The Hemolymph of the Ascidian *Styela plicata* (Chordata-Tunicata) Contains Heparin inside Basophil-like Cells and a Unique Sulfated Galactoglucon in the Plasma

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The hemolymph of ascidians (Chordata-Tunicata) contains different types of hemocytes embedded in a liquid plasma. In the present study, heparin and a sulfated heteropolysaccharide were purified from the hemolymph of the ascidian *Styela plicata*. The heteropolysaccharide occurs free in the plasma, is composed of glucose (~60%) and galactose (~40%), and is highly sulfated. Heparin, on the other hand, occurs in the hemocytes, and high performance liquid chromatography of the products formed by degradation with specific lyases revealed that it is composed mainly by the disaccharides ΔUA(2SO4)-1→4-β-D-GlcN(SO4) (39.7%) and ΔUA(2SO4)1→4-β-D-GlcN(SO4)6SO4) (38.2%). Small amounts of the 3-O-sulfated disaccharides ΔUA(2SO4)-1→4-β-D-GlcN(SO4)3SO4) (9.8%) and ΔUA(2SO4)1→4-β-D-GlcN(SO4)3SO4)6SO4) (3.8%) were also detected. These 3-O-sulfated disaccharides were demonstrated to be essential for the binding of the hemocyte heparin to antithrombin III. Electron microscopy techniques were used to characterize the ultrastructure of the hemocytes and to localize heparin and histamine in these cells. At least five cell types were recognized and classified as uninvaculated and multivaculated cells, amebocytes, hemoblasts, and granulocytes. Immunocytochemistry showed that heparin and histamine co-localize in intracellular granules of only one type of hemocyte, the granulocyte. These results show for the first time that in ascidians, a sulfated galactoglucon circulates free in the plasma, and heparin occurs as an intracellular product of a circulating basophil-like cell.

Heparin is a highly sulfated glycosaminoglycan (GAG) made up of a mixture of polymers with a similar backbone of repeating hexuronic acid (α-L-iduronic acid or β-D-glucuronic acid) linked 1,4 to α-D-galactosamine units. The heparin molecules possess a high heterogeneity, which results from different substitutions on the D-galactosamine (N-acetylated, N-sulfated, O-sulfated at C6 and/or C3) and on the uronic (glucuronic or iduronic) acid residue (O-sulfated at C2) (for reviews, see Refs. 1–4).

In mammals, heparin is synthesized on to a specific protein core, forming the serglycin proteoglycans (PGs) (5–9). These PGs occur in secretory granules of some immunologic cells, such as mast cells and basophils (10–12). In basophils, different from mast cells, the serglycin core protein is substituted exclusively with oversulfated chondroitin sulfate chains (13). Mature mast cells are not found in blood but reside in peripheral mucosa or connective tissue interstice. Basophils, on the other hand, circulate in the blood (14, 15).

In invertebrates, heparin has been reported to occur in different species of mollusks (16–22), crustaceans (23–27), and ascidians (28–30). In the ascidian *Styela plicata* (Chordata-Tunicata), a heparin composed mainly by the disaccharide α-L-iduronic acid 2-sulfate-1→4β-D-GlcN(SO4)6SO4), with a minor contribution (~25%) of the disaccharide α-L-iduronic acid-1→4β-D-GlcN(SO4)6SO4) was detected in intracellular granules of accessory cells, named test cells, that reside in the periviteline space of oocytes (28). Because of the morphological and biochemical similarities between ascidian test cells and mammalian mast cells, we hypothesized that these cells could be evolutionarily related. Other cell types in this ascidian,

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3 The abbreviations used are: GAG, glycosaminoglycan; ΔUA, α-Δ4,5-unsaturated hexuronic acid; ΔUA(2SO4), α-Δ4,5-unsaturated hexuronic acid 2-sulfate; GlcN(SO4), GlcN(SO4)6SO4), and GlcN(SO4)3SO4)6SO4), derivatives of D-galactosamine, bearing a sulfate ester at position N, at both positions N and 6, and at positions N, 3, and 6, respectively; GlcNAc(6SO4); N-acetyl-α-D-glucosamine 6-sulfate; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; PG, proteoglycan; PBS, phosphate-buffered saline; CTA, cetyltrimethylammonium; SAX, strong anion exchange; GlcA, glucuronic acid.
located at the lumen of pharynx and intestine, have also been shown to contain intracellular heparin (28).

The hemolymph of ascidians contains different types of circulating blood cells (31–34). Some of these cells migrate from hemolymph to tissues, where they carry out several immunologic actions, such as phagocytosis of self and non-self molecules, expression of cytotoxic agents, encapsulation of antigens, and also reparation of damaged tissues (35). In the ascidian S. plicata, the hemocytes have been classified by light microscopy by Radford et al. (32). The authors described eight individual cell types: hemoblast, lymphocyte-like cell, signet ring cell, refractile vacuolated cell, nonrefractile vacuolated cell, pigment cell, fried egg cell, and fine granular cell.

As mentioned earlier, in evolved chordates, intracellular GAGs are restricted to immunologic cells that either reside in the tissues (mast cells) or circulate in the blood (basophils) (15). Considering the phylogenetic position of ascidians and taking into account that a heparin-containing cell, similar to a mammalian mast cell, was detected in the tissues of S. plicata, it is possible that a basophil-like cell containing intracellular GAGs circulates in the hemolymph of this invertebrate chordate. To investigate this hypothesis, we submitted the hemolymph of S. plicata to proteolytic digestion before and after separation of plasma and hemocytes and analyzed the extracted and purified sulfated glycans. In addition, ultrastructural and immunocytochemical studies were carried out to characterize and determine which hemocytes express these glycans. Our results reveal the occurrence of two sulfated glycans in the hemolymph of this ascidian: heparin, which is present in intracellular granules of a circulating basophil-like cell, and a sulfated galactoglycan, which occurs free in the plasma. These results show for the first time the presence of heparin in a circulating basophil-like cell in an invertebrate chordate and may contribute toward the understanding of the evolution of the immune system in this phylum.

**EXPERIMENTAL PROCEDURES**

**Materials**

Heparan sulfate from human aorta was extracted and purified as described previously (36). Chondroitin 4-sulfate from whale cartilage, dermatan sulfate and heparin from porcine intestinal mucosa (140 units/mg), twice-crystallized papain (15 units/mg protein), and the standard disaccharides \(\alpha\)-\(\Delta\)UA-1\(\rightarrow\)4-GlcNSO\(_4\)(3\(\rightarrow\)6SO\(_4\)), \(\alpha\)-\(\Delta\)UA-1\(\rightarrow\)4-GlcN-(SO\(_4\))\(_{3\rightarrow6}\), \(\alpha\)-\(\Delta\)UA-1\(\rightarrow\)4-GlcNAc (6SO\(_4\))\(_{3\rightarrow6}\), \(\alpha\)-\(\Delta\)UA-1\(\rightarrow\)4-GlcNAc-(6SO\(_4\))\(_{3\rightarrow6}\), \(\alpha\)-\(\Delta\)UA-1\(\rightarrow\)4-GlcN-SO\(_4\)(6SO\(_4\))\(_{3\rightarrow6}\), and \(\alpha\)-\(\Delta\)UA-2(SO\(_4\))\(_{3\rightarrow6}\) were purchased from Sigma; chondroitin AC lyase (EC 4.2.2.5) from Arthrobacter aurescens, chondroitin ABC lyase (EC 4.2.2.4) from Proteus vulgaris, and heparan sulfate lyase (EC 4.2.2.8) and heparin lyase (EC 4.2.2.7) from Flavobacterium heparinum were from Seikagaku America Inc. (Rockville, MD). For HPLC-SAX experiments, the enzymes from F. heparinum heparinase I (EC 4.2.2.7), heparinase II (no EC number), and heparinase III (EC 4.2.2.8) were obtained from Grampian Enzymes (Aberdeen). Agarose (standard low \(M\)_

**Collection of Tunicates**

Adult individuals of S. plicata were collected at Praia da Urca (Guanabara Bay), Rio de Janeiro, Brazil, and maintained in an aerated aquarium containing filtered sea water at 20 °C until use.

**Isolation of the Hemocytes**

The hemolymph was harvested from the heart by direct puncture and collected into plastic tubes containing an equal volume of marine anticoagulant, containing 0.45 M sodium chloride, 0.1 M glucose, 0.01 M trisodium citrate, 0.01 M citric acid, and 0.001 M EDTA (pH 7.0) (38). After harvesting, the hemocytes were separated from plasma by centrifugation (130 × g for 10 min at room temperature).

**Extraction of the Sulfated Polysaccharides**

The hemocytes were immersed in acetone and kept for 24 h at 4 °C. The dried hemocytes (1 g) were suspended in 20 ml of 0.1 M sodium acetate buffer (pH 5.5), containing 100 mg of papain, 5 mM EDTA, and 5 mM cysteine and incubated at 60 °C for 24 h. The incubation mixture was then centrifuged (2000 × g for 10 min at room temperature), the supernatant was separated, and the precipitate was incubated with papain two more times, as described above. The clear supernatants from the three extractions were combined, and the polysaccharides were precipitated with 2 volumes of 95% ethanol and maintained at 4 °C for 24 h. The precipitate formed was collected by centrifugation (2000 × g for 10 min at room temperature) and freeze-dried. For the extraction of the plasma polysaccharides, after the removal of the hemocytes, the plasma was dialyzed against distilled water, lyophilized, and incubated with papain, as described above.

**Purification of the Polysaccharides**

The glycans obtained from plasma (~8 mg) were applied to a Q Sepharose-FPLC column, equilibrated with 20 mM Tris/HCl buffer (pH 8.0). The glycans were eluted by a linear gradient of 0–2.0 M NaCl (150 ml) at a flow rate of 2.0 ml/min. Fractions of 1.5 ml were collected and checked by a metachromatic assay using 1,9-dimethyl-methylene blue (39). Fractions eluted with different NaCl concentrations were pooled as indicated in Fig. 2A, dialyzed against distilled water, and lyophilized.

The glycans (~2 mg) obtained from hemocytes or porcine heparin (~1 mg) were applied to a Mono Q-FPLC column, equilibrated with 20 mM Tris/HCl buffer (pH 8.0). The glycans...
were eluted by a linear gradient of 0−2.0 M NaCl (45 ml) at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and checked by metachromatic assay using 1.9-dimethyl-methylene blue. Fractions eluted with different NaCl concentrations were pooled as indicated in Fig. 4A, dialyzed against distilled water, and lyophilized.

**Electrophoretic Analysis**

**Agarose Gel**—The crude or purified glycans from plasma (≈10 μg dry weight) or hemocytes (1.5 μg as uronic acid), before or after incubation with specific GAG lyases or deaminative cleavage with nitrous acid were analyzed by agarose gel electrophoresis, as described previously (40). Briefly, the glycans and a mixture of standard GAGs, containing chondroitin sulfate, dermatan sulfate, and heparan sulfate (1.5 μg as uronic acid of each), were applied to a 0.5% agarose gel in 0.05 M 1,3-diaminopropane/acetate (pH 9.0), along with a mixture of standard (Std) glycosaminoglycans, containing chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS).

**Polyacrylamide Gel**—The molecular masses of the purified glycans from plasma and hemocytes were estimated by polyacrylamide gel electrophoresis. Samples (≈10 μg) were applied to a 1-mm-thick 6% polyacrylamide slab gel, and after electrophoresis at 100 V for ~1 h in 0.06 M sodium barbital (pH 8.6), the gel was stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v/v).

**Enzymatic Treatments**

**Chondroitin Lyases**—The ascidian glycans (≈100 μg) were incubated with 0.01 units of chondroitin AC or BC lyase in 0.1 ml of 50 mM Tris-HCl buffer (pH 8.0), containing 5 mM EDTA and 15 mM sodium acetate. After incubation at 37°C for 12 h, another 0.01 units of enzyme was added to the mixture, and the reaction continued for an additional 12-h period.
Ascidian Hemolymph Sulfated Glycans

A

Isobutyric acid:HN₄OH (5:3, v/v)

Origin

GlcA

Glc / Gal

Man

Fuc

GlcN

P2

Std sugars

Butanol:Pyridine:Water (3:2:1, v/v)

GlcA

Gal

Glc

Man

Fuc

P2

Std sugars

B

HOD

Integrals:

0.4

0.6

\[ {^1}\text{H} \text{chemical shift (ppm)} \]

C

\[ {^1}\text{H} \text{chemical shift (ppm)} \]

\[ {^1}\text{H} \text{chemical shift (ppm)} \]
**Heparan Sulfate and Heparin Lyases**—About 50 μg (as dry weight of each) of the glycans extracted from the ascidian tissues were incubated with 0.005 units of either heparan sulfate lyase or heparin lyase in 100 μl of 100 mM sodium acetate buffer (pH 7.0), containing 10 mM calcium acetate for 17 h at 37 °C. At the end of the incubation period, the mixtures were analyzed by agarose gel electrophoresis, as described earlier.

**Hexuronic Acid**—The hexuronic acid content of the glycans from the various tissues was estimated by the carbazole reaction (42).

**Deaminative Cleavage with Nitrous Acid**—Deaminative cleavage with nitrous acid of the sulfated glycans was performed as described by Shievely and Conrad (43).

**Chemical Analyses**—Total hexose was measured by the phenol-sulfuric acid method of DuBois et al. (44). After acid hydrolysis (6.0 N trifluoroacetic acid, 100 °C for 5 h), total sulfate was determined by the BaCl2-gelatin method (45). The proportions of the different hexoses in the acid hydrolysates were determined by paper chromatography in butanol/piridine/water (3:2:1, v/v/v) for 36 h or in isobutyric acid, 1.0 NH4OH (5:3, v/v). The sugars were detected on the chromatogram by silver nitrate staining.

**NMR Spectroscopy**—1H spectra were recorded using a Bruker DRX 600 with a triple resonance probe. About 3 mg of the purified plasma polysaccharide was dissolved in 0.5 ml of 99.9% D2O (CIL).

**Antithrombin III Affinity Chromatography**—The polysaccharide (10 mg) was chromatographed on an ATIII-Sepharose column (15 x 2.6 cm). The column was prepared by binding 100 mg of human ATIII (Hyphen Biomed) on cyanogen bromide-activated Sepharose 4B (Sigma). The polysaccharide fraction was eluted by NaCl solution. The low affinity fraction was eluted out of the column at 0.25 M NaCl solution buffered at pH 7.4 with Tris (10 mM) and desalted on Sephadex G10. The high affinity fraction was eluted with 3 M NaCl, 10 mM Tris and desalted on Sephadex G10.

**Inhibition of Thrombin by Antithrombin in the Presence of Mammalian or Hemocyte Heparins**—These effects were evaluated by the assay of amyloptic activity of thrombin, using chromogenic substrate, as described (28). Incubations were performed in disposable UV semimicrocuvettes. The final concentrations of reactants included 50 nM antithrombin, 15 nM thrombin, and 0–10 μg/ml heparin in 100 μl of 0.02 M Tris/HCl, 0.15 M NaCl, and 1.0 mg/ml polyethylene glycol (pH 7.4) (TS/PEG buffer). Thrombin was added last to initiate the reaction. After a 60-s incubation at room temperature, 500 μl of 100 μM chromogenic substrate Chromogenix TH in TS/PEG buffer was added, and the absorbance at 405 nm was recorded for 100 s. The rate of change of absorbance was proportional to the thrombin activity remaining in the incubation. No inhibition occurred in control experiments in which thrombin was incubated with antithrombin in the absence of heparin; nor did inhibition occur when thrombin was incubated with heparin alone over the range of concentrations tested.

**Conventional Transmission Electron Microscopy**—The hemocytes were fixed in 2.5% glutaraldehyde EM grade, 4% formaldehyde freshly prepared from paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) under microwaves (Laboratory Microwave Processor, Pelco model RFS9MP; 2.45 GHz) for 10 s twice at 45 °C and rinsed twice in cacodylate buffer for 15 min at room temperature. The samples were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.2) in a microwave oven for 10 s, dehydrated through a graded series of acetone, and embedded in epoxy resin Poly/Bed 812 (Polyscience, Inc.). Ultrathin sections (80 nm) were obtained (LKB ultramicrotome) and collected on copper grids (300 mesh). The sections were stained with 2% uranyl acetate for 20 min and 1% lead citrate for 5 min. The samples were observed in a Zeiss 900 EM electron microscope, operated at 80 kV.

**Immunocytochemistry**—Circulating hemocytes were slightly centrifuged from hemolymph and fixed overnight in 4% formaldehyde, 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) and 4% 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma) at 4 °C. The samples were washed with 0.05% sodium borohydride in 0.1 M sodium phosphate buffer (pH 7.2), dehydrated in a graded series of methanol until 95%, and embedded in LR Gold acrylic resin at room temperature. Ultrathin sections (90 nm) were obtained and collected on nickel grids (300 mesh).

The sections were hydrated in phosphate-buffered saline (PBS) (1%) for 10 min, and nonspecific sites were blocked with 1% bovine serum albumin in 1% PBS containing 50 mM ammonium chloride for 10 min. The samples were incubated overnight in a humid chamber with anti-heparin monoclonal antibody (46) or anti-histamine polyclonal antibodies (Chemicon) diluted in 1% PBS. After washing with 1% PBS, the sections were incubated with secondary goat anti-mouse or anti-rabbit 10-nm gold-conjugated IgM antibody (Sigma) for 3 h and washed in 1% PBS containing 1% bovine serum albumin and finally with distilled water. The sections were stained with 1% uranyl acetate for 20 min. The controls were done omitting the incubation with the primary antibody.

**Histamine N-Methyltransferase Activity**—Tissues of *S. plicata* (intestine, hemolymph, and pharynx) as well as a preparation of rat peritoneal mast cells were homogenized with PBS (1%, v/v), subjected to three consecutive cycles of freeze and thaw, and then centrifuged at 1,500 x g for 10 min.
min. Supernatants were then stored at −20 °C until total protein and histamine quantification. Total protein content was spectrophotometrically quantified (540 nm) in the supernatant by means of the Biuret technique (47). Histamine content was estimated in the supernatant by the radioenzymatic technique of Snyder et al. (48), modified by Córrea and Saavedra (49). Briefly, the assay was carried out in a final volume of 60 μl, consisting of 10 μl of standard histamine solution or tissue extract and 50 μl of a freshly prepared mixture containing histamine N-methyltransferase preparation, 0.125 μCi of S-adenosyl[methyl-3H]methionine, and 0.05 M sodium phosphate buffer, pH 7.9. Measurements were made in duplicate, and the blanks were prepared by replacing the sample test with 0.05 M sodium phosphate buffer, pH 7.9. After sample incubation overnight at 4 °C, the enzymatic reaction was stopped by the addition of 0.5 ml of 1 M NaOH containing 10 μl of the unlabeled methylhistamine carrier. The [3H]methylhistamine formed was then extracted into 3 ml of chloroform. After evaporation of the organic phase, the radioactivity was counted with a Beckman LS-100 Scintilograph.

RESULTS

The Hemolymph of S. plicata Contains Different Sulfated Polysaccharides—Ascidian hemolymph contains different types of cells, named hemocytes, embedded in liquid plasma. In order to investigate the presence of sulfated polysaccharides, the hemolymph was subjected to proteolytic treatment with papain, and the extracted material was analyzed by agarose gel electrophoresis (Fig. 1). Two main metachromatic bands with different electrophoretic mobilities, corresponding to two different sulfated polysaccharides, were observed in the gel. The lower mobility band possesses the same migration as standard heparan sulfate, whereas that migrating as chondroitin sulfate originates from plasma. The highermobilitybandinP3representsthechondroitinsulfate,whentanalyzedbyagarosegel electrophoresis (Fig. 1). Two main metachromatic bands with different electrophoretic mobilities, corresponding to two different sulfated polysaccharides, were observed in the gel. The low mobility band possesses the same migration as standard heparan sulfate, whereas that migrating as chondroitin sulfate originates from plasma.

The Polysaccharide from the Plasma Is a Sulfated Galactoglycan—The sulfated polysaccharide from plasma was fractionated on an ion exchange column, as described under “Experimental Procedures.” Three peaks, denominated P1, P2, and P3, were eluted from the column with different NaCl concentrations (Fig. 2A). P2, which eluted from the column at −0.8 M NaCl, displayed a homogeneous metachromatic band, migrating as chondroitin sulfate, when analyzed by agarose gel electrophoresis (Fig. 2B). P3, which eluted from the column with −1.2 M NaCl, showed two metachromatic bands (Fig. 2B). The higher mobility band in P3 represents the chondroitin sulfate-migrating material from P2, which is contaminated with a lower mobility band, corresponding to the heparan sulfate-migrating material from hemocytes.

In order to obtain information about the plasma polysaccharide, the purified polymer obtained from the ion exchange column (P2) was incubated with chondroitinase AC- and ABC-lyase or treated with nitrous acid, and the products were analyzed by agarose gel electrophoresis (Fig. 2C). The plasma polysaccharide resisted the incubations with chondroitin sulfate lyases and the nitrous acid treatment, indicating that it is not chondroitin/dermatan sulfate or heparan sulfate/heparin.

To estimate the size of the plasma polysaccharide, P2 was analyzed by polyacrylamide gel electrophoresis, where its migration was compared with those of known molecular weight standard glycans. As shown in Fig. 2D, P2 migrated slightly behind dextran 8,000 (average Mr, 8,000), which suggests an average molecular weight of ~10,000.

The chemical analysis of the plasma polysaccharide was carried out by paper chromatography on two different buffer systems after acid hydrolysis of the purified polymer (P2). As indicated by the chromatographic analysis on butanol/pyridine/water, the polymer is composed mainly by glucose (60%), followed by galactose (40%). No amino sugar or hexuronic acid was detected (Fig. 3, Table 1) as indicated by the chromatographic analysis on isobutyric acid/NH₄₃. High amounts of sulfate ester, in equimolecular proportions with hexoses, were also detected (Table 1). Structural analysis by one-dimensional (Fig. 3B) and two-dimensional (supplemental Fig. 3C) NMR supports the chromatographic data. Clearly, the one-dimensional ¹H NMR spectrum revealed intensity signals of anomic protons in a proportion of 4:6 for α- and β-forms, respectively (Fig. 3B). This proportion is coincident to the galactose/glucose ratio shown in Table 1 and indicates that the sulfated galactoglycan is composed mainly by α-galactopyranose and β-glucopyranose residues. The COSY spectrum revealed the presence of six connected protons through scalar coupling (cross-peaks), confirming that this compound is a hexose polymer (supplemental Fig. 3C). No evidence of amino or other complex sugars were detected, discarding the possibility of a minor glycosaminoglycan contaminant. Both α- and β-H1 revealed cross-peaks with H2 at ~3.8 ppm, suggesting the same assignment (Table 2). This similarity derives from the equal proton chemical shift of galactose and glucose, which are just C4 epimers. Moreover, the H2 high field resonance certainly indicates that there is no
Ascidian Hemolymph Sulfated Glycans

The Hemocytes of *S. plicata* Contain a Heparin-like GAG—The sulfated glycans extracted from hemocytes were fractionated on an ion exchange column, as described under “Experimental Procedures.” The glycans were eluted in four metachromatic peaks, denominated H1, H2, H3, and H4, at different NaCl concentrations (Fig. 4A). H1 and H2 contain very little material (Fig. 4B) and were discarded. H3, eluted at approximately the same NaCl concentration required to elute porcine intestinal mucosa heparin (∼1.5 m) (Fig. 4A). When analyzed by agarose gel electrophoresis, H3 shows a single metachromatic band migrating as heparan sulfate standard (Fig. 4B). A small amount of this material was eluted at a higher NaCl concentration (H4 in Fig. 4, A and B).

An estimate of the molecular weight of the purified heparin (H3) was carried out by polyacrylamide gel electrophoresis, where its migration was compared with those of known molecular weight standard GAGs. As shown in Fig. 4C, H3 migrated slightly ahead of porcine intestinal mucosa heparin, which suggests an average molecular weight of ∼12,000 (Fig. 2D).

In order to obtain information about the nature of the heparin glycan, the purified polymer from the ion exchange column (H3) was incubated with condroitinase AC/ABC-lyase and heparin/heparan sulfate-lyase, and the products were analyzed by agarose gel electrophoresis (Fig. 5A). The heparin glycan was resistant to the action of chondroitin AC/ABC-lyases as well as heparan sulfate lyase but was almost totally degraded by heparin lyase, indicating that this is a heparin-like GAG. CTA-SAX HPLC analysis of the products formed by the action of heparin-lyase (I, II, and III) on H3 revealed that the heparin glycan is formed mainly by the disaccharides ΔUA(2SO₄)₁→4-ß-D-GlcN(3SO₄)(3SO₄) (39.7%) and ΔUA(2SO₄)₁→4-ß-D-GlcN(6SO₄)(6SO₄) (38.2%). Smaller amounts of the disaccharides ΔUA(2SO₄)₁→4-ß-D-GlcN(3SO₄)(6SO₄) (3.8%) and ΔUA(2SO₄)₁→4-ß-D-GlcN(3SO₄)(3SO₄) (9.8%) were also present (Fig. 5B, Table 3).

The disaccharide ΔUA(2SO₄)₁→4-ß-D-GlcN(3SO₄)(3SO₄) was already identified in depolymerized porcine mucosa heparin (41). The ΔUA(2SO₄)₁→4-ß-D-GlcN(3SO₄)(3SO₄) tentatively structure was attributed according to several analytical indications; the UV maximum at 232 nm is charac-

![FIGURE 4. Isolation and characterization of the sulfated glycan from hemocytes. A, about 2 mg of the sulfated glycan from hemocyte (Std) or mammalian heparin (H3) (∼1 mg) were applied to a Mono Q-FPLC column as described under “Experimental Procedures.” Fractions were assayed for metachromasia and NaCl concentration. B, fractions under the peaks denominated H1, H2, H3, and H4 were pooled, dialyzed against distilled water, lyophilized, and analyzed by agarose gel electrophoresis, as described in the legend to Fig. 1. C, the purified glycan from hemocyte (Sp Hep (H3); ∼10 µg dry weight) and the molecular weight markers dextran 500 (Dx 500), chondroitin 4-sulfate (CS), chondroitin 6-sulfate (CS6), dextran B (Dx 8), and mammalian heparin (Man Hep) (∼10 µg as dry weight of each) were applied to a 1-mm-thick 6% polyacrylamide slab gel, as described under “Experimental Procedures.” Std, standard.

![FIGURE 5. Characterization of the heparin. A, the purified heparin (H3) was incubated with condroitinase AC (Chase AC) or ABC (Chase ABC) lyases or heparin or heparan sulfate lyases, as described under “Experimental Procedures.” B, the disaccharides formed by exhaustive action of heparinase I, II, and III on the heparin glycan were applied to a CTA-SAX HPLC column. The column was eluted with a gradient of NaCl as described under “Experimental Procedures.” Fractions were assayed for metachromasia and NaCl concentration. C, the purified glycan from hemocyte (Sp Hep (H3); ∼10 µg dry weight) and the molecular weight markers dextran 500 (Dx 500), chondroitin 4-sulfate (CS), chondroitin 6-sulfate (CS6), dextran B (Dx 8), and mammalian heparin (Man Hep) (∼10 µg as dry weight of each) were applied to a 1-mm-thick 6% polyacrylamide slab gel, as described under “Experimental Procedures.” Std, standard. monomers identified in the glycan were 4-sulfation (at position 3) in CS6 and 2-sulfation in this compound. The low field resonance at 4.45 ppm for H4 suggests 4-sulfated and/or 4-linked units.

The Hemocytes of *S. plicata* Contain a Heparin-like GAG—The sulfated glycans extracted from hemocytes were fractionated on an ion exchange column, as described under “Experimental Procedures.” Fractions were assayed for metachromasia and NaCl concentration. The eluant was monitored for UV absorbance at 232 nm. The assigned peaks correspond to the disaccharides: ΔII₁s, ΔUA(2SO₄)₁→4-ß-D-GlcN(3SO₄); ΔIs, ΔUA(2SO₄)₁→4-ß-D-GlcN(6SO₄); ΔI₂s, ΔUA(2SO₄)₁→4-ß-D-GlcN(3SO₄)(3SO₄); ΔII₂s, ΔUA(2SO₄)₁→4-ß-D-GlcN(3SO₄)(6SO₄); ΔI₃s, ΔUA(2SO₄)₁→4-ß-D-GlcN(3SO₄)(3SO₄).
**Ascidian Hemolymph Sulfated Glycans**

The disaccharide composition of ascidian and mammalian heparins was determined with a chromogenic substrate as described under “Experimental Procedures.” The polysaccharide fraction was digested with heparin-lyases (I, II, and III) and analyzed by CTA-SAX HPLC (Fig. 6B).

The high affinity fraction, which accounts for 40% of the heparin chains, was eluted out of the ATIII-Sepharose at 3 M NaCl and was desalted on Sephadex G10. This fraction of the heparin glycan was digested with heparin-lyases (I, II, III) and analyzed by CTA-SAX HPLC (Fig. 6C).

In the chromatogram shown in Fig. 6B, it appears clearly that the low affinity fraction of the polysaccharide is basically composed by the disaccharides ΔUA(2SO₄)₁-→4-β-D-GlcN(SO₄)(3SO₄) and ΔUA(2SO₄)₁-→4-β-D-GlcN(SO₄)-3SO₄(6SO₄). The two 3-O-sulfated disaccharides are nearly absent in this part of the heparin glycan.

On the contrary, the presence of the 3-O-sulfated disaccharides is greatly enhanced in the high affinity fraction of the polymer with respect to heparin glycan prior to fractionation (Fig. 6C). Table 4 summarizes these results, and the data clearly demonstrate the key involvement of ΔUA(2SO₄)₁-→4-β-D-GlcN(SO₄)(3SO₄) and ΔUA(2SO₄)₁-→4-β-D-GlcN(SO₄)(6SO₄) in the ATIII affinity of the polymer. However, the presence of a high affinity pentasaccharide-like binding sequence in the heparin glycan remains to be identified and demonstrated in further work.

**Hemocyte Heparin Contains Antithrombin Activity**—Antithrombin-mediated anticoagulant activity is a specific pharmacological characteristic of heparins. In order to investigate if the heparin glycan also

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**TABLE 3**

| Disaccharide | Percentage of the disaccharides |
|--------------|---------------------------------|
|              | Test cell<sup>a</sup> | Granule cell<sup>b</sup> | PIH<sup>ac</sup> |
| ΔUA₁→4-β-D-GlcN(SO₄)(6SO₄) | <1 | 1.4 | <5 |
| ΔUA₁→4-β-D-GlcN(SO₄)(6SO₄) | 25 | 2 | 9–11 |
| ΔUA₁→4-β-D-GlcN(SO₄)(6SO₄) | <1 | 39.7 | 6–8 |
| ΔUA₁→4-β-D-GlcN(SO₄)(6SO₄) | 75 | 38.2 | 60–70 |
| ΔUA₁→4-β-D-GlcN(SO₄)(6SO₄) | <1 | 9.8 | <1 |
| ΔUA₁→4-β-D-GlcN(SO₄)(6SO₄) | 1 | 3.8 | <1 |

<sup>a</sup> Cavalcante et al. (28).

<sup>b</sup> This work.

<sup>c</sup> Porcine intestinal heparin (C. M. de Barros, L. R. Andrade, S. Alldoc, C. Viskov, P. A. Mourier, M. C. M. Cavalcante, A. H. Strauss, H. K. Takahashi, V. H. Pomin, V. F. Carvalho, M. A. Martins, and M. S. G. Pavão, unpublished data).

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**FIGURE 6. Antithrombin activity of the hemocyte heparin and disaccharide analysis of low and high affinity antithrombin fractions.** A, antithrombin activity. Shown is inhibition of thrombin activity in the presence of hemocyte (●) or mammalian (○) heparin. Antithrombin (50 nM) was incubated with thrombin (15 nM) in the presence of various concentrations of heparins. After 60 s, the remaining thrombin activity was determined with a chromogenic substrate as described under “Experimental Procedures.” B and C, disaccharide composition. The purified heparin glycan (10 mg) was chromatographed on an ATIII-Sepharose column (40 × 5 cm). The polysaccharide fraction was eluted by NaCl solution as described under “Experimental Procedures.” Low affinity (B) and high affinity (C) fractions were used with 0.25 and 3 M NaCl, respectively. These fractions were exhaustively digested with a mixture of heparinase I, II, and III and applied to a CTA-SAX HPLC column as described under “Experimental Procedures.” The eluant was monitored for UV absorbance at 232 (black line) and 202–247 nm (gray line). ΔIls, ΔUA(2SO₄)₁→4-β-D-GlcN(SO₄); ΔIls, ΔUA(2SO₄)₁→4-β-D-GlcN(SO₄)(3SO₄); ΔIls, ΔUA(2SO₄)₁→4-β-D-GlcN(SO₄)(3SO₄)(6SO₄); ΔIls, ΔUA(2SO₄)₁→4-β-D-GlcN(SO₄)(3SO₄)(6SO₄) and ΔUA(2SO₄)₁→4-β-D-GlcN(SO₄)(6SO₄) in the ATIII affinity of the polymer. However, the presence of a high affinity pentasaccharide-like binding sequence in the heparin glycan remains to be identified and demonstrated in further work.
TABLE 4
Disaccharide composition of the low and high antithrombin III affinity fractions of the hemocyte heparin

| Disaccharide                                      | Hemocyte heparin | Low affinity fraction | High affinity fraction |
|--------------------------------------------------|------------------|-----------------------|-----------------------|
| ΔUA(2SO₄)-1→4-β-D-GlcN(SO₄)                      | 39.7%            | 42.5%                 | 33%                   |
| ΔUA(2SO₄)-1→4-β-D-GlcN(SO₄)(6SO₄)                | 38.2%            | 45.1%                 | 33.5%                 |
| ΔUA(2SO₄)-1→4-β-D-GlcN(SO₄)(6SO₄)(6SO₄)          | 3.8%             | 0.6%                  | 4.2%                  |
| ΔUA(2SO₄)-1→4-β-D-GlcN(SO₄)(3SO₄)                | 9.8%             | 1.4%                  | 21.2%                 |
| Sum of other minor saccharides                   | 8.5%             | 10.4%                 | 9.1%                  |

FIGURE 7. Conventional transmission electron micrographs showing the ultrastructural features of S. plicata hemocytes. A, univacuolated cell (bar, 0.68 μm). B, multivacuolated cell (bar, 0.66 μm). C, hemoblast or lymphocyte-like cell (bar, 0.66 μm). D, amebocyte (bar, 0.36 μm). E, granulocyte cell (bar, 0.50 μm).

possesses this activity, we measured the inhibition of thrombin by antithrombin in the presence of increasing concentrations of invertebrate or mammalian GAG (Fig. 6A). The rate of thrombin inhibition by antithrombin induced by the hemocyte heparin was similar to that induced by porcine intestinal mucosa heparin, indicating that both mammalian and invertebrate heparins have the same anticoagulant activity.

S. plicata Hemolymph Contains Different Types of Hemocytes—Different types of cells occur in S. plicata hemocyte population. Transmission electron microscopy observations revealed that the hemolymph of this ascidian has at least five recognizable cell types. Fig. 7A shows an univacuolated cell type (5.5–9.5 μm in diameter) containing a huge vacuole that occupies almost the whole volume of the cytoplasm. Dispersed fibrilar material and electron-lucent round vesicles are present within the vacuole. Some of the smaller electron-dense vesicles are seen close to the membrane that surrounds the vacuole with an aspect that suggests a sprouting from the membranes.

A multivacuolated cell type is shown in Fig. 7B. This 6.5–8.0-μm cell has a spherical shape and contains 2–20 regularly sized electron-lucent vacuoles close to small vesicles. Its nucleus is very evident, including the chromatin arrangement.

Another cell type is shown in Fig. 7C. It resembles hemoblasts or lymphocyte-like cells described by others in ascidians (31, 32, 34). This 4.0–5.5-μm cell has small vesicles, sometimes continuous with the nuclear envelope and mitochondria profiles.

Fig. 7D shows the smallest cell type (2.5–5.0-μm diameter). It has a dense cytoplasm with small vesicles, large mitochondria profiles, and a nucleus that occupies most of the cell. This cell type has similar characteristics to the cell type named amebocyte by Fuke and Fukumoto (50).

Finally, Fig. 7E shows the granulocyte cell with a 3.5–6.0-μm diameter. Many granules or vesicles containing a material with varying electron densities can be observed intracellularly. The granules are uniform in the sense that most of them are spherical and with comparable sizes (~0.4 μm).

Heparin Is Restricted to Only One Type of Hemocyte—In order to identify which cells contain heparin, a preparation of the hemocytes was immunogold-labeled with anti-heparin antibody. As shown in Fig. 8, 10-nm gold particles were observed in only one type of cell, named the granulocyte cell. The gold particles associated with the anti-heparin (Fig. 8B) antibody were localized inside electron-dense granules.

In mammals, histamine is associated with heparin in the granules of mast cells and basophils. In the present work, 10-nm gold particles were observed within intracellular granules of ascidian granulocytes (Fig. 8C) and rat peritoneal mast cells (Fig. 8D and E) after immunogold labeling with anti-histamine antibody. The pattern of gold labeling is very similar to that observed when anti-heparin antibody was used. No significant labeling was observed in other regions of the granulocytes or in other hemocytes or in rat peritoneal mast cell when primary antibody was omitted.

In a previous study (20), histamine was detected in the intestine and pharynx of S. plicata, using immunolabeling with anti-histamine antibody. To confirm the presence of histamine in these tissues and also the results of the immunogold labeling described in the present work, the activity of the enzyme histamine N-methyltransferase was measured in homogenates of intestine, pharynx, and hemolymph of S. plicata as well as in
Ascidian Hemolymph Sulfated Glycans

It is interesting to note that the chemical composition of the sulfated glycans in ascidians varies according to the tissue and stage of development. For example, in adult tunic, the main polysaccharide is a high molecular weight sulfated galactan, composed by α-L-galactopyranose residues sulfated at position 1→4 (62, 63). This polymer is synthesized by epidermal cells that epimerize D-glucose, possibly from a trehalose precursor, into L-galactose (64, 65). The larval tunic, on the other hand, possesses a heteropolysaccharide composed mainly of glucosamine and sulfated fucose, with minor amounts of L-galactose (66).

The hemolymph polysaccharide reported in the present study is a low molecular weight sulfated galactoglucan, which has a higher sulfate content (1.0 mol of sulfate/mol of hexose) when compared with adult (0.7 mol of sulfate/mol of hexose) (62) and larval (0.4 mol of sulfate/mol of hexose) tunic (66) glycans. The enantiomeric form of galactose and the position of sulfation of the hemolymph polysaccharide are under investigation. Sulfated GAGs do not occur in the tunic but abound in different organs of ascidians (64, 67). An oversulfated dermatan sulfate composed of IdoA(2SO4)-GalNAc(4SO4) was isolated from the body of the ascidian Phallusia nigra (70, 71).

Previously, we reported the occurrence of heparin, composed of the disaccharides ΔUA(2SO4)-GlcN(SO4) (57%) and ΔUA-4-GlcN(SO4)(6SO4) (25%), in intracellular granules of test cells of S. plicata (28). Now we report in the hemolymph of this ascidian a heparin with a different composition, formed by approximately equal amounts of the disulfated disaccharide ΔUA(2SO4)-GlcN(SO4) and the trisulfated disaccharide ΔUA(2SO4)-GlcN(SO4)(6SO4). Smaller quantities of tri- and tetrarsulfated disaccharides, containing 3-O-sulfated glucosamine, which is required for binding to antithrombin, were also found (72, 73). Overall, these results suggest that the enzymes of the synthesis of heparin in S. plicata are either differently regulated or differ in the test cells and hemocytes.

Because of heparin’s unique binding to antithrombin, involving the specific pentasaccharide sequence GlcNAc(6SO4)2-GlcA-GlcNS(3SO4)-IdoA(2SO4)-GlcNS(6SO4), which contains a unique 3-O-sulfated glucosamine, mammalian heparin is endowed with a potent anticoagulant activity (74, 75). Analysis of the anticoagulant action of the hemocyte heparin revealed an antithrombin activity 10-fold higher than that of test cell heparin and similar to that observed in mammalian heparin (porcine intestinal mucosa). These results are in agree-
ment with the presence of significant amounts of 3-O-sulfated glucosamine residues in the hemocyte heparin, not detected in test cell heparin (28), that could form a pentasaccharide-like sequence with high affinity to antithrombin.

Important data about the morphology of S. plicata hemocytes were revealed by conventional transmission electron microscopy. According to our observations, five types of circulating hemocytes were described: univacuolated and multiculated cells, amebocytes, hemoblasts, and granulocytes. Different from our results, Radford et al. (32) described eight individual hemocyte types in the ascidian S. plicata. This may be due to the different methodology employed in that work, which was based mainly on bright field optical microscopy and cell sorting performed on immunofluorescently stained hemocytes.

The identification of a granulocyte in the hemolymph of S. plicata, morphologically related to vertebrate basophils, was of notice. In the granules of the ascidian granulocyte, a central electron-dense region can be observed. Granules with an electron-dense core are present mainly in granulocytes of higher vertebrates, such as reptiles and mammals (76–78), whereas granules with no electron-dense core have been reported in more primitive vertebrate granulocytes, such as fish and bufonid.

In addition to morphological similarities, S. plicata granulocyte also contains biochemical characteristics common to vertebrate basophils, such as intracellular GAG, in this case heparin and histamine. Mammalian heparin is synthesized onto a specific protein core, forming the serglycin PGs (5–9). These PGSs are resistant to proteolytic degradation (5, 79). In the present work, peptide-free heparin chains were obtained after proteolytic degradation of the hemocytes, suggesting that the granulocyte heparin is probably linked to a core protein different from that of serglycin PGSs.

Histamine was unequivocally detected in the intestine and pharynx of S. plicata and in the hemolymph by measuring the activity of the enzyme histidine N-methyltransferase, which is involved in the catabolism of histamine. We also estimated the content of histamine in rat peritoneal mast cells using this method (~13 pg/cell). It should be emphasized that the histamine assay we have employed for tissues from S. plicata is a sensitive and highly specific method. Its specificity is achieved by employing an enzyme, histamine N-methyltransferase, isolated from guinea pig brains, whereas the sensitivity is accounted for by the use of the S-adenosyl[methyl-3H]methionine as the radioactive cofactor. As far as we know, it is quite unlikely that histidine N-methyltransferase could be using serotonin or dopamine as substrate. The major false positives for histamine found in mammalian tissue samples, mainly concerning fluorimetric assays, are spermine, spermidine, and putrescine. Prior studies have demonstrated that the radioenzymic method for histamine did not mistake the latter for the former as reported (80).

The results presented in the present study suggest that the hemolymph granulocyte may be a primitive counterpart of mammalian basophil, involved in immunological mechanisms, especially when migrating from the blood vessels to perform activities such as encapsulation, phagocytosis, liberation of microbial peptides, triggering of the complement system, and regeneration of tissues.

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