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Biological effect of recombinant IL-1β on the expression of antiviral genes in the gill of rainbow trout, *Oncorhynchus mykiss*

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We have investigated the biological effects of recombinant IL-1β (rIL-1β) on the expression of antiviral genes such as Myxovirus-3 (MX-3) and Interferon regulating factor-1 (IRF-1), which are related to type I interferon. When ten micrograms of rIL-1β were treated, the stimulatory effect we observed on the expression of these antiviral genes. Interestingly, at the early stage of stimulation, these genes were down-regulated and then up-regulated by the results obtained that the expressions of these genes were decreased at day 1 post-injection and gradually increased at day 3 post-injection. Thus, the stimulatory effect of rIL-1β on the expression of MX-3 and IRF-3 gene might be an indirect stimulatory effect because significant up-regulation was delayed until day 3 post-injection.

Key words: Recombinant IL-1β, *Oncorhynchus mykiss*, Myxovirus-3, Interferon regulating factor-1

Introduction

In mammals, the cytokine interleukin-1β (IL-1β) exerts a plethora of localised and systemic biological effects, and is central to initiation and regulation of immune and inflammatory responses by up-regulating other inflammatory cytokines, lymphocyte growth factors, colony-stimulating factors, and mesenchymal growth factor genes (Dinarello, 1996).

In the rainbow trout (*Oncorhynchus mykiss*), IL-1β cDNA has been isolated, and sequenced (Zou *et al.*, 1998) and thus facilitates production of the recombinant protein (rIL-1β), and promotes to perform many researches on the biological activities on immune and antibacterial genes. In the previous papers, it was shown that trout rIL-1β has many of the typical biological activities of IL-1β (Hong *et al.*, 2001 & Hong *et al.*, 2003). Such effects include increased transcript levels of IL-1β, COX-2, MHC class II and lysozyme II *in vitro* and/or *in vivo*, in addition to the effects on the increase of trout leukocyte migration (Peddie *et al.*, 2001), phagocytic activity (Hong *et al.*, 2001) and resistance to pathogenic bacterial disease (Hong *et al.*, 2003). Thus, even though the effects of rIL-1β on the bacterial resistance and immune genes relating initial immune responses are clear, still there is no direct evidence of its effect on antiviral genes such as interferon (IFN).

IFNs are known to intermediate mediators of cellular responses after viral infection. The type I IFN (IFN-α/β) system constitutes the most powerful antiviral defence mechanisms induced in vertebrates during viral infection (Samuel, 1991). Various IFN-α/β-inducible proteins are reported such as interferon regulating factor (IRF)-1, 2-5-A-synthase, Myxovirus (MX) proteins, IL-12, IL-15,

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inducible nitric oxide sythase (iNOS), and interleukin cutting enzyme (ICE) (Halminen *et al.*, 1997; Simon *et al.*, 1991).

Since IFN gene has not been isolated in rainbow trout, detection of IFN induced antiviral genes (i.e. MX-3 and IRF-1) could be an alternative way to investigate whether rIL-1β has the biological effects on antiviral genes. In the studies of mice and humans, MX proteins have proved to be a very specific and sensitive marker for type I IFN induction (Halminen *et al.*, 1997; Simon *et al.*, 1991). Recently, three full length MX (MX-1, 2, 3) cDNAs have been cloned and sequenced from both Atlantic salmon (Robertsen *et al.*, 1997) and rainbow trout (Trobridge and Leong, 1995; Trobridge *et al.*, 1997a). The IRF-1 gene was recently cloned in rainbow trout (Collet *et al.*, 2002). IRF-1 was originally discovered as a transcription factor that plays a role in the regulation of the IFN-β gene (Miyamoto *et al.*, 1988; Fujita *et al.*, 1989a); however, it is also involved in the regulation of other virus- or IFN-inducible genes such as IFN-α (Au *et al.*, 1993). An essential role for IRF-1 is the transcriptional induction of the inducible nitric oxide synthase (iNOS) in macrophages (Kamijo *et al.*, 1994).

The present study aims to analyze the ability of rIL-1β on the expression of antiviral genes (i.e. IRF-1 and MX-3) by a semi-quantitative RT-PCR method in rainbow trout injected intraperitoneally with various IL-1β doses.

Materials and Methods

Fish

Rainbow trout (*Oncorhynchus mykiss*), weighing 200-300g, was purchased from Almondbank (Perthshire, UK), stocked in 250 liter indoor tanks and maintained at 16 ℃, with a constant flow of aerated and dechlorinated water. Fish were acclimated to the tank system for a week prior to use. They were fed a commercial trout pellet diet (EWOS Ltd., UK) twice daily.

Production and purification of recombinant IL-1β

Recombinant rainbow trout IL-1β (rIL-1β) was produced using a method previously reported (Hong *et al.*, 2001). Briefly, rIL-1β was produced in *Escherichia coli* M15 (pREP4) cells after cloning into the pQE30 expression vector (Qiagen, USA). Ni-nitroacetic acid (NTA) metal affinity chromatography was subsequently used for purifying the recombinant protein. The size and purity of the flow-through fractions were determined by SDS-PAGE, whilst the lack of lipopolysaccharide (LPS) contamination was determined by previously documented methodology (Hong *et al.*, 2001).

Tissue sampling

To investigate the effect of rIL-1β on the expression of antiviral genes, four groups of 6 fish were anaesthetized in 1.5 mM ethyl-4-aminobenzoate (benzocaine, BDH, UK), and injected intraperitoneally with 300 $\mu\ell$ of elution buffer or buffer containing rIL-1β (0.1 μg, 1 μg, 10 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, gill tissues were aseptically taken from killed fish, 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.

Reverse transcription-Polymerase Chain Reaction (RT-PCR)

Tissue samples were homogenized in RNAzol (Biogenesis, CS-104, UK) using a glass homogeniser on ice, and total RNA was extracted

Table 1. Primers for specific PCR

Target genes	Direction	Oligonucleotide sequences
β-action	Forward	5′-ATG GAA GAT GAA ATC GCC-3′
	Reverse	5′-CAT GGA GAA GAT CTG GCA-3′
MX-3	Forward	5′-ATG CCA CCC TAC AGG AGA TGA T-3′
	Reverse	5′-CCA CAG TGT ACA TTT AGT TG-3′
IRF-1	Forward	5′-AGG CTG TCT GTG CTG TCT ACT AT-3′
	Reverse	5′-AAT AAC TTC CCG CTC CAT CT-3′

Table 2. The cycling protocol for PCR

Gene	Objects	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
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

and used for the reverse transcription by the method reported previously(Hong *et al.*, 2001). The resulting cDNA was dissolved in DEPC-treated water and stored at -20℃. To detect the expression of immune genes after injection, PCR for the amplification of β-actin, MX-3 and IRF-1 genes was carried out using a pair of primer sets and cycling protocols as summarized in Table 1 and 2. PCR reactions were performed in 25 μℓ reactions containing 5 μℓ of cDNA (diluted in water), 1.25 μℓ (25 pmol) of each primer, 2.5 μℓ of 10X reaction buffer (160 mM $(NH4)_2SO_4$, 670 mM Tris-HCl, 0.1 % Tween-20, pH 8.8), 0.5 μℓ dNTP mixture (2.5 mM for each base), 1.25 μℓ of $MgCl_2$ (50 mM) and 0.125 μℓ (0.625 U) of *Taq* polymerase using a Techne thermocycler (Genius, UK).

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 μℓ) to give constant product yield. The same amount of cDNA was then used for all subsequent PCR reactions as a way of normalising the data in order to give a more quantitative result.

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 (UVP, USA) and UVP Gel-works ID advanced software (UVP, USA). The relative ratios of target gene product to β-actin product were calculated for each of the two genes (MX-3 and IRF-1) and used to quantify inter-group differences in expression levels.

Statistical analysis

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

Results and Discussion

In the present study, the biological effects of rIL-1β on antiviral genes (IRF-1 and MX-3) were analyzed using a semi-quantitative RT-PCR method. RT-PCR is known to be a powerful technique to quantify the mRNA expression of genes such as cytokines, which are often expressed at very low level. Various methodologies on (semi-) quantitative PCR and RT-PCR have been described for measuring cytokine mRNA level (O'Garra and Vieira, 1992; Gilliland *et al.*, 1990; Kanangat *et al.*, 1992; Overbergh *et al.*, 1999).

As shown in Fig. 1 and 3, the expressions of both MX-3 and IRF-1 genes were observed even in control fish, suggesting that these genes are constitutively expressed in the gill tissue of rainbow trout, *Oncorhynchus mykiss*. This result is coincided with the report that these genes are constitutively expressed in gill, head kidney, liver and spleen of rainbow trout (Collet *et al.*, 2002).

It is interesting that the expression of MX-3 and IRF-1 gene was significantly decreased in 24 h after injection with rIL-1β at the doses of 1 and 10 μg (MX-3) or 0.1 μg (IRF-1) ($p<0.05$, by Mann-Whitney test) (Fig. 1 & 3), indicating that these genes were down-regulated by rIL-1β in the early stage of stimulation. This is not coincided with the result reported by Collet *et al.* (2002), showing that both genes are up-regulated in the gill within 24 h after polyinosinic:polycytidylic acid (poly I:C) treatment, interpreting that different stimulants regulate the expression of these genes by different mechanisms. Poly I:C might directly stimulate IFN secretion which induce the up-regulation of MX-3 and IRF-1 while rIL-1β initially stimulates other genes involving in the initial host response.

In biological system, the differential effect of a stimulant on the gene expression is consistent with the requirement of the host undergoing an exogenous or endogenous challenge to boost its natural defences while at the same time to conserve energy by suppressing those constitutively expressed genes not needed for the response (Dinarello, 1996). For example, Warfel *et al.* (1986) reported that LPS treatment of resident mouse peritoneal macrophages suppressed intracellular lysozyme as well as secreted lysozyme. The down-regulation of lysozyme by LPS was abolished within 24 h after removal of exogenous LPS, when normal or even higher lysozyme levels were reached in the peritoneal macrophages, and these levels were maintained for several days. These observations support the concept that activation of inflammatory cells

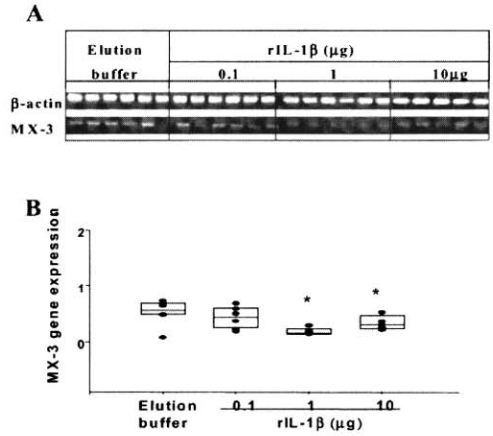

Fig. 1. Effect of rIL-1β on MX-3 gene expression in the gill at day 1 post-injection. A. Patterns of RT-PCR on MX-3 gene. Fish was injected with elution buffer as a negative control, or rIL-1β (0.1, 1, and 10 μg) 24 h before RNA extraction from the gill. B. Levels of MX-3 gene expression from densitometric-scanned values. The box plot represents the median and 95 % confidence intervals for each group while dots show the individual fish data in each group.
* : significant difference from the negative control group in the Mann-Whitney test.

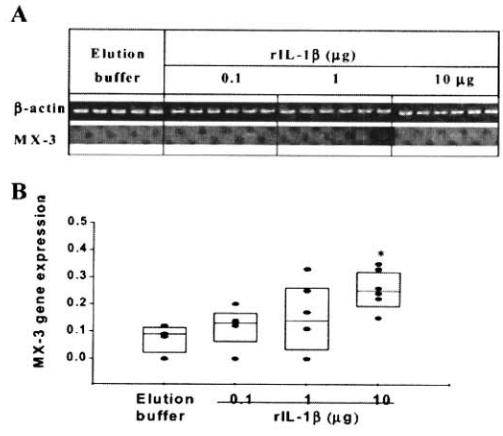

Fig. 2. Effect of rIL-1β on MX-3 gene expression in gill at day 3 post-injection. A. Patterns of RT-PCR on MX-3 gene. Fish was injected with elution buffer as a negative control, or rIL-1β (0.1, 1, and 10 μg) 72 h before RNA extraction from the gill. B. Levels of MX-3 gene expression from densitometric-scanned values. The box plot represents the median and 95 % confidence intervals for each group while dots show the individual fish data in each group.
* : significant difference from the negative control group in the Mann-Whitney test.

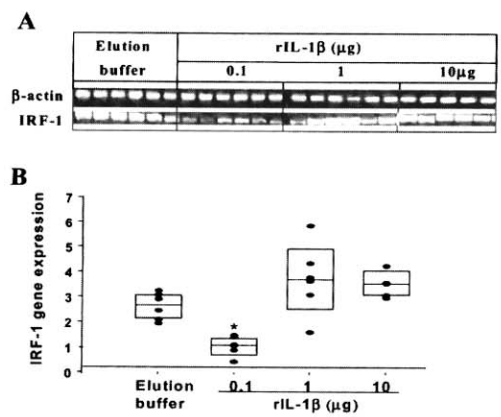

Fig. 3. Effect of rIL-1β on IRF-1 gene expression in gill at day 1 post-injection. A. Patterns of RT-PCR on IRF-1 gene. Fish was injected with elution buffer as a negative control, or rIL-1β (0.1, 1, and 10 μg) 24 h before RNA extraction from the gill. B. Levels of IRF-1 gene expression from densitometric-scanned values. The box plot represents the median and 95 % confidence intervals for each group while dots show the individual fish data in each group.
* : significant difference from the negative control group in the Mann-Whitney test.

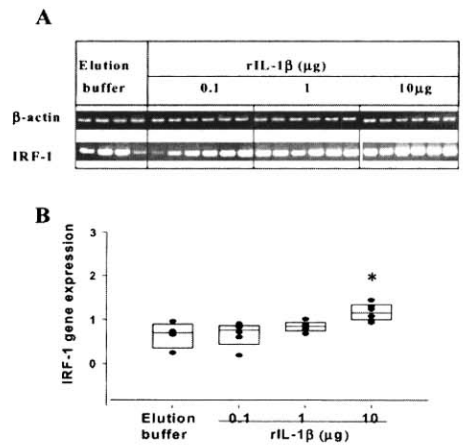

Fig. 4. Effect of rIL-1β on IRF-1 gene expression in gill at day 3 post-injection. A. Patterns of RT-PCR on IRF-1 gene. Fish was injected with elution buffer as a negative control, or rIL-1β (0.1, 1, and 10 μg) 72 h before RNA extraction from the gill. B. Levels of IRF-1 gene expression from densitometric-scanned values. The box plot represents the median and 95 % confidence intervals for each group while dots show the individual fish data in each group.
* : significant difference from the negative control group in the Mann-Whitney test.

may actually be concomitant with the down-regulation of some constitutive functions, which are not needed for initial inflammatory events and can be applicable for the understanding of the results in this study.

It is known that the fish MX genes are up-regulated by a synthetic double-stranded RNA (ds RNA) poly I:C which is a well-known inducer of IFNα/β in higher vertebrates (Robertsen *et al.*, 1997; Trobridge *et al.*, 1997b). Collet *et al.*(2003) have also demonstrated up-regulation of the expression of MX-3 and IRF-1 by polyI:C treatment in rainbow trout; however, it was not clear if IL-1β can stimulate these antiviral gene expression in trout. In the present study, we showed that there was a significant stimulatory effect on the expression of MX-3 gene induced by 10 μg rIL-1β at day 3 post-injection in the gill ($P<0.05$, by Mann-Whitney test)(Fig. 2) after an inhibitory effect of 1 and 10 μg rIL-1β at day 1. This is in agreement with the result reported by Nygaard *et al.* (2000), who demonstrated that the peak expression of MX protein in macrophages occurred in 48 h after induction by poly I:C whereas peak IFN production was observed by 24 h after addition of poly I:C, suggesting that poly I:C may induce the expression of MX protein indirectly through the induction of IFN α/β. Thus, it is suggested that up-regulation of MX-3 gene might have been caused by IFN induced by rIL-1β as if IL-1β is a known inducer of IFN in mammals (Dinarello, 1996).

The IRF-1 gene expression was also significantly increased in gill by 10 μg rIL-1β at day 1 (Fig.3) and at day 3 (Fig. 4) even though 0.1 mg rIL-1β significantly reduced IRF-1 gene expression at day 1. IRF-1 is known to be expressed at low levels or be undetectable in a variety of cell types. However, its expression is inducible by a broader range of stimulants such as viral infection, ssRNA, polyI:C, both Type I and II IFN as well as other cytokines, and activators such as tumor necrosis facto (TNF), IL-1, IL-6, leukaemia inhibitory factor (LIF), concanavalin A (ConA), calcium ionophore A23187 and phorbol 12-myristate 13-acetate (PMA) (Harada *et al.*, 1989; Fujita *et al.*, 1989b; Abdollahi *et al.*, 1991).

Even though trout rIL-1β has a stimulatory effect on the expression of both MX-3 and IRF-3 genes, the expression of IRF-1 and MX-3 genes were delayed and increased. From these results, it is postulated that the stimulatory effect of rIL-1β on the expression of antiviral genes might be indirect. Davidson *et al.* (1999) also reported such delayed up-regulation in a 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 up-regulation after 24 h. Similarly, delayed stimulation of lysozyme production was also reported in Atlantic salmon head kidney macrophages grown in the presence of yeast β-glucan and LPS (Paulsen *et al.*, 2001), and explained by indirect activation of transcription of lysozyme gene. That is, given stimulations first induced the differentiation of the macrophages in culture, then eventually resulting in direct activation of transcription of the lysozyme gene.

In conclusion, rIL-1β administered intrapenitoneally showed a postulated and an indirect stimulating effect on the expression of antiviral genes (MX-3 and IRF-1) followed by IL-1β gene induction since stimulatory effect on these gene expression was detected at day 3 in the gill whilst IL-1β gene expression was induced earlier as detected at day 1 post-injection (Hong *et al.*, 2003). This result could be an indirect evidence that trout rIL-1β might be able to induce IFNs as MX-3 is known to be a sensitive marker for type I IFN induction.

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