Fish & Shellfish Immunology (2008) **24**, 394–399

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Effect of endotoxin on the immunity of Indian major carp, *Labeo rohita*

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Received 25 July 2007; revised 4 September 2007; accepted 21 September 2007
Available online 5 October 2007

KEYWORDS
Endotoxin;
Immunity;
Indian major carp;
Labeo rohita

Abstract Endotoxin, a lipopolysaccharide component of outer cell wall membrane of the Gram-negative bacteria is a factor responsible for a number of biological effects including immunostimulatory activities in different animal species including fish. In this study, *L. rohita* yearlings of weight ranging from 80 to 100 g were injected intraperitoneally with 0.5, 1, 2, 5, 10 and 20 EU/fish dose of endotoxin to find out its effect on the immunity. The *L. rohita* yearlings were found to resist the endotoxin dose up to 20 EU/fish and at the lower doses, i.e., at 1 and 2 EU/fish; it acted as an immune potentiator. Different serum and immune parameters like protein, globulin, lysozyme, respiratory burst activity, myeloperoxidase activity, natural agglutination titre were found to be significantly high ($p < 0.01$) at a dose of 1 EU/fish. While at 10 and 20 EU/fish, most of these parameters were lower thereby indicating the immuno-suppressive nature of the endotoxin at these higher doses.

Introduction

Endotoxin, a lipopolysaccharide (LPS) component of outer cell wall membrane of the Gram-negative bacteria, is the major factor responsible for a number of complications in different animals [1]. No other natural product is ever known to elicit such a great variety of reactions as endotoxins do [2]. The biological activities include pyrogenic, immunogenic, cytotoxic and tumor necrotizing effects; and metabolic disorders like liver disease, vascular complications, sepsis, hypotension, hypoferremia, thrombocytopenia and endotoxic shock [2–9].

Endotoxin has also been reported to have profound effects on the immunity of many animals including fish [10]. Endotoxin can either enhance or suppress the immunity in a host [11]. The effect of endotoxin on T cells, B cells, macrophages, and other immune components has already been reported in many animal species [12]. It can induce the production of antibody, lysozyme, cytokines like interleukin-2 and -6, pro-inflammatory cytokines like IL-1β, tumor-necrosis factor α and several other factors from macrophages [13–17]. Besides, LPS has been reported to have several other effects like pathological, physiological,

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doi:10.1016/j.fsi.2007.09.005

immuno-endocrinological, neuro-immunological, immunomodulatory and even growth stimulatory activities in different fish species [18—24]. Increased oxygen consumption rate, cortisol production and plasma free fatty acid are the common LPS-mediated physiological responses in fish [19]. LPS modifies the activity of the pituitary—interrenal axis [19,22] and induces the elevation of mRNA corticotropin-releasing hormone [23,24]. It also induces apoptosis of follicular cells in fish ovary [25].

Fish, being an aquatic animal, is intimately associated with different Gram-negative bacteria which cause a number of diseases in fish. Although fish are often reported to be resistant to endotoxin [26], the constant exposure to Gram-negative bacteria and their endotoxin can affect the immune system and ultimately the health status of fish. Despite the possibility of severe adverse consequences, very few attempts are being made to study its effect on immune system of fish [27,28]. Therefore, the present investigation was carried out to study the effect of endotoxin on the immunity of Indian major carp, *Labeo rohita*.

Materials and methods

Fish

Indian major carp (*L. rohita*) yearlings of weight ranging from 80 to 100 g were used in the present investigation.

Endotoxin

Pure *Escherichia coli* endotoxin (*E. coli* 055:B5 endotoxin, lot no.: 4L1000) procured from Cambrex (Cambrex BioScience Walkersville, Inc., Walkersville, USA) was used.

Experimental design

L. rohita yearlings were acclimatized in 1000-l cement tanks in the wet laboratory of Fish Health Management Division of Central Institute of Freshwater Aquaculture, Bhubaneswar, India 15 days prior to the start of the experiment. Ten yearlings were maintained per tank and fed with artificial carp diet with daily two-third water exchange.

Six groups containing 10 yearlings in duplicate were intraperitoneally injected with 0.1 ml of endotoxin so that individual fish of a single experimental group received 0.5, 1, 2, 5, 10 and 20 EU of endotoxin while another group without any injection was kept as control group. Blood was collected from all the endotoxin injected and control fish at 7 and 15 days post-injection (dpi) and all serum samples were processed to assay various blood serum and immune parameters. During the entire course of experiment, different water quality parameters like D.O., temperature, pH, total hardness and alkalinity varied from 6.2 to 6.8 mg l^{-1}, 26 to 29 °C, 7.0 to 7.4, 90 to 100 ppm, and 100 to 105 ppm, respectively.

Serum and immune parameters

Total serum protein, albumin and globulin contents

The serum protein content of various groups was estimated as per the standard method [30]. Briefly, 0.2 ml of serum from individual fish of various experimental groups was separately added to 2.0 ml coomassie blue-g-reagent (1% blue-g, 5% ethanol and 10% phosphoric acid) and the absorbance was read at 550 nm. The serum protein content was calculated by regression analysis with bovine serum albumin as standard. The albumin content was estimated spectrophotometrically using standard kits (Glaxo, India). The globulin content was estimated as per the following method. Saturated ammonium sulphate solution (50 μl) was added drop wise to 50 μl serum followed by vortexing. Centrifugation was done at 10,000 × g for 5 min. Then, 20 μl of this sample was dissolved with 80 μl carbonate—bicarbonate buffer (pH 9.3) and the globulin content was determined by estimating the serum protein content as done above.

Myeloperoxidase activity

The myeloperoxidase activity was studied as per the method of Quade and Roth [31]. Briefly, 15 μl of serum was diluted in 135 μl of Hank's balanced salt solution (Ca^{++} and Mg^{++} free) and to it 50 μl of 20 mM 3,3′,5,5′-tetra methyl benzidine and 5 mM H_2O_2 was added. The reaction was stopped after 2 min by adding 50 μl of 4 M sulphuric acid and the optical density (OD) was read at 450 nm.

Respiratory burst assay

The respiratory burst activity was measured by the reduction of nitro blue tetrazolium (NBT) by intracellular superoxide radicals [32]. Briefly, 100 μl of heparinised blood from fish of each group was mixed with 100 μl of 0.2% NBT (Sigma, USA) solution for 30 min at 25 °C. After incubation, 50 μl from the above mixture was added with 1 ml of *N,N*-diethylmethyl formamide (Qualigens, India) and then centrifuged at 3000 × g for 5 min. The optical density of the supernatant was measured at 540 nm.

Lysozyme activity

A turbidometric assay utilizing lyophilized *Micrococcus lysodeikticus* (Sigma, USA) was done to determine the lysozyme activity in serum as described by Sankaran and Shanto [33] and Studnicka et al. [34] with slight modifications. *M. lysodeikticus* at a concentration of 0.2 mg ml^{-1} (in 0.02 M sodium citrate buffer) was added to serum samples at 10:1 ratio. Immediately after adding *M. lysodeikticus*, initial OD was taken at 450 nm. After incubating for 1 h at 24 °C, OD was taken. Lysozyme activity was expressed as units ml^{-1} where one unit is defined as the decrease in absorbance of 0.001 min^{-1}.

Bacterial agglutination activity

The natural bacterial agglutinating activity of the serum samples of all the groups was studied in 'U'-shaped microtitre plates. Two-fold serial dilution of 50 μl serum of fish was made with equal volume of PBS (pH 7.2) in each well, to which 50 μl of formalin-killed *Aeromonas hydrophila* (10^7 CFU ml^{-1}) suspension was added. The plates were incubated overnight at room temperature. The titre was calculated as the reciprocal of the highest dilution of serum showing complete agglutination of the bacterial cells.

Haemagglutination activity

The haemagglutination activity of serum samples from various endotoxin injected and control groups was carried