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Effects of CpG Motifs Present in Synthetic Oligodeoxynucleotides on Nonspecific Immune Responses and Disease Resistance of Olive Flounder (*Paralichthys olivaceus*)

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Effects of synthetic oligodeoxynucleotides (ODNs) containing cytidine-phosphate-guanosine (CpG) motif(s) on nonspecific immune responses of olive flounder (*Paralichthys olivaceus*) and on protection against lethal infection with *Edwardsiella tarda* were investigated. Respiratory burst activities of the head kidney phagocytes in the fish injected either 0.25 or 0.5 μg/fish of ODNs containing CpG motifs (ODN 1826 and ODN 1670) were significantly higher than those injected with an ODN containing a guanosine-phosphate-cytidine (GpC) motif (ODN 1720) or with hanks balanced salt solution (HBSS, control) at 3, 5 and 7 days after injection. The serum lysozyme activities of fish injected with 0.25 μg of ODN 1826 were significantly higher than those injected with ODN 1720 or HBSS at 1 and 7 days after injection. At 7 days after injection, the group of fish injected with CpG ODNs showed higher serum lysozyme activities than fish injected with ODN 1720 or control. The group of fish injected 0.25 or 0.5 μg of CpG ODNs showed higher survival rates than those treated with GpC ODN and the control group after challenge with *Edwardsiella tarda*. The present study proved the ability of synthetic CpG ODN to increase nonspecific immune responses and disease resistance in olive flounder.

Key words: CpG ODN, Olive flounder, Respiratory burst, Lysozyme, Disease resistance, *Edwardsiella tarda*

Introduction

Recent studies indicate that unmethylated cytidine-phosphate-guanosine (CpG) motifs flanked by two 5' purines and two 3' pyrimidines are immunostimulatory in mammals (Klinman et al., 1996 Krieg et al., 1995, 2000). Due to a combination of CpG suppression and CpG methylation, these sequence motifs are rarely present in eukaryotic genomes but are common in prokaryotic genomes (Bird, 1980; 1987). Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs mimic the activity of bacterial DNA, and are recognized as a danger signal in mammalian immune cells (Yamamoto et al. 1992, Krieg et al. 1995, Ballas et al. 1996, Klinman et al. 1996).

In fish, there is limited information concerning the biological effects of CpG ODN. Kanellos et al. (1999) reported that plasmids co-injected with a recombinant protein potentiated antibody responses to the protein in goldfish (*Carassius auratus*). Recently, Jørgensen et al. (2001a,b) demonstrated that plasmid DNA and synthetic CpG-ODN induced production of IFN-like cytokine and IL-1β in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) leucocytes. Oumouna et al. (2002) demonstrated activation of nonspecific cytotoxic cells of catfish (*Ictalurus punctatus*) with synthetic ODN and bacterial genomic DNA. Tassakka and Sakai (2002) reported that intraperitoneal injection of CpG-ODN to carp (*Cyprinus carpio*) enhanced the nonspecific immune responses including phagocytic and nitroblue tetrazolium (NBT) activity in kidney phagocytes and serum lysozyme activity. Meng et al. (2003) observed increase of respiratory burst activity, acid phosphatase and bactericidal activity of grass carp (*Ctenopharyngodon idellus*) head-kidney macrophages by *in vitro* incubation with CpG ODN.

To date, however, effect of CpG ODN on the disease resistance in relation to enhanced nonspecific immune responses of fish has not been

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elucidated so far. In the present study, therefore, we investigated the effects of synthetic CpG ODNs on the nonspecific immune responses including respiratory burst activity of head-kidney phagocytes and serum lysozyme activity, and on the disease resistance in olive flounder (*Paralichthys olivaceus*).

Materials and Methods

Fish

Juvenile olive flounder, weighing 35-40 g, were obtained from a local fish farm. Fish were stocked into fourteen 50 L aquaria at a density of 12 fish per aquarium, and were acclimated 2 weeks prior to the experiment. Fish in 7 aquaria were used for assay of nonspecific immune responses, and fish in the other 7 aquaria were used for challenge test.

Oligodeoxynucleotides (ODNs)

Synthetic ODNs were purchased from Bioneer Inc. (Daejeon, Korea). ODNs were phosphorothioated to increase their resistance to nuclease degradation. ODN 1826 had 2 CpG motifs and the sequence was 5'-TCCATGACGTTCCTGACGTT-3' (CpG motif is underlined.). ODN 1670 had 1 CpG motif and the sequence was 5'-ACCGATAACGTTGAC-3'. ODN 1720 was synthesized by replacing a CpG dinucleotide with a GpC dinucleotide to use as a positive control of CpG motifs, and the sequence was 5'-TCCATGAGCTTCCTGATGCT-3'.

Isolation of head kidney phagocytes

Fish were anaesthetized with tricaine methanesulfonate (MS222; Sigma). The head-kidney was extracted by ventral incision and transferred to L-15 medium (Sigma) supplemented with 2% foetal calf serum (FCS; Sigma), heparin (10 units/mL, Sigma), penicillin (100 μg/mL, Sigma) and streptomycin (100 U/mL, Sigma). The cell suspensions obtained by forcing the organ through a nylon mesh were layered over a 34/51% Percoll density. After centrifugation at 400×g for 30 min at 4℃, the phagocyte enriched interphase was collected and washed three times. Then, the cells were resuspended in culture medium, and dispensed into flat-bottomed 96-well plates. After 2 h at 20℃, wells were washed with culture medium to remove non-adherent cells. The remained phagocytes were detached from the plates by incubating for 1 h at 4℃. The cell viability was examined with tryphan blue exclusion and evaluated to be greater than 95%. The number of phagocytes were adjusted to 1×10^6 cells/mL.

Chemiluminescence (CL) assay

The reactive oxygen species (ROS) produced by stimulated phagocytes was quantified using an automatic photoluminometer (Bio-Orbit 1251, Finland). Each test cuvette contained 0.7 mL luminol (Sigma) made according to the method of Scott and Klesius (1981), 0.4 mL cell suspension, and 0.3 mL zymosan (Sigma), which was added just prior to measurement. The measurements were made for 1 hr, and the assay was made in triplicate.

Effects of ODNs on respiratory burst of phagocytes

Acclimated juvenile olive flounder were divided into 7 groups, each containing 12 fish. ODNs were diluted with hanks balanced salt solution (HBSS) and injected intraperitoneally (i.p.) at a dose of 0.25 or 0.5 μg/fish. Control fish received HBSS alone. Three fish of each group were sampled at 1, 3, 5 and 7 days after injection. Head kidney phagocytes were isolated as described above and analyzed for ROS production by CL.

Effects of ODNs on lysozyme activity of serum

The turbidimetric assay for lysozyme was carried out according to Parry et al. (1965). Briefly, test serum (0.1 mL) was added to 2 mL of a suspension of *Micrococcus lysodeikticus* (0.2 mg/mL, Sigma) in a 0.05 M sodium phosphate buffer, pH 6.2. The reaction was carried out at 25℃ and absorbance at 530 nm was measured after 0.5 and 4.5 min on a spectrophotometer. A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001/min.

Effects of ODNs on disease resistance

Acclimated juvenile olive flounder were divided into 7 groups, either containing 12 fish, and were treated intraperitoneally with each ODN at a dose of 0.25 or 0.5 μg/fish. Control fish received HBSS alone. *Edwardsiella tarda* FSW 910410, isolated from an episode of edwardsiellosis of olive flounder in a local farm of Korea, was obtained from the Laboratory of Fish Diseases Prevention, Pukyong National University. To enhance the virulence, the bacteria had been passaged in five extra-untreated olive flounders by intraperitoneal injection. The bacterium was reisolated from the kidney of moribund fish 3 days later and cultured on TSA (Sigma) plates supplemented with 1.5% NaCl for 24 h at 27℃. The bacterial cell suspension was adjusted to 1.35×10^6 cfu/L. Fish were challenged by immersing into 50

L of the bacterial suspension for 30 min at 2 days post each ODN or HBSS injection. Deaths were recorded over 15 days. Dead fish were collected daily and necropsied. Kidney samples were streaked on TSA to confirm the presence of *E. tarda*.

Statistical analysis

The statistical significance was evaluated using Student's *t*-test of significance, and $P<0.05$ was considered statistically significant.

Results

Effects of ODNs on nonspecific immune responses

Respiratory burst activities of the head-kidney phagocytes of the groups injected either 0.25 or 0.5 μg of ODN 1826 were significantly higher than those in the groups injected with ODN 1720 or HBSS (the control) at 3, 5 and 7 days after injection (Fig. 1). Fish injected with either 0.25 or 0.5 μg of ODN 1670 showed significantly higher CL responses than those injected with ODN 1720 or HBSS. At 7 days post injection, the CL responses were markedly increased in the groups of fish injected with CpG ODNs (ODN 1826 and 1670). The fish injected with ODN 1720 showed no significant differences with the control group in CL responses.

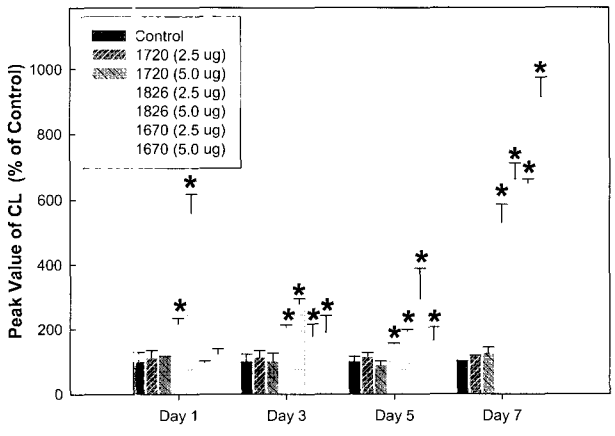

Fig. 1. Respiratory burst activity of head kidney phagocytes of olive flounder (*Paralichthys olivaceus*) injected intraperitoneally with 0.25 or 0.5 μg of synthetic oligodeoxynucleotides (ODN) or HBSS alone (the control). The respiratory burst activity was measured by chemiluminescent response (CL) and analyzed at 1, 3, 5 and 7 days after injection. Values are mean of 3 fish and bars represent standard deviation. (*, Significantly different from control; $P<0.05$).

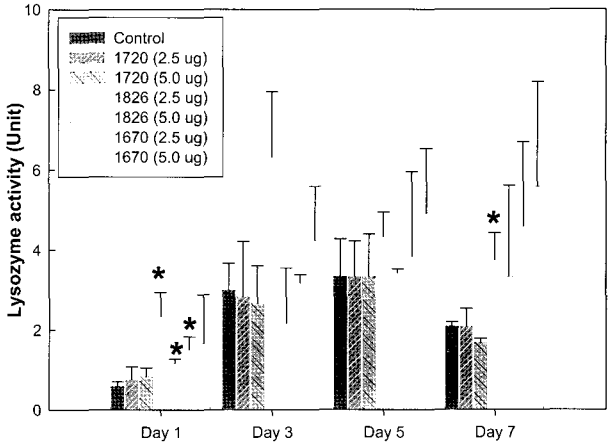

Fig. 2. Serum lysozyme activity of olive flounder (*Paralichthys olivaceus*) injected intraperitoneally with 0.25 or 0.5 μg of synthetic oligodeoxynucleotides (ODN) or HBSS alone (the control). The lysozyme activity was measured by turbidimetric method and analyzed at 1, 3, 5 and 7 days after injection. Values are mean of 3 fish and bars represent standard deviation. (*, Significantly different from control; $P<0.05$).

The serum lysozyme activities of fish injected with 0.25 μg of ODN 1826 were significantly higher than those injected with ODN 1720 or HBSS at 1 and 7 days after injection (Fig. 2). At 7 days after injection, the fish injected with CpG ODN showed higher serum lysozyme activities than those injected with ODN 1720 or the control group.

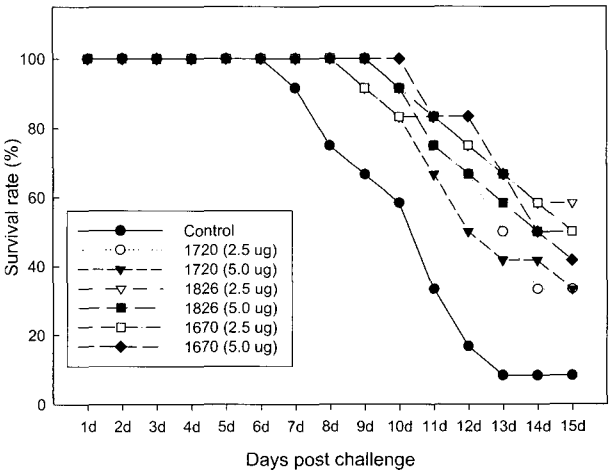

Fig. 3. Survival rate of olive flounder (*Paralichthys olivaceus*) injected intraperitoneally with 0.25 or 0.5 μg of synthetic oligodeoxynucleotides (ODN) or HBSS alone (control) after challenge with *Edwardsiella tarda*.

Effects of ODNs on disease resistance

Survival rates of fish injected with 0.25 or 0.5 μg of ODN 1826 and 1670 showed higher survival rates than thos etreated with ODN 1720 and the control group after challenge with *E. tarda* (Fig. 3). All dead fish were positive for *E. tarda*.

Discussion

The major immunological function of immunostimulants is the increase of phagocytic cells activity, and enhancement of pathogen killing is important in the phagocytes of fish treated with immunostimulants (Sakai, 1999). Reactive oxygen species (ROS) produced by fish phagocytes during the respiratory burst are responsible for the oxygen-dependent killing mechanism, and can be detected by chemiluminescence (Secombes and Fletcher, 1992; Kajita et al., 1990). The present results demonstrate that synthetic ODNs containing CpG motif(s) can enhance respiratory burst activity of fish phagocytes. Fish injected with CpG ODNs showed significantly higher respiratory burst activity of phagocytes. Similarly, Tassakka and Sakai (2002) reported that carp (*Cyprinus carpio*) injected intraperitoneally with CpG ODN showed significantly higher nitroblue tetrazolium (NBT) activity in kidney phagocytes. In the present study, injection of ODN containing GpC motif (ODN 1720) did not influence on respiratory burst activity. This result suggests that the enhancement of respiratory burst activity of fish phagocytes is not by nonspecific unmethylated DNA but by ODN containing CpG motif(s).

Lysozyme is widely distributed on body surface, skin, gill, intestinal tract, and in serum of fish as a protection agent against bacterial infection (Yano, 1996). In the present results, the serum lysozyme activity was increased by i.p. injection of CpG ODNs. In addition to direct antibacterial effect, lysozyme promotes phagocytosis as an opsonin, or by directly activating polymorphonuclear leucocytes and macrophages (Klockars and Roberts, 1976; Jollès and Jollès, 1984). In the present study, therefore, the markedly increased CL response after 7 days of injection with CpG ODNs might be partly attributed to the high lysozyme activity at 7 days post injection.

The rapid induction of an innate immune response is critical in controlling the early spread of intracellular pathogens. Although previous studies showed that CpG ODNs have significant *in vitro* biological activity, the *in vivo* significance of these effects has been unclear. The present study demonstrated that treatment of fish with ODNs containing CpG motifs confers protection against lethal bacterial infection. Fish treated with CpG ODNs showed considerably higher survival rates than fish in the control group against *E. tarda* challenge. Thus, the stimulation of innate protective immunity by host recognition of synthetic ODN containing CpG motif would contribute to the higher survival rate. The cause of higher survival rates of fish injected with GpC ODNs than those in the control, however, could not be explained explicitly.

In conclusion, the present study proved the ability of synthetic CpG ODNs to increase nonspecific immune responses and disease resistance in olive flounder (*P. olivaceus*).

Acknowledgements

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