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Due to an interest in the evolutionary origins of vertebrate mechanisms of immunorecognition, researchers have frequently turned to deuterostome invertebrates as model organisms. The purple sea urchin, Strongylocentrotus purpuratus (Echinodermata: Echinoidea), was selected for this study of putative immunorecognition molecules in a deuterostome invertebrate.

Hemagglutinins, molecules which agglutinate red blood cells, have been reported in most invertebrates including echinoderms. Most relevant studies have characterized hemagglutinins as lectins, carbohydrate-binding molecules, and for this reason these substances have been proposed as primitive recognition molecules. The work presented here describes the occurrence and range of hemagglutinins in the coelomic fluid of Strongylocentrotus purpuratus, and attempts to characterize the sugar-binding

capabilities of these molecules and their responses to various chemical and enzyme treatments.

The results of screening and cross-adsorption experiments with vertebrate red blood cells are described. The existence of at least two hemagglutinating activities in the coelomic fluid of S. purpuratus is demonstrated. Results presented for sugar-inhibition tests support this finding. Glycoproteins were found to be the best inhibitors of all hemagglutinating activity in coelomic fluid. This information suggests that the binding sites for S. purpuratus hemagglutinins may be more complex than can be revealed by mono- and simple oligosaccharide inhibition experiments. The agglutinating activities were unaffected by trypsin, pronase, or periodate treatments, and were relatively heat-stable. In general, the information presented in this thesis shows that multiple hemagglutinating activities, that appear to act as lectins, are present in the coelomic fluid of Strongylocentrotus purpuratus.

Characterization of Putative Recognition Components of Echinoderm Coelomic Fluid: Agglutinins in *Strongylocentrotus purpuratus*

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INTRODUCTION

Organisms maintain an aseptic environment within the body in several ways. One example is the possession of an environmental barrier such as a body wall. When such defenses fail, internal mechanisms must be available to eliminate the invading pathogens or other foreign material.

The ability of an organism to respond defensively to foreign substances, its immunocompetence, has been described in many ways. It has been proposed (Hildemann, Clark, and Raison, 1981) that three criteria must be met to qualify as an immune response. These include selective or specific response to an antigen, anamnesis or enhanced second reaction on re-exposure to an antigen, and concurrent cytotoxic or antagonistic reaction to an antigen. Several levels of immunocompetence have been described for the invertebrates and vertebrates. Vertebrate immune systems are considered highly immunocompetent, and integrate several mechanisms to deal with foreign antigens. The level of complexity of these systems varies among vertebrate groups, with a general trend towards a decrease in complexity in lower vertebrates, e.g. fewer types of immunoglobulin.

Deuterostome invertebrates provide excellent research models for investigating more primitive immune recognition mechanisms and molecules which effect vertebrate immune responses such as lymphokines, complement components, and immunoglobulins. Despite research, immunoglobulins have not been discovered in any invertebrate phylum.

Invertebrates are considered to be immunocompetent on the basis of several criteria acceptable to immunologists. Tissue transplantation techniques are used to determine the nature and specificity of immune responses in various invertebrates. Responses to grafts in both the invertebrates and vertebrates depend on the immunocompetence of the recipient and the extent of genetic similarity between recipient and donor. Graft rejection is generally accompanied by cellular infiltration of the transplanted tissue and subsequent cytotoxic events (Hildemann and Dix, 1972; Karp and Hildemann, 1976). Rejection of allogeneic and/or xenogeneic tissues is demonstrated in sponges (Hildemann and Johnston, 1979; Hildemann, Bigger, Johnston, and Jokiel, 1980a; Van de Vyver, 1980), cnidarians (Hildemann, Jokiel, Bigger, and Johnston, 1980b), nemertines (Langlet and Bierne,1977), arthropods (Lackie, 1983), annelids (Cooper, 1968, 1969; Valembois, Roch, and

Boiledieu, 1980), echinoderms (Ghiradella, 1965; Hildemann and Dix,1972; Karp and Hildemann, 1976; Coffaro and Hinegardner, 1977; Coffaro, 1980), and tunicates (Scofield, Schlumpberger, West, and Weissman, 1982). Transplantation studies have demonstrated that specific immunological memory, evidenced by enhanced reaction on re-exposure to a graft, occurs in several invertebrate phyla, including cnidarians (Hildemann et al, 1980b) annelids (Cooper, 1970) and echinoderms (Karp and Hildemann, 1976, Coffaro and Hinegardner, 1977, Coffaro, 1980).

In addition to transplantation reactions, bacterial or other particle clearance studies demonstrate immunorecognition in invertebrates. The body fluids of these animals are generally aseptic (Bang and Lemma, 1962; Yui and Bayne, 1983); therefore, some mechanism for elimination of potentially infectious agents must be in operation. The ability to clear inorganic particles or foreign cells (e.g., blood cells, yeast, or bacteria) was demonstrated in annelids (Dales and Dixon, 1980; Fitzgerald and Ratcliffe, 1982), arthropods (Pistole and Britko, 1978; Smith and Ratcliffe, 1980), molluscs (Bayne, 1973a, b; Renwrantz and Mohr, 1978), echinoderms (Bang and Lemma, 1962; Brown, 1967; Hilgard and Philips, 1968; Johnson, 1969; Coffaro, 1980; Bertheussen,1981; Yui

and Bayne, 1983), tunicates (Wright and Cooper, 1975), and cephalochordates (Rhodes, Ratcliffe, and Rowley, 1982). Phagocytosis, the first line of internal defense in both vertebrates and invertebrates, was identified as a major mediator in clearance processes (Bang and Lemma, 1962; Smith and Ratcliffe, 1980; Fitzgerald and Ratcliffe, 1982; Yui and Bayne, 1983). Humoral factors (bacteriocidins and agglutinins) may aid in aggregation or opsonization of foreign material and thus aid phagocytosis (Wardlaw and Unkles, 1978; Sminia, van der Knaap, and Edelenbosch, 1979; Renwrantz and Stahmer, 1983).

Transplantation and clearance studies suggest that specific immunorecognition and some degree of anamnestic response are widespread in invertebrates. However, neither immunoglobulins nor lymphocytes are found in invertebrates. Lymphocyte-like cells (Wright and Cooper, 1975; Warr, Decker, Mandel, Deluca, Hudson, and Marchalonis, 1977) and molecules with structural homologies to Beta-2-microglobulin (Shalev, Greenberg, Logdberg, and Bjork, 1981) and to immunoglobulins (Carton, 1974; Vasta and Marchalonis, 1983) have been described in several invertebrates, but there is no evidence for their potential roles in immunity. The question remains, how do invertebrates achieve the

specificity of recognition implied by graft rejection and clearance studies?

The invertebrates comprise an extrememly diverse group of phyla with widely ranging habitats, life histories, and a very long evolutionary history. Therefore, it is possible to suggest that invertebrates probably use several mechanisms of immunorecognition. Researchers have frequently turned to deuterostome invertebrates in the search for evolutionary origins of vertebrate mechanisms of immunorecognition. Several phyla compose the deuterostome invertebrates and within this group, echinoderms hold a pivotal position and are thus an important group for investigations of primitive recognition molecules that may have homologues in vertebrate immune systems.

The purple sea urchin, Strongylocentrotus purpuratus (Echinodermata: Echinoidea), was selected for this study of putative immunorecognition molecules in deuterostome invertebrates. There are several advantages to using S. purpuratus in a study of this kind. These animals are abundant and easily collected on the Oregon coast and can be maintained in a circulating sea water system for several weeks in apparent good health. They also provide ample volumes of coelomic fluid and can be repeatedly bled.

Echinoderms display both memory and specificity in response to allogeneic transplants (Hildemann and Dix, 1972; Karp and Hildemann, 1976; Coffaro, 1980) and specific response to foreign material injected into the coelom (Bang and Lemma, 1962; Hilgard and Philips, 1968; Yui and Bayne, 1983). Echinoderm coelomic fluids contain various humoral factors, such as bacteriocidins (Johnson, 1969; Messer and Wardlaw, 1979), hemolysins (Ryoyama, 1973; Parrinello, Rindone, and Canicatti, 1979; Bertheussen, 1983), and agglutinins/hemagglutinins (Tyler, 1946; Brown, Almodovar, Bhatia, and Boyd, 1968; McKay, Jenkin, and Rowley, 1969; Ryoyama, 1974; Bertheussen, 1983).

Agglutinins are proposed as primitive recognition molecules (Burnet, 1974). Hemagglutinins, molecules that agglutinate red blood cells (rbcs), are reported in most invertebrates (Tyler, 1946; McKay, Jenkin, and Rowley, 1969; Anderson, 1980; see review, Yeaton, 1981) including echinoderms (Tyler, 1946; Brown et al, 1968; Ryoyama, 1974; Parinello, Rindone, and Canicatti, 1979; Bertheussen, 1983). Many studies characterize hemagglutinins as lectins, multivalent carbohydrate-binding molecules. Lectins recognize and reversibly bind to carbohydrate determinants that are widespread on cell surfaces, and for this reason are

proposed as primitive recognition molecules (Acton and Weinheimer, 1974; Marchalonis, 1977; Stein and Cooper, 1982).

In some invertebrates, agglutinins/lectins are described that have the ability to agglutinate bacteria (Cornick and Stewart, 1968; Pauley, Krassner, and Chapman, 1971) and eukaryotic parasites (Bang, 1967; Lackie, 1981; Pereira, Andrade, and Ribeiro, 1981; Loker, Yui, and Bayne, 1984), as well as function as opsonins (Tripp, 1966; Tyson and Jenkin, 1974; Harm and Renwrantz, 1980; Renwrantz and Stahmer, 1983).

The specificity and number of agglutinins in invertebrate body fluids is usually determined by agglutination of vertebrate rbcs and by cross-adsorption experiments with these cells. Echinoderms, specifically echinoids, possess agglutinins that bind to a number of vertebrate rbc types, and this activity is due to more than one agglutinin (Ryoyama, 1974).

The majority of red blood cell surface determinants are glycoprotein or glycolipid in nature; therefore sugar inhibition assays provide a means to determine the potential carbohydrate-binding specificities of agglutinins. Sialic acid (N-acetylneuraminic acid), a common component of many biologically important glycosylated molecules, is known to be a potent inhibitor

of several invertebrate agglutinins (Hall and Rowland, 1974; Anderson and Good, 1975; Vasta, Warr, and Marchalonis, 1982). In addition to sialic acid, a number of agglutinins with binding specificities for galactose (Parinello and Canicatti, 1982; van der Knaap, Doderer, Boerrigter, and Sminia, 1983), N-acetylated sugars (Garte and Russell, 1976; Vasta, Warr, and Marchalonis, 1982; Boswell and Bayne, 1984), and others (lactose: Parinello and Canicatti, 1983; fucose: Bertheussen, 1983) are described.

For some hemagglutinins, a specific carbohydrate inhibitor has not been described. Several hemagglutinins are inhibited by complex glycoproteins, such as fetuin, mucin, and serum glycoproteins (Vasta, Warr, and Marchalonis, 1982; Renwrantz ans Stahmer, 1983), and therefore may recognize subterminal carbohydrate structures, complex branched oligosaccharides, or amino-linked carbohydrates (Lis and Sharon, 1981).

Most agglutinins, including those of vertebrates (Simpson, Thorne, and Loh, 1978), are proteins or glycoproteins, but several anomalous molecules have been described that appear to be carbohydrate in nature. A hemagglutinin in the tunicate, Styela plicata , is described which is heat stable, trypsin-resistant, and inactivated by periodate treatment (Fuke and Sugai,

1972). These observations led Fuke and Sugai (1972) to suggest that the agglutinating activity in S. plicata coelomic fluid may be a mucopolysaccharide or polysaccharide. A non-protein agglutinin may also occur in the echinoderms Anthocidaris crassipina and Pseudocentrotus depressus , since agglutinating activity is completely destroyed by 25mM periodate and is unaffected by proteolytic enzymes (Ryoyama, 1974).

A wide variety of agglutinins, with the potential of being primitive recognition molecules, exist in most invertebrate phyla including the echinoderms. I have therefore investigated the spectrum of hemagglutinating activities found in the purple sea urchin, Strongylocentrotus purpuratus , and attempted to biochemically characterize these activities in whole coelomic fluid.

MATERIALS AND METHODS

1) Experimental Animals and Coelomic Fluid Collection

Strongylocentrotus purpuratus were collected from Yaquina Head, Newport, Oregon approximately every three weeks. Animals were maintained at 15 C in a 400 liter tank associated with the 22,700 liter recirculating sea water system at Oregon State University, Corvallis, Oregon.

Coelomic fluid (CF) was acquired from sea urchins by peristomial puncture with a 20 gauge needle and drawing approximately 5 ml of the coelomic fluid into a syringe. Coelomic fluid was immediately filtered gently through a 5 micrometer Millipore filter plus prefilter, and then finally through a 0.45 micrometer sterile Millipore filter. This rapid removal of coelomocytes precluded any need for anticoagulants. Each sample was a pool of filtered coelomic fluid samples from five urchins.

All CF pools were incubated for 15 min. at 37 C in order to inactivate hemolytic activity present in coelomic fluid (unpublished observation). Heat-treated coelomic fluid (HT-CF) was then stored at 4 C until use.

2) Hemagglutination Assays

Samples of normal blood from a variety of vertebrate species were obtained from several sources available at Oregon State University and the City of Corvallis, OR. Whole blood in Alsever's solution (anticoagulant and blood storage solution) was centrifuged at maximum in an International Clinical Centrifuge for 5 minutes to pellet cells. Erythrocytes were washed three times in a phosphate hemagglutination buffer (0.066 M Phosphate, 0.85 % NaCl, pH 7.3, after Wheeler, Luhby, and Scholl, 1950), and then prepared as a 10% suspension in TBS (tris-buffered saline: 100 mM tris-HCl, 0.15 M NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.02% sodium azide, pH 7.3). Stock suspensions of rbcs were stored at 4 C.

Enzyme treatments of rbcs have been shown to increase availability of binding sites for agglutinins and give higher and more consistent titers (Uhlenbruck, Pardoe, and Bird, 1969). Pronase treatment of rbcs consisted of mixing a packed volume of washed rbcs with two times their volume of 0.1% pronase (Sigma Chemical Co.,Type XIV, protease from Streptomyces griseus) in TBS, pH 7.3. The erythrocyte and pronase mixture was incubated for 30 min. at 37 C. Cells were then washed three times with TBS and stored as a 10% suspension.

Agglutination tests were performed in 96 well microtiter plates (well volume 250 ul). Serial two-fold

dilutions of HT-CF (50 ul) were prepared in microtiter plates and an equal volume of a 2% suspension of rbcs was added. Well contents were mixed for 10 seconds and the plates incubated for one hour at room temperature, then stored at 4 C. The titer was recorded as the reciprocal of the highest dilution giving agglutination. Titers were read at 2 and 24 hours (no change in titers was observed between these two times). Control wells contained TBS and rbcs, but no HT-CF.

A) Cross-Adsorption Tests

HT-CF was diluted 1:2 with TBS and mixed with one third its volume of washed, packed, enzyme treated or untreated rbcs for one hour at room temperature or until titers to the adsorbing blood cell was zero. Erythrocyte-HT-CF mixtures were centrifuged at maximum for 5 minutes in an International Clinical Centrifuge and the supernatant removed and used in a hemagglutination assay as described above.

B) Hemagglutination-Inhibition Tests

For inhibition of activity to untreated rbcs, coelomic fluid was two-fold serially diluted with TBS (50 ul/well) in microtiter plates and an equal volume of a 200 mM solution of inhibitor (working concentration 100 mM) was added to each well. Mono- and oligosaccharides were dissolved in TBS at concentrations up to 200 mM, and

polysaccharides and glycoproteins up to 1% (w/v). All inhibitor solutions were adjusted to pH 7.3. Plates were incubated for one hour at room temperature and an equal volume of a 2% suspension of rbcs was added to each well.

Inhibition tests using pronase-treated rbcs were patterned after Vasta, Warr, and Marchalonis (1983). Hemagglutinating titers were determined for each test rbc type. The titer is equivalent to the reciprocal of the highest dilution giving agglutination. HT-CF was diluted with TBS to one-half of the original titer (therefore, diluted to twice the concentration of HT-CF in the final dilution giving agglutination). Inhibitors were two-fold serially diluted with TBS in 96 well microtiter plates and an equal volume of diluted coelomic fluid was added. Plates were incubated for one hour at room temperature after which an equal volume (50 ul) of a 2% suspension of rbcs was added to each well. Titers and inhibiting concentrations were recorded after two hours. Controls included replacement of HT-CF or inhibitor by TBS. Inhibition corresponded to elimination of agglutinating activity in any test well. The inhibiting concentration is the lowest concentration of a sugar which gave inhibition.

In order to determine if sialic acid residues on glycoproteins were responsible for inhibition of

agglutinating activity, desialyzed glycoproteins were prepared following the method of Vasta, Warr, and Marchalonis (1982). Glycoproteins were dissolved in 0.1 N H_2SO_4 in 0.85% NaCl at a concentration of 10 mg ml^{-1}. Reaction mixtures were incubated for one hour at 80 C. Protein solutions were then dialyzed extensively against TBS at 4 C before use.

3) Coelomic Fluid Treatments

The effect of temperature on hemagglutinating activity was investigated. Samples of HT-CF were incubated for 30 min. in a water bath adjusted to 25, 37, 56, 65, 75, 85, or 100 C. Individual HT-CF samples were exposed to only one test temperature. The heat-treated samples were then tested for their ability to agglutinate pronase-treated rabbit rbcs.

In order to determine if hemagglutinating activity in HT-CF was mediated by proteins, HT-CF was digested with both trypsin and pronase. HT-CF (0.5 ml) was incubated with 0.5 ml of either trypsin (0.5, 2.0, or 5.0 mg ml0-1 in TBS, pH 7.3, Sigma Chemical Co., Type IX; after Ryoyama, 1974) or pronase (0.5, 2.0, or 5.0 mg ml^{-1} in TBS, pH 7.3, Sigma Chemical Co. Type XIV; after Ryoyama, 1974) for one hour at 37 C. Reaction mixtures were brought to room temperature, then serially

two-fold diluted with TBS, and an equal volume of a 2% suspension of pronase-treated rbcs was added. Incubation was done at 4 C to minimize further ezymatic activity. Controls included replacement of enzyme or HT-CF with TBS. Proteolytic activity of enzyme preparations was confirmed by azocasein hydrolysis (Fisher and Allen, 1958).

In order to determine if carbohydrate configuration might be important to hemagglutinating activity, periodate oxidation of coelomic fluid was done. The reaction mixture was as follows: 1.0 ml of HT-CF; 0.3 ml of 0.2 M acetate buffer, pH 5.4; 0.2 ml of various concentrations of sodium metaperiodate (0.005, 0.010, 0.015, 0.025, 0.05, 0.10 M) adjusted to pH 5.4. HT-CF-periodate was incubated for 3 hours at 25 C and then dialyzed against TBS overnight. After dialysis, the hemagglutinating activity of the treated coelomic fluid was tested. As a control for periodate activity, a 1.0 ml sample of a hemagglutinin from the gastropod snail, Biomphalaria glabrata , whose activity is destroyed by 10mM periodate treatment (Boswell and Bayne, 1984) was treated in parallel and tested for its ability to agglutinate pronase-treated rabbit rbcs.

In order to determine if Ca^{2+} or Mg^{2+} was a requirement for activity, HT-CF was dialyzed against

calcium- and magnesium-free TBS (TBS-CMF) containing 50mM EGTA or EDTA, and then dialyzed extensively against TBS-CMF and tested for hemagglutinating activity. To test for reversibility of any loss of activity, EGTA- or EDTA-treated HT-CF was serially two-fold diluted in TBS-CMF and an equal volume of a 2.5, 5.0, 10.0, 20.0, 30.0, or 50.0mM calcium- or magnesium-containing tris-buffered saline was added. An equal volume of a 2% suspension of rbcs in TBS-CMF was then added to each well, and titers were determined.

RESULTS

Screening for Hemagglutinins in *Strongylocentrotus purpuratus* Coelomic Fluid

1) Spectrum of Hemagglutinating Activity for Untreated and Enzyme-Treated Rbcs

S. purpuratus coelomic fluid contains hemagglutinating activity for a number of vertebrate rbcs. Only three of the untreated rbc types tested (rabbit, rat, pig) are obviously agglutinated by *S. purpuratus* coelomic fluid, and even for these, the titers are consistently strong for only rabbit and pig. In some HT-CF pools, titers for even these cells may be low. After pronase treatment, a much wider variety of cells were agglutinated. Except for horse rbcs, which were not agglutinated in any test, pronase treatment increased titers for all rbcs tested. The data presented in Table 1 represents ranges derived for each blood cell type tested with a minimum of three coelomic fluid pools.

2) Cross-Adsorption

In order to determine if the agglutinating activity for untreated and enzyme-treated rbcs was due to more than one agglutinin, titers were determined for a panel

of rbcs after all activity to one had been removed by adsorption (cross-adsorption tests). The coelomic fluid of S. purpuratus appears to contain at least two agglutinating specificities. Adsorptions with untreated rbcs (Table 2) indicate that an agglutinin is present for determinants shared by pig, rabbit, and rat rbcs, and weakly suggests an additional agglutinating activity to rabbit rbcs alone. The results of one experiment are presented in Table 2, and this experiment was replicated three times producing the same adsorption pattern.

This picture is further clarified by cross-adsorption tests with pronase-treated rbcs (PR-rbcs) (Table 3). This approach confirms the existence of at least two agglutinating specificities in the coelomic fluid of S. purpuratus. An agglutinin which recognizes PR-rabbit, PR-rat, and PR-pig rbcs is suggested by adsorptions done with these cells. PR-rat rbcs adsorb all activity for PR-pig rbcs, and the reverse of this adsorption is also true. PR-rabbit rbcs remove all agglutinating activity from the coelomic fluid, thus confirming that PR-rabbit, PR-rat, and PR-pig rbcs share receptor for an agglutinin in the coelomic fluid. Neither rat nor pig can remove all the activity for rabbit rbcs and, therefore, an additional agglutinating activity for PR-rabbit rbcs, separate from that previously described,

must be retained in the coelomic fluid. Adsorption with any type of PR-human rbc removes the activity to any other type of PR-human rbc, and leaves intact activity for PR-rabbit, PR-rat, and PR-pig rbcs. Pr-rat rbcs adsorb activity for PR-pig, but leave some activity for PR-rabbit and PR-human O type cells. The fact that PR-rabbit rbcs remove activity for PR-human rbcs indicates that an agglutinin for PR-rabbit and PR-human rbcs exists in the coelomic fluid. This experiment was replicated three times and results were consistent for all repetitions. An initial serial adsorption of coelomic fluid with PR-rat rbcs and then PR-human rbcs suggests the existence of an agglutinin to only rabbit cells, but further studies are necessary to confirm this observation.

3) Sugar-Inhibition Experiments

Inhibition of agglutinins to untreated rbcs was demonstrated for rabbit, rat, and pig cells. Sugars tested included D-galactose, D-glucose, L-fucose, L-rhamnose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, L-arabinose 2-deoxyglucose, galactosamine HCl, D-melibiose, cellobiose, trehalose, digitoxose, and melezitose. Activity to rabbit rbcs was inhibited by 2-deoxyglucose (100mM) and D-galactose

(100mM), and activity to rat and pig was inhibited by melibiose (50.0mM and 12.5mM respectively). Further inhibition studies were pursued with PR-rbcs, since the hidden determinants revealed by pronase treatment provided a clearer picture of the agglutinating activity in S. purpuratus coelomic fluid.

Inhibition of agglutinating activities to PR-rbcs by various carbohydrates and glycoproteins is presented in Table 4. None of the mono-, di-, or oligosaccharides tested consistently inhibited hemagglutinating activity for PR-rabbit and PR-human rbcs. All of the glycoproteins tested, including desialyzed porcine stomach mucin, bovine submaxillary mucin, and fetuin, inhibited activity to these cell types. Glycoproteins were also good inhibitors of activity to PR-rat rbcs. Fetuin, porcine stomach mucin, and thyroglobulin were all good inhibitors of hemagglutinating activity to PR-rat rbcs. The lack of inhibition by N-acetylneuraminic acid and colominic acid (a homopolymer of 2,8 linked N-acetylneuraminic acid residues) and the excellent inhibition seen with desialyzed glycoproteins for activity to PR-rabbit and PR-human rbcs indicates that this hemagglutinin is not sialic acid-specific. Melibiose did not inhibit agglutinating activity for PR-rabbit and PR-human rbcs, but was effective in inhibiting activity for PR-rat rbcs.

Each carbohydrate or glycoprotein was tested for its ability to inhibit agglutination a minimum of three times. Only those compounds tested that produced consistent inhibition are reported as positive inhibitors of agglutination.

Chemical Properties of Agglutinating Activity in S. purpuratus Coelomic Fluid

Hemagglutinating activity in S. purpuratus coelomic fluid is relatively heat-stable. Greater than 50% of the agglutinating activity to PR-rabbit rbcs was retained after HT-CF was exposed to 75 C for 30 min.; however, all activity was destroyed at 85 C. These results were consistent for the three coelomic fluid pools tested.

Trypsin and pronase digestion of coelomic fluid only slightly affected hemagglutinating activity for PR-rabbit cells. Azocasein hydrolysis confirmed a high degree of proteolytic activity in the enzyme preparations.

Periodate oxidation with up to 100mM periodate did not affect hemagglutinating activity for PR-rabbit rbcs. The activity of the agglutinin from Biomphalaria glabrata was depleted by parallel treatment with 50mM periodate, which indicates that the periodate was

effective under the experimental conditions used (Boswell and Bayne, 1984). The S. purpuratus hemagglutinating activity was not affected by either the experimental pH (5.4) or dialysis against TBS.

Results with EGTA and EDTA treatment of coelomic fluid produced inconsistent results. While treatment with either EGTA or EDTA at levels of 50mM decreased agglutinating activity in some HT-CF pools, this was not always consistent for all pools tested. Furthermore, activity was not consistently restored by addition of Ca^{2+} or Mg^{2+}.

All chemical treatments were tested using a minimum of three coelomic fluid pools and results achieved were consistent for all experiments.

DISCUSSION

Hemagglutinins have been described in a number of echinoderms (Tyler, 1946; Brown et al, 1968), but the number of agglutinins and their binding specificities have been described for only a few members of this phylum (Ryoyama, 1974; Bertheussen, 1983).

As shown by the results of screening experiments with a variety of vertebrate rbcs, Strongylocentrotus purpuratus agglutinins bind a number of the rbcs tested. It appears that determinants on rbcs are made more available to the hemagglutinins by pronase treatment. This suggests that these pronase-resistant determinants may be intrinsic glycoproteins or glycolipids (Vasta, Warr, and Marchalonis, 1982). These results also indicate the importance of using enzyme- or chemically-altered rbcs when attempting to investigate the range of hemagglutinating activities in invertebrate body fluids.

The existence of at least two hemagglutinating activities is demonstrated by cross-adsorption experiments with untreated rbcs. Adsorptions done with pronase-treated rbcs confirm this, and suggest the possibility of a third hemagglutinin in S. purpuratus coelomic fluid. One hemagglutinin recognizes shared determinants on PR-rabbit, PR-rat, and PR-pig rbcs, and

another recognizes PR-rabbit and PR-human cells. A potential third hemagglutinating activity may bind to PR-rabbit rbcs only.

Sugar inhibition tests support the pattern of hemagglutinating activity described above. Inhibition of activity against untreated rabbit, rat, and pig rbcs indicates that a similar sugar structure may be part of the binding site for their common agglutinin. The rabbit inhibitor, D-galactose, and the pig and rat inhibitor, melibiose, both share common structural features. Melibiose is a disaccharide composed of a galactose residue alpha-1,6 linked to D-glucose. Therefore, the arrangement of hydroxyl groups at carbons 3,4,5 and 6 of both D-galactose and the D-galactose residue of melibiose could provide a common binding site for an agglutinin which recognizes rabbit, rat, and pig rbcs. The inhibition by 2-deoxyglucose may correspond to the additional activity to rabbit cells as suggested by cross-adsorption experiments.

The inhibition patterns seen with PR-rbcs indicate that the binding sites for the agglutinins in S. purpuratus coelomic fluid cannot be precisely defined by simple sugar inhibition tests. The only mono-, di-, tri-, or tetrasaccharide tested that inhibited activity for PR-rbcs was melibiose, which was an effective inhibitor

of PR-rat rbcs and of untreated rat and pig rbcs. In order to confirm melibiose as an inhibitor of the agglutinin that recognizes pig and rat rbcs, inhibition tests between PR-pig rbcs and melibiose would be desirable. The lack of consistent inhibition by melibiose for rabbit rbcs, which bear receptors for all of the described agglutinins in S. purpuratus, may be due to interference from residual hemagglutinating activities unaffected by melibiose.

Neither agglutinin was inhibited by N-acetylneuraminic acid or colominic acid (a homopolymer of N-acetylneuraminic acid). Furthermore, desialyzation of glycoproteins did not reduce their ability to inhibit agglutinating activity. The irrelevance of sialic acid to hemagglutinating activity in S. purpuratus contrasts with the situation in many other invertebrates (Gilbride and Pistole, 1979; Vasta, Warr, and Marchalonis, 1982).

The binding sites for S. purpuratus agglutinins are more complex than can be revealed by mono- and disaccharide inhibition. Like a number of lectins from invertebrates (Roche and Monsigny, 1974; Vasta, Warr, and Marchalonis, 1982; Vasta and Marchalonis, 1983), those in S. purpuratus are effectively inhibited by glycoproteins. Many of these lectins also show specificity for galactose and N-acetylgalactosamine,

which are major constituents of the oligosaccharides associated with most described glycoproteins (Sharon, 1974). Though agglutinins in S. purpuratus coelomic fluid could not be inhibited with N-acetylgalactosamine, inhibition by galactose and melibiose, as well as by glycoproteins rich in galactosyl residues, would appear to fit the pattern seen with other invertebrate lectins having similar specificities. The observation that sialic acid, a common terminal sugar on the oligosaccharide chains of many glycoproteins, does not inhibit agglutination supports the idea that S. purpuratus agglutinins may recognize some non-terminal portion of these glycoproteins. Lectins have also been reported which bind carbohydrates linked to amino acid or peptide portions of glycoproteins (see review, Lis and Sharon, 1981), and it may be that S. purpuratus agglutinins recognize determinants very close to the rbc membrane.

In general, inhibition experiments suggest that at least one of the agglutinins in S. purpuratus coelomic fluid, most likely the PR-rabbit, PR-rat, PR-pig agglutinin, is inhibited by galactose-containing sugars. However, all agglutinating activities were inhibited by the glycoproteins tested. This information indicates a more complex pattern of inhibition and binding specificities. A conservative approach must be taken when

attempting to determine binding specificities of agglutinins tested in whole coelomic fluid. The presence of several agglutinins interacting with the same blood cell can create confusing patterns of inhibition that may be clarified by further studies on a purified form of a specific agglutinin (Vasta, Warr, and Marchalonis, 1982).

Hemagglutinating activity to PR-rabbit rbcs was unaffected by trypsin, pronase, or periodate treatments, as well as being relatively heat-stable. This information provides little insight into the structural nature (protein, carbohydrate, or lipid?) of hemagglutinins in S. purpuratus coelomic fluid, but it does follow a very similar pattern to that seen for the sea urchin, Hemicentrotus pulcherrimus (Ryoyama, 1974). The agglutinin described for H. pulcherrimus is also a heat-stable (to 100 C) molecule whose activity is resistant to trypsin, bromelain, and periodate treatment.

A number of echinoderm lectins are reported to be Ca^{2+}-dependent (Ryoyama, 1974; Bertheussen, 1983). Results reported here do not clarify whether hemagglutinating activity in S. purpuratus coelomic fluid is dependent on divalent cations. The presence of multiple lectins may confuse results, since the divalent cation requirements of several agglutinating activities in a single species may not be the same.

In addition to the proposal that agglutinins may act as primitive recognition molecules in invertebrates (Acton and Weinheimer, 1974; Burnet, 1974), Balding and Gold (1976) suggested that lectins, a family of molecules to which most or all invertebrate agglutinins probably belong, may confer on an organism a "natural immunity" to pathogens. No biological function has been ascribed definitively to echinoderm agglutinins; however, in other invertebrates, agglutinins are known to act as opsonins (Harm and Renwrantz, 1980; Renwrantz and Stahmer, 1983). Echinoderms clearly display immune responses to foreign material and have some level of specific immunologic memory (Karp and Hildemann, 1976; Coffaro, 1980), and therefore must possess reasonably sophisticated immunorecognition capabilities. Carbohydrate-based recognition systems probably cannot achieve the level of specificity seen with vertebrate immunoglobulins, but sugar-specific proteins, which occur in vertebrates as well, may represent an evolutionarily ancient surveillance mechanism. Lectins with similar sub-unit structure and binding specificities are reported for many invertebrates and vertebrates (Balding and Gold, 1976). There is an important need to do further functional studies on echinoderm and other invertebrate agglutinins. Research on purified agglutinins would greatly clarify

similarities between invertebrate agglutinins and vertebrate immune recognition molecules, such as immunoglobulins. Studies into the nature of recognition molecules described in deuterostome invertebrates, and especially those described for echinoderms, may contribute significantly to our understanding of the phylogenetic development of immunorecognition mechanisms and immune responses in general.

Table 1. The Titers[a] of Hemagglutinating Activity in *Strongylocentrotus purpuratus* Coelomic Fluid

Rbc Type [b]	Untreated	PR-treated [c]
Human A	0-2	64-512
B	0-2	32-512
AB	0-2	64-512
0	0-2	64-1024
Rabbit	8-256	256-4096
Pig	8-256	128-256
Rat	8-32	64-128
Horse	0	0

(a) Titers are reported as the reciprocal of the highest dilution giving agglutination.
(b) Rbc types tested but non-reactive (all untreated): Cow, sheep, mouse (Swiss-Webster), newt (*Taricha granulosa*), chicken, Ring-necked pheasant, dog, cat, goat. All cell types were tested with three or more coelomic fluid pools.
(c) PR= pronase-treated

Table 2. Residual Hemagglutinin Titers After Adsorption of Coelomic Fluid Against Untreated RBCs

Adsorbing rbc	Residual Hemagglutinin Titer [a]		
	Rabbit	Pig	Rat
None	32	16	16
Rabbit	0	0	0
Pig	0	0	0
Rat	2	0	0

(a) Titers are reported as the reciprocal of the highest dilution giving agglutination.

Table 3. Residual Hemagglutinin Titers After Adsorption of Coelomic Fluid Against Pronase-treated RBCs

Adsorbing rbc	Residual Hemagglutinin Titers [a]			
	PR-Ra	PR-P	PR-Rt	PR-Ho [b]
None	2048	256	128	128
PR-Ra	0	0	0	0
PR-P	64	0	0	ND
PR-Rt	64	2	0	32
PR-Ho	512	64	64	0

(a) Titers are reported as the reciprocal of the highest dilution giving agglutination.
(b) PR= pronase-treated, Ra= rabbit rbcs, P= pig rbcs, Rt= rat rbcs, Ho= Human O rbcs.

Table 4. Inhibition of Hemagglutinating Activity for Pronase-treated RBCs by Carbohydrates and Glycoproteins

Test Compound [a]	Inhibiting Concentrations		
	PR-Ra	PR-H	PR-Rt [b]
NANA[b]	-	-	-
Melibiose	-	-	12.5mM
Colominic Acid	-	-	-
Fetuin	.06%	.03%	.125%
Porcine stomach mucin	.03%	.03%	.01%
Bovine thyroglobulin	.03%	.03%	.01%
Bovine submaxillary mucin	.01%	.01%	ND
Bovine glycoprotein frac.IV	.125%	.125%	ND
Desialyzed BSM[b]	.01%	.01%	ND
Desialyzed PSM[b]	.01%	.01%	ND
Desialyzed Fetuin	.01%	.01%	ND

(a) Carbohydrates tested which do not consistently inhibit hemagglutination: D-galactose, D-glucose, L-fucose, D-mannose, D-glucuronic acid, N-acetylgalactosamine, N-acetylglucosamine, Alpha-methylmannosamine, L- and D-arabinose, Beta-methylneuraminic acid, xylose, rhamnose, cellobiose, gentibiose, and stachiose.
(b) Abreviations: NANA= N-acetylneuraminic acid, PR-Ra= Pronase-treated rabbit rbcs, PR-H= Pronase-treated human rbcs, PR-Rt= Pronase-treated rat rbcs, BSM= Bovine Submaxillary Mucin, PSM= Porcine stomach mucin.

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APPENDIX

APPENDIX A. Preliminary Purification of Hemagglutinins from *Strongylocentrotus purpuratus* Coelomic Fluid

Preliminary attempts were made to acquire a preparation of purified hemagglutinin from *Strongylocentrotus purpuratus* coelomic fluid.

Numerous lectins have been purified using affinity columns prepared by coupling a specific sugar or glycoprotein to a Sepharose matrix and then eluting with either the specific inhibitory sugar or some non-specific eluant such as high salt, EGTA, or a pH gradient (Levine, Kaplan, and Greenway, 1972; Renwrantz and Stahmer, 1983). The hemagglutinins in *S. purpuratus* coelomic fluid are inhibited by a number of glycoproteins; therefore, a mucin-Sepharose 4B matrix was used as a means of affinity purification for these molecules. The following includes a description of the method used and a brief discussion of results achieved.

An affinity matrix was prepared by coupling 25mg porcine stomach mucin (an excellent inhibitor of hemagglutinating activity) to 5ml Cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the instructions of the manufacturer. The matrix was equilibrated with a

Tris-buffered saline (TBS: 0.05 M Tris-HCl, 0.15 M NaCl, 0.010 M $CaCl_2$ and $MgCl_2$, 0.02% sodium azide, pH 7.3).

Batch preparation methods were used for initial attempts at purification of the hemagglutinins. Fresh, titered, heat-treated coelomic fluid (HT-CF) was incubated with the mucin-Sepharose matrix (MS-matrix) in a ratio of 10:1 (HT-CF:MS-matrix, v/v) overnight at 4 C. After incubation, the hemagglutinin coupled MS-matrix (HA-MS-matrix) was centrifuged at approximately 100g, and the supernatant removed and tested for hemagglutinating activity and protein concentration. The supernatant contained approximately 12.5% of the original hemagglutinating activity. Protein concentrations were determined using a BioRad protein assay. The HA-MS-matrix was then washed 5 times with 5 times its volume of TBS. No detectable hemagglutinating activity could be found in any of these washes.

Initial work had indicated that hemagglutinating activity in S. purpuratus coelomic fluid might be dependent on Ca^{2+}. Therefore, 100 mM EGTA in divalent cation-free TBS (CMF-TBS) was used as an eluant. A volume of this EGTA solution, equivalent to that of the coelomic fluid originally added to the MS-matrix, was added to the HA-MS-matrix which was then incubated for 1 hour at room

temperature on a rocking platform. The HA-MS-matrix was then centrifuged at approximately 100g and the supernatant removed. The supernatant was dialyzed extensively at 4 C against TBS and tested for hemagglutinating activity to pronase-treated rabbit and pronase-treated human rbcs. The protein concentrations of the original coelomic fluid, the MS-matrix adsorbed supernatant, and the EGTA eluted fraction were determined and the specific activity with respect to hemagglutinating activity calculated.

Results of these initial experiments looked promising. The prepared MS-matrix bound 87.5% of the hemagglutinating activity to pronase-treated rbcs available in the original sample. The EGTA eluted fraction contained at least a 50% return in hemagglutinating activity. A clear decrease in protein concentration between the original HT-CF sample and the EGTA fraction was observed. This decrease in protein concentration reflected a substantial (12-fold) increase in specific activity for pronase-treated rbcs in the EGTA fraction when compared to the original HT-CF.

Analysis by SDS-PAGE (SDS polyacrylamide electrophoresis, after Laemmli, 1970) (Figure A1) shows that while many bands seen in whole coelomic fluid have been eliminated, a large number of proteins stick to the

MS-matrix and are eluted with EGTA. A band that migrates along with the 205K molecular weight marker has been shown not to be involved with hemagglutinating activity on SDS-PAGE analysis of rbc-adsorbed coelomic fluid samples.

While this method of affinity purification was effective in providing samples with significantly greater specific activity, numerous proteins not associated with hemagglutinating activity appear to co-purify using the MS-matrix. Since mucin is an effective inhibitor of all hemagglutinating activities in the coelomic fluid, it would be hard to identify which bands on SDS-PAGE might correspond with a single protein or agglutinin.

Several things could be done to improve on this method. An attempt at a more specific elution scheme should be tried. One of the agglutinins appears to be inhibited by melibiose, and this disaccharide in high concentration could be used to remove a single hemagglutinin. The coelomic fluid could also be adsorbed with a rbc type recognized by one agglutinin, thus removing it from the coelomic fluid, and this adsorbed-HT-CF preparation incubated with the MS-matrix. Both of these treatments would limit the number of hemagglutinating activities interacting with the MS-matrix and might decrease the number of non-specific

proteins which might bind. The affinity matrix itself might also be improved by preparing melibiose coupled to epoxy-activated Sepharose 4B.

A number of other methods for affinity purification of a single agglutinating activity are available and should be pursued. Among these is the use of pronase-treated rbcs themselves as an affinity matrix. A number of agglutinins have been purified in this way (Reitherman, Rosen, and Barondes, 1974). While the method mentioned above was not immediately successful, further work in this area should realize more purified preparations of S. purpuratus hemagglutinins.

Figure A1: SDS-PAGE Analysis of Strongylocentrotus purpuratus Coelomic Fluid and EGTA Fraction From a mucin-Sepharose matrix. The gel was stained with silver stain (after Merril, Goldman, Sedman, and Ebert, 1981). Lanes described as follows (Specific activity: hemagglutination units ug^{-1} protein)- Lane 1: Molecular weight markers; Lane 2: Whole coelomic fluid, pool 1 (512 units ug^{-1}); Lane 3: Whole coelomic fluid, pool 2 (512 unit ug^{-1}); Lane 4: EGTA fraction from mucin-Sepharose 4B matrix (6139 units ug^{-1}). Arrow denotes band not involved with hemagglutinating activity.

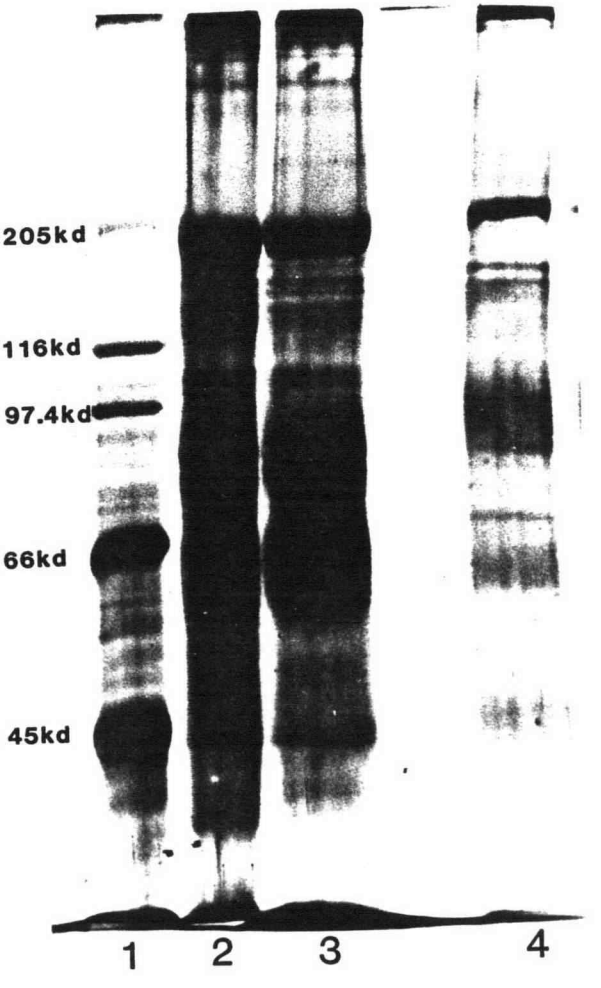

FIGURE A1

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