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Abstract

The activation of head kidney phagocytes was studied in rainbow trout injected with an immunopotentiator muramyl dipeptide (MDP). Phagocytes showed dose-dependent migratory response to zymosan-treated rainbow trout serum in chemotaxis assay using Nuclepore membrane. Phagocytes of MDP-injected fish showed higher degree of chemotactic response than those from control fish. Phagocytic activity against hydrophilic plastic beads was enhanced when zymosan-treated trout serum was added to cultures. The activity increased after injection of MDP to trout. Increased level of superoxide generation was observed in phagocytes from MDP-injected fish compared with those from control fish when the cells were stimulated either with phorbol 12-myristate 13-acetate, *Aeromonas salmonicida* cell lysate or opsonized zymosan in cytochrome C reduction test.

Introduction

Various immunostimulants such as Freund's adjuvant[1,2], levamisole[3-5], FK565[6] and β-1, 3-glucans[7] have been investigated in fish for their ability to enhance non-specific host defense. In mammals, muramyl dipeptide (MDP), a constituent of bacterial cell wall peptidoglycan, enhances host resistance to infection, and the mechanisms of the defense provided by MDP are well elucidated. MDP activates macrophages and polymorphonuclear leukocytes both *in vivo*[8,9] and *in vitro*[10], and humoral factors (monokines) which stimulate lymphocytes are produced by MDP-activated macrophages[11]. The cells of monocyte-macrophage lineage and polymorphonuclear leukocytes are important as effectors and mediators in the cellular defense against invading microorganisms in fish[12-16]. Though resistance to infection in fish treated with MDP has been tested in a pilot trial[2], immunological effect of MDP on fish phagocytes is not known. The present study was designed to define non-specific activation of head kidney phagocytes by MDP by assessing chemotactic and phagocytic activities, and superoxide production.

Materials and Methods

Fish Rainbow trout weighing 100 to 150g were purchased from a commercial farm.

Fish were acclimatized to laboratory conditions for several weeks in 400l plastic aquaria which were filled with dechlorinated tap water (flow rate 60l/hr; water temperature 15°C) and aerated.

MDP *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-*N*-stearoyl-L-lysine [MDP-Lys (L18)] was kindly provided by Dr. A. Inoue (Daiichi Seiyaku Co., Tokyo, Japan). It was dissolved in phosphate buffered saline (PBS; 0.15M, pH7.2) at a concentration of 200 μg/0.5 ml. MDP was injected into fish intraperitoneally at a dose of 200 μg/100 g body weight. Fish were used for experiments 2 days after the MDP injection.

Separation of head kidney phagocytes Head kidney phagocytes were separated as described previously[17] by using continuous Percoll gradient density centrifugation[18].

Chemotaxis assay Chemotactic activity of head kidney phagocytes was examined using Blindwell Chambers (Nuclepore Co., Pleasanton, CA). Zymosan-treated trout serum was used as chemotactic agent[17]. Amount of 0.2 ml of zymosan-treated serum was diluted with Eagle's minimum essential medium (MEM; pH7.4, Nissui Seiyaku Co., Tokyo, Japan), and then added to the lower cell of the Blindwell Chamber. Nuclepore membranes (pore size 5 μm, Nuclepore Co.) were placed over the cells. After screwing the membrane retainer, 0.2 ml of phagocytic cell suspension from MDP-injected or PBS-injected control fish (adjusted to 5×10^5 viable cells/ml MEM containing 10% fetal calf serum (FCS)) was added to upper cell. Chemotactic chambers were incubated at 20°C for 90 min in a incubator with a 5% CO_2 and 95% humidified atmosphere. After the incubation, non-migrated cells on the top surface of the membrane were removed by washing with warm MEM. Membranes were then removed carefully, air dried, and fixed with methanol. Membranes were stained with Giemsa solution, and the number of cells which had migrated to the opposite side of the membrane were counted with a microscope ($\times$400). Ten fields were randomly screened using an eyepiece grid (totally 0.625 mm^2).

Phagocytosis assay Tissue culture plates (24-well plate, Corning, Corning, NY) containing a 15 mm round cover glasses in each well were seeded with 1×10^6 phagocytes/0.5 ml FCS-free MEM. Cells were incubated at 20°C for 4 h. Non-adherent cells were removed by washing cells gently with MEM twice, and adherent cells were cultured for 18h in MEM containing 10% FCS. After cultivation, cells were washed with MEM once, and zymosan-treated rainbow trout serum[17] (final concentrations of 2.5%) was added. Hydrophilic microspheres (2 μm diameter, Lumispheres, Toray Techno Co., Shiga, Japan) were added to each well (microsphere concentration of 1×10^7 in 25 μl of medium) and the plates were further incubated at 20°C for appropriate time. To stop the phagocytosis, 10 μl of 100 mM NaN_3 was added. The cover glasses were washed with PBS and were stained with Fuchsin by dipping into 0.01% Fuchsin solution (Katayama Chemical Industries Co., Osaka, Japan). The extent of phagocytosis was assessed by light microscope ($\times$400). Extracellular Lumispheres were stained with Fuchsin, but beads ingested in cells were not stained. More than 200 cells were assessed, and the number of cells phagocytizing Lumispheres was counted. The phagocytic activity was estimated as the percentage of cells those ingested more than 6 Lumisphere beads.

Assay for superoxide production Phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO) was suspended at a concentration of 20 μg/ml in dimethylsulfoxide and stored at -80°C until use. A cell lysate of *Aeromonas salmonicida* (Y8309-5 strain) and opsonized zymosan were prepared as described previously[17]). The stock solutions were diluted in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-saline (150 mM NaCl, 5 mM HEPES, 1 mM $CaCl_2$, 2 mM glucose; pH7.4) for use. Head kidney phagocytes were suspended in HEPES-saline at a concentration of 10^6/ml in microcell

cuvettes. They were added 5μl of cytochrome C (Sigma, type V) to make final concentration of 80 μM, and were incubated at 20°C for 5 min. PMA (final concentration of 100 *n*g/ml), opsonized zymosan (50 μg/ml) or cell lysate of *A. salmonicida* (23 μg/ml) was added to cuvettes, and increase of optical density (OD) was measured in a spectrophotometer (Hitachi, U-3210, Tokyo, Japan) at a wavelength of 550 nm.

Statistical analysis Data were analyzed statistically by the Student's *t*-test.

Results

Chemotaxis Relation between chemotactic activities of head kidney phagocytes and concentration of zymosan-treated trout serum was examined. Chemotaxis was dose-dependent to the serum added to lower cells, showing the highest activity at concentration of 6.25% (Fig. 1 and 2). Phagocytes collected from MDP-injected fish showed higher chemotactic activity than those from PBS-injected fish ($p<0.05$ to <0.001 at serum concentrations of 0.10 to 12.5%). Migrated cells were determined as macrophages or polymorphonuclear leukocytes by morphological observation under microscope (Fig. 2).

Phagocytosis Phagocytic activities of phagocytes from MDP-injected and PBS-injected fish are shown in Figure 3. Phagocytosis of Lumisphere beads was enhanced in both phagocyte groups by addition of zymosan-treated serum to cultures. Phagocytes from MDP-injected fish showed higher phagocytic activity than those of control fish with or without zymosan-treated serum ($p<0.01$ and <0.05, respectively at 6 h).

Superoxide production When phagocytes were stimulated by either PMA, opsonized zymosan or *A. salmonicida* sonicate, reduction of cytochrome C occurred, showing

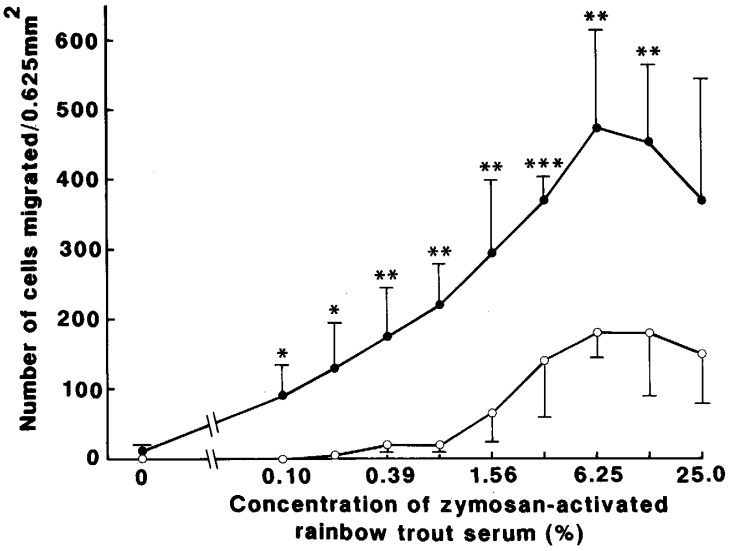

Fig. 1 Effect of zymosan-treated trout serum on chemotaxis of head kidney phagocytes. Chemotaxis of phagocytes from fish 2 days after injection of MDP (●——●) was compared with that of control fish (○——○) against different concentrations of the serum. Each symbol and vertical line represents mean number and SE from 5 paired experiments. $p<0.001$ (***), <0.01 (**) and <0.05 (*).

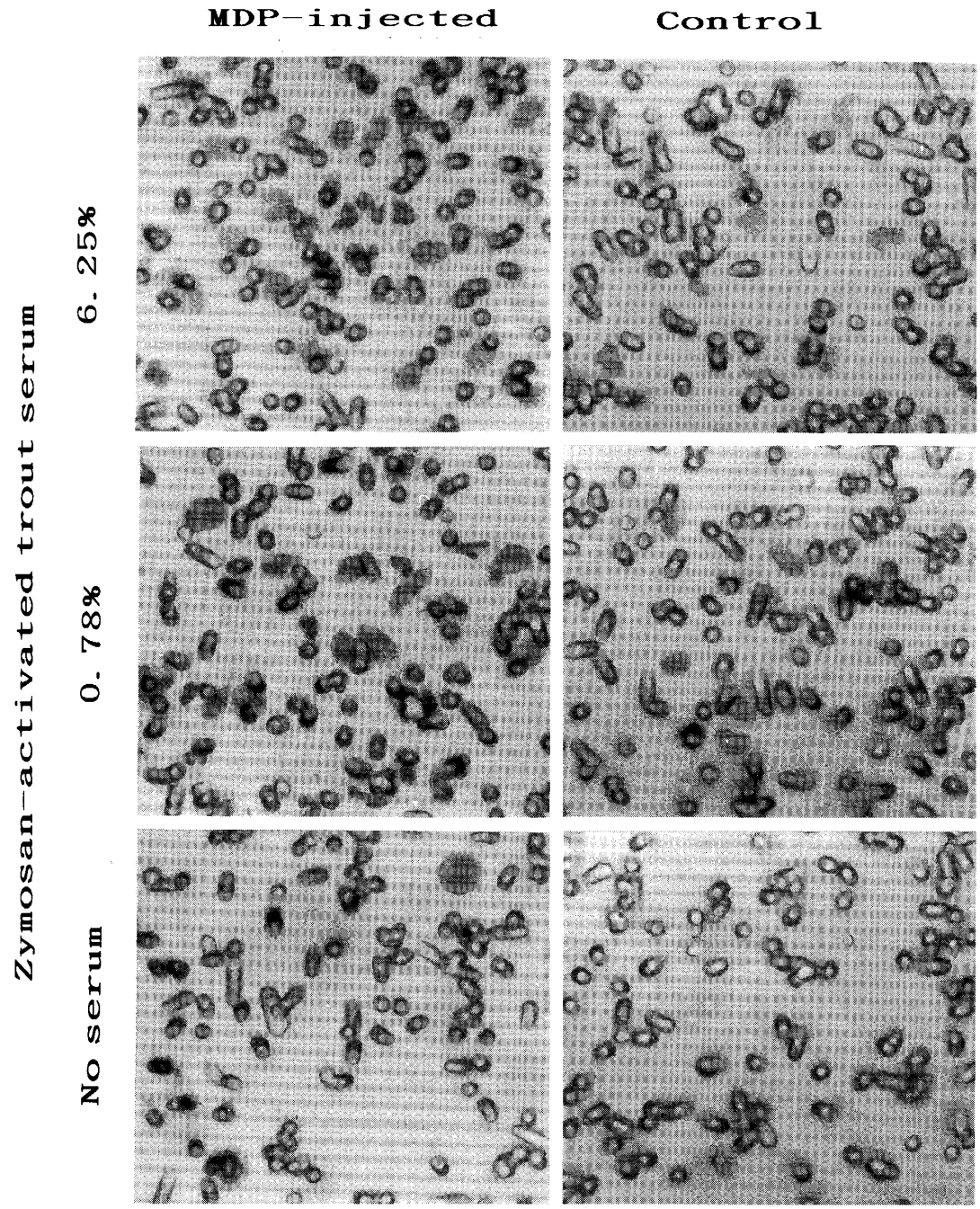

Fig. 2 Migration of rainbow trout phagocytes across Nuclepore membranes. The effect of MDP injection and concentrations of zymosan-treated serum were examined. (×500).

production of superoxide (Fig. 4). In each stimulation, phagocytes from MDP-injected fish showed higher production of superoxide than those from control fish.

Discussion

The present study demonstrated that intraperitoneally injected MDP significantly increased the activities of head kidney phagocytes of rainbow trout. Activation of fish phagocytes through recognition of bacterial cell wall antigen such as MDP play an

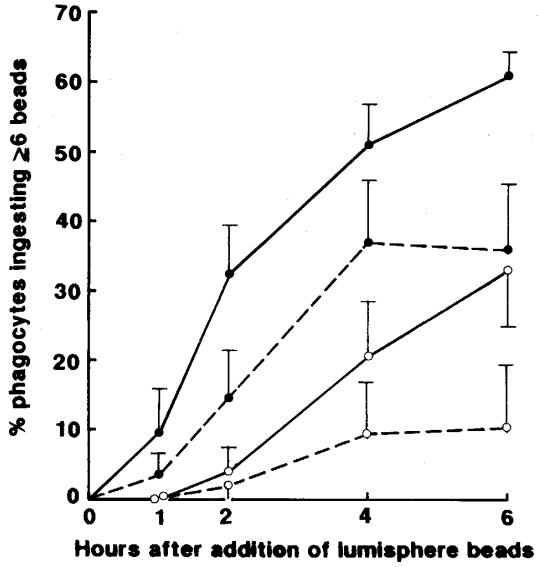

Fig. 3 Time course study of the phagocytosis of Lumisphere beads by phagocytes from MDP-injected (●——●; in the presence of 2.5% zymosan-treated serum, or ○——○; absence of the serum) and control (●·····●; presence or ○·····○; absence of serum) fish. Each symbol and vertical line represents mean percentage of phagocytes ingested more than 6 beads and SD from 6 paired experiments.

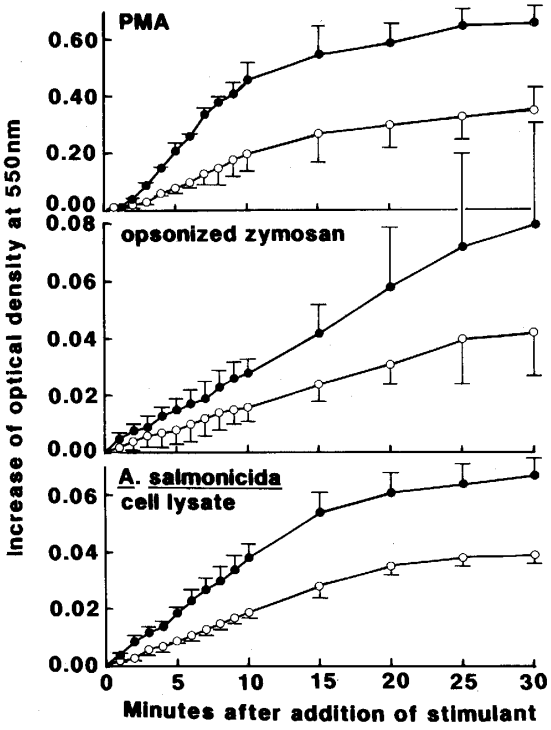

Fig. 4 Production of superoxide in head kidney phagocytes measured by cytochrome C reduction test. Phagocytes from MDP-injected (●——●) or control (○——○) fish were stimulated either with PMA, opsonized zymosan or *A. salmonicida* sonicate. Each symbol and vertical line represents mean value and SD from 5 paired experiments.

important role in the non-specific defense especially in an early stage of bacterial infection, since infiltration of phagocytes into areas of infection followed by phagocytosis and intracellular killing of bacteria are crucial steps of the defense.

Though chemotactic or chemokinetic migration of fish phagocytes has been reported[19–22], the relationship between the increase of migratory activity and the injection of immunopotentiator such as MDP has never been investigated. Migration of phagocytes was dose-dependent of the concentration of zymosan-treated serum present in lower cells of chemotaxis chambers (Figs. 1 and 2). Checkerboard titration has been shown that the migration is due to chemotactic reaction[17]. Phagocytes from MDP-injected fish showed enhanced chemotactic activity compared to those from control fish,

indicating that MDP activated phagocytes and the activated cells accumulated actively in the site of infection or inflammation *in vivo*.

In the phagocytosis assay of glass-adherent cells against hydrophilic Lumisphere beads, zymosan-treated rainbow trout serum greatly enhanced *in vitro* phagocytosis (Fig. 3). Phagocytes from MDP-injected fish showed higher degree of phagocytosis than those from control fish. Although opsonically active fragment of complement such as C3 was consumed by treatment with various polysaccharide[7], other complement fragments generated in zymosan-treated serum might act for enhancing phagocytosis observed in the present study. Fresh rainbow trout serum added to cultures did not show significant opsonic activity (data not shown), indicating that Lumisphere by itself did not activate complement system efficiently.

Head kidney phagocytes generated superoxide when they were stimulated with particulate and soluble stimulants (Fig. 4). Injection of MDP in rainbow trout resulted in the enhancement of superoxide production in phagocytes. Oxidative burst (production of superoxide and hydrogen peroxide) is initiated by both particles (*e. g.* bacteria, zymosan) and soluble (PMA) agents[23], and the products contribute to oxygen-dependent killing of target[24]. It has been reported that different stimuli, priming and triggering are required to cause efficient oxidative burst[25]. In the present study, fish phagocytes exposed to MDP are activated to elicit the chemotaxis and antigen recognition ("priming"). The oxidative burst cycle may be "triggered" effectively when thse "primed" phagocytes are exposed to membrane stimulant (PMA, *A. salmonicida* lysate or zymosan) and therefore phagocytes from MDP-injected fish produced larger amount of superoxide than normal phagocytes.

The mechanisms of phagocyte activation and production of phagocyte-activating factor by MDP, and the role of MDP-activated phagocytes in resistance to bacterial infection are described elsewhere[17].

Acknowledgements

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