Praca oryginalna *Original paper*

Effect of different immunomodulators on the percentage of CD4+ and CD8+ T lymphocytes and the antibody titres in pigeons immunised against PPMV-1*)

TOMASZ STENZEL, BARTŁOMIEJ TYKAŁOWSKI, MARCIN ŚMIAŁEK, AGNIESZKA KWIATKOWSKA-STENZEL*, ANDRZEJ KONCICKI

Department of Poultry Diseases, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowskiego 13, 10-957 Olsztyn, Poland
**Departement of Animal Hygiene and Environmental, Faculty of Animal Bioengineering, University of Warmia and Mazury, Oczapowskiego 5, 10-718 Olsztyn, Poland

Stenzel T., Tykałowki B., Śmiałek M., Kwiatkowska-Stenzel A., Koncicki A.

Effect of different immunomodulators on the percentage of the CD4+ and CD8+ T lymphocytes and the antibody titres in pigeons immunised against PPMV-1

Summary

As a consequence of frequent immunosuppression in pigeons, with resultant decreased post-vaccination immunity and deteriorated health of the birds, a study was taken up in order to determine the effect of three immunomodulators (β-glucans, metisoprinol and levamisole) on the percentage of CD4+ and CD8+ T lymphocytes in peripheral blood and spleen, and the titre of anti-NDV antibodies in the serum of pigeons in four groups (A, B, C, D), 20 birds in each. The birds in each group were immunized against paramyxovirosis in week 6 and 9 of their lives, and water for injection was given intramuscularly 1 day before each injection (group A – control), metisoprinol was given intramuscularly for 3 days at a dose of 300 mg/kg of body weight (group B), levamisole was given as a 7.5% solution of levamisole hydrochloride at a dose of 2 mg/kg of body weight intramuscularly, 1 day (group C), or β-glucans were given 10 days before vaccination per os at a dose of 5 mg/kg of body weight (group D). The immunological examinations were carried out by flow cytometry and the ELISA test. The results indicate that levamisole and β-glucans at the doses used in the study stimulate an increase in the anti-NDV antibody titre and the percentage of CD4+ T lymphocyte subpopulation in peripheral blood (levamisole) along with an increase in the percentage of CD8+ T lymphocytes in the spleen of pigeons vaccinated against paramyxovirosis. The absence of such an effect following the administration of metisoprinol at a dose of 300 mg/kg of body weight for 3 days may have resulted from an excessively high dose.

Keywords: pigeons, immunomodulation, levamisole, β-glucans, metisoprinol

The rapid development of sport pigeon breeding has necessitated the development of prevention programs based on vaccinations and screening tests in flocks. One of the viral diseases that have caused immense losses in pigeon flocks is paramyxovirosis. The first cases of the disease were recorded in 1977 and the disease has spread around the world since then, competitive pigeon racing being a favoring factor (5). The rapid spread of the virus among pigeons and the huge losses that it has caused in flocks caused researchers in the early 1980s to undertake intensive work to obtain a vaccine to enable effective immunization of the birds. Initial attempts which involved using live anti-NDV (Newcastle Disease Virus) provided a certain level of immunity, but due to some technical difficulties with using live vaccines in pigeon flocks, it was decided to develop an inactivated vaccine with an oil adjuvant (1, 17). Such vaccines have been commonly and safely applied to date (both in prevention programs and in intervention vaccinations) and anti-NDV vaccinations can be combined with vaccinations against other viral and bacterial diseases.

Recently, paramyxovirosis has been frequently detected in field conditions in pigeons that have already

*) Supported by the Ministry of Science and Higher Education, Grant Nr NN 308 22 12 33.

been vaccinated against it. This can be attributed to the fact that the birds are frequently infected with immunosuppressive viruses, such as pigeon circovirus (PiCV) (19). Immunosuppression in pigeons is obviously a complex phenomenon, which is a combination of the action of numerous pathogens and some factors related to the specific breeding conditions (transport stress, improper zoohygienc conditions).

Immunomodulation is one of the factors that can be helpful in combating immunosuppression. However, it is relatively difficult to apply in practice. Difficulties arise mainly from the limited possibility of applying immunostimulating preparations and from some dosage problems; they are also associated with the choice of the route and time of administration. There have been reports in the literature about the application of immunostimulants in various poultry species (4, 10, 11, 13-17); however, there have been no reports about using immunity-boosting preparations in pigeons.

Therefore, a research study was taken up aimed at determining the effect of three immunomodulators (β-glucans, metisoprinol and levamisole) on the percentage of $CD4^+$ and $CD8^+$ T lymphocyte subpopulation with respect to vaccinations against paramyxovirosis. The experiment plan put great emphasis on the practical aspect of the study by following the recommended anti-NDV vaccination schedule (immunomodulators applied in prevention).

Material and methods

The experiment with pigeons was conducted with the approval of the Local Ethical Committee for Animal Experiments. Four groups of pigeons, 20 birds in each group, were used in the experiment. The pigeons at the age of 5 weeks were pre-selected (to be uniform in terms of body build and mass) and randomly divided into groups. Each group contained the same number of male and female birds. Subsequently, the birds were immunomodulated. Pigeons in group A were used as a control and were given water for injection intramuscularly 1 day before vaccination. The birds in group B were given metisoprinol as Isoprivet (10% solution, VetAgro Lublin, Poland) at a dose of 300 mg/kg of body weight by intramuscular injection for 3 days before each vaccination. Pigeons in group C were given *i.m.* levamisole as a 7.5% solution of levamisole hydrochloride (Vètoquinol Biowet, Poland) at the dose of 2 mg/kg of body weight 1 day before each vaccination, whereas the birds in group D were given β-glucans *p.o.* as Mrdimune (Medpet, RPA) tablets (5 mg of β-glucans/kg of body weight) for 10 days before each vaccination. The pigeons were vaccinated against paramyxovirosis in accordance with the recommended vaccination schedule in week 6 and 9 of life with the PM-VAC vaccine (Biowet Puławy) at a dose of 0.2 ml s.c. The doses of levamisole and metisoprinol used in this study were determined based on data from literature (15, 16) concerning other bird species. The use of such doses of β-glucans was necessitated by the available commercial preparation. Subsequently, on day 15, 42 and 63 of the experiment, the percentage of the T lymphocyte subpopulation in peripheral blood and spleen as well as the anti-NDV antibodies titre were determined.

The percentage of the T lymphocyte subpopulations was determined with the use of anti-$CD4^+$ and $CD8^+$ monoclonal antibodies (Mouse anti-chicken FITC, Southern Biotech, USA) by flow cytometry on an EPICS XL apparatus (Beckmann coulter, USA). Blood was drawn directly to K_2EDTA coated tubes while spleens were taken during the anatomopathological examination. Blood samples were prepared in accordance with the procedure described by Dudek (9); spleen leucocytes for cytometry were obtained in accordance with the procedure described by Stenzel et al. (18). The percentage of $CD4^+$ and $CD8^+$ Tcells were read out in a lymphocyte gate and the results were analyzed with the System II software.

The titre of specific anti-NDV antibodies was determined by the ELISA test (IDEXX, USA). The serum was diluted 1 : 5. The absorbance of the solution was measured with an Elx800 spectrophotometer (Biotek) at a wavelength of 650 nm.

The results were analyzed statistically by carrying out a bi-factorial analysis of variance, by calculating the standard deviation, the mean value and the significance of differences at $p \leq 0.05$ and $p \leq 0.01$, and the coefficient of variance (CV%) was calculated for the antibody titre. The statistical analysis was performed with the Statistica 8.0 program by means of a Duncan test.

Results and discussion

Changes in the percentages of the lymphocyte T subpopulation with the $CD4^+$ and $CD8^+$ surface markers were analyzed qualitatively and quantitatively in this study. $CD4^+$ T cells in birds induce and support the cellular immune mechanisms by secreting cytokines which affect other cells of the immune system. They play an important role in regulating humoral response by inducing activation and proliferation of B cells. T cells with the CD8 surface receptor are regarded as cytotoxic lymphocytes (Cytotoxic T Lymphocytes, CTL) (2, 6-8).

However, one should bear in mind that the results of immunological tests in pigeons carried out by means of flow cytometry are illustrative rather than quantitative and they are not too accurate, which can be attributed to the antibody clones used in the tests, their species-related specificity (mouse anti-chicken) and incomplete binding of antibodies with cell receptors (12). Such antibodies were used because anti-pigeon lymphocyte antibodies were unavailable. However, the results can be scientifically valuable provided the same conditions of cytometry are maintained, leucocytes are isolated from pigeons in different groups in the same way and tests are always carried out by the same person.

The results of studies of behavior of $CD4^+$ and $CD8^+$ T lymphocyte subpopulations in the blood and spleen of pigeons that were immunomodulated with β-glucans, levamisole and metisoprinol are provided in

tab. 1 and 2. The data in tab. 1 show that giving levamisole *i.m.* at the dose of 2 mg/kg of body weight stimulates a significant ($p \leq 0.01$) increase in the percentage of $CD4^+$ T lymphocytes in blood. The increase was the highest in sampling III (3 weeks after the second vaccination). However, this study did not find any statistically significant differences in the percentage of the $CD8^+$ T lymphocyte subpopulation in the blood of pigeons treated with different immunomodulators. A significant ($p \leq 0.01$) increase in the population in sampling II (4 days after the second vaccination) was recorded in pigeons in all the test groups.

The results obtained in this study are close to the findings of the study conducted by Dudek (9), although the values presented by the author are a little higher. This can be attributed to the different age of the birds used in this study and the different pigeon breeds since differences were found in the percentages of different subpopulations of T lymphocytes in birds depending on the breeding line and age (3).

The data presented in tab. 2 show that the percentage of $CD8^+$ T lymphocytes in the pigeon spleen was affected by giving them β-glucans for 10 days before each vaccination. There are no data in the literature regarding the percentage of $CD8^+$ T lymphocytes and the effect of β-glucans on them. However, there are data which indicate an increase in the percentage of $CD8^+$ T lymphocytes in the intestinal endothelium and in chickens' peripheral blood, which are given the immunomodulator with fodder (4, 10). The lowest ($p \leq 0.01$) percentage of the $CD8^+$ T lymphocyte subpopulation was found in the spleens of pigeons in the group which was given metisoprinol *i.m.* at the dose of 300 mg/kg of body weight for 3 days before each vaccination. The percentage was significantly ($p \leq 0.01$) lower as compared to pigeons in the other groups, which may have been caused by too high a dose of the immunomodulator. These findings correspond to those obtained by Stenzel et al. (18), who observed a suppressive effect of the immunomodulator in turkeys following administration *in ovo* at a dose of 20 mg/embryo.

The values of anti-NDV antibody titre (PPMV-1 is its variant and it cross-reacts with NDV) (15, 17) are given in tab. 3. The data in the table show that the antibody titre was the highest in the serum of pigeons vaccinated and immunomodulated with levamisole and β-glucans as compared with the titre in pigeons in the other groups. The antibody titre increased as the time from the last vaccination passed, whereas the CV% value gradually decreased.

The results obtained in this study indicate that β-glucans and levamisole can be used as adjuvants with the vaccine against paramyxovirosis in pigeons; the absence of such an effect following the administration of metisoprinol could result from too high a dose (300 mg/kg of body weight). Therefore, further studies should be carried out to determine the effect of different doses of metisoprinol on the parameters under study.

Tab. 1. Percentage of $CD4^+$ and $CD8^+$ T lymphocytes in peripheral blood of pigeons immunomodulated and immunised against PPMV-1 ($\bar{x} \pm SD$)

Cell surface receptor	Group				Sampling		
	A	B	C	D	I	II	III
$CD4^+$	11.4^A 2.5	13.4^A 8.1	16.0^B 7.6	13.5^A 3.8	12.5^A 3.7	10.5^A 2.4	21.1^B 8.4
$CD8^+$	8.0 3.0	9.2 4.3	8.6 4.7	9.6 3.3	6.3^A 2.1	11.0^B 4.4	9.9^B 2.5

Explanation: A, B – statistical differences significant at $p \leq 0.01$

Tab. 2. Percentage of $CD4^+$ and $CD8^+$ T lymphocytes in the spleens of pigeons immunomodulated and immunized against PPMV-1 ($\bar{x} \pm SD$)

Cell surface receptor	Group				Sampling		
	A	B	C	D	I	II	III
$CD4^+$	52.0 14.1	49.1 10.8	56.5 7.8	60.1 9.8	57.7 9.2	52.21 3.2	53.7 11.5
$CD8^+$	34.9^A 8.6	28.0^B 7.3	33.7^{AB} 5.7	44.4^C 6.8	35.1 9.1	38.1 8.4	33.4 10.0

Explanation: A, B, C – statistical differences significant at $p \leq 0.01$

Tab. 3. Titre of anti-NDV antibodies (ELISA) in the serum of pigeons immunomodulated and immunized against PPMV-1

NDV titre ELISA	Group				Sampling		
	A	B	C	D	I	II	III
$\bar{x}$	966.6^a	852.3^a	1214.5^b	1139.8^{ab}	52.7^A	1098.9^B	2008.5^C
CD4+	741.2	706.0	915.9	866.3	21.1	286.3	567.3
CV%	76.6	82.8	75.4	76	40.0	26.0	28.2

Explanations: A, B, C – significant at $p \leq 0.01$

References

1. *Alexander D. J., Parsons G., Marshall R.*: Avian paramyxovirus type 1 infections of racing pigeons: 4 laboratory assessment of vaccination. Vet. Rec. 1986, 118, 262-266.
2. *Arstila T. P., Vainio O., Zasilla O.*: Central role of CD4+ in avian immune response. Poult. Sci. 1994, 73, 1019-1026.
3. *Birdle B. W., Julian R., Shewen P. E., Vaillancourt J.-P., Kaushik A. K.*: T lymphocyte subpopulations diverge in commercially raised chickens. Canad. J. Vet. Res. 2006, 70, 183-190.
4. *Chae B. J., Lohakare J. D., Moon W. K., Lee S. L., Park Y. H., Hahn T. W.*: Effects of supplementation of β-glucan on the growth performance and immunity in broilers. Res. Vet. Sci. 2006, 80, 291-298.
5. *Cross G.*: Paramyxovirus-1 infection (Newcastle Disease) of pigeons. Sem. in Avian Exotic Pet Med. 1995, 4, 92-95.

6. *Czekaj H., Samorek-Salamonowicz E., Bednarek D., Kozdruń W., Król K.*: Subpopulations of T lymphocytes in the blood of chickens vaccinated against Marek's disease. Medycyna Wet. 2008, 64, 681-683.
7. *Czekaj H., Samorek-Salamonowicz E., Kozdruń W., Bednarek D., Król K.*: Analysis of T lymphocyte populations in peripheral blood of chickens infected with reoviruses. Medycyna Wet. 2005, 61, 703-705.
8. *Davison F., Kaspers B., Schat K. A.*: Avian Immunology. Academic Press (Elsevier) 2008, 13-51, 91-107, 159-203, 299-339.
9. *Dudek K.*: Wpływ lipopolisacharydu na wystąpienie i przebieg gorączki, kształtowanie się tolerancji pirogenowej oraz wskaźniki immunologiczne i zapalne u gołębi. Praca doktorska, AR, Lublin 2007.
10. *Guo Y., Ali R. A., Quershi M. A.*: The influence of β-glucan on immune response in broiler chicks. Immunopharmacol. Immunotoxicol. 2003, 25, 461-472.
11. *Huff G. R., Huff W. E., Rath N. C., Tellezt G.*: Limited treatment with β-1.3/1.6-glucan improves production values of broiler chickens challenged with Escherichia coli. Poult. Sci. 2006, 85, 613-618.
12. *Jeurissen S. H., Janse E. M.*: The use of chicken-specific antibodies in veterinary research involving three other avian species. Vet. Q. 1998, 20, 140-143.
13. *Lowry W. K., Farnell M. B., Ferro P. J., Swaggerty C. L., Bahl A., Kogut M. H.*: Purified β-glucan as an abiotic feed additive up-regulates the innate immune response in immature chickens against Salmonella enterica serovar Enteritidis. Int. J. Food Microbiol. 2005, 98, 309-318.
14. *Morales-López R., Auclair E., García F., Esteve-Garcia E., Brufau J.*: Use of yeast cell walls; β-1, 3/1, 6-glucans; and mannoproteins in broiler chicken diets. Poult. Sci. 2009, 88, 601-607.
15. *Moya P., Alonso M. L., Baixeras E., Ronda E.*: Immunomodulatory activity of Isoprinosine on experimental viral infections in avian models. Int. J. Immunopharmacol. 1984, 6, 339-343.
16. *Singh K. C., Dhawedkar R. G.*: Immunomodulating effects of levamisole in chicks immunocompromised by infectious bursal disease virus. Trop. Anim. Health Prod. 1993, 25, 11-14.
17. *Stone H. D.*: Efficiacy of oil-emulsion vaccines prepared with pigeon paramyxovirus-1, Ulster and LaSota Newcastle disease viruses. Avian Dis. 1989, 33, 157-162.
18. *Stenzel T., Tykałowski B., Andrzejewski M., Koncicki A.*: The influence of methisoprinol applied in ovo on the percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T-lymphocyte subpopulations in turkeys. Medycyna Wet. 2008, 64, 1157-1160.
19. *Wieliczko A., Piasecki T., Houszka M.*: Infection of circovirus in pigeons. Medycyna Wet. 2005, 61, 94-97.

Corresponding author: Dr. Tomasz Stenzel, Oczapowskiego 13, 10-957 Olsztyn; e-mail: ptasiarz@wp.pl