J. Fish. Sci. Tech. 5(4), 258~262, 2002

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY


# Production of Phagocyte Activating Supernatants by Olive Flounder (*Paralichthys olivaceus*) Leucocytes Stimulated with Genomic DNA of *Escherichia coli*

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(Received June 2002, Accepted October 2002)

Effects of *Escherichia coli* genomic DNA on the production of phagocyte activating supernatants by the head kidney leucocytes isolated from olive flounder (*Paralichthys olivaceus*) were investigated. Phagocyte activating activity of the supernatants was estimated by measuring reactive oxygen species (ROS) production in target head kidney phagocytes. All supernatants from olive flounder head kidney leucocytes-stimulated with *E. coli* DNA induced significantly ($P<0.01$) higher ROS production from target pagocytes than the unstimulated control supernatant. Maximum enhancement of chemiluminescent response was observed 5.0~10.0 $\mu g$ $mL^{-1}$ of bacterial DNA while the increment ability was decreased significantly ($P<0.01$) at the concentration of 20.0 $\mu g$ $mL^{-1}$. The results demonstrate that olive flounder head-kidney leucocytes stimulated with bacterial DNA release a soluble phagocyte activating cytokines capable of enhancing the respiratory burst activity from target phagocytes.

Key words: Bacterial DNA, Phagocyte activating factor, Olive flounder, Respiratory burst

## Introduction

The vertebrate immune system recognizes bacterial DNA as a danger signal on the basis of recognition of so-called CpG motifs, unmethylated cytidine-guanosine dinucleotides within a specific pattern of flanking bases (Krieg et al., 1995; 2000). CpG dinucleotides are underrepresented (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; 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).

In mammals, CpG motifs in bacterial DNA and synthetic oligodeoxynucleotides (sODNs) promote B cell proliferation, Ig production (Krieg et al., 1995) and the secretion of various cytokines, such as IL-1, IL-6, IL-12, IFNs from macrophages and NK cells (Stacey et al., 1996; Chase et al., 1997; Lipford et al., 1997).

To date, there is limited information concerning biological effects of bacterial DNA 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$\beta$ in Atlantic salmon and rainbow trout leucocytes. Oumouna et al. (2002) demonstrated activation of nonspecific cytotoxic cells of catfish (*Ictalurus punctatus*) with sODNs and bacterial genomic DNA.

In the present study, we evaluated *in vitro* effects of *Escherichia coli* genomic DNA on phagocyte activating supernatants production from olive flounder head kidney leucocytes. The activating activity was

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assessed by measuring reactive oxygen species (ROS) production in target head kidney phagocytes.

## Materials and Methods

### Fish

Olive flounder (*Paralichthys olivaceus*) weighing 150~200 g were obtained from a local fish farm in Korea. Fish were allowed to acclimatize for 2 weeks at 20℃ in a 500 L fiberglass aquarium.

### Bacterial DNA preparation

Bacterial DNA was prepared from late log phase cultures of *E. coli* as described by Birren et al. (1997). The DNA pellet was washed in icecold 70% ethanol, dried in air and suspended in Hank's balanced salt solution (HBSS; Sigma Chemical Co., St Louis, MO, USA).

### Production of phagocyte activating supernatants

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). Cell suspensions were obtained by forcing the organ through a nylon mesh and layered over a 51% Percoll density gradient (Sigma). After centrifugation at 400 g for 30 min at 4℃, the leucocyte fraction was removed from the Percoll-medium interface, washed 3 times, counted and adjusted to $5\times10^6$ live cells $mL^{-1}$. Tissue culture flasks (25 $cm^2$) were then seeded with 5 mL of the leucocytes suspension, and the cells pulsed for 3 h at 20℃ with 1.25, 2.5, 5.0, 10.0 and 20.0 μg $mL^{-1}$ of *E. coli* DNA. After then, the cells were gently washed three times with phosphate buffered-saline (0.15 M, pH 7.2) to remove any residual bacterial DNA, and then cultured in L-15 medium containing 10% FCS, penicillin (100 μg/mL) and streptomycin (100 U/mL) for 48 h. The supernatants were then harvested, centrifuged and stored at −70℃ until use. A supernatant collected from leucocytes that had not been pulsed with bacterial DNA but were cultured for the appropriate times was used as a control.

### Phagocyte activating activity in supernatants

The phagocyte activating activity in supernatants was evaluated by measuring their ability to increase target phagocyte ROS production. Phagocytes were isolated as described by Secombes (1990). Head kidney leucocyte suspensions obtained as described above, were enriched for phagocytes using a 34~51% Percoll density gradient. After centrifugation at 400 g for 30 min at 4℃, the phagocyte enriched interphase was collected and washed three times. Then, phagocytes were resuspended in culture medium, and dispensed into wells of flat-bottomed 96-well plates. After 2 h at 20℃, cells were washed with culture medium, removing unattached cells. The remained phagocytes were detached from the plates by incubating for 1 h at 4℃, and adjusted to $1\times10^6$ cells $mL^{-1}$ with culture medium.

The supernatants were diluted 1:4 or 1:8 in culture medium, and were added to phagocyte monolayers in culture plates for 12 h or 24 h at 20℃.

The ROS production of phagocytes was measured by luminol-enhanced chemiluminescent response. After incubation with the supernatants, phagocytes were detached from the plates and washed twice with HBSS. The 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 phagocyte suspension, and 0.3 mL zymosan, which was added just prior to measurement. The measurements were made for 100 min and the light emission was recorded as mV. The assay was carried out in triplicate.

### Statistical analysis

The statistical significance was evaluated using the Mann-Whitney's *U*-test of significance, and $P<0.05$ was considered statistically significant.

## Results

All supernatants from olive flounder head kidney leucocytes stimulated with *E. coli* DNA induced significantly ($P<0.01$) higher ROS production of target phagocytes compared to the unstimulated control supernatant (Fig. 1). Head kidney phagocytes

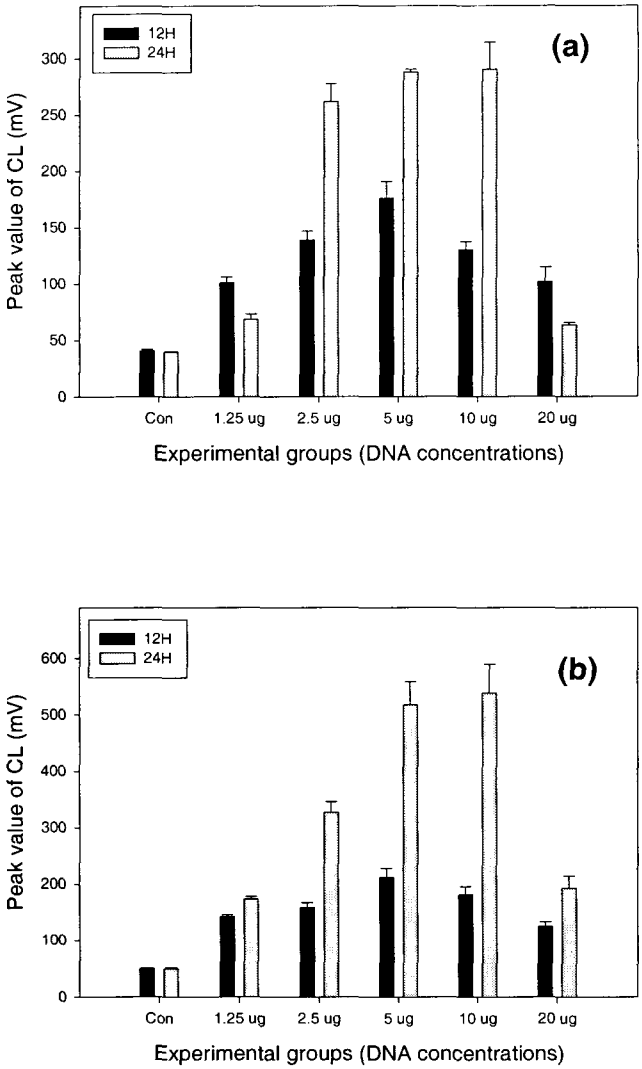


**Fig. 1. Peak chemiluminescent response (CL) by target phagocytes after incubation for 12 or 24 h with 1:8 dilution (a) or 1:4 dilution (b) of supernatants prepared from *E. coli* genomic DNA (0, 1.25, 2.5, 5.0, 10.0 and 20.0 $\mu$g mL$^{-1}$) stimulated kidney leucocytes of olive flounder, *P. olivaceus*. Results are means of triplicate samples and bars represent standard deviations.**

stimulated with a 1/4 dilution of the supernatant and incubated for 24 h showed higher CL increases than those stimulated with a 1/8 dilution of the supernatant and incubated for 12 h, respectively. Maximum enhancement of CL was observed at 5.0~10.0 $\mu$g mL$^{-1}$ of bacterial DNA while the increment ability was decreased significantly ($P<0.01$) at 20.0 $\mu$g mL$^{-1}$ concentration.

## Discussion

The present results demonstrate that olive flounder head-kidney leucocytes stimulated with bacterial DNA release a soluble phagocyte-activating factor (s) able to enhance the respiratory burst activity of target phagocytes. This is the first report of reactive oxygen species (ROS) production of fish phagocytes stimulated by bacterial DNA induced-supernatant. Several reports have shown that macrophage activating factors (MAF) secreted by rainbow trout T-cells induce respiratory burst and microbicidal activity in trout macrophages (Mosmann et al., 1986; Secombes, 1987; Chung and Secombes, 1987). To date, the molecular characteristics of MAF produced by mitogen-stimulated leucocytes remain to be elucidated, but it has been suggested that the molecule may be similar to mammalian IFNγ (Secombes, 1987). IFNγ, a lymphokine secreted by TH1 cells in mammals, is known to induce reactive oxygen species and nitrogen intermediates in mammalian macrophages (Nathan et al., 1983; Murray, 1988). Jørgensen et al. (2001b) reported that CpG ODNs induced IL-1$\beta$ expression and production of interferon-like cytokines in rainbow trout head-kidney macrophages. Therefore, the increased CL responses of olive flounder head-kidney phagocytes by stimulation with the supernatant in this study suggest that cytokines are induced from olive flounder leucocytes by *E. coli* DNA. Higher CL increases by a low dilution (1/4) of supernatant and a long stimulation (24 h) indicate that respiratory burst activity of the supernatant-induced phagocytes is dose- and time-dependent.

In the present study, magnitude of CL response increment of phagocytes stimulated with the supernatant induced by high bacterial DNA concentration (20 $\mu$g mL$^{-1}$) was significantly lower than those of macrophages stimulated with the supernatant induced by lower concentrations (5~10 $\mu$g mL$^{-1}$). 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$^{-1}$ than that at 3.0 $\mu$g mL$^{-1}$. Moreover, it has been demonstrated that MAF supernatants often show inhibitory effects on macrophage respiratory burst activity, possibly due to the pre-

sence of suppressive factors (Graham and Secombes, 1990a, b; Mulero and Meseguer, 1998). As shortage of knowledge on the fish cytokines relative to mammals, the exact causes of the decrease of phagocyte activating ability at a high bacterial DNA concentration in the present results could not known, and this inhibitory mechanism should be investigated further.

Activated phagocytes have the ability to kill virulent microorganisms (Graham & Secombes, 1988) and it is thus conceivable that the induction of phagocyte activating supernatant production by bacterial DNA contributes to stimulation of protective mechanisms against invading pathogens.

## Acknowledgement

This study was supported in part by a grant from NRL project.

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