Fish & Shellfish Immunology 37 (2014) 108–114

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Passive protective effect of chicken egg yolk immunoglobulins against experimental *Vibrio anguillarum* infection in ayu (*Plecoglossus altivelis*)

Chang-Hong Li, Xin-Jiang Lu, Deng-Feng Li, Jiong Chen*

Laboratory of Biochemistry and Molecular Biology, School of Marine Sciences, Ningbo University, Ningbo 315211, China

ARTICLE INFO

Article history:
Received 19 November 2013
Received in revised form
21 January 2014
Accepted 21 January 2014
Available online 29 January 2014

Keywords:
Bacterial burden
Cytokine gene expression
Macrophage phagocytosis
Plecoglossus altivelis
Yolk immunoglobulin

ABSTRACT

Oral administration of chicken egg yolk immunoglobulins (IgY) has attracted much attention as a means for controlling infectious diseases caused by microorganisms. This study evaluated the protective effect of IgY against *Vibrio anguillarum* infection in ayu, *Plecoglossus altivelis*. IgY was isolated from egg yolks laid by hens initially immunized with formalin-inactivated *V. anguillarum*. Lower mortality of ayu was observed in groups treated with anti-*V. anguillarum* IgY (aVIgY), compared with those treated with saline or with nonspecific IgY (nspIgY). All fish in saline-treated groups died within seven days after bacterial inoculation. The bacterial load in blood, liver, and spleen was significantly lower in fish treated with aVIgY than in fish treated with nspIgY. aVIgY treatment significantly reduced tumor necrosis factor-α (*PaTNF-α*), interleukin-1β (*PaIL-1β*), transforming growth factor-β (*PaTGF-β*), and leukocyte cell-derived chemotaxin-2 (*PaLECT2*) transcript levels in the head kidney, spleen, and liver of ayu challenged by *V. anguillarum*, compared with nspIgY treatment. The phagocytic activity of macrophages for *V. anguillarum* in the presence of specific IgY was significantly higher than that seen for nonspecific IgY. These results suggest that passive immunization by oral intubation with pathogen-specific IgY may provide a valuable treatment for *V. anguillarum* infection in ayu.

1. Introduction

Ayu (sweetfish), *Plecoglossus altivelis*, the sole member of the Osmeriformes family Plecoglossidae, is an important cultured freshwater fish in Japan, China, and Korea. Bacterial diseases have been a major cause of losses in the ayu culture industry in Asian countries, with *Vibrio anguillarum* identified as one of the most harmful pathogens [1–3]. Over the last ten years, ayu culture has rapidly increased in China, and diseases caused by *V. anguillarum* pose a serious threat [3].

Antibiotics have been used extensively for treatment of bacterial infections in aquatic animals for many years. However, antibiotic overuse contributes to the growing number of bacterial species that are becoming resistant to antibacterial medications, which is a concern worldwide. In order to reduce the risk of overuse of antibiotics, a number of alternative measures have been explored and applied in animal culture, including the use of antimicrobial peptides [4,5], application of probiotics [6,7], immunostimulation [8–10], vaccination [11,12], and administration of chicken egg yolk immunoglobulins (IgY) [13,14].

Chicken egg yolk immunoglobulin (IgY) is the major circulating antibody found in chickens, but it is also actively transported to the egg in a manner similar to the placental transfer of IgG in mammals [15]. As a passive, inexpensive, and easily produced antibody, IgY has attracted much attention and is recognized to be efficient in both therapy and prevention of various diseases [16]. IgY is environmentally friendly and elicits no side effects, disease resistance, or toxic residues [17]. Studies have successfully elucidated the protective effect of specific IgYs against a variety of microbial pathogens in mammals. For example, specific chicken egg IgYs have been used in mastitic cows against *Escherichia coli* and *Staphylococcus aureus* [18,19]. In aquaculture, chicken egg IgYs have also been used in rainbow trout (*Oncorhynchus mykiss*) against *Yersinia ruckeri* and *V. anguillarum* [20,21], in shrimp crayfish (*Procambius clarkiaii*) and (*Penaeus chinensis*) against WSSV [13,22], and in small abalone (*Haliotis diversicolor supertexta*) against *Vibrio alginolyticus* [14]. The IgY administered in these studies showed effective protection in a dose-dependent fashion against infection. However, further research is needed to better understand the mechanisms by which IgY confers protection [23].

In the present study, the effect of anti-*V. anguillarum* IgY on the mortality, bacterial burden, and tissue cytokine gene expression in ayu challenged by *V. anguillarum* was determined. The phagocytic

* Corresponding author. Tel.: +86 574 87609571; fax: +86 574 87600167.
E-mail addresses: jchen1975@163.com, chenjiong@nbu.edu.cn (J. Chen).

1050-4648/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2014.01.018

activity of macrophages for *V. anguillarum* in the presence of specific IgY was also evaluated.

2. Materials and methods

2.1. Preparation of antigen

V. anguillarum strain ayu-H080701 isolated from diseased ayu [3] was used in this study. Overnight cultures of *V. anguillarum* were separated by centrifugation, washed, and suspended in sterile saline (0.9 w/v NaCl) at a density of 10^8 CFU/ml. Formaldehyde was added at a final concentration of 0.5% (v/v), and the suspension was incubated at approximately 37 °C for 24 h. Cells were washed twice with sterile saline to remove the formaldehyde and resuspended in sterile saline. Complete killing of the *V. anguillarum* suspensions was confirmed by culture. Bacterial suspensions were stored at approximately 4 °C before use.

2.2. Development of anti-V. anguillarum antibodies in chickens

All of the experiments were performed according to Experimental Animal Management Law of China and approved by Animal Ethics Committee of Ningbo University. A group of Hyline White laying hens, 24 weeks old, were kept for immunization and egg production in the Central Animal Facility of Ningbo University. Antigen suspensions comprising formalin-killed *V. anguillarum* were diluted to 10^8 CFU/ml in sterile 0.9% (w/v) NaCl. Hens were injected intramuscularly in breast muscle with 0.5 ml of antigen suspension mixed with an equal volume of complete Freund's adjuvant for the first immunization. After initial immunization, two additional booster injections each consisting of 0.25 ml of antigen suspension mixed with an equal volume of incomplete Freund's adjuvant were given on days 10 and 20. Eggs were then collected daily from the immunized hens and control hens from the same flock and stored at 4 °C until use.

2.3. Isolation and purification of IgY

IgY isolation procedures were carried out by salt precipitation (ammonium sulphate in solid form) followed by centrifugation and dialysis [23]. Briefly, the egg yolk was separated from the white, mixed with nine volumes of sterile water, and frozen at −20 °C overnight. The mixture was then thawed, centrifuged at 2500 g (4 °C, 45 min) and the pellet was discarded. Supernatant was clarified by filtration through a 0.4-μm filter before adding ammonium sulphate (up to 40 percent). After 18 h at 4 °C, the solution was centrifuged at 5000 g (4 °C, 45 min), and the supernatant was discarded. The pellet was resuspended with PBS and dialyzed against PBS. Anti-*V. anguillarum* IgY (aVIgY) was extracted from the immunized eggs, while nonspecific IgY (nspIgY) was extracted from the control. The purity of IgY was investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions by using a 5% stacking gel and a 12% resolving gel. The gel was stained with Coomassie brilliant blue R-250 staining solution (Bio-Rad Laboratories, Hercules, CA, USA).

2.4. Fish

Healthy juvenile ayu weighing 20–25 g each were obtained from pathogen-free stocks and maintained in stock tanks supplied with filtered water at 20–22 °C. Fish were fed a diet of commercial fish food.

2.5. Survival

The 50% lethal dose (LD_{50}) of *V. anguillarum* ayu-H080701 was determined to be about 5.4×10^5 CFU/kg of fish in this study. Two concentrations (100 mg/kg or 200 mg/kg) of aVIgY or nspIgY were orally intubated to ayu that were injected intraperitoneally (ip), simultaneously with 1.2×10^4 CFU *V. anguillarum* per fish. Another group of ayu without IgY treatment was injected ip with *V. anguillarum*. Morbidity was monitored for 8 days after challenge, and the results were recorded every 24 h.

In order to determine the time course of aVIgY, 4 mg aVIgY were orally intubated to ayu before or after bacterial challenge. For this, two groups of ayu were injected ip with 1.2×10^4 CFU *V. anguillarum* per fish at either 7 or 14 d after aVIgY treatment. Two additional groups of ayu were orally intubated with 4 mg aVIgY per fish at either 1 or 2 d post-injection. Another group of ayu was injected ip with *V. anguillarum* without IgY treatment. Morbidity was monitored for 8 days after challenge, and the results were recorded every 24 h.

2.6. Bacterial burden assay

aVIgY or nspIgY at 200 mg/kg was orally intubated to ayu that were injected ip simultaneously with *V. anguillarum*. A bacterial burden assay was performed as previously described [24]. Briefly, the blood, liver, spleen, and head kidney were harvested aseptically from ayu at 24 h post-infection (hpi). Each liver, spleen, or head kidney was homogenized in 1 ml of sterile PBS (pH7.2). Homogenates and blood were serially diluted in sterile PBS (pH7.2) and plated onto separate Luria–Bertani agar plates. After incubation for 18 h at 28 °C, bacterial colonies in the plates were counted separately for each sample. Liver, spleen, and head kidney samples were normalized to organ weight, and blood samples were normalized to blood volume.

2.7. Alteration of cytokine mRNA expression related to IgY administration

Either aVIgY or nspIgY at 200 mg/kg was orally intubated to ayu that were injected ip simultaneously with 1.2×10^4 CFU *V. anguillarum* per fish. Two additional groups of ayu were injected ip with either 1.2×10^4 CFU *V. anguillarum* or the same volume of the saline per fish, without IgY treatment. Liver, kidney, and spleen were collected aseptically at 12 hpi, frozen in liquid nitrogen, and stored at −70 °C until use.

Changes of *PaTNF-α*, *PaIL-1β*, *PaTGF-β*, and *PaLECT2* mRNA expression in ayu liver, kidney, and spleens were determined by real-time quantitative PCR (RT-qPCR) as previously described [25]. Specific primers were designed according to the sequences of *PaTNF-α*, *PaIL-1β*, *PaTGF-β*, and *PaLECT2* (Table 1). As an internal

Table 1
Oligonucleotide primers used in this work.

Primer	Gene	Accession number	Nucleotide sequence (5′ → 3′)	Amplification of length
PaTNF-αF	*TNF-α*	JP740414	ACATGGGAGCTGTGTTCCTC	115 bp
PaTNF-αR			GCAAACACACCGAAAAAGGT	
PaIL-1βF	*IL-1β*	HF543937	TACCGGTTGGTACATCAGCA	104 bp
PaIL-1βR			TGACGGTAAAGTTGGTGCAA	
PaTGF-βF	*TGF-β*	JP742920	CTGGAATGCCGAGAACAAAT	101 bp
PaTGF-βR			GATCCAGAACCTGAGGGACA	
PaLECT2F	*LECT2*	FM253748	CAGTCTGGTCCTTGCAGAGC	440 bp
PaLECT2R			ACTTGGTGGGGTCAGACTTG	
pActin2F	*β-actin*	AB020884	TCGTGCGTGACATCAAGGAG	231 bp
pActin2R			CGCACTTCATGATGCTGTTG	