



## Echinoderm immunity and the evolution of the complement system

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### Abstract

Our understanding of inflammatory responses in humans has its roots in the comparative approach to immunology. In the late 1900's, research on echinoderms provided the initial evidence for the importance of phagocytic cells in reactions to foreign material. Studies of allograft rejection kinetics have shown that echinoderms have a non-adaptive, activation type of immune response. Coelomocytes mediate the cellular responses to immune challenges through phagocytosis, encapsulation, cytotoxicity, and the production of antimicrobial agents. In addition, a variety of humoral factors found in the coelomic fluid, including lectins, agglutinins, and lysins, are important in host defense against pathogens and other foreign substances. Recently, a simple complement system has been identified in the purple sea urchin that is homologous to the alternative pathway in vertebrates. The sea urchin homologue of C3, is inducible by challenge with lipopolysaccharide, which is known to activate coelomocytes. Complement components have been identified in all vertebrate classes, and now have been characterized in protochordates and echinoderms indicating the primordial nature of the complement system. Because it is thought that the complement system evolved from a few primordial genes by gene duplication and divergence, the origin of this system appears to have occurred within the common ancestor of the deuterostomes. © 1999 Elsevier Science Ltd. All rights reserved.

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### 1. Historical background

The comparative approach to immunology

had its origin in early studies of the causes and mechanisms of inflammation. Elie Metchnikoff, the chief proponent of the cellular theory of immunity, asserted that inflammation was the most important phenomenon in pathology and proposed that the primary effectors of the immune response were circulating, amoeboid-like, phagocytic cells [1]. In sup-

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port of his cellular theory of inflammation, Metchnikoff observed that under natural conditions, the bipinnaria larvae of the sea star, *Astropecten pentacanthus*, would respond to both physical injury and to infection by marine algae. These initial observations led Metchnikoff to perform the benchmark experiments in cellular immunology which included introducing rose prickles and glass rods into a bipinnaria larva and finding that mesodermal cells migrated to the injury site and encapsulated the prickle (for review see Ref. [1]). Furthermore, he demonstrated that this phenomenon also occurred when bacteria were introduced into the larva, noting that the bacteria were 'devoured alive' by the phagocytic cells. In 1908, Metchnikoff, along with Paul Ehrlich, was awarded the Nobel Prize for this groundbreaking work using echinoderms as a model, thus beginning the field of comparative cellular immunology.

## 2. Allograft rejection

For nearly half a century after Metchnikoff, the comparative approach was largely ignored until William Hildemann became interested in how sessile animals on a crowded coral reef defended themselves against overgrowth and fusion. He and his colleagues investigated echinoderm immunity by employing allograft rejection to assess immune capabilities in these organisms. Sea stars (*Protoreaster nodosus* and *Dermasterias imbricata*) and a sea cucumber (*Cucumaria tricolor*) were used to demonstrate the capability of these animals to differentiate between self and allogeneic tissues [2,3]. Results indicated that they would reject first set skin allografts in a chronic manner, that repeated allografts (2nd set and 3rd set) were rejected more quickly, and that autografts healed in and remained intact. Unfortunately, third party allografts were either not done or were inconclusive. Histological analyses of rejecting allografts had a significant cellular infiltrate that increased with time [2–4] and appeared to correspond to the phagocytes in the sea star larva that were observed by Metchnikoff.

These studies were the first to demonstrate that echinoderms could respond to allogeneic tissue although the specificity of the response was unclear.

To clarify the non-self recognition capabilities of echinoderms and to characterize their rejection kinetics, primary, secondary and third party allografting experiments were performed on the sea urchin, *Lytechinus pictus* [5–7] and to a lesser extent on the sea urchin, *Strongylocentrotus purpuratus* [6]. Results indicated that sea urchins lacked specific non-self recognition and true immunological memory since 2nd set and 3rd party tissues were rejected at the same rate [6,8].

## 3. Coelomocyte functions

The defense capabilities of echinoderms were defined from allograft rejection experiments, but research has also concentrated on characterizing and understanding the functions of the coelomocytes, the cells that mediate these responses. Coelomocytes are found in the fluid filled coelom and, depending on the species, can be a mixture of several morphologically different types. Sea urchins of the genus *Strongylocentrotus* have four types of coelomocytes which include phagocytes (present in all echinoderms, also called bladder, petal or filiform amoebocytes), red spherule cells (also called eleocytes, morula cells and pigment cells), colorless spherule cells (also called white morula cells) and vibratile cells [9–12].

### 3.1. Coelomocyte infiltrates in injury and infection

Coelomocytes have long been considered to be mediators of the immune response, in part, because of their presence surrounding injuries and infections in several echinoderm species and their appearance in grafted tissues. Skin infections become ringed with black or dark red tissues that are accumulations of red spherule cells in the sea urchin, *Paracentrotus lividus* [13] and in *S. purpuratus* (Smith, unpublished observation). Infiltrates of red spherule cells have also been documented as red spots on gonadal tissue of the sea urchin *Strongylocentrotus intermedius*

that surround metacercaria of a parasitic worm [14]. Cellular infiltrates consisting of phagocytes and red spherule cells have also been noted around broken, infected, and regenerating spines of *S. purpuratus* [15]. Red pigmented cells accumulated at sites of surgical injuries during the initial phase of tissue transplantation in *L. pictus* [5] and histological analysis of allografts in the sea star, *D. imbricata* revealed mixed cellular infiltrates in which cell densities increased during the chronic rejection process [3,4].

### 3.2. Coelomocyte responses to foreign substances

The survival of echinoderms in the microbe-rich marine environment is dependent on their ability to defend themselves against microbial invasion. Combating infections must include rapid, efficient, and sometimes selective clearance of foreign invaders in order for an animal to survive. The capability of echinoderms to eliminate injected pathogens, foreign cells, and other types of particles has been well documented. Injections of bacteria into the coelomic cavity of the sea star *D. imbricata* [16] or into the sea urchins, *Echinus esculentus* [17], *S. purpuratus*, [18] or *Strongylocentrotus droebachiensis* [19] were efficiently cleared in a few hours to a few days. In contrast to the sterile coelomic fluid of sea urchins, pre-injection analysis of coelomic fluid from the sea cucumber, *Parastichopus californicus*, revealed the presence of a natural bacteria flora with as many as  $10^8$  bacteria per milliliter of coelomic fluid. Consequently, bacteria injected into the coelomic cavity of *P. californicus* were either not cleared or were cleared at varying rates which depended on whether the bacteria were isolated from the gut of the sea cucumber or were obtained from other sources [20].

Echinoderms are also capable of clearing xenogenic cells, foreign non-cellular particles and proteins. Injection of cells from the sea urchin, *Arbacia punctulata* into the coelom of the sea star, *Asterias vulgaris* resulted in clumping of the sea star phagocytes, trapping or phagocytosis of the sea urchin cells, and rapid clearance of the injected cells from the coelomic fluid [21]. The sea cucumber, *Holothuria polii* efficiently phago-

cytosed and encapsulated injected red blood cells (RBCs) [22], as did the sea urchin *S. droebachiensis* [23]. The sea urchin, *L. pictus*, efficiently cleared T4 bacteriophage [24] and carmine particles injected into the coelomic cavity of *P. californicus* resulted in agglutination, encapsulation and brown body formation followed by the excretion of the brown bodies into the cloaca [20]. Even latex beads were quickly cleared from the coelomic cavity of the sea urchin, *S. droebachiensis* [23].

In addition to using repeated allografting to assess the specificity of immune memory in echinoderms, repeated injections of foreign substances have also been performed. However, no differences have been noted in clearance rates of bacteria injected multiple times into the coelomic cavity of *S. purpuratus*, regardless of the interval between inoculations [18]. Similarly, accelerated clearance rates were not demonstrated when xenogenic cells were injected a second time into *A. vulgaris* [21], nor after repeated injections of T4 bacteriophage into *L. pictus* [24]. These results corresponded with those obtained from allografting experiments, which had demonstrated the absence of specific immunological memory in echinoderms.

The activities and functions of coelomocytes have also been studied in vitro, where the coelomic fluid from a number of echinoderms has been shown to be bactericidal. Coelomic fluid from *P. lividus* exhibited higher bactericidal activity in vitro when coelomocytes were present than after the cells had been removed [25]. Similar results were reported for coelomocytes from *E. esculentus*, which were found to be bactericidal against *Pseudomonas* sp. and had a wide range of antibacterial activity against both gram negative and gram positive marine bacteria [17,26]. Lysates of phagocytes and red spherule cells from *P. lividus* were bactericidal against both *Vibrio* spp. and *Photobacterium* spp. suggesting that lysozymes and echinochrome (the pigment of red spherule cells) produced by these cells might mediate the bactericidal activity [27]. In addition, echinochrome-A from *E. esculentus* had effective bactericidal activity against marine bacteria in vitro [28].

### 3.3. Cytotoxicity

The phagocyte appears to be the cell type involved in cytotoxic reactions in mixtures of coelomocytes *in vitro*. When phagocytes from the sea urchin *S. droebachiensis* were co-cultured with phagocytes from either *E. esculentus* or *Strongylocentrotus pallidus*, 90% of the cells were killed, and in allogeneic mixtures of *S. droebachiensis* phagocytes, 70% of the cells were killed [29]. However, there was significant variability in the amount of cell killing between different combinations of cells, and Dales [30] was unable to repeat this result using coelomocytes from sea stars and different species of sea urchins. This indicated that either that phagocytes do not have cytotoxic capabilities, or that this method of analyzing coelomocyte function had a number of technical difficulties. On the other hand, others were able to isolate cytolytic granules from phagocytes of *P. lividus*, suggesting that these cells mediated their killing function through the release of this cytolytic material [31].

### 3.4. Phagocytosis

A subpopulation of coelomocytes from echinoderms are defined by their amoeboid behavior and their abilities to engulf foreign cells and particles. Phagocytes from two sea urchin species, *Strongylocentrotus franciscanus* and *S. purpuratus*, were noted to chemotax towards marine bacteria, with gram positive bacteria being phagocytosed more readily than gram negative bacteria [10]. Human and sheep RBCs were taken up within 30 min *in vitro* by phagocytes from the sea urchin, *Strongylocentrotus nudus*, and RBCs opsonized with coelomic fluid from animals that had been pre-injected with RBCs enhanced the phagocytic rate compared to non-opsonized red cells [32].

An important function of the phagocytic cells is degradation of phagocytosed material. Circulating phagocytes from the sea cucumber, *H. polii*, contain a rich selection of lysosomal enzymes, including acid and alkaline phosphatases,  $\beta$ -glucuronidase, aminopeptidase, acid and alkaline protease, and lipase [33]. The presence of

lysozyme and acid phosphatase has also been documented in the sea cucumber *P. californicus* [20]. In addition, arylsulphatase, a lysosomal hydrolase known to play a role in inflammatory phenomena by catalyzing the hydrolysis of sulfate bonds, has been biochemically detected in coelomocyte lysate preparations of seven different echinoderms [34] and a cDNA from *S. purpuratus* coelomocytes matched to arylsulphatase by expressed sequence tag (EST) analysis [35].

## 4. Humoral factors

### 4.1. Cytolytic, bactericidal, and agglutinating factors

The capacity of humoral factors to damage target cells has been shown for a number of echinoderms. Hemolysins have been detected from coelomic fluid of the sea star, *A. forbesi* [36], from several sea urchin species [25,37], as well as from the sea cucumber, *H. polii* [38]. The hemolysin from *P. lividus* binds to erythrocytes, zymosan particles, lipopolysaccharide (LPS), and laminarin surfaces, but not to self or allogeneic cell membranes [39]. Factors in the coelomic fluid of the sea cucumber *P. californicus*, were variably effective at killing bacteria *in vitro* depending on the source of the bacteria [20].

Another type of humoral factor, agglutinins, appear to be involved in maintaining body integrity after injury and in encapsulating foreign invaders. Divalent cation-dependant hemagglutinins have been found in three sea urchin species [40], the sea star, *Asterina pectinifera* [41], and *H. polii* [38]. The hemagglutinin in *P. lividus*, a heterotrimeric complex that binds rabbit RBCs, enhances the adhesive properties of autologous coelomocytes and may be involved in cell–cell and cell–matrix interactions such as clotting, wound repair, opsonization, and encapsulation [42].

Lectins are, in part, responsible for agglutination reactions by binding to specific carbohydrate structures on cells and on extracellular matrices. Lectins have been isolated from the coelomic fluid of several echinoderms and are thought to be important in identifying foreign

cells through opsonization. Two different  $\text{Ca}^{2+}$ -dependent (or C-type) lectins have been identified in the sea cucumber, *Stichopus japonicus* [43] and three lectins from the sea star *A. pectinifera* have different binding abilities; one preferentially agglutinates rabbit RBCs, another binds human RBCs, and the third is a bacterial agglutinin [41]. Echinoidin, a C-type lectin from the sea urchin, *Anthocidaris crassispina*, also has an RGD sequence [44] that can mediate cell adhesion between mammalian cancer cells [45]. In fact, two ESTs with sequence similarities to the carbohydrate binding region of echinoidin have been identified from a coelomocyte cDNA library from *S. purpuratus* [35]. The C-type lectin, CEL-III, from the sea cucumber *Cucumaria echinata*, is another hemagglutinin that lyses rabbit and human RBCs by a novel pore-forming mechanism and may be toxic to foreign microbes [46]. In echinoderms, lectins play important roles in defense by performing opsonization and lytic functions as well as functioning in clot formation and wound repair.

#### 4.2. Other humoral immune effectors

The phenoloxidase system (PO) is important in immune defense in several groups of invertebrates. In arthropods, the PO system is well characterized and is part of an immune surveillance cascade (for review see Ref. [47]). In echinoderms, PO activity has been identified in coelomic fluid and in circulating coelomocytes from certain echinoderms, including the sea star *Asterias rubens* and the sea urchin, *Diadema antillarum* [48,49]. A proposed model of activation, based on the arthropod system, involves the activation of a pro-enzyme by trypsin, to yield an intermediate which, in turn, is stimulated by calcium to form the active phenoloxidase tetramer [50,51]. Another common defense mechanism in plants and animals is the production of reactive oxygen intermediates, although at present the phagocytes from the sea urchin, *S. nudus* are the only echinoderm cells known to produce hydrogen peroxide in vitro when co-incubated with RBCs [32].

In vertebrates, a well studied family of immune

effector molecules are the cytokines, such as interleukin-1, which have wide spread effects on proliferation of immune cells and other stimulatory effects, resulting in rapid and efficient responses to immune challenges. Interleukin-1-like activity has been found in *A. forbesi* [52] and cytokine activity has also been observed in the sea star, *Pisaster ochraceus* [53]. Of interest is the heterodimer known as sea star factor from *A. forbesi*, which appears to have cytokine-like properties and may function to regulate the sea star immune response [54–56]. When minute quantities of sea star factor were injected into the coelomic cavity of *A. forbesi*, it functioned like a non-mitogenic lectin, stimulating rapid but temporary, localized, tight aggregations of circulating coelomocytes. Although none of the cytokine-like molecules from echinoderms have been sequenced and definitively shown to be interleukin homologues, the systemic responsiveness of echinoderms to minimal and localized immune challenges infers that some type of molecular signaling system exists in these animals.

Based on the data reviewed above, echinoderms appeared to have a typical invertebrate type of immune system that functioned through non-adaptive mechanisms. Immune responses were based on coelomocyte activity (chemotaxis and phagocytosis) working in parallel with a variety of humoral factors (lectins, agglutinins, opsonins, bactericidal and probably fungicidal agents) that reacted directly with invading pathogens. No aspect of the echinoderm defense response was homologous to any subsystem of the vertebrate immune response. This changed when a homologue of a vertebrate complement component was identified in the sea urchin, *S. purpuratus* [35].

## 5. Molecular immunology of the purple sea urchin

### 5.1. Coelomocyte activation

Coelomocytes mediate host responses to immune challenges through activities that include increased chemotaxis and phagocytosis, and the formation of cellular clots. These activities require significant changes in cell shape which

are mediated by changes in the actin cytoskeleton [57]. Profilin is a major actin binding and cytoskeletal modifying protein that plays a central role in either inhibiting or promoting the polymerization of actin monomers into filaments [58] thereby modulating the shape of the cell cytoskeleton. An increase in profilin gene expression was noted in coelomocytes from *S. purpuratus* responding to injury [59] and to injections of LPS [60]. This suggested that a change in profilin message was an indication of coelomocyte activation during immune responses. This correlation was supported by studies of profilin expression during gastrulation, where increases in profilin transcripts occurred with the onset of migratory behavior of the primary mesenchyme cells and the formation of filopodia on secondary mesenchyme cells [61]. Consequently, titration of the profilin transcript content in coelomocytes was used to infer the activation status of the sea urchin immune response [59,60].

### 5.2. Homologues of complement components

A random primed, directionally cloned cDNA library was constructed from coelomocytes that had been analyzed for their level of activation after injection of LPS [35,60]. This library was used in an EST study to identify genes that were transcribed in activated cells and that encoded immune effector proteins [35]. Two ESTs of particular interest appeared similar to members of the vertebrate complement cascade. The first, EST064, matched to the thioester protein family which is defined by the presence of a highly reactive carbonyl that is formed between cysteine and glutamine residues within the conserved thioester site that is capable of forming covalent bonds with hydroxyl or amino groups on target proteins. The thioester protein family includes complement components C3, C4, C5 and  $\alpha$ 2-macroglobulin ( $\alpha$ 2M). Although homologues of the thioester proteins have been identified in many vertebrates, homologues of  $\alpha$ 2M have also been characterized in invertebrates including arthropods and molluscs [62]. The second clone, EST152, matched to several short consensus repeats (SCRs) or

complement control protein modules. SCRs are small domains that are typical of proteins involved in the vertebrate complement system including complement receptors, factor B (Bf) and C2, and a number of complement regulatory proteins, but have also been found in non-complement proteins such as factor C from the horseshoe crab *Limulus* [63], factor XIIIb, a blood clotting protein in vertebrates, [64] and the interleukin 2 receptor [65]. These two ESTs, being expressed by the immune cells of the sea urchin, *S. purpuratus*, suggested that a simple complement system was present in this invertebrate and was composed of, at least, a thioester containing protein and a complement receptor or regulatory protein. This was the first molecular evidence that an echinoderm had an element within its immune system that was homologous to a system found only in vertebrates; evidence that inferred homology of immune systems within the deuterostomes [35].

The existence of an echinoderm complement system had been hinted at previously, in that phagocytes from *S. droebachiensis* showed augmented phagocytosis of RBCs opsonized by human C3, suggesting that coelomocytes had a receptor for C3b or C3bi [23,66–69]. Furthermore, inhibitors of complement opsonization decreased or blocked phagocytosis of RBCs by sea urchin phagocytes [70]. Others investigating the hemolytic activity in coelomic fluid from *A. forbesi* and *H. polii* also suggested that a complement-like system functioned in echinoderms [36,71]. However, comparisons of the holes produced by the hemolytic factor from two species of sea cucumber and those resulting from mammalian complement showed significant differences by electron microscopy, indicating that they were most likely produced by different mechanisms [39,72]. These early results suggested that echinoderms might have an alternative pathway of complement that lacked a terminal pathway. However, the lectin pathway of complement activation [73,74] or a lectin opsonin was not considered, even though certain sugars were shown to block the reaction [70].

### 5.3. *SpC3*, a homologue of vertebrate *C3*

The first definitive identification of a complement component in an invertebrate came when the sequence of EST064 was completed. Analysis of the deduced amino acid sequence indicated that the encoded protein, SpC3, was a new member of the thioester complement protein family, and was a homologue of the vertebrate complement component C3 [75]. The homology to vertebrate C3 was based on several conserved regions identified from the alignment between SpC3 and members of the thioester protein family and included the thioester site,  $\beta\alpha$  junction (no  $\alpha\gamma$  junction), C3 convertase site, two factor I cleavage sites, and cysteines in conserved positions including those involved in forming the interchain disulfide bridge [Fig. 1(A)]. On reducing gels, SpC3 revealed two chains with sizes similar to those in other C3 proteins. The gene (Sp064) is expressed specifically by the coelomocytes, the protein is present in coelomocytes and in coelomic fluid. The phylogenetic analysis of the thioester family of proteins indicated that SpC3 is the most ancient member, implying that the alternative pathway of the complement system was present in the common ancestor of the deuterostomes rather than the common ancestor of the vertebrates, as had been previously assumed [75].

### 5.4. *SpC3* is induced in coelomic fluid by LPS

Since the SpC3 protein was present in coelomic fluid of *S. purpuratus* [75], we were interested to know if the amount of SpC3 protein would change after challenge with LPS. Sea urchins used for this experiment were maintained in the controlled environment of a large marine aquarium for approximately 1.5 yr before they were challenged. We determined that these animals had become immunoquiescent since the concentration of SpC3 in the coelomic fluid had fallen to undetectable levels and could serve as excellent controls for studies of immune activation. Using Western blots, animals receiving a single injection of LPS showed detectable SpC3 in circulation 15 min post-injection (Fig. 2).

SpC3 concentration peaked at approximately 2 hr, gradually declining after 4 hr, and returning to control levels by 24 hr post-injection [Fig. 2(A)]. Animals receiving injections of filter sterilized sea water did not show a sharp peak in SpC3 concentration at 2 hr, but instead showed a slow increase in circulating SpC3, which became identical, at 24 hr, to animals receiving LPS [Fig. 2(B)]. Animals receiving no injections showed no discernible SpC3 in the coelomic fluid [Fig. 2(C)]. The densitometric scans of the Western blots show these changes more clearly [Fig. 2(D)]. The rapid appearance of SpC3 in coelomic fluid after LPS injection may have been due to the rapid secretion of stored SpC3 and/or to the induction of SpC3 gene transcription, followed by translation of the message and secretion of the newly produced protein. The appearance of SpC3 in coelomic fluid was transient perhaps because of the minimal challenge posed by a single injection of LPS (see legend to Fig. 2). After five injections of LPS, SpC3 appears in circulation in much greater amounts and is present for at least 90 days (Clow, Gross, Shih and Smith, unpublished).

### 5.5. *SpBf*, a homologue of vertebrate factor *B*

The completed analysis of cDNAs that included the EST152 sequence indicated that the encoded protein was a homologue of vertebrate Bf, and was called SpBf [Fig. 1(B)] [76]. Like other members of the Bf/C2 family, SpBf has a mosaic structure which includes five SCRs, a von Willebrand factor (vWF) domain, and a serine protease domain. The gene encoding SpBf (Sp152) is expressed specifically in coelomocytes. The alignment between SpBf and vertebrate Bf/C2 proteins revealed amino acids in conserved positions for serine protease activity, a conserved factor D cleavage site, and  $Mg^{2+}$  binding sites that, in vertebrate Bf proteins, function in interactions with C3b during the formation of C3 convertase. The presence of five SCRs in SpBf was unusual since all other Bf/C2 proteins have three SCR domains. Sequence alignments and phylogenetic analyses of SCR domains from all the Bf/C2 proteins were used to identify similarities among these domains and to predict any

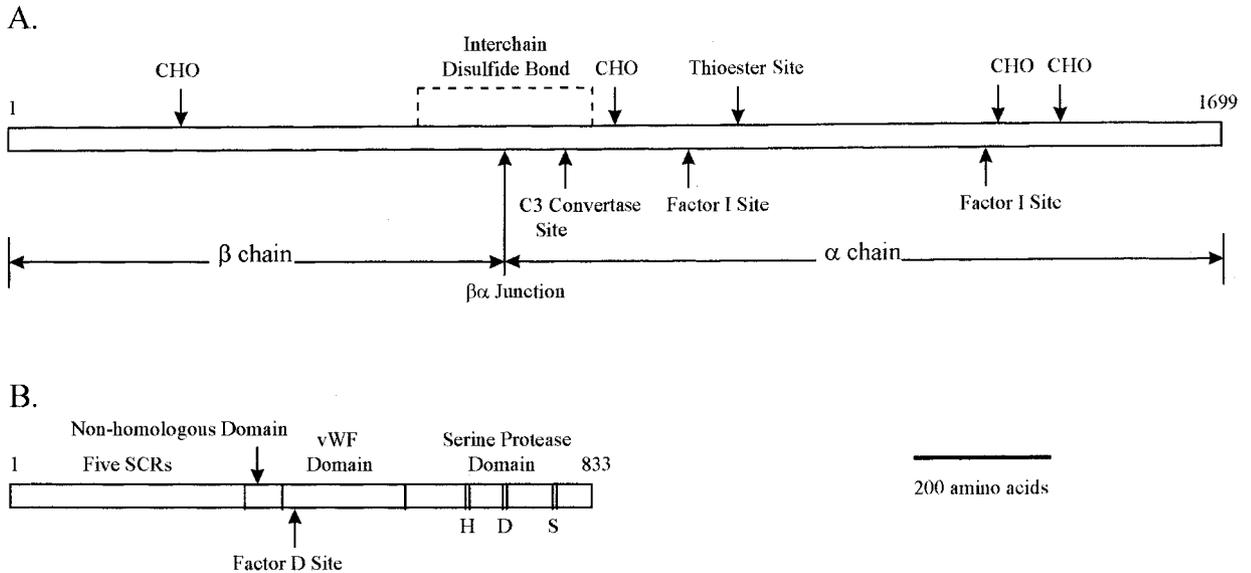


Fig. 1. Schematic representations of SpC3 and SpBf (75, 76). A. The 210 kD SpC3 protein (total length = 1699 amino acids) is composed of two chains,  $\alpha$  (130 kD) and  $\beta$  (80 kD). The  $\beta\alpha$  junction, thioester site and cleavage sites for a putative factor I and C3 convertase are positioned to scale. Four of the five consensus *N*-linked glycosylation sites (CHO) appear to carry sugars (Al-Sharif and Smith, unpublished) and are shown. The positions of the putative interchain disulfide bond is indicated by dotted lines and is based on conserved positions of cysteines in SpC3 that correspond to cysteines involved in disulfide bonding in other C3 proteins (90; see Figure 3 in Ref. [75]). To receive an alignment of all the thioester family proteins including SpC3 by e-mail, send a request to Netserv@ebi.ac.uk with the message; GET ALIGN:DS31395.DAT. B. The SpBf protein is a mosaic protein typical of the Bf/C2 protein family. It is composed of five SCRs, a von Willebrand Factor domain, and a serine protease domain. The conserved cleavage site for a putative factor D is indicated in addition to the amino acids involved in the protease function. To receive an alignment of the Bf/C2 protein family including SpBf by e-mail, send the request to Netserv@ebi.ac.uk with the message; GET ALIGN:DS33817.DAT.

functional significance of five SCRs in SpBf. Based on known functional importance of individual SCRs in Bf for binding to C3b in vertebrates [77–79], results suggested that the three SCRs closest to the vWF domain in SpBf might be more important for binding than the two N-terminal SCRs. Furthermore, SpBf was found to be the most primitive member of the Bf/C2 protein family, and having five SCRs appeared to be the primitive state for the Bf/C2 protein family [76].

### 5.6. The feed-back loop of the alternative pathway

Together, SpC3 and SpBf are among the first complement proteins to be described from an invertebrate, and they appear to be the central components of a primitive complement system

that is homologous to the alternative pathway. Previously, Lachmann [80] proposed a sequence of evolutionary steps that began with an ‘archeo-complement system’ which functioned essentially the same as the alternative pathway, and culminated in the higher vertebrate complement system. The archeo-complement system consisted of a C3-like protein with a thioester site, and a factor B-like protein containing SCRs and a serine protease domain and would be capable of auto-amplification through a positive feedback loop, generating large numbers of activated C3 molecules that would quickly opsonize a pathogen (or any other surface lacking protection against complement attack). A model of how the sea urchin complement system might function, is based on the components that have been identified in the sea urchin thus far. It is feasible that

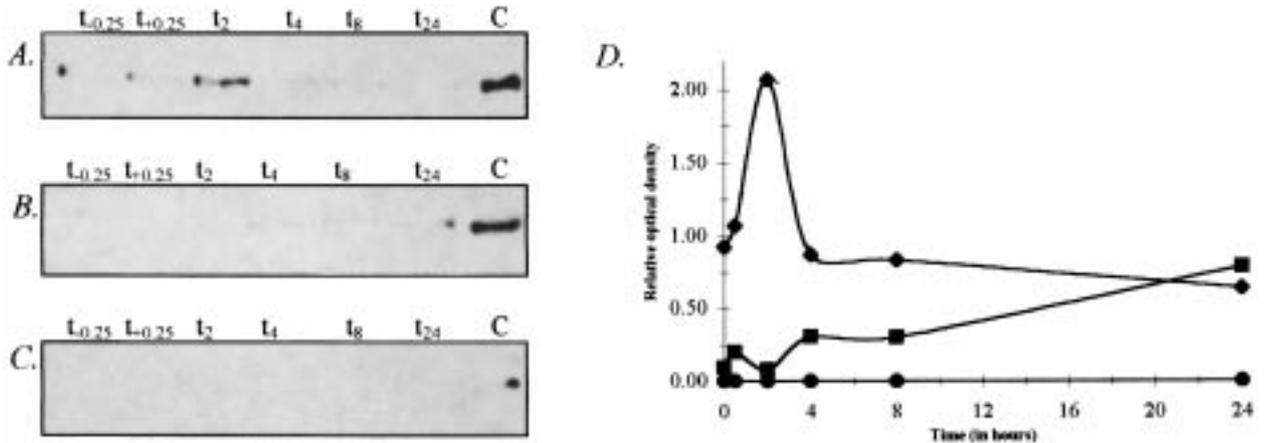


Fig. 2. Western blot analysis of SpC3 in coelomic fluid from *S. purpuratus* after challenge with LPS. Immunoquiescent sea urchins were injected with 2  $\mu$ g LPS/ml coelomic fluid (A), 2  $\mu$ l sterile sea water/ml coelomic fluid (B), or received a needle injury only (C). The volume of coelomic fluid was estimated according to Smith et al. [60]. Coelomic fluid (300  $\mu$ l) was withdrawn into a syringe (pre-loaded with 300  $\mu$ l of ice cold  $\text{Ca}^{2+}$ / $\text{Mg}^{2+}$ -free sea water with 30 mM EDTA and 50 mM imidazole) by inserting the needle through the peristomeum into the coelomic cavity. Samples were taken 15 min prior to injection ( $t_{-0.25}$ ), 15 min post injection ( $t_{+0.25}$ ), 2 hr post injection ( $t_2$ ), 4 hr post injection ( $t_4$ ), 8 hr post injection ( $t_8$ ), and 24 hr post injection ( $t_{24}$ ). Coelomocytes were pelleted immediately and fluid samples were stored at  $-70^\circ\text{C}$  until use. To prevent general immune activation of all the animals from LPS contamination in the water, sea urchins were maintained in small individual aquaria with aeration, at  $16^\circ\text{C}$ , for the duration of the experiment. Coelomic fluid was mixed 2:1 with lysis buffer (lysis buffer = 4% SDS, 20%  $\beta$ -mercaptoethanol, 20% glycerol, 0.1 M Tris), loaded onto SDS-polyacrylamide gels (4.5% stacking, 8% separating) and run under reducing conditions. Proteins were electroblotted to nitrocellulose (BioRad), blocked with 5% milk in Tris buffered saline, incubated with rabbit anti-SpC3- $\alpha'$  chain antibody, followed by goat-anti-rabbit HRP antibody (Pierce) and visualized on film by ECL (Pierce). The primary antibody was produced according to Al-Sharif et al. [75] and directed to a peptide (SGDGGGEQNAAVKVRDDFRETWFFD) that corresponds to the N-terminus of the  $\alpha$  chain after cleavage at the putative C3 convertase site. The control lane (C) on all blots represents coelomic fluid from a single animal that was maintained in a sea water system open to the Pacific Ocean. D. Relative densitometric scans of bands from the western blots corrected to the signal from the control (C) lane. ◆ = LPS challenged animal, ■ = SSW challenged animal, ● = needle injury only animal.

the echinoderm complement system might function like the proposed archeo-complement system in which activation would be initiated either spontaneously by the 'tick-over' mechanism of SpC3 [81], or by the lectin pathway that has been identified in vertebrates and ascidians [73,74,82,83] and probably functions in echinoderms as well. Activated SpC3, with an exposed thioester in the fluid phase would bind to an appropriate unprotected surface such as a microbe. Next, SpBf would bind to SpC3, rendering the bound SpBf susceptible to cleavage and activation by a putative factor D. The resulting complex would then function as a C3 convertase through the activity of the serine protease domain on SpBf. More SpC3 would be activated by the convertase which might also bind to the pathogen forming more convertase creating the

positive feedback loop. Eventually, the pathogen would become covered with complement complexes, stimulating efficient phagocytosis or encapsulation by phagocytes. In addition, a factor I-like homologue would be a necessary inhibitor/regulator to cleave and degrade activated SpC3 in the presence of certain cofactors on cell surfaces and in circulation (for review of complement regulation, see Ref. [84]). This type of opsonization system would be remarkably efficient, and therefore, more valuable in host defense than simple opsonins. At present there is no direct evidence for echinoderm homologues of factor D, factor I (and cofactors), or a complement receptor, however, our preliminary data suggests that the sea urchin complement system may be activated and regulated by mechanisms that are similar to those that function in the

Pathway	Component	Echinoderms	Protochordates	Agnathans	Elasmobranchs	Teleosts and Tetrapods
Terminal	C9				X <sup>104</sup>	X
	C8				X <sup>104</sup>	X
	C7					X
	C6					X
	C5				X <sup>104</sup>	X
Classical	C4				X <sup>100-103</sup>	X
	C2					X
	C1				X <sup>100-103</sup>	X
Lectin	MASP		X <sup>83</sup>	X <sup>74</sup>	X <sup>74</sup>	X <sup>106-107</sup>
	MBL		X <sup>91</sup>			X <sup>105</sup>
Alternative	factor D					X
	factor B	X <sup>76</sup>	X <sup>90</sup>	X <sup>97</sup>	X <sup>98-99</sup>	X
	C3	X <sup>75</sup>	X <sup>89</sup>	X <sup>92-96</sup>	X <sup>98-99</sup>	X

Fig. 3. Phylogeny of Complement in Deuterostomes. The appearance of complement components in deuterostome phylogeny suggests origins in the echinoderms with duplications of genes and pathways in more advanced animals. The information was compiled from the following references which are also cited within the table: [74–76, 83, 89–107]. No citations are included for the classical, alternative, and terminal pathway components in higher vertebrates.

alternative pathway in vertebrates (Al-Sharif and Smith, unpublished).

## 6. The evolution of complement in deuterostomes

The theory that the complement cascades in higher vertebrates evolved from a few primordial genes through gene duplication and subsequent divergence of function [85] is based on similarities in protein sequence and function (e.g., the thioester protein family; the C2/Bf family; members of the terminal pathway), clustered organization of some complement genes (regulators of complement activation cluster, C2 and Bf linkage in mammals) and parallels between pathways (e.g., the alternative and classical pathways; the lectin pathway and the C1 complex) [86,87]. Complement systems of varying complexity have been identified in all vertebrate classes [88] and with the identification of C3 homologues in the sea urchin [75] and in a tunicate [89], this expands the range of animals in which comp-

lement has been found to include the entire lineage of deuterostomes.

The number of complement pathways in an organism and the complexity of each pathway correlates with phylogeny; more complex systems being found in more advanced deuterostomes (Fig. 3). All deuterostomes have, at minimum, a simple alternative pathway with ancient opsonin function, and most deuterostomes can activate the complement system through the lectin pathway. In the elasmobranchs, where immunoglobulins are first identified, the number of components, pathways (the classical and terminal pathways appear), and functional capabilities (antibody effector system) of the complement system expands significantly. In higher vertebrates (teleosts and tetrapods), the alternative and classical pathways are expanded further, adding more components to complete the system (Fig. 3). Analysis of the complement system in animals throughout the deuterostome lineage can be imagined as 'snap shots of complement evolution' in which several of the evolutionary steps have been

preserved, making investigations of the molecular evolution of the complement system accessible in living organisms, rather than being lost to extinction.

The phylogenetic analyses of SpC3 and SpBf indicate that these echinoderm proteins are the most primitive of the thioester and Bf/C2 families of complement components, respectively [75,76]. The sea urchin system conforms to the ‘archeo-complement system’ suggested by Lachmann [80] as the simplest recognizable complement system. Thus, the sea urchin complement system may bear similarities to the system that functioned in the ancestral deuterostome and that gave rise to the complement cascades in the higher deuterostomes [75]. Future investigations of the complement components in echinoderms may provide keys to the primitive beginnings of the deuterostome complement system, which may allow us to identify homologous functions in higher vertebrates and to piece together how the multiple subsystems in the mammalian immune response came to function together.

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