GENERAL INTRODUCTION

Aside from its function of encoding the genetic material, DNA can have direct immune stimulatory effects. The specific immunostimulatory effect of bacterial genomic DNA was first reported by Tokunaga *et al.* (1984) who demonstrated that bacterial DNA activates natural killer (NK) cells and induces interferon (IFN) production as well as tumor regression in some mouse models, but vertebrate DNA does not (Yamamoto *et al.*, 1988; Yamamoto *et al.*, 1992). Other investigators independently found that bacterial DNA but not vertebrate DNA activate B cell proliferation and immunoglobulin secretion (Messina *et al.*, 1991). *In vitro* studies demonstrated that IFN production induced by mycobacterial DNA requires both adherent and non-adherent cell populations and results from palindromic motifs that occur in the mycobacterial DNA (Yamamoto *et al.*, 1992). Using sequence-specific synthetic oligonucleotides (sODNs) to trigger B-cell proliferation, Krieg *et al.* (1995) defined unmethylated CpG motifs displaying 5'Pu-Pu- CpG-Pyr Pyr 3' nucleotide sequences as biologically active. CpG content and methylation distinguish vertebrate DNA and bacterial DNAs. In vertebrate genomes, CpG dinucleotides are "suppressed"; they are present only about one quarter as often as would be predicted if base utilization was random (Bird, 1987). In contrast, CpG dinucleotides are not generally suppressed in bacterial genomes. A second difference in the CpGs between vertebrate and microbial genomes is that in vertebrates, CpGs are highly methylated, while in bacteria, viruses, and retroviruses, the CpGs are not methylated. This suggested the possibility that the immune system may have evolved a defense mechanism based on the recognition of unmethylated CpG dinucleotides, which could be a sign of foreign DNA.

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 interleukin-12 (IL-12), tumor necrosis factor α (TNF α) and IFN α / β (Chase *et al.*, 1997; Stacey *et al.*, 1996). 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 (Chan *et al.*, 1991; Trinchieri, 1995).

In fish, so far, the immunostimulatory effects of CpG-DNA are poorly described. Kanellos *et al.* (1999) defined an adjuvant effect of plasmid DNA containing CpG-ODNs in goldfish (*Carrasius auratus* L), where plasmids containing CpG motifs co-injected with a protein subunit vaccine potentiated antibody responses to the protein. Both plasmid DNA and synthetic ODNs containing CpG-motifs induced production of interferon-like cytokines in Atlantic Salmon leucocytes (Jørgensen *et al.*, 2001). A recent work demonstrated that rainbow trout macrophages not only produce IFN-like cytokines, but also express IL-1 β when stimulated with CpG ODNs (Jørgensen *et al.*, 2001).

There are no reports concerning the immunostimulatory effects of CpG motifs on the non-specific immune response. Since immunostimulant enhanced the non-specific defense mechanisms on fish, these studies were undertaken to examine the immunostimulatory effects of synthetic oligodeoxynucleotides containing CpG motifs in the non-specific immune response of common carp (*Cyprinus carpio* L.).

CHAPTER I

The *in vitro* effects of CpG oligodeoxynucleotides on the phagocytic activity of common carp (*Cyprinus carpio* L.)

I.1. Introduction

Phagocytes are cells principally dedicated to the recognition and elimination of invading organisms and damaged tissue. Those described in fish are the granulocytes (particularly neutrophils) and mononuclear phagocytes (tissue macrophages and circulating monocytes) (Secombes and Fletcher, 1992).

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*. Anderson (1992) reported that the *in vitro* assays have advantages over *in vivo* immunization studies in that the immune response in fish tissues can be temperature controlled and optimized for sampling time and fewer animals are needed for an experiment. Variations in the immune response are less with organ sections from the same individual, than among different fish needed for *in vivo* experiments.

Bacterial DNA and synthetic oligodeoxynucleotides containing unmethylated CpG-motifs in a particular sequence context activate human and vertebrate leucocytes *in vitro*. CpG DNA is a very strong activator of human leucocytes *in vitro,* including B cells (Liang *et al.*, 1996), monocytes (Hartmann *et al.*, 1999) and DCs (Bohle *et al.*, 1999; Hartmann *et al.*, 1999). In mice, CpG DNA promotes *in vitro* antibody responses to model thymus-independent antigens (Chelvarajan *et al.*, 1999). In fish, *in vitro* experiments of CpG oligodeoxynucleotides has been shown to stimulate

macrophages to produce IFN-like cytokines (ILC) and express IL-1 β (Jørgensen *et al.*, 2001).

Since there are no available data on the *in vitro* effect of CpG oligodeoxynucleotides on fish phagocytic cells, the aim of the present study was to examine whether this immunostimulant has such an effect on the phagocytic activity of common carp.

I.2. Materials and Methods

I.2.1. Fish

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

I.2.2. The preparation of CpG Oligodeoxynucleotides (ODNs)

Oligodeoxynucleotides (ODNs) were purchased from SAWADY (Japan) with the following sequences :

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

F = TCC ATG AGC TTC CTG ATG CT (The non-CpG ODN and altered CpG-motifs are underlined).

The oligodeoxynucleotides (ODNs) were suspended in saline (1 μ g/ml).

I.2.3. Isolation of head kidney cells

The head kidney phagocytic cells of carp were isolated according to the modified method described by Braun-Nesje *et al.* (1982). The cells were removed and pushed through a nylon mesh with RPMI 1640 medium (Nissui, Japan) containing 1% streptomycin/penicillin (Sigma) and 0.2% heparin (Sigma) and 10% carp serum (CS). The cell suspension was placed on 34/51% Percoll gradient and centrifuged at 400 X g

for 40 min at 4 °C. The macrophage-enriched cells from 34/51% Percoll interface were separated and centrifuged at 500 X g for 5 min and washed three times the medium. Viable phagocytic cells including neutrophils (about 10%) and macrophages (about 90%) were counted by trypan blue exclusion.

The direct effects of CpG-ODNs on fish phagocytic cells were analyzed by the superoxide anion assay (nitroblue tetrazolium: NBT) and phagocytosis, using six individual fish. This experiment were repeated two times and similar results were obtained.

For the superoxide anion assay, the viable cells were adjusted to 10^7 cells/ml Hank's balanced salt solution (HBSS; Nissui). One hundred microlitres of this suspension were added to the wells of 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.

For phagocytosis, 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 mm X 22 mm) for 1 h after which non-adherent cells were removed by washing with HBSS. Thus, the phagocytic cells were reacted with 10 ng ODNs in RPMI 1640 containing 10% CS overnight at 20 °C.

I.2.4. Detection of superoxide anion in phagocytic cells

The superoxide anion from phagocytic cells was determined by the reduction of the redox dye nitroblue tetrazolium (NBT) as described by Chung & 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 includes phorbol myristate acetate (1 mg/ml) (PMA, Sigma) was added to each well and incubated for 60 min at 20℃. The reduction was stopped by the addition of methanol, after removal of the medium from the cells. The formazan in each well was dissolved in 120 μl of 2 M KOH and 140 μl DMSO, and the optical density was measured by a multiscan spectrophotometer (Pharmacia, Sweden) at 620 nm.

I.2.5. Phagocytic activity

The phagocytic activity of carp kidney leucocytes was examined as described by Yoshida *et al.* (1993). The number of cells was adjusted to 10^7 cells/ml in RPMI 1640 medium containing 10 % carp serum (CS) using haemocytometer. The cells were allowed to adhere to a glass cover-slip (22 mm X 22 mm) for 1 h and non-adherent cells were removed by washing with the medium.

The latex particles (0.85 μ m)(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℃. Then, the cover-slip were picked up using forcep and were 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 x 10^5 cells per cover-slip and the number of phagocytic cells per 300 adhered cells was counted microscopically. The phagocytic activity (PA) was determined using formula:

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

I.2.6. Statistical analysis

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

I.3. Results

I.3.1. The production of superoxide anion

The effect of ODNs on production of superoxide anion in carp phagocytic cells is shown in Fig.1. The production of superoxide, examined by NBT reduction, was significantly higher in cells isolated from fish incubated with CpG-ODNs A and C at concentration of 10 ng/ml, and CpG-ODN B at concentration of 1000 ng/ml ($*P < 0.05$). However, the maximum difference between ODNs-incubated groups and control groups showed in the leucocytes of fish incubated with non-CpG ODN F at concentration of 10 ng/ml ($**P < 0.01$).

I.3.2. Phagocytosis

The phagocytic activity of the kidney leucocytes of carp incubated with CpG-ODN C was significantly higher than that of the leucocytes from control fish (Fig.2). However, the highest activity was observed in the leucocytes of fish treated with non-CpG ODN F ($*P < 0.05$).

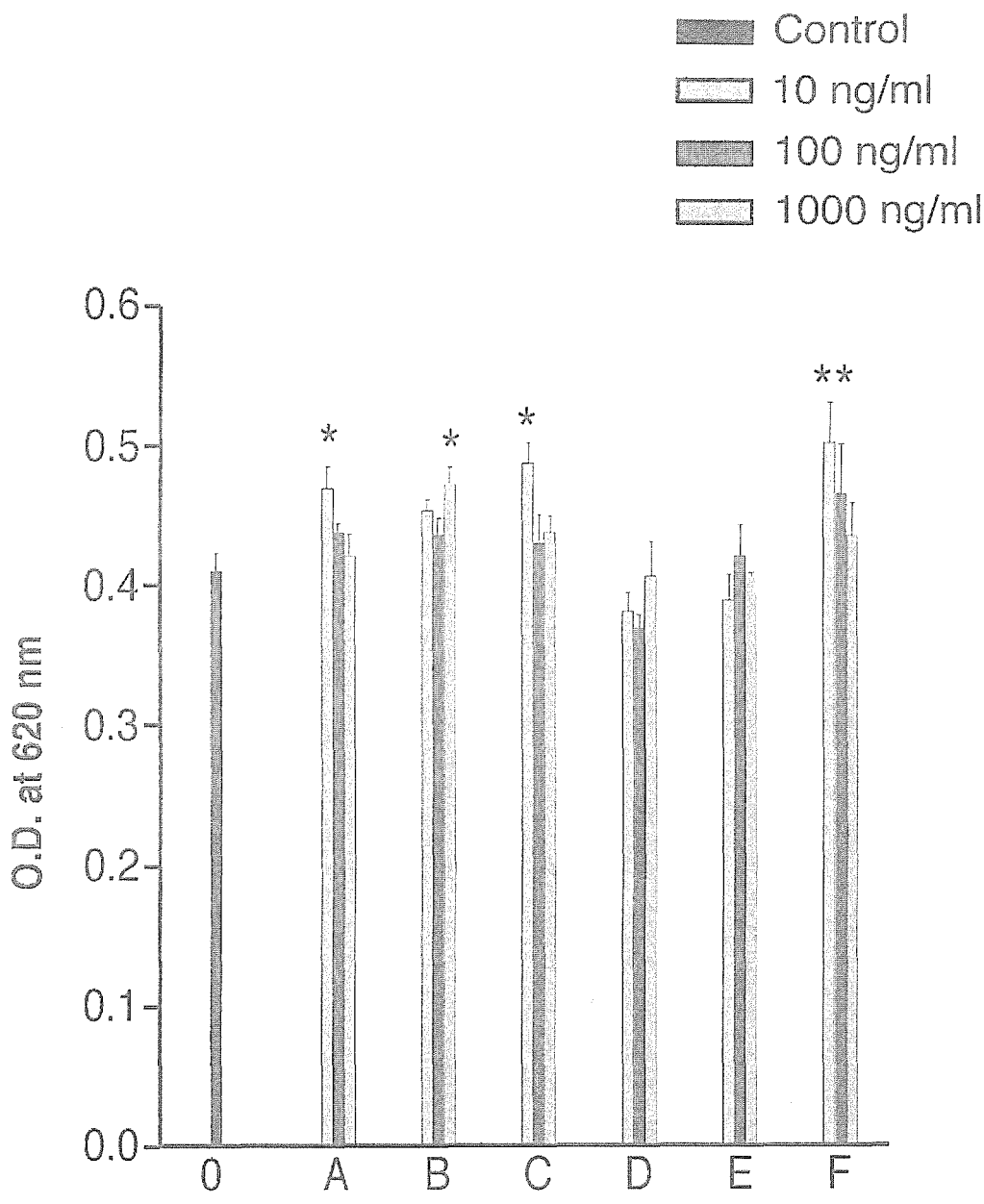

Fig. 1. The production of superoxide anion in carp phagocytic cells treated with different concentrations of CpG-pligodeoxynucleotides as measured by NBT. Values are mean ± SD at 620 nm above spontaneous NBT reduction after 60 min incubation measured in six fish. $^{*}P<0.05$, $^{**}P<0.01$.

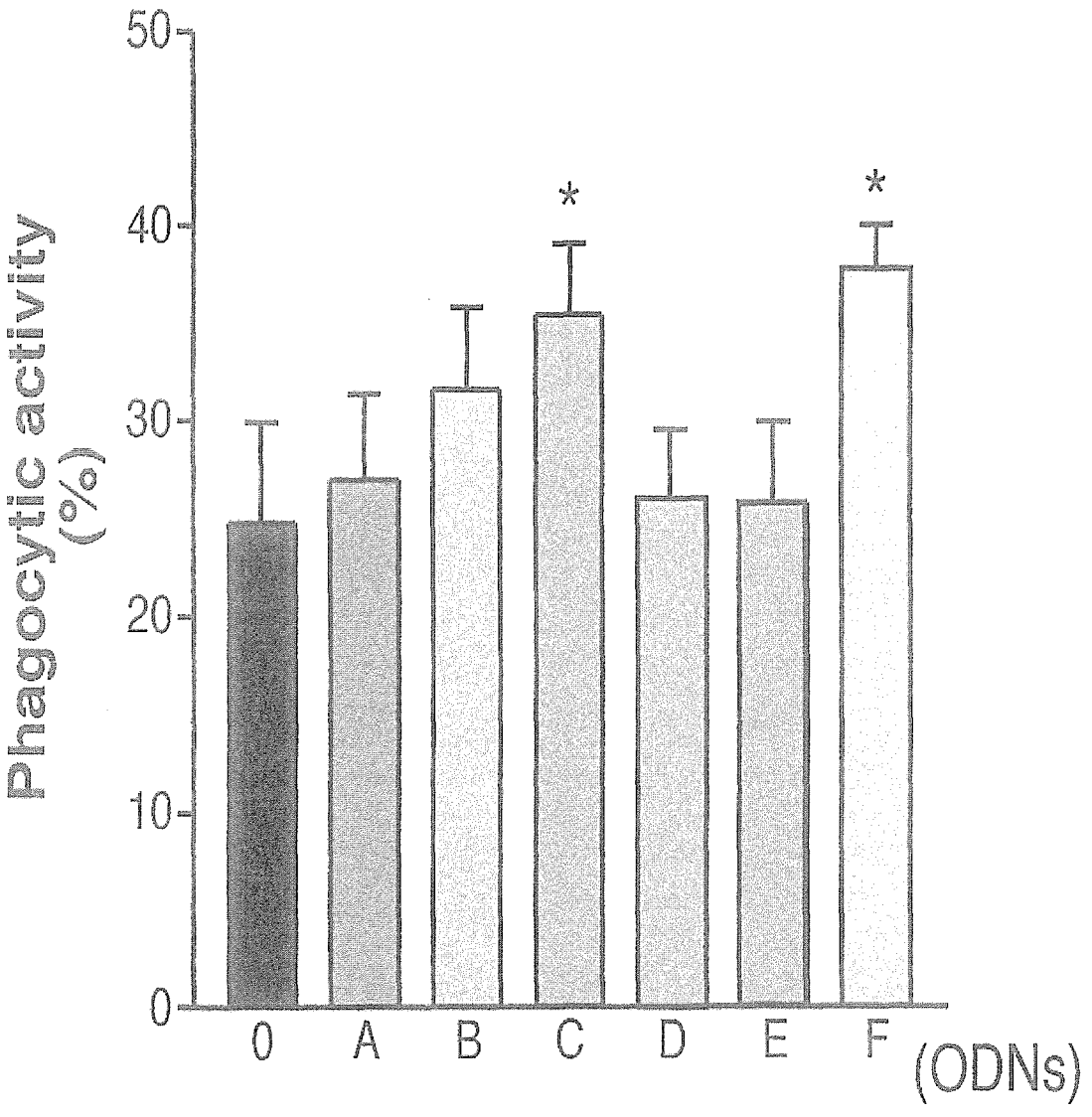

Fig.2. The phagocytic activity of common carp phagocytic cells treated with 10 ng/ml of CpG oligodeoxynucleotides. Values are means SD of the percentage of cells phagocytized latex particles. *$P<0.05$

I.4. Discussions

Phagocytes are clearly important accessory and effector cells in the immune system of fish. They can respond to a wide variety of host and pathogen derived molecules, altering their ability to act as accessory cells, to migrate, phagocytose, and to kill (Secombes and Fletcher, 1992). 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 and autonomic neurotransmitters (alpha and cholinergic receptor agonists)(Chung and Secombes, 1987; Smith *et al.*, 1982; Secombes, 1986; Bayne and Levy, 1991; Flory and Bayne, 1991). In addition, the *in vitro* studies of hormones such as lactoferrin, growth hormone and prolactin has been shown to enhance the production of superoxide anion by leucocytes in fish (Sakai, 1999).

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 *in vitro*, the sequence motif(s) responsible for inducing production of superoxide and phagocytosis in carp macrophages. Krieg (2000) reported that CpG DNA directly activates mouse macrophages (MPs) and DCs to make cytokines that create a Th1-like milieu in lymphoid tissues (Fig.3). NK cells are co-stimulated by CPG DNA and the APC-derived cytokines, leading to an increase in their innate immune activities, as well as to an IFN-γ-dependent feedback loop enhancing APC activation. These Th1-like

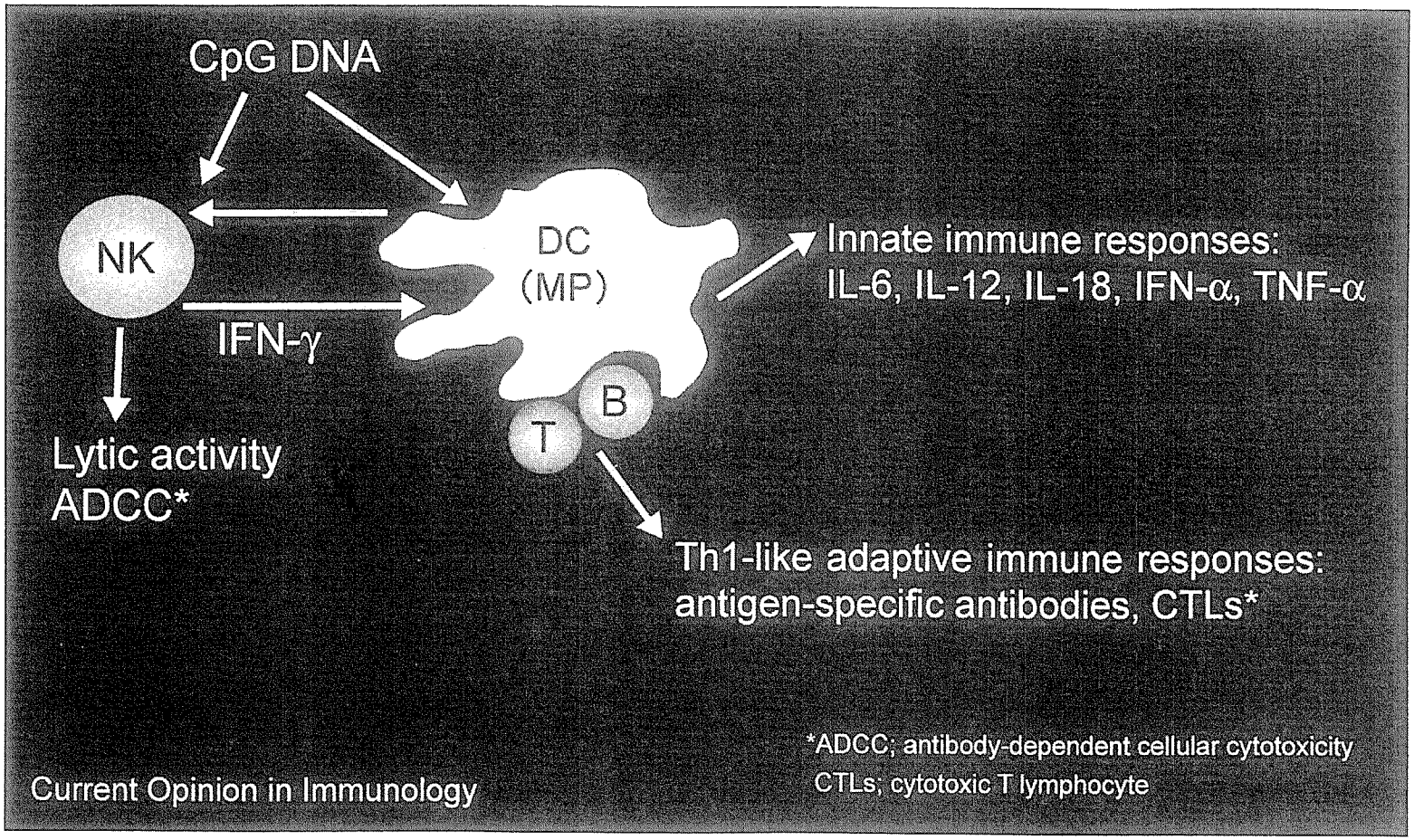

Fig. 3. CpG DNA directly activates DCs and macrophages (MPs) to make cytokines that create a Th1-like milieu in lymphoid tissues.

effects produce a broad spectrum immune resistance to challenge with intracellular infectious agents or tumor cells, and create an environment that opposes allergen sensitization. The DCs also have increased antigen processing and express increased levels of costimulatory molecules, leading to the enhanced generation of Th1-like antigen-specific acquired immune responses.

The kidney cells incubated with ODNs A, B and C enhanced the production of superoxide anions in carp macrophages. This stimulation was seen at concentration of 10 ng/ml for ODNs A and C, and at concentration of 1000 ng/ml for ODN B. However, the maximum response was shown in kidney cells incubated with non-CpG ODN F at concentration of 10 ng/ml

The activation of those ODNs A, B and C is due to the presence of fish-immunostimulatory motifs 5'-AACGTT-3' (B and C) and 5'-GACGTT-3' (A and B). Synthetic oligodeoxynucleotides containing the motif 5'-AACGTT-3' and 5'-GACGTT-3' induced production of antiviral cytokine activity in Atlantic salmon leucocytes. In contrast, the non-CpG ODN or ODNs with an inverted motif (GpC) did not have a stimulatory effect on Atlantic salmon leucocytes (Jørgensen *et al.*, 2001).

The same phenomenon was observed in phagocytic activity of carp leucocytes. The maximum difference between ODNs incubated groups and control groups showed in the leucocytes of fish incubated with the non-CpG ODN F. It is not known what caused this activation. The present findings indicate that more studies are needed to investigate this phenomenon in all vertebrates including fish. In addition, CpG-ODN C added *in vitro* to carp macrophages at concentration 10 ng/ml increased the phagocytic activity of these cells. Sakai (1999) reported that fish treated with immunostimulants usually show enhanced phagocytic cell activities. 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 NBT test. The immunostimulants such as levamisole (Siwicki *et al.*, 1990) and LPS (Solem *et al.*, 1995) increase the superoxide production and phagocytic activity in fish leucocytes by *in vitro* treatment.

From a panel of synthetic oligodeoxynucleotides, ODN C had a better stimulatory capacity than other CpG-ODNs (A, B, D, and E). 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).

The *in vitro* effects of CpG DNA also been reported in mammals. CpG DNA stimulate proliferation of murine lymphocyte *in vitro* (Messina *et al.*, 1991). *In vitro* treatment of spleen cells with CpG DNA can activate autoreactive Th1 effector cells specific for myelin basic protein, triggering the development of experimental autoimmune encephalomyelitis (EAE) (Segal *et al.*, 1997). DNA fragments from sera of patients with systemic lupus erythematosus proliferate mononuclear cells *in vitro* (Sato *et al.*, 1999).

Many questions remain concerning the molecular mechanism with which CpG DNA induces its potent immune stimulatory effects, most notably the identity of the CpG receptor. Nevertheless, the consequences of this immune activation have recently become much clearer. Although some investigators initially expected that there would be a cell surface receptor for CpG DNA, it appears instead that the recognition of CpG

motifs is accomplished through one or more intracellular CpG binding protein (Krieg *et al.*, 1995 and Krieg, 2001). Although many cell types are able to bind DNA on their surface, this binding appears to be non-sequence specific (Zhao, *et al.*, 1994). DNA is taken up by cells, also in a non-sequence specific fashion, into an endosomal compartment where the DNA is acidified and digested by nucleases (Tonkinson *et al.*, 1994; Bennett *et al.*, 1985; Krieg *et al.*, 1993). It appears that this endosomal acidification of CpG DNA may be required for its immune stimulatory activities since inhibition of endosomal maturation with specific inhibitors such as choloroquine completely blocks the downstream signaling pathways induced by CpG (Yi A-K *et al.*, 1998; MacFarlane *et al.*, 1998).

CHAPTER II.

The *in vivo* effects of CpG-ODNs on the non-specific immune responses of common carp (*Cyprinus carpio* L.)

II.1. Introduction

There is increasing evidence that immunostimulants have an ability to increase resistance to disease by enhancing non-specific and specific defense mechanisms. In fish, the immunostimulatory effects of substances such as EF203 (Sakai *et al.*, 1995), growth hormone (Sakai *et al.*, 1997), 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 and these substances play a promising role in aquaculture by enhancing the resistance of cultured fish against diseases.

Recently, it was shown that bacterial DNA and synthetic oligodeoxynucleotides containing CpG motifs are powerful activators of innate immune defences in mammals (Lipford *et al.*, 1998). The *in vivo* studies of CpG DNA motifs are shown to exhibit immunostimulatory effects on leucocytes from mammals. CpG motifs in oligodeoxynucleotides and bacterial DNA induces natural killer activity in murine and human cells (Ballas *et al.*, 1996). *In vivo* experiments demonstrate that CpG-containing oligodeoxynucleotides augment antigen-specific serum antibody levels by up to tenfold, and IFN γ production by up to sixfold. These effects were optimized by physically linking the CpG-containing motifs to the immunogen (Klinman *et al.*, 1999).

The published data about *in vivo* effects of CpG DNA on fish leucocytes are scarce. An adjuvant effect of CpG DNA was recently shown to work in goldfish

(*Carrasius auratus* L.), where plasmids containing CpG-motifs co-injected with a protein subunit vaccine potentiated antibody responses to the protein (Kanellos *et al.*, 1999). It is necessary to investigate the immunostimulatory effect of CpG oligodeoxynucleotides on fish leucocytes. In the present study, common carp were injected with CpG oligodeoxynucleotides in order to investigate the effect of CpG-containing oligodeoxynucleotides on the non-specific immune responses of common carp.

II.2. Materials and Methods

II.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 outdoor tanks with running fresh water at 16 °C for two weeks and fed commercial diets twice daily.

II.2.2. CpG oligodeoxynucleotides (ODNs)

Synthetic oligodeoxynucleotides containing the fish-immunostimulatory motifs 5'- GACGTT-3' and 5'-AACGTT-3'(Kanellos *et al.*, 1999 and Jørgensen *et al.*, 2001) were purchased from SAWADY (Japan), with the following sequences:

B = GCT AGA CGT TAA CGT T

C = ATC GAC TCT CGA ACG TTC TC

The oligodeoxynucleotides were suspended in saline (1 μ g/100 μ l) and injected into carp at a dose of 0.1, 1 and 10 μ g/fish intraperitoneally. Control fish received an equal dose of phosphate buffer saline (PBS) (1 μ g/100 μ l) by intraperitoneal injection. Six fish of each group were sampled at 1, 5 and 7 days after injection.

II.2.3. Isolation of head kidney cells

Carp head kidney was removed and pushed through a nylon mesh with RPMI 1640 medium (Nissui, Japan) containing 1 % streptomycin/penicillin (S/P, Gibco, USA) and 0.2 % heparin (Sigma, USA). The cell suspension was then centrifuged at 500 x g for 5 min and washed three times with the medium. Viable phagocytic cells, including neutrophils and macrophages, were counted by Trypan Blue Exclusion.

II.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.2.5. Phagocytosis 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 mm X 22 mm) for 1 h and non-adherent cells were removed by washing with the medium.

The latex particles (0.85 μ m)(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℃. Then, the cover-slip were picked up using forcep and were 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 x 10^5 cells per cover-slip and the number of phagocytic cells per 300 adhered cells was counted microscopically. The phagocytic activity (PA) was determined using formula:

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

II.2.6. Detection of superoxide anion in phagocytic cells

The superoxide anion from phagocytic cells was determined by the reduction of the redox dye nitroblue tetrazolium (NBT) as described by Chung & Secombes (1988). The kidney cells suspended in RPMI 1640 containing 10 % CS and HEPES were collected as described above. One hundred microliters of this suspension was

added to each well of a 96 well microtiter plates (Nunc, Denmark). After 2 h at 20℃ of incubation the cells were washed by RPMI 1640 medium to remove non-adherent cells. The total adhered cell number per well was about 10^5 cells. One hundred μ l of NBT solution (1 mg/ml in RPMI 1640 medium) and phorbol myristate acetate (1 mg/ml) (PMA, Sigma) were added to each well and incubated for 60 min at 20℃. 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 μ l of 2 M KOH and 140 μ l DMSO, and the optical density was measured by a multiscan spectrophotometer (Pharmacia, Sweden) at 620 nm.

II.2.7. Lysozyme activity

The lysozyme activity was assayed by the turbidimetric method described by Parry *et al.*, (1965). The serum were diluted in the phosphate buffer saline (PBS) with different concentrations (1/250, 1/350, and 1/500). A suspension of *Micrococcus lysodeikticus* (0.2 mg/ml phosphate buffer pH 6.2) was measured by a UV spectrophotometer (530 nm). One hundred microliters of the diluted serum were mixed with 200 μ l of bacterial solution and were added to each well of a 96 well microtiter plates. The reaction was carried out at 37 °C and the absorbance was measured by a multiscan spectrophotometer (550 nm) after 5, 10, 30, and 60 minutes. A unit of lysozyme activity was defined as the amount of sample causing a decrease in absorbance of 0.001/min.

II.2.8. Statistical analysis

The data was expressed as mean $\pm$SD. The data was analysed using Student's

t-test.

II.3. Results

II.3.1. The production of superoxide anion

The production of superoxide, examined by 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.4). The maximum difference between ODNs treated groups and control groups showed in the leucocytes of fish treated with 10 μ g/fish of ODN C.

The production of superoxide anion by leucocytes from fish treated with 10 μ g/fish ODN C significantly increased 5 to 7 days after treatment (Fig.5).

II.3.2. Phagocytosis

The phagocytic activity of the kidney leucocytes from carp treated with CpG ODNs B & C was significantly higher than that of the leucocytes from control fish at 1 day after injection (Fig.6). The maximum stimulation of phagocytic cells was demostrated in the leucocytes of fish treated with 1 μ g/fish of CpG ODN C.

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

II.3.3. Lysozyme activity

The serum lysozyme activity of fish treated with ODNs B & C significantly increased at 5 days after treatment (Fig.8). The highest activity was observed in the serum of fish treated with 10 μ g/fish of ODN C.

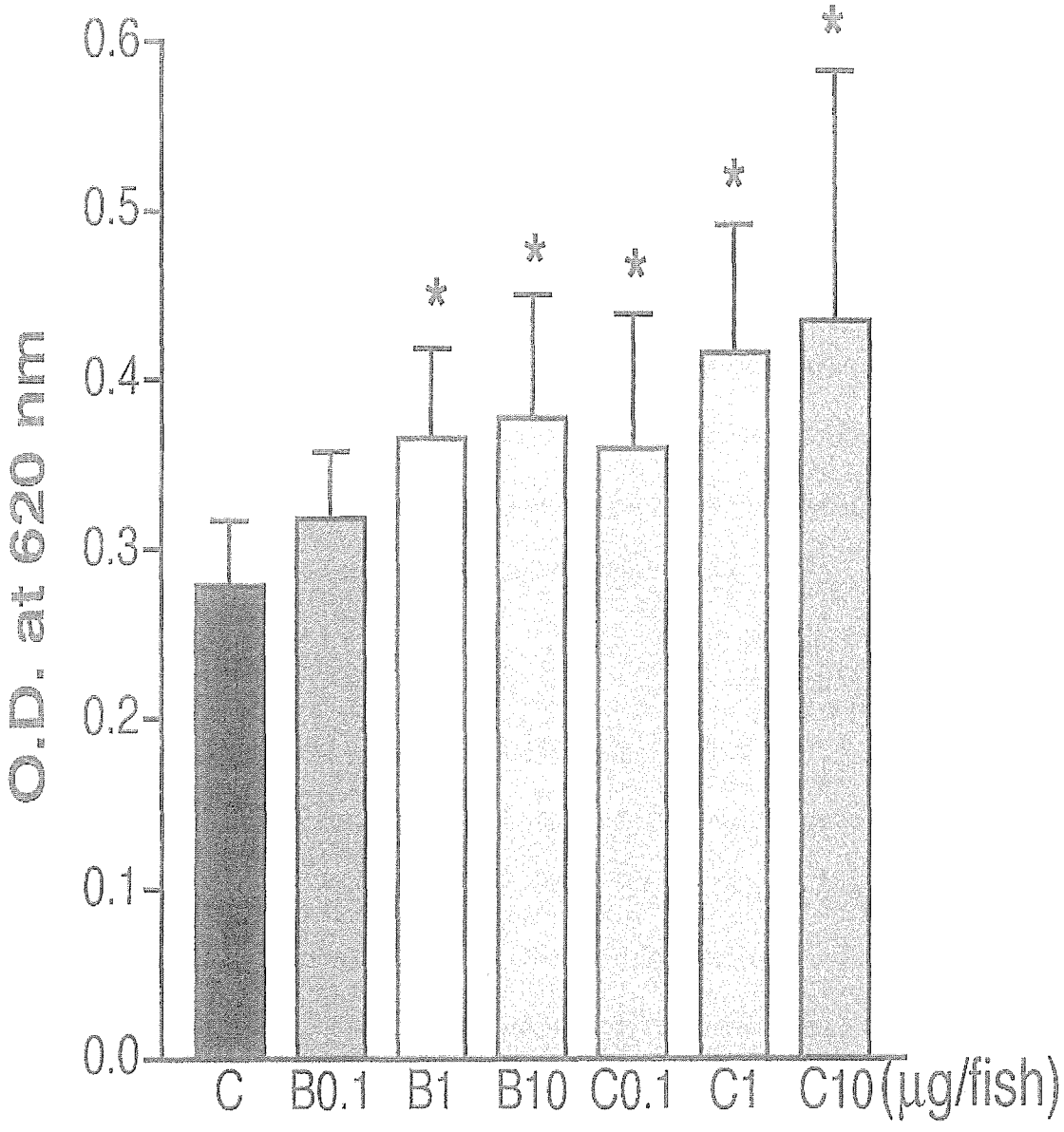

Fig. 4.The dose responses of the production of superoxide anion in leucocytes o carp injected with CpG-ODNs as measured by nitroblue tetrazolium (NBT). The NBT reduction was examined 5 day after injection. Values are mean ± SD at 620 nm above spontaneous NBT reduction after 60 min incubation measured in 6 fish. Asterisks indicate statistically significant differences between control and different concentrations of CpG oligodeoxynucleotides. * $P < 0.05$.

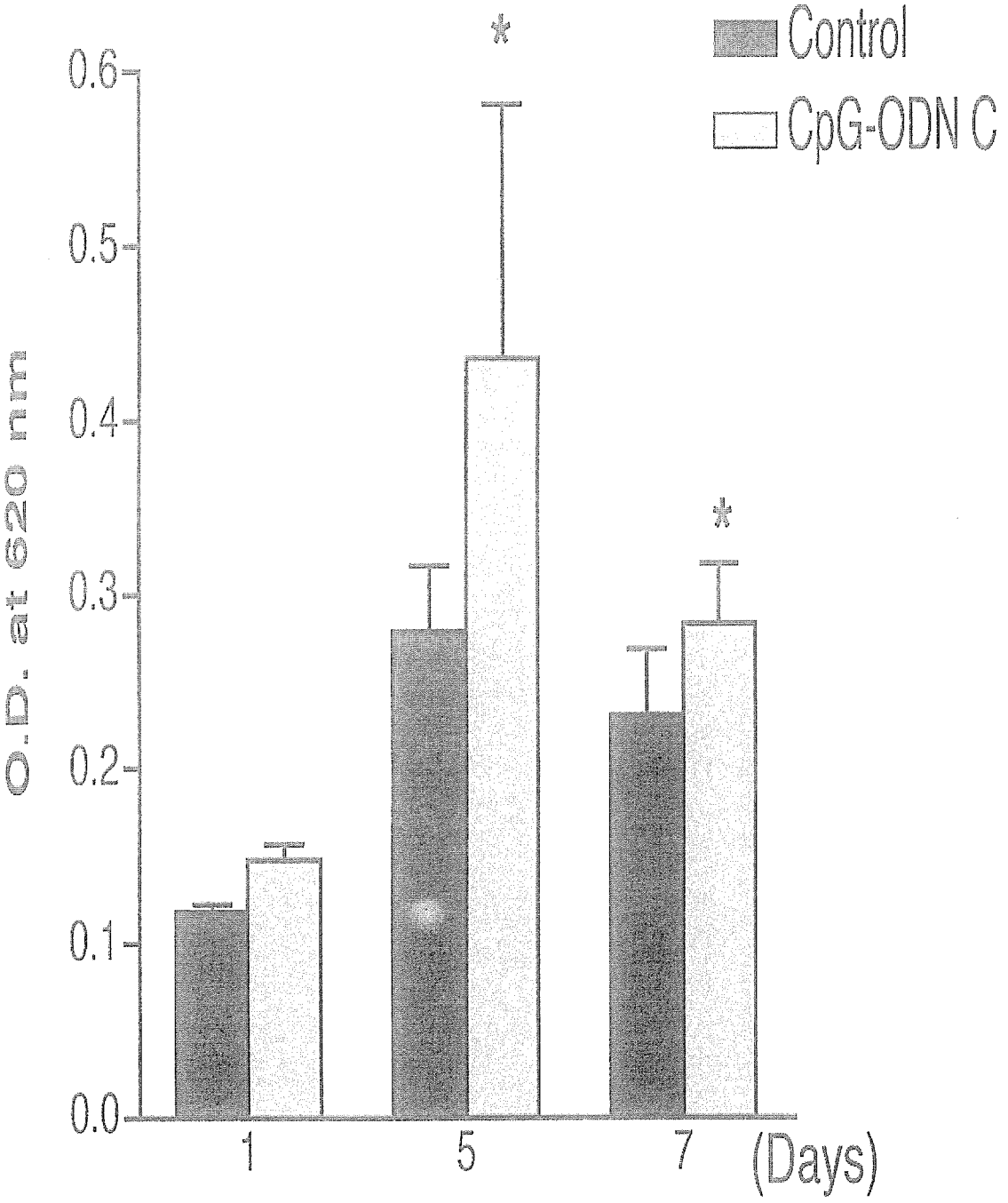

Fig. 5.The production of superoxide anion in leucocytes of carp injected with 10 μg of CpG ODN C as measured by nitroblue tetrazolium (NBT). The NBT reduction was examined 1, 5 and 7 days after injection. Values are mean ± SD at 620 nm above spontaneous NBT reduction after 60 min incubation measured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

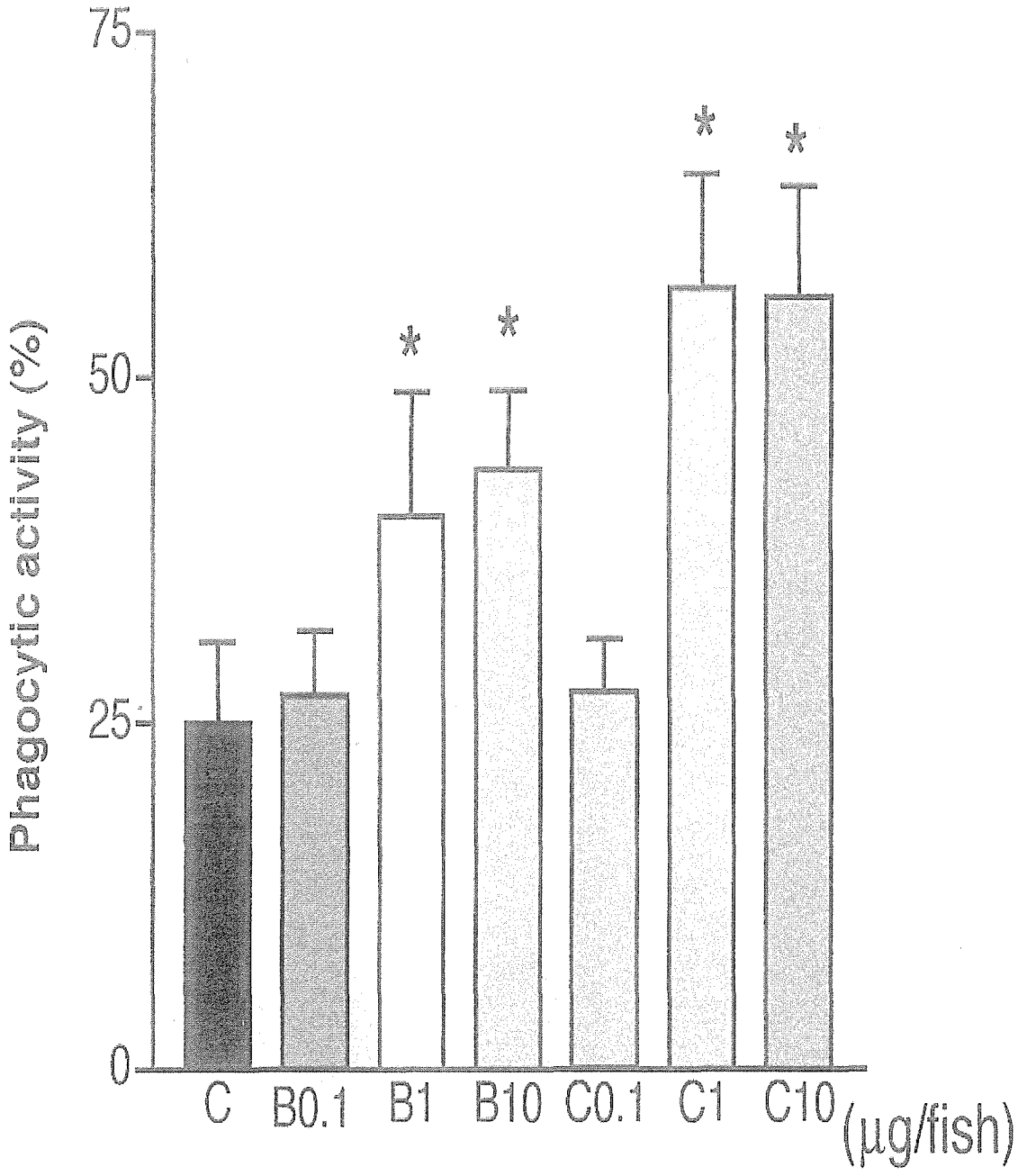

Fig. 6. The dose responses in the phagocytic activities of leucocytes of carp injected with CpG oligodeoxynucleotides. The phagocytic activity was examined 1 day post injection. Values are mean ± SD in 6 fish. Asterisks indicate statistically significant differences between control and different concentrations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

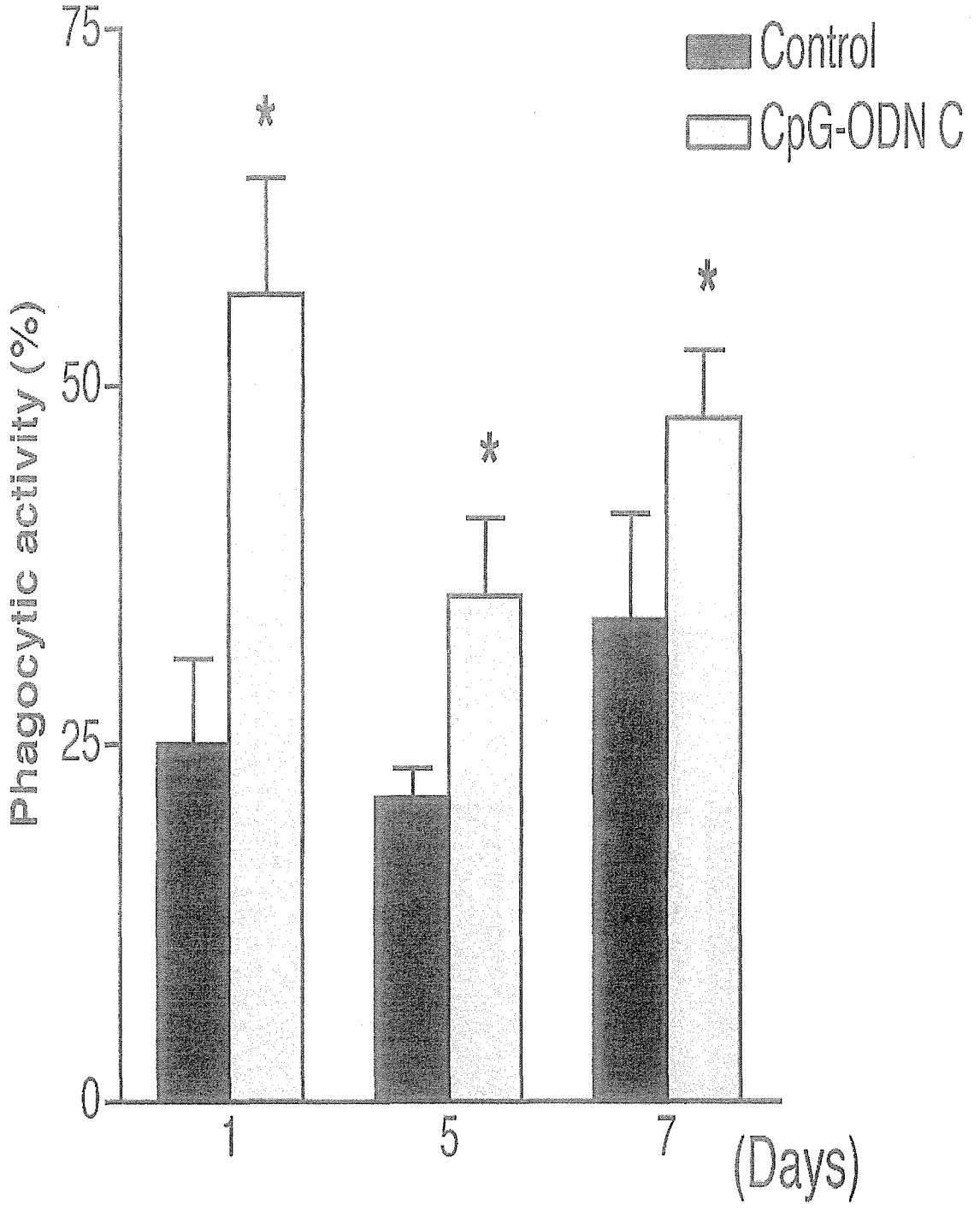

Fig. 7. The phagocytic activity in leucocytes of carp injected with 1 μg of CpG ODN C. The phagocytic activity was examined 1, 5 and 7 days post injection. Values are mean ± SD in 6 fish. * $P < 0.05$.

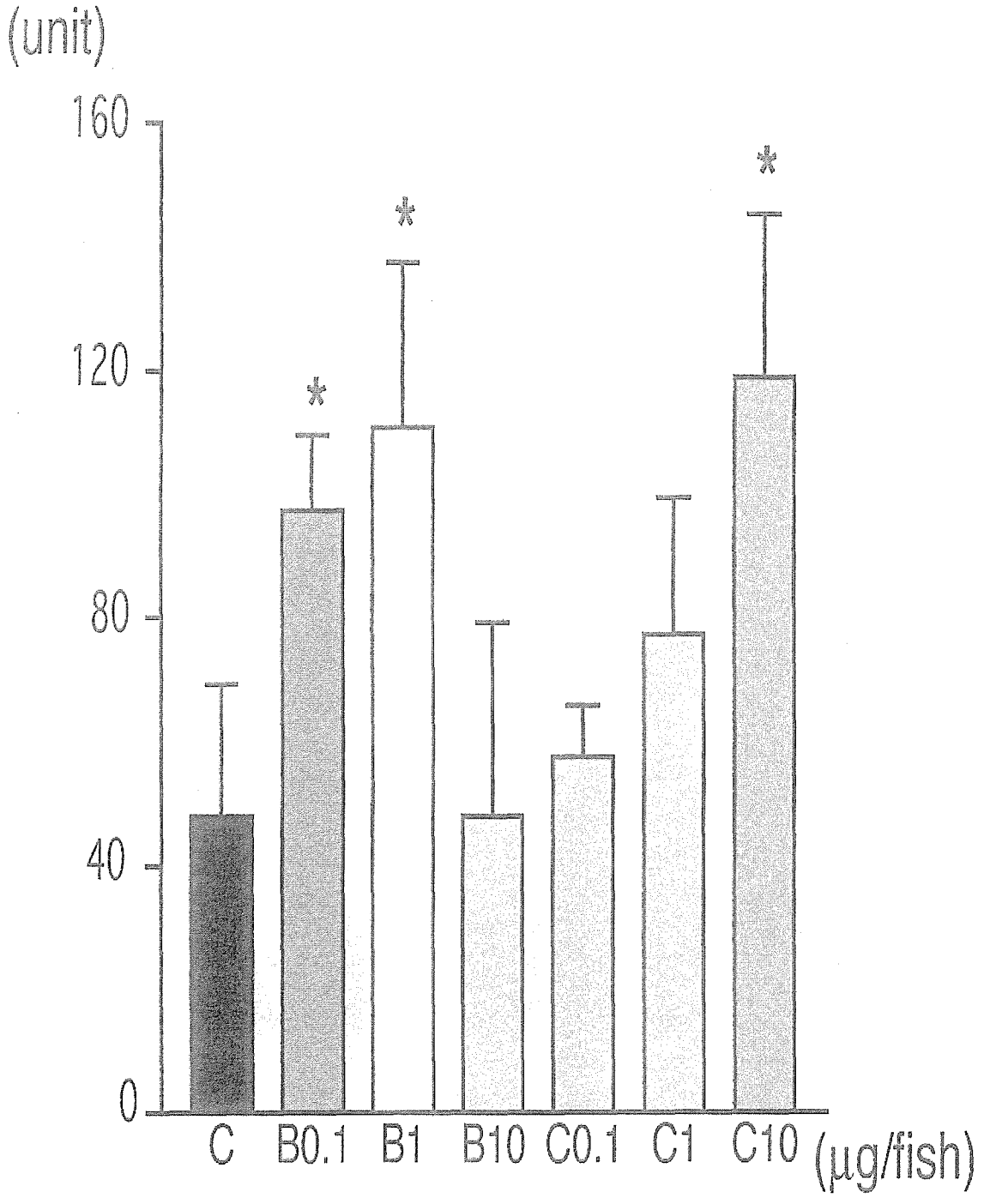

Fig. 8. The dose responses in the lysozyme activity in serum of carp treated with CpG-ODNs. The lysozyme activity was examined 5 days after treatment. Values are mean ± SD in 6 fish. Asterisks indicate statistically significant differences between control and different concentrations of CpG oligodeoxynucleotides. * $P < 0.05$.

Figure 9 shows the duration of increased serum lysozyme activities in fish treated with ODN C at 10 μ g/fish. The maximum stimulation was evident 5 days after injection.

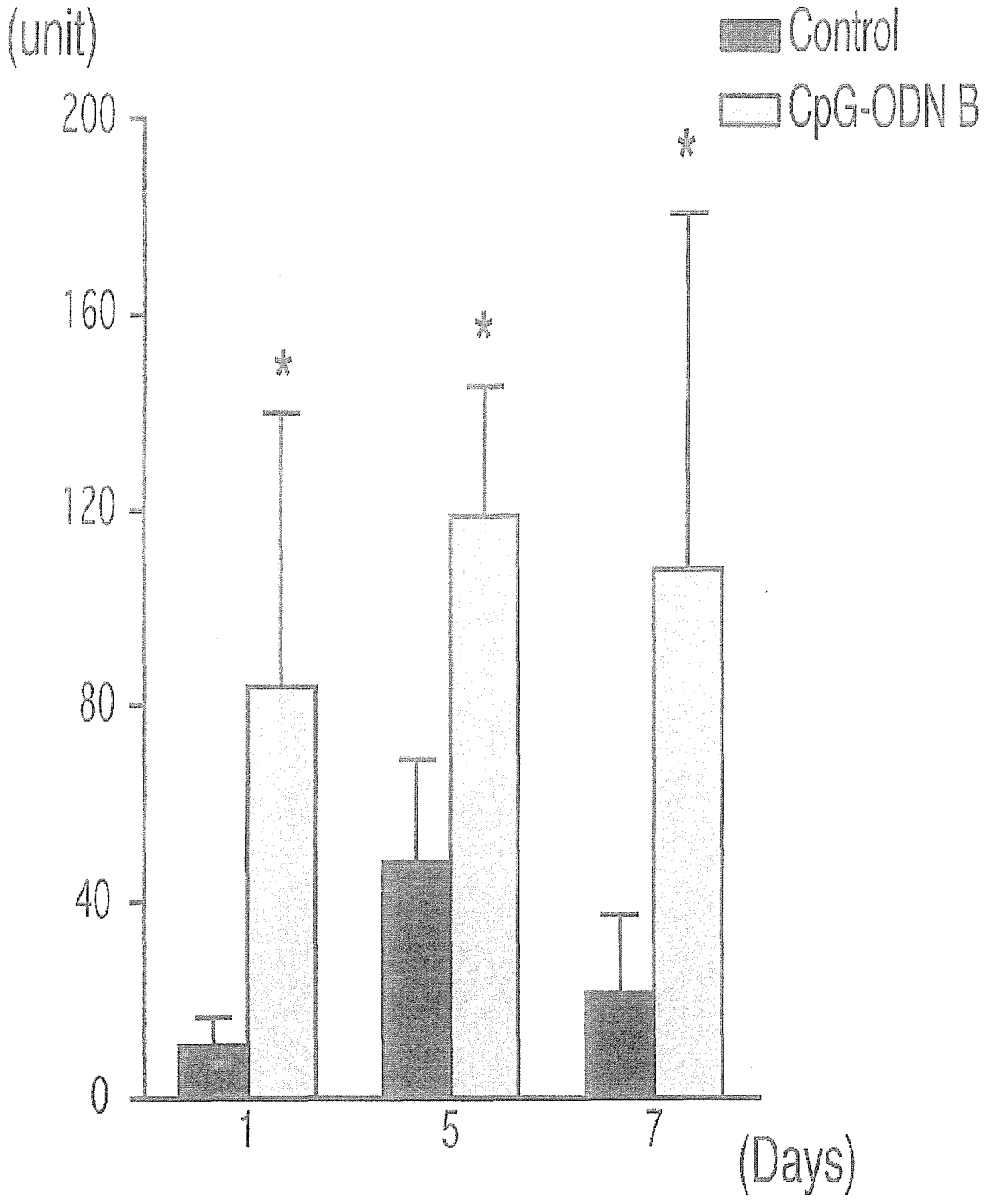

Fig.9. The lysozyme activity in serum of carp injected with 10 μg of ODN B. The lysozyme activity was examined 1, 5 and 7 days after injection. Values are mean ± SD in 6 fish. * $P < 0.05$.

II.4. Discussions

It is well known that injection of fish intraperitoneally (i.p.) with substances such as protease-peptone, casein, glycogen, liquid paraffin, or mineral oil elicits phagocytes into the peritoneal cavity (Chung and Secombes, 1987; Nash *et al.*, 1987; Olivier *et al.*, 1986; Sakai, 1984; Hamaguchi *et al.*, 1989). Experiments suggest that phagocytes, activated or inhibited *in vivo* prior to challenge, are protective against pathogens. Thus, injection of fish i.p. with bacteria in Freund's adjuvant, which elicits and activates cells in the peritoneum, confers non specific protection against bacterial *(A. salmonicida, A. hydrophila, V. ordali and V. anguillarum*) and helminth (*D. spathaceum*) diseases injected via this route (Whyte *et al.*, 1990; Kodama *et al.*, 1989; Olivier *et al.*, 1985). Thus, it is possible to use immunostimulants to induce protective nonspecific responses in fish by up-regulating phagocyte activity.

The biochemical and genetic properties of DNA have been thoroughly investigated, yet only recently has it been appreciated that DNA carries more information than simply a blueprint for the regulation and construction of proteins. Indeed, the immune systems of vertebrates appear to have evolved the ability to distinguish the foreign DNA of bacteria and certain viruses from the self-DNA of the host, a new twist on the self vs non-self detection system already well-known for foreign proteins. Specifically, the unmethylated CpG motifs (CpG denotes covalently linked CG dinucleotides, not C:G base pairs) is extensively suppressed in vertebrates, including mammals (by at least 20-fold)(Bird, 1987), whereas it is found at the usual frequency (1/16) in most bacterial and viral DNA. There have now been several reports that bacterial DNA or synthetic oligodeoxyribonucleotides (ODNs) containing CpG motifs, stimulate the immune systems of mice and humans to first mount innate,

and then antigen specific (when foreign antigen is present), Th1-type responses (Lowrie and Whalen, 2000).

There is evidence from mammalian models that CpG DNA act as an immunostimulant. In contrast, very little published data about CpG DNA effects on fish are available. In a recent published work it was shown that CpG DNA act as an adjuvant in goldfish (Kanellos *et al.*, 1999). A eukaryotic plasmid DNA carrying the AACGTT motif caused an increase in the specific antibody titres of fish and mice after immunization with β-galactosidase (β-gal), while a synthetic oligonucleotide which contains the GACGTT motif, potentiated antibody responses to co-administered β-gal protein in mice, but not in fish. It was demonstrated that lower and higher vertebrates recognize different unmethylated CpG motifs as 'danger' signals. This study has shown that unmethylated CpG DNA enhanced the non-specific immune responses in fish.

This study shows that CpG DNA motifs (ODN B & C) lead to enhance the production of superoxide anion production in carp. 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 μ g/fish of ODN C at day 5. The immunostimulant such as chitosan (Anderson *et al.*, 1995) and glucan (Jeney and Anderson, 1993) increased the superoxide production in fish leucocytes by intraperitoneal injection.

The same phenomenon was observed in the phagocytic cells in carp. Phagocytosis against latex particles in carp leucocytes also enhanced *in vivo* by CpG-ODNs. 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 μ g/fish of ODN C at 1 day after injection. The phagocytic activity also enhanced in carp leucocytes injected with levamisole (Siwicki, 1987, 1989), chitin (Sakai *et al.*, 1992) and LPS (Salati *et al.*, 1987).

CpG ODNs (B & C) activated serum lysozyme activity of carp. Ellis (1990) reported that lysozyme has antibacterial activities (particularly on Gram positive bacteria) by causing lysis and may also act as an opsonin. Lysozyme is present in the serum and mucus of fish but is particularly associated with leucocytes and leucocyte-rich tissues *e.g.* kidney, spleen and gut. Monocytes, macrophages and neutrophils are thought to be the main source of lysozyme (Murray and Fletcher, 1976). 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 (Lie *et al.*, 1989). Members of the cod family have the lowest levels (Lie *et al.*, 1989;Grinde *et al.*, 1988). Sakai (1999) reported that fish treated with immunostimulants increased the lysozyme activity. It was shown that intraperitoneal injection of yeast glucan (Engstad *et al.*, 1992; Jørgensen *et al.*, 1993 and Thompson *et al.*, 1995), and scleroglucan (Matsuyama *et al.*, 1992) enhanced the lysozyme activity in fish.

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 non-specific immune response in carp. Kanellos *et al.* (1999) reported that the motif 5'-AACGTT-3' that was contained in a eukaryotic plasmid DNA caused an increase in the specific antibody titres of goldfish after immunization with β-galactosidase (β-gal). This motif also induced production of antiviral cytokine activity in Atlantic salmon leucocytes (Jørgensen *et al.*, 2001). In addition, the ODNs containing the motif 5'-GACGTT-3'

stimulated that rainbow trout macrophages to produce IFN-like cytokines, and express IL-1 β (Jørgensen *et al.*, 2001).

The ODN C had a better stimulatory capacity than ODN B. The presence of 2 distal CGs in 5' end of ODN C (ATC GAC TCT CGA ACG TTC TC) might have contributed to the enhanced stimulatory capacity of the ODN C. Jorgensen *et al.* (2001) 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 A 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.

Not all CpG motifs are immunostimulatory. Not only is the particular sequence context of the unmethylated CpG dinucleotide important, but there are species-specific motifs (Krieg, 1999). Many motifs which work well on mouse cells do not stimulate human cells, whereas those which do stimulate human cells will also stimulate, at least to some degree, mouse cells. Mouse cells respond optimally to the motif GACGTT (single-letter code for amino acids is used) and human cells to the motif GTCGTT (when present in a nuclease-resistant phosphorothioate backbone). An interesting insight into the evolutionary history of immune recognition of CpG DNA is provided by the finding that fish not only respond to CpG DNA, but may recognize different CpG motifs to mice (Kanellos *et al.*, 1999). In addition, the best motif depends on the backbone used; the best CpG motif with a phosphorothioate backbone

ODN will not necessarily be the best with a phosphodiester backbone (Lowrie and Whalen, 2000). Although the phosphodiester oligodeoxynucleotides are rapidly degraded by nucleases, such ODNs used in this study have shown to have an immunostimulatory effect in fish. According to Krieg (2001), CpG ODN can be divided into several families with distinct immune effects, depending on their structural characteristics (Table 1).

The discovery that the immune system has apparently evolved a detection mechanism for CpG DNA implies that the activation of this mechanism should led to a useful outcome for the host. The utility of CpG DNA as a vaccine adjuvant has been demonstrated in studies using model antigens such as hen egg lysozyme (Chu *et al.*, 1997), ovalbumin (Lipford *et al.*, 1997), heterologous γ globulin (Sun *et al.*, 1998), and β - galactosidase (Roman *et al.*, 1997). Other studies have shown the ability of CpG DNA to act as an adjuvant for infectious disease vaccine such as hepatitis B vaccine in mice (Davis *et al.*, 1998) and orangutans (Davis *et al.*, 2000).

Many of the adjuvants used in fish vaccines, and in particular oil-based adjuvants, contribute to good protection, but at the same time they give serious side-effects (Midtlyng *et al.*, 1996). CpG DNA used as an adjuvant is reported to induce stronger immune responses with less toxicity than other adjuvants when tested in murine models (Weeratna *et al.*, 2000). According to Krieg (2001), as a vaccine adjuvant, CPG ODN have multiple advantages over other adjuvants. ODN are easy to synthesize in large quantities under GMP conditions and are quite economical. The ODN are extremely stable with a shelf life measured in years and can be formulated with essentially any vaccine in aqueous or oil emulsion including live viruses. CpG ODN can be administered through essentially any route including mucosal and oral

routes of vaccination. Finally, in preclinical studies CpG ODN appear to be extremely well tolerated and safe.

CONCLUSIONS

Unmethylated CpG oligodeoxynucleotides causes direct stimulation of several components of the vertebrate immune system. In fish, so far the immunostimulatory effect of CpG DNA are poorly described. Synthethic oligodeoxynucleotides containing "CpG motifs" enhance the production of superoxide anion and phagocytosis of common carp leucocytes *in vitro* and *in vivo*. The serum lysozyme activity also increase in the carp injected with CpG oligodeoxynucleotides. Since the non CpG ODNs had an expected immunostimulatory effect of carp phagocytic cells, more studies are needed to investigate this phenomenon in all vertebrates including fish. Multiple CpGs resulted in greater stimulatory capacity, although CpGs located at the terminus of an ODN were ineffective. The potent immunostimulatory activities of CpG DNA reported for fish in this and other studies points to CpG DNA as an interesting adjuvant to be tested in fish vaccines.

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