The expression of immune-regulatory genes in rainbow trout, *Oncorhynchus mykiss*, during a natural outbreak of proliferative kidney disease (PKD)

J. W. HOLLAND[1], C. R. W. GOULD[2], C. S. JONES[1], L. R. NOBLE[1] *and* C. J. SECOMBES[1]*

[1] *Scottish Fish Immunology Research Centre, School of Biological Sciences, University of Aberdeen, Zoology Building, Tillydrone Avenue, Aberdeen, AB24 2TZ, UK*
[2] *Schering-Plough Aquaculture, Aquaculture Centre, Saffron Walden, Essex, CB10 1EJ, UK*

SUMMARY

Proliferative kidney disease (PKD) is a parasitic infection of salmonid fish characterized by an apparently abnormal immune response to the presence of the myxozoan parasite, *Tetracapsuloides bryosalmonae*. In order to examine the nature of the immune response at the molecular level, the expression of a range of immune regulatory genes, including cytokines and cyclooxygenase (COX)-2 was examined in naive unexposed fish and in naive fish exposed to parasite-infected water at three points during the course of a natural outbreak of PKD. Since fish with advanced PKD pathology generally exhibit increased susceptibility to secondary infections which is typical of stress/cortisol-mediated immune suppression, a further aim of this work was to examine *in vitro* the influence of the glucocorticoid cortisol on the bacterial lipopolysaccharide (LPS)-induced expression of the trout cytokine genes studied. Two weeks after the initial sampling, naive exposed fish showed a specific profile of up-regulated tumor necrosis factor (TNF)-α2, COX-2 and, to a lesser extent, transforming growth factor (TGF)-β1 expression. As the disease pathology increased, TNF-α2 and COX-2 expression returned to normal levels. Stress levels of cortisol suppressed the LPS inducibility of pro-inflammatory cytokine genes, although TGF-β1 and TNF-α2 appeared to be refractory. These data demonstrate that specific immune responses at the molecular level are affected during PKD infection, with the cortisol suppression of cytokine expression *in vitro* providing a possible link to PKD-mediated cytokine down-regulation and immune suppression.

Key words: Rainbow trout, cytokine, expression, PKD, cortisol, stress.

INTRODUCTION

Mammalian parasitic infection models are typically characterized by mutually inhibitory T helper cell (Th)1 or Th2 cytokine profiles, the nature of which depends on the class of parasite in question. The Th1 pro-inflammatory cytokines, interferon (IFN)-γ and interleukin (IL)-2, along with the monocyte/macrophage-derived cytokines, IL-12, TNF-α and, in some cases, IL-1β, control cellular immune responses to parasite infections (Sher & Coffman, 1992; Laurent *et al*. 2001). The anti-inflammatory Th2 cytokines, IL-4, IL-10 and IL-13 promote humoral immune responses during infection (DiPiro, 1997; Jankovic, Sher & Yap, 2001).

The immune/inflammatory stress associated with parasitic infection stimulates the neuroendocrine system leading to the activation of the hypothalamic-pituitary-adrenal (HPA) stress axis. The subsequent release of immune suppressive glucocorticoids promotes the switching of Th1 responses to Th2, thus facilitating control over the ensuing pro-inflammatory response (Elenkov & Chrousos, 1999; Sapolsky, Romero & Munck, 2000). Further control over Th1 processes is provided by the expression of the anti-inflammatory cytokine, TGF-β1 and immune regulatory prostaglandins generated by COX-2 (Herschman, 1996; Letterio & Roberts, 1998). However, chronic inflammation associated with persistent infection may lead to dysregulation between the immune and neuroendocrine systems causing an imbalance in Th1/Th2 homeostasis which can lead to a dysfunctional Th1 response and increased disease susceptibility (Elenkov & Chrousos, 1999).

Proliferative kidney disease in fish is now known to be due to infection with the spores of *Tetracapsuloides bryosalmonae* (Canning *et al*. 2002), formerly *Tetracapsula bryosalmonae*, a myxozoan organism derived from parasitized bryozoan hosts (Canning *et al*. 1999; Feist *et al*. 2001). The characteristic kidney pathology observed during the development of the disease has been attributed to lymphocyte hyperplasia, formation of granulomatous lesions and renal atrophy (Foott & Hedrick, 1987; Chilmonczyk, Monge & De Kinkelin, 2002). Clinical PKD has been recently shown to suppress key elements of the innate immune response in rainbow trout resulting in immune suppression and increased susceptibility to secondary infections (Chilmonczyk *et al*. 2002).

Enormous progress has been made in recent years in the sequencing and characterization of fish

* Corresponding author. Tel: +44-1224-272872. Fax: +44-1224-272396. E-mail: c.secombes@abdn.ac.uk

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cytokine genes, including the production and bioactivity testing of the corresponding recombinant proteins (Hong *et al*. 2001; Secombes *et al*. 2001; Holland, Pottinger & Secombes, 2002). The most studied fish cytokine gene to date, IL-1β, has been shown to be induced by bacterial LPS and recombinant IL-1β *in vitro* and by bacterial infection *in vivo* (Zou, Cunningham & Secombes, 1999*a*; Zou *et al*. 2000). In addition, both cortisol and psychological stress have been found to suppress the LPS-induced expression of the trout IL-1β gene (Zou *et al*. 2000; Holland *et al*. unpublished). Although a recent study reported the induction of trout cytokine genes in macrophages experimentally infected with *Renibacterium salmoninarum* (Grayson *et al*. 2002), no study to date has examined the *in vivo* expression profiles of genes encoding fish cytokines during the natural course of a specific disease condition.

In order to gain further insight into the nature of the immune response to *T. bryosalmonae*, the present study was undertaken to examine the expression profiles of known trout cytokine genes and the immune-regulatory gene, COX-2 during a natural outbreak of PKD. Additional *in vitro* studies were conducted to further examine the influence of stress levels of cortisol on the expression of fish cytokine genes in head kidney leucocytes incubated in the presence of bacterial LPS.

MATERIALS AND METHODS

Sampling and monitoring of fish exposed to a natural outbreak of PKD infection

The kinetics of the gene expression of major pro-inflammatory cytokines and related molecules were examined in kidney samples from trout exposed to a natural outbreak of PKD. Two groups of fish from the same egg source (*ca*. 50–100 g each) were sampled. Firstly, a naive control group from a farm with no history of PKD infection (Group 1) and secondly, a group that consisted of naive fish exposed to parasite-infected water (Group 2). Fish from group 1 were sampled once at the beginning of the sampling regime, whereas group 2 fish were sampled on three dates during the course of the infection. A suitable time to start the sampling of naive parasite-exposed fish (26-06-01) was determined by the appearance of low numbers of *T. bryosalmonae* cells in kidney smears. Subsequent samplings were carried out on the 10-07-01 and 01-08-01.

In all fish examined, approximately 100 mg of kidney tissue were removed from the area of the posterior kidney immediately below the dorsal fin, an area of the kidney normally associated with the onset of pathology (Gould, 1995). Tissue samples were placed into 1 ml of Trizol reagent (Invitrogen) and stored at -80 °C prior to the extraction of total RNA. As a means of assessing the severity or level of clinical pathology, each fish examined was assigned a kidney swelling index (from 0 to 4) using a system devised previously (Clifton-Hadley, Bucke & Richards, 1987; Gould, 1995). By the time sampling of the naive exposed group was underway, fish were beginning to exhibit low-level disease pathology (kidney swelling index; 0–1). By the third sampling time (01-08-01), kidney swelling indices had reached values of 3–4, indicative of advanced/severe PKD pathology. Therefore, kidney tissue taken from all 3 sampling points covered the full range of pathology normally encountered during a PKD outbreak. As a matter of farm routine, checks for other parasite infestations were conducted throughout the PKD season. With respect to the kidney itself, swabs were taken under asceptic conditions at the time of sampling and streaked onto standard TSA plates (Becton-Dickinson) as a means of checking for the presence of opportunistic bacterial pathogens (e.g. *R. salmoninarum* and *Aeromonas salmonicida*). Streaked plates were incubated at 20–22 °C for 48 h and examined for any bacterial growth.

Incubation of isolated head kidney leucocytes in the presence of LPS and/or cortisol

Mixed populations of trout head kidney leucocytes were obtained as described previously (Zou *et al*. 2000). Briefly, head kidney tissue was disrupted through 100 μm nylon mesh under asceptic conditions. After two washes in L-15 medium, cells were resuspended in L-15 containing 100 units/ml penicillin, 100 μg/ml streptomycin and 1·5% foetal calf serum (Gibco BRL.). Cell suspensions from three individuals were pooled and seeded to a density of 2×10^7 cells/ml prior to a 1 h pre-incubation with cortisol (150 or 300 ng/ml, Sigma) or vehicle only (0·03% ethanol) at 22 °C. The cells were then stimulated for a further 3 h with *Escherichia coli* 0127 : B8 LPS (Sigma) at a concentration of 10 μg/ml to induce gene expression of those genes not expressed constitutively. To ensure the duration of cell stimulation was maintained across all conditions, the additions of cortisol/vehicle and LPS were staggered appropriately. At the end of the LPS incubation period, cell culture dishes from each condition, in turn, were decanted and the culture medium retained. Adherent cells were harvested into 1 ml of Trizol reagent (Invitrogen, The Netherlands) per sample, whilst non adherent cells present in the culture medium were pelleted and combined with the appropriate Trizol sample following the removal of the culture supernatant. All samples were stored at -80 °C prior to the extraction of total RNA.

RNA extraction and RT-PCR analysis

Total RNA was purified from tissue samples stored in Trizol reagent according to the manufacturer's

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Table 1. Primers used to amplify rainbow trout immune-regulatory genes

Gene/primer name	Size of PCR product (bp)	Primer sequence (5′–3′)	Accession no.
β-actin-F	254	ATGGAAGATGAAATCGCC	AF157514
β-actin-R		TGCCAGATCTTCTCCATG	
IL-1β1-F8	462	CGAATTCTCTGAGAACAAGTGCTG	AJ223954
IL-1β1-R3		CTTAGTTGTGGCGCTGGATGGTG	
IL-1β2-F4	320	ACTACAAAACAGCCAACTACAAACC	AJ245925
IL-1β2-R8		CTCTGCTGCTGGCTTCAGT	
IL-8-F3	226	GGATGTCAGCCAGCCTTGTC	AJ310565
IL-8-R3		TCCAGACAAATCTCCTGACCG	
TNF-α1-EF2	241	CAAGAGTTTGAACCTCATTCAG	AJ277604
TNF-α1-ER2		TTTCATCCTGCATGGTTGACG	
TNF-α2-EF3	260	CAAGAGTTTGAACCTTGTTCAA	AJ401377
TNF-α2-ER5$^+$		TTCCGTCCTGCATCGTTGCCA	
TGF-β1-F5	356	GAAGAAACGACAAACCACTAC	X99303
TGF-β1-R8		GACATGTGCAGTAATTCTAGC	
COX-2-F	476	CCTGGAGCACAGCAGAGAC	AJ238307
COX-2-R		GAACACCTGGGTTCATGTCACT	

instructions. With respect to the *in vivo* experiment, thawed kidney tissues present in Trizol were maintained on ice and broken up by sonication (4×30 s bursts). The purity and quantity of the resulting RNA was determined spectrophotometrically by measuring the optical density at 260/280 nm. Five μg total RNA were reverse transcribed using Bioscript reverse transcriptase (Bioline) as described previously (Zou *et al*. 2000). The resulting cDNA was adjusted to a total volume of 70 μl with PCR-grade water (Sigma) and stored at $-20\,^{\circ}$C prior to PCR analysis.

Amplification was performed on ice in 25 μl reactions under standard PCR conditions (Zou *et al*. 2000). For both *in vitro* and *in vivo* expression analysis, the cycling number was optimized for each gene examined to ensure that the PCR reaction was in the linear phase of amplification, thus allowing any differences in gene expression levels between individual fish to be easily detected. At each sampling point, amplified fragments of the housekeeping β-actin gene from cDNA representing 8 parasite-exposed fish were compared to β-actin signals from cDNA representing 8 naive control fish. The β-actin signals in both groups were titrated by adjusting the volume of cDNA, thus ensuring that any differences in the expression of immune genes would not be due to differences in cDNA loading. Specific primers were used to examine the expression of a range of trout cytokine genes and a proinflammatory gene, namely IL-1β1, IL-1β2, IL-8, TNF-α1, TNF-α2, TGF-β1 and COX-2. All primer sets were designed so that amplification covered at least 1 intron, thus ensuring that cDNA products would be distinct from any potential genomic DNA contamination (Table 1). Control reactions were set up by substituting PCR water for cDNA template in order to account for any potential false positives due to contamination from pipettes, airborne plasmid or from primer dimer effects.

The relative levels of cDNA were visualized on 2·5% agarose gels post stained with ethidium bromide (0·25 μg/ml) and the size of each PCR product was verified using 1 kb DNA ladder (Bioline). Quantification of PCR products was achieved by densitometric scanning using a UVP gel imaging system and UVP Gelworks ID advanced software. Since all PCRs undertaken were normalized with respect to β-actin, the relative level of gene expression was subsequently expressed in terms of pixel density for each gene examined.

Statistical analysis

The Kruskal–Wallis non-parametric test was used on data from naive fish displaying PKD infection in order to examine whether there was an overall significant effect of swelling index on the median and spread of pixel density values for genes exhibiting up-regulated expression. Further non-parametric analysis was performed using the Mann–Whitney test at 95% confidence.

RESULTS

Gene expression in kidney tissue from naive fish exhibiting PKD pathology

With the cycling regime employed, PCR analysis revealed up-regulated, albeit variable expression profiles for COX-2, TNF-α2 and TGF-β1 in posterior kidney tissue samples taken from naive parasite-exposed fish sacrificed on the second sampling date, 10-07-01, relative to the naive unexposed control group, in which kidney pathology was generally found to exhibit a swelling index of 2. Out of 8 fish

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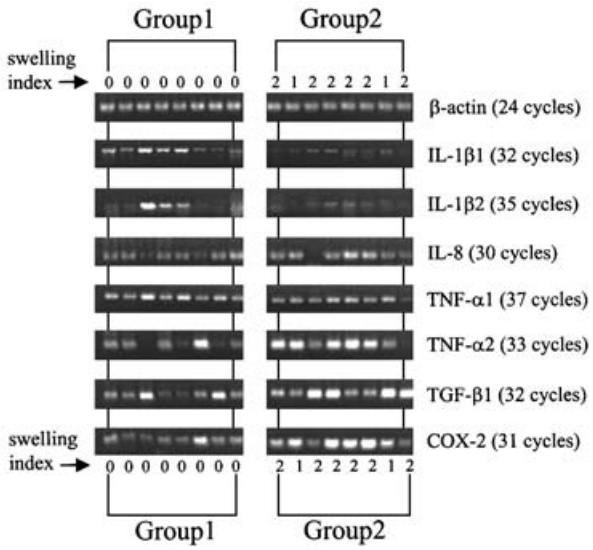

Fig. 1. Expression of immune-regulatory genes in kidney tissue samples from naive control fish (group 1) and naive parasite-exposed fish (group 2) sampled during the clinical stages of infection (10-07-01). Total RNA was extracted and reverse transcribed. cDNA templates from 8 fish per group were titrated by reference to the β-actin gene prior to the amplification of immune-regulatory genes by PCR. Swelling indices for each fish per group are illustrated. The number of PCR cycles required to optimize each gene are indicated in brackets.

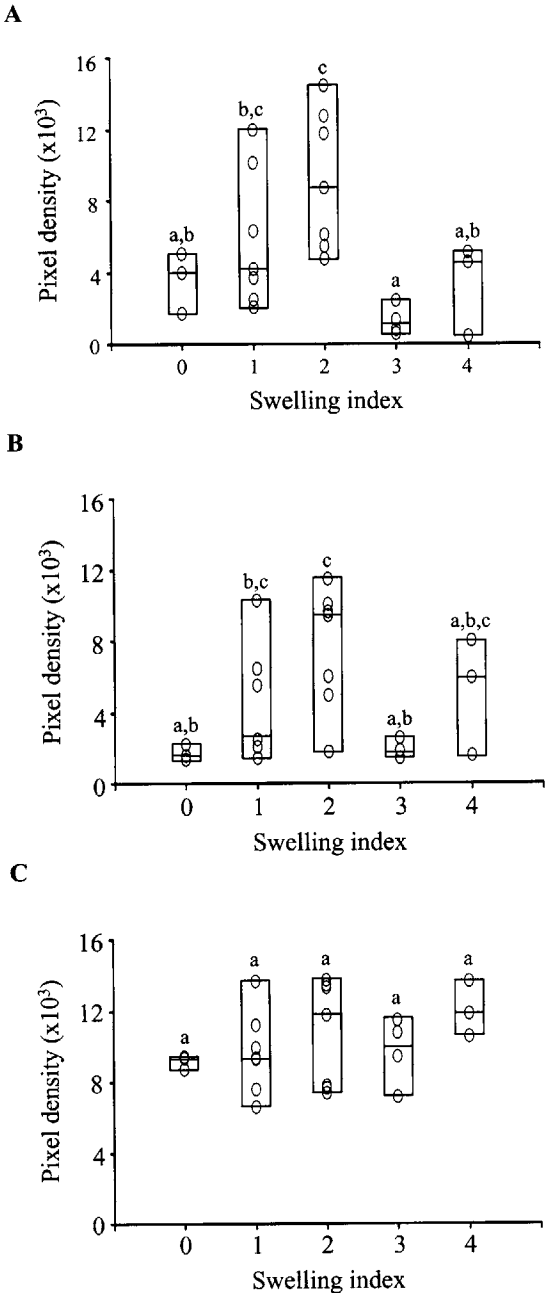

Fig. 2. Boxplots illustrating the median and spread of pixel density values, against swelling index, of bands detected following PCR with specific primers for (A) COX-2, (B) TNF-α2 and (C) TGF-β1 using cDNAs generated from the sampling of naive parasite-exposed fish. Eight fish were sampled and assigned swelling indices on the 26-06-01, 10-07-01 and 01-08-01 respectively. Significant differences between swelling indices are denoted by boxes with no letters (a,b,c) in common ($P<0{\cdot}05$).

examined 5 expressed strong up-regulation of COX-2 and TNF-α2, whereas 4 expressed up-regulated TGF-β1 expression (Fig. 1). In contrast, there was no such up-regulation in COX-2, TNF-α2 and TGF-β1 expression in kidney tissue taken from naive parasite-exposed fish on the first (26-06-01; swelling index; 0–1) and last sampling points (01-08-01; swelling index; 3–4) (results not shown). Agar plates prepared from kidney swabs did not reveal the presence of other microbial pathogens which suggests that the observed gene up-regulation is due to the presence of *T. bryosalmonae*. At all 3 sampling times, variable constitutive expression was evident in naive controls for all genes examined.

Pixel densities from COX-2, TNF-α2 and TGF-β1 PCRs were compiled from all 3 sampling times and plotted against the corresponding swelling index (Fig. 2). In the case of COX-2, there was an overall significant effect ($P=0{\cdot}012$) of swelling index on the median and spread of pixel density values. Further analysis revealed significant differences between the following swelling indices: 0 and 2; 1 and 3; 2 and 3; and 2 and 4 ($P<0{\cdot}05$). There was no overall significant effect of swelling index versus the spread of pixel densities ($P=0{\cdot}056$) from the TNF-α2 data. However, significant differences were noted between swelling indices, 0 and 2 and 2 and 3 ($P<0{\cdot}05$). No significant effects between any groups were observed following TGF-β1 analysis. As to the remaining genes (IL-1β1, IL-1β2, IL-8 and TNF-α1), there was no evidence of modulated expression attributed to the kidney pathology found in fish at all 3 sampling times, with IL-1β1 and IL-1β2 gene expression in naive parasite-exposed fish being barely detectable in all PCR profiles examined.

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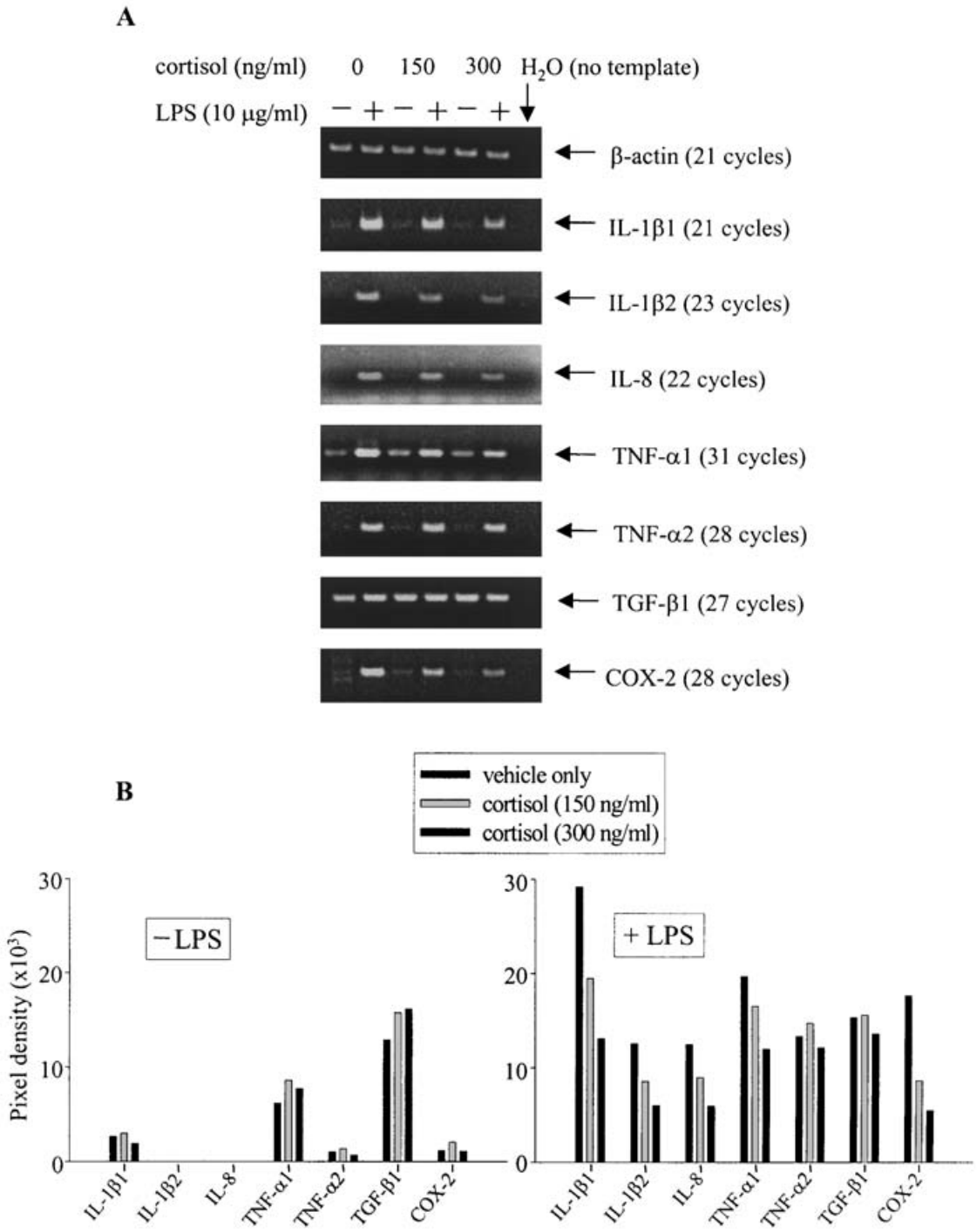

Fig. 3. The impact of cortisol on the LPS-induced expression of immune-regulatory genes in head kidney leucocytes *in vitro*. Cells pooled from three individual trout were incubated with or without exogenous cortisol (150 or 300 ng/ml) for a 1 h pre-incubation followed by a further 3 h incubation in the presence or absence of 10 μg/ml LPS at 22 °C. Following β-actin titration, immune-regulatory genes were amplified by PCR (A). Pixel density values of bands detected in the presence (+) and absence (−) of LPS were plotted against the respective gene amplified by PCR (B).

Cortisol-mediated suppression of LPS-induced gene expression in vitro

Generally, gene expression was higher *in vitro* than *in vivo* with cycling numbers varying from 21–31 *in vitro* (Fig. 3) in comparison to 24–37 *in vivo* (Fig. 1). The exogenous administration of cortisol *in vitro* at concentrations akin to acute stress levels *in vivo* (100–300 ng/ml), suppressed the LPS induction of IL-1β1. In addition, specific primers for the second isoform of IL-1β (IL-1β2), IL-8, TNF-α1 and COX-2 revealed a similar trend of suppressed LPS inducibility in a mixed population of head kidney leucocytes (Fig. 3). Interestingly,

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however, TNF-α2 induction did not appear to be sensitive to cortisol-mediated down regulation, whereas TGF-β1 was apparently refractory to both cortisol and LPS treatment. No constitutive expression was observed following IL-1β2 and IL-8 PCRs, with very low levels detected in the case of IL-1β1, TNF-α2 and COX-2. Conversely, relatively high constitutive expression was observed in TNF-α1 and particularly TGF-β1 PCRs.

DISCUSSION

Molecular examination of the kidney pathology associated with PKD revealed up-regulated expression of TNF-α2, COX-2 and, to a lesser extent, TGF-β1, but not IL-1β1, IL-β2, IL-8 and TNF-α1. The variable gene expression profiles observed in the current study may be explained by either kinetic differences between individual immune genes during clinical PKD or may simply illustrate the relative lack of involvement of certain genes in this disease. In support of the latter, recent data from LPS-induced IL-1β, IL-8 and TNF-α expression in trout leucocytes suggests that these cytokines follow similar expression profiles (Zou *et al*. 2000; Laing *et al*. 2002; Zou *et al*. 2002). Similarly, TNF-α and IL-1β are concomitantly expressed in carp HK leucocytes exposed to the parasite, *Trypanoplasma borreli* (Saejj *et al*. 2003). Furthermore, bacterial challenges *in vivo*, have shown concomitant up-regulation of IL-1β and COX-2 in trout tissues (Zou *et al*. 1999*a*, *b*). Early inflammatory events in mammals are typically characterized by the expression of TNF-α and IL-1β,which in turn induces down stream genes including COX-2 (Vilcek & Lee, 1991; Herschman, 1996). It is, therefore, likely that the absence of up-regulation of certain genes in PKD-infected fish may illustrate the lack of direct involvement of these genes rather than differences in the dynamics of gene expression.

Mammalian parasitic infections frequently result in T cell responses involving a polarization towards either Th1 or Th2 cytokine profiles. IL-1β, although important in various disease phenotypes (Dinarello, 1997), does not appear to have a major role in the control of parasitic infections characterized by a dominant lymphocytic response (Sher & Coffman, 1992). Macrophage recruitment/proliferation during protozoan infections, on the other hand, involves the up-regulation of IL-1β (Laurent *et al*. 2001). Similarly, *in vitro* infection with *R. salmoninarum*, the causative agent of bacterial kidney disease (BKD) in trout, elicits dominant macrophage proliferation and up-regulation of the IL-1β, TNF-α and COX-2 genes (Wiens & Kaattari, 1999; Grayson *et al*. 2002). Therefore, it seems that the cytokine profiles elicited by infection depend on the nature of the pathogen and the dominant immune cells involved in the host response. Recent examination of the hyperplastic response to PKD infection in trout demonstrated the dominance of *in situ* lymphocyte proliferation over macrophage populations (Chilmonczyk *et al*. 2002). These observations may, to some extent, allow an explanation for the specific nature of the cytokine profiles in the present study, although Th1 and Th2 cytokine genes (currently not available to fish immunologists) may play a major role in the pathology of this disease. Nevertheless, since the genes examined in the present study have been well characterized in mammals, analysis of their expression during PKD may corroborate recent observations at the cellular level. The chemokine, IL-8 attracts T cells and macrophages to the site of parasitic infection (Laurent *et al*. 1997; Mukaida, Harada & Matsushima, 1998). The lack of up-regulation of IL-8 in this study supports the premise that the host response to PKD is due to an aberrant proliferation of leucocytes rather than through leucocyte infiltration. COX-2 and TGF-β, expressed primarily by antigen presenting cells, play a central role in the control of general inflammatory responses, in that they regulate the balance between protection offered by pro-inflammatory mediators and over-expression of such mediators during parasite exposure (Herschman, 1996; Ramaswamy, Kumar & He, 2000; Omer, Kurtzhals & Riley, 2000). However, the precise nature of the expression of these genes during PKD remains to be determined. TNF-α, expressed by B and T cells as well as macrophages, plays a key role in the lymphocytic responses to parasite infection (Goldfield & Tsai, 1996). The clear difference in the expression profiles of TNF-α1 and TNF-α2 in fish exhibiting PKD pathology is intriguing. Although, only one copy of the gene exists in mammals, a single polymorphism in the TNF-α promoter is believed to convey differential susceptibility to parasitic disease (McGuire *et al*. 1994; Goldfield & Tsai, 1996). Similarly, the isoforms of trout TNF-α exhibit marked differences in both the promoter region and the 3′ UTR, which are likely to account for the observed differences during PKD infection.

A further interesting aspect of the current study is the apparent concomitant down-regulation of COX-2 and TNF-α2 during advanced pathology. In mammals, down regulation of pro-inflammatory mediators is generally associated with switching from Th1 to Th2 immune responses. Dysregulation towards Th2 may account for the recently observed suppression of innate immune responses and increased susceptibility to secondary infections in PKD-infected trout (Chilmonczyk *et al*. 2002). This is further corroborated by evidence of enhanced humoral immunity during clinical PKD, phenomena indicative of Th2 dominance (Olesen & Jorgensen, 1986; Saulnier & De Kinkelin, 1996).

As with Th1/Th2 processes, the suppression of innate immune responses and increased disease susceptibility are classical signs of dysregulation between the immune and neuroendocrine systems and the release of immune suppressive glucocorticoids (Elenkov & Chrousos, 1999; Sapolsky *et al*. 2000). Indeed, recent studies in fish have demonstrated that cortisol is a key factor involved in stress-associated increased susceptibility to parasite infection (Harris, Soleng & Bakke, 2000; Davis, Griffon & Gray, 2002). Glucocorticoids in mammals are known to bias immune responses towards Th2 processes via the induction of anti-inflammatory cytokines, whilst hindering the expression of pro-inflammatory cytokines (Sapolsky *et al*. 2000). Therefore, stress levels of cortisol attributed to advanced disease pathology and compromised kidney function may account for the down-regulation of COX-2 and TNF-α2 as the kidney swelling index increases. However, an assessment of plasma cortisol levels in fish infected with PKD along with the examination of the effects of parasite exposure on cytokine expression *in vitro* are currently confounded by the inability to generate sufficient quantities of pure parasite material. Therefore, an analogous *in vitro* model demonstrating the impact of cortisol on the LPS-up-regulated expression of immune genes was undertaken. To date, *in vitro* cortisol studies in rainbow trout have demonstrated the dose dependent suppression of LPS-induced IL-1β gene expression by Northern blot analysis (Zou *et al*. 2000). Data from the current study not only support these earlier observations but also extend the influence of cortisol-induced suppression to a host of other pro-inflammatory cytokine genes, including COX-2. In contrast, TGF-β1 appears to be refractory towards LPS and cortisol, which may be due to the highly cell-type specific nature of this cytokine (Letterio & Roberts, 1998). Interestingly, the difference between TNF-α1 and TNF-α2 may again reflect the marked differences in their promoter and 3′ UTR regions. As for TNF-α2 expression in PKD infected fish, neuroendocrine mechanisms other than glucocorticoid-induced immune suppression may account for the observed down-regulation.

In conclusion, with only a relatively small number of fish cytokines sequenced, the present study begins to illustrate the specific nature of PKD infection at the molecular level. However, considering the specificity of cytokine expression in mammalian parasitic diseases, further elucidation of the immune processes associated with PKD infection is highly dependent on the cloning of new fish cytokine genes. Furthermore, since cytokine transcriptional control can be dissociated from post-transcriptional regulation during infection (Dinarello, 1997; Letterio & Roberts, 1998), future progress is also dependent on the development of assays to detect cytokine expression at the protein level.

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