

Effects of CpG Oligodeoxynucleotides on Immune Responses and Expression of Cytokine Genes in Cultured Olive Flounder *Paralichthys olivaceus*

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The induction of cellular and humoral immunity and cytokine gene expression by synthetic CpG oligodeoxynucleotides (CpG-ODNs) has not been investigated systematically in olive flounder *Paralichthys olivaceus* in vivo. We optimized the proper concentration of CpG-ODNs using an in vitro assay for the superoxide anion (O_2^-). CpG-ODNs induced O_2^- and nitric oxide (NO) production, lysozyme activity, and the proinflammatory cytokine gene expression of IL-1 β and TNF- α in olive flounder significantly in vivo, whereas non-CpG-ODNs did not produce these effects or produced them to a lesser extent. This implied that CpG-ODNs could stimulate cellular and humoral immunity and cytokine gene expression in olive flounder. This is the first evidence of NO production and the first study on the mRNA expression of the proinflammatory cytokine genes IL-1 β and TNF- α in olive flounder in response to CpG-ODNs. Comparison of the variation in NO production and lysozyme activity to that of other studies led us to postulate that a group-specific difference exists in the immune responses of olive flounder against CpG-ODNs. Furthermore, the detailed immunostimulatory spectrum of CpG-ODNs in olive flounder could be a useful index with which to analyze the effect of CpG-ODNs against the challenge test prior to field applications.

Key words: *Paralichthys olivaceus*, Olive flounder, CpG-ODNs, IL-1 β , Lysozyme, Nitric oxide, Superoxide anion, TNF- α

Introduction

Bacterial DNA and synthetic oligodeoxynucleotides (ODNs) containing CpG motifs are powerful activators of innate defenses in mammals (Lipford et al., 1998; Asmi and Sakai, 2005). In contrast to vertebrate DNA, bacterial DNA has a relative abundance of unmethylated CpG motifs, which contribute to the immunostimulatory activity (Lipford et al., 1998; Häcker et al., 2002; Kreig, 2002).

In mammals, CpG-ODNs stimulate antigen-presenting cells (APCs) to secrete interleukin-1 (IL-1), IL-6, IL-12, tumor necrosis factor α (TNF- α), and interferon γ (IFN- γ) (Klinman et al., 1996; Lipford et al., 1997; Sparwasser et al., 1998; Sun et al., 1998; Yi et al., 1998).

Yamamoto et al. (1992) reported that bacterial DNA enhances the lytic activity of natural killer (NK)

cells and induces IFN- γ production; this activity was attributed to the palindromic sequences present in bacterial DNA.

In vitro studies in fish have revealed that oligodeoxynucleotides containing CpG motifs have immunostimulatory effects (Jørgensen et al., 2001a,b; Tassakka and Sakai, 2003). Kanellos et al. (1999) found an adjuvant effect of plasmid DNA containing CpG-ODNs in gold fish *Carrasius auratus* L. in vivo. The effects of CpG-ODNs on the immune responses of fish in vitro and in vivo have been reviewed by Tassakka and Sakai (2005). In olive flounder *Paralichthys olivaceus*, Lee et al. (2003a,b) investigated the in vitro and in vivo immunostimulatory effects of synthetic CpG-ODNs within a limited scope. Thus, the in vivo immunostimulatory effects of CpG-ODNs in olive flounder have thus far received little attention.

We systematically investigated the in vivo effects of CpG-ODNs containing CpG motifs that differed in

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structure and repeats on the production of bactericidal free radicals such as the superoxide anion (O_2^-) and nitric oxide (NO), and more specifically, on the induction of serum lysozyme activity as the basic element of immune response. We also examined their effects on the expression of the proinflammatory cyto-kine genes IL-1 β and TNF- α in *P. olivaceus*.

Materials and Methods

Fish

Olive flounder (approximately 200-300 g in body weight) were obtained from a local fish farm. Fish were stocked in 50 L tanks at four fish per tank and allowed to acclimate for at least 2 weeks. Seawater was supplied to the tanks at ambient temperature, which ranged from 18 to 22°C during the course of the study. The fish were handfed daily to satiation until 48 h prior to the start of experiments.

Oligodeoxynucleotides (ODNs)

Non-CpG-ODN 1720 and CpG-ODNs 1668, 1670, and 1826 were purchased from Takara Co. (Tokyo, Japan; Table 1). Various concentrations of CpG-ODNs diluted in 100 μ L of phosphate buffered saline (PBS; pH 7.5) were used for in vitro and in vivo experiments.

Isolation of head kidney leukocytes

The head kidney of *P. olivaceus* was excised and placed on a 60 mm Petri dish containing 1 mL of L-15 medium (Gibco, Grand Island, NY, USA) supplemented with penicillin G-streptomycin sulfate at 10,000 units/mL. The head kidneys were homogenized with sterile nylon mesh. The cell suspension was placed onto the top layer of a 51% Percoll gradient and centrifuged at 1,600 rpm for 30 min at 4°C. The leukocyte layer was collected with a syringe and washed twice with L-15 medium. The pellet was resuspended in L-15 medium and cell viability was examined using the trypan blue exclusion test. Cells were adjusted to 1×10^6 cells/mL PBS (pH 7.5).

Superoxide anion (O_2^-) production

The O_2^- production was examined via the reduction of the redox dye nitroblue tetrazolium (NBT) as described by Chung and Secombes (1988). For this test, 100 μ L of lipopolysaccharide (LPS; 1 μ g/mL) was added to 100 μ L of the cell suspension. After incubation for 1 h at 20°C, NBT (1 mg/mL in PBS, pH 7.5) was added to each well and incubated for 1 h at 20°C. The supernatant was removed carefully by a pipette, and the remaining cells were washed with 70% methanol. Cell lysis was achieved by adding 120 μ L of 2 M KOH and 160 μ L of dimethyl sulfoxide (DMSO) and mixing vigorously by pipetting. NBT reduction was then measured at 630 nm.

Nitric oxide (NO) production

Leukocytes were stimulated with 100 μ L of LPS (1 μ g/mL) and incubated for 48 h at 20°C. A 100 μ L aliquot of the supernatant was placed onto a 96-well plate, and an equal volume of Greiss reagent (Sigma, St. Louis, MO, USA) was added. After incubation for 10 min at room temperature, the absorbance was measured at 540 nm.

Lysozyme activity

The lysozyme activity of serum was determined by the turbidimetric method (Parry et al., 1965). A suspension of *Micrococcus lysodeikticus* (0.2 mg/mL) in PBS (pH 6.0) was mixed with 100 μ L of serum to give a final volume of 2 mL. The reaction was carried out at room temperature and the absorbance was read at 530 nm at 0.5 and 4.5 min. The unit of lysozyme activity was defined as the amount of lysozyme that caused a decrease in absorbance of 0.001/min.

Total RNA extraction from tissue preparation

The head kidney of *P. olivaceus* was excised and minced with a micropestle, after which the total RNA was extracted according to the procedure recommended by the manufacturer of the RNA extraction kit (Invitrogen, Carlsbad, CA, USA). Total RNA was suspended in 50 μ L of diethylpyrocarbonate (DEPC)-treated water, and formaldehyde-1.2% agarose gel electrophoresis was performed to maintain RNA-gel

Table 1. The ODNs used and their in vivo effects on the immune response in olive flounder *Paralichthys olivaceus*

ODNs	Sequence	CpG motif(No.)	O	N	L	I	T
1720	TCCATGAGCTTCCTGATGCT	Non-CpG	-	-	-	+/-	+/-
1668	TCCATGACGTTTCCTGATGCT	GACGTT(1)	+	-	-	+	+
1670	ACCGATAACGTTGCCGGTGACG	AACGTT(1)	-	-	+	+	+
1826	TCCATGACGTTTCCTGACGTT	GACGTT(2)	+	-	+	+/-	+/-

Abbreviations: O, O_2^- ; N, NO; L, Lysozyme; I, IL-1 β ; T, TNF- α .
Symbols: -, no induction; +/-, weak induction, +, good induction.

integrity. The RNA concentration was determined spectrophotometrically by measuring the absorbance at 260 nm in a microcuvette using an MBA 2000 spectrophotometer (PerkinElmer, Wellesley, MA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA from the head kidney was used for cDNA synthesis using an Advantage[®] RT-for-PCR kit according to the manufacturer's protocol (BD Bioscience Clontech, Palo Alto, CA, USA). Genespecific primers for *P. olivaceus* were chosen from the GenBank database: IL-1 β (AB-070835), forward 5'-ATGGAATCAAGATGGAATGC-3', reverse 5'-AACATCCAGCATCAGAGTTAA-3'; TNF- α (AB040448), forward 5'-ATGGTCAAATACACAAGTGCA-3', reverse, 5'-TCAAAGTGCAAAGACACCGAA-3'; and GAPDH (AB029337), forward 5'-CCCATGTTCGT CATGGGCG-3', reverse 5'-GAGCTCAGGGATGA CCTT-3'. All PCR reactions were performed according to the following protocol. A 2- μ L aliquot (5 μ g) of cDNA was mixed with 2.5 μ L of 10 \times buffer, 2 μ L of dNTPs (2.5 μ M of each dNTP), 0.05 μ L of Ex Taq polymerase (5 units/ μ L), 1 μ L of each genespecific primer, and 16.45 μ L of sterile water to give a final volume of 25 μ L. For semiquantitative analysis, the PCR was run for 21 cycles at 95 $^{\circ}$ C for 45 s, 58 $^{\circ}$ C for 45 s, and 72 $^{\circ}$ C for 1 min, with the denaturation step prolonged to 5 min in the first cycle and the DNA synthesis step prolonged to 10 min in the last cycle. The PCR reactions were performed using DNA Engine, DYAD[™] (MJ Research, Inc., Watertown, MA, USA), and the PCR products were electrophoresed on 1.5% agarose gel to detect the specific bands.

Statistics

One-way of analysis variance (ANOVA) followed by Turkey multiple comparisons were used to evaluate the level of significance of all measurements. The results were considered significant at $P < 0.05$.

Results

In vitro assay

To determine the optimal concentrations of ODNs, 0.2, 1, 2.5, and 5 μ M concentrations of the ODNs were used directly to stimulate the head kidney leukocytes, and the production of O₂⁻ was measured by the NBT reduction assay. Compared to the control and non-CpG-ODN 1720, significantly higher O₂⁻ production was recorded in CpG-ODN 1668 at 1,

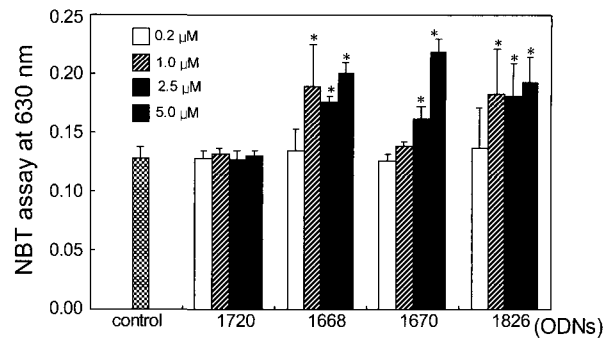


Fig. 1. In vitro superoxide anion (O₂⁻) production in head kidney cells of olive flounder *Paralichthys olivaceus* exposed to 0.2, 1, 2.5, or 5 μ M synthetic non-CpG-ODN 1720 or CpG-ODN 1668, 1670, or 1826 for 60 min at 20 $^{\circ}$ C. The level was measured using the NBT reduction assay. Values are the means \pm standard deviation of four fish (* $P < 0.05$).

2.5, and 5 μ M (all $P < 0.05$), in CpG-ODN 1670 at 2.5 μ M ($P < 0.05$) and 5 μ M ($P < 0.01$), and in CpG-ODN 1826 at 1, 2.5, and 5 μ M (all $P < 0.05$; Fig. 1).

In vivo assay

To examine the immune responses of *P. olivaceus* to various ODNs in vivo, the optimal concentration of 5 μ M ODN, determined from the in vitro assay (Fig. 1), was injected into the intraperitoneal cavity of the fish. After 3 and 5 days, head kidney tissues were obtained from various treated individuals as described in the Materials and Methods, and the O₂⁻ production of the head kidney leukocytes (1 \times 10⁶ cell/mL) was measured. CpG-ODN 1668 significantly induced O₂⁻ production at 3 days ($P < 0.05$), but the other CpG-ODNs did not (Fig. 2A). In contrast, CpG-ODN 1826 significantly induced O₂⁻ production at 5 days ($P < 0.05$), whereas the other CpG-ODNs did not (Fig. 2B).

Similarly, CpG-ODN 1668 significantly induced NO production at 3 days ($P < 0.05$), but the other CpG-ODNs did not (Fig. 3A). However, at 5 days, CpG-ODN 1668 still maintained significant NO production, and CpG-ODN 1670 induced some NO production, but the difference from the control was not significant (Fig. 3B).

To examine the lysozyme activity within the serum of *P. olivaceus* at 3 and 5 days after ODN injection, the blood was obtained from treated fish and the serum was collected. CpG-ODN 1670 significantly induced lysozyme activity at 3 days ($P < 0.01$), but the other CpG-ODNs did not (Fig. 4A). CpG-ODN 1826 significantly induced lysozyme activity at 5 days ($P < 0.01$; Fig. 4B).

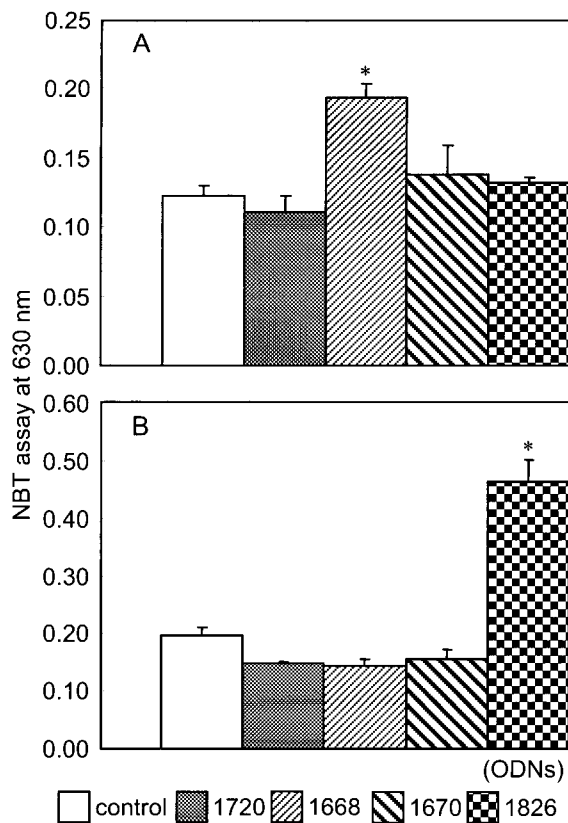


Fig. 2. In vivo superoxide anion (O_2^-) production in head kidney cells of olive flounder *Paralichthys olivaceus* injected with 5 μ M synthetic non-CpG-ODN 1720 or CpG-ODN 1668, 1670, or 1826. The level was measured using the NBT reduction assay at (A) 3 and (B) 5 days. Values are the means \pm standard deviation of four fish (* $P < 0.05$).

Induction of cytokine gene expression by ODNs

To examine the expression of the cytokine genes IL-1 β (743 bp) and TNF- α (780 bp) in the head kidney of *P. olivaceus*, total RNA was isolated from the head kidney after 1, 2, and 3 days of injection with ODN. The induction of IL-1 β mRNA expression by CpG-ODNs 1668 and 1670 was stronger than that by non-CpG-ODN 1720. CpG-ODN 1826 weakly induced IL-1 β mRNA expression at 1, 2, and 3 days (Fig. 5). The induction of TNF- α mRNA expression by CpG-ODNs 1668 and 1670 was relatively stronger than that by non-CpG-ODN 1720 at 1, 2, and 3 days; the induction of TNF- α mRNA expression by CpG-ODN 1826 was much lower, although somewhat higher than that by non-CpG-ODN 1720 (Fig. 5).

Discussion

CpG-ODNs can enhance superoxide anion (O_2^-) production in vitro and in vivo, nitric oxide (NO)

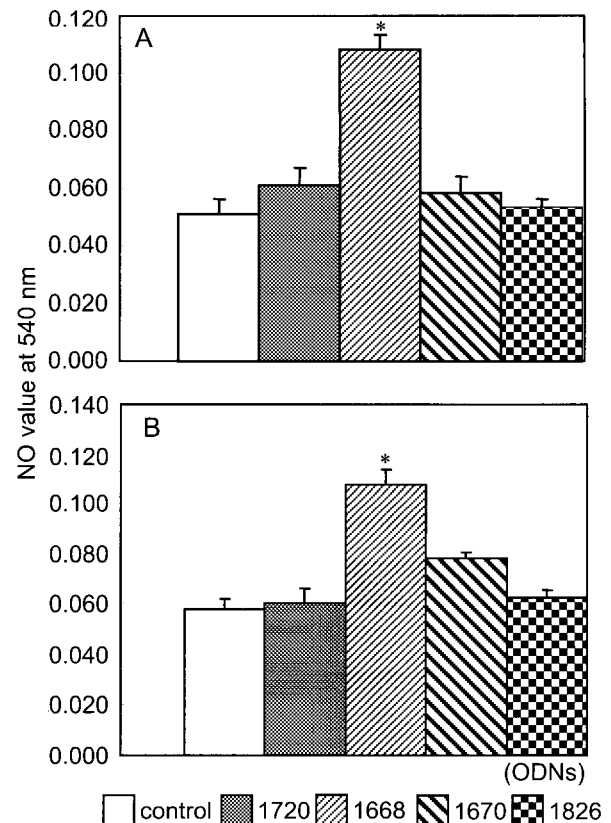


Fig. 3. In vivo nitric oxide (NO) production in head kidney cells of olive flounder *Paralichthys olivaceus* injected with 5 μ M synthetic non-CpG-ODN 1720 or CpG-ODN 1668, 1670, or 1826. The level was measured using Greiss reagent at (A) 3 and (B) 5 days. Values are the means \pm standard deviation of four fish (* $P < 0.05$).

production in leukocytes, serum lysozyme activity, and the expression of the proinflammatory cytokine genes IL-1 β and TNF- α in vivo in *P. olivaceus*. The in vitro and in vivo patterns of the induction of O_2^- production by CpG-ODNs differed. In vitro, all CpG-ODNs directly induced O_2^- production; however, in vivo, only CpG-ODN 1668 induced O_2^- production at 3 days and only CpG-ODN 1826 induced it at 5 days. Therefore, we postulate that the difference in O_2^- production patterns induced by CpG-ODNs depend on the differences in the stimulation and induction systems between in vitro and in vivo systems in *P. olivaceus*.

No previous study has reported that CpG-ODN induces NO production in *P. olivaceus* (Lee et al., 2003a,b). However, we observed that CpG-ODN 1668 significantly induced NO production in head kidney cells in vivo. Therefore, we suggest that differences may exist in NO induction by CpG-ODNs

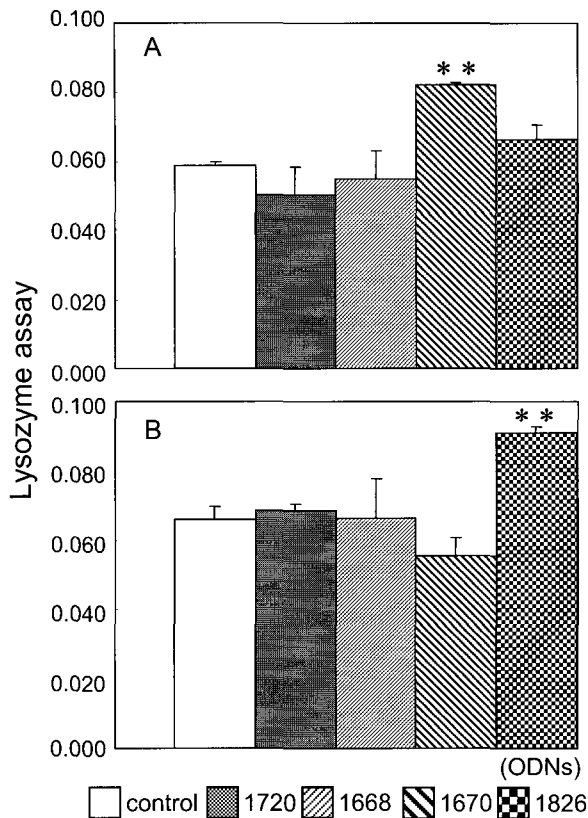


Fig. 4. In vivo lysozyme activity in the blood serum of olive flounder *Paralichthys olivaceus* injected intraperitoneally with 5 μ M synthetic non-CpG-ODN 1720 or CpG-ODN 1668, 1670, or 1826. Lysozyme activity was examined at (A) 3 and (B) 5 days. Values are the means \pm standard deviation of four fish (** $P < 0.01$).

in different groups of *P. olivaceus*.

CpG-ODNs also increased the lysozyme activity. Lee et al. (2003b) reported that 0.25 μ g of CpG-ODN 1826 significantly induced lysozyme activity at 1 and 7 days in *P. olivaceus*, whereas other concentrations of CpG-ODNs 1826 and 1670 resulted in enhanced

lysozyme activity at 1, 3, 5, and 7 days, with fluctuations. However, we found that lysozyme activity in *P. olivaceus* injected with CpG-ODNs 1670 and 1826 was significant only at 3 and 5 days, respectively. Therefore, we suggest that group- or individual-level differences may exist in lysozyme induction by CpG-ODNs in *P. olivaceus*.

CpG-ODNs are reported to induce the secretion of various inflammatory cytokines such as IL-1 β and IFN-like protein in rainbow trout macrophages (Jørgensen et al., 2001b); however, the effects had not been examined in *P. olivaceus*. The CpG-ODNs 1668 and 1670 strongly induced the expression of IL-1 β and TNF- α mRNA over 3 days in head kidney cells. Under the same PCR reaction (i.e., 21 cycles), IL-1 β expression induced by various CpG-ODNs over 3 days was stronger than that of TNF- α . Thus, IL-1 β gene expression was stimulated more strongly by CpG-ODNs than that of TNF- α . We postulate that CpG-ODNs induce proinflammatory cytokines related to the innate immune system by stimulating head kidney macrophages in *P. olivaceus*.

The structural characteristics of the CpG motifs within the CpG-ODNs induced the innate immune response of head kidney cells in *P. olivaceus*. The differences in O $_2^-$ and NO production except the cytokine genes between non-CpG-ODN 1720, which contains the GAGCTT motif, and CpG-ODN 1668, which contains the GACGTT motif, were remarkable, despite their having the same flanking base regions (Table 1).

Jørgensen et al. (2001a) reported that CpG-ODN 1670, which contains the AACGTT motif, was superior to CpG-ODN 1668, which contains the GACGTT motif, in inducing the production of interferon-like cytokines at 2 μ M, but not 5 μ M in vitro in 0.5-kg Atlantic salmon (*Salmo salar* L.). They suggested that the enhanced stimulatory

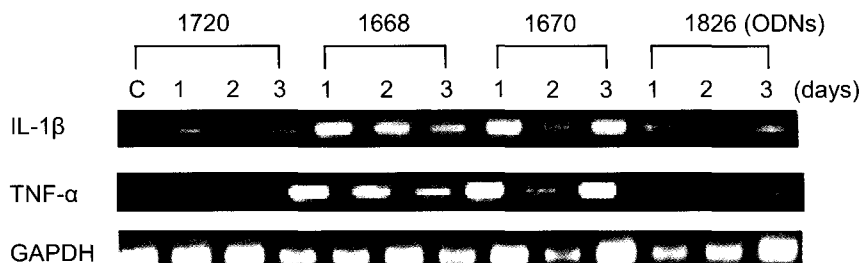


Fig. 5. Molecular expression of IL-1 β (743 bp) and TNF- α (780 bp) in head kidney cells of olive flounder *Paralichthys olivaceus* injected with 5 μ M synthetic non-CpG-ODN 1720 or CpG-ODN 1668, 1670, or 1826. The expression of cytokines was examined using reverse transcription polymerase chain reaction at 1, 2, and 3 days. The GAPDH gene was used as a positive control.

capacity of ODN 1670 might be associated with the presence of one distal CpG at the 5'-end and two distal CpGs at the 3'-end of ODN 1670. However, these two ODNs at 5 μ M in vivo in 0.25-kg *P. olivaceus* showed very different immunostimulatory spectra in terms of O₂⁻ and NO production and lysozyme activity, but resulted in a similar level of expression in proinflammatory cytokine genes.

In addition, CpG-ODN 1826, which contains two GACGTT motifs based on CpG-ODN 1668, resulted in a different immunostimulatory spectrum of NO production and lysozyme activity, and resulted in weak expression of cytokine genes compared to CpG-ODN 1668. A comparison of the expression of the cytokine genes induced by these two ODNs indicates that the two GACGTT motifs reduced the immune response of head kidney cells. Meng et al. (2003) also reported that ODN-D, which contains two re-peats motifs as in 1670, was not more efficient than 1670 in vitro. Therefore, repeated CpG motifs are not always more efficient than a single CpG motif in stimulating immune responses.

Non-CpG-ODN 1720 induced the immune response of head kidney cells in terms of the expression of the cytokine genes over that of the control, but the induction was less than that achieved by the other CpG-ODNs. Thus, non-CpG-ODN 1720 plays a role in triggering and modulating cytokine responses in the head kidney cells of *P. olivaceus* to a certain degree. This type of cytokine induction with a low stimulatory effect by non-CpG-ODN 1720 has been reported in rainbow trout macrophages (Jørgensen et al., 2001b).

Overall, the structure of CpG-ODNs may induce the different spectra and speed of stimulatory effect in the in vivo immune responses of *P. olivaceus*. It is evident that the sequence and structural characteristics of the CpG motif can influence the immune response of head kidney cells and serum in *P. olivaceus*. In olive flounder, CpG-ODN 1668 was the most effective cellular immunostimulant among the tested CpG-ODNs (i.e., 1668, 1670, and 1826), CpG-ODN 1670 was the most rapid humoral immunostimulant, and CpG-ODNs 1668 and 1670 were effective in stimulating cytokine gene expression. For optimal application of the immune effects of CpG-ODNs in the aquaculture of *P. olivaceus*, the immunostimulatory spectra of the CpG-ODNs should be characterized in vivo for specific groups of *P. olivaceus*. This will allow analysis of the effects of CpG-ODNs against the results of challenge tests prior to field application.

Acknowledgments

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References

- Asmi, C.M.A.R.T. and M. Sakai. 2005. Current research on the immunostimulatory effects of CpG oligodeoxynucleotides in fish. *Aquaculture*, 246, 25-36.
- Chung, S. and C.J. Secombes. 1988. Analysis of events occurring within teleost macrophages during the respiratory burst. *Comp. Biochem. Physiol.*, 89B, 539-544.
- Häcker, G., V. Redecke and H. Häcker. 2002. Activation of immune system by bacterial CpG-DNA. *Immunology*, 105, 245-251.
- Jørgensen, J.B., A. Johansen, B. Stenersen and A.I. Sommer. 2001a. CpG oligonucleotides 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., J. Zhou, A. Johansen and C.J. Secombes. 2001b. Immunostimulatory CpG oligonucleotides stimulate expression of IL-1 β and interferon-like cytokines in rainbow trout macrophages via a chloroquine-sensitive mechanism. *Fish Shellfish Immunol.*, 11, 673-682.
- Kanellos, T.S., I.D. Sylvester, V.L. Butler, A.G. Ambali, C.D. Partidos, A.S. Hamblin and P.H. Russel. 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.
- Klinman, D.M., A.K. Yi, S.L. Beaucage, J. Conover and A.M. Kreig. 1996. CpG motif present in bacterial DNA rapidly induces lymphocytes to secrete interleukin-6, interleukin-12, and interferon- γ . *Proc. Natl. Acad. Sci. USA*, 93, 2879-2784.
- Kreig, A.M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.*, 20, 709-760.
- Lee, C.H., H.D. Jeong, J.K. Chung, H.H. Lee and K.H. Kim. 2003a. CpG motif in synthetic ODN primes respiratory burst of olive flounder *Paralichthys olivaceus* phagocytes and enhances protection against *Edwardsiella tarda*. *Dis. Aquat. Organ.*, 56, 43-48.
- Lee, C.H., S.R. Kwon, E.H. Lee and K.H. Kim. 2003b. Effects of CpG motifs present in synthetic oligodeoxynucleotides on nonspecific immune responses and disease resistance of olive flounder (*Paralichthys olivaceus*). *J. Fish. Sci. Technol.*, 6, 125-129.
- Lipford, G.B., T. Sparwasser, M. Bauer, S. Zimmermann, E.S. Koch, K. Heeg and H. Wagner. 1997. Immuno-

- stimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. *Eur. J. Immunol.*, 27, 3420-3236.
- Lipford, G.B., K. Heeg and H. Wagner. 1998. Bacterial DNA as an immune cell activator. *Trends Microbiol.*, 6, 496-500.
- Meng, Z., J. Shao and L. Xiang. 2003. CpG oligodeoxynucleotides activate grass carp (*Ctenopharyngodon idellus*) macrophages. *Dev. Comp. Immunol.*, 27, 313-321.
- Parry, R.M., R.C. Chandau and R.M. Shahani. 1965. A rapid and sensitive assay of muramidase. *Proc. Soc. Exp. Biol. Med.*, 119, 384-386.
- Sparwasser, T., E.S. Koch and R.M. Vablues. 1998. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.*, 28, 2045-2054.
- Sun, S., X. Zhang, D.F. Tough and J. Sprent. 1998. Type I interferon-mediated stimulation of T cells by CpG DNA. *J. Exp. Med.*, 188, 2335-2342.
- Tassakka, A.C.M.A.R. and M. Sakai. 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. and M. Sakai. 2005. Current research on the immunostimulatory effects of CpG oligonucleotides in fish. *Aquaculture*, 246, 25-36.
- Yamamoto, S., T. Yamamoto, T. Kataoka, E. Kuramoto, O. Yano and T. Tokunaga. 1992. Unique palindromic sequences in the oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J. Immunol.*, 148, 4072-4076.
- Yi, A.K., R. Tuetken, T. Redford, M. Waldschmidt, J. Kirsch and A.M. Kreig. 1998. CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species. *J. Immunol.*, 160, 4755-4761.

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