VETERINARSKI ARHIV 80 (1), 61-69, 2010

Evaluation of CD45$^+$ cells kinetics in the blood of fattening chickens immunized with live or inactivated Newcastle disease vaccine

Maja Popović[1]*, Mirta Balenović[2], Anamaria Ekert Kabalin[3], Vladimir Savić[2], Nada Vijtiuk[4], Ksenija Vlahović[1], and Ivica Valpotić[1]

[1]Department of Biology, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

[2]Poultry Centre, Croatian Veterinary Institute, Zagreb, Croatia

[3]Department of Animal Husbandry, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

[4]Teacher Education Faculty, University of Zagreb, Zagreb, Croatia

POPOVIĆ, M., M. BALENOVIĆ, A. EKERT KABALIN, V. SAVIĆ, N. VIJTIUK, K. VLAHOVIĆ, I. VALPOTIĆ: Evaluation of CD45$^+$ cells kinetics in the blood of fattening chickens immunized with live or inactivated Newcastle disease vaccine. Vet. arhiv 80, 61-69, 2010.

ABSTRACT

Since insight into the effects of Newcastle disease vaccination on cellular immune responses has been insufficient to date, this study follows the leukocyte dynamics in the blood of fattening chickens inoculated against Newcastle disease with lyophilised live vaccine of the La Sota virus strain (experimental group E1) and/or inactivated vaccine produced from the same strain (experimental group E2). Blood was treated with labelled antibody for the CD45$^+$ leukocyte marker and examined by flow citometry. In bouth experimental a statistically significant increase ($P<0.01$) of CD45$^+$ cells was observed in relation to the control group of chickens, and the increase was more intensive and faster in the chickens vaccinated with lyophilised live vaccine than it was in chickens vaccinated with inactivated vaccine. The group treated with live vaccine had a significantly increased number of CD45$^+$ cells 6, 12, and 24 hr, and 7, 14, 21 and 28 days after inoculation. But on the 14th day after vaccination, a significantly increased number of CD45$^+$ cells ($P<0.05$) was found in the chickens inoculated with inactivated vaccine. It can be concluded that the lyophilised live vaccine was more efficient and cellular immunity was achieved sooner than with the inactivated vaccine during the observed period of 28 days after inoculation.

Key words: cellular immunity, flow cytometry, fattening chickens, Newcastle disease, vaccines

*Corresponding author:

Prof. Dr. Maja Popović, Department of Biology, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10 000 Zagreb, Phone: +385 1 2390 140; Fax: +385 01 2390 145; E-mail: mpopovic@vef.hr

ISSN 0372-5480
Printed in Croatia

Introduction

Since there are many understandings which can clarify the role of immunity in many diseases in poultry, research of the immune system, including the immune response, is crucial for planning and development of imunnoprophylaxis in poultry production (LILLEHOJ, 1991). Thus a prerequisite for successful intensive poultry production is an identification of immunophenotypes of immune cells from the peripheral blood and determining of their quantity relations (TIZZARD, 2002). The last couple of years, interest have been raised worlwire for researching the poultry immune system (POPOVIĆ et al., 2008). While reviewing the scientific and professional literature, it was clear that flow cytometry has become important in the evaluation of the poultry immune status through the distribution of different leukocyte immunophenotypes of healthy poultry during the production period (BALENOVIĆ et al., 2007).

Flow cytometry is a modern analytical method for quality and quantity determining of biological and physical features of cells by applying specific monoclonal antibodies for membrane and/or intercellular glycoproteins (markers) for determining relative proportions of individual cell subpopulations of lymphocytes in the total leukocyte population (ORMED, 2000). As a method, it is recommended for estimating measures and manners of prevention, making a diagnosis, selection of the most efficient therapy and monitoring recovery of ill animals in the area of veterinary immunology (TARRANT, 2005).

Poultry possess anatomically differentiated organs, Bursa of Fabricius and thymus, which represent key lymphoid organs in regulation of development and direction of humoral and cellular immunity. Namely, stem cells, produced in the bone marrow, migrate to the Bursa of Fabricius and differentiate into B-lymphocytes, while differentiation of T-lymphocytes occurs in the thymus (ERF, 2004). Lymphocytes B, as the source of humoral immunity in the peripheral blood of the poultry, are make up around 30 % in the total leukocyte population, while T-lymphocytes as the source of cellular immunity make up approximately 40 % (NAGLIĆ and HAJSIG, 1993). In birds and in the mammals, B-lymphocytes are the source of plasma cells that produce antibodies, while T-lymphocytes have different regulatory and effector roles. Glycoprotein antigens on leukocyte surfaces serve as "biological markers" and are denominated as CD (cluster of differentiation) (SAALMÛLLER et al., 1997; GLICK, 2000). Depending on a cell population, differentiation of cells within the same cell population and their functional maturity, it is possible to differentiate leukocyte markers which compose a heterogeneous molecular group. Although their variety is large, CD45 is a marker of all leukocytes (PARMITHIOTIS et al., 1991). Based on the information from the literature, it is known that a special role in the

activation of lymphocytes is played by $CD45^+$, which is located on the surface of T and B lymphocytes, but also all other leukocytes supporting the activation of those after binding antigens (ANDREIS et al., 1998).

The first objective of this research was to estimate full blood $CD45^+$ leukocytes using flow cytometry for broiler chickens through a certain time period. The second objective was to determine the impact of certain types of vaccines against Newcastle disease at the cell-mediated immune (CMI) response in broiler chickens showing values of leukocytes in the full blood after immunization.

Materials and methods

Animals and experimental design. The study was carried out on 30 fattening chickens originating from a commercial parent flock (Vindija d.d., Varaždin). One-day-old chickens were placed together until the 14th day, that is, until the inoculation day. On the day of inoculation, the chickens were separated into three groups of 10 each: a control group (C group), experimental group E1 and experimental group E2. Chickens in the C group were vaccinated oculonasally (by dropping one drop in an eye and one drop in the nasal opening) with 100 µL of physiological solution and i/m with 500 µL of neutral emulsion. Chickens from group E1 were vaccinated oculonasally with lyophilised live vaccine of' the La Sota virus strain, Pestikal® La Sota SPF (Veterina d.o.o., Croatia) resuspended in 100 µL of distilled water per vaccine dose and inoculated with 500 µL neutral emulsion i/m per chicken. Chickens from group E2 were inoculated with 500 µL of inactivated vaccine of' the La Sota virus strain, Pestikal® (Veterina d.o.o., Croatia) i/m per chicken and administered 100 µL of physiological solution oculonasally per chicken. Each group then was housed separately on deep litter in line with conventional conditions for intensive breeding. Chickens were fed standard broiler feed. During the experiment, food and water were available to chickens *ad libitum*. Blood was collected (100 µL) by heart puncture into citrate anticoagulant 6, 12 and 24 hours and 7, 12 and 28 days after inoculation.

Flow cytometry. The number of leukocytes in 100 µL was determined by flow cytometry (Coulter® Epics® XL™, Beckham Coulter, USA). Blood samples (100 µL) were suspended in 1× PBS of working solution to the leukocyte number of $5.0\text{-}9.7\times10^9$ L. After that, in 100 µL of dissolved blood, 50 µL of fluorescein-labelled monoclonal antibody (mAb) for chicken CD45 (Southern Biotechnology Associates, Inc., USA) was added and incubated for 20 minutes at room temperature. After incubation, the samples were washed with 1 mL 1× PBS and centrifuged during 5 minutes at 2000 rpm. To the sedimented cells 0.5 mL of buffer solution (NH_4Cl, pH 7.3) was added and the suspension was incubated in the dark at room temperature for 10 minutes. Then the samples were washed with 1 mL 1x PBS by centrifugation for 5 minutes at 2000 rpm. The sedimented

cells were suspended in 1 mL 1× PBS analyzed by flow cytometry. All samples were prepared in triplicate, and for each triplicate.

Collected data were analysed with Statistica 7.1 (StatSoft, Inc., 2006). Basic data processing was made with descriptive statistics procedures. Normal distribution of each experimental group was confirmed with Kolmogorov-Smirnov test. Differences between the control group and experimental groups was determined by One-way ANOVA. For determining sequential analyses of samples, analysis of variance for repeated measurements was employed with Unequal N HSD test used for post-hoc analysis.

Results

By applying the computer program of Epics XL to the cytogram based on the presence of CD45$^+$ markers on their surface, a sample of the leukocyte type was isolated and its proportion was calculated from the total analyzed cells per sample of the full blood. In experimental groups (E1) and (E2) statistical processing was implemented on 9 samples because of mortality by the 12th hour (E1) and 24th hour (E2). Values of total leukocytes in relation to the presence of CD45$^+$ markers on their surface in the total cell population of heterogeneous sample of the full blood in three observed groups of fattening chickens are presented in Table 1. Although several samples over time were statistically significantly different for the control group, the means were did not increase as they did in both experimental groups and the means at the end of the experiment was not different from the beginning. The observed difference among these assays are probably due the lower count among the controls, and hence not biologically significant.

While observing the two experimental groups, statistically significant higher values ($P<0.01$) of CD45$^+$ leukocytes were observed in both of them in relation to determined values in the chickens of the control group during the complete observation period. Also, during and at the end of the experiment, statistically significant higher values ($P<0.01$) of CD45$^+$ leukocytes were observed in the chickens of the group E1 in relation to the chickens from the E2 group. Also, in Table 1, it is clear that the value of CD_{45}^+ leukocytes on the 14. day of the observation period in the group of chickens E1 was statistically significantly higher ($P<0.05$) in relation to the value of CD45$^+$ leukocytes of the chickens from E2 group in the same period. During the entire observed period of the chickens from group P1, a continued growth of leukocytes was observed in relation to the presence of the CD45$^+$ marker in total blood (Table 1). Namely, in the chickens from group E1, a statistically significant increase of CD45$^+$ leukocytes was observed in the 12th hour of the observation period in relation to the value in the 6th hour and after 24 hours, in each period, i.e. the number of CD45$^+$ cells was statistically significantly increasing so that, during the complete period, the value determined at the end of the observation period was

statistically significantly higher (P<0.01) in relation to the value determined in 6th hour (Table 1).

Table 1. Determined leukocyte values (CD45^{+}) in the control group, the group inoculated with lyophilised live vaccine with the La Sota strain against Newcastle disease (E1) and the group inoculated with inactivated vaccine of the NB virus (E2) during the observation period

Time of blood draw	Number of leukocytes (CD45^{+}) in the total cell population of the heterogeneous sample in the full blood of fattening chickens		
	Control group (n =10) Mean ± SD	Experimental group 1 (n = 9) Mean ± SD	Experimental group 2 (n = 9) Mean ± SD
6. hour	7309.00 ± 88.31	7422.00 ± 71.76^{a,b}	7325.00 ± 63.21^{a}
12. hour	7042.00 ± 62.76	7768.00 ± 64.38^{a,b}	7642.00 ± 56.33^{a}
24. hour	7104.00 ± 39.55	7945.00 ± 24.76^{a,b}	7729.00 ± 47.64^{a}
7. day	7215.00 ± 24.35	8234.00 ± 67.56^{a,b}	7949.00 ± 33.24^{a}
14. day	7099.00 ± 63.79	9120.00 ± 56.23^{a,bb}	8933.00 ± 78.31^{a}
21. day	7322.00 ± 72.42	9546.00 ± 73.17^{a,b}	9125.00 ± 37.17^{a}
28. day	7341.00 ± 63.32	9847.00 ± 36.13^{a,b}	9367.00 ± 89.78^{a}

^{a} statistically significant higher value (P<0.01) in relation to the value determined in the control group; ^{b} statistically significant higher value (P<0.01) in relation to the value determined in the experimental group 2; ^{bb} statistically significant higher value (P<0.05) in relation to the value determined in the experimental group 2

In the chickens from group E2, a milder increase of CD45^{+} leukocytes was observed than in the chickens of E1 group, that is, a statistically significant (in relation to the control group) increased value of CD45^{+} cells 6 and 24 hours after inoculation, while a sudden increase of value of CD45^{+} cells was observed on the 14th day. After that period, as well as in the case of E1 group, in each observed period, the value of CD45^{+} cells was statistically significantly increasing so that, during this complete period, the value determined at the end of the observing period was statistically significantly higher (P<0.01) in relation to the value determined in the 6th hour (Table 1).

Discussion

Newcastle disease is one of the most significant contagious viral diseases in the modern poultry production, affecting poultry and many avian species. It is a highly contagious disease which, depending on the virulence of a virus strain and the type of host, can be manifested from sub-clinical symptoms to 100% mortality. In many countries in which poultry is commercially bred, poultry production depends on vaccination to control this disease. Today, satisfactory protection of poultry against the Newcastle disease (ND) provides live attenuated or inactivated vaccines of' the La Sota virus strain. But in spite of the systematic inoculation, ND presents a significant obstacle in the poultry production in many countries (SAVIĆ, 2003). Namely, knowledge about the effects of the ND viruses to the cell immune response in the poultry, especially of different types of vaccines to the stimulation of immune response are unsatisfactory (REYNOLDS and MARAQA, 2000a; 2000b). However, with development of immune and molecular methods, it has been made possible to monitor the kinetics of immune cells and molecules through a certain time period to the stimulation with immunogen. Flow cytometry is of great use, as presented in this study, for quality and quantity analysis of $CD45^+$ cells. Based on the literature, it is known that $CD45^+$ molecules support the activation of T and B lymphocytes after binding antigens (ANDREIS et al., 1998). Furthermore, GARG et al. (2004) state that the total number of lymphocytes in the peripheral blood of broiler chickens is an indicator of functional ability of lymphoid organs and that the lymphocytes are an important factor in the cellular immune response. Thus, in this study, with the application of flow cytometry to broiler chickens treated with lyophilised live vaccine of the La Sota virus of Newcastle disease or inactivated vaccine against ND, a statistically significant expansion of the number ($P<0.01$) of $CD45^+$ cells in circulating blood was observed, which was more pronounced in chickens that were treated with lyophilised live vaccine of the La Sota virus against Newcastle disease. Values of $CD45^+$ cells in whole blood of the control group were consistent to available literature information, that is, gained values are within the same reference presented by GARG et al. (2004), CHANDRA NAIK et al. (2005) and BALENOVIĆ et al. (2007). According to the mentioned literature, we can conclude that the total number of leukocytes in the blood of broiler chickens in the control group was within the referent values for the poultry as species, while for broiler chickens as a specific category, no referent values have been found in the available literature.

Kinetics of $CD45^+$ leukocytes changed significantly in the experimental groups, which showed the activation of the cellular immune response regarding the multiplication of immunogen in live vaccine and less so in the group with inactivated vaccine containing inert immunogen. With the cytometric estimation of $CD45^+$ leukocytes in both experimental groups, it was shown that the chickens treated with live vaccine against the

Newcastle disease in the 6th, 12th and 24th hour, and 7, 14, 21 and 28 days after inoculation had statistically significantly more CD45+ leukocytes. However, on the 14th day after inoculation, a significantly higher value ($P<0.05$) was found for CD45+ leukocytes in the group of chickens inoculated with inactivated vaccine in relation to the control group. Based on the obtained cytometric analyses of the kinetics of CD45+ leukocytes, it can be concluded that the lyophilised live vaccine was more efficient and faster in achieving cellular immunity than inactivated vaccine. Differences in the kinetics and oscillation of leukocytes in the chickens vaccinated with live vaccines Hitchner B1 and Scrubs strains of NDV and inactivated A.G.68 and Northampton strains, were observed by TIMMS and ALEXANDER (1977) while determining an initial cellular immune response. Using inhibition of hemaglutination and inhibition of leukocyte migration, an increase of antibodies and a level of individual subpopulations was observed on the first inoculation day and the greatest increase in the first and the second week, and a gradual decline was observed during the third and fourth week after inoculation. Cytometric studies have not been performed heretofore on chickens vaccinated for Newcastle disease.

The immunological statues of broiler chickens following their immunization with two types of vaccines against ND may be assessed by flow cytometry, and accordingly the immunogenicity of vaccine candidate strains could be validated.

Acknowledgements

This study was supported by the grants nos. 053-0532265-2255, 0530532265-2238, 053-0531863-1861 and 048-0481153-1136 from the Ministry of Science, Education and Sport of Croatia.

References

ANDREIS, I., F. ČULO, M. MARUŠIĆ, M. TARADI (1998): Imunologija (peto izdanje), Medicinska naklada, Zagreb.

BALENOVIĆ, M., M. POPOVIĆ, V. SAVIĆ, D. KEZIĆ, K. VLAHOVIĆ, A. DOVČ, G. BEZROK, I. POPOVIĆ, I. VALPOTIĆ (2007): Quantitative immunophenotypisation of T-lymphocytes from peripheral blood of laying hens (In Croatian). Praxis Vet. 55, 33-39.

CHANDRANAIK, B. M., Y. HARIBABU, G. S. MAMATHA (2005): The immunomodulatory role of calf thymus extract on humoral and cell mediated immune response in chicken vaccinated against New Castle Disease virus. Inter. J. Poultry Sci. 4, 580-583.

ERF, G. F. (2004): Cell-mediated immunity in poultry. Poultry Sci. 83, 580-590.

GARG, U. K., A. K. PAL, G. J. JHA, S. B. JADHAO (2004): Haemato-biochemical and immuno-pathophysiological effects of chronic toxicity with synthetic pyrethroid, organophosphate and chlorinated pesticide in broiler chicks. Int. Immunopharm. 4, 1709-1722.

GLICK, B. (2000): Immunophysiology. In: Sturkie′s Avian Physiology, 5th ed. (Whittow, G. C., Ed.), Academic Press, London, pp. 657-667.

LILLEHOJ, H. S. (1991): Lymphocytes involved in cell-mediated immune responses and methods to assess cell-mediated immunity. Poultry Sci. 70, 1154-1164.

NAGLIĆ, T., D. HAJSIG (1993): Veterinarska imunologija. Školska knjiga, Zagreb.

ORMEROD, M. G. (2000): Flow Cytometry. A practical approach. 3th ed. Oxford University Press.

PARMITHIOTIS, E., L. TKALEC, M. J. H. RATCLIFFE (1991): High levels of CD45 are co-ordinately expressed with CD4 and CD8 on avian thymocytes. J. Immunol. 147, 3710-3717.

POPOVIĆ, M., N. VIJTIUK, M. BALENOVIĆ, I. POPOVIĆ, H. VALPOTIĆ, D. POTOČNJAK, K. VLAHOVIĆ, I. VALPOTIĆ (2008): Effect of caponisation on the expresion of CD molecules on roosters immune cells. Tierärztl. Umschau 63, 566-569.

REYNOLDS, D. L., A. D. MARAQA (2000a): Protective immunity against Newcastle disease: The role of antibodies specific to Newcastle disease virus polypeptides. Avian Dis. 44, 138-144.

REYNOLDS, D. L., A. D. MARAQA (2000b): Protective immunity against Newcastle disease: The role of cell-mediated immunity. Avian Dis. 44, 145-154.

SAALMÛLLER, A., M. J. REDEHASSE, H. J. BUHRING, S. JONJIĆ, U. H. KOSZINOWSKI (1997): Stimultaneous expression od CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. Eur. J. Immunol. 17, 1297-1301.

SAVIĆ, V. (2003): Genetic characterisation of Newcastle disease virus strains isolated in Croatia and neighbouring countries. Dissertation, University of Zagreb, Faculty of Science, Zagreb.

TARRANT, J. M. (2005): The role of flow cytometry in companion animal diagnostic medicine. Vet. J. 170, 278-288.

TIMMS, L., D. J. ALEXANDER (1977): Cell-mediated immune of chickens to Newcastle disease vaccines. Avian Pathol. 6, 51-59.

TIZARD, I. R. (2002): Veterinary Immunology 7th ed. (W.B. Saunders, Ed.), Philadelphia.

Received: 4 July 2008
Accepted: 22 December 2009

POPOVIĆ, M., M. BALENOVIĆ, A. EKERT KABALIN, V. SAVIĆ, N. VIJTIUK, K. VLAHOVIĆ, I. VALPOTIĆ: Procjena kinetike CD45+ leukocita pune krvi tovnih pilića imuniziranih živim ili inaktiviranim cjepivom protiv newcastleske bolesti. Vet. arhiv 80, 61-69, 2010.

SAŽETAK

Budući da su dosadašnje spoznaje o djelovanju cjepnog virusa newcastleske bolest na stanični imunosni odziv u peradi nedostatne, u ovom radu praćena je dinamika leukocita u punoj perifernoj krvi tovnih pilića cijepljenih protiv newcastleske bolest liofiliziranim živim cjepivom proizvedenim od soja La Sota virusa (pokusna skupina E1) i/ili inaktiviranim cjepivom proizvedenim od istog soja (pokusna skupina E2). U tovnih

pilića cijepljenih liofiliziranim živim cjepivom ili inaktiviranim uočen je statistički značajan porast ($P<0,01$) CD45^{+} leukocita u punoj perifernoj krvi, pri čemu je taj porast bio intenzivniji i brži u pokusnoj skupini tovnih pilića koja je bila tretirana liofiliziranim živim cjepivom. Promatrajući obje pokusne skupine, skupina cijepljena živim cjepivom protiv newcastleske bolest 6., 12., 24. sata, 7., 14., 21. i 28 dana nakon cijepljenja je imala statistički značajno veću vrijednost za CD45^{+} leukocita. Međutim, 14. dana nakon cijepljenja znatan porast vrijednosti ($P<0,05$) za CD45^{+} leukocite ustanovljen je u skupini tovnih pilića cijepljenih inaktiviranim cjepivom. Može se zaključiti da je liofilizirano živo cjepivo pokazalo bolju učinkovitost i brže postizanje stanične imunosti od inaktiviranog cjepiva tijekom promatranog razdoblja od 28 tjedana nakon cijepljenja.

Ključne riječi: stanična imunost, protočna citometrija, tovni pilići, newcastleska bolest, cjepiva