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D-GALACTOSE BINDING LECTINS FROM THE TUNICATE ASCIDIA MALACA: SUBUNIT CHARACTERIZATION AND HEMOCYTE SURFACE DISTRIBUTION

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ABSTRACT D-galactose specific lectins purified from Ascidia malaca serum contain a major protein component with an apparent molecular weight of about 58,000 daltons, which moves more rapidly under non-reducing conditions. Intramolecular disulfide linkages can explain this behaviour, suggesting a compact protein structure. Membrane lectins have been demonstrated on the surface of about 34% hemocytes by immunofluorescent methods using a rabbit antiserum against the isolated serum lectins. Small, medium and large hemocytes can be positive, as also shown by binding on Sepharose spherules or by rosette formation with sheep and rabbit erythrocytes. Binding is inhibited by the same sugars specific for the serum lectins. Finally, antibodies to the serum lectins specifically agglutinate the hemocytes. This evidence supports the hypothesis that a lectin with the same specificity and certain structural similarities can be found free in the serum and present on hemocyte surfaces.

INTRODUCTION

Although invertebrates possess self non-self recognition systems, they do not produce immunoglobulins, and the nature of their recognition structures cannot be defined in terms of specific antibody-antigen complementarity (1, 2). Studies of

invertebrate blood show that the action of phagocytes towards foreign material might be selective, and that the interaction between a complementary non-self determinant and cell surface receptor molecules may intervene in encapsulation (3) and recognition immunity mechanisms (2, 4).

Molecular mechanisms based on protein-carbohydrate interaction at the cell surface level have been proposed to explain cellular recognition activity (2, 4-9). Molecules which might be "opsonic" have been found in the blood of several invertebrate species. In a number of cases opsonic functions have been attributed to naturally occurring lectins almost always present in the hemolymph (3, 5, 10). These molecules are sugar specific proteins and are identified by their capacity to agglutinate erythrocytes (2, 11-18), characteristics they share with agglutinins identified in several vertebrate tissues (19, 20). Lectins have also been identified on hemocyte surfaces in some species and might thus be used as receptors to bind directly with foreign particles or to identify self-markers on cell surfaces (5, 21-23). Evolutionary relationships among invertebrate and vertebrate lectins remain an open question. Lectins have been found in the serum of all tunicate species (11-15). Studies of these ancestral chordates might yield information on the presence of homologs or functional analogs of certain vertebrate recognition molecules in a phylogenetically critical taxon (24).

In previous papers we have shown that the binding site of anti-rabbit erythrocytes as well as of anti-human ABO lectins from Ascidia malaca and Phallusia mamillata serum react with membrane sugars characterized by the presence of a D-galacto configuration. We exploited this sugar-specificity to purify them by means of affinity chromatography on Sepharose (13, 14). Hemagglutination sugar inhibition experiments suggest that the Ascidia malaca lectin has a combining site corresponding to a disaccharide in which carbons C2, C4, C5 and C6 of D-galactose are probably involved. Moreover absorption experiments showed that two distinct molecules with similar sugar specific ranges were responsible for rabbit and human erythrocyte agglutination (13). In the present paper dealing with Ascidia malaca we report further data on the subunit molecular weight and distribution on hemocyte surfaces of anti-rabbit and sheep erythrocyte lectins.

MATERIALS AND METHODS

Animals, serum and hemocyte preparation, and assays

Ascidia malaca Traust were collected in the Gulf of Palermo and blood was obtained by cardiac puncture. Serum was separated from cells by centrifuging the pooled blood at 500g, dialyzing with phosphate buffered saline solution pH 7.4 (PBS) and storing it at -75°C. To separate hemocytes, blood was withdrawn from the heart with a sterile syringe in the presence (1:10) of Ca^{++}- and Mg^{++} - free sea water containing 0.1M disodium EDTA (FSWEDTA, pH corrected to 7.4). After centrifuging at 500g for 15 minutes

at 4°C, cells were suspended in the same medium and washed three times in 20 times their volume. The microhemagglutination and sugar inhibition tests were as previously described (13, 14).

Purification of lectins and preparation of lectin antiserum

Affinity chromatographic purification of the D-galactose specific serum lectins (sL) on a Sepharose-HCl column has been described in a previous paper (14). Rabbit immune serum to the purified lectin (anti-sL) was prepared as follows. The D-galactose eluted fractions obtained by affinity chromatography were exhaustively dialyzed in PBS and tested for anti-rabbit erythrocyte (RE) agglutinating activity. Those fractions showing hemagglutinating titres from 2 to 4 (reciprocal of the scalar dilution) were pooled (protein content 95.0 ± 0.9µg/ml ES, n=3) and divided into 5 ml aliquots for use as antigens. An intramuscular injection of antigen emulsified with an equal volume of complete Freund's adjuvant (Difco) was given, followed by two subcutaneous injections, on days 30 and 60, of antigen prepared in incomplete Freund's adjuvant. Blood was obtained by cardiac puncture on day 70 and the immune serum was kept in individual 2 ml tubes and stored at -75°C.

Rabbit immunoglobulin fractionation

Rabbit immunoglobulins were fractionated from the serum by ion exchange chromatography on a DEAE-Sephacel (Pharmacia) column (1.6 x 30 cm) according to Mollison's method (25), as modified by Pharmacia Fine Chemicals (26).

Immunodiffusion and immunoelectrophoresis

Immunodiffusion was carried out on 75 x 25 mm slides covered with 1% agarose gel in Tris-buffered (0.03 M) saline (NaCl 0.1 M) pH 7.2 final concentrations of 0.05 M D-galactose to prevent binding of the lectin to agarose (27); merthiolate was added (0.01%) as preservative. Microimmunoelectrophoresis was performed as previously described (13, 14).

Indirect immunofluorescence analyses

Immunofluorescence staining reactions were accomplished indirectly by the sandwich technique at 4°C (28). The hemocytes washed in FSW-EDTA containing 5% bovine serum albumin (FSW-EDTABSA) were incubated for 60 min with rabbit IgG from anti-sL immune serum, then washed three times in the same medium and incubated with anti-rabbit IgG prepared in goats and conjugated with rhodamine (Bio-Yeda). After washings in the usual manner, a few drops of cell suspension were pipetted onto slides. The dried smears were mounted in glycerol solution (9:1 in PBS, pH 7.4). The observations were carried out using an incident light fluorescence microscope (Reichert-Jung, Microstar).

Absorption

Immune serum aliquots were absorbed by mixing them (v/v) with

purified 5 fold concentrated (Diaflo equipped with UM2 membrane, Amicon Corp., Lexington, Mass.) lectin solution, and the absorbed serum was tested by immunodiffusion. Absorption was also performed with pellets containing hemocyte membranes. Lectins did not react with nonspecific rabbit immunoglobulins, as shown by absorption with a rabbit normal serum and immunodiffusion tests. Hemocyte lysate was prepared from 10^8 hemocytes collected from different specimens and suspended in 10 ml of 0.1% FSW-EDTA,, accurately suspended in 0.5 ml immune serum and shaken continuously. The suspension was then diluted to 10 times its volume in distilled water. After standing for about 10 minutes cell lysis occurred, and the pellet containing membranes was collected by centrifuging at 27,000g for 30 minutes and washed three times with FWS-EDTA. The serum sample was absorbed twice. The reaction mixtures were incubated at room temperature for 1 and 12 hours at 4°C and centrifuged at 27,000g for 30 min.

SDS-polyacrylamide gel electrophoresis and immunoblotting

SDS-PAGE in slab gels was performed according to Laemmli (29). Gels were calibrated with molecular weight marker proteins (Bio-Rad), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). The log molecular weights of standard proteins were plotted against their electrophoretic migration to obtain standard curves. Average values were obtained from eight independent experiments, and standard deviations estimated. Prior to SDS-PAGE some lectin solution dialyzed against Tris-HCl 0.55 M, pH 8.2 was reduced (2-mercaptoethanol 0.75 M final concentration) and alkylated with iodoacetamide according to Stanworth and Turner (30).

Western protein immunoblotting was performed essentially as described by Towbin et al.(31). After electrophoretic transfer of the ascidian lectin (Bio-Rad trans-blot cell) the nitrocellulose sheets were quenched by washing three times for 20 minutes with 5% BSA (Sigma) in PBS. Both rabbit antibodies to serum lectin and the developing goat antibodies (anti-rabbit IgG peroxide conjugated, Bio-Rad) were made up in 0.1% BSA prepared in Tris-NaCl and incubated with the nitrocellulose sheets for at least 60 minutes. 4-chloro1-naphthol (Merck) was used as the substrate.

Hemocyte specific binding to Sepharose or erythrocytes

Reaction mixtures (0.3 ml) containing $0.5\text{-}1.0 \times 10^5$ hemocytes and 0.1 ml packed Sepharose-HCl or $0.5\text{-}1.0 \times 10^4$ erythrocytes were incubated for 30 minutes at room temperature. At the end of this time the settled cells were gently mixed with a pipette and transferred to plastic discs for microscopic observations using a Diavert microscope (Leitz).

Protein content estimation

Quantitive protein analyses were performed using the method of Lowry et al., with BSA as a standard (32).

RESULTS

Molecular weight determination of serum lectin purified by affinity chromatography on Sepharose gel

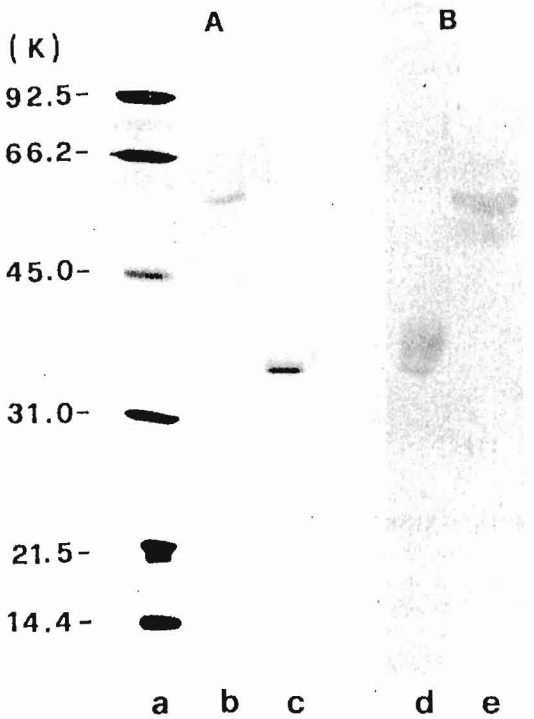

FIG. 1 A - SDS-polyacrylamide gel electrophoresis of Ascidia malaca D-galactose binding serum lectin isolated by affinity chromatography; (a) standard proteins (BioRad), (b) under reducing conditions (2-mercaptoethanol), (c) under non-reducing conditions. B - Western immunoblotting of SDS-PAGE separated anti-RE serum lectin probed with immune serum against the isolated lectin (d) under non-reducing conditions, (e) under reducing conditions. The same electrophoretic pattern was obtained even if the sample was alkylated (0.2 M iodoacetamide) after reduction before SDS-PAGE.

When the isolated D-galactose binding lectins were analyzed by SDS-PAGE under reducing conditions, a major component with an MW of 58,400 ± 3,860 (SD, n=8) was detected (Fig. 1, lane b). When the sLs were subjected to electrophoresis under nonreducing conditions the MW of the major component was 35,500 ± 1,162 (SD, n=8) (Fig. 1, lane c). The reducing agent employed was 2-mercaptoethanol or 0.1 M 1,4-dithiothreitol.

Immunological properties of the isolated lectins

In immunodiffusion and immunoelectrophoresis of serum lectins eluted from Sepharose, rabbit specific antiserum produced a single precipitation band (Fig. 2A, B). Immunoelectrophoretic patterns indicate anodal properties of the molecules (Fig. 2 A),

while western blot analyses demonstrate that the immunological features did not change, owing to reduction of the molecules (Fig. 1B, lanes d, e). SDS-PAGE denaturing conditions probably cause additional bands which are only revealed by immunoblotting.

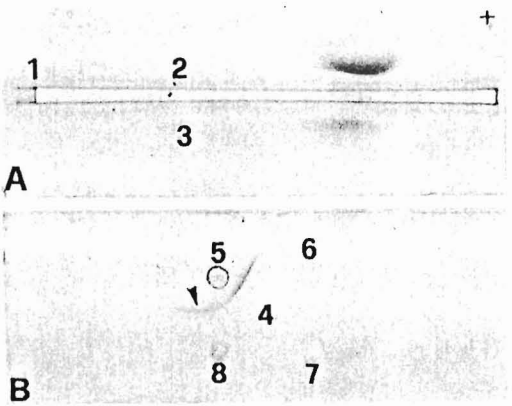

FIG. 2 A - Immunoelectrophoretic analyses of Ascidia malaca D-galactose binding serum lectin isolated by affinity chromatography: 1) anti-lectin rabbit immune serum; (2) isolated lectin; (3) whole A. malaca serum. B - Ouchterlony immunodiffusion of antilectin immune serum before or after absorption: (4) isolated lectin; (5) anti-lectin immune serum; (6) anti-lectin immune serum absorbed with hemocyte membranes; (7) rabbit non-immune serum; (8) anti-lectin immune serum absorbed with ten times concentrated lectin. A precipitation line (arrow) occurs where excess lectins contained in the absorbed immune serum (well 8) diffuse against specific antibodies (well 5).

Hemocyte specific binding to Sepharose or erythrocytes

Hemocytes were bound to Sepharose forming bridges between spherules (Fig. 3b), but they did not adhere to Sepharose beads in the presence of D-galactose (25 mM). When the reaction mixture contained erythrocytes two interaction products could be observed: 1 - clumps of aggregates composed of hemocytes and rabbit or sheep (SE) erythrocytes; 2 - rosettes (Fig. 3, c,d,e), which were observed when more dilute suspensions of erythrocytes were used (0.5-1.0 x 10^3 cells in 0.2 ml). Hemocytes with not less than three erythrocytes attached were considered as positive rosettes; clumps of erythrocytes were very rare. In both cases the intensity of the reactions (larger clumps or more numerous rosettes) depended on the hemocyte number, as observed when serial two-fold dilutions were used.

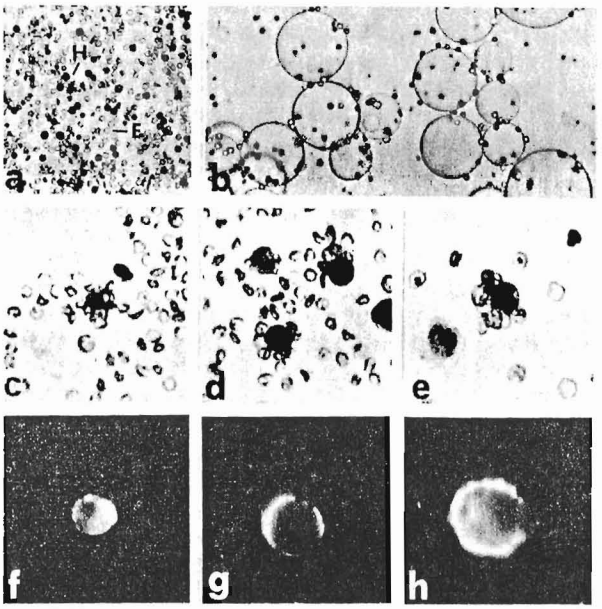

FIG. 3 Ascidia malaca hemocytes binding to Sepharose spherules or rabbit erythrocytes. (a) Hemocytes (H) and rabbit erythrocytes (E) in the presence of D-galactose (25mM), x128; (b) hemocyte binding to Sepharose-HCl spherules, x90 - unbound Sepharose beads in the presence of D-galactose were not shown; (c, d, e) small, medium and large hemocytes forming rosettes with RE, vitally stained with 0.001% neutral red, x410; (f, g, h) small, medium and large hemocytes after immunofluorescence staining by indirect method: primary rabbit immunoglobulins to serum lectin (dilution 1:10); secondary goat rhodamine-conjugated antibodies (dilution 1:1000), x1,120. Unstained negative hemocytes are not shown.

We distinguished positive and negative hemocytes according to their ability to bind erythrocytes. Moreover, hemocytes were small (4.5-4.9µm), medium (7.4-8.5µm) and large (9.5-11.0 µm) rosette-forming cells (RFCs), some of which were vitally stained with 0.001% neutral red (Fig. 3, c, d, e). Supernatants obtained by centrifuging cell suspensions in FSW-EDTA at 0°C and 800g for 15 minutes did not show hemagglutinating activity towards RE and SE even after 2 hours incubation at room temperature. This suggests that hemocytes do not secrete an erythrocyte-aggregating factor.

Competitive inhibition experiments employing sugars

Since the anti-RE lectin isolated from A. malaca serum possesses binding specificities for sugars containing a D-galacto configuration (melibiose, raffinose, galactose, lactose, lactulose), experiments were performed to determine whether

these sugars and D-glucose could inhibit hemocyte-Sepharose or hemocyte-erythrocyte bindings as indicated by clump or rosette formation. The results suggested that a receptor with a binding site similar to that of the serum lectin is distributed on the hemocyte membrane surfaces. In fact, melibiose and raffinose were the best inhibitors and active up to a 3.6 mM final concentration. D-galactose inhibits at 25mM final concentration whereas D-glucose, used as a control, was inactive at 100mM (Fig. 3a).

Inhibition of hemocyte binding

Experiments were also performed to inhibit hemocyte binding ability by anti-sL rabbit immunoglobulins. In three experiments different cell concentrations (3-10 x 10^6 per ml) were incubated for 30 minutes at 37°C with decreasing anti-sL IgG dilution or normal rabbit IgG. Hemocytes were aggregated in different degrees depending on the IgG dilution, however a few fine aggregates were found in the presence of normal rabbit serum (Table 1). Antisera absorbed with sL or hemocyte membranes lost their aggregating ability, whereas hemocytes of the reaction mixtures did not; they reacted with RE by forming large aggregates and rosettes.

Membrane lectins on hemocytes

Immunofluorescent staining reactions were performed using 10,500 hemocytes from eight different animal lots (about 80 specimens). Positivity was defined on the basis of the granular appearance of fluorescence on cell surfaces. Immunological specificity of the fluorescence after staining was established by absorbing the anti-sL IgG with purified lectins or hemocyte membranes (Table 1). The absorbed immune sera were monitored by immunodiffusion (Fig 2B). Controls against non-specific fluorescence were performed by substituting the specific IgG with PBS or IgG from unimmunized rabbits.

As shown in Table 1, significant differences were found between percentage of positive cells (43.3 ± 4.7%) after anti-sL antibodies incubation and percentage of positive cells after primary incubation with: 1) immune serum absorbed with purified sL (11.0 ± 2.7% positive cells); 2) immune serum absorbed with hemocyte membranes (6.0 ± 1.4% positive cells); 3) normal rabbit IgG (9.0 ± 2.0% positive cells); 4) goat rhodamine-conjugated IgG anti-rabbit IgG (0.1 ± 0.01% positive cells); 5) PBS (no positive cells). Evidently antibodies which cross-react with some hemocyte receptors are present in normal rabbit serum.

Although we did not distinguish different cell populations we found, on the basis of cell dimension, that hemocytes similar to those indicated as RFCs can be stained by immunofluorescence (Fig. 3, f, g, h). Hemocytes capped for anti-sL antibodies were observed after warming the incubation mixtures to room temperature.

TABLE I

Membrane lectin of the Ascidia malaca hemocytes demonstrated by anti serum-lectin antibodies.

Rabbit antibodies	Aggregation activity against hemocytes (3-10 x 10^6/ml) (1)	Hemocytes positive by immuno-fluorescence (average %±S.D.) (2)
Anti-sL	10-15	43.3 ± 4.7
Non-immune	<2-2	9.0 ± 2.0
Anti-sL absorbed with sL	<2-2	11.0 ± 2.7
Anti-sL absorbed with hemocyte membranes	0	6.0 ± 1.4
--- (3)	0	0.1 ± 0.01 (4)

(1) Aggregation titers obtained from repeated tests and expressed as reciprocal of last immune serum dilution causing aggregation of hemocytes. (2) Indirect method by goat rhodamine-conjugated anti-rabbit Ig secondary antibodies; a total of 10,500 hemocytes from eight different animal lots (about 80 specimens). (3) FSW-EDTA or FSW-EDTA-BSA without rabbit antibodies. (4) Secondary antibodies alone.

DISCUSSION

Lectins with binding specificity for several different saccharides are widely distributed in invertebrates (11, 13, 14, 16-18). Their biological function is unclear although their involvement in defense mechanisms has often been proposed, but not clearly supported by rigorous experiments (2, 3, 5-7, 15). However, lectins accessible on the cell surface allow us to postulate a significant role for them in cellular recognition in which glycosylate membrane proteins may interact with lectins on other cells or express a receptor function using surface oligosaccharides in order to phagocytose foreign particles (3, 4).

In a previous paper we showed that the blood of Ascidia malaca contains galactose specific serum lectins which can be purified by affinity chromatography on Sepharose-HCl; the purified fraction contains a protein with an anodal electrophoretic mobility (13). We have now demonstrated that the molecule has a major component with an apparent molecular weight of about 58,000, determined by SDS-PAGE, which moves more rapidly under non-reducing conditions. Accordingly, "shrinkage" could depend on the intramolecular disulfide linkage which would produce a more compact protein structure. This agrees with the high resistance to thermal denaturation (inactivated at 100°C)

and the inactivation by mercaptoethanol already demonstrated (12). Moreover, the lectin might not be disulfide-linked, neither to itself nor to other polypeptide molecules.

This serum lectin contains immunological determinants common to hemocyte membrane components, demonstrated by producing an immune serum against the isolated serum fraction and testing it on hemocyte surfaces by indirect immunofluorescence. Membrane lectin is indicated in about 34% of the hemocytes, whether small, medium or large cells. This percentage allows for the fact that the rabbit serum may contain antibodies which cross-react with hemocyte membrane components producing misleading positivity.

The capacity of some of the hemocytes to bridge with RE and SE confirms the presence of surface lectin on these blood cells. Moreover, the hemocyte binding with Sepharose-HCl spherules, and the results of the sugar inhibition tests sustain the hypothesis that a lectin with the same specificity, and some structural similarities, can be found free in the serum, and present on hemocyte surfaces. The poor ability of D-galactose to block binding, and the stronger inhibition power of D-melibiose and D-raffinose, probably indicate that the membrane lectin, like the serum one, recognizes a determinant formed of several monosaccharides provided with a D-galacto configuration.

Even if the positive hemocyte populations have not yet been characterized morphologically, the fact that hemocytes of different sizes can be RFCs, and are stained by immuno-fluorescent methods seems to indicate that different cell types contain receptor sites, although some of these observations could also be attributable to a single type which appears at different maturation stages. Further evidence of hemocyte surface lectin is given by the agglutinating power of the anti-sL antibodies (dilution ratio 1:15) in comparison with normal rabbit serum (dilution 1:2), versus hemocytes. Absorptions with purified sL or hemocyte membranes significantly lowered titers of the agglutinating antibodies, while, after treatment, hemocytes retained their capacity to bind erythro-cytes, and produce cell agglutinates or rosettes.

Preliminary observations suggest that sugar binding lectins are also distributed on hemocyte surfaces in another ascidian Phallusia mamillata (33). It should be noted that lectin mole-cules accessible on cell surfaces may play a significant role in cellular recognition or communication, in which glycosylated membrane proteins interact with lectins on other cells (34). This possibility is substantiated by the finding that lectins with binding specificity for several different saccharides have been detected on the membranes of a variety of mammalian cells including lymphocytes (19, 20). In particular, it has been established that proteins can be isolated from lymphocyte membranes by their affinity binding to fetuin glycopeptides (19).

Studies are in progress to isolate and characterize membrane lectins from A. malaca and Ph. mamillata hemocytes (33).

Furthermore, the information on the occurrence of D-galactose binding lectin on cell surfaces provides the basis for fractionation studies employing Sepharose affinity chromatography of hemocyte populations. This approach will be useful for the future in identifying different cell types and characterizing their biological importance.

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