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Stimulatory Effects of Extracellular Products of *Mycobacterium* spp. and Various Adjuvants on Non-specific Immune Response of Nile Tilapia, *Oreochromis nilotica*

Sang Hoon Choi and Chan-Ho Oh[1]*

Department of Marine Biomedical Science, Kunsan National University, Kunsan 573-400, Korea;
[1]Division of Biotechnology and Environmental Engineering, Woo-Suk University, Chonju 565-701, Korea

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In the present paper, the immunostimulatory effects of the extracellular products (ECP) from *Mycobacterium* spp. and various adjuvants on the non-specific immune responses of Nile tilapia, *Oreochromis nilotica*, were examined. Nile tilapia were immunized by injecting ECP of *Mycobacterium* spp. (strain TB40, TB267 or the type strain *Mycobacterium marinum*) into their swim bladders. A variety of adjuvants like as Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA) and Titremax were similarly injected into additional groups of tilapia. The number of nitroblue tetrazolium (NBT)-positive cells observed in the swim bladder of the immunized fish was significantly increased by the fourth day post-immunization. By day 8, the numbers of NBT-positive cells were fewer in fish immunized with ECP from mycobacteria strains TB40 or TB267 than those immunized with ECP from *M. marinum* or fish injected with FCA or FIA. The level of lysozyme activity detected in the serum of fish 4 d after immunization with ECP from various *Mycobacterium* spp. was also significantly higher than that found in the serum of the control fish. Head kidney macrophages showed enhanced reduction of NBT when cultured *in vitro* with 1 μg/ml of ECP. Concentrations greater than this (10 or 100 μg/ml) were found to suppress the reduction of NBT by the macrophages. ECP from *Mycobacterium* spp. and the various adjuvants used in the study all appear to be good activators of the non-specific immune responses of Nile tilapia.

Fish rely on both specific and non-specific mechanisms to protect themselves against invading pathogens. The non-specific defenses include physical barriers, such as epithelial shield of scales, skin and mucus. Once a pathogen has managed to penetrate these initial barriers, chemical defenses such as serum lysozyme, lectins and complement components may coat the pathogen and opsonize them for further destruction (Fletcher, 1981).

Other components of non-specific immune system are also activated by invading stimuli (Ellis, 1981). For example, phagocytic cells play an important role in the defense mechanisms of the host by adhering to and engulfing invading particles. Such cells include tissue macrophages, circulatory monocytes and neutrophils. There are numerous reports of microbial products which stimulate the phagocytic activity of these cells. Such immunostimulants include Bacille Calmette-Guerin (BCG) (Sher, et al., 1993), *Corynebacterium parvum* (Berd, 1978) endotoxin (Dubos and Schaedler, 1959), glucan (Reynolds et al., 1980) *Nocardia rubra* cell wall skeleton (Masuno et al., 1979) and synthetic products such as muramyl dipeptide and its analogues (Parant et al., 1980; Fraser-smith et al., 1983; Saiki et al., 1983).

Cipriano and Pyle (1985) showed increased protection in eastern brook trout, *Salvelinus fontinalis* (Mitchell), after injection with Freund's incomplete adjuvant (FIA) and EcF1, a chromatographic fraction of *Aeromonas salmonicida*. Similarly, modified Freund's complete adjuvant (FCA) in combination with *Aeromonas salmonicida* formalin-killed bacterin increased protection in coho salmon, *Oncorhynchus kisutch* (Walbaum). Olivier et al. (1985) demonstrated that the adjuvant alone was able to protect coho salmon against *A. salmonicida* infection. Activation of macrophages by the adjuvant was believed to be responsible for this increase in disease resistance. Extracellular products (ECP) of *A. salmonicida* have also been reported to increase macrophage activity in the rainbow trout, *Oncorhynchus mykiss* (Walbaum), *in vitro* (Francis and Ellis, 1994).

*To whom correspondence should be addressed.
Tel: 82-63-290-1431, Fax: 82-63-63-9493
E-mail: choh@core.woosuk.ac.kr

Mycobacterium spp. is the causative agent of mycobacteriosis or fish tuberculosis, a chronic bacterial disease infecting a wide range of fresh water and marine fish (Frerichs, 1983). Presently, three pathogenic agents have been reported to cause the disease: *Mycobacterium marinum, M. fortuitum* and *M. chelonae.* They are acid-fast, aerobic, non-spore forming bacilli and their cell walls are particularly rich in complex lipids. Previous studies have shown that ECP are produced by mycobacteria cultured *in vitro* (Chen et al., 1997) and ECP from *Mycobacterium* spp. mixed with FIA resultes in elevation of the immune response of rainbow trout (Chen et al., 1996).

In this report, we examined the immunostimulatory effect of *Mycobacterium* ECP on the non-specific immune responses of Nile tilapia, *Oreochromis nilotica,* and compared the non-specific immune responses occurring between fish injected with a variety of adjuvants. Tilapia was chosen as a fish model since it is susceptible to mycobacteriosis (Noga, 1990). Stimulation of tilapia macrophages with *Mycobacterium* ECP *in vitro* was also performed.

Materials and Methods

Preparation of extracellular products (ECP)

Mycobacteria strains used to produce extracellular products are listed in Table 1. ECP was prepared from *Mycobacterium* spp. cultured in modified Sauton's medium as previously reported by Chen et al. (1996). Briefly, *Mycobacterium* spp. were cultured in modified Sauton's medium (MSM) which contained 0.05% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4.7H_2O$, 0.005% (w/v) ferric ammonium citrates, 0.2% (w/v) citric acid, 0.4% (w/v) sodium glutamate, and 3.0% (v:v) glycerol. A small volume (5 ml) of bacterial culture 500 ml of MSM was inoculated and cultured at 28℃ for 48 h. Bacteria were heat-killed in water bath at 75℃ for 15 min. The suspension was centrifuged at 7,000×g for 1 h at 4℃, and bacteria were removed by sequential filtration through a Whatman number 1 filter paper and then a Millipore filter (0.2 m). The supernatants were concentrated with polyethylene glycol (MW 8K; Sigma, Chemical) and samples were dialysed against phosphate-buffered saline (PBS; pH 7.2) overnight at 4℃. The supernatants were then centrifuged at 12,000×g for 1 h at 4℃, harvested, and stored at -70℃.

Fish

Nile tilapia, *Oreochromis nilotica,* (40-50 g) were obtained from a fish farm in Kunsan National University, Kunsan. They were held in 50 L tanks, supplied with aerated water at 25℃ and maintained on a commercial diet (Ewos No. 2). Seven groups of fish (eight fish per group) were placed in separate tanks. Groups 1-6 were immunized with either ECP from TB40, TB267 or *M. marinum* (50 μg/fish), or with Freund's complete adjuvant (FCA) (Sigma), Freund's incomplete adjuvant (FIA) (Sigma), or Titremax (Vaxcel™, Inc.), respectively. The seventh group injected with PBS was used as control. The fish were immunized by injecting 0.5 ml of the appropriate substance into their swim bladder (Endo et al., 1995).

Adherence/NBT assays

Blood samples were taken from the caudal vein of fish anaesthetized 4 and 8 d after immunization. At the same time, cells were harvested from the swim bladder by first disinfecting the ventral surface with 70% (v/v) ethanol, and then injecting 2 ml of cold Dulbecco's modified essential medium (DMEM) (Sigma) containing 10 IU heparin, 100 U penicillin (pen) and 100 μg streptomycin (strep) into the swim bladder. The cells were harvested with a 23 gauge needle. To stain the cells, a drop of blood or cell suspension was mixed with a drop of nitroblue tetrazolium (NBT) (Sigma) (0.2% (w/v) in saline) on a coverslip. This was then placed in a humidified chamber (a Petri dish containing a piece of moist paper towel) for 30 min at 22℃. The coverslip was gently washed with PBS. Excess PBS was removed and the coverslip was turned upside down onto a drop of the NBT solution on a microscope slide. Adherent cells stained in blue were counted in 10 fields of the microscope (X 400).

Lysozyme assay

Lysozyme activity was measured by a turbidimetric assay described by Parry et al. (1965). Lyophilized *Micrococcus lysodekticus* (0.2 mg/ml) in 0.04 M sodium phosphate buffer at pH 5.75 was used as a substrate for the serum lysozyme. Test serum (40 μl) was added to 3 ml of the bacterial suspension and reduction in absorbance at 540 nm was measured after 0.5 and 4.5 min at 22℃. One unit of lysozyme activity was defined

Table 1. Mycobacteria strains used to prepare extracellular products

Stain	Origin
Mycobacterium chelonae	NCIMB[a] 1474, tortoise, *Testudo* sp.
Mycobacterium fortuitum	NCIMB 1294, A.J. Ross/Kidney of jubenile chinook salmon, *Oncorhynchus tshawytscha* (Walbaum)
Mycobacterium marinum	NCIMB 1298, neon tetra, *Paracheirodon innesi* (Myers)
TB 1	AAHRI[b], Siamese fighting fish, *Betta splendens* (Regan)
TB 40	AAHRI, Siamese fighting fish, *Betta splendens*
TB 267	AAHRI, Snakehead fish, *Channa striata* (Bloch)
TB 268	AAHRI, Snakehead fish, *Channa striata*

[a]NCIMB, National Collection Of Industrial and Marine Bacteria, Scotland.
[b]AAHRI, Aquatic Animal Health Research Institute, Bankok, Thailand.

as reduction in absorbance of 0.001/min.

Reduction cells of NBT-positive reduction in tilapia head kidney macrophages after culture with mycobacteria ECP

Head kidney macrophages from non-vaccinated Nile tilapia were incubated with ECP of *Mycobacterium* spp. before assaying their ability to reduce NBT-positive cells by the method described by Secombes (20). Briefly, macrophages were isolated on 51%/34% Percoll gradients and monolayers macrophage were prepared. The cell number was adjusted to 1.0×10^7/ml in Leibovitz medium (L-15) (Sigma) containing 1% (v/v) penicillin/streptomycin (pen/strep) and 0.1% (v/v) fetal calf serum (FCS), and 100 µl/well was added to the wells of a 96-well tissue culture plate (Nunc). In order to obtain sufficient numbers of macrophages to carry out the test in one tines of assay, cells from six control fish were pooled. Macrophage monolayers were incubated at 22℃ for 2 h before washing the plate three times in L-15 medium to remove non-adherent cells. L-15 medium containing 1% (v/v) pen/strep and 5% (v/v) FCS was added to the wells (100 µl/well) and the plates were incubated at 22℃ overnight. On the following day, culture medium was removed from the macrophage monolayers and replaced with medium containing the additives described above, to which 1, 10 or 100 µg/ml ECP from the mycobacteria had been added. Diluted ECP was added to triplicate wells (100 µl/well).

After culturing the macrophages with the various ECP for 48 h at 22℃, the respiratory burst of the macrophage was assayed using reduction of NBT reaction (Secombes 1993). Culture media were removed from the wells and macrophage monolayers were incubated for 30 min at 22℃ with PBS containing phorbol myristate acetate (PMA) (1 mg/ml) (Sigma). The reaction was stopped by fixing the cells in methanol. After washing twice with 70% (v/v) methanol, the wells were air-dried and the resulting insoluble formazan was dissolved by adding 120 µl 2 M KOH and 140 µl dimethyl sulfoxide (Sigma). The contents of each well were carefully mixed and air bubbles removed with a needle. The absorbance was determined at 610 nm with a plate reader (Dynatech). The number of macrophages per well was assessed for two control wells. The number of nuclei released after incubating the cells with lysis buffer (0.1 M citric acid, 1% Tween 20 and 0.05% crystal violet) was determined using a hemacytometer. The results were adjusted to give an absorbance at 610 nm per 10^5 cells.

Statistics

The results are presented as mean±SE. Student's *t*-test was used to calculate *P* and data were considered significant at $P<0.05$ (*) and $P<0.001$ (**).

Results

NBT-staining of cells activated by ECP or adjuvants

To investigate the effect of ECP from the various *Mycobacterium* spp. and conventional adjuvants on a non-specific immune response of *Nile tilapia*, blood and swim bladder neutrophils were activated by the ECP of the various *Mycobacterium* spp. and the adjuvants followed by NBT-staining. Fig. 1 and 2 respectively show the NBT-positive cell numbers in the peripheral blood and the swim bladder of Nile tilapia previously sensitized by mycobacterial ECP or adjuvants. NBT-positive cells increased both in the blood and in the swim bladder of the fish 4 d after the administeration of the various ECP and adjuvants compared with control fish injected with PBS, with the exception of neutrophils from the blood of fish injected with FCA and FIA. By the eighth day, fewer NBT-positive cells were found in the blood of fish immunized with each kind of ECP or adjuvants than in the blood of normal fish. In the case of the swim bladder, mycobacteria strains TB40, TB267 and Titermax failed to activate the cells, while only FCA considerably stimulated the cells compared with any other groups including negative control.

Lysozyme activity of cells activated by ECP or adjuvants

It has been previously shown that serum lysozyme concentration in fish increases following exposure to a foreign material (Fletcher and White, 1973). Thus, we examined the level of serum lysozyme activity in the cells activated by ECP or adjuvants. The level of lysozyme activity 4 d after immunization was found to

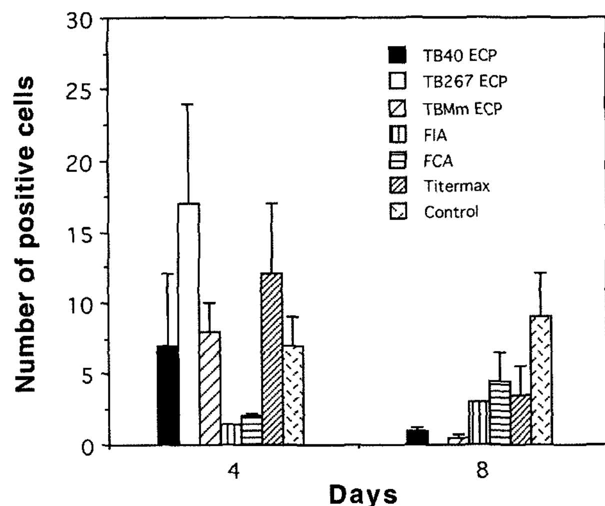

Fig. 1. Number of NBT-positive cells in the peripheral blood of Nile tilapia injected with ECP from mycobacteria or adjuvants into their swim bladders. Results are expressed as the mean number±SD for four fish. Control fish were injected with phosphate-buffered saline. ECP, extracellular products; Mm, *Mycobacterium marinum*; FIA, Freund's incomplete adjuvant; FCA, Freund's complete adjuvant; NBT, nitroblue tetrazolium.

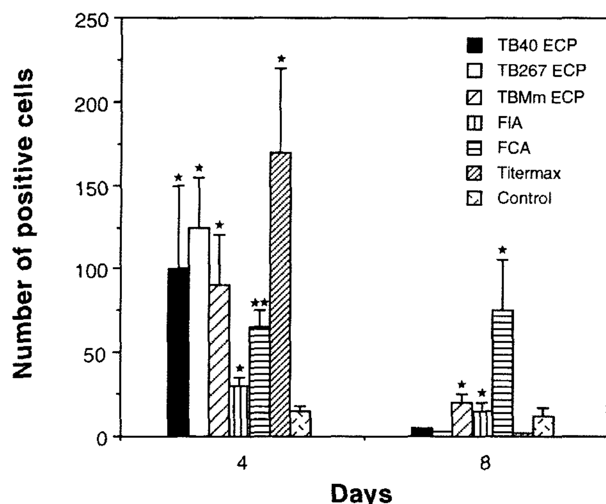

Fig. 2. Number of NBT-positive cells in the swim bladder of Nile tilapia injected with ECP from mycobacteria or adjuvants into their swim bladders. Results are expressed as the mean number + SD for four fish. Control fish were injected with phosphate-buffered saline. ECP, extracellular products; Mm, *Mycobacterium marinum*; FIA, Freund's incomplete adjuvant; FCA, Freund's complete adjuvant; NBT, nitroblue tetrazolium. *, $P< 0.05$; **, $P< 0.001$.

be significantly higher in the serum of fish immunized with ECP from the different mycobacteria or various adjuvants than control fish (205 *vs* 135 unit/ml) (Fig. 3). At day 8, the lysozyme activity of fish immunized with TB40 or FIA was still greater than in the control fish.

In vitro activation of cells with ECP or adjuvants

To investgate whether ECP or adjuvants can induce stimulation of macrophages from Nile tilapia *in vitro*, NBT reduction assay was performed. Fig. 4 shows the ability of ECP from a variety of mycobacteria and of various adjuvants to stimulate head kidney macrophages

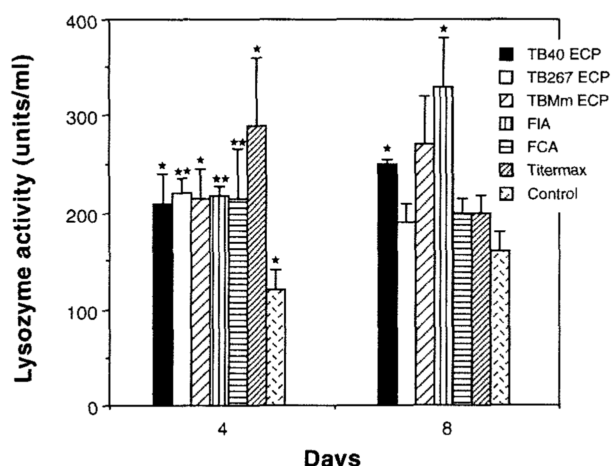

Fig. 3. Serum lysozyme activity of Nile tilapia injected with ECP from mycobacteria or adjuvants into their swim bladders. Results are expressed as the mean units/ml ± SD for four fish. Control fish were infected with phosphate-buffered saline. ECP, extracellular products; Mm, *Mycobacterium marinum*; FIA, Freund's incomplete adjuvant; FCA, Freund's complete adjuvant. *, $P< 0.05$; **, $P< 0.001$.

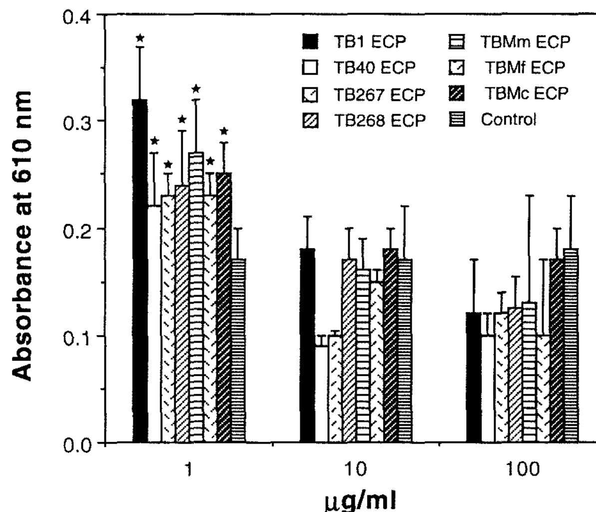

Fig. 4. NBT reduction by head kidney macrophages from Nile tilapia after culturing *in vitro* with ECP from an array of Mycobacterium spp. Results are expressed as the mean absorbance per 10^5 cells at 610 nm for triplicate samples + SD for four fish. ECP, extracellular products; NBT, nitroblue tetrazolium; Mm, *M. marinum*; Mf, *M. fortuitum*; Mc, *M. chelonae*. *, $P< 0.05$.

after culturing *in vitro* for 48 h. The reduction of NBT by the head kidney macrophages was used as a measure of cell activation. Supplementing ECP into the culture medium at 1 µg/ml resulted in a significant increase in NBT reduction by the macrophages. This was found to be the case with all mycobacteria strains examined. However, increasing the ECP concentration to 10 or 100 µg/ml resulted in an immunosuppressive effect on the macrophages (Fig. 4).

Discussion

The swim bladder was used in this study as a site for administering antigens (Endo et al., 1995) and the non-specific immune response to the antigens was subsequently examined. The injection, sited above the pelvic fin and midway between the pelvic fin and lateral line, allowed entry into the swim bladder without injury to the other visceral organs. Using this method, it was easier to collect exudative cells from the swim bladder than from the intraperitoneal cavity. Administration of antigen via the swim bladder has the advantage of allowing a precise dose to be delivered, reducing injury to other organs and allowing slow antigen absorption. This slow absorption however produced inflammation of the swim bladder wall. This was not due to the injection, but rather due to the ECP or the adjuvant, since the PBS control showed no signs of inflammation.

The non-specific immune response in fish is often reported to be a function of macrophage activity such as phagocytosis and chemotaxis (Weeks and Warinner, 1986). Neutrophil activity, measured in the present study, can also be an indicator of the non-specific response. These cells become more adherent to tissue

cell surfaces by the production of adhesion proteins, which facilitate their migration from the capillaries to the sites of injury (Kishimoto et al., 1989; Magnuson et al., 1989). They also exhibit increased production of oxygen radicals (O_2^-, OH^-) during the oxidative burst process. These reactive species are capable of destroying the invading pathogens (Hassett and Cohen, 1989). The ability of neutrophils to adhere to glass allows NBT to be used as a differential staining to measure the level of reactive oxygen species in the cytoplasm of cells. In the presence of oxygen radicals, the soluble dye changes from yellow to the insoluble dark blue of formazan (Anderson, et al., 1992).

In the present paper, the numbers of NBT-positive cells significantly increased in the blood and in the swim bladder 4 d after mycobacterial ECP injection. It is interesting to note that the numbers of NBT-positive cells in the blood of all immunized groups were lower than those of the control group at day 8. It may be that neutrophils migrated from the blood to the swim bladder in the immunized groups, thus reducing their numbers in the blood. Serum lysozyme activity detected 4 and 8 d after the immunization was also significantly increased compared with control fish. Chen et al. (1996) reported that a primary intraperitoneal (IP) vaccination of ECP from *Mycobacterium* spp. (strains TB40, TB267 or *Mycobacterium marinum*) mixed with FIA, followed by a secondary IP injection at 8 wk, resulted in elevation of both the non-specific and specific immune responses of rainbow trout. Increased NBT reduction and phagocytic activity were observed in these fish, with peaks in activity at weeks 2 and 6 post-primary immunization and with a third peak at week 10 post-primary immunization. Lysozyme activity, on the other hand, peaked at week 2 and again at week 8 post-primary immunization, with the exception of the TB40 immunized group. A third peak of lysozyme activity was observed at week 10 post-primary immunization. Type II lysozyme has been found to have a strong antibacterial effect in rainbow trout against four Gram-negative fish pathogens, *Vibrio anguillarum, V. salmonicida, A. salmonicida* and *Yersinia ruckeri* (Grinde, 1989). While the usual response to stress and handling of fish caused a decrease in lysozyme concentration, immunization with ECP from *Mycobacterium* spp. resulted in an increase in this activity.

Culturing macrophages *in vitro* with media supplemented with low levels of *Mycobacterium* ECP (1 μg/ml) enhanced the macrophages' ability to reduce NBT. Increasing these levels, however, had an immunosuppressive effect on the macrophages. The outer membrane proteins of *A. salmonicida* (1 μg/ml) have also been reported to produce a stimulatory effect on the respiratory burst of head kidney macrophages from Atlantic salmon, *Salmo salar* L., measured by NBT reduction (Francis and Ellis, 1994). Rainbow trout head kidney macrophages similarly exhibited an enhanced NBT reduction when cultured *in vitro* with media supplemented with 1 μg/ml of *Mycobacterium* ECP (data not shown).

Because of the sensitivity of the fish's immune system, immunological markers can be used to monitor the health of a fish population. Neutrophil activity can indicate the health status of fish. NBT activity is positively correlated with phagocytosis and killing activity of neutrophils and macrophages. Establishing the level of neutrophil activation by NBT staining after vaccination may help to verify the effectiveness of immunization (Anderson et al., 1992).

ECP from *Mycobacterium* spp. and the adjuvants used in the present study all appear to be good stimulators of the non-specific immune responses of Nile tilapia. Increases in both NBT reduction and lysozyme activity were induced when they were administered via the swim bladder. Mycobacteria are able to function as intracellular parasites, surviving within macrophages (Armstrong and Hart, 1971). Studies are continuing to investigate the intracellular survival of mycobacteria in the fish macrophages and the administration of antigen via the swim bladder.

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References

Anderson DP, Moritamo T, and Grooth R (1992) Neutrophil, glass-adherent, nitroblue tetrazolium assay gives early indication of immunization effectiveness in rainbow trout. *Vet Immunol Immunopathol* 30: 419-429.

Armstrong JA and Hart D'Arcy P (1971) Response of cultured macrophages to *Mycobacterium tuberculosis*, with observation on fusion of lysosomes with phagosomes. *J Exp Med* 134: 713-740.

Berd D (1978) Effects of *Corynebacterium parvum* on immunity. *Pharmacol Therap* 2A: 373-395.

Chen SC, Adams A, and Richards RH (1997) Extracellular products from *Mycobacterium* spp. in fish. *J Fish Dis* 20: 19-25.

Chen SC, Yoshida T, Adams A, Thompson KD, and Richards RH (1996) Immune response of rainbow trout, *Oncorhynchus mykiss*, to extracellular products of *Mycobacterium* spp. *J Aquat Anim Health* 8: 216-222.

Cipriano RC and Pyle SW (1985) Adjuvant-dependent immunity and the agglutinin response of fishes against *Aeromonas salmonicida*, the cause of furunculosis. *Can J Fish Aquat Sci* 42: 1290-1295.

Dubos R and Schaedler RW (1956) Reversible changes in the susceptibility of mice to bacterial infections. *J Exp Med* 104: 53-65.

Ellis AE (1981) Non-specific defense mechanism in fish and their role in disease processes. *Dev Biol Stand* 49: 337-352.

Endo M, Yoshida T, Sakai M, and Iida T (1995) Swim bladder as a site for administration of chemical agents: application to fish immunology. *Fish Shell Fish Immunol* 5: 85-88.

Fletcher TC (1981) The identification of non-specific humoral factors in the plaice (*Pleuronectes platessa* L.). *Dev Biol Stand* 49: 321-327.

Francis CH and Ellis AE (1994) Production of a lymphokine (macrophage activating factor) by salmon (*Salmo salar*) leukocytes stimulated with outer membrane protein antigens of *Aeromonas salmonicidas. Fish Shell Fish Immunol* 4: 489-497.

Fraser-Smith EB, Eppstein DA, Larsen MA, and Matthew TR (1983) Protective effect of muramyl dipeptide analog encapsulated in or mixed with liposomes against *Candia albicans* infection. *Infec Immun* 172-178.
Fletcher TC and White (1973) Lysozyme activity in the plaice (*Pleuronectes platessa* L.). *Experientia* 29: 1283-1285.
Frerichs GN (1983) Mycobacteriosis, nocardiosis. In: Inglis V, Roberts RJ, and Bromage NR (eds), Bacterial Diseases of Fish, Blackwell Scientific Publications, Oxford, pp 219-233.
Grinde B (1989) Lysozyme from rainbow trout, *Salmo gairdneri* Richardson, as an antibacterial agent against fish pathogens *J Fish Dis* 12: 95-104.
Hassett DJ and Cohen MS (1989) Bacterial adaptation to oxidative stress: implications for pathogenesis and interaction with phagocytic cells. *Fed Am Soc Exp Biol* 3: 2574-2581.
Kishimoto TK, Jutila MA, Berg EL, and Butcher EC (1985) Neutrophil MAC-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. *Science* 245: 1238-1241.
Magnuson DK, Weintraub A, Pohlman TH, and Maier RV (1989) Human endothelial cell adhesiveness for neutrophils, induced by *Escherichia coli* lipopolysaccharide *in vitro*, is inhibited by *Bacteroides fragilis* lipopolysaccharide. *J Immunol* 143: 3024-3033.
Masuno T, Ito M, Ogura T, Hirano F, Yamawaki M, Azuma I, and Yamamura Y (1979) Activation of peritoneal macrophage by oil-attached cell-wall skeleton of BCT and *Nocardia rubra*. *Gann* 70: 223-227.
Noga EJ, Wright JF, and Pasarell L (1990) Some unusual features of mycobacteriosis in the cichlid fish *Oreochromis mossambicus*. *J Comp Pathol* 102: 335-344.
Oliver G, Evelyn TPT, and Lallier R (1985) Immunity to *Aeromonas salmonicida* in coho salmon (*Onchorhynchus kitsutch*) induced by modified Freund's complete adjuvant: its non-specific nature and the probable role of macrophages in the phenomenon. *Dev Comp Immunol* 9: 419-432.
Parant M, Audibert F, Chedid L, Level M, Lefrancier P, Choay J, and Lederer E (1980) Immunostimulant activities of lipophilic muramyl dipeptide derivatives and of desmuramyl eptidolipid analogs Infect. *Immunity* 27: 826-831.
Parry RM, Chandan RC, and Shahani KM (1965) A rapid and sensitive assay of muramidase. *Proc Soc Exp Biol Med* 119: 384-386.
Reynolds JA, Kastello MD, Harrington DG, Crabbs CL, Peters CJ, Jemski JV, Scott GH, and Luzio RD (1980) Glucan-induced enhancement of host resistance to selected infectious diseases. *Infec Immun* 30: 51-57.
Saiki I, Tokushima Y, Nishimura Y, Nishimura K, Yamamura Y, and Azuma I (1983) Activation of macrophages by quinonyln-acetyl-muramyl dipeptide. *Infec Immun* 40: 622-628.
Secombs DJ (1983) Techniques in Fish Immunology. SOS Publications, USA, pp 137-152
Sher NA, Chaparas SD, Greenberg LE, and Bernard S (1975) Effect of BCG, *Corynebacterium parvum*, and methanol extraction residue in the reduction of mortality from *Staphylococcus aureus* and *Candida albicans* infection in immunosuppressed mice. *Infec Immun* 12: 1325-1330.
Weeks BA and Warinner JE (1986) Functional evaluation of macrophages in fish from a poluted estuary. *Vet Immunol Immunopathol* 12: 313-320.

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