The Immunomodulatory Effects of Levamisole on Rainbow Trout, *Oncorhynchus mykiss*

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The immunomodulatory effects of levamisole were investigated using rainbow trout, *Oncorhynchus mykiss*. Fish sampled 5 days after intraperitoneal injection of levamisole showed increased protection against *Vibrio anguillarum* when a single dose (0.5 mg/kg) was used. Phagocytic activity, chemiluminescence responses and natural killer cell activity in fish treated with levamisole were also increased, compared with those in the saline treated fish. Furthermore, levamisole activated the alternative complement pathway of rainbow trout, although serum bactericidal activities against *V. anguillarum* were not enhanced by the treatment of levamisole. These results indicate that levamisole has immunomodulatory effects in rainbow trout.

The intensive fish culture system is a highly stressful environment for fish. This stress in fish suppresses the immune responses, and fish kept under these conditions become highly susceptible to fish diseases. Vaccination is a very useful method to increase resistance to disease, but it elicits only specific immunity to one disease. Thus, the increase of nonspecific immunity in stressed fish is important for resistance against many fish diseases.

The immunostimulants have an ability to increase resistance to disease by enhancing nonspecific defense mechanisms, and many substances have been reported for mammals (CHIHARA, 1980). In fish, the immunostimulant effects of substances such as modified FREUND's complete adjuvant (containing *Mycobacterium butyricum*) (OLIVIER *et al.*, 1985, 1986), a lactoyl tetrapeptide (FK-565) isolated from *Streptomyces olivaceogriseus* cultures (KITAO and YOSHIDA, 1986) and β -1, 3-glucans (YANO *et al.*, 1989) have been reported, and these substances may be expected to control fish diseases in fish culture.

Levamisole was introduced in 1965 as an anthelmintic for the treatment of nematode infections in man and a variety of animals. Incidental observations suggested a state of enhanced resistance to various kinds of infection in animals that had received such treatment. Animal studies

also suggested a stimulatory effect on tumor immunity.

In this study, the immunostimulant effects of levamisole were examined in rainbow trout infected with vibriosis experimentally, and the elicitation of nonspecific defense mechanism by levamisole was investigated.

Materials and Methods

Fish

Rainbow trout were supplied by the Iwate Prefectural Inland-Water Fisheries Station, Matsuo, Iwate. The mean weights were 10 g and 50 g. The 50 g rainbow trout were used in immunological assays and the 10 g trout in the experimental infection with vibriosis, respectively. These fish were kept at 14°C and were fed daily with commercial dry pellets.

Bacteria

Vibrio anguillarum (strain PT479), a gram-negative fish pathogenic bacterium, was used in the infection experiment. This strain was cultured at 25°C for 2 days on Tryptosoya agar (TSA) (Nissui) with 1% NaCl.

Administration of levamisole

Levamisole (Takeda) was suspended in saline

and injected into rainbow trout at a dose of 0.1 mg to 5 mg per kg of body weight intraperitoneally. Control fish received an equal volume of saline by the intraperitoneal injection. The fish were kept at 14°C with food supplied, and each experiment was carried out 5 days after injection. In the preliminary experiments, the optimal stimulatory dose of levamisole was determined to be 0.5 mg/kg by using the chemiluminescent response of kidney leukocytes. In this study, a single dose of 0.5 mg levamisole/kg was used for experiments.

LD₅₀ determination

Trout injected with levamisole or saline were challenged by intraperitoneal injection ranging from 10 to 10⁷ cells/fish with a 10-fold stepwise increase of viable *V. anguillarum* cells suspended in saline. Six individuals were used in each challenge group. After the challenge, fish were maintained at 14°C and dead fish were recorded daily for 14 days. All the moribund fish were necropsied and kidney materials were inoculated on TSA with 1% NaCl.

Serum bactericidal activity

Serum bactericidal activity was determined by the modified method of IIDA and WAKABAYASHI (1983). The pooled sera from five fish injected with levamisole or saline were diluted 3 times with 0.1% gelatin-veronal buffer (GVB²⁺) (pH 7.5, containing 0.5 mmol/ml Mg²⁺ and 0.15 mmol/ml Ca²⁺) and then mixed with *V. anguillarum* (1 × 10⁶ CFU/ml) suspended in the same buffer at a 1:1 mixing ratio. The mixtures were incubated and shaken for 90 min at 20°C, and the number of viable bacteria was calculated by counting the colonies on TSA with 1% NaCl.

Complement assay

The serum complement activity was determined by assaying the alternative complement activity (ACH₅₀) (YANO *et al.*, 1988). The pooled sera from five fish injected with the levamisole or saline were incubated with rabbit red blood cells (RaRBC) (4 × 10⁷ cells) in 10 mm EGTA (ethylene glycol-bis tetraacetic acid)-10 mm MgCl₂-GVB²⁺ (pH 7.0) at 20°C for 90 min. The hemolytic activity was measured at O. D. 414 nm by means of an EIA reader (Bio-Rad) and ACH₅₀ was

determined.

Phagocytosis assay

Five individual fish were used as an experimental or a control group. Phagocytosis assays were examined by the method of SAKAI *et al.* (1989). Cells were harvested from the fish kidney and minced in RPMI 1640 (Nissui). Cell suspension was centrifuged at 500 × *g* for 5 min and washed with the medium. Viable phagocytic cells, including neutrophils and macrophages, were counted by trypan blue exclusion, and the cell number was adjusted to a concentration of 10⁷ cells/ml with RPMI 1640 medium containing 5% fetal bovine serum (FBS) (Gibco). Phagocytic cells were stuck onto a coverslide for 1 h and loose cells were removed by washing with RPMI 1960 medium. The 10⁸ latex particles (0.81 μm, Difco), suspended in RPMI 1640 medium (5% FBS), were added to this coverslide and incubated for 2 h at 20°C. The coverslide was fixed with methanol and stained with GIESSA. The number of phagocytizing cells per 300 cells was counted microscopically and the statistical difference was determined by STUDENT's *t*-test.

Chemiluminescence (CL) assay

Kidney cells were prepared by the method described in the phagocytosis assay, and the cell concentration was adjusted to 10⁷ cells in MEM medium without phenol red (Nissui) containing 5 mm HEPES (Wako). 20 mg zymosan A (Sigma) was mixed rainbow trout sera of each group, and reacted at 20°C for 30 min. The sera was removed by centrifugation (800 × *g*) and zymosan A was suspended in 5 mm HEPES-MEM medium. Luminol (Katayama) was used to enhance CL response. CL assay was examined according to the modified method of MORITOMO *et al.* (1988). CL activity was measured with 1250 Luminometer (Wallac) for 30 min at room temperature. In the primary experiment, individual variations were observed, and the kidneys from the individual fish of each group were pooled and CL activities were tested.

Natural killer (NK) cell assay

In this experiment, three individual fish from each group were used and leukocytes were obtained from the kidney, spleen and peripheral blood

as effector cells. Leukocytes in each organ were collected by the method described by CLELAND and SONSTEGARD (1987) and viable leukocytes were counted by trypan blue dye exclusion. A mouse cell line (P815) (H-2b mouse mastocytoma) was used as a target cell line for assaying killer cell activity. The cell line was cultured with RPMI 1640 medium with 10% FBS.

The same volumes of the target cells (5×10^4 cells/ml) and the individual effector cells (5×10^6 cells/ml), suspended in RPMI 1640, were added to culture tubes and reacted for 6 h at 20°C. The percent cytotoxicity was determined by counting dead P815 cells with trypan blue dye exclusion.

% Cytotoxicity =

$$\frac{[(\text{Number of P815 killed by leukocytes} - \text{number of spontaneously dying P815 cells}) / (\text{Number of total P815 cells} - \text{Number of spontaneously dying P815 cells})] \times 100}{}$$

Results

LD₅₀ against V. anguillarum

LD₅₀ values of rainbow trout treated with levamisole or saline against *V. anguillarum* are shown in Table 1. The LD₅₀ for fish treated with levamisole was about 50 times higher than for saline-treated fish.

Serum bactericidal activities against V. anguillarum

The serum bactericidal activities of fish treated with levamisole or saline are indicated in Table

Table 1. LD₅₀ of *Vibrio anguillarum* PT479 in rainbow trout with levamisole

Group	LD ₅₀ (CFU/fish)
Levamisole	$2.3 \times 10^{6.17}$
Control	$2.3 \times 10^{4.5}$

Table 2. Bactericidal activities against *Vibrio anguillarum* PT479 in serum of rainbow trout injected with levamisole

Group	Number of bacterial cells (CFU)
Levamisole	1.1×10^5
Control	1.9×10^5

Table 3. ACH₅₀ values in serum of rainbow trout injected with levamisole

Group	ACH ₅₀ (units/ml)
Levamisole	565 ± 21
Control	354 ± 50

Table 4. Phagocytic activities against latex particles in kidney phagocytic cells of rainbow trout injected with levamisole

Group	Phagocytic activity (%) (n=5)	Significance
Levamisole	37.85 ± 4.40	$p < 0.005$
Control	19.64 ± 2.59	

2. The serum bactericidal activities against *V. anguillarum* were not enhanced in the sera of fish treated with levamisole.

ACH₅₀

The ACH₅₀ value of levamisole-treated fish was 565 ± 21 , and that of saline-treated fish was 354 ± 50 (Table 3). The statistical difference of hemolytic activity is demonstrated by the ACH₅₀ values observed with experimental fish and control fish.

Phagocytic activities

The phagocytic activities of kidney cells were 37.85 ± 4.40 for levamisole-treated fish, and 19.65 ± 2.59 for saline-treated fish (Table 4). The kidney phagocytic cells from fish injected with levamisole showed the increased phagocytic activity ($P < 0.005$).

CL response

The mean maximum level of the CL response of phagocytic cells in experimental fish reached 208 mV (Fig 1). This value was about 2 times the CL response of cells from saline treated fish.

NK cell assay

The NK cell activities of kidney and peripheral blood leukocytes significantly increased in rainbow trout treated with levamisole (Table 5). However, in splenic leukocytes of fish treated with

Fig. 1. Chemiluminescent (CL) response in kidney leucocytes of rainbow trout injected with levamisole. 0.1 mg/kg (1), 0.5 mg/kg (2), 1 mg/kg (3), 5 mg/kg (4), Control (5).

Table 5. Natural killer cell activities against P815 cells in kidney, spleen, or peripheral blood leukocytes of rainbow trout injected with levamisole

Lymphocyte	Group	Natural killer cell activity (%) (n=3)	Significance
Kidney	Levamisole	28.80±4.15	$P<0.005$
	Control	22.25±1.25	
Spleen	Levamisole	18.80±3.48	N. S. ¹⁾
	Control	19.89±1.96	
Peripheral blood	Levamisole	20.73±4.20	$P<0.005$
	Control	13.67±2.32	

N. S.¹⁾; No Significance

levamisole, the enhancement of the activity was not observed. The statistical differences between treated and control groups were demonstrated by the STUDENT *t*-test ($P<0.005$).

Discussion

Levamisole in mammals enhances the metabolic and phagocytic activity of neutrophils, and increases the number of phagocytic cells and

leukocytes and the level of lysozyme in serum (SYMOENS and ROSENTHAL, 1977). In fish, the same effects have already been reported by SIWICKI (1987, 1989). He described that carp injected with levamisole at doses of 5 and 10 mg/kg body weight intraperitoneally showed enhanced phagocytic activity and myeloperoxidase activity in neutrophils, and increased leukocyte numbers and serum lysozyme levels. These immunomodulatory effects lasted for 3 months.

In this study, the LD₅₀ of rainbow trout injected with levamisole against *V. anguillarum* was 50 times higher than of saline-injected fish. This result suggests the possibility of application of levamisole to the control of the disease. OLIVIER *et al.* (1985) also reported that coho salmon injected with levamisole showed the enhanced protection against *Aeromonas salmonicida*. This enhanced protection must be examined in the other fish diseases, including viral diseases.

It is well known that phagocytic cells play an important role in protection against infectious fish diseases (ELLIS *et al.*, 1976, GRIFFIN, 1983). KODAMA *et al.* (1989) reported that the activation of macrophages in carp was demonstrated by a significant increase of the CL response and phagocytic activity when the fish was immunized with *V. anguillarum*. Our results suggest that the phagocytic activities and CL response of phagocytic cells are significantly increased in rainbow trout injected with levamisole. Therefore, it is considered that fish phagocytic cells are activated by levamisole.

Recently, the importance of NK cell in tumor immunity has been indicated. NK activity is increased by agents such as BCG and *Corynebacterium parvum* which stimulate macrophages (ROITT 1984). NK activity also significantly increased in rainbow trout injected with levamisole. Although the function of NK in fish is still unclear, it might afford the increased protection against viral infection in levamisole-treated fish.

We investigated the hemolytic activity against RaRBC (ACH₅₀) and bactericidal activity against *V. anguillarum* in the serum of fish injected with levamisole as an indicator of nonspecific humoral immunity. Levamisole activated the alternative complement pathway of rainbow trout, although the bactericidal activity against *V. anguillarum* was not enhanced in sera. This activated complement may play an important role in the non-specific defense mechanism through the promotion of immunological reactions such as opsonic complement effects.

In this study, we indicated that levamisole activated the protection to vibriosis in rainbow trout. The results show that levamisole may be a possible immunomodulatory substance for the immune enhancement to fish disease. Further studies will address oral administration and

adjuvant effect of immune response in this substance.

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