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Effect of Quaternary Ammonium Compounds(QACs) on the Immune Response of eel(*Anguilla japonica*) *In vitro*

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The immunostimulatory effects of quaternary ammonium compounds(QACs) were investigated in leucocytes of eel(*Anguilla japonica*) in vitro. Proliferation of peripheral blood lymphocytes(PBLs) was no significantly affected by QACs, regardless of mitogen(PHA, ConA and LPS) and the concentration of QACs added. QACs heightened the leucocytes function such as respiratory burst activity, phagocytosis and pinocytosis, resulting in significantly increased the bactericidal activity of macrophages. These results suggested that QAC might modulate the immune responses by activation of leucocytes function but not by increment of immunocompetent cell numbers.

Key words : QACs, Phagocytosis, Bactericidal, Lymphocyte, Immunostimulator

Introduction

In recent years, the intensive fish culture system is a highly stressful environment for fish. Under these condition, infectious disease of fish has increased(McClay *et al.*, 1977; Alderman 1990). To overcome this problem, large quantities of antibiotics as well as vaccination of individual fish have been employed(Aoki *et al.*, 1989; Miguel *et al.*, 1994; Saltai *et al.*, 1984). But these measures are costly, time consuming and may have serious side effects, not least of an ecological nature.

On this background, it is pertinent to look for other means to increase of nonspecific immunity in stressed fish against various fish disease. The immunostimulatants have an ability to increase resistance to disease by enhancing non specific defence mechanism, and many substances have been reported for fish, such as modified Freund's complete adjuvant(Oliver *et al.*, 1986), FK-565(Kitao and Yoshida, 1988), β-1, 3-glucans(Yano *et al.*, 1989), levamisole(Kajita *et al.*, 1990) and glycyrrhizin(Jang *et al.*, 1995) have been reported, and these substances may be expected to control fish diseases in fish culture.

QACs(quaternary ammonium compounds) was introduced as a disinfects that have also been used as an antiseptics to treat skin and gill infectious disease, such as bacterial gill disease. QACs act as surfactants, removing excess mucus that contains parasites and bacteria (Scott, 1993; Warren, 1981; Piper, 1982). QACs exerts a immunostimulating activity in rainbow trout(Jeney and Anderson, 1993). However, it is unclear how QACs affects on the immune effecter cells directly.

These experiment were conducted to evaluate the effect of QACs on the immune response such as the phagocytosis, pinocytosis and respiratory burst activity of kidney leucocytes, and proliferation of peripheral blood lymphocytes.

Materials and methods

Animals

Eel(*Anguilla japonica*) were obtained from a local fish farm. The mean weights were 150 g. Eel were acclimated in 50-liter, circular, flow-through tanks and maintained at 27°C and during the experiment the eels were not feed.

Bacterial preparations

E. tarda were plated on tryptic soy agar(TSA, Difco,) and an isolated colony from each culture was expanded in brain heart infusion broth(BHIB, Difco). Bacteria were cultured for 24 h at 27°C. Cells were collected, centrifuged at 800 g for 20 min and the pellets washed once in calcium-magnesium free Hank's balanced salt solution, pH 7.2(HBSS, Gibco).

Reagents

All reagent purchased from Sigma Co. A stock solution of 1 mg phobol myristate(PMA) and dimethylsulfoxide(DMSO)was prepared and stored in small aliquots at -20°C. Nitroblue tetrazolium (NBT) was suspended in HBSS and kept frozen at -20°C until use. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide(MTT) was suspended in sterile distilled water at a concentration of 5 mg MTT ml^{-1} and stored at -20°C, Quaternary ammonium compounds(QACs) was suspended in HBSS, Stock solutions of lipopolysaccharide(LPS), phytohaemagglutinin(PHA) and concanavalin A(ConA) were resuspended and diluted in RPMI 1640 medium without serum. All mitogens were then stored at -70°C until use.

Isolation of kidney leucocytes

Kidney leucocytes were isolated following the method previously described by Secombes(1990). Briefly, the anterior kidney was removed aseptically and scrapped the organ through a wire grid. After filtering out debris through a 100 μm nylon mesh using RPMI 1640(Gibco). The cell suspension was placed on a 34/51% percoll density gradient. The gradients were centrifuged at 500 g for 30 min at 4°C, The interface was harvested. Viable phagocytic cells were counted by trypan blue exclusion, and the cell number was adjusted to a concentration of 10^7 cells ml^{-1} with RPMI 1640 medium containing 5% fetal calf serum(FCS, Gibco). Nonadherent cells were removed by washing with RPMI 1640 medium.

Phagocytosis assays

phagocytosis assays were examined by the method of Sakai *et al.*(1989). The leucocytes were pretreated for 1 h at 27°C with various concentration of QACs(0.01, 0.05, 0.1, 0.5 and 1 μg ml) and washed with medium. Leucocytes suspensions (0.1 ml of 5×10^6 cells ml) were placed in 96 plate wells and incubated with *E. tarda*(0.1 ml of 5×10^8 cells ml) for 2 h on a shaker, at 27°C before a drop was placed on a slide. After drying, the smear was overlaid with Giemsa stain for 30 min. The results of phagocytosis assays are presented as a direct reading.

(percentage of cells with engulfed bacteria-phagocytosis ratio) and as an index(number of engulfed bacteria/phagocytic cell-phagocytic index).

PI = total number of bacteria engulfed by the 100 cells examined/100

Respiratory burst activity assay

Macrophage respiratory burst activity was assessed by monitoring their ability to reduce nitroblue tetrazolium(NBT, sigma) as a measure of superoxide anion release by the method of Rook *et al.*(1985). Briefly, One hundred ml aliquots of kidney macrophage(5×10^6 cells ml) in RPMI 1640 containing 5% FCS were dispensed into wells of flat-bottomed 96 well microtiter plates. Then cells were triggered with 100 μl of a mixture of 0.2% NBT(1 mg ml^{-1}) containing 1 μg ml^{-1} PMA and incubated for 1 h. After incubation, the NBT solution was removed, the cells were fixed with methanol, and reduced formazan was solublised by adding 120 μl of 2 M KOH and 140 μl of DMSO. The NBT reduction was measured using a multiscan spectrophotometer at 600 nm.

Pinocytosis assay

Pinocytosis of leucocytes were evaluated by uptake of neutral red dye according to the methods of Weeks *et al.*(1987). Duplicated 1 μl aliquots of leucocyte suspensions at a concentration of 2×10^6 cells ml^{-1} were added to the Ependorf tubes contain-

ing 20 μl of a 200 μg ml^{-1} neutral red solution of PBS(4 μg dye per tube) and allowed to incubate for 2 h at 27°C. After incubation, cells were centrifuged at 4200 g for 5 min, washed once in PBS, and resuspended in 500 μl of acid alcohol(3% HCl in 95% ethanol) to lyse the cells and release internalised dye. An equal volume of E-PBS was added and cells were resuspended. Cell debris was removed by centrifugation at 4200 g for 5 min and the absorbance at 533 nm recorded for the supernatant against a blank of PBS : acid alcohol(1 : 1). Standard curves were prepared from serial dilutions of neutral red at 9, 1.25, 2.5, 5, and 10 μg ml^{-1} in E-PBS : acid alcohol(1 : 1). Results are adjusted to report pinocytic activity as accumulation of neutral red dye in μg per 1×10^6 cells ml.

Bactericidal test

Bactericidal activity was assayed by using a plate count method to determine colony forming units(CFU). The leucocytes were pretreated for 1h at 27°C with various concentration of QACs(0.01, 0.05, 0.1, 0.5 and 1 μg ml) and washed with medium. Leucocytes suspensions(0.1 ml of 5×10^6 cells ml) were placed in 96 plate wells and incubated with *E. tarda*(0.1 ml of 5×10^8 cells ml) for 2 h on a shaker, at 27°C. The calculation of CFU was based on an effective concentration in phagocytic assays ranging from 1×10^7 cells ml^{-1}, at times 2 h a 20 ml sample was diluted in 80 μl of sterile diluted water to release engulfed but living bacteria and all adsorbed bacteria. The sample was rapidly diluted in HBSS to a final dilution of 5×10^4 cells ml^{-1}. Two tryptic soy agar(TSA) plates were each spread with 20 μl of the sample, incubated for 48h at 27°C and colonies counted. The number of bacteria surviving phagocytosis was calculated as follows: CFU/ml = $(2.5\times10^4)\times$ average number of CFU on two plates.

Peripheral blood preparation

Peripheral blood was prepared as described by Iida and Wakabayashi(1995). In brief, the blood was taken from the bulbous arterious with a heparinized syringe. The cells were then resuspended to twice the original volume with RPMI 1640(Gibco) and then carefully layered over the Ficoll-Paque(density 1.077, Pharmacia) and centrifuged at 450 g for 30 min at 4°C. The interface cells were collected and washed three times at 500 g for 5 min at 4°C in RPMI 1640 contaning 0.1% FCS. The viable cell determined by trypan blue dye exclusion test. The peripheral mononuclear cells(PMC) were resuspended in 50 ml tissue culture flask(Nunc)and incubated RPMI 1640 containing with 0.1% FCS for 3 h at 27°C. The non adherent cells were harvested and washed by centrifugation at 500 g for 10 min twice and monolayer were maintained with RPMI 1640 with 5% FCS.

Lymphocyte proliferation assay

Lymphocyte proliferation assay was performed by colorimetric MTT assay(Daly *et al.*, 1995). Briefly, after the appropriate time period of lymphocyte culture(38 hs) 20 μl of MTT(5 mg ml^{-1} HBSS) was added to each well and left to further incubate for 10h at 27°C. After this time, the tissue culture plates were centrifuged at 500 g for 10 min and then the supernatant was carefully removed without disturbing the cell pellet or formazan precipitate. The formazan crystals were dissolved by the addition to each well of 200 μl of DMSO followed by 25 μl of glycine buffer. The contents of the wells were then throughly mixed with a multichannel pipette. Ten minutes later, formazan development was read at 600 nm using the Titer Multiscan plate reader. Stimulation indices were calculated using the formula.

Mean optical density of lymphocyte wells with test mitogen at time x/mean optical density of lymphocyte wells with test mitogen at time x-1.

Results

Proliferation response

The proliferation activities of the peripheral blood lymphocytes were not observed in medium with

alone or in medium containing mitogen(LPS, PHA and Con A) with various concentrations of QACs(Fig. 1).

Phagocytic activities

The phagocytic activity was increased overall. The increased region was reached using concentrations of QACs between 0.01 and 0.1 μg ml^{-1}, depending on dose relationship(Fig. 2).

Pinocytosis of neutral red dye

Pinocytic activity of kidney leucocytes were increased in all QACs treated group(Fig 4.). Although there were no correlation to dose depen-

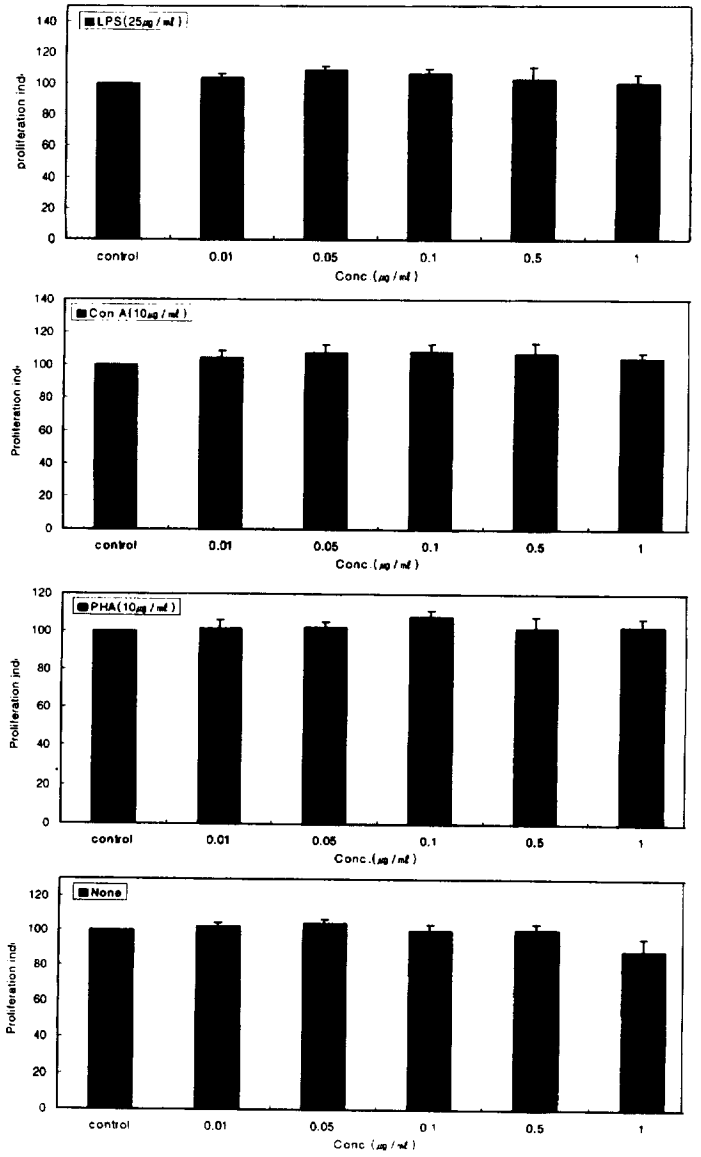

Fig. 1. The effect of QACs on proliferation of blood lymphocytes. blood lymphocytes were incubated in medium(control) or in medium containing mitogen(LPS, PHA and Con A) with various concentrations of QACs for 48 hrs. proliferation was measured by the MTT-method and expressed as $O.D._{600}$ per 10^6 lymphocytes. Results are means±S.D. of triplicate counting expressed as MTT reduction index compared to the control, respectively. Results are means±S.D. of triplicate readings expressed as a stimulation index(SI).

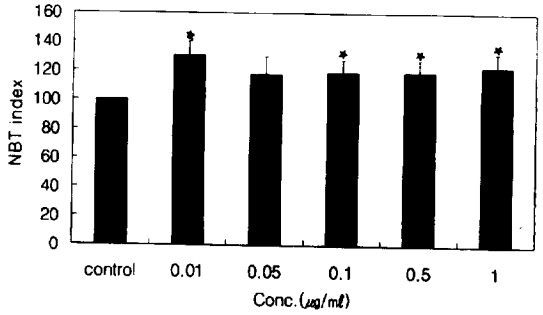

Fig. 2. The effect of QACs on the respiratory burst activity of kidney leucocytes. Leucocytes were incubated in medium with various concentrations of QACs for 1 hrs. O_2. production was measured by the NBT-method and expressed as $O.D._{620}$ per 106 macrophages. Results are means±S.D. of triplicate counting expressed as NBT reduction index compared to the control leucocytes, respectively.

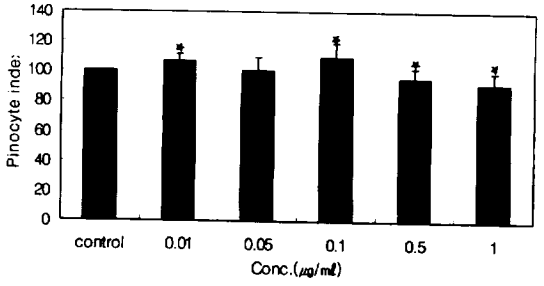

Fig. 3. The effect of QACs on the pinocytosis. Leucocytes were incubated in medium with various concentrations of QACs for 3 hrs. The pincytosis was measured by the colorimetric neutral red dye method and expressed as $O.D._{530} \times 10^6$ leucocytes. Results are adjusted to report pinocytic activity as accumulation of neutral red dye in μg per 1×10^6 cells. Results are means±S.D. of triplicate counting expressed as pinocytic index compared to the control leucocytes, respectively.

dent, A slight peak in pinocytic activity elicited on 0.01 and 0.1 μg ml^{-1} among QACs treated group.

Respiratory burst activity

Production of oxygen radicals by kidney leucocytes as measured by the NBT test was significantly increased in all QACs treated groups in compared control(Fig. 3). Especially, a significantly increased respiratory burst activity was manifested in 10 μg ml^{-1} concentration.

Bactericidal activity

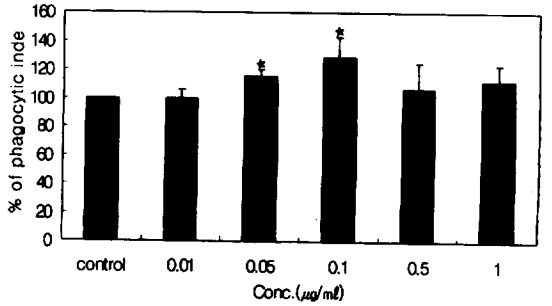

Fig. 4. The effect of QACs on the phagocytosis. leucocytes were pretreated for 1h at 27°C with various concentration of QACs before the addition of the bacteria. Leucocytes were incubated in medium with various concentrations of QACs for 3 hrs. The phagocytosis of bacteria was determined by counting 200 macrophages. Results are means±S.D. of triplicate counting expressed as phagocytic index compared to the control macrophages.

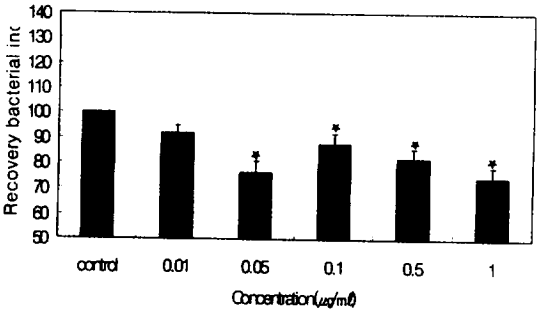

Fig. 5. The effect of QACs on bactericidal activity of blood leucocytes. leucocytes were pretreated for 1h at 27°C with various concentration of QACs before the addition of the bacteria. The bactericidal activity was assayed by using a plate count method to determine colony forming units(CFU). Two TSA plate were each spread with 20 μl of the sample, incubated for 48hrs at 27°C and colonies counted. Results are normalized to the values obtained from QACs free control group and are expressed as % of control.

Bactericidal activity of kidney leucocytes by using a plate count method to determine colony forming units(CFU) was significantly increased (Fig. 5). Generally, the enhancement of the bactericidal activity was dose dependent.

Discussion

Immunostimulants comprise a group of biological and synthetic compounds that enhance the nonspe-

cific defense mechanism in animals. The non specific defense mechanism are more important than the specific immune response. As the former include phagocytosis and the production of oxidative radicals, are quickly activated by the immunostimulants and are rapidly prepared to protect the fish against pathogen. However, the latter requires a longer time for antibody build up and specific celluar activation (Anderson *et al.*, 1992).

QACs act as surfactants, removing excess mucus that contains parasites and bacteria(Gudz and Pisko, 1988). It is a synthetic compounds which its physical and chemical properties are strictly characterized. Therefore, the biological activities of QACs are completely reproducible(Dorson and Michel, 1987; Aliev *et al.*, 1990). The mode of action of QAC in increasing the protective abilities of the fish is unclear.

In these report we have examined the effect of QAC on lymphocyte proliferation and on leucocytes function. We found that QACs did not affect on the proliferation, regardless of mitogen (PHA, ConA and LPS)and the concentration of the QACs added(Fig. 1). These results indicate that QACs does not increased the number of immune competent cells.

It is well known that phagocytic cells play an important role in protection against infectious disease(Secombs *et al.*, 1990; Solem *et al.*, 1995; Baibor *et al.*, 1973). In this study, the macropgage function such as respiratory burst activity, phagocytosis, and pinocytosis were augmented by QACs added(Figs. 2, 3, 4), although not in a dose dependent, resulting significantly increased the bactericidal activity(Fig. 5). These results suggested that QACs might promote the differentiation of premature cells into active mature cells. Jeney and Anderson(1993) also reported that the activation of blood phagocytes in rainbow trout was demon- strated by a significant increase of non-specific defence response such as NBT cells, phagocyic ratio and index and leucocytes counts when the fish bathed in QACs. Therefore, it is considered that fish phagocytic cells are activated by QACs.

In this study, we indicated that QACs might modulate the host immune responses by activation of the leucocytes function but not by increment of immuno-competant cell numbers. Further studies on how the oxygen-independent systems released from phagocytes of QACs participate in bacteri- cidal activity are necessary.

Acknowledgment

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*In vitro*에서 Quaternary Ammonium Compounds(QACs)이 뱀장어의 면역반응에 미치는 영향

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Quaternary ammonium compounds(QACs)가 뱀장어의 면역반응에 미치는 영향을 측정하기 위해서 뱀장어의 혈액으로부터 림프구를 분리하여 증식능을 측정하였으며 또한 신장으로부터 탐식세포를 분리하여 탐식능, pinocytosis 및 superoxide anion활성능 등을 측정하였다. 림프구의 증식능은 mytogen(T-cell mitogen: ConA and PHA, B-cell mitogen: LPS) 및 QACs처리농도에 따라 별다른 차이를 보이지 않았다. 한편 신장유래의 대식세포의 세균살해능은 QACs첨가농도에 따라 유의성 있게 증가되었다. 이러한 결과는 QACs이 작동대식세포로의 분화 및 활성을 촉진시켜서 탐식능, pinocytes 및 superoxide생성 등의 분비를 증가시킨 것으로 사료된다. 이상의 결과로 미루어 QACs가 어체의 면역반응에 관여하는 작용기전은 면역작동세포의 수를 증가시키기보다는 세포의 기능활성을 증강시킴을 알 수 있다.

Key words: QACs, Phagocytosis, Bactericidal, Lymphocyte, Immunostimulator