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Immune gene expression following LPS exposure in the gill of rainbow trout, *Oncorhynchus mykiss*

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In the present study, immune gene expression of rainbow trout, *Oncorhynchus mykiss*, against bacterial endotoxin (LPS) was studied *in vivo*. The expression of proinflammatory cytokine genes (IL-1β and TNF-α) and IFN-related genes (IRF-1, Mx-3) at gill was assessed by RT-PCR at different time point of day1 and day 3 post-injection. It was shown that the proinflammatory cytokine gene expression at gill was induced 1 day after LPS injection but the expression was not sustained until day 3. Meanwhile upregulated expression of IFN-related genes was found to be only at day 3 post injection, indicating indirect effect of LPS on these genes.

Key words: Immune, *Oncorhynchus mykiss,* Lipopolysaccharide, Gill, Interleukin-1β, TNF-a, Mx-3, IRF-1

Introduction

There is a plenty of information on mammal immune system including the response against bacterial invasion. However, less information exists on fish immune system, especially at molecular level. Thus in this study an attempt was tried to elucidate the immune response of fish against LPS (Lipopolysaccharide) by assessing the immune gene expression at gill. LPS is an endotoxic component of the outer membrane in gram-negative bacteria, consisting of three covalently linked regions: lipid-A-coreoligosaccharide-O-specific chain and known to induce strong inflammatory and immuno stimulatory responses. According to Sergey *et al*. (1998), LPS is a potent inducer of endogenous IL-1, a proinflammatory cytokine, and the effects induced by LPS are similar to those observed following IL-1 β administration. Indeed, LPS-induces effects can be blocked or ameliorated by the IL-1 receptor antagonist and IL-1β-deficient mice exhibit lower fever following LPS administration.

A model of cytokine release during infection with a pathogen has been proposed (Campos *et al*., 1993) to explain the sequence of events that result in pathogenesis and immunity to that pathogen. The initial response to pathogens is generally characterized by early inflammatory indications, followed by infiltration and aggregation of inflammatory cells and then the initiation of specific immune responses mediated by T and B lymphocytes (Campos *et al*., 1993). These responses appear to be orchestrated by the production of cytokines in a specific manner in response to invading pathogen.

According to their release following infection, the cytokines can be classified into two categories: early and late cytokines (Hughes and Babiuk, 1998). Generally, early cytokines are produced at the site of infection and are responsible for the early inflammatory response, regulation of adhesion mol-

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ecules, and infiltration of lymphoid cells (Hughes and Babiuk, 1998). These cytokines include the proinflammatory cytokines such as IL-1, TNF-α, and IL-6, as well as the chemotactic cytokines such as IL-8, the *gro* family of cytokines, and other chemoattractants (Hughes and Babiuk, 1998). Meanwhile, late cytokines, such as IL-2, IL-4, IL-5, and IFN-γ are produced by T lymphocytes following recognition of antigen in association with MHC molecules present on the surface of antigen-presenting cells (Hughes and Babiuk, 1998). These late cytokines are responsible for the differentiation and clonal expansion of reactive cells, e.g. phagocytes and lymphocytes, as well as the regulation of the immune response (Hughes and Babiuk, 1998).

In this study it was examined the effect of LPS on proinflammatory cytokines (IL-1β, TNF-α) and IFN-related genes (IRF-1, Mx-3) using a semi-quantitative RT-PCR method.

Materials and Methods

Tissue sampling and total RNA isolation

To investigate the effect of LPS on immune gene expression, two groups of 6 fish were anaesthetized in 25 μg ethyl-4-aminobenzoate (benzocaine, BDH, Poole, UK)/ml water, and injected intraperitoneally with 300 $\mu\ell$ of PBS or LPS (100 μg). Fish were allowed to recover from anaesthesia in an aerated recovery tank, and then moved back to the original tank. At 1 and 3 days post-injection, the gill tissue samples were aseptically taken from killed fish and wrapped individually in aluminium foil, immediately frozen in liquid nitrogen, and stored at -70℃. The gill tissue was chosen because it is a major immune organ and represents a mucosal site. Tissue samples were homogenized in RNAzol (Biogenesis, CS-104) using a glass homogeniser on ice, and total RNA was extracted and reverse transcribed using previously documented methodology (Hong *et al*., 2001), with the resultant cDNA dissolved in DEPC-treated water and stored at -20 ℃.

RT-PCR analysis

To detect expression (mRNA) of immune genes following injection, PCR was carried out using different primer sets (Table 1) and different conditions (Table 2) for β-actin, IL-1β, TNF-α, Mx-3 and IRF-1 genes. PCR reactions were performed in 25 $\mu\ell$ reactions containing 5 $\mu\ell$ of cDNA (diluted in water), 1.25 $\mu\ell$ (25 pmol) of each primer, 2.5 $\mu\ell$ of 10X reaction buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, 0.1% Tween-20, pH8.8, Bioline), 0.5 $\mu\ell$ dNTP mixture (2.5 mM for each base, Bioline), 1.25 $\mu\ell$ of $MgCl_2$ (50 mM, Bioline) and 0.125 $\mu\ell$ (0.625 U) of *Taq* polymerase (Bioline), using a Techne thermocycler (Genius).

The first PCR in each case was for β-actin, and the amount of cDNA used in each sample was titrated (between 1 and 3 $\mu\ell$) to give constant product yield. The same amount of cDNA was then used for all subsequent PCR's as a way of normalising the data in order to give a more quantitative result. PCR conditions were preliminary optimised for each gene and the optimum number of cycles was chosen to detect gene expression level in the log phase of amplification.

PCR products were visualised on a 2% agarose gel containing 0.1 μg/ml ethidium bromide in TBE buffer. The relative levels of RNA were quantified for each gene by densitometric scanning of agarose gel images using a UVP gel imaging system and UVP Gel-works ID advanced software. The relative ratios of gene product to β-actin product were calculated for each of the immune genes and used to quantify inter-group differences in expression levels.

Table 1. Primers for specific PCR

β-actin	Forward	5'- ATG GAA GAT GAA ATC GCC
AJ438158*	Reverse	5'- CAT GGA GAA GAT CTG GCA
IL-1β	Forward	5'- ACA GAC ATG GAT TTT GAA TCA
AJ278242*	Reverse	5'- TTC AGC AGT ACA TCA CAG TAT GAG
TNFα2	Forward	5'- CAA GAG TTT GAA CCT CAT TCA
AJ277604*	Reverse	5'- GGG TAA TCT ACT TAG CGG GGT A
Mx-3	Forward	5'- ATG CCA CCC TAC AGG AGA TGA T
U47946*	Reverse	5'- CCA CAG TGT ACA TTT AGT TG
IRF-1	Forward	5'- AGG CTG TCT GTG CTG TCT ACT AT
AF332147*	Reverse	5'- AAT AAC TTC CCG CTC CAT CT

* : Gene bank accession Nos.

Table 2. The cycling protocol for PCR

Gene		Temp. (℃)	Time	No. of cycles
β-actin	Denaturising	94	5 min	1
	Amplification	94	45 sec	24
		58	45 sec	
		72	30 sec	
	Extension	72	10 min	1
IL-1β	Denaturising	94	5 min	1
	Amplification	94	45 sec	35
		58	45 sec	
		72	30 sec	
	Extension	72	10 min	1
TNFα2	Denaturising	94	5 min	1
	Amplification	94	30 sec	40
		62	20 sec	
		72	20 sec	
	Extension	72	10 min	1
Mx-3	Denaturising	94	5 min	1
	Amplification	94	45 sec	35
		52	45 sec	
		72	45 sec	
	Extension	72	10 min	1
IRF-1	Denaturising	94	2 min	1
	Amplification	94	30 sec	30
		60	30 sec	
		72	20 sec	
	Extension	72	10 min	1

Statistical analysis

Gene expression data were analysed using Kruskal-Wallis ANOVA and Mann-Whitney U-test since the data were not normally distributed.

Results and Discussion

A semi-quantitative RT-PCR method was used to analyse rainbow trout proinflammatory cytokines (IL-1β, TNF-α2) and IFN-related genes (IRF-1, Mx-3) playing an important role in immune regulation. Minor differences in input RNA quantity and efficiency of cDNA synthesis were corrected by normalization of the β-actin products and subsequent expression of the immune gene products as a ratio relative to β-actin.

In this study, LPS significantly increased TNFα and IL-1β mRNA level at the doses of 100 μg (Fig. 1). It is known that stimuli that induce NF-$\kappa\beta$ translocation to the nucleus and binding to the promoter region of TNFα and IL-1β are potent inducers of these genes. LPS is a strong inducer of NF-$\kappa\beta$ in macrophages, and phorbol myristate acetate (PMA), the classic inducer of proteinase C activity, and TNF itself, also recruit NF-$\kappa\beta$ in the nucleus (Myers *et al*., 1994).

Even though, LPS significantly increased TNFα and IL-1β mRNA level at day 1 but the effect was not sustained to day 3 (Fig. 2). This result is consistent with other studies. Adarns *et al*. (1990) have reported that intravenous administration of LPS into both cattle and pigs induces a rapid and transient

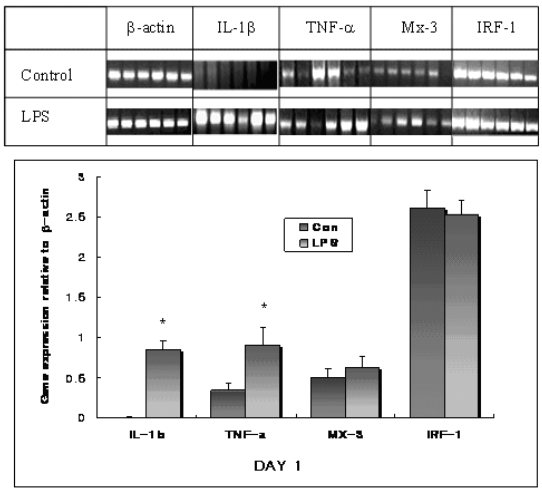

Fig. 1. Effect of LPS on immune gene expression in gill at day 1 post-injection. (a) Fish, 6 Rainbow trout for each group, were injected with PBS buffer as a negative control, or LPS (100 μg) 24 h before RNA extraction from the gill. cDNA from 5 μg RNA was titrated to give a constant β-actin product and then used for PCR using specific primers for IL-1β, TNF-α, Mx-3 and IRF-1. (b) Levels of immune gene expression were described as a ratio to that of β-actin gene expression from densitometric-scanned values. The plot represents the mean and S.E for each group. * : significant difference from the negative control group in the Mann-Whitney test.

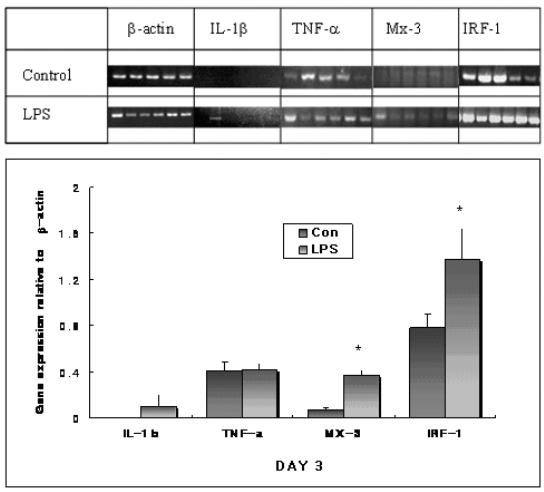

Fig. 2. Effect of LPS on immune gene expression in gill at day 3 post-injection. (a) Fish, 6 Rainbow trout for each group, were injected with PBS buffer as a negative control, or LPS 100 μg) 72 h before RNA extraction from the gill. cDNA from 5 μg RNA was titrated to give a constant β-actin product and then used for PCR using specific primers for IL-1β, TNF-α, Mx-3 and IRF-1. (b) Levels of immune gene expression were described as a ratio to that of β-actin gene expression from densitometric-scanned values. The plot represents the mean and S.E for each group. * : significant difference from the negative control group in the Mann-Whitney test.

elevation of TNFα in the blood as detected in bioassay. TNFα levels were proportional to the amount of LPS administered, peaked at about 1-2 h, and were no longer detectable at approximately 4 h. In fish, the TNFα2 gene is also likely to be an early response gene. Zou *et al*. (2002) demonstrated that TNFα2 expression was increased significantly by LPS from 2 h post-stimulation, reached the highest values at 4 h, decreased from 8 h, and returned to the level in unstimulated cells within 36 h, in head kidney cells.

Both Mx-3 and IRF-1 genes showed a similar pattern of expression when stimulated by LPS in this study (Fig. 1 & Fig. 2). Effects on IFN-related gene (Mx-3 and IRF-1) expression were delayed until day 3, implying an indirect effect of LPS may be occurring. It was reported that the peak expression of the Mx protein in salmon macrophages induced by poly I:C occurred after 48 h whereas peak IFN production was observed by 24 h after addition of poly I:C, suggesting that poly I:C induces the Mx protein indirectly through the activity of type I IFN (Nygaard *et al*., 2000). Apparently, the increase of those gene expression in LPS injected group at day 3 seem to be due to the lower expression of control group compared to day 1. However, it is not difficult to compare day 1 and day 3 data since they were detected by semi-quantitative method.

The delayed increase of IRF-1 and Mx-3 gene expression is postulated to be due to an indirect effect of LPS, since they are genes known to be induced by other cytokines like IFN. Davidson *et al*. (1999) also reported the delayed upregulation of another IFN-inducible gene, trout low molecular weight polypeptide (LMP) 2, where there was no difference after stimulation with PHA for 4 h *in vitro* but upregulation after 24 h, compared to controls.

In conclusion, LPS administered intrapenitoneally showed significant stimulatory effects on inflammatory gene expression i.e. IL-1β, TNF-α and showed a possible indirect stimulatory effect on IFN-related (IRF-1 and Mx-3) gene expression in the gill.

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