Fish & Shellfish Immunology 46 (2015) 436–441

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Differential innate immune response of European seabass (*Dicentrarchus labrax*) against *Streptococcus iniae*

F. El Aamri, F. Real, F. Acosta*, J. Bravo, L. Román, S. Déniz, D. Padilla

University of Las Palmas de Gran Canaria, 35413 Arucas, Spain

ARTICLE INFO

Article history:
Received 22 March 2015
Received in revised form
19 May 2015
Accepted 24 May 2015
Available online 20 June 2015

Keywords:
Streptococcus iniae
Respiratory burst
Cytokines
Leukocytes
Sea bass

ABSTRACT

Streptococcus iniae is a Gram-positive bacteria that causes invasive infections with severe septicemia and meningitis, producing high economic losses in marine and continental aquaculture. Head kidney leukocytes of European sea bass (*Dicentrarchus labrax*) were used to measure the differential innate immune response upon infection with *S. iniae*. The complete inhibition in the production of intracellular superoxide radicals and total peroxidase content was observed in infected cells. This study also elucidates changes in the relative expression of some immune-related genes. Interleukin 1β, tumor necrosis factor-α and interleukin-6 reached a peak of expression at 4–8 h post-infection, subsequently decreasing significantly up to 48 h post-infection. However, interleukin-10 and Mx protein increased over time, reaching the pick of expression at 48 h post-infection, whereas caspase-3 showed down regulation until 48 h post-infection. The *in vivo* study of immune related genes show the same kinetics of mRNAs expression as *in vitro* experience. The proinflammatory cytokines mRNA transcription levels peaked at an earlier time *in vivo* than *in vitro* system. Our findings indicate that there is a direct relationship between the dissemination of bacteria and the resulting infection-associated inhibition of respiratory burst, apoptosis, and the pro- and anti-inflammatory gene expression profiles.

1. Introduction

Streptococcus iniae is important Gram-positive bacteria associated with acute and chronic mortality in marine and continental aquaculture [1], affecting more than 30 fish species [2,3]. *S. iniae* causes invasive infections with severe septicemia and meningitis, producing high economic losses in aquaculture. *S. iniae* is also a zoonotic pathogen causing soft tissue infections and sepsis in humans [4].

Innate immune response in fish is the first line of defense against tissue damage or pathogen interaction, and macrophages are the predominant phagocytic cells type in head kidney. In a process known as respiratory burst activity (RBA), stimulation of the phagocyte cell membrane, with accompanying activation of the membrane associated NADPH-oxidase, initiates the increased oxygen consumption and the production of reactive oxygen intermediates with microbicidal activity [5]. In addition, neutrophils possess myeloperoxidase in their cytoplasmic granules, which in the presence of halide and hydrogen peroxide, kill bacteria by halogenation of the bacterial cell wall [6]. In zebra fish during inflammation process, leukocyte-delivered myeloperoxidase cell-autonomously down-regulates tissue-generated wound H_2O_2 gradients *in vivo* [7]. Inhibition of the respiratory burst of leukocytes has been found to be a virulence mechanism of some pathogens of mammals [8] and fish [9]. This inhibition allows the bacteria to survive inside phagocytes and eventually induces their death by activating of apoptosis-related caspases [10].

Cytokines have essential roles in the development, differentiation, and function of the immune response. Several fish cytokine genes have been identified and characterized in recent years, and researchers have used their mRNA expression as a tool for measuring immune responses [11]. In particular, pro-inflammatory cytokines, including interleukin-1β (IL-1β), Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are commonly used immune-regulatory genes in fish [12]. It is generally agreed that inflammation is regulated through a fine balance between pro- and anti-inflammatory cytokines [13]. The function of IL-10 in teleosts is still unclear but it may down-regulates production of pro-inflammatory cytokines [14]. Caspase-3 (casp-3) plays an important role as prominent mediator of apoptosis and participates in the cell death-signalling cascade in all vertebrates [15]. Generally, the

* Corresponding author.
E-mail address: felix.acosta@ulpgc.es (F. Acosta).

http://dx.doi.org/10.1016/j.fsi.2015.05.054
1050-4648/

induction of Mx mRNA gene expression is related with viral infection, but it has been demonstrated also to be up regulated following injection of vibrio bacterin [16], in Atlantic salmon parr and in Japanese flounder by *Edwardsiella tarda* [17]. In tilapia, Taylor had demonstrated that the infection by *S. iniae* could produce a microenvironment where apoptosis down regulates the immune function, reducing necrosis and eventually suppressing the local inflammatory response [18].

The aim of this study is to investigate the effect of *S. iniae* in the non-specific immune response of European sea bass.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Three different *S. iniae* strains, IUSA-1 a high virulent clinical strain isolated in red porgy (*Pagrus pagrus*) and gilthead seabream (*Sparus aurata*) [3], CIP 103769 (reference strain from the Pasteur Institute isolated in the brain of tilapia) and CLFP *iniae*-1 (clinical isolate in gilthead seabream from University of Zaragoza, Spain) were used. Strains were grown at 22 °C for 20 h in trypticase soy broth (Pronadisa) supplemented with 0.5% (v/v) yeast extract.

2.2. Animals

70 European sea bass (120 g mean body weight) (*Dicentrarchus labrax*) were provided by Parque Científico Tecnológico Marino of Las Palmas de Gran Canaria University (ULPGC, Spain). Fish were maintained in 500 l stocking tanks and fed with a commercial diet administrated ad libitum twice a day.

3. *In vitro* assays

3.1. Isolation of head kidney leukocytes

Head kidney leukocytes (HK leukocytes) were isolated from 20 European sea bass following the method described by Secombes with some modifications [19]. Briefly, head kidney was excised, cut into small fragments and transferred to 8 mL of cell culture medium (Leibovitz L-15) (Gibco, Gaithersburg, MD, U.S.A). After centrifugation at 400 × g for 10 min, cell suspension was layered on a Ficoll gradient (Limphoprep) and centrifuged at 1100 × g for 30 min at 4 °C. After 3 h of incubation at 22 °C, non adherent cells were removed and the culture media replaced with L-15 supplemented with P/S/G (penicillin/streptomycin/gentamycin) and 2% fetal bovine serum. Finally, the monolayer was incubated overnight at 22 °C.

3.2. In vitro infection

Leukocytes cells were infected with *S. iniae* strains with a multiplicity of infection (MOI) of 1:50. The kinetics of the immune response related genes IL-1β, TNF-α, IL-6, IL-10, Mx and casp-3 were determined for 2, 4, 8, 24 and 48 h post infection by qRT-PCR.

3.3. Quantitative RT-PCR

From *in vitro* and *in vivo* assays, RNA was extracted from HK leukocytes using Total RNA kit I (Promega) according to the manufacturer's instructions. RNA was quantified by nanodrop, and total RNA (0.5 μg) isolated from each sample was used as a template for cDNA synthesis (Script cDNA synthesis kit) (Biorad). Quantitative RT-PCR (qRT-PCR) was performed with MyiQ Biorad using a SYBR Green Supermix (Biorad). The sequences of the primers to investigate the immune response are shown in Table 1. To assess that the primer and probe pairs were quantitative within the working range, serial dilutions in 10-fold increment of cDNA were used, and efficiency for the primer pairs was calculated (Table 1). The results were expressed as relative amounts of the target gene using β-actin as the inner reference gene content in each sample using the comparative C_t method ($2^{-\Delta\Delta Ct}$).

3.4. Respiratory burst assays

The production of intracellular superoxide radicals by leukocytes was determined by the reduction of nitro-blue tetrazolium (NBT) according to previously methods [19,9]. 100 mL of NBT at 1 mg mL^{-1} in Hank's Balanced Salt Solution was added to the monolayers at 22 °C for 1 h. In this assay, cultured *S. iniae* was added to each culture well to give a multiplicity of infection (MOI) of approximately 1:50 (phagocyte:bacteria) to determine the response of phagocytes against bacteria at 45 min post-infection. All experiments were carried out by triplicate, and the respiratory burst activity of isolated phagocytes was expressed as stimulation index, which was calculated as the ratio between the absorbance obtained with phagocytes from fish incubated with bacterial cells and the absorbance of the control phagocytes incubated with PMA.

3.5. Peroxidase content

The total peroxidase content present inside leukocytes was measured according to Quade and Roth [20]. To estimate the leukocyte peroxidase content, 10^6 HK leukocytes in L-15 medium per well were dispensed and fixed into round-bottomed 96-well plates. Bacteria were adjusted in phosphate-buffered saline (PBS) to 10^6 cfu mL^{-1} and 100 mL of each suspension was added to samples of 100 mL of HK leukocytes and incubated for 30 min. After incubation, leukocytes were lysed with 75 mL of 0.02% cetyltrimethylammonium bromide (Sigma–Aldrich, Germany). Afterwards, 50 mL of 10 mM 3, 3′,5, 5′-tetramethylbenzidine hydrochloride (Sigma–Aldrich) and 25 mL of 5 mM H_2O_2 were added producing a colour-change reaction. This reaction was stopped after 2 min by adding 50 mL of 2 M sulphuric acid and the optical density was read at 450 nm in a multiscan spectrophotometer. Controls consisted of HK leukocytes incubated with 100 mL of PBS with no bacterial cells.

4. *In vivo* assay

25 fishes were injected intraperitoneally with *S. iniae* IUSA-1 strain at a dose of 2×10^4 cfu/fish, and a control group (25 fishes) with 100 μl of phosphate-buffered saline (PBS). Head kidney was obtained at different time point and processed for real-time RT-PCR as described in *in vitro* assay.

5. Statistical analysis

All statistical analyses were carried out using the statistical software SPSS program version 17. (SPSS, Inc, Chicago, IL, USA). All bioassays were made in triplicate and all measurements were performed on three replicates. Data were analyzed by analysis of variance (ANOVA) and Tukey's test. Differences were considered statistically significant when $P < 0.05$.

6. Results

6.1. Peroxidase content and respiratory burst assays

Infection of HK leukocytes with *S. iniae* resulted in inhibition of the respiratory burst and peroxidase content for the three strains