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Comparison of phagocytosis in three Caribbean Sea urchins

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ABSTRACT

In 1983 large numbers of the sea urchin *Diadema antillarum* unexplainably began showing signs of illness and dying in the Caribbean, and over the next year they came close to extinction, making it one of the worst mass mortality events on record. Present evidence suggests a water-borne pathogen as the etiological agent. Decades later *Diadema* densities remain low, and its near extinction has been a major factor in transforming living coral reefs in the Caribbean to barren algae-covered rock. In the ensuing decades, no solid explanation has been found to the questions: what killed *Diadema*; why did *Diadema* succumb while other species of urchins on the same reefs did not; and why has *Diadema* still not recovered? A recent hypothesis posited by our lab as to *Diadema's* vulnerability was directed at possible compromised immunity in *Diadema*, and experimental results found a significantly impaired humoral response to a key component of gram-negative bacteria. Here we use flow cytometry to examine the cellular arm of invertebrate immunity. We performed cytotoxicity and phagocytosis assays as a measure of the cellular immune responses of cells from *Diadema* and two other species of sea urchins not affected by the die-off. Despite our previous findings of in impaired humoral response, our study found no apparent difference in the cellular phagocytic response of *Diadema* compared to the other urchin species studied.

1. Introduction

In the 1980's, the black-spined sea urchin *Diadema antillarum* suffered one of the most extensive mass mortality events on record for a marine invertebrate. *Diadema* was one of the most abundant and ecologically important herbivores on Caribbean coral reefs, maintaining reef-building coralline algae by their grazing of fleshy algae (Carpenter, 1981; Ogden and Lobel, 1978; Sammarco, 1982, 1980; Sammarco and Levinton, 1974). In 1983, *Diadema* began showing signs of illness, and quickly died (Lessios et al., 1984). Over the course of a year the outbreak spread over 3.5 million square kilometers, with 95% mortality and no populations of *D. antillarum* spared (Hughes et al., 1985). No causative agent has been discovered, but the outbreak followed water currents and mortality did not decrease with distance, suggesting a water-borne pathogen (Lessios, 1988). Two species of bacteria capable of killing *Diadema* were associated with dying urchins in the laboratory, but no bacteria have been isolated from wild individuals (Bauer and Agerter, 1987).

No other species of urchins were affected, nor were Pacific *Diadema* populations of *D. mexicanum* near the initial outbreak, suggesting that the etiological agent was a species-specific pathogen. The loss of this keystone grazer facilitated a phase shift from hard coral to algal dominated reefs throughout the Caribbean (Carpenter, 1990; Hughes, 1994), and *Diadema* densities have failed to recover in the ensuing thirty years, remaining more than 85% below their pre-epizootic levels (Lessios, 2015).

Along with *Diadema*, we examined the immune response of two other major cosmopolitan species of sea urchins in the Caribbean Sea (*Tripneustes ventricosus* and *Echinometra lucunter*). The phylogenetic position of echinoderms makes them an important group for comparative immunological studies (Binyon, 1972; Lin et al., 2001). Sea urchins are in the phylum Echinodermata, class Echinoidea (Harvey, 1956; A. B. Smith and Kroh, 2013). The phylogenetic relationships based on 18S-like small and 28S-like large rRNA show the Diadematoida order of *Diadema* to be one of the more basal, with order Carinacea diverging from Diadematoida and radiating into the large Echinacea clade (A. B. Smith and Kroh, 2013). From within Echinacea were splits that included the Toxopneustidae clade that one of our experimental species, *Tripneustes* ventricosus, belongs to, and another branch that later gave rise to the Echinometridae clade of our experimental *Echinometra lucunter* (and still later the Strongylocentrotidae clade) (A. B. Smith and Kroh, 2013).

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Sea urchins clear pathogens from coelomic fluid (CF) efficiently and CF cells (coelomocytes) mount immune responses (Lin et al., 2001). Progress has been made on deciphering the immune response of sea urchins (Lin et al., 2001; Materna and Cameron, 2008; Rast and Messier-Solek, 2008; Rast et al., 2006; L. C. Smith et al., 2006), and with the sequencing of the genome of the purple sea urchin *Strongylocentrotus purpuratus*, new information will follow (Hibino et al., 2006; Materna and Cameron, 2008; Sodergren et al., 2006). It has been hypothesized that ~5% of the genes in the sea urchin are related to the immune response (Hibino et al., 2006; Rast et al., 2006; L. C. Smith et al., 2006; Sodergren et al., 2006).

Innate immunity involves anatomical, physiological, phagocytic and inflammatory barriers deployed before infection, is capable of rapid response to pathogens, and reacts in mostly the same way to repeated infections (Akira et al., 2006; Medzhitov and Janeway, 2002). These innate responses in invertebrates are activated when microbes breach anatomical barriers. Innate immunity exists in vertebrates, invertebrates, and to a certain extent in plants, however for invertebrates it is the only line of defense. Invertebrate immunity is nonspecific [variable immune-like molecules, and alternative anticipatory and memory-like immune mechanisms are found in some invertebrates (Cerenius and Soderhall, 2013)], based on activation of immune effector cells, and is mediated to a large extent by circulating coelomocytes (Bochud et al., 2007; Lin et al., 2001; Medzhitov and Janeway, 2002). Even though innate immunity lacks the elegance of genetic recombination and shows no memory, the view of innate immunity inconsequentiality is out of date (Akira et al., 2006; Medzhitov and Janeway, 2002). As in vertebrates, the innate response of invertebrates has both a molecular (secreted protein) and cell-mediated component (Beck and Habicht, 1996; Lin et al., 2001).

Sea urchins (as most invertebrates) have an open circulatory system, and the blood cells of their coelomic fluid are referred to as coelomocytes (Chia and Xing, 1996). Their importance in immunity has been well described (Chia and Xing, 1996; Coffaro and Hinegardner, 1977; de Faria and da Silva, 2008; Mangiaterra and Silva, 2001; Matranga et al., 2005; Silva, 2000; V. J. Smith, 1981). There has not been consistent nomenclature and classification of coelomocytes among types of echinoderms (Boolootian and Giese, 1958; Chia and Xing, 1996; Coulter, 1956, 1953; Edds, 1993; Gross et al., 1999; Matranga et al., 2005, 2006; L. C. Smith et al., 2010; V. J. Smith, 1981), but sea urchins are generally described as having at least four types of coelomocytes, with variable population percentages in different species of sea urchins (L. C. Smith et al., 2010). Historically these have often been called phagocytes (sometimes further divided between large and small phagocytes, and also called petaloid/filopodial phagocytes, and phagocytic or bladder amoebocytes), with populations given as between 40 and 80%, red spherule (or morula) cells (sometimes called pigment cells) (4–40%), colorless or white spherule (or morula) cells (5–25%), and vibritile cells (8–20%) (Bertheussen and Seijelid, 1978; Chia and Xing, 1996; Edds, 1993; Gross et al., 2000; Johnson, 1969; Mangiaterra and Silva, 2001; L. C. Smith et al., 2010; V. J. Smith, 1981; Standerholen et al., 2014; Terwilliger et al., 2004).

While often referred to as phagocytic amoebocytes, phagocytic coelomocytes cells have been reported to not be very mobile, and thus 'amoebocytes' may be a misnomer (Matranga et al., 2006, 2005). Conversely, the lack of motion generally reported for red and white cells is thought by some investigators to be due to EDTA in cell preparations, as fresh preparations show them to be so swiftly mobile that these are the cells that should correctly be called amoebocytes (Matranga et al., 2006, 2005). Spherule cells are also reported as not actually being spherical in fresh preparations, and their description as such is likely due to fixatives or anticoagulants (Matranga et al., 2006, 2005).

Phagocytic cells are reported to exist in three forms, large discoidal and polygonal forms responsible for phagocytosis, and a small filopodial form involved in clotting (Brockton et al., 2008; Gross et al., 2000; Majeske et al., 2014; V. J. Smith, 1981), and that possibly these are one cell type that transitions under environmental influences (Edds, 1993, 1985; Henson et al., 1999). Phagocytes are the only cells found to be capable of phagocytosis (Matranga et al., 2006; L. C. Smith et al., 2010), with red and white cells believed to be responsible for releasing humoral components (Matranga, 1996; Matranga et al., 2006, 2005; L. C. Smith et al., 2010). Some reports state phagocytes are also the only cells capable of clotting (Matranga et al., 2006), while others suggest white cells may also be involved (L. C. Smith et al., 2010).

Phagocytosis (the engulfment of foreign material by specialized phagocytic cells) is the predominant cellular defense mechanism in vertebrates and invertebrates (Beck and Habicht, 1996; Cooper, 1976; Marchalonis and Schluter, 1990a). In his studies of invertebrates in the late 19th century, Metchnikoff established the role of phagocytes in host defense, studies that began the field of cellular immunology (Beck and Habicht, 1996). Some of his earliest studies were on the response of echinoderm phagocytes to injury. He proposed that all animals use phagocytosis as a general defense mechanism (Beck and Habicht, 1996; Binyon, 1972; Flajnik and Pasquier Du, 2004).

Phagocytes and cytotoxic cells have been reported in most every invertebrate phylum (Beck and Habicht, 1991; Beck et al., 1993; Blanco et al., 1997; Franceschi et al., 1991). Bertheussen showed that coelomocytes of the sea urchins *Echinus esculentus* and *S. droebachiensis* were cytotoxic towards allogenic coelomocytes (Bertheussen, 1979). Hemocyte-mediated cytotoxicity may involve ROI and/or lysosomal enzymes (Peddie and Smith, 1994). We have shown that coelomocytes from the purple sea urchin (*Arbacia punctulata*) exhibit cellular cytotoxic activity against vertebrate target cells *in vitro* (Lin et al., 2001). We have also demonstrated that phagocytic amebocytes are the effector cells of the cytotoxic activity (Lin et al., 2001). In addition, we have shown that these cells are phagocytic and can be stimulated to release host defense molecules (Beck et al., 1993; Lin et al., 2001).

Previously we asked whether an impaired immune response could be responsible for the susceptibility of *Diadema* to some pathogen. In that study, we employed several assays (reactive oxygen and nitrogen intermediate generation, phenoloxidase responses, release of antimicrobial peptides, and iron sequestration) to investigate the humoral immune response of the 3 cosmopolitan sea urchin species (*Diadema, T. ventricosus* and *E. lucunter*). We found that *Diadema's* humoral response to LPS, a component of gram-negative bacteria, was absent (Beck et al., 2014, 2008). In the present study, we examine *Diadema*'s cellular immune response by assessing cytotoxicity and phagocytosis using flow cytometry.

2. Materials and methods

2.1. Harvesting of sea urchin coelomocytes

For testing cellular immune responses (phagocytosis and cytotoxicity), coelomic fluid (CF) was drawn from sea urchins (thirty *D. antillarum,* five *E. lucunter,* and four *T. ventricosus*) collected from seven study sites on St. Croix (Beauregard Bay, Butler Bay, Cramer's Point, DIVI, Gentle Winds, Grassy Point, Sprat Hole) (Beck et al., 2014, 2008). The soft tissue surrounding Aristotle's lantern was pierced with a 20-gauge needle and 5–8 ml CF was drawn into a 10-mL syringe containing 4 ml of ice-cold anticoagulant [Marine Alsever's solution (0.12 M glucose, 0.03 M sodium citrate, 9 mM EDTA, 0.38 M NaCl), (pH 7.4)] (Bachere et al., 1988; Bowdish and Gordon, 2009; Sarrias et al., 2004; Whelan et al., 2012). All sea