

Lori A. Clow · Paul S. Gross · Chi-Schin Shih
L. Courtney Smith

Expression of SpC3, the sea urchin complement component, in response to lipopolysaccharide

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Abstract The homologue of the vertebrate complement component C3 that is expressed in the coelomocytes of the purple sea urchin, *Strongylocentrotus purpuratus*, designated SpC3, was investigated for changes in response to immune challenge or injury. Immunoreactive animals were used in this study because they have reduced or no detectable SpC3 in their coelomocytes or coelomic fluid (CF). Animals were injected with lipopolysaccharide (LPS) or sterile sea water (SSW, injury control). Changes in the amounts of SpC3 in coelomic fluid and in coelomocytes were then followed over time by Western blots and ELISA. Changes in mRNA from the SpC3 gene (*Sp064*) were also followed by RT-PCR. Although all animals responded to injury with increased levels of SpC3 in

the coelomic fluid, those challenged with LPS had greater amounts of SpC3 in both CF and coelomocytes than those receiving SSW. In most of the animals receiving LPS, initial increases in SpC3 were observed within 1 h post-injection, while the earliest response in the animals receiving SSW was 6 h. The appearance of SpC3 in the coelomocytes was delayed compared to its appearance in CF, and was first detected several days after challenge. Changes in mRNA from the *Sp064* gene paralleled the appearance of SpC3 in the coelomic fluid. Increases in the number of coelomocytes per milliliter of CF and in the percentage of coelomocytes that were SpC3⁺ also occurred after challenge with LPS or in response to injury, with a slightly greater increase in response to LPS. Although the changes in SpC3 were not as great as those identified previously for human C3 expressed in macrophages, the kinetics of the response are similar to that of acute-phase reactants in mammals.

L.A. Clow and P.S. Gross contributed equally to this work

L.C. Smith (✉)
Department of Biological Sciences,
George Washington University, 334 Lisner Hall,
2023 G Street NW, Washington, DC 20052, USA
E-mail: csmith@gwu.edu
Phone: +1-202-9949211
Fax: +1-202-9946100

L.A. Clow · L.C. Smith
The Graduate Program in Genetics,
Institute of Biomedical Sciences, George Washington University,
Washington, DC 20052, USA

P.S. Gross
Department of Biological Sciences,
George Washington University, Washington, DC 20052, USA

C.-S. Shih
The School of Medicine, George Washington University,
Washington, DC 20052, USA

Present address:

P.S. Gross
Department of Biochemistry,
Medical University of South Carolina, 173 Ashley Avenue,
P.O. Box 250509, Charleston, SC 29425, USA

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Introduction

The immune system in the sea urchin has been characterized as an innate immune response which lacks adaptive capabilities. Evidence for nonadaptive immunity in this deuterostome invertebrate has come primarily from studies of allograft rejection kinetics and functions of the coelomocytes, cells that mediate host defense functions and that are found in the coelom of adult animals (reviewed in Gross et al. 1999). Allograft rejection kinetics demonstrated that sea urchins can differentiate between self and nonself, but they cannot distinguish between different allogeneic tissues. The rejection rates for second-set allografts were accelerated relative to first-set rejections, but they were not different from rejections for third-party allo-

grafts (Coffaro 1980; Coffaro and Hinegardner 1977). This defines the sea urchin immune response as non-specific and perhaps similar to the innate system in higher vertebrates (reviewed in Smith and Davidson 1992). Sea urchin coelomocytes mediate the host response to immune challenge in several ways, including chemotaxis, phagocytosis, encapsulation, and secretion (reviewed in Gross et al. 1999; Smith and Davidson 1994). Coelomic fluid (CF) from the purple sea urchin, *Strongylocentrotus purpuratus*, contains a mixture of at least four types of coelomocytes (Booltian and Geise 1958; Johnson 1969) including a subpopulation of macrophage-like, phagocytic cells that can be further subdivided into a number of subsets based on differences in morphology and gene expression (Edds 1993; Gross et al. 2000).

The gene for profilin was the first to be shown to have enhanced expression in coelomocytes activated by injury or lipopolysaccharide (LPS) (Smith et al. 1992, 1995). Profilin is a small, cytoplasmic, actin-binding protein, which has a central role in actin filament modification, and mediates changes in cell shape (Schlüter et al. 1997). Increases in profilin message content in coelomocytes were identified and quantitated using transcript titration methods (Smith et al. 1992) and this information was used to infer general activation of the sea urchin immune response (Smith et al. 1995), since cellular clot formation, amoeboid movement, and phagocytosis require significant changes in coelomocyte shape (Edds 1977). Phagocytes undergo dramatic cytoskeletal reorganization when they alter their pseudopodia from petaloid to filipodial during the process of activation (Edds 1977). These shape changes also occur in response to decreases in salt concentrations (Edds 1979) as well as during phagocytosis and encapsulation (Bertheussen and Seljelid 1978; Johnson 1969). They involve Ca^{2+} fluxes (Henson and Schatten 1983; Hyatt et al. 1984) and a number of proteins including calmodulin, calmodulin-binding proteins (Venuti and Edds 1986), spectrin (Edds and Venuti-Henderson 1982), fascin (DeRosier and Edds 1980; Otto et al. 1979), and perhaps, unconventional myosin (D'Andrea et al. 1994). Consequently, quantitation of increases in profilin message content was used as a marker to identify the level of activation for coelomocytes from individual sea urchins after immune challenge with LPS (Smith et al. 1995). Using this method to assess animals with activated immune responses, genes that encoded proteins involved in host protection were identified from an expressed sequence tag (EST) study (Smith et al. 1996). Two ESTs were characterized as encoding homologues of mammalian complement components. The first, SpC3, is a homologue of the central complement component, C3 (Al-Sharif et al. 1998) and the second, SpBf, is a homologue of factor B (Bf) (Smith et al. 1998). The presence of complement homologues in all major deuterostome phyla that have been investigated, namely echinoderms, protochordates, and

chordates (for a review see Smith et al. 1999) suggest that there is homology among the innate immune systems within this animal lineage (Smith et al. 1996).

The complement system is a major mediator of innate immunity in deuterostomes. In higher vertebrates, it is composed of four pathways; the three initiating pathways, classical, alternative, and lectin, converge to activate the terminal pathway (for a review see Volanakis 1998). The initiating pathways are activated by different mechanisms. The classical pathway is initiated by antigen-antibody complexes, while the alternative pathway is activated by the direct binding of C3 to microbial surfaces. The lectin pathway is initiated by the interaction of mannose-binding lectin with microbial polysaccharides (Endo et al. 1998; Zhang et al. 1999). The three initiation pathways merge with the activation of C3, which binds to microbial surfaces or immune complexes leading either to augmented phagocytosis of the opsonized microbe or particle, or to initiation of the terminal lytic pathway, the formation of the membrane attack complex, membrane destabilization, and cell lysis.

The biosynthesis and expression of complement proteins in mammals have been studied for many years, particularly with respect to the function and expression of the central component of the complement system, C3. Vertebrate C3 is produced in the liver (Alper et al. 1969), but is also expressed at a number of extrahepatic sites, the most important being macrophages (Colten et al. 1986; Einstein et al. 1977; Fischer et al. 1998; Whaley 1980). In response to immune challenge, serum C3 increases 2- to 3-fold as a result of hepatic production (Colten 1992). However, macrophages have been shown to increase production of C3 in vitro as much as 50- to 80-fold in 8–24 h with corresponding increases in mRNA (Botto et al. 1992; Jiang et al. 1995). Based on this responsiveness, C3 has been classified as an acute-phase reactant and is important in the induction of systemic and localized inflammatory responses in mammals.

Previous studies on SpC3 have not addressed changes in gene or protein expression in response to immune challenge (Al-Sharif et al. 1998). If the sea urchin immune system is homologous to aspects of the innate system of mammals, then the functions and expression patterns of SpC3 should be similar to patterns characterized for acute-phase proteins in mammals. Therefore, we monitored gene and protein expression in sea urchins after challenge with LPS or injury using RT-PCR, Western blots analyzed by densitometry, and enzyme-linked immunosorbent assay (ELISA). We report here that the amount of SpC3 was increased in CF and in coelomocytes from animals challenged with LPS, and was enhanced to a lesser extent in animals challenged with sterile sea water (SSW). Changes in the message content in coelomocytes from the *Sp064* gene reflected changes in protein levels in both the cells and the CF. We found that the appearance of SpC3 in coelomocytes from sea urchins

responding to LPS was significantly delayed compared to the appearance of SpC3 in CF, suggesting that coelomocytes may not be the only source of SpC3 production. Furthermore, challenge with LPS and injury resulted in an increase in the number of SpC3⁺ coelomocytes in the CF. These data suggest that the sea urchin homologue of C3 may function as an acute-phase reactant that increases significantly in response to a perceived bacterial infection.

Materials and methods

Animal housing

Purple sea urchins, *S. purpuratus*, were collected and housed as described elsewhere (Gross et al. 2000). Animals had been kept for more than 18 months and had become immunorespondent (Gross et al. 1999).

Injections and sample collection

Sea urchin wet weight was used to calculate the amount of antigen to inject according to the following formula:

$$\text{animal weight (g)} \times 0.219 = \text{total CF (ml)} \times 2 = \text{micrograms LPS to inject}$$

This resulted in an antigenic challenge of about 2 µg LPS/ml CF (LPS was from *Vibrio cholerae*; lot L5262; Sigma, St. Louis, Mo.) as previously described (Smith et al. 1995). Similarly, volumes of SSW given to the control animals were estimated to be 2 µl/ml CF, and were calculated to mimic the volume of injected LPS [1 µg/µl artificial sea water (ASW); Humphreys 1963]. Injected animals were kept in individual aquaria containing 1.5 l of aerated seawater and placed in an incubator set to 14°C. Water was changed daily until the experiment was terminated.

Whole CF (wCF; 300 µl) was withdrawn into a 1-cm³ syringe fitted with a 23-gauge needle and preloaded with 150 µl of calcium/magnesium-free seawater containing 30 mM EDTA and 50 mM imidazole (CMFSW-EI; pH 7.4, 460 mM NaCl, 10.0 mM KCl, 7.0 mM Na₂SO₄, 2.4 mM NaHCO₃) (Humphreys 1963, as modified by Gross et al. 1999). Immediately following collection of CF, an additional 150 µl of CMFSW-EI was added to the syringe and mixed with the CF to block clotting and cell lysis. Half of the sample was used to prepare cell-free CF. Pepstatin A (10 mM; Sigma) and phenylmethanesulfonyl fluoride (2 µM; Sigma) were added and the cells were removed by centrifugation at 11,000 g for 2 min and stored at -70°C. The remaining wCF sample (300 µl) was diluted with 700 µl CMFSW-EI, the cells were counted, pelleted, and resuspended in 2×lysis buffer (4% SDS, 20% glycerol, 20% β-mercaptoethanol, 0.1 M Tris pH 6.8) to yield 10,000 cells/µl. Lysates were vortexed for 8–12 min, boiled for 2 min, and stored at -70°C.

Antisera

A polyclonal rabbit antiserum (αSpC3-6H) was raised against a fragment of the SpC3 α chain that was expressed in bacteria and to which had been added six, N-terminal histidines for the purposes of affinity isolation (Gross et al. 2000). Briefly, a 1400-bp fragment encoding *M_r* 51,400 of the N-terminal region of the α chain of SpC3 (including the α' region and the thioester site) was amplified from the cDNA by PCR and cloned into the pQE32 bacterial expression vector (Qiagen, Valencia, Calif.). The fusion protein was isolated by Ni²⁺ affinity chromatography and injected into two rabbits as described elsewhere (Gross et al. 2000).

A second antibody was provided by Dr. John Lambris, University of Pennsylvania, that was directed to the 26 amino acids located at the N-terminus of the α' chain from SpC3 (anti-SpC3-α'). The peptide was conjugated to keyhole limpet hemocyanin and injected into rabbits (Al-Sharif et al. 1998; Gross et al. 1999, 2000). The pre-immune serum and antiserum were affinity purified with protein A (Pierce, Rockford, Ill.), aliquoted and stored at -70°C.

Western blots

Both CF and cell lysates were electrophoresed on SDS-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose as described by Gross and co-workers (2000). Briefly, filters were blocked, incubated with αSpC3-6H (diluted 1:18,000 to 1:25,000, see below) followed by goat anti-rabbit immunoglobulins conjugated to horse radish peroxidase (GαRIg-HRP, diluted 1:10⁵). After washing, filters were immersed in Super Signal Chemiluminescent Substrate (Pierce) and exposed to X-OMAT X-ray film (Eastman Kodak, Rochester, N.Y.). Films were analyzed densitometrically using Kodak Digital 1D Image Analysis software. Data for each animal were normalized by subtracting the value of the -15 min time point from all data points thereby essentially setting the -15 min time point to zero. Means and SEs for each treatment group were calculated and plotted using Excel 97 (Microsoft).

Enzyme-linked immunosorbent assay

Serial dilutions of CF (from 1:10 to 1:2560 in ASW) were incubated in 96-well plates (Corning, Corning, N.Y.) for 2 h at room temperature (rt) to bind the proteins to the wells. After washing with ASW followed by ASW with 0.03% Tween 20 (ASW-Tween), wells were incubated overnight at 4°C with 60 µl of blocking solution [Tris-buffered saline (TBS; 200 mM Tris, 500 mM NaCl) with 1% normal goat serum (Sigma), 3% bovine serum albumin (Fisher Scientific), 0.05% Tween 20]. After washing with TBS-Tween, 50 µl of αSpC3-6H (1:1000 in block) was added to the wells and incubated for 2 h. Plates were washed and incubated for 1 h with 50 µl of GαRIg conjugated to alkaline phosphatase (GαRIg-AP; Sigma) diluted 1:6400 in blocking solution. After three washes with TBS-Tween, the substrate, p-nitrophenyl phosphate (Southern Biotechnology Associates, Birmingham, Ala.) was added and incubated until the yellow color developed. The reaction was stopped with NaOH (1.5 N) and read at 405 nm on a SpectraMax 340 plate reader (Molecular Devices, Sunnyvale, Calif.). The dilution series of CF undertaken for each time point for each animal was analyzed to characterize the relationship between CF concentration and spectrophotometric readout. The region of the CF dilution series (three dilutions between 1:20 and 1:80) showing a linear relationship between OD and 5 µl of CF was used to calculate an average OD for each sampling time point. The average OD for each time point for each sea urchin was normalized by subtracting the value obtained for the -15 min time point from all subsequent time points, thus generating net values. Data for each group, LPS- and SSW-challenged sea urchins, were combined and means and SEs were calculated and plotted using Excel 97 (Microsoft).

Reverse transcription-polymerase chain reaction

Coelomic fluid was collected in CMFSW-EI, as described above, and cells were pelleted and lysed for total RNA isolation with RNazolB (Leedo Medical Labs, Houston, Tex.) according to the manufacturer's instructions. RT-PCR was carried out as described elsewhere (Gross et al. 2000). To compare samples from individual sea urchins, RT-PCR bands were analyzed by densitometry and normalized to the amount of actin in each sample.

Analysis of SpC3⁺ phagocytes

Cells (10^5) were collected as described above, centrifuged onto slides at 620 *g* using a cytospin rotor (Eppendorf) and stained for SpC3 using anti-SpC3- α' antiserum followed by G α RIg conjugated to either fluorescein isothiocyanate (G α RIg-FITC; Pierce) (Gross et al. 2000) or to horseradish peroxidase (G α RIg-HRP; Pierce). The substrate reaction for HRP employed diaminobenzadine (DAB) and was performed according to the manufacturer's instructions (Vector, Burlingame, Calif.). Cells were observed by phase contrast microscopy (DAB staining) or fluorescence microscopy (FITC staining) using a Zeiss Axioscope microscope (Zeiss, Oberkochen, Germany). Stained cells were counted and the percent SpC3⁺ cells was calculated.

Results

Previous reports have shown that expression of the gene for profilin increases after challenge with LPS or injury (Smith et al. 1992, 1995); however, there were several problems with the methods used to obtain these data. Initially, RNA used for profilin transcript titrations was isolated from coelomocytes that were pooled from six animals (Smith et al. 1992). It became apparent during the course of those studies that data from single animals were important to obtain, so a protocol to isolate coelomocyte RNA from single animals was developed (Smith et al. 1992, 1995). However, we found that interpretation of the results was still complicated because wild-collected animals are all genetically different and responded somewhat differently to experimental challenge (Smith et al. 1992). In addition, sea urchins were housed in a facility that was very carefully tended, but designed with an open sea water system. Ocean water was pumped into the Caltech Kerckhoff Marine Laboratory from the mouth of the harbor at Newport, Calif., where the facility was located. This meant that the animals were continuously exposed to microbes and other challenges from the environment and that their immune systems were at least partially activated before any experiment was initiated.

These problems were addressed by modifying several of the protocols that had been used previously for the current study. First, by miniaturizing the assays to use as little as 300 μ l of CF, single animals could be sampled repeatedly and responses to immune challenge or injury could be followed over time by either Western blot, ELISA, or RT-PCR. Changes in both protein levels and message content before and after challenge could be analyzed from a single animal in addition to comparing the differences between the experimental and control groups. Second, sea urchins were housed in closed aquaria with a water sterilization system and multiple filtering systems for more than 18 months before the study was initiated. We had found that animals kept for long periods of time in a benign environment became immunoquiescent (Gross et al. 1999), and these animals were used in the current study to generate baseline data for com-

parison to data from the same animals activated by immune challenge and/or injury.

Time course of SpC3 appearance in CF and in coelomocytes

To identify changes in SpC3 level in CF and coelomocytes in response to immune challenge, animals were injected with either LPS ($n=6$) or SSW ($n=3$, injury control) every 24 h over the course of 5 days. Samples taken 15 min prior to the first injection were used to establish the baseline level of SpC3 in the immunoquiescent sea urchins. Additional samples were collected 15 min after each injection and, on the first day of the experiment, samples were also taken at 3 and 6 h post-injection. Between days 2 and 9, samples were collected once a day, followed by sample collections once every 3 days from day 9 to 33. After that, animals were sampled weekly and then bimonthly, until day 90. Two animals that had received injections of LPS and one that had received SSW were sampled again on day 258. All samples were analyzed by Western blots using the α SpC3-6H antiserum that recognized the α chain of the SpC3 protein.

SpC3 in the CF

In three of the six animals that received injections of LPS (LPS2, LPS4, LPS6; Fig. 1), the amount of SpC3 in the CF increased sharply between the pre-injection time point and 15 min post-injection. Two other LPS-injected animals (LPS1, LPS5; Fig. 1) showed increases in SpC3 in CF between day 3 and days 8 or 9, although LPS1 showed a delay in the appearance of detectable SpC3 until day 2. The sixth LPS-injected animal (LPS3; Fig. 1) showed variable changes in SpC3, with no clear increases in the CF during the first 9 days of the experiment. Overall, these six animals showed a maximum amount of SpC3 in CF between days 4 and 7. After day 9, SpC3 levels generally decreased in LPS-injected animals until day 15 when it increased again until day 21 (data not shown). After day 21, LPS-injected animals showed variable levels of SpC3 in CF until day 90. In comparison, the animals injected with SSW generally responded with lower levels of SpC3. One of the three SSW-injected animals (SSW8; Fig. 1) showed increased levels of SpC3 in the CF 15 min post-injection which then changed very little afterwards. A second SSW-injected animal (SSW9; Fig. 1) did not show significant changes in the amount of SpC3 in CF during the first 9 days of the experiment. In the third animal that received SSW (SSW7; Fig. 1), SpC3 was undetectable in CF until day 2 but showed no increases thereafter. From day 9 until day 90, animals that received SSW showed relatively constant, but lower levels of SpC3 in the CF compared to animals that received LPS (data not

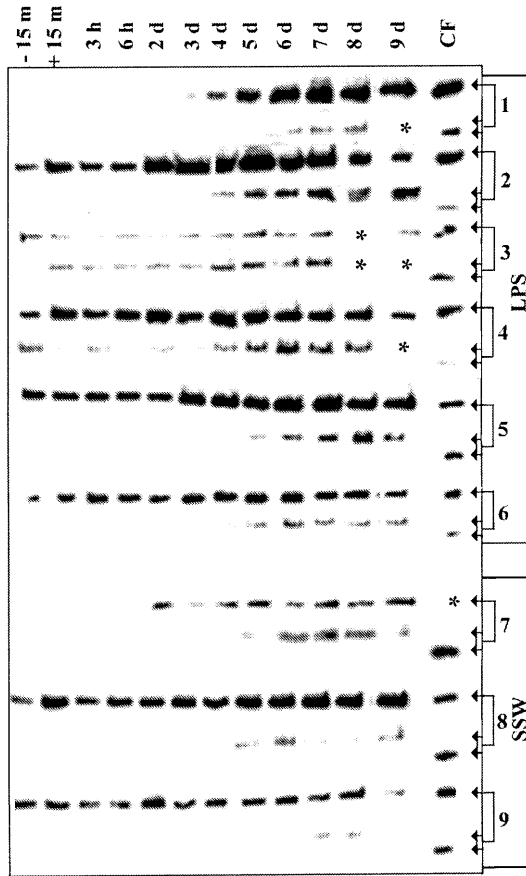


Fig. 1 Expression of SpC3 in lipopolysaccharide (LPS)- and sterile sea water (SSW)-injected animals. Samples of proteins from coelomocytes and coelomic fluid (CF) were separated by SDS-PAGE, and Western blots were probed with α SpC3-6H. The *brackets* on the right indicate whether animals received injections of LPS or SSW. For each animal, the SpC3 detected in CF is shown above the proSpC3 detected in the corresponding coelomocyte samples for each animal. The figure is a composite of 18 different gels showing only the first 9 days of the experiment during which animals received five daily injections and were sampled until day 258. The M_r 130,000 band in the samples from CF represents the cleaved α -chain protein, whereas the M_r 210,000 band in the coelomocyte samples represents the full-length, uncleaved proSpC3. *Arrowheads*, to the right, indicate both sizes; the M_r 210,000 band is above and the M_r 130,000 band is below for coelomocytes. For CF samples, the arrow indicates the M_r 130,000 band. The control for SpC3 (labeled as CF in the last lane to the right) was a sample of CF taken from an activated animal that was used repeatedly as a size standard on each gel. An *asterisk* indicates missing data

shown). No SpC3 was detected in any CF sample taken on day 258.

SpC3 in coelomocytes

Coelomocytes were also analyzed for their SpC3 content by Western blot (Fig. 1). Coelomocyte samples did not have the M_r 130,000 α chain present in the CF samples, but rather, had a M_r 210,000 band equal in size to the unreduced SpC3 (Fig. 1; compare coelomo-

cyte samples to the lane labeled CF, and see figure legend). The M_r 210,000 band has been reported previously from coelomocytes and represents proSpC3 that has not undergone final processing to cleave the $\beta\alpha$ junction that separates the α and β chains (Gross et al. 2000). Mammalian cells that have been induced to produce C3 also show a large proC3 band on protein gels (Beuscher et al. 1987; Goldman et al. 1991; Haga et al. 1996; Katz et al. 1989; Strunk et al. 1985; Sutton et al. 1986). Changes in the amount of proSpC3 in coelomocytes paralleled changes in the α chain in the CF for each sample from each animal, although the amount of proSpC3 was much less than that noted for the secreted α chain in the CF (Fig. 1; compare all coelomocyte samples to all corresponding CF samples). Of the nine animals analyzed for responses to LPS and SSW, eight did not have detectable proSpC3 in the coelomocytes before the first injection (Fig. 1; only LPS4 shows detectable proSpC3 at -15 min). For animals that received LPS, one (LPS3; Fig. 1) showed a significant increase in proSpC3 15 min post-injection, and four others (LPS1, LPS2, LPS5, and LPS6; Fig. 1) did not show detectable proSpC3 until days 2–5. None of the SSW-injected animals showed detectable proSpC3 until day 3–5 (SSW7, SSW8, and SSW9; Fig. 1). Overall, during the first 9 days of the experiment, the amount of proSpC3 in coelomocytes from LPS-injected animals peaked between day 6 and 9 and was present in greater amounts than seen in animals responding to the SSW injury. Animals that received SSW showed no discernible peak. In samples taken between days 9 and 15, LPS-injected animals maintained a relatively constant amount of proSpC3 in coelomocytes which paralleled levels in CF and which was maintained until day 90 (data not shown). Animals that received SSW had variable amounts of SpC3 in coelomocytes between days 9 and 33 followed by a steady decline until day 90 (data not shown). Coelomocyte samples taken on day 258 did not have detectable proSpC3.

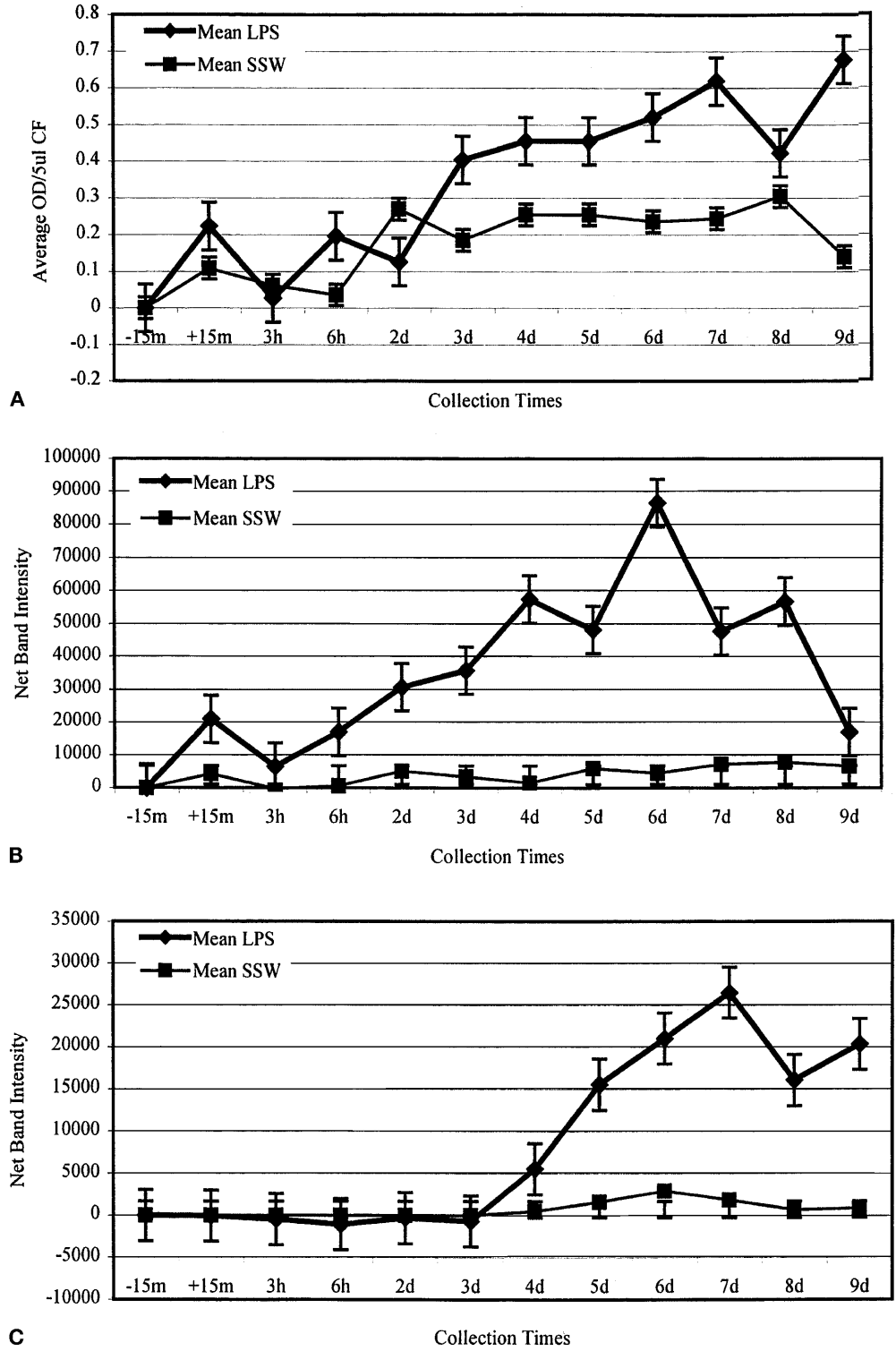
The results from this long-term experiment demonstrated several things. First, we found that when small amounts of CF were taken, sea urchins could be sampled repeatedly over long periods of time without obvious deleterious effects. Consequently, these results are the first to follow immune reactivity in individual sea urchins over time, rather than relying on comparisons between different animals (for example, see Smith et al. 1992, 1995). Second, the amount of proSpC3 in coelomocytes was either very low or undetectable in most of the immunoquiescent sea urchins before immune challenge or injury. Third, swift and dramatic increases were identified in most animals; however, those responding to LPS produced and maintained higher levels of SpC3 more quickly and for longer than animals responding to injury. Finally, when animals were not manipulated for at least 168 days in a closed aquarium, the amount of SpC3 in CF and coelomocytes returned to immunoquiescent levels.

Analysis of SpC3 in CF by ELISA

To compare changes in the amount of SpC3 in CF from animals injected with LPS with those receiving SSW using a different method, samples were also analyzed by ELISA. Results from a dilution series of the CF for each time point for each animal indicated

that sea urchins responded to LPS or SSW 15 min post-injection with increased amounts of SpC3 (Fig. 2A). The amount of SpC3 in CF from all sea urchins fluctuated between +15 min and 2 days. The amount in LPS-injected animals generally increased after 2 days while remaining relatively constant in SSW-injected animals. By 3 days, animals that received LPS

Fig. 2 **A** ELISA analysis of SpC3 in CF. The relative amount of SpC3 in CF from LPS-injected ($n=6$) and SSW-injected ($n=3$) sea urchins that were analyzed by Western blot (see Fig. 1; LPS1-6 and SSW7-9), were confirmed by ELISA. Data shown are for the first 9 days of the experiment that continued until day 258. Each point represents the mean OD from a dilution series (1:20-1:80). **B** Densitometry of SpC3 in CF. Data shown are for the first 9 days and each point represents the mean and SE net band intensity of SpC3 in CF samples for each set of sea urchins as measured using Kodak 1D Scientific Analysis Software. **C** Densitometry of SpC3 in coelomocytes. The assay was done as in **B**



showed significantly elevated levels of SpC3 compared to those that received SSW. These data agree with those from Western blot analysis and suggest that sea urchins responded to both immune challenge and to injury by producing SpC3, but that responses to a combination of LPS plus injury resulted in greater increases.

Analysis of SpC3 in CF and coelomocytes by densitometry

The protocol discussed above was a direct ELISA. This method depends on the efficiency of the antigen to bind to plastic, which may be affected by the concentrations of other proteins in the sample. Although there are no published data to indicate that there are increases in other proteins in the CF of sea urchins undergoing immune activation that might interfere with SpC3 binding to plastic, this may occur. Therefore, we analyzed the Western blots by densitometry (Fig. 2B). The results obtained were similar to those generated by ELISA (compare Fig. 2A,B), and show an increase in SpC3 content in CF in LPS-challenged animals compared to those that received SSW.

The coelomocyte samples shown in Fig. 1 were also analyzed by densitometry (ELISA analysis could not be done on these samples due to interference from the SDS in the gel lysis buffer) (Fig. 2C). Animals injected with LPS showed a sharp increase in proSpC3 beginning on day 4 while animals injected with SSW showed a slight increase in proSpC3 that peaked on day 6. Overall, the densitometry reflected the results shown in Fig. 1.

Early responses: SpC3 protein content and Sp064 expression in response to LPS or injury

SpC3 protein expression

Results from the Western blots (Fig. 1), ELISAs (Fig. 2A), and densitometry (Fig. 2B,C) for the first 9 days of the long-term experiment, indicated that interesting changes in SpC3 occurred during the very early time points. Therefore, the experiment was redesigned to concentrate our observations on the first 2 days, and to track simultaneously changes in SpC3 in the CF and *Sp064* message content in the coelomocytes. Sea urchins were injected twice (0 and 24 h) with either LPS ($n=6$) or SSW ($n=6$), and samples of wCF were collected before (-15 min) and after injection [+15 min or 1 h, 3 or 6 h, 24 h (just prior to the second injection), 36 h, 48 h] and processed for RT-PCR and Western blots. Of the 12 animals analyzed, results from representative animals are shown in Fig. 3. In five of the six animals that received LPS, the amount of SpC3 in wCF increased dramatically 1 h after the first injection compared to the pre-injection level (Fig.

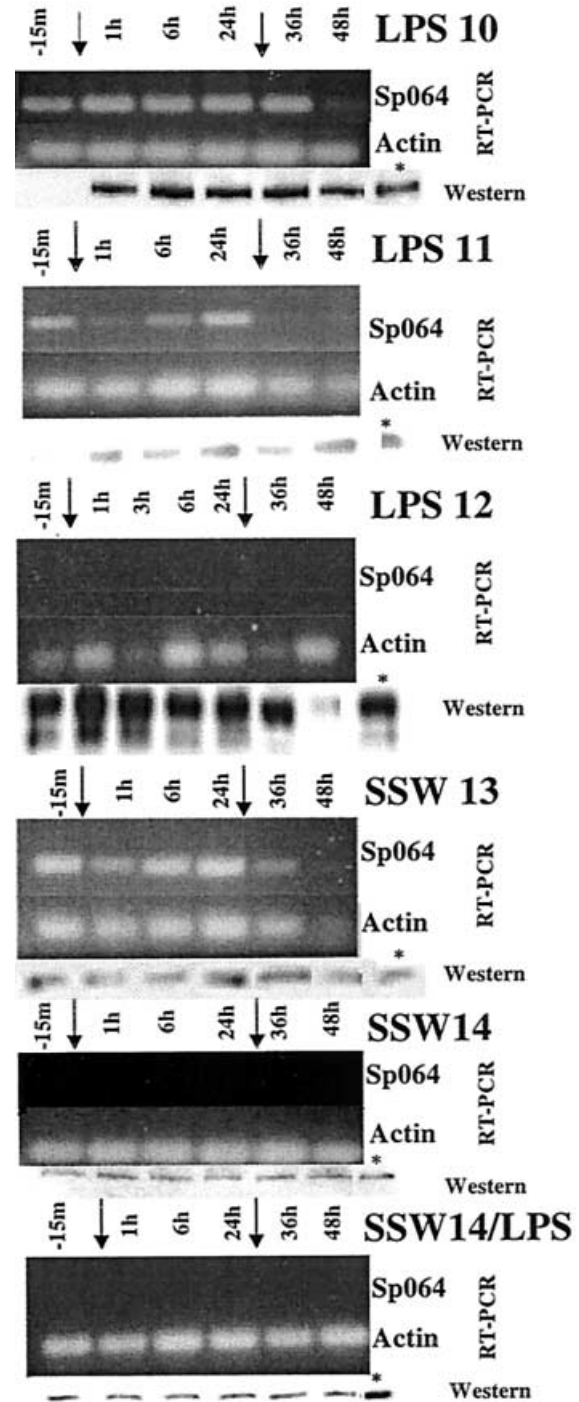


Fig. 3 Early changes in *Sp064* and SpC3 expression in coelomocytes responding to either LPS or SSW (injury). Samples from sea urchins were analyzed by both RT-PCR and Western blots. Injections were performed after the -15 min time point and after the 24 h time point (arrows). Coelomocytes were processed for RT-PCR and wCF was processed for SDS-PAGE and Western blot analysis with α SpC3-6H. Data from individual animals are grouped, with RT-PCR results shown above Western blots. Primers used for RT-PCR amplified messages from *Sp064* (upper panel for each animal) and the actin gene (lower panel). Results from Western blot analysis are shown in the bottom panel for each animal. Time points are indicated above each series and correspond to both RT-PCR and Western blots. The CF control for each Western blot is shown to the right and indicated by an asterisk

3; see Western blots for LPS10 and LPS11, and LPS12). In general, the second injection of LPS given at 24 h resulted in no differences in SpC3 detected in wCF from any animal; however, samples were taken 12 h after the second injection, which presumably did not reveal quick responses. For five of the six animals that received SSW, no increase in the amount of SpC3 by 6 h post-injection was noted compared to the pre-injection time points. Data, including Western blots, from two representative animals that received SSW are shown in Fig. 3 (SSW13 and SSW14). Overall, these data indicate that sea urchins respond to a greater degree and more quickly to challenge from LPS than to injury. These results agree with those shown in Fig. 1.

Sp064 gene expression

Analysis of *Sp064* gene expression in coelomocytes from the 11 sea urchins used in this experiment showed two types of results (representative examples of gels are shown in Fig. 3). From our original sample size of 12, actin bands could not be detected in 1 LPS-injected animal, indicating that the RNA was degraded and, therefore, the data from that animal were not included in the analysis. For the remaining 11, amplified bands for *Sp064* could be detected in 4 that had received LPS and in 3 that had received SSW. To illustrate our results, 3 representative animals with detectable *Sp064* bands are shown in Fig. 3 (LPS10, LPS11, and SSW13). Several other animals (2 LPS-injected animals and 3 SSW-injected animals) had undetectable *Sp064* gene expression even though SpC3 was present in wCF (representative examples are shown in Fig. 3; compare RT-PCR to Western blots for LPS12 and SSW14). This result suggested that either *Sp064* was not being transcribed, or that the ethidium bromide was not sensitive enough to show the amplified bands in the gels. To increase the sensitivity, gels were blotted onto GeneScreen Plus (Dupont/NEN) and probed with a ^{32}P -labeled fragment generated from an SpC3 cDNA using the same primers employed in the RT-PCR amplification of coelomocyte messages. The results demonstrated that, in fact, all animals were expressing the *Sp064* gene, but at very low levels (data not shown). Overall, when densitometry was used to normalize the *Sp064* bands to the actin bands on gels and Southern blots, four of the five animals that received LPS had increased *Sp064* band intensities between +15 min and 24 h post-injection. On the other hand, only three of six animals that received SSW showed increases in *Sp064* between +15 min and 3 h post-injection, while the remaining three animals either had no change in *Sp064* band intensity, or the band intensity decreased compared to the pre-injection time point (Southern blots are not shown; an example of a gel is shown in Fig. 3; SSW13).

One of the SSW-injected animals shown in Fig. 3 (SSW14) did not respond to injury with detectable bands from the amplified *Sp064* message. Therefore, to see if gene expression could be induced, it was subsequently injected with LPS and samples of wCF were processed for Western blot and RT-PCR analysis. Results showed that actin message was present in all samples but that expression from *Sp064* could not be induced to detectable levels on the gel (Fig. 3, SSW14/LPS). However, analysis of the gel by Southern blot showed that the animal was expressing *Sp064* at very low levels, and that there was a slight increase in the amount of the amplified band 1 h post-injection (data not shown).

This series of experiments was designed to monitor the levels of SpC3 in wCF and the expression of *Sp064* in coelomocytes in immunoinactive sea urchins that were responding to immune challenge by LPS compared to injury controls. The results suggest that sea urchins were capable of responding quickly to challenge by LPS and that increases in protein content and gene expression occurred between 15 min to 24 h after challenge. In general, more striking responses were seen in the animals responding to LPS in contrast to those responding to injury.

Total coelomocytes and SpC3⁺ coelomocytes increased after challenge

Results from RT-PCR, Western blots, and ELISA (Figs. 1, 2, and 3) suggested that increases in SpC3 in CF were due to increased *Sp064* gene transcription and increased production and secretion of SpC3. However, these results could also be explained by an increase in the total number of coelomocytes and/or an increase in the number of SpC3⁺ cells in CF. To determine whether cell numbers varied in response to immune challenge or injury, coelomocyte samples were taken from sea urchins 24 h after receiving a single injection of either LPS ($n=8$) or SSW ($n=8$) and total numbers of coelomocytes per milliliter of CF were compared to pre-injection samples, using the paired *t*-test. The results indicated that the total number of coelomocytes per milliliter of CF in individual sea urchins increased significantly for both the experimental and control animals (LPS, $P<0.012$; SSW, $P<0.0016$). When fold increases in coelomocyte numbers from individual animals were calculated and used to compare changes in the two sets of animals, the increases were found not to differ between the two sets (LPS, 1.5 ± 0.3 fold increase; SSW, 1.7 ± 0.7 fold increase).

The above analysis for total coelomocytes was repeated to understand changes in the SpC3⁺ coelomocytes. We used the anti-SpC3- α' antiserum to identify positive cells in the pre-injection and the 24-h post-injection samples and the percent positive cells were calculated. A paired *t*-test used to compare the

pre- and post-injection samples for individual sea urchins indicated that there were significant increases in the SpC3⁺ cells in both sets of animals (LPS, $P < 0.005$; SSW, $P < 0.009$). When the fold increase in SpC3⁺ coelomocytes was calculated for individual sea urchins and the means and standard deviations were calculated for each set of animals, we found that the number of SpC3⁺ cells in animals 24 h after receiving LPS was increased 7.36 ± 9.3 fold, while animals that received SSW showed a 4.07 ± 4.6 fold increase. The large standard deviations were a result of differences in responses among the different sea urchins, which made it difficult to determine whether these two groups were responding differently to LPS compared to SSW. However, the trend suggested that more coelomocytes appeared in CF in sea urchins responding to challenge by LPS compared to those responding to injury. Variations in responses to LPS have also been noted in studies of C3 expression in human macrophages in culture (Strunk et al. 1985) and in in vivo studies of reporter gene expression controlled by the C3 promoter in inbred mice (Varley et al. 1995). In general, increases in the amount of SpC3 in sea urchins after challenge can best be explained as a combination of *Sp064* message accumulation in the coelomocytes, an increase in SpC3 production and secretion, and an increase in the number of SpC3⁺ cells in CF.

Discussion

The mammalian complement system is composed of at least 30 different proteins that function in the activation and terminal pathways or that modulate complement activation by either stabilizing, inhibiting, or promoting the functions of various complement components (for a review see Volanakis 1998). The central component in this complex system is C3, which functions at the intersection of the initiating and terminal pathways and interacts with a large number of molecules. When C3 is activated and cleaved to form C3b it can either activate the terminal pathway through its involvement in the formation of C5 convertase or it can function directly as an opsonin. These activities, which are not mutually exclusive, lead to lysis of foreign cells, or to tagging of foreign cells for uptake and destruction by host cells bearing C3b receptors.

Acute-phase reactants are proteins produced by the liver that increase significantly in serum concentration during an inflammatory response, and that are under the control of the inflammatory cytokines (Bauman and Gauldie 1994; Fearon and Locksley 1996; Mocci et al. 1997). Human C3 has been defined as an acute-phase reactant, which increases two- to threefold in the serum following production by the liver (Baumann 1989). Mononuclear phagocytes are also an important source of complement components, including C3, and their production and secretion at localized sites are

important for initiating local inflammatory responses that act to control local infections (Colten and Strunk 1993; Colten et al. 1986). The time course of C3 synthesis and secretion from cultured monocytes, macrophages, polymorphonuclear leukocytes, and fibroblasts increased between 2 and 12 h after incubation with LPS (Botto et al. 1992; Katz et al. 1989; Strunk et al. 1985). However, expression of a reporter gene under the control of the C3 gene promoter was detected in vivo more quickly, that is, 3 h after challenge with LPS (Varley et al. 1995). Data presented here for SpC3 production in vivo for 8 of 12 sea urchins responding to LPS showed maximal increases in CF between 15 min and 1 h post-injection (Figs. 1, 2, and 3). Although variable responses to LPS were noted in sea urchins, similar variability has been noted in studies of C3 production in vitro by monocytes from different human donors (Strunk et al. 1985), as well as in in vivo studies of mice transformed with a C3-reporter construct expressed in the liver (Varley et al. 1995). The differences in reporter protein production were due to variability in cytokine expression levels (Varley et al. 1995). However, the basis for variable expression of C3 in genetically different, outbred animals such as sea urchins and humans (Strunk et al. 1985; Sutton et al. 1986) is not known.

The appearance of proSpC3 in coelomocytes was significantly delayed (as late as 2 days post-challenge) when compared to SpC3 in CF (appeared as early as 15 min post-challenge), or compared to the appearance of proC3 in mammalian cells (24 h post challenge) (Beuscher et al. 1987; Goldman et al. 1991; Katz et al. 1989; Strunk et al. 1985; Sutton et al. 1986). This difference suggests that there may be other sites of SpC3 production in sea urchins besides the coelomocytes in the CF. Possible sources could include (a) coelomocytes not present in the CF such as emarginated cells and/or coelomocytes that have not been released from their (unknown) site of proliferation and differentiation, (b) fibroblast cells, (c) epithelial cells, or (d) an as yet unidentified liver-like tissue. In mammals, both fibroblasts and endothelial cells have been shown to secrete localized C3 (Katz and Strunk 1989; Katz et al. 1989; Lappin et al. 1992; Strunk et al. 1994). Although we have suggested previously that the phagocyte type of coelomocyte is the only major tissue source of SpC3 (Gross et al. 2000; Al-Sharif et al. 1998), the body wall, coelomic lining, and axial organ were not analyzed, and therefore these structures are possible candidates for production of SpC3. Each of these tissues could presumably contain fibroblastic and endothelial cells or they may support hematopoietic center(s) that produce most or all of the new coelomocytes (reviewed by Edean 1966). Any of these tissues may secrete SpC3.

The amount of *Sp064* mRNA in coelomocytes responding to LPS showed maximal increases at 15 min–24 h post-injection compared to pre-injection levels. Although mammalian cells have not been

analyzed for C3 gene expression as early as 15 min post-challenge, induction of primary macrophage cultures with LPS showed that C3 mRNA was present in these cells before induction, and that increased amounts of C3 mRNA were noted 24 h post-induction (Botto et al. 1992; Haga et al. 1996; Strunk et al. 1994). Examples of other rapid responses to immune challenge from bacteria include the induction of gene expression for the sea urchin homologue of nuclear factor κ B and SpRunt in coelomocytes after 6 h (Pancer et al. 1999), and the expression of antibacterial genes in insect hemocytes and fat body also occur within 1–2 h of bacterial challenge (Lemaitre et al. 1997; Wicker et al. 1990). In addition, responses to heat and cold stress in the sea urchin, *Paracentrotus lividus*, have been shown to result in the induction of heat shock proteins (hsp70) in coelomocytes after 30 min–1 h (Matranga et al. 2000). Increases in *Sp064* mRNA content in coelomocytes could also be due to message stabilization, with or without increased transcription from the *Sp064* gene. Mammalian macrophages in culture show increases in the half-life of C3 mRNA upon activation after 24 h of high doses of interferon- γ (Mitchell et al. 1996). This type of post-transcriptional regulation is thought to occur through protein interactions with the 3' untranslated region (UTR) of mRNAs that contain repeats of the AUUUA motif (Caput et al. 1985). Although the "AU repeat" is not present in the human C3 mRNA, multiple copies have been identified in the 3'UTR of *Sp064* (Al-Sharif et al. 1998) and increases in messages containing these repeats have been demonstrated in coelomocytes from activated sea urchins (Asson-Batres et al. 1994).

Changes in SpC3 protein levels in CF that occur as quickly as 15 min post-challenge are probably due to a combination of mechanisms which include de novo synthesis and mass secretion of the protein after swift and efficient gene transcription and message stabilization, all of which may be amplified by increased numbers of SpC3⁺ cells. Coelomocytes of the large phagocyte class contain SpC3 localized in granules throughout the cytosol (Gross et al. 2000). The morphology of these phagocytes is consistent with SpC3 storage in transport or secretory vesicles that, upon immune challenge, would fuse with the plasma membrane and secrete their contents into the CF. If this exocytotic mechanism is employed by sea urchins to increase rapidly the amount of SpC3 in CF, coelomocytes with detectable quantities of proSpC3 before injection should be evident followed by a significant reduction in proSpC3 at the 15-min post-injection time point. This was observed in one animal injected with LPS (LPS4; Fig. 1). Finally, increases in the relative number of SpC3⁺ coelomocytes in the CF 24 h after challenge might also contribute to increased SpC3 levels in both cells and CF. Although neither the source nor proliferation rate of SpC3⁺ cells is known, if these cells are released from noncirculating

sites into the CF, their relative increase within the coelomocyte population would change the levels of SpC3 in samples from both the cells and the CF. In summary, sea urchins likely use multiple mechanisms, perhaps to varying degrees in different animals, to maximize the amount of SpC3 that is available quickly for protection against a perceived infection.

Few studies on immune responses in invertebrates have followed immune activation over time. In a study on another sea urchin species, *Lytechinus pictus*, rates of allograft rejection were analyzed to determine the length of time between first- and second-set allografts until the second-set allograft was rejected at the same rate as the first set, i.e., did not show accelerated rejection (Coffaro 1979; reviewed in Smith and Davidson 1992, 1994). At least 6 months were required before the accelerated second-set rejection rates began to increase, meaning that the activation of the immune system of the animals in response to the first-set graft had started to abate. We have found that the time required for SpC3 to return to pre-injection levels in either CF or coelomocytes, i.e., to become undetectable (and for the animals to become immunologically quiescent), was about 8 1/2 months when measured from the first of the five injections of LPS. Based on the differences in the activating substances, a solid-tissue graft versus a component of the Gram-negative bacterial outer membrane, the duration of immune activation in these two species of sea urchins is remarkably similar. In the absence of an adaptive immune response in echinoderms, prolongation of the innate response may be the only mechanism available to induce protection against an immune challenge that might re-occur within a short time span. The benefit of an activated but nonspecific system is that all nonself contacts would be neutralized at an accelerated rate. On the other hand, in a relatively benign environment that did not continuously present immune challenges, the disadvantage of the nonspecific response would mean that an immune challenge that re-occurred after 6–8 months, would be handled as a new contact. For a long-lived animal such as the purple sea urchin, this appears to be an adequate solution for host protection.

In higher vertebrates, the innate immune system activates and directs the adaptive immune system for optimal host defense that is specifically directed at the general type of challenge presented. The first and essential task of the innate system is to discriminate between self and nonself. This is accomplished through pattern recognition receptors that are germline encoded and have been selected through evolution for broad recognition capabilities of unique and invariant structures common to large classes of microbes (Janeway 1989). Examples of pattern recognition receptors in mammals include the mannose-binding lectin (Stahl et al. 1998), the Toll receptor that was first characterized in insects (Lemaitre et al. 1996), and more recently in humans (Medzhitov et al.

1997; Rock et al. 1998; for review see Kopp and Medzhitov 1999), and the alternative pathway of complement that includes the capability of C3 to bind to nonself (Carroll and Fischer 1997; Janeway 1989). The role of adjuvant has been recognized as an important function of C3 and is based on augmented capabilities of the adaptive immune system to produce specific antibodies against very small quantities of antigen when bound with C3d, the fragment of C3 that includes the thioester site (Dempsey et al. 1996). The covalent attachment of C3 fragments to foreign particles and cellular pathogens significantly improves the abilities of cells to detect the antigen and is based on the presence of the complement receptor (CR) type 2 on B cells, and CR1 and/or CR3 on phagocytic cells (reviewed in Carroll and Fischer 1997; Law 1988). The end result of nonself identification mediated by C3 is efficient antigen uptake and presentation, and the efficient activation of the adaptive immune system.

The "broad spectrum" recognition capabilities that are characteristic of pattern recognition receptors in higher vertebrates are typical of immune responses in sea urchins and in invertebrates in general. Sea urchin SpC3 (Al-Sharif et al. 1998; Smith et al. 1996) is typical of such pattern recognition receptors. The thioester on the α chain of SpC3 is typical of other thioester-containing proteins, and is capable of binding methylamine (Smith, in press), opsonizing yeast for augmented phagocytosis (L.A. Clow and L.C. Smith, unpublished data) and appears to function as a typical C3 homologue for nonself recognition. However, SpC3 function is limited compared to vertebrate C3 in that it acts to recognize nonself but does not act as an adjuvant because there is no adaptive immune system in echinoderms. To fully understand the instructional activation that is imposed on the adaptive system by the innate system in higher vertebrates, additional information will be needed to predict the state of the ancestral innate immune system present in the ancestor of jawed vertebrates at the time of the invention of adaptive immunity through the appearance of rearranging genes. What were the components and characteristics of that innate system that enabled the ancestral *RAG* genes (Agrawal et al. 1998; Hiom et al. 1998; Schatz 1999) to become established in the genome of the ancestral vertebrate? The complexity of the ancestral system was presumably limited compared to the modern innate system in mammals. This is evident from comparisons between the complex complement system of higher vertebrates and the simple complement system in the sea urchin. A significant expansion of complement components and complement pathways in higher vertebrates occurred concurrently with the appearance of immunoglobulins, T-cell receptors, and major histocompatibility molecules in the sharks and rays (reviewed in Gross et al. 1999). This amplification may have been due to individual gene duplications that created small gene families from ancestral complement genes (Bentley 1988;

Dodds and Day 1993) and may also have been due to genome duplications that duplicated complement pathways (Kasahara 1999). One of the best examples of genomic evolutionary history is the complex innate system in mammals, with more components, systems, and functions than the sea urchin system, which may be very similar to the innate system that functioned in the ancestral deuterostome. Therefore, the sea urchin system may be very useful for identifying the underlying, and perhaps central components and functions of the mammalian innate system as it is today. A case in point, is the position of central importance held by the mammalian complement system in host defense. Future investigations of the simple sea urchin immune system may well identify other components with homologues in the mammalian innate immune system and provide a picture of the immune system that functioned in the vertebrate ancestor and to which the adaptive system was added and integrated.

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References

- Agrawal A, Eastman QM, Schatz DG (1998) Transposition mediated by RAG1 and RAG2 and its implications for the evolution of the immune system. *Nature* 394:744–51
- Alper CA, Johnson AM, Birtch AG, Moore FD (1969) Human C3: evidence for the liver as the primary site of synthesis. *Science* 163:286–288
- Al-Sharif WZ, Sunyer JO, Lambris JD, Smith LC (1998) Sea urchin coelomocytes specifically express a homologue of the complement component C3. *J Immunol* 160:2983–2997
- Asson-Batres MA, Spurgeon SL, Diaz J, DeLoughery TG, Bagby GC (1994) Evolutionary conservation of the AU-rich 3' untranslated region of messenger RNA. *Proc Natl Acad Sci USA* 91:1318–1322
- Baumann H (1989) Hepatic acute phase reaction in vivo and in vitro. *In Vitro Cell Dev Biol* 25:115–126
- Baumann H, Gauldie J (1994) The acute phase response. *Immunol Today* 15:74–80
- Bentley DR (1988) Structural superfamilies of the complement system. *Expl Clin Immunogenet* 5:69–80
- Bertheussen K, Seljelid R (1978) Echinoid phagocytes in vitro. *Exp Cell Res* 111:401–412
- Beuscher HU, Fallon RJ, Colten HR (1987) Macrophage membrane interleukin 1 regulates the expression of acute phase proteins in human hepatoma Hep 3B cells. *J Immunol* 139:1896–1901
- Booltian RA, Geise AC (1958) Coelomic corpuscles of echinoderms. *Biol Bull* 115:53–63
- Botto M, Lissandrini D, Sorio C, Walport MJ (1992) Biosynthesis and secretion of complement component (C3) by activated human polymorphonuclear leukocytes. *J Immunol* 149:1348–1355
- Caput D, Beutler B, Hartog K, Thayer R, Brown-Shimer S, Cerami A (1985) Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators. *Proc Natl Acad Sci USA* 83:1670–1674

- Carroll MC, Fischer MB (1997) Complement and the immune response. *Curr Opin Immunol* 9:64–69
- Coffaro KA (1979) Transplantation immunity in the sea urchin. PhD thesis, University of California, Santa Cruz
- Coffaro KA (1980) Memory and specificity in the sea urchin *Lytechinus pictus*. In: Manning MJ (ed) Phylogeny of immunological memory. Elsevier/North Holland, New York, pp 77–80
- Coffaro KA, Hinegardner RT (1977) Immune response in the sea urchin *Lytechinus pictus*. *Science* 197:1389–1390
- Colten HR (1992) Tissue-specific regulation of inflammation. *J Appl Physiol* 72:1–7
- Colten HR, Strunk RC (1993) Synthesis of complement components in liver and at extrahepatic sites. In: Whaley K, Loos M, Weiler JM (eds) Complement in health and disease. *Immunol Med* 20:127–158
- Colten HR, Strunk RC, Perlmutter D, Cole FS (1986) Regulation of complement protein biosynthesis in mononuclear phagocytes. *Ciba Found Symp* 118:141–154
- D'Andrea, Danon MA, Sgourdas G, Bonder EM (1994) Identification of coelomocyte unconventional myosin and its association with in vivo particle/vesicle motility. *J Cell Sci* 107:2081–2094
- Dempsey P, Allison MED, Akkaraju S, Goodnow CC, Fearon DT (1996) C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* 271:348–350
- DeRosier DJ, Edds KT (1980) Evidence for fascin cross-links between the actin filaments in coelomocyte filopodia. *Exp Cell Res* 126:490–494
- Dodds AW, Day AJ (1993) The phylogeny and evolution of the complement system. *Immunol Med* 20:39–88
- Edds KT (1977) Dynamic aspects of filopodial formation by reorganization of microfilaments. *J Cell Biol* 73:479–491
- Edds KT (1979) Isolation and characterization of two forms of a cytoskeleton. *J Cell Biol* 83:109–115
- Edds K (1993) Cell biology of echinoid coelomocytes. I. Diversity and characterization of cell types. *J Invert Pathol* 61:173–178
- Edds KT, Venuti-Henderson J (1982) Coelomocyte spectrin. *J Cell Motil* 3:691–693
- Einstein LP, Hansen P, Ballow M, Davis AE, Davis J, Alper CA, Rosen FS, Colten HR (1977) Biosynthesis of the third component of complement (C3) in vitro by monocytes from both normal and homozygous C3-deficient humans. *J Clin Invest* 60:963–969
- Endean R (1966) The coelomocytes and coelomic fluids. In: Boolotian RA (ed) Physiology of Echinodermata. Intersciences, New York, pp 301–328
- Endo Y, Takahashi M, Nakao M, Sekin H, Matsushita M, Nonaka M, Fujita T (1998) Two lineages of mannose-binding lectin-associated serine protease (MASP) in vertebrates. *J Immunol* 161:4924–4930
- Fearon DT, Locksley RM (1996) The instructive role of innate immunity in the acquired immune response. *Science* 272:50–53
- Fischer MB, Ma M, Hsu NC, Carroll MC (1998) Local synthesis of C3 within the splenic lymphoid compartment can reconstitute the impaired immune response in C3-deficient mice. *J Immunol* 160:2619–2625
- Goldman M, Knovich M, Goldman JN (1991) Immunologic control of C3 gene expression in tissue macrophages. *J Immunol* 147:4248–4255
- Gross PS, Al-Sharif WZ, Clow LA, Smith LC (1999) Echinoderm immunity and the evolution of the complement system. *Dev Comp Immunol* 23:439–442
- Gross PS, Clow LA, Smith LC (2000) SpC3, the complement homologue from the purple sea urchin, *Strongylocentrotus purpuratus*, is expressed in two subpopulations of the phagocytic coelomocytes. *Immunogenetics* DOI 10.1007/s002510000234
- Haga S, Aizawa T, Ishii T, Ikeda K (1996) Complement gene expression in mouse microglia and astrocytes in culture: comparisons with mouse peritoneal macrophages. *Neurosci Lett* 216:191–194
- Henson JH, Schatten G (1983) Calcium regulation of the actin-mediated cytoskeletal transformation of sea urchin coelomocytes. *Cell Motil* 3:525–534
- Hiom K, Melek M, Gellert M (1998) DNA transposition by the RAG1 and RAG2 proteins: a possible source of oncogenic translocations. *Cell* 94:463–70
- Humphreys T (1963) Chemical dissolution and in vitro reconstruction of sponge cell adhesions. I. Isolation and functional demonstration of the components involved. *Dev Biol* 8:27–47
- Hyatt HA, Shure MS, Begg DA (1984) Induction of shape transformation in sea urchin coelomocytes by the calcium ionophore A23187. *Cell Motil* 4:57–71
- Janeway C (1989) Cross-linking and conformational change in T-cell receptors: role in activation and in repertoire selection. *Cold Spring Harbor Symp*. 54:57–66
- Jiang S-L, Samols D, Rzewnicki D, Macintyre SS, Greber I, Sipe J, Kuchner I (1995) Kinetic modeling and mathematical analysis indicate that acute phase gene expression in Hep 3B cells is regulated by both transcriptional and posttranscriptional mechanisms. *J Clin Invest* 95:1253–1261
- Johnson PT (1969) The coelomic elements of sea urchins (*Strongylocentrotus*) I. *In vitro* reaction to bacteria. *J Invert Pathol* 13:42–62
- Kasahara M (1999) The chromosomal duplication model of the major histocompatibility complex. *Immunol Rev* 167:17–32
- Katz Y, Strunk RC (1989) IL-1 and tumor necrosis factor: similarities and differences in stimulation of expression of alternative pathway of complement and IFN-beta 2/IL-6 genes in human fibroblasts. *J Immunol* 142:3862–3867
- Katz Y, Revel M, Strunk RC (1989) Interleukin 6 stimulates synthesis of complement proteins factor B and C3 in human skin fibroblasts. *Eur J Immunol* 19:983–988
- Kopp EB, Medzhitov R (1999) The Toll-receptor family and control of innate immunity. *Curr Opin Immunol* 11:13–18
- Lappin DF, Guc D, Hill A, McShane T, Whaley K (1992) Effect of interferon-gamma on complement gene expression in different cell types. *Biochem J* 281:437–442
- Law A (1988) C3 receptors on macrophages. *J Cell Sci Suppl* 9:67–97
- Lemaitre B, Nicolas E, Michaut L, Reichart J-M, Hoffmann JA (1996) The dorsoventral regulatory cassette *spätzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell* 86:973–983
- Lemaitre B, Reichhart JM, Hoffmann JA (1997) *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proc Natl Acad Sci USA* 94:14614–14619
- Matranga V, Toia G, Bonaventura R, Müller WEG (2000) Cellular and biochemical responses to environmental and experimentally induced stress in sea urchin coelomocytes. *Cell Stress Chaperones* 5:158–165
- Medzhitov R, Preston-Hurlburt P, Janeway CA (1997) A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388:394–397
- Mitchell TJ, Naughton M, Norsworthy P, Davies KA, Walport MJ, Morley BJ (1996) IFN-gamma up-regulates expression of the complement components C3 and C4 by stabilization of mRNA. *J Immunol* 156:4429–4434
- Mocci S, Dalrymple SA, Nichinakamura R, Murray R (1997) The cytokine stew and innate resistance to *L. monocytogenes*. *Immunol Rev* 158:107–114
- Otto JJ, Kane RE, Bryan J (1979) Formation of filopodia in coelomocytes: localization of fascin, a 58,000 dalton actin cross-linking protein. *J Cell* 17:285–293
- Pancer Z, Rast JP, Davidson EH (1999) Origin of immunity: transcription factors and homologues of effector genes of the vertebrate immune system expressed in sea urchin coelomocytes. *Immunogenetics* 49:773–786
- Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF (1998) A family of human receptors structurally related to *Drosophila* Toll. *Proc Natl Acad Sci USA* 95:588–593

- Schatz DG (1999) Transposition mediated by RAG1 and RAG2 and the evolution of the adaptive immune system. *Immunol Res* 19:169–182
- Schlüter K, Jockusch BM, Rothkegel M (1997) Profilins as regulators of actin dynamics. *Biochem Biophys Acta* 1359:97–109
- Smith LC (in press) The complement system in sea urchins. In: Beck G, Sugumaran M, Cooper E (eds) *Phylogenetic perspectives on the vertebrate immune system*. Plenum, New York
- Smith LC, Davidson EH (1992) The echinoid immune system and the phylogenetic occurrence of immune mechanisms in deuterostomes. *Immunol Today* 13:356–362
- Smith LC, Davidson EH (1994) The echinoderm immune system: characters shared with vertebrate immune systems and characters arising later in deuterostome phylogeny. *Ann N Y Acad Sci* 712:213–226
- Smith LC, Britten RJ, Davidson EH (1992) SpCoel1: a sea urchin profilin gene expressed specifically in coelomocytes in response to injury. *Mol Biol Cell* 3:403–414
- Smith LC, Britten RJ, Davidson EH (1995) Lipopolysaccharide activates the sea urchin immune system. *Dev Comp Immunol* 19:217–224
- Smith LC, Chang L, Britten RJ, Davidson EH (1996) Sea urchin genes expressed in activated coelomocytes are identified by expressed sequence tags: complement homologues and other putative immune response genes suggest immune system homology within the deuterostomes. *J Immunol* 156:593–602
- Smith LC, Shih C-S, Dachenhausen S (1998) Coelomocytes specifically express SpBf, a homologue of factor B, the second component in the sea urchin complement system. *J Immunol* 161:6784–6793
- Stahl P, Allen R, Ezekowitz B (1998) The mannose receptor is a pattern recognition receptor involved in host defense. *Curr Opin Immunol* 10:50–55
- Smith LC, Azumi K, Nonaka M (1999) Complement systems in invertebrates: the ancient alternative and lectin pathways. *Immunopharmacology* 42:107–120
- Strunk RC, Whitehead AS, Cole FS (1985) Pretranslational regulation of the synthesis of the third component of complement in human mononuclear phagocytes by the lipid A portion of lipopolysaccharide. *J Clin Invest* 76:985–990
- Strunk RC, Fleischer JA, Katz Y, Cole FS (1994) Developmentally regulated effects of lipopolysaccharide on biosynthesis of the third component of complement and factor B in human fibroblasts and monocytes. *Immunology* 82:314–320
- Sutton MB, Strunk RC, Cole FS (1986) Regulation of the synthesis of the third component of complement and factor B in cord blood monocytes by lipopolysaccharide. *J Immunol* 136:1366–1372
- Varley AW, Coulthard MG, Meidell RS, Gerard RD, Munford RS (1995) Inflammation-induced recombinant protein expression in vivo using promoters from acute-phase protein genes. *Proc Natl Acad Sci USA* 92:5346–5350
- Venuti J, Edds K (1986) Calmodulin and calmodulin-binding proteins in the morphological transformation of sea urchin coelomocytes. *Cell Motil Cytoskel* 6:604–619
- Volanakis JE (1998) Overview of the complement system In: Volanakis JE, Frand MM (eds) *The human complement system in health and disease*. *Immunol Med* 20:9–32
- Whaley K (1980) Biosynthesis of the complement components and the regulatory proteins of the alternative complement pathway by human peripheral blood monocytes. *J Exp Med* 151:501–516
- Wicker C, Reichhart J-M, Hoffman D, Hultmark D, Samakovlis C, Hoffmann JA (1990) Insect immunity: characterization of a *Drosophila* cDNA encoding a novel member of the dipterin family of immune peptides. *J Biol Chem* 265:22493–22498
- Zhang Y, Suankratay C, Zhang X-H, Lint TF, Gewurz H (1999) Lysis via the lectin pathway of complement activation: minireview and lectin pathway enhancement of endotoxin-initiated hemolysis. *Immunopharmacology* 42:81–90