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Study of the Effect of Levamisole on the Nonspecific Immune Response in Carp (*Cyprinus carpio* L.)*

Badania nad wpływem lewamisolu na nieswoistą odpowiedź immunologiczną u karpia (*Cyprinus carpio* L.)

The immune status of fish is negatively affected by many factors: polietiological stress, environment pollution, some chemoterapeutics. The effects of this factors diminishing fish immunity along with the accumulation of various flora in aquatic environment are conductive to the occurrence of infectious diseases often of a mixed ethiology. Therefore, it is of a great importance to study the early detection of immune deficiencies and the stimulation of the nonspecific cellular and humoral immunity.

The use of immunostimulants for boosting the defense mechanisms and protection against diseases in fish is of increasing interest to fish culturists. Several promising adjuvants, drugs and biological response modifiers have been tested in experiments with fish (1, 5, 6, 7, 13). Immunostimulatory factors enhance the cellular and humoral immune response in both nonspecific and specific ways. Out of many substance displaying immunostimulatory activity, especially levamisole can be applied in immunodeficient organisms (4, 18). It is true also for fish, both when levamisole is applied with food, into the bath and by interaperitoneal injection (2, 3, 11, 12, 14).

The *in vitro* studies showed that levamisole stimulate cell-mediated immunity in fish by influencing macrophage and T-lymphocyte differentiation, increasing the responsiveness of cells to antigens and mitogen, and stimulating the activity of effector lymphocytes (15, 16). In these studies, we continue the experiments with levamisole in carp (*Cyprinus carpio*) or after injections of *Yersinia ruckeri* O-antigen.

MATERIAL AND METHODS

Animals. Three hundred and fifty healthy carp, weighing 100-150 g, were examined. Experimental fish were kept at 20-22 C and fed with pellets containing 25 % protein.

Antigen and immunostimulant. The *Yersinia ruckeri* O-antigen (A) was prepared in National Fish Health Research Laboratory, Kearneysville, WV, USA. Levamisole (LV) was obtained from Rfone Merieux, France. The product was diluted in phosphate buffered saline for dose at 5 mg/per kg of fish.

Experimental design. Five groups of fish were placed on separate time regiments of administration of the immunostimulant in relation to the single dose 10 μg of the *Y. ruckeri* O-antigen. Levamisole doses were 5 mg/kg of fish at each injection time. Group I was given the antigen dose of levamisole 2, 4 and 6 days after (A – LV). Group II was given one dose of levamisole simultaneously with antigen (LV + A). Group III was injected 6,

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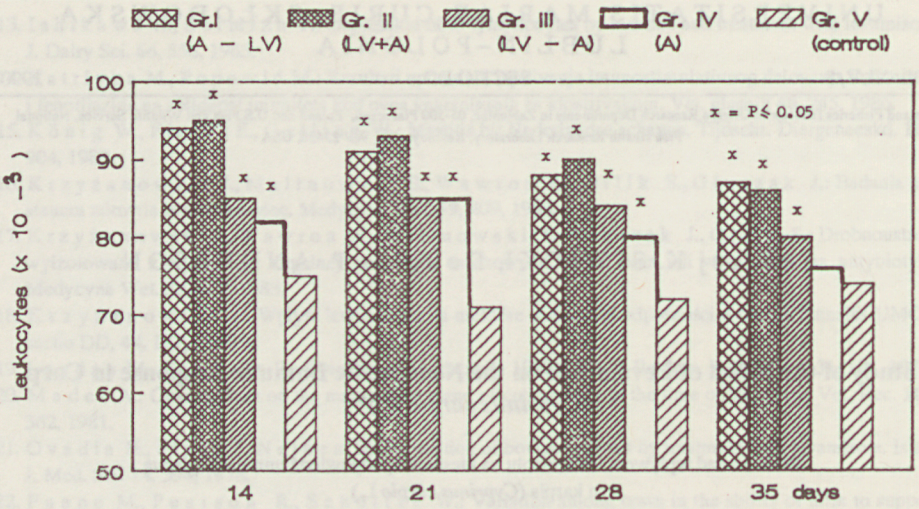

Fig. 1. Kinetics of leukocyte number after injection of *Y. ruckeri* O-antigen (for explanations Figs 1-8)
Kinetyka zmian w liczbie leukocytów po iniekcji O-antygenu *Y.ruckeri* (objaśnienia dotyczą ryc. 1-8)

4 and 2 days before the antigen (LV – A). Group IV was stimulated with antigen only (A). Group V was a negative control – only given PBS (no immunostimulant, no antigen).

Assays of nonspecific immune response. Fish were sampled on days 14, 21, 28 and 35 after antigen exposure, and blood samples were collected by cardiac puncture into plastic tubes, using heparinized syringes. The total leukocyte numbers, relative leukocyte count, phagocytic ability of neutrophils (NBT index), percent of NBT-positive PMN cells, phagocytic index, myeloperoxidase (MPO) activity in PMN cells, lysozyme level in serum, haemagglutinins and haemolysins titre in serum were examined.

The NBT index was performed according to the method of Siwicki et al. (9). Simultaneously, the total number of the leukocytes was assessed using a haemocytometer, and stained blood smears were prepared and examined in order to calculate the absolute numbers of neutrophils in the blood. Percent of NBT-positive polymorphonuclear (PMN) cells was determined by cytochemistry method (14). Phagocytic index was determined by cytochemistry method (14). *Staphylococcus aureus* 209P suspension was used. Myeloperoxidase activity in PMN cells was determined by cytochemistry method (8).

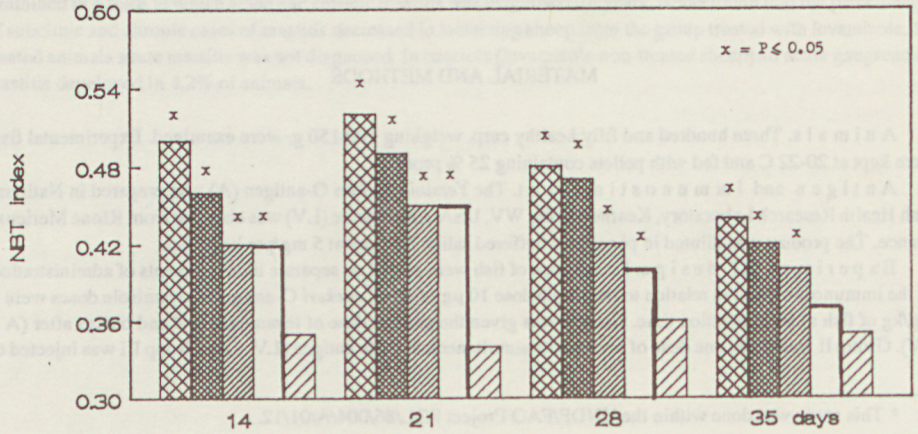

Fig. 2. Kinetics of NBT index
Kinetyka zmian indeksu NBT

The lysozyme level in serum was measured in the turbidimetric assay (17). The standard used was hen egg white lysozyme (Sigma) and *Micrococcus lysodeikticus* suspension in acetate buffer. Haemagglutinins and haemolysins titre in serum ware determined by a micro-method (10).

Results are presented as mean and S.E. Statistical analysis was performed by the Student's t-test. Mean differences with $P < 0.05$ were considered statistically significant.

RESULTS

The kinetics of alteration in the nonspecific defense parameters in relationship to the timing of the injection of levamisole and the antigen are presented in Figs. 1 - 8. On days 14, 21, 28 and 35 after injection antigen, the leukocyte number (Fig. 1), NBT index (Fig. 2), % NBT-positive PMN cells (Fig. 3), phagocytic index (Fig. 4), lysozyme level in serum (Fig. 5), MPO activity in PMN cells (Fig. 6), haemagglutinin and haemolysin titre in serum (Fig. 7, 8) significantly

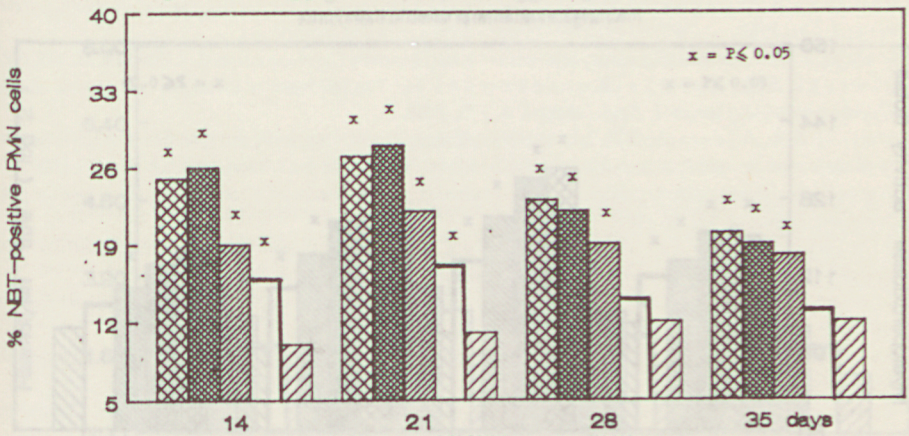

Fig. 3. Kinetics of % NBT-positive PMN
Kinetyka zmian w % PMN komórek NBT dodatnich

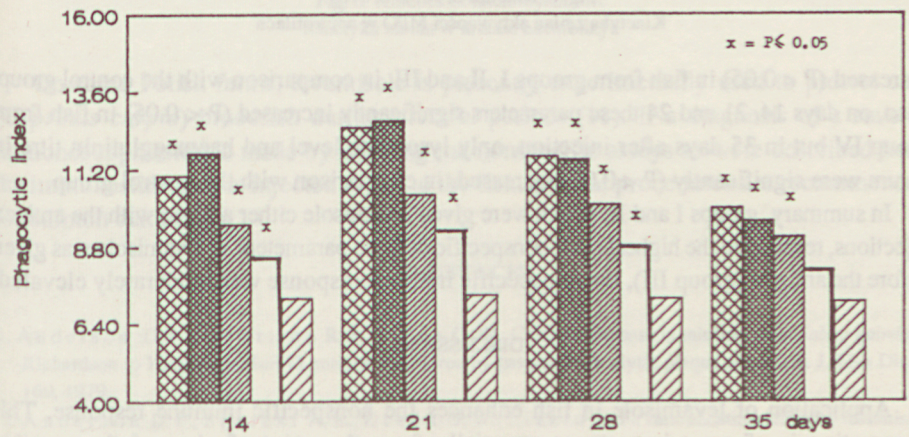

Fig. 4. Kinetics of phagocytic index
Kinetyka zmian indeksu fagocytarnego

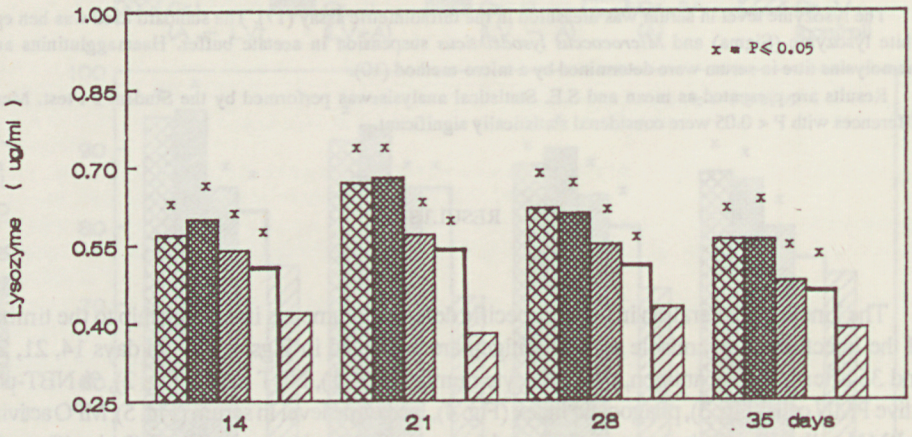

Fig. 5. Kinetics of lysozyme level
Kinetyka zmian w poziomie lizozymu

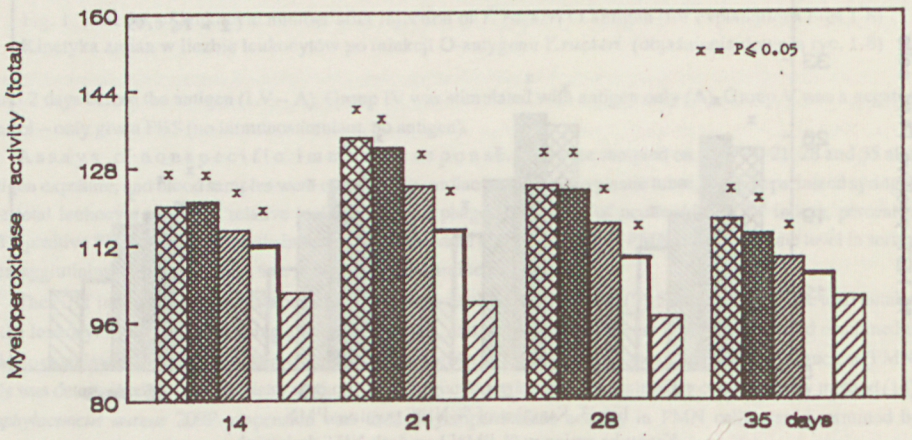

Fig. 6. Kinetics of MPO activity
Kinetyka zmian aktywności MPO w neutrofilach

increased ($P < 0.05$) in fish from groups I, II and III, in comparison with the control group. Also, on days 14, 21 and 24 these parameters significantly increased ($P < 0.05$) in fish from group IV but in 35 days after injection, only lysozyme level and haemagglutinin titre in serum were significantly ($P < 0.05$) increased, in comparison with the control group.

In summary, groups I and II, which were given levamisole either after or with the antigen injections, responded the highest in all nonspecific defense parameters. If levamisole was given before the antigen (group III), the nonspecific immune response was moderately elevated.

DISCUSSION

Application of levamisole in fish enhances the nonspecific immune response. This observation is of great importance especially from the point of view of the possible prophylactic measures during spring season and therapy of the bacterial diseases.

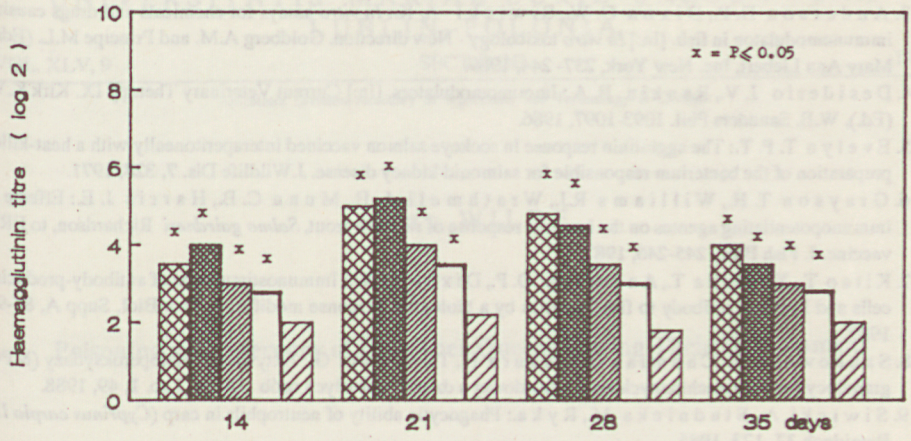

Fig. 7. Kinetics of haemagglutinin titre
Kinetyka zmian w mianie haemaglutynin

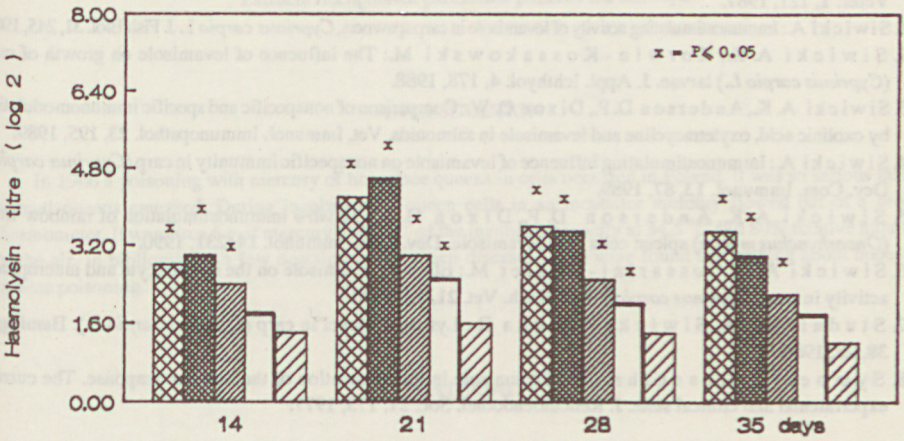

Fig. 8. Kinetics of haemolysin titre
Kinetyka zmian w mianie haemolizyn

On some Polish farms, levamisole is presently experimentally used to protect carp (*Cyprinus carpio*) broodfish during stressful periods (11). If a diagnosis of a state of immunosuppression is made by utilizing the nonspecific assays as were described here, the immunostimulant is injected or fed to the fish. Similar procedures are recommended for salmon culture (2).

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STRESZCZENIE

Badania miały na celu określenie wpływu lewamisolu na nieswoistą odpowiedź immunologiczną u karpia (*Cyprinus carpio*) po podaniu O-antygenu *Yersinia ruckeri*. Badania wykonano na 350 karpiach o masie ciał 100-150 g. Użyty do badań O-antygen *Yersinia ruckeri* podawano rybom w iniekcji dootrzewnowo w dawce 10 μg/0,2 ml PBS. Lewamisol w dawce 5 mg/kg masy ciała ryb stosowano w iniekcji dootrzewnowej: 3- krotnie w odstępach 2-dniowych przed podaniem antygenu (LV i A); jednorazowo równocześnie z antygenem (LV+A) oraz 3-krotnie w odstępach 2-dniowych po podaniu antygenu (A i LV). Badania immunologiczne obejmujące określenie liczby leukocytów i leukogramu, zdolności fagocytarnej neutrofili (NBT index), procentu PMN komórek NBT dodatnich, indeksu fagocytarnego, aktywności mieloperoksydazy w neutrofilach, poziomu lizozymu i przeciwciał naturalnych w surowicy wykonano 14, 21, 28 i 35 dni po podaniu antygenu, każdorazowo u 20 ryb każdej grupy. Kontrolę stanowiły ryby, którym podano dootrzewnowo PBS oraz O-antygen. U badanych ryb z grupy LV i A, LV+A oraz A i LV stwierdzono statystycznie istotny wzrost wszystkich oznaczonych parametrów immunologicznych w porównaniu z rybami, które otrzymały tylko O-antygen i PBS. Podwyższone wskaźniki oznaczonych parametrów utrzymywały się do końca doświadczenia (35 dzień).