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Effects of synthetic oligodeoxynucleotides containing CpG motifs on respiratory burst activity of olive flounder (*Paralichthys olivaceus*) phagocytes

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Effects of synthetic cytidine-phosphate-guanosine oligodeoxynucleotide (CpG ODN) on respiratory burst activity of olive flounder (*Paralichthys olivaceus*) head kidney phagocytes were investigated. Phagocytes precultured with CpG ODNs showed significantly higher CL responses than phagocytes precultured with guanosine-phosphate-cytidine (GpC) ODN or culture medium alone (control) at all concentrations. Supernatants produced from leucocytes, which were coincubated or pulsed with CpG ODNs, induced significantly higher respiratory burst activity than supernatants produced by GpC ODN or culture medium alone. The present *in vitro* experiments have demonstrated the ability of synthetic CpG ODN to increase phagocytes respiratory burst activity of olive flounder.

Key words : Synthetic ODN, Olive flounder, Respiratory burst activity

Introduction

The vertebrate immune system recognizes bacterial genomic DNA as a danger signal on the basis of recognition of unmethylated cytidine-phosphate-guanosine (CpG) dinucleotides flanked by specific bases (Krieg *et al.*, 1995 and 2000). CpG dinucleotides are under-represented (1/60) and selectively methylated on the 5' position of the cytosine in vertebrate DNA, but are present at the expected frequency (1/16) and are unmethylated in bacterial DNA (Bird, 1980 and 1987). A possible molecular mechanism whereby bacterial DNA activates immune cells is revealed with the discovery of Toll-like receptor 9 (TLR9) in mice, a transmembrane receptor capable of recognizing unmethylated CpG oligonucleotides in bacterial DNA (Hemmi *et al.*, 2000). Synthetic oligodeoxynucleotides (ODN) containing CpG motifs have been used to further define the immunostimulatory activity in several vertebrate species.

To date, there is limited information concerning immunomodulatory effects of CpG ODNs in fish. 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 ODNs containing CpG motifs induced production of IFN-like cytokine and IL-1 in Atlantic salmon and rainbow trout leucocytes. Oumouna *et al.* (2002) demonstrated activation of nonspecific cytotoxic cells of catfish (*Ictalurus punctatus*) with synthetic ODNs and bacterial

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genomic DNA. Tassakka and Sakai (2002) reported that intraperitoneal injection of CpG ODNs to carp (*Cyprinus carpio*) enhanced the nonspecific immune responses including phagocytic and nitroblue tetrazolium (NBT) activity in kidney phagocytes and serum lysozyme activity.

In the present study, we evaluated *in vitro* effects of synthetic oligodeoxynucleotides containing CpG motifs on respiratory burst activity of head kidney leukocytes in olive flounder, *Paralichthys olivaceus*.

Materials and Methods

Fish

A total of 30 olive flounder (*P. olivaceus*), weighing 350-400 g, obtained from a local fish farm, was kept in 500 ℓ fiberglass tanks containing filtered, aerated sea water at a temperature of 20±2℃. Fish were acclimated to these conditions for at least 2 weeks before use and fed commercial flounder pellets.

Oligodeoxynucleotieds (ODNs)

Synthetic ODNs were purchased from Bioneer Inc. (Daejeon, Korea). ODNs were phosphotioated 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 leukocytes

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/㎖, Sigma), penicillin (100 ㎍/㎖, Sigma) and streptomycin (100 U/㎖, Sigma). To get leucocytes, the cell suspensions obtained by forcing the organ through a nylon mesh were layered over a 51% Percoll (Sigma). After centrifugation at 400 g for 30 min at 4℃, the leucocytes fraction was removed from the Percoll-medium interface, washed 3 times, counted and adjusted to 5×10^6 cells/㎖. To get phagocytes, head kidney cell suspensions obtained as described above, 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 with L-15 medium. 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/㎖.

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 ㎖ luminol (Sigma) made according to the method of Scott and Klesius (1981), 0.4 ㎖ cell suspension, and 0.3 ㎖ zymosan (Sigma), which was added just prior to measurement. The measurements were made for 1 h and the assay was carried out in triplicate.

Direct priming effects of CpG-ODN on respiratory burst activity of phagocytes

Head kidney phagocytes (1×10^6 cells/$m\ell$) obtained as described above, were precultured for 6, 12 and 24 h with 0 (control), 1.25, 2.5, 5 and 10 $\mu g/m\ell$ of ODNs at 20°C. Cells were then washed twice with Hank's balanced salt solution (HBSS, Sigma) to remove any residual ODN and analyzed for ROS production by CL as described above.

Priming effects of supernatants produced from leucocytes coincubated with CpG ODN on respiratory burst activity of phagocytes

1.5 $m\ell$ of the leucocytes suspension (2×10^6 cells/$m\ell$) were seeded into 24 well-tissue culture plates, and were coincubated for 6, 12 and 24 h at 20°C with 1.25, 2.5, 5.0 and 10.0 $\mu g/m\ell$ of ODNs. At each coincubation time, the supernatants were harvested, centrifuged and stored at -70°C until use. A supernatant collected from leucocytes that had not been coincubated with ODN was used as a control. The phagocytes obtained as described above, were incubated with the supernatants for 3 h and then stimulated with zymosan for analyzing ROS production.

Priming effects of supernatants produced from leucocytes pulsed with CpG ODN on respiratory burst activity of phagocytes

5 $m\ell$ of the leucocytes suspension (5×10^6 cells/$m\ell$) were seeded into tissue culture flasks (25 cm^2), and were pulsed for 3 h at 20°C with 1.25, 2.5, 5.0 and 10.0 $\mu g/m\ell$ of ODNs. After then, the cells were gently washed three times with HBSS to remove any residual ODN, and cultured in L-15 medium for 48 h. The supernatants were then harvested, centrifuged and stored at -70°C until use. A supernatant collected from leucocytes that had not been pulsed with ODN was used as a control. The supernatants were diluted 1:16 with culture medium, and were added to phagocytes monolayer in culture plates for 4, 8, 12 and 24 h at 20°C. At each time point, the cells were washed twice with HBSS and analyzed for ROS production by CL.

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

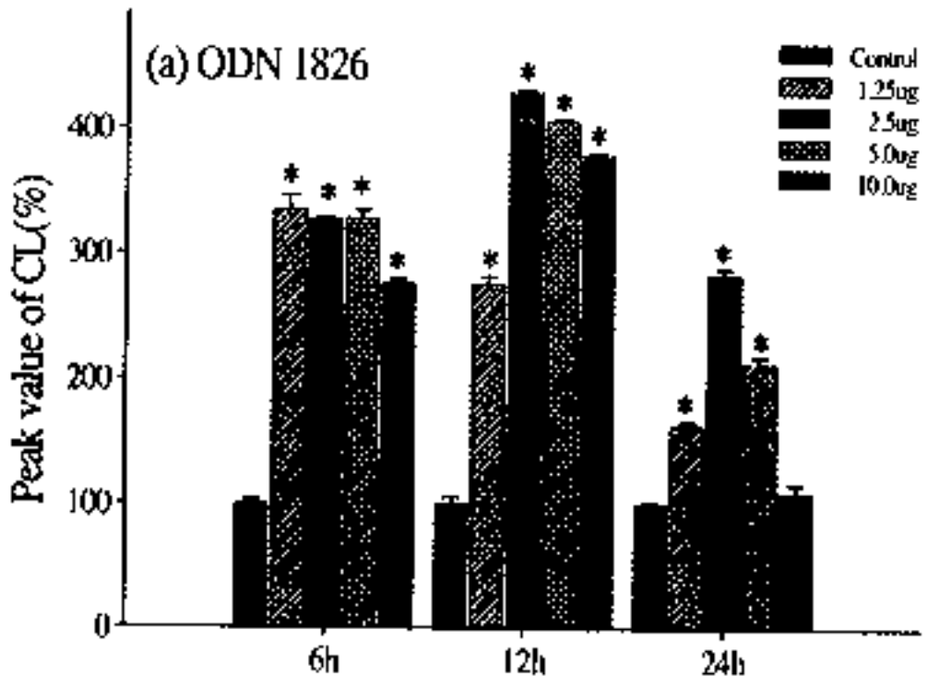
Results

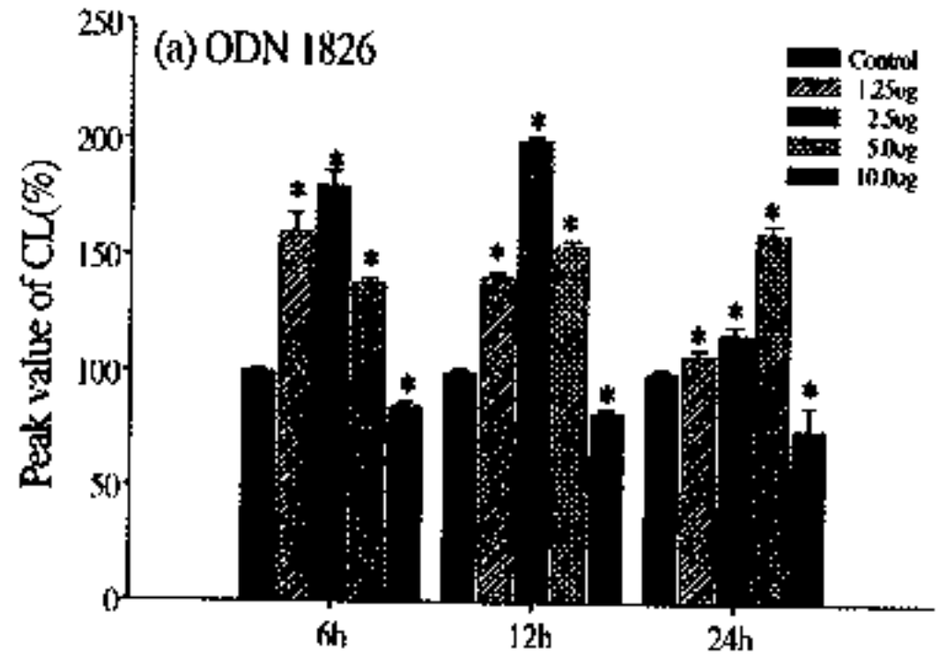
Direct priming effects of CpG ODN on respiratory burst activity of phagocytes

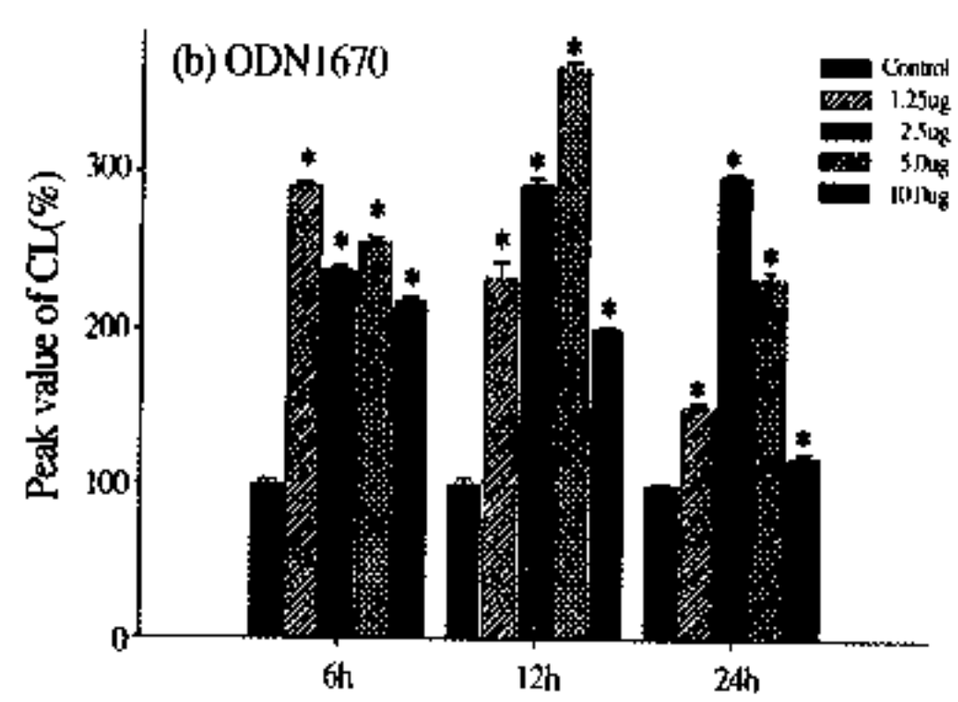
Head kidney phagocytes precultured with CpG ODNs (ODN 1826 and 1670) for 6, 12 and 24 h showed significantly higher CL responses than phagocytes precultured with culture medium alone (control) at all concentrations (Fig. 1). Phagocytes primed with 10 $\mu g/m\ell$ of CpG ODNs for 24 h showed significantly lower CL responses than those primed with lower concentrations of CpG ODNs. Although phagocytes precultured with GpC ODN for 6 h at all concentrations and for 12 or 24 h at 1.25 $\mu g/m\ell$ showed significantly higher CL responses than controls, the extent of CL enhancement was extremely low compared to CpG ODNs.

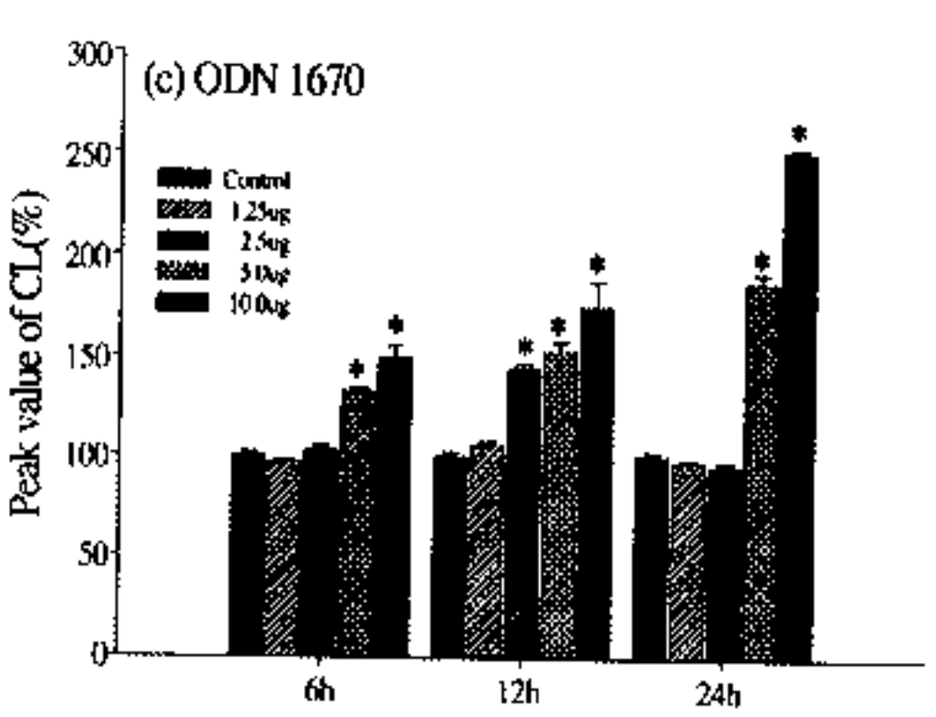
Priming effects of supernatants produced from leucocytes coincubated with CpG ODN on respiratory burst activity of phagocytes

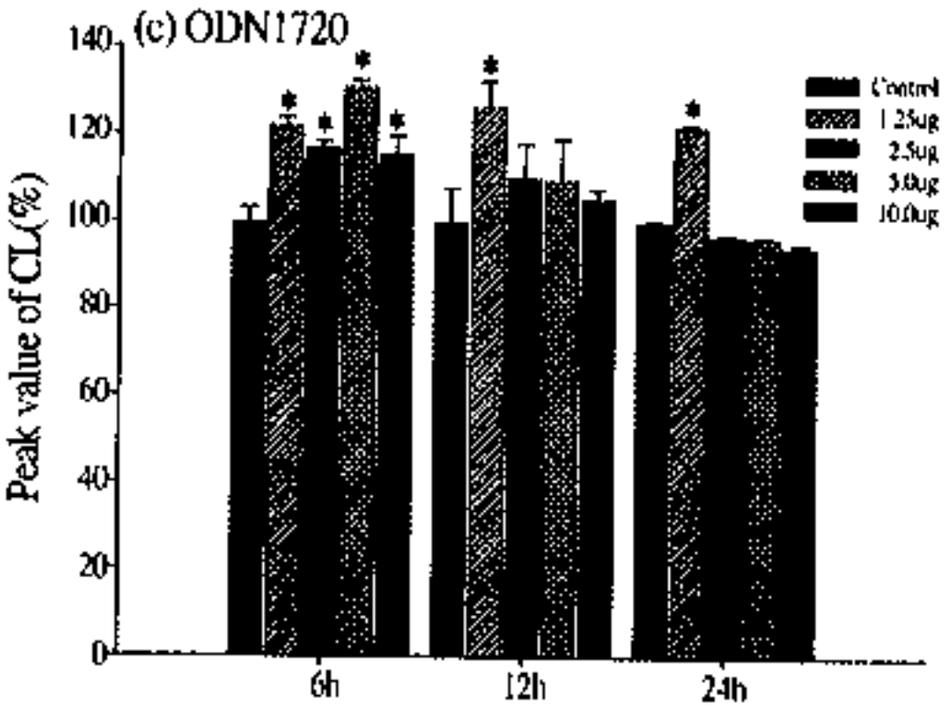
Phagocytes exposed to the supernatants, which were produced from leucocytes coincubated with 1.25-5.0 $\mu g/m\ell$ of ODN 1826 and with 5.0-10.0 $\mu g/m\ell$ of ODN 1670 for 6, 12 and 24 h, showed significantly higher CL responses than cells exposed to the culture medium alone (Fig. 2). However, phagocytes exposed to the supernatants derived from 10.0 $\mu g/m\ell$ of ODN 1826 showed significantly lower CL responses than control at all assayed times. The priming effect of supernatants produced by ODN











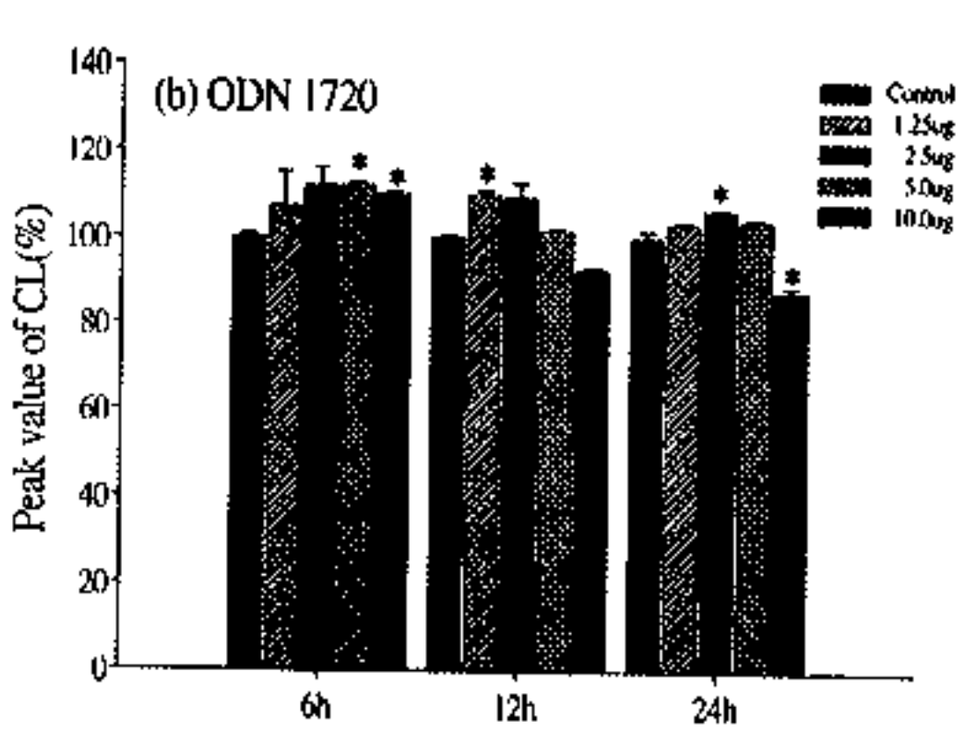


Fig. 1. Effects of synthetic oligodeoxynucleotides (ODNs) on direct priming of respiratory burst activity of olive flounder (*Paralichthys olivaceus*) head kidney phagocytes. Phagocytes were precultured with various concentrations (1.25, 2.5, 5.0 and 10.0 $\mu g/m\ell$) of each ODN (a, b, c) or medium alone (control) for 6, 12 and 24 h, then analyzed for reactive oxygen species (ROS) production by chemiluminescence (CL) assay. Results are mean of triplicate samples and bars represent standard deviation (*, Significantly different from control; $P<0.05$).

Fig.2. Effects of supernatants produced from leucocytes coincubated with synthetic oligodeoxynucleotides (ODNs) on the priming of respiratory burst activity of olive flounder (*Paralichthys olivaceus*) head kidney phagocytes. The supernantants were produced by coincubation of leucocytes with various concentrations (1.25, 2.5, 5.0 and 10.0 $\mu g/m\ell$) of each ODN for 6, 12, and 24 h. Target phagocytes were incubated with each supernatant for 3 h and then stimulated with zymosan. Results are mean of triplicate samples and bars represent standard deviation (*, Significantly different from control; $P<0.05$).

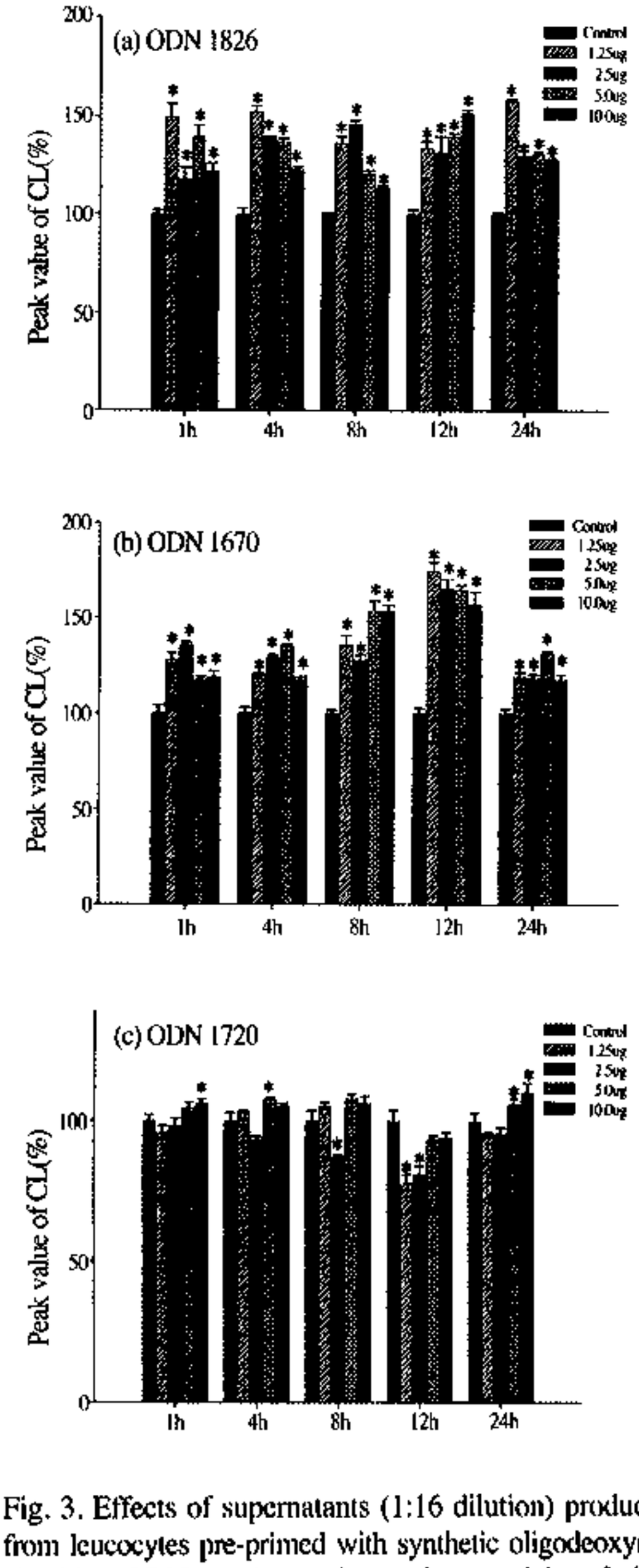


Fig. 3. Effects of supernatants (1:16 dilution) produced from leucocytes pre-primed with synthetic oligodeoxynucleotides (ODN) on the respiratory burst activity of olive flounder (*Paralichthys olivaceus*) head kidney phagocytes. Leucocytes were pulsed with various concentrations (1.25, 2.5, 5.0 and 10.0 $\mu g/m\ell$) of each ODN or medium alone (control) for 3 h, and then washed gently. Supernatants were harvested afterincubation of the washed leucocytes for 48 h at 20℃. Target phagocytes were incubated with each diluted supernatant for 4, 8, 12 and 24 h and then, analyzed for reactive oxygen species (ROS) production by chemiluminescence (CL) assay. Results are mean of triplicate samples and bars represent standard deviation (*, Significantly different from control; $P<0.05$).

1720 were weaker than that of CpG ODNs.

Priming effects of supernatants produced from leucocytes pulsed with CpG ODN on respiratory burst activity of phagocytes

Supernatants produced from leucocytes pulsed with various concentrations of CpG ODNs increased significantly CL responses of head kidney phagocytes when compared to control, irrespective of assay times (Fig. 3). In contrast, supernatants produced by GpC ODN did not enhance significantly or enhanced slightly CL responses of phagocytes.

Discussion

In this study, olive flounder head kidney phagocytes precultured with various concentrations of CpG ODNs for certain times (6, 12 and 24 h) showed significantly higher CL responses than phagocytes precultured with GpC ODN or with medium alone. This result suggests that synthetic ODNs containing CpG motif(s) can enhance respiratory burst activity of fish phagocytes and this priming effect was not by nonspecific unmethylated DNA but by ODN containing CpG motif(s). As the phagocytes were isolated by two-step manipulation including Percoll gradient centrifugation and plate adherence, phagocytes of fish are considered as effective target cells to respond to CpG ODN. In a recent published paper, it was demonstrated that cellular responses to CpG ODN are mediated by a Toll-like receptor, TLR9 (Hemmi *et al.*, 2000), and the internalization of the DNA is required for activity (Krieg, 2000). Moreover, endosomal acidification and/or maturation in the signaling pathways triggered by CpG DNA are critical for the immune stimulatory effects of CpG DNA (Hacker *et al.*, 1998; Yi and Krieg, 1998). Therefore, certain times enough to produce immunostimulatory factors are

needed to get priming effects of CpG DNA in phagocytes. In the present results, a 6 h was enough to prime phagocytes by exposure to CpG ODNs. As the phagocytes were washed after incubation with CpG ODNs in this experiment, the enhancement of CL responses suggests that phagocytes were primed by the medium containing secreted substances from the leucocytes during 6 h. CpG motifs in bacterial DNA and synthetic ODNs promote the secretion of various cytokines, such as IL-1, IL-6, IL-12, IFNs from mammalian macrophages and NK cells (Stacey *et al.*, 1996; Chase *et al.*, 1997; Lipford *et al.*, 1997). Recently, it has been reported that synthetic ODNs containing CpG motifs induced production of IFN-like cytokine activity in Atlantic salmon leucocytes (Jørgensen *et al.*, 2001b). Therefore, the priming effect of CpG ODNs in the present results should be mediated by the cytokines including IFN-like cytokines secreted from leucocytes. No priming effect of GpC ODN on phagocyte respiratory burst in this result indicates clearly that this priming effect was not by nonspecific unmethylated DNA but specifically flanked CpG motifs.

Induction of phagocytes activaing factor secretion by CpG ODNs was well demonstrated in the present study. Supernatants produced by coincubation of leucocytes with CpG ODNs for 6, 12 or 24 h enhanced respiratory burst activity of phagocytes stimulated with zymosan. However, supernantants produced by GpC ODN did not show any enhancing effects. Jørgensen *et al.* (2001b) reported that supernatants harvested from CpG ODN stimulated macrophages for 8 h expressed higher IL-1β than those stimulated for 24 h.

The present results demonstrate that olive flounder head-kidney leucocytes pre-pulsed with CpG ODNs for 3 h release soluble phagocyte-activating factors, which are able to enhance the respiratory burst activity of target phagocytes. This stimulating effect was similar to that of macrophage activating factor (MAF) produced by various mitogens, in that both prime the respiratory burst response of phagocytes after stimulation (Nagakawara *et al.*,1982; Graham and Secombes, 1988;1990a,b). In the present study, supernatants produced by the highest concentration (10 $\mu g/ml$) of CpG ODN 1826 showed lower priming activity on phagocytes than control. Klinman *et al.* (1996) reported that production of cytokines including IL-12 and IFN-γ from murine leucocytes induced by phosphorothioate ODN containing CpG motifs was higher at a concentration 0.10-0.33 $\mu g/ml$ than that at 3.0 $\mu g/ml$. Additionally, it has been demonstrated that MAF supernatants often show inhibitory effects on macrophage respiratory burst activity, possibly due to the presence of suppressive factors (Graham and Secombes, 1990a,b; Mulero and Meseguer, 1998).

In conclusion, the present *in vitro* experiments have demonstrated the ability of synthetic CpG ODNs to increase respiratory burst activity of phagocytes in olive flounder (*Paralichthys olivaceus*).

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