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REGULAR ARTICLE

Inducible lectins with galectin properties and human IL1α epitopes opsonize yeast during the inflammatory response of the ascidian *Ciona intestinalis*

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Abstract Studies on inducible ascidian lectins may shed light on the evolutionary emergence of cytokine functions. Here, we show that the levels of opsonins, with IL1α-epitopes, increase in *Ciona intestinalis* hemolymph as a response to an inflammatory stimulus and, in particular, to intratunic injection of lipopolysaccharide (LPS). The inflammatory agent promptly (within 4 h) enhances Ca^{2+}-independent serum hemagglutinating and opsonizing activities, which are both inhibited by D-galactose and D-galactosides (α-lactose, N-acetyl-D-lactosamine, thio-digalactoside), suggesting that anti-rabbit erythrocyte lectins with galectin properties are involved as opsonins. Inducible galectin molecules contain interleukin-1α (IL1α) epitopes, and their activities are specifically inhibited by anti-human recombinant IL1α antibody. Analysis by SDS-polyacrylamide gel electrophoresis has revealed that the density of the bands of several serum proteins increases within 4 h after LPS injection, correlated with the enhanced serum activity. Moreover, Western blot patterns demonstrate that several serum proteins (59, 37, 30, 23, 15 kDa) cross-react with the antibody as early as 4 h post-injection. Although we have not been able to establish whether, in adition to galectins, various types of D-galactose-specific lectins are contained in the serum, we show, for the first time in invertebrates, that galectin molecules with opsonic properties can be enhanced in response to a non-specific inflammatory stimulus, and that their release can be further stimulated by LPS. Finally, we reveal that multiple galectins share human IL1α epitopes, probably because of steric configuration and the oligomerization process.

Keywords Evolution · Inflammatory response · Phagocytosis · Opsonins · Lectins · IL1α-like galectins · Ascidian, *Ciona intestinalis* (Tunicata)

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Introduction

In the invertebrate immune system, phagocytosis, cell proliferation, and chemotaxis can be regulated by cytophilic humoral molecules with functional similarities to vertebrate cytokines (Beck and Habicht 1996; Beck et al. 1993; Beschin et al. 2004). These molecules modulate defense responses to exogenous and endogenous insults, tissue repair, and the recovery of homeostasis through binding to specific cell-surface ligands and receptors. Many mammalian cytokines are bifunctional molecules containing a receptor-binding domain and an evolutionarily conserved carbohydrate recognition domain (CRD) that is typical of lectins and that associates with carbohydrates for biological activity (Beschin et al. 2004). In this regard, interleukins IL1α and β can be considered as lectins (Cebo et al. 2001a, b, 2002) directly interacting with various pathogens through lectin-like interactions and contributing to pathogen elimination via opsonization and/or leukocyte activation (Zanetta et al. 1996, 1998; George 1994; Luo et al. 1993; Lucas et al. 1994; Magez et al. 2001). CRDs with various ranges of sugar

binding characterize many invertebrate and vertebrate lectins and form an ancestral protein-carbohydrate recognition system that has been implicated in inflammation and several immune responses (White 1987; Söderhall and Smith 1986; Vasta et al. 2004).

Lectins, identified by their hemagglutinating activity (HA) and sugar specificity, are divided into structurally related protein families and superfamilies (Drickamer and Taylor 1993; Dodd and Drickamer 2001; Sharon and Lis 2003). In general terms, two main animal lectin types have been distinguished: Ca^{2+}-dependent lectins (C-type) with a diverse sugar specificity and whose cysteine residues form disulfide bridges; Ca^{2+}-independent lectins (S-type/galectins), which are small soluble lectins that have CRDs with affinity for β-galactosides, and which contain cysteine residues that do not form disulfide bridges. 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 such as α-lactose, N-acetyl-D-lactosamine (Lac-NAC), and thio-digalactoside (TDG) T-disaccharide (Galβ1, 3GalNAc; Dodd and Drickamer 2001; Sharon and Lis 2003; Cooper and Barondes 1999).

Since tunicates are considered a key group in chordate phylogenesis (Hori and Osawa 1987; Field et al. 1988; Zeng and Swalla 2005; Delsuc et al. 2006), studies on inducible ascidian lectins with cytokine functions may shed light on the evolutionary emergence of inflammatory molecules, which may be related to similar molecules in vertebrates. In this respect, multiple naturally occurring soluble ascidian lectins, usually named "tunicate" cytokines, mediate broad recognition and effector immune responses by presenting some functional cytokine properties (Vasta et al. 2004; Beck et al. 1989; Quesenberry et al. 2003). In the hemolymph of the ascidians *Styela clava* and *S. plicata*, "cytokine-like" proteins stimulate cell proliferation in pharyngeal explants (Nair et al. 2001; Raftos and Cooper 1991) and modulate phagocytic activity of tunicate hemocytes (Beck et al. 1993). Both 17-kDa *tun*IL1 and 14-kDa molecules have been identified as Ca^{2+}-dependent C-type lectins that bind galactosyl moieties and opsonize yeast (Beck et al. 1993; Nair et al. 2001; Kelly et al. 1992). The *tun*IL1 of *S. clava* also stimulates the proliferation of mouse thymocytes and L-929 fibroblasts (Raftos and Cooper 1991), exerting its IL1-like activity through a galactosyl-binding site (Raftos 1996; Raftos and Nair 2004). C-type lectins of *Clavelina picta* and *Halocynthia roretzi* modulate cellular activity by enhancing cell proliferation and phagocytosis (Raftos et al. 2001); furthermore, *H. roretzi* lectin stimulates superoxide anion production by hemocytes (Abe et al. 1999).

Several ascidian galectins have been identified. In *C. picta*, a prototype galectin has been characterized (Vasta et al. 2004). In *Didemnum candidum*, a galactosyl-binding lectin shows mitogenic activity toward murine thymocytes (Vasta et al. 1986). Finally, a "galectin-like" opsonin has been reported in colonial *Botryllus schlosseri* (Ballarin et al. 1994, 2000).

In mammals, various galectin immunomodulative activities have been established; galectins appear to be pro-inflammatory cytokines released when immune responses are triggered. Therefore, the release of lectin in invertebrate inflammatory events represents an additional functional approach to studying ascidian immunomodulatory molecules. To date, we know that a *S. plicata* C-type lectin appears to act in a pro-inflammatory manner and can be enhanced as an acute-phase antigen-recognition protein by zymosan injection, which causes an inflammatory response resembling that of mammalian collectins (Green et al. 2003), whereas no data have been reported on the modulation of galectin release.

In *Ciona intestinalis*, naturally occurring C-type and S-type lectins are contained in the hemolymph (Parrinello and Patricolo 1975; Wright 1981). Moreover, C-type lectins and galectins have been recorded in the genome of *C. intestinalis* (Dehal et al. 2002), and a 36-kDa β-galactoside-binding lectin gene is expressed in hemocytes (Shida et al. 2003). This ascidian shows a strong inflammatory reaction just after erythrocytes or foreign proteins are injected into the tunic. Parrinello and collaborators (Parrinello 1981; Parrinello et al. 1984a, b) have reported that numerous hemocytes of various types, including phagocytes, promptly infiltrate the tunic tissue, and a capsule becomes visible by eye at the injection site within 2–3 days. The inflammatory reaction has also been revealed by tunic tissue injury that might mask the capsule in a percentage of treated ascidians depending on the dose of inflammatory agent (Parrinello et al. 1984a, b) and by massive degranulation of hemocytes (Parrinello et al. 1990).

Among experimental approaches used to identify invertebrate cytokine molecules, antibodies neutralizing the activity of mammalian cytokines have been used to screen for cross-reactivity with factors in hemolymph, immunocytes, neuroendocrine cells, and tissues (for a review, see Beschin et al. 2004). In eight North American species of tunicates, molecules with an IL1 function have been neutralized by polyclonal anti-human IL1 antisera (Beck et al. 1989).

In the present paper, we show that lipopolysaccharide (LPS) injection induces a capsule in the *C. intestinalis* tunic, whereas in the hemolymph, the concentration of lectins showing IL1α epitopes and the opsonizing property is promptly (within 4 h) augmented as a response to the non-specific inflammatory stimulus. LPS further enhances serum HA and opsonizing activities, revealing its inflammatory effect in triggering the release of the active

molecules. The D-galactoside specificity and Ca^{2+}-independent activities of naturally occurring and inducible hemagglutinins and opsonins suggest that they possess galectin properties. Since specific antibodies abolish opsonic activity and significantly decrease HA, we suggest that galectin-like serum fractions are provided with IL1α epitopes. Finally, electrophoretic and immunoblotting patterns support the idea that multiple serum proteins with IL1α epitopes can be enhanced in the hemolymph, correlating with serum activity, as a response to LPS challenge.

Materials and methods

Tunicates

C. intestinalis were collected from Sciacca Harbor (Sicily, Italy). Prior to experimentation, tunicates were held for up to 2 days in refrigerated glass aquaria (60 l, 15°C, filtered seawater) and fed every second day with a marine invertebrate diet.

LPS injection

LPS (*Escherichia coli* 055:B5) solution was prepared in sterile marine solution (MS: 12 mM $CaCl_2.6H_2O$, 11 mM KCl, 26 mM $MgCl_2.6H_2O$, 45 mM TRIS, 38 mM HCl, 0.45 M NaCl, pH 7.4). A volume of 100 μl of 1 mg/ml LPS solution (100 μg LPS per animal) was injected into the tunic tissue at the median body region, whereas untreated and MS (100 μl)-treated ascidians served as controls. Before injection, the tunic surface was thoroughly cleaned. All the solutions were filtered through a 0.22-μm filter (Millipore, Millex) and were autoclaved.

Cell-free hemolymph (serum) preparations

The animals were blotted dry to remove any excess seawater and bled by removal of the tunic and puncture of the heart. The hemolymph was withdrawn from the heart with a sterile syringe and immediately centrifuged at 800*g* (10 min, 4°C). The supernatant (serum) was filtered through a 0.45-μm filter (Millex GV, Millipore). At every time-point, serum pools from ten individuals were prepared, divided into small aliquots, and kept at −80°C.

Yeast preparation and 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 (approximately 1×10^8 yeast/ml), autoclaved for 15 min, washed twice by centrifugation at 2,000*g* (5 min, 4°C), and finally incubated for 2 h at 20°C with a solution of eosin-Y at a final concentration of 0.05% (Cammarata and Arizza 1994). After repeated washes, the yeast were suspended in sterile calcium-free and magnesium-free MS (f-MS) at a final concentration of 0.125% and used immediately.

For opsonization, yeast were incubated with serum (0.125% w/v) for 1 h at 20°C, washed in f-MS (3 times), and finally suspended in f-MS at the initial concentration. After this treatment, the yeast appeared to be agglutinated, forming small clumps, but they were easily re-suspended by washes with f-MS.

To verify the role of divalent cations in opsonin-yeast binding, opsonization was carried out in the presence of 20 mM ethylenediaminetetraacetic acid (EDTA). Furthermore, the effect of added Ca^{2+} was estimated with TRIS-buffered saline (TBS) containing 10 mM $CaCl_2$. For the phagocytosis assay, 200 μl hemocyte suspension (1×10^6 cells in MS, indicated as He) from unchallenged ascidians was mixed with 100 μl yeast preparation (10:1 yeast/hemocyte ratio) and incubated in 1-ml plastic test-tubes under gentle stirring for 90 min at 20°C. Then, 50 μl 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 Leica Diaplan Epifluorescence microscope (Leica, Wetzlar, Germany). Hemocytes (about 1,000 for every assay, and at least 200/slide) were counted at 800× magnification. Serum opsonizing capacity was expressed as the percentage of hemocytes showing ingested yeast over the total number of hemocytes. Results were compared with the percentage of phagocytes with engulfed yeast in a reaction mixture in which hemocytes from unchallenged ascidians were mixed with non-opsonized yeast.

Hemagglutination assay

Rabbit erythrocytes (RE) were obtained from the Instituto Zooprofilattico della Sicilia (Palermo). The erythrocyte pellet was washed with phosphate-buffered saline (PBS, pH 7.4), centrifuged at 500*g* for 10 min at 4°C, and then resuspended in TBS (TRIS-HCl 50 mM, NaCl 0.15 M, pH 7.4) to obtain a 1% suspension.

As previously reported (Parrinello and Canicattì 1982), serum HA was determined in 96-well round-bottomed microtiter plates by using TBS containing 0.1% gelatin as the dilution medium and an equal volume of 1% RE in TBS. To increase the erythrocyte sensitivity to the hemagglutination assay, trypsin-treated erythrocytes (try-RE) were prepared by resuspending an RE pellet (obtained from 1.0 ml blood) in 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. The try-RE were washed with TBS and resuspended (1%) in TBS. 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 titer of HA (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.

Sugar inhibition of serum activities

Serum was incubated for 30 min at 20°C with decreasing sugar concentrations (starting from a 100-mM final concentration), avoiding serum dilution. The treated serum was assayed for HA. The lowest sugar concentration (in mM) that abolished the HA of the serum was recorded. For opsonization, yeast were maintained for 1 h in sugar-containing serum and then washed (twice) with f-MS before the phagocytosis assay. The lowest sugar concentration (in mM) giving significant inhibitory activity was recorded. D-galactose, α-lactose, D-mannose, D-glucose, L-fucose, N-acetyl-D-lactosamine (Lac-NAc), and thio-digalactoside (TDG) were assayed.

To examine the effect of sugar added to the yeast suspension, 25 μl opsonizing serum containing 100 mM sugar was mixed on a slide with 25 μl yeast suspension and, after a 1.5-h incubation in a moist chamber, were observed by using a microscope equipped with Nomarski differential interference contrast optics (Leica). The sugar effect was observed by comparing the significant decrease (up to 10%) in the percentage of phagocytes showing ingested yeast.

Absorption of serum by yeast, erythrocytes, and anti-human recombinant interlekin-1 antibodies

Serum was absorbed by packed yeast or erythrocytes by incubating (1 h at 20°C) 50 μl serum with an equal volume of the target pellet. After centrifugation of the mixture at 500*g* (15 min at 4°C), the absorbed sera were used for opsonization and hemagglutination assays.

To check for IL1α epitopes, commercial antibodies raised in rabbit against human recombinant interleukin-1α (IgG fraction of antiserum; anti-*hr*IL1α) were used in sterile PBS (7 mg/ml IgGs). Serum (50 μl) was mixed (v/v) with various dilutions of IgGs (3.5 mg/ml, 1.75 mg/ml, 0.87 mg/ml final concentration), incubated at room temperature for 1 h and overnight at 4°C, and then centrifuged at 27,000*g* (30 min, 4°C).

The opsonizing capacity of the absorbed serum was compared with the activity of a two-fold diluted serum from untreated ascidians.

Specificity of antibody reaction

To check whether non-specific rabbit IgGs cross-reacted with serum proteins, specific antibodies were replaced by the following rabbit IgG preparations: (1) anti-*C. intestinalis* type IX-like collagen (anti-*Ci*IXcollagen) antiserum prepared by Sigma Genosis by using a synthetic peptide as the antigen (Vizzini et al. 2000); (2) anti-fish fucolectin whole rabbit antiserum (anti-*Dl*FL) prepared by Sigma Genosis by using a synthetic peptide of a sequenced fish (*Dicentrarchus labrax*) fucolectin as the antigen (Cammarata et al. 2001); (3) anti-goat IgGs (2.3 mg/ml).

Finally, 100 μl anti-*hr*IL1α IgGs (700 μg) were absorbed overnight at 4°C with 10 μl *hr*IL1α (0.4 μg; Sigma-Aldrich) in PBS and centrifuged at 27,000*g* for 45 min at 4°C. Non-absorbed anti-*hr*IL1α IgG at the same dilution of the absorbed IgG was used as a control in Western blot assays.

In silico sequence analysis

To check the *hr*IL1α epitopes contained in galectins reported in the genome of *C. intestinalis* (JGI v 2.0), a pairwise alignment algorithm (Water program of the EMBOSS package; Rice et al. 2000) was used for comparing the whole length of IL1α (excluding the propeptide) and the galectin deduced amino acid sequences.

Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 15%) was performed according to the method of Laemmli (1970); proteins were stained with Coomassie Blue. Gels were calibrated with molecular mass markers (Sigma-Marker low range 6.5-66 kDa), and the molecular weight was calculated as the mean of five distinct analyses. Before electrophoresis, samples were boiled for 5 min in sample buffer containing 5% β-mercaptoethanol as a reducing agent.

Western blot analysis with anti-hrIL1α

Following SDS-PAGE, the gel was soaked in transfer buffer (20 mM TRIS, 150 mM glycine, pH 8.8) for 10 min, and the proteins were transferred (1 h at 210 mA) to a nitrocellulose sheet in transfer buffer. After being blotted, membranes were blocked overnight with 5% w/v bovine serum albumin (BSA) in PBS containing 0.05% Triton X-100 for 2 h. Blocked membranes were incubated with anti-*hr*IL1α antibodies (1:1,000 in PBS) for 1 h, washed with blocking buffer, and incubated (1 h) with alkaline-phosphatase-conjugated mouse anti-rabbit IgG (1:8,000 in blocking buffer). Blots were washed with PBS and developed with

5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium (BCIP/NBT) according to the manufacturer's instructions. Primary antibodies were omitted in controls.

Protein determination

Quantitative protein analysis was performed according to Bradford (1976), using BSA as a standard. Each value was expressed as the means of three measurements (±SE).

Chemicals and preparation of sterile solutions

Unless otherwise indicated, all chemicals were from Sigma-Aldrich (St Louis, Mo.). Before use, salt solutions and media were filtered with a 0.22-μm filter (Millex GV, Millipore) and autoclaved.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA). Comparisons between different groups were carried out by the Tukey *t*-test. $P<0.05$ was considered as being statistically significant. Standard deviations were calculated based upon 10 experiments.

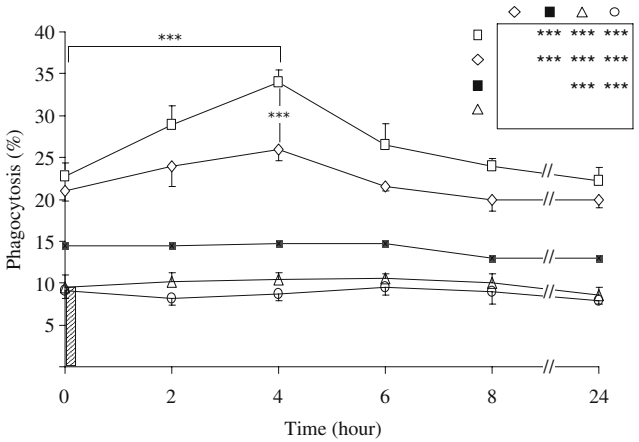

Fig. 1 Time course of opsonizing capacity of *Ciona intestinalis* serum against yeast, after injection of marine solution (MS) or LPS (100 μg/ascidian), compared with the serum activity of untreated ascidians. Hemocytes from untreated ascidians were used in a phagocytosis assay (*Phagocytosis (%)* per cent hemocytes containing ingested yeast, *hatched bar* percent hemocytes from unchallenged ascidians that spontaneously ingested non-opsonized yeast [He], *open squares* sera prepared after LPS [100 μg/ascidian] injection, *open diamonds* sera prepared after MS injection, *filled squares* sera from untreated ascidians held under the same experimental conditions as the injected animals [S], *open triangles* sera from ascidians injected with LPS and opsonins absorbed with rabbit erythrocytes treated with trypsin [try-RE], *open circles* sera from ascidians injected with MS and opsonins absorbed with try-RE). *Inset*: Tukey *t*-test comparison between the various groups. ***$P<0.001$

Results

Inflammatory response following challenge by LPS injection

Within 4–24 h post-injection (p.i.) with LPS, a whitish halo (capsule, 0.5–1.0 cm wide) was visible at the injection site through the transparent tunic. In ten separate experiments on a total of 800 ascidians, an average of 52.6±15% of 800 ascidians showed a capsule after 4–12 hours p.i., whereas tunic tissue injury was observed in 18.5±8% tunicates. Ascidians (200 in total) injected with MS alone presented no visible response throughout the experiments. Variability in the time of the response, reaction intensity, and percent of responders was observed between experiments.

MS or LPS injection promptly enhances serum opsonizing capacity against yeast, and serum HA against try-RE

As shown in Fig. 1, 10% (mean value) of hemocytes from untreated ascidians (He) spontaneously ingested non-opsonized yeast. When the targets were opsonized with serum from untreated ascidians (henceforth indicated as S), a greater number of hemocytes contained engulfed yeast, and the percentage significantly increased by up to 15% ($P<0.05$ vs He). The percentage of hemocytes with ingested yeast reached 21%–23% (significant at $P<0.001$ vs He) when yeast were opsonized with sera prepared from ascidians just after injection of MS (indicated as MS-0h) or LPS (indicated as LPS-0h). Although these last-mentioned results are referred to as being obtained at time 0 in Fig. 1, 5–15 min had elapsed while the animals were bled, and the serum was prepared.

In ten experiments, the response profile of ascidians treated with MS or LPS revealed that the mean serum opsonizing activity was higher at 2 h p.i., reached a maximum at 4 h p.i. (serum indicated as LPS-2h and LPS-4h, respectively, in text) and, within 24 h, decreased to the level registered at the beginning of the response. This last level was maintained until 48 h (data not shown), whereas S showed a lower activity (12%–15%) throughout the duration of the experiments. The inflammatory effect of LPS was revealed by the significantly ($P<0.001$) higher opsonizing capacity of LPS-4h (34%) compared with that of serum prepared at 4 h from ascidians injected with MS (24%; indicated as MS-4h in text; Fig. 1).

The Ca^{2+}-independence of the opsonizing activity was shown by treating yeast with S, MS-4h, or LPS-4h in the presence of 20 mM EDTA or TBS/10 mM $CaCl_2$. In all cases, no differences were observed between samples with or without Ca^{2+} (data not shown). Finally, opsonins were

absorbed by treating MS-4h or LPS-4h with packed try-RE added (v/v) to serum (Fig. 1).

As demonstrated in Fig. 2, the mean HT significantly ($P<0.01$) increased from the value of 2 obtained for serum from untreated ascidians up to the value of 3 found for serum prepared just after LPS or MS injection. Higher values, close to 4 HT ($P<0.001$), were observed for serum prepared at 2 h from MS injection (MS-2h) and for LPS-2h. Finally, the highest titers (close to 5) were found for LPS-4h. High titers (about 4) were maintained until 48 h (data not shown). The serum HA of untreated ascidians did not change throughout the experiment (24 h). Although a wide variability characterized HT values from different experiments, the enhancing effect of LPS was revealed by the significant ($P<0.05$) difference between the HTs observed for the serum from LPS-treated ascidians and those found for the serum from MS-treated ascidians.

Absorption of S, MS-4h, and LPS-4h by yeast significantly ($P<0.001$) decreased the HA (Fig. 2), whereas the presence of 20 mM EDTA or 10 mM $CaCl_2$ did not affect the HTs (data not shown).

D-galactose and D-galactosides inhibit serum opsonizing activity and HA

The opsonic capacity of LPS-4h was inhibited after treatments with 50.0 mM D-galactose, 50.0 mM α-lactose, 50.0 mM D-mannose, 25.0 mM N-Lac-NAc, and 17.5 mM TDG, whereas D-glucose and L-fucose at 100 mM did not exert any inhibitory effect (Table 1). To ascertain whether the decreased serum opsonizing capacity could be attributed to an effect of the sugars on targets, yeast were incubated (30 min, 20°C) with 100 mM sugar in f-MS before opsonizing treatment. None of the tested sugars affected the opsonization.

The HA of LPS-4h was abolished by 22.5 mM D-galactose, 10.5 mM α-lactose, 9.4 mM LacNAc, and 0.7 mM TDG, whereas it was not affected by 100 mM D-mannose, D-glucose, or L-fucose (Table 1). The same sugar concentrations inhibited any opsonizing activity and HA of S and MS-4h.

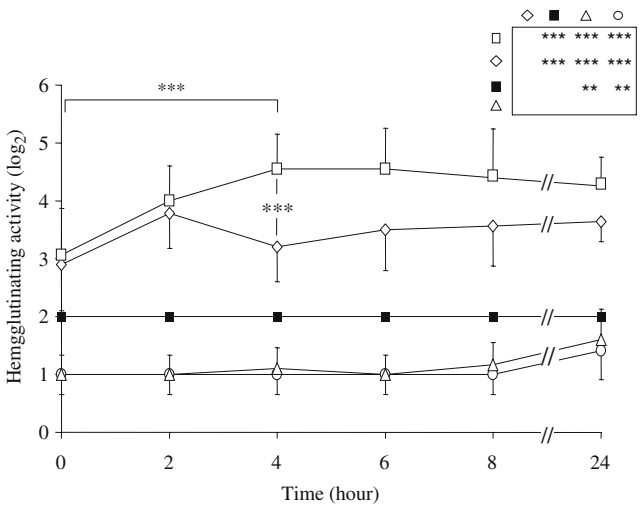

Fig. 2 Time course of hemagglutinating activity (HA) of *C. intestinalis* serum against try-RE after injection of MS or LPS (100 μg/ascidian) compared with the activity of serum from untreated ascidians *open squares* sera prepared after LPS [100 μg/ascidian] injection, *open diamonds* sera prepared after MS injection, *filled squares* sera from untreated ascidians held under the same experimental conditions as the injected animals, *open triangles* sera from ascidians injected with LPS and opsonins absorbed with try-RE, *open circles* sera from ascidians injected with MS and opsonins absorbed with try-RE. *Inset*: Tukey *t*-test comparison between the various groups. ***$P<0.001$, **$P<0.01$

Table 1 Sugar inhibition of *Ciona intestinalis* serum opsonizing and hemagglutinating activities (*HA* hemagglutinating activity, *Gal* D-galactose, *Lac* α-lactose, *Man* D-mannose, *Glu* D-glucose, *Fuc* L-fucose, *LacNac* N-acetyllactosamine, *TDG* thiodigalactoside, *N.I.* no inhibition)

Sugar	Phagocytosis LPS-4h[a]	HA
Gal	50.0±3.1	22.5±4.3
Lac	50.0±1.2	10.5±2.8
Man	50.0±2.5	N.I.
Glu	N.I.	N.I.
Fuc	N.I.	N.I.
LacNAc	25.0±1.5	9.4±4.4
TDG	17.5±2.7	0.7±0.2

[a] LPS-4h (serum at 4 h from LPS injection): the lowest sugar concentration (mM) that abolished the HA of the serum against rabbit erythrocytes or giving significant inhibitory activity of yeast opsonization was recorded (starting sugar concentration: 100 mM)

Anti-hrIL1α polyclonal antibodies abolish serum opsonizing activity and decrease HA

Antibodies (3.5 mg/ml total rabbit IgGs) abolished serum opsonizing activity and significantly decreased HA. The LPS-4h and MS-4h opsonizing capacity dropped down to the He level (Fig. 3), and this effect was dose-dependent (Fig. 4). Inhibition was not an effect of anti-*h*rIL1α antibodies on the yeast suspension because, after pre-treatment with anti-*h*rIL1α IgGs, the yeast suspension showed no clumps (microscopic observations), and the same yeast could be opsonized later (data not shown). Likewise, the antibody treatment significantly decreased the HA of MS-4h and LPS-4h (Fig. 3). Rabbit immune serum toward *Ci*IX collagen or fish fucolectin (*Dl*FL) and rabbit IgGs did not inhibit the opsonizing activity and HA (Fig. 4). In addition, both activities were maintained by treating LPS-4h with anti-*h*rIL1α antibody preparation absorbed with *h*rIL1.

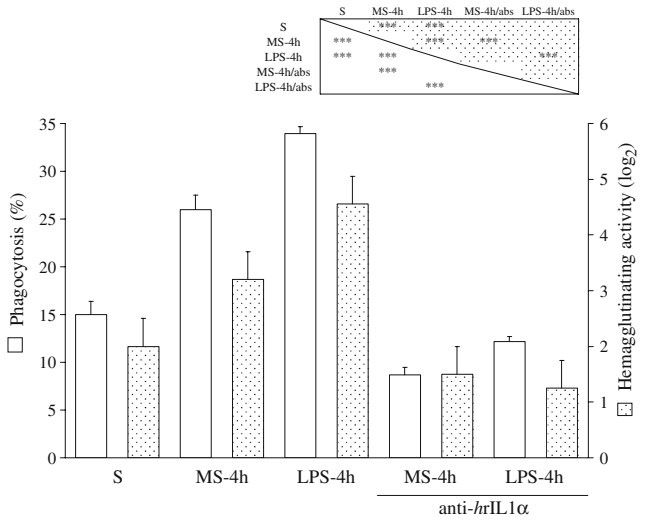

Fig. 3 Inhibitory effect of anti-human recombinant IL1α antibodies (*anti-hIL1α* 3.5 mg/ml total rabbit IgG preparation) on HA and opsonizing activity of *C. intestinalis* serum at 4 h from an injection of MS or LPS (100 μg/ascidian) compared with the activities of serum from untreated and treated ascidians (*S* serum from untreated ascidians, *MS-4h* serum from ascidians treated with MS, *LPS-4h* serum from ascidians treated with LPS, *MS-4h/anti-hrI-Lα*, *LPS-4h/anti-hrI-Lα* serum from MS or LPS treated ascidians absorbed with the antibodies, *Phagocytosis (%)* per cent hemocytes containing ingested yeast). ***$P<0.001$

SDS-PAGE and immunoblotting analyses reveal inducible serum proteins provided with IL1α epitopes at 4 h after LPS injection

Sera from LPS-treated ascidians, examined by SDS-PAGE, revealed several protein components scarcely visible in the serum pattern of untreated ascidians (Fig. 5a). Although all samples were adjusted to a 4 μg protein content, the LPS-4h pattern presented several proteins (59.2±0.3, 37.1±0.9, 30.5±0.7, 27.4±0.5, 23.3±0.3, 20.1±0.4, 15.1±0.6, 8.7±0.4 kDa; n=5) characterized by a differently increased band density as compared with the S and MS-4h (bands scarcely visible), LPS-2h (low band density), and LPS-8h (density significantly decreased) patterns (Fig. 5b). For LPS-4h, the 37.1-kDa and 15.1-kDa bands were mainly enhanced, and minor bands (59.2±0.3 and 43.0±0.1 kDa) were barely visible.

In immunoblotting analysis, some serum proteins that formed the SDS-PAGE pattern of LPS-4h (59.2, 37.1, 30.5, 23.3, 15.1 kDa) cross-reacted with anti-*h*rIL1α antibodies (Fig. 5c). The density of these bands appeared to be visibly augmented compared with those at LPS-2h, LPS-8h, and LPS-12h; among them, the 37.1-kDa and 15.1-kDa bands presented the highest density. For the MS-4h serum, only

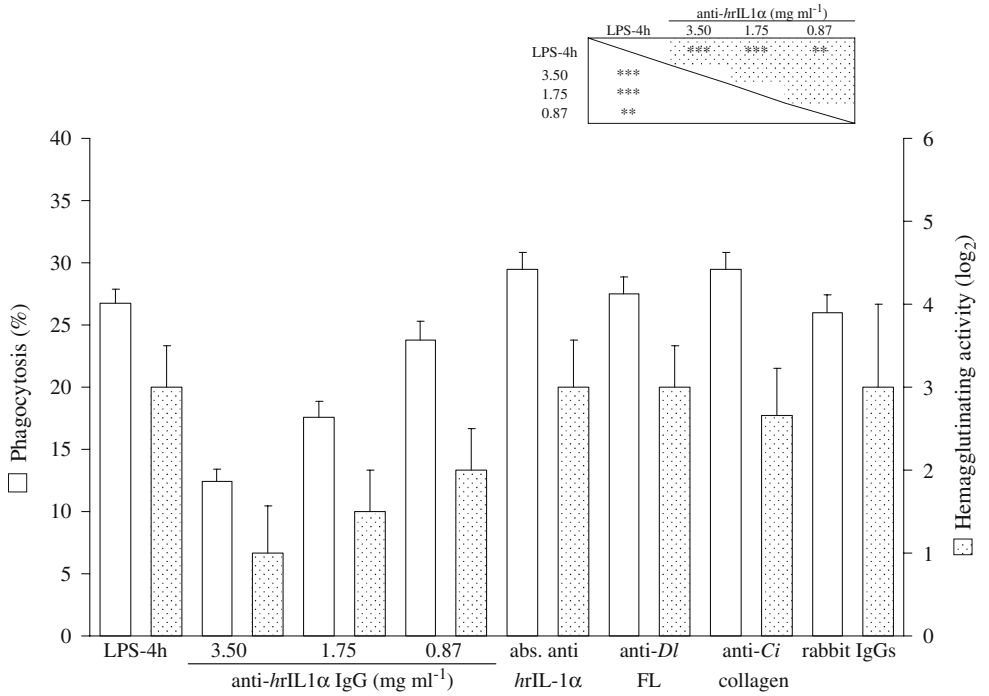

Fig. 4 Specificity of the anti-human recombinant IL1α antibodies (*anti-hIL1α* 3.5 mg/ml total rabbit IgG preparation) reaction with *C. intestinalis* serum opsonins/hemagglutinins enhanced by LPS (100 μg/ascidian) compared with the effect of unspecific antibodies on HA and opsonizing activity of serum at 4 h from an injection of LPS (*LPS-4h* serum at 4 h from LPS injection, *anti-hIL1α IgG* 3.5 mg ml^{-1} and two-fold dilutions [1.75, 0.87] of rabbit anti-*h*rIL1α IgGs, *abs. anti-hrIL1α* antibodies absorbed with specific antigen *h*rIL1α, *anti-DlFL* rabbit antiserum against a fucolectin from *Dicentrarchus labrax*, *anti-CiIX collagen* rabbit antiserum against *C. intestinalis* type IX-like collagen, *rabbit IgGs* IgGs from rabbit). *Inset* Significant differences (**$P<0.05$, ***$P<0.001$) between the activities of LPS-4h serum treated with the highest antibody concentration and decreasing antibody dilutions

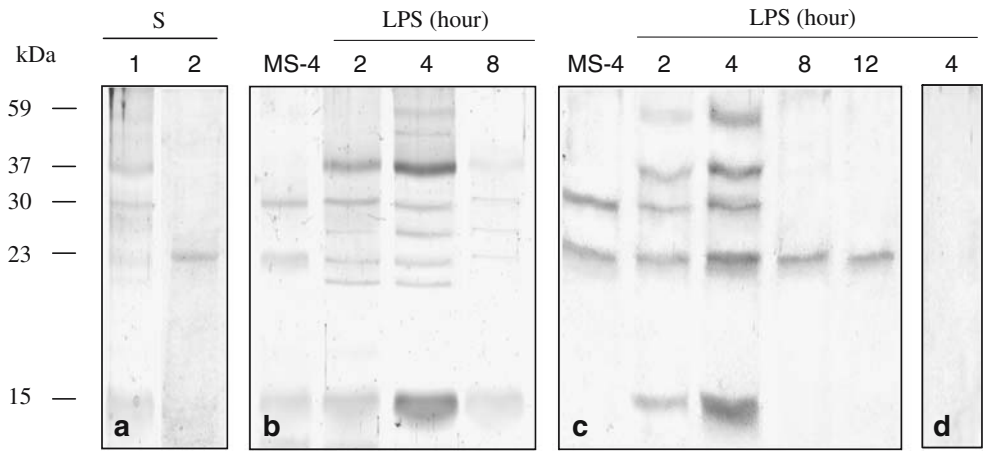

Fig. 5 SDS-PAGE (15%, under reducing conditions) and Western blotting analyses with anti-*h*rIL1α antibodies of serum proteins in *C. intestinalis* hemolymph at various times (*hour*) from LPS injection compared with serum from untreated or MS-treated ascidians. (*S* serum from untreated ascadians, *MS-4* 4 h from MS injection, *LPS* (*hour*) serum samples at 2, 4, 8, and 12 h after LPS injection. **a** SDS-PAGE and Western blot analyses of serum from ascidians. **b** SDS-PAGE of serum from treated ascidians. **c** Western blot analysis of serum from MS- and LPS-treated ascidians. **d** Western blot analysis of ascidian serum at 4 h from LPS injection, with non-specific rabbit IgGs or anti-hrIL1α antibodies absorbed with hrIL1α. Gels calibrated with Sigma-Marker low range 6.5-66 kDa

23.3-kDa and 30.5-kDa bands were clearly visible (Fig. 5c), whereas for the serum from untreated ascidians, only the 23.3-kDa band was observed (Fig. 5a). To check for the specificity of the antibody reaction, a control immunoblot performed with rabbit immune antisera against fish fucolectin (*Dl*FL), *Ci*IX collagen, rabbit IgGs, and anti-*h*rIL1α IgGs absorbed with *h*rIL1α revealed no reaction with serum components of LPS-4h (Fig. 5d).

Two traits of deduced hIL1α amino acid sequences show similarity with galectin CRDs

Whole-length alignment of the deduced human IL1α and *C. intestinalis* galectin amino acid sequences (propeptide excluded) showed similarity of 62.5% between the IL1α 110-117 trait and 39–46 trait of the fgenesh3_pg. C_scaffold_7960000001 (galectin ID 235601, CRDI; Table 2). A further similarity (66.7%) was observed between IL1α 213–223 trait of the motif 2 and 204–214 trait of the fgenesh3_pg. c_chr_06q000046 (galectin ID 216812, CRDII).

Discussion

In ascidians, cytokine-like molecules have been discovered by functional cell proliferation and opsonization studies, but the cross-reactivity of anti-mammal IL1 antibodies with ascidian hemolymph humoral components (Beck et al. 1989) remains unclear. Recent reports of a genome-wide analysis of *C. intestinalis* have provided a comprehensive picture of immune-related genes (Dehal et al. 2002; Shida et al. 2003; Azumi et al. 2003; Terajima et al. 2003) and shown that IL1 genes are not contained in the genome, whereas the recorded C-type lectin and galectin genes can be expressed by hemocytes from untreated ascidians (Shida et al. 2003; Azumi et al. 2003).

Table 2 Pairwise best-region alignment of deduced amino acid sequence between human interleukin 1α mature peptide and putative galectin genes from the genome of *C. intestinalis* (*CRD* carbohydrate recognition domain, *Motif 2* conserved element from IL1α sequence [PRINTS-S database accession no. PR00264]) obtained by using the Water program of the EMBOSS package [human interleukin 1α (GenBank accession no. NP-000566), *C. intestinalis* genome (DOE Joint Genome Institute version 2.0) assigned name: galectin ID 216812 (fgenesh3_pg.c_chr_06Q000046); galectin ID235601 (fgenesh3_pg.c _scaffold_7960000001)]

Sequence name	Alignment			Similarity/identity (%)	Region
Interleukin 1α	110	kprsapfs	117		
		||.|..||		62.5/62.5	
Galectin (235601)	39	KPSSEGFS	46		CRD I
Interleukin 1α	213	titgsetnllf	223		Motif 2
		||.|..|...|		54.5/45.5	
Galectin (216812)	204	TIIGRATAHMF	214		CRD II

The inflammatory reaction in the tunic of *C. intestinalis* (Parrinello 1981; Parrinello et al. 1984a, b, 1990) is a suitable model for the examination of inducible humoral cytokine-like factors. We have therefore stimulated a response to LPS in this ascidian that induces cytokine expression and release in mammals. In accordance with previous results (Parrinello 1981; Parrinello et al. 1984a, b), MS injected into the tunic does not cause any visible inflammatory reaction, whereas within few hours (4–24 h), LPS induces the formation of a visible capsule in a large percentage of the treated ascidians, and, less significantly, tissue injury that represents a more intense response that masks the capsule. The variability in time and intensity of the response might be related to the different ascidian batches gathered at various times during the course of our research.

According to Parrinello et al. (1990), the early inflammatory response of *C. intestinalis* recruits phagocyte populations into the inflamed tissue (Parrinello et al. 1984a, b); consequently, mitogenic, chemotactic, and opsonic factors are probably responsible for cell proliferation in the hemopoietic nodules under the tunic, for hemocyte recruitment, and for the stimulation of inflammatory responses, including enhanced phagocytosis and encapsulation. Pinto et al. (2003) have reported that, following intratunic LPS injection in this ascidian, chemotactic activity is exerted by a "C3a-like" peptide expressed by tunic hemocytes following the activation of a complement-like pathway.

The serum hemolymph from untreated *C. intestinalis* displays a naturally occurring opsonizing capacity with respect to yeast, which, even after repeated washes, become more susceptible to in vitro phagocytosis by hemocytes from untreated ascidians. Immediately after the injection of MS or 100 μg LPS into the tunic, a prompt humoral inflammatory response occurs, as revealed by the increased Ca^{2+}-independent serum opsonizing activity and HA. The opsonizing activity reaches its highest levels within 2–4 h p.i. and then decreases within 12 h, although a high level of activity is maintained until the end of the experiment (24 h). A similar enhanced response characterizes the serum HA against RE. The low titer of Ca^{2+}-independent naturally occurring hemagglutinins increases significantly just after MS or LPS injection, reaching their highest values within 4 h. Following this early response, the HA does not significantly decrease, and high values are maintained until the end of the experiment, suggesting multiple lectin roles that may include wound repair. In this respect, the inflammatory effect of MS injection could be attributable to the wound stimulus in the tunic matrix, although the induction could also be a result of bacteria inadvertently being introduced into the tunic during injections.

Apparently, a timely (within a few minutes) lectin/opsonin response is stimulated by the trauma caused by injecting a medium into the tunic, whereas a further significant inflammatory effect of LPS is manifest (within 4 h), supporting the possibility that serum hemagglutinins with opsonic properties are upregulated by this inflammatory agent. At 4 h p.i., the opsonizing capacity is significantly higher in the serum from LPS-treated ascidians, and at that time, the hemagglutinating response is enhanced ($P<0.05$). Both hemagglutinin and opsonin display a binding capacity to the same target, as shown by absorbing the serum by erythrocytes or yeast.

The galectin nature of inducible lectins is established by considering both Ca^{2+}-independent activities and D-galactose-binding and D-galactoside-binding (α-lactose, LacNAc, TDG) specificity. According to Drickamer and Taylor (1993), Ca^{2+}-independent galectins are characterized by D-galactoside-specific binding, which is primarily exerted by LacNAc and TDG, and less so by Lac (Vasta et al. 2004; Cooper and Barondes 1999).

A controversial result was obtained with D-mannose, which inhibited the galectin opsonic activity without affecting the HA. With regards to this, galectins could occur as oligomers presenting multiple CRDs characterized by different sugar specificities (Dodd and Drickamer 2001). CRD variants could be contained in the galectin repertoire that, like mammalian galectin 10 (Swaminathan et al. 1999), might interact with mannose rather than galactose. Thus, the possibility exists that, in addition to galectin opsonizing activity, a mannose receptor is expressed on *C. intestinalis* phagocytes.

Since antibodies raised against mammalian IL1 were used to identify "IL1-like" invertebrate molecules, the sera showing the highest opsonizing and HA were assayed after treatment with anti-human recombinant IL1α polyclonal antibodies. Specific antibodies abolished the opsonizing activity of S, MS-4h, and LPS-4h and significantly diminished the HA. The specificity of the antibody reaction was ascertained by substituting the specific antibodies with non-specific rabbit IgGs or antisera or with anti-*h*rIL1α IgGs absorbed with the specific antigen *h*rIL1α. The serum opsonizing capacity never decreased after treatment with non-specific rabbit IgGs or anti-*h*rIL1α IgGs absorbed with *h*rIL1α, whereas a residual HA could be observed by naturally occurring non-inducible galactose-specific lectins, which did not share IL1α epitopes.

An alignment comparison between deduced whole-length *h*IL1α (excluding propeptide) and galectin amino acid sequences reported for the *C. intestinalis* genome was not adequate to identify shared epitopes. However two sequence traits (110–117 and 213–223) of deduced IL1α amino acid sequence fitted into CRDs of two distinct *C. intestinalis* galectins: galectin 235601 CRDI (62.5% similarity) and galectin 216812 CRDII (54.5 % similarity), respectively. Although very short sequence traits were found,

the possibility exists that they contribute to galectin steric configuration, thereby exposing epitopes presumably related to CRDs. Furthermore, IL1α is known to recognize oligosaccharide structures by a Ca^{2+}-independent mechanism (Cebo et al. 2001a, b) suggesting a functional homology with galectin CRDs.

As shown by SDS-PAGE and immunoblotting analyses, several serum proteins appear to be modulated in their expression by the LPS-induced inflammatory response. Among them, those at 59, 37, 30, 23, and 15 kDa (mean values, omitting SD) have mainly been identified with the anti-*h*rIL1α antiserum, as in the LPS-4h Western blot pattern.

The 59-kDa, 37-kDa, and 15-kDa cross-reacting proteins appeared to be mostly upregulated by the inflammatory response as indicated by their increased density at various times; density was low at 2 h (LPS-2h), higher at 4 h (LPS-4h), and not detected in the sera at 8 h and 12 h p.i. The increase in density of the 37-kDa and 15-kDa proteins was also visible in the LPS-4h SDS-PAGE pattern. In immunoblots, the 23-kDa band was the only band always visible (MS-4h, LPS-2 to LPS–12h, and S). These results suggest that proteins presenting IL1α epitopes increase their concentration in the serum just after an LPS challenge (within 2 h), peak at 4 h, and are promptly involved in the inflammatory response as indicated by the decreased density of their bands at 8 h and 12 h. The electrophoretic pattern of the sera prepared after MS injection (MS-4h) revealed 30-kDa, 23-kDa, and 15-kDa bands at low density, whereas the smallest band was not visible by immunoblotting analysis. Since serum proteins of the 6.5-66 size range were examined by SDS-PAGE, no strict relationship between the equal total protein content of the various samples and the upregulation of serum components can be drawn. Finally, the ladders on SDS-PAGE and Western blot analysis suggested that enhanced lectins were assembled into functional highly oligomeric structure. A similar oligomerization process has been reported for the humoral opsonins of the ascidian *Pyura stolonifera* (Pearce et al. 2001) and a salmon serum lectin (Stratton et al. 2004).

Although we have not been able to identify the lectins with opsonic properties, our results indicate that the injection procedure can induce an inflammatory response by a release of serum proteins that could be related to the enhanced activity of D-galactoside-specific lectins with opsonin activity. In addition, a further inflammatory effect is exerted by LPS that also increases proteins presenting IL1α epitopes presumably related to an oligomerization process.

In general terms, the evidence reported here is in accord with known mammalian cytokine properties of galectins: (1) galectins are secreted/released from cells when immune responses are triggered; (2) once released, galectins can modulate immune responses by cross-linking galectin ligands on immune cells and targets. In addition, *C. intestinalis* galectins probably display pleiotropy and redundancy, acting as inflammatory cytokines.

In conclusion, *C. intestinalis* galectins having the activity of cytokines may be responsible for a functionally conserved primitive recognition mechanism based on CDMs contributing to innate defense and inflammatory reactions in invertebrate and vertebrate immune-evolution. Research is in progress to isolate, sequence, and characterize further these inducible *C. intestinalis* inflammatory galectins.

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