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The specifically enhanced cellular immune responses in Pacific oyster (*Crassostrea gigas*) against secondary challenge with *Vibrio splendidus*

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ABSTRACT

The increasing experimental evidences suggest that there are some forms of specific acquired immunity in invertebrates, but the underlying mechanism is not fully understood. In the present study, Pacific oyster (*Crassostrea gigas*) stimulated primarily by heat-killed *Vibrio splendidus* displayed stronger immune responses at cellular and molecular levels when they encountered the secondary challenge of live *V. splendidus*. The total hemocyte counts (THC) increased significantly after the primary stimulation of heat-killed *V. splendidus*, and it increased even higher ($p < 0.01$) and reached the peak earlier (at 6 h) after the secondary challenge with live *V. splendidus* compared with that of the primary stimulation. The number of new generated circulating hemocytes increased dramatically ($p < 0.01$) at 6 h after the pre-stimulated oysters received the secondary stimulation with live *V. splendidus,* and the phagocytic rate was also enhanced significantly ($p < 0.01$) at 12 h after the secondary stimulation. Meanwhile, the enhanced phagocytosis of hemocytes was highly specific for *V. splendidus* and they could distinguish *Vibrio anguillarum*, *Vibrio coralliilyticus*, *Yarrowia lipolytica*, and *Micrococcus luteus* efficiently. In addition, the mRNA expression of 12 candidate genes related to phagocytosis and hematopoiesis were also monitored, and the expression levels of *Cg*Integrin, *Cg*PI3K (phosphatidylinositol 3-kinase), *Cg*Rho J, *Cg*MAPKK (mitogen-activated protein kinase kinase), *Cg*Rab32, *Cg*NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, *Cg*Runx1 and *Cg*BMP7 (bone morphogenetic protein 7) in the hemocytes of pre-stimulated oysters after the secondary stimulation of *V. splendidus* were higher ($p < 0.01$) than that after the primary stimulation, but there was no statistically significant changes for the genes of *Cg*PKC (protein kinase C), *Cg*Myosin, *Cg*Actin, and *Cg*GATA 3. These results collectively suggested that the primary stimulation of *V. splendidus* led to immune priming in oyster with specifically enhanced phagocytosis and rapidly promoted regeneration of circulating hemocytes when the primed oysters encountered the secondary challenge with *V. splendidus*.

1. Introduction

Animals have developed canonical mechanisms to combat invading foreign particles, including innate (non-specific) immunity and adaptive (specific, acquired or memorial) immunity. Invertebrates, which lack the lymphocytes and immunoglobulin, have always been considered to possess only innate immune system (Turvey and Broide, 2010). However, recent experimental evidences (Dong et al., 2006; Kurtz and Franz, 2003; Lemaitre et al., 1997; Little et al., 2003; Pham et al., 2007; Roth et al., 2010; Vierstraete et al., 2004; Wang et al., 2009; Witteveldt et al., 2004; Zhang et al., 2004), molecular evolutionary analysis (Flajnik and Du Pasquier, 2004; Kurtz, 2004; Ottaviani, 2011; Saha et al., 2010; Ziauddin and Schneider, 2012) and data from a new field of ecological immunology (Little and Kraaijeveld, 2004; Rolff and Siva-Jothy, 2003) suggest that the immune response of invertebrates also exhibit adaptive characteristics, described as immune priming. But the mechanisms underlying the specific protection of immune priming have not been well characterized.

Invertebrate immune system, same as vertebrate immune system, is based on both cellular and humoral components. The

Abbreviations: PI3K, phosphatidylinositol 3-kinase; MAPKK, mitogen-activated protein kinase kinase; PKC, protein kinase C; NADPH, nicotinamide adenine dinucleotide phosphate; BMP, bone morphogenetic protein; EF, elongation factor.

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cellular immune responses against an invasion of pathogen are generally immediate, while humoral responses seemingly emerge several hours after an infection (Rosales, 2011). Consequently, the circulating cells (coelomocytes or hemocytes) are considered to be the primary effector components in immune responses of invertebrates. These cells interact with numerous foreign particles, leading to the subsequent activation of cellular immune reactions such as phagocytosis, nodule formation, encapsulation, and cytotoxicity, etc. (Lackie, 1980). Phagocytosis is initially triggered to respond to invaders before nodule formation and encapsulation (Dunn, 1986), and it is fundamentally important to invertebrates' survival. Recently, there are accumulating reports about the involvement of cellular immune responses during immune priming. Phagocytes were found to be the critical performers in the priming response of the primed *Drosophila*, and the enhanced phagocytosis was specific to kill the pathogen (Pham et al., 2007). The similar results have also been reported in woodlouse (Roth and Kurtz, 2009) and shrimp (Pope et al., 2011). In Mosquitoes *Anopheles gambiae*, the immune priming aroused quantitative and qualitative differentiation of hemocytes and circulating granulocytes to mediate the enhanced antiplasmodial immunity. The proliferation and differentiation of hemocytes was suspected to be necessary to initiate the innate immune memory (Rodrigues et al., 2010). It has been suggested that the cellular immune response might be one of the most important immune parameters in invertebrate immune priming (Pham et al., 2007; Pope et al., 2011; Roth and Kurtz, 2009). But the detailed mechanism of specific cellular immunity in immune priming is still far from well understood.

The phylum Mollusca is one of the largest and most various groups in the invertebrate animals and some of them are important fishery and aquaculture species. In recent decades, the mechanisms of molluscan immune defense have been investigated for their important position in evolution, and some sporadic phenomena of immune priming have also been observed. In scallop *Chlamys farreri*, a short-term immersion with *V. anguillarum* aroused the scallops with enhanced phagocytosis and acid phosphatase activity against the secondary challenge with *V. anguillarum* (Cong et al., 2008). In addition, C-lectins might be involved in the immune priming of scallop (Wang et al., 2013). In Pacific oyster *Crassostrea gigas*, Poly I:C can induce a protective antiviral immune priming response against the secondary challenge with *Ostreid herpesvirus* (Green and Montagnani, 2013). Recently, the whole genome sequence of Pacific oyster was released (Zhang et al., 2012), and it provided a golden opportunity to expound molecular mechanisms of molluscan immunity including the underlying mechanisms of immune priming in marine invertebrates.

In the present study, the Pacific oysters were immunized with heat-killed *Vibrio splendidus* in the primary stimulation and then challenged with live *V. splendidus* for the secondary stimulation. The cellular responses, including the changes of total hemocyte counts (THC), regeneration of circulating hemocytes and the ability of phagocytosis were measured to investigate whether heat-killed *V. splendidus* could arouse immune priming in oyster. Meanwhile, the expression level of some important genes involved in phagocytosis and hemopoiesis were also monitored to find out the underlying mechanisms of immune response elicited by the primary and secondary stimulations of *V. splendidus*.

2. Materials and methods

2.1. Oysters

Pacific oysters *C. gigas*, about 2 years old, were obtained from National oceanographic Center, Qingdao, China. Animals were cultured in tanks with continuously oxygenated, filtered seawater at 20 °C for 1 week before processing.

2.2. Bacteria and experimental stimulation

Vibrio splendidus, isolated from lesion-like niduses of moribund scallop *Patinopecten yessoensis* (Liu et al., 2013), was applied as stimuli agent in this study. The bacteria was cultured in 2116E media at 18 °C for 24 h, and harvested by centrifuged at 4000*g*, 25 °C for 10 min. Then it was washed and re-suspended in filter-sterilized (0.22 μm pore size) sea water (FSSW) and adjusted to the final concentration of 2×10^8 CFU mL^{-1}.

The oysters were stimulated by heat-killed or live *V. splendidus* according to the previous description (Labreuche et al., 2006) with minor modification. A narrowed notch was sawed in the closed side of the oyster shell, adjacent to the adductor muscle, and then the oysters were acclimated for 1 week to be available for the following experimental stimulation. One hundred and twenty oysters were equally divided into two subgroups designed as FSSW (received an injection of 100 μL FSSW containing 50 mM BrdU) and HK-Vs (received an injection of 100 μL heat-killed *V. splendidus* containing 50 mM BrdU). Two hundred and forty oysters were employed and divided equally into four different subgroups designed as FSSW + FSSW, FSSW + Vs, HK-Vs + FSSW, HK-Vs + Vs, meaning that oysters received an 100 μL injection (containing 50 mM BrdU) with FSSW or heat-killed *V. splendidus* for the primary stimulation at 0 h and an 100 μL injection (containing 50 mM BrdU) with FSSW or live *V. splendidus* (containing 50 mM BrdU) for the secondary stimulation at 168 h. The oysters in blank and control groups did not receive the injection of BrdU. All the animals were returned to seawater tanks and maintained under static conditions after handling.

2.3. Hemocytes collection

Hemolymph samples were randomly collected at time points of 0, 6, 9, 12, 24 and 48 h after the first and secondary stimulations from each subgroup to examine the hemocyte cellular parameters. Five hundred microliter hemolymph of each oyster was aseptically withdrawn from the posterior adductor muscle sinus using a 23-gauge needle attached to a 2-mL syringe containing 1 mL anti-aggregant solution (0.5% g mL^{-1} EDTA in PBS). The hemolymph from three oysters were pooled together (about 4.5 mL) as one sample, and three samples (including nine oysters) were collected for each sampling points. Each sample was mixed in 10 mL tubes held on ice to minimize cell clumping, and then divided into three aliquots. One aliquot was used for quantifying THC, another was used for measuring regeneration of circulating hemocytes and the ability of phagocytosis, and the last one for analyzing gene expression by real-time PCR.

2.4. Total hemocyte counts (THC)

Three hundred microliter of each hemolymph sample was fixed by adding 100 μL absolute formaldehyde, and 10 μL of the mixture was placed in a hemocytometer to measure the THC using a microscope (Olympus BX51, Tokyo, Japan).

2.5. BrdU incorporation assay

Five hundred microliter of each hemolymph sample was centrifuged at 800*g*, 4 °C for 10 min, and the pellet of hemocytes was resuspended in 1 mL FSSW. The suspension was used for hemocytes regeneration analysis based on the method described in a previous report (Sun et al., 2012). A drop of hemocytes of each sample was deposited on a clean glass slide treated with poly-L-lysine and kept in a wet chamber at room temperature for 1 h. After hemocytes set-