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Short communication

Secretion of a collectin-like protein in tunicates is enhanced during inflammatory responses

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Abstract

The sub-cellular and humoral concentrations of a collectin-like protein from the solitary tunicate, *Styela plicata*, were measured after in vivo challenge with the inflammatory agent, zymosan. Tunicates were injected with zymosan before hemocytes and serum were harvested, subjected to western blotting and immunostained with an anti-*S. plicata* collectin antibody to determine the relative titers of collectin-like proteins. Concentrations of the predominant 43 kDa collectin polypeptide were found to decrease in hemocytes immediately after zymosan injection, before rising to levels that were six times higher than controls within 96 h. Similarly, immunohistochemistry showed that the frequency of collectin-positive hemocytes in the circulating hemolymph increased significantly within 96 h of injection. Levels of the 43 kDa polypeptide in serum mirrored those of hemocytes. Humoral collectin concentrations decreased immediately after zymosan injection before rising, within 96 h post-injection, to levels three times higher than controls. This response to an inflammatory stimulus resembles that of mammalian collectins like mannose-binding lectin. The data suggest that, like its mammalian counterparts, the tunicate collectin acts as an acute phase antigen recognition protein.

Keywords: Tunicate; Ascidian; Mannose-binding lectin; Innate immunity; *Styela plicata*; Collectin

1. Introduction

It is now apparent that the vertebrate immune system relies on a variety of recognition molecules in addition to antibodies. One of these alternative recognition molecules is mannose binding lectin (MBL)—an acute phase protein that recognizes patterns of carbohydrates on the surface of non-host cells [1,2]. MBL is a member of a sub-family of C-type lectins called collectins, which are characterized by the inclusion of four discreet domains [2–4]. These include a calcium dependent, C-type carbohydrate recognition domain (CRD), a coiled neck region joining the CRD to a large collagen domain, and a short amino terminal tail region [3,5]. The quaternary structure of native MBL has been said to resemble a 'bunch of tulips' in which oligomers are constructed

Abbreviations: BSA, bovine serum albumin; CRD, carbohydrate recognition domain; FSW, filtered seawater; MBL, mannose binding lectin; MASP, mannose binding lectin-associated serine protease; MAC, marine anticoagulant buffer; galNAc, *N*-acetyl-D-galactosamine; PBS, phosphate buffered saline; NBT/BCIP, nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate; p.i., post-injection; TBS, Tris buffered saline.

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from up to six homotrimers of collectin polypeptides. The final oligomeric structure of MBL aids its recognitive function by affording greater affinity and specificity for carbohydrate ligands [3,6]. Carbohydrate binding by MBL directly induces the phagocytosis of opsonized cells, and can also initiate a lectin-mediated pathway of complement activation in conjunction with MBL-associated serine proteases (MASPs) [8,9].

One of the most important roles for human MBL occurs neonatally, in the period between birth and the development of a competent antibody repertoire [7]. At this time, some authors suggest that MBL represents the predominant recognition molecule of the immune system, a proposition supported by the susceptibility of individuals carrying mutant MBL alleles to infection [7]. In later life, MBL expression can be greatly enhanced in the acute phase of infection prior to the proliferation of antibody-based antigen recognition systems [3].

The early evolution of collectins may help to explain why proteins like MBL can initiate immune effector activities in mammals that are independent of antibodies. Recently, we have identified a collectin-like protein in tunicates—invertebrate chordates that are closely related to vertebrates [10,11]. The tunicate collectin functions as an opsonin and appears to have the same domain structure as mammalian MBL. It forms large MBL-like oligomers based on homotrimeric sub-units. Serine proteases homologous to vertebrate MASPs and homologues of the central complement component, C3, are also evident in tunicates, suggesting that these animals, like mammals, express a collectin-mediated complement activation pathway [11–16].

In this study, we use an antibody directed against the tail region of the *Styela plicata* collectin to test the effects of antigenic challenge on the relative concentrations of protein. We show that the titer of collectin in both *S. plicata* serum and hemocytes is significantly enhanced in vivo by injecting a common inflammatory agent—zymosan. The data indicate that, like its mammalian counterparts, the tunicate collectin contributes to rapidly induced host defense reactions akin to acute phase responses.

2. Materials and methods

2.1. Anti-collectin antibody

A 20 amino acid synthetic peptide to the N-terminal sequence of the tunicate collectin (SNELMVCRRADSTNTEVIIG [10]) was synthesized, conjugated to keyhole limpet hemocyanin as a carrier and used to immunize New Zealand White rabbits by Bethyl Laboratories (Michigan, USA). Immunized rabbits were exanguinated and anti-collectin polyclonal antibodies were affinity purified from serum using the immunizing peptide conjugated to CNBr-activated Sepharose 4B.

2.2. Tunicates and hemolymph collection

S. plicata were collected from boat moorings in Sydney Harbor with the permission of the New South Wales Fisheries Service (Marine Research Permit # 95067). The tunicates were kept in 40L aerated tanks (15 °C) and fed every second day with Marine IVF Invertebrate Diet (New Wave).

Serum was obtained by harvesting hemolymph on ice from incisions in the buccal siphon. The hemolymph was immediately centrifuged (2000 × *g*, 7 min, 4 °C) before the supernatant (serum) was decanted and filtered (0.45 μm, Costar, Pleasanton, CA).

To collect hemocytes, hemolymph was harvested into a threefold excess of ice-cold marine anti-coagulant buffer (MAC; 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 10 mM EDTA, 0.45 M sodium chloride, pH 7.0) and centrifuged (12,000 × *g*, 5 s). The supernatants were removed and the hemocytes re-suspended in either SDS–PAGE sample buffer (5×10^6 cells/ml) or filtered seawater (FSW; 0.45 μm filter, Costar, Pleasanton, CA).

2.3. Antigen injection

Tunicates were weighed to determine correct titer of antigen (zymosan A, Sigma-Aldrich, Sydney, NSW) for injection. Zymosan doses were based on previous calculations which indicated that hemolymph constitutes approximately 20% of the wet body weight of an individual (unpublished data). Injections were performed with the aid of a dissecting

microscope. A sterile scalpel blade was used to shave away a section of tunic to reveal the underlying mantle surface. A 1 ml syringe fitted with a $26\frac{1}{2}$ G needle was then used to inject, immediately under the surface of the mantle, either FSW (controls) or sufficient zymosan (prepared in FSW) to yield a final concentration in hemolymph of 20 μg/ml. The needle was kept in place for 5 min to allow the wound to seal by muscle contraction.

Hemolymph was extracted from tunicates 24, 48, 96 and 192 h after injection for analysis by western blotting or immunohistochemistry.

2.4. Western blotting

SDS–PAGE was performed according to the method of Laemmli [17]. Gels incorporated a lower resolving layer of 10% acrylamide and an upper 4% stacking gel. Molecular mass markers (wide range, 6.5–205 kDa; Sigma-Aldrich) were used to determine the relative molecular masses of unknown proteins. Prior to electrophoresis, samples (serum or 5×10^6 hemocytes/ml) were boiled for 5 min in sample buffer containing 10 mg/ml dithiothreitol as a reducing agent.

Electrophoresed proteins were blotted onto nitrocellulose membranes (0.2 μm; Trans-Blot transfer medium; Bio-Rad, Regents Park, NSW) using the semi-dry method of Kyhse-Andersen [18]. Proteins were blotted at 12 V for 45 min. After blotting, membranes were blocked with 5% w/v bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline (TBS; 0.15 M NaCl, 0.03 M Tris-HCl, pH 7.5) for 2 h. Blocked membranes were incubated overnight with anti-collectin antibody (1:1000 in TBS), and then with alkaline phosphatase-conjugated anti-rabbit IgG (1:10,000 in TBS; Sigma-Aldrich) for 2 h. Blots were developed with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche Diagnostics, Castle Hill, NSW) according to the manufacturer's instructions.

The relative intensities of different bands were determined from digital images of blots using Scion Image software (US National Institutes of Health, Bethesda, MA). The intensity of staining in all blots was standardized by including known concentrations of purified collectin. Anti-collectin-positive bands in these purified collectin preparations were arbitrarily allocated a relative intensity of 1.0. Collectin was purified from whole hemolymph (with hemocytes) containing 2 % v/v Triton X-100 by *N*-acetyl-D-galactosamine (galNAc) affinity chromatography according to the method of Nair et al. [10].

2.5. Immunohistochemistry

Hemocytes (30 μl, 1×10^6 cells/ml in FSW) were loaded into a cytocentrifuge (Shandon, NSW) and centrifuged at room temperature for 5 min ($200 \times g$). Cytocentrifuge preparations were then removed from the sample chambers and allowed to air dry before being fixed with 100% v/v methanol at 4 °C for 20 min. All subsequent incubations were carried out in a humidified chamber at room temperature, and slides were washed for 5 min with phosphate buffered saline (PBS; 130 mM sodium chloride, 2 mM potassium chloride, 10 mM sodium hydrogen orthophosphate, 1.7 mM potassium dihydrogen orthophosphate, pH 7.2) between each step. Cytocentrifuged hemocytes were first blocked for 20 min with PBS containing 5% v/v BSA (PBS–BSA). PBS–BSA was removed before the cells were incubated with anti-collectin (1:100 in PBS) for 30 min. Slides were then incubated for 30 min with alkaline phosphatase-conjugated anti-rabbit IgG (1:200 in PBS; Sigma-Aldrich), before being stained with NBT/BCIP.

Bright field microscopy was used to determine the frequency of positively immunostained hemocytes among 100 randomly selected cells per slide. Negative controls (no primary antibody or normal sheep Ig as primary antibody) were included in all experiments to confirm the specificity of anti-collectin immunostaining.

2.6. Statistical analysis

Statistical analysis was performed with the Microsoft Excel software package (Microsoft Corporation, Mountain View, CA). The statistical significance of differences, as determined by Student's two-tailed *t*-test, were considered to be significant for probabilities (p) of less than 5.0%.

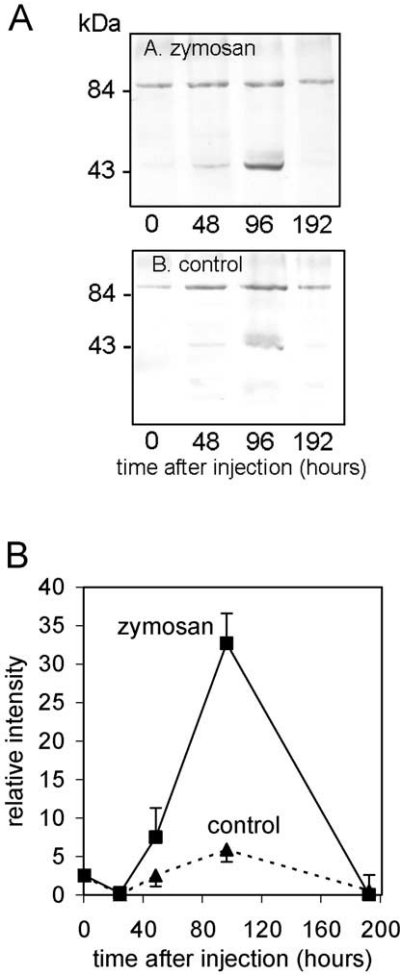

Fig. 1. Effects of zymosan injection on the titers of anti-collectin reactive polypeptides in *S. plicata* hemocytes. (A) Western blots of *S. plicata* hemocytes harvested from tunicates at various times after the injection of zymosan or FSW (controls). The position of molecular mass markers (kDa) is shown on the left. (B) Relative intensities of the 43 kDa collectin polypeptide over time in hemocytes from tunicates that had been injected with zymosan or FSW (controls). Bars = standard error, $n \geq 3$.

3. Results

Fig. 1A shows that the anti-collectin antibody could identify two discrete polypeptides (43 and 90 kDa) in western blots of *S. plicata* hemocytes under reducing conditions. The 43 kDa polypeptide in hemocytes corresponded precisely in terms of molecular mass to the predominant form of collectin present in serum [10] (Fig. 3A).

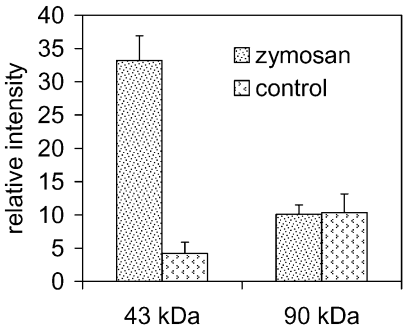

Fig. 2. Relative intensities of the 43 and 90 kDa anti-collectin reactive polypeptides in hemocytes harvested from tunicates 96 h after the injection of zymosan or FSW (controls). Bars = standard error, $n \geq 3$.

Densitometric analysis of anti-collectin western blots revealed that the mean relative intensity of the 43 kDa polypeptide in hemocytes decreased slightly, but still significantly ($p < 0.05$ vs. 0 h time point), during the first 24 h after zymosan challenge (Fig. 1B). The titer of the 43 kDa band then rose within 96 h post-injection (p.i.) to a level that was 15 times greater than that evident in hemocytes from non-injected tunicates (0 h time point), before returning to an intensity comparable to that of non-injected animals within 192 h p.i. ($p > 0.05$ vs. 0 h time point).

The injection of FSW, as opposed to zymosan, also significantly increased the intensity of the 43 kDa band in hemocytes harvested after 96 h p.i. ($p < 0.05$ vs. 0 h time point). However, this increase was not of the same magnitude as that following zymosan injection, so that the intensity of the 43 kDa polypeptide in zymosan-injected animals was still sixfold greater than that of FSW-injected animals after 96 h p.i. ($p < 0.05$).

Even though zymosan injection significantly increased the levels of the 43 kDa polypeptide in hemocytes, it had no effect, when compared to FSW-injected controls, on the relative intensity of the 90 kDa polypeptide that was identified by the anti-collectin antibody. Fig. 2 shows that the intensity of the 90 kDa band did not vary between hemocytes harvested from zymosan- and FSW-injected tunicates at 96 h p.i. ($p > 0.05$), even though zymosan injection significantly increased the intensity of the 43 kDa band at the same time point.

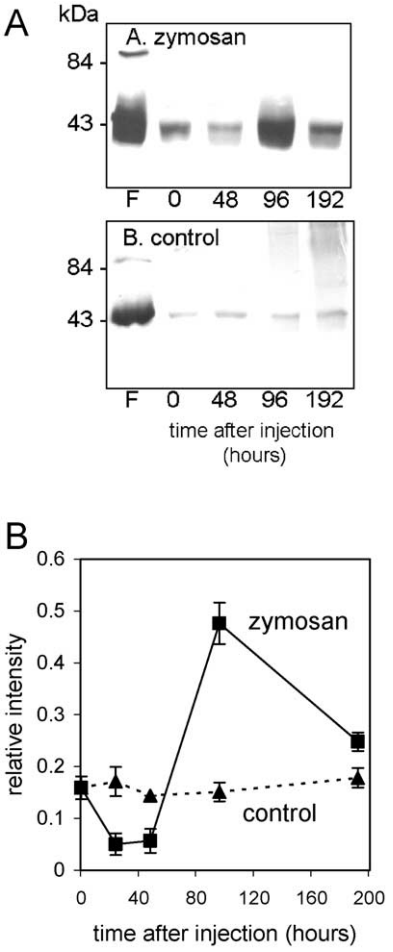

Fig. 3. Effects of zymosan injection on the titers of anti-collectin reactive polypeptides in serum from *S. plicata*. (A) Western blots of serum harvested from tunicates at various times after the injection of zymosan or FSW (controls). Lane F is a carbohydrate affinity-purified collectin fraction from whole hemolymph. The position of molecular mass markers (kDa) is shown on the left. (B) Relative intensities of the 43 kDa collectin polypeptide over time in serum from tunicates that had been injected with zymosan or FSW (controls). Bars = standard error, $n \geq 3$.

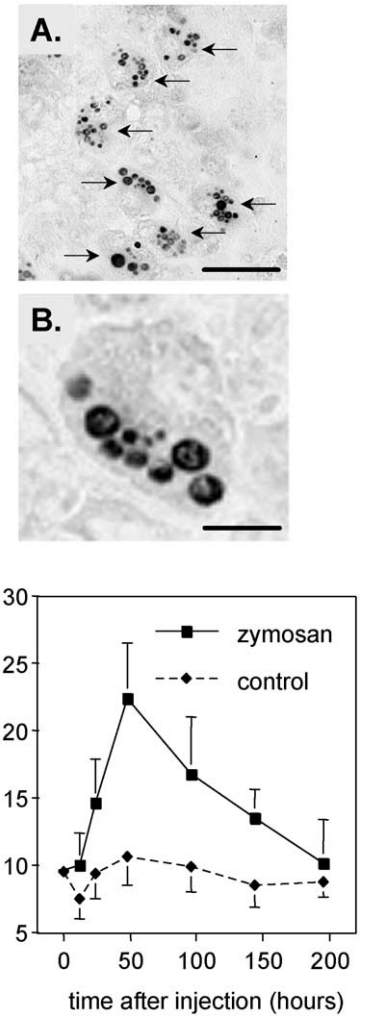

Fig. 4. Effects of zymosan injection on the relative frequency of anti-collectin-positive hemocytes in hemolymph. (A) Low power, bright field micrograph of hemocytes from a zymosan-injected tunicate that had been immunohistochemically stained with anti-collectin. Arrows identify collectin-positive hemocytes. Bar = 30 μm. (B) High power, bright field micrograph of a hemocyte from a zymosan-injected tunicate showing that anti-collectin immunostaining is restricted to numerous large sub-cellular vesicles. Bar = 10 μm. (C) Relative frequencies of anti-collectin positive hemocytes in hemolymph harvested from tunicates at various times after the injection of zymosan or FSW (controls). Bars = standard error, $n = 3$.

Western blotting of serum with anti-collectin identified only the 43 kDa polypeptide in samples harvested from tunicates injected with either FSW or zymosan (Fig. 3). The relative intensity of this collectin polypeptide from zymosan challenged animals decreased significantly ($p < 0.05$), when compared to both non-injected tunicates (0 h time point) and FSW-injected controls, within the first 48 h p.i. However, after 96 h p.i., the collectin polypeptide was three times more intense in the zymosan challenged animals than in FSW-injected controls ($p < 0.05$). Even though the level of collectin in serum from zymosan-injected tunicates began to

decrease after 96 h p.i., it remained significantly greater ($p < 0.05$) than those of FSW-injected controls and non-injected animals (0 h time point) when measured at the final, 192 h p.i. time point.

Immunohistochemistry revealed that zymosan injection increased the frequency of anti-collectin-positive hemocytes in *S. plicata* hemolymph. Figs. 4A and B show that anti-collectin immunostaining was restricted to large vesicles within a restricted sub-set of hemocytes. In contrast, negative controls prepared either with irrelevant primary antibodies (normal rabbit Ig) or secondary antibody alone (anti-Ig-AP) remained unstained (data not shown). The collectin-positive hemocytes were classified as refractile vacuolated cells according the hemocyte classification scheme of Radford et al. [19].

In non-injected tunicates, collectin-positive hemocytes comprised approximately 9% of the hemocyte population (Fig. 4C). Within 48 h of injecting zymosan, this frequency had increased to approximately 22%, before returning to levels that were not significantly different ($p > 0.05$) from those of non-injected tunicates within 192 h p.i. The frequencies of collectin-positive hemocytes in FSW-injected animals did not differ significantly ($p < 0.05$) from those of non-injected animals at any of the time points tested.

4. Discussion

This study demonstrates that in vivo challenge with a common inflammatory agent, zymosan, increases the concentration of a collectin-like protein in both the serum and circulating hemocytes of *S. plicata*. The data suggest that the tunicate collectin responds to mock infection in a similar manner to its mammalian counterpart, MBL, and so contributes to an inducible host defence system.

Even though the concentration of the tunicate collectin in serum was substantially increased 48–96 h p.i., its concentration decreased during the first 48 h after zymosan injection. This fluctuating response to inflammatory stimulus is similar to that reported for mammalian MBL. Serum MBL titers in mammals decrease immediately after the onset of infection, followed by a sharp rise in concentration to levels that far exceed those of naive individuals [20]. In both mammals and tunicates, the initial decrease in humoral concentrations can be explained by the opsonic activities of collectins. The binding of humoral collectins to antigenic particles like zymosan, and their subsequent phagocytosis, is likely to result in a rapid depletion of existing extracellular molecules.

A similar early depletion was also evident among collectin molecules within *S. plicata* hemocytes. The levels of intracellular collectin underwent an initial, albeit subtle, decline within the first 24 h p.i. This suggests that pre-existing intracellular stores of collectin, the presence of which was confirmed by immunohistochemistry, are rapidly secreted from circulating hemocytes in response to antigenic stimulation.

Subsequent increases in the concentration of collectin in serum, to levels that greatly exceeded those of naive animals and FSW-injected controls, can also be explained by the secretion of collectin from circulating hemocytes. Immunohistochemistry showed that hemocytes with internal reserves of collectin increase in frequency, relative to other cell types, 24–48 h after zymosan injection. As a result, intracellular levels of collectin mirrored the increased concentrations of these molecules in serum.

The increased frequency of collectin-positive hemocytes in circulation after zymosan injection has two plausible explanations. It may reflect the release of hemocytes with pre-existing stores of collectin from non-circulating compartments of the hemolymph, such as the parietal endocarps (membranous extensions of the body wall) or the pharynx, both of which are known to contain large numbers of hemocytes [21,22]. Alternatively, the increased frequency of collectin-positive cells may be due to the induced biosynthesis of collectin in circulating hemocytes that do not normally maintain large intracellular stores of the protein. Even though our data clearly identify an increased level of collectin within the circulating hemocyte population, it cannot resolve these two alternative explanations.

Our experiments also provide little direct information about the molecular stimuli that initiate increased collectin levels. They do, however, demonstrate that the concentration of collectin in hemocytes from FSW-injected controls is also elevated relative to non-injected tunicates. This suggests that wounding alone can induce increased

sub-cellular collectin concentrations, even though the levels of collectin in FSW-injected tunicates remained substantially lower than those of zymosan-injected animals.

It is also apparent that the zymosan-induced increases in intracellular collectin concentrations were due exclusively to higher levels of the 43 kDa form of the protein. Another, 90 kDa, collectin-like molecule that was detected in hemocytes (but not hemolymph) remained unaffected by zymosan challenge. The 90 kDa polypeptide could be co-purified with the 43 kDa collectin polypeptide by carbohydrate (galNAc) affinity chomatography of hemolymph lysates, and it is recognized by the anti-collectin antibody. This suggests that the 90 kDa molecule incorporates both a galNAc-specific carbohydrate recognition domain, and a collectin-like tail region. However, confirmation of this protein's identity awaits further analysis.

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