



Molecular cloning and expression analysis of carp (*Cyprinus carpio*) interleukin-1 β , high affinity immunoglobulin E Fc receptor γ subunit and serum amyloid A

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Suppression subtractive hybridisation (SSH) is a powerful means to identify genes of cytokines and other genes that express small amount of mRNA. In this study, cDNA of normal fish (carp) head kidney cells (HKC) was subtracted from pooled cDNA of HKC and peritoneal cell (PC) obtained from fish which had been injected with sodium alginate (SA) and scleroglucan (SG) 3–48 h earlier. This subtraction produced 248 clones of cDNA fragments. After sequencing some of the fragments of interest were used as probes, and yielded full-length cDNAs homologous to mammalian interleukin-1 β (IL-1 β), the γ subunit of high affinity Fc receptor for IgE (Fc ϵ RI γ) and serum amyloid A (SAA); these were cloned and sequenced. Carp IL-1 β shows 21.8–24.7% amino acid identities to mammalian mature IL-1 β , and lacks a signal sequence, which is consistent with mammalian IL-1 β . Carp Fc ϵ RI γ , which was the first cloned non-mammalian Fc receptor subunit, shows 39.3–40.4% amino acid identities to mammalian Fc ϵ RI γ , and contains the immunoreceptor tyrosin-based activation motif characteristic of the signal transduction subunit of antigen- and Fc-receptors. Carp SAA is most similar to mammalian acute phase responsive type SAA with 53.0–55.3% amino acid identities. Both SA-elicited and SG-elicited PC expressed higher amounts of IL-1 β and SAA mRNA compared to saline-injected fish HKC and PC, indicating that these proteins are associated with inflammatory responses, similar to mammalian homologues. Fc ϵ RI γ was constitutively expressed in leucocytes and not immunopotentiator-responsive, but this indicates that Fc receptor including Fc ϵ RI γ subunit is likely functional in the carp immune system.

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Key words: carp, IL-1 β , Fc ϵ RI γ , Serum amyloid A, suppression subtractive hybridisation, inflammation.

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The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases, with the following accession numbers AU052025–AU052112, AB010701, AB013381 and AB016524.

The accession numbers of sequences retrieved in this study are as follows: rainbow trout IL-1 β , AJ223954; human (hu) IL-1 β , P01584, mouse (mo) IL-1 β , P10749; pig IL-1 β , P26889; sheep IL-1 β , P21621; huIL-1 α , P01583; moIL-1 α , P01582; pig IL-1 α , P18430; sheep IL-1 α , Q28579; huIL-1RA, P18510; moIL-1RA, P25085; pig IL-1RA, Q29056; bovine IL-1RA, O77482; huFc ϵ RI γ , P30273; moFc ϵ RI γ , P20491; rat Fc ϵ RI γ , P20411; guinea pig Fc ϵ RI γ , Q07249; rainbow trout SAA, CAA67766; huSAA1, P02737; huSAA2, 2144881; huSAA3, P22614; huSAA4, P35542; moSAA1, P05366; moSAA2, P05367; moSAA3, P04918; moSAA4, P31532; carp 40S ribosomal protein S11, AB012087.

I. Introduction

In teleosts, molecular cloning of the major immune-related components including immunoglobulins (Pilström & Bengtén, 1996; Wilson *et al.*, 1997), complement components (Sunyer & Lambris, 1998), T cell receptors (Partula *et al.*, 1995, 1996; Wilson *et al.*, 1998) and major histocompatibility complex molecules (Dixon *et al.*, 1995) has been progressively increasing. Compared to these components, most of the genes encoding proteins which mediate inflammatory responses of leucocytes, particularly cytokines, are still not identified in spite of their importance in the teleost immune system.

Using suppression subtractive hybridisation (SSH), we recently performed cDNA subtraction between carp leucocytes collected before (head kidney) and after (peritoneal cells) dynamic migration elicited by sodium alginate (Fujiki *et al.*, 1999). We identified a variety of immune-related genes including those encoding cytokines and cytokine receptors. This confirmed the high efficiency of SSH in this system. The SSH-generated sequences included those for genes encoding leucocyte migration-associated proteins, reflecting the selection of tester (to be subtracted) and driver (to subtract) cells. Hence changes of stimulants and a combination of tester and driver cells would lead to identification of other novel genes.

In the present work, we modified the previous SSH by using scleroglucan (SG) to synergise with the immunostimulating effect of SA, and the stimulated fish head kidney cells (HKC) were added to the peritoneal cells (PC) for use as tester cells. SG is known to be an effective immunopotentiator in teleosts as well as in mammals (Yano *et al.*, 1991). SG elicits less influx of leucocytes to the peritoneum than does SA, but the responding cells have higher phagocytic activities than those of SA-elicited PC; SG also increases phagocytic activity of head kidney leucocytes (unpublished data). By means of this SSH we have identified 19 putative immune-functional gene fragments. In this paper we report the full-length cDNAs homologous to mammalian interleukin-1 β , high affinity immunoglobulin E Fc receptor γ subunit, and serum amyloid A.

II. Materials and Methods

FISH

Common carp *Cyprinus carpio* (L.) were purchased from a fish farm in Kumamoto Prefecture, Japan, and acclimated for 2 weeks to laboratory conditions at 22°C in a 60 l aquarium containing recirculating well water. During acclimation, fish were fed with commercial pellets.

PREPARATION OF THE TESTER AND DRIVER CELLS

The tester (to be subtracted) cells were prepared from a mixture of HKC and PC from carp injected with both SA and SG. Briefly, 15 fish (27–40 g) were given an intraperitoneal injection of a 0.85% NaCl solution (saline) containing both 0.4% SA and 0.2% SG in a dose of 2 mg SA and 1 mg SG 100 g body weight⁻¹. At 3, 6, 12, 24 and 48 h after the injection, HKC and PC were harvested from three of the fish as previously described (Fujiki & Yano, 1997);

the HKC and PC were separately pooled. The pooled HKC (9×10^7 leucocytes) and PC (9×10^7 leucocytes) were mixed together and used for poly(A)⁺RNA preparations.

The driver (to subtract) cells were prepared from the head kidney of a single nontreated fish (950 g) as described previously (Fujiki & Yano, 1997).

cDNA SYNTHESIS AND SUBTRACTIVE HYBRIDISATION

Total RNA was extracted from the driver and the tester cell suspensions using ISOGEN LS (Nippon Gene, Toyama, Japan) according to the manufacturer's protocol. Poly(A)⁺RNA (2 μ g) isolated with Oligotex-dT30 <Super> (TaKaRa, Shiga, Japan) were reverse-transcribed to cDNA and subjected to subtractive hybridisation and selective PCR amplification, using PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, U.S.A.) according to the manufacturer's protocol.

CLONING AND SEQUENCING OF THE SUBTRACTED cDNA FRAGMENTS

The cDNA fragments amplified after the subtractive hybridisation were subcloned into pBluescript vector (Stratagene, La Jolla, CA, U.S.A.) and sequenced using dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kits and an Applied Biosystems 377 sequencer (Perkin-Elmer, Norwalk, CT, U.S.A.). The nucleotide sequences were analysed by the BLASTX search (Gish & States, 1993) against the SWISS-PROT protein database.

CLONING AND SEQUENCING OF THE FULL-LENGTH SUBTRACTED cDNA

The ZAP Express (Stratagene) cDNA library constructed from the tester cell poly(A)⁺RNA was transferred to Hybond N membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) and hybridised at 60°C for 16 h with digoxigenin (DIG)-labelled cDNA probes prepared from the SSH fragments of interest, using PCR DIG Probe Synthesis Kit (Boehringer-Mannheim, Mannheim, Germany). The hybridised clones, detected using alkaline phosphatase-conjugated anti-DIG, X-phosphate and NBT, were plaque-purified and subcloned into pBK-CMV by *in vivo* excision according to the manufacturer's protocol (Stratagene). The nucleotide sequence was determined at least once for both strands.

SEMIQUANTITATIVE REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION ASSAY

A group of five carp were intraperitoneally injected with saline (0.5 ml 100 g⁻¹), SA (2 mg 100 g⁻¹) or SG (1 mg 100 g⁻¹), and HKC and PC were collected 48 h later. Their total RNA prepared as described above was reverse-transcribed with Molony mouse leukemia virus (MMLV) reverse transcriptase (RT) using the Advantage RT-for-PCR Kit (Clontech). The synthesized first strand cDNA was diluted 10-fold in H₂O. PCR was conducted in 20 μ l which contained 1 μ l of the diluted first strand cDNA, 1 μ l each of PCR primers and 17 μ l of PCR master mixture provided by Nippon Gene. PCR was

performed in a T3-Thermocycler (Biometra, Göttingen, Germany), using the following parameters: 95° C, 5 min; 20–33 cycles at (95° C for 30 s, 55° C for 30 s, 72° C for 1 min) and a final extension at 72° C for 5 min. Oligonucleotides used as PCR primers are as follows: carp 40S ribosomal protein S11 (433 bp) sense primer 5'-TACAGAACGAGAGGGCTTATC (13–33), antisense primer 5'-TTG GTGACCTTCAGGACATTG (425–445); carp IL-1 β (574 bp) sense primer 5'-ACC ACTGGATTGTGTCAGAAG (76–96), antisense primer 5'-ACAGGGGAAGAACC ATCTAAC (629–649); carp Fc ϵ RI γ (370 bp) sense primer 5'-TCCCTGTGT-C TGTCATTTTCAG (20–40), antisense primer 5'-CCCTGCTATAGTTTGCTTCCT (369–389); carp SAA (352 bp) sense primer 5'-CTTATTCTTGCTGTGCTGGTG (22–42), antisense primer 5'-GAAGGCCCTCGGTCTGTAGC (353–373). The numbers represent the nucleotide numbers of each cDNA sequence submitted to GenBank. An aliquot (5 μ l) of the PCR product was electrophoresed on a 1.5% agarose gel containing 0.5 μ g ml⁻¹ of ethidium bromide and photographed on a UV illuminator.

III. Results

SSH-GENERATED cDNA FRAGMENTS

The SSH applied to the cDNA from the SA-, SG-costimulated and the non-stimulated fish generated about 700 clones in total. Of these, sequencing of 248 randomly selected clones gave 108 distinct sequences with significant similarity to known proteins, determined by *E*-values (Gish & States, 1993) lower than the threshold (1×10^{-5}). These sequences were deposited in GenBank with accession numbers AU052025–AU052112. Among these clones were 19 cytokine and other immune-related genes (Table 1). Clone c-44 (306 bp) showed similarity to human fibroblast growth factor 4 in the BLASTX search with *E*-value of 0.079. A clone c-109 (357 bp) showed high similarities (*E*-values *c.* 7.2×10^{-17}) to mammalian γ subunit of high affinity Fc receptor for immunoglobulin E (Fc ϵ RI γ). A clone c-307 (368 bp) showed extremely high similarities (*E*-values *c.* 2.4×10^{-52}) to mammalian serum amyloid A (SAA).

CLONING OF THE FULL-LENGTH cDNA SIMILAR TO INTERLEUKIN-1 β

Though SSH fragment c-44 has relatively lower *E*-value (0.079) to fibroblast growth factor 4, we cloned the full-length cDNA (C-44) corresponding to the c-44 because it showed similarity to a cytokine and since we had an interest in cytokines we wanted to further investigate this clone. The clone C-44 is 1231 bp-long including 5' and 3' untranslated regions and contains the entire coding region of 276 aa (Fig. 1). The 3' untranslated region also contains a single consensus mRNA destabilising sequence UUAUUUA(U/A)(U/A) (Lagnado *et al.*, 1994). Though c-44 is similar to fibroblast growth factor 4, the overall aa sequence (C-44) shows higher similarities to mammalian interleukin-1 (IL-1 β) with *E*-values of *c.* 9.0×10^{-13} . The aa sequence shows higher identities to IL-1 β (21.8–24.7%) than to IL-1 α (17.4–21.7%) and

Table 1. Results of BLASTX searches with *Rsa* I-digested fragments of sodium alginate- and scleroglucan-injected carp head kidney and peritoneal cell cDNA after subtraction by normal fish head kidney cDNA.

Clone*	Similar to	Species	Accession number
c-14	epidermal differentiation-specific protein	newt	AU052029
c-38	IFN-inducible protein 1-8U	human	AU052032
c-44	fibroblast growth factor-4	human	AB010701†
c-61	<i>Ras</i> -related protein Rab-14	rat	AU052039
c-87	fibrillin 2	human	AU052046
c-105	cathepsin S	human	AU052048
c-109	high affinity IgE Fc receptor γ subunit	guinea pig	AB013381†
c-120	Gi1 protein α -1 subunit	human	AU052051
c-136	CD18	human	AB013074†
c-191	microfibril-associated glycoprotein 4	human	AU052071
c-212	P22 phagocyte B-cytochrome	pig	AU052078
c-249	54 kD progesterone receptor-associated immunophilin	human	AU052084
c-263	RHO GDP-dissociation inhibitor 2	mouse	AU052087
c-279	proteasome component C13	rat	AU052090
c-307	serum amyloid A	wallaby	AB016524†
c-309	L-plastin	mouse	AU052092
c-328	c-Myc binding protein MM-1	human	AU052094
c-349	cathepsin D	chicken	AU052098
c-357	immunophilin FKBP12	bovine	AU052103

*Clones which showed *E*-values below 1×10^{-5} in BLASTX search results and are related to cytokines or other components of the immune system are listed, except for c-44 which showed *E*-value of 0.079. †Accession number of the corresponding full-length cDNA clone.

IL-1RA (16.6–17.6%) especially when the mature proteins are compared to the corresponding region of the C-44 protein (Fig. 2). In addition, C-44 lacks a signal sequence like mammalian IL-1 β (Fig. 2). On the basis of these results, C-44 was assigned as a putative carp homologue of IL-1 β . Amino acid (aa) identities of mammalian IL-1 β to carp IL-1 β are almost the same as those to rainbow trout IL-1 β (data not shown). Carp IL-1 β shares only 22.3% overall aa homology with rainbow trout IL-1 β (Fig. 2).

CLONING OF THE FULL-LENGTH cDNA SIMILAR TO FC ϵ RI γ CHAIN

The full-length cDNA named C-109 is 540 bp long including 5' and 3' untranslated regions and encodes a 89 aa sequence (Fig. 3a). The deduced aa sequence shows 39.3–40.4% identities to mammalian Fc ϵ RI γ and shows lower identities (18.2–18.8%) to mammalian CD3 ζ which is derived from a common ancestral gene with Fc ϵ RI γ (Wegener *et al.*, 1992) (Table 2). As shown in (Fig. 3b), C-109 contains a single immunoreceptor tyrosin-based activation motif (ITAM) which is a characteristic of transmembrane signal pathway subunits of T- and B-cell receptors and Fc receptors (Isakov, 1997), and is found as a single and as triple motifs in mammalian Fc ϵ RI γ and CD3 ζ , respectively. From these results, we concluded that C-109 is a carp homologue of Fc ϵ RI γ .

-53	GAACAACACAGCTCAGCAATTCAGAGAGTGAACGCACCAACACAGAACGATGGCATGCCATGAATATGTACACAGCTGGATTGTGAGAA	42
	M A C H E Y V H Q L D L S E	14
	GCATTCGAGACAGACAGTCAATTTACTCTGACAGTCCGCACTCTGATGAACGGATGCCAGATCCACAATCCATGAGTGGCAATGCGACATG	138
	A F E T D S A I Y S D S A D S D E L D C P D P Q S M S C Q C D M	46
	CATGACATCAAACTGGAGCTGTCTTCGCATCTCAGAGCATGAGGAGGTGGTGAACATCATCATTGCTGTGGAAGACTGAAGCACATCAAAAAAT	234
	H D I K L E L S S H P H S M R Q V V N I I I A V E R L K H I K N	78
	ATGCTCTTGGCAAGTTTTGTGATGAGGAGCTGCTCGGCTTCATCTTGGAGAATGTGATCGAAGAGCGTCTGGTGAAGCCGCTTAATGAAACACCA	330
	M S S G K F C D E E L L G F I L E N V I E E R L V K P L N E T P	110
	ATTTACAGTAAGACCGCTGACCTTGACGTGCACATTTGTGATAAGTACAAAAAGACTATGGTGAGAGCAACAACTAAGTGACGAGCCTCTT	426
	I Y S K T S L T L Q C T I C D K Y K K T M V Q S N K L S D E P L	142
	CATTTGAAGGCTGTGACGCTGAGTGTGGAGCAATGCAATCAAAAGTTCAATTCAGTATGTCAACATTCGTGTGAGTGCAACACAAAAGGAAGCA	522
	H L K A V T L S A G A M Q Y K V Q F S M S T F V S A T Q K E A	174
	CAGCCTGTGTGGCTGGGAATTTCCAACAGCAACCTCTACCTTGCTGTACCCAGTTAGATGGTCTTCCCTGTCTGATCTTGAAGGAGGCCAGT	618
	Q P V C L G I S N S N L Y L A C T Q L D G S S P V L I L K E A S	206
	GGCTCTGTGAACACCATTAAGCCGGTGACCCGAATGACAGCCTCTCTTCTTTCAGGAAAGAACCGGCACAGTTTCAACACTTTTGAATCTGTT	714
	G S V N T I K A G D P N D S L L F R K E T R Y N T F E S V	238
	AAATATCTGGATGGTTTATTAGCACAGCTTTTGTGATTGGGAGAAGGTGGAAATGAACAGATGCCAACACCCGACCAACAACTTACACTT	810
	K Y P G W F I S T A F D D W E K V E M N Q M P T T R T T N F T L	270
	GAGGATCAAAAACGTATCTAGTTAAGAAATCAGATTTGATCCAGAAACATATTTCCAGAGAGTATGACTATGTACAGTTTATAATCTGGATTATT	906
	E D Q K R I *	276
	CAGCGTTACAAACCTGTATGAGTTGATGTATCGACAGTTTTTTTGTGTTGTTATTGTTCTGTCTATTATATGTGTAAGGTTTGTGTTGGGATTG	1002
	GTAATTCATGTTGAATCTTTTAAGTACGAACATTTGCCTATGTGCTTATTATAATTTTAAATTTTAAATTTTAAATTTTAAATTTTAAATTTTAAAT	1098
	CAGACTTATTTTACACTGCAATGATTATTTTATTTTATTTTAAATAAACAAGACATGGATGTTAAAAA	1178

Fig. 1. Nucleotide and deduced amino acid sequences of the clone C-44. Nucleotide and amino acid numbers, starting from the putative methionine initiation codon, are presented on the right side. Asterisk indicates termination codon. The putative polyadenylation signal is underlined. Consensus mRNA destabilising motif UUAUUUA(U/A)(U/A) is boxed.

CLONING OF THE FULL-LENGTH cDNA SIMILAR TO SERUM AMYLOID A

The full-length cDNA clone C-307 contains 621 bp including 5' and 3' untranslated regions and encodes 123 aa (Fig. 4a). Though the complete sequence of rainbow trout SAA is not available (Jensen *et al.*, 1997), carp C-307 shows 64.4% aa identity within the overlapping region of rainbow trout SAA. C-307 shows 53.0–55.3% aa identities to human SAA1, SAA2 and mouse SAA1, SAA2, SAA3 all of which are acute phase type SAAs, while C-307 shows 41.7% aa identity with human SAA4 which is constitutively expressed (Fig. 4b). Human SAA3 and mouse SAA4 are pseudogenes (Steel & Whitehead, 1994). These results indicate that C-307 may be a carp homologue of mammalian acute phase type SAA.

CHANGES IN THE MRNA EXPRESSIONS OF IL-1 β , FC ϵ RI γ AND SAA AFTER SA- AND SG-INJECTIONS

RT-PCR was used to evaluate expression of IL-1 β , FC ϵ RI γ and SAA mRNA in HKC and PC 48 h after saline-, SA- and SG-injections. The number of PC in the absence of injection was approximately 5×10^5 cells fish⁻¹, and saline injection did not elicit an influx of PC. In contrast, SA- and SG-injections yielded 9×10^7 and 2×10^7 cells fish⁻¹, respectively. As shown in Fig. 5, expression of ribosomal protein S11 mRNA was nearly constant in all experimental samples, indicating that the total RNA concentrations were

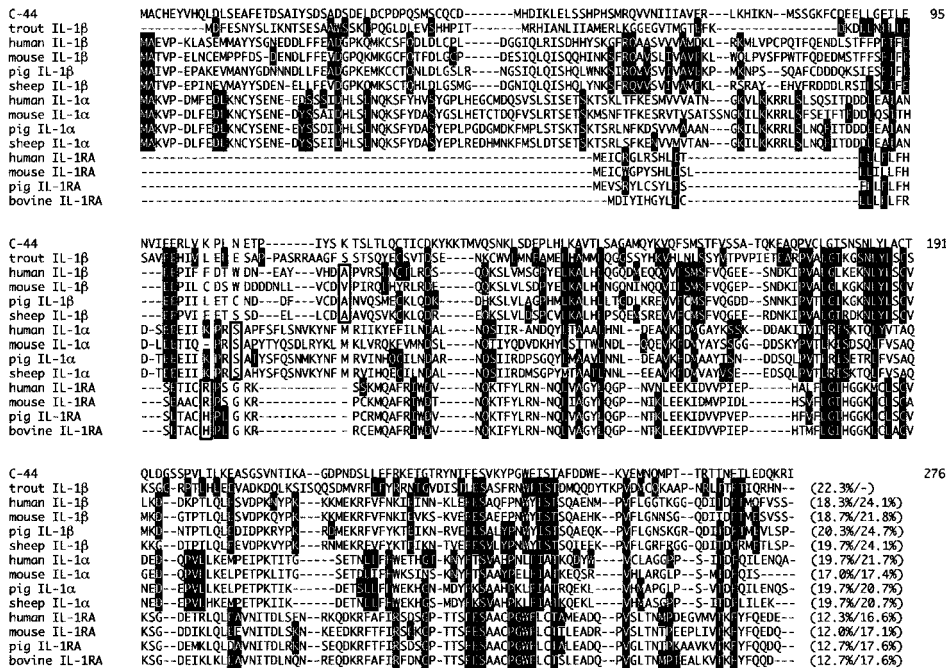


Fig. 2. Alignment of the deduced amino acid sequence of clone C-44 with rainbow trout IL-1 β and mammalian IL-1 α , IL-1 β and IL-1 receptor antagonist (IL-1RA). The numbers presented on the right side are amino acid numbers of clone C-44. Amino acid residues identical with the C-44 sequence are shaded. Dashes indicate gaps introduced for optimal alignment. Amino terminals of the mature forms of the mammalian IL-1 α , IL-1 β and IL-1RA are boxed. The percentages in parenthesis indicate the amino acid identities of overall sequence (left) and mature protein (right).

almost the same among the samples. In HKC, a steady state level of IL-1 β expression was observed in saline-injected control fish, and IL-1 β expression was not significantly changed by SA- or SG-injections. IL-1 β expressions in both SA- and SG-elicited PC were higher than that in the saline-injected fish HKC. Fc ϵ RI γ expression was constitutive in control head kidney cells, and it was not affected by the injection of either SA or SG in HKC and PC. SAA expression in control HKC was either not present or was undetectable. The expression was increased after SA-injection and SG-injection. The levels of this transcript were much higher in PC of SA-injected and SG-injected fish than those in HKC. There was no significant difference between the effectiveness of SA and SG on the SAA expression. A variation of the expression of IL-1 β , Fc ϵ RI γ and SAA between individuals in the case of saline-injected fish peritoneal cells is probably due to a lack of uniformity in the populations of the component cells which are considered to be identical to resident peritoneal cells. The resident peritoneal cells are not well characterised in carp. Fish to fish variation after stimulation may be due to differences in genetic background because the fish used were not inbred.

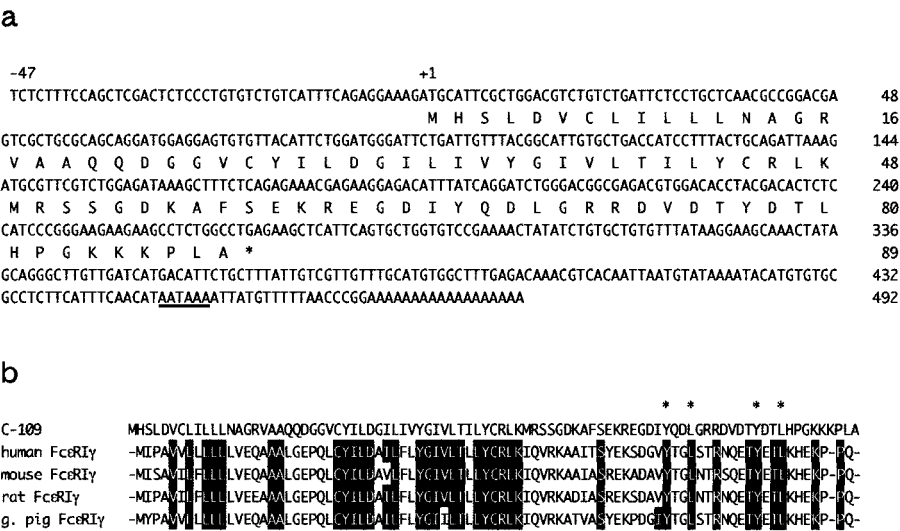


Fig. 3. (a) Nucleotide and deduced amino acid sequences of the clone C-109. Nucleotide and amino acid numbers, starting from the putative methionine initiation codon, are presented on the right side. Asterisk indicates termination codon. The putative polyadenylation signal is underlined. (b) Alignment of the deduced amino acid sequence of clone C-109 with human, mouse, rat and guinea pig γ subunit of high affinity IgE Fc receptor (Fc ϵ RI γ). Identical amino acid residues with the C-109 sequence are shaded. Dashes indicate gaps introduced for optimal alignment. Asterisks indicate the immunoreceptor tyrosine-based activation motif (ITAM), twice-repeated Y-X-X-L(I) sequence flanking six to eight residues.

Table 2. Identities of the deduced amino acid sequence of clone C-109 with mammalian high affinity immunoglobulin E Fc receptor γ subunit (Fc ϵ RI γ) and T cell receptor ζ chain (CD3 ζ)

Clone	Identity (%)*					
	human FcεRI γ	mouse FcεRI γ	rat FcεRI γ	guinea pig FcεRI γ	human CD3 ζ	mouse CD3 ζ
C-109	40.4	39.3	40.4	40.4	18.2	18.8

*The identities were calculated on the basis of the multiple sequence alignment in which clone C-109 was aligned separately with mammalian Fc ϵ RI γ and CD3 ζ by clustal W.

IV. Discussion

IL-1 is the prototypic multifunctional cytokine. The IL-1 gene family is comprised of IL-1 α , IL-1 β and IL-1 receptor antagonist, all of which are considered to have been diverged from the common ancestor. Neither IL-1 α nor IL-1 β contain signal peptides. In contrast to IL-1 α which shows full biological activity in the precursor form, IL-1 β needs cleavage by IL-1 β converting enzyme (ICE) to exert biological activities (Dinarello, 1998). IL-1 β cDNA was first cloned from mouse in 1984 (Lomedico *et al.*, 1984) and subsequently other mammalian homologues have been cloned (Auron *et al.*,

a

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-15      +1
GCCAGCTCTGTAATCATGAAGCTTATTCTGCTGCTGCTGGTCTGGCGCTGGTTTTGGTGGTCGAGACTCAGGCTCAATGGCACCCTACCCAGGACAA 84
      M K L I L A V L V L A L V L V V E T Q A Q W H R Y P G Q 28
GCCATTGGAGGTGCAAGGACATGTGGCTGCTTACCGGATATGAGGAAGGCCCACTGGAAGGCCGAGACAAGTATTTCCATGCACGTGGGAACATAT 183
A I G G A K D M W R A Y R D M R K A N W K G A D K Y F H A R G N Y 61
GATGCTGCAAGAGGGGCCCCGAGGCAGATGGCGAGCCAAAGTAATCAGTAATGGAAGAGAGGCTCTGCAGGGAATCTCTCGCCGCGGTAAATTCAGAT 282
D A A R R G P G G R W A A K V I S N G R E A L Q G L S R R G N S D 94
GCTGCAGCAGACCAGCCGCAATCGCTGGGGACGTAACCGTGGTAACCCCAACCGCTACAGACCAGGGGGCCTTCCCAAAAGTACTGAAAAGAAGAG 381
A A A D Q A A N R W G R N G G N P N R Y R P R G L P K K Y * 115
CATCACTGCAACAGAAATGACTGAAATCATTTTCTCTAGAACTGCTTATTCAATTTTGCATAAAACCATTTACGCTGTTGTTTTTTCAGTCTTGA 480
CAATTATCTTGCATTAATCTGCACATTATGACAAAATGCAATCTTTTAAGTTTAGCATCATTTAGCCTATGTAACACACTCTGGTTTGAAATAAGGA 579
AGTAACCTCAAAAAAAAAAAAAAAAAAAAA 606

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b

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C-307      MKLILAVLVLAVLVVETQAQNHRYPGQAIIGGAKDMWRAYRDMRKANWKGADKYFHARGNYDAARRGPGGRWAQKVIS 78
trout SAA      -----M L I L V V G A A Q N H R Y P G Q A I I G G A K D M W R A Y R D M R K A N W K G A D K Y F H A R G N Y D A A R R G P G G R W A A K V I S
human SAA1      MKL LTGLVFCSLVIGVSSR-SFFSFLGAEFDGARDMWRAYSDMREANYIGSDKYFHARGNYDAARRGPGGWAQKVIS
human SAA2      MKL LTGLVFCSLVIGVSSR-SFFSFLGAEFDGARDMWRAYSDMREANYIGSDKYFHARGNYDAARRGPGGWAQKVIS
human SAA3      MKL STGIIFCSLVIGVSSG-GILTLFKANGQAKMWRAYSDMREANYIGSDKYFHARGNYDAARRGPGGWAQKVIS
human SAA4      YRLFTGIVFCSLVIGVTSSE-SRVSFFKEALQGVGAGRAYWIMISLHONSRLYARCNYYDAARRGPGGWAQKVIS
mouse SAA1      MKL LTSLVFCSLVIGVCHG-GFFSFLGAEFDGARDMWRAYSDMREANYIGSDKYFHARGNYDAARRGPGGWAQKVIS
mouse SAA2      MKL LTSLVFCSLVIGVCHG-GFFSFLGAEFDGARDMWRAYSDMREANYIGSDKYFHARGNYDAARRGPGGWAQKVIS
mouse SAA3      MKPSTATILCITLIGVDSQ-RVQVFMKEAGGSRDMWRAYSDMRKANWNSDKYFHARGNYDAARRGPGGWAQKVIS
mouse SAA4      YRLATVIVLCSLVIGVSGD-GIYSFFREIVQITLWLRAYRDNLEANYQNALQYTHARCNYDAARRGPGGWAQKVIS

C-307      NGREALQ-----GLSRRGNSDAADQAANRWGRNGGNPNRYRPRGLPKKY- 123
trout SAA      NCREMID-----GNGEGHESAAADKANWGRNGGDPNRRFPDCLPKKY- (64.4%)
human SAA1      DARENID-----RFFGHGAESLADQAAAEHRSCKDPNHRPAELPKKY- (53.0%)
human SAA2      DARENID-----RITGHGAESLADQAAAGCRSDRDPNHRPAELPKKY- (55.3%)
human SAA3      DARENVC-----RITGDHAEESLADQATNGHGSCKDPNHRPAELPKKY- (50.8%)
human SAA4      RSRVYLQGLIDYYLFNSSTVLESKSNKEEWEERSCKDPRFRPDCLPKKY- (41.7%)
mouse SAA1      DGRFAFE-----EFFGRCHETIADQEAHRSCKDPNYYRPPCLPKKY- (53.8%)
mouse SAA2      DARESFQ-----EFFGRCHETIADQEAHRSCKDPNYYRPPCLPKKY- (52.3%)
mouse SAA3      DAREAVG-----KFTGHGAESRADQFAHWEERSCKDPNHRPAELPKRY- (55.3%)
mouse SAA4      TSKRYFGLLNRYYFGIRNHLETLOATKKEEWEERSCKDIPNHRPEGLPERF- (43.2%)

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Fig. 4. (a) Nucleotide and deduced amino acid sequences of the clone C-307. Nucleotide and amino acid numbers, starting from the putative methionine initiation codon, are presented on the right side. Asterisk indicates termination codon. The putative polyadenylation signal is underlined. (b) Alignment of the deduced amino acid sequence of clone C-307 with rainbow trout, human and mouse serum amyloid A (SAA). Identical amino acid residues with the C-307 sequence are shaded. Dashes indicate gaps introduced for optimal alignment. The percentages in parenthesis indicate the overall amino acid identities.

1984; March *et al.*, 1985; Gray *et al.*, 1986; Mori *et al.*, 1988; Maliszewski *et al.*, 1988; Nishida *et al.*, 1989; Fiskerstrand & Sargan, 1990; Andrews *et al.*, 1991; Huether *et al.*, 1993). Molecular aspects of the IL-1 β homologues from non-mammalian species were not understood until the rainbow trout IL-1 β was recently cloned (Secombes *et al.*, 1998). Like rainbow trout IL-1 β , carp IL-1 β also has no aa sequence corresponding to the ICE-cleavage site, after Asp¹¹⁶ which is conserved in other mammalian homologues. The existence of an ICE homologue in teleosts and whether teleost IL-1 β needs enzymatic cleavage to become biologically active remains to be seen. Since it has been shown that extracellular proteases including trypsin, elastase, chymotrypsin, a mast cell

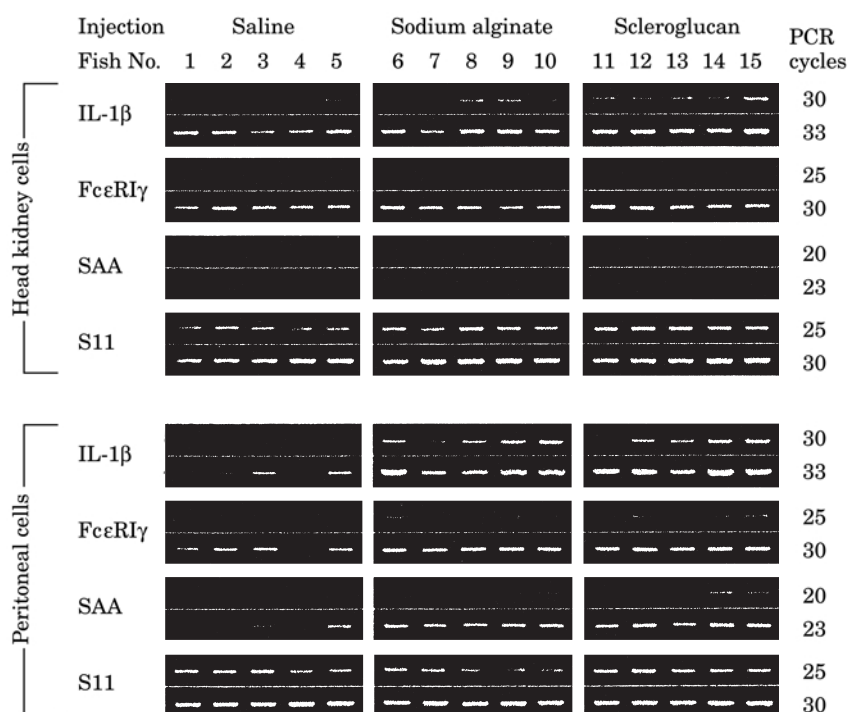


Fig. 5. RT-PCR analysed mRNA expressions of carp IL-1 β , Fc ϵ RI γ and SAA in the head kidney and peritoneal cells of fish injected with saline, sodium alginate or scleroglucan 48 h earlier. For each mRNA, PCR amplification was performed using two numbers of cycles to show that at least under the lower numbers of cycles amplification did not reach the plateau. The fish numbers indicate the individual fish. Injections were given intraperitoneally at 2 mg 100 g⁻¹ (sodium alginate) and 1 mg 100 g⁻¹ (scleroglucan). S11 indicates 40S ribosomal protein S11 used to confirm equal total RNA concentrations.

chymase and granzyme A can also cleave precursor IL-1 β to produce biologically active IL-1 β in mammals (Dinarello, 1998), carp IL-1 β may be activated by cleavage by an enzyme distinct from an ICE homologue. Though carp IL-1 β and rainbow trout IL-1 β show almost the same amino acid identities to mammalian IL-1 β , the identity between these two fish IL-1 β s is low. This is probably because these two families of fish diverged from their common ancestor 150 million years ago. Also both species have undergone tetraploidisation, therefore there has been ample opportunity for duplicated homologues to diverge. A phylogenetic analysis using carp and trout IL-1 β s and mammalian IL-1 superfamily amino acid sequences did not provide sufficient information to hypothesise a common ancestral gene (data not shown).

In mammals, increased expression of IL-1 β by SA and SG has not been reported, though bacterial poly-D-mannuronate that is structurally similar to a mannuronate block in alginate, and the β -1,3-glucan lentinan, which has a similar molecular structure to SG, have been shown to increase IL-1 β expression (Fruehauf *et al.*, 1982; Arinaga *et al.*, 1992; Halaas *et al.*, 1997). In

the present study, IL-1 β expression was higher in SA- and SG-injected fish PC than in saline-injected fish HKC. As the numbers of PC were small in the non-treated and saline-treated fish, most of the PC in the SA- and SG-injected fish were probably composed of leucocytes mobilised from the head kidney, a haemopoietic/lymphoid tissue (Zapata *et al.*, 1996) and a major source of PC in fish (Gruca *et al.*, 1996). In addition, since HKC IL-1 β expression was not significantly affected by these immunopotentiators, the effects on PC may be indirect or affected by other factors present only in the peritoneum. In any case, IL-1 β expression is up-regulated in association with inflammatory leucocyte migration elicited by SA and SG.

In teleostean fish, IgM and chimeric IgD have been cloned (Pilström & Bengtén, 1996; Wilson *et al.*, 1997), however no Fc receptor has been cloned. Carp Fc ϵ RI γ is the first cloned fish Fc receptor cDNA. In mammals, the existence of specific Fc receptors for five classes of immunoglobulins (Fc α R, Fc δ R, Fc ϵ R, Fc γ R and Fc μ R) has been recognised, and Fc α R, Fc γ R and Fc ϵ R have been cloned (Gessner *et al.*, 1998). Fc μ R was identified in mouse at the protein level (Ohno *et al.*, 1990), but extensive study has not been done thereafter. Fc δ R is less characterised. The mammalian Fc ϵ RI γ chain is present in not only the high affinity Fc receptor for IgE (Fc ϵ RI), but also in the low affinity Fc receptor for IgG (Fc γ RIIA). It appears reasonable to suggest that Fc ϵ RI γ may also be shared by Fc μ R and Fc δ R. Thus the existence of a Fc ϵ RI γ homolog in carp may be taken to imply that teleost Fc μ R or Fc δ R includes the Fc ϵ RI γ chain. Identification of subunits of the each Fc receptor in addition to Fc ϵ RI γ would be highly interesting because it will lead to a fuller understanding of the phylogeny of Fc receptors, and Fc receptor function in teleosts.

Like C-reactive protein and serum amyloid P, SAA is one of the major acute phase proteins. The SAA superfamily consists of multiple genes. In humans, SAA1 and SAA2 are acute phase reactants, SAA3 is a pseudogene and SAA4 is a constitutively expressed gene, whereas murine SAA1–3 are acute phase type and SAA4 is a pseudogene (Steel & Whitehead, 1994). Acute phase type SAAs are produced by mainly hepatocytes, but murine SAA3 (Meek *et al.*, 1992) and human SAA-1 and SAA-2 (Urieli-Shoval *et al.*, 1994) are also produced extrahepatically by macrophages; in both cases the increase is dramatic. Carp SAA mRNA was very low in saline-injected fish, and it increased in response to SA- and SG-injections, indicating carp SAA is an acute phase type SAA. In the experiment where arctic char hepatic SAA mRNA was detected in Northern blot analyses using a rainbow trout SAA probe, SAA mRNA first appeared 48 h after pathogenic *Aeromonas salmonicida* infection and continued to increase up to 120 h (Jensen *et al.*, 1997). In carp, the levels of SAA mRNA detected 48 h after SA- and SG-injections were not as high as that of mammalian SAA in response to similar stimuli. Thus the SAA response in poikilothermic vertebrates is probably slower than that in mammals, as has been reported for other inflammatory responses in teleosts (Suzuki & Iida, 1992; Wilson & Warr, 1992).

SAA was formerly considered to be immunosuppressive. However, chemo-attractant activity was found in human SAA-1 to monocytes, neutrophils and T-lymphocytes (Badolato *et al.*, 1994; Xu *et al.*, 1995). SAA production by macrophages in inflammatory lesions may lead to accumulation of leucocytes.

SA and SG are potent PC eliciting agents compared with other mammalian leucocyte-eliciting agents, though the PC influx induced by SG is considerably lower than that by SA (Fujiki & Yano, 1997). Our previous report indicated that a carp chemokine similar to mammalian monocyte chemotactic protein was included in the leucocyte migration elicited by SA (Fujiki *et al.*, 1999). However, it is likely that other pro-inflammatory mediators are also associated with the leucocyte influx. SAA may be one of the mediators responsible for the SA- and SG-elicited influx because SAA expression in the PC was notably high, and it has been shown that pro-inflammatory mediators are produced by mobilised inflammatory cells (Baggiolini *et al.*, 1994; Fujiki *et al.*, 1999).

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References

- Auron, P. E., Webb, A. C., Rosenwasser, L. J., Mucci, S. F., Rich, A., Wolff, S. M. & Dinarello, C. A. (1984). Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. *Proceedings of the National Academy of Sciences USA* **81**, 7907–7911.
- Andrews, A. E., Barcham, G. J., Brandon, M. R. & Nash, A. D. (1991). Molecular cloning and characterization of ovine IL-1 α and IL-1 β . *Immunology* **74**, 453–460.
- Arinaga, S., Karimine, N., Takamuku, K., Nanbara, S., Nagamatsu, M., Ueo, H. & Akiyoshi, T. (1992). Enhanced production of interleukin 1 and tumor necrosis factor by peripheral monocytes after lentinan administration in patients with gastric carcinoma. *International Journal of Immunopharmacology* **14**, 43–47.
- Badolato, R., Wang, J. M., Murphy, W. J., Lloyd, A. R., Michiel, D. F., Bausserman, L. L., Kelvin, D. J. & Oppenheim, J. J. (1994). Serum amyloid A is a chemo-attractant: induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. *Journal of Experimental Medicine* **180**, 203–209.
- Baggiolini, M., Dewald, B. & Moser, B. (1994). Interleukin-8 and related chemotactic cytokines-CXC and CC chemokines. *Advances in Immunology* **55**, 97–179.
- Dinarello, C. A., (1998). Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist. *International Reviews of Immunology* **16**, 457–499.
- Dixon, B., van Erp, S. H. M., Rodrigues, P. N. S., Egberts, E. & Stet, R. J. M. (1995). Fish major histocompatibility complex genes: an expansion. *Developmental and Comparative Immunology* **19**, 109–133.
- Fiskerstrand, C. & Sargan, D. (1990). Nucleotide sequence of ovine interleukin-1 beta. *Nucleic Acids Research* **18**, 7165.
- Fujiki, K. & Yano, T. (1997). Effects of sodium alginate on the non-specific defence system of the common carp (*Cyprinus carpio* L.). *Fish & Shellfish Immunology* **7**, 417–427.
- Fujiki, K., Shin, D.-H., Nakao, M. & Yano, T. (1999). Molecular cloning of carp (*Cyprinus carpio*) CC chemokine, CXC chemokine receptors, allograft inflammatory factor-1 and natural killer cell enhancing factor by use of suppression subtractive hybridisation. *Immunogenetics* **49**, 909–914.
- Fruehauf, J. P., Bonnard, G. D. & Herberman, R. B. (1982). The effect of lentinan on production of interleukin-1 by human monocytes. *Immunopharmacology* **5**, 65–74.
- Gessner, J. E., Heiken, H., Tamm, A. & Schmidt, R. E. (1998). The IgG Fc receptor family. *Annals of Hematology* **76**, 231–248.

- Gish, W. & States, D. J. (1993). Identification of protein coding regions by database similarity search. *Nature Genetics* **3**, 266–272.
- Gray, P. W., Glaister, D., Chen, E., Goeddel, D. V. & Pennica, D. (1986). Two interleukin 1 genes in the mouse: Cloning and expression of the cDNA for murine interleukin 1 β . *Journal of Immunology* **137**, 3644–3648.
- Gruca, P., Chadzinska, M., Lackowska, B., Plytycz, B. (1996). Analysis of peritoneal and head kidney phagocytes during thiogly collate-elicited peritoneal inflammation in the goldfish, *Carrasius auratus*. *Folia biologica* (Kraków) **44**, 137–142.
- Halaas, O., Olsen, W. M., Veiby, O. P., Lovhaug, D., Skjak-Braek, G., Vik, R. & Espevik, T. (1997). Mannuronan enhances survival of lethally irradiated mice and stimulates murine haematopoiesis *in vitro*. *Scandinavian Journal of Immunology* **46**, 358–365.
- Huether, M. J., Lin, G., Smith, D. M., Murtaugh, M. P. & Molitor, T. W. (1993). Cloning sequencing and regulation of an mRNA encoding porcine interleukin-1 β . *Gene* **129**, 285–289.
- Isakov, N. (1997). Immunoreceptor tyrosine-based activation motif (ITAM), a unique module linking antigen and Fc receptors to their signaling cascades. *Journal of Leukocyte Biology* **61**, 6–16.
- Jensen, L. E., Hiney, M. P., Shields, D. C., Uhlar, C. M., Lindsay, A. J. & Whitehead, A. S. (1997). Acute phase proteins in salmonids: evolutionary analyses and acute phase response. *Journal of Immunology* **158**, 384–392.
- Lagnado, C. A., Brown, C. Y. & Goodall, G. J. (1994). AUUUA is not sufficient to promote poly(A) shortening and degradation of an mRNA: the functional sequence within AU-rich elements may be UUAUUUA(U/A)(U/A). *Molecular and Cellular Biology* **14**, 7984–7995.
- Lomedico, P. T., Gubler, U., Hellmann, C. P., Dukovich, M., Giri, J. G., Pan, Y. C., Collier, K., Semionow, R., Chua, A. O. & Mizel, S. B. (1984). Cloning and expression of murine interleukin-1 cDNA in *Escherichia coli*. *Nature* **312**, 458–462.
- Maliszewski, C. R., Baker, P. E., Schoenborn, M. A., Davis, B. S., Cosman, D., Gillis, S. & Cerretti, D. P. (1988). Cloning, sequence and expression of bovine interleukin 1 α and interleukin 1 β complementary DNAs. *Molecular Immunology* **25**, 429–437.
- March, C. J., Mosley, B., Larsen, A., Cerretti, D. P., Braedt, G., Price, V., Gillis, S., Henney, C. S., Kronheim, S. R., Grabstein, K., Conlon, P. J., Hopp, T. P. & Cosman, D. (1985). Cloning sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature* **315**, 641–647.
- Meek, R. L., Eriksen, N. & Benditt, E. P. (1992). Murine serum amyloid A3 is a high density apolipoprotein and is secreted by macrophages. *Proceedings of the National Academy of Sciences USA* **89**, 7949–7952.
- Mori, S., Goto, F., Goto, K., Ohkawara, S., Maeda, S., Shimada, K. & Yoshinaga, M. (1988). Cloning and sequence analysis of a cDNA for lymphocyte proliferation potentiating factor of rabbit polymorphonuclear leukocytes: Identification as rabbit interleukin 1 β . *Biochemical and Biophysical Research Communications* **150**, 1237–1243.
- Nishida, T., Nishino, N., Takano, M., Sekiguchi, Y., Kawai, K., Mizuno, K., Nakai, S., Masui, Y. & Hirai, Y. (1989). Molecular cloning and expression of rat interleukin-1 α cDNA. *Journal of Biochemistry (Tokyo)* **105**, 351–357.
- Ohno, T., Kubagawa, H., Sanders, S. K. & Cooper, M. D. (1990). Biochemical nature of an Fc μ receptor on human B-lineage cells. *Journal of Experimental Medicine* **172**, 1165–1175.
- Pilström, L. & Bengtén, E. (1996). Immunoglobulin in fish – genes, expression and structure. *Fish & Shellfish Immunology* **6**, 243–262.
- Partula, S., de Guerra, A., Fellah, J. S. & Charlemagne, J. (1995). Structure and diversity of the T cell antigen receptor β -chain in a teleost fish. *Journal of Immunology* **155**, 699–706.
- Partula, S., de Guerra, A., Fellah, J. S. & Charlemagne, J. (1996). Structure and diversity of the TCR α -chain in a teleost fish. *Journal of Immunology* **157**, 207–212.

- Secombes, C. J., Zou, J., Daniels, G., Cunningham, C., Koussounadis, A. & Kemp, G. (1998). Rainbow trout cytokine and cytokine receptor genes. *Immunological Reviews* **166**, 333–340.
- Steel, D. M. & Whitehead, A. S. (1994). The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. *Immunology Today* **15**, 81–88.
- Sunyer, J. O. & Lambris, J. D. (1998). Evolution and diversity of the complement system of poikilothermic vertebrates. *Immunological Reviews* **166**, 39–57.
- Suzuki, Y. & Iida, T. (1992). Fish granulocytes in the process of inflammation. *Annual Review of Fish Diseases* **2**, 149–160.
- Urieli-Shoval, S., Meek, R. L., Hanson, R. H., Eriksen, N. & Benditt, E. P. (1994). Human serum amyloid A genes are expressed in monocyte/macrophage cell lines. *American Journal of Pathology* **145**, 650–660.
- Wegener, A. M., Letourneur, F., Hoeveler, A., Brocker, T., Luton, F. & Malissen, B. (1992). The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules. *Cell* **68**, 83–95.
- Wilson, M. R. & Warr, G. W. (1992). Fish immunoglobulins and the genes that encode them. *Annual Review of Fish Diseases* **2**, 201–221.
- Wilson, M., Bengtén, E., Miller, N. W., Clem, L. W., Du Pasquier, L. & Warr, G. W. (1997). A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD. *Proceedings of the National Academy of Sciences USA* **94**, 4593–4597.
- Wilson, M. R., Zhou, H., Bengtén, E., Clem, L. W., Stuge, T. B., Warr, G. W. & Miller, N. W. (1998). T-cell receptors in channel catfish: structure and expression of TCR α and β genes. *Molecular Immunology* **35**, 545–557.
- Xu, L., Badolato, R., Murphy, W. J., Longo, D. L., Anver, M., Hale, S., Oppenheim, J. J. & Wang, J. M. (1995). A novel biologic function of serum amyloid A. Induction of T lymphocyte migration and adhesion. *Journal of Immunology* **155**, 1184–1190.
- Yano, T., Matsuyama, H. & Mangindaan, R. E. P. (1991). Polysaccharide-induced protection of carp, *Cyprinus carpio* L., against bacterial infection. *Journal of Fish Diseases* **14**, 577–582.
- Zapata, A. G., Chibá, A. & Varas, A. (1996). Cells and tissues of the immune system of fish. In: *The fish Immune System: Organism, Pathogen, and Environment* (Iwama, G. & Nakanishi, T., eds) pp 1–62. San Diego, CA: Academic Press.