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Immunostimulatory Effect of Levamisole on the Nonspecific Cellular and Humoral Defence Mechanisms in Chickens

Immunostymulujący wpływ lewamisolu na nieswoiste komórkowe i humoralne mechanizmy obronne u kurcząt

INTRODUCTION

Numerous reports have suggested that the antihelmintic drug, levamisole, can affect humoral and cellular response in man and in variety of animals (1, 2, 3, 4, 5, 6, 9, 10, 11, 12, 13, 14, 15, 17, 19). Although the effect of levamisole on the immune response has been varied, ranging from a decreased response in some cases to an enhanced response in other instances, it has become accepted that the observed responses depend on the dosage used, the time of administration and the immunological status of the animal (1, 20, 21, 22, 26). Thus levamisole treatment of immunocompromised animals generally results in the restoration of immunity to normal levels but cannot enhance immunity in immunologically normal individuals (14, 17, 21, 26). At present the exact mechanism whereby levamisole alters cellular activity is not known but it is proposed that by increasing cyclic guanosine monophosphate and decreasing cyclic adenosine monophosphate levamisole exacts its immunomodulatory effects on the immune system (26).

Intensive chickens cultures are negatively affected on the immune status. The indications of polyetiological stress have the immunosuppressing influence on the nonspecific and specific defence mechanisms. This situation predisposes to developing the bacterial and viral diseases (1, 3, 13, 15).

In food animal production, especially in chickens, it is a common practice to use antibiotics at subtherapeutic and therapeutic levels to increase feed efficiency, promote growth, and control diseases. Studies in man and animals have shown that several antibiotics suppress immune functions by their ability to interfere with protein or immunoglobulin synthesis (7, 8, 18, 25). Immunosuppression is also attributed to rapid elimination of antigen (7, 8), competition for antigenic receptor sites, and interference with phagocytic function. Antibiotics may also suppress or eliminate the normal microbial flora which is essential for the maturation of gut-associated lymphoid tissues (GALT). Literature on antibiotic-induced immunosuppression in domestic and food animals is sparse except for some on domestic poultry (18).

Intensive and prolonged use of antibiotics in commercial poultry is a common practice for protection and therapy infectious diseases. The experimental study presented that chicken and turkeys treated *in ovo* and in early life with various antibiotics at therapeutic levels did not respond as efficiently to immunizations with *Brucella abortus, Salmonella pulorum* antigens and Newcastle diseases virus as did untreated controls. In addition, antibiotics treatment caused a decrease in the number of immunoglobulin bearing cells in the small intestine, cecal tonsils, large intestine and the bursa of Fabricius (7, 8, 18, 25). In addition, IgM, Ig G and IgA levels in the blood serum of treated chickens were lower than in the untreated controls (18). Gentamicin or oxytetracycline given subcutaneously to chickens immunized with *Salmonella pulorum* bacteria also suppressed synthesis of antibodies (18).

The use of natural and synthetic immunostimulants for modulation of immune status and protection against infectious diseases is an increasing interest of chicken culturist and veterinary services.

In the present paper, we study the effect of levamisole on the nonspecific cellular and humoral defense mechanisms in chickens.

MATERIALS AND METHODS

Animals. Two hundred healthy Astra L chickens, 5 days old, were used in experiment. The chickens were distributed into two groups of 100 animals each and all the experimental time were fed with commercial pellets with Witazol AD_3E and Polfamix Z.

Experimental design. The chickens from group I were applied levamisole (Biowet Gorzów) added to the drink water 4 times every 3 days, at doses 2 mg per kg of body weight and chickens from group II were administered levamisole-free water.

To study the effects of levamisole on the nonspecific defence mechanisms the blood was collected from the wing vein using heparin-treated (150 units/ml) syringe on day 7, and also 6 and 12 weeks after the last application of levamisole. The remaining blood was dispensed into a 1.5 ml plastic tube and immediately a 100 ul sample was pipetted into another tube containing 0.9 ml Eagles's minimal essential medium with 2% fetal calf serum for cell counts using a hemocytometer. Simultaneously, the stained blood smears were prepared and examined in order to calculate the absolute numbers of neutrophils in the blood.

In this study, the leukocyte levels, phagocytic ability of neutrophils in blood (NBT index), percent PMN cells NBT positive, phagocytic index, lysozyme activity and total protein (Tp) with gamma-globulin (Ggl) level in serum were examined.

The NBT-spectrophotometric oxidative radical production assay (NBT index) used in this study was a modification of the method outlined by Sigma (Sigma Chemical Company) and adapted for lower vertebrates by Siwicki and Anderson (23). The 0.2% NBT in phosphate buffered saline (PBS) solution and NN-dimethyl formamide (Sigma) were used. Similar NBT solution in PBS to study the percent PMN cells NBT positive were used in cytochemistry method (23). The cells were counted by Computer CCD Color Video Camera System 1011P, Sony, Japan.

A modification of the technique by Siwicki and Anderson (23) was used to measure phagocytic index. The *Staphylococcus aureus* suspended in RPMI 1640 (Sigma) was added to total blood in the microtiter well. The combination was incubated at 37°C for 15 min and centrifugated to ensure contact of bacteria with blood phagocytes. For colorisation the 0.1% safranin solution was used for 10 min. The phagocytic cells were observed and engulfed bacteria were counted by Computer CCD Color Video Camera System 1011P, Sony, Japan for calculation of a phagocytic index.

The micro-turbidimetric assay was used to determine lysozyme activity in plasma with modification by Siwicki and Anderson (24). The suspension of *Micrococcus lysodeikticus* in phosphate buffer (pH 6.5) was used and the kinetics of lysis of bacteria in micro-reader (Dynatech, USA) at 630 nm with computer system were determined.

Total plasma protein was determined by modification of the Lowry et al. (12) colorimetric method, combining 5 µl of serum, 25 µl of reagent A and 200 µl of reagent B (Bio-Rad, Hercules, CA) in a microtiter well. The combination was mixed briefly using an automatic micro-pipette, and after 15 min of incubation measurements were taken using the micro-reader at 630 nm.

The gamma-globulin (Ggl) level in serum was also measured, using the Lowry method (12), with modification by Siwicki and Anderson (24) by first precipitating the gamma-globulin fraction out of the plasma with polyethylenic glycol (10,000 kDa).

For statistical analysis, means and standard deviations for all test values were calculated and Student's t-test was used to determine whether differences existed between two groups. The significance level used was $P<0.05$.

RESULTS

The results of this experiment demonstrate the kinetics of nonspecific cellular and humoral defence mechanisms parameters for chickens after 4-time application *per os* (in water) of the levamisole at doses 2 mg/kg body weight. One week after the last dose of levamisole the statistically significant ($P<0.05$) immunostimulating effects on cellular defence mechanisms parameters were observed. Throughout the time of the experiment (between 1 to 12 weeks), the statistically significant levamisole increased the activity of phagocytic cells compared to the control. Only the total level of leukocytes to the end of this study was similar, compared to the control group.

After application of levamisole the statistically significant stimulating effects were observed. The lysozyme and gamma-globulin levels in serum significantly increased ($P<0.05$) when at this time the total level of protein was similar with the control group to the end of experiment.

DISCUSSION

The problem with the present antibiotic, drug, and chemical treatments to prevent diseases in chickens set the stage for a new concept in disease prevention – immunostimulants. The classic substances for treating chickens diseases are therefore frequently used to control the viral and bacterial diseases, which is expensive, provides only short term benefit, and risks generating antibiotic-resistant strains of the causative bacterium. While each chemotherapeutant is at least partially effective in the treatment of a particular disease, problems arise with accumulation of these substances in the organism and environment as well as the emergence of resistant pathogenic strains when using antibiotics. These shortcomings, combined with a strong popular sentiment against the use of antibiotic in chickens culture continue to make the developing of a more effective specific protection by vaccines or nonspecific protection by immunostimulants. The immunological approach to preventing chicken diseases has been by

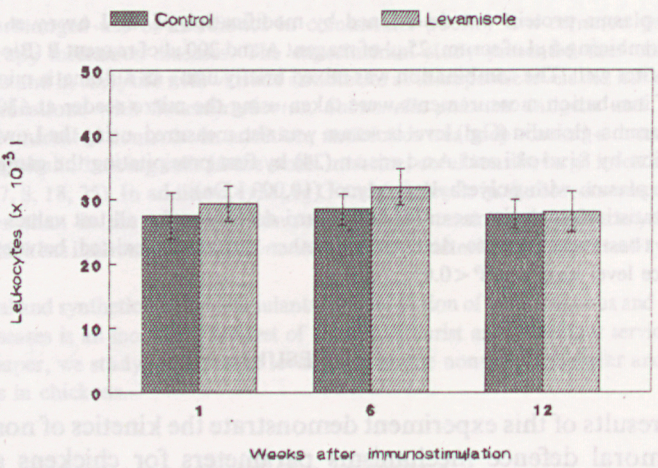

Fig. 1. Effect of levamisole on the leucocytes level in the chicken

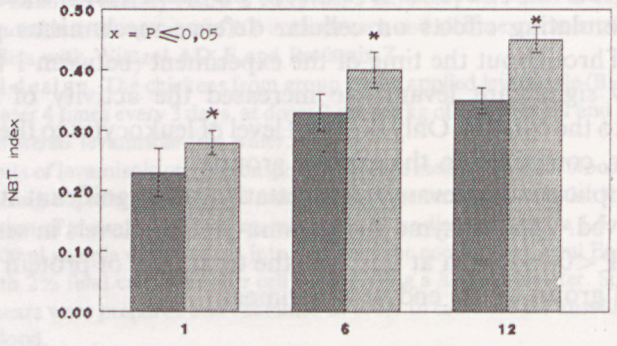

Fig. 2. Effect of levamisole on the NBT index in chicken

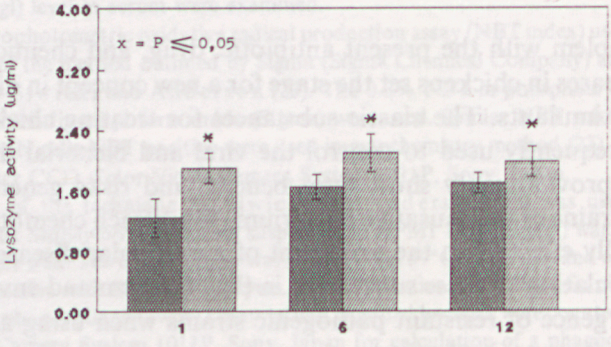

Fig. 3. Effect of levamisole on the percent PMN cells NBT positive in chicken

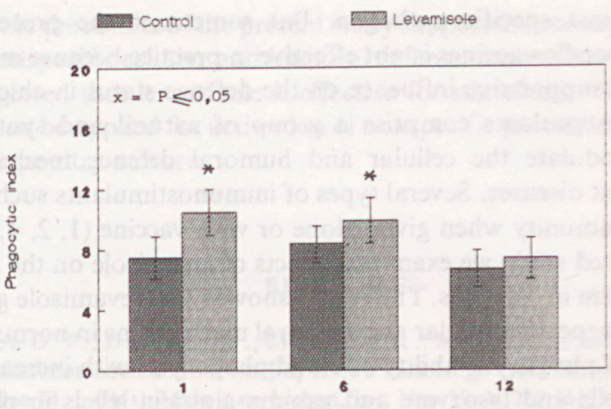

Fig. 4. Effect of levamisole on the phagocytic index in chicken

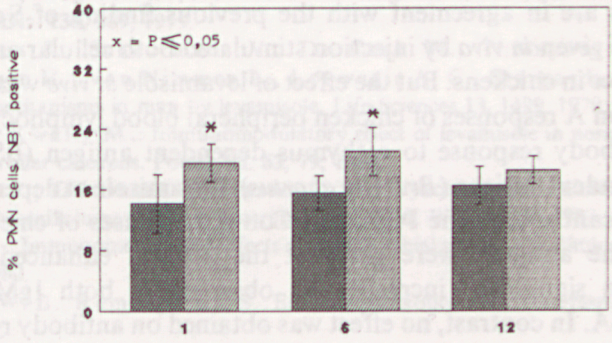

Fig. 5. Effect of levamisole on the lysozyme activity in serum of chicken

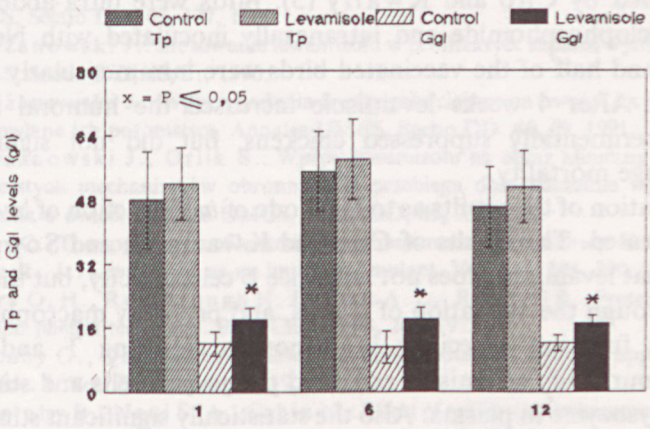

Fig. 6. Effect of levamisol on the total protein (Tp) and gammoglobulin (Ggl) levels in serum of chicken

vaccination against specific pathogen. But sometimes the protection after application of specific vaccines is not effective in practice because many factors have an immunosuppressive influence on the defence status in chickens (18).

The immunostimulants comprise a group of natural and synthetic compounds that modulate the cellular and humoral defence mechanisms and protection against diseases. Several types of immunostimulants such as levamisole modulate immunity when given alone or with vaccine (1, 2, 4, 11, 13).

In the presented study we examined effects of levamisole on the nonspecific defence mechanism in chickens. The results showed that levamisole given *in vivo* enhanced the nonspecific cellular and humoral mechanisms in normal chickens. The activation of phagocytic ability of blood phagocytes with increased percent of phagocytic cells and lysozyme and gamma-globulin levels in plasma were observed. The statistically significant immunostimulating effect was observed three months after application.

These results are in agreement with the previous finding of Soppi et al. (25). Levamisole given *in vivo* by injection stimulated both cellular and humoral immune responses in chickens. But the effect of levamisole *in vivo* was studied on the PHA and Con A responses of chicken peripheral blood lymphocytes and on the in vivo antibody response to a thymus dependent antigen (BSA) and to a thymus independent antigen (*Brucella abortus*). Levamisole at dependent doses increased significantly both the PHA and Con A responses of chickens blood lymphocytes. The antigens were given at the time of enhanced mitogenic responses and a significant increase was observed in both IgM and IgG antibodies to BSA. In contrast, no effect was obtained on antibody responses to *Brucella abortus* antigen (25).

The immunomodulatory effect of levamisole in immunosuppressed chickens were also observed by Cho and Kwally (5). Birds were intra-abdominally injected with cyclophosphomide and intranasally inoculated with Newcastle disease vaccine and half of the vaccinated birds were intramuscularly injected with levamisole. After 4 weeks levamisole increased the humoral immune response in experimentally suppressed chickens, but did not significantly influence challenge mortality.

The interpretation of the results as to the mode of *in vivo* action of levamisole is highly complicated. The results of Cho and Kwally (5) and Soppi et al. (25) suggested that levamisole does not influence B cells directly, but the effects are mediated through the activation of T cells, and probably macrophage and phagocytic cells function especially in responses requiring T and B cell cooperation. In our study levamisole activated phagocytic cells and stimulated the secretion of lysozyme in plasma. Also the statistically significant stimulating effect on gamma-globulin level in plasma, three months after application of levamisole was observed.

As a whole the results of the present study support the concept that levamisole activates nonspecific cellular and humoral defense mechanisms in chickens. Our results suggested that levamisole is effective for stimulation of defence mechanisms and can be applied for activation of nonspecific protection against diseases in intensive poultry culture.

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STRESZCZENIE

Celem badań było określenie wpływu lewamizolu na nieswoiste mechanizmy obrony komórkowej i humoralnej u kurcząt. Badania wykonano na 200 kurczętach rasy Astra „L" w wieku 5 dni. Lewamizol w dawce 2 mg/kg m.c. podano 100 kurczętom czterokrotnie w odstępach 3-dniowych *per os* z wodą do picia. Kontrolę stanowiło 100 kurcząt przebywających w tych samych warunkach i identycznie karmionych jak kurczęta grupy doświadczalnej.

Badania obejmujące określenie liczby leukocytów i leukogramu, zdolności fagocytarnej neutrofili (NBT indeks), procentu PMN komórek NBT dodatnich, indeksu fagocytarnego, aktywności lizozymu, poziomu białka całkowitego i kompleksu gammaglobulin, wykonano w 7 dniu oraz w 6 i 12 tygodniu po ostatnim podaniu lewamizolu. Krew do badań pobierano każdorazowo od 10 losowo wybranych kurcząt grupy doświadczalnej i od 10 kurcząt grupy kontrolnej. U kurcząt, którym podano lewamizol stwierdzono statystycznie istotny wzrost zdolności fagocytarnej neutrofili i monocytów, wzrost odsetka komórek fagocytujących oraz wzrost aktywności lizozymu i poziomu gammaglobulin w surowicy krwi. Uzyskane wyniki badań wskazują, że lewamizol podany 5-dniowym kurczętom w dawce 2 mg/kg m.c., aktywuje nieswoiste mechanizmy obronne zarówno komórkowe, jak i humoralne przez okres co najmniej 3 miesięcy.