CpG oligodeoxynucleotides stimulate the immune system of common carp (*Cyprinus carpio* L.)

DNA の CpG モチーフはコイの非特異免疫応答を活性化させるか

A dissertation submitted to
The united Graduate School of Agricultural Sciences,
Kagoshima University, Japan

In partial fulfillment of the requirement for the degree of
DOCTOR OF PHILOSOPHY (Ph.D)
in Fisheries Science

Presented by

A.R. Tassakka Asmi Citra Malina

MIYAZAKI, JAPAN

2005

The undersigned certify that they have read and recommended to the Kagoshima University United Graduate School of Agricultural Sciences for acceptance a dissertation entitled:

CpG oligodeoxynucleotides stimulate the immune system of common carp (*Cyprinus carpio* L.)

(DNA の CpG モチーフはコイの非特異免疫応答を活性化させるか)

Submitted by : A.R. Tassakka Asmi Citra Malina

Date of Final Oral Examination : January 13, 2005

The student has satisfactorily completed and passed the doctoral oral examination in partial fulfillment of the requirements for the dergree of Doctor of Philosophy (Ph.D) in Fisheries Science.

Reading Committee,

Prof. Dr. Tadashi SAKAI.....

Prof. Dr. Masahiro SAKAI.....

Prof. Dr. Taizo SAKATA.....

Prof. Dr. Toshiaki ITAMI.....

Prof. Dr. Hidemasa MIKI.....

（学位第３号様式）

学 位 論 文 要 旨

氏　名	A. R. Tassakka Asmi Citra Malina
題　目	CpG oligodeoxynucleotides stimulate the immune system of common carp (*Cyprinus carpio* L.) (DNAのCpGモチーフはコイの非特異免疫応答を活性化させるか)

An immunostimulant is a substance or action, which causes an innate immune response or increases an adaptive immune response. Bacterial DNA has long been known to be able to activate immune system, whereas mammalian is not. An important difference between bacterial and mammalian DNA is the suppression of unmethylated CpG motifs in mammalian DNA. Synthetic oligodeoxynucleotides containing unmethylated CpG motifs have been shown to mimic the effect of bacterial DNA in exerting immune responses.

The study mainly focused on the investigation of immunostimulatory effects of CpG oligodeoxynucleotides (CpG-ODNs) on the immune system of common carp (*Cyprinus carpio* L.). The effects of a panel of synthetic oligodeoxynucleotides on the immunocompetence cell activity in common carp were examined. *In vitro* addition of CpG-ODNs enhanced phagocytic function (reduction of nitroblue tetrazolium, phagocytosis) from stimulated kidney phagocytes. The CpG-ODNs also induced lymphocyte proliferation in the fish kidney cells. These results show that CpG-ODNs could potentially improve immunocompetence cell activity in fish through increased cell proliferation and functionality. Based on the results from *in vitro* study in which CpG-ODNs with the mice and fish motif (GA/AA)CGTT, were shown to stimulate phagocyte and lymphocyte activities in common carp head kidney leucocytes, *in vivo* studies were undertaken to examine the capacity of these CpG-ODNs to stimulate phagocytic and serum lysozyme activities in the carp leucocytes. Intraperitoneal injection of CpG-ODNs to fish daily, 3 days, resulted in enhanced responses of phagocytic and nitroblue tetrazolium (NBT) activities in kidney phagocytic cells. This activation of kidney cells was observed for at least 7 days post-injection. The serum lysozyme activity also increased in the fish treated with CpG-ODNs. These results indicated that unmethylated CpG DNA enhance the innate immune response of carp.

Analysis of expressed sequence tags of 88 clones from CpG-ODNs stimulated head kidney of carp was carried out. Several immune-related genes were identified during this analysis, including granulin 2, CCAAT/enhancer binding protein, immunoglobulin heavy chain variable region, lectin, lysozyme C, interleukin-4 receptor alpha chain, cathepsin L preproprotein, CD9 protein and Granulin 1. Using head kidney macrophages of common carp as an *in vivo* model, the effects of CpG-ODNs on expression of a number of immune-related genes in fish macrophages were investigated. CpG-ODNs augmented expression of interleukin (IL)-1β, CXC and CC-chemokines at 1, 5 and 7 days post-treatment. CpG-ODN also increased the lysozyme-C gene expression at 7 days post-injection. Using *in vitro* approaches, CpG-ODNs were shown to up-regulate the expression of IL-1β, CXC and CC-chemokines, and tumor necrosis factor (TNF)-α genes in the head kidney of carp. The high level expressions of these genes were seen at an earlier stage of CpG-ODNs stimulation. The IL-10 gene expressions were not significantly enhanced by CpG-ODNs and the non-CpG ODNs also failed to stimulate the expression of these cytokine genes.

These studies have shown that CpG-ODNs are effective immune stimulators for fish immune cells, suggesting that CpG-ODNs may be useful in enhancing the immune responses in veterinary applications for fish.

（学位第３号様式）

学 位 論 文 要 旨

氏　　名	タサカ・アスミ・シットラ・マリナ
題　　目	DNAのCpGモチーフはコイの非特異免疫応答を活性化させるか (CpG oligodeoxynucleotides stimulate the immune system of common carp (*Cyprinus carpio* L.))

近年、非メチル化・CpGジヌクレオチド（ODN）を含んでいるDNA分子は、哺乳類においてすぐれた免疫賦活作用があることが報告されている。

しかし、これらのDNAモチーフが魚類の免疫系に及ぼす作用についてはほとんど研究されていない。そこで本研究では、コイを用いてDNAのCpGモチーフがコイの白血球の活性に与える影響を調べた。次にCpG-ODNs投与魚で発現する遺伝子をEST解析で調べ、さらにCpGモチーフがコイの白血球のサイトカイン（インターロイキン（IL）-1β、IL-10、TNF-α、CXC-およびCC-ケモカイン）遺伝子の発現に及ぼす影響についても検討を行った。

ODNsは哺乳類で免疫賦活作用のあるモチーフとして知られている5種類のCpG-ODNs (A=TCC ATG ACG TTC CTG ATG CT, B=GCT AGA CGT TAA CGTT, C=ATC GAC TCT CGA ACG TTC C, D=GAA CCT TCC ATG CTG TTC CG, E=GCT AGA TGT TAG CGT)を合成し、本研究に用いた。

試験管内でコイの腎臓の白血球をCpG-ODNsで刺激すると、CpG-ODN BとCが貪食能とNBT活性を有意に増強した。さらにコイのリンパ球の幼若化を有意に促進した。

試験管内で有意な活性を示したCpG-ODN BまたはCをコイの筋肉内に1, 10, 100 μg/kg/fish注射した。その後、腎臓の白血球の活性化を貪食能とNBT法で、また血清中のリゾチーム活性についても測定を行った。

CpG-ODN BまたはCを注射したコイの腎臓の白血球の貪食能は、対照区に比べて有意な増加が確認された。さらに白血球のNBT活性も有意に増加した。また、血清中のリゾチーム活性もCpG-ODN BまたはCを注射した区において有意な増加が認められた。

次にこれらのコイの腎臓組織より作成したcDNAライブラリーより88クローンについてEST解析を行った結果、granulin 2, CCAAT/enhancer binding protein, immunoglobulin heavy chain variable region, lectin, lysozyme C, interleukin-4 receptor alpha chain, cathepsin L preproprotein, CD9 protein, Granulin 1などの免疫系に関与する遺伝子を分離した。

同様に処理したコイの腎臓由来白血球のサイトカイン遺伝子を経時的に半定量解析した結果、炎症性のサイトカインであるIL-1β、CXCおよびCC-ケモカイン遺伝子の発現量が増加した。またCpG-ODN Cはlysozyme-C遺伝子の発現量を増加させた。試験管内においてCpG-ODN BまたはCで処理されたコイの白血球からもIL-1β、CXCおよびCC-ケモカイン、TNF-α遺伝子の発現量増加が確認された。これらサイトカイン遺伝子の発現はCpG-ODNs処理後、速やかに高い発現量を示した。一方、抗炎症性サイトカインであるIL-10遺伝子の発現量については、CpG-ODNs処理による発現量の有意な変化は確認されなかった。また、non-CPG-ODNsではサイトカイン遺伝子の発現量の増加は確認されなかった。

これらの結果よりCpG-ODNモチーフはコイの免疫応答を増強する作用があることが示された。

Table of contents

Chapter I

General Introduction

I-1. Introduction

During the last decades, there has been a continuous growth in the aquaculture industry all over the world. The most significant growth has been seen in carp production as well as in the production of other cyprinids and even more so for carnivorous fish species such as salmonids, which are farmed in intensive conditions. The projections for the next decade are that there will be a shortage in supply of marine proteins from wild caught fish and this gives reason to believe that the growth in aquaculture will continue. One should assume that new marine fish species will be added to the existing spectrum of fish species grown in aquaculture.

However, any intensive bioproduction -whether on land or at sea- will experience disease problems. Infectious diseases that occur as a sporadic event in wild fish populations may cause high mortality when present in intensive fish farming. An increase in the susceptibility of fish to disease is a direct consequence of immunosuppression induced by physical stress associated with handling, vaccination, grading, net changing, salt water transfer, anti-parasite bath treatments, etc., as well as pressures related to other natural events such as environmental changes and social conflicts (Burrels et al., 2001). For the aquaculture industry to prosper in the future it is a prerequisite that the losses caused by diseases and the use of antibiotics are kept at a minimum. Immunoprophylaxis recognized by stimulation of innate and adaptive immunity is consequently the foundation for developing aquaculture into a sustainable bioproduction in the aquatic ecosystems.

Whilst vaccination is the method of choice over antibiotic treatments for the control of many fish diseases, vaccines for others are unavailable or, at best, in the early stages of their development. In recent years in the aquaculture industry, increasing consideration has been given to the use of immunostimulants as adjuncts to vaccination and as a potential route to the reduction in the widespread use of antibiotics.

Bacterial DNA is known as a potent immune stimulator in vertebrates (Messina et al., 1991). Bacterial DNA stimulates the innate immune system cells such as macrophages/monocytes and dendritic cells to produce various inflammatory cytokines including interleukin (IL)-1, IL-6, IL-8, IL-12, tumor necrosis factor (TNF)-α and interferon (IFN)-γ (Klinman et al., 1996; Bohle et al., 1999; Akhtar et al., 2003). It was later discovered that unmethylated 5'-CpG-3' dinucleotides with certain flanking bases are responsible for the immunogenicity of the bacterial DNA (Krieg et al., 1995). Synthetic oligodeoxynucleotides containing this CpG motif (CpG-ODNs) have similar immunostimulatory effects to bacterial DNA (Krieg et al., 1995). In fish knowledge about CpG-ODN effects on the immune system is scant, yet emerging. The aim of this doctoral thesis was to gain an insight into effects of CpG oligodeoxynucleotides on the immune system of teleost fish.

I-2. Review of Literature

I-2.1. *Immunostimulants*

An immunostimulant is a substance or action, which causes an innate immune response or increases an adaptive immune response. For the past ten years the research of immunostimulant in fish has gained increased acceptance because the wide range of parasites, fungi, bacteria and virus that affect the fish production, causing economic losses (Anderson, 1992). Recently, Sakai (1999) reviewed the current status of research into the use of immunostimulants in fish. Mainly, substances such as glucan, chitin, lactoferrin, and levamisole, as well as nutritional factors like vitamins B and C, growth hormone and prolactin are immunostimulatory because of their direct positive influence on innate immune elements such as phagocytic cell activity, natural killer cell activity, lysozyme levels and total immunoglobulin (Ig) levels. However, some of the immunostimulants could not be used because of various disadvantages, such as high cost, limited effectiveness upon parenterally administration, etc.

The most widely studied of these immunostimulants are the glucans, particularly yeast glucans (ß-1,3- and ß-1,6 linked glucans) and their use in fish has been reviewed (Robertsen et al., 1994; Robertsen, 1999; Sakai, 1999). The majority of reports have described increased resistance to mainly bacterial infections such as *Vibrio anguillarum*, *V. salmonicida* or *Yersinia ruckeri* (Robertsen et al., 1990). LaPatra et al. (1998) also reported increased resistance to challenge with infectious haematopoietic necrosis (IHN) virus. Increases in innate resistance have been demonstrated to challenge-infections with *V. anguillarum* and *V. salmonicida* (Raa et al., 1992) and *Aeromonas salmonicida* (Nikl et al., 1993). There is also a paucity of information regarding the effectiveness of orally administered ß-glucans against disease organism other than

bacteria. Besides ß-glucans, chitin or vitamin is also well documented. However, the use of whole organisms instead of their isolated components has hardly been evaluated. In this way, whole yeast cells (mainly *Saccharomyces cerevisiae*), which represent a major commercial source of ß-glucans have recently been described as good immunostimulants in fish (Siwicki et al., 1994; Ortuno et al., 2002).

Immunostimulants will be most effective for short-lived fish living in cool and cold water because the development of an adaptive immune response is temperature-dependent. Advantages of immunostimulants in aquaculture:

- Effective and friendly user substances
- No side effects
- Develop resistance against various diseases
- Provide wide range of protection
- Locally available

Immunostimulants act through following mechanisms:

- Stimulators of T-lymphocytes-levamisole, Freund's complete adjuvant, glucans, muramyl dipeptide.
- Stimulators of B-cells-lipopolysaccharides.
- Inflammatory agents inducing chemotaxis-silica and carbon particles.
- Cell membrane modifiers-detergents, sodium dodecylsulphate, quaternary ammonium compounds, saponins.
- Nutritional factors-vitamins C and E.
- Cytokines-leukotriene, interferon
- Heavy metals-cadmium
- Animal and fish extracts, mitogen.

In general, immunostimulants enhance the activity of macrophages, complements, phagocytes, lymphocyte, and non-specific cytotoxic cells, resulting in resistance and protection to various diseases.

I-2.2. *CpG oligodeoxynucleotides*

Aside from its function of encoding the genetic material, DNA can have direct immune stimulatory effects. The immunostimulatory effects of bacterial DNA have now been known for almost two decades (Tokunaga et al., 1984; Tokunaga et al., 1988; Tokunaga et al., 1992; Yamamoto et al., 1992; Yamamoto et al., 1994). In subsequent studies, it was shown that synthetic oligodeoxynucleotides were also immunostimulatory and their activity was attributed to unmethylated CpG nucleotides flanked by specific bases (Tokunaga et al., 1992; Yamamoto et al., 1994; Krieg et al., 1995). Unlike bacterial DNA, mammalian DNA is not immunostimulatory, due to structural differences. For example, mammalian DNA has a much lower frequency of CpG nucleotides (CpG suppression) and the nucleotides present are usually methylated (Bird, 1986; Krieg et al., 1995).

In vitro and *in vivo* studies have shown that CpG-ODNs are potent activators of the immune system in numerous species including humans, nonhuman primates, mice, cattle, sheep, pigs, horses, dogs, cats, chickens and fish (Brown et al., 1998; Jørgensen et al., 2001a; Rankin et al., 2001; Pontarollo et al., 2002; Wernette et al., 2002). CpG-ODNs stimulate the innate immune system and have been shown to be protective against a variety of pathogens including bacteria, viruses and protozoa in various animal models (Zimmermann et al., 1998; reviewed in Mutwiri et al., 2004). CpG-ODNs are also potent enhancers of antigen-specific immune responses and the adjuvant activity of

CpG-ODN has been demonstrated in humans and numerous animal species (Chu et al., 1997; Davis et al., 1998). Clinical studies have been initiated in humans to evaluate CpG-ODN therapy against infectious disease, cancer, asthma and allergy (Krieg, 2002).

I-2.2.1. *Species selectivity and the importance sequences of CpG-ODNs*

In addition to the presence of unmethylated CpG dinucleotides, the sequences flanking the CpG dinucleotide also play a role for the induction of immunostimulatory activity. The DNA sequences containing an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines such as 'GACGTT', were found to activate the mouse immune system efficiently (Krieg et al., 1995). Early studies indicated that optimally active CpG motif vary from species to species. CpG motifs that most effectively activate mice immune cells are poorly stimulatory in human, due to evolutionary divergence in CpG recognition between species (Bauer et al., 1999; Hartmann and Krieg, 2000; Verthelyi et al., 2001). Human peripheral blood mononuclear cells (PBMCs) are potentially activated by ODN containing 'GTCGTT', 'AACGTT' or 'TTCGTT' motifs (Krieg, 2002). The terms mouse and human motifs were adopted despite the fact that the human motif also has some stimulatory effects on mouse cells. CpG-ODNs that activate PBMCs of primates were classified into three types, the D-, K- and C-types (**Table 1**). The K- (also known as B) type ODNs have a phosphorothioate backbone and express multiple CpG motifs (Verthelyi et al., 2001; Hartmann et al., 2000; Hartmann and Krieg, 2000). D-type ODNs [also referred to as A (Krug et al., 2001) type] have mixed phosphodiester-phosphorothioate backbones and contain a single hexameric purine/pyrimidine/CpG/purine/pyrimidine motif flanked by self-complementary bases that form a stem-loop structure capped at the 3' end by a

Table 1. Comparison of D-, K-, and C-type oligodeoxynucleotides (Reviewed in Klinman, 2004)

ODN type	Example	Structural characteristics	Immunostimulatory activity
D	GGTGCATCGATGCAGGGGGG	Mixed phosphodiester/ phosphorothioate backbone Single CpG motif (bold) CpG flanking region forms a palindrome (underlined) Poly G tail at 3' end	APC maturation; Preferentially stimulates pDCs to secrete IFN-α
K	ATCGACTCTCGAGCGTTCTC	Phosphorothioate backbone Multiple CpG motifs (bold) TCpGT/ApT 5' motif most stimulatory	pDC maturation and production of TNF Triggers B-cell proliferation, and IgM and IL-6 production
C	TCGTCGTCGTTCGAACGACGTTGAT	Phosphorothioate backbone Multiple CpG motifs (bold) TCG dimer at 5'end CpG motif imbedded in a central palindrome (underlined)	Stimulates B cells to produce IgM and IL-6 Activates pDCs to secrete IFN-α

poly-G tail (Verthelyi et al., 2001). The C-type ODNs resemble K-type in being composed entirely of phosphorothioate nucleotides. C ODNs were originally described as expressing a 'TCGTCG' at the 5'end and frequently contain an internal K-type motif (such as 'GTCGTG') imbedded in a palindrome sequences (Hartmann et al., 2003; Marshall et al., 2003). It was subsequently shown that the 'GTCGTT' motif is optimal for the *in vitro* stimulation of lymphocyte proliferation in several domestic species, including cattle, sheep, goats, horses, pigs, dogs, cats and chickens, while the 'GACGTT' motif was optimal only for inbred rabbit and mice (Mutwiri et al., 2003). *In vitro* and *in vivo* studies in fish have revealed that the oligodeoxynucleotides containing 'GACGTT', 'GTCGTT' or 'AACGTT' motifs have the immune effects in fish [Jørgensen et al., 2001a, 2001b; Jørgensen et al., 2003 (Summarized in **Table 4 & 5**)]. Recent studies have suggested that the TLR9 receptor present in the immune cells of different host species confers the sequence selectivity (Takeshita et al., 2001; Bauer et al., 2001).

The presence and position of a CpG dinucleotide and the sequences that flank it are critical for immunostimulatory activity (Kandimalla et al., 2003). It has been shown that a number of structural modifications and chemical modifications, including ribose modifications, phosphate modifications, deletions of certain nucleobases or substitution of certain flanking nucleotides with linkers influenced the activity of CpG DNA (Chong et al., 2003). Recent study of stimulating human PBMC observed that mixtures of ODNs expressing 3-4 different CpG motifs induce stronger immune responses in a greater fraction of PBMC donors than ODNs expressing single motif and the location of these motifs is important, with those at the 5'-end exerting the greatest influence on ODN activity (Klinman and Currie, 2003). Furthermore, a study on mice demonstrated

that an accessible 5'-end of a CpG DNA is required for receptor recognition and subsequent immune stimulation and the receptor read the DNA sequence from the 5'-end (Kandimalla et al., 2003). New evidence was provided in human that CpG DNA contains a secondary structure at the 3'-end, refer to as self-stabilized CpG DNA, cause human pDCs produce higher levels of IFN-α than CpG DNA without 3'-secondary structures (Chong et al., 2003).

I-2.2.2. *Recognition and signaling of CpG DNA*

The discovery and characterization of the Toll-like receptor (TLR) family facilitated the understanding of the innate immune system. Accumulating evidence demonstrates that individual TLRs identify determinants known as pathogen-associated molecular patterns (PAMPs) that are expressed at high frequency by infectious microorganisms but rarely (if at all) by host cells (Underhill and Ozinsky, 2002). For example, lipopolysaccaride (LPS) present in the surface membrane of Gram-negative bacteria engages the TLR4/MD-2 complex, while peptidoglycan (PGN) and bacterial lipoproteins (BLPs) present in the cell wall of Gram-positive bacteria engage TLR2 (Takeuchi et al., 1999). The interaction of a TLR with its PAMP ligand mediates an intracellular signal that results in the production of versatile chemical mediators and cell surface molecules directing innate as well as acquired immune responses (Underhill and Ozinsky, 2002; Schnare et al., 2001; Vasselon and Detmers, 2002).

All functionally characterized TLRs signal through a common pathway involving myeloid differentiation marker 88 (MyD88), IL-1 receptor I-associated protein kinase (IRAK), TNF receptor-associated factor 6 (TRAF6), transforming growth factor (TGF)-β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1) and 2

(TAB2) and the kinases of IκB (IKK), IκB, and NF-κB [Shuto et al., 2001; Jiang et al., 2002 (**Fig 1**)]. Thus, TLRs are regarded as pattern recognition receptors (PRR) that sense PAMPs (Underhill and Ozinsky, 2002). A series of studies support the conclusion that unmethylated CpG motifs present at high frequency in bacterial DNA, but rare in mammalian DNA also act as PAMPs. The likelihood that CpG motifs act as PAMPs is further supported by evidence that the immune response they induce closely resembles that stimulated by LPS and PGN, and that responsiveness to CpG DNA is completely lost in MyD88 knock out (KO) mice (Hackers et al., 2000). Definitive evidence that CpG recognition is mediated by TLR9 was provided by studies involving TLR9 KO mice (Hemmi et al, 2000). Further evidence from humans also indicates that TLR9 specifically recognizes CpG DNA (Takeshita et al., 2001). Among microbial products and related compounds, CpG DNA is the only ligand for TLR9. TLR9 appears to be differentially expressed in humans and mice. In humans, TLR9 is expressed in B cells and $CD123^+$ dendritic cells (plasmacytoid dendritic cells), whereas in mice TLR9 is expressed in myeloid lineage including myeloid dendritic cells, monocytes, and macrophages (Bauer et al., 2001; Hornung et al., 2002).

Unlike other TLR receptors, which are present on the cell membrane, TLR9 is present on the endosome membrane. CpG DNA is taken up by immune cells via receptor-mediated endocytosis and it interacts with TLR9 present in endocytic vesicles (Hemmi et al., 2000). This is a highly specific interaction, with the triggering of cells being abrogated by elimination of the CpG motif through either inversion or methylation (Hemmi et al., 2000; Takeshita et al., 2001). Co-localization of CpG DNA with TLR9 in endosomal vesicles and the accompanying maturation and movement of those vesicles, seem to be involved in signaling initiation (Takeshita et al., 2004).

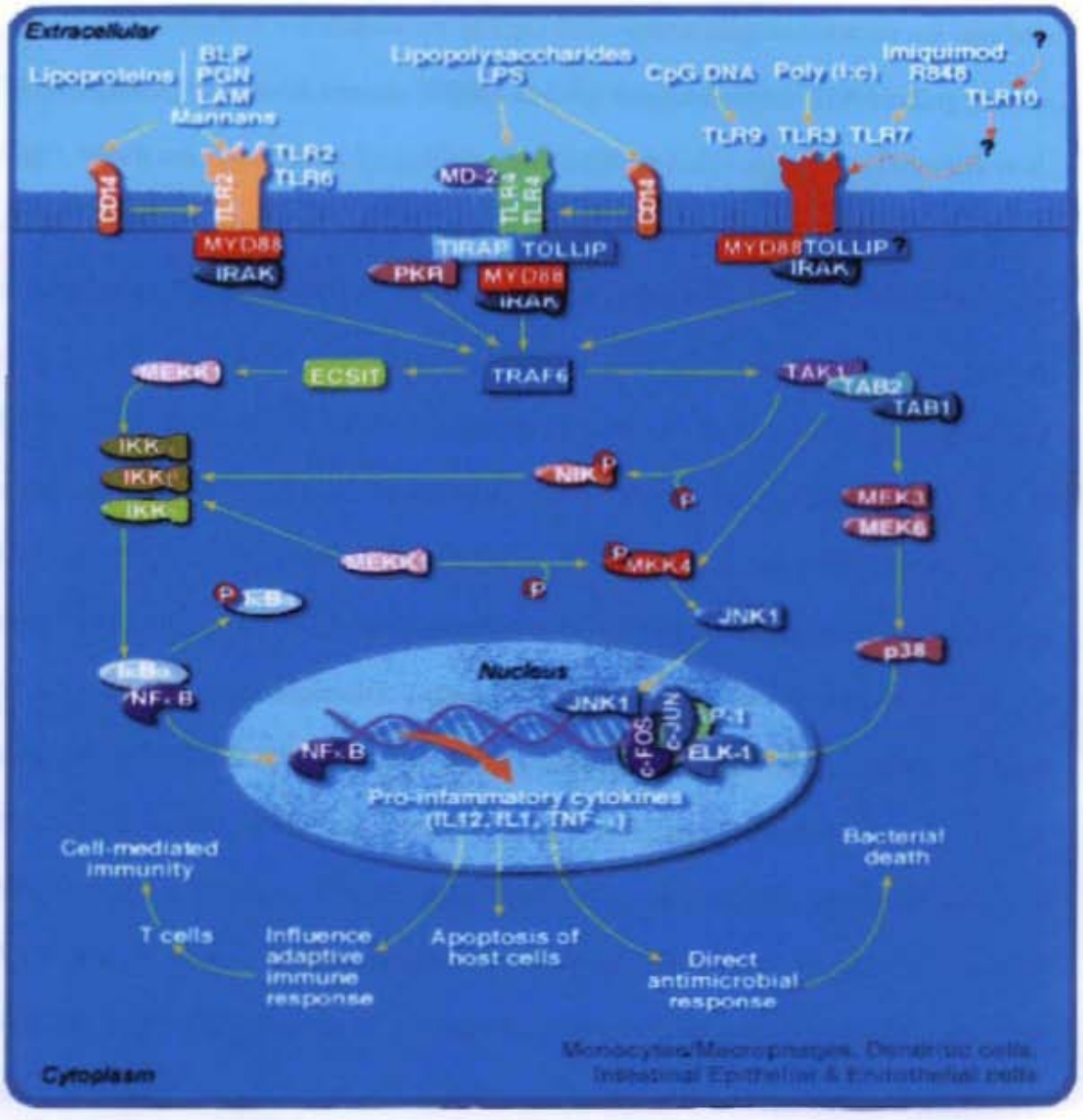

Fig. 1. The innate immune response responds in a general manner to factors present in invading pathogens. Bacterial factors such as lipopolysaccharides (LPS, endotoxin), bacterial lipoproteins, peptidoglycans and also CpG nucleic acids activate innate immunity as well as stimulating the antigen-specific immune response and triggering the inflammatory response. Members of the toll-like receptor (TLR) gene family convey signals stimulated by these factors, activating signal transduction pathways that result in transcriptional regulation and stimulate immune function. TLR2 is activated by bacterial lipoproteins, TLR4 is activated by LPS and TLR9 is activated by CpG DNA. The downstream signaling pathways used by these receptors are similar to that used by the IL-1 receptor, activating the IL-1 receptor associated kinase (IRAK) through the MyD88 adaptor protein and signaling through TRAF-6 and protein kinase cascades to activate NF-κB and c-Jun. NF-κB and c-Jun activate transcription of genes such as the pro-inflammatory cytokines IL-1 and IL-12, which stimulate both the innate immune system and antigen-specific immune response by lymphocytes.

The formation and maturation of CpG DNA-containing endosomes are regulated by phosphatidylinositol 3 kinases (PI3K) and the Ras-associated GTP-binding protein, Rab5, which are essential for the initiation of TLR9-mediated signaling (Takeshita et al., 2004). Rab-5 mediated recruitment of class III PI3K [PI3K (III)] leads to the production of PI (3)P in the endosomal membrane, which binds to the FYVE domain of early endosome antigen 1 (EEA1), recruiting it on to the membrane. The recruited EEA1 also associates with Rab5 and regulates homotypic fusion and trafficking of early endosomes [Backer, 2000; Siddhanta et al., 1998; Vieira et al., 2002 (**Fig 2**)]. The PI (Schnare et al., 2001; Vasselon and Detmers, 2002; Shuto et al., 2001) P3, product of class I PI3K [PI3K (I)], has been demonstrated to activate a signaling cascade consisting of 3-phosphoinositide-dependent kinase-1 (PDK1) and the protein kinase Akt/protein kinase B [AKT/PKB (Ozes et al., 1999; Vanhaesebroeck and Alessi, 2000)]. Ligand-induced association of TLR2 ICD and PI3K (I) was reported to activate the AKT/PKB-NF-κB pathway (Arbibe at al., 2000). CpG DNA also induces phosphorylation of AKT/PKB thereby inhibiting apoptosis in DCs, an effect that is reversed by a PI3K inhibitor, LY294002 (Park et al., 2002). However, recent data demonstrate that DN-p85a, which specifically blocks the function of PI3K (I), but neither DN-PDK1 nor DN-AKT/PKB, inhibits TLR9-mediated NF-κB activation in HEK293 cells (Takeshita et al., 2004). This suggests that (1) PI3K (I) also regulate vesicular trafficking of CpG DNA and TLR9 and/or (2) another pathway mediated by PI3K (I) but not through the PDK1-AKT/PKB pathway is involved in TLR9-mediated NF-κB activation in HEK293. PI3Ks and their second messengers therefore seem to play pivotal roles at distinct steps (i.e. vesicular trafficking for the association between CpG DNA and TLR9 and the signaling pathway directing AKT/PKB activation) in CpG DNA/TLR9-mediated

cellular activation.

Following endocytosis, CpG-ODN co-localizes with the majority of intracellular TLR9 (Hacker et al., 2000; Takeshita et al., 2001) and TLR9 signaling is then mediated through the adaptor protein MyD88 (Hacker et al., 2000; Hemmi et al., 2000), which then activates the IRAK-TRAF6-TAK 1 pathway (Shuto et al., 2001; Jiang et al., 2002; Takeshita et al., 2001). The TLR9 signaling cascade involves mitogen-activated protein kinases (MAPKs), such as p38, c-Jun NH_2-terminal kinase (JNK), extracellular receptor kinase (ERK) and NF-κB-inducing kinase (NIK)-IKK-IκB pathways (Hacker et al., 1999; Yi et al., 2002; Hartmann and Krieg, 2000). The signaling cascades culminates in the activation of several transcription factors including NF-κB, activating protein-1 (AP-1), CCAAT/enhancer binding protein (C/EBP), and camp-responsive element-binding protein (CREB), which directly up-regulate cytokine/chemokine gene expression (Yi et al., 2002; Hacker et al., 1998; Takeshita et al., 2000; Takeshita and Klinman, 2000).

While TLR9 is the only known CpG-ODN receptor to date, a recent report indicating that different classes of ODN utilize different signaling pathways suggests that other receptors or co-receptors may be involved (Kerkmann et al., 2003). In addition, while CpG ODN strongly activates cells that express TLR9 and has essentially no direct effect on cells that do not express TLR9, ODN can increase activity of these latter cells indirectly via PDC-derived cytokines such as IFN α and β (Hornung et al., 2002; Rothenfusser et al., 2001).

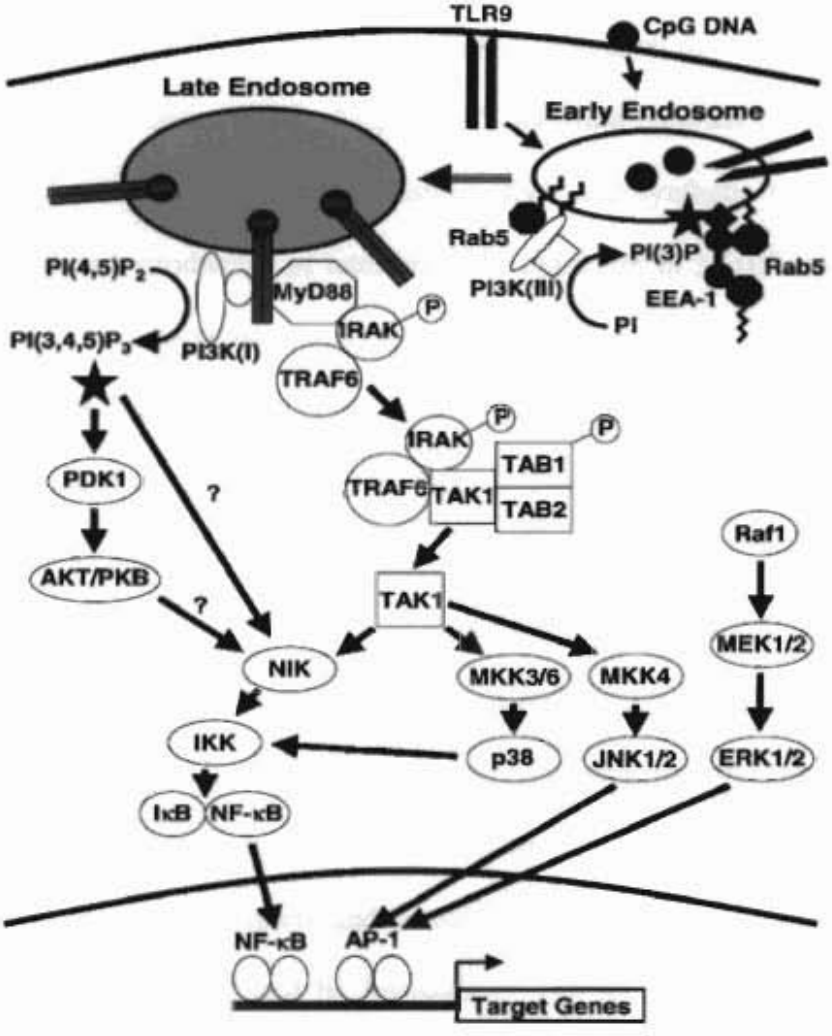

Fig. 2. Scheme of CpG DNA/TLR9-mediated cellular signaling. Class III PI3K (PI3K (III)), EEA1, and Rab5 mediate the trafficking and maturation of endosomes containing CpG DNA and TLR9, by which TLR9 transduces intracytoplasmic signal. The signal initiates with the recruitment of MyD88 to the TIR, which then activates IRAK-TRAF6-TAK1 complex. This leads to the activation of both MAPKs (JNK1/2 and P38) and IKK complex, culminating up regulation of transcription factors including NF-κB and AP-1. Raf1-MEK1/2-ERK1/2-AP-1 pathway is involved in CpG DNA-induced IL-10 production in macrophages. The alternative pathway mediated by class I PI3K (PI3K (I))-PDK1-AKT/PKB is also suggested to be involved in TLR9-mediated cellular activation.

I-2.2.3. *Investigation of the immune effects of CpG-ODNs in fish*

To date, the published data about CpG DNA effects on fish cells are scarce. The specific immunostimulatory effect of CpG-ODN was first reported by Jørgensen et al. (2001a) who demonstrated that both plasmid DNA and synthetic ODNs containing CpG-motifs induced production of interferon-like cytokines in Atlantic Salmon (*Salmo salar* L) leucocytes. Jørgensen et al. (2001b) also demonstrated that rainbow trout (*Oncorhynchus mykiss*) macrophages not only produce IFN-like cytokines, but also express IL-1 β when stimulated with CpG-ODNs. The strong activating effect of CpG-ODNs on fish leucocytes suggests the use of CpG-ODN as an immunostimulant in fish. Although the immune system and associated mechanism in fish have not been studied as thoroughly as those in mammals, the macrophage has been known to play an important role in defence against invading microorganism in fish. More research is needed to investigate the effect of CpG-ODNs on the immune system of fish.

In order to gain a complete understanding of the immune status of a test fish, many immune parameters should be investigated that are representative of different components (innate and adaptive, as well as humoral and cellular reactions) of the immune system. Köllner et al. (2002) propose an experimental set up, which includes functional assays, such as activation and proliferation of leucocyte populations, macrophage phagocytosis and respiratory burst, secretion of antigen-specific antibodies, specific cell-mediated cytotoxicity, as well as challenge models with bacterial and viral pathogens. The head kidney (HK) is an important organ for innate and adaptive immune defenses. Several immune-defence related peptides and proteins including antimicrobial agents, activators and regulators of the immune system were detected in the head kidney leucocytes.

In the thesis at hand, effects of CpG-ODNs on the innate immune system of common carp (*Cyprinus carpio* L.) were investigated (Chapter II). These studies report for the first time that CpG-ODNs activate the cell-mediated immunity in fish. In addition, the effects of CpG-ODNs on the expression of immune-relevant genes in the head kidney of carp were also investigated by Expressed Sequence Tags (EST) approach (Chapter III) and Semi-quantitative analysis (Chapter IV).

Chapter II

The immunostimulatory effects of CpG oligodeoxynucleotides on the innate immune responses of common carp

Mammalian immunity relies on two branches to defend against invading microbial pathogens: innate and adaptive immunity. The latter systems are composed of cells with clonally distributed, highly specific receptors that mediate pathogen recognition. These receptors undergo somatic reorganization to ensure the development of a large set of immune cells with distinct antigen specificity. Although this receptor diversity enables the immune system to recognize pathogens in a very specific mode it has the disadvantages that each receptor specificity is only expressed on a limited number of cells. Thus, cells of the adaptive immune system first have to proliferate after antigenic contact to ensure an appropriate immune response and these results in the delay of adaptive immune response.

In contrast, innate immunity provides a first line of defense with immune mechanisms that can immediately be activated. Nevertheless, innate immunity also has to cope with the task to ensure a specific activation only in response to foreign pathogens. In this regard, it was the elegant concept of Charles Janeway which shed light on pathogen recognition mechanisms within the innate immune system (Medzhitov and Janeway, 1997). According to this concept, which has now been proven experimentally by many groups pathogen-associated molecular patterns (PAMPs) are recognized by a limited set of pattern recognition receptors [PRRS (described in the chapter I)]. Such patterns mainly comprise bacterial cell wall components, yet viral, fungal and parasite structures have also been classified as PAMPs. PAMPs therefore

comprise essential and evolutionary conserved microbial structures. According to Janeway's concept innate immunity is able to recognize the whole set of microbial pathogens with only a limited set of receptors which is expressed on most of the innate cells thus avoiding the necessity of clonal proliferation after primary microbial contact.

Among the PRRs Toll-like receptors have now been identified to play a most important role, and natural ligands have been identified for most of the 10 TLRs [Takeda et al., 2003; Tabeta et al., 2004 (**Fig 1**)]. TLR activation in general results in the activation of innate immunity including secretion of pro-inflammatory cytokines and induction of antimicrobial effector mechanisms. In addition, TLR triggering induces up-regulation of co-stimulatory (CD80, CD86, CD40) and secretion of cytokines (IL-12) in antigen-presenting cells, increases antigen processing and leads to increased peptide-MHC complex stability. As a result the antigen-presenting machinery is activated (Medzhitov, 2001) and therefore induction and shaping of subsequent adaptive immune responses is critically controlled by TLRs (Schnare et al., 2001). With this knowledge in mind it became feasible to utilize synthetic TLR ligands to modulate innate as well as adaptive immune responses. Among such synthetic TLR ligands oligonucleotides with immunostimulative CpG motif that trigger TLR9 have gained special interest.

CpG-ODNs mimic the direct immunostimulatory effects of native bacterial DNA and activate multiple cell types including macrophages, dendritic cells, Natural Killer (NK) cells, and B lymphocytes (Tokunaga et al., 1984; Messina et al., 1991; Krieg et al., 1995; Stacey et al., 1996; Klinman et al., 1996; Hartmann et al., 1999). The diverse immunostimulatory effects of synthetic CpG-ODNs are well established in mice and humans. Based on the success results obtained from mouse models, clinical trials

continuing at various stages in humans to evaluate CpG ODN therapy against infectious disease, cancer, asthma and allergy (Verthelyi and Klinman, 2003). Accumulated evidence has revealed that CpG-ODNs can also activate the immune systems in nonhuman primates (Verthelyi and Klinman, 2003), domestic animals including cattle, sheep, pigs, horses, dogs, cats, and chickens (Mutwiri et al., 2003). To date, knowledge about effects of CpG-ODNs on the fish immune system is still sparse. The study at hand has investigated the *in vitro* and *in vivo* effects of CpG-ODNs on the innate immune responses of common carp.

Chapter II-1

In vitro effects of CpG oligodeoxynucleotides on the cell-mediated immunity of common carp

II-1.1. Introduction

Like mammals, fish rely on both innate and adaptive mechanisms to protect themselves against invading pathogens. In fish, the primary lines of innate defences are the skin and mucus. When pathogens enter the body, cellular and humoral innate defences are mobilized. Phagocytosis is one of the main mediators of innate immunity to pathogens including bacteria, viruses, and parasites in fish. The most important cells involved in this defence are the phagocytes. The activities of phagocytic cells can be detected by phagocytosis, killing and chemotaxis. Enhancement of pathogen killing is most important in the macrophages of fish treated with immunostimulant. Killing mechamisms of macrophages can be broadly categorized as oxygent-dependent or oxygen-independent. Oxygen-dependent killing mechanisms as mediated by reactive oxygen species (ROS) can be detected by the chemiluminescence and the nitroblue tetrazolium (NBT) test (Sakai, 1999). These ROS such as the superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^1O_2$), play an important role in the antimicrobial activity of phagocytic cells.

Nitroblue tetrazolium (NBT) is a yellow water-soluble nitro-substituted aromatic tetrazolium compound that reacts with cellular superoxide ions to form formazan derivative that can be monitored spectrophotometrically (Baehner et al, 1976; Armstrong et al, 2002). The cytoplasmic NADPH, which is produced by oxidation of glucose through the hexose monophosphate shunt, serves as an electron donor. The

oxidase system available in the cytoplasm helps transfer electrons from NADPH to NBT and reduce NBT into formazan (Baehner et al, 1976). Thus, the NBT reaction indirectly reflects the ROS-generating activity in the cytoplasm of cells and therefore can help determine the cellular origin of ROS in a heterogeneous suspension. This was the reason to justify development of the NBT test and use of NBT staining in individual cells, that is, the leukocytes and measurement of the formazan precipitation due to NBT reduction.

For the initiation of cellular and humoral response mechanisms, proliferation of B and T lymphocytes play an important role in fish as in higher vertebrates. The extent of lymphocyte proliferation after antigenic or mitogenic stimulation may be used as a parameter to assess immunocompetence (Lunden and Bylund, 2000).

As described earlier in the chapter I, CpG-ODNs stimulate the innate immune system and have been shown to be protective against a variety of pathogens in various animal models. There are no reports concerning the immunostimulatory effects of CpG-ODNs on the cell-mediated immunity in fish. The aim of present study was to determine if CpG-ODNs could affect *in vitro* on the head kidney phagocyte and lymphocyte activities in common carp.

II-1.2. Materials and Methods

II-1.2.1. *Fish*

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

II-1.2.2. *Preparation of CpG-ODNs*

A panel of synthetic oligodeoxynucleotides (**Table 2**) was provided by SAWADY (Japan). The ODNs were solubilized in sterile water at a concentration of 1 μg/ μl and stored at –20 °C.

II-1.2.3. *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). The kidneys were removed and pushed through a nylon mesh with RPMI 1640 medium (Nissui, Japan) containing 1 % streptomycin/penicillin (Sigma), 0.2 % heparin (Sigma) and 10 % carp serum (CS).

For the NBT and phagocytic assays, the cell suspension was placed on 34/51 % Percoll gradient and centrifuged at 400 × g for 40 min at 4 °C. The macrophage-enriched cells from 34/51 % Percoll interface were separated and centrifuged at 500 × g for 5 min and washed three times with the medium. Viable phagocytic cells including neutrophils (about 10 %) and macrophages (about 90 %) were counted by trypan blue exclusion.

For the lymphocyte proliferation assay, after cell clumps and debris had been removed by sedimentation for 1 h, the kidney cell suspension was centrifuged at 500 × g

for 5 min, resuspended in RPMI 1640 medium and adjusted to the required cell concentration of 5×10^6 lymphocytes/ml.

II-1.2.4. *Nitroblue tetrazolium (NBT) assay*

The viable cells were adjusted to 10^7 cells/ml in Hank's balanced salt solution (HBSS; Nissui). One hundred microlitres of this suspension were added to each well of the microtitre plates (Nunc, USA). After 2 h at 20 °C, unattached cells were washed off with HBSS and monolayers were fed with 0.9 ml RPMI 1640 supplemented with 10 % CS, 1 % S/P and 0.01 ml ODNs (10, 100, 1000 ng/ml) and maintained at 20 °C overnight.

Determination of phagocytic activity by NBT test was performed using a method described by Chung & Secombes (1988). The phagocytic cell monolayers were washed two times with HBSS after ODNs treatment and 100 µl of NBT solution (1 mg/ml in RPMI 1640 medium) which included phorbol myristate acetate [1 mg/ml (PMA, Sigma)] was 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 (O.D.) was measured by a multiscan spectrophotometer (Pharmacia, Sweden) at 620 nm.

II-1.2.5. *Phagocytic activity*

The number of cells was adjusted to 10^7 cells/ml in RPMI 1640 medium containing 10% CS and cells were allowed to adhere to a glass coverslip (22 × 22 mm) for 1 h after which non-adherent cells were removed by washing with HBSS. The

phagocytic cells were allowed to react with 10 ng ODNs in RPMI 1640 containing 10 % CS overnight at 20 °C. The phagocytic activity of carp kidney leucocytes was examined as described by Yoshida et al. (1993).

Latex particles (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 slips were picked up using forceps and washed with the medium for 1 min. Cells were fixed with methyl alcohol, air-dried and stained with Giemsa. The number of adhered cells was about 5×10^5 cells/cover slip and the number of phagocytic cells per 300 adhered cells was counted microscopically. The phagocytic activity (PA) was determined using the formula:

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

II-1.2.6. ***Proliferation assay***

To test the proliferative ability of the leucocytes, Aliquots of 100 μl of cell suspension (5×10^6) were added to wells of 96-well tissue culture plates containing 10, 100 and 1000 ng/ml of synthetic oligodeoxynucleotides or 60 μg/ml of Phytohaemagglutinin (PHA). Controls without mitogen were also included. The plates were incubated at 20 °C for 4 days before addition of 50 μl XTT solution (Roche, Germany). After 16 h (at 20 °C), the optical density was measured by a multiscan spectrophotometer (Pharmacia, Sweden) at 620 nm. The results of the stimulation tests were expressed as a stimulation index (SI), which was calculated according to the

formula:

$$SI = \frac{\text{Mean O.D. of stimulated culture}}{\text{Mean O.D. of non-stimulated control}}$$

II-1.2.7. *Statistical analysis*

The data was expressed as mean ± SD. The data was analyzed using the Student's *t*-test.

Table 2. Synthetic oligodeoxynucleotides (ODNs) used in the study

ODN[a]	Sequence (5'- 3')[b]	Number of C - G[c] in ODN
A	TCC AT**G ACG TT**C CTG ATG CT	1
B	GCT A**GA CGT TAA CGTT**	2
C	AT**C GA**C TCT **CGA ACG TT**C C	3
D	GAA CCT TCC ATG CTG TTC **CG**	1 (terminus)
E	GCT AGA TGT TAG **CG**T	1 (terminus)
F	CTG GTC TTT CTG GTT TTT TTC TGG	0 (non-CpG)
G	TCC AT**G AGC TT**C CTG ATG CT	0 (non-CpG-ODN and altered CpG motifs are underlined)

[a] ODN are listed alphabetical order.

[b] Nucleotide sequence of ODN. The **GACGTT** motif is strongly immunostimulatory in mice and fish. The **AACGTT** motif is strongly immunostimulatory in fish.

[c] Number of CpGs in ODN. CpGs located at the terminus of ODNs D and E, is known ineffective in mice. The non-CpG-ODNs F and G used as control.

II-1.3. Results

II-1.3.1. *Nitroblue tetrazolium (NBT) assay*

The effect of ODNs on the reduction of NBT in carp phagocytic cells is shown in **Fig. 3**. The NBT reduction was significantly higher in cells incubated with CpG-ODNs A/1668 and C at a concentration of 10 ng/ml and CpG-ODN B at a concentration of 1000 ng/ml ($*P < 0.05$).

The maximum difference between ODNs treated groups and control groups was in the leucocytes treated with CpG-ODN C at a concentration of 10 ng/ml ($*P < 0.05$). The cells incubated with non-CpG ODN F did not show a significant increase in NBT reduction.

II-1.3.2. *Phagocytic activity*

Fig. 4 shows the phagocytic activities of kidney leucocytes incubated with ODNs. In comparison with the control leucocytes, the cells incubated with CpG-ODN C showed significantly enhanced phagocytosis ($*P < 0.05$), whereas the cells incubated with ODNs D, E and F did not. Phagocytosis of cells incubated with CpG ODN A/1668 and B were higher than that of the controls, but not significantly.

II-1.3.3. *Proliferation assay*

The results are presented in **Fig. 5**. Stimulation indices were significantly higher than the controls in head kidney cells treated with CpG-ODN A/1668 at concentrations of 10 and 100 ng/ml, CpG-ODN C and D at a concentration of 100 ng/ml ($*P < 0.05$), and CpG-ODN B at a concentration of 1000 ng/ml ($**P < 0.01$). The stimulation indices were significantly lower in cells treated with varying concentrations of CpG-

ODN E ($^{\#}P < 0.05$).

The maximum stimulation of mitotic activity was demonstrated in the leucocytes of fish treated with PHA at a concentration of 60 μg/ml ($^{**}P < 0.01$).

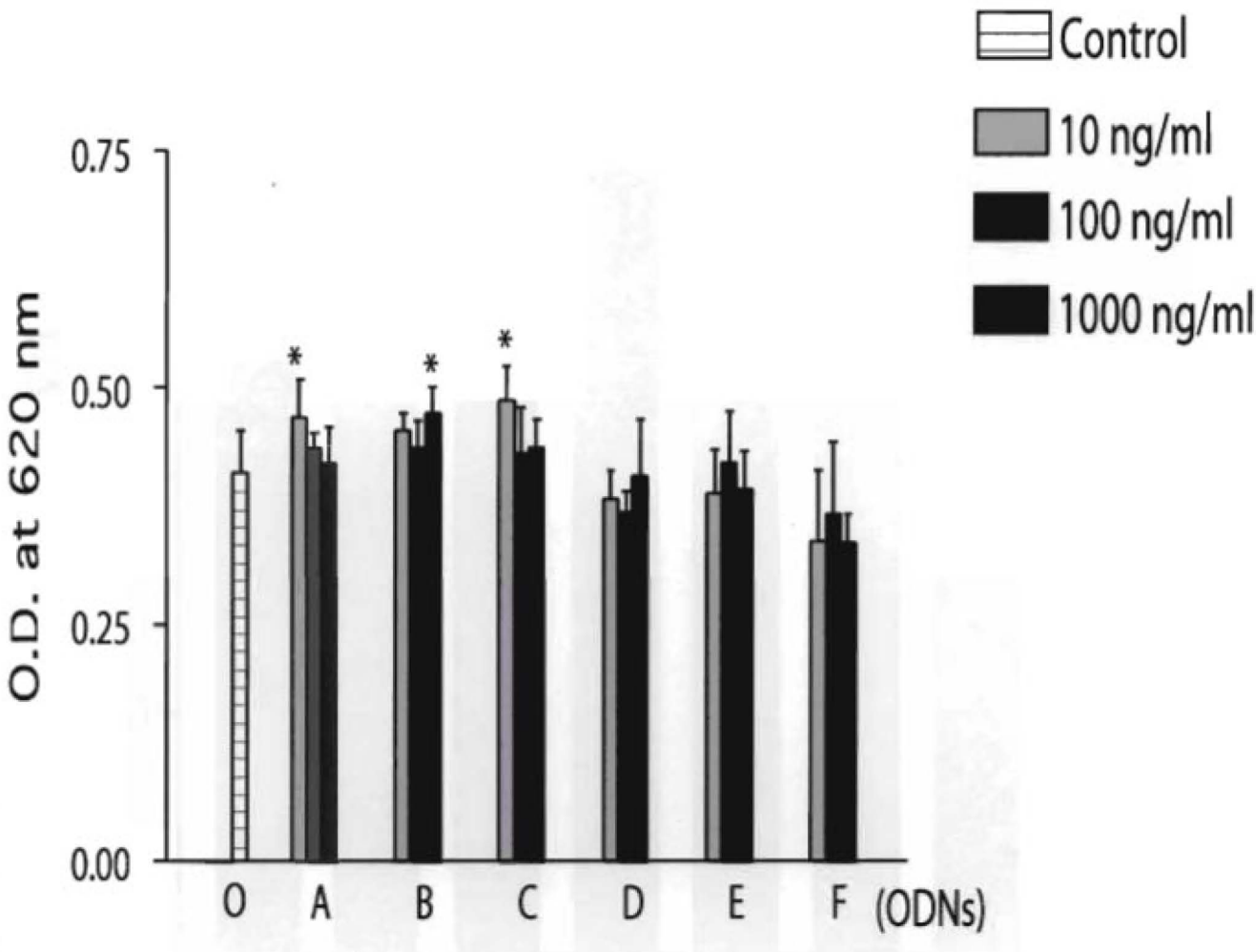

Fig. 3. The nitroblue tetrazolium (NBT) activity in carp phagocytic cells treated with different concentrations of CpG oligodeoxynucleotides. Values are mean ± S.D. at 620 nm above spontaneous NBT reduction after 60 min incubation measured in six fish. $^{*}P < 0.05$.

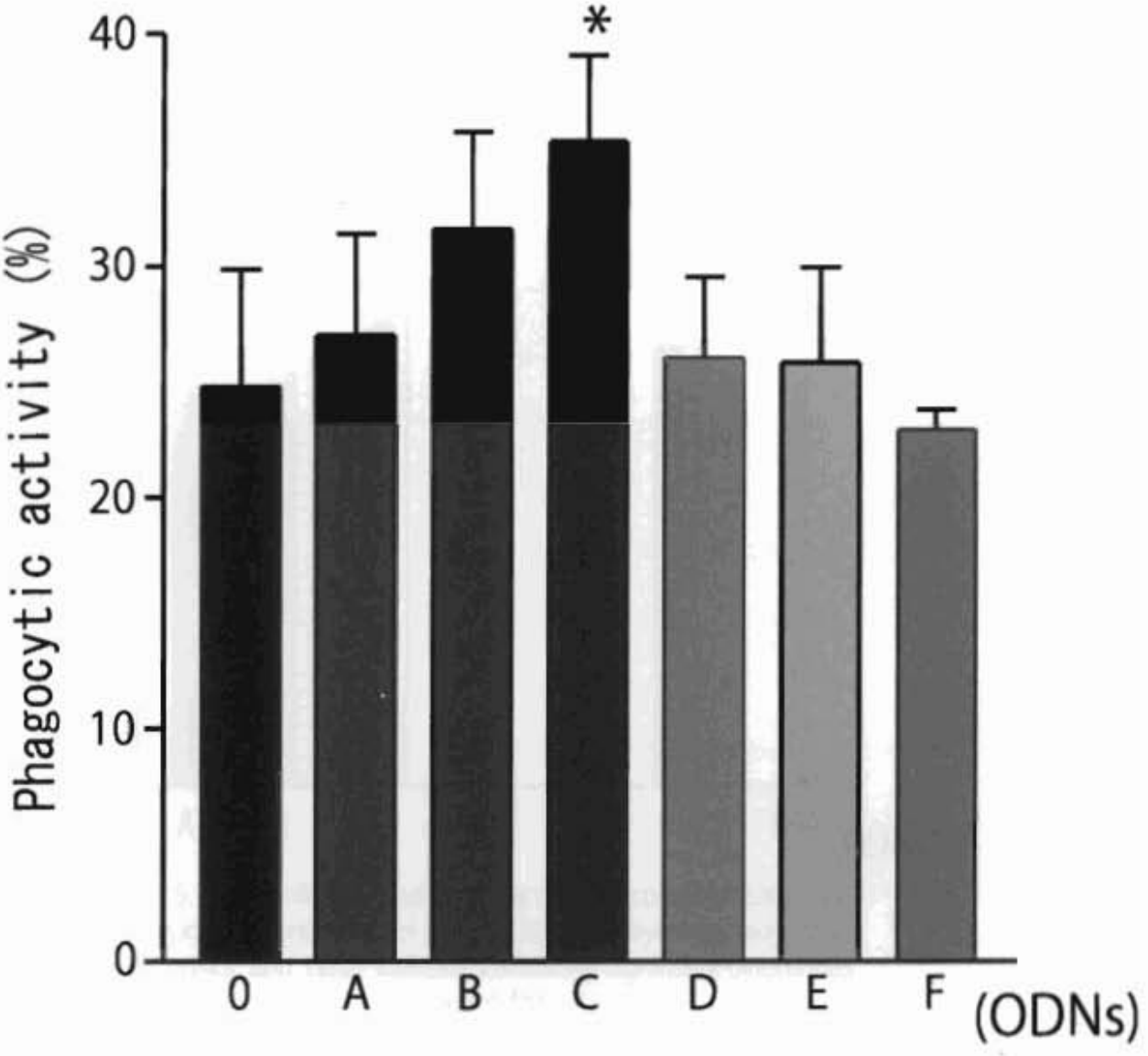

Fig. 4. The phagocytic activity of common carp phagocytic cells treated with 10 ng/ml of CpG oligodeoxynucleotides. Values are mean ± S.D. of the percentage of cells with phagocytized latex particles. Asterisks indicate statistically significant differences compared to untreated control fish. $^{*}P < 0.05$.

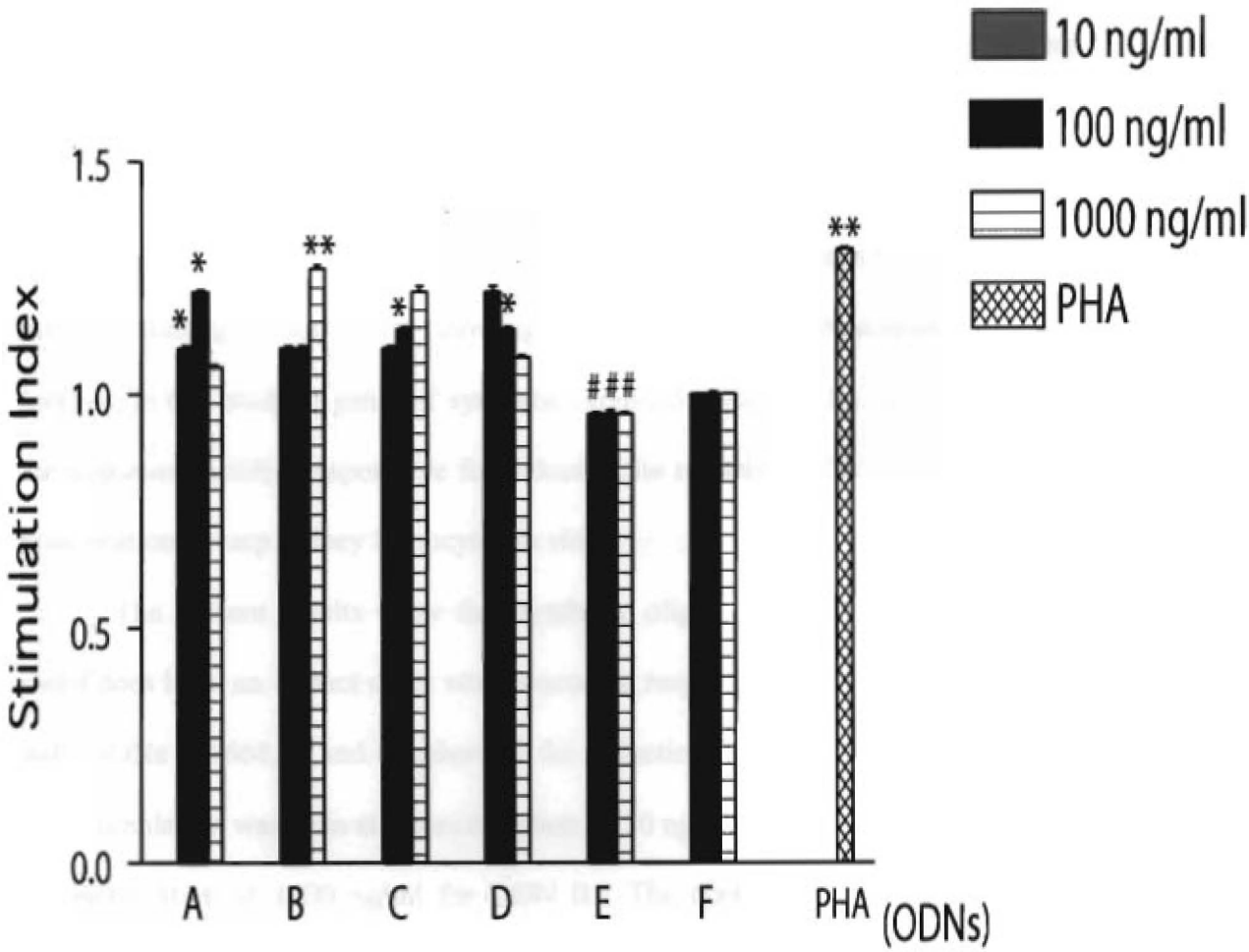

Fig 5. The proliferation of common carp leucocytes treated with varying concentrations of CpG-ODNs and phytohaemagglutinin. Asterisks and hash indicate statistically significant differences compared to untreated control fish. $^{*}P < 0.01$, $^{**}P < 0.01$, #P < 0.05.

II-1.4. Discussion

In vitro immunoassays using fish organ sections have many applications: 1. testing and comparing bacterins and vaccines, 2. showing the effects of toxicants, contaminants or pollutants on the immune response, 3. determining media preferences for fish cells undergoing an immune response, 4. studying variability of the immune response among fishes and 5. showing the effects of immunostimulants (Anderson, 1992). In this study, a panel of synthetic oligodeoxynucleotides was used to examine the sequence motif(s) responsible for inducing the reduction of NBT, phagocytosis and proliferation in carp kidney leucocytes *in vitro*.

The present results show that synthetic oligodeoxynucleotide containing CpG motif does have an impact on *in vitro* leucocyte responses. The kidney cells incubated with ODNs A/1668, B and C enhanced the reduction of NBT in carp phagocytic cells. This stimulation was seen at a concentration of 10 ng/ml for ODNs A/1668 and C and at a concentration of 1000 ng/ml for ODN B. The maximum response was shown in kidney cells incubated with CpG-ODN C at a concentration of 10 ng/ml. The activation of those ODNs A, B and C is associated with the presence of fish-immunostimulatory motifs 5'-AACGTT-3' (B and C) and 5'-GACGTT-3' [A and B (**Table 2**)]. Membrane stimulation, such as the adherence of particle to macrophage or neutrophil membrane or contact with soluble activating agent phorbol myristate acetate (PMA), triggers the production of microbicidal oxygen free radicals or reactive oxygen species (ROS) as well as an increase in their oxygen consumption by a phenomenon termed the respiratory burst (Sakai et al., 1996; Secombes et al., 1988; Sharp & Secombes, 1993). Carp phagocytic cells such as macrophages or neutrophils produce superoxide anion after stimulation with PMA as detected by NBT reduction assay. The superoxide anion

producing the respiratory burst is a powerful bactericidal agent in the phagocytic cells.

The CpG-ODN C containing palindromic sequence 5'-AACGTT-3'added *in vitro* to carp kidney leucocytes at concentration 10 ng/ml increased phagocytosis against latex particles. This study provides new evidence that CpG-ODNs stimulate phagocytic cell activities. Sakai (1999) reported that fish treated with immunostimulants usually show enhanced phagocytic cell activities. The immunostimulants such as levamisole (Siwicki et al., 1990) and LPS (Solem et al., 1995) increase the reduction of NBT and phagocytosis in fish phagocytic cells by *in vitro* treatment. In addition, many studies in fish have shown that phagocyte spreading and respiratory burst activity can be increased *in vitro* by the addition of substances such as glucans, muramyl dipeptide, mitogens (Con A) and autonomic neurotransmitters [alpha and cholinergic receptor agonists (Chung and Secombes, 1987; Smith and Braun-Nesje, 1982; Secombes, 1986; Bayne and Levy, 1991; Flory and Bayne, 1991)].

Studies on fish lymphocytes have concentrated on the general characteristics of cell-mediated and humoral immunity which are now relatively well established. The existence of distinct B and T lymphocyte populations is supported by experiments incorporating mitogen responsiveness, the presence of cell surface immunoglobulin, physical separation characteristics and cellular co-operation in generating antibody responses *in vitro* (reviewed in Rowley et al., 1988). Fish lymphocytes are known to proliferate in response to many stimulants including the classical T and B cell mitogens, PHA and LPS respectively, *A. salmonicida* antigens (Marsden et al., 1995) and blood fluke antigens (Richards et al., 1996). In this study, the CpG-ODN A, B and C induced a direct proliferative response on carp kidney leucocytes *in vitro*. However, maximal responses were obtained in cells treated with mitogen PHA at a concentration of 60

μg/ml. The responsiveness of common carp leucocytes in the present research is rather similar to that described for other fish in the literature. Galeotti et al. (1999) reported that, in some cases, the *in vitro* mitogenic responses of leucocytes of different fish species vary with the type of mitogen used. These differences might reflect differences in mitogen receptors on leucocytes or differences in leucocyte populations in the lymphoid organs of different species.

Since fish cells may respond to CpG motifs different from those of previously tested animals, recently, Jørgensen et al. (2003) examined the ability of a panel of synthetic oligodeoxynucleotides to stimulate Atlantic salmon leucocytes *in vitro*. In other species it has been shown that CpG DNA are mitogenic for peripheral blood leucocytes (PBL) and assays to measure the proliferation have been used to screen for activity (Hartman et al., 2000; Krieg et al., 1995). Therefore, in an introductory experiment, they examined proliferative responses of fish PBL to CpG-ODNs. The results showed that Atlantic salmon PBL respond to CpG-ODNs in a sequence specific manner and proliferative responses varied among different CpG-ODNs, so this type of assay was found to be suitable for activity screening and it was combined with the IFN assay. Based on previous results in which ODN 1668 and 1670, with the murine motif (GA/AA)CGTT, was shown to stimulate antiviral activity in Atlantic salmon leucocytes (Jørgensen et al., 2001a; Jørgensen et al., 2001b), they tested the capacity of a panel of different CpG phosphothiorate ODN to stimulate proliferation of PBL and to induce production of interferon-like factors in head kidney leucocytes. The results showed that the sequence and number of the CpG motifs as well as the lengths of the ODN contribute to their stimulatory activity and ODN with the 6-mer CpG motif (GTCGTT) showed the highest stimulatory activity. These results verify what is described in other

studies, that different species differ in their response to specific CpG motifs and that the motif 'GTCGTT' is the most effective in most species, including cattle, primates, cats, and dogs (Mutwiri et al., 2003). Whilst most of published data about CpG-ODN effects in fish are focused on head kidney leucocytes, these studies report for the first time that CpG-ODN could activate peripheral blood leucocytes in fish.

From a panel of synthetic oligodeoxynucleotides (A, B, C, D, E and F), CpG-ODN C has the highest stimulatory capacity. CpG-ODN C at a concentration of 10 ng/ml enhanced the reduction of NBT and phagocytosis of carp phagocytic cells. CpG-ODN C at a concentration of 100 ng/ml induced proliferation of the carp lymphocytes. Multiple CpGs generally resulted in greater stimulatory capacity (C), although CpGs located at the terminus of an ODN were ineffective [D and E (Klinman et al., 1996)].

In conclusion, the results of the current *in vitro* studies indicate that CpG-ODNs could potentially improve immunocompetence cell activity in fish through increased cell proliferation and functionality.

Chapter II-2

The *in vivo* effects of CpG oligodeoxynucleotides on the innate immune responses of common carp

II-2.1. Introduction

The innate immune system is comprised of physical barriers to infection, humoral defenses, and phagocytic cells. Teleosts are protected from infection by a variety of barriers, including a mucous coat which has been shown to contain lysozyme, complement and trypsin-like proteases which can degrade gram-negative bacteria and complement proteins. Lysozyme is a widely distributed enzyme, located in the serum, mucus, and many other tissues of fish and other higher vertebrates. Lysozyme contributes the innate immunity of animals by its bactericidal and anti-inflammatory properties (Paulsen et al., 2003).

There are many examples of substances able to enhance phagocyte activity. Two approaches have been adopted generally; administration of substances *in vivo* followed by testing *in vitro* or direct addition to isolated phagocytes *in vitro*. The latter approach eliminates the problems of inter-fish variation and potential indirect effects caused by stimulating a mixed cell population. However, the former approach may be more relevant when considering ways to increase *in vivo* disease resistance (Secombes and Fletcher, 1992).

The evidences from *in vitro* studies have shown that CpG-ODNs stimulate the cell-mediated immunity of carp (Chapter II-1). However, there are no reports about the *in vivo* effects of CpG-ODNs on the cellular and humoral innate reponses in fish. Based on previous results from *in vitro* study in which CpG-ODN B and C, with the mice and

fish motif (GA/AA)CGTT, was shown to stimulate phagocyte and lymphocyte activities in carp head kidney leucocytes, *in vivo* studies were undertaken to examine the capacity of these CpG-ODNs to stimulate phagocytic and serum lysozyme activities in carp.

II-2.2. Materials and methods

II-2.2.1. *Fish*

A total of 200 common carp (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.

II-2.2.2. *Preparation of CpG-ODNs*

The CpG-ODN B & C (**Table 2**) were suspended in phosphate buffer saline [PBS (10 μg/100 μl)] and injected into carp at a dose of at a dose of 0.1, 1 and 10 μg/fish intraperitoneally. Control fish received an equal dose of PBS alone by an intraperitoneal injection. Six fish of each group were sampled at 1, 5 and 7 days after injection.

II-2.2.3. *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 (Refer chapter II-1, Materials and Methods II-1.2.3)].

II-2.2.4. *Preparation of carp serum*

Serum obtained from the caudal vessels of carp treated by CpG-ODNs and from fish used as control was used for lysozyme assay.

II-2.2.5. *Nitroblue tetrazolium (NBT) assay*

Determination of phagocytic activity by NBT test was performed using a method

as described by Chung & Secombes [1988 (Refer chapter II-1, Materials and Methods II-1.2.4)].

II-2.2.6. *Phagocytic activity*

Six 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 × 22 mm) for 1 h and non-adherent cells were removed by washing with the medium. The phagocytic activity of carp kidney leucocytes was examined as described by Yoshida et al. [1993 (Refer chapter II-1, Materials and Methods II.1.2.5)].

II-2.2.7. *Serum lysozyme activity*

The lysozyme activity was assayed by the turbidimetric method described by Parry et al. (1965). Serum of six fish from each group was assayed individually 1, 5 and 7 days post treatment.

II-2.2.8. *Statistical analysis*

The data was expressed as mean ± SD. The data was analysed using Student's *t*-test.

II-2.3. Results

II-2.3.1. *NBT reduction of leucocytes*

The NBT reduction was significantly higher in cells isolated from fish treated with ODNs B & C than those isolated from the control fish at day 5 sampling times [$*P < 0.05$ (**Fig. 6**)]. The maximum difference between ODNs treated groups and control groups showed in the leucocytes of fish treated with 10 μg/fish of ODN C.

The NBT reduction by leucocytes from fish treated with 10 μg/fish ODN C significantly increased 5 to 7 days after treatment (**Fig. 7**).

II-2.3.2. *Phagocytic activity*

The phagocytic activity of the kidney leucocytes from carp treated with CpG-ODNs B & C was significantly higher than the leucocytes from control fish at 1 day after injection (**Fig. 8**). The maximum stimulation of phagocytic cells was demostrated in the leucocytes of fish treated with 1 μg/fish of CpG ODN C.

The duration of increased phagocytosis in fish treated with CpG-ODN C at 1 μg/fish are shown in **Fig. 9**. The maximum difference was evident 1 day after the administration of ODN C. This stimulation continued at least 7 days post-treatment.

II-2.3.3. *Serum lysozyme activity*

The serum lysozyme activity of fish treated with ODNs B & C significantly increased at 5 days after treatment (**Fig. 10**). The highest activity was observed in the serum of fish treated with 10 μg/fish of ODN C.

Fig. 11 shows the duration of increased serum lysozyme activities in fish treated with ODN C at 10 μg/fish. The maximum stimulation was evident 5 days after injection.

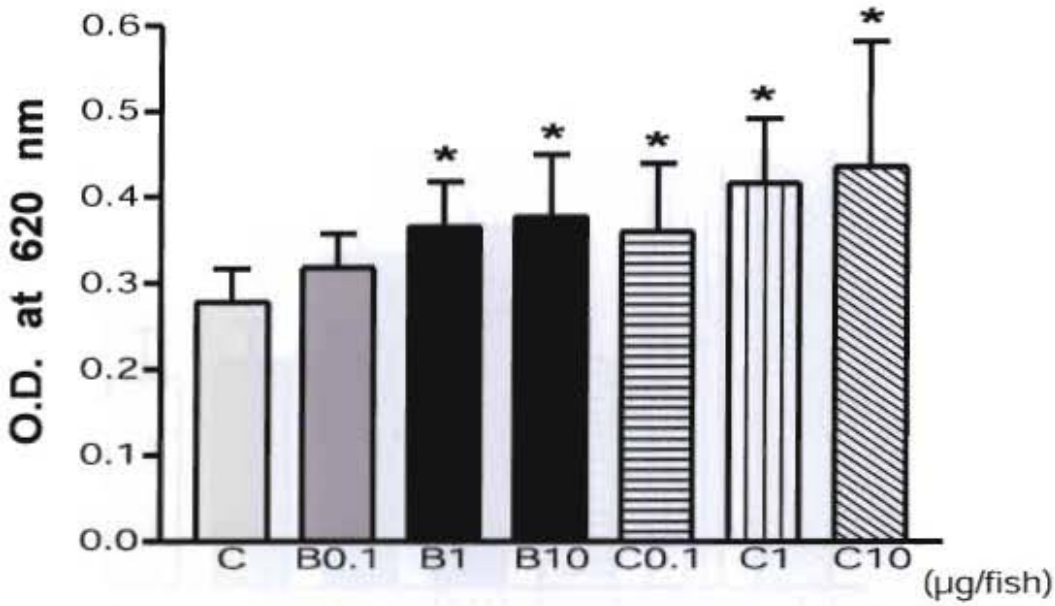

Fig. 6. The dose responses of nitroblue tetrazolium (NBT) activity in leucocytes of carp injected with CpG-ODNs. The NBT reduction was examined 5 days after injection. Values are mean ± S.D. at 620 nm above spontaneous NBT reduction after 60 min incubation measured in 6 fish. Asterisks indicate statistically significant differences between control and different concentrations of CpG oligodeoxynucleotides. * $P < 0.05$.

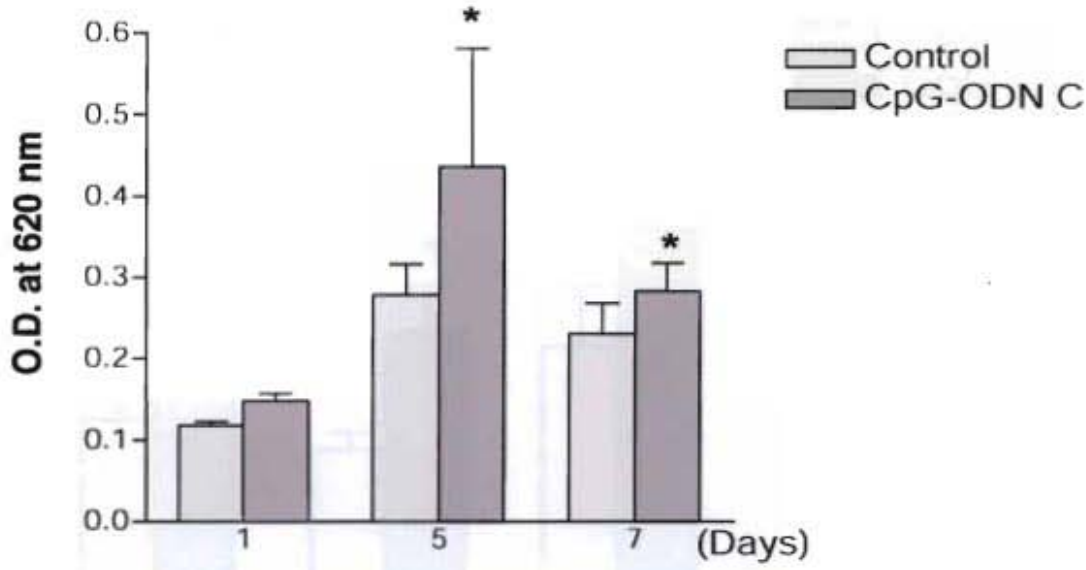

Fig. 7. The nitroblue tetrazolium (NBT) activity of carp injected with 10 μg of CpG-ODN C. The NBT reduction was examined 1, 5 and 7 days post-injection. Values are mean ± S.D. at 620 nm above spontaneous NBT reduction after 60 min incubation measured in 6 fish. * $P < 0.05$.

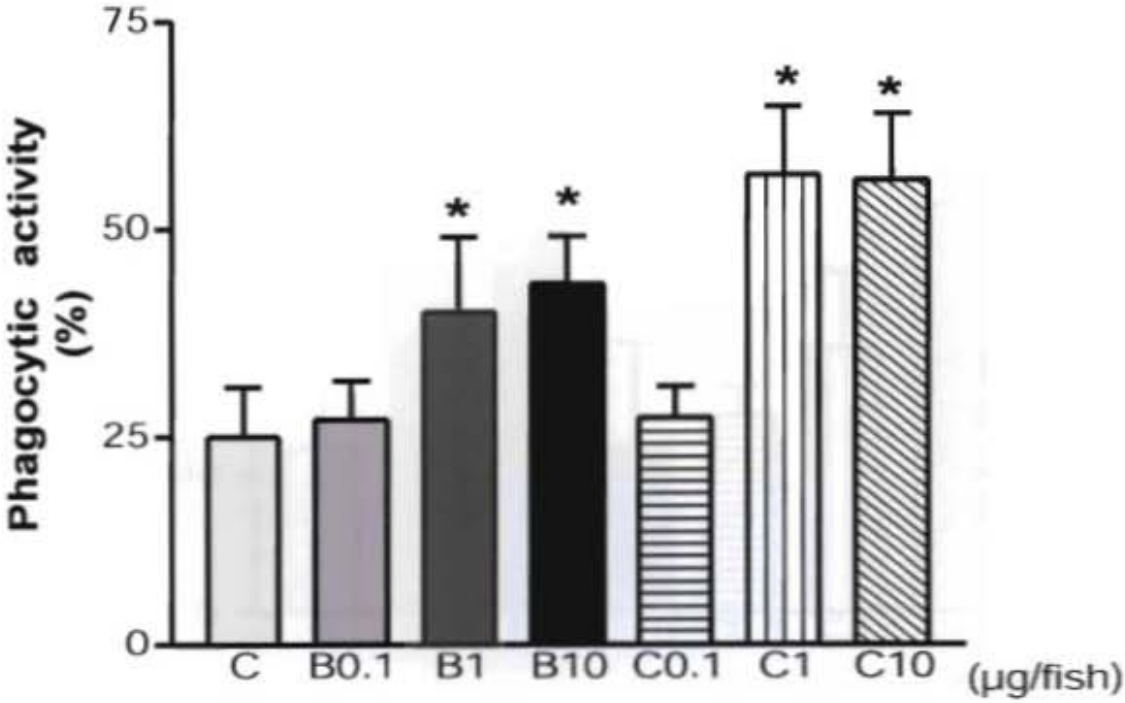

Fig. 8. The dose responses in the phagocytic activities of leucocytes of carp injected with CpG oligodeoxynucleotides. The phagocytic activity was examined 1 day post-injection. Values are mean ± S.D. in 6 fish. Asterisks indicate statistically significant differences between control and different concentrations of CpG oligodeoxynucleotides. * $P < 0.05$.

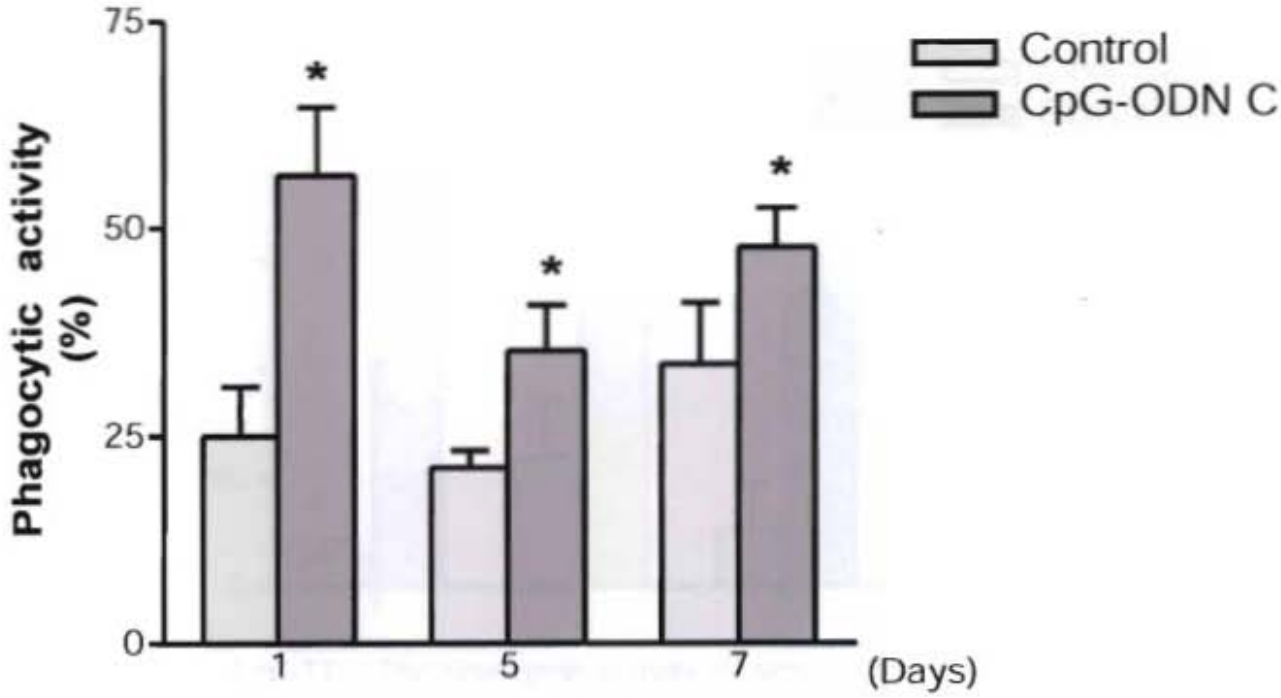

Fig. 9. The phagocytic activity in leucocytes of carp injected with 1 μg of CpG ODN-C. The phagocytic activity was examined 1, 5 and 7 days post-injection. Values are mean ± S.D. in 6 fish. * $P < 0.05$.

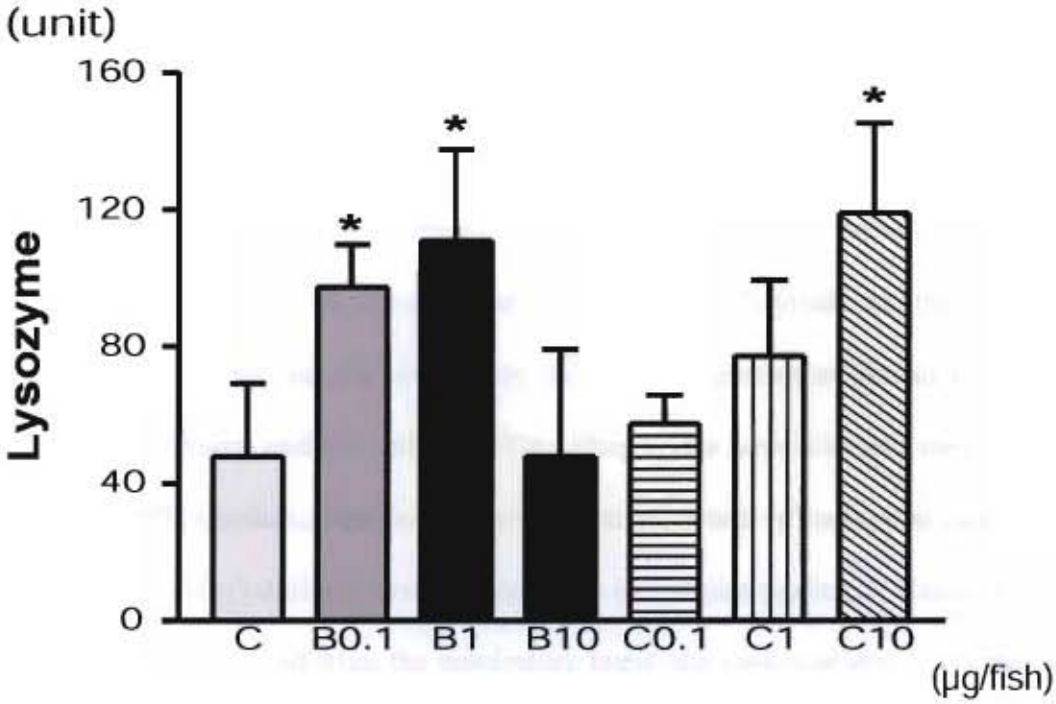

Fig. 10. The dose responses in the lysozyme activity in serum of carp treated with CpG-ODNs. The lysozyme activity was examined 5 days after treatment. Values are mean ± S.D. in 6 fish. Asterisks indicate statistically significant differences between control and different concentrations of CpG oligodeoxynucleotides. * $P < 0.05$.

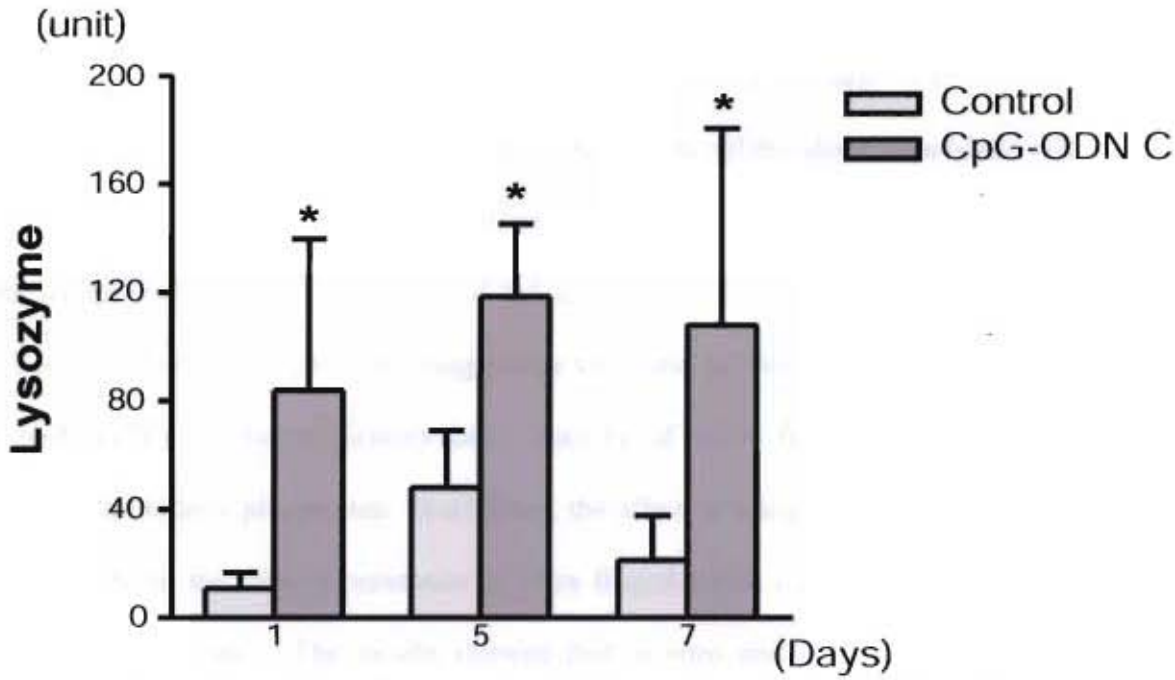

Fig. 11. The lysozyme activity in serum of carp injected with 10 μg of CpG-ODN C. The lysozyme activity was examined 1, 5 and 7 days after injection. Values are mean ± S.D. in 6 fish. * $P < 0.05$.

II-2.4. Discussion

Fish have several types of phagocytic leucocytes which are present in the blood, the peritoneal cavity and a variety of tissue locations. Phagocytosis and the production of oxygen free radicals via the respiratory burst are important events in bactericidal pathway in fish (Sharp and Secombes, 1993). Phagocytes have a unique membraneous enzyme, NADPH oxidase, capable of one-electron reduction of molecular oxygen into superoxide anion (O_2^-) during a process known as the respiratory burst. Since O_2^- is the first product to be released from the respiratory burst, the measurement of O_2^- has been accepted as a direct and accurate way of measuring respiratory burst activity (Secombes, 1990). Dugenci et al. (2003) used two main methods to measure O_2^- : The reduction of ferricytochrome *c* to determine extracellular O_2^- and the reduction of the redox dye NBT to determine intracellular O_2^-.

The present work demonstrates that CpG-ODNs (ODN B & C) induced increased levels of NBT activity in head kidney leucocytes of carp when injected intraperitoneally into the fish. This enhancement was ODNs-dose dependent and continued to 7 days. The maximum difference between ODNs-treated groups and control groups showed in the leucocytes of fish treated with 10 μg/fish of ODN C at day 5. . Recently, Lee et al. (2003) investigated *in vitro* and *in vivo* effects of CpG-ODN 1668 (GACGTT) on the respiratory burst activity of olive flounder (*Paralichthys olivaceus*) head kidney phagocytes. In addition, the effect of intraperitoneal injection of this CpG-ODN on the disease resistance in olive flounder was examined by challenge with *Edwardsiella tarda*. The results showed that *in vitro* and *in vivo* treatments of CpG-ODN 1668 increased phagocytes respiratory burst activity and disease resistance in olive flounder. The immunostimulant such as chitosan (Anderson et al., 1995) and

glucan (Jeney and Anderson, 1993) increased NBT activity in fish leucocytes by intraperitoneal injection.

The same phenomenon was observed in the phagocytic activity in carp. The CpG-ODNs enhanced phagocytosis against latex particles in carp leucocytes. This enhancement was ODNs-dose dependent and continued to 7 days. The maximum stimulation of phagocytic cells was demonstrated in the leucocytes of fish treated with 1 μg/fish of ODN C at 1 day after injection. The phagocytic activity also enhanced in carp leucocytes injected with levamisole (Siwicki, 1989), chitin (Sakai et al., 1992) and LPS (Salati et al., 1987).

CpG-ODNs (B & C) activated serum lysozyme activity of carp. Sakai (1999) reported that fish treated with immunostimulants increased the lysozyme activity. Lysozyme is a cationic enzyme that attacks the β–1,4 glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan of bacterial cell walls. This enables lysozyme to lyse certain Gram-positive bacteria and in conjunction with complement even some Gram-negative bacteria (Alexander and Ingram, 1992). In addition, lysozyme appears to have antibacterial properties independent of the enzyme activity. The presence of lysozyme activity in plasma, skin mucus, organs and fish egg has been well documented in various fish species (Engstad et al., 1992; Paulsen et al., 2003). In rainbow trout and Japanese flounder, the lysozyme gene is transcribed in different organs and two types of cDNA have been identified in trout kidney and liver (Dautigny et al., 1991; Hikima et al., 1997). Different species of fish have different amounts of lysozyme in their tissues and tissue fluids. Rainbow trout appear to have the highest concentrations which are about 15 times higher than the concentrations found in brown trout and Atlantic salmon. Members of the cod family have the lowest levels. It

was shown that intraperitoneal injection of yeast glucan (Engstad et al., 1992; Jorgensen et al., 1993 and Thompson et al., 1995) and scleroglucan (Matsuyama et al., 1992) enhanced the lysozyme activity in fish. Recently, Paulsen et al. (2003) demonstrated that LPS and β-glucan induce plasma lysozyme activity in Atlantic salmon and it was suggested that this induction originate from macrophages in the different organs. The head kidney is likely to be the main supplier of plasma lysozyme considering its high contents of macrophages. Their work supported the notion that microbial compounds containing phylogenetically conserved structures (LPS and ß-glucan) are able to stimulate the non-specific defence animals against infection by enhancing the lysozyme expression. The overall effect of these two compounds on lysozyme induction is thus likely to be due to engagement of pattern recognition receptors such as the Toll family of receptors. Since CpG DNA is also recognized by TLR 9, the induction of CpG-ODNs on serum lysozyme activity in the present study is also likely to be due to engagement of the TLR 9.

Both the ODN B containing 2 motifs 5'-GACGTT-3' and 5'-AACGTT-3', and the ODN C containing the motif 5'-AACGTT-3' increased the innate immune response in carp. Recently, Meng et al. (2003) reported that the CpG-ODNs included the optimal motifs: the ODN-1826 (GACGTT) and 2006 (GTCGTT) for the mice and human cells, the ODN-1670 (AACGTT) used in Atlantic salmon could activate grass carp (*Ctenopharyngodon idellus*) macrophages by increasing the levels of superoxide anion, hydrogen peroxide, acid phosphatase and bactericidal activity. The ODN C had a better stimulatory capacity than ODN B. The higher stimulatory of the ODN C might be due to the presence of 2 distal CGs in 5' end of ODN C (ATCGACTCTCGAACG TTC TC). Jørgensen et al. (2001a) reported that since sequences outside the 6-mer core motif also

influences the biological outcome has been reported, the distal of CGs in the 5' end or 3' end might have contributed to the enhanced stimulatory capacity. Therefore incorporating 3 CpGs into a single ODN C caused a greater stimulatory capacity compared to a single ODN B with 2 CpGs. Klinman et al. (1996) reported that ODNs containing the dinucleotide CpG consistently triggered cytokine release, whereas ODNs lacking this motif did not. Multiple CpGs generally resulted in greater stimulatory capacity of cytokine release in mice.

In this study, the immunostimulary effects of CpG DNA motifs are seen in fish. This is the first report to show the *in vivo* stimulation of CpG-ODNs in the fish immune system, suggesting that CpG-ODN may be useful in enhancing the innate immune response in veterinary application for fish.

Chapter III

Analysis of expressed sequence tags (EST) obtained from common carp head kidney cells after stimulation by CpG oligodeoxynucleotides

III.1. Introduction

Expressed sequence tags (ESTs) analysis, which survey sequences contained in cDNA libraries, is a powerful approach for identifying new genes and profiling gene expression in tissue or cells (Gong et al., 1997). Sequence information from ESTs could also been used in many other applications such as the generation of physical maps of chromosomes and the development of genetic linkage maps. ESTs are important resources for discovery of polymorphic markers by microsatelite tagging as well as by identification of single nucleotide polymorphism (SNP). In addition, ESTs are great resources for development of microarrays for functional genomics. In recent years, EST sequence resources are rapidly growing in molecular databases. However, ESTs from teleosts account for less than 2% of all ESTs in the databases. Over 50% of the teleosts were generated from model fish, such as zebrafish and fugu, while EST resources are lacking for most fish species. The first fish EST survey included tissues of healthy winter flounder (*Pleuronectes americanus*), lumpfish (*Cyclopterus lumpus*) and halibut [*Hippoglossus hippoglossus* (Gong et al., 1994)]. Further EST analysis have been performed on immune-relevant tissues from species such as winter flounder (Douglas et al., 1999), Atlantic salmon (Davey et al., 2001; Martin et al., 2002) and red sea bream [*Chrysophrys major* (Chen et al., 2004)]. In order to maximize the discovery of genes with roles in immunity, ESTs have also been obtained from leucocytes of carp treated with a mitogen (Savan and Sakai, 2002), kidney of Japanese flounder (*Paralichthys*

olivaceus) treated with peptidoglycan (Kono and Sakai, 2001), kidney of rainbow trout and Japanese flounder infected with viruses (Kono et al., 2000; Nam et al., 2000) and catfish (*Ictalurus punctatus*) infected with bacteria (Kocabas et al., 2002). The lack of EST resources in cultured fish species prohibits use of modern functional genomic approaches for the study of growth, development, stress biology and molecular breeding.

Common carp is a widely cultured marine fish species in Asian countries and a cheap source of protein. However, diseases of the cultured fish have occurred frequently and losses due to infectious diseases limit profitability and development of aquaculture. The use of antibiotics has partially solved the problem but has raised concerns of antibiotic residues in fish and polluting environment and antibiotic resistance development. There is extensive interest in enhancing resistance of cultured fish to diseases. The screening for immune-relevant functional genes in fish is very important for identifying the molecular mechanism for disease resistance.

There are evidences stating that oligodeoxynucleotides (ODNs) containing cytosine-phosphodiester-guanine (CpG) motifs are very potent inducers of the innate immune system. There are no reports about EST study in the CpG-ODNs stimulated carp. This initiated us to construct a cDNA library from mRNA isolated from head kidney cells of carp that have been stimulated by CpG-ODNs and to identify the DNA sequences of 88 clones.

III.2. Materials and Methods

III.2.1. *Fish and Immunostimulant*

Common carp (mean weight=100g) was obtained from Sunaso Fisheries farm in Miyazaki, Japan. The fish were acclimated in aerated fresh water tank at 20 °C under natural photoperiod and fed for two weeks prior to the experiment.

The CpG-ODN B & C (**Table 2**) were suspended in phosphate buffer saline [PBS (10 μg/100 μl)] and used for administration.

III.2.2. *Administration of CpG oligodeoxynycleotides*

Fish were injected with CpG-ODN B and C at a dose of 10 μg/fish intraperitoneally. Six fish of each group were sampled for the RNA isolation at 1, 5 and 7 days after injection.

III.2.3. *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 (Refer chapter II-1, Materials and Methods II-1.2.3)]. The stimulated head kidney cells were pooled together and stored in ISOGEN (Nippon Gene, Japan) at –80 °C for mRNA extraction.

III.2.4. *mRNA extraction*

Total RNA was isolated from tissues using ISOGEN (Nippon Gene, japan) acoording to the manufacturer's instructions. Poly (A) RNA was purified from total RNA using a quick prep micro mRNA kit (Amersham Pharmacia Biotech, Sweden) according to manufacturer's protocol. Briefly, 20 μl of total RNA was mixed with 380

µl of extraction buffer and loaded into oligo(dT)-cellulose spin column and mixed for 10 min and centrifuged for 2 min at 1000 rpm. The column was washed thrice with 3 ml high salt buffer [10mMTris-HCl (pH7.4) I mM EDTA, 0.5 M NaCl] and twice with low salt buffer [10mMTris-HCl (pH7.4) I mM EDTA, 0.1 M NaCl] and thrice eluted with 0.25 ml elution buffer preheated at 65 °C. The mRNA was then quantified and immediately used for cDNA synthesis.

III.2.5. *cDNA library construction*

cDNA synthesis was performed using a cDNA synthesis kit (Invitrogen, USA). The library was constructed in a pSPORT cloning vector.

III.2.5.1. *First strand synthesis*

The mRNA is converted into first strand using SUPERSCRIPT II reverse transcriptase (RT). Two µl of *Not* I primer-adaptor was taken in a sterile microcentrifuge tube. Five µg of mRNA was added to the tube and the total contents were made up to 7 µl. The mixture was heated at 70 °C for 10 min and chilled on ice immediately. After a brief centrifugation, the components for the first strand synthesis (Invitrogen, Japan) were added and gently vortexed to mix and centrifuged to collect the contents. The tube was incubated at 37 °C for 2 min.

SUPERSCRIPT II reverse transcriptase (RT) was added depending on the amount of starting material of mRNA as standardized in the manufacturer's protocol. SUPERSCRIPT II RT of 5 µl volume was added to the reaction mixture and incubated for 1 h at 37 °C. The reaction was terminated by keeping on ice.

III.2.5.2. *Second strand synthesis*

The second strand synthesis components (Invitrogen, Japan) were added to the first strand reaction product and incubated for 2 h at 16 °C. The mixture was incubated for a further 5 min at 16 °C and the reaction was terminated by adding 10 µl of 0.5M EDTA. The reaction was extracted in 50 µl phenol: chloroform: isoamylalcohol [PCI (25:24:21)] by centrifugation at 14,000 × g after a brief vortex to mix the solution. The aqueous layer was then transferred to a fresh 1.5 ml micro-centrifuge tube. The reaction mixture was then precipitated in 0.5 ml of 70.0 % ethanol by centrifugation at 14000 × g and dried at 37 °C for 10 min.

III.2.5.3. *Sal I adaptor edition*

The *Sal I* adaptor components (Invitrogen, Japan) were added in the order as give below and incubated for 16 h at 16 °C. The reaction was extracted in 50 µl PCI (25:24:21) by vortexing briefly and centrifuging at 14,000 × g. The aqueous layer was then transferred to a fresh 1.5 ml micro-centrifuge tube. The reaction mixture was then precipitated in 0.5 ml of 70.0 % ethanol by centrifugation at 14,000 × g and dried at 37 °C for 10 min.

III.2.5.4. *Not I digestion*

Not I digestion was carried out at 37 °C for 2 h adding *Not* I digestion components (Invitrogen, Japan). The reaction was extracted by 50 µl PCI (25:24:21) by vortexing briefly and centrifuging at 14,000 × g. The aqueous layer was then transferred to a fresh 1.5 ml micro-centrifuge tube. The reaction mixture was then precipitated in 0.5 ml of 70.0 % ethanol by centrifugation at 14000 × g and dried at 37

°C for 10 min.

III.2.5.5. *Column chromatography*

The cDNA pellet was dissolved in 100 µl of TEN buffer and hydrated on ice. The column was drained and equilibrated with 0.8 ml of TEN buffer. The hydrated cDNA was passed through the column and the effluent was collected in 1.5 ml tube labeled 1. A further 100 µl of TEN buffer was added and the effluent collected (a single drop) in tube 2. This procedure is continued until 18 drops have been collected from tubes 3 to 20. The tube 11 was then precipitated in 0.5 ml of 70 % ethanol and centrifuged at 14,000 × g and dried at 37 °C for 10 min and resuspended in a 10 µl of TEN buffer.

III.2.5.6. *Ligation of cDNA to the pSPORT I vector*

The ligation reaction was carried out overnight at 4 °C adding the components.

III.2.6. *Transformation in E. coli (DH5α) by transformation*

A 100 µl of MAX EFFICIENCY DH5α was thawed on ice for 5 min. Five µl of the ligation reaction was mixed carefully with the compotent cells and stored on ice for 20 min. The competent cells were subjected to heat shock for 55 sec at 42 °C and kept immediately on ice for 2 min. One ml of pre-warmed (37 °C) SOC medium was added to the competent cells and incubated at 37 °C for 1h on a shaker water bath. The transformants were plated by serially diluting it onto an LB-agar plates containing 100 µg/ml ampicillin (Gibco, USA) and grown at 37 °C overnight.

III.2.7. *Colony PCR to screen the transformants for inserts*

The colonies were subjected to PCR reaction in a 50 µl tube containing 20 µl reaction mixtures. PCR was carried out in a PTC -200 machine (MJ research, USA) using T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACT ATAGAA-3') primers (Sawady Technology, Japan). A 30 cycles of reaction was conducted for 1.0 min at 94 °C, 2.0 min at 50 °C and 2 min at 72 °C.

III.2.8. *Agarose gel electrophoresis*

Electrophoresis was carried out on a 1.5 % agarose gel with 0.5 % ethidium bromide. The gel was run on a 0.5 × TBE, with 10 µl of sample along with 2 µl sample loading buffer. Electrophoresis was performed at a constant voltage of 5V/cm. The gel was then observed for the PCR products on a UV-transilluminator and analysed by a gel documentation and analysis system.

III.2.9. *Isolation of plasmids from E. coli*

The protocol for manual isolation of plasmid DNA is modified from alkaline lysis method (Sambrook et al., 1989). For the agarose gel electrophoresis of plasmid DNA, two µl of plasmid solution was mixed with 0.5 µl of sample loading buffer and run on a 1.2 % agarose gel in TBE-buffer and the bands documented by gel documentation and analysis software.

III.2.10. *Cycle sequencing PCR*

The cDNA clones were subjected to pre-sequencing reaction using Thermo sequenase cycle sequencing kit (Amersham Pharmacia Biotech, UK).

Briefly, 2 µl of A, C, G and T reagents were added in four different tubes with 1 µl of IRD labeled primer [2 pmol/µl (T7 or Sp6; Nisshinbo, Japan)] and 5 µl of template DNA. This mixture was subjected to sequencing PCR reaction in a PCR machine PTC-200 (MJ research, USA). The PCR was performed in a PCR machine with 30 reaction cycles of 0.5 min at 95 °C, 0.5 min at 50 °C and 1 min at 70 °C. An initial denaturation of 5 min at 95 °C and a final delay of 5 min at 72 °C were allowed. Immediately after the PCR 4 µl of formamide loading dye (Amersham, Pharmacia Biotech, UK) was incorporated.

III.2.11. *Preparation of sequencing gels*

The gels prepared as mentioned below is brought to room temperature and degassed in a vacuum apparatus. 40 ml gel is mixed with 400 µl of 10.0 % Ammonium per-sulphate (APS; Pharmacia Biotech, Sweeden) and 40 µl of N,N,N',N'-Tetramethyl-ethyl ethylenediamine (TEMED; Wako Japan) and poured into gel plates and allowed to polymeraze for 4 to 5 h and sequenced on an automated DNA sequencer LIC-4200L (Li-Cor, USA).

III.2.12. *Sequence analysis using public sequence database*

The sequences were compared with sequences in the database using the BLASTX algorithm (Altschul et al., 1990). Assignment of putative identities for EST's required a minimum p value of 10^{-5}. EST's with known gene matches were categorized into different functional groups according to categories described by Hwang et al. (1997). Relative levels of gene expression were computed by summing the number of EST's matching to the particular gene and dividing the sum total of EST's that match to

known genes (Hwang et al., 1997). Some genes appeared repeatedly among the sequences.

III.3. Results

A total of 88 cDNA clones isolated from a cDNA library of kidney cells of common carp injected with CpG-ODNs were partially sequenced. The number genes putatively associated with each category was calculated. A summary of results is shown in **Table 3**. Out of 88 clones 84 (95.5 %) matched with nucleic acid and/or amino acid sequences, whereas the remaining 4 (4.5 %) clones did not show any significant homology to the sequences in the databases. The proportion of transcripts associated with each of the broad categories is as follows: Cell organ/defense (14.3 %), Cell structure/motility (5.9 %), Cell signaling/cell communication (50.0 %), metabolism (16.7 %), and unclassified (13.1 %). The cDNA sequences reported in the present paper have been deposited into DNA Data Bank of Japan (DDBJ) with accession number from AU312478-AU312561.

Among all identified genes, 79 genes appeared only once and 5 genes were present more than once. The first three most frequently identified clones were α-globin (n=5), lysozyme-C (n=4) and β–globin (n=3). For genes related to cell/organ defense, granulin 2 (AU312488), CCAAT/enhancer binding protein (AU312493), immunoglobulin heavy chain variable region (AU312495), lectin (AU312498), lysozyme-C (AU312507), interleukin-4 receptor alpha chain (AU312530), cathepsin L preproprotein (AU312537), CD9 protein (AU312538) and Granulin 1 (AU312558) were identified.

Table 3. List of identified EST's from head kidney cDNA of common carp stimulated with CpG oligodeoxynucleotides

Clone number	Putative identification	Accession number	Closest species	Accession number of closest species	Identity	Frequency
	Cell/organism defense					
HK11	Granulin2	AU312488	Zebrafish	Q90ZC9	17/27 (62%)	1
HK16	CCAAT/enhancer binding protein	AU312493	Zebrafish	2808261B	76/234 (32%)	1
HK18	Immunoglobulin heavy chain variable region	AU312495	Rabbit	AF177003 1	15/30 (50%)	1
HK21	Lectin	AU312498	tomato	3002272A	19/52 (36%)	1
HK30	Lysozyme C	AU312507	Common carp	Q9IBG5	143/145(98%)	4
HK53	Interleukin 4-receptor alpha-chain	AU312530	Horse	Q8MIR9	18/48 (37%)	1
HK60	Cathepsin L preproprotein	AU312537	Common carp	Q75SZ8	216/237(91%)	1
HK61	CD9 protein	AU312538	Rainbow trout	2905394B	39/103 (37%)	1
HK81	Granulin1	AU312558	Zebrafish	Q90ZD0	30/92 (32%)	1
	Cell structure/motility					
HK9	Cytochrome C oxidase subunit I	AU312486	Carp	Q85E84	124/225(55%)	1
HK25	Myosin IC heavy chain	AU312502	Amoeba	O61080	42/144 (29%)	1
HK29	extensin-like protein	AU312506	tomato	2720335A	21/48 (43%)	1
HK49	Cytochrome C oxidase polypeptide III	AU312526	Common carp	COX3 CYPCA	185/203(91%)	1
HK72	Cytochrome C oxidase subunit II	AU312549	Common carp	Q85BZ2	164/228(71%)	1
	Cell signaling/ Cell communication					
HK2	Ribosomal protein L23a	AU312479	Catfish	Q90YU4	101/108(93%)	1
HK3	ribosomal protein S11	AU312480	Chicken	Q98TH5	85/91 (93%)	1
HK6	Tax-responsive element binding protein 107	AU312483	Chicken	2803253A	76/89 (85%)	1
HK7	Adenine nucleotide translocase	AU312484	African clawed frog	2711156A	65/97 (67%)	1
HK12	AAT-1 protein	AU312489	human	2824403A	21/56 (37%)	1
HK13	Ribosomal protein S17	AU312490	House mouse	BC002044 1	96/105 (91%)	1
HK14	Vimentin (growth-related gene)	AU312491	Zebrafish	2503277A	69/130 (53%)	1
HK15	60S ribosomal protein L39	AU312492	Catfish	RL39 ICTPU	46/51 (90%)	1
HK17	40S ribosomal protein S18	AU312494	Zebrafish	Q8JGS9	105/106(99%)	1
HK20	Retinal homeobox protein	AU312497	Fruit fly	RX DROME	12/26 (46%)	1
HK23	40S ribosomal S3a	AU312500	Catfish	Q90YS1	35/81 (43%)	1
HK24	60S ribosomal L35	AU312501	Zebrafish	Q8JHJ1	74/86 (86%)	1
HK27	16 ribosomal protein	AU312504	House mouse	MUSRPS16 1	79/116 (68%)	1
HK28	ribosomal protein	AU312505	Dog	Q9XSU3	69/77 (89%)	1
HK31	40S ribosomal protein S15	AU312508	Catfish	Q90YQ9	97/145 (66%)	1
HK32	Insulin receptor substrate-2	AU312509	Human	IRS2 HUMAN	20/48 (41%)	1
HK33	Nucleolar GTP-binding protein 1	AU312510	Human	Q9BZE4	86/160 (53%)	1
HK35	Ubiquitin and ribosomal protein S27a	AU312512	Zebrafish	Q7SXA3	79/136 (58%)	1
HK36	Proline-rich proteoglycan	AU312513	Norway rat	B48013	15/32 (46%)	1
HK37	60S ribosomal protein L23	AU312514	Human	RL23 HUMAN	131/140(93%)	1
HK38	40S ribosomal protein S12	AU312515	African clawed frog	RS12 XENLA	49/105 (46%)	1
HK39	Similar to ribosomal protein L13a	AU312516	African clawed frog	Q7ZY48	52/136 (38%)	1
HK40	Ribosomal protein L27a	AU312517	Catfish	2905383K	71/187 (37%)	1
HK41	60S ribosomal protein L13a	AU312518	Salmon	R13A SALTR	133/146(91%)	1
HK42	60S ribosomal protein L8	AU312519	Zebrafish	AAH65432	200/210(95%)	1
HK43	FK506-binding protein	AU312520	Tobacco hawkmoth	Q9U4Z3	65/82 (79%)	1
HK45	Zinc transporter-like3 protein	AU312522	House mouse	2816292A	20/57 (35%)	1
HK48	Keratin-associated protein	AU312525	Human	27134320	26/77 (33%)	1
HK50	Sperm histone P2 precursor	AU312527	House mouse	HSP2 MOUSE	26/104 (25%)	1
HK55	Ribosomal protein L4	AU312532	Catfish	2905383E	178/218(81%)	1
HK56	Ribosomal protein L7a	AU312533	Catfish	2905383K	192/248(77%)	1
HK57	Guanine nucleotide binding protein	AU312534	Pig	GBAK CAVPO	242/268(90%)	1
HK63	Transcription factor ken	AU312540	Fruit fly	T00119	18/54 (33%)	1
HK64	ribosomal protein L23a	AU312541	Zebrafish	Q6Q416	24/34 (70%)	1
HK67	Sterol regulatory element binding protein	AU312544	Chicken	Q90ZMS	20/47 (42%)	1
HK69	FR01 and FR02-like protein	AU312546	Mouse	Q9FGS9	16/38 (42%)	1
HK71	40S ribosomal protein S2	AU312548	Catfish	RS2 ICTPU	48/93 (51%)	1
HK73	Ribosomal protein L5b	AU312550	Catfish	2905383G	132/206(64%)	1
HK74	Elongation factor 1-alpha	AU312551	Common carp	Q800W9	157/198(79%)	1
HK76	Proline-rich protein MP4	AU312553	Mouse	S19560	16/33 (48%)	1
HK83	40S ribosomal protein S2	AU312560	Spoon worm	RS2 URECA	159/172(92%)	1
HK84	Profilaggrin	AU312561	Human	CAD39823	45/173 (26%)	1
	Metabolism					
HK1	Alpha-globin	AU312478	Common carp	013136	80/93 (86%)	1
HK5	Beta-globin	AU312482	Common carp	Q98851	100/109(91%)	2
HK22	Harmonin a1	AU312499	Rat	Q6PPF3	26/81 (32%)	1
HK34	Alpha-globin	AU312511	Common carp	013139	108/108 (100%)	3
HK46	Aspartate-semialdehyde dehydrogenase	AU312523	Pseudomonas	DHAS PSEAE	18/41 (43%)	1
HK47	ATP synthase 6	AU312524	Barbus	Q9B1G8	166/206(80%)	1
HK54	Beta-globin	AU312531	Common carp	P70073	100/109(91%)	1
HK59	ATP synthase 6	AU312536	Barbus	Q9B7L6	106/173(61%)	1
HK62	NADPH flavin reductase	AU312539	Human	2006199A	119/203(58%)	1
HK66	Membrane alanyl aminopeptidase	AU312543	Norway Rat	A32852	96/180 (53%)	1
HK79	Alpha-globin	AU312556	Common carp	013138	48/63 (76%)	1
	Unclassified					
HK10	Cys-rich protein	AU312487	Protozoa	1806371A	13/33 (39%)	1
HK19	Hypothetical protein C27D6.4 in Chromosome II	AU312496	Nematode	Q09636	16/46 (34%)	1
HK26	Hypothetical protein	AU312503	Japonica cultivar group	Q6YUW2	14/28 (50%)	1
HK58	Male enhanced antigen 1	AU312535	Mouse	Q9DOQO	44/94 (46%)	1
HK65	Hypothetical protein	AU312542	Nematode	T34010	18/52 (34%)	1
HK68	Hypothetical protein	AU312545	Pyrococcus	C75154	25/70 (35%)	1
HK70	Sequence 1773 from patent EP 1270724	AU312547	Human	AX647581 1	23/88 (26%)	1
HK77	Hypothetical protein SC04250	AU312554	Streptomyces	Q9L0P1	41/132 (31%)	1
HK82	Novel protein	AU312560	Zebrafish	BX942844 4	194/229(84%)	1
HK78	Hypothetical protein	AU312555	Zebrafish	AAH59454	129/216(59%)	1
HK80	Hypothetical alpha/beta hydrolases structure containing protein	AU312557	Mouse	Q8BHA3	20/23 (86%)	1

III.4. Discussion

EST analysis is an efficient and fast method for gene discovery. Like in mammals, the immune system of fish is also composed of non-specific and specific immune defense. The head kidney is an important organ for specific and non-specific immune defenses. This tissue also encodes for house keeping genes including ribosomal proteins, translation factors and major structural proteins of the cytoskeleton. In the present study, carp injected with CpG-ODNs were used for EST analysis with the expectation that the expression of biodefence related genes due to the immunostimulant treatment would be detected. A cDNA library of carp HK was constructed and the 88 ESTs were analyzed to identify immune-related genes. Of the 84 known genes, 9 immune-relevant genes were identified: granulin 2, CCAAT/enhancer binding protein, immunoglobulin heavy chain variable region, lectin, Lysozyme-C, interleukin-4 receptor alpha chain, cathepsin L preproprotein, CD9 protein and Granulin 1 were identified.

Fish treated with immunostimulants are likely to have activated macrophages and lymphocytes. In this study, most of identified immune-related genes might be related to the activation of macrophages or lymphocytes. Several immune-related genes that have been identified during this analysis are described below:

a. CCAAT/enhancer binding proteins (C/EBP) are a family of leucine zipper transcription factors that are critically involved in the regulation of normal cellular differentiation and function in multiple tissues. Since the original isolation and cloning of the first C/EBP protein in 1998, there have been many reports on the functions of C/EBP in higher organism, reflecting their significance and importance in normal biological process. C/EBP β and ε in vertebrates have direct roles in the

mediation of the immune system and regulate expression of acute phase (AP) proteins, particularly with the up regulation of cytokines and/or the terminal differentiation and function of macrophages (C/EBP β) and granulocytes (C/EBP ε). Recently, Tucker et al. (2002) have cloned C/EBP in Japanese flounder. They found that C/EBP β and C/EBP ε were expressed in Japanese flounder haemapoietic organs and leucocytes. They also suggested that C/EBP β has a more general role in inflammation as expression of C/EBP β was greatly induced in a number of tissues following inflammatory stimuli.

In this study, stimulation by CpG-ODNs induced the expression of C/EBP β suggesting its role in the mediation on the inflammatory response. The EST study of leucocytes from HRV infected fish (Aoki et al., 1999) detected C/EBP ε and β indicating that these C/EBP genes may have a similar immune related function to those reported for mammalian species. As described in the Chapter I, C/EBP is also activated in the signaling mechanism of CpG DNA by TLR 9, which directly up-regulate cytokine/chemokine gene expression.

b. Lectins are a range of diverse molecules, broadly classified as calnexin, C-, L-, P-, I-, R-, and S-type lectins, with C-type lectins classified into various sub-groups. Lectins are believed to mediate pathogen recognition, which can lead to neutralization of the invading organism (Vasta et al., 1994) during the early stages of an infection. As lower vertebrates and invertebrates do not posses a strong adaptive immunity, their survival depends on the strong non-self recognition molecules. Most recent works have emphasized the possible role of lectins as non-self recognition molecules in vertebrate and invertebrate immunity. Savan et al. (2004) have cloned a new C-type lectin from common carp.

c. Lysozyme is important to inhibit the growth and invasion of infectious pathogens as well as the complement, interferon, C-reactive protein, transferring and lectin located in mucus, serum, and many organs fish. Several types of lysozymes have been described, like the C (Chicken), G (goose), phage, bacteria, plant and i (invertebrate) types. Although several types of lysozymes exist, only C and G types are reported in vertebrates. Lysozyme-C was the most abundantly expressed immune-related genes during this analysis. In fish, lysozyme-C and its variants have been cloned in carp (Fujiki et al., 2000b; Savan and Sakai, 2002), Japanese flounder (Hikima et al., 1997) and Zebrafish (Liu and Wen, 2002).

d. One particularly useful group of molecules that is not presented by many teleost equivalents is the cluster of differentiation (CD) markers. Antibodies to these cell surface molecules are extremely useful for identifying and isolating sub-populations of lymphocytes that cannot be differentiated by morphology alone. In addition, these molecules often paly integral roles in cell adgesion, signaling and activation. CD9 has been cloned and characterized in Atlantic salmon and rainbow trout (Fujiki et al., 2002). CD9 is a tetraspanin family member with a wide tissue distribution that seems to have a specific role in leucocytes, particularly granulocytes, macrophages, pre-B cells and T cells (Fujiki et al., 2002).

It is clear from the present study that CpG-ODNs could stimulate the immune-related genes in fish. In another report on the analysis of expressed genes in Japanese flounder injected with peptidoglycan (Kono and Sakai, 2001), immune-related genes isolated included CC chemokine, hepatic lectin, and immunoglobulin heavy and light chains. The difference in expression of immune-related genes compared to the present study probably relates to the kind of immunostimulants used and the time of sampling.

Chapter IV

Expression analysis of immune-related genes in the common carp head kidney cells after stimulation by CpG oligodeoxynucleotides

Application of vaccines has been an important method in prevention of fish against bacterial diseases. Some vaccines and immunization techniques when actually applied to hatchery conditions are not as effective as they should be. Therefore, research is concentrating on how to improve the potency and efficacy of the antigens and how to optimally activate the non-specific defence mechanisms and specific cellular and humoral immune response. One of the most frequent uncertainties regarding the use of vaccines is effective protection over a long time. The use of adjuvants and immunostimulants in fish culture offers a wide range of attractive methods for inducing and modulating protection against diseases. Adjuvants and immunomodulators used to enhance the non-specific defence mechanisms and a specific immune response has been divided into general group of function. Adjuvants are usually mixed and injected with an antigen preparation, which elevates specific immune response. The application of immunostimulants for the activation of the effectiveness of vaccines is a promising new development in aquaculture. Natural and synthetic immunostimulants activate the non-specific cellular and humoral defence mechanisms and specific immune response if they are administered before, with, or after vaccines. Various immunostimulants like EF203 (Sakai et al., 1995), several polysaccharides (Wang and Wang, 1997), dimerized lysozyme [KLP-602 (Siwicki et al., 1998)] and leaf extract of *Ocimum sanctum* Linn. (Logambal et al., 2000) have been reported to be good immunostimulators.

However, to develop an effective immunostimulant or a vaccine, it is essential to

understand the specific and non-specific immune factors. Although molecular biology has accelerated the research area of immunology in mammals, fish largely remains unexplored and the number of fish genes that have been identified is limited. Genes related to specific and non-specific immune systems have been isolated over the last few years. The inflammatory response-related genes like cytokines have been isolated in carp (Fujiki et al., 1999, 2000a; Savan et al., 2003), rainbow trout (Dixon et al., 1998; Zou et al., 1999), sea bass *Dicentrarchus labrax* (Scapigliati et al., 2001) and Japanese flounder (Kono et al., 2003). Moreover, the expression of the innate immune-related genes by stimulation with bacterial components such as peptidoglycan (Kono & Sakai, 2001) and lipopolysaccharides (Pelegrin et al., 2001; Laing et al., 2001; Paulsen et al., 2003; Savan and Sakai, 2002) have been also reported in fishes. The functional characterization of these genes is important for the development of vaccines, immunostimulants and diagnostic kits to determine the fish health status.

There is evidence that CpG-ODNs act as an immunostimulant in fish. However, very little published data about CpG-ODNs effects on the expression of immune-related genes in fish are avalaible. In this chapter, the *in vivo* effects of CpG oligodeoxynucleotides on the expression of immune-related genes are described for the first time in fish.

Chapter IV-1

In vivo stimulation of CpG oligodeoxynucleotides on the expression of immune-related genes in the common carp head kidney cells

IV-1.1. Introduction

Teleost fish preceded mammals by millions of years and studies on their immune system will give valuable insights into the evolution of defence mechanisms within vertebrates and may have relevance for disease control in fish farming, where losses due to infectious disease are of major economic importance. It is important to investigate the primary expression patterns of the immune-related genes in various tissues of fish. Whilst major advances in some areas of fish immunology have been seen in recent years, studies of cytokine are meager, in common with most non-mammalian vertebrates. Cytokines play significant roles in initiating and regulating the inflammatory process, which is one of the important defenses in innate immunity.

CpG-ODNs are bacterial DNA sequences that induce inflammatory signals via TLR9 (Hemmi et al., 2000). In mammals, studies have shown that the CpG-ODNs are potent inducers of the central nervous system (CNS) inflammation; strong activation of microglia and astrocytes were detected upon intrathecal injection of the CpG-ODNs. Furthermore, it was reported that the CpG-ODNs activate microglia and astrocytes *in vitro*, which leads to the production of various cytokines and chemokines (Takeshita et al., 2001). In fish, there are no reports concerning the *in vivo* effects of CpG-ODNs on the expression of immune-relevant genes.

This study presents an investigation of the ability of synthetic oligodeoxynucleotides containing CpG motifs, to stimulate the the expression of

immune-related genes, such as IL-1β, CXC and CC-chemokines and lysozyme-C in the common carp head kidney leucocytes.

IV-1.2. Materials and Methods

IV-1.2.1. *Fish*

A total of 200 common carp (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 a commercial diet twice daily.

IV-1.2.2. *Preparation of CpG-ODNs*

The CpG-ODN B & C (**Table 2**) were suspended in phosphate buffer saline [PBS (10 μg/100 μl)] and injected into carp at a dose of 10 μ g/fish intraperitoneally. Control fish received an equal dose of PBS alone by an intraperitoneal injection. Four fish from each group were sampled at 1, 5 and 7 days after injection.

IV-1.2.3. *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 (Refer chapter II-1, Materials and Methods II-1.2.3)]. The stimulated head kidney cells were pooled together and stored in ISOGEN (Nippon Gene, Japan) at –80 °C for mRNA extraction.

IV-1.2.4. *RT-PCR analysis*

Two groups containing four fish in each group were injected with 10 μg of the CpG-ODNs and fish in the control group were injected with PBS intraperitoneally. Total RNA was isolated from head kidney of carp using ISOGEN (Nippon Gene, Japan) according to the manufacturer's instructions. Poly (A) RNA was purified using a quick prep micro mRNA kit (Amersham Pharmacia Biotech, Sweden). cDNA synthesis was

performed using ReverTra Dash (Toyobo, Japan).

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'-TTGCTGATCCACATCTGCTG-3') were used as positive control for RT-PCR, since the gene is constitutively expressed. Expression levels of the IL-1β (Genbank accession number: AB010701-1), CXC-chemokine (AB082985), CC-chemokine (AB010469-1), and lysozyme-C (AB027305) in carp were examined using the gene specific primers. These primers were designed using highly conserved regions for IL-1β (Fw: 5'-CAACATTCGTGTCGAG-3' and Rv: 5'-AAGTTTGTGGTTCGGG-3'), CXC-chemokine (Fw: 5'-ATGAAAATCATTACCGCTGTG-3' and Rv: 5'-TGGATTGA AGCATTTCTGCTC-3'), CC-chemokine (Fw: 5'-AATGGAGACACGCAGGATCCT-3' and Rv: 5'-GCTCAGTCACTAATAGATGATGC-3') and lysozyme-C (Fw: 5'-GTGT CTGATGTGGCTGTGCT-3' and Rv: 5'-TATCCCAGGTGTCCCATGAT-3'). The PCR was performed in a PCR apparatus (MJ Research, USA) following the profile of denaturation (94 °C, 30 sec), annealing [52 °C (actin), 48 °C (interleukin-1β), 51 °C (CXC- chemokine), 63 °C (CC-chemokine), 60 °C (lysozyme-C), 1 min] and elongation (72 °C, 1 min). PCR products were electrophoresed on a 1.5 % agarose gel to detect the specific bands.

IV-1.2.5. *Semi-quantitave analysis of RT-PCR products*

Semi-quantitative analysis was carried out according to the method described

by Kono et al. (2003). In order to obtain an optimum semi-quantitative approach to analyse carp immune-related genes expression, both carp immune-related genes and β-actin genes were amplified using a series of cycle numbers (20-30) under the above conditions (2.4). By reducing the cycle number from 30 to 20, it was possible to select a cycle number that just gave a clear product, which in the case of cDNA of carp was 27 cycles for both β-actin and carp immune–related genes. After determining the optimal cycle number, specific PCR was conducted three times.

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 immune-related genes/β-actin product were subsequently calculated for each gene of interest and used to assess the differences in expression levels between control and CpG-ODNs injected group.

IV-1.2.6. *Statistical analysis*

The data was expressed as mean ±SD. The data was analysed using Student's *t*-test.

IV-1.3. Results

Expression analysis of immune-related genes

IL-1β, CXC and CC-chemokines expressions were significantly increased ($^{*}P < 0.05$) in the groups injected with CpG-ODN B and C and lysozyme-C expression was significantly enhanced ($^{*}P < 0.05$) only in the groups injected with CpG-ODN C.

IV-1.3.1. *Expression analysis of IL-1β*

The expression of IL-1β in the kidney leucocytes of carp injected with 10 μg of CpG-ODNs/fish is shown in **Fig. 12**. In comparison with the control leucocytes, the cells injected with CpG-ODN B and C showed a significantly enhanced IL-1β expression on day 1 and 5 ($^{*}P < 0.05$). On day 7, IL-1β expression in the kidney of carp was significantly enhanced by CpG-ODN C ($^{*}P < 0.05$), while downregulated by CpG-ODN B ($^{\#}P < 0.05$).

IV-1.3.2. *Expression analysis of CXC-chemokine*

The expression of CXC-chemokine in head kidney of carp injected with CpG-ODN B and C demonstrated a significantly higher level of expression ($^{*}P < 0.05$) than those of controls at all the stimulation time periods (**Fig. 13**).

IV-1.3.3. *Expression analysis of CC-chemokine*

The kidney cells isolated from fish treated with CpG-ODNs were also found to express CC-chemokine. It was shown that CC-chemokine (**Fig. 14**) demonstrated a similar pattern of expression as that seen for CXC-chemokine (**Fig. 13**). CC-chemokine expression was significantly higher in the cells isolated from fish treated with CpG-

ODN B and C than those isolated from the control fish at day 1, 5 and 7 sampling times ($^{*}P < 0.05$).

IV-1.3.4. *Expression analysis of Lysozyme-C*

The expression of lysozyme-C in the kidney of carp injected with CpG-ODN B and C is shown in **Fig. 15**. Lysozyme-C expression was significantly decreased ($^{\#}P < 0.05$) in the kidney of carp treated with CpG-ODN B on day 5. However, in the kidney of carp injected with CpG ODN C, an induction ($^{*}P < 0.05$) in lysozyme-C gene expression was apparent when compared to the samples from fish injected with PBS at 7 days post-treatment.

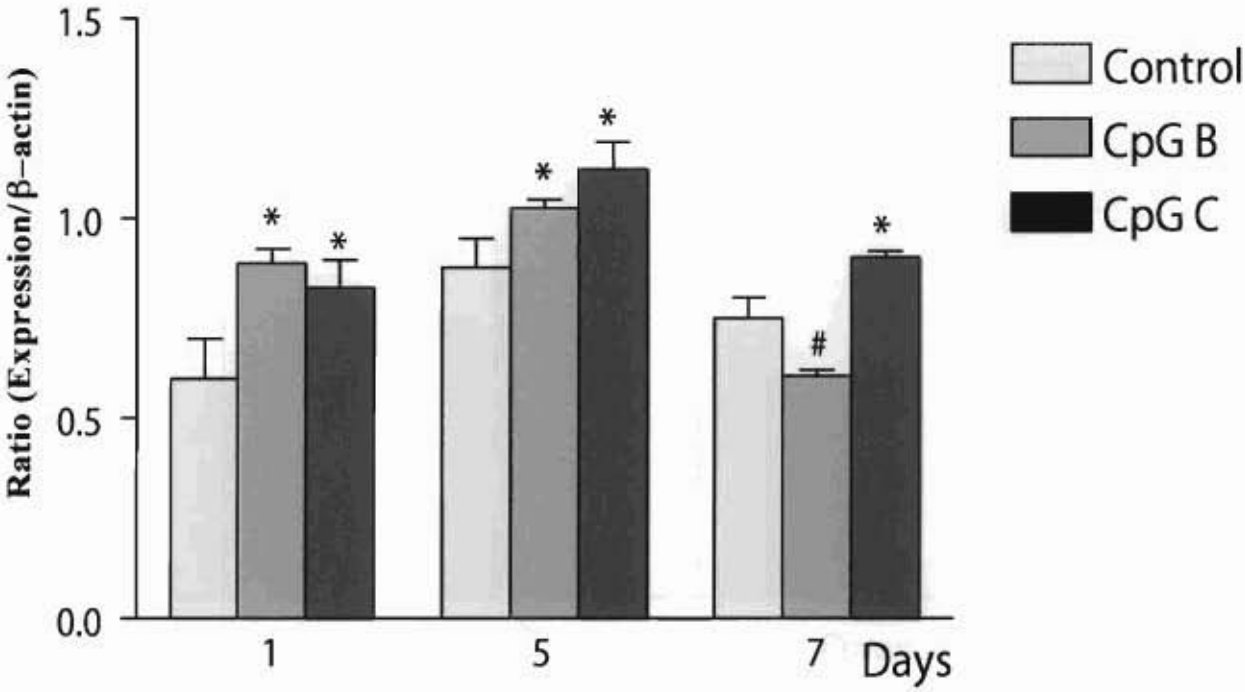

Fig. 12. Densitometric quantification of IL-1β expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish injected (intraperitoneally) with PBS and CpG-ODNs treated fish. Leucocytes were harvested at 1, 5 and 7 days post injection. Values are mean ± S.D. in 4 fish. $*P < 0.05$, $\#P < 0.05$.

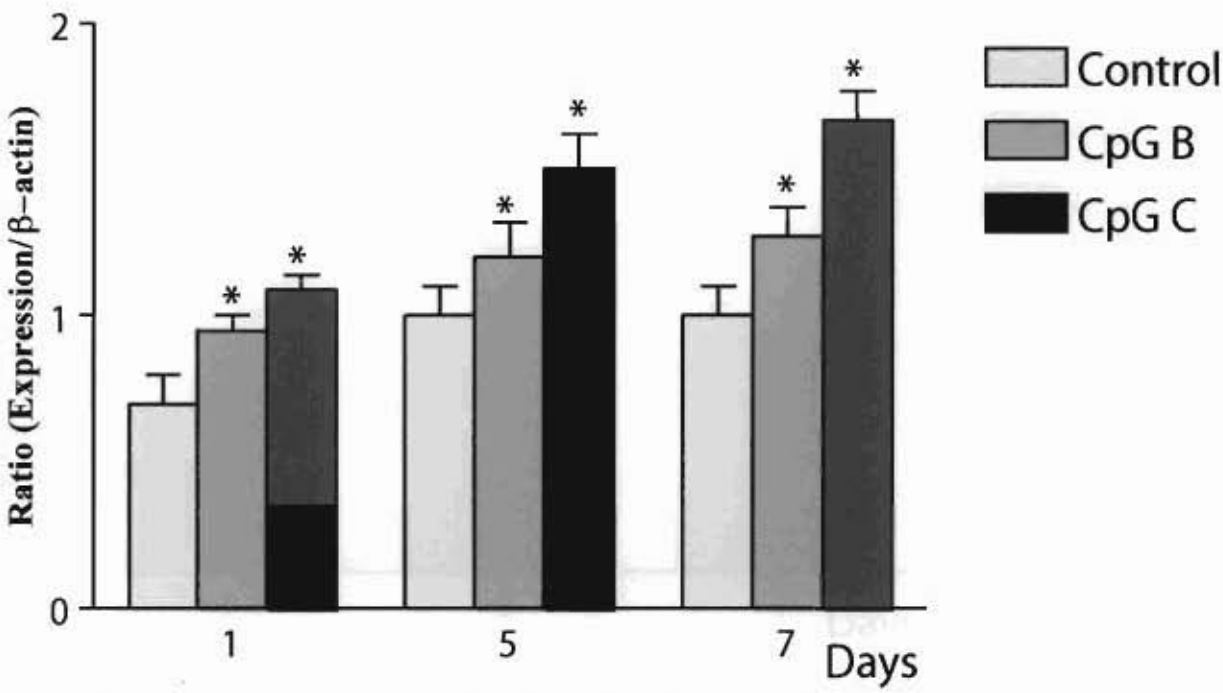

Fig. 13. Densitometric quantification of CXC-chemokine expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish injected (intraperitoneally) with PBS and CpG-ODNs treated fish. Leucocytes were harvested at 1, 5 and 7 days post-injection. Values are mean ± S.D. in 4 fish. $*P < 0.05$.

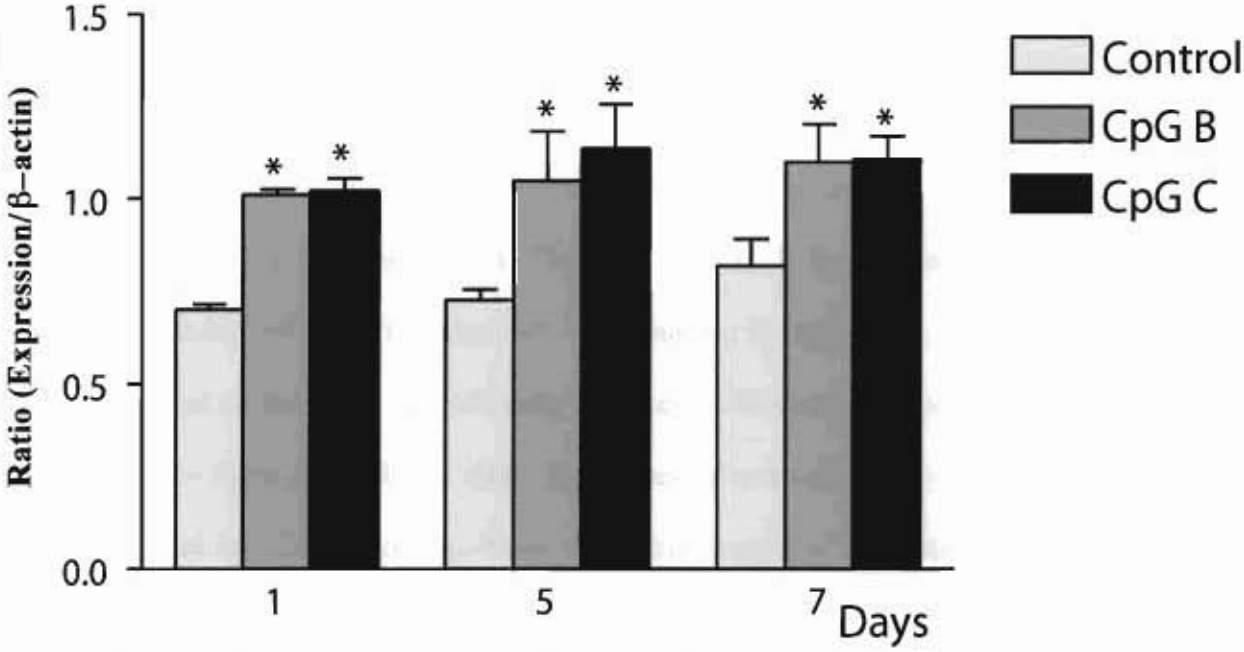

Fig. 14. Densitometric quantification of CC-chemokine expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish injected (intraperitoneally) with PBS and CpG-ODNs treated fish. Leucocytes were harvested at 1, 5 and 7 days post-injection. Values are mean ± S.D. in 4 fish. $*P < 0.05$.

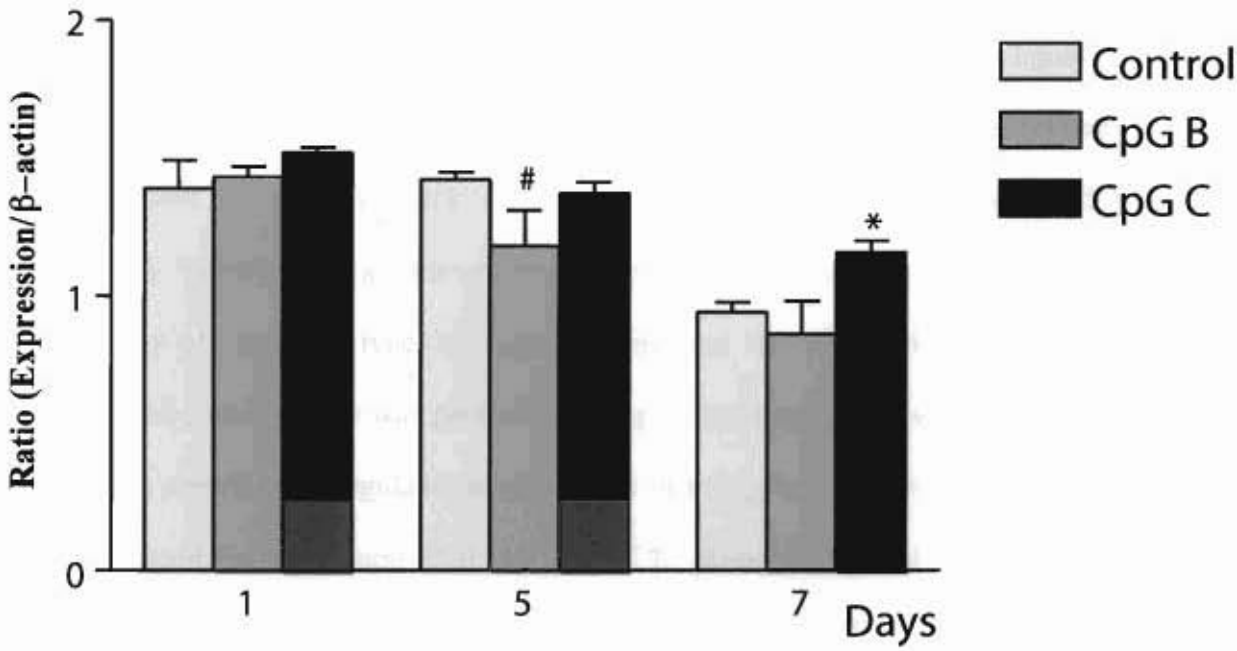

Fig. 15. Densitometric quantification of Lysozyme-C gene expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish injected (intraperitoneally) with PBS and CpG-ODNs treated fish. Leucocytes were harvested at 1, 5 and 7 days post-injection. Values are mean ± S.D. in 4 fish. $*P < 0.05$, $\#P < 0.05$.

IV-1.4. Discussion

Cytokines are simple polypeptides or glycoproteins that act as signaling molecules within the immune system (Thomson, 1994). This is a group of molecules, which is subdivided into families like interleukins, lymphokines, growth factors, interferons and chemokines. In fish, only a few cytokines and chemokines are known. The cytokines cloned in fish are either by expressed sequence tag (EST) analysis or PCR mediated homology cloning. Several cytokines such as CC (Dixon et al., 1998; Fujiki et al., 1999; Kono et al., 2003) and CXC-chemokines (Savan et al., 2003; Huising et al., 2003), interleukin-1β (Zou et al., 1999; Fujiki et al., 2000a; Scapigliati et al., 2001; Engelsma et al., 2003), tumor necrosis factor-α (Hirono et al., 2000), transforming growth factor (Laing et al., 2000; Yin & Kwang, 2000), interferon (Long et al., 2004) and interleukin-10 (Savan et al., 2003) have been cloned in fish.

IL-1β is a key mediator in response to microbial invasion and tissue injury and can stimulate immune responses by activating lymphocytes or by inducing the release of other cytokines that are able to activate macrophages, NK cells and lymphocytes (Low et al., 2003). Macrophages are the primary source of IL-1β although it is produced by a wide variety of other cell types including B-cells and NK cells. Analysis of IL-1β expression in common carp was performed using *in vivo* approaches and CpG-ODN B and C were shown to up-regulate the expression of this gene. However, CpG-ODN B down regulated the expression of IL-1β on day 7. Kono et al. (2002a) revealed that injection of peptidoglycan (PG) stimulates the production of IL-1β in serum of carp. A recent study demonstrated that infection with the ectoparasitic monogenean *Gyrodactylus derjavini* induces IL-1β expression in rainbow trout skin (Lindenstrøm et al., 2003). Low et al. (2003) revealed that increased phagocyte activity may result from

an increased IL-1β production. Both specific and non-specific immune responses could be affected by this marked increase in IL-1β transcription. Kono et al. (2002b) demonstrated that injection of IL-1β cDNA using a DNA injection method increased the activation of lymphocyte and phagocytic cells of common carp, suggesting that the carp IL-1β gene has a function similar to that of mammalian IL-1β.

Chemokines are small, inducible, secreted proteins which cause the migration of leucocytes towards injury or infection sites (Dixon et al., 1998). Chemokine is an acronym for 'chemotactic cytokine' and reflects their discovery and characterization as important chemoattractants in the pro-inflammatory phase of the immune response. The functions like chemotaxis, integrin activation, granule enzyme release, lipid mediator biosynthesis and superoxide radical production have been reported (Oppenheim, at al., 1991; Baggiolini and Dahinden, 1994; Bacon and Schall, 1996). Based on the pattern and spacing of four conserved cysteine residues that determine tertiary structure by virtue of two disulphide bridges, chemokines are subdivided into four classes; CXC (α), CC (β), C (γ) and CX_3C (δ) [Yoshie et al., 2000]. This study provides the first information concerning CpG-ODN induced expression of CXC and CC-chemokines. Expression of CXC and CC-chemokines were found at a higher level in the kidney cells treated with CpG-ODN B and C in all stimulation time periods. Thus CpG-ODNs can be added to the list of substances that stimulate the expression of these important cytokines in fish. The immunostimulatory effects of CpG-ODN on specific cell populations, either directly or through induction of cytokine expression, make these compounds a potentially valuable technology as immunotherapeutic agents and as adjuvants in vaccines (Pontarollo et al., 2002). The immunostimulant such as Ergosan augmented the expression of IL-1β, IL-8 and TNF-α genes in peritoneal leucocytes of

rainbow trout at 1 day post-injection (Peddie et al., 2002). Recently, infection with viral haemorrhagic septicemia virus (VHSV) induced IL-1β and CXC-chemokine IL-8 in rainbow trout spleen (Tafalla, in press).

Lysozymes are considered to be potent innate immunity molecules. Fish lysozyme is mainly distributed in kidney, which is rich in leucocytes and gills or skin where the risk of bacterial invasion is very high, which implies that fish lysozyme plays an important role in host defense mechanisms against infectious diseases. The ability of lysozyme to digest the peptidoglycan of bacteria, makes it an important player in defense against bacterial pathogens. Recently, lysozymes have gained importance not only as a defense molecule, but also as a major digestive enzyme in the stomach of ruminants. As described in the chapter III, lysozyme-C and its variants have been cloned in carp (Fujiki et al., 2000b; Savan and Sakai, 2002), Japanese flounder (Hikima et al., 1997) and Zebrafish (Liu and Wen, 2002). CpG-ODN C induced the lysozyme-C gene expression in carp kidney leucocytes at 7 days after injection. There was a delay in lysozyme-C gene activation. This might be related to the differentiation/maturation of kidney macrophages. As the head kidney constitutes a blood-cell forming organ in fish (Paulsen et al., 2003), head kidney macrophages probably represent a population of variably differentiated cells. During differentiation of macrophages, the lysozyme gene is continuously activated from a low level of expression in precursors such as myeloblasts to a high level of expression in mature macrophages (Cross et al., 1988). Paulsen et al. (2003) revealed that the rather late increase in lysozyme production induced by β-glucan and LPS in Atlantic salmon may be explained by stimulation of differentiation of the macrophages eventually combined with direct activation of transcription of the lysozyme gene. CpG-ODN B down-regulated lysozyme-C

expression in carp kidney leucocytes at 5 days post-treatment. Low et al. (2003) found that lysozyme expression significantly decreased in the spleen and kidney of turbot (*Scophthalmus maximus*) fed a nucleotide-supplemented diet. Lipopolysaccharides and concanavalin A (Savan and Sakai, 2002) induced the expression of lysozyme type C.

In conclusion, *in vivo* approaches of CpG oligodeoxynucleotides augment the expression of IL-1β, CXC and CC-chemokines and lysozyme-C genes in the common carp head kidney leucocytes.

Chapter IV-2

In vitro stimulation of CpG oligodeoxynucleotides on the expression of cytokine genes in the common carp head kidney cells

IV.-2.1. Introduction

As described earlier, innate immune mechanism act as first line of defense against infections. Inflammation typifies an innate immune response, and is seen when the epithelial barriers are breached by an infectious agent. Upon breaching of this barrier, the invading pathogen is met by a whole array of soluble factors like anti-bacterial peptides, proteases, lysozyme, complement factors and acute phase proteins. At the same time the cellular component of innate immunity is activated upon recognition of pathogen derived PAMPs including LPS and double-stranded RNA as well as by host derived cytokines. The latter group includes typical pro-inflammatory cytokines like IL-1β and TNF-α as well as chemokines that are of pivotal importance in recruiting leucocytes to the site of inflammation.

Bacterial DNA and synthetic oligonucleotides with CpG motifs have been demonstrated *in vitro* and *in vivo* to have the capacity to activate and to induce maturation of several cell subsets, including B cells, macrophages and NK cells (Krieg et al., 1995; Pisetsky, 1996; Sparwasser et al., 1998). Macrophages are directly stimulated to secrete a variety of cytokines, such as IL-12, TNF- α and IFN- α / β . These cytokines have downstream effects on other immune cells, and IL-12 in particular, is a major stimulator of IFN- γ production by T cells and NK cells.

There is evidence from *in vivo* study that CpG-ODNs could stimulate the expression of cytokine genes in head kidney of carp. To date, there have been very few

reports about the *in vitro* effects of CpG-ODNs on the expression of cytokine genes in fish. Therefore, in this study we investigated the *in vitro* effects of CpG-ODNs on the cytokine gene expressions in the common carp head kidney cells.

IV-2.2. Materials and Methods

IV-2.2.1. *Fish*

A total of 200 common carp (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 a commercial diet twice daily.

IV-2.2.2. *CpG oligodeoxynucleotides*

The CpG-ODN B & C and the non-CpG-ODN F & G (**Table 2**) were solubilized in sterile water at a concentration of 1 μg/μl and stored at –20 °C.

IV-2.2.3. *Isolation of head kidney cells and stimulation by ODNs*

The head kidney phagocytic cells of the carps were isolated according to the modified method described by Braun-Nesje et al. [1982 (Refer chapter II-1, Materials and Methods II-1.2.3)]. HK cells of 24 fish were stimulated by treatment with 10 ng/ml ODNs for 0.5, 1, 2, 4, 12, and 24 h in RPMI 1640 (Nissui, Japan) medium supplemented with 5 % carp serum and 1 % Streptomycin/Penicillin (S/P, Gibco, USA). The HK cells stimulated for different time points were pooled together and stored in ISOGEN (Nippon Gene, Japan) at – 80 °C for further RNA isolation.

IV-2.2.4. *RT-PCR analysis*

Total RNA was isolated from head kidney of carp using ISOGEN (Nippon Gene, Japan) according to the manufacturer's instructions. Poly (A) RNA was purified using a quick prep micro mRNA kit (Amersham Pharmacia Biotech, Sweden). cDNA synthesis was performed using ReverTra Dash (Toyobo, Japan).

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'-TTGCTGATCCACATCTGCTG-3') were used as positive control for RT-PCR, since the gene is constitutively expressed. Expression levels of the IL-1β (Genbank accession number: AB010701-1), CXC-chemokine (AB082985), CC-chemokine (AB010469-1), TNF-α (AB112424) and IL-10 (AB110780) in carp were examined using the gene specific primers. These primers were designed using highly conserved regions for IL-1β (Fw: 5'-CAACATTCGTGTCGAG-3' and Rv: 5'-AAGTTTGTG GTTCGGG-3'), CXC-chemokine (Fw: 5'-ATGAAAATCATTACCGCTGTG-3' and Rv: 5'-TGGATTGAAGCATTTCTGCTC-3'), CC-chemokine (Fw: 5'-AATGGAGACA CGCAGGATCCT-3' and Rv: 5'-GCTCAGTCACTAATAGATGATGC-3'), TNF-α (Fw: 5'-GCTGCTGTCTGCTTCACG-3' and Rv: 5'-CGTGTTGTACCACAGAT CAT-3') and IL-10 (Fw: 5'-TGATGATTTGGAACCATTATTGAA-3' and Rv: 5'-CAC CTTTTTCCTTCATCTTTTCAT -3'). The PCR was performed in a PCR apparatus (MJ Research, USA) following the profile of denaturation (94 °C, 30 sec), annealing [52 °C (actin), 48 °C (interleukin-1β), 51 °C (CXC- chemokine), 63 °C (CC-chemokine), 60 °C (TNF-α . and IL-10), 1 min] and elongation (72 °C, 1 min). PCR products were electrophoresed on a 1.5 % agarose gel to detect the specific bands.

IV-2.2.5. *Semi-quantitave analysis of RT-PCR products*

Semi-quantitative analysis was carried out according to the method described by Kono et al. (2003). In order to obtain an optimum semi-quantitative approach to

analyse carp immune-related genes expression, both carp immune-related genes and β-actin genes were amplified using a series of cycle numbers (20-33) under the above conditions (2.4). By reducing the cycle number from 33 to 20, it was possible to select a cycle number that just gave a clear product, which in the case of cDNA of carp were 26 cycles for β-actin and 33 cycles of carp immune–related genes. After determining the optimal cycle number, specific PCR was conducted three times.

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 immune-related genes/β-actin product were subsequently calculated for each gene of interest and used to assess the differences in expression levels between control and CpG-ODNs treated group.

IV-2.2.6. *Statistical analysis*

The data was expressed as mean ±SD. The data was analysed using Student's *t*-test.

IV-2.3. Results

IV-2.3.1. *Expression analysis of IL-1β*

The expression of IL-1β in the head kidney leucocytes of carp incubated with 10 ng of ODNs is shown in **Fig. 16**. In comparison with the control leucocytes, the cells incubated with CpG-ODN B showed a significantly enhanced IL-1β expression at 0.5 and 12 h post-stimulation ($*P < 0.05$). At 2 and 24 h post-stimulation, IL-1β expression in the head kidney of carp was significantly enhanced by CpG-ODN C ($*P < 0.05$). The non- CpG-ODNs F and G did not significantly increase these gene expressions (**Fig. 17**).

IV-2.3.2. *Expression analysis of CXC-chemokine*

The expression of CXC-chemokine gene in head kidney of carp incubated with CpG-ODN C demonstrated a significantly higher level of expression ($*P < 0.05$) than those of controls at 2 h after stimulation (**Fig. 18**). The CXC-chemokine gene expression in the head kidney cells incubated with CpG-ODN B (**Fig. 18**) and non-CpG-ODNs F and G did not show a significantly higher level of expression than those of controls (**Fig. 19**).

IV-2.3.3. *Expression analysis of CC-chemokine*

The head kidney cells isolated from fish incubated with CpG-ODNs B & C were also found to express CC-chemokine. It was shown that CC-chemokine (**Fig. 20**) demonstrated a similar pattern of expression as that seen for IL-1β. However, CC-chemokine expression was lower in the cells isolated from fish incubated with CpG-ODN B at 12 h post-treatment. The non-CpG-ODNs F and G did not significantly increase the expression of these genes (**Fig. 21**).

IV-2.3.4. *Expression analysis of TNF-α*

The expression of TNF-α genes were significantly increased in the head kidney of carp incubated with CpG-ODN B at 2 and 4 h and CpG-ODN C at 2 h post-stimulation [**Fig. 22** (*$P < 0.05$)]. The non-CpG-ODN F and G did not significantly increase the expression of these genes (**Fig. 23**).

IV-2.3.5. *Expression analysis of IL-10*

The expression of IL-10 in the head kidney of carp incubated with ODNs is shown in **Fig. 24** and **25**. IL-10 expressions were not significantly increased in the head kidney of carp incubated with all ODNs.

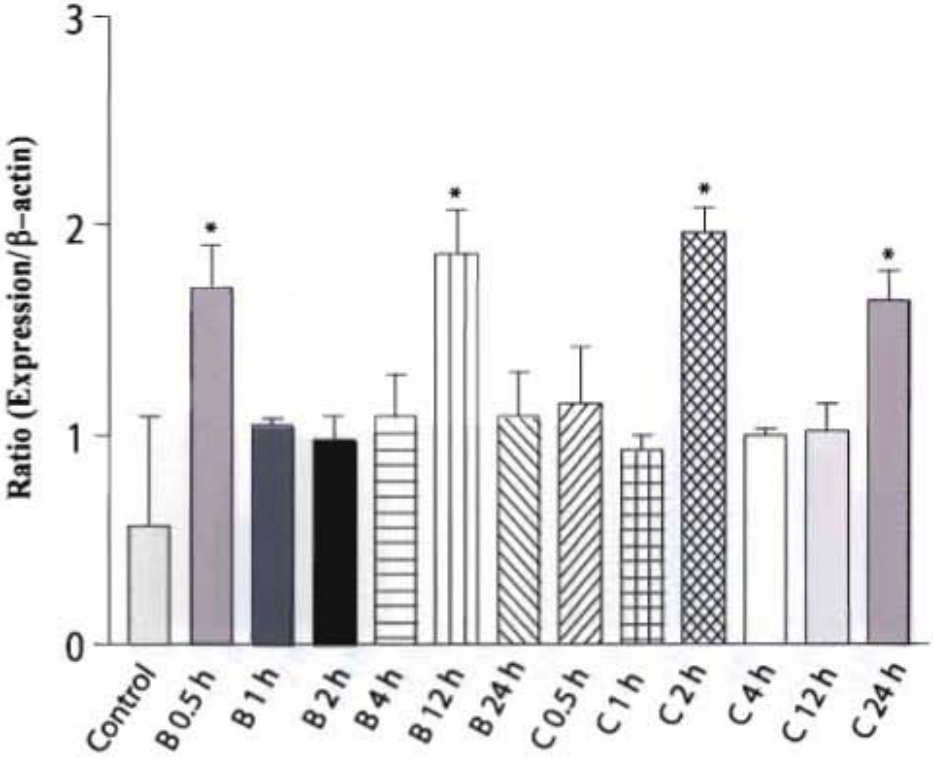

Fig. 16. Densitometric quantification of IL-1β expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish incubated with PBS and CpG-ODNs treated fish (0.5, 1, 2, 4, 12, and 24 hrs). Values are mean ± SD in 4 fish. $^{*}P < 0.05$.

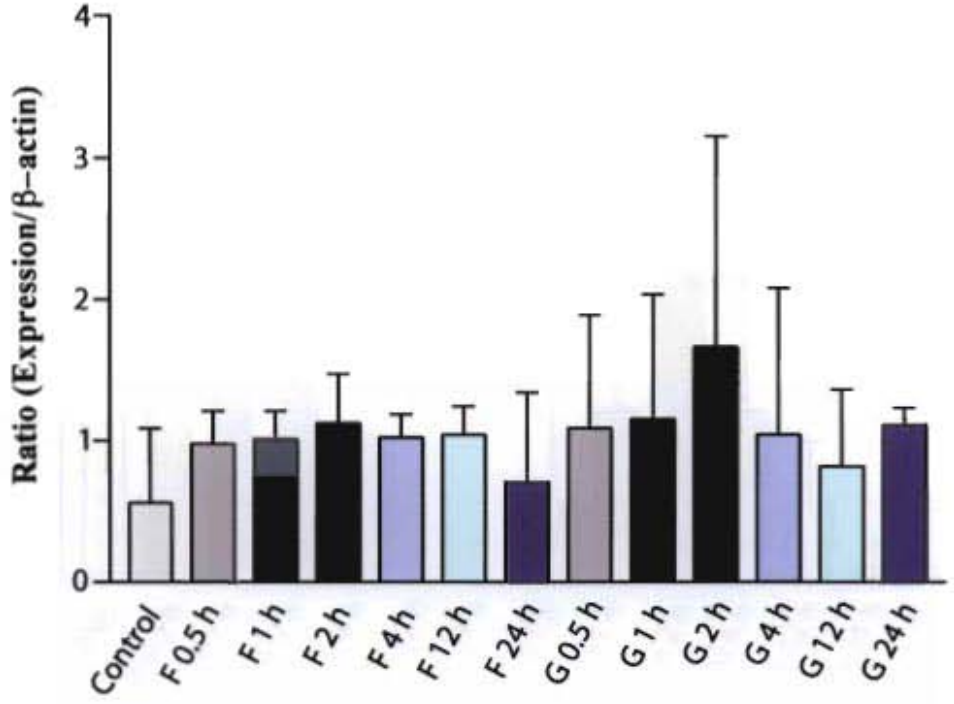

Fig. 17. Densitometric quantification of IL-1β expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish incubated with PBS and non-CpG-ODNs treated fish (0.5, 1, 2, 4, 12 and 24 hrs). Values are mean ± SD in 4 fish.

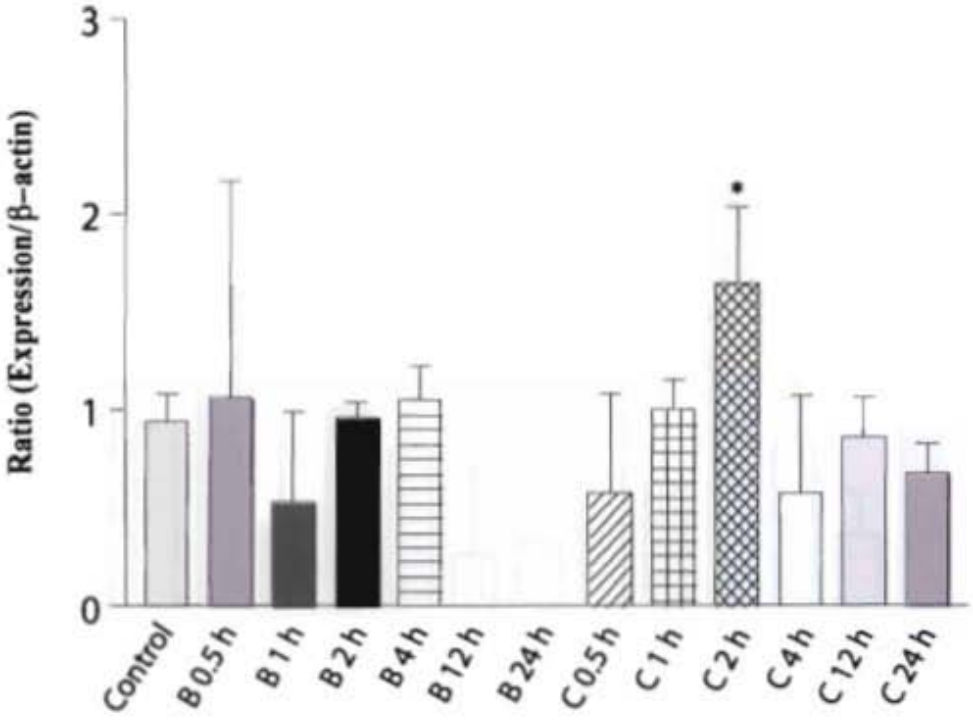

Fig. 18. Densitometric quantification of CXC-chemokine expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish incubated with PBS and CpG-ODNs treated fish (0.5, 1, 2, 4, 12 and 24 hrs). Values are mean ± SD in 4 fish. $^{*}P < 0.05$.

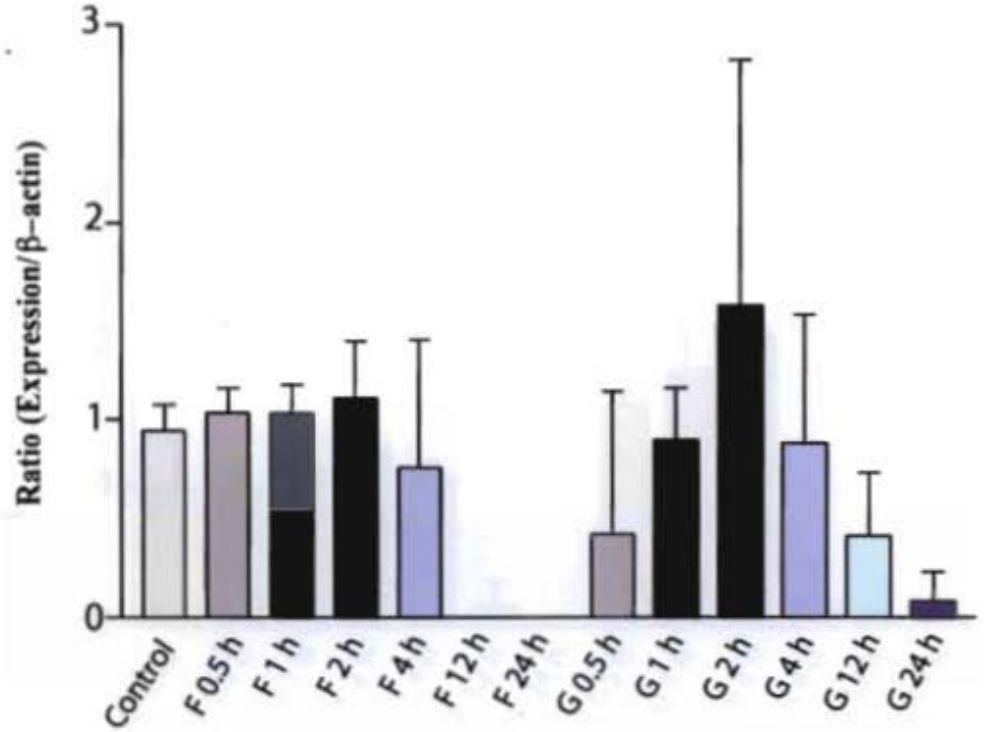

Fig. 19. Densitometric quantification of CXC-chemokine expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish incubated with PBS and non-CpG-ODNs treated fish (0.5, 1, 2, 4, 12 and 24 hrs). Values are mean ± SD in 4 fish.

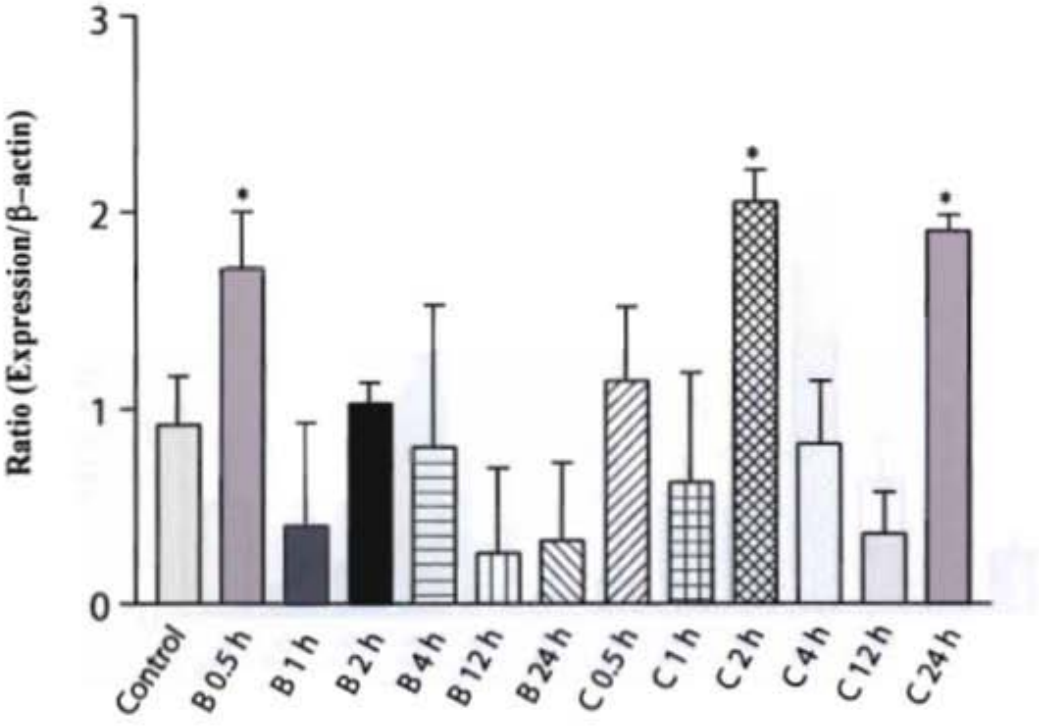

Fig. 20. Densitometric quantification of CC-chemokine expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish incubated with PBS and CpG-ODNs treated fish (0.5, 1, 2, 4, 12 and 24 hrs). Values are mean ± SD in 4 fish. $^{*}P < 0.05$.

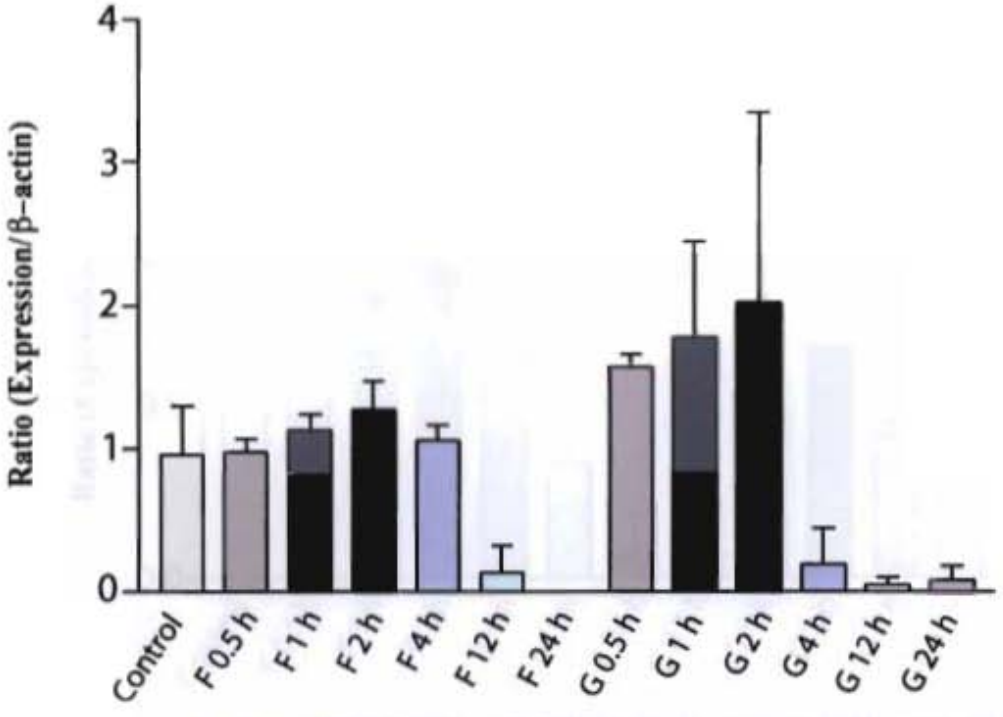

Fig. 21. Densitometric quantification of CC-chemokine expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish incubated with PBS and non-CpG-ODNs treated fish (0.5 , 1, 2, 4, 12 and 24 hrs). Values are mean ± SD in 4 fish.

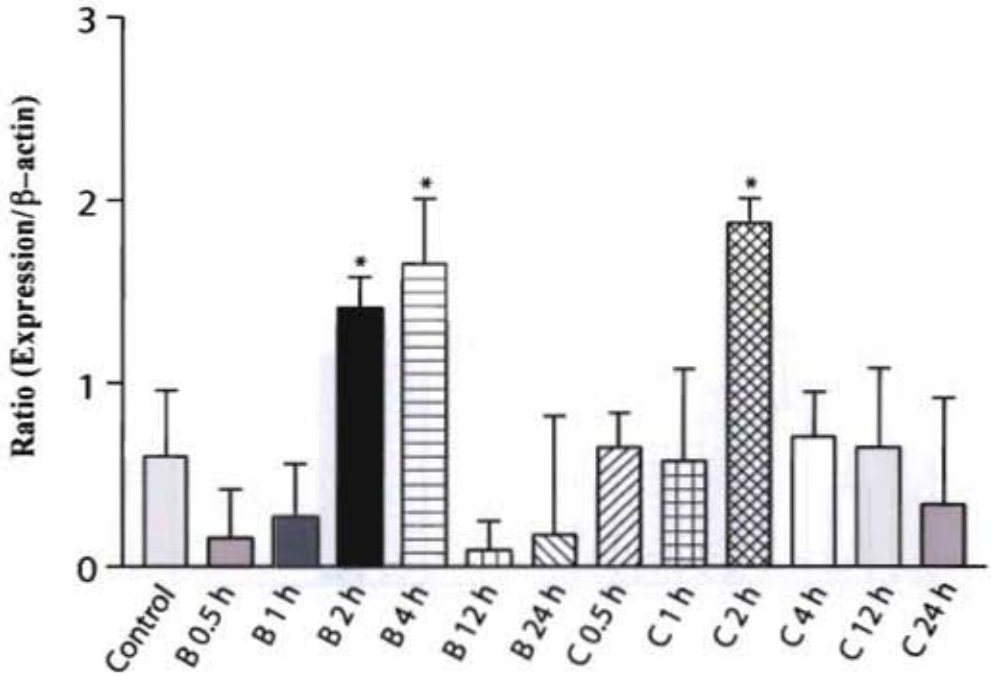

Fig. 22. Densitometric quantification of TNF-α expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish incubated with PBS and CpG-ODNs treated fish (0.5, 1, 2, 4, 12 and 24 hrs). Values are mean ± SD in 4 fish. $^{*}P < 0.05$.

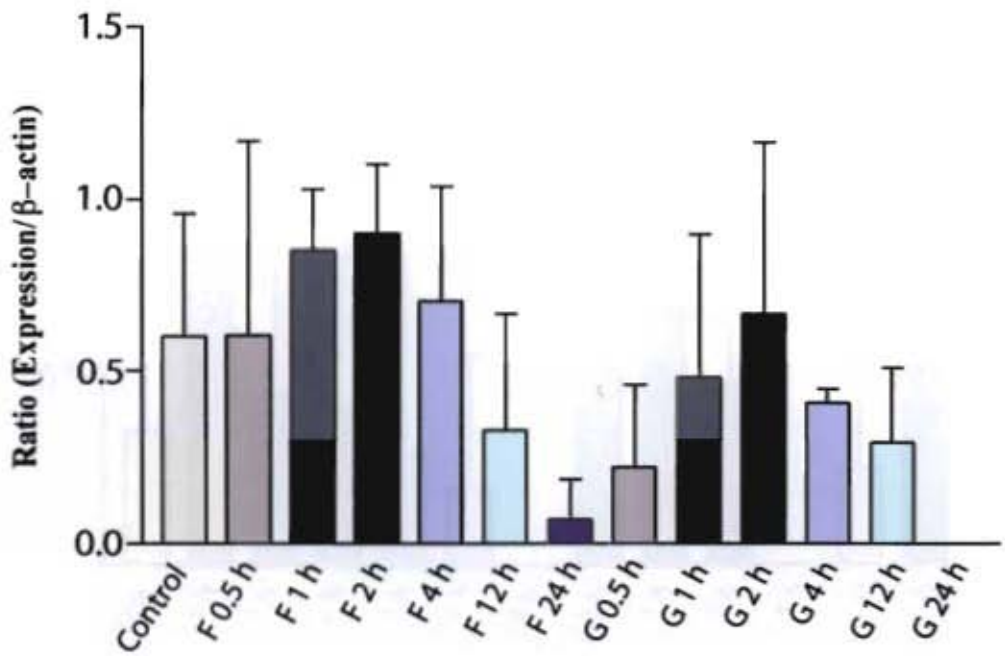

Fig. 23. Densitometric quantification of TNF-α expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish incubated with PBS and non-CpG-ODNs treated fish (0.5, 1, 2, 4, 12 and 24 hrs). Values are mean ± SD in 4 fish.

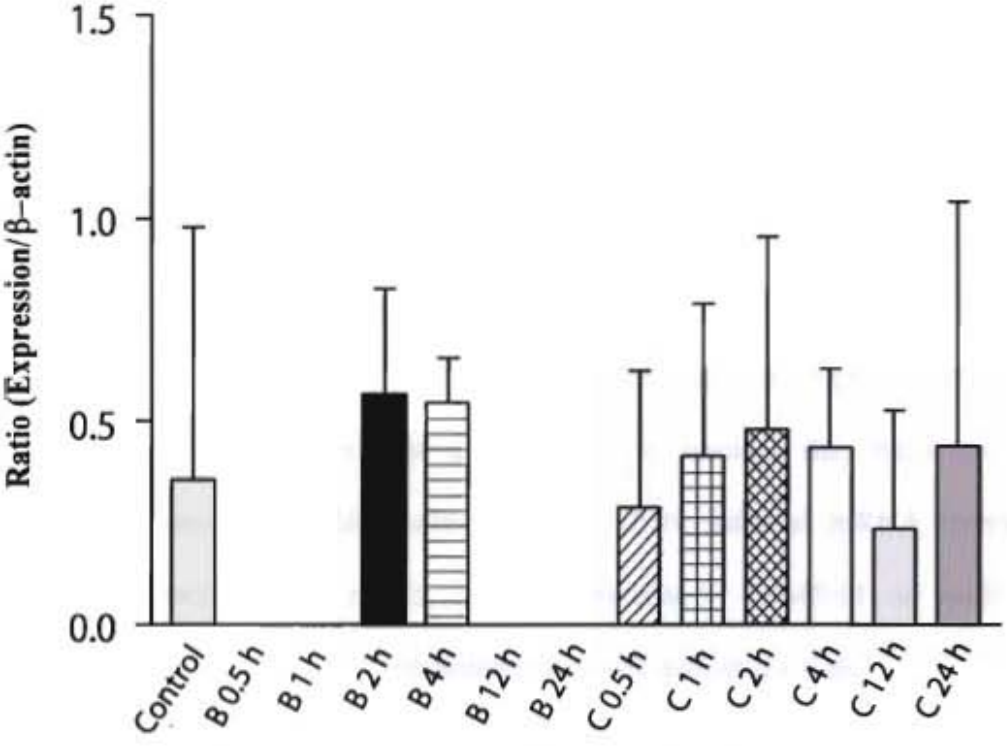

Fig. 24. Densitometric quantification of IL-10 expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish incubated with PBS and CpG-ODNs treated fish (0.5, 1, 2, 4, 12 and 24 hrs). Values are mean ± SD in 4 fish.

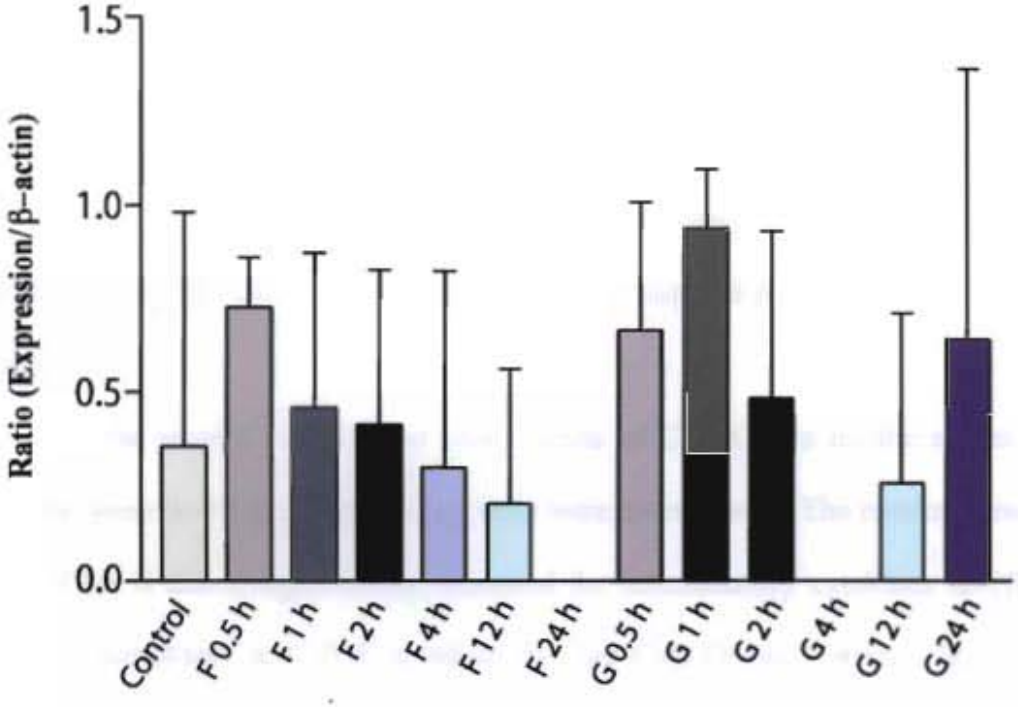

Fig. 25. Densitometric quantification of IL-10 expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish incubated with PBS and non-CpG-ODNs treated fish (0.5, 1, 2, 4, 12 and 24 hrs). Values are mean ± SD in 4 fish.

IV-2.4. Discussion

The cytokine network is an important homeostatic system with potent activites in immune surveillance, growth, developmental and repair processes. Expression of cytokine genes can be increased by using various immunostimulants like PG, LPS, Con A, PHA, etc. CpG-ODNs have gained attention because they have strong innate immune response stimulating effects. CpG-ODN induced mRNA expression of adhesion molecules and matrix metalloproteinase-9 (MMP-9) as well as pro-inflammatory cytokines and chemokines, in mouse astrocytes (Lee et al., 2004). CPG-ODN stimulation in astrocytes induced the activation of IKK and JNK. JNK activation is essential for the induction of cytokine and chemokine gene expression. Phosphorothioate–modified oligodeoxynucleotides (PS-ODNs) of the CpG-ODN 1826 stimulate TNF-α gene expression, TNF-α promoter activity, IκB degradation, and NF-κB activation at higher levels compared with its phosphodiester ODN (PO-ODN). My88 and TRAF6 are commonly required for activation of the TNF-α promoter by various CpG-ODNs with different potencies (Kwon et al., 2003). These results strongly suggest a possibility to optimally activate the innate immune responses by modulating the potency of CpG-ODNs via sequence rearrangement and phosphorothioate backbone modification.

In the present study the *in vitro* effects of CpG-ODNs on the expression of cytokine genes in the carp head kidney cells were investigated. The results showed that CpG-ODNs B and C significantly increased the inflammatory cytokines IL-1β, CXC and CC-chemokines and TNF-α, while the non-CpG-ODNs F and G had minimal stimulatory activity. These data confirm the findings of others (Krieg et al., 1995) and shows that the immunostimulatory effects of CpG motifs are sequence specific and that

a base change from CG to GC in the core motif abolishes the activity. CpG-ODNs challenge at various time intervals indicated that those genes are induced at an early time point and the message is strong in the early period of induction.

IL-1 is one of the pivotal early response pro-inflammatory cytokines that enables organisms to respond to infectious non-self challenges and induces a cascade of effects leading to inflammation. Many of these effects are mediated indirectly through up- or down regulation of other cytokine (Dinarello, 1997). A cytokine analogous to IL-1β was cloned in several teleost fish, including rainbow trout (Zou et al., 1999) and carp (Fujiki et al., 2000a). The IL-1β, IL-1 receptor, IL-6 receptor and IL-8 receptor were cloned by EST analysis in Japanese flounder (Aoki et al. 1999; Nam et al. 2000, 2001). Previous *in vivo* study (Chapter IV-1) has shown that CpG-ODNs B and C activate the expression of IL-1β gene in the head kidney of carp. *In vitro* study demonstrated that CpG-ODN 1668 activates trout macropages to express IL-1β and IFN-like cytokines, while the non-CpGs tested were ineffective (Jørgensen et al., 2001b). Furthermore, IL-1β was induced with similar kinetics by LPS and CpG-ODN, but the CpG response was lower. Hirono and Aoki (2002) noted that the expression of IL-1β in Japanese flounder was apparent after 1 h stimulation with Con A and PMA, but no expression was found after that. On the other hand, in the presence of LPS, the maximum level of IL-1β was expressed at 3 h as compared to 1 h, 6 h after stimulation. Pleguezuelos et al. (2000) similarly demonstrated that IL-1β expression significantly increased between 2.5 and 4 h after the start of stimulation with LPS.

Chemokines are small proteins that derive their name from their chemotactic properties. Recently, three novel CXC-chemokines were identified in common carp through homology cloning (Husising et al., 2004). Phylogenetic analyses show that one

of the three CXC-chemokines is an unambiguous orthologue of CXCL14, whereas both others are orthologues of CXCL12 and were named CXCL12a and CXCL12b. All three novel carp CXC-chemokines are expressed during early development, in contrast to established immune CXC-chemokines. These chemokines must play key roles in the patterning and maintenance of the (developing) vertebrate central nervous system. CC-chemokine genes have been cloned in carp (Fujiki et al., 1999), rainbow trout (Dixon et al., 1998) and Japanese flounder (Kono et al., 2003). The activations of CXC and CC-chemokine genes in the present study confirm the results from *in vivo* study (Chapter IV-1) that CpG-ODNs B and C increased the expression of these genes in the common carp head kidney cells. Savan et al. (2003) noted that the carp CXC-chemokine transcripts were expressed after 1 h of LPS and Con A stimulation. CC-chemokine gene expressions were detected in blood and in the muscle of Japanese flounder injected by plasmid DNA [pCMV-CCC (Kono et al., 2003)].

Similar to IL-1β and chemokines, TNF-α is an important component of early inflammatory events. TNF-α is a 17-kDa protein that is synthesized by different cells types upon stimulation with endotoxin, inflammatory mediators, or cytokines such as interleukin-1 and, in autocrine manner, upon stimulation with TNF itself (Hirono and Aoki, 2002). Multiple isoforms of TNF-α have been cloned in common carp; TNF-1α, TNF-2α (Saeij et al., 2003) and TNF-3α (Savan and Sakai, 2004). Activity of tumor necrosis like factor has also been reported in fish. Recently, there is a report showing enhanced leucocytes migration and phagocytic activity of rainbow trout macrophages when incubated with recombinant trout TNF proteins [rTNF (Zou et al., 2003)]. Pro-inflammatory expression also increased significantly by the rTNF protein. In this study, the expression of TNF-α genes were significantly increased in the head kidney of carp

incubated with CpG-ODN B at 2 and 4 h and CpG-ODN C at 2 h post-stimulation. The present study is the first to report the stimulation of CpG-ODNs on the expression of TNF-α genes in fish. In mice, pretreatment with CpG ODN enhanced the production of TNF-α and type-1 cytokines, including IL-12, IFN-γ and the IFN-γ-dependent ELR-CXC chemokines IFN-γ-inducible protein-10 and monokine induced by IFN-γ in response to Klebsiella challenge (Deng et al., 2004). It has been shown that TNF-α can be induced in rainbow trout and carp macrophages by stimulation with LPS (Zou et al., 2002; Saeij et al., 2003).

IL-10 initially known as cytokine synthesis inhibitory factor, is a multifunctional and demonstrates immunosuppressive function. The main function seems to be the regulation of the immunity and inflammatory response, thereby, minimizing the damage to the host by pathogen or self immune system. IL-10 down-regulates AntiViral Responses by inhibiting: the production of IFN-γ, Antigen Presentation, and Macrophage production of IL-1, IL-6, and TNF-α. IL-10 is also very important in B-cell activation. In mice, CpG-ODNs induced IL-10 from APCs, DCs and B-cells. In the present study, both CpG-ODNs and the non-CpG-ODNs did not enhance the expression of IL-10 genes in the carp head kidney cells. Gallagher et al. (2000) noted that strong expression of IL-10 was not seen until 2 to 4 hours after LPS stimulation in human monocytes.

In conclusion, CpG-ODNs stimulate an early expression of IL-1β, CXC and CC-chemokines and TNF-α genes in the head kidney of common carp. The non-CpG-ODNs fail to enhance the expression of these cytokine genes.

Chapter V

General Discussion

Bacterial DNA has long been known to be able to activate immune system, whereas mammalian is not. An important difference between bacterial and mammalian DNA is the suppression of unmethylated CpG motifs in mammalian DNA. Synthetic oligodeoxynucleotides containing unmethylated CpG motifs have been shown to mimic the effect of bacterial DNA in exerting immune responses.

Innate immunity has received attention not only because its participates in the prevention of microbial infection but also because it is capable of shifting host immunity away from Th2 toward Th1. Human molecules mainly responsible for innate immunity are called Toll-like receptors (TLR). Among them, TLR9 recognizes CpG motifs from bacterial DNA and CpG ODN.

The immunostimulatory effects of CpG-ODNs in mammalian cells are reviewed as follows:

In mice, CpG-ODN-induced gene expression clearly indicates that immunostimulatory CpG motifs act directly on a small subset of cells. The cells stimulated by CpG ODN include B lymphocytes, monocytes, macrophages, dendritic cells, NK cells and even mast cells and depending on the cell type proliferate, up-regulate MHC I and II, B7-1 and B7-2 co-stimulatory molecules, or express a broad range of cytokines including IL-1, IL-6, IL-10, IL-12, IFN-α, IFN-γ and TNF-α (Mutwiri et al., 2003). Most of the immunostimulatory effects of CpG-ODN seen in mice also occur in human cells.

In human and nonhuman primates, K- and D-type elicit very different types of

immune response (**Table 1**). K-type ODN primarily stimulates B-cells and monocyte proliferation, and IgM, IL-10 and IL-6 secretion (Krieg, 2002). The distinct effects of K and D ODNs are particularly evident on plasmacytoid dendritic cells (pDCs). K-type ODNs act on pDCs to promote TNF-α and IL-8 secretion, increased survival and maturation (defined by upregulation of CD80, CD86, and MHC II) but low and transient secretion of type I interferon (IFN) (Verthelyi and Zeuner, 2003). By contrast, D-type ODNs trigger limited pDC maturation but induce the secretion of IFN-α, which indirectly supports the subsequent maturation of APCs (Verthelyi et al., 2001; Krug et al., 2001; Hemmi et al., 2003). C-type ODNs are capable of directly stimulating B-cells and pDCs to produce IFN-α (Hartmann et al., 2003; Marshall et al., 2003).

CpG-ODN-induced gene expression in veterinary species seems to closely parallel the situation in mice though most work has been done with bovine cells. CpG-ODNs induce IL-6 and IL-12 in bovine B cells, monocytes and macrophages *in vitro* (Zhang et al., 2001). The similar effects of CpG-ODN were seen in the porcine PMBC (Mutwiri et al., 2003). CpG-ODNs also stimulate lymphocyte proliferation in PBMC (Rankin et al., 2001) spleen and lymph node cells (Wernette et al., 2002) of canine and feline. *In vivo* stimulatory effects of a CpG-ODN have been reported for the first time on the innate immune responses of two ruminant species. This study reported that the same CpG-ODN (CpG-ODN 2007) have different biological effects in two closely related species, sheep and cattle (Nichani et al., 2004). CpG-ODN 2007 induced an acute phase response in both sheep and cattle that was characterized by a transient increase in body temperature, a mild increase in circulating neutrophils and elevated serum haptoglobin levels. However, elevated serum 2'5'-A synthetase activity was detected in sheep but not in cattle.

The study mainly focused on the investigation of immunostimulatory effects of CpG oligodeoxynucleotides on the immune system of common carp. A panel of synthetic oligodeoxynucleotides on the immunocompetence cell activity in common carp was examined. *In vitro* addition of CpG-ODNs enhanced phagocytic function (reduction of nitroblue tetrazolium, phagocytosis) from stimulated kidney phagocytes. The CpG-ODNs also induced lymphocyte proliferation in the fish kidney cells. The ODNs containing multiple CpGs generally resulted in greater stimulatory capacity, although CpGs located at the terminus of an ODN were ineffective. These results show that CpG-ODNs could potentially improve immunocompetence cell activity in fish through increased cell proliferation and functionality. Based on the results from *in vitro* study in which CpG-ODN B and C, with the mice and fish motif (GA/AA)CGTT, was shown to stimulate phagocyte and lymphocyte activities in common carp head kidney leucocytes, *in vivo* studies were undertaken to examine the capacity of these CpG-ODNs to stimulate phagocytic and serum lysozyme activities in the common carp leucocytes. Intraperitoneal injection of CpG-ODNs to fish daily, 3 days, resulted in enhanced responses of phagocytic and nitroblue tetrazolium (NBT) activities in kidney phagocytic cells. This activation of kidney cells was observed for at least 7 days post injection. The serum lysozyme activity also increased in the fish treated with CpG-ODNs. These results indicated that unmethylated CpG DNA enhance the innate immune response in carp.

Expressed sequence tags (ESTs) analysis, which survey sequences contained in cDNA libraries, is a powerful approach for identifying new genes and profiling gene expression in tissue or cells (Gong et al., 1997). The screening for immune-relevant functional genes in fish is very important for identifying the molecular mechanism for

disease resistance. A representative cDNA library from mRNA obtained from CpG oligodeoxynucleotides-induced head kidney cells of carp was constructed. 88 single pass and partially sequenced clones were generated from EST and these were searched for homology in the DDBJ/GENBANK with blastN and blastX programs. Clones matching known genes were classified according to their function and distribution. 84 genes showed homology with known genes in databases, whereas 4 clones did not show any significant homology to sequences in the public database. 12 clones encoding 9 different sequences, were identified as biodefense genes, associated with an immune response. The proportion of transcripts associated with each of the broad categories is as follows: Cell organ/defense (14.3 %), Cell structure/motility (5.9 %), Cell signaling/cell communication (50.0 %), metabolism (16.7 %), and unclassified (13.1 %). The cDNA sequences reported in the present paper have been deposited into DNA Data Bank of Japan (DDBJ) with accession number from AU312478-AU312561. Among all identified genes, 79 genes appeared only once and 5 genes were present more than once. The first three most frequently identified clones were α-globin (n=5), Lysozyme-C (n=4) and β–globin (n=3). For genes related to cell/organ defense, granulin2 (AU312488), CCAAT/enhancer binding protein (AU312493), immunoglobulin heavy chain variable region (AU312495), lectin (AU312498), Lysozyme-C (AU312507), interleukin-4 receptor alpha chain (AU312530), cathepsin L preproprotein (AU312537), CD9 protein (AU312538) and Granulin 1 (AU312558) were identified. C/EBP is also activated in the signaling mechanism of CpG DNA by TLR 9, which directly up-regulate cytokine/chemokine gene expression.

Fish have non-specific and specific immune mechanism, with humoral and cellular mechanisms to resist infectious diseases. However, the biodefense and

immune-related genes in fish are poorly described. Using head kidney macrophages of common carp as an *in vivo* model, the effects of synthetic oligodeoxynucleotides containing CpG on expression of a number of immune-related genes in fish macrophages were investigated. Genes analysed included cytokines and lysozyme. Both CpG-ODN B (GCTAGACGTTAACGTT) and ODN C (ATCGACTCTCGAACGTTCTC) augmented expression of interleukin-1β, CXC and CC-chemokines at 1, 5 and 7 days post-treatment. CpG-ODN C increased the lysozyme-C gene expression at 7 days post-injection. *In vitro* study has shown that CpG-ODNs B and C significantly increased the pro-inflammatory cytokines IL-1β, CXC and CC-chemokines and TNF- α , while the non-CpG-ODNs F and G had minimal stimulatory activity. CpG-ODNs challenge at various time intervals indicated that those genes are induced at an early time point and the message is strong in the early period of induction. Both CpG-ODNs and the non-CpG-ODNs did not enhance the expression of IL-10 genes in the carp head kidney cells.

Toll-like receptors (TLR) have been recognized to play a fundamental role in the recognition of microbial pathogens and the activation of innate immunity. There is new evidence that TLR9 and some adaptor proteins that involve in a signaling pathway occur in fish. Recently, the members of TLRs and interleukin receptors (IL-R) and associated adaptor proteins containing a TIR domain have been identified in pufferfish (*Fugu rubripes*) and Zebrafish (*Danio rerio*) genomes (Oshiumi et al., 2003; Jault et al., 2004; Meijer et al., 2004). Results indicate that fish TLRs are pattern-recognition receptors rather than receptors for Spatzle-like products and thus fall into the mammalian-type (M-type). *Fugu* TLR1, 2, 3, 5, 7, 8, 9, 21, and 22 are interpreted existed in the ancestral genome common to fish and mammals, and TLR 4 was lost in

the fish lineage, while TLR21 and 22 were lost in the mammalian lineage (Oshiumi et al., 2003). Jault et al. (2004) have identified in zebrafish 19 putative TLR variants, the orthologs of mammalian TLR2-5, 7-9, a fish specific receptor type group and three putative splice variants. In contrast to the pufferfish, two receptors homologous to TLR4 were found in zebrafish, showing that lack of TLR4 is not general for fish. Toll/interleukin-1 receptor (TIR)-domain containing adaptors MyD88 and SARM were present in zebrafish, showing that TLR adaptor molecules are highly conserved in evolution. A similar study in zebrafish also demonstrated the presence of one or more counterparts for the human TLR1, 2, 3, 4, 5, 7, 8, 9, IL-1R and IL-18R genes and two genes that are highly similar to human TLR4 (Meijer et al., 2004). Furthermore, this study has shown that zebrafish and *Fugu* contain conserved homologues of four of the TIR domain adaptor proteins that have been identified in the human genome (My88, MAL, TRIF and SARM), suggesting that the signaling pathways employed by the different TLR and IL-R receptors of mammals and teleosts are likely to be highly conserved. A study examining the expression of the zTLR genes in response to *Mycobacterium marinum* infection indicates that a subset of the TLR genes including zTLR9 was expressed at higher levels in infected fish (Meijer et al., 2004). This induction is not surprising, since TLR9 has been implicated in the recognition of unmethylated CpG dinucleotide motifs that are commonly found in viral as well as bacterial genomes.

Recently, it has been found that chloroquine can inhibit the expression of IL-1β and production of interferon-like cytokines in rainbow trout macrophages (Jørgensen et al., 2001b). It indicates that CpG-ODNs stimulate the cells via a chloroquine-sensitive mechanism and endosomal maturation is essential for CpG signaling. Inhibitors of

endosomal acidification such as bafilomycin A and chloroquine completely block CpG-mediated cell activation, indicating that cellular uptake and subsequent endosomal maturation are necessary (Yi et al., 1998; Hacker et al., 1998). The presence of TLR9 and the here reported endosomal maturation is essential for CpG signaling suggest that molecular mechanism of CpG DNA in fish is similar with mammals. However, an unexpected response of catfish nonspecific cytotoxic cells (NCC) to CpG DNA suggests a differential mechanism from that which occurs in higher vertebrates (Oumouna et al., 2002). This may be related to the different receptors on the different type of cells and it is possible that there would be an unusual pathway of recognition of CpG-ODNs on NCC (Meng et al., 2003).

The immunostimulatory effects of CpG-ODNs in fish immune cells *in vitro* and *in vivo* were recently reported and the responding cells were primarily macrophages. The *in vitro* and *in vivo* effects of CpG-ODNs in fish immune cells (including the results of the present study that have been published) are summarized in **Tables 4 & 5** and reviewed as follows: The first report of the *in vitro* immunostimulatory effects of CpG-ODNs in fish was based on a work by Jørgensen et al. (2001a), where they showed that the ODN 1670 containing the 'AACGTT' motif, was superior to the 'GACGTT' motif (ODN 1668) for inducing production of interferon-like cytokines in Atlantic salmon . The enhanced stimulatory capacity of ODN 1670 has been associated with the existence of one distal CG in the 5'end and two distal CGs in the 3'end of ODN 1670. Further, the ODN 1668 that stimulates the immune mechanism in higher vertebrates has been reported to activate rainbow trout macrophages to express IFN-like cytokines and IL-1β (Jørgensen et al., 2001b). In addition, we have shown that *in vitro* treatment of the ODN 'A'/1668 (GACGTT), ODN 'B' [(GACGTT) and (AACGTT)] and ODN 'C'

(AACGTT) increased the NBT activites and the lymphocyte proliferation in the head kidney cells of common carp (Tassakka and Sakai, 2003). The CpG-ODN 'C' containing multiple CpGs also increased the phagocytic activity of these cells and has the highest stimulatory capacity. This confirms the results from studies in human, that ODNs expressing 3-4 different CpG motifs are strongly stimulatory (Klinman and Currie, 2003). Oumouna et al. (2002) observed an unexpected response of catfish non-specific cytotoxic cells (NCC) to CpG DNA. The results demonstrated a hierarchy for activation of cytotoxicity. ODNs comprised of GpC motifs had the highest activity followed by the palindrome sequence 5'-AACGTT-3'. NCC thus recognise both cytosine and guanosine. Further, non-specific cytotoxic cell receptor (NCCRP)-1 type gene has been isolated in head kidney of tilapia (*Oreochromis niloticus*) after stimulation with CpG-ODN 'B' [(GACGTT) and (AACGTT)] (Ishimoto et al., 2004). Using head kidney macrophages of grass carp as an *in vitro* model, several CpG-ODNs have been investigated in fish immunocytes (Meng et al., 2003). The CpG-ODNs included the optimal motifs: the ODN-1826 (GACGTT) and 2006 (GTCGTT) for the mice and human cells, the ODN-1670 (AACGTT) used in Atlantic salmon, the ODN-D containing two repeats motifs of those in 1670 and the ODN-R with an inverted CpG. The above results showed that CpG has an immunomodulatory role in grass carp. Furthermore, all the ODNs except the ODN-R could activate macrophages by increasing the levels of superoxide anion, hydrogen peroxide, acid phosphatase and bactericidal activity. Moreover, there were no significant differences among the ODNs tested and the ODN-D was not more efficient than 1670. These finding indicate that CpG-ODNs could be useful tools for understanding the important anti-bacterial defense mechanism in fish. Since fish cells may respond to CpG motifs different from those of

previously tested animals, Jørgensen et al. (2003) examined the ability of a panel of synthetic oligodeoxynucleotides to stimulate Atlantic salmon leucocytes *in vitro*. In other species it has been shown that CpG DNA are mitogenic for peripheral blood leucocytes (PBL) and assays to measure the proliferation have been used to screen for activity (Hartman et al., 2000; Krieg et al., 1995). Therefore, in an introductory experiment, they examined proliferative responses of fish PBL to CpG-ODNs. The results showed that Atlantic salmon PBL respond to CpG-ODNs in a sequence specific manner and proliferative responses varied among different CpG-ODNs, so this type of assay was found to be suitable for activity screening and it was combined with the IFN assay. Based on previous results in which ODN 1668 and 1670, with the murine motif (GA/AA)CGTT, was shown to stimulate antiviral activity in Atlantic salmon leucocytes (Jørgensen et al., 2001a; Jørgensen et al., 2001b), they tested the capacity of a panel of different CpG phosphothiorate ODN to stimulate proliferation of PBL and to induce production of interferon-like factors in head kidney leucocytes. The results showed that the sequence and number of the CpG motifs as well as the lengths of the ODN contribute to their stimulatory activity and ODN with the 6-mer CpG motif (GTCGTT) showed the highest stimulatory activity. These results verify what is described in other studies, that different species differ in their response to specific CpG motifs and that the motif 'GTCGTT' is the most effective in most species, including cattle, primates, cats, and dogs (Mutwiri et al., 2003). Whilst most of published data about CpG-ODN effects in fish are focused on head kidney leucocytes, these studies report for the first time that CpG-ODN could activate peripheral blood leucocytes in fish.

The *in vivo* immunostimulatory effects of CpG-ODN on the immune cells of fish are poorly investigated. Recent works from our laboratory demonstrated that

intraperitoneal injection of CpG-ODN 'B' and 'C' (ODNs used for *in vitro* stimulation) into common carp enhanced the NBT activites, phagocytic activity and serum lysozyme activity in the head kidney of common carp (Tassakka and Sakai, 2002). Furthermore, these ODNs also augmented the expression of immune-related genes, such as IL-1β, CXC and CC-chemokines and lysozyme-C in the kidney leucocytes of carp (Tassakka and Sakai, 2004). This study provides the first information concerning CpG-ODN induces expression of CXC and CC-chemokines.

It is therefore clear from *in vitro* and *in vivo* studies reported so far that CpG-ODN can stimulate immune cells of fish and the stimulation of CpG-ODN act directly on a small subset of cells.

In fish, there are few studies reporting the use of CpG-ODNs as an adjuvant (**Table 5**). Kanellos et al. (1999) defined an adjuvant effect of plasmid DNA containing CpG-ODNs in goldfish, where plasmids containing CpG-motifs (AACGTT) co-injected with a recombinant protein potentiated antibody responses to the protein. This motif elicited a stronger effect than an ODN containing 'GACGTT' (optimal motif for mice), suggesting that there may be fish-specific immunostimulatory property. Rhodes et al. (2004) demonstrated a slight improvement in survival in response to an acute *Renibacterium salmoninarum* challenge among chinook salmon (*Oncorhynchus tshawytscha*) receiving the CpG-containing ODN adjuvant, ODN 1826, compared to those receiving the control ODN adjuvant, ODN 1745 and there was no difference in kidney ELISA values of survivors from the ODN treatment groups, indicating that CpG-ODN 1826 containing 'GACGTT' motif failed to stimulate a non specific immune response in chinook salmon.

Although the adjuvant effect of CpG DNA has been demonstrated in goldfish,

the potential for using CpG DNA as a prophylactic treatment in fish has not been thoroughly investigated. Bridle et al. (2003) have shown for the first time the *in vivo* ability of CpG-ODNs to enhance resistance to disease in fish (**Table 5**). They reported that intraperitoneal injection of CpG-ODN 1826 (GACGTT) was able to increase resistance to amoebic gill disease (AGD) in Atlantic salmon. ODN with the 6-mer CpG motif (GTCGTT) were shown to induce protection against infectious pancreatic necrosis virus when injected in Atlantic salmon (Jørgensen et al., 2003). Furthermore, expression of the Mx transcript, as an indicator of alpha/beta interferon induction, was induced in the CpG-injected fish. These results suggest that CpG DNA in fish induces early, non-specific antiviral protection.

Table 4. *In vitro* activity of CpG-ODNs

Species	CpG motif	Effect on immune reponses											References
		IFN-α/β	IL-1β	Leucocytes proliferation	NBT	Phagocytosis	Hydrogen peroxide	Acid phosphatase	Bactericidal activity	Respiratory burst	NCC	NCCRP	
Atlantic salmon	AACGTT,	+											Jørgensen et al. (2001a)
	GACGTT	+											
	GTCGTT	+		+									Jørgensen et al. (2003)
Rainbow trout	GACGTT	+	+										Jørgensen et al. (2001b)
Common carp	GACGTT,			+	+								Tassakka &Sakai (2003)
	AACGTT			+	+	+							
Catfish	AACGTT										+		Oumouna et al. (2002)
Tilapia	GACGTT,											+	Ishimoto et al. (2004)
	AACGTT											+	
Grass carp	GACGTT,				+		+	+	+				Meng et al. (2003)
	GTCGTT,				+		+	+	+				
	AACGTT				+		+	+	+				
Olive flounder	GACGTT									+			Lee et al. (2003)

Table 5. *In vivo* activity of CpG-ODNs

Species	CpG motif	Effect on immune reponses												References
		IFN-α /β	IL-1β	Leucocytes proliferation	NBT	Phagocytosis	Lysozyme activity	Respiratory burst	CXC	CC	Lysozyme C	Mx	Adjuvant activity	Protective agents	
Atlantic salmon	GACGTT													+	Bridle et al. (2003)
	GTCGTT	+		+								+		+	Jørgensen et al. (2003)
Common carp	GACGTT,		+		+	+	+		+	+	+				Tassakka & Sakai (2002),
	AACGTT		+		+	+	+		+	+	+				Tassakka & Sakai (2004)
Goldfish	AACGTT												+		Kanellos et al. (1999)
Olive flounder	GACGTT							+						+	Lee et al. (2003)

Summary

1. CpG oligodeoxynucleotides could potentially improve immunocompetence cell activity in common carp through increased cell proliferation and functionality.
2. CpG oligodeoxynucleotides stimulate the innate immune responses of common carp as evidenced by increases in Nitroblue tetrazolium (NBT), phagocytic and serum lysozyme activites in the carp leucocytes
3. A cDNA library from head kidney stimulated with CpG oligodeoxynucleotides was constructed and 88 clones were sequenced. Several immune-related genes were identified during this analysis such as Granulin 2, CCAAT/enhancer binding protein, immunoglobulin heavy chain variable region, lectin, lysozyme-C, interleukin-4 receptor alpha chain, CD9 protein and Granulin 1.
4. CpG oligodeoxynucleotides augment the expression of immune-related genes in the head kidney of common carp. *In vivo* approaches of CpG-ODNs stimulate the expression of immune genes such as IL-1β, CXC and CC-chemokines and lysozyme-C. *In vitro* approaches also up-regulate the expression of IL-1β, CXC and CC-chemokines and TNF-α genes.

References

Akhtar, M., Watson, J.L., Nazli, A., McKay, D.M., 2003. Bacterial DNA evokes epithelial IL-8 production by a MAPK-dependent, NF-kappaB-independent pathway. FASEB J. 17, 1319-1321.

Alexander, J.B., Ingram, G.A., 1992. Noncellular nonspecific defence mechanisms of fish. Annu. Rev. Fish Dis. 2, 249-280.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403-410.

Anderson, D.P., 1992. Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. Annu. Rev. Fish Dis. 2, 281-307.

Anderson, D.P., Siwicki, A.K., Rumsey, G.L., 1995. Injection or immersion delivery of selected immunostimulants to trout demonstrate enhancement of non-specific defense mechanisms and protective immunity. In: Sharrif, M., Subasighe, R.P., Arthur, J.R. (Eds.), Diseases in Asian Aquaculture Vol. 11. Fish Health Section, Asian Fisheries Society, Manila, Philippines, pp. 413-426.

Aoki, T., Nam, B., Hirono, I., Yamamoto, E., 1999. Sequence of 596 cDNA clones (565.977 bp) of Japanese flounder (*Paralichthys olivaceus*) leucocytes infected with Hirame Rahbdovirus. Mar. Biotechnol. 1, 477-488.

Arbibe, L., Mira, J.P., Teusch, N., Kline, L., Guha, M., Mackman, N., 2000. Toll-like receptor 2-mediated NF-kB activation requires a Rac1-dependent pathway. Nat. Immunol. 1, 533–540.

Armstrong, J.S., Bivalacqua, T.J., Chamulitrat, W., Sikka, S., Hellstrom, W.J., 2002. A comparison of the NADPH oxidase in human sperm and white blood cells. Int. J.

Androl. 25, 223–229.

Backer, J.M., 2000. Phosphoinositide 3-kinases and the regulation of vesicular trafficking. Mol. Cell Biol. Res. Commun. 3, 193–204.

Bacon, K.B, Schall, T.J., 1996. Chemokines as mediators of allergic inflammation. Int. Arch. Aller. Immunol. 109, 97-109.

Baehner, R.L., Boxer, L.A., Davis, J., 1976. The biochemical basis of nitroblue tetrazolium reduction in normal human and chronic granulomatous disease polymorphonuclear leucocytes. Blood 48, 309–313.

Baggiolini, M., Dahinden, C.A., 1994. CC-chemokines in allergic inflammation. Immunol. Today 15, 127-133.

Bauer, M., Heeg, K., Wagner, H., Lipford, G.B., 1999. DNA activates human immune cells through a CpG sequence-dependent manner. Immunology 97, 699-705

Bauer, S., Kirschning, C.J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., Lipford, G.B., 2001. Human TLR9 confers responsiveness to bacterial DNA via species specific CpG motif recognition. Proc. Natl. Acad. Sci. USA. 98, 9237-9242.

Bayne, C.J., Levy, S., 1991. The respiratory burst of rainbow trout, *Oncorhynchus mykiss* (Walbaum), phagocytes is modulated by sympathetic neutrotransmitters and the 'neuro' peptide ACTH. J. Fish Biol. 38, 609-619.

Bird, A.P., 1986. CpG-rich islands and the function of DNA methylation. Nature 321, 209-213.

Bohle, B., Jahn-Schmid, B., Maurer, D., Kraft, D., Ebner, C., 1999. Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN-gamma production in cells from allergic individuals and inhibit IgE synthesis *in vitro*. Eur. J. Immunol. 29, 2344-2353.

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

Bridle, A.R., Butler, R., Nowak, B.F., 2003. Immunostimulatory CpG oligodeoxynucleotides increase resistance against amoebic gill disease in Atlantic salmon, *Salmo salar* L. J. Fish Dis. 26, 367-371.

Brown, W.C., Estes, D.M., Chantler, S.E., Kegerreis, K.A., Suarez, C.E., 1998. DNA and a CpG oligonucleotide derived from Babesia bovis are mitogenic for bovine B cells. Infect. Immun. 66, 5423-5432.

Burrels , C., Williams, P.D., Forno, P.F., 2001. Dietary nucleotides: a novel supplement in fish feeds 1. Effects on resistance to disease in salmonids. Aquaculture 199, 159-169.

Chen, S-L., Xu, M-Y., Hu, S-N., Li, L., 2004. Analysis of immune-relevant genes expressed in red sea bream (*Chrysophrys major*) spleen. Aquaculture 115-130.

Chong, Y-P., Song, S.S., Bhagat, L., Pandey, R.K., Yu, D., Kandimalla, E.R., Agrawal, S., 2003. Self-stabilized CpG DNAs optimally activate human B cells and plasmacytoid dendritic cells. Biochem. Biophys. Res. Commun. 310, 1133-1139.

Chu, R.S., Targoni, O.S., Krieg, A.M., Lehmann, P.V., Harding, C.V., 1997. CpG oligodeoxynucleotides act as adjuvants that switch on Th1 immunity. J.Exp. Med. 186, 1623-1631.

Chung, S., Secombes, C.J., 1987. Activation of rainbow trout macrophages. J. Fish Biol. 31A, 51-56.

Chung, S., Secombes, C.J., 1988. Analysis of events occurring within teleost macrophages during the respiratory burst. Comp. Biochem. Physiol., 89b, 539-544.

Cross, M., Mangelsdorf, I., Wedel, A., Renkawitz, R., 1988. Mouse lysozyme M gene:

isolation, characterization, and expression studies. Proc. Natl. Acad. Sci. U.S.A. 85, 6232-6236.

Dautighny, A., Prager, E.M., Pham-Dinh, D., Jolles, J., Pakdel. F., Grinde, B., Jolles, P., 1991. cDNA and amino acid sequences of rainbow trout (*Onchorhynchus mykiss*) lysozymes and their implications for the evolution of lysozyme and lactalbumin. J. Mol. Evol. 32, 187-198.

Davey, G.C., Caplice, N.C., Martin, S.A., Powell, R., 2001. A surfey of genes in the Atlantic salmon (*Salmo salar*) as identified by expressed sequence tags. Gene 263, 121-130.

Davis, H.L., Weeratna, R., Waldschmidt, T.J., Tygrett, L., Schorr, J., Krieg, A.M., 1998. CpG DNA is a potent adjuvant in mice immunized with recombinant hepatitis B surface antigen. J. Immunol. 160, 870-876.

Deng, J.C., Moore, T.A., Newstead, M.W., Zeng, X., Krieg, A.M., Standiford, T.J., 2004. CpG oligodeoxynucleotides stimulate protective innate immunity against pulmonary Klebsiella infection. J. Immunol. 173, 5148-5155.

Dinarello, C.A., 1997. Role of pro- and anti-inflammatory cytokines during inflammation: experimental and clinical findings. J. Biol. Regul. Homeost. Agents. 11, 91-103.

Dixon, B., Shum, B., Adams, E.J., Magor, K.E., Hedrick, R.P., Muir, D.G., Parham, P., 1998. CK-1, a putative chemokine of rainbow trout (*Oncorhynchus mykiss*). Immunol Revs. 166, 341-348.

Douglas, S.E., Gallant, J.W., Bullerwell, C.E., Wolff, C., Munholland, J., Reith, M.E., 1999. Winter flounder expressed sequence tags: establishment of an EST database and identification of novel fish genes. Mar. Biotechnol. 1, 458-464.

Engelsma, M.Y., Stet, R.J.M., Saeij, J.P., Verburg-van Kemenade, B.M.L., 2003. Differential expression and haplotypic variation of two interleukin-1 β genes in the common carp (Cyprinus carpio L.). Cytokine 21, 1-12.

Engstad, R.E., Robertsen, B., Frivold, E., 1992. Yeast glucan induces increase in activity of lysozyme and complement-mediated haemolytic activity in Atlantic salmon blood. Fish Shellfish Immunol. 2, 287-297.

Flory, C.M., Bayne, C.J., 1991. The influence of adrenergic and cholinergic agents on the chemiluminescent and mitogenic responses of leucocytes from the rainbow trout, *Oncorhynchus mykiss*. Dev. Comp. Immunol. 15, 135-142.

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 substractive hybridization. Immunogenetics 49, 909-914.

Fujiki, K., Nakao, M., Shin, D., Yano, T., 2000a. Molecular cloning and expression analysis of carp (*Cyprinus carpio*) interleukin-1 β, high affinity immunoglobulin E Fc receptor γ subunit and serum amyloid A. Fish Shellfish Immunol. 10, 229-242

Fujiki, K., Shin, D.H., Nakao, M., Yano, T., 2000b. 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 Shellfish Immunol. 10, 643-650.

Fujiki, K., Gauley, J., Bols, N., Dixon, B., 2002. Cloning and characterization of cDNA clones encoding CD9 from Atlantic salmon (Salmo salar) and rainbow trout (*Oncorhynchus mykiss*). Immunogenetics 54, 604-609.

Galeotti, M., Volpatti, D., Rusvai, M., 1999. Mitogen induced *in vitro* stimulation of

lymphoid cells from organs of sea bass (*Dicentrarchus labrax* L.). Fish Shellfish Immunol. 9, 227-232.

Gallagher, G.., Dickensheets, H., Eskdale, J., Izotova, L.S., Mirochnitchenko, O.V., Peat, J.D., Vazquez, N., Petska, S., Donnelly, R.P., Kotenko, S.V., 2000. Cloning, expression and initial characterization of interleukin-19 (IL-19), a novel homologue of human interleukin-10 (IL-10). Genes Immun. 1, 442-450.

Gong, Z., Hu, Z., Gong, Z.Q., Kitching, R., Hew, C.L., 1994. Bulk isolation and identification of fish genes by cDNA clone tagging. Mol. Mar. Biol. Biotechnol. 3, 243-251.

Gong, Z., Yan, T., Liao, J., lee, S.E., He, J., Hew, C., 1997. Rapid identification and isolation of zebrafish cDNA clones. Gene 201, 87-98.

Hacker, H., Mischak, H., Miethke, T., Liptay, S., Schmid, R., Sparwasser, T., Heeg, K., Lipford, G.B., Wagner, H., 1998. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. EMBO. J. 17, 6230-6240.

Hacker, H., Mischak, H., Hacker, G., Eser, S., Prenzel, N., Ullrich, A., 1999. Cell type-specific activation of mitogen-activated protein kinases by CpG-DNA controls interleukin-12 release from antigen-presenting cells. EMBO. J. 18, 6973-6982.

Hacker, H., Vabulas, R.M., Takeuchi, O., Hoshino, K., Akira, S., Wagner, H., 2000. Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. J. Exp. Med. 192, 595–600.

Hartmann, G., Weiner, G.J., Krieg, A.M., 1999. CpG DNA: a potent signal for growth, activation and maturation of human dendritic cells. Proc. Natl. Acad. Sci. U S A.

96, 9305-9310.

Hartmann, G., Krieg, A.M., 2000. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. J. Immunol. 164, 944-953.

Hartmann, G., Weeratna, R.D., Ballas, Z. K., Payette, P., Blackwell, S., Suparto, I., Rasmussen, W.L., Waldschmidt, M., Sajuthi, D., Purcell, R.H., Davis, H.L., Krieg, A.M., 2000. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses *in vitro* and *in vivo*. J. Immunol. 164, 1617-1624.

Hartmann, G., Battiany, J., Poeck, H., Wagner, M., Kerkmann, M., Lubenow, N., Rothenfusser, S., Endres, S., 2003. Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-α induction in plasmacytoid dendritic cells. Eur. J. Immunol. 33, 1633-1641.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., Akira, S., 2000. A Toll-like receptor recognizes bacterial DNA. Nature 408, 740-745.

Hemmi, H., Kaisho, T., Takeda, K., Akira, S., 2003. The roles of Toll-like receptor 9, MyD88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets. J. Immunol. 170, 3059-3064.

Hikima, J., Hirono, I., Aoki, T., 1997. Characterization and expression of c-type lysozyme cDNA from Japanese flounder (*Paralichthys olivaceus*). Mol. Mar. Biol. Biotechnol. 6, 339-344.

Hirono, I., Nam, B-H., Kurobe, T., Aoki, T., 2000. Molecular cloning, characterization, and expression of TNF cDNA and gene from Japanese flounder *Paralichthys olivaceus*. J. Immunol. 165, 4423-4427

Hirono, I., Aoki, T., 2003. Immune-related genes of the Japanese flounder, *Paralichthys olivaceus*. Aquatic Genomics. Springer-Verlag Tokyo, 286-300.

Hornung , V., Rothenfusser, S., Brtisc, S., Krug, A., Jahrsdorfer, B., Giese, T., Endres, T., Hartmann, G., 2002. Quantitative expression of Toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunol. 168, 4531-4537.

Huising, M.O., Stolte, E., Flik, G., Savelkoul, H.F.J., Verburg-van Kemenade, B.M.L., 2003. CXC-chemokines and leukocyte chemotaxis in common carp (*Cyprinus carpio* L.). Dev. Comp. Immunol. 27, 875-888.

Huising, M.O., van der Meulen, T., Flik, G., Verburg-van Kemenade, B.M., 2004. Three novel carp CXC chemokines are expressed early in ontogeny and at nonimmune sites. Eur. J. Biochem. 271, 4094-4106.

Hwang, D. M., Dempsey, A.A., Wang, R.X., Rezvani, M., Barrans, J.D., Dai, K.S., Wang, H.Y., Ma, H., et al. 1997. A genome-based resource for molecular cardiovascular medicine: Towards a compendium of cardiovascular genes. Circulation 96, 4146-4203.

Ishimoto, Y., Savan, R., Endo, M., Sakai, M., 2004. Non-specific cytotoxic cell receptor (NCCRP)-1 type gene in tilapia (*Oreochromis niloticus*): its cloning and analysis. Fish Shellfish Immunol. 16, 163-172.

Jault, C., Pichon, L., Chluba, J., 2004. Toll-like receptor gene family and TIR-domain adapters in *Danio rerio*. Mol. Immunol. 40, 759-771.

Jeney, G., Anderson, D.P., 1993. Glucan injection or bath exposure given alone or in combination with a bacterin enhance the non-specific defense mechanisms in raibow trout (*Oncorhynchus mykiss*). Aquaculture 116, 315-329.

Jiang, Z., Ninomiya-Tsuji, J., Qian, Y., Matsumoto, K., Li, X., 2002. Interleukin-1 (IL-1) receptor-associated kinase-dependent IL-1-induced signaling complexes phosphorylate TAK1 and TAB2 at the plasma membrane and activate TAK1 in the cytosol. Mol. Cell. Biol. 22, 7158–7167.

Jørgensen, J.B., Lunde, H., Robertsen, B., 1993. Peritoneal and head kidney cell response to intraperitoneally injected yeast glucan in Atlantic salmon, *Salmo Salar* L. J. Fish Dis. 16, 313-325.

Jørgensen, 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. Dev. Comp. Immunol. 25, 313-321.

Jørgensen, 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 Shellfish Immunol. 11, 673-682.

Jørgensen, J.B., Johansen, L-H., Steiro, K., Johansen, A., 2003. CpG DNA induces protective antiviral immune responses in Atlantic salmon (*Salmo salar* L.). J. Virol. 77, 11471-11479.

Kandimalla, E.R., Bhagat, L., Cong, Y-P., Pandey, R.K., Yu, D., Zhao, Q., Agrawal, S., 2003. Secondary structures in CpG oligonucleotides affect immunostimulatory activity. Biochem. Biophys. Res. Commun. 306, 948-953.

Kanellos, T.S., Sylvester, I.D., Butler, V.L., Ambali, A.G., Partidos, C.D., Hamblin, A.S., 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, 507-510.

Kerkmann, M., Rothenfusser, S., Hornung, V., Towarowski, A., Wagner, M., Sarris, A., Giese, T., Endres, S., Hartmann, G., 2003. Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells. J. Immunol. 170, 4465-4474.

Klinman, D.M., Yi, A., Beaucage, S.L., Conover, J., Krieg, A.M., 1996. CpG motifs expressed by bacterial DNA rapidly induce lymphocytes to secrete IL-6, IL-12, and IFNg. Proc. Natl. Acad. Sci. USA. 93, 2879-2883.

Klinman, D.M., Currie, D., 2003. Hierarchical recognition of CpG motifs expressed by immunostimulatory oligodeoxynucleotides. Clin. Exp. Immunol. 133, 227-232.

Kocabas, M.M., Li, P., Cao, D., Karsi, A., He, C., Patterson, A., et al, 2002. Expression profile of the channel catfish spleen: analysis of genes involved in immune functions. Mar. Biotechnol. 4, 526-536.

Köllner, B., Wasserrab, B., Kotterba, G. & Fischer, U. 2002. Evaluation of immune functions of rainbow trout (*Oncorhynchus mykiss*) - how can environmental influences be detected? Toxicol. Letters. 131, 83-95.

Kono, T., Sakai, M., Lapatra, S.E., 2000. Expressed sequence tag analysis of kidney and gill tissues from rainbow trout (*Oncorhynchus mykiss*) infected with infectious hematopoietic necrosis virus. Fish Shellfish Immunol. 2, 493-498.

Kono, T., Sakai, M., 2001. The analysis of expressed genes in the kidney of Japanese flounder, *Paralichthys olivaceus*, injected with the immunostimulant peptidoglycan. Fish Shellfish Immunol. 11, 357-366.

Kono, T., Watanuki, H., Sakai, M., 2002a. The activation of interleukin-1β in serum of carp, *Cyprinus carpio*, injected with peptidoglycan. Aquaculture 212, 1-10.

Kono, T., Fujiki, K., Nakao, M., Yano, T., Endo, M., Sakai, M., 2002b. The immune responses of common carp, *Cyprinus carpio* L., injected with carp interleukin-1β gene. J. Interferon Cytokine Res. 22, 413-419.

Kono, T., Kusuda, R., Kawahara, E., Sakai, M., 2003. The analysis of immune responses of a novel CC-chemokine gene from Japanese flounder *Paralichthys olivaceus*. Vaccine 21, 446-457.

Krieg, A.M., Yi, A., 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 374, 546-548.

Krieg, A.M., Hartmann, G., Yi, A.K., 2000. Mechanism of action of CpG DNA. Curr. Top. Microbiol. Immunol. 247, 1-21.

Krieg, A.M., 2002. CpG motifs in bacterial DNA and their immune effects. Annu. Rev. Immunol. 20, 709-760.

Krug, A., Rothenfusser, S., Hornung, V., Jahrsdorfer, B., Blackwell, S., Ballas, Z.K., Endres, S., Krieg, A.M., Hartmann, G., 2001. Identification of CpG oligonucleotide sequences with high induction of IFNa/b in plasmacytoid dendritic cells. Eur. J. Immunol. 31, 2154-2163.

Kwon, H.J., Lee, K.W., Yu S.H., Han, J.H., Kim, D.S., 2003. NF-κB-dependent regulation of tumor necrosis factor-α gene expression by CpG-oligodeoxynucleotides. Biochem. Biophys. Res. Commun. 311, 129-138.

Laing, K.J., Cunningham, C., Secombes, C.J., 2000. Genes for three different isoforms of transforming growth factor-β are present in plaice (*Pleuronectes platessa*) DNA. Fish Shellfish Immunol. 10, 261-271

Laing, K.J., Wang, T., Zou, J., Holland, J., Hong, S., Bols, N., Hirono, I., Aoki, T.,

Secombes, C.J., 2001. Cloning and expression analysis of rainbow trout *Oncorhynchus mykiss* tumour necrosis factor-alpha. Eur. J. Biochem. 268, 1315-1322.

Lapatra, S.E., Lauda, K.A., Jones, G.R., Shewmaker, W.S., Bayne, C.J., 1998. Resistance to IHN virus infection in rainbow trout is increased by glucan while subsequent production of serum neutralizing activity is decreased. Fish Shellfish Immunol. 8, 435-466.

Lee, C.H., Jeong, H.D., Chung, J.K., Lee, H.H., Kim, K.H., 2003. CpG motif in synthetic ODN primes respiratory burst of olive flounder *Paralichthys olivaceus* phagocytes and enhances protection against *Edwardsiella tarda*. Dis. Aquat. Org. 56, 43-48.

Lee, S., Hong, J., Choi, S.Y., Oh, S.B., Park, K., Kim, J.S., Karin, M., Lee, S.J., 2004. CpG oligodeoxynucleotides induce expression of proinflammatory cytokines and chemokines in astrocytes: the role of c-Jun N-terminal kinase in CpG ODN-mediated NF-κB activation. J. Neuroimmunol. 153, 50-63.

Lindenstrøm, T., Buchmann, K., Secombes, C.J., 2003. *Gyrodactylus derjavini* infection elicits IL-1β expression in rainbow trout skin. Fish and Shellfish Immunol. 15, 107-115.

Liu, F., Wen, Z., 2002. Cloning and expression pattern of the lysozyme-C Gene in Zebrafish. Mech. Dev. 113, 69-72

Logambal, S.M., Venkatalakshmi, S., Michael, R.D., 2000. Immunostimulatory effect of leaf extract of *Ocimum sanctum* Linn. in *Oreochromis mossambicus* (Peters). Hydrobiologia. 430, 113-120.

Long, S., Wilson, M., Bengten, E., Bryan, L., Clem, L.W., Miller, N.W., Chinchar, V.G.,

2004. Identification of a cDNA encoding channel catfish interferon. Dev. Comp. Immunol. 28, 97-111.

Low, C., Wadsworth, S., Burrells, C., Secombes, C.J., 2003. Expression of immune genes in turbot (*Scophthalmus maximus*) fed a nucleotide-supplemented diet. Aquaculture 221, 23-40

Lunden, T., Bylund, G., 2000. The influence of *in vitro* and *in vivo* exposure to antibiotics on mitogen induced proliferation of lymphoid cells in rainbow trout (*Onchorhynchus mykiss*). Fish Shellfish Immunol. 10, 395-404.

Marsden, M.J., Hamdani, S.H., Secombes, C.J., 1995. Proliferative responses of rainbow trout, *Onchorhynchus mykiss*, T and B cells to antigens of *Aeromonas salmonicida*. Fish Shellfish Immunol. 5, 199-210.

Marshall, J.D., Fearon, K., Abbate, C., Subramanian, S., Yee, P., Gregorio, J., Coffman, R.L., Van Nest, G., 2003. Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. J. Leukoc. Biol. 73, 781-792.

Martin, S.A, Caplice, N.C., Davey, G.C., Powell, R., 2002. EST-based identification of genes expressed in the liver of adult Atlantic salmon (*Salmo salar*). Biochem. Biophys. Res. Commun. 293, 578-585

Matsuyama, H., Mangindaan, R.E.P., Yano, T., 1992. Protective effect of schizophyllan and scleroglucan against *Streptococcus* sp. infection in yellowtail (*Seriola quinqueradiata*). Aquaculture 101, 197-203.

Medzhitov, R., Janeway, C.A., 1997. Innate immunity: the virtues of nonclonal system of recognition. Cell 91, 295-298.

Medzhitov, R., 2001. Toll-like receptors and innate immunity. Nat. Rev. Immunol. 1, 135-145.

Meijer, A.H., Krens, S.F.G., Rodrigues, I.A.M., He, S., Bitter, W., Snaar-Jagalska, B.E., Spaink, H.P., 2004. Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. Mol. Immunol. 40, 773-783.

Meng, Z., Shao, J., Xiang, L., 2003. CpG oligodeoxynucleotides activate grass carp (*Ctenopharyngodon idellus*) macrophages. Dev. Comp. Immunol. 27, 313-321.

Messina, J.P., Gilkeson, G.S., Pisetsky, D.S., 1991. Stimulation of *in vitro* murine lymphocyte proliferation by bacterial DNA. J. Immunol. 147, 1759-1764.

Mutwiri, G., Pontarollo, R., Babiuk, S., Griebel, P., van Drunen Littel-van den Hurk, S., Mena, A., Tsang, C., Alcon, V., Nichani, A., Ioannou, X., Gomis, S., Townsend, H., Hecker, R., Potter, A., Babiuk, L.A., 2003. Biological activity of immunostimulatory CpG DNA motifs in domestic animals. Vet. Immunol. Immunopathol. 91, 89-103.

Mutwiri, G.K., Nichani, A.K., Babiuk, S., Babiuk, L.A., 2004. Strategies for enhancing the immunostimulatory effects of CpG oligodeoxynucleotides. J. Control Release 97, 1-17.

Nam, B.H., Yamamoto, E., Hirono, I., Aoki, T., 2000. A surfey of expressed genes in the leucocytes of Japanese flounder, *Paralichthys olivaceus*, infected with Hirame rhabdovirus. Dev. Comp. Immunol. 24, 13-24.

Nichani, A.K., Mena, A., Popowych, Y., Dent, D., Townsend, H.G.G., Mutwiri, G.K., Hecker, R., Babiuk, L.A., Griebel, P.J., 2004. *In vivo* immunostimulatory effects of CpG oligodeoxynucleotide in cattle and sheep. Vet. Immunol. Immunopathol. 98, 17-29.

Nikl, L., Evelyn, T.P.T., Albright, L.J., 1993. Trials with an orally and immersion-administered β-1,3 glucan as an immunoprophylactic against *Aeromonas salmonicida* in juvenile Chinook salmon *Onchorhynchus tshawytscha*. Dis. Aquat. Org. 17, 191-196.

Oppenheim, J.J., Zachariae, C.O., Mukaida, N., Matsushima, K., 1991. Properties of the novel pro-inflammatory supergene"intercrine" cytokine family. Ann. Rev. Immunol. 9, 617-648

Ortuno, J., Cuesta, A., Rodrigues, A., Esteban, M.A., Meseguer, J., 2002. Oral administration of yeast, *Saccharomyces cerevisiae*, enhances the cellular innate immune response of gilthead seabream (*Sparus aurata* L.). Vet. Immunol. Immunopathol. 85, 41-50.

Oshiumi, H., Tsujita, T., Shida, K., Matsumoto, M., Ikeo, K., Seya, T., 2003. Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome. Immunogenetics 54, 791-800.

Oumouna, M., Jaso-Friedmann, L., Evans, D.L., 2002. Activation of non-specific cytotoxic cells (NCC) with synthetic oligodeoxynucleotides and bacterial genomic DNA: binding, specificity and identification of unique immunostimulatory motifs. Dev. Comp. Immunol. 26, 257-269

Ozes, O.N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M., Donner, D.B., 1999. NF-kB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 401, 82–85.

Park, Y., Lee, S.W., Sung, Y.C., 2002. Cutting Edge: CpG DNA inhibits dendritic cell apoptosis by up-regulating cellular inhibitor of apoptosis proteins through the phosphatidylinositide-3-OH kinase pathway. J. Immunol. 168, 5–8.

Parry, R.M. Jr., Chandan, R.C., Shahani K.M., 1965. A rapid and sensitive assay of muramidase. Proc. Soc. Exp. Biol. Med. 119, 384-386.

Paulsen, S.M., Lunde, H., Engstad, R.E., Robertsen, B., 2003. *In vivo* effects of β-glucan and LPS on regulation of lysozyme activity and mRNA expression in Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol. 14, 39-54

Peddie, S., Zou, J., Secombes, C.J., 2002. Immunostimulation in the rainbow trout (*Oncorhynchus mykiss*) following intraperitoneal administration of Ergosan. Vet. Immunol. Immunopathol. 86, 101-113.

Pelegrin, P., Garcia-Castillo, J., Mulero, V., Meseguer, J., 2001. Interleukin-1β isolated from a marine fish reveals up-regulated expression in macrophages following activation with lipopolysaccharide and lymphokines. Cytokine 21, 67-72.

Pisetsky, D.S., 1996. Immune activation by bacterial DNA: a new genetic code. Immunity. 5, 303-310.

Pisetsky, D.S., 2000. Mechanisms of immune stimulation by bacterial DNA. Springer Semin. Immunopathol. 22, 21-33.

Pleguezuelos, O., Zou, J., Cunningham, C., Secombes, C.J., 2000. Cloning, sequencing, and analysis of expression of a second IL-1β gene in rainbow trout (*Oncorhynchus mykiss*). Immunogenetics. 51, 1002-1011.

Pontarollo, R.A., Rankin, R., Babiuk, L.A., Godson, D.L., Griebel, P.J., Hecker, R., Krieg, A.M., van Drunen Little-van den Hurk, S., 2002. Monocytes are required for optimum *in vitro* stimulation of bovine peripheral blood mononuclear cells by non-methylated CpG motifs. Vet. Immunol. Immunopathol. 84, 43-59.

Raa, J., Rørstad, G., Engstad, R., Robertsen, B., 1992. The use of immunostimulants to increase resistance of aquatic organisms to microbial infections. In: Sharif, M.I.,

Subasinghe, R.P., Arthur, J.R.. (Eds.), Diseases in Asian Aquaculture. Fish Health Section, Asian Fisheries Society, Manila, Philippines, pp. 39-50.

Rankin, R., Pontarollo, R., Ioannou, X., Krieg, A.M., Hecker, R., Babiuk, L.A., van Drunen Littel-van den Hurk, S., 2001. CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. Antisense Nucleic Acids Drug Dev. 11, 333-340.

Rhodes, L.D., Rathbone, C. K., Corbett, S.C., Harrell, L.W., Strom, M.S., 2004. Efficacy of cellular vaccines and genetic adjuvants against bacterial kidney disease in chinook salmon (*Oncorhynchus tshawytscha*). Fish Shellfish Immunol. 16, 461-474

Richards, D.T., Hoole, D., Lewis, J., Ewens, E., Arme, C., 1996. Stimulation of carp *Cyprinus carpio* lymphocytes *in vitro* by the blood fluke *Sanguinicola inermis* (Trematoda: Sanguinicolidae). Dis. Aquat. Org. 25, 87-93.

Robertsen, B., Rørstad, G., Engstad, R., Raa, J., 1990. Enhancement of non-specific disease resistance in Atlantic salmon, *Salmo salar* L., by a glucan from *Saccharomyces cerevisiae* cell walls. J. Fish Dis. 13, 391-400.

Robertsen , B., Engstad, R.E., Jørgensen, J.B., 1994. β-glucans as immunostimulants in fish. In: Stolen, J., Fletcher, T.C. (Eds), Modulators of Fish Immune Responses. SOS publications, Fair Haven, NJ, pp. 83-89..

Robertsen, B., 1999. Modulation of non-specific defence of fish by structurally conserved microbial polymers. Fish Shellfish Immunol. 9, 269-290.

Rothenfusser, S., Hornung, V., Krug, A., Towarowski, A., Krieg, A.M., Endres, S., Hartmann, G., 2001. Distinct CpG oligonucleotide sequences activate human gamma delta T cells via interferon-alpha/-beta. Eur. J. Immunol. 31, 3525-3534.

Rowley, A.F., Hunt, T.C., Page, M., Wainwaring, G., 1988. Fish. In Vertebrate Blood Cells (A. F. Rowley & N. A. Ratcliffe, eds) pp. 19-75. Cambridge: Cambridge University Press.

Saeij, J.P., Stet, R.J., de Vries, B.J., van Muiswinkel, W.B., Wiegertjes, G.F., 2003. Molecular and functional characterization of carp TNF: a link between TNF polymorphism and trypanotolerance? Dev. Comp. Immunol. 27, 29-41.

Sakai, M., Kamiya, H., Ishii, S., Atsuta, S., Kobayashi, M., 1992. The immunostimulating effects of chitin in rainbow trout, *Oncorhynchus mykiss*. In: Sharrif, M., Subasighe, R.P., Arthur, J.R. (Eds.), Diseases in Asian Aquaculture Vol. 1. Fish Health Section, Asian Fisheries Society, Manila, Philippinnes, pp. 413-417

Sakai, M., Yoshida, T., Kobayashi, M., 1995. Influence of the immunostimulant, EF203, on the immune responses of rainbow trout, *Oncorhynchus mykiss*, to *Renibacterium salmoninarum*. Aquaculture 138, 61-67.

Sakai, M., Miyama, K., Atsuta, S., Kobayashi, H., 1996. The chemiluminescence responses of phagocytic cells of coho salmon, rainbow trout and carp against *Renibacterium salmoninarum*. Fish Shellfish Immunol. 6, 71-73.

Sakai, M., 1999. Current research status of fish immunostimulants. Aquaculture 172, 63-92.

Salati, F., Hamaguchi, M., Kusuda, R., 1987. Immune response of red sea bream to *Edwardsiella tarda* antigens. Fish Pathol. 22, 93-98.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Savan, R., 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. Comp. Biochem. Physiol. B 131, 71-82

Savan, R., Kono, T., Aman, A., Sakai, M., 2003. Isolation and characterization of a novel CXC chemokine in common carp (*Cyprinus carpio* L.). Mol. Immunol. 39, 829-834.

Savan, R., Daisuke, I., Sakai, M., 2003. Cloning, characterization and expression analysis of common carp *Cyprinus carpio* L. interleukin-10. Eur. J. Biochem. 270, 4647-4654

Savan, R., Endo, M., Sakai, M., 2004. Characterization of a new C-type lectin from common carp *Cyprinus carpio*. Mol. Immunol. 41, 891-899.

Savan, R., Sakai, M., 2004. Presence of multiple isoforms of TNF alpha in carp (*Cyprinus carpio* L.): genomic and expression analysis. Fish Shellfish Immunol. 17, 87-94.

Scapigliati, G., Buonocore, F., Bird, S., Zou, J., Pelegrin, P., Falasca, C., Prugnoli, D., Secombes, C.J., 2001. Phylogeny of cytokines: molecular cloning and expression analysis of sea bass *Dicentrarchus labrax* interleukin-1β. Fish Shellfish Immunol. 11, 711-726

Schnare, M., Barton, G.M., Holt, A.C., Takeda, K., Akira, S., Medzhitov, R., 2001. Toll-like receptors control activation of adaptive immune responses. Nat. Immunol. 2, 947–950.

Secombes, C.J., 1986. Immunological activation of rainbow trout macrophages induced *in vitro* by sperm autoantibodies and factors derived from testis sensitised leucocytes. Vet. Immunol. Immunopathol. 12, 193-201.

Secombes, C.J., Chung, S., Jeffries, A.H., 1988. Superoxide anion production by rainbow trout macrophages detected by the reduction of ferricytochrome C. Dev. Comp. Immunol. 12, 201-206.

Secombes, C.J., 1990. Isolation of salmonid macrophages and analysis of their killing activity. In *Techniques in Fish Immunology*. 137-154. Fair Haven, NJ: SOS publications.

Secombes, C.J., Fletcher, T.C., 1992. The role of phagocytes in the protective mechanisms of fish. Annu. Rev. Fish Dis. 2, 53-71.

Sharp, G. J. E., Secombes, C. J., 1993. The role of reactive oxygen species in the killingof the bacterial fish pathogen *Aeromonas salmonicida* by rainbow trout macrophages. Fish Shellfish Immunol. 3, 119-129.

Shuto, T., Xu, H., Wang, B., Han, J., Kai, H., Gu, X.X., 2001. Activation of NF-kB by nontypeable Hemophilus influenzae is mediated by Toll-like receptor 2-TAK1-dependent NIK-IKKa/b-IkBa and MKK3/6-p38 MAP kinase signaling pathways in epithelial cells. Proc. Natl. Acad. Sci USA 98, 8774–8779.

Siddhanta, U., McIlroy, J., Shah, A., Zhang, Y., Backer, J.M., 1998. Distinct roles for the p110a and hVPS34 phosphatidylinositol 3-kinases in vesicular trafficking, regulation of the actin cytoskeleton, and mitogenesis. J. Cell Biol. 143, 1647–1659.

Sigh, J., Lindenstrøm, T., Buchmann, K., 2004. Expression of pro-inflammatory cytokines in rainbow trout (*Oncorhynchus mykiss*) during an infection with Ichthyophthirius multifiliis. Fish Shellfish Immunol. 17, 75-86.

Siwicki, A.K., 1989. Immunostimulating influence of levamisole on non-specific immunity in carp (*Cyprinus carpio*). Dev. Comp. Immunol. 13, 87-91.

Siwicki, A.K., Anderson, D.P., Dixon, O.W., 1990. *In vitro* immunostimulation of

rainbow trout (*Oncorhynchus mykiss*) spleen cells with levamisole. Dev. Comp. Immunol. 14, 231-237.

Siwicki, A.K., Anderson, D.P., Rumsey, G.L., 1994. Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection aginst furunculosis. Vet. Immunol. Immunopathol. 41, 125-139.

Siwicki, A.K., Klein, P., Morand, M., Kiczka, W., Studnicka, M., 1998. Immunostimulatory effects of dimerized lysozyme (KLP-602) on the non-specific defense mechanisms and protection against furunculosis in salmonids. Vet. Immunol. Immunophatol. 61, 369-378.

Smith, P.D., Braun-Nesje, R., 1982. Cell mediated immunity in the salmon: Lymphocyte and macrophage stimulation, lymphocyte/macrophage interactions, and the production of lymphokine-like factors by stimulated lymphocytes. Dev. Comp. Immunol. Suppl. 2, 233-238.

Solem, S.T., Jorgensen, J.B., Robertsen, B., 1995. Stimulation of respiratory burst and phagocytic activity in Atlantic salmon (*Salmo salar* L.) macrophages by lipopolysaccharide. Fish Shellfish Immunol. 5, 475-491.

Sparwasser, T., Koch, E., Vabulas, R.M., Heeg, K., Lipford, G.B., Ellwart, J.W., Wager, H., 1998. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur. J. Immunol. 28, 2045-2054.

Stacey, K.J., Sweet, M.J., Hume, D.A., 1996. Macrophages ingest and are activated by bacterial DNA. J. Immunol. 157, 2116-2122.

Tabeta, K., Georgel, P., Janssen, E., Du, X., Hoebe, K., Crozat, K., Mudd, S., Shamel, L., Sovath, S., Goode, J., et al., 2004. Toll-like recptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection.

Proc. Natl. Acad. Sci. USA 101, 3516-3521.

Tafalla, C., Coll, J., Secombes, C.J. Expression of genes related to the early immune response in rainbow trout (*Oncorhynchus mykiss*) after viral haemorrhagic septicemia virus (VHSV) infection. Dev. Comp. Immunol. In press.

Takeda, K., Kaisho, T., Akira, S., 2003. Toll-like receptors. Annu. Rev. Immunol. 21, 335-376.

Takeshita, F., Klinman, D.M., 2000. CpG ODN-mediated regulation of IL-12 p40 transcription. Eur. J. Immunol. 30, 1967-1976.

Takeshita, F., Ishii, K.J., Ueda. A., Ishigatsubo, Y., Klinman, D.M., 2000. Positive and negative regulatory elements contribute to CpG oligonucleotide-mediated regulation of human IL-6 gene expression. Eur. J. Immunol. 30, 108–116.

Takeshita, F., Leifer, C.A., Gursel, I., Ishii, K., Takeshita, S., Gursel, M., Klinman, D.M., 2001. Cutting Edge: role of toll-like receptor 9 in CpG DNA-induced activation of human cells. J. Immunol. 167, 3555-3558.

Takeshita, F., Gursel, I., Ishii, K.J., Suzuki, K., Gursel, M., Klinman, D.M., 2004. Signal transduction pathways mediated by the interaction of CpG DNA with Toll-like receptor 9. Semin. Immunol. 16, 17-22.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., 1999. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. Immunity 11, 443–451.

Tassakka, A.C.M.A.R., Sakai, M., 2002. CpG oligodeoxynucleotides enhance the non-specific immune responses on carp, *Cyprinus carpio*. Aquaculture 209, 1-10

Tassakka, A.C.M.A.R., Sakai, M., 2003. The *in vitro* effect of CpG Oligodeoxynucleotides on the innate immune response of common carp, *Cyprinus*

carpio L. Aquaculture 220, 27-36

Tassakka, A.C.M.A.R., Sakai, M., 2004. Expression of immune-related genes in the common carp (*Cyprinus carpio* L) after stimulation by CpG oligodeoxynucleotides. Aquaculture 242, 1-12.

Thompson, K.D., Cachos, A., Inglis, V., 1995. Immunomodulating effects of glucans and oxytetracycline in rainbow trout, *Oncorhynchus mykiss*, on serum lysozyme and protection. In: Sharrif, M., Subasighe, R.P., Arthur, J.R. (Eds.), Diseases in Asian Aquaculture Vol. 11. Fish Health Section, Asian Fisheries Society, Manila, Philippinnes, pp. 433-439.

Thomson, A., 1994. The cytokine Handbook, 2nd edition. Academic Press, London, pp 1-211.

Tokunaga, T., Yamamoto, H., Shimada, S., Abe, H., Fukuda, T., Fujisawa, Y., Furutani, Y., Yano, O., Kataoka, T., Sudo, T., 1984. Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG: Isolation, Physicochemical characterization, and antitumor activity. J. Natl. Cancer Inst. 72, 955-962.

Tokunaga, T., Yamamoto, S., Namba, K., 1988. A synthetic single-stranded DNA, poly (dG, dC), induces interferon-alpha/beta and –gamma, augments natural killer activity, and suppresses tumor growth. Jpn. J. Cancer Res. 79, 682-686.

Tokunaga, T., Yano, O., Kuramoto, E., Kimura, Y., Yamamoto, T., Kataoka, T., Yamamoto, S., 1992. Synthetic oligonucleotides with particular base sequences from the cDNA encoding proteins of *Mycobacterium bovis* BCG induce interferons and activate natural killer cells. Microbiol. Immunol. 36, 55-66.

Tucker, C. S., Hirono, I., Aoki, T., 2002. Molecular cloning and expression of

CCAAT/enhancer binding protein in Japanese flounder *Paralichthys olivaceus*. Dev. Comp. Immunol. 26, 271-282.

Underhill, D.M., Ozinsky, A., 2002. Toll-like receptors: key mediators of microbe detection. Curr. Opin. Immunol. 14, 103–110.

Vanhaesebroeck, B., Alessi D.R., 2000. The PI3K-PDK1 connection: more than just a road to PKB. Biochem. J. 346, 561– 576.

Vasta, G. R., Ahmed, H., Fink, N.E., Elola, M.T., Marsh, A.G., Snowden, A., Odom, E.W., 1994. Animal lectins as self/non-self recognition molecules. Biochemical and genetic approaches to understanding their biological roles and evolution. Ann. N.Y. Acad. Sci. 712, 55-73.

Vasselon, T., Detmers, P.A., 2002. Toll receptors: a central element in innate immune responses. Infect. Immun. 70, 1033–1041.

Verthelyi, D., Ishii, K.J., Gursel, M., Takeshita, F., Klinman, D.M., 2001. Human peripheral blood cells differentially recognize and respond to two distinct CpG motifs. J. Immunol. 166, 2372-2377.

Verthelyi, D., Klinman, D.M., 2003. Immunoregulatory activity of CpG oligonucleotides in humans and nonhuman primates. Clin. Immunol. 109, 64-71.

Verthelyi, D., Zeuner, R.A., 2003. Differential signaling by CpG DNA in DCs and B cells: not just TLR9. Trends Immunol. 24, 519-522.

Vieira, O.V., Botelho, R.J., Grinstein, S., 2002. Phagosome maturation: aging gracefully. Biochem. J. 366, 689–704.

Wang, W.S., Wang, D.H., 1997. Enhancement of the resistance of tilapia and grass carp to experimental *Aeromonas hydrophila* and *Edwardsiella tarda* infections by several polysaccharides. Comp. Immunol. Microbiol. Infect. 20, 261-270.

Wernette, C.M., Smith, B.F., Barksdale, Z.L., Hecker, R., Baker, H.J., 2002. CpG oligodeoxynucleotides stimulate canine and feline immune cell proliferation. Vet. Immunol. Immunopathol. 84, 223-236.

Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O., Tokunaga, T., 1992. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. J. Immunol. 148, 4072-4076.

Yamamoto, T., Yamamoto, S., Kataoka, T., Tokunaga, T., 1994. Lipofection of synthetic oligodeoxyribonucleotide having a palindromic sequence of AACGTT to murine splenocytes enhances interferon production and natural killer activity. Microbiol. Immunol. 38, 831-836.

Yi, A.K., Tuetken, R., Redford, T., Waldschmidt, M., Kirsch, J., Krieg, A.M., 1998. CpG motifs in bacterial DNA activate leucocytes through the pH-dependent generation of reactive oxygen species. J. Immunol. 160, 4755-4761.

Yi, A.K., Yoon, J.G., Yeo, S.J., Hong, S.C., English, B.K., Krieg, A.M., 2002. Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th1 response. J. Immunol. 168, 4711-4720.

Yin, Z., Kwang, J., 2000. Molecular isolation and characterization of carp transforming growth factor β 1 from activated leucocytes. Fish Shellfish Immmunol. 10, 309-318.

Yoshida, T., Sakai, M., Kitao, T., Khill, S.M., Araki, S., Saito, R., Ineno, T., Inglish, V., 1993. Immunomodulatory effects of the fermented products of chicken egg, EF203 on rainbow trout, *Oncorhynchus mykiss*. Aquaculture 109, 207-214.

Yoshie, O., Nomiyama, N., Miyasaka, M., 2000. Chemokine Handbook: Cell

Technology. Shujunsha Co. Ltd., Tokyo

Zhang, Y., Shoda, L.K., Brayton, K.A., Estes, D.M., Palmer, G.H., Brown, W.C., 2001. Induction of interleukin-6 and interleukin-12 in bovine B lymphocytes, monocytes, and macrophages by a CpG oligodeoxynucleotide (ODN 2059) containing the GTCGTT motif. J. Interferon Cytokine Res. 21, 871-881.

Zimmermann, S., Egeter, O., Hausmann, S., Lipford, G.B., Rocken, M., Wagner, H., Heeg, K., 1998. CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. J. Immunol. 160, 3627-3630.

Zou, J., Grabowski, P.S., Cunningham, C., Secombes, C. J., 1999. Molecular cloning of interleukin-1 β from rainbow trout *Oncorhynchus mykiss* reveals no evidence of an ICE cut site. Cytokine 11, 552-560.

Zou, J., Wang, T., Hirono, I., Aoki, T., Inagawa, H., Honda, T, et al, 2002. Differential expression of two tumor necrosis factor genes in rainbow trout, *Oncorhynchus mykiss*. Dev. Comp. Immunol. 28, 229- 237.

Zou, J., Peddie, S., Scapigliati, G., Zhang, Y., Bols, N.C., Ellis, A.E., Secombes, C.J., 2003. Functional characterisation of the recombinant tumor necrosis factors in rainbow trout, *Oncorhynchus mykiss*. Dev. Comp. Immunol. 27, 813-822.

Acknowledgements

All praises are to the almighty who bestowed on me the courage, patience and mental peace so that this piece of work could come to completion.

I wish to express sincere gratitude to my advisory supervisor, Professor Masahiro SAKAI for his invaluable guidance, assistance, constructive criticism and encouragements through out this study.

I am very grateful to acknowledge to Professor Tadashi SAKAI of University of Miyazaki, Professor Taizo SAKATA of Kagoshima University, Professor Toshiaki ITAMI of University of Miyazaki and Professor Hidemasa MIKI of Kagoshima University for their valuable and important advices and suggestions.

I am grateful to my laboratory members, Dr. Tomoya Kono, Dr. Hironobu Watanuki, Dr. Ram Savan, I. Gunimala Devi, M. F. Sc, H. Sakata, A. Aman, S. Hama, D. Igawa, Y. Itou, R. Sogabe for their collaboration and assistance.

I also gratefully acknowledge contributions to staffs of the United Graduate School of Agricultural Sciences of Kagoshima University, staffs of student affairs section and library of University of Miyazaki for their kind assistance.

I would like to thankful to Ministry of Education, Culture, Sports, Science and Technology of Japan (MONBUSHO) for the supporting a scholarship throughout the period of study in Japan.

I extend genuine thanks and the very best wishes to my friends Adi Izhar, Syafura, Anirban chakraborthy, Top Neth, Madura, Ma Menglin and other friends for their care and support in all situations of my life.

I feel a particular pride in acknowledging the unconditional love and undiluted supported by my family members, my father H.A.R. Tassakka, mother H. Raehana, my father-in-law Kol. A. Bagenda Ali and my brothers and sisters. My profound gratitude goes to my dear husband A. Fachry, B. F. Sc for his endless understanding, sacrifices and persistent support that permit me to complete this study successfully and to my little daughter, Bunga Aqilah Juniyazaki and my baby Alya Nadhrah Marenazaki for being the source of strength in my life and also for enduring my long hours of absence from home due to research work.