Developmental and Comparative Immunology 40 (2013) 142–147

Contents lists available at SciVerse ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

The response of mRNA expression upon secondary challenge with *Vibrio anguillarum* suggests the involvement of C-lectins in the immune priming of scallop *Chlamys farreri*

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ARTICLE INFO

Article history:
Received 12 November 2012
Revised 1 February 2013
Accepted 7 February 2013
Available online 19 February 2013

Keywords:
C-lectin
Immune priming
Chlamys farreri
Vibrio anguillarum

ABSTRACT

The enhanced immunity against a second encounter with the particular pathogen has suggested the presence of "immune priming" in scallop. In the present study, the survival rate and expression patterns of five C-lectin isoforms from scallop *Chlamys farreri* were explored after "vaccination" of heat-killed *Vibrio anguillarum* or successively challenge with *V. anguillarum* and *Micrococcus luteus*. When scallops were challenged with live bacteria, the survival rate increased significantly only in the group firstly "vaccinated" with inactivated *V. anguillarum* and then challenged with live *V. anguillarum* compared with naive scallops (from 41% to 63.6%, $P < 0.05$), showing enhanced protective effects of inactivated bacteria with "specificity". When scallops received the challenge with *V. anguillarum*, the mRNA expression level of five C-lectins in scallops which were immuned previously with heat-killed *V. anguillarum* peaked significantly higher (26.7-, 121.7-, 60.1-, 27.4-, 16.3-fold to 0 h, respectively, $P < 0.01$) than that in non-immuned scallops (7.6-fold, $P < 0.05$; 6.4-, 3.9-fold, $P > 0.05$; 5.7-fold, $P < 0.05$; 11.7-fold, $P < 0.01$, to 0 h, respectively). A significantly higher peak and 3–9 h earlier response of all C-lectins mRNA expression were observed after challenge with live *V. anguillarum* (26.7-, 121.7-, 60.1-, 26.4- and 16.3-fold to 0 h, respectively, $P < 0.01$), compared with those only received first injection with heat-killed *V. anguillarum* (1.6-fold, $P > 0.05$; 8.3-fold, $P < 0.05$; 5.2-fold, $P > 0.05$; 14.5-fold, $P < 0.01$; 4.3-fold, $P > 0.05$, to 0 h, respectively). The response of mRNA expression to the secondary encounter with the same bacteria was stronger than that of successively encounter with the different class of bacteria. It was obvious that the mRNA expression of C-lectins in scallops was significantly enhanced by the successive challenge of same species of bacteria with a certain degree of specificity. All the results suggested that C-lectins might be involved in some form of immune priming, and it might provide new insights into mechanism of invertebrate immune priming.

1. Introduction

The invertebrates are always considered entirely relying on innate (non-specific) immunity to fight against pathogens, because they lack the "true" lymphocytes and functional antibody (Janeway and Medzhitov, 2002). However, the recent studies on insectan or crustacean have revealed that a second encounter with the particular pathogen resulted in enhanced immunity. Increased survival rate or fecundity after secondary encounter with the same pathogen were reported in some tested animals, such as *Drosophila melanogaster* (Pham et al., 2007) and *Bombus terrestris* (Sadd and Schmid-Hempel, 2006). These observations favored the existence of some form of specific immunity in invertebrates (Hauton and Smith, 2007), which have been referred as "immune priming" (Moret, 2006), "specific immune priming" (Pham et al., 2007; Roth and Kurtz, 2009), "trained immunity" (Netea et al., 2011) or "line specific immune memory" (Kurtz and Franz, 2003). The underlying mechanism for this phenomenon of priming in invertebrates is sophisticated and far to know.

In recent years, research efforts have been made on the molecules that contribute to specific immune recognition and immune priming in invertebrates. Recent discovery suggests that a lot of immune molecules may be involved in this mechanism, such as receptors with high genetic diversity (Schulenburg et al., 2007). Pathogen recognition receptors (PRRs) were proteins on cell surface which can recognize and bind the pathogen-associated

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http://dx.doi.org/10.1016/j.dci.2013.02.003

molecular patterns (PAMPs), and they were suspected to establish comparatively short-term immunological memory (Kvell et al., 2007). The specific recognition of pathogens could be greatly enhanced by increasing the presence of relevant receptors, and this dosage-dependent specificity was expected to be associated with immunological priming (Schulenburg et al., 2007). C-type lectins are a superfamily of diverse proteins with one or more carbohydrate recognition domains (CRDs) of ~130 amino acid residues. As PRRs, C-lectins can specifically bind PAMPs on the surfaces of many pathogens (Kilpatrick, 2002; Devi et al., 2010), and involve in non-self recognition (Geijtenbeek et al., 2004; Cambi et al., 2005; Akira et al., 2006). In invertebrates, large number of C-lectins have been identified, and proved to play vital roles in immune recognition, agglutination, induction of phagocytosis and encapsulation (Zelensky and Gready, 2005; Ao et al., 2007; Wang et al., 2009b). Considering the multi-function of invertebrate C-lectins, it would be interesting and meaningful to survey their response during the successive bacteria challenge, and understand their contribution in the immune priming.

Zhikong scallop, *Chlamys farreri*, is one of the most important cultured mollusks in China, and the enhanced immune protection upon secondary encounter with *Vibrio anguillarum* has been observed in our previous study (Cong et al., 2008). The knowledge on molecular mechanism of this phenomenon will be helpful to better understand the immune defense system of scallop and conducive to develop the strategy for disease control. In the present study, 5 isoforms of C-lectin which were previously identified from scallop *C. farreri* and named as Cflec-1, Cflec-2, Cflec-3, Cflec-4, Cflec-5 (Wang et al., 2007; Zheng et al., 2008; Zhang et al., 2009a,b, 2010) were selected (1) to monitor their temporal mRNA expression in the hemocytes of immuned and non-immuned scallops response against *V. anguillarum* challenge; (2) to examine their mRNA expression level in hemocytes after scallops were secondary encountered with the same pathogen *V. anguillarum*; (3) to investigate the specificity of their mRNA response to different microorganism challenge and (4) to find any possible involvement of C-lectin in "specific" or "selective" recognition of immune priming.

2. Materials and methods

2.1. Animals and bacteria

Healthy Zhikong scallops *C. farreri*, averaging about 25.65 g in wet weight, were collected from a farm in Qingdao, Shandong province, China, and maintained in aerated tanks at 15 °C for two weeks before use. The seawater was changed 100% daily.

Two bacteria, Gram-negative bacteria *V. anguillarum* kindly provided by Dr. Zhaolan Mo, and Gram-positive bacteria *Micrococcus luteus* purchased from Microbial Culture Collection Center (Beijing, China), were used in this experiment. *V. anguillarum* and *M. luteus* were incubated in marine broth 2216E at 28 °C and LB medium at 37 °C to logarithmic growth phases respectively. The bacterial cells were harvested by centrifugation at 8000 rpm at 4 °C for 5 min, washed three times with filtered seawater, and resuspended in the same seawater at a density of 1×10^8 CFU mL^{-1} for the following experiments. Bacteria used for the first immunization were inactivated by boiling for 0.5 h (Pham et al., 2007).

2.2. Bacteria challenge and sampling

A pretest of the pathogenicity in which naive scallops (named N_0) were injected with 1×10^8 CFU mL^{-1} of live *V. anguillarum* or *M. luteus* was performed to estimate the survival rate after 24 h.

The tracking of survival rate and sampling for RNA extraction after bacteria exposure were operated with the same experimental design according to previous report (Pope et al. 2011), except that the survival rate was only recorded at 24 h. Take RNA sampling for example, 10 scallops kept in the seawater without any treatment were set as blank. Sw and Va group were included in the "vaccination" process and Sw–Sw, Va–Va, Sw–Va and Va–Ml group were included in the challenge process. In detail, during the first injection for "vaccination", one group including 120 scallops received an injection of 50 μL seawater as control (also named as Sw group), and another 120 scallops received an injection with the same volume of heat killed *V. anguillarum* suspension at the concentration of 1×10^8 CFU mL^{-1} (named as Va group). At 3, 6, 12, 24, 168 h after the initial immunization, 6 individuals of scallop were sampled for hemolymph collection from control and Va groups.

During the second injection for challenge at 168 h after the initial immunization, 40 scallops from Sw group received an injection with 50 μL seawater as control (also named as Sw–Sw group); another 50 scallops from Sw group received an injection of 50 μL live *V. anguillarum* (named as Sw–Va group). Fifty scallops from Va group received an injection of 50 μL of live *V. anguillarum* suspension (named as Va–Va group), and the left 40 scallops received an injection of live *M. leuteus* suspension (named as Va–Ml group). Six scallops were sampled at 3, 6, 12, 24, 48 h after the challenge for hemolymph collection.

2.3. RNA extraction and cDNA synthesis

The hemolymph from the blank, control and challenged groups were extracted with a syringe from the adductor muscle and centrifuged at 800*g*, 4 °C for 10 min to harvest the hemocytes. The pellets were resuspended in 1 ml Trizol reagent (TaKaRa, Japan) and stored at −80 °C.

Total RNA from hemocytes was isolated according to the manufacturer's protocol. The concentrations and quality of RNAs were measured by spectrophotometry (Genequant, Amersham Biosciences, USA), and their integrity was checked by electrophoresis in 1% agarose gel. After digestion with RQ1 RNase-free DNase (Promega) to eliminate the genomic DNA contamination, cDNAs were synthesized with reverse transcription kit (Promega) with oligo(dT) primer. The reaction was carried out at 42 °C for 60 min and inactivated at 95 °C for 5 min. PCR with a β-actin primer set (Table 1) including an intron region was performed to evaluate if there was any genomic DNA contamination. The cDNAs were diluted 50-fold and stored at −20 °C till use.

2.4. Quantitative real-time PCR

Six specific PCR primer sets for the β-actin and five C-lectin genes were designed using the primer 5 program based on the

Table 1
Primers used for real-time PCR analysis.

Primer name	Sequence(5′–3′)	Accession number
Cflec-1(forward)	CAACCTGTTCTATATCTGCGAG	DQ209290
Cflec-1(reverse)	GATCTGTTGGCTGATTTCAC	
Cflec-2(forward)	CTGTCAGACTGGGAGTTTGAGG	DQ209289
Cflec-2(reverse)	GTGAGTGGGCATTCTCTGTGTT	
Cflec-3(forward)	ACTTTGCGTGTCGTCTTGGC	DQ209291
Cflec-3(reverse)	CCATTCAACCCAGTCCCTTTC	
Cflec-4(forward)	GGGATACTGGGAATACGATACG	DQ209292
Cflec-4(reverse)	TCCCTCCGCCATTGTCTCAC	
Cflec-5(forward)	CGTTTGAGTAAGAGCCTATTCCT	GU002543
Cflec-5(reverse)	TATTAGTCATTTCACGGTATCCTC	
β-Actin(forward)	CAAACAGCAGCCTCCTCGTCAT	
β-Actin(reverse)	CTGGGCACCTGAACCTTTCGTT	