Immunostimulation in the Common Carp (*Cyprinus carpio* L.) Following Injection of CpG Oligodeoxynucleotides

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ABSTRACT

The immunostimulatory effect of synthetic oligodeoxynucleotides containing unmethylated cytidine-phosphate-guanosine (CpG) was evaluated in the common carp (*Cyprinus carpio* L.). Daily intraperitoneal injection of CpG oligodeoxynucleotides (CpG-ODN) for 3 days resulted in increased responses of phagocytic activity and production of superoxide anion in kidney phagocytic cells. This activation of kidney cells was apparent up to 7 days post-treatment. A single dose of 10 µg significantly augmented expression of interleukin (IL)-1β, CXC and CC-chemokines at 1, 5 and 7 days post-injection. CpG-ODN also stimulated expression of lysozyme C at 7 days post-treatment.

INTRODUCTION

The innate immune system recognizes synthetic oligodeoxynucleotides (ODNs) and bacterial DNA containing unmethylated (CpG) dinucleotides in the context of particular base sequences (Krieg *et al*., 2000). Bacterial DNA, containing 20-fold more CpG-dinucleotides than vertebrate DNA, activates immunocytes in a CpG dependent manner. The immunostimulatory effects of bacterial DNA could be mimicked by synthetic oligodeoxynucleotides containing proper CpG-motif (CpG-ODNs). A possible molecular mechanism whereby bacterial DNA activates immune cells is revealed with the discovery of Toll-like receptor 9 (TLR9) in mice, a transmembrane receptor capable of recognizing unmethylated CpG oligonucleotides in bacterial DNA (Akira *et al*., 2001).

In mammals, CpG-ODN have been shown to directly stimulate B-cell proliferation and induce secretion of Ig (Krieg *et al*., 1995), IL-6 (Yi *et al*., 1996), and IL-10 (Redford *et al*., 1998). CpG-ODN also directly activates monocytes, macrophages, and dendritic cells *in vitro* to secrete IL-12 ((Jakob *et al*., 1998), TNF-((Stacey *et al*., 1996), and IFN-α (Ballas *et al*., 1996).

However, little is known on the effects of CpG-ODN in fish. Kanellos *et al*. (1999) reported that plasmids co-injected with a recombinant potentiated antibody responses to the protein

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in goldfish (*Carrasius auratus* L). J∅rgensen *et al*. (2001a, b) demonstrated that plasmid DNA and synthetic ODNs containing CpG-motifs induced production of interferon-like cytokines and IL-1βin Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) leucocytes. A recent work from our laboratory demonstrated that CpG-ODNs enhance the innate immune response of carp (*Cyprinus carpio*) *in vivo* (Tassakka and Sakai, 2002) and *in vitro* (Tassakka and Sakai, 2003).

In the present study we investigated the *in vivo* effects of synthetic CpG-ODNs on phagocytic cells of common carp. In addition, the effect of CpG-ODN on the expression of immune-related genes was also examined. Genes analysed included those involved in non-specific immune responses, such as cytokine and lysozyme. Carp specific molecular primers were designed to a number of important immune genes, including interleukin (IL)-1β, CXC and CC-chemokines, and lysozyme-C.

MATERIALS AND METHODS

Fish

A total of 200 common carp *Cyprinus carpio* (mean weight=100g) was obtained from Sunaso Fisheries farm in Miyazaki, Japan. Fish were maintained in out-door tanks with running fresh water at 16°C for two weeks and fed commercial diets twice daily.

CpG oligodeoxynucleotides (ODNs)

Synthetic oligodeoxynucleotides containing CpG motifs were purchased from SAWADY (Japan), with the following sequences:

A = ATC GAC TCT CGA ACG TTC TC

B = GCT AGA CGT TAA CGT T

The oligodeoxynucleotides were suspended in saline (10μg/100μl) and injected into carp at a dose of 10 μg/fish intraperitoneally. Control fish received an equal volume of phosphate buffer saline (PBS) alone by an intraperitoneal injection. Four fish of each group were sampled at 1, 5 and 7 days post-injection.

Isolation of head kidney cells

The head kidney phagocytic cells of the carps were isolated according to the modified method described by Braun-Nesje *et al*. (1982). Carp head kidney was removed and pushed through a nylon mesh with RPMI 1640 medium (Nissui, Japan) containing 1% streptomycin/penicillin (S/P, Gibco, USA) and 0.2% heparin (Sigma, USA). The cell suspension was then centrifuged at 500 X g for 5 min and washed three times with the medium. Viable phagocytic cells, including neutrophils and macrophages, were counted by Trypan Blue Exclusion.

Phagocytic activity

Four individual fish were used in this experiment. The number of cells was adjusted to 10^7 cells/ml in RPMI 1640 medium containing 10% carp serum (CS) using haemocytometer.

The cells were allowed to adhere to a glass cover-slip (22 mm X 22 mm) for 1 h and non-adherent cells were removed by washing with the medium.

The latex particles (diameter 0.85 µm) (10^9 particles/ml) (Difco,USA) were suspended in RPMI 1640 medium (10% CS) and were added to the cover-slip and incubated for 2 h at 20°C. Then, the cover-slip was picked up using forceps and washed with the medium for 1 min to remove non-ingested latex particles. Cells were fixed with methyl alcohol, air-dried and stained with Giemsa. The number of adhered cells was about 5×10^5 cells per cover-slip and the number of phagocytic cells per 300 adhered cells was counted microscopically. The phagocytic activity (PA) was determined using formula:

$$PA = \frac{\text{Number of phagocytizing cells}}{\text{Total number of cells}} \times 100$$

Detection of superoxide anion in phagocytic cells

The superoxide anion from phagocytic cells was determined by the reduction of the redox dye nitroblue tetrazolium (NBT) as described by Chung and Secombes (1988). The kidney cells suspended in RPMI 1640 containing 10% CS and HEPES were collected as described above. One hundred microliters of this suspension was added to each well of a 96 well microtiter plates (Nunc, Denmark). After 2 h incubation at 20°C the cells were washed by RPMI 1640 medium to remove non-adherent cells. The total adhered cell number per well was about 10^5 cells. One hundred microliters of NBT solution (1 mg/ml in RPMI 1640 medium) and phorbol myristate acetate (1 mg/ml) (PMA, Sigma) were added to each well and incubated for 60 min at 20°C. The reduction was stopped by the addition of methanol, after removal of the medium from the cells. The formazan in each well was dissolved in 120 µl of 2 M KOH and 140 µl DMSO, and the optical density was measured by a multiscan spectrophotometer (Pharmacia, Sweden) at 620 nm.

RT-PCR analyses

Four individual fish were injected intraperitoneally with 10 µg of CpG-ODNs. Total RNA extracted from the kidney was used for cDNA synthesis by Rever Tra Dash (Toyobo, Japan) according to the manufacturer's protocol.

The cDNA was then used for PCR. All PCR reactions were performed according to the following protocol: 1 µl of cDNA was mixed with 5 µl dNTPs (10 µM of each dNTP), 0.5 Taq polymerase (5 units/µl), 5 µl of each gene-specific primer and 27.5 µl of water. Primers for β-actin (Fw: 5'-ACTACCTCATGAAGATCCTG-3' and (Rv: 5'-TTGCTGATCCA CATCTGCTG-3') were used as a positive control for RT-PCR, since the gene is constitutively expressed. Gene specific primers for carp were designed using highly conserved regions for IL-1β (Fw: 5'-CAACATTCG TGTCGAG-3' and Rv: 5'-AAGTTTGTGGTTCGGG-3'), CC -chemokine (Fw: 5'-AAT GGAGACACGCAGGATCCT-3' and Rv: 5'-GCTCAGTCAC TAATAGATGATGC-3'), CXC-chemokine (Fw: 5'-ATGAAAATCATTACCGCTGTG-3' and Rv: 5'-TGGATT GAAGCATTTCTGCTC-3'), and lysozyme C (Fw: 5'-GTGTCTGAT GTGGCTGT GCT-3' and Rv: 5'-TATCCCAGGTGTCCCATGAT-3'). The PCR was performed in a PCR apparatus (MJ Research, USA) with 27 reaction cycles of 0.5 min at

94°C, 1 min at 52°C (actin), 51°C (CXC- chemokine), 48°C (Interleukin-1β) 63°C (CC-chemokine), 60°C (Lysozyme-C) and 1 min at 72°C. PCR products were electrophoresed on a 1.5 % agarose gel to detect the specific bands.

Semi-quantitave analysis of RT-PCR products

The relative levels of RNA were quantified for each gene by densitometry, which was performed by measuring the photostimulated luminescence values using Science Lab99 Image Gauge software (Fujifilm, Japan). Ratios of cytokine (Lysozyme) product: β-actin product were subsequently calculated for each gene of interest and used to asses the differences in expression levels between control and CpG-ODNs injected group.

Statistical analysis

Results were expressed as mean ± SEM. A student's t-test was used to test for statistical significance of differences between controls and CpG A or CpG B treated groups. A level of $P < 0.05$ was considered significant.

Table 1. Phagocytic activities in kidney leucocytes of common carp injected with 10 mg CpG ODNs/fish against latex particles.

RESULTS AND DISCUSSION

	Days after injection		
	1	5	7
Control	25 ± 1.3	21.3 ± 1.2	33.8 ± 3.2
CpG A	56.4 ± 4.4*	35.3 ± 3.1*	47.8 ± 2.1*
CpG B	40 ± 2.0*	29.7 ± 1.3	45.8 ± 1.9*

The phagocytic activity of the kidney leucocytes from carp treated with 10 μg/fish of CpG-ODNs is shown in Table 1. Head kidney leucocytes from carp treated with CpG-ODNs A and B for 1 day showed a significantly higher phagocytic activity than that of the leucocytes from control fish (*$P < 0.05$). This stimulation continued at least 7 days after treatment.

The NBT reduction by carp phagocytic cells treated with CpG-ODNs A & B significantly increased in comparison to the control cells at day 5 (*$P < 0.05$; Fig 1). Enhanced NBT reduction was still apparent up to 7 days after injection.

The expression of immune-related genes such as IL-1β, CXC and CC-chemokines and Lysozyme-C were significantly increased (*$P < 0.05$) in the CpG-ODNs injected groups (Fig. 2). The expression of IL-1β in the kidney leucocytes of carp injected by 10 μg/fish of CpG-ODNs is shown in Fig 3. IL-1β expression was significantly higher in the cells isolated from fish treated with CpG-ODNs A & B than those isolated from the control fish at 1 and 5 days post-injection (*$P < 0.05$). Increased expression was still apparent up to day 7 in the kidney cells of fish treated with CpG-ODN A. CXC-chemokine expression in head kidney cells of carp injected with CpG-ODNs A and B demonstrated a significantly higher level of expression (*$P < 0.05$) than those of controls at day 1, 5 and 7 (Fig. 4). The same phenomenon was observed in CC-chemokine expression in carp. The expression was also enhanced by CpG-ODNs at all the stimulation time periods (*$P < 0.05$; Fig 5). The expression of lysozyme-C in the kidney of carp injected with CpG-ODN A and B is shown in Fig 6. This expression had a significant effect only in the fish injected with CpG ODN A at 7 days post-treatment (*$P < 0.05$).

In this study, we observed the immune activation in the common carp kidney leucocytes

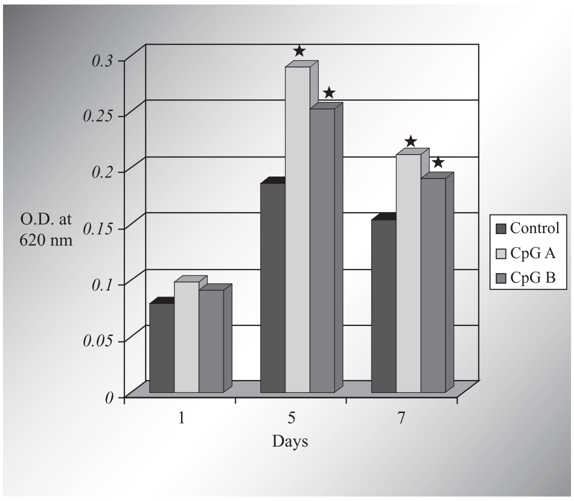

Figure 1. The production of superoxide anion in leucocytes of carp injected with 10 mg of CpG-ODN A and B as measured by NBT. The NBT reduction was examined 1, 5 and 7 days after injection. Values are mean ± SE at 620 nm

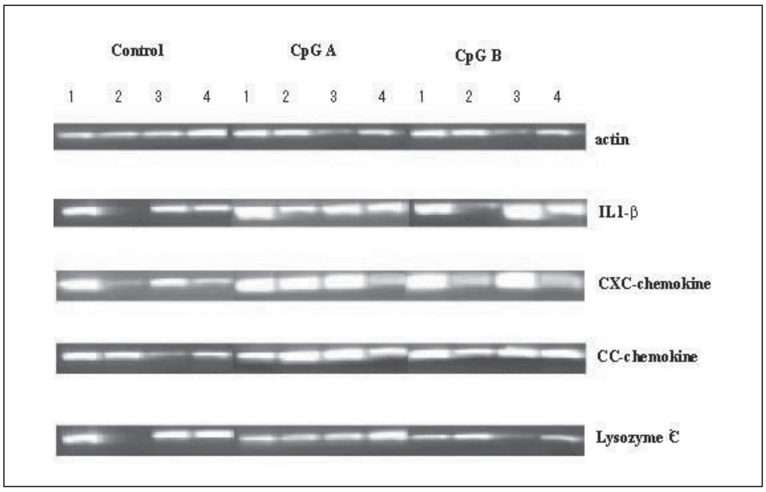

Figure 2. RT-PCR analysis of cytokine and lysozyme gene expression in head kidney leucocytes from control fish injected (intraperitoneally) with PBS and CpG-ODNs treated fish. Leucocytes were harvested at 5 days post-injection for IL1-b and CC-chemokine expression, and at 7 days post-injection for CXC-chemokine and Lysozyme C expression.

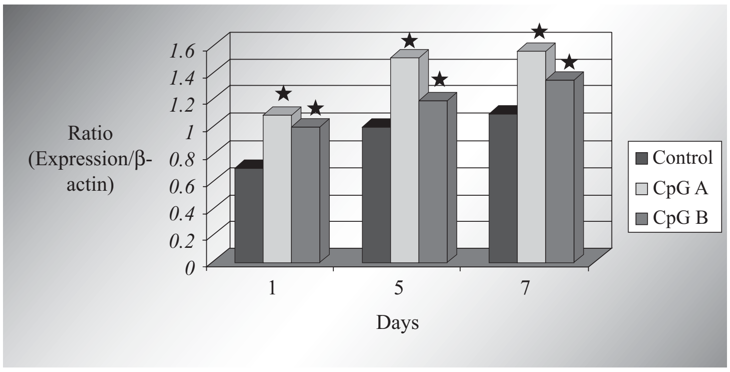

Figure 3. Densitometric quantification of IL-1b expression relative to the b-actin transcript in head kidney leucocytes isolated from control fish injected (intraperitoneally) with PBS and 10 mg CpG-ODNs treated fish. Leucocytes were harvested at 1, 5 and 7 days post injection. Values are mean ± SE in 4 fish. *$P < 0.05$.

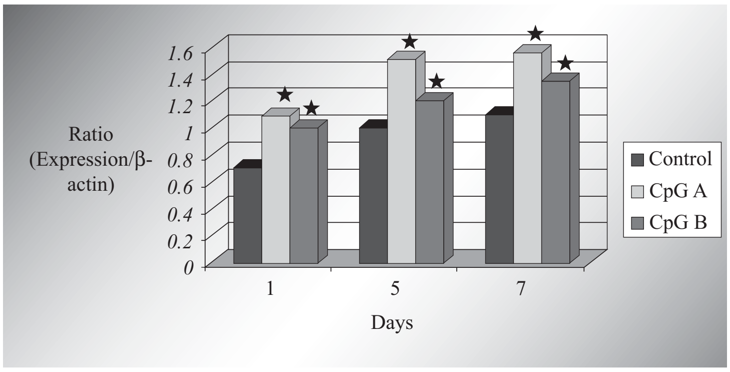

Figure 4. Densitometric quantification of CXC-chemokine expression relative to the b-actin transcript in head kidney leucocytes isolated from control fish injected (intraperitoneally) with PBS and 10 mg CpG-ODNs treated fish. Leucocytes were harvested at 1, 5 and 7 days post injection. Values are mean ± SE in 4 fish. *$P < 0.05$.

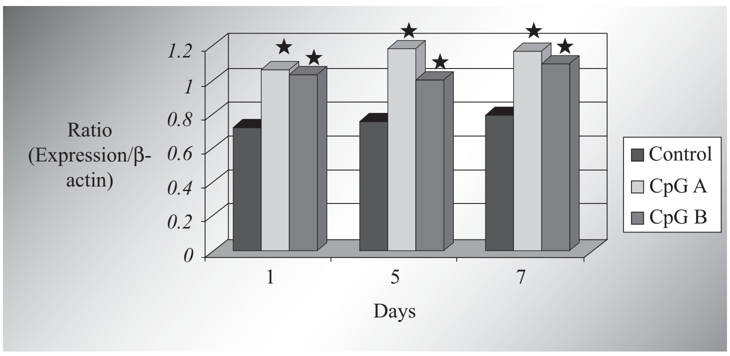

Figure 5. Densitometric quantification of CC-chemokine gene expression relative to the b-actin transcript in head kidney leucocytes isolated from control fish injected (intraperitoneally) with PBS and 10 mg CpG-ODNs treated fish. Leucocytes were harvested at 1, 5 and 7 days post injection. Values are mean ± SE in 4 fish. *$P < 0.05$.

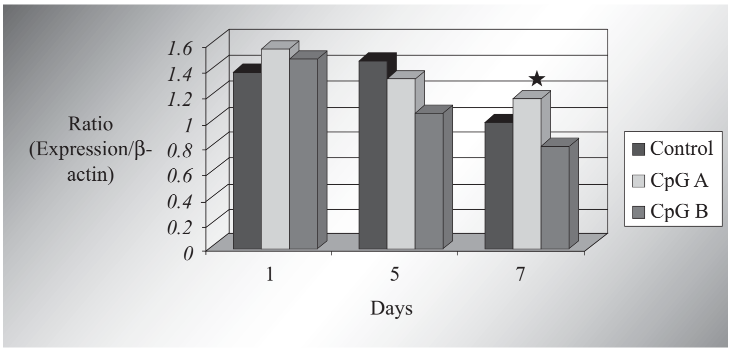

Figure 6. Densitometric quantification of lysozyme-C gene expression relative to the b-actin transcript in head kidney leucocytes isolated from control fish injected (intraperitoneally) with PBS and 10 mg CpG-ODNs treated fish. Leucocytes were harvested at 1, 5 and 7 days post injection. Values are mean ± SE in 4 fish. **P* < 0.05.

following injection of CpG-ODNs. Intraperitoneal injection of CpG-ODNs (ODN A and B) lead to induced phagocytosis against latex particles, the production of superoxide anion and expression of cytokine and lysozyme genes in carp leucocytes. Recently published studies have shown that CpG-ODNs activate fish leucocytes (Oumouna *et al*., 2002; Meng *et al*., 2003; J∅rgensen *et al*., 2003). The strong activating effect of CpG-ODNs on fish leucocytes suggests the use of CpG-ODNs as an immunostimulant in fish.

This study provides new evidence that CpG-ODN induced expression of cytokines (CXC and CC-chemokine) genes in fish. Cytokines are a group of molecules that play significant role in initiating and regulating the inflammatory process (Thomson, 1994). IL-1β, CXC and CC-chemokines are three cytokines that regulate immune responses. IL-1β is a member of the β-trefoil family of cytokines. The major functions of IL-1β are activation of the proliferation of such lymphocytes as T cells and B cells, activation of cytotoxic activity in macrophage and natural killer (NK) cells, and induction of immunoglobulin (Ig) secretion. Phagocytes are important sources for the synthesis and release of IL-1 for co-stimulation of T cell activation (Dower and Sims, 1994). The chemokines are a superfamily of approximately 40 different small secreted cytokines that direct the migration of immune cells to sites of infection (Secombes *et al*., 2001). These molecules act as chemo-attractants causing an influx of neutrophils, monocytes, T cells and basophils in humans. The functions like chemotaxis, integrin activation, granule enzyme release, lipid mediator biosynthesis, and superoxide radical production have been reported (Oppenheim, *et al*., 1991; Schall and Bacon, 1994; Baggiolini and Dahinden, 1994; Bacon and Schall, 1996). Four distinct subgroups make up the chemokine superfamily. These are designated as CXC (α), CC (β), C (γ) and CX_3C (δ) that are defined by the arrangement of the first two cysteine residues within the protein (Yoshie *et al*., 2000). In fish, only a few cytokines and chemokines have been known. A novel CXC chemokine was identified for the first time in fish from common carp (Savan *et al*., 2003). This gene was obtained from the head kidney stimulated with LPS and Con A.

It was reported that CpG-ODNs are capable to up-regulate the expression of cytokine genes in Atlantic salmon and rainbow trout leucocytes (J∅rgensen *et al*., 2001a,b). Both plasmid DNA and synthetic ODNs containing CpG-motifs induced production of interferon-like cytokines in Atlantic salmon leucocytes (J∅rgensen *et al*., 2001a). Rainbow trout macrophages not only produce IFN-like cytokines, but also express IL-1β when stimulated with CpG-ODNs (J∅rgensen *et al*., 2001b). Thus, CpG-ODNs can now be added to the list of substances that stimulate the expression of these important cytokines in fish. The immunostimulatory properties of CpG-ODNs have allowed their use as therapeutic agents for a abroad spectrum of disease indications including cancers, viral and bacterial infections, and inflammatory disorders and as adjuvant in immunotherapy.

CpG-ODN A induced lysozyme type C expression in carp kidney leucocytes at 7 days after injection. Lipopolysaccharides and Concavalin A (Savan and Sakai, 2002) induced the expression of lysozyme type C. Lysozymes are considered to be potent innate immunity molecules. They act as a non-specific bio-defense molecule in the skin, mucus and serum of fish, protecting against the invasion of pathogenic bacteria. Recently, lysozymes have gained importance not only as a defense molecule, but also as a major digestive enzyme in the stomach of ruminants. In fish, lysozyme C and its variants have been cloned in carp (Fujiki *et al*., 2000; Savan and Sakai, 2002) and Japanese flounder (*Paralicthys olivaceus*) (Hikima *et al*., 1997).

In conclusion, the immunostimulatory property of intraperitoneally injected CpG-ODNs has been demonstrated in common carp. A dose of 10 (g CpG ODN augmented macrophage activation, as evidenced by increases in phagocytosis, NBT reduction, cytokine and lysozyme gene expressions.

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REFERENCES

Akira, S., Takeda, K. and Kaisho, T. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nature Immunology 2, 675.

Bacon, K.B. and Schall, T.J. 1996. Chemokines as mediators of allergic inflammation. International Archives of Allergy and Immunology 109.

Baggiolini, M. and Dahinden, C.A. 1994. CC chemokines in allergic inflammation. Immunology Today 15, 127.

Ballas, Z.K., Rasmussen, W.L. and Krieg, A.M. 1996. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. Journal of Immunology 157, 1840.

Braun-Nesje, R., Kaplan, G. and Sejelid, G. 1982. Rainbow trout macrophages in vitro: morphology and phagocytic activity. *Developmental and Comparative Immunology 6*, 281-291.

Chung, S. and Secombes, C.J. 1988. Analysis of events occurring within teleost macrophages during the respiratory burst. Comparative Biochemistry and Physiology 89b, 539.

Dower, S.K. and Sims, J.E. 1994. Interleukin-1 receptor antagonist (IL-1α, IL-1β, and IL-1Ra). *In* Nicola, N.A. (ed.). Guidebook to Cytokines and Their Receptors. Oxford University Press, New York. pp. 17.

Fujiki, K., Shin, D.H., Nakao, M. and Yano, T. 2000. Molecular cloning of carp (*Cyprinus carpio*) leucocyte cell-derived chemotaxin 2, glia maturation factor, CD45 and lysozyme C by use of suppression subtractive hybridisation. Fish and Shellfish Immunology 10, 643.

Hikima, J., Hirono, T. and Aoki, T. 1997. Characterization and expression of c-type lysozyme cDNA from Japanese flounder (*Paralichthys olivaceus*). Molecular Marine Biology and Biotechnology 6, 339.

Jakob, T., Walker, P.S., Krieg, A.M., Udey, M.C. and Vogel, J.C. 1998. Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. Journal of Immunology 161, 3042.

J⌀rgensen, J.B., Johansen, A., Stenersen, B., Sommer, A.I. 2001a. CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (*Salmo salar* L.) leucocytes to produce supernatants with antiviral activity. Developmental and Comparative Immunology 25, 313.

J⌀rgensen, J.B., Zou, J., Johansen, A., Secombes, C.J. 2001b. Immunostimulatory CpG oligodeoxynucleotides stimulate expression of IL-1β and interferon-like cytokines in rainbow trout macrophages via a chloroquine-sensitive mechanism. Fish and Shellfish Immunology 11, 673.

J⌀rgensen, J.B., Johansen, L.-H., Steiro, K. and Johansen, A. 2003. CpG DNA induces protective antiviral immune responses in Atlantic salmon (*Salmo salar* L.). Journal of Virology 77, 11471.

Kanellos, T.S., Sylvester, I.D., Butler, V.L., Ambali, A.G., Partidos, C.D., Hamblin, A.S. and Russel, P.H. 1999. Mammalian granulocyte-macrophage colony-stimulating factor and some CpG motifs have an effect on the immunogenicity of DNA and subunit vaccines in fish. Immunology 96, 507.

Krieg, A.M., Yi, A.K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A., Klinman, D.M. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature (Lond.) 374, 546.

Krieg, A.M., Hartmann, G. and Yi, A.K. 2000. Mechanism of action of CpG DNA. Current Topics in Microbiology and Immunology 247, 1.

Meng, Z., Shao, J. and Xiang, L. 2003. CpG oligodeoxynucleotides activate grass carp (*Ctenopharyngodon idellus*) macrophages. Developmental and Comparative Immunology 27, 313-321.

Oppenheim, J.J., Zachariae, C.O., Mukaida, N. and Matsushima, K. 1991. Properties of the novel proinflammatory supergene “intercrine” cytokine family. Annual Review of Immunology 9, 617.

Oumouna, M., Jaso-Friedmann, L. and Evans, D.L. 2002. Activation of nonspecific cytotoxic cells (NCC) with synthetic oligodeoxynucleotides anhd bacterial genomic DNA: binding, specificity and identification of unique immunostimulatory motifs. Developmental and Comparative Immunology 26, 257-269.

Redford, T.W., Yi, A.K., Ward, C.T., and Krieg, A.M. 1998. Cyclosporin A enhances IL-12 production by CpG motifs in bacterial DNA and synthetic oligodeoxynucleotides. Journal of Immunology 161, 3930.

Savan, R. and Sakai, M. 2002. Analysis of expressed sequence tags (EST) obtained from common carp, *Cyprinus carpio* L., head kidney cells after stimulation by two mitogens, lipopolysaccharide and concavalin-A. Comparative Biochemistry and Physiology B Biochemistry and Molecular Biology 131, 71.

Savan, R., Kono, T., Aman, A., and Sakai, M. 2003. Isolation and characterization of a novel chemokine in common carp (*Cyprinus carpio* L.). Molecular Immunology 39, 829.

Schall, T.J. and Bacon, K.B. 1994. Chemokines, leucocyte trafficking and inflammation. Current Opinion in Immunology 6, 865.

Stacey, K.J., Sweet, M.J. and Hume, D.A. 1996. Macrophages ingest and are activated by bacterial DNA. Journal of Immunology 157, 2116.

Secombes, C.J., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K.J., Cunningham, C. and Zou, J. 2001. Cytokines and innate immunity of fish. Developmental and Comparative Immunology 25, 713.

Tassakka, A.R., Malina, A.C. and Sakai, M. 2002. CpG oligodeoxynucleotides enhance the non-specific immune responses on carp, *Cyprinus carpio*. Aquaculture 209, 1.

Tassakka, A.R., Malina, A.C. and Sakai, M. 2003. The in vitro effect of CpG Oligodeoxynucleotides on the innate immune response of common carp, *Cyprinus carpio* L. Aquaculture 220, 27.

Thomson, A. 1994. The Cytokine Handbook, 2nd ed. Academic Press, London. Pp. 1.

Yi, A.K., Klinman, D.M., Martin, T.L., Matson, S. and Krieg, A.M. 1996. Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. Journal of Immunology 157, 5394.

Yoshie, O., Nomiyama, N. and Miyasaka, M. 2000. Chemokine Handbook: Cell Technology. Shujunsha Co. Ltd, Tokyo.