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Levamisole Enhances the Natural Cytotoxic Cell Activity of Japanese Flounder (*Paralichthys olivaceus*) Head Kidney Leukocytes

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Japanese flounder (*Paralichthys olivaceus*) head kidney (HK) leukocytes were incubated with 10^3 to 10^{-3} ng levamisole/ml for 4, 24 or 48 h and then assayed for their natural cytotoxic activity against xenogeneic tumor cells. This activity was slightly increased after 24 h of incubation. In a second experiment, fish were fed 0, 75, 150 or 300 mg levamisole/kg diet for 10 consecutive days. The fish were then fed a commercial non-supplemented diet and sampled 0, 1, 2, 3, 4 or 6 weeks post-administration of levamisole. The cytotoxic activity was found to be increased with increasing levamisole dose and remained greatly enhanced until the end of the experiment. In conclusion, levamisole enhanced flounder natural cytotoxic cell activity both *in vitro* and *in vivo* and had a great lasting action when administered by feeding.

Immunostimulants are natural or synthetic substances capable of activating non-specific and/or specific immune responses (Anderson, 1992). The use of natural substances for fish farming purposes has been widely described as they are slowly replacing antibiotics (because of their negative effects on the environment) and vaccines (because of their limited effect on fish living in cool or cold water). The actual number of synthetic substances or drugs used in fish farming is small, and more emphasis is now given to natural replacements. However, synthetic substances, such as levamislol, which has been widely used as an antihelmintic agent in medicine and veterinary sciences (Sakai, 1999), seem to be highly efficient as immunostimulants. Although levamisole is a registered and accepted drug for the European Community and the US Food and Drug Administration, its metabolism is not well known. Its toxicity and tissue residues have been documented in some animals (Babish et al., 1990), but not in fish. A deeper knowledge in levamisole treatment regarding suitable dosage administration time is needed to clarify whether or not levamisole is a good immunostimulant for animals and more specifically, for farmed fish.

In some fish species, levamisole has been demonstrated to be a good enhancer of non-specific or specific immune responses when given alone or with a vaccine (acting as an adjuvant) (Anderson, 1992). The *in vitro* treatment of fish leukocytes with levamisol enhanced phagocytosis, respiratory burst, lymphocyte proliferation and plaque-forming cells (Siwicki and Cossarini-Dunier, 1990; Siwicki et al., 1990). In addition, *in vivo* experiments have shown that injection, immersion or dietary administration of levamisole, with or without a vaccine, increase serum lysozyme and complement activities, specific antibody levels, the number of plaque-forming cells, phagocytic cell activities (chemotactic activity, phagocytosis, respiratory burst and myeloperoxidase activity) or NK cell activity in carp, rainbow trout and coho salmon (Olivier et al., 1985; Siwicki, 1987, 1989; Anderson et al., 1989; Kajita et al., 1990; Anderson and Jeney, 1992; Baba et al., 1993; Jeney and Anderson, 1993; Siwicki et al., 1994). Fish also demonstrated markedly increasing resistance to administered pathogens after administration of levamisole, demonstrating its effect on the specific immune system (Siwicki, 1987, 1989; Baba et al., 1993; Kajita et al., 1990; Olivier et al., 1985; Mulero et al., 1998a,b). It has previously been shown that the dietary intake of levamisole stimulates the immune responses of gilthead seabream (complement activity, phagocytosis, respiratory burst actvity and macrophages activating factor production) and enhances the resistance to vibriosis (Mulero et al., 1998a). However, no such response was observed when it was administered *in vitro* to head-kidney (HK) leukocytes (Mulero et al., 1998b).

It has been suggested that the target cells of levamisole treatment are lymphocytes (Renoux, 1980), which are the principal cytotoxic cells involved in fish defenses against protozoa, tumor cells and virus infected

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cells. However, there is little detailed knowledge about its effect on such activity. Taking the above into account, this study aimed to test the role of levamisole in the natural cytotoxic cell (NCC) activity of Japanese flounder (*Paralichthys olivaceus*) HK leukocytes.

Materials and Methods

Fish

One hundred and forty specimens (150 g mean weight) of the seawater teleost Japanese flounder (*Paralichthys olivaceus*), obtained from Kunsan fish farm, were kept in 450 liter fiber-glass running seawater aquaria, 28‰ salinity, at 20°C and a 12 h dark photoperiod. The fish were fed a commercial pelleted diet at rate of 1% body weight per day.

Sample collection

The fish were anaesthetized with 100 mg/l of MS222 (Sandoz). Briefly, blood was taken and HK leukocytes were isolated using a 48% Percoll density gradient (Pharmacia), washed and finally resuspended in sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride (to adjust the medium's osmolarity to flounder plasma osmolarity, 353.33 mOs), 2% fetal calf serum (FCS), 100 I.U./ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco)] (Cuesta et al., 1999). Cell viability was higher than 98%, as determined by the trypan blue exclusion test.

Incubation of leukocytes with levamisole

To study the *in vitro* effect of levamisole on HK leukocyte natural cytotoxic activity, 2.5×10^6 cell per well were cultured in flat-bottomed 48-well plates (Nunc) with levamisole at 0 (control) or different concentrations ranging from 10^{-3} to 10^3 ng/ml for 4, 24 or 48 h at 22°C prior to evaluating their natural cytotoxic activity. After incubation, the viability of the leukocytes was monitored by flow cytometry.

Dietary administration of levamisole

For *in vivo* experiments, 140 specimens were randomly divided into four groups and each group was fed a diet consisting of the above commercial pelleted diet (control group) or the diet supplemented with 75, 150 or 300 mg levamisole/kg of diet. The levamisole-supplemented diets were prepared as follows: pelleted commercial diet was crushed, mixed with 75, 150 or 300 mg powdered levamisole hydrochloride (Sigma) per kg and repelleted. The non-supplemented diet (control) was equally treated but had no levamisole added. The diets were then kept in cool and dark place. Fish were fed at a rate of 10 g dry diet/kg biomass (1% per day for 10 consecutive days. After this, fish were fed with the commercial diet (free of levamisole) and sampled 0, 1, 2, 3, 4, 5 or 6 weeks post-treatment. The biomass of fish in each aquarium was measured before the experiment and the daily ration was adjusted accordingly after each sampling.

Target cells

A mouse lymphoma cell line (L-1210, ATCC CCL-219) was used as target cells in the cytotoxic assays. The cells were cultured in RPMI-1640 culture medium supplemented with 10% FCS, 100 I.U./ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (Gibco) at 37°C, 85% relative humidity in a 5% CO_2 atmosphere and maintained in exponential growth.

To label the cells, they were incubated in RPMI-1640 culture medium with 10 µg/ml of 3,3-dioctadecyloxacarbocyanine perchlorate (DiO, Sigma) for 3 h in a light-protected micro-environment. After labeling, free DiO was removed by washing three times in PBS and uniformity of cell staining was examined by flow cytometry.

Cytotoxic activity

The natural cytotoxic or tumoricidal activity of flounder HK leukocytes (effectors) after *in vitro* incubation or after feeding levamisole supplemented diets was evaluated using a flow cytometric technique based on double-fluorescent labeling (Cuesta et al., 1999). Each cytotoxic assay was carried out in duplicate. Briefly, the leukocytes were transferred to 5 ml tubes (Falcon, Becton Dickinson), to which, 50 µl of DiO-labeled L-1210 cells (targets) (10^6 cells/ml in sRPMI) were added (final effector:target ratio of 50:1). The samples were centrifuged (400×g, 2 min, 22°C) and incubated at 22°C for 3 h. Samples at 0 h were used as controls to determine initial target viability. The viability of targets maintained at 22°C in sRPMI culture medium for 3 h was also monitored.

At the end of the incubation time, 30 µl of propidium iodide (400 µg/ml, Sigma) were added to each sample and gently mixed before analysis in a FACScan (Becton Dickinson) flow cytometer adjusted to obtain optimal discrimination of the target cell population. The FACS only accepted the positive FL1 region, which corresponded to DiO-labeled target cells. Standard samples of DiO-labeled target cells or HK leukocytes were included in each cytotoxic assay. The percentage of dead or non-viable target cells showing green and red fluorescence was related with the cytotoxic activity of flounder leukocytes.

Cytotoxic activity, a parameter describing the percentage of non-viable target cells, was calculated by the formula:

cytotoxic activity (%)

= (% sample − % control)/(100 − % control)×100

Statistical analysis

A quantitative study of the flow cytometric results was made using the statistical option of the Lysis Software Package (Becton Dickinson). Data were presented as means±S.E. and analyzed by one way analysis of variance (ANOVA). When the ANOVA test pointed to statistically significant ($p<0.05$) differences between groups (control and levamisole), a comparison of means was applied.

Results

In vitro effect of levamisole on the NCC activity

The NCC activity of flounder HK leukocytes on mouse lymphoma cells was significantly increased after incubation with levamisole at the concentrations of 10^1 to 10^2 ng /ml for 24 h (more than 20%) (Fig. 1). However, the significant effect disappeared after additional 24 h incubation. Incubation of leukocytes with levamisole slightly increased the NCC activity (Fig. 1).

In vivo effect of levamisole on the NCC activity

The NCC activity of flounder HK leukocytes was greatly enhanced after feeding levamisole-supplemented diets (Fig. 2). Even fish sampled immediately after levamisole administration for 10 days showed a statistically significant increase in NCC activity in the case of specimens fed 150 or 300 mg/kg diet. Moreover, such activity remained high until the end of the experimental period, i.e., 6 weeks after termination of levamisole treatment. Maximum NCC activity was found at one week after treatment in the 300 mg/kg diet fed group and was up to four times higher than the activity found in control specimens.

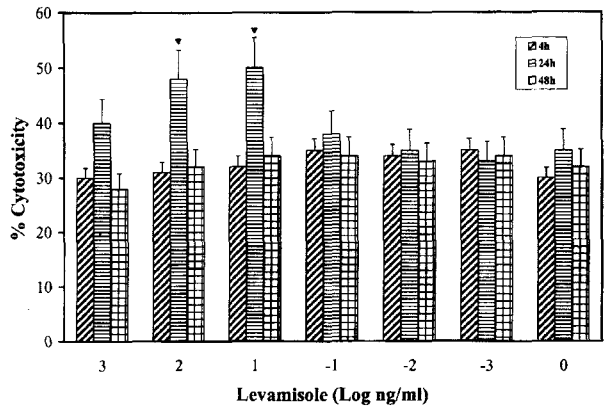

Fig. 1. Natural cytotoxic activity of flounder HK leukocytes after incubation with levamisole-supplemented RPMI-1640 culture medium for 4, 24 or 48 h. Bars represent mean+S.E. (n=6). Inverted triangles denote statistically significant differences (P<0.05) between control leukocytes and those incubated with levamisole-supplemented culture medium.

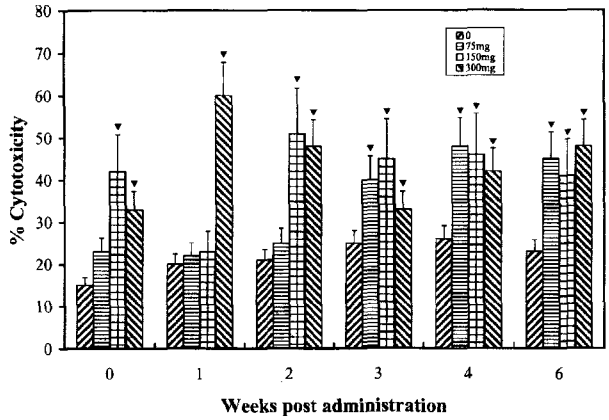

Fig. 2. Natural cytotoxic activity of HK leukocytes from flounder fed levamisole supplemented diet. Bars represent mean+S.E. (n=6). Inverted triangles denote statistically significant differences (P<0.05) between leukocytes from control fish and those from fish fed incubated with levamisole-supplemented diet.

Discussion

Levamisole is a synthetic compound classically used as an antihelmintic agent in animals. Moreover, levamisole has been proven for its immunostimulant properties. In mammals, levamisole has been shown to increase serum lysozyme activity, serum antibody titers after immunization, the number of leukocytes, phagocyte activities, the expression of cytokines by macrophages, lymphocyte proliferation and anti-tumor responses (Woods et al., 1974; Symoens and Rosenthal, 1977; Moertel et al., 1990; Kimball et al., 1992; Tempero et al., 1995; Holcombe et al., 2001). Levamisole enhances the innate immune response as it does with the acquired response (acting as an adjuvant). Cells treated with levamisole enhanced their cGMP level, which also increased microtubular assembly and cell mobility (Anderson et al., 1976). However, the mechanism by which levamisole stimulates the immune system cells is not well known.

Fish culture has become an important industry throughout the world. Antibiotics, vaccines and immunostimulants have been widely used to prevent or control fish diseases (Anderson, 1992), of which immunostimulants have received most of the attention during the last two decades. One such immunostimulant is levamisole which has been demonstrated to stimulate carp (Siwicki, 1987, 1989; Baba et al., 1993), rainbow trout (Kajita et al., 1990), coho salmon (Olivier et al., 1985) and gilthead seabream (Mulero et al., 1998a,b) immune responses and disease resistance. In the above mentioned studies, humoral (complement and lysozyme activities as well as the specific antibody titers) and cellular (phagocytic cell activities, NCC activity and plaque-forming cells) immune responses were enhanced after *in*

vitro or *in vivo* treatments. Levamisole has even been indicated as a growth promoting factor for sheep (Cabaj et al., 1995) and fish (Mulero et al., 1998a; Siwicki and Korwing-Kossakowski, 1998). Although positive effects on the immune system have been demonstrated (depending on administration time and dosage) some authors have reported negative or immunosuppressive effects (Siwicki et al., 1990). Some information is available on the effects of levamisole on fish phagocytic cell activities but only one study exists on its effect on fish cytotoxic activity although T-lymphocytes and NK cells have been suggested as its main targets (Renoux, 1980; Holcombe et al., 2001). The scarce information available regarding fish innate cytotoxic activity and its importance in the fish defense mechanism against protozoa, tumor cells and virus-infected cells led us to carry out this study. Differences in the NCC and phagocytic-cell activities were brought up due to the lymphocytes implication on the former.

Firstly, we investigated the *in vitro* effect of levamisole on non-specific cytotoxic cells in flounder HK. The results indicated that their activity was enhanced to a statistically significant degree after 24 h incubation with levamisole at 10^1 to 10^2 ng/ml. None of the other treatments affected the NCC activity. Other fish immune responses have been also seen to increase with *in vitro* levamisole treatment. For example, carp lymphocyte proliferation was increased (Siwicki and Cossarini-Dunier, 1990), and, in rainbow trout, immunization of spleen cells cultured with levamisole (5 μg/ml) enhanced their phagocytic activity, respiratory burst and the number of plaque-forming cells (Siwicki et al., 1990). In contrast, higher levamisole doses (25 or 50 μg/ml) suppressed some of the tested immune responses. In flounder, none of the phagocytic functions were enhanced by levamisole *in vitro* (Mulero et al., 1998b).

Although injection is the most rapid and effective way of administering immunostimulants (Esteban et al., 2001), incorporation in the diet is the most convenient way for fish-farmers (Siwicki et al., 1994). In the present experiment, flounder specimens were fed a levamisole-supplemented diet, which resulted in greatly enhanced NCC activity. Moreover, the effect of administering levamisole for 10 d lasted until the end of the experiment at 6 weeks. NCC or NK cell activity was also increased in rainbow trout injected with levamisole (Kajita et al., 1990). However, the degree of stimulation and duration of the effect were less than those found in this experiment for NCC activity. Furthermore, the levamisole dosages at which the maximum activities were obtained in the above experiments were higher than those tested in this experiment. Such findings have previously been documented for other immunostimulants (Esteban et al., 2000, 2001; Cuesta et al., 2002). It is known that the gilthead seabream NCC can be formed by lymphocytes, monocyte-macrophages and acidophilic granulocytes (Mulero et al., 1994; Meseguer et al., 1996; Cuesta et al., 1999), while the phagocytic functions are carried out only by non-lymphocytic cells. The direct involvement of lymphocytes in the NCC activity could be the reason for such differences, as has been observed in the *in vitro* assays.

To conclude, flounder HK leukocyte NCC activity was enhanced by the *in vitro* or dietary administration of levamisole. The *in vitro* study revealed that the incubation time with levamisole was a very important factor affecting the NCC activity. *In vivo*, the NCC activity of levamisole-fed fish increased from the very beginning of the experiment to the end (after 6 weeks). This is the greatest difference from other immunostimulants, which lead to a punctual stimulation, which do not last in time. The differences in the effect of levamisole on NCC and phagocytic activities could be due to the fact that lymphocytes are its target.

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Corrigendum

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Authors of abstract number 746: Hyun hee Kim, Byung Yong Kang, Young Wook Park, Yoon Jung Hwang, and Pyung Sub Yoon