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Changes in holothurian coelomocyte populations following immune stimulation with different molecular patterns

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ABSTRACT

Echinoderms possess a variety of cells populating the coelomic fluid; these cells are responsible for mounting defense against foreign agents. In the sea cucumber *Holothuria glaberrima*, four different coelomocyte types were readily distinguished using morphological, histochemical and physiological (phagocytic activity) parameters: lymphocytes, phagocytes, spherulocytes and "giant" cells (listed in order of abundance). Monoclonal antibodies generated against sea cucumber tissues and one polyclonal against sea urchin mayor yolk protein (MYP) were also used to characterize these cell populations. The effects of several pathogen-associated molecular patterns (PAMPs): Lipopolysaccharides from *Escherichia. coli* (LPS), heat-killed *Staphylococcus aureus* (SA) and a synthetic dsRNA were studied on coelomocyte cell populations. PAMPs increased the phagocytic activity of the holothurian coelomocytes, and were able to induce selective immune responses in several of these populations, demonstrating the ability of the sea cucumber to respond to a different variety of immune challenges. Overall, these results show the variety of cells that populate the coelomic fluid of the holothurian and demonstrate their involvement in immune reactions. These animals represent an untapped resource for new findings into the evolution and development of the immune response not only in invertebrates but also in phylogenetically shared reactions with vertebrates.

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1. Introduction

The study of echinoderm immunity has a long history, extending from the discovery of cellular immunity in starfish [1] to the groundbreaking work of sequencing the genome of the sea urchin *Strongylocentrotus purpuratus* [2–4]. In the late 19th century, the Russian Nobel laureate Ilya Metchnikoff, discovered the presence of phagocytic cells, when he inserted a foreign body (a rose prickle) into the larva of the starfish *Astropecten pentacanthus* and observed how cells tried to engulf it [1]. Later studies showed the ability of several echinoderm species to differentiate self from non-self tissues through allograft rejection experiments [5,6]. These observations along with studies in other sea urchins, defined the basics of echinoderm immunity, showing the importance of the immune cells and the different roles they play in immunological defense [7,8].

The effector cells of the echinoderm immune system are the coelomocytes; they are the primary mediators of allograft rejection [5,7], response to injury or infection, and the clearance of foreign substances and bacteria [9–13]. In general, morphologically distinguishable cell types have been described in the coelomic fluid of echinoderms [14]. However, not all types are necessarily present in every echinoderm species [15]. Moreover, their classification can be sometimes confusing when comparing different species. Five cellular types have been reported to be present in holothurians, i.e. hemocytes, phagocytes, spherule cells, lymphocytes and crystal cells, as normal components of the coelomic fluid. The first four are thought to be the ones involved in immune reactions [16]. These cell types have been identified and characterized using mainly classical histological methods. Nowadays, the availability of new techniques (e.g. immunofluorescence) represents an important tool to gain insight into the characterization of these cells.

Echinoderm coelomocytes possess different roles, for example, phagocytes as their name implies act as an efficient clearance mechanism due to their recognition, ingestion and efficient degradation of ingested particles [12,17]. They also can produce reactive oxygen species (ROS) inducible by stimulation with non-self materials [18]. During wound healing, they accumulate at the injury site and engulf cellular debris [19]. Both phagocytes and spherule cells appear to be involved in cell clumping and the formation of capsules around ingested particles. It is possible that spherule cells release bactericidal substances, including, lipase,

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peroxidase and serine proteinase [20] that cause the breakdown of phagocytized material [21]. The role of other cell types, however, remains unclear or completely unknown. For example, lymphocytes, have been proposed to be circulating progenitor cells [22]; vibratile cells which are thought to be involved in coelomic fluid movement and clotting reactions [23]; and finally, crystal cells whose role is not yet totally understood [22].

The echinoderm immune system can recognize a diverse set of non-self molecules as determined by allograft rejection studies [5,6], and their ability to initiate a response when challenged with pathogen-associated molecular patterns (PAMPs). PAMPs represent common molecular features of potential pathogens, like bacteria (lipopolysaccharides for gram-negative bacteria, and peptidoglycan for gram-positive), viruses (dsRNA) or fungi (mannan, chitin), which are recognized by molecules known as pathogen recognition receptors (PRRs) [24,25]. In the sea urchin, several PAMPs have been shown to induce immune responses, e.g. lipopolysaccharides (LPS) [26], β1-3-glucan and dsRNA can induce the expression of the 185/333 transcripts, a well-known family of sea urchin immune response genes [27].

To understand the proper function of the immune response in other non-echinoid echinoderms, it is important to determine their coelomocyte composition. The present study shows a first approach towards the characterization of the immune responses of the sea cucumber *Holothuria glaberrima*. Using classical histological methods and immunological markers we have identified several populations of coelomocytes and shown how they change upon stimulation with three PAMPs, i.e. *Escherichia coli* LPS, heat-killed *Staphylococcus aureus* (SA) and dsRNA. We have found distinct coelomocyte populations that can be recognized using specific markers and have defined the response of these populations to the different PAMPs. Our data increases the available knowledge of echinoderm immunity and shows a hitherto unexpected level of complexity.

2. Materials and methods

2.1. Animals

Adult sea cucumbers 10–12 cm long (*H. glaberrima*) were collected from the rocky shores of northeastern Puerto Rico (18°28′12.23“N, 66°7′8.99”W). Animals were kept in seawater aquaria at 20–24 °C for a week for acclimation before initiating the experiments.

2.2. Coelomocyte preparation

Coelomocytes were collected into ice-cold calcium- and magnesium-free artificial seawater with 30 mM EDTA and 50 mM Imidazole pH 7.4 (CMFSW-EI) as described previously [28–30]. Cells were centrifuged at 450 g for 10 min at 4 °C then washed twice with 5 ml CMFSW-EI and fixed in 4% paraformaldehyde diluted in CMFSW-EI for 15 min. Fixative was removed by pelleting cells and washing them twice with CMFSW-EI. Fresh coelomocyte preparations were also used to observe cell morphology of live cells. Briefly, 10 ul of freshly collected coelomic fluid was drawn onto a hemocytometer and allowed to settle for 10 min, after which microscopic observations were made.

2.3. Immunological challenge

Sea cucumbers were immunologically activated by injections of: 0.5 mg of LPS from *E. coli* (L2630, Sigma, St Louis, MO), 1×10^9 cells of heat-killed *S. aureus* or 6.5 ug of dsRNA. Each PAMP was diluted in filtered seawater to a final volume of 100 uL. Double stranded RNA was prepared with the MEGAscript® kit (Ambion/Applied biosystems, Austin, TX) using an empty pBluescript plasmid as a template to generate a 400 bp dsRNA, plasmid sequence and the primers used to amplify this region are presented in Supplementary material S1. Control animals were injected with equivalent volumes of filtered seawater. Three animals were used per treatment.

2.4. Phagocytosis assays

Phagocytic activity (PA) was determined as described in a previous publication [28]. Briefly, 48 h after the injection of the PAMPs, the animals were injected with 500 uL of a 1/1000th dilution of fluorescent beads (Polysciences, Warrington, PA) in filtered seawater. Animals were then left undisturbed for 2 h, after which coelomocytes were extracted as mentioned in Section 2.3. The phagocytic activity (PA) was determined by dividing the number of cells with incorporated fluorescent beads by the total number of cells and multiplying by 100. *T*-tests were conducted to determine the statistical significance of the difference between control and experimental animals.

2.5. Morphological studies

Cells were observed under DIC microscopy and classified according to their size and morphology. Cell numbers were determined using a hemocytometer. Morphological classification was done by staining coelomocyte cell spreads with toluidine blue. Additional morphological characterization was also done by staining the cell spreads with fluorescent phalloidin-tetramethylrhodamine B isothiocyanate (TRITC) conjugate from *Amanita phalloides* (cat No. 77418, Sigma, St Louis, MO). Phalloidin binds to filamentous actin, allowing the visualization of the actin cytoskeleton and thus of the overall cellular morphology. Nuclei were stained using DAPI mounting media (buffered glycerol with 2 ug/mL DAPI). Digital micrographs were taken on a Nikon Eclipse E600 fluorescent microscope with FITC, R/DII and DAPI filters and the Metaview software (V 6.0; Universal Imaging) was used for image analysis and processing.

2.6. Immunohistochemistry

The immunohistochemical procedures have been described in a previous publication [31]. Briefly, coelomocyte cell spreads were left to air dry for 5 min and then treated with goat serum (1:50) for 1 h, then permeabilized with 0.5% Triton X-100 and incubated overnight in a humid chamber with the corresponding primary antibody. Anti-MYP was kindly donated by Dr. Gary M. Wessel and used accordingly [32]. Monoclonal antibodies were obtained from fusions of spleens from mice immunized with various antigens. The immunogens used were as follows: for Sph1 and Sph4, homogenates of intestinal tissue [33]; Sph2, an acid-extracted and semipurified holothurian collagen fraction [34]; and for Sph3, a Holothurian homeobox peptide [35] coupled to bovine serum albumin. For Sph5, an aqueous extraction of body wall coelomic epithelium of a 7 days regenerating neuromuscular wound [36]. For Coel1, an ethanol-extraction of coelomic epithelium of non-regenerating intestinal tissue, and for Ly1, homogenates of intestinal tissue. Anti-Ly1, anti-Coel1, anti-Sph2, anti-Sph3, anti-Sph4 and anti-Sph5 supernatants were applied undiluted to the cell spreads. The following day, the slides were rinsed in PBS and incubated 1 h with Cy3-conjugated goat-anti rabbit and goat-anti mouse secondary antibodies (1:2000) (Biosource/Invitrogen). Slides were rinsed again in PBS and mounted in buffered glycerol with DAPI (2 ug/ml).

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