Fish & Shellfish Immunology 34 (2013) 521–528

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

Inflammatory response of rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) larvae against *Ichthyophthirius multifiliis*

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ARTICLE INFO

Article history:
Received 3 September 2012
Received in revised form
26 November 2012
Accepted 27 November 2012
Available online 20 December 2012

Keywords:
Ontogeny
Rainbow trout
Larvae
Ichthyophthirius multifiliis
Inflammatory response

ABSTRACT

At hatching, the immune system of the rainbow trout larva is not fully developed. The larva emerges from the egg and is exposed to the aquatic freshwater environment containing pathogenic organisms. At this early stage, protection from disease causing organisms is thought to depend on innate immune mechanisms. Here, we studied the ability of young post-hatch rainbow trout larvae to respond immunologically to an infection with *Ichthyophthirius multifiliis* and also report on the localization of 5 different immune relevant molecules in the tissue of infected and uninfected larvae. Quantitative RT-PCR (qPCR) was used to analyze the genetic regulation of IL-1β, IL-8, IL-6, TNF-α, iNOS, SAA, cathelicidin-2, hepcidin, IL-10, IL-22, IgM and IgT. Also, a panel of 5 monoclonal antibodies was used to investigate the presence and localization of the proteins CD8, SAA, MHCII, IgM and IgT. At 10 days (84 degree days) post-hatching, larvae were infected with *I. multifiliis* and sampled for qPCR at 3, 6, 12, 24, 48 and 72 h post-infection (p.i.). At 72 h p.i. samples were taken for antibody staining. The first of the examined genes to be up-regulated was IL-1β. Subsequently, IL-8 and cathelicidin-2 were up-regulated and later TNF-α, hepcidin, IL-6, iNOS and SAA. Immunohistochemical staining showed presence of CD8 and MHCII in the thymus of both infected and non-infected larvae. Staining of MHCII and SAA was seen at sites of parasite localization and weak staining of SAA was seen in the liver of infected larvae. Staining of IgT was seen at site of infection in the gills which may be one of the earliest adaptive factors seen. No positive staining was seen for IgM. The study illustrates that rainbow trout larvae as young as 10 days (84 degree days) post-hatch are able to regulate important immune relevant cytokines, chemokines and acute phase proteins in response to infection with a skin parasitizing protozoan parasite.

1. Introduction

While the fish larva develops inside the egg the chorion forms an efficient barrier shielding the larva against pathogens in the environment. At hatching, the larva is immediately and directly exposed to the environment and therefore needs to possess some means of resisting or overcoming invasion by pathogenic organisms. The immune organs of rainbow trout only fully matures after the time of hatching [1] and this is reflected in the immature immune response to allografting of early larval stages of rainbow trout [2] and a refractory period of the specific antigen response [3]. Studies have shown the presence of maternally transferred immune molecules in salmonids [4,5] but due to the low amount and concentration of these molecules and also the long time span of the egg phase in rainbow trout, it remains uncertain if these molecules have any protective effect in the hatched larva. The ability of the fish larvae to resist or overcome infection at this early stage is thought to depend on innate immune mechanisms present a hatching. To this end, macrophages and neutrophils have been shown to be an important part of cell mediated defenses in fish with the ability to engulf particles [6] and migrate to sites of inflammation and clear bacteria [7,8].

The skin parasite *Ichthyophthirius multifiliis* infects by penetrating into the epidermis of the host where it settles and feeds before exiting the skin as a mature tomont [9]. Rainbow trout infected with sub-lethal doses of *I. multifiliis* are able to respond immunologically [10,11] and generate protection to subsequent infection [12,13]. These earlier reports have focused on older stages of rainbow trout and it is not known if the ability to respond against an infection of *I. multifiliis* has been established in young post-hatch larvae.

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http://dx.doi.org/10.1016/j.fsi.2012.11.036

In the present work we have studied the initial response of rainbow trout larvae to infection with *I. multifiliis*. The aim of the study was to investigate the competences of early post-hatch larvae to respond against a natural infection of *I. multifiliis* and regulate genetic transcription of immune relevant genes. Further, using a panel of available mAbs, we have studied the localization of 5 different immune related proteins and effector molecules in the tissue of infected and uninfected larvae.

2. Materials and methods

2.1. Larvae and experimental setup

Rainbow trout larvae from Bækkelund fish farm (Jutland) were hatched at Bornholms lakseklækkeri and transported to Faculty of Life Sciences, University of Copenhagen. Two days prior to start of the experiment four 8 L aquaria were set up with 6 L of 1/1 tap water/demineralized water. Each aquarium was aerated and set up with 75 larvae. The aquaria were temperature controlled to 10 °C (±0.5°) and pH was between 7.78 and 8.02. The larvae were 10 days (84 degree days) post-hatch (dph) at startup of the experiment.

2.2. Infection procedure and experimental sampling

A rainbow trout heavily infected with *I. multifiliis* was sacrificed by an overdose of MS222 (200 mg/l) and left in 1 L of aquarium water for 4 h at 12–13 °C to let the trophonts leave the fish skin. The fish was removed and the shed tomonts were left for 48 h to produce theronts. The concentration and number of theronts were estimated by counting theronts in 20 × 200 μl and calculating the number of theronts/l. To infect the trout larvae, they were kept for 1 h in 3 L of water containing an estimated 500 theronts/fish (~12,500 theronts/l). After one hour the larvae were relocated to the experimental aquaria. The larvae in the two control replicates were submitted a sham infection with no parasites. Sampling of infected and control larvae were done at 3, 6, 12, 24, 48 and 72 h post-infection (p.i.). At each sampling point 5 larvae from each aquarium were sampled for qPCR and 4 larvae were sampled at 72 h post-infection for immunohistochemistry. No mortality of the larvae was seen during the experiment.

2.3. Extraction of total RNA and cDNA production

Total RNA was extracted and purified from whole larvae using GenElute™ Mammalian Total RNA kit (Sigma–Aldrich, Cat. No. RTN350, Denmark). Homogenization of the rainbow trout larvae was done in 700 μl lysis buffer on ice using a sonic dismembrator (ARTEK model 300, Bie & Berntsen, Denmark) and RNA was isolated on columns according to the manufacturer's protocol. The purified total RNA was DNase treated (DNase I, Fermentas Cat. No EN0521, Denmark) to remove any residual genomic DNA. The purity and concentration (599–4828 ng/μl; x; = 2464 ng/μl) of the RNA was measured on a spectrophotometer (NanoDrop, Saveen & Werner ApS, Denmark) and the integrity of RNA was verified by gel electrophoresis on a 1.5% agarose gel. Purified RNA was kept at −80 °C for later use. Prior to generation of cDNA, all RNA samples were diluted to 400 ng/μl using RNase/DNase free water (Invitrogen Cat. No 10977, Denmark). For reverse transcription (RT) and first strand cDNA generation, the TaqMan® Reverse Transcription kit with random hexamer primers was used (Applied Biosystems Cat. No. N8080234, Denmark). For each 20 μl reaction, 2 μl of total RNA was used (800 ng RNA/reaction). The resulting solution of 20 μl cDNA was diluted 1:10 by addition of 180 μl RNase/DNase free water. The RT-PCR reactions were run on a T3 Thermocycler (Biometra, Germany) under the following conditions: 25 °C for 10 min, 37 °C for 60 min and 95 °C for 5 min.

2.4. Real time quantitative PCR

The primers and probes for the genes studied by qPCR are shown in Table 1. The qPCR reactions were set up using 6.25 μl Brilliant® II master mix (Agilent Technologies, cat. No. 600804), 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM), 0.5 μl fluorescent probe (5 μM), 2.25 μl RNase/DNase free water and 2.5 μl cDNA template. The 12.5 μl qPCR reactions were run in 96

Table 1
List of genes, primers and probes used to evaluate gene expression by qPCR.

Target gene	Primers	Probes	Amplicon	Accession no.
IL-1β	FWD: ACATTGCCAACCTCATCATCG REV: TTGAGCAGGTCCTTGTCCTTG	CATGGAGAGGTTAAAGGGTGGC	91	AJ223954
IL-8	FWD: AGAATGTCAGCCAGCCTTGT REV: TCTCAGACTCATCCCCTCAGT	TTGTGCTCCTGGCCCTCCTGA	69	AJ279069
IL-6	FWD: ACTCCCCTCTGTCACACACC REV: GGCAGACAGGTCCTCCACTA	CCACTGTGCTGATAGGGCTGG	91	DQ866150
[a]TNF-α	FWD: GGGGACAAACTGTGGACTGA REV: GAAGTTCTTGCCCTGCTCTG	GACCAATCGACTGACCGACGTGGA	75	AJ277604
iNOS	FWD: ACCAGAAGGAGGGTCACTT REV: TGGGTGAGGGTGATGCCAA	ATGTGTGTGGGGGTGTGAACATGG	109	AJ300555
SAA	FWD: GGGAGATGATTCAGGGTTCCA REV: TTACGTCCCCAGTGGTTAGC	TCGAGGACACGAGGACTCAGCA	79	AM422446
Cathelicidin-2	FWD: AAAGATTCCAAGGGGGGT REV: CAAAGGGTGTGTTGTGCTGT	GCTCTCGTCCTGGGTTTGGCTCC	135	AY360356
Hepcidin	FWD: GAGGAGGTTGGAAGCATTGA REV: TGACGCTTGAACCTGAAATG	AGTCCAGTTGGGGAACATCAACAG	95	AF281354
IL-10	FWD: CGACTTTAAATCTCCCATCGAC REV: GCATTGGACGATCTCTTTCTTC	CATCGGAAACATCTTCCACGAGCT	70	AB118099
IL-22	FWD: ATGACCACCACCACAGCATT REV: ATTCCTTTCCCCTCCTCCAT	CTTTCCGCAAGAAGTTGTCCGAG	64	AM748537
IgM	FWD: CTTGGCTTGTTGACGATGAG REV: GGCTAGTGGTGTTGAATTGG	TGGAGAGAACGAGCAGTTCAGCA	72	S63348
IgT	FWD: AGCACCAGGGTGAAACCA REV: GCGGTGGGTTCAGAGTCA	AGCAAGACGACCTCCAAAACAGAAC	73	AY870265

[a] The primers overlap both isotype 1 and 2.