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To cite this version:

Ak Siwicki, M Cossarini-Dunier. Effect of levamisole on the lymphocyte and macrophage activity in carp (Cyprinus carpio). Annales de Recherches Vétérinaires, INRA Editions, 1990, 21 (2), pp.95-100. <hal-00901926>

HAL Id: hal-00901926
https://hal.archives-ouvertes.fr/hal-00901926
Submitted on 1 Jan 1990

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Effect of levamisole on the lymphocyte and macrophage activity in carp (Cyprinus carpio)

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(Received 8 March 1989; accepted 2 June 1989)

Summary — Levamisole, an anthelminthic drug which is often used in cattle, is known for its immunomodulatory effect in several species. In the present work we investigated the in vitro effects of different concentrations of levamisole on lymphocyte proliferation with mitogen in carp. At low concentrations, ie 12.5 to 0.1 μg/ml, levamisole stimulated proliferation with phytohemagglutinin P (PHA-P) and Con A, had no effect at medium concentrations and acted as a suppressor at high concentrations, ie 100 μg/ml PHA-P and 100 to 10 μg/ml for Con A. Spleen and kidney macrophages were in contact with levamisole for 2 h before phagocytosis of opsonised Yersinia ruckeri. Doses of 200 and 100 μg/ml were found to be suppressive, whereas 6.2–0.8 μg/ml were stimulatory, as observed by chemiluminescence.

levamisole / immunostimulation / lymphocyte / macrophage / Cyprinus carpio

INTRODUCTION

Studies on human and animals have shown the influence of levamisole on the activity of immune cells, mainly during impaired immune function (Fidler and Spitler, 1975; Pabst and Crawford, 1975; Renoux and Renoux, 1977; Simoens and Rosenthal, 1977; Richard et al, 1979; Espinasse, 1980).

Levamisole, the levo-isomer of tetramisole, has become widely recognized as an effective anthelminthic in animals. It has a broad range of activity and is able to re-

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move 90–100% of nematodes. The widespread use of levamisole may be due to the availability of an injectable preparation that facilitates administration of the drug. In addition to its anthelmintic activity, levamisole is known as an immunomodulator, and this effect has been studied intensively in several species. Levamisole is effective in restoring the cellular immune response when T-lymphocyte and phagocytic activities are depressed (Simoens and Rosenthal, 1977). In most cases it is considered that levamisole does not affect B lymphocytes directly, but can still influence humoral responses indirectly by affecting macrophages and T lymphocytes (Simoens and Rosenthal, 1977; Soppi et al, 1979). Levamisole increases cyclic guanosine monophosphate concentration in T cell-enriched mouse spleen cells (Hadden et al, 1975). The evidence strongly suggests that levamisole administration restores the normal function of phagocytes and T lymphocytes in the organism (Simoens and Rosenthal, 1977).

Several promising adjuvants, drugs and biological response modifiers have been tested on fish. Evelyn (1971) injected Freund's complete adjuvant with formalin-killed *Renibacterium salmoninarum* and found an elevated antibody titer compared to control fish. The phagocytic activity was increased by the drug FK 565, and caused a slight elevation in numbers of antibody-producing cells and serum antibody titers when administered with specific bacterins (Kitao et al, 1987). BCG (Calmette Guerin Bacillus) extract and saponin Quil-A also have been shown to stimulate the non-specific immune response in rainbow trout (Grayson et al, 1987). The immune response was enhanced in fish injected intraperitoneally with levamisole (Siwicki, 1987). Application of levamisole in food enhances the metabolic and phagocytic activity of neutrophils in carp (Siwicki, 1989). *In vitro* and *in vivo*, levamisole acts on the non-specific defense mechanisms of rainbow trout as shown by the increased non-specific immune response. The specific immune system was elevated with the addition of levamisole with or after antigen injection (Anderson et al, 1989; Siwicki et al, 1989).

In this study, we continue to examine the effect of levamisole on carp (*Cyprinus carpio*), and in particular whether levamisole has an *in vitro* effect on carp lymphocyte proliferation and macrophage activity.

**MATERIALS AND METHODS**

**Animals**

Carp (*Cyprinus carpio*) weighing 100–150 g were purchased from local fish farms (La Dombes, Ain, France). They were maintained in 150-l tanks with a continuous flow of dechlorinated water, thermoregulated at 20 °C. Fish were fed daily with carp commercial pellets (Aqualim, Charente, France) at 1% of body weight. Carp were submitted to a standard photoperiod of 12 h daylight.

**Chemicals and vaccine**

Levamisole chloride was obtained from Rhône-Mérieux, Toulouse, France; the *Yersinia ruckeri* vaccine utilised was *Yersivax*, from Rhône Mérieux IFFA Laboratory, Lyon, France.

**Isolation of phagocytes**

Pronephros and spleen from each carp were removed and single cell suspensions obtained by teasing the tissues in medium through a nylon mesh. The cells were washed in ice-cold heparinized Hank's balanced salt solution (HBSS); 2 ml were carefully layered on top of a 30-ml Percoll gradient (Pharmacia) according to
Bayne (1985), then spun in a swing-out rotor at 350 g for 30 min at 4 °C. Macrophages were found together with lymphocytes and thrombocytes in the 1.055–1.070 density band. Living cell counts were made with Trypan blue 0.5% in a haemocytometer after 3 washings in MEM-Dulbecco without Phenol red. Macrophages were then diluted in 5.75 ml at 1.14 X 10^7 cells per ml and 0.25 ml of this suspension was mixed with 0.25 ml of a solution of levamisole at different concentrations in polystyrene cuvettes, and then left for 2 h.

**Phagocytosis**

*Yersinia ruckeri* vaccine (*Yersivax®*) was washed twice and 0.75 ml of the pure bacterial pellet was mixed with 0.7 ml hyperimmune sera (1:100 agglutination), with 0.9 ml fresh sera for complement and 8 ml of MEM. Opsonisation was performed for 2 h at room temperature before contact with macrophages contaminated with different concentrations of levamisole or controls and 0.5 ml of luminol (1.77 mg/ml dimethyl sulphoxide diluted to 1:20 in MEM).

**Chemiluminescence (CL) assay**

CL was measured in triplicate with a thermoregulated automatic photoluminometer (LKB Wallac Luminometer 1251, Wallac OY, Turku, Finland) with a mixing control system allowing for continuous registration. An Apple IIe computer controlled test conditions calculated the rate of change of light emission with time (mV), the integral representing the total amount of light emitted during a particular period. The baseline response was defined as the amount of CL obtained in the absence of any stimulation.

**Lymphocyte proliferation**

Lymphocytes from pronephros and spleen were purified by the same technique as previously described for phagocytes. Lymphocytes were distributed in 96 well plates at 5 x 10^5 cells/well (50 µl) then various dilutions of levamisole were distributed (50 µl) in 10 wells for each concentration. Phytohemagglutinin P (PHA-P) Gibco (10 µg/ml) or Concanavalin A Serva (1 µg/ml) was then added (100 µl) to the wells and the plates were incubated for 7 days at 28 °C in 5% CO_2 before pulsing with 1 µCi/well of ^3_H-thymidine (CEA, France) according to Avtalion and Shahrabani (1975). Sixteen hours after pulsing, the individual wells were harvested onto glass filters with a Skatron Automated Cell Harvester (Skatron Inc, Norway). The filters were dried and the contents from individual wells were placed into mini-vials containing 2 ml of scintillation fluid (Beckman) and counted for 1 min in a Beckman LS 3801 liquid scintillation counter. Results were expressed as mean counts per min (cpm) per group of 10 wells and an analysis of variance performed to compare different concentrations to control.

**Statistical analysis**

One-way analysis of variance (Snedecor F values) and comparison of means, by Scheffe’s test was performed successively on all results, particularly on the areas under the kinetic curves; results are given with Student’s test, P < 0.05.

**RESULTS**

**Spleen and kidney macrophages phagocytosis (fig 1)**

The two doses 200 and 100 µg/ml had a suppressive effect (P < 0.05) when 12.5–50 µg/ml were equivalent to control. From 6.2–0.8 µg/ml, levamisole had a statistically significant stimulatory effect (P < 0.05).

**Proliferation of spleen and kidney lymphocytes with PHA-P (fig 2)**

After 8 days of culture with different concentrations of levamisole, a strong sup-
pressive effect on proliferation was observed with 100 μg/ml (P < 0.05) when 50 and 25 μg/ml was equivalent to control. Between 0.1–12.5 μg/ml levamisole has a high stimulatory effect on lymphocyte proliferation (P < 0.05). At concentrations lower than 0.01 μg/ml, the effect was similar to control.

**DISCUSSION**

In the present work we observed an immunomodulatory effect of levamisole in vitro on 2 major functions of the immune system in fish, *i.e.* the specific mechanism of lymphocyte proliferation and the non-specific mechanism, phagocytosis. For both functions levamisole followed a dose–effect law, acting as a suppressor at high concentrations and as an immunostimulator at low concentrations.

The development of the *in vitro* immune response assays to study immunomodulators in fish is an effective way of carrying out preliminary screening of candidate drugs or biological response modifiers be-

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**Fig 1.** Effect of levamisole after 2 h contact on spleen and kidney macrophages on phagocytosis of opsonized *Yersinia ruckeri* studied by chemiluminescence.

**Fig 2.** Proliferation of spleen and kidney lymphocytes with 10 μg/ml PHA-P with different concentrations of levamisole in culture medium for 8 days.

**Proliferation of spleen and kidney lymphocytes with ConA (fig 3)**

Doses of 100 μg/ml and 10 μg/ml were immunosuppressive (P < 0.05) when 1 μg/ml was equivalent to control. Doses of 0.1 μg/ml and 0.01 μg/ml had a strong stimulatory effect (P < 0.05).

**Fig. 3.** Proliferation of spleen and kidney lymphocytes with 1 μg/ml Con A with different concentrations of levamisole in culture medium for 8 days.
fore performing more extensive in vivo experiments. Other advantages of this in vitro model compared to the in vivo assays include the utilisation of fewer fish, and less time taken for the experimental run.

In a previous work we studied the effect of in vitro contamination of levamisole on immune response of spleen cells from rainbow trout. All non-specific and specific immune defense parameters were elevated for 5 μg/ml levamisole. Doses of 25 μg/ml resulted in a slight elevation in non-specific levels but suppressed the specific immune response, while 50 μg/ml suppressed the stimulatory effects of the bacterin in all parameters measured (Siwicki et al, 1989).

Levamisole administered in vivo after or with antigen caused a heightening of the response of spleen cell phagocytic index, phagocytic ability of neutrophils, number and indexes of adherent cells. The number of specific antibody-producing cells was also greater in these fish when either antigen was injected (Anderson et al, 1989).

Similar immunostimulatory effects of levamisole have been observed in humans and animals (Levo et al, 1975; Pabst and Crawford, 1975; Schmidt and Douglas, 1976; Simoens and Rosenthal, 1977). Levamisole administered to animals or added to polymorphonuclear (PMN) cells and macrophages increased phagocytosis in these cells (Anderson et al, 1976; Simoens and Rosenthal, 1977). Levamisole may stimulate cell-mediated immunity by influencing T-lymphocyte differentiation, increasing the responsiveness of cells to antigens and mitogen, and stimulating the activity of effector lymphocytes (Purswell, 1988; Brunner and Muscoplat, 1980).

The in vitro results showed that levamisole may be utilised for the enhancement of immune functions in fish. The use of levamisole for stimulating the defense mechanisms and for protection against diseases in fish is of increasing interest to fish culturists.

ACKNOWLEDGMENTS

We are most grateful to Rhône-Mérieux Toulouse for providing the levamisole and Rhône-Mérieux IFFA Laboratory Lyon for the Yersivax® vaccine. Analysis by LKB Luminometer was kindly carried out by Dr J Descotes at the Pharmacy Faculty in Lyon 8.

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