

CHANGES IN LYMPHATIC ORGANS OF LAYER CHICKENS FOLLOWING VACCINATION AGAINST MAREK'S DISEASE: HISTOLOGICAL AND STEREOLOGICAL ANALYSIS

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The aim of this study was to investigate histomorphometrical characteristics of the thymus, bursa of Fabricius and spleen in the chickens vaccinated with a vaccine against Marek's disease. For this purpose, we used newly hatched chickens of the light hybrid line, obtained from a local hatchery. The chickens were vaccinated on the 5th day after hatching with a bivalent cell-associated Marek's disease vaccine (PFU-2000 per dose). On day 13 both vaccinated chickens and unvaccinated controls were sacrificed, and thymus, bursa and spleen were removed and processed for light microscopy. The serial tissue sections, hematoxylin-eosine stained, were used for histomorphometric analysis. Vaccination against Marek's disease decreased the relative mass of the lymphoid organs, and caused significant damage of the thymus and spleen in experimental chicken. In addition, vaccination, similar to Marek's disease virus, induced morphometric changes in the lymphoid organs. Namely, it significantly decreased the diameter and volume of lymphoid follicles, volume of follicular medulla and number of cells in the follicular cortex in the bursa of Fabricius. In the thymus, vaccination reduced the thymus volume and the absolute number of thymocytes. However, vaccination against Marek's disease caused an increase in the diameter, number and volume of lymphoid follicles in the spleen.

The present data suggest that vaccination against Marek's disease was able to induce the immune response in processed organs, although it reduced the mass and number of lymphocytes in the major lymphoid organs.

Key words: bursa of Fabricius, Marek's disease, morphometry, spleen, thymus, vaccine

INTRODUCTION

Marek's disease (MD) is a contagious disorder of chickens, worldwide spread, caused by Marek's disease virus (MDV), an *Alpha herpes virus* that induces T cell lymphomas, polyneuritis, immunosuppression and rarely atherosclerosis (Churchill and Biggs, 1967; Calnek, 2001; Brown *et al.*, 2006). The outcome of infection is dependent on the virus oncogenicity and the genetic resistance of chicken. Numerous studies have demonstrated the existence of MDV strains of different pathogenicity, ranging from a naturally avirulent chicken herpes virus and a naturally avirulent turkey herpes virus (HVT) (serotypes 2 and 3), to highly virulent strains (serotype 1) in poultry populations (Witter, 1998). Genetic resistance has been linked, partly, to class I major histocompatibility complex (MHC) antigens (Powell and Lombardini, 1986). In addition, the incidence of Marek's disease is influenced by factors associated with environmental conditions (Witter *et al.*, 1984).

Although natural infection with MDV occurs by inhalation of cell-free MDV in feather dander (Beasley *et al.*, 1970), the respiratory tract does not appear to be an early site of virus replication (Adidinger and Calnek, 1973). Marek's disease virus enters the primary lymphatic organs via macrophages (Barrow *et al.*, 2003). The virus is usually detectable on the 4th day post infection by the viral internal antigens and virus isolation occurs in the spleen, bursa of Fabricius and thymus (Calnek *et al.*, 1984; Islam *et al.*, 2002). Marek's disease is characterized by: (1) an early cytolytic infection of B lymphocytes, and a small percentage of activated T lymphocytes; (2) latent infection of both B and T lymphocytes; (3) late cytolytic infection of mainly T lymphocytes; and (4) transformation of lymphatic cells (T cells), leading to lymphoid tumor formation and death (Venugopal and Payne, 1995; Calnek *et al.*, 1998; Calnek, 2001).

Having in mind that MD causes severe death losses and condemnation of the affected flocks, prevention of MD can be accomplished by monovalent, polyvalent or recombinant vaccines. Previously published reports indicate that different vaccine types have different efficiency on the immune system of chickens (Lee *et al.*, 1978; Calnek *et al.*, 1979; Friedman *et al.*, 1992; Fynan *et al.*, 1995; Mičić *et al.*, 2000; Tischer *et al.*, 2002). Cell-mediated immunity, in particular the activity of cytotoxic T lymphocytes (mainly CD8+ cells), has an important role in the vaccinal protective mechanism against MD lymphoma (Omar and Schat, 1996). Vaccination is usually protective against the development of clinical manifestations of MD, but does not prevent MDV infection (Purchase *et al.*, 1971; Lee *et al.*, 1999; Witter, 2001; Islam *et al.*, 2001; 2002).

However, fast changes of Marek's disease virus genome towards greater virulence may explain increased poultry losses from MD even in vaccinated flocks (Witter, 1998). Vaccine efficacy might be affected by many factors, such as the B haplotype (Bacon and Witter, 1993, 1994a, b), maternal antibodies (Witter and Lee, 1984; Witter *et al.*, 1995; Sonoda *et al.*, 2000), vaccine dose and so on.

Having in mind the data above, the aim of this study was to identify and quantify changes in the lymphatic organs of vaccinated chickens, by histological and stereological methods.

MATERIALS AND METHODS

Experimental animals and tissue preparation

Newly hatched chicks of light hybrid line were obtained from a local hatchery. They were given commercial feed and water *ad libitum*. The chicks were divided randomly into two groups. The first group was vaccinated 5 days after hatching with a bivalent cell-associated Marek's disease vaccine (Bio Marek bivalent), containing at least 2000 plaque forming units of turkey herpes virus FC-126 and chicken herpes virus HPRS-16 per dose. The second group of unvaccinated chicks, was used as the control. On day 13 after hatching, ten chicks from each group were sacrificed and lymphatic organs (bursas, thymuses and spleens) were removed for further analysis. The chicks and matching bursas, thymuses and spleens were weighted. The removed organs were fixed in Bouin's solution for 12 h. Paraffin sequential serial sections (5 μm thickness) of the bursa, thymus and spleen were stained with hematoxylin-eosin. We used the multipurpose test system M42 for morphometric measurements.

Morphometric measurements

Histoquantitative studies were performed using the multipurpose test system M42 (Weibel, 1979). Each 10th section was analyzed under a Reichert Biovar (Austria) microscope with an eyepiece fitted with Weibel's M 42 test system. The test area was always chosen at random, and the size of the final sample was calculated for each parameter separately to provide results within the confidence interval of 95% percent.

Volume density (V_v) of the thymic cortex and medulla, bursal follicles, follicular cortex and medulla, and lymphocytic follicles in the spleen were determined under a magnification $\times 6.3$, using a point-counting method (Weibel, 1979), and calculated according to the equation:

$$V_v = P_f/P_t$$

Where P_f = the number of test points falling on the analyzed structure; P_t – the total number of test points (Kališnik, 1985).

Absolute values of examined structures were calculated from their volume density and absolute volume of the whole organ.

Numerical density of the lymphocytic follicles in the bursa and spleen, as well as lymphocytes in the thymic compartments and bursal lymphocytic follicles per volume unit were calculated according to the following equation:

$$N_v = NA/(D + G_o)$$

The number of follicles/lymphocytes per surface unit of the test area (NA) was obtained from the relationship between the number of follicles/lymphocytes per test area (N) and the size of the test area (A_t).

The mean diameter (D) of the follicles/lymphocytes represents the relationship between volume density (V_v) and surface density (S_v), where S_v represents the relationship between the number of intersections of the test lines

with the follicles/plasma membrane of lymphocytes and the total length of the test line (Kališnik, 1985).

Depth sharpness (G_o) was estimated from the light wavelength (λ), coefficient of diffraction of the immersion oil (n) and numerical aperture of the objective lens (N_a).

The total number of the thymic and bursal thymocytes/lymphocytes was calculated from NV of thymocytes/lymphocytes in the thymic/bursal compartments and absolute volume of these compartments.

Statistical analysis

All results are expressed as mean \pm SD. Comparisons among groups were conducted by analysis of variance (ANOVA) test. In all cases, Scheffe test was used as a post hoc analysis. The level of significance was considered at $p < 0.05$.

RESULTS

Body and organ weight

The results of this study showed that the bivalent vaccine against Marek's disease significantly decreases the relative mass of the thymus ($p < 0.001$), bursa of Fabricius ($p < 0.05$) and spleen ($p < 0.01$) compared to corresponding controls (Figure 1), while body weight remained unchanged (Figure 2). A significant volume decrease of the thymus ($p < 0.001$), bursa of Fabricius ($p < 0.05$) and spleen ($p < 0.01$), in the vaccinated chicken group, was observed, as well (Figure 1). Calculation of the lymphatic organs body index (relationship between the relative weight of the bursa/thymus/spleen in vaccinated and control chickens) revealed significant damage of the thymus and spleen in the vaccinated chicks. However, the decrease of the bursa body index was not significant (Figure 1).

Histological and quantitative analysis

Bursa of Fabricius

Histological analysis, done by light microscopy, revealed that the size of bursal plicae did not change in the chicks vaccinated against Marek's disease. The bursae of vaccinated birds contained follicles distinguished by their size and the apparent cortical and medullar structure. In addition, within the bursal plicae an increased visibility of the stroma was noted (Figure 3a). In the lymphocytic follicles the reduced number of lymphocytic cells in the follicular cortex, and enlarged density of cells in the follicular medulla, was evident (Figure 3b). Stereological analysis confirmed that vaccination induces a significant decrease of lymphocytic follicles diameter and volume, volume of the follicular medulla and number of cells in the follicular cortex. On the other hand, vaccination against Marek's disease raises the number of lymphocytic cells in the follicular medulla, number of lymphocytic follicles per unit area, as well as their absolute number. Likewise, vaccination alters the relationship between follicular cortex and medulla (Table 1).

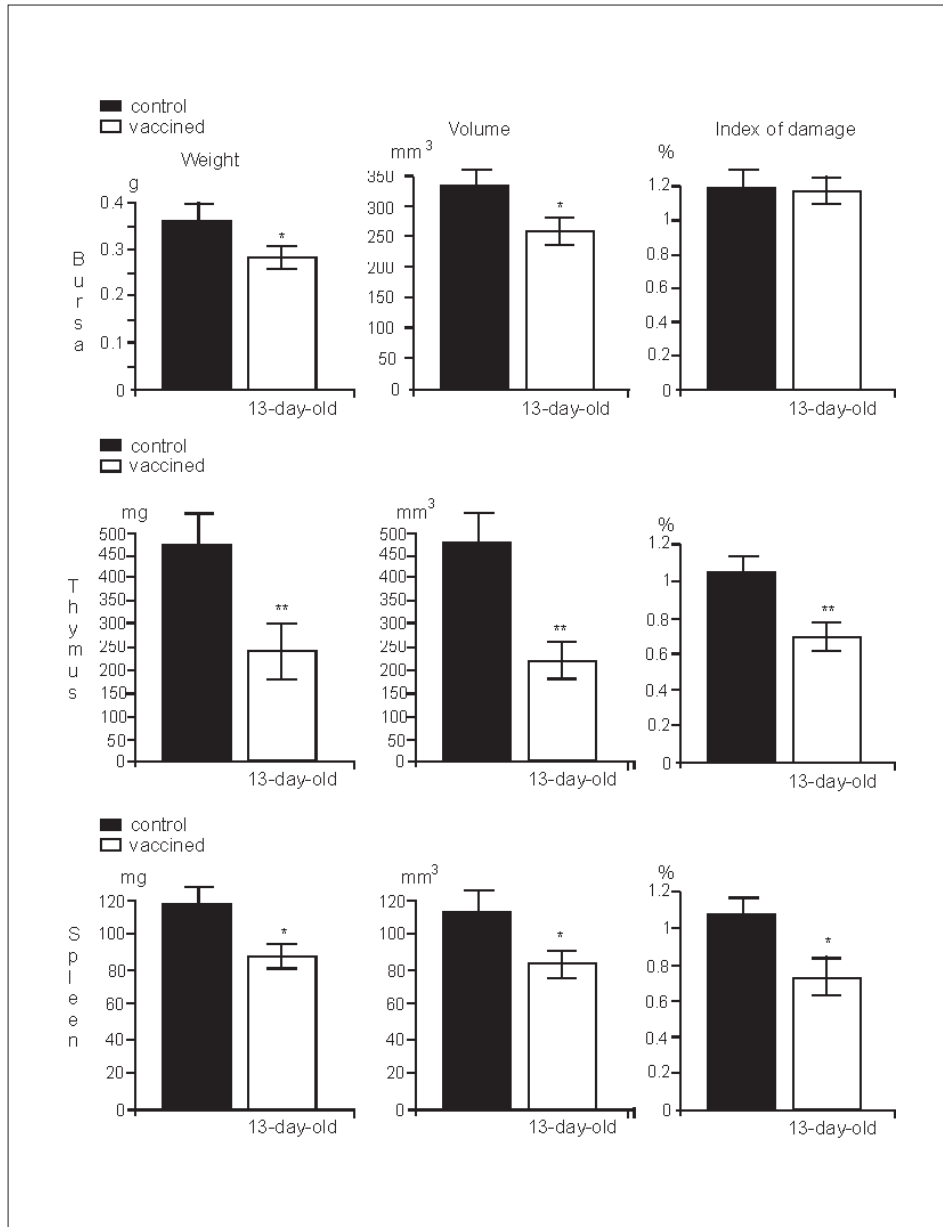


Figure 1. Changes of stereological parameters are shown as graphics: weights, volumes and index of damage of the lymphatic organs (bursae, thymus, spleen) in the control and vaccinated chickens. Values represent mean \pm S.D. ** $p < 0.01$; * $p < 0.05$; vs. control

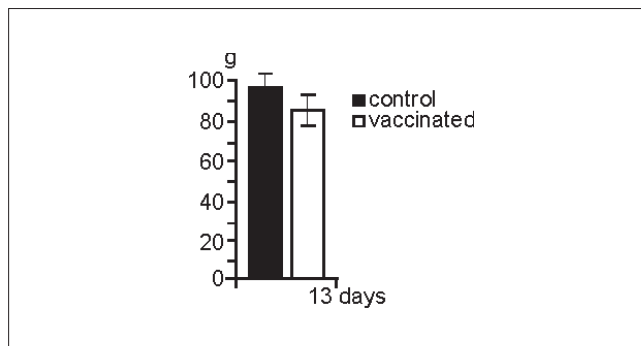


Figure 2. Body weight of the control and vaccinated chickens

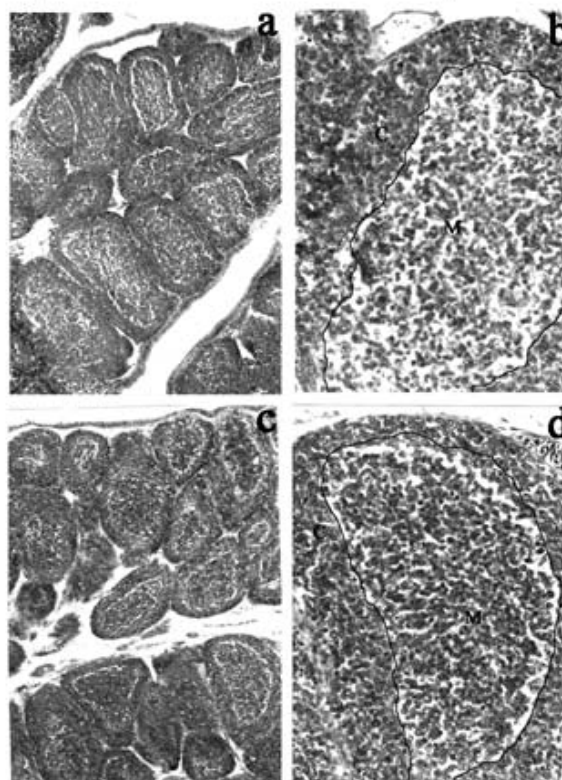


Figure 3. The structure of bursal compartments in vaccinated chickens is well preserved, although the follicles are smaller (c, d). Control (a, b) and vaccinated (c, d) chickens. Original magnification 10x (a, c) and 40x (b, d)

Table 1. Changes in the compartments of the bursa of Fabricius in vaccinated and control chickens obtained by the stereological method

Parameters	Experimental groups	
	Control	Vaccinated
Volume (mm ³)		
Follicles	0.26 ± 0.02	0.19 ± 0.01*
Follicular cortex	0.115 ± 0.005	0.103 ± 0.001
Follicular medulla	0.138 ± 0.004	0.092 ± 0.002*
Cortex/medulla	0.8: 1	1.1: 1
Numerical density (mm ⁻³)		
Follicles x 10 ⁻³	10 ± 0.2	16 ± 0.5*
Lymphocytes x 10 ⁻³		
Follicular cortex	8.5 ± 0.5	6.8 ± 0.6
Follicular medulla	16 ± 0.8	18 ± 0.09*
Total number x 10 ⁶		
Follicles	32 ± 2	43 ± 3*
Cells		
Follicular cortex	5.0 ± 0.5	3.2 ± 0.3*
Follicular medulla	10.0 ± 0.2	13.0 ± 0.04*

Volumes of the follicles, follicular cortex and medulla represent parts of bursal volume occupied with the analyzed structures. Total number of the follicles and cells within the cortex and medulla was calculated from the numerical density of thymocytes and absolute volume of the analyzed structures. Numerical density (NV) of the follicles and lymphocytes in the follicular cortex and medulla, expressed as number of thymocytes per volume unit of test area. The results are presented as mean values ± SD, n = 10. *p<0.05; vs. control.

Thymus

Analysis of thymus sections demonstrated that vaccination induces constriction of the thymus cortex with scant cell numbers (Figure 4). Considering the importance of thymus compartments in the process of thymocyte differentiation, we investigated whether application against Marek's disease evoked morphometrical changes in the thymus compartments. Our results showed that the vaccine reduces the thymus volume (Figure 1), as well as the volume of the thymic cortex and medulla. In addition, numerical density and number of cells in the thymic cortex and medulla and total number of thymocytes were decreased, too (Table 2).

Table 2. Changes in the thymic compartments volume, number and the numerical density of thymocytes in the vaccinated and control chickens

Parameters	Experimental groups	
	Control	Vaccinated
Absolute number of thymocytes x 10 ⁶	16.5 ± 0.6	3.00 ± 0.5***
Cortex		
Absolute volume (cm ³)	0.29 ± 0.05	0.14 ± 0.04**
Number of thymocytes (x 10 ⁶)	14.5 ± 0.1	3.6 ± 0.3**
Numerical density of thymocytes (x 10 ⁻³)	0.50 ± 0.02	0.37 ± 0.01*
Medulla		
Absolute volume (cm ³)	0.13 ± 0.03	0.07 ± 0.01**
Number of thymocytes (x 10 ⁶)	3.9 ± 0.1	1.54 ± 0.02**
Numerical density of thymocytes (x 10 ⁻³)	0.30 ± 0.004	0.22 ± 0.02*
Interlobular connective tissue		
Absolute volume (cm ³)	0.03 ± 0.002	0.01 ± 0.001

Volumes of the cortex, medulla and interlobular connective tissue represent parts of tissue volume occupied with the analyzed structure. Total number of the thymocytes in the cortex and medulla was calculated from the numerical density and the absolute volume of the analyzed structure. Numerical density (NV) of the thymocytes in the outer cortex, deep cortex and medulla is expressed as number of thymocytes per volume unit of test area. The results are presented as mean values ± SD, n = 10. ***p<0.001; **p<0.01; *p<0.05; vs.control

Spleen

Histological analysis showed that vaccination induces visible changes only in the spleen follicles (Figure 5). Namely, it raises their diameter, number and volume (Table 3).

Table 3 Changes in the volume of spleen lymphocytic follicles, their diameter and numerical density in the vaccinated and control chickens

Parameters	Experimental groups	
	Control	Vaccinated
Follicles		
Diameter (µm)	76 ± 6	230 ± 15**
Volume (mm ³)	0.027 ± 0.001	0.051 ± 0.004**
Numerical density x 10 ⁻³	1.1 ± 0.15	1.65 ± 0.2*

The results are presented as mean values ± SD, n = 10. **p<0.01; *p<0.05; vs.control

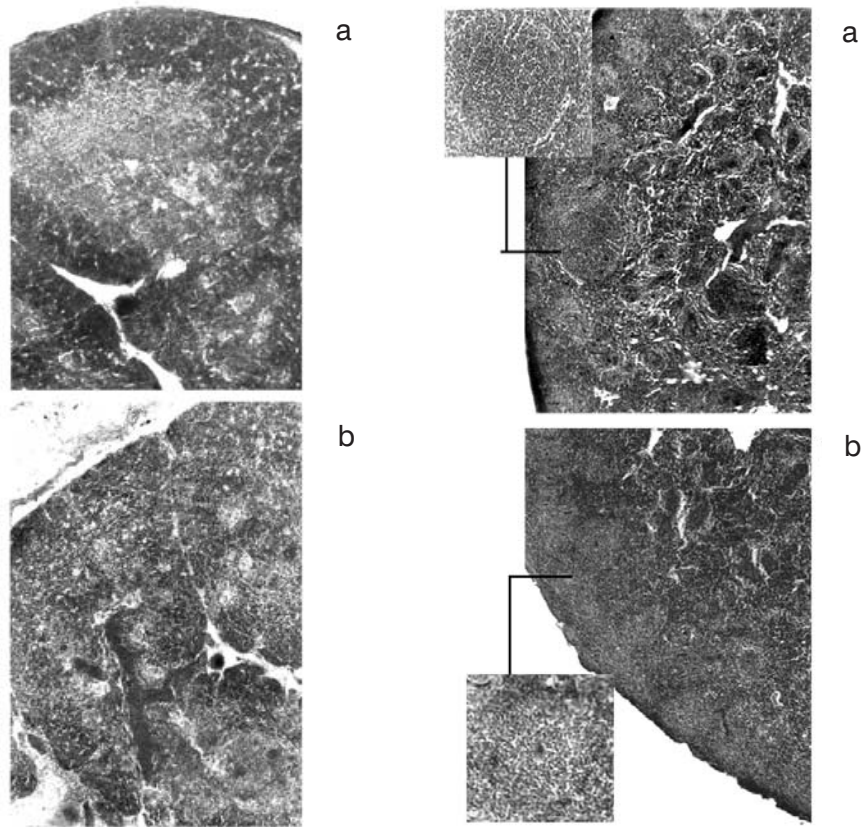


Figure 4. The thymus of the chickens vaccinated against Marek's disease (a) and control animals. Original magnification 10x

Figure 5. The spleen of vaccinated (a) and control (b) chickens. Original magnification 10x, and 40x (insert: lymphocytic follicles)

DISCUSSION

The aim of this study was to examine the changes in the lymphatic organs of vaccinated layer chickens with bivalent cell-associated Marek's disease vaccine. Our results revealed that this vaccine is immunosuppressive to commercial chickens, as evidenced by reduction of relative bursal and thymic weights, thymic index of damage, as well as B-cell and T-cell numbers. In agreement with our results are the findings of a mild depletion of T- and B-lymphocytes after vaccination by other authors (Lee *et al.*, 1999; Islam *et al.*, 2002). Considering that the primary cell targets of MDV are the lymphocytes, and that the early effects are

mainly seen in lymphatic organs such as the bursa of Fabricius, thymus and spleen (Venugopal and Payne, 1995; Calnek *et al.*, 1998; Calnek, 2001), the results obtained in this study were to be expected. In addition, MDV-induced immunosuppression, including both the humoral and cell-mediated immunity, most likely associated with lymphopaenia due to cytolysis of B and T lymphocytes, (Calnek *et al.*, 1998) is in line with the reduction of the cell number in the thymus and bursal follicular cortex. A transient depletion of B-lymphocyte activity of varying intensity has also been observed following the vaccination with bivalent (HVT + SB-1) or MDV serotype 1 (Rispen) vaccines (Friedman *et al.*, 1992). Consequences of this immunosuppression may be linked with the reduced resistance to concurrent infection (Biggs *et al.*, 1968; Abbassi *et al.*, 1999, 2000), but the extent of immunosuppression varies according to several host resistance factors and the virus pathotype (Calnek *et al.*, 1998; Baigent and Davison, 1999).

The vaccine against Marek's disease increases the number of bursal lymphatic follicles, while it reduces their volume and diameter, as a consequence of the follicular medulla reduction. Interestingly, this vaccine reduces the cell number in the follicular cortex and increases their number in the medulla. Separated maturation of B cells in the follicular cortex and medulla (Sayegh *et al.*, 2000), rapid proliferation and gene conversion of the cortical B-cells (Reynaud *et al.*, 1987; Masteller *et al.*, 1995), and emigration of the cells predominantly directly from the cortical population to the periphery. Paramithiotis and Ratcliffe, 1994 support changes notified in the follicular cortex. In addition, the rapid cell proliferation, presumably due to selective expression of a functional B-cell receptor, may be the reason for the cell loss by apoptosis in this dynamic structure of bursa follicles (Sayegh *et al.*, 1999; 2000). On the other hand, the changes in the follicular medulla may be explained by the medullar communication to the gut lumen via the lumen of the bursa, through specialized follicle-associated epithelium, which has the ability to transfer particulate matter from the lumen into the medulla (Sorvari *et al.*, 1975) and most likely has an antigen-presenting function (Sorvari *et al.*, 1997). The extent to which this may be involved in the generation of immune response is not clear.

Our results showed that vaccination reduces the thymus mass, as well as the volume of the thymic cortex and medulla, and significantly damages the thymus. In addition, it decreases the absolute number of thymocytes, and the numerical density and the number of the cells in the thymic cortex and medulla. The reduction of thymic weight was also described in MDV challenged chickens (Calnek *et al.*, 1998) and a direct correlation between the degree of thymic/bursal atrophy, following MDV infection, and the virulence or pathotype of virus strains has been reported (Calnek *et al.*, 1998). These results are accordant with results of Morimura and his collaborates (1996), who described thymic atrophy as a consequence of massive CD4+CD8+ thymocyte apoptosis and reduction in circulating CD4+ lymphocytes.

Finally, considering the cell number decrease in the thymus and bursa cortex it is to be expected that vaccination suppresses specific immune response in the spleen. However, the increased number and diameter of lymphocytic follicles in the spleen indicates that vaccination against Marek's disease has no

effect on specific immune responses. This is consistent with the observation that vaccinal HVT failed to affect humoral antibody responses (Reddy *et al.*, 1996). A similar result has been reported following vaccination with vaccine serotype 2 (Calnek *et al.*, 1979).

The results obtained in this study suggest that the vaccinal Marek virus induced a decrease of the cell number in the bursal cortex and, especially, in the thymus. This most likely consequently increased the emigration of cells to the periphery, or cell lyses. On the other side, although vaccination has suppressive effects on the B and T cell numbers, it has no effect on the specific immune response.

Abbreviations: Marek's disease - MD; Marek's disease virus – MDV; Herpes virus turkey - HVT

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**PROMENE U LIMFATIČNIM ORGANIMA KOMERCIJALNIH PILIĆA NAKON
VAKCINACIJE PROTIV MAREKOVE BOLESTI: HISTOLOŠKA I STEREOLOŠKA
ANALIZA**

MILJKOVIĆ BILJANA, RAKIN ANA, AŠANIN RUŽICA, DIMITRIJEVIĆ LJILJANA
i MIČIĆ MILEVA

SADRŽAJ

Cilj ovih ispitivanja su bile histomorfometrijske karakteristike timusa, burze Fabricii i slezine pilića vakcinisanih protiv Marekove bolesti. Za istraživanja su korišćeni jednodnevni pilići lake hibridne linije, gajeni u standardnim uslovima. Pilići su vakcinisani 5. dana od izleganja, bivalentnom vakcinom protiv Marekove bolesti (PFU 2000 po dozi). Trinaestog dana od izleganja kontrolni i vakcinisani pilići su žrtvovani, uzeti su im timus, burza Fabricii i slezina koji su pripremljeni za svetlosno mikroskopsku analizu. Za analizu su korišćeni serijski preseki limfatičnih organa, bojani metodom hematoksilin-eozin. Dobijeni rezultati su ukazali da vakcinacija protiv Marekove bolesti dovodi do smanjenja relativne mase ti-

musa, burze i slezine, kao i povećanja indeksa oštećenja timusa i slezine. Takođe je ustanovljeno da vakcinacija dovodi do promena morfometrijskih parametara u limfatičnim organima. U burzi Fabricii je uočeno značajno smanjenje dijametra i zapremine limfocitnih folikula, zapremine medule folikula i broja ćelija u korteksu folikula. U timusu vakcinacija dovodi do redukcije zapremine timusa i apsolutnog broja timocita. Međutim, vakcinacija protiv Marekove bolesti u slezini dovodi do povećanja dijametra limfocitnih folikula, kao i do povećanja njihovog broja i zapremine. Rezultati ove studije su ukazali da vakcinacija pilića protiv Marekove bolesti, iako redukuje broj T i B limfocita, indukuje razvoj imunskog odgovora.