Developmental and Comparative Immunology 65 (2016) 258–267

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Leukocyte susceptibility and immune response against *Vibrio parahaemolyticus* in *Totoaba macdonaldi*

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ARTICLE INFO

Article history:
Received 20 June 2016
Received in revised form
26 July 2016
Accepted 26 July 2016
Available online 31 July 2016

Keywords:
Totoaba
Vibrio parahaemolyticus
Cellular immune response
Gene expression
Phagocytic activity
Apoptosis

ABSTRACT

Vibrio parahaemolyticus is a serious pathogen that affects aquaculture. Nonetheless, to the best of our knowledge, no studies have focused on its immunological implications in *Totoaba macdonaldi*. Thus, the early immune response to *V. parahaemolyticus* in juveniles of totoaba was studied at 24 h post-infection with an *in vivo* study. In addition, changes in cellular innate immune parameters - phagocytosis, respiratory burst activity and viability (annexin V/propidium iodide) - were evaluated *in vitro* in head-kidney, spleen and thymus leukocytes at 6 and 24 h after bacterial stimulation by flow cytometry. Simultaneously, the expression levels of two immune-relevant genes (IL-1β and IL-8) were measured by using real time PCR. During *in vivo* study, mRNA transcripts of IL-1β were highly expressed in spleen, thymus and intestine and down-regulated in liver after 24 h post-infection. IL-8 gene expression was upregulated in spleen, intestine and liver compared to that of non-infected fish and down-regulated in thymus after 24 h post-infection. Generally, the results showed a significant decrease in cellular immune responses during the infection, principally in phagocytic ability and respiratory burst. The survival or viability of stimulated leukocytes was significantly reduced causing necrosis and apoptosis, indicating a robust killing response by *V. parahaemolyticus*. Finally the *in vitro* analysis showed that transcript levels of IL-1β and IL-8 were up-regulated during stimulation with *V. parahaemolyticus* in head-kidney, spleen and intestine and down-regulated in thymus at any time of the experiment. Although *V. parahaemolyticus* has been reported to be an important pathogen for many aquatic organisms, to our knowledge this might be the first report of early-immune response in juvenile totoaba and these immune parameters may be reliable indicators and can be useful in the health control of this species.

1. Introduction

In Mexico, totoaba (*Totoaba macdonaldi*) is an endemic marine fish of the Gulf of California, which is considered the largest member of the Sciaenidae family (Cisneros-Mata et al., 1997). Totoaba was an important species for commercial fisheries in Northwest México, however, due to unregulated capture and habitat loss, it has been classified as critically endangered since 1975 (Baillie and Groombridge, 1996; Fishbase, 2004; Bobadilla et al., 2011), causing the Mexican Government to close the fishery of this specie (Barrera-Guevara, 1990). A breeding program successfully implemented in Baja California, México has allowed its introduction in aquaculture with relative success in broodstock management and juvenile production, so now the species is being evaluated as a potential aquaculture candidate due to its fast growth and good adaptation to culture conditions (True and Castro, 1997). Nowadays, the studies on totoaba biology are more significant, so the infectious and disease status can be better identified. Moreover, the identification of health markers and immunotherapeutics are critical studies to develop an improved totoaba aquaculture. Currently, only a few studies have been devoted to its population status (Lercari and Chavez, 2007; Marquez-Farías and Rosales-Juárez, 2013), biological performance (Minjarez-Osorio

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http://dx.doi.org/10.1016/j.dci.2016.07.016
0145-305X/

et al., 2012; Mata-Sotres et al., 2015) and dietary evaluation on hematological and biochemical status (López et al., 2015). Understanding the immune defense mechanisms of fish against pathogens is important in terms of control and prevention (Sahoo et al., 2008). For example, an experimental challenge with a known pathogen provides valuable information on the integrated performance of the immune system. Therefore, to cultivate totoaba, information about its immune system under infection conditions is necessary, and disease management is essential to the successful production of this marine fish species. *Vibrio parahaemolyticus*, a Gram-negative bacterium, causes vibriosis of fish, shellfish, and other aquatic animals and is the causative agent to one of the most severe infectious diseases worldwide (Broberg et al., 2011). In this regard, immune responses evoked by pathogens, such as *Vibrio* spp., have been examined in yellow croaker, orange-spotted grouper, zebrafish and Pacific red snapper illustrated by broad changes in immune signaling mechanisms during infection (Huang et al., 2011; Pang et al., 2016; Zhang et al., 2016; Cardenas et al., 2016). Moreover, the fish innate immune system is complex and has different mechanisms that act against a broad range of pathogens. One of these mechanisms is the production of cytokines including several groups, such as tumor necrosis factor-alpha (TNF-α), interferons (IFNs), and pro-inflammatory interleukins (i.e. IL-1β and IL-8) involved in the innate immune system that also participate in the adaptive immune system (Secombes et al., 1996; Trichet, 2010). On the other hand, the ability of leucocytes to phagocytize and kill microorganisms represents the immediate responses to invading pathogens, and further, the cells ability to present antigens is the central link to the specific humoral immune response (Haugland et al., 2014).

Since the immune responses of totoaba against bacterial infections have not yet been investigated, and description on its cell types and functions has not been performed on isolated leukocytes, the objective of this study was to evaluate the immune response of totoaba after infection with *V. parahaemolyticus* under experimental conditions. Firstly, we evaluated the expression levels of principal cytokines (IL-1β and IL-8) in infected and non-infected totoabas; and secondly, in an *in vitro* experiment using head-kidney, spleen and thymus leukocytes, we measured the cellular immune parameters and changes in cell populations by flow cytometry after live *V. parahaemolyticus* challenge at different time points. Finally, we studied the gene expression of IL-1β and IL-8 in leukocytes at 6 and 24 h post-stimulation with *V. parahaemolyticus* to better understand their potential roles in totoaba immune responses. In addition, to our knowledge, it is the first time that blood cell populations in totoaba (*Totoaba macdonaldi*) have been isolated and characterized.

2. Materials and methods

2.1. Vibrio parahaemolyticus conditions

The *V. parahaemolyticus* strain used in this study was provided by Centro de Investigaciones Biologicas del Noroeste (CIBNOR, Mexico) from its bacterial collection. Briefly, *Vibrio parahemolyticus* was cultured in TSB (tryptic soy broth, BD #211825) supplemented with 2.5% NaCl and incubated at 28 °C for 24 h. *V. parahaemolyticus* cultures was then centrifuged at 8000*g* at 4 °C for 20 min. The supernatant was removed and the bacterial pellet was suspended in sterile 0.9% PBS to 1×10^8 cell ml^{-1}.

2.2. Experimental fish

Healthy juvenile totoabas without history of bacterial or parasitic infections were provided by the marine hatchery of Earth Ocean Farms Pichilingue Unit in La Paz, B.C.S., México where this study was performed. This trial was carried out in an indoor closed recirculation system. During the trial, temperature was maintained at 23.0 ± 1.0 °C, salinity averaged 35 ± 0.5‰ and photoperiod was maintained on a 12:12 light:dark schedule. Oxygen concentration was kept higher than 6 mg l^{-1} (Mata-Sotres et al., 2015).

2.3. In vivo study

Twelve totoabas (50 ± 5 g mean body weight) were randomly placed in six seawater tanks (2 fish per tank). Specimens were divided into two groups by triplicate: the control group and *V. parahaemolyticus* group. Fish immune challenge was performed by intraperitoneal injection of 100 µl with *V. parahaemolyticus* (1×10^8 cell ml^{-1}). Fish injected with 100 µl PBS (pH, 7.4) were used as controls. After 24 h post-infection, fish from each tank were sampled. Fish were anesthetized in diluted clove oil solution at the concentration of 100 mg l^{-1}. Spleen, thymus, liver, and intestine were sampled and immediately stored at −80 °C in TRIzol Reagent (Invitrogen) for RNA extraction.

2.4. In vitro study

2.4.1. Isolation of head-kidney, spleen and thymus leukocytes

For the *in vitro* study, head kidney, spleen and thymus samples of 6 healthy totoaba (50 ± 5 g mean body weight) were used to separate leukocytes under sterile conditions, following Lee et al. (2014). Briefly, tissues were removed and passed through 100 micrometer cell strainers (BD Falcon, Franklin Lakes, NJ, USA) in sRPMI [RPMI-1640 culture medium (Gibco) with 0.35% sodium chloride, 100 IU ml^{-1} penicillin (Flow), 100 mg ml^{-1} streptomycin (Flow), 10 IU ml^{-1} heparin (Sigma), and 5% fetal bovine serum (Gibco)]. The volume of the cell suspension was adjusted to 2 ml. All samples were carefully layered over 51% Percoll gradient (Sigma-Aldrich, USA) and centrifuged at 500*g* for 45 min at 23 °C without brake. The leucocyte layer was collected, and washed with sRPMI medium by centrifuging at 800*g* for 10 min at 23 °C. Head kidney, spleen and thymus leukocytes cells were observed and counted with a TC20 Coulter Particle Counter (BioRad, Hercules, CA, USA) and adjusted to 10^6 cells ml^{-1} of sRPMI. A drop of leukocyte suspension was stained with trypan blue (Sigma, Cat. T-8154) to calculate viability, and leukocyte suspension with a viability of more than 95% was used for *in vitro* experiment (Reyes-Becerril et al., 2016).

2.4.2. Stimulation test

One milliliter of head-kidney, spleen or thymus leukocytes were dispensed into wells of flat-bottomed 24-well microtitre plates (Nunc) containing 1×10^6 cells ml^{-1} by wells. Leukocytes were stimulated with 20 µl of *V. parahaemolyticus* (1×10^8 cells ml^{-1}) resuspended in PBS at 22 °C, with 85% relative humidity and 5% CO_2 atmosphere. Control samples consisted of leukocytes incubated with culture medium alone plus 20 µl of PBS. Cells were collected at 6 and 24 h post-incubation with *V. parahaemolyticus* and centrifuged at 11,000g at 10 °C for one minute. For gene expression, supernatant was discarded and cells were resuspended in one ml Trizol reagent (Invitrogen, USA), mixed well by vortexing for one minute, and stored at −80 °C for RNA extraction. In parallel per treatment, 500 µl of leukocytes were taken for triplicate. Samples were filtered and placed in 5 ml tubes (Falcon, Becton Dickinson) for flow cytometry analysis in S3e Cell Sorter (Bio-Rad).

2.5. Flow cytometry analysis

Before using flow cytometry, head-kidney, spleen and thymus