefore it is not possible to assign the content in cytosolic lectin molecules to discrete hemocytes. The highest hemagglutinating titres were found in B2-HLS that mainly contained hyaline amoebocytes (~ 57 %) and a lower proportion of granulocytes (~ 20 %). Likewise the high titre that characterized B1-HLS, composed with 76 % hyaline amoebocytes in the absence of granular amoebocytes, emphasized the effect of the enrichment in hyaline amoebocytes as lectin-containing cells. On the other hand, also granular amoebocytes enriched in B3 (~ 71 % granular amoebocytes, and 22 % hyaline amoebocytes) could be responsible of the high titre registered in their lysate supernatant. Finally, a low level was found in the lysate from morula cells enriched (85 %) in B6. Cytosolic lectins appeared to be contained in several hemocyte types, supporting their role in multiple cell functions. In addition, the high content of lectins in inflammatory hemocytes, like hyaline and granular amoebocytes, suggested their involvement in inflammatory reactions.

Accordingly, a similar hemagglutin titre profile was observed by examining the activity of supernatant from B1-B6 hemocyte cultures even if the titres were lower than those observed in the hemocyte

lysate supernatants, probably due to a lesser number of hemocytes, as yielded from separated bands, cultured for a short time. The highest activity was found by culturing hemocytes from B1 and B2 mainly enriched in hyaline amoebocytes that have a role as phagocytes and presumably release lectins to be involved in opsonization. However, the low hemagglutinating activity of supernatants from enriched granular amoebocyte population (B3) does not exclude their involvement in lectin-dependent immune functions. In fact, the experimental conditions and the short time we used for preparing culture supernatants could affect the lectin release from this hemocyte type.

Further research is required to uncover the molecular structure of hemocyte β-galactoside-specific lectins and ascertain whether they belong to the galectin family as identified in the *C. intestinalis* genome.

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