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SpC3, the complement homologue from the purple sea urchin, *Strongylocentrotus purpuratus*, is expressed in two subpopulations of the phagocytic coelomocytes

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Abstract The lower deuterostomes, including the echinoderms, possess an innate immune system that includes a subsystem with similarities to the vertebrate complement system. A homologue of the central component of this system, C3, has recently been identified in the purple sea urchin, *Strongylocentrotus purpuratus*, and is called SpC3. We determined previously that coelomocytes specifically express the SpC3 gene (*Sp064*); however, the sea urchin has at least four different types of coelomocytes: amoeboid phagocytes, red spherule cells, colorless spherule cells, and vibratile cells. To determine which of these subpopulations expresses *Sp064* and produces SpC3, coelomocytes were separated by discontinuous gradient density centrifugation. Relatively homogenous fractions were obtained consisting of the four major cell types in addition to two types of amoeboid phagocytes with different densities and distinct morphologies. Analysis of proteins from separated cell subpopulations by Western blot and analysis of gene expression by RT-PCR revealed that phagocytes express the gene and contain the protein. Immunolocalization showed that $SpC3^+$ phagocytes are present as subsets of both the low- and high-density subpopulations of phagocytes; however, the subcellular localization of SpC3 is different in these two subpopulations.

Key words Complement · C3 · Coelomocyte · Evolution · Innate · Lipopolysaccharide

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Introduction

Unlike the immune system of higher vertebrates, which consists of a complex of interlocking adaptive and innate recognition and effector functions, that of the sea urchin is simpler, and similar to the innate system of higher deuterostomes. Members of the echinoderm phylum (sea urchins, sea stars and others) do not exhibit specificity in immune recognition or immune memory, which defines the nonadaptive character of their immune system (reviewed in Gross et al. 1999; Smith and Davidson 1992, 1994). Swift defense functions in sea urchins are mediated by circulating coelomocytes, cells located within the fluid-filled coelomic cavity. These cells are extremely sensitive to minimal injury and to injections of lipolysaccharide (LPS) as assayed by increases in the number of *profilin* transcripts in responding coelomocytes (Smith et al. 1992, 1995). Profilin is a key mediator of changes in the actin cytoskeleton of amoeboid cells in response to either internal or external stimuli (Bubb et al. 1998; reviewed in Schlüter et al. 1997). Therefore, increases in *profilin* message imply cytoskeletal modifications or changes in cell shape which correspond to immune activation (Smith et al. 1995). However, the molecular mechanisms and cellular mediators that underlie invertebrate innate immune responsiveness are largely unknown.

The two major phyla within the deuterostome lineage of the animal kingdom, the echinoderms and the chordates (including vertebrates and others) are monophyletic, which means that their evolutionary origins are linked, including the evolution of the immune response and, specifically, the evolution of innate immune functions. In higher vertebrates, an important component of innate immunity is the complement system. It is composed of a large number of cell surface and humoral proteins organized into four pathways with specific initiation, amplification, and regulatory mechanisms. The three initiating pathways (classical, alternative, and lectin) culminate by activating the central protein of the complement system, C3, which, in vertebrates, leads to the activation of the terminal pathway and the production of the membrane attack complex that destabilizes membranes resulting in lethal cytosolic leakage (for reviews, see Liszewski et al. 1996; McPhaden and Whaley 1993; Volanakis 1998). The antibody-independent, alternative pathway is initiated when C3 is cleaved to form C3a, a potent anaphylatoxin and chemoattractant, and C3b, which binds to or opsonizes foreign cells. The feedback loop of the alternative pathway results in the quick and efficient activation of more C3 molecules that quickly coat pathogenic cells with C3b molecules. These C3b molecules either initiate the terminal pathway resulting in cell lysis, or they tag the pathogen for phagocytosis and subsequent destruction by phagocytes bearing receptors for C3b.

The complement system appears to have evolved by gene and genome duplications (reviewed in Kasahara 1999; Rached et al. 1999). This view is based on significant sequence similarities among members of the complement gene families, and on similarities in structure and function of the complement components encoded by these genes (see Fig. 4 in Campbell et al. 1988; reviewed in Bentley 1988; Reid and Day 1989). The ancestral or "archaeo" complement system was thought to have been composed of a protein with a thioester site like C3, a protein with a serine protease domain like factor B (Bf), and a complement receptor on phagocytes (Lachmann 1979). This prediction has been partially confirmed with the identification of a C3 homologue, SpC3 (Al-Sharif et al. 1998; Smith et al. 1996), and a Bf homologue, SpBf (Smith et al. 1998), in the purple sea urchin.

In mammals, the *C3* gene is expressed in liver cells and the protein is secreted into the blood (Alper et al. 1969; Lambris 1988). Although the liver is the major source of C3, cells such as macrophages (Haga et al. 1996; Lambris 1988; Rothman et al. 1989; Strunk et al. 1985) in addition to a number of other cell types (Colten and Strunk 1993) also produce C3. We have found previously, that the coelomocytes of the purple sea urchin express *Sp064*, while the gut, ovary, and testis are negative (Al-Sharif et al. 1998). Since no liver-like organ has been defined in the sea urchin, the coelomocytes may serve as the major source of SpC3 in these animals. In the purple sea urchin, there are four morphologically different circulating coelomocytes, each with presumably different functions: (1) amoeboid phagocytes (which are present in all echinoderms), (2) red spherule cells (also called eleocytes or morula cells), (3) colorless spherule cells (also called white morula cells), and (4) flagellated vibratile cells (Boolootian and Giese 1958; Johnson 1969; reviewed in Gross et al. 1999). The phagocytes are considered to be the major mediators of the immune system based on their amoeboid, chemotactic, and phagocytic activities (Johnson 1969). We report here that all $SpC3^+$ cells were of the phagocyte type, and that only about 16% of all coelomocytes were $SpC3^+$. We have also found that two subpopulations of phagocytes could be separated by discontinuous density gradient centrifugation: those that are large, low-density cells (discoidal phagocytes), and small, higher-density cells (polygonal phagocytes), as well as a third subpopulation of phagocytes that were a minor component of both phagocyte fractions and had a unique morphology (small phagocytes). Within the two phagocyte fractions, 22% of the cells in the high-density fraction and 34% of the cells in the low-density fraction were $SpC3^+$. SpC3 in the polygonal phagocytes was localized predominately in a perinuclear distribution with a speckled distribution in the peripheral cytosol. The discoidal phagocytes did not have a perinuclear concentration of SpC3, but an even, speckled distribution throughout the cytosol. These results show that there are at least four subpopulations of phagocytes defined by a combination of density, morphology, *Sp064* gene expression, and SpC3 protein synthesis.

Materials and methods

Animals

Purple sea urchins, *Strongylocentrotus purpuratus*, were collected by SCUBA from shallow waters off the southern California coastline, and maintained in 100-gallon marine aquaria at the George Washington University animal facility. The aquaria were maintained at 15 °C on a 12/12 h light/dark cycle, were well aerated, and equipped with physiological and biological filters, a protein skimmer, and a UV-water sterilizer system. Sea water was prepared using Instant Ocean Synthetic Sea Salt (Mentor, Ohio) mixed into deionized water that was corrected for salinity and pH. A small volume of water (5–10%) was exchanged weekly. Animals were fed once a week with commercially obtained rehydrated kelp.

Coelomocyte and coelomic fluid preparations

Aliquots of whole coelomic fluid (wCF, fluid plus coelomocytes) were withdrawn by inserting a 23-gauge needle through the peristomeum into the coelomic cavity and withdrawing the fluid into a syringe preloaded with an equal volume of ice-cold Ca^{2+}/Mg^{2+}-free seawater containing 70 mM EDTA and 50 mM imidazole, pH 7.5 (CMFSW-EI) (Humphreys 1963; as modified in Gross et al. 1999) to prevent clotting and cell lysis. Cell concentrations from aliquots of wCF from individual animals were determined with a hemocytometer.

Discontinuous density gradient centrifugation

Coelomocyte cell types were separated by discontinuous gradient centrifugation. The gradient consisted of five concentrations of CMFSW-EI mixed with iodixinol (Optiprep; Nycomed, Oslo, Norway), so that the concentration of iodixinol was 5, 10, 20, 30, and 70%. The gradient was formed by underlayering 5 ml of successively denser solutions into a 35-ml centrifuge tube on ice. After forming the gradient, 5 ml of wCF in CMFSW-EI was overlayered and centrifuged at 1500 *g* for 30 min at 4 °C in a swinging-bucket rotor using soft start and no brake (model 5403; Eppendorf, Engelsdorf, Germany). Cell fractions were removed with a pasteur pipette from the surfaces of the upper to lower gradient steps. For the fraction collected at the 30–70% iodixinol interface (red spherule cell fraction), an equal volume of CMFSW-EI was added to the recovered samples to dilute the iodixinol and ensure osmotic equilibrium. Differential cell counts were performed with a hemocytometer to determine the major cell type for each faction plus the percentage of contaminating cell types.

RNA isolation and RT-PCR

Cells (2×10^6) from each fraction were pelleted and total RNA was recovered using RNAzolB (Leedo Medical Labs, Houston, Tex.) according to the manufacturer's instructions. Isolated RNA was further purified with a subsequent phenol:SEVAG (SEVAG is 24:1 chloroform:isoamyl alcohol) extraction and quantified by spectrophotometric analysis. Total RNA (3 µg) was used for reverse transcription in a 25-µl reaction with a random hexamer primer (2.8 µM) in a reaction containing 0.8 mM dNTPs, 8 mM dithiothreitol, 20 units RNAsin (Promega, Madison, Wis.) that was heated for 5 min at 70 °C followed by an ice chill. Superscript II reverse transcriptase (200 units; Gibco/BRL, Bethesda, Md.) plus company-supplied buffer were added and the reaction was incubated at 37 °C for 60 min, followed by a 5-min denaturation at 90 °C. The cDNA was stored at –70 °C without further analysis or processing.

Coelomocyte cDNA (1.0 µl of the reverse transcriptase reaction) was amplified using *Taq* polymerase (0.1 units; Promega) in 20-µl reactions that included company-supplied buffer, 2.0 mM $MgCl_2$, 0.15 mM dNTPs plus pairs of primers (0.5 µM) specific for either *Sp064* or *actin*. Primers for *Sp064* were designed from the cDNA sequence (Al-Sharif et al. 1998): forward, 5′-ACT TAC AGG GCT CAA ACA GGT GGT GAA CG-3′; reverse, 5′-TCC TTC CCG GTC AAA TCT TGT ATA TGG CC-3′, and amplified a 352-bp fragment. *Actin* primers were designed from the coding region identified in EST278 (GenBank accession number R62049; Smith et al. 1996): forward, 5′-ACG ACG ATG TTG CCG CTC TTG TCA T-3′: reverse, 5′-GCT GTC CTT CTG TCC CAT ACC GAC CA-3′, and amplified a 152-bp fragment. The *actin* primers were used as a positive control for RT-PCR to demonstrate that equal amounts of template were added to each reaction. The two sets of primers were designed to work with the same PCR thermocycler program: 94 °C for 5 min followed by 25 cycles of 94 °C for 30 s, 68 °C for 2 min, 2 min ramp to 72 °C with a hold at 72 °C for 40 s, and finishing with a hold at 4 °C. Amplified products were resolved on 1.5% agarose gel containing ethidium bromide and photographed with a Polaroid (Cambridge, Mass.) camera under UV illumination.

Antisera

A polyclonal rabbit antiserum was produced from a histidine-tagged SpC3 fusion protein (SpC3-6H). A 1400-bp PCR fragment encoding M_r 51,400 of the N-terminal region of the α chain of SpC3 (including the putative C3a fragment and the thioester site) was amplified from the cDNA by PCR [5′ primer with a *Bam*HI site (underlined), 5′-CGG GAT CCG ATT GAC AGG GAT CAA; 3′ primer with a *Pst*I site (underlined), 5′-ATT GGA GCT GTA AAG GCT GCA GAA]. The fragment was digested and ligated in frame into the polylinker of the pQE31 bacterial expression vector (Qiagen, Valencia, Calif.). Upon reaching the proper density, transformed bacterial cultures were induced to express SpC3-6H by adding isopropyl thio-β-D galactoside (1 mM) and incubating the culture at 37 °C for an additional 4 h. Bacterial pellets were lysed in binding/lysis buffer (8 M urea, 10 mM Tris, pH 8, 0.1 M NaH_2PO_4) and the DNA was sheared by passage through an 18-gauge and a 22-gauge needle. Cell fragments were pelleted and the supernatant was passed through a Ni affinity column, washed, and the bound fusion proteins were eluted at pH 4.5. Fractions containing SpC3-6H were identified by SDS-PAGE, pooled, rebound, and re-eluted from the Ni column. Fractions containing SpC3-6H were pooled, dialyzed against 10 mM phosphate buffer pH 7.4, and injected into two rabbits. Antiserum (αSpC3-6H) was collected according to protocols established by Lampire Biological Laboratories (Pipersville, Pa.), aliquoted, and stored at –70 °C.

A second polyclonal antiserum (kindly provided by Dr. John Lambris at the University of Pennsylvania) was produced from a peptide that corresponds to the N-terminus of the putative α′ chain of SpC3 using previously published methods (Al-Sharif et al. 1998; Gross et al. 1999). Briefly, the synthetic peptide (SGGDGGEQNAAVKVRDDFRETWFFDC) was conjugated to keyhole limpet hemocyanin using the cysteine and injected into rabbits. Preimmune and anti-SpC3-α′ peptide antisera (anti-SpC3-α′) were aliquoted and stored at –70 °C. To reduce significantly the background staining, both the anti-SpC3-α′ and the preimmune serum were affinity purified on a protein A column (Pierce, Rockford, Ill.) according to the manufacturer's instructions and the eluted antibodies were aliquoted and stored at –70 °C.

Western blots

Coelomocytes (~10^5 cells) were pelleted and lysed in 100 µl of 2×lysis buffer (4% SDS, 20% β-mercaptoethanol, 20% glycerol, 0.1 M Tris, pH 6.8), vortexed for 2 min and boiled for 2 min. Coelomocyte proteins (equivalent of 1.8×10^4 cells) were resolved by SDS-PAGE (4.5% stacking, 10% resolving) under reducing conditions. Proteins were electroblotted onto nitrocellulose in a Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, Calif.) for a minimum of 3 h at 300 mA in a chilled water bath (Neslab Instruments, Portsmouth, N.H.) in blotting buffer (20% methanol, 20 mM Tris pH 8.8, 0.15 M glycine, 0.05% SDS). After transfer, filters were stained with PonceauS (0.1% w/v in 5% acetic acid) to mark the standards and the positions of the lanes. Filters were blocked in "blotto" (5% nonfat dried milk in TBS-Tween, 200 mM Tris pH 7.4, 140 mM NaCl, 0.1% Tween 20) with constant agitation for at least 12 h at room temperature (RT), followed by incubation for 3 h with αSpC3-6H (1:15,000 in blotto) at RT. After washing three times in TBS-Tween and TBS with constant agitation, filters were incubated with goat anti-rabbit Ig conjugated with horse radish peroxidase (GαR-Ig-HRP; 1:80,000 in blotto; Pierce), washed in TBS-Tween and TBS with constant agitation, and briefly incubated in Super Signal Enhanced Chemiluminescent Substrate System (Pierce) before exposure to X-OMAT AR X-ray film (Eastman Kodak, Rochester, N.Y.).

Immunocytology

Coelomocytes were centrifuged onto poly-L-lysine- (50 mg/ml in 10 mM Tris pH 8) coated slides using a cytology rotor (Eppendorf) at approximately 1000 *g* for 7 min at 4 °C. Cells were fixed for 5 min with 4% paraformaldehyde in CMFSW-EI, washed in CMFSW-EI, and blocked for 60 min with cytology blocking buffer (CBB; 10% normal goat serum, 10% bovine serum albumin in CMFSW-EI). After blocking, slides were incubated for 60 min with affinity-purified anti-SpC3-α′ or preimmune serum

(diluted 1:50 in CBB) followed by fluorescein-labeled GαR-Fab [GαR-Fab-FITC, 1:1000 in CBB; Pierce]. Cells were counterstained with propidium iodide (3.5 μg/ml CMFSW-EI), examined and photographed under both phase contrast and fluorescence microscopy, using a Zeiss Axioscope microscope (Oberkochen, Germany).

Confocal microscopy

Phagocytes were isolated and processed as described above for fluorescence microscopy except that the secondary antibody was GαRIg conjugated to alexin fluorochrome (GαRIg-A; Molecular Probes, Eugene, Ore.) diluted 1:5000 in CBB. Cells were washed with CMFSW-EI (30 mM EDTA) and the intensity of alexin emission was augmented using Slowfade (Molecular Probes) in the final mounting solution. Cells were observed with an Olympus IMT2-RFC inverted microscope (Olympus, Melville, N.Y.) at ×600 magnification and images were captured with a Bio-Rad MRC 1024 Confocal Laser Scanning System attached to the microscope. Final color plate formatting was done with Adobe Photoshop v.4.0 (Adobe Systems, San Jose, Calif.).

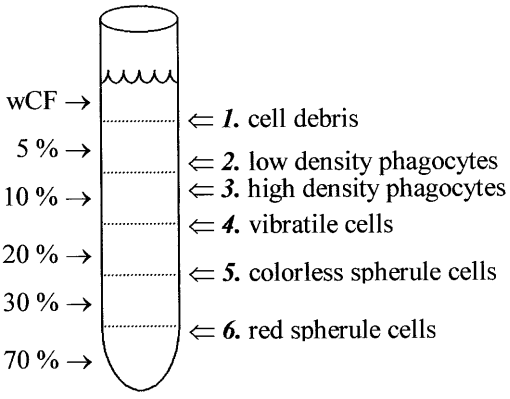


Fig. 1 Relative locations of five coelomocyte cell fractions in a discontinuous density gradient. Iodixinol (Optiprep) layers were generated by mixing with CMFSW-EI. Whole coelomic fluid was diluted 1:1 with CMFSW-EI and overlayered onto the gradient. Approximate locations of the coelomocyte fractions within the gradient are shown

Results

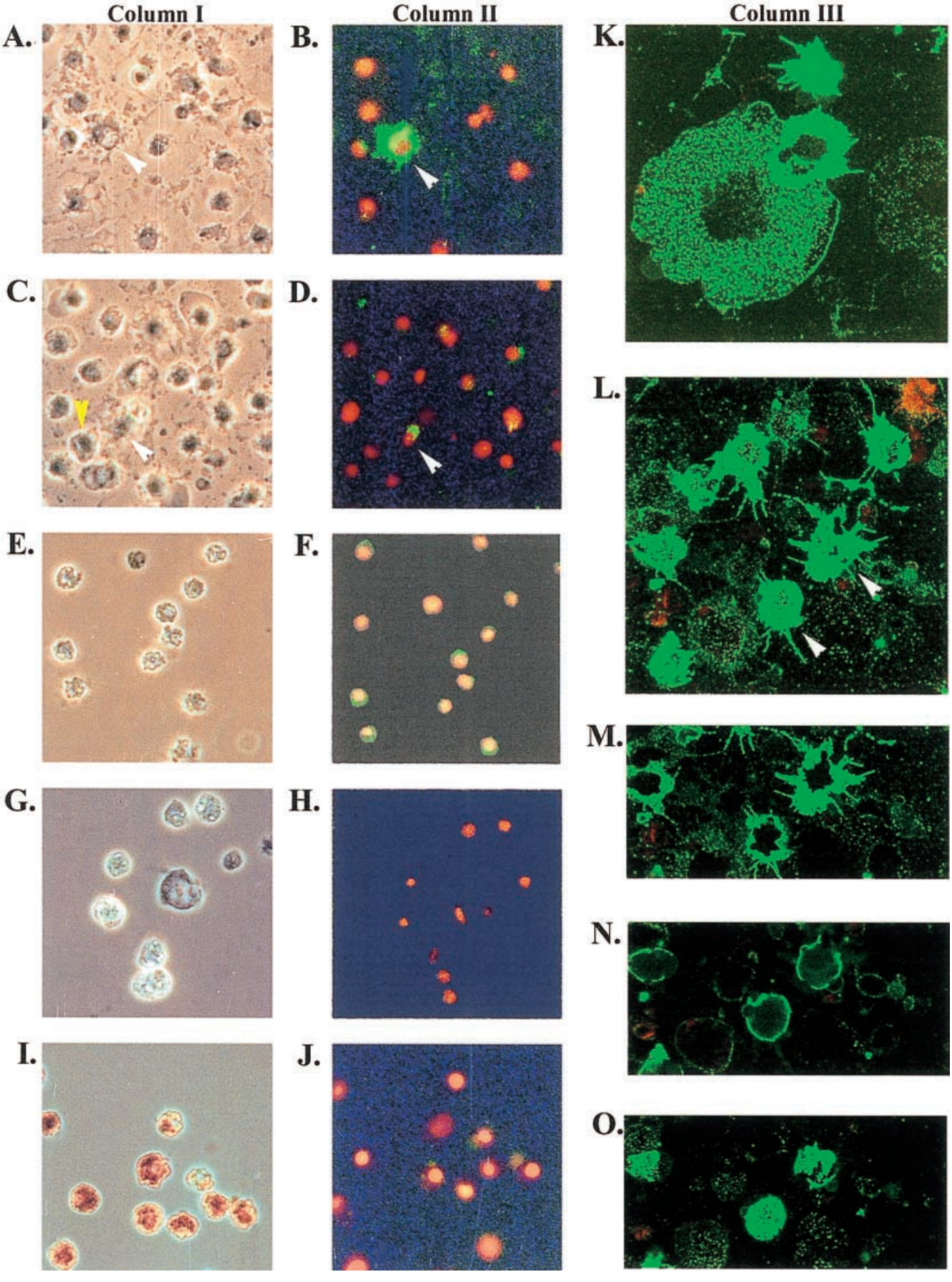
Four major coelomocyte cell types and three morphologically unique subpopulations of phagocytes were identified in S. purpuratus

Discontinuous density centrifugation has been used previously to separate morphologically different types of coelomocytes into different fractions employing sucrose gradients (Edds 1977, 1993), a mixture of Ficoll, and Triosil (Messer and Wardlaw 1979) or sodium metrizoate (Gerardi et al. 1990; Matranga et al., in press). More recently, step gradients were generated with Percoll (Smith et al. 1992) which is a slurry of polyvinylpyrrolidone-coated silica particles and which may be a poor choice for use with phagocytic cells. Potentially, phagocytic coelomocytes may take up the Percoll particles which could alter their morphology or general behavior. Furthermore, Percoll is formulated for mammalian cells and has to be extensively dialyzed against CMFSW-EDTA to correct the osmolality and to chelate the divalent cations before it can be used with sea urchin cells (Smith et al. 1992). Therefore, a cell separation procedure was developed using Optiprep (iodixinol) which is not particulate and can be adapted easily for use with cell types that function at nonmammalian salinity. With this medium, and under optimal conditions, coelomocytes could be separated into five, fairly homogeneous fractions that were similar to fractions previously reported for Percoll gradients (Smith et al. 1992).

An example of a density gradient and the positions to which the different types of coelomocytes migrate is shown in Fig. 1. The most dense cells, the red spherule cells, migrated to the surface of the 70% Optiprep cushion, the colorless spherule cells migrated to the 30% Optiprep layer, and the vibratile cells migrated to the 20% layer. In some cases, however, the colorless spherule cells and the vibratile cells comigrated and appeared as a mixture on the 20% layer. Unlike Percoll gradients, where only a single fraction of phagocytes was reported, iodixinol gradients separated phagocytes into two distinct layers near the 5–10% interface. The denser phagocytes migrated slightly into the 10% Optiprep layer, while the less dense fraction migrated well into the 5% Optiprep layer (Fig. 1).

Two distinct subpopulations of phagocytes, separable by density gradient separation, have been reported previously for *S. droebachensis* and *S. purpuratus*, using sucrose gradients (Edds 1993). The low-density cells were described as discoidal, while the high-density cells were described as polygonal. These descriptive terms were based on the morphology of the cells when spread on a microscope slide. We obtained similar results for *S. purpuratus* using iodixinol, where the phagocytes fractionate into two distinct densities; however, microscopic examination of the phagocyte fractions yielded three distinct morphologies. The first two phagocyte types corresponded to those described by Edds (1993): low-density discoidal cells and high-density polygonal cells (Fig. 2A,B). The third heretofore undescribed phagocytic cell type was rarely observed, found in both density fractions, had little cytoplasm and was much smaller in diameter than either the discoidal or polygonal phagocytes. We have called these cells small phagocytes (Fig. 2C, yellow arrowhead).

To determine the relative abundance of each cell type in CF, coelomocytes were taken from sea urchins (n=10) and differential cell counts were performed (Table 1). Most of the coelomocytes (76.6%) are of the phagocyte class, although variability between animals in all cell subpopulations was reflected in the ranges, and was similar to what has been reported previously (Smith et al. 1992). There are no data in the literature regarding differences in coelomocyte subpopulations from sea urchins, yet these differences


Column I
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A.
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Table 1 Subpopulations of coelomocytes. Coelomocyte subpopulations from sea urchins (n=10) are shown as the percentage of total coelomocytes. Different sea urchins had slightly different percent compositions of subpopulations and those variations are represented by the range shown in *parentheses* (*nd* not determined). Two sea urchins were used for cell separations studies. Coelomocytes were separated by discontinuous gradient centrifugation, stained for SpC3 with anti-SpC3-α′, and three representative regions of the slides for each cell fraction from each animal were photographed under both phase contrast and fluorescence microscopy, and the percentage of cells that were $SpC3^+$ were calculated as follows: (number of $SpC3^+$ cells/total number of cells)×100=percent $SpC3^+$ cells per fraction. The percentage of $SpC3^+$ coelomocytes was calculated from data in the second and third columns

Cell type	Coelomocyte subpopulations (%)	Coelomocyte subpopulations used in separation studies (%)	$SpC3^+$ cells in each coelomocyte subpopulation (%)	$SpC3^+$ cells in total coelomocyte (%)
Phagocyte	76.6 (60.0–82.6)	63.5	25.9	16.4
Low density	nd	26.2	22.2	5.8
High density	nd	37.3	34.2	12.7
Vibratile	11.9 (2.8–21.0)	20.0	0.0	0.0
Colorless spherule	3.7 (0.7–6.3)	3.5	0.0	0.0
Red spherule	7.8 (1.8–16.0)	13.0	0.0	0.0

were found even in animals housed for long periods in our closed aquaria where the environment was regulated and all animals presumably experienced the same external stimuli. Differential cell counts were also performed on the two animals that were used for density separations and analysis of $SpC3^+$ cells and included data on the high- and low-density fractions of phagocytes (Table 1). Results from these two animals fell within or close to the ranges found for the larger sample.

Sp064 was expressed in phagocytic coelomocytes

When cells were separated for analysis of *Sp064* message and SpC3 protein by RT-PCR and Western blot, respectively, the colorless spherule cells and vibratile cells did not separate and formed a single fraction. Furthermore, to obtain sufficient numbers of phagocytes for Western blot analysis, the two phagocyte fractions (low- and high-density fractions) were pooled. Therefore, three fractions were used in the analyses and none were pure separations, although percentages of contaminating cell types were determined (see legend to Fig. 3). We found, however, that the problems encountered with the gradient (or with the cell densities from the individual sea urchin) did not adversely affect the results. Analysis of *Sp064* message by RT-PCR showed an intense band amplified from phagocyte cDNA (Fig. 3, lane 1) while very minor bands were amplified from the other cell types (Fig. 3, lanes 2, 3). These data indicated that the phagocytes expressed *Sp064* and that either the other cell types expressed this gene at very low levels, or the source of the amplified band was cDNA from phagocytes contaminating the other two fractions (fraction 2 had 2.1% phagocyte contamination, fraction 3 had 1.5% phagocyte contamination). Amplification of the *actin* message served as a control to assess the amount and

Fig. 2A–O Phase contrast (*Column I*), fluorescence (*Column II*), and confocal (*Column III*) micrographs of separated coelomocyte cell types. **A–J** Paired phase and fluorescence micrographs taken of the same field: low-density phagocytes (**A,B**), high-density phagocytes (**C,D**), vibratile cells (**E,F**), colorless spherule cells (**G,H**), red spherule cells (**I,J**). *Arrowheads* (**A–D**) indicate relative position of the same cells within the fields that are positive. Cells were separated by discontinuous density gradient centrifugation, spun onto slides, fixed and stained with anti-SpC3-α′ antiserum followed by GαR-Fab-FITC and counterstained with propidium iodide (Sigma) nucleic acid stain. Phase contrast and fluorescence micrographs were taken on a Zeiss Axioplan fluorescence microscope at ×400 magnification. Major cell types and contaminating cell types from each fraction were as follows – fraction 1: discoidal phagocytes 100%; fraction 2: polygonal phagocytes 97.8%, vibratile cells 2.2%; fraction 3: vibratile cells 94%, phagocytes 5.4%, colorless spherule cells 0.6%; fraction 4: colorless spherule cells 77.8%, red spherule cells 22.2%; fraction 5: red spherule cells 86.5%, colorless spherule cells 13.5%. *Yellow arrowhead* indicates a small phagocyte (**C**). **K–O** Confocal micrographs. Discoidal phagocytes, Z series (**K**); polygonal phagocytes, Z series (**L**). Arrowheads indicate the cells shown in **M–O** – a series of single optical sections: near the bottom of the cells (**M**), through the middle of the cells (**N**), near the top of the cell over the nucleus (**O**). Cells were processed as described for fluorescence microscopy except that the secondary antibody was GαRIg-A and propidium iodide was not used

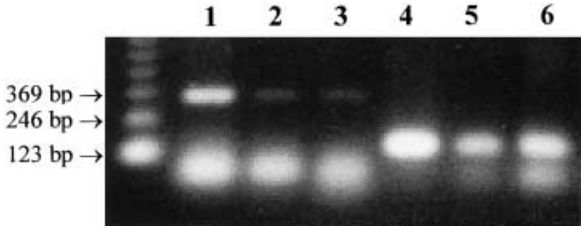

Fig. 3 RT-PCR amplification of *Sp064* from cDNA of separated coelomocyte cell types. Cells were separated by discontinuous density gradient centrifugation, and RNA recovered from each cell fraction was reverse transcribed and amplified by PCR. *Lanes 1–3* amplified with *Sp064* primers, *lanes 4–6* amplified with *actin* primers (control). *Lanes 1, 4* phagocytes (82.6% phagocytes, 13.9% vibratile cells, 2.6% colorless spherule cells, 0.9% red spherule cells); *lanes 2, 5* vibratile cell and colorless spherule cell mixture (2.1% phagocytes, 63.2% vibratile cells, 34.7% colorless spherule cells); *lanes 3, 6* red spherule cells (1.5% phagocytes, 98.4% red spherule cells). Size standards are shown to the right

quality of input cDNA in each reaction. Although the phagocyte fraction appeared to contain greater amounts of cDNA than the other two fractions (Fig. 3, compare lane 4 to lanes 5 and 6) based on the more intense *actin* band, phagocytes are known to have extensive cytoskeletons containing large amounts of actin. These cells have been used as a model system to investigate cytoskeletal shape changes including analysis of the proteins involved (Edds 1977; Hyatt et al. 1984; Otto et al. 1979). Consequently, we believe that the strong *actin* band in the phagocyte lane did not accurately represent the amount of cDNA used in the PCR amplifications. When comparing the band intensities of *actin* and *Sp064* between the phagocyte fraction and the other types or coelomocytes, these data indicate that the phagocytes specifically expressed the sea urchin complement gene, *Sp064*.

Phagocytes produced the SpC3 protein

The presence of a message within a cell does not always indicate that the message is transcribed. Alternatively, a protein may be taken up by a cell type that does not produce it. Therefore, we were interested in identifying the coelomocyte type(s) that contained, and presumably produced SpC3. The same cell fractions used in RT-PCR were analyzed by Western blot to determine whether the phagocyte fraction which expressed *Sp064* also produced the protein. Results show that only the pooled phagocyte fraction had detectable amounts of SpC3 (Fig. 4, lane 1), whereas the mixture of vibratile cells and colorless spherule cells (Fig. 4, lane 2) and the red spherule cells (Fig. 4, lane 3) were negative. These results agree with the RT-PCR detection of *Sp064* expression in the phagocytes and suggest that the source of minor bands seen by PCR in the nonphagocyte cell fractions was due to phagocyte contamination. These results also show that cells that do not express the gene do not acquire the protein exogenously.

Inspection of Fig. 3, lane 1, shows that SpC3 from phagocytes appeared as two bands that were significantly larger (M_r 200,000 and 210,000) than that routinely identified for the secreted form of the SpC3 α chain (M_r 130,000) that was included as a control (Fig. 4, compare lanes 1 and 4, and see below). The large size of SpC3 in phagocytes corresponded with the size of unreduced SpC3 isolated from CF (see Fig. 5 in Al-Sharif et al. 1998) and indicated that SpC3 from coelomocytes had not undergone final processing to separate the α and β chains before being secreted into the CF.

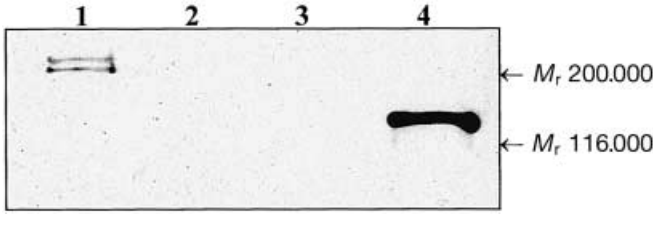

Fig. 4 SpC3 protein is produced by phagocytes. The same fractions described in the legend for Fig. 3 were analyzed for SpC3 expression by Western blot. Between 1.18×10^6 and 6.63×10^6 cell equivalents were loaded per lane, and the filter was incubated with αSpC3-6H followed by GαRIg-HRP. *Lane 1* phagocytes, *lane 2* mixture of vibratile cells and colorless spherule cells, *lane 3* red spherule cells, *lane 4* cell-free coelomic fluid. Size standards are indicated on the right

Two subpopulations of phagocytes expressed SpC3

Cell fractions that are either mixtures of cell types or that contain contaminating cell types make it difficult to assess whether all or only some of the cells within the fraction express any given gene or protein. In addition, very low levels of protein expression, or expression by only a few cells within a fraction may not be detected by Western blot methods. Although the phagocyte fraction was shown to be positive for both *Sp064* transcripts and SpC3, phagocytes were found not to be a homogeneous population of cells (see above). To determine whether all or only some of the phagocytes were $SpC3^+$, we prepared separated fractions for observation by fluorescence microscopy. Discontinuous gradient centrifugation yielded five fractions of coelomocytes (see Fig. 1). Cells were spun onto slides and incubated with anti-SpC3-α′. Pairs of images are shown in Figure 2 (A–J), with phase contrast in column I and fluorescence in column II. Cells positive for SpC3 were only identified in the phagocyte fractions, in agreement with Western blots and RT-PCR. A subset of discoidal phagocytes (Fig. 2B) and a subset of polygonal phagocytes (Fig. 2D) were positive for SpC3. The results also confirm that the vibratile cells (Fig. 2F), colorless spherule cells (Fig. 2H), and red spherule cells (Fig. 2J) were negative for SpC3. The minor staining observed for the vibratile cells (Fig. 2F) was also observed when preimmune serum was used, suggesting that rabbit serum nonspecifically bound to these cells. All other cells were negative for staining with preimmune serum (data not shown). Counts of $SpC3^+$ cells indicated that 16.4% of the total coelomocytes expressed SpC3, that most of the $SpC3^+$ cells were polygonal cells (12.7% of total coelomocytes) although many discoidal cells were also positive (5.8% of total coelomocytes) (Table 1). In addition, some small phagocytes were $SpC3^+$ (not shown); however, counts were not performed due to the rarity of this cell type.

To characterize the subcellular localization of SpC3, polygonal and discoidal phagocytes were imaged by confocal microscopy (Fig. 2, column III). Not only were the morphologies of the two cell types different, but their patterns of SpC3 localization also differed. Of the three cells shown in Figure 2K, the more brightly staining, small cell near the top of the

image is a contaminant of the high-density, polygonal phagocyte type and will be discussed below. The other two, one slightly smaller than the other, are less brightly staining, discoidal phagocytes (Fig. 2K). These cells were very flat, with a vertical dimension of about 1.2 μm based on capturing four optical sections of 0.3 μm each on the confocal microscope. Localization of SpC3 revealed a granular appearance consistent with SpC3 being contained within vesicles. These vesicles appeared to be evenly spaced throughout the cytosol, and the concentration of vesicles or staining intensity in the two discoidal cells was much less than in the polygonal cell (Fig. 2K). The discoidal phagocytes in Fig. 2K were so flat, and so little cytoplasm was present above and below the nucleus, that the Z series (combined image of all optical sections) showed that these regions were negative for SpC3. In contrast, the polygonal phagocytes were "taller" cells, encompassing eight optical slices of about 0.5 μm or about 4 μm in vertical dimension (Fig. 2L). The distribution of SpC3 was less uniform in this cell type in which SpC3 was concentrated in the perinuclear region of the cytosol compared to the speckled appearance of the peripheral pseudopodia. This distribution was more easily seen in single optical sections (Fig. 2M–O) taken from the Z series depicted in Fig. 2L. The bottom optical section (Fig. 2M) shows thin pseudopodia that extended away from the negative nuclear region along the surface of the slide. The middle optical section (Fig. 2N) shows the thin cytoplasm that surrounded the sides of the nucleus, and the top optical section (Fig. 2O) shows the cytoplasm that covered the top of the nucleus. Within these sections, the perinuclear region could most easily be seen in Fig. 2M, and the amount of SpC3 appeared greater around the irregular, negative nucleus compared to the cell periphery and the pseudopodia. Differences in subcellular localization of SpC3 in these two types of phagocytes may indicate differences in cellular function.

Discussion

Boolootian and Giese (1958) were the first to describe multiple, morphologically different cell types in the coelomic fluid of the sea urchin genus, *Strongylocentrotus*. Four distinct cell types have been identified: amoeboid phagocytes, red spherule cells, colorless spherule cells, and vibratile cells (Johnson 1969; Karp and Coffaro 1980; Smith 1981). In mammals, there are many different functional types of tissue-based (e.g., Kupffer cells, mesangial cells, Langerhans cells, microglial cells) and circulating (e.g., monocytes, macrophages, granulocytes, leukocytes) phagocytic cells. However, in echinoderms, phagocytes have been assumed to be a homogeneous population of cells. We show here that, like mammals, sea urchin phagocytes are a complex of different cells that consists of three major subtypes that are identifiable by morphology, can be separated by density, and have differential expression of SpC3.

In *S. purpuratus* and its congener, *S. droebachiensis*, two phagocyte subpopulations have been identified based on morphology and sucrose density centrifugation (Edds 1993). The cells were distinguished by cytoskeletal morphology when spread on a flat substrate (i.e., discoidal and polygonal) and by differing amounts of cytoskeletal proteins. These two cells types appear to correspond to the two major types found in our iodixinol gradients. The low-density cells have the same morphology as the discoidal cells, and the high-density cells may correspond to the polygonal cells identified by Edds (1993). The small phagocyte identified in this study, which was not mentioned by Edds (1993), was not found in large numbers, was not adequately screened for SpC3 expression, and was therefore not included in the data presented in Table 1. However, we did find that some of the small phagocytes were $SpC3^+$ (data not shown). Each morphologically different class of coelomocyte has been assumed to be functionally uniform. For example, Johnson (1969) reported that all phagocytes display chemotactic and phagocytic reactions towards bacteria, Ito et al. (1992) reported that phagocytes opsonized red blood cells in vitro, and Bertheussen (1979) implied that all phagocytes were involved in cytotoxic reactions in vitro in allogeneic mixtures of coelomocytes. The identification of subsets of phagocytes that are based on differences in SpC3 expression implies that these cells may be different with respect to host defense functions. The data presented here show that there are clearly four different phagocyte subpopulations, based on morphology and SpC3 expression, and there may be as many as six, if small phagocytes that are $SpC3^+$ and $SpC3^-$ are taken into consideration.

A few cDNAs isolated from coelomocytes from the purple sea urchin, other than *Sp064*, have been either partially (Smith et al. 1996) or fully sequenced. Fully sequenced genes include those for a second complement component SpBf (Smith et al. 1998), three transcription factors, SpNFkB, SpGATAc and SpRUNT, and a complex of genes encoding proteins with scavenger receptor cysteine-rich (SRCR) repeats (Pancer et al. 1999), and profilin (Smith et al. 1992). Some of these genes, such as that for profilin, are expressed in other adult cell types besides coelomocytes, and many are expressed in embryos. Only two genes, *profilin* and *Sp064*, have been analyzed for their expression in coelomocyte subpopulations, and while *profilin* is expressed in all coelomocytes (Smith et al. 1992), *Sp064* is the first gene to show differences in expression within coelomocyte subpopulations.

Analysis of mammalian cells that have been induced to produce C3 show a high molecular-weight band that is the unprocessed proC3 form of C3 (Botto et al. 1992; Goldman et al. 1991; Haga et al. 1996; Strunk et al. 1985; Sutton et al. 1985). However, that

two, high molecular-weight proSpC3 bands were present in phagocyte samples (Fig. 4, lane 1) was unexpected. One band (M_r 210,000) corresponds in size with nonreduced SpC3 isolated from CF (Al-Sharif et al. 1998), and the smaller band may represent an incompletely glycosylated form and reflect different stages of proSpC3 processing. The polygonal phagocytes showed a higher concentration of SpC3 in the perinuclear region (Fig. 2M), which is typically the location of the Golgi apparatus. If the polygonal cells were actively producing SpC3 at the time of collection, some of the SpC3 may have been present in the Golgi in an incompletely processed form. On the other hand, the discoidal $SpC3^+$ phagocytes showed an evenly speckled appearance with no concentration of signal in the perinuclear regions (Fig. 2K). These cells may not have been actively producing SpC3 at the time of sampling, and the SpC3 may have passed through the Golgi apparatus, packaged into vesicles and stored in the proSpC3 form. Alternatively, SpC3, in its final form, may be differentially glycosylated. We have noted recently that closely spaced, double bands of the α chain (M_r 130,000) can be identified on Western blots (double bands are not discernible in Fig. 4, lane 4).

The M_r 130,000 α chain was not detected in coelomocytes when the cells were completely separated from the proteins in the CF upon entering the density gradients during centrifugation. This result suggested two things. First, final processing and cleavage at the $\beta\alpha$ junction of SpC3 probably occurs when the protein is secreted into the CF. Maintaining SpC3 in its pro form would be an efficient mechanism for keeping the thioester inactive while the protein is within the cytoplasmic vesicle. Second, the speckled appearance of SpC3 in phagocytes is not due to uptake of SpC3 into endocytotic vesicles from the CF. If this occurred, SpC3 in its final processed form would have been identified, i.e., the M_r 130,000 α chain would have been present on the Western blots of coelomocytes. Furthermore, cell surface localization of SpC3 was not found when live cells were incubated with the antisera (not shown).

Although we have suggested that two types of phagocyte produce SpC3, there is an alternative explanation. The polygonal and discoidal $SpC3^+$ phagocytes may be the same cell type, but at distinct stages of differentiation. The polygonal cell may be less differentiated and actively producing SpC3, and it may differentiate into the larger discoidal morphology with SpC3 stored in secretory vesicles in the cytoplasm. The cells with the discoidal morphology may be collectively prepared to secrete SpC3 en mass in response to a local or systemic immune challenge (see Clow et al. 2000).

The production of complement components, and C3 in particular, in non-liver cells such as monocytes and macrophages in mammals has been documented extensively (reviewed in Colten and Strunk 1993; McPhadden and Whaley 1993). The production of both C3 and Bf by the same types of cells at local sites of infection promotes the activation of the alternative pathway which functions to tag foreign pathogens and to generate a local inflammatory reaction. This could occur quickly, before serum (liver-derived) proteins could enter the region (Colten and Strunk 1993), and may be similar to the activities of the $SpC3^+$ phagocytes. Since a liver-like organ has not been identified in sea urchins, all host defense reactions, including those that are complement mediated, may be initiated and perhaps generally mediated at the level of local infection or injury. Without a closed circulatory system, local defense reactions in a sea urchin might be initiated or mediated by complement proteins, which might quickly result in general immune activation. General immune activation has been observed based on increased levels of *profilin* message in coelomocytes, quantitated from sea urchins responding to local injury, e.g., a single needle hole in the peristomial membrane (Smith et al. 1992) or to minimal (2 μg LPS/ml CF) injections of LPS (Smith et al. 1995). Similarly, we have identified increases in SpC3 levels in CF and coelomocytes from sea urchins responding to injury or LPS, which occurs as quickly as 15 min post-injection and lasts for as long as 90 days in individual sea urchins (Clow et al. 2000).

The few genes that encode immune effector proteins in coelomocytes, the complement proteins (Al-Sharif et al. 1998; Smith et al. 1996, 1998) and the SRCR receptors (Pancer et al. 1999), in addition to the lack of immune memory in sea urchins (Coffaro 1979; Smith and Davidson 1992), indicate that the echinoderm immune system functions as an innate system and is similar in some respects to the innate immune system in mammals. Although the vibratile cells and spherule cells from the sea urchin do not appear to correspond to any cell type in higher vertebrates, the phagocytes bear striking similarities to the macrophage/monocyte class of hematopoetic cells. Homologous tissues and cell types can be defined not only by their embryological source but also by the genes they express and the effector or structural proteins they produce which, in turn, define their behavior and function. Although the developmental source of coelomocytes in the embryo, the larva, the adult rudiment, and the source of replacement coelomocytes in the adult sea urchin are not known, phagocytes share a number of significant characteristics with mammalian monocytes and macrophages. Phagocytic coelomocytes and macrophages have similar morphology, express similar reactions toward foreign antigens including chemotaxis and phagocytosis, they express complement, NF-κB and GATA transcription factors, and SRCR-containing proteins (Al-Sharif et al. 1998; Pancer et al. 1999; Smith et al. 1998). These similarities are probably not coincidental, and are evidence for evolutionarily derived homologies between phagocytic coelomocytes and mammalian macrophages. Continued investigations of phagocytes aimed at

understanding the mechanisms of host defense that are mediated by the few effector proteins that have been identified will not only define how the sea urchin immune response functions, but will aid in understanding the evolutionary underpinnings of the vertebrate innate immune system. To define the form of the ancient defense system that existed in the vertebrate ancestor prior to the invention of gene rearrangement and the evolutionary appearance of the adaptive immune response will assist us in understanding the importance of the innate system in defense responses in mammals today.

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