J. Fish. Sci. Tech., 7(2), 70-75, 2004

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY

Survey of the Expression Pattern and Immuno Stimulatory Effect of DNA Vaccine Using β-Galactosidase Reporter System in Olive Flounder (*Paralichthys olivaceus*)

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The CMV promoter driven *lacZ* reporter gene (pcDNA-lacZ) was constructed and used for DNA immunization study. The expression of the *lacZ* gene was confirmed *in vitro* using RTG-2 cell line before using for *in vivo* study in olive flounder (*Paralichthys olivaceus*). In the dose response study, the maximum expression of the *lacZ* gene was found in the group injected with 5 μg of the plasmid DNA. Kinetic study showed a significantly increased expression of β-galactosidase gene at 7 days after injection. Effects of DNA vaccine on specific and nonspecific immune responses such as antibody and NO production were studied and the significant effect was found in olive flounder injected with 10 and 15 μg DNA (sub optimal dose for *lacZ* gene expression). Two proinflammatory cytokine genes, IL-1β and TNF-α, were also found to be up regulated in the muscle injected with the plasmid, suggesting an induction of local inflammatory response.

Key words: LacZ, *Paralichthys olivaceus*, Nitric oxide, Antibody, IL-1β, TNF-α, DNA vaccine

Introduction

DNA based vaccination could circumvent several problems associated with traditional methods of immunization. Thus many studies have recently been done to test efficacy of DNA introduction in fish using reporter genes. They have shown strong expression of reporter genes, such as luciferase, β-galactosidase, and chloramphenicol acetyltransferase (CAT) genes in muscle cells of fish injected with plasmid DNA (Hansen et al., 1991; Rahman and Maclean, 1992; Anderson et al., 1996; Gomez-Chiarri et al., 1996). However, most of studies in fish have been focused on fresh water fish while rarely studied in marine fish including olive flounder.

Olive flounder (*Paralichthys olivaceus*) is one of the major species in Korean aquaculture industry. At present disease control is one of the major concerns in olive flounder culture as well as other fish species, but there is no vaccine available for the prevention of viral and parasitic diseases in olive flounder. Tucker et al. (2001) have demonstrated the expression of green fluorescent protein (GFP) and CAT gene in olive flounder following DNA bombardment. However, there is still insufficient information on the exogenous gene expression in olive flounder yet since they have only focused on the duration of expression and the pathological effect in the injected site.

We studied to provide information on the expression of the plasmid encoding heterogeneous gene in olive flounder. For this purpose we used the CMV promoter driven *lacZ* reporter gene that expresses *Esherichia coli* β-galactosidase, which is widely used as a model for DNA immunization studies in mice and fish (Kanellos et al., 1999a,b). The β-galactosidase expression from the *lacZ* reporter gene can be easily analyzed by assay kit and antibody of high titre can be readily detected by ELISA in serum. The *lacZ* reporter gene expression has already been examined in some freshwater fish including gold fish (Russel et al., 1998, Kanellos et al., 1999a,b), rainbow trout,

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and zebra fish (Heppell et al., 1998), but not in olive flounder.

In addition to the expression of the *lacZ* gene encoding plasmid, we also examined the effect on nonspecific immune response (e.g nitrogen species production) and cytokine gene expression (e.g. interleukin-1, tumor necrosis factor α) as well as antibody production to test the availability of DNA vaccination in olive flounder. DNA vaccines are known to trigger both nonspecific and specific immune mechanisms (via the expression of antigen) in other fish species (Hepplle and Davis, 2000).

Materials and Methods

DNA construct

A plasmid encoding β-galactosidase (*lacZ*) gene was constructed for this study. The *lacZ* gene was obtained from the pSV-β-galactosidase vector (Promega) with the restriction endonucleases of *Kpn*I and *Eco*RI, and inserted into a eukaryotic expression vector (pcDNA3, Invitrogen) at the downstream of the CMV promoter to create a new recombinant plasmid called pcDNA-lacZ. The constructed plasmid was multiplied in *E. coli* strain DH5α cells grown in Luria-Bertani (LB) media, and plasmid DNA was purified with anion-exchange chromatography columns (Plasmid Midi-Kit, Qiagen).

In vitro expression of the constructed plasmid

The pcDNA-lacZ plasmid was transfected into rainbow trout gonad (RTG)-2 cells in a 24 well cell culture plate using the Effectene transfection reagent (Gibco) according to manufacture's instruction. After incubation with the DNA-Effectene complexes, the complexes were removed and 350 μL of fresh growth medium was added. The transfected cells were cultured for 72 h and examined for β-galactosidase expression using a commercial assay kit (Promega). Briefly, cultured cells were lysed and incubated with the equal volume of 2X assay buffer for 3 h at 37℃. The β-galactosidase activity was measured at 420 nm.

Plasmid injection

Olive flounder (30-50 g) were anaesthetized with MS-222 (3-aminobenzoic acid ethyl ester, Sigma) and intramuscularly injected with plasmid DNA diluted in sterile D.W. to a depth of 0.5 cm on the back below the dorsal fin using a 0.5 cc insulin syringe. The amount of DNA per fish varied according to the different treatment. For the study of dose response, 0, 0.1, 1, 5, 15 or 30 μg of pcDNA-lacZ was injected in a volume of 20 μL and the injected fish were maintained for a week before being sacrificed. For kinetics of expression, 10 μg of pcDNA-lacZ DNA was injected in 20 pL and the fish were sacrificed at 0, 1, 3, 7 days after injection. To study the effect on antibody production, olive flounder were injected with 0, 0.1, 1, 10 or 30 μg of pcDNA-lacZ and bled after 3 weeks.

Detection of the β-galactosidase gene expression

Anaesthetized olive flounder (30-50 g) were killed and dissected into injected muscle (i.e. a piece of muscle tissue approximately 0.4 cm^3 including the injected site). Samples were put in 1.5 mL microtubes with 500 μL of chilled cell lysis reagent (Reagent Lysis Buffer; RLB, Promega) and homogenized using micropestles (Kontes Co.). Homogenized tissues were further lysed by 3 cycles of frozen and thaw processes and centrifuged at 15,000×g for 15 min at 4℃. The supernatant was stored at -70℃ until used for the enzymatic assay. β-galactosidase activity was examined using a β-galactosidase assay kit (Promega). Briefly, 50 μL of the supernatants were mixed with *o*-nitrophenyl-β-galactopyranoside (ONPG) to 1:1 ratio, triplicate in a 96-well plate. After 3 h incubation at 37℃, β-galactosidase activity was measured at 420 nm.

ELISA for antibody to β-galactosidase protein

A sandwich ELISA was used to detect antibody titre in serum from olive flounder injected with plasmid. Briefly, β-galactosidase (Sigma) was coated on a 96 well plate at 20 μg/mL and reacted with two fold dilutions of fish sera at 1:50 to 1:12,800 overnight at 4℃. The bound antibody was detected using the biotin labeled β-galactosidase (Sigma) at 5 μg/mL for 2 h and Extraavidin-horseradish peroxidase (Sigma Co.) at 1:1,000 dilutions for 1 h at room temperature. The reaction was visualized by applying 0.05 M phosphate-citrate (pH 5.0) substrate solution (Sigma) containing urea hydrogen peroxide and 2.2'-Azino-bis(3-Ethylbenzen-thiazoline-6-Sulfonic acid) for 45 min at room temperature and the optical density (O.D.) was determined at 405 nm using a plate reader (Tecan Co.).

Effect on nonspecific immune response

Effect of pcDNA-lacZ DNA injection on nonspecific immune response was assessed by measuring

the production of the reactive nitrogen intermediate, nitrite (NO_2^-), using a Griess reaction assay (Green et al., 1982). Head kidneys from 4 fish were pooled and macrophage cells were isolated. The attached macrophage cells on a 96-well plate were stimulated with 100 μL of Poly I:C (0.1 μg/mL) for 48 h at 18℃. One hundred μL of supernatant was transferred into a new well of 96-well plate, and equal volume of Greiss reagent (Sigma) was added. After 10 min incubation at room temperature, the O.D. was measured at 540 nm.

RT-PCR analysis

Proinflammatory cytokine gene (IL-1β and TNF-α) expression following plasmid DNA injection was observed in the injected muscle site by RT-PCR analysis. Total RNA was extracted from muscle using TRIzol reagent (Invitrogen) and reverse transcribed using reverse transcriptase, with the resultant cDNA dissolved in DEPC-treated water and stored at 20℃. Specific primer sets were designed (IL-1β: 5'-CTCT CCACTGACTACCACAG and TCAGCTGAACTG ATCTGGTG-3', TNF-1α: 5'-ATGGTGAAATACA CAAGTGCA and TCAAAGTGCAAAGACACCG AA-3') based on the nucleotide sequences of the IL-1 β and TNF-α gene of olive flounder (Gene Bank Accession numbers: AB070835 and AB040448, respectively). The PCR was run for 25 cycles and the cycling conditions were 95℃ for 45 sec, 58℃ for 45 sec and 72℃ for 1 min, with the denaturation step prolonged to 5 min in the first cycle and the DNA synthesis step to 10 min in the last cycle. PCR reactions were performed in 25 μL reactions containing 5 μL of cDNA (diluted in water), 1.25 μL (25 pmol) of each primer, 2.5 μL of 10X reaction buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, 50 mM $MgCl_2$, 0.1% Tween-20, pH 8.8, Takara), 2 μL dNTP mixture (2.5 mM for each base, Takara) and 0.125 μL (0.625 U) of Ex*Taq* polymerase (Takara), using a thermocycler (Perkin-Elmer). PCR products were visualised on a 2% agarose gel containing 0.1 μg/mL ethidium bromide in TAE (Tris-Acetic acid-EDTA) buffer.

Statistical analysis

Kruskal-Wallis test was performed to know whether there are differences between samples and followed by Mann-Whitney comparison test to find where the difference exists ($P<0.05$).

Results and Discussion

CMV promoter driven *lacZ* gene coding vector was constructed to study exogenous gene expression in olive flounder and the expression was confirmed *in vitro* before using for *in vivo* study. RTG-2 cells were used for the transfection since olive flounder cell line is not available at the present. The constructed vector (pcDNA-lacZ) was successfully expressed in the transfected RTG-2 cell line (Fig. 1), suggesting that the construct was fully operated.

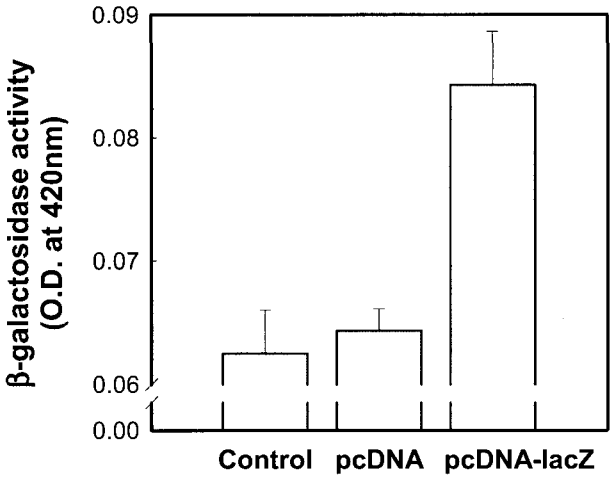

Fig. 1. β-galactosidase expression in RTG-2 cells transfected with pcDNA-lacZ vector or pcDNA vector as a negative control. pcDNA-lacZ vector was conjugated with the effectene reagent and transfected into RTG-2 cells. The transfected cells were lysed after 72 h and assayed for β-galactosidase expression. β-galactosidase activity was measured at optical density (O.D.) 420 nm. *Significantly higher than control analyzed by Mann-Whitney test ($p<0.05$).

Therefore, the pcDNA-lacZ plasmid was used to determine the optimal dose of DNA that induces the highest expression of the injected gene in olive flounder. The lacZ gene expression in the injected site of muscle increased in a dose dependent manner as doses increased from 0.1 to 5 μg. Injection of the greater amount of DNA (15, 30 μg) did not significantly increase activity further (Fig. 2). This is in agreement with the report of Heppell et al. (1998) where demonstrated a dose dependent increase of luciferase expression in trout and zebra fish injected with 0.1-1 μg of plasmid and no further increment of activity at DNA amount greater than 10 μg.

Kinetic study showed that β-galactosidase activity in the injected muscle was significantly increased up to 7 days after injection (Fig. 3). In a study counting

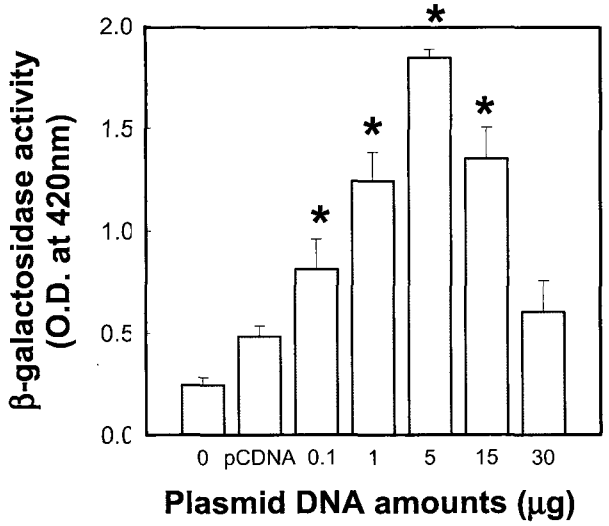

Fig. 2. Dose response of β-galactosidase expression in olive flounder injected intramuscularly with different doses of pcDNA-lacZ plasmid (0.1-30 μg). Enzymatic activity was measured at day 7 post-injection. Bars represent the mean O.D. at 420 nm (n=4) and T bars represent S.E. of the mean. *Significantly higher than control analyzed by Mann-Whitney test ($p<0.05$)

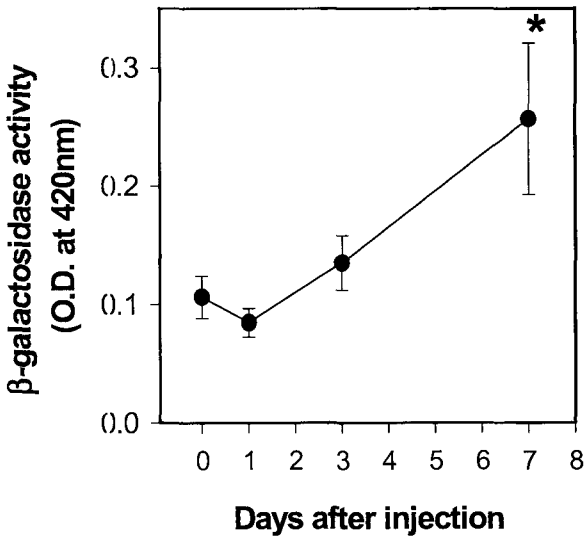

Fig. 3. Kinetics of β-galactosidase expression in muscles of olive flounder injected intramuscularly with 10 μg pcDNA-lacZ plasmid. Enzymatic activity was measured at 0, 1, 3, and 7 days after injection. Bars represent the mean O.D. at 420 nm (n=4) and T bars represent S.E. of the mean. *Significantly higher than control analyzed by Mann-Whitney test ($p<0.05$)

β-gal positive muscle fibres at different times after intramuscular administration of pCMV-lacZ, numbers of β-gal positive muscle fibres started to increase from 7 days and became stable until 21 days (Kanellos et al., 1999b). However, peak activity of another reporter gene, luciferase, when injected into fish muscle varied upon fish species or researchers. For example, maximum expression of luciferase gene was found at 7 days in rainbow trout (Anderson et al., 1996) or 2.5 days in rainbow trout, *Xiphophorus* sp. and zebra fish (Heppell et al., 1998).

Most of DNA vaccine studies have focused on the effect of DNA vaccine on antibody production, but effects on non-specific immune response are little known. In this study, we tried to elucidate the effect of intramuscular injection of pcDNA-lacZ plasmid on the modulation of the nonspecific immune responses such as NO production in head kidney macrophages using Griess reagent. Even though statistically significant increase was not found in any group, the group injected with 15 μg of DNA showed much higher NO production than other groups (Fig. 4).

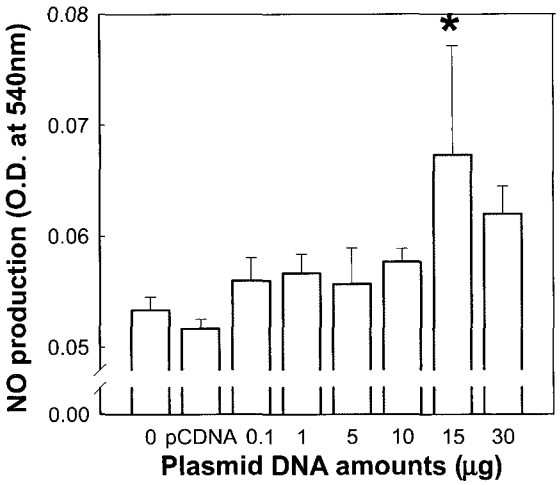

Fig. 4. Dose response of nitric oxide (NO) production in head kidney macrophage cells isolated from olive flounder injected with pcDNA-lacZ plasmid (0.1-30 μg) intramuscularly. The NO production was examined in 7 days after injection. Bars represent the mean O.D. at 540 nm (n=3) and T bars represent S.E. of the mean. *Significantly higher than control analyzed by Mann-Whitney test ($p<0.05$)

In addition to non-specific immune response, two proinflammatory cytokine (i.e. IL-1β and TNF-α) genes were also found to be up regulated in pcDNA -lacZ plasmid injected site (Fig. 5), suggesting an induction of local inflammatory response to initiate

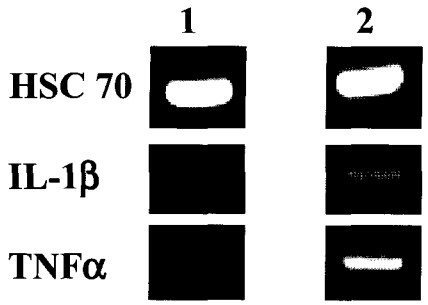

Fig. 5. Proinflammatory cytokine gene (IL-1β and TNF-α) expression was analyzed by RT-PCR method. Olive flounder was intramuscularly injected with 10 mg plasmid DNA (lane 1; pcDNA and lane 2; pcDNA -lacZ). Total RNA was isolated from injected site and transcribed into cDNA. The produced cDNA was titrated and normalized relative to the expression of the housekeeping gene, HSC70.

immune response. IL-1β and TNF-α are major pro- in response to the invasive stimuli such as bacterial, viral, fungal, parasitic or neoplastic agents. They induce many gene products involved in inflammation and immune responses (Sidhu et al., 1993).

The effect of DNA vaccination on specific immune response was also assessed in this study and significant antibody production was detected in fish injected with 10 or 30 μg of pcDNA-lacZ plasmid (Fig. 6). Maxi-

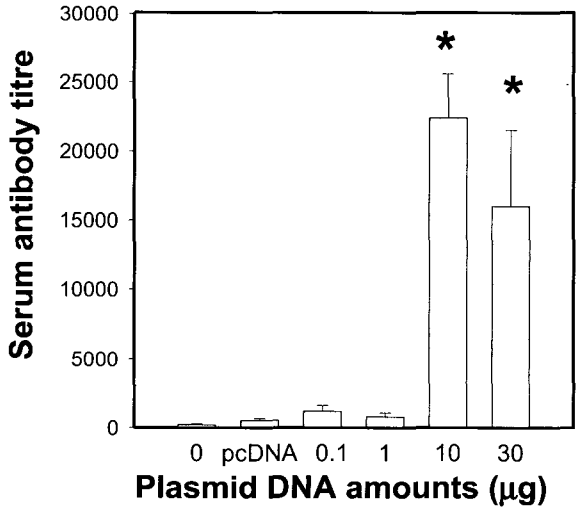

Fig. 6. Dose response of antibody production in fish sera injected with pcDNA-lacZ plasmid (0.1-30 μg) intramuscularly. The serum was collected 3 weeks after injection and antibody titer was determined by ELISA. Bars represent the mean serum titer (n=4) and T bars represent S.E. of the mean. *Significantly higher than control analyzed by Mann-Whitney test ($p<0.05$)

inflammatory cytokines produced by many cell types including lymphocytes, neutrophils, and astrocytes mum antibody production occurred in 10 μg treatment group, but higher amount of DNA than 10 μg did not induce further antibody production. This amount is much smaller than the other study in gold fish where a dose dependent increase of serum antibody titer after 1-125 μg of pCMV-lacZ plasmid injection has reported and the highest level was detected in 125 μg injected group (Kanellos et al., 1999b). This difference could be due to the difference in species. In mammals, the strength and longevity of antibody responses induced by DNA vaccination depend on the animal and antigen model (Davis et al., 1996; Donnelly et al., 1999).

In conclusion, the constructed pcDNA-lacZ plasmid was used as a model to study the effect of DNA vaccine in olive flounder. The plasmid was successfully expressed recombinant protein in olive flounder muscle, induced local inflammation by upregulating proinflammatory cytokine genes (IL-1β and TNF-α), and stimulated systemic nonspecific immune response within 1 week. This systemic and local early immune response could induce the specific immune response by producing high level of antibody against the recombinant protein. The optimal dose of the plasmid DNA seems to be 10-15 μg as a successful DNA vaccine in olive flounder.

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(Received October 2003, Accepted June 2004)